Diverse Mechanisms of Endothelium-Derived Hyperpolarizing Factor-Mediated Dilatation in Small Myometrial Arteries in Normal Human Pregnancy and Preeclampsia

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ABSTRACT

This ex vivo study focuses on the mechanisms of endothelium-dependent dilatation in the uterine circulation of normal pregnancy (n = 12) and in women with preeclampsia (n = 12). Arteries (internal diameter, ~250 μm) isolated by myometrial biopsy from women undergoing planned cesarean delivery or delivery as a result of the deterioration of preeclampsia were studied using a wire myograph. Bradykinin-induced dilatation was assessed in the presence and/or absence of pharmacological inhibitors to determine the contribution of nitric oxide and endothelium-derived hyperpolarizing factor (EDHF), as well as that of EDHF-mediated pathways such as myoendothelial gap junctions (MEGJs) and products of arachidonic acid, H₂O₂ and cytochrome P450 2C9 (CYP2C9). Transmission electron microscopy was used to visualize morphological prerequisites for MEGJs. In normal pregnancy, EDHF through MEGJs appeared to be a predominant mediator conferring endothelium-dependent relaxation in small myometrial arteries. In preeclampsia, bradykinin-induced relaxation was reduced via compromised EDHF-type responses, in which the contribution of MEGJs became negligible. The attenuated role of MEGJs to endothelium-dependent relaxation was partly compensated through the contribution of H₂O₂ or other endothelium-derived relaxing factors. CYP2C9 products of arachidonic acid had no effect on EDHF-type relaxation in arteries of women with normal pregnancy or with preeclampsia. We suggest that EDHF-type responses via MEGJs are primarily targeted in small myometrial arteries in women with preeclampsia. This could significantly contribute to the impaired uteroplacental blood flow in this disorder.

ENDOTHELUM-DERIVED HYPERPOLARIZING FACTOR, GAP JUNCTIONS, MYOMETRICAL ARTERIES, NITRIC OXIDE, PREGNANCY

INTRODUCTION

Up to 5% of women during a first pregnancy develop preeclampsia (PE), a severe, hypertensive complication in the second half of pregnancy. PE places women at risk for kidney [1], liver, and heart failure as well as for seizures and/or stroke, and it is usually accompanied by a higher rate of growth-restricted neonates and increased perinatal mortality [2]. Evidence is accumulating for a pathogenic model of PE, whereby a deficiency in the trophoblast invasion of spiral arteries in the placental bed leads to a poorly perfused uteroplacental unit and secretion of factors by the placenta into the maternal circulation. These factors can cause “activation” of the vascular endothelium, with the clinical syndrome of PE resulting from widespread changes in endothelial cell (EC) maintenance [3, 4].

Although generalized maternal endothelial dysfunction is considered to be a hallmark of PE, functional investigations of small arteries have provided equivocal results. Because it is assumed that PE is instigated via an abnormal uteroplacental setting, the myometrial arteries could serve as a primary target. Hypothetically, endothelial dysfunction in these arteries might be the most severe, and because uterine circulation is of unique importance during pregnancy, the abnormalities in the myometrial arteries might further aggravate the disease process. The results of ex vivo studies are controversial, however, with reported findings ranging from almost total abolishment [5–7] to reduction [8, 9] to preservation [7, 10] of endothelium-dependent responses.

Several endothelium-derived vasodilator substances—nitric oxide (NO), prostacyclin (PGI₂), and endothelium-derived hyperpolarizing factor (EDHF)—are involved in endothelium-dependent relaxation at the level of resistance vasculature. Considering the many ways by which availability and/or synthesis of NO could be reduced in PE (e.g., enhanced generation of superoxide anion could scavenge NO), the hypothesis regarding up-regulation of “backup” endothelial pathways like EDHF seems to be reasonable. However, data about the compromised pathways in endothelium-dependent dilatation at the level of small arteries in PE are also rather conflicting, indicating the possible involvement of NO [11–13], PGI₂ [14, 15], and particularly, EDHF [10, 16], the contribution of which is increasingly appreciated for small artery maintenance [17, 18].

The EDHF-type responses may be mediated simultaneously by several factors or pathways, depending on the type of vasculature, the species, and the physiological environment [17]. We recently showed that myoendothelial gap junctions (MEGJs), either alone or in combination with H₂O₂ and/or cytochrome P450 2C9 (CYP2C9) products of arachidonic acid, are involved with EDHF-mediated responses in small subcutaneous arteries in PE [16], whereas MEGJs alone conferred the EDHF pathway in women with a normal pregnancy (NPG) [19]. Although the contribution of MEGJ in the endothelium-dependent response to bradykinin (BK) has been suggested in myometrial arteries from an NPG [20], to our knowledge...
nobody has clarified the mechanisms behind EDHF-type responses in the uterine circulation in PE.

Based on our previous findings in the peripheral circulation, we hypothesize that EDHF is primarily targeted in PE, and that pathways conferring EDHF-type responses in small myometrial arteries in this disorder differ from those obtained in arteries from NPG. Therefore, in the present study, we aimed 1) to compare the pattern of endothelium-dependent responses in small arteries from uterine circulation between NPG and PE, 2) to assess the contribution of EDHF versus NO in these responses, and 3) to determine the mechanisms involved in EDHF-mediated relaxation.

**MATERIALS AND METHODS**

**Subjects**

The present study was approved by the Ethics Committee at the Karolinska University Hospital, Huddinge, and all women gave informed consent before participation. PE was defined as blood pressure of 140/90 mmHg or higher and proteinuria exceeding 300 mg/24 h or ≥2+ on a random urine sample in the absence of urinary tract infection after 20 weeks of gestation in previously normotensive, nonproteinuric women. Exclusion criteria for women with PE and women with NPG included diabetes, established atherosclerosis, chronic hypertension, malignancy, hepatic or renal failure, systemic infection, autoimmune diseases, and recent surgery or trauma.

Twelve women with PE undergoing caesarean delivery for deterioration of PE were included. During the operation, myometrial biopsy samples from the upper edge of the transverse uterine incision were collected and immediately placed in ice-cold physiological salt saline (PSS). Myometrial biopsy specimens were also obtained from 12 women with NPG undergoing planned caesarean section because of breach presentation (n = 2), previous caesarean section (n = 6), and psychological indications (n = 4).

**Experimental Setup**

Using a stereomicroscope, small myometrial arteries were dissected from the biopsy specimens. From each specimen, two or more arteries of similar size were isolated and mounted on two stainless-steel wires (diameter, 25–40 μm) in the organ baths of a four-channel wire myograph (multimyograph, model 610; Danish Myo Technology) as described previously using isolated small subcutaneous arteries from women with NPG and women with PE [16, 19].

**Experimental Protocols**

Norepinephrine (NE; 3 μmol/L) or vasopressin (VP; 3 nmol/L) were used for preconstriction. Arteries that did not produce a sustained, steady constriction to NE upon the mechanical responses at the final bath concentrations [19] were preconstricted again, and a second concentration-response curve for BK was established whether adequate NOS inhibition was achieved with L-NAME, a reversible inhibitor of nitric oxide synthase (NOS) inhibitor Nω-nitro-arginine-methyl ester (l-NAME; 300 μmol/L) and the cyclooxygenase (COX) inhibitor indomethacin (Indo; 10 μmol/L) to block the production of NO and PGI₂, respectively. When and subsequent arteries were preconstricted again, and a second concentration-response curve for BK was obtained. As in previous publications using isolated subcutaneous arteries [16, 19, 21], the term EDHF in the present study refers to the l-NAME- and Indo-insensitive component of endothelium-dependent vasodilatation to BK. To establish whether adequate NOS inhibition was achieved with l-NAME, a concentration-response curve for BK was assessed in a separate set of arteries used in different experimental protocols. The force (mN) developed per length (mm) of artery segment during incubation with different agents in arteries used in different experimental protocols was determined by one-way ANOVA. Mann-Whitney U-test was used to compare clinical data between two groups. All data are presented as the mean ± standard error of the mean or median and the range.

**Chemicals**

The composition of PSS was as follows: 119 mmol/L of NaCl, 4.7 mmol/L of KCl, 2.5 mmol/L of CaCl₂, 1.17 mmol/L of MgSO₄, 25 mmol/L of NaHCO₃, 1.18 mmol/L of KH₂PO₄, 0.026 mmol/L of ethylenediaminetetraacetic acid, and 5.5 mmol/L of glucose. The chemicals were obtained from Sigma. To prepare stock solution, the substances were dissolved in distilled water. Indo was dissolved in ethanol, and 18-GA was dissolved in dimethyl sulfoxide. All concentrations represent the final steady-state concentrations in the chamber. Our previous study showed that the solvent used has no effect upon the mechanical responses at the final bath concentrations [19].

**Data Analysis**

The force (mN) developed per length (mm) of artery segment during application of each concentration of vasoactive compound was calculated using Myodata (Danish Myo Technology). All absolute measurements were corrected for the baseline force developed by the arteries. The relaxation to BK was calculated as a percentage of the contraction. Negative log concentration required to cause 50% of the maximum response (pEC₅₀) was calculated by non-linear-regression analysis. Data were then transferred to STATISTICA (version 8.0; StatSoft), in which all statistical analyses were performed. One-way ANOVA for repeated measures was used to compare concentration-response curves before and after incubation with different pharmacological inhibitors in NPG and PE. Statistical differences in pEC₅₀ values before and after incubation with different agents in arteries used in different experimental protocols were determined by one-way ANOVA. Mann-Whitney U-test was used to compare clinical data between two groups. All data are presented as the mean ± SEM; unless indicated in the text, n represents the number of patients. Significance was set at the 5% level for all comparisons.

**RESULTS**

Clinical data for the women with PE and the women with NPG are presented in Table 1. As expected, the gestational
length was shorter, and the infant birth weight was lower, in the PE group compared to NPG group.

In total, 43 myometrial arteries with an internal diameter of 259 ± 15 µm were dissected from 12 biopsy specimens of women with PE, and 38 arteries with an internal diameter of 257 ± 11 µm were dissected from 12 biopsies specimens of women with NPG. No differences were found in the magnitude of contraction to high-K⁺ (124 mmol/L) PSS (2.5 ± 0.2 mN/mm in PE vs. 2.2 ± 0.2 mN/mm in NPG) or in preconstriction level with NE (2.2 ± 0.2 mN/mm [n = 16] in NPG) or VP (2.7 ± 0.2 mN/mm [n = 29] in PE vs. 2.2 ± 0.2 mN/mm [n = 22] in NPG) between the arteries used in different experimental protocols. The type of vasoconstrictor used did not influence the response to BK (relaxation at 3 µM of BK: 98 ± 1 after preconstriction with NE (n = 5) vs. 97 ± 1 after preconstriction with VP [n = 7] in NPG [Fig. 1], and 80 ± 7 [n = 4] vs. 70 ± 7 [n = 8], respectively, in PE; P > 0.05), thereby ruling out any specific contribution of the individual contractile pathway to our results.

**BK-Induced Relaxation in NPG and PE**

Myometrial arteries from women with NPG relaxed substantially, up to 96–100% (Fig. 2), in response to increasing concentrations of BK. The time-control experiments showed a slight reduction in tone after preconstriction and following a 20-min time interval of less than 8% in NPG and up to 11% in PE, which was comparable between the groups (Fig. 2). Incubation with L-NAME plus Indo resulted in a significant reduction of relaxation to BK compared with that obtained with PSS (pEC₅₀: 7.0 ± 0.1 in L-NAME plus Indo vs. 8.0 ± 0.1 in PSS; P < 0.001). The concentration of NOS inhibitor was sufficient to inhibit NO synthesis, because endothelium-
Mechanism of EDHF-type Responses in NPG Versus PE

The gap junction inhibitor 18-αGA markedly reduced BK-induced relaxation in NPG arteries after incubation with L-NAME (relaxation at 3 μmol/L of BK: 18% ± 8% in 18-αGA plus L-NAME plus Indo vs. 76% ± 4% in L-NAME plus Indo; P < 0.001) (Fig. 3). The contribution of MEGJs, when assessed by incubation of myometrial arteries with 18-αGA, to EDHF-type responses was reduced in women with PE compared with NPG (residual relaxation at 3 μmol/L of BK: 45% ± 10% in PE vs. 18% ± 8% in NPG; P < 0.001) (Fig. 3).

Catalase, which dismutates H₂O₂ to form water and oxygen, significantly attenuated the response of myometrial arteries to BK after NOS and COX inhibition in PE (Fig. 4A); however, it had no effect in arteries from women with NPG (Fig. 4B). Thus, EDHF-type responses after incubation with catalase were reduced in arteries from women with PE versus those from women with NPG (42% ± 10% in PE vs. 72% ± 7% in NPG; P < 0.05) (Fig. 4), suggesting an enhanced contribution of endogenous H₂O₂ to EDHF-mediated relaxation in PE.

Sulfaphenazole, an inhibitor of CYP2C9 epoxygenase, was used to assess the contribution of arachidonic acid metabolites, most probably 11,12-epoxyeicosatrienoic acid, to EDHF-mediated relaxation in arteries from women with PE and women with NPG. Sulfaphenazole did not influence EDHF-type relaxation in either group (46% ± 8% after sulfaphenazole plus L-NAME plus Indo vs. 56% ± 5% after L-NAME plus Indo in PE; 82% ± 5% and 76% ± 4%, respectively, in NPG; P > 0.05) (Fig. 5).

Transmission Electron Microscopy

The diameter of arteries used for morphological evaluation was compatible to that used for functional studies. Analysis of the transmission electron microscopic (TEM) images focused on morphological prerequisites for the gap junctions between ECs and smooth muscle cells (SMCs) in myometrial arteries from women with PE and women with NPG. TEM images relevant for the main study objectives are presented in Figure 6. The main criteria for identification of MEGJs was the presence of the characteristic pentalaminar membrane structure at points of cell-cell contact, wherever the central region had a higher electron opacity than the inner parts [22].

In myometrial arteries, ECs and SMCs were separated by the relatively large internal elastic lamina. ECs of myometrial arteries sent protrusions toward SMCs, the majority of which were rather short, resulting in rarely observed EC-SMC contacts (Fig. 6, A–D). No cases of observed EC-SMC contacts had signs of the characteristic pentalaminar structures, or these signs were unable to be detected because of an ill-defined electron opacity (Fig. 6, E and F). Therefore, it is
feasible that rarely observed contacts between two types of cells within the vascular wall of myometrial arteries should be referred to as myoendothelial associations rather than as MEGJs.

In the endothelial layer, long sections of well-defined gap junctions between ECs were observed in myometrial arteries from women with NPG and women with PE (Fig. 6G).

**DISCUSSION**

Here, we report that endothelial dysfunction in PE, defined as reduced vasodilator response to the endothelium-dependent agonist BK, is apparent in the isolated small arteries from women with NPG and women with PE (Fig. 6G).

**FIG. 4.** Concentration-response curves to BK obtained after incubation with L-NAME in combination with Indo (L-NAME+Indo) alone or in combination with catalase (Catalase+L-NAME+Indo) in myometrial arteries isolated from PE (A) and NPG (B) women. *P < 0.05, L-NAME+Indo vs. Catalase+L-NAME+Indo.

**FIG. 5.** Concentration-response curves to BK obtained after incubation with L-NAME in combination with Indo (L-NAME+Indo) alone or in combination with sulfaphenazole (Sulph+L-NAME+Indo) in myometrial arteries isolated from PE and NPG women. *P < 0.05, PE vs. NPG.
indicates that in this disorder, endothelial disturbances primarily involve the EDHF-mediated pathway. In the present study, we dissect the mechanisms behind the reduced contribution of EDHF to endothelium-dependent dilatation in these arteries and show, to our knowledge for the first time, that disturbances at the level of MEGJs and the enhanced contribution of H$_2$O$_2$ may play an important role.

We found that an almost 100% relaxation to the endothelium-dependent agonist BK in myometrial arteries from women with NPG could serve as an indicator for high vasodilator capacity of uterine resistance vasculature, which is associated with a substantial decrease in vascular resistance and an increase in blood flow to the uterus in these women [23]. This observation could be strengthened by our previous report [24], in which an evidently lower (55%) endothelium-dependent dilatation to BK was observed in isolated myometrial arteries from nonpregnant women. In addition, a comparison with our previous investigations in subcutaneous arteries on endothelium-dependent dilatation to BK in women with NPG [16, 19] may imply that the uterine circulation is more sensitive to this agonist, an observation in line with the fact that in these women, the relative decrease in uterine vascular resistance greatly exceeds the relative fall in systemic vascular resistance [23]. Our results contrast with those of studies from another group [5, 6, 8, 25, 26], in which the response to 1 µM BK in myometrial arteries ranged from 55% to 75%, but our results concur with more recent data showing a compatible relaxation not only from BK [27] but also from acetylcholine (ACh) and substance P [7]. The reasons behind this discrepancy are far from clear, although differences in preconstriction level, size of the artery used, levels of technical skills, and startup normalization procedure using a wire-myography setup all could have influenced the extent of relaxation [28–31]. However, our previous reports support the hypothesis of EDHF malfunction in PE. Although under pressurized conditions, demonstrated no impairment of overall endothelium-dependent dilatation in PE [10]. The latter observation therefore is more relevant to our previous finding in subcutaneous arteries, in which BK-induced relaxation was diminished only after incubation with L-NAME plus Indo and, as such, strongly supports the hypothesis of EDHF malfunction in PE. Although one previous study suggested an impairment of EDHF-type responses in myometrial arteries in PE, those vessels, which were under pressurized conditions, demonstrated no impairment of overall endothelium-dependent dilatation in PE [10].

It is generally accepted that the vasodilatory capacity of the endothelium is achieved by release of endothelium-derived vasodilators: NO, PGI$_2$, and EDHF. In the present study, we focus on the relative contribution of NO versus EDHF, deliberately omitting evaluation of COX products because of minor contribution (if any) to BK-induced relaxation in myometrial arteries [10]. Consistent with earlier reports, we also found that NO is involved in BK-induced relaxation of myometrial arteries from women with NPG [10, 20, 27], although EDHF seems to be a predominant mediator for endothelium-dependent response. Indeed, the contribution of EDHF, estimated as residual BK-induced relaxation after incubation with L-NAME plus Indo, was up to 78% of the total response.

By using a pharmacological approach (i.e., 18-zGA), we have confirmed our previous finding for the potential role of MEGJs in EDHF-type responses in small arteries in NPG [16, 20]. It should be noted that some debate exists with respect to the specificity of 18-zGA when uncoupling gap junction channels [28–31]. However, our previous reports support the absence of nonspecific effects [16, 21]. Thus, 18-zGA was equally potent in virtual reduction of EDHF-type responses in myometrial and, as reported previously, in subcutaneous arteries [19], suggesting a crucial contribution of MEGJs to BK-induced relaxation in NPG in both uterine and peripheral circulations. Whereas characteristic pentalaminar membrane structures in TEM images further strengthened our pharmaco-

logical support for MEGJs in subcutaneous arteries [16], these structures were not apparent in myometrial arteries, a morphological observation that concurs with a previous report [32]. In this respect, speculation exists that at the level of MEGJs, only individual gap-junctional channels, rather than plaques, are responsible for cell-cell communication. This structural feature could aggravate the visualization of MEGJs at the TEM level [33]. This explanation concurs with the general belief that a remarkably limited number of MEGJs and an extremely low density of the gap-junctional channels detected morphologically in the TEM images could serve as an additional explanation for failure to visualize MEGJs by electron microscopy in spite of intact functional coupling [34].

Comparison of endothelium-dependent relaxations between myometrial arteries in women with and without PE revealed an obvious endothelial malfunction in the uterine circulation in PE. This contrasts with our previous report on subcutaneous vessels, in which reduced response to BK was observed only after incubation with NOS and COX inhibitors [16]. Thus, it could be anticipated that myometrial arteries are particularly susceptible to the toxic environment that occurs in PE (e.g., pro-oxidative milieu and misbalance of pro- and antiangiogenic factors). The enhanced susceptibility for impairment in these vessels concurs with observations in which ex vivo incubation with PE plasma had an inhibitory effect on BK-induced dilatation in myometrial, but not omental, arteries from women with NPG [35] and with those studies of PE demonstrating severe reduction, or even total abolishment, of endothelium-dependent relaxation to ACh [7], BK [5, 6, 8, 25], or flow [9, 36] in this particular vascular bed.

In PE, the main compromised pathway of endothelium-dependent relaxation is still a matter of debate. Whereas NO seems to be the most attractive candidate to be compromised in PE, particularly with stimulus as shear stress [11, 36], in general, its role is still uncertain [4, 10, 16, 37, 38]. The present study indicates that EDHF-type response rather than NO-mediated relaxation in response to BK serves as the main compromised pathway in PE. Indeed, in myometrial arteries, the reduced response to BK persists to the same extent after incubation with L-NAME plus Indo and, as such, strongly supports the hypothesis of EDHF malfunction in PE. Although one previous study suggested an impairment of EDHF-type responses in myometrial arteries in PE, those vessels, which were under pressurized conditions, demonstrated no impairment of overall endothelium-dependent dilatation in PE [10].

In PE, incubation with 18-zGA had only a small effect (if any) on EDHF-type responses in myometrial arteries, indicating disturbances at the level of MEGJs as an important contributory factor to endothelial dysfunction in PE. It is premature to speculate if MEGJ malfunction could play an underlying role in the genesis of vascular abnormalities and, eventually, clinical features of PE, although a potential role of connexin proteins (Cx), which constitute MEGJs, for blood pressure control has recently been supported by experiments in Cx knockout mice [39, 40]. Importantly, even if abolishment of the MEGJ contribution occurs in myometrial arteries in PE, the EDHF-type responses are preserved, corresponding to as much as 65% of overall relaxation in response to BK (Fig. 2). This indicates the occurrence of alternative pathways, a finding that...
concerns with our recently reported heterogeneity in the mechanisms responsible for the EDHF-mediated component of endothelium-dependent relaxation in subcutaneous arteries from women with PE [16]. In myometrial arteries, such an alternative pathway predominantly occurs via $H_2O_2$, which accounted for a residual relaxation after inhibition of NOS and COX. Because catalase did not induce a complete abolishment of residual relaxation, involvement of other mechanisms in EDHF-type responses in PE, including those yet unknown, could be suggested.

Although the $H_2O_2$ candidacy as EDHF has been discussed in several publication [41–43], its mechanism of action has not been completely defined [44, 45]. Nevertheless, independently from the exact mechanism by which $H_2O_2$ could contribute to endothelium-dependent relaxation, our novel finding of its role in EDHF-type responses in myometrial arteries in PE warrants further research. Despite consistent evidence for a state of oxidative stress in PE [46, 47], modification of the prooxidant and antioxidant milieu may thus disturb the preparations of vascular tone in uterine circulation, with a corresponding reduction of blood supply to the fetoplacental unit that eventually might affect the fetal growth. These thoughts are reasonably relevant to explain, in part, the negative outcome of the “Vitamins In Preeclampsia” (VIP) trial, which found an increased rate of low birth weight babies among those supplemented with vitamins C and E [48], although the balance between beneficial and harmful roles of reactive oxygen species in the pathogenesis of PE warrants more thorough research.

The present study and our recent report on subcutaneous arteries [16] provide one of the first systematic analyses of vascular bed-specific characteristics of endothelial function at the level of vasculature resistance in NPG and PE. We show here that in PE, myometrial arteries demonstrate a significantly reduced response to the endothelium-dependent agonist BK and that EDHF-type, rather than NO-mediated, responses are impaired in myometrial and subcutaneous [16] arteries isolated from women with PE. The contribution of MEGJs as a common pathway of EDHF-type responses in arteries from women with NPG became reduced in subcutaneous arteries [16] and even more severely impaired in myometrial arteries from women with PE. However, the attenuated role of MEGJs in PE is partly compensated through contribution of $H_2O_2$. Future studies clarifying the role of reactive oxygen species in vascular maintenance and elaborating potential improvement of EDHF-pathway in PE are important.

REFERENCES


