Proteomic Assessment of a Preclinical Model of Preeclampsia

Use of Traditional and Proteomic Methods in the Assessment of a Preclinical Model of Preeclampsia

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ABSTRACT

Recent studies have demonstrated downregulation of breast cancer resistance protein (BCRP/ABCG2) in placenta obtained from women with preeclampsia (PE). BCRP is highly expressed in placenta and plays an important role in preventing xenobiotics from entering the fetal compartment. While PE is often therapeutically managed with drugs that are substrates of BCRP, there are limited studies on the impact of PE on fetal drug exposure. Due to ethical concerns, use of preclinical models is an important approach. Thus, by using proteomic and traditional methods, we characterized transporter changes in an immunological rat model of PE to determine its utility and predictive value for future drug disposition studies. PE was induced by daily administration of low-dose endotoxin (0.01-0.04 mg/kg) to rats on gestational days (GD) 13 to 16, urine was collected, and rats were sacrificed on GD17 or GD18. PE rats shared similar phenotype to PE patients, including proteinuria, and increased levels of TNF-α and IL-6. Transcript and protein levels of Bcrp were significantly downregulated in placenta of PE rats on GD18. Mdr1a, Mdr1b and Oatp2b1 mRNA were also decreased in PE. Proteomics revealed activation of various hallmarks of PE including immune activation, oxidative stress, endoplasmic reticulum stress and apoptosis. Overall, our results demonstrated that the immunological PE rat model exhibits numerous similarities to human PE along with dysregulation of placental transporters. Therefore, this model may be useful in examining the impact of PE on the maternal and fetal disposition of BCRP substrates.
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**Significance Statement:**

Fully characterizing preclinical models of disease is necessary in order to determine their validity to human conditions. Combining traditional and proteomic methods of model characterization, we identified numerous phenotypic similarities between our model of PE and human disease. The alignment with human pathophysiological changes allows for more confident use of this preclinical model.
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INTRODUCTION

Preeclampsia (PE) is a pregnancy-specific disease that is characterized by hypertension, proteinuria, and edema. It affects 3-8% of pregnant women worldwide and is the leading cause of maternal and fetal morbidity (Abalos et al., 2013). Although the pathogenesis of PE is not fully understood, there is increasing evidence that suggests similarities to autoimmune diseases. Studies have shown an exaggerated maternal inflammatory response in PE, with activation of both innate and adaptive immunity, resulting in Th17 cell imbalance, systemic inflammation and increased serum and placental levels of IL-6, and TNF-α (Xia and Kellems, 2009; Lau et al., 2013; Anvari et al., 2015; Molvarec et al., 2015). The heterogeneity of the disease has led to the identification of two distinct subtypes of PE: early- and late-onset, in which PE develops before or after 34 weeks respectively. Although both subtypes have overlapping features, they differ with respect to many biochemical markers and clinical attributes.

During pregnancy, the placenta plays an important role in nutrient and waste exchange between mother and fetus. Several ATP-Binding Cassette (ABC) efflux and Solute Carrier (SLC) uptake transporters are highly expressed in the placenta and can impact the passage of endogenous and exogenous compounds into the fetus. Our laboratory recently reported that PE is associated with a dysregulation of drug transporters in human placenta (Kojovic et al., 2020). In particular, a pronounced downregulation of the breast cancer resistance protein (BCRP/ABCG2) was seen in placentas obtained from women with PE. BCRP is the most highly expressed efflux transporter in human placenta and is thought to play a fetoprotective role by preventing placental transfer of potentially toxic substrates, including many clinically important drugs (Anoshchenko et al., 2020). PE patients are often maintained on medication to manage the disease, including...
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antihypertensive drugs, anticonvulsants, and corticosteroids. As dysregulation of placental transporters in PE can result in altered transport of these drugs, it is important to understand how disease-mediated changes in their expression impact fetal drug exposure and the associated risks.

Over the past several decades, numerous animal models have been generated and used to investigate the pathogenesis and therapeutic management of PE. These include models where experimental PE is induced by immunological, genetic, pharmacological, or surgical manipulation (Taylor and George, 2022). While each model focuses on specific aspects of the disease, most models differ from the human condition. The frequently used immune-mediated model of PE involves treatment of pregnant rats with low doses of endotoxin, resulting in increased levels of pro-inflammatory cytokines, proteinuria, and elevated blood pressure, key symptoms in PE patients (Cotechini et al., 2014a). The impaired spiral artery remodeling and fetal growth restriction observed in this model indicate that the model shares similar features to early-onset PE (Cotechini et al., 2014a; Cotechini et al., 2014b).

Full characterization of preclinical disease models is desirable as it allows researchers to be confident in the predictive validity of the model. There are many ways such characterization can be performed. Traditional methods of characterization typically involve examining underlying pathophysiological and biochemical processes. Targeted gene and protein analysis is often performed using tools such as PCR and immunodetection. However, such analyses are confined by limited knowledge of the disease and scarce availability of valid biomarkers. For instance, levels of sFlt-1 and endoglin could be considered important biomarkers to assess preclinical models of PE, however this only considers angiogenic components of PE, thus excluding a
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multitude of other proteins and pathways which contribute to the disease phenotype (Leaños-Miranda et al., 2019; Kojovic et al., 2020). Untargeted global proteomics, which is the study of the entire proteome of an organism, is a tool that shows promise in the characterization of these models because of its ability to take a global protein “snapshot” (Deracinois et al., 2013).

Proteomics is a powerful method to characterize various aspects of different animal models, organisms, and diseases. For instance, proteomics has been used to validate biomarkers and reveal potential druggable targets in a preclinical stroke model; it has also been used to compare protein expression in human tissues to that of preclinical models (Zheng et al., 2020, Linscheid et al., 2021). Similar tactics can be used when holistically characterizing PE animal models for their merit in preclinical studies, particularly when human studies pose ethical issues, such as those which exist for pregnant women. To our knowledge, this is the first instance in which the technique is used to characterize a preclinical model for PE. Therefore, our aim was to use proteomics, alongside traditional techniques, to further assess changes in both drug disposition and pathogenic parameters in this model and better understand its usefulness for preclinical pharmacokinetic-pharmacodynamic studies.

METHODS

Animal studies

Timed pregnant Sprague-Dawley rats (Charles River Laboratories) were received on GD11 and housed using a 12-hour light-dark cycle with free access to water and food. Pregnant rats were injected intraperitoneally with either endotoxin or saline. Pregnant rats in the PE group received a bolus intraperitoneal 0.01 mg/kg dose of endotoxin (lipopolysaccharide, *Escherichia coli*).
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serotype 0111:B4; Sigma-Aldrich), dissolved in saline (0.9% NaCl) on GD 13, followed by daily 0.04 mg/kg doses on GD 14, 15 and 16. Controls received intraperitoneal doses of saline alone (n= 7-8 per group). Dams were placed in metabolic cages and urine was collected on GD 13, 16, 17 and 18, either immediately before endotoxin injection or sacrifice. Rats were anesthetized and sacrificed on GD 17 or GD 18 (24 h or 48 h after the last injection). The weight of pregnant dams, placentas, and fetuses are shown in Table S2. Maternal livers, kidneys, placentas, and fetuses were collected, fetal heads were separated from torsos, and all tissues were weighed and snap-frozen in liquid nitrogen for storage at -80°C for future analysis. Cardiac puncture was used to collect maternal blood, followed by centrifugation at 1500g and 4°C to isolate plasma. Maternal plasma and urine samples were snap-frozen in liquid nitrogen and stored at -80°C for future analysis. All animal studies were approved by the Office of Research Ethics at the University of Toronto and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Plasma and Urine Analysis

Protein expression of IL-6 and TNF-α in maternal plasma was quantified using the rat IL-6 Quantikine ELISA Kit (#R600B, R&D Systems) and the rat TNF-α Quantikine ELISA Kit (#RTA00, R&D Systems) following the manufacturer’s protocols. Vascular endothelial growth factor (VEGF) was measured by Eve Technologies (Calgary, AB Canada) using a rat cytokine 27-plex discovery assay (n= 4 /group).
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To assess urinary protein levels, total protein concentration was measured in collected urine using a protein assay (Bio-Rad Laboratories) and was normalized against total urinary creatinine concentration using a Creatinine Colorimetric Assay Kit (Cayman Chemical Company).

**Gene and Protein Expression**

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, tissues were homogenized in TRIzol, followed by addition of chloroform and isopropanol. RNA pellets were washed using 75% ethanol and resuspended in DEPC water. RNA concentration and purity were measured by a NanoDrop 1000 spectrometer (Thermo Fisher Scientific). Isolated RNA (2 μg) was treated with DNase I (Thermo Fisher Scientific) and reverse transcribed to cDNA using a High-Capacity cDNA RT Kit (Applied Biosystems). The cDNA was quantified using the Power SYBR Green detection system (ABI 7900HT). The primer sequences used are listed in Table S1. For each gene, the comparative threshold cycle method (ΔΔCₜ) was used to determine the relative mRNA expression. mRNA expression was normalized to that of the housekeeping gene GAPDH.

Individual placentas (approximately 0.3 g) were homogenized in a RIPA buffer (0.1mol/L Tris-HCl, pH 7.5; 3μL/mL protease inhibitor cocktail protease inhibitor; 50μg/mL PMSF). The protein concentration of lysates was quantified using a Bradford protein assay (Bio-Rad Laboratories) with BSA as a standard. Total protein (50 μg) was mixed with Laemmli buffer and 10% β-mercaptoethanol for each sample. Samples were separated on 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. Blots were blocked for 1.5h at room temperature in 5% skim milk Tris-buffered saline solution containing Tween (TBST), and
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incubated overnight at 4°C with primary mouse monoclonal anti-ABCG2 (BCRP) antibody (1:100; #sc-58222, Santa Cruz Biotechnology Inc) or anti-ABCB1 (P-glycoprotein) antibody (1:50; C219, Enzo Life Sciences). Following washes with TBST, blots were incubated with horseradish peroxidase-conjugated or anti-mouse (1:30000; #NA931, Jackson ImmunoResearch Laboratories) secondary antibody for 1.5h. Protein expression was normalized to the internal control β-actin (1:75000; #A1978, Sigma-Aldrich). Protein bands were detected using enhanced chemiluminescence SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using the ChemiDoc MP Imaging System (Bio-Rad).

Data from qRT-PCR and immunodetection was analyzed using Prism 8 (GraphPad Software). Results are presented as mean ± standard deviation (S.D.) Statistical significance between PE and controls was measured by a two-tailed Student’s t-test for unpaired experimental values.

**Proteomic Analysis**

Placental samples from control and PE rats (n=3 per group) were obtained and prepared for LC-MS/MS-based global proteomics using a filter-aided sample preparation method, as previously described by Wiśniewski et al. (2009). Briefly, placentas were homogenized in lysis buffer containing Tris-HCl (pH 7.6), DTT, and protease inhibitor, and SDS was added following homogenization to achieve a final percentage of 2%. Samples were sonicated, centrifuged at 4°C and the supernatant was collected. Protein concentration in each sample was determined using a BCA assay (ThermoFisher Scientific) and 100ug of protein was subject to sample cleanup. 8M Urea was added to the protein and SDS was depleted from the samples using 30 kDa filters (Sigma Aldrich, MRCF0R030). Proteins were denatured, alkylated using iodoacetamide, and
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digested overnight using Trypsin- LysC (Promega, V5073) and ultrafiltration. Peptides were then acidified, desalted and quantified using the Pierce Colorimetric Peptide Assay (ThermoFisher cat# 23275). 1ug of protein was loaded onto the ThermoFisher Q-Exactive Orbitrap HPLC-MS/MS alongside a digested BSA control. The ThermoFisher Easy Spray PEPMAP C18 reverse phase column (2 μm x 50 cm) and Nanoviper C18 precolumn (75 μm x 70 mm) were used and a data-dependent acquisition (DDA) method was employed.

Following LC-MS/MS runs, the sample raw files were uploaded onto MaxQuant (v. 2.0.3.1) alongside the Rattus Norvegicus proteome (Uniprot, UP000002494) and analyzed using standard label-free quantification methods (Tyanova et al., 2016). Once complete, the ProteinGroups.txt output was loaded onto Perseus software (v. 1.6.5.0). Data was filtered, transformed, and proteins that were significantly differentially expressed between the control and PE groups were identified. These proteins were then subject to a functional enrichment analysis using G-profiler software and visualized using Cytoscape’s Enrichment Map and AutoAnnotate plugins (v.3.9.1) using the pathway enrichment analysis workflow described by Reimand et al. (2019).

RESULTS

PE model induces phenotypic changes

Preeclampsia is associated with proteinuria and increased maternal and placental levels of pro-inflammatory cytokines. We detected high levels of protein in the urine obtained from the PE group after beginning endotoxin injections (Figure 1). While baseline measurements on GD13 were not different between groups, the urinary ratio of total protein to creatinine was significantly increased in urine collected from the PE group on GD16, 17 and 18. We also detected elevated levels of pro-inflammatory cytokines in the PE group. As compared to
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controls, maternal plasma levels of IL-6 and TNF-α were significantly increased in PE rats on GD 17 and 18 (Figure 2A). Likewise, PE was associated with increased transcript levels of IL-6 and TNF-α in placenta and increased IL-6 in fetal brains on GD17 and 18 (Figure 2B, C). In addition, VEGF was in trend of decreasing in PE rats (controls = 648 ± 114 pg/mL; PE = 406 ± 44 pg/mL; p=0.09).

**Impact of PE on Transporters**

Induction of PE was associated with significant alterations in the mRNA expression of both influx and efflux transporters on GD17 and GD18 in placental and fetal brains (Figure 3A, B). As compared to controls, the PE group had significant decreases of 35-55% in placental mRNA levels of Bcrp on GD17 and GD18 (Figure 3A). A corresponding 60% decrease in protein levels of Bcrp were seen in PE placenta on GD18 (Figure 4). Placental transcript levels of Mdr1a and Oatp2b1 were also decreased in PE on GD17 and GD18, while Mdr1b was only decreased on GD18 (Figure 3A). Transcript levels of Bcrp and Oatp2b1 were decreased in fetal brains of PE dams on GD 17 and 18 and levels of Mdr1b were decreased on GD18 (Figure 3B). Immunodetection of Pgp by Western blotting appeared fainter in the PE group but were not quantitated due to the presence of multiple bands (data not shown).

In maternal liver, we observed a significant increase in Bcrp mRNA in PE rats on GD17 while Mdr1b was significantly increased on GD18 (Figure S1, Supplemental data). No significant changes in mRNA expression of Bcrp, Mdr1a or Mdr1b were seen in maternal kidney (data not shown).
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Proteomic analysis shows dysregulation of proteins in PE Rats

A total of 3007 proteins were identified by LC-MS/MS based proteomics. Principal component analysis (PCA) demonstrated clear effects of the endotoxin treatment on the proteome (Figure S2, Supplemental data) and hierarchical clustering analysis confirmed these findings. Of the identified proteins, 326 were differentially expressed between the control and PE group (p-value cut-off of 0.05, FDR of 0.05 (S0= 0.1), Figure 5). More specifically, 211 proteins were upregulated while 115 were downregulated; the full list of these proteins is provided in the supplementary materials (Excel Table S3, Supplemental data). Included within the dysregulated proteins were heat shock proteins, integrins, peroxiredoxins, glycoproteins, protein disulfide isomerases and complement cascade regulators. Of note, several proteins which had been previously identified to be differentially expressed in the placental proteome of women with PE were also found to be changed in rat PE placental proteome (Table 1). Some transporters were identified in this analysis; most importantly, Pgp was found to be significantly downregulated by 67% in the PE group, consistent with the fainter bands seen on immunoblots.

Pathway enrichment analysis reveals involvement of canonical PE pathways

Functional enrichment analysis, performed using G-profiler software, demonstrated that the top 20 affected pathways were highly involved in endoplasmic reticulum processes, protein folding, response to oxygen levels and carbohydrate metabolism (p-value <0.05, g:SCS significance threshold). These pathways, specific to KEGG and GO terms, are captured with their corresponding p-values in Figure 6. Further examination of all pathways identified were performed using the Enrichment Map plugin in Cytoscape. This clustered related pathways into groups, revealing high-level functions affected by the treatment. A subset analysis of pathways
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implicated as key drivers to PE pathogenesis was performed for a more in-depth analysis, revealing significant involvement in the PE group. As visualized in Figure 7, these pathways include oxidative stress, apoptosis, inflammation, vascularization, and endoplasmic reticulum stress.

DISCUSSION

The major goal of our study was to further characterize the immunological model of PE to determine its suitability for preclinical studies, with respect to both phenotypic and pathophysiological considerations. This model has been used extensively by other groups and was previously shown to exhibit both proteinuria and hypertension (Chau et al., 2022). In PE patients, proteinuria and hypertension are criteria for diagnosis, making them essential features to recapitulate for model validity (Dong et al., 2017). In this study, we confirmed proteinuria in the model, reflective of renal damage secondary to disease. Additionally, a downward trend in expression of the angiogenic VEGF was noted in the maternal plasma of the PE rats. VEGF plays a role in development of normal placental vasculature, and attenuation of its action can cause hypertension (Sircar et al., 2015; Mäki-Petäjä et al., 2021). It has been demonstrated that VEGF protein levels are decreased in the plasma of PE women; demonstrating consistency of our PE model with human disease. While these diagnostic features are well-characterized by our group and others, there are a multitude of other traits that could impact the suitability of this PE model in preclinical studies.

Traditionally, defective spiral artery remodeling is referred to as the key driver of PE progression, classifying it as a disorder of the placental vasculature (Uzan et al., 2011; Burton et
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al., 2019). Studies have shown, however, that two subtypes of PE, coined early- and late-onset PE, have differing triggers to pathogenesis (Raymond and Peterson, 2011; Staff et al., 2018). While poor placentation is associated with early-onset PE, other maternal factors are thought to be responsible for late-onset PE. As such, other pathways such as oxidative stress, inflammation, and apoptosis are widely accepted hallmarks of the disease, particularly in late-onset cases (Mary et al., 2017; Burton et al., 2019; Nguyen et al., 2019; Navajas et al., 2021). These pathways are intricately linked and create a positive feedback loop towards the progression of PE, emphasizing the importance of capturing the heterogenic phenotypic qualities of this disease. The use of traditional methods to evaluate specific markers, along with global proteomic analysis, enables more comprehensive investigation into the phenotype of preclinical models.

PE is associated with chronic immune activation, resulting in a sustained inflammatory state, (Auer et al., 2010; Harmon et al., 2016). We observed increased levels of pro-inflammatory cytokines in the maternal circulation and fetal tissues which are consistent with the literature on human PE. Likewise, proteomic analysis of PE rat placenta revealed upregulation of several proteins that are commonly implicated in immune activation, such as the complement components. Indeed, functional pathway analysis identified 9 key clusters of immune-related processes that were significantly affected in the PE group including upregulation of processes such as MHC Class 1 loading (necessary for presentation of foreign antigen and recruitment of immune cells). Moreover, upregulation of the immune-related Jak-STAT signalling pathway was observed. Overall, our data indicates that immune activation occurs in our PE model, consistent with human PE.
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Inflammation can profoundly alter the expression of proteins involved in drug disposition pathways, including many transporters (Staudinger, 2013; Cressman et al. 2012). We previously demonstrated that BCRP expression is significantly decreased in placentas obtained from women with PE (Kojovic et al., 2020). Similar to this, we observed significant decreases in the mRNA and protein expression of Bcrp in placenta isolated from our PE dams. Moreover, our PE model was associated with decreased placental expression of Pgp protein, as detected by mass spectrometry. Bcrp and Pgp are known to play critical roles in the fetal to maternal transplacental efflux of many diverse substances including many clinically important drugs and endogenous compounds (Taggi et al., 2022). Therefore, dysregulation of Bcrp and Pgp could result in increased fetal accumulation of potentially harmful substrates. Indeed, inflammation-mediated decreases in the placental expression of Bcrp and Pgp have been previously seen in rodent models of infection and inflammation, along with increased fetal accumulation of their substrates (Petrovic & Piquette-Miller, 2015; Petrovic & Piquette-Miller, 2010; Petrovic et al., 2008). Therefore, this PE model could have great utility in examining the impact of PE on fetal exposure to important endogenous and drug substrates. It is interesting to note that targeted immunoblotting failed to detect significant changes in Pgp expression in human PE and posed issues with our rat placental samples, likely due to issues with the commercial antibody used. However, the greater sensitivity offered by proteomic analysis enabled more robust characterization of Pgp downregulation, consistent with observed trends of decreased Pgp expression seen in human PE placenta (Kojovic et al., 2020). Additionally, the observed decrease of Oatp2b1 mRNA levels is opposite to the increased levels seen in human PE placenta; however, this could be attributed to species differences in regulation or due to therapeutic management of women with PE (Kojovic et al., 2020).
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Inflammation is most often associated with alterations in the hepatic expression of drug transporters and metabolizing enzymes (Morgan et al., 2008). Hence, we also examined the hepatic and renal expression of transporters in our model. Interestingly, we observed increased rather than decreased transcript levels of Bcrp and Mdr1b in the livers of dams with PE while renal expression was unaffected. This suggests that tissue-specific regulatory mechanisms are occurring in the PE model, and that maternal drug disposition could be affected by PE. Interestingly, while proteomics confirmed the significant downregulation of Pgp at the protein level, other key drug transporters were not detected by our LC-MS/MS analysis. This could be a result of the global extraction method used and the relative abundance of other proteins, which could “mask” the identification of membrane proteins in a label-free quantification workflow. Therefore, targeted traditional techniques offered greater sensitivity for investigation into drug disposition parameters such as transporters or drug metabolizing enzymes.

Beyond targeted analysis of drug disposition proteins, proteomic evaluation of the PE model allowed for examination into a broader range of biomarkers and disease pathways implicated in PE. Oxidative stress has been identified as an essential component to the pathophysiology of PE (Can et al., 2014; Aouache et al., 2018). Decreased placental perfusion and subsequent ischemia reperfusion injury results in hypoxia and increased production of reactive nitrogen and oxygen species, thereby increasing oxidative stress and apoptosis (Uzan et al., 2011; Navajas et al., 2021, Can et al., 2014). Our proteomic results revealed model-mediated imbalances in various proteins involved in both hypoxia and oxidative stress. Downregulation of antioxidant enzymes, with concurrent increases in the hypoxia regulated proteins, indicate altered oxidative state (Mary et
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al., 2017). Functional pathway analysis identified that responses to hypoxia and oxidative stress were highly affected processes. This clearly demonstrates oxidative stress and hypoxia play a significant role in the phenotype of the PE rats, as observed in humans.

Likewise, high levels of endoplasmic reticulum (ER) stress are postulated to be a common feature of PE and is closely linked to oxidative stress (Burton and Yung, 2011; Nguyen et al., 2019). ER stress signalling pathways, such as the ER-associated proteasomal degradation pathway (ERAD) and the unfolded protein response (UPR), are thought to play a role in the pathogenesis of early PE (Yung et al., 2008, 2019; Burton and Yung, 2011). We found that ER stress was highly upregulated in placenta of PE dams and evidence of both ERAD and UPR activation was clear. Of the top 20 dysregulated pathways uncovered using functional enrichment analysis, half of them involved the ER and its role in protein processing. Furthermore, pathway enrichment analysis identified 10 clusters of biological processes involved in ER-related functions, one of which encompassed pathways directly correlated to ERAD.

Proteomic analysis also implicated apoptosis as a component of the model phenotype. Several apoptotic mediators were upregulated in the PE group and functional pathway analysis revealed significant alterations in apoptotic cleavage of proteins and regulation of apoptotic processes. Increased trophoblast apoptosis is commonly observed in human PE, contributing to insufficient spiral artery remodelling (Can et al., 2014; Lokeswara et al., 2021) and is a source of hypertension and inflammation. Altered apoptosis is therefore involved in multiple aspects of PE pathogenesis (Levy, 2005). Thus, upregulation of apoptotic processes in our PE model is
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characteristic of the PE phenotype in humans and reflects the multifactorial aspects of this disease.

Overall, similarities between the biochemical and proteomic features of women with PE, and those observed in this immunological PE model, provides promising support for its validity. Phenotypic characteristics of the model clearly recapitulates the five key hallmarks of PE (hypoxia, oxidative stress, inflammation, ER stress and apoptosis) and shares the general disease phenotype, in addition to the placental dysregulation of drug transporters. Due to the inability to study pregnant populations in clinical trials, accurate recapitulation of altered pharmacokinetic pathways is pivotal to improving therapy recommendations. As a disorder unique to humans, establishment of a good animal model is paramount to the execution of scientifically accurate preclinical work that allows for investigation into potential intervention. To our knowledge, this is the first study to use proteomic methods to achieve holistic PE model characterization. This expanded our investigation into the model and allowed a look at the broad phenotype of the treated animals, beyond proteinuria and hypertension.

CONCLUSION

Traditional targeted methods were used in combination with novel proteomic techniques in order to evaluate the suitability of an immunological rodent model of PE for preclinical studies. Overall, results demonstrated that the model shared phenotypic similarities to human disease such as proteinuria, immune activation, and dysregulation of BCRP. Therefore, this model may be useful in examining PE-mediated changes in maternal and fetal exposure to transporter
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substrates. Furthermore, this study underscores the potential utility of proteomics analysis in the assessment of preclinical disease models.

Authorship Contributions:

Participated in research design: Dai, Pollinzi, Piquette-Miller

Conducted experiments: Dai, Pollinzi

Performed data analysis: Dai, Pollinzi, Piquette-Miller

Wrote or contributed to writing the manuscript: Dai, Pollinzi, Piquette-Miller
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Footnotes:

No author has an actual or perceived conflict of interest with the contents of this article.

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**TABLES**

**Table 1. Summary Comparison of Differential Protein Expression of PE Rat Model to Human PE.** List of proteins significantly* changed in our PE model that have been reported to be significantly changed in human PE and the key pathway(s) involved. The direction of protein dysregulation in human tissues, including placenta (1Mary et al., 2017, 2Baig et al., 2014, 3Tarca et al., 2019, 4Kojovic et al., 2020) and blood (5Blumenstein et al., 2009, 6Kolla et al., 2012, 7Park et al., 2012, 8Auer et al., 2010) are reported, alongside the percent change seen in placenta of our PE model.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Related Pathway</th>
<th>Dysregulation in Human PE</th>
<th>% Change in PE Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoplasmic Reticulum BiP</td>
<td>Apoptosis</td>
<td>↑ in placenta1,2</td>
<td>↑ 28%</td>
</tr>
<tr>
<td>Peroxiredoxin-5</td>
<td>Apoptosis</td>
<td>↓ in placenta1</td>
<td>↓ 35%</td>
</tr>
<tr>
<td>Peroxiredoxin-5</td>
<td>Oxidative stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-1-acid glycoprotein</td>
<td>Inflammation</td>
<td>↑ in placenta1</td>
<td>↑ 210%</td>
</tr>
<tr>
<td>Protein Disulfide-Isomerase A4</td>
<td>ER Stress</td>
<td>↓ in placenta1</td>
<td>↑ 39%</td>
</tr>
<tr>
<td>Protein Disulfide-Isomerase A3</td>
<td>ER Stress</td>
<td>↑ in placenta1</td>
<td>↑ 24%</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>PE Biomarker</td>
<td>↑ in plasma5,6</td>
<td>↑ 56%</td>
</tr>
<tr>
<td>Complement C4</td>
<td>Inflammation</td>
<td>↓ in sera7</td>
<td>↓ 59%</td>
</tr>
<tr>
<td>Facilitated glucose transporter member 1</td>
<td>Transporter</td>
<td>↓ in placenta1</td>
<td>↓ 33%</td>
</tr>
<tr>
<td>Integrin subunit alpha 2b</td>
<td>Coagulation</td>
<td>↑ in placenta3</td>
<td>↓ 48%</td>
</tr>
<tr>
<td>Glutathione S-transferase P</td>
<td>Apoptosis</td>
<td>↑ in placenta1</td>
<td>↑ 91%</td>
</tr>
<tr>
<td>Glutathione peroxidase 3</td>
<td>Oxidative stress</td>
<td>↓ in sera8</td>
<td>↓ 32%</td>
</tr>
<tr>
<td>Endoplasmin</td>
<td>ER Stress</td>
<td>↑ in placenta1</td>
<td>↑ 39%</td>
</tr>
<tr>
<td>Transferrin receptor protein 1</td>
<td>Hypoxia</td>
<td>↑ in placenta1</td>
<td>↓ 73%</td>
</tr>
<tr>
<td>Pregnancy specific beta-1-glycoprotein 1</td>
<td>Pregnancy</td>
<td>↓ in sera7</td>
<td>↑ 60%</td>
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<tr>
<td>Hypoxia upregulated protein 1</td>
<td>Hypoxia</td>
<td>↑ in placenta1</td>
<td>↑ 35%</td>
</tr>
<tr>
<td>P-glycoprotein (ABCB1)</td>
<td>Transporter</td>
<td>↑ in placenta4</td>
<td>↓ 67%</td>
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Proteomic Assessment of a Preclinical Model of Preeclampsia

FIGURE CAPTIONS

**Figure 1.** Concentration ratios of total protein: creatinine in urine. Urine was collected from PE and control rats (n=7-8/group) at baseline on GD13, and after induction of PE on GD16, GD17 and GD18. Data shown as mean ± S.D. relative to control values and analyzed using Student’s unpaired t-test (* p < 0.05, ** p<0.01).

**Figure 2.** Inflammatory cytokines in (A) maternal plasma, (B) placenta, and (C) fetal brain on GD17 and GD18. Maternal plasma concentrations measured by ELISA; mRNA expression measured in placenta and fetal brain using qRT-PCR, normalized to GAPDH. Data is presented as mean ± S.D. (n=7-8 per group). Significance was determined using Student’s unpaired t-test (*p < 0.05, **p<0.01, ***p<0.001).

**Figure 3.** Transporter mRNA expression in (A) placenta and (B) fetal brain on GD17 and GD18. Relative mRNA levels measured by qRT-PCR, normalized to GAPDH, and presented as mean ± S.D. relative to controls (n=7-8 per group). Significance was determined using Student’s unpaired t-test (*p < 0.05, **p<0.01, ***p<0.001).

**Figure 4.** Placental protein expression of BCRP on GD18. Protein expression in PE and control dams was measured by western blot assay and normalized to β-actin level. Data is presented as mean± S.D. relative to controls (n=7-8 per group). Significance was determined using Student’s unpaired t test (**p<0.01). Representative blot shown below graph.

**Figure 5.** Volcano plot depicting protein expression fold-change between PE and controls. Fold change is depicted on the x-axis while the -log of the p-value is on the y-axis. Significantly differentially expressed proteins, determined using a t-test as the sample statistic, are portrayed by purple points above the curves (p-value<0.05) with upregulated proteins on the right and downregulated proteins on the left; n=3, run in technical duplicates. A false discovery rate of <0.05 was used and the plot was generated using Perseus software.

**Figure 6.** Top 20 most significantly affected biological processes in PE placenta. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms were used as pathway identifiers. Processes, functions, and cell compartments in the PE rats are plotted against the –log of their p-values. High involvement of ER-related processes, alongside carbohydrate and protein metabolism, hypoxia, and immune processes is exhibited. Data generated using functional enrichment analysis in G-profiler and the g:SCS significance threshold (p-value <0.05).

**Figure 7.** Enrichment map of dysregulated pathways related to key PE features. GEM output from G-profiler was uploaded to Cytoscape and the Enrichment Map plugin was used. Filters were applied to the resulting network to only show pathways related to PE hallmarks. This subnetwork was then AutoAnnotated to generate large clusters of related pathways and manually organized to cluster related nodes based on specific PE features. These organized
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clusters are connected using lines to indicate relationships; nodes are similarly connected based on shared proteins. Enrichment Map was created using an FDR Q<0.05, and Jaccard Overlap combined coefficient >0.375 with combined constant = 0.5, using Cytoscape.
Figure 1
Figure 2

Maternal Plasma

![Graph showing plasma concentration of IL-6 and TNF-α in GD17 and GD18 for control and PE groups.]

Placenta

![Graph showing mRNA expression (relative to controls) of IL-6 and TNF-α in GD17 and GD18 for control and PE groups.]

Fetal Brain

![Graph showing mRNA expression (relative to controls) of IL-6 and TNF-α in GD17 and GD18 for control and PE groups.]

* Indicates a significant difference between control and PE groups.
** Indicates a highly significant difference between control and PE groups.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7