Contribution of placental 11β-HSD2 to the pathogenesis of preeclampsia

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Abstract

Background and purposes Preeclampsia(PE) is associated with abnormal function of various factors in placentas. 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is abundantly expressed in placenta and controls the local availability of glucocorticoids. We aimed to elucidate the role of 11β-HSD2 in the pathogenesis of PE. Experimental approach Pregnant rats were administrated with 11β-HSD2 inhibitor carbenoxolone (CBX) subcutaneously or by placenta-targeted delivery system. The blood pressure, renal and placental morphology, placental blood flow and circulatory levels of fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PlGF) were subsequently examined. Cultured human trophoblasts were used to investigate the role of 11β-HSD2 in migration and invasion function and sFlt1 release in vitro. Key results Subcutaneous administration and placenta-targeted delivery of CBX resulted in the hallmark of PE-like features including hypertension, proteinuria, renal damages, elevated circulatory sFlt1 level and increased sFlt1/ PlGF in pregnant rats. These animals displayed reduced trophoblast invasion in uterus, impaired spiral artery remodeling and reduced placental blood flow. In vitro study showed that 11β-HSD2 dysfunction inhibited migration and invasion of the extravillous trophoblasts and promoted sFlt1 release in syncytiotrophoblasts. Mechanically, sFlt1 release induced by 11β-HSD2 dysfunction is mediated by enhancement of a disintegrin and metalloprotease (ADAM)17 transcription in placenta.

Experimental approach

Pregnant rats were administrated with 11β-HSD2 inhibitor carbenoxolone (CBX) subcutaneously or by placenta-targeted delivery system. The blood pressure, renal and placental morphology, placental blood flow and circulatory levels of fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PlGF) were subsequently examined. Cultured human trophoblasts were used to investigate the role of 11β-HSD2 in migration and invasion function and sFlt1 release *in vitro* .

Key results

Subcutaneous administration and placenta-targeted delivery of CBX resulted in the hallmark of PE-like features including hypertension, proteinuria, renal damages, elevated circulatory sFlt1 level and increased sFlt1/ PlGF in pregnant rats. These animals displayed reduced trophoblast invasion in uterus, impaired spiral artery remodeling and reduced placental blood flow. *In vitro* study showed that 11β-HSD2 dysfunction inhibited migration and invasion of the extravillous trophoblasts and promoted sFlt1 release in syncytiotro-

phoblasts. Mechanically, sFlt1 release induced by 11β-HSD2 dysfunction is mediated by enhancement of a disintegrin and metalloprotease (ADAM)17 transcription in placenta.

Conclusion and implication

Our data indicate that 11β-HSD2 is a crucial factor for placental development and maintaining balance release of pro- and anti-angiogenic factors in placenta. We reveal previously unrecognized role of placental 11β-HSD2 dysfunction in the pathogenesis of PE and immediately highlight innovative targets to counteract PE.

Keywords: preeclampsia; 11β-HSD2; glucocorticoids; ADAM17; placenta

Abbreviations:

11β-HSD, 11β-hydroxysteroid dehydrogenase; PE, preeclampsia; CBX, carbenoxolone; sFlt1, fms-like tyrosine kinase 1; PlGF, placental growth factor; ADAM, a disintegrin and metalloprotease; GC, glucocorticoids; IUGR, intrauterine growth retardation; ICG, indocyanine green; ICG-NPs, ICG-loaded nanoparticles; CSA, chondroitin sulfate A; ICG-CSA-NPs, ICG-NPs linked to CSA binding peptide; CBX-NPs, CBX-loaded nanoparticles; CBX-SCR, CBX-NPs linked to scramble peptide; CBX-CSA, CBX-NPs linked to CSA binding peptide; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; EVT, extravillous cytotrophoblasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GD, gestational day; BP, blood pressure; VEGF, vascular endothelial growth factor; DEX, dexamethasone; MT, mesometrial triangle; GREs, glucocorticoid response elements; MMP, matrix metalloprotein; MAP, mean arterial pressure; TBC, trophoblast; SA, spiral artery.

Bullet point summary

What is already known:

11β-HSD2 expression is downregulated in PE placentas, which is implicated be associated with IUGR in PE patients. The role of 11β-HSD2 in PE pathogenesis is unknown.

What this study adds:

Placental 11β-HSD2 is a crucial factor for placental development and maintaining balance release of pro- and anti-angiogenic factors in placenta.

Clinical significance:

We reveal the critical role of placental 11β-HSD2 in the pathogenesis of PE and uncover a novel target for therapeutic management of PE.

Introduction

Preeclampsia (PE) is a major human pregnancy-specific disorder that leads to maternal and fetal morbidity and mortality (Roberts & Cooper, 2001; Sibai et.al., 2005). The pathogenesis of PE remains largely unknown, however, it is widely accepted that PE is initiated by shallow invasion of trophoblasts into the uterine wall and thereafter results in abnormal placentation followed by increased release of placenta-produced factors into the maternal circulation(Seki, 2014; Szpera-Gozdziewicz & Breborowicz, 2014). These in turn cause dysfunction of the maternal endothelium, leading to the preeclamptic clinical symptoms. In the past decades, many efforts have therefore been made to uncover the critical factors relevant to placenta development and pathogenesis of PE in placenta.

Glucocorticoids (GCs) are involved in many events during pregnancy including embryo implantation, growth and development of the fetus and placenta as well as initiation of parturition (Fowden & Forhead, 2015; Zannas & Chrousos, 2015). The gestational tissues including placentas, fetal membranes and deciduas are the targets of GCs. Interestingly, most of target tissues of GCs usually express 11β-hydroxysteroid dehydrogenase (11β-HSD) which catalyzes the rapid metabolism of cortisol (corticosterone in rodents) to inert 11-keto derivatives (cortisone, 11-dehydrocorticosterone), thereby controlling local availability of active

GCs (Hunter & Bailey, 2015). This enzyme exists as, at least, two isoforms; 11β-HSD1 (which is bidirectional but favors reduction of inactive cortisone to active cortisol) and 11β-HSD2 (which operates essentially as a unidirectional dehydrogenase, converting cortisol to cortisone)(Burton & Waddell, 1999; Hunter & Bailey, 2015; Patel et al., 1999). In placenta, both of 11β-HSD1 and 2 have been identified, however, 11β-HSD2 is predominant (Brown et al., 1996; Burton & Waddell, 1999; Patel et al., 1999). Thus, 11β-HSD2 maintains a concentration gradient of active GC levels between the mother's and the fetus's compartments and constitutes a specific barrier protecting the fetus from the maternal GCs. Placental 11β-HSD2 expression and activity are found to be significantly downregulated in PE patients, which is associated with higher GC level in placentas of these patients(Alfaidy et al., 2002; Brown et al., 1996; Causevic & Mohaupt, 2007; He et al., 2014; Schoof et al., 2001). As GCs have a detrimental proapoptotic effect when they are in excess, downregulated 11β-HSD2 in placenta is implicated to be associated with intrauterine growth retardation (IUGR) in PE patients (Causevic & Mohaupt, 2007; Hofmann et al, 2001). To our knowledge, whether 11β-HSD2 dysfunction is involved in the pathogenesis of PE has not been reported. Of note, recent studies have shown that synthetic GCs can modulate placental trophoblast function including invasion and proliferation *in vitr* o (Kisanga et al., 2018; Mandl et al.,2006; Pavek et al., 2007). Thus, it would be of great interest to explore the effects of excessive GCs caused by placental 11β-HSD2 downregulation on pregnant outcome.

The objectives of the present study were to explore the potential role of placental 11β-HSD2 in the pathogenesis of PE and underlying mechanisms using animal and human models. Given that carbenoxolone (CBX) has been proven to be able to inhibit 11β-HSD2 activity both *in vitro* and *in vivo* (Duax et al., 2000; Lindsay et al., 1996; Sanna et al., 2016; Stewart, et al.,1990), we firstly investigated the effects of subcutaneous administration of CBX on the pregnant outcome in pregnant rats. Since a drug delivery system of placenta-targeted nanoparticles has recently been developed (Zhang et al., 2018), we then examined the effects of specific delivering CBX to placenta on pregnant outcome in rats in order to eliminate the potential effect of CBX on other organs which might affect the outcomes. Furthermore, we elucidated the potential mechanisms underlying 11β-HSD2 linked to PE pathogenesis using animal models and *in vitro* human tissue and cell models. Here, we revealed an important role of 11β-HSD2 in placental development and balance release of pro- and anti-angiogenic factors in placenta and immediately identified a novel target for therapeutic management of PE development.

Methods

Placental tissue acquisition

The placenta tissues were obtained at Maternity and Child Health Hospital of Pudong New District, Shanghai. Collections of tissues were performed with the approval of Ethics Committee of Medical Research of Maternity and Child Health Hospital of Pudong New District. Informed consent was obtained from all patients.

There were 43 pregnant women with normotension at term (37-40weeks) and 43 women with PE (36-40 weeks) included in the present study. Human subject data were summarized and indicated in Tab. S1. PE was defined as pregnancy-induced hypertension (blood pressure [?]140/90 mmHg) and proteinuria ([?]0.3 g/24 h or [?]1 + according to a routine urinalysis) in women who were normotensive before pregnancy and had no other underlying clinical problems such as renal disease. Pregnant women with multiple pregnancies, intrahepatic cholestasis of pregnancy and those with type 1, type 2 or gestational diabetes mellitus, a history of kidney disease, or autoimmune disease were excluded. All of the placentas were collected within 1 hour of cesarean birth, and six small pieces of tissues from separate lobules were randomly taken in each placenta. The tissues were washed with cold saline, immediately frozen in liquid nitrogen and then stored at -80.

Animal models

All animal procedures were carried out in accordance with the guidelines for the use of laboratory animals published by the People's Republic of China Ministry of Health (January 25, 1998) with the approval of the Ethical Committee of Experimental Animals of Second Military Medical University as well as Ethical Committee of Experimental Animals of Central South University.

Adult Sprague-Dawley rats (weight 200-220g) were obtained from Shanghai SLAC Laboratory Animal Co (Shanghai, China). The rats were housed in specific pathogen free animal house, and in social groups of 3–5 in a cage with regular light-dark cycles (lights on at 7:.m., lights off at 7:00p.m.) under controlled temperature (22±) and humidity (50±10%), and were given standard diet and water ad libitum. Breeding females were handled daily for 1 week. The female was placed with a male at 3:00p.m. The male was removed the following day at 8:00a.m. to its social group, and the female was transferred to a new cage. Pregnant rats with confirmation by microscopic analysis of vaginal smears for the presence of sperm. In the following experiments, a random number table method was used to assign animals. In the first experiment, pregnant rats were administered (s.c) with CBX (Sigma-Adrich, St.Louis, MO) at 0.6mg/kg,1.2mg/kg,2.4mg/kg or saline once every day from gestational day (GD) 7.5 to 17.5. In second experiment, pregnant rats were intravenously administrated with saline, CBX (0.6 mg/kg), CBX-loaded nanoparticles (CBX-NPs), CBX-NPs linked to scramble peptide (CBX-SCR), and CBX-NPs linked to chondroitin sulfate A (CSA) binding peptide (CBX-CSA) every two days from GD7.5 to GD 17.5. The dosage of CBX which was loaded in CBX-NPs, CBX-SCR and CBX-CSA was equivalent to 0.6 mg/kg. In the third experiment, pregnant rats were administered (s.c) with saline and dexamethasone (DEX,Sigma-Adrich) at the dosage of 0.1mg/kg once every day from GD7.5 to GD 17.5. The dosage of CBX and DEX were chosen based on the literature(Lindsay et al., 1996; Sanna et al., 2016) and our preliminary study. All the rats of above experiments were received measurement of blood pressure (BP) by the noninvasive tail-cuff system every two days from GD 7.5 to 19.5. On GD 20.5, the right carotid artery of the rats was catheterized for BP measurement under anaesthetization (urethane 800 mg/kg and alpha-chloralose 40 mg/kg, i.p.). Then, the animals were sacrificed by deep anesthetization, and maternal blood, pups, placentas, uterus and kidney tissues were collected. The above experiments were blinded to operators.

Measurement of blood pressure

In conscious rats, mean arterial pressure (MAP) was simultaneously monitored by noninvasive tail-cuff system (ALC-NIBP, Shanghai Alcott Biotech) as described by previously (Fraser et al., 2001; Zha et al., 2013). The rats were restricted by a recording chamber for 15 minutes before measurement of MAP to make the rat acclimate the holding device, and to keep rat quiet and comfortable during the whole process. In addition, retaining the ambient temperature of 37degC in chamber with controlled warming plate under the rats is beneficial to for vasodilation of the tail artery. The values resulted from averaging of at least six consecutive measurements, and MAP was measured every week or every 2 days after treatments.

On GD 20.5, arterial blood pressure measurement was performed as described previously (Zha et al., 2013). Briefly, the pregnant rats were anaesthetized (urethane 800 mg/kg and alpha-chloralose 40 mg/kg, i.p.), and then the right carotid artery was catheterized. The systolic blood pressure was determined by the PowerLab system (AD Instruments, Australia).

Doppler ultrasonography

Doppler ultrasonography was used to assess the uteroplacental and umbilicoplacental hemodynamic alterations on GD 19.5 based on the method described by Renaud et al.(2011). Briefly, the dams were anaesthetized using 3% isoflurane in air by face mask and hair in abdomen was removed by shaving followed by a chemical depilatory. Maternal breathing and heart rate were monitored during the measurement. For Doppler mode, a high frame rate 22 mHz scanhead (SL3116, Esaote Mylab twice, Italy) was used to obtain the waveform recording for offline analysis. For each dam, measurements were performed on 2-4 implantation sites those were located close to the bladder or ovary. In each implantation site, the Doppler waveforms of 2-3 spiral arteries (SAs), maternal canal and umbilical artery were recorded, and the peak systolic velocity (PSV) was measured. Finally, PSV of umbilical artery and maternal canal from two implantation sites and PSV of spiral arteries from four implantation sites were obtained in each pregnant rat.

Preparation of placenta-targeted nanoparticles and confirmation of the targets of nanoparticles

Placenta-targeted nanoparticles were prepared as described previously (Zhang et al., 2018). Briefly, the first step was preparation of CBX-loaded nanoparticles (CBX-NPs). CBX-NPs were synthesized from PLGA,

soybean lecithin, CBX and DSPE-PEG (2000)-COOH using a previously reported single-step sonication method (Zhang et al., 2018). The second step is conjugation of chondroitin sulfate A (CSA)-binding peptide to CBX-NPs (CBX-CSA) and conjugation of the scramble peptide (SCR) to CBX-NPs (CBX-SCR). The conjugation efficiency of the peptides was confirmed via the bicinchoninic acid (BCA) assay. The third step is to define characterization of the nanoparticles. The size, surface charge, polydispersity, and size distribution of the NPs were determined using a Beckman Coulter Delsa TM Nano C (Beckman, USA) at room temperature. The morphology and particle size of these NPs were further analyzed using a transmission electronic microscope (TEM, JEM-100XII, JEOL, Japan) with the negative stain method (Zhang et al., 2018). Finally, the encapsulation efficiency and loading efficiency of CBX in the NPs was determined. A dialysis experiment was performed to analyze CBX release from the different CBX formulations. The CSA-binding peptide-conjugated nanoparticles loaded with indocyanine green (ICG-CSA-NPs) and ICG without peptide conjugation (ICG-NPs) were prepared according to the same procedures.

Pregnant rats at GD13.5 were intravenously administered ICG-CSA-NPs and ICG-NPs (5mg/kg). Forty-eight hours after injection, the animals were anaesthetized, and ICG signal in uteri was imaged using a Bruker In-Vivo Xtreme system (Perkin Elmer) as described previously (Zhang et al., 2018). Then, the animals were sacrificed, the fetus, placenta, lung, kidney, heart, spleen and liver were collected and fixed by PAF(4%). The tissues were embedded into OCT and freezing sliced (15μm). ICG fluorescence images were captured using a Zeiss LSM 780 confocal microscope. This experiment was blinded to the operators.

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Placental tissues (0.1–0.2g) were homogenized in ice-cold 10 mM sodium phosphate buffer (pH 7.0) containing 0.25 M sucrose. The homogenate was used immediately in the following assays. Protein concentration was determined by BCA kit (BoGuang Ltd, Shanghai) protein assay kit with BSA as standard. The 11β-HSD dehydrogenase activity was determined by measuring the rate of conversion of cortisol to cortisone(Alfaidy et al., 2002; Schoof et al., 2001). Briefly, the assay tubes were added with placental tissue homogenate (containing 20–50 mg protein), cortisol (0.5 mM) and cofactor NAD or NADP (250 mM) and then incubation at 37 for 30 min. Cortisol and cortisone concentration was determined by using liquid chromatography tandem mass spectrometry as described previously (Fahlbusch et al., 2013;2015) and performed by APT. Co. Ltd (Shanghai, China).

Analysis of protein and creatinine concentration in urine

The urine was collected on GD 18.5–19.5 from the dams housed individually in metabolic cages in the absence of food. Total protein concentration was measured by using a protein assay (BCA protein assay kit) and was normalized against total creatinine concentration (Westang Biotech Co Ltd, Shanghai, China).

Histological analysis of kidneys and placentas

Kidneys and placentas of rats were harvested from the dams on GD 20.5. The tissues were then fixed in 10% buffered formalin overnight at 4°C. Processed tissues were embedded in paraffin. Serial sections (5 μm) were cut from each uteroplacental unit and serial sections (3 μm) were cut from each kidney. The sections of kidney were deparaffinized, rehydrated and stained with Hematoxylin and Eosin and periodic acid-Schiff for histopathological assessment of renal alterations. The degree of glomerular pathology was assessed by a blinded observer based on criteria and scoring methods adapted from prior studies (Li et al., 2007; Strevens et al., 2003) and after consultation with an experienced renal pathologist at Xiangya Hospital. For each kidney, 20 glomeruli were individually scored and the severity of glomerular pathology was determined from the mean score for each kidney. The sections of placentas were de-waxed, hydrated, and stained with Hematoxylin and Eosin, and the morphology was examined by analyzing 10 fields in 5 sections from 3 placentas in each rat under light microscopy.

Identification of trophoblasts and smooth muscle cells and assessment of interstitial trophoblast invasion

Trophoblasts and smooth muscle cells in placenta and uterus were identified by immunocytochemistry. Im-

munocytochemistry were performed as described previously (Bridgman, 1948; Cotechini et al., 2014; He et al., 2014). Paraffin sections (5 μm) of the rat placentas were prepared, de-waxed, and hydrated, and endogenous peroxides were quenched with 0.3% H_2O_2. After heat-induced antigen retrieval, the sections were incubated with antibodies against cytokeratin (1:500; Dako, Cat# M515;Clone ID, AE1/AE3) or α-actin (1:500; Abcam, Cat# ab179467; RRID: AB_2737344; Clone ID: EPR16769) overnight at 4. Negative controls consisted of the sections in which the primary antibody was substituted with an equal concentration of mouse IgG (DAKO). The bound antibodies were detected with the biotin–streptavidin–peroxidase system (UltraSensitive-SP-kit, MaiXin Biotechnology, Fuzhou,) using diaminobenzidine (Sigma-Aldrich,) as chromogen. Counterstaining was performed with hematoxylin.

Assessment of interstitial trophoblast invasion was performed on slides scanned at 20× using Nikon ImageScope system and software. Interstitial trophoblast invasion was calculated by imageJ system as the total area occupied by cytokeratin-positive interstitial trophoblast cells within the mesometrial triangle (MT). Only sections exhibiting the "break through" of interstitial trophoblast cells through the giant cell layer were used to quantify interstitial invasion for consistency (Bridgman, 1948; Cotechini et al., 2014).

Immunofluorescent analysis

Paraffin sections (5μm) of placentas were rehydrated and incubated in sodium citrate buffer (pH 6.0) for 10min to retrieve antigens. After blocking in 3% H_2O_2, the slides were incubated with antibody against laminin (1:200; Abcam, Cat# ab11575; RRID: AB_298179) at 4°C overnight. After washing 3 times with PBS, the sections were incubated with Alexa fluor 488 donkey anti-mouse IgG(H+L) (1:1,000 dilution) for 2h in dark at room temperature. Then the slides were mounted with cover glass by mounting solution, and the picture was taken under Zeiss LSM 780 confocal microscope (Carl Zeiss Inc., Jena, Germany).

Placental explants and cell culture

Placental explants culture was performed as described previously(Hu et al., 2015). Briefly, cotyledons from the central part of the placenta were removed under sterile conditions. Explants (approximately 30-50mg) were dissected and cultured in 24-well plates containing DMEM/F12 (Gibco, Thermo Fisher Scientific, Rockford, IL) with 10% FCS at 37 in 5% CO_2-95% air. Culture medium was replaced every 24h. On day 4, explants were treated with increasing concentration of cortisol (10^{-8}-10^{-6}M) in absence and presence of CBX (10^{-6}-10^{-5}) in FCS-free DMEM/F12 for 24h. Culture media and tissues were collected and stored at -80oC.

Primary placental cell cultures were performed by a modified Kliman's method (He et al., 2014). Briefly, cotyledons were removed from the maternal side and dispersed with trypsin (Sigma-Aldrich) and deoxyribonuclease I (Sigma-Aldrich). A purified fraction of cytotrophoblasts was obtained following Percoll (GE healthcare, Uppsala, Sweden) gradient centrifugation. The cells were then plated into 12-well plates (Corning-Costar Inc., Corning, NY) at a density of $1.2x10^6$/well and grown in phenol red-free DMEM (Gibco) with 10% FCS at 37 in 5% CO_2-95% air. After 48 h of plating, cells were treated with cortisol (Sigma-Aldrich) in absence and presence of CBX at the indicated concentrations for 24h. Control cultures were maintained without additives. Each treatment was performed in triplicate for each preparation of cells. Culture media and cells were collected and stored at -80oC.

HTR8, an extravillous cytotrophoblasts (EVT) cell line, was a gift from Prof. Charles H. Graham (Queen's University, Canada). HIPEC65, a cell line established from a primary culture of transformed by T-SV40, was kindly provided by Dr. Thierry Fournier (INSERM, France). The above cells were grown in DMEM/F12 supplemented with 10% FCS, 100 UI/ml penicillin and 100 mg/ml streptomycin at 37oC in 5% CO_2-95% air.

RNA interferences

The siRNAs for ADAM17 and 11β-HSD2 were designed and synthesized by GenePharma Corporation (Shanghai, China). Control siRNA was scrambled sequence without any specific target. The sequences for targeting human negative control are: sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. The sequences for targeting human ADAM17 are: sense: 5'-CACAUGUAGAAACACU

-3', antisense: 5'-AGUAGUGUUUCUACAUGUIGTT-3'. The sequences for targeting human 11β-HSD2 are: sense: 5'-CCACUUUCCGUAGCUGCAUTT-3', antisense: 5'-AUGCAGCUACGGAAAGUGGTT-3'; Transfection of siRNA was performed by using Lipofectamine ™ 3000 (Invitrogen, Thermo Fisher Scientific, Rockford, IL) as described previously (He et al., 2014). The efficiency of ADAM17 and 11β-HSD2 were determined by western blotting.

In vitro **trophoblast migration and invasion assessment**

The migration function of HTR8 and HIPEC65 cells was examined in Boyden chamber with an 8-μm pore size (Corning-Costar)(Chen, 2005). The cells were seeded into the top chamber, and were allowed to migrate toward the bottom chamber containing conditioned media in which cortisol (10^{-6}M) was added. After 24h incubation, cell moved to the underside of the membrane were stained with DAPI (Beyotime Biotechnology). The number of the cells on the membrane from 3 random fields at ×200 magnification was counted under the microscope. In some cases, scramble siRNA and 11β-HSD2 siRNA mixed with Lipofectamine™3000 were added into the cells in order to knockdown of 11β-HSD2. After 12h incubation, the culture media were changed into fresh media containing cortisol (10^{-6}M). After incubation for 24h, cell moved to underside of the membrane were stained with DAPI. The number of the cells on the membrane was counted as described above.

Invasion function was assessed by the ability of cells to digest and invade the Matrigel-coated 8 μm pore size polycarbonate membrane Transwell inserts (Corning-Costar) as described previously (Yang et al., 2012). Briefly, HIPEC65 or HTR8 cells seeded in the inserts and treated with cortisol for 24h. Noninvaded cells on the top of the filter were scraped off using a cotton swab, and cells were fixed and stained with DAPI for microscopic analysis. Invaded cells from 3 random fields at ×200 magnification were counted under the microscope. In some cases, cells were treated with scramble siRNA and 11β-HSD2 siRNA mixed with Lipofectamine™ 3000. After 12-h incubation, the culture media were changed into fresh media containing cortisol (10^{-6}M). Then the cells were assessed for invasion activity.

Quantitative real-time RT-PCR and western blotting analysis

Extraction of total RNA of tissues and cells, quantitative real-time PCR and western blotting were performed as described previously (He et al., 2014). For quantitative real-time RT-PCR, total RNA of placental tissues and cells were extracted by TRIzol reagent (Invitrogen). 2 μg RNA was reverse transcribed to generate cDNA by superscript reverse transcriptase (Invitrogen). Quantitative real-time PCR was carried out using MiniOpticon Real-Time PCR Detection System (BioRad, Hercules, CA). Real-time PCR reaction solution consisted of 2.0 μl diluted cDNA, 0.2 μM of each paired primer and 1×PCR Master Mix (TaKaRa, Otsu, Japan). SYBR Green (Roche Ltd, Basel, Switzerland) was used as detection dye. Amplification of the housekeeping genes β-actin was measured for each sample as an internal control for sample loading and normalization. The primers used were listed as followings: sFlt1 (NM_001159920), sense: TTGGGACTGTGGGAAGAAAC; anti-sense: TTGGAGATCCGAGAGAAAACA. β-actin (NM_001101), sense: TGTGTTGGCGTACAGGTCTTTG; anti-sense: GGGAAATCGTGCGTGACATTAAG. The temperature range to detect the melting temperature of the PCR product was set from 60–95. The specificity of PCR products was examined by melting curve at the end of the amplification and subsequent sequencing. To determine the relative quantitation of gene expression for both sFlt1 and β-actin genes, the comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta^{\hat{}}\tau}$) was used.

For the western blotting analysis, 70 mg of tissues were homogenized in cold RIPA lysis buffer containing protease inhibitor cocktail tablet (Roche, Indianapolis, IN) and primary cultured cells were scraped off the plate in the presence of RIPA lysis buffer containing protease inhibitor cocktail tablet. The protein concentration was quantified by BCA kit. Then lysates were quickly sonified in ice bath, boiled 5 min at 100^{o}C, and stored at -80^{o}C. 30 μg of protein samples were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After blockage in 5% skim milk powder in 0.1% Tris-buffered saline/Tween 20 (TBST), membranes were incubated with primary antibodies for 11β-HSD1 (Santa Cruz, Cat# sc-19259, RRID: AB_2119513, Clone ID: C-17), 11β-HSD2 (1:2000; Abcam, Cat# ab80317, RRID: AB_1658782C), a

disintegrin and metalloproteinase (ADAM)17 (1:2000; Abcam, Cat# ab2051 RRID: AB_302796) , ADAM10 (1:2000; Abcam, Cat# ab124695 RRID:AB_10972023, Clone ID: EPR5622) and GAPDH(1:2000; Abcam, Cat# ab181602, RRID:AB_2630358, Clone ID: EPR16891) at 4 °C overnight. Then, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody (Proteintech Inc,WuHan,China) for 1 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence Western blotting detection system (Santa Cruz). The chemiluminiscent signal from the membranes was quantified by image J software. To control sampling errors, the ratio of band intensities to GAPDH was obtained to quantify the relative protein expression level.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of fms-like tyrosine kinase 1 (sFlt1) and placental growth factor (PlGF) were determined with specific ELISA kit from Westang Biotech Co Ltd (Shanghai, China). A commercial ELISA kit from Cayman Chemical (catalogue #: 501320) was used to measure the corticosterone levels in rat serum and placental tissues according to the manufacturer's instruction.

Determination of ADAM17 gene promoter activity

Wild type and mutant GRE ADAM17 promoter (-150 to +200bp) were cloned into pGL3-luciferase reporter vector (GenePharma Corporation). Primary trophoblasts were transfected with the pGL3-luciferase reporter DNA and pRL-TK-Renilla-luciferase plasmid (Promega Corp., Beijing, China) using Lipofectamine^{TM} 3000 for 12h (He et al., 2014). Cells were then treated with DEX (10^{-6}M) for 24h. Luciferase assays were carried out using the dual luciferase assay kit (Promega).

Statistical analysis

All data are expressed as mean +- SEM. Statistical analyses were performed using IBM SPSS Statistics 20. Normal distribution was assessed by Shapiro-Wilk test. Statistical significance was determined according to sample distribution and homogeneity of variance. Statistical comparisons between two groups were determined by two-tailed Student's t test and Mann-Whitney U test. One-way ANOVA following by Bonferroni's post hoc test, two-way ANOVA with simple effect test and Kruskal-Wallis test with Dunn's post hoc test were performed for comparisons among multiple groups. $P < 0.05$ was considered statistically significant. The group sizes of the individual experiments were chosen based on our preliminary experiments. It was shown that the significance was reached when the number in each group was 3 or 4. In our study, n =5-8 fulfilled the statistical requirements. The exact statistical method and exact group size for the individual experiments are shown in the corresponding figure legends. No samples were excluded from the analyses.

Materials

Carbenoxolone disodium salt, dexamethasone-21-phosphate disodium salt and cortisol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbenoxolone disodium salt and dexamethasone-21-phosphate disodium salt were dissolved in sterile saline to achieve the final concentration as indicated. Cortisol was dissolved in ethanol as a stock and then diluted by culture media to final concentration.

Results

Σψστεμις αδμινιστρατιον οφ 11β-ΗΣΔ2 ινηιβιτορ ρεσυλτς ιν ΠΕ-λικε φεατυρες ιν πρεγναντ ρατς

Subcutaneous administration of CBX (0.6-2.4mg/kg) from GD7.5 to GD17.5 resulted in a decrease in 11β-HSD2 expression and activity in the placentas (Fig.1A). The significance was achieved in CBX (1.2 and 2.4 mg/kg) groups compared with vehicle group. In contrast, 11β-HSD1 expression level in the placentas was not significantly differed among vehicle and CBX groups (supplemental Fig.S1). As expected, a significant increase in placental corticosterone level was exhibited in CBX (1.2 and 2.4 mg/kg) groups (Fig.1B). Maternal circulatory level of corticosterone was significantly increased from GD 15.5 and reached the peak on GD 19.5 in the rats with CBX (1.2 and 2.4 mg/kg) treatment (Fig.1C).

The pregnant rats with CBX treatment (1.2 and 2.4 mg/kg) displayed PE-like features. As shown in Fig1D,

MAP of these rats was significantly elevated from GD 15.5 compared with control rats. They also showed a significant increase in SBP measured on GD 20.5 (Fig.1E). The protein/creatinine in urine was also elevated in the rats with CBX treatment (1.2 and 2.4 mg/kg) (Fig.1F). Histology analysis of kidney showed mesangial hypercellularity and occlusion of capillary loops and the urinary space in the glomeruli (Fig.1G) and a higher histopathological score in CBX groups compared with vehicle group (Fig.1H). IUGR was found in CBX (1.2 and 2.4 mg/kg) groups as evidenced by decreased fetal weight (Fig.1I). Moreover, a significant increase in circulatory sFlt1 level and sFlt1/ PlGF ratio occurred in CBX (1.2 and 2.4 mg/kg) groups (Fig.1J), but circulatory PlGF and VEGF levels were not significantly changed in these animals (supplemental Fig.S2).

Σπεςιφις ινηιβιτιον οφ πλαςενταλ 11β-ΗΣΔ2 αςτιτψ αλσο λεαδς το ΠΕ-λικε φεατυρες ιν πρεγναντ ρατς

As mentioned, the placenta-targeted drug delivery system has just recently been developed in pregnant mice (Zhang et al., 2018). To explore whether this system works in rats, the targets of placenta-targeted nanoparticles in the pregnant rats were examined. At 48h after administration of ICG-CSA-NPs, ICG signals were mainly accumulated in the placenta (supplemental Fig S3). In contrast, very few ICG signals were found in the placentas of the rats with administration of ICG-NPs. Moreover, ICG fluorescence was localized in labyrinth zone of the placentas in the group of ICG-CSA-NPs. These data suggest that this system also specifically target to the labyrinth zone of placenta in rats.

Then, we studied the effects of administrating this system loaded with CBX (0.6mg/kg) on pregnant outcome. We found that specific delivery of CBX to placenta, ie, administration of CBX-CSA, could also lead to a significant decrease in 11β-HSD2 expression and activity (Fig.2A) whilst had no impact on 11β-HSD1 expression level (supplemental Fig.S4). Administration of CBX-NPs and CBX-NPs-linked to scramble peptide (ie, CBX-SCR) did not affect 11β-HSD1 and 11β-HSD2 expression (Fig.2A&supplemental Fig.S4). The levels of corticosterone in placentas were significantly increased in CBX-CSA group whereas they were not significantly changed in CBX, CBX-NPs and CBX-SCR groups compared with vehicle (Fig.2B). Maternal circulatory levels of corticosterone were significantly elevated in CBX-CSA group from GD 15.5 compared with vehicle group (Fig.2C). PE-like features also occurred in CBX-CSA group. As shown in Fig.2D&E, MAP was significantly elevated from GD15.5, and consistently, SBP measured on GD 20.5 was also elevated. These rats showed an increase in urine protein/creatinine (Fig.2F) and abnormal morphology in glomeruli (Fig.2G&H). Administration of CBX-CSA also suppressed fetal growth as evidenced by a significant decrease in fetal weight (Fig.2I) and led to a significant increase in sFlt1 level and sFlt1/PlGF in maternal circulation (Fig.2J), but did not affect circulatory PlGF and VEGF levels (supplemental Fig.S5). The rats of CBX-NPs and CBX-SCR groups did not display abnormal BP and renal morphology as well as abnormal sFlt level and sFlt1/PlGF in maternal circulation.

Systemic administration of synthetic glucocorticoid induces PE-like features in pregnant rats

We then explored whether excess GCs mediate PE-like features caused by placental 11β-HSD2 downregulation. As shown in Fig.3A&B, administration of DEX from GD 7.5 and 17.5 could result in a significant increase in SBP and protein/creatinine in urine. The rats with DEX treatment also displayed abnormal renal morphology including hypercellularity in mesangial and occlusion of capillary loops and the urinary space (Fig.3C) and a higher histopathological score compared with control rats (Fig.3D). As expected, elevated circulatory sFlt1 level and decreased fetal weight were also found in DEX-treated dams (Fig.3E&F).

Πλαςενταλ 11β-ΗΣΔ2 δοωνρεγυλατιον ρεσυλτς ιν δεφισιεντ ινασιον οφ ιντερστιτιαλ τροπηοβλαστς, ιμπαιρεδ ενδοασςυλαρ ΣΑ ρεμοδελινγ ανδ ρεδυςεδ πλαςενταλ βλοοδ φλοω

The area of interstitial trophoblast invasion in MT was measured for evaluation of trophoblast invasion in uterus (Fig.4A). It was significantly reduced in CBX (1.2 and 2.4 mg/kg) groups compared with vehicle group (Fig.4B). Less trophoblasts infiltrated in SA higher α-actin staining intensity in SA were found in CBX (1.2 and 2.4 mg/kg) groups compared with vehicle group (Fig.4C). Abnormal morphology of placenta was found in CBX (1.2 and 2.4 mg/kg) groups as evidenced by disorganization in the labyrinth zone (Fig.4D) and reduced laminin staining intensity compared with vehicle group (Fig.4E).

Specific delivery of CBX to placenta also led to impaired interstitial trophoblast invasion and SA remodeling. The area of interstitial trophoblast invasion was significantly decreased in CBX-CSA group compared with vehicle. Higher α-actin staining intensity and less trophoblast infiltration in SA were displayed in CBX-CSA group (Fig.5C). Obvious morphological disturbance with disorganization in the labyrinth zone and reduced laminin staining intensity in the placentas were also exhibited in CBX-CSA group (Fig.5D&E). The animals of CBX-NPs and CBX-SCR groups did not show abnormal interstitial trophoblast invasion, trophoblast infiltration and placental morphology.

The placental blood flow was examined by using Doppler ultrasonography (Fig.6A&B). The placental blood flow was significantly decreased in the pregnant rats with CBX (1.2 and 2.4mg/kg) treatment as evidenced by a significant reduction in peak systolic velocity (PSV) of SA, maternal canal and umbilical artery compared with control rats (Fig.6C). As shown in Fig.6D, CBX-CSA group also displayed a significant decrease in PSV of SA, maternal canal and umbilical artery. The rats of CBX-NPs and CBX-SCR groups did not show reduced PSV of SA, maternal canal and umbilical artery compared with control rats.

11β-ΗΣΔ2 δοωνρεγυλατιον συππρεσσεστροπηοβλαστ μιγρατιον ανδ ινασιον ανδ προμοτες σΦλτ-1 ρελεασε ιν τηε μοδελς οφ ςυλτυρεδ ηυμαν τροπηοβλαστς

To extent our rodent studies, we investigate the role of 11β-HSD2 in PE pathogenesis using human placental tissue and trophoblast models*in vitro* . At first, we examined whether 11β-HSD2 is involved in regulation of the migration and invasion of extravillous trophoblast (EVT) cell lines, HTR8 and HIPEC60. It was found that cortisol (10^{-9}M-10^{-6}M) treatment did not significantly affect the migration and invasion function (supplemental Fig. S6). However, cortisol (10^{-6}M) significantly suppressed migration and invasion in the cells transfected with 11β-HSD2 siRNA (supplemental Fig.S6&7).

We then examined the role of 11β-HSD2 in regulation of PlGF and sFlt1secretion in human placental explants and cells. In the presence of 11β-HSD2 inhibitor CBX (10^{-6}-10^{-5}M), cortisol (10^{-6}M) treatment significantly increased sFlt1 release (Fig.7A) but did not significantly affect PlGF secretion in placental explants (supplemental Fig. S8). Cortisol treatment also significantly increased sFlt1 output in cultured primary syncytiotrophoblasts with CBX treatment (Fig.7B). In the syncytiotrophoblasts transfected with 11β-HSD2 siRNA, cortisol could also promote sFlt1 secretion (Fig.7C). We then examined the effect of cortisol on sFlt1 mRNA expression in order to study whether cortisol promotes sFlt1 output via modulating sFlt1 transcript. However, cortisol treatment had no impact on sFlt1 mRNA expression (supplemental Fig. S8).

11β-ΗΣΔ2 δοωνρεγυλατιον λεαδς το υπρεγυλατιον οφ ΑΔΑΜ17 εξπρεσσιον

Increased sFlt1 release is associated with either increased sFlt-1 transcripts or increased shedding of sFlt1 from membrane Flt1. ADAM10 and ADAM17 are the sheddases for shedding of sFlt1form Flt1 (Zhao et al., 2010). Cortisol (10^{-6}M) treatment significantly increased ADAM17 but not ADAM10 protein expression in placental explants in the presence of CBX (supplemental Fig.S9). Further, we found that cortisol (10^{-6}M) treatment significantly enhanced ADAM17 expression in primary syncytiotrophoblasts transfected with 11β-HSD2 siRNA (Fig.7D). We then confirmed that sFlt1 release was mediated by ADAM17 by showing that sFlt1 secretion was significantly decreased in syncytiotrophoblasts with ADAM17 knockdown compared with the cells transfected with scramble siRNA (Fig.7E).

In the classic model of steroid hormone action, upon binding a ligand steroid receptors interact with the DNA at specific hormone response elements to modulate gene transcription. Bioinformatic analysis shows that there are seven putative glucocorticoid response elements (GREs) in the upstream (2kb) of*ADAM17*gene. We therefore investigated whether GCs have an impact on *ADAM17*gene transcriptional activity and the role of these elements in GC regulation of *ADAM17* transcription in placental cells. As shown in Fig.7F, DEX (10^{-6}M) significantly increased transcriptional activity of ADAM 17 reporter. When these elements were mutated, DEX did not affect transcriptional activity of the mutant GRE ADAM17 reporter in primary syncytiotrophoblasts.

In the animal models, we found that systemic administration of CBX (1.2 and 2.4 mg/kg) led to a significant increase in ADAM17 expression in the placenta compared with vehicle treatment (Fig.8A). Specific inhibition of placental 11β-HSD2 by administration of CBX-CSA could also significantly promote ADAM17 expression in placentas compared with vehicle control (Fig.8B). Administration of CBX-NPs and CBX-SCR had no effect on ADAM17 expression in placenta.

We then examined ADAM17 expression in human placental samples recruited from a Shanghai hospital. It was shown that ADAM17 levels were significantly increased in PE placentas compared with normal ones (Fig.8C). In addition, we also determined 11β-HSD2 protein expression in placenta and confirmed that placental 11β-HSD2 level was significantly decreased in PE patients.

Discussion

In the present study, we demonstrated for the first time that placental 11β-HSD2 dysfunction leads to hallmark of PE features, deficient trophoblast invasion, abnormal placentation and reduced placental blood flow in pregnant rats. Further, using cultured human placental cells, we revealed that 11β-HSD2 downregulation suppresses migration and invasion of EVTs and promotes sFlt1 release. These data strongly indicate that dysregulation of placental 11β-HSD2 play critical roles in PE development.

Prior studies have demonstrated that placental 11β-HSD2 expression and activity are suppressed by systemic administration of CBX (Lindsay et al., 1996; Sanna et al., 2016). Similarity, we showed that systemic administration and placenta-targeted delivery of CBX resulted in a decrease in 11β-HSD2 expression and activity in placentas. In accordance with that 11β-HSD2 converts corticosterone into 11-hydroxycorticosterone, an increase in corticosterone concentration in placenta was exhibited in the pregnant rats with administration of CBX (1.2mg/kg & 2.4mg/kg) or CBX-CSA, indicating that inhibition of 11β-HSD2 lead to excess GC in placenta. As expected, systemic administration of CBX could lead to increased corticosterone concentration in maternal circulation because 11β-HSD2 in maternal peripheral tissues besides placenta was inhibited. Of note, our data also demonstrated that specific inhibition of placental 11β-HSD2 by CBX-CSA could increase corticosterone level in maternal circulation in late gestation. It indicates that a large amount of corticosterone is metabolized by the placenta in late gestation, and confirms that placental 11β-HSD2 constitutes a barrier for glucocorticoids across placenta.

As mentioned, local GC concentration in tissues is controlled by 11β-HSD1 and 11β-HSD2(Hunter & Bailey, 2015). Some studies have demonstrated that 11β-HSD1 in fetal membranes regulates prostaglandin synthesis and metabolism through control of local GC concentration (Li et al., 2014; Mirazi et al., 2004). Since no specific GC receptor antagonist is commercially available, we investigated the effect of DEX administration on pregnant outcome in order to mimic the effects of excess GCs. PE-like features including hypertension, nephropathy and increased level of sFlt1 in maternal circulation were induced in the pregnant rats with DEX administration. In the cultured human placental cells, reduced migration and invasion and increased sFlt-1 secretion were induced by cortisol in the cells with 11β-HSD2 knockdown. Together, it strongly suggests that excess GCs mediate the development of PE caused by placental 11β-HSD2 downregulation.

Maternal endothelial dysfunction in PE mainly results from the factor(s) released by the ischemic placenta. Among them, imbalance release of sFlt1 and PlGF plays critical roles in PE development (Seki, 2014; Szpera-Gozdziewicz & Breborowicz, 2014). Many studies have shown that sFlt1/PlGF ratio is markedly increased in PE patients and in women with pre-existing conditions predisposing or mimicking PE (Hodel et al., 2019; Saleh et al.,;2017; Zeisler et al., 2016). In consistence with these studies, we demonstrated that an increased circulatory sFlt-1/PlGF ratio was found in the rats with PE-like features induced by placental 11β-HSD2 downregulation. Moreover, we found that 11β-HSD2 downregulation promoted sFlt1 release but did not affect PlGF release, indicating that increased sFlt1 secretion from placenta is one of the mechanisms of the PE development induced by placental 11β-HSD2 dysfunction.

In the present study, we demonstrated that 11β-HSD2 modulates sFlt1 release through proteolytic process of Flt1 in placenta. Membrane anchored metalloproteases of the ADAM family assume central functions in the living cell by the controlled cleavage and release of biologically active proteins and peptides from

the membrane surface (Jones et al, 2016; Zunke &Rose-John,2017). ADAM10 and ADAM17, the well characterized members of the ADAM family, have been shown to mediate the release of a number of cytokines and various receptors from cell membrane in human placentas (Hung et al., 2008; Ma et al.,2011; Yang et al., 2012; Zhao et al., 2010). Our previous study has shown that ADAM10 can mediate sFlt1 release in human placenta (Hu et al., 2015). In the present study, we confirmed that ADAM17 is also involved in sFlt1 secretion in placenta. The regulatory mechanisms responsible for ADAM10 and ADAM17 expression in placentas remain to be elusive. In the present study, we firstly proved that excess GCs caused by 11β-HSD2 dysfunction could upregulate ADAM17 but not ADAM10 mRNA expression. As bioinformatic analysis shows seven putative GREs in *ADAM17* gene promoter, we then demonstrated that these elements conferred GCs upregulation of*ADAM17* gene transcription using the molecular biological approaches. Of note, Yang et al (2012) have proposed that increased expression of ADAM10 and ADAM17 is associated with oxidative stress, a common phenomenon in PE placenta. Interestingly, our previous study has shown that decreased H_2S production contributes to increased ADAM10 expression in PE placentas (Hu et al., 2015). The present study demonstrated that excessive GCs could promote ADAM17 expression in placentas. All together, it may indicate that ADAM10 and ADAM17 are the final paths of various factors linked to PE development.

As mentioned, the first stage of PE is attributed to impaired functions of EVTs. In the cultured human cell lines of EVTs, we demonstrated that 11β-HSD2 regulates migration and invasion function of EVTs. In the animal model, we showed that placental 11β-HSD2 maintains normal interstitial trophoblast invasion, endovascular SA remodeling, placental blood flow and placental morphology, indicating that placental 11β-HSD2 is crucial for normal placentation and placental development. Of note, a number of prior studies have proposed that IUGR in rodents caused by administration of CBX is attributed to excess active GCs because GCs have been known to inhibit fetal growth (Murphy et al., 2012; Welberg et al., 2000). In human studies, it has been assumed that downregulation of placental 11β-HSD2 is associated with IUGR because GCs have detrimental effects on fetal growth (Causevic & Mohaupt, 2007; Hofmann et al, 2001). Our findings indicate that abnormal placentation and reduced placental blood flow caused by excess GCs due to 1β-HSD2 dysfunction also contribute to IUGR.

The limitation of this study is that we did not elucidate the mechanisms responsible for excessive GC suppression of migration and invasion function in EVTs. It is known that invasion function is associated with expression of MMPs in EVTs, in particular, MMP2 and MMP9 are crucial for invasion function of EVTs (Peng et al., 2016; Zhang et al., 2013). Interestingly, some studies have shown that synthetic GCs can regulate MMP2 and MMP9 expression in trophoblast cell lines (Mandl et al., 2006; Pavek et al., 2007). More recently, Kisanga et al (2018) have shown that synthetic GCs can suppress migration and invasion of EVTs and lead to more than 3,000 genes changed, suggesting that GCs modulate EVT function via various signaling pathways. Nevertheless, the mechanisms underlying GCs regulation of trophoblast invasion remains to be further investigated.

In conclusion, our study has demonstrated a previously unrecognized role of 11β-HSD2 in placental development and function. Excessive active GCs caused by placental 11β-HSD2 downregulation can lead to impaired invasion function of EVTs, abnormal placentation and increased sFlt1 release from placenta via elevated ADAM17 expression. Thus, our data provide *in vivo* evidence that dysregulation of placental 11β-HSD2 plays a critical role in pathogenesis of PE. It is therefore valuable to develop strategies to treat PE by using specific activators of 11β-HSD2 or specific inhibitor to block GCs in placenta.

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Figure legends

Φιγ. 1. ˜ΒΞ τρεατμεντ συππρεσσες πλαχενταλ 11β-ΗΣΔ2 εξπρεσσιον ανδ αχτιιτψ ανδ ινδυχες τηε φεατυρες οφ ΠΕ ιν πρεγναντ ρατς. Pregnant rats were administrated (s.c) with CBX at dosage of 0.6,1.2 and 2.4mg/kg from GD7.5 to GD17.5. Control rats were injected with same volume of saline. Urine was collected from GD18.5 to GD19.5. After determination of arterial BP, the rats were sacrificed on GD20.5 for collection of blood and tissues. A, 11β-HSD2 expression and activity in placentas. B, corticosterone levels in placentas. C, corticosterone levels in maternal circulation. D, MAP measured from GD 7.5 until GD 19.5. E, SBP measured on GD20.5. F, protein/creatinine in urine. F, morphology of glomeruli stained by hematoxylin and eosin (400x). G, histopathological score of glomerular pathology. H, fetal weight measured on GD 20.5. I, the circulatory sFlt-1 level and sFlt-1/PIGF. Data are expressed as mean±SEM (n=8). Statistic was performed by one-way ANOVA(A,C,D,E,F,H&J) and Kruskal-Wallis test (B&I). MAP: mean arterial pressure; SBP: systolic blood pressure.

Fig. 2. Placenta-targeted delivery of CBX leads to hallmark of PE in pregnant rats. Pregnant rats were intravenously injected with CBX, CBX-NPs, CBX-SCR and CBX-CSA every two days from GD7.5 to GD17.5. Control rats were injected with same volume of saline. The dosage of CBX was 0.6mg/kg. Urine of all the animals was collected from GD18.5 to GD19.5. After determination of arterial BP, the rats were sacrificed on GD20.5 for collection of blood and tissues. A, 11β-HSD2 expression and activity in placentas. B, corticosterone level in placentas. C, corticosterone levels in maternal circulation. D, MAP measured from GD 7.5 until GD 19.5 in pregnant rats. E, SBP measured on GD20.5. F, protein/creatinine in urine. G, morphology of glomeruli stained by hematoxylin and eosin and periodic acid-Schiff (400x). H, histopathological score of glomerular pathology. I, fetal weight on GD 20.5. J, the circulatory sFlt-1 level and sFlt-1/PIGF in the pregnant rats. Data are expressed as mean±SEM(n=6). Statistic was performed by one-way ANOVA (A-F,H&J) and Kruskal-Wallis test (I). MAP: mean arterial pressure; SBP: systolic blood pressure.

Fig.3. DEX administration induces the features of PE in pregnant rats. Pregnant rats were administrated (s.c) with DEX at dosage of 0.1mg/kg from GD7.5 to GD17.5. Control rats were injected with same volume of saline. Urine was collected from GD18.5 to GD19.5. After determination of arterial BP, the animals were sacrificed on GD20.5 for collection of blood and tissues. A, SBP measured on GD20.5. B, protein/creatinine ratio in urine. C, morphology of glomeruli stained by hematoxylin and eosin and periodic acid-Schiff (400×). D,histopathological score of glomerular pathology. E, the circulatory sFlt-1 level. F, fetal weight measured on GD 20.5. Data are expressed as mean±SEM (n=8). Statistic was performed by two-tailed Student's *t*test. SBP: systolic blood pressure.

Fig. 4. CBX treatment leads to reduced interstitial trophoblast invasion,impaired endovascular SA remodeling and abnormal placentation. Pregnant rats were administrated (s.c) with CBX at a dosage of 0.6, 1.2 and 2.4mg /kg/d from GD7.5 to GD17.5. The rats were sacrificed on GD20.5, and placental tissues were collected for histological analysis. A, the representative image of the total area occupied by CK-positive interstitial trophoblast cells in MT (20×). B, cumulative data of interstitial trophoblast invasion in MT of the pregnant rats with administration of CBX. C, representative CK staining of trophoblast invasion, and α-actin staining of vascular smooth muscle in spiral arteries of the pregnant rats with systemic administration (200×). D, H&E staining were performed to evaluated the morphology of placental labyrinth of the pregnant rats with systemic administration (upper panel:400×, lower panel:100×). E, immunofluorescence analysis of laminin staining (400×). Data are expressed as mean±SEM (n=8). Statistic was performed by one-way ANOVA. TBC: trophoblast cells.

Fig.5. Placenta-targeted delivery of CBX results in deficient interstitial trophoblast invasion,

impaired endovascular SA remodeling and abnormal placentation in pregnant rats. Pregnant rats were intravenously injected with CBX, CBX-NPs, CBX-SCR and CBX-CSA every two days from GD7.5 to GD17.5. Control rats were injected with same volume of saline. The dosage of CBX was 0.6mg/kg. The rats were sacrificed on GD20.5, and placental tissues were collected for histological analysis. A, the representative image of the total area occupied by CK-positive interstitial trophoblast cells in MT (20×). B, cumulative data of interstitial trophoblast invasion in MT of the pregnant rats with administration of CBX. C, representative CK staining of trophoblast invasion, and α-actin staining of vascular smooth muscle in spiral arteries of the pregnant rats with systemic administration (200×). D, H&E staining were performed to evaluated the morphology of placental labyrinth of the pregnant rats with systemic administration (upper panel:400×, lower panel:100×). E, immunofluorescence analysis of laminin staining (400×). Data are expressed as mean±SEM (n=6). Statistic was performed by one-way ANOVA. TBC: trophoblast cells.

Fig.6. Systemic administration and placenta-targeted delivery of CBX lead to reduced uteroplacental perfusion in pregnant rats. Pregnant rats were administrated (s.c) with CBX every day from GD7.5 to GD17.5, or injected with CBX, CBX-NPs, CBX-SCR and CBX-CSA every two days from GD7.5 to GD17.5. The rats were anesthetized for ultrasound biomicroscopy. A, the representative images of the vasculatures in implantation sites (spiral arteries and maternal channels) and fetal umbilical artery visualized by ultrasound biomicroscopy. B, the representative images of doppler flow waveform of umbilical artery, maternal channel, spiral artery. C, cumulative data of the PSV of the umbilical artery, maternal channel and spiral artery in the pregnant rats with systemic administration of CBX. D, cumulative data of the PSV of the umbilical artery, maternal channel and spiral artery in the pregnant rats with placenta-targeted administration of CBX. Data are expressed as mean±SEM (n=6). Statistic was performed by Kruskal-Wallis test (C) and one-way ANOVA (D).

Φιγ.7. 11β-ΗΣΔ2 μοδυλατες σΦλτ-1 ρελεασε ια μοδυλατιον οφ ΑΔΑΜ17 εξπρεσσιον ιν πλαζενταλς. A, 11β-HSD2 modulates sFlt1 release in cultured human placental explants. Placental explants were treated with cortisol (10^{-6}M) in the presence or absence of CBX (10^{-5} or 10^{-6}M) for 24h. The media were then collected for determination of sFlt1 concentration. B&C, 11β-HSD2 modulates sFlt1 release in primary syncytiotrophoblasts. Human primary syncytiotrophoblasts were treated with cortisol(10^{-9}-10^{-6}M) in the presence or absence of CBX(10^{-5}-10^{-6}M) for 24h (B), or the cells were transfected with control siRNA and 11β-HSD2 siRNA, and then treated with cortisol (10^{-6}M) for 24h (C). The level of sFlt-1 in culture supernatant was determined by ELISA. D, 11β-HSD2 modulates ADAM17 expression in primary syncytiotrophoblasts. Primary syncytiotrophoblasts were transfected with control siRNA and11β-HSD2 siRNA, and then treated with cortisol (10^{-6}M) for 24h. Cells were harvested for determination of ADAM17 expression by western blotting. Representative bands of ADAM17 were shown on the top of the cumulative data. E, the role of ADAM17 in sFlt-1 release in primary syncytiotrophoblasts. Cells were transfected with control siRNA and ADAM17 siRNA for 24h. Culture media were harvest for determination of sFlt-1 content. F, GCs stimulates ADAM17 gene transcriptive activity in syncytiotrophoblasts. Placental trophoblasts were transfected with pGL3-luciferase reporter containing ADAM17 promoter or mutant ADAM17 promoter and combination with pRL-TK-Renilla-luciferase plasmid for 12hrs. Cells were then treated with DEX (10^{-6}M) for 24hrs. Luciferase assays were performed using the dual luciferase assay kit. Data are expressed as mean±SEM (n=5 independent cultures in each figure). Statistic was performed by two-way ANOVA (A,C,D,F), two-tailed Student's t test(E) and one-way and two-way ANOVA (B).

Fig.8. The effects of CBX administration on ADAM17 expression in placentas of pregnant rats and ADAM17 and11β-HSD2 levels in placentas of PE patients. A, pregnant rats were administrated (s.c) with CBX at a dosage of 0.6,1.2 and 2.4mg /kg/d from GD7.5 to GD17.5. Placental tissues were collected on GD 20.5 for determination of ADAM17 protein levels by western blotting. Representative bands of ADAM17 were shown on the top of the cumulative data. Data are expressed as mean±SEM (n=8). Statistic was performed by one-way ANOVA. B, the rats were intravenously injected with CBX, CBX-NPs, CBX-SCR and CBX-CSA every two days from GD7.5 to GD17.5. The rats were sacrificed on GD20.5, and placental tissues were collected for determination of ADAM17 protein levels by western blotting. Representative bands of ADAM17 were shown on the top of the cumulative data. Data are expressed as mean±SEM (n=6). Statistic was performed by

and Kruskal-Wallis test. C, ADAM17 and 11β-HSD2 in normal and PE placentas recruited from a Shanghai hospital. Representative bands of ADAM17 and 11β-HSD2 were shown on the top of the cumulative data. Data are expressed as mean±SEM (n=43 in each group). Statistic was performed by Mann-Whitney U test.