

ORIGINAL ARTICLE

Glucocorticoid exposure induces preeclampsia via dampening 1,25-dihydroxyvitamin D₃

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The pathogenesis of preeclampsia (PE) involves a number of biological processes that may be directly or indirectly affected by glucocorticoid (GC) and vitamin D. GC exposure increases the risk of PE, and 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) deficiency may result in PE. The purpose of the present study was to confirm the involvement of GC/1,25-(OH)₂D₃ axis in the pathogenesis of PE. In the study, cortisol levels of PE patients were found to be higher than that of non-complicated pregnancies, while 1,25-(OH)₂D₃ were decreased in both PE women and GC-induced PE rats. Mechanically, GC reduced 1,25-(OH)₂D₃ levels via disturbing its biosynthetic and catabolic enzymes, including *Cyp3a1*, *Cyp24a1* and *Cyp27b1*, especially enhancing the expressions of *Cyp3a1*, the dominant enzyme for vitamin D degeneration. Moreover, replenishing 1,25-(OH)₂D₃ ameliorated the symptoms and placental oxidative stress of GC-induced rat PE. The protective actions of 1,25-(OH)₂D₃ might be explained by its roles in antagonizing the effects of GC on trophoblast proliferation and apoptosis. Together, these findings suggest that GC exposure could lead to PE via dampening 1,25-(OH)₂D₃ biosynthesis, and GC/1,25-(OH)₂D₃ axis might represent a common pathway through which PE occurs.

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INTRODUCTION

Preeclampsia (PE), characterized by maternal hypertension, proteinuria and other systemic disorders occurring after 20 weeks of gestation, is a leading cause of maternal and fetal morbidity and mortality.^{1–3} Despite active research, the etiological mechanism of this disorder remains elusive. Increasing evidence indicates that stress or glucocorticoid (GC) exposure may result in PE.^{4–8} László *et al.*⁷ showed that stress in the first trimester of pregnancy might increase the risk of early-onset PE. Moreover, Takiuti *et al.*⁹ confirmed that stress in early pregnancy could induce PE in rat. We demonstrated previously that stress-mediated increase in GC activity is involved in spontaneous miscarriages¹⁰ and PE,⁵ and GC exposure in mid-late gestation could lead to rat intrauterine growth restriction.¹¹ Interestingly, we confirmed recently that GC exposure in early placentation could induce PE in rats.⁶

The pathogenesis of PE involves a number of biological processes that may be directly or indirectly affected by vitamin D, including immune dysfunction, placental implantation, abnormal angiogenesis, excessive inflammation and hypertension.^{12,13} In fact, PE has been demonstrated to be associated with low circulating levels of 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D₃

(1,25-(OH)₂D₃).^{14,15} A case-control study showed a significant association between 25(OH)D concentrations in early pregnancy and subsequent PE.¹⁶ Moreover, expression and activity of 25-hydroxyvitamin D-1 alpha-hydroxylase (CYP27B1) are restricted in cultures of syncytiotrophoblast cells from preeclamptic pregnancies.¹⁷ Therefore, a deficiency of 1,25-(OH)₂D₃ may result in PE.

The balance between bioactivation and degradation of 1,25-(OH)₂D₃ is critical for ensuring its appropriate biological effects and is tightly controlled *in vivo*. Biosynthesis of vitamin D involves the action of CYP27B1, leading to the synthesis of hormonally active 1,25-(OH)₂D₃.¹⁸ 1,25-(OH)₂D₃ is catabolized by CYP24A1, which is followed by sequential metabolism, yielding the terminal product calcitroic acid.¹⁹ It also undergoes CYP3A4-dependent 24- and 25-hydroxylations.²⁰ CYP3A1 (the rodent ortholog of CYP3A4) represents a major component of CYP system in the rat placenta, highly expressed throughout pregnancy.²¹ Interestingly, previous findings indicated that CYP3A4, not CYP24, dominated the hydroxylation of 1,25-(OH)₂D₃ in human liver and intestine, and SXR/CYP3A4 has a key role in mediating vitamin D catabolism and drug-induced osteomalacia.¹⁹ Synthetic GC dexamethasone (Dex)

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could induce CYP3A1 expression in rat liver,²² and Dex could transactivate PXR and induce CYP3A1 expression in rat hepatocytes.²³ Importantly, CYP3A4 induction could exert an important role in decreasing 1,25-(OH)₂D₃ levels.²⁴ We showed that serum cortisol levels of PE women are higher than that of normal pregnancies.⁵ Moreover, we confirmed recently that GC exposure in early placentation could induce PE in rats,⁶ which may involve the loss of 1,25-(OH)₂D₃.

The present study, therefore, was undertaken to confirm the involvement of GC/1,25-(OH)₂D₃ axis in the pathogenesis of PE and to explore the underlying mechanisms. This study would provide a clue for better understanding of the pathogenesis of PE and contribute to develop potential therapeutic strategies in preventing PE.

METHODS

Reagents and antibodies

Dex, Dex sodium phosphate, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 1,25-(OH)₂D₃ and Hoechst 33258 were from Sigma Aldrich (Allentown, PA, USA). Anti-hypoxia-inducible factor 1- α (HIF1 α , sc-10790) and Lamin B (sc-374015) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Patient samples

Blood samples were obtained from 26 women: 13 women with PE defined by hypertension (systolic and diastolic blood pressures higher than 140/90 mm Hg) and proteinuria (0.3 g per day)²⁵ were recruited from the Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, China; as a comparative group, 13 pregnant women were originally selected with characteristics similar to those presented by the preeclamptic patients, including body mass index, gestational age, eliminated high blood pressure, kidney disease, diabetes and so on. Blood and placenta samplings were obtained from diagnosed patients after informed consent and approved by the Ethical Committee of the Medical Faculty of Tongji Medical College, Huazhong University of Science and Technology in accordance with the Declaration of Helsinki.

The patients had not taken any medications before specimen collection. Blood extracted from the pregnant women stood for 20 min at room temperature. After centrifugation, serum was collected and stored at -80 °C until use.

Animals and experimental protocol

Female Sprague-Dawley rats (10–12 weeks old, weighing 220–250 g) were purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. Animals were housed individually in plastic cages with wood chips as bedding under pathogen-free conditions, in a controlled environment of temperature at 20–25 °C and 12 h cycles of light and dark. Rats were fed a standard laboratory diet and water *ad libitum*. Pregnancy was obtained by mating female rats with fertile male rats at a ratio of 2:1 overnight. Daily vaginal smears were observed, and appearance of spermatozoa in vaginal smear was defined as gestational day (GD) 1. All animal work was conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All studies involving rats were approved by Animal Care and Use Committee of Huazhong University of Science and Technology.

Experimental protocol 1. Pregnant rats were randomly divided into control and treatment groups. Treatment rats were injected s.c. with Dex sodium phosphate (2.5 mg kg⁻¹ per day) from GD7 to 13, while controls were injected s.c. with equal saline. On GD21 (pregnancy time = 22 days), rats were fully anesthetized with chloral hydrate and placental specimens were rapidly extracted. Blood samples were drawn by heart puncture and centrifuged at 3000 r.p.m. for 20 min at 4 °C to obtain the serum.

Experimental protocol 2. Pregnant rats were injected s.c. with different doses of Dex sodium phosphate (1, 2.5, 5 and 10 mg kg⁻¹) on GD13, while controls

were injected s.c. with equal saline. At 24 h post Dex or saline injection, the rats were fully anesthetized with chloral hydrate, and the placenta, kidney and liver specimens were rapidly collected.

Experimental protocol 3. Pregnant rats were randomly divided into three groups: control; Dex; and Dex plus 1,25-(OH)₂D₃. From GD7 to 13, controls were injected s.c. with equal saline, rats in Dex group were injected s.c. with Dex sodium phosphate (2.5 mg kg⁻¹ per day) and rats in Dex plus 1,25-(OH)₂D₃ group were injected with Dex sodium phosphate (2.5 mg kg⁻¹ per day) s.c. as well as 1,25-(OH)₂D₃ (1.2 μ g kg⁻¹ per day) i.p. On GD21, rats were fully anesthetized with chloral hydrate and the uterus was removed and placed in a chilled dish. Placenta and pup were rapidly extracted. All of the pups and placentas were weighed, and litter size noted. Kidney was also removed. All samples were analyzed individually.

Here 2.5 mg kg⁻¹ of Dex is selected because we referenced a previous report indicating that this dose could result in circulating GC levels in rats over time that reproduce the circulating levels of GC in rats observed during and after exposure to acute stress.²⁶ Our previous work has also reported that this dose of Dex could induce PE development in pregnant rats.⁶ Moreover, 2.5 mg kg⁻¹ of Dex was administered as we observed in the initial experiments that 1 mg kg⁻¹ or lower doses of Dex was insufficient to significantly induce typical manifestations of PE in pregnant rats, while 5 mg kg⁻¹ Dex contributed to fetal loss or death rather than PE development. A unit of 1.2 μ g kg⁻¹ of 1,25-(OH)₂D₃ per day is selected because we referenced a previous report indicating that 5 μ g kg⁻¹ every other day was safe and tolerated by the mice, having no major side effects while maintaining its activity.²⁷ Importantly, the most advantage of 1,25-(OH)₂D₃ is its fast metabolic clearance *in vivo*.

Measurement of systolic blood pressure

At indicated time (initial non-pregnant status, GD3-5, GD14 and GD20), the systolic blood pressure was determined in conscious, restrained pregnant rats. An automated system with a photoelectric sensor linked to a dual channel recorder (BP-98A, Softron, Tokyo, Japan), tail cuff and sphygmomanometer was used to obtain indirect blood pressure measurements, which have been previously demonstrated to be closely correlated with direct arterial measurements.²⁸ The measurements were repeated three times for each rat, with the mean value recorded.

Determination of urinary albumin excretion

For 24 h urine collection, on GD5 and GD20, the pregnant rats were placed in metabolic cages. To avoid contaminating the collected urine, rats were restricted from food; however, they were allowed free access to water. To avoid the adverse effects of fasting, rats were fed in other cages for 30 min every 6 h. Urine samples were centrifuged at 3000 r.p.m. for 20 min at room temperature, and the supernatant was collected for urinary albumin analysis. Urine protein concentrations were determined by using pyrogallol red in an automatic biochemical analyzer (ADVIA 2400 Chemistry System, Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA).

Hematoxylin and eosin staining

For histological evaluation, placenta and kidney were fixed in neutral-buffered formalin. Hematoxylin and eosin staining was performed on 4 μ m paraffin sections of placenta and kidney specimens for conventional morphological evaluation under light microscope (Olympus BX60, Tokyo, Japan).

Measurement of thiobarbituric acid reactive substances

Placental thiobarbituric acid reactive substances (TBARS) was measured by using a commercially available kit (QuantiChrom TBARS Assay Kit, DTBA-100) according to the manufacturer's instruction (BioAssay Systems, Hayward, CA, USA). Briefly, placentas (~20 mg) were placed into 200 μ l ice-cold phosphate-buffered saline with protease inhibitors. The tissues were first homogenized thoroughly and then sonicated for 20 s on ice. Samples were then centrifuged at 3000 r.p.m. for 10 min at 4 °C. A 20 μ l aliquot was removed for protein analysis. The resultant absorbances were read at 535 nm. TBARS levels were expressed as nmol mg⁻¹ protein.

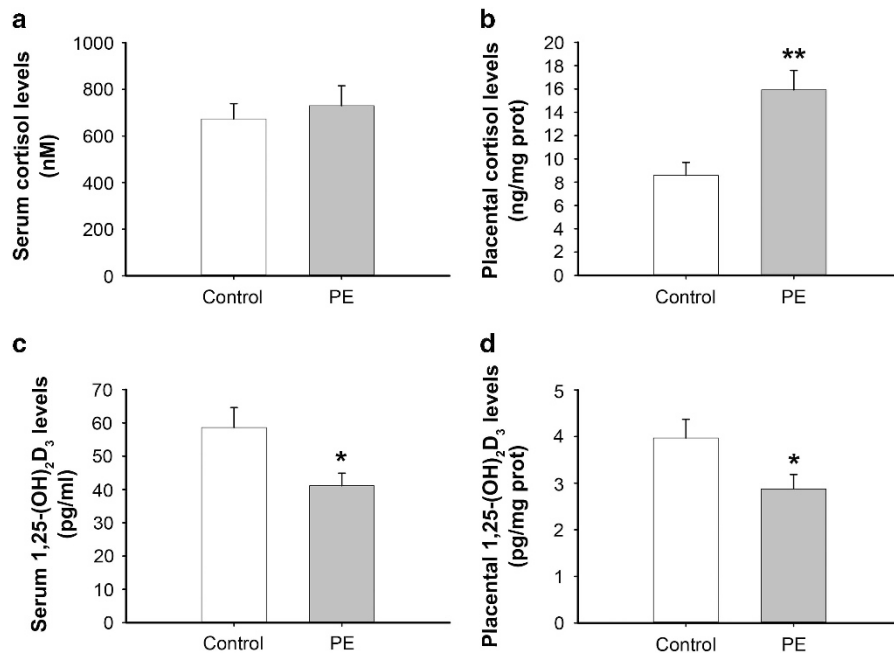


Figure 1 Expressions of cortisol and 1,25-(OH)₂D₃ in human preeclampsia (PE). (a) Serum cortisol levels in PE women; (b) placental cortisol levels in PE women; (c) serum 1,25-(OH)₂D₃ levels in PE women; (d) and placental 1,25-(OH)₂D₃ levels in PE women. The levels of cortisol were detected by enzyme chemiluminescence immunoassay. 1,25-(OH)₂D₃ was determined by enzyme-linked immunosorbent assay. Results are expressed as means ± s.e.m. ($n = 13$ in control and PE group). * $P < 0.05$ and ** $P < 0.01$ vs. control group, two-tailed Student's t -test.

Cell culture and treatment

Human first-trimester trophoblast cell line HPT-8 was kindly provided by Fourth Military Medical University (Xi'an, China). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂ in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 25 mM HEPES, 100 U ml⁻¹ penicillin G and 100 U ml⁻¹ streptomycin. Cells were treated with Dex (10⁻⁷ mol l⁻¹), 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) or Dex (10⁻⁷ mol l⁻¹) plus 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) for MTT cell proliferation assay, and Dex (10⁻⁶ mol l⁻¹), 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) or Dex (10⁻⁶ mol l⁻¹) plus 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) for Hoechst cell apoptosis assay.

Cell proliferation and apoptosis assay

Cell proliferation was determined by a colorimetric method based upon metabolic reduction of the soluble yellow tetrazolium dye MTT to its insoluble purple formazan. Approximately, 5000 cells per well were grown in 96-well plates and incubated overnight in 200 μl of culture medium. After cells were treated with indicated conditions for 24 h, each well was added with 20 μl MTT (0.5 mg ml⁻¹) and incubated for 4 h before supernatant was removed. After plate was placed at 37 °C for 15 min in 150 μl dimethylsulfoxide, the absorbency was measured with a micro ELISA reader (Amersham Biosciences, Pittsburgh, PA, USA) at a wavelength of 492 nm.

Cell apoptosis was examined by Hoechst staining. Cells were incubated with the Hoechst dye for 15 min before examination under fluorescence microscope. Apoptotic cells were characterized by characteristic nuclear fragmentation; only those nuclei showing evidence of DNA fragmentation without plasma membrane damage were taken to be apoptotic cells.

RNA isolation and real-time PCR

Total RNA was isolated from placenta, kidney or liver using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. The concentration and purity of RNA was determined by the ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). cDNA synthesis was performed using ReverTra Ace cDNA Synthesis kit (FSQ-101, TOYOBO, Osaka, Japan) according to the manufacturer's instruction. Quantitative PCR analysis was carried out on a Bio-Rad CFX Connect Real-Time PCR

Detection System using iQaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Richmond, CA, USA). Each sample was quantified in triplicate ($n = 3$). PCR conditions were 30 s at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at 60 °C. Data were analyzed using the Bio-Rad CFX Manager 2.1 software, and normalized to GAPDH. The sequences of primers were as follows: rat *Cyp24a1*, forward 5'-CCGCTGATGACAGACGGTGA-3' and reverse 5'-TACTCTGCCACGGTGTCTGTG-3'; rat *Cyp27b1*, forward 5'-GCTACGGCGGATGGTGAAG-3' and reverse 5'-CCTTAGTCGTCGCACGAGGT-3'; rat *Cyp3a1*, forward 5'-TGGAGAAAGCCAAGAAGCTCTT-3' and reverse 5'-TCATATACTGGCGTGAGGAATGG-3'; rat *Pxr*, forward 5'-GACGGCAGCATCTGGAACACTAC-3' and reverse 5'-TGATGACGCCCTTGAACATG-3'; rat *Gapdh*, forward 5'-GGCACAGTCAAGGCTGAGAATG-3' and reverse 5'-ATGGTGGTGAAGACGCCAGTA-3'.

Western blotting

Nuclear protein of placental tissue was extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of Biotechnology, Shanghai, China) for assessing HIF1A expression. Protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Then, regular western blotting assay was performed. Briefly, equal amounts of protein (40 μg) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% (w/v) non-fat dried milk and incubated with primary antibodies overnight at 4 °C, followed by incubation with a secondary horseradish peroxidase-conjugated antibody. The bound antibody was detected by an enhanced chemiluminescence kit (Millipore) on X-ray film. Lamin B served as internal control.

Measurement of cortisol, 25(OH)D and 1,25-(OH)₂D₃

The levels of cortisol were detected by enzyme chemiluminescence immunoassay in chemiluminescence analyzer (ADVIA Centaur XP Immunoassay System, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). The concentrations of total 25(OH)D were measured by electrochemical luminescence immunoassay in chemiluminescence analyzer (Roche cobas e 601, Roche Diagnostics GmbH, Mannheim, Germany). 1,25-(OH)₂D₃ was determined using

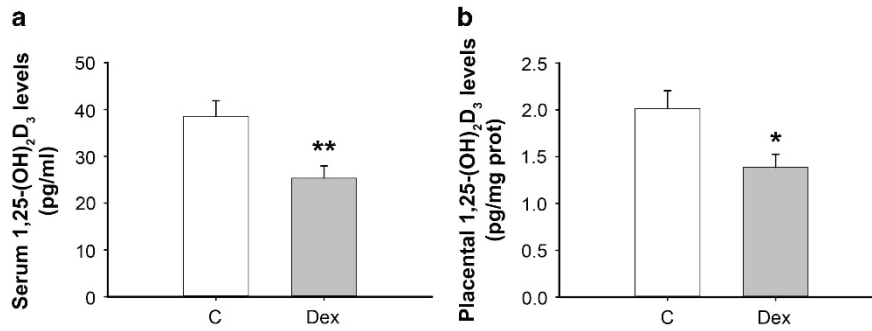


Figure 2 Glucocorticoid downregulates 1,25-(OH)₂D₃ levels in experimental preeclampsia rats. Rats were treated with Dex according to Experimental protocol 1. (a) Effect of dexamethasone (Dex) on serum 1,25-(OH)₂D₃ levels in pregnant rats; and (b) effect of Dex on placental 1,25-(OH)₂D₃ levels in pregnant rats. 1,25-(OH)₂D₃ levels were determined by enzyme-linked immunosorbent assay. Results are expressed as means ± s.e.m. ($n=7$ rats in each group). * $P<0.05$ and ** $P<0.01$ vs. control group, two-tailed Student's *t*-test.

1,25-(OH)₂D₃ ELISA kit (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were done using the SPSS 19.0 software (IBM, Armonk, NY, USA). The results were expressed as means ± s.e.m. of multiple independent experiments. The means of the different groups were compared by using either Student's *t*-test or one-way analysis of variance followed by *S-N-K post hoc* test. A value of $P<0.05$ was considered significant.

RESULTS

Upregulation of cortisol and downregulation of 1,25-(OH)₂D₃ in human PE

To determine whether GC and 1,25-(OH)₂D₃ are correlated with PE, we measured the levels of cortisol and 1,25-(OH)₂D₃ in serum and placenta of both normal pregnancy and PE women. As shown in Figure 1a, the levels of serum cortisol had no significant difference between PE and normal women, while the levels of placental cortisol in PE women showed significant increase compared to normal pregnancies (Figure 1b). As expected, serum 1,25-(OH)₂D₃ levels of PE women decreased significantly compared to normal pregnancies (Figure 1c). In line with this, placental 1,25-(OH)₂D₃ levels of PE women were significantly lower than those of normal pregnancies (Figure 1d). Furthermore, serum total 25-(OH)D levels of PE women decreased significantly compared to controls (Supplementary Figure 1). These clinical data suggest that cortisol activity is upregulated while 1,25-(OH)₂D₃ level is downregulated in human PE.

GC downregulates 1,25-(OH)₂D₃ in experimental PE rats

To confirm the involvement of GC/1,25-(OH)₂D₃ axis in the pathogenesis of PE, we detected the 1,25-(OH)₂D₃ levels of both serum and placenta in GC-mediated rat PE. As shown in Figures 2a and b, 1,25-(OH)₂D₃ of both serum and placenta were strikingly downregulated by Dex in experimental PE rats. Furthermore, Dex significantly decreased serum total 25-(OH)D levels of experimental PE rats compared to controls (Supplementary Figure 2). These results suggest that GC exposure may result in PE via inhibiting 1,25-(OH)₂D₃ expression.

GC disturbs 1,25-(OH)₂D₃ synthesis and catabolism in pregnant rats

To explore related molecular mechanisms underlying the effects of GC on 1,25-(OH)₂D₃ levels, we detected the expressions of *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* in liver, kidney and placenta of pregnant rats after GC treatment. As shown by real-time PCR in Figure 3a, the

mRNA expressions of liver *Cyp24a1* and *Pxr* were strikingly downregulated by Dex, while liver *Cyp27b1* and *Cyp3a1* expressions were strikingly upregulated by Dex in pregnant rats. Downregulation of *Cyp24a1* and upregulation of *Cyp27b1* and *Cyp3a1* were observed in kidney of Dex-treated pregnant rats (Figure 3b). The mRNA expressions of placental *Cyp24a1*, *Pxr* and *Cyp3a1* were enhanced by Dex in pregnant rats (Figure 3c). Therefore, GC-mediated disturbance of 1,25-(OH)₂D₃-synthesizing and -catabolizing enzymes may be the reason for lower levels of 1,25-(OH)₂D₃ in PE.

1,25-(OH)₂D₃ treatment improves symptoms of GC-mediated rat PE

To validate the involvement of GC/1,25-(OH)₂D₃ axis in the pathogenesis of PE, a GC-induced rat PE model was constructed as previously,⁸ and, meanwhile, the rat PE model was treated with 1,25-(OH)₂D₃. As shown in Figure 4a, systolic blood pressure was significantly elevated after Dex exposure, while blood pressure conditions were obviously improved relative to Dex group after 1,25-(OH)₂D₃ treatment, but they could not be dropped to the control levels. The urine protein level was significantly higher in Dex group on GD20, and 1,25-(OH)₂D₃ treatment significantly reduced the excretion of urine protein (Figure 4b). The fetal and placental weight of Dex group was significantly lower than that of control and 1,25-(OH)₂D₃ groups (Figure 4c). In addition, Dex-treated rats displayed significant structural abnormalities in placenta and kidney characterized by thickening of vessel walls in labyrinth and glomerular endotheliosis, while 1,25-(OH)₂D₃ treatment significantly ameliorated placental and renal impairment (Figure 4d). These data suggest that 1,25-(OH)₂D₃ is effective in relieving rat PE-related symptoms induced by GC.

1,25-(OH)₂D₃ treatment ameliorates placental oxidative stress of GC-mediated rat PE

To further address the question of how 1,25-(OH)₂D₃ prevents GC-mediated PE, the focus was turned to placental oxidative impairment. Placenta oxidative impairment is the fundamental abnormality leading to PE.²⁹ HIF1A, overexpressed in hypoxic placentas of preeclamptic women, is implicated in the pathogenesis of PE.^{1,2,30} On GD21, levels of placental oxidative impairment and HIF1A expression were detected by TBARS assay kit and western blotting respectively. As shown in Figure 5a, Dex administration resulted in increased placental TBARS levels, suggesting placental oxidative damage. HIF1A was overexpressed in placenta of Dex-treated rats vs. controls (Figure 5b). But after 1,25-(OH)₂D₃ treatment, both

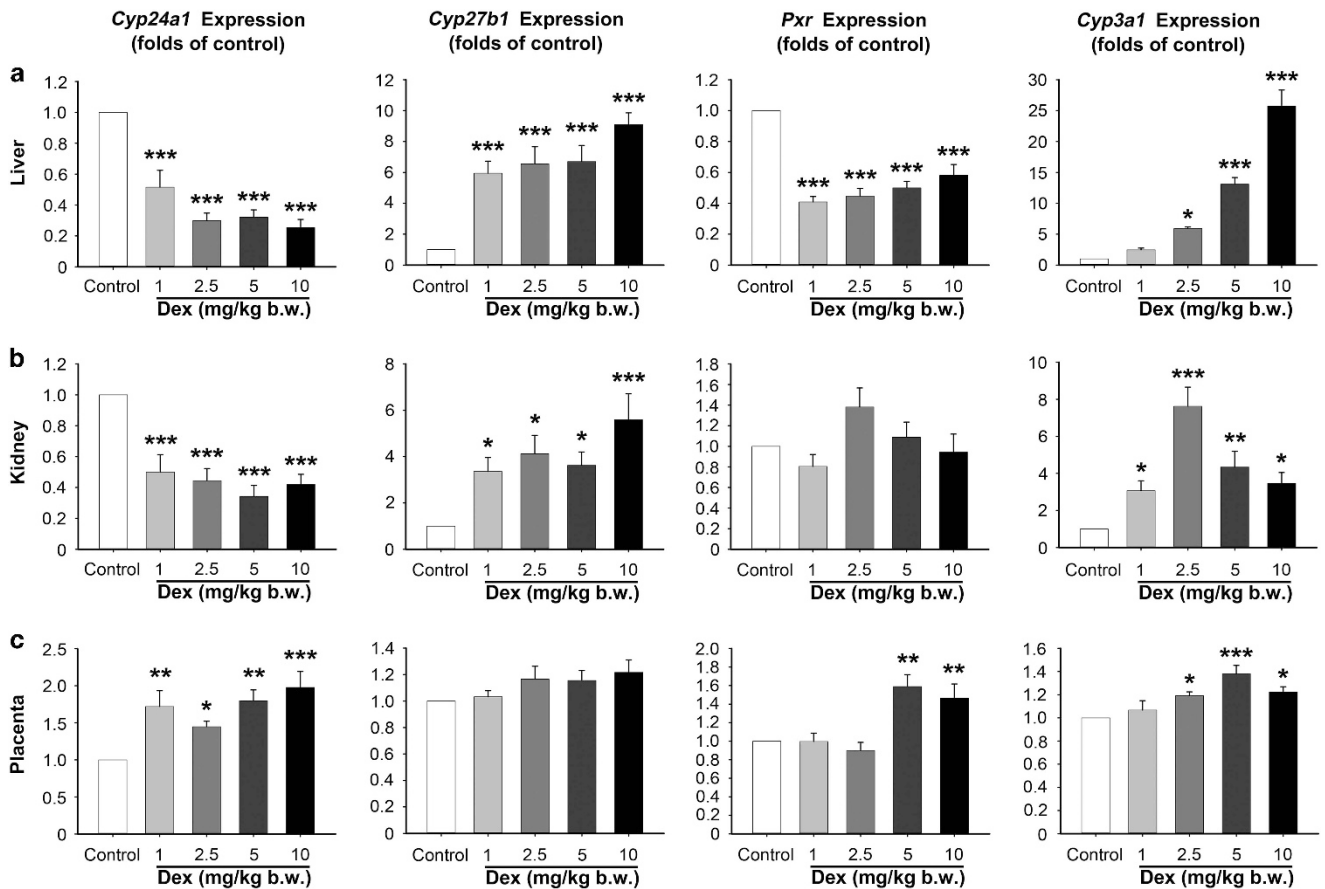


Figure 3 Glucocorticoid disturbs 1,25-(OH)₂D₃-synthesizing and -catabolizing enzymes in pregnant rats. Rats were treated with dexamethasone (Dex) according to Experimental protocol 2. (a) Effect of Dex on *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* gene expression in liver of pregnant rats; (b) effect of Dex on *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* gene expression in kidney of pregnant rats; and (c) effect of Dex on *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* gene expression in placenta of pregnant rats. Gene expression was detected by real-time PCR. Results are expressed as means±s.e.m. (*n*=7 rats in each group). **P*<0.05, ***P*<0.01 and ****P*<0.001, one-way analysis of variance with S–N–K post test.

placental TBARS level and HIF1A expression were downregulated (Figures 5a and b).

1,25-(OH)₂D₃ antagonizes the effects of GC on trophoblast proliferation and apoptosis *in vitro*

Dex significantly decreased the proliferation of trophoblast cell line HPT-8, while Dex triggered HPT-8 apoptosis *in vitro* (Figures 6a and b). Importantly, 1,25-(OH)₂D₃ treatment antagonized the effects of Dex on HPT-8 proliferation and apoptosis (Figures 6a and b). These results suggest that 1,25-(OH)₂D₃ could antagonize the inhibitory effects of Dex on trophoblast development *in vitro*, supporting the protective actions of 1,25-(OH)₂D₃ on trophoblast against GC.

DISCUSSION

To date, the molecular mechanisms underlying PE remain elusive. Our data support that GC/1,25-(OH)₂D₃ axis might be a common pathway through which PE occurs. The present study provides evidence that GC might result in PE via downregulating 1,25-(OH)₂D₃, an endogenous protective mediator during pregnancy. Our results also reveal the molecular mechanism underlying the effect of GC on 1,25-(OH)₂D₃.

This study first revealed novel findings regarding the GC levels and 1,25-(OH)₂D₃ concentrations in serum and placenta of PE women. The serum cortisol levels of PE women had an increase

(not significant), while the placental cortisol levels showed significant increase compared to normal pregnancies. In contrary, 1,25-(OH)₂D₃ levels of both serum and placenta decreased significantly in PE women compared to controls. These clinical data suggest the increase of GC activity and decrease of 1,25-(OH)₂D₃ levels in PE. Hence, this study reports the clinical data of involvement of GC/1,25-(OH)₂D₃ in PE.

Accumulating evidence suggest the adverse effects of stress and GC exposure on pregnancy.^{7,10,11,31} Previous works reported that stress could induce PE in pregnant rats and women.^{7,9} We demonstrated recently that GC exposure in early placentation could lead to PE-related manifestations in pregnant rats,⁶ supporting that GC exposure is a potential stimulus for PE development. Here GC-treated pregnant rats were characterized by reduced 1,25-(OH)₂D₃ expression, suggesting these symptoms may have occurred, at least in part, in response to 1,25-(OH)₂D₃ downregulation caused by GC as 1,25-(OH)₂D₃ is an endogenous protective mediator during pregnancy. In fact, 1,25-(OH)₂D₃ concentrations of both serum and placenta were strikingly downregulated by GC in experimental PE rats.

The biosynthesis and degradation of 1,25-(OH)₂D₃ is controlled by several enzymes. CYP24A1 is important in the catabolism of 1,25-(OH)₂D₃, while CYP27B1 is involved in its biosynthesis. PXR/CYP3A1 also has the dominant role in hydroxylation of 1,25-(OH)₂D₃. To clarify the effects of GC on 1,25-(OH)₂D₃-synthesizing and -catabolizing enzymes, we studied the expressions

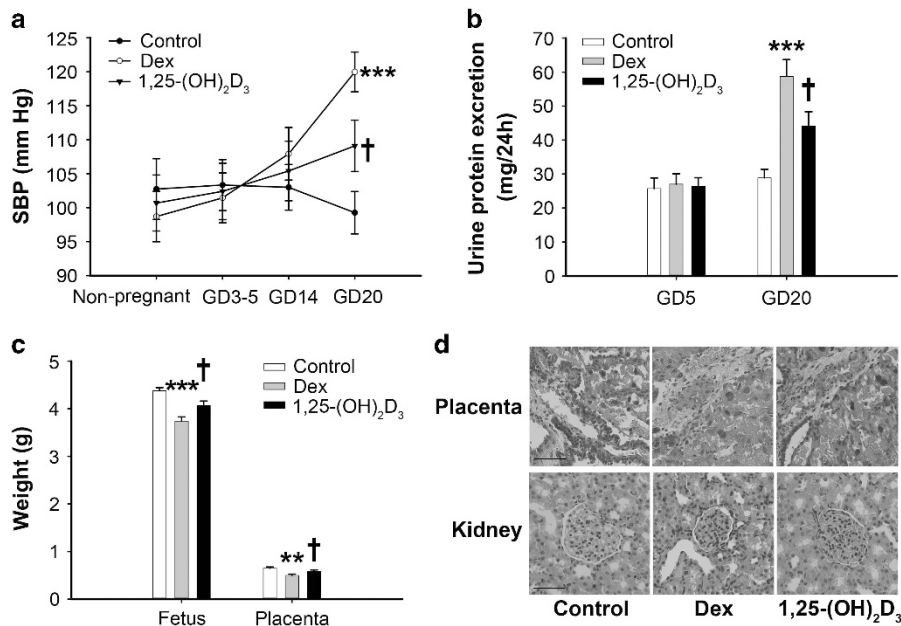


Figure 4 1,25-(OH)₂D₃ ameliorates the symptoms of glucocorticoid-mediated rat preeclampsia. Rats were treated with dexamethasone (Dex) according to Experimental protocol 3. Systolic blood pressure (SBP), 24 h urinary protein excretion and fetal and placental weight are presented in **a–c**, respectively. Results are expressed as means ± s.e.m. ($n=7$ rats in each group). ** $P<0.01$ and *** $P<0.001$ vs. control group, † $P<0.05$ vs. Dex group, one-way analysis of variance with S–N–K post test. **(d)** Effect of 1,25-(OH)₂D₃ on Dex-mediated placental and renal impairment. Representative hematoxylin and eosin staining images of placenta and kidney are showed, the magnification is ×400, bar = 50 μm. A full color version of this figure is available at the *Hypertension Research* journal online.

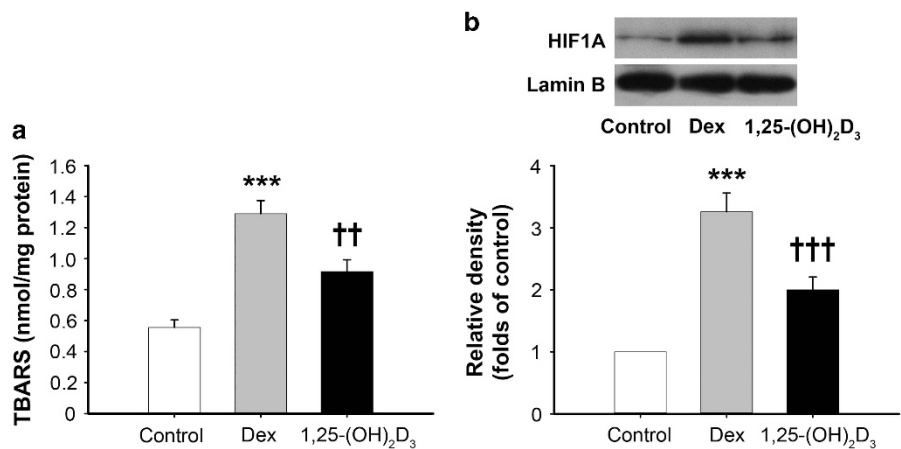


Figure 5 1,25-(OH)₂D₃ improves placental oxidative stress of glucocorticoid-mediated rat preeclampsia. Rats were treated with dexamethasone (Dex) according to Experimental protocol 3. **(a)** Effect of 1,25-(OH)₂D₃ on Dex-mediated placental lipid peroxidation. Levels of lipid peroxidation were determined by using a commercially available thiobarbituric acid reactive substances (TBARS) kit. The measurements of lipid peroxidation represent the degree of placental oxidative stress. Results are expressed as means ± s.e.m. ($n=7$ rats in each group). **(b)** Effect of 1,25-(OH)₂D₃ on Dex-mediated placental HIF1A expression. HIF1A was detected by western blotting. The histogram represents means ± s.e.m. of the densitometric scans for protein bands ($n=7$ rats in each group), normalized by comparison with Lamin B and expressed as a percentage of control. *** $P<0.001$ vs. control group, †† $P<0.01$ and ††† $P<0.001$ vs. Dex group, one-way analysis of variance with S–N–K post test.

of *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* in liver, kidney and placenta of GC-treated pregnant rats systematically. The results showed that GC downregulated the expressions of *Cyp24a1* and *Pxr*, while enhanced *Cyp27b1* and *Cyp3a1* expressions in liver of pregnant rats. Moreover, downregulation of *Cyp24a1* and upregulation of *Cyp27b1* and *Cyp3a1* were observed in kidney of GC-treated pregnant rats. Furthermore, the expressions of placental *Cyp24a1*, *Pxr* and *Cyp3a1* were enhanced by GC. Therefore, GC-mediated enhancement of liver and renal

Cyp3a1 expression, not *Cyp24a1*, may be the dominant reason for lower serum 1,25-(OH)₂D₃ levels in PE, which is supported by previous findings.¹⁹ Here both liver and renal *Cyp27b1* expressions were upregulated by GC, the reason of which may be the remove of 1,25-(OH)₂D₃-mediated inhibition on *Cyp27b1* promoter. It is also possible that secondary to the 1,25-(OH)₂D₃ degeneration, *Cyp27b1* upregulation observed here may occur as a compensatory response against 1,25-(OH)₂D₃ reduction. Further studies are planned to

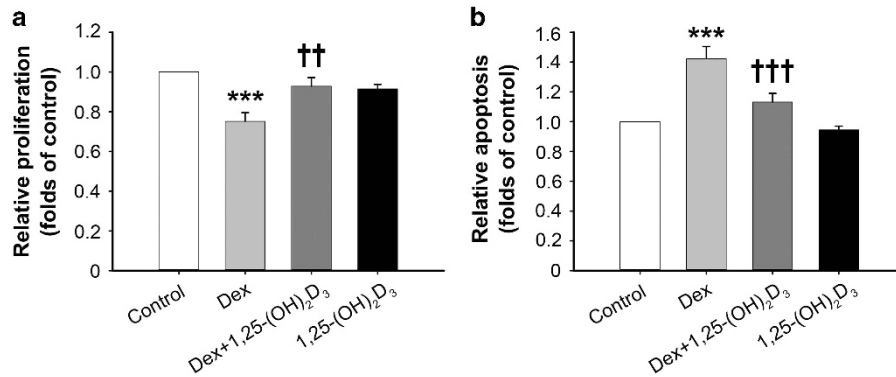


Figure 6 1,25-(OH)₂D₃ antagonizes the effects of glucocorticoid on trophoblast proliferation and apoptosis *in vitro*. HPT-8 cells were treated with dexamethasone (Dex; 10⁻⁷ mol l⁻¹), 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) or Dex (10⁻⁷ mol l⁻¹) plus 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) for 24 h in proliferation assay (a) and treated with Dex (10⁻⁶ mol l⁻¹), 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) or Dex (10⁻⁶ mol l⁻¹) plus 1,25-(OH)₂D₃ (10⁻⁹ mol l⁻¹) for 24 h in apoptosis assay (b). (a) 1,25-(OH)₂D₃ reverses the inhibitory effect of Dex on HPT-8 proliferation; and (b) 1,25-(OH)₂D₃ inhibits the promotion effect of Dex on HPT-8 apoptosis. Results are expressed as means ± s.e.m. from six independent experiments. ****P*<0.001 vs. control group, ††*P*<0.01 and †††*P*<0.001 vs. Dex group, one-way analysis of variance with S–N–K post test.

investigate the detailed reasons. Indeed, a previous report demonstrated that Dex induces the rat hepatic CYP27 gene expression.³² However, GC-mediated enhancement of placental *Cyp24a1* and *Pxr/Cyp3a1* expression may represent the main reason for local decrease of 1,25-(OH)₂D₃ levels in PE placenta. Plasma concentrations of 1,25-(OH)₂D₃ are tightly controlled via feedback regulation of renal CYP27B1 and CYP24A1, while placenta-specific methylation of the *CYP24A1* gene occurs in pregnancy.³³ Our findings may suggest that epigenetic regulation of CYP24A1 by GC has an important role in disturbing 1,25-(OH)₂D₃ bioavailability at the fetomaternal interface. Taken together, GC decrease 1,25-(OH)₂D₃ via modulating the expressions of *Cyp24a1*, *Cyp27b1*, *Pxr* and *Cyp3a1* in liver, kidney and placenta differentially.

In this study, Dex exposure contributed to hypertension, proteinuria, restricted placenta and fetus, placental abnormalities and renal impairment, all classic hallmarks of PE. Interestingly, 1,25-(OH)₂D₃ supplementation significantly improved PE-related manifestations in Dex-induced PE rats, suggesting the effectiveness of 1,25-(OH)₂D₃ in relieving symptoms of GC-mediated PE. Furthermore, 1,25-(OH)₂D₃ treatment ameliorated placental oxidative stress in GC-mediated rat PE. Placenta oxidative stress is the fundamental abnormality leading to PE.²⁹ We demonstrated here decreased placental oxidative stress after 1,25-(OH)₂D₃ treatment in Dex-mediated rat PE model that may support the protective effects of 1,25-(OH)₂D₃ against adverse actions of GC on placental function. HIF1A, overexpressed in hypoxic placentas of preeclamptic women, is implicated in the pathogenesis of PE.^{1,2,30} Importantly, we reported that 1,25-(OH)₂D₃ treatment could inhibit HIF1A overexpression in Dex-treated placenta. Here 1,25-(OH)₂D₃-mediated inhibition of HIF1A in Dex-treated placenta provides an important mechanistic explanation for the antagonism of 1,25-(OH)₂D₃ on GC in PE.

We demonstrated that Dex could inhibit trophoblast proliferation while promote trophoblast apoptosis *in vitro*. However, 1,25-(OH)₂D₃ antagonized the effects of Dex on trophoblast proliferation and apoptosis. These results demonstrate that 1,25-(OH)₂D₃ could antagonize the inhibitory effects of Dex on trophoblast development, supporting the protective actions of 1,25-(OH)₂D₃ against GC in placentation, providing another important mechanistic explanation for the counterregulation of 1,25-(OH)₂D₃ on GC in PE placenta.

By using clinical data, the present study first showed the involvement of GC/1,25-(OH)₂D₃ in human PE. Furthermore, relying on the

results gathered by using rat model and trophoblast model, the study provides evidence that GC/1,25-(OH)₂D₃ axis is involved in the pathogenesis of PE. However, PE is thought to be a multifactorial disorder, involving complicated mechanisms in its pathogenesis. A common pathophysiology of PE is endothelial dysfunction and angiogenic/angiostatic imbalance, involving sFlt-1, PlGF and sEng.³⁴ In addition, a recent report showed that PE onset is associated with visfatin polymorphisms and levels.³⁵ Interestingly, 1,25-(OH)₂D₃ receptor VDR has been demonstrated to be linked to the development of PE.³⁶ Therefore, this study just proposes one of the important mechanisms underlying the occurrence of PE. It is also noteworthy that there may be other causative factors in addition to dysregulated 1,25-(OH)₂D₃ that are responsible for Dex-induced PE, and may act in concert to stimulate placental oxidative stress, HIF1A overexpression and PE development reported in the present study. In the study, rat was selected as the animal model to study whether Dex could induce PE-related symptoms. It is reasonable for us to conclude that GC exposure is a potential cause for PE development in rat. However, further studies are needed to study other potential molecular pathways, and, meanwhile, confirm whether GC-induced PE is rodent specific or not. Clinically, Dex is used to improve hellp syndrome in PE women, but increasing evidence indicates that Dex has several side effects and even aggravates clinical conditions after withdrawal. In fact, our previous work confirmed that prior exposure to Dex could induce PE development in rats.⁶ Here we further found that Dex exposure induces PE via dampening 1,25-(OH)₂D₃ by using this model.

In conclusion, relying on the results gathered by using GC-mediated rat PE model and *in vitro* trophoblast model, the present study provides further evidence that GC/1,25-(OH)₂D₃ axis is involved in the pathogenesis of PE. This study proposes a common mechanism underlying the occurrence of PE, providing new insights into the understanding of PE. Our data suggest that the avoidance of stress/GC exposure and administration of 1,25-(OH)₂D₃ might be potential strategies to prevent PE, and also contribute to develop other potential therapeutic strategies in preventing PE.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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