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Research paper

Hyper-methylation of *AVPR1A* and *PKCB* gene associated with insensitivity to arginine vasopressin in human pre-eclamptic placental vasculature

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ABSTRACT

Background: Pre-eclampsia is a leading cause of maternal mortality and morbidity. Although the exact mechanisms that cause pre-eclampsia remain unclear, it is undeniable that abnormal placental function and circulation are a center for initiation pre-eclampsia. As a potent vasoconstrictor, arginine vasopressin (AVP) has long been implicated in controlling placental vascular tone and circulation; its secretion is grossly elevated in pre-eclamptic circulation. However, little is known about the reactivity of AVP in pre-eclamptic placental vasculature.
Methods: To reveal the special features of placental vascular regulations with placental pathophysiological changes, as well as the corresponding molecular mechanisms under pre-eclamptic conditions, vascular function and molecular assays were conducted with placental vessel samples from normal and pre-eclamptic pregnancies.
Findings: The present study found that vasoconstriction responses of placental vessels to AVP were attenuated in pre-eclampsia as compared to in normal pregnancy. The insensitivity of AVP was correlated with the down-regulated AVP receptor 1a (AVPR1A, *AVPR1A* gene) and protein kinase C isoform β (PKCβ, *PKCB* gene), particularly the hyper-methylation-mediated *AVPR1A* and *PKCB* gene down-regulation, respectively.
Interpretation: The findings collectively revealed that aberrant DNA methylation-mediated gene expressions are correlated with vascular dysfunction in pre-eclamptic placental circulation.
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SUBJECT CODES

Vascular biology
Placental vascular dysfunction
Pre-eclampsia.

1. Introduction

As the most common medical syndrome of human pregnancy, pre-eclampsia (PE) affects millions of women worldwide each year [1–3]. PE is a major cause of immediate and long-term maternal-fetal morbidities such as maternal-fetal death, fetal growth restriction, and future diseases for mother and child. Although the ultimate etiology of PE is still unknown, the consensus that a complex interplay among immune dysfunction, vascular dysfunction, oxidative stress, and angiogenesis mechanisms is involved in the development of PE has been widely accepted [2–5]. In the non-pregnant state, arginine vasopressin (AVP) has been associated with each of these four mechanisms through actions at its receptors [6–8]; meanwhile, non-pregnant low-renin hypertensive disorders often exhibit an elevated AVP secretion [9,10]. These previous studies have suggested that AVP possibly plays a central role

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Research in context

Evidence before this study

It is undeniable that abnormal placental function and blood circulation (particularly placental ischemia) are a center for initiation pre-eclampsia. Previous studies have suggested that, as a potent vasoconstrictor, arginine vasopressin possibly plays an important part in regulating placental vascular tone and circulation. The potent vasopressor effects of arginine vasopressin have been investigated previously, but information regarding its vascular effects on pre-eclamptic placental units is very limited.

Added value of this study

- The vasoconstriction responses of placental vessels to arginine vasopressin were attenuated in pre-eclampsia, correlated with the down-regulated AVPR1A and PKCβ.
- A hyper-methylation-mediated transcription repression of *AVPR1A* and *PKCB* was observed in pre-eclamptic placental vasculature.
- To the best of our knowledge, our study is the first to reveal the attenuated sensitivity of arginine vasopressin and its underlying epigenetic mechanism in pre-eclamptic placental vessels.

Implications of all the available evidence.

Our study not only offered new information for understanding the pathological features of pre-eclampsia, but also underlined a crucial role of the epigenetic-mediated gene expression in pre-eclamptic placental vascular dysfunctions.

in the pathogenesis of PE. Indeed, significant information regarding the relationship between AVP and PE is clear from these data: 1) An assessment of maternal plasma copeptin (a stable protein by-product and clinically useful biomarker of AVP secretion) revealed that AVP secretion is grossly elevated in the first few weeks of pregnancy and that these women eventually develop PE [11]. 2) Maternal plasma copeptin is significantly predictive of the development of PE, irrespective of clinical covariates, in at least as early as the sixth week of pregnancy [12–14]. 3) A chronic infusion of AVP during gestation in rodents is sufficient to phenocopy essentially all maternal and fetal symptoms of human PE [11,15].

It is undeniable that abnormal placental function and blood circulation (particularly placental ischemia) is a center for initiation PE [12,16,17]. As it is a feto-maternal vascular organ, normal placental function and circulation are dependent on sufficient placental perfusion and adequate blood flow via placental circulatory systems [17]. Because placental vessels lack autonomic innervation [18], circulating and locally synthesized vasoactive substances are important for controlling vaso-activities and the blood flow in the placental circulation. AVP, a potent vasoconstrictor, has long been implicated in controlling the vascular tone via activation of the protein kinase C (PKC) pathway by binding on smooth muscle receptors [mainly classified into V1a (AVPR1A), V1b (AVPR1B), and V2 (AVPR2) subtypes] in vascular smooth muscle cells (SMCs) [19,20]. As early as 30 years ago, AVP was reported to induce robust contraction responses in placental vessels [21–23], suggesting that AVP could play an important role in regulating the placental vascular tone and circulation. The potent vasopressor effects of AVP have been investigated previously, but information regarding the vascular effects of AVP on pre-eclamptic placental units is very limited. Do high AVP values in circulation cause remarkable vasoconstrictions in a pre-eclamptic placental system? Does AVP play the same physiological role in pre-eclamptic placental vessels as it does in the normal ones? Therefore, in the present study, we investigated the contractile responses of AVP in normal and pre-eclamptic placental vessels to reveal the special features of placental vascular regulations with placental pathophysiological changes, as well as the molecular mechanisms of AVP under pre-eclamptic conditions.

2. Methods

2.1. Sample collection

Healthy normal pregnant (NP, $N = 42$) and pre-eclamptic women (PE, $N = 40$) were recruited from the local hospitals, Suzhou, China. The Ethics committee of First Hospital of Soochow University approved all procedures in this work, and all participants were given informed consent, conformed to the principles outlined in the Declaration of Helsinki. Healthy pregnancy was defined as blood pressure < 120/90 mmHg and no clinically significant complications. Preeclampsia was defined as blood pressure > 140/90 mmHg and significant proteinuria after the 20th weeks of pregnancy [1,24]. Women with essential hypertension or medical complications, such as diabetes and renal disease, were excluded from the study. The demographic characteristics of all participants were detailed in Supplementary Table S1.

2.2. Vascular functional studies

Placenta was immediately acquired from normal and pre-eclamptic pregnancy after vaginal delivery within 1 h. The middle parts of placenta were kept in iced Krebs solution (containing in mmol/L: NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.0, glucose 11.0, and $CaCl_2$ 2.5), and bubbled with 95% O_2 and 5% CO_2. Human placental vessels (HPV-A1/A2, First- and second-order branches of umbilical vessels in the placenta, the main stem villous arteries; HPV-A3, branch of the main stem villous arteries with diameter around 150 um, mainly chorionic plate placental arteries) were carefully isolated. HPV-A1/A2 rings were cut into rings approximately 4–5 mm in length and suspended in a 5 mL organ bath with 5 mL Krebs solution, and continuously with a mixture of 95% O_2 and 5% CO_2. Under the tension of 2 g, HPV-A1/A2 rings were allowed to balance for 2 h. Then the contraction of potassium chloride (KCl, 120 mol/L) was used to gain maximum reaction degree. The contraction induced by AVP was standardized through comparing with the maximal tension by KCl. HPV-A3 was dissected from placenta and cut into 3–4 mm rings. Isolated HPV-A3 rings were suspended in organ chambers filled with HEPES-PSS solution (mmol/L: NaCl 141.85, KCl 4.7, $MgSO_4$ 1.7, KH_2PO_4 1.17, $CaCl_2.2H_2O$ 2.79, EDTA 0.51, glucose 5.0 and HEPES 10.0; pH 7.4), gassed continuously with 95% O_2 and 5% CO_2. Wire myograph (Danish Myo Technology, Midtjylland, Denmark) was used to measure vascular responses. Half hour was allowed for equilibration. KCl (60 mmol/L) was applied for each experiment in order to detect the tissue maximum response. HPV-A1/A2 and HPV-A3 rings were contracted by the addition of incremental doses of AVP (10^{-10}-10^{-4} mol/L) at 3-min intervals. Between continuous concentrations of AVP, there was at least three minutes reaction time, during which time the reaction of preceding concentration reached equilibrium phase. In the subsequent experiments, SR49059 (inhibitor of AVPR1A, 10umol/L) or GF109203X (inhibitor of PKC, 100umol/L) were used for pretreating HPV-A1/A2, or HPV-A3 for 30 min before application of AVP, and the vessel responses were recorded. All drugs were freshly prepared and purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Quantitative real-time PCR (qRT-PCR) and western blot analysis

Total RNA was isolated from human placental vessels (HPV) using Trizol reagent (Invitrogen) according to manufacturer's instructions, and was then reversed transcribed using the first-strand cDNA

Synthesis Kit (Toyobo Corp., Shanghai, China). qRT-PCR was performed using SYBR Green Supermix Taq Kit (Takara Biotechnology Co., Ltd., Dalian, China) and analyzed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences were listed in Supplementary Table S2. ΔΔCt method was used to comparatively quantify the amount of mRNA levels. The protein abundance of AVPR1A, AVPR2, and PKC (α, and β) in HPV was measured with Western blot normalized to β-actin. The primary antibodies were the rabbit polyclonal antibody (Santa Cruz Biotechnology) against AVPR1A, AVPR2, PKCα, PKCβ and β-actin (all 1:1000). The secondary antibody was the goat anti-rabbit antibody (1:1000; Beyotime Biotechnology, Jiangsu, China). Immuno-signals were visualized using UVP imaging system (Tianneng, Shanghai, China). Imaging signals were calculated and analyzed, and then the ratio of band brightness to β-actin was acquired to measure the relative protein expression level. Analyses was performed as previously described [25,26].

2.4. DNA isolation and targeted bisulfite sequencing assay

Genomic DNA was extracted from HPV rings by standard phenol/chloroform technique as previously described [26], and subjected to bisulfite conversion using EZ DNA Methylation™-GOLD Kit (Zymo Research) according to manufacturer's protocols. DNA was quantified and then diluted to a working concentration of 10-20 ng/μL for BiSulfite Amplicon Sequencing (BSAS) [27]. CpG islands located in the proximal promoter of *AVPR1A* and *PKCB* were selected according to the following criteria: 1) ≥200 bp length; 2) ≥50% GC content; 3) ≥60% ratio of observed/expected dinucleotides CpG [28]. Based on the genomic coordinates of the candidate CpG sites, we carefully designed the BSAS primers in order to detect them in a panel (Supplementary Table S2). After PCR amplification, products were sequenced by Illumina Hiseq 2000. Methylation level at each tested CpG site was calculated as the percentage of the methylated cytosines over the total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the *AVPR1A* or *PKCB* gene.

2.5. PKC levels in human placental vessels

HPV rings were immersed in liquid nitrogen and then homogenized into five packed volumes of extraction buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, and 1 mM EDTA,) with protease inhibitors (5 mM DTT, 1 mM PMSF, 1 mg/L leupeptin, and 1 mg/L pepstatin) at 4 °C for 1 h with occasional vortexing. The homogenate was centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant of the extract was assayed. Solid phase enzyme-linked immuno-absorbent (ELISA) assay was used to determine PKC levels according to the manufacturer's instructions (Shanghai Haling Biotechnology Co., Ltd. China). The minimum detectible dose of PKC for this assay was 0.5 ng/g. The intra-assay coefficient of variation was <6% and the inter-assay coefficient of variation was <10%. Samples and data were handled in a blind manner.

2.6. Data analysis and statistics

All data were expressed as the mean ± s.e.m. Significance ($P < .05$) was ascertained by Student's *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni's test. Concentration-dependent response curves were performed by computer assisted nonlinear regression (Graph Pad Prism software CA, USA). DNA methylation/mRNA correlation plots were identified by causal inference test (SigmaPlot 10.0).

3. Results

3.1. AVP-induced contractions in human placental vessels

In both HPV-A1/A2 (Fig. 1a; $P = .770$) and HPV-A3 (Fig. 1d; $P = .746$), no significant differences were observed in the KCl-induced maximal contraction between the NP group and the PE group. In contrast, compared with the normal, the Emax (AVP-induced contraction at 10^{-4} mol/L) and pD2 (−log[50% effective concentration]) values significantly decreased in the cases of pre-eclamptic HPV-A1/A2 (Fig. 1b-c; $P < .05$) and HPV-A3 (Fig. 1e-f; $P < .05$). These data indicated that pre-eclamptic placental vessels were significantly insensitive to AVP.

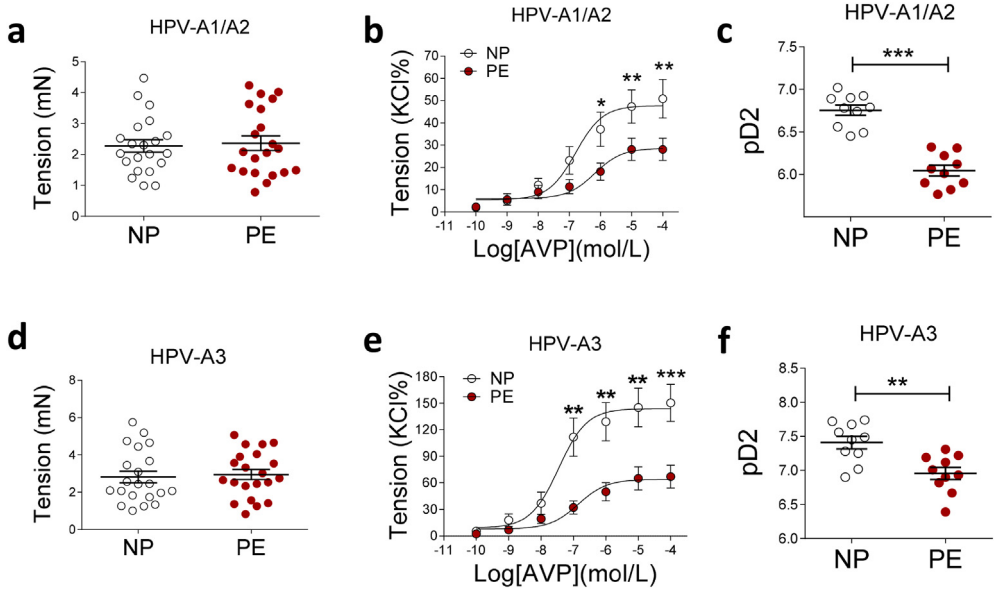

Fig. 1. AVP-mediated vascular reactivity in human placental vessels. (a and d) KCl-induced maximal contractions in HPV-A1/A2 ($N = 20, n = 20$) and HPV-A3 ($N = 20, n = 20$) rings; $P > .05$. (b and e) Concentration-response curves of AVP-induced dose-dependent contractions in HPV-A1/A2 ($N = 28, n = 38$) and HPV-A3 ($N = 29, n = 37$) rings; $P < .05$. (c and f) pD2 (−log[50% effective concentration]) values of AVP-induced dose-dependent contractions in HPV-A1/A2 ($P < .001$) and HPV-A3 ($P = .0078$). KCl, potassium chloride; NP, normal pregnancy; PE, preeclampsia; HPV-A1/A2, first-, second-order branch of placental vessels; HPV-A3, third branch of placental vessel (micro-vessels with diameter around 100 um). Error bars denote s.e.m. Statistical significance was calculated by Student's *t*-test (a, c, d, and f) or two-way ANOVA (b and e). *, $P < .05$; **, $P < .01$; ***, $P < .001$. N, number of participants; n, number of vessel rings.

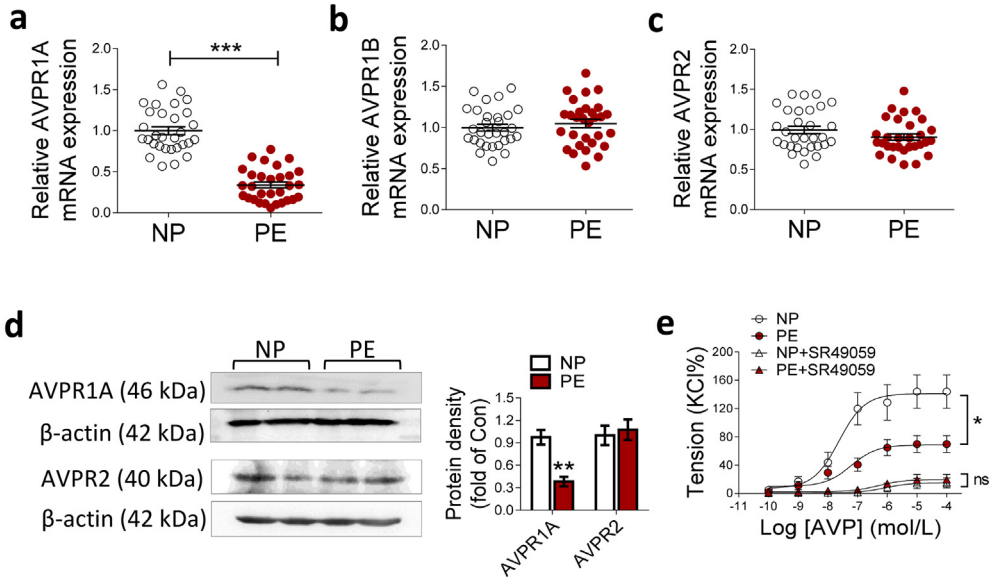

Fig. 2. Expression of AVP receptors in human placental vessels. (a-d) mRNA and protein levels of AVP receptors in HPV were determined by qRT-PCR and Western blot ($N = 30$, $n = 30$ each group). (e) Effects of SR49059 on AVP-mediated vasoconstrictions in HPV ($N = 13$, $n = 25$ each group). SR49059, AVPR1A-specific antagonist. HPV, placental vessels. Error bars denote s.e.m. Statistical significance was calculated by Student's t-test (a-c) or two-way ANOVA (d and e). *P < .05; ***P < .01; ns, nonsiginificanse. N, number of participants; n, number of vessel rings.

3.2. Expression of AVP receptors in human placental vessels

In the vasculature, AVP receptors are divided into AVPR1 (AVPR1A and AVPR1B) and AVPR2 [20]. To investigate whether the decreased AVP-mediated vasoconstrictions correlated with AVP receptor expression in pre-eclamptic placental vessels, the AVP receptor mRNA and protein levels were determined. As shown in Fig. 2a-c, compared with those in the NP group, the mRNA levels of AVPR1A (Fig. 2a; $P <$ .001) were decreased in the PE group, whereas no significant differences were observed in AVPR1B (Fig. 2b; $P = .454$) and AVPR2

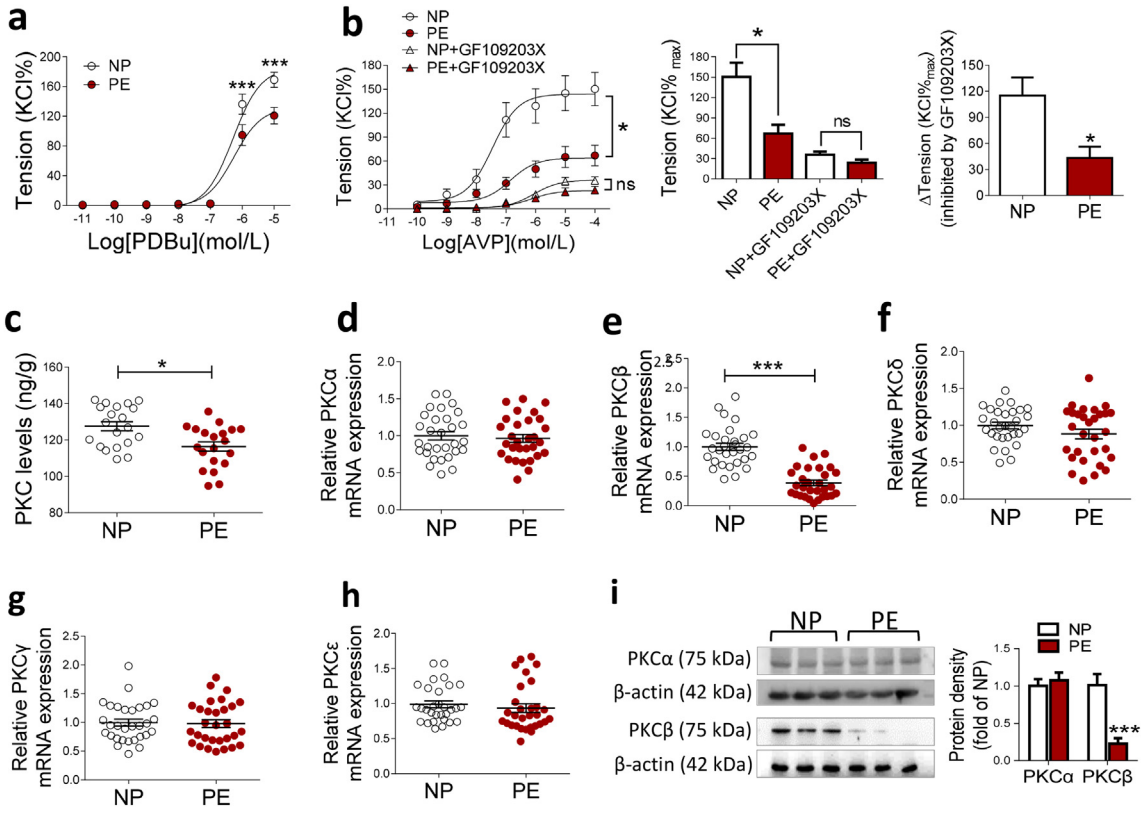

Fig. 3. The decreased sensitivity of AVP was also dependent on PKC pathway. (a) PDBu induced vasoconstrictions in HPV rings ($N = 15$, $n = 26$ each group); P < .05. (b) The inhibitory effect of GF109203X on AVP-induced contractions in HPV ($N = 16$, $n = 27$ each group). (c) PKC levels in HPV determined by ELISA (N = 30, n = 30 each group); $P = .014$. (d-h) The mRNA levels of PKCα, PKCβ, PKCγ, PKCδ, and PKCε in HPV determined by qRT-PCR (N = 30, n = 30 each group). (i) The protein levels of PKCα, and PKCβ in HPV determined by Western blot. GF109203X, PKC antagonist; PDBu, Phorbol 12, 13-dibutyrate (PKC activator). Error bars denote s.e.m. *P < .05; ***P < .001; ns, nonsiginificanse. N, number of participants; n, number of vessel rings.

(Fig. 2c; $P = .152$) between the NP and the PE groups. Consistently, there was a significant decrease in the protein of AVPR1A, not AVPR2, in the PE group (Fig. 2d; $P < .01$). To further determine whether the reduced AVPR1A was associated with the decreased AVP-mediated vasoconstrictions in the PE group, vascular rings were pre-treated with SR49059 (AVPR1A-specific antagonist). As shown in Fig. 2e, SR49059 almost completely blocked the AVP-mediated contractions in both the NP and the PE groups, and no differences were observed in the AVP-induced vasoconstrictions between the NP and the PE groups after pretreatment with SR49059 ($P > .05$). These data indicated that the decreased sensitivity of pre-eclamptic placental vessels to AVP was related to the down-regulated AVPR1A.

3.3. Dependence of decreased sensitivity of AVP on the PKC pathway

In vascular SMCs, AVP-AVPR1A induces vasoconstrictions by activating the PKC pathway [19]. PKC activation can phosphorylate CPI17 (protein phosphatase 1 regulatory inhibitor), which in turn inhibits myosin light chain (MLC) phosphatase, increases MLC phosphorylation, and enhances vascular SMC contraction [19,29-31]. We found that the PKC agonist (PDBu) caused weaker dose-dependent contractions in HPV from PE than that in the NP group (Fig. 3a; $P < .05$). To investigate whether the decreased AVP-mediated vasoconstrictions in the PE group were also related to the PKC pathway, vascular rings were pre-treated with GF109203X (PKC-specific antagonist). GF109203X could restrain AVP-induced vasoconstrictions in both the NP and the PE groups, without significant differences in the AVP-mediated vasoconstrictions the two groups after pretreatment with GF109203X (Fig. 3b; $P > .05$). Meanwhile, GF109203X produced a weaker attenuation of the AVP-mediated vasoconstrictions in the PE group (Fig. 3b; $P = .011$). Furthermore, the ELISA results revealed a decreased PKC level in the pre-eclamptic HPV (Fig. 3c; $P = .014$). In the vasculature, PKC mainly includes α, β, γ, δ, and ε isoforms [31]. As shown in Fig. 3d-h, in the case of HPV, no significant differences were observed in the PKCα (Fig. 3d; $P = .662$), PKCδ (Fig. 3f; $P = .149$), PKCγ (Fig. 3g; $P = .864$) and PKCε (Fig. 3h; $P = .492$) mRNA levels between the two groups; however, the mRNA levels of PKCβ (Fig. 3e; $P < .001$) were significantly decreased in the PE group compared with those in the NP group. The protein levels of PKCβ were also significantly down-regulated in the PE group (Fig. 3i; $P < .001$). These data indicated that the reduced AVP-mediated vasoconstrictions in the PE group were also related to the PKC pathway.

3.4. DNA methylation of CpG locus within AVPR1A gene promoter in human placental vessels

One CpG island contains 14 CpG sites within exons of the *AVPR1A* gene (Fig. 4a). *AVPR1A* is located on chromosome 12q14.2. Supplementary Table S3 shows the CpG labels. We validated the methylation status of the 14 CpG sites with targeted bisulfite sequencing. The bisulfite conversion rate of each sample was higher than 99%, and no significant difference was found between the NP and the PE groups, indicating that the bisulfite conversion was efficient and reliable in the experiments

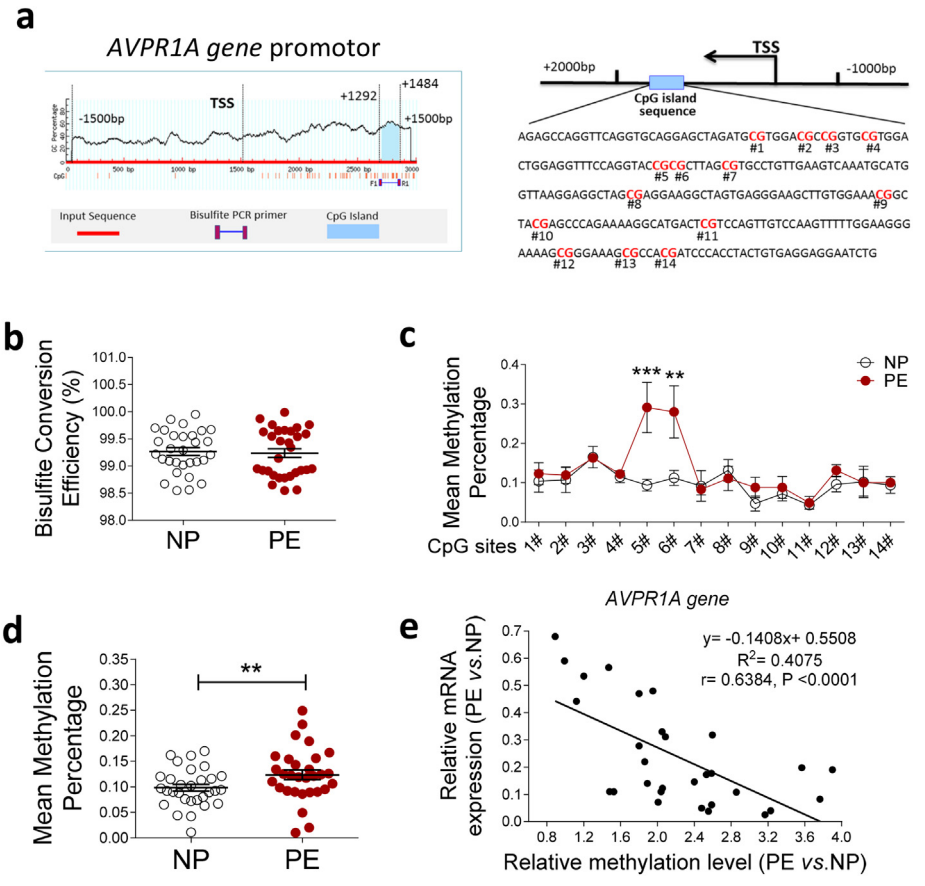

Fig. 4. DNA methylation of CpG locus at *AVPR1A* gene promoter in human placental vessels. (a) Bioinformatic analysis of CpG islands of *AVPR1A* gene from upstream −1.5 kb to downstream +1.5 kb region. Sequence analysis identified one CpG island in exon that contains 14 CpG sites, located at positions +1292 to +1484 from the translation start site (TSS, defined as position 1) in *AVPR1A* gene promoter. (b) Represent image of bisulfite conversion efficiency between NP and PE group (N = 30 each group); $P = .782$. (c and d) The mean methylation status of CpG locus (the total and each tested) at AVPR1A gene promoter in HPV (N = 30 each group). (e) Expression analysis of *AVPR1A* gene and its correlation with methylation levels of CpG sites (5 and 6); $P < .001$. DNA methylation/mRNA correlation plots for *AVPR1A* gene identified by causal inference test (N = 30 each group). The Y-axis represents the relative expression level of *AVPR1A* gene which was detected with qRT-PCR method. The X-axis represents the relative mean methylation level of the CpG sites (5 and 6) at AVPR1A gene promoter in HPV. r: Pearson correlation coefficient. Error bars denote s.e.m. Statistical significance was calculated by Student's t-test (b and d) or two-way ANOVA (c). *$P < .05$; **$P < .01$; ***$P < .001$. N, number of participants.

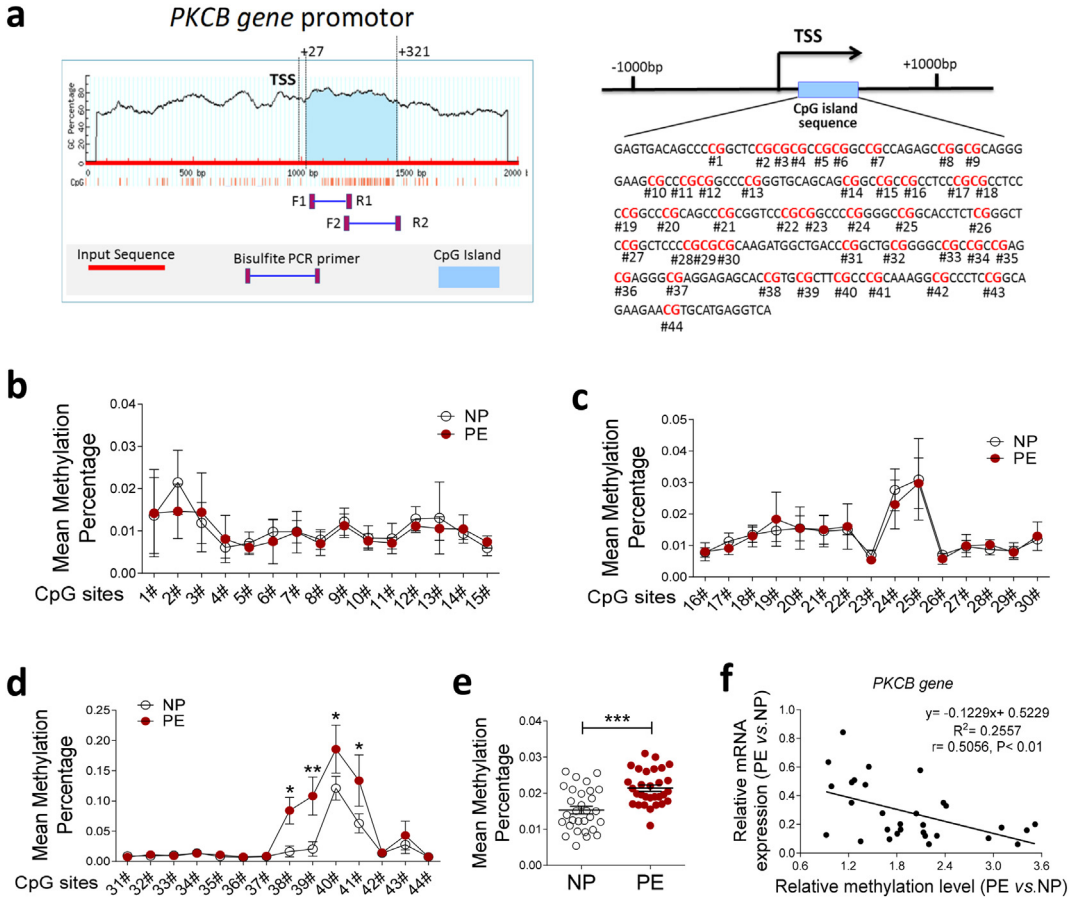

Fig. 5. DNA methylation of CpG locus at *PKCB* gene promoter in human placental vessels. (a) Sequence analysis identified a CpG island that contains 44 CpG sites, located at positions +27 to +321 from TSS at *PKCB* gene promoter. (b-e) The mean methylation status of CpG locus (the total and each tested) at *PKCB* gene promoter in HPV (N = 30 each group). (f) DNA methylation (the mean methylation status of CpG sites (38–41)) /mRNA correlation plots for *PKCB* gene identified by causal inference test ($N = 35$ each group); $P < .001$. Error bars denote s.e.m. Statistical significance was calculated by Student's t-test (e) or two-way ANOVA (b-d). *P < .05; ***P < .001. N, number of participants.

(Fig. 4b; $P = .782$). By using targeted bisulfite sequencing, we found that the mean methylation percentage of the 14 CpG sites within the CpG island at the *AVPR1A* gene promoter (Fig. 4 d; $P = .005$) in the pre-eclamptic placental vessels significantly increased at specific CpG sites 5 and 6 (Fig. 4 c; $P < .01$) (Supplementary Table S4). A correlation analysis between the *AVPR1A* gene methylation and expression was also conducted. As shown in Fig. 4e, a significantly inverse correlation was obtained between the methylation statuses of the CpG sites (5 and 6) in the *AVPR1A* gene promoter and the *AVPR1A* gene expression ($P < .001$).

3.5. DNA methylation of CpG locus within PKCB gene promoter in human placental vessels

A sequence analysis identified one CpG island that contains 44 CpG sites within the exons of the PKCB gene (Fig. 5a). PKCB is located on chromosome 16p12.2. Targeted bisulfite sequencing showed that compared with NP, the mean methylation percentage obviously increased at specific CpG sites (38, 39, 40, and 41) within the CpG islands at the PKCB gene promoter in the PE group (Fig. 5d; $P < .05$), whereas no significant difference between the NP and the PE groups was found at other specific CpG sites (Fig. 5b-c; $P > .05$). Consistently, there was a significant increase in the mean methylation percentage of the 44 CpG sites within the CpG islands at the PKCB gene promoter in the PE group (Fig. 5e; $P < .001$). The position and the methylation levels of the 44 CpG sites are listed in Supplementary Tables S5 and S6. After a careful analysis of the DNA methylation and expression data, we concluded that there was a significantly inverse correlation between the methylation statuses of CpG sites 38–41 in the PKCB gene promoter and its expression (Fig. 5f; $P < .001$).

4. Discussion

The main findings of this study were as follows: AVP exhibited a potent contractile effect in placental vessels, suggesting that AVP could be critically involved in regulation of placental circulation. The vasoconstriction responses to AVP were attenuated in the pre-eclamptic placental vessel related to the down-regulated AVPR1A-PKCβ axis, particularly the deactivated transcription of *AVPR1A* and *PKCB*. The *AVPR1A* and *PKCB* transcription repression was, respectively, associated with increased DNA methylation within the *AVPR1A* and *PKCB* gene promoter. To the best of our knowledge, this study first revealed a hypermethylation of certain CpG islands in the *AVPR1A* and *PKCB* gene promoter was associated with the insensitivity of pre-eclamptic placental vessels to AVP.

A complex interplay among immune dysfunction, vascular dysfunction, oxidative stress, and angiogenesis mechanisms was involved in the pathogenesis of PE [2,4,5]. In the non-pregnant state, AVP has been associated with each of these four mechanisms [6,7]. Meanwhile, maternal AVP levels were increased in pre-eclamptic women, and AVP hypersecretion could be used as a predictive marker for PE in as early as the sixth week of pregnancy [11,32]. This body of the literature indicated a relationship between AVP hypersecretion and the pathogenesis of PE. As aforementioned, local vaso-activators are important for maintaining the balance of placental blood circulation. Functional abnormalities in the placental vasculature could be a root cause for abnormal

placental function, eventually leading to an abnormal course of pregnancy such as PE [17,33]. In the present study, an in vitro investigation of the contractile properties of AVP in isolated fetoplacental vascular branches was performed using the myograph technique. Even though two studies have investigated the vasoreactivity of AVP in the placental vasculature between women with PE and normal pregnancy [22,23]; unfortunately, the conclusions reached were inconsistent. For example, Wareing et al. reported that the maximal vasoconstriction induced by AVP was reduced in the pre-eclamptic placental vessels [22]. In contrast, Ong et al. showed that the constriction responses to AVP did not differ between pre-eclamptic and normal placental vessels [23]. Although the same experimental materials and methods were used in these studies, the results and conclusions were different. These differences might be attributed to the fact that the sample size in these studies was very small and could not capture the huge variations in human subjects. In the present study, a relatively large size of human samples (75 independent vessel tests and >40 placentas) was used for the functional analysis. At least 80% of the subjects in the 75 independent tests showed that the AVP-induced contractions were significantly decreased in the pre-eclamptic placental vessels.

Our experiments showed that with respect to the normal, the curve of the AVP-mediated constrictions was shifted to the right in the PE group with decreased maximal contraction and pD2, indicating that the reduced AVP-produced constrictions were associated with the number of AVP receptors or the sensitivity of the vascular SMCs to AVP. AVP has long been implicated in the regulation of vascular tone by binding to vascular receptors (mainly classified into AVPR1A, AVPR1B, and AVPR2 subtypes) [19,20]. We found that the mRNA and protein abundances of AVPR1A, not AVPR1B and AVPR2, were significantly decreased; meanwhile, an AVPR1A-specific antagonist could completely block AVP-induced constrictions in the placental vessels. These data therefore indicated that the decreased sensitivity of AVP in the pre-eclamptic placental vessels correlated with the down-regulated AVPR1A, particularly the deactivated transcription of *AVPR1A*. In addition, the downregulated AVPR1A were also observed in both of pre-eclamptic placenta villi and amnion (Supplementary fig. 1a-c). These data indicated the downregulated AVPR1A was prevalent in the pre-eclamptic placenta. Interestingly, AVPR2 was upregulated in pre-eclamptic amnion. The pathological changes and possible clinical importance of AVPR2 in pre-eclamptic placental amnion deserves further exploration.

AVP–AVPR1A axis can induce vasoconstrictions mainly via activation PKC pathway [19,29]. The present study found that PKC agonist caused weaker dose-dependent contractions; PKC antagonist produced a weaker attenuation of the AVP-mediated vasoconstrictions in the PE group, indicating that the decreased AVP-mediated vasoconstrictions in the pre-eclamptic placental vessels were also correlated with PKC pathway. This finding was further supported by the following data: PKC levels, as well as PKCβ, not the other isoforms of PKC, were significantly decreased in the pre-eclamptic placental vessels. Together, these data indicated that the abnormal AVP–AVPR1A–PKCβ axis was responsible for the insensitivity of pre-eclamptic placental vasculature to AVP.

The present study indicated that the abnormal AVP–AVPR1A–PKCβ axis could be ascribed to the deactivated transcription of *AVPR1A* and *PKCB* in the pre-eclamptic placental vasculature. DNA methylation is a major epigenetic mechanism that regulates gene transcription [28]. Studies on rodents have shown that the transcription of *AVPR1A* was partly regulated by the DNA methylation [34–36]. A number of studies on humans and laboratory animals have suggested that the promoter methylation levels are important for the transcriptional regulation of *PKCB* [37–40]. Our sequence analysis identified a critical CpG island that contains 14 CpG sites in the *AVPR1A* gene promoter and 44 CpG sites in the *PKCB* gene promoter. First, we evaluated DNA methylation status of CpG sites within the gene promoter and found that the mean methylation percentage of CpG sites within the CpG islands in both the *AVPR1A* and *PKCB* gene promoter was obviously increased in the pre-eclamptic placental vasculature. Second, we conducted a correlation analysis and found a significantly inverse correlation between the DNA methylation statuses of the gene promoter and the gene transcription levels. Together, the present study indicated that the transcription of *AVPR1A* and *PKCB* was regulated by DNA methylation in the human placental vasculature and revealed that the hypermethylation in the *AVPR1A* and *PKCB* gene promoter was associated with the decreased sensitivity of the pre-eclamptic placental vessels to AVP. DNA methylation-mediated gene expression has been considered to be a contribution to the development of placental insufficiency and PE [41,42]. The present study first indicated that DNA methylation-mediated gene expression could also be critically involved in the pathogenesis of vascular dysfunction in the pre-eclamptic placental circulation. Therefore, the pathological and possible clinical importance of DNA methylation in pre-eclamptic placental vasculature units deserves further exploration.

Pre-eclamptic placental vascular responses to AVP were decreased. This result is very interesting in light of the previous findings of an elevated AVP secretion in PE case. The attenuation of the AVP–AVPR1A–PKCβ signaling in placental vessels appears to protect the normal placental vascular tone from AVP hypersecretion in the pre-eclamptic circulation, which was beneficial for maintaining normal placental blood circulation. In contrast, why does the AVP secretion increase during PE? These questions have puzzled doctors and scientists for many years without precise answers. The present study also provided a new insight into it: the reduced AVP-mediated vasoconstrictions in the placental circulation might cause compensatory responses, resulting in an increased AVP in the circulation that can eventually induce PE. Such an increased AVP may be a part of a compensatory mechanism to offset the pathological effects of the pre-eclamptic placental vasculature. The physiological and possible clinical importance of the present findings deserves further investigation.

In general, PE cases exhibited reduced gestational weeks at delivery. Gestational age may be an important confounder when studying changes in placental tissue [43–45]. Ideally, both NP and PE groups should have been matched for gestation at delivery, but in reality, babies are very rarely delivered pre-maturely if the pregnancy is normal. This is a limitation of all studies of PE when collecting umbilical-placental samples at birth and comparing them with samples from a normal full-term pregnancy. In this study, we had considered that kind of concerns and carried out correlation analysis. For example, there were no correlation between gestational age (weeks) and AVPR1A and PKCB mRNA expressions in PE group (Supplementary Fig. 2), suggesting that the difference observed in this study, was not attributed to a significant difference in gestation at delivery. In addition, placental vascurture includes fetoplacental and uteroplacental vessels. This present study was conducted with placental chorionic plate arteries (fetoplacental vessels), because of such vessels is mainly undertaken placenta vascular resistance, and contributes to control of placental circulation. We understand it should be better to use uteroplacental as close to maternal side due to the nature of PE. Thus, we also determined the expression levels of AVPR1A and PKCB in uteroplacental vessels (Supplementary Fig. 3). Similarly, the downregulated AVPR1A and PKCB were also observed in pre-eclamptic uteroplacental vessels, indicating the downregulated AVPR1A and PKCB was prevalent in both of pre-eclamptic fetoplacental and uteroplacental vessels.

In conclusion, AVP is critically involved in the pathogenesis of PE. However, the nature of the vascular effects of AVP in the placental units, as well as the mechanism underlying the AVP-mediated vascular functional changes in PE, was unknown. To the best of our knowledge, the present study is the first one to clarify the AVP effects on placental vessels and solve some of the prevalent confusion in the field. Importantly, the present study revealed the attenuated sensitivity of AVP and its underlying epigenetic mechanism in pre-eclamptic placental vessels. The findings not only offered new information for understanding the pathological features of PE but also underlined a crucial role of the epigenetic-mediated gene expression in pre-eclamptic placental

vascular dysfunctions. This new and important information may be helpful in understanding the pathogenesis of PE as well as for the development of new approaches and treatments for PE.

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Conflicts of interests

All authors have no competing interests to declare, financial or otherwise.

Author contributions

QG processed the data and figures, and performed vessel experiments with HL, FX, TX, JT, YL, XC, and XZ. QG and HD processed the data and performed molecular studies. QG, and HL preformed data analysis. QG, JT, and ZX prepared human samples, design the study, and drafting the article. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.056.

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