Co-regulation of AS and eNOS phosphorylation by calcium

Protein Kinase Cα Phosphorylates a Novel Argininosuccinate Synthase Site at Serine 328 During Calcium-Dependent Stimulation of Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells

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*Running Title: Co-regulation of AS and eNOS phosphorylation by calcium

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Background: Argininosuccinate synthase (AS) is critical for endothelial nitric oxide production, yet little is known about its regulation.

Results: AS Ser328 Phosphorylation increased with calcium stimulation and decreased with PKCα interference.

Conclusion: PKCα phosphorylates AS at Ser328 under calcium-dependent stimulatory conditions to support nitric oxide production.

Significance: Knowledge of how AS is regulated is essential in understanding nitric oxide homeostasis.

SUMMARY

Endothelial nitric oxide synthase (eNOS) utilizes L-arginine as its principal substrate, converting it to L-citrulline and nitric oxide (NO). L-citrulline is recycled to L-arginine by two enzymes, argininosuccinate synthase (AS) and argininosuccinate lyase (AL), providing the substrate arginine for eNOS and NO production in endothelial cells. Together, these three enzymes, eNOS, AS and AL, make up the citrulline-NO cycle. Although AS catalyzes the rate-limiting step in NO production, little is known about the regulation of AS in endothelial cells beyond the level of transcription. In this report, we showed that AS Ser328 phosphorylation was coordinately regulated with eNOS Ser1179 phosphorylation when bovine aortic endothelial cells (BAECs) were stimulated by either a calcium ionophore or thapsigargin to produce NO. Furthermore, using in vitro kinase assay, kinase inhibition studies, as well as protein kinase C alpha (PKCα) knockdown experiments, we demonstrate that the calcium-dependent phosphorylation of AS Ser328 is mediated by PKCα. Collectively, these findings suggest that phosphorylation of AS at Ser328 is regulated in accordance with the calcium-dependent regulation of eNOS under conditions that promote NO production, and are in keeping with the rate-limiting role of AS in the citrulline-NO cycle of vascular endothelial cells.

Nitric oxide (NO) production is strictly regulated in vascular endothelial cells. Impairment of this control is associated with risk factors that compromise endothelial function. Arginine directed to eNOS for purposes of NO production is maintained by the recycling of citrulline to arginine, creating a distinct pool of arginine in endothelial cells (1-4). This intracellular arginine pool that is sequestered from the bulk cytosolic arginine is generated through the action of the arginine recycling enzymes argininosuccinate synthase (AS) and argininosuccinate lyase (AL), where catalysis by AS is rate-limiting for NO production in endothelial cells (5). Since the endothelium represents a unique environment under the continuous influence of...
Co-regulation of AS and eNOS phosphorylation by calcium

signaling cascades that integrate responses to physiologic cues, it was logical to propose that acute or immediate regulation of AS would be necessary to accommodate regulatory responses already known for eNOS. For example, VEGF and insulin signaling have been shown to coordinate regulation of two phosphorylation sites of eNOS, increasing phosphorylation at Ser1179 and decreasing phosphorylation at Thr497 to affect an overall increase in eNOS activity (6). This change in eNOS phosphorylation that results from either VEGF or insulin signaling is mediated largely by calcium-independent kinases, such as Akt (7).

Despite the fact that relatively recent reports have shown that AS is a phosphoprotein (8,9), no physiologically relevant phosphorylation sites have been reported. In 2008, an automated phosphoproteome analysis revealed that AS is phosphorylated at serine 352 in HeLa cells, but the physiologic significance was not established (9). Later that same year, Corbin et al also demonstrated that AS was a phosphoprotein, and that VEGF stimulation of NO production affected the phosphorylation of AS via a PKA pathway, but no phosphorylation site was identified (8). In this report, we now show that phosphorylation of AS at serine 328 (Ser328) occurs in response to the calcium-dependent activation of eNOS, and is mediated by PKCα.

EXPERIMENTAL PROCEDURES

Cell Culture—BAECs were isolated from whole bovine aorta as described in (10) with modifications. Briefly, aortas were harvested, washed with PBS without magnesium or calcium to remove blood, and luminal surfaces were exposed by longitudinal dissection followed by incubation with 0.1% collagenase for 10 minutes. Endothelial cell isolation was achieved by rolling sterile swabs across surface, then releasing cells by twirling swabs in 5ml enriched Dulbecco’s modified eagle medium (DMEM, 1g/L glucose, Invitrogen) containing 10% FBS (HyClone) with 100 IU/ml penicillin and streptomycin and 250 ng/ml amphotericin B (Cellgro) in 15 ml conical tubes. Cells were pelleted, resuspended in 6ml DMEM + 10% FBS and plated in 25cm² treated cell culture flasks. Authentication of isolation of endothelial cells in culture was confirmed by flow cytometry.

Prior to treatment confluent BAEC (between passage 3 and 9) were serum starved for 16 hours in DMEM (1g/L glucose, minus phenol red, Invitrogen) supplemented with 0.1% BSA and 1mM glutamine. Treatments included incubation with 100nM insulin (Sigma), 100ng/ml VEGF (R & D Systems), 0.5µM calcium ionophore A23187 (Sigma), 10µM bradykinin (Sigma), 42µM rottlerin or 2.5µM bisindolylmaleimide I (Sigma) for PKC inhibition, or 50µM BAPTA (Calbiochem). Incubation times are indicated in figure legends. Controls were treated with serum starved media only.

Generation of AS variants and Transient Transfections: AS cDNA was cloned into pcDNA 3.1/ V5-His, B expression vector (Invitrogen). The AS construct was then subjected to site directed mutagenesis via the QuikChange kit (Stratagene) per manufacturer’s instructions. Briefly, Ser328 was mutated to alanine (A) to mimic a non-phosphorylated state and to aspartic acid (D) to mimic a constitutively phosphorylated state. Primers used for Ser328A mutation were sense: ACGGGTTTCTGCGACGCGCCGAGTGTGAATTT and antisense: AAATTCACACTCGGGCGCTGGCCAGAAACCGT. Primers used for Ser328D mutation were sense: ACGGGTTTCTGCGACGCGCCGAGTGTGAATTT and antisense: AAATTCACACTCGGGGTCGTGCCAGAAACCGT. Experimental plasmids (1 μg DNA/well of a 6 well dish) were transiently transfected into BAEC using Lipofectamine 2000 (Invitrogen) in serum free Opti-MEM I (Invitrogen). After 4 h, media was replaced with DMEM containing 10% serum and cells were cultured for 6 or 24 h. Cells were stimulated with calcium ionophore A23187 and sodium orthovanadate for 4 hours then culture media was assayed for NO production via the DAN assay.

Mass spectrometry analysis—BAEC transiently transfected with AS were incubated 24 hours, serum starved for 16 hours, and treated with 10μM bradykinin or 50nM okadaic acid for 30 minutes. Overexpressed AS was purified by its fused 6X-His tag using Ni-NTA agarose magnetic beads (Qiagen). Proteins were separated by SDS-PAGE to identify the AS band (51 kD). A duplicate gel was run to confirm expression of the AS plasmid by western blot. Gel bands of interest
were excised and destained. The protein disulfides were reduced with tris(carboxymethyl)phosphine and the cysteines were alkylated with iodoacetamide. In-gel trypsin digestion was used for proteolysis. Nanoflow reverse phase liquid chromatography was used to separate the peptides by hydrophobicity (LC Packings, Dionex, Sunnyvale, CA). Online detection was accomplished with an electrospray linear ion trap mass spectrometer (LTQ, Thermo, San Jose, CA). Tandem mass spectra were assigned to peptide sequences using Mascot and Sequest database search algorithms. Sequence assignments were validated by manual inspection of the data.

Argininosuccinate Synthase Phosphoserine Ser328 Specific antibody- A phospho-specific antibody against pSer328 AS was generated by 21st Century Biochemicals using the phosphopeptide AS sequence 322-335: RR-Ahx-YGFWH[pS]PECEFVR-amide. The phospho-AS antibody specificity was determined by performing phospho-peptide competition of immunoprecipitations and western blotting.

Western blot analysis- BAEC were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Pierce) followed by scraping for collection. Whole cell lysates were clarified and total protein was quantitated using the BCA protein assay (Pierce). Equal amounts (20µg) of protein were resolved on 4-15% Tris-HCl TGX precast gels (Bio-Rad) and blotted onto Immobilon-P polyvinylidene difluoride membranes. Western blotting was performed as previously described (11). Membranes were blocked in 100% Starting Block (Pierce) and incubated with primary antibody, 1:2500 anti-AS (BD Transduction Labs), in 20% Starting Block. Secondary antibody used was peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Labs) at 1:50,000 dilution. Blots were visualized by chemiluminescence using West Pico reagent (Pierce) and exposed to film. Band intensities were quantitated using Quantity-One software (Bio-Rad).

Nitric oxide determinations- Nitrite was measured in the medium as an indicator of cellular NO using the fluorescent 2,3-diaminonaphthalene (DAN) (Sigma) assay as described previously (12). Reactions were carried out in 96 well black plates and fluorescence was read on a BMG FLUOstar Galaxy spectrofluorometer platereader exciting at 360nm and detecting emission at 405nm. Data are described as pmol nitrite per microgram protein.

PKCa knockdown assay- BAEC, 90% confluent, were transfected for 48 hours with DsirNA (dicer-substrate siRNA) directed against the 5th exon of PKCa (IDT) using lipofectamine 2000 (Invitrogen) performed as described previously for AS constructs. The duplex sequence used to silence the gene expression was the following: 5'-rCrGrA rGrGrA rGrCrA rArGrC rArCrA rArGrU rUrCrA rArGA T-3' 5'-rArUrC rUrUrG rArArC rUrUrG rUrGrC rUrUrG rCrUrC rCrUrC rGrGrG-3'. Transfected BAEC were then treated with calcium ionophore (A23187, Sigma) and lysed followed by western blotting. Relative amounts of indicated proteins were quantitated as described above.

In Vitro Kinase Assay- Assays were performed as described by Corbin et al. (8). Breifly, purified recombinant AS was generated via subcloning bovine AS (NP_776317) into the pET-28(c)+ vector (Novagen) and expressed in E. coli. The protein was subsequently purified via the His-bind resin per the manufacturer’s instructions (Novagen). Successful purification was verified by SDS-PAGE. The positive control used to determine kinase activity for each kinase contained applicable peptide substrate, while negative control contained all components except peptide. Addition of 33P-ATP to each reaction mixture initiated the assays incubated at 30°C for 30 minutes. Assay termination was achieved by reaction spotting on phosphocellulose P81 plates followed by 3 washes then radioactivity read via scintillation counting. Imaged in vitro kinase reactions were terminated by addition of sample buffer then fractionated on 10% polyacrylamide gels.

RESULTS

Identification of the argininosuccinate synthase Ser328 phosphorylation site- In order to establish biologically significant sites of phosphorylation in the AS sequence, we carried out a proteomic analyses by applying mass spectrometry (LC-MS/MS) on overexpressed AS purified from cultured BAECs treated with bradykinin or okadaic acid (PP2A and PP1 phosphatase inhibitor). Mass spectrometry analysis revealed three highly conserved serine/threonine
phosphorylation sites that were distinguished from background ion signal. These included threonine 131 (Thr131)/serine 134 (Ser134), serine 180 (Ser180)/serine 189 (Ser189), and serine 328 (Ser328) (Figure 1). The proximity of Thr131 to Ser134 and Ser180 to Ser189 made these individual signals indistinguishable and therefore described as Thr131/Ser134 and Ser180/Ser189.

Of the sites identified, Ser328 showed the greatest change in signal between cells treated with phosphatase inhibitor (okadaic acid) and non-treated cells. In addition, Ser328 showed substantial change in signal with bradykinin treatment relative to non-treated control cells (Figure 1). Furthermore, in silico modeling showed that the most accessible sites for kinase interaction were Thr131/Ser134 and Ser328 while Ser180/Ser189 appeared buried near the active site of the enzyme (Data not shown). Based on these results, Ser328 was selected as being the strongest candidate for phosphorylation in vivo. Consequently, a phospho-specific antibody directed against pSer328 was developed for further investigation (see EXPERIMENTAL PROCEDURES).

Calcium-independent stimulation of BAECs decreases phosphorylation at Ser328- To determine whether phosphorylation at Ser328 had physiological significance, changes in Ser328 phosphorylation were examined relative to non-treated control cells (Figure 1). Furthermore, in silico modeling showed that the most accessible sites for kinase interaction were Thr131/Ser134 and Ser328 while Ser180/Ser189 appeared buried near the active site of the enzyme (Data not shown). Based on these results, Ser328 was selected as being the strongest candidate for phosphorylation in vivo. Consequently, a phospho-specific antibody directed against pSer328 was developed for further investigation (see EXPERIMENTAL PROCEDURES).

Calcium-dependent stimulation of BAECs increases phosphorylation at Ser328- Insulin and VEGF mediate eNOS phosphorylation at Ser1179 via the PI3K/Akt pathway which is largely calcium-independent (18-20). Since calcium-independent signaling mediated by either insulin or VEGF promoted the apparent dephosphorylation of AS Ser328, we then questioned whether phosphorylation at Ser328 was involved with calcium-dependent signaling. To initially explore this question, site-directed mutagenesis analysis was carried out. Three constructs were used, one containing wild type (WT) AS, one containing an alanine (Ala) substitution for Ser328 (phospho-null), and one containing an aspartate (Asp) substitution for Ser328 (phospho-mimetic). As shown in Figure 3A, the overexpression of phospho-mimetic Ser328D AS enhanced NO production similar to WT AS when stimulated with calcium ionophore. Conversely, overexpression of phospho-null Ser328A AS failed to enhance NO production in response to the same treatment. Consistent expression of the constructs is shown by western blotting (Figure 3B). The observation that only Ser328D overexpression increased NO production to the level of WT suggested that phosphorylation at Ser328 supports arginine delivery to eNOS under calcium-dependent stimulatory conditions.

Since these findings were consistent with a calcium-dependency, experiments were then carried out to determine whether calcium-dependent changes in Ser328 could be demonstrated for endogenous AS. For these experiments, BAECs were treated with either the calcium ionophore, A23187, or the endoplasmic reticulum ATPase inhibitor, thapsigargin. Both agents are known to promote an intracellular increase in calcium (21) and stimulate NO production. As shown in Figures 4A and 4B, treatment with either agent promoted phosphorylation of eNOS at Ser1179 by nearly 50% with a subsequent decrease in phosphorylation of eNOS at Thr497 by 40%, consistent with reported values (21,22). However, in this case, treatment with calcium ionophore or thapsigargin demonstrated an increase in phosphorylation at AS Ser328 (~60%) (Figure 4A,B). To further substantiate the calcium
dependency of AS Ser328 phosphorylation, BAECs were treated with the intracellular calcium chelator BAPTA-AM. As expected, calcium ionophore and thapsigargin rescued phosphorylation at eNOS pSer1179 that diminished with BAPTA-AM treatment alone. Importantly, as shown in Figure 4A, treatment with 50 µM BAPTA-AM completely abolished phosphorylation at Ser328, as well as the phosphorylation at eNOS Ser1179. Thus, phosphorylation of AS Ser328 appeared to be coordinately regulated with the phosphorylation of eNOS Ser1179 through calcium-dependent signaling.

To demonstrate physiological relevance, NO measurements were taken after cells were treated with BAPTA-AM for 30 minutes followed by stimulation with calcium ionophore A23187 for 20 minutes. A23187 and thapsigargin significantly increased NO production relative to non-treated or BAPTA-AM treated BAECs (Figure 4C). In addition, BAPTA-AM treatment significantly decreased A23187 or thapsigargin stimulated NO production by 2.5 and 2-fold respectively.

**PKC is involved in mediating Ser328 phosphorylation in BAECs**- In order to further elucidate the signaling pathway involved in the calcium-dependent phosphorylation of AS at Ser328, a series of experiments were carried out using kinase inhibitors.

Since the classical protein kinase C (PKC) isotypes are calcium-dependent kinases and are not involved in insulin or VEGF signaling (23,24), we hypothesized that a member of the PKC family may be involved in the calcium-dependent phosphorylation of AS at Ser328. To test this hypothesis, non-isotype specific PKC inhibition studies were carried out. As a first approach, the effects of PKC inhibition were directly measured by examining AS Ser328 phosphorylation. As shown in Figure 5A and B, treatment of BAECs with either the PKC inhibitor rottlerin or bisindolylmaleimide I, followed by stimulation with calcium ionophore, decreased AS Ser328 phosphorylation by approximately 60% relative to non-treated controls. These results were taken to be consistent and supportive of the role of a classical PKC isotype promoting phosphorylation of AS at Ser328.

Interestingly, phosphorylation at Thr497 or Ser1179 of eNOS also decreased with treatment of rottlerin by approximately 60%, irrespective of calcium stimulation. In contrast, when BAECs were treated with bisindolylmaleimide I alone, phosphorylation at Ser1179 and Thr497 of eNOS decreased by only 20%. However, upon calcium stimulation, phosphorylation at eNOS Ser1179 increased to levels similar to calcium ionophore treatment alone. Nevertheless, phosphorylation at Ser328 was unaffected by calcium in presence of bisindolylmaleimide I (Figure 5A).

In order to demonstrate physiological relevance of the system in response to inhibitors, we measured NO production for each treatment (Figure 5C). PKC inhibition with bisindolylmaleimide I has little effect on NO production by BAECs, however rottlerin significantly reduced calcium ionophore stimulated NO production by over 2-fold. These results suggest that rottlerin may affect the activity of several kinases, and bisindolylmaleimide I clearly decreased phosphorylation at eNOS Thr497 and thus promoted NO production similar to calcium ionophore alone.

To further examine the potential involvement of other calcium-dependent kinases that could phosphorylate Ser328, additional kinase inhibition studies were carried out. ERK1/2 was inhibited with PD 98059 and CaMKII was inhibited with KN93. In either case, no inhibitor effect was observed on AS Ser328 phosphorylation when BAECs were co-treated with calcium ionophore or thapsigargin (data not shown). These results suggest that PKC, but not ERK1/2 or CaMKII, is responsible for the calcium stimulated phosphorylation of AS Ser328.

Since the α isotype of PKC (PKCα) is a calcium-dependent kinase that has been shown to be active during calcium-dependent stimulation of eNOS (25), we sought to determine whether this is the isotype responsible for phosphorylating Ser328 of AS. To test this, we examined the effects of direct knockdown of PKCα with dicer substrate siRNA (DsiRNA). DsiRNA is different from traditional siRNA used for gene silencing in that the RNA duplex used to silence not only mimics dicer products, but is also optimized for dicer processing by the RISC complex to result in a more potent knockdown (26). As shown in Figure 6, significant knockdown of PKCα was achieved. DsiRNA directed against PKCα allowed for 50% and 75% knockdown at 1nM and 5nM
DsiRNA, respectively. Knockdown of PKCα followed by treatment with calcium ionophore showed a decrease in phosphorylation of Ser328 by 50% and 60% with 1nM or 5nM, respectively, and prevented increased phosphorylation in response to calcium ionophore treatment. Phosphorylation of eNOS at Ser1179 was largely unaffected by PKCα knockdown under these conditions, while eNOS Thr497 dropped approximately 60% when PKCα was knocked down by 80%. These results suggest that PKCα is a kinase that phosphorylates Ser328 of AS.

**PKCα directly phosphorylates AS** - Since an observed decrease in phosphorylation due to kinase knockdown may be the result of inhibition of an upstream kinase in a signaling cascade, a targeted in vitro kinase assay was performed to determine whether PKCα directly phosphorylates AS. Akt1 was used as a negative control based on the lack of a consensus Akt phosphorylation motif (R-X-R-X-X-S/T) (27) in the AS protein sequence.

As shown in Figure 7A, PKCα clearly demonstrated the capacity to phosphorylate AS in vitro. Although there was an increase in counts above the blank reaction with Akt1, this was below the threshold considered significant. SDS-PAGE fractionation of [³²P]-labeled kinase reaction products and visualization by autoradiography film exposure confirmed that PKCα, but not Akt1, phosphorylates AS (Figure 7B).

**DISCUSSION**

Vascular endothelial NO production is a dynamic and targeted process, where multi-level regulation of eNOS is well established (28). Since AS is required for NO production, it was logical to speculate that the post-translational regulation of AS would play a significant role in maintaining NO homeostasis. Indeed, recent work using mass spectrometry analysis showed that AS is phosphorylated at site S352 (human sequence) (9); however, the biological significance of this phosphorylation was not defined. In addition, Corbin et al showed that phosphorylation of AS was altered in response to VEGF treatment (8), yet the phosphorylation site was unknown. In this report, we demonstrated that AS Ser328 phosphorylation supports the calcium-dependent stimulation of NO production in vascular endothelial cells, and is mediated by PKCα.

The involvement of AS in catalyzing the rate-limiting step of the arginine recycling side of the citrulline-NO cycle is critical in sustaining NO production in endothelial cells (29). Consistent with the established transcriptional co-regulation between AS and eNOS (30-32), work described in this report, using western blot analysis, showed that AS is co-regulated post-translationally with eNOS under conditions where the stimulation of NO production is dependent on calcium mediated signaling. Further studies showed that calcium stimulation of BAECs overexpressing the phospho-mimetic (Ser328D) AS increased NO production to that of the overexpressed WT AS; whereas, the overexpressed phospho-null (Ser328A) AS was unable to support this increase in NO production relative to the WT control (Figure 3). Moreover, the finding that under basal conditions both the null and phospho-mimetic AS mutant constructs were able to enhance NO production to that of the wild-type construct also verified that both overexpressed AS mutants were metabolically active (data not shown). Finally, through kinase inhibitor studies and knockdown analyses, we were able to establish that the phosphorylation of AS Ser328 under calcium mediated stimulation of NO production was facilitated by PKCα.

Considering the essential role that AS plays in NO production, and the spatial and temporal manner that eNOS is regulated by calcium levels, it is not surprising that AS is susceptible to a regulatory mechanism governed by changes in calcium. Indeed, the fact that the calcium-independent activation of eNOS by VEGF or insulin resulted in a decrease in phosphorylation at AS Ser328 further differentiated AS Ser328 phosphorylation to the calcium-dependent pathway for eNOS activation.

Previously, our laboratory had shown that AS resides in caveolae with eNOS (33). Since PKCα is associated with the plasma membrane when activated (34), a local functional relationship between PKCα and AS may be supported. In addition, it has been reported that changes in intracellular calcium promote the cytosolic re-localization of eNOS (35). Therefore, it is also tempting to speculate that the phosphorylation of AS at Ser328 may promote cytosolic AS re-localization in a manner consistent with eNOS in
response to calcium mediated signaling. This would then ensure maintenance of the functional relationship between arginine recycling and the translocated eNOS. Experiments are underway to test this hypothesis.

In summary, these results represent the first demonstration of a biologically relevant phosphorylation site for AS, and are consistent with previous findings demonstrating the coordinate regulation of eNOS and AS relative to vascular endothelial NO production. Given the critical role of AS in vascular endothelial NO production and previous work from our laboratory showing that the overall phosphorylation of AS changes in response to VEGF treatment (8), these results also suggest that other sites of phosphorylation are involved in AS regulation which merit further consideration.

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Co-regulation of AS and eNOS phosphorylation by calcium


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FOOTNOTES

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3The abbreviations used are: AS, argininosuccinate synthase; eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; thap, thapsigargin; BAEC, bovine aortic endothelial cells

FIGURE LEGENDS

**Figure 1.** Ser328 (S328) is an exposed site accessible by kinases and shows change in phosphorylation relative to treatment. BAECs were transiently transfected with AS expression vector then treated with 10µM bradykinin or 50nM okadaic acid. AS was His-tag purified then subjected to SDS-PAGE. The band corresponding to AS was excised and trypsin digested before tandem mass spectrometry analysis. Results represent one experiment with samples analyzed in triplicate with SE represented by error bars.

**Figure 2.** Calcium-independent stimulation of NO production decreased phosphorylation at argininosuccinate synthase Ser328 (S328). BAECs were treated with insulin at 100nM (A) or VEGF at 100 ng/mL (C) for 20 min. Phosphorylation was assayed by Western blotting using phospho-specific antibodies and reprobed with eNOS and AS specific antibodies. Blots are representative of 3 separate experiments. Densitometric values (B and D) represent percent change from non-treated (NT) controls normalized to total eNOS or AS respectively. Densitometric changes are significant with SE represented by error bars (n=3, p< 0.05). (E) Culture media from BAECs treated with insulin or VEGF was assayed for nitrite with the DAN assay with SE represented by error bars (* n=3, p<0.05).

**Figure 3.** Overexpressed Ser328 phospho-null mutation to alanine produced significantly less stimulated NO than wild type (WT) or phospho-mimetic (S328D). (A) BAECs were transiently transfected with wild
type (WT), phospho-null serine to alanine 328 (S328A), or phospho-mimetic serine to aspartate (S328D) AS then stimulated with 0.5µM calcium ionophore A23187 and 50µM sodium orthovanadate. Results are representative of 4 different experiments with SE noted by error bars. (* p<0.05 relative to WT; † p<0.05 relative to S328D). (B) Representative western blot confirming WT, S328A, and S328D overexpression.

**Figure 4.** Calcium-dependent stimulation of NO production increased phosphorylation at argininosuccinate synthase S328. (A) Western blot analysis of changes in phosphorylation relative to 20 minute treatment with calcium ionophore (A23187), thapsigargin (Thap), in presence or absence of intracellular calcium chelator BAPTA-AM. Blots are representative of 5 separate experiments. (B) Densitometric values represent percent change from non-treated (NT) controls normalized to total eNOS or AS respectively. Densitometric changes are significant with SE represented by error bars. (n=3, p<0.05, significantly increased from NT * , p<0.05, significantly decreased from A23187 alone ‡ p<0.05, significantly decreased from Thap alone † p<0.05).

**Figure 5.** Non-isotype specific inhibition of PKC with 42µM rottlerin or 2.5µM bisindolylmaleimide I decreased phosphorylation at AS S328. BAECs were pre-treated with 42µM rottlerin (Rott) or 2.5µM bisindolylmaleimide I (Bis) for 30 minutes prior to stimulation with calcium ionophore (A23187) for 20 minutes. (A) Representative western blot of 3 experiments. (B) Densitometric values represent percent change from non-treated (NT) controls normalized to total eNOS or AS respectively. Densitometric changes are significant with SE represented by error bars (n=3, p<0.05). (C) Culture media from treated BAECs was assayed for nitrite with the DAN assay. A23187 significantly increased NO production with or without bisindolylmaleimide I (significance relative to NT: * n=3, p<0.05; significance relative to A23187: ‡ n=3, p<0.05).

**Figure 6.** Knockdown of PKCα decreased phosphorylation at S328 but not eNOS S1179. BAECs were treated with 1nM or 5nM dicer substrate RNA (DsiRNA) directed against the 5th exon of PKCα and allowed to express for 24 hours, followed by stimulation with calcium ionophore A23187 for 20 minutes and compared to non-treated (NT) BAECs. (A) Representative western blot of 3 experiments. (B) Densitometric values represent percent change from non-treated (NT) controls normalized to total eNOS or AS respectively. Densitometric changes are significant (except for A2317 effect on PKCα expression or 1nM DsiRNA treatment on pS1179 eNOS) with SE represented by error bars (n=3, p< 0.05). (C) Culture media from treated BAECs was assayed for nitrite using the DAN assay (significance relative to NT or DsiRNA alone: * n=3, p<0.05)

**Figure 7.** PKCα, but not Akt phosphorylate AS in vitro. (A) In vitro kinase assays. Gray bars represent the incubation of purified AS minus kinase (Blank). Black bars represent the incubation of purified AS with the indicated kinase (Kinase). Kinase activity is represented as counts per minute (CPM) with error bars representing SE (n = 3*, p < 0.05). (B) Representative autoradiograph of in vitro kinase reactions separated by SDS-PAGE as described in (A).
Figure 1
Co-regulation of AS and eNOS phosphorylation by calcium

Figure 2

[Diagram showing phosphorylation of various proteins and figures representing percent change from NT]
Figure 3

(A) Comparison of nitrite production per µg protein among WT, S328A, and S328D. * and † denote statistical significance.

(B) Western blot analysis showing expression of AS and GAPDH in WT, S328A, and S328D.
Co-regulation of AS and eNOS phosphorylation by calcium

Figure 4

A

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C

Graph showing the effect of calcium modulation on eNOS and AS phosphorylation.
Co-regulation of AS and eNOS phosphorylation by calcium

Figure 5
Figure 6

A

A23187  -  +  -  +  -  +
PKCα DsiRNA (nM)  -  -  1  1  5  5
pT497 eNOS  

B

A23187  +  -  +  -  +  +
PKCα DsiRNA (nM)  -  1  1  5  5  5

Percent change from NT

-100  -50  0  50  100  150

- pS328 AS
- pS1179 eNOS
- pT497 eNOS
- PKCa
Figure 7
Protein Kinase C α Phosphorylates a Novel Argininosuccinate Synthase Site at Serine 328 During Calcium-Dependent Stimulation of Endothelial Nitric Oxide Synthase in Vascular Endothelial Cells

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