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Regulation of the Transcription Factor
Nuclear Factor *kappa* B in the
Neuroendocrine Response to Stress

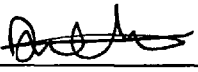
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degree of Doctor of Philosophy

Declaration

I hereby declare that the work presented in this thesis is my own. I have acknowledged the help and contribution of others (page 31).



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Abstract

Lipopolysaccharide (LPS) activates the hypothalamo-pituitary adrenal (HPA) axis, possibly by acting on the anterior pituitary gland. Whilst the mechanisms governing this response are unclear, LPS induces many of its effects via the glucocorticoid sensitive pro-inflammatory transcription factor, nuclear factor *kappa* B (NF κ B). The present study tested the hypothesis that LPS promotes adrenocorticotrophin (ACTH) release via an NF κ B-dependent mechanism and may be a target for the negative feedback actions of glucocorticoids. Two murine pituitary cell lines, which were found to express an intact LPS signalling system, were utilised alone and in co-culture to model folliculostellate (TtT/GF cells) and corticotroph (AtT20 D16:16 cells) inter-communication. TtT/GF cell-derived interleukin 6 (IL-6) release was induced by LPS via an NF κ B-dependent mechanism and inhibited by dexamethasone. Although LPS did not directly affect basal ACTH secretion from the AtT20 D16:16 cells, recombinant IL-6 potentiated the stimulatory effect of CRH, supporting the premise that LPS increases ACTH release via actions of folliculostellate cell-derived IL-6. In contrast, LPS-induced NF κ B activity in the AtT20 D16:16 cells inhibited CRH-induced ACTH release. In the co-culture studies, CRH increased ACTH secretion directly and indirectly by stimulating IL-6 release from the TtT/GF cells, possibly via actions of AtT20 D16:16 cell-derived mediator(s). These findings support the hypothesis that NF κ B signalling in folliculostellate cells promotes ACTH secretion and is sensitive to the inhibitory effects of glucocorticoids, whilst corticotroph NF κ B signalling inhibits CRH-induced ACTH secretion.

Whilst NF κ B inhibitors show potential as therapeutic agents for anti-inflammatory disorders, the *in vitro* studies suggest such compounds may impair the HPA response to stress. We therefore examined the effects of a novel NF κ B inhibitor, PHA781535E, upon the HPA responses to immunological (LPS) and psychological (restraint) stress in rats. In the LPS model, PHA781535E treatment attenuated the stress-induced rises in plasma ACTH, IL-6 and tumour necrosis factor *alpha* (TNF α), but not corticosterone. In parallel, increased NF κ B activity in the anterior pituitary gland was also reduced, supporting the premise that, in addition to the stimulatory actions of circulating NF κ B-dependent pro-inflammatory cytokines, NF κ B signalling in the anterior pituitary gland is important in mediating the ACTH response to LPS. Furthermore, it was shown for the first time that restraint stress activates NF κ B in the anterior pituitary gland and that impairment of NF κ B signalling reduces restraint stress-induced ACTH and corticosterone release. The study has identified, for the

first time, an important role for NF κ B in mediating the HPA responses to immunological and psychological stress. Together, these findings suggest that, whilst NF κ B inhibitors may be beneficial for treating chronic inflammatory disorders, such treatment may be compromised by impaired stress-induced HPA activity.

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Abbreviations

3V	Third ventricle
11β-HSD 1	11- <i>beta</i> -hydroxysteroid dehydrogenase Type 1
11β-HSD 2	11- <i>beta</i> -hydroxysteroid dehydrogenase Type 2
12(S)-HETE	12(S)-hydroxyeicosatetraenoic acid
α-MSH	<i>alpha</i> -melanocyte stimulating hormone
AA	Ascorbic acid
A-MuLV	Abelson leukaemia virus
Ab	Antibody
ABC	Adenosine tri-phosphate-binding cassette
ABCA1	Adenosine tri-phosphate-binding cassette transporter family of proteins A1
Acryl-40®	Acrylamide
ACTH	Adrenocorticotrophin hormone
ACTH-R	Adrenocorticotrophin hormone receptor (or melanocortin receptor 2)
AMV RT	Avian Myeloblastosis virus reverse transcriptase
ANOVA	Analysis of variance
ANXA1	Annexin 1
AP-1	Activator Protein 1
ArcN	Arcuate nucleus
AtT20	Murine corticotroph-like cell line
ATP	Adenosine tri-phosphate
AVP	Arginine vasopressin
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
BIS-2®	acrylamide/bisacrylamide
BSA	Bovine serum albumin
C	Control
Ca²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CD14	Cluster of differentiation 14
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
C/EBP-β	CCAAT/enhancer binding protein <i>beta</i>

CLIP	Corticotrophin-like intermediate lobe peptide
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO₂	Carbon dioxide
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CREB	Cyclic adenosine monophosphate response element binding protein
CRH	Corticotrophic releasing hormone
CRH-R1	Corticotrophic releasing hormone receptor type 1
CRH-R2	Corticotrophic releasing hormone receptor type 2
CT-1	Cardiotrophin 1
CVO	Circumventricular organs
DD	Death domains
DEX	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene glycolbis (2-aminoethylether)-N,N,N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EMSA	Electrophoretic mobility shift assay
EtOH	Ethanol
FCS	Fetal calf serum
FSH	Follicle-stimulating hormone
GABA	<i>gamma</i> -aminobutyric acid
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GC	Glucocorticoid
GFAP	Glial fibrillary acid protein
GH	Growth hormone
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
GRα	Glucocorticoid receptor <i>alpha</i>
GRE	Glucocorticoid response element

h	Human
H₂O₂	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPA	Hypothalamo-pituitary-adrenal
HRP	Horse radish peroxidase
i.p.	Intraperitoneal
IFNγ	Interferon <i>gamma</i>
IgG	Immunoglobulin type G
Igκ	Immunoglobulin <i>kappa</i> light chain
IκB	Inhibitor of <i>kappa</i> B
IκBα	Inhibitor of <i>kappa</i> B <i>alpha</i>
IKK	Inhibitor of <i>kappa</i> B kinase
IKKC	Inhibitor of <i>kappa</i> B kinase complex
IKKα	Inhibitor of <i>kappa</i> B kinase <i>alpha</i>
IKKβ	Inhibitor of <i>kappa</i> B kinase <i>beta</i>
IKKγ	Inhibitor of <i>kappa</i> B kinase <i>gamma</i>
IL-1	Interleukin 1
IL-1β	Interleukin 1 <i>beta</i>
IL-1Rα	Interleukin-1 receptor <i>alpha</i>
IL-1ra	Interleukin 1 receptor antagonist
IL-6	Interleukin 6
IL-6Rα	Interleukin 6 receptor <i>alpha</i>
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-11	Interleukin 11
IRAK 1	Interleukin 1 receptor associated kinase 1
IRAK 4	Interleukin 1 receptor associated kinase 4
IRMA	Two-site solid phase immunoradiometric assay
JAK 1	Janus activated kinase 1
JAK 2	Janus activated kinase 2
Jnk	Janus N kinase
Jurk	Jurkat cells
KCl	Potassium chloride
KH₂PO₄	Dipotassium phosphate
LBP	Lipopolysaccharide binding protein
LC	Locus coeruleus

LH	Lutenizing hormone
LIF	Leukaemia inhibitory factor
LIF-Rα	Leukaemia inhibitory factor receptor <i>alpha</i>
LPS	Lipopolysaccharide
m	Murine
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MC-R	Melanocortin receptor
MD-2	Myeloid differentiation factor 2
ME	Median eminence
Met	Methylcellulose-tween-saline
MHC	Major histocompatibility complex
MIF	Macrophage inhibitory factor
MgCl₂	Magnesium chloride
M-MLV	Moloney murine leukaemia virus
mPOA	Medial preoptic area
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
MSH	Melanocyte stimulating hormone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response protein 88
MW	Molecular weight marker
NaCl	Sodium chloride
NaF	Sodium fluoride
NaHCO₃	Sodium hydrogen carbonate
Na₂HPO₄	Disodium phosphate
NaN₃	Sodium azide
Na₃VO₄	Sodium vanadate
NP-40	Nonionic detergent p40
N-POMC	N-terminal proopiomelanocortin
ND	Not detected
NE	Noradrenergic
NF-IL6	Nuclear factor interleukin 6
NFκB	Nuclear factor <i>kappa</i> B
NO	Nitric oxide

NOS-1	Nitric oxide synthase 1
NOS-2	Nitric oxide synthase 2
NOS-3	Nitric oxide synthase 3
NPY	Neuropeptide Y
ns	Non-specific
NTS	Nucleus tractus solitarius
OC	Optical chiasm
OD	Optical density
OSM	Oncostatin M
p38α-MAPK	p38 α -mitogen activated kinase
PACAP	Pituitary adenylate cyclase-activating peptide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDFS	Pituitary-derived human folliculostellate cell line
PDTC	Pyrrolidine dithiocarbonate
PeN	Periventricular nucleus
PG	Prostaglandin
PGD₂	Prostaglandin D ₂
PGE₂	Prostaglandin E ₂
PGF_{2α}	Prostaglandin E ₂ <i>alpha</i>
PHA781535E	Specific inhibitor of inhibitor of <i>kappa</i> B kinase <i>beta</i> (IKK β) subunit
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase
PLA2	Phospholipase A2
PBMC	Peripheral blood monocyte cells
PMN	Polymorphonuclear leukocytes
PMSF	Phenyl methyl sulfonyl fluoride
p.o.	Oral administration
POMC	Proopiomelanocortin
PRL	Prolactin
Pro-γ-MSH	Pro- <i>gamma</i> - melanocyte stimulating hormone
PVN	Hypothalamic paraventricular nucleus
RAW 264.7	Murine macrophage-like cell line
RIA	Radioimmunoassay

rh	Recombinant human
rIL-6	Recombinant interleukin 6
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Specific
Sal	Saline
SC-514	Specific inhibitor of inhibitor of <i>kappa</i> B kinase <i>beta</i> (IKK β) subunit
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SEM	Standard error
SH2 domain	Src homology domain type 2
SOC	Suppressor of cytokine signalling
SOC-3	Suppressor of cytokine signalling 3
SP	Substance P
SPC	Subtilisin/kexin-like endoproteases (proprotein convertase)
STAT	Signal transducer and activator of transcription
STAT 1	Signal transducer and activator of transcription 1
STAT 3	Signal transducer and activator of transcription 3
Symph. sys.	Sympathetic system
T₃	Tri-iodo-L-asparagine
TAB1	TAK1 binding protein (activator protein) 1
TAB2	TAK1 binding protein (adaptor protein) 2
TAB3	TAK1 binding protein (adaptor protein) 3
TAK1	Member of the mitogen-activated protein kinase kinase kinase family
TBS	Tris buffered saline
TBE	Tris-Borate-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TIR	Toll/interleukin-1 receptor
TIRAP	Toll/interleukin-1 receptor domain containing adaptor protein
Tlr	Toll-like receptor
Tlr 2	Toll-like receptor 2
Tlr 4	Toll-like receptor 4
TNFα	Tumour necrosis factor <i>alpha</i>
TRAF	Tumour necrosis factor receptor associated factor

TRAF6	Tumour necrosis factor receptor associated factor 6
TRIS	Tris (hydroxymethyl) aminomethane
TRIS HCL	Tris (hydroxymethyl) aminomethane hydrochloride
TSH	Thyroid-stimulating hormone
TTBS	Tween Tris buffered saline
TtT/GF	Murine folliculostellate-like cell line
Tyk2	Tyrosine kinase 2
Ucn	Urocortin
V	Vehicle
VEGF	Vascular endothelial growth factor
VLM	Ventrolateral medulla
VMN	Ventromedial nucleus

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1 Introduction

1.1 The stress response

In response to an imbalance in, or a threat to, homeostasis in an individual, a general alarm is initiated followed by a series of co-ordinated adaptations aimed at restoring homeostasis. These co-ordinated responses, collectively known as the stress response, are composed of alterations in behaviour, autonomic function and the secretion of multiple hormones, including adrenocorticotrophin (ACTH), cortisol/corticosterone and adrenal catecholamines (adrenaline and noradrenaline), to enable the individual to cope with the situation (Chrousos 1988; Van De Kar & Blair 1999). The autonomic responses alter the physiological state of the individual, through changes in respiratory rate and intermediate metabolism (e.g. gluconeogenesis and lipolysis), elevations in core temperature, enhancement of cardiac output, the redirection of blood flow to provide highest perfusion to the brain and skeletal muscles and shunting oxygen and nutrients to the central nervous system, skeletal muscles and the stressed body site(s). These changes occur primarily to promote both the availability of vital substrates and redirect energy to enable the individual to cope with the stress, whilst detoxification functions to rid the individual of unnecessary metabolic products (Chrousos 1988; Gold *et al.* 1988b; Gold *et al.* 1988a). The behavioural changes depend on the nature of the stressor and the previous experience of the individual, and include aggression, increased alertness and arousal, improved cognition, immobility, appetite suppression as well as euphoria or dysphoria. The neuroendocrine changes include alterations of the hypothalamo-pituitary adrenal (HPA) axis (section 1.2). Activation of the HPA axis involves the release of corticotrophin releasing hormone (CRH) from the hypothalamus which in turn acts on the anterior pituitary gland to stimulate ACTH secretion. The principal target of ACTH is the adrenal cortex, which releases glucocorticoids that act in a negative feedback manner to restore homeostasis. The stress response is therefore an essential physiological response and although in itself it is no threat to the individual, if sustained and the individual fails to cope, the response may lead to illness.

The stress response is subserved by both the central nervous system (CNS) and the periphery, and the CNS receives and integrates a diversity of neurosensory (i.e. higher cortical, limbic, visual, auditory, olfactory, gustatory, somatosensory, nociceptive and visceral) and blood borne signals (i.e. hormones, prostaglandins,

cytokines and other mediators) that arrive via distinct pathways to converge on the hypothalamus (Chrousos & Gold 1992). The hypothalamus and the brainstem are important regions of the stress system. They include neurones of the paraventricular nuclei (PVN) of the hypothalamus and neurones of the paraventricular and parabrachial nuclei of the medulla as well as the locus coeruleus (LC) and other noradrenergic (NE) cell groups of the medulla and pons (Chrousos & Gold 1992). The peripheral limbs of the stress system include the pituitary and adrenal glands, together with the efferent sympathetic/adrenomedullary system, and components of the parasympathetic nervous system.

1.2 The Hypothalamo-pituitary adrenal axis

The HPA axis serves as the primary effector of the stress response, influencing virtually every cell in the body in response to a stressor. Following exposure to stress, cells of the PVN secrete CRH into the hypophyseal blood supply, which in turn act on the anterior pituitary gland to stimulate the synthesis of proopiomelanocortin (POMC) and the release of ACTH, a product of POMC, into the bloodstream. Arginine vasopressin (AVP) is also released from the PVN and acts synergistically with CRH to stimulate ACTH release, but not POMC synthesis (Gillies *et al.* 1982; Antoni 1993). ACTH primarily targets the adrenal glands to stimulate the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents). The HPA axis is self-regulated to prevent its over-activation, which could be detrimental to the individual. This is achieved through the actions of the released glucocorticoids, which feed back on to the pituitary gland, the hypothalamus and the higher centres of the brain (long feedback actions) which are activated by stress and send projections which converge onto the hypothalamus (see section 1.2.1), and also by suppression of the generation of mediators of the immune response (see section 1.3.1). Together, these actions down-regulate the HPA axis (figure 1.1) and thus restore homeostasis in the individual (Habib *et al.* 2001).

Basal HPA activity is influenced by the circadian timing system and therefore exhibits pronounced daily rhythmicity. Its activity is also regulated by the negative feedback actions of glucocorticoids, which is facilitated by the binding of glucocorticoids to glucocorticoid receptors (GRs; see section 1.3.2) in the hypothalamus, pituitary, and extra-hypothalamic regions (e.g. hippocampus). As a result, glucocorticoid levels show a robust diurnal rhythm that is in phase opposition with CRH under normal conditions (i.e. in humans peaking around the time of awakening and dropping to

their lowest levels in the evening whereas the opposite is true for CRH (Suemaru *et al.* 1995; Lightman *et al.* 2000; Wong *et al.* 2000).

1.2.1 Ultra short feedback loops in the regulation of CRH and ACTH release

In addition to the long feedback actions of glucocorticoids, short and ultrashort feedback loops also exist between the hypothalamus and the pituitary gland which serve to regulate CRH and ACTH release (figure 1.1). CRH is able to augment its own secretion during stress through an ultrashort positive feedback loop (Ono *et al.* 1985a; Ono *et al.* 1985b). The site of action for the stimulatory effect of CRH on its own release is not clear, but may involve cross-talk between the PVN and the locus coeruleus-noradrenergic (LC-NE) system as projections of CRH-secreting neurones from the lateral PVN to the central sympathetic systems in the brain stem stimulates the secretion of noradrenaline through its specific receptors, which in turn stimulates CRH secretion primarily through α 1-adrenergic receptors (Calogero *et al.* 1988b; Chrousos & Gold 1992; Chrousos 1995). Autoregulatory, ultrashort negative feedback loops are also present in these neurones, with CRH and noradrenaline collateral fibres acting in an inhibitory fashion on pre-synaptic CRH and α 2-adrenoreceptors, respectively. The CRH, AVP, and noradrenaline neurones receive stimulatory input from the serotonergic, cholinergic, and histaminergic systems and inhibitory input from the gamma-aminobutyric acid and opioid peptide neuronal systems of the brain.

Animal studies have also provided evidence that circulating ACTH acts to restrain its own secretion (Motta *et al.* 1965; Vernikos-Danellis & Trigg 1967). Whether this postulated ACTH autoregulation takes place directly at the pituitary level as an ultrashort negative feedback loop or is mediated via inhibition of CRH secretion from the hypothalamus, thus participating as a short negative feedback loop, remains uncertain. Direct self inhibition by pituitary ACTH has been suggested by *in vitro* studies using mouse pituitary tumour cells producing ACTH (Richardson 1978), and in clinical studies (Boscaro *et al.* 1988) and short-loop negative feedback effects of the short POMC derived peptides, ACTH, α -melanocyte stimulating hormone (α -MSH) and corticotrophin-like intermediate lobe peptide (CLIP) on CRH secretion has been demonstrated *in vitro* (Motta *et al.* 1965; Suda *et al.* 1986; Suda *et al.* 1987; Calogero *et al.* 1988b), although the source of POMC gene-derived peptides involved in this short negative feedback loop *in vivo* has not been fully elucidated. Pituitary ACTH, which is unable to cross the blood-brain barrier (BBB) (Allen *et al.* 1974) may

suppress hypothalamic CRH secretion by acting at the median eminence (ME), one of five brain structures not protected by the BBB (Moore 1986). However, the concentrations of POMC peptides in the hypophyseal portal blood of adult monkeys are not affected by hypophysectomy (Newman *et al.* 1984), suggesting that the most likely source of these peptides is the brain (Smith *et al.* 1982).

1.2.2 Adrenal glands

The adrenal glands are paired glands, situated close to the anterior part of the kidney. Each gland comprises a central medulla and a surrounding cortex, which have different functions and embryonic origins. The adrenal cortex comprises three layers, the zona glomerulosa, which secretes mineralocorticoids (aldosterone) (Gill 1979) the zona fasciculata, and the zona reticularis, which release glucocorticoids (cortisol or corticosterone) and androgens respectively (Gill 1979). The cortex contains low levels of stored glucocorticoids and efficiently synthesises hormone when required. The primary stimulus for the synthesis and release of glucocorticoids is ACTH (Gill 1979) which triggers the production of pregnenolone from cholesterol in the mitochondria (Simpson & Waterman 1988). Pregnenolone is then further converted to glucocorticoids by a series of distinct P450 enzymes (Simpson & Waterman 1988). ACTH binds to its specific receptor, the ACTH receptor (ACTH-R; also known as melanocortin receptor 2) which is a seven transmembrane domain receptor belonging to the G-protein coupled receptor (GPCR) super-family and is expressed in all three zones of the adrenal cortex (Mountjoy *et al.* 1992). The binding of ACTH to ACTH-R leads to the activation of adenylate cyclase and causes an increase in cyclic adenosine 3',5'-monophosphate (cAMP) levels and the activation of cAMP dependent protein kinases (Buckley & Ramachandran 1981; Beuschlein *et al.* 2001), subsequently resulting in the synthesis of glucocorticoids, the final product of the HPA axis.

1.2.2.1 Functions of glucocorticoids

Glucocorticoids exert powerful regulatory actions over a wide range of physiological functions. One of the first identified of these was the regulation of intermediary metabolism, through the up-regulation of gluconeogenesis in the liver, kidney and skeletal muscle and the elevation of blood glucose levels. Other roles of glucocorticoids include modulating fat, protein and glutamine metabolism and bone turnover. They are also essential for the regulation of several homeostatic mechanisms in the body, including the cardiovascular system, maintaining metabolic

homeostasis and modulating the host defence system. Glucocorticoids also have diverse effects on CNS functions including memory, cognition and attention and their control over the behaviour of the individual is aimed to limit homeostatic disturbance, an example of this being the synchronisation and co-ordination of circadian events such as food intake and sleep. The primary role of glucocorticoids in the physiological stress response is to prepare the body to respond to stress and facilitate the recovery of homeostasis after stress. This is achieved by altering several parameters including maintaining blood glucose levels, acting as powerful immunosuppressive/anti-inflammatory agents and restoring homeostasis. However, in situations where the levels of glucocorticoids are exacerbated, many components of the immune system, such as pro-inflammatory mediator expression and leukocyte migration, are inhibited (Vincent *et al.* 1977; Keller *et al.* 1981; Zhang *et al.* 1998; Elenkov & Chrousos 1999; Bauer *et al.* 2001) which can result in suppression of the immune system. The consequences of immuno-suppression include increased susceptibility and frequency of disease, prolonged healing times, and a greater incidence of secondary health complications associated with infection (Bailey *et al.* 2003).

1.2.3 Pituitary gland

The pituitary gland, which is highly vascularised, is comprised of two distinctive parts termed the posterior lobe (neurohypophysis) and the anterior lobe (adenohypophysis). In many mammals, there is also an intermediate lobe which lies between the anterior and posterior pituitary but is nearly indistinguishable from the anterior lobe. The pituitary gland is located immediately below the ME of the hypothalamus to which it is connected via the pituitary stalk. The hypophyseal portal vein system transports hormones secreted by the neurones terminating in the ME to the anterior pituitary gland. The pituitary stalk contains both efferent fibres from the hypothalamus which project to the cells of the posterior lobe and the portal vasculature connecting the ME and the anterior pituitary gland. The anterior pituitary is the largest and most important functional part of the pituitary gland. At least five distinct endocrine cell types exist in the adenohypophysis. These endocrine cells are corticotrophs, which produce ACTH and comprise of approximately 5% of the total cell population, gonadotrophs (10% of cell population) which release follicle-stimulating hormone (FSH) and lutenizing hormone (LH), lactotrophs (15-25%) which produce prolactin (PRL), somatotrophs (50%) which are growth hormone (GH) releasing cells and thyrotrophs (5%) which produce thyroid-stimulating hormone

(TSH). These subtypes can easily be identified by classical histological methods [reviewed in (Pelletier 1991)]. The remaining cell types include endothelial and folliculostellate cells (see section 1.7.2.1).

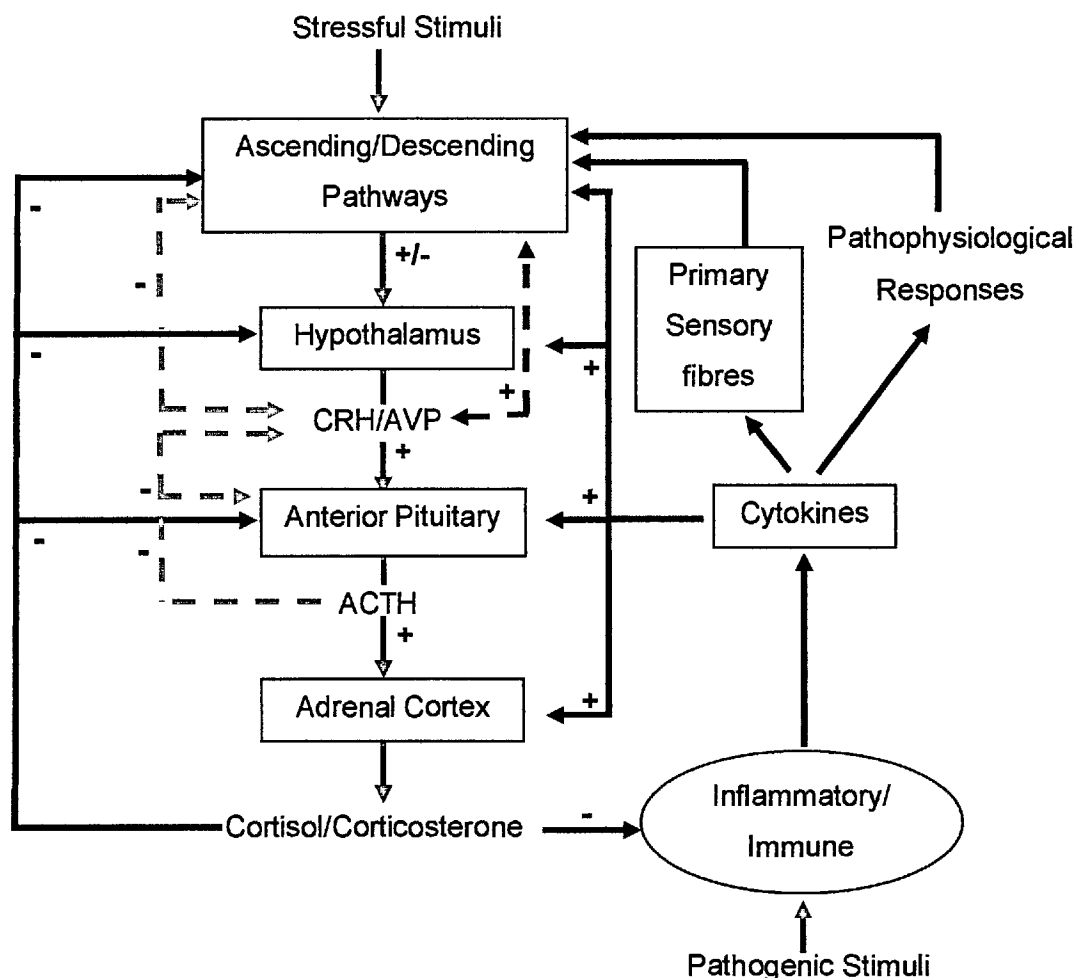


Figure 1.1 The auto-regulation of the HPA axis by glucocorticoid negative feedback following exposure to stress and its interactions with the immune system. The diagram depicts the major mediators involved in communication between the hypothalamus, anterior pituitary gland and adrenal cortex to regulate the stress response and the interactions between pro-inflammatory mediators released by immune/inflammatory cells and the HPA axis. Solid black lines represent interaction of the immune system with the HPA axis, solid red lines represent stimulatory pathways within the HPA axis and solid blue lines represent negative feedback effects of cortisol/corticosterone. Pink and blue dashed lines represent short and ultra-short feedback loops involved in the regulation of ACTH and CRH release. ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; CRH, corticotrophin releasing hormone; +, stimulatory effect; -, inhibitory effect.

Activation of the HPA axis results in the increased release of ACTH from the anterior pituitary gland. ACTH is initially synthesised as an inactive precursor, POMC, which is processed post-translationally by a family of subtilisin/kexin-like endoproteases generally known as proprotein convertases (SPCs), of which seven distinct proprotease convertase genes have been identified to date [reviewed by (Bergeron *et al.* 2000)]. In the pituitary, gene expression of SPC1 (Day *et al.* 1993), SPC2, SPC3 (Day *et al.* 1992), SPC4 and SPC6 (Dong *et al.* 1995) has been detected. POMC is cleaved by SPC2, which is abundantly expressed in the intermediate lobe of the pituitary gland (Benjannet *et al.* 1991; Bloomquist *et al.* 1991; Seidah *et al.* 1991), to produce β -endorphin and α -MSH or by SPC3, which is expressed in the anterior pituitary gland (Dong & Day 2002), to generate pro-ACTH and β -lipotrophin (Benjannet *et al.* 1991; Thomas *et al.* 1991). Pro-ACTH is then cleaved in two places to produce ACTH and N-terminal POMC (N-POMC), as well as a joining peptide between the two and β -lipocortin is further cleaved to produce β -endorphin and γ -lipotrophin. Several isoforms of N-POMC have been identified, of which the major circulating N-POMC peptide is pro- γ -MSH (human 1-76 POMC). The biological activity of each of these peptides is distinct as also are the biological responses they induce (Estivariz *et al.* 1980; Estivariz *et al.* 1982; Lu *et al.* 2002; Coll *et al.* 2006), and the concentrations of these circulating ACTH precursors can be as high as that of ACTH (White & Gibson 1998).

Removal of endogenous glucocorticoids by adrenalectomy results in an increase in SPC3 mRNA expression in corticotrophs, an effect which is attenuated with the administration of the synthetic glucocorticoid dexamethasone (Dong & Day 2002). These findings may present a mechanism by which glucocorticoids regulate POMC processing. Furthermore, studies in adrenalectomised rats revealed a time-dependent increase in the nuclear transportation of mature POMC mRNA to the cytoplasm, an effect which was inhibited by dexamethasone administration (Autelitano *et al.* 1989), suggesting glucocorticoids also regulate POMC gene expression at the post-transcriptional level.

1.2.3.1 Pituitary folliculostellate cells

The pituitary cell population includes a number of non-endocrine cell types such as resident macrophages, endothelial cells and folliculostellate cells. Of particular interest for the regulation of pituitary function are the folliculostellate cells which were previously designated either follicular cells or folliculostellate cells (Ishikawa *et al.*

1983; Girod *et al.* 1985). The first description of follicular cells was made by Farquhar in 1957, who identified a population of cells that form small follicles and are distributed throughout the anterior pituitary gland (Farquhar 1957 414). Due to their star-shaped morphology (Bergland & Torack 1969) and long cellular processes that project to neighbouring endocrine and endothelial cells, these cells were renamed folliculostellate cells (Ishikawa *et al.* 1983).

Folliculostellate cells comprise up to 10% of the anterior pituitary cell population (Matsumoto *et al.* 1993) and, since their discovery, efforts have been made to assign their origin and their function in the pituitary gland. The population of folliculostellate cells does not consist of a uniform cell-type, but comprises subpopulations of different immuno-phenotypes. To date, five sub-types of folliculostellate cells have been identified in the anterior pituitary, and they have been categorised by the different markers they express. A proportion of cells have been found to co-express S-100 and glial fibrillary acid protein (GFAP) (Velasco *et al.* 1982) both of which are markers of neuroectodermal origin. Another subset express both S-100 and keratin (Tachibana & Yamashima 1988) and the third group co-express vimentin and S-100 or GFAP (Nakajima *et al.* 1980; Velasco *et al.* 1982; Hofler *et al.* 1984). The fourth subset, described by Yamashita *et al.*, show expression of keratin and weak expression of S-100 (Yamashita *et al.* 2005). The final subset co-expresses S100 and MHC-class II determinants (an immune marker) and may thus be derived from a monocyte/dendritic cell/macrophage lineage (Allaerts *et al.* 1991; Allaerts *et al.* 1993; Allaerts *et al.* 1996). This subset has both IL-6 secretory and T-cell stimulatory capacities, further supporting their similarities to lymphoid dendritic cells (Allaerts *et al.* 1997). However, further studies are required to ascertain whether these subsets represent distinct cell types or populations at different stages of development and/or activation.

The origin of the folliculostellate cells has been the subject of debate since the discovery that they are immunoreactive for the S-100 protein (Cocchia & Miani 1980; Nakajima *et al.* 1980; Allaerts & Vankelecom 2005). As dendritic cells in lymphoid and non-lymphoid tissues also express S-100 (Zeid & Muller 1993), it has been suggested that S-100 positive folliculostellate cells are of myeloid origin (Allaerts *et al.* 1996; Hoek *et al.* 1997; Herkenham 2005). Others have proposed that the folliculostellate cells may be part of a renewal or stem cell system in the anterior pituitary gland (Horvath & Kovacs 2002; Inoue *et al.* 2002). While it has been suggested that stem cells are required for the replenishment of endocrine cells

(Carbajo-Perez & Watanabe 1990; Taniguchi *et al.* 2002) no stem cells have been identified in the pituitary gland to date. These observations have led to the suggestion that folliculostellate cells may differentiate into other pituitary cell types, although evidence for such a phenotypical change is still required.

In contrast to endocrine cells, folliculostellate cells are multifunctional, providing structural support within the anterior pituitary gland, and are involved in intra-pituitary cell-to-cell communication, modulating the response of endocrine cells to neuroendocrine and immunological stimuli through gap junction connections to these hormone-producing cells (Mira-Moser 1975; Morand *et al.* 1996). Folliculostellate cells do not secrete classical pituitary hormones but provide a source of growth factors and cytokines such as vascular endothelial growth factor (VEGF) (Ferrara & Henzel 1989; Gospodarowicz & Lau 1989), follistatin (Gospodarowicz & Lau 1989), basic fibroblast growth factor (bFGF) (Ferrara *et al.* 1987; Amano *et al.* 1993), NO (Ceccatelli *et al.* 1993), macrophage inhibitory factor (MIF) (Tierney *et al.* 2005), LIF (Ferrara *et al.* 1992) and IL-6 (Vankelecom *et al.* 1989; Renner *et al.* 1998). Folliculostellate cell derived IL-6 acts in an autocrine/paracrine manner to stimulate PRL, GH, LH and FSH release from primary pituitary cultures (Arzt *et al.* 1999; Gloddek *et al.* 2001). Studies have focused on elucidating the mechanism by which folliculostellate cells communicate with each other and endocrine cells over long distances and it has been proposed that this communication occurs through the electro-coupling of folliculostellate cells via calcium waves which are then propagated through gap junctions within the folliculostellate cell and endocrine cell network (Fauquier *et al.* 2001).

Functional studies examining folliculostellate cell subpopulations have been possible with the establishment of immortalised cell lines, in particular, the TtT/GF cell line, which was established from a murine thyrotropic pituitary tumour (Inoue *et al.* 1992). The TtT/GF cell line exhibits many characteristics assigned to folliculostellate cells including the production of IL-6 and the positive expression of S-100 and GFAP after repeated passages (Inoue *et al.* 1992). It is likely that the TtT/GF cell line represents a sub-population of folliculostellate cells of the anterior pituitary as approximately 30% of folliculostellate cells co-express S-100 and GFAP (Tachibana & Yamashima 1988).

1.2.4 Hypothalamus

The hypothalamus plays a vital role in the maintenance of homeostasis, forming extensive links with other brain areas to control bodily processes important for both autonomic and behavioural functions. These bodily functions include blood pressure, body temperature, fluid balance and metabolism and are maintained by the hypothalamus either through neural signals to the autonomic nervous system or via neuroendocrine signals to the pituitary gland. The hypothalamus is located below the thalamus on either side of the third ventricle and contains neurosecretory cells which are arranged in discrete nuclei. Some of these cells have projections which lead to a specialised region on the floor of the third ventricle, known as the ME, and it is from here that various hypothalamic factors [which include CRH, AVP, LH releasing hormone, FSH releasing factor, dopamine, γ -aminobutyric acid (GABA) and somatostatin] produced by the neurosecretory cells are stored and released into the hypophyseal-portal blood system via a distinct group of capillary loops (hypophyseal-portal blood vessels) that leave the brain and descend along the pituitary stalk to terminate at the pituitary (Pelletier 1991; Marubayashi *et al.* 1999; Kiss *et al.* 2006). The main hypothalamic nuclei involved in the neuroendocrine axis are the arcuate nucleus (ArcN), medial preoptic area (mPOA), periventricular nucleus (PeN), ventromedial nucleus (VMN) and the paraventricular nucleus (PVN) (figure 1.2). The PVN contains both magnocellular and parvocellular cells; the magnocellular PVN cells project to, and release hormones from, the posterior pituitary, whereas the parvocellular cells of the PVN release hormones which target the anterior pituitary in response to stressful stimuli (Antoni 1986; Aguilera 1994).

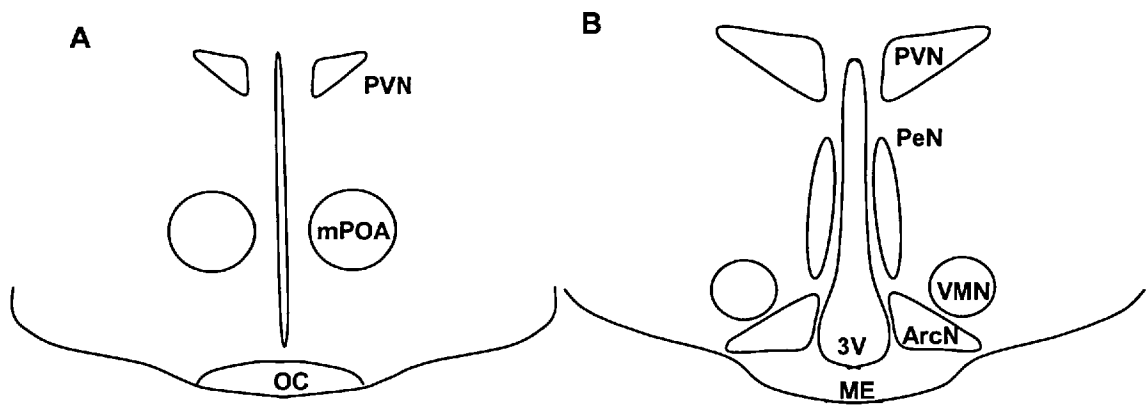


Figure 1.2 Diagrammatic representation of the locations of key hypothalamic nuclei from coronal sections of the rat hypothalamus. (A) Rostral section of the hypothalamus positioned at -1.4mm with respect to bregma and (B) caudal section of the hypothalamus positioned at -2.1mm with respect to bregma. 3V, third ventricle; ArcN, arcuate nucleus; ME, median eminence; mPOA, medial preoptic area; OC, optic chiasm; PeN, periventricular nucleus; PVN, paraventricular nucleus; VMN, ventromedial nucleus [taken from (Paxinos 1986)].

1.2.4.1 Arginine vasopressin

The synthesis and release of AVP by magnocellular neurones are stimulated in response to stressors such as haemorrhage, hypotension and hypoglycaemia (Baylis 1987; Laszlo *et al.* 1991) and the peptide released has an important role in osmoregulation and modulating blood volume and pressure via its anti-diuretic and vasoconstrictor actions. AVP was initially believed to be the principal factor involved in hypothalamic control of ACTH secretion (McCann & Brobeck 1954). However, following the discovery of CRH (Vale *et al.* 1981), which was found to stimulate the release of ACTH and β -endorphin *in vitro* (Vale *et al.* 1983) and *in vivo* (Rivier *et al.* 1982; Donald *et al.* 1983), AVP was shown to act primarily by potentiating the actions of CRH (Gillies *et al.* 1982). The regulatory effects of AVP occur through interactions of the peptide with specific plasma membrane receptors of which there are three subtypes; V_{1a} , V_2 and V_{1b} (also known as V_3). V_{1a} receptors are widely distributed on blood vessels and have been detected in the CNS (Ostrowski *et al.* 1994), and the V_2 subtype is predominantly expressed by cells of the renal collecting system (De Wied *et al.* 1991). The third subtype, V_{1b} (V_3), is predominantly expressed by pituitary corticotrophs (Jard *et al.* 1986; Rene *et al.* 2000) as well as in multiple brain regions and a number of peripheral tissues including kidney, heart, lung and breast (Lolait *et al.* 1995).

1.2.4.2 Corticotrophin releasing hormone

CRH is a 41 amino acid peptide found in both the brain and peripheral organs such as the adrenal gland (Thompson *et al.* 1987; Valentino *et al.* 1995) and ovaries (Mastorakos *et al.* 1993b; Mastorakos *et al.* 1994). In addition to its role in activating the HPA axis, CRH also participates in the inflammatory response as a locally expressed pro-inflammatory factor (Karalis *et al.* 1991; Van Tol *et al.* 1996; Webster *et al.* 1998). CRH exerts its actions via its receptors CRH-R1 and CRH-R2, which are expressed in the anterior pituitary, throughout the brain and in various peripheral organs such as the adrenal medulla, prostate, gut and spleen (Habib *et al.* 2001). In the pituitary however, CRH predominantly exerts its effects through CRH-R1 [reviewed by (Aguilera *et al.* 2004)]. The binding of CRH to CRH-R1 causes an increase in cAMP levels, a rapid release of pre-existing ACTH into the circulation and an increase in POMC mRNA levels (Kalin *et al.* 1983; Suda *et al.* 1988).

In addition to the stimulatory effect of CRH upon ACTH synthesis and release, CRH also acts through the CNS to mediate both behavioural and autonomic responses to stress (Fisher 1989; Cole & G.F. 1991; Davis 1992). The autonomic changes induced by CRH include increased blood pressure, heart rate, plasma catecholamine levels and glucose levels (Brown *et al.* 1982; Fisher *et al.* 1983; Brown & Fisher 1985; Kurosawa *et al.* 1986; Bakke *et al.* 1990; Diamant & De Wied 1991; Korte *et al.* 1993) and behavioural changes include changes in locomotor activity, rearing, grooming and feeding (Sutton *et al.* 1982; Koob *et al.* 1984; Brown & Hayden 1985; Sherman & Kalin 1987; Dunn & Berridge 1990; Cole & G.F. 1991; Diamant & De Wied 1991; Glowa & Gold 1991; Bray 1992; Korte *et al.* 1993; Wiersma *et al.* 1995). The structures involved in the effects of CRH upon such behavioural and autonomic changes include the LC, the PVN, the hippocampus, the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Lee & Davis 1997; Koob & Heinrichs 1999; Nijssen *et al.* 2001).

1.2.4.3 Implications of CRH and AVP in depression

AVP has only weak ACTH secretagogue activity alone (Lamberts *et al.* 1984) but acts synergistically with CRH to potentiate ACTH release in comparison with levels achieved following CRH administration alone (Gillies *et al.* 1982; Rivier & Vale 1983; Lamberts *et al.* 1984; Murakami *et al.* 1984; Antoni 1986). However, AVP does not affect POMC gene transcription, peptide synthesis or storage (Scott & Dinan 1998). Despite its weak stimulatory effects upon ACTH release during acute/novel stressor

exposure, recent work suggests that HPA axis dysregulation in chronic stress paradigms and depression may be associated with a shift towards increased vasopressinergic control of the axis (Kiss *et al.* 1984; Sawchenko *et al.* 1984; De Goeij *et al.* 1991; De Goeij *et al.* 1992a; De Goeij *et al.* 1992b; De Goeij *et al.* 1992c; Holsboer & Barden 1996; Dinan *et al.* 1999).

Recent studies utilising models of both chronic psychosocial and immobilisation stressors have shown alterations in AVP and CRH mRNA expression (Hauger *et al.* 1987; Whitnall *et al.* 1987), namely an increase in AVP synthesis in CRH-producing neurones of the PVN, and in AVP storage in the CRH-containing nerve terminals in the external zone of the ME (De Goeij *et al.* 1991; Bartanusz *et al.* 1993; Bartanusz *et al.* 1994). In contrast, CRH production was decreased, with an associated down-regulation of the CRH receptor and the upregulation of the V_{1b} receptor. Such findings suggest that HPA axis dysregulation in chronic stress paradigms and depression is associated with a shift from CRH to AVP controlled pituitary-adrenal activation (Kiss *et al.* 1984; Sawchenko *et al.* 1984; Hauger *et al.* 1987; Tilders 1989; De Goeij *et al.* 1991; De Goeij *et al.* 1992a; De Goeij *et al.* 1992b; De Goeij *et al.* 1992c; Holsboer & Barden 1996; Dinan *et al.* 1999).

1.2.5 Higher control of the HPA axis in response to cognitive and non-cognitive stress

The release of CRH and AVP from the hypothalamus is controlled by ascending pathways from the brainstem and descending pathways from the higher centres that converge onto the hypothalamus. These higher centres include limbic-associated structures which are also targeted by the negative feedback actions of glucocorticoids. The brain circuits that initiate and maintain the stress response are illustrated in figure 1.3. The main limbic structures include the hippocampus, the medial prefrontal cortex and the amygdala but these structures do not regulate the HPA axis in response to stressors which lack a cognitive component, such as immunological challenge (e.g. endotoxin exposure) or physical trauma (e.g. haemorrhage) (Chrousos 1988). Direct input from these limbic-associated structures influences the stress responsive CRH and/or AVP neurones of the PVN. The hippocampus exerts a tonic inhibitory effect on CRH and AVP mRNA expression as shown by rodent hippocampal lesion studies (Herman *et al.* 1989; Sapolsky *et al.* 1989; Herman *et al.* 1992; Herman *et al.* 1995b). The hippocampus is also involved in terminating the stress response, whereby hippocampal lesioning in the rodent

resulted in prolonged release of corticosterone and/or ACTH following exposure to a number of psychological stressors (Sapolsky *et al.* 1989; Herman *et al.* 1995b). This inhibition may be mediated via neurones in the ventral subiculum, which is the principal source of hippocampal efferents (Herman *et al.* 1995b).

The medial prefrontal cortex is also involved in modulating HPA axis activation, as bilateral lesions of the anterior cingulate or pre-limbic components of the medial prefrontal cortex resulted in an enhanced ACTH and corticosterone response to restraint stress (Diorio *et al.* 1993; Brake *et al.* 2000). Such findings support the premise that dorsal subregions of the medial prefrontal cortex selectively inhibit HPA axis responses to anticipatory stressors. However, unlike the hippocampus, dorsal and ventral medial prefrontal cortex lesions do not affect the morning or evening basal levels of ACTH or corticosterone, indicating the prefrontal cortex is involved in selective modulation of stress-induced HPA axis activity (Diorio *et al.* 1993; Figueiredo *et al.* 2003).

In contrast to the hippocampus and prefrontal cortex, the amygdala is involved in activating the HPA axis, with amygdala lesions resulting in a decrease in both ACTH and corticosterone plasma levels following restraint stress (Beaulieu *et al.* 1986). The amygdala has little direct contact with the PVN, which is limited to scattered fibres present in pre-autonomic cell groups (Gray *et al.* 1989; Marcilhac & Siaud 1997). Its actions occur via its rich connections to the brain stem structures, including the bed nucleus of the stria terminalis, which in turn innervate the PVN (Schwaber *et al.* 1982; Van Der Kooy *et al.* 1984).

Non-cognitive stressors, such as immune challenge, changes in blood pressure (hypotension and hypertension), pain, cold, heat or trauma also increase CRH/AVP secretion from the PVN by signalling through brain stem nuclei (Berkenbosch *et al.* 1987; Sapolsky *et al.* 1987; Uehara *et al.* 1987; Rivest & Rivier 1991). In response to such stressors, there is afferent input by sensory neurones to the brain stem that activates the LC, leading to CRH and probably AVP release (McCann *et al.* 2000a). Studies have provided evidence for the involvement of at least two brain stem nuclei, the nucleus tractus solitarius (NTS) and ventrolateral medulla (VLM), in signalling to the PVN through reciprocal connections following systemic immune challenge (Swanson & Kuypers 1980; Sawchenko & Swanson 1982; Schwanzel-Fukuda *et al.* 1984; Van Der Kooy *et al.* 1984; Luiten *et al.* 1985; Ericsson *et al.* 1994; Portillo *et al.* 1998; Shafon *et al.* 1998; Pyner & Coote 1999; Toth *et al.* 1999; Buller *et al.* 2001;

Hardy 2001; Buller *et al.* 2003). The VLM is important in the tonic and reflex control of arterial blood pressure (Pyner & Coote 1999), with bilateral lesions in the VLM reducing blood pressure (Guertzenstein & Silver 1974), whilst injection of excitatory amino acids increase blood pressure (Dampney *et al.* 1982; Dampney 1994). Regulation of blood pressure by the VLM involves signalling input from the NTS (Krukoff *et al.* 1995; Masubuchi *et al.* 2004) and it has been suggested that an imbalance of activity between the two nuclei can lead to hypertension (Johnson *et al.* 1996; Masubuchi *et al.* 2004). From these findings, it is likely that the activation of the HPA axis in response to hypertension involves reciprocal communication between the NTS, the VLM and the PVN (Guertzenstein & Silver 1974; Coote 1995; Pyner & Coote 1999). In conditions of hypotensive haemorrhage, Fos immunoreactive neurones have been visualized in autonomic regions of the brain stem, including the NTS, VLM, lateral parabrachial nucleus and LC (Chan & Sawchenko 1994), in the hypothalamic supraoptic nucleus, and in magnocellular and parvocellular neurones of the PVN (Badoer *et al.* 1993; Petrov *et al.* 1995), a region which is known to contain neurones which project to brainstem autonomic regions and spinal cord sympathetic preganglion neurones (Badoer *et al.* 1993; Blair *et al.* 1996). Furthermore, the ACTH response to blood loss is prevented by lesions in the NTS (Darlington *et al.* 1986) and the LC (Lefcort *et al.* 1984) suggesting that PVN CRH neurones receive cardiovascular afferent information conveyed from multiple brainstem sites (Chan & Sawchenko 1994; Van De Kar & Blair 1999).

These findings support the premise that the CNS responses to non-cognitive stress involve complex reciprocal connections between the PVN and the brainstem, whereby brainstem cell populations could essentially act as integratory sites for descending and ascending signals.

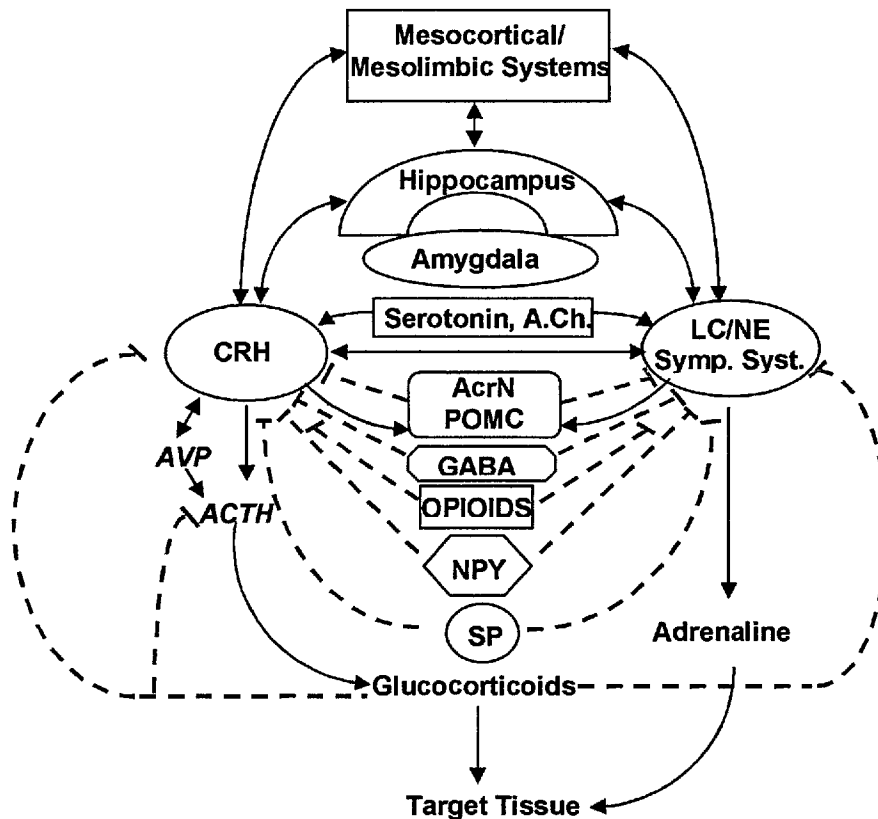


Figure 1.3 A simplified representation of the central and peripheral components of the stress system with their functional interactions to other central pathways involved in the stress response. A.Ch., acetylcholine; ACTH, adrenocorticotrophic hormone; ArcN, arcuate nucleus; AVP, arginine vasopressin; CRH, corticotrophic releasing hormone; GABA, γ -aminobutyric acid; LC/NE Symp. Syst., locus coeruleus/adrenaline-sympathetic system; NPY, neuropeptide Y; POMC, proopiomelanocortin; SP, substance P. Activation is represented by *solid blue lines*, and inhibition is represented by *dashed red lines* [Adapted from (Habib *et al.* 2001)].

1.3 Glucocorticoids

1.3.1 Negative feedback actions of glucocorticoids

Hypersecretion of glucocorticoids is prevented by a complex network of negative feedback systems through which the steroids act at multiple sites to suppress the release of ACTH and its hypothalamic releasing factors (figure 1.1). There are three phases of glucocorticoid feedback; (1) rapid, (2) early delayed and (3) late delayed feedback. These phases are the consequence of at least three separate molecular mechanisms of glucocorticoid action at feedback-sensitive sites. The fast phase of

glucocorticoid feedback occurs in the first few minutes of the stress response and does not involve protein synthesis and thus does not occur through genomic actions (Jones *et al.* 1972). This phase involves inhibition of the release of preformed ACTH and CRH/AVP stored in granules in the corticotrophs and in the terminals of CRH/AVP neurones respectively (Keller-Wood & Dallman 1984). These 'early' inhibitory effects play a crucial role in the regulation of basal plasma ACTH levels (Windle *et al.* 1997), and in determining the magnitude and duration of the stress response (Engeland *et al.* 1977).

The early delayed feedback phase emerges approximately thirty minutes after a rise in glucocorticoids and is usually maximal at approximately two hours. However, this phase can persist for up to twenty four hours (Jones & Stockham 1966; Hodges & Sadow 1967) and is believed to be dependent upon the actions of second messengers such as the glucocorticoid-inducible protein annexin 1 (ANXA1) which acts to inhibit the release, but not the synthesis of, ACTH and its hypothalamic releasing factors (Buckingham 1992). The late phase of feedback is apparent after several hours of exposure to glucocorticoids and during this phase, glucocorticoids inhibit the synthesis and release of both hypothalamic CRH/AVP and pituitary ACTH (Jingami *et al.* 1985; Herman *et al.* 1995a; Young *et al.* 1995; Keller-Wood & Dallman 1984). Hence early feedback occurs through a non-genomic mechanism and is primarily involved in the inhibition of pre-formed hormone, whilst a genomic mechanism is predominantly responsible for the inhibition of both pre-formed hormone release and its synthesis during the late delayed feedback phase.

1.3.2 Genomic effects of glucocorticoids

Glucocorticoids exert many of their effects through the GR, a member of the type I nuclear hormone receptor super-family, which, following hormone activation, acts as a transcription factor. The nuclear receptors contain five domains designated A to E. Domain C is the DNA binding site and contains 8 cysteine residues arranged in two groups of four. These groups facilitate the binding of two zinc atoms, which result in the formation of two zinc fingers that protrude from the molecule and interact with DNA. The C-terminus (domain E) contains a ligand binding site and also acts as the homodimerisation site. There are two forms of GR; GR α and GR β and they are both produced from the same gene by alternate splicing (Hollenberg *et al.* 1985). Expression of GR β is located in the liver, lungs and thymus and GR α is expressed in every tissue in the body and to a greater extent than GR β . The GR α variant of GR is

the predominantly biologically active form and in the absence of glucocorticoid hormone, GR α resides in the cytoplasm in an inactive state. Ligand binding facilitates translocation of the GR-complex to the nucleus, which can then interact with glucocorticoid response elements (GRE) within the DNA to activate appropriate glucocorticoid responsive genes. This results in increased transcription of genes coding for anti-inflammatory proteins, e.g. ANXA1, IL-10 and IL-1 receptor antagonist (IL-1ra), and the down-regulation of pro-inflammatory genes e.g. IL-8, IL-6 and TNF α (Nyhlen *et al.* 2000; Li *et al.* 2001), the enzymes responsible for their biosynthesis and regulation (e.g. nitric oxide synthase 2; NOS-2) (Barnes 1998), and for hypothalamic (e.g. AVP and CRH) and pituitary-derived (e.g. POMC) hormones. However, not all these pro-inflammatory cytokines contain GREs and the down regulation of these cytokines can occur through GR-mediated inhibition of pro-inflammatory transcription factors responsible for cytokine transcription. Such transcription factors include activating protein 1 (AP-1) (Karin & Chang 2001) and nuclear factor *kappa* B (NF κ B) (Scheinman *et al.* 1995b). In addition, glucocorticoids inhibit pro-inflammatory cytokine synthesis by altering the stability of mRNA and thus the translation rate of several glucocorticoid responsive proteins (Ing 2005). They also influence the secretion rates of specific proteins and alter the electrical potential of neuronal cells through unelucidated mechanisms (Ing 2005). Glucocorticoids are also able to mediate their effects through their binding to another member of the nuclear receptor family, the mineralocorticoid receptor (MR). Glucocorticoids display a ten-fold higher affinity for MR binding compared to GR binding and the mechanism of glucocorticoid-MR interaction is similar to that of glucocorticoid-GR binding (Marver *et al.* 1974; Arriza *et al.* 1987; Beato *et al.* 1995; Reul *et al.* 2000).

Access of glucocorticoids to their receptors is regulated by the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD), of which there are two isoforms - 11 β -HSD1 and 11 β -HSD2. 11 β -HSD catalyses the inter-conversion of hormonally active glucocorticoid (cortisol, corticosterone) and its inactivate form (cortisone, 11-dehydrocorticosterone) (Stewart & Krozowski 1999). In human tissues, 11 β -HSD1 converts cortisone to the active form cortisol and 11 β -HSD2 converts cortisol to the inactive form, cortisone. 11 β -HSD1 is widely distributed, but found most abundantly in the pituitary gland, the liver and in adipose tissue whereas 11 β -HSD2 is predominantly expressed in mineralocorticoid target tissues and the kidney and colon, where it serves to protect MR from excess binding of glucocorticoids (Korbonits *et al.* 2001; Quinkler & Stewart 2003). MR can also bind to GREs leading

to speculation over the extent of functional specificity of glucocorticoid and mineralocorticoid action (Beato *et al.* 1995). Unlike GR, MR is expressed in a specific pattern, located in the distal colon, kidney, hippocampus and CNS (Fuller & Verity 1990; Funder 1996; Zennaro *et al.* 1997; Watzka *et al.* 2000).

1.3.3 Non-genomic effects of glucocorticoids

The actions of glucocorticoids do not solely occur through intra-cellular receptor-mediated alterations in gene transcription. There are a multitude of rapid, glucocorticoid-mediated effects that cannot be explained via a genomic mechanism. With respect to the feedback actions of glucocorticoids within the HPA axis, non-genomic actions have been associated principally with the initial rapid phase of feedback (Jones *et al.* 1972). The speed of action of glucocorticoids during this phase is less than ten minutes, which is too early to account for a genomic action. Inhibition of DNA and protein synthesis utilizing the DNA-dependent RNA synthesis inhibitor, actinomycin D and the protein synthesis inhibitor, cyclohexamide had no effect upon this initial feedback response *in vivo* (Liu *et al.* 1995). The non-genomic actions of glucocorticoids are likely to involve specific membrane receptors, yet to be identified, which in turn modulate the protein kinase C (PKC) and the protein kinase A (PKA) signalling pathways that result in a reduction in intracellular Ca^{2+} concentration in neural-glial cells and pituitary cells respectively (Chen & Qiu 1999).

Di *et al.* recently identified a non-genomic mechanism of rapid glucocorticoid feedback inhibition at the level of hypothalamic neurones. This mechanism involves glucocorticoid-activated release of a retrograde endocannabinoid messenger from CRH neurones via the activation of a postsynaptic G-protein coupled membrane receptor. These cannabinoids were able to decrease the excitatory synaptic drive to parvocellular neuroendocrine cells of the PVN (including CRH expressing cells), by acting locally on pre-synaptic type I cannabinoid receptors expressed on afferent glutamergic axon terminals (Di *et al.* 2003).

Glucocorticoids also exert rapid inhibitory effects on neurohypophysial oxytocin and AVP secretion from hypothalamic magnocellular neurones of the supraoptic nucleus and PVN via a similar mechanism to the parvocellular cells (Di *et al.* 2005). These findings provide an appropriate mechanism for the rapid phase feedback actions of glucocorticoids on the activity of CRH neurones and similar mechanisms may occur elsewhere in the brain, such as the hindbrain, midbrain and limbic sites, which have

been implicated as sites of action for the rapid effects of glucocorticoids. Endogenous cannabinoids also exert robust effects on hormone secretion from the pituitary, leading to the suppression of pituitary hormone release (e.g. TSH, gonadotropin, GH and PRL) (Hillard *et al.* 1984; Murphy *et al.* 1998; Wenger & Moldrich 2002). In addition to the ability of cannabinoids to inhibit pituitary-derived hormone release via the hypothalamus, cannabinoids may also exert their inhibitory effects directly at the level of the pituitary. Conversely, exogenous cannabinoids are able to stimulate the HPA axis (Puder *et al.* 1982; Weidenfeld *et al.* 1994; Brown & Dobs 2002; Patel *et al.* 2004). A possible explanation for the opposing effects of endogenous versus exogenous cannabinoids in the PVN is that the stimulatory effects of exocannabinoids occur upstream from the hypothalamus and are relayed synaptically to the CRH neurones in the PVN (Tasker 2004). However, the receptor(s) and second messengers(s) involved in this mechanism still need to be identified.

At the level of the pituitary, glucocorticoids have been shown to up-regulate the expression of the second messenger ANXA1 (Buckingham & Flower 1997), which has been shown to inhibit both CRH and ACTH release both *in vitro* and *in vivo* (Loxley *et al.* 1993; Taylor *et al.* 1993; Sudlow *et al.* 1996).

1.3.3.1 Annexin 1

ANXA1 is a 37kDa protein of the annexin family of phospholipid and Ca²⁺ binding proteins and contributes to the anti-inflammatory effects of glucocorticoids (Flower & Rothwell 1994). Within the HPA axis, ANXA1 expression is confined to discrete cell types. In the hypothalamus, ANXA1 is localised in the ependymal lining of the third ventricle and in microglia (Woods *et al.* 1990; Smith *et al.* 1993; McKanna & Zhang 1997) and inhibits the release of hypothalamic CRH (Loxley *et al.* 1993; Sudlow *et al.* 1996; Pompeo *et al.* 1997). In the anterior pituitary gland, ANXA1 is expressed mainly in the non-endocrine folliculostellate cells, particularly in projections that lie in close proximity with endocrine cells (Traverso *et al.* 1999; Chapman *et al.* 2002), and suppresses the release of ACTH and other pituitary hormones (Taylor *et al.* 1993). ANXA1 also acts as a mediator of the anti-inflammatory effects of glucocorticoids both peripherally and locally at sites of inflammation (Taylor *et al.* 1993; Green *et al.* 1998). The inhibitory actions of glucocorticoid-induced ANXA1 upon ACTH release is involved in the non-genomic actions of glucocorticoids (Taylor *et al.* 1993; Buckingham *et al.* 2003). Studies conducted by our laboratory have shown that the

steroid-induced exportation of ANXA1, from folliculostellate cells, occurs via a non-classical GR-dependent mechanism that requires mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and Ca²⁺-dependent PKC pathways (John & Buckingham 2003; Solito *et al.* 2003). However, the externalisation of ANXA1 does not occur by the classical regulated secretory pathway (Comera & Russo-Marie 1995; Philip *et al.* 1998) as ANXA1 lacks a cleavable signal sequence at its N-terminal (Wallner *et al.* 1986). A likely mechanism for ANXA1 externalisation in folliculostellate cells has recently been shown to involve a member of the ATP-binding cassette (ABC) transporter family of proteins A, ABCA1 (Chapman *et al.* 2003; Omer *et al.* 2006).

1.4 Stressors activating the HPA axis

The pathways involved in the activation of the HPA axis depend largely on the nature of the stressor. Some of the most potent activators of the HPA axis and the autonomic nervous system are psychological in nature, with consequent increments in plasma glucocorticoids (Keim & Sigg 1976) and catecholamines (Kvetnansky *et al.* 1971). Intrinsic to psychological stress is a cognitive assessment of the stimulus, and common forms of psychological stress include loss of control of an individual's environment, threats to self-esteem and perceived confinement. A well-established model of mild psychological stress is the restraint technique (Pare & Glavin 1986; Glavin *et al.* 1994). This model is widely used for the elucidation of drug effects, where the effects of various agents can be examined on restraint stress-induced pathology and the HPA axis.

As outlined earlier (see section 1.2.5.2), the HPA axis is also sensitive to non-cognitive stimuli such as hypotension, hypoglycaemia and immunological challenge (e.g. endotoxin exposure). In the case of immune insults, the primary driving force for glucocorticoid release is pro-inflammatory mediators released by activated immune and inflammatory cells (e.g. eicosanoids and cytokines). These mediators are capable of acting directly at all levels of the HPA axis, or indirectly, by acting at the site of inflammation to stimulate nociceptive afferents which in turn stimulates the HPA axis via central pathways (see figure 1.1).

1.4.1 Psychological stress

Emotions are processed in the limbic system and frontal cortex which is composed of nuclei including the sensory thalamic nuclei, the basolateral and central amygdala,

the nucleus accumbens, the LC, the hippocampus and associated cortical areas such as the medial prefrontal cortex (Glavin *et al.* 1983; Chrousos & Gold 1992). Projections from these higher centres converge on the hypothalamus, both directly and indirectly [e.g. PVN CRH neurones receive input from the central amygdala both directly and through the bed nucleus of the stria terminalis (Gray *et al.* 1989; Cullinan *et al.* 1993; Gray *et al.* 1993)] to stimulate the release of CRH, and are activated in response to restraint stress (Sheng & Greenberg 1990; Schreiber *et al.* 1991; Arnold *et al.* 1992; Kononen *et al.* 1992; Chowdhury *et al.* 2000).

Exposure to chronic restraint has been associated with a variety of central and peripheral changes including hypothermia (Murakami *et al.* 1985), atrophy of the thymus and spleen and adrenal hypertrophy (Seyle 1936). Chronic stress increases both AVP mRNA expression in the PVN, to a greater degree than CRH mRNA, and POMC mRNA expression in the anterior pituitary gland (Harbuz & Lightman 1989; Bartanusz *et al.* 1993), which is associated with elevated plasma levels of ACTH and corticosterone (Pare & Glavin 1986).

Restraint stress is also associated with increased plasma levels of the pro-inflammatory cytokines IL-1 and IL-6 (Zhou *et al.* 1993; Merlot *et al.* 2002), although this effect appears to be dependent on the severity and length of the stressor (Li *et al.* 2000). Interestingly, non-immune induced elevation of these cytokines occurs independently of endotoxaemia, tissue damage or inflammation (Lemay *et al.* 1990; Takaki *et al.* 1994; Besedovsky & Del Rey 1996). Studies have shown that psychological stress induces gut permeability, resulting in the release of intestinal endotoxins, which may stimulate the production of cytokines (Hart & Kamm 2002). Similarly, restraint stress causes an increase in the production of glucocorticoids, which, in turn, adversely affects cutaneous permeability (Denda *et al.* 2000; Kao *et al.* 2003).

1.4.2 Immunological stress

Endotoxin is also a potent activator of the HPA axis (Van Amersfoort *et al.* 2003). In animals, lipopolysaccharide (LPS), a component of the outer wall of gram negative bacteria, stimulates the release of ACTH and corticosterone (Butler *et al.* 1989; Feuerstein *et al.* 1990; Ramachandra *et al.* 1992; Schotanus *et al.* 1994; Tilders *et al.* 1994). In addition, many cytokines produced during an immune/inflammatory response e.g. IL-6, interleukin 1 *beta* (IL-1 β) and TNF α are also capable of provoking

widespread pathological effects, which are perceived as stressful and can therefore stimulate the HPA axis (Buckingham *et al.* 1996). Activation of the HPA axis by inflammatory mediators is not exclusive to cytokines, but is also induced by other products of the immune system including platelet activating factor, epidermal growth factor and prostaglandin E₂ (PGE₂). PGE₂ is synthesised by the inducible enzyme cyclooxygenase 2 (COX-2; which converts arachidonic acid into prostaglandins) (Ribot *et al.* 2003), an enzyme which is activated by nitric oxide (NO) (Mohn *et al.* 2005; Mollace *et al.* 2005) and these products all exhibit strong CRH releasing properties (Calogero *et al.* 1988a; McCoy *et al.* 1994; Rivier 1998; Uribe *et al.* 1999; Bugajski *et al.* 2003; Ribot *et al.* 2003; Gadek-Michalska & Bugajski 2004; Cragnolini *et al.* 2006). The importance of prostaglandins in activating the HPA axis following LPS treatment was shown by Gadek-Michalska *et al.*, whereby inhibition of COX-2 caused a significant reduction in LPS-induced ACTH and corticosterone levels (Gadek-Michalska & Bugajski 2004). However, in contrast to the stimulatory effects of phospholipase A2 (PLA2; a key enzyme involved in the release of arachidonic acid from the cell membrane) and its metabolites arachidonic acid and PGE₂ on ACTH release (Cowell *et al.* 1991; Brooks 1992), incubation of primary pituitary cultures with prostaglandin D₂ (PGD₂) and prostaglandin F₂ *alpha* (PGF₂ α) attenuated CRH-stimulated, but not tonic, ACTH release, indicating differential roles of PLA2 and eicosanoids in the regulation of ACTH release (Vlaskovska *et al.* 1984; Cowell *et al.* 1991).

1.4.3 Cytokine activation of the HPA axis

The most notable immune-derived mediators which activate the HPA axis are pro-inflammatory cytokines (e.g. TNF α , IL-1 β and IL-6). Cytokines are large polypeptides which do not cross the BBB readily. Therefore, the mechanisms through which inflammatory cytokines access the central AVP and CRH secretory neurones are not clear. However, several structures, such as the axon terminals of the PVN neurones that project into the ME and the posterior pituitary gland, are exposed to circulating cytokines in the blood stream. Furthermore, the circumventricular organs (CVOs) also lack a BBB and are exposed to the chemical environment of the systemic circulation (Krisch *et al.* 1978), in particular the choroid plexus, the vascular organ of the lamina terminalis and the subfornical organ (Harre *et al.* 2002). The expression of immune cytokines has also been detected in these structures. For example, an increase in IL-1 β and IL-6 mRNA has been observed in response to LPS stimulation, suggesting that cytokines may contribute to HPA axis activation through pathways

originating in the CVOs (Grinevich *et al.* 2001). Cytokines are also synthesised in the brain and it is possible that during stress, brain derived cytokines may augment peripheral cytokine expression (De Simoni *et al.* 1990). In addition, the pituitary and adrenal glands are also capable of producing IL-6 (Vankelecom *et al.* 1989; Spangelo *et al.* 1990a; Spangelo *et al.* 1990c; Tatsuno *et al.* 1991; Judd 1998) and ACTH has been shown to stimulate the release of IL-6 from cultured zona glomerulosa cells (Judd & Macleod 1992).

In addition, cytokines are capable of initiating a cascade of paracrine and autocrine events which results in the sequential secretion of pro-inflammatory mediators by non-fenestrated endothelial cells, glial cells and cytokine responsive neurones which are able to act on AVP and CRH secreting neurones. Alternatively, LPS may act directly on the endothelium of the blood brain vessels, which express the LPS signalling receptor, toll-like receptor 4 (Tlr4) and the co-receptor, CD14, to trigger a series of signalling events leading to the development of an inflammatory response in the brain (Singh & Jiang 2004). The pro-inflammatory cytokines produced in response to LPS signalling also up-regulate CD14 and Tlr4 expression and may act in a paracrine manner to activate adjacent cells within the brain. Indeed, Rivest *et al.* showed that intracerebroventricular TNF α injection cause a robust and rapid production of CD14 mRNA in the rat brain (Rivest *et al.* 2000).

Several studies have provided evidence that peripherally produced cytokines, including IL-1 β and IL-6, may cross the BBB at sites of increased permeability in response to bacterial infection, to stimulate AVP/CRH secreting neurones (Banks *et al.* 1994; Gutierrez *et al.* 1994; Boje 1995; De Vries *et al.* 1996; Banks *et al.* 2001). However, other laboratories have found LPS does not alter the permeability of the BBB (Bickel *et al.* 1998), or has only a modest effect to allow permeability to smaller molecular weight compounds (Singh & Jiang 2004). Singh *et al.* demonstrated peripherally administrated LPS does not cross the BBB, but accumulates in brain endothelial cells which express receptors for LPS signalling (Singh & Jiang 2004). The binding of LPS to CD14 may trigger a series of signalling events leading to the release of eicosanoids and pro-inflammatory mediators, including NO and IL-1 β (Lacroix *et al.* 1998). In particular, NO production has been demonstrated in the PVN following LPS challenge (Uribe *et al.* 1999) and NO is a likely candidate for modulating ACTH release, both at the hypothalamic and pituitary level.

Cytokines are also able to indirectly activate the HPA axis through the stimulation of central noradrenergic pathways, or by acting at the sites of inflammation to stimulate peripheral nociceptive, somatosensory and visceral afferent fibres leading to activation of the LC/NE and PVN CRH neuronal systems via ascending spinal pathways [reviewed in (Chrousos 1995)]. However, not all signals from the immune system are stimulatory to the stress system; for example, transforming growth factor β (TGF β) exerts negative effects on HPA axis activity (Raber *et al.* 1997).

Immune cytokines are also expressed in the hippocampus, the hypothalamus and the pituitary gland (Ray & Melmed 1997; Vitkovic *et al.* 2000) and increased expression of these cytokines in response to insult influences the release of hormones from endocrine tissue. For example, IL-1 receptor (IL-1R) expression has been detected in the murine AtT20 pituitary corticotroph-like cell line (Bristulf *et al.* 1991) and is up-regulated by CRH treatment, as well as by other cAMP inducers including forskolin and isoprenaline (Ray & Melmed 1997).

1.4.4 Effects of nitric oxide on the HPA axis

NO is synthesised from L-arginine by a family of enzymes called NO synthases (NOS). The biochemical pathways leading to NO formation involves three distinct NOS isoenzymes: neuronal NOS (NOS-1), inducible NOS (NOS-2) and endothelial NOS (NOS-3) (Knowles & Moncada 1994; Nathan & Xie 1994; Dawson & Dawson 1996). NOS-1 and NOS-3, which are constitutively expressed in neurones, endothelial cells and astrocytes, produce NO in a rapid and Ca^{2+} -dependent manner (Lamas *et al.* 1992; Knowles & Moncada 1994; Nelson *et al.* 1997). NOS-2, which was first described in cells of the immune system (Xie *et al.* 1992), is a Ca^{2+} -independent isoform and is expressed in the brain by cell types including glial cells and microvascular endothelial cells (Nelson *et al.* 1997).

The expression of NOS has been localised to the anterior pituitary (Ceccatelli *et al.* 1993) and hypothalamic regions controlling hypophyseal secretions (Bredt *et al.* 1990; Siaud *et al.* 1994; Uribe *et al.* 1999) and in the immediate proximity of neuroendocrine nerve terminals to the external zone of the ME (Prevot *et al.* 2000). Studies have shown that LPS induced NOS activity increases the expression of CRH and AVP mRNA expression in the PVN and the release of ACTH, an effect which is reversed by NOS inhibition (Lee *et al.* 1999; Uribe *et al.* 1999; Akasaka *et al.* 2006). LPS also increases PVN NOS-2 expression, but this effect was only evident several

hours after the peak in ACTH secretion, implying that this isoform is not involved in the acute ACTH response, but may play a role in sustaining ACTH levels (Wong *et al.* 1996b). The stimulatory effects of NO upon hypothalamic peptide synthesis and release may represent a mechanism by which peripheral LPS stimulates ACTH release, without directly crossing the BBB (Van Dam *et al.* 1998; Uribe *et al.* 1999; Rivest *et al.* 2000)

Conversely, NO localised to corticotrophs (Ohta *et al.* 1993) and folliculostellate cells (Ceccatelli *et al.* 1993), has been shown to inhibit ACTH release (Vankelecom *et al.* 1992; McCann *et al.* 2000b), as well as the release of other pituitary hormones, including PRL, GH and LH (Vankelecom *et al.* 1990; Kato 1992; Ceccatelli *et al.* 1993). The inhibitory role of NO in the pituitary is further supported by studies which have shown that IFN γ increases folliculostellate derived NOS-2 expression in both primary pituitary monolayer and aggregate cultures, resulting in the attenuation of ACTH release (Vankelecom *et al.* 1990; Vankelecom *et al.* 1997). The opposing effect of NO in the pituitary, compared to at the hypothalamic level, may reflect a pathway by which folliculostellate cells regulate ACTH secretion in response to immunological stimulation.

1.5 Innate immunity and inflammation

1.5.1 The inflammatory response

In response to viral or bacterial infection or injury, a cascade of events is initiated by the host, termed the inflammatory response. This response can lead to inflammation at the local site of injection/injury as well as systemic inflammation, fever, leukocytosis and activation of the HPA axis.

The inflammatory response is a protective response and functions as part of a normal host surveillance mechanism to destroy or quarantine both harmful agents and damaged tissue. Following insult, components of the immune system are recruited to initiate the production of pro-inflammatory mediators, including cytokines synthesised by activated macrophages. The immediate and sequential release of these mediators causes vasodilatation, increased blood flow and raises vascular permeability (Laroux 2004; Rankin 2004). This results in an increase in leukocyte trafficking to the inflammatory site due to the induction of endothelial and leukocyte cell adhesion molecule expression and chemoattractant production (Carlos & Harlan

1994; Springer 1994). During this stage of the response, there is a predominance of polymorphonuclear leukocytes at the inflamed site, which like monocytes and tissue macrophages, are phagocytic cells that engulf and eliminate foreign micro-organisms (Robbins S. 1989).

Following the removal of the infectious agent, the inflammatory response progresses to the resolution ('healing') phase, which involves restoration of tissue function and repair of any damage caused. Removal of the causative agent leads to the clearing of inflammatory cells by apoptosis and lymphatic drainage (Savill 2001) and anti-inflammatory mediators involved in the repair process are produced until the insult is resolved (Spector & Willoughby 1968).

1.5.2 Glucocorticoids as anti-inflammatory agents

Glucocorticoids are powerful anti-inflammatory agents, which act to inhibit pro-inflammatory cell recruitment, down-regulate the production of both pro-inflammatory cytokines and prostanoids, and up-regulate the production of anti-inflammatory mediators (Van Der Burg & Van Der Saag 1996; Chivers *et al.* 2004; Schramm & Thorlacius 2004; Yeager *et al.* 2004). An important mechanism by which glucocorticoids exert their anti-inflammatory effects is through the inhibition of NF κ B and AP-1. In the nucleus, activated GRs modulate transcriptional events by directly associating with DNA elements compatible with the GREs. The binding of GR to its response element allows inhibition of the activity of pro-inflammatory transcription factors (Almawi & Melemedjian 2002). Glucocorticoids also mediate gene transcription through the up-regulation of ANXA1 (Flower & Rothwell 1994). ANXA1 inhibits the expression and activity of pro-inflammatory enzymes such as NOS-2 in macrophages (Wu *et al.* 1995) and COX-2 in microglia (Minghetti *et al.* 1999), and is also involved in the inhibition of neutrophil and macrophage migration at local sites of inflammation (Walther *et al.* 2000; Oliani *et al.* 2001).

1.5.3 The LPS signalling pathway

Bacterial lipopolysaccharides, the major outer surface membrane components present in almost all gram-negative bacteria, act as potent stimulators of innate or natural immunity. LPS consist of a poly- or oligosaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A, which is the primary immuno-stimulatory centre of LPS. With respect to immuno-activation in mammals, the classical group of strongly agonistic forms of LPS has

been shown to be comprised of a similar set of lipid A types [reviewed by (Seydel *et al.* 2003)]. These agonistic forms of LPS trigger numerous physiological immunostimulatory effects in mammalian organisms, but exposure to higher doses can also lead to pathological reactions such as the induction of septic shock.

The LPS signalling pathway involves the recognition of LPS by the host, which is initiated by binding of LPS and its binding protein (LBP) to either the membrane-bound or the soluble form of the co-receptor CD14 (Schumann *et al.* 1990; Wright *et al.* 1990; Hailman *et al.* 1994). Extracellular toll-like receptor 4 (Tlr4) acts as a heterodimer with the accessory protein myeloid differentiation 2 (MD-2), which goes on to interact with the LPS/LBP/CD14 complex [reviewed in (Beutler 2000) and (Anderson 2000)]. When engaged by LPS, the complex transduces a signal detected by the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), which is then recruited and facilitates the association of interleukin-1-receptor-associated kinase 4 (IRAK4) (Muzio *et al.* 1998; Kawai *et al.* 1999). The binding of MyD88 to IRAK4 leads to IRAK4-mediated phosphorylation of IRAK1 and the binding of tumour necrosis factor receptor-associated factor 6 (TRAF6) to this complex. The IRAK1-TRAF6 complex disengages from the receptor and interacts at the plasma membrane with another preformed complex consisting of TAK1 (a member of the mitogen-activated protein kinase kinase kinase family; MAPKKK) and the TAK1 binding proteins, TAB1 (activator protein) and TAB2 (adaptor protein) or TAB3 (He *et al.* 2003). The formation of this complex results in the phosphorylation of TAB2/3 and TAK1 which translocate to the cytoplasm where TAK1 is activated. This leads to the activation of the inhibitor of *kappa* B ($\text{I}\kappa\text{B}$) kinases (IKKs) and the phosphorylation and degradation of $\text{I}\kappa\text{B}$ for subsequent release of $\text{NF}\kappa\text{B}$ (Akira & Takeda 2004) (figure 1.4).

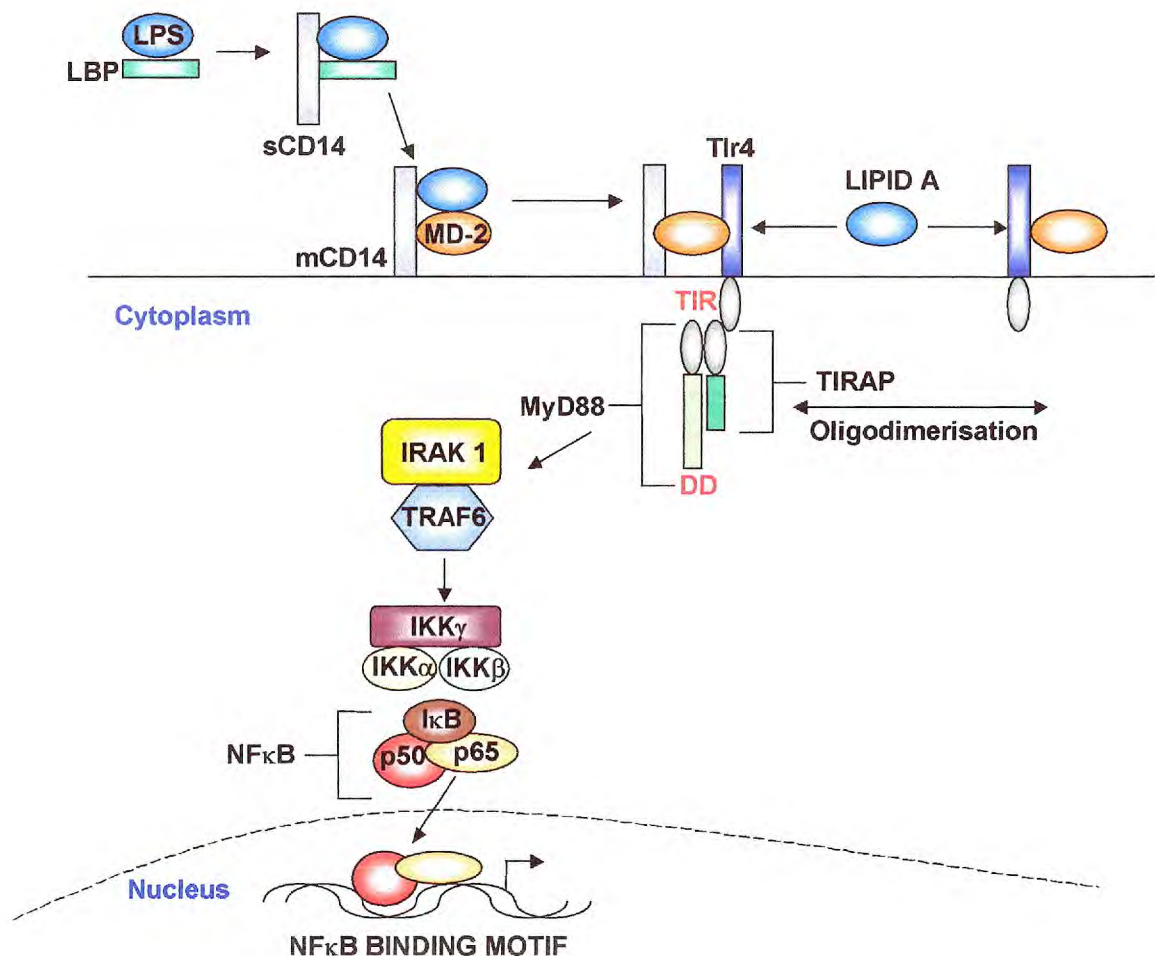


Figure 1.4 The LPS signalling pathway. CD14, cluster of differentiation factor 14; DD, death domain; IκB, inhibitor of *kappa* B; IKK, IκB kinase; IRAK1, interleukin-1-receptor-associated kinase 1; LPS, lipopolysaccharide; LBP, LPS-binding protein; mCD14, membrane bound CD14; MD-2, myeloid differentiation 2; MyD88, myeloid differentiation primary response protein 88; NFκB, nuclear factor *kappa* B; sCD14, soluble CD14; TIR, toll/interleukin-1 receptor; TIRAP, TIR-domain-containing adaptor protein; Tlr4, Toll-like receptor 4; TNF-receptor-associated factor 6; TRAF6.

1.5.4 The Toll-like receptor family

The innate immune system includes a family of trans-membrane receptors that mediate the recognition of conserved microbial structures to activate the host immune response, known as the toll-like receptor (Tlr) family. Signalling through the Tlr family was originally identified in *Drosophilla* as part of the embryonic dorso-ventral patterning system (Belvin & Anderson 1996) and subsequently found to

mediate the innate immune response against fungal infection (Lemaitre *et al.* 1996). Homologues of Toll have been found and eleven members of the Tlr family have been identified in mammals to date. The Toll receptor structure is similar to that of type I transmembrane receptors, with an ectodomain containing leucine-rich repeats and a conserved intracellular region and the Toll/interleukin-1 receptor (TIR) domain that is required for downstream signal transduction (Slack *et al.* 2000).

The various members of the Tlr family are activated by different ligands leading to the activation of NF κ B and the release of pro-inflammatory mediators (Akira & Takeda 2004). The Tlr4 receptor is required for mediating a pro-inflammatory response to the bacterial endotoxin LPS, a potent activator of host cells such as monocytes, macrophages and neutrophils (Chow *et al.* 1999; Shimazu *et al.* 1999). In addition to LPS signalling through Tlr4, gram-positive bacteria (which do not contain LPS, but carry surface teichoic acids, lipoteichoic acids and peptidoglycan instead) also trigger the release of pro-inflammatory cytokines, although subsequent signalling occurs through Tlr2 rather than Tlr4 (Akira 2000). The Tlr2 receptor is also expressed in both mouse and human adrenal glands under basal conditions and is involved in mediating the adrenal stress response (Bornstein *et al.* 2004b). The absence of Tlr2 has been associated with an enlargement of the adrenal gland and reduction of corticosterone levels and impairment of the HPA axis (Bornstein *et al.* 2004b).

1.6 Nuclear factor *kappa* B

1.6.1 NF κ B signalling

NF κ B is a ubiquitous dimeric transcription factor comprising members of the Rel protein family. To date, the Rel family members identified include the mammalian proteins p65 (also known as Rel A), Rel B, c-Rel, p50/p105 and p100/p52. The p50/p52 subunits are proteolytic cleavage products of precursor proteins that possess both a Rel region and an I κ B-like region. Rel proteins possess a highly conserved region of approximately 300 amino acids known as the Rel homology domain. Contained within this domain are DNA recognition/binding sites involved in the dimerisation and nuclear localisation functions of NF κ B. These Rel proteins have the ability to homodimerise or heterodimerise with other Rel proteins to form DNA binding-competent NF κ B factors with different sequence specificities. Although not all combinations of NF κ B dimers have been shown to form or to have relevant physiological actions, several transcriptionally active NF κ B dimers have been

identified, including Rel B/p52, p65/p50, p65/p65 and p50/p50. The composition of NF κ B dimers determines their DNA sequence specificity by combining different DNA recognition loops in the Rel homology domains of different subunits, for example p65 homodimers preferentially bind DNA enhancer motifs that are not well recognised by p65/50 heterodimers or p50/p50 homodimers. The variety of dimerisation may contribute to the cell type specificity of the NF κ B response. The prototypical and most extensively studied NF κ B dimer is the p65/p50 heterodimer and is considered the “classical NF κ B” (Karin 1999).

In resting cells, NF κ B is found in an inactive form in the cytoplasm bound to an inhibitor protein of the I κ B family (Baeuerle & Baltimore 1988), which conceals the nuclear localisation sequence and thus prevents NF κ B from translocating to the nucleus. The most extensively studied protein in this family is I κ B α . Pro-inflammatory stimuli activate the inhibitor of *kappa* B kinase (IKK) complex (IKKC), which contains two catalytic subunits (IKK- α and IKK- β) and one regulatory subunit (IKK- γ). Activation of IKKC signals the ubiquitination, phosphorylation and degradation of the I κ B α protein in the proteasome, exposing the nuclear translocation signal of NF κ B. This causes the release of the active NF κ B dimer, allowing its translocation to the nucleus. In the nucleus, NF κ B binds with a consensus sequence (5'-GGGACTTCC-3') in the promoter region of various genes and activates their transcription (Karin & Delhase 2000). The phosphorylation activity of IKKs are greater when I κ B α is bound to NF κ B, explaining how free I κ B α can accumulate in cells in which IKK is still active, thus contributing to the termination of NF κ B activation (Zandi *et al.* 1998).

The classical pro-inflammatory pathway involves I κ B α complexed to p65/p50 NF κ B and activation of the IKKC, in particular IKK β , allowing ‘free’ NF κ B to transcribe genes encoding proteins including the cytokines IL-8 and IL-6, intercellular adhesion molecule 1 and the inducible enzymes NOS-2 and COX-2. A key component of the resolution phase of the inflammatory response is the inhibition of NF κ B activity, and hence a down-regulation of inflammatory mediators. This action is an important target for glucocorticoids in their anti-inflammatory role.

1.6.2 Immunological stress activation of NF κ B

NF κ B was identified more than a decade ago by Sen and Baltimore as an enhancer-binding protein controlling immunoglobulin *kappa* light chain (Ig κ) gene expression in

B cells. Despite being initially perceived as a tissue-restricted transcription factor, NF κ B was later found to be expressed in other immune cells as an inactive protein sequestered in the cytoplasm and to be activated in response to pro-inflammatory stimuli such as LPS or TNF α . Its role is of particular importance to immune cells, where it has been shown to regulate a wide variety of genes involved in mammalian immune and inflammatory responses [reviewed in (Liou 2002)]. NF κ B is characteristically activated by cellular factors including oxidative stress, cytokines and growth factors, which induce nuclear translocation of NF κ B in endothelial and smooth muscle cells, monocytes and macrophages, and the renal epithelium (Hofmann *et al.* 1999; Valen *et al.* 2001; Wardle 2001). Upon nuclear translocation, NF κ B is able to up-regulate the expression of target genes including adhesion molecules and pro-inflammatory cytokines. These cytokines are also able to further stimulate NF κ B activation (Zhou *et al.* 1993; Shintani *et al.* 1995; Merlot *et al.* 2002).

NF κ B also controls the gene expression of all known anti-apoptotic genes and is therefore important in promoting cell survival as well as modulating inflammatory responses (Collins & Cybulsky 2001). In addition to their roles in immune and inflammatory responses, NF κ B family members are also crucial regulators of cell development, proliferation and differentiation (Guttridge *et al.* 1999; Weih & Caamano 2003), apoptosis (Grimm *et al.* 1996; Chen *et al.* 2000) and oncogenesis (Cavin *et al.* 2004). Along with its previously described function in neurological disorders and apoptotic processes within the brain, NF κ B also has a confirmed role in normal physiological function of the CNS at both cellular and behavioural levels, for example, studies have shown that the inhibition of NF κ B results in an impairment of long term memory (Merlo *et al.* 2002; Meffert & Baltimore 2005). NF κ B signalling in the brain is clearly very important but more research is required to fully understand its multi-functional roles. For example, little work has been done in elucidating the signal transduction mechanisms leading to NF κ B activation in neuronal or glial cells and it is important to investigate the potential roles of the NF κ B family members in the development of the CNS.

1.6.3 Psychological stress-induced activation of NF κ B

The activation of NF κ B was primarily believed to occur in response to immunological stress. However, psychological stressors have also been associated with an increase in cellular activation and cytokine release. Although no clear mechanism has yet

been elucidated to account for the interaction between psychological stress and immune activation, NF κ B is a likely candidate.

Studies have found that psychosocial stress induces the activation of NF κ B in peripheral blood mononuclear cells (PBMCs) through the interaction of noradrenaline with α_1 - and β -adrenergic receptors, which results in activation of the MAPK pathway and subsequent NF κ B nuclear translocation (Bierhaus *et al.* 2003). Removal of the stressor resulted in the expression of nuclear NF κ B protein returning to basal levels, although the time frame for restoration of basal levels varied between the subjects, implying that the individual's perception of stressful situations modulates the extent and duration of NF κ B activation (Bierhaus *et al.* 2003). Restraint stress is also associated with NF κ B activation, whereby nuclear translocation of NF κ B is detected in the cerebral cortex after four hours of stressor exposure (Garcia-Bueno *et al.* 2005). NF κ B is an important regulator of NOS-2 and COX-2, which are both involved in the production of reactive oxygen species (ROS), and their expression has been detected in the brain following restraint stress (Olivenza *et al.* 2000; Madrigal *et al.* 2002; Garcia-Bueno *et al.* 2005). ROS are potent stimulators of NF κ B and indirect activation of NF κ B by restraint induced NOS-2 and COX-2 production may present a feedback loop for increasing NF κ B activation and pro-inflammatory cytokine release.

The stimulation of receptors for the neurotransmitter glutamate also leads to NF κ B activation in neurones (Guerrini *et al.* 1995; Kaltschmidt *et al.* 1995; Madrigal *et al.* 2002), and glutamate released following restraint stress may also trigger nuclear translocation of NF κ B.

Although it is clear that NF κ B is activated in response to psychological stressors, such as restraint stress, further studies are required to ascertain the importance of NF κ B signalling in modulating the HPA axis response to restraint stress.

1.7 Interactions between the CNS and the immune system

The immune system and the CNS system mount a variety of essential, coordinated responses to danger and they communicate with each other through three types of systems. In the first, the CNS system acts reciprocally with the immune system, in the second, the CNS drives immunity through both neuroendocrine and neuronal pathways and in the third, the immune system regulates the CNS through neuronal

and humoral routes, via immune mediators, cytokines and their respective receptors. Thus, these two systems, which are involved in both sensing danger and mounting a counterattack to these threats, are inextricably linked.

1.7.1 Cytokine activation of the HPA axis

Immune cytokines are a large and diverse group of pleiotropic and redundant polypeptides. They are rapidly induced in response to tissue injury, infection and inflammation. In addition to acting locally at sites of injury/infection, cytokines also act in the brain to regulate the HPA axis, through several mechanisms as outlined in section 1.3.3. The most extensively studied cytokine in regard to regulation of the HPA axis is IL-6, particularly in respect to hormone secretion from the pituitary (Bethin *et al.* 2000). IL-6 is a member of the IL-6-family of cytokines, comprising of IL-11, leukaemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor and cardiotrophin 1. Members of this family all share a common signal-transducing mechanism and thus elicit similar, overlapping physiological responses. IL-6 signalling involves association of IL-6 with its membrane-bound receptor, IL-6R α (the *alpha* subunit of the functional receptor), which induces the association of IL-6R α with gp130 (Arzt 2001). The IL-6R α may also occur in a soluble form through proteolytic cleavage of the membrane form or as a result of alternative mRNA splicing, resulting in a transcript encoding only the extracellular domain portion and a modified C-terminal sequence. These modified forms of IL-6R α retain the same binding affinity for IL-6 and the ability to complex with the membrane-bound gp130 receptor (Montero-Julian 2001). The soluble form of gp130 acts as an antagonist for the IL-6/IL-6R α complex as it is able to bind to the complex, but it is unable to fulfil its role as initiator of the downstream signalling pathway (Montero-Julian 2001). The binding of IL-6/IL-6R α to gp130 leads to the rapid homodimerisation of gp130. This homodimerisation is necessary for signal transduction through the IL-6R α complex and also for other related complexes involving the other receptor α subunits such as for LIF. Signalling with LIF is similar to that of IL-6, but involves binding of LIF to its LIF receptor α subunit (LIF-R α), which results in induction of the heterodimer LIFR/gp130 (Jones *et al.* 1994). Signalling through gp130 is mediated by tyrosine kinases of the Janus-activated kinase (JAK) family and transcription factors of the signal transducers and activator of transcription (STAT) family.

Activation of the JAK/STAT pathway is initiated following homo/heterodimerisation of gp130. Thereafter, JAKs (JAK1, JAK2 and Tyk2) are activated and in turn,

phosphorylate gp130 at several residues, providing docking sites for SH2 domain containing molecules such as the STAT1 or STAT3 members of the STAT family. Upon phosphorylation, the STATs are translocated as dimers to the nucleus and bind to promoter regions of their specific response genes (Arzt 2001). In order to prevent excessive hyperactivation of the HPA axis in terms of ACTH release, a tightly regulated mechanism is in place in pituitary corticotrophs, in addition to the negative feedback actions of circulating glucocorticoids. This mechanism involves the inhibitory actions of suppressor of cytokine signalling (SOC). SOC-3 is an early response gene which is induced in response to a variety of cytokines including IL-6 and gp130 in the pituitary (Starr *et al.* 1997; Auernhammer *et al.* 1999). SOC-3 inhibits corticotroph JAK/STAT signalling, rendering the cell resistant to further stimulation by gp130 cytokines and thus inhibits cytokine-mediated POMC synthesis and ACTH release (Starr *et al.* 1997; Auernhammer *et al.* 2000; Nicholson *et al.* 2000).

1.7.2 Interleukin 6

Originally, IL-6 was identified as a factor which induces immunoglobulin synthesis in B cells, promotes B-cell hybridoma and plasmacytoma growth, stimulates acute-phase protein synthesis in hepatocytes and plays a role in haematopoiesis and cytotoxic T-cell differentiation (Van Snick 1990). Progressively, the biological role of IL-6 has expanded to its involvement in inflammation (Hirano 1992), viral infection (Akira & Kishimoto 1992), autoimmunity (Kishimoto 1992) and development (Lee 1992). IL-6 is upregulated in response to a variety of stimuli such as restraint stress (Zhou *et al.* 1993; Takaki *et al.* 1994; Li *et al.* 2000; Merlot *et al.* 2002), proinflammatory cytokines (Ammit *et al.* 2002), prostaglandins (Denizot *et al.* 1998) and LPS (Spangelo *et al.* 1990b; Schobitz *et al.* 1993), and is inhibited by anti-inflammatory agents including IL-10 (De Waal Malefyt *et al.* 1991) and glucocorticoids (Ray *et al.* 1990; Carmeliet *et al.* 1991).

Research into the role of IL-6 in modulating the HPA axis has developed substantially over the last few years. Receptors for IL-6 have been detected at all levels of the HPA axis, particularly in the pituitary and adrenal glands (Path *et al.* 1997; Vallieres & Rivest 1997; Kurotani *et al.* 2001) and IL-6 has been shown to stimulate the release of CRH (Navarra *et al.* 1991; Vallieres & Rivest 1999) and promote glucocorticoid release both *in vitro* and *in vivo* (Tominaga *et al.* 1991; Mastorakos *et al.* 1993a; Franchimont *et al.* 2000). It is during the later phase of endotoxaemia that IL-6

appears to be required, to prolong the activation of neural cells throughout the brain and maintain CRH expression in the PVN neurones (Vallieres & Rivest 1999). This is consistent with studies that have examined the temporal profile of cytokine release and HPA axis responsiveness in LPS stimulated rodents, wherein both PVN and plasma levels of IL-6 increased after maximal levels of ACTH and corticosterone had been reached (Givalois *et al.* 1994; Kakizaki *et al.* 1999). IL-6 also stimulates the release of ACTH through a CRH-independent mechanism, to promote glucocorticoid synthesis and release (Naitoh *et al.* 1988; Fukata *et al.* 1989; Mastorakos *et al.* 1993a; Bethin *et al.* 2000). The later increase in IL-6 is likely to contribute to the continued release of ACTH after LPS exposure, thus maintaining glucocorticoid levels. This is further supported by studies which demonstrated IL-6 deficient mice did not have an altered response to LPS after one hour exposure, but levels of circulating glucocorticoid were significantly lower in the knockout animals compared to their wild type counterparts thereafter (Fattori *et al.* 1994; Turnbull *et al.* 2003).

IL-6 is also synthesised in the brain following endotoxin exposure and is likely to act as a long-term regulator of plasma ACTH and glucocorticoid levels by acting directly at the level of the anterior pituitary gland (Muramami *et al.* 1993). The primary source of IL-6 has been located to the folliculostellate cells of the pituitary (Vankelecom *et al.* 1989; Vankelecom *et al.* 1993), a site which is sensitive to the negative feedback actions of glucocorticoids (Carmeliet *et al.* 1991; Grosset *et al.* 1999; Lohrer *et al.* 2000). IL-6, synthesised and released by folliculostellate cells, is able to act in an autocrine and paracrine manner to further stimulate its production and to promote the release of ACTH from corticotrophs, which express the IL-6R/gp130 receptors and are up-regulated in response to LPS (Bethin *et al.* 2000; Kurotani *et al.* 2001; Gautron *et al.* 2003). Regulation of IL-6 gene expression is controlled by the binding of several transcription factors to known consensus sequences within the promoter region. These include *cis*-acting binding elements for GR, AP-1, cAMP response element binding protein (CREB), CCAAT enhancer-binding protein- β (C/EBP- β), nuclear factor IL-6 (NF-IL6) and NF κ B (Ray *et al.* 1988; Tanabe *et al.* 1988; Akira *et al.* 1990; Libermann & Baltimore 1990). Binding of IL-6 to its receptor and activation of the JAK/STAT pathway leads to translocation of the activated STAT-3 complex to the nucleus and binding to its response element on the POMC promoter, with subsequent transcription of POMC and, ultimately, ACTH release (Bousquet *et al.* 1997; Bousquet *et al.* 2000).

1.7.3 Modulation of NFκB signalling in the pituitary gland by cytokines

Utilising macrophage, monocytic, osteoblastic, fibrosarcoma and uterine cell lines, stimulators such as LPS, IL-1 and TNFα have been shown to critically require intact NF-IL6 and NFκB binding sites within the IL-6 promoter in order to stimulate IL-6 release (Matsusaka *et al.* 1993; Galien *et al.* 1996; Deshpande *et al.* 1997; Ray *et al.* 1997; Vanden Berghe *et al.* 1998). Concurrent with these findings, Lohrer *et al.* found that LPS directly stimulated NFκB activation, which was associated with an increase in IL-6 release from TtT/GF cells and primary murine folliculostellate cell cultures and involved the p38α (p38α) MAPK pathway (Lohrer *et al.* 2000). However, the involvement of NFκB activation in pituitary-derived IL-6 release appears to be dependent on the stimulus as demonstrated by Nagashima *et al.* whereby TtT/GF cells exposed to pituitary adenylate cyclase-activating peptide (PACAP) released IL-6 independently of NF-IL6 and NFκB, and required AP-1 and CREB (Nagashima *et al.* 2003).

Recent studies support the premise that LPS stimulated IL-6, and possibly LIF, requires an intact NFκB signalling pathway (figure 1.5). Such evidence includes the demonstration of NFκB DNA binding activity in murine pituitary tissue in response to LPS stimulation (Parnet *et al.* 2003) and the subsequent release of IL-6 which was inhibited with a specific p38 MAPK inhibitor (Lohrer *et al.* 2000). The activation of NFκB in the pituitary and the subsequent production of pro-inflammatory mediators may present an additional pathway by which ACTH synthesis and release occurs.

1.7.4 Glucocorticoid and NFκB interactions

The yeast two-hybrid system has allowed investigators to study the interactions between transcription factors and has led to the identification of a variety of enhancer binding transcription factors that interact with a wide range of nuclear proteins. These nuclear proteins act as transcriptional co-factors that repress or enhance the activity of transcription factors. The interaction between GR and NFκB provides an example of this, both having opposite functions in the regulation of immune and inflammatory responses. The activity of NFκB is involved in up-regulating the transcription of pro-inflammatory cytokines involved in immunity and inflammation, whereas GR inhibits the expression of many of these cytokines. The absence of GREs on these NFκB inducible genes initially made it difficult to ascertain the mechanism through which

glucocorticoids repressed these genes. It is now understood that these transcription factors can physically interact with one another to function as mutual transcriptional antagonists. One mechanism by which glucocorticoids inhibit NF κ B has been shown to be through the direct interaction of GR with the p65 subunit of NF κ B, thus preventing p65 from binding to its target gene sequences (Ray & Prefontaine 1994; Scheinman *et al.* 1995b; De Bosscher *et al.* 1997). Additionally, glucocorticoid mediated inhibition of NF κ B can also occur indirectly, by inducing the expression of I κ B α protein, which inactivates the transcriptional activity of NF κ B by sequestering it in the cytoplasm (Auphan *et al.* 1995, Scheinman, 1995 #113). These genomic mechanisms of NF κ B inhibition may contribute to the early delayed and late delayed phases of glucocorticoid feedback but not to the rapid phase of feedback, which occurs through non-genomic pathways. The anti-inflammatory ANXA1 protein is constitutively expressed in the hypothalamus (Loxley *et al.* 1993; Taylor *et al.* 1993; Buckingham & Flower 1997) and the pituitary gland (Traverso *et al.* 1999) and is induced by glucocorticoids. The induction of this protein may present a non-genomic glucocorticoid pathway through which NF κ B activity is inhibited (Solito *et al.* 2003; John *et al.* 2004).

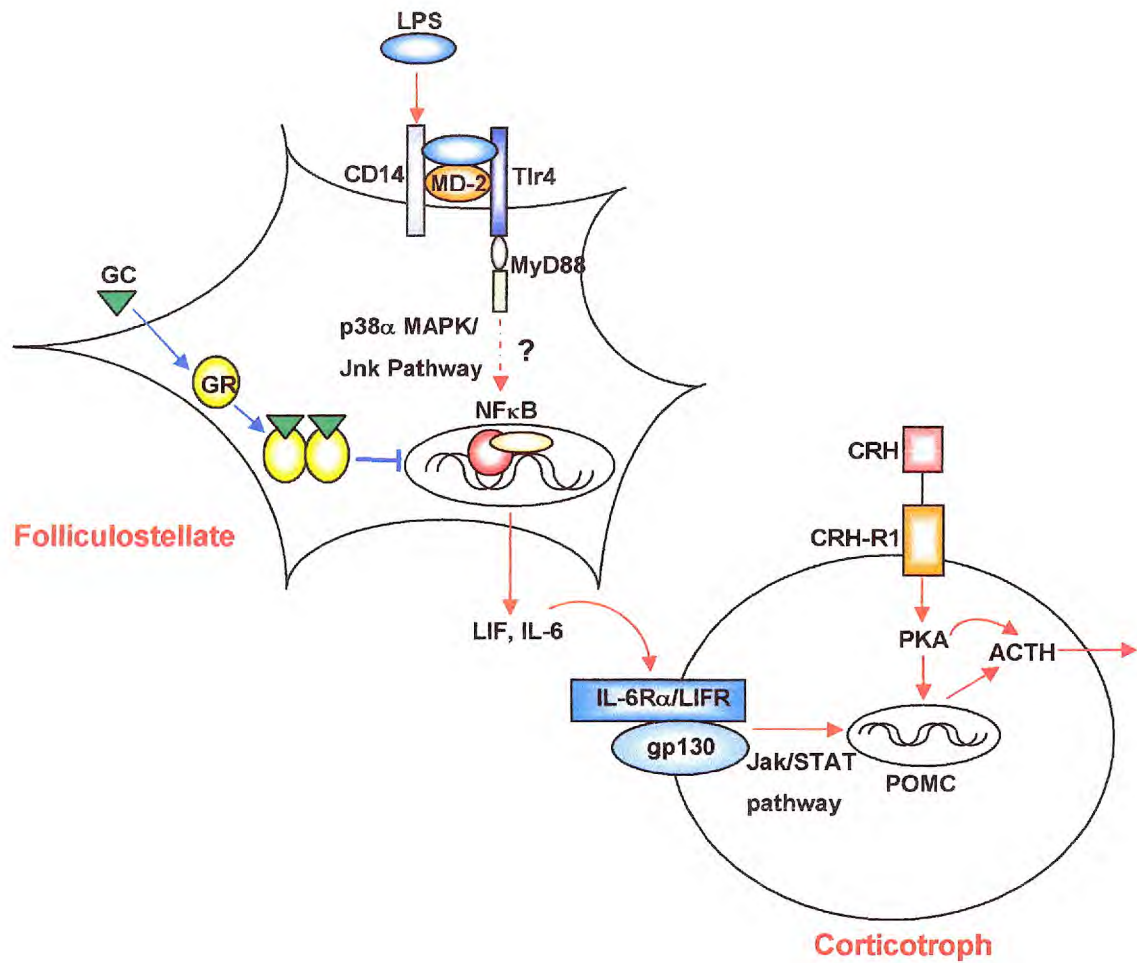


Figure 1.5 Paracrine communications in the anterior pituitary gland. Diagrammatic representation illustrating the proposed mechanisms by which LPS acts on folliculostellate cells in the anterior pituitary gland to trigger the cytokine-dependent synthesis and release of ACTH. Red arrows indicate pathways leading to increased ACTH synthesis and release; blue lines indicate glucocorticoid-dependent inhibition of NFκB activation and the subsequent synthesis and release of ACTH. ACTH, adrenocorticotrophic hormone; CD14, cluster of differentiation 14; CRH, corticotrophin releasing hormone; CRH-R1, corticotrophin-releasing hormone receptor 1; GC, glucocorticoid; GR, glucocorticoid receptor; IL-6, interleukin 6; IL-6Rα, interleukin-6 receptor *alpha*; Jak/STAT, janus activated kinase/signal transducer and activator of transcription; Jnk, Janus N kinase; LIF, leukaemia inhibitory factor; LIFR, leukaemia inhibitory factor receptor; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MD-2, myeloid differentiation factor 2; MyD88, myeloid differentiation primary response protein 88; NFκB, nuclear factor *kappa* B; PKA, protein kinase A; POMC, proopiomelanocortin; Tlr4, Toll-like receptor 4.

1.8 Hypothesis

The activation of the HPA axis during inflammation is an important protective mechanism for the host as the neuroendocrine stress response, and resultant induction of glucocorticoids, suppresses the immune reaction to prevent its over-activation, and assists in progression to the resolution phase. The pituitary derived pro-inflammatory cytokine IL-6 has been shown to sustain activation of the HPA axis in response to stressors such as bacterial infection, and understanding both the importance of intra-pituitary-derived IL-6 in mediating ACTH, and the pathways involved in its synthesis, are of great interest for the treatment of inflammatory disorders and impaired glucocorticoid function.

As part of the classical neuroendocrine response to stress, we hypothesise that the production of ACTH and subsequent glucocorticoid release is dependent, in part, on an NF κ B signalling pathway, involving pituitary folliculostellate cells. The transcriptional activity of NF κ B is likely to be of particular importance in mediating the HPA axis response to immune insults and possibly psychological stress. Signalling through NF κ B is therefore a potentially significant component of a co-ordinated multi-factorial process, which facilitates an optimal neuroendocrine response to immune/inflammatory insults and other stressors.

NF κ B signalling in the folliculostellate cells of the pituitary following stress stimulation is responsible for mediating the synthesis and release of IL-6, which in turn stimulates the release of ACTH. We propose that pituitary NF κ B signalling may be sensitive to the actions of glucocorticoids, and that it is through the inhibition of NF κ B that glucocorticoids mediate part of their negative feedback effects upon the HPA axis.

NF κ B is an important target for the treatment of inflammatory diseases such as rheumatoid arthritis. Yet little is known about the importance of NF κ B signalling in the HPA axis, and hence the possible side effects that may arise following its inhibition. Therefore, the aim of this thesis is to investigate the role of NF κ B in the activation of the HPA axis following both physiological (bacterial invasion) and psychological (restraint) stress and whether compounds inhibiting NF κ B activity will impair HPA axis function.

1.9 Aims and objectives

Whilst previous studies support the premise that bacterial endotoxin (LPS) can act directly at the anterior pituitary level to induce ACTH release, little is understood about the mechanisms involved in this response. Evidence suggests that the stimulatory effects of LPS occur through the release of pro-inflammatory cytokines (e.g. IL-6) from anterior pituitary folliculostellate cells which in turn act in a paracrine manner on neighbouring corticotrophs to stimulate the synthesis and release of ACTH. NF κ B is an important modulator of IL-6 gene expression in immune cells (e.g. macrophages) and it is likely that this transcription factor is involved in LPS-stimulated IL-6 release from folliculostellate cells. Although normal corticotrophs express LPS signalling receptors, to our knowledge, no effect of LPS upon ACTH release from corticotrophs has been reported and it is unclear whether LPS-induced NF κ B activity in corticotrophs affects CRH-stimulated ACTH secretion. Furthermore, whilst CRH is a potent stimulator of ACTH release, this peptide does not affect the release of folliculostellate cell-derived IL-6.

Thus, the first aim of this thesis is to investigate the mechanisms involved in LPS and CRH-induced ACTH release utilising transformed cell lines of folliculostellate cells and corticotrophs. The effects of LPS and CRH stimulation upon IL-6 (folliculostellate cells) and ACTH release (corticotrophs) will be examined and the effects of inhibiting NF κ B on the release of these factors will be investigated. The second aim of the thesis is to investigate whether the responses of these cell-types are altered when they are co-cultured together. These studies will provide information as to whether endotoxin-induced ACTH release is dependent upon interactions between folliculostellate cells and corticotrophs. The third aim is to extend the *in vitro* findings to *in vivo* models to examine whether HPA responses to immunological and psychological stress are affected by NF κ B inhibition. The *in vivo* studies will use rats treated with LPS as the immunological stressor and rats subjected to restraint stress as the psychological stressor. The HPA responses will be assessed by measuring plasma ACTH and corticosterone release, and NF κ B activity in the anterior pituitary gland.

2 Methods

2.1 Animals

All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 under project licence PPL 70/5593.

2.1.1 Rats

Male Sprague-Dawley rats were obtained from a commercial supplier (Harlan Olac, Oxfordshire, UK) and maintained in standard, wire-top cages in the Comparative Biology Unit at Hammersmith Hospital (Faculty of Medicine, Imperial College London), in conditions of controlled lighting (lights were on between 08.00h and 20.00h), temperature (21°C-23°C) and humidity (60-65%) with standard rat chow and water provided *ad libitum*. On arrival, the rats were acclimatised to their new environment for 48-72 hours and they were housed in groups of five per cage. The experiments were always started between 09.00h and 11.00h to minimise complications associated with the circadian rhythm. For one week prior to the stress experiments, rats were gently handled between 09:00 and 12:00 daily.

2.2 Cell Lines

2.2.1 TtT/GF cell line

The TtT/GF cell line (a gift from Dr C McArdle, University of Bristol, UK) was used as a model of murine folliculo-stellate cells of the anterior pituitary. The cell line was established from a murine pituitary thyrotrophic tumour and possesses characteristics similar to those of folliculostellate cells, i.e. the presence of many lysosomes and numerous intermediate filaments in the cytoplasm, phagocytic activity, follicle formation, and stains positively for GFAP and S-100 protein (Inoue *et al.* 1992).

2.2.2 PDFS cell line

The PDFS cell line (a gift from Dr A Klibansky and Dr D Danila, Harvard Medical School, Massachusetts, USA) was used as a model of human folliculostellate cells of the anterior pituitary. This cell line spontaneously developed from a clinically non-functioning pituitary macroadenoma and possesses characteristics similar to those of folliculo-stellate cells, i.e. the cells exhibit an epithelial morphology with long

cytoplasmic projections and frequent intercellular junctions including desmosomes. These cells express vimentin, S-100 protein, follistatin and activin A, all of which are characteristic of folliculo-stellate cells (Danila *et al.* 2000).

2.2.3 AtT20 (D1 and D16:16) cell lines

The AtT20 cell line was used as a model of corticotroph cells due to its ability to secrete ACTH. These cells also synthesise and secrete the ACTH/ β -lipotrophin family of peptides, including β -endorphin, which are derived from POMC. The D1 subclone (a gift from Dr S Guild, University of St Andrews, Fife, UK) is a non-adherent cell line that was first cloned by Buonassisi *et al.* and established after alternate passage of mouse pituitary tumour cells as tumours in animals and in cell culture (Buonassisi *et al.* 1962). The pituitary tumours were originally removed from LAF1 mice by Gadsden *et al.* (Gadsden & Furth 1953). The D16:16 (a gift from Dr S Guild, UK) cell line is a subclone of the D16v cell line (Yasumura 1968), a clonal variant of the original D1 clone and grows as an adherent monolayer with spindle-shaped morphology. The AtT20 D16:16 cells are responsive to CRH and sensitive to the inhibitory effects of glucocorticoid treatment (Woods *et al.* 1992; Tierney *et al.* 2003).

2.2.4 RAW 264.7 cell line

The RAW 264.7 cell line (purchased from American Type Culture Collection, Manassas, VA, via LGC Promochem, Middlesex, UK) was used as a model of murine macrophage cells. This cell line was established from the ascites of a tumour induced in a male mouse by the intraperitoneal injection of Abelson leukaemia virus (A-MuLV) (Ralph & Nakoinz 1977). These cells are phagocytic and express receptors for both complement and immunoglobulin.

2.2.5 Maintenance of cell lines

All cell lines were grown in 75ml ventilated flasks (T75; Costar, New York, USA) at 37°C and 5% CO₂ (Heraeus Incubator; Kendro Laboratory Products, Hertfordshire, UK) in Dulbecco's Modified Eagle Media (DMEM) containing 44mM NaHCO₃, 111 μ M sodium pyruvate and 4mM L-alanyl-L-glutamine (Invitrogen Ltd, Paisley, UK), 100 μ M L-alanine, 98 μ M L-asparagine, 8.8 μ M L-aspartic acid, 10 μ M L-glutamic acid, 1 μ M glycine, 10mM L-proline and 10 μ M L-serine; Invitrogen Ltd, UK) and supplemented with 10% heat inactivated fetal calf serum (FCS; PAA Laboratories Ltd, Yeovil, UK)

and 105µM gentamicin (Sigma, Poole, UK). For the TtT/GF and AtT20 (D16:16) cell lines, the medium was further supplemented with 5mg/l insulin, 5mg/l transferrin, 116µM sodium selenite (Invitrogen Ltd, UK) and 30pM tri-iodo-L-thyronine sodium salt (T_3 ; Sigma, UK). The PDFS cells are a non-adherent cell line, therefore, the flasks were pre-coated with poly-L lysine (prepared as 0.1% (w/v) in distilled water; Sigma, UK), which is an effective adhesive reagent due its ability to interact with the anionic sites of both tissue and cells. The PDFS cells were cultured in the same supplemented medium as the TtT/GF and AtT20 (D16:16) cell lines, but without T_3 .

2.2.6 Passage of cells

Once the TtT/GF cells, PDFS cells and AtT20 (D16:16) cells reached approximately 90% confluency, they were washed with Dulbecco's phosphate-buffered saline (PBS) containing 2.7mM KCl, 1.5mM KH_2PO_4 , 137mM NaCl and 6.5mM Na_2HPO_4 (Invitrogen Ltd, UK) and incubated with 0.5g/l trypsin (Invitrogen Ltd, UK) and 684µM tetrasodium ethylenediaminetetraacetate (EDTA; Invitrogen Ltd, UK) for 2 minutes at 37°C to detach them from the flask wall. The cells were plated at 1.5×10^6 cells per T75 flask in DMEM, supplemented as previously mentioned (see section 2.2.5). For the passage of the suspension AtT20 (D1) cell line, the medium containing the cells was centrifuged at 200g for 5 minutes. The cell pellet was washed in PBS, resuspended in fresh medium and plated at 3×10^6 cells per T75 flask. Once the RAW 264.7 cells reached approximately 90% confluency, they were washed in PBS, detached by gentle scraping with a rubber scraper (Costar, USA) and seeded at 3×10^6 cells per T75 flask. The cell lines used did not exceed a passage number of 30.

2.3 In vitro experiments

2.3.1 Single cell cultures

2.3.1.1 Experimental conditions for determining NFκB activation

For the detection of NFκB activation, the cells were plated in their respective culture medium (section 2.2.5) for 24 hours prior to stimulation at a density of: 2.5×10^5 (TtT/GF), 6×10^5 (RAW 264.7), or 6×10^5 (AtT20 D16:16) cells per cm^2 . For stimulation, the cells were transferred to fresh medium containing the appropriate drugs and incubated for periods ranging from 0 to 24 hours. They were collected by gentle scraping with a rubber scraper (Costar, USA) and subjected to nuclear and cytosolic

protein extraction (section 2.5.3). The cytosolic and nuclear extracts were prepared for detection of p65 and I κ B α protein expression by western blot analysis (section 2.5.4) and the nuclear extracts were prepared for the detection of NF κ B DNA-binding activity by electrophoretic mobility shift assay (EMSA; section 2.5.5) and/or p65 TransAMTM assay (section 2.5.7).

2.3.1.2 Experimental conditions for measuring IL-6 release

For the measurement of IL-6, the cells were plated in their respective culture medium (section 2.2.5) [at a density of 1.3×10^5 cells per cm²] for 24 hours prior to stimulation. Cells were then transferred to fresh medium containing appropriate drugs and incubated for periods ranging from 0 to 24 hours. Following incubation, the medium was collected, concentrated (section 2.5.8) and assayed for IL-6 by enzyme linked immunosorbant assay (ELISA; section 2.5.9)

2.3.1.3 Experimental conditions for measuring ACTH release

For the measurement of ACTH, the cells were incubated in their respective culture medium (section 2.2.5) [at a density of 1.3×10^5 cells per cm²] for 24 hours prior to stimulation. Cells were then transferred to fresh medium containing appropriate drugs and incubated for periods ranging from 0 to 24 hours. Following incubation, the medium was collected and assayed for ACTH release by radioimmunoassay (RIA; section 2.5.11).

2.3.1.4 Experimental conditions for stimulation with LPS and CRH

Within the anterior pituitary gland, folliculostellate cells provide an exclusive source of IL-6 (Vankelecom *et al.* 1989; Renner *et al.* 1998) and LPS injection increases intra-pituitary IL-6 production *in vivo* (Grinevich *et al.* 2001). Therefore, the cell lines utilised in the current *in vitro* studies were incubated with 100ng/ml LPS, a concentration previously shown to stimulate IL-6 release from folliculostellate cells (Lohrer *et al.* 2000; Tierney *et al.* 2005). The cells were stimulated with LPS (100ng/ml; *Escherichia coli* serotype 011:B4; Sigma, UK) and/or graded concentrations of CRH (1-100nM; Tocris Biosciences, Avonmouth, UK) over a 24 hour period to determine the optimal time point for NF κ B activation and IL-6 and ACTH release by ELISA and RIA (sections 2.5.9 and 2.5.11 respectively). From these experiments, the optimal time for NF κ B activation following LPS and/or CRH stimulation was 30 minutes for the TtT/GF cells and 1 and 7 hours for the AtT20

D16:16 cells. The optimal time point for IL-6 release from the TtT/GF cells following LPS stimulation was 16 hours and for the AtT20 D16:16 cells, the optimal ACTH response was 7 hours following CRH stimulation (see chapter 3).

2.3.1.5 Experimental conditions for stimulation of AtT20 D16:16 cells with murine recombinant IL-6

The AtT20 D16:16 cells were stimulated with murine recombinant IL-6 (1nM; R&D systems, Abingdon, UK) in the presence and absence of graded concentrations of CRH (1-100nM; Tocris Biosciences, Avonmouth, UK). The medium was collected and the concentration of ACTH was determined by RIA (section 2.5.11).

2.3.2 NF κ B inhibition studies

For the NF κ B inhibition studies, the following compounds were utilised: 1) the proteasome inhibitor Mg132 [dissolved in 2mM ethanol; Tocris Biosciences, UK; (Chen *et al.* 1997)], 2) the synthetic glucocorticoid dexamethasone [dexamethasone 21-phosphate disodium salt, dissolved in culture medium; Sigma, UK; (Jeon *et al.* 2000)] or 3) the specific IKK β inhibitor SC514 [dissolved in 0.1% DMSO; Pfizer Ltd, Kent, UK; (Kishore *et al.* 2003)].

2.3.2.1 Experimental conditions for the treatment of TtT/GF cells with Mg132

The TtT/GF cells were pre-treated for 1 hour with Mg132 (1-100 μ M) prior to the addition of LPS (100ng/ml; Sigma, UK). The cells were collected for cytosolic and nuclear extraction (section 2.5.3) and the nuclear extracts were subjected to NF κ B EMSA (section 2.5.5). For the measurement of IL-6, the cells were pre-treated with Mg132 for 1 hour prior to the addition of LPS (100ng/ml; Sigma, UK). The medium was collected, concentrated (section 2.5.8) and assayed for IL-6 (section 2.5.9).

2.3.2.2 Experimental conditions for the treatment of TtT/GF cells with dexamethasone

The TtT/GF cells were incubated concomitantly with dexamethasone (1-100nM; Sigma, UK) and LPS (100ng/ml; Sigma, UK). Following incubation, the cytosolic and nuclear fractions were extracted from the cells (section 2.5.4) for the detection of NF κ B activity by EMSA (section 2.5.5) and I κ B α protein expression by western blot

(section 2.5.4). The medium was collected, concentrated (section 2.5.8) and assayed for IL-6 (section 2.5.9).

2.3.2.3 Experimental conditions for the treatment of TtT/GF and AtT20 D16:16 cells with the IKK β inhibitor SC-514

The TtT/GF and D16:16 cells were incubated for 1 hour with SC-514 (50-300 μ M) prior to the addition of LPS (100ng/ml; Sigma, UK) and/or graded concentrations of CRH (1-100nM; Tocris Biosciences, UK). The medium from the TtT/GF cells was collected following incubation, concentrated (section 2.5.8) and assayed for IL-6 (section 2.5.9) and the medium from the AtT20 D16:16 cells was collected and assayed for ACTH release (section 2.5.11). For the detection of NF κ B activity, the cytosolic and nuclear fractions were extracted from the cells (section 2.5.3) and subjected to either NF κ B EMSA (TtT/GF cells; section 2.5.5) or p65 TransAMTM assay (AtT20 D16:16 cells; section 2.5.7).

2.3.3 Co-cultures of TtT/GF and AtT20 D16:16 cells

To investigate further the effects of LPS and CRH stimulation upon IL-6 and ACTH release from the TtT/GF and AtT20 D16:16 cells respectively, when these two cell types are in contact with one another, a co-culture model was utilised.

2.3.3.1 Monolayer co-cultures

For mono-layer co-cultures of the TtT/GF and AtT20 D16:16 cells, the cells were plated at a 1:4 ratio of TtT/GF:AtT20 D16:16 cells (see chapter 3) and left for 24 hours to adhere. The following day, the medium was changed and the cells for left for a further 24 hours. On the third day, the cells were stimulated, in new medium.

2.3.3.1.1 Estimation of cell numbers

To determine the number of TtT/GF and AtT20 D16:16 cells in the monolayer co-cultures, the cells were plated at a ratio of 1:4 (TtT/GF:AtT20 D16:16 cells) and incubated in culture medium (see section 2.2.5). On the third day, the cells were incubated with 0.5g/l trypsin (Invitrogen Ltd, UK) and 684 μ M tetrasodium ethylenediaminetetraacetate (EDTA; Invitrogen Ltd, UK) for 2 minutes at 37°C to detach them from the plate. The cells were then counted under a light microscope using a haemocytometer. The shape of the detached TtT/GF cells was distinct from the AtT20 D16:16 cells, thus enabling the two cells types to be easily distinguished.

2.3.3.2 Aggregate co-cultures

For aggregate co-cultures, the cells were plated at ratio of 1:4 (TtT/GF:AtT20 D16:16 cells) in non-adherent 24-well culture plates and placed over night on a rocking platform under the conditions outlined in section 3.2.5, to allow clusters of the TtT/GF and AtT20 D16:16 cells to form. The following day, the medium was changed and the cells were incubated for a further 24 hours on the rocking platform prior to treatment.

2.3.3.3 Experimental conditions for measuring IL-6 and ACTH release

For the measurement of IL-6 and ACTH release, the cells were plated at a density of 3.3×10^4 (TtT/GF cells) and 1.3×10^5 (D16:16 cells) per cm^2 . The cultures were stimulated with LPS (100ng/ml; Sigma, UK) in the presence and absence of graded concentrations of CRH (1-100nM; Tocris Biosciences, UK) and the medium was collected and either concentrated (section 2.5.8) and assayed for IL-6 release (section 2.5.9) or stored for the measurement of ACTH (section 2.5.11).

2.3.3.4 Experimental conditions for the inhibition of NF κ B

For the NF κ B inhibition studies, the co-cultures were pre-treated for 1 hour with SC-514 (100 μ M; Pfizer Ltd, UK) prior to the addition of LPS (100ng/ml; Sigma, UK) and/or CRH (1-100nM; Tocris Biosciences, UK). The medium was collected following incubation and either concentrated (section 2.5.8) for IL-6 determination (section 2.5.9) or stored for ACTH measurement (section 2.5.11).

2.4 In vivo experiments

These experiments were undertaken to explore the role of NF κ B in mediating the HPA responses to psychological (restraint) or immunological (LPS) stress.

2.4.1 Application of restraint stress

Adult male rats (section 2.1.1), weighing approximately 200-250g, were placed into cylindrical Broome rodent restrainers that allowed for a close fit (barrel internal diameter 50.8mm; Harvard Apparatus Ltd, Kent, UK) for 15 or 30 minutes. Control animals were not subjected to stress. Animals were either killed by decapitation immediately following restraint stress (15 and 30 minutes) or, returned to their housing cages for a 30 minute or 1 hour period of recovery and then killed by decapitation. The brains were quickly removed, frozen in liquid nitrogen and stored at

-80°C. The trunk blood was collected into EDTA-coated tubes, allowed to clot on ice prior to centrifugation at 3130g for 15 minutes and the plasma was stored at -80°C until required for detection of ACTH (section 2.5.12), corticosterone (section 2.5.13), IL-6 (section 2.5.9) and TNF α (section 2.5.10). The anterior pituitary, thymus and adrenal glands were quickly removed and subjected to cytosolic and nuclear extraction (section 2.5.3) for the detection of NF κ B activation by EMSA (section 2.5.5).

2.4.2 Pre-treatment with the IKK β inhibitor, PHA781535E, prior to restraint stress

Two hours prior to the onset of restraint stress (duration of 15 minutes) adult males rats were treated with the NF κ B inhibitor, PHA781535E (a kind gift from Pfizer Ltd, Kent, UK), orally in a volume of 400 μ l, at a dose of 25 or 50mg/kg, or an equivalent volume of vehicle (400 μ l, methycellulose-tween-saline). The dose and pharmacokinetic parameters of PHA781535E were established by Pfizer Ltd (*unpublished observations, confidential*). The animals were killed by decapitation and the trunk blood, brain, anterior pituitary, thymus and adrenal glands were collected and processed as described in section 2.4.1.

2.4.3 Treatment with LPS

Adult male rats (see section 2.1.1) weighing approximately 200-250g were injected intraperitoneally (i.p.) with either vehicle (400 μ l, sterile physiological saline; Baxter International Inc., Glasgow, UK) or LPS (dissolved in sterile physiological saline; Escherichia coli; serotype 055:B5; Sigma, UK) in a volume of 400 μ l (5mg/kg). The dose of LPS was selected based on previous studies examining the release of pro-inflammatory cytokine release and ACTH and corticosterone release in rats receiving LPS i.p. (Kakizaki *et al.* 1999; Hannibal *et al.* 1999). Control animals were not subjected to vehicle or LPS administration. Animals were killed by decapitation either 2 or 4 hours after LPS administration. Trunk blood, brain, anterior pituitary, thymus and adrenal glands were collected and processed as described in section 2.4.1.

2.4.4 Pre-treatment with the IKK β inhibitor, PHA781535E, prior to LPS treatment

Two hours prior to the administration of LPS (i.p., 400 μ l, 50mg/kg; Sigma, UK), adult males rats were treated with the NF κ B inhibitor, PHA781535E (Pfizer Ltd, Kent, UK),

orally in a volume of 400µl, at a dose of 25 or 50mg/kg, or an equivalent volume of vehicle (400µl, methycellulose-tween-saline). Two hours after LPS injection, the animals were killed by decapitation and the trunk blood, brain, anterior pituitary, thymus and adrenal glands were collected and processed as described in section 2.4.1.

2.5 Analytical techniques

2.5.1 RNA extraction

The RNA was extracted from the TtT/GF, RAW 264.7, PDFS and AtT20 (D1 and D16:16) cells for reverse transcriptase polymerase chain reaction (RT-PCR) analysis (section 2.5.2). The adherent cells (approximately 5×10^6 cells) were removed from the flask wall either by trypsinisation (PDFS, D16:16 and TtT/GF cells; see section 2.2.6) or by scraping (RAW 264.7 cells; see section 2.2.6). The D1 cells, which grow in suspension, were collected in their medium and centrifuged at 200g for 5 minutes. The cells were washed twice in PBS and the RNA was extracted immediately using the Qiagen RNeasy kit (Qiagen, West Sussex, UK) as described below.

Tissue and cell pellets were resuspended in 350µl RLT[®] buffer (as provided in the Qiagen RNeasy kit, RLT is the given name of the buffer by the company) containing 100mM β-mercaptoethanol, transferred to a Qiagen shredder column and centrifuged at 15000g for 2 minutes at 4°C. Following the addition of 35% ethanol (VWR International Ltd, Poole, UK), the lysate was transferred to an RNeasy column and centrifuged at 8500g for 1 minute at 4°C. The column was washed with RW1[®] buffer (as provided in the Qiagen RNeasy kit, RW1 is the given name of the buffer by the company) and centrifuged at 8500g for 1 minute at 4°C. The column was washed through with RPE[®] buffer (as provided in the Qiagen RNeasy kit, RPE is the given name of the buffer by the company) and centrifuged at 8500g for 1 minute at 4°C. The column was washed again with RPE buffer and centrifuged at 8500g for 2 minutes at 4°C. To elute the RNA, the column was transferred to a collection tube and 30µl RNase-free water (Ambion Incorporation, Texas, USA) was added directly onto the RNeasy silica-gel membrane. The column was centrifuged for at 8500g for 1 minute at 4°C. This step was repeated to ensure all the RNA was eluted from the membrane. The RNA extracts were then diluted 1:50 in RNase-free water and the optical density (OD) was measured at 260nm and 280nm using a spectrophotometer (Jenway 6305 UV/Visual Spectrum; Jencon PLS, Forest Row, East Sussex, UK). The

reading at 260nm allows the calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 40µg/ml for single stranded RNA (Davis *et al.* 1986). The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid (Davis *et al.* 1986). Pure preparations of RNA have OD_{260}/OD_{280} values between 1.8 and 2.0 (Davis *et al.* 1986).

2.5.2 Detection of mRNAs by reverse transcriptase polymerase chain reaction

2.5.2.1 Preparation of cDNA by reverse transcription

The RNA extracted from the TtT/GF, RAW 264.7, PDFS and AtT20 (D1 and D16:16) cells (see section 2.5.1) was reverse transcribed (RT) by avian myeloblastosis virus reverse transcriptase (AMV RT; Promega Ltd, UK) and the resulting cDNA was amplified by polymerase chain reaction (PCR; section 2.5.2.2).

Reverse transcription was performed on RNA samples (3µg) incubated with 0.05µg/µl oligo (dT)₁₅Primer (Promega Ltd, Southampton, UK) and made up to a final volume of 28µl with RNase-free water. The RNA was denatured for 5 minutes at 65°C on a thermal cycler (Omnigene; Thermo Electron Co., UK) and chilled on ice for 2 minutes. Reverse transcriptase solution containing a dNTP mix (0.6mM dATP, 0.6mM dCTP, 0.6mM dGTP and 0.6mM dTTP; Promega Ltd, UK), 1U/ml rRNasin®Ribonuclease inhibitor (Promega Ltd, UK), 5U/ml AMV RT (Promega Ltd, UK) and 5U/ml Moloney murine leukaemia virus (M-MLV) buffer [57mM Tris-HCl, pH 8.3, 85mM KCl, 3mM MgCl₂, 11mM dithiothreitol (DTT); Promega Ltd, UK] was added to 12µl of denatured RNA and incubated for 2 hours at 42°C. The cDNA was stored at -20°C.

2.5.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR; Techne TC-312; Jencons PLS, UK) was performed on the complementary DNA (cDNA) generated by reverse transcription (section 3.5.2.1) with specific primers for the genes of interest (Table 2.1). The PCR mixture was composed of PCR buffer (50mM KCl, 10mM Tris-HCl, pH 9.0 at 25°C, 0.1%Triton®x-100; Promega Ltd, UK), 0.2mM dNTP mix (Promega Ltd, UK), 1.5mM MgCl₂ (Promega Ltd, UK), 0.05U/µl *Taq* DNA polymerase (Promega Ltd, UK) and forward and reverse primers (Applied Biosystems, North Warrington, UK) specific for each reaction. Table 2.2 shows the optimal concentrations used for each PCR

reaction, determined by preliminary studies in which graded concentrations of primers were used (10 μ M, 25 μ M and 50 μ M). To the PCR mixture, 5 μ l cDNA was added and the final volume was made up to 50 μ l with RNase-free water. The cycling parameters consisted of a denaturing step for 5 minutes at 95°C followed by 35 PCR cycles (the optimal PCR cycle number was determined from preliminary experiments using a series of cycle numbers – 15, 25, 30 and 35 cycles) of denaturation for 1 minute at 95°C, annealing of primers at specific temperatures (Table 2.2) for 1 minute and an extension step for 1 minute at 72°C. The PCR products were subjected to a final extension step of 72°C for 15 minutes following completion of the cycles and were stored at 4°C. For the detection of amplified cDNA, 5 μ l of PCR product and 5 μ l of PCR marker (Promega Ltd, UK; molecular weight bands corresponded to 1000, 750, 500, 300, 150 and 50 base pairs) were mixed with 1 μ l 6x loading dye each (Promega Ltd, UK) and were loaded into separate wells of a 2% agarose gel (Gibco-BRL, Paisely, UK) stained with 0.02% ethidium bromide (Sigma, UK). The gel was subjected to electrophoresis for 30 minutes at 100V in EDTA buffer (TAE; Invitrogen, UK). Analysis of the RT-PCR product was performed under ultraviolet light with camera image analysis (Herolab GmbH Laborgeräte, Germany) and Multi-Genius Bio-Imaging System software (Syngene, Cambridge, UK). The amplification of the correct sequences was confirmed by the product length.

2.5.3 Extraction of nuclear and cytoplasmic protein

For preparation of nuclear and cellular extracts, tissue or cell samples were resuspended in 200 μ l buffer A (containing 10mM HEPES (Sigma, UK), 10mM KCl (Sigma, UK), 0.1mM EDTA (Invitrogen, UK), 0.1mM EGTA (Sigma, UK), 1mM DTT (Sigma, UK), 0.5mM phenylmethylsulfonyl fluoride (PMSF; Sigma, UK), 1mM Na₃VO₄ (Sigma, UK), 1mM NaF (Sigma, UK), 1 μ g/ml aprotinin (Sigma, UK), 1 μ g/ml pepstatin and 1 μ g/ml leupeptin (Sigma, UK), pH 7.4 at 4°C). The primary tissue samples were gently sonicated in 200 μ l buffer A and all samples (cell lines and tissue) were allowed to swell on ice for 15 minutes prior to the addition of 0.4% NP-40 (Sigma, UK). Samples were vortexed for 10 seconds and centrifuged for 5 minutes at 8500g at 4°C. The supernatant containing the cytoplasmic fraction was collected and the concentration was measured using the Bio-Rad protein assay system (Bio-Rad Laboratories, California, USA; section 2.5.4.1). The nuclear pellet was resuspended in 100 μ l cold buffer C (20mM HEPES, 0.4mM NaCl (Sigma, UK), 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 1mM Na₃VO₄, 1mM NaF, 1 μ g/ml aprotinin, 1 μ g/ml

pepstatin and 1µg/ml leupeptin, pH 7.4 at 4°C) and vigorously agitated at 4°C for 15 minutes. The samples were centrifuged at 8500g for 5 minutes at 4°C and the supernatant containing the nuclear fraction was collected. The concentration of the nuclear extracts was measured by the Bio-Rad protein assay system (section 2.5.4.1). The cytoplasmic and nuclear extracts were stored at –80°C until required for western blotting (section 2.5.4), EMSA (section 2.5.5) or p65 TransAM™ assay (section 2.5.7).

Table 2.1 Primer sequences. CD14, cluster of differentiation 14; MD-2, myeloid of differentiation factor 2; Tlr2, Toll-like receptor 2; Tlr4, toll-like receptor 4.

Target	Primer Sequence*
CD14 (mouse)	5' ATGGAGCGTGTGCTTGGCTTGTTGCTGTTG 3' GCGGCTTAAACAAAGAGGCGATCTCCTAGG
Tlr2 (mouse)	5' GCGGCGGAACACTTTTCATCCGATGCCCCG 3' GCGGCAGCCTGGTGACATTCCAAGACGGA
Tlr4 (mouse)	5' GCGGCGGGTCAAGGAACAGAAGCAG 3' GCGGCGCTCATTTCTCACCCAGTCC
MD-2 (mouse)	5' CTGAATCTGAGAAGCAACAGTGG 3' CAGTCTCTCCTTTCAGAGCTCTGC
CD14 (human/mouse)	5' GCCCTGCGTGTGCT 3' CAGGATTGTCAGACAGG
Tlr2 (human)	5' GGCTTCTCTGTCTTGTGACC 3' GGGCTTGAACCAGGAAGACG
Tlr4 (human)	5' TTGTATTCAAGGTCTGGCTGG 3' GCAACCTTTGAAACTCAAGCC
MD-2 (human)	5' CTGCAACTCATCCGATGCAAG 3' CAGTCTCTCCTTTCAGAGCTCTGC
GAPDH (human/mouse)	5' AAGGTGAAGGTCGGAGTCAACG 3' GGCAGAGATGATGACCCTTTTGGC

*Gene sequences were identified through GenBank® and primer sequences were calculated using the BioMath Primer3 programme (<http://fodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/>). Sequences that matched the gene of interest with ≥95% identity were chosen. Accession numbers of gene sequences are: mCD14, NM_009841; mTlr2, NM_011905; mTlr4, NM067272; mMD-2, AB018550; mGAPDH, NM_008084; hCD14, NM_000591; hTlr2, U88878; hTlr4, U88880; hMD2, AB018549; hGAPDH, M33197.

Table 2.2 RT-PCR conditions for mRNA detection of Tlr2, Tlr4, MD-2 & CD14. CD14, cluster of differentiation 14; MD-2, myeloid of differentiation factor 2; Tlr2, Toll-like receptor 2; Tlr4, toll-like receptor 4.

Target	Concentration of primers (pmol/ μ l)	Annealing temperature ($^{\circ}$ C) *
Tlr2 (mouse)	50	47
Tlr4 (mouse)	50	30
MD2 (mouse)	50	56
CD14 (mouse)	25	50
Tlr2 (human)	50	54
Tlr4 (human)	50	50
MD2 (human)	50	56
CD14 (human/mouse)	50	47

*Annealing temperatures recommended by Applied Biosystems, UK

2.5.4 Sodium dodecyl sulphate western blot analysis

2.5.4.1 Protein concentration determination (Bradford assay)

The binding of Coomassie blue to tyrosine residues causes a shift in the absorption maximum of the dye from 465nm to 595nm. The increase in the absorption maximum is measured at 595nm against a standard curve ranging from 0-1mg/ml BSA. The lysed cell samples (section 2.5.3) were diluted to ensure they fell within the linear portion of the standard curve. The samples and the standards were added in duplicate into a 96-well microtitre plate (10 μ l/well) followed by the addition of Bio-Rad protein assay reagent (100 μ l/well; stock solution diluted 1:5 with distilled water; Bio-Rad, USA). After 1 minute colour equilibration, the plate was read on an Anthos htll plate reader (Anthos Labtec Instruments GmbH, Austria) at 595nm. The sample protein concentrations were calculated by linear regression from the standard curve (figure 2.1).

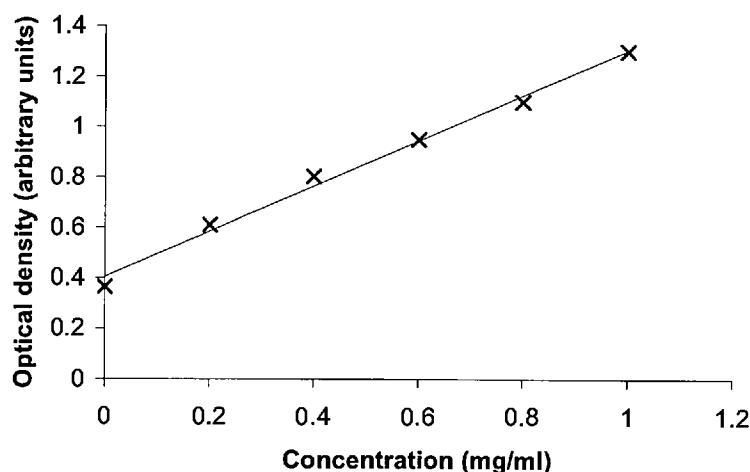


Figure 2.1 Typical standard curve for protein estimation

2.5.4.2 Electrophoretic separation of proteins

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using acrylamide gels [10% v/v acrylamide, (Bio-Rad Laboratories, California, USA), 377mM Tris-HCl (Sigma, UK), 1mM sodium dodecyl sulfate (SDS; Sigma, UK), 0.5mM N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma, UK) and 0.5mM ammonium persulfate (Sigma, UK), pH 8.8]. The stacking gel consisted of 25mM acrylamide/bis solution, 132mM Tris-HCl, 1mM SDS, 1mM TEMED and 5.2mM ammonium persulfate at pH 6.8. The cytoplasmic and nuclear protein extracts (section 2.5.3) were resuspended in triple concentrate sample buffer (192mM Tris-base, 150mM 2-mercaptoethanol, 300mM glycerol, 60mM sodium dodecyl sulfate and 0.03mM bromophenol) and heated to 100°C for 2 minutes. The samples and molecular weight marker (Santa Cruz Biotechnology Inc., California, USA; consisting of molecular weights of 132, 90, 55, 43, 34 and 23 kDa) were loaded in individual wells. The proteins were resolved in non-denaturing conditions (running buffer: 100mM Tris/Bicine and 1mM SDS at pH 8.3) at 100V for 180 minutes.

2.5.4.3 Western blot with chemiluminescent detection

The separated proteins were transferred onto a nitrocellulose membrane (Hybond-C; Amersham Biosciences Ltd, Chalfont St Giles, UK), which was pre-soaked in transfer buffer (25mM Tris-HCl (Sigma, UK), 200mM glycine (Sigma, UK) and 200mM ethanol (BDH Chemicals Ltd, Poole, UK) at pH 8.3), by electrophoresis at 50mA (per blot; 2117 MULTIFOR II, Pharmacia LKB, Bromma, Sweden) for 120 minutes. The

nitrocellulose membrane was washed for 10 minutes in 50mM Tris-buffered saline (TBS; 50mM Tris-HCL, 150mM NaCl at pH 7.0), containing 1% Tween (TTBS), and incubated in TTBS supplemented with 5% non-fat milk powder (Marvel, Knighton Edbaston, Stafford, UK) for 2 hours at room temperature on a rocking platform. The membrane was washed 4 times for 15 minutes in TTBS and incubated at 4°C overnight under agitation with either goat anti-rabbit p65 polyclonal antibody (Santa Cruz Biotechnology Inc., USA) or goat anti-rabbit I κ B α polyclonal antibody (Santa Cruz Biotechnology Inc., USA) at a 1:1000 dilution in TTBS containing 5% non-fat milk powder and 1% BSA. The membrane was washed a further 4 times (15 minutes each) with TTBS and incubated with horseradish peroxidase (HRP) conjugated rabbit anti-goat IgG antibody (Santa Cruz Biotechnology Inc., USA) at 1:2000 in TTBS containing 5% milk powder and 1% BSA for 1 hour at room temperature under agitation.

The membrane was washed for a further hour (4 x 15 minute washes) and antibody binding was visualised by enzymatic chemiluminescence (ECL) according to manufacturer's instructions (ECL Plus; Amersham Biosciences Ltd, UK). To ensure equal amounts of protein were loaded, the membrane was then washed again and incubated in TTBS supplemented with 5% non-fat milk powder for 1 hour at room temperature on a rocking platform. The membranes were washed and incubated with mouse monoclonal anti-mouse β -actin antibody (Abcam Ltd, Cambridge, UK) at 1:5000 in TTBS containing 5% milk powder for 1 hour at room temperature. The membrane was washed again and incubated with HRP-conjugated rabbit anti-mouse IgG antibody (Sigma, UK) at 1:1000 in TTBS containing 5% milk powder for 1 hour at room temperature on a rocking platform. The membrane was washed and the bands were visualised by ECL Plus detection (Amersham Biosciences Ltd, UK).

The proteins were visualised by exposure to X-ray film (Amersham Biosciences Ltd, UK) for up to 5 minutes, for a permanent record using an automatic developer (Konica medical film processor, model: SRX – 101A, Konica UK Ltd, UK) and the developed blots were scanned onto a PC (HP Scanjet 5200C with Adobe Photodeluxe Business Edition version 1.1). Because comparisons between separate gels can not be made, the experiments were repeated at least two times to ensure reproducibility and densitometric analysis of the bands on the same gel were performed using Scion Image (version Beta 4.02, Frederick, Maryland, USA). The

data were presented as a percentage of control (per gel) to show changes in protein expression more clearly (non-quantitative).

2.5.4.4 Determination of NF κ B DNA binding activity by electrophoretic mobility shift assay

The nuclear proteins extracted from the tissue samples and cell lines (section 2.5.3) were analysed for NF κ B DNA binding activity by electrophoretic mobility shift assay (EMSA). NF κ B consensus oligonucleotides (3.5pmol; 5'-AGT TGA GGG GAC TTT CCC AGG C-3', 5'-CCT GGG AAA GTC CCC TCA ACT-3'; Promega Ltd, UK) were end labelled with [γ -³²P]ATP (0.19MBq; Amersham Biosciences Ltd, UK) using T4 polynucleotide kinase (10-20U) in commercially supplied T4 polynucleotide kinase buffer (Promega Ltd, UK). The mixture was incubated at 37°C for 10 minutes before the reaction was halted by addition of 0.5M EDTA and made up to a final volume of 100 μ l with diethylpyrocarbonate (DEPC) pre-treated distilled water. Unincorporated nucleotides were removed from the DNA probe by passage through a G-25 Sephadex® column containing 10mM Tris-base, 1mM EDTA, 100mM NaCl (pH 8.0; Boehringer Mannheim, Ingelheim, Germany). The activity of the labelled DNA was measured using a liquid scintillation counter (model WALLAC 1409B; LKB WALLAC, Sweden) with probes of activities between 2x10⁵ and 4 x10⁵ counts per minute being used.

Nuclear protein extract (5-10 μ g) was incubated with labelled probe in commercially supplied binding buffer (containing 5mM Tris-HCl, 0.5mM MgCl₂, 0.25mM EDTA, 0.25mM DTT, 25mM NaCl, 0.025 μ g/ μ l poly(dI-dC), 2% glycerol, at pH 7.5; Promega Ltd, UK) for 30 minutes at room temperature. In control reactions, unlabelled consensus NF κ B (1.75pmol; specific competitor) or AP-1 non-specific competitor (1.75pmol; 5'-CGC TTG ATG AGT CAG CCG GAA-3', 5'-TTC CGG CTG ACT CAT CAA GCG-3'; Promega Ltd, UK) oligonucleotides were added to the nuclear extracts in buffer and incubated for 10 minutes on ice. To the control and sample reactions, [γ -³²P]ATP-labelled double-stranded probe was added and the reactions were incubated for 30 minutes at room temperature.

All extracts were resolved on a 4% non-denaturing polyacrylamide gel containing 5% v/v Tris-Borate-EDTA (TBE; consisting of 100mM Tris base, 100mM boric acid and 2mM EDTA at pH 8.3; Promega Ltd, UK) buffer, 15% v/v acrylamide/bisacrylamide (Bis-2®; Amresco, Ohio, USA), 3.5% v/v acrylamide (Acryl-40®; Amresco, USA), 3.5%

v/v glycerol at 200V in 0.5x TBE buffer. The gel was fixed in 10% v/v methanol (VWR International, Leicestershire, UK), 10% v/v acetic acid (VWR International, UK) for 10 minutes before being transferred to Whatman filter paper (Whatman®, Maidstone, UK) and dried using a Scie-Plas gel dryer system (Model GD5040; Warwickshire, UK). The gel was then exposed to X-ray film for 1-2 days (Kodak Biomax MR film; Anachem, Luton, UK) for a permanent record and the films were scanned onto a PC (HP Scanjet 5200 with Adobe Photodeluxe Business Edition version 1.1) and subsequently, densitometric analysis of the bands on each gel were performed using Scion Image (version Beta 4.02, Frederick, Maryland, USA). As direct comparisons between separate gels could not be made, the experiments were repeated at least two times to ensure reproducibility and the data were presented as the mean percentage of control to show changes in NF κ B DNA-binding activity more clearly (non-quantitative). Where possible, three or more samples from the same treatment group were loaded on the same gel and following densitometric analysis, non-parametric (semi-quantitative) statistical analysis was performed (see section 2.5.13).

2.5.5 Determination of p50 and p65 subunits of NF κ B by supershift assay

For the identification of the NF κ B DNA-binding complexes containing the transcriptionally active p65 subunit, supershift assays were performed on LPS (100ng/ml) stimulated TtT/GF cells (figure 2.2), AtT20 D16:16 cells (figure 2.3) and pituitary and adrenal tissue extracted from LPS (5mg/kg, i.p.) treated rats (figure 2.4). The nuclear extracts of the AtT20 D16:16 cells (10 μ g) were pre-incubated with antibody directed against p65 (0.5 μ g, goat anti-rabbit p65 polyclonal antibody; Santa Cruz Biotechnology Inc., USA) and nuclear extracts of the TtT/GF cells (10 μ g) and anterior pituitary and adrenal tissues (5 μ g) were pre-incubated with either an antibody directed against p65 (0.5 μ g; Santa Cruz Biotechnology Inc., USA) or antibody directed against p50 (0.5 μ g; goat anti-rabbit p50 polyclonal antibody; Santa Cruz Biotechnology Inc., USA) for 10 minutes on ice prior to the addition of radioactive label and followed by EMSA (section 2.5.5). A supershift or a decrease in the intensity of the NF κ B band in the presence of the antibodies was considered to be indicative of the presence of that protein in the DNA-binding complex. Densitometry analysis (see section 2.5.5) was performed on bands identified as the p65/p50 heterodimer by supershift in the cells/tissues (figure 2.2 - 2.4).

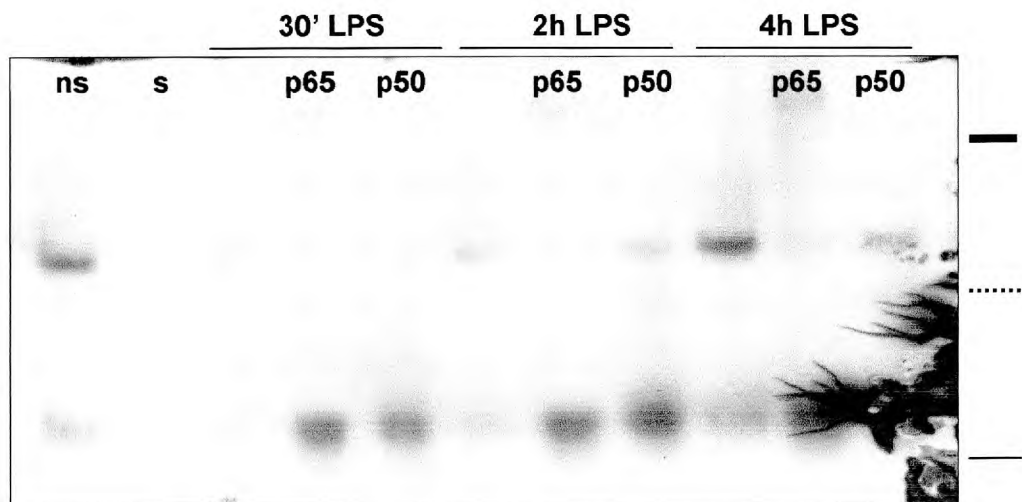


Figure 2.2 Identification of NF κ B binding proteins in TtT/GF cells stimulated with LPS (100ng/ml, 30 minutes – 4 hours) by antibody supershift electrophoretic mobility shift assay (EMSA). The heavy line identifies the DNA binding complexes super-shifted by p65 antibody and the dotted and fine lines indicate specific NF κ B binding complexes. A reduction in the bands labelled with a dotted line in the presence of p65 and p50 antibodies indicate the NF κ B DNA-binding complex is the p65/p50 heterodimer. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B.

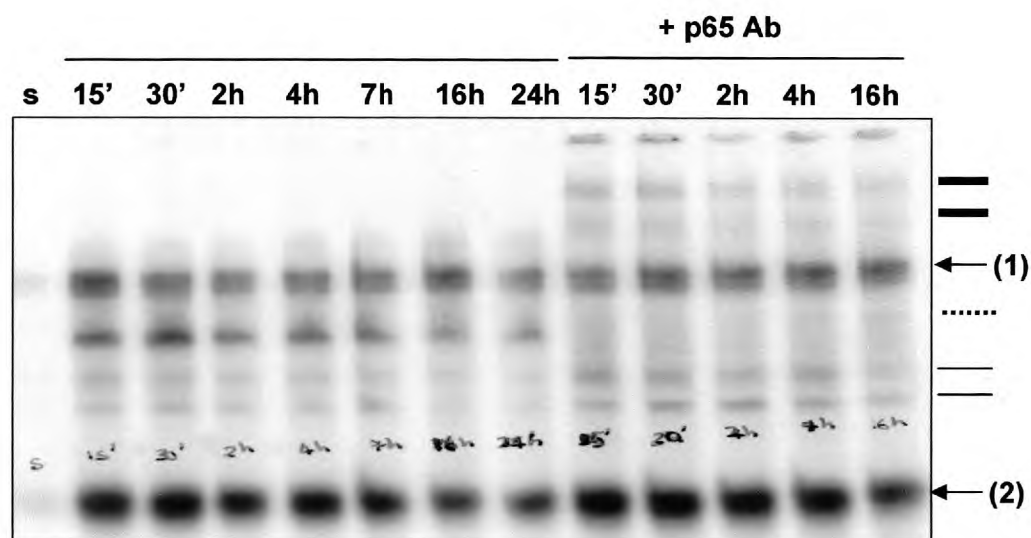


Figure 2.3 Identification of NFκB binding proteins in AtT20 D16:16 cells stimulated with LPS (100ng/ml, 15 minutes – 24 hours) by antibody supershift electrophoretic mobility shift assay (EMSA). The heavy lines identify the DNA binding complexes super-shifted by the p65 antibody and the dotted and fine lines indicate specific NFκB binding complexes. A reduction in the bands labelled with a dotted line in the presence of p65 antibody indicates p65 containing NFκB DNA-binding complexes. (1) and (2) indicate non-specific binding due to the continued presence of these bands in the competitive reaction containing 100 molar excess of the NFκB oligonucleotide (s, specific). Ab, antibody; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B.

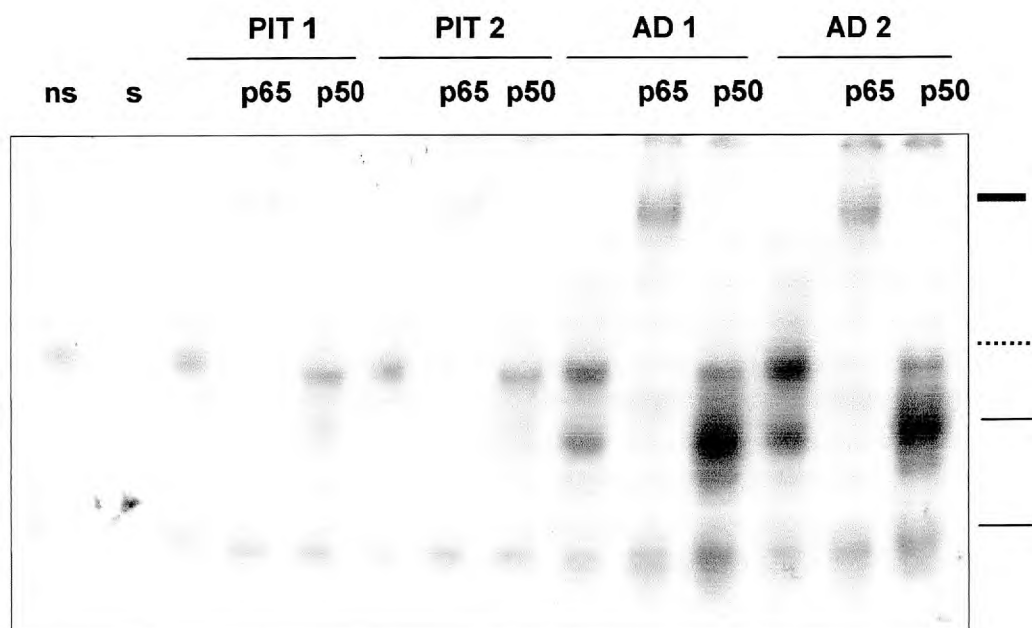


Figure 2.4 Identification of NF κ B binding proteins in anterior pituitary and adrenal tissue extracted from LPS (5mg/kg, 2 hours) treated rats by antibody supershift electrophoretic mobility shift assay (EMSA). The heavy line identifies the DNA binding complexes super-shifted by p65 antibody and the dotted and fine lines indicate specific NF κ B binding complexes. A reduction in the bands labelled with the dotted line in the presence of p65 and p50 antibodies indicate the NF κ B DNA-binding complex is the p65/p50 heterodimer. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). AD, adrenal tissue; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; PIT, anterior pituitary tissue;

2.5.6 Determination of NF κ B DNA binding activity by TransAMTM

The prepared AtT20 D16:16 nuclear extracts (section 2.5.3) were analysed for NF κ B DNA binding activity by a TransAMTM kit (Active Motif, Rixensart, Belgium). The assay was carried out according to the manufacturer's specifications and all reagents were supplied by the manufacturer. Nuclear protein extract (2.5 μ g; diluted in lysis buffer containing 5mM DTT and 10 μ l protease inhibitor cocktail; as provided in the kit) was added to a 96-well plate coated with an immobilised oligonucleotide containing the NF κ B consensus site (5'-GG ACT TTC-3') for 1 hour, at room

temperature, under gentle agitation (100rpm on a rocking platform), in the presence of 30µl commercially supplied binding buffer (as provided in the kit; containing 2mM DTT and 10ng/µl Herring sperm DNA). In control reactions, 30µl of binding buffer containing either wild-type (20pmol; specific competitor; provided in the kit) or mutated (20pmol; non-specific competitor; provided in the kit) consensus oligonucleotides were added to the nuclear extracts prior to incubation.

Following incubation, the wells were washed three times with wash buffer (200µl/well; provided in the kit). The samples were incubated for 1 hour at room temperature with p65 primary antibody (details of antibody not specified by the manufacturer, 100µl/well; antibody was diluted 1:100 in antibody binding buffer as provided in the kit). The wells were washed three times with wash buffer and 100µl of anti-rabbit HRP-conjugated IgG secondary antibody (details of antibody not specified by the manufacturer) was added to each well (diluted 1:100 in antibody binding buffer as provided in the kit) and incubated for 1 hour at room temperature. To each well, 100µl of developing solution (as provided in the kit) was added and incubated for 2-10 minutes in the dark, until the colour of the samples had developed from a range of medium to dark blue. To stop the reaction, 100µl of stop solution (as provided by the kit) was added to each well and the optical density was measured at 450nm and 655nm. The values obtained from the 655nm reading were subtracted from the 450nm readings to correct for optical imperfections in the plate. As separate assays were not standardised to each other, direct comparisons could not be made. Therefore, where possible, the assay was repeated twice (using samples taken from repeated experiments) to ensure the results were reproducible. The mean optical density detected each treatment group was expressed as a percentage of control to show changes in NFκB DNA-binding activity more clearly (non-quantitative). The assay specifically detects bound NFκB p65 in human, mouse and rat extracts and the detection limit of the assay was 0.5µg.

2.5.7 Concentration of culture medium

The supernatant collected from incubated cell lines was concentrated by micon centrifugal filter devices (according to manufacturer's instructions; Millipore, USA) with a 10kDa cut-off point for the measurement of IL-6 by ELISA (section 2.5.9).

The medium was applied to the concentrator columns and centrifuged at 6000g at 10°C for 1 hour. The filters were placed into 2ml collection tubes and centrifuged for

a further 5 minutes at 8500g to collect the retained medium. Equal volumes of retained medium were collected following concentration (600µl) and stored at -80°C until required.

2.5.8 Measurement of IL-6 by enzyme-linked immunosorbant assay

The rat plasma (section 2.4) and the concentrated media (section 3.5.8) collected from incubated cell lines were subjected to an enzyme-linked immunosorbant assay (ELISA) to measure the concentration of (i) mouse IL-6 released from TtT/GF, AtT20 (D16:16 subclone) and RAW 264.7 cells, (ii) human IL-6 released from PDFS cells and (iii) rat plasma IL-6. Specific DuoSet ELISA development kits were obtained from R&D Systems (Abingdon, UK) and each ELISA was performed according to the manufacturer's specifications.

A 96 well titre plate was coated with 100µl of capture antibody per well (for mouse specific IL-6 ELISA, the capture antibody was rat anti-mouse IL-6 (2µg/ml); human – mouse anti-human IL-6 (2µg/ml); rat - mouse anti-rat IL-6 (4µg/ml)) and sealed and incubated overnight at room temperature. The capture antibody was aspirated and the plate was washed four times with Tween washing buffer (0.05% Tween-20 in PBS, pH 7.2 – 7.4) and drained. Any remaining buffer was removed by blotting the plate against a paper towel. To reduce non-specific binding, 300µl of block buffer for the mouse ELISA (1% BSA, 5% sucrose, 0.05% NaN₃ in PBS, pH 7.2 – 7.4) or 300µl of Reagent Diluent for the human and rat ELISA (1% BSA in PBS, pH 7.2 - 7.4; as provided by IL-6 ELISA kit) was added and incubated for 1 hour at room temperature, after which the plate was washed four times as previously described. In duplicate, 100µl of sample or recombinant mouse IL-6 standard for the mouse ELISA (concentrations ranging from 3.9 to 1000pg/ml in Reagent Diluent), 100µl of sample or recombinant human IL-6 standard for the human ELISA (concentrations ranging from 9.3 to 600pg/ml in Reagent Diluent) or 100µl of sample or recombinant rat IL-6 standard for the rat ELISA (concentrations ranging from 31.25 to 8000pg/ml in Reagent Diluent) was added to each well and incubated at room temperature for 2 hours. Two wells containing 100µl of Reagent Diluent were added for reference and an intra-assay standard (mouse - 100pg/ml; human – 75pg/ml; rat – 500pg/ml) was also included for each assay.

Following the incubation period, the plate was washed four times and any remaining buffer was removed. To each well, 100µl biotinylated IL-6 detection antibody (200ng/ml goat anti-mouse in Reagent Diluent for mouse ELISA; 200ng/ml goat anti-human for human ELISA; 100ng/ml goat anti-rat for rat ELISA) was added and the plate was incubated for 2 hours at room temperature. The plate was washed four times and 100µl streptavidin conjugated to horse radish peroxidase (as provided by IL-6 ELISA kit, R&D systems, UK) was added to each well (and incubated for 20 minutes at room temperature in the dark. The plate was washed a further four times and 100µl substrate solution (1:1 mixture of colour reagent A; H₂O₂ and colour reagent B; tetramethylbenzidine, as provided by IL-6 ELISA kit, R&D systems, UK) was added to each well. To stop the enzymatic reaction, 50µl of 2M H₂SO₄ was added to each well and the plate was tapped gently to ensure thorough mixing. The optical density was measured at 450nm and 570nm. The values obtained from the 570nm reading were subtracted from the 450nm readings to correct for optical imperfections in the plate. The concentration of IL-6 was calculated by linear regression from the standard curve (a representative graph is illustrated in figure 2.5). The inter-assay and intra-assay coefficients of variation for the mouse ELISA were 5.7% and 3.9% respectively, 6.2% and 7.8% for the human ELISA and 9.8% and 14% for the rat ELISA. The detection limit for the mouse ELISA was 7.8pg/ml, 9.2pg/ml for the human ELISA and 31.25pg/ml for the rat ELISA.

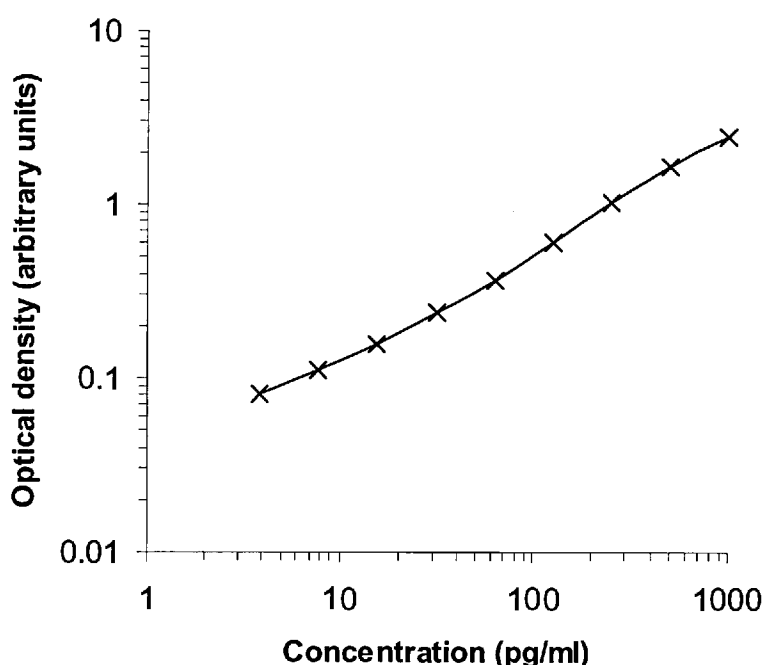


Figure 2.5 Typical standard curve for IL-6 ELISA. ELISA, enzyme-linked immunosorbant assay; IL-6, interleukin 6.

2.5.9 Measurement of TNF α by enzyme-linked immunosorbant assay

The plasma collected from the rats (see section 2.4.1 and 2.4.3) was subjected to an ELISA for the measurement of TNF α . Specific DuoSet ELISA development kits were obtained from R&D Systems (Abingdon, UK) and each ELISA was performed according to the manufacturer's specifications.

The TNF α ELISA was conducted as outlined in section 2.5.8, with the following changes: 100 μ l of capture antibody (mouse anti-rat TNF α ; 4 μ g/ml) was added to a 96-well microtitre plate and incubated overnight at room temperature. The plate was washed with wash buffer (see section 2.5.8) and 300 μ l of Reagent Diluent (1% BSA in PBS, pH 7.2 - 7.4; as provided by TNF α ELISA) was added to each well and incubated for 1 hour at room temperature, after which the plate was washed four times as previously described. In duplicate, 100 μ l of sample or recombinant TNF α standard (concentrations ranging from 31.25 to 4000pg/ml in Reagent Diluent) was added to each well and incubated at room temperature for 2 hours. Two wells containing 100 μ l of Reagent Diluent were added for reference and an intra-assay standard (500pg/ml) was also included for each assay.

Following the incubation period, the plate was washed four times and 100 μ l biotinylated TNF α detection antibody (100ng/ml goat anti-rat TNF α in Reagent Diluent) was added to each well for 2 hours at room temperature. The plate was then developed with streptavidin conjugated to horse radish peroxidase (100 μ l/well; as provided by TNF α ELISA kit) and substrate solution (100 μ l; 1:1 mixture of colour reagent A; H₂O₂ and colour reagent B; tetramethylbenzidine, as provided by TNF α ELISA kit) under the conditions detailed in section 2.5.8 and the optical density was measured at 450nm and 570nm. The values obtained from the 570nm reading were subtracted from the 450nm readings to correct for optical imperfections in the plate. The concentration of TNF α was calculated by linear regression from the standard curve (a representative graph is illustrated in figure 2.6). The inter-assay and intra-assay coefficients of variation for the assay were 4.7% and 2.8% respectively.

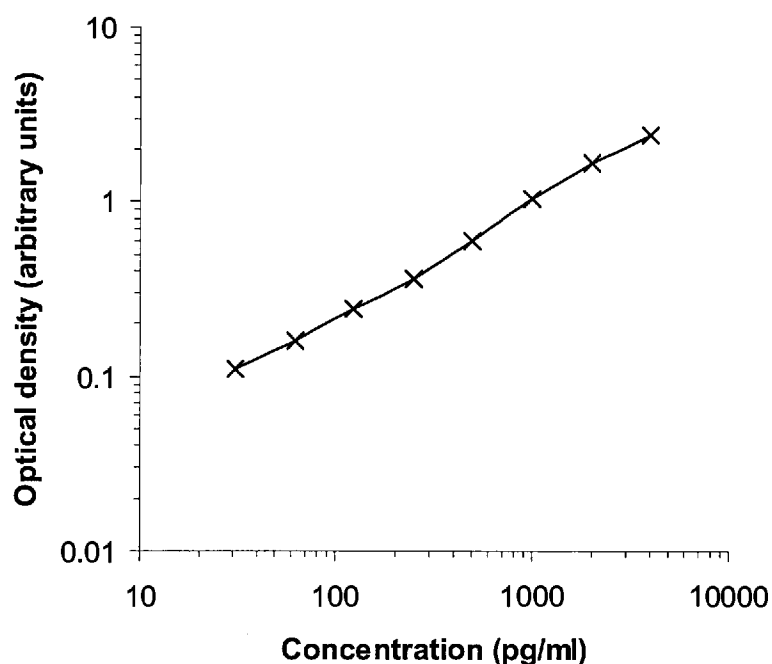


Figure 2.6 Typical standard curve for TNF α ELISA. ELISA, enzyme-linked immunosorbant assay; TNF α , tumour necrosis factor *alpha*.

2.5.10 Measurement of ACTH release by radioimmunoassay

The release of ACTH into the medium of incubated cell lines was determined in duplicate by RIA using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK, California, USA). The primary ACTH antibody was raised in rabbit against human ACTH₁₋₃₉ (code: AFP6328031 National Hormone and Pituitary Program, California, USA) and provided in 2% normal rabbit serum in phosphate buffered saline (PBS), lyophilized and reconstituted in 1ml distilled water. The antibody was directed against the 1-39 region of the peptide and did not cross-react with corticotrophin-like intermediate lobe peptide (CLIP), α -melanocyte-stimulating hormone (α -MSH), LH or prolactin (PRL). The reference preparation was synthetic human ACTH₁₋₃₉ (NIDDK, USA) and provided in 1% BSA in PBS. Standard solutions of this peptide were prepared in triplicate by serial dilution of a stock solution (1 μ g/ml in 0.1M HCl) containing 10mM ascorbic acid, to produce solutions ranging from 19.5 to 10, 000pg/ml. The assay buffer used contained 12.7mM EDTA 68.2mM Na₂HPO₄, 3mM sodium azide, 2mM DTT, 0.1% v/v Triton X-100 and 0.033% w/v BSA in distilled water at pH 7.4 (Sigma, UK). Tubes containing 100 μ l test solution and 200 μ l ACTH antibody (diluted 1:48 000) were prepared in duplicate.

Tubes containing 300µl assay buffer were used to determine non-specific binding and tubes containing 100µl assay buffer and 200µl ACTH antibody were used to determine specific binding. To each tube, 100µl of ^{125}I -labelled human ACTH (NIDDK, USA), which had been diluted in assay buffer to give approximately 10 000 counts per minute, was added. To determine the total number of counts, tubes containing only 100µl of ^{125}I -labelled ACTH were included. The tubes were then vortexed and incubated at 4°C for 4 days. On the fourth day, the bound and free peptides were separated by the addition of 100µl of goat anti-rabbit decanting suspension (Pharmacia Upjohn, Buckinghamshire, UK) to each tube, except for the total counts tubes, and incubated for 2 hours at room temperature. To each tube, 500µl of 0.1% triton X-100 in PBS and 500µl of 30% PEG in 0.05M phosphate buffer was added (excluding the total counts tubes) and centrifuged at 1760g for 1 hour. The supernatant fluid was aspirated and the bound fraction counted on an automatic gamma counter (Perkin-Elmer LAS (UK) Ltd., Beaconsfield, UK) using a 2 minute count time. The concentration of ACTH was calculated from the standard curve (a representative graph is shown in figure 2.7). The inter-assay and intra-assay variations were 11.1% and 10.7% respectively. The detection limit of the assay was 40pg/ml.

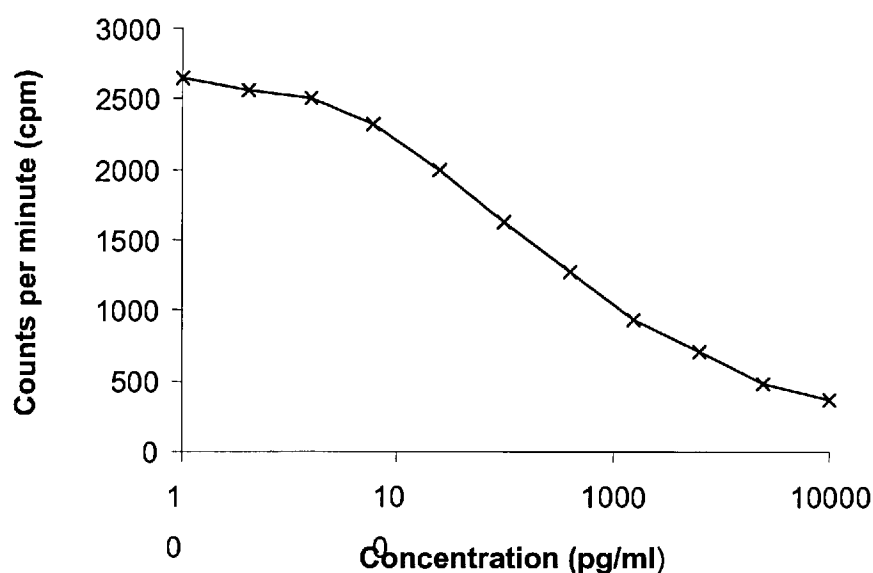


Figure 2.7 Typical standard curve for ACTH RIA. ACTH, adrenocorticotrophin; RIA, radioimmunoassay.

2.5.11 Measurement of plasma rat ACTH by a two-site solid phase immunoradiometric assay

Plasma levels of ACTH in rat was determined in duplicate by an IRMA kit supplied EURO-Diagnostica AB (Malmö, Sweden), The IRMA method has high accuracy and specificity and is a useful and convenient system for quantifying plasma ACTH. The assay involved the use of two polyclonal antibodies in excess. These antibodies recognise different binding sites on the antigen. The first antibody (lyophilised and reconstituted in 12.5ml distilled water) is a highly purified monospecific radioiodinated sheep IgG and recognises the N-terminus of the ACTH molecule. The second antibody reacts non-competitively with the C-terminal region of the ACTH molecule and is immobilised and coupled to primary sheep anti-rabbit coated tubes.

Standard solutions containing lyophilised ACTH were provided in the kit and stored in human plasma matrix with preservative and stabiliser. Each standard was reconstituted in 1ml of distilled water and prepared in duplicate in C-terminal anti-ACTH, coupled to sheep anti-rabbit, coated tubes. The range of standards provided was 0 – 1250pg/ml.

Tubes containing 200µl standard and test plasma were prepared in coated tubes, in duplicate. Non-coated tubes containing 400µl standard A (0pg/ml) were used to determine non-specific binding and coated tubes containing 200µl standard A were used to determine specific binding. To each tube, 200µl ¹²⁵I ACTH antibody was added. To determine the total number of counts, non-coated tubes containing only 200µl ¹²⁵I ACTH antibody were included. The tubes were vortexed gently and incubated overnight at room temperature. During this incubation step, both antibodies react with the ACTH in the sample. A sandwich-type complex is formed, which binds to the tube wall.

The remaining excess of tracer (¹²⁵I Sheep-anti-human ACTH) was removed from each tube (except for the total counts tubes) by washing the coated tubes twice with 2ml of wash buffer (as provided in the kit; reconstituted with 300ml distilled water) and the liquid was aspirated completely. The radioactivity in the tube was counted on an automatic gamma counter (Perkin-Elmer LAS (UK) Ltd., Beaconsfield, UK) using a 2 minute count time. The concentration of ACTH in the plasma was calculated from the standard curve (a representative graph is shown in figure 2.8). The inter-assay

and intra-assay variations were 3.6% and 3.4% respectively. The detection limit of the assay was 3pg/ml.

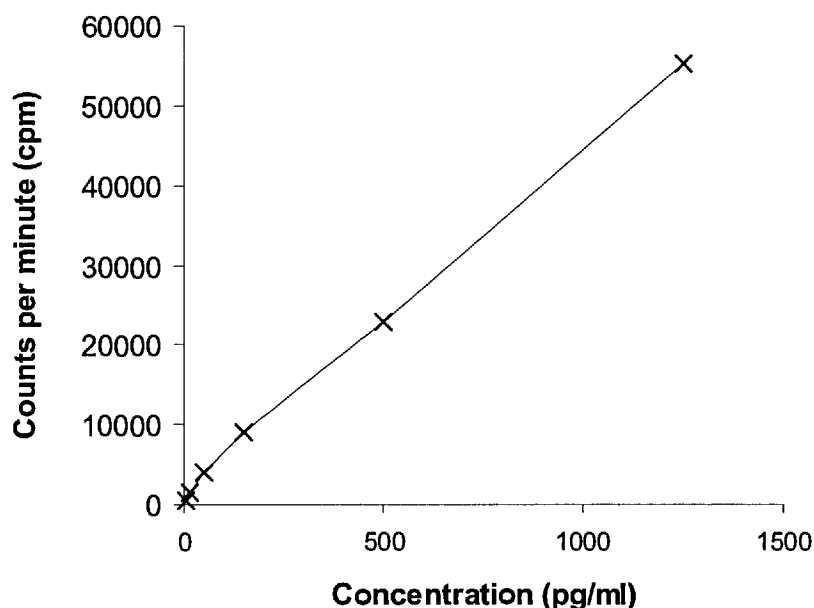


Figure 2.8 Typical standard curve for IRMA ACTH assay. ACTH, adrenocorticotrophin; IRMA, two-site solid phase immunoradiometric assay.

2.5.12 Measurement of plasma rat corticosterone by radioimmunoassay

Plasma concentrations of corticosterone were determined in duplicate using a double antibody RIA kit provided by Immunodiagnostic Systems Ltd (Tyne and Wear, U.K). A polyclonal rabbit anti-corticosterone antiserum was used as the primary antibody in this assay and provided in assay buffer (PBS containing gelatine and a preservative supplied by the manufacturer). Corticosterone standard solutions were prepared in duplicate by serial dilution of a stock solution (1000ng/ml, provided in assay buffer), to produce concentrations ranging from 0.5 – 62.5ng/ml. Tubes containing 50µl of standards and test (plasma) solution, in duplicate, were incubated with 50µl corticosterone antiserum. Tubes containing 100µl assay buffer were used to determine non-specific binding and tubes containing 50µl assay buffer and 50µl corticosterone antiserum were used to determine specific binding. To each tube, 50µl of ¹²⁵I-labelled corticosterone was added. To determine the total number of counts, tubes containing only 50µl of ¹²⁵I-corticosterone ACTH were included. The tubes were then vortexed and incubated at 4°C for 16-24 hours. Following the incubation

period, 50 μ l secondary antibody (goat anti-rabbit gamma globulin in assay buffer) was added to all the tubes, except the total counts tube, and incubated for 1 hour at room temperature. To all the tubes (except total counts), 500 μ l of saline was added and the tubes were centrifuged at 1760g for 15 minutes. The supernatant fluid was aspirated and the bound fraction counted on an automatic gamma counter (Perkin-Elmer LAS (UK) Ltd., Beaconsfield, UK) using a 2 minute count time. The concentration of corticosterone in the plasma was calculated from the standard curve (figure 2.9). The inter-assay and intra-assay variations were 2.0% and 2.3% respectively. The detection limit of the assay was 0.39ng/ml.

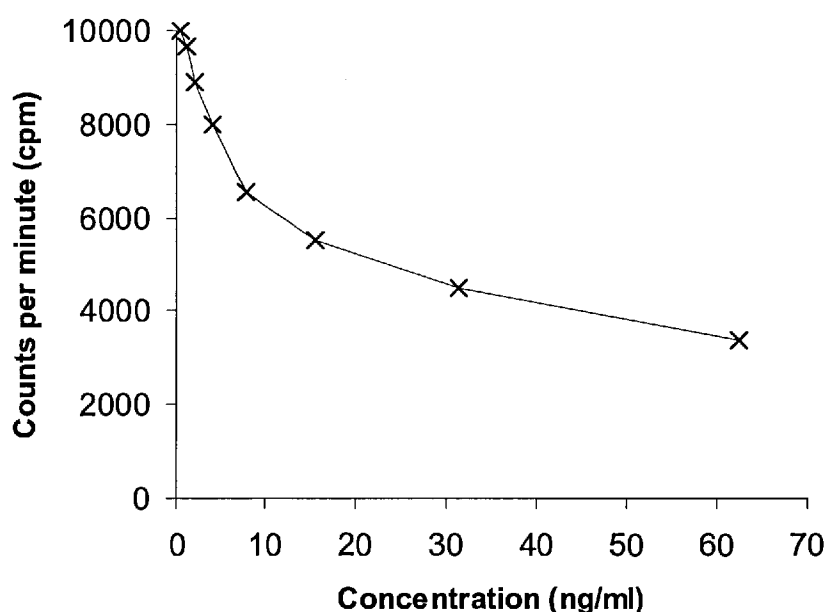


Figure 2.9 Typical standard curve for corticosterone RIA. RIA, radioimmunoassay.

2.5.13 Statistical analysis

All data are expressed as the mean $\pm$ standard error of the mean (SEM). All statistical analyses were carried out using Jandel SigmaStat 3.0 software (Jandal Corporation, San Ramon, CA). Preliminary analysis was undertaken to show that data were normally distributed (Kolmogorov-Smirnov test) and that variances were equal (Levene's median test). Statistical analysis was carried out using one-way ANOVA, two-way ANOVA or three-way ANOVA (as appropriate), with *post hoc*

analysis performed using Bonferroni's corrected Student's *t*-test. For statistical analysis of densitometric results from EMSA gels (chapter 5), the comparison of two groups (n=5, restraint vs control) was performed by the non-parametric Mann-Whitney rank sum test and the comparison of three groups (n=4, untreated control, saline vehicle control and LPS) was performed by Kruskal-Wallis one-way ANOVA followed by the Student-Newman-Keul method. In all cases, differences were considered to be significant at $p < 0.05$.

2.5.14 Drugs and reagents

Table 2.3 **Drugs and reagents.** The following drugs and reagents were used:

Gentamicin	Sigma, Poole, UK
Thyronine sodium salt (T_3)	Sigma, Poole, UK
Oligo (dT) ₁₅ Primer	Promega Ltd, Southampton, UK
rRNasin® Ribonuclease inhibitor	Promega Ltd, Southampton, UK
AMV reverse transcriptase	Promega Ltd, Southampton, UK
<i>Taq</i> DNA polymerase	Promega Ltd, Southampton, UK
Aprotinin	Sigma, Poole, UK
Leupeptin	Sigma, Poole, UK
Goat anti-rabbit p65 polyclonal antibody	Santa Cruz Biotechnology, California, USA
Goat anti-rabbit I κ B α polyclonal antibody	Santa Cruz Biotechnology, California, USA
Goat anti-rabbit IgG antibody	Santa Cruz Biotechnology, California, USA
Mouse monoclonal anti-mouse β -actin antibody	Abcam Ltd, Cambridge, UK
Rabbit anti-mouse IgG antibody	Sigma, Poole, UK
Aprotonin	Bayer UK Ltd, Berkshire, UK
NF κ B consensus oligonucleotide	Promega Ltd, Southampton, UK
AP-1 consensus oligonucleotide	Promega Ltd, Southampton, UK
[γ - ³² P]ATP	Amersham Biosciences Ltd, Chalfont St Giles, UK

T4 polynucleotide kinase	Promega Ltd, Southampton, UK
Rat anti-mouse IL-6 antibody	R&D Systems, Abingdon, UK
Biotinylated goat anti-mouse antibody	R&D Systems, Abingdon, UK
Recombinant mouse IL-6 standard	R&D Systems, Abingdon, UK
Rabbit anti-human ACTH ₁₋₃₉ peptide	National Hormone and Pituitary Program, California, USA
Synthetic human ACTH ₁₋₃₉ peptide	National Hormone and Pituitary Program, California, USA
¹²⁵ I-labelled human ACTH	National Hormone and Pituitary Program, California, USA
LPS (<i>Escherichia coli</i> 0111:B4)	Sigma, Poole, UK
LPS (<i>Escherichia coli</i> 055:B5)	Sigma, Poole, UK
Dexamethasone 21-disodium phosphate	Sigma, Poole, UK
SC-514	Provided by Pfizer Ltd, Kent, UK
Mg132	Tocris Bioscience, Avonmouth, UK
PHA718535E	Provided by Pfizer Ltd, Kent, UK
Recombinant mouse IL-6	Tocris Biosciences, Avonmouth, UK
Recombinant human CRH	R&D Systems, Abingdon, UK
Corticosterone 21-acetate	Sigma, Poole, UK

3 LPS signalling in pituitary-derived cell lines

Immunological challenge with LPS potently activates the HPA axis, causing a release of ACTH from the corticotrophs in the anterior pituitary gland (Butler *et al.* 1989; Feuerstein *et al.* 1990; Ramachandra *et al.* 1992; Schotanus *et al.* 1994; Tilders *et al.* 1994). This activation occurs, in part, through the upregulation of systemically produced IL-6, which has been shown to induce ACTH secretion independently of CRH (Bethin *et al.* 2000). Intra-pituitary IL-6 released from folliculostellate cells (Vankelecom *et al.* 1989; Renner *et al.* 1998) may also act directly on corticotrophs to influence ACTH secretion (Gloddek *et al.* 2001). Glucocorticoids, which are the end products of the activated HPA axis, may therefore target folliculostellate cell derived IL-6 as part of their negative feedback actions, resulting in the inhibition of ACTH synthesis and release (see section 1.7.2).

The aim of this study was to characterise the LPS signalling pathway(s) in the anterior pituitary gland utilising *in vitro* models of folliculostellate cells (murine TtT/GF and human PDFS cell lines) and corticotrophs (murine AtT20 D1 and D16:16 subclones). Further work involved analysis of the functional consequence of LPS signalling by detecting NF κ B activation (by western blot analysis, EMSA and p65 TransAMTM assay; see sections 2.5.4.3, 2.5.5 and 2.5.7 respectively), IL-6 (by ELISA; section 2.5.9) and ACTH release (by RIA; see section 2.5.11), and investigating the possible modulatory role of glucocorticoids upon this system (using the synthetic glucocorticoid, dexamethasone).

3.1 Detection of LPS signalling components in cell lines by RT-PCR

To ensure the cell lines used in these studies were appropriate for examining LPS signalling, expression of mRNAs for the major components necessary for a cellular response to LPS (Tlr4, CD14 and MD-2) were investigated by reverse transcriptase polymerase chain reaction (RT-PCR; see section 3.5.2). The expression of Tlr2, the receptor involved in the recognition of gram-positive bacteria and fungal stimuli, was also explored as signalling through this receptor, as with Tlr4, occurs via CD14 and leads to NF κ B activation. The LPS signalling pathway has been well established in the macrophage-like RAW 264.7 cell line and therefore this cell line was included as a positive control (Du *et al.* 1999).

The detection of mRNA for the housekeeping gene GAPDH in the cell lines was also included in the RT-PCR studies, using the same cDNA for each reaction, to verify that equal amounts of mRNAs were used (a representative PCR gel is shown in figure 3.1E).

3.1.1 TtT/GF cells

In the TtT/GF cells, a band at 1100bp, which corresponds to the expression of CD14 mRNA, was detected (figure 3.1A). The TtT/GF cells also expressed mRNAs for MD-2 and Tlr4, as indicated by bands of 293bp (figure 3.1B) and 300bp (figure 3.1C) respectively. In addition, Tlr2 mRNA was detected in these cells (490bp; figure 3.1D).

3.1.2 PDFS cells

The human derived folliculostellate PDFS cell line expressed mRNAs for CD14 (detected at 300bp; figure 3.1A) and Tlr2 (294bp; figure 3.1D). However, mRNAs for MD-2 (389bp; figure 3.1B) or Tlr4 (438bp; figure 3.1C) were not detected, indicating that the PDFS cells do not possess an intact LPS signalling system and thus do not exhibit all the characteristics assigned previously to folliculostellate cells (Danila *et al.* 2000).

3.1.3 AtT20 D1 and D16:16 subclones

The expression of CD14 mRNA was detected in the D16:16 subclone (1100bp), but not in the D1 subclone (figure 3.1A). However, both subclones expressed mRNAs for MD-2, Tlr4 and Tlr2 (figures 3.1B, C and D respectively).

3.1.4 RAW 264.7 cells

The RAW 264.7 cell line, which was included in the RT-PCR studies as a positive control, expressed mRNAs for CD14, MD-2, Tlr4 and Tlr2 (figures 3.1A, B, C and D).

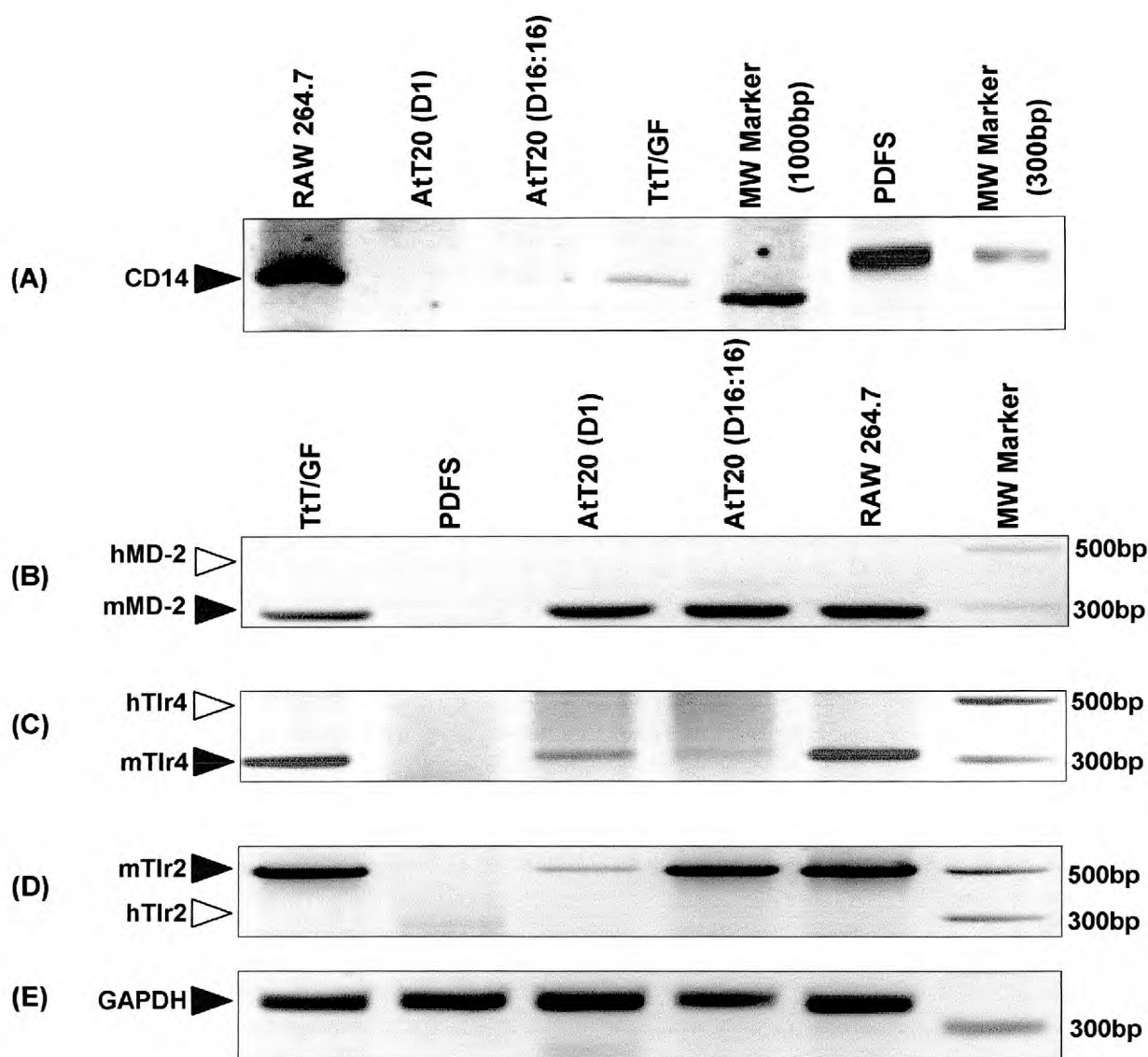


Figure 3.1 Detection of mRNA for the LPS signalling components (A) CD14, (B) MD-2, (c) Tlr4 and (d) Tlr2 in folliculostellate (TtT/GF and PDFS), corticotroph (AtT20 D1 and D16:16 subclones) and macrophage (RAW 264.7) cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR). Representative PCR gel for GAPDH mRNA expression is shown in (E). Data are representative of 3 separate PCR reactions. bp, base pair; h, human; m, murine; MW marker, molecular weight marker; Tlr, Toll-like receptor.

3.2 Examination of the functional effects of LPS stimulation in the TtT/GF, PDFS, AtT20 (D1 and D16:16) and RAW 264.7 cell lines – IL-6 release

Following the confirmation of the presence of mRNAs for the important LPS signalling components in the TtT/GF cell line, the functional relevance of LPS signalling was examined. LPS is a potent stimulator of NF κ B-mediated IL-6 release (Tanabe *et al.* 1988; Akira *et al.* 1990; Libermann & Baltimore 1990; Schobitz *et al.* 1993), which is characteristically produced by folliculostellate cells (Vankelecom *et al.* 1989; Vankelecom *et al.* 1993). Therefore, both NF κ B activation and IL-6 release were examined in the TtT/GF cell line following LPS stimulation. Previous studies have shown that 100ng/ml LPS (in the presence of 10% fetal calf serum) induces a sub-maximal IL-6 response in TtT/GF cells (Lohrer *et al.* 2000) and this was confirmed in our laboratory (Tierney *et al.* 2005). The PDFS cell line was also included in the study to examine whether these cells would respond to LPS despite not expressing Tlr4 or MD-2 mRNAs. Although previous reports have found that corticotrophs do not release IL-6, the presence of the LPS signalling components in the AtT20 subclones prompted further investigation as to whether these corticotroph-like cell lines produced IL-6. For a positive comparison, the profile of NF κ B activation and IL-6 release in LPS-stimulated RAW 264.7 cells was also examined.

3.2.1 TtT/GF cells

The stimulation of the TtT/GF cells with LPS (100ng/ml) resulted in a significant time-dependent increase in IL-6 release, with the effect being first evident after 4 hours contact ($p < 0.001$ vs unstimulated control) and maximal at 16 and 24 hours ($p < 0.01$ vs unstimulated control and preceding LPS stimulated time point; figure 3.2A).

3.2.2 RAW 264.7 cells

The RAW 264.7 cells readily responded to LPS stimulation with an increase in IL-6 release. Elevated amounts of IL-6 were measured in the medium after 4 hours of LPS exposure ($p < 0.001$ vs unstimulated control) being maximal after 16 hours ($p < 0.001$ vs unstimulated control and preceding LPS stimulated time point; figure 3.2B). The concentration of LPS-stimulated IL-6 release in the medium of the RAW 264.7 cells was significantly higher than that measured in the medium of the TtT/GF cells from 4 hours up to 24 hours post stimulation ($p < 0.001$), with the maximal IL-6

concentrations from the RAW 264.7 cells (figure 3.2B) being 50 times greater than that of TtT/GF cells (figure 3.2A).

3.2.3 PDFS cells

The effects of LPS (100ng/ml, 0-24 hours) stimulation on IL-6 release from the PDFS cells were investigated. Unlike the TtT/GF cells, the PDFS cells did not produce detectable amounts of IL-6 at any of the time points investigated (Table 3.1), confirming our suggestion that this cell line will not respond to LPS due to the absence of the Tlr4 receptor, a requisite for LPS signalling.

3.2.4 AtT20 D1 and D16:16 subclones

The AtT20 D1 and D16:16 subclones also failed to produce detectable amounts of IL-6 (AtT20 D1; Table 3.2 and AtT20 D16:16; Table 3.3), despite expressing Tlr4, MD-2 and CD14 mRNA (figure 3.1A, B and C), further supporting their similarity to primary corticotrophs, which do not release IL-6 (Yeung *et al.* 2006).

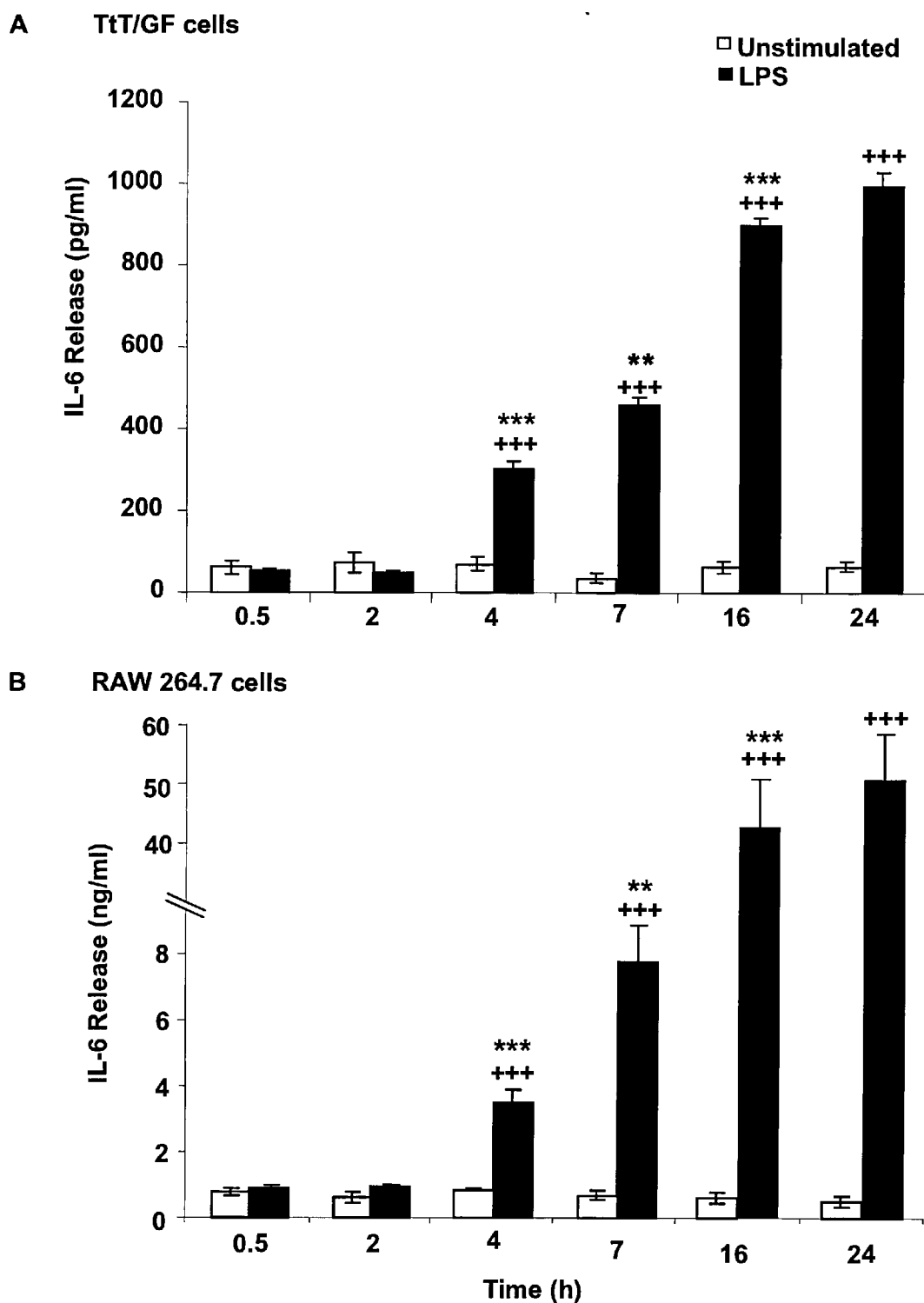


Figure 3.2 Time-dependent effects of LPS (100ng/ml, 30 minutes - 24 hours) on IL-6 release from (A) TtT/GF and (B) RAW 264.7 cells. Each value is a mean $\pm$ SEM of four samples. The data are representative of three separate experiments. *** $p < 0.01$ vs unstimulated control, ** $p < 0.01$, *** $p < 0.001$ vs preceding time points. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. IL-6, interleukin 6; LPS, lipopolysaccharide.

Table 3.1 Time-dependent effect of LPS (100ng/ml, 30 minutes - 24 hours) stimulation on IL-6 release from PDFS cells. The detection limit of the human IL-6 ELISA assay was 4.6pg/ml. ELISA, enzyme linked immunosorbant assay; IL-6, interleukin 6; LPS, lipopolysaccharide.

Time (h)	IL-6 release (pg/ml)	
	Unstimulated	LPS (100ng/ml)
0.5	undetectable	undetectable
2	undetectable	undetectable
4	undetectable	undetectable
7	undetectable	undetectable
16	undetectable	undetectable
24	undetectable	undetectable
Known standard (100pg/ml)	103.6 ± 3.8	-

Table 3.2 Time-dependent effect of LPS (100ng/ml, 30 minutes - 24 hours) stimulation on IL-6 release from AtT20 D1 cells. The detection limit of the mouse IL-6 ELISA was 3.9pg/ml. ELISA, enzyme linked immunosorbant assay; IL-6, interleukin 6; LPS, lipopolysaccharide.

Time (h)	IL-6 release (pg/ml)	
	Unstimulated	LPS (100ng/ml)
0.5	undetectable	undetectable
2	undetectable	undetectable
4	undetectable	undetectable
7	undetectable	undetectable
16	undetectable	undetectable
24	undetectable	undetectable
Known standard (100pg/ml)	64.7± 2.9	-

Table 3.3 Time-dependent effect of LPS (100ng/ml, 30 minutes - 24 hours) stimulation on IL-6 release from AtT20 D16:16 cells. The detection limit of the mouse IL-6 ELISA was 3.9pg/ml. ELISA, enzyme linked immunosorbant assay; IL-6, interleukin 6; LPS, lipopolysaccharide.

Time (h)	IL-6 release (pg/ml)	
	Unstimulated	LPS (100ng/ml)
0.5	undetectable	undetectable
2	undetectable	undetectable
4	undetectable	undetectable
7	undetectable	undetectable
16	undetectable	undetectable
24	undetectable	undetectable
Known standard (100pg/ml)	85.41± 1.9	-

3.3 Effects of LPS stimulation upon NF κ B activity in TtT/GF and RAW 264.7 cells

The nuclear translocation of the functionally active p65 subunit of NF κ B was examined by western blot analysis in the TtT/GF and RAW 264.7 cells, following LPS challenge (100ng/ml, 0-24 hours). The expression of the cytosolic inhibitory protein I κ B α was also examined, as NF κ B activation is associated with I κ B α degradation. In parallel, the functional activation of NF κ B (p65/p50) was investigated, by assessing the DNA binding activity of the transcription factor to its consensus DNA sequence by EMSA.

3.3.1 Nuclear translocation of p65 and I κ B α protein degradation in LPS stimulated TtT/GF cells

The p65 subunit of NF κ B was readily detected in cytoplasmic extracts of TtT/GF cells (figure 3.3). The intensity of the band corresponding to p65 was reduced by exposure of the cells to LPS (100ng/ml) for 30 minutes and 2 hours. Within 4 hours, cytoplasmic p65 protein expression had increased to above control levels (figure 3.3A). The decrease in cytoplasmic p65 protein was paralleled by an increase in p65 protein expression in the nuclear extracts compared to unstimulated cells, where p65 expression was barely detectable in the nucleus (figure 3.3B). A further decrease in cytoplasmic p65 was evident in the cells after 16 hours stimulation with LPS, but with levels higher than control at 24 hours (figure 3.3A). However, the nuclear levels of p65 expression remained unchanged at 16 and 24 hours (figure 3.3B).

I κ B α protein expression was readily detected by western blot analysis in cytosolic fractions of TtT/GF cells. The degradation of cytosolic I κ B α was evident after 30 minutes exposure to LPS and thereafter returned to control levels (figure 3.4). The time frame of I κ B α degradation correlated with the increased levels of nuclear p65 expression (figure 3.3B).

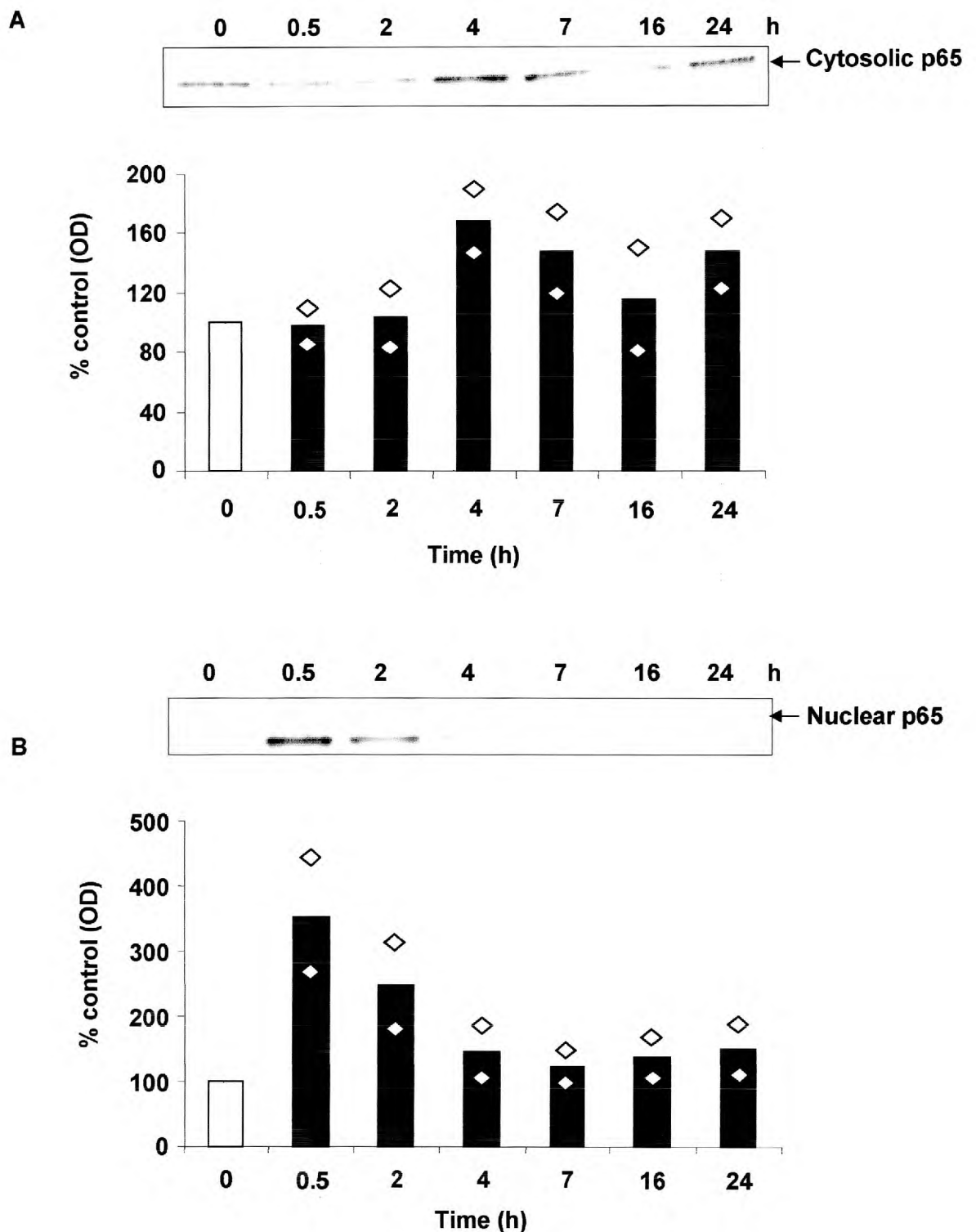


Figure 3.3 Time-dependent effects of LPS (100ng/ml, 0-24 hours) on p65 protein expression in (A) cytosolic (20 μ g) and (B) nuclear extracts (20 μ g) of TtT/GF cells, detected by western blot analysis. The data are representative of three separate experiments and the histogram represents the mean optical density of the bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. LPS, lipopolysaccharide; OD, optical density.

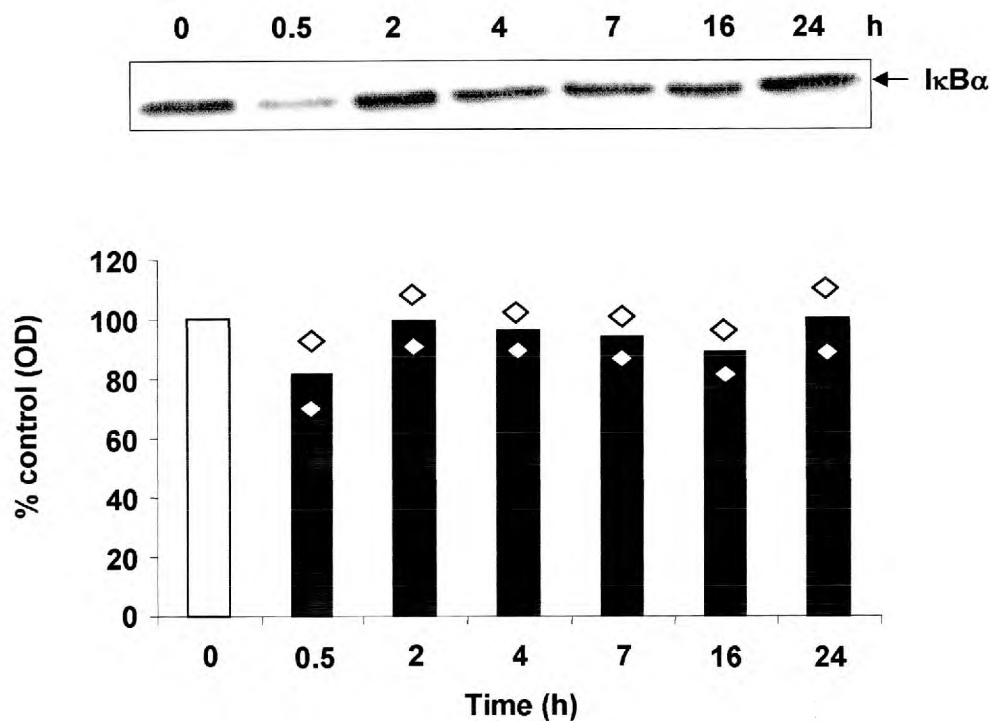


Figure 3.4 Time-dependent effects of LPS (100ng/ml, 0-24 hours) on I κ B α protein expression in cytosolic (20 μ g) extracts of TtT/GF cells, detected by western blot analysis. The data are representative of three separate experiments and the histogram represents the mean optical density of the bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. I κ B α , inhibitor of *kappa* B *alpha*; LPS, lipopolysaccharide; OD, optical density.

3.3.2 Detection of NF κ B DNA binding activity in LPS stimulated TtT/GF cells by EMSA

To verify the activation of NF κ B by LPS (100ng/ml; 0-24 hours) stimulation, the DNA binding activity of NF κ B was assessed in nuclear extracts of TtT/GF cells by EMSA (section 4.4.12). Two bands were detected, the higher band (1, figure 3.5A) representing the larger p65/p50 heterodimer and the lower band representing the p50/p50 homodimer (2, figure 3.5A; see section 2.5.6 in methods). The DNA-binding activity of the p65/p50 heterodimer, detected by EMSA (figure 3.5B), corresponded with the profile of nuclear p65 protein expression detected by western blot analysis (figure 3.3). NF κ B (p65/p50) DNA binding was increased following 30 minutes and 2 hours LPS stimulation in the TtT/GF cell line, further confirming our findings that NF κ B activation in the TtT/GF cell line is a rapid response (figure 3.5).

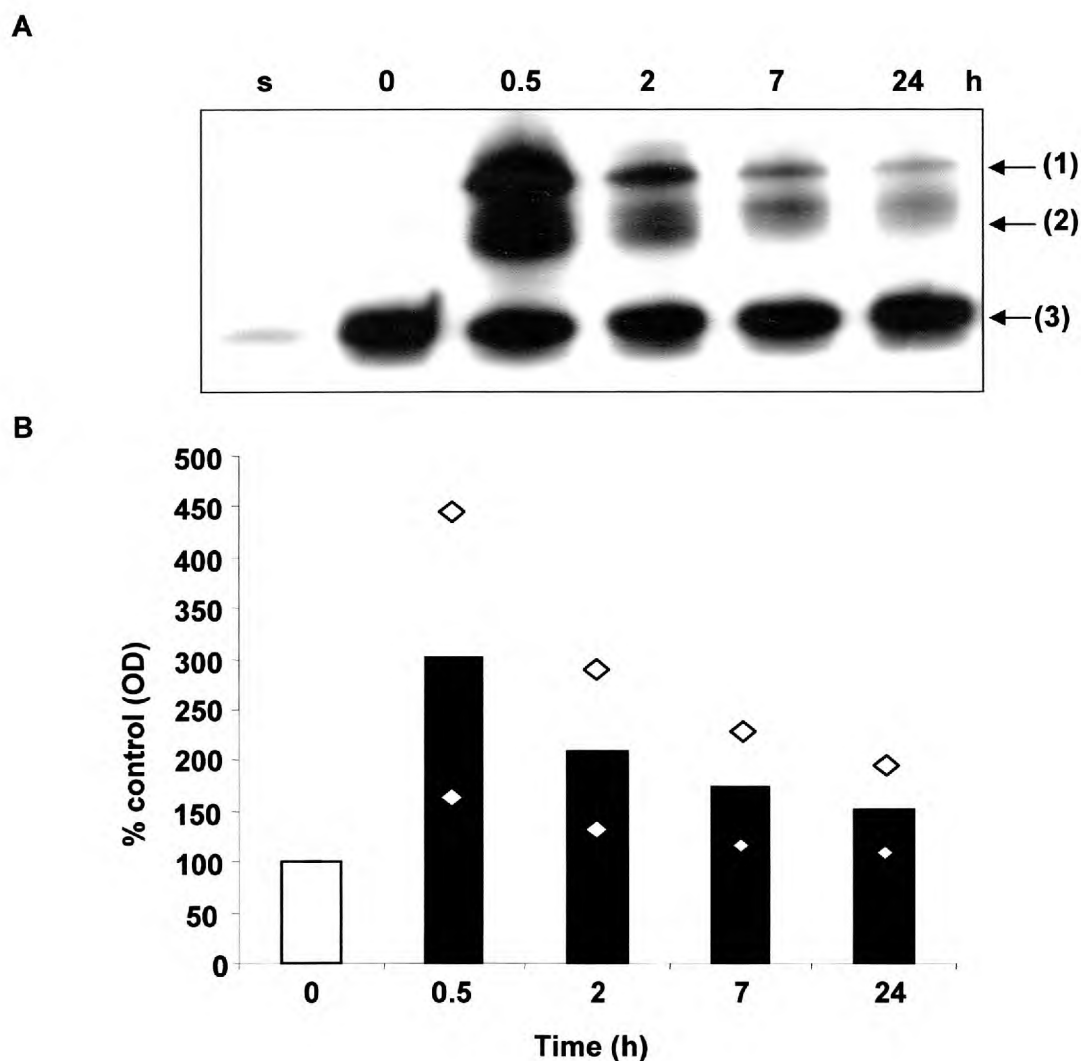


Figure 3.5 Time-dependent effects of LPS (100ng/ml, 0-24 hours) on NF κ B DNA binding activity in nuclear extracts (10 μ g) of TtT/GF cells, detected by (A) electrophoretic mobility shift assay (EMSA). To confirm NF κ B binding, a competitive binding reaction was performed with 100 fold molar excess of unlabelled NF κ B consensus oligonucleotides (lane s). Upper band (1) represents p65/p50 heterodimer and lower band (2) represents p50/p50 homodimer. Bands labelled as (3) represent non-specific binding. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; OD, optical density; s, competitive NF κ B specific binding.

3.3.3 Effects of LPS stimulation on the p65 nuclear translocation and I κ B α protein degradation in LPS stimulated RAW 264.7 cells

In contrast to the profile of NF κ B (p65/p50) activity seen in the TtT/GF cells, the RAW 264.7 cells exhibited a prolonged activation of NF κ B in response to LPS stimulation. A decrease in cytoplasmic p65 protein expression was evident with 30 minutes of LPS stimulation compared to control levels (figure 3.6A). The levels declined progressively with time, such that the protein was barely detectable at 24 hours (figure 3.6A). The decrease in cytoplasmic p65 was associated with an increase in nuclear p65 protein expression, which persisted for 7 hours, with p65 expression returning to levels comparable to those of the unstimulated control at 16 hours and 24 hours (figure 3.6B). The LPS-induced increase in nuclear p65 protein in the RAW 264.7 cells was accompanied by a decrease in cytoplasmic I κ B α protein, which persisted for 24 hours (figure 3.7).

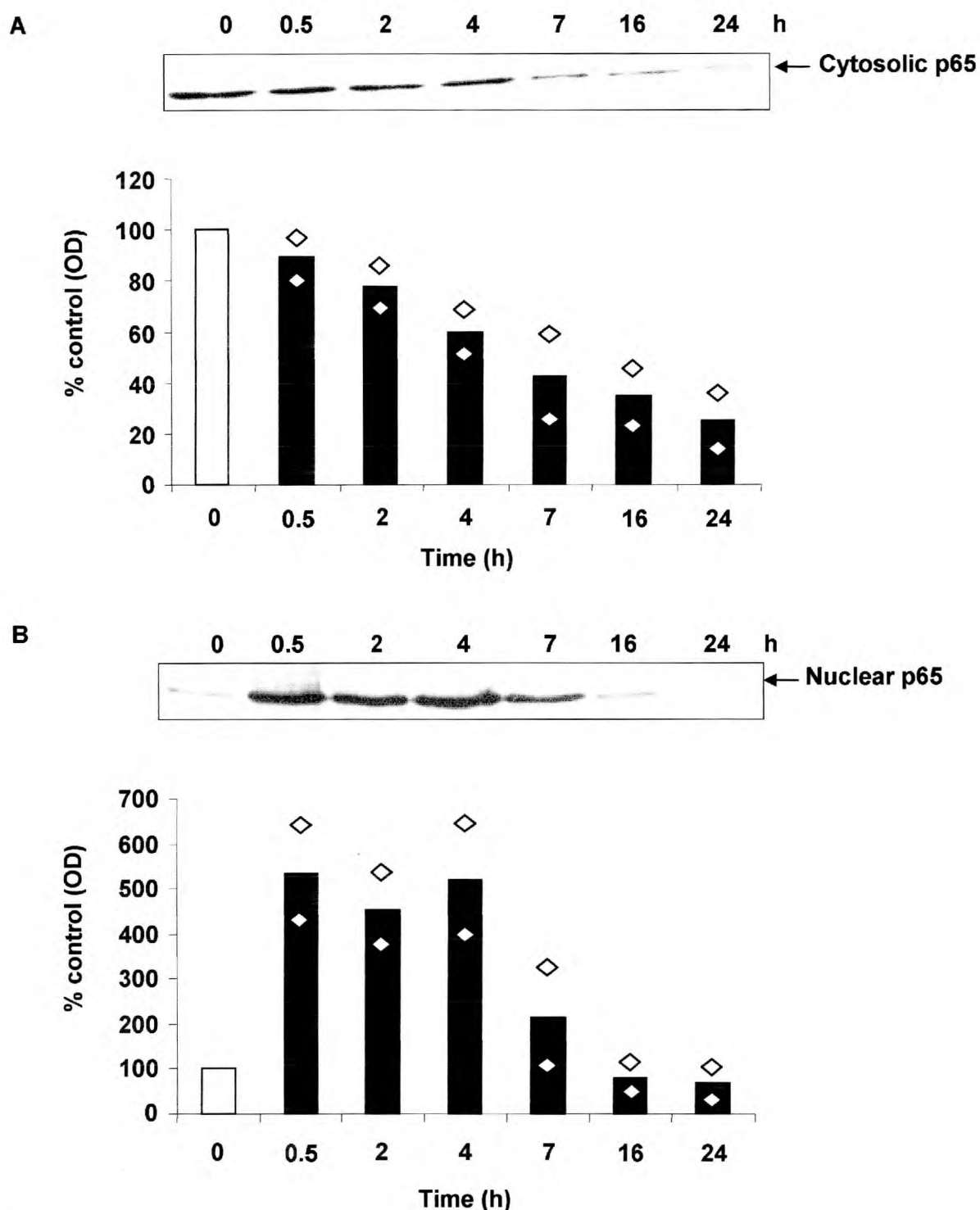


Figure 3.6 Time-dependent effects of LPS (100ng/ml, 0-24 hours) stimulation on protein expression of p65 in (A) cytosolic (20 μ g) and (B) nuclear (20 μ g) extracts of RAW 264.7 cells, detected by western blot analysis. The data are representative of two separate experiments and the histogram represents the mean optical density of the bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. LPS, lipopolysaccharide; OD, optical density.

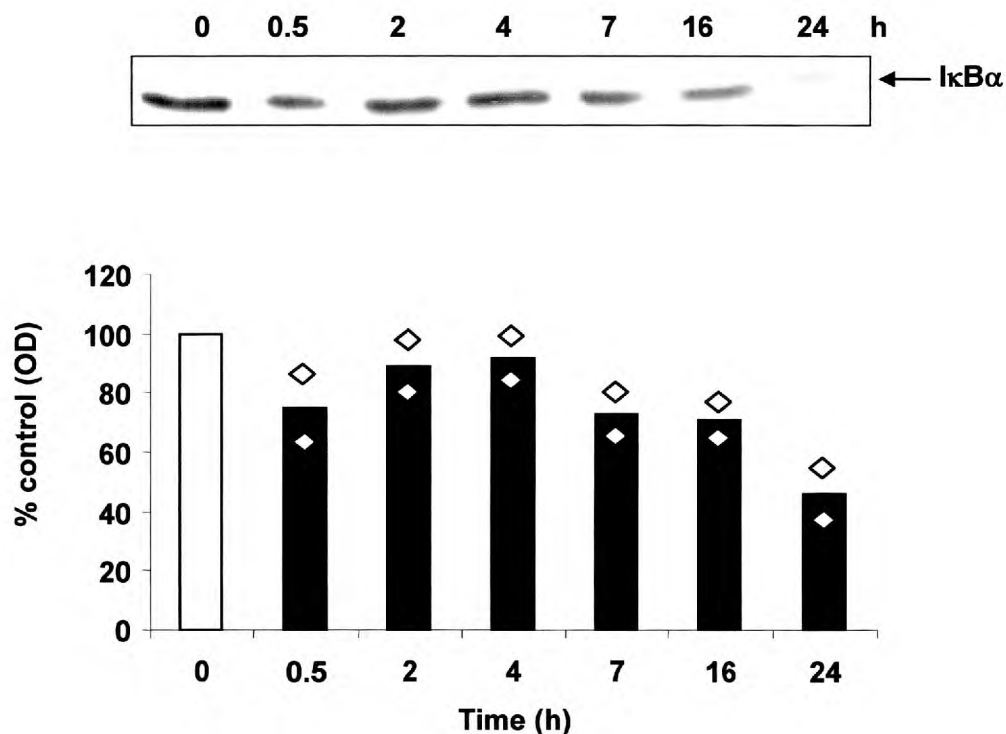


Figure 3.7 Time-dependent effects of LPS (100ng/ml, 0-24 hours) stimulation on protein expression of IκBκ in cytosolic (20μg) extracts of RAW 264.7 cells, detected by western blot analysis. The data are representative of two separate experiments and the histogram represents the mean optical density of the bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. IκBα, inhibitor of *kappa* B *alpha*; LPS, lipopolysaccharide; OD, optical density.

3.3.4 Detection of NF κ B DNA binding activity in LPS stimulated RAW 264.7 cells by EMSA

The time-dependent changes in the DNA binding activity of the p65/p50 heterodimer detected by EMSA (1, figure 3.6A) in the LPS (100ng/ml; 0-24 hours) stimulated RAW 264.7 cells correlated with the profile of nuclear p65 protein expression detected by western blot analysis (figure 3.6B), with increased NF κ B (p65/p50) activity observed at all the time points examined over the 7 hour stimulation period (figure 3.8B). This activity was reduced at 24 hours, but still higher than the control (figure 3.8B).

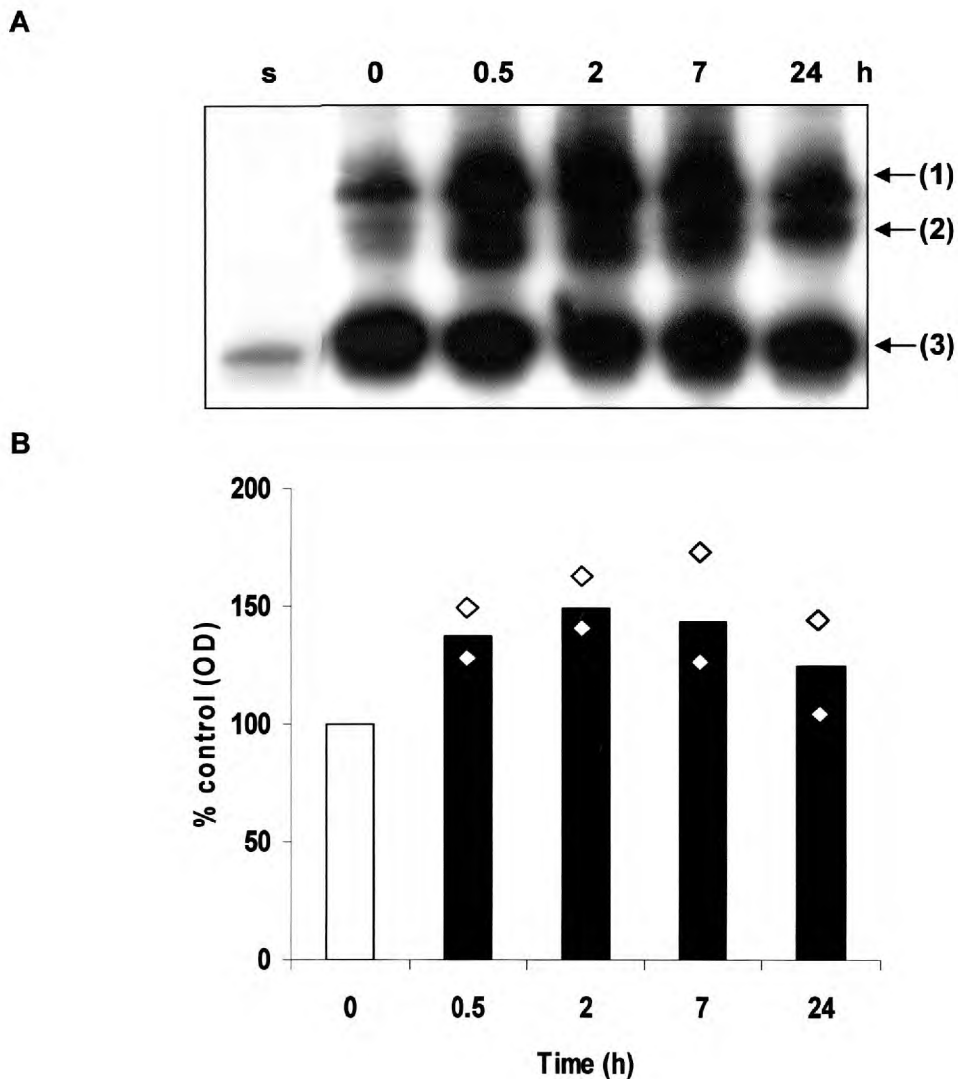


Figure 3.8 Time-dependent effects of LPS (100ng/ml, 0-24 hours) on NF κ B DNA binding activity in nuclear extracts (10 μ g) of RAW 264.7 cells, detected by (A) electrophoretic mobility shift assay (EMSA). To confirm NF κ B binding, a competitive binding reaction was performed with 100 fold molar excess of unlabelled NF κ B consensus oligonucleotides (lane s). Upper band (1) represents p65/p50 heterodimer and lower band (2) represents p50/p50 homodimer. Bands labelled as (3) represent non-specific binding. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; OD, optical density; s, competitive NF κ B specific binding.

3.4 Effects of NF κ B inhibition upon LPS stimulated IL-6 release from TtT/GF cells

Whilst the IL-6 promoter is known to be regulated by multiple transcription factors (see section 1.5.2.3), the relative importance of each of these varies, depending on the cell type (Akira *et al.* 1990; Matsusaka *et al.* 1993; Galien *et al.* 1996; Deshpande *et al.* 1997; Ray *et al.* 1997). To examine whether IL-6 transcription is regulated by NF κ B in the TtT/GF cell line, the effect of inhibition of NF κ B activity upon the release of LPS-stimulated IL-6 was investigated, using the specific IKK β inhibitor SC-514 and the proteasome inhibitor Mg132. To examine whether IL-6 release and NF κ B activation in the TtT/GF cells are sensitive to glucocorticoid action, the synthetic glucocorticoid dexamethasone was included in the medium.

Our previous studies have shown LPS induces a time-dependent increase in IL-6 from TtT/GF cells (figure 3.2A), with optimal concentrations measured after 16 hours incubation. Therefore, we used this time point to investigate the effects of including SC-514 (50-300 μ M), Mg132 (1-100 μ M) or dexamethasone (1-100nM) in the medium of TtT/GF cells on the release of IL-6 induced by LPS (100ng/ml, 16 hours). As an increase in NF κ B (p65/p50) activity was detected after 30 minutes LPS challenge in the TtT/GF cells, (figure 3.3 and 3.5), the effect of these compounds on LPS stimulated NF κ B (p65/p50) activity was investigated at this time point.

3.4.1 Effect of SC-514 treatment on NF κ B activity and IL-6 release from LPS stimulated TtT/GF cells

Incubation of the TtT/GF cells with either SC-514 (25-100 μ M, 30 minutes) or the vehicle solution (0.2% DMSO, 30 minutes) did not alter basal NF κ B (p65/p50) DNA-binding activity compared to unstimulated control samples (1, figure 3.9A and B). NF κ B (p65/p50) activation induced by LPS stimulation was not affected by the presence of DMSO, thus any effects seen with SC-514 were due to the compound and not the DMSO vehicle (figure 3.9A and B). As shown by densitometric analysis, LPS stimulated NF κ B (p65/p50) activation was attenuated by SC-514 treatment in a concentration-dependent manner (figure 3.9B).

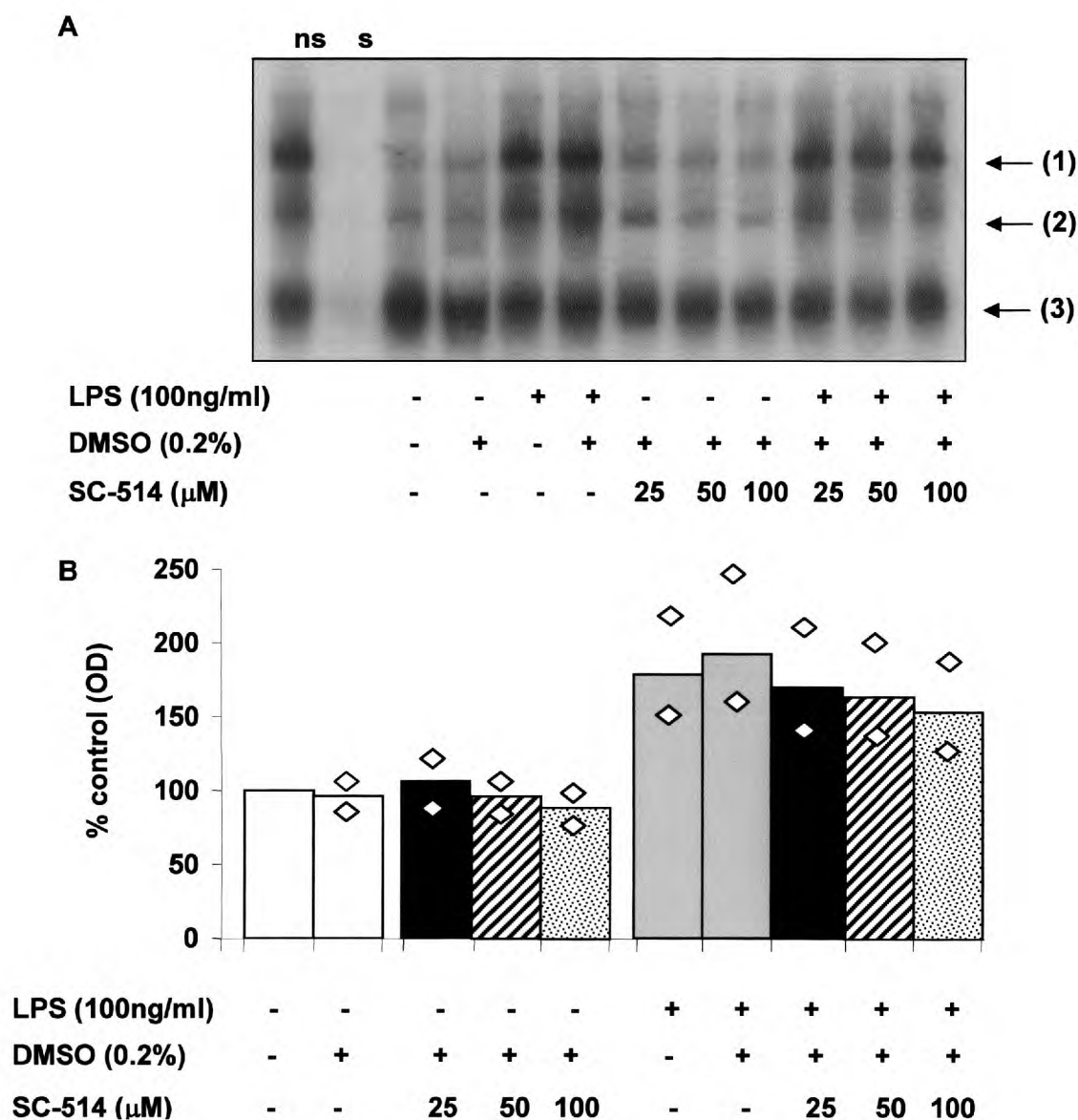


Figure 3.9 Effect of the IKK β inhibitor, SC-514 (25-100 μ M, 90 minutes), on LPS (100ng/ml, 30 minutes) stimulated NF κ B activity in nuclear extracts (10 μ g) of TtT/GF cells, detected by (A) electrophoretic mobility shift assay (EMSA). Bands labelled (1) represent p65/p50 heterodimer, (2) p50/p50 homodimer and (3) non-specific binding. Cells were treated with SC-514 for 1 hour prior to LPS stimulation and the drug was kept in the medium throughout the 30 minute incubation period with LPS. (B) The data are representative of three separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (untreated control). The points on the histogram represent the range values. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). DMSO, dimethylsulfoxide; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; OD, optical density.

The vehicle solution, (0.2% DMSO, 16 hours) did not affect basal ($p=1.00$) or LPS (100ng/ml, 16 hours) stimulated ($p=0.512$) IL-6 release from the TtT/GF cells (figure 3.10). Treatment with SC-514 (50-300 μ M, 16 hours) alone did not affect IL-6 release ($p=1.00$; figure 3.10), but incubation of the cells with 50 and 100 μ M SC-514 resulted in an attenuation of LPS-stimulated IL-6 release ($p<0.01$ vs LPS; figure 3.10). In contrast, the higher concentration of SC-514 (300 μ M) was without effect ($p=0.229$; figure 3.10).

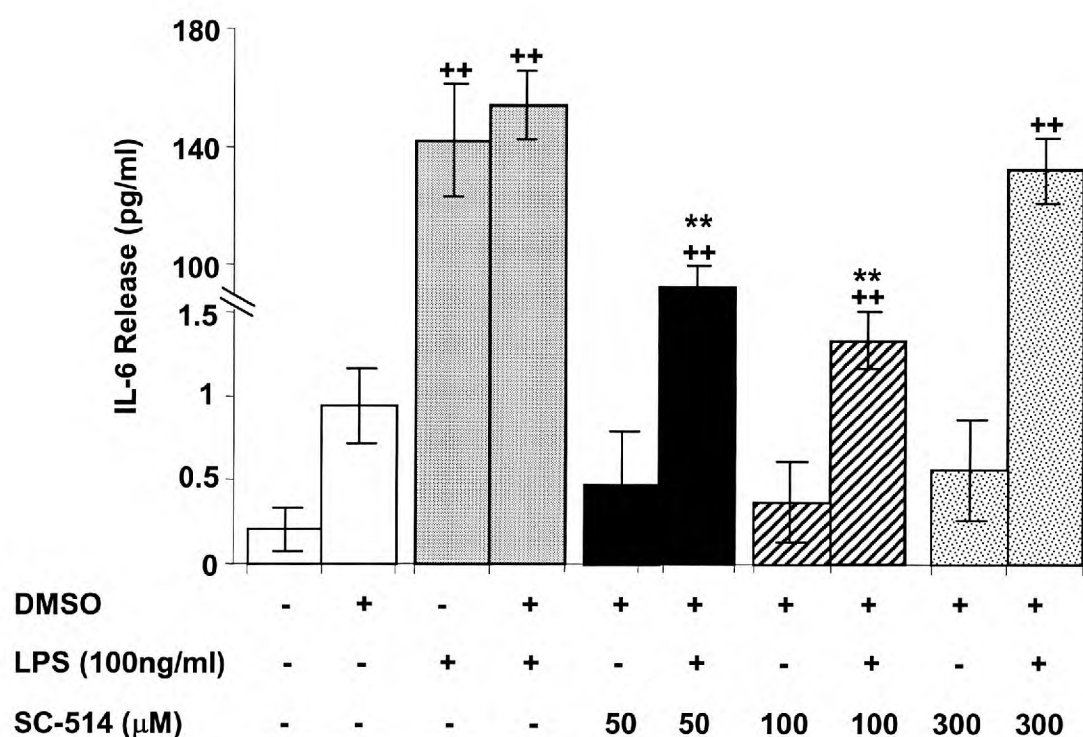


Figure 3.10 Effect of the IKK β inhibitor, SC-514 (50-300 μ M, 17 hours), on LPS (100ng/ml, 16 hours) stimulated IL-6 release from TtT/GF cells. The cells were incubated with SC-514 or vehicle (0.2% DMSO) for 1 hour prior to the addition of LPS and the drug was kept in the medium throughout the 16 hour incubation period with LPS. Control and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. ** $p<0.01$ vs unstimulated control (Con), *** $p<0.001$ vs LPS alone. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. IL-6, interleukin 6; LPS, lipopolysaccharide.

3.4.2 Effects of the proteasome inhibitor Mg132 on LPS stimulated NF κ B activity and IL-6 release from TtT/GF cells

The effects of the proteasome inhibitor Mg132 (1-50 μ M, 90 minutes) on LPS (100ng/ml, 30 minutes) induced NF κ B activity in the TtT/GF cells were examined by EMSA (figure 3.11A and B). Several bands representing NF κ B DNA-binding complexes were detected by EMSA (figure 3.11A). The lower two bands (labelled as 3 and 4) are likely to represent non-specific binding as they were still highly expressed in the competitive reaction (lanes s; figure 3.11A) compared to the non-competitive reaction (lane ns; figure 3.11A). The higher two bands, labelled (1) and (2) represent the different dimer compositions of NF κ B, with the higher of the two being the p65/50 heterodimer (1) and the lower being p50/p50 (2) (figure 3.11A; see section in 2.5.6).

Basal levels of NF κ B (p65/p50) activity were increased in the TtT/GF cells following 90 minutes incubation with a low (1 μ M) concentration of Mg132 but higher concentrations (10 and 50 μ M) were without effect (figure 3.11B). The vehicle (ethanol, 2mM) did not affect resting levels of NF κ B (p65/p50) but it caused a small increase in LPS induced NF κ B activity (figure 3.11B). Mg132 (1 and 10 μ M) decreased LPS-stimulated NF κ B activation, but this effect was more pronounced when the concentration was raised to 50 μ M (figure 3.11B).

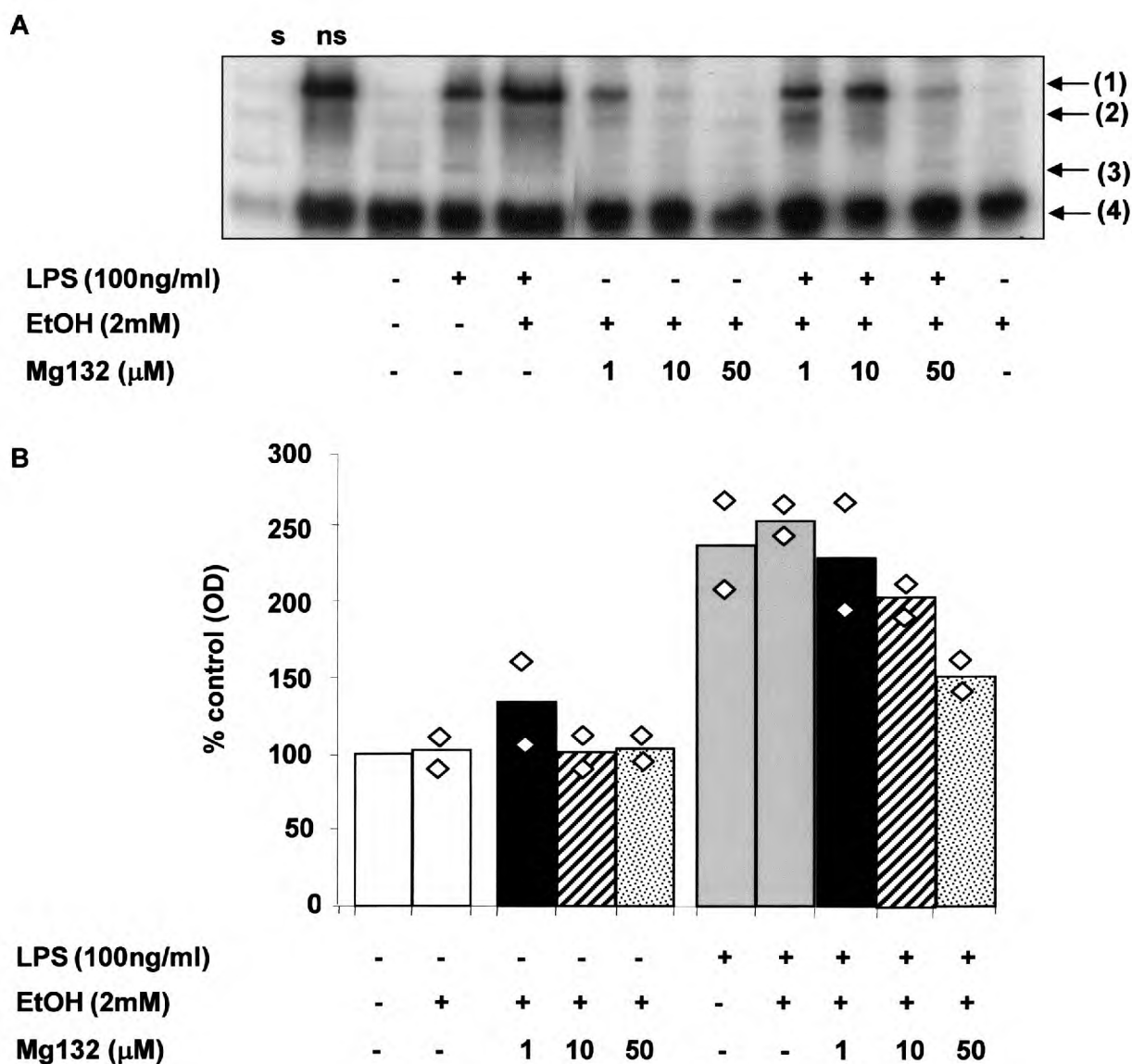


Figure 3.11 Effect of the proteasome inhibitor, Mg132 (1-50μM, 90 minutes), on LPS (100ng/ml, 30 minutes) stimulated NFκB activation in TtT/GF cells, detected by (A) electrophoretic mobility shift assay (EMSA). The cells were incubated with Mg132 for 1 hour prior to the addition of LPS and the drug remained in the medium in the presence of LPS. Bands labelled (1) represent the p65/p50 heterodimer; (2), p50/p50 homodimer and (3) and (4) represent non-specific binding. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (untreated control). The points on the histogram represent the range values. Competitive reactions were included with 100 fold molar excess of the NFκB oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). EtOH, ethanol; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B; OD, optical density.

Treatment with Mg132 (25 μ M, $p=0.183$) did not affect basal IL-6 release and the vehicle (2mM EtOH, $p=1.00$) did not influence the stimulatory effect of LPS (figure 3.12). However, treatment with 50 μ M Mg132 alone potentiated basal IL-6 release ($p<0.05$ vs control). Mg132 treatment (25 μ M) significantly reduced LPS-stimulated IL-6 release ($p<0.001$ vs LPS), with a 2-fold higher concentration being less potent (50 μ M; $p<0.01$ vs 25 μ M Mg132 + LPS; figure 3.12).

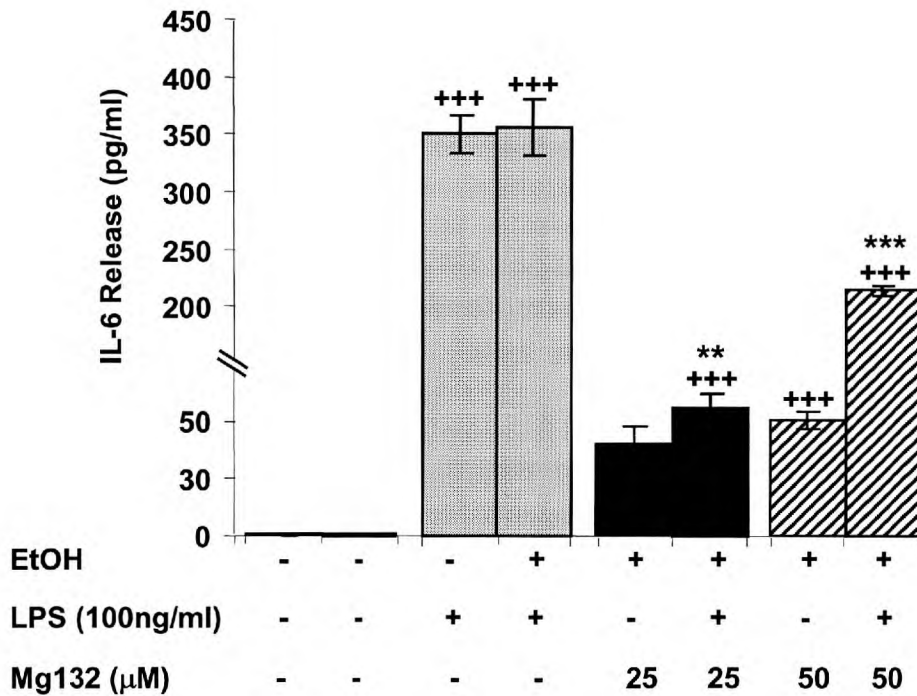
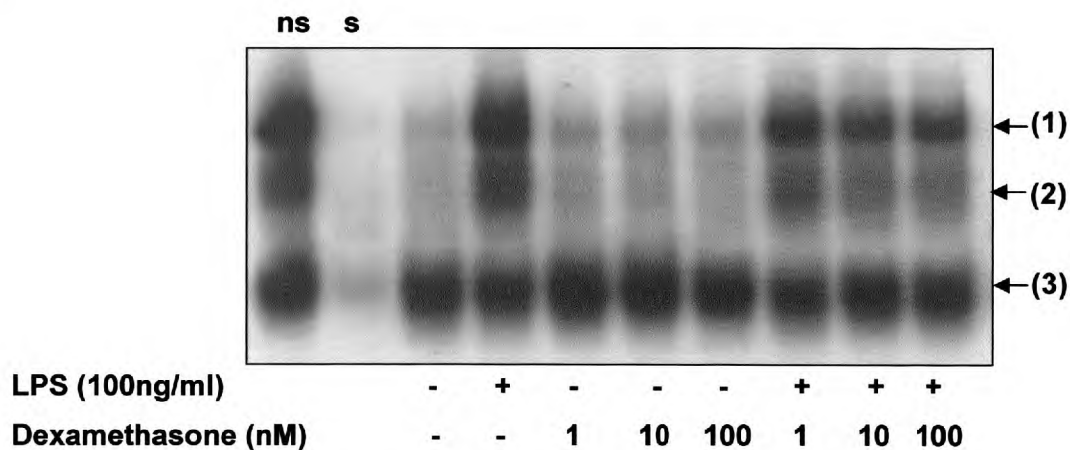


Figure 3.12 Effect of proteasome inhibitor, Mg132 (25 and 50 μ M), on LPS (100ng/ml, 16 hours) stimulated IL-6 release from TtT/GF cells. The cells were incubated with Mg132 or vehicle (2mM ethanol) for 1 hour prior to the addition of LPS. Control and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. *** $p<0.001$ vs unstimulated control (Con), ** $p<0.01$, *** $p<0.001$ vs LPS alone. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. EtOH, ethanol; IL-6, interleukin 6; LPS, lipopolysaccharide.

3.4.3 Effects of dexamethasone upon LPS stimulated NF κ B activity and IL-6 release from TtT/GF cells

The treatment of the TtT/GF cells with dexamethasone alone (1-100nM) did not alter the basal level of NF κ B (p65/p50) activity (labelled at 1; figure 3.13A), but it reduced the stimulatory effect of LPS (100ng/ml, 30 minutes) in a concentration-dependent manner (figure 3.13A and B). The inhibitory effects of glucocorticoids upon NF κ B activity have been shown to occur, at least in part, through the upregulation of I κ B α (Auphan *et al.* 1995, Scheinman, 1995 #112). To investigate whether dexamethasone increased the expression of I κ B α in the TtT/GF cells, the protein levels of cytosolic I κ B α were detected by western blot analysis in samples that had been treated with dexamethasone (10nM, 30 minutes) in the presence and absence of LPS (100ng/ml, 30 minutes; figure 3.14A). LPS stimulation decreased the protein expression of I κ B α compared to control levels (figure 3.14A and B). In contrast, dexamethasone treatment alone and with LPS increased I κ B α protein expression compared to the control and LPS stimulated samples (figure 3.14A and B).

A



B

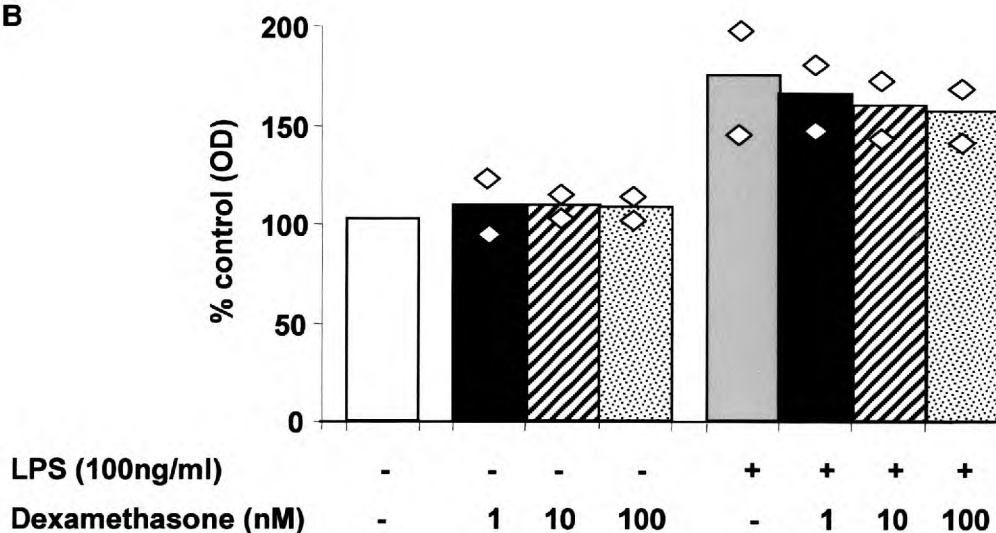
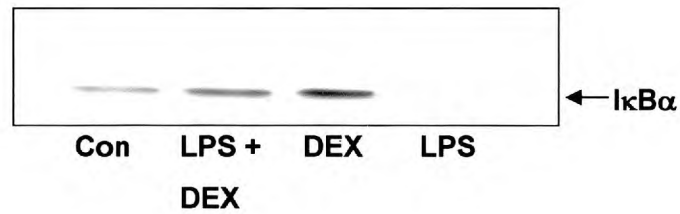


Figure 3.13 Effect of the synthetic glucocorticoid, dexamethasone (1-100nM, 30 minutes), in the presence and absence of LPS (100ng/ml, 30 minutes), on NF κ B activity in nuclear (10 μ g) TtT/GF cells, detected by (A) electrophoretic mobility shift assay (EMSA). (1) p65/p50 heterodimer; (2) p50/p50 homodimer and (3) non-specific binding. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (untreated control). The points on the histogram represent the range values. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; OD, optical density.

A



B

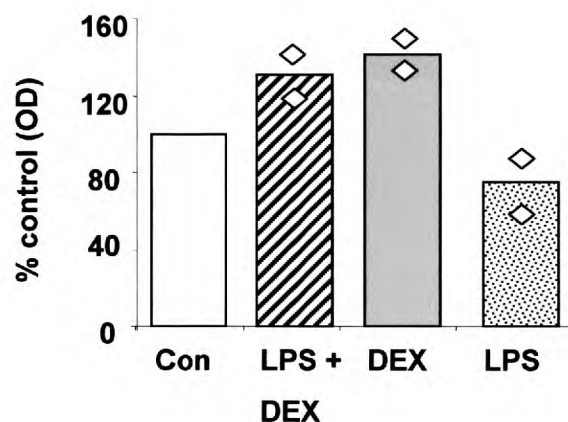


Figure 3.14 Effect of the synthetic glucocorticoid, dexamethasone (10nM, 30 minutes), on cytosolic IκBα (20μg) protein expression in LPS (100ng/ml, 30 minutes) stimulated TtT/GF cells. IκBα protein expression was detected by (A) western blot analysis and (B) densitometric analysis of IκBα protein expression (expressed as percentage of control). The data are representative of two separate experiments and the histogram represents the mean optical density of the bands, expressed as a percentage of control (untreated). The points on the histogram represent the range values. Con; unstimulated control, DEX, dexamethasone, IκBα; inhibitor of *kappa B alpha*; LPS; lipopolysaccharide, OD; optical density.

IL-6 was undetectable in the medium of both unstimulated and dexamethasone treated (1-100nM, 16 hours) cells (figure 3.15). Inclusion of LPS (100ng/ml) for 16 hours induced a robust increase in IL-6 release ($p<0.001$ vs control; figure 3.15). Dexamethasone attenuated the stimulatory effect of LPS in a concentration dependent manner (10 and 100nM; $p<0.001$ vs LPS), with the lowest concentration used having no effect (1nM; $p=0.058$; figure 3.15).

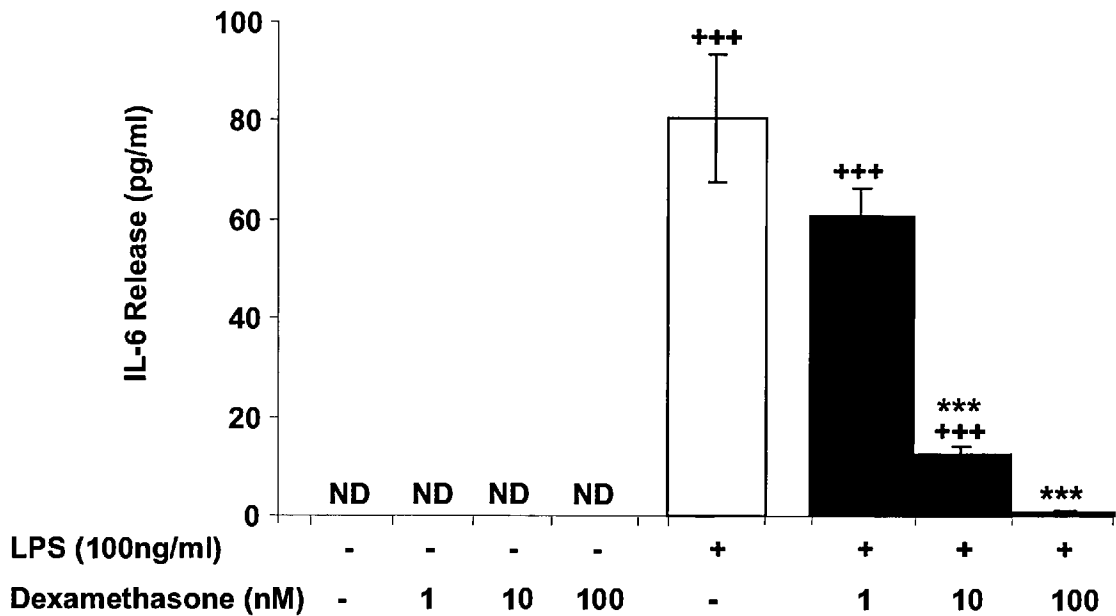


Figure 3.15 Effect of the synthetic glucocorticoid, dexamethasone (1-100nM, 16 hours), on LPS (100ng/ml, 16 hours) stimulated IL-6 release from TtT/GF cells. The concentrations of IL-6 in the medium of the unstimulated control and dexamethasone (1, 10 and 10nM) alone treated cells were undetectable (the detection limit of the IL-6 ELISA was 3.9pg/ml). Control and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. *** $p<0.001$ vs unstimulated control (Con), *** $p<0.001$ vs LPS-stimulated group. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. Con, control; IL-6, interleukin 6; LPS, lipopolysaccharide; ND, not detected.

3.4.4 Modulation of IL-6 release from LPS stimulated TtT/GF cells by CRH

Hypothalamic CRH is a potent stimulator of ACTH release from corticotrophs. However, the influence of CRH upon IL-6 release from folliculostellate cells has not been previously examined to our knowledge. Therefore, the effects of CRH (1 and 10nM; 16 hours) stimulation on the release of IL-6 from the TtT/GF cells, in the presence and absence of LPS (100ng/ml, 16 hours), were examined (section 2.3.1.2).

The release of IL-6 from the TtT/GF cells was significantly increased in the presence of LPS, compared to unstimulated controls ($p < 0.001$ vs control; figure 3.16). Treatment with the vehicle solution (10 μ M ascorbic acid) did not affect basal levels of IL-6 ($p = 1.00$) or interfere with the stimulatory effect of LPS on IL-6 release ($p = 1.00$; figure 3.16). Incubation of the cells with CRH (1 and 10nM) did not affect basal or LPS stimulated IL-6 release 1nM, $p = 1.00$; 10nM, $p = 0.195$; figure 3.16).

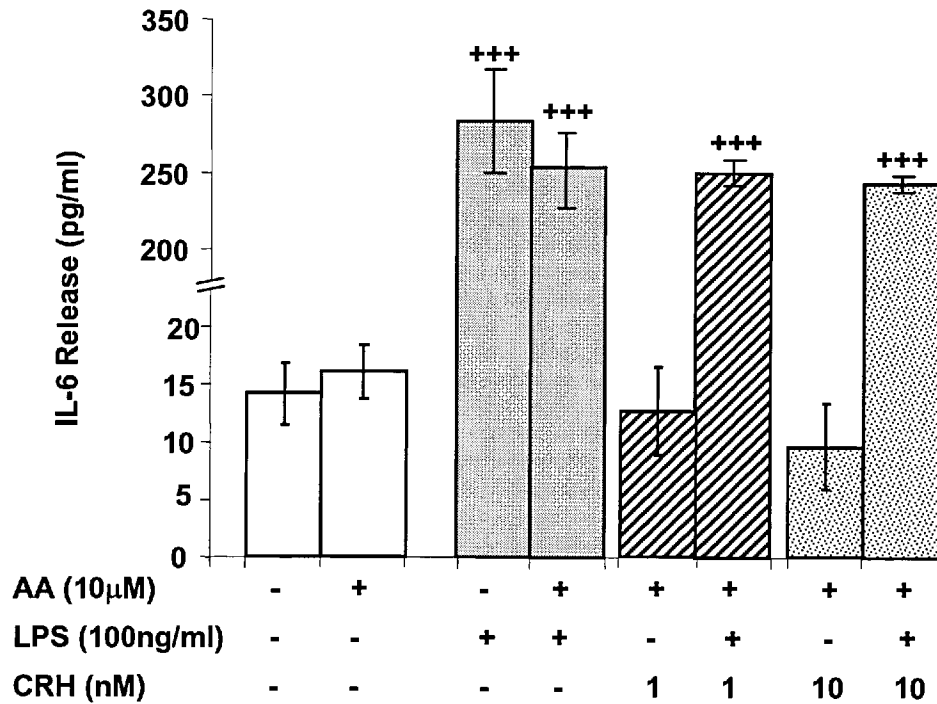


Figure 3.16 Effect of CRH upon LPS stimulated IL-6 release from TtT/GF cells. The release of IL-6 was measured from cells stimulated with CRH (1 and 10nM) and/or LPS (100ng/ml) for 16 hours. Control (vehicle only - 10µM AA) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. *** $p < 0.01$ vs vehicle (AA). Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; CRH, corticotrophin releasing hormone; IL-6, interleukin 6; LPS, lipopolysaccharide.

3.5 LPS signalling in AtT20 D16:16 cells

The effects of LPS stimulation upon corticotroph-derived ACTH release were compared to those induced by CRH, a potent stimulator of ACTH secretion. For these studies, the AtT20 D16:16 cell line was used as the model of corticotrophs as previous studies have shown that this AtT20 subclone responds more readily to CRH stimulation compared to the AtT20 D1 subclone (Woods *et al.* 1992). Additionally, the AT20 D16:16 cells are ideal for co-culture studies (section 3.6) due to their adherent nature, rather than the AtT20 D1 suspension cell line. In parallel the DNA-binding activity of NF κ B in these cells, following either LPS or CRH stimulation, was examined by EMSA (section 2.5.4.4) and p65 TransAMTM assay (section 2.5.6). Further work entailed examining the interactions between CRH and LPS upon both ACTH release and NF κ B activity and the consequence of inhibiting NF κ B (using SC-514) upon these parameters.

3.5.1 Effects of CRH on ACTH release from AtT20 D16:16 cells

The AtT20 D16:16 cells released ACTH spontaneously and a time-dependent accumulation of ACTH into the medium was observed over 24 hours, with significant increases measured 16 hours after the addition of new medium ($p < 0.01$ vs 7 hours; figure 3.17). Stimulation with 10nM CRH resulted in a marked increase in ACTH release after 7 hours exposure compared to basal levels ($p < 0.01$ vs 7 hours control), whilst 1nM CRH did not affect the basal accumulation of ACTH into the medium ($p = 1.00$ vs respective control time point; figure 3.17).

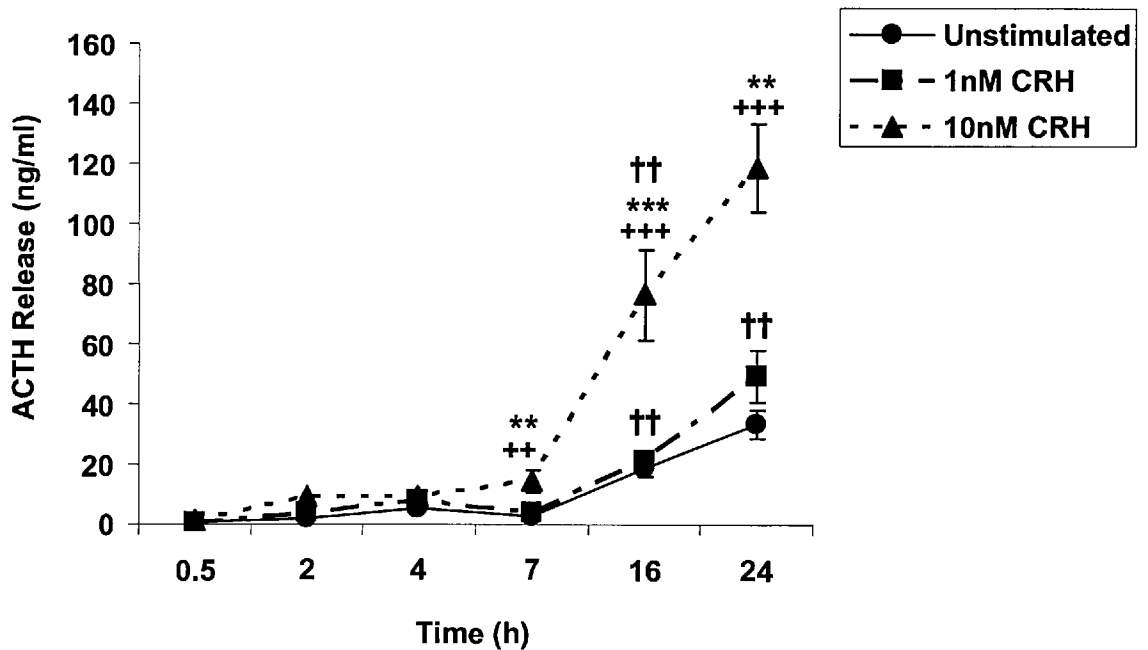


Figure 3.17 Time-dependent effect of CRH (1-10nM, 30 minutes - 24 hours) upon ACTH release from AtT20 D16:16 cells. Control (vehicle - 10 μ M ascorbic acid) stimulated groups are the mean of six samples $\pm$ SEM. All samples contained vehicle. The data are representative of three separate experiments. **p<0.01, ***p<0.001 vs respective time point of unstimulated control, **p<0.01, ***p<0.001 vs respective time point of 1nM CRH, ††p<0.01 vs preceding time point. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide.

3.5.2 Effects of CRH on NF κ B activity in AtT20 D16:16 cells

NF κ B (p65/p50) activity in the AtT20 D16:16 cells following CRH (10nM) stimulation was increased after 30 minutes challenge (figure 3.18A and B) and the activity was reduced after 2 hours to levels comparable to control (2 hour unstimulated control; figure 3.18A and B). A later increase in activity was observed from 4 hours up to 16 hours post-stimulation (figure 3.18A and B).

Because the constitutive activity of NF κ B in the AtT20 D16:16 cells were high, in comparison to the TtT/GF cells (figure 3.5), changes in activity in the AtT20 D16:16 cells after stimulation with CRH were difficult to detect by EMSA (figure 3.18A). NF κ B activity was thus also measured using the more sensitive p65 TransAMTM assay (see section 2.5.6). In concurrence with the EMSA results, an increase in NF κ B (p65) activity was detected 30 minutes after the onset of stimulation with CRH and activity was reduced at 2 hours (10nM; figure 3.19). However, although an increase in activity was still observed at 4 hours by EMSA (figure 3.18A), data from the p65 TransAMTM assay showed a reduction in NF κ B activity at 4 hours CRH challenge, and was comparable to control at 7 hours (figure 3.19). The differences in the findings from the two assays highlight the difficulties in detecting small changes in NF κ B activity when using less sensitive assays such as the EMSA. Therefore, further studies of NF κ B activity in the AtT20 D16:16 cells by EMSA were verified by the p65 TransAMTM assay.

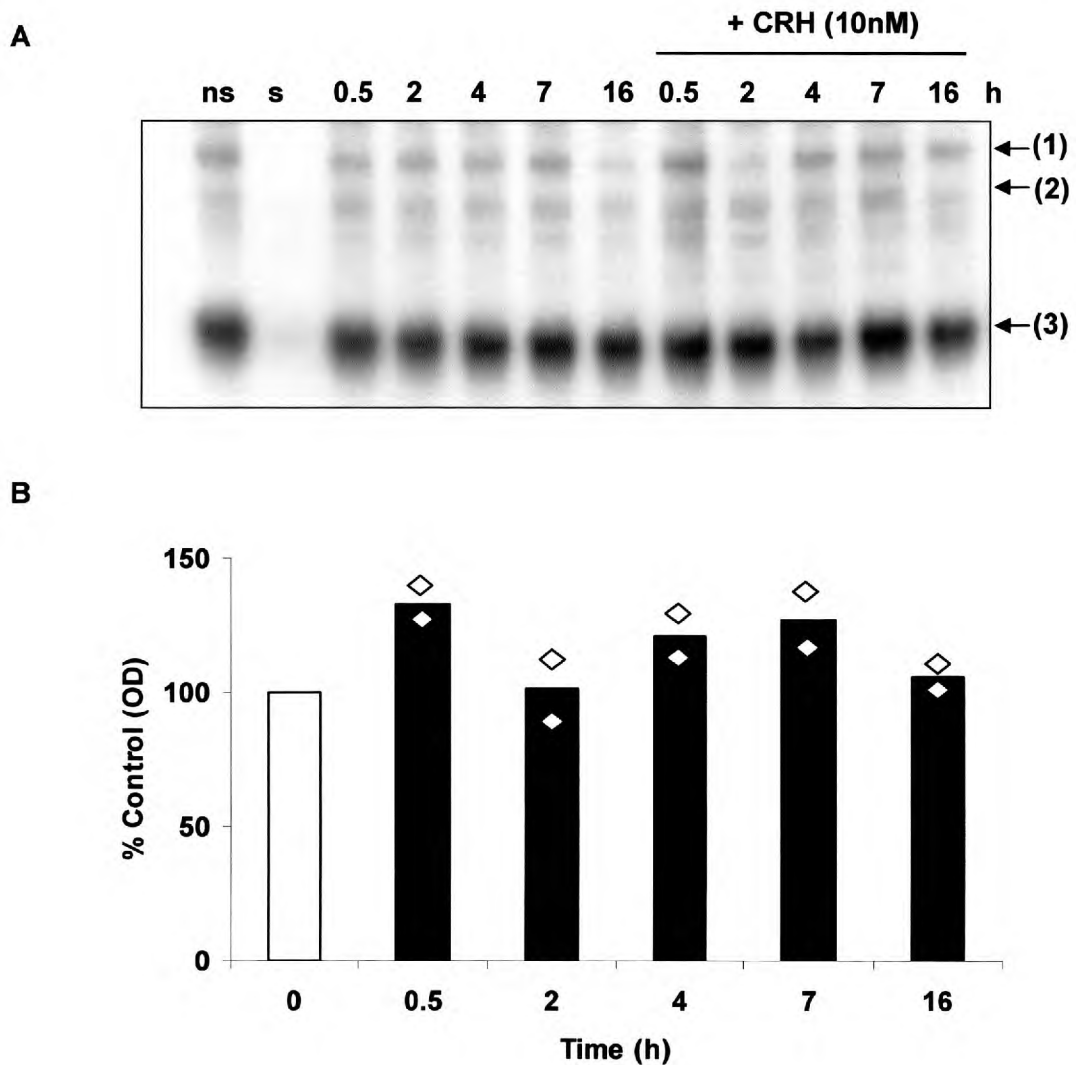


Figure 3.18 Effect of CRH (10nM, 30 minutes - 16 hours) on NF κ B activity in nuclear extracts (10 μ g) of AtT20 D16:16 cells, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer (expressed as percentage of control). (1) p65/p50 heterodimer; (2) p50/p50 homodimer and (3) non specific binding. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). CRH, corticotrophin-releasing hormone; NF κ B, nuclear factor *kappa* B; OD, optical density.

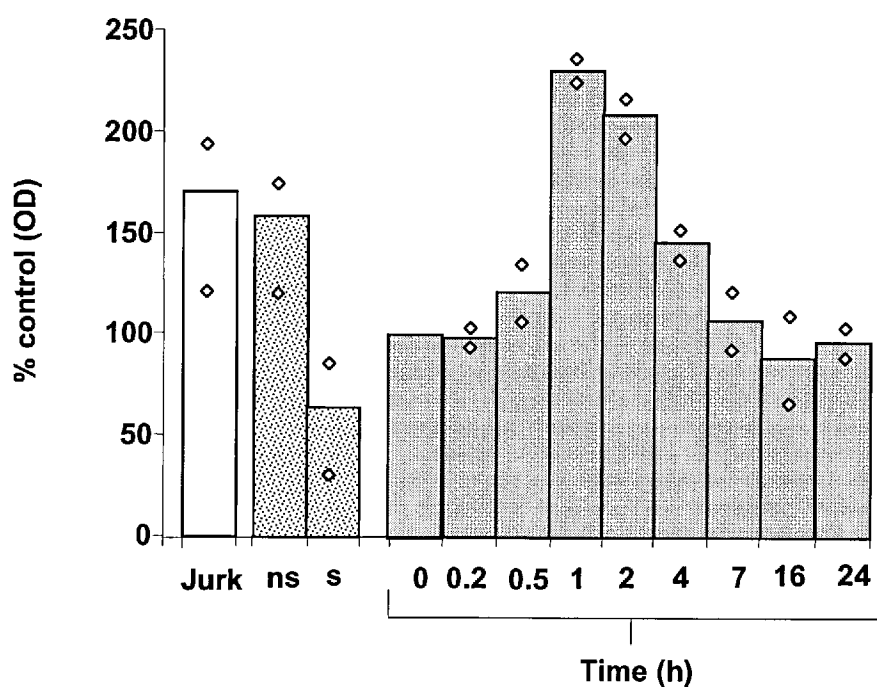


Figure 3.19 Effect of CRH (10nM, 0-24 hours) stimulation upon NF κ B (p65) activity in nuclear extracts (2.5 μ g) of AtT20 D16:16 cells, detected by p65 TransAMTM assay. The experiment was repeated twice and the data presented is the mean optical density for each treatment group [expressed as percentage of control (0 hour)]. The points on the histogram represent the range values. ns, non-specific competitor; s, specific competitor; Jurk, jurkat nuclear extract (positive control); NF κ B, nuclear factor *kappa* B; CRH, corticotrophin releasing hormone; OD, optical density.

3.5.3 Effect of LPS stimulation on ACTH release from AtT20 D16:16 cells

LPS (100ng/ml; 30 minutes - 24 hours) did not affect the spontaneous release of ACTH from AtT20 D16:16 cells (figure 3.20).

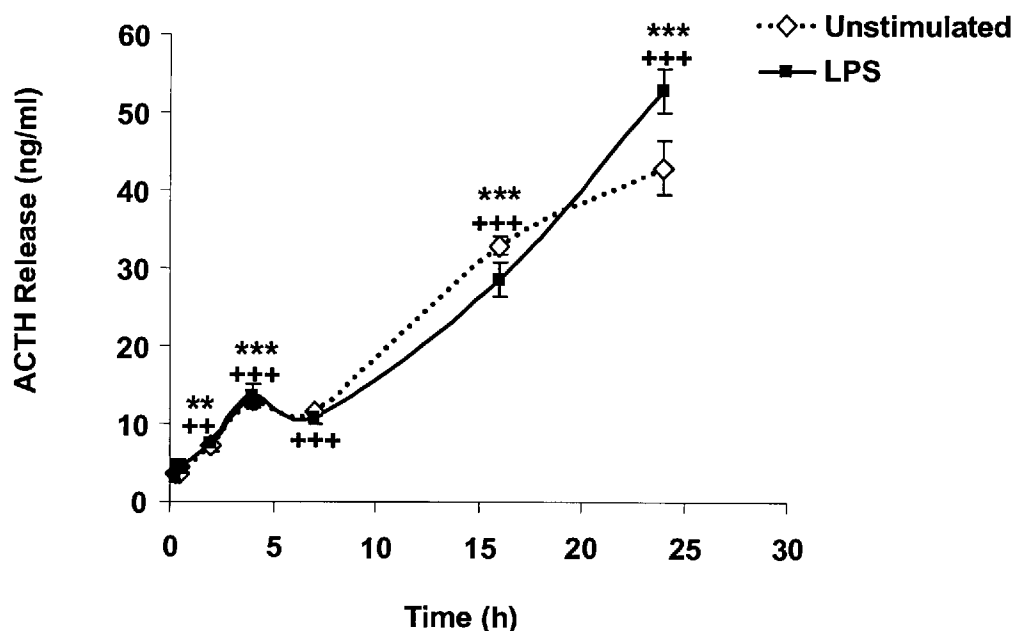


Figure 3.20 Effect of LPS (100ng/ml, 30 minutes – 24 hours) on ACTH release from AtT20 D16:16 cells. Control (unstimulated) and stimulated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. ** $p < 0.01$, *** $p < 0.001$ vs control (0 hour); ** $p < 0.01$, *** $p < 0.001$ vs preceding time point. Analysis for comparison of time points within each group was performed by one-way ANOVA with *post hoc* comparison using Bonferroni's t-test. Analysis for the comparison of LPS vs unstimulated control at each time point was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH, adrenocorticotrophin; LPS, lipopolysaccharide.

3.5.4 Effect of LPS stimulation on NF κ B activity in AtT20 D16:16 cells

The constitutive and LPS stimulated activity of NF κ B was initially detected by EMSA in AtT20 D16:16 cells (30 minutes to 16 hours; figure 3.21A). LPS (100ng/ml) increased basal NF κ B (p65/p50) activity 30 minutes after contact, with activity decreasing after 2 hours exposure (figure 3.21B). A later increase in NF κ B activity was evident 4 hours following LPS stimulation, with activity progressively decreasing thereafter and returning to basal levels at 16 hours (figure 3.21B).

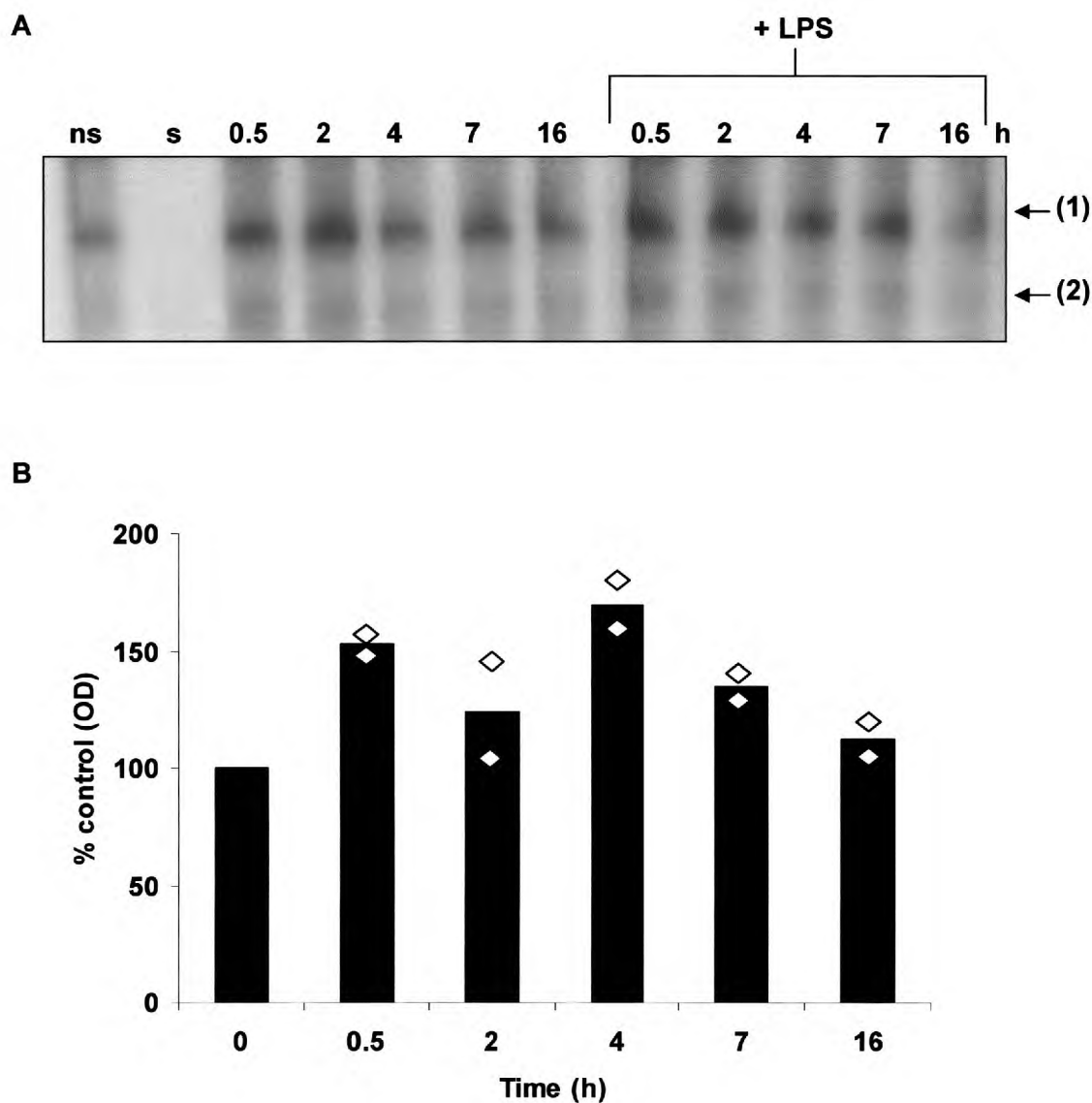


Figure 3.21 Time-dependent effect of LPS (100ng/ml, 30 minutes - 16 hours) stimulation upon NF κ B activity in nuclear protein extracts (10 μ g) of AtT20 D16:16 cells, detected by (A) electrophoretic mobility shift assay. (1), p65/p50 heterodimer and (2), p50/p50 homodimer. (B) The data are representative of two separate experiments and the histogram represents the mean optical density of the p65/p50 bands, expressed as a percentage of control (0 hours). The points on the histogram represent the range values. Competitive reactions were included with 100 fold molar excess of the NF κ B oligonucleotide (s, specific) and AP-1 oligonucleotide (AP-1; ns, non-specific). LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; OD, optical density.

To verify the EMSA findings, NF κ B activity was also measured by the p65 TransAM™ assay. In contrast to the EMSA results (figure 3.21A and B), whereby increased NF κ B activity was detected following 30 minutes stimulation with LPS (100ng/ml), increased NF κ B activity in the AtT20 D16:16 cells was not detected by the TransAM™ assay until 1 hour contact with LPS, with peak activity detected at 7 hours (figure 3.22). The discrepancies in the time frame of NF κ B activation, detected by the two assays, is likely to be attributed to the high constitutive activity of AtT20 D16:16 NF κ B, thus making it difficult to discriminate between changes in NF κ B activity by non-quantitative methods such as the EMSA.

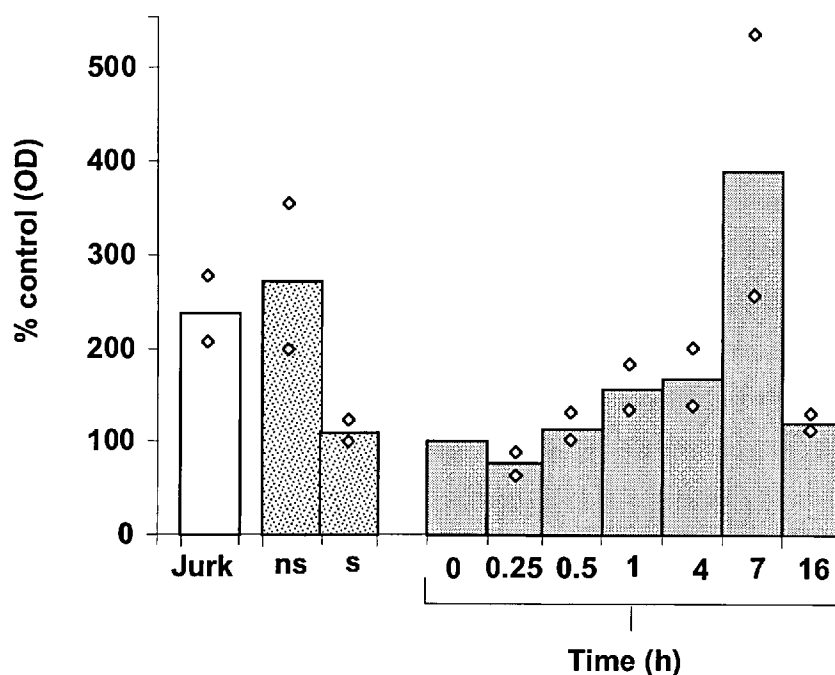


Figure 3.22 Time-dependent effect of LPS (100ng/ml, 15 minutes - 16 hours) stimulation upon NF κ B activity in nuclear extracts (2.5 μ g) of AtT20 D16:16 cells, detected by p65 TransAM™ assay. The experiment was repeated twice and the data presented is the mean optical density for each treatment group [expressed as percentage of control (0 hour)]. The points on the histogram represent the range values. Jurk; Jurkat nuclear extract; LPS, lipopolysaccharide; NF κ B, nuclear factor κ B; ns, non-specific competitor; OD, optical density; s, specific competitor.

3.5.5 Effects of murine recombinant IL-6 upon basal and CRH stimulated ACTH release from AtT20 D16:16 cells

One of the mechanisms through which LPS increases ACTH release is through the induction of IL-6 (Bethin *et al.* 2000; Gloddek *et al.* 2001). As folliculostellate cells reside near corticotrophs and provide a source of IL-6, it was investigated whether murine recombinant IL-6 (rIL-6, 1nM, 7 hours) at concentrations comparable to those produced by TtT/GF cells following 7 hours stimulation with LPS (100ng/ml) would influence ACTH release from AtT20 D16:16 cells, in the presence and absence of graded concentrations of CRH (1-100nM).

CRH (10 and 100nM, 7 hours) stimulation induced significant increases in ACTH release ($p < 0.001$ vs control) from the AtT20 D16:16 cells, but a lower concentration of CRH (1nM) was without effect ($p = 1.00$; figure 3.23). Whilst IL-6 alone (1nM, 7 hours) did not affect ACTH release ($p = 0.173$), when combined with the lower concentration of CRH (1nM) it increased basal ACTH release ($p < 0.01$ vs control). In contrast, rIL-6 (1nM) did not affect the increase in ACTH release induced by higher concentrations (10 and 100nM) of CRH (10nM, $p = 0.584$ and 100nM, $p = 0.918$; figure 3.23).

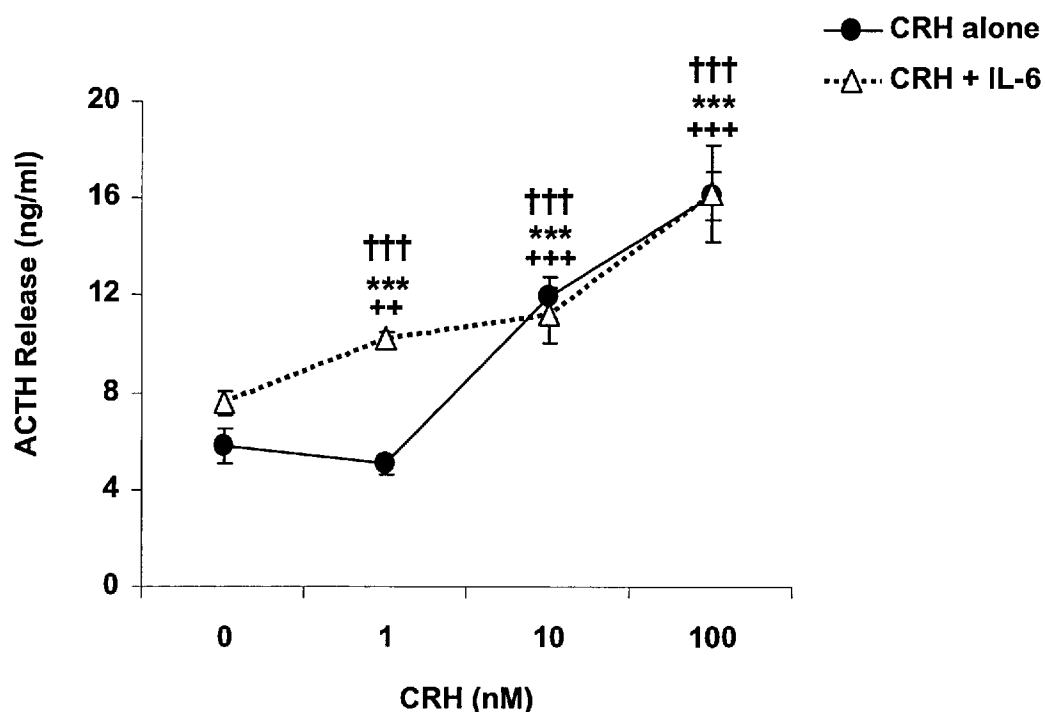


Figure 3.23 Effects of rIL-6 (1nM, 7 hours) and/or CRH (1-100nM, 7 hours) upon ACTH release from AtT20 D16:16 cells. Control (vehicle - 10 μ M ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. All samples contained vehicle. The data are representative of three separate experiments. ** p <0.01, *** p <0.001 vs vehicle, *** p <0.001 vs rIL-6 alone, ††† p <0.001 vs 1nM CRH alone. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's *t*-test. ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone; rIL-6, recombinant IL-6.

3.5.6 Effect of LPS upon CRH stimulated ACTH release from AtT20 D16:16 cells

The release of ACTH induced by CRH stimulation was examined in the presence of LPS. Whilst LPS (100ng/ml, 7 hours) challenge alone did not affect the basal ACTH release from the AtT20 D16:16 cells ($p=0.827$), it attenuated the stimulatory effect of low CRH (1nM, $p<0.01$ vs 1nM alone and 10nM, $p<0.001$ vs 10nM alone) but not high (100nM, $p=0.169$) concentrations of CRH (figure 3.24).

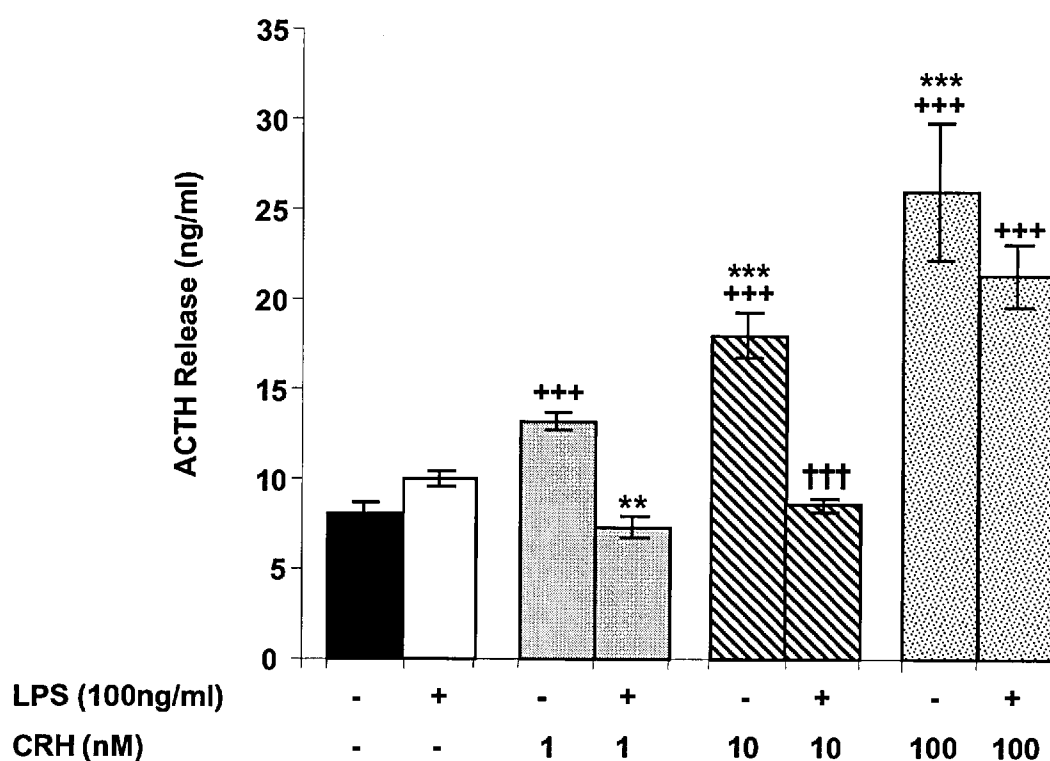


Figure 3.24 Effects of CRH (1-100nM, 7 hours) and/or LPS (100ng/ml, 7 hours) stimulation on ACTH secretion from AtT20 D16:16 cells. Control (vehicle only – 10 μ M ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of four separate experiments. *** $p<0.001$ vs vehicle, ** $p<0.01$, *** $p<0.001$ vs 1nM CRH, ††† $p<0.001$ vs 10nM CRH. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide.

3.5.7 Effect of the IKK β inhibitor, SC-514, on CRH stimulated ACTH release from AtT20 D16:16 cells

Whilst both CRH (10nM) and LPS (100ng/ml) increased NF κ B activity in the AtT20 D16:16 cells (CRH; figure 3.18 and 3.19 and LPS; figure 3.21 and 3.22), LPS attenuated the stimulatory effect of CRH (10nM) upon ACTH secretion from these cells (figure 3.24). Therefore, the effect of the NF κ B inhibitor, SC-514 (100 μ M) upon AtT20 D16:16-derived ACTH in the presence and absence of CRH (1-10nM) and/or LPS (100ng/ml) was examined (figure 3.25-3.27).

Primary analysis of the results (performed by 3-way ANOVA), indicated CRH and SC-154 induced significant main effects on ACTH release, but that LPS did not (figures 3.25-3.27). Significant interactions were also detected between 1) CRH and SC-514, 2) CRH and LPS and 3) CRH and SC-514 and LPS. Therefore, further analysis of the individual group differences were performed by a second-order one-way ANOVA with *post hoc* comparison using Bonferroni's t-test.

LPS (100ng/ml, 7 hours) and SC-514 (100 μ M, 7 hours) alone or in combination had no effect on the basal release of ACTH (LPS, $p=1.00$ and SC-514, $p=1.00$; figures 3.25-3.27). CRH (1nM, 7 hours) alone also failed to induce ACTH release ($p=1.00$, figure 3.25). However, when given in combination with SC-514, a significant increase ($p<0.01$ vs control) in peptide release was apparent (figure 3.25). Higher concentrations of CRH (10 and 100nM) induced significant increases in ACTH release ($p<0.001$ vs control), which were further increased by SC-514 (10nM, $p<0.001$ vs 10nM CRH alone; figure 3.26 and 100nM, $p<0.01$ vs 100nM CRH alone; figure 3.27). LPS attenuated the stimulatory effect of CRH (10nM, $p<0.001$ vs CRH alone; figure 3.26) upon ACTH secretion and this effect was reversed by SC-514 ($p<0.001$ vs CRH + LPS; figure 3.26).

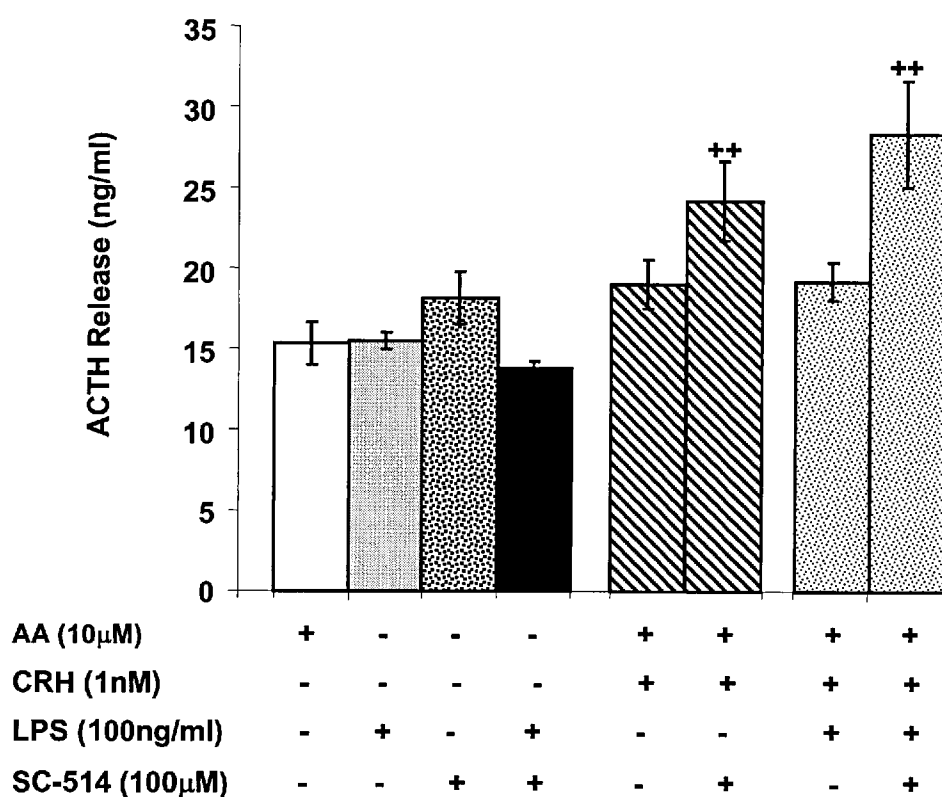


Figure 3.25 Effects of the IKK β inhibitor, SC-514 (100 μ M, 8 hours), upon ACTH release from AtT20 D16:16 cells in the presence and absence of CRH (1nM, 7 hours) and/or LPS (100ng/ml, 7 hours). The cells were incubated with SC-514 for 1 hour prior to the addition of CRH and/or LPS and the drug was included throughout the incubation period (7 hours). Control (vehicle only - 10 μ M AA) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. ** p <0.01, *** p <0.001 vs vehicle (AA). Analysis was performed by three-way ANOVA followed by a second order one-way ANOVA with *post hoc* comparison using Bonferroni's *t*-test. AA, ascorbic acid; ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone.

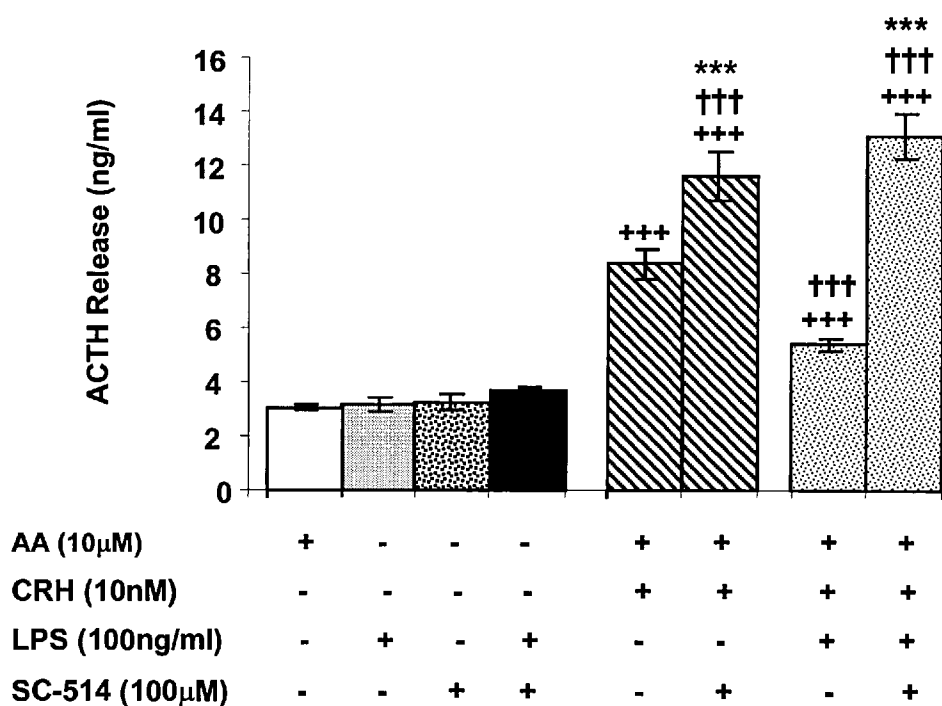


Figure 3.26 Effects of the IKK β inhibitor, SC-514 (100 μ M, 8 hours), upon ACTH release from AtT20 D16:16 cells in the presence and absence of CRH (10nM, 7 hours) and/or LPS (100ng/ml, 7 hours). The cells were incubated with SC-514 for 1 hour prior to the addition of CRH and/or LPS and the drug was included throughout the incubation period (7 hours). Control (vehicle only - 10 μ M AA) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. $^{+++}p<0.001$ vs vehicle (AA), $^{\dagger\dagger\dagger}p<0.001$ vs CRH, $^{***}p<0.001$ vs CRH + LPS. Analysis was performed by three-way ANOVA followed by a second order one-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone.

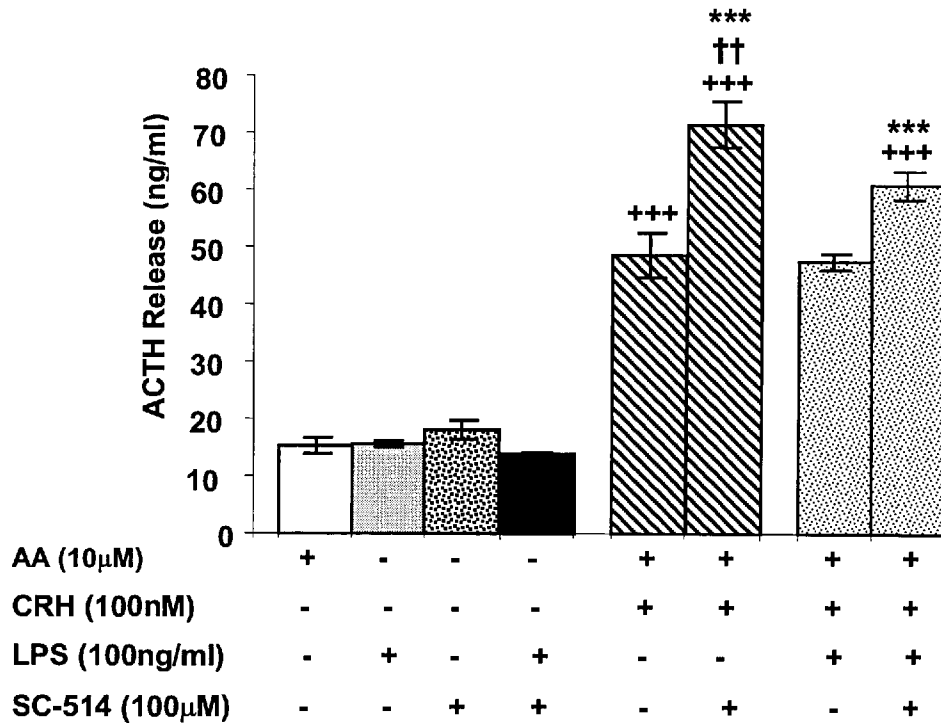


Figure 3.27 Effects of the IKK β inhibitor, SC-514 (100µM, 8 hours), upon ACTH release from AtT20 D16:16 cells in the presence and absence of CRH (100nM, 7 hours) and/or LPS (100ng/ml, 7 hours). The cells were incubated with SC-514 for 1 hour prior to the addition of CRH and/or LPS and the drug was included in the medium throughout the incubation period (7 hours). Control (vehicle only - 10µM AA) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. $^{+++}p < 0.001$ vs vehicle (AA), $^{\dagger\dagger}p < 0.01$ vs CRH, $^{+++}p < 0.001$ vs CRH + LPS. Analysis was performed by three-way ANOVA followed by a second order one-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone.

3.5.8 Effects of the IKK β inhibitor, SC-514, on CRH and LPS stimulated NF κ B activity in AtT20 D16:16 cells

CRH (10nM; figures 3.18 and 3.19) and LPS (100ng/ml; figures 3.21 and 3.22) were previously shown to increase NF κ B activity in the AtT20 D16:16 cells after 1 and 7 hours respectively. Therefore, the effects of 1 and 7 hours stimulation with LPS (100ng/ml) and CRH (10nM), in the presence and absence of SC-514 (100 μ M) on NF κ B activity in the AT20 D16:16 cells were examined by p65 TransAMTM assay.

CRH (10nM, 1 hour) induced a robust increase in NF κ B activation (figure 3.28A). Similar but less pronounced effects were induced by LPS (100ng/ml, 1 hour; figure 3.28A). The effects of CRH and LPS upon NF κ B activity appeared to be additive (figure 3.28A). SC-514 (100 μ M, 1 hour) reduced both basal and CRH and/or LPS stimulated NF κ B activity (figure 3.28A).

A later increase in activation was evident with LPS (100ng/ml, 7 hours), whilst the stimulatory effect of CRH (10nM) alone was markedly reduced compared to its effect after 1 hour stimulation (figure 3.28A). Inclusion of CRH did not affect LPS-induced NF κ B activity (figure 3.28B). SC-514 (100 μ M, 7 hours) attenuated LPS and CRH induced NF κ B activation, to levels comparable to control (figure 3.28B).

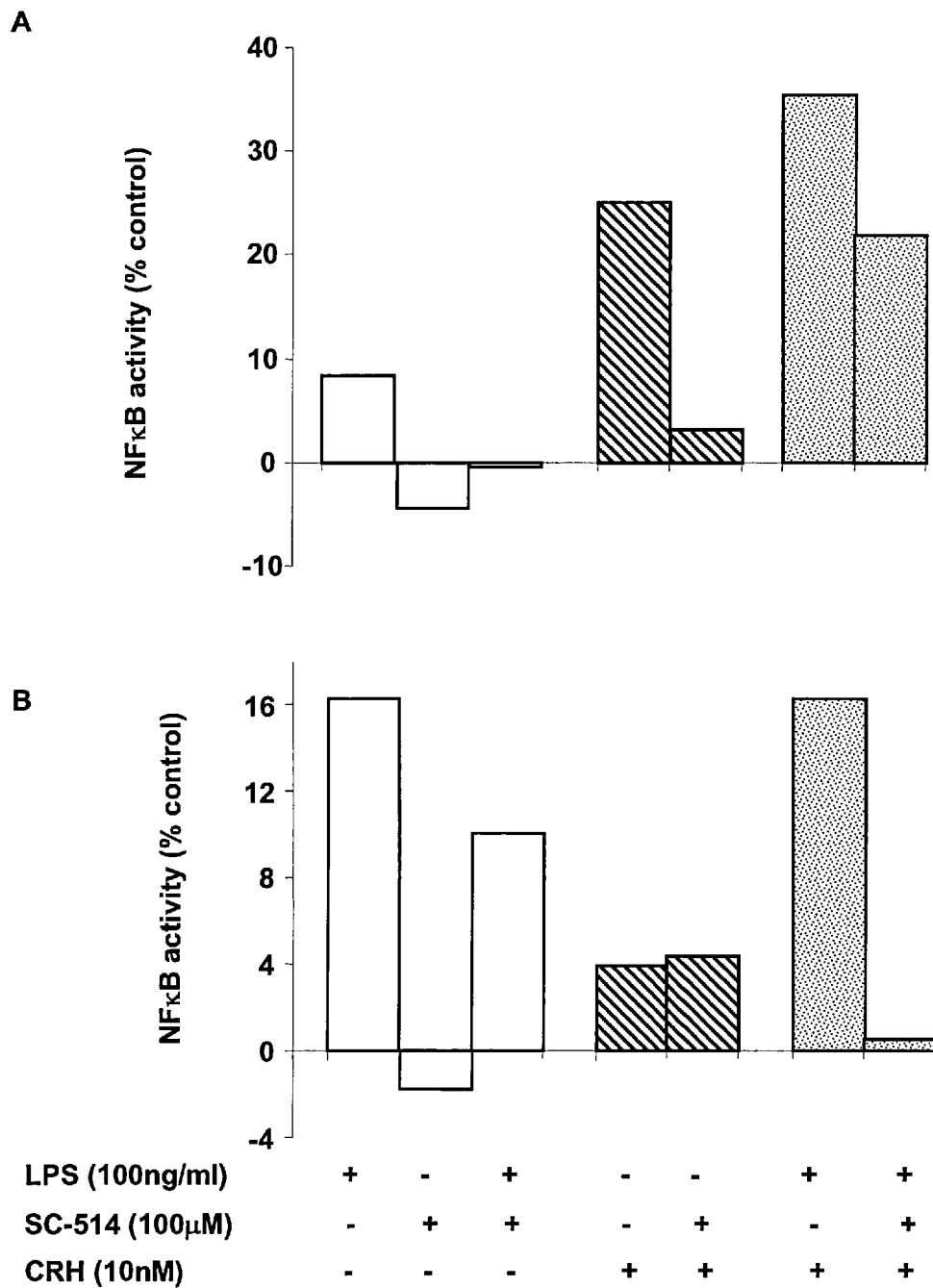


Figure 3.28 Effects of the IKK β inhibitor, SC-514 (100 μ M), upon CRH (10nM) and/or LPS (100ng/ml) stimulated NF κ B activity in nuclear extracts (2.5 μ g) of AtT20 D16:16 cells, detected by p65 TransAMTM assay. Cells were pre-treated for 1 hour with SC-514 prior to stimulation with CRH and/or LPS for (A) 1 hour and (B) 7 hours. The drug was included in the medium throughout the incubation periods (1 and 7 hours). The experiment was performed once and the data are expressed as percentage of control (untreated). Con, control; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B.

3.6 Discussion

3.6.1 Models and methods

3.6.1.1 Cell lines

The current studies utilised the murine TtT/GF and human PDFS cell line as models of folliculostellate cells and the murine AtT20 (D1 and D16:16 subclones) cell line as a model of corticotrophs. In addition, as folliculostellate cells have been described as “glial-like” and show some characteristics similar to that of macrophages (Stokreef *et al.* 1986), initial studies examining LPS signalling in the murine macrophage-like RAW 264.7 cell line were also conducted.

3.6.1.1.1 Folliculostellate cells

Previous studies examining the function and properties of folliculostellate cells of the anterior pituitary have used purified preparations of folliculostellate cells established by density gradients (Van Der Schueren *et al.* 1982; Allaerts & Denef 1989; Hentges *et al.* 2000) or a primary culture system (Ferrara *et al.* 1986). Although these *in vitro* systems have contributed to the understanding of the function of folliculostellate cells, problems using such model systems include unpure preparations and limited viability of the cells (Pryor-Jones *et al.* 1989; Alexander *et al.* 1991; Alexander *et al.* 1995). The generation of the folliculostellate-like cell lines have omitted these limitations (Inoue *et al.* 1992; Danila *et al.* 2000) and have allowed studies of folliculostellate cell function to expand considerably (Matsumoto *et al.* 1993; Traverso *et al.* 1999; Lohrer *et al.* 2000; Perez Castro *et al.* 2000; Chapman *et al.* 2002; Chapman *et al.* 2003; Nagashima *et al.* 2003; Tierney *et al.* 2003; Tierney *et al.* 2005). A well-characterised folliculostellate-like cell line is the TtT/GF cell line, which spontaneously developed from a murine thyrotrophic tumour (Inoue *et al.* 1992). This cell line exhibits many of the characteristics of primary folliculostellate cells, i.e. they possess many lysosomes and numerous intermediate filaments in the cytoplasm, they exhibit phagocytic activity and follicle formation and they express the glial and neuronal proteins, GFAP and S-100 (Cocchia & Miani 1980; Nakajima *et al.* 1980; Velasco *et al.* 1982), as well as releasing IL-6 (Inoue *et al.* 1992).

More recently, a human derived folliculostellate cell line, designated PDFS, developed spontaneously from a clinically non-functioning pituitary macroadenoma, (Danila *et al.* 2000). These cells, which show an epithelial-like morphology with long

cytoplasmic processes and stain positively for S-100 protein expression, provide an ideal tool for studying folliculostellate cells of human origin (Danila *et al.* 2000; Solito *et al.* 2003). However, unlike the TtT/GF cell line, it is unclear whether PDFS cells release IL-6, particularly in response to endotoxin (LPS), a key feature of folliculostellate cells (Inoue *et al.* 1999; Gloddek *et al.* 2001; Inoue *et al.* 2002).

3.6.1.1.2 Corticotrophs

Due to the low proportion of corticotrophs in the anterior pituitary gland and the difficulty in isolating these cells, the current study utilised the AtT20 cell line derived from a radiation-induced anterior pituitary tumour (Furth *et al.* 1953). This cell line currently has three known strains (D1, D16v and D16:16). The D1 subclone was first derived from the tumour and maintained as a suspension culture (Yasumura 1968), the D16v strain was derived from the D1 cells and grows as a monolayer (Yasumura 1968; Tashjian 1979) and the D16:16 strain was subcloned from the D16v cells on the basis of high intracellular β -endorphin content (Sabol 1980). The AtT20 D16:16 cell line has maintained many characteristics of the normal corticotroph, such as CRH-responsiveness and glucocorticoid suppressibility (Woods *et al.* 1992) and the current study has detected mRNA expression of the LPS signalling receptor toll-like receptor 4 (Tlr4), another characteristic recently identified in primary corticotrophs (Tichomirowa *et al.* 2005). Therefore, due to their similarity to normal corticotrophs, the AtT20 D16:16 cell line was employed as an *in vitro* model of corticotrophs for studying ACTH secretion. AtT20 cells have relatively high rates of basal ACTH secretion which is likely to be due to the frequent spontaneous membrane depolarisations and neuron-like action potentials observed in electrophysiological studies (Surprenant 1982; Adler *et al.* 1983; Korn *et al.* 1991). In concurrence with this, the current study identified an accumulation of basal ACTH secretion from the AtT20 D16:16 cells. Therefore, to avoid high basal ACTH levels masking any alterations in the response of these cells to treatment, maximal incubation times of 7 hours were used, before significantly elevated levels of basal ACTH were measured.

3.6.1.1.3 Macrophages

The RAW 264.7 cell line, a widely used model of macrophages, was established from the ascites of a tumour induced in a male mouse by the intraperitoneal injection of Abelson leukaemia virus (A-MuLV) (Raschke *et al.* 1978). Both the LPS signalling pathway and NF κ B dependent IL-6 synthesis and release have been well characterised in these cells, (Le Page *et al.* 1996; Ishii *et al.* 2003; Kim *et al.* 2006).

As such, the RAW 264.7 cells were included in the study as a positive control for examining LPS signalling.

Although studies using transformed cell lines are not without their limitations, they are extremely versatile, easily available and do not require the amount of material or time required for preparing primary cell populations. Additionally, these cell lines omit the problem of heterogeneous populations of cells present in primary cultures, which can interfere with the function of the cells of interest, and result in misleading interpretations.

3.6.1.2 Experimental methods

3.6.1.2.1 Detection of mRNA for the LPS signalling components by RT-PCR

To examine whether the TtT/GF, PDFS and AtT20 subclones were appropriate cell lines for investigating LPS signalling, the expression of mRNA for the LPS signalling components CD14, Tlr4 and MD-2 (and the gram positive recognition receptor Tlr2) were initially examined by RT-PCR. However, the positive expression of such components is not necessarily an indication that these cells express the functional proteins. Although this problem can be overcome by detecting the protein of interest by western blot analysis, specific and reliable antibodies for detecting CD14, MD-2 and Tlr4 protein are not currently available and as such, detection of these components was limited to PCR analysis in the study. However, the PCR findings complemented the functional studies (NF κ B activation), which showed that the TtT/GF and AtT20 D16:16 cells were both responsive to LPS stimulation.

The RT-PCR method does not quantitatively measure changes in mRNA expression. However, the purpose of the current study was not to quantify mRNA expression in the cells, but to confirm either the presence or absence of the LPS signalling components. Several initial studies were required to optimise the PCR conditions, such as the number of cycles and annealing temperatures of the oligonucleotides and positive and negative controls were included in the study to ensure that amplified bands were not representative of non-specific sequences.

3.6.1.2.2 Detection of NF κ B activity

3.6.1.2.2.1 Western blot analysis

In the current study, NF κ B activity was examined using three methods. Initially, the expression of the transcriptionally active subunit of NF κ B, p65, was detected in cytosolic and nuclear cell fractions by western blot analysis. This enabled the translocation of p65 from the cytoplasm into the nucleus to be determined. As I κ B α degradation is associated with increased NF κ B activity, the expression of this protein was also examined. Whilst the technique of western blot analysis is a widely utilised and accepted method for detecting protein expression, a major draw back to the method is the limited power of quantifying protein expression. The majority of the western blot studies employed in the current study examined time-dependent changes in protein expression in cytosolic and nuclear fractions and due to the limited number of samples that can be used for each gel (maximum of nine samples), the inclusion of duplicate samples for statistical analysis was not possible. Additionally, because comparisons of the density of the bands representing p65 could not be made on samples from different gels, and cytosol and nuclear fractions were loaded on separate gels, direct comparisons in changes in protein expression between the two fractions were limited. However, because only activated and unsequestered p65 can translocate to the nucleus, very low levels of p65 protein expression were detected in untreated control samples, allowing increases in protein levels to be easily detected. Additionally, observed decreases in I κ B α protein expression in the cytosol are also indicative of NF κ B activation. To ensure that the changes in protein expression detected by western blot analysis were reproducible, the experiments were repeated at least two times. To show the changes in protein expression more clearly, densitometric analysis of the bands on the gel were performed and the data was expressed as a percentage of control (non-quantitative).

3.6.1.2.2.2 EMSA analysis

An important limitation of evaluating NF κ B activation by western blot analysis is that this method does not discriminate between non-transcriptional and transcriptionally active nuclear NF κ B. Therefore, the EMSA method was employed. This assay allows the transcriptional activity of NF κ B to be detected by incubating nuclear samples with a consensus NF κ B sequence. The principal of the assay is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when

subjected to non-denaturing polyacrylamide electrophoresis. If NF κ B present in the nucleus is transcriptionally active, it will bind to the DNA sequence and the heavier complex will be detected as a higher band. Several bands may be detected with this method due to the existence of several hetero- or homo-dimers of NF κ B. Up to 6 complexes can be detected although the two predominant ones are the p65/p50 heterodimer and the p50/p50 homodimer. The other complexes include (in order of size, beginning with the largest and thus, the highest bands): p65/p65, p49/p65, p52/40 and p49/p49. Increased binding of p65/p65 heterodimers can also be observed *in vitro* in conditions of increased nuclear p65 and multiple complexes generally indicate protein degradation or partially pure or crude nuclear extracts. To ensure that the detected bands were not due to non-specific binding reactions, specific competitive (excess unlabelled NF κ B oligonucleotide was added to the samples in the presence of the labelled NF κ B sequence) and non-specific competitive reactions (excess unlabelled AP-1 oligonucleotide was added) were included in the assay. Any bands that represent specific NF κ B binding would still be detected in the non-specific reaction, but not the specific reaction. Similarly to the western blot assay, there is a limited power of quantifying DNA-binding activity. Multiple samples from the same treatment could not be loaded on the same gel for statistical analysis due to the limited number of samples that can be used for each gel (maximum of twelve samples). Additionally, comparisons of the density of the bands representing NF κ B (p65/p50) DNA-binding activity could not be made on samples from different gels as they were not normalised to each other. Therefore, the experiments were repeated at least two times to ensure reproducibility and data was presented as the mean percentage of control following densitometric analysis, to show changes in NF κ B DNA-binding activity more clearly (non-quantitative).

3.6.1.2.2.3 Supershift assay

Whilst there are characteristic shifts caused by binding of specific protein(s) to the target DNA, the change in relative mobility, detected by EMSA, does not identify the bound protein. Identification of bound protein is accomplished by including an antibody, specific for the DNA-binding protein (e.g. p65), to the reaction. Binding of the antibody to the complex will result in either a further shift in the band. In some cases, the antibody may disrupt the protein:DNA interaction resulting in the loss of the characteristic shift, but no super-shift. A weak shift or a reduction in the intensity of the band usually implies the presence of low levels of the protein in the complex. The p65 protein is the transcriptionally active subunit of the NF κ B dimer and the

p65/p50 heterodimer is the most extensively studied form. Therefore, the bands representing the p65/p50 heterodimer in nuclear extracts of LPS stimulated TtT/GF and AtT20 D16:16 cells were identified by supershift assays (section 2.5.6) and used as a reference for further assays.

Detecting NF κ B DNA-binding by EMSA is a lengthy process and requires the use of radioactive ^{32}P . As described earlier (3.6.1.2.2.2), this assay has similar limitations to the western blot technique, i.e. the inability to compare DNA-binding complexes between separate gels and quantifying protein:DNA binding activity for statistical analysis. Therefore, the experiments were repeated to ensure the changes in binding activity were reproducible and correlated with the western blot findings.

3.6.1.2.2.4 p65 TransAMTM assay

Although western blot and EMSA techniques are commonly used for detecting NF κ B activation, these assays do not provide quantitative results and therefore, unless basal NF κ B activity is relatively low and there are obvious alterations in observed protein expression or binding activity, it is difficult to be sure of any induced changes. The ELISA-like p65 TransAMTM assay is a non-radioactive and quantitative method of measuring NF κ B activity which overcomes the limitations of the western blot and EMSA techniques. This assay has been shown to be 10-fold more sensitive than EMSA (by tests carried by the manufacturer, Active Motif. U.K.), and can detect transcriptionally active NF κ B using low nuclear protein concentrations (0.5-2.5 μg), compared to the western blot (20 μg) and EMSA (10 μg) methods, thus reducing background levels. Examining NF κ B activity by western blot and EMSA in the TtT/GF and RAW 264.7 cells was sufficient as both cell lines exhibited low levels of basal activity and obvious alterations were detected. However, the basal activity in the AtT20 D16:16 cells were much higher and thus, changes in NF κ B activity following LPS and CRH stimulation were difficult to detect. Therefore, the p65 TransAMTM assay was employed for measuring NF κ B (p65) DNA-binding activity in these cells. However, although the assay can be used to quantitatively measure NF κ B DNA-binding activity, in the current study, the number of samples per treatment group that could be included in each assay were limited (n=2) and comparisons between separate assays could not be made as they were not standardised. Therefore, statistical analysis of the results obtained could not be performed. However, where possible, the experiments were repeated to ensure reproducibility and the optical

density for each treatment group was expressed as the mean percentage of control to show changes in activity more clearly (non-quantitative).

3.6.1.2.3 Measuring IL-6 release by ELISA

The release of the cytokine IL-6 from primary and transformed cell lines is routinely detected by ELISA. The current study employed an ELISA system that utilised a primary antibody specific for murine IL-6 for cell lines of a mouse origin (TtT/GF, AtT20 D1 and D16:16 subclones and RAW 264.7 cells) or an antibody specific for human IL-6 for the detection of IL-6 release from the human derived PDFS cell line. The murine ELISA showed no cross-reactivity to either rat, human or porcine IL-6 and the human ELISA did not cross-react with either rat or mouse IL-6 or the human recombinant cytokines IL-11, IL-12, ciliary neurotrophic factor, soluble gp130, LIF and oncostatin M (OSM). The measurement of IL-6 by ELISA is advantageous over other assays such as western blot analysis and RT-PCR as it quantitatively measures the concentrations of secreted IL-6. It also allows high numbers of samples to be analysed (approximately 40 per plate) so that appropriate duplicates can be included, thus allowing the results to be analysed statistically. The IL-6 ELISAs used in the current study were also highly sensitive, with detection limits of 3.9pg/ml and 9.3pg/ml for the mouse and human assays respectively. Each assay included positive and negative controls.

3.6.1.2.4 Measuring ACTH release by RIA

The concentration of immunoreactive ACTH in the medium of incubated cell lines was determined by RIA (section 2.5.11). RIAs are a widely used technique for detecting ACTH *in vitro* due to their high sensitivity and specificity. The RIA used in the *in vitro* studies utilised a primary antibody directed against the 1-39 region of ACTH (ACTH₁₋₃₉), which did not cross react with other molecules that are biosynthesised with ACTH, namely CLIP, α -MSH, LH or PRL. However, because the RIA only detects molecules of ACTH that are specific to the antibody, a limitation of the RIA is that the measurements obtained do not necessarily reflect the amount of bioactive ACTH released (due to the presence of fragments of the ACTH₁₋₃₉ peptide), which varies between ACTH precursors (Zehnder *et al.* 1995). Therefore, caution must be applied when interpreting results in regard to bioactivity and hormone release. Nevertheless, the current study limited the observations made by the RIA results to the effects of CRH and LPS administration upon ACTH secretion, and not ACTH bioactivity. The number of samples analysed by the assay were not limited,

allowing high sample output, and comparisons could be made between profiles of hormone release, with repeated experiments (at least three times) ensuring that results were reproducible.

3.6.1.3 Compounds for studying NF κ B signalling

3.6.1.3.1 Synthetic glucocorticoid - dexamethasone

The synthetic glucocorticoid, dexamethasone, is a readily available compound used for the treatment of inflammatory disorders, due to its potent anti-inflammatory effects. Dexamethasone is also commonly used in studies of the functions of glucocorticoids in modulating the stress response and, as such, was included in the current study to investigate the effects of glucocorticoids on NF κ B signalling and IL-6 release from folliculostellate cells.

3.6.1.3.2 Specific IKK β inhibitor - SC-514

The IKK β inhibitor, SC-514, was employed in the study as a specific NF κ B inhibitor for investigating NF κ B signalling in the modulation of IL-6 and ACTH release from TtT/GF and AtT20 D16:16 cells respectively. Studies using non-specific NF κ B inhibitors (Lohrer *et al.* 2000), such as inhibitors of the p38 α mitogen activated kinase (MAPK) pathway (e.g. SB203580) or glucocorticoids (e.g. dexamethasone) cannot attribute any cellular effects solely to NF κ B as such compounds would also interfere with other signalling pathways that may not involve NF κ B. For example, the p38 α MAP kinase pathway regulates the activity of other transcription factors involved in IL-6 synthesis such as AP-1 (Guha & Mackman 2001; Katsoulidis *et al.* 2005). The SC-514 compound is more applicable to studying NF κ B signalling as it specifically inhibits activation of the IKK β subunit which is responsible for activating NF κ B (p65/p50) and does not interfere with upstream signalling pathways (Kishore *et al.* 2003). However, this compound is not effective *in vivo* and its use is therefore limited to *in vitro* studies (Kishore *et al.* 2003).

3.6.1.3.3 Proteasome inhibitor – Mg132

For comparative studies of NF κ B inhibition, the cells were also treated with the proteasome inhibitor, Mg132, which is a non-specific NF κ B inhibitor. This compound is a useful tool for studying NF κ B signalling as NF κ B activation is associated with degradation of its inhibitory protein I κ B α via the proteasome. However, some studies

have found that proteasome inhibitors can also increase NF κ B activity by phosphorylating the IKK α / β subunits, leading to degradation of I κ B α (Dolcet *et al.* 2006). Therefore, using proteasome inhibitors as blockers of NF κ B activation is not always appropriate. Additionally, the vehicle solution the compound is dissolved in (ethanol), can also activate NF κ B and induce a pro-inflammatory response (Yuan *et al.* 2006), and as such, vehicle controls were included in the experiments. Although Mg132 treatment was effective in inhibiting LPS-induced NF κ B activation in the TtT/GF cells, the resting levels of NF κ B were increased by low concentrations of Mg132, which correlates with previous studies that have reported NF κ B activating properties of Mg132 in other cell systems (Pritts *et al.* 2002; Dolcet *et al.* 2006).

3.6.2 LPS signalling in folliculostellate cells

3.6.2.1 Characterisation of folliculostellate cell lines

The cell lines were initially characterised by RT-PCR for the expression of mRNAs for the LPS signalling components, CD14, Tlr4 and MD-2. As CD14 signalling also occurs through interaction with the receptor involved in recognition of gram-positive bacteria, leading to NF κ B activation, expression of the mRNA for Tlr2 was also examined in the cells. Like the RAW 264.7 cells, and in agreement with previous studies, the TtT/GF cells expressed mRNA for CD14 and Tlr4 (Lohrer *et al.* 2000). Furthermore, mRNAs for the accessory protein, MD-2, and Tlr2, were detected in the TtT/GF cells, providing evidence for the first time, to our knowledge, of an intact LPS-signalling system in these cells.

In contrast, mRNAs for Tlr4 and MD-2 were not detectable in the PDFS cells, suggesting that these cells do not respond to LPS and therefore, may not be as representative of folliculostellate cells as previously believed (Danila *et al.* 2000). However, studies of folliculostellate cells have identified at least five different subtypes (see section 1.7.3). It is unclear whether all these subtypes are responsive to endotoxin, and as such, it is possible that the PDFS cell line may represent a different subtype of folliculostellate cells in comparison to the TtT/GF cell line. However, as the current study used LPS as an immunological stimulus, the TtT/GF cell line was therefore the appropriate cell line to use as an *in vitro* model of folliculostellate cells.

3.6.2.2 Characterisation of corticotroph cell lines

Whilst both the AtT20 subclones, D1 and D16:16, expressed mRNAs for Tlr4, MD-2 and Tlr2, only the AtT20 D16:16 cells expressed mRNAs for CD14. Although these findings do not discount the responsiveness of the AtT20 D1 cells to LPS, as LPS signalling can still occur via interaction with the soluble receptor form of CD14 which is present in serum, the AtT20 D16:16 cells were employed for further studies of corticotrophs as, in addition to being used extensively in other studies (Woods *et al.* 1992; Tierney *et al.* 2003; Tierney *et al.* 2005), this subclone expressed mRNAs for all the LPS signalling components known to be present in primary corticotrophs (Tichomirowa *et al.* 2005).

3.6.2.3 Functional consequences of LPS signalling in TtT/GF, PDFS and RAW 264.7 cells - NF κ B activation and IL-6 release

In agreement with our suggestion that the PDFS cells would be unresponsive to LPS stimulation due to the absence of mRNAs for LPS signalling, the PDFS cells did not show an increase in IL-6 release when challenged with LPS. As these cells express Tlr2, it is possible that they are responsive to gram-positive bacteria or fungal stimulation, which activates cellular responses via this receptor. However, as this study focused on the use of LPS as the stimulus for NF κ B activation, further studies of folliculostellate cells were carried out on the TtT/GF cells. These findings highlight that initially characterising cell lines is extremely important for determining whether they are appropriate models for the intended study.

In concurrence with the presence of mRNAs for the LPS signalling components, the TtT/GF cells readily responded to LPS challenge in a time-dependent manner with increased IL-6 release evident within 4 hours of contact. The increase in IL-6 release was preceded by activation of NF κ B and followed by a secondary decrease in cytoplasmic p65 protein expression, detected by western blot analysis after 16 hours of LPS exposure. However, the reduction in cytoplasmic p65 was not associated with changes in nuclear p65 expression or the transcriptional activity of NF κ B activity and is therefore likely to be related to its degradation. The subsequent restoration of cytoplasmic p65 to control levels at 24 hours may reflect *de novo* synthesis of the protein.

The RAW 264.7 cells also responded to LPS stimulation with increases in IL-6 release which were considerably more pronounced than those observed in the TtT/GF cells. The high levels of IL-6 released from the RAW 264.7 cells correlated with a rapid and prolonged period of NF κ B activation, which persisted up to 24 hours after LPS stimulation. These findings are in agreement with previous studies reporting NF κ B-dependent regulation of IL-6 release from macrophages (Libermann & Baltimore 1990; Matsusaka *et al.* 1993; Deshpande *et al.* 1997). Because macrophages play an important role in mediating an inflammatory response, it is not surprising that the RAW 264.7 cells exhibit a sustained period of NF κ B activity, with high amounts of IL-6 release compared to the TtT/GF cells. In contrast, if comparable to primary tissue, the early and rapid response of the TtT/GF cells to LPS may reflect the existence of a tightly regulated system within the anterior pituitary gland, which ensures appropriate concentrations of IL-6 are produced rapidly to induce ACTH release in conditions of endotoxaemia. It is likely that the RAW 264.7 cells release high amounts of IL-6 release in response to LPS because, in addition to macrophage-derived cytokines being released locally at sites of infection, they are also released into the circulation to exert widespread systemic effects. In comparison, the low levels of IL-6 produced by the TtT/GF cells compared to the RAW 264.7 cells may reflect the fact that folliculostellate cell-derived factors generally act locally within the anterior pituitary gland and are not therefore diluted in the circulation before reaching their targets (Inoue *et al.* 1999).

3.6.2.4 NF κ B dependent IL-6 release from TtT/GF cells

In conditions of endotoxaemia, evidence supports a paracrine, stimulatory role for folliculostellate cell-derived IL-6 on ACTH release from neighbouring corticotrophs, in both the presence and absence of hypothalamic CRH (Renner *et al.* 1998; Bethin *et al.* 2000; Gloddek *et al.* 2001). IL-6 release from folliculostellate cells may therefore present a potential target for the negative feedback actions of glucocorticoids. As mentioned previously, in immune cells, such as macrophages and monocytes, IL-6 synthesis is regulated by NF κ B, a transcription factor that is inhibited by glucocorticoids (Auphan *et al.* 1995; Heck *et al.* 1997; Almawi & Melemedjian 2002). Therefore, the mechanism through which glucocorticoids inhibit ACTH release may be effected, in part, through the inhibition of NF κ B activity in folliculostellate cells. As outlined in the sections below, using both non-specific (section 3.6.2.4 and 3.6.2.5) and specific (section 3.6.2.6) NF κ B inhibitors, the current study has provided firm

evidence that an NF κ B-dependent pathway is involved in the regulation of IL-6 release from TtT/GF cells.

3.6.2.5 Effects of the proteasome inhibitor, Mg132, on LPS stimulated NF κ B activity and IL-6 release from TtT/GF cells

In the current study, treatment with Mg132 inhibited LPS-stimulated NF κ B activity and IL-6 release from the TtT/GF cells. Interestingly, whilst the vehicle solution in which Mg132 was dissolved (ethanol) did not affect basal activity of NF κ B in the TtT/GF cells, it further increased LPS stimulated NF κ B activity. Ethanol is a potent stimulator of NF κ B (Lee & Rivier 2005) and therefore, it is possible that whilst the concentrations (2mM) used in the current study were not sufficient to induce an independent increase in NF κ B activity, they may have been sufficient to potentiate the effects of LPS.

However, whilst Mg132 was shown to decrease NF κ B activity, incubation of the TtT/GF cells with lower concentrations of Mg132 (1 and 10 μ M) resulted in an increase in basal NF κ B activity, an effect that cannot be attributed to the vehicle. Previous studies have reported increased NF κ B activity induced by proteasome inhibitors such as Mg132 (Jobin *et al.* 1998; Lee *et al.* 2006), which was associated with activation of the heat shock response (Jobin *et al.* 1998). Therefore, in the resting state, low concentrations of Mg132, which do not completely block proteasome activity, may increase NF κ B activity in the TtT/GF cells through the activation of heat shock proteins. Concurrent with this, Mg132 treatment also increased basal IL-6 release from the TtT/GF cells, an effect which supports previous studies demonstrating increased NF κ B activity and IL-6 release from other cell types following Mg132 treatment (Pritts *et al.* 2002).

In LPS stimulated cells, treatment with 25 μ M Mg132 was more effective at inhibiting IL-6 release than a two-fold higher concentration of Mg132 (50 μ M). As 50 μ M Mg132 sufficiently reduced LPS induced NF κ B activity, the abrogated inhibitory effect of this agent on IL-6 suggests that in response to endotoxin, compensatory mechanisms may be activated to facilitate IL-6 release in conditions of repressed NF κ B activity, such as activation of other IL-6 transcription factors including AP-1, NF-IL6 and C/EBP (Akira *et al.* 1990; Bretz *et al.* 1994; Galien *et al.* 1996; Ray *et al.* 1997; Xiao *et al.* 2004). Screening of the activity of these transcription factors under these

conditions is required to confirm whether this is the case. Whether the stimulatory effects induced by Mg312 on IL-6 release in the TtT/GF cells involves an upregulation in heat shock proteins or other transcription factors involved in IL-6 synthesis, warrants further investigation and may present an additional signalling pathway through which IL-6 release is induced in these cells. However, because Mg132 is not a selective inhibitor of NF κ B, the inhibitory effects it exerted on IL-6 release from the TtT/GF cells cannot be assumed to occur through the suppression of NF κ B alone. Therefore, the effects of the specific NF κ B inhibitor, SC-514, on IL-6 release from LPS-stimulated TtT/GF cells were also examined.

3.6.2.6 Effects of the IKK β inhibitor, SC-514, on LPS stimulated NF κ B activation and IL-6 release from TtT/GF cells

The IKK complex is the convergence point for the activation of NF κ B in response to LPS. The IKK complex is composed of two catalytic subunits, IKK α and IKK β , and the regulatory subunit IKK γ (Didonato *et al.* 1997; Zandi *et al.* 1997; Hu & Wang 1998; Rothwarf *et al.* 1998; Yamaoka *et al.* 1998; Karin & Ben-Neriah 2000). Gene knockout studies have clearly demonstrated that the IKK β and IKK γ subunits are both required for NF κ B activation by most pro-inflammatory stimuli, including LPS (Li *et al.* 1999a; Li *et al.* 1999b). Although NF κ B activation is associated with increased IL-6 release from the TtT/GF cells, the study aimed to investigate the importance of this transcription factor in the regulation of IL-6 synthesis, utilising the IKK β inhibitor, SC-514.

The SC-514 drug, which has been used extensively by others as a selective NF κ B inhibitor for *in vitro* studies (Kishore *et al.* 2003; Buss *et al.* 2004; Gomez *et al.* 2005; Jeong *et al.* 2005; Peng *et al.* 2005; Hwang *et al.* 2006), was characterised by Kishore *et al.* and shown to inhibit all forms of recombinant human IKK β including the recombinant human (rh) IKK β homodimer, rhIKK α /IKK β heterodimer, as well as the constitutively active form of the rhIKK β (Ser¹⁷⁷-Glu, Ser¹⁸¹-Glu), with IC₅₀ values in the 3-12 μ M range (see figure 3.29A). Furthermore, SC-514 also inhibited the activated, native IKK complex immunoprecipitated from IL-1 β stimulated rheumatoid arthritis-derived synovial fibroblasts with similar potency (see figure 3.29B). However, the SC-514 inhibitor did not interfere with the other IKK isoforms - rhIKK α , rhIKK ϵ or the rhTBK-1 or other serine-threonine and tyrosine kinases (see figure 3.29C).

The current study showed that inhibition of NF κ B resulted in a significant decrease in LPS-stimulated IL-6 release from the TtT/GF cells. The inhibitory effect of SC-514 was no longer apparent at the higher concentration of 300 μ M, an effect which was also observed with increasing concentrations of Mg132. Although this could be due to toxic effects of the compound, it is likely that the transcriptional activity of other IL-6 transcription factors (e.g. NF-IL6 and C/EBP) come into effect in conditions of repressed NF κ B activity.

However, these findings provide compelling evidence that, NF κ B, which is normally associated with immune/inflammatory cells, also plays an important role within the anterior pituitary gland, through the regulation of IL-6 release from this folliculostellate cell line (figure 3.30). These findings are concurrent with previous studies that have demonstrated increased NF κ B activity in the TtT/GF cells and pituitary reaggregate co-cultures following LPS exposure (Baur *et al.* 2000; Lohrer *et al.* 2000) and studies that have suggested the involvement of an NF κ B-dependent pathway in the regulation of IL-6 from LPS-stimulated TtT/GF cells through the use of p38 α -MAPKinase inhibitors, as activation of the p38 α -MAPKinase pathway leads to NF κ B activation (Lohrer *et al.* 2000).

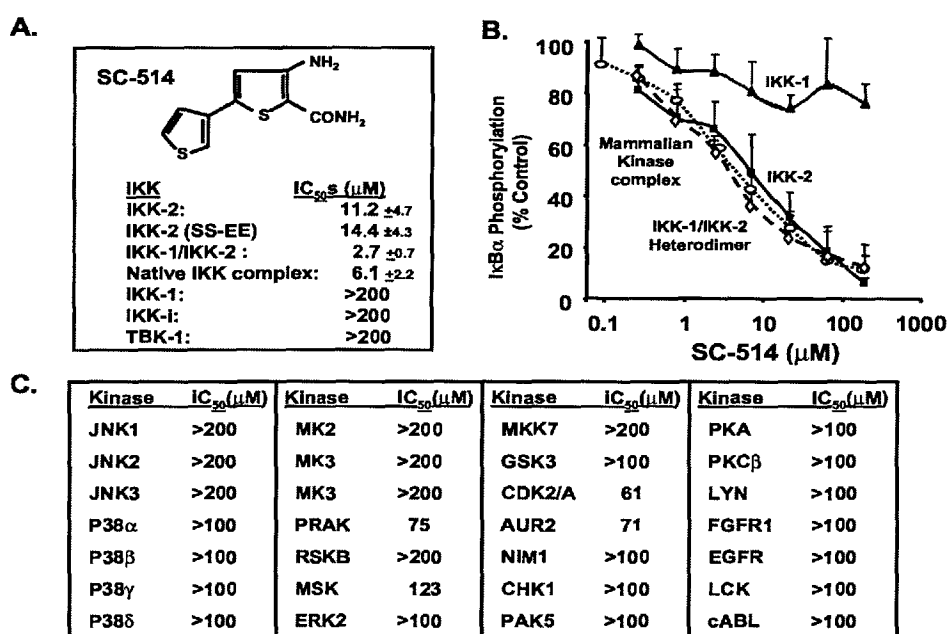


Figure 3.29 SC-514 is a potent and selective inhibitor of IKK β . (A) structure of SC-514 drug and (B) IC₅₀ values of SC-514 against IKK β and its isoforms. (c) IC₅₀ values of SC-514 against a panel of tyrosine and serine-threonine kinases [Taken from (Kishore *et al.* 2003)].

3.6.2.7 Inhibitory effects of the synthetic glucocorticoid, dexamethasone, on LPS stimulated NF κ B activity and IL-6 release from TtT/GF cells

Glucocorticoids are able to inhibit ACTH release through the upregulation of the secondary messenger ANXA1 (Buckingham & Flower 1997; Traverso *et al.* 1999; John *et al.* 2004) and through binding to glucocorticoid response elements (GREs) within the POMC promoter (Keller-Wood & Dallman 1984; Dallman *et al.* 1987; Nakai *et al.* 1991; De Kloet *et al.* 1998). In agreement with a previous study (Lohrer *et al.* 2000), our data showed for the first time that LPS-stimulated IL-6 release from the TtT/GF cells is sensitive to the inhibitory effects of glucocorticoids and this inhibitory effect is mediated, at least in part, through the inhibition of NF κ B by increasing the expression of I κ B α , identifying at least one mechanism through which dexamethasone inhibits NF κ B activity in the TtT/GF cells (Auphan *et al.* 1995; Scheinman *et al.* 1995a) (figure 3.30). If folliculostellate derived IL-6 is a paracrine mediator of ACTH release from corticotrophs, which there is strong evidence for (Gloddek *et al.* 2001), the findings from the current study suggest that NF κ B signalling in folliculostellate cells is an additional target for glucocorticoid-mediated inhibition of ACTH release from the anterior pituitary gland.

3.6.2.8 IL-6 response of TtT/GF cells to CRH stimulation

Previous studies have shown that the TtT/GF-derived factors such as MIF and IL-6 are not influenced by CRH stimulation (Tichomirowa *et al.* 2005; Tierney *et al.* 2005). However, to our knowledge, the effects of CRH stimulation on LPS-activated folliculostellate cells has not been previously examined and thus, the current study addressed this utilising the TtT/GF cell line. In concurrence with previous reports (Tichomirowa *et al.* 2005; Tierney *et al.* 2005), the TtT/GF cells used in this study did not display altered responses to LPS-stimulated IL-6 release in the presence of CRH. Classically, CRH stimulates POMC synthesis and ACTH release through its interaction with the CRH-R1 subtype expressed by corticotrophs [reviewed by (Aguilera *et al.* 2004)]. To the best of our knowledge, CRH receptor expression has not been examined in either primary folliculostellate cells or the TtT/GF cell line and, thus, we cannot exclude the possibility that the apparent inactivity of CRH in the TtT/GF cells reported here and elsewhere (Tichomirowa *et al.* 2005; Tierney *et al.* 2005) reflects a lack of functional CRH receptors, thus discounting the possibility that CRH may also stimulate ACTH release indirectly by acting on TtT/GF cells to increase IL-6 release (figure 3.30).

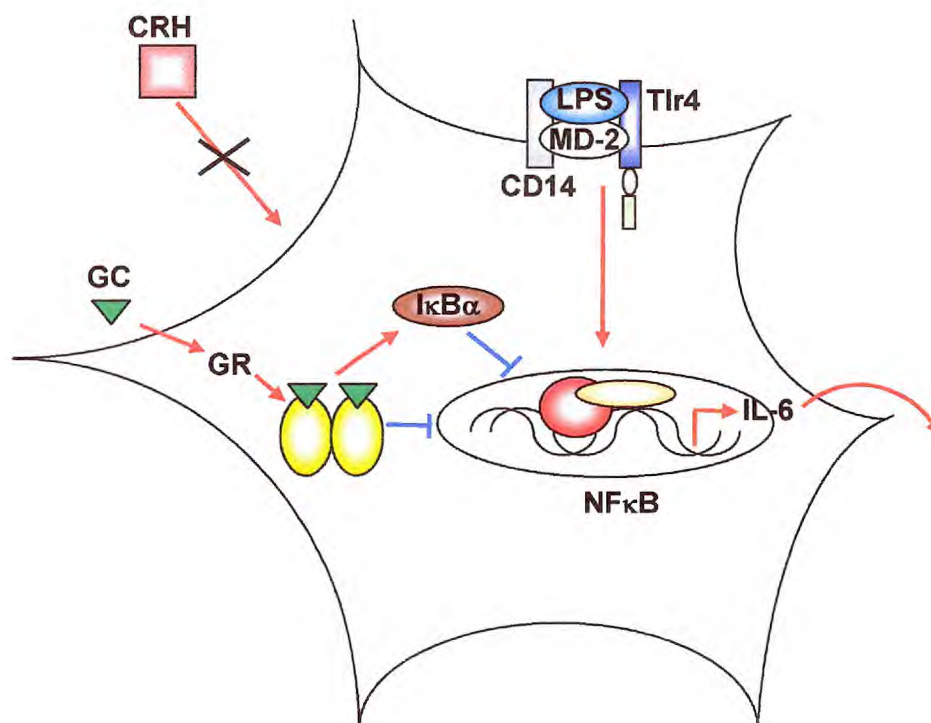


Figure 3.30 Regulation of IL-6 release from TtT/GF cells. Diagrammatic representation illustrating the proposed mechanisms by which LPS stimulates IL-6 synthesis and release from TtT/GF cells. Red arrows indicate pathways leading to increased IL-6 release and blue lines indicate inhibitory pathways leading to decreased IL-6 production. CD14, cluster of differentiation 14; CRH, corticotrophin releasing hormone; GC, glucocorticoid; GR, glucocorticoid receptor; $I\kappa B\alpha$, inhibitor of *kappa B alpha*; IL-6; interleukin 6; LPS, lipopolysaccharide; MD-2, myeloid differentiation factor 2; $NF\kappa B$, nuclear factor *kappa B*; Tlr4, toll-like receptor 4.

3.6.3 LPS signalling in corticotrophs

3.6.3.1 Modulation of ACTH release from AtT20 D16:16 cells by CRH and LPS

Although basal ACTH release by AtT20 D16:16 cells is modest, there was an accumulation of the ACTH in the medium over the time course studied; with significant increases evident after 7 hours incubation in fresh medium. Therefore, subsequent studies with the AtT20 D16:16 cells used a maximum incubation time of 7 hours to prevent high basal amounts of ACTH masking any effects of drug treatment. In agreement with previous studies, the AtT20 D16:16 cells readily

responded to CRH stimulation in a concentration-dependent manner (Woods *et al.* 1992). Despite expressing mRNAs for the LPS signalling components, the cells did not exhibit an ACTH response to LPS stimulation alone, which is in agreement with previous findings (Tichomirowa *et al.* 2005). However, in the study by Tichomirowa *et al.*, the effects of LPS were only examined on basal ACTH secretion (Tichomirowa *et al.* 2005). As endotoxin-induced plasma ACTH release is associated with increased levels of CRH, which has been detected both in the ME of the hypothalamus and in rat brain extracellular fluid (Ma *et al.* 2000; Beishuizen & Thijs 2003), this suggests that the stimulatory effects of LPS on ACTH release from the anterior pituitary corticotrophs may also involve CRH. Therefore, the effects of LPS upon CRH stimulated ACTH release from the AtT20 D16:16 cells were examined. The current study demonstrated an inhibitory effect of LPS on ACTH release in the presence of low, but not high, concentrations of CRH, suggesting that the inhibitory effects of LPS occur during the initial stages of endotoxaemia, when CRH concentrations are low. Furthermore, an indirect mechanism through which LPS inhibits ACTH release has been demonstrated recently (Solito *et al.* 2006). ANXA1, a glucocorticoid-induced protein which is released from folliculostellate cells and acts as a paracrine and juxtacrine mediator of ACTH release from corticotrophs (Tierney *et al.* 2003), is upregulated in folliculostellate cells by LPS challenge *in vitro* (Solito *et al.* 2006). Together these findings support the premise that LPS may target corticotrophs in the anterior pituitary to inhibit ACTH release as part of the protective mechanism that allows the pro-inflammatory host defence response to develop, before it is contained within appropriate limits by the hyper-secretion of the anti-inflammatory glucocorticoids (Munck & Naray-Fejes-Toth 1994).

3.6.3.2 NF κ B mediated ACTH release from AtT20 D16:16 cells

A recent report demonstrated an inhibitory effect of CRH on oxidative stress-induced NF κ B in corticotrophs *in vitro*, which was associated with increased POMC synthesis (Karalis *et al.* 2004). However, whilst this study supports the premise that corticotroph NF κ B is targeted by CRH as part of its stimulatory effect on ACTH synthesis, the mechanisms through which this occurs is unclear (Karalis *et al.* 2004). In the current study, LPS challenge attenuated the stimulatory effect of CRH on ACTH release from the AtT20 D16:16 cells and this was associated with increased NF κ B activity. Inhibition of LPS induced NF κ B reversed the inhibitory effect of LPS, suggesting that inhibition of ACTH release by LPS may occur via an NF κ B-mediated mechanism. CRH also stimulated NF κ B activity in the AtT20 D16:16 cells used in the

current study, which conflicts with the inhibitory effects of CRH reported by Karalis *et al.* There are several explanations for the conflicting findings; firstly, the culture conditions outlined in the previous study differed from this study – which included the absence of serum in the experimental conditions and the absence of proteins such as insulin and transferrin in the culture medium. These differences in culture conditions can lead to variations in cell phenotype and as such, cells can exhibit altered responses. Concurrent with this, previous studies have demonstrated that serum in the culture medium is required to effect LPS signalling *in vitro* (Lohrer *et al.* 2000). The study conducted by Karalis *et al.* did not include serum in the culture medium of the AtT20 cells and as LPS (at a concentration 50 times higher than that used in the current study) did not induce a noticeable increase in NFκB activation in these cells, compared to the AtT20 D16:16 cells used in the current study, this response is likely to be attributed to the impaired LPS signalling. However, the inhibitory effect of CRH observed upon NFκB activity in the AtT20 cells reported by Karalis *et al.* contradicts the stimulatory effect of CRH observed in the AtT20 subclone used in the current study. It is therefore possible that the conflicting findings are a result of different AtT20 subclones used in these studies. It is likely that the cell line used in the study conducted by Karalis *et al.* was the D1 sub-type which does not express the CD14 receptor and, due to the absence of serum in the experimental conditions, also lacked the presence of soluble CD14 for effective LPS signalling. These observations illustrate the potential difficulties of using different subclones of transformed cell lines, as the variations in phenotypes can result in false interpretations and comparisons being made between studies, hence the need for characterisation of cell lines to investigate their suitability.

However, both studies have identified an inhibitory effect of NFκB activity on ACTH release from corticotrophs *in vitro*, with the current study showing that inhibition of CRH-induced NFκB activity resulted in a potentiation of CRH stimulated ACTH release. CRH induced an early increase in resting levels of NFκB activity, with a later increase evident at 24 hours, suggesting a bi-phasic effect of CRH upon NFκB activation. Immunohistochemical analysis at the electron microscopic level has localised MIF to granules in the corticotrophs, with some granules containing MIF only, whereas others contain both MIF and ACTH (Nishino *et al.* 1995). Furthermore, functional studies using the AtT20 D16:16 cell line identified a stimulatory role of CRH upon MIF release (Nishino *et al.* 1995). It is therefore possible that the later increase in NFκB activity following CRH stimulation is due to autocrine actions of

factors such MIF, which both induces, and is induced by, NF κ B (Roger *et al.* 2001; Onodera *et al.* 2004; Cao *et al.* 2005; Amin *et al.* 2006; Cao *et al.* 2006).

The stimulatory effect of CRH upon NF κ B in the current corticotroph model system may reflect a counter-regulatory mechanism which is in place to prevent the over-production of ACTH release and ensure that levels of glucocorticoids produced are maintained within appropriate limits (figure 3.31). However, as concentrations of CRH increase, this inhibitory effect is less apparent, suggesting that when sufficient concentrations of endogenous CRH are released, the inhibitory effects of LPS, via NF κ B activation, are no longer effective. This effect would initially prevent high levels of glucocorticoids, which are potent anti-inflammatory agents, being released prematurely, before a sufficient inflammatory/immune response has been mounted.

The mechanism through which CRH induces NF κ B activation in the AtT20 D16:16 cells is unclear but, in contrast to CRH signalling through CRH-R1 to induce ACTH secretion [reviewed in (Aguilera *et al.* 2004)], CRH-R2 may be involved in CRH-stimulated NF κ B activation. This is supported by studies reporting that the pro-inflammatory effects exerted by peripherally-acting CRH involves increasing NF κ B activity in immune cells, via a CRH-R1-independent mechanism (Zhao & Karalis 2002). Whilst it is understood that the predominant receptor expressed by corticotrophs is the CRH-R1 subtype (Perrin & Vale 1999), CRH-R2 mRNA expression has been detected in the anterior pituitary gland, albeit at low levels (Kageyama *et al.* 2003) and it is possible that stimuli such as LPS can increase expression of this receptor, as has been reported in mast cells (Papadopoulou *et al.* 2005).

These findings support the premise that in addition to the classically perceived role of NF κ B in the inflammatory response, this transcription factor is also involved in the regulation of ACTH secretion from the anterior pituitary gland, through its activity in corticotrophs. However, the mechanisms through which NF κ B inhibits ACTH release are unclear, and may involve interference of this transcription factor with other known regulators of POMC synthesis, including AP-1 and the orphan nuclear receptor Nurr77 (Drouin *et al.* 1987; Drouin *et al.* 1989; Drouin *et al.* 1993; Phillips *et al.* 1997).

3.6.3.3 IL-6 increases CRH stimulated ACTH release from AtT20 D16:16 cells

The regulation of ACTH secretion from the anterior pituitary gland is complex, and the current findings have added to this complexity by showing that LPS differentially influences the secretion of IL-6 and ACTH from pituitary derived cell lines, via NF κ B-dependent mechanisms. LPS-induced NF κ B activation in the AtT20 D16:16 cells was shown to attenuate CRH-stimulated ACTH release. Paradoxically, it has been reported that LPS-stimulated IL-6 release from folliculostellate cells, which was shown in the current study to also occur via an NF κ B-dependent mechanism, stimulates ACTH secretion from corticotrophs via paracrine actions (Gloddek *et al.* 2001).

To investigate whether IL-6 stimulates ACTH release from the AtT20 D16:16 cells in this study, the effects of murine recombinant IL-6, at concentrations reflecting those produced by the TtT/GF cells after LPS stimulation, upon ACTH release were examined. Recombinant IL-6 potentiated ACTH release from the AtT20 D16:16 cells only in the presence of low concentrations of CRH, with this synergistic effect no longer being evident in the presence of increasing concentrations of CRH. This supports the premise that, in response to endotoxin exposure, folliculostellate-derived IL-6 acts to potentiate corticotroph-derived ACTH release in conditions of low CRH levels and possibly, to sustain ACTH levels as concentrations of CRH increase (figure 3.31).

3.7 Conclusion

The findings reported here have provided compelling evidence that the role of anterior pituitary-NF κ B signalling in the regulation of ACTH release is dependent on the cell population it is expressed in. In corticotrophs, NF κ B signalling negatively regulates ACTH release, whilst a positive effect upon ACTH release is exerted through its activity in folliculostellate cells, via the production of IL-6.

The purpose of this study was to examine the role of NF κ B in the regulation of ACTH release from the anterior pituitary gland, and whether its inhibition would mimic the effects induced by glucocorticoids in regard to attenuating ACTH release. The results obtained, using single cell-type cultures of TtT/GF and AtT20 D16:16 cells as models of folliculostellate cells and corticotrophs respectively, have shown that NF κ B, an important regulator of the cellular effects induced by LPS, acts in a cell-specific

manner to regulate ACTH release. Whilst its expression in the TtT/GF cells increases IL-6 release, which acts synergistically with CRH to further increase ACTH secretion, its activation in the AtT20 D16:16 cells has been shown to inhibit CRH-stimulated ACTH release. However, the mechanism through which this inhibitory effect occurs – whether it is directly at the transcriptional level of POMC synthesis or via the upregulation of other NF κ B-dependent mediators which interfere with ACTH release - remains unclear.

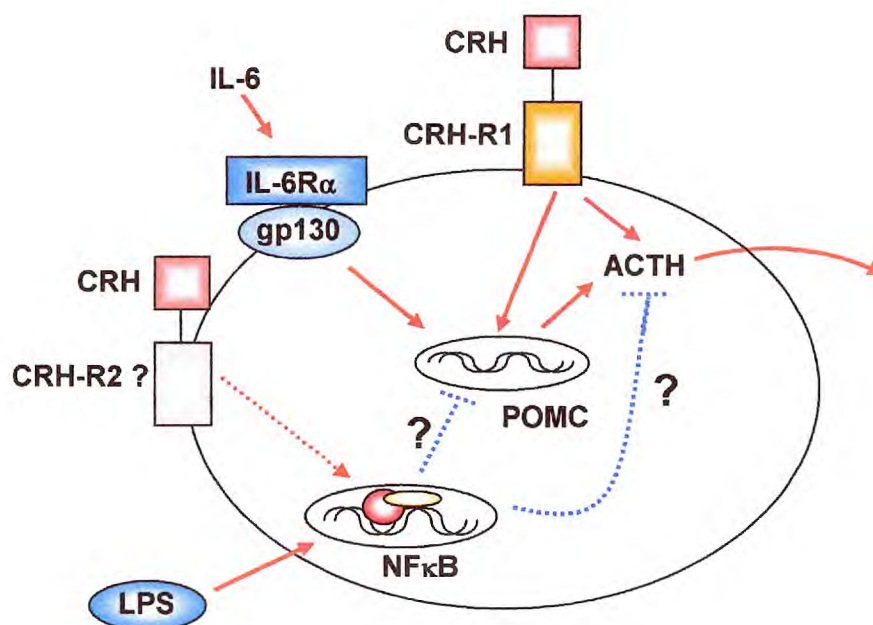


Figure 3.28 Regulation of ACTH release from AtT20 D16:16 cells. Diagrammatic representation illustrating the proposed mechanisms by which LPS acts on AtT20 D16:16 cells to inhibit ACTH synthesis and release. Red arrows indicate pathways leading to increased ACTH synthesis and release; dotted red lines indicate proposed pathways leading to ACTH synthesis and release and blue lines indicate inhibitory pathways leading to decreased ACTH synthesis and release. ACTH, adrenocorticotrophic hormone; CRH, corticotrophin releasing hormone; CRH-R, corticotrophin releasing hormone receptor; IL-6, interleukin 6; IL-6R α , interleukin 6 receptor *alpha*; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; POMC, proopiomelanocortin.

3.8 Future studies

The mechanisms involved in CRH-induced activation of NF κ B in the AtT20 D16:16 cells are unclear and warrant further investigation. It has been reported that the pro-inflammatory effects of CRH occur through the CRH-R2 receptor subtype (Zhao & Karalis 2002), whilst ACTH release by CRH is mediated via the CRH-R1 subtype (Perrin & Vale 1999). To identify the receptor subtype involved in CRH-mediated NF κ B activation, specific CRH-R1 and R2 receptor antagonists can be employed (Mousa *et al.* 2003; Mastorakos *et al.* 2006).

Although the current findings have identified a dual role for NF κ B signalling in the regulation of ACTH release, these studies were performed in single cell-type cultures of folliculostellate cells and corticotrophs. The effects of inhibiting NF κ B on IL-6 and ACTH release following LPS and/or CRH stimulation when these two cell types are cultured together, and thus, able to communicate with one another through direct physical contact and exposure to the mediators released by both cell types, needs to be addressed.

Whilst we are aware of the limitations of studying transformed cells, such co-culture systems can provide valuable information regarding the interactions between these two cell types in conditions of immunological stress and allow the elucidation of the molecular mechanisms involved. Co-cultures of transformed cell lines representing folliculostellate cells and corticotrophs were used to obtain a clear understanding of the interactions between these two cell-types. This approach is advantageous over primary pituitary tissue cultures, as the cells would not be exposed to factors released from other cell-types of the anterior pituitary, which could also influence ACTH secretion. For example, 50% of the cells of the anterior pituitary are somatotrophs, which release growth hormones. Growth hormones also stimulate ACTH release, through CRH-dependent and independent mechanisms (Ghigo *et al.* 1999). Additionally, the incubation times for treatment of the cells were relatively long (16 hours for IL-6 release and 7 hours for ACTH release). This may be problematic when using primary tissue cultures as their viability is limited and the effects detected may be attributed to cell death. As such, the role of NF κ B signalling in folliculostellate cell and corticotroph co-cultures was examined using the TtT/GF and AtT20 D16:16 cell lines.

4 Interactions between folliculostellate cells and corticotrophs in a co-culture system

4.1 Co-cultures of TtT/GF and AtT20 D16:16 cells

Folliculostellate cells form close connections with corticotrophs in the anterior pituitary gland and influence the secretion of ACTH by releasing pro-inflammatory cytokines, including IL-6 (Gloddek *et al.* 2001). The previous chapter focused on the responses of the TtT/GF and AtT20 D16:16 cells to LPS and CRH stimulation when cultured alone (chapter 3). However, because folliculostellate cells are positioned near corticotrophs in the anterior pituitary gland, this chapter examined the interactions between these cell types when they were cultured together. Imaging studies showed that the morphology of the TtT/GF cells differs from that of the AtT20 D16:16 cells (figure 4.1A and B), allowing the distinction of the cells when cultured together. Figure 4C shows a phase contrast image of the projections from the TtT/GF cells forming connections with each other and with the AtT20 D16:16 cells in monolayer co-cultures.

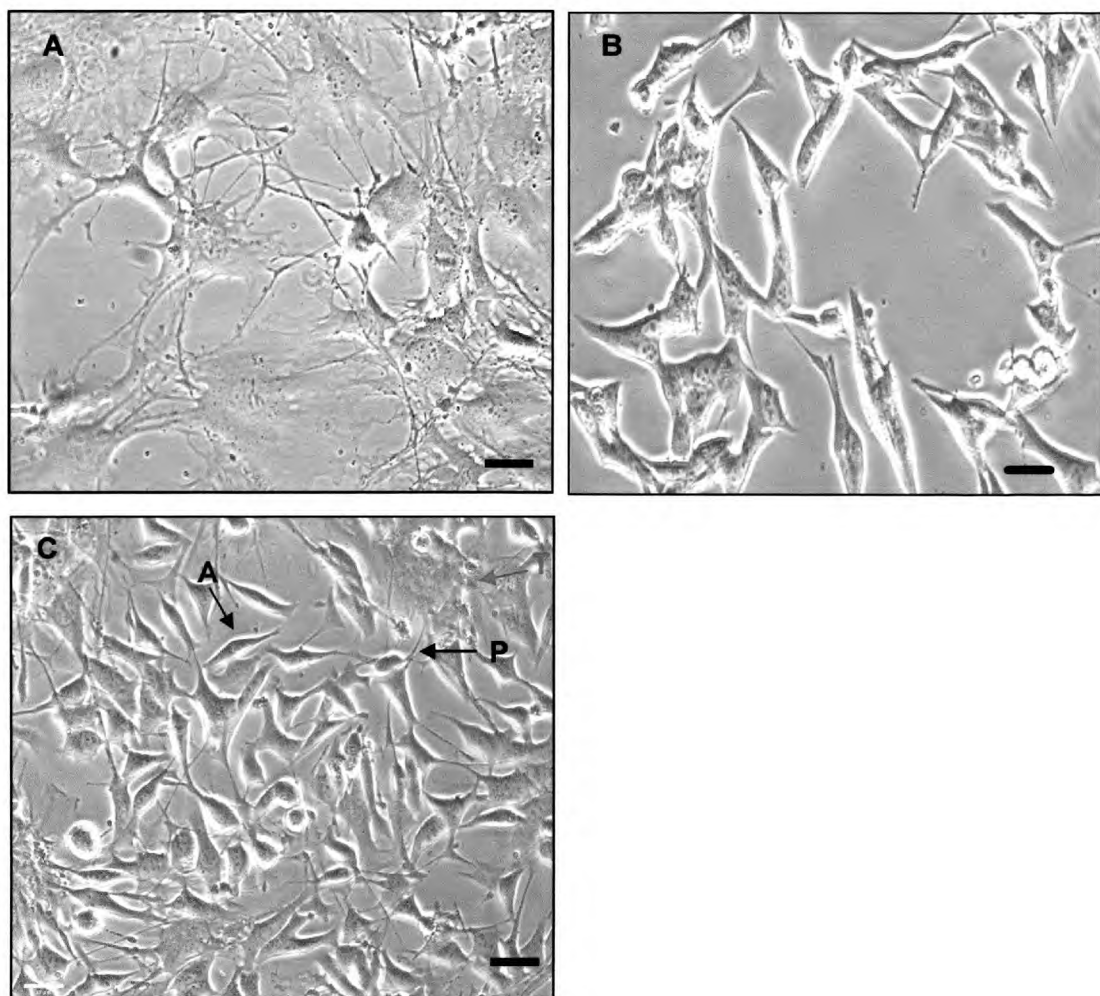


Figure 4.1 Phase contrast images of TtT/GF and AtT20 D16:16 cells. (A) TtT/GF cells, (B) AtT20 D16:16 cells and (C) monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. The co-cultures contained 200, 000 AtT20 D16:16 and 50, 000 TtT/GF cells. Scale bar is equal to 60 μ m. A, AtT20 D16:16 cell; T, TtT/GF cell; P, point of contact between a projection from a TtT/GF cell and an AtT20 D16:16 cell.

4.2 LPS and CRH signalling in co-cultures of TtT/GF and AtT20

D16:16 cells

The findings described in chapter 3 identified the transcription factor, NF κ B, as an important mediator of LPS stimulated IL-6 release from TtT/GF cells (section 3.4). In contrast, CRH (1, 10 and 100nM) failed to influence either basal or LPS (100ng/ml) stimulated IL-6 release from the TtT/GF cells (section 3.4.4).

The AtT20 D16:16 cells readily responded to CRH (1, 10 and 100nM) stimulation with significant increases in ACTH release (section 3.5.1). Whilst LPS (100ng/ml) failed to affect basal ACTH secretion (section 3.5.3), it attenuated the stimulatory effects of CRH (1 and 10nM) via an NF κ B dependent mechanism (sections 3.5.6 and 3.5.7).

The focus of this chapter was to investigate the IL-6 and ACTH responses of TtT/GF and AtT20 D16:16 cells to LPS and CRH when they are cultured together, and thus able to communicate with each other, and to examine the effect of the NF κ B inhibitor, SC-514, on these responses. Because both these cell types adhere to the flask wall, a monolayer co-culture system was utilised for these studies.

4.2.1 Determination of cell numbers of TtT/GF and AtT20 D16:16 cells plated in monolayer co-culture for optimal ACTH response to CRH stimulation

Initially, the optimal number of TtT/GF cells cultured with the AtT20 D16:16 cells was determined by culturing different ratios of the two cell-types together and measuring their ACTH responses to CRH stimulation. Figure 4.2 shows the effects of CRH (10nM) stimulation on ACTH secretion from the AtT20 D16:16 cells, when cultured alone (50, 000 or 200, 000 cells) and in the presence of TtT/GF cells at ratios of 1:1 (50, 000 TtT/GF cells cultured with 50, 000 AtT20 D16:16 cells), 1:4 (50, 000 TtT/GF cells cultured with 200, 000 AtT20 D16:16 cells) and 4:1 (200, 000 TtT/GF cells cultured with 50, 000 AtT20 D16:16 cells). CRH (10nM, 7 hours) significantly increased ACTH release from the AtT20 D16:16 cells (50, 000 cells, $p < 0.001$ vs unstimulated AtT20 D16:16 cells and 200, 000 cells, $p < 0.001$ vs unstimulated AtT20 D16:16 cells) and the co-cultures (1:1, 4:1 and 1:4, $p < 0.001$ vs respective unstimulated control; figure 5.2). Both basal [1:1, $p < 0.001$ vs AtT20 D16:16 (50, 000 cells) control group, 4:1, $p < 0.01$ vs AtT20 D16:16 (50, 000 cells) control group and 1:4, $p < 0.001$ vs AtT20 D16:16 (200, 000 cells) control group] and CRH-stimulated

[1:1 and 4:1, $p < 0.001$ vs AtT20 D16:16 (50, 000 cells) CRH-stimulated group and 1:4, $p < 0.001$ vs AtT20 D16:16 (200, 000 cells) CRH-stimulated group] ACTH release were increased in the presence of the TtT/GF cells compared to the AtT20 D16:16 single cultures (figure 4.2). CRH stimulation induced the greatest ACTH response from the 1:4 co-cultures ($p < 0.001$ vs 4:1 CRH-stimulated group, $p < 0.01$ vs 1:1 CRH-stimulated group; figure 4.2). These findings concurred with previous studies conducted in the laboratory (Tierney *et al.* 2003) and therefore, all further experiments were performed on co-cultures containing 50, 000 TtT/GF cells and 200, 000 AtT20 D16:16 cells.

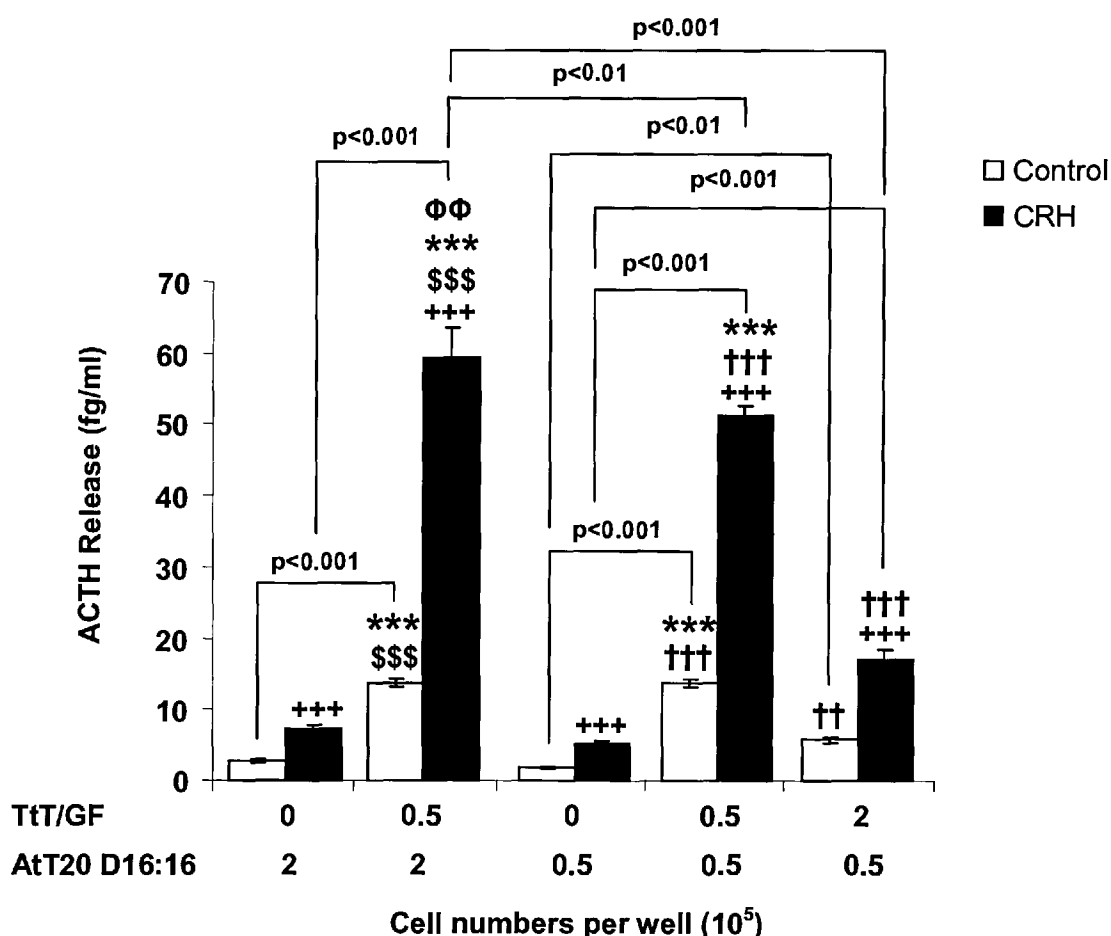


Figure 4.2 Effect of CRH (10nM, 7 hours) stimulation on ACTH release from AtT20 D16:16 cells and monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. AtT20 D16:16 cells (50, 000 or 200, 000 cells per well) were cultured alone or in the presence of TtT/GF cells (50, 000–200, 000 cells per well). *** $p < 0.001$ vs control (within corresponding culture group), $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$ vs corresponding treatment group of AtT20 D16:16 cells alone (50, 000 cells), $\text{\$}\text{\$}\text{\$} p < 0.001$ vs corresponding treatment group of AtT20 D16:16 cells alone (200, 000 cells), $\text{\$}\text{\$}\text{\$} p < 0.001$ vs corresponding treatment group of 200, 000 TtT/GF cells cultured with 50, 000 AtT20 D16:16 cells (4:1), $\Phi\Phi p < 0.01$ vs 50, 000 TtT/GF cells cultured with 50, 000 AtT20 D16:16 cells (1:1) CRH stimulated group. Control groups were incubated in medium containing vehicle (10 μ M ascorbic acid). Each value is a mean $\pm$ SEM of six samples. Representative example of three separate experiments. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH adrenocorticotrophin; CRH, corticotrophin-releasing hormone.

4.2.2 Rate of cell proliferation of single cell and monolayer co-cultures of TtT/GF and AtT20 D16:16 cells plated at a ratio of 1:4

To examine whether the rate of cell proliferation of the TtT/GF and AtT20 D16:16 cells was changed when the cells were cultured together, the number of TtT/GF and AtT20 D16:16 cells cultured alone and together were counted on the day of plating (day 1) and the day of stimulation (day 3). The number of AtT20 D16:16 cells in the single culture increased by 45% after 3 days in culture, compared to a 39% increase in cell growth when they were cultured with the TtT/GF cells (figure 4.3). Overall, the number of AtT20 D16:16 cells in the single culture was significantly higher than the number of cells in the co-cultures ($p < 0.001$; figure 4.3). The TtT/GF cell number increased by 78% when cultured alone, compared to an increase of 54% when cultured with the AtT20 D16:16 cells (figure 4.3). Similarly to the AtT20 D16:16 cells, the number of TtT/GF cells in the single cultures was significantly higher than in the co-cultures ($p < 0.001$; figure 4.3)

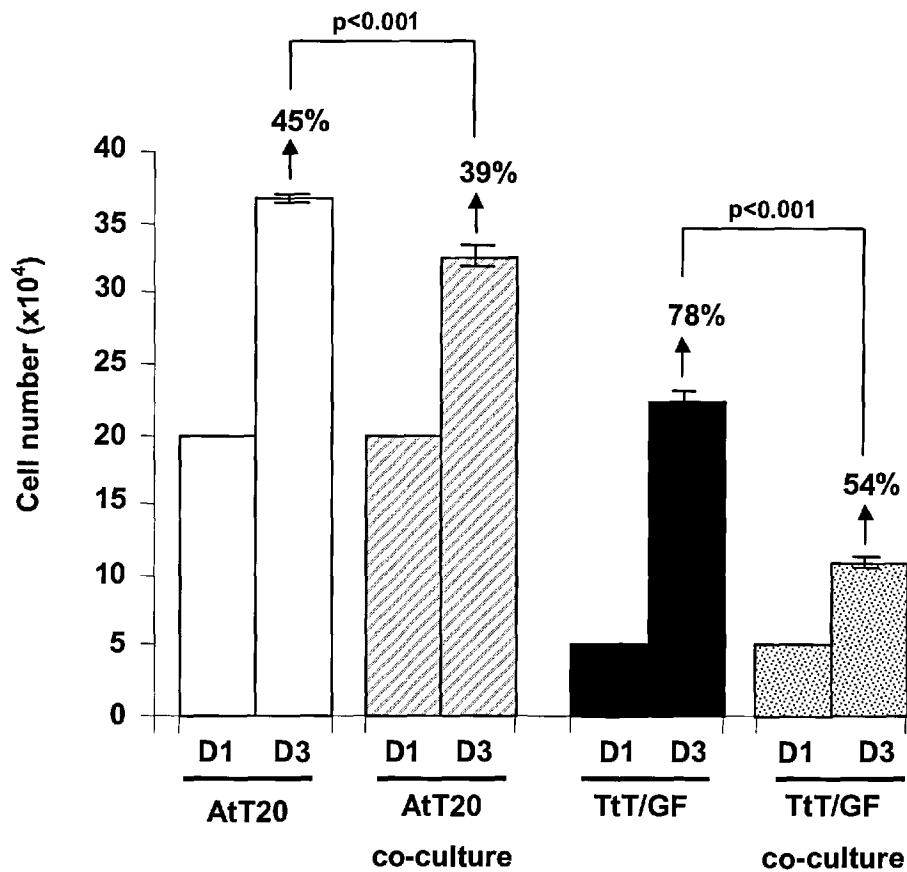


Figure 4.3 Cell proliferation of single cultures and monolayer co-cultures of TtT/GF and AtT20 D16:16 cells after 3 days in culture. The number of TtT/GF and AtT20 D16:16 cells in single and co-cultures was counted on the day of plating (day 1, D1) and the day of stimulation (day 3, D3). Each value is a mean $\pm$ SEM of five samples in a single experiment. Analysis was performed by one-way ANOVA with *post hoc* comparison using Bonferroni's t-test.

4.2.3 Effects of CRH on LPS-stimulated IL-6 release from monolayer co-cultures of TtT/GF and AtT20 cells plated at a ratio of 1:4

The effect of CRH (1 and 10nM, 16 hours) on basal and LPS (100ng/ml, 16 hours) stimulated IL-6 release from the co-cultures was examined. LPS stimulation induced a significant increase ($p<0.001$ vs control) in IL-6 release (figure 4.4). Whilst CRH alone (1 and 10nM) had no effect on basal IL-6 release ($p=1.00$), it potentiated the stimulatory effect of LPS in a concentration-dependent manner (1nM CRH, $p<0.05$ vs LPS and 10nM, $p<0.001$ vs LPS; figure 4.4).

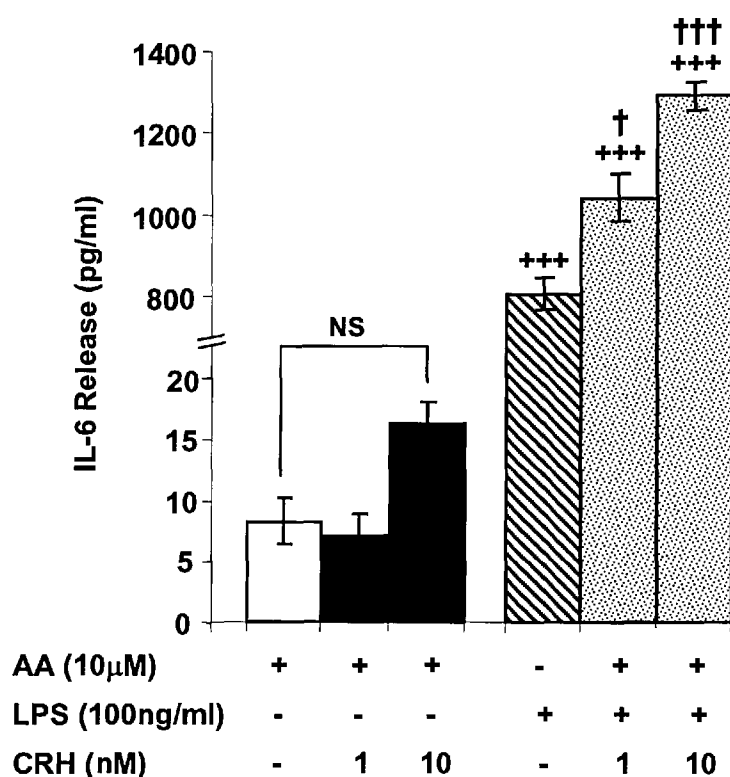


Figure 4.4 Effects of LPS (100ng/ml, 16 hours) and CRH (1 and 10nM, 16 hours) on IL-6 release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. Control (10µM ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. *** $p<0.001$ vs control (AA), $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.001$ vs LPS, *** $p<0.001$ vs CRH + LPS. Analysis performed by two way ANOVA with *post hoc* comparison using Bonferroni's t-test. CRH, corticotrophin releasing hormone; IL-6, interleukin 6; LPS, lipopolysaccharide; NS, not significant.

4.2.4 Effects of LPS on CRH-stimulated ACTH release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells plated at a ratio of 1:4

The effect of LPS (100ng/ml, 7 hours) on CRH (1 and 10nM, 7 hours) stimulated ACTH release from co-cultures of TtT/GF and AtT20 D16:16 cells were examined. Basal ACTH release was significantly increased with 10nM CRH stimulation ($p<0.001$ vs control), but not at the lower concentration of 1nM CRH ($p=0.113$). Although LPS did not affect basal secretion of ACTH ($p=0.867$), it potentiated the stimulatory effects of CRH ($p<0.01$ vs 1nM CRH alone, $p<0.001$ vs 10 nM CRH; figure 4.5).

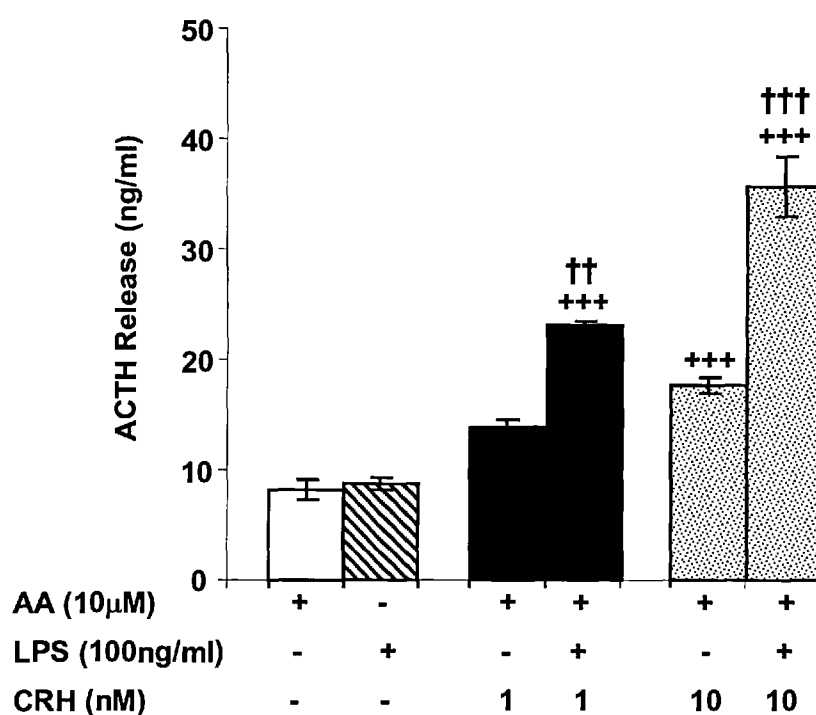


Figure 4.5 Effects of LPS (100ng/ml, 7 hours) on CRH (1 and 10nM, 7 hours) stimulated ACTH release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. Each value is a mean $\pm$ SEM of six samples. Representative example of three separate experiments. $^{***}p<0.001$ vs control (10 μ M ascorbic acid), $^{††}p<0.01$, $^{†††}p<0.001$ vs corresponding CRH only stimulated group. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; ACTH adrenocorticotrophin; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide.

4.3 Effects of NF κ B inhibition upon ACTH and IL-6 release from co-cultures of TtT/GF and AtT20 D16:16 cells

The findings from the single culture studies (chapter 3) support a dual role for NF κ B signalling in the regulation of ACTH release, exerting a positive effect through its activity in the folliculostellate-like TtT/GF cells and an inhibitory effect through its activity in the corticotroph-like AtT20 D16:16 cells. To examine the role of NF κ B in mediating IL-6 and ACTH release when these two cell-types are cultured together, the specific NF κ B inhibitor, SC-514, was used.

4.3.1 Effects of the IKK β inhibitor, SC-514, on IL-6 release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells

The release of IL-6 from the co-cultures was significantly increased by treatment with LPS (100ng/ml, $p < 0.001$ vs unstimulated control; figure 4.6A and B). Whilst treatment with SC-514 (100 μ M) alone did not affect basal IL-6 ($p = 1.00$), it significantly attenuated the stimulatory effect of LPS ($p < 0.01$ vs LPS; figure 4.6A and B). CRH alone (1 and 10nM) did not affect basal IL-6, either in the presence or absence of SC-514 ($p = 1.00$; figure 4.6A and B). However, in the presence of LPS, both concentrations of CRH potentiated the stimulatory effect of LPS ($p < 0.001$ vs LPS; figure 4.6A and B). This increase was attenuated by inclusion of SC-514 in the medium ($p < 0.01$ vs LPS + 1nM CRH and $p < 0.001$ vs LPS + 10nM CRH), although the amounts of IL-6 released remained significantly higher than the LPS-stimulated group ($p < 0.01$ vs LPS and $p < 0.001$ vs LPS; figure 4.6A and B).

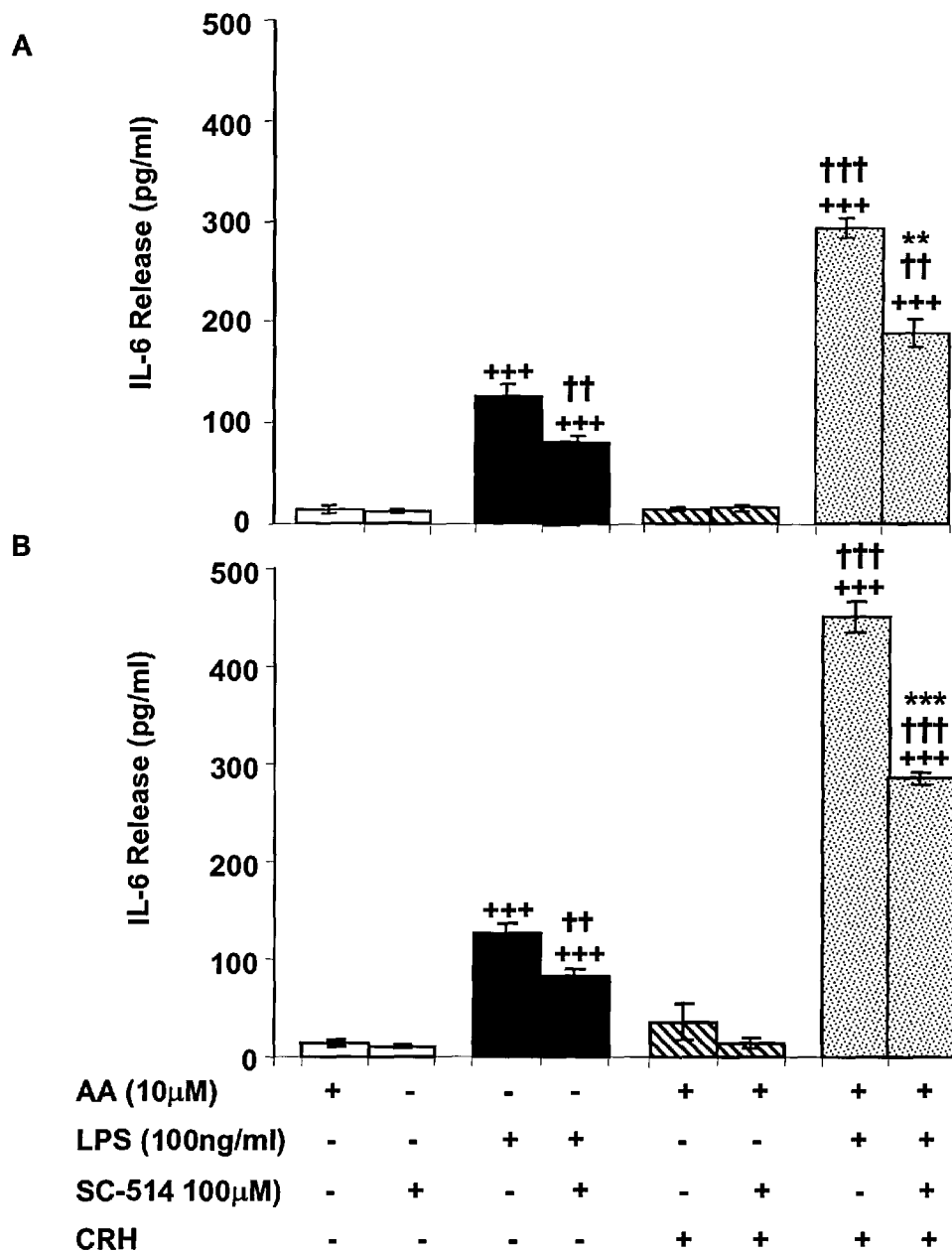


Figure 4.6 Effects of SC-514 treatment (100 μ M, 17 hours) upon LPS (100ng/ml, 16 hours) and (A) 1nM CRH (16 hours) and (B) 10nM CRH (16 hours) stimulated IL-6 release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. The cultures were incubated with SC-514 for 1 hour prior to the addition of LPS and CRH. Control (10 μ M ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. ** p <0.01, * p <0.001 vs control (AA), †† p <0.01, ††† p <0.001 vs LPS, ** p <0.01, *** p <0.001 vs CRH + LPS. Analysis was performed by three-way ANOVA followed by a second order one-way ANOVA with *post hoc* comparison using Bonferroni's *t*-test. AA, ascorbic acid; CRH, corticotrophin releasing hormone; IL-6, interleukin 6; LPS, lipopolysaccharide.**

4.3.2 Effects of the IKK β inhibitor, SC-514, on ACTH release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells

The basal release of ACTH from the co-cultures was significantly increased following stimulation with CRH for 16 hours (1nM CRH, $p < 0.01$ vs control; figure 4.6A and 10 and 100nM CRH, $p < 0.001$ vs control; figure 4.7B and C). Although incubation with LPS (100ng/ml, 16 hours) did not affect basal ACTH release ($p = 1.00$), it potentiated the stimulatory effect of 1nM CRH ($p < 0.05$ vs 1nM CRH alone; figure 4.7A) and 10nM CRH ($p < 0.01$ vs 10nM CRH alone; figure 4.7B), but not 100nM CRH ($p = 0.093$; figure 4.7C). SC-514 (100 μ M, 17 hours) treatment also potentiated the stimulatory effect of 10nM CRH ($p < 0.01$ vs 10nM CRH; figure 4.7B) and 100nM CRH ($p < 0.01$ vs 100nM CRH, figure 4.7C), but not of 1nM CRH ($p = 0.061$; figure 4.7A). Treatment with SC-514, followed by combined stimulation with LPS and 10nM CRH, but not 100nM CRH (SC-514 + LPS + 100nM CRH vs SC-514 + 100nM CRH; $p = 0.130$), resulted in a further increase in ACTH release ($p < 0.001$ vs CRH + LPS, $p < 0.01$ vs CRH + SC-514; figure 4.7B).

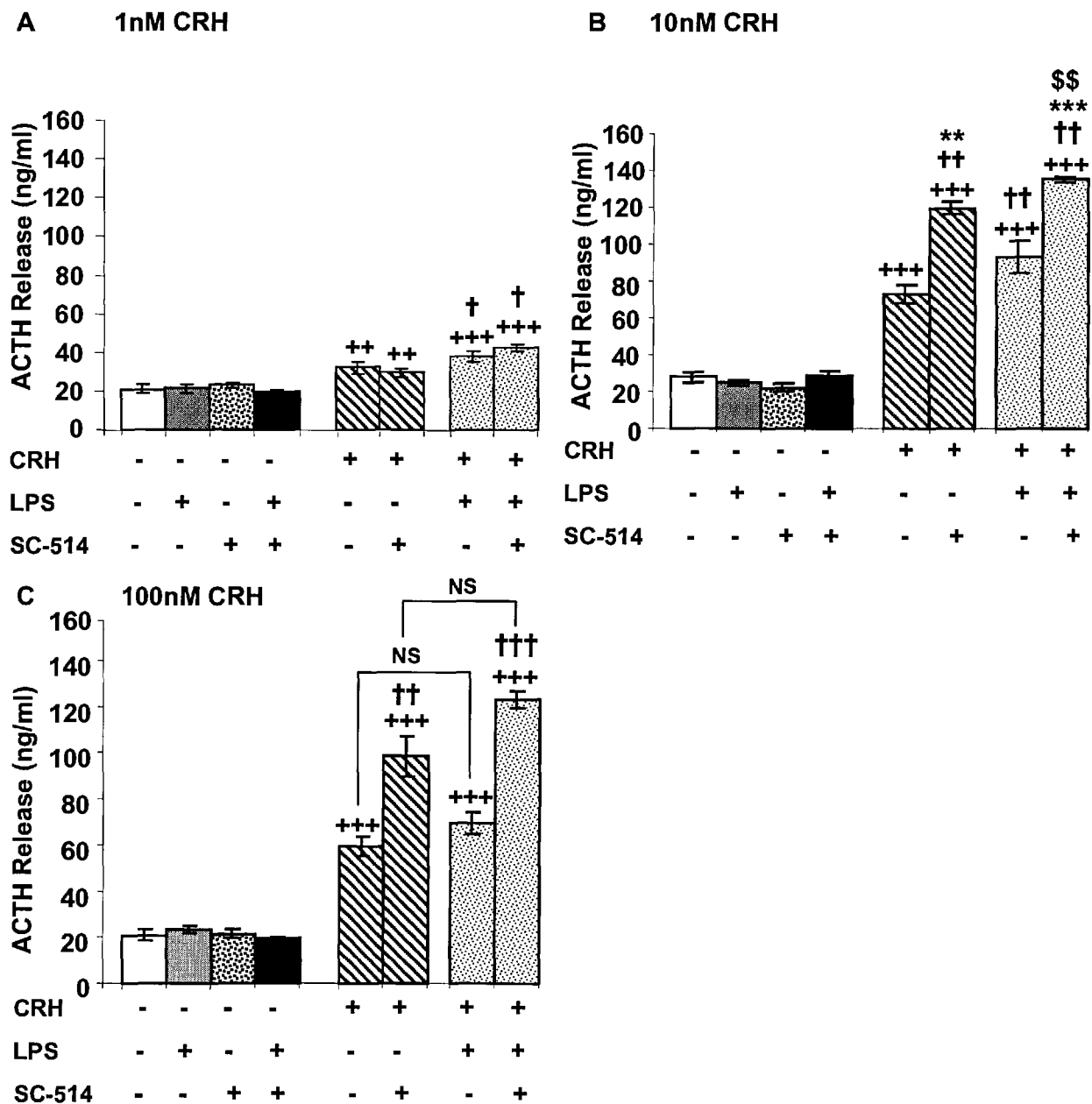


Figure 4.7 Effects of the NF κ B inhibitor, SC-514 (100 μ M, 8 hours), on LPS (100ng/ml) and (A) 1nM, (B) 10nM and (c) 10nM CRH stimulated ACTH release from monolayer co-cultures of TtT/GF and AtT20 D16:16 cells. Co-cultures were incubated with SC-514 1 hour prior to addition of LPS (7 hours) and CRH (7 hours). Control (10 μ M ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. The data are representative of three separate experiments. ++ p <0.01, +++ p <0.001 vs control, $\dagger p$ <0.05, $\dagger\dagger p$ <0.01, $\dagger\dagger\dagger p$ <0.001 vs CRH, ** p <0.01, *** p <0.001 vs CRH + LPS, \$\$ p <0.01 vs CRH + SC-514. Analysis was performed by three-way ANOVA followed by a second order one-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide; NS, not significant.

4.3.3 Effects of the IKK β inhibitor, SC-514, on ACTH release from aggregate co-cultures of TtT/GF and AtT20 D16:16 cells

The aforementioned experiments utilised monolayer co-cultures of TtT/GF and AtT20 cells D16:16 cells. To examine whether the co-cultures would behave differently if cultured as three-dimensional structures, the effects of SC-514 (100 μ M, 8 hours) treatment on LPS (100ng/ml, 7 hours) and CRH (10nM, 7 hours) stimulated re-aggregate co-cultures (containing 50, 000 TtT/GF cells cultured with 200, 000 AtT20 D16:16 cells) was compared. Unlike the monolayer cultures (figure 4.7B), LPS induced a small, but significant increase in ACTH release from the aggregate cultures ($p < 0.05$ vs control; figure 4.8). LPS exposure also potentiated the stimulatory effect of CRH ($p < 0.001$ vs CRH; figure 4.8), similarly to the monolayer cultures (figure 4.7B). Incubation of the aggregates with SC-514 and CRH resulted in a similar ACTH response to the monolayer cultures (figure 4.7B), with a further increase in ACTH release ($p < 0.001$ vs CRH), but this increase was not significantly different from that observed with CRH and LPS stimulation ($p = 1.00$; figure 4.8). However, incubation with SC-514 and LPS significantly increased CRH-stimulated ACTH release compared to the CRH + SC-514 ($p < 0.001$) and CRH + LPS ($p < 0.001$) treated groups (figure 4.8), a response which was also seen in the monolayer co-cultures (figure 4.7B).

4.3.4 Effects of conditioned medium from TtT/GF cells on the release of ACTH from AtT20 D16:16 cells

To examine whether the synergistic interactions of LPS and CRH upon ACTH release from the co-cultures was dependent on cell to cell contact between the TtT/GF and AtT20 cells, or whether it was attributed to mediators released from the TtT/GF cells acting on the AtT20 D16:16 cells, the effects of incubating the AtT20 D16:16 cells with conditioned medium, collected from TtT/GF cells stimulated with LPS (100ng/ml) and CRH (10nM), upon ACTH secretion was investigated. As SC-514 (100 μ M) treatment potentiated the stimulatory effect of LPS (100ng/ml) and CRH (10nM) on ACTH release from the co-cultures (monolayer co-cultures; figure 4.7B and aggregate co-cultures; figure 4.8), the effect of medium collected from TtT/GF cells, treated with SC-514 (100 μ M), on ACTH release from AtT20 D16:16 cells was further explored.

The TtT/GF cells were stimulated with LPS (100ng/ml) in the presence and absence of CRH (10nM) and/or SC-514 (100 μ M: pre-incubation of 1 hour prior to addition of LPS and/or CRH stimulation) for 7 hours. The AtT20 D16:16 cells were incubated with the conditioned medium collected from the TtT/GF cells for a further 7 hours. For comparison, the AtT20 D16:16 cells were incubated for 7 hours with unconditioned medium, which had been treated under the same conditions as the TtT/GF cells.

The conditioned medium had no effect on the basal release of ACTH from the AtT20 D16:16 cells ($p=1.00$; figure 4.9). The basal release of ACTH from the AtT20 D16:16 cells incubated with conditioned or unconditioned medium was also unaffected by inclusion of LPS in the medium (unconditioned medium, $p=0.885$ and conditioned medium, $p=0.771$) and (unconditioned medium, $p=0.624$ and conditioned $p=0.493$) /or (unconditioned medium, $p=0.239$ and conditioned $p=0.874$) SC-514 (figure 4.9).

The release of ACTH from the AtT20 D16:16 cells incubated in conditioned and unconditioned medium was increased to the same degree by CRH ($p<0.001$ vs basal) and the effects of the peptide were potentiated by SC-514 in both conditions ($p<0.01$ vs CRH alone; figure 4.9). In the unconditioned medium, LPS attenuated CRH-stimulated ACTH release ($p<0.01$ vs CRH) and this effect was overcome in the presence of SC-514 ($p<0.01$ vs LPS and CRH; figure 4.9). In contrast, the attenuating effect of unconditioned medium containing LPS upon ACTH release was

reversed when the cells were incubated with conditioned medium containing LPS ($p < 0.01$ vs unconditioned medium containing LPS and CRH; figure 4.9).

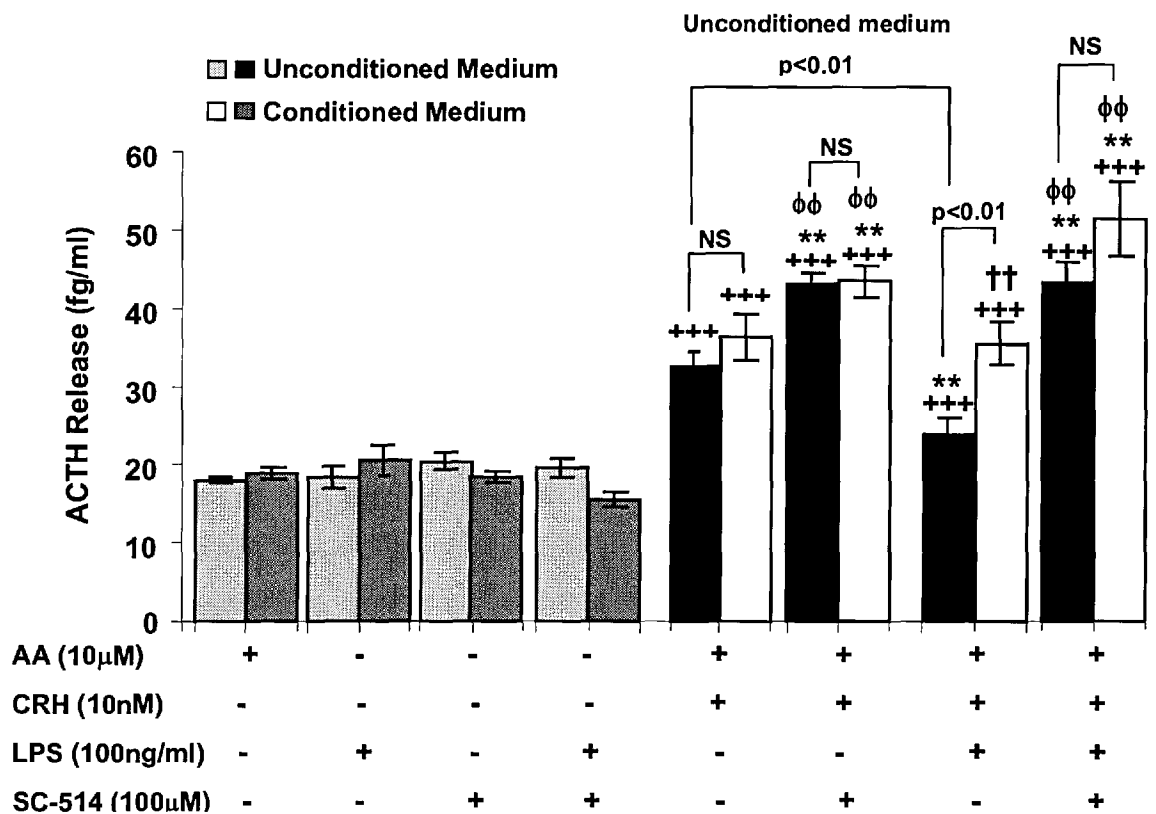


Figure 4.9 Effects of conditioned medium collected from TtT/GF cells following LPS (100ng/ml, 7 hours), CRH (10nM, 7 hours) and SC-514 (100 μ M, 8 hours) stimulation upon ACTH release from AtT20 D16:16 cells. TtT/GF cells (50, 000) were pre-incubated with SC-514 for 1 hour prior to the addition of LPS and/or CRH (7 hours). Following incubation, the medium from the TtT/GF cells was collected (medium still contained LPS, CRH and/or SC-514) and added to AtT20 D16:16 (200, 000) cells. The AtT20 D16:16 cells were then incubated with the TtT/GF cell-conditioned medium for a further 7 hours. The ACTH response of the AtT20 D16:16 cells to conditioned medium were compared to their response to unconditioned medium (AtT20 D16:16 cells were incubated for 7 hours in unconditioned medium). The unconditioned medium was treated under the same conditions as the conditioned medium, i.e. the unconditioned medium, containing LPS and/or CRH and SC-514 were placed in empty wells and incubated for 7 hours under the same conditions as the TtT/GF cells prior to addition to the AtT20 D16:16 cells. Control (10 μ M ascorbic acid) and treated groups are the mean of six samples $\pm$ SEM. +++p<0.001 vs control, $\dagger\dagger$ p<0.01 vs unconditioned medium (within the same treatment group), **p<0.01 vs CRH stimulated conditioned medium group), $\phi\phi$ p<0.01 vs CRH + LPS. Analysis was performed by three-way ANOVA followed by a second order two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. AA, ascorbic acid; ACTH, adrenocorticotrophin; CRH, corticotrophin releasing hormone; LPS, lipopolysaccharide; NS, not significant.

4.4 Discussion

4.4.1 Monolayer co-cultures of TtT/GF and AtT20 D16:16 cells

Given the complexity of intra-pituitary signalling, direct investigation of communication between two specific cell types is not possible in an *in vivo* model. Similarly, studies on primary pituitary tissue *in vitro* are limited in this regard because of the presence of multiple cell types. Several workers have attempted to purify specific pituitary cell populations to address these problems and whilst the preparations of enriched cell populations of a variety of pituitary types, including folliculostellate cells and corticotrophs, have met with some success, the cell populations are not homogenous and, thus, the results cannot be attributed solely to the cell types under investigation (Pryor-Jones *et al.* 1989; Alexander *et al.* 1991; Alexander *et al.* 1995; Gloddek *et al.* 2001). Furthermore, these preparations require the use of large numbers of animals and have limited viability *in vitro* (Alexander *et al.* 1991; Alexander *et al.* 1995). An alternative approach is to use cell lines as models. Accordingly, in the present study we used two pituitary cell lines, the murine folliculostellate-like TtT/GF cell line (Inoue *et al.* 1992; Lohrer *et al.* 2000; Gloddek *et al.* 2001) and the murine corticotroph-like AtT20 D16:16 cell line (Woods *et al.* 1992), to model interactions between folliculostellate cells and corticotrophs.

4.4.1.1 Increased basal ACTH release from AtT20 D16:16 cells in the presence of the TtT/GF cells

The current study showed that both basal and CRH-stimulated ACTH release from the AtT20 D16:16 cells are significantly enhanced in the presence of TtT/GF cells, thus confirming a previous report from our laboratory (Tierney *et al.* 2003). Since TtT/GF cells produce growth factors which are known to increase corticotroph proliferation, e.g. bFGF (Ferrara *et al.* 1987; Amano *et al.* 1993; Van Wijk *et al.* 1995), we hypothesised that this differential effect may reflect increased proliferation of the AtT20 D16:16 cells. However, our data showed that, when grown in the presence of TtT/GF cells, the rate of proliferation of the AtT20 D16:16 cells was reduced, as indeed was that of the TtT/GF cells. This surprising finding may be attributed to the relative decrease in the surface area available for cell adhesion in the co-cultures vs. single cell cultures, as the total number of cells in the co-culture (5×10^4 TtT/GF cells cultured with 2×10^5 AtT20 D16:16 cells) was greater compared to the total number of cells in the single cultures (5×10^4 TtT/GF cells cultured alone and 2×10^5 AtT20 D16:16 cells cultured alone). To examine this, further studies are

required using varied culture conditions and more precise measurements of proliferation, e.g. [³H]-thymidine uptake or measures of DNA content.

Several factors released from folliculostellate cells are known to exert a positive effect on ACTH secretion from corticotrophs and, in particular, to enhance the response to CRH (Auernhammer & Melmed 1999). Of particular importance are IL-6 and other cytokines such as LIF, which act via gp130 receptors (Ferrara *et al.* 1992; Gearing 1993; Bousquet *et al.* 1997). Although measurements of cytokine release were not made in the study in which we compared single cell cultures and co-cultures directly, it is feasible that the increased ACTH release observed in the presence of TtT/GF cells reflects the actions of these agents (Chesnokova *et al.* 1998; Chesnokova & Melmed 2000) which may accumulate at the junctions between TtT/GF and AtT20 D16:16 cells at high concentrations.

4.4.1.2 Increased ACTH response of monolayer co-cultures of TtT/GF and AtT20 D16:16 cells to LPS and CRH stimulation

In contrast to the attenuating effect of LPS upon CRH-induced ACTH release from the AtT20 D16:16 cells (section 3.5.6), the current study has provided evidence that the ACTH-stimulating effects of LPS at the anterior pituitary level are mediated through its actions on folliculostellate cells, with LPS potentiating CRH-induced ACTH secretion. Our previous studies on single cell cultures (section 3.5.5) indicated that at concentrations akin to those found in medium from TtT/GF cells challenged with LPS, IL-6 alone does not increase ACTH *per se* from AtT20 D16:16 cells, although it potentiates the stimulatory effect of low concentrations of CRH, suggesting that this cytokine is important in the ACTH response during the early stages of endotoxaemia, when CRH concentrations are low. Therefore, unless the concentration of IL-6 increases in the intercellular spaces between the TtT/GF and AtT20 D16:16 cells to amounts sufficient to stimulate ACTH release, the potentiating effects of LPS observed in the current study are likely to involve other TtT/GF cell-derived mediators induced by LPS, e.g. LIF (Wang *et al.* 1996). The section below discusses such potential TtT/GF cell-derived factors.

4.4.1.3 Potential ACTH-stimulating cytokines released from folliculostellate cells

In vivo studies show that peripherally administered LPS rapidly and massively activates NFκB transcriptional activity in cells throughout the anterior and posterior

pituitary lobes (Quan *et al.* 1998; Whiteside *et al.* 1999). In addition, mRNAs for cytokines such as IL-1 β and TNF α are more discretely induced in both lobes, with separate induction time courses for each lobe and cytokine (Whiteside *et al.* 1999). The phenotypes of the expressing cells are not known, but, on the basis of their widespread and scattered locations (Quan *et al.* 1998; Whiteside *et al.* 1999), it appears that a portion of them could be folliculostellate cells (Herkenham 2005). Therefore, in addition to LPS-induced IL-6 production, other cytokines produced by these cells could be involved in the increased ACTH response to LPS observed in the current study (Kobayashi *et al.* 1997). Furthermore, folliculostellate cells produce LIF (Inoue *et al.* 2002), which potently induces POMC gene transcription and ACTH secretion (Auernhammer *et al.* 1998; Butzkueven *et al.* 2002; Mynard *et al.* 2002) and potentiates CRH induction of POMC gene expression *in vitro* (Bousquet *et al.* 1997; Bousquet *et al.* 2000). Moreover, LIF is strongly induced in the anterior pituitary gland in response to inflammation *in vivo* (Wang *et al.* 1996; Chesnokova & Melmed 2000; Chesnokova *et al.* 2002). Thus, it is possible that the increased effects of LPS upon ACTH release measured in the current study may also be attributed to TtT/GF cell-derived LIF release. Furthermore, LIF production is regulated by NF κ B signalling in human endometrial cells (Laird *et al.* 2000) and LIF also induces IL-6 gene expression in mononuclear phagocytes through transcriptional activation of NF κ B (Gruss *et al.* 1992). Therefore, in addition to the direct stimulatory effect of LIF upon ACTH release, which occurs via the same signalling pathway as IL-6 (Mynard *et al.* 2002), it is possible that this cytokine is also involved in increasing ACTH release by acting in an autocrine manner to activate NF κ B, subsequently leading to increased IL-6 release from folliculostellate cells.

4.4.2 Aggregate co-cultures of TtT/GF and AtT20 D16:16 cells

The effective actions of the aforementioned folliculostellate cell-derived mediators upon corticotrophs may be enhanced by the increased cell to cell contacts which form in the three-dimensional structure of re-aggregate cultures. In view of this, a previous study identified discrepancies between the ACTH responses of primary pituitary cells grown as monolayer and re-aggregate cultures (Gloddek *et al.* 2001). These findings suggest that the multiple cell to cell contacts formed in the re-aggregates may allow relatively high concentration of mediators to accumulate in the intracellular spaces, thus permitting effective paracrine or juxtacrine communication between the cells. In monolayer co-cultures, cell to cell contacts are inevitably more limited and the concentration of mediators may be insufficient to affect hormone

release. In the present study, we therefore compared the ACTH responses of monolayer co-cultures of TtT/GF and AtT20 D16:16 cells to aggregate co-cultures of TtT/GF and AtT20 D16:16 cells.

4.4.2.1 Increased ACTH response of aggregate co-cultures of TtT/GF and AtT20 D16:16 cells to LPS

In accordance with the studies by Gloddek *et al.*, LPS alone increased ACTH release from the AtT20 D16:16 cells in the presence of TtT/GF cells in the aggregate co-cultures, but not in the monolayer co-cultures (Gloddek *et al.* 2001). The different responses elicited by these co-culture models therefore support the premise that a three-dimensional structure optimises the communication between folliculostellate cells and corticotrophs in conditions of endotoxaemia. These findings raise the possibility that single cell type cultures grown as a monolayer may also behave differently when cultured as aggregates. For example, the previous single culture studies used monolayer cultures of TtT/GF or AtT20 D16:16 cells to examine the effects of LPS and CRH upon IL-6 and ACTH release (chapter 3). As these cell types also form connections when they are cultured separately (see section 4.1), it is possible that altered responses to LPS may be elicited from these cells when cultured as aggregates. However, our studies indicate that only the LPS responsiveness of these cells is enhanced, as the response to combined CRH and LPS stimulation were similar in both models. As such, monolayer co-cultures of TtT/GF and AtT20 D16:16 cells is a valid model for investigating the role of NF κ B signalling in the interactions between these two cell types.

4.4.3 Role of NF κ B signalling in the ACTH response to LPS

A dual role for NF κ B signalling in the regulation of ACTH release was suggested in the previous chapter, with a potentiating effect exerted through TtT/GF cell-derived IL-6 (section 3.5.5) and an inhibitory effect exerted through its activity in the AtT20 D16:16 cells (section 3.5.7). To examine this further, the effects of NF κ B blockade, using the specific NF κ B inhibitor, SC-514, upon the ACTH responses of monolayer and aggregate co-cultures of TtT/GF and AtT20 D16:16 cells to LPS and CRH stimulation was investigated; the responses were qualitatively similar in both culture conditions.

In the presence of the TtT/GF cells, NF κ B blockade continued to augment the ACTH response to lower concentrations of CRH, despite reduced IL-6 production. This suggests that the stimulatory effect of CRH upon the AtT20 D16:16 cells is partially masked by a paradoxical NF κ B-dependent inhibitory effect of the peptide on ACTH release, as was seen in the AtT20 D16:16 cells alone (section 3.5.7). Alternatively, NF κ B activity may be involved in repressing the synthesis and/or release of an AtT20 D16:16 cell-derived mediator(s) involved in increasing ACTH release from corticotrophs and/or IL-6 release from folliculostellate cells. Both mechanisms would explain the effects of the NF κ B inhibitor on the response to LPS although the latter would predict that the inhibitor would augment the release of IL-6, which was not shown in the current study. Furthermore, the responses to the NF κ B inhibitor suggest that the NF κ B-dependent inhibitory actions of LPS and CRH on ACTH release may be of considerable importance in tempering the neuroendocrine responses to LPS and thus, this may represent an important mechanism by which ACTH release is limited during endotoxaemia to facilitate an effective inflammatory response.

However, LPS stimulation further potentiated CRH-induced ACTH release in conditions of suppressed NF κ B activity. Initially these findings appear to contradict the premise that reduced NF κ B-dependent IL-6 release from the TtT/GF cells would result in a reduction, or complete removal, of the stimulatory effect of LPS. However, upon further investigation, it was shown that in the presence of AtT20 D16:16 cells, LPS-induced IL-6 release from the TtT/GF cells was potentiated further by CRH stimulation, with the concentration of IL-6, even after SC-514 treatment, remaining significantly higher than that induced by LPS alone. Therefore, in line with the current findings, the concentration of IL-6 under these conditions may have remained sufficient to augment ACTH secretion, thus accounting for the potentiating effect of LPS, even after NF κ B blockade. Furthermore, these findings have provided evidence for the first time, to our knowledge, that a CRH-induced mediator(s) released from AtT20 D16:16 cells may act on the TtT/GF cells to augment LPS-stimulated IL-6 release. The co-operative interactions between these two cell types to augment ACTH release, as part of the neuroendocrine response to bacterial LPS, may therefore be attributed to the paracrine/juxtacrine actions of released mediators from both cell types. The sections below consider potential corticotroph-derived factors effecting communication between these two cell types to enhance ACTH release.

4.4.4 Potential IL-6 secretagogues released from AtT20 D16:16 cells

A potential IL-6 secretagogue is the pro-inflammatory cytokine, MIF, which has been localised to secretory granules in corticotrophs (Nishino *et al.* 1995) and is released from AtT20 D16:16 cells in response to CRH and LPS stimulation (Nishino *et al.* 1995; Tierney *et al.* 2005). MIF stimulates IL-6 release from human monocytes, dendritic cells and fibroblast-like synoviocytes (Murakami *et al.* 2002; Santos *et al.* 2004), whilst macrophages from MIF knockout mice exhibit decreased IL-6 production in response to LPS (Bozza *et al.* 1999). Thus it is possible that the paracrine actions of CRH-induced MIF release from corticotrophs may increase IL-6 production from folliculostellate cells (figure 4.10). MIF exerts some of its biological effects through binding to its cognate receptor, CD74 (Renner *et al.* 2005), but it is not known whether the TtT/GF cells express this receptor. However, MIF may also signal via a nonclassical endocytic pathway in the absence of CD74 (Renner *et al.* 2005). MIF signalling is associated with activation of the extracellular signal-regulated kinase 1/2 (ERK-1/2), promoting cell growth and activating Ets transcription factors which are critical for the expression of Tlr4 (Mitchell *et al.* 1999; Roger *et al.* 2001; Renner *et al.* 2005). (Renner *et al.* 2005). Therefore, MIF may also increase the responsiveness of folliculostellate cells to LPS and thus, CRH-induced MIF release from corticotrophs may present an additional mechanism through which the ACTH response to endotoxin is enhanced. The mechanism(s) through which CRH induces MIF release is unclear, although NF κ B signalling may be involved as the *mif* promoter region contains an NF κ B DNA-binding site (Renner *et al.* 2005). However, since the concentrations of CRH required to induce MIF release are well below those required for maximal release of ACTH (Calandra *et al.* 1994; Nishino *et al.* 1995; Tierney *et al.* 2005), and as the affinity of CRH for CRH-R2, the receptor implicated in NF κ B activation (Zhao & Karalis 2002), is lower than that for CRH-R1 (Kishimoto *et al.* 1995; Chalmers *et al.* 1996; Perrin & Vale 1999), direct activation of NF κ B by CRH is unlikely to be involved.

The expression of urocortin, a mammalian member of the CRH family, has been detected in the mouse (Chen *et al.* 2003) and rat pituitary gland (Wong *et al.* 1996a; Oki *et al.* 1998) and is released from rat primary pituitary cultures in response to CRH (Nemoto *et al.* 2007). Urocortin exhibits a high affinity for CRH-R2 (Vaughan *et al.* 1995) and therefore, CRH may increase NF κ B-mediated MIF release indirectly through the upregulation of urocortin. Furthermore, the function of urocortin in the anterior pituitary gland is unclear, since it does not appear to affect POMC synthesis

or ACTH release (Nemoto *et al.* 2007). Thus it is possible that CRH-induced urocortin may act via CRH-R2 to activate NF κ B and increase MIF synthesis in our system. However, the receptor involved in CRH-induced urocortin release has not yet been determined (Nemoto *et al.* 2007) and it remains to be investigated whether CRH stimulates urocortin release from AtT20 D16:16 cells at concentrations sufficient to stimulate MIF release.

In addition to NF κ B, MIF is regulated by other CRH-induced transcription factors, namely AP-1 and CREB (Boutillier *et al.* 1995; Waeber *et al.* 1998; Becquet *et al.* 2001; Kasagi *et al.* 2002; Kasckow *et al.* 2003; Mynard *et al.* 2004; Renner *et al.* 2005), and activation of CREB by CRH has previously been shown to occur through the CRH-R1 subtype (McEvoy *et al.* 2002; Kasckow *et al.* 2003). Moreover, a previous study has demonstrated impaired CRH-induced MIF release from AtT20 cells containing a mutated CRE site within the *mif* promoter region (Waeber *et al.* 1998). It is therefore plausible that CRH-induced MIF release from the AtT20 D16:16 cells is regulated by transcription factors such as CREB, at least during early endotoxaemia when CRH concentrations are low. During later stages of immune stimulation, when concentrations of CRH increase, MIF regulation by NF κ B via CRH-R2 may come into effect, possibly via the induction of urocortin. This is supported by the finding that whilst LPS-stimulated IL-6 release from the TtT/GF cells is mediated by NF κ B (see section 3.4.1), inhibition of NF κ B only partially attenuated the synergistic effects of CRH and LPS upon IL-6 release, with the percentage reduction in IL-6 release being similar in both the presence and absence of CRH. Together, these findings support the premise that the attenuating effect of the NF κ B inhibitor upon IL-6 release can be attributed to its direct effects on the TtT/GF cells, whilst the potentiating effect of CRH occurs predominantly via an NF κ B-independent mechanism.

4.4.4.1 Eicosanoids as potential AtT20 D16:16 cell-derived IL-6 secretagogues

Eicosanoids have been implicated in the regulation of ACTH secretion, exerting both inhibitory and stimulatory effects, depending on both the locus of action and the species of eicosanoid. At the hypothalamic level, prostanoids (e.g. PGA₁, PGB₁ and PGE₁) increase CRH release (Hedge 1972; Hedge 1976; Navarra *et al.* 1991), whilst, conversely, in the pituitary gland prostanoids are inhibitory to ACTH release. For example, stimulation of primary pituitary tissue with PGA₁, PGB₁, PGE₁ and PGE₂ depresses ACTH release, whilst treatment with the non-selective COX inhibitor,

indomethacin, reverses this inhibitory effect (Hedge 1977; Vlaskovska *et al.* 1984; Cowell *et al.* 1991; Wilson & Guild 2002). However, PGE₂, which is formed in the rat anterior pituitary gland in response to CRH and AVP stimulation (Vlaskovska *et al.* 1984; Knepel *et al.* 1985), stimulates the release of IL-6 from primary pituitary cells (Spangelo *et al.* 1990a). As corticotrophs are the primary target of CRH, it is possible that CRH-induced PGE₂ originates from this cell type, possibly through NFκB-mediated synthesis of COX-2, the enzyme responsible for prostaglandin synthesis (Bukata *et al.* 2004) (figure 4.10), and acts on folliculostellate cells to induce IL-6 release. Furthermore, PGE₂ also increases CD14 gene expression and activates AP-1, a regulator of IL-6 transcription in murine macrophages (Iwahashi *et al.* 2000). Therefore, if PGE₂ exerts similar effects on folliculostellate cells, this may present a potential mechanism through which CRH indirectly increases IL-6 production in the anterior pituitary gland.

Whilst studies using both the AtT20 D16:16 cell line and dispersed rat pituitary cells have demonstrated an inhibitory effect of PGE₂ upon ACTH release (Vlaskovska *et al.* 1984; Wilson & Guild 2002), the preparation of dispersed anterior pituitary cells would result in the loss of folliculostellate cells and of the three-dimensional cell network, which in turn may impair their function (Christian *et al.* 1997). Therefore, such studies may not be a reliable representation of the true effects of PGE₂ on the anterior pituitary gland (Vlaskovska *et al.* 1984). However, these studies have demonstrated a direct inhibitory effect of PGE₂ at the corticotroph level and thus, in combination with its stimulatory effects upon folliculostellate cell-derived IL-6, the actions of PGE₂ within the anterior pituitary gland may represent a regulatory mechanism through which exacerbated ACTH release in response to endotoxin is prevented. Furthermore, NFκB-dependent PGE₂ synthesis in corticotrophs may represent a mechanism through which NFκB activity in corticotrophs inhibits ACTH release (figure 4.10).

In addition to prostaglandins, potential corticotroph-derived mediators include other metabolites of arachidonic acid, which are generated via the cytochrome P450 and lipoxygenase pathways, such as 12(S)-hydroxyeicosatetra-enoic acid [12(S)-HETE]. These metabolites increase ACTH release from primary pituitary cells (Luini & Axelrod 1985; Cowell & Buckingham 1989; Cowell *et al.* 1991), and inhibition of the lipoxygenase pathway impairs CRH-induced ACTH release from AtT20 cells (Luini & Axelrod 1985). Furthermore, 12(S)-HETE also increases ACTH secretion *in vivo*

(Won & Orth 1994) and is one of the major lipoxygenase metabolites identified in the rat anterior pituitary gland, although it is not known whether it is produced by corticotrophs. 12(S)-HETE increases both the mRNA and protein expression of IL-6 and TNF α in macrophages via PKC, p38 MAP Kinase and JNK signalling pathways (Wen *et al.* 2006) and stimulates LIF release from human bone marrow stromal cells (Denizot *et al.* 1998). Therefore, if products of the lipoxygenase pathway originating in corticotrophs act via a similar mechanism in folliculostellate cells to augment cytokine generation, they could indirectly potentiate CRH-induced ACTH secretion (figure 4.10).

4.4.5 Effects of conditioned medium from TtT/GF cells on the ACTH responses of the AtT20 D16:16 cells

Our findings have provided novel evidence for reciprocal communication between the TtT/GF and AtT20 D16:16 cells via the actions of released mediators. To examine whether these mediators exert their effects through a paracrine and/or juxtacrine manner, we examined the effects of TtT/GF cell-conditioned medium upon the ACTH response of the AtT20 D16:16 cells. The conditioned medium did not affect the ability of the NF κ B inhibitor to potentiate the response to CRH, thus supporting the view that CRH exerts two opposing actions directly on the AtT20 D16:16 cells to regulate ACTH release. The conditioned medium did, however, overcome the inhibitory effects of LPS upon CRH-driven ACTH release from the AtT20 D16:16 cells, thus supporting the premise that the synergistic effects of LPS and CRH in the co-cultures are dependent upon the release of a TtT/GF-derived mediator(s), which could be IL-6, LIF or a combined effect of the two cytokines, although the concentration of these cytokines and other potential mediators (i.e. IL-1 β , TNF α , IL-11 and OSM) need to be measured in the conditioned medium to confirm this. Although the conditioned medium overcame the direct inhibitory effect of LPS upon ACTH release, it did not further potentiate the effects of CRH. This is likely to be because the conditioned medium was obtained from TtT/GF cells that were not cultured in the presence of the AtT20 D16:16 cells, which have been shown to cause increased TtT/GF cell-derived IL-6 release, possibly via a CRH-induced mediator(s), even in the presence of the NF κ B inhibitor. Therefore, the concentration of IL-6 in the conditioned medium may have been too low to potentiate CRH-induced ACTH release, supporting the premise that bi-directional communication between the two cell types is an important requisite for the ACTH response to endotoxin. Moreover, although LPS potentiated CRH-stimulated ACTH release from the AtT20 D16:16 cells when co-cultured with the

TtT/GF cells, the conditioned medium from LPS-stimulated TtT/GF cells wherein NF κ B activity was inhibited failed to have an effect, providing further evidence that the actions of TtT/GF cell-derived mediators induced by LPS are dependent on NF κ B activation.

Whilst these findings support the involvement of paracrine-acting mediators in regulating the IL-6 and ACTH responses to LPS and CRH stimulation, the close contact the cells have when they are cultured together is also likely to be of importance. As the TtT/GF cell-conditioned medium, containing LPS-induced factors which stimulate ACTH release, was incubated for 7 hours prior to its addition to the AtT20 D16:16 cells, it is possible that such mediators were degraded, as cytokines have a relatively short half-life (Noguchi *et al.* 1996). In contrast, the AtT20 D16:16 cells were exposed to TtT/GF cell-derived mediators rapidly, i.e. upon release, and they were situated close to the TtT/GF cells so that the mediators were not diluted in the medium before reaching the AtT20 D16:16 cells. This problem can be avoided with the use of Transwell® cell culture inserts which allows two cell types to be cultured separately but in the same culture medium. The use of such a system would allow the reciprocal communication between the two cell types to be further investigated and provide valuable information as to the importance of cell to cell contact between the TtT/GF and AtT20 D16:16 cells.

Together, these findings support the premise that both folliculostellate cells and corticotrophs interact with one another in response to endotoxin, to increase ACTH release. Moreover, in addition to the classical actions of CRH on corticotrophs to increase ACTH secretion, CRH also indirectly increases ACTH release through the up-regulation of IL-6 release from folliculostellate cells. This is the first time, to our knowledge, that corticotrophs have been implicated in the modulation of folliculostellate cell-derived mediators (figure 4.10).

4.5 Conclusion

It is understood that LPS acts directly at the pituitary level to induce ACTH release, as well as acting indirectly via the induction of pro-inflammatory cytokines released from immune/inflammatory cells (Haddad *et al.* 2002). However, the mechanisms governing this response are unclear, although a role for folliculostellate cells has been identified (Gloddek *et al.* 2001). The current study has provided novel evidence that LPS-induced ACTH release, through its direct actions on the anterior pituitary

gland, involves interactions between the TtT/GF and AtT20 D16:16 cells and these interactions are modulated, at least in part, through paracrine-acting mediators. Potential mediators released from the TtT/GF and AtT20 D16:16 cells, and the pathways through which they act, are summarised in figure 4.10.

The study has provided conclusive evidence that the potentiating effect of LPS upon ACTH release is mediated by NF κ B-dependent factors released from the TtT/GF cells. We have also shown for the first time, to our knowledge, an additional pathway through which CRH increases ACTH release, i.e. through the production of corticotroph-derived mediators which, in turn, act on neighbouring folliculostellate cells to increase IL-6 production. The potentiating effects of IL-6 upon ACTH release in the presence of low concentrations of CRH suggest that this cytokine is important in activating the HPA axis during early endotoxaemia. However, the inhibitory effect of CRH upon ACTH release, via activation of corticotroph-NF κ B, may occur to ensure the ACTH response is contained, allowing an effective pro-inflammatory response to be mounted prior to increased glucocorticoid release. Together, these findings have provided further information as to how the extremely complex interactions that occur at the level of the anterior pituitary ensure the HPA response to endotoxin is tightly regulated.

4.6 Future studies

Although an important role for folliculostellate cell-derived IL-6 in mediating the ACTH response to endotoxin has been demonstrated, the studies indicate the involvement of other mediators, possibly including LIF and other gp130 cytokines. Further work is required to ascertain whether LPS induces LIF release from the TtT/GF cells and whether LIF, alone and in combination with IL-6, potentiates CRH-induced ACTH release in the current model. Further work is also required to identify potential corticotroph-derived mediator(s) (which may include MIF) that are involved in increasing LPS-stimulated IL-6 release from folliculostellate cells. Although studies of immune cells *in vitro* have demonstrated that CRH induces NF κ B activation through CRH-R2 (Zhao & Karalis 2002), it is unclear whether the signalling mechanism is the same in corticotrophs. Therefore more work is required to identify the receptor through which CRH activates NF κ B in the AtT20 D16:16 cells (see section 3.8), which can be achieved using specific CRH-R inhibitors [reviewed in (Hillhouse & Grammatopoulos 2006)]. The premise that urocortin, which preferentially binds to CRH-R2, may activate NF κ B in corticotrophs to induce potential IL-6 secretagogues e.g. via COX-2 dependent prostaglandin synthesis or MIF (section 4.4.4), warrants further investigation to further our understanding into the mechanisms through which NF κ B is activated in corticotrophs.

Within the AtT20 D16:16 cells, NF κ B signalling has been shown to play an inhibitory role in CRH stimulated ACTH release. Thus, targeting NF κ B for therapeutic treatment of inflammatory disorders may lead to increased levels of ACTH release and thus, a hyper-active HPA response if the individual is under immunological stress. Furthermore, treatment with NF κ B inhibitors may impair the negative feedback actions of glucocorticoids, leading to prolonged hypercortisolemia and dysregulation of the HPA axis which in turn can lead to an array of diseases, including severe forms of depression (Gibbons & Mc 1962; Carpenter & Bunney 1971; Gillespie & Nemeroff 2005), type II diabetes and cardiovascular disease (Bjorntorp *et al.* 1999). It is clear from these findings that NF κ B is involved modulating the HPA response to immunological stress, providing a link between the interactions between the endocrine and immune systems, at least at the level of the anterior pituitary gland. Additionally, NF κ B signalling has been demonstrated throughout the brain, specifically in limbic regions, hypothalamic nuclei and within brain stem nuclei associated with cranial nerves (Joseph *et al.* 1996; Lee & Rivier

2005). Therefore, the effects of NF κ B blockade upon the HPA response to immunological stress need to be investigated, using *in vivo* models, to further understand the extent to which this transcription factor is involved in regulating the HPA response to stress.

Although activation of the HPA axis in response to psychological stress does not involve an immune component, restraint stress, which is regarded as a form of psychological stress (Pare & Glavin 1986; Glavin *et al.* 1994), increases NF κ B activity in the rat brain (Garcia-Bueno *et al.* 2005). Furthermore, activation of NF κ B in human PBMCs in response to psychosocial stress, which may occur through interactions of noradrenaline with α_1 - and β -adrenergic receptors, leads to activation of the MAPKinase pathway and subsequent NF κ B nuclear translocation (Bierhaus *et al.* 2003). Therefore, in addition to investigating the role of NF κ B in the HPA response to immunological stress, its role in psychological stress warrants further investigation. To this end, the following chapter examines the effects of inhibiting NF κ B upon ACTH, corticosterone and pro-inflammatory cytokine release (IL-6 and TNF α) from rat models subjected to either immunological (LPS) or psychological (restraint) stress.

5 Role of NFκB in the regulation of the HPA axis

5.1 Introduction

Immunological stress is a potent activator of the HPA axis, ultimately leading to restoration of physiological homeostasis through the anti-inflammatory actions of released glucocorticoids [reviewed by (Buckingham 1996)]. The mechanisms underlying the interplay between the immune system and the HPA axis are now understood to involve the actions of pro-inflammatory cytokines, in particular IL-6. Injection of the endotoxin LPS, *in vivo* increases the plasma concentrations of IL-6 (Kakizaki *et al.* 1999) which, in turn, stimulate the release of ACTH (Fukata *et al.* 1989; Bethin *et al.* 2000). In addition to the stimulatory effect of IL-6 released from activated immune cells into the systemic circulation, IL-6 is also produced within the anterior pituitary gland by folliculostellate cells. Transcriptional regulation of the IL-6 gene in many cell types (e.g. macrophages, endothelial cells, microglial cells, dendritic cells) is dependent in part on NFκB signalling (Takatsuna *et al.* 2005). Thus, the activation of the HPA axis by immunological stress may be, at least in part, dependent on NFκB (Ji *et al.* 2004).

The previous chapters have provided evidence that LPS-induced ACTH release is positively regulated by NFκB signalling in folliculostellate cells and negatively regulated by NFκB signalling in corticotrophs (chapters 3 and 4). However, the involvement of NFκB in activating the HPA axis may not be confined to conditions of immunological stress. Indirect evidence for a role of NFκB in mediating cellular effects in response to psychological stress stems from animal studies describing stress-induced NFκB activation and subsequent NFκB-dependent gene expression in the brain cortex of rats exposed to immobilisation stress (Madrigal *et al.* 2002). Increased NFκB activity has also been detected in blood lymphocytes of females stressed by the experience of breast biopsy (Nagabhushan *et al.* 2001). Furthermore, *in vivo* studies have described increased plasma concentrations of IL-6 in response to psychological stress such as footshock and immobilisation stress, which may be attributed to activation of NFκB (Zhou *et al.* 1993). These findings provide strong evidence of a role for NFκB in modulating HPA responses to both immunological and psychological stress.

NF κ B is an important mediator of the immune response to stress, and its activation results in increased expression of many genes involved in the inflammatory response [reviewed in (Ozato *et al.* 2002)]. Therefore drugs, which target NF κ B activation in patients with chronic inflammatory disorders, such as rheumatoid arthritis, could provide potential therapeutic treatment (Carlsen *et al.* 2004; Kulms & Schwarz 2006). However, whilst such compounds may be effective in treating such disorders, the additional side effects NF κ B inhibition may have upon the HPA axis have not been addressed. Therefore, the aim of this study was to investigate the effects of NF κ B inhibition upon the HPA responses to both immunological and psychological stress.

5.2 Experimental design

5.2.1 Immunological stress – LPS

For the immunological stress model (section 2.4.3), male Sprague-Dawley rats (section 2.1) were injected with either vehicle (400 μ l i.p., sterile physiological saline) or LPS (5mg/kg i.p., *Escherichia coli* serotype 055:B5). Control animals were not subjected to any treatment. Initial time course studies were performed to examine the optimal time for ACTH (IRMA; section 2.4.17) and corticosterone (RIA; section 2.4.18) responses following LPS challenge. Plasma IL-6 and TNF α concentrations (ELISA; section 2.4.15) were measured as a marker of immunological activation and the effects LPS on NF κ B activity in the anterior pituitary gland were examined by EMSA (section 2.4.12). For comparison, DNA binding activity of NF κ B was also examined in thymic tissue.

5.2.2 Effects of the IKK β Inhibitor, PHA781535E, on LPS-induced HPA activation

Following the determination of the optimal time point for immunological (IL-6; figure 5.2) and HPA (ACTH and corticosterone; figure 5.1A and B) activation in response to LPS, the effects of suppressing LPS induced NF κ B activation upon these parameters were examined (section 2.4.4). Rats were treated with either the NF κ B inhibitor, PHA781535E (25 or 50mg/kg, p.o.), or the vehicle solution [400 μ l, methylcellulose (0.5% w/v) tween (0.1% w/v) p.o.] orally, 2 hours prior to injection of LPS (5mg/kg, i.p., *Escherichia coli* serotype 055:B5) or saline (400 μ l i.p., physiological saline). Blood and tissues were collected 2 hours later for analysis. The control animals were not subjected to any treatment. Elevated concentrations of the pro-inflammatory

cytokine TNF α (which is both regulated by, and stimulates, NF κ B activation) have been reported in models of endotoxaemia (Kakizaki *et al.* 1999) and therefore, plasma TNF α concentrations were also examined (ELISA; section 2.4.16) as an indicator of immunological stress induced by LPS, and NF κ B inhibition by PHA781535E administration.

5.2.3 Psychological stress – restraint

For the psychological stress model, male Sprague-Dawley rats were subjected to restraint stress (section 2.4.1). Initial studies examined the optimal time point for HPA activation by measuring ACTH (IRMA; section 2.4.17) and corticosterone (RIA; section 2.4.18) plasma concentrations at intervals after stress. Rats were restrained either for (a) 15 or 30 minutes and killed immediately or for (b) 30 minutes followed by either a 30 or 60 minute recovery period (rodents were returned to their housing cage for the recovery period). For the assessment of cytokine activation, plasma IL-6 concentrations (ELISA; section 2.4.15) were measured. Although NF κ B activation following psychological stress has been reported in the brain cortex (Madrigal *et al.* 2002) and PBMCs (Nagabhushan *et al.* 2001), to our knowledge, its activity in the anterior pituitary gland under these conditions has not. Therefore, the effect of restraint stress upon NF κ B activity in the anterior pituitary gland were assessed (EMSA; section 2.4.12). For a comparison of NF κ B activity in distal organs, NF κ B DNA-binding activity was also examined in thymic and adrenal tissue.

5.2.4 Effects of the IKK β inhibitor, PHA781535E, on restraint stress-induced HPA activation

Following the determination of the optimal time point for HPA activation in response to restraint stress, the effects of NF κ B inhibition upon these parameters were examined (section 2.4.2). Male adult rats were treated either with the vehicle solution (400 μ l, methylcellulose-tween, p.o.) or with PHA781535E (25 or 50mg/kg, p.o.) 2 hours prior to being subjected to 15 minutes of restraint stress, the optimal time of restraint exposure where both ACTH and corticosterone concentrations were significantly elevated (figure 5.17A and B). Psychological stress exposure has also been associated with increased plasma TNF α concentrations (Yamasu *et al.* 1992; Nukina *et al.* 1998), and therefore, the effects of restraint stress, in the presence and absence of PHA781535E, upon plasma TNF α concentrations (ELISA; section 2.4.16) were also included in the study.

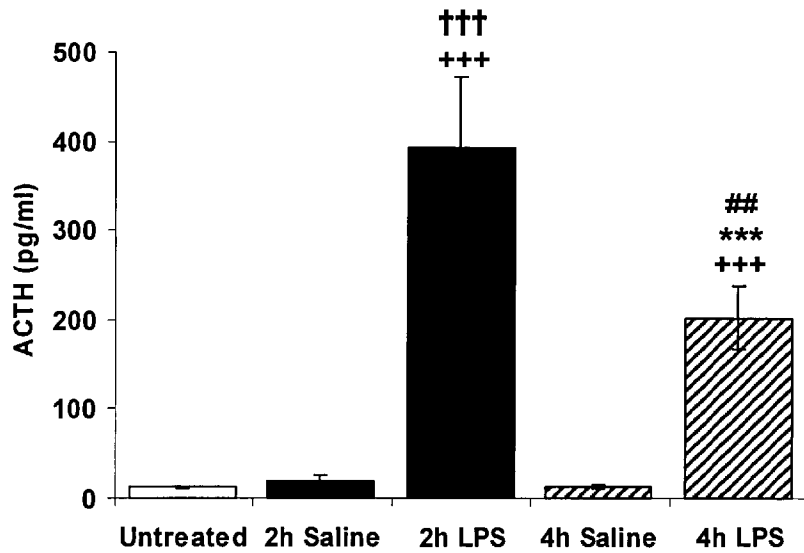
5.3 Results – Immunological stress (LPS)

5.3.1 Effects of LPS upon the plasma ACTH and corticosterone concentrations

The LPS vehicle (saline; 400µl, i.p.) did not affect basal plasma concentrations of ACTH (2 and 4 hours, $p=0.966$), but LPS (5mg/kg, i.p.) challenge resulted in a significant increase in plasma ACTH concentration (2 hours, $p<0.001$ vs untreated control; figure 5.1A). Four hours after the injection, the plasma ACTH concentration had declined ($p<0.01$ vs 2 hours), although it remained higher than the basal concentration ($p<0.001$; figure 5.1A).

The basal plasma concentration of corticosterone was unaffected by injection of saline (400µl, i.p.) 2 or 4 hours ($p=0.899$; figure 5.1B) previously. LPS (5mg/kg, i.p.) administration induced a significant increase in plasma corticosterone concentration within 2 hours ($p<0.001$; figure 5.1B) which was maintained for at least a further 2 hours (2 hours LPS vs 4 hours LPS, $p=0.110$; figure 5.1B).

A



B

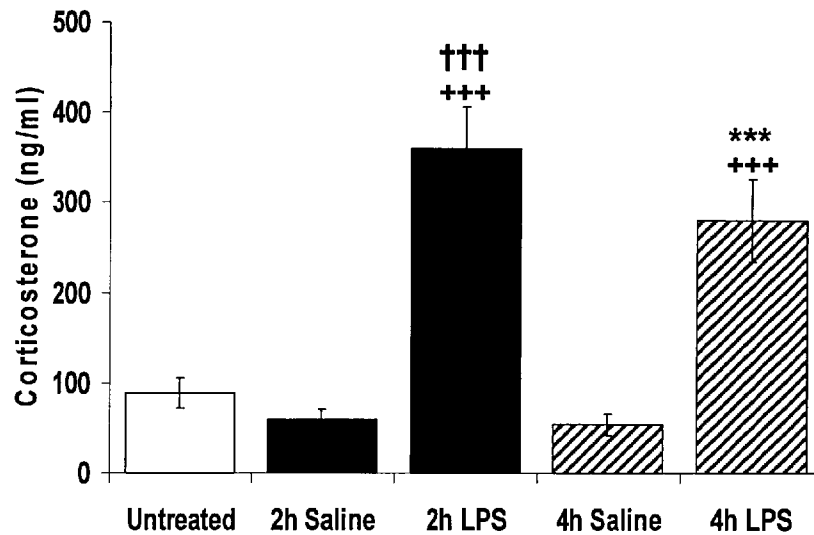


Figure 5.1 Time-dependent effects of LPS (5mg/kg i.p.) on the plasma (A) ACTH and (B) corticosterone concentrations. Rats in the untreated group remained in their housing cages until collection and vehicle treated groups were injected with saline (400 μ l i.p.) for 2 or 4 hours. Untreated (control) and treated groups are the mean of five samples $\pm$ SEM. *** p <0.001 vs untreated control, ††† p <0.001 vs 2 hour saline, *** p <0.001 vs 4 hour saline, ## p <0.01 vs 2 hour LPS. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH, adrenocorticotrophin; LPS, lipopolysaccharide.

5.3.2 Effect of LPS upon the plasma IL-6 concentration

IL-6 was undetectable by ELISA in the plasma collected from untreated rats or rats treated with saline 2 or 4 hours previously (figure 5.2). By contrast, IL-6 was readily detectable in the plasma of rats challenged with LPS (figure 5.2). Marked elevations in the plasma IL-6 concentration were thus evident with 2 hours of the injection ($p<0.001$ vs control; figure 5.2). These declined over the next 2 hours (2 hours LPS vs 4 hours LPS, $p<0.01$), but remained significantly higher than basal ($p<0.001$; figure 5.2).

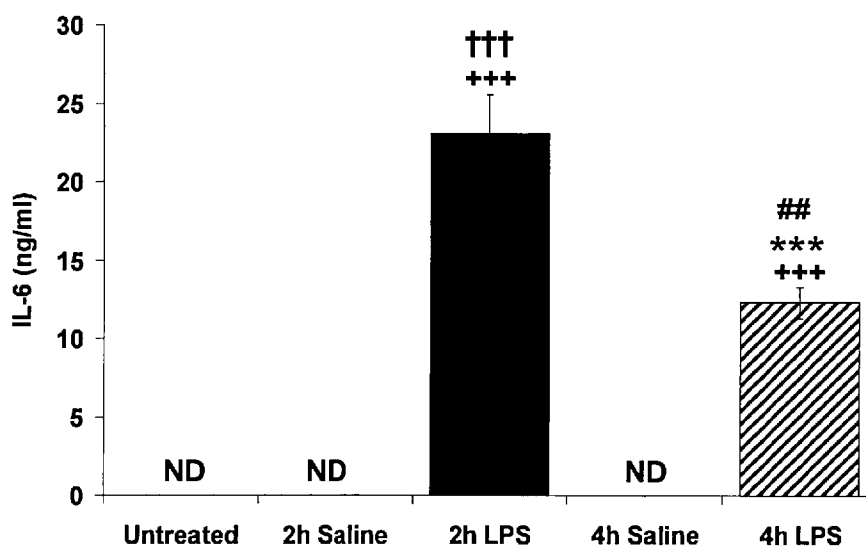


Figure 5.2 Time-dependent effects of LPS (5mg/kg, i.p.) on the plasma IL-6 concentration. Rats in the untreated group remained in their housing cages until collection. Blood was collected 2 or 4 hours after the injection of saline (400 μ l) or LPS (5mg/kg). Untreated (control) and treated groups are the mean of five samples $\pm$ SEM. *** $p<0.001$ vs untreated control, ††† $p<0.001$ vs 2 hour saline, *** $p<0.001$ vs 4 hour saline, ## $p<0.01$ vs 2 hour LPS. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. IL-6, interleukin 6; LPS, lipopolysaccharide; ND, not detectable.

5.3.3 Effect of LPS treatment *in vivo*, on NF κ B activity in rat anterior pituitary tissue

Basal DNA-binding activity of NF κ B was low in pituitary tissue extracted from untreated rats (lanes 1-4; figure 5.3A and 5.4A). There was no detectable change in NF κ B activity 2 hours (lanes 5-8; figure 5.3A) or 4 hours (lanes 5-8; figure 5.4A) after the injection of saline. By contrast, LPS (5mg/kg) caused a marked increase in NF κ B DNA-binding at 2 hours ($p < 0.05$ vs control and 2 hour saline; 2 hour LPS - lanes 9-12, untreated control - lanes 1-4 and saline-injected control - lanes 5-8; figure 5.3A and B) and 4 hours ($p < 0.05$ vs control and 4 hour saline; 4 hour LPS - lanes 9-12, untreated control - lanes 1-4 and saline-injected control - lanes 5-8; figure 5.4A and B).

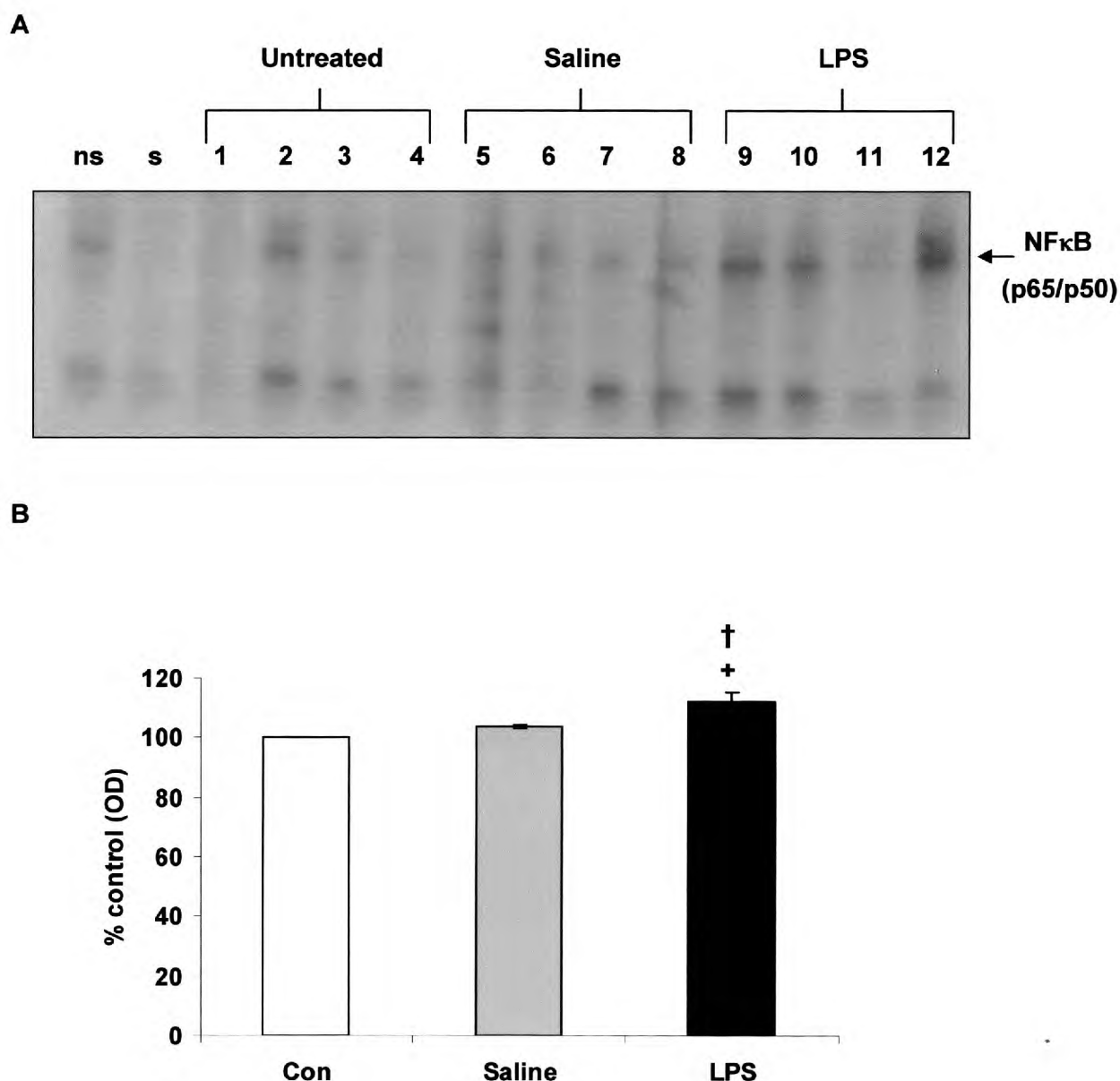


Figure 5.3 Detection of NFκB DNA-binding activity in anterior pituitary tissue extracted from LPS (5mg/kg, i.p., 2 hours) and saline (400μl, i.p., 2 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples $\pm$ SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. [†] $p < 0.05$ vs untreated control, [†] $p < 0.05$ vs 2 hour saline. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NFκB).

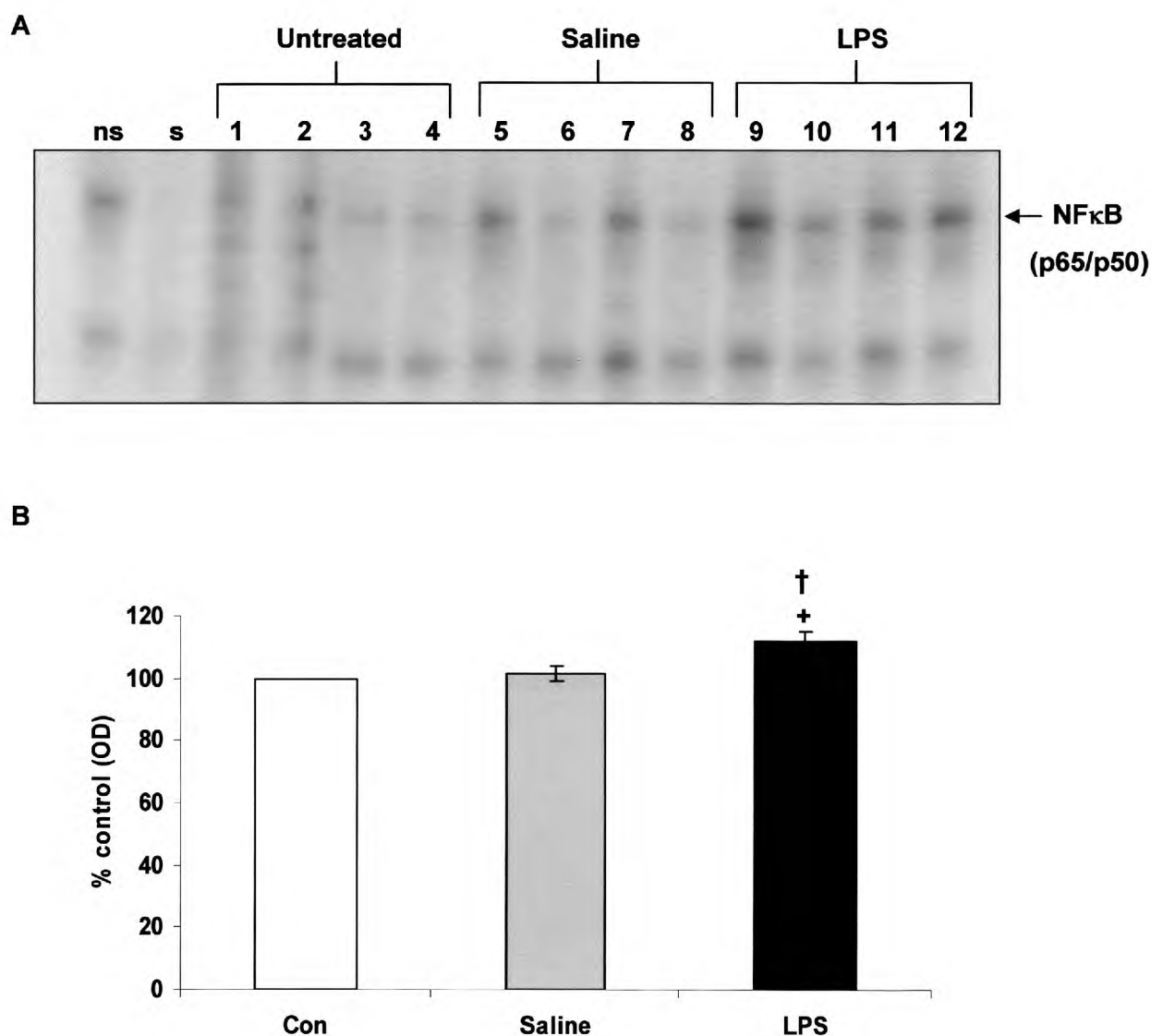


Figure 5.4 Detection of NF κ B DNA-binding activity in anterior pituitary tissue extracted from LPS (5mg/kg, i.p., 4 hours) and saline (400 μ l, i.p., 4 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples $\pm$ SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. [†]p<0.05 vs untreated control, [†]p<0.05 vs 4 hour saline. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NF κ B).

5.3.4 Effect of LPS treatment *in vivo*, on NF κ B activity in rat thymic tissue

The basal NF κ B DNA-binding activity (lanes 1-4; figure 5.5A and 5.6A) was unchanged 2 hours ($p>0.05$, lanes 5-8; figure 5.5A and B) and 4 hours after saline injection ($p>0.05$, lanes 5-8; figure 5.6A and B). LPS injection increased NF κ B activity (lanes 9-12) in the thymus gland at both 2 and 4 hours (2 hours, $p<0.05$ vs untreated control and $p<0.05$ vs saline control and 4 hours, $p<0.05$ vs saline control; figure 5.5A and B and 5.6A and B).

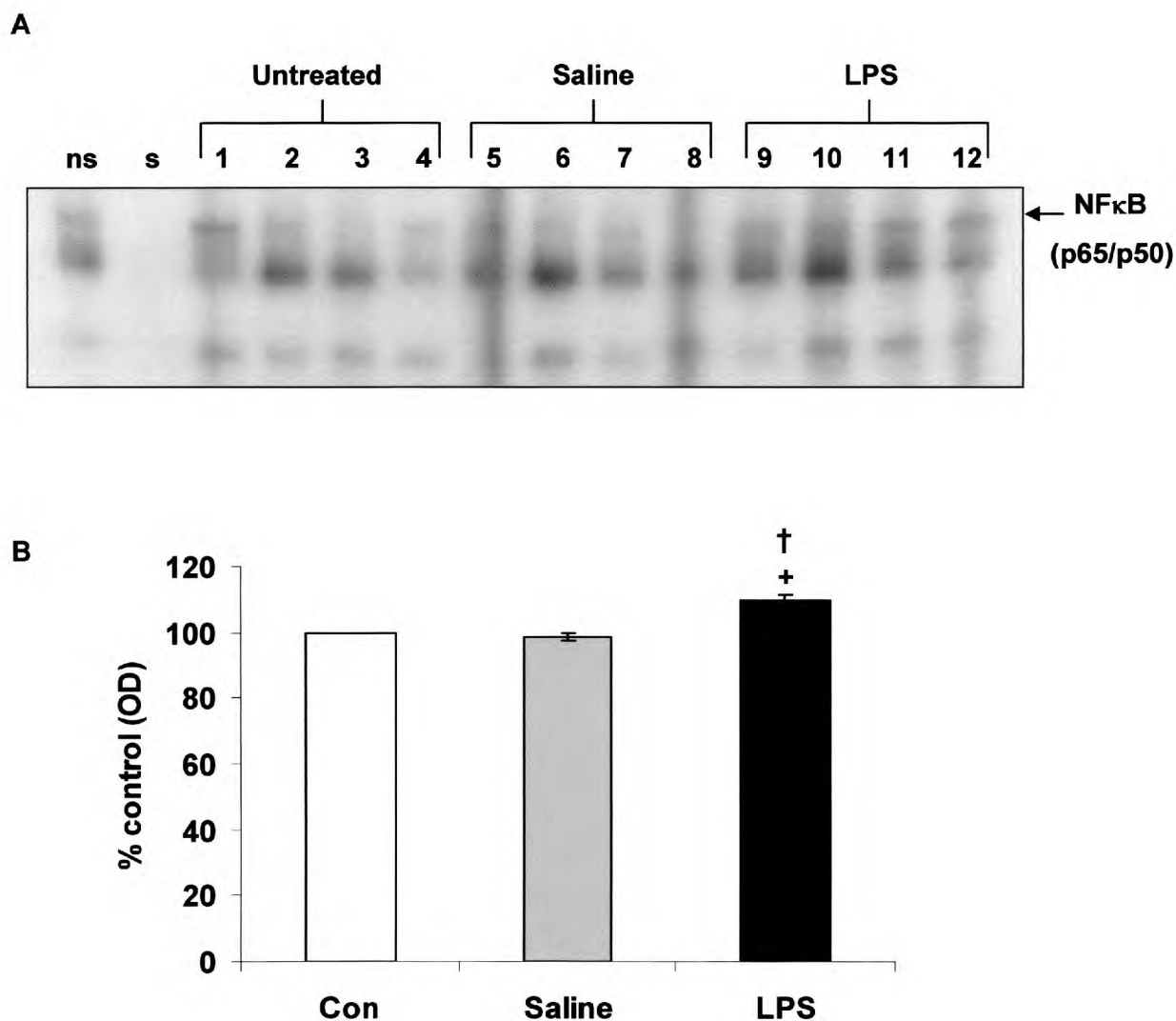
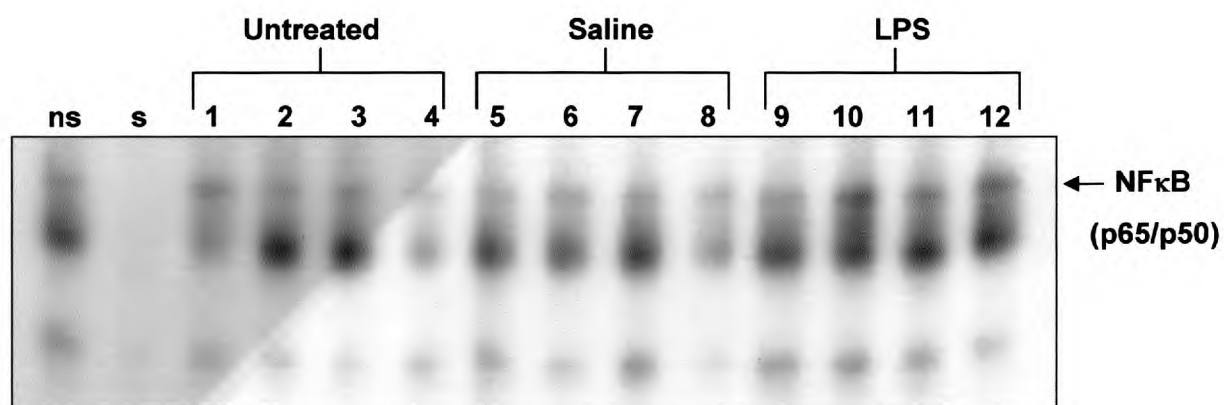


Figure 5.5 Detection of NFκB DNA-binding activity in thymic tissue extracted from LPS (5mg/kg, i.p., 2 hours) and saline (400μl, i.p., 2 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples ± SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. [†]p<0.05 vs untreated control, [†]p<0.05 vs 2 hour saline. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B; NS, not significant; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NFκB).

A



B

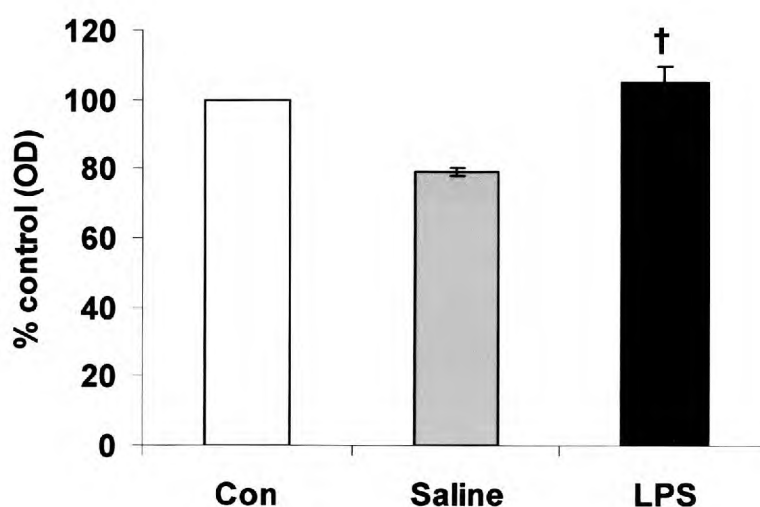
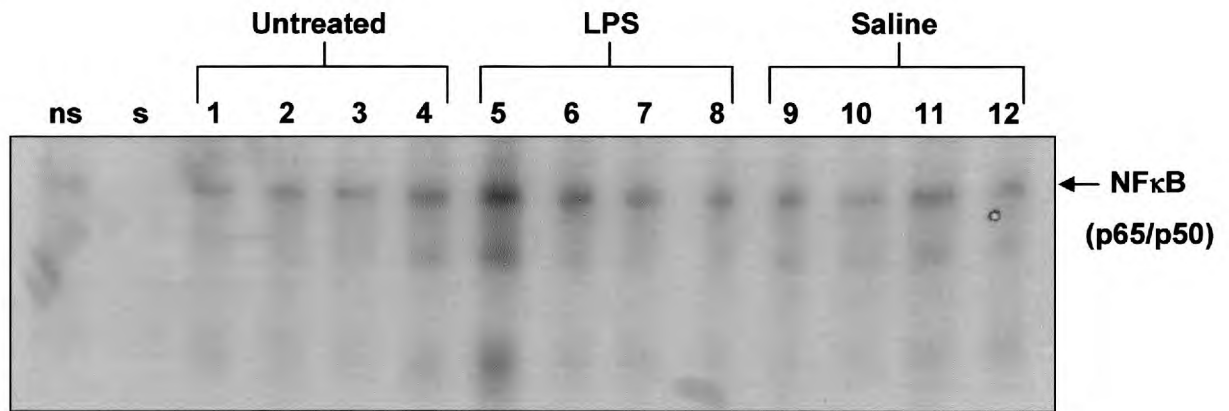


Figure 5.6 Detection of NF κ B DNA-binding activity in thymic tissue extracted from LPS (5mg/kg, i.p., 4 hours) and saline (400 μ l, i.p., 4 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples $\pm$ SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. $^{\dagger}p < 0.05$ vs 4 hour saline. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; NS, not significant; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NF κ B).

5.3.5 Effects of LPS treatment *in vivo*, on NF κ B activity in rat adrenal tissue

The basal DNA-binding activity of NF κ B in adrenal tissue extracted from rats was not affected by saline treatment at 2 hours ($p>0.05$, lanes 9-12, figure 5.7A and B) or at 4 hours ($p>0.05$, lanes 5-8, figure 5.8A and B). In contrast, LPS exposure for 2 hours (lanes 5-8; figure 5.7A), but not 4 hours (lanes 9-12; figure 5.8A), resulted in an increase in NF κ B activity compared to basal activity ($p<0.05$ vs control, lanes 1-4; figure 5.7B).

A



B

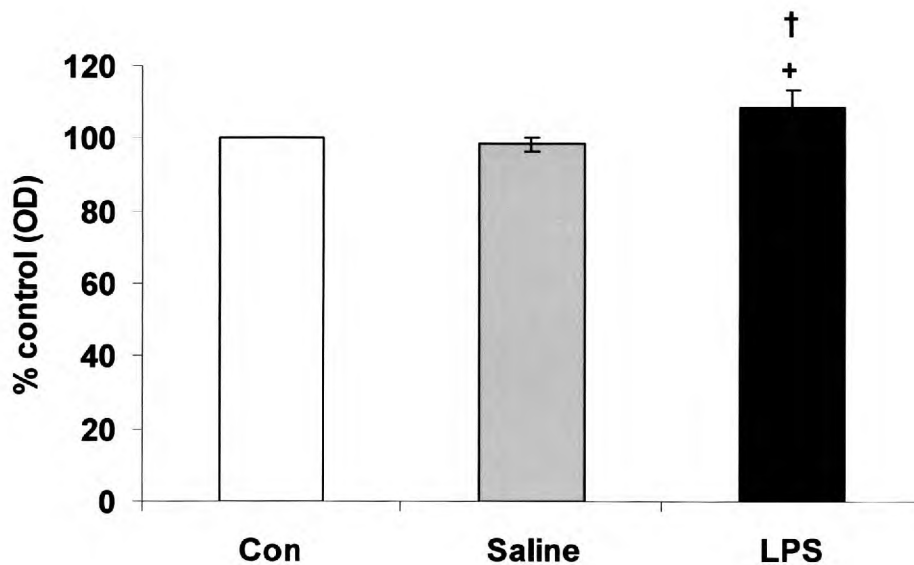
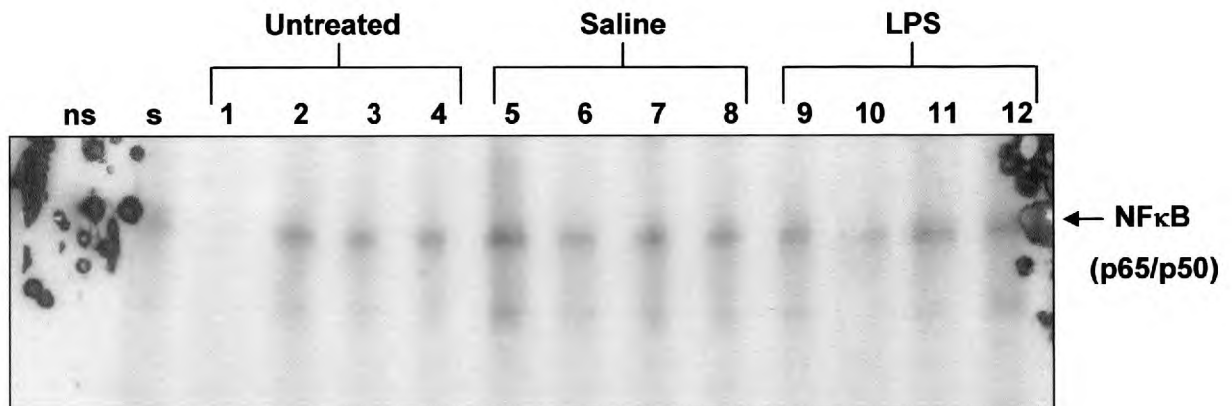


Figure 5.7 Detection of NF κ B DNA-binding activity in adrenal tissue extracted from LPS (5mg/kg, i.p., 2 hours) and saline (400 μ l, i.p., 2 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples $\pm$ SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. $^*p < 0.05$ vs untreated control, $^\dagger p < 0.05$ vs 2 hour saline. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NF κ B).

A



B

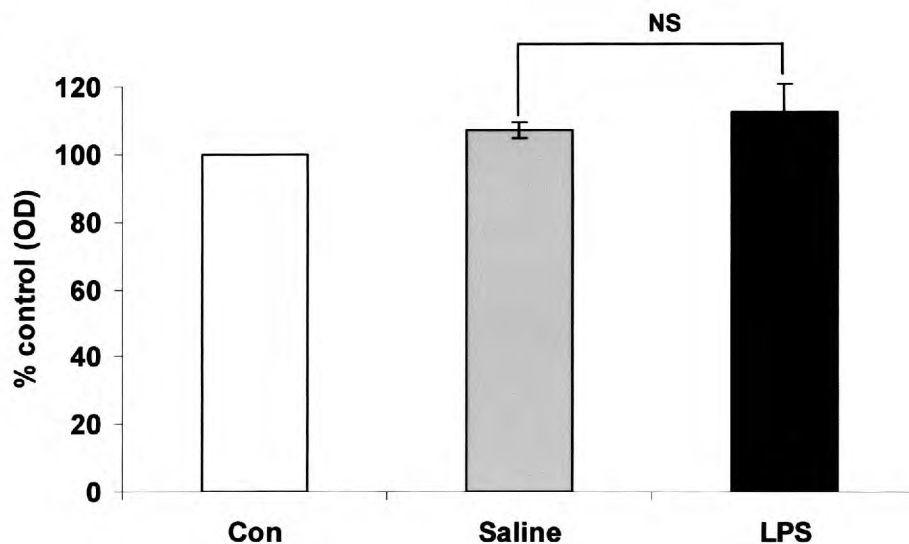


Figure 5.8 Detection of NFκB DNA-binding activity in adrenal tissue extracted from LPS (5mg/kg, i.p., 4 hours) and saline (400μl, i.p., 4 hours) treated rats by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [each group represents the mean optical density of four samples $\pm$ SEM and is expressed as percentage of control (untreated)]. Lanes 1-4, untreated; lanes 5-8, saline; lanes 9-12, LPS. Analysis of densitometric results was performed by Kruskal-Wallis one-way ANOVA on ranks with the Student-Newman-Keuls *post hoc* method. Con, untreated control; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B; NS, not significant; NS, not significant; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NFκB).

5.3.6 Effects of the IKK β inhibitor, PHA781353E, on LPS stimulated plasma ACTH and corticosterone concentrations

The findings outlined in sections 5.3.1 and 5.3.2 showed the optimal time point for increased plasma concentrations of ACTH (figure 5.1A), corticosterone (figure 5.1B) and IL-6 (figure 5.2) following LPS (5mg/kg, i.p.) injection was 2 hours. LPS (5mg/kg, i.p.) also induced, within 2 hours, an increase in NF κ B activity in the anterior pituitary (figure 5.3), thymus (figure 5.5) and adrenal glands (figure 5.7). Therefore, the effects of the NF κ B inhibitor, PHA781353E (25 and 50mg/kg, p.o.), upon these parameters was investigated in rats challenged with LPS (5mg/kg, i.p.) for 2 hours (see section 3.4.3).

5.3.6.1 Effects of treatment with PHA781353E and LPS vehicles on the plasma ACTH and corticosterone concentrations

Administration of the PHA781353E vehicle, methylcellulose-tween (400 μ l, p.o.), 2 hours prior to administration of the LPS vehicle (physiological saline, 400 μ l, i.p.) had no significant effect on the plasma ACTH or corticosterone concentrations (ACTH, $p=0.319$ vs saline; corticosterone, $p=0.663$ vs saline; figure 5.9A and B). Therefore, any changes in ACTH and corticosterone concentrations following treatment with PHA781353E and/or LPS could not be attributed to the effects of either vehicle.

5.3.6.2 Effect of PHA781353E treatment upon LPS-induced plasma ACTH concentrations

LPS caused a highly significant increase in plasma ACTH concentration ($p<0.001$ vs basal; figure 5.9A). Whilst PHA781353E treatment alone did not affect the resting plasma concentration of ACTH (25mg/kg, $p=1.00$; 50mg/kg, $p=1.00$) at both doses tested, 25mg/kg ($p<0.001$) and 50mg/kg ($p<0.001$), it attenuated the stimulatory effect of LPS on ACTH release (figure 5.9A).

5.3.6.3 Effect of PHA781535E treatment upon LPS-induced plasma corticosterone concentrations

LPS caused a highly significant increase in the plasma concentration of corticosterone ($p < 0.001$ vs basal; figure 5.9B) which was unaffected by PHA781535E treatment at both doses tested (25mg/kg, $p = 1.00$; 50mg/kg, $p = 0.780$; figure 5.9B). However, whilst treatment with the lower dose of 25mg/kg PHA781535E alone did not alter the basal plasma corticosterone concentration ($p = 1.00$ vs basal), treatment with the higher dose (50mg/kg) significantly increased the basal corticosterone concentration ($p < 0.01$ vs basal; figure 5.9B).

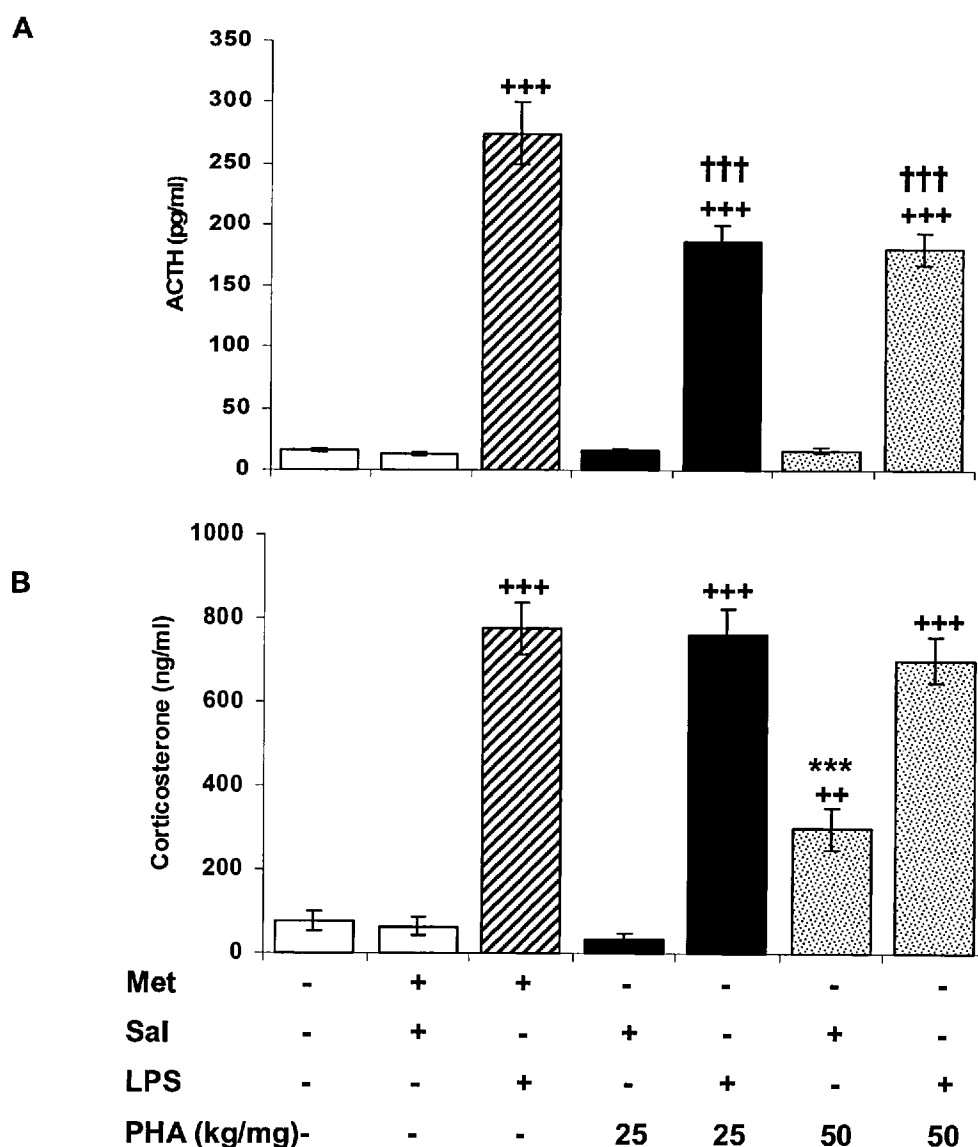
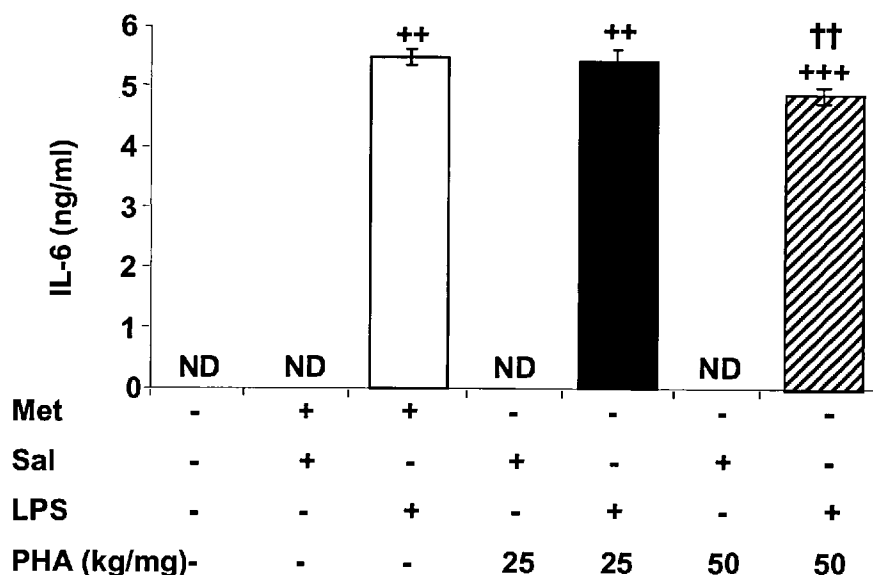


Figure 5.9 Effects of the IKK β inhibitor, PHA781535E (25 and 50mg/kg, p.o., 4 hours), on plasma (A) ACTH and (B) corticosterone concentrations in LPS (5mg/kg, i.p., 2 hours) treated rats. Rats were treated with either PHA781535E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) for 2 hours prior to LPS or saline (400 μ l; i.p.) administration. Untreated (control) and treated groups are the mean of five samples $\pm$ SEM. ** p <0.01, *** p <0.001 vs methylcellulose-tween + saline treated controls, ††† p <0.001 vs methylcellulose-tween + LPS, *** p <0.001 vs 25mg/kg PHA781535E + saline. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. ACTH, adrenocorticotrophin; LPS, lipopolysaccharide; met, methylcellulose-tween; PHA, PHA781535E; Sal, saline.

5.3.7 Effects of the IKK β inhibitor, PHA781353E, on plasma IL-6 and TNF α concentrations in LPS treated rats

The concentrations of IL-6 and TNF α in the plasma of untreated and vehicle treated groups (methylcellulose tween and/or saline) were below the detection limit of the ELISA assays, but treatment with LPS for 2 hours induced a significant increase in the plasma concentrations of IL-6 ($p < 0.001$ vs control; figure 5.10A) and TNF α ($p < 0.001$ vs control; figure 5.10B). Treatment with PHA781353E dose-dependently inhibited the LPS-induced increase in plasma TNF α concentration (25mg/kg, $p < 0.01$ vs LPS and 50mg/kg, $p < 0.001$ vs LPS; figure 5.10B). Similarly, the higher (50mg/kg, $p < 0.01$ vs LPS), but not the lower (25mg/kg, $p = 0.885$) dose of PHA781353E attenuated the increase in plasma IL-6 concentration induced by LPS (figure 5.10A).

A



B

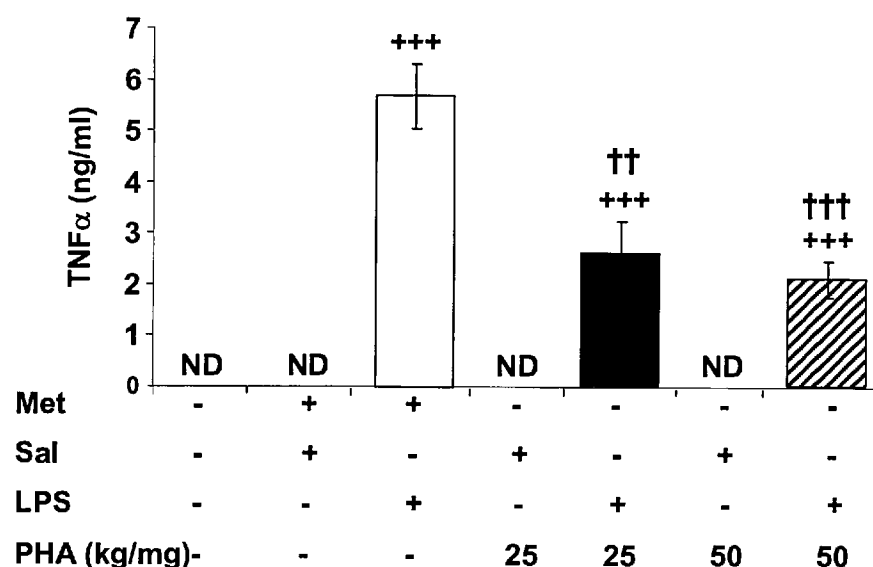


Figure 5.10 Effects of the IKK β inhibitor, PHA781535E (25 and 50mg/kg, p.o., 4 hours), on the plasma concentrations of (A) IL-6 and (B) TNF α in LPS (5mg/kg, i.p., 2 hours) treated rats. Rats were treated with either PHA781535E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) for 2 hours prior to LPS or saline (400 μ l, i.p.) administration. Untreated (control) and treated groups are the mean of five samples $\pm$ SEM. IL-6 and TNF α concentrations were undetectable in control, vehicle treated and PHA781535E treated groups. *** p <0.001 vs methylcellulose-tween + saline treated control, ** p <0.01, *** p <0.001 vs methylcellulose-tween + LPS. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's t-test. IL-6, interleukin 6; LPS, lipopolysaccharide; ND, not detected; PHA, PHA781353E; TNF α , tumour necrosis factor *alpha*.

5.3.8 Effect of the IKK β inhibitor, PHA781353E, on NF κ B activity in anterior pituitary tissue extracted from LPS treated rats

The activity of NF κ B in nuclear extracts of pituitary tissue from rats that were pre-treated with either vehicle (methylcellulose-tween, 400 μ l, p.o.) or the NF κ B inhibitor, PHA781353E (25 and 50mg/kg, p.o.) 2 hours prior to saline (400 μ l, i.p.) or LPS (5mg/kg, i.p.) injection was investigated 2 hours after the final injection.

Pituitary tissue from rats treated with either the vehicle (methylcellulose-tween, lanes 1-2; figure 5.11A and B) or PHA781353E, followed by saline injection (lanes 6-8; 25mg/kg; figure 5.11A and 50mg/kg; figure 5.12A), exhibited a decrease in basal NF κ B DNA-binding activity (figure 5.11A and B and 5.12A and B). LPS injection increased NF κ B activity (lanes 3-5; figure 5.11A and B and 5.12A and B) compared to the untreated (lane c) and vehicle treated groups (lanes 1-2; figure 5.11A and B and 5.12A and B). Pre-treatment with PHA781353E reduced LPS-stimulated NF κ B activity (lanes 9-11; 25mg/kg; figure 5.11A and B and 50mg/kg; figure 5.12 A and B).

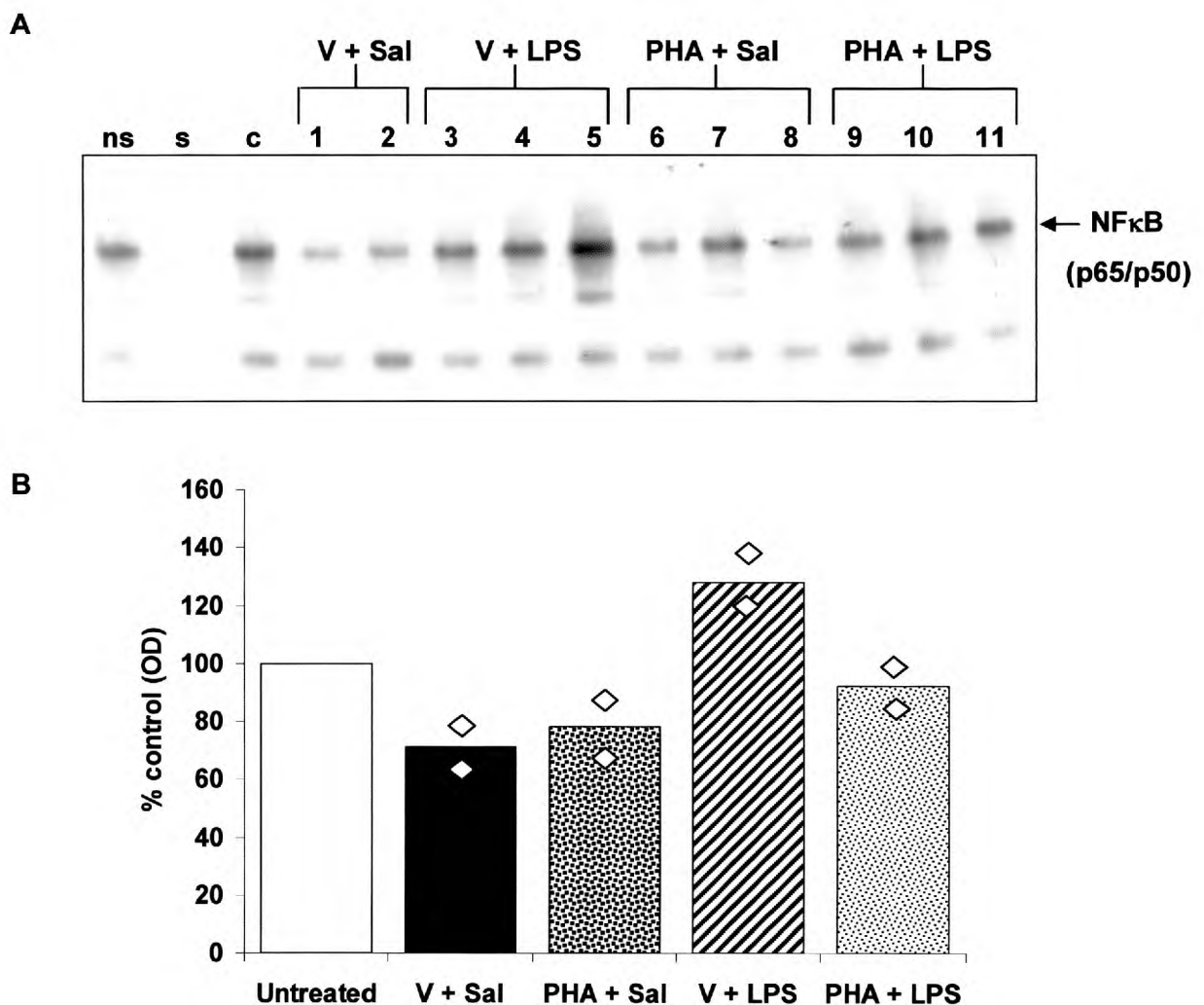


Figure 5.11 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o., 4 hours), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in anterior pituitary tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-5, methylcellulose-tween + LPS; lanes 6-8, PHA781353E + saline; lanes 9-11, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

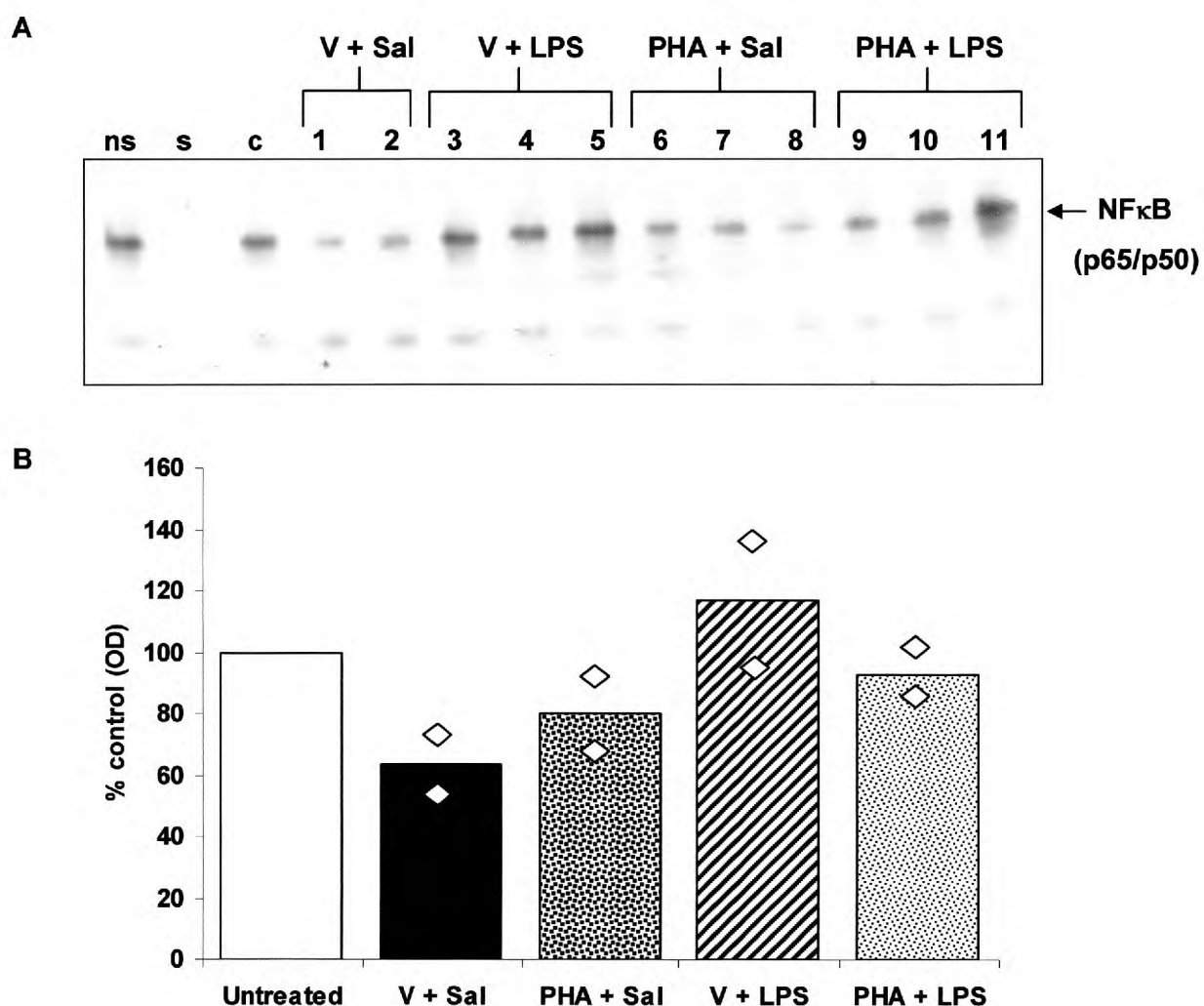


Figure 5.12 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o., 4 hours), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in anterior pituitary tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control group is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-5, methylcellulose-tween + LPS; lanes 6-8, PHA781353E + saline; lanes 9-11, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

5.3.9 Effect of the IKK β inhibitor, PHA781535E, on NF κ B activity in thymic tissue extracted from LPS treated rats

The effects of PHA781535E (25 and 50mg/kg, p.o.) treatment on LPS (5mg/kg, i.p., 2 hours) induced NF κ B activity in excised thymic tissue was also investigated. Administration of methylcellulose-tween (400 μ l, p.o.) or saline (400 μ l, i.p.) increased thymic NF κ B activity (lanes 1-2; figure 5.13A and B and 5.14A and B) compared to the untreated control (lane c; figure 5.13A and B and 5.14A and B). LPS failed to cause a further increase in NF κ B activity (lanes 3-6; figure 5.13A and B and lanes 3-5; figure 5.14A and B compared to the vehicle and saline treated groups, lanes 1-2; figure 5.13A and B and 5.14A and B). However, PHA781535E (25mg/kg PHA781535E + saline, lanes 7-9 and 25mg/kg PHA781535E + LPS, lanes 10-12; figure 5.13A and B and 50mg/kg PHA781535E + saline, lanes 6-8 and 50mg/kg PHA781535E + LPS, lanes 9-11; figure 5.14A and B) reduced NF κ B activity to levels comparable to the control in both LPS and saline-treated animals (figure 5.13A and B and 5.14A and B).

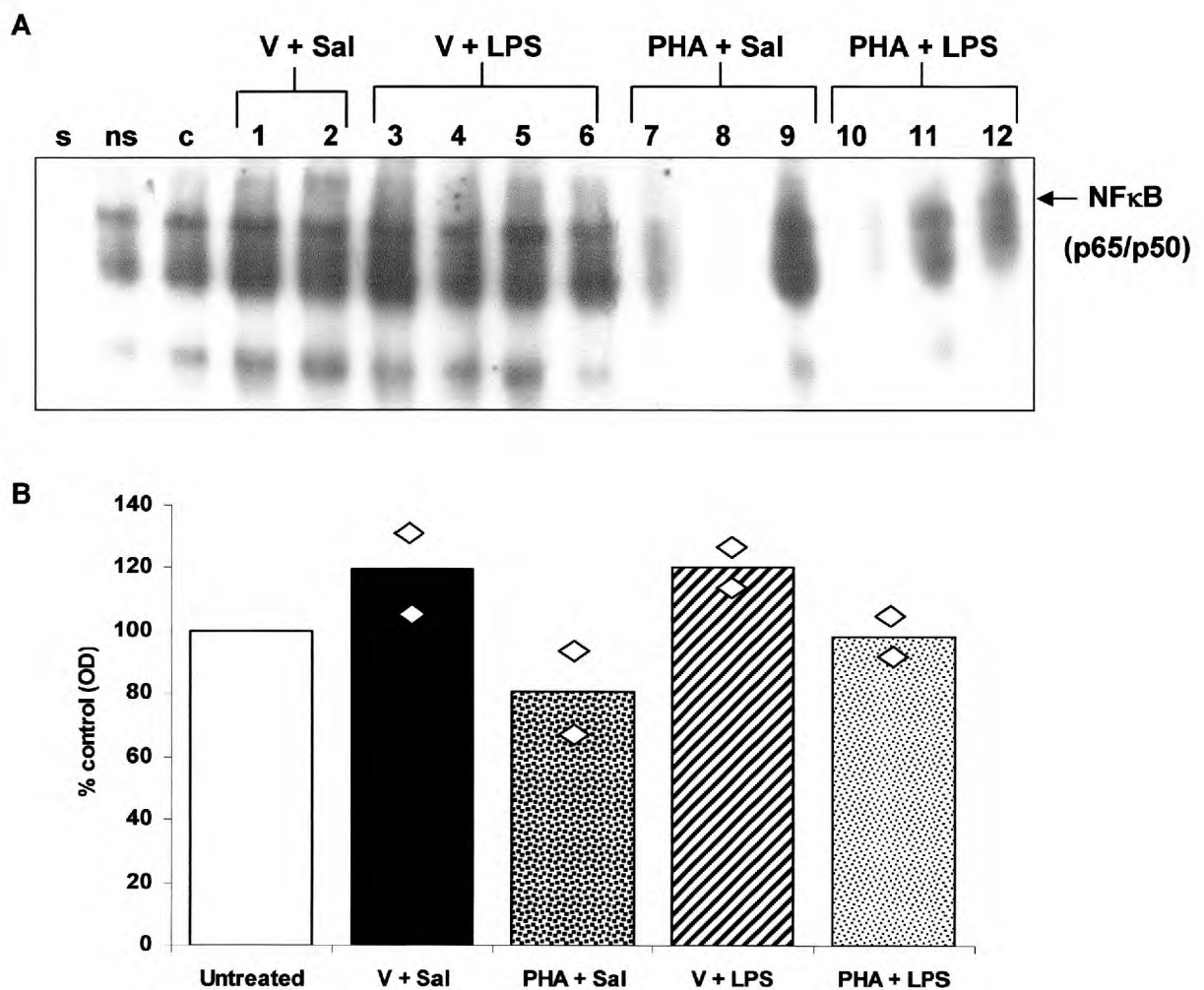


Figure 5.13 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o., 4 hours), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in thymic tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control group is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-5, methylcellulose-tween + LPS; lanes 6-8, PHA781353E + saline; lanes 9-11, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

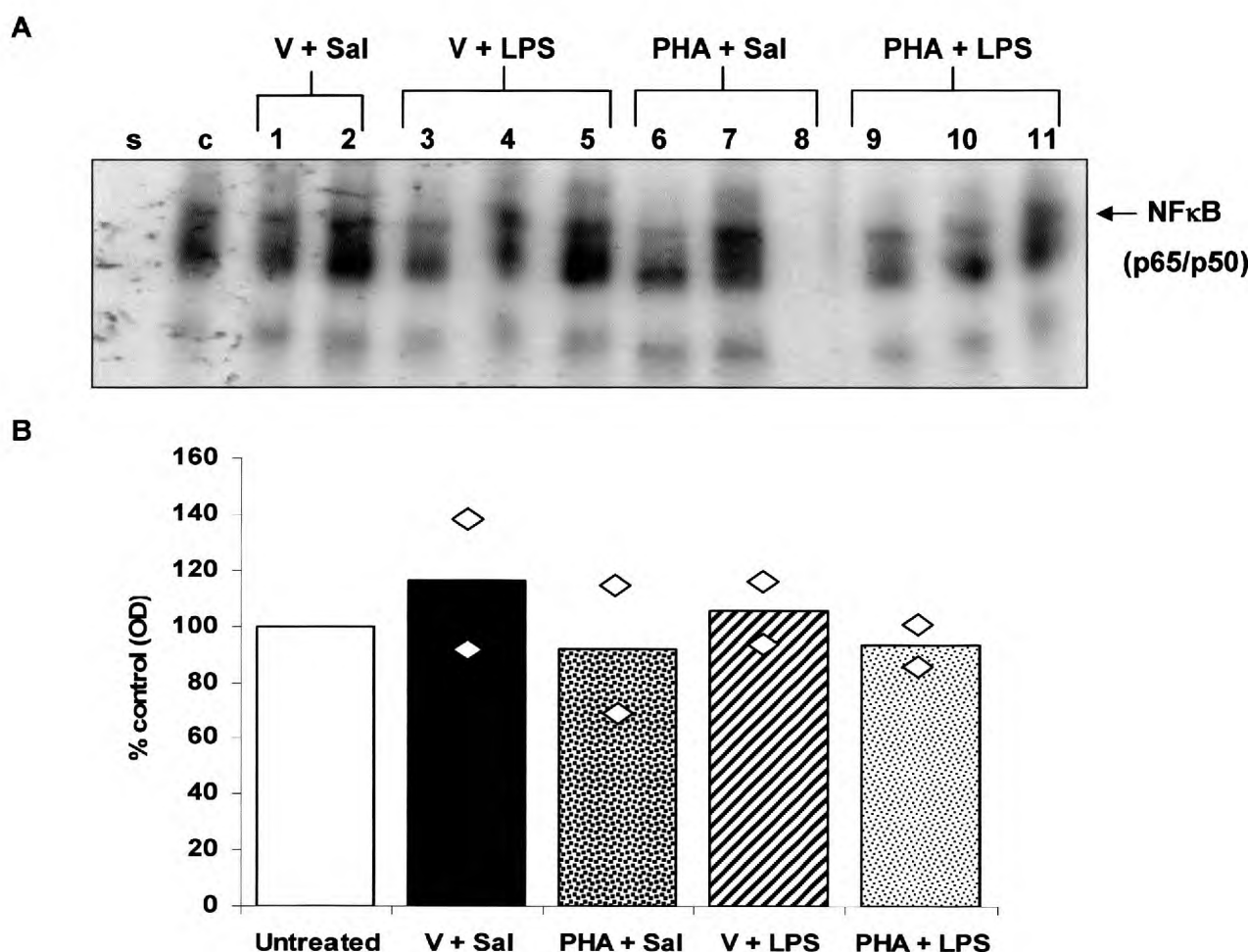


Figure 5.14 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o., 4 hours), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in thymic tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control group is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-5, methylcellulose-tween + LPS; lanes 6-8, PHA781353E + saline; lanes 9-11, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

5.3.10 Effect of the IKK β inhibitor, PHA781353E, on NF κ B activity in adrenal tissue extracted from LPS treated rats

NF κ B DNA-binding activity in the adrenal tissue extracted from untreated (lane c), or vehicle treated groups (lanes 1-2) was barely detectable (figure 5.15A and B and 5.16A and B). Both PHA781353E and saline (lanes 1-2) and vehicle and LPS (lanes 3-4; figure 5.15A and lanes 3-5; figure 5.16A) increased basal NF κ B activity to the same degree (figures 5.15B and 5.16B), but pre-treatment of LPS-injected rats with 25mg/kg PHA781353E resulted in a slight reduction in NF κ B activity (lanes 8-10; figure 5.15A and B), whilst 50mg/kg PHA781353E failed to have an effect (lanes 8-10; figure 5.16A and B).

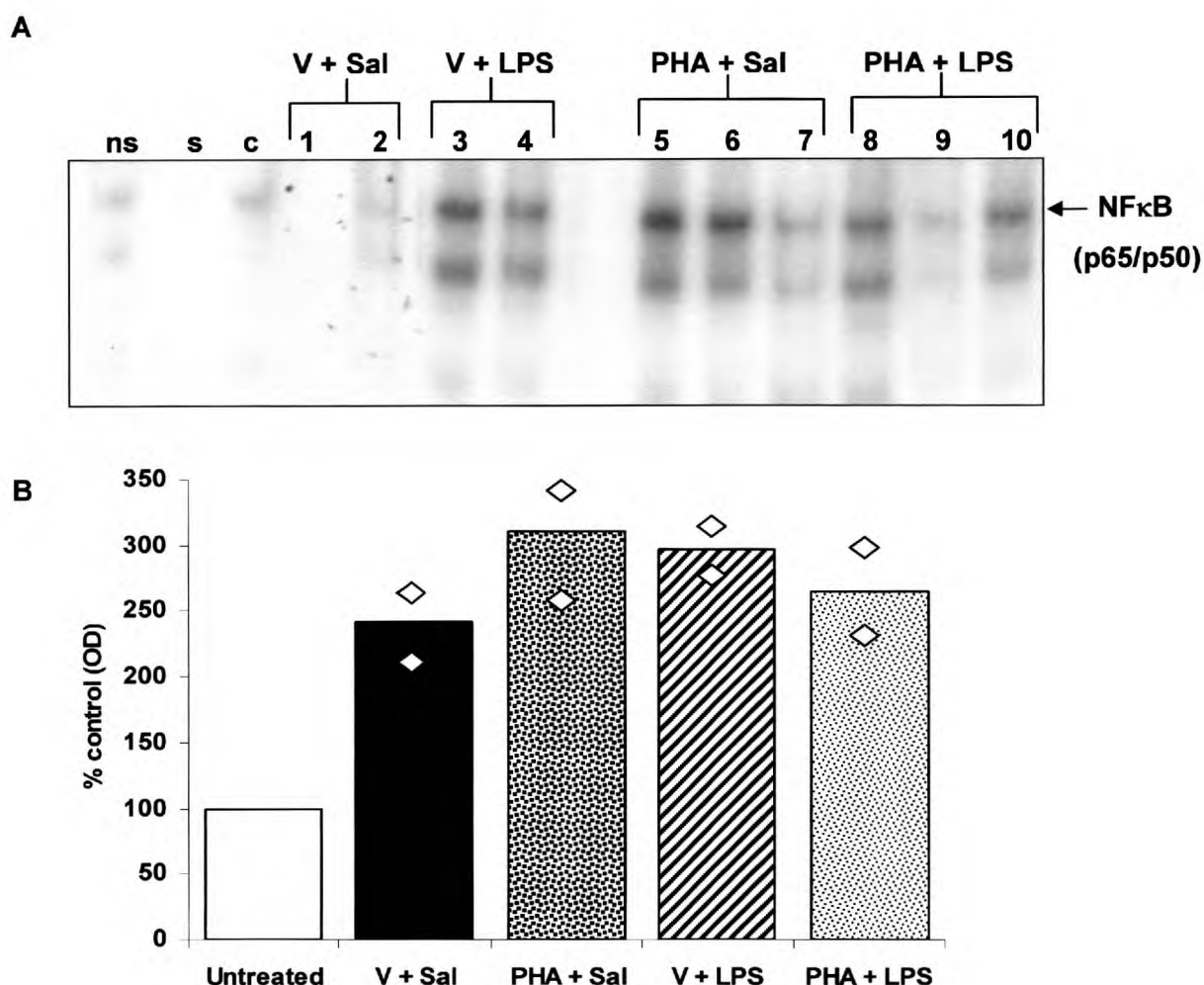


Figure 5.15 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o., 4 hours), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in adrenal tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control group is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-4, methylcellulose-tween + LPS; lanes 5-7, PHA781353E + saline; lanes 8-10, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

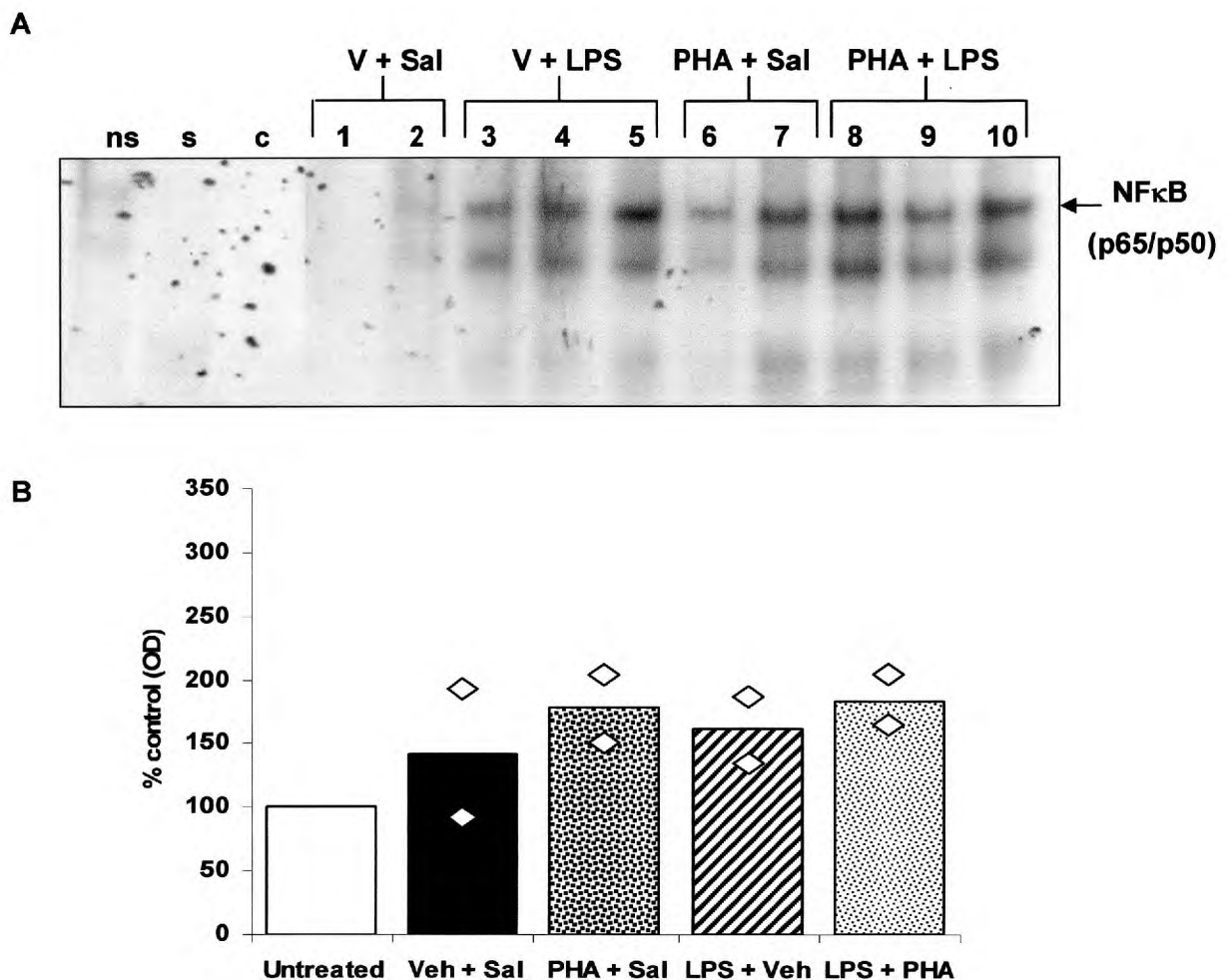


Figure 5.16 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o.), on LPS (5mg/kg, i.p., 2 hours) stimulated NF κ B activity in adrenal tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 homodimer [control group is based on the optical density of 1 sample, vehicle + saline group represent the mean optical density of 2 samples and LPS and PHA781353E treated groups represent the mean optical density of 3 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or the vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to LPS or saline (400 μ l, i.p.) injection. Lane c, untreated control; lanes 1-2, methylcellulose-tween + saline; lanes 3-4, methylcellulose-tween + LPS; lanes 5-7, PHA781353E + saline; lanes 8-10, PHA781353E + LPS. LPS, lipopolysaccharide; NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA781353E; s, specific binding reaction (NF κ B); Sal, saline; V, vehicle (methylcellulose-tween-saline).

5.4 Results - Psychological stress (restraint)

5.4.1 Effects of restraint stress upon plasma ACTH and corticosterone concentrations

The effects of the mild psychological stressor, restraint stress, upon the plasma ACTH and corticosterone concentrations were examined to elucidate the optimal time point for maximal HPA axis activation. Following 15 minutes of restraint stress, plasma ACTH concentrations were significantly and maximally elevated compared to basal levels ($p < 0.001$; figure 5.17A). After 30 minutes of restraint stress, plasma ACTH concentrations were not significant from control levels ($p = 0.449$; figure 5.17A). The plasma concentrations of ACTH from rats that were restrained for 30 minutes followed by a 30 minute and 60 minute recovery period were also comparable to basal (30 minutes recovery, $p = 1.00$ and 60 minutes recover, $p = 1.00$; figure 5.17A).

The plasma corticosterone concentration was increased in rats that had been restrained for 15 minutes ($p < 0.01$ vs control) and maximal after 30 minutes restraint stress ($p < 0.001$ vs control; figure 5.17B). The plasma corticosterone concentration in rats that were allowed a recovery period after 30 minutes restraint stress were significantly reduced and comparable to the unrestrained control group (30 minutes recovery, $p = 1.00$ and 60 minutes recovery, $p = 1.00$; figure 5.17B).

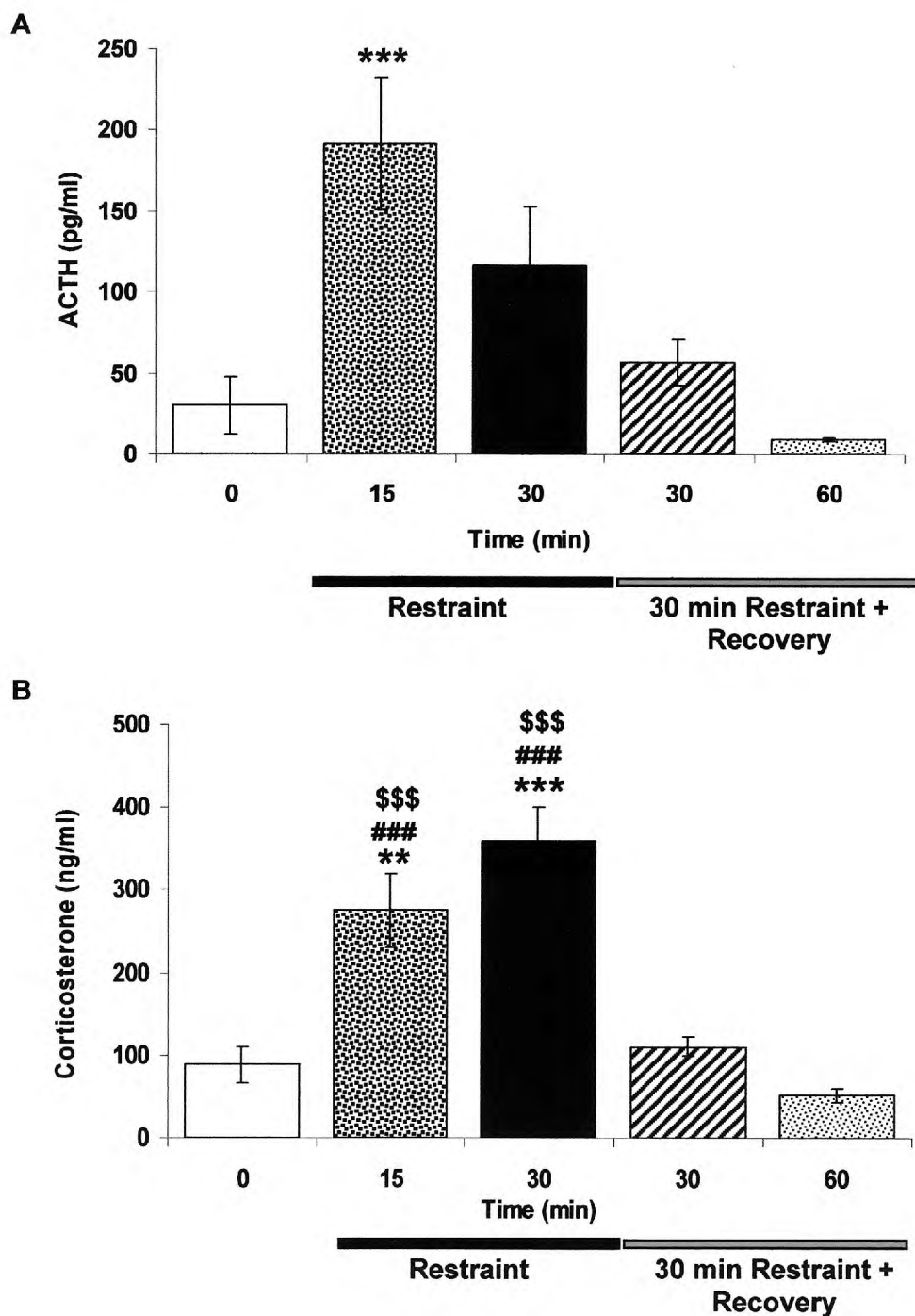


Figure 5.17 Time-dependent effects of restraint stress on the plasma (A) ACTH and (B) corticosterone concentrations. Male adult rats were restrained for 15 or 30 minutes followed by a recovery period of 0, 30 or 60 minutes. Each group is a mean of five animals $\pm$ sem. ** $p < 0.01$, *** $p < 0.001$ vs control (0 min), ### $p < 0.001$ vs 30 min restraint + 30 min recovery, \$\$\$ $p < 0.001$ vs 30 min restraint + 60 min recovery. Analysis was performed by one way ANOVA followed by Bonferroni's *post hoc* t-test. ACTH, adrenocorticotrophin.

5.4.2 Effects of restraint stress on the plasma IL-6 concentration

Previous studies have reported that rats exposed to psychological stressors, such as immobilization stress and footshock, exhibit elevated concentrations of plasma IL-6. However, rats exposed to restraint stress in this study did not have detectable concentrations of plasma IL-6 (Table 5.1).

Table 5.1 Detection of plasma IL-6 concentration from rats subjected to 15 or 30 minutes restraint stress or 30 minutes restraint stress followed by a 30 or 60 minute recovery period by enzyme linked immunosorbant assay (ELISA).

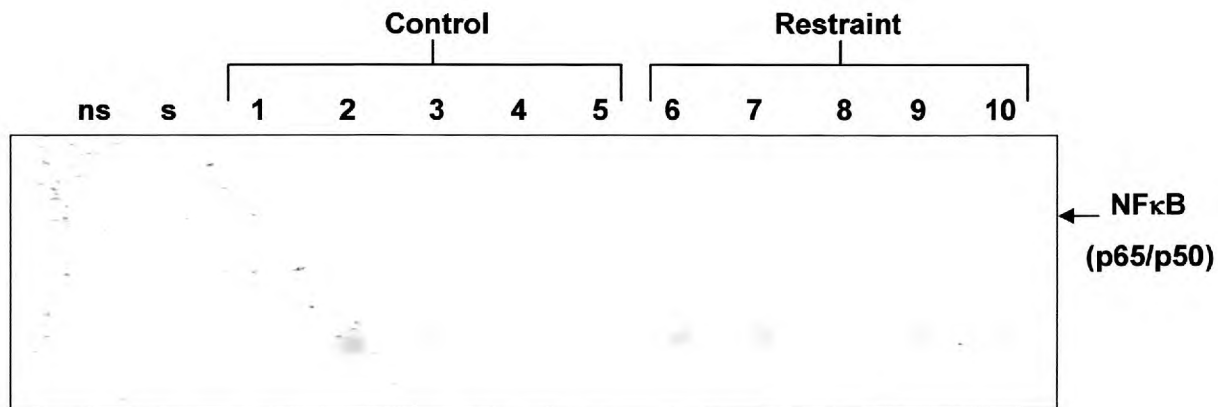
The detection limit of the rat IL-6 ELISA was 31.25pg/ml. IL-6, interleukin 6.

Sample	IL-6 (pg/ml)
Unrestrained	Undetectable
15 minutes restraint	Undetectable
30 minutes restraint	Undetectable
30 minutes restraint + 30 minutes recovery	Undetectable
30 minutes restraint + 60 minutes recovery	Undetectable
Known standard (1000pg/ml)	1168.46±267

5.4.3 Effects of restraint stress on NFκB activity in rat anterior pituitary tissue

In the pituitary nuclear extracts, 15 minutes restraint stress induced an increase in NFκB DNA-binding activity ($p < 0.05$ vs control, lanes 6-10; figure 5.18A and B) compared to the unrestrained control samples (lanes 1-5; figure 5.18A and B). The increase in NFκB DNA-binding activity was still evident after 30 minutes restraint stress ($p < 0.05$ vs control; lanes 5-10; figure 5.19A and B) and after 30 minutes restraint stress followed by a 30 minute recovery period ($p < 0.05$ vs control; lanes 6-10; figure 5.20A and B). The activity of NFκB was not examined in the anterior pituitary tissue of rats subjected to 30 minutes restraint stress followed by a 60 minute recovery period.

A



B

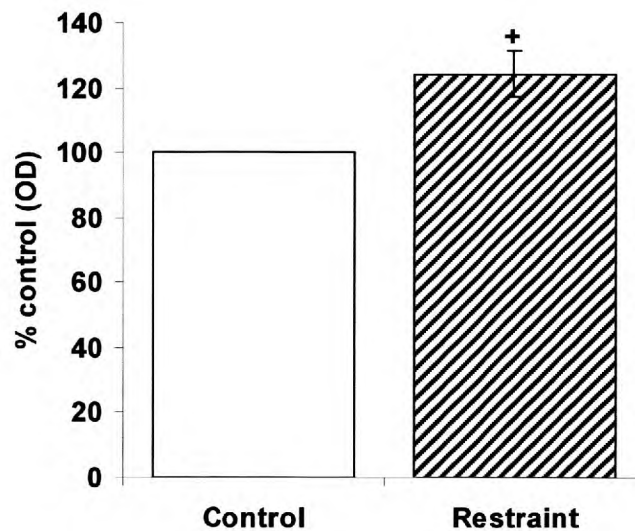


Figure 5.18 Effect of restraint stress (15 minutes) on NFκB activity in anterior pituitary tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [optical density values are the mean of 5 samples $\pm$ SEM and expressed as percentage of control (untreated)]. Lanes 1-5; unrestrained control samples; lanes 6-10, restrained samples. $^+p<0.05$ vs control. Analysis of densitometric results was performed by Mann-Whitney rank sum test. NFκB, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NFκB).

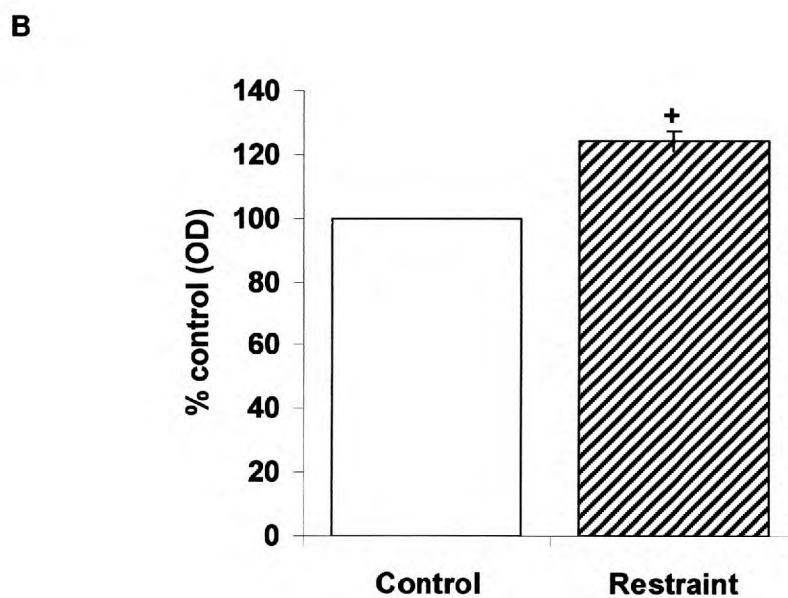
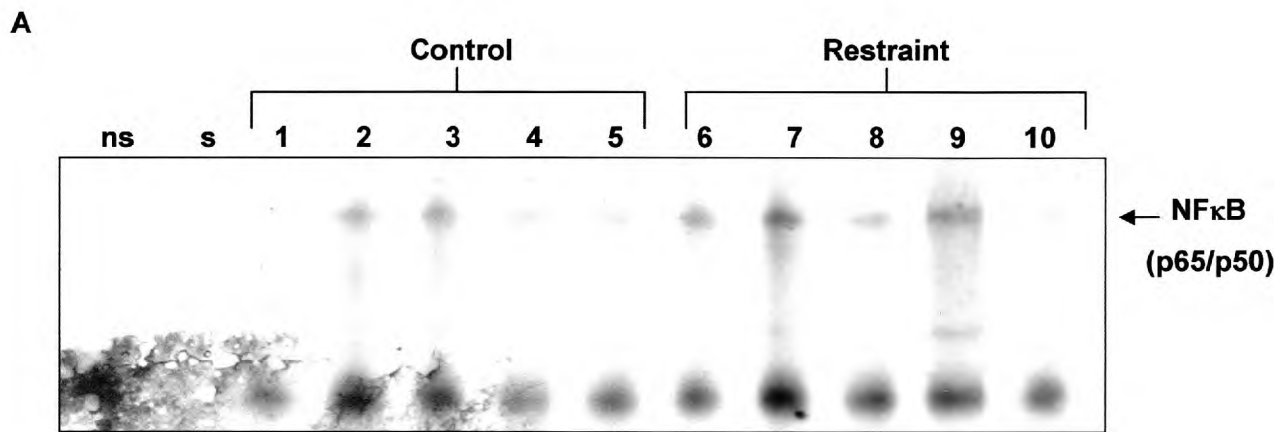


Figure 5.19 Effect of restraint stress (30 minutes) on NFκB activity in anterior pituitary tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [optical density values are the mean of 5 samples $\pm$ SEM and expressed as percentage of control (untreated)]. Lanes 1-5; unrestrained control samples; lanes 6-10, restrained samples. * $p < 0.05$ vs control. Analysis of densitometric results was performed by Mann-Whitney rank sum test. NFκB, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NFκB).

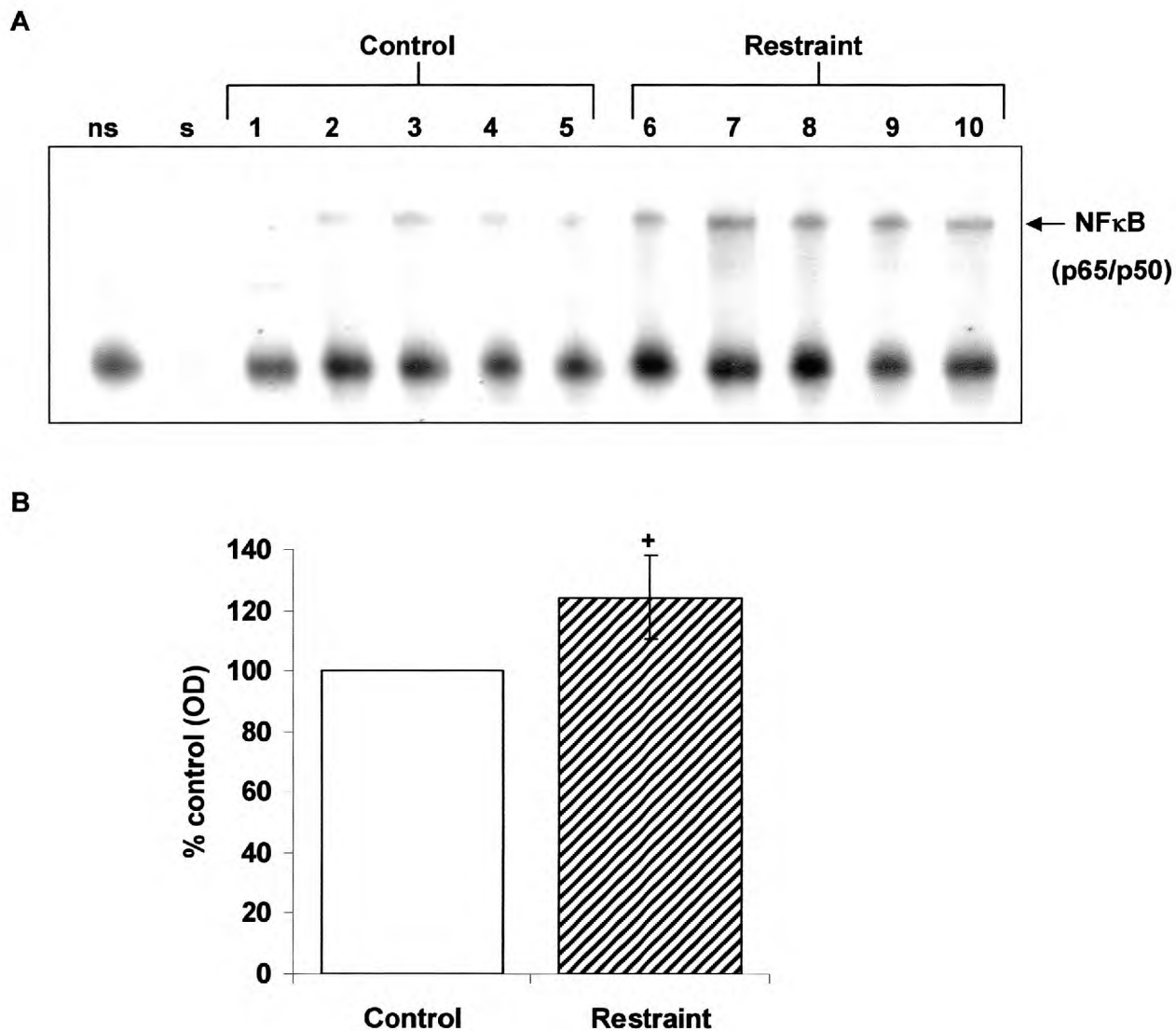


Figure 5.20 Effect of restraint stress (30 minutes restraint followed by a 30 minute recovery period) on NF κ B activity in anterior pituitary tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [optical density values are the mean of 5 samples $\pm$ SEM and expressed as percentage of control (untreated)]. For the recovery period, rats were returned to their housing cages. Lanes 1-5; unrestrained control samples; lanes 6-10, restrained samples. ⁺ $p < 0.05$ vs control. Analysis of densitometric results was performed by Mann-Whitney rank sum test. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NF κ B).

5.4.4 NF κ B activity in thymic tissue extracted from restraint stressed rats

The profile of NF κ B DNA-binding activity in thymic tissue extracted from restraint stressed rats was examined. An increase in NF κ B DNA-binding activity was not detected until 30 minutes restraint stress (lanes 6-8; figure 5.21A and B), compared to the control group (lanes 1-2; figure 5.21A and B). This increase was still evident after 30 minute restraint stress and the rats were allowed to recover for a further 30 minutes (lanes 9-10) and 60 minutes (lane 11; figure 5.21A and B) in their housing cages.

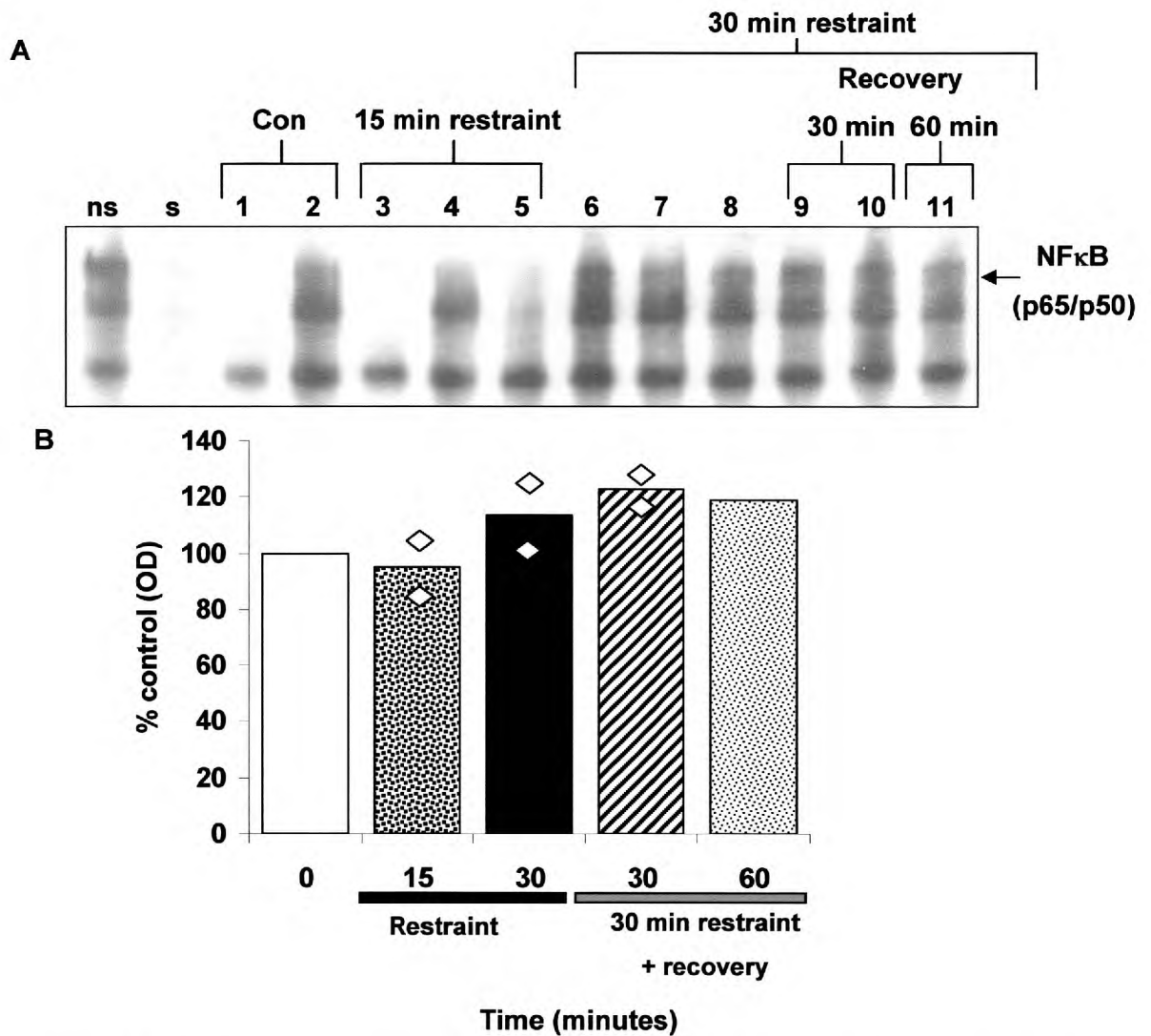


Figure 5.21 Effects of restraint stress (15 - 30 minutes, followed by 30 - 60 minutes recovery period) on NF κ B activity in thymic tissue, detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [control and 30 minutes restraint + 30 minutes recovery group represent the mean optical density of 2 samples, 15 and 30 minute restraint group represent the mean optical density of 3 samples and 30 minutes restraint + 60 minutes recovery group is based on the optical density of 1 sample. Data are expressed as percentage of control (unrestrained)]. The points on the histogram represent the range values. Lanes 1-2, unrestrained control samples; lanes 3-5, 15 minutes restraint stress; lanes 6-8, 30 minutes restraint stress; lanes 9-10, 30 minutes restraint stress followed by 30 minutes recovery period and lane 11, 30 minutes restraint stress followed by 60 minutes recovery. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; s, specific binding reaction (NF κ B).

5.4.5 Effects of the IKK β inhibitor, PHA781535E, on plasma ACTH and corticosterone concentrations in restraint stressed rats

Significantly elevated plasma ACTH and corticosterone concentrations were measured in rats exposed to 15 minutes restraint stress (section 5.4.1). Similarly, increased NF κ B activity was detected in anterior pituitary tissue after 15 minutes restraint stress (section 5.4.3). Therefore, the effects of treatment with the NF κ B inhibitor, PHA781535E on these parameters were investigated in rats exposed to 15 minutes restraint stress.

5.4.5.1 Effects of PHA781535E treatment on restraint stress-induced plasma ACTH concentrations

Pre-treatment with the PHA781535E vehicle, methylcellulose-tween (400 μ l; p.o.), did not affect basal ($p=0.081$) or restraint induced plasma ACTH concentrations ($p=0.203$; figure 5.22A). Whilst treatment with PHA781535E alone (25mg/kg, $p=1.00$ and 50mg/kg, $p=1.00$) had no effect on basal plasma concentrations of ACTH, the NF κ B inhibitor significantly attenuated the elevated plasma ACTH concentrations induced by restraint stress (25 and 50mg/kg PHA781535E, $p<0.01$ vs restraint), with no significant difference in the inhibitory effect induced by 25mg/kg and 50mg/kg PHA781535E ($p=0.901$; figure 5.22A).

5.4.5.2 Effects of PHA781535E treatment on restraint stress-induced plasma corticosterone concentrations

Similarly to the ACTH response, the vehicle (methylcellulose-tween) did not affect basal ($p=0.645$) or restraint induced plasma corticosterone concentrations ($p=0.288$; figure 5.22B). Exposure to 15 minutes restraint significantly increased concentrations of plasma corticosterone compared to the unrestrained control ($p<0.001$) and pre-treatment with both doses of PHA781535E attenuated this increase ($p<0.001$ vs restraint), with there being no significant difference in the effect induced by 25mg/kg and 50mg/kg PHA781535E ($p=1.00$; figure 5.22B). However, whilst treatment with 25mg/kg PHA781535E alone did not affect basal corticosterone concentrations ($p=1.00$), 50mg/kg PHA781535E significantly increased the basal plasma corticosterone concentration ($p<0.01$ vs unrestrained control; figure 5.22B).

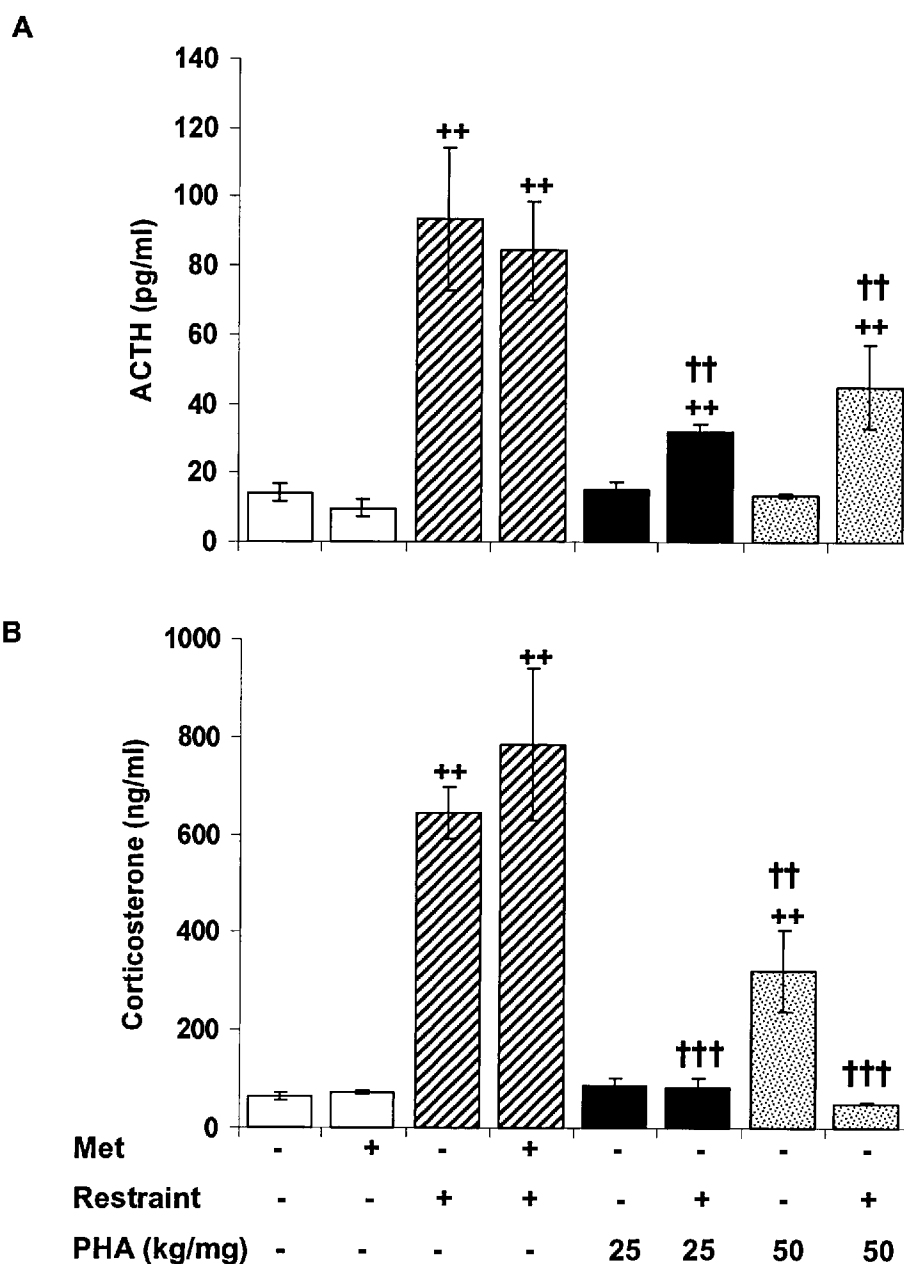


Figure 5.22 Effects of the IKK β inhibitor, PHA781353E (25 and 50mg/kg, p.o.), on plasma (A) ACTH and (B) corticosterone concentrations in rats subjected to 15 minutes restraint stress. Rats were treated with PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Control (untreated/unrestrained) and treated/restrained groups are the mean of five samples $\pm$ SEM. ** p <0.01, *** p <0.001 vs untreated control, †† p <0.01, ††† p <0.001 vs 15 minutes restraints stress. Analysis was performed by two-way ANOVA with *post hoc* comparison using Bonferroni's *t*-test. ACTH, adrenocorticotrophin; PHA, PHA781353E; Met methylcellulose-tween.

5.4.6 Effects of the IKK β inhibitor, PHA781353E, on plasma IL-6 and TNF α concentrations from rats subjected to restraint stress

The concentrations of IL-6 and TNF α were undetectable in the plasma collected from both the control and restrained groups (Table 5.2 and 5.3). Treatment with methylcellulose-tween or PHA781353E alone or prior to restraint stress also failed to induce detectable concentrations of these cytokines (Table 5.2 and 5.3).

Table 5.2 Detection of the plasma IL-6 concentration. Rats were treated with either vehicle (methylcellulose-tween; 400 μ l, p.o.) or the NF κ B inhibitor, PHA781535E (25 and 50mg/kg, p.o.), 2 hours prior to 15 minutes restraint stress by enzyme linked immunosorbant assay (ELISA). The detection limit of the rat IL-6 ELISA was 31.25pg/ml. IL-6, interleukin 6; vehicle, methylcellulose-tween.

Sample	IL-6 (pg/ml)
Control	Undetectable
Vehicle	Undetectable
PHA781535E (25mg/kg)	Undetectable
PHA781535E (50mg/kg)	Undetectable
15 minutes restraint + vehicle	Undetectable
15 minutes restraint + PHA781535E (25mg/kg)	Undetectable
15 minutes restraint + PHA781535E (50mg/kg)	Undetectable
Known standard (500pg/ml)	775.07 $\pm$ 110.24

Table 5.3 Detection of the plasma TNF α concentration. Rats were treated with either vehicle (methylcellulose-tween; 400 μ l, p.o.) or the NF κ B inhibitor, PHA781535E (25 and 50mg/kg, p.o.), 2 hours prior to 15 minutes restraint stress by enzyme linked immunosorbant assay (ELISA). The detection limit of the rat TNF α ELISA was 31.25pg/ml. TNF α , tumour necrosis factor *alpha*; vehicle, methylcellulose-tween.

Sample	TNF α (pg/ml)
Control	Undetectable
Vehicle	Undetectable
PHA781535E (25mg/kg)	Undetectable
PHA781535E (50mg/kg)	Undetectable
15 minutes restraint + vehicle	Undetectable
15 minutes restraint + PHA781535E (25mg/kg)	Undetectable
15 minutes restraint + PHA781535E (50mg/kg)	Undetectable
Known standard (125pg/ml)	136.47 $\pm$ 15.66

5.4.7 Effect of the IKK β inhibitor, PHA781353E, on restraint stress-induced NF κ B activity in anterior pituitary tissue

Restraint stress (15 minutes) increased basal NF κ B activity in pituitary tissue nuclear extracts (lanes 1-3; figure 5.23A and B and figure 5.24A and B). Treatment with the vehicle (methylcellulose-tween, 400 μ l, p.o.) slightly reduced basal NF κ B (lane V; figure 5.23A and B and figure 5.24A and B) and administration of 25mg/kg PHA781353E induced a further reduction (lanes 4-5; figure 5.23A and B). The higher dose of 50mg/kg PHA781353E did not have an observable effect on basal NF κ B activity (lanes 5-6; figure 5.24A and B). However, pre-treatment with PHA781353E (lanes 7-10; 25mg/kg PHA781353E + restraint; figure 5.23A and B and 50mg/kg PHA781353E + restraint; figure 5.24A and B) resulted in a decrease in restraint stress-induced NF κ B activation.

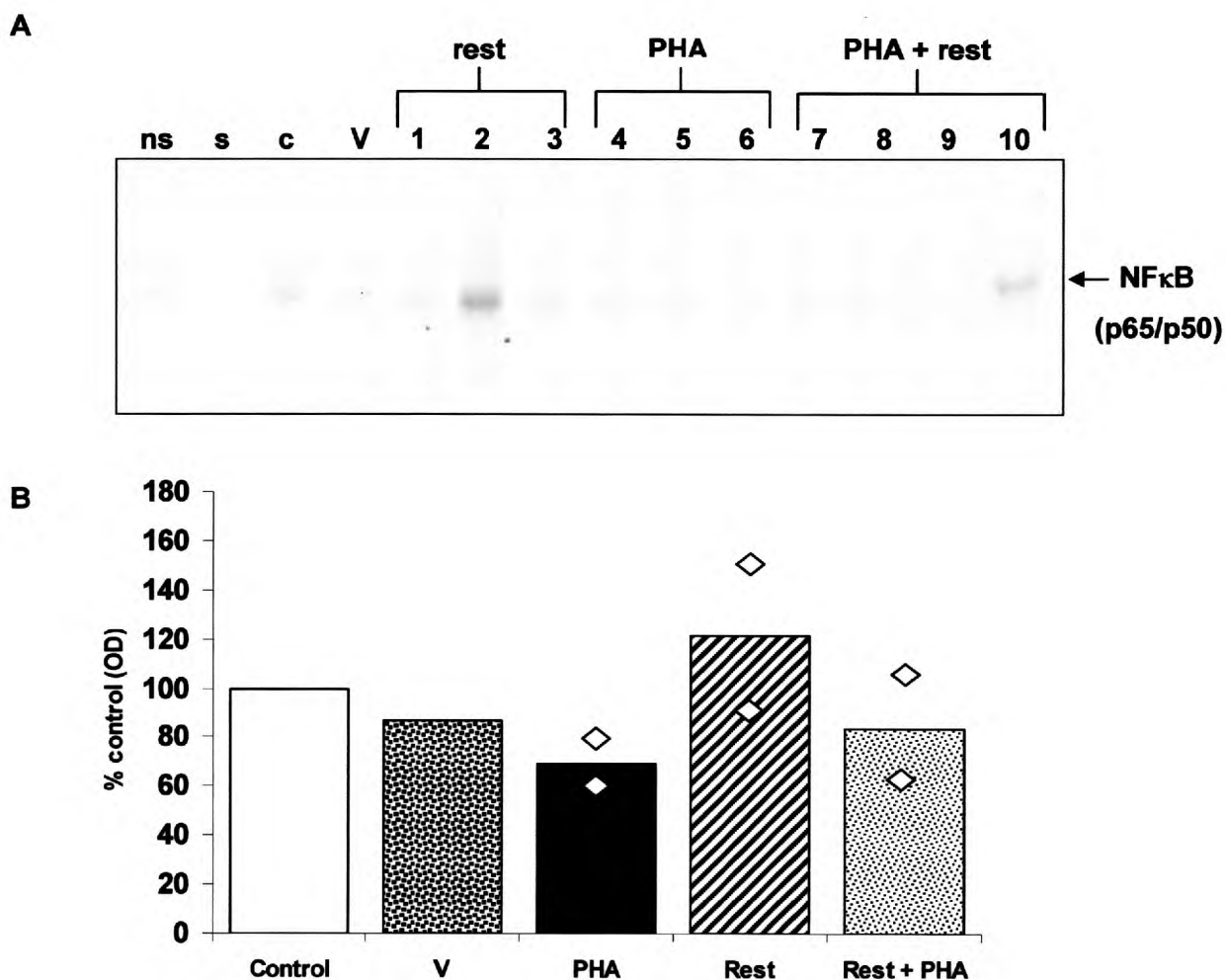


Figure 5.23 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o.), on NF κ B activity in anterior pituitary tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [control and vehicle groups are based on the optical density of 1 sample, PHA781353E only treated and restraint only groups represent the mean optical density of 3 samples and restraint + PHA781353E treated group represent the mean optical density of 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

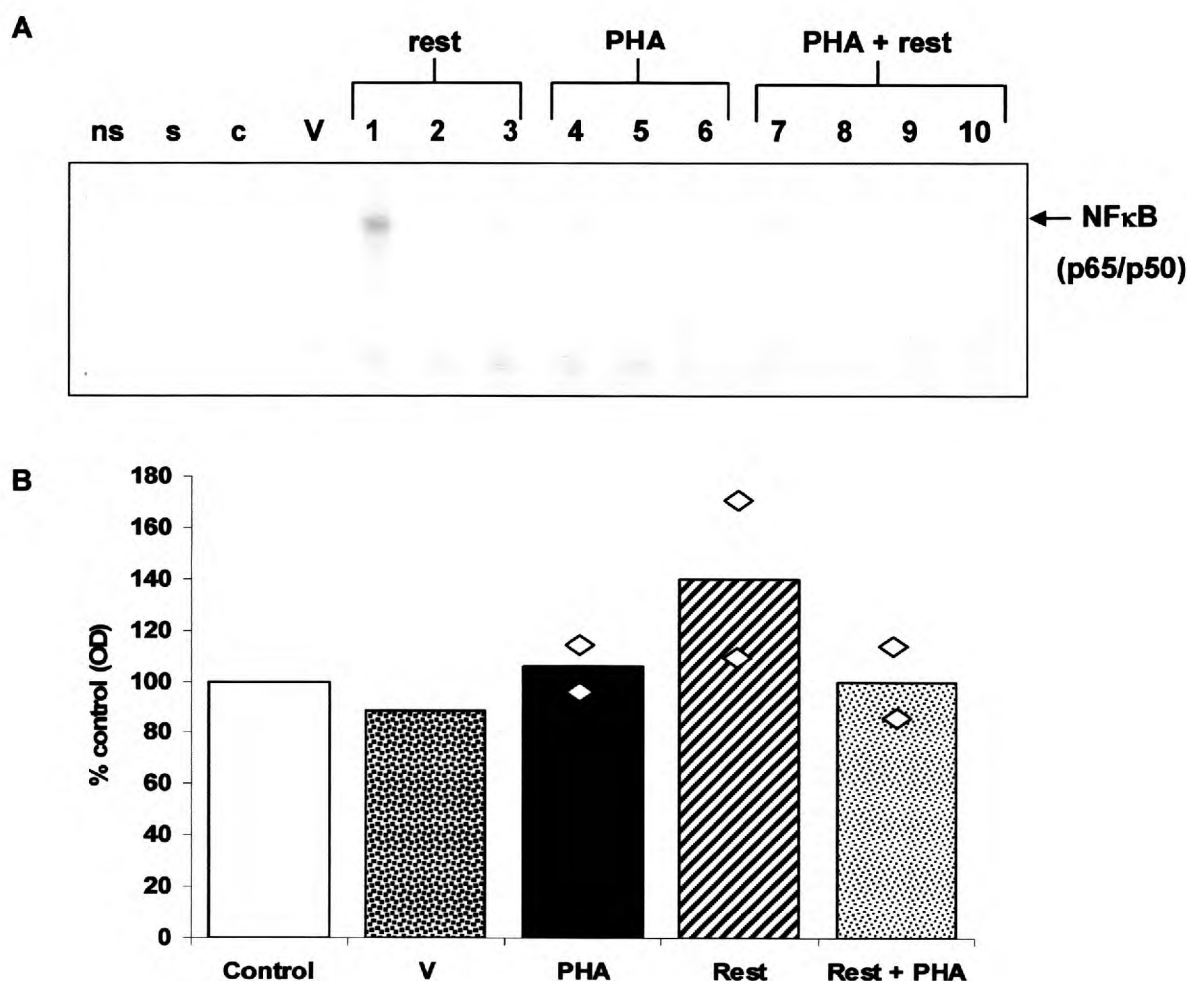


Figure 5.24 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o.), on NF κ B activity in anterior pituitary tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [control and vehicle treated groups are based on the optical density of 1 sample, PHA781353E only treated and restraint only groups represent the mean optical density of 3 samples and restraint + PHA781353E treated group represent the mean optical density of 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

5.4.8 Effect of the IKK β inhibitor, PHA781353E, on restraint stress-induced NF κ B activity in thymic tissue

The PHA781353E vehicle (methylcellulose-tween, 400 μ l, p.o.) increased basal activity (lane c, control and lane V, vehicle) of NF κ B in thymic tissue extracted from unrestrained rats (figure 5.25A and B and 5.26A and B). However, treatment with PHA781353E did not induce an observable effect on basal NF κ B activity (lanes 4-6; 25mg/kg; figure 5.25A and B and 50mg/kg; figure 5.26A and B). Restraint stress increased NF κ B DNA-binding activity compared to both unrestrained and vehicle induced levels (lanes 1-3; figure 5.25A and B and 5.26A and B) and pre-treatment with 25mg/kg PHA781353E attenuated this increase to levels comparable to control (lanes 7-10; figure 5.25A and B). Treatment with 50mg/kg did not affect increased NF κ B induced activity by restraint stress (lanes 7-10; 50mg/kg; figure 5.26A and B).

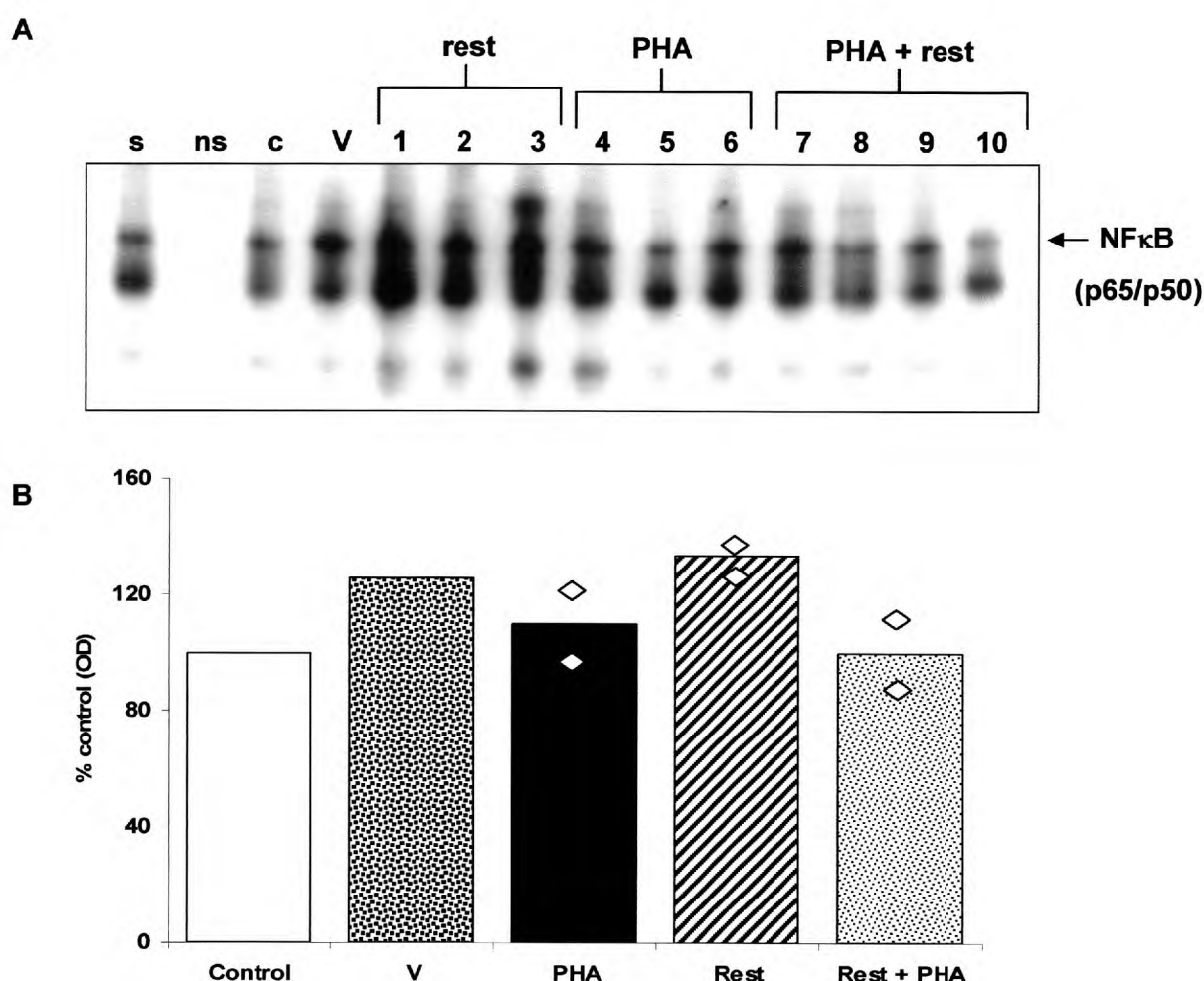


Figure 5.25 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o.), on NF κ B activity in thymic tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [control and vehicle treated groups are based on the optical density of 1 sample, PHA781353E only treated and restraint only groups represent the mean optical density of 3 samples and restraint + PHA781353E treated group represent the mean optical density of 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

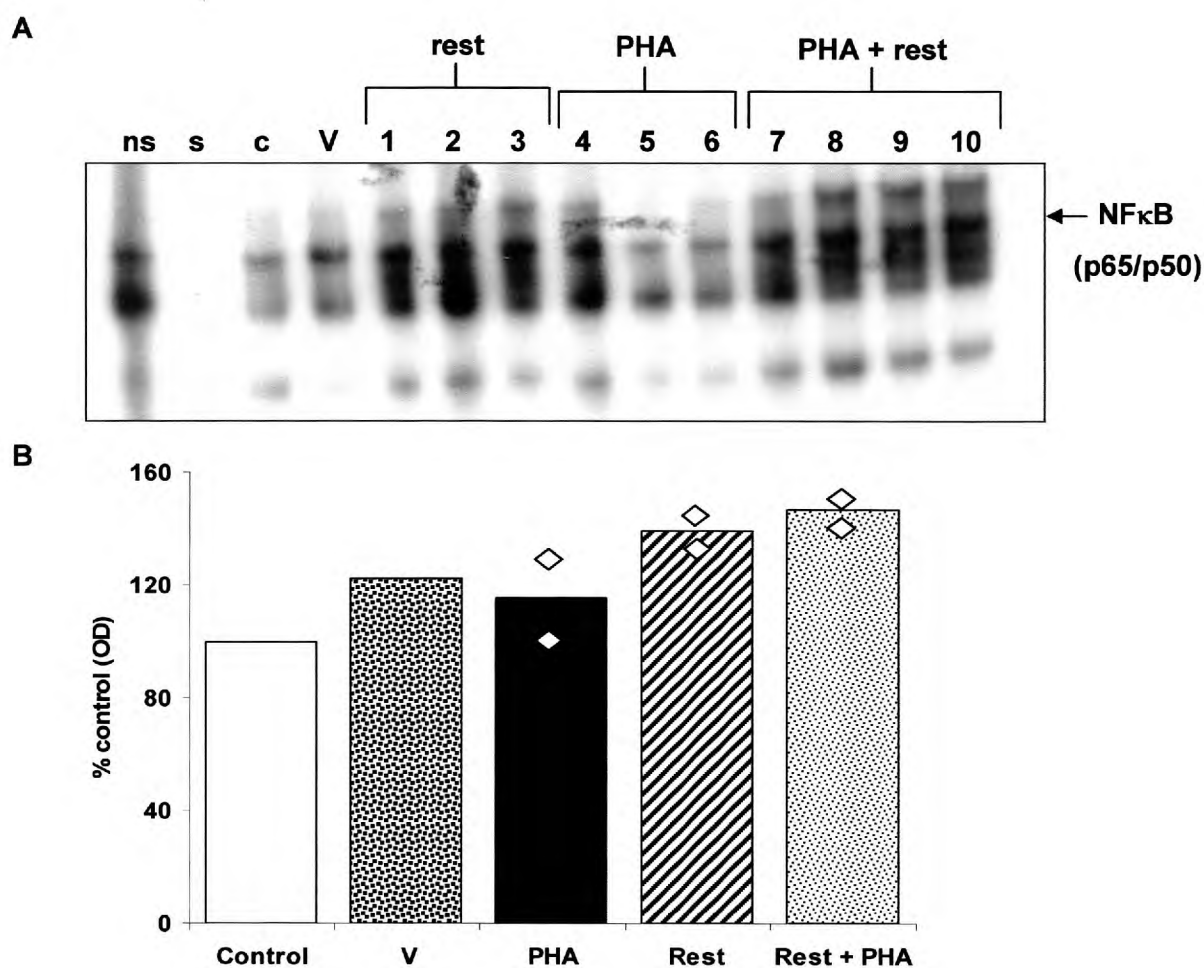


Figure 5.26 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o.) on NF κ B activity in thymic tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [optical density for the control and vehicle treated groups are representative of 1 sample, PHA781353E only treated and restraint only groups represent the mean optical density of 3 samples and PHA781353E + restraint group represent the mean optical density for 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

5.4.9 Effect of the IKK β inhibitor, PHA781353E, on restraint stress-induced NF κ B activity in rat adrenal tissue

In adrenal tissue, NF κ B activity appeared to be reduced by both vehicle (methylcellulose-tween, 400 μ l, p.o., lane V; figure 5.27A and B and 5.28A and B) and PHA781353 treatment (lanes 4-6; 25mg/kg; figure 5.27A and B and 50mg/kg; figure 5.28A and B). NF κ B activity also appeared to be attenuated by restraint stress samples (lanes 1-3; figure 5.27A and B and figure 5.28A and B). Treatment with 25mg/kg PHA781353E following restraint stress increased NF κ B activity (lanes 7-10; figure 5.25A and B) compared to the restraint group (lanes 1-3; figure 5.27A and B) but DNA-binding activity was still less than control levels (lane c; figure 5.27A and B). Treatment with the higher dose of 50mg/kg PHA781353E prior to restraint stress did not have an observable effect on NF κ B activity (lanes 7-10; figure 5.28A and B) compared to the restraint group (lanes 1-3; figure 5.28A and B).

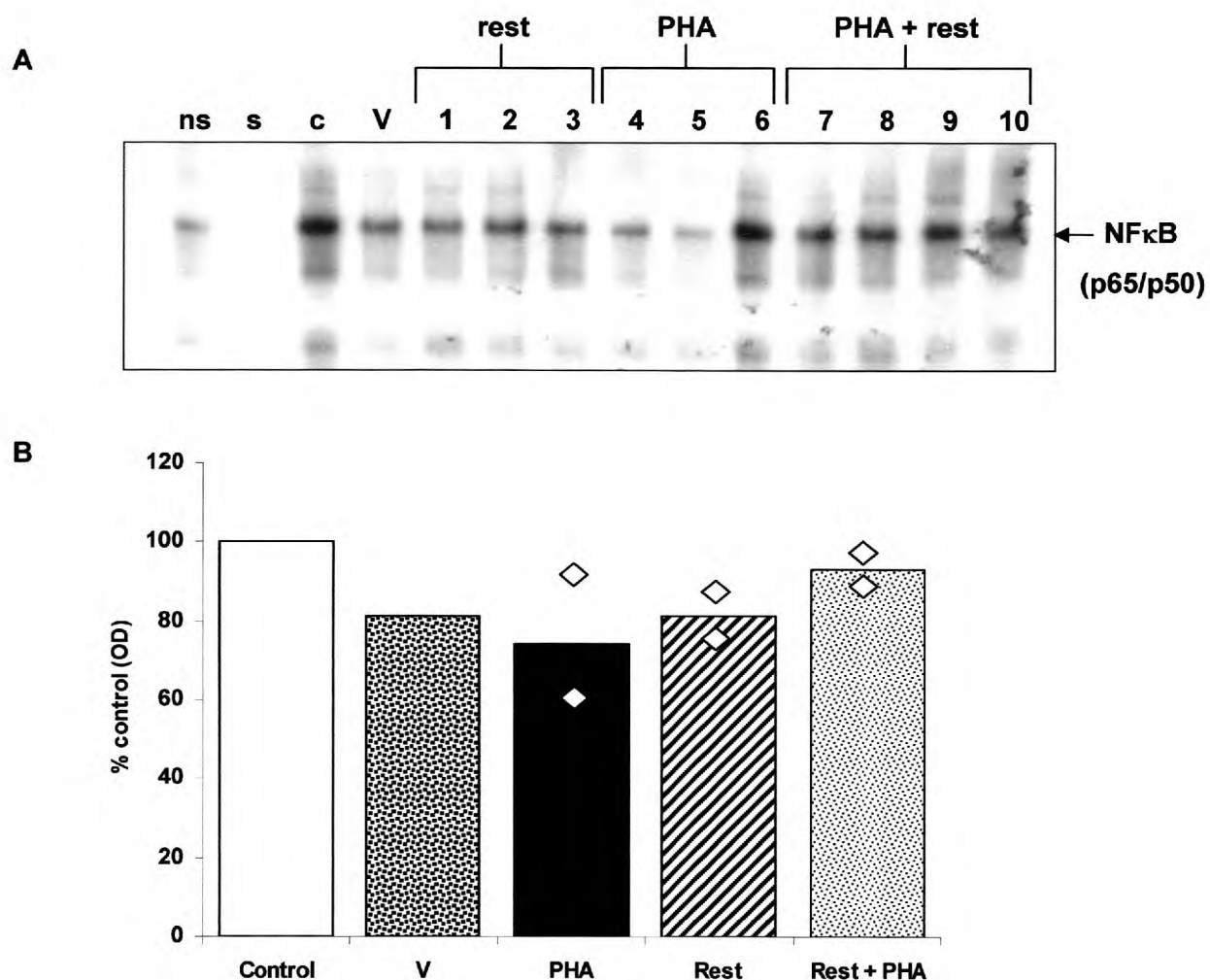


Figure 5.27 Effect of the IKK β inhibitor, PHA781353E (25mg/kg, p.o.), on NF κ B activity in adrenal tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometric analysis of the bands representing the p65/p50 heterodimer [control and vehicle treated groups are based on the optical density of 1 sample, PHA781353E only treated and restraint only groups represent the mean optical density of 3 samples and restraint + PHA781353E treated group represent the mean optical density of 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

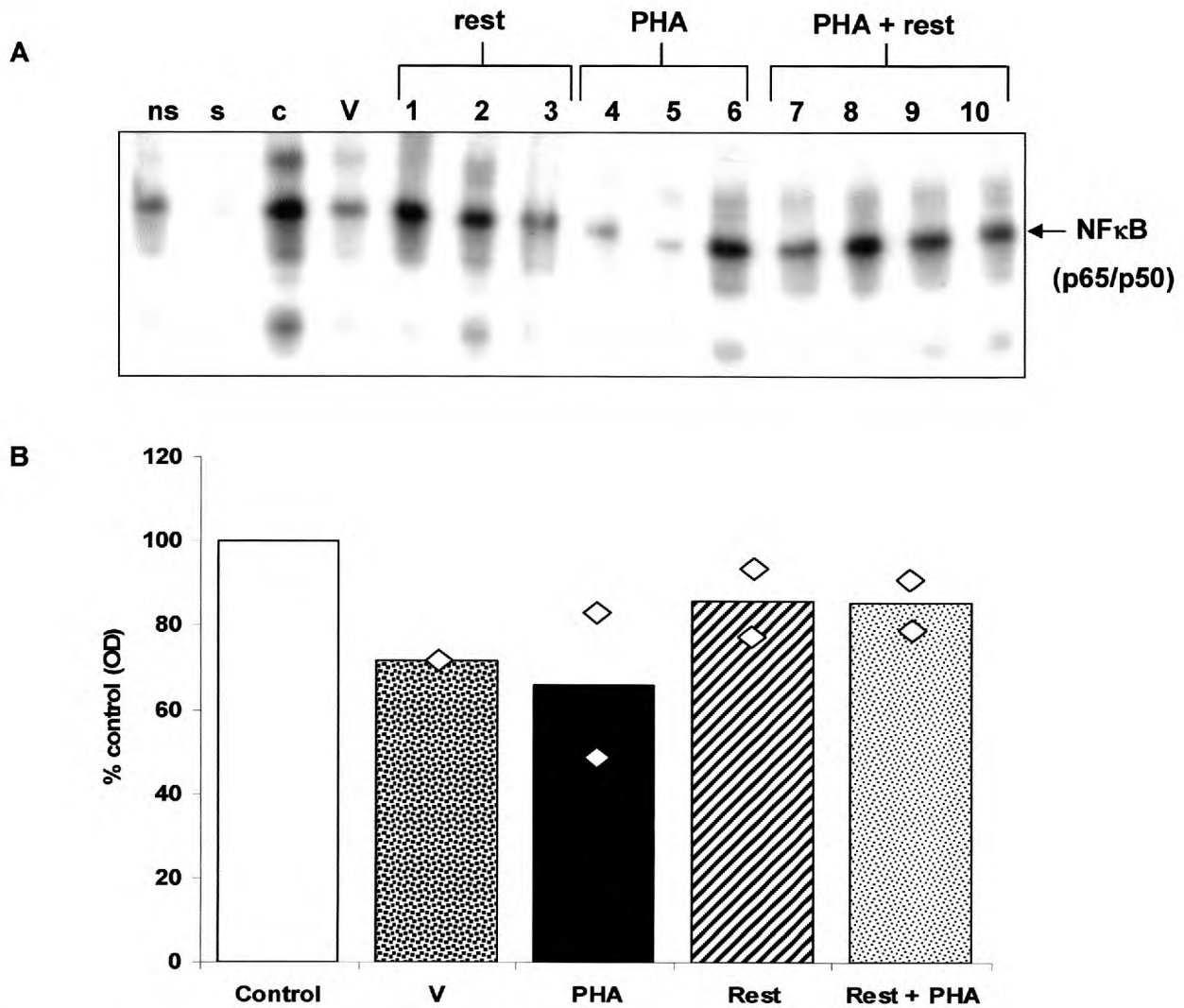


Figure 5.28 Effect of the IKK β inhibitor, PHA781353E (50mg/kg, p.o.), on NF κ B activity in adrenal tissue extracted from rats subjected to restraint stress (15 minutes), detected by (A) electrophoretic mobility shift assay (EMSA). (B) Densitometry analysis of the bands representing the p65/p50 heterodimer (control and vehicle treated groups are based on the optical density of 1 sample, PHA781353E only treated and restraint only groups represent the optical density of 3 samples and restraint + PHA781353E treated group represent the mean optical density of 4 samples. Data are expressed as percentage of control (untreated)]. The points on the histogram represent the range values. Rats were treated with either PHA781353E or vehicle (methylcellulose-tween; 400 μ l, p.o.) 2 hours prior to restraint stress. Lane c, unrestrained control; lane V, vehicle (methylcellulose-tween); lanes 1-3, restraint stress; lanes 4-6, PHA781353E treatment; lanes 7-10; PHA781353E treatment + restraint stress. NF κ B, nuclear factor *kappa* B; ns, non-specific binding reaction (AP-1); OD, optical density; PHA, PHA781353E; rest, restraint; s, specific binding reaction (NF κ B); V, vehicle (methylcellulose-tween).

5.5 Discussion

The HPA axis is readily activated by immunological or psychological stress. ACTH release from the anterior pituitary gland is thus increased, as too is the synthesis and release of glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex (section 1.2). The HPA axis plays a key role in regulating the body's response to stress and restoring homeostasis. Its activity is normally maintained within limits by the negative feedback actions of glucocorticoids (see section 1.3), but perturbations of cortisol secretion are not uncommon in clinical medicine and are now thought to be a major contributing factor to the pathogenesis of a variety of diseases.

The pro-inflammatory transcription factor, NF κ B, is an important mediator of the inflammatory response and regulates the gene expression of a variety of pro-inflammatory enzymes and mediators, including the inducible enzymes NOS-2 and COX-2 and the pro-inflammatory cytokines TNF α and IL-6 (see section 1.6). Therefore, targeting NF κ B may provide potential therapy for the treatment of chronic inflammatory disorders such as rheumatoid arthritis. However, our studies (see chapter 3 and 4) and those of others (Lohrer *et al.* 2000) have identified a role for pituitary-derived NF κ B in mediating the release of ACTH induced by endotoxin. Therefore, inhibition of NF κ B in patients with chronic inflammatory disorders as a form of treatment may result in adverse side effects such as impaired HPA responses to immunological stress. Furthermore, as NF κ B is also activated in human PBMCs and rodent brain cortex tissue by psychological stress (see section 1.6.3), NF κ B blockade may also affect the HPA responses to psychological stress.

5.5.1 Models and methods

5.5.1.1 Animal model for immune stress (LPS)

To study the effects of a novel NF κ B inhibitor (PHA818535E; section 5.5.3.1) upon the rat HPA responses to immunological stress, adult male Sprague-Dawley rats were injected intraperitoneally (i.p.) with the bacterial endotoxin, LPS. LPS, a major component of the outer surface membrane of almost all gram-negative bacteria, acts as a powerful stimulator of innate or natural immunity and is commonly used experimentally to induce immunological responses *in vivo* [reviewed in (Beishuizen & Thijs 2003)]. However, as high doses of LPS can lead to severe pathological reactions such as the induction of septic shock (Beishuizen & Thijs 2003), care must

be taken in selecting the dose. Previous studies have shown that i.p. administration of 5mg/kg LPS (*Escherichia coli*; serotype 055:B5) in adult male Sprague-Dawley rats induces an immunological response with increased plasma TNF α and IL-6 concentrations, without causing lethality, and an associated increase in HPA activity (Fernandez-Martinez *et al.* 2004). Therefore, the current study used this dose of LPS for the model of immunological stress.

Recent studies have demonstrated that, in addition to stimulating the HPA axis indirectly through the release of pro-inflammatory cytokines from immune cells (e.g. macrophages), LPS also acts directly at the level of the anterior pituitary gland to stimulate ACTH release (Lohrer *et al.* 2000; Gloddek *et al.* 2001). The *in vitro* experiments conducted in the current study (chapters 3 and 4) provide evidence that this effect is mediated by NF κ B. Therefore, the effects of LPS injection upon NF κ B activity in the anterior pituitary gland were examined by EMSA. For a comparison, NF κ B activity was also examined in distal organs (thymus and adrenal glands) after LPS injection.

Initially, the profiles of NF κ B activity in anterior pituitary tissue and the plasma ACTH and corticosterone concentrations were examined in rats 2 or 4 hours after treatment with LPS. We found robust increases in plasma ACTH and corticosterone concentrations 2 hours after LPS injection, together with increases in plasma IL-6 and TNF α (which were measured to determine activation of the immune system) and anterior pituitary NF κ B activity. Therefore, we selected this time point to investigate the effects of the NF κ B inhibitor, PHA781535E, upon the HPA responses to LPS.

Vehicle-treated and untreated control groups were included to ensure that the effects induced by LPS could be distinguished from the stress of injecting the rats with a needle and from the vehicle. The untreated control groups were left in their housing cage on the day of the study until tissue collection and vehicle-treated controls received an equivalent volume of the vehicle solution (sterile physiological saline; 400 μ l, i.p.) in place of LPS. For the NF κ B inhibition studies, the control groups included rats treated with the PHA781535E vehicle (methylcellulose-tween, 400 μ l, p.o.) and the LPS vehicle (physiological saline, 400 μ l, i.p.) and rats treated with PHA781535E (25 or 50mg/kg, p.o.) and the LPS vehicle (physiological saline, 400 μ l, i.p.) so as to ensure that any effects were attributed to PHA781535E and not the vehicles or the stress of oral dosing or i.p. injection.

5.5.1.2 Animal model for psychological stress (restraint)

The current study aimed to investigate the role NF κ B in mediating the HPA response to stress. Emerging evidence suggests that in addition to contributing to the responses to immunological stress [reviewed in (Liou 2002)], NF κ B also has a role in effecting the responses to psychological stress (Bierhaus *et al.* 2003; Garcia-Bueno *et al.* 2005). Therefore, we examined the effects of psychological stress upon the HPA axis (plasma ACTH and corticosterone concentrations) and the immune system (plasma IL-6 and TNF α concentrations and NF κ B activity in anterior pituitary, thymic and adrenal tissues) and explored how these effects were modified by NF κ B blockade. Restraint stress is widely accepted as a model of psychological stress (Pare & Glavin 1986; Glavin *et al.* 1994). The procedure involves confining the rat in a cylindrical plastic tube for periods ranging from 5 minutes to several hours. The restraint tubes used in the present study were sized according to the weight of the rats to ensure that the animals were unable to move once placed inside the tube. Other models used for the study of psychological stress include immobilisation stress (which involves restraining the rat on a board in a supine position by taping the limbs to holders without anaesthesia), water immersion, conditioned fear responses, whole-body vibration, loud noise, foot-shock and forced swimming. Unlike restraint, these are considered intense forms of psychological stress (Pare & Glavin 1986; Glavin *et al.* 1994). Restraint stress was used in the current study because, in addition to this technique being less severe than the immobilisation method, its effects upon the HPA axis are well documented (Seltzer *et al.* 1986; Tan & Nagata 2002). Furthermore, restraint stress also increases plasma IL-6 concentrations in the rat (Zhou *et al.* 1993; Nukina *et al.* 1998) and mouse (Nukina *et al.* 2001; Huang *et al.* 2003). The source IL-6 in the plasma of restraint stressed rats is unclear and does not appear to be attributable to secretion by splenic cells or PBMCs (Zhou *et al.* 1993). As explanted adrenal cells release IL-6 *in vitro* and ACTH can stimulate IL-6 release from cultured adrenal zonal glomerulosa cells (Judd & Macleod 1992; Judd & Macleod 1995), it is possible that the adrenal glands provide an important source of IL-6 during restraint stress.

Increased NF κ B activity has been reported in rat brain cortex tissue following restraint stress, possibly as a consequence of activation of glutamate receptors following the release of excitatory amino acids during stress (Guerrini *et al.* 1995; Kaltschmidt *et al.* 1995; Madrigal *et al.* 2003). These findings led us to hypothesise that NF κ B is an important effector of the HPA response to restraint stress. To explore

this hypothesis, we examined the effects of NF κ B blockade on the responses to restraint stress. Initial studies were designed to optimise the period of restraint stress required to increase plasma ACTH and corticosterone concentrations. In light of reports that restraint stress increases IL-6 release (Zhou *et al.* 1993; Nukina *et al.* 1998), plasma IL-6 concentrations were also determined. As detailed in chapters 3 and 4, LPS-induced NF κ B signalling plays a role in regulating corticotroph-derived ACTH release. To our knowledge, the effects of psychological stress upon NF κ B activity in the anterior pituitary gland have not been investigated and thus, the present study examined the effects of restraint stress upon anterior pituitary NF κ B activity. For comparison, NF κ B activity was also examined in the thymus and adrenal glands.

5.5.2 Experimental methods

5.5.2.1 Detection of plasma ACTH by IRMA

The concentration of ACTH in the plasma collected from the trunk blood of the rats was determined in triplicate by an immunoradiometric assay (IRMA; section 2.5.12). ACTH is a single chain polypeptide consisting of 39 amino acids. The first 24 N-terminal amino acids are essential for its biological activity and are identical in many mammalian species (White & Gibson 1998). The IRMA used in the *in vivo* studies is designed for quantitative determination of intact ACTH (ACTH₁₋₃₉) in plasma and uses two polyclonal antibodies recognising different binding sites on the antigen. The first antibody is a highly purified monospecific radioiodinated sheep IgG and recognises the N-terminus of the ACTH molecule and the second antibody reacts non-competitively with the C-terminal region of the ACTH molecule and is immobilised and coupled to primary sheep anti-rabbit coated tubes. During the incubation step, both antibodies react with the molecules of the sample and a sandwich-type complex is formed.

The IRMA assay is specific for the ACTH₁₋₃₉ peptide and does not detect ACTH₁₋₂₄, ACTH₁₈₋₃₉, ACTH₂₂₋₃₉, ACTH₃₄₋₃₉, CLIP, α -MSH, β -MSH and β -endorphin. The quantification of ACTH by IRMA is advantageous over more conventional RIAs as the use of monoclonal antibodies increases the sensitivity of the assay (3pg/ml) and avoids cross-reaction with metabolites and related peptides. However, because most anti-ACTH antibodies currently available have slow association kinetics and poor affinity constants, this level of sensitivity is often achieved after an incubation of 20 hours. Despite this, the incubation period of the samples in the IRMA assay is faster

than that required for RIAs (four days) (Kertesz *et al.* 1998). The IRMA assay also has increased reproducibility compared to RIAs and the number of samples analysed per assay are not limited, thus allowing a high sample output.

5.5.2.2 Detection of plasma corticosterone by RIA

The concentration of corticosterone (4-pregnen-11, 21-diol-3, 20-dione) in the plasma collected from the trunk blood of the rats was determined by RIA (section 2.5.13). As described previously (section 3.6.1.2.4), RIAs are routinely used for measuring plasma concentrations of hormones, including corticosterone, due to their high sensitivity and specificity and the corticosterone assay used in the current study had a detection limit of 0.39ng/ml. The assay employed is based on the ability of a limited quantity of antibody to bind to a fixed amount of radiolabelled antigen. The percentage of bound radiolabelled antigen decreases as a function of increasing concentration of unlabelled antigen in the test sample. The relative percent of cross-reactivities of the assay are outlined in table 5.4. The RIA employed is designed to measure corticosterone concentrations in plasma samples (plasma is the virtually cell-free supernatant of blood containing anticoagulant obtained after centrifugation) unlike most other assays which require serum (serum is the undiluted, extracellular portion of blood after adequate coagulation is complete). Several stages are required for the collection of serum samples, which include incubating whole blood samples for up to 30 minutes to allow for coagulation. In contrast, plasma samples are collected in the presence of an anti-coagulant, such as EDTA, and can be separated immediately from whole blood by centrifugation. This process is more efficient in comparison to serum collection and also results in larger volumes of plasma, compared to serum, collected from the same volume of blood. Additionally, the use of plasma samples omits coagulation-induced changes which can arise from serum samples. These include changes in the concentration of numerous constituents of the extra-cellular fluid which can be induced by the following mechanisms: (a) increase in the concentrations of platelet components in serum as compared to plasma (e.g. potassium, phosphate, magnesium, aspartate aminotransferase, lactate dehydrogenase, serotonin, neurone-specific enolase, zinc), (b) decrease in the concentration of constituents in serum as a result of cellular metabolism and the coagulation process (e.g. glucose, total protein, platelets) and (c) activation of the cell lysis of erythrocytes and leukocytes in non-coagulated blood (e.g. cell-free haemoglobin, cytokines, receptors) (Uges 1988). Although the disadvantages of using plasma samples in some assay systems include the unknown influence of the

anticoagulant on the assay system, on the protein binding and on the stability of the sample, the RIA employed in the current study was designed specifically for plasma samples. Furthermore, the current study also measured ACTH concentrations in the plasma (section 5.5.2.1) and therefore, the samples collected could be used for both assays.

5.5.2.3 Detection of plasma IL-6 and TNF α by ELISA

Plasma concentrations of IL-6 and TNF α are routinely measured by ELISA. The ELISAs used in the present study utilised primary antibodies specific for rat IL-6 and TNF α respectively. The rat anti-IL-6 antibody showed no cross-reactivity with human or porcine recombinant IL-6 or with recombinant ciliary neutrophilic factor (CNTF), but weak (0.7%) cross reactivity with mouse recombinant IL-6. The anti-TNF α antibody showed no cross-reactivity with either human recombinant TNF α or TNF β or porcine recombinant TNF α , but moderate (2.4%) cross reactivity with mouse recombinant TNF α . The measurement of IL-6 and TNF α by ELISA is quantitative and thus has advantages over qualitative methods such as western blot analysis. It also allows a high number of samples to be analysed (approximately 40 per plate) in parallel so that appropriate duplicates can be included, thus allowing the results to be analysed statistically. The ELISAs used in the current study were also highly sensitive, with a detection limit of 31.25pg/ml. Each assay included positive and negative controls to ensure the assays were working correctly.

Table 5.4 The relative percentage of cross-reactivity of the anti-corticosterone antibody with other hormones. The relative percentage of cross-reactivity by weight of highly purified corticosterone and various other hormones was determined from the amount of purified corticosterone required to reduce the binding of ^{125}I corticosterone by 50%, relative to the amount of steroid being tested that is required to do the same. Specificity of the assay was performed by IDS Ltd, UK.

Analyte	% Cross reactivity
Aldosterone	0.20
Cortisol	0.40
Cortisone	<0.10
Deoxycorticosterone	3.30
Deoxycortisol	<0.10
Dexamethasone	0.05
DHEA	0.03
Oestradiol	0.01
Oestriol	0.01
Oestrone	0.03
17 α -hydroxypregnenolone	0.01
17 α -hydroxyprogesterone	0.20
20 α -hydroxyprogesterone	0.20
20 β -hydroxyprogesterone	0.20
Prednisone	0.04
Prednisolone	0.20
Pregnanediol	<0.01
5 α -pregnanedione	0.40
5 β -pregnanedione	1.00
Pregnanetriol	0.01
Pregnenolone	0.04
Progesterone	0.60
Testosterone	0.10
Tetrahydrocortisol	0.09
Tetrahydrocortisone	0.02

5.5.2.4 Detection of NFκB DNA-binding activity in animal tissue

5.5.2.4.1 EMSA analysis

The DNA-binding activity of NFκB in nuclear extracts (5μg) of anterior pituitary, thymic and adrenal tissue extracted from the rats was examined by EMSA. As described previously (see section 3.6.1.2.2.2), EMSA is an ideal method for detecting NFκB DNA-binding activity because, unlike western blot analysis, the EMSA method discriminates between non-transcriptional and transcriptionally active nuclear NFκB. Several bands may be detected by this method due to the existence of several hetero- or homo-dimers of NFκB, although the two predominant ones are the p65/p50 heterodimer and the p50/p50 homodimer. To ensure that the detected bands were not due to non-specific binding reactions, competitive (excess unlabelled NFκB oligonucleotide was added to the samples in the presence of the labelled NFκB sequence) and non-competitive (excess unlabelled AP-1 oligonucleotide was added to the samples in the presence of the labelled NFκB sequence) reactions were included in the assay. Bands that represent NFκB binding would be detected in the non-specific reaction, but not the specific reaction. The limitations of the EMSA include the length of time required for the assay to be completed and the requisite for radioactive ³²P. Additionally, this method does not allow direct comparisons of the DNA-binding complexes between separate gels and quantifying protein:DNA binding activity on each gel is difficult due to the limited number of samples that can be used for each gel (maximum of 12) so that duplicate samples could not always be assayed together. However, most assays were conducted with at least 2 samples per group assayed together and the mean optical density of the bands per treatment group were expressed as a percentage of control to show changes in NFκB DNA-binding activity more clearly (non-quantitative). Where four or five samples per treatment group were loaded on the same gel, non-parametric statistical analysis was performed (semi-quantitative; see section 2.5.13).

It has been noted that in some cases the variability between samples within a treatment group was high. This could be attributed to several factors, for example the tissues used in the current study (anterior pituitary gland, thymus and adrenal glands) are highly vascularised and it is possible that blood-borne cells which express NFκB activity may have contributed to the overall activity detected by EMSA. Although this could be avoided by using perfused tissue, the aim of the current study was to investigate the HPA response to stress (immunological and psychological) and the

consequences of inhibiting NF κ B upon these parameters. Furthermore, the *in vitro* findings described earlier (chapters 3 and 4) support the premise that NF κ B signalling in folliculostellate cells and corticotrophs of the anterior pituitary gland is important in mediating the ACTH response to LPS. Because these tissues are vascularised, it is possible that NF κ B activity in cells from the blood may in turn influence the secretion of factors from these glands. As such, removal of the blood from the tissues may result in reduced NF κ B activity detected by EMSA and thus misleading interpretations into the importance of NF κ B signalling in the stress response.

A more likely explanation for the variability in the NF κ B DNA-binding activity detected in the tissues is variations in the nuclear protein concentrations used in the assay, since the method for measuring the nuclear protein concentrations (Bradford assay; section 2.5.4.1) is an estimation. Unlike western blot analysis, whereby detection of a house-keeping gene can be used to ensure consistent protein concentrations between the samples, this can not be applied to the EMSA. Therefore, as described above, where possible, three or more samples from each group were assayed on the same gel to reduce the risk of large variations in protein concentrations causing ambiguous results. However, the main drawback to this method is the limited power of quantification as direct comparisons between the absolute values obtained for each sample by densitometric analysis can only be made on the same gel. Together, these features inherent to EMSA present problems for statistical analysis. Therefore non-parametric tests were adopted where four or more samples within each group were used on the same gel. For a comparison of two groups (i.e. control vs restraint stress), the Mann-Whitney rank sum test was employed and for comparisons of three groups (control vs vehicle vs LPS), the Kruskal-Wallis one-way ANOVA on ranks followed by the Student-Newman-Keuls *post hoc* method was used.

5.5.2.4.2 Supershift assay

Whilst there are characteristic shifts caused by binding of specific protein(s) to the target DNA, the change in relative mobility detected by EMSA does not identify the bound protein. Identification of bound protein is accomplished by including an antibody specific for the DNA-binding protein (e.g. p65) to the reaction. Binding of the antibody to the complex results in a further shift in the band, although in some cases the antibody may disrupt the protein:DNA interaction resulting in the loss of the characteristic shift, but no super-shift. A weak shift or a reduction in the intensity of

the band usually implies the presence of low levels of the protein in the complex. The p65 protein is the transcriptionally active subunit of the NF κ B dimer and the p65/p50 heterodimer is the most extensively studied form. Therefore, the bands representing the p65/p50 heterodimer in nuclear extracts of anterior pituitary and adrenal tissues were identified by supershift assays (section 2.5.6) and used as a reference for further assays.

5.5.3 Compounds for studying NF κ B signalling

5.5.3.1 PHA781535E

The IKK β inhibitor, PHA781535E (2-amino-[6-[2-hydroxy-6-(2-methylpropoxy)phenyl]-4-(3-piperidinyl)]-3-pyridinecarbonitrile), was employed in the study to investigate the importance of NF κ B signalling in mediating the rat HPA responses to immunological (LPS) and psychological (restraint) stress. Studies using non-specific NF κ B inhibitors, such as inhibitors of the p38 α MAPKinase pathway (e.g. SB203580) (Dong *et al.* 2006) or glucocorticoids (e.g. dexamethasone) (Unlap & Jope 1997) cannot attribute any cellular effects solely to NF κ B as such compounds would also interfere with other signalling pathways that may not involve NF κ B. For example, the p38 α MAPKinase pathway regulates the transcriptional activity of other transcription factors involved in IL-6 synthesis such as AP-1 (Guha & Mackman 2001; Katsoulidis *et al.* 2005).

Other methods for inhibiting NF κ B include (a) targeting NF κ B protein expression by siRNA (Guo *et al.* 2004; Kalota *et al.* 2004), (b) interference with NF κ B nuclear translocation and DNA-binding using NF κ B decoys or proteasome inhibitors [reviewed in (Montagut *et al.* 2006)], (c) interference with IKK complex formation using specific IKK inhibitors (Hideshima *et al.* 2006) or vectors encoding a superrepressor form of I κ B α (Batra *et al.* 1999; Widera *et al.* 2006; Tamura *et al.* 2007), (d) blockade of upstream activators and (e) inhibition of NF κ B transcriptional activity (p65 kinases) [reviewed in (Burke 2003)]. Inhibition of NF κ B by siRNA has been demonstrated in tumour cells *in vivo* (Guo *et al.* 2004). However, despite these promising findings, gene-specific knockdown at the RNA level is a relatively new technology and requires significant refinement. The disadvantages to this technique include difficulties in the efficiency of delivery, duration of action, improved specificity, and establishment of safety (Kalota *et al.* 2004). Although interference with the degradation of I κ B through the use of proteasome inhibitors, such as Mg132, is an

effective way to inhibit NF κ B [reviewed in (Montagut *et al.* 2006)], the proteosome is involved in the degradation of all poly-ubiquitinated proteins. As such, proteosome inhibition impacts many signalling pathways and thus, the specificity of the response is unclear. Furthermore, there are reports that Mg132 can also activate NF κ B by inducing the heat shock response, suggesting that such compounds may not be reliable NF κ B inhibitors (Jobin *et al.* 1998; Lee *et al.* 2006).

Inhibitors of the NF κ B pathway are being developed by the pharmaceutical industry for potential therapeutic use and much effort is focused on the discovery of selective small molecule inhibitors of IKK β [reviewed in (Karin *et al.* 2004)], such as SC-514 (Kishore *et al.* 2003). However, SC-514, which was used for the *in vitro* studies (see chapters 3 and 4), is not effective *in vivo* due to its limited bioavailability (2%) and poor half-life (0.2 hours) (Kishore *et al.* 2003). PHA781535E, which was generously provided by Pfizer Ltd, is a small molecule which effectively inhibits NF κ B *in vivo* (Murata *et al.* 2004) (see figure 5.29). This compound is more applicable to studying NF κ B signalling as it specifically inhibits activation of the IKK β subunit which is responsible for activating NF κ B (p65/p50) and thus, does not interfere with upstream signalling pathways (see table 5.5) (Murata *et al.* 2004).

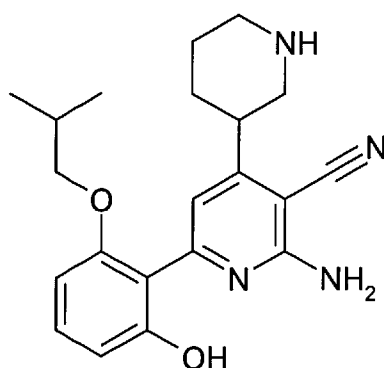


Figure 5.29 Structure of PHA781535E (2-amino-[6-[2-hydroxy-6-(2-methyl propoxy)phenyl]-4-(3-piperidinyl)]-3-pyridinecarbonitrile). [Taken from (Murata *et al.* 2004)].

Table 5.5 Pharmacokinetics of the specific IKK β inhibitor, PHA781535E.

The compound was generously provided by Pfizer Ltd (unpublished data). IL-1 β , interleukin 1 *beta*; IL-8, interleukin 8; IKK β , inhibitor of *kappa* B Kinase *beta*; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; TNF α , tumour necrosis factor *alpha*.

	IC ₅₀ (μ M)
IKK β (Resin Assay)	0.031
IL-1 β stimulated synovial fibroblasts (IL-8)	0.576
IKK β alamar blue (synovial fibroblasts)	>30
IKK β LPS treated PBMC TNF α release assay	0.021
IKK β alamar blue (PBMC)	>1.0
LPS stimulated human whole blood (TNF α)	1.5

Acute rat i.p. LPS model – 98.4% inhibition of TNF α at 100mg/kg
Acute rat i.p. LPS model – 95.3% inhibition of TNF α at 10mg/kg
Protein binding – 97%
T _{max} – 0.44 hours
C _{max} – 20.9 μ g/ μ l/kg
Oral bio-availability – 99.3%

5.5.4 Effects of LPS on the HPA axis

5.5.4.1 ACTH and corticosterone responses to LPS

The current study confirmed reports that LPS activates the HPA axis, inducing robust increases in the plasma concentrations of ACTH and corticosterone within 2 hours of treatment (Besedovsky *et al.* 1975; Lenczowski *et al.* 1997; Takemura *et al.* 1997; Conde *et al.* 1998; Whiteside *et al.* 1999; Dunn 2000). The finding that the ACTH concentration had declined, albeit not to basal, 4 hours after LPS injection but that the increase in plasma corticosterone concentration was sustained may reflect a reduction in the intensity of the stress, with corticosterone taking longer to return to basal due to the time required for corticosterone synthesis and the different half lives of the two hormones (Cook *et al.* 1972; Keller-Wood *et al.* 1983; Lu *et al.* 1983; Sainio *et al.* 1988). Alternatively, it may be explained by the negative feedback actions of corticosterone upon ACTH release being effective at this time. Previous studies have documented that corticosterone typically returns to baseline concentrations 8 hours after LPS challenge in rats (Givalois *et al.* 1994). However, as the aim of this study was to determine the optimal time point for HPA activation to

examine the effects of NF κ B inhibition on this system, time points exceeding 4 hours were not examined. As such, the length of time required for plasma corticosterone concentrations to return to basal was not determined in the current LPS model.

5.5.4.2 Role for NF κ B signalling in the HPA response to LPS

The plasma concentrations of the pro-inflammatory cytokines TNF α and IL-6, which act at all levels of the HPA axis including the anterior pituitary gland to stimulate ACTH release (Arzt *et al.* 1999; Bethin *et al.* 2000), were increased 2 hours after treatment with LPS. These findings agree with previous reports documenting the temporal profile of TNF α and IL-6 release in rats and other species, with an early phase (90 minutes after LPS treatment) characterised by increased plasma TNF α , rising plasma IL-6 (Givalois *et al.* 1994; Kakizaki *et al.* 1999) and peak ACTH secretion (Kakizaki *et al.* 1999), and the later phase characterised by maximal levels of IL-6 (Givalois *et al.* 1994; Kakizaki *et al.* 1999). IL-1 β is also released in response to LPS and reaches maximum plasma concentrations after TNF α , but before IL-6 (Givalois *et al.* 1994; Kakizaki *et al.* 1999). In the current model, the increase in the plasma concentration of ACTH in LPS treated rats is likely to be attributable, at least in part, to the direct and indirect stimulatory actions of systemic TNF α , IL-1 β and IL-6 on the release of CRH, and possibly also AVP, from the hypothalamus (Navarra *et al.* 1991; Spinedi *et al.* 1992; Beishuizen & Thijs 2003). The ME of the rat hypothalamus expresses the receptors involved in LPS signalling, namely CD14 and Tlr4 (Laflamme & Rivest 2001) and thus LPS itself may directly act at the hypothalamic level to augment the secretion of the releasing factors.

The anterior pituitary gland also expresses CD14 and Tlr4 (Gloddek *et al.* 2001; Breuel *et al.* 2004; Tichomirowa *et al.* 2005) and may therefore also be targeted directly by LPS as indicated by our *in vitro* studies (chapter 4) which show that pituitary cell lines respond to LPS with increases in NF κ B activity. In accordance with these findings, our *in vivo* study showed that the LPS-induced rise in plasma ACTH concentration is paralleled by an increase in NF κ B DNA-binding activity in the anterior pituitary gland. These data concur with previous reports of LPS-induced increases in NF κ B activity in the mouse (Parnet *et al.* 2003) and rat (Yang *et al.* 2002) pituitary gland. Together these findings provide evidence that, in addition to the stimulatory effects of circulating pro-inflammatory cytokines on the release of CRH and AVP *in vivo*, LPS-induced NF κ B activity within the anterior pituitary gland may be

involved in mediating the hypersecretion of ACTH in conditions of endotoxaemia. Therefore, to determine the importance of NF κ B signalling in the ACTH response to LPS treatment, we examined the effects of a novel NF κ B inhibitor, PHA781535E, on the HPA responses to the toxin (see section 5.5.3.1).

5.5.4.3 Evidence of PHA781535E as an effective NF κ B inhibitor

As we have had no previous experience of using this compound, we included measures to verify the effectiveness of PHA781535E as an NF κ B inhibitor. In accord with reports that LPS-induced IL-6 and TNF α release from immune cells such as macrophages is mediated by NF κ B (Libermann & Baltimore 1990; Laird *et al.* 2000; Corsini *et al.* 2006; Kim *et al.* 2006; Martin *et al.* 2006), the present data show that NF κ B blockade attenuates the LPS-induced increases in the plasma concentrations of both cytokines. Interestingly, the reduction in plasma IL-6 was less pronounced than that of TNF α and was evident only with the higher dose of PHA781535E tested, perhaps because transcription factors other than NF κ B which are involved in the regulation of IL-6 synthesis (e.g. AP-1, CREB, C/EBP β and NF-IL6) come into effect to compensate for the repression of NF κ B activity (Ray *et al.* 1988; Tanabe *et al.* 1988; Akira *et al.* 1990).

Since previous studies using molecular imaging techniques have demonstrated a robust increase in NF κ B activity in the thymus gland within 2 hours of LPS treatment (Carlsen *et al.* 2004), we also examined the effects of PHA781535E treatment upon NF κ B activity in this organ. NF κ B activity was readily detected in the thymus gland of untreated rats by the EMSA in our study. This was not surprising as NF κ B plays an important role in thymocyte development and activation, with impaired NF κ B activity resulting in a reduction in T cell numbers in peripheral tissue and increased apoptosis of thymocytes (Liu *et al.* 2006). We also observed an increase in NF κ B DNA-binding activity in thymic tissue 2 and 4 hours after LPS treatment compared to the untreated and saline treated controls. Importantly, the effect of LPS on thymic NF κ B activity was attenuated by PHA781535E. These findings, together with the evidence that the increases in plasma IL-6 and TNF α concentrations induced by LPS were decreased by PHA781535E treatment, support the premise that this compound is an effective NF κ B inhibitor *in vivo* (Murata *et al.* 2004).

5.5.4.4 Potential NF κ B-dependent mechanism mediating LPS-stimulated ACTH secretion from the anterior pituitary gland

In accordance with the premise that NF κ B drives the ACTH response to LPS, treatment with the NF κ B inhibitor attenuated LPS-stimulated ACTH release. The inhibitor did not however completely abolish the adrenocorticotrophic response to the toxin, even at the higher dose tested, raising the possibility that, in addition to NF κ B, other factors contribute to the increase in ACTH release.

The role of circulating cytokines in mediating the hypothalamo-anterior pituitary response to LPS is well documented (Martin *et al.* 2006) and one possible explanation for the inhibitory effects of PHA781535E on LPS-induced ACTH secretion would be blockade of the release of TNF α , IL-6 and possibly IL-1 β into the systemic circulation. Surprisingly however, PHA781535E had only a very small effect on the plasma IL-6 concentration in comparison to its effects on ACTH, suggesting that reduced availability of circulating cytokines does not account entirely for the inhibition of ACTH secretion. A further possibility is that PHA781535E acts within the brain to attenuate the stimulatory actions of NF κ B-dependent cytokines on the release of CRH and AVP, possibly by suppressing the induction of COX-2 or NOS-2, and hence, the generation of prostaglandins and NO in the hypothalamus (Gadek-Michalska & Bugajski 2004; Lee *et al.* 1999; Uribe *et al.* 1999). In support of this, a growing body of data suggests that NF κ B signalling contributes to the hypothalamic response to stress. For example, NF κ B activity in the rat hypothalamus is increased by oxidative stress (Lezoualc'h *et al.* 2000) and alcohol vapour exposure (Lee & Rivier 2005), whilst inhibition of hypothalamic NF κ B attenuates alcohol-induced ACTH release (Lee & Rivier 2005). Whether PHA781535E crosses the BBB to reach potential targets such as the perikarya of the CRH/AVP cells is unknown, although it is possible that the inhibitor may attenuate CRH and AVP release by acting on structures of the hypothalamus that are exposed to the blood stream (e.g. the axon terminals of the PVN neurones that project into the ME) or cross the BBB at sites of increased permeability induced by LPS (Banks *et al.* 1994; Gutierrez *et al.* 1994; Boje 1995; De Vries *et al.* 1996; Banks *et al.* 2001).

As the reduction in ACTH release effected by NF κ B blockade was also paralleled with a decrease in NF κ B activity in the anterior pituitary gland, it seems likely that intra-pituitary NF κ B signalling is involved in mediating the ACTH response to

endotoxin *in vivo*. Furthermore, the data correlate with the findings obtained from the co-culture studies (chapter 4) which suggest an important role for NFκB in mediating the ACTH response to LPS. Therefore, it is likely that the inhibition of LPS-induced ACTH release in PHA781535E treated rats is attributable to the combined effects of blocking intra-pituitary NFκB signalling and the inhibition of the release of immune derived cytokines, including TNFα and IL-6, into the systemic circulation.

Although the current findings support the hypothesis that the stimulatory effect of LPS upon ACTH secretion *in vivo* is mediated, at least in part, by its direct actions on the anterior pituitary gland, the mechanisms governing this response are unclear. The previous chapters provided evidence for both direct inhibitory (in corticotrophs) and indirect stimulatory (in folliculostellate cells) effects of NFκB-signalling in the regulation of ACTH release *in vitro* (chapters 3 and 4). Therefore, as folliculostellate cells and corticotrophs predominantly reside within the anterior portion of the pituitary gland (Rennels & Herbert 1979; Matsumoto *et al.* 1993), the current study examined NFκB DNA-binding activity in this region. As the measurements were made in tissue extracts, we were not able to distinguish differences between the anterior pituitary cell-types in the activation of NFκB. However, it is possible that, in accordance with the *in vitro* findings, the effects of NFκB upon ACTH release are cell-specific *in vivo*. Hence, activation of NFκB in corticotrophs by CRH may exert an inhibitory effect upon ACTH release, whilst its activation in folliculostellate cells by LPS may increase ACTH release via a paracrine mechanism involving the release of IL-6 and possibly other cytokines. If, as published evidence suggests, CRH is released much more rapidly than IL-6 in response to LPS (Naitoh *et al.* 1988; Kakizaki *et al.* 1999), the effects of LPS at the pituitary level on ACTH release would be phasic with an initial inhibition and subsequent rise in secretion. Such a mechanism would temper glucocorticoid release in the early phase of infection, thus allowing an effective pro-inflammatory response to be mounted, but subsequently induce a rise in glucocorticoids to contain the inflammatory response and thus restore homeostasis (Munck & Naray-Fejes-Toth 1994). The notion that glucocorticoid release is initially inhibited in response to immunological stress, to allow an inflammatory response to be mounted to fight the infection, is further supported by a recent study from our laboratory on folliculostellate cells, which demonstrated a rapid and transient increase in the release from folliculostellate cells of ANXA1 (Solito *et al.* 2006), a protein which is known to be a powerful inhibitor of ACTH release (Taylor *et al.* 1993). To explore further the possibility of cell-specific NFκB activation *in vivo*,

nuclear translocation of NF κ B (p65 subunit) in the folliculostellate cells and corticotrophs could be identified at different time points after the administration of LPS, using immunohistochemical techniques [i.e. co-staining anterior pituitary tissue from LPS-treated rats with antibodies specific for NF κ B (p65) and specific markers for folliculostellate cells (S100) and corticotrophs (ACTH or POMC)] and the functional effects of NF κ B signalling in each cell type could be identified using a transgenic approach (e.g. by targeted deletion of the NF κ B or I κ B α genes or by generation of mice which overexpress NF κ B or I κ B α in a cell-specific manner).

5.5.4.5 Role of NF κ B in mediating the corticosterone response to LPS

As ACTH is the primary stimulus for corticosterone release and the increase in the plasma corticosterone concentration induced by LPS treatment is associated with a marked increase in ACTH release, it would appear that the adrenocortical response to LPS is secondary to the rise in ACTH. However, there is evidence that LPS acts directly at the level of the adrenal glands, which express the LPS signalling receptor Tlr4 (Vakharia *et al.* 2002; Bornstein *et al.* 2004a; Zacharowski *et al.* 2006), to stimulate cortisol release (Vakharia *et al.* 2002) and, as our data demonstrate, the LPS-induced increase in corticosterone release was also associated with a rise in adrenal NF κ B activity. Together, these data raise the possibility that LPS acts at the adrenal level to modulate corticosterone release via an NF κ B-dependent mechanism. Our studies in animals treated with PHA781535E do not however support this premise and are difficult to interpret for several reasons. Firstly, at both doses tested, PHA781535E itself increased NF κ B activity in the adrenal gland and failed to modify the response to LPS; it thus failed to act as a NF κ B inhibitor in this tissue. Secondly, while at the lower dose tested, the inhibitor effectively attenuated the ACTH response to LPS, it failed to modify the LPS-induced rise the corticosterone. Although it can be argued that, despite the inhibitory effects of PHA781535E on the LPS-induced release of ACTH, the plasma ACTH levels attained may still be sufficient to cause maximal steroidogenesis, an alternative explanation is that PHA781535E activates NF κ B at the adrenal level and thereby augments corticosterone secretion. Such an argument is supported by our observation that the higher dose of PHA781535E augmented the basal plasma corticosterone concentration. Furthermore, although this dose reduced the LPS-induced increment in plasma corticosterone, the absolute levels of corticosterone attained after LPS treatment were unaffected by the higher dose of PHA781535E.

As PHA781535E treatment did not affect the basal plasma concentrations of IL-6, TNF α and ACTH or basal pituitary NF κ B activity, the unusual effects of the drug do not appear to be systemic, but rather due to direct actions on the adrenal glands. However, the underlying mechanism(s) by which PHA781535E increases adrenal NF κ B activity and corticosterone release is unclear. Although PHA781535E was shown to inhibit NF κ B-dependent signalling pathways in the current study (see section 5.5.4.3), there is limited information available on the effects of this compound upon other signalling pathways. Studies by Pfizer Ltd have demonstrated lethality in rats treated with doses above 50mg/kg (unpublished observations, Pfizer Ltd). Therefore it is possible that, whilst the highest dose tested in the present study was not lethal, this dose may activate NF κ B in the adrenal glands through the local production of inducers of NF κ B, such as NO, heat shock proteins (Schreck *et al.* 1992) or the pro-inflammatory cytokines TNF α , IL-1 and IL-6, thus resulting in the subsequent release of corticosterone (Judd & Macleod 1995; Swain & Maric 1996; Judd 1998; Wang *et al.* 2003).

5.5.4.6 Possible NF κ B-dependent mechanisms involved in mediating corticosterone synthesis and release

The current findings suggest that adrenal NF κ B is involved in the control of corticosterone secretion. Previous reports have provided indirect evidence of a potential role for NF κ B in the pathways leading to corticosterone biosynthesis, including the detection of increased mRNA expression of the NF κ B-dependent enzyme COX-2 in the adrenal glands of LPS-treated rats (Cover *et al.* 2001). COX-2 is an important regulator of prostaglandin synthesis [reviewed in (Simmons *et al.* 2004)] including the prostaglandin, PGE₂, which is released from the adrenal glands in response to LPS (Vakharia & Hinson 2005). Interestingly, in conditions of endotoxaemia, PGE₂ release precedes the increase in cortisol secretion, with COX-2 inhibition attenuating the concentrations of both PGE₂ and cortisol. These findings suggest that the formation of prostaglandins is an important step in the adrenal response to LPS (Vakharia & Hinson 2005) (figure 5.30).

In addition to the stimulatory effects of IL-6 upon glucocorticoid release from the adrenal glands (Path *et al.* 1997), which express the IL-6 signalling receptor, IL-6R α (Zubelewicz *et al.* 1999; Willenberg *et al.* 2002), the adrenal glands also provide a source of IL-6 (Judd & Macleod 1992; Judd & Macleod 1995; Judd 1998). Therefore increased adrenal NF κ B activity induced by PHA781535E treatment may result in the

local synthesis and release of IL-6, which in turn acts in an autocrine manner to further stimulate corticosterone release (Path *et al.* 1997) (see figure 5.30).

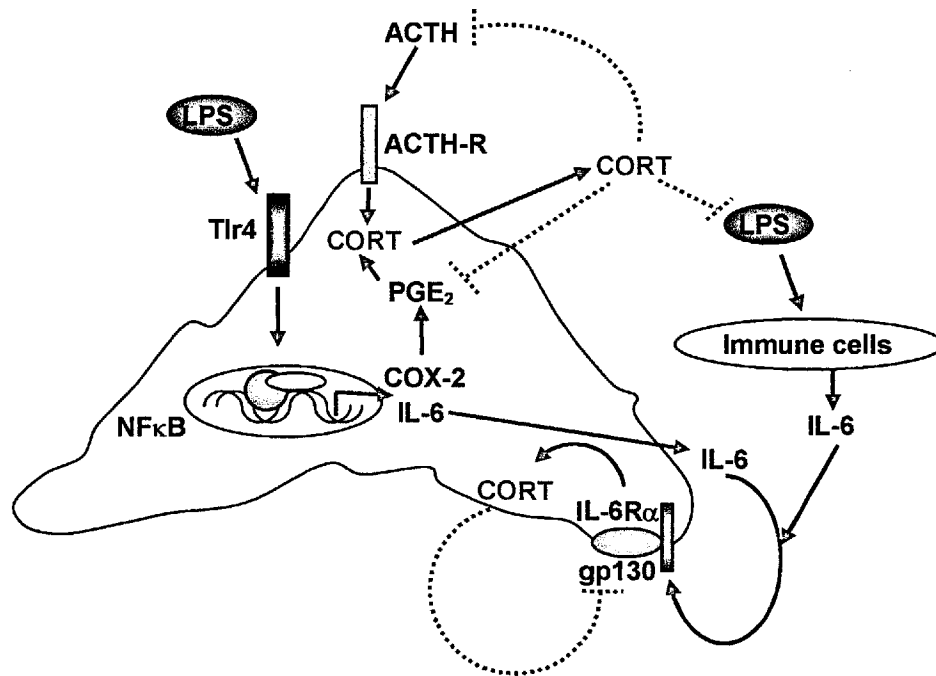


Figure 5.30 Hypothetical representation of the stimulatory effects of LPS upon corticosterone synthesis/release from the adrenal gland. LPS-induced NFκB-mediated COX-2 expression in the adrenal gland increases prostaglandin synthesis (e.g. PGE₂), which in turn stimulates corticosterone release. LPS-induced NFκB activity increases adrenal-derived IL-6 release which acts in an autocrine manner to stimulate corticosterone release, in addition to the effects of IL-6 produced by immune cells (e.g. macrophages). Red arrows depict activation pathways and dashed blue lines represent inhibitory pathways. ACTH, adrenocorticotrophin; ACTH-R, adrenocorticotrophin receptor; CORT, corticosterone; COX-2, cyclooxygenase 2; IL-6, interleukin 6; IL-6Rα, interleukin 6 receptor *alpha*; LPS, lipopolysaccharide; NFκB, nuclear factor *kappa* B; PGE₂, prostaglandin E₂; Tlr4, toll-like receptor 4.

5.5.5 Role of NF κ B in the HPA response to psychological stress

In addition to the systemic effects of NF κ B signalling (i.e. increased production of circulating pro-inflammatory cytokines), the current study has identified an important role for NF κ B signalling within the anterior pituitary, and possibly the adrenal glands, in mediating the HPA response to immunological stress. However, whilst NF κ B activation is normally associated with immunological stress, recent studies show that NF κ B activity in the rat cerebral cortex (Garcia-Bueno *et al.* 2005) and human PBMCs (Bierhaus *et al.* 2003) is increased by psychological stress. Therefore, we also examined the effects of NF κ B blockade upon the rat HPA responses to restraint stress, using PHA781535E.

5.5.5.1 ACTH and corticosterone responses to restraint stress

In accordance with previous reports (Seltzer *et al.* 1986; Ginsberg *et al.* 2003; Bomholt *et al.* 2005; Hesketh *et al.* 2005) and similarly to the effects of LPS, restraint stress induced robust increases in the plasma concentrations of ACTH and corticosterone. Whereas the increase in ACTH was transient (maximal after 15 minutes stress), the rise in corticosterone was maintained throughout the period of the restraint stress (15 to 30 minutes) and declined when the animal was returned to its home cage. The decline in the plasma ACTH concentration at 30 minutes restraint may reflect a reduction in the intensity of the stress to the rat and thus, reduced sensory input to the hypothalamus. As corticosterone has a longer half-life than ACTH (Cook *et al.* 1972; Keller-Wood *et al.* 1983; Lu *et al.* 1983; Sainio *et al.* 1988), it is not unusual that the corticosterone concentration remained elevated when the ACTH concentration had declined. Alternatively, the temporal profile of ACTH and corticosterone release may be attributable to the effective negative feedback actions of corticosterone at this time.

Elevated cytokine concentrations have been reported in animals subjected to severe forms of psychological stress (see section 1.6.3), such as prolonged (3-4 hours) immobilisation (Zhou *et al.* 1993; Nukina *et al.* 1998; Nukina *et al.* 2001; Merlot *et al.* 2002). However, in contrast to these findings and to our studies in which LPS was used as a stress, the plasma concentrations of IL-6 and TNF α remained undetectable in rats subjected to restraint stress. As the short period of restraint (30 minutes) used in the current study is a less intense form of psychological stress than 3-4 hours immobilisation, this suggests that the activation of the immune system by

psychological stress is dependent on the severity and duration of the stressor (Li *et al.* 2000). Furthermore, IL-6 released in response to chronic stress may be an important factor in sustaining corticosterone release as administration of recombinant IL-6 prolongs the elevation in plasma corticosterone induced by restraint (Zhou *et al.* 1996).

5.5.5.2 Role of anterior pituitary NF κ B signalling in mediating the ACTH response to restraint stress

Activation of the HPA axis during psychological stress involves input from several CNS regions, including the hippocampus, the medial prefrontal cortex and the amygdala which converge on the hypothalamus to regulate CRH and AVP release (see section 1.2.5). In the present study we did not explore the potential role for NF κ B signalling in the brain in mediating the release of these neuropeptides. Hence, we cannot exclude the possibility that the inhibitory effects of the NF κ B-inhibitor, PHA781535E, on ACTH release reported here are exerted, at least in part, in the brain. Indeed, evidence that psychological stress increases NF κ B activity (Garcia-Bueno *et al.* 2005) and the expression of the NF κ B-dependent proteins, NOS-2 and TNF α , in the cerebral cortex of adult male rats (Madrigal *et al.* 2002) would favour such an action. However, the present data suggest that NF κ B signalling in the anterior pituitary gland is also important in this regard. Certainly, NF κ B activity in the anterior pituitary gland was increased by restraint stress, and remained so 30 minutes after the removal of the stressor. Furthermore, the stress-induced increments in anterior pituitary NF κ B activity and plasma ACTH concentration were effectively inhibited by treatment with the NF κ B inhibitor. Although psychological stress increases NF κ B activity in the rat cerebral cortex (Garcia-Bueno *et al.* 2005), to our knowledge this is the first time that activation of this transcription factor has been detected in the anterior pituitary gland of rats subjected to a psychological stress or implicated in mediating the associated increase in ACTH release.

The mechanisms through which psychological stress induces NF κ B activity are unclear, since this transcription factor is normally activated by immunological stimulants. Furthermore, as the plasma concentrations of IL-6 and TNF α remained undetectable after restraint, the activation of anterior pituitary NF κ B could not be attributed to the stimulatory effects of these circulating cytokines. Previous studies have shown that CRH activates NF κ B in macrophages (Agelaki *et al.* 2002), as also

did our *in vitro* studies on AtT20 D16:16 cells (chapter 3). However, CRH-induced NF κ B activation would be unlikely to increase ACTH release because, as our *in vitro* data demonstrate, CRH-driven NF κ B activation in corticotrophs inhibits ACTH release. A more likely explanation is that psychological stress activates NF κ B in the folliculostellate cells, promoting the release of factors such as IL-6 which augment ACTH release via a paracrine mechanism. How this is triggered requires further investigation.

5.5.5.3 Role of adrenal NF κ B signalling in mediating the corticosterone response to restraint stress

Although restraint stress did not affect NF κ B activity in the adrenal glands, treatment with PHA781535E significantly attenuated the stress-induced increase in corticosterone release. From these findings, it appears that the effects of the drug upon corticosterone release are likely to be due to attenuated ACTH release. However, as in our previous study in which LPS was used as a stressor, the higher dose of PHA781535E caused a significant increase in the basal corticosterone concentration without affecting ACTH release. Together these findings suggest that PHA781535E may also exert a direct action at the adrenal level, although the underlying mechanism is unknown. In contrast to our study with LPS which demonstrated a positive effect of PHA781535E on NF κ B activity in the adrenal gland, in our study on restraint stress, PHA781535E appeared to depress basal adrenal NF κ B activity. This discrepancy may reflect small differences in the experimental conditions and/or the small sample size ($n=3-4$). However, our studies on the thymus also suggest that the actions of the higher dose of PHA781535E may be non-specific. Thus, while restraint stress increased NF κ B activity in the thymus gland and this activity was attenuated by the lower dose of PHA781535E (25mg/kg), the higher dose of the inhibitor (50mg/kg) was ineffective in this regard. As described earlier, (section 5.5.4.5), high doses of PHA781535E may activate the heat shock response or ROS formation, both of which induce NF κ B activation (Tacchini *et al.* 1995; Kurata *et al.* 1996; Basu *et al.* 2000). Therefore, whilst the effects of PHA781535E at the lower dose tested may be attributable to blockade of NF κ B activation, interpretation of the responses to the higher dose may be confounded by non-specific actions, which include increased NF κ B activity.

5.6 Conclusion

The purpose of this study was to examine the role of NF κ B in mediating the HPA responses to two different types of stressors – immunological and psychological. The data show that inhibition of NF κ B at the anterior pituitary level suppresses stress-induced ACTH release. In the case of psychological stress, the adrenocortical response is also inhibited. However, the adrenocortical response to LPS was retained in rats treated with PHA781535E and, at the higher dose tested, the inhibitor also increased the basal corticosterone concentration. Further studies are necessary to determine the potential relevance of these findings to the proposed clinical usage of NF κ B inhibitors as anti-inflammatory drugs. As discussed earlier, it seems likely that the elevation in basal corticosterone induced by the higher dose of PHA781535E reflects non-specific action. However, if it extends to drugs used in clinical practice, then patients may be at risk of developing an array of disorders associated with hypercortisolemia, including type II diabetes mellitus, hypertension, coronary heart disease, osteoporosis and depression. Conversely, the suppression of the HPA responses to acute psychological stress may precipitate a potentially fatal adrenal crisis.

5.7 Future studies

Although the current study has demonstrated that NF κ B plays an important role in mediating the HPA response to immunological and psychological stress, it is unclear whether adrenal NF κ B signalling is involved in these responses. To explore this possibility, studies on the effects of NF κ B blockade upon resting and ACTH-stimulated corticosterone release from primary adrenal tissue, in the presence and absence of LPS, are required. In the current study, changes in NF κ B activity in the adrenal and thymus tissue were difficult to detect by EMSA as the sample numbers were small, and thus statistical analysis was limited. Therefore, more quantitative methods of measuring NF κ B activity need to be employed, such as the p65 TransAMTM assay which also allows a high sample output, to confirm whether high doses of PHA781535E stimulate NF κ B activity. It is also possible that the non-specific effects of the high dose of PHA781535E used in the current study activate pathways involved in corticosterone biosynthesis and release (see section 5.1.4.8). To examine this possibility, further studies are required to investigate the effects of this compound upon mediators involved in steroidogenesis, which include NO, COX-2 and PGE₂.

The current findings support the premise that anterior pituitary-derived NF κ B is involved in mediating stress-induced ACTH release, and subsequent corticosterone release. However, the cell-types within the anterior pituitary that are involved in this regulation need to be identified to corroborate the *in vitro* findings of a dual-role for NF κ B signalling in the regulation of ACTH release. The overall decrease in ACTH release induced by the NF κ B inhibitor may also be attributable in part to effects of the compound at the hypothalamic level, possibly by impairing CRH and AVP secretion. As such, the effects of the NF κ B inhibitor at the hypothalamic level could be determined by measuring CRH and AVP mRNA expression (rat brains were also collected in the study and are currently stored, awaiting analysis). In addition, previous studies have demonstrated elevated pro-inflammatory cytokine release in response to severe and prolonged psychological stress, which may involve action of NF κ B (see section 1.4.1). Thus while the relative mild restraint stress employed in the present study did not increase plasma IL-6 or TNF α release, it did increase NF κ B activity in the anterior pituitary and thymus glands, suggesting the immune system is activated by psychological stressors and that more severe stressors may evoke systemic immunological responses. Therefore, there would be merit in examining the effects of NF κ B inhibition upon the HPA responses to more severe psychological stressors (e.g. immobilisation stress, foot shock, water immersion), to provide further insight to the role of NF κ B signalling in the activation of the immune system by psychological stressors.

6 General discussion

The HPA axis is activated by immunological stress (e.g. endotoxaemia), with consequent release of glucocorticoids from the adrenal cortex. Glucocorticoid synthesis and release are largely dependent on the stimulatory actions of ACTH, which is secreted from the anterior pituitary gland in response to the hypothalamic neuropeptides CRH and AVP. LPS, a component of gram negative bacteria, activates the HPA axis through the actions of circulating immune-derived mediators, which include the pro-inflammatory cytokines, $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 (Navarra *et al.* 1991; Besedovsky & Del Rey 1996; Vallieres & Rivest 1997; Beishuizen & Thijs 2003). However, recent studies suggest that LPS also acts directly on the anterior pituitary gland to stimulate the secretion of ACTH, and subsequent glucocorticoid release, independently of the actions of CRH (Elenkov *et al.* 1992a; Elenkov *et al.* 1992b; Yang *et al.* 2002). Although the mechanisms governing the effects of LPS upon ACTH release are unclear, a key role has been identified for the paracrine actions of IL-6 (Gloddek *et al.* 2001), which is synthesised and released from anterior pituitary-derived folliculostellate cells (Vankelecom *et al.* 1989; Vankelecom *et al.* 1993). The pro-inflammatory transcription factor $\text{NF}\kappa\text{B}$, which is activated by LPS, is an important regulator of IL-6 gene transcription in immune cells, such as macrophages, and its activity is inhibited by glucocorticoids (Ray & Prefontaine 1994; Auphan *et al.* 1995; Scheinman *et al.* 1995a; De Bosscher *et al.* 1997). As folliculostellate cells exhibit similar characteristics to macrophages, $\text{NF}\kappa\text{B}$ may also regulate the synthesis and release of IL-6 from these pituitary-derived cells. Therefore folliculostellate cell-derived $\text{NF}\kappa\text{B}$ signalling could be both potentially important in mediating the ACTH response to LPS and an additional target for the negative feedback actions of glucocorticoids. If this premise is true, the utilisation of specific $\text{NF}\kappa\text{B}$ inhibitors, which are currently being developed by the pharmaceutical industry to treat chronic inflammatory diseases such as rheumatoid arthritis, may induce adverse side effects associated with impaired HPA activity, by mimicking the negative feedback actions of glucocorticoids.

Therefore, the current study examined the importance of $\text{NF}\kappa\text{B}$ signalling in mediating the ACTH response to LPS *in vitro*, using transformed murine cell lines as models of folliculostellate cells (TtT/GF cells) and corticotrophs (AtT20 D16:16 cells). Both the TtT/GF and AtT20 D16:16 cells expressed the LPS signalling components CD14, Tlr4 and MD-2 and responded to LPS stimulation with increased $\text{NF}\kappa\text{B}$

activity. Within the TtT/GF cells, LPS-induced IL-6 release was dependent on NF κ B activation and this cytokine, at concentrations reflecting those released from the TtT/GF cells, potentiated the stimulatory effect of low concentrations of CRH upon ACTH release from the AtT20 D16:16 cells. Taken together, these findings support the premise that folliculostellate cell-derived IL-6 increases ACTH secretion when CRH concentrations are low, and possibly sustains ACTH release as CRH concentrations increase. Furthermore, NF κ B-dependent IL-6 release from the TtT/GF cells was inhibited by the synthetic glucocorticoid, dexamethasone, which concurs with the hypothesis that NF κ B activity in folliculostellate cells is a target for the negative feedback actions of glucocorticoids.

LPS and CRH stimulated NF κ B activity in the AtT20 D16:16, with inhibition of NF κ B paradoxically potentiating the stimulatory effects of low concentrations of CRH upon ACTH secretion. Although the *in vitro* studies indicate that the inhibitory effects of LPS upon ACTH release occur when CRH concentrations are low, CRH release is an immediate response, occurring within minutes following stressful exposure. Thus it appears unlikely that the inhibitory effects of LPS at the anterior pituitary level would be as rapid *in vivo*. However, the current *in vivo* studies demonstrated increased NF κ B activity in the anterior pituitary gland at 2 hours following LPS injection (although it is unknown whether activation may occur earlier since the earliest time point examined in the current study was 2 hours) and previous studies have demonstrated increased CRH mRNA expression at 6 hours following LPS injection (Grinevich *et al.* 2001). Thus, if the *in vitro* studies are translated to the *in vivo* model, it is possible that within the first few hours of endotoxaemia, LPS may exert a partial inhibitory effect upon CRH-induced ACTH release at the corticotroph level as part of a mechanism through which the hyper-secretion of ACTH is prevented, thus allowing a sufficient immune response to be mounted. However, as the response develops and the concentration of CRH increases, the inhibitory actions of LPS may no longer be effective, to allow restoration of homeostasis.

To investigate further a potential dual-role for intra-pituitary NF κ B signalling in mediating LPS and CRH induced ACTH release, co-cultures of TtT/GF and AtT20 D16:16 cells were used to mimic the interactions between folliculostellate cells and corticotrophs. In agreement with previous work (Gloddek *et al.* 2001) and the data from the current single cell culture studies (chapter 3), the co-culture studies demonstrated a potentiating effect for LPS-induced folliculostellate cell-derived IL-6

upon CRH-induced ACTH release. Furthermore, the study also provided novel evidence that CRH-induced mediator(s) released from corticotrophs act on folliculostellate cells to potentiate LPS-induced IL-6 release. Therefore, in addition to the direct actions of CRH on corticotrophs, the paracrine effects of corticotroph-derived mediator(s) may present an additional pathway through which CRH increases ACTH release in conditions of immunological stress. Although NFκB blockade enhanced the stimulatory effects of CRH upon ACTH release, the concentrations of IL-6 in the medium of cells treated with LPS, CRH and the NFκB inhibitor were higher than those induced by LPS alone. This implies that the synthesis of corticotroph-derived IL-6 secretagogues is mediated through NFκB-independent mechanisms. Together these findings have provided novel evidence that NFκB signalling plays an important role in regulating the ACTH response to LPS, with a positive effect exerted through its activity in folliculostellate cells, and a negative effect exerted through its expression in corticotrophs.

Although the *in vitro* studies demonstrated blockade of NFκB within the anterior pituitary gland alters the ACTH response to CRH and LPS stimulation, to address the issue of whether inhibition of NFκB alters the stress response in an intact HPA system, the study in chapter 5 utilised *in vivo* models. In addition to immunological stress, the study also addressed the role of NFκB in the HPA response to psychological stress through the use of two stress models - LPS (immunological) and restraint (psychological) stress. Inhibition of NFκB *in vivo* attenuated the ACTH response to LPS treatment and restraint, thus mimicking the actions of glucocorticoids. Furthermore, the decrease in ACTH release correlated with reduced NFκB activity in the anterior pituitary gland in both models, supporting the premise that intra-pituitary NFκB is important in mediating ACTH secretion. The partial reduction in ACTH release in conditions of suppressed NFκB activity, 2 hours after LPS injection, is in agreement with the *in vitro* findings; folliculostellate cell-derived NFκB increases ACTH secretion, whilst in corticotrophs NFκB inhibits ACTH release. Alternatively, it may reflect the fact that LPS also acts via NFκB-independent mechanisms to augment ACTH release. As the NFκB inhibitor did not affect the corticosterone response to LPS, it is likely that NFκB is primarily important in mediating the HPA response to LPS at the anterior pituitary level. Surprisingly, NFκB blockade exerted a powerful effect upon the HPA response to psychological stress, decreasing both ACTH and corticosterone to near basal concentrations. Psychological stress is modulated by the higher brain centres that converge on the

hypothalamus (see section 1.2.5), and although the effects of the NF κ B inhibitor focused on the anterior pituitary and adrenal glands in the current study, it is possible that the reduced ACTH and corticosterone plasma concentrations are attributable to impaired NF κ B signalling in the hypothalamus and possibly in the higher brain structures.

Together these findings have identified, for the first time, that in addition to its role in the inflammatory response, NF κ B signalling in the anterior pituitary gland is also important in mediating the HPA response to stress. As such, the use of NF κ B inhibitors for the treatment of chronic inflammatory disorders may impair the individual's ability to cope with stress, whether it is immunological or psychological in nature. Further to this, the NF κ B inhibitor used in the study increased the plasma concentration of basal corticosterone, but not that of ACTH. Although the site(s) of action and the mechanisms involved in this response are unclear, these findings suggest that, in addition to impairing the HPA responses to stress, NF κ B inhibitors may also alter basal corticosterone release in the absence of stress, thus increasing susceptibility to disorders associated with hypercortisolemia.

In conclusion, this thesis has provided evidence for the first time that NF κ B is involved in the bi-directional communication between folliculostellate cells and corticotrophs in regulating IL-6 and ACTH release. Furthermore, NF κ B signalling has been identified as an important mediator in the HPA responses to both immunological and psychological stress.

7 Bibliography

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