The Akt kinase signals directly to endothelial nitric oxide synthase

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Endothelial nitric oxide synthase (eNOS) is an important modulator of angiogenesis and vascular tone [1]. It is stimulated by treatment of endothelial cells in a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent fashion by insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) [2,3] and is activated by phosphorylation at Ser1177 in the sequence RIRTQS^{1177}F (in the single-letter amino acid code) [4]. The protein kinase Akt is an important downstream target of PI 3-kinase [5,6], regulating VEGF-stimulated endothelial cell survival [7]. Akt phosphorylates substrates within a defined motif [8], which is present in the sequence surrounding Ser1177 in eNOS. Both Akt [5,6] and eNOS [9] are localized to, and activated at, the plasma membrane. We found that purified Akt phosphorylated cardiac eNOS at Ser1177, resulting in activation of eNOS. Phosphorylation at this site was stimulated by treatment of bovine aortic endothelial cells (BAECs) with VEGF or IGF-1, and Akt was activated in parallel. Preincubation with wortmannin, an inhibitor of Akt signalling, reduced VEGF- or IGF-1-induced Akt activity and eNOS phosphorylation. Akt was detected in immunoprecipitates of eNOS from BAECs, and eNOS in immunoprecipitates of Akt, indicating that the two enzymes associate *in vivo*. It is thus apparent that Akt directly activates eNOS in endothelial cells. These results strongly suggest that Akt has an important role in the regulation of normal angiogenesis and raise the possibility that the enhanced activity of this kinase that occurs in carcinomas may contribute to tumor vascularization and survival.

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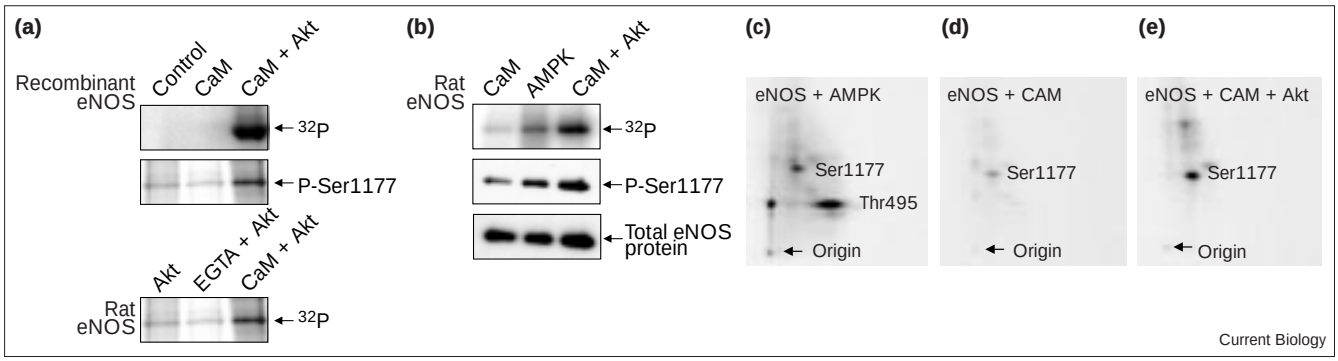
Results and discussion

Angiogenesis is impaired in eNOS knockout mice following ischemic challenge and is not restored by VEGF treatment, indicating that eNOS lies downstream of VEGF [1]. Release of NO stimulated by treatment of endothelial cells with VEGF or IGF-1 is approximately halved by the PI 3-kinase inhibitors wortmannin and LY2940002 [2,3], but the mechanism of PI 3-kinase-mediated activation of eNOS has not been characterized. We previously observed that the AMP-activated protein kinase (AMPK) catalyzes phosphorylation of eNOS on Ser1177 resulting in activation of the enzyme [4]. The sequence surrounding Ser1177 closely resembles the consensus Akt substrate-recognition motif identified using synthetic peptides Arg-X-Arg-Z-Z-Ser/Thr-Hyd (where X is any amino acid, Z is a small residue other than glycine and Hyd is a bulky hydrophobic residue) [8]. This raises the possibility that Akt may directly phosphorylate and activate eNOS. We found that recombinant eNOS, co-expressed in bacteria with calmodulin (CaM) to maintain its stability, was readily phosphorylated by purified Akt conjugated to glutathione-S-transferase (GST–Akt), as was partially purified rat heart eNOS (Figure 1a). Rat heart eNOS was used for all subsequent experiments because it is myristoylated and palmitoylated [4] and therefore localized to the plasma membrane. This form of eNOS contains some associated CaM and thus Akt catalyzes a low level of eNOS phosphorylation, which is significantly reduced by removal of CaM with EGTA and stimulated more than 10-fold by the addition of Ca^{2+}–CaM (Figure 1a, lower panel and 1b). Binding of Ca^{2+}–CaM to eNOS has been proposed to induce a conformational change in eNOS, allowing phosphorylation at Ser1177 [4]. Under these conditions, the stoichiometry of phosphorylation was estimated to be approximately 50%, from Coomassie blue staining of the eNOS preparation following SDS–PAGE (data not shown).

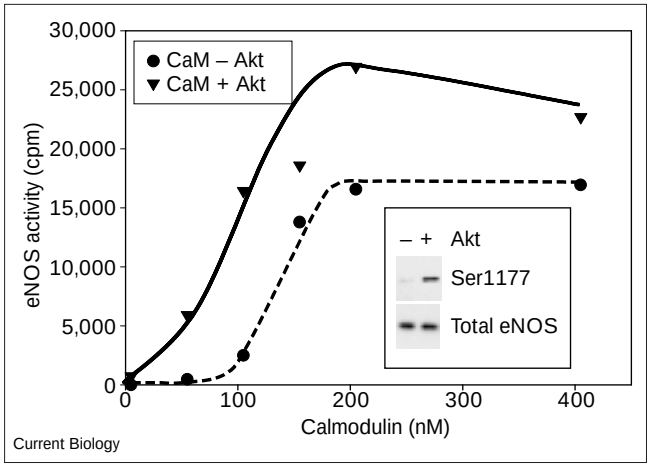
In parallel, eNOS was phosphorylated by AMPK in the absence of Ca^{2+}–CaM, a condition that favours phosphorylation at an inhibitory site, Thr495, as well as at Ser1177 [4]. Immunoblotting with a phospho-Ser1177-specific antibody revealed that both kinases catalyzed phosphorylation at this site (Figure 1b). To further characterize the specificity of these reactions, the phosphorylated eNOS was analyzed by two-dimensional tryptic phosphopeptide mapping (Figure 1c–e). AMPK phosphorylated eNOS at Ser1177 and Thr495, as previously described [4]. Phosphorylation by Akt was largely confined to Ser1177, as shown by comparison of the phosphopeptide maps following

Figure 1



Phosphorylation of eNOS by Akt and AMPK. The active GST–Akt fusion protein was purified by glutathione–Sepharose chromatography of extracts from transiently transfected HEK 293 cells [17] treated with 100 μM pervanadate as described previously for fibroblasts [18]. AMPK and recombinant and rat heart eNOS were purified as described previously [4,19]. Phosphorylation of eNOS (30 nM) was carried out in the presence or absence of GST–Akt (Akt), AMPK, 100 μM $CaCl_2$ with 500 nM CaM and/or 1 mM EGTA as indicated, in the presence of kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 0.05% Triton X-100) containing 20 μM [γ-^{32}P] ATP (10,000 cpm/pmol). After a 30 min incubation at 22°C, the reactions were stopped with SDS–PAGE sample buffer and subjected to SDS–PAGE on a 10% slab gel. (a,b) Proteins were transferred onto a polyvinyl difluoride (PVDF) membrane. Phosphorylated eNOS (^{32}P) was visualized by Phosphorimage analysis and the phosphorylation at Ser1177 and total eNOS were detected by immunoblot analysis. To detect phosphorylation of Ser1177, blots were probed with anti-phospho-eNOS antibodies directed to RIRTQSpFSLQER (Ser1177) [4]. The top and middle panels in (a) refer to the recombinant eNOS and are labeled at the top of the figure. The control lane is phosphorylated in the absence of added CaM or Akt. The other panels in (a,b) refer to rat heart eNOS. (c–e) eNOS gel bands from duplicate reactions were excised and digested with trypsin. Phosphopeptides were isolated from in-gel tryptic digests and phosphopeptide mapped as described previously [20].

Figure 2



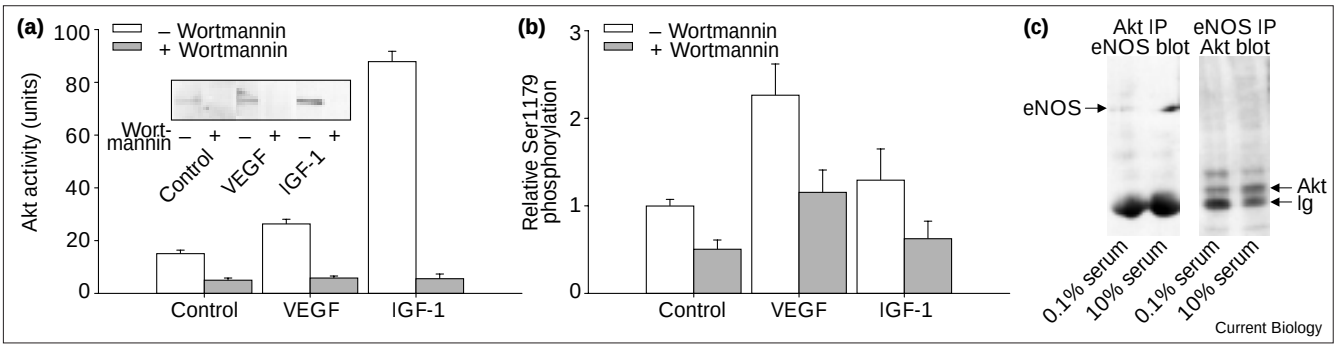
Effect of Akt-catalyzed phosphorylation on eNOS activity. Phosphorylation assays were performed in kinase assay buffer in the presence of 200 μM ATP, 2 μM $CaCl_2$ and 500 nM CaM with or without Akt. After phosphorylation, eNOS was diluted 1:100 and the change in eNOS activity was determined by monitoring the eNOS-catalyzed conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as a function of the CaM concentration [4]. The inset shows the relative amounts of eNOS phosphorylated at Ser1177, determined by immunoblot analysis. Blots were probed with anti-phospho-Ser1177 antibody and then stripped and reprobed for total eNOS to adjust for variations in protein recovery and gel loading. Re-plotting of this data with eNOS activity expressed as a percentage of the maximum achieved in either the presence or the absence of Akt also revealed that phosphorylation enhances eNOS sensitivity to CaM (see Supplementary material).

phosphorylation by the two kinases (Figure 1c,e). This determination was confirmed by phosphate-release analysis, which showed phosphorylation at cycle 3 during amino-terminal Edman degradation of the peptide TQS*FSLQER (data not shown). In the absence of Ca^{2+}–CaM, Akt-mediated phosphorylation of Ser1177 proceeded at a significantly slower rate and no Thr495 phosphorylation was observed (data not shown).

Akt phosphorylates and activates eNOS, rendering the enzyme 40% more sensitive to CaM (changing the relative $K_{0.5}$, the concentration of added CaM required for half-maximal enzyme activity read from the dose-response curves, from 135 to 85 nM) and increasing the maximum activity (V_{max}) by 50% (Figure 2). Immunoblotting confirmed that this activation was associated with phosphorylation of Ser1177. Phosphorylation at this site complemented and enhanced the regulation of eNOS by Ca^{2+}–CaM, allowing increased NO production at lower intracellular Ca^{2+} concentrations. We have proposed previously that this was due to phosphorylation-induced relief of an autoinhibitory function of the carboxyl terminus of eNOS [4]; supporting this hypothesis, truncation mutagenesis at Ser1177 renders eNOS constitutively active (B.J.M., I.R-C., P.R. O. de M. and B.E.K., unpublished observations).

To assess the relationship between Akt and phosphorylation of eNOS *in vivo*, we examined extracts from BAECs treated with VEGF or IGF-1 with and without pretreatment

Figure 3



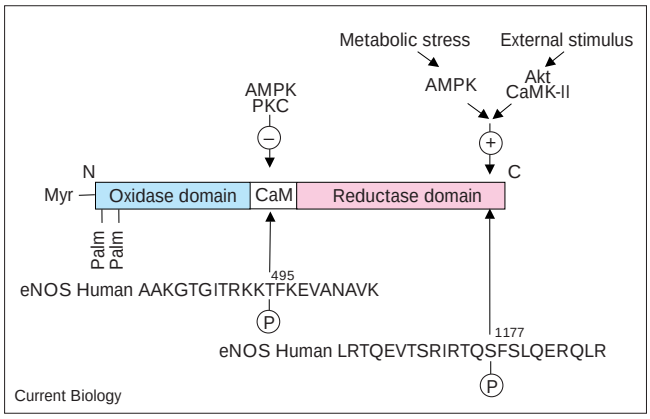
Regulation of Akt activity and phosphorylation of eNOS in endothelial cells. BAECs were maintained in culture in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS). Cells were serum-starved in DME containing 0.1% FBS for 20 h before experimental treatment (control cells). BAECs were incubated with 50 ng/ml VEGF for 5 min or 50 ng/ml IGF-1 for 15 min, with or without 200 nM wortmannin pretreatment for 30 min, as indicated. The different times of treatment with VEGF and IGF-1 were chosen from time-course experiments of eNOS activation (see Supplementary material). After experimental treatment, BAECs were washed twice with PBS pH 7.4, harvested on ice in 0.8 ml lysis buffer containing 20 mM Hepes, pH 7.4, 2 mM EDTA, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM dithiothreitol (DTT), 2 μg/ml aprotinin, 2 μg/ml leupeptin, 10 μg/ml soya bean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (w/v) NP40 and frozen in liquid nitrogen. Thawed lysates were then clarified by centrifugation at 20,000 × *g* for 5 min at 4°C. (a) Endogenous Akt activity was assayed using the specific peptide substrate RPRAATF [8]. Lysate (5 μl) was incubated for 10 min at 30°C in 25 mM Tris pH 7.5, 0.5 mM DTT, 0.5 mM benzamidine, 50 μM [γ-^{32}P]ATP (10,000 cpm/pmol), 10 mM $MgCl_2$, 150 μM peptide substrate. The incubation was terminated by addition of trichloroacetic acid to 10% (w/v). Following 20 min on ice precipitated protein was pelleted by centrifugation for 20 min at 13,000 × *g*. The supernatant containing substrate peptide was spotted onto P81 paper and radioactive inorganic phosphate ($^{32}P_i$) incorporated into peptide was quantitated as described previously [18]. The units of activity are pmol P_i transferred per minute per mg protein. These results were confirmed by immunoblotting with Akt-phospho-Ser473-specific antibody (New England Biolabs) following SDS–PAGE of 20 μg of the relevant lysates on a 10% slab gel (inset). Phosphorylation at this site reflects the activity state of the enzyme [14]. (b) The eNOS was isolated from extracts by ADP–Sepharose chromatography. The extracts were mixed with 50 μl ADP–Sepharose resin for 1–2 h and the beads were washed in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol, 0.1% Triton X-100. The relative amounts of phosphorylated Ser1179 were determined by immunoblot analysis as described in Figure 2 and expressed as mean ± standard error (n = 4). (c) BAECs either maintained in 10% FBS or serum-starved in DME containing 0.1% FBS for 20 h were harvested as above. Extracts of one 10 cm plate of 60% confluent cells were immunoprecipitated with antisera raised to the carboxy-terminal 16 residues of Akt or with the anti-eNOS monoclonal antibody 1030–1209 (Transduction Laboratories). Immunoprecipitates (IP) of either Akt or eNOS were washed extensively in PBS containing 2% Triton X-100, subjected to SDS–PAGE on a 10% slab gel and immunoblotted with the reciprocal antibody as indicated (blot). Ig indicates immunoglobulin.

with the PI-3-kinase inhibitor wortmannin (Figure 3a,b). Akt was stimulated by each growth factor and this activation was strongly inhibited by wortmannin. Concomitant phosphorylation of eNOS at Ser1179 (equivalent to Ser1177 in rat eNOS) was seen in each case but, in keeping with previous data, this phosphorylation was only partially wortmannin sensitive. The eNOS and Akt proteins co-immunoprecipitated from both starved and serum-supplemented BAECs (Figure 3c), indicating a direct interaction of the two enzymes *in vivo*. Treatment of BAECs with rapamycin, a specific inhibitor of p70 S6 kinase, the other major downstream target of PI 3-kinase, had no effect on IGF-1-induced phosphorylation of eNOS at Ser1179 (see Supplementary material). It is not possible to rule out some involvement of two other PI 3-kinase effectors, serum and glucocorticoid-inducible kinase (SGK) and the atypical protein kinase Cs (PKCs), although stimulation of PKCζ with phorbol ester does not result in phosphorylation of Ser1179 (B.J.M. and B.E.K., unpublished observations).

We anticipated that there would be PI-3-kinase-independent pathways capable of activating eNOS because of the partial wortmannin insensitivity of Ser1179 phosphorylation. It is likely that these pathways will, at least in part, involve signaling via Ca^{2+}–CaM. Ca^{2+}–CaM-dependent eNOS activity and phosphorylation is detected in the absence of added kinase (Figure 2 and data not shown) and eNOS is activated by treatment of endothelial cells with the calcium ionophore ionomycin and inactivated by Ca^{2+} chelation and/or calmodulin antagonism [3]. Furthermore, the motif surrounding Ser1179 contains the Arg-X-X-Ser-Hyd sequence motif favored by CaM-dependent protein kinase II (CaMK-II; [10]). Interestingly, although IGF-1 is a more potent activator of Akt, VEGF promotes greater phosphorylation of eNOS on Ser1179. One interpretation of these data is that VEGF signals more strongly than IGF-1 by an alternative pathway, perhaps by increasing intracellular Ca^{2+} through the activation of phospholipase Cγ [11].

We have shown that Akt phosphorylates and activates eNOS, indicating that Akt has an important role in the regulation of angiogenesis and maintenance of vascular tone [1,9]. Note that, during the revision stage of this

Figure 4



Regulation of eNOS by phosphorylation at Thr495 and Ser1177. Metabolic stress (AMPK) or growth factor receptor (Akt) mediated signal transduction pathways target eNOS. A number of protein kinases including Akt, AMPK [4], PKC (B.J.M., K.I.M. and B.E.K., unpublished observations and [21]) and CaM-K-II [22] cause activation or inhibition of eNOS as indicated by a plus or minus sign, respectively, by phosphorylating Ser1177 or Thr495. Abbreviations: Palm, palmitoylation; Myr, myristoylation; CaM, CaM-binding domain; N, amino terminus; C, carboxyl terminus.

manuscript, two other groups have shown direct activation of eNOS by Akt [12,13]. This role is distinct from its well-documented roles in glucose metabolism involving phosphorylation of glycogen synthase kinase 3 [14] and cell survival and apoptosis involving phosphorylation of Bad, procaspase 9 and a forkhead transcription factor [15,16]. The deregulation of the Akt pathway, especially through defects in the tumor suppressor PTEN, is implicated in the generation of many human cancers [15]. Thus, in addition to disrupting cell-death pathways, tumor cells may exploit the activation of Akt to enhance vascularization and tumor survival. It is significant that the AMP-activated protein kinase, which senses metabolic stress, also activates eNOS by phosphorylation at Ser1177/1179. Tumors experience metabolic stress, which also promotes neovascularization. It is apparent that Ser1177/1179 in eNOS is a major intersection point of regulatory pathways for growth factors, metabolic stress and calcium (Figure 4).

Supplementary material

Supplementary material including a graph of Ser1179 phosphorylation in BAECs and a time course of eNOS activation is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

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Supplementary material

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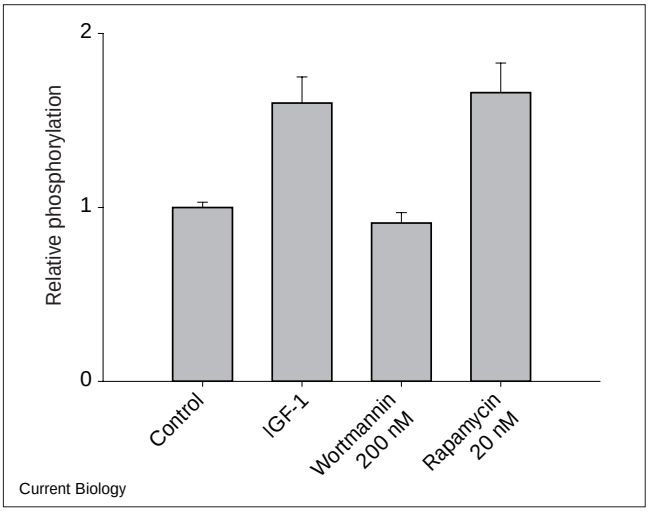
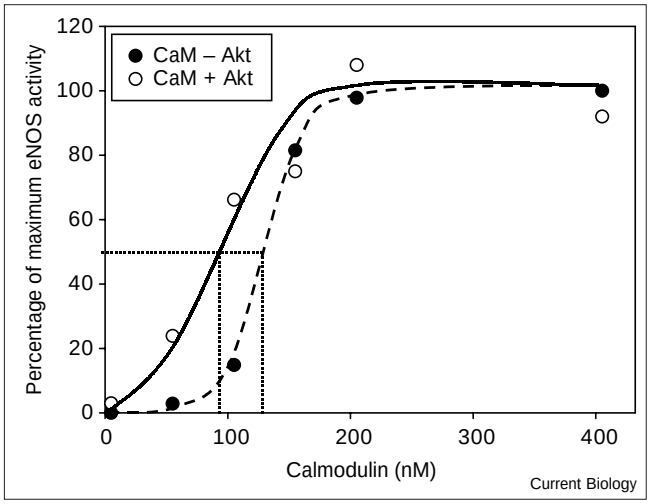
Figure S1



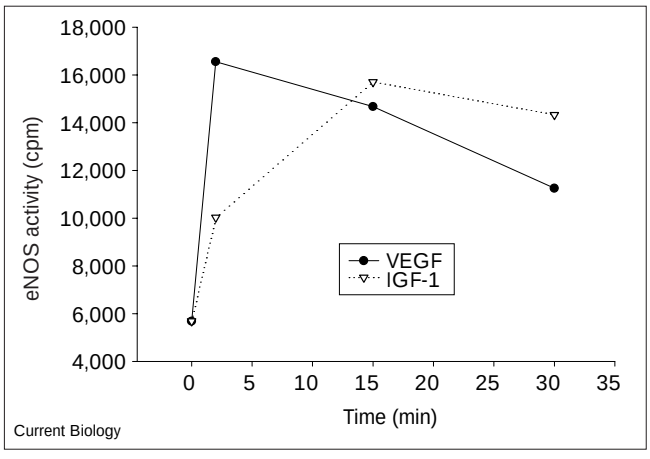
Ser1179 phosphorylation in BAECs. BAECs were serum-starved in DME containing 0.1% FBS for 20 h before incubation with 50 ng/ml IGF-1 for 15 min, with or without pretreatment for 30 min with 200 nM wortmannin, or 20 nM rapamycin, as indicated. After experimental treatment, cells were washed and harvested as described in Figure 3. eNOS was isolated from extracts by ADP–Sepharose chromatography as described in Figure 3b and the relative amounts of phosphorylated Ser1179 were determined by immunoblot analysis as described in Figure 2 and expressed as mean ± standard error (n = 4).

Figure S2



Effect of Akt-catalyzed phosphorylation on eNOS activity. Phosphorylation assays and eNOS activity determination were carried out as described in Figure 2. The eNOS activity is expressed as percentage of the maximum achieved in either the presence or the absence of Akt. The $K_{0.5}$ values for added CaM are 90 nM and 130 nM, respectively.

Figure S3



Time course of eNOS activation. Serum-starved BAECs were treated with 50 ng/ml VEGF or IGF-1 for the time periods indicated. eNOS was isolated from the clarified lysates as described in Figure 3 and its activity was determined as described in Figure 2.