Dissociation of endothelial nitric oxide synthase phosphorylation and activity in uterine artery endothelial cells

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Submitted 6 September 2005; accepted in final form 1 November 2005


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The mechanisms controlling expression and activation of endothelial nitric oxide (NO) synthase (eNOS) in vascular endothelium have been intensely studied since this protein was first characterized. All NO synthases form homodimers and bind calmodulin (CaM) to catalyze a reaction of L-arginine, NADPH, and oxygen to L-citrulline, NADP$^+$ and nitric oxide (NO). Our previous work demonstrated that phosphorylation of at least five eNOS amino acid residues typically occurs after endothelial stimulation (ovine numbering: S116, T497, S617, S635, and S1179). Diphosphorylation of S116 and T497 and phosphorylation of S617, S635, and S1179 generally associate with increased eNOS activity (1, 5, 14, 15, 18, 28, 38–40). However, endothelial cells from different vascular beds and species display different patterns and mechanisms of phosphorylation. For example, bradykinin stimulates dephosphorylation of T495 in human umbilical vein endothelial cells (HUVEC) and T497 in BAEC; however, protein phosphatase type 1 (PP1) was determined to be responsible for dephosphorylation in the former case, and protein phosphatase type 2B (PP2B) in the latter (15, 38). In BAEC, dephosphorylation of T497 was not observed to occur at all during 30 min of stimulation by VEGF (25). In addition, the vast literature on phospho (p)-S1179 implicates Akt as the kinase responsible for phosphorylation of this site and activation of the enzyme (17, 18). Despite this, we and others have determined that inhibition of phosphatidylinositol 3-kinase (PI3-K)/Akt signaling does not decrease p-S1179 or eNOS activity (5, 12, 51).

Understanding the regulation of eNOS activity is critical for many physiological states, including pregnancy. During pregnancy, increased uterine artery vasodilation is, in part, responsible for increased blood flow to the uteroplacental unit and maintenance of normal pregnancy. Enhancement of endothelial responses to many vasoactive factors occurs during pregnancy, resulting in lowered systemic and uterine vascular resistance (53). This, in part, appears to be a programming response, with adaptation occurring at the level of cell signaling (2, 12, 20, 55). This programming, in turn, confers on the uterine artery greater resistance to vasoconstricting agents (52, 54). A corresponding increase in both magnitude and duration of NO release from uterine artery endothelium is observed (2, 12, 55) and thought to be necessary for proper maintenance of uterine vascular tone during a normal healthy pregnancy (29). Our
ACTIVATION AND PHOSPHORYLATION OF eNOS IN P-UAEC AND COS-7 CELLS

The purpose of this study was to determine whether changes in the known sites of eNOS phosphorylation were critical to or closely correlated with stimulated activity in P-UAEC. In addition, we previously isolated the ovine eNOS cDNA and described a COS-7 ovine eNOS cell model (8) to which we now compare UACe phosphorylation and activation of eNOS. Use of the COS-7 eNOS model also allowed us to further determine how a phosphomimetic mutation of T497 affects phosphorylation of the other known eNOS sites and to compare its activity to eNOS with two mutations predicted to be detrimental to activity (T497D/S1179A). Initially, the current studies correlated well with current assumptions widely held in the literature. However, as we further investigated eNOS activation, our observations revealed added complexity between the relationship of eNOS activation and phosphorylation and underscored the necessity of completeness when studying eNOS regulation by phosphorylation in endothelial or, indeed, any cell model.

MATERIALS AND METHODS

Cell culture. Isolated P-UAEC were obtained as previously described (2). Briefly, freshly isolated cells (passage 0) were plated to 35-mm dishes in growth medium (MEM containing 20% fetal bovine serum, 1% penicillin streptomycin, 1% gentamicin). Cells were grown to ~70% confluence and then passaged (passage 1) to 60-mm dishes. Cells were grown to ~70% confluence and then transferred (passage 2) to T75 flasks. Cells were again grown to ~70% confluence and passed (passage 3) to growth medium (containing 10% DMSO) and frozen in liquid nitrogen. In subsequent experiments, cell were recovered and grown in T75 flasks to 70% confluence and subcultured (passage 4) for the indicated experiment.

COS-7 cells (American Type Culture Collection; Manassas, VA) were cultured in Dulbecco’s modified Eagle medium, high glucose (Invitrogen) with 10% fetal bovine serum and 1.0 U/ml penicillin, 1.0 µg/ml streptomycin, and 4 µg/ml gentamicin. COS-7 cells were used between passages 3–6. Cells were serum withdrawn for experiments as noted. COS-7 cells were transfected (see Gene transfection) at ~60% confluence and further grown for 1–2 days before experiments were performed. Cells were typically 80–90% confluent at the time the experiments were performed. A23187 (Sigma-Aldrich; St. Louis, MO) was initially dissolved in DMSO, such that final DMSO concentration in cell treatment was ≤0.1%. We have previously established that DMSO concentrations <1% do not detectably alter cell signaling or eNOS activation in COS-7 or P-UAEC studies.

Site-directed mutagenesis. QuikChange Site-Directed Mutagenesis kit (Stratagene; La Jolla, CA) was used to mutate ovine eNOS cDNA (pBK-CMV) (8) Thr497 to Asp (T497D: sense oligo 5’-GCG CCA TCA CCA GGA AGA AGG ACT TTA AGG AAG T-3’), Thr497 to Ala (T497A: sense oligo 5’-GCG CAG CCA TCA CCA GGA AGA AGG CCT TTA AGG AAG T-3’), Ser1179 to Ala (S1179A: sense 5’-GCC GTA TAC GTA CCC AGG CCT TTT CCC TGC AGG AG-3’). In addition, the double mutant (T497D/S1179A) was generated by subjecting T497D to a second round of mutagenesis with the S1179A primers. The mutant sequences were verified by sequence analysis performed at the University of Wisconsin Biotechnology Center after ABI Big-Dye fluorescent-labeled dideoxy termination sequence generation and sequence cleanup with Auto-Seq G-50 (Amersham; Piscataway, NJ).

Gene transfection. The pBK-CMV plasmid (Stratagene) containing the eNOS clone was propagated in XL-10 Gold Ultracompetent (Stratagene) E. coli. Transfection quality plasmid DNA was purified by using Qiagen EndoFree Plasmid Maxi kit (Qiagen). With the use of the GeneJammer reagent (Stratagene), plasmids were transiently transfected into COS-7 cells. Following the manufacturer’s protocol, we used 6-to-1 (vol/wt) ratio of GeneJammer to plasmid DNA. DNA (0.5 µg) was used per each 22-mm well and 2 µg per each 60-mm dish. Cells were allowed to recover for 48 h (as noted in figure legends) before execution of the experiments.

eNOS activity assay. COS-7 cells were plated in 12-well dishes (22-mm wells) and allowed to attach overnight. Forty-eight hours before the activity assay, COS-7 cells were transfected as described and maintained in growth medium overnight. In the case of UACe, cells were plated in 12 well dishes, and experiments were performed the following day. Before stimulation, cells were washed twice and incubated for 1 h in 400 µl Krebs buffer [containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 1 KH2PO4, 6 glucose, 25 HEPES, and 2 CaCl2 (Calbiochem; San Diego, CA), pH 7.4]. [3H]arginine (1.5 µCi/22-mm dish, Amersham) and treatments or control were added, and cells were incubated for the indicated times. Reactions were stopped with ice-cold 15% perchloric acid (PCA) to a final concentration of 5% PCA. [3H]citrulline dpm values were determined by 10.220.32.247 on September 21, 2017 http://ajpheart.physiology.org/ Downloaded from
buffer [containing (in mM) 4 NaP2O7·H2O, 50 HEPES, 100 NaCl, 10 EDTA, 10 NaF, and 2 Na3VO4 (pH 7.5) with added 1 mM PMSF, 1% Triton X-100, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] before brief sonication (Sonifier Cell Disruptor, W185, Heat Systems, Ultrasound). Solubilized protein was quantified in cell lysates by BCA assay procedure (Sigma-Aldrich) before SDS-PAGE and Western blot analysis.

**SDS-PAGE and Western blot analysis.** Whole cell lysates from each protein sample (10 μg for COS-7 and 15 μg for P-UAEC) were applied to SDS-PAGE: 7.5% acrylamide-Tris·HCl gels were run for 200 V, 1 h in Bio-Rad Criterion tank (Bio-Rad, Hercules, CA). After the transfer to Immobilon P membrane (100 V, 1 h in Bio-Rad Transblot with plate electrodes and cooling), blots were blocked in 5% milk in TBS-Tween (TBST, 0.1%). Primary antibodies against human p-eNOS S116 [Upstate Biotechnology; polyclonal antibody (pAb); UAEC, 1:1,000; COS-7, 1:2,500] human p-eNOS T495 [Upstate Biotechnology; pAb; UAEC, 1:5,000; COS-7, 1:5,000], bovine p-eNOS S617 [Upstate Biotechnology; pAb; UAEC, 1:2,000; COS-7, 1:3,000], bovine p-eNOS S635 [Upstate Biotechnology; pAb; UAEC, 1:10,000; COS-7, 1:20,000], human p-eNOS S1177 (Cell Signaling Technology; pAb; UAEC, 1:1,000; COS-7, 1:5,000), or nonphosphospecific eNOS (BD Transduction Labs; mAb; UAEC, 1:2,500; COS-7, 1:5,000) were diluted in 1% BSA-TBST. According to each manufacturer, the phosphospecific eNOS antibodies are all immunoreactive with at least bovine antigens, as well as other species, in some cases. Because the bovine and bovine protein sequences share 100% homology (8), the antibodies would be predicted to react equally well with the bovine antigen. Horseradish peroxidase-conjugated secondary antibodies (SoM Fab2, Amersham, and GoR, Cell Signaling Technology) were used for chemiluminescent detection with enhanced chemiluminescence (ECL) reagent (Amersham). Blots were exposed to Hyperfilm ECL (Amersham), and autoradiography images were quantified by using Hewlett-Packard DeskScan and Molecular Analyst Software v1.4 (Bio-Rad). Data collected with phosphorylation state-dependent antibodies were normalized to signal obtained from phosphorylation state-independent antibodies and then to signal from time-matched control data points. Normalized intensity data were then expressed as fold of time 0 control.

**Intracellular Ca2+ detection.** P-UAEC or COS-7 cells were plated on 35-mm dishes with glass coverslips (Intracellular Imaging; Cincinnati, OH). After desired confluence was achieved, cells were incubated in Krebs buffer (as for activity assay) and loaded with 5 μM fura-2 AM (Molecular Probes; Eugene, OR) in the presence of 0.05% pluronic acid F127 (Molecular Probes) for 45 min at 37°C. The cells were then washed twice and covered in 1 ml of Krebs buffer and then incubated for at least 15 min (COS-7) or 30 min (P-UAEC) at room temperature and in the dark to allow complete ester hydrolysis. Cells were washed again and incubated in 1 to 2 ml of Krebs buffer. Fura-2 loading was verified by viewing at 380-nm UV excitation on a Nikon inverted microscope (InCyt Im2, Intracellular Imaging). In each experiment comparing ATP treatment to ATP + PMA, 40–80 individual cells at 80–100% confluency were recorded for 30 min using alternate excitation at 340 nm and 380 nm at 1-s intervals and measuring emitted light at 510 nm by using a pixel fly video camera. In the studies with nifedipine (Calbiochem) and BAY K-8644 (Calbiochem), a group of 10–20 cells was recorded in each experiment and illuminated with alternate wavelengths of 340 nm and 380 nm at 50-ms intervals. A photomultiplier tube connected to InCyt Im2 software quantified average emissions at 510 nm from the cell groups before and after treatment with the designated agonists (Sigma-Aldrich). From the ratio of emission at 510 nm detected at the two excitation wavelengths and by comparison with a standard curve established for the same settings using buffers of known free [Ca2+], the intracellular free [Ca2+] was calculated in real time using the InCyt Im2 software. For all experiments, cells were recorded for 30 s to 1 min before agonist addition to establish the basal [Ca2+].

**Data analysis.** Data are representative of n = 4 separate experiments and presented as means ± SE. ANOVA or Student’s t-test was used to determine differences between treatments and controls or between constructs, when appropriate. Results were considered significant at P < 0.05.

**RESULTS**

We have previously shown that UAEC obtained from the uterine arteries of pregnant versus nonpregnant ewes and maintained in culture for 14 days show similar levels of eNOS expression yet still display key differences in agonist-sensitive activation. This enhanced activation occurs in response to agonists of heptahelical receptors coupled to PLC as well as to growth factors. In particular, we have studied the effects of ATP, which is known to activate P-UAEC through a P2Y2 receptor (12), and induced both enhanced MAPKK/ERK1/2 activation and a rapid and sustained increase in Ca2+ (2, 12, 20). Surprisingly, in contrast to other endothelial cell types, ATP does not potently stimulate Akt phosphorylation and activity (12), even though it is a potent activator of eNOS, and, as such, this cell model may give us an opportunity to unmask other potential regulatory mechanisms of eNOS activation.

Treatment of P-UAEC with 100 μM ATP maximally activated eNOS by 5 min, and no additional activation was observed beyond that time point (Fig. 1A). ATP alone stimulated a small but significant decrease in T497 phosphorylation in P-UAEC, but p-S1179, as well as p-S617 and p-S635, clearly increased within the first 5 min, i.e., during the time at which activation was maximal (Fig. 1B). In P-UAEC, S116 immunoblotting produced a faint signal in only one of the four cell preparations used (data not shown). Because this antibody easily detected p-S116 on ovine eNOS expressed in COS-7 cells (see Fig. 3), we interpret the low levels of P-UAEC p-S116 to indicate that this residue is not abundantly phosphorylated in the P-UAEC in response to ATP treatment.

Treatment of P-UAEC with 10 nM PMA alone failed to achieve detectable activation of eNOS (Fig. 1A) yet stimulated phosphorylation of the three known activating sites: S1179, S617, and S635 (Fig. 1B). The lack of eNOS activation by PMA was not due to phosphorylation of T497, because this remained near control levels throughout the time course (Fig. 1B). Our finding that PMA did not stimulate eNOS activity, given significantly increased phosphorylation of these residues, illustrates clearly that phosphorylation of these residues alone is not sufficient for eNOS activation in P-UAEC. Nonetheless, there is another aspect to consider, namely, elevation of Ca2+.

We have shown previously that ATP activates kinases but also sensitizes eNOS to the Ca2+ activation of P-UAEC with PMA activates the same kinases but does not achieve detectable activation of eNOS (Fig. 1A) yet stimulated phosphorylation of the three known activating sites: S1179, S617, and S635 (Fig. 1B). In addition, PMA tended to increase ATP-stimulated p-S617 at early time points but neither enhanced nor inhibited ATP-stimulated p-S635. As for treatment with ATP alone, ATP with PMA stimulated small but significant decreases in T497 phosphorylation yet still displayed key differences in agonist-sensitive activation.

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 phosphorylation; however, by 30 min of treatment, ATP plus PMA increased p-T479 significantly over levels seen with ATP alone, although not above time 0 control levels.

The comparison of effects of ATP alone with that of ATP plus PMA suggested that changes in activation do not always relate directly to changes in phosphorylation of previously described key regulatory amino acid residues. Nonetheless, as mentioned above, Ca^{2+} is a key part of the signaling response to ATP, and inhibition of Ca^{2+} mobilization does result in up to a 60% loss in eNOS activation (47). PMA has been observed
to decrease both histamine-induced intracellular calcium release in human aortic endothelial cells (44) and 2-methylthio-ATP (a potent agonist of the P2Y1 receptor) stimulated peak and sustained phase calcium in bovine pulmonary endothelial cells (9). Therefore, we tested whether PMA altered ATP-induced increases in [Ca\(^{2+}\)]\(_i\), using imaging with fura-2 to determine whether decreased [Ca\(^{2+}\)]\(_i\) explained the attenuation of ATP-stimulated eNOS activity by PMA. Because PMA decreased ATP-stimulated eNOS activity in P-UAEC by \(-30\%\) (Fig. 1), a comparable decrease in the magnitude of ATP-stimulated intracellular Ca\(^{2+}\) release would implicate this as a mechanism for eNOS inhibition. As we have previously reported, during the first few minutes of ATP treatment in P-UAEC, an acute rise or peak in [Ca\(^{2+}\)]\(_i\) is followed by a sustained phase of elevated [Ca\(^{2+}\)]\(_i\), (12). In the current study, 10 nM PMA did not significantly alter 100 μM ATP stimulation of Ca\(^{2+}\) release in the initial minutes of the recording period (Fig. 2). Over the extended recording period (from about 5 to 30 min), a slight attenuation of average [Ca\(^{2+}\)]\(_i\) was observed; however, the decrease was not significant (Fig. 2). During extended recordings of P-UAEC, after peak and sustained Ca\(^{2+}\) release, transient bursts of Ca\(^{2+}\) are commonly observed in individual confluent cells (Fig. 2). These bursts are difficult to appreciate in the averaged tracings; therefore, the number of bursts was quantified for each individual cell in each experiment. Decreased Ca\(^{2+}\)-burst activity was observed in P-UAEC when PMA was combined with ATP (Fig. 2); however, the decrease in Ca\(^{2+}\) bursts occurred after the difference in eNOS activity was already established between the two treatments. The mechanism controlling the Ca\(^{2+}\) bursts in P-UAEC is currently under investigation and may have implications for long-term eNOS activity. Nonetheless, the disparity between the effects of PMA on eNOS activity and eNOS phosphorylation in the initial 5-min period still stands and is not apparently due to changes in Ca\(^{2+}\) signaling.

One issue in the interpretation of our data from UAEC is that we are dealing with an ovine eNOS sequence, so we must consider whether species-specific sequence difference account for our unique findings. We (8) have previously reported the amino acid sequence is essentially that of bovine eNOS. Nonetheless, further mutagenesis studies would shed more light on whether these findings are a characteristic of the UAEC signaling environment or the amino acid sequence itself. To address this, we used an alternative approach by

![Fig. 2. Intracellular Ca\(^{2+}\) changes stimulated by ATP alone or combined with PMA in P-UAEC. A: P-UAEC plated to dishes with glass coverslips were allowed to grow to confluence. After loading with fura-2 AM and recovery were complete, cells were imaged for 1 min to record basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) before stimulation. Cells were treated (arrow) with final concentration of 100 μM ATP alone (black line) or 100 μM ATP and 10 nM PMA (gray line) and were recorded for a total of 30 min. Only responding cells were used to calculate the [Ca\(^{2+}\)]\(_i\) traces. Data represent means of 8 average recordings for each treatment, where 50 –70 cells were recorded in each experiment. B: percentage of cells that responded to agonist treatment in each experiment is depicted and was not different between the two treatments. C: sample Ca\(^{2+}\) trace for single cell is pictured after treatment with 100 μM ATP and exhibiting peak (P), sustained phase (S), and bursts (B). D: Ca\(^{2+}\) bursts were counted for all cells that responded to 100 μM ATP or to 100 μM ATP + 10 nM PMA. Data are expressed as percentage of responding cells that exhibited 0–3 bursts or >3 bursts (means ± SE) for each treatment. *P < 0.05, compared with 100 μM ATP alone.
performing site directed mutagenesis of the ovine sequence and transient transfection in COS-7 cells. This expression model has been used extensively by others to study molecular mechanisms of eNOS regulation. However, reports characterizing phosphorylation and activity of eNOS, in particular, in response to treatment with more physiological receptor agonists in this model system are relatively lacking. We (8) have previously shown that COS-7 cells express caveolin proteins and that expression of ovine eNOS gives rise to activity which is agonist sensitive, is not altered by the use of sepiapterin, and can be fully blocked by N^G-nitro-L-arginine methyl ester (L-NAME) but not N^G-nitro-D-arginine methyl ester (D-NAME). In addition, ATP dose dependently increases peak [Ca^{2+}]_i in COS-7 cells in a manner dependent on intracellular Ca^{2+} stores and similar to that observed in UAE (8). We, therefore, chose this agonist as a physiological calcium mobilizer and to provide a comparative model for the studies in P-UAEC. As in UAEC, the use of a physiological agonist ATP stimulated maximum COS-7 eNOS activity by 5 min of treatment and was not significantly increased beyond this time point (Fig. 3A). ATP on its own also stimulated significant dephosphorylation of p-S116 and p-T497 and increased phosphorylation of p-S617, p-S635, and p-S1179 (Fig. 3B).

Interestingly, treatment of COS-7 cells expressing ovine eNOS with PMA promoted no significant change in eNOS activity in the first 5 min, but the times of 15 min and onward showed a progressive rise in activity becoming significant at 30 min (Fig. 3A). Although no change in activity was detectable in the first 5 min, increases in p-T497, p-S617, p-S635, and p-S1179 were already observed and largely sustained up to 30 min. Nonetheless, these increases in phosphorylation were all less pronounced than seen in response to ATP (Fig. 3B). To our knowledge, this is the first time that PMA has been observed to stimulate p-S1179, p-S617, and p-S635. Previous reports (38) had only suggested that PMA decreased p-S1179 in BAEC and this activity could be reversed with PKC inhibition.

The combination treatment of PMA with ATP significantly attenuated the ATP-stimulated eNOS activity by 30 min, similar to that seen in UAEC (Fig. 3A). Combined treatment did increase p-T497 over control from 10 to 20 min more than the effects of ATP or PMA alone (Fig. 3B). Cotreatment of PMA and ATP did not significantly change the phosphorylation levels of p-S116, p-S617, p-S635, or p-S1179 compared with ATP treatment alone (Fig. 3B). Our data do not rule out the possibility that PMA acts to attenuate ATP-stimulated activation due to enhanced p-T497 phosphorylation in COS-7 cells. Nonetheless, the same questions concerning Ca^{2+} mobilization and possible changes in the presence of PMA apply as above. Although PMA appeared to lengthen the peak Ca^{2+} response to ATP (up to 1.5 min from the beginning of peak; Fig. 4) and tended to decrease the sustained phase of the response between 1.5–12.5 min after start of the peak response, these differences were not significant (Fig. 4). Interestingly, PMA has been shown to alter Ca^{2+} currents through L-type calcium channels (36, 48). Although L channels have no known role in Ca^{2+} entry into P-UAEC, L-type calcium channels appear to play a small role in the sustained phase of elevated [Ca^{2+}]_i in COS-7 cells, as evidenced by partial inhibition of ATP-stimulated [Ca^{2+}]_i by nifedipine, an L-type calcium channel inhibitor (Fig. 4). BAY K 8644, an L-type calcium channel agonist, stimulates small rises in intracellular calcium but not to the degree that ATP does (Fig. 4). To determine whether the tendency of PMA to inhibit Ca^{2+} release (from 2.5 to 12.5 min) is due to alterations in L-type calcium channels is beyond the scope of this study, and our only intention here is to illustrate its potential involvement in ATP-stimulated calcium response. Inhibition of ATP-stimulated [Ca^{2+}]_i by PMA was not profound nor statistically significant, suggesting again that PMA inhibition of eNOS activity in COS-7 cells could indeed occur via increased p-T497 or a parallel mechanism. Nonetheless, the relationship so far is strictly correlative, but by using the ovine eNOS cDNA expression model, we can also further test this with site-directed mutagenesis.

We reasoned, based on similar studies using clones from other species, that expression of eNOS with T497 mutated to alanine would withstand inhibition when PMA is added to ATP if T497 phosphorylation is the main mechanism decreasing activity in response to physiological agonists, as has been shown for pharmacological agents. Indeed, significant attenuation of ATP-stimulated activity by PMA occurred with wild-type (WT) eNOS but not T497A eNOS (Fig. 5), indicating that PMA inhibition of ATP-stimulated activity was indeed largely due to the phosphorylation of T497 in COS-7. We did not, however, expect also to observe decreased activity in the T497A mutant under both basal conditions and after stimulation with ATP or A23187. According to Lin et al. (31), T497A eNOS decreased basal NO_2⁻ production when expressed in COS-7 cells, while concomitantly increasing superoxide production. In contrast, they observed that T497A eNOS exhibited significantly increased activity over WT under V_{max} conditions (a broken cell assay replete with cofactors, substrate, CaM, and supraphysiological Ca^{2+}). However, basal NO_2⁻ production in intact cells was significantly decreased in T497A compared with WT but did not differ after either ATP or ionomycin treatment. In our experiments, eNOS protein expression was equal between WT and T497A; however, T497A eNOS exhibited ~54% of ATP-stimulated WT-eNOS activity (Fig. 5) and ~78% of WT for A23187-stimulated activity (data not shown). Relevantly, interdependence of phosphorylation sites was recently observed by using phosphomimetic and phosphonull mutants expressed in COS-7 cells (1, 23). Like Greif et al. (23), we also observed a decrease in basal and A23187-stimulated p-S1179 when T497 is mutated to aspartate (Fig. 6A). Here we further demonstrate for the first time that this mutation also decreased eNOS p-S1179 after 5 min of treatment with ATP (10 μM), a physiological Ca^{2+} mobilizing agent. Basal p-S1179 of T497D WT eNOS decreased to 40 ± 5% of WT eNOS and A23187-stimulated p-S1179 to 45 ± 5% of WT eNOS (1.8 fold of T497D control), whereas ATP-stimulated p-S1179 was only reduced to 74 ± 4% of WT (3.4-fold of T497D control). Whereas both ATP and A23187 increase [Ca^{2+}], A23187 acts independently of a receptor and achieves near \( \frac{V_{max}}{V_{max}} \) levels (supraphysiological) levels of activation. ATP stimulates more physiological levels of intracellular calcium mobilization via purinergic G protein-coupled receptors that also independently stimulate additional kinase activation. This alternate kinase signaling event may explain the ability of ATP to stimulate p-S1179 despite the T497D mutation. Mutation of eNOS to T497D did not alter A23187- or ATP-stimulated p-S116, p-S617, or p-S635, despite small reductions in basal p-S116 and p-S617. We interpret these findings to mean that phosphorylation of S1179 is influenced by T497 phosphorylation...
state, and this occurs by spatial and temporal mechanisms that are distinct from those controlling other residues.

The initial characterization of T497 revealed that mutation of this equivalent site in human eNOS to aspartate decreased CaM sensitivity and resulting activity under \( V_{\text{max}} \) conditions (15). When this mutant is expressed in COS-7 cells, basal, ATP-, and ionomycin-stimulated NO production is reduced (31), and A23187-stimulated eNOS activity from intact cells is

Fig. 3. Ovine eNOS (oeNOS) activation in COS-7 cells. A: COS-7 cells plated to 12-well dishes were transfected with 0.5 \( \mu \)g of pBK-CMV-oeNOS/well. After 1.5 days of eNOS expression, cells were serum withdrawn in 0.01% BSA-DMEM overnight. After cells were rinsed with and incubated in Krebs buffer for 1 h, \([^{3}H]\)arginine was added, and cells were treated with vehicle, 10 \( \mu \)M ATP, 10 nM PMA, or combination for up to 30 min. Data are expressed as means \( \pm \) SE in femtomoles of \([^{3}H]\)citrulline generated at each time point minus time-matched control and represent \( n \) = 4 experiments. B: oeNOS phosphorylation in COS-7 cells. COS-7 cells plated to 60-mm dishes were transfected with 2 \( \mu \)g of pBK-CMV-oeNOS/dish. After 1.5 days of eNOS expression, cells were serum withdrawn in 0.01% BSA-DMEM overnight and then treated with vehicle, 10 \( \mu \)M ATP, 10 nM PMA, or combination for up to 30 min. Cell lysates were collected and p-eNOS was detected in whole cell lysates as described in MATERIALS AND METHODS. Representative strip blots for each antibody and treatment are depicted with graphed data. Data are expressed as fold change of arbitrary densitometry units from control \( \pm \) SE and represent \( n \) = 4 experiments. * \( P < 0.05 \), compared with time 0 control; \( +P < 0.05 \) ATP + PMA vs. ATP from same time point.
also decreased (23). In our own studies, basal T497D eNOS activity was reduced to 60% of WT (data not shown). A23187- and ATP-stimulated T497D eNOS activity (with basal activity subtracted) was reduced to 43.8 ± 6.1% and 19.3 ± 5.1% of WT eNOS, respectively (Fig. 6B). We hypothesized that because T497D dramatically reduced p-S1179 and because others have proposed that phosphorylation of this site allows maximal activation under many conditions, double mutation of the enzyme (T497D/S1179A) should be further detrimental to its activation. Nonetheless, no further reduction of ATP- or A23187-stimulated eNOS activity was observed with the double T497D/S1179A mutant (Fig. 6B) compared with T497D with the same treatments. Because combining S1179A with T497D does not further reduce eNOS activity with these Ca2+ mobilizing agents, p-S1179 status may not affect eNOS activity when T497 is mutated to aspartate.

Although p-S1179 does not appear necessary for eNOS activation under Vmax conditions (14), Akt-mediated NO production is greatly enhanced when p-S1179 is allowed to proceed in certain cell types (7, 18). In the current study, ATP-, but not A23187-, stimulated eNOS activity was decreased with the S1179A mutant (Fig. 6B). The necessity of p-S1179 in the face of extreme increases in [Ca2+]i after A23187 stimulation is unlikely and confirmed by this experiment. In contrast, the results with ATP stimulation of this mutant expressed in COS-7 cells indicate that this physiological mobilizer of intracellular calcium depends somewhat on p-S1179 for full stimulation of eNOS catalytic activity.

DISCUSSION

Phosphorylation of five eNOS amino acid residues has been described to date, with the most extensive information available to date, focusing on S1179 regulation. S1179 phosphorylation is thought to release a COOH-terminal autoinhibitory loop, increasing reductase activity (30) and decreasing depen-
agonists that stimulate eNOS activity have been observed to trigger dephosphorylation of p-S116 by PP2B in BAEC (28). The relevance of this residue to eNOS activation and the molecular mechanism influencing catalytic activity has not yet been elucidated. Clearly, the combination of all five phosphorylation events occurring simultaneously and superimposed on intracellular trafficking of the enzyme, as well as the protein-protein interactions, considerably complicates understanding of eNOS regulation.

Although others have shown that increased [Ca\(^{2+}\)], influences eNOS phosphorylation, detailed studies are lacking in the literature. Several compounds are available that increase [Ca\(^{2+}\)], by physiological (ATP, histamine, and bradykinin) or pharmacological (A23187, ionomycin, and thapsigargin) means. ATP is a relevant agonist for endothelial cells because primary HUVEC have been shown to release ATP when subjected to shear stress, substantially elevating local extracellular ATP concentrations (4). In addition to ATP, we studied PMA, which activates several PKC isoforms and has been shown to cause phosphorylation of eNOS T497 (15, 38), to decrease p-S1179 levels (38), and to inhibit glucose-stimulated NO production in murine glomeruli (11). In P-UAEC, at least three PKC isoforms are present (α, β, and γ) (20), and 10 nM PMA stimulates ERK1/2 phosphorylation (T183/Y185) to about threefold of control levels (2), likely via a PKC signaling pathway. In addition to increasing p-T497, PMA has been shown to decrease levels of p-S1179 in BAEC, thereby utilizing two phosphorylation events to inhibit eNOS activity (38).

In the current study, we investigated whether P-UAEC behaves like other characterized endothelial cells or cell models with regard to eNOS activation and phosphorylation. We unexpectedly observed that PMA stimulated phosphorylation of S1179 in P-UAEC but also that this was not associated with any increase in eNOS activity as measured by citrulline production. It may be argued that the use of an indirect assay failed to detect activation due to confounding factors such as changes in arginine transport, effects of tetrahydrobiopterin, and perhaps competing reactions. However, we have previously shown that dose-dependency analysis of ATP-stimulated eNOS activity by measurement of nitrate/nitrite versus arginine consumption. We must, therefore, conclude that the apparent lack of effect of PMA on activity is real and that the choice of assay is not the cause of this finding. In addition, significant increases in p-T497 did not occur after PMA treatment of P-UAEC as they did in COS-7 cells. This finding was quite surprising considering data established in other endothelial cell types. The lack of phosphorylation is not an artifact of amino acid sequence because the same ovine sequence shows detectable alterations in phosphorylation when expressed in COS-7 cells. We must conclude that a lack of T497 dephosphorylation in response to agonist indicates either that this residue is phosphorylated on a small fraction of eNOS molecules and therefore exerts little if any control over eNOS activity or that a pool of eNOS molecules phosphorylated on this residue may be sequestered from the pool activated with ATP treatment. The latter argument may be dismissed by recent data revealing that eNOS targeted to plasma membrane
Fig. 6. Phosphorylation and activity of mutant oeNOS expressed in COS-7 cells. A: COS-7 cells plated to 60-mm dishes were transfected with 2 μg of WT oeNOS or T497D oeNOS/dish. After 1.5 days of eNOS expression, cells were serum withdrawn in 0.01% FBS-DMEM overnight and then treated with vehicle, 10 μM A23187, or 10 μM ATP for 5 min. Cell lysates were collected and p-eNOS was detected in whole cell lysates as described in MATERIALS AND METHODS. Representative strip blots for each antibody and treatment are depicted directly under corresponding graph. Data are expressed as fold change of arbitrary densitometry units from WT oeNOS control and represent 4 experiments.

B: COS-7 cells plated to 12-well dishes were transfected with 0.5 μg of pBK-CMV-oeNOS/well. After 1.5 days of eNOS expression, cells were serum withdrawn in 0.01% BSA-DMEM overnight. In the activity assay, cells were treated with vehicle, 10 μM A23187, or 10 μM ATP for 15 min. Data are expressed as percentages of WT oeNOS activity after subtracting basal eNOS activity.

*P < 0.05, compared with time 0 control; +P < 0.05, compared with WT oeNOS.
has relatively high p-S1179 and p-T497, as well as robust activity in COS-7 cells (17). However, in these studies, as well as our own, phosphorylation state-specific eNOS antibodies do not reveal the proportion of total cellular eNOS protein phosphorylated on a given amino acid, because only relative changes can be measured. Another quandary that this method does not resolve is the degree to which a single eNOS molecule is phosphorylated on both S1179 and T497 at any given time.

The COS-7 model is used extensively in eNOS studies, particularly for characterization of phosphomimetic or phosphonull mutants. Using COS-7 cells expressing ovine eNOS to model P-UAEC, we observed similar patterns of activity when using ATP and PMA. However, after interpreting the phosphorylation data, we must infer that different signaling pathways are present or active in a different context between these two cell types because PMA stimulated p-T497 only in COS-7 and enhanced p-S1179 and p-S617 exclusively in P-UAEC. We previously determined by Western blot analysis that COS-7 cells express three PKC isoforms (α, γ, and δ) (8), differing slightly from the profile observed in P-UAEC. From this analysis we conclude that COS-7 may not provide a faithful model of eNOS phosphorylation in P-UAEC as it currently exists. It does, however, provide a valuable and widely used model system to host ovine eNOS modified by mutagenesis to facilitate structure/function studies and compare those findings directly to those from other species. Our observations using WT eNOS showed clearly that COS-7 cells treated with PMA alone could display increased phosphorylation of stimulatory residues, despite minimal activation of the enzyme. Because the cells were preloaded with [3H]arginine, we do not believe that differences in arginine uptake caused differential activation in PMA-containing treatments. Indeed, in COS-7 cells, PMA-stimulated phosphorylation of T497 was responsible for decreased ATP-stimulated activity.

PMA activates several isoforms of PKC, including those we previously detected in both P-UAEC and COS-7 cells. Partovian et al. (42) recently demonstrated that overexpression of PKC-α in BAEC increased p-S1179 as well as NO production. The authors did not observe increased p-T497, implying that PKC-α was not responsible for phosphorylation of this amino acid. Our data support the idea that there is a divergence between the responsibilities of PKC isoforms phosphorylating eNOS if indeed directly acted on by these kinases. We observed that PMA stimulated phosphorylation of S1179 in both P-UAEC and COS-7 cells, and because both cell types express PKC-α, it is reasonable to conclude that this isoform is responsible for this observation in both cell types. In P-UAEC, this phosphorylation event did not result in increased eNOS activity, indicating that PMA activates signaling pathways other than PKC-α that results in a net lack of eNOS activation. Because PMA also led to p-T497 phosphorylation in COS-7 cells, a different PKC isoform could be responsible for this activity, and the most likely culprit would be PKC-δ; however, further investigation will be necessary to determine the signaling pathway responsible. In BAEC, 100 nM PMA treatment resulted in dephosphorylation of S1179 by activation of PP2A (38). This phenomenon does not appear to occur in either P-UAEC or COS-7 cells, which could result from different PKC isoforms or phosphatases present in the different cell types.

Mutagenesis experiments determined no difference in stimulated eNOS activation between the T497D and T497D/S1179A mutants, which raises important questions about current assumptions of the role of p-S1179 on eNOS activation and doubts over the substitution of p-S1179 as a measure of activity. Further studies will be necessary in transfected endothelial cells to determine how activation of T497D compares with T497D/S1179A after treatment with VEGF, an agonist proposed to require p-S1179 for full eNOS activation in several cell types.

The phosphonull mutant T497A was unexpectedly less active than WT when stimulated by ATP and A23187, contradicting previously published data (31). The discrepancy may result from the two different methods used to analyze eNOS activity: measurement of arginine conversion to citrulline versus analysis of NO metabolites accumulated in media. Although both reflect eNOS activity, neither method is a direct measure of NO production. More studies are needed to address why T497A exhibited decreased oxygenase activity in intact cells when it would be expected to have greater activity both basally and after stimulation, provided that all other conditions remain as for WT eNOS. Lin et al. (31) proposed that this mutation uncouples the enzyme, favoring increased production of superoxide that could also be occurring in P-UAEC. Extending the existing studies of eNOS phosphorylation and activity of T497D, A23187 and ATP stimulated activity to ~44% and 19% of WT, respectively, despite ATP stimulating much higher p-S1179. Another interesting finding was that the double mutant T497D/S1179A was no more detrimental to eNOS activity than T497D under control or ATP- and A23187-stimulated conditions. It remains to be determined whether the double mutant (T497D/S1179A) exhibits lower eNOS activity than T497D when stimulated by agents like VEGF, which have been shown to require phosphorylation of this amino acid residue in certain cell types (13, 45).

This study, while characterizing eNOS phosphorylation and activity in the COS-7 cell model and the more physiological P-UAEC, illustrates a divorce from previously held beliefs regarding site-specific phosphorylation of eNOS and activity. Although we do not intend to dispute the fine work that has been done in this field, our findings, as well as those of others, indicate that in both P-UAEC and other standard models changes in phosphorylation of known residues, alone or together, are not always a predictor of changes in activity. Although the phosphorylation of eNOS clearly does influence activity, the possible interaction between phosphorylation of multiple sites combined with the influence of Ca²⁺ and cofactor association, as well as subcellular localization, precludes inference of activity via investigation of any one, specifically S1179, or even multiple phosphorylation events.

ACKNOWLEDGMENTS

The authors thank Masako Ochiai for excellent technical contributions to this work.

GRANTS

This work was supported by National Institutes of Health (NIH) Grants HD-38843 and HL-64601. J. M. Cale was supported by NIH T32 Training Award (HD-41921), and this work formed part of her doctoral studies in the Endocrinology Reproductive Physiology Training Program at the University of Wisconsin.
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