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Research Article

The dual role of endothelial differentiation-related factor-1 in the cytosol and nucleus: modulation by protein kinase A

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Abstract. Endothelial differentiation-related factor (EDF)-1 is involved in the repression of endothelial cell differentiation and is the first studied calmodulin (CaM)-binding protein in endothelial cells. Here we report that (i) EDF-1 is in vitro and in vivo phosphorylated by protein kinase A (PKA); (ii) EDF-1/CaM interaction is modulated by the phosphorylation of EDF-1 by PKA; (iii) forskolin stimulates nuclear accumulation of EDF-1, and (iv) PKA phosphorylation enhances EDF-1 interaction with the TATA-binding protein. CaM modulates the activity of several enzymes, among which is nitric oxide synthase (NOS). EDF-1, but not phosphorylated EDF-1, inhibits the activity of NOS. Accordingly, we detected an increase in NOS activity in cells that express low amounts of EDF-1. Our results indicate that EDF-1 serves two main functions in endothelial cells: (i) it regulates CaM availability in the cytosol, and (ii) it acts in the nucleus as a transcriptional coactivator.

Key words. Endothelium; EDF-1/MBF-1; differentiation.

New blood vessels develop from pre-existing vessels [1] or from circulating endothelial progenitor cells [2] in response to angiogenic factors. Angiogenesis promotes development, wound healing and the female reproductive cycle as well as solid tumor growth and metastatization, rheumatoid arthritis and neovascular eye diseases [1]. While a notable amount of information is available about the mechanisms of endothelial growth, less is known about the differentiation of endothelial cells, a pivotal step in the formation of functional vessels.

Endothelial differentiation-related factor-1 (EDF-1), a novel 16-kDa polypeptide, has been identified as an endothelial differentiation suppressor protein [3]. EDF-1 is a member of the calmodulin (CaM)-binding proteins, and is the first of this family to be studied with such a function in endothelial cells [4]. CaM-binding proteins share a loosely conserved region of about 20 amino acids, designated the IQ motif, which contains a CaM-binding domain and, in some istances, one or more protein kinase (PKC or PKA) phosphorylation site(s) [5]. The IQ motif was originally identified in neuromodulin, which concentrates CaM at specific sites in neurons [6]. Since then, several other proteins have been shown to possess this motif. CaM, which is the classical Ca^{2+} receptor protein inside cells, mediates calcium regulation of a number of enzymes such as adenyl cyclases, kinases and phosphatases which are important components of signal transduction systems implicated in cell cycle progression and cytoskeletal rearrangement [7]. In endothelial cells, we have demonstrated that EDF-1/CaM interaction is tightly regulated by the levels of Ca^{2+} and the activation of PKC both in vitro and in vivo. In addition, we have described a dramatic increase in nuclear-associated EDF-1 in response to the phorbol ester TPA. EDF-1 is highly homologous to multiprotein bridging factor (MBF)-1 [8], a tran-

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scriptional coactivator in *Bombyx mori*. EDF-1 (also denominated human MBF-1) has recently been shown to serve as a cofactor for non-steroid nuclear receptors involved in lipid metabolism [9], among which is PPAR-gamma, also implicated in cell differentiation and in the modulation of the angiogenic process [10]. We have previously shown that native and PKC-phosporylated EDF-1 interact with the TATA-binding protein (TBP) to a similar extent [4].

Here, we show that phosphorylation by PKA disrupts EDF-1/CaM interaction and stimulates EDF-1 accumulation in the nucleus where its binding to TBP is enhanced. Since nitric oxide synthase (NOS) is a CaM-dependent enzyme, we also provide evidence that the phosphorylation of EDF-1 correlates with the activation of NOS in endothelial cells.

Materials and methods

Cell culture

HUVECs were cultured as described elsewhere [3]. HUVECs were transiently transfected with an EDF-1 antisense cDNA using lipofectin according to the manufacturer's instructions.

Cloning of the antisense cDNA

Antisense EDF-1 was generated by PCR on human cDNA using the following oligomers: 5′-GGGTCGACATGGCCGAGAGCG-3′; 5′-CTAGGTACCCAAGGGGAACCGGCGGAA-3′. The PCR product was cloned into a pCI vector (Promega), in the *Kpn*I/*Sal*I sites, and sequenced. The cDNA was analyzed in the NCBI gene bank and shown to be specific.

Immunofluorescence staining

HUVECs were seeded on gelatin-coated coverslips. When subconfluent, cells were treated with forskolin (100 nM) or TPA (50 nM), washed and fixed in PBS containing 3% paraformaldehyde and 2% sucrose. After extensive washing, cells were permeabilized with Hepes-Triton, incubated with anti-EDF-1 immunopurified IgGs and stained with TRITC-labeled swine immunoglobulins against goat [4]. Staining with mouse or rabbit non-immune IgGs did not yield any significant signal.

Site-directed mutagenesis

EDF-$1_{S87\rightarrow D}$ was generated using the following primers: 5′-GGT CGGCAGGACAAGGGGCTTACG-3′; 5′-AAGCCCCTTGTCCTGCCGACCTTG-3′. The EDF-1_{D3} was generated using EDF-$1_{S87\rightarrow D}$ as template with the following oligomers: 5′-CAGATCCACCCTGTCATGGTGCT CCTCGTCCTC-3′; 5′-GAGGACGAGGAGCTGCACCATGACAGGGTGGATCTG-3′. The external primers used to generate restriction sites were: 5′-CTAGGTACCCAAGGGGAACCGGCGAAC-3′; 5′-CTAGGATCCGCCATGGCC GAGAGCGAC-3′. PCR was performed as described previously [4]. Mutations were confirmed by sequencing. The mutants as well as the wild-type protein were purified as described elsewhere [4].

Western blot and immunoprecipitation

Samples were resolved by SDS-PAGE, transferred to nitrocellulose sheets at 150 mA for 16 h, and probed with anti-EDF-1 immunopurified IgGs (0.1 µg/ml). Secondary antibodies were labeled with horseradish peroxidase (Amersham). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins [3]. To coimmunoprecipitate EDF-1 and other interacting proteins, HUVECs were lysed in ice-cold RIPA buffer and immunoprecipitated with anti-CaM (Santa Cruz) or anti-EDF-1 IgGs. The immunocomplexes were bound to protein A-Sepharose, washed, eluted in Laemmli buffer at 95 °C for 5 min and separated on SDS-PAGE. After transfer onto nitrocellulose, Western blot was performed as described above using anti-EDF-1 immunopurified IgGs, anti-TBP, or anti-CaM antibodies. All the results shown were reproduced at least four times. In some experiments, anti-actin or anti-histone antibodies (Santa Cruz) were used.

Anti-endothelial NOS antibodies were from Santa Cruz.

In vitro and in vivo phosphorylation

Recombinant EDF-1 was phosphorylated at 28 °C for 30 min by incubation with 10 µg/ml PKA, 5 µCi γ^{32}P-ATP, 25 mM HEPES-NaOH, pH 7.3, at 25 °C. The reaction was terminated by the addition of Laemmli sample buffer. The samples were run on 12% SDS-PAGE. Dried gels were exposed for autoradiography. Subconfluent HUVECs were washed twice with DMEM without phosphate and incubated for 45 min in the same medium. The medium was replaced and the cells incubated for 3 h at 37 °C with phosphate-free DMEM containing 50 µCi/ml carrier-free ^{32}P to label the endogenous ATP pool [4]. Then, forskolin (100 nM) was added for 15 min. The reaction was stopped by removing the medium, and rapidly washing with ice-cold PBS. The cells were lysed and immunoprecipitated with anti-EDF-1 antibodies as described above. Cell fractionation was performed as described previously [8]. The experiments were repeated three times with reproducible results.

Binding to CaM-agarose

EDF-1 and its mutants were loaded on a CaM-agarose (Pharmacia) column and extensively washed with a buffer containing 20 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1% NP40, and 5 mM EGTA. Elution was performed in 20 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1% NP40, and 2 mM $CaCl_2$. Eluates were resolved on SDS-PAGE and processed for Western blot using anti-EDF-1

IgGs as described [4]. EDF-1 mutants were incubated with CaM-agarose in the aforementioned buffer, washed, centrifuged and eluted with Laemmli buffer to be analyzed by Western blot.

In vitro EDF-1/TBP interaction

EDF-1, EDF-$1_{S87\rightarrow D}$, EDF-1_{D3}, and EDF-1_{D4} (100 ng/ml), and TBP (30 ng/ml, Promega) were incubated for 30 min on ice in a buffer containing 20 mM HEPES-KOH (pH 7.9), 20% glycerol, 0.5 mM EDTA, 60 mM $MgCl_2$, 0.1% NP40, 5 mM 2-mercaptoethanol, and 1 mM PMSF. The samples were then immunoprecipitated with a monoclonal antibody against TBP (Santa Cruz) and blotted with anti-EDF-1 IgGs [4]. The experiment was repeated three times with reproducible results.

NOS activity

NOS activity was measured by monitoring the conversion of ^{3}H-arginine to ^{3}H-citrulline as previously described [11]. Due to the unavailability of commercial recombinant e-NOS, we utilized nNOS in our studies, since this isoform is also CaM dependent. For the in vitro assay, 2 µg of NOS were incubated with different concentrations of EDF-1 or its mutants in a reaction mix (10 mM NADPH, 1 µCi/µl ^{3}H-arginine, 25 mM Tris-HCl, 3 µM BH_4, 1 µM FAD, 1 µM FMN, 100 nM CaM, and 60 µM $CaCl_2$), for 1 h at room temperature [11]. The assay was terminated by the addition of stop buffer (50 mM HEPES pH 5.5, 5 mM EDTA). One hundred microliters of pre-equilibrated resin (DOWEX 50WX8 200–400 mesh, H form; Sigma) were added to each sample, shaken, and centrifuged. The flow-through was collected and the radioactivity was quantified in a scintillation counter. An in vivo assay was performed on 40 µg of cell extracts using the same reaction buffer.

Results

Phosphorylation of EDF-1 by PKA

We have previously shown that PKC phosphorylates EDF-1 and this event inhibits its interaction with CaM [4]. Since EDF-1 contains three conserved PKA phosphorylation sites, one of which lies within the IQ domain, we examined whether the phosphorylation of EDF-1 by PKA affects its binding to CaM. EDF-1 was in vitro phosphorylated by PKA. Phosphorylated EDF-1 did not interact with calmodulin agarose (fig. 1A, lane 1) and the ^{32}P-labeled protein was all recovered in the flow-through fraction of the column (fig. 1A, lane 2). An equal amount of the corresponding recombinant protein was loaded on a SDS-PAGE for autoradiography (fig. 1A, lane 3). We also determined whether Ser87, which is the PKA phosphorylation site within the IQ motif, plays a role in modulating EDF-1 interaction with CaM. To this end, because

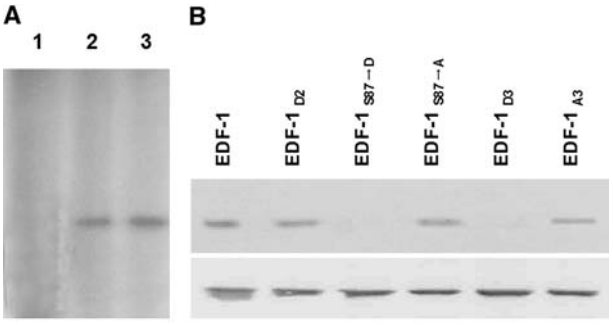

Figure 1. Phosphorylation of EDF-1 by PKA prevents its binding to CaM-agarose. (*A*) EDF-1 was in vitro phosphorylated using recombinant PKA as described (lane 3). PKA-phosphorylated EDF-1 was incubated with CaM-agarose, washed, centrifuged and the resin was eluted with $CaCl_2$ (2 mM). The flow-through (lane 2) and the elution from the resin (lane 1) were separated on SDS-PAGE and autoradiographed. (*B*) EDF-1 and its mutants EDF-1_{D2}, EDF-$1_{S87\rightarrow D}$, EDF-$1_{S87\rightarrow A}$, EDF-1_{D3}, and EDF-1_{A3} were incubated with CaM-agarose, washed, centrifuged and the resin was eluted in sample buffer. The eluates were analyzed by Western blot (upper panel). An equal amount of the corresponding recombinant proteins was loaded on a gel and processed by Western blot using anti-EDF-1 antibodies (lower panel).

the introduction of negative charges mimics a phosphorylated residue, the Ser87 → Asp mutation was introduced (EDF-$1_{S87\rightarrow D}$). We also mutated two additional putative PKA phosphorylation sites present throughout the sequence of EDF-1 into Asp, generating EDF-1_{D3}, which fully mimics PKA phosphorylation of EDF-1. Another mutant was obtained in which only the two PKA phosphorylation sites outside the IQ region (Thr65 and Thr74) were mutated to Asp (EDF-1_{D2}). While the substitution of Ser87 to Ala (EDF-$1_{S87\rightarrow A}$) did not affect EDF-1/CaM interaction, conversion of Ser87 to Asp markedly reduced the affinity of EDF-1 for CaM. Indeed, no EDF-$1_{S87\rightarrow D}$ bound CaM (fig. 1B). EDF-1_{D2} interacted with CaM as much as the wild-type protein. The substitution of the three PKA phosphorylation sites (EDF-1_{D3}) prevented binding, whereas the mutation of three sites into Ala (EDF-1_{A3}) did not affect the interaction (fig. 1B).

We evaluated whether EDF-1 is phosphorylated in vivo by metabolically labeling HUVECs with ^{32}P. Cells were exposed to forskolin (100 nM) for 15 min and immunoprecipitated with an anti-EDF-1 antibody. Figure 2A shows that EDF-1, which is unphosphorylated in untreated HUVECs, was readily phosphorylated upon exposure to forskolin. Forskolin-induced phosphorylation was completely abrogated by H89, a selective inhibitor of PKA (data not shown). To evaluate whether or not in vivo-phosphorylated EDF-1 bound to CaM, we coimmunoprecipitated EDF-1 and CaM in HUVECs exposed to forskolin or TPA (50 nM). As shown in figure 2B, a lower amount of EDF-1 coimmunoprecipitated with CaM in forskolin, as in TPA-treated cells, when compared to controls (five- and fourfold reduction, respectively).

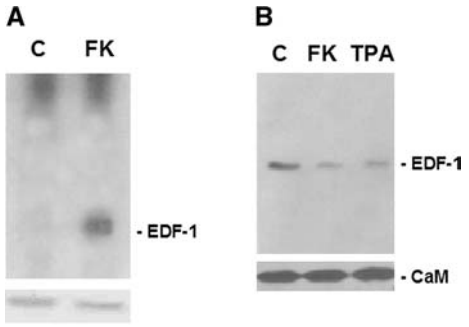

Figure 2. Forskolin stimulates the phosphorylation of EDF-1 in HUVECs. (*A*) HUVECs were metabolically labeled with 50 µCi/ml carrier-free ^{32}P. Then, forskolin (100 nM) was added for 15 min. The cells were lysed and immunoprecipitated with anti-EDF-1 IgGs. Immunoprecipitates were divided in two aliquots. One aliquot was utilized for autoradiography (upper panel) and the remaining one was used in Western analysis with anti-EDF-1 antibodies (lower panel). C, control; FK, forskolin. (*B*) HUVECs were treated with forskolin (100 nM) or TPA (50 nM) for 20 min. Cells were lysed, immunoprecipitated with a monoclonal anti-CaM antibody and Western blot was performed using anti-EDF-1 IgGs (upper panel) or an anti-CaM polyclonal antibody (lower panel).

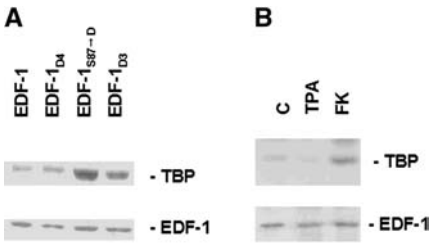

Figure 4. PKA-phosphorylated EDF-1 binds TBP. (*A*) EDF-1, EDF-$1_{S87\rightarrow D}$, EDF-1_{D3}, and EDF-1_{D4} were incubated for 30 min in the presence of TBP. The samples were then immunoprecipitated with anti-EDF-1 IgGs and blotted utilizing a monoclonal antibody against TBP (upper panel) or with anti-EDF-1 antibodies (lower panel). (*B*) Forskolin- and TPA-treated HUVEC lysates were immunoprecipitated with anti-EDF-1 IgGs and blotted with an anti-TBP monoclonal antibody. The membrane was stripped and Western blot was performed using anti-EDF-1 antibodies.

Subcellular localization of EDF-1

While localized both in the nucleus and the cytosol in control cells, EDF-1 was mainly associated with the nucleus of forskolin-treated cells as detected by immunofluorescence (fig. 3A). In TPA-treated cells, EDF-1 was detectable mainly in the nucleus, although a signal could also be observed in the cytosol. To evaluate the localization of phosphorylated EDF-1, HUVECs were metabolically labeled with ^{32}P, treated with forskolin or TPA, and cell fractionation was performed. We found that, while after PMA treatment phosphorylated EDF-1 was located both in the nucleus and the cytosol, in forskolin-treated cells, phosphorylated EDF-1 was mainly nuclear (fig. 3B).

EDF-1 interaction with TBP

EDF-1 interacts with TBP [4]. Interestingly, the binding to TBP was enhanced when we utilized the mutants EDF-1_{D3} and EDF-$1_{S87\rightarrow D}$ (5- and 3.5-fold induction, respectively), while we detected no increase in the interaction between TBP and EDF_{D4} which fully mimics PKC phosphorylation [4] (fig. 4A). We also determined whether this interaction occurred in vivo in HUVECs treated or not with forskolin (100 nM) or TPA (50 mM) for 20 min. We showed that EDF-1 and TBP co-immunoprecipitate (fig. 4B). This interaction was enhanced (2.3-fold) after activation of PKA by forskolin, while no modulation was observed in cells exposed to TPA.

Modulation of NOS activity by EDF-1

NOS is a Ca^{2+}/CaM-dependent enzyme [11]. Since EDF-1 binds CaM, we wondered whether it may affect NOS activity. We measured NOS activity in vitro in the presence of EDF-1 and its mutants. As shown in figure 5,

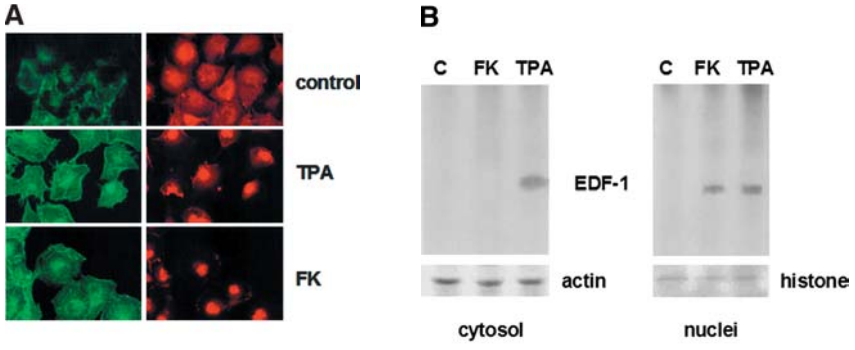

Figure 3. Cellular localization of EDF-1. (*A*) HUVECs were cultured for 48 h on gelatin-coated coverslips and then treated with forskolin (100 nM) or TPA (50 nM) for 20 min. Cells were then fixed and stained with rabbit anti-EDF-1 immunopurified antibodies, followed by rhodamine anti-rabbit IgGs. (*B*) ^{32}P-labeled HUVECs were treated with forskolin (FK, 100 nM) and TPA (50 nM) for 20 min and fractionated to separate nuclei and cytosols. An aliquot was used for autoradiography (upper panel). The other aliquot was processed by Western blot using anti-actin antibodies for the cytosol and anti-histone for the nuclei to confirm that the same amounts of protein had been loaded per lane (lower panel).

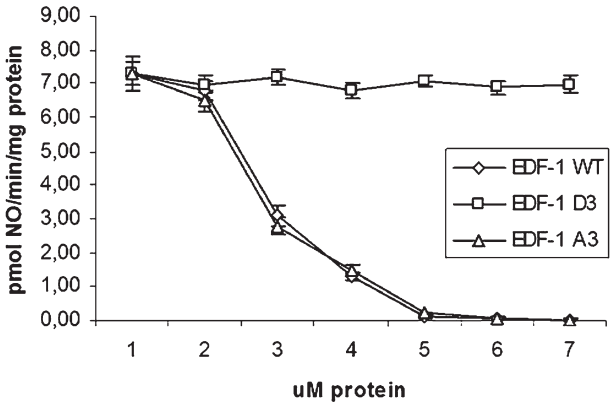

Figure 5. In vitro modulation of NOS activity by EDF-1 and its mutants. NOS activity was measured by monitoring the conversion of ^{3}H-arginine to ^{3}H-citrulline in the presence of different concentrations of EDF-1 and its mutants as described. Results are shown as the mean ± SD.

EDF-1 inhibits NOS activity. The inhibition was completely reversed by the mutant EDF-1_{D3}, which does not bind CaM and leaves CaM available to activate the enzyme (fig. 5). To extend these studies in vivo, HUVECs were transiently transfected with an antisense cDNA targeted against EDF-1 for 24 and 48 h. Figure 6A shows that the antisense cDNA prominently lowered EDF-1 expression, while it did not affect the total amount of NOS as evaluated by Western blot. Figure 6B shows that NOS activity was higher in antisense-transfected HUVECs than in mock-transfected cells.

Discussion

We have previously shown that PKC phosphorylates EDF-1, initially described as a factor involved in the repression of human endothelial cell differentiation [3]. PKC phosphorylation of EDF-1 inhibits its interaction with CaM [4]. In this report, we show that EDF-1 is also phosphorylated by PKA and this prevents the protein from binding CaM. Our results indicate that the impact of EDF-1 phosphorylation on cell function is mediated at least in part through the regulation of CaM availability. Indeed, we found that EDF-1 inhibits the in vitro activity of NOS, a CaM-dependent enzyme, while phosphorylated EDF-1 does not. We therefore hypothesize that PKA phosphorylation disrupts the EDF-1/CaM complex so that CaM is then available to activate NOS. Accordingly, we demonstrate an increase in NOS activity in anti-EDF-1 antisense-transfected endothelial cells, in the absence of any modulation in the total amount of the enzyme. These results further complicate the complex regulation of NOS, an enzyme implicated in the regulation of vascular physiology. Of note is that NO is not only a potent vasodilator, thus regulating arterial pressure, but also contributes to the angiogenic program by triggering endothelial cell growth and differentiation [12]. More studies are required to establish the relationship between EDF-1 and NOS activity and how this is implicated in vascular disease and angiogenesis.

We also showed that, upon PKA activation in HUVECs, EDF-1 is translocated to the nucleus where it may exert its function as a transcriptional coactivator through its binding to TBP. While PKC-mediated phosphorylation did not modulate the extent of EDF-1/TBP interaction [4], PKA activation enhances this binding. This result indicates a differential modulation of the nuclear function of EDF-1 by PKA and PKC, thus suggesting that PKA activation may modulate endothelial transcription by a direct effect on EDF-1/TBP interactions. Indeed, EDF-1 has no intrinsic transcriptional activity and does not possess any of the histone modifying activities which have been associated with the modulation of the transcriptional response [9]. The human protein not only binds TBP [4], but it also interacts with the nuclear receptor SF-1/Ad4BP (steroidogenic factor/adrenal 4 binding protein), which regulates steroid hormone synthesis [13]. Recently, EDF-1 has been demonstrated to enhance the transcriptional activity of three nuclear receptors involved in lipid metabolisms, i.e.,

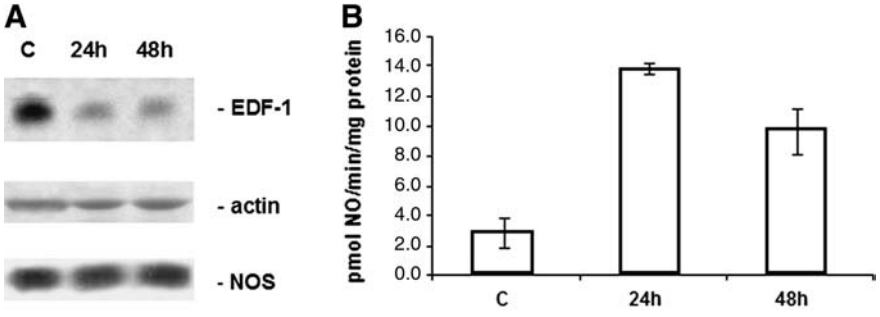

Figure 6. The activity of NOS in HUVECs negative for EDF-1 expression. (*A*) Cell extracts (200 µg) from HUVECs transfected with the antisense cDNA against EDF-1 were evaluated by Western blot with anti-EDF-1, anti-actin and anti-endothelial NOS antibodies as described. (*B*) NOS activity was measured on 40 µg of cell extracts obtained from mock- and antisense-transfected cell as described. Results are shown as the mean ± SD.

liver receptor homolog 1 (LRH-1), liver X receptor (LXR)-alpha, and peroxisome proliferator-activated receptor (PPAR)-gamma [9]. Of note is that LRH-1, LXR-alpha, and PPAR-gamma are all expressed in endothelial cells and we anticipate that their activity could be dependent, in part, on the availability of nuclear EDF-1. In conclusion, we propose two distinct roles for EDF-1 in endothelial cells: (i) in the cytosol, the regulation of CaM availability and, consequently, the activation of a large array of enzymes, some of which play a crucial role in the maintenance of vascular integrity, and (ii) in the nucleus, participation in the regulation of transcription through its interaction with TBP.

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