JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 84(3):41–46 JULY 2009

OXYTOCIN RECEPTOR (OXTR) MRNA EXPRESSION IN HUMAN AMNION-DERIVED WISH CELLS

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ABSTRACT—Parturition is regulated largely by the action of oxytocin and prostaglandins on the myometrium. An important source of myometrial contractile prostaglandins is the amnion. Oxytocin (OXT) stimulates amnion prostaglandin biosynthesis and directly stimulates myometrial contractions. Tissue responsiveness to OXT is determined by the level of oxytocin receptor (OXTR) expression. In this study, human amnion-derived WISH cells were used to model the effects of estradiol-17β (0.1 nM), forskolin (10 μM), and interleukin-1β (10 nM) on OXTR mRNA expression by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). We hypothesized that these agents would up-regulate OXTR mRNA. None of the agents at the concentrations tested demonstrated a consistent, significant effect on OXTR mRNA expression in these cells; thus, the data do not support our hypothesis.

Parturition, the process by which the fetus is expelled from the uterus, is largely stimulated by increased expression of uterine oxytocin receptors (OXTR) and increased synthesis of intrauterine prostaglandin (PG) E_2 and $F_{2\alpha}$ (Kniss and Iams, 1998; Challis et al., 2000). An increase in myometrial OXTR number toward term is well established (Fuchs et al., 1982). It has also been demonstrated that chorio-decidual and amnion tissues have increased OXTR expression toward term (Benedetto et al., 1990; Takemura et al., 1994). This increased expression of OXTR renders the uterine environment more sensitive to the uterotonic effect of oxytocin (OXT). Intrauterine PGE_2 and $PGF_{2\alpha}$, acting in a paracrine fashion, also promote myometrial contractions. An important source of PGE_2 at term is the amnion, the innermost fetal membrane (Mitchell et al., 1978; Okazaki et al., 1981; Olson et al., 1983). Oxytocin is one of several factors that stimulate amnion PGE_2 production (Moore et al., 1988; Mitchell and Lundin-Schiller, 1990). Thus, OXT directly and indirectly—through increasing PG biosynthesis—stimulates myometrial contractions at term.

Because OXTR protein levels closely follow OXTR mRNA levels in studies which have measured both, current thought is that OXTR expression is mainly regulated at the level of transcription (Kimura, 1998; Jeng et al., 1998). The regulatory region of the human OXTR gene displays binding sites for several transcription factors, such as growth associated binding protein, CCAAT/enhancer-binding protein (C/EBP), activator protein-1 (AP-1), Sp-1, nuclear factor-κB (NK-κB), and NF-interleukin-6 (Kimura et al., 1992; Inoue et al., 1994). The regulatory region also includes several half-palindromic estrogen response elements (ERE) and a cAMP response element/AP-1 like motif (Kimura et al., 1992; Inoue et al., 1994). The complexity of this regulatory/promoter region portends the complexity of the physiological conditions leading to up- or down-regulation of the receptor. Indeed, there is a vast literature, often contradictory, on the regulation of OXTR expression in various tissues (e.g., Bale and Dorsa, 1995; Ostrowski and Lolait, 1995; Schmid et al., 2001; Terzidou et al., 2006; Bussolati and Cassoni, 2001). Classical thought on uterine OXTR is that it is strongly up-regulated by estrogens and down-regulated by progesterone (Ivell and Walther, 1999). This is in keeping with estrogens' and progesterone's roles in promoting and inhibiting, respectively, uterine contractions. Paradoxically, the OXTR gene does not display a classical ERE. Instead, it contains partial EREs that may be important in mediating the estrogen response. In addition, the Sp-1 site may interact with estrogen receptors (ER) and/or estrogens may act through non-classical pathways including stimulation of cAMP synthesis to affect some target tissues (Bjornstorm and Sjoberg, 2005; Fleming et al., 2006).

In addition to the classical regulation by steroids, there is a growing body of evidence that likens the events within the uterus at term to an immune response, marked by an increase in cytokine synthesis and an influx of neutrophils into the uterus (Mitchell et al., 1993; Gilmour et al., 1998; Keelan et al., 2003). Since the OXTR gene regulatory region contains cytokine inducible transcription factor binding sites for C/EBP and NF-κB, other researchers have hypothesized that cytokines are important in regulating uterine OXTR (Schmid et al., 2001; Terzidou et al., 2006). Indeed, Terzidou et al. (2006) found that interleukin-1β (IL-1β) increased OXTR mRNA concentrations in myometrial cells, *in vitro,* by activating AP-1 C/EBPβ and NF-kB gene regulatory proteins.

There is a paucity of data regarding the regulation of human amnion OXTR expression. We have employed a human amnion-derived cell line, WISH (Wistar Institute Susan Hayflick), to examine the effect of estradiol-17β (E2), forskolin (a pharmacological agent which elevates intracellular cAMP), and IL-1β on OXTR mRNA expression. We hypothesized that these substances would increase the expression of OXTR mRNA compared to untreated cells. WISH cells retain a number of important physiological similarities to primary cultures of human amnion (Hayflick, 1961; Harris et al., 1988; Pavan et al., 2003; Ackerman et al., 2005; Tyson-Capper and Europe-Finner, 2006). For example, WISH cells, like primary

cultures of amnion, increase PGE_2 production when stimulated with E2, OXT, and IL-1β (Pavan et al., 2000; Pavan et al., 2001; Allport et al., 2000).

MATERIALS AND METHODS

Cell Culture—Human amnion-derived WISH cells were obtained from American Type Culture Collection (#CCL-25). Cells were seeded in 35 mm culture plates at 2.5×10^5 cells/plate and grown to 80% confluence in Dulbecco's Modified Eagle Medium:F12 minus phenol red (Invitrogen Corp., Carlsbad, California) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 0.15% sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, St. Louis, Missouri), and 2% antibiotic/antimycotic solution (AbAm, Invitrogen #15240-096). Medium was changed every 48 h. Cells were incubated at 37°C in 5% CO_2 in air. At 80% confluence cells were maintained for another 16 h in the absence of FBS. Cultures were then treated in triplicate for 3 and 6 h with either control (vehicle only), 0.1 nM estradiol-17β (Sigma-Aldrich), 10 μM forskolin (Sigma-Aldrich), or 10 nM interleukin-1β (R&D Systems, Inc., Minneapolis, Minnesota) in DMEM:F12 minus phenol red plus AbAm and $NaHCO_3$. The experiment was conducted on three separate passages of cells. Following treatment, RNA was extracted from cell cultures using Tri Reagent RNA Isolation Kit (Applied Biosystems, Austin, Texas) and quantified using a NanoDrop® ND1000 spectrophotometer. Isolated RNA was reverse transcribed.

Reverse Transcription (RT)—RT reactions contained: 1 μg of RNA, 50 pM reverse OXTR primer (5′-GGGTTGCAG CAGCTGTTGA-3′, Applied Biosystems), 50 pM reverse β-actin primer (5′-GCCCTGAGGCACTCTTCC A-3′, Integrated DNA Technologies, Coralville, Iowa), 1 μL RNase-Out, 1 μL 10 mM dNTP mix, 4 μL 5× first-strand buffer, 1 μL reverse transcriptase (all from Invitrogen), and PCR grade water to a final volume of 25 μl. Reverse transcription was carried out under the following thermocycler conditions: 65°C for 5 min, 42°C for 2 min, 42°C for 50 min, and 72°C for 15 min.

Quantitative Polymerase Chain Reaction (qPCR)—cDNA obtained from RT reactions was amplified using the ABI 7500 Sequence Detection System and SYBR® green chemistry. β-actin served as the endogenous control. Three replicates of each sample for OXTR mRNA detection and three replicates of each sample for β-actin mRNA detection were prepared in a 96-well plate. Each well held a 25 μL reaction consisting of 300 nM of appropriate forward and reverse primer, 2.5 μL cDNA, 0.1 μL ROX reference dye, 12.5 μL SYBR® green master mix (Applied Biosystems), and PCR grade water. Thermocycler conditions were 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The cycle threshold (Ct) values were obtained and analyzed as described below. PCR products were confirmed by gel electrophoresis or by analysis of melting curves.

qPCR Primers—Both primer sets span exon-exon junctions to ensure that no genomic DNA is amplified. The OXTR primer set was based on Vignozzi et al. (2004) [Forward primer: 5′-CAACGCGCCCAAGGAAG- 3′; Reverse primer: 5′-GGGTTGCAGCAGCTGTTGA-3′]. The PCR product using these primers is a 72 base pair fragment. The β-actin primer set was designed using Primer Express software (Applied Biosystems) [Forward primer- 5′- GCCCTGAGG CACTCTTCCA-3′, Reverse primer – GCATCCTGTCGG CAATGC-3′]. The PCR product using these primers is a 171 base pair fragment.

Optimal primer concentrations were determined after running qPCR reactions with a primer matrix composed of varying concentrations of forward and reverse primers (50, 300, and 900 nM) on a pooled cDNA sample and reaction conditions as described above. Gel electrophoresis confirmed the presence of the target products. The 300 nM forward and 300 nM reverse primer combination gave the lowest Ct, and these primer concentrations were used in all subsequent reactions.

Comparative Ct Method or ΔΔCt Method—Comparative Ct Method depends upon the target gene and endogenous control gene having similar amplification efficiencies. To measure relative efficiency, amplifications were performed in triplicate on the same diluted samples, using primers for β-actin and OXTR. The average Ct was calculated for the genes and ΔCt ($Ct_{OXTR} - Ct_{\beta\text{-actin}}$) was determined. Log of RNA concentration versus ΔCt was graphed. If the absolute value of the slope was close to zero ($m < 0.1$), the efficiencies of OXTR and β-actin amplifications were similar, and thus the ΔΔCt calculation could be applied (Bookout and Mangelsdorf, 2003).

Calculations of fold-induction based on Cts were carried out as outlined by Bookout and Mangelsdorf (2003). Briefly, the mean, standard deviation (SD), and coefficient of variation (CV) were determined for the triplicate Ct values of each sample. Outlier wells were removed (> 4% CV). Fold-inductions for treatments over control were calculated from mean Cts as follows: $\Delta Ct = Ct_{OXTR} - Ct_{\beta\text{-actin}}$; $\Delta\Delta Ct = Ct_{Treatment} - Ct_{Control}$; fold-induction $= 2^{(-\Delta\Delta Ct)}$. Adapting statistical methods outlined by Yuan et al. (2006), the hypothesis that ΔΔCts are equal to zero was tested for each treatment using Student's *t*-test, Wilcoxon signed-rank two-tailed test, and JMP® 6 Statistical Software (SAS Institute, 2005). *P*-values ≤ 0.05 are interpreted to mean the treatment has a significant effect on OXTR mRNA expression compared to control. Mean fold-induction (fold-induction $= 2^{(-\Delta\Delta Ct)}$) and standard error of mean (SEM) were calculated for all samples within a treatment across all experiments and presented graphically. In a final analysis, the hypothesis that fold-inductions are equal to one was also tested for each treatment.

RESULTS

Graphical analysis of relative efficiencies of OXTR and β-actin amplification indicated similar efficiencies (Fig. 1). Thus, relative quantification methods were employed in the study of the OXTR mRNA. Gel electrophoresis of samples amplified with OXTR primers indicated a single 72 base pair band, and samples amplified with β-actin primers indicated a single 171 base pair band. Dissociation curves for samples amplified with OXTR primers displayed a strong peak at 82.5°C; samples amplified with β-actin displayed a strong peak at 87°C (Fig. 2). These data suggest that a single amplicon was produced for each primer set during the qPCR reaction.

Mean fold-inductions were calculated for each cell passage (Fig. 3) and for all cell passages combined (Fig. 4). A fold-induction of 1.0 represents no difference from control cell expression. Fold-inductions greater than 1.0 would indicate a

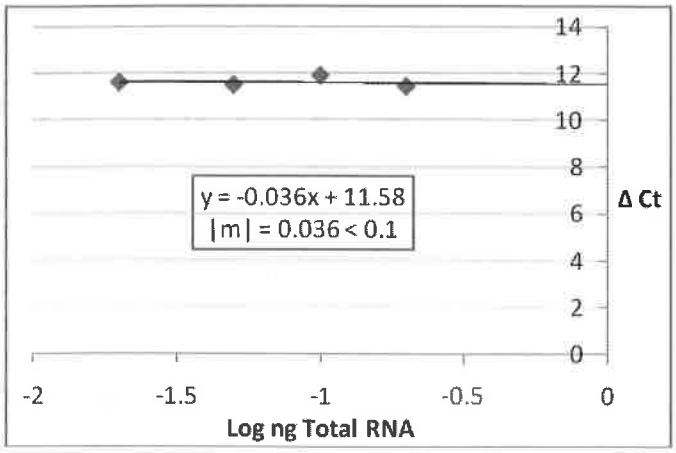


FIG. 1. Graphical analysis of relative OXTR and β-actin amplification efficiency. The average Ct at each dilution of RNA was calculated from triplicate determinations − average Ct_{OXTR} − average $Ct_{\beta\text{-actin}}$ = ΔCt. The absolute value of the slope (m) was 0.036 (< 0.1).

higher level of expression than control and fold-inductions less than 1.0 would indicate lower levels of expression than control. Estradiol-17 β treatment showed no significant effect on OXTR mRNA expression compared to control. Forskolin treatment in cell passage 1 at 3 h displayed a significantly higher OXTR mRNA expression compared to control cells (P = 0.0039, Student's t-test of ΔΔCt analysis only, Fig. 3). However, forskolin did not alter OXTR mRNA in cells at passages 2 or 3. Interleukin-1β treatment in cell passage 2 at 6 h significantly lowered OXTR mRNA expression compared to that of control cells (P = 0.0124, Student's t-test of ΔΔCt analysis, and P = 0.0084, Student's t-test of fold-induction analysis; Fig. 3). Neither of these treatments was significantly different from control cells using Wilcoxon signed-rank test. The Wilcoxon test was used as a nonparametric alternative to the t-test. When all fold inductions were averaged across all passages for all treatments, there was no significant effect on the OXTR mRNA levels compared to untreated cells (Fig. 4).

DISCUSSION

Increased sensitivity to OXT via OXTR up-regulation causes activation of the uterine tissues at term. There is a paucity of data regarding the regulation of human amnion OXTR expression. In the present study, we tested the effect of E2 (0.1 nM), forskolin (10 μM), and IL-1β (10 nM) on OXTR mRNA expression in human amnion-derived WISH cells at 3 and 6 h and report that none of the agents, at the concentrations tested, had a significant effect on OXTR mRNA expression. Forskolin and IL-1β at these concentrations and within this time range have been shown in other cells to up-regulate OXTR mRNA (Forskolin: Jeng et al., 1998; IL-1β:Terzidou et al., 2006). The E2 concentration of 0.1 nM was chosen as a starting point for these studies based on work in our laboratory showing this level of E2 stimulated proliferation in estrogen receptor positiveT47D cells (Datta and Lundin-Schiller, 2008).

In many tissues, the OXTR is up-regulated by E2 (e. g., Umscheid et al., 1998; Engstrom et al., 1999; Amico et al., 2002; Fleming et al., 2006). For example, E2 (10^{-7} M) has been shown to cause an increase in OXTR mRNA expression

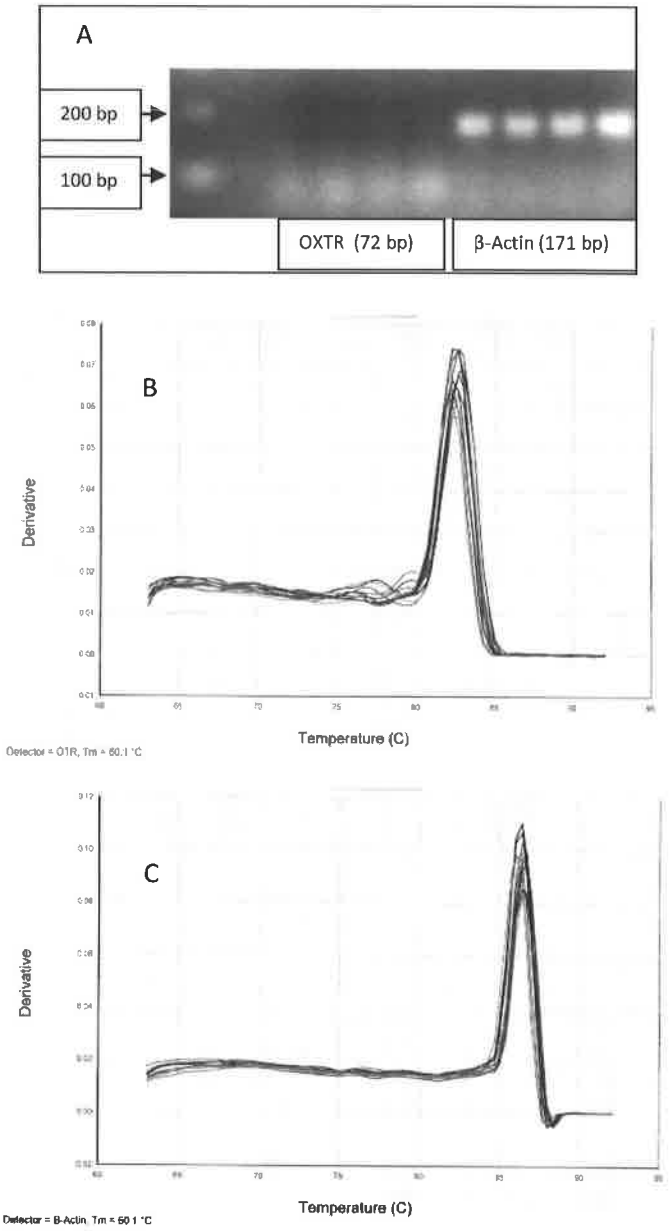
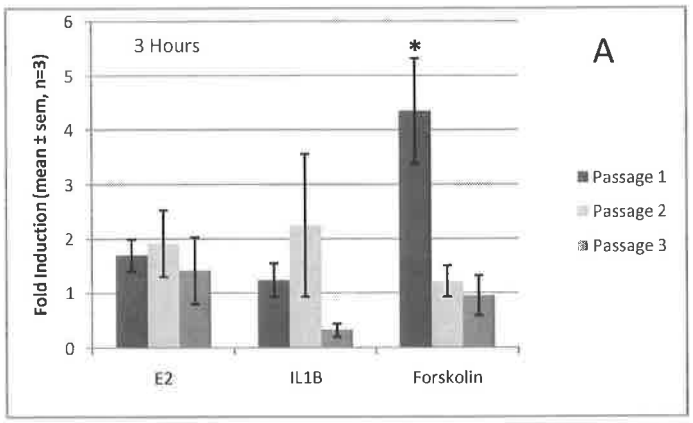


FIG. 2. Panel A: Electrophoretic analysis of OXTR and β-actin amplification products. Lane 1 100 base pair (bp) ladder, lane 2 empty, lanes 3–6 OXTR amplicon (72 bp), lanes 7–10 β-actin (171 bp) amplicon. Panel B: Representative dissociation curves for OXTR amplification product. Panel C: Representative dissociation curves for β-actin amplification product.

in MCF7 human breast cancer cell line as measured by RT-PCR (Amico et al., 2002). The ovine gene was shown to be up-regulated in reporter gene assays by E2 (10^{-8} M) and the activated estrogen receptor (ER) interacted with the GC rich Sp-1 binding site of the OXTR regulatory region (Fleming et al., 2006). To achieve a significant effect in these studies, concentrations greater than 1 nM had to be used, suggesting that a higher concentration of E2 than that used in our experiments is warranted. Furthermore, ERs are often down-regulated in estrogen deficient media (Castles et al., 1997). Thus, it is possible that our serum starvation for 16 h prior to and during treatment created a cell culture milieu that did not



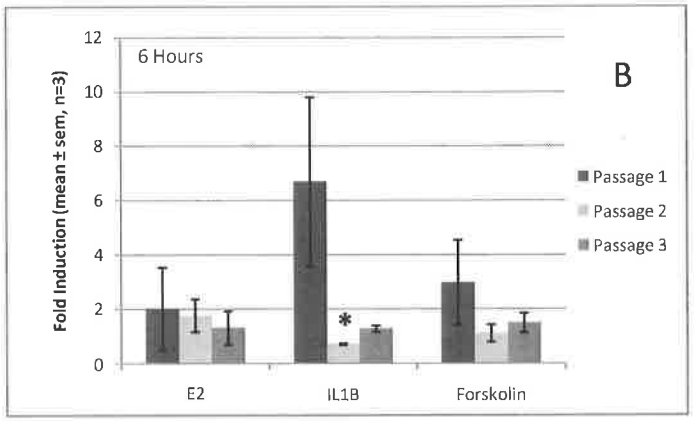


FIG. 3. Fold-induction of OXTR mRNA over control cells induced by 0.1 nM estradiol-17β (E2), 10 nM interleulin-1β (IL1B), and 10 μM forskolin for either 3 (Panel A) or 6 (Panel B) h. Data are presented as mean ± SEM (n=3 plates; except for E2 - Passage 1–3 h where one sample was lost). An asterisk (*) indicates a statistically significant difference from control.

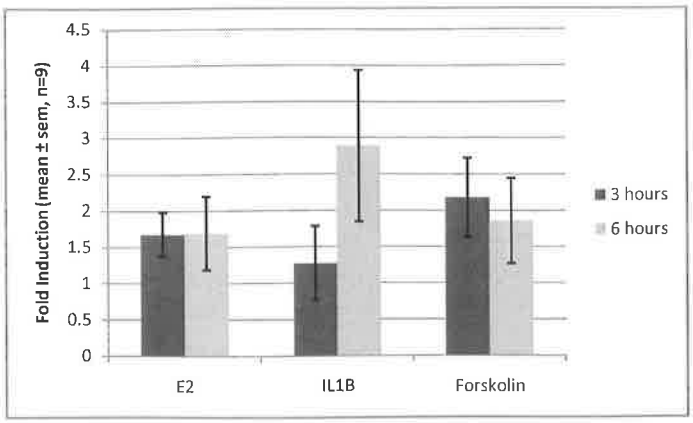


FIG. 4. Combined fold-induction (mean ± SEM, n=9 plates) for all plates and all cell passages of OXTR mRNA over control cells induced by 0.1 nM estradiol-17β (E2), 10 nM interleulin-1β (IL1B), and 10 μM forskolin at 3 and 6 h. When statistical analyses were conducted on combined ΔΔCt or fold-induction data from the three cell passages for each treatment, no statistically significant effect was detected.

maintain all necessary cellular proteins to allow the cells to respond to an E2 treatment.

Hinko and Soloff (1993) demonstrated that forskolin and $(Bu)_2$cAMP increased the number of OXTR binding sites and OXT-stimulated PGE_2 biosynthesis in primary cultures of rabbit amnion. Further studies indicated that cortisol could also up-regulate OXTR binding sites in rabbit amnion cell cultures (Hinko and Soloff, 1993). In the 1993 study and a later study (Jeng et al., 1998), the Soloff laboratory found that the effect of cortisol was potentiated by concomitant treatment with forskolin (25 μM). Using nuclear run-on assays, they showed that forskolin and cortisol increased the transcription rate of the OXTR gene. Interestingly, the maximal effect of forskolin (100-fold increase) was seen at 4 h and was followed by an increase in OXTR mRNA instability which could be reversed by protein synthesis inhibitors. Cortisol induced a slower, progressive increase in OXTR transcription without an increase in mRNA stability. Forskolin and cortisol together had a synergistic effect on OXTR transcription. Regarding forskolin in WISH cells, Pavan et al. (2001) have shown that forskolin up-regulated E2 binding sites and modulated E2 stimulated PGE_2 release.

Each agent was tested separately in this experiment. If changes occur in response to a single agent but the degree of change is relatively slight or transient, it may be missed. As shown by Jeng et al. (1998) for cortisol and by Pavan et al. (2001) for E2, forskolin has the ability to synergize the effect of steroids. Based upon the findings from these two laboratories, it may be necessary to pre-treat or co-treat the WISH cells with forskolin to observe optimal effects of E2 on OXTR mRNA, and E2 treatment may be needed to abrogate any forskolin induced OXTR mRNA instability. Combining bioactive agents would also more closely resemble physiological conditions since, at term, the amnion is bathed on one side by amniotic fluid in which a variety of substances are known to increase in concentration as gestation proceeds including cortisol, estrogens and estrogen precursors, prostaglandins, and inflammatory mediators (Tan et al., 1976; Forest et al., 1980; Dray and Frydman, 1976; Opsjon et al., 1993).

The ability of IL-1β to up-regulate OXTR mRNA or protein appears to be quite dependent on details of culture conditions and cell source. Schmid et al. (2001) reported that both IL-1β and IL-6 caused decreased OXTR mRNA in a human myometrial cell line (ULTR) as measured by ribonuclease protection analysis. In a later study, Terzidou et al. (2006) found that IL-1β stimulated OXTR mRNA and OXTR binding site expression in primary cultures of human myometrial cells obtained from biopsy at the time of elective cesarean section. They measured a 3-fold increase in OXTR mRNA and a 135% increase in OXTR binding sites over control. The effect was significant by 2 h and maximal at 4 h. Terzidou et al. (2006) suggested that the difference in the results from the two studies was the cell source and further suggested that the ULTR cell line is not a good model for pregnant myometrium. The WISH cell line and primary cultures of amnion do respond similarly to IL-1β in terms of PGE_2 production (Mitchell et al., 1993; Allport et al., 2000). Specifically, in both culture types IL-1β induces cyclooxygenase 2 expression leading to increased conversion of arachidonic acid to PGH the precursor of PGE_2. We are unaware of any studies to date comparing the effect of IL-1β on OXTR expression in WISH and primary amnion cultures. It is our ultimate goal to provide that information.

Finally, an alternative explanation for our results is that in this cell line and in human amnion a basal level of OXTR expression is genomically "programmed" and cannot respond to agents which, in other tissues, trigger increased OXTR gene transcription. Indeed, in contrast to Benedetto et al. (1990), Fukai et al. (1984) have reported that, while human amnion expresses the OXTR, there is no change in expression level toward term.

Acknowledgments

We thank G. Pitts (APSU) for assisting in the design the β-actin primers and for sharing wisdom while we navigated qPCR. We also thank C. Baskauf and J. Schiller (APSU) for helpful discussions about statistical methods. This work was funded by the APSU Presidential Research Scholarship and the Department of Biology.

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