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Estradiol and Progesterone-Mediated Regulation of P-gp in P-gp Overexpressing Cells (NCI-ADR-RES) and Placental Cells (JAR)

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Abstract: The effect of progesterone and estrogen treatment on the expression and function of P-glycoprotein (P-gp) was evaluated in JAR cells and a P-gp overexpressing cell line, NCI-ADR-RES. Western blot analysis and real-time Q-PCR were used to evaluate P-gp protein and MDR1 mRNA expression respectively in the cells following incubation with progesterone (P4) and/or β-estradiol (E2). Cellular uptake studies of the P-gp substrates, saquinavir and paclitaxel, were performed to evaluate function. Treatment with either E2 or P4 resulted in a significant increase in P-gp protein levels in the NCI-ADR-RES cells at concentrations of or greater than 100 nM or 10 nM, respectively. JAR cells also had increased levels of P-gp with 100 nM of P4 but were much more sensitive to E2 showing increased P-gp at a concentration of 1 nM. Furthermore, E2 or P4 treatment resulted in a significant decrease in cellular uptake of the P-gp substrates tested in these cells lines. Based on mRNA quantitation, a transient increase (2-fold) in MDR1 levels was observed at 8 h postincubation with either E2 or P4, while MDR1 levels remained high in the JAR cells treated with E2 for 72 h postincubation. The addition of actinomycin D, a transcription inhibitor negated the increase in P-gp by P4 and E2. P4 and E2 increase P-gp expression and function in NCI-ADR-RES and JAR cells with the ERα-expressing cells (JAR) much more sensitive to E2. Furthermore, transcriptional regulation by E2 and P4 likely contributes to the modulation of P-gp levels.

Keywords: Hormones; pregnancy; estradiol; progesterone; JAR cells; NCI-ADR-RES cells; regulation

Introduction

P-glycoprotein (P-gp, ABCB1) is a member of the ATP-binding cassette (ABC) superfamily and is encoded by the multidrug resistance type I gene (MDR1) with a molecular weight of ~170−180 kDa. P-gp functions as an ATP-dependent efflux transporter with broad substrate specificity. P-gp has been shown to be expressed in numerous tissues including the epithelial cells of the intestine, liver, kidney, and placenta as well as capillary endothelial cells which make up the blood−brain barrier and blood−testis barrier.[1−3] It is thought that a key role of P-gp is to protect the body against naturally occurring xenotoxins. What's more, P-gp has been identified as a barrier for the effective delivery of

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drugs for many diseases including cancer, epilepsy and HIV. Understanding the regulatory mechanisms of P-gp may allow for strategies designed to improve the therapeutic efficacy of the many drugs that are substrates of P-gp.

Our recent work, as well as work by others, has shown that P-gp protein levels *in vivo* vary with gestational status.[4−7] We recently showed that P-gp levels in both the placenta and brain of mice are greater at mid-gestation relative to late-gestation.[8] Importantly, P-gp protein levels have also been shown to decline in the human placenta as pregnancy progresses.[4−6] These alterations in placental P-gp protein levels are likely significant since P-gp has been shown to play a role in the fetal transport of P-gp substrates. For example, many HIV protease inhibitors exhibit low permeability to the fetus, likely due to the activity of efflux transporters such as P-gp and MRP.[9,10] Understanding the role of P-gp in the fetal uptake of drugs as well as the regulatory mechanisms of P-gp activity is important in order to design therapeutic agents with the appropriate permeability characteristics for the placental or blood−brain barriers.

P-gp activity is controlled by a variety of endogenous and environmental stimuli including hormones, cytotoxic agents, heat shock, irradiation, genotoxic stress, inflammation, inflammatory mediators, cytokines and growth factors.[11−13] Two hormones of particular interest are progesterones and estrogens. Progesterone (P4) and 17β-estradiol (E2) are two dynamic hormones that are essential for pregnancy. During pregnancy, E2 and P4 hormone levels can increase by as much as 100-fold.[14−16] It has also been postulated that these hormones may be responsible for the observed pregnancy-induced changes in P-gp protein expression in the placenta.[4] For example, investigators have shown that E2 and P4 have the ability to regulate the expression of ABC transporters.[12,13,17−20] Furthermore, there is evidence that expression of the estrogen receptor (ERα) plays a role in the regulation of these transporters.[13] The goal of this study was to evaluate the effect of β-estradiol (E2) and progesterone (P4) on P-gp protein levels and function in an ERα negative P-gp overexpressing cell line, NCI-ADR-RES and an ERα positive cell line. This study will complement and enhance what is known about P-gp expression in ERα expressing and nonexpressing cells and provide new insight into the regulation of P-gp by E2 and P4.

Materials and Methods

Chemicals. P4 (P-8783) and E2 (E-2758) were purchased from Sigma (St. Louis, MO). Ethanol was purchased from American Bioanalytical (Rockford, IL). [^{3}H]Saquinavir (1.1 Ci/mmol) and [^{3}H]paclitaxel (0.1 Ci/mmol) were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Vera-

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Table 1. Sequences of Primers Used for Quantitative Real-Time PCR

gene	accession no.	sequence 5′ to 3′
MDR1	NM_011076	GCTTACAGCCAGCATTCTCCGTAA (f)
		CCCTTTCACTTGAGCAGCATCGTT(r)
gapdh	BC_096440[a]	CCCATCACCATCTTCCAGGAG (f)
	NM_014364	GTTGTCATGGATGACCTTGGC (r)
	NM_017008	

[a] Human gapdh primer designed.

pamil was purchased from Sigma (St. Louis, MO). Universol scintillation cocktail was purchased from Perkin-Elmer (Waltham, MA) and hydrochloric acid from American Bioanalytical (Rockford, IL). Protease inhibitor cocktail tablets (Complete) were purchased from Roche (Mannheim, Germany), and DC protein assay kit was purchased from Bio-Rad (Hercules, CA). The anti-P-gp antibody (C219), anti-β-actin, and HRP-labeled antimouse antibodies were purchased from ID Laboratories (London, ON, Canada), Sigma, and Kirkegaard and Perry Laboratories (Gaithersburg, MD), respectively. Tris-buffered saline was purchased from Quality Biological Inc. (Gaithersburg, MD) and Tween 20 from American Bioanalytical (Natick, MA). Supersignal West Pico chemiluminiscent substrates were purchased from Pierce (Rockford, IL). The Laemmli sample buffer and 2-mercaptoethanol were purchased from Bio-Rad (Hercules, CA). The DNase I kit and the RNeasy MiniElute kit were purchased from Qiagen (Valencia, CA). iScript reverse transcription (RT) kit and iQ SYBR Supermix were purchased from Bio-Rad (Hercules, CA). Forward and reverse primers for MDR1 and GADPH (Table 1) were designed using Beacon Software and synthesized by Integrated DNA Technologies (Coralville, IA). The NCI-ADR-RES cell type was a gift from Douglas Ross, University of Maryland. The JAR cells were a gift from Peter Swaan, University of Maryland. Phenol-red free DMEM, charcoal stripped fetal bovine serum, penicillin/streptomycin, sodium pyruvate, glutamine, HEPES buffer, and insulin were all purchased from Invitrogen (Carlsbad, CA). Triton X-100 was purchased from American Bioanalytical (Natick, MA). Actinomycin D, MG-132 and cyclohexamide were all purchased from Sigma (St. Louis, MO).

Cell Culture. A P-gp overexpressing human ovarian cancer cell line (NCI-ADR-RES; previously misnomered as MCF-7^{adr} cells) was maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, HEPES buffer, sodium pyruvate, and insulin at 37 °C in a humidified incubator with 5% CO_2. The placental JAR cells were maintained in F-12K media supplemented with 10% FBS, nonessential amino acids and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO_2. The medium was replaced with fresh medium every 2−3 days. For hormonal treatment, the cells were then seeded on 12-well plates (~150,000 cells/cm^2) and cultured for 24 h. Medium was then replaced with phenol-red free DMEM and charcoal stripped FBS, and cells were cultured for approximately 72 h (80−90% confluence). Medium was changed daily throughout the study. The medium was then replaced with fresh medium containing various concentrations of E2 and P4. Stock solutions of E2 and P4 were prepared in ethanol, and the concentration of ethanol used in these experiments was 0.1% (v/v). Medium was changed daily until the cells were harvested for analysis or used for functional studies. Cells were harvested for protein or mRNA quantitation at either 8, 24, 48, or 72 h postincubation.

The whole cell lysates were prepared by washing cells twice with ice-cold PBS and adding 300 μL of lysis buffer (10 mM Tris-HCl, 1% Triton X-100, 1% SDS, 0.5% deoxycholic acid, complete protease inhibitor) to each well. The cells were allowed to detach and suspended in the lysis buffer by vigorous pipetting. The sample was then placed on ice and sonicated briefly and protein content measured using the DC protein assay kit using albumin as a standard. SDS Laemmli loading buffer was then added to the membrane protein samples (1:1 v/v), and the samples were frozen at −80 °C until analysis.

Quantification of P-gp Levels by Immunoblot Analysis (Western Blotting). Protein samples were heated at 60 °C for 5 min, and membrane proteins (40−60 μg) were loaded onto an 8% SDS−polyacrylamide gel. Electrophoresis was conducted for 2.5 h at 30 mA. Proteins were transferred at 25 V (constant) and 160 mA (initial) for 2 h onto polyvinylidene membrane (PVDF). Nonspecific binding sites were blocked for 1 h with 5% nonfat dry milk in TBS-Tween 0.1%. PVDF membrane was incubated with P-gp antibody, C219 (1:1500) or ERα (1:500) or ERβ (1:500) or PR (A and B) (1: 500) at 4 °C overnight and/or β-actin antibody (1:1500) for 1 h at room temperature, washed with TBS-T (3 × 15 min) and then incubated with a horseradish peroxidase-linked secondary antibody (1:1500 to 1:10000) for 1 h, and then rinsed with TBS-T. Detection of the protein−antibody complexes was accomplished via the use of ECL reagents and chemiluminescent detection. P-gp band intensity was measured by densitometric analysis software and normalized to the β-actin band intensity.

RNA Isolation and Purification. After treatment of the NCI-ADR-RES cells as described above, total cellular RNA was isolated and purified from the cells using the RNeasy MiniElute kit according to manufacturer's instructions. For greater purity, samples were also digested with DNase I according to manufacturer's instructions. Total RNA concentration was determined by ultraviolet (UV) spectroscopy at 260 nm and RNA integrity and purity confirmed by 260/280 ratio (>1.8) and separation on a 1% agarose gel followed by visualization with ethidium bromide.

Real Time Quantitative RT-PCR. MDR1 and GAPDH (internal control) mRNA levels were quantitated using a SYBR based real time quantitative PCR assay on the iCycler instrument. One microgram of total RNA was converted to cDNA in a final reaction volume of 25 μL using the iScript reverse transcription (RT) kit. Quantitative PCR (Q-PCR) reactions were carried out in a total volume of 25 μL with ten percent of the volume of the RT reactions and using the iQ SYBR Supermix and final primer concentrations of 200

nM. Primers (Table 1) for Q-PCR were designed using the NCBI database and Beacon Designer software (Premier Biosoft International, Palo Alto, CA). Primers were tested for specificity using an alignment search (BLAST) against the whole human genomic plus transcript database. Cycling parameters were as follows: denaturing at 95 °C for 30 s and then annealing and extending at 60 °C for 45 s. Specific amplification was confirmed by melting curve analysis as well as evaluation of the Q-PCR products on a 3% agarose gel. The threshold cycles (Ct) were calculated by the iCycler iQ real time detection system software. The Ct for each sample was normalized against that of GAPDH. Fold changes were determined relative to the control group using the comparative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$).

Intracellular Uptake Studies. Intracellular uptake of [^{3}H]saquinavir and [^{3}H]paclitaxel was determined in NCI-ADR-RES or JAR cells following E2 (10^{-7}), P4 (10^{-7}) or E2/P4 treatment. Cells were grown as described in the Cell Culture section. Upon study commencement, the medium was removed and cells were washed with ice-cold PBS. For cell uptake studies, medium spiked with either [^{3}H]saquinavir (0.1, 1.0, or 125 μM), [^{3}H]paclitaxel (0.5 μM) or verapamil (100 μM) was then added to the cells cultured on the 12-well plates. Cells were incubated at 37 °C for 20, 60, or 90 min. At the desired collection time, medium was removed and washed twice with ice cold PBS. Cells were then lysed with 1 N NaOH and incubated at 37 °C for 2 h. The cells were then neutralized with hydrochloric acid. Cell lysate (250 μL/well) was then added to scintillation vials containing 5 mL of scintillation cocktail and analyzed for radioactivity using a Beckman Coulter liquid scintillation counter (LS6500). To determine protein content, 5 μL of the remaining preparation was used with the DC protein assay kit. Total radioactivity was divided by protein content to determine radioactivity per μg of protein.

MG-132 and Actinomycin D Study. NCI-ADR-RES cells were cultured as described in the Cell Culture section. Once the cells reached ~80−90% confluence (72 h postseeding), the medium was then replaced with fresh medium containing no hormones (control), E2 (10^{-7}) or P4 (10^{-7}) and either MG-132 (5 μM) or actinomycin D (4 μM). Cells were incubated for 24 h, and samples were collected for immunoblot analysis as described above.

Statistical Analysis. Data were analyzed for statistically significance differences using ANOVA analysis followed by Dunnett's post hoc analysis or Student's *t* test (GraphPad Prism, La Jolla, CA). A *p*-value less than 0.05 was considered statistically significant.

Results

Characterization of Hormone Receptors in NCI-ADR-RES and JAR Cells and Effect of Cell Culture Conditions on P-gp Expression. A P-gp basal expression study was conducted to determine the appropriate conditions to assess the effect of E2 and P4 on P-gp expression. As expected, the level of P-gp was much higher in the NCI-ADR-RES cells over the JAR cells. We unexpectedly observed that the expression of P-gp increased significantly in these cells for up to three days after seeding and switching to charcoal stripped media (Figure 1). This increase in P-gp level was greater in NCI-ADR-RES cells. Since the increase in P-gp level was minimal after 3 days of culture, cells were incubated for 72 h before testing the effects of E2 and P4 on P-gp levels in the NCI-ADR-RES and JAR cells. Because the presence or absence of hormone receptors is expected to play a key role in the biological effects of their respective hormone, we tested for the presence of ERα, ERβ, and PR (PR-A and PR-B) in these cell lines using immunodetection. The receptor ERβ was detected in both the NCI-ADR-RES and JAR cells at high levels (Figure 1). However, ERα was present at much higher levels in the JAR cells relative to NCI-ADR-RES cells. As for the PR, a stronger band was detected with the NCI-ADR-RES cells compared to the JAR cells (Figure 1).

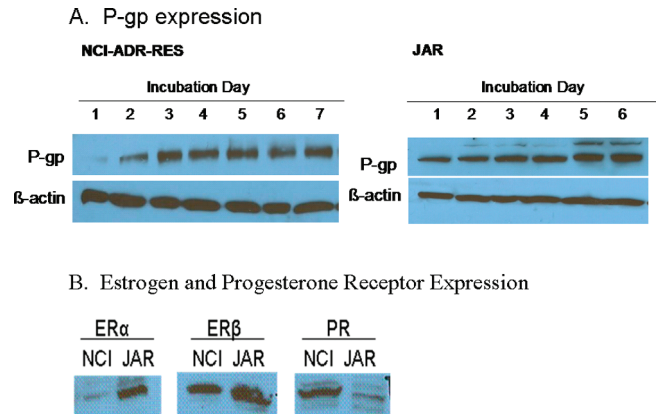

Figure 1. (A) Effects of incubation time on P-gp expression in NCI-ADR-RES and JAR cells. Representative immunodetection of P-gp expression (~170 kDa) in whole cell lysates isolated from NCI-ADR-RES and JAR cells. β-Actin (43 kDa) in the corresponding lanes. NCI-ADR-RES and JAR cells were seeded on 12-well plates on day 0. (B) Presence of ERα, ERβ, and PR in NCI-ADR-RES and JAR cells 72 h following seeding and cell culture using the phenol-red free and charcoal stripped medium. Representative immunodetection of ERα (~65 kDa), ERβ (~60 kDa) and PR (~100 kDa) in whole cell lysates isolated from NCI-ADR-RES and JAR cells. For all lanes, protein extracts (20 μg for JAR and 60 μg for NCI-ADR-RES) were separated by electrophoresis on an 8% sodium dodecyl sulfate−polyacrylamide gel and transferred onto a polyvinylidene membrane. The membrane was incubated with C219 (P-gp) or anti-β-actin antibody followed by incubation with horseradish peroxidase-linked secondary antibody. Detection was made using ECL reagents.

Effect of E2 and P4 on MDR1 mRNA Levels in NCI-ADR-RES and JAR Cells. To begin to evaluate the effects of P4 or E2 on P-gp expression, mRNA levels were determined in NCI-ADR-RES and JAR cells following E2 or P4 treatment. For this study, MDR1 mRNA levels were measured in NCI-ADR-RES cells following treatment with

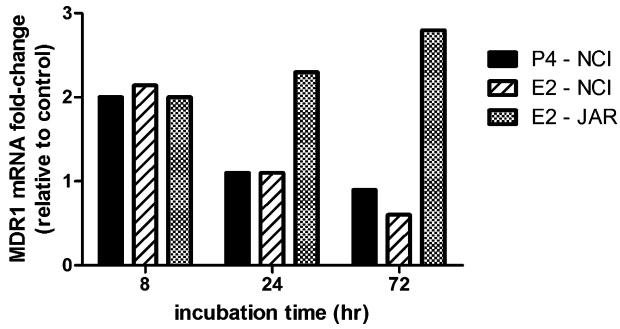

Figure 2. Effects of P4 and E2 treatment on MDR1 mRNA expresion in NCI-ADR-RES and JAR cells. Fold changes in MDR1 levels in NCI-ADR-RES and JAR cells in the presence or absence of P4 (10^{-7} M) or E2 (10^{-7} M) ($n = 2$). Quantitative real-time PCR was used to determine cycle threshold values for MDR1 and GADPH. Relative analysis using the comparative threshold cycle (Ct) method (2-ΔΔCt) was then employed, and MDR1 levels were normalized for GADPH and fold changes determined relative to control.

10^{-7} M P4 or E2 (Figure 2). A 2-fold increase in MDR1 mRNA levels was observed in NCI-ADR-RES and JAR cells 8 h post incubation with E2 (Figure 2). Following P4 treatment, an increase in MDR1 levels was also observed 8 h post incubation to NCI-ADR-RES cells but not JAR cells. Furthermore, MDR1 levels in the NCI-ADR-RES cells were returned to baseline by 72 h post incubation. However, unlike the NCI-ADR-RES cells, MDR1 levels in the JAR cells treated with E2 remained elevated for the duration of the study (72 h).

Progesterone and 17β-Estradiol Upregulate P-gp Protein Expression in the MDR1 Overexpressing Cell Line, NCI-ADR-RES and the JAR Cell Line. The effect of P4 and E2 on P-gp expression was evaluated in NCI-ADR-RES and JAR cells. P4 treatment resulted in a dose-dependent increase in P-gp expression in the NCI-ADR-RES and JAR cells following 24 and 72 h incubation periods (Figure 3). Under these conditions, P4 concentrations of 10^{-8} M and higher resulted in significant upregulation of P-gp protein levels in NCI-ADR-RES cells. The highest level of induction (2.6-fold) was observed at a P4 concentration of 10^{-8} after 24 h of treatment. A slightly different concentration effect was observed for the 24 and 72 h incubation time points. In the JAR cells, P4 concentrations of 10^{-7} M and higher were required for significant upregulation of P-gp protein levels with the highest induction of 3.4-fold the result of 10^{-7} M P4 concentration 24 h postincubation. As observed in NCI-ADR-RES cells, the concentration effect of P4 on P-gp levels varied slightly for the 24 and 72 h incubation times.

E2 treatment of NCI-ADR-RES cells also resulted in a dose-dependent increase in P-gp protein levels, but to a lesser extent than P4 (Figure 4). Significant increases in P-gp protein levels were evident at concentrations of E2 of 10^{-7} M and 10^{-6} M. The highest level of induction was approximately 2-fold by 10^{-6} M of E2 after 24 h. Unlike with the NCI-ADR-RES cells where P4 caused a greater increase in P-gp levels, E2 caused a greater induction of P-gp in JAR cells. Significant upregulation of P-gp was observed at E2 concentrations of 10^{-9} M with the highest level of induction (~6-fold) resulting from incubation with 10^{-7} M of E2. Thus, these results indicate that both P4 and E2 cause significant increases in the total level of P-gp in these cells with the level of increase being higher with P4 in NCI-ADR-RES cells and E2 in JAR cells.

Effect of MG-132 and Actinomycin D on P-gp Protein Expression. To investigate the mechanism of induction of P-gp protein levels by P4 and E2, the effects of the transcriptional inhibitor, actinomycin D and proteasome inhibitor, MG-132 on the induction by P4 and E2 were investigated in the NCI-ADR-RES and JAR cells. For these studies, cells were treated with either P4 (10^{-7} M) or E2 (10^{-7} M) in the presence or absence of these inhibitors. The data indicated that addition of MG-132 had no significant effect on the induction of P-gp levels by E2 or P4 (Figure 5A) in both NCI-ADR-RES cells and JAR cells. However, the addition of actinomycin D eliminated the induction of P-gp by both P4 and E2 (Figure 5B).

Progesterone and 17β-Estradiol Decrease Cellular Uptake of P-gp Substrates in the MDR1 Overexpressing Cell, NCI-ADR-RES. As reported above, treatment of NCI-ADR-RES cells with 10^{-7} M concentrations of P4 or E2 resulted in significant increases in P-gp protein levels (Figure 3 and Figure 4). To determine the functional significance of this increase, the cellular uptake of P-gp substrates ([^{3}H]saquinavir and [^{3}H]paclitaxel) was also evaluated using these concentrations of P4 or E2. The addition of P4 resulted in significant decreases in cellular uptake of both saquinavir and paclitaxel (Figure 6). Following P4 treatment, the cellular uptake of [^{3}H]paclitaxel decreased by approximately 40% while [^{3}H]saquinavir concentrations decreased by approximately 30%. The cellular uptake of these agents was also evaluated after incubation with E2 (Figure 6). Similar to P4 treatment, significant decreases in [^{3}H]paclitaxel and [^{3}H] saquinavir uptake (~40%) were also observed when the cells were treated with E2. Furthermore, a kinetics evaluation was conducted in which NCI-ADR-RES cells incubated with [^{3}H]paclitaxel and E2 or P4 resulted in 40−50% decreases in [^{3}H]paclitaxel uptake at 20, 60, and 90 min postincubation (data not shown). The use of verapamil confirmed the presence of functioning P-gp. The addition of verapamil (100 uM) to the control cells resulted in approximately 10-fold greater uptake of [^{3}H]paclitaxel and 6-fold greater uptake of [^{3}H]saquinavir compared to control (Figure 6).

17β-Estradiol Decreases Cellular Uptake of P-gp Substrates in the JAR Cells. Similar results reported for the NCI-ADR-RES cells were obtained when uptake studies were conducted with JAR cells treated with P4 or E2. As reported above, treatment of JAR cells with 10^{-7} M concentrations of P4 or E2 resulted in significant increases in P-gp protein levels (Figure 3 and 4). The cellular uptake of the P-gp substrate, saquinavir, was evaluated using these

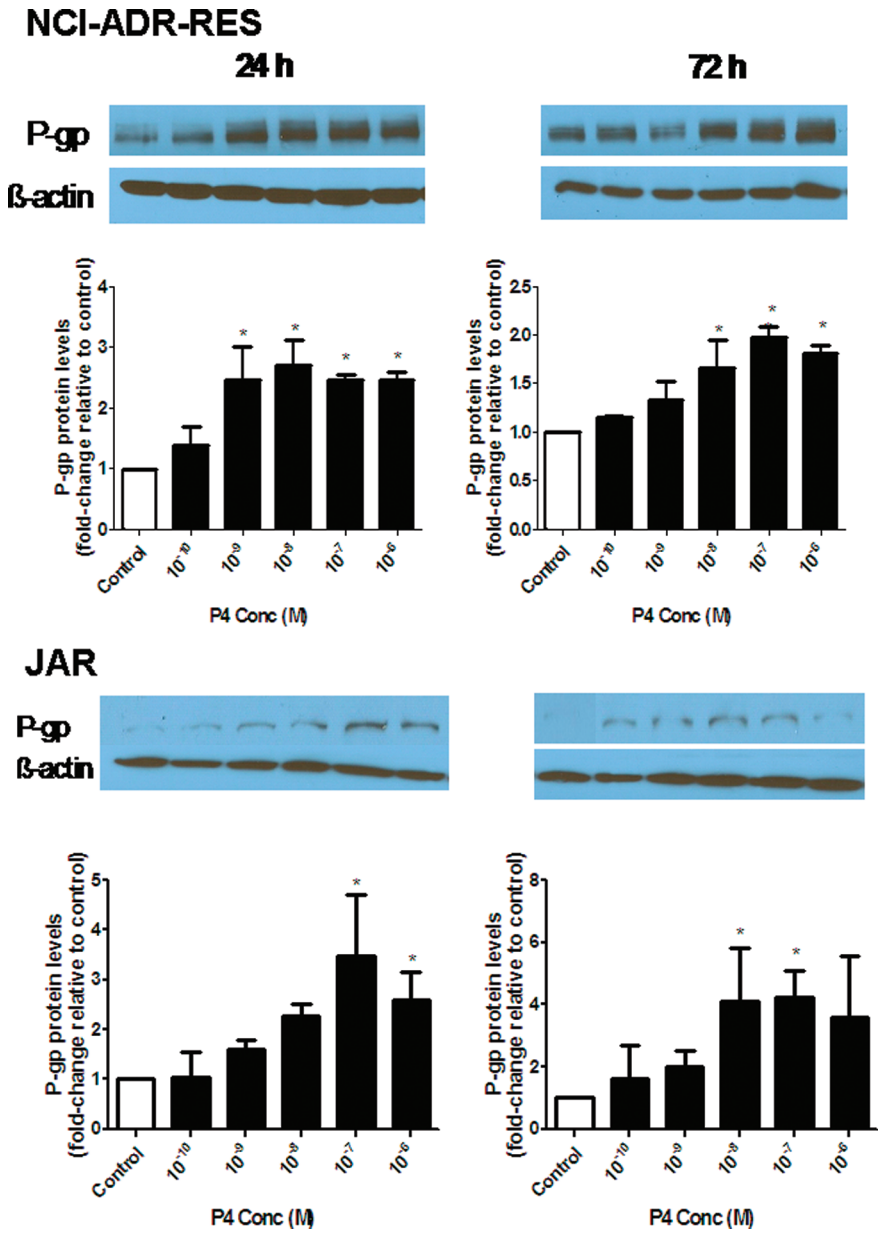

Figure 3. Dose dependence of P4 on P-gp expression in NCI-ADR-RES and JAR cells. Representative immunodetection of P-gp expression (~170 kDa) in whole cell lysates isolated from NCI-ADR-RES and JAR cells in the presence or absence of various concentrations of P4 at 24 and 72 h postdose. β-Actin (43 kDa) in the corresponding lanes. For all lanes, protein extracts were separated by electrophoresis on an 8% sodium dodecyl sulfate−polyacrylamide gel and transferred onto a polyvinylidene membrane. The membrane was incubated with C219 (P-gp) or anti-β-actin antibody followed by incubation with horseradish peroxidase-linked secondary antibody. Detection was made using ECL reagents. P-gp expression was measured by densitometric anlaysis software ($n = 3$). * indicates significant difference at $p < 0.05$ compared to control.

concentrations of P4 and E2. The addition of E2 resulted in significant decreases (~30%) in cellular uptake of saquinavir at both concentrations tested (Figure 7). Following P4 treatment, the cellular uptake of [^{3}H]saquinavir was significantly decreased by approximately 30% when 1 μM saquinavir was evaluated but was not significantly changed by 0.1 μM saquinavir. The use of verapamil confirmed the presence of functioning P-gp. The addition of verapamil (100 μM) to the control cells resulted in approximately 50% and 30% greater uptake of 0.1 and 1 μM [^{3}H]saquinavir, respectively compared to control (Figure 7). Incubation of verapamil with the NCI-ADR-RES cells resulted in a 6-fold increase in saquinavir uptake. This difference in substrate uptake highlights the contribution of P-gp in the transport of these substrates in the P-gp overexpressing cells.

Discussion

The regulation of P-gp expression by estrogen and progesterone has been previously investigated.[12,13,17] These reports demonstrate that these hormones are capable of

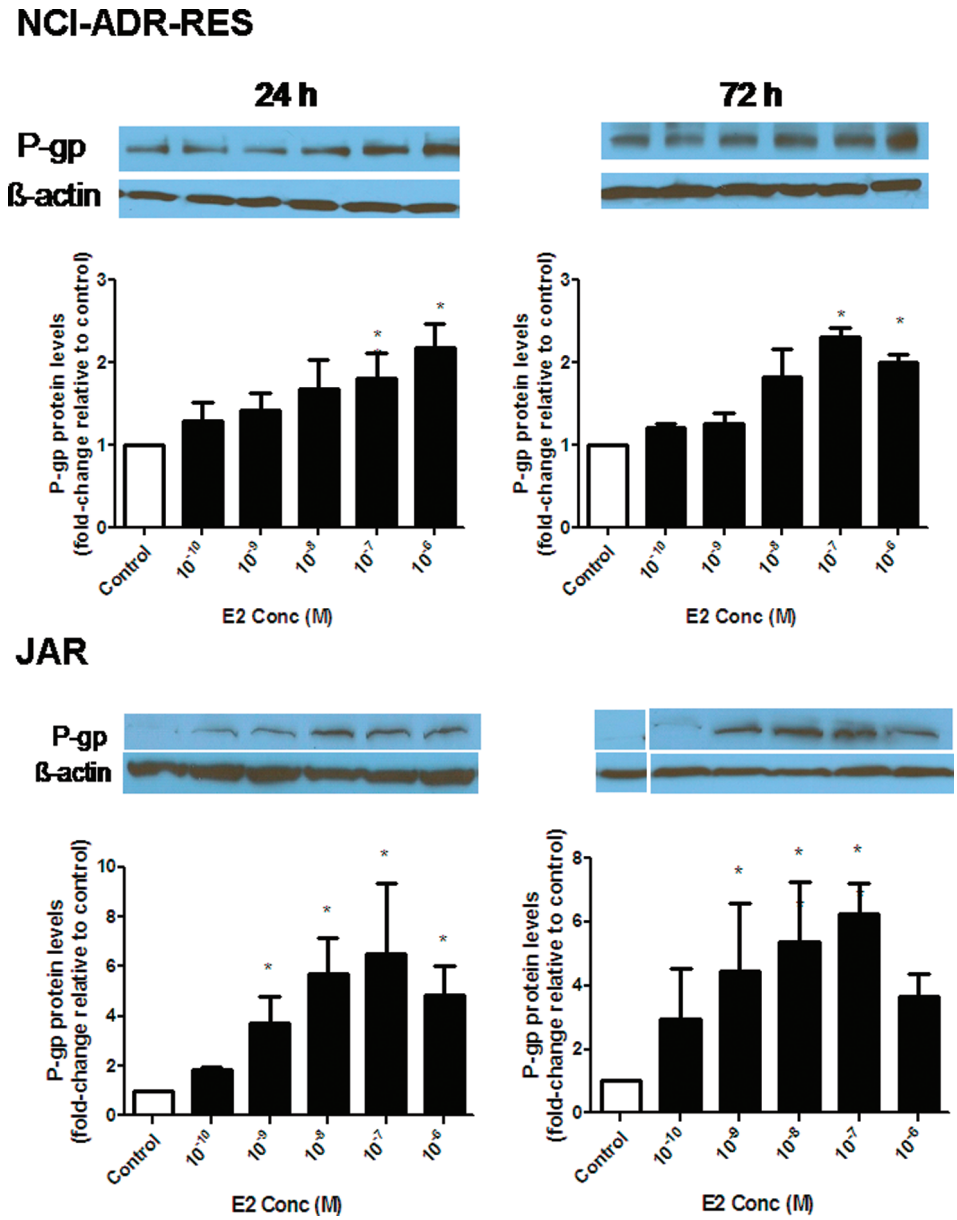

Figure 4. Dose dependence of E2 on P-gp expression in NCI-ADR-RES and JAR cells. Representative immunodetection of P-gp expression (~170 kDa) in whole cell lysates isolated from NCI-ADR-RES and JAR cells in the presence or absence of various concentrations of E2 at 24 h (A) and 72 h (B) postdose. β-Actin (43 kDa) in the corresponding lanes. For all lanes, protein extracts were separated by electrophoresis on an 8% sodium dodecyl sulfate−polyacrylamide gel and transferred onto a polyvinylidene membrane. The membrane was incubated with C219 (P-gp) or anti-β-actin antibody followed by incubation with horseradish peroxidase-linked secondary antibody. Detection was made using ECL reagents. P-gp expression was measured by densitometric anlaysis software ($n = 3$). * indicates significant difference at $p < 0.05$ compared to control.

regulating the expression of P-gp as well as other ABC-transporters. However, the magnitude and direction appear to depend on the protein, cell type, hormone concentrations, and possibly even cell culture conditions. To further characterize hormonal regulation of P-gp expression, we examined the effect of E2 and P4 on P-gp expression and function in the human ovarian carcinoma cell line, NCI-ADR-RES and human placental carcinoma cell line, JAR.

Our results revealed that the level of P-gp in the ERα negative NCI-ADR-RES cells was unchanged at low concentrations of E2 (from 10^{-10} to 10^{-8} M), but that at higher concentrations of E2 (10^{-7} and 10^{-6} M), a significant increase in P-gp level (up to 2.4-fold) was observed. With JAR cells, which contain high levels of both ER_α and ER_β, an induction of P-gp level was observed at an E2 concentration as low as 10^{-9} M. Induction of P-gp level by E2 has been previously reported in LS180 human colon carcinoma cell line,[21] in primary trophoblast cells[12] and in MCF-7 cells.[19] A cor-

(21) Kim, W. Y.; Benet, L. Z. P-glycoprotein (P-gp/MDR1)-mediated efflux of sex-steroid hormones and modulation of P-gp expression in vitro. *Pharm. Res.* **2004**, *21* (7), 1284–93.

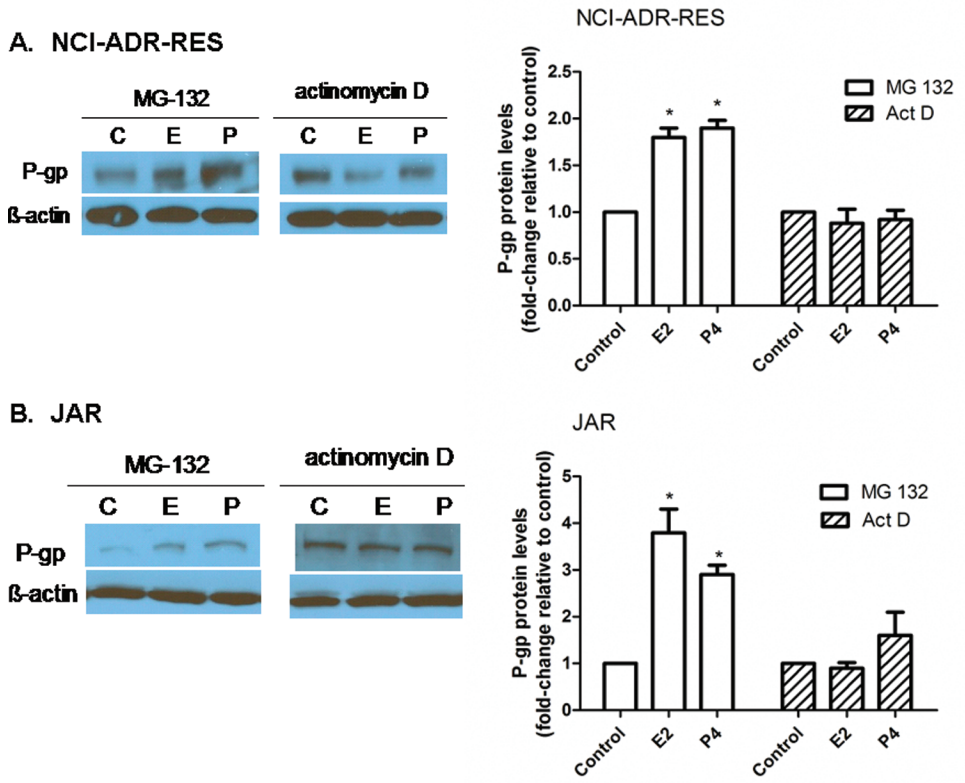

Figure 5. Effects of MG-132 and actinomycin D on P-gp expression in NCI-ADR-RES cells in the presence or absence of P4 and E2. Representative immunodetection of P-gp expression (~170 kDa) in whole cell lysates isolated from NCI-ADR-RES and JAR cells in the presence or absence of various concentrations of P4 or E2 and either MG-132 (5 μM) or actinomycin D (4 μM). Protein was collected for analysis 24 h thereafter. β-Actin (43 kDa) in the corresponding lanes. For all lanes, 40 μg (NCI-ADR-RES) and 60 μg (JAR) of protein extracts were separated by electrophoresis on an 8% sodium dodecyl sulfate−polyacrylamide gel and transferred onto a polyvinylidene membrane. The membrane was incubated with C219 (P-gp) or anti-β-actin antibody followed by incubation with horseradish peroxidase-linked secondary antibody. Detection was made using ECL reagent. P-gp expression was measured by densitometric analysis software (n = 3). * indicates significant difference at $p < 0.05$ compared to control.

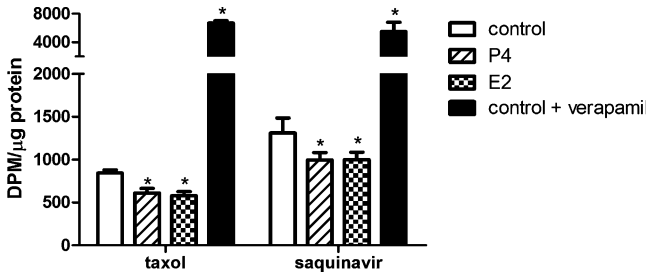

Figure 6. [^{3}H]Paclitaxel and [^{3}H]saquinavir uptake in NCI-ADR-RES cells 60 min in the presence or absence of P4 (10^{-7} M), E2 (10^{-7} M), or verapamil (100 μM) for 72 h. Data presented as mean + SD for n = 4. * indicates significant difference at $p < 0.05$ compared to control.

relation between ERα expression and E2 induction of P-gp level was noted by Zampieri et al. (2002) which we also observe at lower E2 concentrations.[19] Mutoh et al. reported that E2 downregulated P-gp in MCF-7-MDR1-transduced cells which contain ERα but did not find any change in the ERα negative NCI-ADR-RES cells with 10 nM concentrations of E2.[13] However at greater E2 concentrations ($\geq 10^{-7}$), P-gp level can still be induced in the absence of ERα. Since NCI-ADR-RES cells express ERβ at a relatively high level, the induction of P-gp protein level at high E2 concentrations may be mediated by ERβ. In this study, a consistent effect of E2 in the NCI-ADR-RES cells was not observed until 100 nM E2 concentrations were reached at which a dose-dependent upregulation of P-gp was observed. These data suggest that, even for cells which have only low levels of ERα, regulation of P-gp by E2 is possible albeit at high concentrations of E2. E2 has also been shown to increase P-gp protein levels in MCF-7 cells, primary trophoblast cells and MDCK.[12,19,21] Although 100 nM concentrations of E2 are quite high they may have clinical significance. For example, pregnant women may attain these concentrations during pregnancy (~50−100 nM at term).[14] Importantly, the distribution of drugs given at parturition such as HIV antiretrovirals or anesthetics may be impacted by changes in transporter expression. Additionally, it is possible that intracellular concentrations for example, at a tumor site, may be significantly higher. Although 100 nM doses were required for NCI-ADR-RES cells, 1 nM concentrations

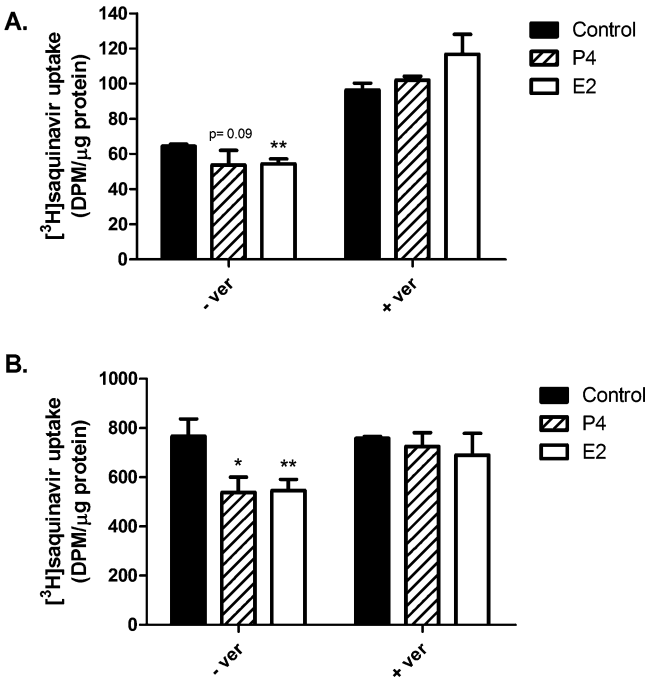

Figure 7. Effects of E2 and P4 treatment on P-gp protein expression and function in JAR cells. [^{3}H]Saquinavir uptake in JAR cells 60 min in the presence or absence of P4 (10^{-7} M), E2 (10^{-7} M), P4 and E2 (10^{-7} M each), or verapamil (100 μM) for 72 h. Saquinavir concentrations of 0.1 μM (A) and 1.0 μM (B) were evaluated. Data presented as mean + SD for $n =$ 4. Data presented as mean + SD for $n = 4$. * indicates significant difference at $p < 0.05$ compared to control.

resulted in significantly increased P-gp in the placental cells, which is the model system which parallels our *in vivo* work. Importantly, 1 nM concentrations are obtained early and throughout pregnancy.

Like E2, P4 also enhanced P-gp protein levels in the two cell lines to different extents. In the NCI-ADR-RES cells, which express relatively high level of progesterone receptor, low concentrations of P4 (starting from 10^{-9} M) increased P-gp levels (~3-fold). On the other hand, JAR cells, which express relatively low level of PR, were less sensitive to P4, requiring concentrations of 10^{-7} M or more for a significant induction of P-gp levels. P4 has also been shown to increase P-gp levels in cells such as porcine granulose, MDCK and primary trophobast cells.[12,13,20,21] Our data is consistent with the notion that PR mediates the induction of P-gp level by P4 and that the level of PR is important in the sensitivity to P4 activity. We also cannot rule out that other biochemical differences between the cells, such as presence of transcriptional cofactors of PR, may account for the difference in sensitivity to P4.

Our results reveal that, in addition to the increase in P-gp protein levels by E2, this hormone elevated the *MDR1* mRNA levels 2-fold in both the NCI-ADR-RES and JAR cells. However, the kinetics of induction by E2 were different, with the elevated *MDR1* mRNA level returning to baseline by 24 h postincubation in the NCI-ADR-RES cells while the elevated mRNA level lasted up to 72 h in the JAR cells. It is possible that this difference could be due to the presence of ERα in JAR cells and its absence in NCI-ADR-RES cells. The addition of 10^{-7} M P4 to the NCI-ADR-RES also resulted in upregulation of MDR1 mRNA levels with kinetics similar to E2 incubation, whereas, while P-gp protein levels were increased, MDR1 upregulation was not observed in JAR cells incubated with this concentration of P4. It is plausible that PR expression differences in these two cell lines play a role. A similar finding was also reported by Evseenko et al. in which significant increases in P-gp protein level in primary trophoblast tissue were noted with no changes in MDR1 levels.[12] This suggests that induction of P-gp with this treatment is due to post-transcriptional mechanisms. As demonstrated by MG-132 treatment, proteosome degradation does not appear to change with hormone treatment. Other explanations include changes in RNA degradation and protein synthesis or that the concentration of P4 evaluated (10^{-7} M) was too low as upregulation of P-gp protein required 10^{-8} M concentrations in some instances to significantly increase P-gp protein levels.

The induction of *MDR1* mRNA levels by E2 and P4 can be the result of the transcriptional activation of the *MDR1* gene or alternatively, the lengthening of the half-life of the *MDR1* mRNA by these hormones. To test these two possibilities, we cotreated these two cell lines with either the proteasome inhibitor MG-132, as negative control, or the transcriptional inhibitor, actinomycin D, along with either E2 or P4. The results indicate that MG-132 had no discernible effect on the induction of P-gp by these two hormones in both of these cells where as actinomycin D completely prevented induction of P-gp levels in these two cells by both hormones. Taken together, our data indicates that E2 and P4 induce the level of P-gp in these two cells by stimulating the transcription of the *MDR1* gene through their respective receptors. Additionally, in the absence of ERα, ERβ may be able to substitute for it at high E2 concentrations. It has been shown that ERβ can also activate the transcription of various genes. A search of the human *MDR1* gene promoter region using the TRANSFAC database revealed the presence of a putative estrogen response element upstream of the transcriptional initiation site. When NCI-ADR-RES cells were treated with both P4 (100 nM) and E2 (100 nM), a greater increase in P-gp levels resulted compared with control cells. This is in agreement with *in vivo* results where treatment of combination estrogen and progesterone resulted in an increase in mdr1 mRNA and P-gp in the uterine secretory epithelium in mice.[22]

In a previous study, we demonstrated that membrane P-gp level is higher in the brains and placenta of mice at mid-gestation compared to late-gestation.[8] The high level of P-gp corresponds to the levels of E2 in mice which are much

(22) Arceci, R. J.; Baas, F.; Raponi, R.; Horwitz, S. B.; Housman, D.; Croop, J. M. Multidrug resistance gene expression is controlled by steroid hormones in the secretory epithelium of the uterus. *Mol. Reprod. Dev.* **1990**, *25* (2), 101–9.

higher at mid-gestation relative to late gestation. Addition of E2 to the placental JAR cells demonstrates that E2 can upregulate P-gp protein levels, suggesting that the fluctuating levels of E2 can regulate P-gp levels during pregnancy. An apparent discrepancy between the previous and current results is that a difference was not present in the *MDR1* mRNA level between the mid- and late-gestation periods in mice where as the induction of P-gp level in the two cell lines was associated with an increase in mRNA levels. Potential explanations for this difference may be species difference or that other mechanisms of P-gp regulation by these two hormones exist.

The upregulation of P-gp levels by either E2 or P4 also resulted in changes in P-gp function. For the NCI-ADR-RES cells, both saquinavir and paclitaxel uptake was decreased (∼30−40%) with P4 treatment. Saquinavir uptake was also significantly decreased (15−30%) in JAR cells treated with E2. The extents of the decreases in drug uptake did not match the level of P-gp induction (2-fold in NCI-ADR-RES cells and 5-fold in JAR cells), but this was not unreasonable since the drugs likely enter the cell via other routes such as other transporters or passive transport. It is also possible that not all of the additional P-gp proteins are localized to the plasma membrane, thus unable to contribute to the efflux of substrates. It is also possible that differences in basal levels of P-gp in these two cell lines also contribute to the discrepancy in P-gp protein induction and drug uptake. Nonetheless, the significant decrease in intracellular concentration of P-gp substrates indicate that increases in P-gp protein levels by E2 or P4 manifest in significant functional differences. The concentrations of E2 and P4 that upregulated P-gp in our experiments are clinically significant as premenopausal women can attain concentration of 10 nM to 500 nM of P4 during pregnancy.[16] In addition, E2 concentration of up to 100 nM can be reached during pregnancy (∼50−100 nM at term).[14] Additionally, in certain pathological conditions, such as ovarian cancer, there may be localized tissue area of high hormone concentrations. In summary, the steroid hormones P4 and E2 increase P-gp expression and function in NCI-ADR-RES and JAR cells with the ERα-expressing cells (JAR) being more sensitive to E2. Our results indicate that P4 and E2 induce the level of P-gp by activating the transcription of the *MDR-1* gene. These results provide a better understanding of the regulation of P-gp expression in the placenta and ovary during pregnancy and may provide insight into the potential effects of hormone treatment on multidrug resistance during chemotherapy.

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