

TITLE:

Oxytocin activates NF- κ B-mediated inflammatory pathways in human gestational tissues

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ABSTRACT

Human labor, both at term and preterm, is preceded by NF- κ B-mediated inflammatory activation within the uterus leading to myometrial activation, fetal membrane remodelling and cervical ripening. The stimuli triggering inflammatory activation in normal human parturition are not fully understood. We show that the neurohypophyseal peptide, oxytocin (OT), activates NF- κ B and stimulates downstream inflammatory pathways in human gestational tissues. OT stimulation (1pM-100nM) specifically via its receptor (OTR) in human myometrial and amnion primary cells led to MAPK and NF- κ B activation within 15min and maximal p65-subunit nuclear translocation within 30min. Both in human myometrium and amnion, OT-induced activation of the canonical NF- κ B pathway upregulated key inflammatory labor-associated genes including IL-8, CCL5, IL-6 and COX-2. IKK $_{\beta}$ inhibition (TPCA1; 10 μ M) suppressed OT-induced NF- κ B-p65 phosphorylation, whereas p65-siRNA knockdown reduced basal and OT-induced COX-2 levels in myometrium and amnion. In both gestational tissues, MEK1/2 (U0126; 10 μ M) or p38 inhibition (SB203580; 10 μ M) suppressed OT-induced COX-2 expression, but OT-induced p65-phosphorylation was only inhibited in amnion suggesting OT activation of NF- κ B in amnion is MAPK-dependent. Our data provide new insight into the OT/OTR system in human parturition and suggest that its therapeutic modulation could be a strategy for regulating both contractile and inflammatory pathways in the clinical context of term/preterm labor.

HIGHLIGHTS

- In human gestational tissues OT activates NF- κ B via the canonical pathway.
- OT increases expression of NF- κ B mediated inflammatory labor-associated genes.
- Cross-talk exists between the OT-induced activation of MAPKs and NF- κ B signaling cascades in human amnion

KEYWORDS: oxytocin, NF- κ B, inflammation, myometrium, amnion, parturition

ABBREVIATIONS

OT, oxytocin; OTR, oxytocin receptor; MAPK, mitogen activated protein kinase; PG, prostaglandin; PKC, protein kinase C; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol trisphosphate; DAG, diacyl-glycerol; OVT, ornithine vasotocin; GPCR, G-protein coupled receptor; ECM, extracellular matrix.

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1. Introduction

Oxytocin is widely recognised as playing a major role in parturition by promoting myometrial contractility. Currently, oxytocin is the most potent uterotonic available and is extensively used in the clinical management of dysfunctional labor (Wei *et al*, 2013). However, myometrial contractions represent a late event in the cascade leading to labor and are preceded by cervical ripening and fetal membrane activation and remodelling. The onset of human labor resembles an inflammatory reaction and a substantial body of evidence suggests that both preterm and term labor are linked with increased NF- κ B activity within the uterus (Allport *et al*, 2001; Chapman *et al*, 2004; Condon *et al*, 2006). NF- κ B activation occurs in both human myometrium (Khanjani *et al*, 2011) and the fetal membranes (Lim *et al*, 2012) prior to the onset of labor and is associated with the up regulation of pro-labor genes including cyclooxygenase type 2 (COX-2) and the oxytocin receptor (OTR) (Terzidou *et al*, 2011). Consistent with this, inhibition of NF- κ B activity inhibits LPS-induced preterm labor in mice (Condon *et al*, 2004; Pirianov *et al*, 2009) and IL-1 β induced uterine contractions in Rhesus monkeys (Sadowsky *et al*, 2003). The role of the peptide hormone oxytocin (OT) in the modulation of inflammatory pathways preceding labor has been largely overlooked. Just prior to the onset of human labor, uterine sensitivity to OT increases markedly via upregulation of the OTR (Fang *et al*, 1996; Fuchs *et al*, 1982; Soloff *et al*, 1979). OT is an important regulator of PG production in the endometrium, amnion and decidua in several species including humans (Fuchs *et al*, 1981; Hinko & Soloff, 1992; Jeng *et al*, 2000; Lee *et al*, 2012; Milne & Jabbour, 2003; Moore *et al*, 1988; Terzidou *et al*, 2011; Zhang *et al*, 2011). This OT effect on PGs release in the human amnion has been shown to be through up-regulation of COX-2 (the rate limited step of PG biosynthesis) and was suggested to be protein kinase C (PKC)-dependent (Moore *et al*, 1991; Wouters *et al*, 2014).

Amnion is a major site of prostaglandin production in human pregnancy and its activation is critical for cervical ripening and the stimulation of myometrial contractions. Just prior to labor, there is an increase in inflammatory cytokine release from the amnion (Keelan *et al*, 2003; Satoh *et al*, 1979) as well as increased PG synthesis, particularly PGE₂ (Bennett *et al*, 1992; Olson, 2003). Collectively these changes promote cervical ripening, lower uterine segment remodelling and initiation of myometrial contractions (Fletcher *et al*, 1993; Keirse, 1993; Keirse *et al*, 1983; McLaren *et al*, 2000; Olson, 2003). In murine parturition surfactant protein A (SP-A) produced by the maturing fetal lung has been suggested to represent the stimulus for a cascade of inflammatory signaling pathways leading to labor onset (Condon *et al*, 2004). However in human pregnancy the endocrine or mechanical stimuli triggering inflammatory activation are obscure.

NF- κ B is a complex of heterogeneous dimers of various subunits from the Rel/NF- κ B family. The Rel/NF- κ B family consists of RelA (p65), RelB, c-Rel, p100/p52 and p105/p50, which share a Rel homology domain (RHD). The inactivated NF- κ B subunits are present in the cytoplasm as homo- or hetero- dimers and they are tightly regulated by an inhibitory protein from I κ B family, which prevents

nuclear translocation of NF- κ B. The canonical NF- κ B pathway is typically triggered by pro-inflammatory stimuli such as cytokines and lipopolysaccharides (LPS). Following ligand-specific receptor activation, downstream signaling activates I κ B kinase (IKK) complex, comprised of IKK α , IKK β , and NF- κ B essential modulator (NEMO), which is responsible for phosphorylating I κ B α (Traenckner *et al*, 1995). Phosphorylated I κ B α then undergoes tertiary structure changes, which expose motifs recognised by SCF ubiquitin ligases and becomes a target for ubiquitination (Yaron *et al*, 1998). This results in degradation of I κ B α by 26S proteasome and frees NF- κ B dimers (typically p50/p65) to enter the nucleus. The phosphorylation of the p65 subunit is important for initiating transcription (Sasaki *et al*, 2005; Vermeulen *et al*, 2002) and facilitates binding to the promoter region of various contraction associated proteins including OTR (Fuchs *et al*, 1982; Terzidou, 2006), PGF₂ receptor (Olson *et al*, 2003) and COX-2 (Belt *et al*, 1999; Slater *et al*, 1995; Soloff *et al*, 2004) and proinflammatory cytokines IL-6 (Libermann & Baltimore, 1990) and IL-8 (Khanjani *et al*, 2012).

We have previously demonstrated that OT upregulates the expression of COX-2, which itself is NF- κ B dependent, suggesting that OT may activate NF- κ B (Moore *et al*, 1991; Terzidou *et al*, 2011). In this study, we determined the effects of oxytocin on NF- κ B-mediated inflammatory pathways in human gestational tissues. We show that in both human myometrial and amnion cells OT stimulation of its receptor (OTR) drives the sequential activation of specific MAPKs and NF- κ B leading to the production of inflammatory pro-labor cytokines and prostaglandins. Our results demonstrate a previously unrecognised role for oxytocin in modulating NF- κ B-mediated inflammatory pathways thereby promoting the laboring phenotype. These findings provide novel insight into how the OT/OTR system contributes to normal human parturition but also highlights its potential impact in therapeutic treatments using oxytocin or OTR antagonists.

2. Materials and methods

2.1. Cell preparation and culture

All samples were collected with informed consent. Approval was granted by the local ethics committee (Placenta; RREC 2002-6283 and Myometrium; RREC 1997-5089). Fetal membranes and myometrial biopsies were obtained from women undergoing elective caesarean section at term (38⁺⁰ - 39⁺⁶ weeks of pregnancy), prior to the onset of labor. The patients did not have pre-existing medical conditions and had not received uterotonics. Those with pre-eclampsia, or multiple pregnancies were not included in this study. Amnion epithelial cells were prepared from tissue as previously described (Bennett *et al*, 1987). All amnion epithelial cells were primary cultures (no passage) and were cultured for 2 - 4 days prior to treatment.

Myometrial tissues were washed three times in PBS and dissected into fine pieces. Tissue samples were then digested in filter-sterilised collagenase solution with 1mg/ml collagenase 1A (Sigma-Aldrich), 1mg/ml collagenase X (Sigma-Aldrich), and 2mg/ml BSA (Sigma-Aldrich) in 50% serum-free DMEM and 50% DMEM/Nutrient Mixture F-12 HAM (Sigma-Aldrich) for 45min at 37°C. DMEM containing 10% FCS was added to the collagenase solution to inactivate the enzymes. The cell suspension was filtered through a cell strainer (70µm) and centrifuged at 3000 rpm for 5min. The pellet was resuspended in DMEM containing 10% FCS, 2mM LG, and 100U/ml PS and seeded into a T25 culture flask (Corning) to grow at 37°C, 5% CO₂. Once the cells reach ~95% confluence, they were washed in PBS and trypsinised in 0.25% trypsin containing 0.02% EDTA in PBS. DMEM containing 10% FCS was added to inactivate the enzyme and the cell suspension was centrifuged and diluted in fresh DMEM containing 10% FCS, 2mM LG and 100U/ml PS to be re-seeded in cell culture flasks or plates. Cells were used between passage numbers 1-4.

2.2. Real time RT-PCR

Total RNA was extracted by a guanidiumthiocyanate-phenol-chloroform extraction using RNA STAT-60 reagent (AMS Biotechnology, Abingdon, Oxon, UK) according to the manufacturer's specifications. Prior to cDNA synthesis, any DNA contaminations were eliminated by DNaseI treatment (Invitrogen). The DNaseI treated RNA were used for first-strand cDNA synthesis with SuperScriptII first-strand synthesis kit (Invitrogen). Gene expression was verified by real-time PCR performed on ABI StepOne Real Time PCR system (Applied Biosystems) using SYBR Green I Master mix (Applied Biosystems). Amplification was carried out using specific primers for the target DNA, generated using the software Primer Express (Applied Biosystems). The following gene specific primers were used for RT-PCR: L19, 5'- GCGGAAGGGTACAGCCAAT-3' and 5'- GCAGCCGGCGCAAA-3'; COX-2, 5'- TGTGCAACACTTGAGT-GGCT-3' and 5'- ACTTTCTGTACTGCGGGTG-G-3'; IL-8, 5'- GCCTTCCTGATTTCTGCAGC-3' and 5'- CGCAGTGTGGTCCACTCTCA-3'; IL-6, 5'- CCTTCC-AAAGATGGCTGAAA-3' and 5'- AGCTCTGGCTTGTTTCCTCAC-3'; CCL2, 5'- T-CTGTGCCTGCTGCTCATAG-3' and 5'- AGAT-

CTCCTTGGCCACAATG-3'; CCL5, 5'- CCATA-TTCCTCGGACACCAC-3' and 5'- TGTACTCC-GGAACCCATTTC-3'; GAPDH, 5'-TGATGACATCAAGAAGGTGGTGAAG- 3' and 5'-TCCTTGGAGGCCATGTAGGCCAT-3'; SOD2, 5'- TTGGCCAA-GGGAGATGTTAC-3' and 5'-AGTCACGTTTG-ATGGCTTCC-3'. The data were analyzed using Sequence Detector Version1.7 software (Applied Biosystems). Expression levels were assessed using the comparative Ct method and the target Ct values were normalised to ribosomal protein L-19 or GAPDH for analysis.

2.3. Protein Extraction, Western Blot and Immunodetection

For nuclear/cytosolic protein extraction, primary amnion epithelial cells were grown to confluence and they were rinsed in ice-cold PBS, then scraped in a buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM dithiothreitol (DTT), 1% (v/v) Nonidet P-40 (NP-40) alternative and complete protease inhibitor cocktail (Sigma). The cells were lysed by addition of 1% NP-40 alternative and cytosolic protein extracts were obtained by centrifugation of the lysate for 30 seconds at 12,000xg at 4°C. The pellets were resuspended in a buffer containing 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 2mM DTT, 400mM NaCl, 1% (v/v) NP-40 alternative and protease inhibitor cocktail (Sigma). The lysates were shaken vigorously for 15 min on ice. Nuclear protein extracts were obtained in the supernatant after centrifugation for 5 min at 12,000xg at 4°C.

For whole-cell protein, cells were lysed on ice for 10 min in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, 150mM NaCl, 10mM Tris (pH 7.4) and 1mM EDTA with 1mM PMSF, protease and phosphatase inhibitor cocktail (Sigma, Thermo-fisher). After quantification of the protein samples using BioRad protein assay kit, 40µg of protein samples were denatured by boiling for 10min at 90°C and separated by electrophoresis on an 10% SDS-polyacrylamide gel for 80min at 140V. Transfer from gel to PVDF membrane (Millipore) took place in wet-transfer chamber system (BioRad) for 90min at 300mA. The blots were incubated in primary antibodies overnight at 4°C in a fresh blocking buffer (1x PBS, 1% milk protein and 0.1% Tween-20) followed by incubation with HRP-conjugated secondary antibodies (1/2,000; Santa cruz, Cell signaling) the following day. Signal detection was carried out using ECL plus (GE Amersham Biosciences). To confirm equal loading of each well, the membranes were treated with a stripping buffer and re-probed for β-actin for whole cell lysates and TATA binding protein/α-tubulin for nuclear/cytosolic extracts.

2.4. EMSA

Consensus double-stranded oligos (NF-κB consensus; 5'-AAGAGAAGGGGCTTGCCCAAGG-3') were end-labelled with 0.37MBq ³²P (γATP) by incubating for 30 – 60 minutes at 37°C with T4 polynucleotide kinase (Promega). The labelled oligos were cleaned by centrifugation at 3000 rpm for 5 minutes through MicroSpin G-25 sephadex columns. Total of 5 µg nuclear proteins were incubated for 1 h on ice with non-radiolabelled non-specific oligos (Oct-1 consensus; 5'-

TGTCGAATGCAAATCACTAGAA -3') in an EMSA binding buffer containing 20% glycerol (v/v), 5mM MgCl₂, 2mM EDTA, 50mM Tris-HCl (pH 7.5), 250mM NaCl and 54mM DTT. Proteins were then incubated with 0.035pmol ³²P (γATP)-end labelled probes for 40min on ice. The resulting protein/DNA complexes were separated in a 4% non-denaturing acrylamide gel in 0.25xTBE buffer. The gel was then dried and transferred to a filter paper under vacuum at 80°C, and exposed to X-ray film overnight at -80°C. For supershift analysis, 2μg p65 or p50 specific antibodies (Santa Cruz) were incubated with the samples prior to probe binding. Non-radiolabelled oligos were used for specific and non-specific competition for DNA binding.

2.5. siRNA gene silencing

Transfection for gene silencing studies was performed using the Amaxa Nucleofector Technology (Lonza) according to manufacturer's protocol. Primary amnion epithelial cells were harvested by trypsinizing for 10 - 15min. Approximately 1×10⁶ cells were resuspended in 100μl room-temperature Nucleofector Solution and mixed with 30pmol of siGENOME SMARTpool siRNA (Thermo-Fisher). The cell/siRNA suspension was then transferred into certified cuvette and electroporated in the Nucleofector Cuvette Holder with the Nucleofector Program T-020 for amnion epithelial cells and A-033 for myometrial smooth muscle cells. Immediately after electroporation, cells were suspended with 500μl pre-warmed culture medium and plated. The cells were incubated in 5% CO₂, 95% air at 37°C and washed with PBS after 24 h. Total RNA and proteins were extracted for further analysis at 48 h and 72 h respectively.

2.6. ELISA

Concentrations of IL-6, CCL5 and PGE₂ released in supernatant were determined by a standard enzyme-linked immunosorbent assay (ELISA). Primary amnion epithelial cells were grown to confluence and treated for 1 h, 2 h, 4 h and 6 h with OT (100nM). Supernatant was collected and immediately frozen at -20°C for subsequent analysis by ELISA according to manufacturer's instructions (R&D systems).

2.7. Antibodies and Materials

The following antibodies were obtained from Santa Cruz Biotechnologies (Wiltshire, UK): goat anti-COX-2 (C20); mouse anti-α-tubulin; mouse anti-p65; mouse anti-RelB; mouse anti-IκBα; rabbit anti-p50 and HRP-conjugated secondary antibodies raised against goat, rabbit, and mouse IgGs. Rabbit monoclonal antibodies to phospho cPLA₂; phospho p65 (ser536); phospho IKK_{α/β}; phospho MAPK14 (p38 MAPK); phospho MAPK3/1 (ERK1/2 p44/42 MAPK) and MAPK8 (SAPK/JNK) were from Cell Signaling Technology, Inc. The mouse monoclonal anti-β-actin and anti-TATA-binding protein (TATA, TBP) antibodies were from Abcam (Cambridge, UK).

2.8. Statistical analysis

Data sets were tested for normality using the Kolmogorov-Smirnov test. For multiple comparisons of normally distributed data, ANOVA followed by Tukey's or Dunnett's post hoc test was used. For data that were not normally distributed, multiple comparisons were carried out using Friedman's test, followed by Dunn's Multiple Comparisons post hoc test. All data sets were presented with standard error of mean (S.E.M) and probability value of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. OT activates NF- κ B in amnion and myometrium

NF- κ B activation by inflammatory stimuli such as IL-1 β typically occurs via the canonical pathway whereby p65 homodimers or p50-p65 heterodimers translocate to the nucleus and drive transcriptional activity. Non-canonical activation can also occur and involves nuclear translocation of RelB-p52 heterodimers (Lindstrom, 2005). To examine the effect of OT on NF- κ B activation, human primary myometrial cells (n=6) and amnion (n=12) were treated with OT and nuclear translocation of the NF- κ B p65, p50 and RelB subunits was examined by western blotting analysis of cytosolic or nuclear protein extractions. Initial dose response studies (1pM to 100nM) were performed in amnion and myometrial cells (Supplementary Figure 1 and 2). In both cell types the effect of OT upon p65 phosphorylation appeared to be dose-dependent and maximal at 100nM. Subsequent studies were performed using a dose of 100nM.

In myometrial cells, OT stimulation led to an increase in phosphorylation of p65 and nuclear translocation of NF- κ B p65 and p50 within 30min which was sustained after 1h (Fig 1). There was no effect upon RelB. Examination of upstream components of the NF- κ B signaling pathway revealed that OT stimulation led to a significant increase in the activated phosphorylated form of IKK $_{\alpha\beta}$ within 30min. In amnion cells however, OT treatment induced a transient increase in p65 phosphorylation and in nuclear translocation of NF- κ B p65 but not p50. This was associated with phosphorylation of IKK $_{\alpha\beta}$ within 30min and a concurrent I κ B α degradation (Fig 2). As in myocytes there was no RelB nuclear translocation. To confirm that OT does not induce nuclear translocation of p50 in amnion cells, we performed EMSA to examine NF- κ B -DNA binding. As expected, IL-1 β treatment of amnion cells led to binding of both p50 and p65 to NF- κ B consensus sequence however, OT treatment led to binding of only p65 (Fig 2D).

3.2. OT increases expression of NF- κ B mediated inflammatory labor-associated genes

To provide further evidence that OT functionally activates NF- κ B, we studied the effect of OT on downstream NF- κ B-regulated gene expression. Target genes were selected from a list of pro-labor, NF- κ B regulated genes recently identified in human myometrium using a cDNA microarray analysis (Khanjani *et al*, 2011). OT (100nM) induced upregulation of several key inflammatory labor-associated genes in both myocytes and amnion cells including IL-8 (3.3- and 6-fold, respectively), IL-6 (2.2- and 2.5-fold, respectively) and CCL5 (2.6- and 3.2-fold, respectively) and COX-2 (4- and 3.5-fold) ($P<0.05$, compared to non-stimulated vehicle controls) (Fig 3A-D and 4A-D). OT treatment led to significant increase in CCL2 and SOD2 mRNA expression in amnion by 4- and 2.5-fold respectively but had no effect in myometrium (Fig 4E and F). ELISAs in amnion cells confirmed a significant protein increase in IL-6 (3-fold), CCL5 (4-fold) and PGE $_2$ (15-fold) levels ($P<0.05$,

compared to non-stimulated vehicle controls) (Fig 4G-I). These effects were transient following a similar pattern of activation as previously described with IL-1 β (Lee *et al*, 2003; Rauk & Chiao, 2000) with maximal upregulation for IL-8, CCL2, CCL5, IL-6 and SOD2 between 2 and 4 h before returning to basal levels at 24 h.

We and others have previously shown that labor and inflammation are associated with increased amnion sensitivity to OT through increased OTR expression and subsequent PGE₂ synthesis (Terzidou *et al*, 2011). To investigate whether OT drives the expression of other prostaglandin synthetic enzymes amnion epithelial cells were incubated with OT for up to 24h and the expression of PGE₂ synthetic enzymes cPLA₂, COX-2, PGES-1 and PGES-2 was examined by RT-PCR (Supplementary Fig S3). Increased mRNA of all PGE₂ synthetic enzymes was observed within 2h, reaching a maximal response at this time point for cPLA₂, COX-2 and PGES-2 and at 6h for PGES-1. Levels of all enzymes returned to basal levels by 24h. Consistent with our previous findings (Terzidou *et al*, 2011), OT treatment also induced a time-dependent increase in PGE₂ secretion into the culture media (Fig 4I) with maximal response reached after 6h.

3.3. OT induced expression of COX-2 is NF- κ B dependent

To determine whether NF- κ B activation is required for OT-induced COX-2 expression in human myometrium and amnion, pre-labor amnion epithelial cells were treated with TPCA1 (10 μ M), a selective IKK β inhibitor, prior to OT stimulation. TPCA1 treatment inhibited OT-induced p65 activation to basal level in both gestational tissues (Fig 5A and 6A) and in turn suppressed COX-2 expression. This demonstrates that OT requires the activation of IKK β to regulate COX-2 expression. Similarly, IL-1 β induced COX-2 expression was suppressed in the presence of TPCA1 in myometrial cells but was not inhibited in amnion cells (Fig 5B and 6B). Targeted siRNA knockdown studies showed that down-regulation of the NF- κ B p65 subunit significantly reduced basal and OT-induced COX-2 expression in myometrial and amnion cells (Fig 5C and 6C). As expected, knockdown of NF- κ B p65 subunit resulted in suppression of IL-1 β induced COX-2 expression (Supplementary Fig S4). Collectively these results show that OT-induced COX-2 expression is NF- κ B dependant.

3.4. Cross-talk exists between the OT-induced activation of MAPKs and NF- κ B signaling cascades in human amnion, but not myometrium

We have previously shown that OT-induced COX-2 expression in human amnion epithelial cells involves ERK1/2 activation (Terzidou *et al*, 2011). We further examined the dynamics of MAPK activation following OT stimulation and explored how these MAPKs might modulate NF- κ B dependent OT-induced COX-2 expression. ERK1/2, p38 kinase and JNK1/2 phosphorylation were

examined at 15min, 30min, 1 h, 2 h and 4 h post OT treatment in human myometrial and amnion cells. ERK1/2 activation was significantly increased at 15min before returning to basal levels (Fig 7A and 8A). Levels of activated p38 were significantly increased at 15min and 30min post OT exposure in the myometrium and amnion, respectively. JNK activation was increased at 15 and 30min before returning to basal levels at 2 h (Fig 7A and 8A). Pre-treatment of amnion epithelial cells with inhibitors of MEK1/2 (U0126) and p38 kinase (SB203580) resulted in decreased NF-κB p65 phosphorylation (Fig 8B), whereas in myometrial cells NF-κB p65 phosphorylation was not affected following MAPkinase inhibition (Fig 7B). MAPK inhibitor efficacies were confirmed by Western blot (Supplementary Fig S5). The reduction in p65 phosphorylation and therefore NF-κB activity following specific MAPK inhibition suggests MAPK involvement in the NF-κB signaling cascade regulation in human amnion. This effect was further reflected with an attenuation of OT-induced COX-2 protein expression after treatment with inhibitors of MEK1/2 (U0126) and p38 kinase (SB203580) (Fig 7C and 8C). In contrast, pre-treatment with the JNK1/2 inhibitor (SP600125) had no effect on either OT-induced phospho-p65 activation nor COX-2 protein expression levels. Cross talk between MAPKs and NF-κB signaling cascades was not observed upon IL-1β stimulation of NF-κB in either myometrial or amnion cells (Fig 7D-E and 8 D-E).

3.5. Inflammation activation by OT is specifically via OTR

OT typically exerts its effects via OTR but can also act as a ligand for vasopressin receptors, in particular, the V1A receptor (Zingg, 1996). Although the V1A receptor is abundantly expressed in human myometrium, its expression remains unchanged during gestation or labor suggesting it does not play a significant role in labor onset (Maggi *et al*, 1990). RT-PCR revealed the V1A receptor is also expressed in the human amnion (Supplementary Fig S6), and thus we aimed to determine whether OT acts as a ligand to this receptor in myometrium and amnion. Myometrial and amnion cell cultures were pre-treated with a potent, highly specific OTR antagonist, [d(CH₂)₅,Tyr(Me)², Thr⁴, Orn⁸, Tyr-NH₂⁹] vasotocin (OVT) (Manning *et al*, 2001), before being stimulated with OT for indicated time intervals. OT-induced activation of MAPKs and NF-κB were significantly reduced in both myocytes and amnion cells (Fig 9A and 10A), as was subsequent upregulation of COX-2 (Fig 9B and 10B), confirming that OTR specifically mediates OT-stimulated inflammation activation in human myometrium and amnion.

4. Discussion

Our study shows that OT increases the expression of COX-2 and other inflammatory mediators known to be associated with the onset of labor in both the myometrium and amnion via activation of NF- κ B and MAPKs. In myometrium, OT activates the established canonical pathway involving p65/p50 heterodimers with no cross talk between NF- κ B and MAPKs. In amnion however, OT signalling is markedly different. NF- κ B activation involves only p65 nuclear translocation and this is dependent on crosstalk with MAPKs.

OT has been reported to regulate the expression of cPLA₂ and COX-2 genes in both rat (Farina *et al*, 2007) and in human myometrial cells (Molnar *et al*, 1999) via PKC and ERK (Wouters *et al*, 2014) as well as the calcineurin/NFAT pathway (Pont *et al*, 2012). The NFAT transcription factors are also part of the Rel family and they have similar structure to the NF- κ B family. They can bind to overlapping DNA sequence elements and have been previously shown to demonstrate interdependence in mediating cardiac hypertrophic gene expression as NF- κ B nuclear translocation induced by IKK β or p65 enhanced NFAT nuclear localisation (Liu *et al*, 2012). OT has been shown to play a role in prostaglandin production in amnion via activation of MAPKs (Keirse *et al*, 1983; McLaren *et al*, 2000; Moore *et al*, 1988; Terzidou *et al*, 2011). Although Toll like receptors (TLRs) and Interleukin-1R like receptors (ILRs) are the 'classic' receptors upstream of NF- κ B, cross talk between NF- κ B and GPCRs, and regulation of NF- κ B by GPCRs has been established in several other systems (Fraser, 2008; Ye, 2001). Labor is antedated by NF- κ B activation and inflammatory stimulation in both the myometrium and the amnion (Khanjani *et al*, 2011; Lim *et al*, 2012). Our demonstration that OT stimulates NF- κ B in each of these tissues suggests a central signaling role for OT in the convergence of the biochemical events that precede the onset of uterine contractions. OT therefore has a dual role in both stimulation of gene expression associated with amnion and myometrial activation as well as in myometrial contractions.

The inflammatory cascade associated with parturition involves elevation of inflammatory cytokines (eg IL-1 β , IL-6 and TNF α) and chemokines (eg IL-8, CCL2 and CCL5) in amnion and myometrium (Osman *et al*, 2003; Shynlova *et al*, 2013). We show that OT treatment of amnion and myometrial cells activates a similar cassette of inflammatory mediators suggesting that OT acts as an endogenous inflammatory signaling molecule. In amnion, OT-induced activation of NF- κ B and downstream genes is comparable to the levels of activation induced by IL-1 β , a well established stimulus of NF- κ B activation. In myometrium however, IL-1 β results in a stronger level of activation compared to OT. Amnion is a major site of cytokine and prostaglandin production during parturition, thought to be critical for the onset of labor (Smith, 2007). In the context of the normal physiology of parturition, the greater sensitivity of the amnion to induction of inflammation would presumably lead to enhanced activation of inflammatory and pro-labor genes that would be withdrawn following completion of the

third stage of labor involving the delivery of the placenta and fetal membranes. In the myometrium inflammation that persists after birth might be disadvantageous.

We show NF- κ B activation by OT differs between amnion and myometrium and that this is likely due to preferential NF- κ B subunit translocation and differential crosstalk with MAPKs. In amnion the OT-induced activation of NF- κ B partly resembles that of a canonical NF- κ B signaling pathway triggered by IL-1 β stimulation (Lee *et al*, 2003), except that it also requires MAPK activation and OT-induced activation of NF- κ B involves nuclear translocation of p65 but not p50. Lim *et al*. have previously described a physical interaction between Rel-B and p65 in the nucleus of amnion epithelial cells (Lim *et al*, 2012) however, the lack of Rel-B translocation after OT treatment suggests that this interaction is not characteristic of amnion OT induced NF- κ B activation. Cytoplasmic p65 homodimers have been shown to be associated with I κ B α (Ganchi *et al*, 1993; Beg *et al*, 1993). The transcriptional activity of p65 is dependent on its carboxy-terminal region (Fujita *et al*, 1992). Similar to our findings, the pro-inflammatory mediator thrombin has been shown to induce ICAM-1 expression in endothelial cells via the induction of an NF- κ B signaling pathway involving p65 but not p50 (Rahman *et al*, 1999). OT-activation of NF- κ B in the amnion requires ERK1/2 and p38 kinase activity. Inhibition of p38 kinase activity, which is the most downstream kinase of the MAPK pathway, has been shown in other cell systems to significantly inhibit NF- κ B dependent gene expression (Beyaert *et al*, 1996; Schmitz *et al*, 2001; Schulze-Osthoff *et al*, 1997). OT activation of NF- κ B in myometrium involves both p65 and p50 translocation and appears to be identical to the activation caused by cytokines. It is therefore probable that OT regulates a different cassette of genes in amnion than in myometrium, illustrated by our findings that SOD-2 and CCL-2 are differentially regulated.

COX-2 is considered to be a key enzyme regulating the onset of labor (Smith, 2007). NF- κ B activation modulates COX-2 expression in amnion (Allport *et al*, 2001). Down-regulation of the NF- κ B p65 subunit using targeted siRNA inhibited the expression of OT-induced COX-2 at both mRNA and protein levels, further illustrating that OT-induced COX-2 expression in the amnion has an absolute requirement for activation of NF- κ B.

Although oxytocin typically binds to OTR, it has been reported to bind to vasopressin receptors to drive downstream signaling (Zingg, 1996). While we could detect V1A receptor expression in our amnion and myometrial cells, pre-treatment with OVT, a specific OTR antagonist, confirmed that OT signals through OTR to induce its pro-inflammatory effects. OVT treatment inhibited both OT-induced MAPK and NF- κ B activation as well as COX-2 and p-cPLA₂ expression. Our data suggest activation of inflammation by OT is mediated principally by its receptor whereas arginine vasopressin is mediated by both OTR and V1A receptor (Bossmar *et al*, 1994).

A potential weakness of our study is the cell culture model systems used. In the mouse, knockout models of either OT or OTR do not affect the onset of labor. However the role of OT in murine parturition is different to its role in the human; with OT having a significant luteotrophic activity

which is irrelevant in the human. Similarly knock out models of COX-2 or CRH in the mouse do not have a parturition defect phenotype whilst the roles of COX-2 and CRH in the human are firmly established. Mouse models are therefore not useful to study the role of OT in the human (Mitchell & Taggart, 2009). Amnion epithelial cells are easily isolated and cultured from fetal membranes collected after delivery. Sufficient cells can be obtained to undertake experiments without the need for passage and these cells retain their pre-labor/ non-activated or activated/ post-labor phenotype for the duration of experiments. They therefore represent a good model for the *in vivo* cell type. There is no ideal cell culture model for human pregnant uterine myocytes. Immortalised cells have dramatically different expression profiles for GPCR and nuclear receptors and are therefore poor models for our studies. In our cell culture system, primary myometrial cells maintained similar levels of labor-associated proteins, α -smooth actin, PR and OTR expression and calcium influx following OT stimulation up to passage 10 (Bishop, 2013; Mosher *et al*, 2013) thus indicating that they retain their individual functional responsiveness in culture and represent a reasonable model for investigating OT signalling.

In conclusion, our findings describe a novel mechanism of inflammatory activation in human myometrium and amnion mediated by the OT-OTR system, which leads to NF- κ B activation and subsequent upregulation of prostaglandins and inflammatory chemokines and cytokines that are involved in fetal membrane remodelling, cervical ripening and myometrial activation. The role for OT in the onset of human labor exceeds stimulation of myometrial contractions and involves concurrent activation of inflammatory pathways. Accordingly, in drug discovery in the management of preterm and term labor it may be important to consider that OT may exacerbate inflammation. Conversely, inhibition of the OT/OTR system in preterm labor has the potential to both inhibit both contractions and inflammation.

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Figure Legends

Figure 1. Activation of NF- κ B in human myometrial smooth muscle cells upon OT stimulation.

Human pre-labor primary myometrial smooth muscle cells were cultured from biopsies taken from patients undergoing elective caesarean section at term. Myometrial cells were treated with OT (100 nM) for 15 min, 30 min, and 1 h. Western blot analysis of nuclear cytosolic extracts demonstrated nuclear translocation of NF- κ B p65 and p50, but not RelB upon OT stimulation (A). Membranes were probed with α -tubulin and TATA binding protein (TBP) to confirm separation of nuclear and cytosolic extracts. Densitometric plots show significant increase in nuclear p65 and p50 (B) (n=6; * $p<0.05$ ** $p<0.01$ vs NS, ANOVA). Western blot analysis of whole cell extracts was performed to study the effects of OT stimulation on p-p65 p-IKK $_{\alpha\beta}$ and I κ B α (C). Control with β -actin confirmed equal protein loading (n=6; * $p<0.05$ vs NS, ANOVA).

Figure 2. Activation of NF- κ B in human amnion epithelial cells upon OT stimulation.

Human pre-labor primary amnion epithelial cells were treated with OT (100 nM) for 15 min, 30 min, and 1 h. Western blot analysis of nuclear and cytosolic protein extracts illustrates cytosolic and nuclear localisation of different NF- κ B subunits, p65, p50 and RelB following OT treatment (A). Membranes were probed with α -tubulin and TATA binding protein (TBP) to confirm separation of nuclear and cytosolic extracts. Densitometric plots show significant increase in nuclear p65 but not p50 or RelB (B) (n=12; * $p<0.05$ vs NS, ANOVA). Western blot analysis of whole cell extracts was performed to study the effects of OT stimulation on p-p65 p-IKK $_{\alpha\beta}$ and I κ B α (C). Control with β -actin confirmed equal protein loading (n=6; * $p<0.05$ vs NS, ANOVA). EMSA studies demonstrated increase in NF- κ B nuclear protein DNA binding upon OT or IL-1 β (1ng/ml) stimulation (D). Specificity of NF- κ B binding was confirmed by co-incubation with non-labelled non-competing oligos (Oct-1 consensus; 5'-TGTCGAATGCAAATCACTAGAA-3') and non-labelled competing oligos (NF- κ B consensus; 5'-AAGAGAAGGGGCTTGCCCAAGG-3'). Co-incubation with antibodies to NF- κ B p50 and p65 resulted in band supershift in IL-1 β treated samples but only p65 supershift was shown in OT treated samples, indicating increase in NF- κ B p65 DNA binding.

Figure 3. Increase in the expression of pro-labor downstream NF- κ B-regulated genes with OT stimulation in human myometrium.

Human pre-labor primary myometrial cells were treated with OT (100 nM) for 1 h, 2 h, 4 h, 6 h and 24 h, and the expression of downstream NF- κ B-regulated genes, IL-8 (A), IL-6 (B), CCL5 (C), COX-2 (D), CCL2 (E) and SOD2 (F) were analysed using qRT-PCR. Transcript levels were normalised to the housekeeping gene, GAPDH (n=6; * $p<0.05$, ** $p<0.01$ vs NS, ANOVA).

Figure 4. Increase in the expression of pro-labor downstream NF- κ B-regulated genes with OT stimulation in human amnion. Human pre-labor primary amnion cells were treated with OT (100 nM) for 1 h, 2 h, 4 h, 6 h and 24 h, and the expression of downstream NF- κ B-regulated genes, IL-8 (A), IL-6 (B), CCL5 (C), COX-2 (D), CCL2 (E) and SOD2 (F) were analysed using qRT-PCR. Transcript levels were normalised to the housekeeping gene, L19 (n=6; * $p < 0.05$, ** $p < 0.01$ vs NS, ANOVA). The collected culture media were used for measuring secreted levels of IL-6 (G), CCL5 (H) and PGE₂ (I) ELISA (n=3; * $p < 0.05$, ** $p < 0.01$ vs NS, ANOVA).

Figure 5. IKK $_{\alpha/\beta}$ and NF- κ B p65 play a role in OT-induced COX-2 expression in human myometrium. Human pre-labor primary myometrial cells were treated with IL-1 β (1ng/ml) or OT (100nM) for 30min and 6 h in the presence or absence of TPCA1 (10 μ M). Whole cell extracts were subjected to Western blot analyses with antibodies against p-p65 (A) and COX-2 (B) (n = 6; * $p < 0.05$, *** $p < 0.001$ compared with NS, $\neq$ $p < 0.01$ compared with IL-1 β treated, § $p < 0.01$ compared with OT treated, ANOVA). Myometrial cells transfected with non-target siRNA or p65-target siRNA were treated with OT for 6 h. Whole cell extracts were subjected to Western blot analysis for p65 and COX-2 (D) (n = 3; ** $p < 0.01$, *** $p < 0.001$ compared with NS, § $p < 0.01$ compared with non-target siRNA+OT, ANOVA).

Figure 6. IKK $_{\alpha/\beta}$ and NF- κ B p65 play a role in OT-induced COX-2 expression in human amnion. Human pre-labor primary amnion cells were treated with IL-1 β (1ng/ml) or OT (100nM) for 30min and 6 h in the presence or absence of TPCA1 (a selective IKK $_{\beta}$ inhibitor;10 μ M). Whole cell extracts were subjected to Western blot analyses with antibodies against p-p65 (A) and COX-2 (B) (n = 6; * $p < 0.05$, *** $p < 0.001$ compared with NS, $\neq$ $p < 0.01$ compared with IL-1 β treated, § $p < 0.01$ compared with OT treated, ANOVA). Amnion cells transfected with non-target siRNA or p65-target siRNA were treated with OT for 6 h. Whole cell extracts were subjected to Western blot analysis for p65 and COX-2 (D) (n = 3; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with NS, § $p < 0.01$ compared with non-target siRNA+OT, ANOVA).

Figure 7. OT-induced expression of COX-2 in human myometrium requires independent activation of MAPKs and NF- κ B. Human pre-labor primary myometrial cells were treated with OT (100nM) for 15min, 30min, 1 h, 2 h and 4 h and were subjected to Western blot analysis to study the effects of OT stimulation on p-ERK1/2, p-p38 and p-JNK expression (A) (n = 6; * $p < 0.05$, ** $p < 0.01$ compared with NS, ANOVA). Myometrial cells were pretreated with MEK1/2 inhibitor (U0126; 10 μ M), p38 kinase inhibitor (SB203580; 10 μ M) or JNK inhibitor (SP600125; 10 μ M) for 2 h prior to OT (100nM) stimulation for 30min and 6 h. Whole cell extracts were subjected to Western blot

analysis with antibodies against p-p65 (B) and COX-2 (C) . (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with NS, § $p < 0.05$ compared with OT treated, ANOVA). Myometrial cells treated with IL-1 β (1ng/ml) for 30min and 6 h in the presence of MAPK inhibitors, U0126 (10 μ M), SB203580 (10 μ M) or SP600125 (10 μ M) were subjected to Western blot analyses for p65 phosphorylation (D) and COX-2 expression (E) (n = 6; * $p < 0.05$, *** $p < 0.001$ compared with NS, $\neq p < 0.05$ compared with IL-1 β treated, ANOVA).

Figure 8. OT-induced expression of COX-2 in human amnion requires MAPK dependent NF- κ B activation. Human pre-labor primary amnion cells were treated with OT (100nM) for 15min, 30min, 1 h, 2 h and 4 h and were subjected to Western blot analysis to study the effects of OT stimulation on p-ERK1/2, p-p38 and p-JNK expression (A) (n = 6; * $p < 0.05$ compared with NS, ANOVA). Amnion cells were pretreated with MEK1/2 inhibitor (U0126; 10 μ M), p38 kinase inhibitor (SB203580; 10 μ M) or JNK inhibitor (SP600125; 10 μ M) for 2 h prior to OT (100 nM) stimulation for 30 min and 6 h. Whole cell extracts were subjected to Western blot analysis with antibodies against p-p65 (B) and COX-2 (C) . (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with NS, § $p < 0.01$ compared with OT treated, ANOVA). Amnion cells treated with IL-1 β (1ng/ml) for 30min and 6 h in the presence of MAPK inhibitors, U0126 (10 μ M), SB203580 (10 μ M) or SP600125 (10 μ M) were subjected to Western blot analyses for p65 phosphorylation (D) and COX-2 expression (E) (n = 6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with NS, $\neq p < 0.01$ compared with IL-1 β treated, ANOVA).

Figure 9. The activation of inflammation by OT in human myometrium is specifically through OTR. Human pre-labor primary myometrial cells were incubated in OVT (1 μ M) for 30 min prior to 5min, 15min or 30min of OT stimulation (100nM). Pretreatment with OVT for 30min completely inhibits the effect of OT upon phosphorylation of p65 and p38 kinase (A). Western blot analysis of cells treated with OT (100nM) for 2 h, 4 h and 6 h in presence or absence of OVT (1 μ M) demonstrated inhibition of OT-induced COX-2 expression in presence of OVT (B) (n = 6; * $p < 0.05$, ANOVA).

Figure 10. The activation of inflammation by OT in human amnion is specifically through OTR. Human pre-labor primary amnion epithelial cells were incubated in OVT (1 μ M) for 30min prior to 5min, 15min or 30min of OT stimulation (100nM). Pretreatment with OVT for 30min completely inhibits the effect of OT upon phosphorylation of p65, ERK, and p38 kinase (A). Western blot analysis of cells treated with OT (100nM) for 2 h, 4 h and 6 h in presence or absence of OVT (1 μ M)

demonstrated inhibition of OT-induced COX-2 expression in presence of OVT (B) ($n = 6$; * $p < 0.05$, *** $p < 0.01$, ANOVA).

Supplementary figure S1. Activation of MAPKs and NF- κ B by OT in human myometrium is dose-dependent. Human pre-labor primary myometrial cells were treated with OT (1pM-100nM) for 5min, 15min, 30min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analysis with antibodies against p-p65, p-p38, p-ERK1/2 and COX-2 (A). Densitometric analysis show dose-dependent effect of OT, with maximal response at 100nM (B).

Supplementary figure S2. Activation of MAPKs and NF- κ B by OT in human amnion is dose-dependent. Human pre-labor primary amnion epithelial cells were treated with OT (1pM-100nM) for 5min, 15min, 30min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analysis with antibodies against p-p65, p-p38, p-ERK1/2 and COX-2 (A). Densitometric analysis show dose-dependent effect of OT, with maximal response at 100nM (B).

Supplementary figure S3. OT increases expression of PG synthetic enzymes in human amnion. Human pre-labor primary amnion epithelial cells were treated with OT (100 nM) for 1 h, 2 h, 6 h, and 24 h. Total RNA was subjected to gene expression analysis for PG synthetic enzymes; cPLA₂, COX-2, PGES-1 and PGES-2 (n = 6; * $p < 0.05$, ** $p < 0.01$ compared with NS, ANOVA).

Supplementary figure S4. NF- κ B p65 plays a role in IL-1 β -induced COX-2 expression in human amnion. Human pre-labor primary amnion epithelial cells transfected with non-target siRNA or p65-target siRNA were treated with IL-1 β (1ng/ml) for 6 h. Whole cell extracts were subjected to Western blot analysis for p65 and COX-2 (n = 3; * $p < 0.05$, ** $p < 0.01$ compared with NS, $\neq p < 0.01$ compared with non-target siRNA+IL-1 β , ANOVA).

Supplementary figure S5. Efficacy of different MAPK inhibitors. Human pre-labor primary amnion epithelial cells were incubated in the presence of the ERK1/2 inhibitor (U0126; 10 μ M), p38 kinase inhibitor (SB23580; 10 μ M) or JNK inhibitor (SP600125; 10 μ M) for 2 h prior to OT (100 nM) stimulation for 30 min. Whole cell lysates were extracted for Western blot analysis of p-ERK, p-HSP27, and p-JNK. The efficacy of SB23580 was determined by studying the effect on p-HSP27 as SB23580 inhibits the activity of p38 kinase without affecting its phosphorylation state. Control with β -actin confirmed equal protein loading.

Supplementary figure S6. Human amnion expresses arginine vasopressin receptor 1A (V1A). Human pre-labor primary amnion epithelial cells were established from 4 different samples. cDNAs were synthesized using total RNA extracted from non-stimulated amnion cells. Products of PCR reactions with primers specific for arginine vasopressin receptor (V1A) were analysed on 2% agarose gel. PCR products of expected size, 473 bp, were detected. Placental cDNA was used as positive controls (P). The PCR product was purified and subjected to DNA sequencing.

FIGURE 1 (2-column)

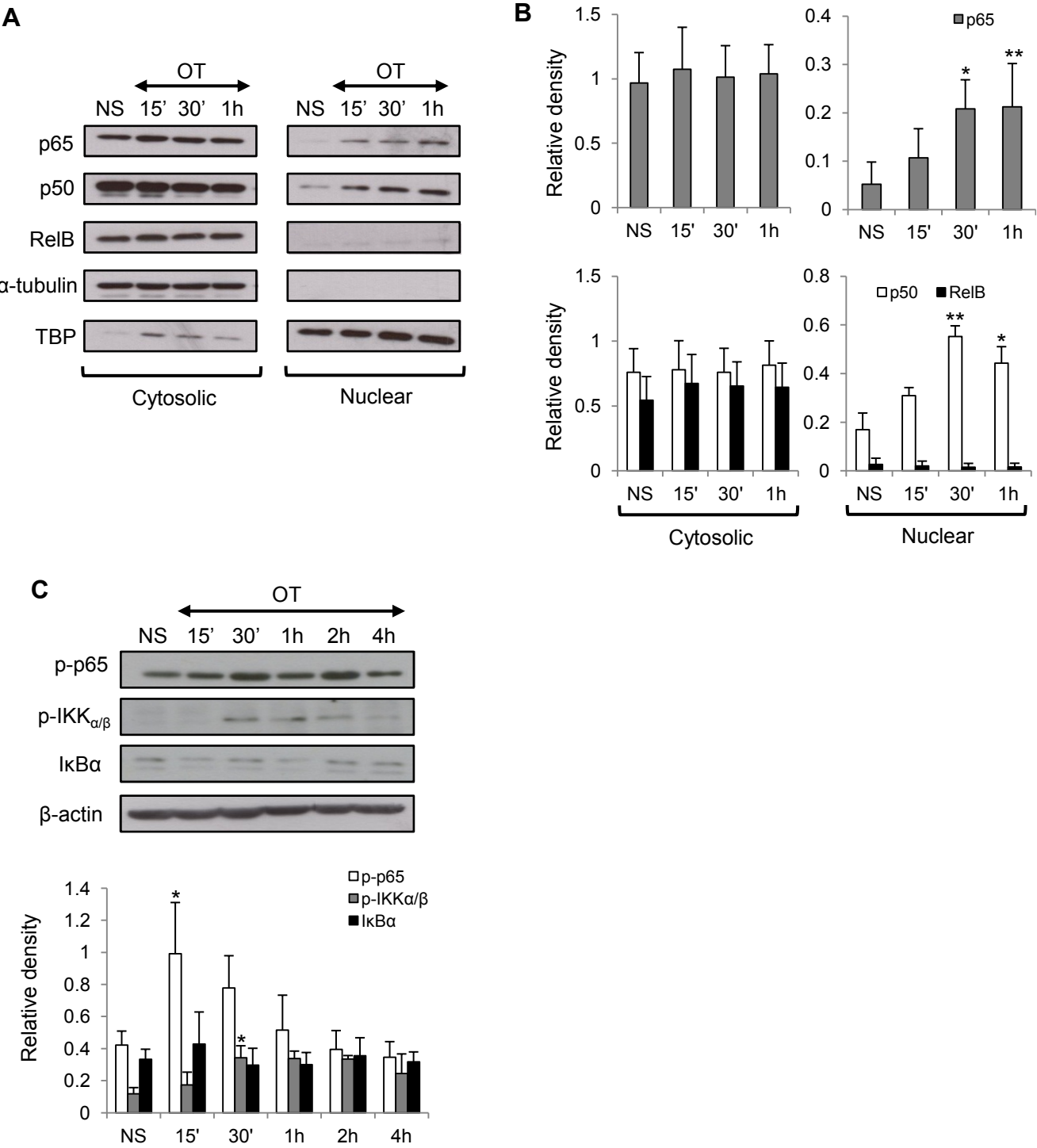
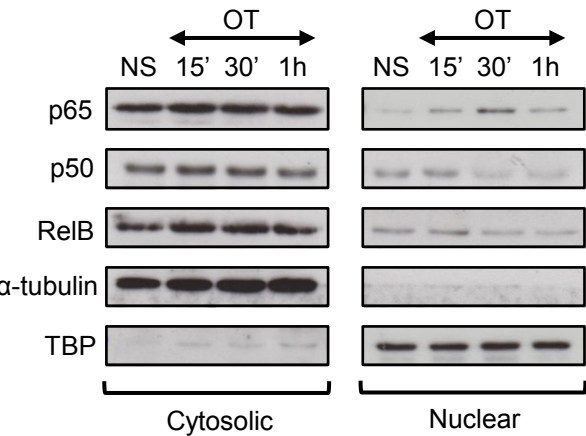
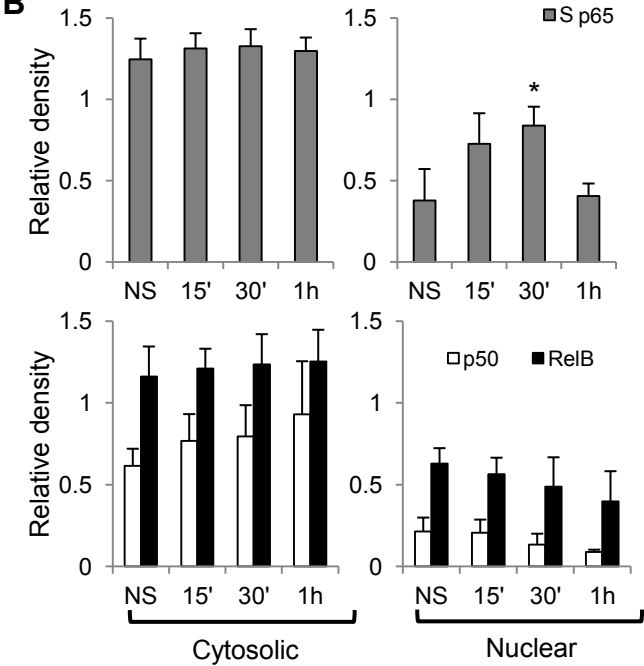


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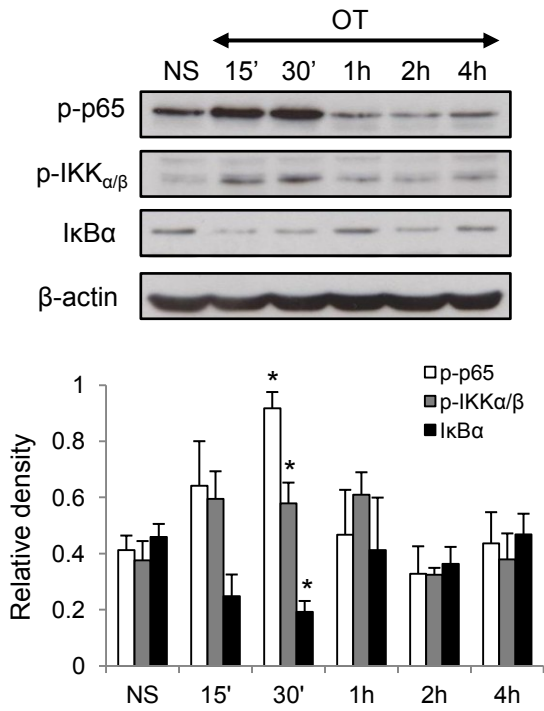
A



B



C



D

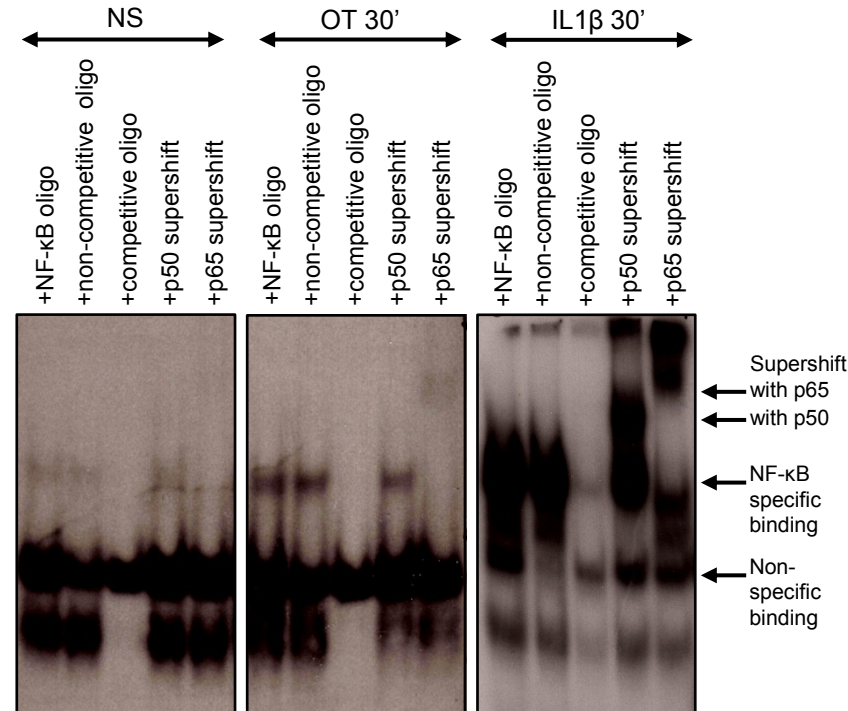


FIGURE 3 (1.5-column)

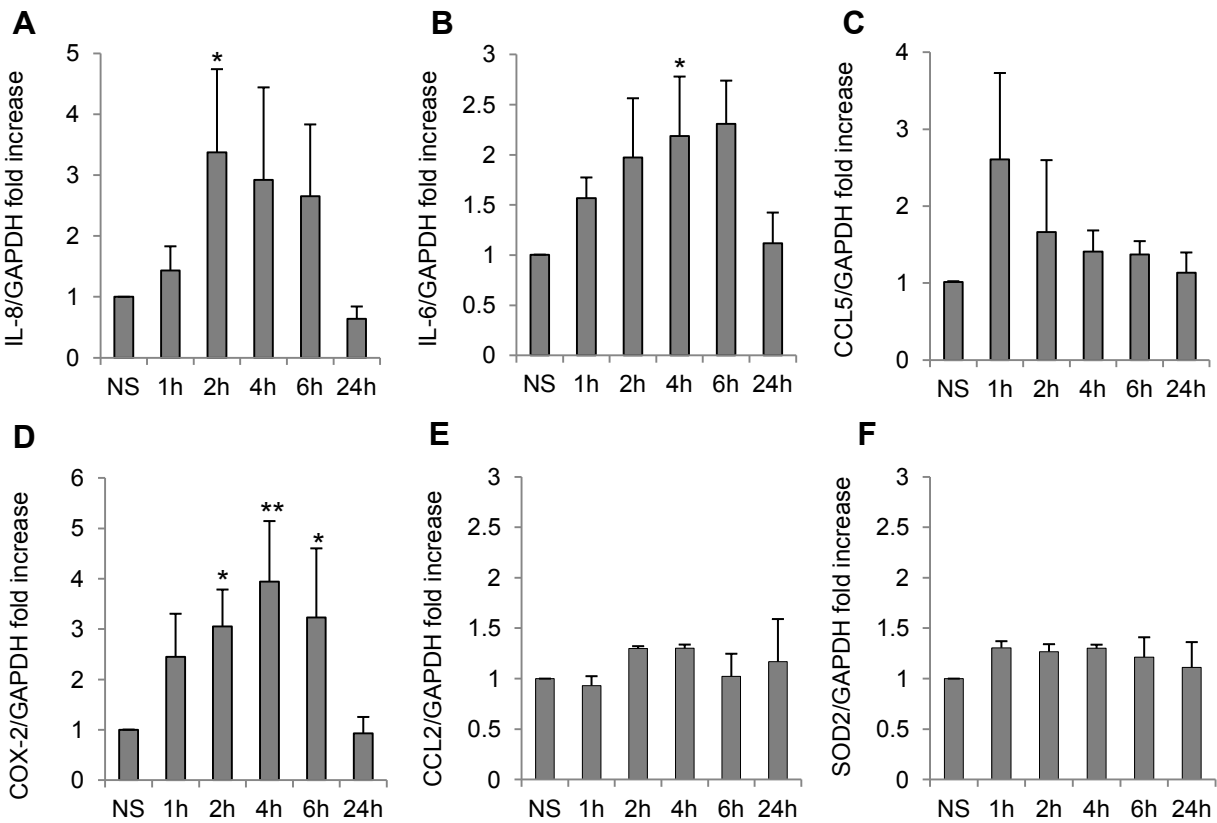


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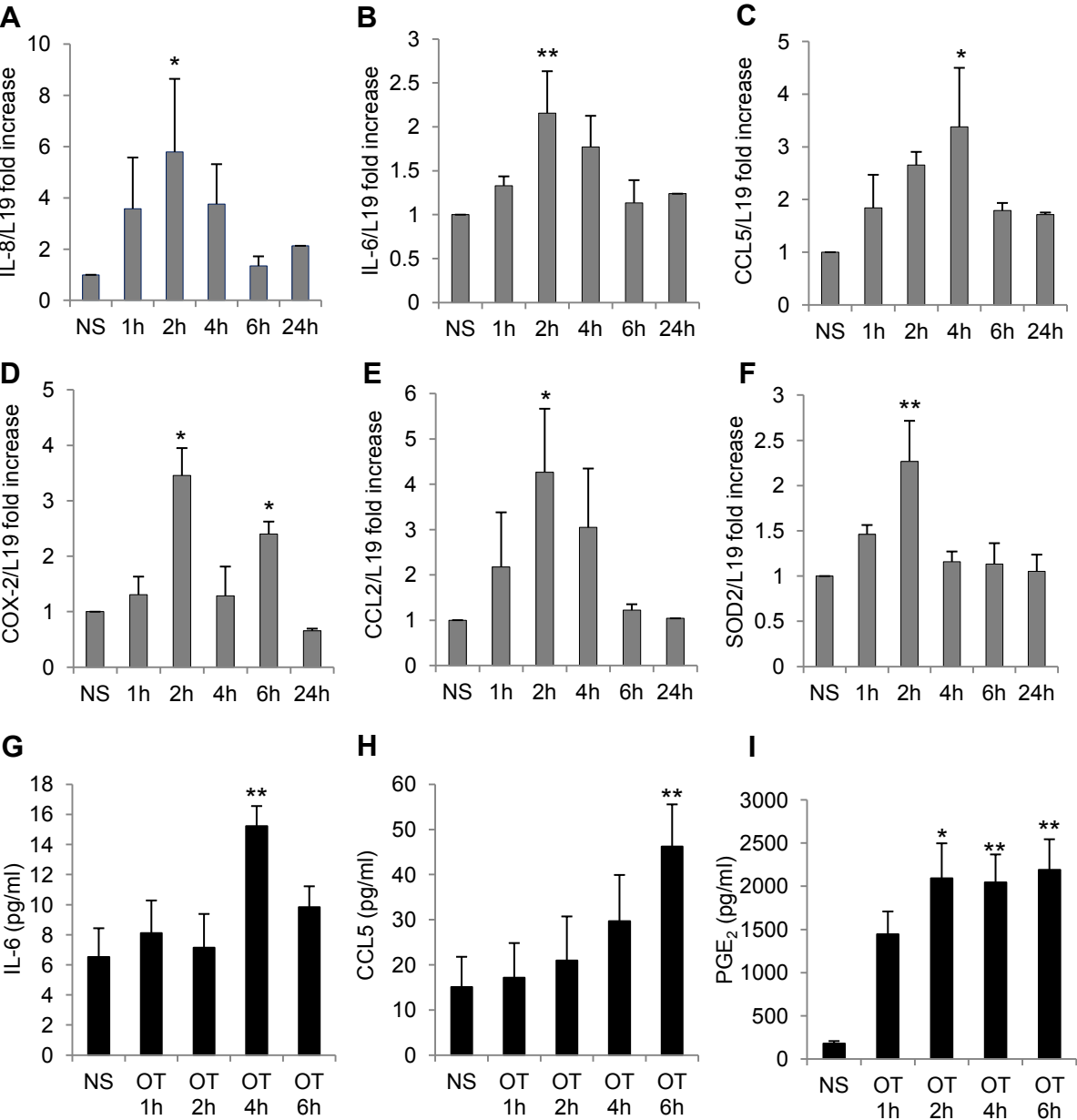


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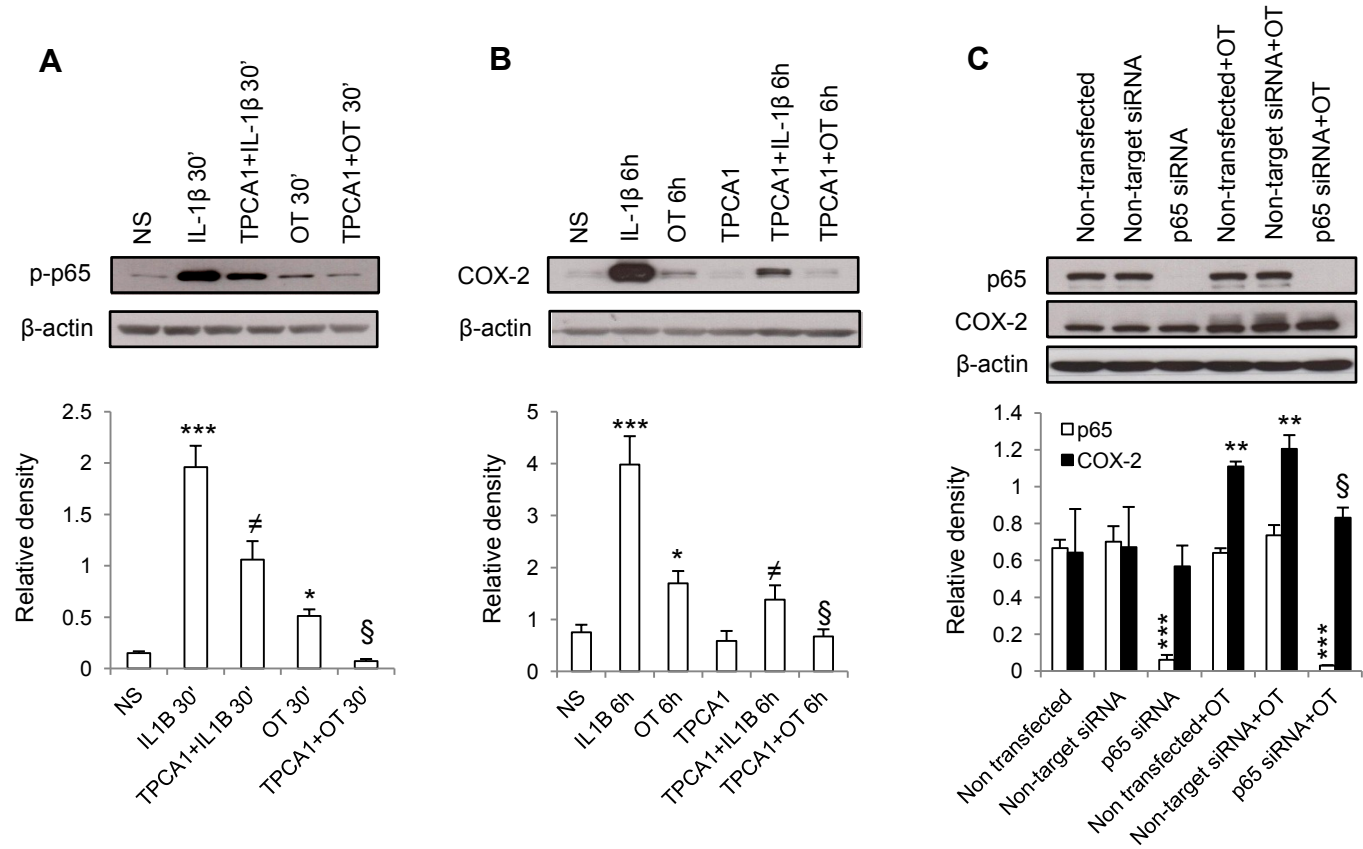


FIGURE 6 (2-column)

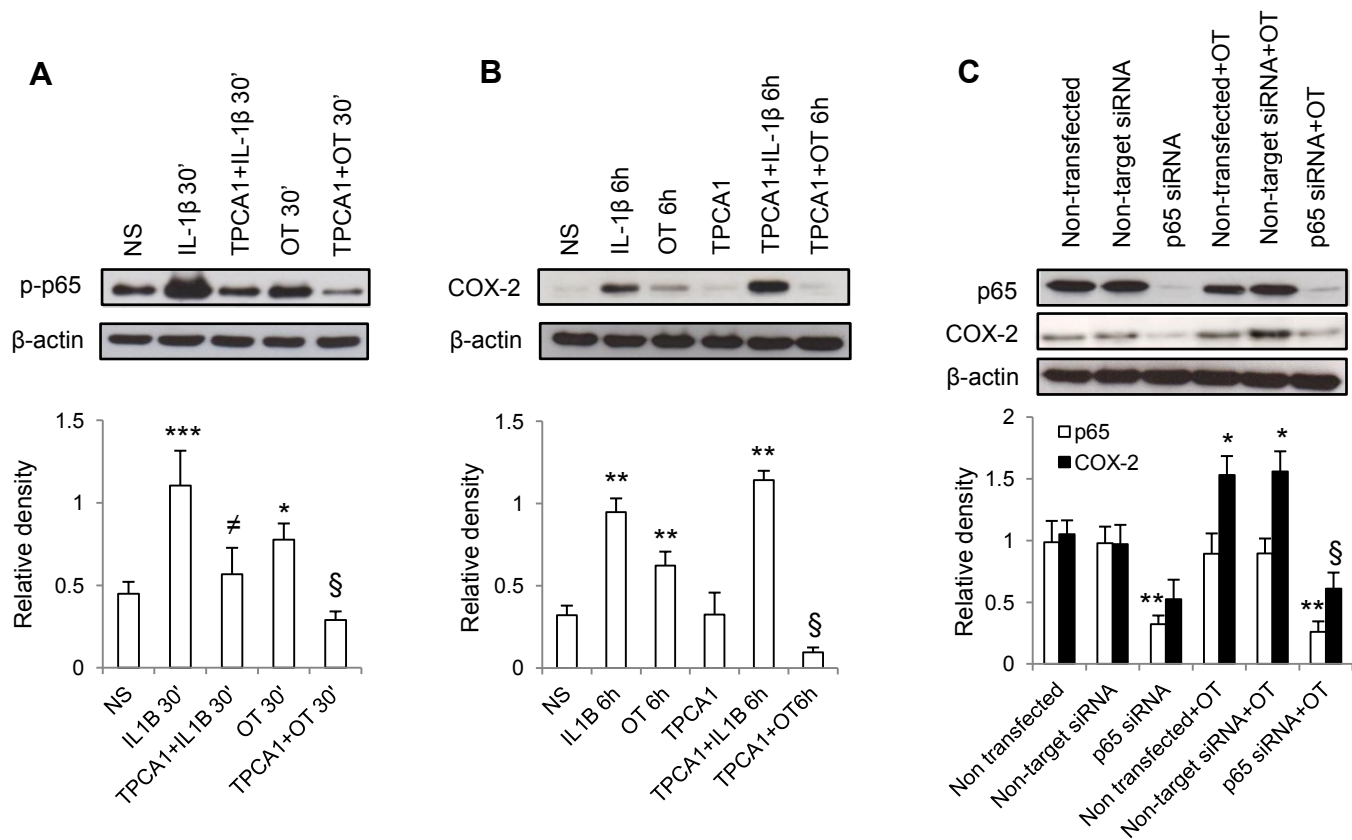


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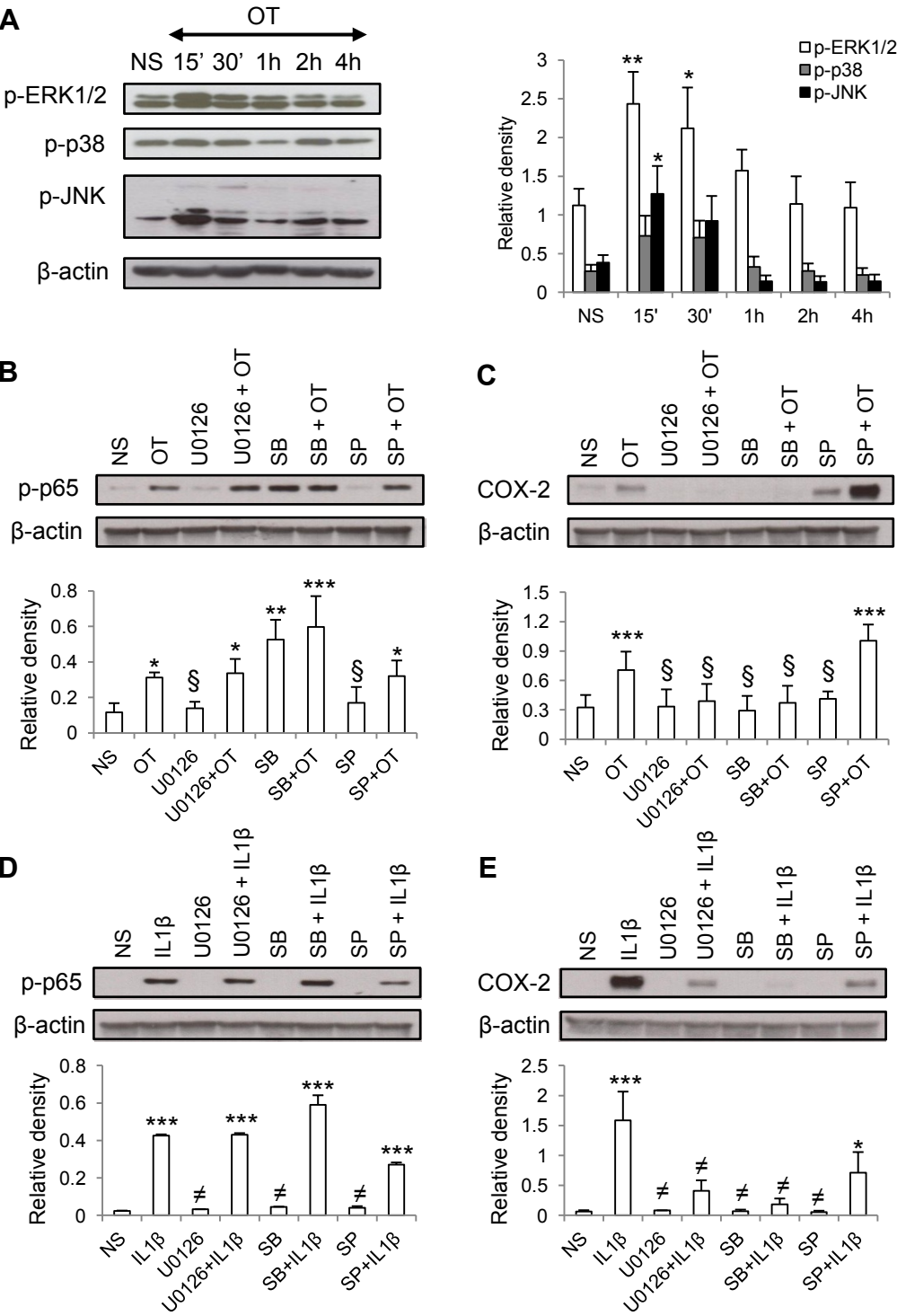


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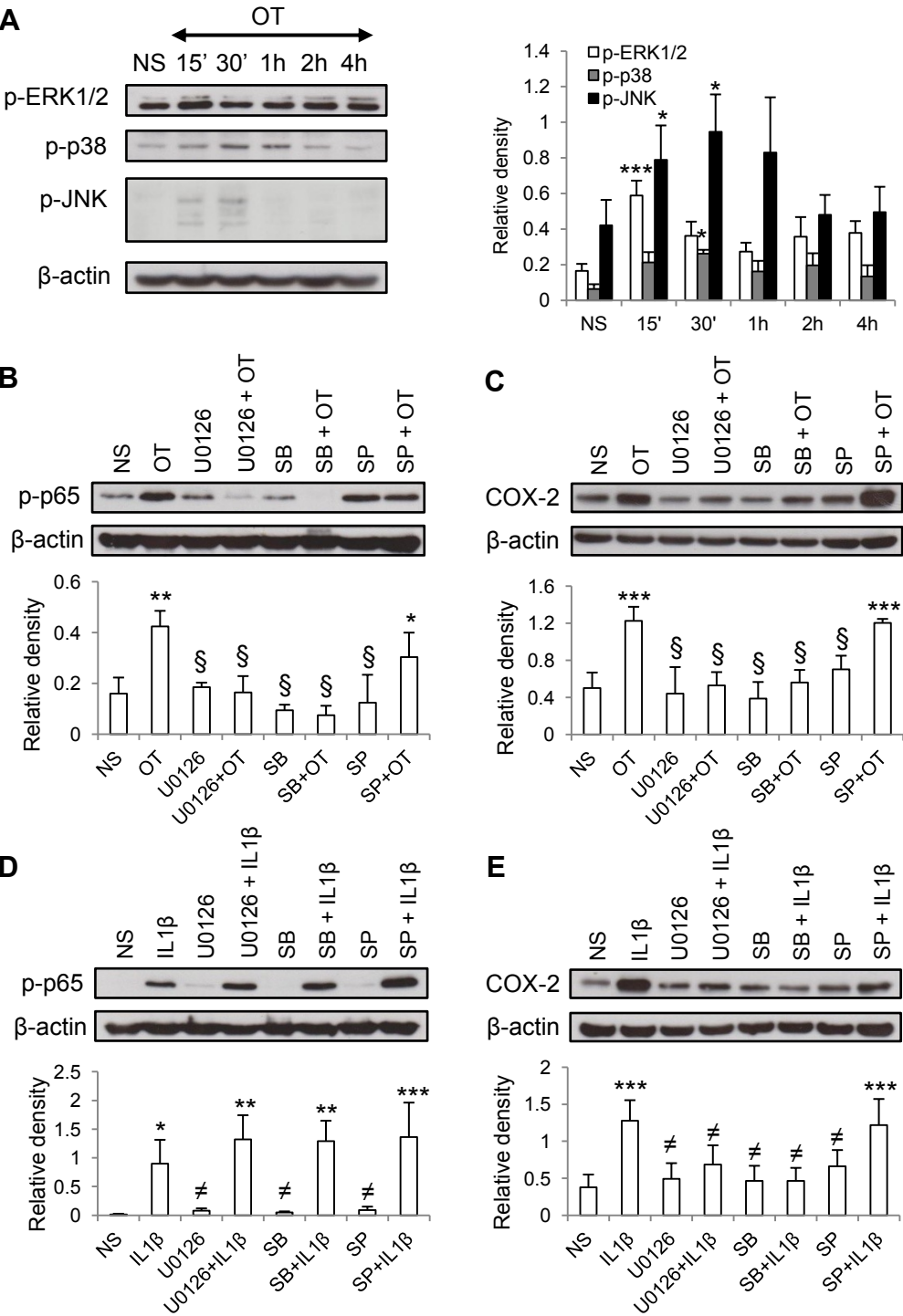
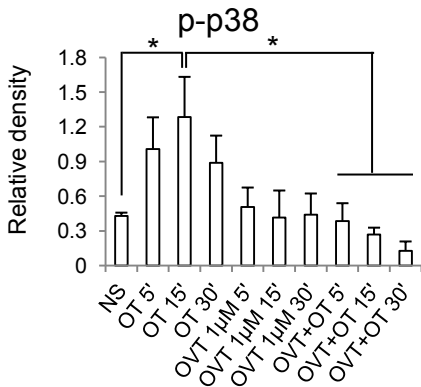
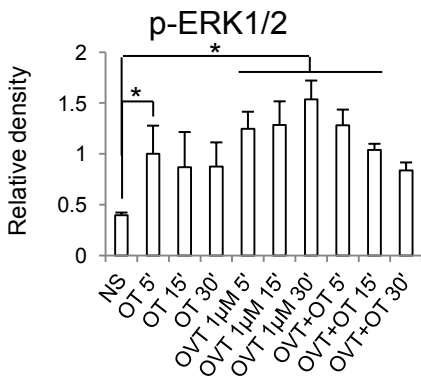
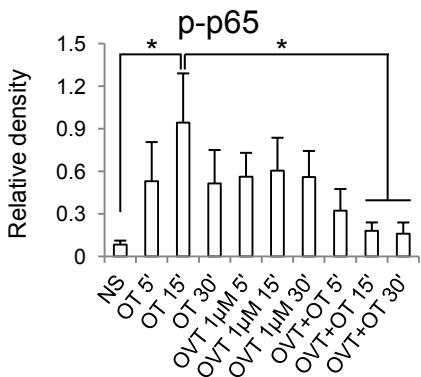
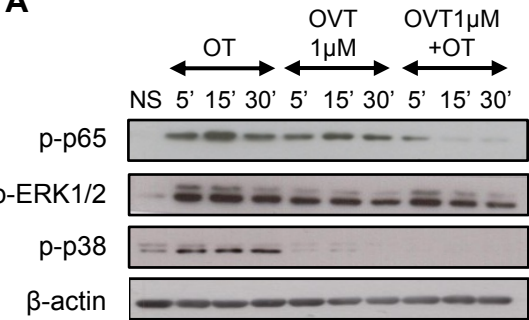


FIGURE 9 (1.5-column)

A



B

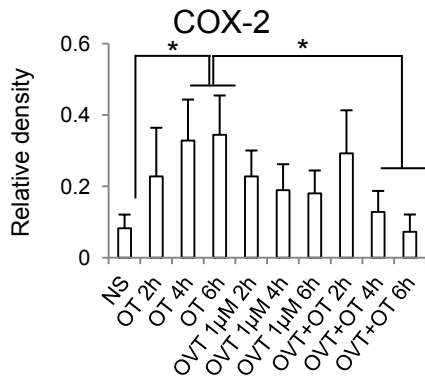
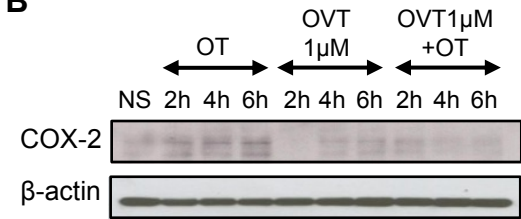
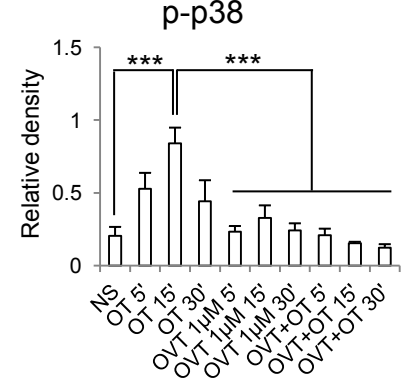
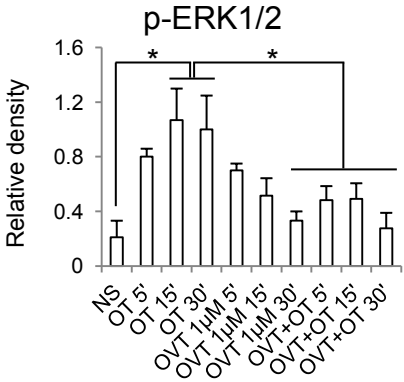
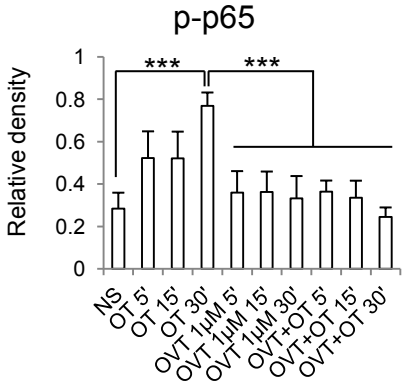
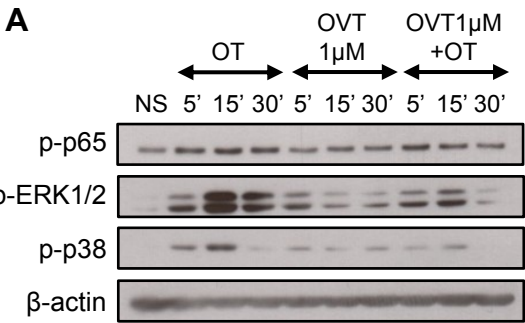
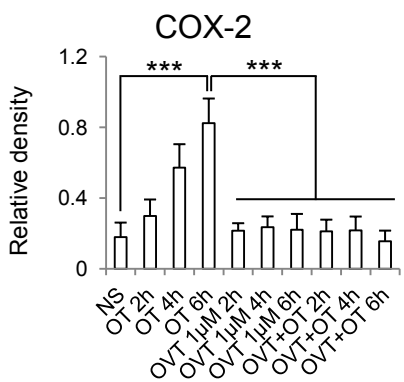
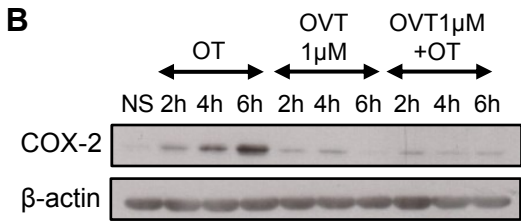


FIGURE 10 (1.5-column)

A

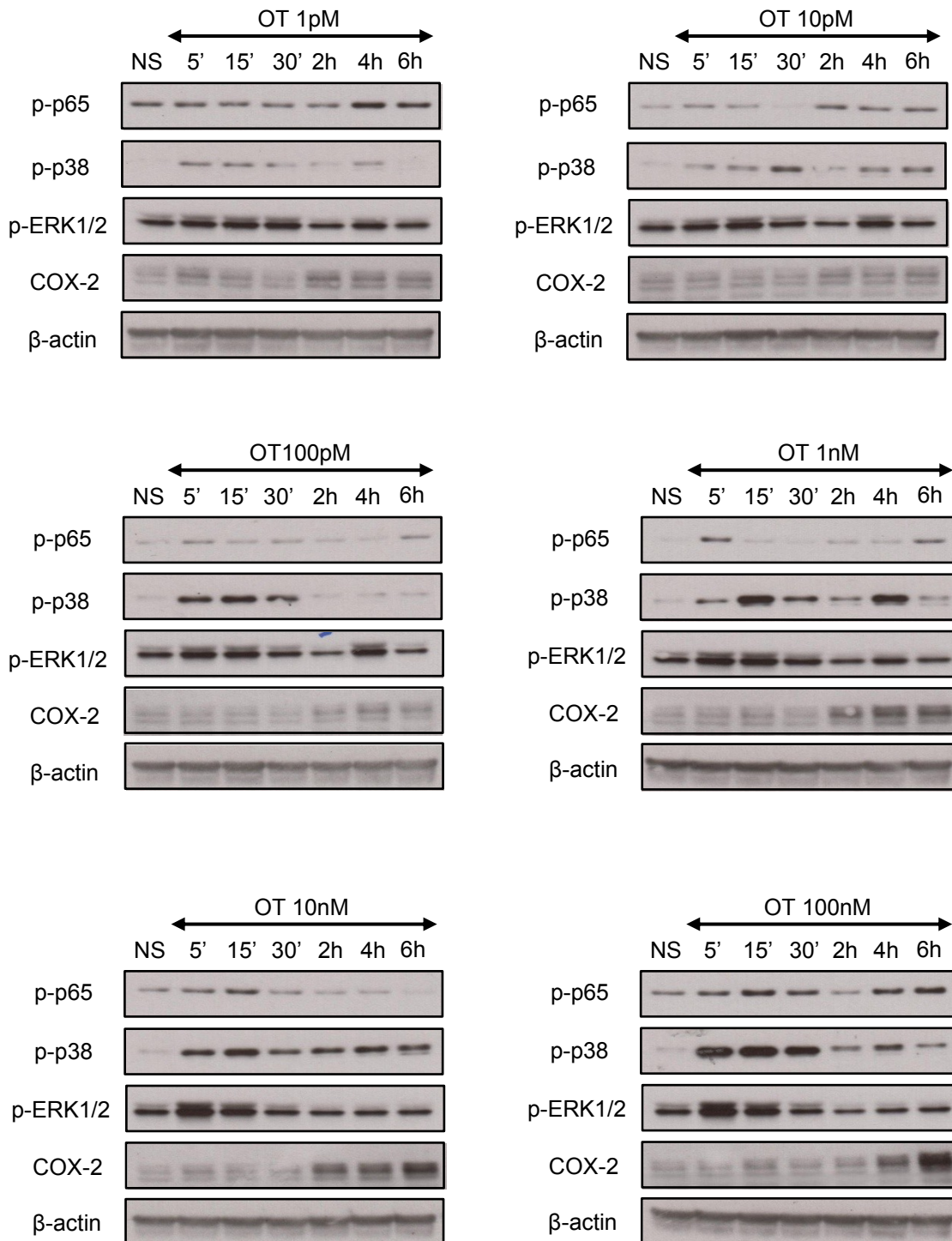


B

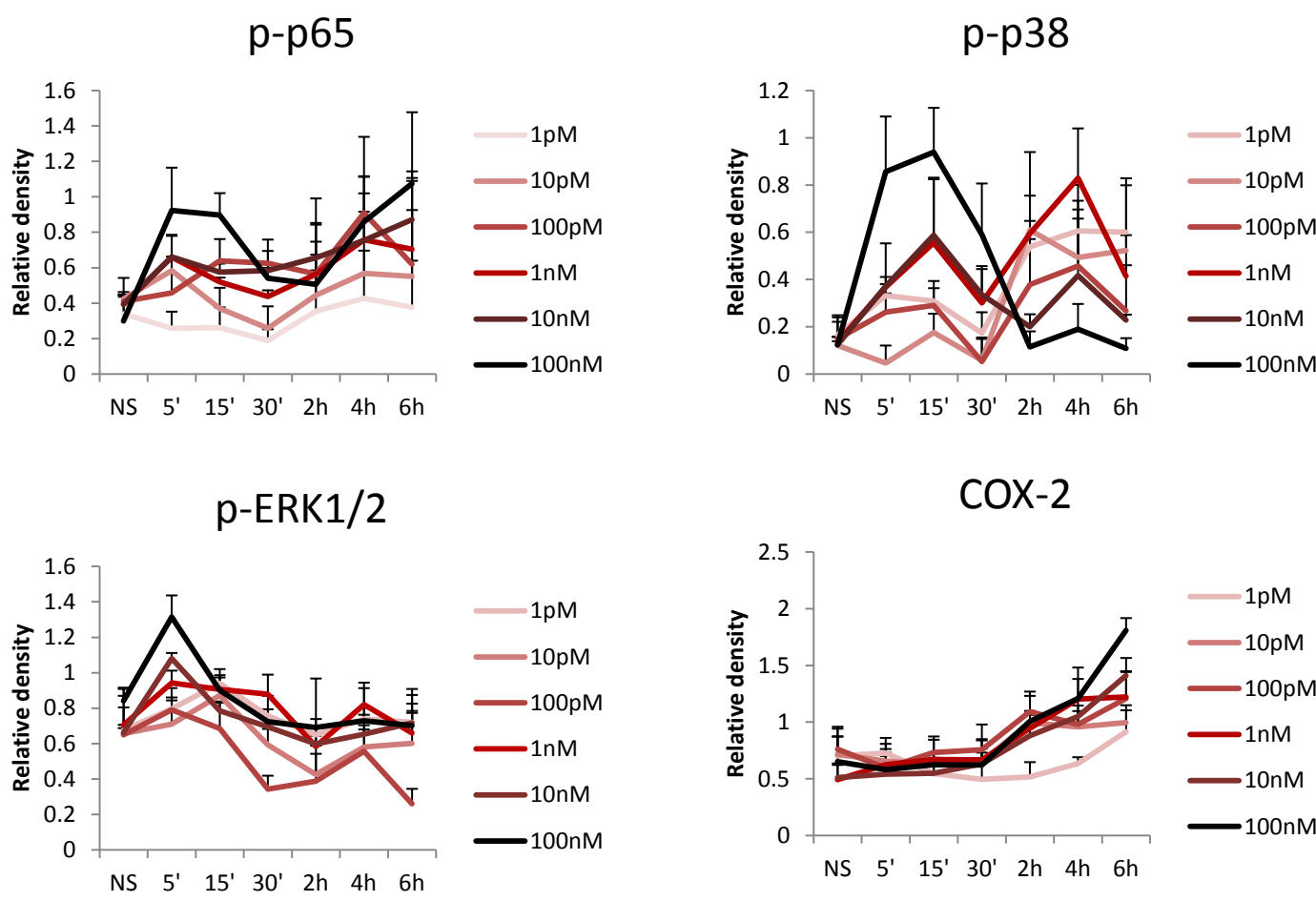


SUPP

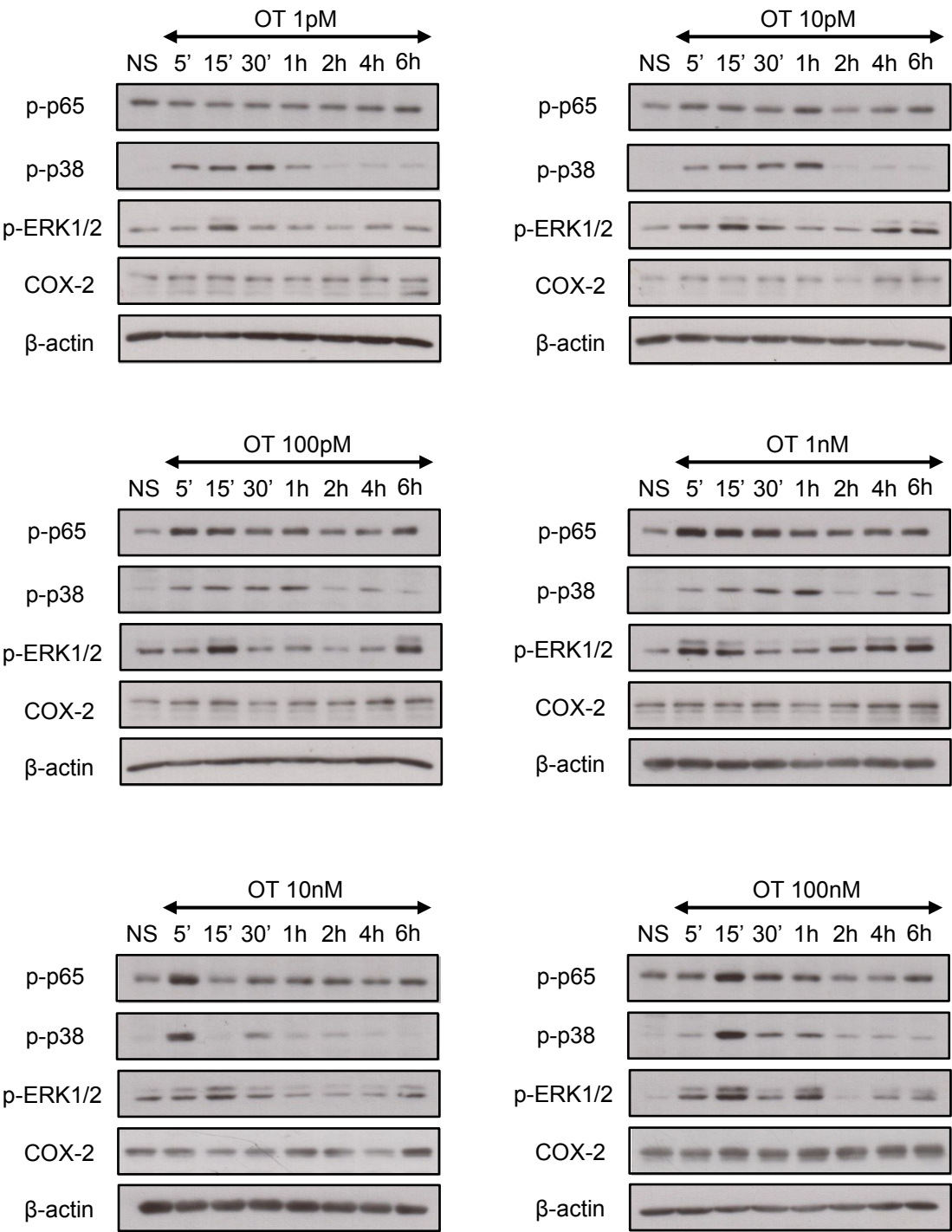
SUPPLEMENTARY FIGURE S1A



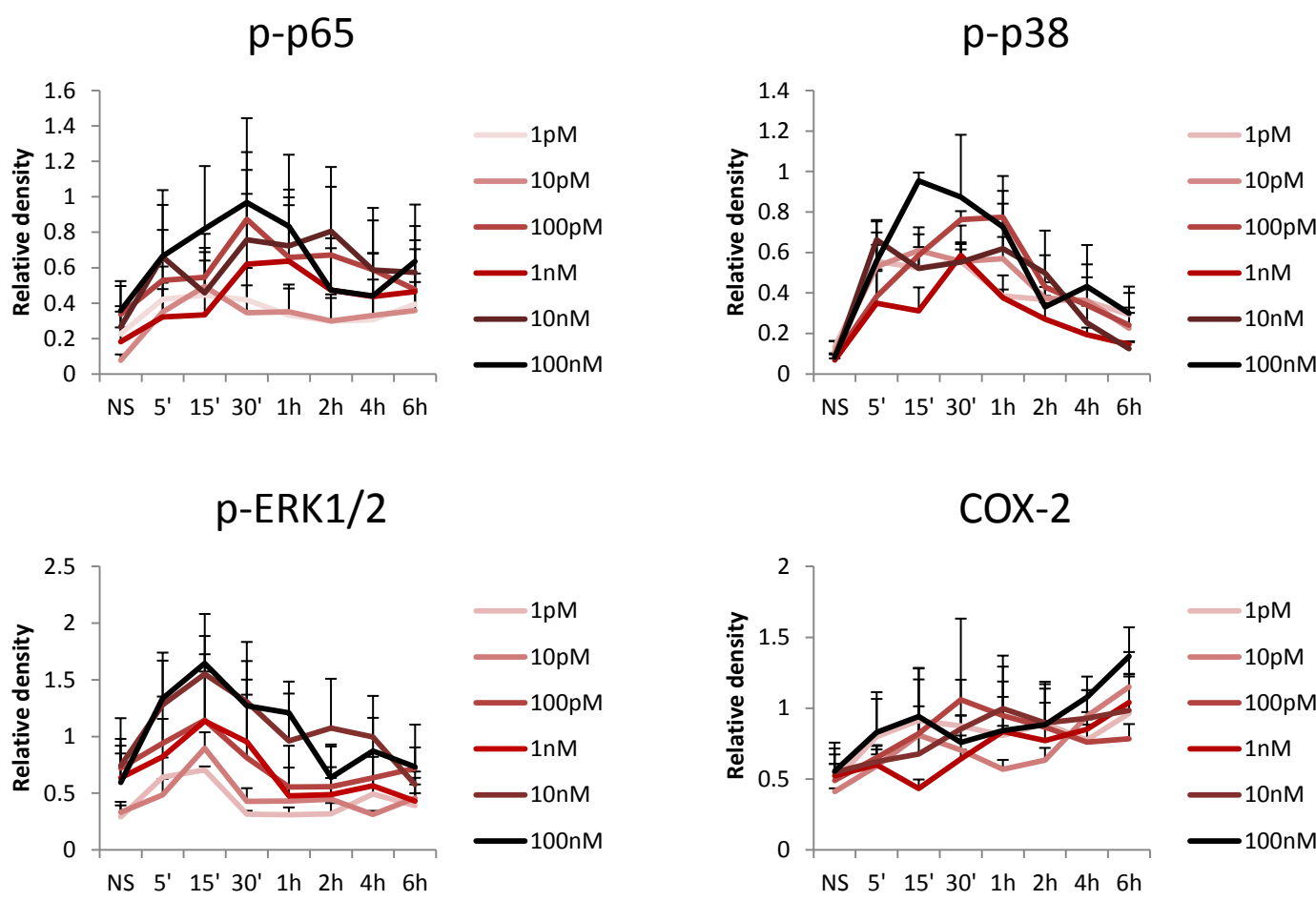
SUPPLEMENTARY FIGURE S1B



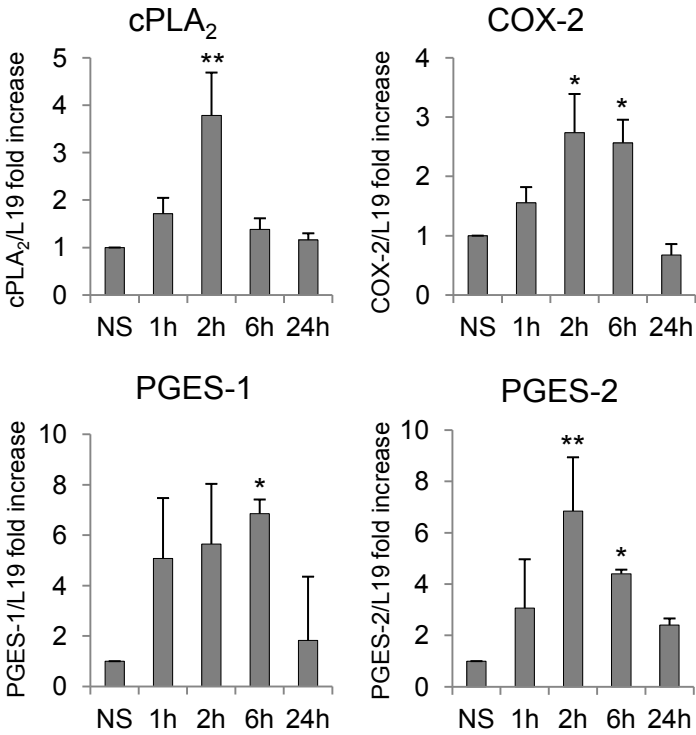
SUPPLEMENTARY FIGURE S2A



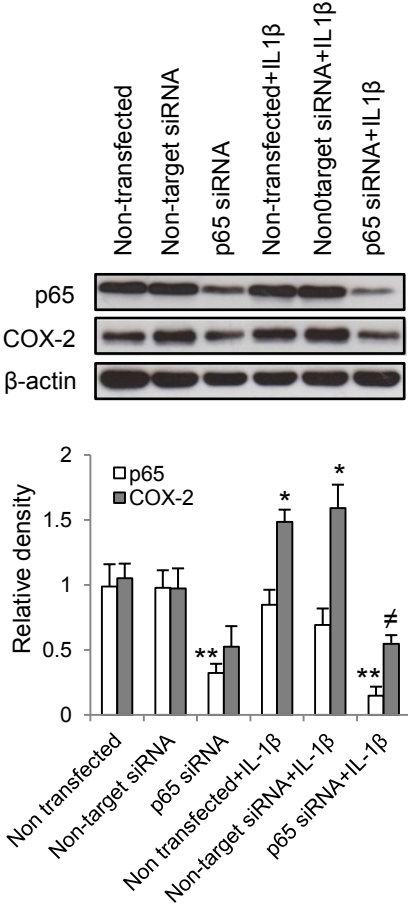
SUPPLEMENTARY FIGURE S2B



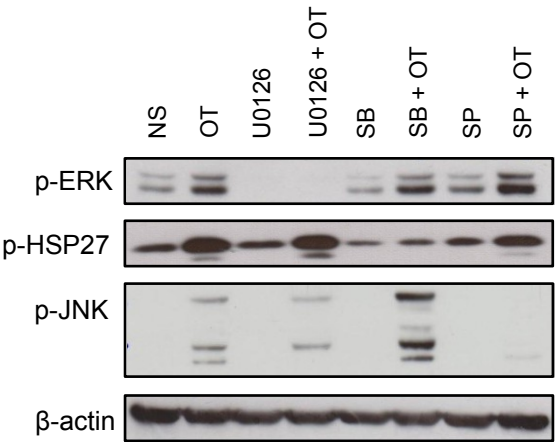
SUPPLEMENTARY FIGURE S3



SUPPLEMENTARY FIGURE S4



SUPPLEMENTARY FIGURE S5



SUPPLEMENTARY FIGURE S6

