Altered VEGF-stimulated Ca^{2+} signaling in part underlies pregnancy-adapted eNOS activity in UAEC

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Abstract

In pregnancy, the uterine vasculature undergoes dramatic vasodilatory adaptations. Previously, vascular endothelial growth factor (VEGF) has been shown to stimulate endothelial nitric oxide synthase (eNOS) in uterine artery endothelial cells (UAECs) derived from pregnant ewes to a greater extent than those from non-pregnant ewes in a manner not fully explained by changes in the phosphorylation of eNOS. In this study, we used Fura-2 Ca^{2+} imaging and arginine-to-citrulline conversion eNOS activity assays to assess the importance of VEGF-stimulated Ca^{2+} responses in pregnancy-related changes in NO production in UAEC. In this study, we show that pregnancy-induced changes in VEGF-stimulated Ca^{2+} responses could account in part for the greater capacity of VEGF to stimulate eNOS in UAECs from pregnant versus non-pregnant animals. VEGF-stimulated Ca^{2+} responses in UAECs from pregnant and non-pregnant animals were mediated through VEGF receptor 2 and were detected in roughly 15% of all cells. There were no pregnancy-specific differences in area under the curve or peak height. UAECs from pregnant animals were more consistent in the time to response initiation, had a larger component of extracellular Ca^{2+} entry, and were more sensitive to a submaximal dose of VEGF. In UAECs from pregnant and non-pregnant animals Ca^{2+} responses and eNOS activation were sensitive to the phospholipase C/inositol 1,4,5-trisphosphate pathway inhibitors 2-aminoethoxydiphenylborane and U73122. Thus, changes in VEGF-stimulated $[Ca^{2+}]_i$ are necessary for eNOS activation in UAECs, and pregnancy-induced changes in Ca^{2+} responses could also in part explain the pregnancy-specific adaptive increase in eNOS activity in UAECs.

Key Words
- VEGF
- endothelium
- pregnancy
- adaptation

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Introduction

Pregnancy is a time of dramatic vascular adaptation. Through initial angiogenesis and then sustained vasodilation, the pregnant uterus achieves the biggest drop in vascular resistance of any organ system. This in turn promotes a dramatic increase in maternal uterine blood flow to meet the needs of the growing fetus (Magness 1998). Impaired vasodilator outputs, including nitric oxide (NO) and prostacyclin, have been implicated

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in hypertensive disorders of pregnancy, such as preeclampsia (reviewed in Sladek *et al.* (1997), Bird *et al.* (2003), Walsh (2004) and Valdes *et al.* (2009)). A further understanding of the causes of vascular adaptation of pregnancy and its failure in associated gestational hypertensive disorders could also be informative with respect to generalized hypertension.

The isolation and culture of ovine uterine artery endothelial cells (UAEC) has allowed further mechanistic studies (Bird *et al.* 2000, Di *et al.* 2001, Cale & Bird 2006, Gifford *et al.* 2006*a*,*b*, Sullivan *et al.* 2006, Grummer *et al.* 2009, Yi *et al.* 2010*a*), and we have developed a mechanistic model of the tightly regulated changes that underlie pregnancy-enhanced vasodilator production in the gravid uterus. Results from our studies to date have indicated that ATP (working through heptahelical P2Y2 receptors) and vascular endothelial growth factor (VEGF) (working through VEGF receptor 2 (VEGFR2)) in particular both stimulate comparatively low NO production in luteal-phase non-pregnant ewes, but relatively higher production in late-gestational pregnant ewes. In the case of ATP, an increase in capacitative Ca^{2+} entry (CCE), mediated by an increase in connexin 43 coupling and taking the form of repeated $[Ca^{2+}]_i$ bursts, is also observed during pregnancy, and this is clearly a major drive for enhanced NO output (Yi *et al.* 2010*a*, 2011). Our previous study of the effects of VEGF in UAEC failed to reveal evidence for activating phosphorylation events sufficient to explain enhanced NO output in response to VEGF during pregnancy (Grummer *et al.* 2009). Given that cell–cell coupling alters the general ability of the cell as a whole to mount CCE responses, other classes of hormones (besides ATP), such as VEGF may also trigger enhanced endothelial NO synthase (eNOS) activation through enhanced capacitative entry responses. If so, an alteration in CCE response with VEGF stimulation could at least partly explain greater NO output.

While VEGF Ca^{2+} signaling has been studied in detail in a number of other cell types, there is little understanding of the changes in endothelial VEGF signaling that relate to eNOS activation during pregnancy adaptation. To that end, our study examines in detail VEGF-driven Ca^{2+} signaling as it relates to NO production in both the non-pregnant and pregnant states. We propose the hypothesis that VEGF stimulates a phospholipase C (PLC)-mediated Ca^{2+} response in UAEC in general, and that a pregnancy-related increase in the VEGF-stimulated Ca^{2+} entry response (i.e. during the CCE phase) occurs. We further propose that enhanced Ca^{2+} entry is causally related to eNOS activation, and may explain the greater eNOS activation in UAEC from pregnant animals as previously observed by Grummer *et al.* (2009). Thus the goals of this study are to establish i) if there is VEGF-stimulated Ca^{2+} signaling in UAEC, ii) the role of VEGFR1 and 2 in any such response in UAEC from non-pregnant and pregnant animals, iii) if the Ca^{2+} entry (CCE) component of such responses is also enhanced by pregnancy, and iv) if such a change is related to eNOS activation and can explain the pregnancy-related increase in eNOS activity in response to VEGF.

Materials and methods

Materials

Fura-2 AM (2-{6-[Bis(carboxymethyl)amino]-5-(2-{2-[bis(carboxymethyl)amino]-5-methylphenoxy}ethoxy)-1-benzofuran-2-yl}-1,3-oxazole-5-carboxylic acid-acetoxymethyl ester) and Pluronic F127 were obtained from Life Technologies, $CaCl_2$ from EMD Millipore (Billerica, MA, USA), and ATP (disodium salt) and all other chemicals, unless noted otherwise, were from Sigma. Also unless noted otherwise, MEM and all other cell culture reagents were purchased from Life Technologies. For $[Ca^{2+}]_i$ imaging studies, 35-mm dishes with glass coverslip windows were purchased from MatTek Corp. (Ashland, MA, USA). VEGF165 and placental growth factor (PlGF) were from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant Orf virus VEGF-E was purchased from Angio-Proteomie (Boston, MA, USA). VEGFR tyrosine kinase inhibitor (VEGFRi; 4-((4′-chloro-2′-fluoro)phenylamino)-6,7-dimethoxyquinazoline, a reasonably selective inhibitor of VEGFR2 over VEGFR1; IC_{50} = 100 nM and 2 μM respectively), 2-aminoethoxydiphenylborane (2-APB; a somewhat selective inositol 1,4,5-trisphosphate receptor (IP3R) inhibitor), and U73122 (a selective inhibitor of PLC activation) were purchased from EMD Millipore. PP2 (a selective inhibitor of SRC family kinases) was purchased from Enzo Life Sciences (Farmingdale, NY, USA), and U0126 (a selective inhibitor of MEK, a kinase known to directly phosphorylate ERK1 and ERK2) was from Promega Corp.

Isolation of UAEC

Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin–Madison Research Animal Care Committees of both the School of Medicine and Public Health and the College of Agriculture and Life Sciences and followed the recommended American Veterinary Medicine Association guidelines for humane treatment and euthanasia of

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laboratory farm animals. Uterine arteries were obtained from mixed Western breed NP sheep and pregnant ewes at 120–130 days of gestation during non-survival surgery, and UAECs were prepared as previously described (Bird *et al.* 2000, Grummer *et al.* 2009) and stored in liquid nitrogen. For all subsequent experiments, the cells thawed for use were grown to near confluence in MEM containing 20% FBS, 1% penicillin–streptomycin, and 4 μg/ml gentamycin.

Fura-2 $[Ca^{2+}]_i$ imaging studies

Pooled passage 4 cells were grown in 35-mm glass-bottom micro well dishes and imaged at 70% confluence. The cells were loaded with 5 μM Fura-2 AM in Krebs buffer at 37 °C for 45 min as performed previously (Yi *et al.* 2010*a*). They were then incubated at room temperature for 30 min to allow the hydrolysis of ester, and imaging was performed exactly as previously described (Yi *et al.* 2010*a*). Pretreatments were 25 min for VEGFR kinase inhibitor; 20 min for PP2, U0126, and U73122; and 5 min for 2-APB.

eNOS activation assay

Passage 3 cells stored in liquid N_2 were plated in T75 flasks. After growing to confluence, as mentioned earlier, the cells were passaged to 12-well dishes and grown for 24 h to approximately 80% confluence. The cells were preincubated in Krebs buffer (25 mM HEPES (pH 7.4), 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 6 mM glucose, and 2 mM $CaCl_2$) and eNOS activity was then assessed using the arginine-to-citrulline conversion assay exactly as used previously (Grummer *et al.* 2009). Following pretreatment with vehicle or antagonists as described for 5-min, cells were treated with or without agonists for 10 min.

Western blotting analyses

UAECs were passaged into 60-mm dishes and maintained for 24–48 h until approximately 80% confluent. Antagonists were added 5 min before the 10-min agonist treatment. At the end of stimulation, media were quickly aspirated and cells were snap-frozen in liquid N_2. The cells were solubilized in lysis buffer (50 mM HEPES (pH 7.5), 4 mM $Na_4P_2O_7$–10 H_2O, 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM $Na_3(VO_4)_2$, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 μg/ml leupeptin, and 5 μg/ml aprotinin), briefly sonicated, and centrifuged at 12 000 ***g*** for 5 min. Subsequently, western blotting of extracted proteins and detection of phosphorylated proteins using phosphorylation-state-specific antibodies were achieved using ECL detection exactly as described previously (Sullivan *et al.* 2006, Grummer *et al.* 2009).

Statistical analysis

Data are presented as means ± S.E.M. and were analyzed by Student's *t*-test or ANOVA, as appropriate. A value of $P<0.05$ was considered statistically significant.

Results

VEGF165 stimulates Ca^{2+} response in UAEC

Treatment of UAEC from pregnant animals (passage 4) with a maximal mitogenic or eNOS stimulatory (Grummer *et al.* 2009) dose of VEGF165 (10 ng/ml), a 165 amino acid splice variant of VEGF-A, consistently revealed a Ca^{2+} response that was detectable by video imaging. A summary of the responses calculated from all cells analyzed and the average data of all the responding cells are given in Table 1. VEGF165 (10 ng/ml) only detectably recruited approximately 15% of cells to respond (Table 1), whereas the prototypical G-protein-coupled receptor agonist, ATP (100 μM), mobilized Ca^{2+} in over 90% of cells (Yi *et al.* 2010*a*), added to Table 1 for comparison). The Ca^{2+} response per cell to 10 ng/ml VEGF165 was also highly variable in its nature (Fig. 1). Overall, VEGF165 caused a singular transient response or multiphasic waves of Ca^{2+} with periodic maxima, but did not return to near basal values between those maxima. Responses occurred at these two extremes as well as variations on those combined themes (Fig. 1A and B). Figure 1C shows that, where observed, VEGF165 commonly gave a delayed response (Fig. 1C) taking from many seconds to minutes to initiate a detectable Ca^{2+} response. There was also no evidence of any synchronous $[Ca^{2+}]_i$ burst responses in UAEC from pregnant animals in response to 10 ng/ml VEGF165 alone, in contrast to results described in our previous report of synchronized burst responses in UAEC from pregnant animals treated with ATP (Yi *et al.* 2010*a*). Nonetheless, the timing of the onset of response was more tightly grouped in UAEC from pregnant animals (Fig. 1C). When responses were further combined to show the average, the VEGF165-stimulated Ca^{2+} response in UAEC from pregnant animals tended to reach more defined early maxima and then return to basal, while in UAEC from non-pregnant animals the average responses tended to peak at a lower level but with a longer plateau (Fig. 1D).

In Fig. 2, we extend our analysis to show dose dependency of the percentage of cells responding to

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Table 1 Measurement parameters for VEGF family peptide-stimulated Ca^{2+} responses in UAEC from non-pregnant animals (NP-UAEC) and UAEC form pregnant animals (P-UAEC). Summary of Ca^{2+} response parameters for 30 min treatment with VEGF isoforms, and ATP as a comparison ($n=5$–8 dishes/treatment). Results are mean ±S.E.M., normalized where stated

				Responders only			All cells		
Treatment	Cell type	Percentage responding	Basal Ca^{2+} (nM)	Peak height (nM above basal)	AUC	AUC normalized to VEGF-165 P-UAEC	Peak height (nM above basal)	AUC	AUC normalized to VEGF165 P-UAEC
VEGF165 (10 ng/ml)	P-UAEC	15.66±3.53	52.51±4.71	138.02±26.92	60 382.62±13 861.95	100.00±22.96	26.38±5.97	7201.94±3817.68	100.00±53.01
	NP-UAEC	16.56±2.53	71.37±3.62‡	105.77±8.12	82 117.51±7322.55	136.00±12.13	21.33±3.78	7947.18±3675.82	110.35±51.04
VEGF-E (100 ng/ml)	P-UAEC	12.82±3.52	52.64±3.06	109.28±20.12	56 949.63±12 841.02	94.31±21.27	17.88±4.19	2201.01±5596.85	37.50±77.71
	NP-UAEC	31.60±3.61*,‡	72.64±3.91‡	133.41±9.23	120 360.87±13 530.75	199.33±22.41‡	43.39±4.34*,‡	35 160.17±5658.94	488.20±78.58*,‡
PlGF (100 ng/ml)	P-UAEC	1.36±0.61*,†	54.38±2.88						
	NP-UAEC	2.31±1.56*,†	73.1±1.68‡						
ATP (100 μM)	P-UAEC	90.40±3.99*,†	63.17±3.88	187.96±39.96	58 353.26±15 715.28	96.64±26.03	175.40±42.54*,†	54 524.37±16 112.26	757.08±223.72*,†
	NP-UAEC	92.92±2.65*,†	63.11±4.36	135.21±28.85	33 518.25±8709.70	55.51±14.42*,†	129.11±28.22*,†	32 736.07±8878.09	454.55±123.27*

Gray boxes indicate no further analysis. Statistics by Student's *t*-test: *$P<0.05$ versus 10 ng/ml VEGF165; †$P<0.05$ versus 100 ng/ml VEGF-E; and ‡$P<0.05$ versus P-UAEC of same treatment. AUC, area under the curve (arbitrary units).

VEGF165. Notably, UAEC from pregnant animals showed a significantly greater rate of observed Ca^{2+} responses to VEGF165 than UAEC from non-pregnant animals at the sub-maximal dose of 1 ng/ml VEGF165, which is closer to the physiological range (Fig. 2). Thereafter, in UAEC from pregnant animals there was no further significant difference between 1 and 10 ng/ml in recruitment of cells to produce a Ca^{2+} response (12 and 15% respectively). In contrast, UAEC from non-pregnant animals treated with 1 ng/ml VEGF165 showed a significantly lower cell recruitment for a clear Ca^{2+} response, although this response increased further up to the level observed for responders in UAEC from pregnant animals as the dose increased beyond the physiological range to 10 ng/ml (5 and 16% respectively, Fig. 2).

VEGFR2 mediates the Ca^{2+} response to VEGF165

To differentiate the role of VEGFR1 and VEGFR2 in the mediation of Ca^{2+} response, we repeated the challenge of Fura-2-loaded UAEC with PlGF and VEGF-E. Whereas VEGF165 binds both VEGFR1 and VEGFR2 (Zachary 2003), PlGF1 binds only VEGFR1 (Zachary 2003), and VEGF-E preferentially binds VEGFR2 (Ogawa *et al*. 1998, Meyer *et al*. 1999). This follows the same strategy employed previously (Grummer *et al*. 2009) to decipher the critical role of VEGFR2 in eNOS phosphorylation and activity. Notably, neuropilin-1 (a transmembrane receptor known to bind VEGF) was not detected in UAEC by western blotting in previous experiments (Grummer *et al*. 2009).

In UAEC from pregnant animals, VEGF-E-treated cells behaved similarly to cells treated with VEGF165 in terms of the percentage of cells producing a Ca^{2+} response (Table 1). Parameters measuring amplitude and duration, such as Ca^{2+} peak height and area under the curve (AUC), were also indistinguishable. In UAEC from non-pregnant animals, the Ca^{2+} response to VEGF-E was significantly greater than the response to VEGF165 both in terms of the percentage of cells responding and the AUC for each responding cell (Table 1). The cumulative effect of this manifested itself in a vastly larger mean AUC when using data from all cells measured, including non-responding cells (Table 1). In contrast to VEGF-E, the VEGFR1-selective PlGF was only able to produce a negligible response in both UAEC from non-pregnant animals and UAEC from pregnant animals. Few cells (<2%) responded to PlGF and as a result descriptive analysis on the response shape was not undertaken. To further investigate the possible negative regulatory effect of VEGFR1 stimulation on UAEC from non-pregnant animals (since VEGF-E gives

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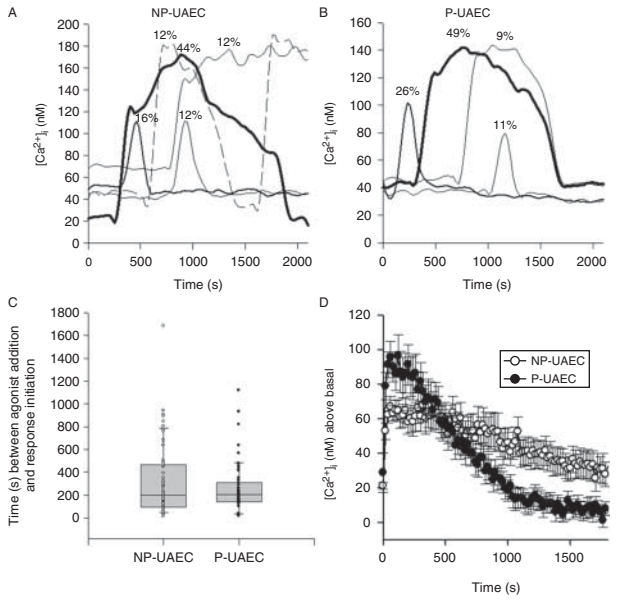

Figure 1
Descriptive characterization of VEGF165-stimulated Ca^{2+} responses in UAEC. UAEC from non-pregnant animals (NP-UAEC) ($n=8$ dishes) and UAEC from pregnant animals (P-UAEC) ($n=8$ dishes) were treated with 10 ng/ml VEGF165 for 30 min. (A and B) Typical variations in Ca^{2+} response shapes. Percentages and line thickness correspond to the proportion of responding cells that can be roughly placed into each category. (C) Response delay time between agonist addition and initiation of a detectable Ca^{2+} response, reaching double the basal levels. (D) Mean Ca^{2+} responses, aligned at the point of response origin and plotted throughout the course of the experiment ($n=64$ responding cells for UAEC from non-pregnant animals $n=58$ cells for UAEC from pregnant animals).

a greater response than VEGF165), we treated UAECs with a combination of VEGF-E and PlGF. There were no differences from VEGF-E stimulation alone for any parameter measured (results not shown). The Ca^{2+} responses to VEGF165, PlGF1, and VEGF-E in UAEC from both non-pregnant and pregnant animals are all fully consistent with previously published results (Grummer *et al.* 2009), indicating that VEGFR2 is the sole mediator of eNOS activation by the VEGF peptide family members. In UAEC from both non-pregnant and pregnant animals the VEGF165-stimulated Ca^{2+} response was blocked by the VEGFRi (Fig. 3), indicating that VEGF165-stimulated Ca^{2+} responses are mediated by the endogenous kinase domain of VEGFR2. VEGFR2 coupling to Ca^{2+} signaling was not inhibited by the MEK–ERK inhibitor U0126 (Fig. 3), indicating that ERK signaling does not play a role in VEGF165-stimulated Ca^{2+} responses. However, a level of inhibition similar to that of the VEGFRi was achieved with PP2, indicating that the activity a Src family kinase member is also crucial for propagation of the VEGFR2-mediated Ca^{2+} response to VEGF165 (Fig. 3).

VEGFR2 is coupled to PLCγ activation to mediate the elevation of $[Ca^{2+}]_i$ in UAEC

Our finding that VEGF165 responses in UAEC typically initiate between 1 and 5 min post-stimulation, and can even occur very late in the 30-min recording time is entirely consistent with PLCγ recruitment (Rameh *et al.* 1998). Removal of added Ca^{2+} from the extracellular buffer also revealed an initial symmetric peak of $[Ca^{2+}]_i$ that was probably from intracellular sources (Fig. 4A), and the aforementioned sustained Ca^{2+} response that was otherwise highly variable was uniformly removed, so suggesting it to be entirely reliant on extracellular Ca^{2+} influx. On average, in UAEC from non-pregnant animals, over 80% of the AUC for the VEGF165-stimulated Ca^{2+} response was dependent on Ca^{2+} influx, but in UAEC from pregnant animals over 90% was dependent on extracellular Ca^{2+} influx (Fig. 4B). An indication that the sustained-phase Ca^{2+} response also requires IP3 formation, and release of an intracellular pool of Ca^{2+} was further examined by determining if the PLC inhibitor U73122 could block both phases of the Ca^{2+} response completely. Dose responses for both percentage of cells responding and reduced AUC are shown in Fig. 5A and B respectively. U73122 efficiently blocked the VEGF165-stimulated Ca^{2+} response at 1 μM in both UAEC from both non-pregnant and pregnant animals, virtually

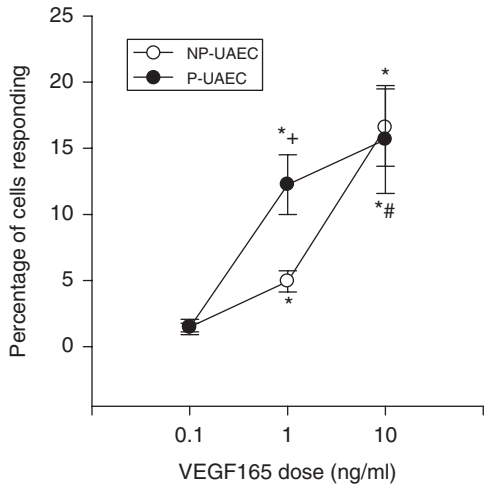

Figure 2
VEGF165 dose–response stimulation of Ca^{2+}. UAEC from non-pregnant and pregnant animals were treated with 0.1 ng/ml ($n=5$ dishes for each condition), 1 ng/ml ($n=5$ dishes for each condition), or 10 ng/ml VEGF165 ($n=8$ dishes for each condition) and imaged for 30 min. Results are the mean percentages of cells giving a detectable Ca^{2+} response per dish at each dose ± S.E.M. $^{*}P<0.05$ versus 0.1 ng/ml of same cell type, $^{\#}P<0.05$ versus 1 ng/ml of same cell type, and $^{+}P<0.05$ versus equivalent dose in UAEC from non-pregnant animals.

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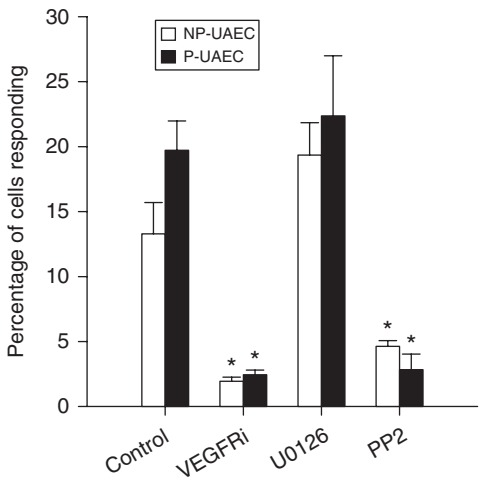

Figure 3
VEGF165-stimulated Ca^{2+} signals through the VEGFR2 kinase domain and is dependent on Src but not ERK activity. UAEC from non-pregnant animals ($n=5$ dishes) and UAEC from pregnant animals ($n=5$ dishes) were treated for 30 min with 10 ng/ml VEGF165, which was preceded by a 25-min treatment with 1 μM VEGFR2 kinase inhibitor (VEGFRi), or a 20-min pretreatment with 10 μM PP2 or 10 μM U0126. Cells were grown to 70% density, loaded with Fura-2, and 50–80 cells/dish were imaged for 30 min. Results are the mean percentages of cells responding ± S.E.M. *$P<0.05$ versus control.

eliminating any ability to mount a Ca^{2+} response at this dose. At lower doses where there was no significant reduction in number of cells responding, there was likewise no reduction in AUC for those cells. This indicates that once threshold levels of IP3 are reached sufficient to trigger a response, the subsequent Ca^{2+} response will continue in its entirety.

The VEGF165-stimulated sustained Ca^{2+} entry response phase is a form of repeated bursting and probably via IP3R/TRPC interaction

To corroborate the findings with U73122 and further address the nature of the Ca^{2+} entry mechanism in the sustained phase of the VEGF165 response, we tested the IP3R 'antagonist' 2APB as we have done previously in examining the response to ATP stimulation (Gifford *et al.* 2006*a*, Sullivan *et al.* 2006). Our former data for ATP indicate that in UAEC 2-APB most effectively blocks IP3R–TRPC interactions. Consistent with this, whereas the initial $[Ca^{2+}]_i$ peak in response to VEGF165 was not inhibited by 2-APB, and in UAEC from non-pregnant animals there was even a dose-dependent increase in the percentage of cells producing the initial transient Ca^{2+} response to VEGF165 (Fig. 5C), UAEC from both non-pregnant and pregnant animals subsequently displayed a dose-dependent decrease in the sustained Ca^{2+} response, indicated by a reduction in AUC response (Fig. 5D), as expected. The occurrence of repeated periodic maxima was also seen to return to near baseline levels in the otherwise sustained-phase response of UAEC from pregnant animals when the maximal dose of VEGF165 was added with 2-APB (Fig. 6). This may further indicate that the apparently continuous sustained phase in response to VEGF165 alone may be comprised of fused bursts. Consistent with this, we have also seen more discretely repeated bursts in response to sub-maximal stimulation when using a lower dose of VEGF165 (≤1 ng/ml) in the absence of 2-APB (Fig. 6).

The Ca^{2+} response antagonists U73122 and 2-APB also inhibit VEGF165-stimulated eNOS activity

To further determine whether the VEGF165-stimulated eNOS activity is indeed dependent on Ca^{2+}, UAEC from both non-pregnant and pregnant animals were again pretreated with the two Ca^{2+} response inhibitors described earlier, U73122 or 2-APB, and VEGF165-stimulated eNOS activity was measured. With both inhibitors, UAEC from both non-pregnant and pregnant animals showed a dose-dependent decrease in eNOS activity (Fig. 7A and B). For U73122, the dose–response curves for percentage inhibition for UAEC from both non-pregnant and pregnant animals were nearly identical. However, a higher dose of U73122 was required to completely block

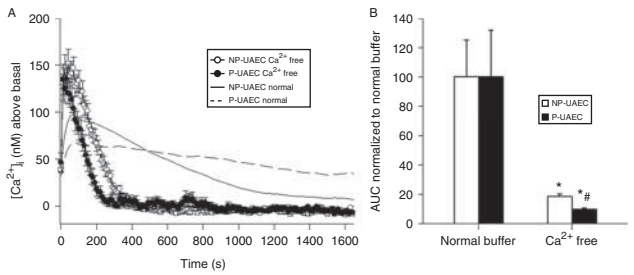

Figure 4
Sustained Ca^{2+} responses to VEGF165 depend on extracellular Ca^{2+} influx. Ca^{2+} was removed from the experimental buffer and UAEC were treated with 10 ng/ml VEGF165 (A, $P<0.001$ for UAEC from non-pregnant animals ($n=5$ dishes) versus UAEC from pregnant animals ($n=5$ dishes) in the first 300 s, analyzed by paired *t*-test and one-way ANOVA). In B, quantification of AUC for UAEC from both non-pregnant and pregnant animals in the presence of Ca^{2+}-free buffer versus normal buffer ($n=5$ dishes per cell type, per treatment) is shown. Data are represented as dish means ± S.E.M. (*$P<0.05$ versus normal buffer of same cell type and #$P<0.01$ versus UAEC from non-pregnant animals of Ca^{2+} free buffer). Significance was determined using Student's *t*-test for B.

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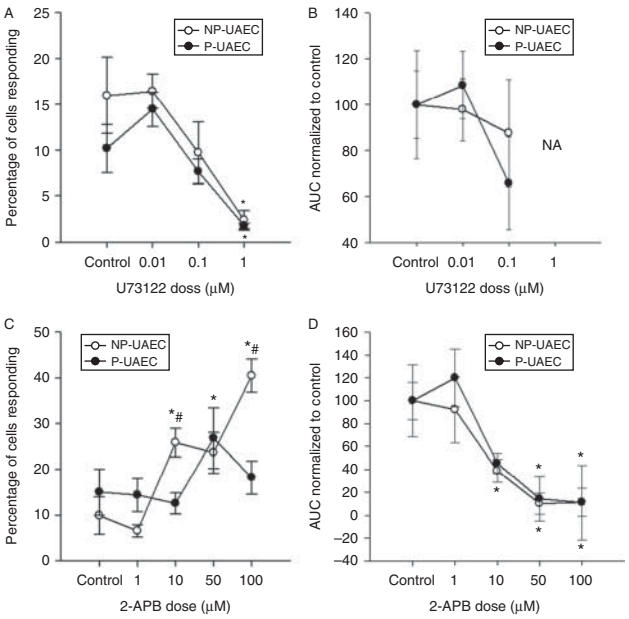

Figure 5
Both initial and sustained Ca^{2+} responses to VEGF165 are IP3-dependent. The dose responses to inhibitors of the IP3 signaling pathway for VEGF165-stimulated Ca^{2+} responses are shown. In A and B, dose responses for pretreatment for 20 min with the PLC inhibitor, U73122, for both UAEC from non-pregnant animals ($n=5$ dishes) and UAEC from pregnant animals ($n=5$ dishes), with 10 ng/ml VEGF165 stimulation, are shown (A, $^{*}P<0.05$ versus control). Too few cells responded to VEGF165 at the 1 μM dose of U73122 to permit statistical analysis for area under the curve (AUC, (B) indicated by NA). In C and D, dose responses for pretreatment for 5 min with the IP3R antagonist, 2-APB, with 10 ng/ml VEGF165 in UAEC from non-pregnant animals ($n=5$ dishes) and UAEC from pregnant animals ($n=5$ dishes) are shown (C, $^{*}P<0.05$ versus control, $^{\#}P<0.05$ versus same dose of 2-APB in UAEC from pregnant animals; D, $^{*}P<0.05$ versus control). Data presented as mean ± S.E.M. for all panels.

eNOS activity (10 μM) than was required to block the Ca^{2+} response (1 μM). Pretreatment with 2-APB also inhibited VEGF165-stimulated eNOS activity for UAEC from both non-pregnant and pregnant animals. At 10 μM, 2-APB caused equivalent reductions in eNOS activity, by roughly 40%. However, at 50 μM, 2-APB only reduced eNOS activity a further 10% in UAEC from pregnant animals whereas in UAEC from non-pregnant animals the response was completely blocked. In comparison, U73122 and 2-APB had minimal effects on eNOS phosphorylation at various sites previously implicated in the regulation of eNOS activity (Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article).

Discussion

Our previous studies reported that UAECs do not display an observable Ca^{2+} response (Bird *et al.* 2000, Gifford *et al.* 2003) to VEGF in primary culture, but this was undertaken using just one cell at a time with the basic photometry apparatus. Since that time we have reexamined this question using video imaging and can monitor many (40–80) Fura-2-loaded cells simultaneously. We have found using video imaging that a subpopulation of cells are indeed capable of mounting detectable Ca^{2+} responses to VEGF, therefore supporting the hypothesis that VEGF165 stimulates Ca^{2+} responses in UAEC. Our initial examination reveals few obvious differences in the gross Ca^{2+} responses to 10 ng/ml VEGF165 observed in NP- and P-UAEC. With respect to the hypothesis that VEGFR1 and 2 have a role in Ca^{2+} response in UAEC from both non-pregnant and pregnant animals, examination of the relative effects of PlGF and VEGF-E with VEGF165, as well as the effect of the VEGR2 kinase inhibitor, confirms the action of VEGF165 activating Ca^{2+} signaling through VEGFR2 in UAEC from both non-pregnant and pregnant animals. Consistent with this, the VEGFR1 agonist PlGF does not cause a reliable Ca^{2+} response on its own, nor does it strongly stimulate eNOS activity (Grummer *et al.* 2009). Nonetheless, while both VEGF165 and VEGF-E behaved almost identically in UAEC from pregnant animals in UAEC from non-pregnant animals twice as many cells responded to VEGF-E than to VEGF165. Those cells that gave a detectable Ca^{2+} response to VEGF-E also tended to give a more prolonged response, as indicated by the larger mean AUC in the subset of cells that responded (Table 1). Because the only difference between VEGF165 and VEGF-E binding to VEGFRs is that VEGF165 binds both VEGFR1 and VEGFR2 and VEGF-E preferentially binds VEGFR2, these results would indicate a co-modulatory

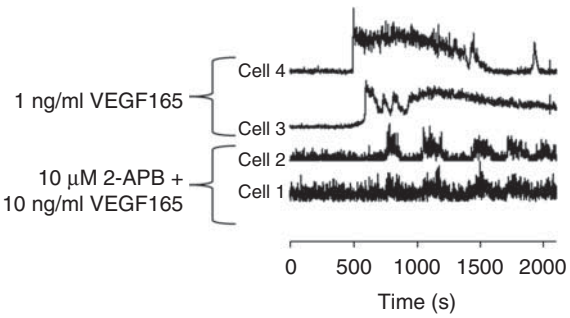

Figure 6
Reducing the VEGF165-stimulated Ca^{2+} response signal reveals Ca^{2+} bursts. Using a submaximal dose of VEGF165 (1 ng/ml, cells 3 and 4) or 10 μM 2-APB pretreatment with 10 ng/ml VEGF165 (cells 1 and 2) revealed that Ca^{2+} burst responses may be fused in continuous Ca^{2+} responses to 10 ng/ml VEGF165 alone in UAEC from pregnant animals. Burst activity is also observed in UAEC from non-pregnant animals under similar conditions.

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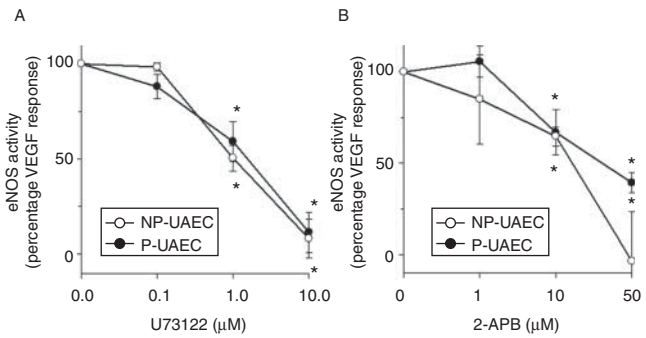

Figure 7
Inhibitors of VEGF165-stimulated Ca^{2+} responses inhibit eNOS activity. eNOS activity was measured by arginine-to-citrulline conversion assay. UAEC from non-pregnant animals ($n=4$/dose) and UAEC from pregnant animals ($n=4$/dose) were pretreated with inhibitor for 5 min, followed by a 10-min treatment with 10 ng/ml VEGF165. (A) Cells were treated with 0.1, 1, or 10 μM U73122. (B) Cells were treated with 1, 10, or 50 μM 2-APB. Data for both panels presented as mean ± S.E.M. (*$P<0.05$ versus control).

role for VEGFR1 over VEGFR2, as previously suggested (Grummer *et al.* 2009). Together, our findings indicate that VEGFR1 may indeed be acting as 'decoy receptor' in UAEC as others have suggested (Park *et al.* 1994). Even though Grummer *et al.* (2009) reported that relative VEGFR1 and VEGFR2 expression levels are similar in UAEC from non-pregnant and pregnant animals, there still may be changes in co-localization that could account for these differences between the non-pregnant and pregnant state. Further studies would be needed to definitively address this question.

Regarding the hypothesis that there is pregnancy-specific enhancement of CCE, even though the Ca^{2+} responses to VEGF overall show no clear differences in AUC between UAEC from non-pregnant and pregnant animals, removal of extracellular Ca^{2+} reveals that CCE plays a greater role in the VEGF Ca^{2+} response in UAEC from pregnant animals. Despite a lack of discrete synchronous bursting by neighboring cells as previously observed in response to ATP (Yi *et al.* 2010*a*,*b*), UAEC from pregnant animals show more coordinated and timely initiation of a Ca^{2+} response to VEGF165 and are more capable of producing a coordinated early Ca^{2+} peak compared with UAEC from non-pregnant animals.

Previously, we used U73122 (a PLC inhibitor) and 2-APB (an IP3R/TRPC activation antagonist) to implicate IP3 as a key mediator in the Ca^{2+} response to ATP (Sullivan *et al.* 2006). As shown here, both inhibitors work in a similar manner to inhibit the VEGF165-stimulated Ca^{2+} response in UAEC as they do with ATP. While U73122 gives a total inhibition of the Ca^{2+} response, 2-APB more selectively inhibits the sustained phase. At the higher doses used in this study, 2-APB even augments cell recruitment in response to 10 ng/ml VEGF165, but Ca^{2+} responses only remained elevated above basal levels for a duration corresponding closely with the initial endoplasmic reticulum Ca^{2+} release alone (in the absence of extracellular Ca^{2+}). These observations alone beg the question of whether VEGF activation of PLCγ may in turn evoke a Ca^{2+} response through interaction of at least one of the IP3R and TRP isoforms already known to be present in UAEC. Others have reported VEGF signaling through TRPC6 (Ge *et al.* 2009), an isoform which is present in UAEC (Gifford *et al.* 2006*a*). One criticism that could be raised is that the co-immunoprecipitation (IP) studies of IP3R2 and TRPC3 coupling observed in response to ATP revealed no such co-IP for VEGF (Gifford *et al.* 2006*a*). However, co-IP may not be capable of identifying IP3R–TRPC interactions in response to VEGF because few cells give a measurable Ca^{2+}response and the Ca^{2+} responses do not synchronize as is otherwise the case for ATP. At any given time, there may simply be too few simultaneous IP3R–TRP channel interactions to detect by co-IP/western blotting analysis. Nonetheless, there is some evidence for at least a bursting-type behavior previously attributed to an IP3R–TRPC interactions in UAEC when we apply VEGF under submaximal conditions. Adding a submaximal dose of VEGF165 alone or stimulating with a maximal dose of VEGF165 but also an incomplete inhibitory dose of 2-APB allow us to reveal that discrete Ca^{2+} bursts are possible in response to VEGF. We must conclude that VEGF does recruit cell machinery capable of producing repeated $[Ca^{2+}]_i$ bursts, but maximal stimulation causes these bursts to fuse into a singular, sustained Ca^{2+} response with only minor periodic maxima. While our data are highly consistent with an IP3R/TRPC burst mechanism previously identified in response to ATP, the subtypes of IP3R and TRPC involved in responses to VEGF remain unknown at this time, and certainly further studies are warranted.

The final hypothesis (in UAEC from pregnant animals challenged with VEGF a greater role for CCE underlies pregnancy-adapted NO output) is the hardest to address. Certainly the eNOS phosphorylation state previously reported (Grummer *et al.* 2009) is not substantially different in between UAEC from non-pregnant and pregnant animals on VEGF challenge. If we also consider the recent description of eNOS sensitivity to elevated $[Ca^{2+}]_i$ in various phosphorylation states (Tran *et al.* 2009), then the minimal $[Ca^{2+}]_i$ levels necessary for eNOS activation would be predicted to be roughly 90–100 nM. As indicated in Fig. 1D, UAEC from pregnant animals are

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more likely to reach these levels in response to VEGF165 than UAEC from non-pregnant animals, and certainly both U73122 and 2-APB inhibition of eNOS activity only begins at doses where these drugs also measurably inhibit the Ca^{2+} response. Therefore, it seems likely that pregnancy-specific changes in the VEGF-stimulated Ca^{2+} response coordinate the Ca^{2+} response in UAEC from pregnant animals, resulting in an earlier and higher maxima, may indeed contribute to the pregnancy adapted NO output (Table 1).

What remains unclear is whether such changes in CCE alone can fully account for pregnancy-adapted changes in eNOS activation, or whether other events such as eNOS phosphorylation may also still contribute. The Ca^{2+} response in UAEC at the submaximal but physiological dose of 1 ng/ml VEGF165 may be important in further addressing this point. According to Grummer *et al.* (2009), 1 ng/ml of VEGF165 was the lowest dose that gave a significant increase in eNOS activation in UAEC from pregnant animals compared with UAEC from non-pregnant animals. Herein, we also show that there is indeed a significant difference in number of Ca^{2+}responders between UAEC from non-pregnant animals and UAEC from pregnant animals on stimulation with the same 1 ng/ml dose of VEGF165 (Fig. 2). In the UAEC from pregnant animals the number of Ca^{2+} signaling responders to 1 ng/ml VEGF165 is not only greater but also closer to maximal than in UAEC from non-pregnant animals. The difference observed at the 1 ng/ml dose alone is in contrast to the eNOS activity data from Grummer *et al.* (2009), which showed clearly that UAEC from pregnant animals were more able to activate eNOS than UAEC from non-pregnant animals at all doses tested equal to or over 1 ng/ml. In addition, while blocking Ca^{2+} responses using U73122 or 2-APB is certainly inhibitory to eNOS activation, it is not fully inhibitory. Taken together, these results indicate that while Ca^{2+} mobilization may be critically important for enabling eNOS activation in response to VEGF165, and is necessary for maximal activation, it is probably not the sole regulator. It should also be remembered Ca^{2+} responses to VEGF-E are observed in far more UAEC from non-pregnant animals than UAEC from pregnant animals and yet eNOS activation is not greater (Grummer *et al.* 2009). Therefore, other mechanisms, such as kinase-mediated eNOS phosphorylation, could still be playing an important role in pregnancy-adaptive programing.

Previous studies by others regarding the role of phosphorylation in regulation of eNOS activation showed that dual phosphorylation optimally increased efficiency of electron transfer through the eNOS dimer in the presence of calmodulin, thereby shifting the $[Ca^{2+}]_i$ sensitivity in the physiological range to the left (Tran *et al.* 2009). Thus, elevated Ca^{2+} and an optimal eNOS phosphorylation state combined can lower the eNOS activation threshold and achieve significant eNOS activity even at more modest $[Ca^{2+}]_i$ elevation. Grummer *et al.* (2009) examined the effect of 10 ng/ml VEGF165 on multiple eNOS phosphorylation sites and established that any modest differences in phosphorylations at multiple eNOS residues were not significant between UAEC from non-pregnant and pregnant animals. It was not determined if those events were paired on the same eNOS molecule, or if this changed between the NP- and P-state. Thus, while it was concluded that eNOS phosphorylation alone is unlikely to be the key regulator of pregnancy-adapted changes in eNOS activation, the results of this study, together with the findings of Grummer *et al.* (2009), indicate subtle changes in dual eNOS phosphorylation combined with subtle changes in the Ca^{2+} response most probably account for pregnancy-specific increases in VEGF activation of eNOS in UAEC.

Conclusion

While it is clear that regulation of pregnancy-adapted changes in eNOS activity in UAEC in response to VEGF may be more complex than originally thought, our findings indicate that pregnancy-induced changes in Ca^{2+} signaling in response to VEGF occur in UAEC from pregnant animals and probably involve changes in sustained-phase capacitative burst responses operating throughout the cell, as reported previously for pregnancy-specific changes in Ca^{2+} response to ATP (Yi *et al.* 2010*a*). Since inhibitors of the Ca^{2+} response (U73122 and 2-APB) were both able to inhibit VEGF165-stimulated eNOS activity, we can also conclude that elevated $[Ca^{2+}]_i$, specifically via CCE, is critically important for eNOS activation in response to VEGF165, just as it is for ATP. However, it is unlikely in the case of VEGF that these changes alone fully explain pregnancy-adapted changes in NO production by UAEC from pregnant animals i.e. such changes in Ca^{2+} signaling may be necessary, but they are not sufficient. Combined with the findings of Grummer *et al.* (2009) it seems most likely that VEGF subtly influences the overall eNOS phosphorylation state to create more dual-phosphorylated eNOS molecules to enhance the $[Ca^{2+}]_i$ sensitivity of NO production, as proposed by Tran *et al.* (2009). Such information indicates that future endothelial-targeted therapies that protect and

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even improve endothelial CCE mechanisms mediated via IP3R/TRPC interaction could not only improve vasodilatory responses to G-protein-coupled activators of PLCβ but also growth factor activation of PLCγ. This type of global rescue of responses may be critical in applications aimed at disease states, such as preeclampsia, where sustained Ca^{2+} responses to ATP (and other agonists) have otherwise failed and associated vasodilation is lacking (Krupp *et al*. 2013).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-14-0252.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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