

**Original citation:**

Kim, Sung Hye, MacIntyre, David A., Hanyaloglu, Aylin C., Blanks, Andrew M., Thornton, Steven, Bennett, Phillip R. and Terzidou, Vasso. (2016) The oxytocin receptor antagonist, Atosiban, activates pro-inflammatory pathways in human amnion via Gαi signalling. *Molecular and Cellular Endocrinology*, 420 . pp. 11-23.

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## The oxytocin receptor antagonist, Atosiban, activates pro-inflammatory pathways in human amnion via $G_{\alpha i}$ signalling



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### ARTICLE INFO

#### Article history:

Received 4 August 2015

Received in revised form

9 November 2015

Accepted 9 November 2015

Available online 14 November 2015

#### Keywords:

Oxytocin

G protein

NF- $\kappa$ B

Amnion

Parturition

### ABSTRACT

Oxytocin (OT) plays an important role in the onset of human labour by stimulating uterine contractions and promoting prostaglandin/inflammatory cytokine synthesis in amnion via oxytocin receptor (OTR) coupling. The OTR-antagonist, Atosiban, is widely used as a tocolytic for the management of acute preterm labour. We found that in primary human amniocytes, Atosiban (10  $\mu$ M) signals via PTX-sensitive  $G_{\alpha i}$  to activate transcription factor NF- $\kappa$ B p65, ERK1/2, and p38 which subsequently drives upregulation of the prostaglandin synthesis enzymes, COX-2 and phospho-cPLA<sub>2</sub> and excretion of prostaglandins (PGE<sub>2</sub>) (n = 6; p < 0.05, ANOVA). Moreover, Atosiban treatment increased expression and excretion of the inflammatory cytokines, IL-6 and CCL5. We also showed that OT-simulated activation of NF- $\kappa$ B, ERK1/2, and p38 and subsequent prostaglandin and inflammatory cytokine synthesis is via  $G_{\alpha i-2}$  and  $G_{\alpha i-3}$  but not  $G_{\alpha q}$ , and is not inhibited by Atosiban. Activation or exacerbation of inflammation is not a desirable effect of tocolytics. Therefore therapeutic modulation of the OT/OTR system for clinical management of term/preterm labour should consider the effects of differential G-protein coupling of the OTR and the role of OT or selective OTR agonists/antagonists in activating proinflammatory pathways.

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### 1. Introduction

Early preterm birth (<34 weeks gestation) accounts for the majority of neonatal morbidity and mortality and is often associated with infection and/or inflammation. While uterine inflammation can activate contractile pathways leading to preterm labour (PTL) it also represents one of the most potent causes of cytokine mediated cerebral injury in the neonate (Thornton et al., 2012). In full term infants, chorioamnionitis and maternal infection are

associated with cerebral palsy and periventricular leucomalacia (Grether and Nelson, 1997; Wu and Colford, 2000). Atosiban is a nonapeptide, desamino-oxytocin analogue and a competitive oxytocin/vasopressin receptor antagonist used clinically to reduce uterine activity in threatened PTL (Goodwin et al., 1994; Usta et al., 2011). Atosiban is generally considered to be a relatively safe tocolytic with an improved adverse drug profile to earlier tocolytics such as ritrodine (Husslein et al., 2006). Its efficacy was established in 6 large randomised controlled double blinded trials (French/Australian Atosiban Investigators, 2001; Goodwin et al., 1994, 1996; Moutquin et al., 2000; Norton et al., 1993; Romero et al., 2000), however a meta-analysis of these trials involving 1695 women suggested that Atosiban does not reduce the incidence of preterm birth or improve the neonatal outcome compared with placebo (Papatonis et al., 2005).

While the precise mechanisms underpinning human labour initiation are not fully understood, it is well established that oxytocin (OT) acting through its G protein-coupled receptor (GPCR)

*Abbreviations:* OT, oxytocin; OTR, oxytocin receptor; MAPK, mitogen activated protein kinase; PG, prostaglandin; GPCR, G-protein coupled receptor; PLC, phospholipase C; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP<sub>3</sub>, inositol triphosphate.

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<http://dx.doi.org/10.1016/j.mce.2015.11.012>

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stimulates and maintains uterine contractions by elevating intracellular calcium levels. Just prior to the onset of human labour, uterine sensitivity to OT increases markedly via up-regulation of the OTR (Fuchs et al., 1982). Binding of OT to its receptor (OTR) leads to the activation of its heterotrimeric G-proteins  $G_{\alpha q/11}$  and  $G_{\alpha i/o}$  resulting in the activation of phospholipase C (PLC), hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) to diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>), leading to an increase in intracellular Ca<sup>2+</sup> for  $G_{\alpha q/11}$ , while signalling through  $G_{\alpha i/o}$  results in inhibition of adenylate cyclase activity and a reduction in intracellular cAMP levels (Busnelli et al., 2012; Gravati et al., 2010; Strakova and Soloff, 1997).

We and others have recently described a role for the OT-OTR system in the onset of human labour, additional to the stimulation of myometrial contractions, that involves increase in the expression of COX-2 and other inflammatory mediators known to be associated with the onset of labour (Kim et al., 2015; Pont et al., 2012). OT-OTR interaction leads to NF- $\kappa$ B activation and subsequent upregulation of prostaglandins, inflammatory chemokines and cytokines that are known to play roles in fetal membrane remodelling, cervical ripening and myometrial activation. Amnion is an important site of prostaglandin production within the human uterus (Bennett et al., 1992; Olson, 2003) and its activation is critical for fetal membrane remodelling, cervical ripening and the stimulation of myometrial contractions (Kim et al., 2015). In this study we show that Atosiban fails to inhibit the proinflammatory effects of OT upon human amnion and itself stimulates inflammatory pathway activation in amnion via agonism of  $G_{\alpha i}$ .

## 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 (RREC 2002-6283). Fetal membranes were obtained from women undergoing elective caesarean section at term (38–39 weeks of pregnancy), prior to the onset of labour. Exclusion criteria included pre-existing medical conditions, multiple pregnancies or treatment with uterotonics. Amnion epithelial cells were prepared from tissue as previously described (Bennett et al., 1987).

### 2.2. Real time polymerase chain reaction (RT-PCR)

Total RNA was extracted by a guanidiumthiocyanate-phenol-chloroform extraction using RNA STAT-60 reagent (AMS Biotechnology) 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'-GCAGCCGGCGCAA-3'; *COX-2*, 5'-TGTGCAACACTTGAGT-GGCT-3' and 5'-ACTTCTGTACTGCGGGT-G-3'; *IL-6*, 5'-CCTTCC-AAA-GATGGCTGAAA-3' and 5'-AGCTCTGGCTTGTCTCTCAC-3'; and *CCL5*, 5'-CCATA-TTCTCGGACACCAC-3' and 5'-TGACTCC-CGAACC-CATTC-3'. The data were analysed using Sequence Detector Version 1.7 software (Applied Biosystems). Expression levels were assessed using the comparative Ct method and the target Ct values were normalised to ribosomal protein *L19* for analysis.

### 2.3. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were performed using Phusion high-fidelity DNA polymerase (New England Biolabs). PCR reaction mix was prepared following the manufacturer's protocol. Template DNA were initially denatured for 30 s at 98 °C, then the reaction was subjected to 30 thermo-cycles of 98 °C for 10 s, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. This was followed by the final extension step of 72 °C for 10 min. PCR products were then analysed by electrophoresis using 1.5–2% (w/v) agarose gels. The size of DNA fragments were estimated by using a hyperladder V (Bioline) containing restriction fragments of known sizes. The bands were imaged using a dark reader (Dark Reader transilluminator, Clare Chemical Research).

### 2.4. Protein extraction and Western blot

Cells were lysed on ice for 10 min in radioimmunoprecipitation assay buffer consisting of 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris (pH 7.4) and 1 mM EDTA with 1 mM of PMSF, protease and phosphatase inhibitor cocktail (Thermo-fisher). Lysed samples were centrifuged at 4 °C for 10 min at 13000 × g. The resulting supernatant was recovered and protein concentration determined using the BioRad protein assay kit. A total of 20 µg of total protein was denatured for 10 min at 80 °C before undergoing electrophoretic separation on a 10% SDS-polyacrylamide gel for 80 min at 140 V. Resolved proteins were then transferred to PVDF membrane (Millipore) using a wet-transfer chamber system (BioRad) for 90 min at 300 mA. Membranes were incubated in primary antibodies (detailed below) overnight at 4 °C followed by incubation with HRP-conjugated secondary antibodies the following day. Signal detection was carried out using ECL plus (GE Amersham Biosciences). To confirm equal loading of each well, the membranes stripped using 0.2 M NaOH for 10 min and re-probed for  $\beta$ -actin.

### 2.5. siRNA gene silencing

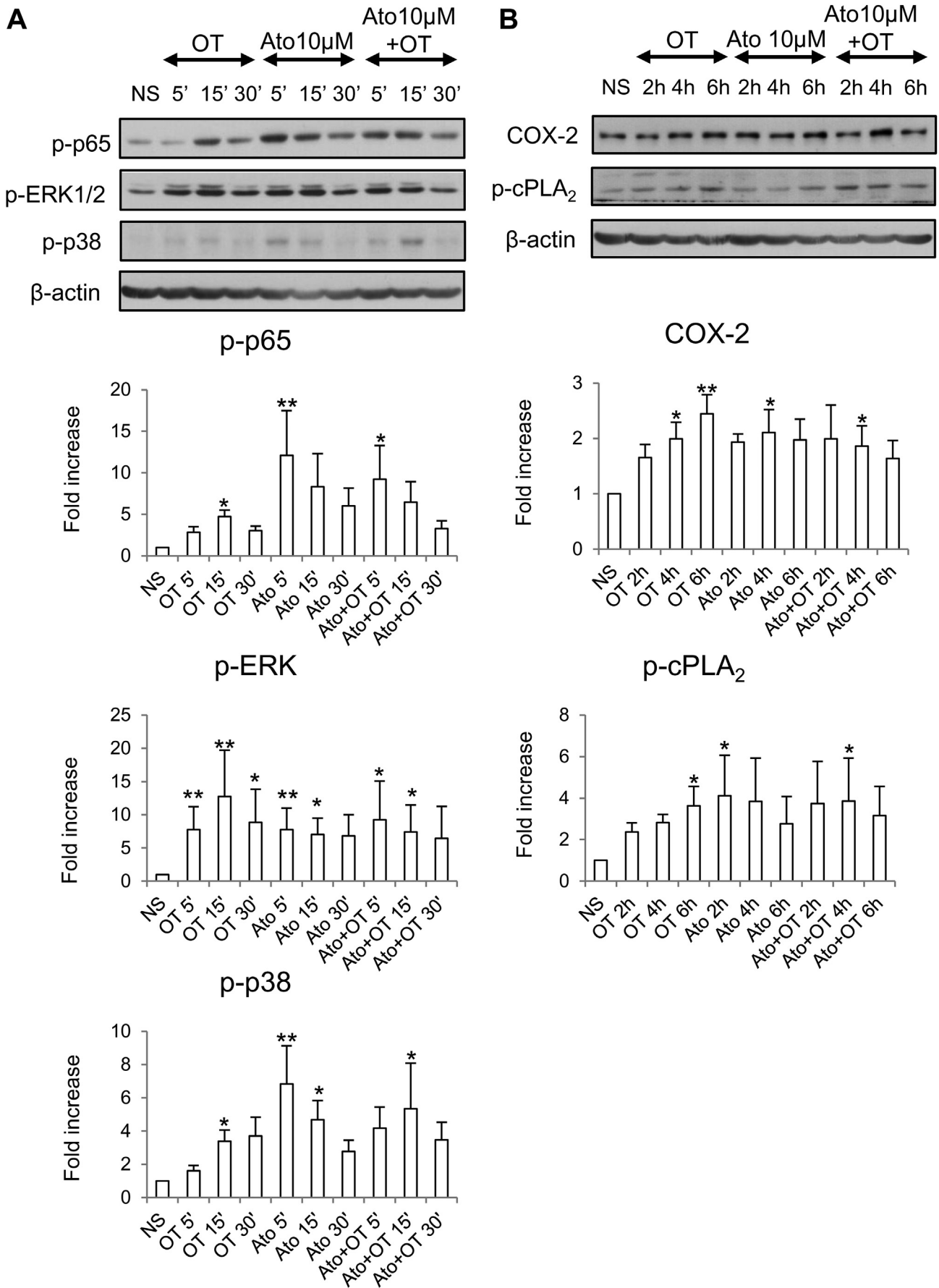
Transfection for gene silencing studies was performed using the Amaxa Nucleofector Technology (Lonza) according to manufacturer's protocol. The cell/siRNA suspension was transfected with 30 pmol of siGENOME SMARTpool siRNA (Dharmacon) via electroporation using the Program T-020. Total proteins from the transfected cells were extracted for further analysis at 72 h.

### 2.6. ELISA

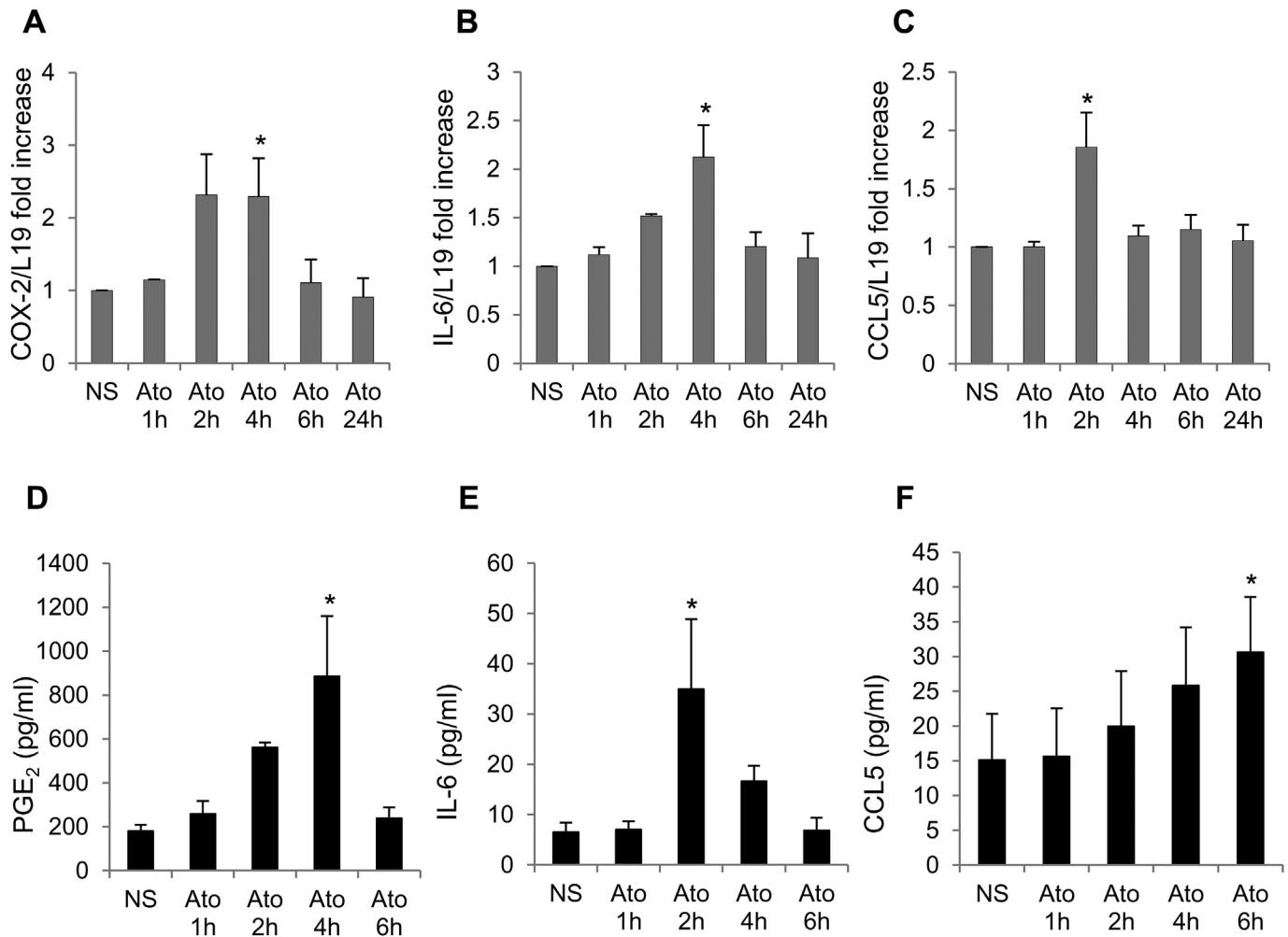
Concentrations of IL-6, CCL5 and PGE<sub>2</sub> released were determined by a standard ELISA. Supernatants were collected from treated amnion cultures and immediately frozen at –20 °C for subsequent analysis by ELISA according to manufacturer's instructions (R&D systems).

### 2.7. Antibodies and materials

The following antibodies were obtained from Santa Cruz Biotechnologies: goat anti-COX-2 (1:2000); anti- $G_{\alpha q}$  (1:1000) and HRP-conjugated secondary antibodies raised against goat (1:2000), rabbit (1:2000), and mouse IgGs (1:2000). Rabbit monoclonal antibodies to p-cPLA<sub>2</sub> (1:2000); phospho-p65 (ser536) (1:5000); phospho-IKK $\alpha/\beta$  (1:1000); phospho-p38 MAPK (1:1000); phospho-ERK1/2 (1:5000) and p-SAPK/JNK (1:2000) were from Cell Signalling Technology. The mouse monoclonal anti- $\beta$ -actin (1:50000); anti- $G_{\alpha i2}$  (1:1000) and anti- $G_{\alpha i3}$  (1:1000) antibodies were from Abcam. Inhibitors PTX and UBO-QIC were purchased from ENZO



**Fig. 1. Atosiban does not inhibit OT-induced proinflammatory effects.** Pre-labour amnion epithelial cells were treated with Atosiban (10  $\mu$ M) and OT (100 nM) for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell proteins were subjected to Western blot analyses with antibodies against different phosphorylated NF- $\kappa$ B p65 subunit, ERK1/2 and p38 MAPK (A), as well as COX-2 and p-cPLA<sub>2</sub> (B), and matching densitometry analyses shown as fold changes compared to non-stimulated (NS) controls have been added below the representative blots. Membranes were probed with  $\beta$ -actin to confirm equal loading (n = 6; \*p < 0.05, \*\*p < 0.01 compared with NS, ANOVA).



**Fig. 2.** Increase in prolabour NF-κB-regulated gene expression with Atosiban stimulation. Pre-labour amnion epithelial cells were treated with Atosiban (10 μM) for 1 h, 2 h, 4 h, and 6 h. Total RNA was subjected to gene expression analysis for downstream NF-κB regulated genes; COX-2 (A), IL-6 (B) and CCL5 (C) (n = 6; \*p < 0.05 compared with NS, ANOVA). PGE<sub>2</sub> (D), IL-6 (E), and CCL5 (F) release was assessed using ELISA (n = 6; \*p < 0.05 compared with NS, Friedman's test, followed by Dunn's Multiple Comparisons *post hoc* test).

Lifesciences and University of Bonn, respectively. ON-TARGETplus SMARTpool siRNAs comprised of 3 different siRNAs for Non-Target (D-001810-01-05), G<sub>αq</sub> (L-008562-00-0005), G<sub>αi-2</sub> (L-003897-00-0005), and G<sub>αi-3</sub> (L-005184-00-0005) were purchased from Dharmacon.

### 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

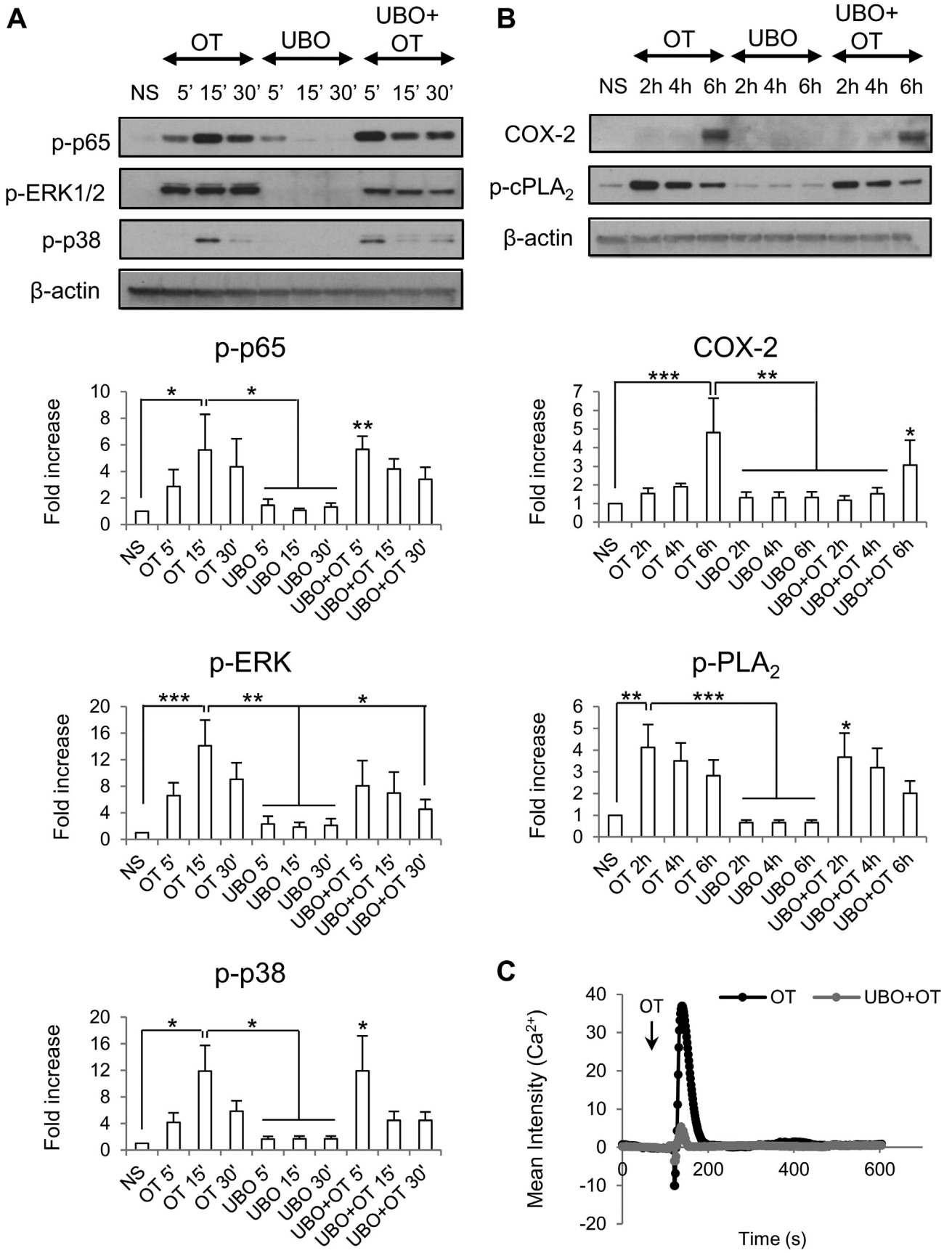
### 3.1. Atosiban fails to inhibit the pro-inflammatory effects of OT in human amnion and instead, stimulates inflammation in human amnion

We have previously shown that the OT/OTR system drives

inflammatory activation in human amnion via NF-κB with subsequent upregulation of prostaglandins and inflammatory chemokines and cytokines (Kim et al., 2015). Incubation of amnion cells with Atosiban had no significant effect upon OT-stimulated activation of NF-κB p65, ERK1/2, or p38 (Fig 1A). Similarly, upregulation of COX-2 and activation of cPLA<sub>2</sub> by OT was unaffected by Atosiban treatments (Fig 1B). Instead incubation of amnion cells with Atosiban alone resulted in activation of NF-κB p65, ERK1/2, and p38 and downstream expression of COX-2 and p-cPLA<sub>2</sub> to a similar degree as seen with OT treatment (Fig 1). We therefore examined the effect of Atosiban on the expression of down-stream NF-κB regulated genes. As demonstrated at the protein level, treatment of amniotic cells with Atosiban alone increased mRNA expression of COX-2 (Fig 2A) as well as IL-6 and CCL5 (Fig. 2B–C). Atosiban treatment also led to increased excretion of prostaglandin (PGE<sub>2</sub>) and cytokines (IL-6 and CCL5) in cell supernatant as assessed by ELISA (Fig. 2D–F).

### 3.2. The pro-inflammatory effect of OT in human amnion is via G<sub>αi</sub>

To gain insight into the molecular mechanisms underlying the pro-inflammatory effects of OT and Atosiban in amnion, we investigated the role of different G proteins potentially involved in OT-OTR signalling. In human myometrium, OTR has been shown to



**Fig. 3.** *G<sub>αq</sub>* inhibitor, UBO-QJC, does not block the pro-inflammatory effects of OT. Pre-labour amnion epithelial cells were incubated in UBO-QJC (1 μM) for 2 h prior to 5 min, 15 min or 30 min of OT stimulation (100 nM). Presence of UBO-QJC suppressed the effect of OT upon ERK1/2 phosphorylation, but not p65 and p38. Denitometry plots show no significant changes of p-p65, p-ERK1/2 and p-p38 in comparison to NS controls (A). Western blot analysis of cells pretreated with UBO-QJC (1 μM) for 2 h prior to 2 h, 4 h or 6 h of OT stimulation (100 nM) showed no significant effect on OT-induced COX-2 expression and phosphorylation of cPLA<sub>2</sub> in presence of inhibitor. Bar graphs show densitometry analysis of COX-2 and p-cPLA<sub>2</sub> (B). Inhibition of calcium response in human myometrial cells were used as a positive control for UBO-QJC (C) (n = 6; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, ANOVA).

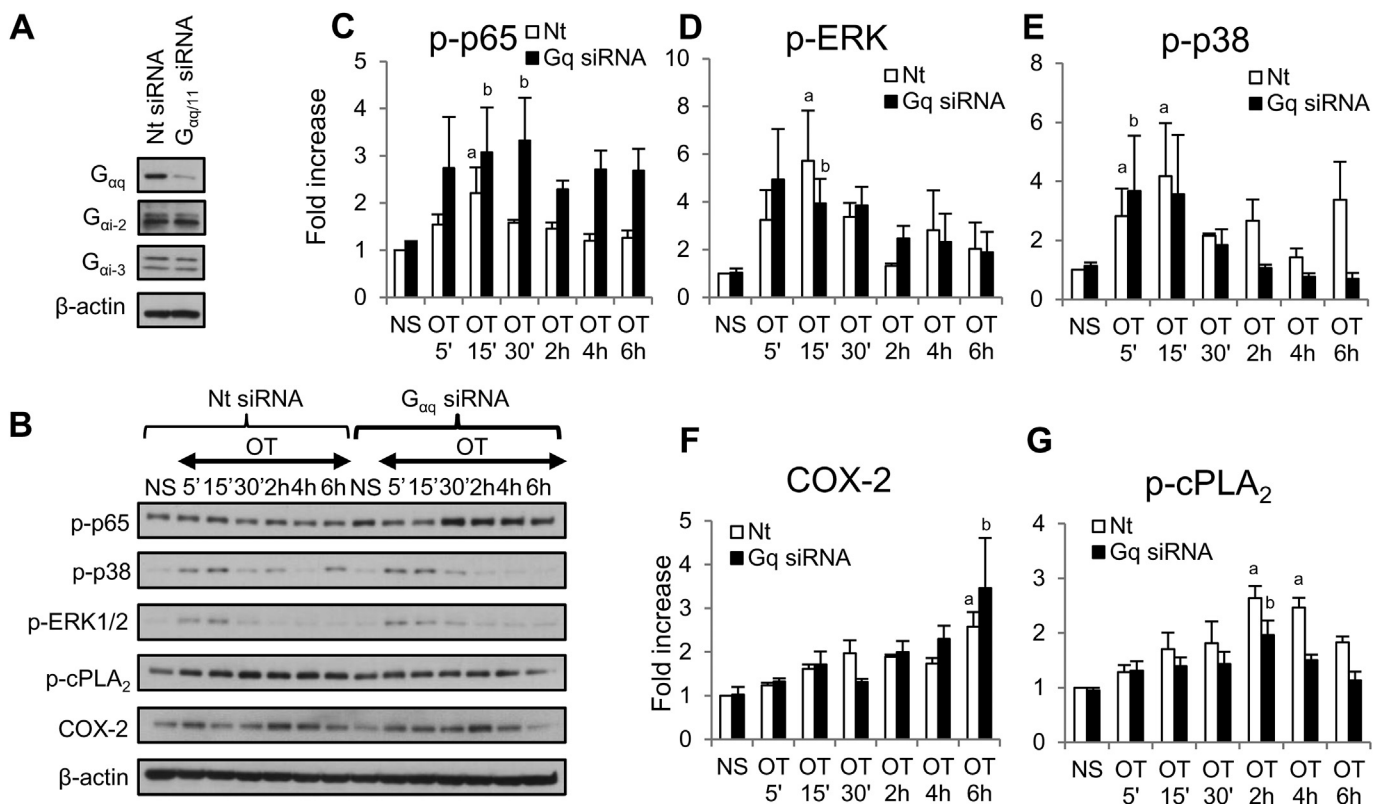
couple to various G proteins including  $G_{\alpha q/11}$  and  $G_{\alpha i}$  (Phaneuf et al., 1993). However, the G protein-coupling of OTR in amnion is unknown. Treatment of amnion samples with the  $G_{\alpha q/11}$  inhibitor, UBO-QIC, had no significant effect on the OT-induced activation of NF- $\kappa$ B p65 or p38 (Fig 3A), or upon COX-2 and p-cPLA<sub>2</sub> expression (Fig 3B). Presence of UBO-QIC resulted in a marginal reduction of OT-induced activation of ERK1/2 (Fig 3A). The efficacy of UBO-QIC was confirmed by demonstrating successful inhibition of calcium influx in human myometrial cells (Fig 3C). These findings were further confirmed using  $G_{\alpha q/11}$  targeted RNAi knockdown whereby down-regulation of  $G_{\alpha q/11}$  protein did not suppress the OT-induced activation of NF- $\kappa$ B and MAPKs, as well as subsequent COX-2 and p-cPLA<sub>2</sub> expression (Fig 4).

In contrast, pre-treatment of amnion cells with the  $G_{\alpha i}$  inhibitor, pertussis toxin (PTX), significantly reduced OT-stimulated NF- $\kappa$ B activation and to a lesser extent MAPK activation (Fig 5A). Presence of PTX inhibited subsequent OT-induced upregulation of COX-2 and p-cPLA<sub>2</sub> protein levels (Fig 5B). Three subtypes of human  $G_{\alpha i}$  proteins have been identified to date;  $G_{\alpha i-1}$ ,  $G_{\alpha i-2}$  and  $G_{\alpha i-3}$  (Hurowitz et al., 2000). RT-PCR analysis of the  $G_{\alpha i}$  subtypes revealed that  $G_{\alpha i-2}$  and  $G_{\alpha i-3}$  are expressed in amnion but not  $G_{\alpha i-1}$  (Fig 6). To determine the role of specific  $G_{\alpha i}$  subtypes in human amnion, cells were transfected with  $G_{\alpha i-2}$  or  $G_{\alpha i-3}$  targeted siRNA. Transient down-regulation of  $G_{\alpha i-2}$  led to the attenuation of OT-induced activation of MAPKs, NF- $\kappa$ B and downstream COX-2 expression (Fig. 7A–G). Down-regulation of  $G_{\alpha i-2}$  also resulted in reduction of OT-induced p-cPLA<sub>2</sub> expression, however this did not reach significance. Similarly, down-regulation of  $G_{\alpha i-3}$  suppressed OT-induced activation of p38 and COX-2 expression, but unlike  $G_{\alpha i-2}$ ,

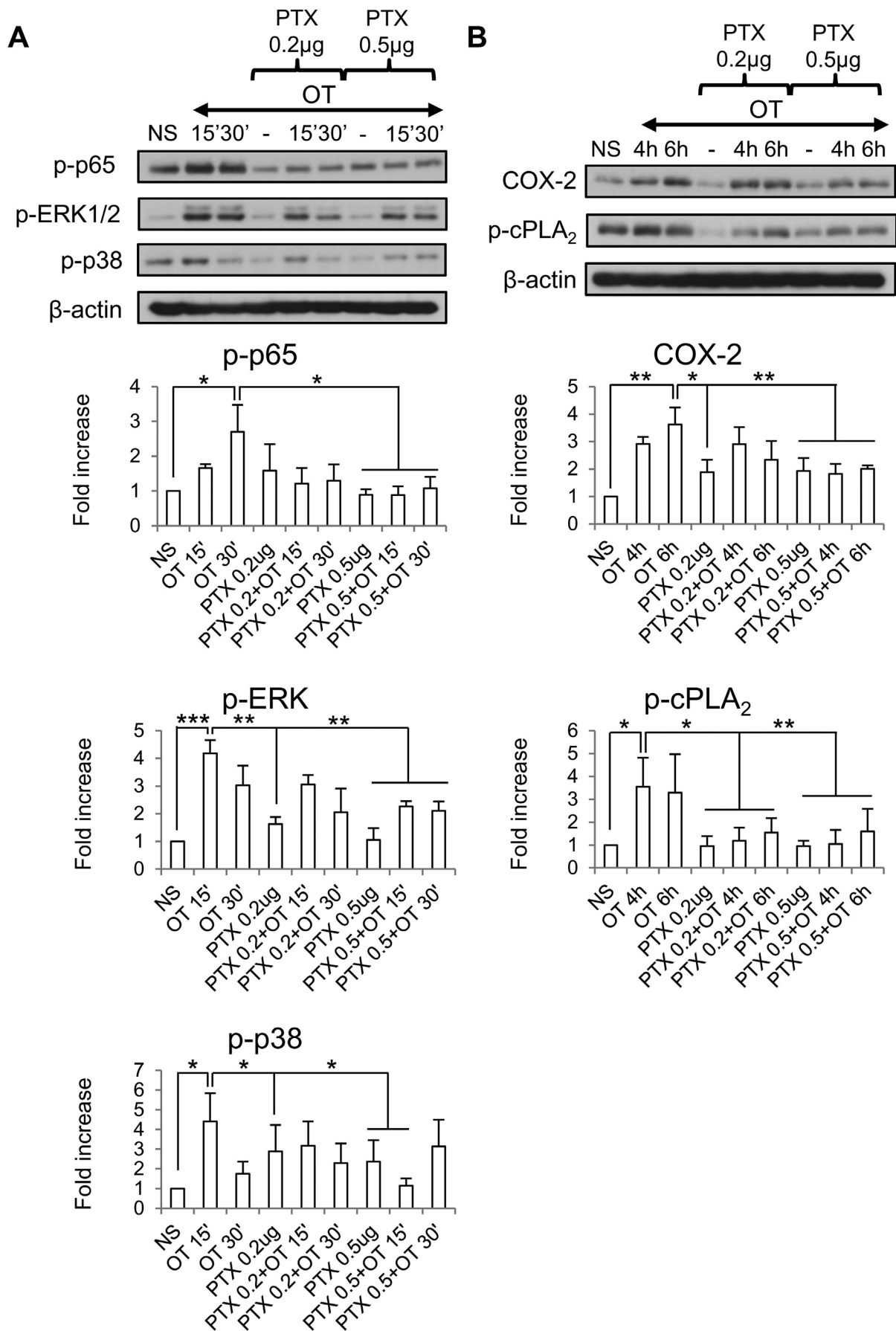
knockdown of  $G_{\alpha i-3}$  showed no significant effect on NF- $\kappa$ B, ERK1/2 and downstream p-cPLA<sub>2</sub> expression (Fig. 8A–G). Inhibition of OT-induced inflammatory activation was observed with both  $G_{\alpha i-2}$  and  $G_{\alpha i-3}$  down-regulation, however, it was most prominent with  $G_{\alpha i-2}$  knockdown suggesting a major role for  $G_{\alpha i-2}$  in OT-mediated inflammatory effects.

### 3.3. G protein signalling bias of Atosiban in human amnion

Recent studies have suggested that Atosiban is a biased ligand that antagonises  $G_{\alpha q/11}$  signalling whilst acting as a  $G_{\alpha i}$  agonist in various cell lines (Busnelli et al., 2012; Reversi et al., 2005). Therefore, we employed Atosiban as a tool to specifically activate OTR-mediated  $G_{\alpha i}$  signalling in human amnion. To demonstrate whether Atosiban exhibits G protein signalling bias in human amnion, we investigated the  $G_{\alpha i}$ -dependence of Atosiban-induced inflammatory responses. Presence of PTX completely blocked Atosiban-induced activation of NF- $\kappa$ B p65 and ERK1/2, and although there was a reduction in p38 activation, it did not reach significance (Fig 9A). PTX also suppressed Atosiban-mediated expression of COX-2 and p-cPLA<sub>2</sub> (Fig 9B). With targeted-knockdown of  $G_{\alpha i-2}$  proteins, the effects of Atosiban on the activation of p38, and expression of COX-2 and p-cPLA<sub>2</sub> appears to be attenuated (Fig. 10A–G). However, only  $G_{\alpha i-3}$  knockdown resulted in significant decreases in Atosiban-induced activation of NF- $\kappa$ B, p38 and downstream COX-2 expression (Fig. 11A–G). These results suggest that  $G_{\alpha i-3}$  protein plays a more significant role in Atosiban-induced activation of inflammatory mediators.

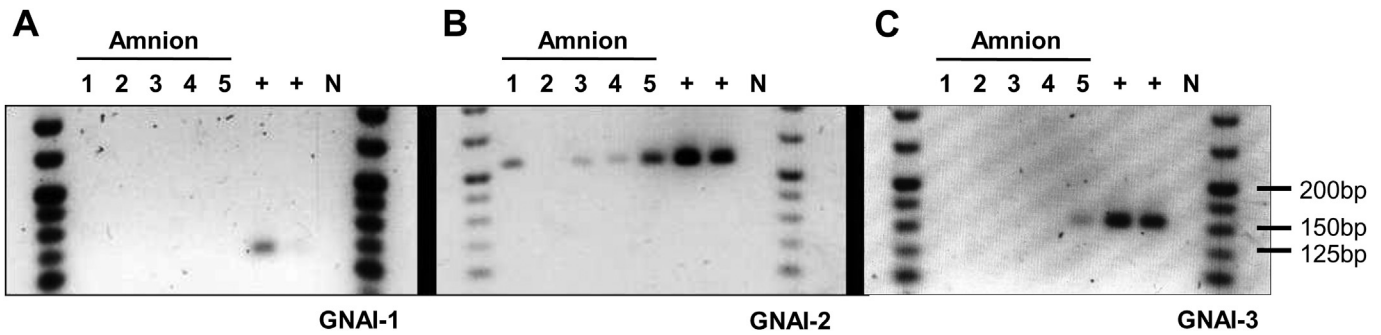


**Fig. 4. Transient knockdown of  $G_{\alpha q}$  does not affect the OT-mediated pro-inflammatory effects.** Pre-labour amnion epithelial cells transfected with non-target siRNA or  $G_{\alpha q}$ -target siRNA were treated with OT (100 nM) for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analyses with antibodies against  $G_{\alpha q}$  (A) to confirm  $G_{\alpha q}$  knockdown with  $G_{\alpha q}$ -target siRNA transfection. siRNA knockdown of  $G_{\alpha q}$  had no effect on OT-induced activation of NF- $\kappa$ B and MAPKs, or expression of COX-2 and p-cPLA<sub>2</sub> (B). Densitometry plots of p-p65 (C), p-ERK1/2 (D), p-p38 (E), COX-2 (F) and p-cPLA<sub>2</sub> (G) show no significant difference in the effects of OT between non-target siRNA and  $G_{\alpha q}$ -target siRNA transfected cells ( $n = 3$ ; **a**  $p < 0.05$  compared with NS of non-target siRNA transfected cells (Nt), **b**  $p < 0.05$  compared with NS of  $G_{\alpha q}$ -target siRNA transfected cells, ANOVA).



**Fig. 5.  $G_{\alpha i}$  inhibitor, PTX, suppresses the pro-inflammatory effects of OT.** Pre-labour amnion epithelial cells were incubated in PTX (0.2  $\mu$ g/ml or 0.5  $\mu$ g/ml) for 30 min prior to 15 min or 30 min of OT stimulation (100 nM). Presence of PTX suppresses the effect of OT upon p65, ERK1/2, and p38 phosphorylation. Densitometry analyses show significant decreases in OT-induced p-p65, p-ERK1/2 and p-p38 expression in presence of PTX (A). Western blot analysis of cells pretreated with PTX (0.2  $\mu$ g/ml or 0.5  $\mu$ g/ml) for 30 min prior to 4 h or 6 h of OT stimulation (100 nM) demonstrated inhibition of OT-induced COX-2 expression and phosphorylation of cPLA<sub>2</sub> in presence of PTX. Bar graphs show densitometry analysis of COX-2 and p-cPLA<sub>2</sub> (B) (n = 6; \*p < 0.05, \*\*p < 0.01, ANOVA).





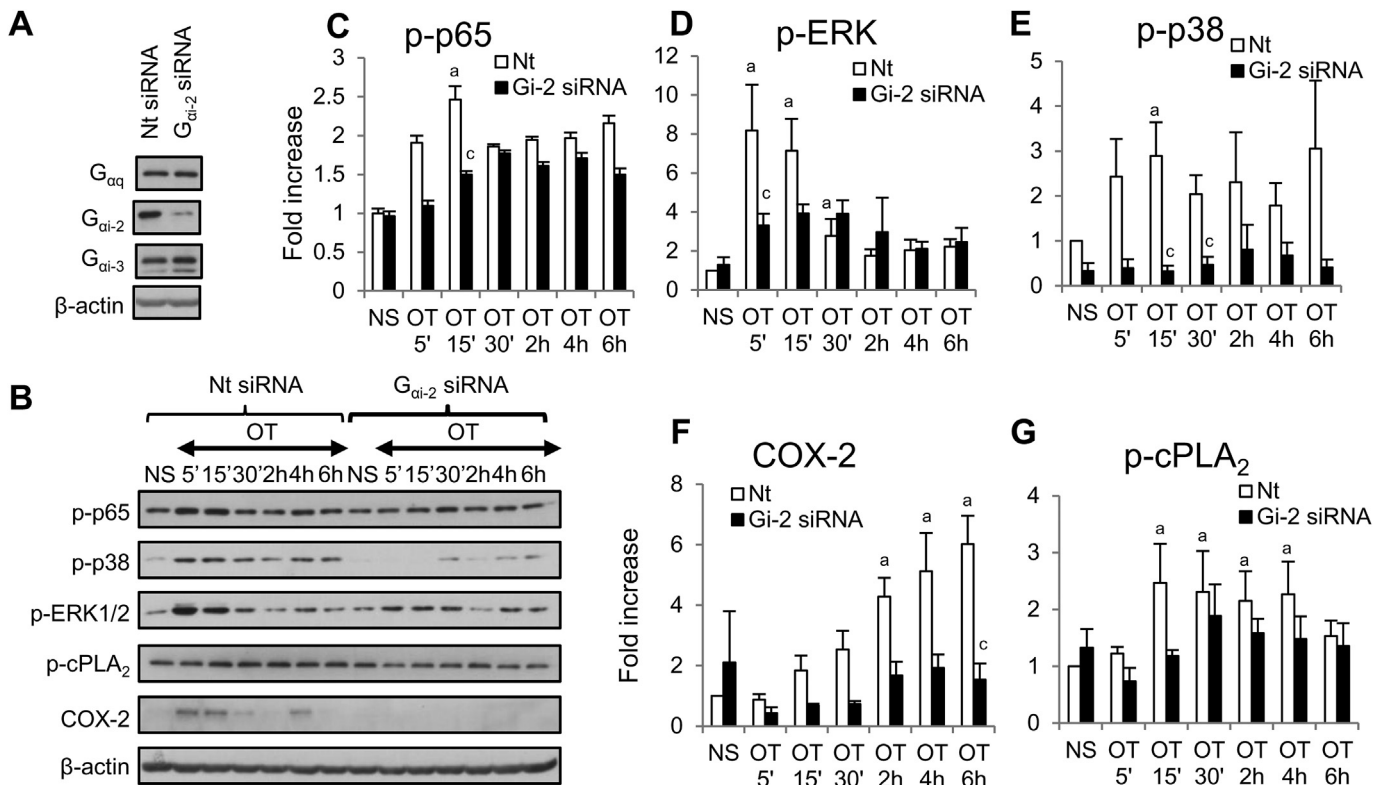
**Fig. 6. Human amnion expresses different subtypes of  $G_{\alpha i}$  proteins.** Pre-labour amnion epithelial cells were established from 5 different samples. cDNAs were synthesized using total RNA extracted from non-stimulated amnion cells. Products of PCR reactions with primers specific for  $G_{\alpha i-1}$ ,  $G_{\alpha i-2}$  and  $G_{\alpha i-3}$  were analysed on 2% agarose gel.  $G_{\alpha i-1}$  (A) was not detectable in amnion, however, the expression of  $G_{\alpha i-2}$  (B) and  $G_{\alpha i-3}$  (C) were observed. Myometrial cDNA was used as positive controls (+) (expected product sizes;  $G_{\alpha i-1}$  115 bp,  $G_{\alpha i-2}$  212 bp and  $G_{\alpha i-3}$  154 bp).

**4. Discussion**

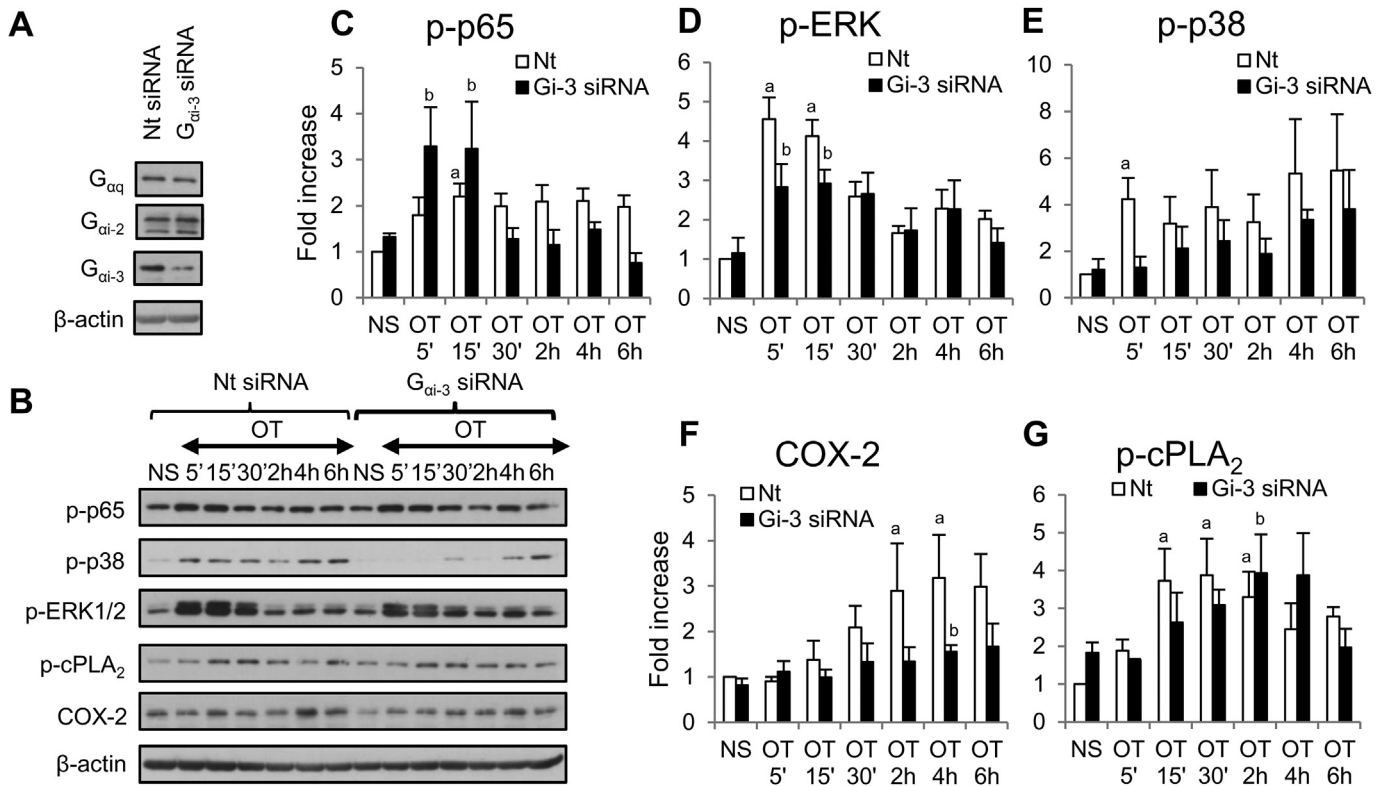
While the OTR antagonist Atosiban has a well-described role in the modulation of myometrial contractility its effect on other gestational tissues is unknown. In this study we demonstrate that Atosiban, a mixed OTR/ $V_{1a}$  antagonist widely used as a tocolytic drug, acts as a biased ligand in amnion cells. It fails to inhibit the activation of pro-inflammatory mediators stimulated by oxytocin, and, in the absence of OT it activates the same pro-labour inflammatory pathways as OT. Treatment of amnion cells with Atosiban had no effect upon OT-mediated activation of NF- $\kappa$ B p65, ERK1/2,

and p38 or expression of COX-2 and cPLA<sub>2</sub>. Moreover, Atosiban treatment alone resulted in activation of NF- $\kappa$ B, ERK1/2 and p38 and downstream expression of COX-2, CCL5, IL-6 and PGE<sub>2</sub>.

Human parturition is associated with an inflammatory cascade that involves elevation of inflammatory cytokines (eg IL-1 $\beta$ , IL-6 and TNF $\alpha$ ) and chemokines (eg IL-8, CCL2 and CCL5) both in amnion and myometrium (Osman et al., 2003; Shynlova et al., 2013). Amnion is a major site of prostaglandin and pro-inflammatory cytokine and chemokine release leading to cervical ripening and fetal membrane remodelling (Kim et al., 2015). Persistent activation of pro-inflammatory pathways in human



**Fig. 7.  $G_{\alpha i-2}$  signalling play a major role in the regulation of OT-induced proinflammatory response.** Pre-labour amnion epithelial cells transfected with non-target siRNA or  $G_{\alpha i-2}$ -target siRNA were treated with OT for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analyses with antibodies against  $G_{\alpha i-2}$  (A) to confirm  $G_{\alpha i-2}$  knockdown with  $G_{\alpha i-2}$ -target siRNA transfection. siRNA knockdown of  $G_{\alpha i-2}$  suppressed OT-induced activation of NF- $\kappa$ B and MAPKs, or expression of COX-2 (B). Densitometry plots of p-p65 (C), p-ERK1/2 (D), p-p38 (E), COX-2 (F) and p-cPLA<sub>2</sub> (G) show significant decrease in OT-induced activation of NF- $\kappa$ B and MAPKs, as well as COX-2 with  $G_{\alpha i-2}$ -target siRNA transfection (n = 3; **a**  $p < 0.05$  compared with NS of Nt, **b**  $p < 0.05$  compared with NS of  $G_{\alpha i-2}$ -target siRNA transfected cells, **c**  $p < 0.05$  Nt vs  $G_{\alpha i-2}$ -target siRNA transfected cells, ANOVA).



**Fig. 8.** The OT-mediated activation of NF- $\kappa$ B and MAPKs, and downstream PG synthetic enzyme expression involve  $G_{\alpha i-3}$  signalling. Pre-labour amnion epithelial cells transfected with non-target siRNA or  $G_{\alpha i-3}$ -target siRNA were treated with OT for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analyses with antibodies against  $G_{\alpha i-3}$  (A) to confirm  $G_{\alpha i-3}$ -knockdown with  $G_{\alpha i-3}$ -target siRNA transfection. siRNA knockdown of  $G_{\alpha i-3}$  reduced OT-induced expression of COX-2 but had a lesser effect on the OT-induced activation of NF- $\kappa$ B and MAPKs (B). Densitometry plots of p-p65 (C), p-ERK1/2 (D), p-p38 (E), COX-2 (F) and p-cPLA<sub>2</sub> (G) show a decrease in OT-induced activation of p38 and expression of COX-2, but not in OT-induced activation of NF- $\kappa$ B or ERK1/2 with  $G_{\alpha i-3}$ -target siRNA transfection ( $n = 3$ ;  $a$   $p < 0.05$  compared with NS of Nt,  $b$   $p < 0.05$  compared with NS of  $G_{\alpha i-3}$ -target siRNA transfected cells, ANOVA).

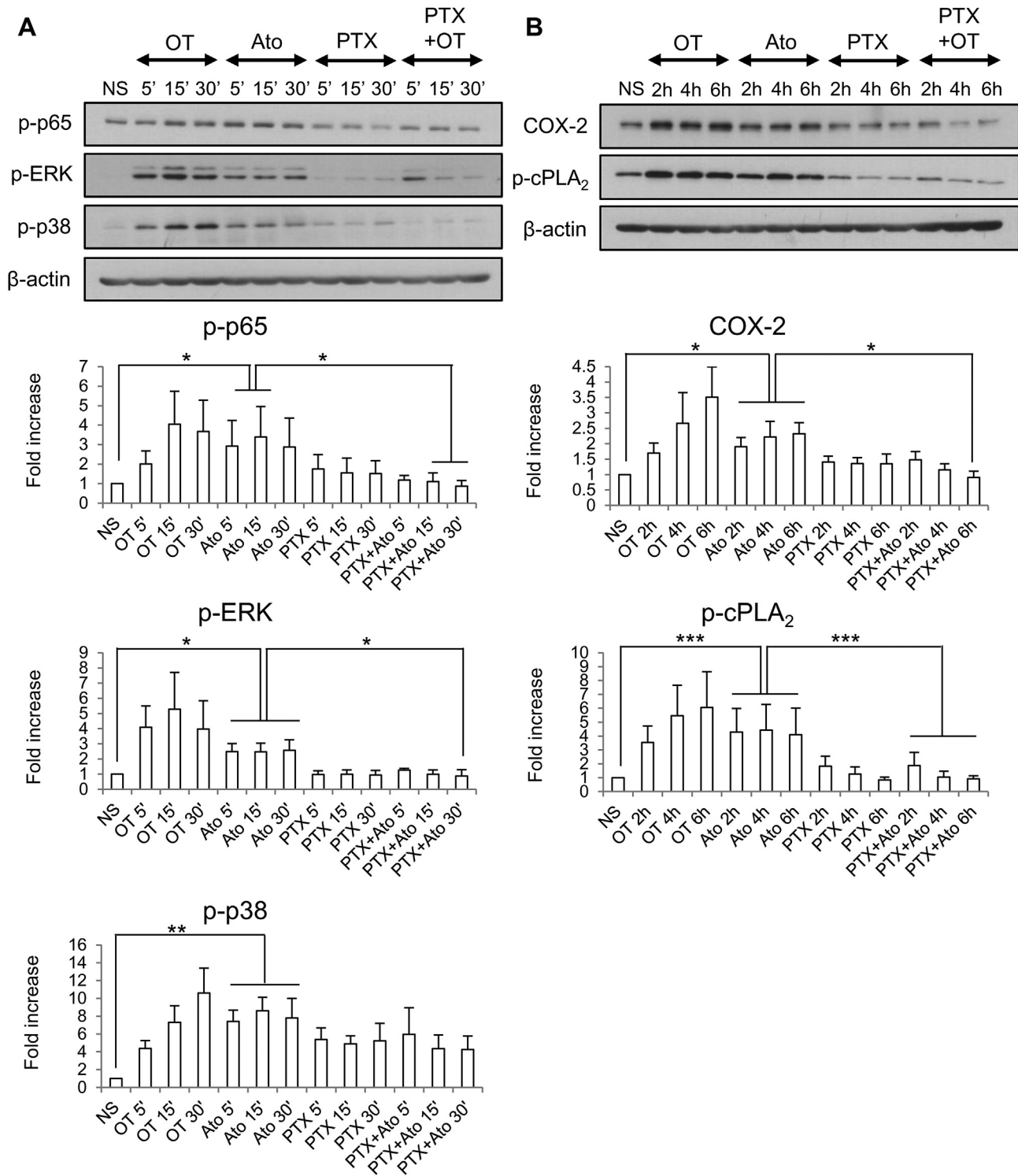
amnion including activation of NF- $\kappa$ B, appears to precede the onset of labour. We have hypothesised that fetal membrane activation is a committing step on the pathway to the onset of labour, resolved only when the fetal membranes are expelled following delivery with the placenta (Lim et al., 2012). We have shown increased expression of OTR in amnion correlates with activation of NF- $\kappa$ B, and that OT treatment of both amnion and myometrial cells activates a similar cassette of inflammatory mediators suggesting that OT acts as an endogenous inflammatory signalling molecule, and potentially plays a role in the activation of amnion which occurs pre-labour (Kim et al., 2015).

OTRs are coupled to both  $G_{\alpha q}$  and  $G_{\alpha i}$  that collectively regulate diverse cellular functions through complex signalling pathways. While the subtypes of G proteins involved in OT-induced COX-2 expression in myometrium are well described (Phaneuf et al., 1996, 1993; Zhou et al., 2006), they have not been investigated in human amnion. A study in rabbit amnion reported that OT-induced Ca<sup>2+</sup> response and activation of ERK1/2 is  $G_{\alpha q}$ -dependent (Jeng et al., 2000). Further studies confirming the significance of Ca<sup>2+</sup> response and  $G_{\alpha q}$  linked Ca<sup>2+</sup> signalling in OT-initiated pro-inflammatory effects in human amnion are required to clarify the tissue-specific signalling properties of OT/OTR system. However, the results in this study indicate that OT-initiated pro-inflammatory signal transduction in human amnion epithelial cells is majorly  $G_{\alpha i}$ -dependent. Under conditions where  $G_{\alpha q}$  proteins were down-regulated by targeted siRNA transfection, OT-induced activation of MAPKs and NF- $\kappa$ B and subsequent COX-2 and p-cPLA<sub>2</sub> expressions were not affected, suggesting that OTR- $G_{\alpha q}$  coupling is not required to propagate OT-induced pro-inflammatory signalling in

human amnion. Reduced concentrations of  $G_{\alpha q}$  proteins are associated with compensatory changes in the levels of other G proteins including  $G_{\alpha i}$  (Krumins and Gilman, 2006). However, this compensatory effect can be excluded as we did not observe any changes in the other G protein expressions with  $G_{\alpha q}$  knockdowns.

The  $G_{\alpha i}$  inhibitor, PTX, has partial inhibitory effects in rat and human myometrium (Phaneuf et al., 1993; Zhou et al., 2006). In our study OT-induced MAPK and NF- $\kappa$ B activation as well as COX-2 and p-cPLA<sub>2</sub> protein levels were markedly reduced by PTX indicating a key role for  $G_{\alpha i}$  in OT signalling. However, as PTX does not discriminate between different  $G_{\alpha i}$  subtypes, we used  $G_{\alpha i}$  targeted siRNA knockdown to definitively demonstrate the functional requirement of  $G_{\alpha i-2}$  and  $-3$  subtypes in response to OT stimulation. Down-regulation of  $G_{\alpha i-2}$  blocked OT-induced activation of NF- $\kappa$ B and MAPK, as well as expression of COX-2. However, OT-mediated ERK activation and p-cPLA<sub>2</sub> expression was not completely inhibited by the knockdown of  $G_{\alpha i-2}$ . This could possibly be due to the involvement of  $\beta$ -arrestin signalling which can activate ERK1/2 independent of G proteins (Shenoy et al., 2006) resulting in p-cPLA<sub>2</sub> expression.  $G_{\alpha i-3}$  targeted knockdown only inhibited the rapid activation of MAPKs and COX-2 expression but to a lesser extent. These data demonstrate that  $G_{\alpha i-2}$  and not  $G_{\alpha q}$  signalling plays a significant role in mediating these OT-induced responses leading to a sequential activation of MAPKs and NF- $\kappa$ B and subsequent inflammatory pathway activation that promotes the labour phenotype in human amnion.

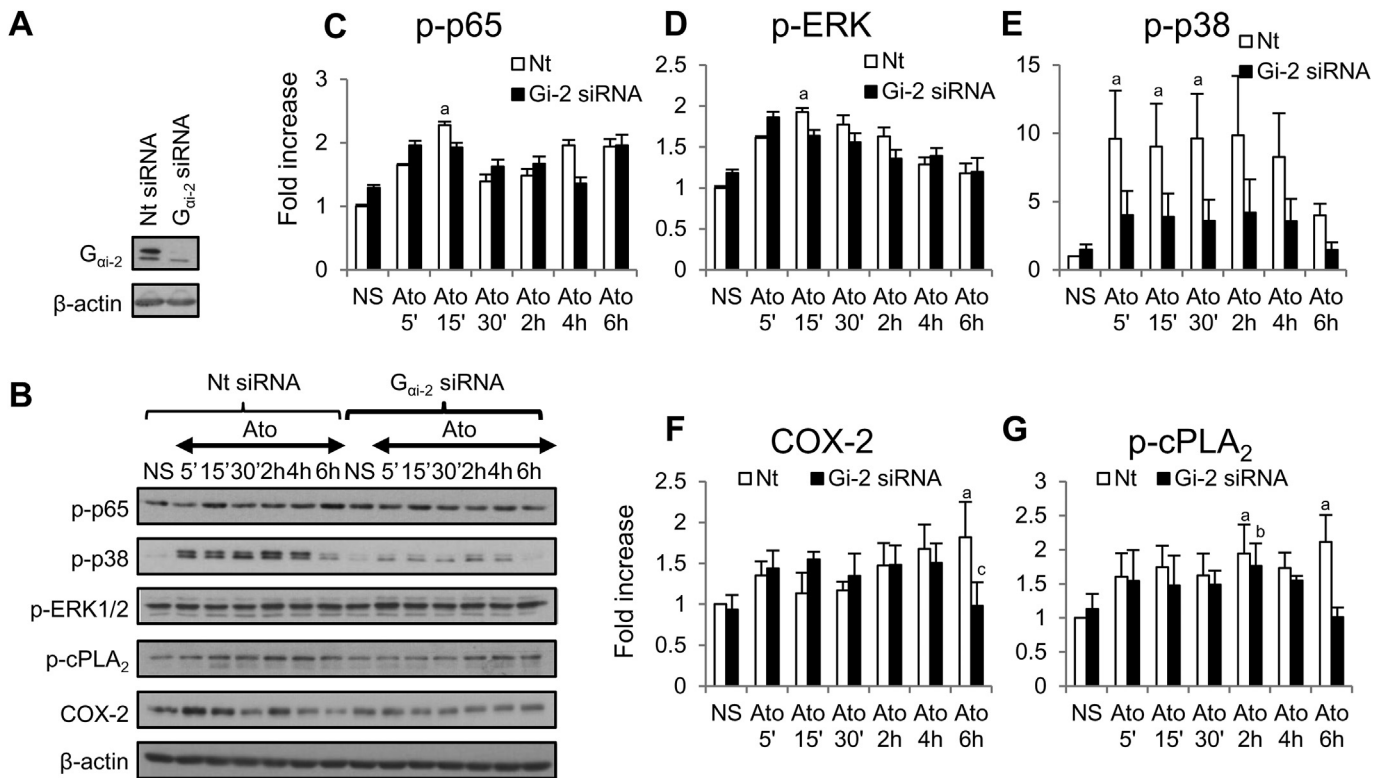
Busnelli et al. have recently reported that Atosiban induces a significant activation of the  $G_{\alpha i-3}$  but not  $G_{\alpha q}$  subunit in HEK293 cell lines stably transfected with the human OTR cDNA



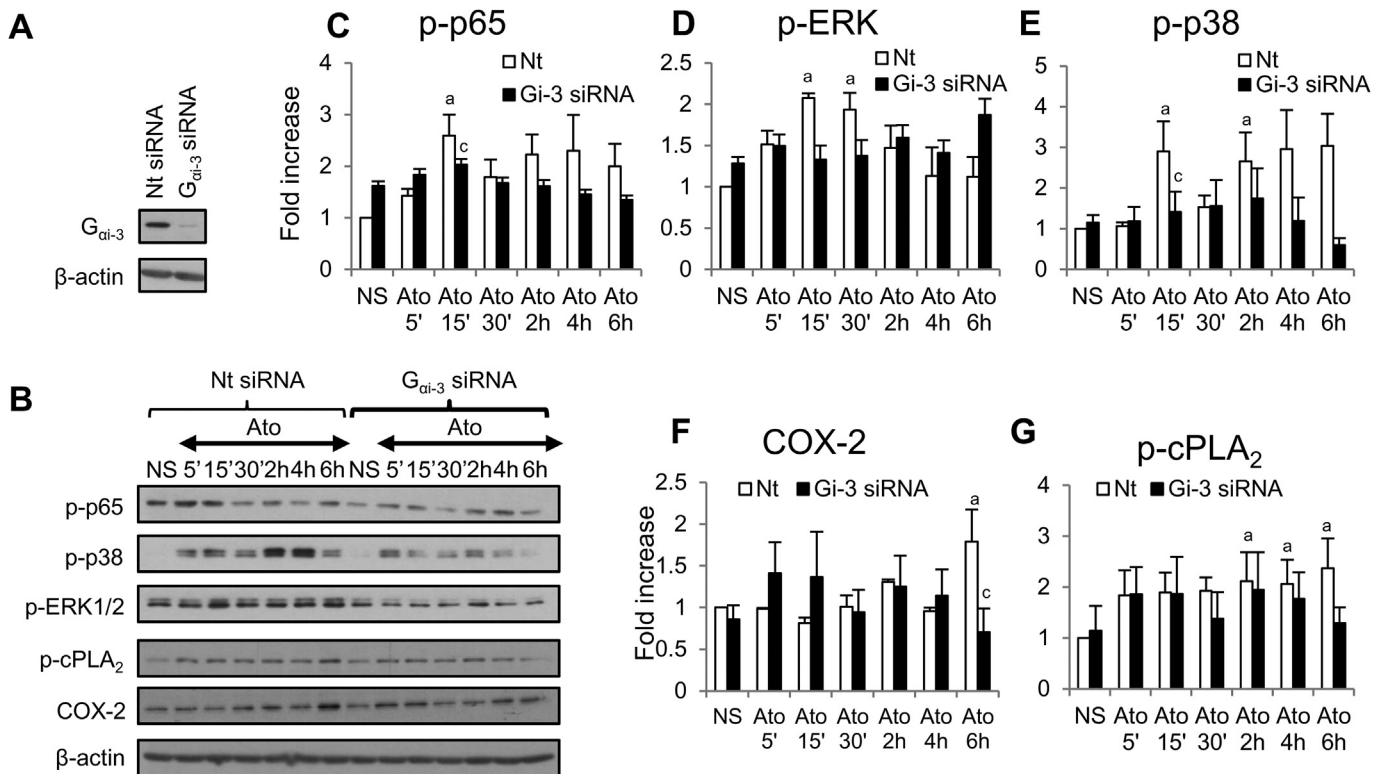
**Fig. 9.**  $G_{\alpha i}$  inhibitor, PTX, suppresses Atosiban-induced activation of NF- $\kappa$ B and MAPKs, as well as COX-2 and p-cPLA<sub>2</sub> expression. Pre-labour amnion epithelial cells were incubated in PTX (0.2  $\mu$ g/ml or 0.5  $\mu$ g/ml) for 30 min prior to 15 min or 30 min of Atosiban stimulation (10  $\mu$ M). Presence of PTX suppresses the effect of Atosiban upon p65, ERK1/2, and p38 phosphorylation. Bar graphs show densitometry analyses of p-p65, p-ERK1/2 and p-p38 (A). Western blot analysis of cells pretreated with PTX (0.2  $\mu$ g/ml or 0.5  $\mu$ g/ml) for 30 min prior to 4 h or 6 h of Atosiban stimulation (10  $\mu$ M) demonstrated inhibition of Atosiban-induced COX-2 expression and phosphorylation of cPLA<sub>2</sub> in presence of PTX. Bar graphs show densitometry analyses of COX-2 and p-cPLA<sub>2</sub> (B) (n = 6; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, ANOVA).

(Busnelli et al., 2012). Consistent with these findings we demonstrate that this also applies to primary amnion cells with endogenous OTR expression. While the primary effect of Atosiban in the myometrium may be OTR antagonism of  $G_{\alpha q}$  mediated signalling and decreased contractility by suppressing PLC activation and IP<sub>3</sub> and DAG production further affecting phosphorylation and

downstream transcriptional activity our results suggest Atosiban concurrently activates inflammation via  $G_{\alpha i}$  in the amnion and consequently promotes a labouring phenotype. Further studies will need to define tissue specific differences in  $G_{\alpha q}$  and  $G_{\alpha i}$  activation. In this study we have found that in amnion the Atosiban-induced activation of NF- $\kappa$ B p65 and ERK1/2 were PTX-sensitive and



**Fig. 10.  $G_{\alpha i-2}$  signalling is involved in Atosiban-induced activation of NF- $\kappa$ B and MAPKs, as well as COX-2 and p-cPLA<sub>2</sub> expression.** Pre-labour amnion epithelial cells transfected with non-target siRNA or  $G_{\alpha i-2}$ -target siRNA were treated with Atosiban for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analyses with antibodies against  $G_{\alpha i-2}$  (A) to confirm  $G_{\alpha i-2}$ -knockdown with  $G_{\alpha i-2}$ -target siRNA transfection. siRNA knockdown of  $G_{\alpha i-2}$  reduced Atosiban-induced expression of COX-2 but had a lesser effect on the Atosiban-induced activation of NF- $\kappa$ B and MAPKs (B). Densitometry plots of p-p65 (C), p-ERK1/2 (D), p-p38 (E), COX-2 (F) and p-cPLA<sub>2</sub> (G) show a decrease in Atosiban-induced activation of p38 and expression of COX-2, but not in Atosiban-induced activation of NF- $\kappa$ B or ERK1/2 with  $G_{\alpha i-2}$ -target siRNA transfection ( $n = 3$ ; **a**  $p < 0.05$  compared with NS of Nt, **b**  $p < 0.05$  compared with NS of  $G_{\alpha i-2}$ -target siRNA transfected cells, ANOVA).



**Fig. 11.  $G_{\alpha i-3}$  signalling plays a major role in the regulation of Atosiban-induced activation of NF- $\kappa$ B and MAPKs, as well as COX-2 and p-cPLA<sub>2</sub> expression.** Pre-labour amnion epithelial cells transfected with non-target siRNA or  $G_{\alpha i-3}$ -target siRNA were treated with Atosiban for 5 min, 15 min, 30 min, 2 h, 4 h and 6 h. Whole cell extracts were subjected to Western blot analyses with antibodies against  $G_{\alpha i-3}$  (A) to confirm  $G_{\alpha i-3}$ -knockdown with  $G_{\alpha i-3}$ -target siRNA transfection. siRNA knockdown of  $G_{\alpha i-3}$  suppressed OT-induced activation of NF- $\kappa$ B and MAPKs, or expression of COX-2 (B). Densitometry plots of p-p65 (C), p-ERK1/2 (D), p-p38 (E), COX-2 (F) and p-cPLA<sub>2</sub> (G) show significant decrease in Atosiban-induced activation of NF- $\kappa$ B and p-p38, as well as COX-2 with  $G_{\alpha i-3}$ -target siRNA transfection ( $n = 3$ ; **a**  $p < 0.05$  compared with NS of Nt, **b**  $p < 0.05$  compared with NS of  $G_{\alpha i-3}$ -target siRNA transfected cells, **c**  $p < 0.05$  Nt vs  $G_{\alpha i-3}$ -target siRNA transfected cells, ANOVA).

down-regulation of  $G_{\alpha i-2}$  or  $G_{\alpha i-3}$  proteins resulted in decreases in Atosiban-induced downstream COX-2 expression.

Our data suggest that Atosiban acts as an anti-labour agent through inhibition of OT-induced contractions in the myometrium but as a pro-labour agent in the amnion resulting in activation of inflammation via differential OTR G-protein coupling. Previous studies have shown that GPCR agonists/antagonists can activate diverse signalling pathways by inducing conformational changes in their receptors (Kenakin, 2001) and can result in alteration of the receptor (possibly via phosphorylation) to a non-active conformational state (Gether et al., 1997). Therefore the biased agonism of Atosiban we describe in the amnion via OTR-specific  $G_{\alpha i}$  coupling could possibly be a result of such OTR structural changes. These contradictory actions of Atosiban may reduce its potency as a tocolytic. Oxytocin receptor antagonists that inhibit both OT induced contractions and inflammation are likely to be more effective.

Fetal concerns regarding the use of Atosiban are discussed in the literature mainly based on the results of the Atosiban versus placebo trial by Romero and co-workers (Romero et al., 2000) who found a higher rate of fetal-infant death in the Atosiban treated group in extremely premature infants. There was however a significant imbalance in randomisation at gestational ages with more very preterm infants being exposed to Atosiban. Atosiban crosses the placenta with an average fetal versus maternal ratio of 0.124 (Valenzuela et al., 1995) and concentrations of Atosiban do not appear to accumulate in the fetus. Romero and colleagues had previously hypothesized that the anti-vasopressin effects of Atosiban could have altered fetal responses to stress and therefore could have contributed to the poor outcome in the extremely preterm infants. However maternal and fetal cardiovascular parameters are not significantly altered when Atosiban is administered in pregnant sheep (Greig et al., 1993) and fetal oxygenation remains the same after Atosiban infusion in chronically instrumented baboons (Nathanielsz et al., 1997). Nevertheless, given the association between inflammation and poor neonatal outcome, activation or exacerbation of inflammation is not a desirable effect of any agent to be used in the context of acute preterm labour. It is therefore critical that therapeutics designed to modulate the OT/OTR system for the management of term and preterm labour take into account the effects of differential G-protein coupling of the OTR and the role of OT and selective OTR agonists/antagonists in the activation of pro-inflammatory pathways.

## Acknowledgements

This work was supported by an Action Medical Research Project Grant (SP4454), Genesis Research Trust (P14754) and the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed are those of the author(s) and not necessarily those of Imperial College, the NHS, the NIHR or the Department of Health.

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