

REGULATION OF ATF3 EXPRESSION IN CARDIOMYOCYTES

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MBBS (Hons)

Submitted for the degree of Doctor of Philosophy of the
Imperial College London

Imperial College London
National Heart and Lung Institute

January, 2008

ABSTRACT

The stress responses of cardiomyocytes are likely to constitute a significant aspect of the development of cardiac pathologies. Oxidative stress is a common theme in the pathophysiology of ischaemic and non-ischaemic cardiomyopathy. On the other hand, hypertrophic agonists (e.g. endothelin-1, ET-1) are important mediators of the hypertrophic response to hemodynamic overloading, and cardiac pathologies such as heart failure are usually preceded by cardiac hypertrophy. Microarray studies indicate that one of the genes most potently induced by H₂O₂ (as an oxidative stress) or ET-1 in cardiac myocytes is the transcription factor ATF3. This thesis examines the regulation and role of ATF3 in neonatal rat ventricular cardiomyocytes.

The increase in expression of ATF3 mRNA was confirmed in neonatal rat cardiomyocytes exposed to H₂O₂ or ET-1 using RT-PCR and Q-PCR. Using immunoblotting, it was confirmed that H₂O₂ or ET-1 increased ATF3 protein expression. ATF3 is an immediate early gene since the upregulation of ATF3 mRNA by ET-1 was not inhibited by cycloheximide (20 µM). Upregulation of ATF3 was inhibited by U0126 (10 µM), suggesting that signalling through ERK1/2 (extracellular signal-regulated kinases 1/2) was required. Other studies suggest that ATF3 downregulates interleukin-6 (IL-6) in human cells. The rat IL-6 promoter possesses an ATF consensus sequence, and chromatin immunoprecipitation (ChIP) analysis indicated that ET-1 or H₂O₂ increased the association of ATF3 with the IL-6 promoter. IL-6 mRNA and protein expression was transiently upregulated in cardiomyocytes exposed to H₂O₂ or ET-1, and this was shown to be an immediate early gene response. Following IL-6 mRNA upregulation, its expression was more rapidly downregulated than that of ATF3. The peak of ATF3

protein expression coincided with the return of IL-6 mRNA to basal levels after stimulation with either ET-1 or H₂O₂. This suggests that ATF3 operates in a negative feedback loop to downregulate IL-6 mRNA expression in cardiomyocytes. This was confirmed by adenoviral-mediated overexpression of full-length ATF3 antisense RNA, which attenuated ATF3 mRNA and protein expression following stimulation with ET-1. Inhibition of ATF3 expression was associated with superinduction of IL-6 mRNA as shown by Q-PCR. Other potential downstream targets of ATF3 were identified from microarray studies using bioinformatics. Those with ATF/CRE consensus sequences in their promoters included epiregulin and leukaemia inhibitory factor (LIF) (also shown to be immediate early genes). Epiregulin and LIF mRNA expression was superinduced by ATF3 antisense RNA. Taken together, these experiments indicate that ATF3 operates in a negative feedback loop by downregulating a cluster of immediate early genes induced by ET-1. Thus, ATF3 may play an important role in the establishment and fine-tuning of an organised and compensatory hypertrophic response in the cardiomyocyte secondary to hypertrophic stimulation.

ACKNOWLEDGEMENTS

I want to dedicate this thesis to my wife, Natalia. Her love and support through the ups and downs, and her comforting words during the most difficult times kept me motivated to be able to complete this work. I'm also grateful of the example and support from my parents Jairo and Yaneth. They always knew I could give more and always pushed me to achieve new goals.

I'm very grateful to my excellent supervisors, Professor Peter Sugden and Dr Angela Clerk, for their continuous support and example of research greatness.

Although this thesis is the result of my own work, the completion of this thesis and the data inside would have never been possible without the help from my friends at my lab in the NHLI, Imperial College London. I particularly wish to express my gratitude to my good friend Sampsa Pikkarainen. He always gave me advice and was always available to discuss the successful experiments and the problematic ones during the daily work at the lab.

STATEMENT OF CONTRIBUTION

Dr Stephen Fuller from our laboratory prepared and purified the adenoviral constructs for ATF3, which I have used in some experiments of this thesis. Cardiomyocyte cultures from neonatal rat hearts were prepared by Ms. Amanda Nercessian from our laboratory. All experimental work and data analyses were performed by myself unless otherwise stated.

PUBLICATIONS AND ABSTRACTS

Publications

Cullingford TE, Markou T, Fuller SJ, Giraldo A, Pikkarainen S, Zoumpoulidou G, Kemp TJ, Dennis J, Game L, Sugden PH, Clerk A. Early transcriptional responses induced by endothelin-1 in neonatal rat cardiomyocytes. *Genome Biology*. 2008 (Under revision).

Markou T, Cullingford TE, Giraldo A, Weiss SC, Amin AA, Fuller SJ, Clerk A, Sugden PH. Glycogen synthase kinases 3 α and 3 β in cardiac myocytes: regulation and consequences of their inhibition. *Cellular Signalling*. 2008;20:206-18.

Clerk A, Giraldo A, Sugden PH. Chemotherapeutic agents and the heart. *Advances in Enzyme Regulation*. 2007;47:140-53.

Clerk A, Cullingford TE, Fuller SJ, Giraldo A, Markou T, Pikkarainen S, Sugden PH. Signalling pathways mediating cardiac myocyte gene expression in physiological and stress responses. *Journal of Cell Physiology*. 2007;212:311-22.

Abstract

Giraldo A, Pikkarainen S, Sugden PH, Clerk A. Regulation of the expression of ATF3 in cardiac myocytes in response to hypertrophic and apoptotic stimulation. *Journal of Molecular and Cellular Cardiology* 2007;42:S83.

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ABBREVIATIONS

4EBP	eIF4E-binding protein
Aldh3a1	Aldehyde dehydrogenase family 3 member a1
ANGII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
ATF	Activating transcription factor
BNP	B-type natriuretic peptide
bp	Base pair
BSA	Bovine serum albumin
CaM	Calmodulin
CaMKII	CaM kinase II
ChIP	Chromatin immunoprecipitation
CRE	cAMP response element
CREB	cAMP response element-binding protein
CT-1	Cardiotrophin 1
Cyp1a1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E64	Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

eIF4E	Eukaryotic initiation factor 4E
EMSA	Electrophoretic mobility shift assay
Ereg	Epiregulin
ERK1/2	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin-1
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FoxO	Forkhead box transcription factors of group O
Gab	Grb2-associated binder
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gp130	Glycoprotein 130
GqPCR	Gq protein-coupled receptor
GSK3	Glycogen synthase kinase 3
GTFs	General transcription factors
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HLH	Helix-loop-helix
HTH	Helix-turn-helix
IEG	Immediate early gene
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
IL-6R α	IL-6 receptor α subunit
Ins(1,4,5)P ₃	Inositol 1,4,5 <i>tris</i> phosphate
JNK	c-Jun N-terminal kinase

kDa	Kilodalton
LIF	Leukaemia inhibitory factor
LIFR β	LIF receptor β subunit
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
MKK	Mitogen-activated protein kinase kinase
MKKK	Mitogen-activated protein kinase kinase kinase
MI	Myocardial infarction
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated-T cells
NF- κ B	Nuclear factor kappa B
Nfkbiz	NF- κ B inhibitor zeta
NOX	NAD(P)H oxidases
p38-MAPK	p38-mitogen-activated protein kinase
p90RSK	90-kDa ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PK1	3-phosphoinositide-dependent protein kinase-1
PE	Phenylephrine
PI3K	Phosphatidylinositol 3'-kinase
PIC	Preinitiation complex
PKA	Protein kinase A

PKB	Protein kinase B
PKC	Protein kinase C
PLB	Phospholamban
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulphonyl fluoride
PP2A	Protein phosphatase 2A
PtdInsP ₂	Phosphatidylinositol 4,5 <i>bis</i> phosphate
PtdInsP ₃	Phosphatidylinositol 3,4,5 <i>tris</i> phosphate
Q-PCR	Quantitative PCR
RGS	Regulator of G-protein signalling
ROS	Reactive oxygen species
RPTK	Receptor protein tyrosine kinase
S6K1	70-kDa ribosomal S6 kinase
SAPK	Stress-activated protein kinase
SH2	Src homology 2
SOD	Superoxide dismutase
Sos	Son of sevenless
SQ-PCR	Semiquantitative PCR
TAF _{II} s	TBP-associated factors
TBP	TATA-binding protein
TBE	Tris-HCl-boric acid-EDTA
TNF α	Tumor necrosis factor α
XO	Xanthine oxidase

Chapter 1

Introduction

1.0 General introduction

In the United Kingdom, cardiovascular diseases account for the death of one in three people each year (36%) (Allender et al., 2007). Heart failure is the end result of most diseases affecting the cardiovascular system and it has been estimated that at least 10 million patients in Europe have symptomatic heart failure with a similar number of patients having asymptomatic left ventricular dysfunction (Swedberg et al., 2005). The prognosis of this disease is very poor if the causes cannot be rectified, with mortality rates of 50% within four years of diagnosis, and this is even worse in patients with severe heart failure in which the mortality is more than 50% within a year (Swedberg et al., 2005).

The clinical syndrome of heart failure is the end result of most disease processes affecting the cardiovascular system, and the pathophysiology of heart failure has proven to be multifactorial and complex. Despite some progress over the past two decades in the diagnosis and the advent of novel pharmacological therapy targeting neurohumoral activation in heart failure, the number of cases is steadily increasing as a result of increased survival of coronary artery disease, and the general trend to higher life expectancy in developed and developing countries with a consequent increase in the aging population worldwide in recent years (Poole-Wilson, 2007). Additionally, mortality rates for this disease are still very high despite careful implementation of guidelines for the management of the disease, mainly because the use of current therapy slows but does not stop the progressive nature of this disease. Therefore there is an urgent need to gain a better understanding of the pathophysiological mechanisms of heart failure, and the development of novel pharmacological agents that hopefully will help to stop the progression of this disease.

1.1 Cardiac hypertrophy and heart failure

1.1.1 Compensatory cardiac hypertrophy is stimulus dependent

In the most general sense, cardiac hypertrophy is an adaptive response to increased haemodynamic demands. Cardiomyocytes are terminally differentiated cells which withdraw from the cell cycle soon after birth (Li et al., 1996; Soonpa et al., 1996), therefore cardiac hypertrophy is the result of increased cellular growth (e.g. increased cellular content of, for example, myofibrils) in the absence of cell division. Immediate early genes (IEGs) are a class of genes whose upregulation is acute and usually transient, preceded by activation of intracellular signalling cascades and occurs in the absence of *de novo* protein synthesis (Clerk et al., 2007). In cardiomyocytes, the hypertrophic phenotype is associated with changes in the expression of IEGs (e.g. *c-jun*, *c-fos*, *c-myc*), and re-expression of a pattern of genes usually expressed during development, the so called “foetal pattern” of gene expression (e.g. atrial natriuretic peptide, ANP; B-type natriuretic peptide, BNP; and β -myosin heavy chain), which are often used as markers of hypertrophy (Hoshijima and Chien, 2002; Dorn and Force, 2005).

Two different types of haemodynamic overload lead to two very well defined patterns of ventricular hypertrophy. Thus, pressure-overload, as occurs in hypertensive states, leads to concentric cardiac hypertrophy in which the ventricular wall thickness is increased with minimal or no increase in the ventricular diameter of the chamber (Grossman, 1975). Due to the circumferential disposition of muscle fibres in the heart, this pattern of hypertrophy correlates with an increase in the cross-sectional area of the cardiomyocytes, as a result of sarcomeric assembly in parallel (Grossman, 1975). In contrast, volume

overload, as occurs in mitral or aortic regurgitation, leads to eccentric hypertrophy which is characterized by an increase in chamber diameter and a proportional increase in wall thickness. At the cellular level, this pattern of hypertrophy correlates with a proportional increase in myocyte cross-sectional area and length, and sarcomeric assembly occurs in series as well as in parallel. These two patterns of hypertrophy are necessary to achieve a compensated state with normal or near normal systolic wall stress in accordance with the law of Laplace (Grossman, 1975).

The distinction between eccentric and concentric hypertrophy is important for several reasons. The specific adaptation of the structure of the ventricle to a particular haemodynamic load virtually forbids aberrant conditions such as the development of eccentric hypertrophy and a predominant increase in myocyte cell length in the setting of pressure-overload. Such a change in the morphology of the ventricle and the cardiomyocytes will be clearly maladaptive. Importantly, cumulative data indicates that intrinsic abnormalities in the cardiomyocytes, which include myocyte elongation and myocyte slippage, and collectively known as cardiomyocyte remodelling (reviewed in Gerdes, 2002), contribute to the process of ventricular remodelling and the progression to heart failure (see also section 1.1.3) (for a recent review, see, for example Opie et al., 2006).

1.1.2 From cardiac hypertrophy to heart failure

Although initially a compensatory response to increased workload, sustained cardiac hypertrophy over a prolonged period of time is usually accompanied by adverse outcomes secondary to arrhythmias, coronary artery disease and the development of heart failure (Levy et al., 1990; Bikina et al., 1993; Gosse, 2005). The current paradigm of the

progression from cardiac hypertrophy to heart failure was proposed by Felix Meerson almost 40 years ago by using a model of acute sub-occlusion of the aorta in dogs (Meerson, 1962; Meerson, 1969; Meerson 1976). Thus, after an initial insult to the heart whether by an increase in its haemodynamic demands (e.g. hypertension) or loss of the contractile tissue of the heart (e.g. myocardial infarction), an initial phase of “transient breakdown” (Stage 1) is observed. If the organism survives to this initial phase, compensatory hypertrophy develops to maintain cardiac output (Stage 2 or “compensatory hyperfunction”). However, when this initial insult persists (e.g. uncontrolled hypertension) or is irreversible as in myocardial infarction, a final phase called “chronic myocardial exhaustion” (Stage 3) will take place over months to years (Meerson 1962; Meerson 1969). This third stage is characterized by progressive left ventricular failure, further hypertrophy and replacement of dead cardiomyocytes by fibrotic tissue (Katz, 1989). The clinical outcome of this process is cardiac decompensation and overt heart failure. From Meerson’s studies it is clear that under conditions of increased haemodynamic demands after a compensated hypertrophic state with a stable period of months or years, invariably the heart will progress to a phase of “chronic exhaustion” which is manifest by heart failure.

One of the important implications of Meerson’s work is the amazing ability of the heart to compensate in response to increased work demands (in contrast to other organs), not only being able to recover from near *in extremis* conditions (transient breakdown) after acute imposition of pressure-overloading, but also in way that allows it to completely recover functional capacity and remain in a compensated state for a prolonged period of time before chronic exhaustion supervenes and heart failure develops. Thus, Meerson also postulated that during the imposition of the pressure-overload, “antibreakdown”

mechanisms should be activated in order to maintain the viability of the animal. This leads to the conclusion that the heart possesses an innate capacity to recover from very precarious haemodynamic conditions, and probably does so not only by activating compensatory mechanisms, but also antibreakdown systems. Although the compensatory mechanisms leading to cardiac hypertrophy have been extensively studied over the past two decades, the antibreakdown systems have not received considerable attention. Of note, some of the pathways involved in the hypertrophic phenotype also promote survival of the myocyte [e.g. the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (see section 1.3.1.6), and the protein kinase B (PKB, also known as Akt) pathway (see section 1.3.2.2)]. However, cardiac hypertrophy from concomitant pressure-overload in the setting of overexpression of G α q subunits from guanine nucleotide proteins (G-proteins), paradoxically leads to heart failure associated with increased apoptosis and eccentric rather than concentric remodelling (Sakata et al., 1998). Thus, it is possible that the hypertrophic phenotype is accompanied by both cytoprotective and pro-apoptotic signalling, and probably the final outcome ultimately depends on the nature of the stimuli and the context of the cells (cardiac milieu) at the time of the stimuli.

1.1.3 Ventricular remodelling: the road to heart failure progression

Changes in the ventricular architecture and the cardiomyocyte size and shape have collectively received the name of ventricular remodelling. The seminal studies of Janice Pfeffer and colleagues, lead to the concept of ventricular remodelling in the late 1970s. Initially, during her PhD studies at Frohlich laboratory, she noticed that with spontaneously hypertensive rats, as the animals aged the shape of the ventricle progressively changed towards a dilated, thinned and spherical morphology rather than the normal elliptical one (Pfeffer et al., 1979a; Pfeffer et al., 1979b). To this change in the

characteristics of the ventricle after a period of compensatory hypertrophy, the name of ventricular remodelling was given. Later, these findings were extended to a model of myocardial infarction in rats, where a similar remodelling process was documented. An initial phase of compensatory hypertrophy was followed by progressive dilatation and thinning of the ventricular cavities (Fletcher et al., 1981). One of the important clinical applications of these studies is the current anti-remodelling strategy following myocardial infarction in patients. Initially, while testing the effects of several vasodilators, remodelling of the ventricle was found to be attenuated by what was then a new class of drug, the angiotensin converting-enzyme (ACE) inhibitors, which had a major effect in ameliorating this process (Pfeffer et al., 1982; Pfeffer et al., 1985). This finally led to the concept of cardioprotection and anti-remodelling therapy, and the current use in clinical practice of antiremodelling drugs such as the ACE inhibitors, which has led to significant reductions in morbidity and mortality after myocardial infarction (Pfeffer et al., 1992; Sutton et al., 1997). This therapy has been extended to improve the prognosis of patients with heart failure (Pfeffer et al., 1988; Kiowski et al., 1996).

The molecular mechanisms underlying the progression to heart failure have been under intensive investigation over the past two decades. The weight of evidence supports the notion that this process is the end result of increased neurohumoral stimulation which activates multiple signalling cascades that change the phenotype of the myocardium by altering the patterns of gene expression controlling growth, survival and apoptosis of cardiomyocytes. The resulting terminal phenotype is characterized by poor contractility, altered myocardial energetics, myocardial fibrosis, and ventricular dilatation with a corresponding increase in myocyte length (Colucci et al., 1997; Hunter and Chien, 1999; Katz, 2003). In the next section the general classification of the different neurohumoral

factors that have been implicated in inducing cardiomyocyte hypertrophy will be summarized. Then in section 1.3 a detailed description of the different signalling pathways involved in cardiomyocyte hypertrophy and which are activated by these neurohumoral factors will be presented.

1.2 Hypertrophic agonists

Haemodynamic stress in the form of pressure-overload and volume overload is associated with the autocrine/paracrine release of a plethora of hypertrophic factors. The majority can be classified in three groups: vasoactive agonists, peptide growth factors and the cytokine members of the interleukin-6 (IL-6) family (Molkentin and Dorn, 2001). In addition to their ability to cause hypertrophic growth of the heart and cardiomyocytes, vasoactive agonists can also act in the smooth muscle of the blood vessels and induce vasoconstriction and as a consequence also raise blood pressure (Sugden, 2002). These include peptide hormones like angiotensin II (ANGII), and endothelin-1 (ET-1), and catecholamines such as noradrenaline. Collectively the effects of these vasoactive agonists are mediated through G-protein-coupled receptors (GPCRs). Thus, ANGII activities are mediated via the AT1 (AT1a/b in rodents) or AT2 receptors. Hypertrophic actions are thought to be mediated principally through the AT1a receptors, although studies in genetically-modified mice suggest a role for the AT2 receptor subtype (Ishiwara et al., 2001). In mammals the actions of ET-1 are mediated via ET-A and ET-B receptors, both of which have been cloned (Davenport, 2000). However, the ET-A receptor predominates in the rodent heart (Hilal-Dandan et al., 1997). The actions of noradrenaline in the cardiovascular system are generally mediated through α_1 , β_1 and β_2

adrenoceptors, but those involved in cardiac hypertrophy are mainly derived from activation of α 1-adrenoceptors. In experimental settings, the most commonly used agonist is phenylephrine (PE) which is a more selective synthetic agonist for α -adrenoceptors compared with noradrenaline.

Of note, although ANGII stimulation of cardiomyocytes usually results in a hypertrophic phenotype that is mediated through the AT1a receptor (Inagami et al., 1999), some studies suggest that the hypertrophic effects on cardiomyocytes are relatively weak compared with agonists like ET-1 or PE (reviewed in Sugden, 2002). Thus, the direct role of ANGII in mediating cardiac hypertrophy has been object of debate for several years (Reudelhuber et al., 2007). It has been postulated that most of the actions of ANGII in cardiac hypertrophy are probably secondary to the release of other autocrine/paracrine hypertrophic factors such as ET-1 (Ito et al., 1993, Xia et al., 2004; Laplante and Champlain 2006), peptide growth factors such epidermal growth factor (EGF) (Shah et al., 2003; Smith et al., 2004), and/or by AT1-mediated transactivation of the EGF receptor (EGFR) (Zhai et al., 2006). With the considerations for ANGII mentioned above, the discussion of the actions derived from the activation of GPCR in cardiac hypertrophy will be centered on the observations made with the α -adrenoceptor agonist PE, and particularly with ET-1 (see section 1.3.1).

Peptide growth factors can promote cardiomyocyte hypertrophy. However, there are significant differences in their relative potency in inducing the response. Thus, EGF, platelet derived growth factor (PDGF) or fibroblast growth factor (FGF) cause significant hypertrophic response in cardiomyocytes (as assessed by measurements of cell area), whereas insulin, insulin-like growth factor-1 (IGF-1) or nerve growth factor (NGF) do not

induce significant hypertrophy of cardiomyocytes (Clerk et al., 2006). Peptide growth factors activate receptor protein tyrosine kinases (RPTK) to mediate their actions. The archetypical model of RPTK signalling is the EGF receptor (EGFR) signalling system which will be discussed in detail in section 1.3.2.

The IL-6 family of cytokines [also known as the gp130 (glycoprotein 130) cytokines], include IL-6, cardiotrophin-1 (CT-1), leukaemia inhibitory factor (LIF), IL-11, oncostatin M, and ciliary neurotrophic factor. The best studied of this family are CT-1, LIF and IL-6 (Pennica et al., 1996; Wollert et al., 1996). These agonists activate signalling cascades by coupling to heteromeric plasma membrane receptor complexes which contain one or more copies of the common signal transducing receptor chain glycoprotein 130 (gp130), (Bravo and Heath, 2000). The hypertrophic effects of this family of cytokines in cardiomyocytes are characterized by a prominent increase in myocyte length, rather than increased cross-sectional area (as occurs with ET-1), thus causing myocyte elongation (Wollert et al., 1996). A detailed description of the mechanisms of signal transduction pathways activated by this family of cytokines will be discussed in section 1.3.3.

1.3 Intracellular signalling pathways activated by hypertrophic agonists

All hypertrophic agonists cause the activation of several intracellular signalling pathways, some of which may be common to many, but their relative potency in term of degree of activation and initial mechanistic steps at the membrane receptor level differ considerably. Here, I will discuss the events leading to the activation of the different

signalling cascades by the different hypertrophic agonists. The supporting evidence of their role in the hypertrophic process will be also discussed.

1.3.1 Signal transduction pathways activated by ET-1 or PE

1.3.1.1 ET-1 or PE activate second messengers at the plasma membrane-cytoplasm interface

In cardiomyocytes, the receptors through which pro-hypertrophic signalling of ET-1 and PE is activated are the ET-A and $\alpha 1$ -adrenergic receptors respectively. These receptors belong to a large superfamily receptors which characteristically consist of seven transmembrane α -helical domains (H1-H7), thus the name heptahelical receptors. The N-terminal domain and three loops that connect the H2-H3, H4-H5 and H6-H7 domains are located facing the extracellular space, whereas the C-terminal domain and three loops that connect the H1-H2, H3-H4 and H5-H6 domains are located facing the intracellular space. The ET-A and $\alpha 1$ -adrenergic receptors are coupled mainly to heterotrimeric G-proteins of the q/11 family (and are therefore collectively known as GqPCRs) which activate the enzyme phospholipase C β (PLC- β).

Heterotrimeric G-proteins are composed of three subunits, namely alpha (α), beta (β) and gamma (γ). In the basal state, the heterotrimeric G-proteins form a complex, with the G α subunit bound to GDP (Linder and Gilman, 1992). Upon ligand binding to their specific GqPCR receptors, a conformational change at the intracellular domain occurs triggering the association of the G-protein complex with third C-terminal loop and the C-terminal tail of the receptor (Conklin et al., 1993). This is followed by rapid exchange of GTP for GDP in the G α q subunit and the dissociation of the heterotrimer into G α q-GTP and G $\beta\gamma$.

(Figure 1.1). Both $G\alpha q \cdot GTP$ and $G\beta\gamma$ are capable of inducing direct and independent activation of PLC- β (Rhee, 2001), which catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5 *bis* phosphate [PtdIns(4,5)P₂] with the formation of the soluble inositol 1,4,5 *tris* phosphate [Ins(1,4,5)P₃] and hydrophobic and membrane-localized diacylglycerol (DAG). The actions of $G\alpha q \cdot GTP$ are terminated by the intrinsic GTPase activity of $G\alpha q$, although this can be accelerated by GTPase-activating proteins (GAPs) known as regulator of G-protein signalling (RGS) proteins (Ross et al., 2000), which can reduce considerably the time of activation of the effectors of $G\alpha q$. Finally, there is re-association of $G\alpha q \cdot GDP$ with $G\beta\gamma$ dimers.

1.3.1.2 Downstream signals activated by DAG in response to ET-1 or PE

Protein kinase C (PKC) enzymes are the best characterized immediate effectors of DAG (Figure 1.2). The PKC family comprises multiple related genes which are serine/threonine kinases, and play an important role in the regulation of cellular growth and viability. PKCs are subdivided into three subfamilies: the classical or conventional cPKCs (α , βI , βII , γ) which are Ca^{2+} -dependent and are activated by both DAG and phosphatidylserine; the novel nPKCs (δ , ϵ , η , θ) which are Ca^{2+} -independent but require DAG and phosphatidylserine for activation; and the atypical aPKCs (ζ , ι/λ) which are Ca^{2+} - and DAG-independent but phosphatidylserine can regulate their functions (Mellor and Parker, 1998; Steinberg, 2004). Of the PKCs that require DAG for their activation, cardiomyocytes express cPKC α (Clerk et al., 1994; Disatnik et al., 1994; Rhode et al., 2000), nPKC δ and nPKC ϵ (Clerk et al., 1994; Disatnik et al., 1994; Clerk et al., 1995). In addition to DAG-dependent PKCs the atypical aPKC ζ and aPKC ι/λ have also been detected in cardiomyocytes (Clerk et al., 1994; Disatnik et al., 1994; Steinberg, 2004).

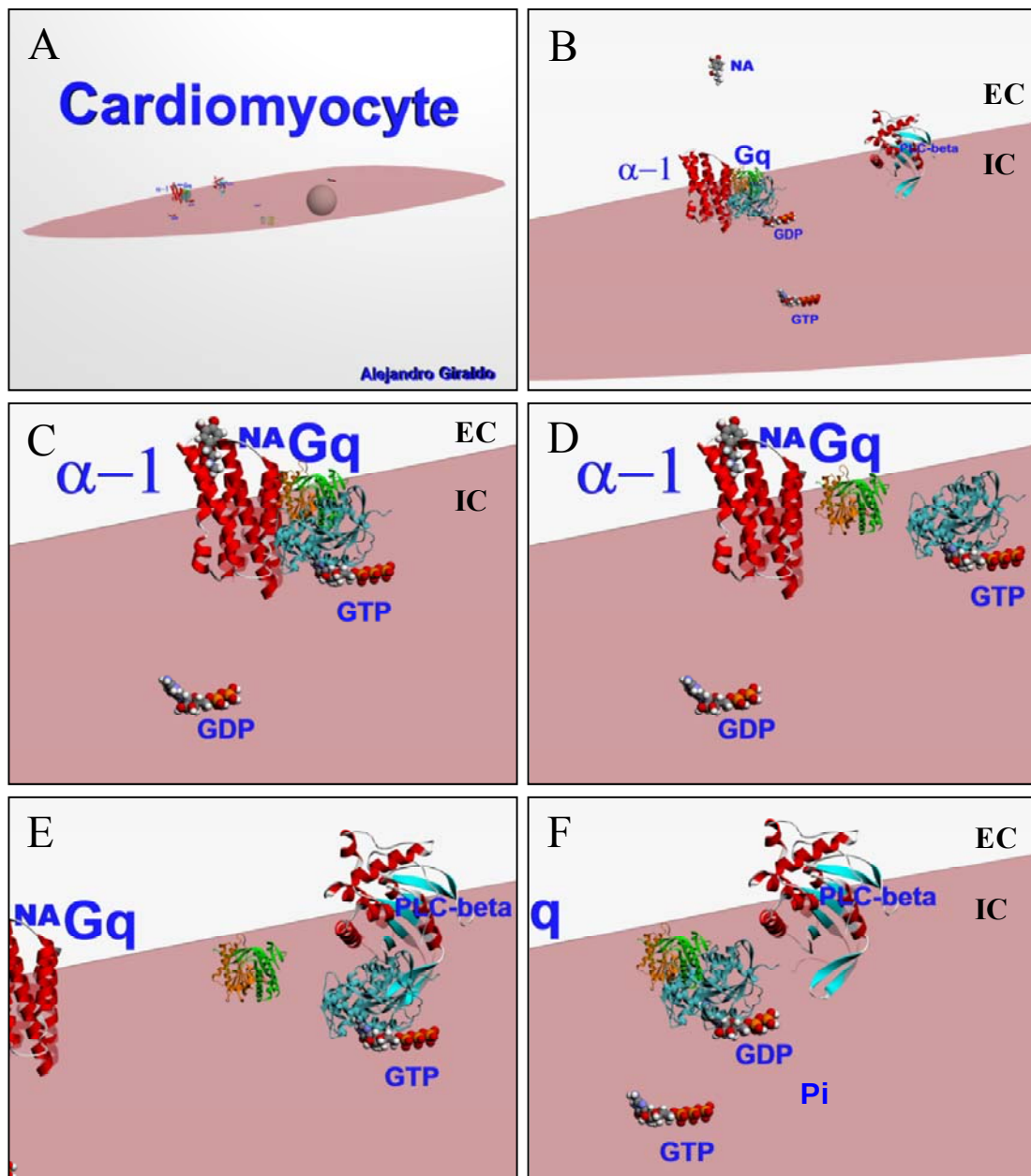


Figure 1.1 Activation of heterotrimeric Gq protein complex by noradrenaline in cardiomyocytes. In the resting cardiomyocyte (A and B), heterotrimeric Gq-proteins remain inactive and bound to GDP. Upon noradrenaline (NA) binding to the α -1 adrenergic receptor (C) a conformational change in the receptor (at the C-terminal) leads to binding of GTP to the G α q-subunit (depicted in blue). This is followed by dissociation of the Gq protein complex into G α q-GTP and G $\beta\gamma$ subunits (green and orange) (D). Then G α q-GTP (or G $\beta\gamma$) binds to and activates the enzyme phospholipase-C β (PLC- β) (E). The activity of G α q is terminated by its intrinsic GTPase activity and this is followed by reassociation of the heterotrimeric Gq protein complex (F). Abbreviations: EC = extracellular space; IC = Intracellular space. Protein data bank (PDB) files obtained from Research Collaboratory for Structural Bioinformatics PDB web site. Figures were created with imported ribbon structures using the software 3D Studio Max ® from Discreet software Inc.

PKCs are soluble kinases that remain in the cytosol in the inactive state and upon stimulation of cardiomyocytes with ET-1, nPKC δ and nPKC ϵ translocate to the membrane fraction to bind to DAG and phosphatidylserine, a response that is indicative of their activation (Clerk et al., 1994). Although a rapid translocation of nPKC ϵ to the membrane also occurs in response to PE, this is significantly less than with ET-1, and translocation of nPKC δ to the membrane fraction is almost undetectable with this agonist (Clerk et al., 1994). Characteristically, nPKC δ and nPKC ϵ begin to return to the cytosolic fraction (retrotranslocation) within 3-5 min (probably because DAG is rapidly phosphorylated by DAG kinases, thus terminating its actions), although a significant proportion of nPKC ϵ remains bound to the membrane fraction up to 15 min (Clerk et al., 1994). The EC₅₀ of ET-1 in promoting translocation of nPKC ϵ to the membrane is about 1 nM whereas that for nPKC δ is significantly greater (10 nM) (Clerk et al., 1994). Interestingly, ET-1 causes rapid translocation of the nPKC δ pool to the membrane fraction (within 30 s) an event that is associated with phosphorylation of Tyr-residues on this kinase (Markou et al., 2006). nPKC δ retains its activity even after retrotranslocation and is therefore presumably involved in phosphorylation of substrates elsewhere (Markou et al., 2006).

With respect to cPKC α , this isoform appears to be up to 8 times more abundant on a molar basis than nPKC ϵ in cardiomyocytes (Rohde et al., 2000) and, assuming similar activities for both isoforms, only a small fraction of the cPKC α may need to be translocated to get a similar change in activity as for complete translocation of nPKC ϵ . However, again noradrenaline, acting through α 1-adrenoceptors only activated nPKC δ and nPKC ϵ , but not cPKC α (Clerk et al., 1994; Rohde et al., 2000). The role of cPKC α in cardiac hypertrophy cannot be dismissed because of this, as downregulation of cPKC α

with antisense oligonucleotides leads to suppression of some of the molecular criteria of hypertrophy induced by PE or ET-1. It also correlates with a reduction in the activation of ERK1/2 by PE, an important cascade involved in cardiac hypertrophy (see section 1.3.1.5), implicating cPKC α as an upstream effector of ERK1/2 activation in cardiomyocytes (Kerkelä et al., 2002). An important role for cPKC α in cardiomyocyte hypertrophy has also been suggested by the demonstration that adenoviral-mediated overexpression of cPKC α results in a hypertrophic phenotype (through downstream activation of the ERK1/2 cascade), whereas overexpression of dominant negative cPKC α blunts the hypertrophic response to PE (Braz et al., 2002; Braz et al., 2004).

1.3.1.3 Downstream signals activated by Ins(1,4,5)P₃ in response to ET-1 or PE

Upon diffusion from the plasma membrane to the cytosolic space, Ins(1,4,5)P₃ binds to Ins(1,4,5)P₃ receptors (IP3R) located in several membranes of organelles including the endoplasmic reticulum, the mitochondria and the nucleus. IP3R operate as Ca⁺² channels and, upon activation by Ins(1,4,5)P₃, mobilize Ca²⁺ ions from the organelle. Although this mobilization of Ca²⁺ ions into the cytosol has been considered of different nature and consequences to the process of “excitation-contraction coupling” that regulates the activation of the myofilaments during systole and diastole in the heart, its physiological significance in cardiomyocytes has remained obscure for several years.

Recently, a process has been described whereby Ins(1,4,5)P₃ signalling by ET-1 or PE results in activation of gene expression secondary to localized mobilization of Ca²⁺ into the nucleus. Ca²⁺ binds to calmodulin (CaM) and increases the activity of Ca²⁺/CaM kinase II (CaMKII). The homologous name “excitation-transcription coupling” has been given to this process (Wu et al., 2006) (Figure 1.2). The consequences of activation of

CaMKII in the perinuclear envelope involves phosphorylation, nuclear export to the cytoplasm of histone deacetylase 5 (HDAC5) which remains bound by inhibitory proteins 14-3-3 (McKinsey et al., 2000). HDAC5 is a negative regulator of the myocyte enhancer factor 2 (MEF2) transcription factor (Wu et al., 2006, reviewed in Backs and Olson, 2006), and therefore activation of CaMKII ultimately leads to derepression of MEF2 (Lu et al., 2000), an important transcription factor in cardiac development and growth (reviewed in Akazawa and Komuro, 2003). HDAC5 belongs to the class II HDAC family which are commonly involved in the negative regulation of gene expression by increasing the coiled structure of DNA into the nucleosomes (chromatin condensation), a widely used mechanism for the control of gene expression by the cells (see section 1.6). The important implications of this novel mechanism of $\text{Ins}(1,4,5)\text{P}_3$ regulation of cellular processes is that cardiomyocytes, and possibly other cells, have the ability to distinguish local mobilization of Ca^{2+} within the nucleus which is involved in the control of gene expression, and global mobilization of Ca^{2+} ions which is involved in contraction and relaxation of the cardiomyocytes.

1.3.1.4 Activation of small G-protein Ras by GqPCR agonists

One of the next events regulated by GqPCR agonists is the activation of the small (21 kDa) guanine nucleotide binding proteins of the Ras family (Figure 1.2). The Ras family belongs to the small G-protein superfamily which is classified into five subfamilies with over 100 members, and it is even possible that more members and subfamilies remain to be identified (Schultz et al., 2000). Three Ras proteins have been the most studied over the years including Harvey (Ha-Ras), Kirsten (Ki-Ras) and N-Ras, and these are the archetypical Ras members (Takai et al., 2001).

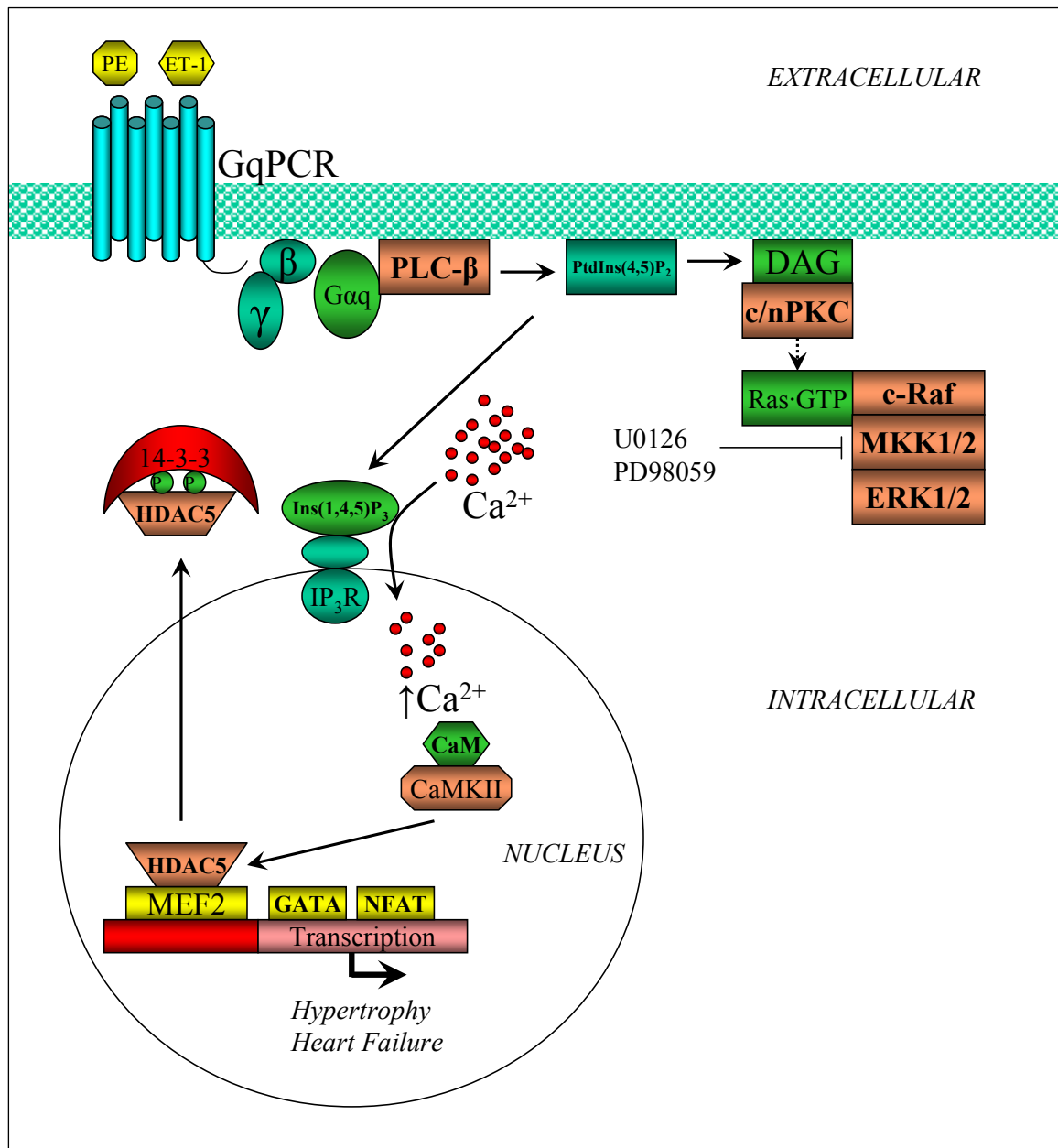


Figure 1.2 Activation of signalling pathways by GqPCR agonists ET-1 or PE in cardiomyocytes. Binding of ET-1 or PE to their GqPCRs, activates Gq-proteins leading to activation of phospholipase C β (PLC- β). This triggers the hydrolysis of PtdIns(4,5)P₂ into soluble inositol 1,4-5-trisphosphate [Ins(1,4,5)P₃] and DAG. Ins(1,4,5)P₃ activates Ins(1,4,5)P₃ receptors (IP₃R) in the nuclear envelope causing a localized increase in Ca²⁺ ions, which activates calmodulin (CaM) and CaM kinase II (CaMKII), this later inducing phosphorylation of histone deacetylase 5 (HDAC5) and its nuclear export to the cytosol and binding to inhibitory proteins 14-3-3, thus derepressing transcription MEF2 activated transcription. DAG remains in the membrane locale where it activates several members of the n/cPKCs (cPKC α , nPKC δ and nPKC ϵ). This result in activation of Ras and a cascade of events including activation of c-Raf, MKK1/2 and finally activation of ERK1/2, which can be blocked by specific inhibitors such as U0126 or PD98059 (see section 1.3.1.5).

In a manner similar to heterotrimeric G-proteins, small G-proteins are inactive when bound to GDP and are activated upon exchange of GDP for GTP, triggering a change in the conformational structure of the Ras effector-binding region, which allows it to interact with its effectors including Raf, the upstream kinase of the ERK1/2 cascade (see next section). Thus, the Ras proteins work as molecular switches for the activation of signalling cascades. The process of GDP for GTP exchange and activation of Ras can be accelerated by guanine nucleotide exchange factors (GEFs) such as Son of sevenless (Sos). The inactivation of Ras is partly dependent on the intrinsic GTPase activity of Ras leading to Ras·GDP and termination of its actions. However, the intrinsic GTPase activity of Ras is very low and it is thought that GTPase activating proteins (GAP) like Ras·GAP, which in most cases are specific for the Ras family, play a major role in the rapid termination of their actions *in vivo* (Boguski and McCormick, 1993). Other proteins modulating the actions of Ras are the guanine nucleotide dissociation inhibitors (GDI) which, by reducing the exchange of GDP for GTP on Ras, prevents their activation (Boguski and McCormick, 1993).

Upon stimulation with ET-1 or PE in cardiomyocytes, total Ras is maximally activated (Ras·GTP loading) within 1-2 min (Chiloeches et al., 1999), and although it has been possible to confirm this for Ha-Ras and N-Ras, whether or not Ki-Ras is also activated in the same way has not been established. However, activation of all Ras members peptide growth factors such as EGF, has been demonstrated recently (Clerk et al., 2006). Because lower concentrations of ET-1 are needed for the rapid activation of nPKC ϵ (EC₅₀ 1.4-1.7 nM) (Clerk et al., 1994), it is most likely that this PKC has a role in the activation of Ras *in vivo* where lower physiological concentrations of ET-1 than those used in experimental settings are usually found. The mechanisms of Ras activation by PKC are not clear, but

given the maximal activation achieved within 1-2 min by GqPCRs (Chiloeches et al., 1999), it is likely that this process involves the activation of a GEF.

1.3.1.5 Activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade by GqPCR agonists

The amino acid residues in proteins that are susceptible of being regulated through phosphorylation by kinases, or dephosphorylation by phosphoprotein phosphatases are Ser, Thr and Tyr. The kinases and phosphoprotein phosphatases are classified in three groups, Ser-/Thr- specific, Tyr- specific, or capable of modifying Ser-/Thr- and Tyr-, in which case they are termed dual-specificity. One of the most important signalling events involved in cardiomyocyte hypertrophy secondary to the stimulation with ET-1 or PE is the activation of ERK1/2 (Figure 1.2), members of the mitogen-activated protein kinase (MAPK) superfamily. The MAPK cascades are highly conserved signal transduction pathways essential for the adaptation, survival and preservation of homeostasis of the organism (Chan et al., 2001). They comprise three-tiered protein kinase cascades culminating in the dual Thr-/Tyr- phosphorylation in the activation loop of the terminal MAPK. Thus, upstream MAPK kinase kinases (MKKKs) phosphorylate and activate intermediate MAPK kinases (MKKs), which in turn finally phosphorylate and activate the effector MAPKs. To date, four MAPK subfamilies have been extensively characterized. There are two subclasses of ERKs: ERK1 and 2 (ERK1/2), and ERK5 or big MAPK (BMK1). ERKs have the activation motif (Thr-Glu-Tyr) (Payne et al., 1991; Mody et al., 2003). The c-Jun N-terminal kinases (JNKs) have the activation motif (Thr-Pro-Tyr) (Lawler et al., 1998), and p38-MAPKs have the motif (Thr-Gly-Tyr) (Han et al., 1994). The different components of these MAPKs cascades are summarized in Figure 1.3.

For the ERK1/2 cascade the upstream MKKKs are members of the Raf family (A-Raf, B-Raf or c-Raf), which activate the intermediate MKK1 or MKK2 by phosphorylating two Ser-residues on these. MKK1 and MKK2 are dual-specificity kinases and phosphorylate a Tyr- and a Thr- residue on the Thr-Glu-Tyr motif of ERK1/2. The activation of Raf (best characterized for c-Raf) is achieved by its translocation to the plasma membrane, which is triggered by activation of Ras. This promotes the interaction of c-Raf N-terminal regulatory domain with Ras with consequent c-Raf activation, although the mechanism involves additional steps. Briefly, in the inactive state the N-terminal regulatory domain of c-Raf appears to bind to and inactivate the C-terminal kinase domain. c-Raf is also phosphorylated at Ser-259 and Ser-621 and remains bound to 14-3-3 inhibitory proteins in the cytoplasm. Upon Ras-GTP loading, the 14-3-3 protein located at the N-terminal domain of c-Raf is displaced by binding of c-Raf by Ras (Rommel et al., 1997), and Ser-259 is dephosphorylated by protein phosphatase 2A (PP2A) (Ory et al., 2003). Further phosphorylations also occur after binding of Ras to c-Raf, the most critical being phosphorylation of Tyr-341 mediated probably by Src tyrosine kinase, and Ser-338 phosphorylation, which is possibly mediated by p21-activated kinase (PAK) or another unidentified kinase (Wellbrock et al., 2004; Dumaz et al., 2005). These phosphorylations occur at the region between the N-terminal and the C-terminal domains (hinge region), thus releasing the inhibition from the N-terminal regulatory domain over the C-terminal kinase domain.

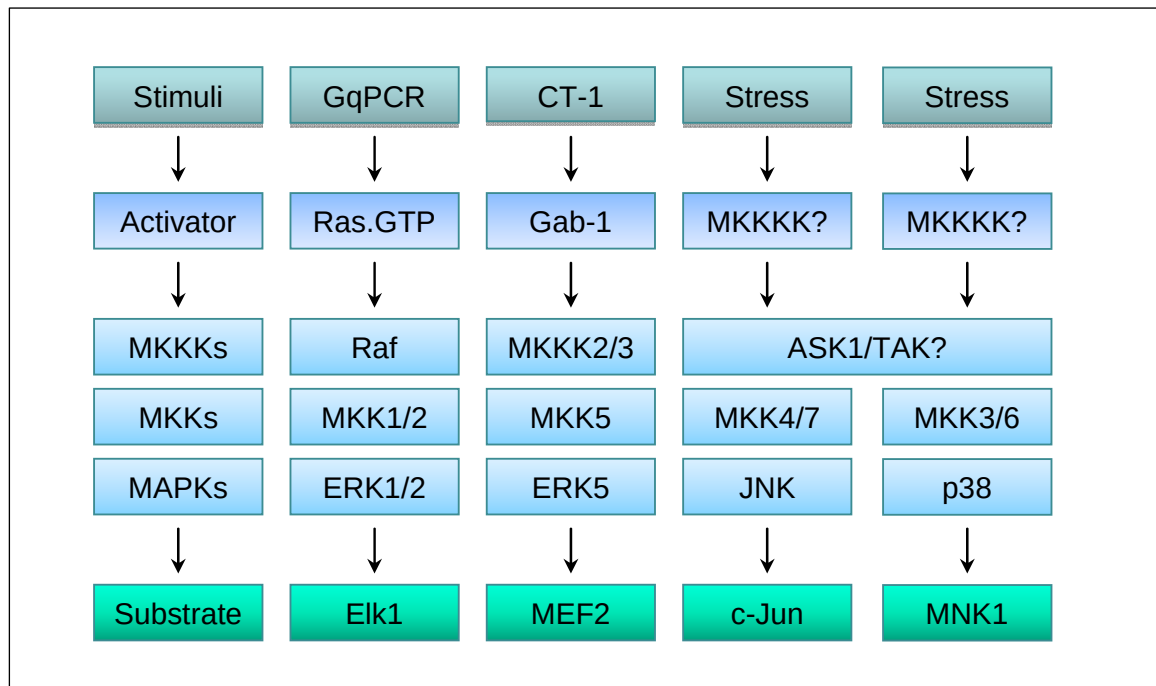


Figure 1.3 Components of the MAPK signalling cascades. MAPKs (mitogen-activated protein kinases) are the final components of three-tiered cascades and phosphorylate and regulate a range of substrates. The best studied MAPKs are ERK1/2, ERK5, p38-MAPKs and JNKs. MAPKs are phosphorylated in their Thr-Xaa-Tyr motif and activated by dual-specificity Thr-/Tyr- MAPKKs (MAPK kinases). MAPKK are in turn phosphorylated and activated by MAPKKKs (MAPK kinase kinases).

As in the case of Ras (Chiloeches et al., 1999), ET-1 stimulation of cardiomyocytes causes a rapid and powerful activation of Raf (c-Raf and A-Raf) which is maximal within 3-5 min (Bogoyevitch et al., 1995a), and also maximal activation of MKK1/2 occurs within 3-5 min (Bogoyevitch et al., 1994). This acute activation of the upstream MKKKs and MKKs of ERK1/2 correlates with the rapid activation of ERK1/2 (maximal within 5 min) following stimulation of cardiomyocytes with ET-1 or PE (Bogoyevitch et al., 1993; Bogoyevitch et al., 1996). The stimulation of cardiomyocytes with phorbol 12-myristate 13-acetate (PMA), a phorbol ester which acts as a pharmacological mimic of DAG, thus producing sustained activation of DAG-sensitive PKCs, causes acute activation of ERK1/2 which is initially detected in the cytoplasm and after 4 min in the nucleus (Chiloeches et al., 1999), suggesting that activated ERK1/2 may influence the phosphorylation state of nuclear proteins such as transcription factors and, by implication, influence transcriptional regulation in cardiomyocytes.

ERK1/2 are Pro- directed Ser-/Thr- kinases which phosphorylate Ser-/Thr- residues usually located towards the N-terminal of Pro- residues (Songyang et al., 1996). However, the Ser-/Thr-Pro- sequence is very common with up to 80% of all proteins exhibiting this sequence, leading to the conclusion that other mechanisms are involved in determining the substrate specificity of ERK1/2. Several of the important factors determining the specificity of the substrates of ERK1/2 may include the presence of docking sites (i.e. short amino acid sequences) on the potential substrate (reviewed in Bardwell, 2006), scaffold proteins such as kinase suppressor of Ras which, in addition to increasing the specificity, also appears to increase the efficiency of activation of the cascade (reviewed in Sacks, 2006), and compartmentalization (e.g. nuclear localization)

of the ERK1/2 signal (reviewed in Kolch, 2005). The substrates of ERK1/2 are not completely characterized but some of them include transcription factors (reviewed in Yoon and Seger, 2006), which is consistent with the nuclear localization of ERK1/2 within 4 min after stimulation with PMA (Chiloeches et al., 1999). As an example, one of these transcription factors is Elk-1, a member of the ternary complex factor subfamily of ETS-domain transcription factors. The phosphorylation of the C-terminal activation domain of Elk-1 induces a conformational change accompanied by the stimulation of DNA binding and transcriptional activity (Marais et al., 1993; Yang et al., 1999). The transcription factor c-Fos, an IEG, is a member of the activated protein 1 (AP-1) family of transcription factors (see section 1.5). It has been shown that upregulation of c-Fos upon stimulation of cardiomyocyte hypertrophy with PE requires the phosphorylation of Elk-1 and binding Elk-1 to the promoter of *c-fos*, all of which is dependent on the activation of the ERK1/2 cascade (Babu et al., 2000).

ERK1/2 are also involved in the regulation of the translational machinery, suggesting that the ERK1/2 cascade is also partly responsible for increased protein synthesis, a characteristic feature of the increased cellular mass observed during the growth response of cardiomyocytes induced by GqPCR agonists. Many mRNAs contain a 7methyl-GTP 5'-cap structure which is required for cap-dependent initiation of translation of mRNA into protein. Translation initiation, the rate limiting step of protein translation, requires the interaction of the 7methyl-GTP 5'-cap structure with the eukaryotic initiation factor 4E (eIF4E) and binding to the initiation complex scaffold eIF4G, thus allowing the formation of active initiation complexes (reviewed in Proud, 2004). The binding of eIF4E and eIF4G is regulated by eIF4E-binding proteins (4EBPs) which, in their dephosphorylated state, bind to and inhibit eIF4E (Gingras et al., 1999a). Several phosphoacceptor sites have

been described for 4EBP1, and phosphorylation of these reduces the ability of 4EBP1 to interact with eIF4E (Gingras et al., 1999b). Phosphorylation of 4EBP1 by ET-1 or PE was recently demonstrated in cardiomyocytes and appears to be mediated through the ERK1/2 pathway acting in concert with other regulatory proteins like the 70-kDa ribosomal S6 kinase (S6K1) and mammalian target of rapamycin (mTOR), thus activating protein synthesis (Wang and Proud, 2002).

1.3.1.6 Role of the ERK1/2 cascade in cardiac hypertrophy

As stated earlier, ERK1/2 are strongly activated by hypertrophic stimuli and appear to be important for the hypertrophic process (Yue et al., 2000; Sugden, 2001; Sugden, 2003). The involvement of the ERK1/2 cascade in cardiomyocyte signalling to hypertrophy has been evaluated using small molecule inhibitors of the cascade such as U0126 and PD98059. These inhibitors, used at a concentration of 10 μ M for U0126 and below 50 μ M for PD98059, are highly selective for inhibition of MKK1/2 in the ERK1/2 cascade in several systems including cardiomyocytes (Alessi et al., 1995; Favata et al., 1998; Clerk et al., 2002). Thus, U0126 selectively inhibits ET-1 or PE-induced activation of ERK1/2, and this is associated with inhibition of the associated increases in protein synthesis and cell size (Yue et al., 2000). Indeed, several characteristics usually associated with cardiomyocyte hypertrophy (i.e. expression of ANP) are attenuated with the use of U0126 following stimulation with ET-1 or PE (Yue et al., 2000). Preincubation of cardiomyocytes for 15 min with the ERK1/2 inhibitor PD98059, followed by exposure to ET-1 or PE over 24 h, results in decreased myofibrillar organization further supporting a role for the ERK1/2 cascade in cardiomyocyte hypertrophy; however, a decrease in cell size was not observed (Clerk et al., 1998a).

Other studies also show that following PE-stimulation of cardiomyocytes, overexpression of constitutively-activated MKK1 augments ANP promoter activity (one of the marker genes of cardiac hypertrophy), whereas a dominant negative form attenuates this response in cardiomyocytes (Gillespie-Brown et al., 1995). Similarly, downregulation of ERK1/2 expression using antisense oligonucleotides attenuates the morphological characteristics of cardiomyocyte hypertrophy (sarcomerogenesis and cell size) and the expression of the ANP (promoter activity and ANP mRNA) following exposure of cardiomyocytes to PE over 48 h (Glennon et al., 1996).

The important role of the ERK1/2 cascade in hypertrophy is also highlighted by studies in genetically-modified mice with cardiospecific overexpression of a constitutively-activated form of MKK1 under the control of α -myosin heavy chain (α -MHC) promoter (Bueno et al., 2000). In this model, the resultant phenotype is mild concentric cardiac hypertrophy characterized by a thicker septum, thicker left ventricular posterior wall and reduced ventricular chambers size. There was also increased contractility in MKK1 transgenic mice compared with wild-type animals at 2 and 6 months of age consistent with a hyperdynamic ventricular function, which suggests a phenotype of compensated cardiac hypertrophy (Bueno et al., 2000). MKK1 transgenic animals also demonstrated increased resistance to ischaemia/reperfusion induced apoptosis (Bueno et al., 2000). Taken together, the studies mentioned above suggest a strong relationship between the activation of the ERK1/2 pathway in mediating the cardiac hypertrophic response both in vitro and in vivo, and this appears to be associated to an intrinsic cytoprotective effect of this pathway in the heart.

1.3.2. Other signalling cascades activated by GqPCR agonists

In addition to the ERK1/2 signalling cascade, other signalling cascades are also activated by GqPCR agonists including the p38-MAPK and JNK cascades, Calcineurin-NFAT (nuclear factor of activated-T cell transcription) pathway, and the PKB also known as Akt pathway. The PKB/Akt pathway is not as strongly activated by GqPCR agonists as it is by growth factors like EGF, insulin and IGF-1, therefore this pathway will be discussed in section 1.4.

1.3.2.1 Stress-activated protein kinase cascades

Other members of the MAPKs also activated by ET-1 or PE are the JNK and p38-MAPKs, commonly termed stress-activated protein kinases (SAPKs) because they are potently activated by cytotoxic stresses (e.g. hyperosmotic shock, oxidative stress, ischaemia/reperfusion) (Figure 1.3). JNKs have the activation motif (Thr-Pro-Tyr) (Lawler et al., 1998), and p38-MAPKs have the activation motif (Thr-Gly-Tyr) (Han et al., 1994). The dual specificity MKKs responsible for JNK activation are MKK4 (also known as SEK-1) and MKK7, and those for p38-MAPK are MKK3 and MKK6. Upstream, several MKKKs analogous to Raf have been described (e.g. apoptosis signalling regulating kinase 1, ASK1) (Kyriakis and Avruch, 2001) (Figure 1.3). Three *Jnk* (*Jnk1-3*) genes generate at least 10 JNK protein isoforms by alternative-splicing (Gupta et al., 1996). JNK1 and JNK2 are expressed in many tissues including the heart (Liang et al., 2003; Kaiser et al., 2005), whereas JNK3 proteins are mainly expressed in the brain (Mohit et al., 1995; Martin et al., 1996). However, as in other tissues, JNK1 is the main JNK activated in cardiomyocytes and is identified as two bands of 46 and 54 kDa by immunoblotting (Bogoyevitch et al., 1995c, Clerk et al., 1998b). Of the four genes encoding p38-MAPKs (α , β , γ and δ), the most highly expressed protein isoforms in

the human heart are the alternatively spliced isoforms p38-MAPK- α 1 and α 2, with little or no detection of the alternatively-spliced p38-MAPK β 1 or β 2 (Lemke et al., 2001). Although p38-MAPK γ (also known as ERK6 or SAPK3) and p38-MAPK δ are also expressed in the heart, their functional role is not clear (Lemke et al., 2001; Court et al., 2002).

Hypertrophic agonists such as ET-1 or PE activate JNKs and p38-MAPKs in cardiomyocytes, but this activity is comparatively less than that obtained by cellular stresses particularly when compared with hyperosmotic shock (0.5 M sorbitol) (Bogoyevitch et al., 1995c; Clerk et al., 1998a). Consistent with an activating pathway through G α q, activation of JNK and p38-MAPK cascades has also been observed in transgenic mice with cardiac overexpression of G α q (D'Angelo et al., 1997). The role of SAPKs in cardiomyocyte hypertrophy or even in cell death is controversial (Sugden and Clerk 1998; Liang and Molkenstein 2003; Petrich and Wang, 2004) and possibly depends on the signalling context and status of the cell.

1.3.2.2 Role of stress-activated protein kinase cascades in cardiac hypertrophy

Initial studies indicating that JNKs and p38-MAPK are activated by GqPCR agonists (Bogoyevitch et al., 1995c; Clerk et al., 1998a) suggested a role for SAPKs in the regulation of cardiomyocyte hypertrophy. Further studies using PE in the isolated perfused rat heart revealed activation of SAPKs as well as ERK1/2 cascades (Lazou et al., 1998) and led to the more general hypothesis that all MAPKs could be involved in the regulation of cardiac hypertrophy. This hypothesis was further supported by studies involving overexpression or inactivation of the different components of these signalling cascades in cultured cardiomyocytes. Thus, overexpression of constitutively-activated

MKK3 or MKK6 (caMKK3 or caMKK6) results in cardiomyocyte hypertrophy (increased cell size and sarcomeric organization) and correlates with the expression of ANP, implicating p38-MAPK in the development of the hypertrophic phenotype (Zechner et al., 1997; Nemoto et al., 1998; Wang et al., 1998a). Similarly, the use of the p38-MAPK α/β inhibitor SB203580 blocks all growth features (increased cell size, and ANP and α -skeletal actin promoter activities) in cardiomyocytes exposed to caMKK6 or PE (Zechner et al., 1997). Although, collectively these studies suggest an important role for the p38-MAPK cascade in mediating cardiac hypertrophy, other studies indicated that blockade of this pathway with the inhibitor SB203580 is not sufficient to attenuate all aspects of ET-1 or PE-induced hypertrophy (Choukroun et al., 1998; Clerk et al., 1998a).

A similar paradigm was also established for the JNK cascade in cardiac hypertrophy. Thus, the hypertrophic agonists, ET-1 or PE, induce activation of JNK in cardiomyocytes (Bogoyevitch et al., 1995c; Ramirez et al., 1997). ANGII infusion in rats cause elevated blood pressure and cardiac hypertrophy. This is associated with increased activity of the JNK cascade, but not the ERK1/2 cascade, implicating JNKs in cardiac hypertrophy secondary to hypertension induced by ANGII (Yano et al., 1998). Overexpression of wild-type MKK7 or constitutively active MKK7 (caMKK7) also leads to cardiomyocyte hypertrophy as evidenced by increased cell size, sarcomeric organization and expression of ANP (Wang et al., 1998b). Consistent with a role for the JNK cascade in cardiomyocyte hypertrophy, overexpression of a dominant negative mutant of MKK4 (dnMKK4) decreases all aspects of hypertrophy in cardiomyocytes exposed to ET-1 or PE (Choukroun et al., 1998; de Windt et al., 2000), as well as cardiac hypertrophy induced in vivo by pressure-overload in rats (Choukroun et al., 1999).

Taken together, all the above data suggest that there is a strong link between activation of SAPKs and cardiac hypertrophy. However, the use of in vivo gain of function studies of JNK or p38-MAPK cascades in transgenic mice overexpressing activated forms of several components of these cascades does not produce compensated cardiac hypertrophy (in contrast to overexpression of components of the ERK1/2 cascade in the heart, see section 1.3.1.6), but are usually associated with decompensated heart failure and myocardial fibrosis (see Table 1.1). Thus, transgenic mice with cardiospecific overexpression of either MKK3 or MKK6 present bilateral atrial enlargement and ventricular interstitial fibrosis without any significant increase in heart-weight to body-weight ratios (Liao et al., 2001). Interestingly, the MKK3 transgenic mice exhibit myocyte atrophy rather than hypertrophy (Liao et al., 2001). Transgenic mice overexpressing MKK7 in the heart, also exhibit bilateral atrial enlargement with little or no ventricular enlargement. These transgenic animals also present with diastolic dysfunction, secondary to increased myocardial stiffness probably as a consequence of extracellular matrix remodelling (e.g. increased fibronectin deposition) (Petrich et al., 2002; 2003; 2004). These studies suggest that unregulated overexpression of upstream MKKs for both p38-MAPK and JNK cascades leads to restrictive cardiomyopathy in the absence of ventricular hypertrophy, which is opposite to the findings of most studies performed in cultured cardiomyocytes.

Consistent with the above in vivo data, other studies using either gene-targeted mice or transgenic overexpression in the heart of dominant-negative proteins for one or more components of the SAPKs cascades have suggested that JNKs and p38-MAPKs may indeed have an antihypertrophic effect in the heart (Table 1.1). For instance, mice expressing dominant-negative mutants of p38-MAPK α (dn-p38-MAPK α), dnMKK3 or dnMKK6, under the control of the α -MHC promoter show selective reduction in p38-

MAPK activity associated with progressive concentric hypertrophy at 2-4 months of age. After 8 months this progresses to lethal cardiomyopathy in most animals (Braz et al., 2003). One mechanistic explanation of the hypertrophic response in these transgenic animals may be attributed to the increased activity of NFAT dependent transcription (Braz et al., 2003) (see section 1.3.2.3).

Similarly, inhibition of the JNK cascade through inactivation of several components of this cascade has also revealed an antihypertrophic and cytoprotective role for this signalling cascade (Table 1.1). Thus, transgenic animals overexpressing dominant-negative forms of JNK1 or JNK2 (dnJNK1 or dnJNK2) or knockout mice with targeted deletion of JNK1^{-/-}, JNK2^{-/-} or three allele JNK1^{+/-}-JNK2^{-/-}, all exhibit selective reduction in JNK activity associated with increased cardiac hypertrophy following TAC (Liang et al., 2003). The morphological pattern found in these animals was consistent with marked eccentric hypertrophic growth (Liang et al., 2003). These data suggest that the JNK cascade probably antagonises adaptive cardiac hypertrophy in vivo. The increased hypertrophic response observed in these genetically-modified mice with reduced JNK activity is linked to increased activity of NFAT transcription factors, demonstrated in vitro as well as in vivo (see section 1.3.2.3). Consistent with this, concomitant targeting of the *calcineurin A β* gene, a regulator of the NFAT transcription factor (see section 1.3.2.3), abrogates the hypertrophic response observed in dnJNK1 and dnJNK2 transgenic animals (Liang et al., 2003).

Collectively the data presented above are consistent with a complex role for the SAPKs in regulating cardiac hypertrophy with opposing results depending on the model studied. As stated earlier, this may represent the intrinsic complexity of the role of these MAPKs in

the regulation of the hypertrophic or even the apoptotic response in cardiomyocytes (see Table 1.1). The final event (hypertrophy vs buffering of hypertrophy or apoptosis vs cytoprotection) may ultimately depend on the signalling context and status of the cardiomyocyte at the time of application of a particular stimulus.

1.3.2.3 Calcineurin/NFAT pathway in cardiac hypertrophy

The Ca^{2+} /calmodulin-activated protein Ser-/Thr-phosphatase calcineurin (also known as protein phosphatase 2B), represents an important signalling pathway in the development of cardiac hypertrophy and heart failure (Heineke and Molkentin, 2006). This signalling pathway and the consequences of its activation were initially described in the context of T cell activation, the classical system of study. Thus, T cell receptor engagement increases intracellular Ca^{2+} which binds to calmodulin, a Ca^{2+} sensor protein, and then the Ca^{2+} -calmodulin complex binds to and activates calcineurin (Crabtree and Olson, 2002; Gallo et al., 2006). Calcineurin is conserved from yeast to humans and is composed of three subunits: a catalytic A subunit (CnA) of approx 57-61 kDa, bound to two small EF-hand calcium binding proteins, the regulatory subunit calcineurin B (CnB) and calmodulin (Klee et al., 1998). In mammals, three different genes encode three separate CnA isoforms (CnA α , CnA β and CnA γ), of which CnA α and CnA β are ubiquitously expressed whereas CnA γ is expressed only in testis and brain (Rusnak and Mertz, 2000; Molkentin, 2000). Two genes encode two different isoforms of the regulatory subunit in mammals (CnB1 and CnB2), of which CnB1 is ubiquitously expressed in many tissues including the heart (Rusnak and Mertz, 2000; Molkentin, 2000).

Table 1.1. SAPKs cascades in the heart – studies of gain and/or loss of function. This table is limited to studies involving MAPKKs, MAPKKs or MAPKs for JNK or p38-MAPK cascades. Commonly-used transcriptional indices of hypertrophy are increased expression of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), skeletal muscle (SkM) α -actin and β -myosin heavy chain (β -MHC); reduced expression of α -myosin heavy chain (α -MHC), sarcoplasmic (endoplasmic) reticulum Ca²⁺-ATPase 2 (SERCA2) and possibly phospholamban (PLB).

Target Gene	Phenotype	Reference
JNK Cascade		
Conditional ¹ or inducible ² expression of activated MKK7.	Development of cardiomyopathy/congestive heart failure and premature death (~7 w). Bilateral atrial but little or no ventricular enlargement. Ventricular extracellular matrix ‘remodelling’ with increased fibronectin deposition and increased ‘stiffness’. Ventricular gap junction ‘remodelling’ and slowed conduction. No increase in apoptosis.	Petrich et al., 2002, 2003, 2004
JNK1 ^{-/-} , JNK2 ^{-/-} , JNK3 ^{-/-} , JNK1 ^{+/-} /JNK2 ^{-/-} . Inhibitory JNK1/JNK2.	Reduction in JNK activity increases hypertrophy in the basal state or following TAC. Antagonised by targeted inactivation of <i>calcineurin A</i> gene. Reduction in JNK activity also protects against I/R damage and apoptosis, and promotes anti-apoptotic phosphorylation of Bad. JNKs interact with the calcineurin-NFAT pathway to promote nuclear export of NFATs thus preventing hypertrophy.	Liang et al., 2003a Kaiser et al., 2005
MKK7-JNK1 chimaeric gene. Activated MKK7.	MKK7-JNK1 antagonises hypertrophy after expression of activated calcineurin. With activated MKK7, lethality before breeding age in the majority of lines. In one line: severe dilated cardiomyopathy at 5 w. One low copy number line: 4-5 fold increase JNK activity with some evidence of ERK1/2 activation. Normal histological examination, slightly hyperdynamic/hypertrophied hearts. Paradoxical protection against I/R damage and myocyte apoptosis, with anomalous decrease in anti-apoptotic phosphorylation of Bad.	Liang et al., 2003a Kaiser et al., 2005
JNK1 ^{-/-} , JNK2 ^{-/-} , JNK3 ^{-/-} .	Hypertrophy following TAC same as wild-type, but JNK1 ^{-/-} shows an early decrease in mechanical performance and an increase in apoptosis. Normalised at later stages. JNK1 protective in the initial stages of pressure-overload, preventing early deterioration.	Tachibana et al., 2006
p38-MAPK Cascade		
Conditional ¹ expression of activated MKK3 or MKK6.	Although producing a slightly different phenotype, each transgene resulted in early mortality (5-7 w) secondary to heart failure with systolic depression. Bilateral atrial enlargement and ventricular interstitial fibrosis without significant increase in Hwt/Bwt. Myocyte atrophy with MKK3 transgene. Hypertrophic pattern of gene expression. Therefore increased activity of p38 MAPK in the heart leads to cardiac remodelling and restrictive cardiomyopathy.	Liao et al., 2001
Inhibitory p38-MAPK, MKK3 or MKK6.	Enhanced increase in Hwt/Bwt at baseline or in response to AAB compared with wild-type. Late lethal cardiomyopathy with inhibitory p38-MAPK or MKK3 genotypes. Increased interstitial fibrosis and decreased fractional shortening. Increased NFAT-dependent transcription. Increased expression of ANF and BNP. Inhibitory p38-MAPK-induced hypertrophy and contractile dysfunction antagonised by targeted inactivation of calcineurin A gene. Protection against I/R damage including apoptosis.	Braz et al., 2003, Kaiser et al., 2004
Conditional ¹ targeted inactivation of p38-MAPK.	Normal heart structure and function. Increase in Hwt/Bwt after TAC identical to control. After TAC or infusion of isoproterenol, targeted inactivation was associated with LV systolic dysfunction, dilatation, myocardial fibrosis and increased apoptosis. After TAC, targeted inactivation also associated with increased JNK and MKK3/MKK6 phosphorylation with no change in ERK1/2 phosphorylation.	Nishida et al., 2004
Overexpression of MKK6.	MKK6-overexpression increased resistance to I/R damage, and decreased apoptosis. Bcl-2 expression was increased.	Martindale et al., 2005

Notes: ¹Conditional gene-targeted inactivation or expression is achieved by loxP site/Cre-mediated recombination with the floxed target gene and Cre expression controlled by a cardiac myocyte-specific, postnatally-expressed promoter (often the α -MHC promoter). ²A chimaeric gene in which the Cre coding region was ligated to a copy of the oestrogen-binding domain of the oestrogen receptor (mER) at both the 5' and the 3' ends was placed under the control of the α -MHC promoter (α -MHC-mER-Cre-mER). Expressed Cre is inactive until tamoxifen administration removes the inhibitory effect of mER. Abbreviations: AAB, banding of the abdominal aorta (to induce haemodynamic pressure-overload); ASK1, apoptosis signal-regulating kinase; Hwt/Bwt, heart weight/body weight ratio; I/R, ischemia/reperfusion; TAC, transverse aortic constriction (to induce haemodynamic pressure-overload). Adapted from Clerk et al. (2007).

In T lymphocytes the major biological responses to calcineurin involve activation of NFAT transcription factors, which are encoded by five genes (NFATc1-NFATc4 and NFAT5) producing multiple alternatively-spliced transcripts (Crabtree and Olson, 2002). In their phosphorylated form, NFAT transcription factors are retained in the cytoplasm possibly bound to 14-3-3 inhibitory proteins (Liao et al., 2005). Upon dephosphorylation by calcineurin, NFATs translocate to the nucleus with subsequent activation of transcriptional activity. In cardiomyocytes, a similar sequence of events has been proposed. Interestingly, in this system, the calcineurin/NFAT pathway represents a mechanism whereby changes in cytosolic Ca^{2+} concentrations can affect growth of the cardiomyocyte. In cardiomyocytes, ET-1 causes dephosphorylation of NFATc1 which translocates to the nucleus. This translocation is prevented by cyclosporine A, an immunosuppressant commonly used to implicate calcineurin in biological responses (Kawamura et al., 2004). Indeed, some aspects of the effects of ET-1 and PE on *calcineurin-A* gene transcription have also been identified (Oka et al., 2005).

Most evidence implicating calcineurin/NFATs in cardiac hypertrophy derives from genetically-modified mice with overexpression of regulatory components of the pathway in the heart. Thus, transgenic mice overexpressing activated forms of calcineurin or NFATc4 in the heart results in a massive form of cardiac hypertrophy which frequently evolves to heart failure and death (Molkentin et al., 1998). However, cumulative evidence suggests that NFATc3 (rather than NFATc4, as was initially proposed) is an important mediator of the hypertrophic response (Molkentin et al., 1998; Wilkins et al., 2002; Wilkins and Molkentin, 2004). Thus, CnA β or NFATc3 null mice, but not NFATc4 null mice, exhibit impaired hypertrophic responses following pressure-overload induced by abdominal aortic constriction, or following ANGII or isoproterenol infusion (Bueno et al.,

2002; Wilkins et al., 2002). The general view with respect to the role of the calcineurin/NFAT signalling cascade in cardiac hypertrophy is that activation of calcineurin in the heart is associated with the development of cardiac hypertrophy which ultimately progresses to decompensated heart failure (Wilkins and Molkentin, 2004).

1.3.2 Signal transduction pathways activated by peptide growth factors in cardiomyocytes

The hypertrophic response of cardiomyocytes has been studied mostly using GqPCR agonists. However, cardiomyocytes also respond to peptide growth factors which activate RPTKs, although there are differences in their relative potency for the activation of the pathways involved in cellular growth in many cell systems including cardiomyocytes. For the purpose of this overview, the model described will be the activation of the EGFR and downstream signalling cascades by the peptide growth factor EGF. The differences in the relative degree of activation of the ERK1/2 and PKB/Akt pathway in cardiomyocytes by several peptide growth factors and their contribution to cardiomyocyte hypertrophy will also be discussed.

1.3.2.1 Activation of the ERK1/2 cascade by peptide growth factors

The general mechanisms of activation of signalling pathways by the EGFR and other RPTKs upon binding by their cognate ligands were elucidated during the 1990s (for reviews see Schlessinger, 2000; Dreux et al., 2006; Iwamoto and Mekada, 2006). With the exception of the insulin receptors, all other RPTKs (i.e EGFR, FGFR) remain as inactive monomers in the cell membrane, and form dimers upon stimulation by the agonists. There are four EGF receptors, known as ErbB1 (EGFR), ErbB2, ErbB3 and

ErbB4. However, only ErbB1 (the archetypical EGFR) and ErbB4 possesses both ligand-binding and tyrosine-kinase activity. In addition to EGF, other EGF-related peptides also bind to the EGFR, including transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (Ereg), amphiregulin and betacellulin.

Upon binding of EGF to the EGFR, there is homo- or heterodimerization of the receptor (e.g. ErbB1 dimerization), thus inducing a conformational change in the receptor resulting in autophosphorylation of the intracellular tyrosine-residues secondary to activation of the tyrosine-kinase domains of the receptor. These tyrosine autophosphorylations occur in regions of the receptor devoid of catalytic activity. These sites function as binding sites for a variety of signalling proteins containing SH2 (Src homology 2) domains or PTB (phosphotyrosine binding) domains, including adaptor proteins such as Grb2 and Shc. In particular, Grb2 links a variety of cell surface receptors to the Ras/ERK1/2 signalling cascade. Thus, Grb2 interacts with the dimerized and activated ErbB1 receptor via its SH2 domain and through its SH3 domain recognizes proline-rich sequences in the Ras GEF, Sos. This places Sos at the plasma membrane where it activates Ras (Pawson, 1995; Schlessinger, 2000) (Figure 1.4). The ERK1/2 cascade is then activated as described in section 1.3.1.5. The ERK1/2 cascade is maximally activated within 5-10 minutes by EGF through Ras/Raf and a similar sequence of events also occurs with FGF (Clerk et al., 2006). However, despite PDGF causes a similar activation of this cascade, PDGF uses an alternative pathway requiring tyrosine phosphorylation of PLC- γ 1 and subsequent activation of DAG-dependent nPKC δ (as evidenced by Tyr- phosphorylation) (Clerk et al., 2006). Although insulin and IGF-1 also activate the ERK1/2 pathway, in cardiomyocytes this is significantly less than that for GqPCR agonists and that for EGF, FGF and PDGF (Clerk et al., 2006).

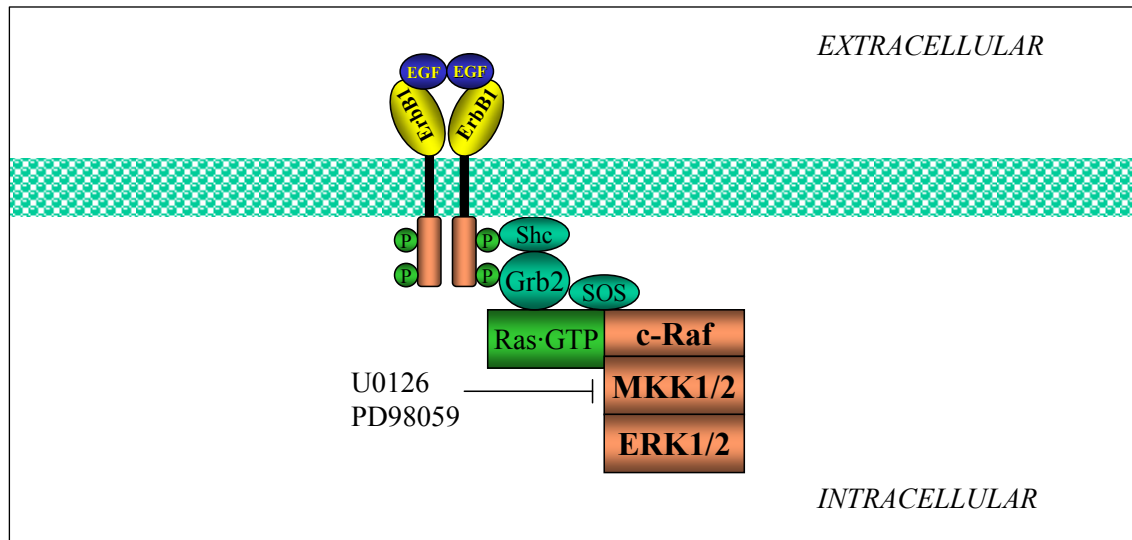


Figure 1.4 EGFR-mediated activation of the ERK1/2 pathway. Upon stimulation with EGF, the EGFR (ErbB1) heterodimerizes and is autophosphorylated on Tyr-residues in the intracellular domains. This allows the binding of adaptor proteins (Shc and Grb2) through their Src homology 2 (SH2) domains. The Grb2 SH3 domain binds to son of sevenles (Sos), thus positioning this guanine exchange factor (GEF) in the membrane where it activates Ras, and the cascade of events that culminates in the activation of ERK1/2.

The transactivation of RPTKs by GqPCRs has also been demonstrated in several systems including cardiomyocytes. For ANGII the mechanism appears to involve the direct transactivation of EGFRs by several residues that have been mapped to the C-terminal domain of AT1 receptors with the Tyr-319 in the motif Tyr-Ile-Pro-Pro (YIPP) critical for this transactivation mechanism (Seta and Sadoshima, 2003). In support of a role for this mechanism of EGFR transactivation in pathological hypertrophy, transgenic mice with cardiac overexpression of a mutant form of the receptor Tyr-319-Phe (Y319F) in the YIPP motif (Tg-Y319F), which is devoid of EGFR transactivating activity, or overexpression of wild-type AT1 receptors (Tg-WT) were compared. The Tg-WT presented with significant cardiac hypertrophy compared with Tg-Y319F, which was enhanced by ANGII infusion (Zhai et al., 2006). Upon infusion of ANGII, Tg-Y319F developed significantly less apoptosis and fibrosis than Tg-WT animals.

1.3.2.2 Activation of the PKB/Akt signalling pathway by peptide growth factors

The initial steps in the activation of the PKB/Akt pathway by growth factors proceed in a similar manner as described above, upon stimulation with EGF, there is formation of receptor dimers and autophosphorylation of the ErbB1 receptor. This promotes the recruitment of the p110 α catalytic subunit of phosphatidylinositol 3'-kinase (PI3K) to the cell membrane, which is triggered by the interaction of SH2 domains of PI3K p85 subunit (the regulatory subunit of PI3K) with tyrosine phosphorylated residues of the active ErbB1 receptor. This is followed by the release of an autoinhibitory constraint on p110 α activating its catalytic activity (Figure 1.5) (Schlessinger, 2000). PI3K is a lipid kinase that initiates cell signalling by phosphorylating the hydroxyl group at position 3 in membrane phosphoinositides. Thus, phosphorylation of phosphatidylinositol 4,5 *bis*

phosphate (PtdInsP₂) produces phosphatidylinositol 3,4,5 *tris* phosphate (PtdInsP₃). Accumulating PtdInsP₃ in the membrane leads to recruitment of PDK1 (3-phosphoinositide-dependent protein kinase-1) and PKB/Akt triggering the activation of PDK1 which phosphorylates and activates PKB/Akt (Figure 1.5) (Vanhaesebroeck and Alessi, 2000).

Downstream effectors of PKB/Akt include glycogen synthase kinase 3 (GSK3 α , GSK3 β). Phosphorylation of an N-terminal residue of GSK3 α (Ser-21) or GSK3 β (Ser-9) by insulin inhibits their activity (Cross et al., 1995). Interestingly, other kinases also phosphorylate these sites including PKA (cAMP-dependent protein kinase) and the 90-kDa ribosomal protein S6 kinases (p90RSKs) (Cohen and Frame, 2001; Frame and Cohen, 2001). Thus, GSK3 appears to be at a convergence point for several signalling pathways. Several potential targets of GSK3 have been described including a number of transcription factors. Thus, phosphorylation of c-Jun by GSK3 mainly at Ser-243 (although Thr-239 and Ser-249 are also phosphorylated by this enzyme), inhibits its DNA-binding and transactivating activity (Boyle et al., 1991; Nikolakaki et al., 1993). Similarly, phosphorylation of NFAT transcription factors within their NFAT homology domain inhibits their DNA-binding activity and results in their nuclear export (Beals et al., 1997; Neal and Clipstone, 2001). In the active (unphosphorylated) state GSK3 α/β inhibits hypertrophy (reviewed in Kerkela et al., 2006).

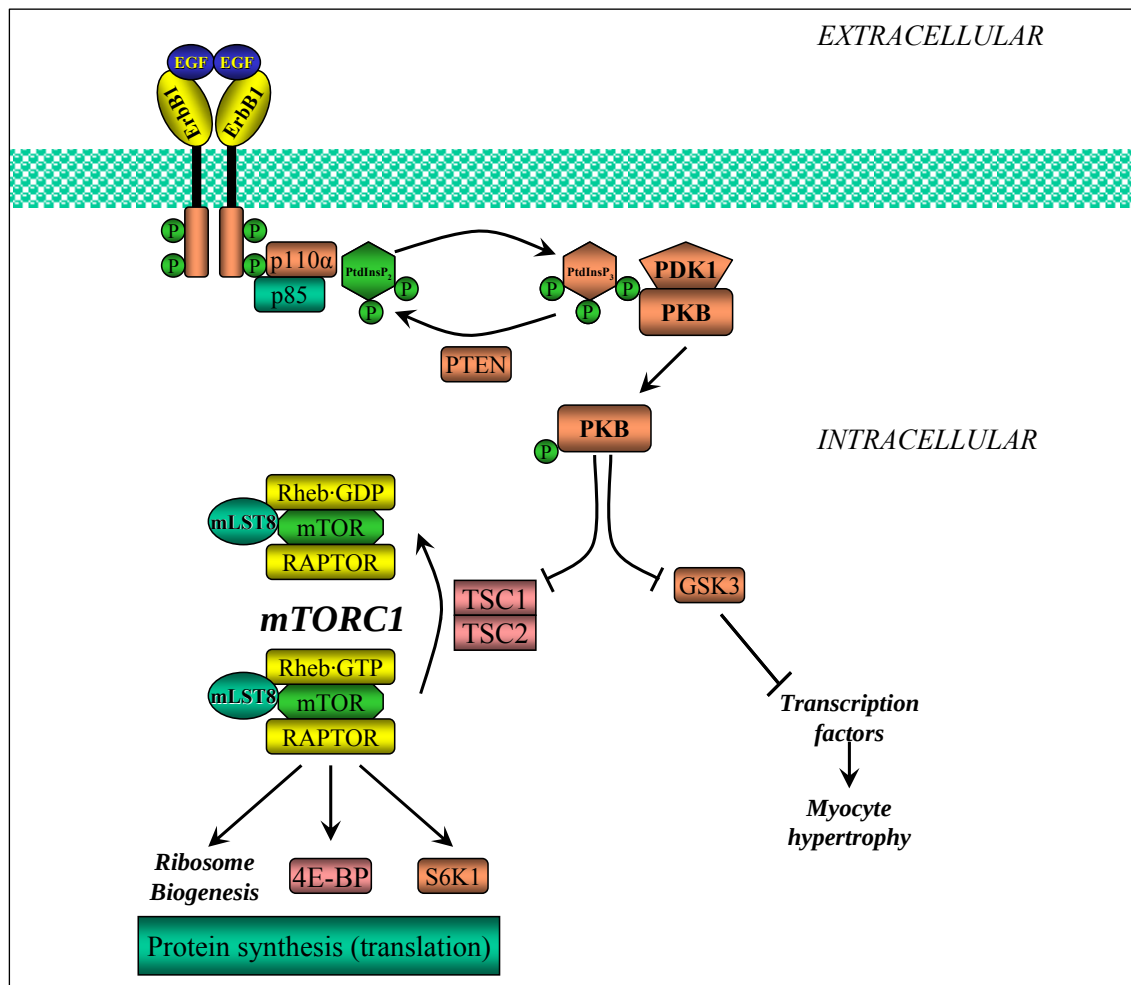


Figure 1.5 Activation of the PKB/Akt pathway by growth factors. Upon activation of the EGFR (ErbB1) by EGF, there is induction of receptor dimerization and autophosphorylation. This triggers the recruitment of PI3K (p110 α catalytic subunit) to the membrane by the regulatory subunit (p85). Activation of PI3K occurs as a result of removal of an autoinhibitory constraint. PI3K then catalyses the formation of phosphatidylinositol 3,4,5 *tris* phosphate (PtdInsP₃) by phosphorylation of phosphatidylinositol 4,5 *bis* phosphate (PtdInsP₂). Accumulating PtdInsP₃ in the membrane leads to recruitment of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PKB/Akt to the membrane, where activated PDK1 phosphorylates and activates PKB/Akt. Activation of PKB/Akt leads to phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3 α/β), which may phosphorylate and inactivate of transcription factors (e.g. NFAT). PKB also promotes inhibition of tuberous sclerosis complex proteins (TSC1/2), GTPase-activating proteins for the small G protein Rheb. This promotes increased activity of Rheb-GTP and subsequent activation of mammalian target of rapamycin complex 1 (mTORC1). This complex, containing mTOR, mLST8 and Raptor, is sensitive to the antifungal macrolide rapamycin. Activation of mTORC1, promotes ribosome biogenesis by phosphorylation and inactivation of 4EBP and phosphorylation of S6K1, thus increasing the rate of protein synthesis.

Consistent with the above, inhibition of GSK3 by ET-1 promotes nuclear accumulation of NFAT transcription factors, which then stimulates cardiac hypertrophy (Haq et al., 2000). It has been suggested that this hypertrophic response is mediated, at least in part, by a PI3K-independent kinase. In support of this, activation of the PI3K/Akt pathway by GqPCR agonists like ET-1 is relatively minor compared with the classical regulators of the pathway insulin and IGF-1 (Clerk et al., 2006). In addition, as mentioned above, other kinases activated by ET-1 via ERK1/2, such as p90RSKs (Frödin and Gammeloft, 1999), promote phosphorylation of GSK3 α/β , suggesting the existence of a PKB/Akt-independent route to GSK3 phosphorylation in cardiomyocytes. Interestingly, inhibition of GSK3 with the small molecule inhibitor 1-azakenpaullone does indeed causes hypertrophic growth in cardiomyocytes. However, this growth response is characterized by increased cell length (elongation) which is accompanied by increased binding of JunB (a member of the AP-1 family of transcription factors, see section 1.5) to the AP-1 consensus binding sequence as demonstrated by electrophoretic mobility shift assay (EMSA) (Markou et al., 2008). Comparatively, ET-1 promotes more marked AP-1 transcription factor binding to the AP-1 consensus than 1-azakenpaullone, and PE induces hypertrophic growth of cardiomyocytes by increasing cell size in both length and width (as is observed in concentric hypertrophy following pressure-overload, see section 1.1). In addition, ET-1 causes significantly less phosphorylation of GSK3 compared with insulin. Therefore, although GSK3 may contribute to some aspects of cardiomyocyte hypertrophy, it does not seem to contribute significantly to this response following ET-1-stimulation of cardiomyocytes (Markou et al., 2008).

In other cell systems, activation of PKB/Akt is associated with increased protein synthesis and cytoprotection (Vanhaesebroeck and Alessi, 2000). The cytoprotective effects of this

pathway result from phosphorylation of proteins involved in the apoptotic process such as the pro-apoptotic Bcl-2 family member Bad (at Ser-136), which results in its inactivation, thus preventing apoptosis (Datta et al., 1997; Pesenti et al., 1997). Also associated with cytoprotection are the changes in gene expression mediated by Forkhead box, group O (FoxO, or FOXO) transcription factors, which are involved in the transcriptional regulation of Fas ligand and other apoptotic proteins. The FoxO transcription factors are located to the nucleus upon proapoptotic stimulation, and their phosphorylation by activated PKB/Akt (e.g. by growth factors) causes their translocation to the cytoplasm, thus preventing apoptosis (Sen et al., 2003).

The effects on protein synthesis are mediated by changes in the activities of existing translation factors and the regulation of ribosome biogenesis. In other cell systems, PKB/Akt associates with mammalian target of rapamycin (mTOR) in different complexes (reviewed in Bhaskar and Hay, 2007). mTOR complex 1 (mTORC1), which is sensitive to the antifungal macrolide, rapamycin, is a complex containing mTOR, mLST8 and Raptor (Figure 1.5). In mTORC1, mTOR is activated downstream of PKB/Akt and appears to be involved in protein synthesis by phosphorylation and inactivation of eIF4E binding protein 4EBP, and phosphorylation of S6K1, thus promoting ribosome biogenesis and increased protein synthesis (see Figure 1.5). Whether or not this process also occurs in the heart remains to be determined.

In cardiomyocytes, EGF, PDGF and IGF-1 induce potent activation of PKB/Akt, which is maximal at 5-10 minutes (Clerk et al., 2006). Direct comparison of agonists demonstrates that insulin promotes the most potent activation of PKB/Akt compared with that of PDGF, EGF, FGF and even IGF-1. In contrast, ET-1 promotes minimal activation of

PKB/Akt, with PMA resulting in no measurable effect (Pham et al., 2000; Clerk et al., 2006). Consistent with the studies in cardiomyocytes, in whole hearts, potent activation of PKB/Akt by insulin has also been reported (Huisamen et al., 2001) and a similar response is also observed in mice with cardiac overexpression of IGF-1 (Li et al., 1997; Yamashita et al., 2001). Inhibition of the pathway in cardiomyocytes can be achieved with selective inhibitors of PI3K such as wortmannin and LY294002. These promote reduced hypertrophy of cardiomyocytes with a reduction in the basal rate of protein synthesis and that induced by insulin (Schülter et al., 1998; Pham et al., 2000). Also, genetic manipulation of the PKB/Akt pathway in vitro and in vivo suggests that activation of PKB/Akt promotes cardiomyocyte survival (reviewed in Matsui and Rosenzweig, 2005 and Heineke and Molkentin, 2006). Although it has been suggested that the PKB/Akt pathway is involved in cardiac hypertrophy, the hypertrophic effect of insulin (the most potent activator of the pathway in cardiomyocytes, see above) is minimal compared with that of ET-1. ET-1, in contrast has a minimal effect on the activation of the PKB/Akt pathway, despite potent induction of cardiomyocyte hypertrophy (Clerk et al., 2006).

Overall, it is possible that the PKB/Akt pathway is an important regulator of cardiomyocyte postnatal maturational growth, as a general growth mechanism, coupling insulin levels to nutritional status. It may also be important in the regulation of cardiac hypertrophy in response to exercise training in athletes, so-called “physiological hypertrophy”. Thus, in genetically-modified mice, cardiac overexpression of constitutively-activated p110 α PI3K, causes cardiac hypertrophy, associated with resistance to apoptosis (Shioi, et al., 2000). The opposite is seen with overexpression in the heart of a dominant-negative form of p110 α PI3K, in which the hypertrophic response to exercise is blunted whereas a normal hypertrophic response is still observed in

response to pressure-overload (McMullen et al., 2003). This is in accord with previous observations that IGF-1 is the direct mediator of growth hormone actions. Thus, upon growth hormone release, there is increased secretion of IGF-1 (formerly known as somatomedin-1), which then acts on growing cells by increasing anabolic functions such as protein synthesis, and promoting cell division (Christoforidis et al., 2005). Mice harbouring an Akt1 knockout exhibit a general reduction in the size, with reduced size of all organs including the heart, despite an otherwise normal phenotype (Cho et al. 2001). Taken together, the overall conclusion is that PKB/Akt pathway may play an important role in myocyte general growth and survival (Heineke and Molkentin, 2006). However, despite a probable important role for this pathway in cardiac postnatal eutrophy and physiological cardiac hypertrophy, the current evidence in favour of a role for the PKB/Akt pathway in cardiac hypertrophy in response to haemodynamic overloading is not yet conclusive.

1.3.3 Signalling pathways activated by gp130 cytokines

Members of the IL-6 family of cytokines which, for the purpose of clarity in this discussion will be collectively referred to as gp130 cytokines, include CT-1, IL-6, LIF, oncostatin M, interleukin-11, and ciliary neurotrophic factor. Since its discovery, CT-1 has been shown to induce myocyte elongation by directing sarcomeric assembly in series, and these observations have been extended to other members of the gp130 cytokines (Wollert et al., 1996). Therefore, since the discovery of CT-1, this and other members of this family of cytokines have been postulated as probable mediators of eccentric hypertrophy and more importantly the dilatation of the heart observed in the progression to heart failure, assuming that the circumferential arrangement of cardiomyocytes in the heart translates myocyte elongation into ventricular cavity dilatation (see section 1.1)

(Wollert et al., 1996, Hunter and Chien, 1999). The most studied members of this family are CT-1, LIF and IL-6, and the relative order of potency for induction of the hypertrophic response is $CT-1 \geq LIF > IL-6$ (Pennica et al., 1996; Wollert et al., 1996). The receptors for this family are multimeric and share the transducing receptor chain gp130 (also known as CD130) (Bravo and Heath, 2000). Intracellular signalling is activated by inducing homodimerization of gp130 (Murakami et al., 1993) or heterodimerization of gp130 with a related transmembrane protein, the LIF receptor β subunit (LIFR β) (Gearing et al., 1991, reviewed in Müller-Newen, 2003). Thus, LIF or CT-1 signal through gp130/LIFR β heterodimers (Gearing et al., 1991, Wollert et al., 1996). Interestingly, IL-6 requires an additional receptor, the IL-6 receptor α subunit (IL-6R α), and the formation of the IL-6/IL-6R α complex then triggers dimerization of gp130 thus forming a heterotrimeric receptor complex for activation of signal transduction (Taga et al., 1989, reviewed in Henrich et al., 2003).

The gp130/LIF β or IL-6R α /gp130 activate a diverse array of signalling cascades, including the Janus kinase (JAK)/STAT (Signal transducer and activator of transcription) pathway, the PKB/Akt pathway, and the ERK1/2 and ERK5 MAPK signalling cascades (Figure 1.6). Whereas all other cascades activated by these agonists are probably involved in resistance to cell stress and survival (reviewed in Hilfiker-Kleiner et al., 2006), ERK5 seems to be responsible for the hypertrophic response involving elongation of cardiomyocytes observed with these agonists (Nicol et al., 2001; Takahashi et al., 2005). The adapter protein Gab1, a member of the Grb2-associated binder (Gab) family, contains a N-terminal pleckstrin homology (PH) domain, and several conserved tyrosine residues that are targets of tyrosine kinases and binding sites for SH2 and SH3 domains (reviewed in Nishida et al., 2003 and Sármay et al., 2006). Recently it was demonstrated that Gab1

phosphorylation and SHP2 (SH2 domain-containing protein tyrosine phosphatase) binding are required for LIF-induced activation of the ERK5 cascade in cardiomyocytes, which ultimately leads to increased sarcomeric organization in series (myocyte elongation) associated with selective marker gene regulation (Nakaoka et al., 2003) (Figure 1.6).

The N-terminal domain of ERK5 which contains the kinase domain and the activation loop is homologous to that of ERK1/2 (Nishimoto and Nishida, 2006). As with other MAPKs, the activation of ERK5 requires upstream MKKs and MKKKs. Thus, MKKK2 or MKKK3 activates the dual-specificity kinase MKK5, which in turn phosphorylates and activates ERK5 (Figure 1.3) (Chao et al., 1999; Sun et al., 2001). In support of a major role for the ERK5 cascade in promoting an elongated phenotype in cardiomyocytes, overexpression of constitutively-active MKK5 elicits serial assembly of sarcomeres, whereas a dominant-negative form of MKK5 blocks LIF-induced elongation of cardiomyocytes (Nicol et al., 2001). Consistent with the in vitro effects of the ERK5 cascade, cardiac overexpression of constitutively-activated MKK5 in transgenic mice results in eccentric hypertrophy, heart failure and sudden death (Nicol et al., 2001).

Although initial studies suggested that the JAK/STAT3 pathway could also be involved in the hypertrophic response to these agonists, this is not supported by a recent study which systematically assessed different components of the cascades activated by the gp130 receptor signalling system (Takahashi et al., 2005). Whereas overexpression of suppressor of cytokine signalling (SOCS) 1 or 3, endogenous inhibitors of the JAK/STAT cascade, inhibit elongation of cardiomyocytes secondary to CT-1 stimulation, overexpression of a dominant-negative STAT3 mutant (STAT3F) does not prevent the elongation in this setting, despite a reduction in STAT3 phosphorylation. This suggests that another pathway apart from STAT3 is involved in elongation of myocytes following CT-1 exposure. In this same study, dominant-negative MKK1 only inhibited the hypertrophic response of cardiomyocytes in response to ET-1, whereas overexpression of dominant-negative MKK5 completely abrogated cardiomyocyte elongation in response to CT-1, thus clearly demonstrating that the ERK5 cascade is responsible for the hypertrophic phenotype observed with CT-1 (Takahashi et al., 2005).

Importantly, myocardial expression of CT-1 and LIF is augmented in animal models of heart failure (Aoyama et al., 2000; Jougasaki et al., 2003) and in explanted hearts from patients with chronic heart failure (Zolk et al., 2002). However, in the study of Zolk et al. (2002), the protein levels of gp130 were decreased, with a concomitant increase in gp130 transcript levels, indicative of augmented gp130 protein turnover, potentially a consequence of adaptive desensitization and degradation with concomitant reduced protein synthesis (Zolk et al., 2002). Consistent with tissue levels, circulating levels of CT-1 are increased in patients with dilated cardiomyopathy (Tsutamoto et al., 2001), and IL-6 and CT-1 circulating levels have been proposed to be important prognostic markers

of death and/or heart failure in patients following myocardial infarction (Takahashi et al., 2003; Fisman et al., 2006). Thus, accumulating evidence implicates gp130 cytokines and the activation of the ERK5 cascade by these agonists in eccentric hypertrophy of the heart which may be responsible for progression to heart failure.

1.4 Oxidative stress and ventricular remodelling

Cardiomyocytes have very limited capacity for regeneration which renders the heart particularly vulnerable to damage induced by, for example, oxidative stress. Cells may die by necrosis and/or apoptosis, secondary to increased production of reactive oxygen species (ROS). ROS production in the heart occurs during ischaemia alone (Ambrosio et al., 1993; Becker et al., 1999), and this is exacerbated by reperfusion (Ambrosio et al., 1993; Du et al., 1998). Even in non-ischemic cardiomyopathy, the increased muscle mass of cardiac hypertrophy (or intrinsic abnormalities during diastole in the pacing-induced heart failure model) renders the heart vulnerable to ischaemia because of reduced coronary reserve, and this is particularly important in the subendocardium due to increased wall stress (Vatner et al., 1990; Vatner et al., 1993; Helmer et al., 1996). ROS production is also implicated in several non-acute processes, whereby increased production of ROS by the mitochondria or outside the mitochondria by enzymes like NAD(P)H oxidase and xanthine oxidase, appear to be involved in the development of cardiac hypertrophy and LV remodelling.

1.4.1 Sources and types of ROS

Several potential sources of ROS in the heart have been identified, including the mitochondria, xanthine oxidases (XO), uncoupled NO synthases, NAD(P)H oxidases (NOX), and infiltrating inflammatory cells. Of note, NOX enzymes appear to be particularly important for redox signalling in the heart and are implicated in processes such as hypertrophy in response to pressure-overload and LV remodelling post MI.

1.4.1.1 Mitochondria

Under normal conditions, up to 1-2% of the mitochondrial O₂ uptake leads to ROS production (Turrens, 1997; Gnaiger and Kuznetsov, 2002) and, as mentioned before, this rate increases during ischaemia and ischaemia/reperfusion (Ambrosio et al., 1993; Du et al., 1998; Becker et al., 1999). These conditions are of high impact to the heart given that up to 40% of the cytoplasmic volume of the cardiomyocyte is occupied by mitochondria (Barth et al., 1992; Ayyetey et al., 1993). Molecular O₂ contains two unpaired electrons on its outermost shell, and is highly reactive. As a consequence, O₂ tends to initiate chain reactions that result in several types of ROS production which may induce irreversible chemical changes in lipids or proteins and therefore cellular damage.

During metabolism, superoxide anion radicals (O₂^{•-}) are a by product of normal respiration through the one-electron reduction of O₂ (Davies 1995; Raha and Robinson, 2000; Genova et al., 2003). Complete reduction of oxygen to water (H₂O), the final event in the electron transport chain requires donation of three more electrons after the formation of superoxide. Superoxide is rapidly metabolized in the mitochondria by the enzyme manganese superoxide dismutase (Mn²⁺-SOD) and in the cytosol by Cu²⁺/Zn²⁺--SOD to form hydrogen peroxide (H₂O₂), a less potent but more stable oxidizing agent

(Kinnula and Capro, 2004). H_2O_2 is usually removed by the actions of catalase, glutathione peroxidase, and peroxiredoxin (Nohl and Jordan, 1980; Halliwell, 1989; Schroder and Ponting, 1998). However, in pathological circumstances in the presence of cuprous or ferrous ions, H_2O_2 forms the highly reactive hydroxyl radical ($\cdot\text{OH}$), a process that could be facilitated by the release of ferrous iron from the iron-sulphur cluster $[\text{Fe}_4\text{S}_4]^{2+}$ containing enzyme aconitase when damaged by $\text{O}_2^{\cdot-}$ (Vasquez-Vivar et al., 2000).

Evidence indicating that mitochondria are an important source of ROS production is derived from experiments in which efflux of H_2O_2 is observed in intact mitochondria as well as from perfused organs, suggesting that mitochondria produce $\text{O}_2^{\cdot-}$ in vivo which then is reduced to H_2O_2 (Chance et al., 1979). $\text{O}_2^{\cdot-}$ production by mitochondria has been attributed to mitochondrial complexes I and III (Raha and Robinson, 2000; St-Pierre et al., 2002; Ide et al., 1999). Increased production of ROS occurs in cardiomyocytes derived from animal models of pacing-induced heart failure and in heart failure secondary to MI in mice (Ide et al., 1999; Ide et al., 2000; Ide et al., 2001). The mitochondria are also a source of ROS (through mitochondrial complex III) in cardiomyocytes exposed to $\text{TNF}\alpha$, and this leads to mitochondrial dysfunction and mitochondrial DNA damage. This suggests that one of the probable pathologic effects of $\text{TNF}\alpha$ during ventricular remodelling is derived, at least in part, from induction of ROS production and secondary mitochondrial dysfunction (Suematsu et al., 2003). Of note, H_2O_2 is implicated as the signalling molecule responsible for apoptotic killing of cells in culture exposed to $\text{TNF}\alpha$ (Pletjuskina et al., 2005).

Although, under normal physiological conditions, the production of ROS by the mitochondria is relatively low (Boveris et al., 1972), a phenomenon of ROS-induced ROS release (RIRR) has been described, whereby mitochondria respond to increased concentrations of ROS (either exogenous or endogenous) by increasing their own ROS production (Nieminen et al., 1997; Zorov et al., 2000; Leach et al., 2002). In the mitochondria, at least two different mechanisms are involved in RIRR, one of them involves the activation of the mitochondrial permeability transition pore (MPTP), and the second mechanism involves opening of the inner mitochondrial anion channel (IMAC) (reviewed in Zorov et al., 2006). The MPTP is a non-specific pore that spans both membranes of the mitochondria and contains the voltage dependent anion channel (VDAC, also known as porin) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner membrane, and cyclophilin D in the matrix, with probably also the peripheral benzodiazepine receptor (PBR), which appears to be in close contact with the VDAC in the outer membrane (Halestrap et al., 2002; Crompton et al., 2002; Galiegue et al., 2003). RIRR through the MPTP leads to an increased burst of ROS production from the electron transport chain, a mechanism which may be involved in the regulation of apoptotic and preconditioning pathways in cardiomyocytes (Shanmuganathan et al., 2005; Davidson et al., 2006). In the second mechanism of RIRR, the opening of the IMAC channel secondary to elevated ROS concentrations also results in an increased transient burst of ROS derived from the electron transport chain. This latter mechanism may be involved in triggering postischaemic arrhythmias (Akar et al., 2005).

Most ROS species like $O_2^{\cdot-}$ and $\cdot OH$ possesses limited diffusion capabilities probably because they are short-lived, so their actions are limited to a short period of time and are confined in space. In contrast, H_2O_2 is a more stable and diffusible form of ROS. Of note,

H₂O₂ could be the key signalling mediator of apoptosis signal propagation in cardiomyocytes through amplification of the response in waves of activation from mitochondrion to mitochondrion via MPTP-mediated RIRR (Lakatta and Sollot, 2002). As mentioned earlier, probably because of its more rapid diffusion, H₂O₂ has been implicated as the signalling molecule responsible for long distance apoptotic killing of cells in culture exposed to TNF α (Pletjuskina et al., 2005).

Exposure to increasing concentrations of H₂O₂ has different effects in cardiomyocytes. Thus, high concentrations of H₂O₂ (> 0.5 mM) cause necrotic cell death (Kwon et al., 2003), whereas intermediate concentrations (0.1 – 0.5 mM) promote an apoptotic phenotype (Cook et al., 1999; Kang et al., 2000). In contrast, low concentrations of H₂O₂ ($\leq$ 0.04 mM) are non-toxic and, interestingly, may be hypertrophic and/or cytoprotective in cardiomyocytes (Kwon et al., 2003). Therefore, H₂O₂ constitutes a physiologically relevant example of oxidative stress that is readily manipulatable in experimental settings.

1.4.1.2 Xanthine oxidases

The enzyme xanthine oxidoreductase (XOR) is encoded by a single gene, but the protein exists as a homodimer in two interconvertible forms, xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (Stirpe and la Conte, 1969). These two forms differ in that XO reduces only oxygen, whereas XDH can also reduce, with higher affinity, NAD⁺ (Waud and Rajagopalan, 1976). Both forms of XOR are reduced during the process of uric acid synthesis from xanthine. The reoxidation process of fully reduced XOR is then carried out in 4 sequential steps. During the first two steps, two electrons are transferred to O₂ molecules, thus generating H₂O₂ (Olson et al., 1974). In the final two steps, the remaining two electrons are transferred separately to O₂ molecules to generate O₂^{•-}. Therefore

during each cycle this enzyme generates two molecules of H_2O_2 and two molecules of $\text{O}_2^{\cdot -}$ (Hille and Massey, 1981). Recent evidence suggest that XOR is expressed at low levels in cardiomyocytes (Cappola et al., 2001), and is probably localized to the sarcoplasmic reticulum (Khan et al., 2004). Elevation of XOR expression and activity has been documented in end-stage human heart failure and in a canine model of pacing-induced heart failure (Cappola et al., 2001; Ukai et al., 2001). Of note, treatment with the XO inhibitor, allopurinol, improves contractility and decreases myocardial oxygen consumption in pacing-induced heart failure in dogs (Ekelund et al., 1999; Ukai et al., 2001).

There is also evidence that allopurinol significantly reduces LV remodelling secondary to experimental MI in mice (Engberding et al., 2004), further supporting a role of increased XO activity in cardiac pathologies. Of note, in a model of pressure-overload induced hypertrophy in rats, the increased XO activity is only detected as congestive heart failure develops, and not during compensated hypertrophy (de Jong et al., 2000). This suggests that, in ventricular remodelling secondary to pressure-overload, the activity of XO is more important at the advanced stages and may play a role in the transition to heart failure (de Jong et al., 2000). Recently, in a model of spontaneously hypertensive rats with established heart failure, treatment with the XO inhibitor oxypurinol for 4 weeks decreased XO activity and superoxide production, all of which correlated with beneficial effects from the treatment as evidenced by reverse remodelling of the left ventricles (Minhas et al., 2006).

1.4.1.3 NAD(P)H oxidases

The membrane-associated NOX enzymes are a major source of ROS in the cardiovascular system. Each enzyme contains a catalytic subunit termed Nox, of which five isoforms have been described (Nox1-5) encoded by separate genes (Lambeth, 2004). Each Nox subunit forms a heterodimer with the p22^{phox} subunit, constituting the heterodimeric catalytic core (or cytochrome b558) where electron transfer from NAD(P)H to molecular O₂ results in the formation of O₂^{•-}. Of note, whereas Nox1 and Nox2 clearly require the association of additional cytosolic regulatory subunits (p47^{phox}, p67^{phox}, p40^{phox} and Rac1) to activate O₂^{•-} production, Nox4 does not require these (Ambasta et al., 2004; Martyn et al., 2006). The tissue distribution of different Nox isoforms varies considerably. Thus, Nox1 is more abundantly expressed in vascular smooth muscle cells (Griendling et al., 2000), whereas Nox2 and Nox4 are abundantly expressed in cardiomyocytes (Li et al., 2002; Xiao et al., 2002), and endothelial cells (Li et al., 2004). In fibroblasts, the expression of Nox2, Nox4 (Colston et al., 2005) and Nox5 (Cucoranu et al., 2005) has been described.

Although during development of cardiomyocytes, Nox4 is the predominant isoform with a role in cardiac differentiation (Li et al., 2006), in the adult heart the predominant isoform is Nox2. Nox2 is implicated in the regulation of redox-sensitive signalling pathways including the modulation of kinase and phosphatase activities (Sabri et al., 2003), gene expression (Wenzel et al., 2001), and ANGII induced superoxide-dependent inactivation of nitric oxide (Kinugawa et al., 2005). Accumulating evidence indicates that NOX enzymes play an important role in cardiac hypertrophy, ventricular remodelling and heart failure. In pressure-overload-induced left ventricular hypertrophy, the expression and activity of Nox2 are increased in cardiomyocytes and endothelial cells (Li et al.,

2002). In humans, the expression of Nox2 in cardiomyocytes is increased after myocardial infarction (Krijnen et al., 2003), and in end-stage heart failure (Heymes et al., 2003). Recently, Nox2 knockout mice (Nox2 ^{-/-}) have provided important information regarding the role of Nox2 in cardiac hypertrophy and remodelling. Nox2 ^{-/-} animals exhibit a blunted hypertrophic response to subpressor doses of ANGII infused over 7 to 14 days, which parallels reduced activation of NOX activity, ANP expression, and reduced myocardial fibrosis compared with wild-type controls (Bendall et al., 2002).

Despite an important role for Nox2 in agonist-induced hypertrophy, the situation may be different during pressure-overload, where Nox2 may play a role in mediating pathological remodelling. Thus, mice deficient in Nox2 subjected to TAC develop cardiac hypertrophy and increased expression of ANP in correlation with increased NOX activity, attributed to Nox4 (Byrne et al., 2003). However, systolic function was relatively preserved with reduction of myocardial interstitial fibrosis (Byrne et al., 2003). More recently, the same group reported a detailed analysis of LV contractile function in mice deficient in Nox2 compared with wild-type animals after TAC. They were able to demonstrate that Nox2 ^{-/-} animals are protected against the LV systolic and diastolic dysfunction in the whole heart as well as in isolated cardiomyocytes (Grieve et al., 2006).

The activity of NOX is significantly increased by several stimuli relevant to the pathophysiology of cardiac hypertrophy, ventricular remodelling and heart failure, including mechanical stretch (Matushita et al., 2001), ANGII (Mollnau et al., 2002), ET-1 (Lund et al., 2005), α -adrenergic agonists (Amin et al., 2001) and TNF α (Suematsu et al., 2003). Membrane translocation of the subunit p47^{phox} is important for the activation of the NOX enzyme in response to ANGII (Li et al., 2003). Consistent with this, p47^{phox} (^{-/-})

mice in which MI was induced exhibit reduced ventricular dilatation with significant reduction in mortality. This is associated with reduced apoptosis, reduced activity of MMP-2 gelatinolytic activity and preserved endothelial NO production compared with wild-type animals (Doerries et al., 2007). Of note, not only NOX activity but also XO were reduced in p47^{phox} (-/-) mice after MI compared with wild-type (Doerries et al., 2007), confirming previous in vitro studies suggesting that XO activity is probably triggered secondary to NOX activation (McNally et al., 2005).

1.4.2 Antioxidant mechanisms

Given the potential deleterious and unspecific actions that increased ROS production may have within cells, an important aspect of ROS production is the tight control that (under normal physiological conditions) is exerted over the systems responsible for its production and buffering systems to remove ROS. There are several cellular mechanisms that regulate ROS levels. Here, two key systems will be discussed: SOD and thioredoxin (Trx).

In the heart, SOD is expressed in two isoforms: Mn²⁺-SOD in the mitochondrial matrix, and Cu²⁺/Zn²⁺-SOD, the cytosolic form (Ohta et al., 1994). The rapid production of H₂O₂ and O₂ from O₂^{•-} by SOD is usually coupled with the actions of catalases (in tissue peroxisomes) glutathione peroxidases (in the cytoplasm) and peroxiredoxins (Nohl and Jordan, 1980; Halliwell, 1989; Schroder and Ponting, 1998; Dhalla et al, 2000). Consistent with this, mice with cardiospecific overexpression of glutathione peroxidase exhibit reduced ventricular remodelling after MI (Shiomi et al., 2004). Similarly, cardiospecific overexpression of Mn²⁺-SOD in mice protects from myocardial ischaemia/reperfusion injury (Chen et al., 1998), whereas post-ischaemic functional

contractile recovery is impaired in mice with heterozygous (-/+)Mn²⁺-SOD knockout (Asimakis et al., 2002).

Thioredoxin1 (Trx1) is a 12 kDa multifunctional protein which limits oxidative stress by direct ROS scavenging and the regeneration of several antioxidant molecules including ascorbic acid, ubiquinone (Q10), and lipoic acid. It therefore constitutes an important defence mechanism against ROS (Yamawaki et al., 2003). Collectively, Trx1, Trx reductase and NAD(P)H are called the Trx system which operates as a potent protein-disulfide oxidoreductase system (Masutani and Yodoi, 2002). Serum levels of Trx1 are elevated in patients with heart failure and coronary artery disease (Kishimoto et al., 2001b). Inhibition of Trx1 in the heart causes increased oxidative stress and cardiac hypertrophy, with further increases upon imposition of pressure-overload. This correlates with increased activation of the ERK1/2 cascade (Yamamoto et al., 2003). In contrast, cardiospecific overexpression of Trx1 reduces oxidative stress and cardiac hypertrophy after pressure-overload (Yamamoto et al., 2003).

Although it would seem desirable to have evolved more robust antioxidant systems, particularly in view that an excess in oxidative stress is at the very heart of degenerative pathologies like heart failure and neoplastic diseases, it is possible that excessive antioxidant activities may completely abolish physiological processes that require controlled amounts and localized production of ROS. It is for this reason that oxidative stress related diseases such as heart failure (the so called “cancer of cardiology”) and cancer are the principal causes of death in the modern aging population with the increased life expectancy gained at least in part as a result of the “post antibiotic era”.

1.5 Regulation of gene expression in cardiomyocytes

The changes in the phenotype of the heart with hypertrophic growth of cardiomyocytes and ventricular remodelling is the end result of alterations of the patterns of gene expression which occur in response to changes in haemodynamic loads (e.g. mechanical stretch) and/or neurohumoral stimulation. The expression of protein-coding genes can be regulated at several stages including transcription (initiation, elongation and termination), mRNA processing and stability, and translation and protein degradation. However, the current belief is that most regulation occurs at the level of initiation of transcription (Maston et al., 2006). In this section, a general overview of the mechanisms controlling gene expression through transcriptional regulation, specifically the initiation of transcription, will be discussed.

1.5.1. Overview of transcriptional regulation

Chromatin is the complex of DNA and protein that forms the chromosomes. Cytological studies distinguish two forms of chromatin. Heterochromatin is a more densely packed form which characteristically stains more darkly (heteropicnotic) and contains multiple repetitive sequences. It is not devoid of genes as it was classically thought (Cosma, 2002). Euchromatin is less densely packed (Elgin, 1996; Richards and Elgin, 2002) and contains protein-coding genes. DNA in a diploid human cell is more than two meters long; but is highly compacted in the nucleus which is only a few microns in size. This means a high level of organization must be maintained. The primary level of organization is into structural units called nucleosomes (Figure 1.7). The nucleosome comprises double-stranded DNA (dsDNA) of 146 bp, wrapped in 1.65 left-handed spherical turns around an octameric core composed of highly basic proteins, the histones [a histone (H3-H4)₂

heterotetramer and two histone H2A-H2B dimers] (Luger et al., 1997, reviewed in Kornberg and Lorch, 1999) (Figure 1.7).

One of the main characteristics of the regulation of gene expression in eukaryotic cells is that in principle it is restrictive, mainly as a result of the dense packaging of DNA into the nucleosomes. Nucleosomes repress general gene expression except for some which are actively transcribed under the influence of very specific positive regulatory mechanisms. Nucleosomes interfere with gene expression by at least three different mechanisms. First, as a direct consequence of the packing of DNA into nucleosomes, the specific sequences on core promoters which are usually recognized by regulatory proteins (e.g. transcription factors) are blocked (Workman and Kingston, 1998). Secondly, chains of nucleosomes can be further coiled into higher-order structures through linker histones such as histone H1 and other chromatin-associated proteins including members of the high mobility group (HMG) proteins, which may even shut down the transcription of entire domains within a chromosome (Ding et al., 1997; Dutnall and Ramakrishnan, 1997; Bell and Fensfeld, 1999). Thirdly, the interaction of nucleosomes with additional chromosomal proteins in heterochromatin appears to cause hereditary repression of gene expression (Grunstein, 1998). This occurs for example in the X chromosome that is inactivated in females, where genes on a randomly selected X chromosome, are condensed and silenced (Richards and Elgin, 2002).



Figure 1.7 Nucleosome structure. Top view of a nucleosome shown colored by histone subunit type (H2A blue, H2B red, H3 yellow, H4 green). DNA structure is shown colored by strand (turquoise and orange). PDB file from Research Collaboratory for Structural Bioinformatics (RCSB) PDB web site. Picture customized and generated with WebLab Viewer Pro 4.0 (Molecular Simulations Inc.).

The restrictive nature of gene expression in eukaryotes makes it a matter of necessity that several proteins in addition to transcription factors interact with the chromatin in a positive and negative way to determine whether a gene is expressed or not. The regulatory proteins required for the control of gene expression can be classified into four groups:

1. Histone acetylation
2. Basal transcriptional machinery which includes general transcription factors
3. Gene activator proteins commonly known as transcription factors
4. Coactivators (cofactors)

1.5.1.1 Histone acetylation

As discussed above, packaging of DNA into nucleosomes is a basic mechanism for restricting the transcription of genes. Therefore, modification of the chromatin is an important initial step in order to allow or prevent access of *trans*-acting regulatory proteins to genomic DNA. Remodelling of chromatin is usually achieved by covalent modifications such as addition of acetyl groups, methyl groups, phosphorylation, and reversible ubiquitination (reviewed in Lee and Young, 2000, Näär et al., 2001 and Shilatifard, 2006). Of these mechanisms, the best studied is acetylation/deacetylation of histones. Modification of core histone proteins by acetylation appears to “relax” the nucleosome structure allowing transcription factors to bind. This process is induced by histone acetyltransferases (HATs), which promote gene expression, and is reversed by histone deacetylases (HDACs) which suppress it.

There are three classes of HDACs, which operate to promote DNA coiling and transcriptional repression. Class II HDACs, appear to be important in the regulation of gene expression during cardiac hypertrophy. These enzymes (compared with other classes) contain N-terminal extensions of approximately 500 amino acid residues which harbour binding sites for other transcriptional regulators. One characteristic of class II HDACs is that they usually form large multiprotein complexes with transcriptional co-repressors (Verdin et al., 2003). The N-terminal extensions of class II HDACs also contain conserved phosphorylation sites and shuttle between the cytoplasm and the nucleus according to their phosphorylation status. For example, (as discussed earlier; see section 1.3.1.3 and Figure 1.2), local alterations in the concentration of Ca^{2+} in the nucleus, secondary to stimulation with ET-1 or PE, promotes activation of CaM and CaMKII, and leads to phosphorylation of the class II HDAC, HDAC5, which translocates from the nucleus to the cytoplasm where it remains sequestered bound to inhibitory 14-3-3 proteins (Wu et al., 2006). HDAC5 is a negative regulator of MEF2 transcription factor, so phosphorylation of HDAC5 with transfer to the cytoplasm promotes transcriptional activity of MEF2. Class II HDACs appear to be particularly important in regulating cardiac specific gene expression and may play a role in cardiac hypertrophy and ventricular remodelling as suggested by studies in HDACs knockout mice (reviewed in Olson et al., 2006). However, paradoxically trichostatin A and scriptaid (small molecule inhibitors of HDAC I and II) significantly reduced the hypertrophic response in mice after TAC, compared with vehicle-treated animals (Kong et al., 2006). Importantly, treatment with these same HDAC inhibitors also prevented ventricular dysfunction and fibrosis without any observable increase in apoptosis, compared with vehicle-treated control animals and sham-operated animals. As expected in vitro experiments revealed that treatment of cardiomyocytes with HDAC inhibitors significantly increased acetylation of

target proteins such as histone 3 (H3) or α -tubulin. Taken together this study suggests that inhibitors of class I and II HDACs may have a promising therapeutic potential in the treatment of heart failure (Kong et al., 2006).

Probably the best characterized HATs are p300 and CBP (cAMP response element binding protein) which were initially thought to be transcriptional co-activators (Kalkhoven, 2004). p300/CBP are recruited to specific promoters by enhancer-specific transcription factors, and both the transcription factors interactions and HAT activities may be influenced by their phosphorylation state. Whether or not their activity is regulated by phosphorylation in cardiomyocytes remains to be defined. However, hyperphosphorylation of p300 via p38-MAPK signalling may target the protein for degradation in cardiomyocytes (Poitza et al., 2005). Importantly, p300 and/or CBP are required for cardiomyocyte hypertrophy (Gusterson et al., 2003).

1.5.1.2 Basal transcriptional apparatus

The enzymes that catalyze the transcription of DNA into RNA are collectively called RNA polymerases, of which there are three RNA polymerase I, II and III. They were originally characterized on the basis of their sensitivity to the poisonous cyclic octapeptide, α -amanitin. Thus, RNA polymerase I is very insensitive to α -amanitin, RNA polymerase II is very sensitive and RNA polymerase III exhibits intermediate sensitivity (Roeder, 1974). From a functional perspective, RNA polymerase II is responsible for transcription of all protein-coding genes into messenger RNA (mRNA), RNA polymerase I is responsible for synthesis of ribosomal RNAs (rRNAs), and RNA polymerase III synthesizes transfer RNA (tRNA) and some small RNAs such as U6 (which is involved in mRNA splicing) (Roeder, 1974).

RNA polymerase II is a large multi-subunit enzyme which requires additional factors for accurate transcription initiation. These with RNA polymerase II are referred to as general transcription factors (GTFs) (reviewed in Roeder, 1996). These GTFs include (in addition to RNA polymerase II) TFIIA, TFIIB, TFIID, TFIIIE, TFIIF and TFIIH, each of which has specific roles such as positioning RNA polymerase II on the promoter (TFIIB) or unwinding of DNA (TFIIH). TFIID is itself a multi-subunit complex and, as the major sequence-specific DNA-binding component of GTFs, is generally involved in promoter recognition. This complex contains the TATA-binding protein (TBP) and several TBP-associated factors (TAF_{II}s) which also appear to be highly promoter selective (reviewed in Green, 2000). The TATA-box is a common theme in the promoter of most, but not all genes and consists of a consensus sequence whose most common sequence is TATA(A/T)A(A/T)(A/G) (Bucher, 1990). It is usually localized 25-35 bp upstream of the transcriptional start site and, characteristically, genes containing a TATA-box frequently start with an adenine nucleotide. Binding of TFIID to the TATA-box is the critical initial step in the formation of the preinitiation complex PIC. After this event, other GTFs including RNA polymerase II assemble on the promoter in an ordered fashion through protein-protein interactions (see section 1.5.1.5). It is currently believed that enhancer specific transcription factors (see next section) interact through their activation domain with one or more components of the basal transcriptional machinery known as the “targets” to stimulate the formation of the PIC. The mechanisms and the “targets” in this activation process are becoming clearer, although they are not yet completely understood (reviewed in Green, 2005).

1.5.1.3 Gene activator proteins (classical transcription factors)

Research into transcriptional control in eukaryotes has identified *cis*-acting regulatory elements in the DNA particularly in the 5' promoter region. This includes the core promoter and proximal regulatory elements, and other distal DNA regulatory elements such as enhancers, silencers, insulators and Locus Control Regions (LCR) (for a review see Maston et al., 2006). The delineation of *cis*-acting regulatory elements can be determined by mutagenesis analysis. Research into transcriptional regulation also identified *trans*-acting DNA-binding regulatory proteins that bind mainly to silencers and enhancers in the promoters and inhibit or stimulate transcription. These are commonly referred to as transcription factors or *trans*-activators. To easily differentiate these transcription factors from the GTFs, I will also use the term enhancer-specific transcription factors, particularly when this distinction is necessary for clarity of the discussion.

Detection of transcriptional activators can be performed by DNA-binding analysis, which has revealed the existence of many types of transcription factors (Luscombe et al., 2000). Transcription factors usually stimulate gene transcription (i.e. the synthesis of RNA from a protein-coding gene) and may be referred to as transcriptional activators, although some transcription factors can inhibit gene transcription and are therefore called transcriptional repressors. These selectively regulate expression of genes in a tissue and cell specific manner, although they can also stimulate transcription in response to specific stimuli (e.g. hypertrophic agonists), or in a temporal fashion (e.g. embryonic vs postnatal expression of genes). One characteristic of transcription factors is their modular structure with both DNA-binding domains and activation domains. The most typical structure found in the DNA-binding domains of most transcription factors is an α -helix (which inserts into the

major groove of DNA) in the proximity of several positively charged amino acids (Luscombe et al., 2000). This domain binds specifically to short segments of DNA usually 5 to 10 bp long, called consensus binding sites. Of note, transcription factors do not usually work alone, but in a co-operative manner, and complex interactions between several transcription factors bound to their specific consensus binding sequences in the promoter region may increase the efficiency of transcription of genes by forming an enhanceosome (Veitia, 2003; Arnosti and Kulkarni, 2005). The activation domain mediates the co-operative binding of transcription factors to DNA-regulatory elements, and postranslational modifications such as phosphorylation is a common mechanism of transactivation of transcription factors by extracellular signals (Hill and Treisman, 1995).

The recurrent structural motifs in the DNA-binding domain of transcription factors form the basis for their classification. Although there are at least eight functional/structural groups of protein-DNA complexes, most transcription factors contain one of four motifs: zinc finger, leucine zipper, helix-turn-helix (HTH) or helix-loop-helix (HLH) (reviewed in Luscombe et al., 2000). Of particular interest for this thesis are the leucine zipper transcription factors which will be briefly described here. Leucine zipper family of transcription factors comprise a single α -helix of 60 or more amino acid residues and are structurally divided into two parts: a dimerization region and a DNA-binding region. The C-terminal region of each zipper protein spans approx 30 amino acids, and contains leucine or another hydrophobic amino acid every seven amino acids in the sequence. These leucine amino acids are essential for the interaction with the corresponding leucine amino acids from a dimerization partner, which results in the formation of a molecular coil that stabilizes (or “zips up”) two proteins containing the same leucine zipper motif. The DNA-binding region is structurally an extension of the α -helix towards the N-

terminal region and is rich in basic amino acids that interact with phosphates within the major groove of DNA.

The correct configuration of a pair of leucine zippers is the formation of dimers that virtually grip the DNA, and these can be identical proteins (homodimers) or two different proteins with the same leucine zipper motif (heterodimers) (Figure 1.8). This leucine zipper motif is found in the transcription factors c-Jun and c-Fos of the activator protein family (AP-1). c-Jun can form homodimers or heterodimerize with c-Fos, and bind to the AP-1 consensus (5'-TGAGTCA-3') (Glover and Harrison, 1995) (Figure 1.8). Also of common occurrence is the formation of heterodimers of members of the AP-1 family with those of the activating transcription factor (ATF)/CRE (cAMP responsive element) family with resulting activating and repressing effects on target genes when bound to AP-1 consensus or ATF/CRE consensus (5'-TGACGTCA-3') (Hai et al., 1999). On the basis of the presence of the amino acid leucine in all leucine zipper family transcription factors, and the invariable presence of basic amino acids at the N-terminal region, the leucine zipper proteins are also called basic (leucine) zippers (bZip).

Transcriptional activators can interact with proteins of the basic transcriptional apparatus and increase the rate of formation of the PIC (see above), or promote a step in the transcription process (e.g. initiation, elongation). Recently, an additional feature of transcription factors has also been described whereby they promote the recruitment of chromatin-modifying proteins (e.g. HATs/HDACs) and influence their activities to facilitate transcription or repress it (reviewed in Green, 2005) (see section 1.5.1.1).

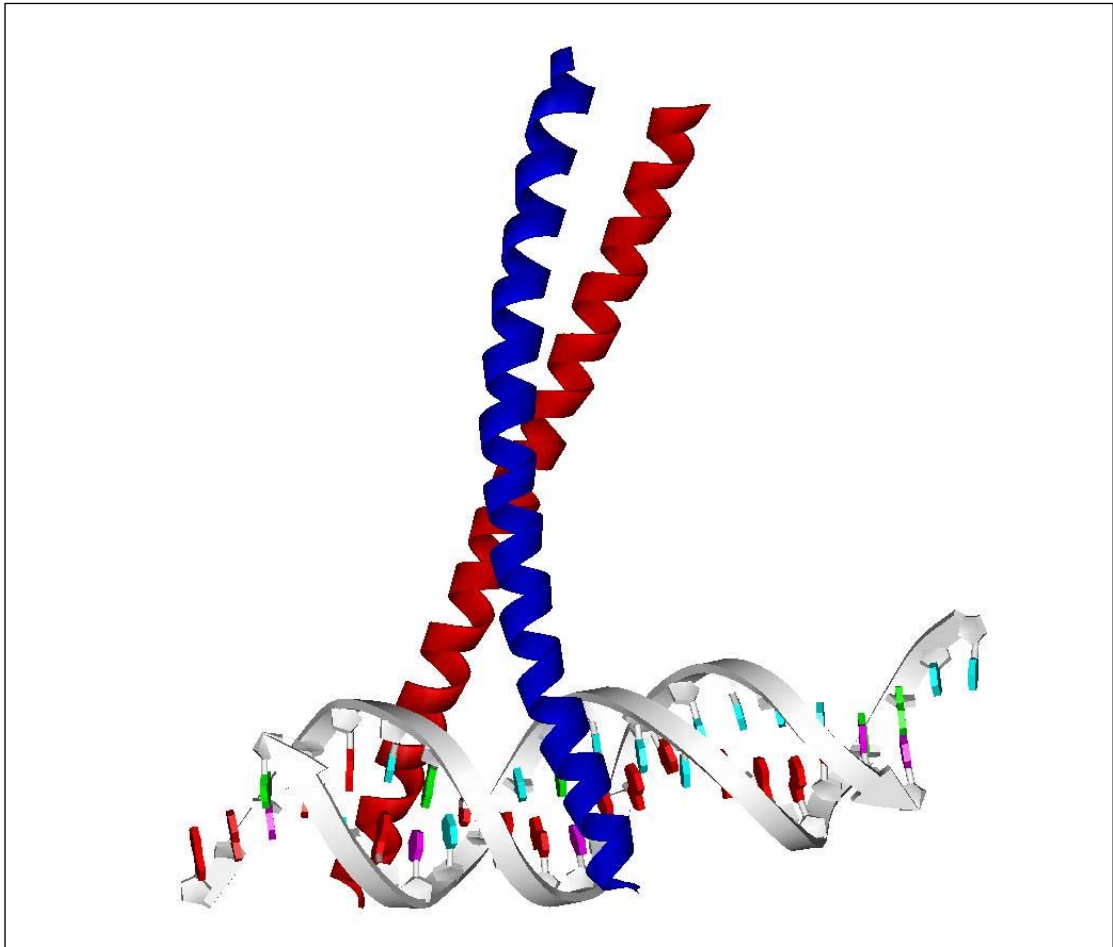


Figure 1.8 c-Jun and c-Fos heterodimer bound to DNA. The α -helix in both c-Jun (shown in Red) and c-Fos (shown in Blue) contains the activation domain (C-terminal) through which leucine zipper transcription factors heterodimerize. The DNA-binding domain (N-terminal domain) is an extension of this same α -helix which interact with DNA and enter the major grooves at both sides of the DNA double helix.

1.5.1.4 Coactivators (cofactors)

Coactivators are proteins that interact with and affect the functions of enhancer specific transcription factors. Amongst the most studied, although not completely understood in higher eukaryotes is the Mediator complex. The Mediator complex is a large multi-subunit coactivator (more than 20 subunits) which was initially characterized in *Saccharomyces cerevisiae* (Hampsey and Reinberg, 1999). More recently, a mammalian Mediator complex has been characterized and the multi-subunit structure and the functional characteristics are evolutionary conserved from yeast to human Mediators. The Mediator complex appears to serve as an interacting protein that facilitate the communication of RNA polymerase II/GTFs within the core promoter with enhancer-specific transcription factors, thus modulating the transcriptional activity (reviewed in Conaway et al., 2005). Of note, several subunits of the Mediator complex also appear to be “targets” of enhancer specific transcription factors, although the mechanistic details are still under investigation (Malik and Roeder, 2005). What is clear, however, is that in vivo transcription by RNA polymerase II requires a Mediator complex (Malik and Roeder, 2005).

1.5.1.5 General overview of the induction of transcription initiation by enhancer specific transcription factors

In this section, an overview of the different steps involved transcription initiation induced by enhancer specific transcription factors will be summarized. Although not yet completely defined in cardiomyocytes, it can be assumed that transcription factors are probably phosphorylated at their activating domain (e.g. AP-1 or ATF/CRE family members) downstream of, for example, the ERK1/2 cascade following stimulation with ET-1 or PE. There is recruitment of HATs such as CBP/p300 to facilitate the remodelling

of chromatin by acetylation of core histone proteins on the nucleosomes. This allows exposure of proximal regulatory elements such as the TATA-box and distal regulatory elements such as the enhancer. Binding of enhancer specific transcription factors to their consensus binding sites and formation of the enhanceosome plus their interaction with poorly-defined “target” proteins of the basal transcriptional machinery promotes the positioning and binding of TBP (TFIID) to the TATA-box element (Figure 1.9) (Roeder, 2005; Green, 2005). The stability of the positioning of TBP in the TATA-box appears to be facilitated by TFIIA which enters the complex at the same time as TFIID (Imbalzano et al., 1994). Once TBP is bound to the TATA-box, TFIIB is able to bind to the core promoter making contact with TBP and the DNA on either side of the TATA-box. The N-terminal domain of TFIIB extends towards the start site, so the main role of TFIIB appears to be the selection of the transcriptional start site (Barberis et al., 1993). After binding, TFIIB then helps direct a preformed complex of RNA polymerase II and TFIIF to the transcriptional start site (Barberis et al. 1993). TFIIF appears to be involved in the suppression of non-specific DNA binding of RNA polymerase II, thus potentially helping to ensure the specificity of binding of the enzyme to the transcriptional start site (Conaway and Conaway, 1993). Finally, TFIIIE enters the forming complex and serves as a docking site for TFIIH, which subsequently binds and completes the formation of the PIC (Figure 1.9). The helicase function of TFIIH then allows the melting of dsDNA at the core promoter and subsequent initiation of transcription by RNA polymerase II (Guzman and Lis, 1999).

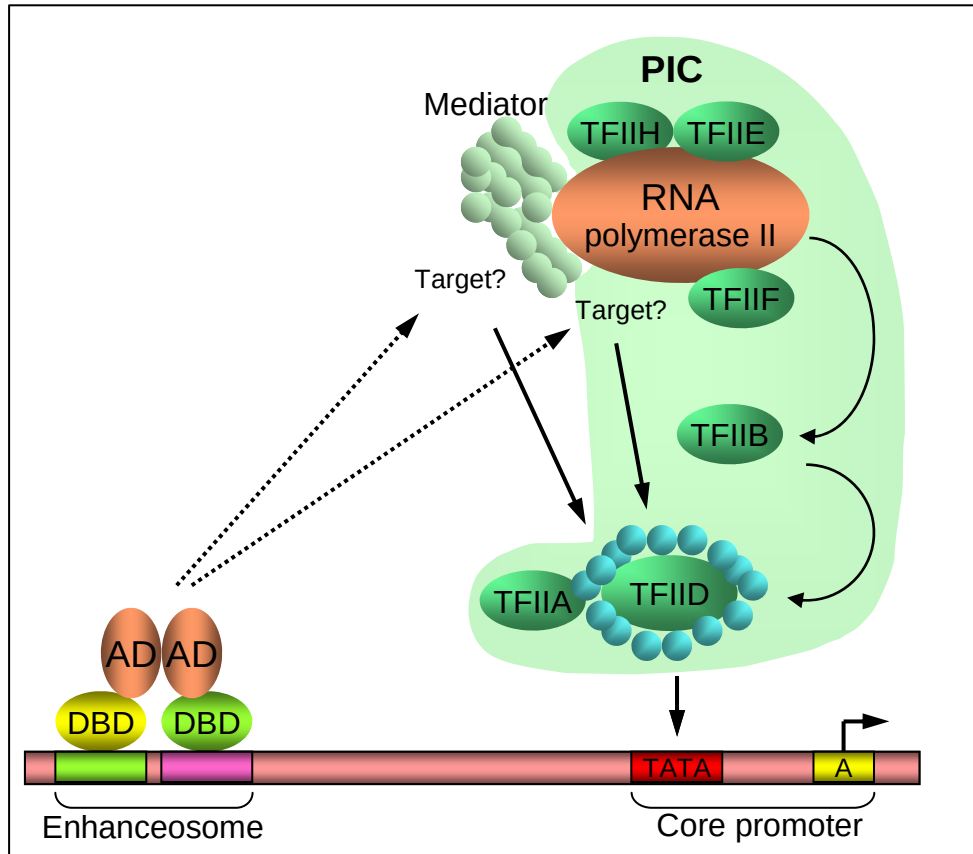


Figure 1.9 Transcription initiation induced by enhancer specific transcription factors. Binding of enhancer specific transcription factors to their specific consensus sites in the distal regulatory elements (enhancers), promotes their interaction with poorly delineated “target” proteins that form part of the GTFs. This interaction promotes the binding of TFIID through its TBP subunit to the TATA-box element in the core promoter. Binding of TFIIB then occurs and, after recognition of the start site by TFIIB, allows the positioning of RNA polymerase to the transcriptional start site (frequently an adenine nucleotide for TATA-box containing genes). The PIC is finally completed by the binding of TFIIIE, which serves as a docking site for TFIIH. Helicase activity of TFIIH melts the core promoter allowing the initiation of transcription by polymerase II. The interaction and communication of enhancer specific transcription factors with “target” proteins from the Mediator complex appear to modulate positively or negatively the transcriptional process, although the details of this process are not completely understood.

The signalling cascades that initiate the hypertrophic response after haemodynamic-overload are set into motion by locally-released neurohumoral factors [e.g. autocrine-paracrine release of ET-1 acting through cognate receptors located at the cell membrane of the cardiomyocytes (section 1.2 and 1.3)]. Therefore, ET-1, PE, and many other agonists are capable of inducing cardiomyocyte hypertrophy (Sugden and Clerk, 1998; Molkentin et al., 2001). The flow of information in these signalling cascades leads to activation of several protein kinases including the mitogen-activated protein kinases MAPKs, which then phosphorylate target proteins. These target proteins include several transcription factors that bind to the promoters of several genes and regulate gene expression. Thus, cardiac myocyte hypertrophy is associated with changes in expression of IEGs (e.g. *c-jun*, *c-fos*), and re-expression of a “foetal pattern” of gene expression (e.g. ANP; β -myosin heavy chain), which are often used as markers of hypertrophy (Hoshijima and Chien, 2002; Dorn and Force, 2005). Many of the genes that are activated during this acute phase are themselves transcription factors that will regulate a second wave of gene expression presumably responsible for the sub-acute and chronic changes in phenotype leading to cardiac hypertrophy (Hoshijima et al., 2002; Clerk et al., 2005; Clerk et al., 2007, Cullingford et al., manuscript submitted). Microarray studies from our group, comparing the changes in transcriptional profiles induced by H₂O₂ or ET-1 in neonatal rat cardiomyocytes identified ATF3 mRNA as being significantly induced by all these agonists (Clerk et al., 2005), and this forms a focus for this thesis.

1.6 Hypothesis

The basic hypothesis is that stimulation of cardiomyocyte hypertrophy or apoptosis stimulate intracellular signalling pathways leading to early changes in gene expression which propagate the response. One such gene identified by microarrays is ATF3 which is induced by hypertrophic or apoptotic stimuli. This thesis explores the regulation and role of ATF3 in cardiomyocytes. Further studies examine the effects of inhibitors of protein kinase signalling on cardiomyocyte gene expression.

Chapter 2

General Methods

2.1 Preparation of primary cultures of neonatal rat cardiomyocytes

Primary cultures of ventricular cardiomyocytes from neonatal (2-4 days) Sprague-Dawley rats (purchased from Harlan) were used for all the experiments and were prepared by Ms. Amanda Nercessian. The yield was, on average, 4×10^6 cells per heart. Myocytes were dissociated from the ventricles by a previously described adaptation (Bogoyevitch et al., 1995) of the method of Iwaki et al. (1990). Neonatal rats were culled by cervical dislocation and then decapitated. The hearts were removed immediately under sterile conditions. Ventricles were separated from atria, opened and washed in ice-cold sterile digest buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM Na_2HPO_4 , 5.6 mM glucose, 5.4 mM KCl and 0.8 mM MgSO_4 , pH: 7.35). Ventricular tissue was cut into pieces and cardiomyocytes were dissociated by serial digestion with 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin in digest buffer.

Digests were performed in a sterile 100 ml Schott bottle with agitation in a water bath at 37°C. After the first digest (5 min, 160 cycles/min), the supernatant, containing blood and digestion enzymes, was removed and discarded. Following the second digest (20 min, 136 cycles/min) the cell suspension was transferred to a sterile 50 ml Falcon tube containing 2 ml of foetal calf serum (FCS) (Invitrogen) to quench the digestion. Cells were centrifuged (5 min, $60 \times g$), resuspended and stored in the incubator. Remaining cardiomyocytes were dissociated from heart tissue with 4 sequential digestions (2×25 min, 112 cycles/min; 1×20 min, 136 cycles/min; 1×10 min, 112 cycles/min). Cells were centrifuged, resuspended as described above and added to the cells from the second digest. Pooled cells were recovered by centrifugation (5 min, $60 \times g$) and the cell pellet resuspended in plating medium [Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) / Medium 199 (Invitrogen) (4:1 v/v), containing 15% (v/v) FCS (Invitrogen), 100 units/ml

penicillin and 100 units/ml streptomycin (Invitrogen)]. The resulting cell suspension was pre-plated (4 ml per dish) for 30 min (37°C, 5% CO₂) on 60 mm Primaria culture dishes to remove non-myocytes (e.g. fibroblasts and endothelial cells), which attach to the culture plates and were discarded. Non-adherent cardiomyocytes were collected and counted using a haemocytometer [75 µl myocyte suspension, 175 µl plating medium and 250 µl Trypan blue solution (Invitrogen)] ignoring blue-stained non-viable cells. Cardiomyocytes were suspended in plating medium (10⁶ myocytes/ml) and plated (4 ml per 60 mm dish) onto Primaria culture dishes which had been pre-coated with sterile 1% (w/v) gelatin in water. Myocytes were confluent and beating spontaneously within 18 h. After 24 h the plating medium was replaced by serum-free maintenance medium [DMEM/Medium 199 (4:1 v/v), containing 100 units/ml penicillin and streptomycin (Invitrogen)] for a further 24 h.

2.2 Agonists and inhibitors used to treat cardiomyocytes

Inhibitors (Table 2.1), H₂O₂ (Sigma) and ET-1 (Sigma) were added directly to the cardiomyocytes in serum-free medium. For a full list of reagents and chemicals used in the experiments of this thesis see Appendix A.

Table 2.1 Inhibitors used to treat cardiomyocytes (all dissolved in dimethyl sulphoxide except cycloheximide which was dissolved in H₂O).

Inhibitor	Supplier	Concentration	Pre-incubation time	Target
Cycloheximide	Sigma	20 µM	10 min	Protein translation
SB203580	Alexis Biochemicals	10 µM	15 min	p38-MAPK α/β
SP600125	Alexis Biochemicals	10 µM	15 min	JNKs
PD98059	Alexis Biochemicals	25 µM	15	MKK1/2
U0126	Alexis Biochemicals	10 µM	15 min	MKK1/2
LY294002	Alexis Biochemicals	50 µM	15	PI3K

2.3 Extraction of total RNA

Cardiomyocytes (4×10^6 per sample) were scraped into ice-cold RNazol Bee™ (1000 µl), incubated with chloroform on ice (200 µl, 5 min) and centrifuged (4°C, 10 min, $15,300 \times g$). The upper phase was incubated with an equal volume of isopropanol on ice (1 h). RNA was pelleted by centrifugation (4°C, 10 min, $15,300 \times g$), washed (70% ethanol) and resuspended in 15 µl Milli-Q filtered water. RNA concentrations were determined by spectrophotometry ($\lambda = 260$ nm). The purity was assessed from A_{260}/A_{280} . Values of 1.9–2.1 were considered acceptable.

2.4 DNase treatment

In order to exclude any DNA contamination during RT-PCR reactions, RNA preparations were treated with DNase to remove genomic DNA. Total RNA (1 µg) was mixed with 1 µl of 10X DNase I reaction buffer (200 mM Tris-HCl, pH 8.4, 20 mM MgCl₂, 500 mM

KCl) (Invitrogen); 1 µl DNase I Amp Grade (1 U/µl) (Invitrogen); and DEPC (diethyl pyrocarbonate)-treated water to final volume of 10 µl. Following incubation (room temperature, 15 min), the reaction was terminated by addition of 1 µl EDTA (25 mM) and heating (65°C, 10 min).

2.5 cDNA synthesis

RNA (1 µg) was mixed with 0.5 µg random hexamers and dATP, dCTP, dGTP and dTTP (0.74 mM each) in a total volume of 13.5 µl. Following incubation (65°C, 5 min), 6.5 µl solution A [15 mM dithiothreitol (DTT), 4 µl commercial 5 × 1st strand buffer (Invitrogen), 200 units M-MLV reverse transcriptase (Invitrogen) and 0.5 µl RNaseOut® (Invitrogen)] were added and samples were incubated (37°C, 1 h). The reaction was terminated (65°C, 3 min).

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Primers for semiquantitative (SQ-PCR) and/or quantitative (Q-PCR) were designed for established genes using published rat sequences (see Table 2.2). Where possible, these were designed across exon boundaries.

Table 2.2 Primers used for Q-PCR and SQ-PCR

Gene	Accession number	Q-PCR primers		
		Size (bp)	Forward primer	Reverse primer
ATF3	NM_012912	181	GAGCGAAGACTGGAGCAAAA	AAGGTGCTTGTCTGGATGG
Ereg	NM_021689	113	ACTGCAGATGTGAAGTGGGC	GAGGAAAACGAGAATCACGG
IL-6	NM_012589	136	CCGGAGAGGAGACTTCACAG	CAGAATTGCCATTGCACAAC
LIF	NM_022196	128	TCAACTGGCTCAACTCAACG	ACCATCCGATACAGCTCGAC
c-Jun	NM_021835	140	GATCATCCAGTCCAGCAATG	TATTCTGGCTATGCAGTTCAG
CT-1	NM_017129	139	GGAGGGAAGTCTGGAAGACC	CTGCACATATTCTCCAGCA
GAPDH	NM_017008	83	GCTGGCATTGCTCTCAATGACA	TCCACCACCCTGTTGCTGTA
Gene	Accession number	SQ-PCR primers		
		Size (bp)	Forward primer	Reverse primer
ATF3	NM_012912	388	GCTGCCAAGTGTGAAACAAG	CAGTTTCCAATGGCTTCAGG
IL-6	NM_012589	428	CCGGAGAGGAGACTTCACAG	GAGCATTGGAAGTTGGGGTA
GAPDH	NM_017008	452	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

2.6.1 Quantitative Real time PCR (Q-PCR)

Q-PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems). A master-mix containing (per reaction) 12.5 µl Sybr-Green Jump Start Taq Readymix (Sigma-Aldrich) and 5 µl oligonucleotides (5 pmol each of forward and reverse primers) was aliquoted into Optical 96-well reaction-plates (Applied Biosystems), and the cDNA template added (7.5 µl, 1/15 dilution in Milli-Q filtered water). PCR conditions for all primer pairs were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 59°C for 60 s. Following Q-PCR, a dissociation curve analysis was performed to check for aberrant amplification products (Figure 2.1). Q-PCR analysis of GAPDH was performed in each 96-well plate as an endogenous control and the relative quantitation protocol was used.

2.6.2 Semi-Quantitative RT-PCR (SQ-PCR)

PCR reactions were performed in 50 µl of 1X Buffer IV® (ABgene) containing 0.5-1.0 ng cDNA, 1 unit Taq polymerase, dATP, dCTP, dGTP and dTTP (0.2 mM each) and 100 pmol forward and reverse primers (see Table 2.2). Samples were heated (94°C, 2 min), then subjected to 22-32 cycles of denaturation (94°C, 30 s), annealing (59°C, 30 sec), and extension (72°C, 30 s), heated again (72°C, 2 min) and finally cooled to 4°C. Samples were mixed with PCR sample buffer [0.25% (w/v) bromophenol blue, 40% (w/v) sucrose] at 10% (v/v) and subjected to electrophoresis (90 V, 20 min) on 2% (w/v) agarose gels containing 100 µg/l ethidium bromide in 1X TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA). Bands were detected under UV light. Band sizes were estimated by comparison to a øX174 RF DNA *Hae* III digest (ABgene) or 100 bp DNA ladder (Invitrogen).

Densitometric analysis was performed using Imagemaster 1D Prime, version 3.0 (GE Healthcare). Samples were normalised to the RT-PCR product for the housekeeping gene, GAPDH. For genes displaying constitutive expression, it was confirmed that the PCRs were semi-quantitative. This was achieved by performing PCR over a range of cycles with a single sample, followed by densitometric analysis and construction of amplification curves (Figure 2.2). The PCR cycle numbers employed in experiments lay within the linear sections of the curves.

2.7 Preparation of protein extracts

2.7.1 Total protein extracts

Cardiomyocytes (4×10^6 per sample) were rinsed twice in ice-cold phosphate-buffered saline (PBS) (Invitrogen). For preparation of total protein extracts, cardiomyocytes were scraped into 150 μ l ice-cold Buffer G [20 mM glycerophosphate pH 7.5, 50 mM NaF, 2 mM EDTA, 0.2 mM Na_3VO_4 , 10 mM benzamide, 0.2 mM leupeptin (Sigma), 10 μ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64) (Sigma), 5 mM DTT (Sigma), 0.3 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma), 2 μ M microcystin (Alexis Biochemicals), 1% (v/v) Triton X-100]. Samples were centrifuged (4°C, 5 min, $10,600 \times g$) and the supernatants retained. A volume of supernatant (2 μ l) was used to determine the protein concentration (see below) and the remaining supernatant was boiled with 0.33 volume of immunoblotting sample buffer [0.33 M Tris-HCl (pH 6.8), 10% (w/v) sodium dodecyl sulphate (SDS), 13% (w/v) glycerol, 133 mM DTT, 0.01% (w/v) bromophenol blue]. Immunoblotting samples were stored at -20°C.

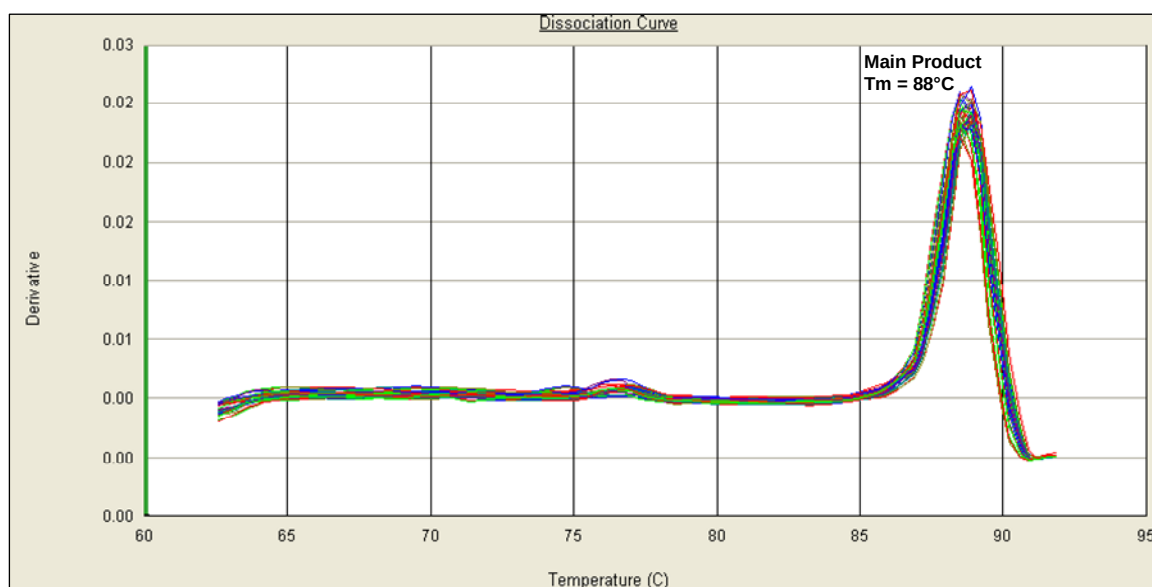


Figure 2.1 Q-PCR dissociation-curve analysis. Following Q-PCR, optical 96-well reaction plates containing the amplified products were subjected to dissociation-curve analysis to detect the presence of non-specific amplification. The presence of a uniform main product ($T_m = 88^\circ\text{C}$) in the Q-PCR run (as shown here) indicates the absence of primer dimers (typical $T_m = 75^\circ\text{C}$) during the Q-PCR reactions.

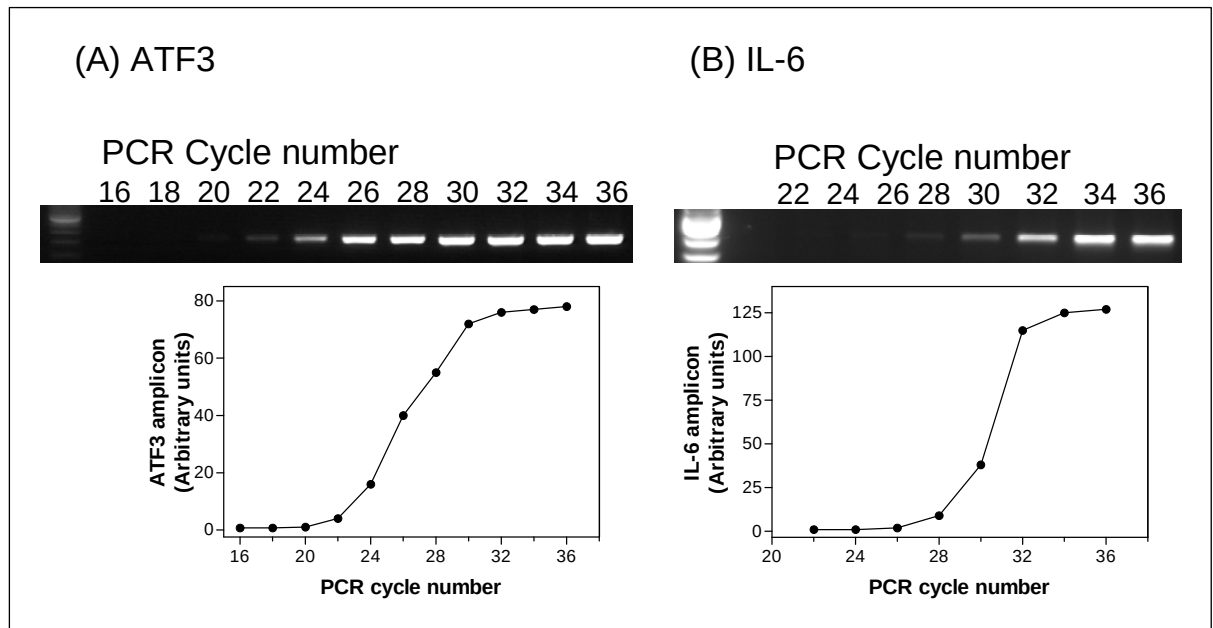


Figure 2.2 Amplification plots of ATF3 and IL-6 genes. PCRs were performed for increasing cycle numbers with a single cDNA sample. Plotting of the densitometric analysis demonstrated that amplification followed the expected sigmoid curve. The PCR cycle numbers used in semi-quantitative RT-PCR lay within the linear sections of the curves. The images (upper panels) and the densitometric analysis (lower panels) shown are representative of two independent experiments.

2.7.2 Nuclear extracts

Cardiomyocytes (4.0×10^6 cells per sample) were rinsed in PBS and scraped into 150 μ l ice-cold buffer A [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl_2 , 0.3 mM Na_3VO_4] containing freshly-added protease and phosphatase inhibitors [200 μ M leupeptin, 10 μ M E64, 300 μ M PMSF, 2 μ M microcystin, 5 mM DTT]. Samples were centrifuged (4°C, 5 min, $10,600 \times g$) and the pellets washed in 100 μ l buffer A containing 0.1% (v/v) Nonidet P40 (NP40) with added phosphatase and protease inhibitors. Samples were centrifuged (4°C, 5 min, $10,600 \times g$) and the pellets resuspended in 50 μ l buffer C [20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25% (v/v) glycerol, 0.3 mM Na_3VO_4] containing phosphatase and protease inhibitors. Suspensions were incubated on ice (1 h), centrifuged (4°C, 5 min, $10,600 \times g$) and supernatants transferred to Eppendorf tubes and stored at -80°C.

2.7.3 Bradford protein assay

Protein concentrations were determined by Bradford assay (Bradford 1976). BioRad Bradford reagent [1/5 dilution in double-deionised water, 1 ml] was added to samples (2 μ l) in a final volume of 100 μ l. Absorbance at $\lambda=595$ nm was measured and the protein concentration determined by comparison to a range of bovine serum albumin (BSA) dilutions (1-15 μ g).

2.8 Immunoblotting

Proteins (25 μ g) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) polyacrylamide resolving gels and 6% (w/v) stacking gels in BioRad Protean II mini-gels (200 V, 45 min) with running buffer [25 mM Tris-HCl, 192 mM

glycine, 2% (w/v) SDS]. Proteins were transferred electrophoretically to nitrocellulose membranes using BioRad Trans-Blot semi-dry transfer cells (10 V, 1 h) with transfer buffer [25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol]. Membranes were incubated (room temperature, 20 min, with agitation) in blocking buffer [5 % (w/v) non-fat milk powder in Tris-buffered saline [20 mM Tris-HCl (pH 7.6), 137 mM NaCl] with 0.1% (v/v) Tween 20 (TBST), washed in TBST (3 × 5 min, with agitation), and washes repeated between each step afterwards. Membranes were incubated (4°C, overnight) with primary antibodies specific for ATF3 (rabbit polyclonal, SC-188, Santa Cruz Biotechnology Inc.) and IL-6 (goat polyclonal, SC-1266, Santa Cruz Biotechnology Inc.) or ATF2 antibody (rabbit polyclonal, SC-187, Santacruz Biotechnology Inc.) [diluted 1/1000 in TBST containing 5% (w/v) BSA]. Blots were then incubated (room temperature, 60 min) with horseradish peroxidase-conjugated secondary antibodies (polyclonal goat anti-rabbit immunoglobulins for ATF3; rabbit anti-goat immunoglobulins for IL-6, DakoCytomation) [1/5000 in TBST containing 1% (w/v) non-fat milk powder]. Immunoreactive bands were detected by enhanced chemiluminescence (Santa-Cruz Biotechnology Inc.). Blots were exposed to Hyperfilm and the bands analysed by semi-quantitative scanning densitometry (Imagemaster 1D Prime version 3.0).

2.9 Slot-blotting

After treatments, the media from cardiomyocyte cultures were collected and loaded (500 µl) onto a 24-well slot-blot microfiltration manifold (SHM-24, Camlab) preloaded with a nitrocellulose membrane pre-soaked in PBS. After filtration of the samples, the slots were washed and filtered three times with 250 µl PBS. Membranes were air-dried and then

fixed with 25% propanol / 10% acetic acid (room temperature, 2 min), washed once with PBS (room temperature, 2 min, with agitation) and air-dried again. Membranes were incubated (room temperature, 20 min, with agitation) in blocking buffer (as above, section 2.8), and washed in TBST (3×5 min, with agitation). Immunodetection of IL-6 was then performed as described above (section 2.8).

2.10 Chromatin Immunoprecipitation (ChIP) assay

After stimulation, cardiomyocytes (4×10^6 per sample; 60 mm dishes) were fixed in formaldehyde at a final concentration of 1% (10-12 min at room temperature). Cross-linking was terminated by the addition of glycine (pH 2.5, 125 mM final concentration). After 10 min, cells were washed with ice-cold PBS, scraped into 1 ml PBS supplemented with protease and phosphatase inhibitors [0.2 mM leupeptin, 10 μ M E64, 5 mM DTT, 0.3 mM PMSF and 2 μ M microcystin] and pelleted by centrifugation ($3,000 \times g$, 4°C, 5 min). The supernatants were removed by aspiration and pellets lysed for 15 min in 500 μ l L1 buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.1% NP40, 10% glycerol) supplemented with protease and phosphatase inhibitors. Resulting nuclei were pelleted ($3,000 \times g$, 4°C, 5 min) and resuspended in 200 μ l L2 buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA). Four samples from each condition were pooled into one Eppendorf tube at this stage.

Samples in L2 buffer containing the chromatin were sheared by sonication (4×30 s at amplitude 30) (Vibracell sonicator). The lysates were centrifuged ($4,000 \times g$, 4°C, 5 min), and the supernatants (750 μ l) transferred to another Eppendorf tube for immunoprecipitation. A sample (35 μ l) was retained to determine inputs. The samples from immunoprecipitation were diluted 1/10 in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.5%

NP40, 0.2 M NaCl, 0.5 mM EDTA). Extracts were precleared for 15 min with 20 μ l of a 50% (v/v) suspension of protein A-Sepharose in RIPA buffer. Immunoprecipitations were carried out overnight by incubation with gentle mixing by rotation (4°C overnight) with 10 μ g of anti-ATF3 antibody (rabbit polyclonal, SC-188X, Santa Cruz Biotechnology Inc.) or without antibody (control). Immune complexes (antibody-protein-DNA) were collected with 100 μ l Protein A-Sepharose [50% (v/v) suspension in RIPA buffer containing 1 μ g/ml sonicated salmon sperm DNA], by incubation with gentle mixing by rotation (4°C, 2 h).

Protein A-Sepharose was recovered by centrifugation (1,000 $\times$ g, 4°C, 3 min) and washed (4°C, 3 min, with gentle mixing by rotation) in 1 ml high salt buffer (20 mM Tris, pH 8.0, 0.1% SDS (w/v), 1% (v/v) NP40, 2 mM EDTA, 500 mM NaCl). Protein A-Sepharose was again recovered by centrifugation (4°C, 1 min, 200 $\times$ g) and then washed (4°C, 3 min, with mixing by rotation) in 1 ml low salt buffer [1X Tris-HCl/EDTA (TE)]. Immune complexes were extracted in 250 μ l elution buffer (1X TE containing 1% (w/v) SDS), heated (10 min, 65°C) then vortex agitated and centrifuged (1 min, room temperature, 200 $\times$ g). Supernatants were transferred to Eppendorfs. The extraction was repeated once more in the same manner, and the extracts pooled.

The input samples (made up to 500 μ l in TE) and the immunoprecipitated extracts (500 μ l) were uncross-linked by incubation (65°C, overnight) with 20 μ l 5 M NaCl (final concentration 0.2 M NaCl). Input and extract samples were then incubated with 500 μ l phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) (Sigma) (on ice, 5 min). Aqueous and organic phases were separated by centrifugation (15,300 $\times$ g, 4°C, 10 min). The upper aqueous phase was transferred to an Eppendorf tube and incubated with 500 μ l

isopropanol (-80°C, 3 h). DNA was pelleted by centrifugation (15,300 × g, 4°C, 10 min), precipitated by addition of 70% (v/v) ethanol and pelleted by centrifugation (15,300 × g, 4°C, 10 min). The DNA was air-dried (4°C) and resuspended in 25 µl Milli-Q filtered water.

PCR amplification was performed as described for RT-PCR (see above) in a final volume of 20 µl to which 2 µl of DNA were added. Promoter-specific primers were used for rat IL-6 (sense 5'- TGC TCA AGT GCT GAG TCA CT -3'; antisense: 5'- AGA CTC ATG GGA AAA TCC CA -3'). The number of cycles used for each primer set was 32. The conditions for PCR amplification were as follows: denaturing at 95°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 1 min. For semi-quantitative analysis PCR was also performed with genomic DNA obtained from input samples. Samples were mixed with PCR sample buffer [0.25% (w/v) bromophenol blue, 40% (w/v) sucrose] at 10% (v/v) and subjected to electrophoresis (90 V, 20 min) on 2% (w/v) agarose gels containing 100 µg/l ethidium bromide in 1X TBE (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA). Bands were detected under UV light.

2.11 Replication-deficient adenovirus

2.11.1 Cloning strategy and production of ATF3 adenoviral vectors

Replication-deficient adenoviruses expressing either full-length wild-type rat ATF3 (WT-ATF3) or full-length rat ATF3 antisense (AS-ATF3), were prepared using the AdEasy™ XL Adenoviral Vector System (Stratagene). Both adenovirus constructs were made and amplified by Dr Stephen Fuller in our laboratory. Constructs were cloned into a shuttle vector (pShuttle-CMV, see Figures 2.3 and 2.4A) which was then linearized with restriction enzyme *Pme* I and transformed into BJ5183-AD-1 competent cells (see Figure

2.4A). These cells are pre-transformed with the pAdEasy-1 plasmid which contains most of the human adenovirus serotype 5 (Ad5) genome but with genes E1 and E3 deleted, and possess the machinery necessary for homologous recombination between the shuttle vector and pAdEasy-1 (see Figures 2.4B and 2.5). The removal of the E1 and E3 viral genes creates space for foreign DNA (up to 7.5 kb) and eliminates self-replication capabilities. After recombination the ampicillin resistance gene of pAdEasy-1 is exchanged for the kanamycin resistance gene of pShuttle-CMV, enabling antibiotic selection of recombinants for kanamycin resistance which were then screened by restriction digestion with Pac1 (see Figures 2.4B and 2.6A). After identification, the recombinant pAdEasy-1 was produced in bulk in a recombination-deficient bacterial cell line (e.g. XL10-Gold[®]). Purified recombinant pAdEasy-1 plasmid DNA was linearised by digestion with Pac1 to expose the inverted terminal repeats (ITRs) and used to transfect human embryonic kidney cells HEK293 cells (see Figure 2.4C). Recombinant adenoviruses were then generated within 7-10 days.

To facilitate the identification, intracellular location and isolation of virally infected proteins, the WT-ATF3 construct was tagged with a FLAG sequence (DYKDDDDK) at its N-terminus (See Figure 2.6B). The FLAG sequence was introduced between the BglII and KpnI restriction sites of the multiple cloning site (MCS) of the pShuttle-CMV vector by synthesis of an oligonucleotide cassette containing the FLAG sequence preceded by an ATG start codon and flanked by appropriate restriction sites (see Figure 2.6B). The ATF3 coding sequences were isolated by PCR from rat cDNA using Pfu polymerase and primers designed to the 5' ATG start site and 3' stop codon regions (See Figure 2.6A). Primers were designed to include restriction enzyme sites to facilitate insertion into the multiple cloning site (MCS) downstream to and in-frame with the inserted FLAG

sequence (see Figure 2.6B). A similar strategy was used for AS-ATF3 constructs, using ATF3-FLAGpShuttle as a template for PCR and inserting the product into pShuttle-CMV vector (see Figure 2.3) but without the FLAG sequence.

2.11.2 Adenoviral infections

WT-ATF3 recombinants were tested for appropriate expression in neonatal cardiomyocytes by western blotting using anti-ATF3 antibody (SC-188, Santa Cruz Biotechnology) and anti-FLAG antibody (SC-807, Santa Cruz Biotechnology). Adenoviral infection of cardiomyocytes was performed at a multiplicity of infection (moi) of 10-100 in 4 ml (60 mm culture dishes) of serum-free maintenance medium [DMEM/Medium 199 (4:1 v/v) overnight at 37 °C in a humidified, 5 % CO₂ incubator].

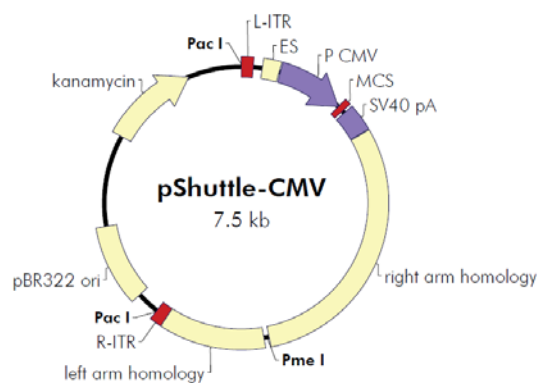


Figure 2.3 pShuttle-CMV vector map. The vector pShuttle-CMV contains a multiple cloning site (MCS) sandwiched between the CMV promoter and the SV40 polyadenylation signal and is suitable for insertion of a large cDNA (up to 6.6 kb). The regions indicated as arms are the stretches of sequence homology with pAdEasy-1 where the homologous recombination occurs. The R-ITR and L-ITR regions are short inverted terminal repeats (Left and Right) which have a role in replication of the viral DNA. Taken from instruction manual AdEasy™ Adenoviral vector system (Stratagene).

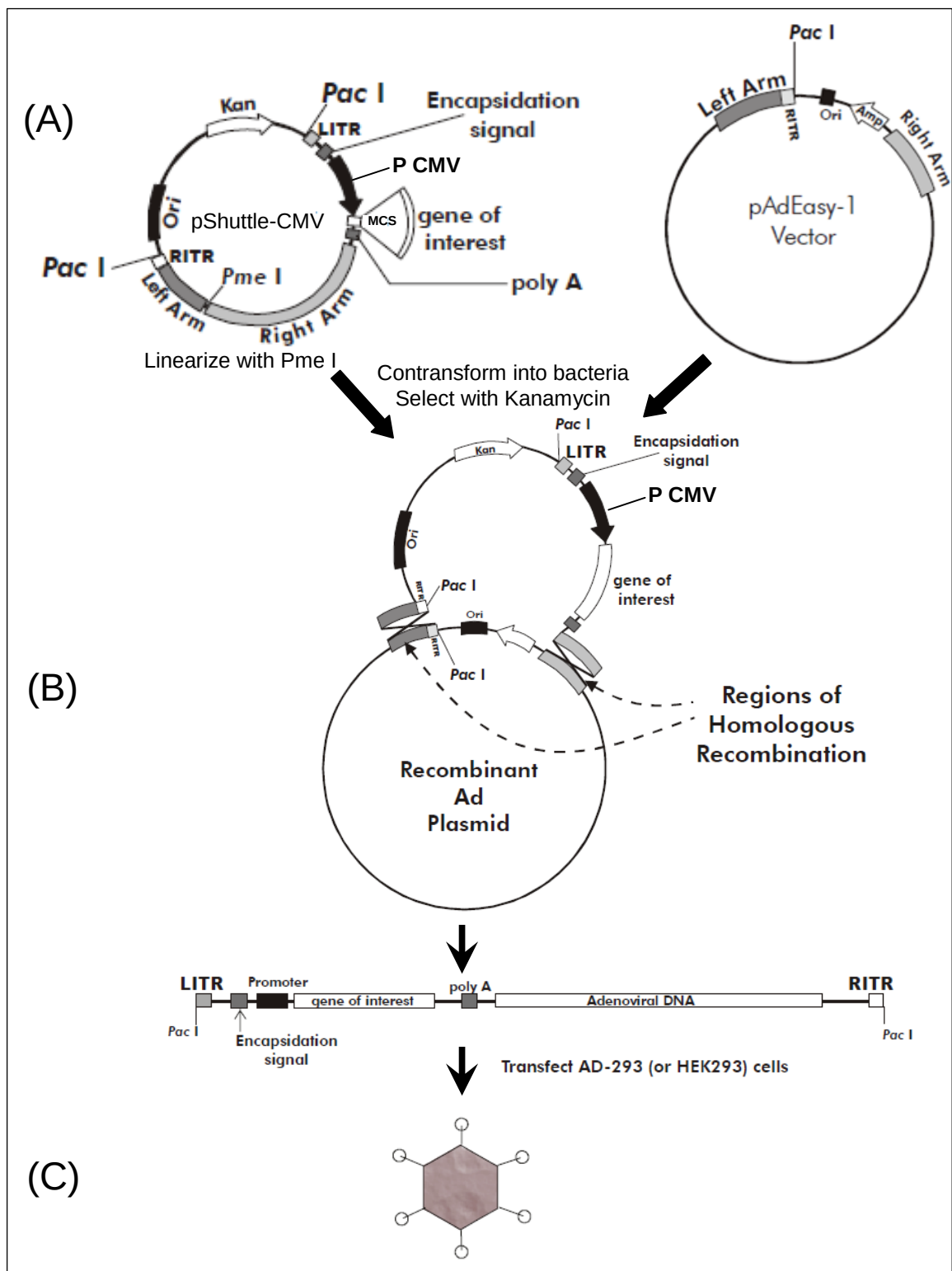


Figure 2.4 Schematic representation of the generation of recombinant adenovirus. (A) Cloning of the gene of interest into a Shuttle Vector (e.g. pShuttle-CMV). (B) Resultant plasmid is linearized with restriction endonuclease Pme I, and subsequently co-transformed into *E. coli* BJ5183-AD-1 cells with an adenoviral backbone plasmid (e.g. pAdEasy-1). Recombinants are selected for kanamycin resistance. (C) The linearized recombinant plasmid was transfected into adenovirus packaging cell lines (e.g. HEK293 cells). Recombinant adenoviruses were then generated. Adapted from instruction manual AdEasy™ Adenoviral vector system (Stratagene).

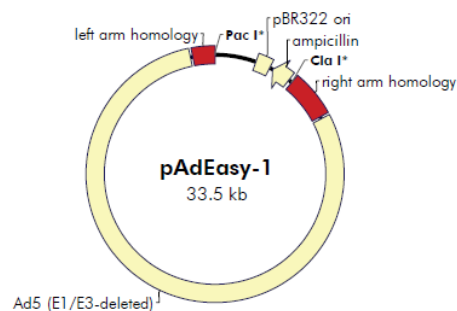


Figure 2.5 pAdEasy-1 vector map. The plasmid pAdEasy-1, containing most of the human adenovirus serotype 5 (Ad5) genome, is deleted for the genes E1 and E3. The removal of these two viral genes creates space for foreign DNA (up to 7.5 kb) and eliminates self-replication capabilities. pAdEasy-1 carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector. Taken from instruction manual AdEasy™ Adenoviral vector system (Stratagene).

(A)

Rat ATF3 (coding sequence 546 bp)

CCCTGGCCGCCCCGAGCGAAGACTGGAGCAAA**ATGATGCTTCAACATCCAGGCC**CAGGTCTCTGCCTCAGAAG
TCAGCGGACCGCCATCGTCCCCTGCCTCTCACCTCCTGGGTCACTGGTGTGTTGAGGATTTTGCTAACCTGA
CACCTTTTGTCAAGGAAGAGCTGAGATTCGCCATCCAGAACAAGCACCTTTGCCATCGGATGTCCTCTGCGC
TGGAGTCAGTCACCATCAACAACAGACCTCTGGAGATGTCAGTCACCAAGTCTGAGGTGGCCCCTGAAGAAG
ATGAGAGAAAAAGGAGGCGGCGGGAAAGAAACAAAATTGCTGCTGCCAAGTGTGAAACAAGAAAAAGAGA
AGACAGAGTGC**CTGCAG**AAGGAGTCAGAGAACTGGAGAGTGTGAATGCCGAACGAAGGCCAGATCGAGG
AGCTGAAGAATGAGAAGCAGCATCTGATTTACATGCTCAACCTGCACCGGCCACGTGTATCGTCCGGGCTC
AGAACGGGCGGACGCCGGAAGACGAGAGGAACCTTTTATCCAACAGATAAAAG**GAAGGAACATTGCAGAGCT**
AAGCAGAGGTGGCATGGGGGCAAT

Sense primer 5' - **ATGATGCTTCAACATCCAGGC** -3'

Antisense primer 5' - **TTAGCTCTGCAATGTTCTTC** -3'

(B)

KpnI

5' - **GACGACAAGGGTACC****ATG**ATGCTTCAACATCCAGGC -3' ATF3FwdKpnI

Asp.Asu.Lys.Gly.Thr... = end of FLAG sequence

5' - **CTTATCTAGAAGCTT****TTA**AGCTCTGCAATGTTCTTC -3' ATF3RevHindIII

.....HindIII.. = reverse MCS in pShuttle

Figure 2.6 Rat ATF3 cloning strategy. (A) PCR was performed using rat cDNA with fully complementary primers (location of primers within ATF3 sequence is shown in bold underlined text) for the coding region. Start and termination codons are shown in red. A PstI restriction site is shown in blue, which gives products of 342 and 204 bp to help confirm the identity of the PCR product. (B) PCR was performed from rat cDNA with ATF3FwdKpnI and ATF3RevHindIII primers and the product was then cut with KpnI / HindIII and ligated into pShuttleFLAG.

Chapter 3

Expression of ATF3 in cardiomyocytes in response to endothelin-1 or oxidative stress

3.1 Introduction

3.1.1 Activating transcription factor 3 (ATF3)

As previously discussed (section 1.5), the induced expression of protein-coding genes is regulated by transcription factors that bind to specific consensus binding sites in the promoters of target genes. The response of cardiomyocytes to hypertrophic agonists such as ET-1 and stress stimuli such as oxidative stress is likely to cause alterations in their phenotype (e.g. hypertrophy or cell death) by inducing changes in the patterns of gene expression that regulates these processes in the cell. Microarray studies from our laboratory identified ATF3 as one of the most potently induced transcription factors following stimulation of cardiomyocytes with ET-1 or H₂O₂ (Clerk et al., 2005) (Table 3.1). ATF3 is a member of the ATF/CREB (cyclic AMP-responsive element binding protein) family of basic leucine zipper-type transcription factors (section 1.5.1.3).

ATF3 is upregulated rapidly (usually within 2 h) and transiently after many stress-inducing stimuli. These stimuli include ischaemia alone and ischaemia/reperfusion in the heart (Yin et al., 1997), ischaemia/reperfusion in the kidney (Yin et al., 1997), partial hepatectomy (Hsu et al., 1991), seizure in the brain (Hai et al., 1999), axotomy of peripheral nerves (Tsujino et al., 2000), cycloheximide and FGF in hepatocytes (Weir et al., 1994), the antineoplastic drug doxorubicin and TNF α in cardiomyocytes and endothelial cells respectively (Nobori et al., 2002; Kawauchi et al., 2002), along with many other stimuli in many tissues and cells (reviewed in Hai et al., 1999). The transient induction of ATF3 by many stress signals has led to the conclusion that this transcription factor is important for the cells to cope with stress in the early stages of the stress response (Hai et al., 2001). However, the biological consequences of ATF3 induction in

these systems are still incompletely understood. Overexpression studies intended to mimic the upregulation of ATF3 in cells suggest that ATF3 could be involved in preventing cellular damage in neurons (Nakagomi et al., 2003) or cardiomyocytes (Nobori et al., 2002) (see below), or in the removal of damaged cells (Yan et al., 2005). However, unregulated overexpression of ATF3 in the pancreas (Hartman et al., 2004) or heart (Nobori et al., 2002) contributes to the dysfunction of these organs. Thus, despite cumulative evidence suggest that ATF3 plays an important role in the cellular response to stress, in general, its role in cell biology is not well understood and there is very little information about its physiological function in cardiomyocytes and the heart.

Table 3.1 Upregulation of ATF3 in cardiomyocytes by ET-1 or H₂O₂ (microarray analysis).

ATF3	
Affymetrix probe set 1369268_at	
ET-1 (100 nM)	
Time	Fold stimulation
30 min	3.9
1 h	6.2
2 h	3.2
4 h	0.9
24 h	1.1
H₂O₂ (0.2 mM)	
Time	Fold stimulation
2 h	20.9
4 h	6.6
24 h	1.8

Cardiomyocytes were exposed to ET-1 or H₂O₂ for the indicated times. RNA was extracted, cRNA was prepared and samples hybridized to Affymetrix Rat Genome 230 2.0 arrays. Array data were analysed by MicroArray Suite 5.0 followed by Genespring 7.31. Results are expressed as fold stimulation relative to unstimulated control (n = 3).

3.1.1.2 Structure, function and isoforms of ATF3

ATF3 was initially isolated from HeLa cells treated with PMA, as one of eight cDNA clones which encoded transcription factors that were able to bind to the ATF consensus sequence (5'-TGACGTCA-3') (Hai et al., 1989). ATF3 is a protein of 181 amino acids which contains a basic leucine zipper (b-Zip) DNA binding domain (residues 88–142) (Figure 3.1) responsible for homo- and heterodimer formation with other members of the b-Zip family (Figure 3.2). This domain also contains the basic amino acids which interact with ATF/CRE (5'-TGACGTCA-3') and activated protein-1 (AP-1) consensus sequences (5'-TGAGTCA-3') on target genes (Hai et al., 1999). In vitro studies have shown that ATF3 can form homodimers (ATF3/ATF3) or heterodimers with Jun family proteins. ATF3 homodimers are negative regulators of transcription of its own promoter (Chen et al., 1994; Wolfgang et al., 2000), and the promoters of other genes such as the proapoptotic transcription factor CHOP [C/EBP (CCAAT/enhancer binding protein) homologous protein] (Wolfgang et al., 1997), and the Ca²⁺ regulatory protein PLB among others (Gao et al., 2004). Heterodimers such as c-Jun/ATF3 or JunD/ATF3 activate transcription from promoters containing ATF/CRE, AP-1 and related transcription factor binding sites (Hsu et al., 1992; Chu et al., 1994), whereas the JunB/ATF3 heterodimer can either activate or repress transcription depending on the promoter context (Hsu et al., 1992).

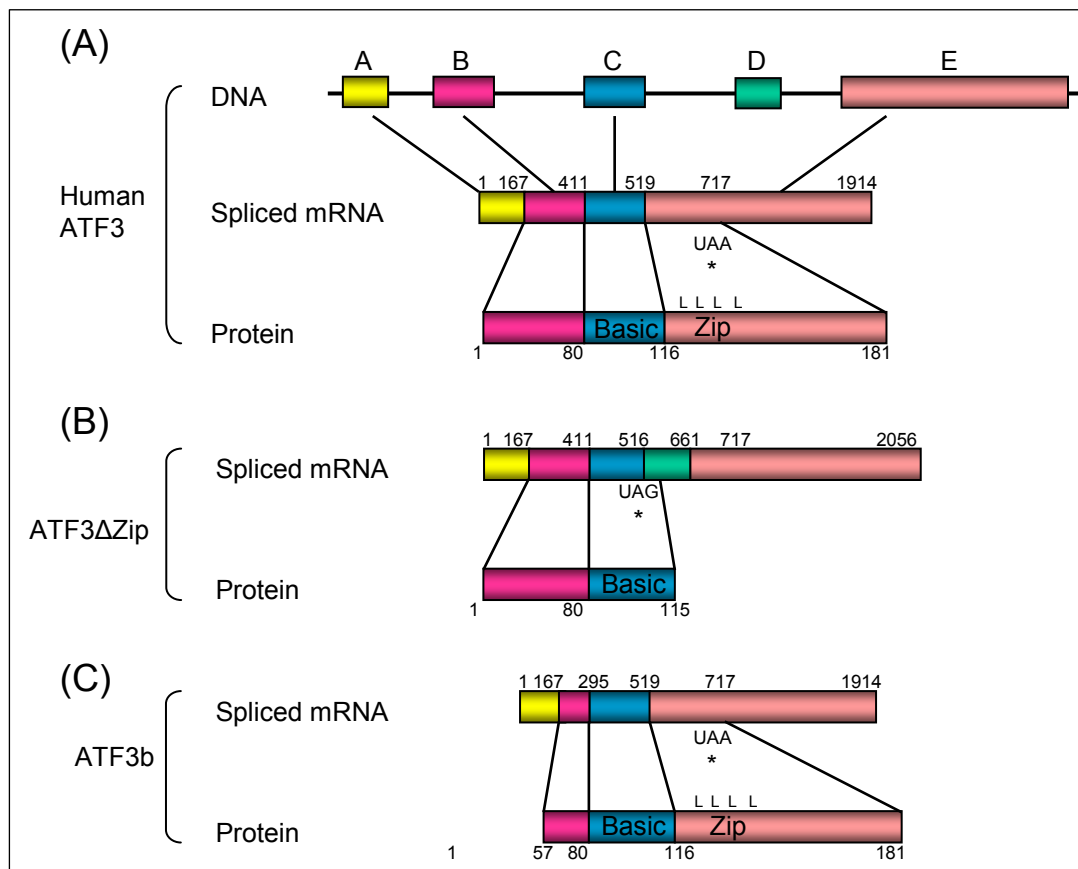


Figure 3.1 Human ATF3 and the alternatively-spliced ATF3ΔZip and ATF3b isoforms. (A) The human ATF3 gene has 5 exons (A-E) some of which are spliced into mRNA encoding the full-length ATF3. Exon A corresponds to the 5' untranslated region, exon B encodes the 80 N-terminal region, exon C encodes the basic region, and exon E encodes the leucine zipper domain. (B) Alternative-splicing of ATF3 containing all 5 exons results in an in-frame termination codon after the initial leucine of the leucine zipper domain. This leads to a 115 amino acid protein known as ATF3ΔZip, which is incapable of DNA binding as a consequence of lacking the leucine zipper domain. (C) The ATF3b isoform is formed as a consequence of an intron-like deletion of 116 bases from exon B, producing a shorter protein lacking 39 amino acids of the weak activation region (1–39 amino acids) and 18 amino acids of the repression region (40–84 amino acids). This protein retains DNA binding capabilities as the basic and leucine zipper regions are preserved. (A) and (B) adapted from Liang et al. (1996); (C) adapted from Wang et al. (2003).

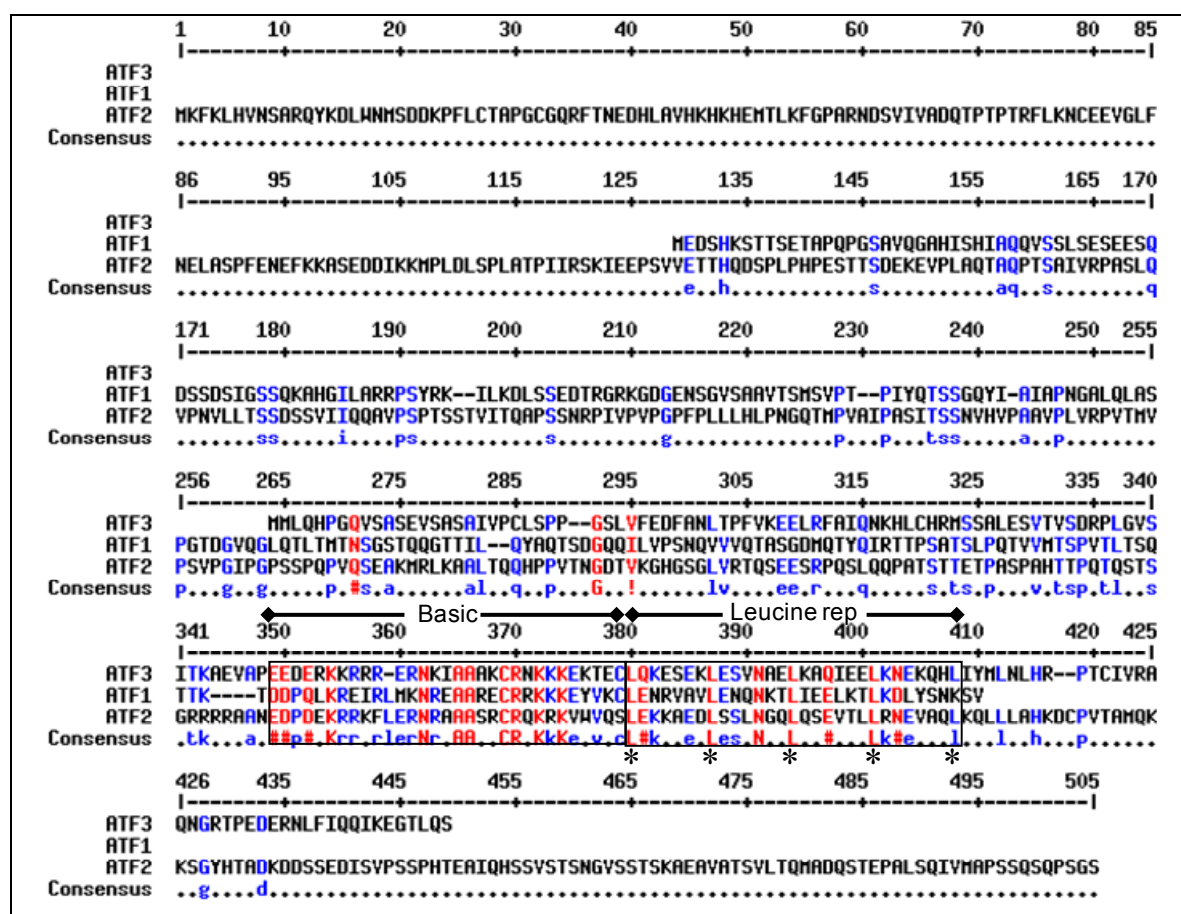


Figure 3.2 ATF3 shares significant homology with other ATF/CRE transcription factors. ATF3 has significant homology with ATF1 and ATF2 predominantly in the basic region (Basic) and the leucine zipper domain (leucine repeat) as indicated in boxed sequences. Like most leucine zipper transcription factors, leucine amino acids of the zipper domain are repeated every 7 amino acids as indicated by (*). Human ATF3: NP_001025458; Human ATF1: NP_005162; Human ATF2: NP_001871; graphic was generated using MultiAlin tool (Corpet, 1988) <http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html>

The amino acid sequence homology of mouse ATF3 protein is 99% and 98% with that of rat and human proteins respectively (Drysdale et al., 1996). The human ATF3 gene contains 5 exons (exons A-E) which encodes a protein of 181 amino acids following splicing of the RNA and removal of exon D (Liang et al., 1996) (Figure 3.1A). Thus, exon A encodes the 5' untranslated region, exon B encodes the N-terminal 80 amino acids, exon C encodes 36 amino acids which corresponds to the basic region, and exon E encodes the C-terminal 65 amino acids which contain the leucine zipper residues (Liang et al., 1996) (Figure 3.1A). The rat ATF3 was initially designated liver regenerating factor 1 (LRF1) and also contains 5 exons. In a similar manner to the human orthologue, rat ATF3 mRNA encodes a protein of 181 amino acids which is also a product of exons B, C, and E (Hsu et al., 1991; Chen et al., 1994). Several isoforms of ATF3 have been described in humans, which are the result of alternative-splicing of the transcribed RNA. However, to date such isoforms have not been described in rodents. The ATF3 Δ zip splice variant contains an additional 145 bases (exon D) between exons C and E (Figure 3.1B) (Chen et al., 1994). This replacement results in an in-frame termination codon immediately after the first leucine residue in the leucine zipper domain. The protein is therefore devoid of the leucine zipper dimerization domain and is incapable of binding to DNA (Figure 3.1B) (Chen et al., 1994). Recently two alternatively-spliced isoforms ATF3 Δ zip2a/b encoding the same protein of 135 amino acids were also described (Hashimoto et al., 2002). These isoforms also lack the leucine zipper domain and are unable to form dimers or bind to DNA consensus. Another isoform is ATF3b (Figure 3.1C) which lacks 39 amino acids of the weak activation region (1–39 amino acids) and 18 amino acids of the repression region (40–84 amino acids), as a consequence of an intron-like deletion of 106 bases from exon B. This isoform retains the intact bZip DNA binding domain and therefore has the ability to form dimers with other bZip family

members. Interestingly, ATF3b has a higher binding affinity for the ATF/CRE consensus than the long isoform (Wang et al., 2003).

3.1.2 ATF3 in cancer

Accumulating evidence suggests that ATF3 is an important adaptive response of the cell to genotoxic agents and that its function in this context may be related to the prevention of cell transformation and carcinogenesis. Thus, ATF3 is known to induce or accelerate apoptosis in tumour cells treated with chemotherapeutic agents (Yu et al., 1996; Mashima et al., 2001; Yan et al., 2005). As discussed in section 1.4.2, the PKB/Akt signalling pathway has an important role in regulating cell growth in several cell systems and has been proposed as a mediator of cardiac hypertrophy. However, activation of several components of this pathway correlates with the development of several forms of cancer, including breast cancer (Hutchinson et al., 2001) thyroid carcinoma (Ringel et al., 2001) and colon adenocarcinoma (Khaleghpour et al., 2004). Therefore, the blockade of this pathway is actively being considered as an important target for the development of novel antineoplastic drugs (reviewed in Kim et al., 2005). The PI3K inhibitor LY294002 exerts antitumorigenic effects and induction of apoptosis in human colon cancer cells both in vitro and in vivo (Semba et al., 2002). Interestingly, treatment of colorectal cancer cells with the PI3K inhibitor LY294002, leads to ATF3 upregulation secondary to phosphorylation of the transcription factor, early growth response 1 (EGR-1). Knockdown of the ATF3 gene using small interference RNA (siRNA) suppresses LY294002-induced apoptosis in colorectal cancer cells (Yamaguchi et al., 2006). This suggests that ATF3 could be responsible for some of the antineoplastic effects of LY294002 (Yamaguchi et al., 2006). One important observation derived from several microarray studies is that ATF3 expression levels appear to be downregulated in many

human cancers, including cancers of colon (Notterman et al., 2001), liver (Chen et al., 2002), kidney (Higgins et al., 2003), lung (Beer et al., 2002), breast (Sorlie et al., 2001) and prostate (Lapointe et al., 2001). Using a cancer profiling array, ATF3 expression levels were found to be downregulated by more than 50% in kidney, lung, breast, bladder, cervical and ovarian cancers (Yan and Boyd, 2006).

Recently a link between ATF3 and the tumour suppressor p53 was described that has shed some light on the possible role of ATF3 in cancer. The transcription factor p53 is an important regulator of cell fate and regulates cell cycle arrest and apoptosis (Levine, 1997). In normal cells, the protein levels of p53 are low as a consequence of a rapid turnover (half-life ~20 min) through the ubiquitin proteasome system that quickly degrades the protein (Horn and Vousden, 2007). Upon cellular stress by, for example, genotoxic agents or oxidative stress, the protein levels of p53 are upregulated via an increase in the stability of the protein. This leads to upregulation of p53-dependent genes which will promote cell cycle arrest to allow DNA repair, or commit the cells to apoptosis to eliminate damaged cells (Horn and Vousden, 2007). The importance of p53 as an antitumor gene is highlighted by the finding of mutations of the p53 gene in almost 50% of human cancers (Levine, 1997). One of the main regulators of p53 is the MDM2 (mouse double minute 2) protein (also known as HDM2 in humans), which binds to the N-terminal domain of p53 and acts as an ubiquitin ligase, thus adding ubiquitin moieties to several lysine residues of the C-terminal domain of the protein (Fang et al., 2000). This promotes p53 shuttling from the nucleus to the cytoplasm and its degradation by proteasomes (Lohrum et al., 2001). Recently, ATF3 was shown to be induced by the same genotoxic stress stimuli that also upregulate p53 (e.g. doxorubicin, camptothecin and actinomycin D) (Yan et al., 2005). Under these conditions, ATF3 binds to the C-terminal

domain of p53 and this binding leads to concealment of the lysine residues that are targeted by MDM2 for the addition of ubiquitin moieties, suggesting that ATF3 prevents degradation and increases the stability and activity of p53 by interfering with MDM2 (Yan et al., 2005). Thus, ATF3 appears to be a regulator of the stability and activity of p53, and in this way protect cells from transformation.

A dual role for ATF3 in cancer cells has been recently described whereby ATF3 overexpression enhances apoptosis of untransformed mammary epithelial cells (MCF10A). However, ATF3 protects the aggressive MCF10CA1a cancer cells, from apoptosis and even increases their motility, thus increasing cell viability and metastatic potential (Yin et al., 2007). This correlates with the finding of more than two copies of the ATF3 gene in up to 80% of breast tumours with increased expression of ATF3 in 50% of tumours evaluated (Yin et al., 2007). This suggests that ATF3 may have both protective activities against cell transformation and possible permissive effects in the setting of cancer metastasis at least for some types of tumours.

3.1.3 Role of ATF3 in the cardiovascular system

The physiological functions of ATF3 in the cardiovascular system are not entirely clear, and protective as well as harmful effects have been described. ROS production is increased during ischaemia or ischaemia/reperfusion, and this plays an important role in the development of cardiovascular pathologies because of the increased cellular damage to cardiomyocytes secondary to necrosis and/or apoptosis (Clerk et al., 2003). Using microarray analysis, ATF3 was shown to be upregulated in rat hearts subjected to ischemia/reperfusion, suggesting a possible cytoprotective role during oxidative stress

(Simkhovich et al., 2003). Ischaemic preconditioning is a natural phenomenon that occurs in the heart and other organs whereby after several brief periods of ischaemia followed by reperfusion there is increased resistance to cellular damage when further episodes of ischaemia of longer duration are applied (reviewed in Eisen et al., 2004). Recently, one study using microarray analysis confirmed that ATF3 is upregulated by ischaemia/reperfusion (Canatan, 2007). However, after the induction of ischaemic preconditioning by brief periods of ischaemia/reperfusion [3 x (5 min ischaemia followed of 10 minutes reperfusion)], ATF3 levels were downregulated during a lethal period of ischaemia/reperfusion (Canatan, 2007). The significance of the downregulation of ATF3 after ischaemic preconditioning in the heart is not clear, but suggests that under these conditions of limited cellular damage, ATF3 upregulation is probably not required by the cell. ATF3 expression is also increased in the heart by ischaemia alone and ischaemia coupled to reperfusion, which appears to depend on JNK activation (Yin et al., 1997).

Increased ROS production has also been associated with the proapoptotic effects of the chemotherapeutic drug doxorubicin in cardiomyocytes (Kumar et al., 2002). Under these conditions, ATF3 appears to play a cytoprotective role in the cardiovascular system. Thus, ATF3 mRNA and protein is upregulated by doxorubicin and adenovirus-mediated overexpression of ATF3 protects cardiomyocytes from doxorubicin-induced apoptosis by downregulating the transcription factor p53 (Nobori et al., 2002). Adenovirus-mediated overexpression of ATF3 in endothelial cells represses the transcription of the *p53* gene and protects these cells from TNF α induced apoptosis (Kawauchi et al., 2002).

Although some of the above evidence suggest that ATF3 is cytoprotective in the cardiovascular system, unregulated expression of ATF3 also appears to be harmful. Thus, cardiospecific overexpression of ATF3 in mice induces some degree of cardiac hypertrophy (which is more prominent in the atria than the ventricles, suggesting diastolic dysfunction, which correlates with a reduced relaxation rate), increases myocardial fibrosis, reduces cardiac contractility and increases arrhythmogenesis (Okamoto et al., 2001). Some in vivo studies have also suggested an important but ill-defined role for ATF3 in the heart. Microarray studies of the gene expression profiles induced in a model of myocardial infarction in rats found that among the genes expressed after 24 h of coronary artery ligation, ATF3 is induced in the area remote to the infarct but not within the necrotic area (LaFramboise et al., 2005). In contrast, 28 days after the occlusion, ATF3 is expressed in both the necrotic and remote areas of the affected heart (LaFramboise et al., 2005). Microarrays were also used to evaluate the transcriptional profiles of end-stage ischaemic and non-ischaemic cardiomyopathy in humans, and demonstrated that ATF3 is downregulated in the ischaemic group (Kittleson et al., 2005). However, the specific role of ATF3 was not further investigated in these studies. Nevertheless, all the above evidence suggests that ATF3 may be cytoprotective or harmful in the cardiovascular system depending on the precise circumstances.

Microarray studies in cardiomyocytes, comparing the changes in the transcriptional profile induced by H₂O₂, and the GqPCR agonist ET-1 (Table 3.1), identified ATF3 as one of the genes most potently induced by all these stimuli (Clerk et al., 2005). In this chapter, I have characterized the time course of ATF3 mRNA and protein expression in cardiomyocytes exposed to the hypertrophic agonist ET-1, or stress-inducing stimuli such

as H₂O₂ or doxorubicin). The signalling pathways involved in ATF3 expression were also explored in the setting of stimulation by ET-1.

3.2 Methods

3.2.1 RT-PCR

Cardiomyocytes were exposed to ET-1 (100 nM for 0-6 h or 0-100 nM for 30 min), or to H₂O₂ (0.2 mM for 0-6 h or 0-1 mM for 30 min), or to doxorubicin (3 µM for 0-6 h or 0-10 µM for 30 min). In some experiments, cardiomyocytes were preincubated for 10 min with 20 µM cycloheximide before the addition of ET-1, or preincubated for 15 min with 10 µM U0126, 10 µM SP600125 or 10 µM SB203580 prior to the addition of ET-1. Total RNA was extracted and cDNA synthesized (sections 2.3 and 2.5). SQ-PCR and Q-PCR were performed (section 2.6) to assess expression of *Atf3* or *Gapdh* mRNA, using primers designed across exon boundaries (see Table 2.2). For SQ-PCR 28 cycles of amplification for ATF3 and 22 cycles for GAPDH were performed.

3.2.2 Immunoblotting

Cardiomyocytes were exposed to 100 nM ET-1 or 0.2 mM H₂O₂ for 0-6 h. Nuclear proteins were extracted and immunoblotting was then performed (sections 2.8.2 and 2.9). Briefly, proteins (25 µg) were subjected to SDS-PAGE with a 12% polyacrylamide resolving gel and 6% stacking gel. Proteins were transferred to nitrocellulose membranes and probed with primary antibody to ATF3 (SC-188, Santa Cruz Biotechnology Inc.). Immunoreactive bands were detected by enhanced chemiluminescence and analysed semi-quantitatively by scanning densitometry. Values are expressed as arbitrary units.

3.3 Results

3.3.1 ATF3 mRNA is upregulated by endothelin-1

Using microarrays, ATF3 was identified as one of the genes most potently induced in cardiomyocytes by H₂O₂ or ET-1 (Table 3.1) (Clerk et al., 2005). To confirm these data and examine the time course of induction in more detail, ATF3 mRNA expression was evaluated in cardiomyocytes exposed to 100 nM ET-1 using SQ-PCR. ATF3 mRNA was expressed under basal conditions, but was significantly induced in response to ET-1 (Figure 3.3A). The maximal expression of ATF3 mRNA was at 30 min and rapidly returned to near basal levels within 2 h. These results were also validated for 100 nM ET-1 (0-8 h) using Q-PCR. A similar time course of expression with peak levels of mRNA at 30 min was observed (Figure 3.3B). The amplification plots (product vs number of cycles) for both ATF3 and GAPDH are shown in Figure 3.4. These demonstrate clearly the changes in the amount of ATF3 mRNA as a result of the different expression levels at different time points, with homogeneous amplification of GAPDH.

The effects of concentrations of ET-1 (30 min) on the expression levels of ATF3, were examined. ET-1 caused an increase in ATF3 mRNA expression at concentrations between 10 nM to 100 nM, whereas concentrations below 10 nM did not cause significant induction (Figure 3.4).

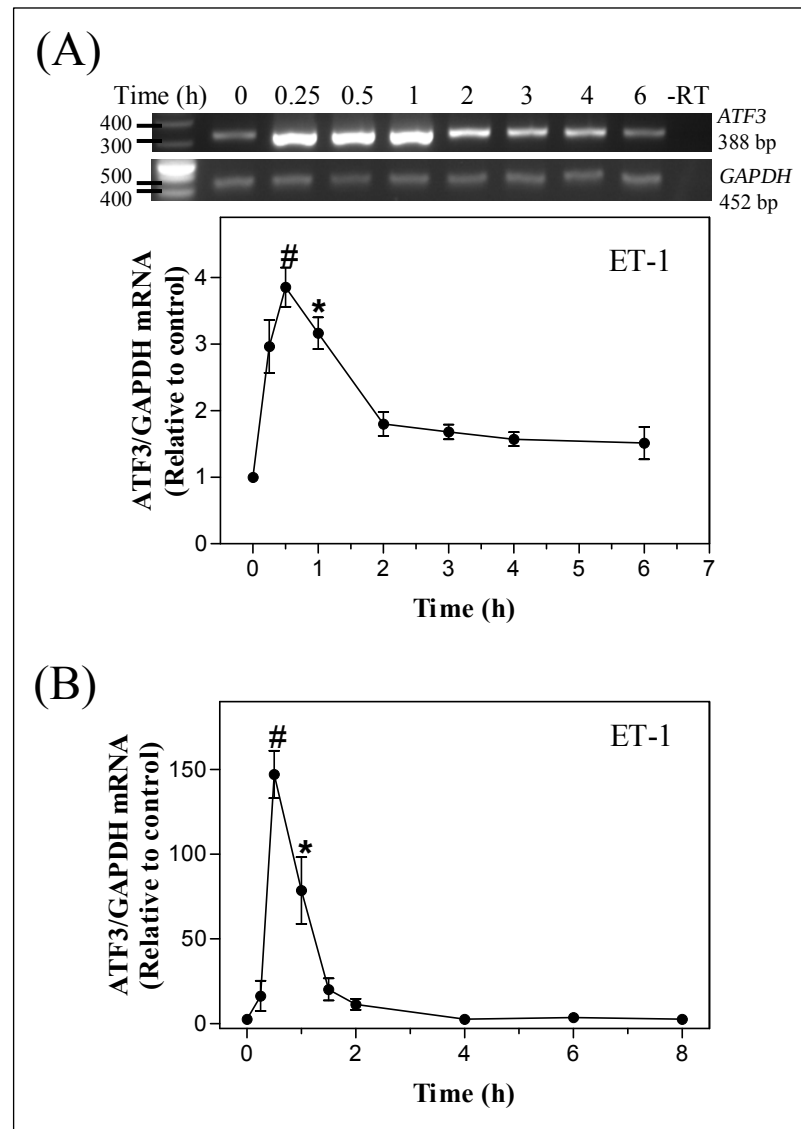


Figure 3.3 ET-1 promotes up-regulation of ATF3 mRNA in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to ET-1 (100 nM), for the indicated periods of time. In (A), the expression of ATF3 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panels. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH. In (B), the expression of ATF3 mRNA was analysed by Q-PCR. Results are means $\pm$ S.E.M. for four independent cardiomyocyte preparations. (* $p < 0.05$; # $p < 0.01$, relative to control, one-way ANOVA with Tukey post test).

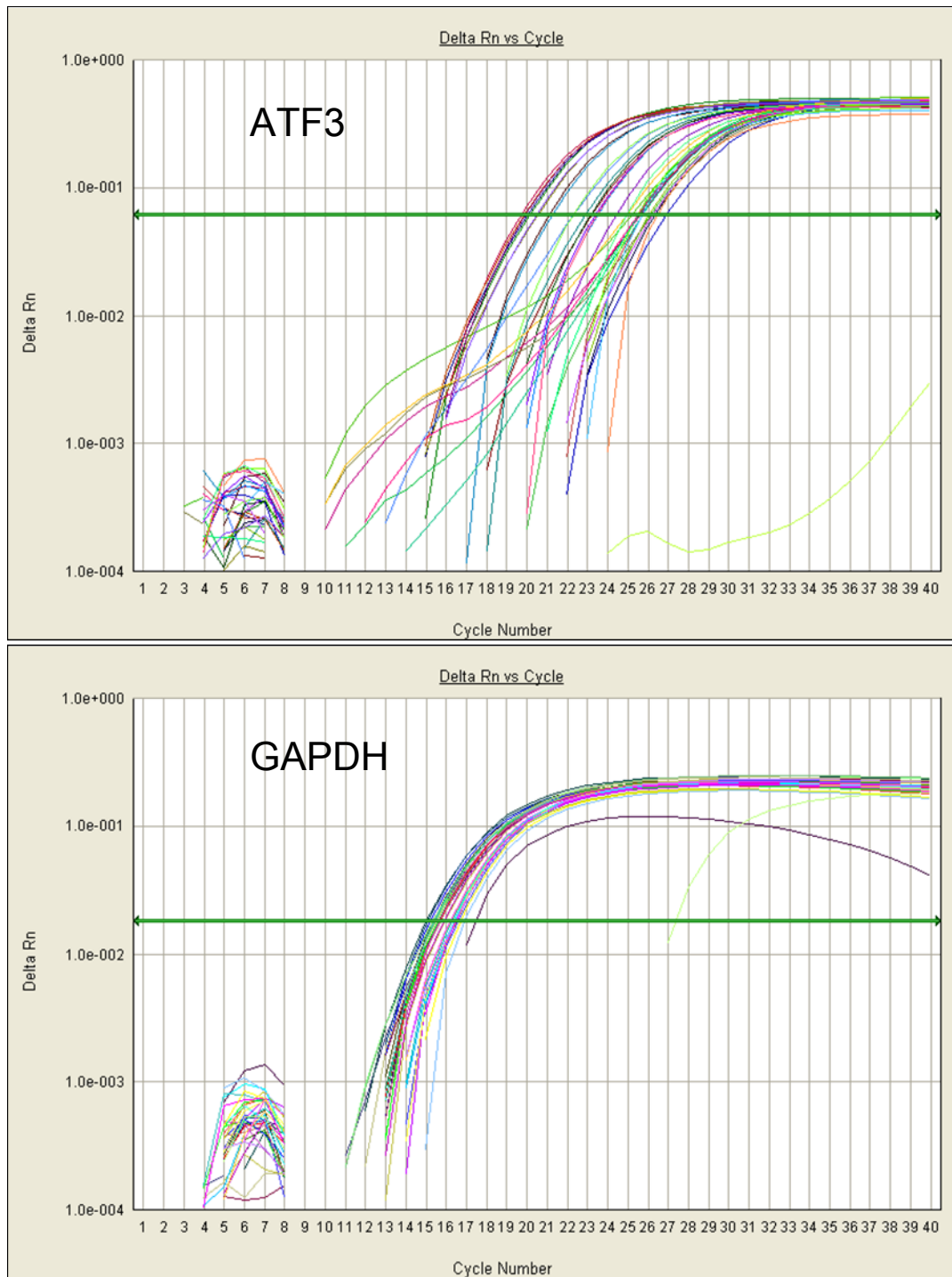


Figure 3.4 Q-PCR amplification plots for ATF3 and GAPDH. Expression of ATF3 (top panel) was analysed by Q-PCR, with simultaneous analysis of the housekeeping gene GAPDH (lower panel) in each 96-well optical plate as an endogenous control. Notice the variance of the amplification plot (product vs number of cycles) for ATF3 compared with the homogeneity of the amplification plot for GAPDH, indicating the homogeneous levels of housekeeping gene GAPDH, and the different levels of ATF3 expression that are quantified with the different treatment conditions.

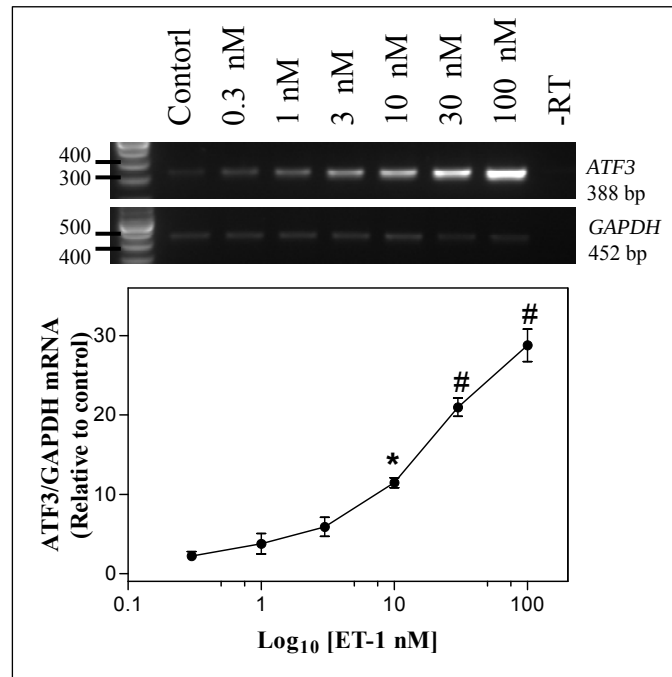


Figure 3.5 Effects of ET-1 concentration on the expression of ATF3 mRNA. Cardiomyocytes were unstimulated (controls, zero time) or exposed to ET-1 for 30 min with the concentrations indicated. The expression of ATF3 mRNA was analysed by SQ-PCR and a representative image from a single experiment is shown in the upper panel. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations (* $p < 0.01$; # $p < 0.001$, one way ANOVA with Tukey post test).

3.3.2 ATF3 is an immediate-early gene upregulated by endothelin-1 through the ERK1/2 cascade

The induction of IEGs occurs in the absence of *de novo* protein synthesis and thus cannot be blocked by protein synthesis inhibitors (e.g. cycloheximide) (Woodget et al., 1989; Platenik et al., 2000). Given the transient expression of ATF3 in cardiomyocytes in response to ET-1 and that activation of the ERK1/2 pathway by GqPCR agonists such as ET-1 plays an important role in the development of cardiomyocyte hypertrophy (sections 1.3.1.5 and 1.3.1.6) (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994; Bogoyevitch et al., 1995a; Gillespie-Brown et al., 1995; Bogoyevitch et al., 1996; Glennon et al., 1996; Clerk et al., 1998a; Yue et al., 2000), I then examined whether ATF3 is an IEG, and whether or not this was mediated through the ERK1/2 cascade.

Incubation of cardiomyocytes for 30 min with ET-1 in the presence of the protein synthesis inhibitor cycloheximide did not reduce the expression levels of ATF3 mRNA (Figure 3.6). Indeed, on the contrary, cycloheximide caused superinduction of ATF3 mRNA expression compared with ET-1 alone (Figure 3.6), suggesting that protein synthesis is not involved in ATF3 mRNA upregulation. This confirms that ATF3 is an IEG upregulated in cardiomyocytes in response to ET-1. Preincubation of cardiomyocytes with the MEK1/2 inhibitor U0126 significantly attenuated the expression of ATF3 mRNA induced by ET-1 (Figures 3.6 and 3.7), indicating that the ERK1/2 pathway is involved in the upregulation of ATF3 mRNA by ET-1.

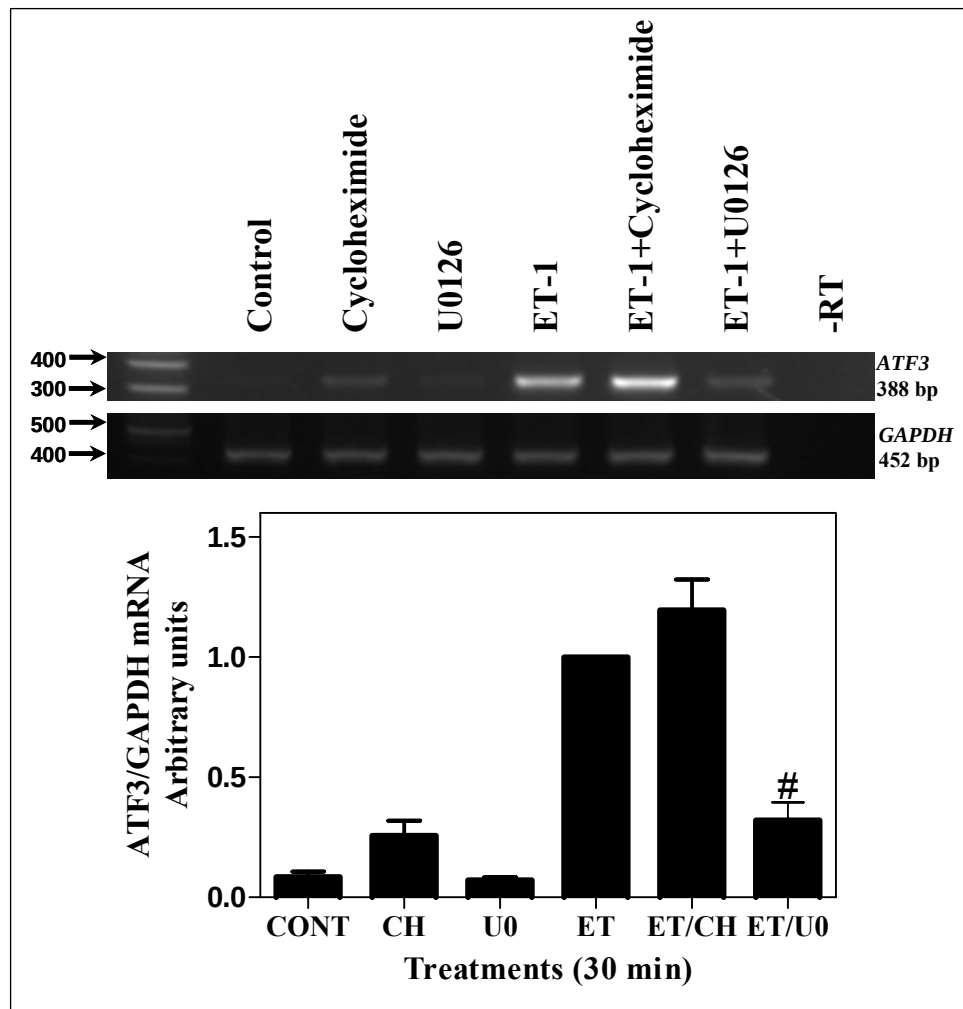


Figure 3.6 ATF3 is an IEG upregulated in cardiomyocytes by ET-1 through the ERK1/2 pathway. Cardiomyocytes were unstimulated (control) or exposed to ET-1 (100 nM) for 30 min, in the absence or presence of cycloheximide (20 μ M), or U0126 (10 μ M). The expression of ATF3 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panel. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for five independent cardiomyocyte preparations (# $p < 0.001$ relative to ET-1, one way ANOVA with Tukey post test) (CH= cycloheximide; CONT= control; U0= U0126).

3.3.3 JNKs but not p38-MAPKs are also implicated in ATF3 mRNA upregulation in response to ET-1

Previous studies have shown that ATF3 upregulation by the doxorubicin in cardiomyocytes could be regulated, at least in part, by the JNK pathway (Nobori et al., 2002). In other cell systems, p38-MAPK appears to be the main MAPK pathway responsible for ATF3 upregulation by agonists such as the protein synthesis inhibitor anisomycin, cytokines such as IL-1 β , and TNF α and oxidative stress in the form of H₂O₂ (Lu et al., 2007). Therefore, I examined whether p38-MAPK or JNK were involved in ATF3 mRNA upregulation in response to ET-1 in cardiomyocytes.

Preincubation of cardiomyocytes for 15 min with 10 μ M SB203580, which inhibits p38-MAPKs in cardiomyocytes (Clerk et al., 2002), did not significantly affect the induction of ATF3 mRNA by ET-1, suggesting that this pathway is not necessary for the upregulation of ATF3 by ET-1 (Figure 3.7). In contrast, pretreatment of cardiomyocytes with 10 μ M SP600125 which inhibits JNKs in cardiomyocytes (Clerk et al., 2002), or with U0126, an inhibitor of the ERK1/2 pathway, significantly blunted the upregulation of ATF3 mRNA induced by ET-1 (Figure 3.7).

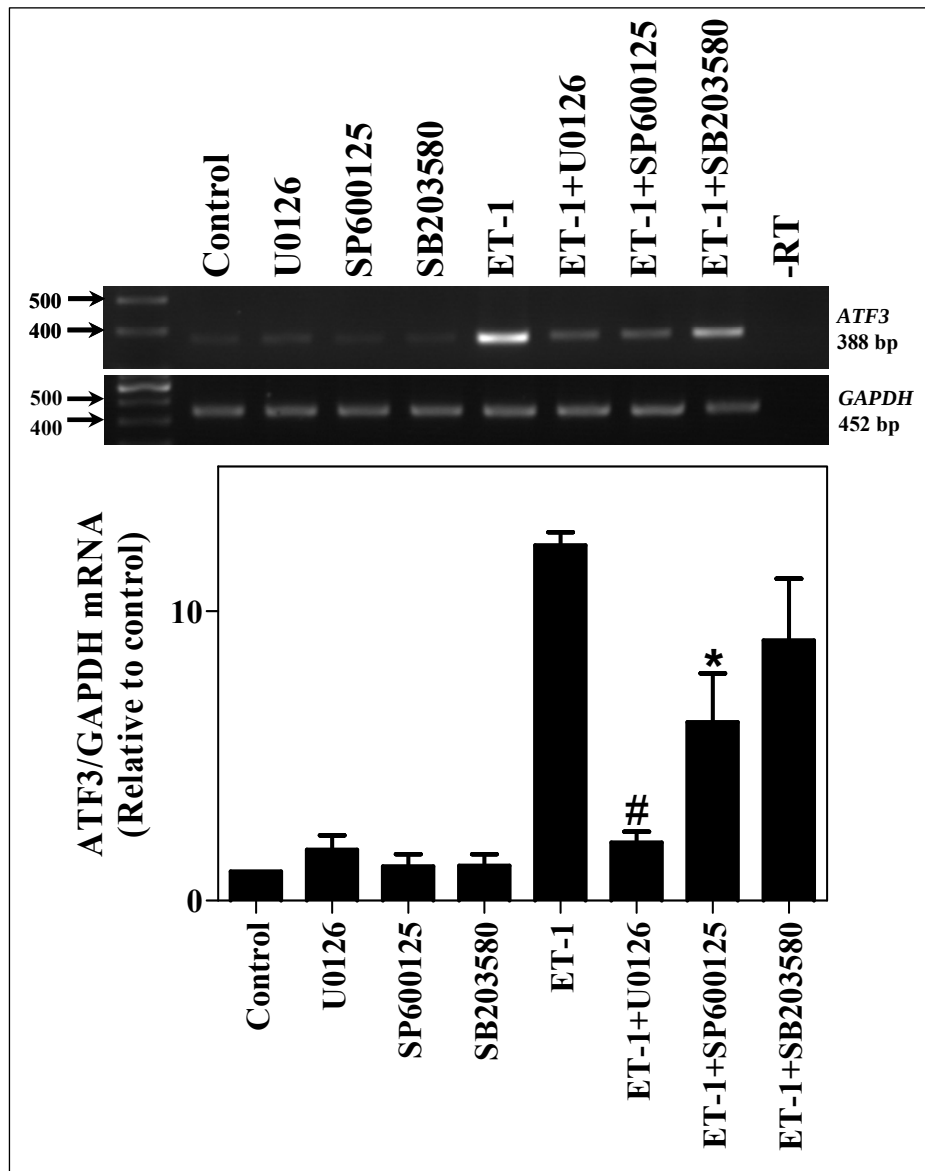


Figure 3.7 ERK1/2 and JNKs but not p38-MAPKs, are involved in the upregulation of ATF3 mRNA in cardiomyocytes in response to ET-1. Cardiomyocytes were unstimulated (Control) or exposed to 100 nM ET-1 for 30 min in the absence or presence of 10 μ M U0126, 10 μ M SP600125 or 10 μ M SB203580. Cardiomyocytes were also exposed to inhibitors alone. The expression of ATF3 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panel. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations (* $p < 0.05$; # $p < 0.001$, relative to ET-1, one way ANOVA with Tukey post test).

3.3.4 ATF3 protein is upregulated by ET-1 in cardiomyocytes

To confirm that the increase in ATF3 mRNA was reflected by an increase in ATF3 protein, the time course of expression of ATF3 protein was studied by western blotting of cardiomyocyte nuclear extracts. ATF3 was detected mainly as a band of 21 kDa but also as an additional fainter band of slower mobility (~23 kDa) on some occasions. This latter band may correspond with a phosphorylated form of ATF3. Consistent with the expression of ATF3 mRNA, ATF3 protein was present at low abundance under basal conditions and its expression was increased in response to ET-1 (Figure 3.8A). Maximal expression was at 1 h. Although the protein levels remained elevated for over 2 h with ET-1, expression of ATF3 had declined significantly by 4 h, and levels returned to basal by 6 h (Figure 3.8A). To confirm that the input from the ERK1/2 cascade on mRNA levels of ATF3 in response to ET-1 was also reflected at the protein level, I examined the effect of U0126 on the induction of ATF3 by ET-1. Preincubation of cardiomyocytes for 15 min with 10 μ M U0126 significantly attenuated the expression of ATF3 protein induced by 100 nM ET-1 (1 h) (Figure 3.8B).

3.3.5 ATF3 mRNA is upregulated by H₂O₂

I then investigated the time course of expression of ATF3 mRNA by H₂O₂, another stimulus that in previous microarray studies from our laboratory was shown to potently induce ATF3 (Clerk et al., 2005). ATF3 mRNA expression was evaluated in cardiomyocytes exposed to 0.2 mM H₂O₂ using SQ-PCR. ATF3 mRNA was expressed under basal conditions, but was induced in response to H₂O₂ (Figure 3.9A). The peak of expression of ATF3 mRNA was at 1-2 h (Figure 3.9A) and levels returned to basal after 6 hours.

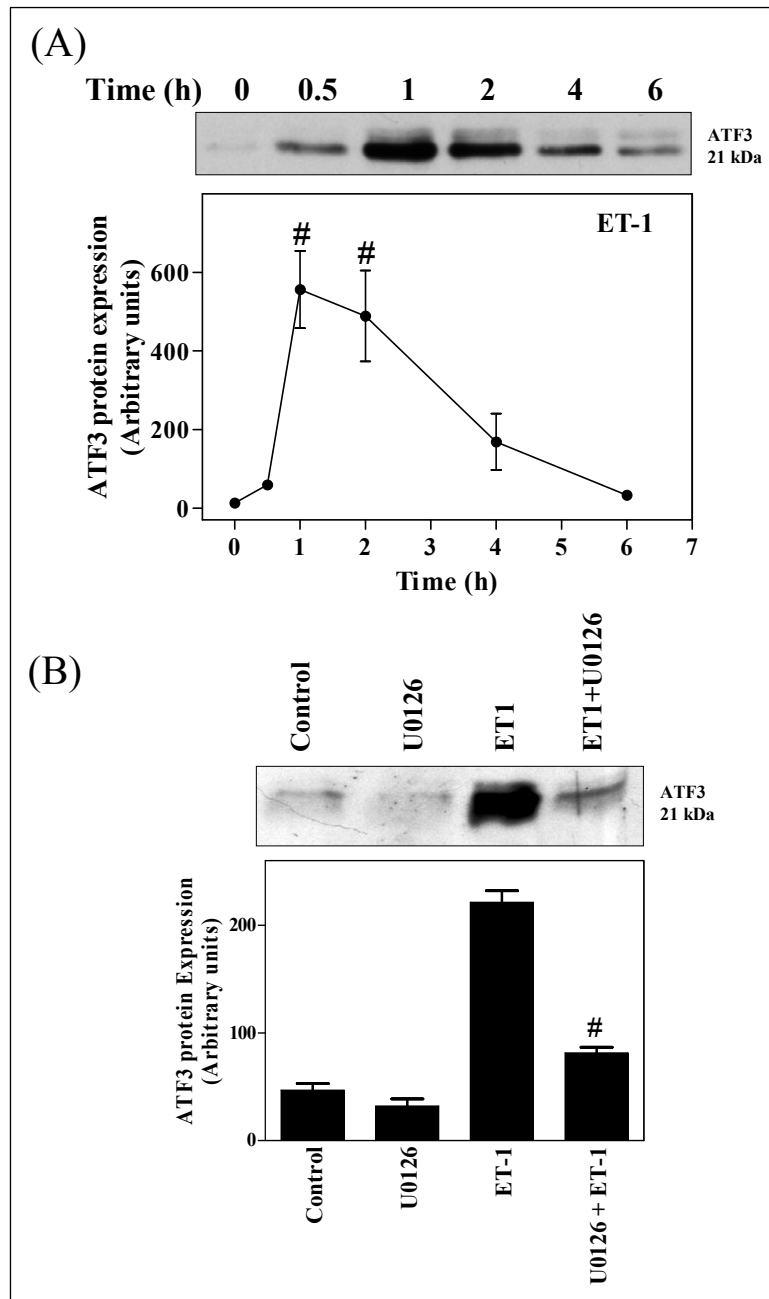


Figure 3.8 ATF3 protein expression is upregulated by ET-1 through the ERK1/2 cascade. In (A), cardiomyocytes were unstimulated (control, zero time), exposed to 100 nM ET-1 for the indicated times. In (B), cardiomyocytes were unstimulated (control) or exposed to ET-1 (100 nM) for 1 h, in the absence or presence of U0126 (10 μ M), or exposed to U0126 alone. Nuclear extracts (25 μ g) were immunoblotted with antibodies to ATF3 and representative images are shown in the upper panels. The blots were analysed by scanning densitometry and the combined data are shown in the lower panels as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (In (A) # $p < 0.001$ relative to control; in (B) # $p < 0.001$ relative to ET-1, one-way ANOVA with Tukey post test).

The effects of H₂O₂ concentration (1 h) on the expression levels of ATF3 were also examined. H₂O₂ caused an increase in ATF3 mRNA expression at a concentration of 0.1 mM with a similar level of induction up to at least 1 mM (Figure 3.9B). In contrast, concentrations below 0.1 mM did not cause any significant increase in ATF3 mRNA expression (Figure 3.9B).

3.3.6 ATF3 protein is upregulated by H₂O₂

I also confirmed that the increase in ATF3 mRNA was reflected by an increase in protein, and explored the time course of expression of ATF3 protein by immunoblotting of cardiomyocyte nuclear extracts. Consistent with the expression of ATF3 mRNA (Figure 3.9A), ATF3 protein was present at low abundance under basal conditions and was rapidly induced from 1 h with maximal expression at 2 h (Figure 3.10). The protein levels then declined by 4 h returning to near basal levels at 6 h (Figure 3.10).

3.3.7 ATF3 mRNA is downregulated by doxorubicin in cardiomyocytes

Doxorubicin is a very effective anticancer drug that is associated with high incidence of cardiomyopathy, probably as a consequence of increased production of reactive oxygen species and apoptosis of cardiac cardiomyocytes (Kumar et al., 2002). A previous study has shown that doxorubicin induces the expression of ATF3 at both mRNA and protein levels in cardiomyocytes (Nobori et al., 2002). I sought to confirm the effects of doxorubicin on the expression of ATF3. ATF3 mRNA expression was evaluated in cardiomyocytes exposed to 3 µM doxorubicin using SQ-PCR. ATF3 mRNA was expressed under basal conditions but, although there was a tendency towards increased expression by 30 min, this was not statistically significant (Figure 3.11A). In contrast,

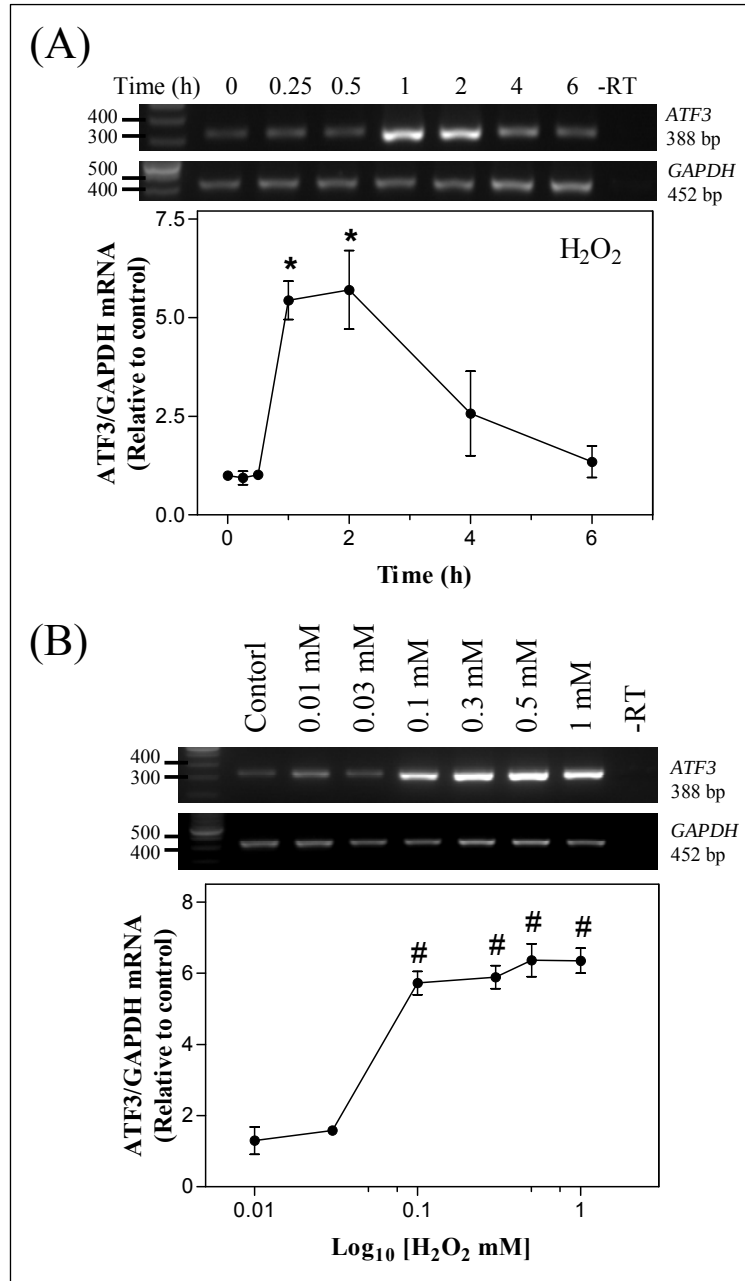


Figure 3.9 ATF3 mRNA expression is upregulated by H₂O₂ in cardiomyocytes. In (A), cardiomyocytes were unstimulated (control, zero time) or exposed to 0.2 mM H₂O₂ for the indicated periods of time. In (B), cardiomyocytes were unstimulated (Control) or exposed to H₂O₂ for 1 h at the concentrations indicated. The expression of ATF3 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panels. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations (* $p < 0.01$; and # $p < 0.001$, one way ANOVA with Tukey post test).

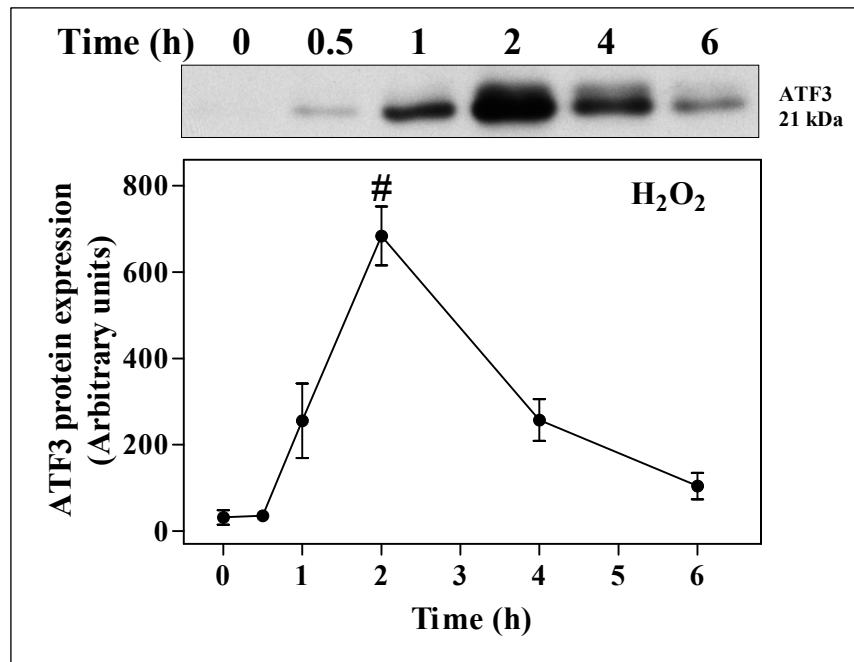


Figure 3.10 ATF3 protein expression is upregulated by H₂O₂. Cardiomyocytes were unstimulated (control, zero time) or exposed to 0.2 mM H₂O₂ for the indicated times. Nuclear extracts (25 µg) were immunoblotted with antibodies to ATF3 and a representative image are shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panel as means ± S.E.M. for three independent preparations of cardiomyocytes. (# p< 0.001 relative to control, one-way ANOVA with Tukey post test).

ATF3 mRNA expression not only declined after 1 h, but the mRNA was significantly downregulated within 3 hours and remained downregulated for 6 h following doxorubicin exposure (Figure 3.11A). I then explored the effects of different concentrations of doxorubicin on the expression levels of ATF3 mRNA (30 min). Doxorubicin had a tendency towards induction of ATF3 mRNA levels only at a concentration of 3 μ M, however this effect was not statistically significant (Figure 3.11B).

3.3.8 ATF3 protein levels are transiently upregulated and then downregulated below basal levels by doxorubicin in cardiomyocytes

I then evaluated the time course of ATF3 protein expression by doxorubicin using immunoblotting. As in other experiments (see sections 3.3.4 and 3.3.6), ATF3 protein was present at low abundance under basal conditions, and was significantly induced at 30 min (Figure 3.12). Interestingly, as with the mRNA levels of ATF3 (see above) the protein levels quickly declined and were downregulated below basal levels after 1 h and this was significant at the 6 h time point (Figure 3.12).

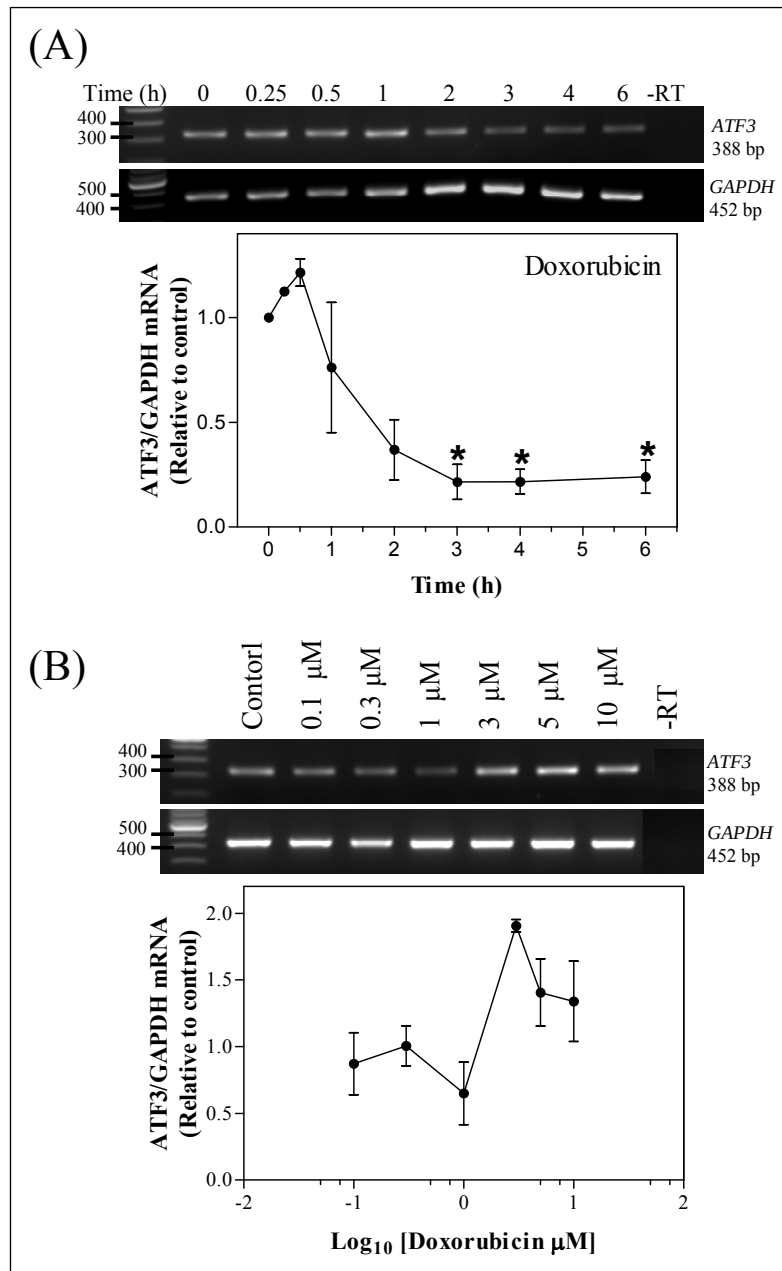


Figure 3.11 Doxorubicin downregulates the expression of ATF3 mRNA in cardiomyocytes. In (A), cardiomyocytes were unstimulated (control, zero time) or exposed to 3 μ M doxorubicin for the indicated periods of time. In (B), cardiomyocytes were unstimulated (Control) or exposed to doxorubicin for 30 min at the concentrations indicated. The expression of ATF3 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panels. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte. (* $p < 0.05$ relative to control, one-way ANOVA with Tukey post test).

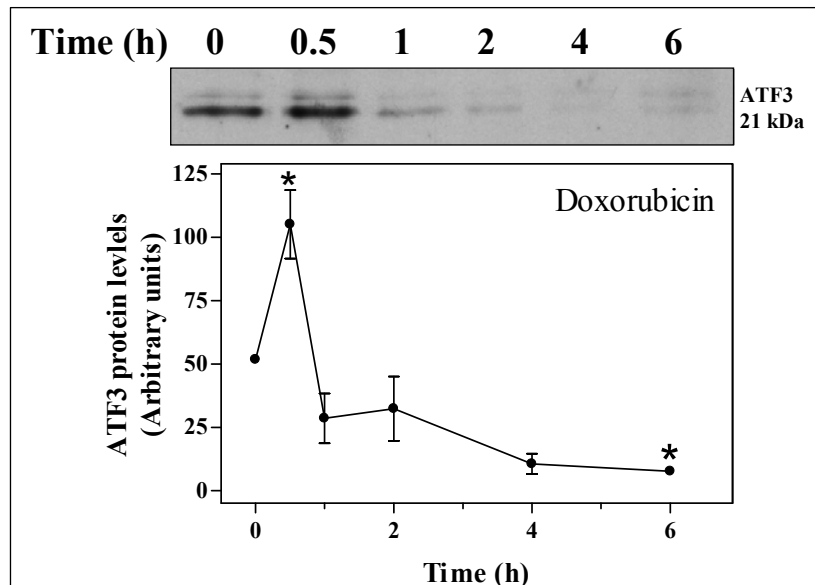


Figure 3.12 Doxorubicin transiently upregulates ATF3 protein levels, and then downregulates its expression below basal levels. Cardiomyocytes were unstimulated (control, zero time) or exposed to 3 μ M doxorubicin for the indicated periods of time. Nuclear extracts (25 μ g) were immunoblotted with antibodies to ATF3 and a representative image is shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panel as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (* $p < 0.05$ relative to control, one-way ANOVA with Tukey post test).

3.4 Discussion

3.4.1 Expression of ATF3 mRNA and protein in cardiomyocytes

ATF3 is upregulated in response to many stress stimuli in several tissues and cell systems (Hai et al., 1999). It has been postulated that one of the main functions of this transcription factor is to provide the cell with the means to cope with stress during the early stages of the response (reviewed in Hai et al., 2001). Previous studies in cardiomyocytes have documented acute upregulation of ATF3 mRNA and protein levels in response to the anticancer drug doxorubicin, which appears to be cytoprotective under these conditions (Nobori et al., 2002). However, the effects on ATF3 gene expression caused by stimuli also associated with cardiac pathologies such as hypertrophic stimulation by GqPCR agonists (e.g. ET-1, PE) or oxidative stress in the form of H₂O₂ has not been studied in these cells.

The experiments in this chapter demonstrate that ATF3 mRNA and protein abundance are increased in cardiomyocytes in response to ET-1 or H₂O₂ (Figures 3.3-3.9). As assessed by SQ-PCR and Q-PCR, the expression of ATF3 mRNA was potently induced by either H₂O₂ or ET-1 (Figures 3.3, 3.5 and 3.9), in agreement with previous microarray studies from our laboratory (Clerk et al., 2005). The effects of either agonist on the expression of ATF3 mRNA in cardiomyocytes were concentration-dependent (Figures 3.5 and 3.9). At the range of concentrations which induce ATF3 mRNA expression (10-100 nM), ET-1 promotes cardiomyocyte hypertrophy and this is mediated through signalling events dependent on the ERK1/2 pathway, a signalling cascade which also appears to be cytoprotective in cardiomyocytes (Yue et al., 2000). However, the concentrations of H₂O₂ which induce ATF3 expression (0.1-1 mM) (Figure 3.9) cause apoptosis (0.1-0.3 mM) or necrosis (> 0.5 mM) of cardiomyocytes (Cook et al. 1999; Kwon et al., 2003).

In addition to increasing the abundance of ATF3 mRNA, ET-1 or H₂O₂ also caused an increase in the expression of ATF3 protein (Figures 3.8 and 3.10). The induction of ATF3 mRNA and protein by ET-1 in cardiomyocytes was statistically significant. Although there are no previous reports of ET-1 induction of ATF3 in this or other cell systems, it has been reported that peptide growth factors or PMA, do induce such a response in other cell systems. Thus, treatment of the RAW 264.7 mouse macrophage cell line with PMA (3 h) induces the expression of ATF3 mRNA (Farber, 1992). In addition, EGF or hepatic growth factor potentially induce the expression of ATF3 mRNA in rat hepatocytes (maximal at 1 h) (Weir et al., 1994). Similarly, FGF induces the expression of ATF3 (4 h) in neuroblastoma cells (Tan et al., 1994).

The effect of H₂O₂ on ATF3 induction was also statistically significant and in agreement with previous studies in other cell systems and in cardiomyocytes. Thus, direct exposure of rat (r-RIN) and mouse (β TC6) pancreatic islet cells to 0.1 mM H₂O₂ for 1 h leads to increased expression of ATF3 mRNA (Allen-Jennings et al., 2001). However, the time course of expression was not explored in this study. The anticancer drug doxorubicin, appears to cause apoptosis, at least in part as a consequence of increased ROS production (Bachur et al., 1979; Yu et al., 1996). In human H9 leukaemia cell line treated with doxorubicin the expression of ATF3 was upregulated within 1 h and remained elevated over 6 h (Bachur et al., 1996). This induction of ATF3 expression correlated with JNK activation, increased *c-jun* mRNA expression and induction of apoptosis, suggesting that ATF3 may be involved in the elimination of cancer cells by cytotoxic agents like doxorubicin.

Importantly, the adverse effects of doxorubicin and related compounds in the heart frequently cause a severe form of cardiomyopathy. This cardiomyopathy is dependent on the cumulative dose administered to the patient (Minotti et al., 2004), and also appears to be mediated through the increased production of superoxide radical anion ($O_2^{\cdot-}$), which is quickly converted into H_2O_2 or peroxynitrite (Dorr, 1996; Kumar et al., 2002). Consistent with this, doxorubicin and related compounds such as daunorubicin cause apoptosis of cardiomyocytes secondary to increased production of ROS (Sawyer et al. 1999; reviewed in Minotti et al., 2004). Interestingly, previous reports have shown that doxorubicin induces the expression of ATF3 mRNA and protein in cardiomyocytes, with maximal expression at 1 h and 2 h, respectively (Nobori et al., 2002). In these settings, increased expression of ATF3 has been postulated to be cytoprotective. Thus, adenovirally-mediated overexpression of ATF3 in these cells prevents doxorubicin-induced apoptosis (Nobori et al., 2002). Using SQ-PCR, I also observed that ATF3 mRNA abundance tends to increase in cardiomyocytes exposed to doxorubicin for 30 min, but this does not appear to be statistically significant. However, subsequent downregulation of the expression of ATF3 mRNA below basal levels is statistically significant (Figure 3.9). Our studies also show a significant (though small) increase in expression of ATF3 protein levels following doxorubicin stimulation (Figure 3.10). However, this was followed by significant downregulation of ATF3 protein below basal levels. Although the reasons for these discrepancies between the data presented here and those of Nobori et al. (2002) are not clear, it is possible that the methodology employed (Northern blot (Nobori et al., 2002) vs SQ-PCR here) or doxorubicin concentration (1 μ M (Nobori et al., 2002) vs 3 μ M here) may have influenced these observations. However, the concentration dependence suggests that this is not the case, because at the 1 μ M concentration I still did not observe any increase in the transcript levels of ATF3 (Figure 3.9).

3.4.2 ATF3 is an IEG

My experiments show that ATF3 is an IEG which is rapidly and transiently induced by ET-1 in a process that does not require *de novo* protein synthesis (Figure 3.5). Although this has not been previously shown in cardiomyocytes, previous studies in other cell systems and tissues have suggested that ATF3 is indeed an IEG which may be activated by growth or stress signals. Thus, ATF3 mRNA levels are low under basal conditions, but dramatically increase upon stimulation by serum (Mohn et al., 1991; Chen et al., 1994). The 3' untranslated region (3' UTR) of the ATF3 mRNA contains several AU-rich sequences, which is a characteristic of many IEGs (Chen et al., 1994; Hsu et al., 1994), and this also appears to be responsible for the short half life of these so called "labile mRNAs" (Zubiaga et al., 1995; Barreau et al., 2006). In fact, this may also explain at least in part the transient expression of ATF3 observed upon ET-1 and H₂O₂ (Figures 3.3, 3.8, 3.9, and 3.10). It was also evident from experiments from Figure 3.6 that cycloheximide alone induces the expression of ATF3 mRNA which correlates with the induced expression of ATF3 previously reported in hepatocytes (Weir et al., 1994). A common characteristic of many IEGs is the superinduction of their expression in the presence of cycloheximide (Woodgett, 1989), and this was also observed in our experiments (Figure 3.5), although this was not statistically significant.

ATF3 has been previously described as a negative regulator of transcription (Chen et al., 1994). Thus, another potential explanation for the transient expression of ATF3 in these experiments is that once ATF3 message is translated into protein, the active ATF3 may feedback into its own promoter and cause transcriptional repression of the message. In agreement with this hypothesis, there also is evidence that ATF3 causes autorepression of

its own transcription and several ATF/CRE consensus sequences located in the promoter of ATF3 are required for efficient repression (Wolfgang et al., 2000).

3.4.3 Regulation of ATF3 expression by ET-1 is mediated through the ERK1/2 and JNK signalling pathways

There are suggestions in the literature that ATF3 activity and/or expression is regulated by protein kinases, but how this occurs is not yet completely understood. Of the different kinase superfamilies, MAPKs are the most likely. Thus, many stress responses that are implicated in ATF3 expression are known to activate one or more of the MAPKs. For example, ischaemia or ischaemia/reperfusion cause increased expression of ATF3 in the heart and the kidney (Yin et al., 1997), and this same stress causes activation of all MAPK cascades in the heart, although most prominently JNKs and p38-MAPKs (Bogoyevitch et al., 1996; Clerk et al., 1998). Recently, ATF3 gene and protein expression was shown to be induced in cardiomyocytes by doxorubicin, and the phosphorylation of JNK, (but not p38-MAPKs or ERK1/2) was documented as emulating the time course of expression of ATF3, which was interpreted as indirect evidence for a role of JNK in ATF3 regulation (Nobori et al., 2002).

A recent report studied the involvement of the MAPKs cascades on the expression of ATF3 in several transformed cells lines. Using a variety of agonists (anisomycin, IL-1 β , TNF α or H₂O₂) appear to promote ATF3 mRNA and protein expression via the p38-MAPK signalling cascade (Lu et al., 2007). In contrast, neither ERK1/2 nor JNKs seem to be necessary or sufficient for ATF3 induction by these agonists (Lu et al., 2007). However, studies in other systems have led to different conclusions. Thus, induction of ATF3 expression by the non-steroidal anti-inflammatory drug sulindac in colon cancer

cells is mediated through the increase in the transcriptional activity of Egr-1 on the ATF3 promoter (Bottone et al., 2005). This effect was inhibited by overexpression of a dominant negative ERK1 (dn-ERK1) and by the ERK1/2 cascade inhibitor PD98059, indicating the importance of this pathway in regulating ATF3 expression in this system (Bottone et al., 2005). In endothelial cells, JNKs play an important role in upregulation of ATF3 by homocysteine (Cai et al., 2000) or by TNF α (Inoue et al., 2004). In contrast, activation of the ERK1/2 pathway by TNF α may downregulate ATF3 levels in this same system (Inoue et al., 2004). This suggests that upregulation of ATF3 by a particular MAPK cascade may depend on the cellular context.

ET-1 and other GqPCR agonists potently activate the ERK1/2 cascade and this signalling cascade plays a major role in cardiac hypertrophy both in vitro and in vivo (sections 1.3.1.5, 1.3.1.6) (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994; Bogoyevitch et al., 1995a; Gillespie-Brown et al., 1995; Bogoyevitch et al., 1996; Glennon et al., 1996; Clerk et al., 1998a; Bueno et al., 2000; Yue et al., 2000). Given the importance of the ERK1/2 pathway in cardiac hypertrophy, and the potency of GqPCR in activating this pathway, I explored the role of the 3 major subfamilies of MAPKs in the regulation of ATF3 expression in response to ET-1. My experiments demonstrated that ATF3 is not only an IEG induced by ET-1 (see above), but also the upregulation of this IEG is mediated particularly through the ERK1/2 cascade (Figure 3.6). Thus, the upregulation of ATF3 mRNA and protein in response to ET-1 was significantly inhibited by the ERK1/2 inhibitor U0126 (Figure 3.7 and 3.8B) and to a lesser extent but also significant ATF3 mRNA levels by the JNK inhibitor SP600125 (Figure 3.7). This suggests that ATF3 upregulation by ET-1 in cardiomyocytes is mediated mainly through the ERK1/2 cascade with some input from the JNKs, although the relative lack specificity of SP600125 for

inhibition of the JNK cascade may be partially responsible for the effect observed on ATF3 upon ET-1 stimulation (Figure 3.7) (Bain et al., 2007). However, in contrast to other studies that suggests p38-MAPK is the main regulator of ATF3 mRNA and protein in several cell lines (Lu et al., 2007), the p38-MAPK pathway does not appear to have a significant involvement in the regulation of ATF3 in cardiomyocytes following stimulation by ET-1 (Figure 3.7).

ATF3 is a basic leucine zipper member and similarity with other members of this family of transcription factors has been described. Thus, ATF3 and c-Fos (another IEG) share homology in the b-Zip region and some authors have proposed that they probably evolved from a common ancestor (Meyer and Habener, 1993). In addition, human ATF3 and c-Jun dimerization protein 2 (JDP2) have 90% homology at the b-Zip motif (Katz and Aronheim, 2002). However, despite the fact that the conserved b-Zip region of ATF3 derived from several species possesses an identical putative phosphoacceptor site to JDP2 (Thr-148), ATF3 does not appear to be phosphorylated by any of the MAPKs, whereas phosphorylation of this site is crucial for JDP2 phosphorylation by JNKs (Katz and Aronheim, 2002). Therefore it is unlikely, that ERK1/2 or JNKs may influence the transactivating activity of ATF3, and probably their role in the regulation of ATF3 in cardiomyocytes is restricted to influence the signalling events leading to the upregulation of this IEG.

Interestingly, although it may be coincidental, JNKs, p38-MAPK or ATF3 cardiospecific over-expressing transgenic mice produce similar phenotypes, consisting of conduction disturbances, myocardial fibrosis and heart failure (Liao et al., 2001; Okamoto et al., 2001; Petrich et al., 2002; Liang and Molkentin 2003; Petrich et al., 2003; Petrich et al.,

2004). The cardiac hypertrophic phenotype in these transgenic mice is not as marked as in other models [e.g. MKK1 overexpression (Bueno et al., 2000)], and all these studies describe bilateral atrial enlargement after MKK7, MKK3, MKK6 or ATF3 overexpression. An additional finding common to the phenotypes derived from cardiac overexpression of ATF3, p38-MAPKs and JNKs is that most studies are inconclusive with respect to their apoptotic effects, with both pro- and anti-apoptotic effects described, suggesting a context dependent outcome for their effects on apoptosis (Liao et al., 2001; Okamoto et al., 2001; Petrich et al., 2002; Liang and Molkentin 2003; Petrich et al., 2003; Petrich et al., 2004; Mitchell et al., 2006).

Upregulation of ATF3 in cardiomyocytes in response to doxorubicin appears to be cytoprotective (Nobori et al., 2002). It is possible that technical differences (e.g. concentration used) prevented me from replicating the previously described upregulation of ATF3 with doxorubicin. Nevertheless, the data of Nobori et al. (2002) suggest that ATF3 induction in response to oxidative stress in the form of doxorubicin is cytoprotective. The experiments presented here suggest that ATF3 may play an important role in the regulation of the cellular response to oxidative stress and hypertrophic stimulation. However, the specific role of ATF3 in response to ET-1 or H₂O₂ remains to be established. Further experiments will evaluate the physiological consequences of ATF3 induction in cardiomyocytes in response to oxidative stress and ET-1.

Chapter 4

Regulation of interleukin 6 expression by ATF3 in cardiomyocytes

4.1 Introduction

In chapter 3, I have demonstrated that the transcription factor ATF3 is an IEG that may play an important role in the hypertrophic or stress response of cardiomyocytes as indicated by the potent induction of its mRNA and protein by ET-1 or H₂O₂. The availability of the complete sequence of the human genome and several others such as the rat and mouse, has led to the cataloguing of thousands of genes (~25,000) which are protein-coding genes (International Human Genome Sequencing Consortium, 2004). The major task now is to understand how the changes in the expression of genes in different cells and tissues lead to alterations in the structure and function of organs such as the heart during adaptive responses such as cardiac hypertrophy or in pathological ventricular remodelling following myocardial infarction. Accordingly, to characterize further the physiological function of ATF3 in cardiomyocytes exposed to ET-1 or H₂O₂, it is important to identify the potential downstream targets.

As discussed in chapter 1 the gp130 cytokines (also known as IL-6 family of cytokines) appears to play an important role in disease states such as heart failure (section 1.3.3). The most studied members of this family in the heart include CT-1, LIF and IL-6 (Pennica et al., 1996; Wollert et al., 1996). It is well-established that this family of cytokines acting on their cognate receptors activate several signalling pathways including the ERK5 signalling cascade, which cause elongation of cardiomyocytes by directing sarcomeric assembly in series (Wollert et al., 1996). Due to the circumferential disposition of cardiomyocytes in the ventricle, myocyte elongation may ultimately translate into eccentric hypertrophy and ventricular dilatation (section 1.1) (Grossman, 1975). It is for this reason that, since the

discovery of cardiotrophin-1, this and other members of the gp130 cytokines have been postulated as direct mediators of the alterations in the dilatation of the heart observed in the progression to heart failure (Wollert et al., 1996; Hunter and Chien, 1999). Other signalling cascades activated by the IL-6 family upon binding to their receptors are the ERK1/2 cascade, the PKB/Akt pathway and the JAK/STAT pathway, all of which are probably involved in cytoprotection and prevention of apoptosis in the cardiomyocyte following oxidative stress (reviewed in Hilfiker-Kleiner et al., 2006).

IL-6 has an important role in the regulation of the immune response defence of the organism. Thus, upon stimulation with foreign antigens from invading bacteria, such as bacterial lipopolysaccharide (LPS), the activation of receptors (e.g. Toll-like receptors) in the cells of the innate immune system (e.g. monocytes, the first line of defence of the organism) leads to their activation and removal of the invader (Royet et al., 2005). Under these conditions, the mounting of a proper defence response not only involves destruction and removal of the pathogenic agents (e.g. bacteria), but also the preservation of the integrity of the organism by avoiding excessive inflammation and tissue damage. This process is positively regulated, at least in part, by cytokines such as IL-6 secreted by activated monocytes, and there is evidence that increased expression of IL-6 leads to chronic inflammation and autoimmune diseases (reviewed in Rose-John et al., 2007). Recently, IL-6 was identified as one of the direct targets of ATF3 in monocytes activated with LPS. Thus, during the initial phase of the response, LPS induces the expression of IL-6 within a period of 2 h, and this correlates with the activation of monocytes into cytotoxic macrophages. However, after more prolonged exposure, ATF3 is upregulated and this correlates with a reduction in the expression levels of

IL-6 4-6 h following stimulation (Gilchrist et al., 2006). This suggests that ATF3 may contribute to the modulation of the immune response. In agreement with this hypothesis, using chromatin immunoprecipitation (ChIP), ATF3 can be identified as being bound to the promoter of IL-6 within 4 h of stimulation of monocytes with LPS. Injection of LPS into ATF3 ^{-/-} mice, leads to sustained inflammation and increased mortality, which correlates with significantly increased levels of IL-6 in the serum of ATF3-deficient mice compared with wild-type animals. This suggests that ATF3 is involved in the regulation of the LPS-Toll-like receptor-stimulated inflammatory response in a negative feedback loop (Gilchrist et al., 2006).

Parallel studies from our laboratory indicate that IL-6 is a gene whose expression is significantly induced upon stimulation with ET-1 or H₂O₂ in cardiomyocytes at different time points (Table 4.1) (Cullingford et al., manuscript submitted). Because IL-6 and other members of the family may play an important role in cardiac pathologies, I further characterized the expression of IL-6 mRNA and protein, and evaluated the binding of ATF3 to the rat IL-6 promoter using ChIP assays in cardiomyocytes stimulated with ET-1 or H₂O₂.

Table 4.1 Upregulation of IL-6 in cardiomyocytes by ET-1 or H₂O₂ (microarray analysis).

IL-6	
Affymetrix probe set 1369191_at	
ET-1 (100 nM)	
Time	Fold stimulation
30 min	4.6
1 h	2.2
2 h	1.0
4 h	1.1
24 h	1.5

H₂O₂ (0.2 mM)	
Time	Fold stimulation
2 h	15.2
4 h	7.4
24 h	6.4

Myocytes were exposed to ET-1 or H₂O₂ for the indicated times. RNA was extracted, cRNA was prepared and samples hybridized to Affymetrix Rat Genome 230 2.0 arrays. Array data were analysed by MicroArray Suite 5.0 followed by Genespring 7.31. Results are expressed as fold stimulation relative to control (n = 3).

4.2 Methods

4.2.1 RT-PCR

Cardiomyocytes were exposed to ET-1 (100 nM for 0-6 h), or to H₂O₂ (0.2 mM for 0-6 h). In some experiments, cardiomyocytes were preincubated for 10 min with 20 µM cycloheximide or for 15 min with 10 µM U0126, prior to the addition of ET-1. Total RNA was extracted and cDNA synthesized (Sections 2.3 and 2.5). SQ-PCR and Q-PCR were performed (Section 2.6) to assess expression of *IL-6* and *Gapdh* mRNA. For SQ-PCR 29 cycles of amplification for *IL-6* and 22 cycles for GAPDH were performed.

4.2.2 Immunoblotting

Cardiomyocytes were exposed to 100 nM ET-1 or 0.2 mM H₂O₂ (0-4 h) and then immunoblotting of total protein extracts (10 µg) performed (Sections 2.8.1 and 2.9). The primary antibodies used were to IL-6 (SC-1266, Santa Cruz Biotechnology). Bands were analysed by semi-quantitative scanning densitometry.

4.2.3 Slot-blotting

Cardiomyocytes were exposed to 100 nM ET-1 or 0.2 mM H₂O₂ (0-4 h), conditioned media was collected and slot-blotting performed (section 2.10). Immuno-detection of IL-6 was then performed as described in section 2.9. The primary antibody used was to IL-6 (SC-1266,

Santa Cruz Biotechnology Inc.). Bands were analysed by semi-quantitative scanning densitometry.

4.2.4 Promoter analysis

The analysis of the promoter sequence of IL-6 was performed using the tool TRANSFAC 4.0 available at the website address:

<http://www.gene-regulation.com/pub/programs/alibaba2/index.html?>

4.2.5 Chromatin immunoprecipitation (ChIP) assay

Cardiac myocytes were unstimulated or exposed to 100 nM ET-1 (1 h) or 0.2 mM H₂O₂ (2 h) and ChIP assays were performed (Section 2.11). The ATF3 antibody (10 µg) used in the immunoprecipitation was from Santa Cruz Biotechnology Inc (SC-188X). The primers used for PCR were specific to the *IL-6* promoter at the boundaries of the ATF/CRE consensus (Sense 5'- TGC TCA AGT GCT GAG TCA CT -3'; Antisense 5'- AGA CTC ATG GGA AAA TCC CA -3') (see also section 4.3.4), or to an arbitrary downstream sequence lying 2000 bp 3' to the *IL-6* promoter (Sense 5'- CAC CTC TCC ACC CTG ACA TT -3'; Antisense 5'- CCA ACT AGA CAG CCC AGA GC -3'). PCR products were analysed by scanning densitometry.

4.3 Results

4.3.1 IL-6 mRNA is upregulated in response to ET-1

Using microarray analysis parallel studies from our laboratory indicated that IL-6 is one of the genes upregulated by ET-1 or H₂O₂ in cardiomyocytes (Table 4.1) (Cullingford et al., manuscript submitted). Recent studies have identified IL-6 as a downstream target of ATF3 in other systems (Gilchrist et al., 2006). To confirm the microarray data and delineate the time course of induction of IL-6 mRNA in more detail, I evaluated the expression of IL-6 in cardiomyocytes in response to ET-1 using SQ-PCR. As shown in Figure 4.1A, IL-6 transcripts are present at low abundance under basal conditions, but after stimulation of cardiomyocytes with ET-1 there is a potent induction from as early as 15 min. This is followed by a peak of expression at 30 min, and then a significant decline in the mRNA abundance within 1 h post-stimulation, with return to near basal levels by 2 h (Figure 4.1A). These results were also confirmed by Q-PCR. A similar time course of expression of IL-6 with peak of mRNA at 30 min was observed (Figure 4.1B).

4.3.2 IL-6 is an IEG upregulated by ET-1 through the ERK1/2 cascade

Given the transient nature of the expression of IL-6 following stimulation with ET-1 and the importance of the ERK1/2 cascade in the signalling events activated by GqPCR agonists such as ET-1 during cardiomyocyte hypertrophy (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994; Bogoyevitch et al., 1995a; Gillespie-Brown et al., 1995; Bogoyevitch et al., 1996; Glennon et al., 1996; Clerk et al., 1998a; Yue et al., 2000), I further evaluated whether IL-6 was an IEG and whether or not upregulation of IL-6 mRNA was mediated via the ERK1/2

pathway. Incubation of cardiomyocytes with the protein synthesis inhibitor cycloheximide and ET-1 did not cause a decrease in the expression of IL-6 mRNA abundance at 30 min. On the contrary, this treatment caused a superinduction of IL-6 expression compared with ET-1 alone (Figure 4.2). Even in the absence of ET-1, cycloheximide caused significant induction of IL-6 (Figure 4.2). This confirmed that IL-6 expression does not require *de novo* protein synthesis and therefore exhibits the characteristic pattern of expression of an IEG: acute and transient induction in the absence of *de novo* protein synthesis (Woodget et al., 1989; Platenik et al., 2000). Preincubation of cardiomyocytes with the MEK1/2 inhibitor U0126 significantly attenuated the expression of IL-6 mRNA induced by ET-1 (Figure 4.2), indicating that the ERK1/2 pathway is involved in the upregulation of IL-6 mRNA by ET-1 in cardiomyocytes.

4.3.3 IL-6 protein expression is upregulated in response to ET-1

To confirm that IL-6 mRNA was translated into protein, I evaluated the expression of IL-6 protein by immunoblotting of total protein extracts from cardiomyocytes and also using slot-blotting of the conditioned media from cardiomyocytes in culture since IL-6 should be secreted. IL-6 protein was detected by immunoblotting of cardiomyocyte extracts as one band of 25 kDa. Consistent with the expression of IL-6 mRNA, the protein abundance of IL-6 was low under basal conditions and was induced by ET-1 stimulation (Figure 4.3). Maximal expression of IL-6 protein was at 1 to 2 h and levels then declined after 4 h (Figure 4.3A).

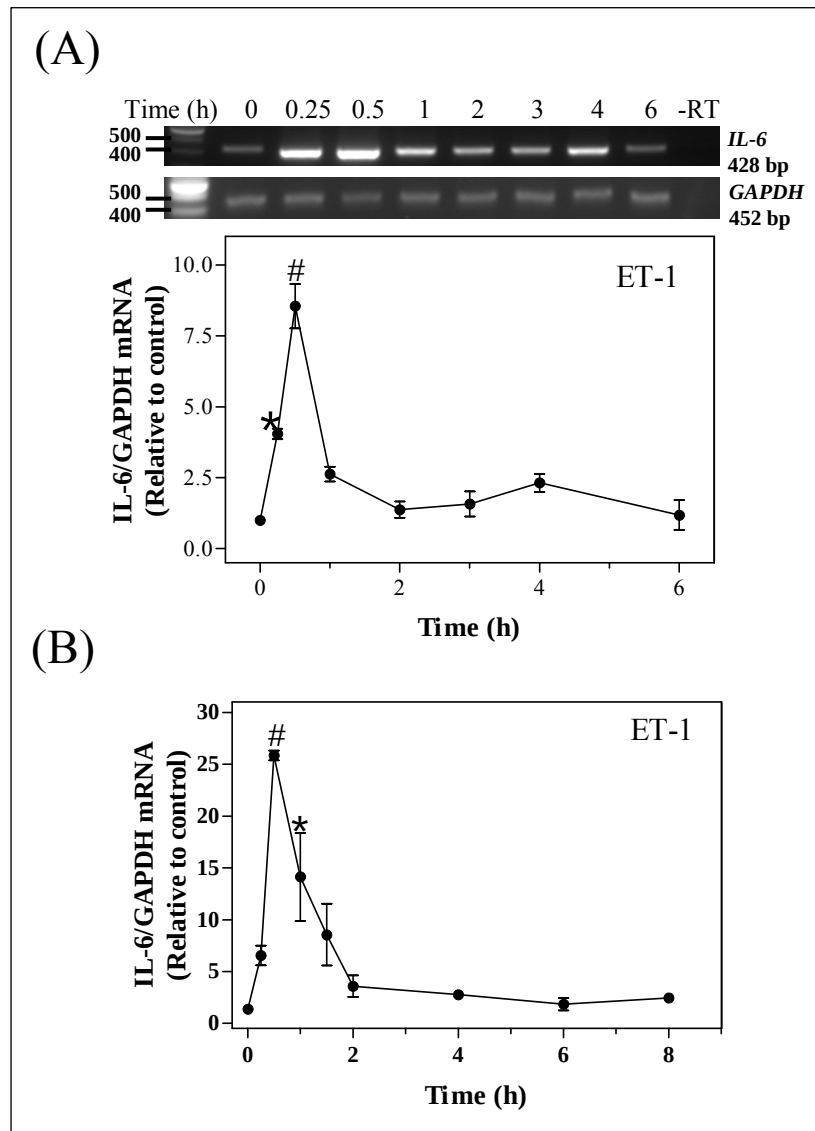


Figure 4.1 Upregulation of IL-6 mRNA by ET-1 in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to 100 nM ET-1 for the indicated period of time. In (A), the expression of IL-6 mRNA was analysed by SQ-PCR and representative images from a single experiment is shown in the upper panel. Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH for three independent cardiomyocyte preparations. In (B), the expression of IL-6 mRNA was analysed by Q-PCR with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for four independent cardiomyocyte preparations. (* $p < 0.01$; # $p < 0.001$ relative to control, one way ANOVA with Tukey post test).

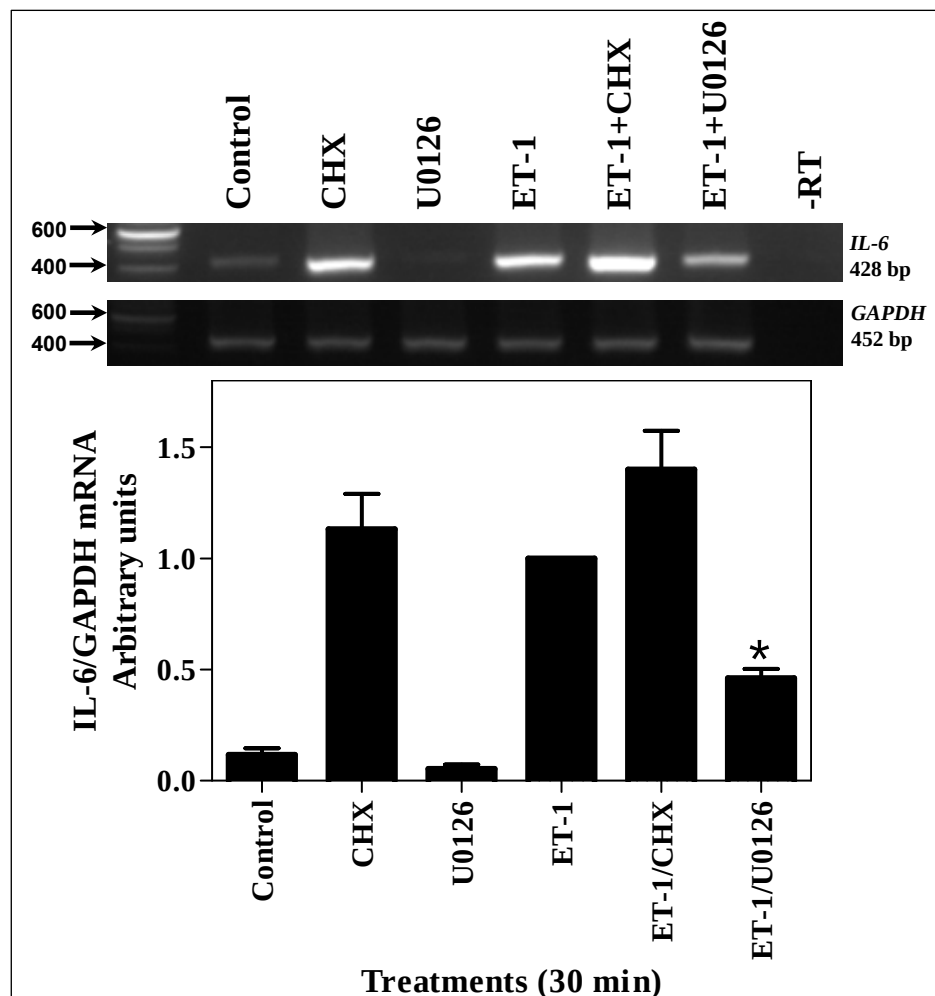


Figure 4.2 IL-6 is an IEG upregulated by ET-1 through the ERK1/2 signalling pathway in cardiomyocytes. Myocytes were unstimulated (control) or exposed to ET-1 (100 nM) for 30 min, in the absence or presence of cycloheximide (CHX) (20 μ M), or U0126 (10 μ M). The expression of IL-6 mRNA was analysed by SQ-PCR and representative images from a single experiment are shown in the upper panel. Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for five independent cardiomyocyte preparations (* $p < 0.01$ relative to ET-1, one way ANOVA with Tukey post test).

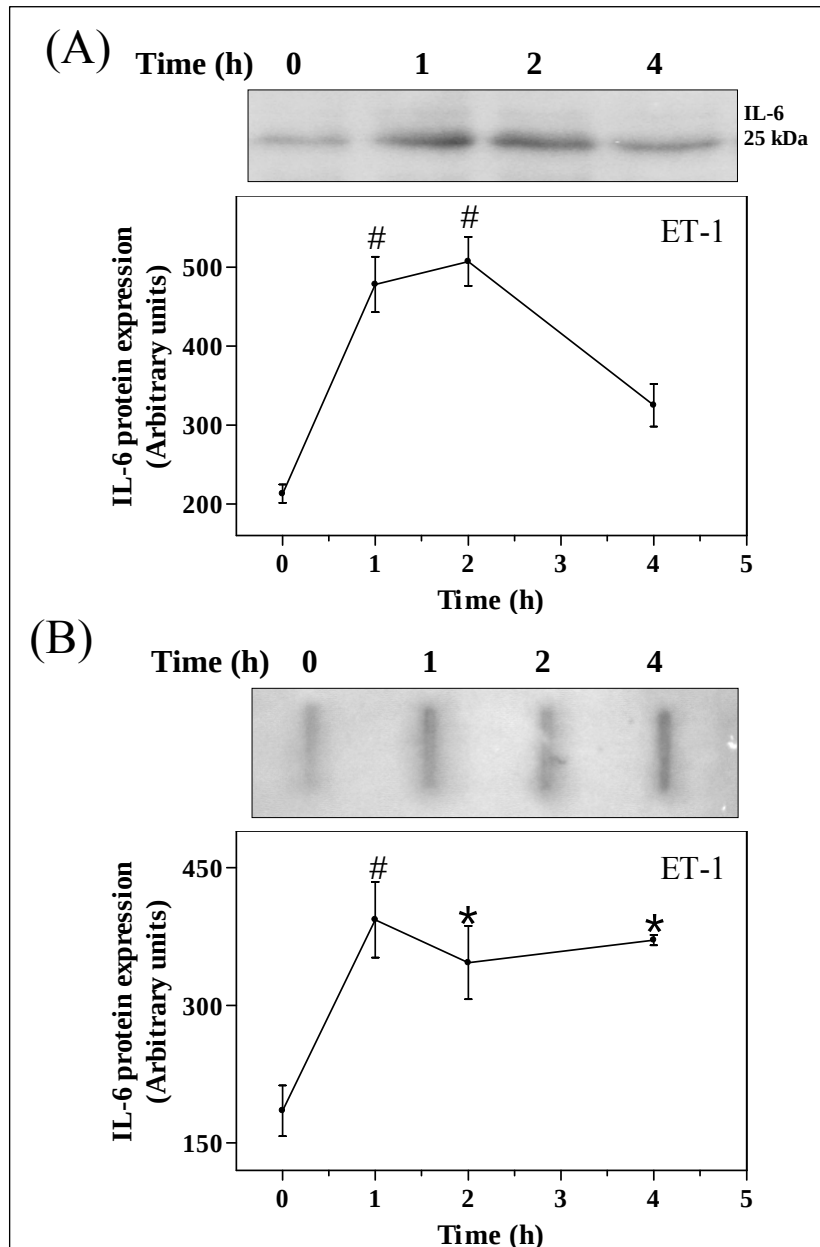


Figure 4.3 IL-6 protein is upregulated by ET-1 in cardiomyocytes. Cardiomyocytes were unstimulated (controls) or exposed to 100 nM ET-1 for the indicated periods of time. In (A), total protein extracts were immunoblotted with antibodies to IL-6 and a representative image is shown in the upper panel. In (B), conditioned media was collected, slot-blotted and then probed with antibodies against IL-6 and a representative image is shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panels as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (* $p < 0.05$; # $p < 0.01$ relative to controls, one way ANOVA with Tukey post test).

In the slot-blotting experiments, there was induction (secretion) of IL-6 protein from 1 h that remained elevated up to 4 h (Figure 4.3B). This suggests that IL-6 was being synthesized within 1 h, as seen on immunoblots of total extracts (Figure 4.3A), and then steadily secreted by the cardiomyocytes to the culture media. To test this hypothesis, I performed further slot-blotting experiments in which, following stimulation of cardiomyocytes, the media was collected and replaced every hour up to 3 h with concomitant collection of the media from unstimulated cardiomyocytes for controls (Figure 4.4). After 1 h (0-1 h) of stimulation of cardiomyocytes with ET-1, the conditioned media contained elevated levels of IL-6 protein compared with controls. However, during the second and third hour there was no significant further increase in the levels of IL-6 protein of stimulated cardiomyocytes as compared with controls, although it was evident that the change in the media has an effect on its own influencing to increase the levels of IL-6 (Figure 4.4). These results suggest that, following ET-1 stimulation of cardiomyocytes in culture, most of the IL-6 protein is synthesized within the first 2 h, as evidenced by immunoblotting (Figure 4.3A), and is steadily released to the media where it remains stable and is probably not degraded to any significant extent (Figure 4.3B and 4.4).

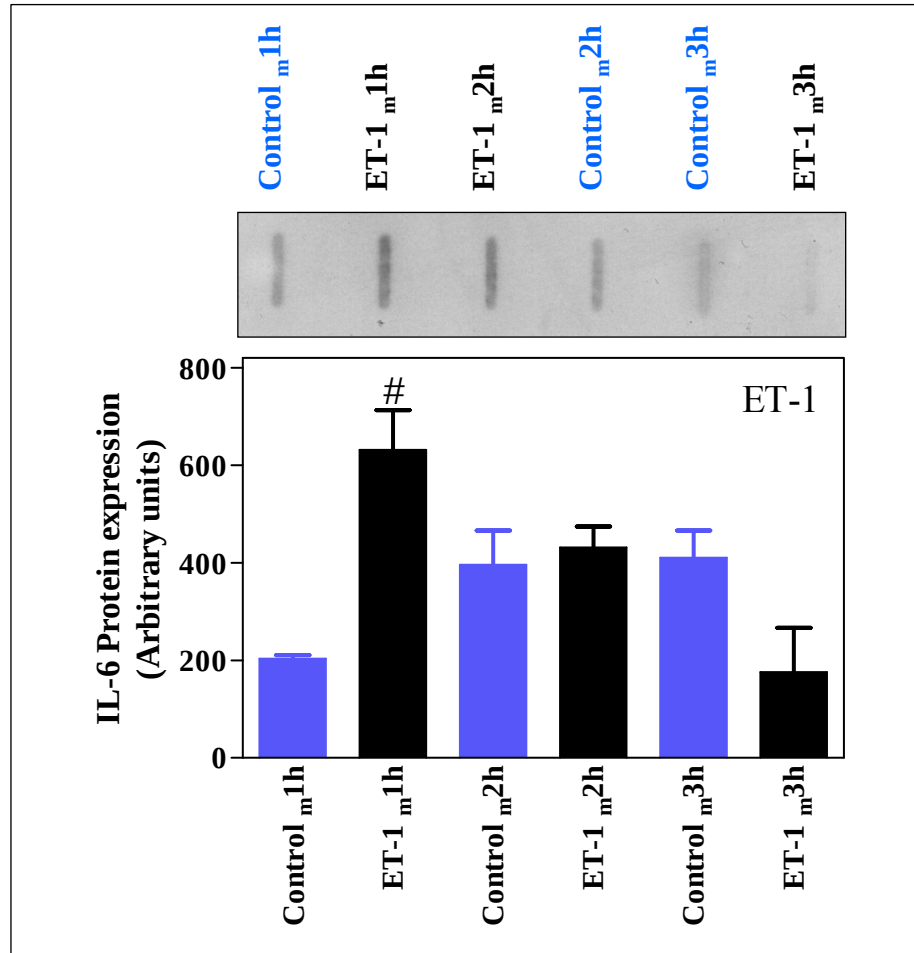


Figure 4.4 IL-6 is secreted by cardiomyocytes shortly after stimulation with ET-1. Cardiomyocytes were unstimulated (Control) or exposed to 100 nM ET-1, and the conditioned medium collected and replaced every 1 h for up to 3 h following the initial stimulation with ET-1. Conditioned media was slot-blotted and probed with IL-6 antibodies. A representative image is shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panels as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (# $p < 0.01$ relative to control_m1h, one way ANOVA with Tukey post test).

4.3.4 IL-6 mRNA and protein are also upregulated in cardiomyocytes by H₂O₂

I then, similarly, evaluated the time course of induction of IL-6 mRNA in cardiomyocytes exposed to H₂O₂ using SQ-PCR. Under basal conditions, IL-6 mRNA abundance was low and remained low up to 1 h following stimulation with H₂O₂. There was then a transient, but potent, induction of IL-6 mRNA, with levels returning to near-basal levels by 2 h (Figure 4.5). To confirm that translation of IL-6 mRNA also occurs in response to H₂O₂, I then evaluated the expression of IL-6 protein by immunoblotting. As shown in Figure 4.6A, IL-6 was identified as one band of ~25 kDa. The protein abundance of IL-6 was low under basal conditions and was induced following stimulation with H₂O₂ with maximal expression at 1 h, although it remained elevated for up to 4 h. To evaluate the secretion of IL-6 protein by cardiomyocytes following stimulation with H₂O₂, slot-blotting was performed using the collected media. As shown in Figure 4.6B, there is increased secretion of IL-6 protein by cardiomyocytes at 1 h and this remained elevated for up to 4 h.

The above studies suggest that IL-6 protein is synthesized principally at 1 h, as shown by immunoblotting, and is secreted by the cardiomyocyte from this early time point, as suggested by slot-blotting. However, it is not clear from these experiments whether IL-6 continues to be secreted after 1 h with a constant rate of degradation or instead it is secreted at earlier time points but not degraded. To clarify this further, the conditioned media was collected every hour for up to 3 h following H₂O₂ stimulation, and the media of unstimulated cardiomyocytes was also collected simultaneously every hour for the same period of time to serve as controls for this experiment. From the data presented in Figure 4.7, it is evident that the change in the media has an effect on its own in the control as well as treated cells.

However, it is also clear that following stimulation of cardiomyocytes with H_2O_2 , IL-6 is mainly secreted by cardiomyocytes at 1 h (figure 4.7). Thus, the observed persistent elevated levels of IL-6 in the media following several hours of stimulation with H_2O_2 as shown in Figure 4.6B, is probably more a reflection of this initial secretion without significant degradation, rather than a continuous secretion with a concomitant degradation.

4.3.5 ATF3 binds to the promoter of IL-6 in response to ET-1 or oxidative stress

My experiments indicate that ATF3 and IL-6 are both IEGs upregulated by ET-1 or H_2O_2 , and upregulation of both appears to require activation of the ERK1/2 cascade (Figures 3.5 and 4.2). Previous studies have suggested that ATF3 is a negative regulator of IL-6 in the mouse immune system thus controlling the inflammatory response (Gilchrist et al., 2006). I therefore examined the rat IL-6 gene promoter for the presence of an ATF/CRE binding site that could be a target binding site for ATF3 in this gene. Using the online TRANSFACT 4.0 tool (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html>) I established the presence of a predicted ATF/CRE consensus binding site within the promoter of IL-6 located 165 bp upstream from the transcriptional start site of the IL-6 gene (Figure 4.8). This suggested that ATF3 could be involved in the regulation of IL-6 gene expression.

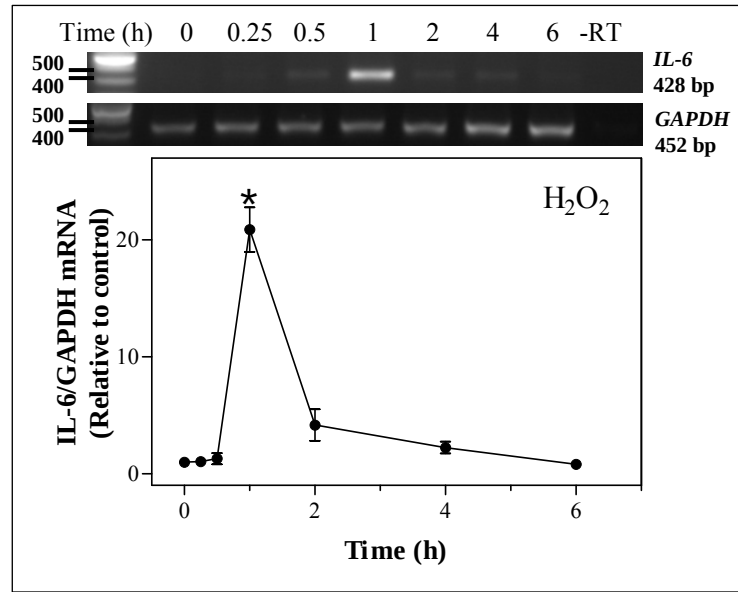


Figure 4.5 IL-6 mRNA is upregulated in cardiomyocytes in response to H₂O₂. Myocytes were unstimulated (controls, zero time) or exposed to 0.2 mM H₂O₂ for the indicated periods of time. The expression of IL-6 mRNA was analysed by SQ-PCR and a representative image from a single experiment is shown in the upper panel. Densitometric analysis of the data is provided in the lower panel and expressed relative to controls after normalization with the housekeeping gene GAPDH for three independent cardiomyocyte preparations. (* p< 0.001 relative to control, one way ANOVA with Tukey post test).

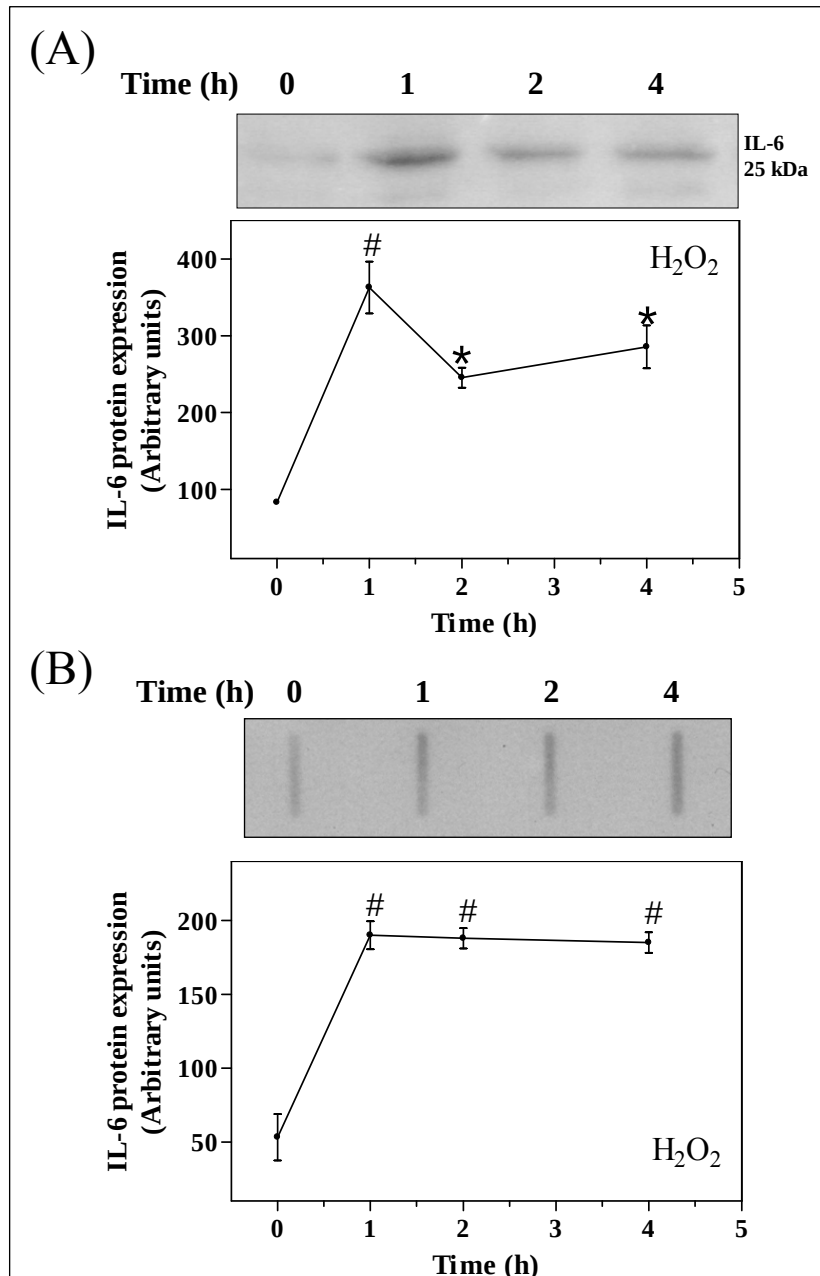


Figure 4.6 IL-6 protein is upregulated in cardiomyocytes by H₂O₂. Cardiomyocytes were unstimulated (controls, zero time), or exposed to 0.2 mM H₂O₂ for the indicated periods of time. In (A), total protein extracts were immunoblotted with antibodies to IL-6 and a representative image is shown in the upper panel. In (B), conditioned media was collected, slot-blotted and then probed with IL-6 antibodies and a representative image is shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panels as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (* $p < 0.01$; # $p < 0.001$ relative to control, one way ANOVA with Tukey post test).

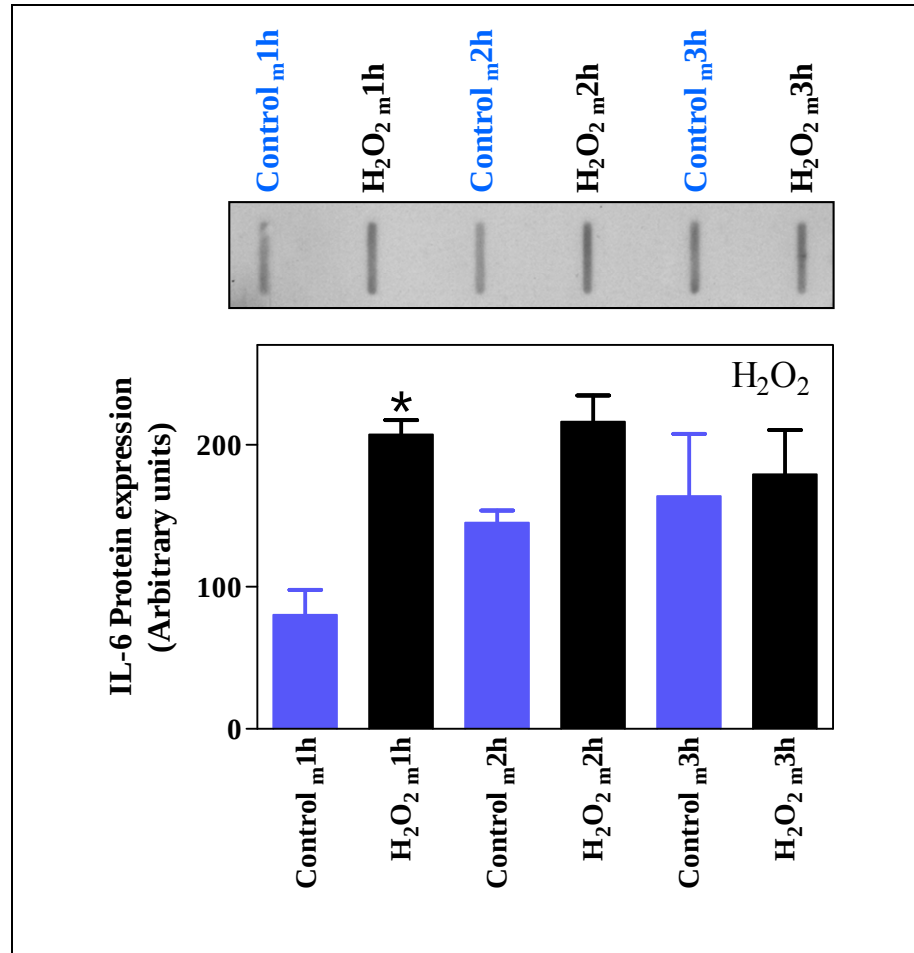


Figure 4.7 IL-6 is secreted by cardiomyocytes shortly after stimulation with H₂O₂. Myocytes were unstimulated (Controls) or exposed to 0.2 mM H₂O₂ and the supernatants collected and then replaced every 1 h for up to 3 h following initial stimulation with H₂O₂. Collected supernatants were slot-blotted and then probed with IL-6 antibodies and a representative image is shown in the upper panel. The blots were analysed by scanning densitometry and the combined data are shown in the lower panel as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (* $p < 0.05$ relative to Control_m1h, one way ANOVA with Tukey post test).

To confirm that ATF3 binds to the promoter of IL-6 at this ATF/CRE binding site, ChIP assays were performed. Using antibodies to ATF3, ATF3 (crosslinked to DNA) was immunoprecipitated from cardiomyocytes which were unstimulated [Control (+)], or stimulated with 100 nM ET-1 (1 h) or 0.2 mM H₂O₂ (2 h), [ET-1 (+) or H₂O₂ (+) respectively]. As an additional negative control, DNA was also extracted from cardiomyocytes stimulated with 100 nM ET-1 (1 h) or 0.2 mM H₂O₂ (2 h) and the immunoprecipitation process performed in the absence of antibodies [ET-1 (-) or H₂O₂ (-) respectively]. The IL-6 promoter DNA was detected by PCR, using primers located 118 bp upstream and 105 bp downstream to the ATF/CRE binding site, which amplifies a product of 223 bp (Figure 4.8). The ATF3 antibodies immunoprecipitated IL-6 promoter DNA from cardiomyocytes stimulated by ET-1 but not from unstimulated cardiomyocytes (Figure 4.9). As expected, IL-6 promoter DNA was not significantly amplified from samples that were not subject to immunoprecipitation with ATF3 antibodies. These results suggest that ATF3 binds to the endogenous promoter of IL-6 in response to ET-1. To confirm the specificity of the ChIP assay, immunoprecipitated DNA samples were subjected to PCR using primers to an arbitrary sequence 2000 bp 3' to the *IL-6* gene promoter and this was compared with those using the input DNA. As shown in Figure 4.10, there were only background levels of amplification of this sequence when immunoprecipitated DNA was evaluated, but significantly more when the input DNA was amplified, thus confirming the specificity of the ChIP assay.

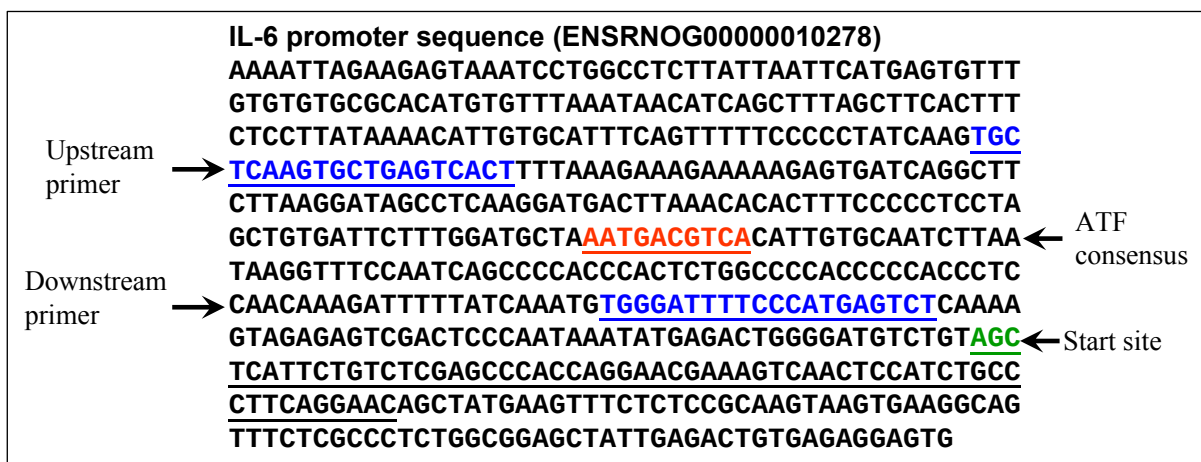


Figure 4.8 The IL-6 promoter possesses an ATF consensus binding site. Promoter analysis was performed using the online tool TRANFACT 4.0. The ATF/CREB consensus is indicated in red. The transcriptional start site and first codon within the first exon is shown in green. The location of primers for the ChIP assay (see also Figures 4-9 and 4-11), which are localized upstream and downstream of the ATF consensus are shown in blue. Rat IL-6 promoter sequence was obtained from Ensembl (ENSRNOG00000010278).

The ATF3 antibodies also immunoprecipitated IL-6 promoter DNA from cardiomyocytes stimulated by H₂O₂, but not from unstimulated cardiomyocytes (Figure 4.11). As expected, IL-6 promoter DNA was not significantly amplified from samples that were stimulated with H₂O₂ but not immunoprecipitated. These results suggest that ATF3 binds to the endogenous promoter of IL-6 under conditions of H₂O₂ stimulation in cardiomyocytes. To confirm the specificity of the ChIP assay, immunoprecipitated DNA samples were subjected to PCR using primers to an arbitrary sequence lying 2000 bp 3' to the *IL-6* gene promoter and compared with that using the input DNA. Again, only background levels of amplification of this sequence were present when immunoprecipitated DNA was subjected to PCR but significantly more when the input DNA was evaluated, which confirms the specificity of the ChIP assay (Figure 4.12).

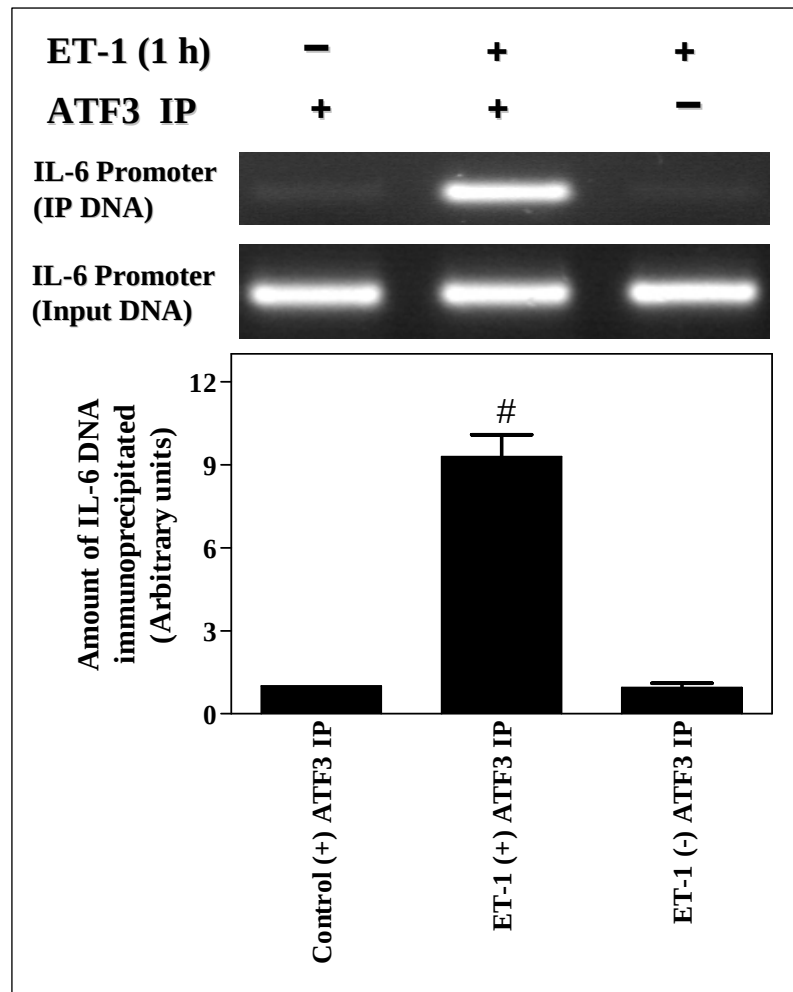


Figure 4.9 ATF3 binds to the promoter of IL-6 in cardiomyocytes stimulated with ET-1. Cardiomyocytes were unstimulated and immunoprecipitated [Control (+)], or stimulated with 100 nM ET-1 for 1 h and immunoprecipitated [ET-1 (+)], or stimulated with 100 nM ET-1 for 1 h but no immunoprecipitated [ET-1 (-)]. DNA crosslinked to ATF3 was immunoprecipitated by ChIP assay using antibodies to ATF3 (IP DNA). *IL-6* promoter DNA was detected by PCR (representative image, top panel). DNA from samples prior to immunoprecipitation (Input DNA) was used as a positive control (representative image, middle panel). Both PCRs were performed for 32 cycles. The densitometric analysis is shown with values normalised to the input DNA in the lower panel. Results are means $\pm$ SEM for three independent experiments [[#] $p < 0.001$ relative to control (+), one way ANOVA with Tukey post test].

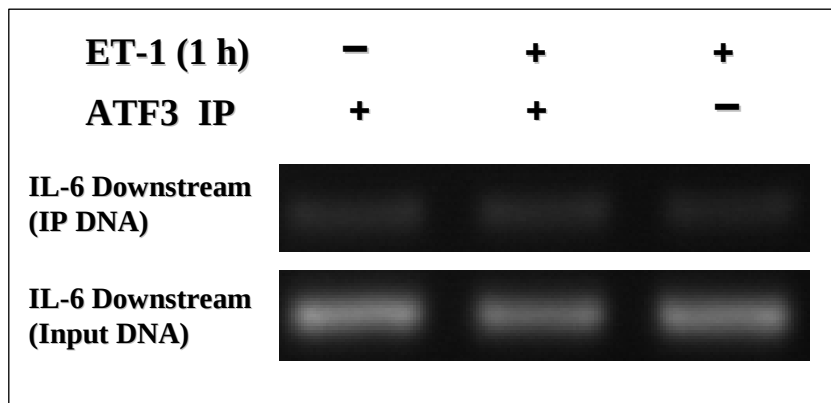


Figure 4.10 Determination of ChIP assay specificity (ET-1). Cardiomyocytes were unstimulated and immunoprecipitated [Control (+)], or stimulated with 100 nM ET-1 for 1 h and immunoprecipitated [ET-1 (+)], or stimulated with 100 nM ET-1 for 1 h but no immunoprecipitated [ET-1 (-)]. DNA crosslinked to ATF3 was immunoprecipitated by ChIP assay using antibodies to ATF3 (IP DNA). PCR was performed to detect an arbitrary DNA sequence 2000 bp 3' to the *IL-6* promoter. DNA from samples prior to immunoprecipitation (Input DNA) was used as a positive control. Both PCRs were performed for 32 cycles. Representative images of three independent experiments are shown.

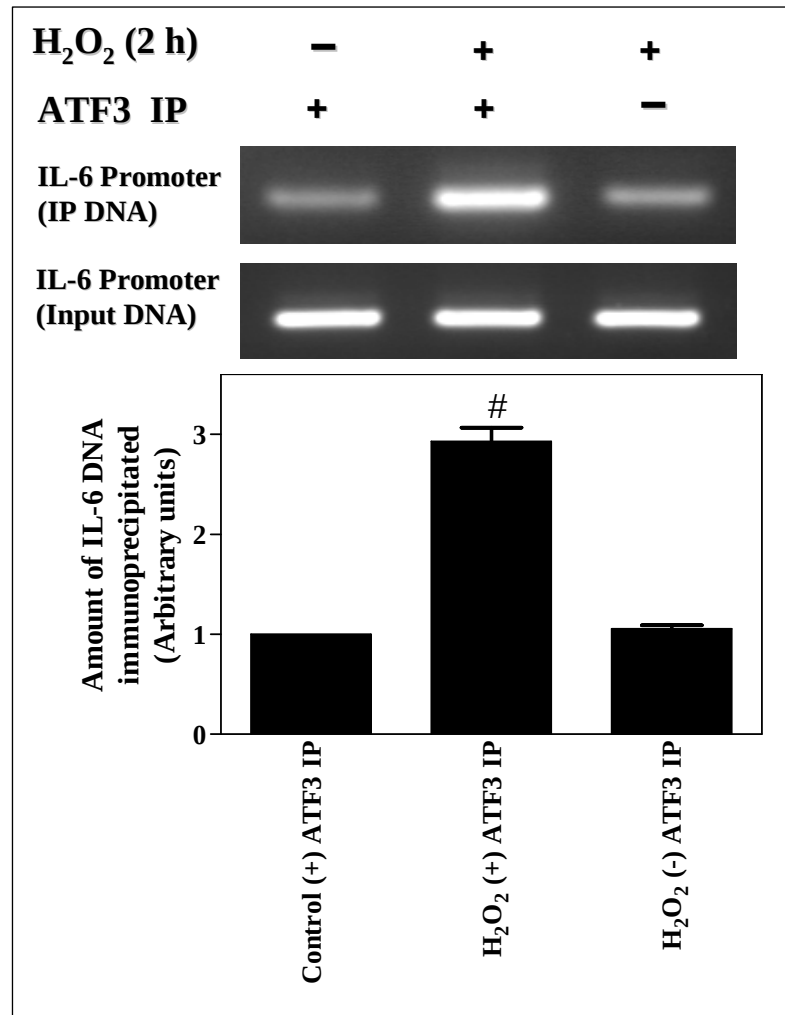


Figure 4.11 ATF3 binds to the promoter of IL-6 in cardiomyocytes stimulated with H₂O₂. Cardiomyocytes were unstimulated and immunoprecipitated [Control (+)], or stimulated with 0.2 mM H₂O₂ for 2 h and immunoprecipitated [H₂O₂ (+)], or stimulated with 0.2 mM H₂O₂ for 2 h but no immunoprecipitated [H₂O₂ (-)]. DNA crosslinked to ATF3 was immunoprecipitated by ChIP assay using antibody to ATF3 (IP DNA). *IL-6* promoter DNA was detected by PCR (representative image, top panel). DNA from samples prior to immunoprecipitation (Input DNA) was used as a positive control (representative image, middle panel). Both PCRs were performed for 32 cycles. The densitometric analysis is shown with values normalised to the input DNA in the lower panel. Results are means $\pm$ SEM for three independent experiments (# $p < 0.001$ relative to control (+), one way ANOVA with Tukey post test).

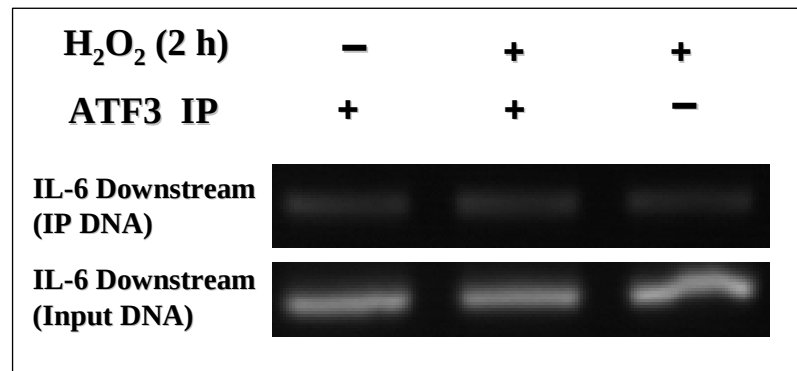


Figure 4.12 Determination of ChIP assay specificity (H₂O₂). Cardiomyocytes were unstimulated and immunoprecipitated [Control (+)], or stimulated with 0.2 mM H₂O₂ for 2 h and immunoprecipitated [H₂O₂ (+)], or stimulated with 0.2 mM H₂O₂ for 2 h but no immunoprecipitated [H₂O₂ (-)]. DNA crosslinked to ATF3 was immunoprecipitated by ChIP assay using antibody to ATF3 (IP DNA). PCR was performed to detect an arbitrary DNA sequence 2000 bp 3' to the *IL-6* promoter. DNA from samples prior to immunoprecipitation (Input DNA) was used as a positive control. Both PCRs were performed for 32 cycles. Representative images of three independent experiments are shown.

4.4 Discussion

4.4.1 *Expression of IL-6 mRNA and protein in cardiomyocytes*

The above studies demonstrate that IL-6 mRNA and protein abundance increased in response to stimulation of cardiomyocytes by ET-1 or H₂O₂. Thus, the expression of IL-6 mRNA was potently induced by both ET-1 and H₂O₂ as assessed by SQ-PCR and Q-PCR (Figures 4.1 and 4.5) in agreement with previous microarray studies from our laboratory (Cullingford et al., manuscript submitted) (Table 4.1). In addition to increasing IL-6 mRNA abundance, ET-1 or H₂O₂ also caused an increase in the expression of the IL-6 protein, suggesting that, after synthesis of mRNA, the rate of translation of IL-6 mRNA into protein is also subsequently increased with concomitant secretion of IL-6 by the cardiomyocytes within the first hour (Figures 4.3, 4.4, 4.6, and 4.7).

The induction of IL-6 by ET-1 was statistically significant and in agreement with previous studies. Thus, using cultured cardiomyocytes, Saito et al., (1999) have previously reported that ET-1 induces the expression of IL-6, as well as LIF, but not CT-1 mRNA levels, with a peak of expression observed at 1 h for both IL-6 and LIF. Similarly, a recent report evaluated the postnatal effects of cardiac overexpression of human ET-1 using a conditional cardiac-specific tetracycline-regulated (tet-off) gene expression system, in which expression of ET-1 is inactivated by the administration of the tetracycline doxycycline (DOX) (Yang et al., 2004). Following DOX withdrawal in transgenic animals, dilated cardiomyopathy (characterized by an interstitial inflammatory cell infiltrate on histological examination, associated with signs of overt heart failure) was observed between 5 to 11 weeks (Yang et al., 2004). Mortality rates were around 50% within 8 weeks following DOX withdrawal, whereas

transgenic animals maintained on DOX did not exhibit a significant increase in mortality compared with control non-transgenic mice. Evaluation of cytokines expressed in the myocardium at 2 week intervals following DOX withdrawal revealed that mRNA levels of IL-6, TNF α , IL-1 and interferon- γ (INF- γ) were all expressed at low levels under basal conditions but significantly increased at 6 to 8 weeks. Histological analysis conducted in parallel revealed that a progressive inflammatory process develops progressively and is widespread at 6 weeks following DOX removal (Yang et al., 2004). This study suggests that cardiac ET-1 overexpression in the heart during the postnatal period causes dilated cardiomyopathy associated with increased expression of inflammatory cytokines including IL-6, thus leading to a pattern of inflammatory cardiomyopathy, heart failure and increased mortality (Yang et al., 2004). Another GqPCR agonist, ANGII not only causes induction of LIF and IL-6 mRNA expression in cardiomyocytes, but also induces CT-1, all with a similar time course of expression as that observed in my studies (i.e. maximal expression of IL-6 at 30 minutes with decline in mRNA abundance after 2 h). A concomitant increase in IL-6 protein secretion at 2 h was also observed (Sano et al., 2000).

ET-1 has also been reported to induce IL-6 expression in other cell systems. Thus, in human vascular smooth muscle cells, ET-1 induces the expression of IL-6 mRNA at 30 minutes with a rapid decline in the levels of expression afterwards (Browatzki et al., 2000). The IL-6 secretion was also evaluated in this same study following 24 h of stimulation with ET-1, and showed a significant increase in the secretion of this cytokine. This study concluded that, in the context of vascular smooth muscle cells, ET-1 appears to be involved in the pathogenesis of atherosclerosis at least in part by inducing the release of proinflammatory cytokines such

as IL-6 (Browatzki et al., 2000). In a rat endothelial cells line (WAE-1), ET-1 causes induction of IL-6 mRNA expression at 6 h in a concentration dependent manner, which correlates with secreted IL-6 protein (Xin et al., 1995). Similarly, in osteoblasts, the immature cells of bone, ET-1 induces the expression of IL-6 mRNA at 1-2 h with concomitant increase in IL-6 protein in supernatant (Perkins et al., 1997). In the settings of osteoblasts, ET-1 appears to increased bone turnover (e.g. resorption) by the upregulation of inflammatory cytokines like IL-6 (Perkins et al., 1997; Kawamura et al., 1999).

The effects of H₂O₂ on the induction of IL-6 mRNA and protein secretion were also statistically significant, and to my knowledge this is the first report of the direct induction of IL-6 by H₂O₂ in cardiomyocytes. A previous report has documented the expression of IL-6 in the heart of dogs following ischaemia or ischemia/reperfusion. In this model, the induction of IL-6 following ischaemia alone for 1-4 h was minimal, whereas 1 h ischaemia coupled with 3 h reperfusion results in a marked increase in IL-6 mRNA levels as assessed by Northern blot analysis (Kukielka et al., 1995). As discussed previously (section 1.6), following ischaemia/reperfusion there is increased production of ROS including H₂O₂ (Ambrosio et al., 1993; Du et al., 1998). Thus, the potent induction by ischaemia/reperfusion of IL-6 is probably secondary to the elevated levels of ROS production in the myocardium which occur during reperfusion, compared with ischaemia alone which induces comparatively less ROS production (Ambrosio et al., 1993; Du et al., 1998; Clerk et al, 2003). In other cell types, there have been reports of increased expression of IL-6 directly by ROS species such as H₂O₂. Thus, H₂O₂ induces IL-6 protein expression/secretion in cardiac fibroblasts following stimulation for over 4-24 h (Colston et al., 2002). More recently, ANGII-induced expression

of IL-6 was reported in cardiac fibroblasts, and this is mediated by ROS production (Sano et al., 2001). Exogenously applied H₂O₂ also induces IL-6 mRNA in the same cell system in a concentration dependent manner, an effect that is mediated by the ERK1/2 and p38-MAPK cascades (Sano et al., 2001).

The role of IL-6 and other members of this family of cytokines in cardiomyocytes exposed to ET-1 is not completely understood. However, as discussed in section 1.3.3, there is ample evidence implicating IL-6, LIF and CT-1 in myocyte elongation and a causal role in eccentric hypertrophy through activation of the ERK5 cascade (Hunter and Chien, 1999; Nicol et al., 2001). Consistent with a role in the induction of myocyte elongation by these cytokines through the ERK5 cascade, adenoviral-mediated overexpression of constitutively active MKK5 causes serial assembly of sarcomeres, whereas a dominant-negative form of MKK5 prevents LIF-induced elongation of cardiomyocytes (Nicol et al., 2001). Similarly, cardiospecific overexpression of constitutively-activated MKK5 in transgenic mice results in eccentric hypertrophy, heart failure and sudden death (Nicol et al., 2001). Additional evidence implicating IL-6 family members such as CT-1 and LIF in the pathophysiology of heart failure is the observation that the myocardial expression of these cytokines is elevated in animal models of heart failure (Aoyama et al., 2000; Jougasaki et al., 2003). The levels of CT-1 mRNA are also elevated in explanted hearts from patients with heart failure, however the protein levels of gp130 are decreased, with a concomitant increase in the mRNA levels of this gene, indicating that gp130 protein turnover is augmented, probably as a consequence of an adaptive desensitization and degradation mechanism with concomitant reduced protein synthesis (Zolk et al., 2002). Consistent with tissue levels, circulating levels of CT-1 are

increased in patients with dilated cardiomyopathy (Tsutamoto et al., 2001). Recently, circulating levels of IL-6 and CT-1 have been proposed as important prognostic markers of death and/or heart failure in patients post-myocardial infarction (Takahashi et al., 2003; Fisman et al., 2006).

In rat hearts subjected to right ventricular (RV) pressure or RV volume overload BNP is upregulated in the RV overloaded ventricles for both stimuli, but comparatively, the levels of IL-6, TNF α , and pre-pro-ET-1 (pp-ET-1) are higher upon volume-overload than pressure-overload (Roncon-Albuquerque Jr, et al., 2006). In addition, local expression levels of BNP, IL-6, TNF α and ppET-1 positively correlate with RV end-diastolic pressures (Roncon-Albuquerque Jr, et al., 2006). Of note, mRNA levels of IL-6 and other cytokines such as TNF α and IL-1 β are significantly increased in the hearts and serum samples of patients that required LVAD therapy (as a bridge to heart transplant due to the rapid deterioration of their disease process), compared with those that are more stable and could await a heart transplant (Birks et al., 2001). This suggests that IL-6 and other cytokines may be involved in the worsening of heart failure at late stages of the disease. Consistent with this idea, in a model of chronic intermittent hypoxia in rats over 4 weeks results in LV concentric cardiac hypertrophy), whereas intermittent hypoxia over 8 weeks leads to eccentric cardiac hypertrophy (Chen et al., 2007). mRNA and protein levels of TNF α , IGF-1, and the activity of p38-MAPK and STAT-1 and STAT3 were elevated in the 4 weeks intermittent hypoxia group compared with normoxic rat controls. However, after 8 weeks of intermittent hypoxia, in addition to the above factors, IL-6 mRNA and protein, and MEK5 and ERK5 activity, were also elevated, indicating that IL-6-gp-130 receptor activation is associated with late

eccentric hypertrophy and dilatation after prolonged hypoxia (Chen et al., 2007). Given the nature of the stimuli, it could be argued that, possibly, alterations in redox systems initially lead to concentric hypertrophy and after sustained hypoxia further alterations in these systems lead to changes in gene expression associated with eccentric hypertrophy and LV dilatation.

Taken together, it seems that increased expression of IL-6 family of cytokines in the heart, and activation of the ERK5 signalling cascade through their cognate receptors are implicated in the development of eccentric hypertrophy and in the process of ventricular remodelling, probably by regulation of cardiomyocyte elongation and subsequent ventricular dilatation. Although the mechanistic details are still unclear, their deregulated expression at later stages of the disease could be secondary to increased oxidative stress and/or neurohumoral activation. Thus, it is likely that the induction of these cytokines is probably part of a maladaptive response to hypertrophic agonists such as ET-1, which in addition to concentric hypertrophy in the initial stages, also has the potential of inducing eccentric hypertrophy and myocardial dysfunction at later stages. However, the final outcome may depend of the particular circumstances influencing the cardiac milieu at the time of expression of these cytokines.

There is also strong evidence indicating that gp130 cytokines, in addition to the ERK5 cascade, also activate the ERK1/2 cascade, JAK/STAT3 signalling and the PKB/Akt pathway, all of which appear to mediate cytoprotective effects in the heart (reviewed in

Hilfiker-Kleiner et al., 2006). Thus, LIF stimulation or adenoviral-mediated overexpression of constitutively-active STAT3 (caSTAT3) in cardiomyocytes significantly reduces the production of ROS species following hypoxia/reoxygenation (Negoro et al., 2001a). This effect appears to be mainly mediated by the induction of Mn²⁺SOD, thus increasing the ROS scavenging capacity of the cardiomyocyte. Similarly, infusion of a complex of IL-6 with the soluble IL-6 receptor inhibits cardiomyocyte apoptosis and limits the infarct size following ischaemia/reperfusion in rats, thus suggesting that IL-6 is cytoprotective under conditions of increased ROS production (Matsushita, et al., 2005). Consistent with a cytoprotective role for the activation of gp130 signalling, mice with cardiospecific-knockout of gp130, although displaying a normal phenotype under basal conditions, exhibit increased apoptosis of cardiomyocytes and myocardial dysfunction early after induction of pressure-overload (Hirota et al., 1999). LIF activation of gp130 signalling through PKB/Akt reduces apoptosis induced by the anticancer drug doxorubicin in cardiomyocytes, suggesting a cytoprotective role for this cascade when activated by IL-6 members under cytotoxic conditions (Negoro et al., 2001b). It has been postulated that the mechanisms of cytoprotection by the gp130 activated pathways may include the ability of several of these cascades to induce the expression or restore the activities of antiapoptotic and cytoprotective proteins such as Bcl-xL, an antiapoptotic Bcl-2 family member (Fujio et al., 1997; Negoro et al., 2001b) or, as described above, the ROS scavenger Mn²⁺SOD in cardiomyocytes (Negoro et al., 2001a). Therefore, stimulation of cardiomyocytes with H₂O₂ and resultant increased expression of IL-6 as has been demonstrated here may represent an attempt by the cell to cope with this stress by the subsequent activation of cytoprotective pathways through the gp130 signalling complex and downstream pathways.

4.4.2 IL-6 is an IEG upregulated in cardiomyocytes in response to ET-1 through the ERK1/2 cascade

I have also shown that IL-6 is an IEG that is upregulated by ET-1 through the ERK1/2 cascade. Thus, IL-6 mRNA expression is rapidly and transiently induced following ET-1 stimulation in a process that does not require *de novo* protein synthesis as suggested by the lack of inhibition of its transcriptional expression by the protein synthesis inhibitor cycloheximide (Figure 4.2). Although this has not been previously shown in cardiomyocytes, there is ample evidence that IL-6 is an IEG in other systems particularly in the settings of the acute defence responses against pathogens in which IL-6 plays a central role in inflammatory and immune processes (Iwamoto and Konicek, 1997; reviewed in Kishimoto, 2006), and in the setting of liver injury with IL-6 facilitating liver regeneration (Laurent et al., 2001; reviewed in Iimuro et al., 2007). That the upregulation of this IEG following ET-1 stimulation is mediated through the ERK1/2 cascade (Figure 4.2) is also in agreement with previous studies. Thus, ET-1 induction of IL-6 synthesis in osteoblasts is mediated by the ERK1/2 cascade (Kawamura et al., 1999). It has also been reported that IL-6 induction by ANGII is mediated by the activation of the ERK1/2 and p38-MAPK cascades in cardiac fibroblasts. Although it is possible that, in cardiomyocytes, activation of other MAPKs such as JNKs or p38-MAPKs is involved in the response leading to IL-6 induction following ET-1 stimulation, I concentrated my studies on the ERK1/2 cascade because of the ample evidence of the prominent involvement of this pathway in the cardiac hypertrophic response both in vitro and in vivo (section 1.3.1.6) (Bueno et al., 2000; Yue et al., 2000; Sugden., 2001; Sugden., 2003).

4.4.3 ATF3 binds to the promoter of IL-6 in response to ET-1 or H₂O₂ stimulation

ChIP assays were performed to assess the binding of ATF3 to the endogenous ATF-CRE consensus present in the promoter of the IL-6 gene under conditions of ET-1 and H₂O₂ stimulation and demonstrated that, following exposure of cardiomyocytes to ET-1 or H₂O₂, ATF3 becomes bound to the promoter of this gene (Figures 4.9 and 4.11). Thus, my results suggest that IL-6 is a downstream target gene of ATF3 following hypertrophic stimulation of cardiomyocytes by ET-1, or upon oxidative stress following H₂O₂ stimulation. This is consistent with a previous study indicating that following stimulation of monocytes with LPS, ATF3 binds to the promoter of IL-6 and is a negative regulator of this gene (Gilchrist et al., 2006). However, the studies in this chapter do not firmly establish whether ATF3 is a positive or negative regulator of IL-6 expression. Experiments involving overexpression or knockdown of the ATF3 protein levels will help to clarify the role of ATF3 in the regulation of IL-6. The observation that, following induction of IL-6 mRNA by either ET-1 or H₂O₂, the time when IL-6 mRNA returns to near basal levels correlates with the time of maximal expression of ATF3 protein for either agonist (Figures 3.7, 3.8, 4.1 and 4.5) (Table 4.2), suggests that ATF3 may be involved in a negative regulatory feedback loop to downregulate IL-6 in cardiomyocytes following ET-1 or H₂O₂ stimulation (Figure 4.13). This is further supported by accumulating evidence suggesting that ATF3 is mainly a negative regulator of transcription in other cell systems (section 3.1.1.2) (Chen et al., 1994; Wolfgang et al., 1997; Wolfgang et al., 2000; Gao et al., 2004; Gilchrist et al., 2006). Further experiments aimed to clarify these findings will be presented in the forthcoming chapter.

Table 4.2 Comparison of the induced levels of expression of ATF3 protein and those of IL-6 mRNA following ET-1 or H₂O₂ stimulation of cardiomyocytes.

Endothelin-1 stimulation			
<i>ATF3 protein</i>		<i>IL-6 mRNA</i>	
control	13.4	control	1
30 min	59.2	30 min	8.5
1 h	556.3	1 h	2.6
2 h	488.6	2 h	1.3
4 h	168.8	4 h	1.9
6 h	33.0	6 h	1.1
H₂O₂ stimulation			
<i>ATF3 protein</i>		<i>IL-6 mRNA</i>	
control	31.6	control	1
30 min	35.7	30 min	1.2
1 h	255.6	1 h	20.8
2 h	683.8	2 h	4.1
4 h	257.2	4 h	2.2
6 h	104.3	6 h	0.8

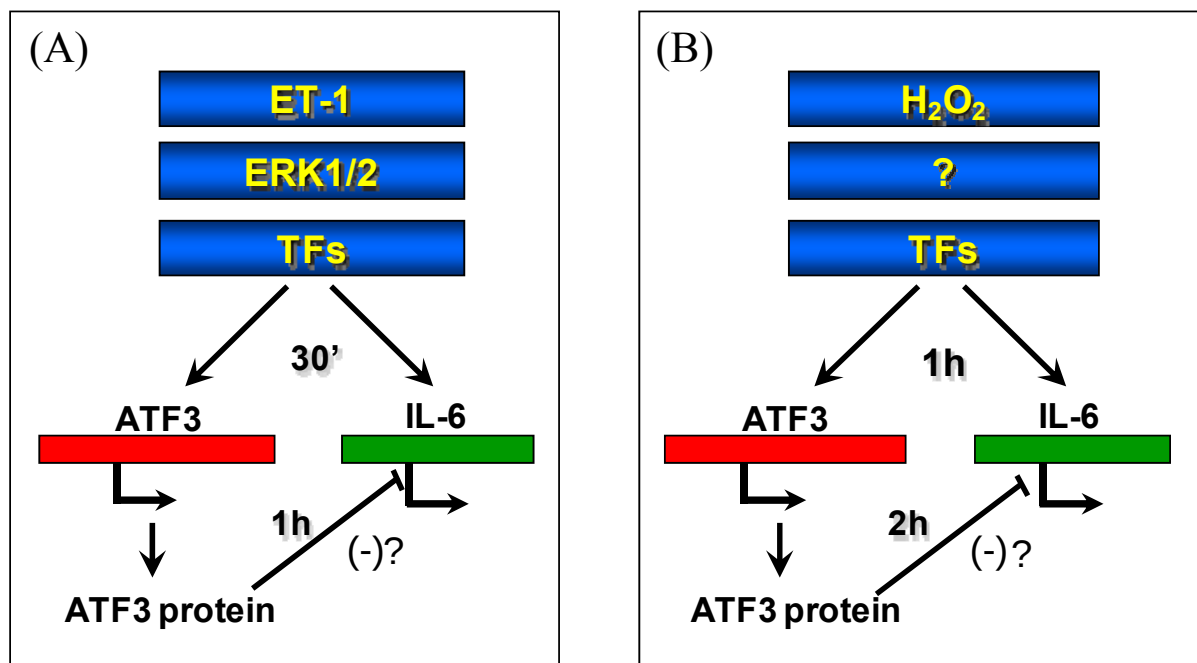


Figure 4.13 ATF3 forms a possible negative feedback loop for IL-6 regulation in cardiomyocytes in response to ET-1 or oxidative stress. In (A), following upregulation of ATF3 and IL-6 mRNA by ET-1 (30 min) through the ERK1/2 cascade, ATF3 mRNA is translated into protein and the active ATF3 then binds to the promoter of IL-6 within 1 h potentially repressing the IL-6 gene. Similarly in (B), following stimulation of cardiomyocytes with H₂O₂ there is upregulation of ATF3 and IL-6 mRNA at 1 h. Then the synthesized ATF3 protein binds to the promoter of IL-6 and potentially represses IL-6 gene expression.

Chapter 5

Identification of novel downstream targets of ATF3 in cardiomyocytes

5.1 Introduction

Cardiomyocytes are terminally-differentiated cells which withdraw from the cell cycle soon after birth. Maturational growth (e.g. eutrophy) is therefore achieved by the enlargement of individual cardiomyocytes in the postnatal period. The establishment of a particular phenotype in the cardiomyocyte (e.g. hypertrophy upon pressure overload) is regulated by complex regulatory mechanisms. As discussed in section 1.2, it is established that many hypertrophic agonists, including ET-1, are capable of regulating the hypertrophic process acting through cognate receptors located at the cell membrane of the cardiomyocytes (Sugden and Clerk, 1998; Molkentin et al., 2001, Sugden, 2003). The flow of information in the signalling cascades activated by hypertrophic agonists leads to rapid downstream activation of protein kinases such as the ERK1/2 (within 5 min), which play an important role in the hypertrophic process as has been demonstrated in vitro and in vivo (Sections 1.3.1.5 and 1.3.1.6). ERK1/2 then phosphorylates target proteins including several transcription factors that subsequently bind to the promoters of several genes. Thus, cardiomyocyte hypertrophy is associated with changes in gene expression including IEGs (e.g. *c-jun*, *c-fos*, *c-myc*) with re-expression of the “foetal pattern” of gene expression (e.g. ANP; BNP, β -myosin heavy chain), which are often used as markers of hypertrophy (Hoshijima and Chien, 2002; Dorn and Force, 2005). Early studies indicated that IEGs are induced rapidly in cardiomyocytes by hypertrophic stimuli (Starksen et al., 1986; Iwaki et al., 1990), suggesting that these promote later changes in gene expression associated with the hypertrophic phenotype. Indeed, many of the genes that are activated during this acute phase are themselves transcription factors that will regulate a second wave of gene expression presumably responsible for the sub-acute and chronic changes of the phenotype leading to cardiac hypertrophy (Hoshijima et al., 2002; Clerk et al., 2005; Clerk et al., 2007).

In chapters 3 and 4 of this thesis, I demonstrated that two of the IEGs upregulated in cardiomyocytes by ET-1 through the ERK1/2 cascade are ATF3 and IL-6, and these same genes are also upregulated in response to H₂O₂. Using ChIP assays, I also demonstrated that ATF3 binds to the promoter of IL-6 following stimulation with ET-1 or H₂O₂, suggesting that ATF3 may be involved in the regulation of IL-6 expression under these conditions (Figures 4.9 and 4.11). It is likely that the regulatory function of ATF3 over the IL-6 gene forms a negative feedback mechanism for modulating the expression of IL-6. In support of this hypothesis, previous studies have shown that ATF3 is a negative regulator of IL-6 in monocytes stimulated with LPS, thus reducing excessive amounts of IL-6. In this way, ATF3 prevents deleterious chronic inflammatory responses (Gilchrist et al., 2006).

Parallel studies from our laboratory have recently explored the initial waves of gene expression in cardiomyocytes stimulated with ET-1 using microarray analysis. These studies were aimed at the identification of temporally-regulated genes in the initial stages of the hypertrophic response (0.5, 1, 2 and 4 hours), before the morphological and physiological changes develop fully (Cullingford et al., manuscript submitted). The clusters of IEGs exhibit differential temporal and in most cases transient upregulation/downregulation. Of the functional groups, very few were associated with end-stage cardiac hypertrophy (Dorn et al., 2003). Instead, many were associated with gene or protein expression, or with signalling (Cullingford et al., manuscript submitted). This suggests that there is a phasic expression of early response genes which is probably required for the propagation of the signal beyond the primary signalling pathways through

transcriptional networks towards the end stage phenotype. It is likely that these networks would modulate cardiomyocyte responsiveness to the cardiac milieu before the changes in the phenotype are completed. Of note, some IEGs were upregulated by ET-1 at 1 h and had reduced expression at 2 h, despite being significantly superinduced by cycloheximide at 2 h or even at 1 h. For these IEGs, synthesis of a negative regulator could be required to suppress the response. Thus I reasoned that one such protein could indeed be ATF3 since most accumulating evidence suggests that this transcription factor acts mainly as a negative regulator of transcription in other systems (section 3.1.1.2) (Chen et al., 1994; Wolfgang et al., 1997; Wolfgang et al., 2000; Gao et al., 2004; Gilchrist et al., 2006; Li et al., 2007). Indeed, our results indicate that by the time the expression of ATF3 protein is maximal following 1 h ET-1, IL-6 mRNA expression is already reduced after a transient induction, supporting a negative regulatory role for ATF3 in IL-6 regulation (Figures 3.7, 4.1 and Table 4.2). Furthermore, as demonstrated through ChIP assays, ATF3 is bound to the IL-6 promoter after 1 h of ET-1 stimulation (Figure 4.9). These observations add support to the hypothesis that ATF3 may be involved in a negative feedback loop probably turning off the transcription of IL-6 and other IEGs following ET-1 stimulation of cardiomyocytes.

In this chapter, the role of ATF3 following stimulation of cardiomyocyte with ET-1 was evaluated. First, I performed an analysis of the promoters of IEGs that were superinduced following treatment with cycloheximide in combination with ET-1 at 2 h, to evaluate the presence of predicted ATF/CRE consensus binding sequences. Then, I performed knockdown of the *ATF3* gene with adenoviral-mediated overexpression of an antisense sequence targeting ATF3 mRNA. Finally, I evaluated the effect of ATF3 knockdown on

the expression levels of IL-6 and other IEGs that could be potential target genes of ATF3, and which also possess ATF/CRE consensus binding sites in their promoters.

5.2 Methods

5.2.1 *Infection of cardiomyocytes with adenovirus containing Flag sequence or ATF3 antisense sequence*

Replication-deficient adenoviruses for expression of a full-length rat ATF3 antisense RNA for the whole coding sequence (AS-ATF3), were prepared by Dr Stephen Fuller, a member of our research group, using the AdEasy™ XL Adenoviral Vector System (Stratagene) (section 2.12). Adenoviral infection of cardiomyocytes was performed overnight at a multiplicity of infection (moi) of 10-100, in 4 ml (60 mm culture dishes) serum-free maintenance medium [DMEM/Medium 199 (4:1, v/v) overnight at 37 °C in a humidified, 5 % CO₂ incubator].

5.2.2 *RT-PCR*

Cardiomyocytes were exposed to 10 nM ET-1 for 1 h. Total RNA was extracted and cDNA synthesized (see sections 2.3 and 2.5). Q-PCR was performed (see section 2.6.1) to assess expression of ATF3, epiregulin (Ereg), c-Fos, FosB, immediate early response 2 (Ier2), IL-6, c-Jun, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta (Nfkbiz), prostaglandin-endoperoxide synthase 2 (Ptgs2), CT-1 and GAPDH mRNA (see Table 2.2 in general methods for primers). For Q-PCR the cycles were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 59°C for 60 sec. Following Q-PCR, a dissociation curve analysis was performed to check for aberrant amplification products. Q-PCR analysis of GAPDH was performed in each 96-well plate as an endogenous control and the relative quantitation protocol was used. Values are expressed as fold stimulation.

5.2.3 Immunoblotting

Cardiomyocytes were exposed to 10 nM ET-1 for 1 h. Nuclear proteins were extracted and immunoblotting was then performed (sections 2.8.2 and 2.9). Briefly, proteins (25 µg) were subjected to SDS-PAGE with a 12% polyacrylamide resolving gel and 6% stacking gel. Proteins were transferred to nitrocellulose membranes and probed with primary antibodies to ATF3 (SC-188, Santa Cruz Biotechnology Inc) or ATF2 antibodies (SC-187, Santa Cruz Biotechnology Inc.). Immunoreactive bands were detected by enhanced chemiluminescence and analysed semi-quantitatively by scanning densitometry. Values are expressed as arbitrary units.

5.2.4 Promoter analysis

The analysis of the promoter sequence of genes was performed using the tool TRANSFAC 4.0 available at the website address (<http://www.gene-regulation.com/pub/programs/alibaba2/index.html?>).

5.3 Results

5.3.1 Several IEGs upregulated by ET-1 contain ATF/CRE sites in their promoters

Since ATF3 is potently induced by hypertrophic stimuli such as ET-1 (Chapter 3 Figures 3.3, 3.4 and 3.7), and this response is mediated through the ERK1/2 cascade (Figures 3.5, 3.6 and 3.7), a kinase cascade that plays an important role in cardiac hypertrophy (Section 1.3.1.4), it is likely that ATF3 plays an important role in the regulation of the hypertrophic response. Using microarray analysis, parallel studies from our laboratory have identified several IEGs which were upregulated by ET-1 at 1 h and whose expression was reduced at 2 h, but which were superinduced when the cells were preincubated with cycloheximide and then treated with ET-1 for 2 h (Table 5.1). The behaviour of the expression of these genes suggested that, under conditions of ET-1 treatment alone for up to 2 h, a negative regulator of their transcription is being expressed. In the absence of the synthesis of this inhibitor (i.e. in the presence of cycloheximide), there was a potent superinduction of their mRNA abundances. I hypothesized that, for at least some of the genes, ATF3 could be the negative regulator. I therefore performed promoter analysis of the gene sequences for all of these IEGs (Table 5.1) with the online tool TRANSFACT 4.0. The analysis indicated that 15 out of 18 of these genes exhibited ATF/CRE or related sequences in their promoters (Table 5.1). In Figures 5.1 to 5.4 the promoter sequences for some of these genes (e.g. LIF, Ereg, FosB and c-jun) is shown, indicating the ATF/CRE consensus or related binding sites. The analysis of the IL-6 promoter sequence which contains an ATF/CRE binding site was already presented in Chapter 4, Figure 4.8.

Table 5.1 IEGs upregulated by cycloheximide alone or in combination with ET-1 (Microarray analysis*).

Gene Symbol	Probeset Number	ATF/ CRE Site	CHX (1 h)	ET-1 (1 h)	ET-1 + CHX (1 h)	CHX (2 h)	ET-1 (2 h)	ET-1+ CHX (2 h)
Axud1	1389402_at	No	6.14	7.14	14.42	11.37	7.93	27.26
Bdkrb1	1369807_at	Yes	3.45	2.16	5.14	11.23	2.20	18.77
Dusp1	1368147_at	Yes	7.19	3.06	11.97	11.27	2.42	16.45
Egr2	1387306_a_at	Yes	5.8	8.46	25.2	10.29	1.35	42.06
Egr3	1392791_at	Yes	10.26	19.94	26.36	20.65	5.03	42.44
Egr4	1387442_at	No	60.18	50.23	927.9	198.79	8.14	1748.96
Ereg	1385150_at	Yes	3.55	3.15	5.07	13.82	1.32	15.43
Fos	1375043_at	Yes	24.39	9.78	52.24	25.35	1.25	67.37
FosB	1373759_at	Yes	49.24	210.90	562.7	26.13	7.06	303.37
Ier2	1372389_at	Yes	9.02	3.43	23.17	12.9	1.70	29.36
Il6	1369191_at	Yes	15.02	5.58	22.07	31.09	2.37	53.37
Jun	1374404_at	Yes	8.79	2.61	14.96	13.34	1.23	20.56
LIF	1393728_at	Yes	11.91	4.88	11.96	20.79	2.62	26.22
Nfkbiz	1378032_at	Yes	6.27	3.05	10.75	12.03	2.29	17.74
Nr4a1	1386935_at	Yes	6.92	17.55	22.38	10.26	3.66	28.89
Nr4a3	1369067_at	Yes	9.07	26.05	43.27	15.35	13.78	65.50
Ptgs2	1368527_at	Yes	10.01	10.56	19.27	19.22	3.42	31.06
Tnfaip3	1385641_at	No	5.45	2.42	7.08	10.43	1.59	13.97

* Cardiomyocytes were exposed to 100 nM ET-1 or 20 μ M cycloheximide (CHX) alone or in combination with ET-1 for the indicated times. RNA was extracted, cRNA was prepared and samples hybridized to Affymetrix rat genome 230 2.0 arrays. Array data were analysed by MicroArray Suite 5.0 followed by Genespring 7.31. Results are expressed relative to unstimulated control (n = 3).

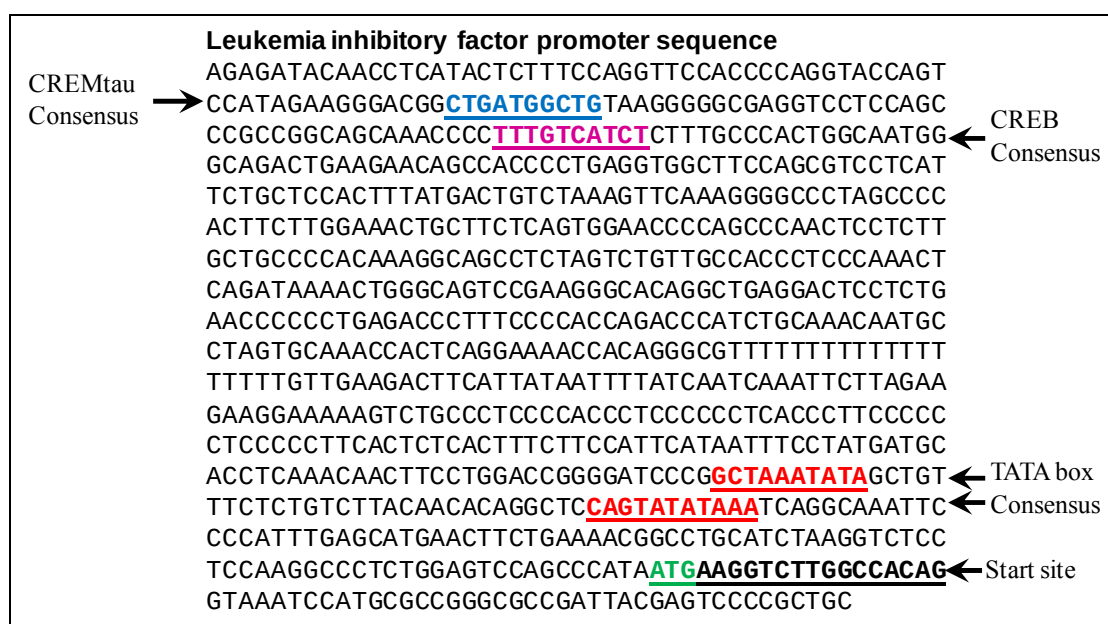


Figure 5.1 ATF/CRE consensus sites in the rat LIF promoter. Promoter analysis was performed using the online tool TRANFACT 4.0. The predicted cAMP-responsive element modulator-tau (CREMtau) consensus is shown in blue, cAMP response element-binding (CREB) consensus is shown in pink. Two TATA box consensus sequences are also shown in red. The transcriptional start site and first codon within the first exon is shown in green. The rat LIF promoter sequence was obtained from Ensembl (ENSRNOG00000007002).

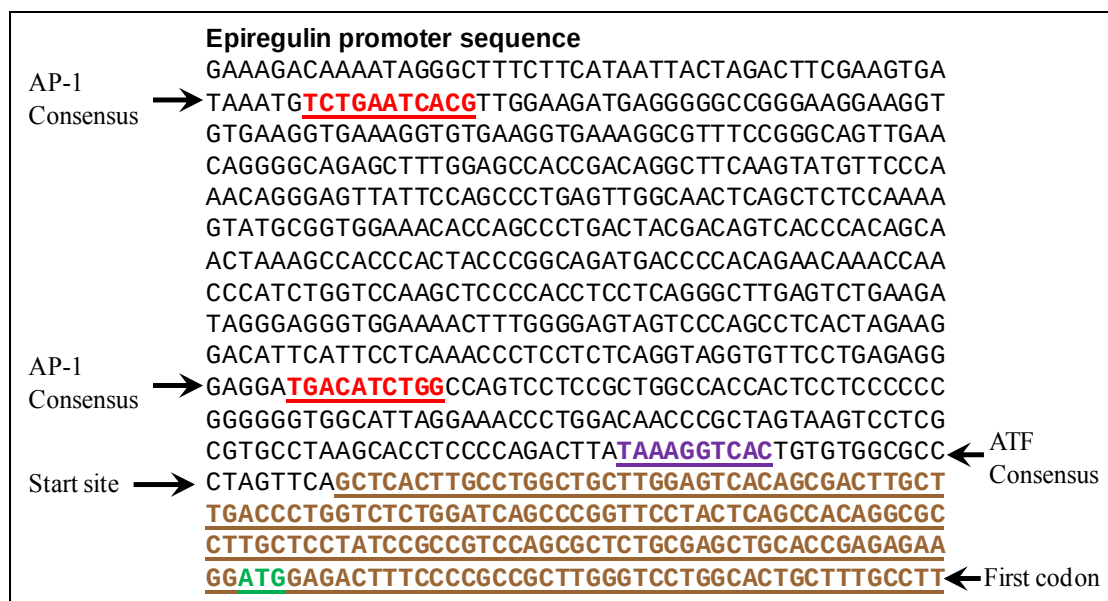


Figure 5.2 ATF/CRE consensus sequences in the promoter of the rat Ereg gene. Promoter analysis was performed using the online tool TRANFACT 4.0. A predicted ATF consensus binding site is shown in purple. Two AP-1 sites are shown in red. The transcriptional start site and first exon are shown in brown with the first codon shown in green. Rat Ereg promoter sequence was obtained from Ensembl (ENSRNOG00000002771).

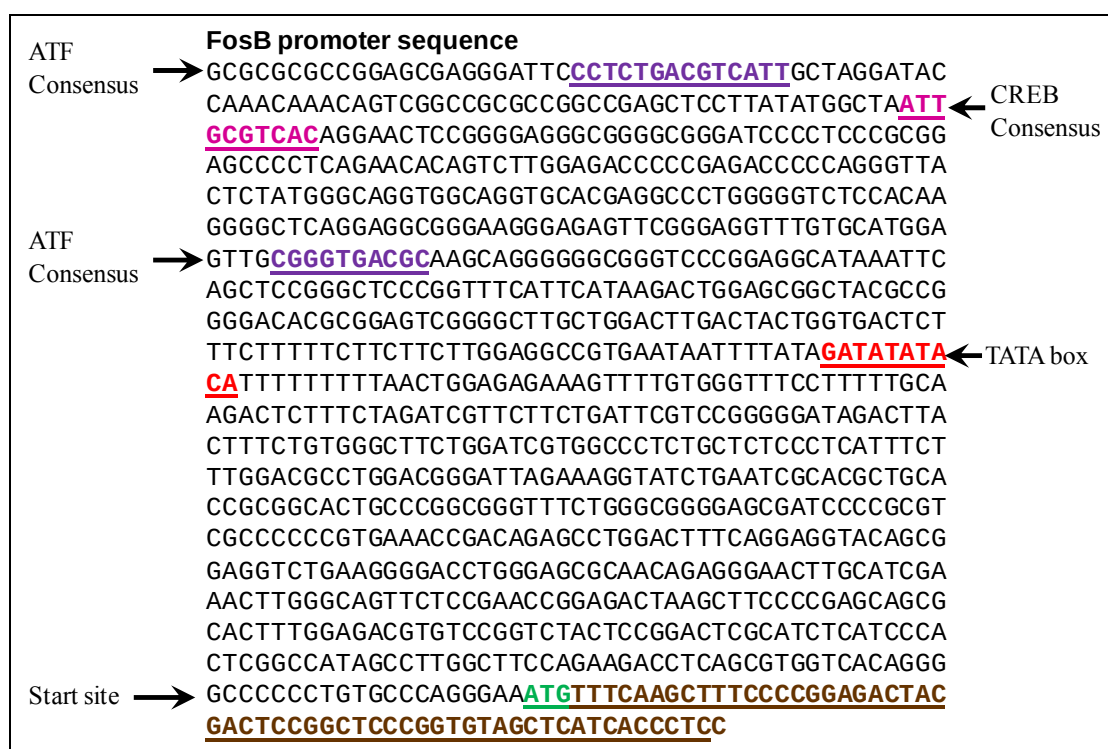


Figure 5.3 ATF/CRE consensus sequence in the promoter of the rat FosB gene. Promoter analysis was performed using the online tool TRANFACT 4.0. Two predicted ATF consensus sequences are shown in purple, a CREB consensus sequence is shown in pink. TATA box sequence is shown in red. The location of the transcriptional start site (which also corresponds to the first codon) within the first exon is shown in green. Rat FosB promoter sequence was obtained from Ensembl (ENSRNOG00000016811).



Figure 5.4 ATF/CRE consensus sequences in the rat c-Jun promoter. Promoter analysis was performed using the online tool TRANSFAC 4.0. Two predicted ATF/CRE sites are indicated in pink. Also an early growth response 1 (Egr-1) binding site is indicated in red and an activator protein 1 (AP-1) site is indicated in blue. The transcriptional start site in the first exon (also corresponding to the first codon) of the c-Jun gene is indicated in green. Rat c-jun promoter sequence was obtained from Ensembl (ENSRNOG00000026293).

5.3.2 Knockdown of the ATF3 gene using adenoviral mediated overexpression of the antisense sequence of ATF3.

A full length ATF3 antisense-containing adenovirus was constructed by Dr Stephen Fuller from our laboratory as described in section 2.12. Infections of cardiomyocytes at a multiplicity of infection (MOI) ~10 – 100 took place overnight with either an ATF3 antisense-containing adenovirus (ATF3-A/S) or with a Flag-Shuttle virus, containing only the p-Shuttle vector and the Flag sequence (DYKDDDDK) (Flag-Shuttle), this later serving as a control for any adenoviral-mediated unspecific effects. The next day infected cardiomyocytes were either left untreated (controls) or were stimulated with 10 nM ET-1 for 1 h. I first evaluated the effects of the adenoviral-mediated overexpression of ATF3 antisense (ATF3-A/S) on the expression levels of endogenous ATF3 using Q-PCR analysis of extracted mRNA, and immunoblotting of nuclear extracts. Adenoviral-mediated overexpression of ATF3-A/S resulted in significant reduction of mRNA levels of the ATF3 gene with respect to Flag-Shuttle infected cardiomyocytes following ET-1 treatment (Figure 5.5A). Adenoviral-mediated overexpression of ATF3-A/S also caused a significant inhibition of ET-1-induced ATF3 protein levels, reducing expression of the ATF3 protein to 43 ($\pm$ 3) % relative to Flag-Shuttle following ET-1 stimulation (Figure 5.5B upper panel). These experiments also demonstrated that infections with Flag-Shuttle alone had no effect on the expression of ATF3, thus confirming that the effect on ATF3 knockdown was mediated by the overexpressed ATF3 antisense sequence and not by non-specific effects as a consequence of adenoviral infection of cardiomyocytes. The protein levels of ATF2, another ATF/CRE family member expressed in cardiomyocytes, were not altered after adenoviral infections under identical treatment conditions (Figure 5.5B middle panel), demonstrating that the effect of ATF3-A/S on cellular proteins was not a consequence of general inhibition of global protein translation. Previous studies have

shown that ATF2 is phosphorylated following ET-1 treatment (Clerk and Sugden, 1997). Consistent with this, the presence of a reduced mobility band for ATF2 was observed following ET-1 stimulation of cardiomyocytes (Figure 5.5B middle panel).

5.3.3 Effects of ATF3 knockdown on the expression of IL-6 in cardiomyocytes stimulated by ET-1.

Previously, I have shown that IL-6 mRNA and protein levels increase in response to ET-1 stimulation (Chapter 4). Furthermore, ATF3 binds to the promoter of IL-6 following ET-1 stimulation and may be involved in a negative feedback loop to repress IL-6 expression (Chapter 4). To evaluate the possibility that ATF3 is a negative regulator of IL-6, I examined the effects of ATF3 knockdown (see above section 5.3.2) on the expression levels of IL-6 using Q-PCR. ATF3 protein knockdown resulted in significant superinduction of IL-6 following stimulation of cardiomyocytes with ET-1 for 1 h (Figure 5.6). This suggests that, under conditions of reduced ATF3 protein levels, following ET-1 stimulation IL-6 mRNA expression becomes de-regulated resulting in superinduction of the mRNA abundance of IL-6. These results also confirm that IL-6 is one of the downstream targets of ATF3 regulation following ET-1 stimulation of cardiomyocytes.

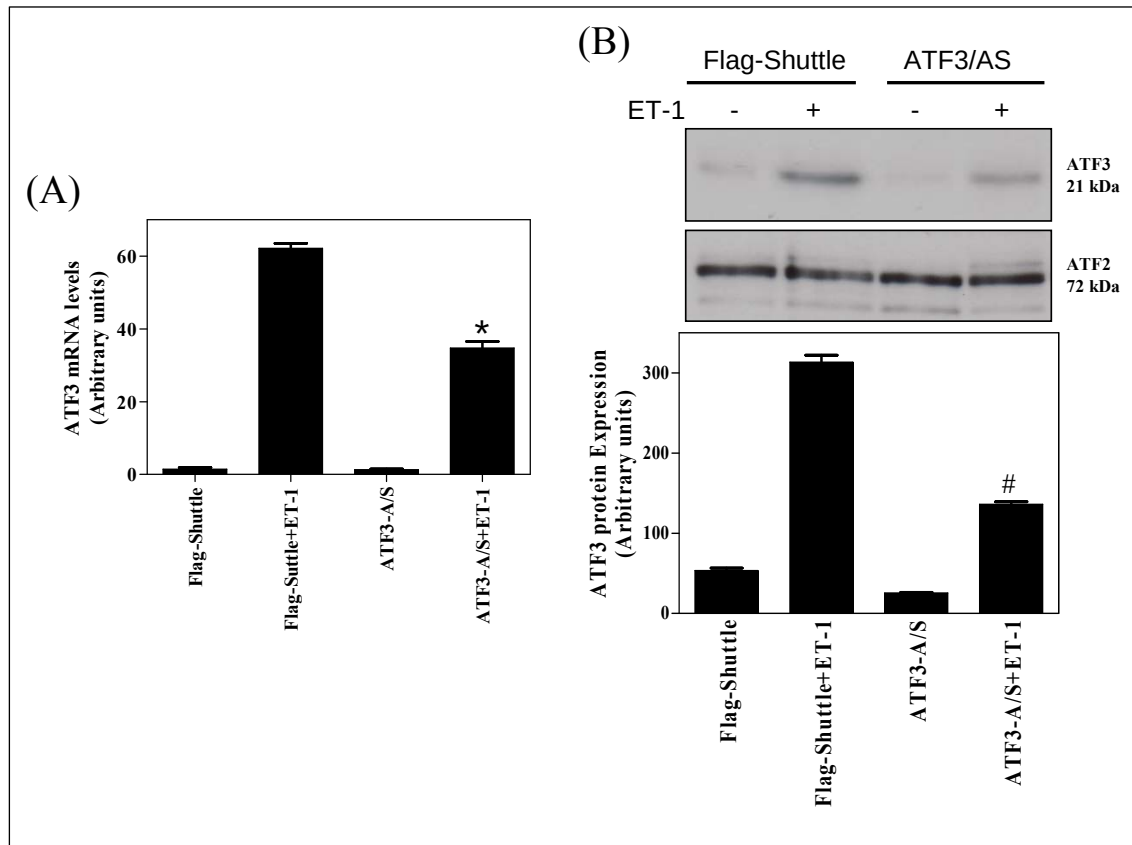


Figure 5.5 Effects of ATF3 knockdown on mRNA and protein levels of ATF3 in cardiomyocytes. Cardiomyocytes were infected overnight with a Flag-Shuttle adenovirus (p-Shuttle vector containing the Flag sequence), or with ATF3-A/S adenovirus (containing the ATF3 antisense sequence). Next day, cardiomyocytes were either left untreated or stimulated with 10 nM ET-1 for 1 h. In (A), the expression of ATF3 mRNA was analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. In (B), nuclear extracts were immunoblotted with antibodies to ATF3 (upper panel) or ATF2 (middle panel). Representative images are shown. The blots of ATF3 were analysed by scanning densitometry and the combined data are shown in the lower panel as means $\pm$ S.E.M. for three independent preparations of cardiomyocytes. (* $p < 0.01$, # $p < 0.001$ relative to Flag-Shuttle+ET-1, one way ANOVA with Tukey post test).

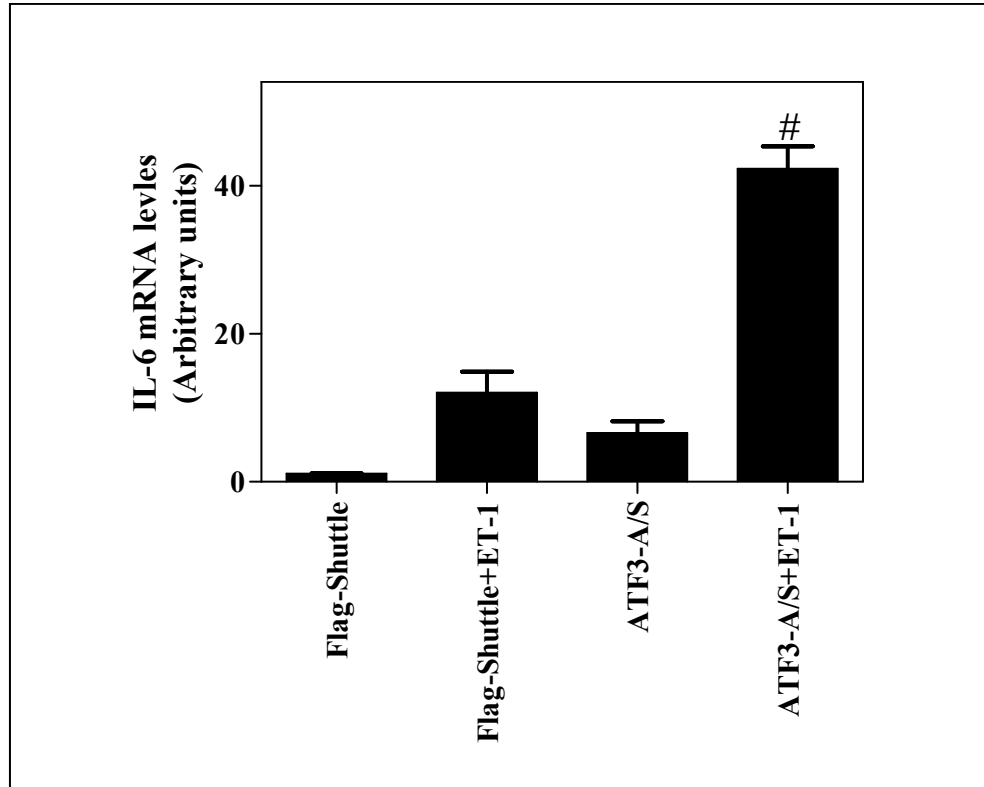


Figure 5.6 Knockdown of ATF3 causes increased expression of IL-6 after ET-1 treatment of cardiomyocytes. Cardiomyocytes were infected overnight with a Flag-Shuttle adenovirus (p-Shuttle vector containing the Flag sequence), or with ATF3-A/S adenovirus (containing the ATF3 antisense sequence). Next day, cardiomyocytes were either left untreated or stimulated with 10 nM ET-1 for 1 h. Unstimulated cells were used as controls for both adenoviral conditions (Flag-Shuttle and ATF3-A/S). The expression of IL-6 mRNA was analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. (# $p < 0.001$, relative to Flag-Shuttle+ET-1, one way ANOVA with Tukey post test).

5.3.4 Several immediate early genes are downstream targets of ATF3

As shown in Table 5.1, 15 out of 18 immediate early genes that are superinduced by preincubation with the protein synthesis inhibitor cycloheximide followed by treatment with ET-1 at 1 and 2 hours, have at least one ATF/CRE consensus or related binding site in their promoters. As shown in the preceding section, knockdown of ATF3 protein results in superinduction of IL-6 mRNA expression levels, a gene that is also one of the IEGs superinduced under these conditions. To test the hypothesis that at least some of the other genes with ATF/CRE consensus sequences in their promoters were also downstream target genes of ATF3 regulation, Q-PCR analysis was performed to interrogate the expression levels of eight selected genes (Ereg, c-Fos, FosB, Ier2, c-Jun, LIF, Nfkbiz, and Ptgs2), using mRNA extracted from cardiomyocytes under conditions of knockdown of ATF3 protein as described above (section 5.3.2). With this approach, I identified six genes that were superinduced by knockdown of ATF3 protein and two whose mRNA levels did not change under these same conditions.

Knockdown of ATF3 protein resulted in significant superinduction of LIF, Ereg and Nfkbiz (Figure 5.7, A to C). This suggests that these genes are probable downstream target genes of ATF3, and that, as with IL-6, ATF3 may operate in a negative feedback loop to repress their transcription following stimulation with ET-1. The genes c-Fos, Ier2 and Ptgs2 were also slightly superinduced by ATF3 knockdown, however this effect did not reach statistical significance (Figure 5.7, D to F). Nevertheless, these genes are possible downstream targets of ATF3 with similar negative regulatory effects on their transcription but it is likely that other transcription factor(s) could also be involved in

their transient expression following ET-1 stimulation (Cullingford et al., manuscript submitted).

5.3.5 The transient pattern of induction of c-jun and FosB is possibly mediated by an alternative mechanism not involving the transcription factor ATF3

I also performed Q-PCR analysis to evaluate the effects of ATF3 knockdown on c-Jun and FosB mRNA abundance following ET-1 stimulation. The analysis indicates that following ATF3 knockdown, the mRNA levels of c-Jun and FosB remain unchanged indicating that these genes are probably not downstream target genes of ATF3 (Figure 5.8).

5.3.6 CT-1 another member of the gp130 cytokines is affected in an opposite manner after knockdown of ATF3 in cardiomyocytes

My analysis indicated that two out of three of the most studied members of the IL-6 family of cytokines, IL-6 and LIF are probable targets of ATF3 downregulation, as indicated by superinduction of their mRNA abundance after knockdown of ATF3 following ET-1 stimulation (Figures 5.6 and 5.7B). CT-1 is another member of the gp130 cytokines with important functions in the pathophysiology of eccentric hypertrophy and also appears to promote dilatation of the heart leading to heart failure (Wollert et al., 1996; Hunter and Chien, 1999).

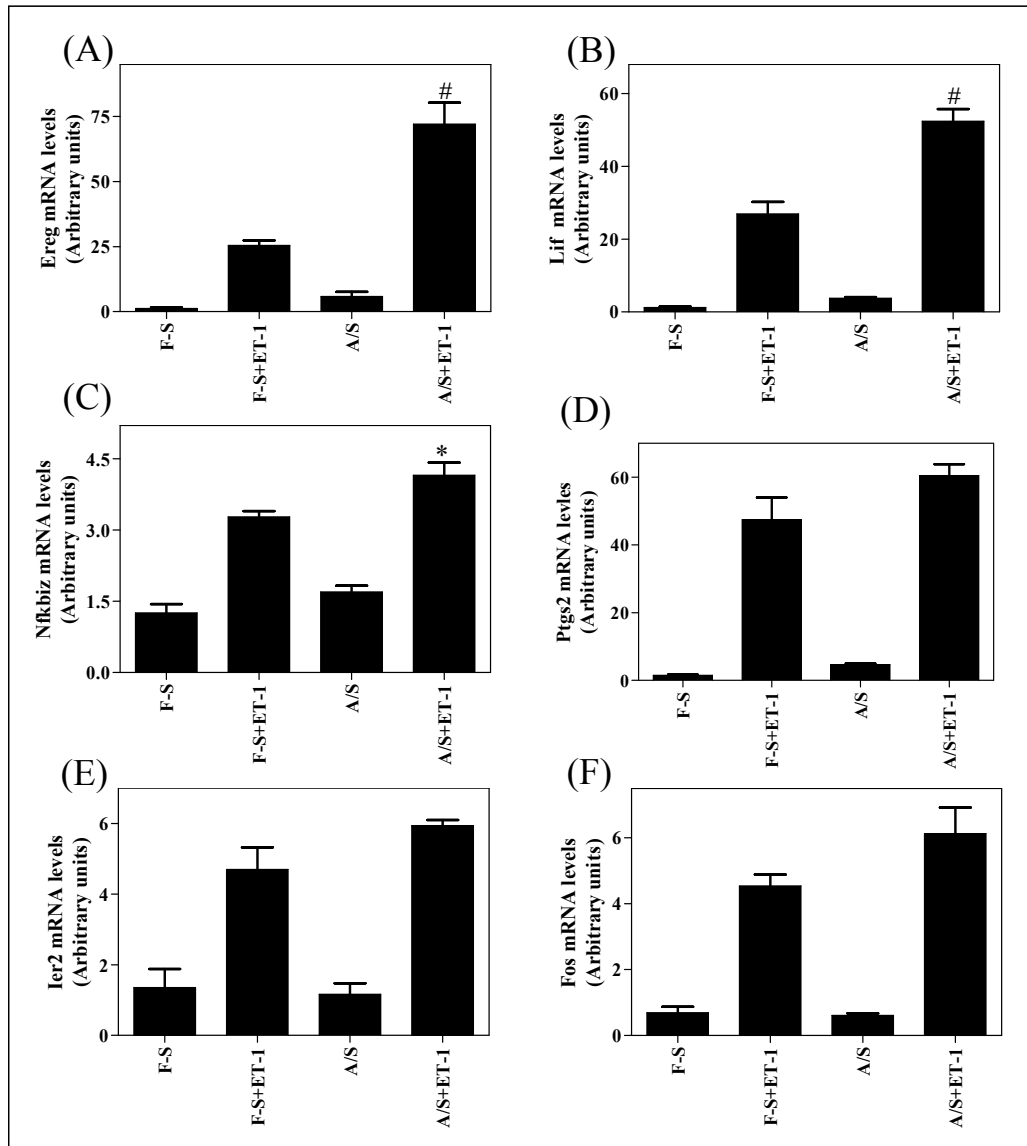


Figure 5.7 Knockdown of ATF3 causes superinduction of LIF, Ereg, Nfkbiz, Ptgs2, Ier2 and Fos mRNA expression in cardiomyocytes upon ET-1 stimulation. Cardiomyocytes were infected overnight with a Flag-Shuttle adenovirus (p-Shuttle vector containing the Flag sequence), or with ATF3-A/S adenovirus (containing the ATF3 antisense sequence). Next day, cardiomyocytes were either left untreated or stimulated with 10 nM ET-1 for 1 h. Unstimulated cells were used as controls for both adenoviral conditions (Flag-Shuttle and ATF3-A/S). The expression of (A) Ereg, (B) LIF, (C) Nfkbiz, (D) Ptgs2, (E) Ier2, and (F) Fos mRNA levels was analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. (* p < 0.05; # p < 0.001, relative to Flag-Shuttle+ET-1, one way ANOVA with Tukey post test).

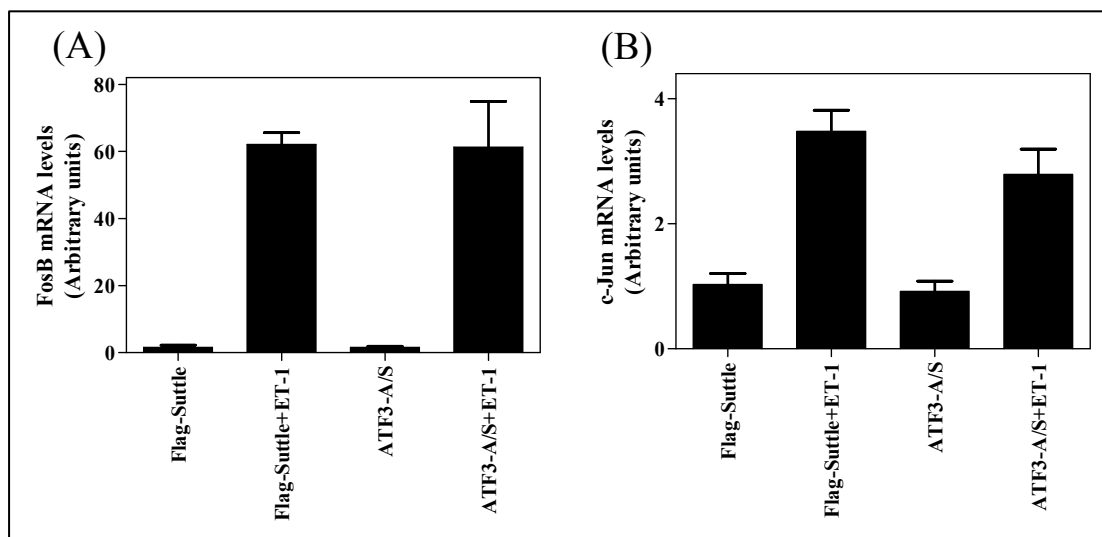


Figure 5.8 Knockdown of ATF3 does not affect the expression levels of the IEGs FosB and c-Jun in response to ET-1. Cardiomyocytes were infected overnight with a Flag-Shuttle adenovirus (p-Shuttle vector containing the Flag sequence), or with ATF3-A/S adenovirus (containing the ATF3 antisense sequence). Next day, cardiomyocytes were either left untreated or stimulated with 10 nM ET-1 for 1 h. Unstimulated cells were used as controls for both adenoviral conditions (Flag-Shuttle and ATF3-A/S). The expression levels of (A) FosB (B) c-Jun mRNA were analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations.

Previous studies have shown that the human *CT-1* gene contains an ATF/CRE consensus sequence in its promoter (Erdmann et al., 1998) (Figure 5.9 and 5.10). The analysis of the rat *CT-1* gene with the tool TRANSFACT 4.0 showed that its promoter does indeed possess a predicted ATF/CRE consensus sequence but in a different location relative to the transcription start site with respect to the human *CT-1* gene (Figures 5.9 to 5.11). Further analysis of the rat and human *CT-1* genes using the multiple alignment tool MultiAlin (Corpet, 1988), reveals that the human ATF/CRE consensus sequence previously described in the literature was not conserved in the rat *CT-1* gene promoter (Figure 5.9). In fact, the analysis of the human *CT-1* gene with the TRANSFACT tool reveals the presence of another ATF/CRE consensus on the human *CT-1* promoter (Figure 5.10). However, even though the ATF/CRE consensus of human and rat do not overlap, I can conclude that the *CT-1* promoters of both species possess ATF/CRE sites which could be bound by ATF3 (Figures 5.9 to 5.11). Interestingly, parallel studies from our laboratory indicate that although IL-6 and LIF are IEGs induced following stimulation of cardiomyocytes with ET-1, *CT-1* is not one of these IEGs. In accordance with this observation, knockdown of ATF3 not only did not induce its expression but on the contrary caused a marked downregulation of the basal levels (Figure 5.12).

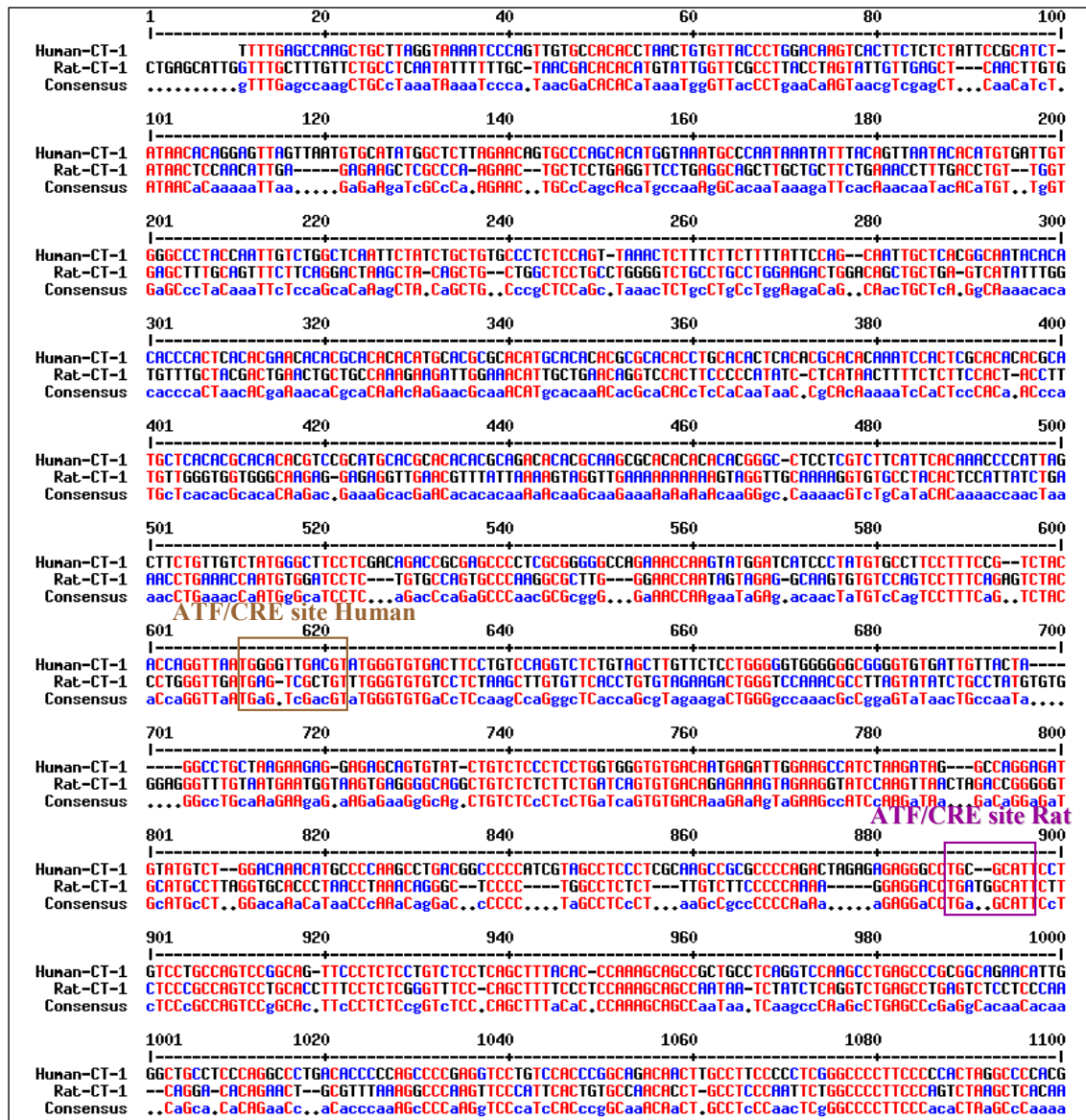


Figure 5.9 The human CT-1 promoter contains an ATF/CRE consensus that differs from the predicted ATF/CRE consensus in the rat CT-1 promoter. The human and rat CT-1 gene promoter regions obtained from Ensembl (Human CT-1: ENSG00000150281; Rat CT-1: ENSRNOG00000018962) were analysed with the MultiAlin tool. The previously described human ATF/CRE consensus is shown boxed in brown, and the rat ATF/CRE site is shown boxed in purple. Both promoter regions differ considerably for each species (see also Figures 5.10 and 5.11).

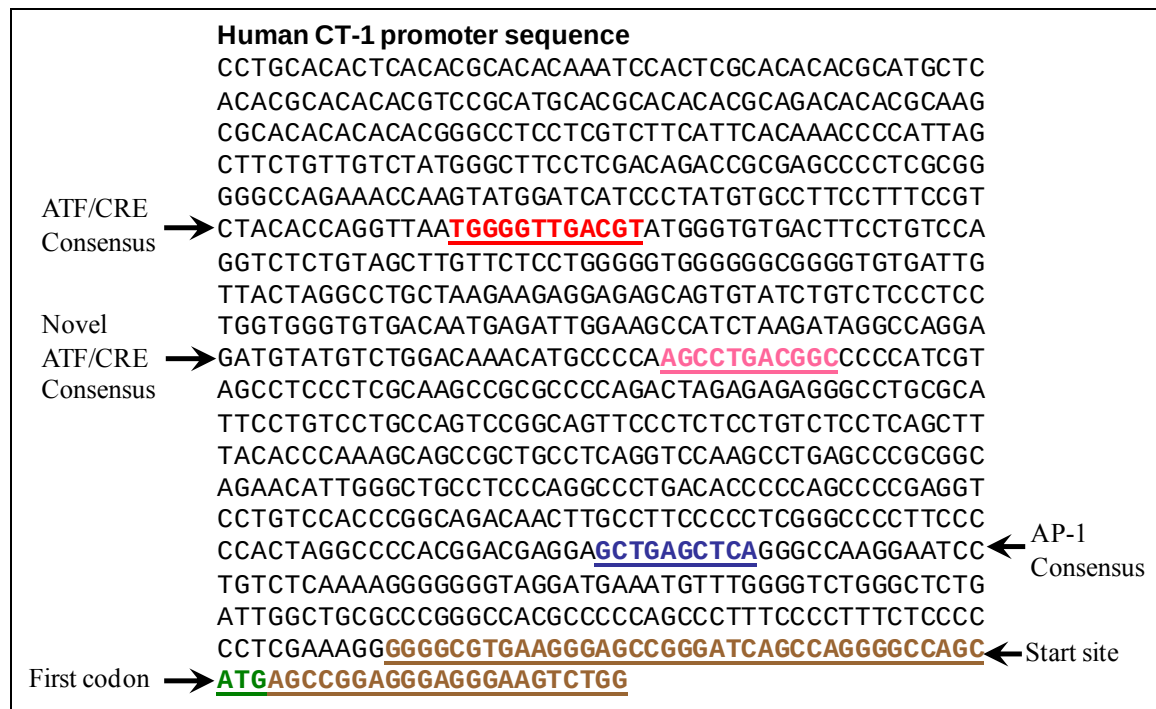


Figure 5.10 ATF/CRE consensus sequences in the human CT-1 gene promoter. Promoter analysis was performed using the online tool TRANFACT 4.0. A previously described ATF/CRE site is shown in red; a novel predicted ATF/CRE site is shown in pink. An AP-1 consensus site is shown in blue, with the transcriptional start site and first codon within the first exon shown in brown and green respectively. The human CT-1 promoter sequence was obtained from Ensembl (ENSG00000150281).

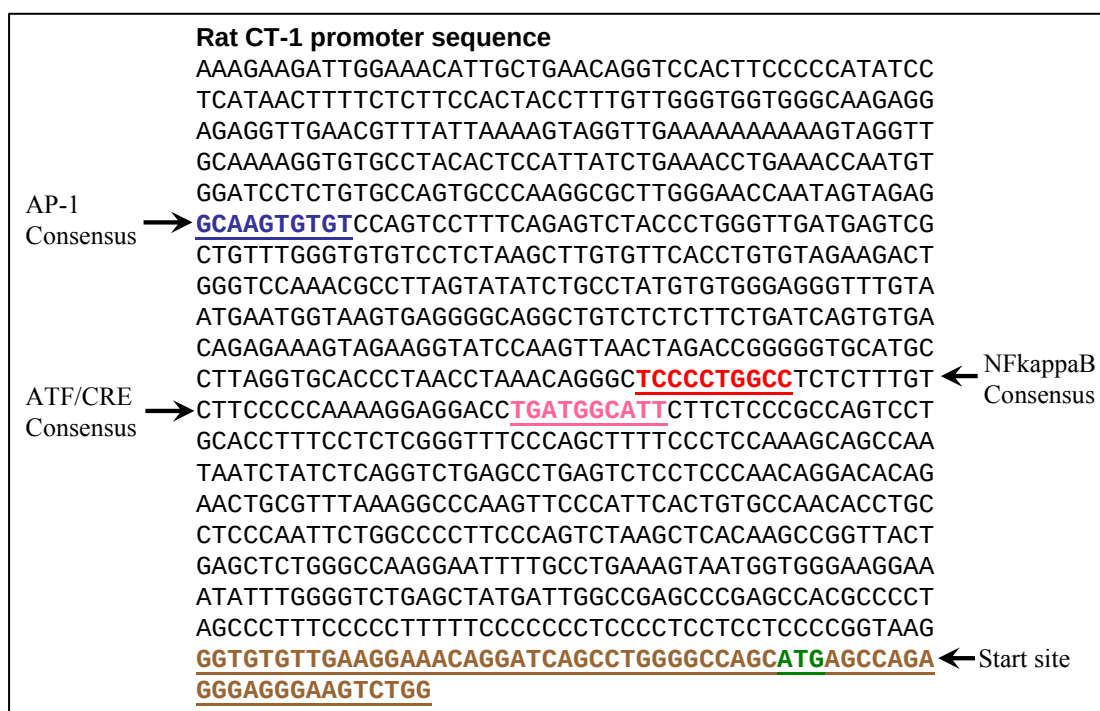


Figure 5.11 ATF/CRE consensus site in the rat CT-1 gene promoter. Promoter analysis was performed using the online tool TRANFACT 4.0. The ATF/CRE consensus is shown in pink, one AP-1 consensus is shown in blue and an NFkappaB (Rel) consensus is shown in red. The transcriptional start site and first codon within the first exon are shown in brown and green respectively. The rat CT-1 promoter sequence was obtained from Ensembl (ENSRNOG00000018962).

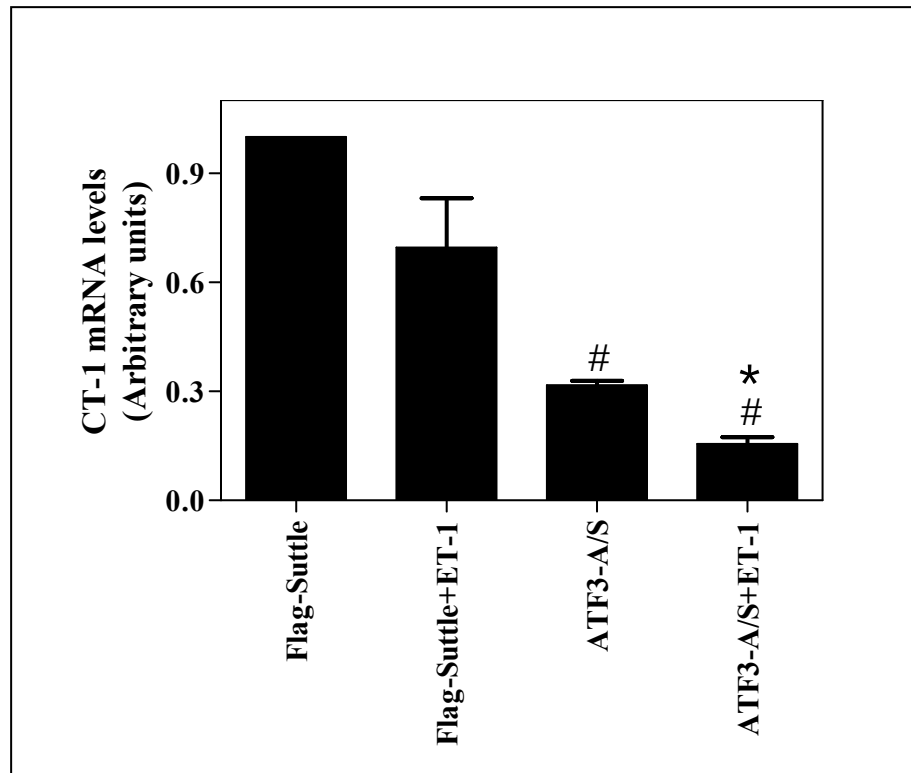


Figure 5.12 Effect of ATF3 knockdown on CT-1 mRNA levels following ET-1 stimulation. Cardiomyocytes were infected overnight with a Flag-Shuttle adenovirus (p-Shuttle vector containing the Flag sequence), or with ATF3-A/S adenovirus (containing the ATF3 antisense sequence). Next day, cardiomyocytes were either left untreated or stimulated with 10 nM ET-1 for 1 h. Unstimulated cells were used as controls for both adenoviral conditions (Flag-Shuttle and ATF3-A/S). The expression levels of CT-1 mRNA were analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. (* $p < 0.01$ relative to Flag-Shuttle+ET-1; # $p < 0.001$ relative to Flag-Shuttle, one way ANOVA with Tukey post test).

5.3.7 Ereg and LIF mRNA expression is transient following stimulation with ET-1

The above experiments indicated that IL-6, LIF, Ereg and Nfkbiz were all significantly superinduced after knockdown of ATF3 followed by ET-1 stimulation, suggesting that ATF3 is involved in their negative regulation. The regulation of these genes by ATF3 is likely to be suppression of their transcriptional activation rather than stability. Therefore, I performed Q-PCR analysis to evaluate the mRNA expression levels of ATF3 (see also Figure 3.3), IL-6 (see also Figure 4.3) LIF and Ereg using mRNA extracted from cardiomyocytes unstimulated (controls) or exposed to 100 nM ET-1 for up to 8 h (Figure 5.13). This analysis indicated that the expression of these genes is also transient as suggested by parallel studies from our laboratory using microarray analysis (Cullingford et al., manuscript submitted). Thus, ATF3 and IL-6 had a peak of expression at 30 minutes with some decline after 1 h and a return to near basal levels after 2 h (Figure 5.13A and B). A similar transient induction was also observed for Ereg and LIF, however the peak of expression of Ereg was observed at 1 h, and that of LIF was observed between 30 min and 1 h but, in both cases, levels returned to basal within 2 h (Figure 5.13 C and D). All of this is consistent with the observation that IL-6, LIF and Ereg were identified as IEGs upregulated in cardiomyocytes by ET-1 and whose transient expression pattern could be the result of a negative regulatory effect of ATF3 on their transcriptional activation.

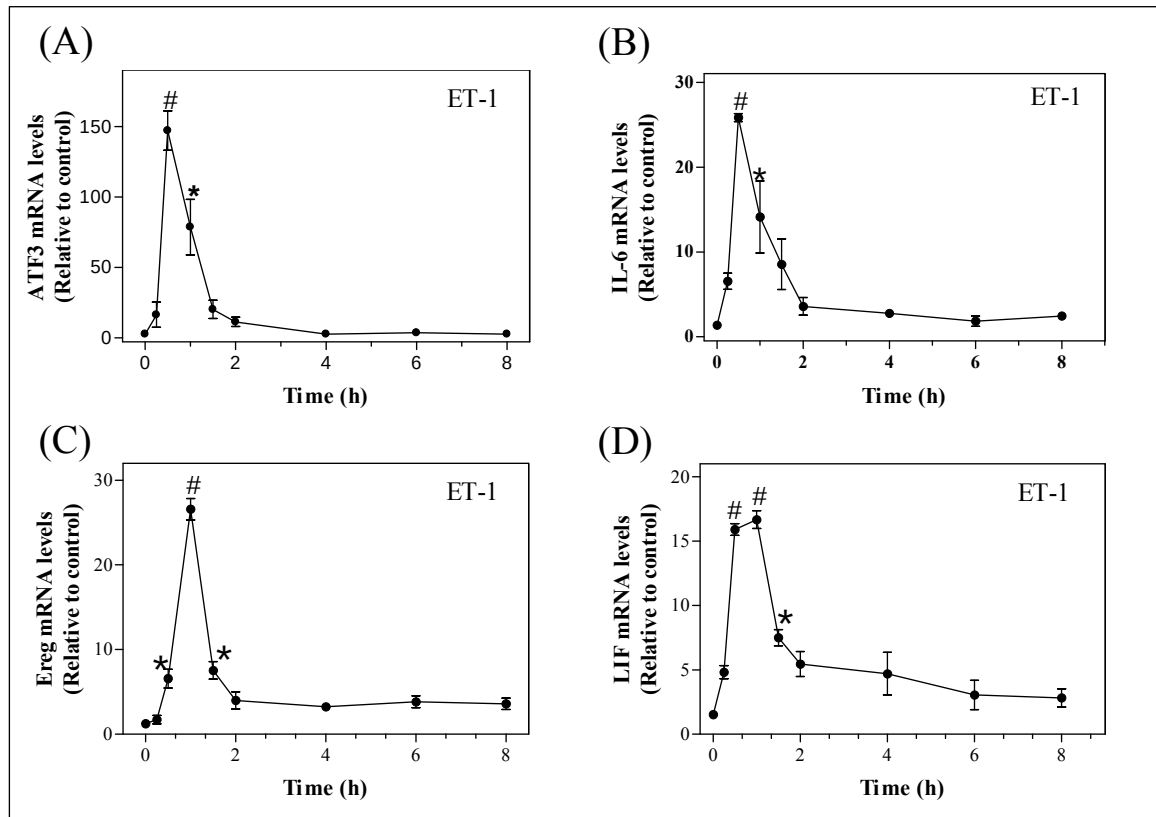


Figure 5.13 Expression of ATF3, IL-6, LIF and Ereg mRNA in response to ET-1 in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to ET-1 (100 nM), for the indicated periods of time. The expression of (A) ATF3, (B) IL-6, (C) Ereg or (D) LIF mRNA was analysed by Q-PCR, with simultaneous analysis of GAPDH in each 96-well plate as an endogenous control using the relative quantification protocol. Results are means $\pm$ S.E.M. for four independent cardiomyocyte preparations. (* $p < 0.05$; # $p < 0.01$, relative to control, one-way ANOVA with Tukey post test).

5.4 Discussion

5.4.1 Role of ATF3 in the transcriptional repression of several IEGs in cardiomyocytes

In vitro studies have shown that ATF3 is an activator of transcription when bound as a heterodimer with for example c-Jun or JunD to specific ATF/CRE or related binding sites (Hsu et al., 1992; Chu et al., 1994). Interestingly, the ATF3/JunB heterodimer can either activate or repress transcription depending on the promoter context (Hsu et al., 1992). In contrast, ATF3 homodimers (ATF3/ATF3) are negative regulators of transcription (Chen et al., 1994; Wolfgang et al., 2000). Although this ability to form heterodimers and homodimers and therefore positively or negatively regulate transcription suggests a versatility in the regulation of gene expression by ATF3, most studies to date have shown that ATF3 acts mainly as a transcriptional repressor in vitro and in vivo. Thus, ATF3 homodimers are negative regulators of transcription of its own promoter (Chen et al., 1994; Wolfgang et al., 2000), as well as the proapoptotic transcription factor CHOP [C/EBP (CCAAT/enhancer binding protein) homologous protein] (Wolfgang et al., 1997). ATF3 has been also implicated in the negative regulation of PLB in cardiomyocytes following its induction by adenylyl cyclase IV or isoproterenol (Gao et al., 2004), and is involved in a negative feedback loop repressing IL-6 or IL-12 expression in monocytes following stimulation with lipopolysaccharide (Gilchrist et al., 2006).

Viral-mediated antisense overexpression in cells, tissues and in whole animals has long been recognized as an effective method to knockdown genes to evaluate the effects of the target gene in biological processes (Green et al., 1986). Thus, overexpression mediated by retroviral vector of an ANGII type 1B (AT_{1B}) receptor antisense resulted in knockdown of

the AT_{1B} receptor and reduced ANGII actions in neurons from hypertensive rats (Lu and Raizada, 1995). This same retroviral AT_{1B}-antisense system was also effective in controlling blood pressure in spontaneously hypertensive rats after a single injection to 5 days old rats (Iyer et al., 1996), and in reducing cardiac hypertrophy secondary to the cardiac actions of ANGII in the renin-transgenic rat following a single intracardiac injection to 5 days old rats (Pachori et al., 2002). To determine the role of ATF3 in cardiomyocytes following ET-1 stimulation, I used adenoviral-mediated overexpression of ATF3 antisense to the full coding sequence. As shown in figure 5.5, ATF3-A/S overexpression resulted in significant reduction of both ATF3 mRNA and protein levels induced by ET-1, and this response was specific as no effect on the levels of ATF3 mRNA or protein was observed with the Flag-Shuttle vector. A further confirmation of the specificity is that ATF3 protein levels were downregulated in the absence of any change in the expression levels of ATF2 protein in nuclear extracts (Figure 5.5B).

To evaluate the effects of ATF3 knockdown following hypertrophic stimuli in the form of ET-1 stimulation of cardiomyocytes, the expression levels of several IEGs was evaluated using Q-PCR analysis (Table 5.1). First I confirmed that IL-6 is a downstream target of ATF3 regulation by showing that ATF3-A/S mediated knockdown of the ATF3 protein results in superinduced expression of IL-6 mRNA (Figure 5.6). This superinduction was statistically significant and in accord with previous studies using bone marrow derived macrophages derived from ATF3 ^{-/-} mice in which stimulation with LPS resulted in a substantial increase in the expression levels of IL-6 compared to cells derived from wild-type animals (Gilchrist et al., 2006; Withmore et al., 2007). In the study of Gilchrist et al., (2006), the in vivo effects of absence of ATF3 following exposure to LPS were even more dramatic. Thus, ATF3 ^{-/-} mice not only produced more IL-6 as measured from

peritoneal macrophages, but also exhibited a more severe endotoxic shock probably secondary to sustained inflammation. 100 % of ATF3 null animals died within 24 h of LPS injection, whereas none of the wild-type mice had succumbed by this time period (Gilchrist et al., 2006).

To extend the observation made with IL-6, I also demonstrated that other IEGs were also superinduced after ATF3 knockdown (Figure 5.7). Among these, LIF, another member of the gp130 cytokines (section 1.3.3), Ereg, a member of the EGF related growth factors (section 1.3.2), and Nfkbiz, also known as inhibitor of nuclear factor kappa B (NF- κ B) zeta (IkB- ζ), were all found to be significantly superinduced by ATF3 protein knockdown following stimulation with ET-1 in cardiomyocytes (Figure 5.7 A to C). Therefore these genes are probable target genes of ATF3. To our knowledge this is the first report of these genes as potential downstream targets of ATF3 in any cell system. I also found that Ptgs2, c-Fos and Ier2 have a tendency to be superinduced under the same ATF3 knockdown conditions (Figure 5.7 D to F). However, this effect did not reach statistical significance. For these genes, I suggest that either the time point selected at 1 h is possibly too early to fully appreciate the effects of ATF3 protein knockdown, or alternatively the effect of ATF3 is shared with another transcriptional repressor that is present despite the reduced levels of ATF3. In contrast, c-Jun and FosB are probably not downstream targets of ATF3 transcriptional repression as indicated by the lack of effect in their mRNA abundance under similar ATF3 protein knockdown conditions (Figure 5.8).

LIF as well as IL-6 and CT-1, is a cytokine capable of inducing eccentric hypertrophy and is therefore considered important for the pathophysiology of heart failure (Hunter and

Chien, 1999; Nicol et al., 2000). The role of Ereg has yet to be established in cardiomyocytes. Studies in other systems indicate that Ereg expression is induced in vascular smooth muscle cells (VSMCs) following stimulation with ANGII, ET-1 or α -thrombin, and is a potent mitogenic factor for these cells (Taylor et al., 1999). Importantly, alterations in the phenotype of VSMCs from a differentiated towards a de-differentiated state are of critical importance in the pathogenesis of vascular diseases such as atherosclerosis (Ross, 1993). Recently, Ereg was reported as a de-differentiation factor in VSMCs a response that is mediated through the activation of the ERK1/2 and p38-MAPK pathways (Takahashi et al., 2003). Ereg is also upregulated following stimulation of VSMCs with lysophosphatidic acid and PDGF-BB, two potent VSMC-de-differentiation factors. These data and the finding of increased Ereg expression in human atherosclerotic lesions and in rat arteries subjected catheter balloon injury, suggests that Ereg is an important factor in the pathophysiology of atherosclerosis (Takahashi et al., 2003). Ereg is also implicated as an important growth regulating factor in several cell types (Toyoda et al., 1997), and the finding of increased levels of this protein in several cancers and epithelial tumour cell lines indicates that it may play a role in the progression of carcinomas (Toyoda et al., 1997; Lindvall et al., 2003; Yamamoto et al., 2004a).

The transcription factor NF- κ B is an important regulator of innate and adaptive immune responses, and also plays a key role in apoptosis, cell proliferation and differentiation (Li and Verma, 2002; Hayden and Ghosh, 2004; Bonizzi and Karin, 2004). Uncontrolled activation of NF- κ B, as seen in severe infections, is in part responsible of life-threatening conditions such as endotoxic (septic) shock. Therefore, the activity of NF- κ B is highly controlled in normal cells. In normal quiescent cells, NF- κ B is tethered in the cytoplasm in a complex with its inhibitory proteins I κ B- α , I κ B- β and I κ B- ϵ (reviewed in Bonizzi and

Karin, 2004). Nfkbiz, the predicted rat orthologue of human I κ B- ζ , was recently characterized as a novel NF- κ B inhibitor, which is strongly induced in response to LPS in several cells and tissues in vitro and in vivo (Kitamura et al., 2000; Haruta et al., 2001; Yamazaki et al., 2001). Unlike other I κ B proteins, I κ B- ζ is localized to the nucleus where it is capable of potentiating as well as repressing the transcriptional activities of NF- κ B (Yamazaki et al., 2001; Motoyama et al., 2005). The role of I κ B- ζ is not completely understood. However, its acute expression in response to inflammatory stimuli and its negative and positive effects on NF- κ B transcription suggest that it is an important regulator of the immune response in vivo. Similarly the role of this novel protein in the cardiovascular system remains to be explored.

Despite the roles of Ereg and Nfkbiz (I κ B- ζ) in cardiomyocytes being currently unknown, studies in other systems indicate that these two genes are important regulators of the expression of IL-6 in cells of the immune system. Thus, bone marrow derived macrophages and dendritic cells (BMDMs and BMDCs) from Ereg $-/-$ mice exhibit reduced production of IL-6 levels following stimulation with the bacterial antigen peptidoglycan, indicating that Ereg has a key role in peptidoglycan-induced proinflammatory cytokine production in these cells (Sugiyama et al., 2005). As described above, I κ B- ζ is a positive regulator of NF- κ B transcriptional activities and this effect has been mapped to its N-terminus (Motoyama et al., 2005). Consistent with a positive effect on NF- κ B activities, overexpression of I κ B- ζ augments IL-6 production in response to LPS (Motoyama et al., 2005). In contrast, peritoneal macrophages from I κ B- ζ deficient mice exhibit severe reduction in the production levels of IL-6 following LPS and IL-1 β stimulation (Yamamoto et al., 2004). This suggests that I κ B- ζ is a key element in the regulation of proinflammatory cytokines such as IL-6 in response to LPS and IL-1 β .

Thus, both Nfkbiz and Ereg act as positive regulators of IL-6 expression in other systems. Taken together, these findings in other systems and the results from our studies suggest that ATF3 is probably involved in a negative regulatory network which targets the repression of genes such as IL-6 and LIF, and also of positive regulators of IL-6 such as Ereg and I κ B- ζ in cardiomyocytes following stimulation with ET-1. This suggests that ATF3 may be a modulator of the hypertrophic response. This effect may be mediated at least in part by preventing the expression of genes that could lead to eccentric hypertrophy such as IL-6 family members, thus probably favouring the development of concentric rather than eccentric hypertrophy following ET-1 stimulation.

Interestingly, my studies of the mRNA expression levels of CT-1 following knockdown of ATF3 indicate that the above generalization is not extensible to all gp130 cytokines. In contrast to the observed superinduction of IL-6 and LIF, knockdown of ATF3 protein resulted in significant downregulation of CT-1 mRNA levels following ET-1 stimulation (Figure 5.12). This effect and the observation that ET-1 alone had a negative effect on the expression levels of CT-1 suggest that, in contrast to IL-6 and LIF, ATF3 does not negatively regulate CT-1 expression, and possibly a negative regulator of CT-1 is expressed following ET-1 stimulation, an effect that is accentuated after ATF3 knockdown. This also suggests that CT-1 could be positively regulated by ATF3 and further experiments are required (e.g. ATF3 overexpression in cardiomyocytes) to clarify this issue. Although CT-1 mRNA levels are upregulated in cardiomyocytes, this effect has been ascribed to catecholamines acting through β -adrenergic receptors (Funamoto et al., 2000; Lowes et al., 2002) and in response to ANGII (Sano et al., 2000) but not by ET-1 stimulation, which is in agreement with our findings of no upregulation of the levels of

this gene in microarray studies from our laboratory (Clerk et al., 2005; Cullingford et al., manuscript submitted).

Chapter 6

Expression of Cyp1a1 and Aldh3a1 in response to MAPK cascade inhibitors or doxorubicin in cardiomyocytes

6.1 Introduction

6.1.1 Chemotherapeutic agents and cardiac toxicity

The anthracycline doxorubicin and related compounds such as daunorubicin and epirubicin, are among the most potent anticancer drugs available today. Their use in combination with other chemotherapeutic drugs, has proved to be very successful in inducing tumour remission and, in some cases, doxorubicin is considered to cure some tumours (e.g. acute lymphoblastic leukaemia, the commonest of all childhood tumours) (Horan et al., 2006). However, doxorubicin and related compounds have a high incidence of cardiac toxicity which usually limits their utility. Most chemotherapeutic drugs in current use target proliferating cells causing their death. However, because their use is systemic (as they are not specifically designed to target cancerous cells), they also affect proliferating cells throughout the body causing many side effects. These drugs also affect the heart and, whereas other tissues in the body have the capacity for regeneration and therefore are able to replace cells lost as a consequence of chemotherapy, the limited capacity for regeneration of cardiomyocytes renders the heart highly vulnerable to this damage. Cumulative doses of doxorubicin below 450 mg/m^2 (administered in bolus of 50 mg/m^2) which are usually considered “safe” and are the goal in most patients receiving doxorubicin could still result in delayed cardiotoxicity manifested as heart failure (Shan et al., 1996).

Anthracycline cardiotoxicity appears to be mediated at least in part through their increased generation of $\text{O}_2^{\cdot-}$, which is quickly converted to H_2O_2 (Dorr, 1996; Kumar et

al., 2002). This is consistent with the observation of increased rates of apoptosis in cardiomyocytes exposed to doxorubicin or daunorubicin secondary to the production of ROS (Sawyer et al. 1999; reviewed in Minotti et al., 2004). All of this makes therapy with doxorubicin a clinical challenge for modern medicine. Novel formulations and related compounds are under investigation, and there is a constant search for potent anticancer drugs with a better profile of adverse reactions.

Targeting of intracellular signalling cascades for the development of novel anticancer drugs is a rapidly evolving area of research in recent years. ERK1/2 are activated by MKK1/2, which, in turn, are activated by the Raf family of MKKKs (section 1.3.1.5). Although, in the heart, activation of the ERK1/2 cascade is associated with hypertrophic growth of cardiomyocytes and cardiac hypertrophy (section 1.3.1.6), in proliferating cells this cascade leads to cell growth, division and survival and has been implicated in the development of cancer (Sebolt-Leopold and Herrera, 2004). Several human tumours have activating mutations in Raf kinases and its upstream activator Ras (Malumbres and Barbacid, 2003; Wellbrock et al., 2004; Aviel-Ronen et al., 2006; Zebisch et al., 2006), and therefore activation of the ERK1/2 cascade is a common characteristic of several types of cancer, with ERK1/2 as the only known substrates for MKK1/2. Recently, B-Raf has been identified as a protooncogene implicated in up to 60% of human cancers (particularly melanoma and thyroid cancers) (Niculescu-Duvaz et al., 2006; Kohno and Pouyssegur, 2006). All of these features make this cascade unique and particularly attractive for the development of new anticancer drugs (Downward, 2003; Sebolt-Leopold et al., 2004; Kohno and Pouyssegur, 2006). There are several compounds that selectively inhibit MKK1/2. The compounds PD184352 (CI-1040), PD0325901 and ARRY-142883 have a pharmacological profile suitable for use in clinical settings and are currently in

different stages of phase I and phase II clinical oncology trials. Although the clinical trials completed so far with PD184352 (Rinehart et al., 2004; LoRusso et al., 2005) show that this drug is well tolerated and have good safety/efficacy profile in the short term, its potential toxicity in long term clinical trials remains unknown. Phase I clinical trials with PD0325901 and ARRY-142883 are still underway.

The PKB/Akt signalling pathway has an important role in regulating cell growth in several cell systems and has been proposed as a mediator of cardiac hypertrophy (1.3.2.2). The PKB/Akt pathway is also particularly implicated in cell survival and prevention of apoptosis in several systems including the heart (Tong et al., 2002; Clerk et al., 2003; Martelli et al., 2007; McCubrey et al., 2007). Activation of several components of this pathway correlates with the development of several forms of cancer, including breast cancer (Hutchinson et al., 2001) thyroid carcinoma (Ringel et al., 2001) and colon adenocarcinoma (Khaleghpour et al., 2004), and therefore the blockade of this pathway is actively being considered as an important target for the development of novel antineoplastic drugs (reviewed in Kim et al., 2005). As an example, the PI3K inhibitor LY294002 exerts antitumorigenic effects and induces apoptosis in human colon cancer cells both in vitro and in vivo (Semba et al., 2002).

RPTKs that respond to peptide growth factors such as EGF activate both the ERK1/2 and the PI3K-PKB/Akt pathways (section 1.4) (Schlessinger, 2000). Overstimulation of the EGF receptor (e.g. overexpression of ErbB2) or mutations that result in its constitutive activation are associated with certain types of cancer including up to 35% of breast cancers (Kauranemi and Kallioniemi, 2006). Therefore, RPTKs are themselves attractive

therapeutic targets for cancer. A recently developed anticancer drug is trastuzumab (Herceptin), a humanized antibody that targets ErbB2 and therefore inhibits EGF receptor signalling probably by preventing ErbB2 dimerization with for example ErbB1, or ErbB4 (section 1.3.2). The use of this drug particularly in association with anthracyclines has been recently reported to cause cardiotoxicity (Suter et al., 2004). Although this cardiotoxicity appears to be reversible in most cases, up to 5% of patients develop systolic cardiac dysfunction and ~1% develops heart failure (Hayes and Pickard, 2006). There is also one report indicating that up to 28% of patients experienced some degree of cardiotoxicity following one year of therapy with trastuzumab, as indicated by a decrease in ejection fraction below normal levels of 50%, decrease in 20 percentage points in the left ventricular ejection fraction compared with baseline, or signs and/or symptoms of heart failure (Guarneri et al., 2006). However, despite the high incidence of cardiotoxicity, discontinuation of therapy resulted in recovery in the functional or haemodynamic status in all but three patients, although there was one patient with cardiac related death during the study (Guarneri et al., 2006). Therefore, although therapy with this novel drug may cause some degree of cardiotoxicity in a high percentage of patients, this appears to be self limited and there is recovery following termination or discontinuation of therapy. However, long term effects of chronic therapy are still unknown.

Studies from our laboratory which used microarray analysis to dissect the role of ERK1/2 in the expression of genes resulting from ET-1 stimulation in cardiomyocytes (Kennedy et al., 2006) identified a subset of genes that are upregulated or downregulated by U0126, a specific inhibitor of MKK1/2 (Favata et al., 1998). Among the six genes which were upregulated, the genes for aldehyde dehydrogenase family 3 member a1 (Aldh3a1), two

members of the cytochrome P450 enzymes (Cyp1a1, Cyp1b1), and NAD(P)H dehydrogenase quinone 1 (NQO1), are classically induced by the transcription factor aryl hydrocarbon receptor (AhR) following binding to their promoter in xenobiotic response elements (XRE) in response to polycyclic aromatic hydrocarbons like dioxin and benzo[a]pyrene (Denison et al, 2002). This suggests that one effect of U0126 in cardiomyocytes is to stimulate XRE, also known as dioxin response element (DRE), in the promoters of these genes, potentially via the AhR. These genes are xenobiotic metabolizing enzymes, and Cyp1a1 induction is considered a marker gene of dioxin-induced toxicity. The consequences of their activation are strongly linked to carcinogenesis and developmental defects in several species including humans (Mandal, 2005). The significance of activation of this response in the heart has not yet been considered, but cardiotoxicity is a potential side effect. In this chapter the expression of the genes Cyp1a1 and Aldh3a1 was evaluated in cardiomyocytes exposed to ERK1/2 inhibitors, and these results extended to evaluation of their expression in response to the JNK cascade inhibitor SP600125, the p38-MAPK cascade inhibitor SB203580, and the PI3K inhibitor LY294002, as well as in response to doxorubicin.

6.2 Methods

6.2.1 SQ-PCR

Cardiomyocytes were exposed to 10 μ M U0126, 25 μ M PD98059, 10 μ M SP600125, 10 μ M SB203580, or 50 μ M LY294002 (for 0-4 h), or exposed to 5 μ M doxorubicin (0-12 hours). Total RNA was extracted and cDNA synthesized (Sections 2.3 and 2.5). SQ-PCR was performed (Section 2.6) to assess expression of Cyp1a1 and Aldh3a1 mRNA.

PCR reactions were performed in 50 μ l of 1X Buffer IV[®] (ABgene) containing 0.5–1.0 ng cDNA, 1U Taq polymerase, 0.2mM each of dATP, dCTP, dGTP and dTTP and 100 pmol forward and reverse primers [cytochrome P450 family 1 member 1 (Cyp1a1): forward 5'-CAA AGC CCA TGT TCC TGT TT-3', reverse 5'-AGC GGT TCA TGA CTG TAC CC-3', 237 bp product; aldehyde dehydrogenase family 3 member 1 (Ald3a1): forward 5'-GGC TGT GTA GGA GTT GCA AT-3', reverse 5'-ACC TAT GAC AAG GAC CAC AC-3', 245 bp product; glyceraldehyde 3' phosphate dehydrogenase (GAPDH): forward 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse 5'-TCC ACC ACC CTG TTG CTG TA-3', 452 bp product)]. For Cyp1a1 and Aldh3a1, samples were subjected to 27 cycles of denaturation (94 °C, 30 s), annealing (59 °C, 30 s) and extension (72 °C, 30 s). For of amplification GAPDH 22 cycles were performed. Bands were detected under UV light and sizes were estimated by comparison with a ϕ X174 RF DNA *Hae* III digest DNA ladder (ABgene) or a 100 bp ladder (Invitrogen).

6.3 Results

6.3.1 Expression of Cyp1a1 and Aldh3a1 with ERK1/2 inhibitors U0126 and PD98059 in cardiomyocytes

Drugs which selectively block the MKK1/2 signalling in the ERK1/2 cascade [e.g. PD184352 (CI-1040), PD0325901 and ARRY-142883] are being evaluated for use in clinical oncology. The completed phase I-II clinical trials so far indicate that PD184352 is generally well tolerated with minor side effects (Rinehart et al., 2004; LoRusso et al., 2005). However, their long term safety and efficacy in patients treated for cancer remains to be established. U0126 is structurally related to PD184352 and PD0325901 (Kohno et al., 2006). According to microarray data (Kennedy et al., 2006), U0126, a specific inhibitor of the ERK1/2 cascade (Favata et al., 1998; Davies et al., 2000) [although it also inhibits the ERK5 cascade at higher concentrations (Mody et al., 2001)], induces the expression of stress-responsive genes, four of which are classically induced by the AhR in response to polycyclic aromatic hydrocarbons like dioxin or Benzo[a]pyrene stimulation. The effect of 10 μ M U0126 on the expression of Cyp1a1 and Aldh3a1 mRNA in cardiomyocytes was confirmed by SQ-PCR. Both Cyp1a1 and Aldh3a1 mRNAs were expressed at low levels under basal conditions, but were highly induced after incubation of the cells with U0126 up to 4 h (Figure 6.1A and B). To determine whether an alternative, structurally related inhibitor of the MKK1/2 has a similar effect, cardiomyocytes were exposed to PD98059 (25 μ M). Cyp1a1 and Aldh3a1 were also induced by PD98059 over 4 h (Figure 6.1C and D), although the response was observed from 1 h, somewhat earlier than with U0126 (Figure 6.1A and B).

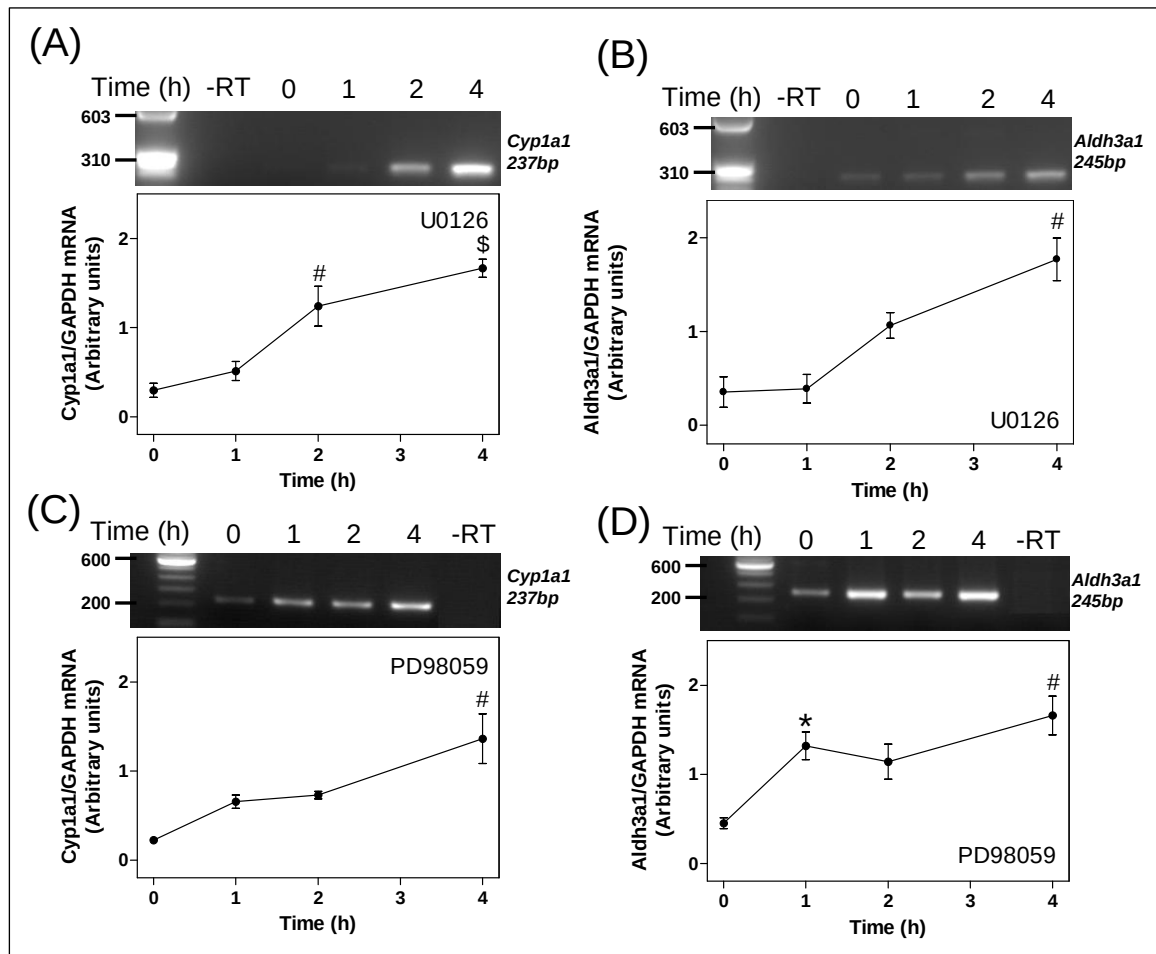


Figure 6.1 Upregulation of Cyp1a1 and Aldh3a1 by ERK1/2 inhibitors in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to 10 μ M U0126 (A, B) or 25 μ M PD98059 (C, D), for the indicated times. The expression of Cyp1a1 and Aldh3a1 mRNA was analysed by SQ-PCR. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. (* $p < 0.05$; # $p < 0.01$; \$ $p < 0.001$, one way ANOVA with Tukey post test).

6.3.2 Doxorubicin and inhibitors of JNKs (SP600125) or p38-MAPKs (SB203580), but not the PI3K inhibitor LY294002, induce the expression of Cyp1a1 and Aldh3a1 in cardiomyocytes.

Dioxin and benzo[a]pyrene (Figure 6.2A and B) possess planar hydrocarbon rings (halogenated in the case of dioxin) which are required for interaction with the AhR (Waller et al., 1995), and it is possible that the planar hydrocarbon rings in U0126 and related compounds (Figure 6.2D-F) interact directly with the AhR in a similar way. Anthracyclines such as doxorubicin also possess planar hydrocarbon rings (Figure 6.2C). The effects of doxorubicin on the expression of Cyp1a1 and Aldh3a1 mRNA in cardiomyocytes were therefore investigated. Doxorubicin (5 μ M) indeed induced the expression of Cyp1a1 and Aldh3a1 (Figure 6.3), although this effect was delayed compared with ERK1/2 inhibitors particularly for Cyp1a1 (Figure 6.3A).

Inhibitors of JNKs and p38-MAPKs are currently being developed for use in the treatment of chronic inflammatory and allergic diseases (Duan et al., 2006; O'Neill., 2006). In experimental settings, the compounds SP600125 (an inhibitor of JNKs) and SB203580 (which inhibits p38-MAPK α/β) are commonly used to delineate the involvement of these cascades in cellular processes. These compounds also possess planar hydrocarbon rings (Figure 6.2G and H), therefore the effects of these drugs on Cyp1a1 and Aldh3a1 expression were assessed. Both Cyp1a1 and Aldh3a1 were induced in cardiomyocytes incubated with either SP600125 or SB203580 (Figure 6.4).

The PI3K pathway is clearly cytoprotective in cardiomyocytes as in other cells (Tong et al., 2002; Clerk et al., 2003; Martelli et al., 2007; McCubrey et al., 2007) and is an alternative target for chemotherapy (Faivre et al., 2006). LY294002 is a selective inhibitor of PI3K (Vlahos et al., 1994) and, like U0126, PD98059, SB203580 and SP600125, it possesses planar hydrocarbon rings (Figure 6.2I). However, in contrast to the effects of inhibitors of the MAPK cascades (see preceding sections), treatment of cardiomyocytes with LY294002 (50 μ M) had a tendency to downregulate the basal levels of Cyp1a1 and Aldh3a1 mRNA expression although this was not statistically significant (Figure 6.5).

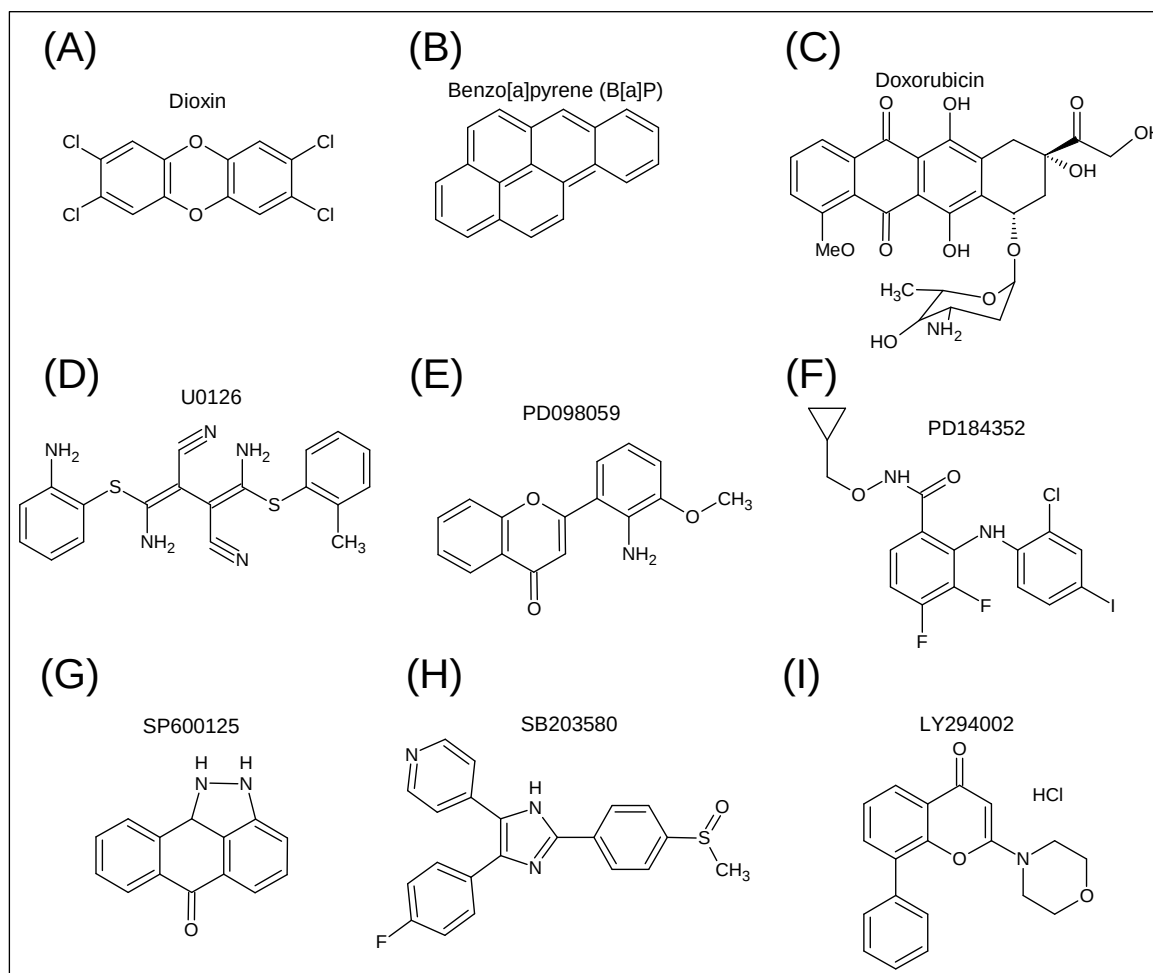


Figure 6.2 Chemical structures. Genotoxic agents 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin) (A) and benzo[a]pyrene (B); chemotherapeutic agent doxorubicin (C). MAPK inhibitors: ERK inhibitors U0126 (D), PD98059 (E) and PD184352; JNK inhibitor SP600125 (G), p38-MAPK inhibitor SB203580 (H). PI3K inhibitor LY294002 (I).

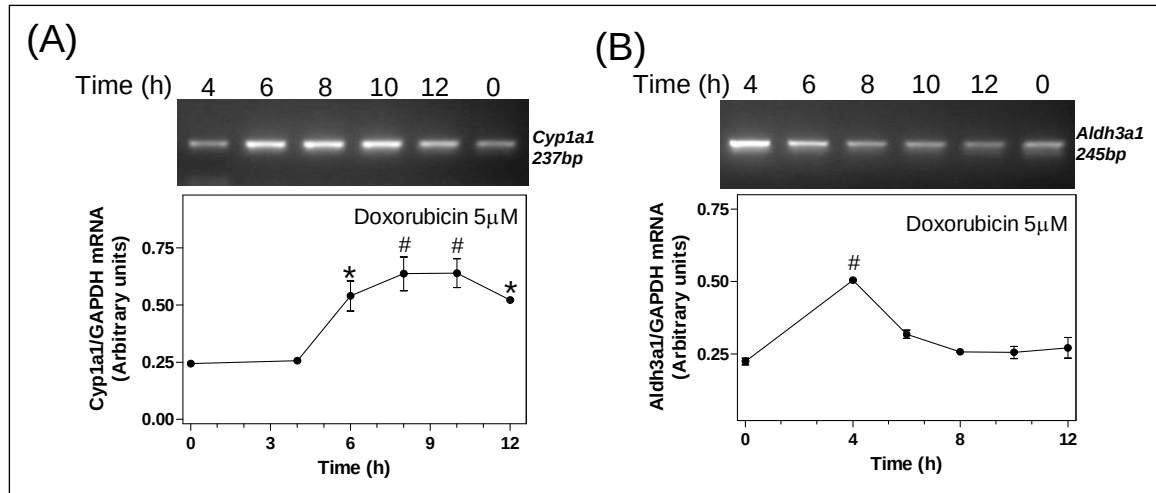


Figure 6.3 Upregulation of Cyp1a1 and Aldh3a1 by doxorubicin in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to 5 μ M doxorubicin, for the indicated times. The expression of Cyp1a1 (A) and Aldh3a1 (B) mRNA was analysed by SQ-PCR. Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations. (* $p < 0.05$; # $p < 0.01$, one way ANOVA with Tukey post test).

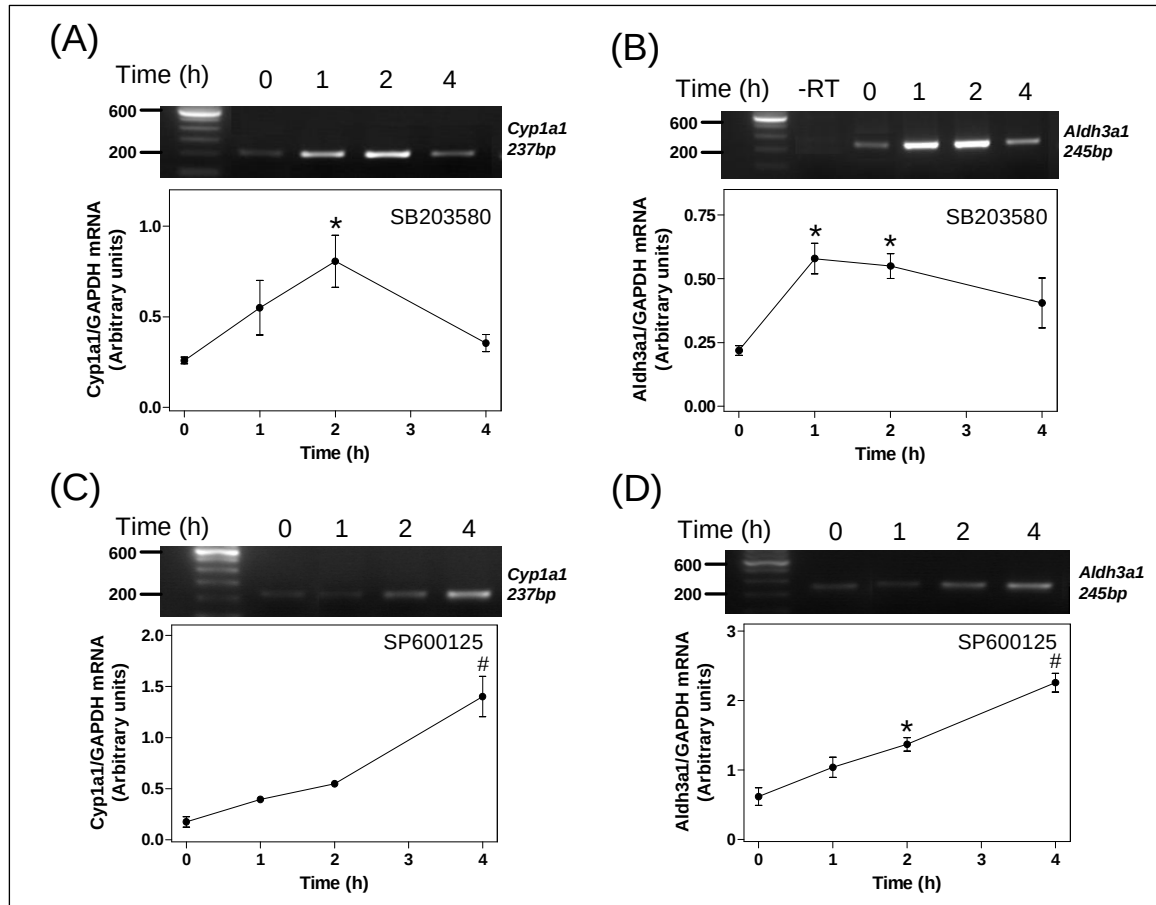


Figure 6.4 Upregulation of Cyp1a1 and Aldh3a1 by inhibitors of JNK and p38-MAPK α/β in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to (A-B) 10 μ M SP600125, or (C-D) 10 μ M SB203580 for the indicated times. The expression of Cyp1a1 and Aldh3a1 mRNA was analysed by SQ-PCR. Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for two independent cardiomyocyte preparations. (* $p < 0.05$; # $p < 0.01$, one way ANOVA with Tukey post test)

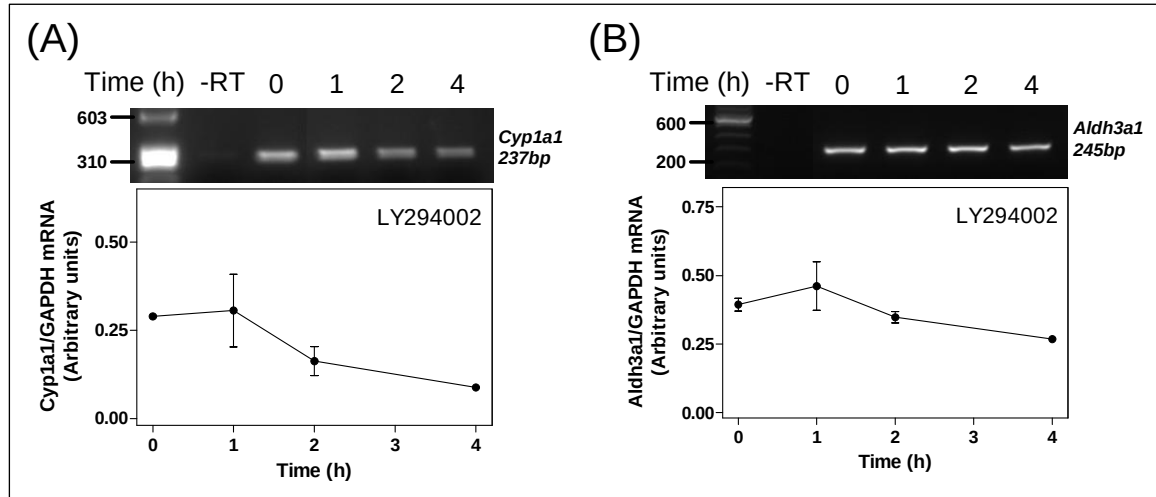


Figure 6.5 Downregulation of Cyp1a1 and Aldh3a1 by LY294002 in cardiomyocytes. Cardiomyocytes were unstimulated (controls, zero time) or exposed to 50 μ M LY294002, for the indicated times. The expression of Cyp1a1 and Aldh3a1 mRNA was analysed by SQ-PCR. Control PCR reactions were carried out in the absence of reverse transcriptase (-RT). Densitometric analysis of the data is provided in the lower panels and expressed relative to controls after normalization with the housekeeping gene GAPDH. Results are means $\pm$ S.E.M. for three independent cardiomyocyte preparations.

6.4 Discussion

6.4.1 Induction of Cyp1a1 and Aldh3a1 by ERK1/2 inhibitors in cardiomyocytes

The genes *Cyp1a1* and *Aldh3a1* which are classically induced in response to AhR were expressed at low levels in cardiomyocytes under basal conditions, but there was an increase in expression of both genes in response to U0126 (Figure 6.1A and B). The classical response to AhR activation by dioxins and other AhR ligands is very often evaluated by the induction of the prototypical gene *Cyp1a1*, and therefore our observations so far are in agreement with what has been previously reported in the literature. For example, in rat hepatocytes and human hepatoma cells, exposure to U0126 leads to significant upregulation in the levels of mRNA, protein abundance, as well as increased enzymatic activity of *Cyp1a1*, an effect which appears to be mediated by activation of AhR (Andreux et al., 2004). Our data therefore suggests that, in cardiomyocytes as in other cells, U0126 stimulates the AhR which then binds to the XRE in the promoter of these genes. This is consistent with the finding that in hepatocytes U0126 and related ERK1/2 inhibitors (PD98059 or PD184352) increase *Cyp1a1* expression, an effect that is probably dependent on inhibition of the basal ERK1/2 signalling which triggers the response secondary to AhR stabilization (Chen et al., 2005). This is also in agreement with the finding that PD98059 also induced the expression of *Cyp1a1* and *Aldh3a1* in cardiomyocytes (Figure 6.1C and D), although this effect was more rapid than for U0126 (Figure 6.1A and B).

However, although the toxic effects of dioxins are well recognized and dioxins promote defects in the developing heart (Goldstone and Stegman, 2006), the significance of the

dioxin response in the adult heart is not obvious. Thus, in some studies, dioxins do not significantly activate the Cyp1a1 promoter in the adult heart (Lund et al., 2003; Galijatovic et al., 2004), suggesting that the AhR itself may not be expressed in the developed heart. Interestingly, deletion of the AhR in mice results in cardiac hypertrophy and fibrosis, but this effect appears to be mediated by secondary elevation of the plasma levels of ANGII and ET-1 (Lund et al., 2003, 2005, 2006). Therefore, it is also possible that the effects of U0126 and or PD98059 on cardiomyocyte AhR-responsive genes are not mediated directly by AhR itself, but by a related factor (Saatcioglu et al., 1990; Luo et al., 1997).

6.4.2 Induction of Cyp1a1 and Aldh3a1 by JNK and p38-MAPK α/β inhibitors in cardiomyocytes

The evaluation of the effects of other MAPK inhibitors on AhR-dependent genes indicated that SP600125 an inhibitor of JNK and SB203580 a commonly used selective inhibitor of p38-MAPK α/β also increased the expression of Cyp1a1 and Aldh3a1 mRNAs (Figure 6.3). In contrast, the PI3K inhibitor LY294002 caused a slight downregulation of the Cyp1a1 and Aldh3a1 mRNAs (Figure 6.5). This is in agreement with previous observations showing that LY294002 suppresses the upregulation of Cyp1a1 in response to dioxin, though potentially independent of any inhibition of PI3K (Guo et al., 2000). Additionally, our results are in contrast with previous observations indicating that SP600125 is an antagonist of the AhR (Joiackim et al., 2003). With our current data, we cannot rule out or confirm the possibility that activation or blockade of the AhR pathway is involved in these responses to MAPK inhibitors and LY294002. Further experiments are required to clarify the involvement of AhR in these responses.

6.4.3 Induction of Cyp1a1 and Aldh3a1 by doxorubicin in cardiomyocytes

Doxorubicin, which (like the protein kinase inhibitors used in this study) possesses planar hydrocarbon rings (Figure 6.2C), also induced the expression of Cyp1a1 and Aldh3a1 (Figure 6.3) although the effect was delayed compared to U0126. To our knowledge, this is the first time that upregulation of these genes in response to doxorubicin has been identified. Doxorubicin is one of the most potent anticancer drugs available. However, a characteristic cardiomyopathy is a frequent side effect with its use in the clinical settings. One of the mechanisms implicated in this cardiotoxicity is the production of ROS and secondary apoptosis of cardiomyocytes (Wu et al., 2002; Minotti et al., 2004). The data presented here may represent an additional mechanism of toxicity in the pathophysiology of doxorubicin induced cardiomyopathy. Cyp1a1 is a monooxidase that utilizes oxygen (via a NAD(P)H-dependent electron transport chain) to oxidize xenobiotics. Importantly, like all cytochrome P450 enzymes the coupling of the reaction to molecular oxygen leads to generation of ROS and therefore to increased oxidative stress in the cell (Zangar et al., 2004). This is consistent with the sustained increase in oxidative stress that has been observed in mice following the intraperitoneal injection of dioxin (Shertzer et al., 1998). Since cardiomyocytes are highly vulnerable to increased oxidative stress (section 1.7), increased expression of Cyp1a1 associated to increased production of ROS might be expected to be detrimental to cardiomyocytes. Given that doxorubicin induces the expression of Cyp1a1, this could also contribute to the increase in oxidative stress and apoptosis in cardiomyocytes exposed to this drug (Minotti et al., 2004).

The following observations also support the hypothesis that induction of Cyp1a1 and Aldh3a1 may represent additional mechanisms through which doxorubicin cause

cardiotoxicity. Resveratrol, an important antioxidant found in the diet and wines (Fremont et al., 2000), has potential beneficial effects as an adjuvant in anticancer therapy (Rezk et al., 2006). The mechanisms by which this compound mediates its effects are not completely understood, but the induction of cellular antioxidants and phase 2 enzymes has been proposed in experimental studies using H9c2 cardiomyocytes (Cao et al., 2004). Interestingly, this compound is also a known AhR ligand and antagonist (Casper et al., 1999; Ciolino et al., 1999), therefore part of its potential beneficial effects could be the negative regulation of AhR-dependent genes in the heart. Whether the effects of induction of Cyp1a1 and Aldh3a1 genes by MAPK inhibitors and doxorubicin as shown herein are detrimental to the heart remain unknown and further experiments are required to clarify this. Another consideration is that several of the protein kinase inhibitors (and related compounds) used in the studies presented herein are being considered for therapeutic use in cancer, chronic inflammatory processes and cardiac diseases, and possible adverse effects could be envisaged since they upregulate AhR-dependent genes.

Chapter 7

Summary of results and general discussion

7.1 Summary of results

7.1. 1. Expression of ATF3 in response to ET-1 or H₂O₂

The mRNA abundance and protein levels of the transcription factor ATF3 were potently and transiently induced in response to ET-1 or H₂O₂ in cardiomyocytes. Upregulation of ATF3 mRNA by ET-1 did not require *de novo* protein synthesis indicating that ATF3 is an IEG. Upregulation of ATF3 mRNA and protein was inhibited by U0126 an inhibitor of the ERK1/2 cascade indicating that its upregulation was mediated primarily through the ERK1/2 cascade. However, ATF3 mRNA expression also required an input from the JNK cascade following ET-1 stimulation.

7.1. 2. Expression of IL-6 in response to ET-1 or H₂O₂

Stimuli such as ET-1 or H₂O₂ caused transient upregulation of the mRNA and protein levels of the cytokine, IL-6. IL-6 protein was secreted by cardiac myocytes shortly after its expression in response to both agonists. IL-6 mRNA induction in response to ET-1 did not require *de novo* protein synthesis and this effect was blocked by the ERK1/2 cascade inhibitor U0126. IL-6 is therefore another IEG that is induced in cardiomyocytes through the ERK1/2 cascade in response to ET-1.

7.1.3. ATF3 binds to the promoter of IL-6 following ET-1 or H₂O₂ stimulation

A bioinformatics approach showed that the rat IL-6 gene possesses an ATF/CRE binding consensus sequence in its promoter region, and ChIP assays demonstrated that ATF3

binding to the promoter of this gene increased following ET-1 or H₂O₂ stimulation. Interestingly, peak levels of ATF3 protein have an inverse relationship with respect to the mRNA levels of IL-6 suggesting that ATF3 may be involved in a negative regulatory feedback repressing IL-6 transcription following ET-1 or H₂O₂ stimulation (Gilchrist et al., 2006).

7.1.4 Role of ATF3 in the regulation of IL-6 and other IEGs following ET-1 stimulation of cardiomyocytes

Using an adenovirus encoding the ATF3 antisense sequence (ATF3-A/S), mRNA and protein levels of ATF3 were successfully and specifically downregulated following ET-1 stimulation. Under these conditions, IL-6 mRNA abundance was potently superinduced indicating that the effect of ATF3 when bound to the promoter of IL-6 was the repression of IL-6 transcription. Similarly, the mRNA abundances of the genes LIF, Ereg and Nfkbiz were also superinduced by knockdown of ATF3 protein, indicating that these immediate early genes are also targets of ATF3 repression in cardiomyocytes.

7.1.5 Induction of Cyp1a1 and Aldh3a1 by MAPK inhibitors and doxorubicin in cardiomyocytes

We have shown that the chemotherapeutic agent, doxorubicin, induces the expression of the xenobiotic enzymes Cyp1a1 and Aldh3a1 in cardiomyocytes, two of the genes regulated by the AhR transcription factor in response to toxic agents such as dioxin and related carcinogenic compounds. Similarly, the ERK1/2 cascade inhibitors U0126 and PD98059 as well as the JNK inhibitor, SP600125, and p38-MAPK α/β inhibitor

SB203580, as well as doxorubicin, all induced the expression of these same genes. In contrast, the PI3K inhibitor, LY294002, did not induce this response but resulted in slightly reduced expression levels of these genes. Whether the expression of these genes in response to doxorubicin or inhibitors of MAPK cascades results in cardiomyocyte toxicity remains to be determined.

7.2 General discussion

The studies presented in this thesis have examined the expression of ATF3 and IL-6 genes in response to ET-1 or H₂O₂ in cardiomyocytes. The effect of ATF3 on the mRNA expression levels of IL-6 and other IEGs that are transiently induced upon ET-1 stimulation were also evaluated. All studies were carried out in cultured cardiomyocytes, a model system that allows the evaluation of changes in gene expression specifically in the contractile cells of the heart, as opposed to whole hearts in which intermixed cell content may obscure the response of the cardiomyocytes.

7.2.1 Expression of ATF3 in cardiomyocytes

In Chapter 3, the expression of ATF3 in response to ET-1 or H₂O₂ was evaluated. The potent and transient induction of its mRNA correlated with its translation into protein with both agonists. ATF3 was also identified as an IEG upregulated by ET-1 whose expression is highly dependent on the ERK1/2 cascade with some input received from the JNK cascade. This effect of ERK1/2 and JNK on the expression of ATF3 is in agreement with some but not all previous reports in other cell systems. Thus, induction of ATF3 expression by the non-steroidal anti-inflammatory drug sulindac in colon cancer cells is

mediated through the increase in the transcriptional activity of Egr-1 on the ATF3 promoter (Bottone et al., 2005). This effect was inhibited by overexpression of a dominant negative ERK1 (dn-ERK1) and by the ERK1/2 cascade inhibitor PD98059, indicating the importance of this pathway in regulating ATF3 expression in this system (Bottone et al., 2005). In endothelial cells, JNKs appear to be involved in the upregulation of ATF3. Thus, in human umbilical vein endothelial cells (HUVECs), ATF3 expression in response to homocysteine is preceded by a sustained increase in JNK phosphorylation. Treatment of HUVEC cells with the p38-MAPK cascade inhibitor SB203580 or the ERK1/2 cascade inhibitor PD98059 has no effect on the expression levels of ATF3. Using reporter assays with the ATF3 1850 bp region of the 5' flanking sequence fused to the luciferase reporter gene, luciferase was induced 2-fold following homocysteine stimulation in HUVEC cells. Incubation of the cells with expression plasmids containing dominant negative mutants of the upstream MKKs of JNK (dn-MKK4 and dn-MKK7) results in complete inhibition of ATF3 luciferase activity, whereas pre-treatment of the cells with SB203580 or PD98059 has no effect on the luciferase activity (Cai et al., 2000). This suggests that ATF3 expression following stimulation of HUVEC cells with homocysteine is mediated through the JNK and not the ERK1/2 or p38-MAPK cascades.

More recently, a bidirectional role for JNKs and ERK1/2 on ATF3 expression in endothelial cells was described in response to TNF α . Thus, in HUVECs ATF3 expression is increased by TNF α and this is abolished by dn-MKK7 expression plasmids, whereas a constitutively-active MKK7 (ca-MKK7) increases expression of ATF3 (Inoue et al., 2004). A dominant negative mutant of the upstream MKK of p38-MAPK (dn-MKK6) does not cause any reduction in the expression of ATF3 following exposure to TNF α , and ca-MKK6 does not cause any induction of ATF3. Interestingly, a dn-mutant of MKK1

(one of the upstream MKKs for ERK1/2) causes a significant increase in ATF3 expression whereas a ca-MKK1 results in reduced expression of ATF3 following TNF α stimulation. Overall these results suggest that ATF3 expression following TNF α stimulation in HUVECs is induced through the JNK cascade, with the ERK1/2 cascade having an opposite effect and downregulating ATF3 expression (Inoue et al., 2004). This was also further confirmed using the ERK1/2 cascade inhibitors PD98059 and U0126, which result in enhanced expression of ATF3 following treatment of HUVECs with TNF α (Inoue et al., 2004).

Recently, one study addressed in some detail the involvement of the MAPKs cascades in the expression of ATF3 in several cell lines using the agonists anisomycin (which was used as a stress model because of its ability to increase the activity of all MAPKs), IL-1 β , TNF α and H₂O₂. This study suggests that ATF3 expression at the mRNA and protein levels is regulated by the p38-MAPK signalling cascade (Lu et al., 2007). Thus, anisomycin-induced expression of ATF3 is not decreased by the ERK1/2 cascade inhibitor PD98059, and there is no increased expression of ATF3 with ca-ERK (resulting from expression of a fusion protein of ERK2 and its upstream MAPK kinase MKK1), indicating that the ERK1/2 cascade is neither necessary nor sufficient to cause ATF3 expression (Lu et al., 2007). Similarly, inhibition of the JNK pathway with the cell permeable peptide JNK-inhibitor (JNKi), which contains the minimal inhibitory region of the human protein JNK interacting protein (JIP) and is linked to a ten amino acid sequence of the HIV-transactivator of transcription (HIV-Tat) sequence which allows rapid cell and nuclear penetration (Schwarze et al., 1999; Bonny et al., 2001), does not decrease the expression levels of ATF3 following anisomycin treatment. In addition, neither JNK1 nor ca-MKK7 expression plasmids result in increased ATF3 gene

expression. This suggests that, as in the case of the ERK1/2 cascade, JNK is neither necessary nor sufficient to cause ATF3 gene expression (Lu et al., 2007). In contrast, preincubation with the p38-MAPK inhibitor, SB203580, results in decreased expression of ATF3 in response to anisomycin as well as to IL-1 β , TNF α and H₂O₂, and similar results were also found with the use of dn-MKK6 expression plasmids. Furthermore, ca-MKK6 causes increased ATF3 gene expression. Taken together these studies indicate that the p38-MAPK cascade is both necessary and sufficient to cause ATF3 expression in response to stress and cytokines in several cell lines (Lu et al., 2007). Although some of the above studies are in agreement with our finding of the involvement of the ERK1/2 and JNK cascades in the expression of ATF3 following stimulation of cardiomyocytes with ET-1, the divergent results found by others using other cell systems and agonists highlight the importance of considering the dependence on the context for the actions ATF3 which is also manifested in the signalling pathways controlling its expression.

The fact that ET-1 or H₂O₂ are capable of potently inducing expression of the ATF3 transcription factor, suggests that it may play an important role in cardiomyocyte hypertrophy following ET-1 stimulation and/or in apoptosis following oxidative stress. Previous studies have suggested that ATF3 may indeed play a cytoprotective role following exposure of cardiomyocytes to the cardiotoxic anticancer drug, doxorubicin (Nobori et al., 2002), a known pro-apoptotic agent in cardiomyocytes and other cell types probably because it induces the formation of ROS (Kumar et al., 2002; Minotti et al., 2004). Induction of ATF3 mRNA and protein in cardiomyocytes by doxorubicin is transient suggesting a role for ATF3 in response to doxorubicin (Nobori et al., 2002). In addition, adenoviral-mediated overexpression of ATF3 correlates with reduced rates of cardiomyocyte apoptosis, indicating that the role of ATF3 under these conditions is

cytoprotective (Nobori et al., 2002). My results on the expression of ATF3 following doxorubicin stimulation contrast for the most part with those previously reported (Nobori et al., 2002), with significant downregulation observed following doxorubicin exposure (Figures 3.10 and 3.11). The reasons for these contrasting results are not evident. However, the methodology that I employed to evaluate mRNA levels (SQ-PCR), differs from that of Nobori et al., (2002), who employed Northern-blot analysis. In addition, I used 3 μ M doxorubicin whereas their study used 1 μ M. My studies of the concentration-dependency of ATF3 expression did not find significant mRNA upregulation with 1 μ M and the greatest effect was observed with 3 μ M. However, this effect did not reach statistical significance either (Figure 3.10). Nevertheless, assuming technical difficulties prevented me from replicating the previously described upregulation of ATF3 with doxorubicin, the data of Nobori et al., (2002) suggest that ATF3 induction in response to oxidative stress in the form of doxorubicin-mediated induction of reactive oxygen species is cardioprotective. In a similar way, our results using H₂O₂ indicate a potent upregulation of this transcription factor under conditions of increased ROS production and therefore ATF3 may play a similar cytoprotective here. The role of ATF3 upregulation following stimulation of cardiomyocytes with ET-1 or H₂O₂ remains to be established with further studies (see below sections 7.2.2 and 7.2.3).

7.2.2 Expression of IL-6 in cardiomyocytes

In Chapter 4, I evaluated the expression of IL-6. My results indicate that mRNA expression of this gene is statistically significant and transient following ET-1 or H₂O₂ stimulation. I was also able to demonstrate that IL-6 is an IEG upregulated in response to ET-1 through the ERK1/2 cascade. IL-6 protein is synthesized by cardiomyocytes after

translation of mRNA into protein. The studies of Chapter 4 also indicate that IL-6 protein is secreted by cardiomyocytes, but this occurs within the first hour following stimulation with ET-1 or H₂O₂. It was then shown that the IL-6 promoter region contains an ATF/CRE consensus binding site, and using ChIP assays, I was able to demonstrate that ATF3 binding to the IL-6 promoter increases following 1 h stimulation with ET-1 or after 2 h stimulation with H₂O₂. It was not clear from these studies whether ATF3 was activating or repressing IL-6 transcription, a versatility that has been previously described for ATF3 actions (section 3.1.1.2). However, evaluation of the expression levels of ATF3 protein in relation to IL-6 mRNA demonstrated an inverse relationship between the peak of expression of ATF3 protein and the return to near basal levels of IL-6 mRNA following transient induction by ET-1 or H₂O₂. This suggested that ATF3 may be involved in a negative regulatory feedback and therefore is involved in downregulation of IL-6 following its induction by these agonists. This was consistent with previous studies that have shown such a repressive effect of ATF3 on IL-6 expression in other systems (Gilchrist et al., 2006). Thus, IL-6 expression was induced in monocytes exposed to bacterial LPS and ATF3 was found to be bound the IL-6 promoter using ChIP assays. Similarly, IL-6 expression was superinduced in monocytes derived from ATF3 ^{-/-} mice exposed to LPS (Gilchrist et al., 2006).

7.2.3 ATF3 is a negative regulator of IL-6 and several other IEGs that are induced by ET-1 in cardiomyocytes

Experiments were then performed to elucidate the role of ATF3 in IL-6 regulation following ET-1 stimulation of cardiomyocytes. In Chapter 5, I was able to successfully downregulate the ET-1-induced expression of ATF3 mRNA and protein using adenoviral-

mediated overexpression of the ATF3 antisense targeting the full coding region. Similarly it was shown that under conditions of ATF3 protein knockdown, IL-6 mRNA levels were significantly upregulated (superinduced) following stimulation of cardiomyocytes with ET-1, thus confirming that ATF3 is a repressor of IL-6 when bound to the IL-6 promoter in response to ET-1. It is likely that this regulatory role of ATF3 over the expression levels of IL-6 in cardiomyocytes could prove to be very important in vivo as suggested by studies in other systems. Thus, ATF3 null mice that received an intraperitoneal injection of LPS exhibited more than tenfold increase in circulating levels of IL-6 compared with wild-type animals and this was accompanied by increased mortality (100 % in null mice, compared with 0% in wild-type animals after 24 h) secondary to severe inflammation (endotoxic shock) (Gilchrist et al., 2006). Similarly, and in support of a pathological role for increased levels of IL-6 in the heart, a recent study showed that transgenic mice with cardiospecific overexpression of ET-1 in the postnatal period leads to the development of a type of cardiomyopathy characterized by an inflammatory infiltrate and increased expression of cytokines including IL-6 expression levels in the heart. This suggests that unregulated expression of ET-1 which also induces increased expression of IL-6 (among other cytokines) in the heart is clearly of pathological significance (Yang et al., 2004).

It is also important to consider other potential pathological effects of IL-6 not in relation to the inflammatory actions of this cytokine. As a whole, the gp130 cytokines have been consistently found to be involved in myocyte elongation in vitro, which results in eccentric hypertrophy of the heart in vivo due to the circumferential disposition of cardiomyocytes in the heart. Thus, IL-6, LIF and CT-1 are all implicated in the development of cardiomyocyte elongation in vitro (Wollert et al., 1996). Therefore, since the discovery of CT-1 this cytokine, as well as IL-6 and LIF, has been implicated in the

development of dilatation of the heart and the pathogenesis of heart failure (Hunter and Chien, 1999). Consistent with this, rats subjected to intermittent hypoxia for 4 weeks develop concentric LV hypertrophy associated with increased mRNA and protein levels of TNF α and IGF-1, as well as phosphorylated p38-MAPK and STAT-1 and STAT3 compared with normoxic controls. Interestingly, more prolonged intermittent hypoxia (8 weeks) leads to the development of LV eccentric hypertrophy and, in addition to the above changes in gene expression and signalling activation, there is also increased expression of IL-6 mRNA and protein with activation of the MKK5-ERK5 pathway (Chen et al., 2007). This suggests that intermittent hypoxia for 4 weeks may cause a redox imbalance that will initially lead to the development of concentric cardiac hypertrophy, but after more prolonged hypoxia this redox imbalance may also lead to the development of eccentric hypertrophy and left ventricular dilatation following the activation of the IL-6-gp-130 signalling system through the MKK5-ERK5 pathway. This is consistent with the observation that the ERK5 pathway is activated by IL-6 family members, and that this leads in vitro to the development of cardiomyocyte elongation, which is replicated by adenoviral-mediated overexpression of MKK5 in cardiomyocytes (Nicol et al., 2001). In fact, transgenic mice with cardiac overexpression of activated MKK5 develop dilated cardiomyopathy secondary to eccentric hypertrophy as the main morphological characteristic (Nicol et al., 2001).

By evaluating the mRNA expression of LIF, Ereg and Nfkbiz (also known as I κ B- ζ) under conditions of ATF3 protein knockdown, I was able to identify these genes as additional IEGs that were significantly superinduced (and by implication targets of ATF3 repression) following ET-1 stimulation of cardiomyocytes. Although the actions of Ereg and I κ B- ζ remain to be determined in cardiomyocytes, these genes have been identified as

important regulators of IL-6 secretion in other systems. Thus, peritoneal macrophages from I κ B- ζ deficient mice exhibited severe reduction in the production levels of IL-6 following LPS and IL-1 β stimulation (Yamamoto et al., 2004), suggesting that I κ B- ζ is a key element in the regulation of proinflammatory cytokines such as IL-6 in response to LPS and IL-1 β . Similarly, bone marrow derived macrophages and dendritic cells (BMDM and BMDC) from Ereg-null mice exhibit reduced IL-6 expression levels following stimulation with the bacterial antigen peptidoglycan compared to those from wild-type animals (Sugiyama et al., 2005), suggesting that Ereg plays an important role in peptidoglycan-induced expression of IL-6 in these cells. Taken together, our data indicate that ATF3 may be involved in a regulatory network that represses the expression of IL-6 and LIF and other positive regulators of IL-6 expression such as Ereg and I κ B- ζ . This suggests that ATF3 may be a modulator of hypertrophy which could be involved in the fine tuning of the response and probably does so in a way that prevents the expression of genes that could lead to a maladaptative form of cardiac hypertrophy (eccentric hypertrophy), and therefore probably favours the development of concentric hypertrophy following ET-1 stimulation. This however is not a generalized effect of ATF3 on all members of the IL-6 family as our data indicate that CT-1, which is not induced by ET-1 in our microarray studies, is not a target of ATF3 regulation in the cardiomyocyte at least following ET-1 stimulation.

7.2.4 Molecular mechanisms of ATF3 functions

Recent studies of the molecular mechanisms of ATF3 action in several cell systems indicate that, in addition to binding to ATF/CRE consensus sites (5'-TGACGTCA-3') and activator protein-1 consensus sequences (5'-TGAGTCA-3') on target genes (Hai et

al., 1999) (section 3.1.1.2), ATF3 also recruits HDACs which results in the increased deacetylation of histones and therefore in repression of gene expression (section 1.5.1.1). ATF3 is involved in a negative feedback loop, downregulating IL-6 and IL-12b and thus modulating the inflammatory response secondary to activation of Toll-like receptors in vitro and in vivo (Gilchrist et al., 2006). In this model, ATF3 inhibits IL-6 and IL-12b transcription by directly recruiting histone deacetylase 1 (HDAC1), a class II HDAC, thus inducing histone deacetylation and thereby restricting access of Rel transcription factors to the promoters of IL-6 and IL-12b (Gilchrist et al., 2006). It is likely that ATF3 could lead to transcriptional repression of IL-6, LIF, Ereg and Nfkbiz at least in part through a mechanism involving the recruitment of HDAC to their promoters. However this should be confirmed through further experiments.

7.2.5 Induction of Cyp1a1 and Aldh3a1 by MAPK inhibitors or doxorubicin in cardiomyocytes

In Chapter 6, I showed that several MAPK cascade inhibitors or doxorubicin were able to induce the expression of the xenobiotic metabolizing enzymes Cyp1a1 and Aldh3a1, which are classically induced in response to AhR, thus confirming and expanding the observations made by previous microarray studies from our laboratory (Kennedy et al., 2006). The classical response to AhR activation by dioxins and other AhR ligands is very often evaluated by the induction of the prototypical gene Cyp1a1, and therefore our observations are in agreement with what has been previously reported in other cell systems. For example, in rat hepatocytes and human hepatoma cells, exposure to U0126 leads to significant upregulation in the levels of mRNA, protein abundance, as well as increased enzymatic activity of Cyp1a1, an effect which appears to be mediated by activation of AhR (Andrieux et al., 2004). Similarly, in hepatocytes U0126 and related

ERK1/2 cascade inhibitors (PD98059 or PD184352) increase Cyp1a1 expression, an effect that is probably dependent on inhibition of the basal ERK1/2 signalling which triggers the response secondary to AhR stabilization (Chen et al., 2005). This is also in agreement with the results presented in Chapter 6 indicating that PD98059 also induces the expression of Cyp1a1 and Aldh3a1, although this effect was more rapid than for U0126.

The evaluation of the effects of other MAPK cascades inhibitors on AhR-dependent genes indicated that the JNK inhibitor SP600125 and the p38-MAPK inhibitor SB203580 increased the expression of Cyp1a1 and Aldh3a1 mRNAs. In contrast, the PI3K inhibitor LY294002 caused a slight downregulation of the Cyp1a1 and Aldh3a1 mRNAs. This is in agreement with previous observations showing that LY294002 suppresses the upregulation of Cyp1a1 in response to dioxin, though potentially independent of any inhibition of PI3K (Guo et al., 2000). Additionally, my results contrast with previous observations made in immortalized human breast epithelial cell line MCF10A indicating that SP600125 is a ligand though antagonist of the AhR (Joiackim et al., 2003). With our current data, we can not rule out or confirm the possibility that activation or blockade of the AhR pathway is involved in these responses to MAPK inhibitors and LY294002, and further experiments are required to clarify this point.

Doxorubicin, which (like the protein kinase inhibitors used in this study) possesses planar hydrocarbon rings (Figure 6.2), also induced the expression of Cyp1a1 and Aldh3a1. To our knowledge, this is the first time that upregulation of these genes in response to doxorubicin has been identified. Doxorubicin is one of the most potent and effective

anticancer drugs available. However, a characteristic cardiomyopathy is a frequent side effect with its use in the clinical settings. One of the mechanisms implicated in this cardiotoxicity is the production of ROS and secondary apoptosis of cardiomyocytes (Wu et al., 2002; Minotti et al., 2004). The data presented here may represent an additional mechanism of toxicity in the pathophysiology of doxorubicin-induced cardiomyopathy. Cyp1a1 is a monoxidase that utilizes oxygen (via a NAD(P)H-dependent electron transport chain) to oxidize xenobiotics. Importantly, like all cytochrome P450 enzymes, the coupling of the reaction to molecular oxygen leads to generation of ROS and therefore to increased oxidative stress in the cell (Zangar et al., 2004). This is consistent with the sustained increase in oxidative stress that has been observed in mouse following the intraperitoneal injection of dioxin (Shertzer et al., 1998). Since cardiomyocytes are highly vulnerable to increased oxidative stress (section 1.4), increased expression of Cyp1a1 associated to increased production of ROS might be expected to be detrimental to cardiomyocytes. Given that doxorubicin induces the expression of Cyp1a1, this could also contribute to the increase in oxidative stress and apoptosis in cardiomyocytes exposed to this drug (Minotti et al., 2004).

The following observations also support the hypothesis that induction of Cyp1a1 and Aldh3a1 may represent additional mechanisms through which doxorubicin cause cardiotoxicity. Resveratrol, an important antioxidant found in the diet and wines (Fremont et al., 2000), has potential beneficial effects as adjuvant in anticancer therapy (Rezk et al., 2006). The mechanisms by which this compound mediates its effects are not completely understood, but the induction of cellular antioxidants and phase 2 enzymes has been proposed in experimental studies using H9c2 cardiomyocytes (Cao et al., 2004). Interestingly, this compound is also a known AhR ligand and antagonist (Casper et al.,

1999; Ciolino et al., 1999), therefore part of its potential beneficial effects could be the negative regulation of AhR-dependent genes in the heart. Whether the effects of induction of Cyp1a1 and Aldh3a1 genes by MAPK inhibitors and doxorubicin as shown herein are detrimental to the heart remain unknown and further experiments are required to clarify this. Another consideration is that several of the protein kinase inhibitors (and related compounds) used in the studies presented in Chapter 6 are being considered for therapeutic use in cancer, chronic inflammatory processes and cardiac diseases, and possible adverse effects could be envisaged since they upregulate AhR-dependent genes.

7.3 Future work

7.3.1 Confirmation of binding of ATF3 to the promoters of LIF, Ereg and Nfkbiz

I have demonstrated that ATF3 protein knockdown leads to upregulation of IL-6 as well as LIF, Ereg and Nfkbiz, and also demonstrated that upon ET-1 stimulation of cardiomyocytes ATF3 is bound to the promoter of IL-6 using ChIP assays. In further experiments it is necessary to confirm that ATF3 binds to the promoters of other potential target genes of ATF3 using ChIP assays under conditions of ET-1 stimulation.

7.3.2 Effect of ATF3 knockdown on cardiomyocyte morphology following ET-1 stimulation

One of the main findings of this thesis is that ATF3 probably effects a repressor activity on several genes of the gp130 cytokines (IL-6 and LIF), as well as in genes that could be

positive regulators of IL-6 expression (Ereg and Nfkbiz). This suggests that ATF3 is probably involved in a regulatory network that favours the development of concentric rather than eccentric hypertrophy following ET-1 stimulation and therefore has a role in fine-tuning of the hypertrophic response by this and may be other agonists. To confirm this hypothesis it could be useful to evaluate the effects of ATF3 knockdown following ET-1 stimulation on the cellular dimensions of the cardiomyocytes using planimetry and morphometric analysis. It is anticipated that increased levels of IL-6 and LIF, as well as Ereg and Nfkbiz could lead to an increase in myocyte length to width ratio. The effects of this morphological change will therefore translate into eccentric hypertrophy in the in vivo system due to the circumferential disposition of cardiomyocytes in the heart. This could be of pathological significance during processes such as ventricular dilatation of the heart following sustained pressure overload as in chronic hypertension, probably leading to the development of heart failure.

7.3.3 Role of ATF3 in cardiomyocytes following exposure to apoptotic concentrations of H₂O₂

In Chapter 4, I showed that ATF3 is potently induced by H₂O₂ and that this transcription factor is bound to the promoter of IL-6 following 2 h exposure of cardiomyocytes to 0.2 mM H₂O₂. However, whether ATF3 is a repressor or activator of IL-6 under these conditions remains to be determined. To further delineate the role of ATF3 on IL-6 regulation, further experiments should evaluate the effects of ATF3 knockdown on IL-6 mRNA levels. In addition, previous studies have shown that increased oxidative stress in the form of doxorubicin leads to ATF3 induction and this appears to be cytoprotective as adenoviral mediated overexpression of ATF3 lead to reduced apoptosis in

cardiomyocytes treated with doxorubicin (Nobori et al., 2002). Dr Stephen Fuller, from our laboratory, has also prepared adenoviruses expressing the full length rat ATF3 sense sequence which will allow overexpression of ATF3 in cardiomyocytes. The effects of adenoviral-mediated overexpression or knockdown on cardiomyocyte apoptosis evaluated using a combination of techniques such as TUNEL, cleavage of caspases, and changes in morphology could be useful in determining the role of ATF3 under increased oxidative stress in the form of H₂O₂.

7.3.4 Identification of other potential target genes regulated by ATF3 following stimulation of cardiomyocytes with ET-1

One of the most important tasks following completion of the genomic sequencing of the human and other genomes (International Human Genome Sequencing Consortium, 2004) is the understanding of how changes in the patterns of expression of genes in different cells and tissues, lead to alterations in the phenotype accompanying adaptive responses such as cardiac hypertrophy or pathological processes such as ventricular remodelling following myocardial infarction or sustained pressure overload. To this end, the identification of additional downstream genes regulated by ATF3 is an important complement to the studies presented in this thesis. The use of microarray studies in conjunction with knockdown of ATF3 following ET-1 stimulation will also help to identify novel target genes of ATF3, and these could be also confirmed using additional ChIP assays under conditions of ET-1 stimulation.

APENDIX A

Reagents and chemicals

Supplier	Reagent
AB Gene	10× Buffer IV (PCR) ØX174 RF DNA <i>Hae</i> III digest DNA ladder Taq polymerase
Alexis Biochemicals	GF109203X LY294002 Microcystin SB203580
Amersham	Hyperfilm Poly di-de sodium salt Rainbow molecular weight marker (RPN 800)
BD Biosciences	Chromaspin columns pore size 10
BDH	Acrylamide Benzamide Bis-acrylamide Boric acid EDTA Ethanol Glucose Glycine Glycerol Glycerophosphate HEPES Isopropanol MgCl ₂ MgSO ₄ Methanol Methylated spirit Nonidet® P40 (NP40) KCl NaCl NaF Na ₂ HPO ₄ Na ₃ VO ₄ Sodium dodecyl sulphate (SDS) Sucrose Tris Triton X100 Tween 20
Beckton Dickenson	Primaria culture dishes

Biogenesis	RNAzol Bee™
BioRad	Bradford Reagent
BOC gases	Liquid N ₂
DAKO	Horseradish peroxidase-conjugated secondary antibodies
Harlan	Neonatal (two day old) Sprague-Dawley rats
Invitrogen	100 bp DNA ladder Agarose dATP, dCTP, dGTP, dTTP DNase I Amp Grade (1 U / µl) DNase I reaction buffer Dulbecco's modified Eagle's medium (DMEM) M199 Foetal Calf serum Horse Serum M-MLV reverse transcriptase Penicillin and streptomycin Phosphate buffered saline (PBS) (10×) RNaseOut® 5× 1 st strand buffer (cDNA synthesis) Trypan blue solution
MWG Biotech	PCR primers
New England Biolabs	Primary antibodies
Promega	Random hexamers
Santa Cruz Biotechnology	Chemiluminescence reagents Primary antibodies
Schleicher and Schuell	Nitrocellulose membrane (Protran®)
Sigma	Ammonium persulphate Bovine serum albumin Bromophenol blue Chloroform Cycloheximide Dithiothreitol (DTT) oligonucleotides Endothelin (ET-1) Ethidium bromide Gelatin H ₂ O ₂ Leupeptin Pancreatin Phenylmethylsulfonyl fluoride Protein A-Sepharose Sonicated salmon sperm DNA TEMED Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane
Waitrose	Non-fat milk powder
Worthington	Collagenase

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