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The loss of sustained Ca\(^{2+}\) signaling underlies suppressed endothelial nitric oxide production in preeclamptic pregnancies: implications for new therapy

Jennifer Krupp, Derek S. Boeldt, Fu-Xian Yi, Mary A. Grummer, Heather A. Bankowski Anaya, Dinesh M. Shah, and Ian M. Bird

1Perinatal Research Laboratories, Department of Obstetrics and Gynecology, School of Medicine and Public Health, University of Wisconsin Madison, Madison, Wisconsin; and 2Division Maternal Fetal Medicine, Department of Obstetrics and Gynecology, School of Medicine and Public Health, University of Wisconsin Madison, Madison, Wisconsin; and 3Division of Maternal Fetal Medicine, Department of Obstetrics and Gynecology, University of Iowa Hospital and Clinics, Iowa City, Iowa

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Krupp J, Boeldt DS, Yi F, Grummer MA, Anaya HA, Shah DM, Bird IM. The loss of sustained Ca\(^{2+}\) signaling underlies suppressed endothelial nitric oxide production in preeclamptic pregnancies: implications for new therapy. Am J Physiol Heart Circ Physiol 305: H969–H979, 2013. First published July 26, 2013; doi:10.1152/ajpheart.00250.2013.—Approximately 8% of pregnancies are complicated by preeclampsia (PE), a hypertensive condition characterized by widespread endothelial dysfunction. Reduced nitric oxide (NO) output in PE subjects has been inferred but not directly measured, and there is little understanding of why this occurs. To address this we have used direct imaging of changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and NO in umbilical vein endothelium of normal and PE subjects that is still intact and on the vessel luminal surface. This was achieved by dissection and preloading with fura 2 and DAF-2 imaging dyes, respectively, before subsequent challenge with ATP (100 µM, 30 min). As a control to reveal the content of active endothelial nitric oxide synthase (eNOS) per vessel segment, results were compared with a maximal stimulus with ionomycin (5 µM, 30 min). We show for the first time that normal umbilical vein endothelial cells respond to ATP with sustained bursting that parallels sustained NO output. Furthermore, in subjects with PE, a failure of sustained [Ca\(^{2+}\)]\(_i\) bursting occurs in response to ATP and is associated with blunted NO output. In contrast, NO responses to maximal [Ca\(^{2+}\)]\(_i\), elevation using ionomycin and the levels of eNOS protein are more similar between groups than the responses to ATP. When the endothelial cells from PE subjects are isolated and allowed to recover in culture, they regain the ability under fura 2 imaging to show multiple [Ca\(^{2+}\)]\(_i\) bursts otherwise seen in the cells from normal subjects. Thus novel clinical therapy aimed at restoring function in vivo may be possible.

calcium ion; endothelium; hypertension

PREGNANCY IS ASSOCIATED with a need to expand maternal blood volume and increase cardiac output to allow for the increased blood flow to the uterus. Normal maternal vascular adaptation also involves enhanced vasodilation, including sustained and pronounced activation of endothelial nitric oxide synthase (eNOS) to make nitric oxide (NO). The greatest effect is seen in the uterus, but enhanced vasodilation is also seen in the systemic circulation (6, 17). Our own detailed studies in this area have shown that increased output of NO by uterine artery endothelium in response to ATP exceeds changes in eNOS expression and is causally related to enhanced endothelial cell Ca\(^{2+}\) signaling in the form of repeated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) bursts [recently termed “adaptive programming” (6, 7)]. In turn, studies of these cells in primary culture show the sustained [Ca\(^{2+}\)]\(_i\), burst response is a form of pregnancy-enhanced capacitative Ca\(^{2+}\) entry (CCE) (9) that is facilitated by a pregnancy-specific increase in connexin 43 (Cx43) gap junction communication between endothelial cells, so facilitating more cells to exhibit burst responses during pregnancy and more synchronous bursts between cells (25, 26). The uterine artery is not alone in showing this pregnancy-enhanced adaptation of Ca\(^{2+}\) signaling; a similar pregnancy-specific increase in the earliest CCE phase of [Ca\(^{2+}\)]\(_i\), response to G protein-coupled receptor (GPCR)-coupled phospholipase C (PLC)-β agonists has also been implicated in maternal human hand vein endothelial cells (HHVEC) (13), and similar CCE profiles are seen in human umbilical vein endothelial cells (HUVEC) of normal umbilical cords (18). In both cases this otherwise sustained CCE response was reported to be lacking in preeclampsia (PE) subjects, although its causal relationship to changes in NO production was not investigated. In both HHVEC and HUVEC studies to date, the short stimulation times also did not allow determination of the duration of sustained [Ca\(^{2+}\)]\(_i\), bursting in pregnancy or how much bursting was reduced in PE subjects.

Currently, antihypertensive treatments for PE are limited to agents that target the vascular smooth muscle and are used alongside MgSO\(_4\) to prevent development of seizures (1). Such approaches have only limited success, and not every patient responds to therapy. New strategies based on new approaches to therapeutic intervention are clearly needed. Untreated, both the mother and fetus are at risk, and fetal growth restriction can result. Given the Barker hypothesis that associates low birth weight with increased risk of adult-onset diseases (4), clearly any new therapy would be of lifelong value for the progeny, and an endothelial therapy would be a unique advance. Such novel advances, however, will only be possible given clear confirmation of the hypothesis that endothelial adaptive signaling in pregnancy at the level of [Ca\(^{2+}\)]\(_i\), in humans is indeed the basis for increased vasodilation, and that such adaptation is lacking in PE subjects. Establishing these facts would also provide a basis to screen potential therapeutic agents in the
powerful technique to directly examine vascular endothelial function and dysfunction in PE subjects. Herein we report for the first time the application of this technique to intact human umbilical vein endothelium (UV Endo) and we extend these studies to reveal responses of the corresponding cultured HUVEC of normal vs. PE pregnancies to establish if the lack of sustained CCE function in PE UV Endo is maintained in culture. The results of this study provide direct evidence that UV Endo function is lacking in PE subjects due to processes including an active suppression of sustained Ca\(^{2+}\) signaling, and further that HUVEC should be considered appropriate models for the study of the human vascular adaptive programming of pregnancy and the mechanisms that may underlie dysfunction that occurs in the endothelial cells of PE subjects.

**MATERIALS AND METHODS**

**Human Subjects Enrollment and Tissue Collection**

The Institutional Review Boards of the University of Wisconsin Hospital and Clinics and Meriter Hospital (both located in Madison, WI) approved this study. All subjects signed a formal consent. To avoid as many confounding variables as possible in the diagnosis of PE, inclusion criteria included maternal age 18 years or older, singleton pregnancy, and clinical diagnosis of PE as appropriate. Exclusion criteria included multiple gestation, gestational age <29 wk, moderate/thick meconium, maternal comorbidities including diabetes (gestational or gestational), chronic hypertension or pregestational antihypertensive use, and chorioamnionitis. A summary of maternal subject data is given in Tables 1 and 2, and corresponding birth weights are shown in Fig. 1. Of note, birth weights for the PE group were not substantially different from that expected for gestational age, confirming the PE was not severe enough to routinely cause intrauterine growth restriction in their offspring in this study population. Umbilical cord segments were obtained from a total of 16 control and 12 PE subjects. Data were obtained from intact UV Endo of nine control and six PE subjects. Alternatively, HUVEC were successfully isolated and cultured from seven control and six PE subjects for experimentation and data analyses.

### Table 1. Control subject data

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Control subject data: n = 9 umbilical vein endothelium (UV Endo) and 7 human umbilical vein endothelial cell (HUVEC) experiments. M, multiparous; P, primiparous. Sixty-two percent of subjects were between the ages of 23 and 37 yr old, whereas 25% were between 33 and 37 yr old, and only 13% were in the age range of 18 to 22 yr old. For efficiency and timing of experiments, 81% of the control subjects were multiparous, and specimens were obtained after they were delivered by scheduled repeat cesarean section. As expected, there were no deliveries before 34 wk gestation, with only 19% of the deliveries between 34 and 38 wk gestation. The majority of the subjects (81%) were delivered after 39 wk gestation. None of these subjects had the following: history of hypertension (HTN) or had ever been on antihypertensive medication, elevated aspartate aminotransferase (AST) or alanine aminotransferase (ALT), low platelets, or seizure.

### Table 2. PE subject data

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<th>BP</th>
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PE subject data: n = 6 UV Endo and 6 HUVEC experiments. BP, blood pressure; Pt, platelets. The age range for the PE subjects was evenly spread among the younger age ranges. Thirty-three percent were between 18 and 22 yr old, 25% were between 23 and 27 yr old, 33% were between 28 and 32 yr old, whereas only 8% were in the age range of 33–37 yr old. Forty-two percent of these subjects were primiparous. The majority of subjects (58%) were delivered between 34 and 38 wk gestation. Twenty-five percent were delivered between 29 and 33 wk gestation with the remainder of the subjects (17%) delivered between 39 and 41 wk gestation. Seventy-five percent of patients were diagnosed with mild preeclampsia (PE), which is defined as gestational blood pressure elevation >140/90 (but <160/110) on two separate occasions at least 4 h apart and proteinuria (>1 + protein on urine dipstick or >300 mg in 24 h) as stated in the Report of National High BP Education Program (2). Five of the 12 subjects were treated with antihypertensive therapy due to severely elevated blood pressure. Two were treated with labetalol (a\(B\) adrenergic blocker), one subject was treated with nifedipine (calcium channel blocker), and two subjects were treated with both of these antihypertensives during the antepartum period before delivery. None of these subjects had low platelets or seizures. Only one subject had elevated AST and ALT.

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Materials

The endothelial cell agonist ATP (disodium salt) and all other general laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO) to at least ACS standards unless stated otherwise. Minimum essential medium (MEM), medium 199 (M199), fetal bovine serum (FBS), fura 2-AM, DAF2-DA, and all other general cell culture reagents were purchased from Life Technologies (Grand Island, NY) unless otherwise noted. Endothelial cell growth supplement was from EMD Chemicals (San Diego, CA). Glass bottom microwell plates (Millipore) and heparin (sodium salt, grade 1A from American Type Culture Collection) were from Life Technologies unless otherwise stated. Antibodies used in this study included anti-eNOS (no. 610297; BD BioSciences, San Jose, CA), Cx43 (no. C6219; Sigma Chemical), and heat shock protein 90 (Hsp90, no. 4874; Cell Signaling Technology, Danvers, MA), with detection using rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated F(ab')2 secondary antibody (no. AQ160P; Millipore) and goat anti-rabbit HRP-conjugated secondary antibody (no. 7074; Cell Signaling Technology). For validation of HUVEC preparations further primary antibodies used were ve-cadherin (no. 36–1900; Life Technologies) and vascular endothelial growth factor receptor (VEGFR2, no. sc-19530; Santa Cruz Biotechnology, Santa Cruz, CA).

METHODS

The protocols for isolating the intact human umbilical vein endothelium and for culturing the human umbilical vein endothelial cells were based on isolation of uterine artery endothelial cells (UAEC) from sheep (19). All standard culture media used for cell isolation or culture (MEM or M199) routinely contained 1% penicillin-streptomycin (Life Technologies), and 4 μg/ml gentamycin (Life Technologies). The established methods of dual imaging of [Ca2+]i, response and simultaneous NO production utilized in intact UA Endo and the [Ca2+]i imaging done utilized in UAEC (25, 26) were used in this study on the intact UV Endo and the cultured HUVEC from control and PE subjects.

Simultaneous imaging of [Ca2+]i, and NO in intact UV Endo. After fine dissection and loading vessels of ~6 mm length with both DAF2-DA (10 μM) and fura 2-AM (10 μM) in standard Kreb’s buffer (in mmol/l: 125 NaCl, 5 KCl, 1 MgSO4, 1 KH2PO4, 6 glucose, 2 CaCl2, and 25 HEPES, pH 7.4) as required for 90 min, the loading dye medium was replaced, and dye hydrolysis was allowed to proceed for 30 min. The vessel chamber was then mounted on an inverted microscope (Diaphot 150; Nikon, Melville, NY) with the ×20 phase/fluor objective focused on the endothelium. Individual endothelial cells (>30 cells/field) were visualized using dual excitation (switching at 340 and 380 nm) for fura 2 and 485 nm excitation DAF-2 (high-speed Lambda 10–2; Sutter Instruments, Novato, CA). Emission was uniformly measured at 535 nm for all excitation wavelengths. The fluorescence images were recorded in real time by a PixelFly camera (Cooke, Romulus, MI). Imaging and analysis software (InCyt Im3; Intracellular Imaging, Cincinnati, OH) was used to acquire, digitize, and store the images and data for offline analysis. Vessel segments were observed under basal conditions or after addition of ATP (100 μM) or ionomycin (5 μM) to fully activate the available pool of eNOS, and recording was continued as indicated in Figs. 1–9. Relative fluorescence was then calculated as the relative measure of intracellular NO, whereas [Ca2+]i was calculated ratiometrically against a prerecorded standard curve (25, 26; CalBiochem, La Jolla, CA). HUVEC cell isolation. The umbilical cord segments (approximate length of 4–5 cm for an unclamped area of the cord) were prepared, and a blunt-end needle was placed in the vein lumen. After thoroughly rinsing the vessel free of blood using ~10 ml M199, the needle in the vessel was then tied off, and the vessel was flushed one more time and incubated for 15 min at 37°C. The vessel was then removed from the incubator and inflated with M199/0.5% BSA containing 2 mg/ml collagenase B (Roche Molecular Biochemicals, Indianapolis, IN) via a luerlock three-way tap before clamping off for digestion. Digestion was allowed to proceed for 25 min at 37°C before flushing the collagenase solution and endothelial cell sheets from the inner surface of the vessels using 10 ml M199/0.5% BSA medium. Recovered cells were pelleted by centrifugation (2 min, 300 g) and resuspended in appropriate media.

HUVEC cell culture. Freshly isolated HUVEC were resuspended in HEH media (MEM-based media containing 20% FBS, endothelial cell growth supplement (3.75 mg/ml), and porcine intestinal mucosa heparin (10 mg/100 ml)) before plating to three wells of a six-well plate (Falcon Primaria; Thermo Fisher Scientific, Waltham, MA). Cell viability was assessed after 4 h to remove unattached cells (including the few vascular smooth muscle cells present). Cells were grown to cover ~70% of the plate surface and were then selectively trypsinized from the dish and replated to three 60-mm dishes. Cells were grown to 80–90% confluence and trypsinized, and the cells from two dishes were frozen in HEH medium plus 10% DMSO (passage 2) while the cells from the remaining dish were passed to a T75 flask. These cells were grown to 95% confluence and trypsinized for freezing in media with 10% DMSO in 35 cryovials at passage 3. Individual cell preparations were independently validated by Western blot before use by growing to confluence in a 35-mm dish, lysed as below (see Western Blot Analysis), and analyzed for consistent expression of eNOS, ve-cadherin, and VEGFR2 as endothelial markers, as well as Cx43 necessary for Ca2+ bursting (26) and Hsp90 as normalization control. Levels of these proteins in HUVEC preparations varied <10% within each group or between control and PE groups, with no significant difference observed (data not shown). Each further vial of HUVEC was then used for plating to four to six glass bottom dishes for fluorescent imaging. Of note, plated cells from all individual donors consistently responded ~95% to ATP (100 μM) and 100% to vascular endothelial growth factor (10 ng/ml, data not shown).

HUVEC imaging. Cultured HUVEC frozen at passage 2 were plated to one well of a six-well dish in HEH media (above) on day 1 and trypsinized at 95% confluence (day 3) and then were passed to six 35-mm glass bottom dishes in HEH media. Cells were imaged at 95% confluence (day 7). On the day of imaging, HUVEC were centrifuged (2 min, 300 g) and resuspended in culture medium to allow for cell growth to cover 50–70% of the dish surface. Cells were grown to cover 80–90% of the dish surface before imaging. A 10-μl droplet of imaging media was added to the tissue culture dish, and imaging was undertaken using the MetaFluor system (Universal Imaging, West Chester, PA). Imaging was undertaken at 20× magnification using the appropriate filter set for each fluorophore.
were incubated in 10 μM of fura 2-AM with 0.05% Pluronic acid F127 (Life Technologies) dissolved in 1 ml HEH media for 1 h at 37°C. The cells were then washed with Kreb’s buffer (above), and 1.8 ml Kreb’s buffer was added to each dish for an additional 30 min at room temperature to allow complete ester hydrolysis. The dish was then placed in the field of view, and fura 2 loading was verified by viewing at 380 nm ultraviolet excitation on a Nikon inverted microscope (above). More than 40 cells/field were identified and circled for analysis. An initial 5-min recording was performed to obtain a steady baseline \([\text{Ca}^{2+}]_i\) before subsequent addition of 100 μM ATP. The intracellular free \([\text{Ca}^{2+}]_i\) for each cell was calculated in real time against an established ratiometric standard curve using the InCyt Im2 software. For all \([\text{Ca}^{2+}]_i\) imaging experiments where baseline correction was calculated to reveal the agonist response above control, data recorded for 30 s before agonist stimulation were used to calculate the basal \([\text{Ca}^{2+}]_i\).

**Western analysis.** Freshly isolated UV Endo cell sheets were washed in protein-free M199 buffer and pelleted before solubilization in protein kinase lysis buffer [50 mM HEPES, pH 7.5, 4 mM Na4P2O7·10H2O, 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM Na3(VO4)2, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. Samples accumulated throughout the study were stored at −70°C. Solubilized protein was quantified using the BCA assay before separation of proteins on 7.5% gels with 10 μg protein/lane and transfer of proteins to an Immobilon-P PVDF membrane. Western analyses were performed as described previously (19, 26). Total eNOS was detected using anti-eNOS (1:1,000) and rabbit anti-mouse IgG HRP-conjugated F(ab’2) secondary antibody (1:2,500). Total Cx43 was detected using anti-Cx43 (1:5,000) and goat anti-rabbit HRP-conjugated secondary antibody (1:3,000). Hsp90 was detected using anti-Hsp90 (1:2,000) and goat anti-rabbit HRP-conjugated secondary antibody (1:3,000).

**Statistical analysis.** For imaging and Western experiments, data from six or more individual subject cell preparations were collected. Where multiple cells were simultaneously analyzed per dish or vessel, responses were first combined as the patient/dish average response and then as mean of means ± SE. Data were subsequently analyzed by Student’s t-test and analysis of variance, as appropriate. A value of \(P < 0.05\) was considered statistically significant.

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**Fig. 2.** Representative tracings from umbilical vein endothelium (UV Endo) individual cells. Representative tracings from DAF-2 and fura 2-AM-loaded UV Endo dissected from the cord from control subjects (A) and PE subjects (B) are shown after vessel segments are treated with the physiological agonist ATP (100 μM at time 0) after obtaining a 5-min baseline. Synchronization of bursts between individual cells in close proximity to each other in the control subjects was also observed (D). As a control, to bypass the receptor signaling apparatus, the vessel segment was treated with a Ca2+ ionophore (ionomycin 5 μM) at time 0 (C). The recording was continued for 30 min in all cases after the vessel segment was treated. \([\text{Ca}^{2+}]_i\), intracellular Ca2+ concentration; NO, nitric oxide.
RESULTS

Imaging of Real-Time Changes in [Ca\textsuperscript{2+}], and NO in Intact UV Endo

Responses to ATP and ionomycin in individual UV Endo cells. Initially we examined the endothelial cells of the intact umbilical vein by direct dual imaging of [Ca\textsuperscript{2+}], and NO production to demonstrate any differences in Ca\textsuperscript{2+} signaling and associated NO output in control and PE subjects. Of note, we have published extensively on the use of DAF-2 as an NO sensor in intact endothelium, including its ability to detect NO generation in vascular endothelium that is \textit{l}-NAME sensitive and requires extracellular Ca\textsuperscript{2+} (25, 28). In the representative tracings shown in Fig. 2, the UV Endo from control subjects (Fig. 2A) shows a normal adaptive [Ca\textsuperscript{2+}], response similar to that seen in previous studies on sheep uterine artery endothelium (25, 27). Following an initial [Ca\textsuperscript{2+}], peak there was a decline followed by repeated [Ca\textsuperscript{2+}], bursts in the sustained phase, which were coincident with a continuous rise in NO production as detected by DAF-2 fluorescence. In comparison, a distinct absence of this adaptation of [Ca\textsuperscript{2+}], burst signaling was observed in the UV Endo from subjects with PE (Fig. 2B), and the associated NO output was also correspondingly diminished. While control subject UV Endo cells also demonstrated synchronous bursting (Fig. 2D), this was not apparent in UV Endo from PE subjects (data not shown).

Averaged responses to ATP and ionomycin in UV Endo cells. Although Fig. 2 shows the data from individual cells, it does not address the overall number of cells (%) responding with bursts, or the average response of all those cells combined, and both have physiological relevance. One method we have used previously to demonstrate differences between physiological groups is to assess the percentage of UV Endo cells showing each given sequential [Ca\textsuperscript{2+}], burst. These data were quantified for individual cells in each image field and combined from multiple subjects, and are shown in Fig. 3. Consistent with our representative tracings from Fig. 2, the percentage of cells with each successive burst was significantly lower in the PE group than those in controls, confirming that cell signaling failure at the level of sustained [Ca\textsuperscript{2+}], bursts is indeed widespread in cells from PE subjects. Another form of analysis reflecting overall endothelial function can be achieved by combining the tracings from the UV Endo from control or PE subjects, and these combined average tracings are shown in Fig. 4. Combining overall data from multiple patients in this way eliminated the obvious [Ca\textsuperscript{2+}], bursts but also reveals the [Ca\textsuperscript{2+}], mobilized by the endothelium as a whole in control and PE subjects. The combined average tracings from the control subjects treated with ATP (Fig. 4A) still showed an initial [Ca\textsuperscript{2+}], peak followed by a slow decline in average [Ca\textsuperscript{2+}], that remained well above basal thereafter. In contrast, the average tracing from the PE subjects (Fig. 4C) began with a similar initial peak of [Ca\textsuperscript{2+}], and showed a much more rapid decline to baseline. A sustained NO output was also clearly observed in the control group, but in the vessels from PE subjects this was once again clearly less rapid in its rise, and the DAF-2 signal plateaued at a much lower level, showing the eNOS response was complete much earlier in these subjects. As a control, treatment of both groups with ionomycin (Fig. 4, B and D) showed consistent elevation of [Ca\textsuperscript{2+}], (as would be expected for an ionophore acting independently of signaling pathways); however, the NO output by the PE group appeared consistently reduced compared with vessels from control subjects, suggesting that the functional eNOS pool has also been reduced in PE subjects. Nonetheless, the extent to which the functional pool of eNOS was reduced in PE subjects did not match the lower NO output in response to ATP in PE subjects. Further verification of this is given when we quantified changes in [Ca\textsuperscript{2+}], and overall NO output at key time points and compared these quantitatively, as shown in Fig. 5. In previous UA Endo studies (25) we have measured [Ca\textsuperscript{2+}], elevation at 900 s in vessel segments from nonpregnant and pregnant states, since this reflects when the response in nonpregnant UA Endo has returned to basal but that in pregnant UA Endo has not. When the average [Ca\textsuperscript{2+}], and NO responses in UV Endo treated with ATP (100 \textmu M) were quantified in the same manner, there was also a significant reduction of sustained phase [Ca\textsuperscript{2+}], at 900 s in the PE subjects (Fig. 5A). The overall NO output in response to ATP was also significantly decreased in parallel to the decline in [Ca\textsuperscript{2+}], response in the PE group. When the UV Endo was treated with ionomycin, the decrease in overall NO production in the subjects with PE (Fig. 5B) was also significant, but the proportionate decline was only two-thirds of that observed in response to ATP (PE subjects show 34% decline in response to ionomycin compared with 54% in response to ATP). Thus changes in cell signaling are clearly contributing to the loss of NO output, even though a change in the amount of available functional eNOS may also play a role and exacerbate the deficit further in vivo.

Expression levels of eNOS and functionally associated proteins in UV Endo of control vs. PE subjects. Western blot analysis was also independently performed, albeit on a limited number of samples, to confirm if the ionomycin observations

Fig. 3. Percent of UV Endo cells showing each successive burst after stimulation with the physiological agonist ATP (100 \textmu M). UV Endo from each treatment group (control \( n = 9 \); PE \( n = 6 \)) treated with ATP (100 \textmu M) were assessed for Ca\textsuperscript{2+}, bursts, and the data were plotted for individual cells in each image field. The data represent means \pm SE of UV Endo from multiple segments from each treatment group. The percentage of cells responding with each of the successive bursts is shown. The percentage of cells with successive bursts in the control group was significantly higher than those in the group with PE (*PE < 0.05).
were paralleled by changes in eNOS protein expression levels in the control and PE UV Endo (Fig. 6). Cx43 is a gap junction protein that is also found in endothelial cells and in uterine artery is required for enhanced \([\text{Ca}^{2+}]_i\) bursting, which underlies increased NO production (26). It is therefore important to know if it shows an altered level of expression in PE subjects. Figure 6 shows the quantification of these proteins normalized to Hsp90 (since this is a molecular weight between Cx43 and eNOS). Clearly the levels of both eNOS and Cx43 expression in PE subjects are not substantially reduced to a degree proportional to the observed fall in the ionomycin-activated pool of functional eNOS in PE subjects shown in Fig. 5. Combined, these data suggest that the protein may indeed be showing elevated levels of dysfunction, perhaps as has been proposed by others through reactive oxygen species-mediated damage in the presence of NO (15).

**Imaging of Real-Time Changes in \([\text{Ca}^{2+}]_i\), in HUVEC Maintained in Primary Culture**

Representative tracings from HUVEC. Figure 7, A and B, shows representative tracings of positive-responding cells from the primary HUVEC derived from a control subject and a PE subject following treatment with 100 μM ATP. HUVEC from control cords and maintained to passage 3 showed an initial peak of \([\text{Ca}^{2+}]_i\), followed by repeated bursting in a manner similar to that reported previously in pregnancy-derived UAEC (26). What is striking, however, is that responses in the cells from PE subjects also showed repeated bursting following limited culture, and the rate was more rapid compared with the HUVEC from a control subject. Nonetheless, the magnitude of the individual \([\text{Ca}^{2+}]_i\) bursts in the PE HUVEC appeared smaller, implying both the average \([\text{Ca}^{2+}]_i\), response of responding HUVEC in general, and the time in which peak \([\text{Ca}^{2+}]_i\) levels were sufficient for full eNOS activation in particular (20) could still be reduced overall. To determine if this was the case, more detailed analysis of the percentage of cells showing each \([\text{Ca}^{2+}]_i\) burst and the average \([\text{Ca}^{2+}]_i\); for all responding cells was calculated as for UV Endo above, and then extended to determine the average burst shape in cells from control vs. PE subjects.

**Average burst probability events.** As for UV Endo, the likelihood of cells showing sequential burst events in the bulk...
population of cells derived from control and PE subjects is shown in Fig. 8. In clear contrast to the burst probabilities from individual UV Endo cells observed ex vivo (Fig. 3), Fig. 8A shows the percentage of isolated HUVEC cells showing successive bursts was now significantly higher (P < 0.05) in the PE group compared with those in the control group.

Changes in average [Ca^{2+}]_i response. The average [Ca^{2+}]_i tracings of the responding HUVEC from the control and PE subject groups were very similar (data not shown), and examination of the average level of [Ca^{2+}]_i at the initial peak or at 900 s is shown in Fig. 8B. Of note the average [Ca^{2+}]_i levels were not significantly different between groups. Further examination of the average level at 600 s, the time when average pregnancy-adapted CCE [Ca^{2+}]_i elevation is maximal in uterine artery (24), also revealed no significant difference between groups. Further quantification of the area under the initial peak or sustained phase response for all cells was calculated (data not shown), and this confirmed the overall Ca^{2+} mobilized was not significantly different between the control and PE subject-derived HUVEC.

Third burst response. To evaluate the magnitude of the [Ca^{2+}]_i burst events for any difference between HUVEC from the control and PE subject groups, a “third burst” analysis was undertaken. The third burst was chosen specifically to avoid any confounding overlap with the initial peak, which is predominantly due to release of Ca^{2+} from the endoplasmic reticulum. The third burst peak is clearly more dependent on the CCE response alone. In Fig. 9, average data for the third burst in the HUVEC response was derived from at least 80 cells/dish and 6 dishes/subject in the control and PE groups. The data were then combined from the patients’ average third peaks and reported as the mean ± SE of the values from control (n = 7) and PE (n = 6) subjects. The data were corrected for respective basal level, and the third bursts for control and PE subjects were aligned by maximal point and averages calculated as shown. The dashed line indicates the level to which [Ca^{2+}]_i would have to rise above basal to achieve activation of eNOS (20). The burst shape above the dashed line indicates that the magnitude of the burst and the length of time the burst spent above the activation threshold line in the HUVEC from PE subjects was consistently lower and narrower than that of the HUVEC from control subjects. Despite the fact that the HUVEC from PE subject bursts more frequently, they failed to spend as long near or above the 100 nM [Ca^{2+}]_i level otherwise necessary to achieve significant activation even for optimally phosphorylated eNOS (20).

DISCUSSION

As previously stated, adequate NO production is necessary for the enhanced vasodilation of the vasculature to support a healthy pregnancy. Studies on UA Endo and the corresponding UAEC from pregnant and nonpregnant sheep have set the stage for our understanding of the importance of optimal cell signaling to promote amplified and sustained (pregnancy adapted) vasodilation in response to multiple agonists. In this study, we have taken the next step in applying cutting-edge imaging methods to analyze human UV Endo cells from normal and PE pregnancies to establish, for the first time, the nature of Ca^{2+} signaling and associated NO output in each group. We have indeed shown directly that the repeated [Ca^{2+}]_i bursting responses observed in UV Endo cells from control subjects are very similar to the responses seen in the maternally adapted
ovine UA Endo from late-pregnant sheep (25), whereas the cells from PE subjects clearly show deleteriously altered function of a nature that is reminiscent of the nonadapted UA Endo from nonpregnant sheep. Given the known sensitivity of eNOS to the CCE phase of $[\text{Ca}^2+]$, entry (12) and the observation that optimally phosphorylated eNOS requires $[\text{Ca}^2+]$ above 80–100 nM for activation and up to 200 nM for maximal activation (20), the absence of sustained synchronous $[\text{Ca}^2+]$ bursting in this range in vessels from PE subjects could fully explain the observed decrease in NO production. Although a decline in NO output in response to ionomycin is also detectable to some extent in PE-derived UV Endo segments, it is by no means as pronounced as the reduced NO response to physiological agonist. Further Western blot analysis, albeit limited in power, still supports these findings, and combined all the data infer that PE is not a disease of altered expression of eNOS, but is instead a disease whereby NO output is decreased predominantly as a consequence of failed or inadequate $\text{Ca}^2+$ signaling adaptation, and this is further aggravated by some degree of eNOS functional damage.

While the UV Endo studies described provide valuable insight into the in vivo environment and endothelial cell function of the intact endothelium, the remaining question is whether the lack of response in PE-derived cords is due to active suppression of $\text{Ca}^2+$ signaling by prior exposure to circulating growth factors and cytokines (5a) or simply a lack of programming for optimal function in the first place. Examination of functional responses in HUVEC at passage 3 shows that both control and PE subjects now display repeated bursting. Had HUVEC from the PE subjects continued to display the same lack of adaptive burst function as seen in the PE-derived UV Endo ex vivo, this would have suggested the cells never developed enhanced bursting function in the first place [much as nonpregnant sheep UAEC have never been pro-

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**Fig. 7.** Representative tracings from human umbilical vein endothelial cells (HUVEC). Representative tracings from HUVEC cells from fura 2-loaded primary HUVEC from the control (A) and PE (B) subject groups. Baseline level of $[\text{Ca}^2+]$, was recorded, and the agonist ATP (100 μM) was added at 300 s. Recording is continued for an additional 30 min. The initial $[\text{Ca}^2+]$, peak is similar in both the control and PE tracings. Of note, the PE tracings consistently show more rapid bursts, but of smaller magnitude as represented in the above tracing. Further statistical analysis is shown in Fig. 8.

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**Fig. 8.** Average burst events and $\text{Ca}^2+$ mobilization in HUVEC from control and PE subjects. A shows the overall percentage of cells showing each successive burst in response to ATP (100 μM). Data from at least 80 cells/dish on 6 combined dishes/patient were combined to give means ± SE from control ($n = 7$) and PE ($n = 6$) subject groups. Bursting probability data in A do not take into account the size of the $[\text{Ca}^2+]$ bursts shown in Fig. 7. The data in B are the average $[\text{Ca}^2+]$, data for all responding cells per patient combined, and bars represent the average $[\text{Ca}^2+]$, reached for each patient group. Black bars represent the initial peak height, light gray represents average $[\text{Ca}^2+]$, at 600 s, and dark gray at 900 s. All data are means ± SE, and significance between control and PE subjects is as indicated (*$P < 0.05$).
grammed and so in turn never show pregnancy-adapted levels of bursting in culture (26). However, the fact the HUVEC from PE subjects maintained to passage 3 showed improved bursting function compared with fresh UV Endo from PE subjects clearly suggested the cells were equipped to respond, and the lack of adaptive burst function detected in intact vessel segments ex vivo was due to active suppression of function, but could also recover once cells were maintained in vitro in standard culture conditions.

The finding that recovery of cell function is possible is good news for clinicians; if at least some degree of recovery of function is possible, then examining new ways to target such recovery of function is worthy of further study. The HUVEC from PE subjects are not, however, responding in quite the same way as those from controls. While bursting is largely restored in HUVEC from PE subjects in primary culture, the increased [Ca^{2+}]_{i} bursting rate is accompanied by a decrease in average burst height and duration, as demonstrated by the third burst analysis. Again, given the observations of Tran et al. (20) on the [Ca^{2+}]_{i} dose dependency of eNOS activation requiring [Ca^{2+}]_{i} to exceed 80–100 nM for activation and 200 nM for full activation (when eNOS is optimally phosphorylated), the average third burst in HUVEC from PE subjects is significantly smaller and so only achieves that activation level of [Ca^{2+}]_{i} for a shorter duration with each burst, even if bursting is otherwise more rapid. Albeit a more limited analysis (stimulation time was insufficient to detect repeated [Ca^{2+}]_{i} bursts), Mahdy et al. (13) have also shown that the initial CCE-mediated Cu^{2+} entry in human HHVEC maintained in culture is enhanced in cells from normal pregnancy and ends earlier in cells from PE subjects. When also considering the reported findings of others that subjects with existing hypertension are more likely to develop PE and those developing PE are more likely to go on to develop hypertension (5, 16), these combined findings beg the question of whether some degree of CCE abnormalities is already preexisting in at least some these patients.

While the mechanistic changes underlying pregnancy adaptation of Ca^{2+} signaling have been defined in the uterine vasculature, neither we nor others have yet studied in detail the molecular basis for the sustained Ca^{2+} CCE response in HUVEC. Nonetheless, the bursting phenomenon in control UV Endo certainly shows remarkable similarities to that reported in the UA Endo of pregnant sheep (25). The derived pregnant sheep UAEC are known to respond to ATP stimulation via the P2Y2 class of purinergic receptor coupled to PLC subtype B3 (PLCB3), and the CCE phase of the bursting response is also known to be mediated by inositol 1,4,5-trisphosphate receptor subtype 2 (IP3R2)/TRPC3 transient receptor potential cation channel, subfamily C interaction that is in turn permissively facilitated by pregnancy-enhanced cell-cell communication via Cx43 (10, 26). Not only are burst numbers per cell facilitated by the upregulation of functional Cx43, but also a corresponding pregnancy-specific increase in synchronization of [Ca^{2+}]_{i}, bursting among groups of cells is also dependent on Cx43 (26). HUVEC have already been shown to express P2Y2 receptors, PLCB3, and similar classes of IP3R and TRPC proteins (reviewed in Ref. 7). HUVEC have also been reported to express Cx43 as well as Cx37 and Cx40 (21). Of note, while there are three Cx isoforms reportedly expressed in HUVEC, it is still Cx43 that best mediates electrical coupling of HUVEC (21). The detection of Cx43 protein in UV Endo cell lysates herein, combined with our report of synchronization of [Ca^{2+}]_{i} bursts in UV Endo from control subjects, certainly is consistent with gap junction-mediated cell-cell communication. Also, while UAEC are maternal and indeed arterial, and HUVEC are both fetal and considered venous, the notion that HUVEC and UAEC may operate under similar functional control to maximize vasodilatation certainly makes functional sense when one
also considers optimal maternal flow to the uterus would have to be balanced to match the very vessel that transports oxygenated blood from the placenta to the fetus. To that end, it should also be noted while HUVEC may by name be venous endothelium, they have also been reported to share microvascular properties (22).

In conclusion, Lin and colleagues (12) have shown that the sustained-phase [Ca\(^{2+}\)] response is important for eNOS activation, and Tran et al. (20) have defined the extent to which [Ca\(^{2+}\)]; must rise to achieve eNOS activation. Herein we now show normal pregnancy UV Endo cell signaling responses to ATP include sustained [Ca\(^{2+}\)]; bursting sufficient to maintain periodic eNOS reactivation and, under dual-imaging conditions using DAF-2, correspondingly sustained NO production is observed. In contrast, in the UV Endo of PE pregnancy, these [Ca\(^{2+}\)]; burst responses are notably absent, and this is associated with a dramatic loss of NO output in response to ATP. Western blot analysis demonstrated there is no obvious difference in eNOS protein expression in the UV Endo between the control and PE subjects, but there is some loss of NO response when UV Endo is treated with ionomycin, suggesting reactive oxygen species damage to the eNOS protein pool may have occurred in PE subjects in vivo. Nonetheless, the finding that the reduction in NO response to ATP is even greater than in response to ionomycin in PE subjects suggests the eNOS pool itself is not the sole limiting factor, and the failure of Ca\(^{2+}\) signaling is equally critical. Strategies to restore [Ca\(^{2+}\)]; bursting in response to GPCR-coupled agonists such as ATP in those with established PE should still result in improved NO production in vivo. Of note, while the technical ability to image changes in real time in individual cells has focused our studies here on NO production, restoration of the Ca\(^{2+}\) burst response may also serve to rescue the parallel decline in PGI\(_2\) production that also occurs in PE subjects (8), given that, like eNOS, the activation of cytosolic phospholipase A\(_2\) is also Ca\(^{2+}\) sensitive and sensitivity to Ca\(^{2+}\) increases in UA Endo during pregnancy (6). Not only could a strategy to immediately restore bursts by direct pharmacological support of IP\(_3\)/R/TRPC-mediated CCE bursts improve the symptoms of a severe PE subject to some degree alone, it could also be used in combination with a smooth muscle relaxant such as the L-channel antagonist nifeditine where nifedipine therapy alone proves insufficient. Even partial success in such severe cases before 28 wk may be enough to delay the otherwise forced delivery of a premature infant purely for the sake of protecting the mother, to a later gestational time when the infant’s survival is also assured and the further impact of premature delivery on lifelong health will be reduced (4).

Of further clinical relevance to the underlying cause of PE, our observation that HUVEC from PE subjects in primary culture can still recover some degree of bursting in vivo suggests the absence of the sustained [Ca\(^{2+}\)]; bursting in vivo is due to active suppression of function, and is entirely consistent with the reports of others that serum from PE subjects can also impair normal HUVEC function (11, 23, 29). Many candidate factors have been suggested to mediate such impairment of function, but to date no single candidate or group of candidates has been found to be capable of accurately predicting PE (14). We have recently proposed that it is the combination of growth factors and cytokines elevated in PE subjects acting together through shared kinase signaling pathways that ultimately converge on the phosphorylation and closure of Cx43 gap junctions, so suppressing endothelial [Ca\(^{2+}\)]; burst function and reversing pregnancy adapted vasodilatory function in PE subjects (5a). Given the outcome of these studies, we now propose that limiting the PE-associated overstimulation of these inhibitory signals in vivo at the first signs of onset of hypertension and proteinuria could moderate the developing hypertensive symptoms. Many challenging studies will now be necessary to establish if such an outcome is achievable without compromising the mother or the child, but given the risks of severe PE to the mother and the lifelong consequences of preterm birth, particularly when associated with growth restriction (4), the benefits of such a therapy could be enormous to the individuals concerned and to society as a whole.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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LOSS OF PREGNANCY ADAPTED SIGNALING IN PREECLAMPSIA


