Original Paper

Reduction of Uterine Perfusion Pressure Induced Redistribution of Endothelin Receptor Type-B Between the Intima and Media Contributes to the Pathogenesis of Pregnancy-Induced Hypertension

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Key Words

Pregnancy-induced hypertension • Endothelin receptor • Reduction of uterine perfusion pressure • NF-κB

Abstract

Background/Aims: Studies have shown that a change in endothelin receptor expression in the artery is related to pregnancy-induced hypertension (PIH). However, the mechanism underlying this change is unclear. **Methods:** To test whether the distribution of endothelin receptor type-A (ET_AR) and type-B (ET_BR) plays an important role in PIH, a reduction of uterine perfusion pressure (RUPP) rat model was used to mimic some of the features of PIH; the resulting arterial endothelin receptor expression was investigated in the media and intima of the aorta. Single vascular smooth muscle cells (VSMCs) were isolated from RUPP and normal pregnant (NP) rats to study the effect of ET_AR and ET_BR in smooth muscle cells. **Results:** Compared with NP rats, RUPP rats had a significant redistribution of ET_BR expression from the intima and media, while there was no significant difference in ET_AR expression between the two groups. ET_BR upregulation in VSMCs enhanced cellular contraction and contributed to the PIH. The TNF-α plasma levels in RUPP rats were two-fold higher than those of NP rats, which upregulated the expression of ET_BR in VSMCs through the NF-κB pathways in RUPP rats. **Conclusion:** Redistribution of ET_BR between the media and intima played an important role in the pathogenesis of PIH.

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Introduction

Preeclampsia is a pregnancy-induced hypertensive disorder that is characterized by severe hypertension, proteinuria, eclampsia and seizures [1]. It is one of the leading causes of maternal and perinatal morbidity; however, the underlying mechanisms are not clear. A normal pregnancy (NP) is associated with decreased vascular resistance, increased cardiac output, and a slight reduction or no change in blood pressure [2, 3]. Instead of these beneficial changes, vascular resistance and arterial pressure are greatly increased in women suffering from preeclampsia.

Placental ischemia induced inflammatory cytokine production during pregnancy is an important initiating event in preeclampsia, because it induces structural and functional alterations in endothelial cells that results in the release of oxidizing free radicals and the formation of lipid peroxides. The lipid peroxides and oxygen radical compounds can directly damage endothelial cells. Moreover, the inflammatory cytokines also promote the formation of a number of endothelial cell substances, which enhance vasoconstriction [3].

Endothelin-1 (ET-1) is a major endothelium-derived factor that is regarded as one of the most potent vasoconstrictors; ET-1 plays an important role in the pathogenesis of preeclampsia [1, 4, 7]. Plasma ET-1 concentrations during preeclampsia are approximately two- to three-times higher than normal levels, and ET-1 has significant long-term effects on arterial pressure regulation [8-10]. However, some studies have reported that there was no elevation in plasma endothelin in preeclampsia patients [11]. In addition, ET-1 is considered to be involved in the progression of preeclampsia rather than the initiation of the disease; thus, the exact role of endothelin is unknown [7].

From in-depth research, it was discovered that ET-1 can activate both endothelin receptors type-A (ET_A R) and type-B (ET_B R) [12]. ET_A R is mainly expressed in vascular smooth muscle cells (VSMCs) to induce vasoconstriction, whereas ET_B R is predominately expressed in endothelial cells (ECs) to induce the release of vasodilator substances as well as aiding ET-1 clearance [13]. ET_A R was previously considered to be primarily responsible for pregnancy-induced hypertension (PIH) and it has been shown that blocking ET_A R attenuates PIH [14, 15]. However, ET_B R upregulation in VSMCs was also found to be associated with many cardiovascular diseases and contribute to vasoconstriction [13, 16-19]. Mazzuca *et al.* reported that downregulation of ET_B R in microvascular endothelial cells is a central vascular mechanism of PIH [20]. Our previous results have demonstrated that ET_B R upregulation in the intima and downregulation in the media were associated with adaptive vasodilation in pregnant rats [16]. Current evidence has shown that ET_B R expression/activity in ECs and VSMCs could be regulated by cytokines such as TNF- α through the protein kinase C (PKC), extracellular-regulated protein kinase 1 and 2 (ERK1/2) and NF- κ B pathways [17, 21-23]. Thus, we speculate that placental ischemia induced inflammatory cytokine production may regulate the redistribution of ET_B R expression in the intima and media of the artery and contribute to the pathogenesis of preeclampsia.

The reduction of uterine perfusion pressure (RUPP) rat model mimics some of the features of preeclampsia, including PIH, endothelial dysfunction, and increased vasoconstriction [24]. The present study aimed to explore the endothelin receptor expression change between the RUPP and NP rat models, which may provide new insights into the underlying molecular mechanisms of PIH.

Materials and Methods

Animals

The animal experiments in the present study were approved by the Animal Care and Use Committee of Yangzhou University. Sprague-Dawley rats (12 weeks of age) were purchased from HFK Bioscience (Beijing, China). Rats were maintained in a pathogen-free barrier facility at Yangzhou University.

On day 14 of pregnancy, rats underwent either sham-operations or surgical procedures to reduce the uteroplacental perfusion pressure. On day 18 of pregnancy, the mean arterial pressure (MAP) was recorded. The RUPP procedure and measurement of the MAP were performed as described previously [20].

Isolation of single vascular smooth muscle cells and measurement of cellular contraction

Single aortic VSMCs were freshly isolated, and cellular contraction was measured as previously described [16]. Freshly isolated aortic VSMCs were placed on a slide and the cell images were acquired by a Nikon digital camera and image acquisition software. After measuring the length of resting cells, the cells were stimulated with ET-1 (10^{-6} M) for 10 min, and the changes in cell length were measured. Then selective ET_AR antagonist BQ123 (10^{-6} M), ET_BR antagonist BQ788 (10^{-6} M) and the ET_BR agonists IRL-1620 (10^{-6} M) were added together with ET-1 separately, and the cell lengths were recorded. The magnitude of cellular contraction was expressed as $(L_i - L_f)/L_i \times 100\%$, where L_i represents the initial cell length and L_f represents the final cell length.

ELISA

The plasma concentration of ET-1 and the related inflammatory factors were determined by ELISA Kit following the manufacturer's protocols: Rat Endothelin 1 ELISA Kit (Abcam, ab46070), Rat ET-1 ELISA kit (Abcam, ab46070), Rat IL-1 ELISA Kit (Abcam, ab100768), Rat IL-2 ELISA Kit (Abcam, ab221334), Rat IL-6 ELISA Kit (Abcam, ab100772), Rat IL-6 ELISA Kit (Elabscience, E-EL-R0560), Rat IL-10 ELISA Kit (Abcam, ab113349), and Rat MCP1 ELISA Kit (Abcam, ab219045). Absorbance was recorded using a microplate reader (Thermo Scientific).

Immunohistochemistry and immunofluorescence staining

Three sections from each graft were stained with HE and the thickness was measured. Immunohistochemistry and immunofluorescence was performed as described previously [16]. The sections were incubated overnight with anti-ET_AR (Abcam, ab85163) or anti-ET_BR (Abcam, ab117529) for immunohistochemistry. To evaluate the expression of ET_AR in endothelial and smooth muscle cells, the sections were double stained by anti-CD 31 (Abcam, ab28364) and anti- α -SMA (Abcam, ab28052) with anti-ET_BR (Abcam, ab117529) by immunofluorescence. Negative controls were included using isotype-matched control antibodies (Abcam). In the control experiment, either the primary antibody or secondary antibody was omitted. The stained arterial segments were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments, Inc, NY, USA) and analyzed by IMAGE-PRO PLUS. For quantification, the mean optical densities of the ET_AR and ET_BR staining in the immunohistochemistry images were determined using IMAGE-PRO PLUS. In each section, the mean optical density was measured at 4 preset areas, and the mean optical density was obtained from the four areas.

Western blot

The aortas were frozen in liquid nitrogen and homogenized in a cell extract denaturing buffer that contained a phosphatase inhibitor cocktail and protease inhibitor cocktail. The cultured cells were resolved using RIPA Lysis Buffer. The antibodies for ET_AR (Abcam, ab85163), ET_BR (Abcam, ab117529), phospho-NF- κ B p65 (Abcam, ab32092) and NF- κ B p65 (Abcam, ab16502) were used as primary antibodies. Secondary antibodies were HRP-conjugated anti-rabbit IgG or rabbit anti-goat IgG (Biosharp). All antibodies were used at a dilution of 1:1,000 and the experiments were repeated 3 times. An anti-GAPDH antibody (Bioworld, AP0063) was used for normalization. The western blot band intensity was determined using IMAGE-PRO PLUS.

Data analysis

Data are expressed as the mean $\pm$ SEM. Unpaired and paired Student's *t* tests were performed for two-group comparisons and one-way ANOVA was used for multi-group comparisons. The Bonferroni correction was used to identify the difference between each two groups after one-way ANOVA test. $P < 0.05$ was considered significant. All experiments were repeated 3-5 times.

Results

The mean arterial pressure was higher in RUPP rats than NP rats without significant vascular remodeling

On gestation day 18, RUPP in pregnant rats resulted in significant increases in MAP, which was approximately 20 mmHg higher than in NP rats (Fig. 1A). The thoracic aortas of RUPP rats and NP rats were harvested for pathological examination. HE staining revealed no significant difference in the thicknesses of the intima and media between the RUPP and

NP rats (Fig. 1B). To investigate the role of ET-1 in the PIH process, the plasma ET-1 levels of RUPP and NP rats were measured by ELISA. However, the ET-1 level in RUPP rats only increased slightly, and there was no statistical significance between the two groups (Fig. 1C).

Thus, it was determined that in advanced stages of PIH, RUPP induced hypertension might be related to the distribution of the ET receptors rather than the concentration of ET-1 or vascular remodeling.

ET_BR expression was elevated in the media of RUPP rats but significantly reduced in the intima, while the ET_AR level remained relatively stable

To investigate the role of ET receptors in the pathogenesis of PIH, the level and location of the two types of receptors were detected in the aortas of RUPP and NP rats by Western blotting and immunohistochemistry staining. Results showed that ET_AR and ET_BR protein levels were slightly, but not significantly, elevated in the aortas of RUPP rats (Fig. 2A). In addition, the intima was detached from the aorta and the protein level was measured again. The ET_BR level was much greater in RUPP rats compared to controls, while the level of ET_AR was similar to the first experiment (Fig. 2B). These results suggested that ET_BR was mainly expressed in the intima of NP rats, was significantly upregulated in the media of RUPP rats, and was downregulated in the intima.

Then, the aortas of RUPP and NP rats were stained by immunohistochemistry staining, and the results confirmed our previous hypothesis. ET_BR was mainly expressed in the intima of NP rats, and few ET_BR positive cells were detected in the intima of RUPP rats. However, ET_BR were significantly elevated in the media of RUPP rats, while ET_AR was detected in both groups without obvious differences (Fig. 2C).

ET_BR expression decreased with the reduction of the endothelial cell density in the intima and significantly increased in media VSMCs of RUPP rats

It has been reported that ET_BR is mainly expressed in the intima of NP rats and is significantly lower in RUPP rats [20]. To investigate the reason for ET_BR downregulation in the intima of RUPP rats, the double immunofluorescence with CD 31 and ET_BR antibodies was performed in the aortas of both groups. Most of the CD 31 positive cells in the intima were positive for ET_BR. The number of CD 31 positive cells in RUPP rats was significantly lower than in the NP rats (Fig. 3A). The aorta sections were also stained with

Fig. 1. (A), The mean arterial pressure in RUPP rats was approximately 140 mmHg, higher than that of NP rats. HE staining revealed no significant difference in the thickness of the intima and media between RUPP and NP rats. (C), The plasma ET-1 level of RUPP rats was slightly higher than that of NP rats, but there was no statistical significance between the two groups. (* P value of t test <0.05).

Fig. 2. (A), There was no significant difference in the protein levels of ET_AR and ET_BR between the aortas of RUPP and NP rats. (B) When the intima was detached from the aorta, the ET_BR level was found to be much greater in RUPP rats than in NP rats, but there was no obvious change in the ET_AR level. (C), ET_BR was mainly expressed in the intima of NP rats, and few ET_BR positive cells were detected in the intima of RUPP rats. However, ET_BR were significantly elevated in the media of RUPP rats, and the ET_AR was detected in both groups without obvious differences. (* P value of t test <0.05).

α-SMA and ET_BR antibodies. The majority of the α-SMA positive cells in RUPP rats were also positive for ET_BR, which was much greater than the numbers observed in NP rats (Fig. 3B).

These results confirmed that ET_BR was mainly expressed by ECs in NP rats, and the expression of ET_BR was correspondingly downregulated in RUPP rats alongside a reduction in the number of intima endothelial cells. However, ET_BR expression was significantly increased in the media VSMCs of RUPP rats.

The increased ET_BR in VSMCs contributed to cellular contraction and enhanced the function of ET_AR

To test the function of ET_AR and ET_BR in VSMCs, single aortic VSMCs were freshly isolated from the aortas of NP and RUPP rats. Spindle-shaped cells ≥ 50 μm in length were selected

Fig. 3. (A), Most of the CD 31-positive cells in the intima were positive for ET_BR, but the number of CD 31-positive cells was significantly lower in RUPP rats than in NP rats. (B), The majority of the α-SMA positive cells in RUPP rats were also positive for ET_BR, which was much greater than in NP rats.

for study. There was no significant difference between the two groups. ET-1 (10^{-6} M) caused greater contraction in VSMCs from RUPP rats than those from NP rats (Fig. 4A). When the VSMCs of RUPP and NP rats were pretreated with the ETAR antagonist BQ123 (10^{-6} M) for 10 min, ET-1-induced contraction was inhibited in both groups. However, the ET-1-induced contraction in VSMCs from RUPP rats was greater than the contraction from NP rats (Fig. 4B). When VSMCs were pretreated with both BQ123 (10^{-6} M) and the ET_BR antagonist BQ788 (10^{-6} M), ET-1-induced contraction was almost negligible in both groups (Fig. 4C).

According to the previous results, we deduced that the upregulated ET_BR in smooth muscle cells enhanced the ET-1-induced contraction in RUPP rats. To test our hypothesis, the ET_BR agonist IRL-1620 (10^{-6} M) was used. It caused a persistent contraction in both groups, which was much more significant in the VSMCs of RUPP rats than in those of NP rats (Fig. 4D).

TNF-α induced upregulation of ET_BR in cultured VSMCs through NF-κB related pathways

The NF-κB signaling pathway has been reported to mediate ET_BR expression in VSMCs of resistance arteries [25]. Thus, we attempted to elucidate whether NF-κB is involved in the upregulation of ET_BR in the aorta VSMCs of RUPP rats. First, the NF-κB and p-NF-κB p65 levels of aortas from RUPP and NP rats without intima were determined by western blotting. The p-NF-κB p65 level was significantly higher in media VSMCs from RUPP rats than those from NP rats (Fig. 5A).

Then, the concentrations of several proinflammatory cytokines related to PIH were measured by ELISA [6]. Similar to previous reports, several cytokines were increased more

Fig. 4. (A), ET-1 caused greater contraction in the VSMCs from RUPP rats than from NP rats. (B), ETAR antagonist BQ123 alleviated ET-1 induced contraction in both groups. However, ET-1-induced contraction was still greater in VSMCs from RUPP rats than from NP rats. (C), BQ123 and the ETBR antagonist BQ788 completely abolished the ET-1-induced contraction in both groups. (D), The ETBR agonist IRL-1620 caused a persistent contraction in both groups, which was much more significant in VSMCs from RUPP rats than from NP rats. (* P value of t test <0.05).

than 1.5-fold in RUPP rats compared with NP rats (Fig. 5B). TNF- α has been reported as an important contributor to the pathogenesis of PIH and it promotes the activation of NF- κ B [10]. Therefore, we postulated that some of the elevated cytokines such as TNF- α could upregulate the expression of ET_BR in VSMCs through the NF- κ B pathway during the PIH process.

To test our speculation, VSMCs from the aortas of NP rats were cultured *in vitro*. The isolated VSMCs from NP rats were co-incubated with TNF- α for 24 hours, and then p-NF- κ B p65 and ET_BR protein levels were measured. Both p-NF- κ B p65 and ET_BR expression increased with an increased concentration of TNF- α . When NF- κ B activation was blocked by an NF- κ B inhibitor (BAY11-7082), TNF- α induced ET_BR upregulation was abolished (Fig. 5C). Thus, we determined that TNF- α upregulated the expression of ET_BR in VSMCs through the NF- κ B signaling pathway.

Discussion

Our results proved that: (1) the increased plasma TNF- α expression in RUPP rats could induce upregulation of ET_BR in VSMCs through the NF- κ B signaling pathway; (2) redistribution of ET_BR but not ET_AR was associated with the pathogenesis of PIH; and (3) the expression of ET_BR in media VSMCs enhances vasoconstriction in RUPP rats.

Fig. 5. (A), Although the level of NF-κB was similar in VSMCs from RUPP rats and NP rats, the p-NF-κB p65 level was significantly higher in RUPP rats. (B), The plasma level of TNF-α, IL-6, IL-8, and MCP-1 increased more than 1.5-fold in RUPP rats compared to NP rats. (C), TNF-α dose-dependently promoted both p-NF-κB p65 and ET_BR expression. When NF-κB activation was blocked by an NF-κB inhibitor (BAY11-7082), the TNF-α-induced ET_BR upregulation was abolished. (* value of t test <0.05, # Bonferroni corrected level of significance <0.05).

Several studies have proved that treatment of RUPP rats with an ET_AR antagonist decreased blood pressure, suggesting that ET_AR was responsible for PIH [15]. However, in our study, there was no significant difference in ET_AR expression between the aortas of RUPP and NP rats. Although endothelial ET_BR induces vasodilation and shows vasoprotective effects in neointima hyperplasia, upregulation of ET_BR in VSMCs mediates the vasoconstricting effects and is thought to be associated with many vascular diseases [13, 16-19, 27, 28]. Mazzuca *et al.* reported that downregulation of ET_BR in endothelial cells played an important role in PIH, yet they also found that ET_BR levels were elevated in endothelium-denuded microvessels of RUPP rats [20]. Our previous results demonstrated that in NP rats, ET_BR expression is upregulated in the intima and downregulated in the media [16]. In contrast, in this study, we found that ET_BR expression was significantly decreased in the intima but increased in media VSMCs of RUPP rats, which enhanced VSMC contractions. Thus, we concluded that the redistribution of ET_BR in the intima and media in the advanced stages PIH was related to the pathogenesis of PIH in RUPP rats. This result may explain, at least in part, why ET-1 plays a greater role in the progression of PIH than in the initiation of the disease.

Several lines of evidence have suggested that ischemic placenta contributes to endothelial and vascular smooth muscle cell activation and dysfunction of maternal circulation by enhancing the synthesis of cytokines, thereby increasing vascular resistance and arterial pressure; however, the underlying mechanism is not clear [4]. In our study, it was also found that the plasma concentration of several cytokines increased. Thus, we tried to investigate whether the increased cytokines in plasma were responsible for the redistribution of ET_BR. TNF-α is an inflammatory cytokine that plays a key role in the pathogenesis of PIH and it reportedly increases approximately 2-fold in the plasma of women with preeclampsia [29]. Davis *et al.* found that chronic infusion of TNF-α into normal pregnant rats significantly increased their mean arterial pressure [29, 30]. Furthermore, LaMarca *et al.* suggested that ET-1 plays an important role in mediating TNF-α induced hypertension in pregnant rats.

However, they found that TNF- α infusion did not increase the plasma levels of endothelin [23, 29]. Thus, it is postulated that TNF- α may be involved in the regulation/redistribution of the ET receptors.

In our study, the plasma level of several cytokines increased in RUPP rats, including TNF- α . To identify whether elevated TNF- α can induce the upregulation of ET_BR in VSMCs, TNF- α was added into the culture medium of VSMCs at different concentrations; ET_BR expression in VSMCs increased significantly with a concentration-dependent trend. The results of the *in vitro* experiments confirmed our hypothesis; consequently, we tried to determine the mechanism through which TNF- α promoted the expression of ET_BR in VSMCs.

NF- κ B is a pivotal transcriptional factor that is essential for the expression of several genes that are involved in inflammation [31, 32]. It has been shown that NF- κ B signaling could mediate ET_BR expression in VSMCs in cardiovascular disease patients [21, 33]. Therefore, we tried to investigate whether NF- κ B was the downstream transcriptional factor for the TNF- α induced ET_BR upregulation in the process of PIH. NF- κ B is composed of p65 and p50 subunits, and is located in the cytoplasm as an inactive complex with the I κ B family. When the I κ B is removed from the I κ B-NF- κ B complex under a variety of stimuli, the p65-p50 heterodimer is released and translocates into the nucleus. There is an alternative pathway for NF- κ B activation which involves phosphorylation and acetylation of p65 [22]. In our study, we found that the level of p-NF- κ B p65 was much higher in RUPP rats, which indicated that the activation of NF- κ B might be related to ET_BR upregulation. In the subsequent experiments, it was found that TNF- α could promote the expression of p-NF- κ B p65 and ET_BR. When NF- κ B activation was blocked by an NF- κ B inhibitor (BAY 11-7082), which inhibited TNF- α induced I κ B phosphorylation, the upregulation of both p-NF- κ B p65 and ET_BR was abolished. Thus, we conclude that NF- κ B mediated ET_BR upregulation in VSMCs during PIH progression.

In addition to the upregulation of ET_BR in VSMCs, downregulation of endothelial ET_BR in the intima of RUPP rats may also be related to TNF- α . In this study, we found that the ET_BR expression decreased with the number of ECs in the intima. It has been shown that TNF- α can induce damage and apoptosis in ECs, which may be a reason for the downregulation of endothelial ET_BR [4].

TNF- α plays an important role in the redistribution of ET_BR between the intima and media, which contributes to RUPP-induced PIH. Nevertheless, it should be noted that NF- κ B is associated with many inflammatory factors, and the mechanism underlying the ET_BR regulation is very complex. For this reason, there may be other cytokines which were also involved in the effects of TNF- α in RUPP rats, and these cytokines should be further investigated in future studies.

Conclusion

RUPP-induced redistribution of ET_BR resulted in decreased vasodilatation and enhanced vasoconstriction, which increased the arterial resistance and thereby arterial pressure. Understanding the underlying molecular mechanisms that are responsible for PIH may provide novel opportunities for treatment.

Disclosure Statement

The authors declare no Disclosure Statement.

Acknowledgements

This work was supported in part by the National Nature Science Foundation of China (81460239), Ningxia Nature Science Foundation (NZ14179), and the Key Research Project of Yangzhou Social Development (YZ2016063).

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