Coordinated Control of Endothelial Nitric-oxide Synthase Phosphorylation by Protein Kinase C and the cAMP-dependent Protein Kinase*

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Endothelial nitric-oxide synthase (eNOS) is an important regulatory enzyme in the cardiovascular system catalyzing the production of NO from arginine. Multiple protein kinases including Akt/PKB, cAMP-dependent protein kinase (PKA), and the AMP-activated protein kinase (AMPK) activate eNOS by phosphorylating Ser-1177 in response to various stimuli. During VEGF signaling in endothelial cells, there is a transient increase in Ser-1177 phosphorylation coupled with a decrease in Thr-495 phosphorylation that reverses over 10 min. PKC signaling in endothelial cells inhibits eNOS activity by phosphorylating Thr-495 and dephosphorylating Ser-1177 whereas PKA signaling acts in reverse by increasing phosphorylation of Ser-1177 and dephosphorylation of Thr-495 to activate eNOS. Both phosphatases PP1 and PP2A are associated with eNOS. PP1 is responsible for dephosphorylation of Thr-495 based on its specificity for this site in both eNOS and the corresponding synthetic phosphopeptide whereas PP2A is responsible for dephosphorylation of Ser-1177. Treatment of endothelial cells with calyculin selectively blocks PKA-mediated dephosphorylation of Thr-495 whereas okadaic acid selectively blocks PKC-mediated dephosphorylation of Ser-1177. These results show that regulation of eNOS activity involves coordinated signaling through Ser-1177 and Thr-495 by multiple protein kinases and phosphatases.

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Phosphorylation of eNOS on Ser-1177 and Thr-495

MALDI-TOF Mass Spectrometry—Phosphatase assays were performed using synthetic phosphopeptides (100 μM) and immunocomplexes in reaction buffer (200 μl) containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% n-octyl-glucoside and 2 mM MnCl₂. At time points, immunocomplexes were pelleted by centrifugation, and an aliquot of peptide (20 μl) was removed and added to 10 μl of 2% trifluoroacetic acid. The peptides were desalted using C18 Zip Tips (Millipore, Bedford, MA) into 5 μl of 2% trifluoroacetic acid. Peptides (0.5 μl) were then loaded onto a MALDI-TOF mass spectrometer sample stage with the matrix, α-cyano-4-hydroxycinnamic acid (0.5 μl). Masses were analyzed using a linear Voyager DE (PerSeptive Biosystems) MALDI-TOF instrument operating in delayed extraction mode.

RESULTS

VEGF and PKA Signaling in Endothelial Cells—Both VEGF and IGF-1 stimulate Akt/PKB kinase in BAEC to phosphorylate and activate eNOS (2). In HUVEC but not in BAEC, we observed that VEGF stimulation led to a transient increase in Ser-1177 phosphorylation that was accompanied by a decrease in Thr-495 phosphorylation (Fig. 1A). Human eNOS Ser-1177 and Thr-495 correspond to bovine eNOS Ser-1179 and Thr-497, respectively. Treatment of BAEC with the phosphodiesterase inhibitor IBMX (Fig. 1B) or forskolin, but not 8-bromo-cGMP (data not shown), caused dephosphorylation of Thr-497 and enhanced phosphorylation of Ser-1179 resulting in increased eNOS activity (Fig. 1C). The PKA-stimulated phosphorylation/dephosphorylation is maintained for at least 30 min (longest period tested) whereas the VEGF-stimulated phosphorylation/dephosphorylation is transient and reverses within 10 min. Thus, signaling through either the VEGF receptor or via PKA activates eNOS by the coordinated phosphorylation of Ser-1179 and dephosphorylation of Thr-497.

PKC Signaling in Endothelial Cells—PMA treatment of BAEC increased phosphorylation of Thr-497 and decreased Ser-1179 phosphorylation (Fig. 2A), inhibiting eNOS activity. The partially specific PKC inhibitor Ro-318220 (17) enhanced Ser-1179 phosphorylation and suppressed Thr-497 phosphorylation while the inactive isomer Ro-310645 did not (Fig. 2B), consistent with PKC involvement. Further, chronic PMA treatment of BAEC decreases Thr-497 phosphorylation and increased Ser-1179 phosphorylation (results not shown), consistent with the down-regulation of expression of the PMA-responsive PKC isoforms (18).

HUVEC were treated with VEGF over a time course of 0, 2, 10, and 30 min with either the PKC inhibitor Ro-318220 or the inactive isomer Ro-310645. Inhibition of PKC prolonged VEGF-induced stimulation of Ser-1177 phosphorylation consistent with inhibition of a PKC-dependent phosphatase responsible for dephosphorylation of Ser-1177. The VEGF-induced dephosphorylation of Thr-495 was also prolonged by inhibition of PKC providing further evidence that PKC phosphorylates this site (Fig. 2C). Thus, signaling through PKC inhibits eNOS activity by phosphorylation of Thr-497 and dephosphorylation of Ser-1179. In contrast to the results obtained in endothelial cells, PKC phosphorylates both Thr-497 and Ser-1179 in vitro. The site phosphorylated depends on the presence of calmodulin, with Thr-497 phosphorylated in the presence of EGTA and Ser-1179 in the presence of Ca²⁺/CaM (Fig. 2D). Phosphoryla-
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A, BAEC were incubated with and without 100 nM PMA or 500 nM IBMX for 5 min. eNOS was purified by ADP-Sepharose chromatography, and Western blots were probed for associated PP1 and PP2A (representative blot, n = 12). B, recombinant eNOS was phosphorylated predominantly on either Thr-497 or Ser-1179 as indicated and then incubated with immunoprecipitates of PP1 and PP2A. Aliquots of eNOS were removed at 0, 10, 30, and 120 min into SDS sample buffer and subjected to SDS polyacrylamide gel electrophoresis and Western blot analysis. C–F, synthetic eNOS phosphopeptides corresponding to the human Ser-1177 and Thr-495 phosphorylation sites were incubated with immunocomplexes of PP2A (C, E) and PP1 (D, F). The theoretical mass of the alkylated phosphopeptides eNOS-(487–504)C486pT495, GITRKKpTFKEVANC was 1730 and eNOS-(1172–1183)C1171pS1177, CRIRTQpSFSLQER was 1760. The observed phosphopeptide and dephosphopeptide masses are indicated. A peak of ~80 m/z units less than the phosphopeptide was produced by loss of phosphate after phosphatase incubation. The position of the phosphopeptide peak is indicated by a solid arrow; the corresponding dephosphopeptide peak by a dashed arrow.

PP1 and PP2A Dephosphorylation of eNOS—Both phosphatases PP1 and PP2A are associated with affinity-purified eNOS. However, there is no detectable change in their association with phosphorylation of eNOS when endothelial cells were treated with either IBMX or PMA (Fig. 3A). Therefore, we investigated whether these phosphatases preferentially dephosphorylate either Thr-497 or Ser-1179. Recombinant eNOS phosphorylated predominantly at either Thr-497 or Ser-1179 was incubated with immunoprecipitates of PP1 and PP2A (Fig. 3B). The Thr-497 site was preferentially dephosphorylated by PP1, whereas Ser-1179 was preferentially dephosphorylated by PP2A. PP1 dephosphorylated the Thr-497 site by more than 80% whereas PP2A caused less than 40% dephosphorylation. In contrast, PP1 dephosphorylated the Ser-1179 site by ~30% whereas PP2A caused more than 70% dephosphorylation.

Syntetic phosphopeptides corresponding to the two phosphorylation sites were also tested as substrates using a MALDI-TOF mass spectrometry assay. The Thr-495 phosphopeptide was dephosphorylated by both phosphatases but more rapidly with PP1 than PP2A (Fig. 3, C and D). The Ser-1177 phosphopeptide was readily dephosphorylated by immunoprecipitates of PP2A but not PP1 (Fig. 3, E and F). The results show that PP1 and PP2A have distinct specificities with PP1 primarily responsible for Thr-495 dephosphorylation and PP2A for Ser-1177 dephosphorylation.

Selective Inhibition of Thr-497 and Ser-1179 Dephosphorylation by Calyculin and Okadaic Acid—Treatment of BAEC with okadaic acid alone increased Ser-1179 phosphorylation ~2-fold (Fig. 4, A and B) and calyculin alone increased Thr-497 phosphorylation 2.5-fold (Fig. 4, C and D). Calyculin A is reported to inhibit PP1 more selectively than PP2A, whereas okadaic acid inhibits PP2A at concentrations up to 1 μM without inhibiting PP1 (19, 20). The selective inhibition of the dephosphorylation of the two sites by okadaic acid and calyculin indicates that PP1 is responsible for dephosphorylation of Thr-497 and PP2A for dephosphorylation of Ser-1179 in full agreement with the specificity of these phosphatases for the respective sites in vitro.

Treatment with okadaic acid blocked the PMA-induced dephosphorylation of Ser-1179 (Fig. 4A) but not the effects of IBMX on Thr-497 phosphorylation even at concentrations up to 500 nM (Fig. 4B) consistent with PP2A dephosphorylating Ser-1179. In contrast, calyculin did not block the dephosphorylation of Ser-1179 (Fig. 4, C and D) but did block the IBMX-induced dephosphorylation of Thr-497 supporting the idea that PP1 dephosphorylates Thr-497. PMA-induced phosphorylation of
Thr-497 is enhanced by calyculin and okadaic acid. The inhibition of PKP1 by calyculin alone causes an increase in Thr-497 phosphorylation and enhanced the PMA effect on Thr-497 phosphorylation (Fig. 4C). Whereas, okadaic acid alone caused a slight reduction in Thr-497 phosphorylation (Fig. 4A) it enhanced the PMA-induced phosphorylation of Thr-497. These results demonstrate that the two phosphatase inhibitors have distinct inhibition patterns for the dephosphorylation of Thr-497 (calyculin) and Ser-1179 (okadaic acid) sites of eNOS.

Treatment of cells with okadaic acid elevated eNOS activity (Fig. 4E) in parallel with the increased phosphorylation of Ser-1179 and reduced Thr-497 phosphorylation (Fig. 4, A and B). In contrast, PMA reduced eNOS activity in endothelial cells (Fig. 4E) in parallel with a 3-fold increase in Thr-497 phosphorylation and a 4-fold decrease in Ser-1179 phosphorylation (Fig. 4, A and C).

**DISCUSSION**

The regulation of eNOS activity by phosphorylation at Ser-1177 and Thr-495 is relatively complex involving at least four protein kinases (Akt, PKA, PKC, and AMPK) and two phosphatases (PP1 and PP2A). Previous studies have shown that Ser-1177 phosphorylation activates eNOS (1–3, 6, 7) whereas Thr-495 phosphorylation inhibits activity as a consequence of this site being present in the CaM binding sequence (1). During signaling events that promote phosphorylation at either of these sites, there is coordinated dephosphorylation at the alternate site. In this way the inhibition of eNOS resulting from PKC phosphorylation of Thr-495 is amplified by the simultaneous dephosphorylation of Ser-1177. Similarly, activation of eNOS in response to PKA signaling involves phosphorylation of Ser-1177 as well as dephosphorylation of Thr-495 (Fig. 5). At present it is not clear how signaling through PKA and PKC causes selective dephosphorylation of eNOS by PP1 and PP2A, respectively. Phosphorylation at one site may not be the trigger for dephosphorylation at the second site because in vitro one or other site is selectively phosphorylated rather than both suggesting that dephosphorylation of one precedes phosphorylation of the other. The dephosphorylation and phosphorylation reactions at the two sites appear independently coordinated.

Because PKA signaling activates PKP1 to dephosphorylate Thr-495, one potential mechanism may involve the inactivation of a phosphatase inhibitor analogous to NIPP-1 that localizes PKP1 that is inactivated by PKA phosphorylation (21). Other phosphatase inhibitors are activated by phosphorylation (inhibitor-1 and CPI-17 activated by PKA and PKC phosphatase respectively, reviewed in Ref. 22). We have not detected PKA or PKC substrates in immunoprecipitates of PP1 or PP2A that could act as phosphatase inhibitors. Cyclosporin A blocks the dephosphorylation of eNOS at Thr-497 in response to bradykinin in early passage (2–6) BAEC as well as NO production (11). However, the dephosphorylation of Thr-497 triggered by PKA signaling observed here was unaffected by preincubation with the calcineurin inhibitor FK506 (1 μM).

VEGF stimulates at least two protein kinases (Akt and PKC) that ensure the tight control of eNOS activation. Signaling through PKC attenuates VEGF-induced stimulation of Ser-1177 phosphorylation by Akt. The PKC-stimulated dephosphorylation of Ser-1177 by PP2A occurs simultaneously with enhanced phosphorylation of Thr-495 and inhibits eNOS activity. In contrast, PKA directly phosphorylates Ser-1179 and stimulates the PP1-dependent dephosphorylation of Thr-497, activating eNOS (Fig. 5). Several other examples of PKC-stimulated dephosphorylation have been reported including dephosphorylation of the cadherin-associated proteins P120 and p100 in epithelial and endothelial cells (24, 25) and in the attenuation of the signaling of activated guanyl cyclase-linked natriuretic peptide receptors, GC-A and -B where PP2A may also be involved (23).

The inhibition of eNOS following activation of PKC by VEGF or phorbol esters illustrates that signaling through PKC can suppress NO production from eNOS. These results add a new dimension to our understanding of the complexities of eNOS regulation (26). Given, that NO plays such a diverse role in the cardiovascular system, it raises the possibility that one of the actions of PKC inhibitors in suppressing the vascular complications of diabetes (27) may be mediated in part by blocking PKC inhibitory signaling to eNOS.

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