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Simultaneous imaging of \([\text{Ca}^{2+}]_{i}\) and intracellular NO production in freshly isolated uterine artery endothelial cells: effects of ovarian cycle and pregnancy

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Yi, Fu-Xian, Ronald R. Magness, and Ian M. Bird. Simultaneous imaging of \([\text{Ca}^{2+}]_{i}\) and intracellular NO production in freshly isolated uterine artery endothelial cells: effects of ovarian cycle and pregnancy. Am J Physiol Regul Integr Comp Physiol 288: R140–R148, 2005. First published August 5, 2004; doi:10.1152/ajpregu.00302.2004.—Pregnancy and the follicular phase of the ovarian cycle show elevation of uterine blood flow and associated increases in uterine artery endothelium (UAE) endothelial nitric oxide (NO) synthase (eNOS) expression. Nonetheless, a role for increased NO production during pregnancy and the follicular phase has only been inferred by indirect measures. The recent development of a uterine artery endothelial cell model further suggests that pregnancy is associated with reprogramming of cell signaling, such that eNOS may become more \(\text{Ca}^{2+}\)-sensitive and be subject to regulation by \(\text{Ca}^{2+}\)-independent kinases. This study describes for the first time the direct and simultaneous monitoring of NO production and intracellular free \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_{i}\)) in freshly isolated UAE from pregnant, follicular, and luteal sheep. The pharmacological agonists ionomycin (calcium ionophore) and thapsigargin (TG; endoplasmic reticulum (ER) 

Nitric oxide (NO) is a ubiquitous intracellular signaling molecule, synthesized from L-arginine by NO synthase in diverse cells and tissues. Endothelial NO synthase (eNOS) first identified in endothelial cells, plays a major role in the control of blood pressure and vascular homeostasis. In the vascular endothelium, production of NO results in vascular smooth muscle relaxation, which, in turn, reduces blood pressure. eNOS is highly dependent on intracellular free \(\text{Ca}^{2+}\) concentration ([\(\text{Ca}^{2+}]_{i}\)) (1, 12) and is activated by [\(\text{Ca}^{2+}]_{i}\)-mobilizing agonists of diverse G-protein-coupled receptors, such as acetylcholine, bradykinin, and extracellular ATP (40). A complication, however, is that the expression of eNOS in vascular endothelium does not automatically mean that NO will be produced once the enzyme is activated since it is also possible for eNOS to generate other products (42).

During pregnancy, the uterine artery (UA) shows increased vasodilation in response to a number of agonists, including ATP, which in turn contributes to the increase in uterine blood flow (UBF) necessary to meet the needs of the growing fetus (3, 32–34). The follicular phase of the ovarian cycle is partially characterized by elevation in UBF, and both pregnancy and follicular phase are associated with increased estrogen levels and eNOS expression in UA endothelium (UA) (34), as well as estrogen enhancement of flow-induced vasodilation (19, 20). To date, only indirect measures have implied an increase in UA NO production (33, 34, 46), and there has been no direct demonstration of NO production in UA or evaluation of whether NO production increases in parallel with increased eNOS expression in these physiological states. This is not only important for the reasons stated above but also because more recent studies in sheep have implied that the pregnancy-associated increase in the endothelium-dependent relaxation of the UA via NO release may not only occur by way of increases in eNOS expression but also by way of reprogramming of kinase signaling (3). As a result, eNOS may be regulated at multiple phosphorylation sites by kinases that function independently of cytosolic [\(\text{Ca}^{2+}\)], and may activate eNOS directly or render eNOS more sensitive to cytosolic [\(\text{Ca}^{2+}\)] (2, 9, 16). Put another way, this means that each cell can produce more NO for the same increase in [\(\text{Ca}^{2+}\)], so functionality is enhanced beyond that due to increased eNOS expression alone. This concept is of fundamental importance to our understanding of the mechanisms underlying control of UBF during pregnancy and yet remains unproven in vivo owing to the lack of direct evidence that NO is produced and that recruitment of a [\(\text{Ca}^{2+}\)]-sensitive protein kinase signaling event contributes

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to the pregnancy-specific increase in NO production over and above that due to changes in eNOS expression.

Experimental studies on [Ca\(^{2+}\)],-dependent signal transduction in cells were greatly facilitated by the development of the fluorescent indicator fura-2 (17). However, the detection of NO is complicated by its low cellular output and its rapid decomposition (14). Due to its high reactivity, most of the methods currently available for detecting the in situ production of NO in cells are indirect (45). Nonetheless, bioimaging of NO using electronic paramagnetic resonance (EPR) (50) and chemiluminescence assays (28) has been reported. The chemiluminescence method provides excellent sensitivity, but its use is limited by serious technical drawbacks (e.g., use of the cytotoxic H\(_2\)O\(_2\)). The use of the EPR method is hampered by a low spatial resolution. Also, the instruments for EPR or even know-how (e.g., EPR specialists) are too costly for an average biomedical laboratory. Recently, Kojima et al. (23, 24) developed a group of fluorescent NO-sensitive dyes, diaminofluoresceins (DAFs), which can be used routinely to directly measure low-output NO (detection limit: 3–10 nM NO) in an average equipped laboratory. Among these dyes, DAF-2 DA and DAF-FM DA can be used for intracellular imaging of NO, and DAF-FM DA has improved sensitivity over DAF-2 DA (24). DAF-FM DA is transformed by intracellular esterases into the highly water-soluble dye DAF-FM, which is essentially nonfluorescent and traps NO produced by NO synthase (24). This N-nitrosation of DAF-FM produces a highly fluorescent green-triazole compound in cells. Because this nitrosation reaction is essentially irreversible (29), DAF fluorescence reflects a sum total of NO production. Thus this dye can be used to directly monitor NO production in response to different stimuli for both temporal and spatial studies. In the present study, by dual-loading DAF and fura-2, we directly compared the simultaneous responses of [Ca\(^{2+}\)]\(_i\) using pharmacological agents (to fully activate endogenous eNOS), with the effects of the physiological agonist ATP on NO, measured directly for the first time. We revealed that increases in eNOS expression are not sufficient to explain pregnancy-enhanced NO production and that additional [Ca\(^{2+}\)]\(_i\)-insensitive mechanisms of stimulation as well as increased Ca\(^{2+}\) sensitivity are indeed responsible for enhancement of NO production by UAE during pregnancy.

MATERIALS AND METHODS

Materials. ATP (disodium salt) and ionomycin were purchased from Sigma (St. Louis, MO), and thapsigargin (TG) was purchased from Calbiochem (San Diego, CA). Unless noted otherwise, MEM D-Val and all other cell culture reagents were purchased from Gibco (Life Technologies, Grand Island, NY); glass-bottom microwell dishes for [Ca\(^{2+}\)]\(_i\) imaging studies were purchased from MatTek (Ashland, MA). PGF\(_{2\alpha}\) was purchased from Dinoprost Tromethamine-Lutalyse (Upjohn, Kalamazoo, MI).

Synchronization of F- and L-phase ewes. Mixed Western breed ewes (50–60 kg) exhibiting normal estrous cycles were implanted with a vaginal progesterone controlled internal drug-releasing device (CIDR; 0.3 g; Latinagro de Mexico, Monterrey, Mexico) for 12–14 days. On the day before CIDR removal, sheep were given two PGF\(_{2\alpha}\) injections (7.5 mg im, 4 h apart). At CIDR removal (experimental day 0), animals were given an intramuscular injection of pregnant mare serum gonadotrophin (Sioux Biochemical, Sioux Center, Iowa). UA samples were obtained from follicular-phase sheep (n = 6) 48 h after CIDR removal at the peak of the estrous-induced rise in UBF (15). UA samples were obtained from L-phase sheep (n = 7) on day 10 of the estrous cycle, i.e., 12 days after CIDR removal and pregnant mare serum gonadotrophin (15). This synchronization protocol results in ewes showing behavioral estrus within ~40–44 h and ovulation 56 h after CIDR removal (13, 15, 21, 31). We visually confirmed that the ovaries of the follicular-phase ewes only had preovulatory follicles (≥6 mm) and regressed corpora lutea (~4–7 mm), whereas the L-phase sheep had one or more large, functional vascular corpora lutea (10–12 mm). We previously have reported the estrogen and progesterone levels using this method (15, 31).

Isolation of UAE. Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin-Madison Research and Animal Care and Use Committees of both the Medical School and the College of Agricultural and Life Sciences, and follow the recommendations of the “Report of the American Veterinary Medical Association Panel on Euthanasia.” Nonpregnant (F, n = 6; L, n = 7) and late pregnant (n = 12, 125 ± 4 days of gestation) ewes were euthanized with pentobarbital sodium (50–70 mg/kg). Briefly, UAs were dissected free of connective tissue, fat, and veins. The arteries were thoroughly rinsed free of blood using medium 199 before arterial branches were tied off, the larger diameter end was clamped off, and arteries were inflated with medium 199 containing 5 mg/ml collagenase B (Roche Molecular Biochemicals) and 0.5% BSA through a luerlock three-way tap. Digestion was allowed to proceed for 50–55 min at 37°C before the collagenase solution and endothelial cell sheets from the inner surface of the vessels were flushed. Isolated endothelium (~20–30 endothelial cells, 100% cell density) were plated directly to 35-mm glass-bottom microwell dishes and incubated in MEM containing 20% FBS for ~18 h for attachment.

Simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and NO production. After attachment, the endothelium patches in 35-mm glass-bottom microwell dishes were washed twice with 2 ml of prewarmed (37°C) Krebs buffer (in mM: 125 NaCl, 5 KCl, 1 MgSO\(_4\), 1 KH\(_2\)PO\(_4\), 6 glucose, 25 HEPES, 2 CaCl\(_2\), pH 7.4). The cells were then covered with 1 ml of Krebs buffer and loaded with both fluorescent indicators fura-2 AM (5 μM, Molecular Probe) and DAF-FM DA (5 μM, Molecular Probe) for 45 min. The cells were washed with Krebs buffer as described above to remove excess probes, covered in 2 ml of Krebs buffer, and incubated for 30 min to allow complete ester hydrolysis. At the end of the 30-min incubation period, the cells were again washed and covered with 2 ml of Krebs buffer. The cell dish was mounted on an inverted microscope (Diaphot 150; Nikon) with a ×20 phase/fluor objective (Nikon Diaphot). The excitation light from a xenon lamp was filtered to provide wavelengths of 340 nm (for fura 2), and 485 nm (for DAF) with a high-speed wavelength switcher (Lambda 10-2; Sutter, Novato, CA). Emission light from endothelial cells was passed through a dichroic mirror (500 nm) and through an emission filter of 535 ± 30 nm. The fluorescence images were recorded by a digital camera (PixelFly, Cooke). InCyt Im2 imaging and analysis software (Intracellular Imaging, Cincinnati, OH) was used to acquire, digitize, and store data for offline processing and statistical analysis. Our previous study with intact artery endothelium showed that there is no interference between fura-2 and DAF signals (49). In our present experiments, when the endothelium was only loaded with fura 2-AM, no signal at 485/535 nm (for NO imaging) was detected even when cells were stimulated with NO donor, deta NONOate (300 μM). In contrast, when the endothelium was only loaded with DAF-FM DA, no detectable signal could be recorded at 340/535 nm wavelengths (for Ca\(^{2+}\) imaging). A single wavelength of 485 nm to excite DAF-FM or only 340 nm to excite fura-2 could not determine absolute NO or calcium levels; therefore, we expressed the intracel-
lutar NO production and \([\text{Ca}^{2+}]\), as relative fluorescence (F/F0) of DAF (for NO) and fura (for \([\text{Ca}^{2+}]\)), where F is fluorescence intensity obtained during experiments and F0 is its basal fluorescence intensity.

**Conversion of DAF fluorescence intensity to rate increase of NO production.** Fura-2 fluorescence reflects a dynamic change in \([\text{Ca}^{2+}]\). Unlike the reversible reaction between fura-2 and \([\text{Ca}^{2+}]\), NO does not dissociate from DAF-FM once this dye reacts with NO. So, the detected NO-sensitive fluorescence with DAF-FM primarily represents a cumulative amount of NO produced within the cells as described above. An obvious disadvantage of this method for continuous NO measurements is that the plateau phase of the NO-DAF fluorescence curve actually represents the termination of NO production within the cells. To more accurately present the time-dependent relationship between eNOS activity and \([\text{Ca}^{2+}]\), in the cells, we performed a differential conversion of the NO-DAF fluorescence curve to calculate rate increases in DAF fluorescence. We first performed regression analysis of the NO-DAF fluorescence curve recordings for a given stimuli to obtain a best-fit curve using SigmaPlot 6.0. From this curve, rate increases in DAF fluorescence per 1.7 s (data collection interval) were derived.

**Statistical analysis.** Data are presented as means ± SE and were analyzed by Student’s t-test and analysis of variance, as appropriate. A value of \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Simultaneous measurement of \([\text{Ca}^{2+}]\), and NO production.** Ionomycin is a \(\text{Ca}^{2+}\) ionophore that can rapidly induce \(\text{Ca}^{2+}\) influx in the presence of extracellular \(\text{Ca}^{2+}\) and has been widely used as an agonist for maximal \(\text{Ca}^{2+}\)-dependent eNOS activation. In the first series of experiments, we verified the ability of ionomycin to simultaneously increase \([\text{Ca}^{2+}]\), and fully stimulate NO production in freshly isolated pregnant UAE. Figure 1 shows representative images and recordings of simultaneously measured \([\text{Ca}^{2+}]\), and NO production in the UAE. Figure 1A shows sample images in which the 5 \(\mu\text{M}\) ionomycin-induced rise of DAF green fluorescence in endothelium is clearly apparent by eye. A digitized online recording of fura wavelength of 340 nm and DAF fluorescence in this arterial endothelium preparation was also monitored throughout the experiment (Fig. 1B) and illustrates that ionomycin immediately increases fura wavelength of 340 nm fluorescence (\([\text{Ca}^{2+}]\)), which was accompanied by a gradual increase in DAF fluorescence (NO production) to a final plateau. As discussed above, because NO and DAF interact irreversibly through covalent modification, this plateau actually signifies the termination of NO production rather than constant association and dissociation of NO and DAF. A differential calculation as presented in Fig. 1C shows the rate of increase of DAF fluorescence/NO production, thereby revealing the time-dependent changes in eNOS activity.

**Ionomycin increases \([\text{Ca}^{2+}]\), and NO production in L-, F-, and P-UAE.** With the use of the simultaneous imaging analysis, we next compared ionomycin-induced \([\text{Ca}^{2+}]\), or NO production among L-, F-, and P-UAE. Figure 2 shows averaged response traces of ionomycin (5 \(\mu\text{M}\))-induced increase in \([\text{Ca}^{2+}]\), and DAF fluorescence (total NO production) and rate increase of DAF fluorescence (NO production rate). There was no difference in the \([\text{Ca}^{2+}]\) response among L-, F-, and P-UAE (Fig. 3). However, NO production in response to ionomycin (500-s stimulation) was 1.95-fold in P-UAE and 1.34-fold in F-UAE compared with L-UAE. Ionomycin-stimulated NO production in P-UAE was 1.45-fold over that in F-UAE (Fig. 3).

**TG increase in \([\text{Ca}^{2+}]\), and NO production in L-, F-, and P-UAE.** TG, a selective inhibitor of the \(\text{Ca}^{2+}\)-ATPase on endoplasmic reticulum, has been shown to elevate \([\text{Ca}^{2+}]\), and activate NO release in artery endothelial cells, as well as stimulate endothelium-specific vasodilation in vessel preparations (26, 27). Therefore, in this experiment, we used TG as an alternative to ionomycin to increase \([\text{Ca}^{2+}]\), and thus stimulate NO production in L-, F-, and P-UAE. As shown in Fig. 4, TG (10 \(\mu\text{M}\)) rapidly increased \([\text{Ca}^{2+}]\), in each case, and \([\text{Ca}^{2+}]\), remained elevated to similar levels in each case. TG also induced an initial rapid DAF fluorescence increase in the first 60 s, which was followed by a more gradual increase of DAF fluorescence. Although in general terms the results were qualitatively similar to those using ionomycin, there were differences most clearly revealed from the differential calculation. After a sharp initial rise in eNOS activation, there was a more rapid decline. Nonetheless, when we compared the increase in
DAF fluorescence after a 500-s exposure to TG (Fig. 5B), differences in NO production in response to TG were similar to those for ionomycin, with a 2.05-fold change in P-UAE and a 1.37-fold change in F-UAE compared with L-UAE. In addition, TG-stimulated NO production in P-UAE was 1.50-fold over that in F-UAE (Fig. 5).

Effect of ATP on 

Although the aforementioned studies with the receptor-independent, pharmacological agents ionomycin and TG show the outcome of maximal eNOS activation at supraphysiological levels of 

ATP, which was known to act via cell surface receptors, was also tested in parallel studies using cells from the same animals and gave distinctly different results. Our laboratory’s previous studies (2, 9) show that ATP stimulated a more sustained 

response and greater NO production in cultured pregnant uterine artery endothelial cell (UAEC) compared with nonpregnant UAEC. In this experiment, we directly and simultaneously imaged 

and NO in L-, F-, and P-UAE. As seen in Fig. 6, there was little difference in the initial ATP-induced 

peak among P-, F-, and L-UAE. However, ATP stimulation of P-UAE caused a more sustained 

response compared with F- or L-UAE, as reported previously (L-UAE was previously referred to as NP-UAE since no F-UAE samples were tested) (2, 9, 16). Overall, NO production was clearly greatest in P-UAE over F-UAE, which was in turn greater than L-UAE. Mathematical derivation of time-dependent changes in eNOS activity revealed that the pattern was more similar to that of TG than ionomycin. An important difference, however, is revealed comparing both the magnitude of the initial burst and the duration of the response in each case. As summarized in Fig. 7, ATP (100 

M) stimulated a 3.43-fold increase in NO production in P-UAE and a 1.90-fold increase in F-UAE compared with L-UAE. Expressing this an alternate way, ATP-stimulated NO production in P-UAE is 1.81-fold over that in F-UAE. Also, the duration of the P-UAE response above basal was in excess of 500 s, whereas that for F-UAE and L-UAE were clearly much less.

Effect of 2-APB on ATP- and ionomycin-induced 

It could be argued that the greater relative effect of ATP on NO in P-UAE compared with F- or L-UAE, which can be explained by differences in eNOS expression alone, is simply a reflection of a more sustained 

response. Our laboratory’s prior studies, however, show that some alteration of cell signaling via kinases also

Fig. 2. Ionomycin (5 

M)-induced 

increase and NO production in the freshly isolated UAE. UAE from pregnant (n = 8 ewes; top) and nonpregnant follicular (n = 6 ewes; middle) and luteal (n = 6 ewes; bottom) ewes were dual loaded with fura 2-AM and DAF-FM and imaged as described. Values are means ± SE. Note the data for DAF imaging (middle) was then subjected to a curve-fit process and time-dependent changes in eNOS activity were derived as shown at right.

Fig. 3. Effects of ionomycin on 

and NO production in L-, F-, and P-UAE. Using the data from Fig. 2, change (Δ) in F/F0 (net increment fluorescence intensity) for fura 2 or DAF signals were determined at 500 s. Data are means ± SE of arteries from 6 to 8 animals. *P < 0.05 vs. luteal. #P < 0.05 vs. follicular.

Fig. 4. Comparison of 

and NO production in the freshly isolated UAE. UAE from pregnant (n = 8 ewes; top) and nonpregnant follicular (n = 6 ewes; middle) and L-UAE (n = 6 ewes; bottom) were dual loaded with fura 2-AM and DAF-FM and imaged as described. Values are means ± SE.
occurs (2, 9, 16). To reveal the extent to which kinases may be
playing a role, we used 2-APB to block any increase in \([\text{Ca}^{2+}]_{i}\)
and reveal the residual NO production that remained. As shown in Fig. 8, in the presence of the inositol 1,4,5-trisphos-
phate (IP3) receptor antagonist 2-APB (50 \(\mu\)M), the ATP-
induced \([\text{Ca}^{2+}]_{i}\), was totally inhibited, consistent with our prior
data that ATP increases \([\text{Ca}^{2+}]_{i}\), mainly via P2Y receptors/
phospholipase C/IP3 pathway. However, 2-APB inhibited
ATP-induced NO production by only 70%, suggesting the
existence of activation of eNOS in P-UAE even at basal
\([\text{Ca}^{2+}]_{i}\) levels and consistent with the prior data in UAEC
suggesting regulation by \([\text{Ca}^{2+}]_{i}\)-independent or \([\text{Ca}^{2+}]_{i}\)-insen-
sitive signaling pathways. To rule out the possibility that
2-APB nonspecifically decreases fura wavelength of 340 nm
and DAF fluorescence, we performed parallel experiments
with ionomycin as a receptor-independent stimulator of both
\([\text{Ca}^{2+}]_{i}\) pools, and NO production. These results, shown in Fig. 8,
revealed that, with a stimulus acting independently of intracel-
lar \([\text{Ca}^{2+}]_{i}\) pools, 2-APB had no effect on either ionomycin-
induced \([\text{Ca}^{2+}]_{i}\), or NO production.

**DISCUSSION**

An adequate increase of UBF throughout gestation is essen-
tial for uterine, placental, and fetal growth. Maternal cardio-
vascular adaptation has to provide the uterine perfusion that is
necessary to meet the requirements of the developing and
growing fetus by providing transport of nutrients and oxygen to
the placenta and the fetus. Thus UBF is inextricably linked to
fetal growth and survival (3). Chronic reductions of UBF can
be observed in preeclampsia and intrauterine growth restric-
tion. In addition to their acute effects, both preeclampsia and

![Fig. 4. Thapsigargin (10 \(\mu\)M)-induced \([\text{Ca}^{2+}]_{i}\), increase and NO production in the freshly isolated UAE. UAE from pregnant (n = 8 ewes; top) and nonpregnant follicular (n = 6 ewes; middle) and luteal (n = 6 ewes; bottom) ewes were dual loaded with fura 2-AM and DAF-FM and imaged as described. Values are means ± SE. Note the data for DAF imaging (middle) were then subjected to a curve-fit process, and time-dependent changes in eNOS activity were derived as shown at right.](http://ajpregu.physiology.org/)

![Fig. 5. Effects of thapsigargin on \([\text{Ca}^{2+}]_{i}\), and NO production in the freshly isolated UAE. A: typical fluorescent microscopic images showing NO-induced DAF green fluorescence within endothelial cells under control condition (left) and after incubation with thapsigargin (10 \(\mu\)M for 500 s; right). B: with the use of the data from Fig. 4, \(\Delta F/F_0\) (net increment fluorescence intensity) for fura 2 or DAF signals were determined at 500 s of treatment with thapsigargin (10 \(\mu\)M) in pregnant, follicular, and luteal UAE. Data are means ± SE of UAE from 6 to 8 ewes. *P < 0.05 vs. luteal. #P < 0.05 vs. follicular.](http://ajpregu.physiology.org/)
intrauterine growth restriction also increase the risk of cardiovascular disease in later life of both mothers and children (38). Investigating the mechanisms that regulate normal vasodilator production in the UA may allow us to understand the pathophysiological etiology of preeclampsia and intrauterine growth restriction. Although growth of new vessels as well as remodeling of existing vessels during early pregnancy contributes to the increased UBF, the fact that the period of greatest increase in UBF occurs after the completion of new vessel growth indicates that the maintenance of vasodilation in existing or newly developed vessels is crucial (41). Indirect evidence suggests that pregnancy- and follicular phase-associated elevation in UBF is at least partially mediated by NO (15, 22, 47). However, the physiological role of NO in pregnancy and follicular phase remains controversial owing to the lack of direct NO measurement (11, 37, 39). By simultaneously monitoring \([\text{Ca}^{2+}]_i\), and NO levels in the freshly isolated endothelium of UAs, we report the first direct evidence that pregnant, follicular, and L endothelial cells have different abilities to produce NO in response to pharmacological and physiological \([\text{Ca}^{2+}]_i\) agonists. Although ionomycin, TG, and ATP all stimulated more NO production in P-UAE relative to F- and L-UAE, the extent was different for pharmacological (receptor independent) vs. physiological (receptor mediated) agonists. For example, NO production for P-UAE over L-UAE in response to ionomycin and TG was similar at 1.95- and 2.05-fold, respectively, at 500 s but was 3.43-fold in response to ATP. If we examine the magnitude of the changes in actual eNOS activity, we also see substantially greater differences in response to ATP than we do for pharmacological agents. The extent to which \([\text{Ca}^{2+}]_i\) is increased by pharmacological agents ionomycin or TG is of course much higher than that increased by a physiological agonist such as ATP, so the NO production by ionomycin or TG in the present study relates more closely to eNOS expression. In contrast, with the physiological agonist ATP using a receptor-mediated mechanism of action, other considerations come into play. In addition to changes in the level of eNOS itself, previous studies in UAEC primary cultures (2, 16) have suggested pregnancy actually programs both a more sustained \([\text{Ca}^{2+}]_i\) phase (confirmed herein) and also recruits other \([\text{Ca}^{2+}]_i\)-insensitive signaling pathways, which may in turn contribute to pregnancy-specific and possibly follicular phase-specific enhancement of NO production. Although we have confirmed recently that alterations in cell signaling initially reported in UEAC at passage 4 are also seen
Fig. 8. Effects of 2-aminoethoxydiphenyl borate (APB; 50 μM) on ATP- and ionomycin-induced increase of [Ca\(^{2+}\)], and NO production in the freshly isolated pregnant UAE. After pretreatment with 2-APB (50 μM) or vehicle for 10 min, UAE was stimulated with ATP (100 μM) or ionomycin (5 μM) for 500 s. Data for fura 2 and DAF signals at 500 s are means ± SE of UAE from 6 animals. *P < 0.05 vs. ATP or ionomycin alone.

in freshly isolated UAE, we were unable at that time to measure the level of eNOS activation since the quantity of cells was insufficient for arginine-citrulline conversion assays. These imaging measurements with freshly isolated endothelium of UAs circumvent these problems and allow direct comparison of [Ca\(^{2+}\)]\(_i\) levels with NO production in the same cells. Such direct imaging studies are also important for other reasons. When concentrations of l-arginine or tetrahydrobiopterin are low, eNOS may generate both NO and superoxide (O\(_2^-\)) (42). Both O\(_2^-\) and NO contain an unpaired electron and rapidly react together, leading to inactivation of NO (7). Preeclampsia is characterized by elevation of maternal plasma levels of reactive oxygen species (38), and particularly O\(_2^-\) inactivation of NO certainly can contribute to endothelial dysfunction (7). Thus, in preeclampsia, decreased NO bioavailability may occur even in the face of increased total NO production. As a result, activity reported by nitrate/nitrite or arginine-citrulline conversion in preeclampsia may not represent the true level of NO achieved. Theoretically, interaction between NO and O\(_2^-\) is more rapid than the chemical reaction between NO and DAF, so, under such conditions, the result with DAF imaging really represents NO bioavailability, a more physiologically relevant parameter.

Despite considerable evidence that eNOS is a Ca\(^{2+}\)-dependent enzyme (1, 12), recent studies indicated that eNOS can produce NO independent of an increase in [Ca\(^{2+}\)]\(_i\) (6, 43). In reality, basal [Ca\(^{2+}\)]\(_i\) is still a prerequisite of this so-called “calcium-independent” activation of eNOS (49), since purified eNOS and broken endothelial cell preparations containing eNOS are unable to produce NO in the absence of Ca\(^{2+}\) in the reaction mixtures (18, 35). Many studies have shown that shear stress releases ATP from endothelial cells (4, 5, 36, 48). The 20- to 50-fold increase in UBF during pregnancy results in a considerable rise in shear stress (3); thus ATP may be one of the most important physiological vasodilators underlying UA adaptation to pregnancy. Our laboratory’s previous studies (9) on the cultured UAEC model have shown that ATP mobilizes [Ca\(^{2+}\)], by binding to a Gq protein-coupled P2Y receptor and activating the phospholipase C pathway. In the present study, ATP produced a simultaneous increase in [Ca\(^{2+}\)], and intracellular NO in UAE. Nonetheless, although the IP\(_3\) receptor antagonist 2-APB abolished ATP-induced [Ca\(^{2+}\)], confirming that ATP increases [Ca\(^{2+}\)], mainly via phospholipase C/IP\(_3\) pathway in UAE (25), it failed to fully block the increase in NO in response to ATP in P-UAE. This confirms that, in P-UAE at least, there is some component of cell signaling that can activate eNOS in a [Ca\(^{2+}\)]-insensitive manner, as predicted by our studies in UAEC. Although we were unable to perform such analysis in F- or L-UAE for lack of a sufficiently large DAF response in the presence of 2-APB, prior studies in UAEC have confirmed that this [Ca\(^{2+}\)]-insensitive component of eNOS activation is not as great or is lacking in NP-UAEC (9), along with a reduced activation of extracellular regulated kinase 1/2.

Having considered the major differences between NP- and P-UAE, it is appropriate to now further consider whether the same changes seen in pregnancy are also observed to a lesser extent between F- and L-UAE. Both follicular phase and pregnancy exhibit increases in estrogen levels (22), and estrogen is believed to bind to the membrane estrogen receptor, leading to rapid activation of eNOS (8). There was no estrogen added to our present experimental solution, so the difference of NO production among P-, F-, and L-UAE could not be due to the acute effect of estrogen on NO production. The levels of sustained [Ca\(^{2+}\)]\(_i\) elevation in response to ionomycin or TG are not significantly different in F-UAE than in L-UAE, but comparison of the relative magnitudes of changes in NO response relative to ionomycin or TG suggest that the responsiveness of F-UAE is significantly greater than L-UAE. This difference, however, is not any greater than could be accounted for by increased eNOS expression, as would be expected for these agents. Responses to ATP also show a progressive increase in the magnitude of NO production with F-UAE greater than with L-UAE. Nonetheless, the relative magnitude of the NO increase in F-UAE relative to L-UAE in response to ATP (1.90-fold) was still greater than that in response to ionomycin (1.34-fold) or TG (1.37-fold). Certainly, the possibility of enhanced cell signaling in F-UAE and whether this is a programmed event that can be retained in culture warrants further investigation.

In addition to the important physiological findings of this study, some closer examination of the nature of the responses to the three stimulatory agents is also informative regarding signaling mechanisms and eNOS activation in UAE. Ionomycin elevates [Ca\(^{2+}\)], by inducing Ca\(^{2+}\) influx in the presence of extracellular Ca\(^{2+}\). TG elevates [Ca\(^{2+}\)], in endothelial cells by specifically inhibiting the Ca\(^{2+}\)-ATPase activity of the endoplasmic reticulum without affecting the plasmalemmal Ca\(^{2+}\)-ATPase, ultimately depleting intracellular Ca\(^{2+}\) stores. This, in turn, leads to capacitative Ca\(^{2+}\) entry (10, 44). In other words, TG-induced Ca\(^{2+}\) mobilization involves both release of Ca\(^{2+}\) from intracellular stores and capacitative Ca\(^{2+}\) entry. Similar to pulmonary artery endothelial cell and COS-7 cell in previous
studies (30), the TG-induced sustained phase of the $[\text{Ca}^{2+}]_i$ transient in UAE was ablated in the absence of extracellular Ca$^{2+}$. Ionomycin (5 $\mu$M) produced a comparable increase in $[\text{Ca}^{2+}]_i$ to TG (10 $\mu$M). However, the shape of ionomycin-induced increase of DAF fluorescence was very different from that produced by TG. Ionomycin gradually increased DAF fluorescence, whereas TG initially induced a rapid increase, followed by a more gradual increase of DAF fluorescence. The initial TG-induced increase in DAF fluorescence may be due to the release of Ca$^{2+}$ from intracellular stores located in close proximity to caveolae, whereas ionomycin was less location specific. Taken together, these results indicate that eNOS was not only dependent on $[\text{Ca}^{2+}]_i$ itself but also dependent on the sources and mechanisms of $[\text{Ca}^{2+}]_i$ increase.

Finally, it is interesting to note that ionomycin or TG stimulated a substantially greater NO production than ATP, which suggests that physiological agonists such as ATP at best only activate a small fraction of total eNOS in the cell. Remapping of intracellular signaling pathways could clearly make activation of the existing eNOS more efficient during pregnancy, so why is it necessary to increase expression? One possible explanation is that such remapping of cell signaling also allows activation of new subpools of eNOS at different locations in the cell, perhaps leading to NO release in different directions or even with different time courses. This has many physiological implications and is clearly an area for future study.

In summary, our data not only provide the first direct measurement for NO in UAE but also demonstrate a powerful method to study $[\text{Ca}^{2+}]_i$-dependent regulation of eNOS. By using fluorescent microscopy to directly and simultaneously monitor NO production and $[\text{Ca}^{2+}]_i$, we compared the ability of NO production among freshly isolated P-, F- and L-UAE. Ionomycin and TG each produced more NO in P-UAE over F-UAE or L-UAE, and in F-UAE over L-UAE, in accordance with previously reported differences in eNOS expression. Our findings with ATP-stimulated eNOS activation further revealed that enhanced activation was achieved beyond the level of eNOS expression and, in pregnancy, involved both $[\text{Ca}^{2+}]_i$-independent/insensitive and $[\text{Ca}^{2+}]_i$-sensitive activation mechanisms.

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