Effect of Prostaglandin E₂ on Multidrug Resistance Transporters in Human Placental Cells^S

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ABSTRACT

Prostaglandin (PG) E₂, a major product of cyclooxygenase (COX)-2, acts as an immunomodulator at the maternal-fetal interface during pregnancy. It exerts biologic function through interaction with E-prostanoid (EP) receptors localized to the placenta. The activation of the COX-2/PGE₂/EP signal pathway can alter the expression of the ATP-binding cassette (ABC) transporters, multidrug resistance protein 1 [P-glycoprotein (Pgp); gene: ABCB1], and breast cancer resistance protein (BCRP; gene: ABCG2), which function to extrude drugs and xenobiotics from cells. In the placenta, PGE₂-mediated changes in ABC transporter expression could impact fetal drug exposure. Furthermore, understanding the signaling cascades involved could lead to strategies for the control of Pgp and BCRP expression levels. We sought to determine the impact of PGE₂ signaling mechanisms on

Pgp and BCRP in human placental cells. The treatment of placental cells with PGE₂ up-regulated BCRP expression and resulted in decreased cellular accumulation of the fluorescent substrate Hoechst 33342. Inhibiting the EP1 and EP3 receptors with specific antagonists attenuated the increase in BCRP. EP receptor signaling results in activation of transcription factors, which can affect BCRP expression. Although PGE₂ decreased nuclear factor κ -light chain-enhancer of activated B activation and increased activator protein 1, chemical inhibition of these inflammatory transcription factors did not blunt BCRP up-regulation by PGE₂. Though PGE₂ decreased Pgp mRNA, Pgp expression and function were not significantly altered. Overall, these findings suggest a possible role for PGE₂ in the up-regulation of placental BCRP expression via EP1 and EP3 receptor signaling cascades.

Introduction

Inflammation has an important role in the pathophysiology of several common and serious pregnancy disorders that are each associated with adverse outcomes and compromised placental function. The inflammatory responses observed during pregnancy are complex and the result is often dependent on the timing, severity, and type of stimulus. An inflammatory response can be triggered by a wide variety of pathologic stimuli, including hypoxia, infection, tissue damage, trauma, and cellular stress (Guo et al., 2010; Bloise et al., 2013). It is increasingly clear

that different stimuli can induce alternative patterns of inflammatory molecules, thereby invoking different regulatory patterns of gene expression (Ho and Piquette-Miller, 2006).

Studies of placental cells and tissue in vitro document their responsiveness to inflammatory stimuli with increased production of cytokines, chemokines, and prostaglandins (PGs) (Keelan et al., 2003). PGs are potent mediators of immune responses (Betz and Fox, 1991; Snijdewint et al., 1993; Roper and Phipps, 1994) and inflammation (Gilroy et al., 1999; Trebino et al., 2003). They play a role in many aspects of pregnancy including maternal-fetal tolerance, parturition, and innate immunity. PGE₂ is the predominant PG produced by the placenta (Myatt, 1990). As a result, levels at the placental interface are likely higher than in the peripheral circulation. PGE₂ is derived from arachidonic acid via the activities of cyclooxygenase (COX)-2. The effects of PGE₂ are facilitated by specific-membrane bound G protein-coupled E-prostanoid (EP) receptors (EP1–EP4), which each have distinct signal transduction profiles and often differing cellular actions (Hata and Breyer, 2004).

The activation of COX-2 affects the ATP-binding cassette (ABC) transporters, multidrug resistance protein (MDR) 1 [P-glycoprotein (Pgp);

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ABBREVIATIONS: ABC, ATP-binding cassette; AH-6809, 6-isopropoxy-9-oxoxanthene-2-carboxylic acid; ANOVA, analysis of variance; AP-1, p activator protein 1; BAY-11-7082, 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile; BCRP, breast cancer resistance protein; calcein AM, calcein acetoxymethylester; COX, cyclooxygenase; C_t, cycle threshold; EP, E-prostanoid; FBS, fetal bovine serum; FCS, fetal calf serum; FTC, fumitremorgin C; hCG, human chorionic gonadotropin; IL, interleukin; L-161982, N-[[4'-[[3-butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4H-1,2,4-triazol-4-yl[methyl]]-1,1'-biphenyl]-2-yl[sulfonyl]-3-methyl-2-thiophenecarboxamide; L-798106, (2E)-N-[[5-bromo-2-methoxyphenyl)sulfonyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide; MDR, multidrug resistance protein; MK-571, 3-[[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl]]-[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid; MRP, multidrug resistance-associated protein; NF- κ B, nuclear factor κ -light chain-enhancer of activated B; NS398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide; PBS, phosphate-buffered saline; PBS-T, PBS-Tween 0.1%; Pgp, P-glycoprotein; PG, prostaglandin; SC-19220, 8-chloro-dibenz[b,f][1,4]oxazepine-10(11H)-carboxy-(2-acetyl)hydrazide; SR 11302, (E,E,Z,E)-3-methyl-7-(4-methylphenyl)-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid.

gene: ABCB1], multidrug resistance-associated protein (MRP) 1 (gene: ABCC1), and breast cancer resistance protein (BCRP; gene: ABCG2) (Surowiak et al., 2008; Liu et al., 2010), which play important roles in extruding drugs and xenobiotics out of cells. COX-2 up-regulates the expression and activity of Pgp and BCRP in several cell types (Fantappiè et al., 2002; Patel et al., 2002). Furthermore, specific COX-2 inhibitors, such as celecoxib and NS398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide), block the COX-2-mediated increase in Pgp and BCRP, suggesting that PGE₂ may be implicated in this response (Arico et al., 2002; Patel et al., 2002; Zatelli et al., 2007; Arunasree et al., 2008; Ko et al., 2008; Zrieki et al., 2008; Roy et al., 2010).

Pgp and BCRP are the most abundant ABC efflux transporters on the apical brush-border membrane of the placenta. These proteins pump compounds back into the maternal circulation and are generally believed to have a protective role (Ceckova-Novotna et al., 2006; Mao, 2008). However, it is easy to see how problems could arise if they were to prevent drug delivery to the fetus where the medication was intended to have a therapeutic effect. In either instance, changes in Pgp and/or BCRP expression in the placenta could affect fetal drug exposure.

The functional expression of Pgp and BCRP can be up- or down-regulated by exposing placental cells to various inflammatory agents. For example, acute inflammation, such as that mediated by microbial products such as lipopolysaccharide, are known to down-regulate the expression of several placental transporters, including Pgp (Chen et al., 2005; Wang et al., 2005; Petrovic et al., 2007, 2008; Bloise et al., 2013). On the other hand, chronic, sublethal exposure to lipopolysaccharide had no effect on overall placental Pgp activity (Bloise et al., 2013). In other examples of chronic inflammation, such as that observed in subjects with rheumatoid arthritis, Pgp expression is increased (Dumoulin et al., 1997; Llorente et al., 2000). We previously documented up-regulation of placenta Pgp and BCRP expression in the presence of histologic chorioamnionitis (Mason et al., 2011). Taken together, the findings suggest there are many interactive and reactive mediators of inflammation that can alter these transporters in the placenta. To date, studies have primarily focused on the effects of proinflammatory cytokines (Ho and Piquette-Miller, 2006; Evseenko et al., 2007). PGs are released by the actions of proinflammatory cytokines. It has been shown that interleukin (IL)-1 β , IL-6, tumor necrosis factor- α , and IL-8 directly stimulate PGE₂ production by cells obtained from term placenta (Furuta et al., 2000). Surprisingly, there is no information on the effects of PGE₂ on ABC efflux transporters in the placenta.

In this investigation, we tested the hypothesis that PGE₂ increases Pgp and BCRP expression and activity in placental cells via distinct EP receptor signaling pathways. Identification of these signaling pathway(s) could provide important information about mechanisms of inflammation on transporter regulation and facilitate the development of strategies to manipulate Pgp and BCRP expression levels via EP receptor inhibition.

Materials and Methods

The PG receptor antagonists SC-19220 (8-chloro-dibenz[*b,f*][1,4]oxazepine-10(11*H*)-carboxy-(2-acetyl)hydrazide), AH-6809 (6-isopropoxy-9-oxoanthene-2-carboxylic acid), and L-161982 (*N*-[[4'-[3-butyl-1,5-dihydro-5-oxo-1-[2-(trifluoromethyl)phenyl]-4*H*-1,2,4-triazol-4-yl]methyl][1,1'-biphenyl]-2-yl]sulfonyl]-3-methyl-2-thiophenecarboxamide) were purchased from Cayman Chemical (Ann Arbor, MI). PGE₂, L-798106 [(2*E*)-*N*-[(5-bromo-2-methoxyphenyl)sulfonyl]-3-[2-(2-naphthalenylmethyl)phenyl]-2-propenamide], verapamil, funitremorgin C (FTC), MK-571 (3-[[[3-[(1*E*)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]propanoic acid), sulprostone, anti-MDR1 monoclonal antibody (clone F4), and Percoll were purchased from Sigma-Aldrich (St. Louis, MO). BAY-11-7082 (3-[(4-methylphenyl)sulfonyl]-(2*E*)-propenenitrile) and SR 11302 [(*E,E,Z,E*)-3-methyl-7-(4-methylphenyl)-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid] were from Cayman Chemical and Tocris Bioscience (R&D Systems, Minneapolis, MN), respectively. The antibody against BCRP

(clone BXP21) was purchased from EMD Millipore (Billerica, MA). All receptor antagonists were dissolved in DMSO as 10 mM stock solutions and stored at -20°C; PGE₂ (1 mg/ml) was dissolved in ethanol. The concentration of DMSO and EtOH after the dilution of compounds to their final concentrations was 0.1 and 0.5%, respectively. Lipofectamine 2000, p-nuclear factor κ -light chain-enhancer of activated B (NF- κ B)-Luc plasmid, and p activator protein 1 (AP-1)-Luc plasmid were purchased from Life Technologies (Carlsbad, CA). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI).

Cell Culture. Jar cells were obtained from American Type Cell Culture (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Human placentas were obtained under a protocol approved by the Institutional Review Board at the University of Kansas Medical Center. Cytotrophoblasts were isolated from healthy placenta delivered at term by cesarean section as previously described (Arroyo et al., 2004) with minor modifications. Briefly, 50 g of villous tissue was digested in Hanks' balanced salt solution media (Sigma-Aldrich) containing 40 mg of DNase (Roche Diagnostics, Indianapolis, IN), 100 mg of trypsin (Sigma-Aldrich), and 500 units of dispase (BD Biosciences, Bedford, MA). Digestion was performed at 37°C for 60 minutes in an orbital shaking incubator at 350g. Digest media were strained to remove tissue fragments and then centrifuged for 5 minutes at 500g. The cell pellet was resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS; Sigma-Aldrich). Cell suspensions were layered on a discontinuous Percoll gradient (5–70%) and spun for 20 minutes at 1200g. Cells between the 40 and 50% Percoll bands were collected and plated at a density of 6×10^6 cells on 60-mm² petri dishes. The cells were grown in M199 media, supplemented with 10% FCS, epidermal growth factor (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% CO₂ humidified atmosphere at 37°C. After 24 hours in culture, the cells were washed with phosphate-buffered saline (PBS), and renewed with media containing 10% FCS and nystatin.

The purity of the isolated cells was determined by immunofluorescence staining using anti-cytokeratin 7 and anti-vimentin antibodies. A proportion of $\geq 90\%$ cytokeratin 7-positive cells (cytotrophoblasts) was considered to be of sufficient purity. Primary trophoblasts from eight different placentas were used for the regulatory experiments, and the data generated were pooled and analyzed collectively.

Assessment of Cytotrophoblast Cell Biochemical Differentiation. The β -subunit of human chorionic gonadotropin (hCG) is produced by terminally differentiated syncytiotrophoblast and was measured as an indicator of cytotrophoblast differentiation in culture after their isolation from term placenta. Culture medium was collected at 24, 48, 72, 96, and 120 hours of cytotrophoblast cell culture, and was stored in aliquots at -80°C. β -hCG was measured by enzyme-linked immunosorbent assay following the manufacturer's instructions (DRG International, Springfield, NJ).

PGE₂ Measurements. Cells were cultured in their respective phenol red free, charcoal-stripped FBS media for 24 hours. Cytotrophoblasts were cultured for 4 days, at which point media were replaced and collected after 24 hours. The PGE₂ concentration in the media was then determined using a PGE₂ enzyme-linked immunosorbent assay kit (Thermo Fisher Scientific, Rockford, IL) following the manufacturer's instructions. The sensitivity of the kit was 15.6 pg/ml, and the intra- and inter-assay coefficients of variation were less than 10%. PGE₂ concentrations were calculated using a four-parameter logistic curve-fitting program in GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Treatment. To examine the effect of PGE₂ on Pgp and BCRP transporter expression, cells were cultured in their respective phenol red free medium supplemented with 10% charcoal-/dextran-stripped FBS for 24 hours. Primary trophoblasts were cultured for 4 to 5 days to allow for the development of syncytialization. The medium was then replaced with fresh medium containing 5 μ g/ml [14.8 μ M] PGE₂ and cell culture continued. For pharmacological interventions of PGE₂ receptor-specific isoforms, Jar cells were incubated with 10 μ M PG receptor antagonists (SC-19220, EP1; AH-6809, EP1 and EP2; L-798106, EP3; and L-161982, EP4) for 1 hour prior to the addition of PGE₂ (5 μ g/ml). The effects of PGE₂ and EP receptor antagonists on mRNA expression were tested at 24 hours. Multiple time points (6–48 hours) were tested in preliminary studies. At 24 hours, we observed clear and consistent changes

in transporter mRNA and protein with PGE₂ treatment. In consecutive studies, cells were exposed to an EP3 receptor agonist (sulprostone, 10 μ M) or an EP1 receptor agonist (17-phenyltrilor PGE₂) for 24 hours prior to RNA isolation. Finally, Jar cells were treated with BAY-110782 (10 μ M) and SR 11302 (10 μ M) for 1 hour prior to the addition of PGE₂. Pgp and BCRP mRNA expression were determined at 24h.

RNA Isolation and Purification. Total cellular RNA was isolated from the cells and purified using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Valencia, CA). Total RNA concentration was determined by UV spectroscopy at 260 nm and RNA integrity and purity were confirmed by a 260/280 ratio (>1.8) and separation on a 1% agarose gel followed by visualization with ethidium bromide.

Quantitative Real-Time Polymerase Chain Reaction. Primer sequences for both Pgp and BCRP amplifications were previously described (Mason et al., 2011). 18s rRNA was used to normalize mRNA expression levels. Each sample was assayed in duplicate. All primer sets were tested to ensure efficiency of amplification over a wide range of template concentrations. SYBR Green (Bio-Rad Laboratories, Hercules, CA) was used for amplicon detection. A melt curve was used after amplification to ensure that all samples exhibited a single amplicon. The average C_t value (cycle threshold for target or endogenous reference gene amplification) was estimated using the software associated with the iCycler real-time polymerase chain reaction detection system (Bio-Rad Laboratories). Relative changes in mRNA expression of the target genes were analyzed using the $\Delta\Delta C_t$ method ($2^{-\Delta\Delta C_t}$) (Livak and Schmittgen, 2001). The average ΔC_t was calculated by subtracting the average C_t value of the endogenous reference gene (18s rRNA) from the average C_t value of the target gene for the treatment and control groups. Fold changes in mRNA expression of target genes from the treatment groups were expressed relative to that of the vehicle control group.

Immunoblot Analysis (Western Blotting). Whole cell lysates were prepared by washing the cells twice with ice-cold PBS and then adding lysis buffer (50 mmol/l Tris-Cl, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Igepal Ca 630, 0.1% SDS, 0.5% sodium deoxycholate, protease inhibitor, phosphatase inhibitor cocktail I, phosphatase inhibitor cocktail II) to each well. The cells were collected in lysis buffer using a cell scraper, and the sample was placed on ice and sonicated briefly. The protein content was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

Protein samples were heated in SDS Laemmli loading buffer [1:1 (v/v)] at 95°C for 5 minutes, and total protein (40 μ g) was loaded onto a 7.5% SDS-polyacrylamide gel. Electrophoresis was conducted at 100 V. The proteins were transferred overnight at 35 V onto polyvinylidene membrane. Nonspecific binding sites were blocked for 1h with 5% nonfat dry milk in PBS-Tween 0.1% (PBS-T). The polyvinylidene membrane was incubated with Pgp antibody (1:500), BCRP antibody (1:1000), and β -actin antibody (1:20,000) at 4°C overnight, washed three times with PBS-T, and then incubated with a horseradish peroxidase-linked secondary antibody for 1 hour at room temperature. The cells were then washed three times with PBS-T. Detection of the protein-antibody complexes was accomplished via the use of enhanced chemiluminescence reagents and chemiluminescent detection.

Functional Activity. Efflux transporter-mediated activity was measured by the intracellular accumulation of fluorescent substrates in the presence or absence of specific inhibitors. Drug efflux activity of cells in monolayer culture was measured by inhibition of the cellular uptake of fluorescent Pgp substrates, calcein acetoxymethyl ester (calcein AM) and Flutax-2 (Oregon green-488 paclitaxel), and BCRP substrate, Hoechst 33342. Calcein AM is a lipophilic, nonfluorescent dye that diffuses into cells where it is cleaved by cytosolic esterase to a green fluorescent dye. Because calcein AM is also a substrate for MRP1, Flutax-2 was used to confirm Pgp activity. Flutax-2 is a commercially available fluorescent derivative of paclitaxel with a high diffusion coefficient to cells (Jang et al., 2001; Díaz et al., 2003). Hoechst 33342 is a dye that fluoresces only when bound to DNA. Thus, the amount of transporter activity is inversely proportional to the accumulation of intracellular fluorescence. The selective Pgp and BCRP inhibitors used were verapamil (100 μ M) and FTC (5 μ M), respectively.

For functional studies, Jar cells were plated on 24-well plates at 1×10^5 cells/well and incubated for 1 day in phenol red free medium containing 10% charcoal/dextran-stripped FBS before study. Primary trophoblasts were seeded at a density of 5×10^5 cell/well and cultured for 4 days in growth medium containing 10% charcoal/dextran-stripped FBS prior to treatment. Cells were

incubated for 24 hours at 37°C with or without PGE₂ (5 μ g/ml). The selective blockers were added for 60 minutes at 37°C prior to treatment with fluorescent substrates. Fluorescent substrates were then added to final concentrations of 0.4 μ M (calcein AM), 0.5 μ M (Flutax-2), and 5 μ g/ml (Hoechst 33342). After a 60-minute incubation period, the cells were washed thrice with cold PBS and lysed in 10 mM Tris-HCl-1% Triton X-100 (pH 7.4). The accumulation of fluorescent substrates was measured on a SpectraMax M5 Microplate reader (Molecular Devices, Sunnyvale, CA) at 485/535 nm for calcein AM and Flutax-2, and 355/460 nm for Hoechst 33342. Relative fluorescent units were normalized by protein concentration and expressed as percentage compared with control.

Luciferase Assay. pNF- κ B-Luc plasmid or pAP-1-Luc plasmid was cotransfected with *Renilla* luciferase plasmid into Jar cells using Lipofectamine 2000 according to the manufacturer's instructions (Life Technologies). The cells were transfected for 24 hours, washed, and then treated with PGE₂ (5 μ g/ml) for another 24 hours. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega) according to the recommended protocol. The luciferase activity in the cell lysates was measured by luminescence using the TD-20/20 luminometer (Promega) and normalized to total protein. Each treatment group was run in triplicate.

Statistical Analyses. All studies were repeated at least three times. Descriptive statistics were performed for each data set. Graphs were plotted, data were transformed, and statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software). β -hCG concentrations were log transformed and analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post test. For expression studies, either the unpaired *t* test or one-way ANOVA followed by the Tukey's post test was performed as appropriate. Differences in the cellular accumulation of fluorescent substrates were analyzed using a one-way ANOVA followed by Dunnett's post test. $P < 0.05$ was considered to be significant.

Results

Differentiation of Cytotrophoblasts in Culture. We confirmed prior reports of cytotrophoblast morphologic and biochemical differentiation in culture. The morphologic changes were accompanied by biochemical differentiation as indicated by increased hCG secretion. Cytotrophoblast β -hCG secretion increased significantly by 72 hours and peaked at 96 hours in culture (Supplemental Fig. 1).

PGE₂ Production by Jar Cells and Primary Trophoblasts. To address the possible effect of endogenous PGE₂ produced in cell culture, we evaluated PGE₂ concentrations in culture media from untreated cells. In a 24-hour culture, Jar cells (5×10^5 cells) produced a mean PGE₂ concentration of 39.7 ± 2.8 pg/ml ($n = 4$). The basal level of PGE₂ produced in 24 hours by differentiated trophoblasts (6×10^6 cells; cultured for 4 days) was 153.4 ± 23.4 pg/ml ($n = 5$).

PGE₂ Differentially Affects BCRP Expression in Human Placental Cells. PGE₂ increased BCRP mRNA expression nearly 1.9-fold in Jar cells ($P < 0.001$) and 1.5-fold in human primary trophoblasts ($P < 0.05$) after 24-hour treatment (Fig. 1A). PGE₂ decreased Pgp mRNA in Jar cells by approximately 50% ($P < 0.05$) compared with the control, but there was no change of expression in human trophoblasts (Fig. 1B).

Compared with the untreated control, 24 hours of PGE₂ produced a 3.3-fold ($P < 0.01$) increase in Jar cell BCRP protein and a 1.6-fold ($P < 0.05$) increase in human primary trophoblasts (Fig. 2A). Pgp protein levels were not statistically altered among cells exposed to PGE₂ and vehicle ($P = 0.29$) (Fig. 2B).

PGE₂ Alters BCRP, But Not Pgp Activity. BCRP functional activity was clearly present in Jar cells and primary trophoblasts, which showed about a 13 and 19% increase, respectively, in Hoechst 33342 accumulation after 60-minute incubation in the presence of the BCRP selective blocker FTC ($P < 0.05$) (Fig. 3A). In agreement with the increased BCRP mRNA and protein levels, PGE₂ treatment (5 μ g/ml; 24 hours) decreased the accumulation of Hoechst 33342 over 20% compared with the control in Jar cells and around 16% in primary trophoblasts ($P < 0.05$; Fig. 3A). Accumulation of the Pgp substrate,

Fig. 1. Relative changes in BCRP (A) and Pgp (B) mRNA expression were determined by real-time polymerase chain reaction in human placental choriocarcinoma Jar cells ($n = 3$) and human primary trophoblast cells ($n = 8$) stimulated with PGE₂ (5 μ g/ml) for 24 hours. Samples were normalized to the endogenous reference gene, 18s rRNA, and expressed relative to the vehicle control. The mean fold change was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. Treatment with PGE₂ increased BCRP mRNA expression. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$; *** $P < 0.001$.

calcein AM, was about 30% higher in the presence of verapamil ($P < 0.05$; Fig. 3B), but there was no difference when cells were treated first with PGE₂. Cells treated with MK 571 also increased cellular accumulation of calcein AM indicating the presence of MRP1 in Jar cells. However, PGE₂ had no effect on the MRP1 mRNA level as determined by quantitative real-time polymerase chain reaction (Supplementary Fig. 2). The treatment of Jar cells with verapamil also increased the cellular accumulation of Flutax-2 by 33% ($P < 0.05$). PGE₂ increased Flutax 12%, but this increase did not achieve statistical significance (Fig. 3B).

PGE₂ Regulates BCRP Expression via EP1 and EP3 Receptors. To determine the EP receptors involved in PGE₂-mediated up-regulation of BCRP expression, cells were pretreated with an EP receptor antagonist before exposure to PGE₂ (5 μ g/ml) for 24 hours. Both the selective EP1 antagonist SC-19220 ($P < 0.05$) and the EP3 antagonist

Fig. 2. The effect of PGE₂ on BCRP (A) and Pgp (B) protein expression was determined by Western blot in human placental choriocarcinoma Jar cells ($n = 3$) and human primary trophoblast cells ($n = 8$). Immunoblots shown are the representative results obtained in typical experiments. Relative protein levels were normalized to β -actin. Band intensity (intensity/mm²) was expressed as a fold change over the average control for 24-hour PGE₂ treatments. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$.

L-798196 ($P < 0.01$) inhibited the induction of BCRP mRNA by PGE₂. Treatment of cells with EP4-selective antagonist L-161982 and EP1/EP2 antagonist AH 6809 slightly reduced BCRP mRNA levels (Fig. 4). All EP receptor antagonists tested failed to fully restore Pgp mRNA levels in Jar cells exposed to PGE₂ (Fig. 5). There was no effect on Pgp or BCRP expression when cells were treated with antagonist alone. It should be noted that neither PGE₂ nor the EP receptor antagonists altered EP1 and EP3 receptor levels (data not shown), indicating that the effect of these agents on transporter expression is not attributable to changes in the cellular expression of EP1 and EP3, but rather the signaling pathway involved. Sulprostone (EP3 agonist; 10 μ M) increased BCRP mRNA expression in Jar cells 1.7-fold ($P < 0.001$), whereas 17-phenyltrilor PGE₂ (EP1 agonist; 10 μ M) increased BCRP expression 1.4-fold ($P < 0.05$; Fig. 6).

PGE₂ Stimulates AP-1 and Represses NF- κ B Activation. To examine whether NF- κ B and AP-1 mediate PGE₂-induced BCRP

Fig. 3. The functional expression of BCRP in Jar cells and primary trophoblasts (A) and Pgp in Jar cells (B) was evaluated after treatment with PGE₂ (5 $\mu\text{g}/\text{ml}$). Drug efflux activity was determined after 24-hour PGE₂ exposure, and selective chemical inhibition of the intracellular accumulation of fluorescent BCRP substrate Hoechst 33342 and Pgp substrates calcein AM and Flutax-2. The selective BCRP and Pgp inhibitors used were FTC (5 μM) and verapamil (100 μM), respectively. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$.

expression in Jar cells, we first transfected the cells with NF- κ B *cis*-reporter plasmid pNF- κ B-Luc or AP-1 *cis*-reporter plasmid pAP-1-Luc. Once complete, PGE₂ treatment repressed NF- κ B activation as reflected in a nearly 2.0-fold decrease in luciferase activity at 24 hours ($P < 0.001$). AP-1 luciferase activity was increased 2.9-fold in the presence of PGE₂ at 24 hours ($P < 0.001$, Fig. 7A).

In subsequent experiments, inhibition of NF- κ B and AP-1 with BAY-11-7082 (10 μM) and SR 11302 (10 μM), respectively, had little effect on PGE₂-induced BCRP up-regulation (Fig. 7B). The addition of BAY 11-7082 or SR 11302 alone had no effect on BCRP mRNA levels (data not shown).

Discussion

Inflammatory responses during pregnancy can trigger altered expression of placenta transporters, but the factors responsible for these changes remain largely unknown. We find that PGE₂, an immunomodulatory lipid associated with the terminal phases of cervical softening and uterine contraction, alters the functional expression of

BCRP in both immortalized and primary placental cells. PGE₂ increased BCRP mRNA and protein expression (Figs. 1 and 2, respectively), albeit to a lesser extent in human placental trophoblasts. The results correlate with increased BCRP activity in Jar cells and primary trophoblasts, which was identified by a decrease in the cellular accumulation of its substrate, Hoechst 33342 (Fig. 3). These findings are supported by prior studies demonstrating that the administration of a COX-2 inhibitor, celecoxib, which would inhibit PGE₂ production, reverses BCRP-related drug resistance via the down-regulation of BCRP mRNA (Ko et al., 2008; Kalalinia et al., 2011).

Activation of COX-2 has also been shown to increase Pgp (Patel et al., 2002; Liu et al., 2010). Thus, we were surprised that PGE₂ decreased mRNA levels of Pgp in Jar cells (Fig. 1). Although there was a trend toward decreased Pgp protein expression, the differences failed to achieve statistical significance ($P = 0.29$) (Fig. 2B). As previously cited, differences at the RNA level do not always closely correlate with ABC protein expression in trophoblast cultures (Evseenko et al., 2006). Furthermore, we found virtually no effect of PGE₂ on calcein AM accumulation in Jar cells. Calcein AM is also an MRP1 substrate, which

Fig. 4. The effects of PGE₂ receptor (EP) antagonism on BCRP mRNA expression in placental Jar cells. The mean fold change in transporter mRNA expression was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. C_t values of the target gene were normalized to the endogenous reference gene, 18s rRNA, and expressed relative to the vehicle control. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, no significant difference.

too, is functionally expressed in Jar cells (Evseenko et al., 2007). Although PGE₂ had no effect on MRP1 transcripts (Supplemental Fig. 2B), MRP1 inhibition with MK-571 increased the cellular accumulation of calcein AM (Supplemental Fig. 2A). Paclitaxel may be a better substrate for Pgp, even though it can also be transported by MRP2 (Lagas et al., 2006). MRP2 is poorly expressed in Jar cells (Evseenko et al., 2006), and the cellular accumulation of Flutax-2 (i.e., fluorescently labeled paclitaxel) is perhaps more representative of Pgp function. PGE₂ modestly increased Flutax-2 accumulation in Jar cells (Fig. 3B). The fact that changes in Pgp gene expression were not followed by similar functional expression indicates that there can be changes in placental Pgp gene expression independent of its activity. A disconnect between Pgp gene expression and activity was previously demonstrated in other placenta models (Petropoulos et al., 2010; Bloise et al., 2013).

There were obvious differences in the extent that PGE₂ altered BCRP and Pgp expression in Jar and primary trophoblast cells. This could be attributed to several different factors including those related to immortalization, culture conditions, metabolic status, transporter expression, and interindividual variability (as in the case of primary cultures). ABC transporter expression varies among the various placental cell lines (Evseenko et al., 2006). The choriocarcinoma Jar cell line has been used to study trophoblast biology and transport in vitro (Atkinson et al., 2003; Evseenko et al., 2006; Serrano et al., 2007;

Coles et al., 2009). We chose the Jar cell line for this study because it expresses both Pgp and BCRP at levels detectable for quantitation and shows evidence of functional expression of these transporters.

The most prominent effect of PGE₂ on transporter expression was observed in the Jar cells. To more closely evaluate the mechanism and identify clear differences, we performed the subsequent studies using these cells. There are several reasons the effect of PGE₂ on transporter expression was more prominent in Jar cells. Differentiation (syncytialization) may explain some or all the observed differences between the two cell models. Jar cells proliferate in culture but do not spontaneously fuse, whereas trophoblast cells form differentiated syncytium by days 4 to 5. β -hCG is produced by terminally differentiated syncytiotrophoblasts and is used to assess cytotrophoblast biochemical differentiation in culture. We found that β -hCG levels dramatically increase around 72 hours in culture (Supplemental Fig. 1). BCRP and Pgp expression appear to change as trophoblasts mature in culture. BCRP levels in primary trophoblasts increased at 120 hours of incubation relative to those at 24 hours, whereas Pgp decreased (Evseenko et al., 2006). Even so, our results were similar for trophoblasts cultured for 1 day (data not shown) or 4 to 5 days prior to PGE₂ treatment.

Intrinsic differences in the functional expression of the PGE₂ machinery (i.e., COX-2, arachidonic acid, EP receptors, etc.) and PGE₂ production could also explain observed differences between the cells. For

Fig. 5. The effects of PGE₂ receptor (EP) antagonism on Pgp mRNA expression in placental Jar cells. The mean fold change in transporter mRNA expression was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. C_t values of the target gene were normalized to the endogenous reference gene, 18s rRNA, and expressed relative to the vehicle control. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. NS, no significant difference.

instance, previous reports failed to detect the production of PGE₂ by JEG-3 choriocarcinoma cells. In contrast with primary trophoblasts, it has been suggested that the limited arachidonate releasing capacity of JEG-3 cells might be responsible for the low PGE₂ production (Premyslova et al., 2006). In theory, this could mean that choriocarcinoma cells, such as Jar, are more sensitive to exogenous PGE₂ stimulation compared with the primary trophoblasts. In the present study, we determined that primary trophoblasts seeded at a density of 6×10^6 cells and differentiated for 4 days produced around 153.4 ± 23.4 pg/ml of PGE₂ in 24 hours. Jar cells were also capable of producing PGE₂. The mean basal concentration of PGE₂ produced in Jar cells seeded at a density of 5×10^5 cells was 39.7 ± 2.8 pg/ml. We cannot rule out the possibility that endogenous PGE₂ may have some effect on transporter expression.

A primary function of BCRP and Pgp is to transport compounds out of placental cells and prevent access to the fetal circulation. In tissue barriers, BCRP and Pgp often act in concert (de Vries et al., 2007), implying that the loss of either transporter alone does not result in an appreciable increase in penetration of dual substrates. For example, in Pgp/Pgpb knockout mice (where *bcrp1* is present), *bcrp1* alone is sufficient to limit brain uptake of xenobiotics (Agarwal et al., 2011). The same can be said of *mdr1a/mdr1b* in *bcrp1* knockout mice. Thus,

the greatest impact on transplacental passage of dual substrates may be seen when both transporters are similarly altered or when one of these transporters is predominately expressed in the cell.

BCRP mRNA levels are highest in placenta compared with other tissues, and BCRP is approximately 10 times greater than Pgp in human term placenta (Ceckova et al., 2006). Many drugs commonly administered to pregnant women such as glyburide (Zhou et al., 2008), nitrofurantoin (Merino et al., 2005), and cimetidine (Pavek et al., 2005) are BCRP substrates, and its induction may affect how these drugs are distributed and cleared during pregnancy. The importance of BCRP in placenta is further recognized by its presence on the fetal blood vessel endothelial cells (Ceckova et al., 2006; Yeboah et al., 2006; Mason et al., 2011), where it could efflux potentially harmful exogenous and endogenous substrates into the fetal circulation. In this case, up-regulation of BCRP in fetal blood vessel endothelial cells could have an impact on transport toward the fetus. Interestingly, Yeboah et al. (2006) found that neither the umbilical cord vein nor arteries express BCRP. They speculated that BCRP expression in the fetal blood vessel endothelial cells may be regulated by the local action of factors derived from the placenta itself. PGE₂ is produced by the placenta and our data link PGE₂ to up-regulation of BCRP,

Fig. 6. The EP1 receptor agonist 17-phenyl trinor PGE₂ and the EP3 receptor agonist sulprostone increase BCRP mRNA expression. The mean fold change in transporter mRNA expression was calculated in each sample by the $2^{-\Delta\Delta C_t}$ method. C_t values of the target gene were normalized to the endogenous reference gene, 18s rRNA, and expressed relative to the vehicle control. Results are expressed as the mean $\pm$ S.E.M. * P < 0.05; *** P < 0.001 compared with the vehicle control.

advocating it as a potentially important factor in terms of placental transport.

The heterogeneity of PGE₂ immunoregulatory effects may depend on distinctive signaling mechanisms mediated by different PGE₂ receptors. All four membrane receptor subtypes (EP1–EP4) have been localized in human placental villous tissue (Grigsby et al., 2006). It is believed that their presence in human placenta may indicate autocrine and paracrine roles for PGE₂ in the signaling pathways associated with parturition (Grigsby et al., 2006). We suspect that specific EP receptors are involved in downstream regulation of BCRP and Pgp. We observed that antagonism of the EP1 and EP3 receptors counteracts the increase in BCRP mRNA induced by PGE₂ (Fig. 4), and that the EP1 and EP3 agonists alone increased BCRP mRNA expression (Fig. 6). Thus, EP1/EP3 receptors may facilitate PGE₂ signals that modulate placental BCRP expression. Further evaluation may reveal that these receptors are potential pharmacological targets, whereby limiting the responsiveness to PGE₂ during pathologic pregnancy could potentially mitigate BCRP overexpression while maintaining its relevant transport function. This could allow adequate delivery of drugs intended for the fetus without compromising the protective function of BCRP in the placenta.

The greatest impact on BCRP mRNA expression occurred with the EP3 receptor. This receptor is unique among the EP subtypes in that it seems to be the only prostanoid receptor present in the syncytiotrophoblast layer of placenta from women at various stages of labor (Grigsby et al., 2006). Its expression corresponds to the apical membrane localization of BCRP in syncytiotrophoblasts, supporting its potential involvement in BCRP regulation. The EP3 receptor has multiple isoforms generated by alternative splicing. These variants differ in tissue expression, constitutive activity, and regulation of signaling molecules (Adam et al., 1994; Negishi et al., 1995; Schmid et al., 1995). Their expression profile in the human placenta is unknown, but suggests the potential for isoform-specific regulation of BCRP transcriptional activation. Targeting placenta-specific isoforms of EP3 to regulate BCRP activity could avoid the ever-present possibility of affecting BCRP levels in other tissues.

EP receptor signaling pathways ultimately lead to activation and translocation of transcription factors involved in the regulation of transporter expression. Within the promoter region of BCRP and Pgp are the putative binding sites for both NF- κ B and AP-1. These proinflammatory transcription factors could play a role in the regulation of PGE₂ on transporter expression. It was previously shown that

Fig. 7. The effect of PGE₂ on the activation of transcription factors NF- κ B and AP-1, and their impact on BCRP transcriptional expression. (A) The effect of PGE₂ on NF- κ B- and AP-1-dependent transcriptional activity was measured by NF- κ B- and AP-1-dependent promoter luciferase reporter gene assay. Cells were cotransfected with pNF- κ B-Luc or pAP-1-Luc for 24 hours, and then exposed to PGE₂ (5 μ g/ml). The luciferase activity was measured after 24-hour administration of PGE₂. Data for the NF- κ B- and AP-1-dependent promoter activity were normalized to luciferase activity from cotransfection of *Renilla* luciferase plasmid. (B) Jar cells were treated with the NF- κ B inhibitor BAY-11-7082 (10 μ M) or the AP-1 inhibitor SR 11302 (10 μ M) for 1 hour prior to stimulation with PGE₂ for an additional 24 hours. BCRP mRNA expression was determined by real-time polymerase chain reaction. Neither BAY-11-7082 nor SR 11302 affected the PGE₂-mediated up-regulation of BCRP. Values are the means $\pm$ S.E.M. * P < 0.05; *** P < 0.001 compared with vehicle control.

COX-2/PGE₂/EP receptor signaling produces dose-dependent changes in the activity of NF- κ B (Hu et al., 2008). Others have observed that NF- κ B inhibits the transcription of Pgp/mdr1a/mdr1b (Ogretmen and Safa, 1999) and induces the transcription of BCRP/Bcrp1 (Pradhan et al., 2010). We found that PGE₂ decreased NF- κ B and increased AP-1 activity (Fig. 7A) in Jar cells, supporting AP-1 activation as a candidate transcriptional pathway for BCRP up-regulation. It is possible that NF- κ B and AP-1 may modulate each other and that activation of AP-1 negatively affects NF- κ B activity (Fujioka et al., 2004). Although this could explain the effect of PGE₂ on NF- κ B activity, inhibition of these transcription factors in PGE₂-treated Jar cells did not interfere with BCRP induction (Fig. 7B). This indicates that BCRP mRNA may not be directly regulated by NF- κ B or AP-1 when stimulated with PGE₂.

Clearly, a broader investigation into the transcription factor profile activated by PGE₂ stimulation would provide insight into those that contribute to the induction of BCRP mRNA.

Both BCRP and Pgp are important elements of barrier function and distinct signaling pathways may regulate their functional expression. This study highlights a potential role of PGE₂ in regulating BCRP expression in human placenta cells. Given that placental PGE₂ levels rise in response to infection, we postulate that BCRP up-regulation is a placental mechanism to dynamically respond to protect the developing fetus from inflammatory insults, and that PGE₂ is a trigger for this cellular response. We cannot rule out the possibility that other PGs, such as PGF_{2α}, may also have an effect on Pgp and BCRP expression. It would be interesting then in future investigations to compare the impact of different PGs on transporter activity.

Authorship Contributions

Participated in research design: Mason, Dong, Zhou, Weiner.

Conducted experiments: Mason, He.

Contributed new reagents or analytic tools: Lee.

Performed data analysis: Mason.

Wrote or contributed to the writing of the manuscript: Mason, Weiner.

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