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Inhibition of MEK/ERK1/2 signalling alters endothelial nitric oxide synthase activity in an agonist-dependent manner

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eNOS (endothelial nitric oxide synthase) activity is post-translationally regulated in a complex fashion by acylation, protein–protein interactions, intracellular trafficking and phosphorylation, among others. Signalling pathways that regulate eNOS activity include phosphoinositide 3-kinase/Akt, cyclic nucleotide-dependent kinases [PKA (protein kinase A) and PKG], PKC, as well as ERKs (extracellular-signal-regulated kinases). The role of ERKs in eNOS activation remains controversial. In the present study, we have examined the role of ERK1/2 in eNOS activation in HUVEC-CS [transformed HUVEC (human umbilical-vein endothelial cells)] as well as a widely used model for eNOS study, transiently transfected COS-7 cells. U0126 pretreatment of HUVEC-CS potentiated ATP-stimulated eNOS activity, independent of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In COS-7 cells transiently expressing ovine eNOS, U0126 potentiated A23187-stimulated eNOS activity, but inhibited ATP-stimulated activity. Compensatory changes in phosphorylation of five key eNOS residues did not account for changes in A23187-stimulated activity. However, in the case of ATP, altered phosphorylation and changes in $[Ca^{2+}]_i$ may partially contribute to U0126 inhibition of activity. Finally, seven eNOS alanine mutants of putative ERK1/2 targets were generated and the effects of U0126 pretreatment on eNOS activity were gauged with A23187 and ATP treatment. T97A-eNOS was the only construct significantly different from wild-type after U0126 pretreatment and ATP stimulation of eNOS activation. In the present study, eNOS activity was either potentiated or inhibited in COS-7 cells, suggesting agonist dependence for MEK/ERK1/2 signalling [where MEK is MAPK (mitogen-activated protein kinase)/ERK kinase] to eNOS and a complex mechanism including $[Ca^{2+}]_i$, phosphorylation and, possibly, intracellular trafficking.

Key words: agonist, Ca^{2+}, COS-7 cell, endothelial nitric oxide synthase, mitogen-activated protein kinase/extracellular-signal-regulated kinase (ERK) kinase (MEK), phosphorylation.

INTRODUCTION

eNOS (endothelial nitric oxide synthase) is regulated by a complex combination of post-translational mechanisms, including acylation, intracellular trafficking, protein–protein interactions, as well as phosphorylation and S-nitrosylation [1–12]. Several signalling cascades and kinases have been implicated in eNOS regulation by phosphorylation, including phosphoinositide 3-kinase/Akt, PKA (protein kinase A), PKC and the ERKs (extracellular-signal-regulated kinases), among several others. Phosphorylation of eNOS on residues Tyr^{83}, Ser^{116}, Thr^{497}, Ser^{617}, Ser^{635} and Ser^{1179} (ovine and bovine numbering) has been demonstrated, and fluctuations in relative levels of phosphorylation on each amino acid residue have been observed after a variety of physiological stimuli [6,10,13–15].

The role of ERK1/2 in eNOS regulation remains controversial and not well understood at a mechanistic level. Several studies have been undertaken investigating how inhibition of this signalling pathway affects eNOS activity [18–30]. These studies have been performed using specific inhibitors of the kinases immediately upstream of ERK1/2, MEK1/2 [where MEK is MAPK (mitogen-activated protein kinase)/ERK kinase]. The compounds PD98059 and U0126 inhibit inactive MEK and both inactive and active MEKs respectively and have been used extensively to study the MEK/ERK1/2 signalling pathway. In addition, several studies of eNOS activation and phosphorylation both *in vivo* and *in vitro* have utilized these compounds to determine whether MEK/ERK1/2 signalling is necessary for eNOS activation.

Oestrogen stimulates acute eNOS activation in many cell types independent of any alteration in eNOS expression [16,17]. In pulmonary artery endothelial cells [18] and in UAEC (uterine artery endothelial cells) [19], inhibition of MEK1/2 with PD98059 attenuated oestrogen-stimulated eNOS activity. Oestrogen-stimulated phosphorylation of Ser^{1179} was also partly attenuated by PD98059 in UAEC. Recently, *in vivo* studies indicated that oestrogen-stimulated NO-dependent vasodilation in femoral and carotid arteries of mice was dependent on MEK/ERK1/2 signalling [20]. In addition to these studies with oestrogen, inhibition of MEK/ERK1/2 signalling has been observed to attenuate eNOS activity stimulated by a variety of factors in several cell lines [21–25]. In contrast, several studies performed in BAEC (bovine aorta endothelial cells) and another study in porcine pulmonary arteries have shown that inhibition of MEK/ERK1/2 signalling results in either no change [26–29] or an enhancement [30] of stimulated eNOS activity. Despite these many studies, a mechanism by which MEK/ERK1/2 signalling affects eNOS activity remains to be determined. It is unclear whether ERK1/2 directly phosphorylates eNOS and on which amino acid this may take place or whether these kinases signal indirectly to eNOS to alter activity.

ERKs phosphorylate many types of proteins including transcription factors, metabolic enzymes, cytoskeletal proteins and

Abbreviations used: BAEC, bovine aorta endothelial cells; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical-vein endothelial cells; HUVEC-CS, transformed HUVEC; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; pAb, polyclonal antibody; PKA, protein kinase A; UAEC, uterine artery endothelial cells; NP-UAEC, UAEC derived from non-pregnant ewes; pS617, phospho-Ser^{617}; P-UAEC, UAEC derived from pregnant ewes; VEGF, vascular endothelial growth factor.

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other protein kinases. They are proline-directed serine/threonine kinases, since their substrates have a proline residue in the S/T + 1 position, where S/T denotes the phosphorylated serine or threonine residue. In the case of ERK1/2 in particular, many substrates contain proline in the S/T –2 position as well. The motif is then PX(S/T)P for ERK1/2 phosphorylation, and ovine eNOS contains seven such motifs.

In the present study, we utilized the COS-7 eNOS model to gain insight into potential molecular mechanisms for the effects of MEK/ERK1/2 signalling on eNOS. The literature contains studies where inhibition of MEK/ERK1/2 stimulates, inhibits or has no effect on eNOS activity, depending on the cell model and the agonists used [18–30]. We examined eNOS activity and changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in HUVEC-CS [transformed HUVEC (human umbilical-vein endothelial cells)] [31] to determine how our results would compare with previously published studies in untransformed HUVEC [22,23]. Little information is available on potential molecular mechanisms controlling the interaction of ERK1/2 with eNOS; therefore we used the COS-7 cells first to determine how inhibition of MEK/ERK1/2 affected A23187- and ATP-stimulated eNOS activity and phosphorylation on previously characterized amino acids. In addition, we determined how ATP stimulation of $[Ca^{2+}]_i$ was altered under the same conditions. Finally, several eNOS alanine mutants were generated, based on the conservative ERK1/2 phosphorylation motif PX(S/T)P described above. Each mutant eNOS construct was transfected into COS-7 cells and stimulated activity in the presence and absence of U0126 was compared with wild-type eNOS. Our findings show that one residue in particular could indeed be a target for ERK1/2 phosphorylation that impacts on eNOS activity.

EXPERIMENTAL

Cell culture

COS-7 cells (A.T.C.C., Manassas, VA, U.S.A.) were cultured in DMEM (Dulbecco's modified Eagle's medium), high glucose (Invitrogen, Carlsbad, CA, U.S.A.) with 10 % (v/v) FBS (fetal bovine serum; Invitrogen) and 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen) and 4 μg/ml gentamicin (Sigma–Aldrich, St. Louis, MO, U.S.A.). Trypsin–EDTA (0.05 % trypsin with EDTA; Invitrogen) was used to passage cells and COS-7 cells were used between passages 3 and 6. Cells were propagated and stored according to A.T.C.C. guidelines. Cells were serum-withdrawn for experiments as noted. Confluency at the time of experimentation varied, and was typically between 70 and 90 %.

HUVEC (HUVEC-C, a spontaneously transformed cell line) were purchased from A.T.C.C. HUVEC-CS, derived from HUVEC-C [31], were plated on to gelatin-coated T75 flasks in MEM (minimal essential medium) D-Val (Gibco BRL) containing 20 % FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 4 μg/ml gentamicin. Following culture to near confluence, cells were seeded to raw plastic for activity assays or glass coverslips for Ca^{2+} imaging.

eNOS activity assay

HUVEC-CS were grown in gelatin-coated T75 flasks and grown to approx. 70 % confluence before seeding to 35 mm dishes and allowing to attach overnight before the activity assay. COS-7 cells were grown, transfected and plated as described above. Arginine-into-citrulline conversion assays were performed in intact cells as previously described [31,32]. The MEK inhibitor, U0126 (10 μM), was added 15 min before the 30 min treatment with A23187 or ATP.

Intracellular Ca^{2+} detection

Cells (COS-7 or HUVEC-CS) were plated on to 35 mm dishes with glass coverslips (MatTek, Ashland, MA, U.S.A.) with regular media changes before imaging was performed at the density stated in the individual experiments. At the time of imaging, cells were incubated in Krebs buffer [125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 6 mM glucose, 25 mM Hepes and 2 mM $CaCl_2$ (Calbiochem, San Diego, CA, U.S.A.), pH 7.4] containing 5 μM fura 2/AM (fura 2 acetoxymethyl ester; Invitrogen) and 0.05 % Pluronic F127 (Invitrogen) for 45 min at 37 °C. The cells were washed twice and covered in 1 ml of Krebs buffer and then incubated for a further 30 min at 37 °C to allow complete ester hydrolysis. Cells were washed again and covered with 1–2 ml of Krebs buffer. fura 2 loading was verified by viewing at 380 nm UV excitation on a Nikon inverted microscope. The cells were recorded with a pixel-fly camera. By comparison with a standard curve established for the settings using buffers of known free $[Ca^{2+}]$ (Molecular Probes), the intracellular free $[Ca^{2+}]$ was calculated in real time. During optimization of $[Ca^{2+}]_i$ imaging for COS-7 cells, it was determined that sham-transfected cells did not respond differently from untransfected cells.

Gene transfection

The pBK-CMV (Stratagene, La Jolla, CA, U.S.A.; CMV is cytomegalovirus) or plasmid containing wild-type [33] or mutant ovine eNOS cDNA was propagated in XL-10 Gold Ultracompetent (Stratagene) *Escherichia coli* cells. Transfection-quality plasmid DNA was purified with EndoFree Plasmid Maxi kit (Qiagen) following exactly the manufacturer's guidelines. Using GeneJammer reagent (Stratagene), plasmids were transiently transfected into COS-7 cells. Following the manufacturer's instructions, a 6:1 (v/w) ratio of GeneJammer to plasmid DNA was employed. Cells were transfected in either 60 mm dishes for studies of phosphoproteins (4×10^5 cells/dish; 12 μl of GeneJammer and 2.0 μg of plasmid DNA) or 100 mm dishes for study of eNOS activity (12×10^5 cells/dish; 36 μl of GeneJammer and 6.0 μg of plasmid DNA). After seeding COS-7 cells to 60 or 100 mm dishes and overnight incubation at 37 °C, cells were ideally 60 % confluent at the time of transfection.

Approximately 48 h after transfection, the cells in 100 mm dishes were trypsinized and seeded to 12-well dishes to allow each treatment of the activity assay to be performed in triplicate. Some of the remaining cells were plated in a 60 mm dish to verify equal eNOS expression between wild-type and the various mutant eNOS constructs. Cells were allowed to attach for 8 h before they were serum-withdrawn in 0.01 % BSA–DMEM overnight.

Investigation of phosphorylated eNOS, cell lysis and protein solubilization

COS-7 cells transiently expressing wild-type eNOS for 1.5 days were serum-withdrawn overnight in 0.01 % BSA–DMEM. Cells were pretreated with vehicle or 10 μM U0126 for 15 min and then treated with 10 μM A23187 or 10 μM ATP for the indicated time. Following treatments as described above, cells were rinsed twice in ice-cold PBS, and frozen at −70 °C. Cells were then solubilized in lysis buffer (4 mM $NaP_2O_7 \cdot H_2O$, 50 mM Hepes, 100 mM NaCl, 10 mM EDTA, 10 mM NaF and 2 mM Na_3VO_4, 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; Ultrasonics). Cell lysates were centrifuged for 5 min at 16000 ***g*** to pellet cell debris. Solubilized whole cell lysate protein concentrations were

determined by the BCA (bicinchoninic acid) assay (Sigma–Aldrich) with comparison with BSA standards.

SDS/PAGE/immunoblotting

Whole cell lysates from each protein sample (10 μg) were size-separated by SDS/PAGE on 7.5 % (w/v) acrylamide/Tris/HCl Criterion gels (Bio-Rad) at 200 V for 1 h in Bio-Rad Criterion tank in 192 mM glycine, 25 mM Tris (pH 8.3) and 0.1 % SDS (Bio-Rad, Hercules, CA, U.S.A.). Following transfer to an Immobilon P membrane at 100 V for 1 h in Bio-Rad Transblot with plate electrodes and cooling coil at 10 °C (150 mM glycine and 25 mM Tris), the blots were blocked in 5 % (w/v) non-fat milk in TBS-T (500 mM NaCl, 25 mM Tris, pH 7.5, and 0.1 % Tween 20). Primary antibodies against the following antigens were used in the experiments and diluted in 1 % BSA–TBST: human p-eNOS Ser^{116} [Upstate Biotechnology; pAb (polyclonal antibody); 1:2500], human p-eNOS Thr^{495} (Upstate Biotechnology; pAb; 1:5000), bovine p-eNOS Ser^{617} (Upstate Biotechnology; pAb; 1:3000), bovine p-eNOS Ser^{635} (Upstate Biotechnology; pAb; 1:20 000), human p-eNOS Ser^{1177} (Cell Signaling Technology; pAb; 1:5000), p-independent eNOS [BD Transduction Laboratories; mAb (monoclonal antibody); 1:5000], p-ERK1/2 Thr^{183}/Tyr^{185} (Promega; pAb; 1:5000), and p-independent ERK1/2 (Cell Signaling Technology; pAb; 1:1000). Horseradish peroxidase-conjugated secondary antibodies (SαM Fab2 1:1000–1:10 000; Amersham; and GαR 1:3000–1:5000; Cell Signaling Technology) were used for chemiluminescent detection with ECL® reagent (Amersham). Blots were exposed to Hyperfilm ECL® (Amersham), and autoradiography images were quantified using HP DeskScan and Molecular Analyst software V1.4 (Bio-Rad). All data collected with phosphorylation-state-dependent antibodies were normalized to the data collected with phosphorylation-state-independent antibodies for the same samples.

Site-directed mutagenesis

The QuikChange® Site-Directed Mutagenesis kit (Stratagene) was used to mutate pBK-CMV ovine eNOS following the manufacturer's instructions exactly as specified. The sense oligonucleotides used for mutagenesis are listed below according to the amino acid substitution and the mutated bases are indicated in boldface: **S33A**, 5′-CAGGGCCCAGCC**G**CCCCGGCACCTGAGCCC-3′; **T46A**, 5′-CCCGCACCCGCC**G**CCCCGCACGCGCCAGAC-3′; **S58A**, 5′-CCAGCTCCCAAC**GC**CCCCACGCTGACCCGG-3′; **T97A**, 5′-GACGGGCCCTGC**G**CTCCCAGGTGCTGCCTG-3′; **S872A**, 5′-CCTCCCCACCC**GC**CCCCCGGCTTCTCCG-3′; **S1085A**, 5′-CCCGGGAACCTGAC**GC**CCCCAAGACCTACG-3′; **T1202A**, 5′-GCCCGGCCCAGAC**G**CCCCCGGCCCCTG-3′.

Data analysis

Data are representative of at least $n = 3$ separate experiments and are presented as the means $\pm$ S.E.M. ANOVA was used for multiple comparisons, while paired Student's t test was used to determine differences between U0126 effects on individual mutants and paired wild-type controls (P values shown in Tables 1 and 2) as appropriate. Results were considered significant at $P < 0.05$ level.

RESULTS

U0126 potentiates ATP-stimulated eNOS activity in HUVEC-CS without significantly altering Ca^{2+} flux

In previous studies of UAEC, we found that MEK/ERK1/2 inhibition significantly elevated ATP-stimulated eNOS activity in NP-UAEC (UAEC derived from non-pregnant ewes) and there was a similar trend in P-UAEC (UAEC derived from pregnant ewes) [34]. In addition, VEGF (vascular endothelial growth factor)-stimulated eNOS activity is enhanced in P-UAEC with U0126 pretreatment and tends towards this in NP-UAEC (M.A. Grummer, personal communication). However, not all endothelial cells share the same cell signalling properties; therefore we chose to investigate how MEK/ERK1/2 inhibition affected eNOS activity in another endothelial cell line commonly used in our laboratory. HUVEC-CS, a transformed cell line of HUVEC, were challenged with ATP with or without U0126 pretreatment to determine whether MEK/ERK1/2 inhibition affects eNOS activity in these cells. ATP-stimulated eNOS activity measured over 30 min was increased in cells pretreated with U0126 by approx. 15 % over control pretreatment (Figure 1A). While this was consistent with results from UAEC, potential differences in cell signalling exist between endothelial cell types, and we were unsure whether increased eNOS activity was due to enhancement of HUVEC-CS $[Ca^{2+}]_i$ response to ATP. Somewhat surprisingly, U0126 appeared to decrease the average $[Ca^{2+}]_i$ trace over a 30 min recording period (Figure 1B); however, average peak $[Ca^{2+}]_i$ was not significantly different for each treatment, nor was the total $[Ca^{2+}]_i$ when calculated as area under the curve (Figure 1C). These results imply that MEK/ERK1/2 potentiation of eNOS activity involves a mechanism dependent on eNOS phosphorylation, protein–protein interactions or eNOS trafficking rather than indirect effects on $[Ca^{2+}]_i$. Further studies are necessary to determine the mechanism or mechanisms responsible in this cell line. In previously published studies performed in primary HUVEC, MEK/ERK1/2 inhibition with PD98059 decreased leptin-stimulated cell migration, an NO-dependent action [22]. In addition, Wyatt et al. [23] determined that cGMP production was inhibited in adenosine-stimulated HUVEC. Our results may contrast with previously published studies because we are working with an immortalized cell line instead of primary cells, but it seems more likely that this is due to a difference in the signalling mechanisms utilized by each of the different receptor classes, combined possibly with the more indirect assay methods used in those studies.

U0126 pretreatment potentiates A23187-stimulated eNOS activity in transfected COS-7 cells without enhancing phosphorylation of key stimulatory residues

To date, no one has studied the role of ERK1/2 on eNOS in COS-7 cells, which is somewhat surprising considering the extensive work that has been done in COS-7 cells to describe and define the role of Akt on eNOS phosphorylation and activation [15,35–37]. After inhibiting endogenous MEK/ERK1/2 signalling in eNOS-transfected COS-7 cells with 10 μM U0126, we measured basal and A23187-stimulated eNOS activity. U0126 potentiated A23187-stimulated activity in COS-7 cells by approx. 30 % (Figure 2A).

To investigate effects of MEK/ERK1/2 inhibition on eNOS phosphorylation, COS-7 cells were transfected with ovine eNOS and experiments similar to the activity assays were performed. Whole cell lysates were collected for SDS/PAGE and Western-blot analysis with phospho-specific antibodies. A23187 stimulated phosphorylation of ERK1/2 on activating sites (pTEpY Thr^{183}/Tyr^{185}) in COS-7 cells over time that was completely inhibited by U0126 (Figure 2B). Phosphorylation of Ser^{116} and Thr^{497} decreased with A23187 treatment, and U0126 pretreatment did not alter these levels (Figure 2C). In addition, increases in pS617 (phospho-Ser^{617}) and pS1179 with A23187 treatment were not changed by inhibition of MEK/ERK1/2 (Figure 2C).

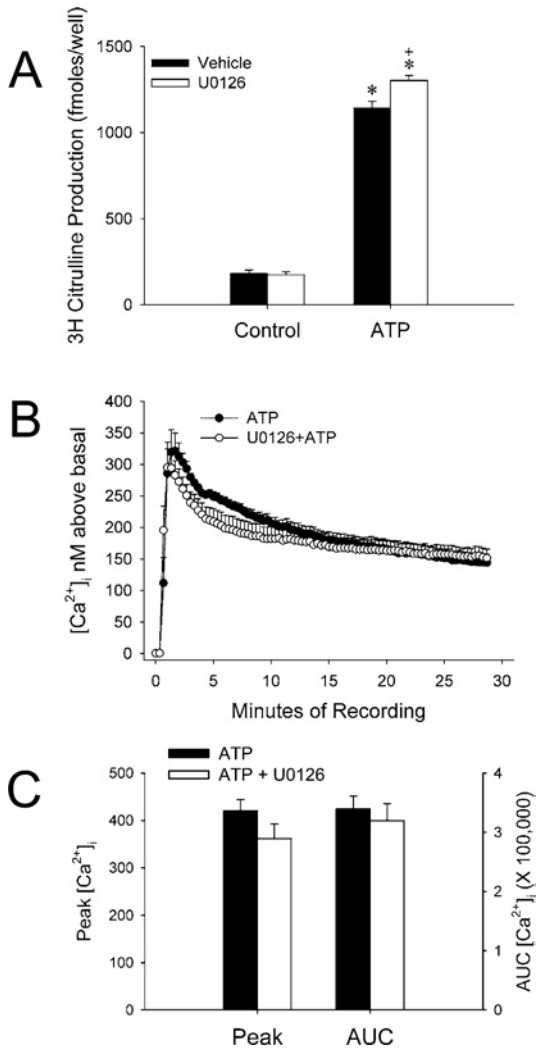

Figure 1 U0126 enhances ATP-stimulated eNOS activity HUVEC-CS despite a tendency towards decreased stimulation of $[Ca^{2+}]_i$

(**A**) HUVEC-CS were seeded to 35 mm dishes and incubated overnight at 37 °C in 20 % FBS/MEM. On the day of experiment, cells were rinsed with and incubated in Krebs buffer for 1 h at 37 °C. Before treatment, cells were incubated in 10 μM U0126 for 15 min at 37 °C. [^{3}H]Arginine and either vehicle or ATP (final concentration 100 μM) in Krebs buffer were added to cells for 30 min. The results are expressed as means $\pm$ S.E.M. for femtomoles of [^{3}H]Citrulline and represent $n = 3$ independent experiments (*$P < 0.05$ when compared with ATP alone). (**B**) Cells were plated on to glass coverslips in 35 mm dishes and allowed to attach overnight. Cells incubated in Krebs buffer were inhibited with vehicle or 10 μM U0126 for 15 min at 37 °C before treatment with 100 μM ATP. The results are expressed as the means $\pm$ S.E.M. for $[Ca^{2+}]_i$ above basal (for each 20th data point) from $n = 5$ (ATP) and $n = 6$ (U0126 + ATP) experiments. (**C**) Data from each recording were averaged for the net peak $[Ca^{2+}]_i$ as well as area under the curve (AUC) for each cell.

In contrast, phosphorylation of Ser^{635} was inhibited both basally and after A23187 stimulation with MEK/ERK1/2 inhibition (Figure 2C).

U0126 pretreatment inhibits ATP-stimulated eNOS activity in transfected COS-7 cells as well as phosphorylation of key stimulatory residues and ATP-stimulated $[Ca^{2+}]_i$

When eNOS-transfected COS-7 cells were treated with ATP after U0126 pretreatment, citrulline production was decreased compared with vehicle pretreatment (Figure 3A). This finding was especially interesting, considering the contrasting results observed with A23187 treatment. ATP stimulated ERK1/2 phosphorylation and was completely inhibited with U0126 pretreatment (Figure 3B). eNOS phosphorylation on Ser^{116} did not vary between U0126 and vehicle-pretreated cells. In contrast, significant differences were observed for at least one time point on the other phosphorylation sites (Thr^{497}, Ser^{617}, Ser^{635} and Ser^{1179}). Importantly, phosphorylation of amino acids associated with increased activity (Ser^{617}, Ser^{635} and Ser^{1179}) was inhibited with U0126 pretreatment.

In addition to phosphorylation of key amino acid residues, we determined whether ATP-stimulated $[Ca^{2+}]_i$ in COS-7 cells was altered with U0126 pretreatment. The sustained phase was somewhat decreased in response to ATP in cells pretreated with U0126 compared with cells stimulated with ATP alone (Figure 4A). Peak $[Ca^{2+}]_i$ was not altered; however, the total area under the curve was indeed decreased with U0126 pretreatment (Figure 4B).

Mutation of putative ERK1/2 phosphorylation sites on eNOS produced a candidate for further characterization with ATP

Seven eNOS alanine mutants were generated based on the PX(S/T)P ERK1/2 phosphorylation substrate motif: S33A, T46A, S58A, T97A, S872A, S1085A and T1202A. Each construct was transfected into COS-7 cells, and citrulline production in intact cells was compared with wild-type eNOS under the same conditions. Consistent expression levels were verified within each experiment by Western blotting (Figure 5).

If ERK1/2 directly phosphorylated eNOS on one amino acid, we might expect to observe a loss of this effect with the target amino acid mutated to alanine. Since MEK/ERK1/2 inhibition potentiated A23187-stimulated eNOS activity, mutation to alanine would be expected to result in a loss of this potentiation with U0126 pretreatment. In contrast, MEK/ERK1/2 inhibition decreased ATP-stimulated eNOS activity; therefore mutation to alanine of a putative substrate amino acid would be expected to result in loss of this inhibition with U0126 pretreatment.

In the case of A23187 (Table 1), none of the mutants was observed to lose U0126 potentiation of A23187-stimulated eNOS activity. In the case of ATP, however, only three of the mutants (S58A, S1085A and T1202A) exhibited inhibition of stimulated activity with U0126 pretreatment comparable with wild-type eNOS (Table 2). Direct comparison of the activity ratios (U0126 + ATP/ATP) demonstrated that only T97A-eNOS significantly differed from wild-type eNOS.

DISCUSSION

Our results have shown that U0126-mediated inhibition of MEK/ERK1/2 enhances ATP-stimulated eNOS activity without altering Ca^{2+} flux in a transformed endothelial cell line, HUVEC-CS. In addition, our studies characterized how inhibition of MEK/ERK1/2 alters eNOS activation and phosphorylation in a commonly used cell model of transiently transfected COS-7 cells treated with A23187 and ATP. The COS-7 eNOS model has been employed many times in past characterizations of molecular mechanisms of eNOS activation by acylation, protein–protein interactions and phosphorylation [1,13,38–42]. While even subtle differences in cell-specific signalling pathways mean that results from the COS-7 model cannot be directly extrapolated to endothelial cells (*in vitro* or *in vivo*) without further validation, these results provide a valuable basis for such future studies.

It is noteworthy that MEK/ERK inhibition attenuates eNOS activation after treatment with such diverse agonists as VEGF, oestrogen, adenosine, HDL (high-density lipoprotein) and leptin [19,21–23,25,43]. These studies were performed in a variety of

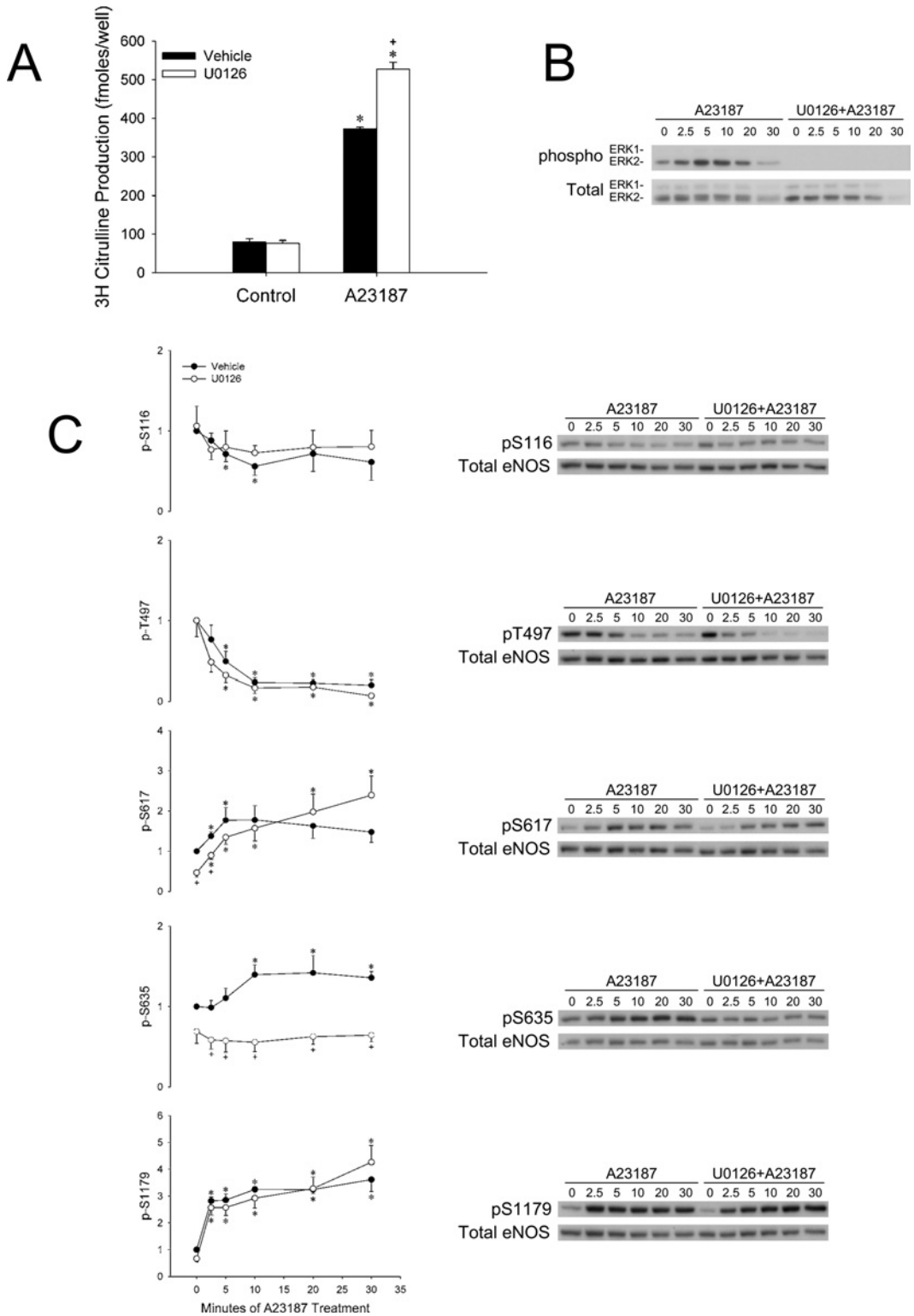

Figure 2 U0126 potentiates A23187-stimulated eNOS activity in COS-7 cells and decreases pS635

(**A**) COS-7 cells were plated on to 100 mm dishes and incubated overnight at 37 °C in 10 % FBS–DMEM. Cells were transfected with 6 μg of pBK-CMV-oeNOS. After 2 days of eNOS expression, cells were trypsinized and subcultured to 12-well plates. After plating for 8 h, cells were serum-withdrawn in 0.01 % BSA–DMEM overnight. On the day of the experiment, the cells were rinsed with and incubated in Krebs buffer for 1 h at 37 °C and then [^{3}H]Arginine was added for a 30 min incubation. Halfway through this incubation, 10 μM U0126 or vehicle was added. Cells were treated with vehicle or 10 μM A23187 for 30 min. The results are expressed as the means $\pm$ S.E.M. for femtomoles of [^{3}H]Citrulline and represent $n = 3$ experiments where each treatment was performed in triplicate ($^+P < 0.05$ compared with A23187 alone; $^*P < 0.05$ compared with control). (**B**, **C**) Ovine eNOS-transfected COS-7 cells (60 mm dishes) were serum-starved overnight in 0.01 % BSA–DMEM. Cells were treated with 10 μM U0126 or vehicle for 15 min and then stimulated with vehicle or 10 μM A23187 for 0–30 min. Whole cell lysates were collected and SDS/PAGE and Western blotting were performed with phospho-specific antibodies for ERK1/2 (**B**) and eNOS [pS116, pT497 (phospho-Thr^{497}), pS617, pS635 and pS1179] (**C**) and also phosphorylation-state-independent antibodies to ERK1/2 and eNOS ($^+P < 0.05$ compared with A23187 alone; $^*P < 0.05$ compared with control).

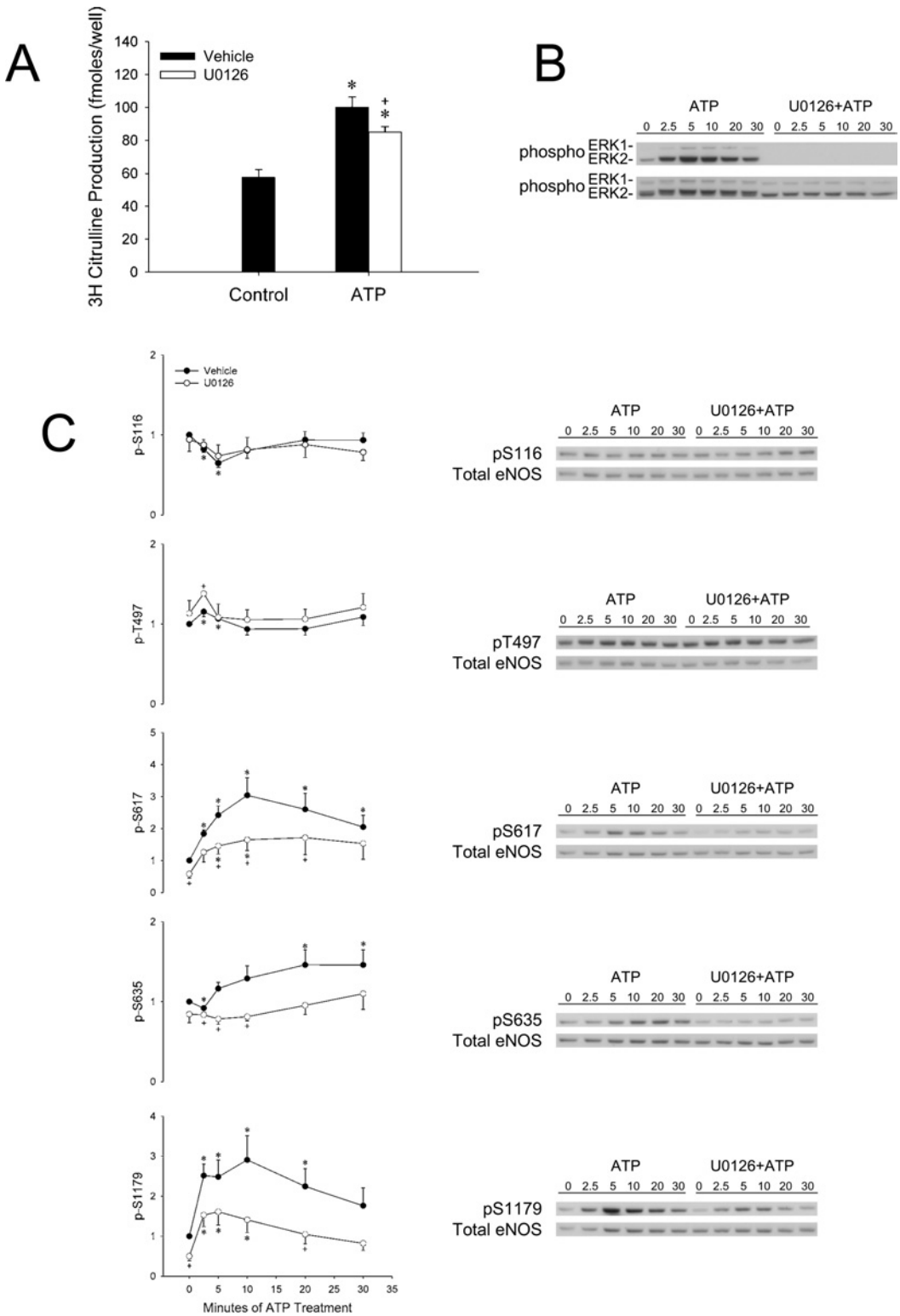

Figure 3 U0126 decreases ATP-stimulated eNOS activity in COS-7 cells and decreases pS617, pS635 and pS1179

(**A**) COS-7 cells were plated on to 100 mm dishes and incubated overnight at 37 °C in 10 % FBS–DMEM. Cells were transfected with 6 μg of pBK-CMV-oeNOS. After 2 days of eNOS expression, cells were trypsinized and subcultured to 12-well plates. After plating for 8 h, cells were serum-withdrawn in 0.01 % BSA–DMEM overnight. On the day of the experiment, the cells were rinsed with and incubated in Krebs buffer for 1 h at 37 °C and then [^{3}H]Arginine was added for a 30 min incubation. Halfway through this incubation, 10 μM U0126 or vehicle was added. Cells were treated with vehicle or 10 μM ATP for 30 min. The results are expressed as means $\pm$ S.E.M. for femtomoles of [^{3}H]Citrulline and represent $n = 3$ experiments where each treatment was performed in triplicate ($+ P < 0.05$ compared with ATP alone; $* P < 0.05$ compared with control). (**B**, **C**) Ovine eNOS-transfected COS-7 cells (60 mm dishes) were serum-starved overnight in 0.01 % BSA–DMEM. Cells were treated with 10 μM U0126 or vehicle for 15 min and then stimulated with vehicle or 10 μM ATP for 0–30 min. Whole cell lysates were collected and SDS/PAGE and Western blotting were performed with phospho-specific antibodies for ERK1/2 (**B**) and eNOS (pS116, pT497, pS617, pS635 and pS1179) (**C**) and also phosphorylation-state-independent antibodies to ERK1/2 and eNOS ($+ P < 0.05$ compared with A23187 alone; $* P < 0.05$ compared with control).

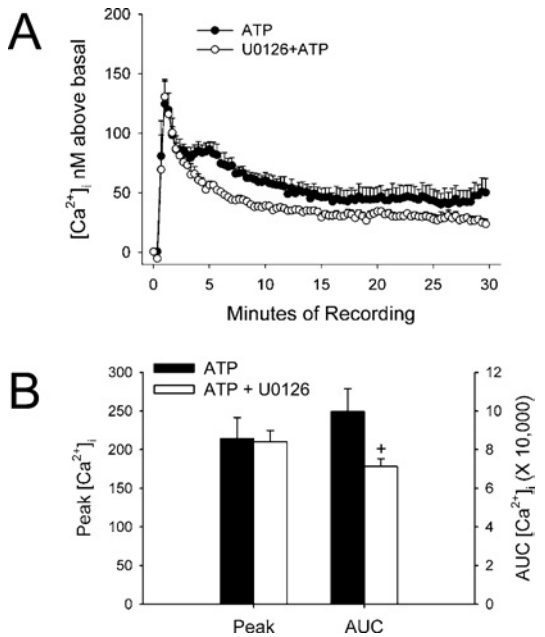

Figure 4 U0126 decreases ATP-stimulated extended phase of ATP stimulation of $[Ca^{2+}]_i$

(**A**) Cells were plated on to glass coverslips in 35 mm dishes and allowed to attach overnight. Cells incubated in Krebs buffer were inhibited with vehicle or 10 μM U0126 for 15 min at 37 °C before treatment with 10 μM ATP. The results are expressed as the means ± S.E.M. for $[Ca^{2+}]_i$ above basal (for each 20th data point) from $n = 5$ experiments. (**B**) Data from each recording were averaged for the net peak $[Ca^{2+}]_i$ as well as area under the curve (AUC) for each cell ($^+P < 0.05$ when compared with ATP treatment alone).

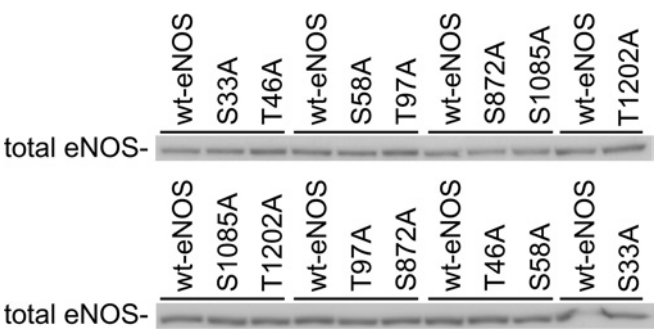

Figure 5 Wild-type and mutant eNOS constructs were evenly expressed within each experiment

Cells were plated, transfected, and experiments were performed as described for Figures 2 and 3. The strip blots indicate equal protein expression of eNOS between the different constructs transiently expressed by COS-7 cells and investigated in the same experiment (indicated by each solid bar over a group of two to three protein bands). The strip blots represent two of a total of eight blots performed to check for equal eNOS expression. wt, wild-type.

cell types, including pulmonary artery endothelial cells, UAEC, HUVEC and even BeWo cells (a placental choriocarcinoma cell line available from A.T.C.C.). In contrast, intact BAEC do not exhibit decreased eNOS activation following MEK/ERK1/2 inhibition [26–28,30].

In the present study, eNOS activity was enhanced by MEK/ERK1/2 inhibition in ATP-stimulated HUVEC-CS and A23187-stimulated COS-7 cells transiently expressing eNOS. One other study observed enhancement of eNOS activity in cell lysates after MEK/ERK1/2 inhibition [30]. Bernier et al. [30] found evidence suggesting that ERK1/2 may directly phosphorylate eNOS *in vitro* on a site that is not Ser^{1179}. They also found that $V_{max,\,eNOS}$ activity

Table 1 U0126 influences A23187-stimulated citrulline production in intact COS-7 cells expressing wild-type and mutant eNOS

Results for citrulline production are means ± S.E.M. for four independent experiments performed in triplicate expressed as percentages of citrulline production after combined U0126/A23187 treatment compared with A23187 treatment alone. $^*P < 0.05$ compared with A23187 treatment alone (100 % citrulline production). The far-right column represents the mean ± S.E.M. of the ratio of wild-type/mutant citrulline production.

Construct	Citrulline production (%): U0126 + A23187/A23187		*P* value (mutant versus wild-type)	Ratio of wild-type/mutant
	Mutant	Wild-type		
S33A	126 ± 4*	130 ± 4*	0.62	1.03 ± 0.05
T46A	127 ± 4*	130 ± 6*	0.40	1.02 ± 0.02
S58A	119 ± 4*	125 ± 7*	0.52	1.05 ± 0.06
T97A	121 ± 3*	124 ± 7*	0.59	1.02 ± 0.04
S872A	120 ± 2*	122 ± 6*	0.72	1.02 ± 0.05
S1085A	119 ± 6*	119 ± 1*	0.97	1.01 ± 0.04
T1202A	119 ± 5*	121 ± 4*	0.39	1.02 ± 0.02

Table 2 U0126 influences ATP-stimulated citrulline production in intact COS-7 cells expressing wild-type and mutant eNOS

Results for citrulline production are means ± S.E.M. for four independent experiments performed in triplicate expressed as percentages of citrulline production after combined U0126/ATP treatment compared with ATP treatment alone. $^*P < 0.05$ compared with A23187 treatment alone (100 % citrulline production). The far-right column represents the mean ± S.E.M. of the ratio of wild-type/mutant citrulline production.

Construct	Citrulline production (%): U0126 + ATP/ATP		*P* value (mutant versus wild-type)	Ratio of wild-type/mutant
	Mutant	Wild-type		
S33A	94 ± 6	84 ± 4*	0.31	0.91 ± 0.07
T46A	94 ± 3	86 ± 4*	0.33	0.92 ± 0.07
S58A	92 ± 2*	86 ± 4*	0.30	0.94 ± 0.05
T97A	101 ± 3	88 ± 1*	0.03	0.87 ± 0.02
S872A	93 ± 3	92 ± 2*	0.74	0.99 ± 0.04
S1085A	88 ± 1*	86 ± 4*	0.82	0.99 ± 0.06
T1202A	88 ± 1*	86 ± 4*	0.67	0.98 ± 0.05

was decreased by incubating eNOS with recombinant ERK. eNOS immunoprecipitated from BAEC treated with bradykinin (with or without PD98059 pretreatment) had higher catalytic V_{max} activity when MEK had been inhibited; however, these studies did not include an analysis of eNOS activity after MEK inhibition in intact cells. Our study is the first to show that MEK inhibition potentiates A23187-stimulated eNOS activity in intact cells, and further that this potentiation of eNOS activity occurs in the absence of compensatory changes in phosphorylation of five key eNOS residues. In fact, even in the face of increased activity, we observed a significant decrease in the level of stimulated Ser^{635} phosphorylation, an event previously correlated to increased eNOS activity [44–46].

Ser^{635} (KRKE**S**SNTDS) is not a consensus site for ERK phosphorylation, implying a more complex role of MEK/ERK1/2 in the regulation of Ser^{635} phosphorylation. Others have determined that this amino acid is phosphorylated in a PKA-dependent manner [44,45]. Phosphorylation of this site may also depend on PKG activity [45]. The mechanistic role of Ser^{635} phosphorylation has not been completely elucidated, and studies of phosphonull and phosphomimetic mutants expressed in COS cells have not simplified the matter [47]. Under V_{max} conditions, S635A exhibited enhanced activity compared with wild-type –eNOS, whereas S635D did not differ significantly from wild-type. In

contrast, assays in intact cells showed that S635A was detrimental for basal NO production (as measured by nitrite accumulation), but did not affect ATP-stimulated NO production, despite increased ATP-stimulated Hsp90 (heat-shock protein 90) binding than wild-type eNOS, typically associated with increased eNOS activity. Finally, in intact cells, S635D-eNOS exhibited increased basal and ATP-stimulated NO production when compared with wild-type. Drawing on mutant experiments and considering that phosphorylation of Ser^{635} increases when the protein is activated, one might logically conclude that phosphorylation of this site augments eNOS activity. Before drawing that conclusion, it is important to consider that Ser^{635} phosphorylation has been shown to occur with a slower time course than Ser^{1179} in response to shear stress, VEGF and 8-bromo-cAMP [44] and ATP [32]. The time-course data are even more intriguing in light of recent experiments using Golgi- or plasma-membrane-targeted eNOS constructs expressed in COS-7 cells [48]. In these studies, the greatest phosphorylation of Ser^{635} occurred when eNOS was targeted to the *trans*-Golgi network and associated vesicles. However, basal NO production was lower than the wild-type with this construct and did not differ from wild-type after cells were stimulated with thapsigargin. Taking into account what is known about eNOS cycling and trafficking in the cell, pS635 may be more a measure of where eNOS is located in the cell rather than a stimulator of catalytic activity.

A key observation in this paper is the opposite effect observed between ATP and A23187 stimulation of eNOS activity in transiently transfected COS-7 cells pretreated with U0126. Whereas with A23187, potentiation of eNOS activity was observed, ATP-stimulated activity was actually decreased with MEK/ERK1/2 inhibition. Several potential explanations for this observation were determined by study of phosphorylation of key residues as well as changes in $[Ca^{2+}]_i$. U0126 decreased the stimulation of Ser^{617}, Ser^{635} and Ser^{1179} phosphorylation by ATP. In addition, the overall change in $[Ca^{2+}]_i$ over the sustained phase of intracellular Ca^{2+} release was decreased, yet this event clearly would have been bypassed when using a receptor-independent stimulus such as the ionophore A23187.

The present study is the first to undertake systematic mutation of conservative putative ERK1/2 phosphorylation sites on eNOS in hopes to find a candidate substrate residue. It has been supposed that Ser^{1179} could be a target for proline-directed kinases [49] due to its +1 P context (TRP<u>S</u>PSGPP). We have determined in the present study that inhibition of MEK/ERK1/2 does not subsequently lead to decreased basal or stimulated pS116 in transfected COS-7 cells, indicating that another proline-directed kinase could be responsible for this event in this cell type. We expected to find that if ERK1/2 directly phosphorylated one of the mutant amino acids, we should be able to see a loss of either potentiation (with A23187) or inhibition (with ATP) of eNOS activity observed with U0126 treatment. In the case of A23187, all of the constructs exhibited unaltered potentiation of activity compared with wild-type eNOS. This strongly suggests that none of these amino acid residues are phosphorylated in a manner that is a hindrance to A23187-stimulated activity. When studying these constructs' responses to ATP, however, the T97A mutant was the only one to exhibit significantly different behaviour compared with wild-type eNOS. Thr^{97} is adjacent to one of the cysteine residues (Cys^{96}) responsible for co-ordination of a zinc ion in the dimer interface [50]. Phosphorylation of Thr^{97} could also affect S-nitrosylation of eNOS on Cys^{96} (evidenced in mutagenesis studies) and Cys^{101} (demonstrated by mass spectroscopy and mutagenesis) [11,12, 51,52]. Nitrosylation in the zinc tetrathiolate cluster is thought to disrupt eNOS dimerization and moderately decrease eNOS activity [11,12], and any substantial charge alteration introduced by phosphorylation or dephosphorylation of Thr^{97} may affect eNOS S-nitrosylation [53].

The crystal structure of eNOS oxygenase dimer [54] shows that the T97 side chain is located on the surface of the eNOS protein, with the nearby (+1) proline accessible for targeting of kinases such as ERK1/2 (see Supplementary Figure at http://www.BiochemJ.org/bj/398/bj3980279add.htm). Thus the amino acid has both a highly appropriate location and accessibility to perform a regulatory role on eNOS activation. Clearly, further work is necessary to determine whether one or both such residues must be phosphorylated in an eNOS dimer for full effect, and whether such phosphorylation events are physiologically relevant *in vivo*. It would also be important to determine whether this mutation or the converse mutation to aspartate alters S-nitrosylation of eNOS.

The role of ERK1/2 in eNOS activation seems to be not only dependent on the cell and tissue in which eNOS activity is measured, but also depends on the agonist used to stimulate activity. While there is some evidence suggesting that eNOS could be directly phosphorylated by ERK1/2 *in vitro* [30], it is equally likely that ERK1/2 may affect eNOS activity by an indirect pathway, altering phosphorylation of eNOS residues or even Ca^{2+} flux. This is illustrated clearly when these studies are attempted in UAEC.

In UAEC, we previously reported that eNOS activation by a variety of agonists acting through growth-factor receptors or G-protein-coupled receptors was strongly correlated with ERK1/2 phosphorylation in P-UAEC, but not in NP-UAEC [55]. Therefore we went on to determine whether inhibition of MEK/ERK1/2 signalling would affect eNOS activity. Initial studies using detection of nitrate/nitrite production suggested that inhibition of MEK could block eNOS activation in response to ATP [56], but this method has been suggested to be prone to several errors [57]. Subsequent studies using arginine-into-citrulline conversion assays (also used herein) have instead shown that ATP-stimulated eNOS activity is somewhat potentiated by treatment with U0126 in P-UAEC and even more so in NP-UAEC [34]. It seems therefore that the same mechanisms operating in HUVEC-CS may be operating in UAEC. Nonetheless, the situation is complicated by effects on $[Ca^{2+}]_i$ since when P-UAEC are used, U0126 has little effect on the Ca^{2+} response, but in NP-UAEC, potentiation of the Ca^{2+} response is clearly observed (F. X. Yi and I. M. Bird, unpublished work). Thus some degree of potentiation of eNOS activation in P-UAEC may be mediated at the level of eNOS itself, but in NP-UAEC, eNOS activation may also be mediated in part by a potentiated Ca^{2+} response. Further studies are now under way to determine at what level MEK inhibition impacts on Ca^{2+} signalling, particularly in NP-UAEC.

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