ORIGINAL ARTICLE

Intracellular calcium ion concentrations in endothelial cells in preeclampsia

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Aim: Preeclampsia (PE) is a disorder characterized by hypertensive symptoms and proteinuria in pregnant women. Previous studies have demonstrated that endothelial cell (EC) dysfunction is involved in the pathogenesis of PE. Intracellular free calcium ions are thought to function as secondary messengers in the EC signaling pathway. The aim of this study is to evaluate factors found in sera of PE patients by measuring their effects on intracellular calcium ion concentration ([Ca²⁺]_i) in ECs.

Methods: ECs obtained from umbilical cords of normal pregnant women were cultured and incubated with Fura-2/acetoxymethyl ester (Fura-2AM). [Ca²⁺]_i values were measured in ECs treated with sera from 29 normal pregnant women, 7 PE patients, and 10 non-pregnant women by monitoring fluorescence using a CAM 220 fluorometer.

Results: [Ca²⁺]_i values were significantly higher in ECs treated with sera from normal pregnant women compared to those from non-pregnant women, and the effects were even stronger when ECs were treated with sera from pregnant women who were in later stages of pregnancy. Furthermore, sera from PE patients significantly increased EC [Ca²⁺]_i compared to those from normal pregnant women in the 3rd trimester.

Conclusions: It is possible that serum factors play important roles in the maintenance of normal pregnancy. Furthermore, EC activation could be associated with the pathogenesis of PE through increases in EC [Ca²⁺]_i.

Introduction

Preeclampsia (PE) is a serious disease that affects pregnant women, often leading to intra-uterine fetal growth retardation or perinatal death. However, the etiology of PE is not yet fully understood. Given the rapid improvement in PE symptoms after delivery, it has been proposed that disordered uteroplacental circulation may be involved in the disease process, with other causative factors circulating throughout the body.¹⁾ Factors derived from endothelial cells (ECs) are altered in PE patients, which suggests that EC activation or dysfunction may play an important role in uteroplacental circulation in PE.^{2,3)} Both EC-derived fibronectin⁴⁾ and von Willebrand factor⁵⁾ are elevated in the serum of PE patients, whereas the EC-derived prostacyclin to thromboxane A2 ratio is reduced.^{6,7)} In addition, ECs have been shown to regulate the vascular tone^{8,9)} and coagulation cascade.⁴⁾ The symptoms of hypercoagulation¹⁰⁾ and vasospasms¹¹⁾ observed in PE patients, therefore, may indicate endothelial dysfunction.

Rodgers et al. reported that placenta-derived factors in maternal circulation decreased rapidly after delivery, and that these factors might be related to PE pathogenesis.¹²⁾ Moromizato et al. found an ouabain-like factor in the plasma of PE patients that induced vasospasms.¹¹⁾

Intracellular calcium ion concentration ([Ca²⁺]_i) regulates endothelial functions such as leukocyte adhesion, an essential element of the inflammatory response.¹³⁾ There are three mechanisms that maintain low levels of [Ca²⁺]_i without stimulation: 1) inhibition of Ca²⁺ influx, 2) activation of Ca²⁺ efflux, and 3) transport of Ca²⁺ into intracellular storage. Endothelial activation alters these mechanisms and affects cytokine production in ECs.¹³⁾

Fura-2 is an established calcium-sensitive fluorescent dye with 30-fold greater fluorescence intensity than other commonly used dyes, such as Quin-2. Fura-2 binds to Ca²⁺ more strongly compared to Mg²⁺ or other divalent ions, and exhibits Ca²⁺-dependent fluorescence changes at particular excitation wavelengths. Therefore, cellular [Ca²⁺]_i can be measured using Fura-2 and two

excitation wavelengths.¹⁴⁾ The aim of this study is to determine $[Ca^{2+}]_i$ in ECs treated with sera derived from non-pregnant women, normal pregnant women, and PE patients.

Materials and methods

Definitions of PE and subjects

PE was defined according to JSSHP criteria.¹⁵⁾ Specifically, PE was defined as sustained, pregnancy induced hypertension with proteinuria after 20 weeks gestation. Hypertension was defined as blood pressure $\geq 140/90$ mmHg. Proteinuria was defined as either a urine protein concentration ≥ 30 mg/dl or two separate readings of $\geq 1+$ on the dipstick test. All patients were normotensive prior to pregnancy and had no complications, such as chronic hypertension or renal disease. Informed consent was obtained from all patients, and the study protocol was approved by the local committee.

Sample collection

Ten milliliters of peripheral blood were collected from 10 non-pregnant women, 29 normal pregnant women (10 in the 1st trimester, 11 in the 2nd trimester, and 8 in the 3rd trimester), and 7 PE patients in the 3rd trimester. All blood samples were placed in heparinized tubes. Sera were obtained by centrifugation of whole blood (at 1,500 rpm for 60 min) and stored at -80°C until use. Obstetrical summaries of the subjects are shown in Table 1.

Primary human umbilical vein EC (HUVEC) cultures

HUVECs were obtained from umbilical veins of normal pregnant women using the method previously described by Jaffe et al.¹⁶⁾ Briefly, veins were incubated for 15 min at 37°C with 0.2% collagenase (Sigma Aldrich Corp.,

MO, USA). HUVECs were collected and washed in minimum essential medium (MEM; Gibco, NY, USA) with 10% fetal bovine serum (FBS) and then plated onto collagen-coated dishes in MEM containing 2% FBS and 0.012 mg/ml bovine brain tissue extract (Clonetics, CA, USA) for 5–7 days, at 37°C , under 5% CO_2 . Primary cultures were used for this investigation.

Sera treatment

Portions of subjects' sera were heated to 56°C for 60 min or fractionated using an octadecyl silica gel (C18) column (YMC Co., Ltd., Kyoto, Japan). C18 columns were initially washed with methanol and distilled water. Sera were applied to the pretreated columns and the flow-throughs were collected.

$[Ca^{2+}]_i$ measurements

HUVECs were made quiescent in phenol-red free MEM with 0.1% FBS for 12 hours. HUVECs were washed with 10 μM HEPES-EBSS buffer, incubated for 60 min in the presence of 5 μM Fura-2-acetoxymethyl ester (Fura-2AM; Chemical-Dojin Co., Ltd., Kumamoto, Japan) at 37°C , washed twice, and re-suspended in 1 ml 10 μM HEPES-HBSS containing 100 $\mu\text{l/ml}$ of treated or untreated serum of individual subjects. HUVECs were viewed at $400\times$ using an inverted microscope (Zeiss Axiovert 35M, Oberkochen, Germany). HUVECs were excited alternatively with wavelengths of 340 nm and 380 nm. The emission filter was set at 500 nm and emission was detected using a calcium flow meter (CAM220, Japan Spectrum Industry, Tokyo, Japan). 4-bromo-Ca ionophore (20 μM ; A23187; Sigma-Aldrich Corp.) and 3 mM ethylene glyco-bis-N, N, N', N'-tetra acetic acid (EGTA; Sigma-Aldrich Corp.) were used for calibration.

Table 1. Characteristics of the study subjects

	non-pregnancy (n = 10)	1st trimester (n = 10)	normal pregnancy 2nd trimester (n = 11)	3rd trimester (n = 8)	preeclampsia (n = 7)
age (yr)	29 $\pm$ 2	30 $\pm$ 1	30 $\pm$ 1	30 $\pm$ 1	30 $\pm$ 1
nulliparity (n)	5	4	5	5	4
gestational age at blood sampling (wk)	—	11 $\pm$ 1	18 $\pm$ 1	34 $\pm$ 1	34 $\pm$ 1
body mass index (kg/m ²)	21.5 $\pm$ 0.6*	22.5 $\pm$ 1.4**	23.8 $\pm$ 0.8* **	23.2 $\pm$ 0.3*	30.2 $\pm$ 1.5
systolic BP (mmHg)	107 $\pm$ 4*	115 $\pm$ 4*	106 $\pm$ 3*	116 $\pm$ 4*	166 $\pm$ 4
diastolic BP (mmHg)	64 $\pm$ 3*	65 $\pm$ 4*	59 $\pm$ 3*	68 $\pm$ 4*	106 $\pm$ 4
proteinuria (n)	—	—	—	—	7
gestational age at delivery (wk)	—	39 $\pm$ 1*	39 $\pm$ 1*	39 $\pm$ 1*	35 $\pm$ 1
birth weight (g)	—	3,441 $\pm$ 96*	3,328 $\pm$ 119*	3,160 $\pm$ 105*	2,006 $\pm$ 276

body mass index = body weight (kg)/body height (m²)

* $P < 0.05$ vs. preeclampsia ** $P < 0.05$ vs. non-pregnancy.

Serum estradiol (E2) and progesterone (P4) concentration measurements

Serum E2 and P4 concentrations were measured using mini VIDAS (bioMérieux Vitek, Inc., MO, USA) according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean $\pm$ standard error (SE). Comparisons between groups were made by one-way analysis of variance (ANOVA), followed by the Bonferroni *post-hoc* test and Mann-Whitney *U* test. Differences were considered significant if $P < 0.05$.

Results

$[Ca^{2+}]_i$ was significantly higher in HUVECs treated with sera from 1st trimester normal pregnant women (16.2 ± 8.1 nM) compared to those treated with sera from non-pregnant women (luteal phase; 5.36 ± 1.74 nM; $P < 0.05$). In addition, the effects on $[Ca^{2+}]_i$ were even stronger when ECs were treated with sera from pregnant women who were in later stages of pregnancy (Figure 1). Sera derived from PE patients significantly increased $[Ca^{2+}]_i$ (140.2 ± 48.1 nM) in HUVECs compared to those derived from 3rd trimester normal pregnant women (60.8 ± 10.9 nM; $P < 0.05$) (Figure 2). The increases in EC $[Ca^{2+}]_i$ by sera from normal pregnant women and PE patients were not affected by heating (56°C , 60 min). On the other hand, the effects on EC $[Ca^{2+}]_i$ by sera derived from normal pregnant women and PE patients were significantly reduced by C18 column treatment (4.9 ± 2.2 nM; $P < 0.05$ and 2.0 ± 1.8 nM; $P < 0.01$, respectively) (Figure 2).

Serum E2 concentrations significantly increased from

Figure 1. Changes in $[Ca^{2+}]_i$ values in ECs treated with sera from normal pregnant women or non-pregnant women.

The effects on $[Ca^{2+}]_i$ were stronger with sera from pregnant women who were in later stages of pregnancy. *: $P < 0.05$ vs. non-pregnancy. **: $P < 0.01$ vs. non-pregnancy.

the luteal phase (0.04 ± 0.03 ng/ml) to the 3rd trimester (19.4 ± 4.4 ng/ml; $P < 0.05$) (Figure 3). However, E2 concentrations did not differ between normal pregnant women and PE patients (20.3 ± 3.7 ng/ml). Serum P4 concentrations also significantly increased from the luteal phase (5.4 ± 1.0 ng/ml) to the 3rd trimester (60.8 ± 4.5

Figure 2. Changes in $[Ca^{2+}]_i$ in ECs treated with sera from normal pregnant women in the 3rd trimester and PE patients after heating or C18 column treatment.

Heating sera did not affect EC $[Ca^{2+}]_i$. However, C18 column treatment of sera significantly decreased EC $[Ca^{2+}]_i$. *: $P < 0.05$ vs. 3rd trimester in normal pregnancy. **: $P < 0.01$ vs. PE. ***: $P < 0.05$ vs. 3rd trimester in normal pregnancy.

Figure 3. Serum E2 and P4 concentrations in normal pregnancy and PE.

Serum E2 concentrations did not differ between normal pregnancy and PE. The average serum P4 concentration was higher in PE patients than in normal pregnant women. Open column represents E2. Closed column represents P4. *: $P < 0.05$ vs. non-pregnancy. **: $P < 0.01$ vs. non-pregnancy. ***: $P < 0.05$ vs. 3rd trimester in normal pregnancy. ****: $P < 0.05$ vs. PE.

ng/ml; $P < 0.01$) (Figure 3). However, the average P4 concentration was significantly higher in PE patients (140.2 ± 24.0 ng/ml; $P < 0.05$). Heating did not influence E2 and P4 concentrations (data not shown), whereas C18 column treatment significantly decreased average P4 concentrations in sera from both normal pregnant women (4.9 ± 1.2 ng/ml; $P < 0.05$) and PE patients (1.8 ± 1.0 ng/ml; $P < 0.05$).

Discussion

The vascular endothelium is critical for the maintenance of circulation during pregnancy, and ECs reduce inflammation and coagulation by producing anticoagulatory factors and cytokines. In addition, ECs modulate vascular tone and maintain vascular homeostasis by producing vasoconstrictors, such as endothelin,^{17,18)} and relaxation factors, such as nitric oxide (NO).^{8,19)} EC-derived factors, such as von Willebrand factor,⁵⁾ endothelin,¹⁷⁾ and fibronectin,⁴⁾ are reportedly higher in sera derived from PE patients. Accordingly, it has been hypothesized that EC dysfunction plays roles in vascular homeostasis and PE pathophysiology, including hypertension and proteinuria.

In the present study, treatment with sera from normal pregnant women and PE patients significantly increased $[Ca^{2+}]_i$ in ECs, suggesting that higher levels of EC $[Ca^{2+}]_i$ may be required for the pathogenesis of PE and the maintenance of normal pregnancy.

Increased EC $[Ca^{2+}]_i$ induces myosin phosphorylation by EC myosin light chain kinase, possibly resulting in cytoskeletal rearrangements and intercellular gap formation.²⁰⁾ Huang et al. reported that increased EC $[Ca^{2+}]_i$ caused morphological changes and separation of inter-endothelial cellular junctions, leading to leukocyte invasion through vascular endothelial activation.¹⁴⁾ Leukocyte-endothelium adhesion and endothelial tissue invasion are important primary signs of endothelial dysfunction. An association between inflammation and the pathogenesis of PE has been suggested. However, the present study demonstrated that sera derived from normal pregnant women and PE patients increased EC $[Ca^{2+}]_i$, suggesting that vascular endothelial activation may play roles in both PE pathogenesis and maintenance of normal pregnancy.

Given that the factors that increased EC $[Ca^{2+}]_i$ were removed by C18 column chromatography, these factors are likely small non-polar molecules, such as prostaglandins, steroid hormones, and peptides. The steroid hormones, E2 and P4, which increase during pregnancy, were thus investigated as candidate factors. Although estrogens, including E2, are known to activate Ca^{2+} -dependent NO production in ECs and lead to vasodilatation,²¹⁾ no significant differences were found

in serum E2 levels between PE patients and normal pregnant women. Bowyer et al. reported that serum P4 concentrations were higher in PE patients than in normal pregnant women,²²⁾ although P4 does not require increased EC $[Ca^{2+}]_i$ as a second messenger in the signaling pathway leading to EC activation. Rather, P4 directly affects vascular smooth muscle through the production of carbon monoxide, which increases cyclic GMP and causes vasodilatation.^{23–26)} Therefore, E2 may be the primary candidate among serum factors that increase EC $[Ca^{2+}]_i$ during normal pregnancy. Yet, it is also possible that neither E2 nor P4 is responsible for increasing EC $[Ca^{2+}]_i$ in PE.

Sera derived from PE patients may stimulate vasoconstriction,²⁷⁾ the coagulation cascade,²⁸⁾ and inflammation²⁹⁾ through EC activation. On the contrary, PE serum could also induce vascular relaxation³⁰⁾ through EC activation. Taken together, the effects of sera derived from pregnant women on ECs remain controversial, and further studies are needed to identify the factors responsible for increasing EC $[Ca^{2+}]_i$.

Our results showed that serum factors from PE patients activated ECs by increasing $[Ca^{2+}]_i$ in these cells. Vasoconstriction in PE patients could be regulated by increases in EC $[Ca^{2+}]_i$, which are related to NO production by ECs. To elucidate the role of EC $[Ca^{2+}]_i$ in the pathogenesis of PE, further investigation is needed to address the roles of serum factors and EC receptors, as well as the mechanisms of Ca^{2+} influx, Ca^{2+} efflux, and Ca^{2+} transport into intracellular storage.

Conflict of interest

None.

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