

Dephosphorylation of Endothelial Nitric-oxide Synthase by Vascular Endothelial Growth Factor

IMPLICATIONS FOR THE VASCULAR RESPONSES TO CYCLOSPORIN A*

Received for publication, May 8, 2002

Published, JBC Papers in Press, June 5, 2002, DOI 10.1074/jbc.M204519200

Ruqin Kou, Daniel Greif‡, and Thomas Michel§¶

From the Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School,

Boston, Massachusetts 02115 and the §Veterans Affairs Boston Healthcare System, West Roxbury, Massachusetts 02132

The endothelial isoform of nitric-oxide synthase (eNOS) is a key determinant of vascular tone. eNOS, a Ca^{2+} /calmodulin-dependent enzyme, is also regulated by a variety of agonist-activated protein kinases, but the role and regulation of the protein phosphatase pathways involved in eNOS dephosphorylation are much less well understood. Treatment of endothelial cells with vascular endothelial growth factor (VEGF), a potent eNOS agonist, leads to the activation of calcineurin, a Ca^{2+} /calmodulin-dependent protein phosphatase. In these studies, we used a phosphorylation state-specific antibody to show that VEGF promotes dephosphorylation of eNOS at serine residue 116 in cultured endothelial cells. Cyclosporin A, an inhibitor of calcineurin, completely blocks VEGF-induced eNOS dephosphorylation; under identical conditions, cyclosporin A also inhibits VEGF-induced eNOS activation. VEGF-induced eNOS dephosphorylation shows an EC_{50} of 2 ng/ml and is maximal 30 min after agonist addition. eNOS phosphorylation at serine 116 is completely blocked by the protein kinase C inhibitor calphostin but is blocked by neither wortmannin (an inhibitor of phosphatidylinositol 3-kinase) nor the MAP kinase pathway inhibitor U0126. A phosphorylation-deficient mutant of eNOS in which serine 116 is changed to an alanine residue (S116A) shows significantly enhanced enzyme activity compared with the wild-type enzyme. Taken together, these findings indicated that VEGF-induced eNOS dephosphorylation at serine 116 leads to enzyme activation. Cyclosporin A is widely used as an immunosuppressive drug for which hypertension is an important dose-limiting side effect. Our results suggest that cyclosporin A-induced hypertension may involve, at least in part, the attenuation of endothelium-derived NO production through a calcineurin-sensitive pathway regulating eNOS dephosphorylation.

The endothelial isoform of nitric-oxide synthase (eNOS)¹ is a key determinant of vascular homeostasis (for review see Ref. 1).

* This work was supported in part by Grant HL46457 from the NHLBI, National Institutes of Health (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a Physician postdoctoral research fellowship from the Howard Hughes Medical Institute.

¶ To whom correspondence should be addressed: Cardiovascular Division, Brigham and Women's Hospital, Thorn Bldg., Rm. 1210A, 75 Francis St., Boston, MA 02115. Tel.: 617-732-7376; Fax: 617-732-5132; E-mail: michel@calvin.bwh.harvard.edu.

¹ The abbreviations used are: eNOS, endothelial isoform of nitric-oxide synthase; VEGF, vascular endothelial growth factor; BAEC, bovine aortic endothelial cells; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-related kinase.

eNOS enzyme activity is closely controlled by diverse extracellular signals that also modulate myriad other regulatory pathways in endothelial cells. Nitric oxide synthesized by eNOS promotes the relaxation of vascular smooth muscle cells, leading to a reduction in blood pressure. eNOS is a Ca^{2+} /calmodulin-dependent enzyme and is activated in response to the stimulation of a variety of Ca^{2+} -mobilizing cell surface receptors. The activity of eNOS also is influenced by phosphorylation at three or more sites in the enzyme: serine 1179 (serine 1177 in the human eNOS sequence, Refs. 2–4); threonine 497 (threonine 495 in the human sequence, Refs. 5–7); and serine 116 (2). Of these phosphorylation sites, the regulation of eNOS serine 1179 has been most extensively characterized; protein kinase Akt (3, 4), the AMP-activated protein kinase (8), and the cyclic AMP-dependent protein kinase (6) among others have been shown to modify eNOS at this residue, thereby potentiating eNOS enzyme activity. The phosphorylation of threonine 497 appears to be modulated by the agonist bradykinin (5, 7), but the intracellular pathways involved in this response remain less well understood. Phosphorylation of eNOS at serine 116 has been observed in intact endothelial cells subjected to hemodynamic shear stress (2), but the regulatory consequences of eNOS phosphorylation at this site are almost entirely unknown. In these studies, we will show that the eNOS agonist vascular endothelial growth factor (VEGF) promotes eNOS activation associated with the dephosphorylation of eNOS at serine 116 in a pathway blocked by the phosphatase inhibitor cyclosporin A.

The principal molecular target of cyclosporin A is the Ca^{2+} /calmodulin-dependent phosphatase calcineurin, which is also known as protein phosphatase PP2B (10). Calcineurin promotes the dephosphorylation of a restricted range of protein substrates of which the best characterized is the transcription factor NF-AT (nuclear factor of activated T cells) (11). Cyclosporin A is in widespread clinical use as an immunosuppressant but frequently causes hypertension; this principal side effect represents an important dose-limiting toxicity (12). There is some evidence that cyclosporin A attenuates endothelium-dependent vasorelaxation (13, 14), but a clear relationship between calcineurin action and eNOS regulation has not yet been clearly established. One agonist known to activate calcineurin in endothelial cells is VEGF (15), which is also one of the key activators of eNOS in the vessel wall.

VEGF treatment of endothelial cells leads to a transient increase in intracellular Ca^{2+} (16) as well as the activation of diverse protein tyrosine kinase and serine/threonine kinase pathways (17). VEGF promotes the activation of eNOS by Ca^{2+} /calmodulin and also stimulates the protein kinase Akt, which directly phosphorylates eNOS on serine 1179 and increases enzyme activity (3, 7). Because VEGF also activates the phosphatase calcineurin in endothelial cells (15), in the present

studies we explored the hypothesis that VEGF modulates eNOS phosphorylation pathways involving calcineurin, and we have therefore analyzed patterns of VEGF-dependent eNOS activation and phosphorylation as modified by the calcineurin inhibitor cyclosporin A.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT); all other cell culture reagents and media were from Invitrogen. VEGF, BAPTA (bis(*O*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid), and wortmannin were from Calbiochem (La Jolla, CA), and the MAP kinase pathway inhibitor U0126 was from Cell Signaling Technologies (Beverly, MA). Cyclosporin A was from Biomol (Plymouth Meeting, PA). Anti-phospho-Ser¹¹⁶-eNOS and anti-phospho-Thr⁴⁹⁷-eNOS antisera were from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Ser¹¹⁷⁷-eNOS antiserum (Ser¹¹⁷⁷ in the human eNOS sequence corresponds to Ser¹¹⁷⁹ in bovine eNOS), anti-phospho-Akt antibody, and anti-phospho-ERK1/2 antibody were from Cell Signaling Technologies. Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). The Super Signal chemiluminescence detection reagents were from Pierce. L-[³H]arginine was from Amersham Biosciences. Protein concentrations were determined with the Bio-Rad protein assay kit. FuGENE 6 was from Roche Molecular Biochemicals. All other reagents were from Sigma.

Plasmids—The eNOS S116A mutant was constructed by standard PCR-based mutagenesis methods using cDNA encoding wild-type bovine eNOS (18) as template, with flanking primers at the *Eco*RI and *Bgl*II sites located in the upstream polylinker and the *Bgl*II site in the middle of the eNOS-coding sequence, respectively. Following amplification, purification, restriction digestion, and molecular cloning of the PCR-generated fragment containing the mutation, the nucleotide sequence of the amplified region of the cDNA was validated by dideoxynucleotide sequencing using standard techniques. For construction of the S116A mutant, the upstream primer pair was comprised of the forward primer 5'-CGCGAATTCGAAGGAGCCACCATGGGCAACTTGAAGAG-3' and the reverse (mutated) primer 5'-AGGTCCCGGGCGGGCCCGGGT-3'; the second primer pair was comprised of the forward primer 5'-ACCCGGCCCGCCCGGGACCT-3' and the reverse primer 5'-CCGAGATCTTACCGCGTTG-3' (the bold character notes the base pair that was altered to change Ser to Ala).

Cell Culture, Transfection, and Drug Treatment—Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%) as described (19). Cells were plated onto gelatin-coated culture dishes and studied prior to cell confluence between passage 5 and 9; cells were serum-starved overnight prior to experimental treatments. COS-7 cells were maintained in culture, transfected with various cDNA constructs using FuGENE6 as described previously (19), and studied 48 h following transfection.

Immunoblot Analysis and NOS Activities Assay—Cell lysates were prepared using a cell lysis buffer containing Nonidet P-40, and the degree of protein expression and phosphorylation were assessed as we previously described in detail (19). eNOS activity in intact cultured cells was quantified as the formation of L-[³H]citrulline from L-[³H]arginine as described previously (19).

Other Methods—All experiments were performed at least three times. Mean values for individual experiments were expressed as mean \pm S.E. Statistical differences were assessed by analysis of variance followed by Student's *t* test. A *p* value $<$ 0.05 was considered statistically significant.

RESULTS

The fact that VEGF treatment of endothelial cells leads to the activation of calcineurin (15) led us to explore the effects of the calcineurin inhibitor cyclosporin A on VEGF-induced eNOS phosphorylation and enzyme activity. We found that pretreatment of BAEC with cyclosporin for 30 min completely blocked the robust increase in eNOS enzyme activity that was seen in response to VEGF treatment in control BAEC (Fig. 1). There was no substantive change in basal eNOS activity seen in BAEC treated with cyclosporin (Fig. 1), and cyclosporin pretreatment failed to block eNOS activation elicited by several other well known eNOS agonists including bradykinin (data not shown). We and others have extensively characterized the VEGF-induced phosphorylation of eNOS on serine 1179, and

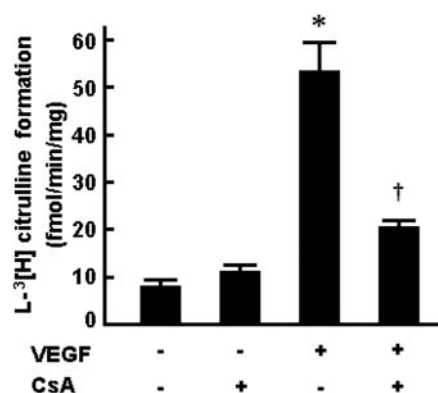


FIG. 1. Attenuation by cyclosporin A of VEGF-induced eNOS activation in endothelial cells. Shown are the results of eNOS activity assays performed in BAEC pretreated either with cyclosporin A or vehicle for 30 min and then treated with VEGF or vehicle for additional 30 min, as indicated in the figure. eNOS activity was quantitated by adding L-[³H]arginine to the cells for the last 10 min of drug treatment and then analyzing the formation of L-[³H]citrulline as described (19). Each data point represents the mean \pm S.E. derived from four independent cell preparations, each performed in triplicate. * indicates *p* $<$ 0.01 for VEGF versus vehicle treatment. † indicates *p* $<$ 0.05 for cyclosporin A versus vehicle pretreatment.

we have found that in immunoblots probed with phosphorylation state-specific antisera cyclosporin pretreatment had no effect on VEGF-induced phosphorylation at serine 1179 (Fig. 2) or at threonine 497 (data not shown). By contrast, VEGF dramatically reduced the phosphorylation of eNOS on serine 116, assessed using an antiserum directed against eNOS phosphorylated at serine 116 (Fig. 2). VEGF-induced eNOS serine 116 dephosphorylation was completely blocked by pretreatment of BAEC with cyclosporin, with no change in the total eNOS protein recovered following these treatments (Fig. 2). As seen in Fig. 2, cyclosporin also markedly reduced VEGF-induced phosphorylation of kinase Akt and also attenuated VEGF-promoted ERK activation.

We next performed a more detailed pharmacological characterization of VEGF-induced eNOS dephosphorylation at serine 116. The time course of VEGF-induced eNOS dephosphorylation at serine 116 showed a maximal effect at 30 min and did not return to basal levels even 1 h after adding VEGF. This response is considerably slower than that seen with the VEGF-promoted phosphorylation of eNOS at serine 1179, which has a maximal response at 5 min and returns to basal levels by 30 min (Fig. 3; see Ref. 16). The dose response to VEGF showed an EC₅₀ of 2 ng/ml (Fig. 3B), consistent with the EC₅₀ observed with other biological responses elicited by VEGF (20, 21).

To more fully understand the consequences of eNOS phosphorylation at serine 116 on enzyme activity, we mutated serine 116 to alanine in the eNOS cDNA (to yield the plasmid S116A-eNOS) and transfected this construct into COS-7 cells. Immunoblots probed with the serine 116-phospho-eNOS antiserum detected a band corresponding to eNOS in COS-7 cells transfected with wild-type eNOS but not in cells transfected with the phosphorylation-deficient S116A eNOS mutant (Fig. 4). Immunoblot analysis revealed that the wild-type and S116A mutant constructs yielded a similar amount of total eNOS expression, with no substantive difference in the level of eNOS serine 1179 phosphorylation between the wild-type and S116A mutant eNOS (Fig. 4) or in the level of phosphorylation at threonine 497 (data not shown). There was no substantive difference in the basal eNOS enzyme activity in COS-7 cells transfected with the wild-type eNOS or the S116A mutant, both of which had eNOS activity measured at \sim 12 fmol L-[³H]citrulline formed/min/mg of protein. However, in re-

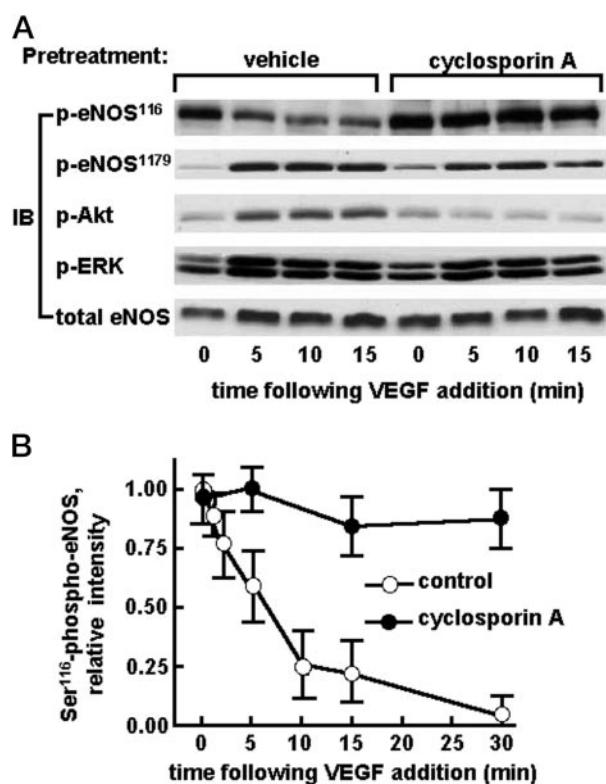


FIG. 2. Attenuation by cyclosporin A of VEGF-induced eNOS dephosphorylation in endothelial cells. *A*, representative immunoblot prepared from BAEC cells pretreated with cyclosporin A (100 nM) for 30 min and then treated with VEGF (20 ng/ml) for the indicated times. Cell lysates were resolved by SDS-PAGE and analyzed in immunoblots (*IB*) probed separately, as indicated, with specific antibodies directed against Ser¹¹⁶-phospho-eNOS, Ser¹¹⁷⁹-phospho-eNOS, phospho-Akt, phospho-ERK1/2, and total eNOS. *B*, data pooled from six similar independent experiments exploring the VEGF-induced change in Ser¹¹⁶-phospho-eNOS in cells pretreated with cyclosporin A or vehicle (*control*) as indicated and then analyzed in immunoblots probed with the Ser¹¹⁶-phospho-eNOS antibody, which were quantitated by measuring the chemiluminescence (ChemiImager, Alpha Innotech) of the Ser¹¹⁶-phospho-eNOS immunoreactive band.

sponse to the calcium ionophore A23187 (Fig. 4), the eNOS enzyme activity of the phosphorylation-deficient S116A mutant was significantly higher than that of the wild-type eNOS at equivalent levels of recombinant protein expression (5.3 ± 0.4 -fold increase in eNOS activity for the S116A mutant compared with a 3.2 ± 0.3 -fold increase seen with wild-type eNOS; mean \pm S.E. of $n = 5$ experiments performed in duplicate, $p < 0.01$).

We next sought to explore the protein kinase pathways that might be involved in the phosphorylation of eNOS on serine 116. The kinase consensus sequence around serine 116 suggests that this residue might represent a site for phosphorylation by ERK1/2 MAP kinases. However, the MAP kinase kinase inhibitor U1026 had no effect on eNOS serine 116 phosphorylation under conditions in which ERK1/2 activation was completely blocked by this inhibitor treatment (Fig. 5A). The phosphatidylinositol 3-kinase inhibitor wortmannin similarly had no effect on eNOS serine 116 phosphorylation under conditions in which phosphorylation of the phosphatidylinositol 3-kinase downstream kinase Akt was completely blocked (Fig. 5A). Of a wide range of kinase inhibitors, we found that the protein kinase C inhibitor calphostin was distinct in its ability to completely block phosphorylation of eNOS at serine 116, accompanied by a significant attenuation in phosphorylation at serine 1179 (Fig. 5B).

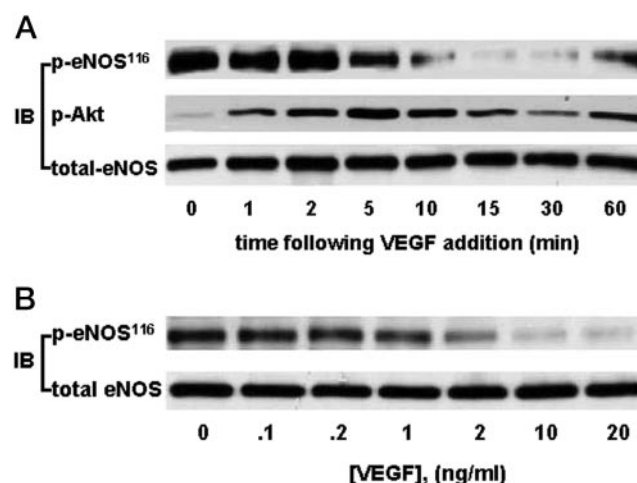


FIG. 3. Time course and dose response for VEGF-induced eNOS serine 116 dephosphorylation. *A*, results of a time course experiment showing immunoblots (*IB*) prepared from VEGF-treated BAEC, harvested at the indicated times following addition of VEGF (20 ng/ml). The upper immunoblot was probed with antiserum specific for Ser¹¹⁶-phospho-eNOS; the middle immunoblot was probed with antiserum directed against activated (phosphorylated) kinase Akt; and the lower immunoblot derives from the same blot as in the upper immunoblot, but it has been stripped and re-probed for total eNOS as a loading control. *B*, a VEGF dose response showing immunoblot analyses of samples prepared from BAEC treated for 30 min with the indicated doses of VEGF. The upper lanes present a blot probed with the antibody directed against Ser¹¹⁶-phospho-eNOS, and the lower lanes show the results obtained when the same blot is stripped and re-probed with antibody against total eNOS.

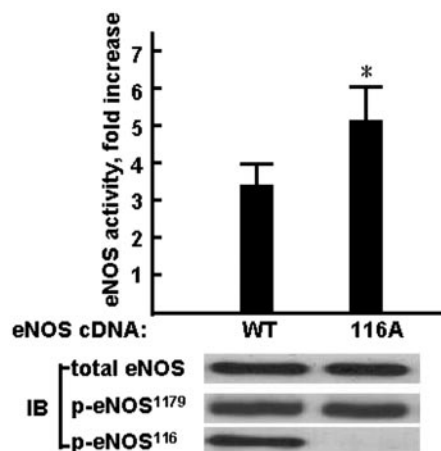


FIG. 4. eNOS enzyme activity in COS-7 cells transfected with wild-type or S116A mutant eNOS. This figure shows the results of an eNOS activity assay performed in COS-7 cells transfected either with wild-type eNOS (*WT*) or an eNOS phosphorylation-site mutant in which serine 116 is changed to alanine (*116A*). 48 h after transfection, NOS activity was assayed by adding L-[³H]arginine with or without calcium ionophore A23187 (10 μ M) to the cells; 10 min later, cells were harvested and lysed, and eNOS enzyme activity was measured as the formation of L-[³H]citrulline. Data are presented as the -fold increase over the NOS enzyme activity seen in the absence of ionophore, which did not differ between the wild-type- and S116A mutant eNOS-transfected cells (~ 12 fmol/min/mg of protein). The data shown are pooled from three experiments, each performed in duplicate; the S116A activity was significantly greater than the wild-type enzyme ($p < 0.01$). The lower panel is a representative immunoblot (*IB*) prepared from cells processed in parallel with the cells used for the activity assay; identical immunoblots were probed with antisera directed against Ser¹¹⁶-phospho-eNOS, Ser¹¹⁷⁹-phospho-eNOS, or total eNOS as indicated.

DISCUSSION

VEGF has been extensively characterized as a key agonist for eNOS activation in the vascular wall and only more recently

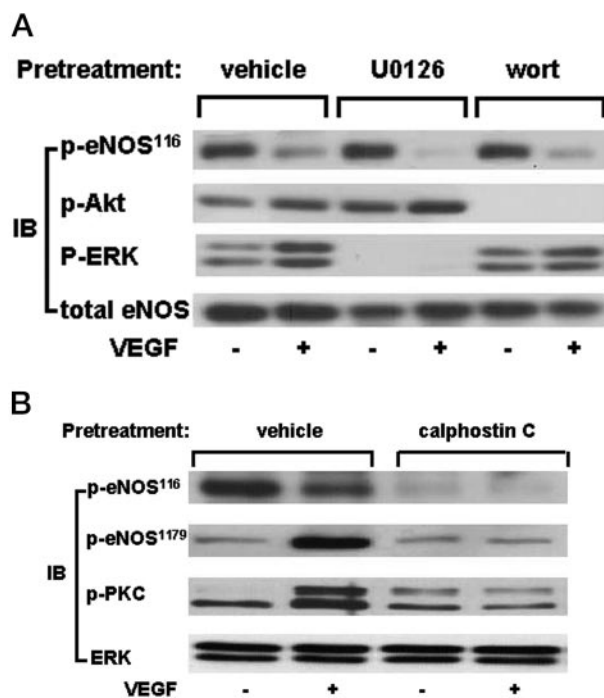


FIG. 5. Effects of protein kinase inhibitors on eNOS phosphorylation of at serine 116. Figure 5 shows a series of immunoblots (IB) prepared from BAEC treated with various protein kinase inhibitors. *A*, BAEC were treated for 30 min with the MAP kinase pathway inhibitor U0126 (10 μ M), with the phosphatidylinositol 3-kinase inhibitor wortmannin (*wort*, 500 nM), or with vehicle and then were treated as indicated for an additional 15 min with VEGF (20 ng/ml) or vehicle before harvesting. Cell lysates were resolved by SDS-PAGE and analyzed in immunoblots probed separately, as indicated, using specific antibodies directed against Ser¹¹⁶-phospho-eNOS, Ser¹¹⁷⁹-phospho-eNOS, phospho-Akt, and phospho-ERK1/2 as well as total eNOS. *B* shows a similarly designed experiment in which cells were pretreated with the protein kinase C inhibitor calphostin C (1 μ M) for 30 min and then treated for 15 min with VEGF or vehicle as indicated, and then the cells were analyzed in immunoblots probed with antibodies directed against Ser¹¹⁶-phospho-eNOS, phospho-Akt, phospho-protein kinase C (*p*-PKC), or total ERK1/2. The results shown are representative of three independent experiments that yielded similar results.

has been identified as a potent activator of the phosphoprotein phosphatase calcineurin in endothelial cells (15). Treatment of endothelial cells with VEGF promotes the dephosphorylation of the transcription factor NF-AT; this response is blocked by cyclosporin A (11). The major phosphoprotein phosphatase involved in NF-AT dephosphorylation is the Ca²⁺/calmodulin-dependent enzyme calcineurin, a phosphatase that is inhibited by immunosuppressant drugs such as cyclosporin A and FK506. The arterial hypertension that accompanies the systemic administration of cyclosporin A represents an important limitation to the use of this clinically important immunosuppressant drug. Our results provide supporting evidence for the hypothesis that cyclosporin A-induced hypertension involves, at least in part, the attenuation of endothelium-derived NO production through a calcineurin-sensitive pathway involving eNOS phosphorylation.

A role for cyclosporin and FK506 in nitric-oxide synthase regulation is consistent with observations reported in several prior studies, although the molecular mechanisms remain incompletely understood. Dawson *et al.* (22) reported that FK506 treatment of cells transfected with cDNA encoding the neuronal isoform of nitric-oxide synthase (nNOS) led to enhanced nNOS phosphorylation and to the inhibition of nNOS enzyme activity. Animal models of cyclosporin treatment have suggested that the drug attenuates endothelium-dependent vasorelaxation (13, 14), suggesting a connection between cyclo-

sporin and eNOS inhibition. More recently, Harris *et al.* (5) found that cyclosporin blocks the bradykinin-induced dephosphorylation of eNOS at threonine 497, associated with a reduction in bradykinin-induced eNOS activation. This finding stands in direct contrast to the results of Fleming *et al.* (7), who found no effect of cyclosporin on bradykinin-induced eNOS activation. Furthermore, a recent study that implicated protein phosphatase PP1, rather than calcineurin, as the relevant phosphatase at phosphothreonine 497 (6). Thus, the role of calcineurin-modulated pathways in regulating eNOS dephosphorylation at threonine 497 remains controversial. In the present studies, we did not see any effect of bradykinin on eNOS dephosphorylation at serine 116; conversely, VEGF did not affect phosphorylation at threonine 497. Therefore, the principal locus of inhibitory effect of cyclosporin on VEGF-induced eNOS activation appears to reflect the ability of the drug to inhibit VEGF-induced serine 116 dephosphorylation. Indeed, it is possible that the robust activation of eNOS elicited by VEGF reflects its additive effect to promote serine 116 dephosphorylation, a response not elicited by other eNOS agonists such as bradykinin and sphingosine 1-phosphate, which are less efficacious activators of eNOS in BAEC (23). It is becoming clear that different eNOS agonists activate distinct protein kinase pathways and differentially modulate eNOS phosphorylation at specific sites (23). Seen in this context, the converse also appears to be true, that different agonists may also promote the activation of distinct phosphoprotein phosphatases that differentially modulate eNOS dephosphorylation at discrete sites on the enzyme.

The time course of VEGF-induced eNOS dephosphorylation is considerably slower than the transient increase in intracellular Ca²⁺ and the rapid activation of eNOS enzyme activity seen in response to this agonist (16). Moreover, the dephosphorylation of eNOS at serine 116 lags behind the time course of VEGF-induced eNOS phosphorylation at serine 1179. It is plausible that the dephosphorylation of eNOS at serine 116 represents a longer term control mechanism for establishing the level of eNOS activity rather than reflecting a pathway involved in rapid responses to extracellular signals. The mechanisms whereby serine 116 phosphorylation attenuates eNOS enzyme activity remain to be determined. Some clues may be derived from inspecting the crystal structure of eNOS (9) in the vicinity of serine 116; this region includes the sites for Zn²⁺ ligation (cysteine 101) and pterin binding (serine 104) and also forms part of the eNOS homodimer interface. Which, if any, of these eNOS structural features are perturbed by the phosphorylation of serine 116 remains to be determined in enzymological studies of the purified enzyme.

It should be emphasized that the fact that cyclosporin inhibits VEGF-induced eNOS serine 116 dephosphorylation does not establish that calcineurin directly dephosphorylates eNOS any more than the inhibitory effects of calphostin on serine 116 phosphorylation establish protein kinase C as the enzyme directly responsible for eNOS phosphorylation at this site. As shown in Fig. 5, we found that calphostin, a protein kinase C inhibitor, also blocks phosphorylation of eNOS at serine 1179, an observation more consistent with a role for protein kinase C in kinase Akt activation (Fig. 5B) than with the hypothesis that the protein kinase C itself directly modifies this eNOS residue. Thus, we may only interpret the effects of the protein kinase C inhibitor calphostin and the calcineurin inhibitor cyclosporin on phosphorylation of serine 116 as providing evidence that protein kinase C- and calcineurin-modulated pathways importantly influence eNOS phosphorylation at this residue. Indeed, the amino acid sequence surrounding serine 116 does not represent a particularly robust consensus sequence either for pro-

tein kinase C-induced phosphorylation or for calcineurin-promoted dephosphorylation. A broadly based experimental approach will be required in order to identify the specific molecular targets that are modulated by protein kinase C and calcineurin in endothelial cells.

The major findings of this study are that VEGF, a key eNOS agonist, promotes the dephosphorylation of eNOS at serine 116, that this VEGF response is blocked by cyclosporin A, and that serine 116 phosphorylation is associated with eNOS inhibition. These observations may have interesting implications for our understanding of the hypertensive effects of systemic cyclosporin treatment. The level of eNOS phosphorylation at serine 116 may represent an important determinant of vascular tone, and the dynamic modulation of kinase and phosphatase pathways that regulate phosphorylation at this site may influence NO-dependent signaling pathways in the vascular wall. A deeper understanding of calcineurin-modulated signaling pathways in the vascular endothelium may lead to the identification of points for pharmacological intervention that might mitigate the dose-limiting hypertension seen with administration of the widely used immunosuppressant cyclosporin A.

Acknowledgments—We thank Drs. Lisa Robinson and Prakash Prabhakar for contributions in some of the early studies of serine 116 phosphorylation and Dr. Junsuke Igarashi for a critical reading of the manuscript and many helpful discussions.

REFERENCES

- Loscalzo, J., and Welch, G. (1995) *Prog. Cardiovasc. Dis.* **38**, 87–104
- Gallis, B., Corthals, G. L., Goodlett, D. R., Ueba, H., Kim, F., Presnell, S. R., Figeys, D., Harrison, D. G., Berk, B. C., Aebbersold, R., and Corson, M. A. (1999) *J. Biol. Chem.* **274**, 30101–30108
- Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *Nature* **399**, 597–601
- Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) *Nature* **399**, 601–605
- Harris, M. B., Hong Ju, H., Venema, V. J., Liang, H., Zou, R., Michell, B. J., Chen, Z., Kemp, B. E., and Venema, R. C. (2001) *J. Biol. Chem.* **276**, 16587–16591
- Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D. A., Sim, A. T., and Kemp, B. E. (2001) *J. Biol. Chem.* **276**, 17625–17628
- Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E., and Busse, R. (2001) *Circ. Res.* **88**, E68–R75
- Chen, Z. P., Mitchellhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., and Kemp, B. E. (1999) *FEBS Lett.* **443**, 285–289
- Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998) *Cell* **95**, 939–950
- Graef, I. A., Chen, F., Chen, L., Kuo, A., and Crabtree, G. R. (2001) *Cell* **105**, 863–875
- Horsley, V., and Pavlath, G. K. (2002) *J. Cell Biol.* **156**, 771–774
- Cifkova, R., and Hallen, H. (2001) *J. Hypertens.* **19**, 2283–2285
- Cairns, H. S., Fairbanks, L. D., Westwick, J., and Neild, G. H. (1989) *Br. J. Pharmacol.* **98**, 463–468
- Auch-Schwellk, W., Bossaller, C., Gotze, S., Thelen, J., and Fleck, E. (1993) *J. Cardiovasc. Pharmacol.* **21**, 435–440
- Armesilla, A. L., Lorenzo, E., Gomez del Arco, P., Martinez-Martinez, S., Alfranca, A., and Redondo, J. M. (1999) *Mol. Cell. Biol.* **19**, 2032–2043
- He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B., and Caldwell, R. B. (1999) *J. Biol. Chem.* **274**, 25130–25135
- Guo, D., Jia, Q., Song, H. Y., Warren, R. S., and Donner, D. B. (1995) *J. Biol. Chem.* **270**, 6729–6733
- Robinson, L. J., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11776–11780
- Igarashi, J., Bernier, S. G., and Michel, T. (2001) *J. Biol. Chem.* **276**, 12420–12426
- Verheul, H. M., Jorna, A. S., Hoekman, K., Broxterman, H. J., Gebbink, M. F., and Pinedo, H. M. (2000) *Blood* **96**, 4216–4221
- Petrova, T. V., Makinen, T., and Alitalo, K. (1999) *Exp. Cell Res.* **253**, 117–130
- Dawson, T. M., Steiner, J. P., Dawson, V. L., Dinerman, J. L., Uhl, G. R., and Snyder, S. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 9808–9812
- Igarashi, J., and Michel, T. (2001) *J. Biol. Chem.* **276**, 36281–36288