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# Vascular endothelial growth factor up-regulates nitric oxide synthase expression in endothelial cells<sup>1</sup>

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## Abstract

**Objective:** Vascular endothelial growth factor (VEGF), secreted by vascular cells and a variety of tumour cells, is a potent angiogenic factor. Since nitric oxide (NO) seems to play a key role in the VEGF-induced proliferation of endothelial cells, the aim of the present study was to determine whether VEGF stimulates endothelial NO synthase (eNOS) expression and hence results in a maintained increase in NO formation. **Methods:** Experiments were performed using cultured human umbilical vein endothelial cells (HUVEC) and isolated rat aortic rings. eNOS expression was assessed by Western blotting and RT-PCR analysis. **Results:** Exposure of either confluent HUVEC or rat aortic rings to VEGF<sub>165</sub> significantly increased eNOS mRNA and protein levels. This stimulatory effect of VEGF<sub>165</sub> on eNOS expression was associated with an elevation in the basal production of cGMP in HUVEC, and with a leftward shift of the concentration–relaxation curve to acetylcholine in aortic rings. The VEGF-induced increase in eNOS mRNA levels was abolished by tyrosine kinase inhibitors suggesting involvement of a tyrosine kinase-dependent pathway. Since eNOS mRNA levels remained elevated in VEGF-treated cells in the presence of actinomycin D, it is likely that the VEGF-induced up-regulation of eNOS expression may be a consequence of a post-transcriptional effect on eNOS mRNA stability. **Conclusion:** The results demonstrate that VEGF enhances the expression of eNOS in native and cultured endothelial cells, an effect which may be important in the process of VEGF-induced angiogenesis. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Nitric oxide synthase; mRNA stability; Protein tyrosine kinase; Endothelium; Human; Rat

## 1. Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is considered to be a key mediator in angiogenesis. VEGF, via its binding to specific receptors predominantly expressed on vascular endothelial cells, promotes cellular events involved in neovascularisation, such as endothelial proliferation and migration as well as the degradation of extracellular matrix components [1]. De novo vessel formation is thought to play a major role not only in tumour growth [2] but also in the supply of oxygen to ischaemic tissue [3,4].

A vasculoprotective role for VEGF has been proposed on the basis of observations made in different experimental

models. For example in balloon-injured arteries, the local administration of recombinant VEGF protein or naked VEGF cDNA was shown to promote re-endothelialisation and to restore endothelium-dependent vasomotor responses and thromboresistance [5,6]. Such protective effects are thought to be related to the VEGF-stimulated growth of endothelial cells. There is experimental evidence to suggest that the angiogenic effect of VEGF is mediated by the sustained formation of nitric oxide (NO). Indeed, NO synthase (NOS) inhibitors have been reported to abolish the VEGF-induced proliferation of cultured venous endothelial cells [7], as well as the formation of network-like structures in HUVEC cultured in three-dimensional collagen gels [8]. In the latter studies it is unlikely that the NO-mediated effects of VEGF on endothelial cell proliferation can be attributed to the transient increase in endo-

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thelial  $[Ca^{2+}]_i$  observed in response to acute VEGF stimulation [9]. Indeed the increase in  $[Ca^{2+}]_i$  elicited by VEGF is a relatively transient phenomenon. Therefore, the aim of the present study was to determine whether VEGF exerts a stimulatory effect on the expression of eNOS and leads to a sustained increase in endothelial NO production.

## 2. Methods

### 2.1. Materials

Chemicals were obtained from either Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Human recombinant vascular endothelial growth factor (VEGF<sub>165</sub>) was provided by Chiron (Emeryville, CA, USA), erbstatin A and genistein from Biomol (Hamburg, Germany), Diclofenac (Voltaren injection solution) from Ciba-Geigy (Wehr, Germany) and Ro-318220 from Roche Products. The  $[\alpha^{32}P]$ dCTP was purchased from Hartmann analytic (Braunschweig, Germany). The cloned bovine eNOS cDNA was kindly provided by Dr. D.G. Harrison (Emory University, Atlanta, USA). The mouse monoclonal human eNOS antibody was purchased from Transduction Laboratories (Affiniti, Exeter, UK) and the mouse monoclonal phosphotyrosine antibody from Upstate Biotechnology (Biomol). Male Wistar rats were obtained from Charles River Wiga Germany. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

### 2.2. Primary cultures of human umbilical endothelial cells

Human umbilical vein endothelial cells, isolated as previously described [10], were seeded in culture dishes containing M-199 medium (Life Technologies, Eggenstein, Germany) and 20% foetal calf serum (FCS, Biochrom, Berlin, Germany) supplemented with penicillin (50 U/ml) and streptomycin (50 µg/ml). All experiments were performed on quiescent confluent cells maintained in serum-deprived M-199 supplemented with 0.1% bovine serum albumin (BSA) for 24 h or as indicated. In order to avoid any change of the endothelial cell phenotype, all experiments were performed on primary cultured cells.

### 2.3. Vascular reactivity studies

The descending thoracic aorta was removed from anaesthetised (60 mg/kg sodium pentobarbitone i.p.) male Wistar rats, cleaned of connective tissue and cut either into rings (3–4 mm in length) for organ chamber experiments or segments (10 mm in length) for Western blot and RT-PCR analyses. In both cases, aortae were incubated for 6 or 24 h in 0.5 ml of minimum essential medium (MEM)

containing 2 mmol/l glutamine, 5 mmol/l N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid NaOH, 5 mmol/l N-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid NaOH (pH 7.3), 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1% BSA and 1 µg/ml polymyxin B, and in the absence and presence of VEGF<sub>165</sub> (100 ng/ml). The segments were then frozen in liquid nitrogen for Western blot and RT-PCR analyses. The rings were suspended in a thermostated (37°C) organ bath (Schuler-Organbad; Hugo Sachs Elektronik) for measurement of isometric force and equilibrated for 30 min under a resting tension of 2 g in Krebs–Henseleit solution (composition in mmol/l: NaCl 144.0, KCl 5.9, CaCl<sub>2</sub> 1.6, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25.0 and D-glucose 11.1, 95% O<sub>2</sub>; 5% CO<sub>2</sub>, pH 7.4) containing the cyclooxygenase inhibitor diclofenac (1 µmol/l). Rings were repeatedly contracted by phenylephrine (1 µmol/l), followed by a 30 min washout and equilibration period, until reproducible contractions were obtained. Thereafter, acetylcholine (1 µmol/l) was added at the plateau of the phenylephrine-evoked contraction to verify the presence of functional endothelium. After a 30 min washout and equilibration period, rings were contracted with phenylephrine prior to obtaining concentration–relaxation curves to acetylcholine (1 nmol/l–3 µmol/l) and sodium nitroprusside (1 nmol/l–1 µmol/l).

### 2.4. Determination of 3', 5'-cyclic guanosine monophosphate (cGMP)

HUVEC were exposed to VEGF<sub>165</sub> (100 ng/ml) for 48 h. Isobutylmethyl xanthine (0.5 mmol/l), a non-specific phosphodiesterase inhibitor, was added to the incubation medium during the last 30 min. The incubation was stopped by removal of the supernatant and the cells were then immediately extracted with 0.6 ml ice-cold trichloroacetic acid (6%) and scraped off. The cell suspension was then sonicated for 10 s before being centrifuged for 5 min at 4000 g. Supernatants were extracted with four volumes of water-saturated diethylether, and the samples were kept frozen (–20°C). The cGMP content of each sample was determined using a cGMP radioimmunoassay including an acetylation step as previously described [11]. Proteins were determined according to Lowry et al. [12].

### 2.5. Analysis of the eNOS mRNA expression by RT-PCR and Northern blot

Total RNAs were extracted according to the method of Chomczynski and Sacchi [13]. Due to the small amount of RNA (4–5 µg/35×10 mm petri dishes), semi-quantitative RT-PCR experiments were performed to assess eNOS transcript levels. For the reverse transcription (RT), 2 µg total RNA were incubated with 200 U reverse transcriptase (Gibco), dNTP (125 µmol/l), oligo(dT) (200 ng) and reaction buffer in a final volume of 20 µl at 37°C for 60 min. In some reaction mixtures, reverse transcriptase or

total RNA were omitted to determine the amplification of contaminating genomic DNA or cDNA. After a final denaturation at 94°C for 7 min, 6 µl cDNA was subjected to PCR consisting in a denaturation at 94°C for 1 min, followed by 90 s annealing at 52°C and 90 s elongation at 72°C for 30 cycles. The last cycle was ended with 7 min elongation at 72°C. In each PCR, the cDNA for eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were co-amplified. The primers used to amplify eNOS were derived from the sequence of the cloned human eNOS cDNA [14,15] (sense primer: 5'GCTGCG-CCAGGCTCTCACCTTC3') (antisense primer: 5'GGCTGCAGCCCTTTGCTCTCAA3') resulting in the amplification of a 540 bp fragment. The primers used to amplify GAPDH were derived from the cloned human and rat GAPDH cDNA [16] (sense primer: 5'TATGACAACCTCCCTCAAGAT3', antisense primer: 5'AGATCCACAACGGATACATT3'), resulting in the amplification of a 320 bp fragment. The PCR contained 0.4 µmol/l of each primer, dNTP (200 µmol/l), MgCl<sub>2</sub> (1 mmol/l) reaction buffer and 2.5 U Taq polymerase (Promega) in a final volume of 50 µl. The amplified cDNAs were size-fractionated by agarose gel electrophoresis, visualised under UV with an ethidium bromide staining, transferred to a nylon membrane (porablot NY amp, Macherey-Nagel, Düren, Germany) and hybridised with a <sup>32</sup>P-labelled eNOS fragment obtained from the cloned bovine eNOS cDNA and with a <sup>32</sup>P-labeled GAPDH fragment isolated from PCR. The eNOS cDNA and the GAPDH cDNA were quantified after autoradiography by scanning densitometry. The eNOS cDNA was normalised by comparison with GAPDH cDNA. Northern blots were performed using 20 µg total RNA extracted from pooled 60×15 mm dishes. RNA were electrophoresed on a 1.2% formaldehyde-denatured agarose gel, visualised with ethidium bromide, transferred to a nylon membrane (porablot NY amp) and hybridised with either <sup>32</sup>P-labeled eNOS fragment obtained from the cloned bovine eNOS cDNA or <sup>32</sup>P-labeled 18S ribosomal RNA fragment. Autoradiographs were then exposed for 4–72 h. Quantification of eNOS mRNA was performed by scanning densitometry, normalised for the ribosomal RNA signal to correct loading irregularities. The autoradiographs were analysed by scanning densitometry using as software IMAGE MASTER 1D (Pharmacia, Freiburg, Germany).

### 2.6. Western blot analysis

Crude protein extracts were obtained after alcoholic precipitation of the phenol phase obtained after the guanidinium isothiocyanate–phenol–chloroform extraction method [13]. Proteins (30 µg), measured according to Lowry et al [12], were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad) as previously described [17]. Prestained molecular mass marker proteins (Bio-Rad) were used as standards for the

SDS-PAGE. A Ponceau staining was performed to verify the quality of the transfer and the equal amount of protein in each lane. Proteins were detected using either eNOS or phosphotyrosine antibodies and were visualised by enhanced chemiluminescence using a commercially available kit (Amersham, Germany). The autoradiographs were analysed by scanning densitometry.

### 2.7. Statistics

Data are expressed as means±S.E.M. Statistical analysis were performed by one-way analysis of variance (ANOVA) followed by a Bonferroni *t* test or the two-tailed Student's *t* test where appropriate. Values of *P*<0.05 were considered statistically significant. EC<sub>50</sub> were calculated for each concentration–relaxation curve as the concentration of compound which elicited a 50% relaxation.

## 3. Results

### 3.1. Effect of VEGF<sub>165</sub> on the eNOS expression and activity in HUVEC

Exposure of confluent HUVEC to VEGF<sub>165</sub> (100 ng/ml) for 48 h led to a 1.9±0.2-fold increase in eNOS protein as assessed by Western blot analysis (Fig. 1A and B). VEGF<sub>165</sub> treatment was also associated with enhanced eNOS activity as assessed by the intracellular production of cGMP. Exposure of confluent HUVEC to VEGF<sub>165</sub> (100 ng/ml for 48 h) resulted in a 3.3-fold increase in the basal cGMP level (Fig. 1C). In contrast, VEGF<sub>165</sub> did not significantly affect the production of cGMP evoked by sodium nitroprusside (30 µmol/l, for 5 min). cGMP levels were 8.00±0.78 pmol/well in VEGF-treated cells vs. 6.87±1.57 pmol/well in solvent-treated cells, indicating that the VEGF-associated enhancement of basal cGMP production was not attributable to an increase in soluble guanylyl cyclase activity and/or expression.

### 3.2. Analysis of the stimulatory effect of VEGF<sub>165</sub> on eNOS expression

To determine whether VEGF<sub>165</sub> increased eNOS mRNA, co-amplification of eNOS and GAPDH cDNA was performed on RNA extracted from HUVEC exposed to VEGF<sub>165</sub> (1–100 ng/ml for 4–48 h). These experiments revealed a concentration- and time-dependent increase in eNOS cDNA. The amount of GAPDH cDNA was not significantly modified following VEGF treatment and was therefore used as internal control for RT-PCR. At the transcript level, a 2-fold increase was observed with 3 ng/ml VEGF<sub>165</sub> and the maximal effect at 30 ng/ml VEGF<sub>165</sub> (data not shown). Therefore, subsequent characterisation of the VEGF<sub>165</sub> effect on the eNOS mRNA expression was performed with 30 ng/ml. In control cells

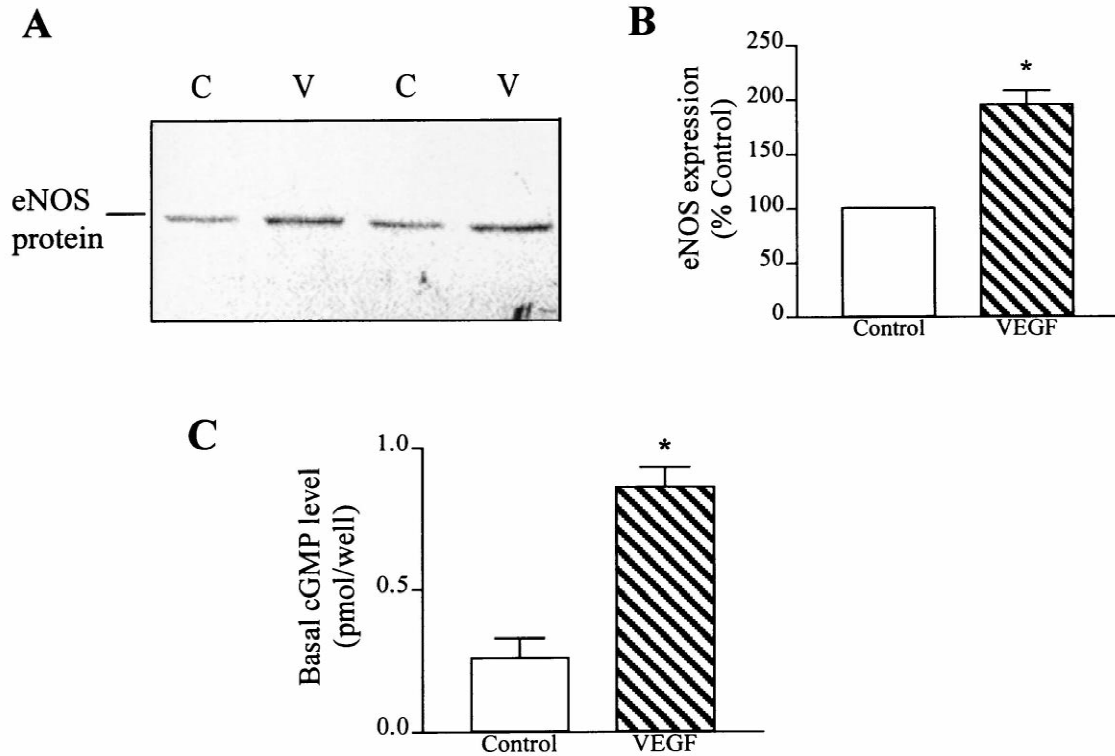


Fig. 1. Stimulatory effect of VEGF<sub>165</sub> on eNOS expression and activity. Confluent HUVEC were incubated in the absence (C) and presence (V) of VEGF<sub>165</sub> (100 ng/ml) for 48 h. Total cellular proteins were analysed by Western blotting with a monoclonal eNOS antibody: (A) Representative Western blot; (B) Statistical summary of densitometric analysis of seven separate experiments; (C) Effect of VEGF<sub>165</sub> (100 ng/ml, 48 h) on intracellular cGMP levels in confluent HUVEC. Data are presented as the mean  $\pm$  SEM of results obtained using four separate primary cultures, \* $P < 0.05$ .

maintained for various times in serum-deprived medium, a time-dependent decrease in eNOS expression was observed (Fig. 2A). VEGF<sub>165</sub> treatment (30 ng/ml) elicited a marked increase in eNOS mRNA levels after 4 h ( $2.6 \pm 0.3$ -fold,  $n=3$ ,  $P < 0.05$ ) which persisted over the next 44 h. Northern blot analysis, performed on cells treated for 8 h with 30 ng/ml VEGF<sub>165</sub> (Fig. 2B), confirmed that the extent of the VEGF<sub>165</sub>-induced increase in eNOS mRNA

levels was similar to that determined by RT-PCR analysis ( $2.75 \pm 0.4$ -fold increase,  $n=3$ ,  $P < 0.05$ ).

### 3.3. Role of protein tyrosine kinases in the stimulatory effect of VEGF<sub>165</sub>

Consistent with previous reports, exposure of HUVEC to VEGF<sub>165</sub> over 4 h enhanced the tyrosine phosphoryla-

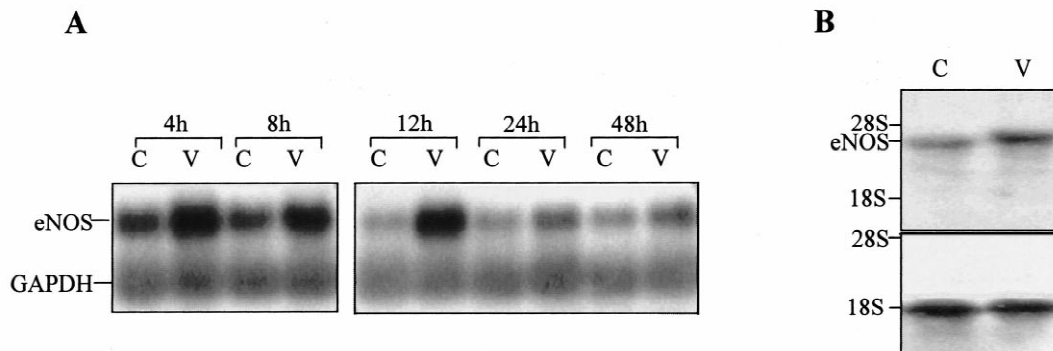


Fig. 2. Time-dependent effect of VEGF<sub>165</sub> on eNOS expression: (A) Confluent HUVEC were incubated in the absence (C) or presence (V) of VEGF<sub>165</sub> (30 ng/ml) for the times indicated (4, 8, 12, 24 and 48 h). Total RNA was analysed by RT-PCR experiments, followed by Southern blotting with eNOS and GAPDH probes. The autoradiograph shown is representative of data obtained in three separate experiments; (B) Analysis of the effect of VEGF on eNOS transcripts by Northern blotting. Confluent HUVEC were incubated in the absence (C) or presence (V) of VEGF<sub>165</sub> (30 ng/ml, 8 h). Total RNA (20  $\mu$ g) was electrophoresed on formaldehyde-denatured agarose gel, transferred to a nylon membrane and hybridised with either <sup>32</sup>P-labeled eNOS fragment obtained from the cloned bovine eNOS cDNA or <sup>32</sup>P-labeled 18S ribosomal RNA fragment. The autoradiograph shown is representative of data obtained in three independent experiments.

tion of several proteins as assessed by Western blot analysis (Fig. 3A). Two of these proteins were identified as the kinases Erk1/2 from the mitogen activated protein (MAP) kinase family ( $M_r$  42 000 and 44 000) (data not shown). The tyrosine kinase inhibitor, genistein (10  $\mu\text{mol/l}$ ), prevented the stimulatory effect of VEGF<sub>165</sub> (Fig. 3A). In parallel with the reduction in tyrosine phosphorylation, genistein abolished the VEGF<sub>165</sub>-induced increase in eNOS mRNA (Fig. 3B). Similar findings were obtained using erbstatin A (10  $\mu\text{mol/l}$ ), a structurally dissimilar tyrosine kinase inhibitor (data not shown). Incubation of HUVEC with the protein kinase C inhibitor Ro-31 8220 (0.1  $\mu\text{mol/l}$

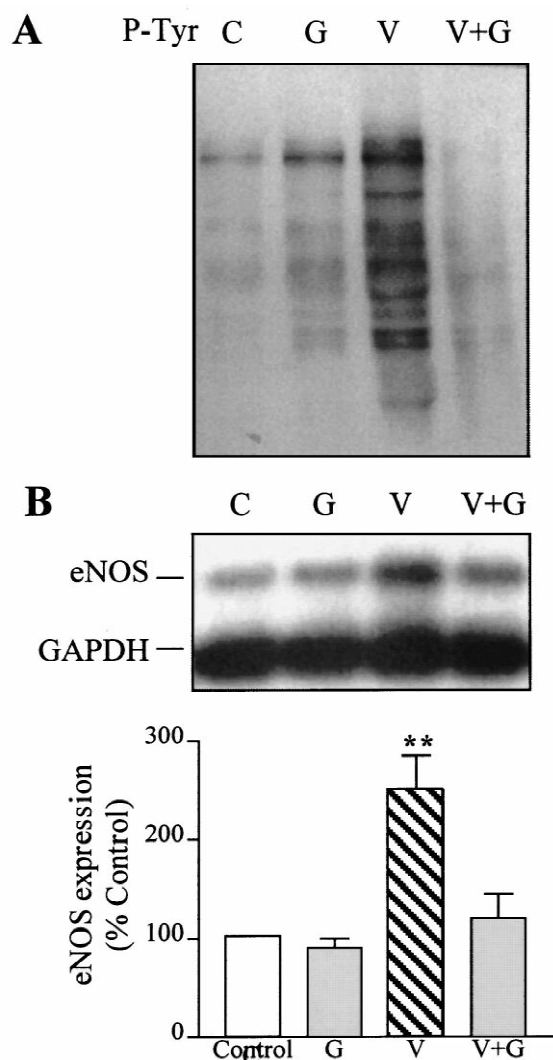


Fig. 3. Effect of genistein on the VEGF-induced tyrosine phosphorylation of endothelial proteins and expression of eNOS mRNA. Confluent HUVEC were incubated for 30 min in the presence of solvent (C) or the tyrosine kinase inhibitor, genistein (G, 10  $\mu\text{mol/l}$ ) prior the addition of VEGF<sub>165</sub> (V, 30 ng/ml, 4 h). (A) Total cellular proteins were analysed by Western blotting with a monoclonal anti-phosphotyrosine antibody. A Western blot representative of four additional experiments is shown; (B) total RNA was analysed by RT-PCR experiments, followed by Southern blotting with eNOS and GAPDH probes. A autoradiograph representative of three independent experiments is shown, \*\* $P < 0.01$ .

l, 4 h) increased the eNOS mRNA level in control cells. Under these experimental conditions VEGF (30 ng/ml, 4 h) further increased eNOS mRNA although the effect was not as pronounced as in cells treated with VEGF<sub>165</sub> alone (Fig. 4). Incubation of HUVEC with the non-selective phosphatidylinositol 3'-kinase inhibitor, wortmanin (1  $\mu\text{mol/l}$ , 4 h) decreased eNOS mRNA levels by about 45% but did not prevent the up-regulation of eNOS mRNA followed by VEGF treatment (30 ng/ml, 4 h, Fig. 4).

### 3.4. Effect of VEGF<sub>165</sub> on eNOS mRNA stability

To determine whether the stimulatory effect of VEGF<sub>165</sub> on eNOS expression was dependent on post-transcriptional mechanisms, experiments were performed with the transcription inhibitor, actinomycin D (4  $\mu\text{g/ml}$ ). The effects of short-term actinomycin D (30 min to 2 h) treatment on the eNOS transcripts were highly variable from cell batch to cell batch, which prevented an accurate determination of eNOS mRNA half-life. However, incubation of HUVEC with actinomycin D for 4 h decreased eNOS mRNA to a level which was below detection in a number of experiments. In cells pretreated with VEGF<sub>165</sub> actinomycin D failed to elicit a decrease in eNOS transcript levels, suggesting that VEGF<sub>165</sub> prevents the degradation of eNOS mRNA (Fig. 5).

### 3.5. Effect of VEGF<sub>165</sub> on the eNOS expression in native endothelial cells

In order to determine whether VEGF<sub>165</sub> affects eNOS expression in native endothelial cells, endothelium-intact rat aortic segments were incubated for either 6 or 24 h in the presence of VEGF<sub>165</sub> (100 ng/ml). Thereafter, relaxation was examined in organ chamber experiments and eNOS mRNA and protein expression by RT-PCR analysis and Western blotting. The Western blot and the RT-PCR analysis performed using aortic segments revealed that eNOS protein and mRNA levels were significantly en-

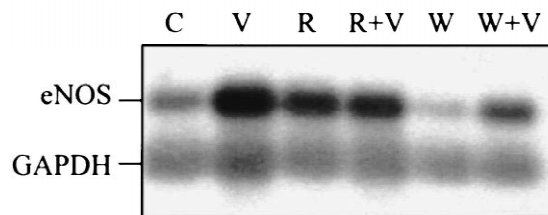


Fig. 4. Effect of protein kinase C and phosphatidylinositol 3'-kinase inhibitors on the VEGF-induced up-regulation of eNOS expression. Confluent HUVEC were incubated for 30 min in the absence (C) and presence of the protein kinase C inhibitor, Ro-318220 (R, 0.1  $\mu\text{mol/l}$ ) or of the non-specific phosphatidylinositol 3'-kinase inhibitor, wortmanin (W, 1  $\mu\text{mol/l}$ ), prior the addition of VEGF<sub>165</sub> (V, 30 ng/ml, 4 h). Total RNA was analysed by RT-PCR experiments, followed by Southern blotting with eNOS and GAPDH probes. Identical data were obtained in two additional independent experiments.

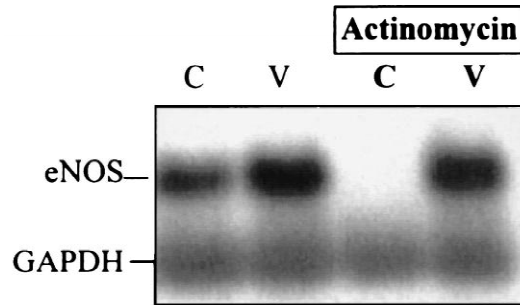


Fig. 5. Effect of VEGF<sub>165</sub> on the actinomycin D-induced decrease in eNOS expression. Confluent HUVEC were exposed either to solvent (C) or VEGF 165 (V, 30 ng/ml) in the absence or presence of actinomycin D (4 µg/ml) for 4 h. Total RNA was analysed by RT-PCR experiments, followed by Southern blotting with eNOS and GAPDH probes. Identical data were obtained in three independent experiments.

hanced (by 72 and 66%, respectively;  $P < 0.05$ ,  $n = 5$ ) following incubation with VEGF<sub>165</sub> for 6 h compared to solvent treated segments (Fig. 6A and B). Similar results were obtained after 24 h incubation with VEGF<sub>165</sub> (data not shown).

The endothelium-dependent relaxation of phenylephrine-precontracted aortic rings to acetylcholine was slightly but significantly greater in vessels pre-treated with VEGF<sub>165</sub>. Indeed, after 24 h pre-treatment with VEGF<sub>165</sub> there was a significant reduction in the EC<sub>50</sub> to acetylcholine (Fig. 6C). In contrast, relaxations to sodium nitroprusside (SNP, 1 nmol/l–1 µmol/l), were identical in rings pre-treated for 24 h with VEGF<sub>165</sub> or solvent (SNP EC<sub>50</sub> 7.2 ± 1.1 nmol/l in control aorta; 5.4 ± 1 nmol/l in VEGF-pretreated aorta,  $n = 3$ ).

#### 4. Discussion

In the present study we have demonstrated that VEGF<sub>165</sub> exerts a stimulatory effect on eNOS expression in endothelial cells resulting in the enhanced generation of bioactive NO. This was demonstrated by a VEGF<sub>165</sub>-induced increase in basal cGMP levels in cultured endothelial cells and a leftward shift of the concentration–response curve to acetylcholine in rat aortic rings. The stimulatory effect of VEGF<sub>165</sub> on eNOS expression involves a tyrosine kinase-dependent signalling pathway(s) and the apparent increase in eNOS mRNA is attributable to an enhancement of its stability.

eNOS is classified as a constitutively expressed gene, the activity of which is partly regulated by  $[Ca^{2+}]_i$ , in contrast to the inducible NOS isoform (iNOS) which is regulated solely at the transcriptional level. However, it has recently been appreciated that eNOS expression can be modulated to a certain extent by physical and hormonal stimuli. For example the two-fold increase in eNOS mRNA reported in chronically exercised dogs, and most likely attributable to enhanced fluid shear stress levels, has been suggested to contribute to the beneficial effect of exercise on the vascular system [18]. We report here that VEGF increases eNOS expression in cultured endothelial cells by about two-fold. Although the extent of the stimulatory effect of VEGF is at first view not pronounced, our observations are consistent with the effects of stimuli already described to affect the expression of eNOS in vitro including transforming growth factor β [19], basic fibroblast growth factor [20,21], fluid shear stress [22] and hypoxia [23]. This modest VEGF-induced increase in eNOS message was associated with a three-fold increase in

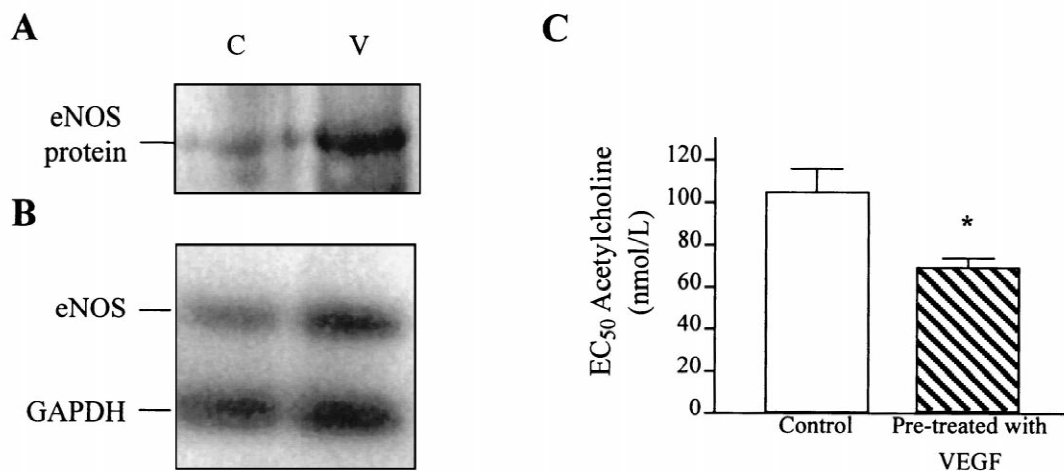


Fig. 6. Effect of VEGF<sub>165</sub> on the expression of eNOS in endothelium-intact rat aortic rings. Aortic rings were incubated in the absence (C) or presence of VEGF<sub>165</sub> (V, 100 ng/ml) for 6 h: (A) Total cellular proteins were analysed by Western blotting with a monoclonal anti-eNOS antibody. A Western blot representative of four additional experiments is shown; (B) total RNA was analysed by RT-PCR experiments, followed by Southern blotting with eNOS and GAPDH probes. An autoradiograph representative of three additional experiments is shown; (C) EC<sub>50</sub> values of the concentration–relaxation curves to acetylcholine (1 nmol/l to 3 µmol/l) in endothelium-intact rat aortic rings pre-treated with either solvent (open column) or VEGF<sub>165</sub> (100 ng/ml, 24 h, shaded column). Results are presented as the mean ± S.E.M. of five independent experiments, \* $P < 0.05$ .

the basal production of cGMP, the major intracellular effector molecule of NO and hence the most appropriate index to assess the generation of biologically active NO. VEGF also led to an up-regulation of eNOS mRNA and protein expression in native endothelial cells from rat aortae, as indicated by RT-PCR and Western blot analysis, and VEGF-treated aortic rings exhibited a leftward shift in the concentration-dependent relaxation elicited by acetylcholine. These results indicate that in addition to the short-term stimulation of eNOS activity already described following an acute increase in  $[Ca^{2+}]_i$  [9], VEGF exerts a long-term stimulatory effect on endothelial NO generation by increasing eNOS expression.

The signalling pathway activated by VEGF which results in the increased expression of eNOS was shown to be sensitive to tyrosine kinase inhibitors. VEGF is known to interact on endothelial cells with at least two types of receptors, Flt1 (VEGFR1) and Flk1/KDR (VEGFR2) [1,24] both of which demonstrate intrinsic tyrosine kinase activity. Activation of Flk1 (VEGFR2) has been associated with mitogenic and chemotactic responses to VEGF whereas the cellular responses associated with Flt1 (VEGFR1) receptor activation have not been precisely defined [1]. The application of VEGF to cultured bovine aortic endothelial cells is reported to enhance the tyrosine phosphorylation of at least 11 proteins including Flt1 (VEGFR1) and Flk1/KDR (VEGFR2) receptors as well as phospholipase C $\gamma$ , phosphatidylinositol 3'-kinase, the Ras GTPase activating protein (GAP) and the oncogenic adapter protein NcK, eventually culminating in a proliferative response [25]. In the present study we similarly observed that VEGF induced the prolonged tyrosine phosphorylation of several cellular proteins in HUVEC, possibly reflecting sustained activation of the growth factor's receptors. Of those tyrosine-phosphorylated proteins, mitogen activated protein kinases Erk1/2 were identified. Since Erk1/2 have been shown in various cell types to be involved in the control of proliferation and differentiation processes, it might be hypothesized that activation of Erk1/2 under VEGF treatment might play a role in the VEGF-induced stimulation of angiogenesis. In addition to the activation of tyrosine kinases, VEGF has been shown to increase the activity of the phosphatidylinositol 3'-kinase in endothelial cells [26]. However, since the non-selective inhibitor of phosphatidylinositol 3'-kinase, wortmanin failed to abrogate the stimulatory effect of VEGF on eNOS expression, this kinase is unlikely to be involved in the intracellular pathway triggered by VEGF. The effect of the protein kinase C inhibitor on the VEGF-induced increase in eNOS mRNA was difficult to evaluate as this substance alone had a stimulatory effect on eNOS expression, a result which is in agreement with a previous report [27].

The VEGF-induced increase in eNOS expression appears to involve a predominantly post-transcriptional mechanism since VEGF reduced the rate of degradation of eNOS mRNA in the presence of actinomycin D. Changes

in the stability of eNOS mRNA have previously been shown to account for the down-regulation of eNOS mRNA by tumour necrosis factor  $\alpha$  [28] and oxidised low density lipoprotein [29]. Destabilisation of eNOS may also account for confluency-related changes in eNOS expression as the half-life of eNOS mRNA was reported to be about 2-fold longer in sub-confluent, proliferating cells than in confluent cells [30]. The effect of VEGF on eNOS transcripts from confluent cells described in the present investigation may indicate that VEGF stabilises mRNA by counteracting constitutively active processes regulating its destabilisation. Amongst potential targets for such an effect, are protein-RNA complexes located in the 3'UTR of eNOS mRNA which have been implicated in the post-transcriptional regulation of its expression [31].

In summary, we have shown that VEGF induces an increase in eNOS mRNA and protein levels and increases basal NO generation, as indicated by the elevation of cGMP levels in cultured endothelial cells and the enhanced receptor-dependent, endothelium-mediated relaxation of aortic rings. This effect is consistent with reports performed on cultured endothelial cells which appeared during the preparation of this manuscript [8,32]. The present study extends these observations to native endothelial cells from rat aorta and spotlights the potential mechanisms involved in the VEGF-induced increase in eNOS transcripts. Such effects of VEGF on eNOS expression may underlie the vascular protective effect of VEGF treatment. Indeed a sustained elevation in endothelial NO formation decreases vascular tone and hence reduces the likelihood of local vasospasm, protects the vessel wall from oxidative stress, maintains vascular smooth muscle cells in a non-proliferative state [33] and promotes the re-growth of injured endothelial cells.

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