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Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids and growth factors

Denis A. Evseenko, James W. Paxton, and Jeffrey A. Keelan

Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand: D.A.E. and J.A.K.

Department of Pharmacology & Clinical Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand: J.W.P. and J.A.K.

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Regulation of placental ABC transporters

Corresponding author:

Jeffrey A. Keelan, PhD

Liggins Institute and Department of Pharmacology and Clinical Pharmacology

University of Auckland

Private Bag 92019

Phone +64 9 3737599 ex 86246

Fax: +64 9 3737497

E-mail: j.keelan@auckland.ac.nz

Number of text pages: 21

Number of tables: 1

Number of figures: 5

Number of references: 42

Number of words in:

> **Abstract - 230**
>
> **Introduction - 799**
>
> **Discussion - 1534**

Non-standard abbreviations:

MRP, multidrug resistance protein; MDR multidrug resistance; BCRP, breast cancer resistance protein; TNF, tumor necrosis factor; IL, interleukin; IGF, insulin-like growth factor; EGF, epidermal growth factor; BCA, bicinchoninic acid; E_2, 17β-estradiol; P_4, progesterone.

Abstract

Placental ABC (ATP binding cassette) transporters protect placental and fetal tissues by effluxing xenobiotics and endogenous metabolites. We have investigated the effects of cytokines and survival/growth factors, implicated in various placental pathologies, on ABC transporter expression and function in primary placental trophoblast cells. Treatment of primary term trophoblasts *in vitro* with TNF-α or IL-1β decreased mRNA and protein expression of apical transporters ABCB1/MDR1 (multidrug resistance gene product-1) and ABCG2/BCRP (breast cancer resistance protein) protein by 40-50% ($P<0.05$). In contrast, IL-6 increased mRNA and protein expression of the basolateral transporter ABCB4/MDR3 ($P<0.05$), while ABCC1/MRP1 expression was unaltered. Pretreatment of trophoblasts with TNF-α over 48 h resulted in significantly decreased BCRP efflux activity (increased mitoxantrone accumulation) with minimal changes in MDR1/3 activity. Epidermal growth factor (EGF) and insulin-like growth factor- II, on the other hand, significantly increased BCRP expression at the mRNA and protein level ($P<0.05$); EGF treatment also increased BCRP functional activity. Estradiol stimulated BCRP, MDR1 and MDR3 mRNA and protein expression by 40-60% and increased MDR1/3 functional activity ($P<0.05$). Progesterone had modest positive effects on MRP1 mRNA and MDR1 protein expression ($P<0.05$). In conclusion, this study demonstrates that pro-inflammatory cytokines, sex steroids and growth factors exert independent affects on expression of apical and basolateral placental ABC transporters in primary trophoblast. Such changes could alter placental drug disposition, increase fetal susceptibility to toxic xenobiotics and impact upon placental viability and function.

Introduction

The human placenta expresses many transport proteins which facilitate the import and efflux of a variety of endogenous substrates and xenobiotics. Among these proteins are several members of the ATP binding cassette (ABC) family, noted for their role as drug transporters (Litman et al., 2001; St-Pierre et al., 2002; Unadkat et al., 2004). Of the xenobiotic transporters expressed on the maternal facing apical surface of the placental syncytiotrophoblast membrane, the most abundant are multidrug resistance gene product-1 (MDR1/ABCB1), also known as P-glycoprotein, and breast cancer resistance protein (BCRP/ABCG2). These proteins play prominent roles in effluxing drugs and toxic metabolites out of the fetoplacental compartment (Lankas et al., 1998; Jonker et al., 2000). Interestingly, BCRP is also expressed in endothelial cells lining the fetal capillaries (Ceckova et al., 2006), suggesting that it may also serve to pump substrates out of the placenta and in to the fetal circulation. The two predominantly basolateral (i.e. fetal-facing) ABC transporters, MDR3/ABCB4 and multidrug resistance protein-1 (MRP1/ABCC1), transport their substrates from mother to fetus, vectorially opposite to MDR1 and BCRP. These transporters are localised to the basement membrane of the syncytium and the fetal capillary endothelium (Nagashige et al., 2003; Evseenko et al., 2006a). In cases where substrate specificity overlaps, net transfer across the placenta will be determined by the balance of activity of maternal- and fetal-facing transporters (Evseenko et al, 2006b).

Little is known of the factors that regulate ABC transporter expression and activity in the placenta. There is some evidence that their expression changes with gestational age, although the data are inconsistent (Patel et al., 2003; Mathias et al., 2005; Zu Schwabedissen et al., 2006). During trophoblast differentiation and syncytialization, BCRP and MRP1 expression is significantly increased, while MDR1 and MDR3 expression is markedly reduced (Pascolo et al., 2003; Evseenko et al., 2006a; Zu Schwabedissen et al., 2006); it is not known, however, whether this change in ABC expression profile is a function of differentiation, or whether it is plays an integral part of the differentiation process. Epidermal growth factor (EGF), which promotes *in vitro* trophoblast differentiation and protection from apoptosis (Morrish et al., 1998), increases BCRP expression in primary trophoblasts (Zu Schwabedissen et al., 2006), consistent with the increase in BCRP expression associated with syncytialisation. Insulin-like growth factor II (IGF-II) is also known to be a major modulator of placental growth and development (Constancia et al., 2002) and is likely to be involved in regulation of apoptosis and cell differentiation (Miller et al., 2005). However, the effects of IGF-II, EGF or other growth factors on placental ABC expression remains undetermined.

The roles of estrogen and progesterone in the regulation of placental BCRP expression have been investigated, although only in the BeWo choriocarcinoma cell model (Ee et al., 2004; Wang et al., 2006). Both a marked increase and decrease in BCRP expression was reported in response to treatment with 17β-estradiol, with progesterone playing a lesser but synergistic role (Ee et al., 2004; Wang et al., 2006). The effects of steroids on ABC transporter expression in primary trophoblast cells have not yet been described.

We hypothesized that, while steroids and growth factors derived from the placenta might be expected to drive placental ABC expression and function, a decline in ABC transporter expression might be predicted under pathological conditions in which placental function is compromised. Conditions such as placental insufficiency/fetal growth restriction, preeclampsia and gestational diabetes are relatively common pregnancy disorders in which elevated cytokine concentrations are believed to play a role (Hamai et al., 1997; Steinborn et al., 1999; Saji et al., 2000; Johnson et al., 2002). Placental expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is elevated in preeclampsia and gestational diabetes (Hamai et al., 1997; Coughlan et al., 2001; Kirwan et al., 2002). Some studies have also found elevated interleukin 1β (IL-1β) and interleukin 6 (IL-6) expression with preeclampsia (Saji et al., 2000; Luppi and Deloia, 2006). Microbial inflammatory activation is also associated with increased placental cytokine expression (Saji et al., 2000). The effects of cytokines on human placental ABC transporter expression have not been studied to date, although findings from other tissues (Lee and Piquette-Miller, 2003; Belliard et al., 2004) and animal models (Wang et al., 2005) would suggest that TNF-α, IL-1β and IL-6 could decrease the expression of MDR1, while IL-6 may stimulate MRP1 expression. There is insufficient data to base any prediction of the effects of cytokines on MDR3 and BCRP expression.

The present study, therefore, was carried out to establish the effects of steroids (estradiol and progesterone), growth factors (EGF and IGF-II) and cytokines (TNF-α, IL-1β and IL-6) on the transcriptional regulation of mRNA and protein expression of MDR1, MDR3, BCRP and MRP1 in primary trophoblast cells in monolayer culture. Functional studies were also carried out to support the physiological/pharmacological interpretation of the most significant findings of the expression studies.

Materials and methods

Reagents. Medium 199 (M199), fetal calf serum (FCS), normal horse serum (NHS), insulin, transferrin, human recombinant EGF, IGF-II, sodium selenite, penicillin/streptomycin, DNase I (molecular grade), and Superscript III cDNA Synthesis Kit were from Invitrogen (Auckland, NZ

and Carlsbad, USA). Power SybrGreen PCR Master Mix was from Applied Biosystems (Warrington, UK). Difco trypsin 250 was obtained from Becton & Dickinson (Sparks, MD, USA). The RNAqueous Kit for total RNA extraction was acquired from Ambion (Huntington, UK). Complete protease inhibitor was from Roche Diagnostics (Mannheim, Germany). Nitrocellulose Hybond membrane was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Monoclonal anti-MDR1 (clone F4) was from Sigma-Aldrich (St. Louis, USA); anti-MDR3 (clone P3 II-26), MRP1 (clone QCRL3), and BCRP (clone BXP21) antibodies were purchased from Chemicon (Temecula, USA). Anti-β actin, peroxidase-conjugated goat anti-mouse antibody, bicinchoninic acid (BCA) reagent, 17β-estradiol and progesterone were purchased from Sigma-Aldrich (St. Louis, USA). TNF-α, IL-1β and IL-6 were purchased from PreProTech (Canton, USA). Transporter substrates [^{3}H] mitoxantrone and [^{3}H] digoxin were from Moravek Biochemicals Inc (Brea, CA, USA) and PerkinElmer (Wellesley, MA, USA), respectively. General chemicals (analytical grade) were obtained from Serva (Heidelberg, Germany), Scharlay Chemie (Barcelona, Spain), or AppliChem (Darmstadt, Germany).

Cytotrophoblast culture. Placentas were obtained with informed consent from women after delivery by caesarean section at term. Cytotrophoblasts were isolated as described by Kliman et al. 1986 (Kliman et al., 1986) with some modifications (Blumenstein et al., 2001). Cytotrophoblasts derived from term placenta were liberated by digestion with 0.25% trypsin (8 digestions in total), supernatants were collected in 50-ml Falcon tubes and centrifuged at 300 *g* for 7 min. Erythrocytes were removed by incubation of a cell pellet in lysis buffer (50 mM NH_4Cl, 10 mM $NaHCO_3$, and 0.1 mM EDTA), and cytotrophoblasts were purified by centrifugation at 1,200 *g* for 20 min on a discontinuous Percoll gradient (20–60%). Cells between the 40 and 50% Percoll bands were collected and plated either in 12-well plates (1 x 10^6 cells/well), or 24-well plates (5 x 10^5 cells/well). The cells were grown for 24 h in M199 media, supplemented with 10% FCS, EGF (10 ng/ml), insulin (5 ng/ml), transferrin (10 ng/ml), sodium selenite (0.2 nM), and penicillin/streptomycin (100 U/ml) in a 95% air/5% CO_2 humidified atmosphere at 37°C. The proportion of cytokeratin-positive cells (cytotrophoblasts) in the cultures was 95.7 ± 3.14% (n=3). After 24 h in culture, cells were washed with PBS, and media were renewed with M199 supplemented with 10% charcoal-stripped FCS. Test substances were added as follows: TNF-α (20 ng/ml), IL-1β (2 ng/ml), IL-6 (20 ng/ml), EGF (10 ng/ml), IGF-II (25 ng/ml), estradiol (100 nM) and progesterone (100 nM). With the exception of TNF-α, these concentrations were selected to achieve levels that approximate maximal physiological concentrations reported in either peripheral or extracellular fluids (Batra and Bengtsson, 1978; Khan-Dawood and Dawood, 1984; Barreca et al., 1998; Kim et al., 2000; Inada et al., 2001; Lee

and Piquette-Miller, 2003). With respect to TNF-α, because of its potential to induce trophoblast apoptotic death, 20 ng/mL was selected for these studies, representing the maximal concentration that could be used without exerting significant (>10%) loss of viability (as assessed by MTT assay) over 48 h of treatment. None of the other test agents had any effects on cell viability. Effects of regulators on mRNA expression were tested at 12 h, the time-point at which both inhibition and stimulation of mRNA expression could be clearly and consistently observed (data from pilot experiments). Protein levels were measured after 48 h exposure to test treatments. This relatively extended time point was selected because of the long half-life (14 to 17 h) of transporter proteins (Muller et al., 1995); incubation for shorter time periods (24 h) was not sufficient to detect reductions in transporter protein expression, although stimulation of expression was observable. Because spontaneous trophoblast fusion and differentiation (syncytialisation) took place during culture, the results obtained represent changes in protein expression and function additional to those caused by responses to differentiation. Five different primary trophoblast extractions were used for regulatory experiments and the data generated were pooled and analysed collectively.

Quantitative real-time PCR. Total RNA from cultured trophoblast cells was extracted using an RNAqueous kit (Ambion, Huntington, UK) according to the manufacturer's instructions and treated with DNase I to remove genomic DNA. First-strand cDNA synthesis with random hexamers primers was carried out using a Superscript III Synthesis kit. Primers for MDR1, MRP1 and BCRP, MDR3 were intron-spanning, based on published cDNA sequences (Table 1); 18S RNA primers were purchased from Applied Biosystems. PCR amplification and detection was performed using an ABI Prism 7900 HT (Applied Biosystems, Warrington, UK). Amplification efficiency for all genes examined was more than 0.95 and comparable to the amplification efficiency for 18s RNA. Amplification of single product for each primer set was confirmed by dissociation curve analysis, and all products were visualized after electrophoresis on 2% agarose gel.

The comparative threshold cycle (Ct) method for relative quantification ($2^{-\Delta\Delta Ct}$) was used to quantitate gene expression according to Applied Biosystem recommendations (7900 HT Real-Time fast and SDS enterprise and database user guide). Expression of target genes was normalized to the level of 18S rRNA and expressed relative to control. The Ct values for the MDR1, MDR3, MRP1 and BCRP PCR reactions ranged from 30-35, 30-35, 25-30, and 22-30, respectively. 18S sRNA Ct values spanned 15-20 cycles (using 1/10th the cDNA).

Immunoblotting. Total cell lysates were prepared by homogenization in the following buffer: 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% SDS, 0.5% deoxycholic acid and complete protease inhibitor. Lysates were maintained for a minimum of 1 h at 4°C. After sonication, insoluble

material was then removed by centrifugation at 14,000 g for 10 min at 4°C and stored at - 80°C. Protein concentration was measured by the BCA assay calibrated to BSA. Protein (20–30 µg) was separated under reducing conditions (MDR1 in non reduced conditions) on a 4–12% BisTris precast polyacrylamide gradient gel (Invitrogen, NZ) and transferred to a nitrocellulose membrane in an XCELL transfer module (Invitrogen, NZ). Membranes were blocked in 2% nonfat milk powder, and incubated overnight with the following monoclonal antibodies: anti-MDR1 (1:200), MDR3 (1:500), MRP1 (1:500) and BCRP (1:500). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse antibody and visualized by enhanced chemiluminescence recorded on CP-BU New X-ray film (Agfa, Westerlo-Heultje, Belgium). Band intensity was quantitated by densitometry using Quantity One software (Bio-Rad Laboratories, Auckland, NZ). Equivalence of protein loading was confirmed by secondary immunoblotting with anti-β actin antibodies.

Accumulation experiments. Drug accumulation in cultured trophoblasts was measured in duplicate in 24 well plates (0.5 x 10^6/ cells per well). From day 2 of culture, cells were pre-treated with either TNF-α (20 ng/ml), EGF (10 ng/ml) or estradiol (100 nM) for 48 h in M199 media containing 10% stripped FCS without other supplements or antibiotics. After washing thoroughly with phosphate-buffered saline (PBS) the media was replaced with 0.5 ml of M199 serum free media containing 100 nM [^{3}H] digoxin (37 Ci/mmol) or 20 nM of [^{3}H] mitoxantrone (3 Ci/mmol). After incubation for 20 and 60 min time periods, previously shown to allow sufficient accumulation of substrate in cultured trophoblast cells (Evseenko et al., 2006a), medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline. The cells were then lysed with 0.25 ml of 1 M NaOH, neutralized with HCl, and radioactivity determined by liquid scintillation counting and expressed as pmol per mg protein. Substrate accumulation studies were carried out in 4 independent primary trophoblasts cultures with similar results.

Statistics. All studies were performed 4-5 times and descriptive statistics were performed for each data set. Graphs were plotted and data transformed with using Graph Pad Prism 3.02 (GraphPad Software, Inc., San Diego, USA). Statistical analysis was performed using Sigmastat software from Systat Software Inc (Richmond, USA). For expression studies one-way ANOVA was applied, followed by Student-Newman-Keuls test. For functional time-course studies, statistical significance was assessed by one-way ANOVA with repeated measures. P value < 0.05 was considered to be significant.

Results

A. *Expression studies*

Effects of pro-inflammatory cytokines

The most significant changes in the ABC transporter mRNA expression occurred after 12h of exposure to test treatments. At this time point, MDR1 mRNA expression was inhibited by TNF-α and IL-1β by almost 45% ($P<0.05$) (**Fig. 1**). Expression of BCRP mRNA was also markedly and consistently (>40%) down regulated by TNF-α and IL-1β ($P<0.05$), whereas exposure to IL-6 had little effect on the expression of the apical transporters. In contrast, the basolateral transporters showed a very different response to cytokine treatment. MDR3 mRNA levels were increased by IL-6 by >70%, although this response was quite variable between experiments. Neither TNF-α nor IL-1β significantly altered expression of MDR3 mRNA (or protein) at any time point tested. However, expression of MRP1 mRNA was significantly ($P<0.05$) increased by TNF-α (64%), IL-1β (43%) and IL-6 (63%) after 12 h treatment **(Fig. 1).**

Little or no effect of cytokine treatment on ABC transporter protein levels was observed after 24 h (data not shown), consistent with their reported long half lives (Muller et al., 1995). However, by 48 h of cytokine treatment, an approximately 50% decrease in MDR1 abundance was observed in response to TNF-α and IL-1 β (**Fig. 2**), consistent with the changes in mRNA expression. IL-6-treated cells exhibited >30% reduction in BCRP and MDR1 protein levels compared to control, but this effect was not statistically significant. However, in contrast to the mRNA data, which showed increased MRP1 expression with cytokine treatment, no significant changes in MRP1 protein levels were observed**.** On the other hand, MDR3 protein expression was significantly increased by >40% in response to IL-6 ($P<0.05$), consistent with the mRNA findings **(Fig. 2).**

Growth factors and steroid hormones

EGF (10 ng/ml) significantly increased BCRP mRNA levels by >120% above control ($P<0.05$) **(Fig. 3A).** IGF-II (25 ng/ml) also significantly increased BCRP mRNA by ~70% ($P<0.05$)**.** EGF treatment resulted in a trend towards increased MDR3 mRNA expression by >40%, but this effect did not reach statistical significance. MDR1 and MRP1 mRNA expression demonstrated no response to either EGF or IGF-II.

17β-estradiol (100 nM) stimulated expression of MDR1 mRNA by ~50% above control and MDR3 by >60% ($P<0.05$) **(Fig. 3B).** Estradiol also increased mean BCRP mRNA expression by >50%, although this did not reach statistical significance, and had no significant effects on MRP1 gene expression. Progesterone (100 nM) stimulated MRP1 mRNA expression by

almost 90% (P<0.05), but had no other significant effects on ABC transporter mRNA expression **(Fig. 3B)**

Changes in protein expression were minimal or modest after 24 h treatment, but after 48 h were more evident and consistent and reached statistical significance. BCRP protein levels were increased by EGF treatment by ~90% above control after 48 h of exposure (*P*<0.05) **(Fig. 4)**. EGF also increased MDR3 protein levels by ~50% (*P*<0.05)**.** IGF-II significantly increased BCRP protein levels by ~50% (*P*<0.05) but had no effects on the protein levels of the other three transporters. Consistent with the mRNA data, MDR1 and MRP1 protein expression was unaltered by either EGF or IGF-II. In contrast, after 48 h of exposure to estradiol, significant increases in both MDR1 and MDR3 protein levels (~60% above control) were observed (P<0.05) **(Fig. 4)**. BCRP protein levels were also significantly increased by >50% by estradiol treatment (P<0.05), supporting the observed trend towards increased mRNA expression. Neither estradiol nor progesterone had any significant effects on MRP1 protein abundance. However, progesterone had a weak stimulatory effect on MDR1 protein expression, increasing levels by ~40% (*P*<0.05) despite a lack of detectable change in mRNA levels.

B. Functional studies.

Short term accumulation studies were undertaken, using radiolabelled substrates for BCRP ([^{3}H] mitoxantrone) and MDR1 and 3 ([^{3}H] digoxin), to confirm that the changes in expression observed were translated into altered efflux rates. Cells were pre-treated for 48 h with TNF-α, EGF and estradiol, since these agents exerted the most significant changes in BCRP and MDR1 protein expression. Substrate accumulation was assessed at two time points, 20 and 60 min. As shown in Figure 5A, trophoblast cells pre-treated with TNF-α demonstrated significantly increased accumulation of [^{3}H] mitoxantrone at the 60 min time point (15.0 ± 3.9 % above control, mean ± SD, n=4). In contrast, treatment with EGF resulted in significantly decreased accumulation of the BCRP substrate at both the 20 and 60 min time points (-17.5 ± 5.9 and -12.2 ± 8.8 % of control, respectively; *P*<0.05) **(Fig. 5A)**. These findings were consistent with the observed negative and positive effects of TNF-α and EGF, respectively, on BCRP expression. Estradiol treatment significantly decreased accumulation of [^{3}H] digoxin at the 60 min time point (-20.9 ± 6.5 % of control), consistent with its ability to up-regulate MDR1 and MDR3 protein **(Fig. 5B).**

Discussion

In this study we have demonstrated for the first time differential regulation of placental apical and basolateral drug transporters by cytokines, growth factors and sex steroids. Most interestingly, we found that expression of the apical efflux transporters MDR1 and BCRP was significantly down-regulated by TNF-α and IL-1β, cytokines associated with several placental pathologies. In contrast, the expression of MDR3 and MRP1, localized predominantly in the basolateral membrane of trophoblast, was either up-regulated or unchanged following cytokine treatment. These data suggest that placental exposure to pro-inflammatory cytokines may decrease fetal protection from xenobiotics and/or create conditions where active transport of drugs to the fetus will be enhanced. In contrast, EGF and estradiol, two factors associated with placental growth and differentiation, were found to up-regulate BCRP, MDR1 and MDR3 expression, consistent with the notion that these transporters reflect the phenotype of the mature syncytiotrophoblast and hence act to sustain syncytial function.

Previously published studies on ABC transporter expression in the human placenta have used a number of different models, paradigms and culture conditions, making comparison of published results difficult. Trophoblast-like carcinoma cells (BeWo, Jar, Jeg3) may be useful for investigating some aspects of ABC transporter regulation, but need to be used with caution (Evseenko et al, 2006a). These cell lines do not undergo spontaneous differentiation and fusion in culture, but proliferate and divide, and respond abnormally to growth/apoptotic regulators. On the other hand, when primary trophoblasts are extracted and cultured, they are inevitably exposed to dramatic environmental changes in concentrations of various modulators, growth factors and steroid hormones. These changes may unintentionally affect the expression of ABC genes and alter the results of regulation studies. In particular, steroid exposure in culture needs to be monitored and controlled. Treating FCS with activated charcoal will significantly reduce levels of estrogens and other steroids in the media and therefore allow a more representative response of ABC genes to test factors. It has been previously demonstrated that if charcoal-stripped serum is used in the culture medium the response of the BCRP gene to estradiol is significantly increased (Ee et al., 2004). The presence of the estrogenic compound phenol red in media may also change the response of ABC genes to estrogens (Ernst et al., 1989).

We also recommend caution when ABC transporter expression and regulation is studied at the RNA level only, as we have previously demonstrated that mRNA levels do not always closely correlate with ABC protein expression in trophoblast cultures (Evseenko et al., 2006a). In the

present studies, in general changes in protein levels at 48 h correlated with changes in mRNA expression at 12 h, the difference in time reflecting approximately two half lives of the proteins concerned (assuming half life data from other tissues can also be applied to trophoblast). Significant posttranslational effects on protein expression cannot be ruled out, however, which may explain the discrepancies observed between mRNA and protein data for MRP1 (cytokine and progesterone regulation).

Exposure of the placenta to elevated cytokine concentration is believed to occur during a number of obstetric disorders associated with placental pathologies. Concentrations of TNF-α in maternal serum are elevated from the first trimester of pregnancy in women who subsequently developed preeclampsia or first trimester spontaneous abortion (Shaarawy and Nagui, 1997). Elevated TNF-α concentrations have also been reported early in pregnancy in women with gestational diabetes (Kirwan et al., 2002; Winkler et al., 2002); placentas from diabetic pregnancies secrete increased amounts of TNF-α in response to glucose (Coughlan et al., 2001). Finally, the placenta is also exposed to high cytokine levels with intrauterine infection (Saji et al., 2000). While concentrations of TNF-α reported in maternal plasma are considerably lower than those used in this study (< 50 pg/mL), local levels in the placenta are likely to be much higher than in the peripheral circulation since the placenta is a source of this cytokine. The regulatory effects of cytokines on expression of drug transporters in trophoblasts have not been studied to date, although in a pregnant rat model LPS administration was recently reported to reduce placental mdr expression and increase fetal uptake of an mdr substrate (Wang et al, 2005); the specific role of cytokines in this response has not been determined, however. In the liver and other organs and tissues, pro-inflammatory cytokines have been implicated in the regulation of the function and expression of several ABC transporters (MDR1, MRPs), demonstrating predominantly inhibitory effects (Tang et al., 2000; Hartmann et al., 2001; Sukhai et al., 2001). Our results suggest that TNF-α and IL-1β have inhibitory effects on expression of the major placental apical drug transporters, while IL-6 can exert either stimulatory or inhibitory effects depending on the transporter. In contrast, basolateral transporters seem relatively insensitive to cytokine regulation.

Trophoblast cells pre-treated with TNF-α demonstrated significantly increased accumulation of BCRP substrate ([^{3}H] mitoxantrone) compared to controls, consistent with the negative effects observed of TNF-α on BCRP mRNA and protein expression. Functional studies showed a very modest effect of TNF-α on functional activity of MDR1/3 in the trophoblasts, in apparent contradiction with the observed reduction of MDR1 mRNA and protein by TNF-α. However, it

should be noted that digoxin is also a substrate for MDR3 (Smith et al., 2000), expression of which was not down-regulated by TNF-α in these studies. Due to the absence of blockers selective for MDR1 or 3, it is at present difficult to study the efflux activity of these transporters independently with confidence.

We and others have recently previously reported that levels of ABC transporters vary markedly with trophoblast differentiation (Pascolo et al., 2003; Evseenko et al., 2006; Zu Schwabedissen et al., 2006). Levels of BCRP and MRP1 increase with trophoblast differentiation, whereas MDR1 and MDR3 expression decrease (Pascolo et al., 2003; Evseenko et al., 2006a; Zu Schwabedissen et al., 2006). In order to minimise the effects of differentiation on our results, we performed our studies on day 2 of culture and omitted the supplements required to drive syncytium formation. However, delayed syncytialisation occurs spontaneously in term trophoblast cells in vitro, so some changes in ABC transporter expression as a result of differentiation are inevitable, contributing an unavoidable background effect to the studies presented. As an additional complication, EGF and estradiol are both inducers of trophoblast differentiation (Morrish et al., 1998; Yashwanth et al., 2006). Hence, we cannot rule out the possibility that these agents indirectly increased MDR1, MDR3 and BCRP expression through their effects on trophoblast differentiation and maturation. However, it should be pointed out we have previously shown that MDR1 and MDR3 expression actually declines with syncytialisation (Evseenko et al., 2006a). Toxicity studies confirmed that exposure to cytokines at the concentrations applied did not significantly reduce cell viability, so the observed negative effects on ABC expression were not secondary to a general toxicity-induced reduction in gene expression.

Effects of growth factors on expression of ABC transporters in the human placenta have not been investigated previously, with the exception of recent study in which EGF was found to stimulate BCRP expression in primary trophoblast (Zu Schwabedissen et al., 2006). We confirmed this finding and also found positive effects of EGF on MDR3 gene and protein expression, while both EGF and IGF-II both stimulated BCRP expression.

The regulatory effects of placental steroids on ABC gene expression are somewhat controversial. It was reported that estradiol exerts a potent stimulatory effect on BCRP expression in BeWo cells (Ee et al., 2004). A more recent study, however, indicated that the same concentrations of estradiol markedly inhibited BCRP production in BeWo cells (Wang et al., 2006). In the same study, progesterone was observed to be a BCRP inducer, but this effect

was detectible only at very high concentrations (>1 µM) (Wang et al., 2006). Circulating estradiol and progesterone concentrations in late pregnancy reach approximately 50 and 500 nM at term, respectively (Batra and Bengtsson 1978; Khan-Dawood and Dawood 1984). While the free fraction is likely to be much less than this (5-50%, depending on the steroid and gestational age), secretion of these steroids by the placenta is likely to result in increased local concentrations. Our data indicated a moderate positive effect of estradiol at physiologically relevant concentrations (100 nM) on placental BCRP, MDR1 and MDR3 expression, but little or no effect of progesterone. However, we did find significant positive effects of progesterone on MRP1 expression. We also presented evidence of increased functional MDR activity (i.e. reduced [^{3}H] digoxin accumulation) by estradiol, supporting the expression data. It is important to note here that these accumulation studies do not take into account directionality of efflux, which is obviously of physiological significance, particularly for chemicals that are substrates for both apical and basolateral transporters (Evseenko et al, 2006b).

In summary, using a combination of real-time PCR, immunoblotting and substrate accumulation assays, we have demonstrated that physiologically relevant mediators such as pro-inflammatory cytokines, steroid hormones and growth factors significantly affect expression of placental ABC transporters in primary trophoblast, but that the response of apical transporters and basal transporters vary. Findings of a reduction of BCRP and MDR1 expression by TNF-α and IL-1β have the most significant clinical implications, suggesting that decreased fetal protection from xenobiotics and increased susceptibility of trophoblast cells to apoptosis may occur in obstetric disorders associated with exposure to elevated cytokine concentrations.

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Footnotes.

Address for reprints: Jeffrey A. Keelan, PhD, Liggins Institute and Department of Pharmacology and Clinical Pharmacology, University of Auckland, Private Bag 92019, Auckland, New Zealand. E-mail: j.keelan@auckland.ac.nz.

[1]Liggins Institute, University of Auckland, Private Bag 92019, Auckland, New Zealand

[2]Department of Pharmacology & Clinical Pharmacology, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

Figure legends

Figure 1

Relative expression of ABC transporter mRNA in primary trophoblast after 12 h exposure to pro-inflammatory cytokines, as determined by real-time PCR. Expression of target genes was normalized to the level of 18s rRNA and expressed as a percentage of control (mean ± SD, n=5). For statistical analysis one-way ANOVA was used, followed by Student-Newman-Keuls test. [*], $P<0.05$.

Figure 2

Immunoblotting analysis of protein expression (mean ± SD, n=5) of ABC proteins in primary trophoblast after 48 h exposure to pro-inflammatory cytokines. **A**, representative series of blots; **B**, average band intensity (n=5 experiments), normalized to β-actin, and expressed relative to control. For statistical analysis, one-way ANOVA was performed, followed by Student-Newman-Keuls test. [*], $P<0.05$.

Figure 3

Relative expression of ABC transporter mRNA in primary trophoblast, determined by real-time PCR, after 12 h exposure to test agents. **A,** EGF and IGF-II; **B,** estradiol (E_2) and progesterone (P_4). Expression of target genes was normalized to the level of 18s rRNA and expressed as a percentage of control (mean ± SD, n=5). For statistical analysis one-way ANOVA was performed, followed by Student-Newman-Keuls test. [*], $P<0.05$.

Figure 4

Protein expression (mean ± SD, n=5) of ABC proteins in primary trophoblast after 48 h exposure to EGF, IGF-II, estradiol and progesterone as determined by immunoblotting. **A**, representative series of blots; **B**, average band intensity (n=5 experiments) normalized to β-actin, and expressed relative to control. For statistical analysis, one-way ANOVA was applied, followed by Student-Newman-Keuls test. [*], $P<0.05$.

Figure 5.

Accumulation of radiolabelled substrates for BCRP (**A**, [^{3}H] mitoxantrone) or MDR (**B**, [^{3}H] digoxin) efflux in primary trophoblasts after 20 and 60 minute incubation. Cells were pre-treated with either TNF-α (20 ng/ml), EGF (10 ng/ml) or estradiol (100 nM) for 48 hours prior to study. Accumulation (as counts per minute [cpm]) was expressed relative to total protein concentration (mean ± SD). A representative experiment, one of four conducted on different trophoblast cultures, is shown. Statistical significance was assessed by one-way ANOVA with repeated measures. *, $P<0.05$.

Table 1. Gene-specific PCR primers used for real time PCR.

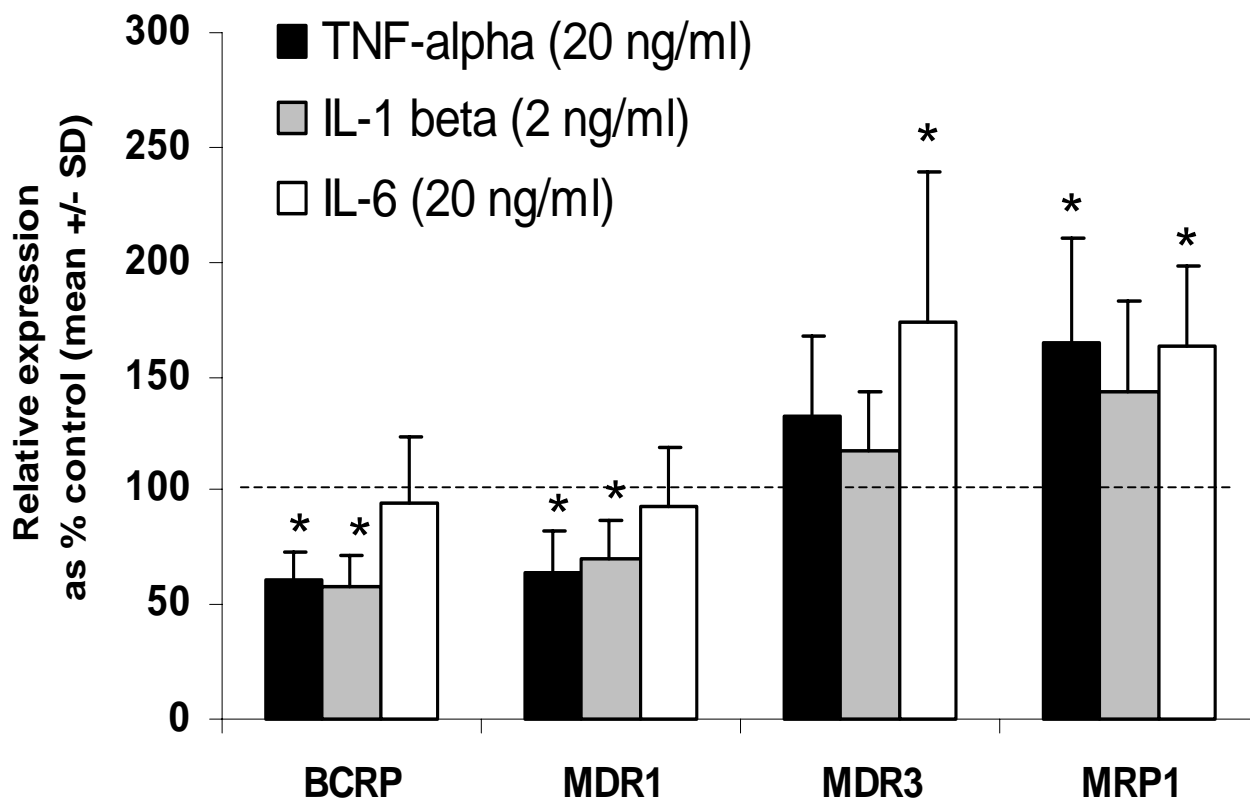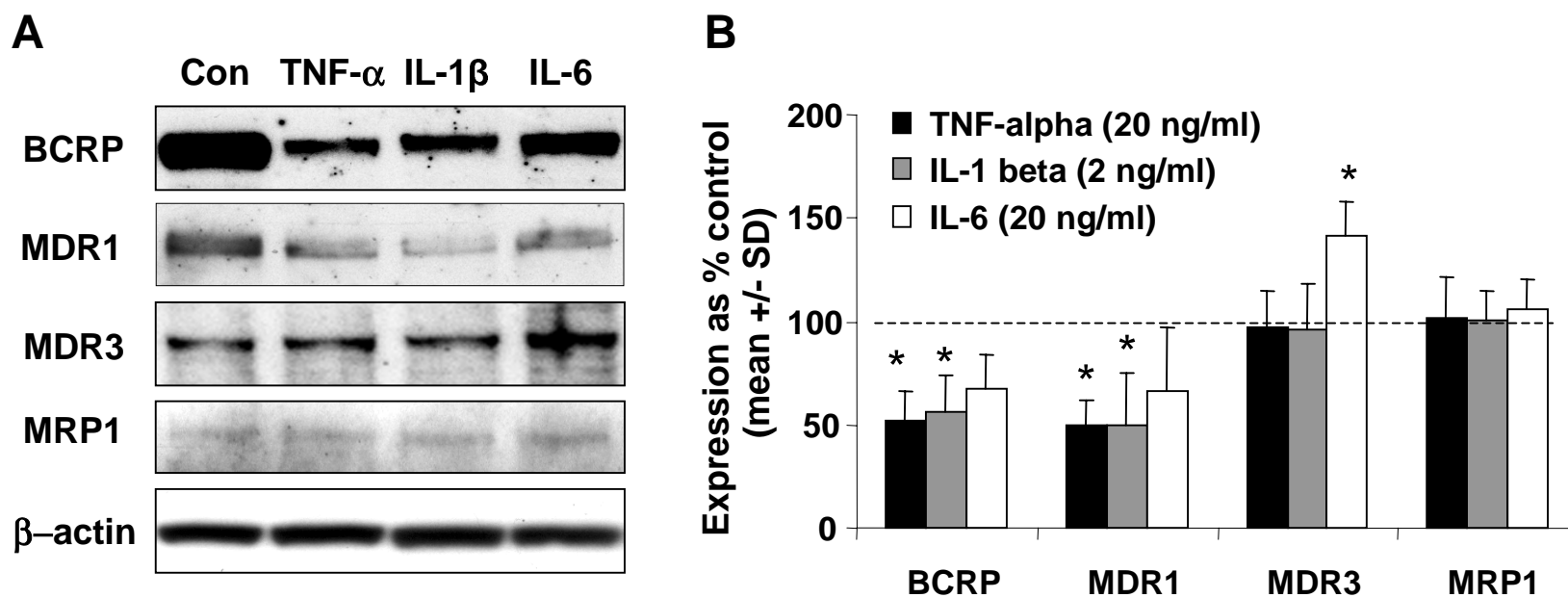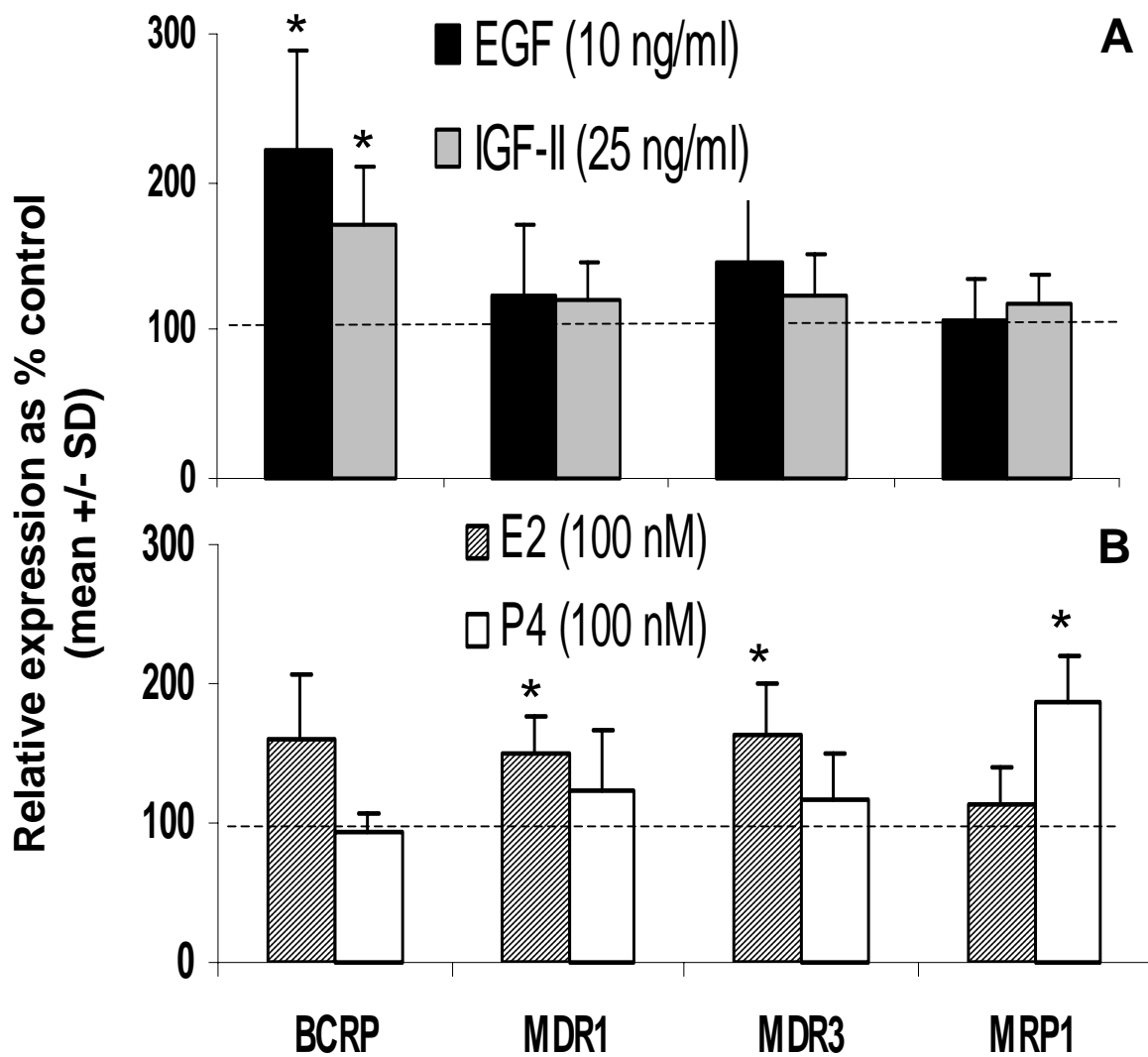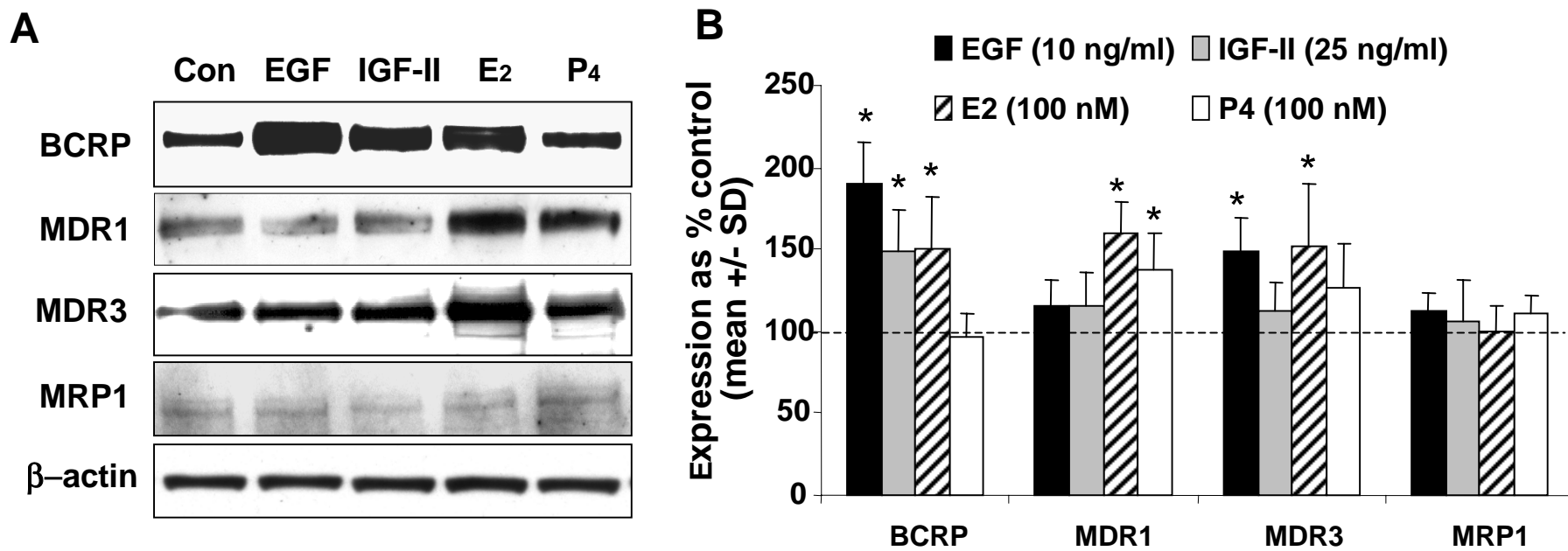| Gene	5' Primer	3' Primer
BCRP/ABCG2	GGATGAGCCTACAACTGGCTT	CTTCCTGAGGCCAATAAGGTG
MDR1/ABCB1	GTGGTGGGAACTTTGGCTG	TACCTGGTCATGTCTTCCTCC
MRP1/ABCC1	GTGTTTCTGGTCAGCCCAACT	TTGGATCTCAGGATGGCTAGG
MDR3/ABCB4	ATCGAGACGTTACCCCACAA	CATTCTGGATGGTGGACAGG

Figure 1

Figure 2

Figure 3

Figure 4

A

	Con	EGF	IGF-II	E₂	P₄
BCRP					
MDR1					
MDR3					
MRP1					
β–actin					

B

Figure 5