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Cardiovascular Research 51 (2001) 773–783

Cardiovascular Research

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Genetic augmentation of nitric oxide synthase increases the vascular generation of VEGF

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Received 17 January 2001; accepted 1 May 2001

Abstract

Objective: Vascular endothelial growth factor (VEGF) induces the release of nitric oxide (NO) from endothelial cells. There is also limited data suggesting that NO may enhance VEGF generation. **Methods:** To further investigate this interaction, we examined the effect of exogenous and endogenous NO on the synthesis of VEGF by rat and human vascular smooth muscle cells (VSMC) by exposing cells to exogenous NO donors, or to genetic augmentation of eNOS or iNOS. **Results:** NO-donors potentiated by 2-fold the generation of VEGF protein by rat or human VSMC. Similarly, rat or human VSMC transiently transfected with plasmid DNA encoding eNOS or iNOS, synthesized up to 3-fold more VEGF than those transfected with control plasmid DNA, an effect which was reversed after treatment with the NOS antagonist L-NAME. Rat VSMC stably transfected with pKeNOS plasmid, constitutively produced NO and released high concentrations of VEGF. In these cells, L-NAME significantly reduced NO synthesis and decreased VEGF generation. The VEGF protein produced by NOS-transfected VSMC was biologically active, as conditioned media harvested from these cells increased endothelial cell proliferation. **Conclusion:** These studies reveal that NO derived from NO-donors or generated by NOS within the cells, upregulates the synthesis of VEGF in vascular smooth muscle cells. Administration of NO donors, or augmentation of endogenous NO synthesis, may be an alternative approach in therapeutic angiogenesis.

Keywords: Endothelial factors; Gene expression; Growth factors; Nitric oxide; Signal transduction

1. Introduction

A number of angiogenic factors, including vascular endothelial growth factor (VEGF), upregulate the expression of eNOS and stimulate the release of endothelium-derived NO [1–6]. The release of NO by these factors plays a critical role in their angiogenic actions. When endothelial cells are grown in a three-dimensional fibrin gel, they produce NO and form capillary-like structures when stimulated by VEGF, effects that are blocked by pharmacological antagonism of NO synthase [4,7]. In the rabbit cornea model of angiogenesis, VEGF-induced angiogenesis is blocked by L-NAME [8]. The angiogenic response to hindlimb ischemia is impaired in the eNOS-deficient mice, an effect that cannot be reversed by VEGF [9]. We have also shown that ADMA, the endogenous inhibitor of NO synthesis, impairs angiogenesis in the apoE deficient mouse [10].

Angiogenesis requires several key processes, including dissolution of matrix, endothelial cell proliferation and migration, and organization into tubes, followed by lumen formation [11]. In all of these processes, NO has been implicated to play a role [4,5,12,13]. In addition, we have recently provided evidence that NO may also positively

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Time for primary review 19 days.

0008-6363/01/$ – see front matter
PII: S0008-6363(01)00344-3

regulate the expression of VEGF [14]. Although our observations are consistent with reports from some groups [15,16], others have suggested that NO downregulates VEGF synthesis [17,18].

Most studies regarding the involvement of NO in VEGF expression have been performed using NO-donors, such as sodium nitroprusside (SNP). The drawback of this approach is that the influence of the released NO may be masked by NO-independent actions of donating compounds or their derivatives. A better strategy may be to manipulate the endogenous generation of NO. Recently we [14] and others [16] have demonstrated that induction of iNOS expression is a prerequisite for cytokine or LPS-induced VEGF synthesis in rat VSMC or murine macrophages.

The principal aim of the present studies was to determine if a similar effect on VEGF synthesis could be achieved in vascular cells by overexpressing endothelial or inducible nitric oxide synthases. Furthermore, we were interested to compare the approach of enhancing endogenous NO production to that of exposing vascular cells to exogenous NO donors. The results strongly support a role for NO in regulation of VEGF.

2. Material and methods

2.1. Reagents

Culture media (DMEM F-12, M-199) and fetal calf serum (FCS) were purchased from Gibco BRL (Warsaw, Poland). NO-donors: SNAP (*S*-Nitroso-*N*-acetyl-D,L-penicillamine), SIN-1 (3-morpholinosydnonimine), DETA (diethylenetriamine), SNP (sodium nitroprusside) and NO-scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetrathylimidizoline-1-oxyl-3-oxide potassium salt) were procured from Alexis Biochemicals (Laufelfingen, Switzerland). QIAfilter Maxiprep Kit was obtained from Qiagen (Hilden, Germany), CytoTox 96 assay, Total RNA Extraction Kit, Taq and Tth DNA Polymerases, pSVβgal plasmid and Tfx-50 liposomes were obtained from Promega (Madison, USA). Primers were synthesized by TIB-MOLBIOL (Poznan, Poland) or ARK (Darmstadt, Germany). Cell proliferation ELISA (colorimetric detection of BrdU incorporation), and the Luciferase Assay System were obtained from Roche Diagnostics (Mainheim, Germany). Human IgG1, blocking antibodies against human VEGF, ELISA kits for human VEGF and for murine/rat VEGF, were procured from R&D (Abingdonm, UK). All other chemicals were purchased from Sigma (St. Louis, USA). Plasmid pKeNOS was kindly provided by Dr Thomas Michel, plasmid pCISiNOS by Drs Edith Tzeng and Timothy Billiar, whereas a construct containing the human VEGF promoter (−2279 to +54) cloned into a luciferase reporter plasmid pGL2 was a generous gift of Dr Hideo Kimura.

2.2. Cell cultures

All experiments were performed according to the guide of the local ethical committee. Rat vascular smooth muscle cells (RVSMC) were isolated from thoracic aorta by collagenase digestion. Human aortic smooth muscle cells (HASMC) were purchased from Clonetics (St. Catharine, Germany). For testing the effect of conditioned media on HUVEC proliferation, the transfected RVSMC or HASMC were incubated in M-199 medium supplemented with 2% FSC, heparin (500 u/ml) and L-glutamine (2 mM).

Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated [19] and grown in M199 medium containing 20% FCS, heparin (500 u/ml), L-glutamine (2 mM) and Endothelial Cell Growth Factor Supplement (ECGF, 150 μg/ml).

2.3. Incubation with NO-donors

RVSMC and HASMC were cultured up to full confluence in medium with 5% FCS. Before each experiment, the cells were starved in DMEM F-12 medium with 0.5% FCS for 24 h. Then fresh medium was introduced, and supplemented with SNAP, SIN-1, or DETA (final concentration 100 μM). In some experiments the cells were co-incubated with NO donors and NO scavenger, carboxy-PTIO (100 μM). After 24 h, the media were collected for measurements of nitrite concentration, lactate dehydrogenase (LDH) release and VEGF production.

2.4. Transient transfections

Expression plasmids, pKeNOS (containing bovine endothelial eNOS cDNA regulated by a CMV promoter), pCISiNOS (harboring rat iNOS cDNA driven by a CMV promoter), control pSVβgal (with bacterial β-galactosidase cDNA gene under the control of a SV40 promoter), and a reporter pGL2VEGF plasmid containing the human VEGF promoter, were multiplied in HB-101 *E. coli* bacteria and isolated on maxiprep columns. The quality of the DNA was assessed by spectrophotometry and by electrophoresis in 1% agarose gel.

RVSMC or HASMC grown to 70–80% confluence were transfected in 24-well plates using 200 μl of serum-free medium mixed with 0.5 μg plasmid DNA and 1.5 μl Tfx-50 liposomes per-well. After 1 h, the transfection mixture was replaced with regular culture medium with or without L-NAME (N^g-nitro-L-arginine-methyl ester, 2 mM) or D-NAME (N^g-nitro-D-arginine-methyl ester, 2 mM). Media were collected after a 48-h incubation period for determination of VEGF, nitrites and LDH. Remaining cells were lysed and subjected to RNA isolation for RT-PCR. RVSMC transfected with pGL2VEGF were exposed to NO-donors 24 h after transfection. After a period of

48-h, the cells were collected, lysed and studied using the luciferase assay.

2.5. Stable transfection

RVSMC were transfected as described above and selected in medium containing geneticin (0.2 mg/ml). The resulted cell line produced NO constitutively and was maintained in standard culture conditions for several months. For experiments, the cells were grown to confluence and starved in medium containing 0.5% FCS for 24 h. Then fresh medium was added; some wells were also treated with L-NAME. VEGF concentrations were measured in media harvested after 48 h incubation. In some experiments, cells were lysed and DNA was isolated for PCR analysis.

2.6. PCR and RT-PCR

Total DNA or RNA were isolated from the cells by acid guanidinium thiocyanate-phenol-chloroform extraction [20]. PCR with primers for human iNOS (5′-CCG AGG CAA ACA GCA CAT TC (sense) and 5′-GGT TGG GGG TGT GGT GAT GT (antisense) or bovine eNOS (5′-GTG ATG GCG AAG CGA GTG AA and 5′-CCG AGC CCG AAC ACA CAG AA) were used to confirm the presence of exogenous cDNA in transfected VSMC using PCR. RT-PCR reactions were performed on 100 ng of total RNA with primers recognizing rat and human VEGF (5′-CAC CGC CTC GGC TTG TCA CAT and 5′-CTG CTG TCT TGG GTG CAT TGG), as well as the GAPDH gene (5′-TCC ACC ACC CTG TTG CTG TA, and 5′-ACC ACA GTC CAT GCC ATC AC). Reverse transcription (RT) was carried out for 40 min at 65°C in 10 μl of RT mixture, with Tth polymerase (2.5 U/10), in the presence of Mn^{2+} (2.5 mM). After addition of chelating buffer (EGTA 750 μm; Mg^{2+}–2.5 mM; total volume of PCR mixture–50 μl) the PCR was performed for 30 cycles using the following protocol: 95°C–40 s, 58°C–40 s, and 72°C–50 s. PCR and RT-PCR products were analyzed by electrophoresis in 3% agarose gel. The product length of the eNOS was 422 bp, for $VEGF_{121}$–431 bp (for rat $VEGF_{120}$–428 bp), $VEGF_{165}$–563 (for rat $VEGF_{164}$–560 bp), and GAPDH–452 bp.

2.7. Measurement of nitrite levels

Measurements were performed without preceded reduction of nitrate in the samples. Release of NO from NO-donors was confirmed by measurement of nitrite accumulation in the culture media using Griess method as described previously [21]. The concentrations of nitrites in the media from transfected cells were measured using a fluorimetric method, sensivity of which ranged from 10 nM to over 10 μM [22].

2.8. Measurement of VEGF release

Concentrations of VEGF protein in the culture media were measured by ELISA.

2.9. Measurement of VEGF mRNA expression

Concentrations of rat VEGF mRNA were measured by ELISA performed on 2.5 μg of total RNA using Quantikine mRNA Base Kit, and Probe and Calibrator Kit for rat VEGF. As an additional control for the amount of total RNA, the concentration of constitutively expressed GAPDH mRNA was measured using a kit specific for rat GAPDH.

2.10. Cell viability assay

Cell viability was assessed by a colorimetric measurement of LDH release.

2.11. Proliferation of endothelial cells

HUVECs were placed into a 96-well plate (3000 cells/well) in routine culture medium. After 6–8 h fresh medium supplemented with 2% FCS, was added. After overnight incubation, the medium was replaced with conditioned medium from VSMC transfected with pCISiNOS, pKeNOS, pSVβgal plasmids, or from non-transfected VSMC. In some experiments, media from pKeNOS transfected human HASMC were preincubated for 1 h at room temperature with anti-VEGF antibodies (100 ng/ml) to block VEGF protein or with non-immune human IgG1 (100 ng/ml). After 48 h, BrdU was added for 2 h and its incorporation was measured by ELISA.

2.12. Statistical analysis

The experiments were made in duplicates or triplicates and were repeated at least three times. Data are presented as means±S.D. Statistical evaluation was done with Student's *t*-test or with ANOVA followed by Tukey test. Differences were accepted as statistically significant at $P<0.05$.

3. Results

3.1. Effect of NO-donors on VEGF generation

Rat or human vascular smooth muscle cells were incubated with several NO donors, namely SIN-1, SNAP, or DETA (100 μM each) for 24 h. This treatment did not influence the cell viability, as assessed by LDH assay (data not shown). The release of NO from NO-donors was confirmed by increased nitrite concentration (up to 20–40 μM).

Control VSMC constitutively produced VEGF (80–150 pg/ml, which amount varied by cell batch). Both in rat (Fig. 1A) and human (Fig. 1B) cells the release of VEGF protein into the culture medium was significantly (1.5 up to 2-fold, $P<0.05$) higher in the presence of SIN-1, SNAP, or DETA NO-donors. This stimulatory effect was significantly reduced in the presence of NO-scavenger, carboxy-PTIO (by 51% and 39% for SNAP and SIN-1, respectively). In contrast to above mentioned NO-donors, SNP did not enhance VEGF synthesis and even exerted an inhibitory effect in rat ([14] and Fig. 2A) and in human VSMC (not shown).

The expression of VEGF mRNA was determined by RT-PCR analysis of rat VSMC. We could detect mRNA for $VEGF_{120}$ and $VEGF_{164}$ in all cell cultures (Fig. 2, inset). In cells exposed to SIN or SNAP (but not SNP), the VEGF mRNA expression was significantly increased, as evidenced by mRNA ELISA (Fig. 2). Therefore we investigated the effect of NO donors on the transcriptional activity of a full-length VEGF promoter in cells transfected with the reporter construct. The transcriptional activity of the VEGF promoter was significantly (1.7–3-fold, $P<0.05$) higher in the presence of NO-donors as assessed by luciferase assay (Fig. 3A). In subsequent experiments, we observed that a similar effect could be achieved by exposing the cells to IL-1β so as to induce iNOS. Under these conditions, the human VEGF promoter was activated, an effect which was abrogated by the NOS inhibitor L-NAME (Fig. 3B).

3.2. Effect of iNOS transfection on VEGF generation

To determine the effect of autocrine NO on the generation of VEGF, we transfected the cells with the pCIS expression plasmid containing human iNOS cDNA driven by a CMV promoter. The presence of iNOS cDNA and mRNA in the transfected cells was confirmed by PCR and RT-PCR, respectively (data not shown). The efficiency of transfection was low, ranging around 5–8%, as determined by in situ staining for β-galactosidase activity in the cells treated with pSVβgal (not shown). After 48 h incubation, we could detect the accumulation of nitrites (2.5–4 μM) in the culture media harvested from the VSMC transfected with pCISiNOS (Fig. 4A). The accumulation of nitrites was blocked by L-NAME, but not by D-NAME (an inactive enantiomer), indicating that nitrites derived from NO were

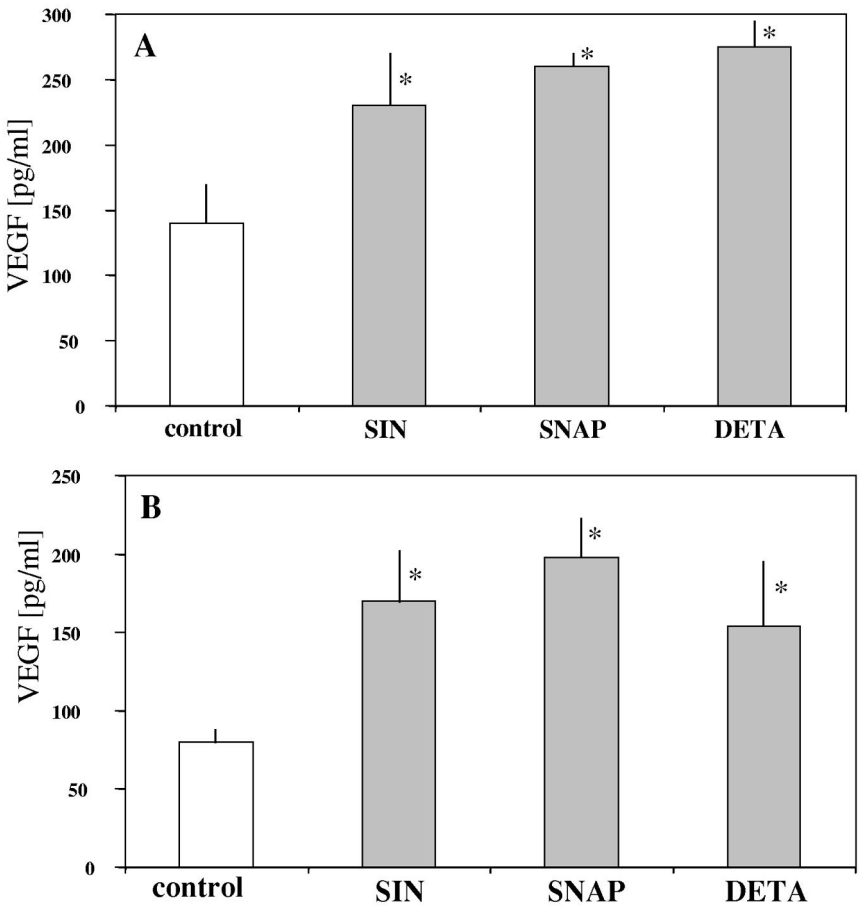

Fig. 1. Effect of NO-donors (100 μM, 24 h) on the generation of VEGF in rat (A) and human (B) VSMC. One of three similar experiments made in triplicates. *$P<0.01$ in comparison to control.

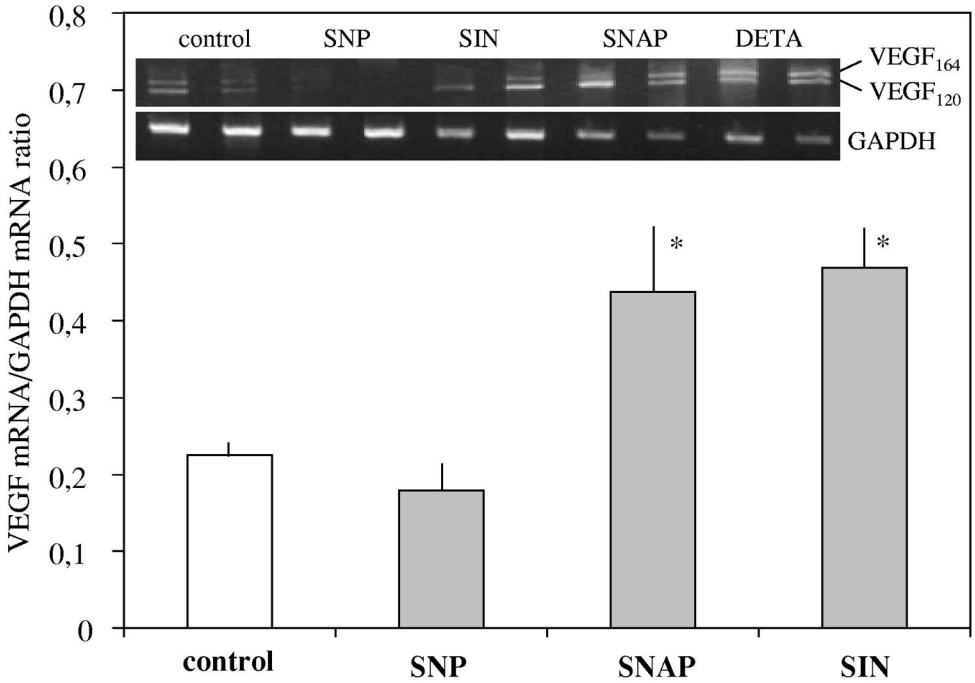

Fig. 2. Effect of NO-donors (100 μM, 24 h) on the expression of VEGF mRNA in rat VSMC (mRNA-ELISA). Mean of four experiments. *$P<0.01$ in comparison to control, Student *t*-test. Inset: representative of four RT-PCR results.

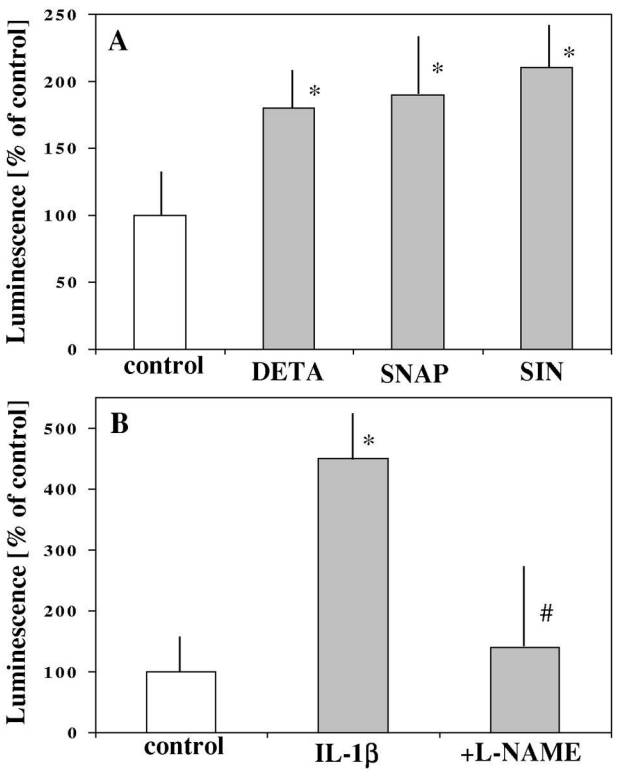

Fig. 3. (A) Effect of NO-donors (100 μM, 48 h) on the activity of full-length VEGF promoter in rat VSMC (luciferase assay). Mean of three experiments made in triplicates. (B) Effect of L-NAME on IL-1β-induced activation of full-length VEGF promoter in rat VSMC after 48 h incubation (luciferase assay). One of three similar experiments. *$P<0.05$ in comparison to control, #$P<0.05$ in comparison to IL-1β-stimulated cells.

produced by nitric oxide synthase. Analysis by ELISA revealed higher concentrations of VEGF in conditioned media harvested from the pCISiNOS-transfected cells, in comparison to conditioned media from pSVβgal-transfectants or from non-transfected VSMC (Fig. 4B). Importantly, this upregulation of VEGF synthesis was reversed by the NOS inhibitor L-NAME, but not by its inactive enantiomer D-NAME.

3.3. Effect of eNOS transfection on VEGF generation

To confirm that the up-regulation of VEGF production was related to NO generation, we repeated the transfection experiments using another expression plasmid, with bovine eNOS cDNA driven by a CMV promoter. Using this construct, we also produced a stably transfected rat VSMC line. The presence of eNOS cDNA and mRNA in the transiently and stably transfected cells was confirmed by PCR and RT-PCR, respectively (data not shown).

Human VSMC transfected with eNOS generated NO and released more VEGF protein into the culture media than non-transfected or pSVβgal-treated cells (Fig. 5). The increase in NO synthesis, and in VEGF generation, was greater in stable versus transient transfectants (Fig. 6). Both in transiently and stably transfected VSMC, the production of NO and the synthesis of VEGF was significantly reduced by L-NAME. Furthermore, the effect of L-NAME could be reversed by supplementation with L-arginine.

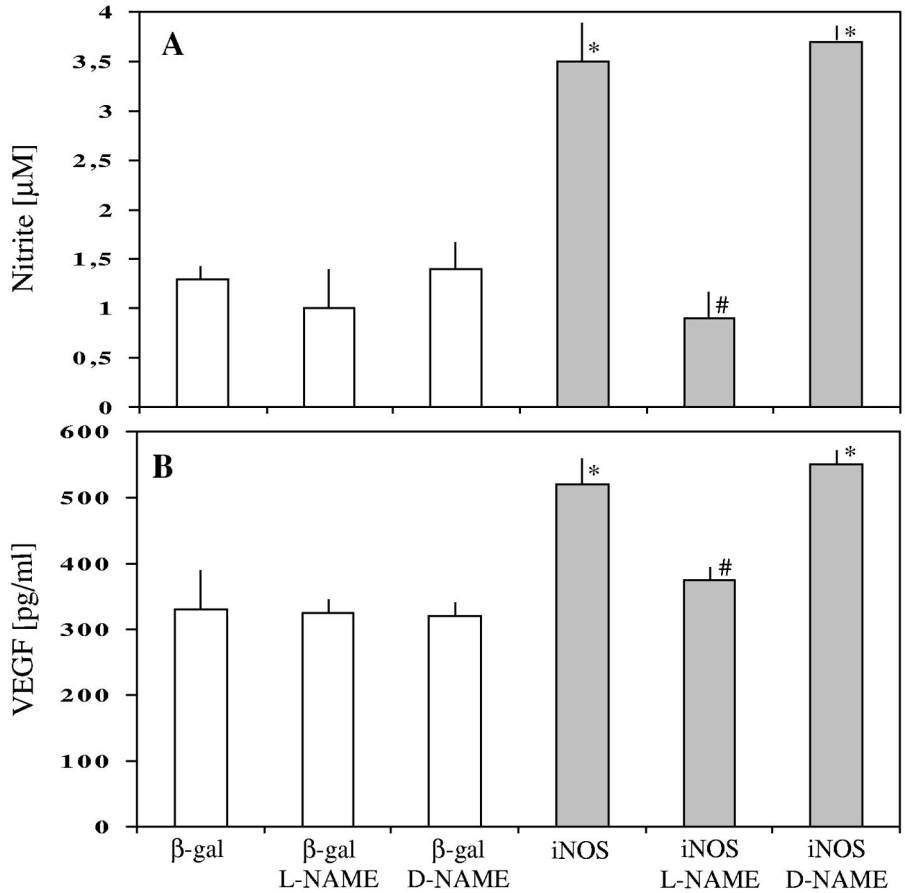

Fig. 4. (A) Effect of transfection of rat VSMC with iNOS on the generation of nitrites after 48 h incubation (fluorimetric method). (B) Effect of transfection of rat VSMC with iNOS on the generation of VEGF after 48 h incubation (ELISA). One of two similar experiments made in duplicates. *$P<0.01$ in comparison to the cells transfected with control β-galactosidase gene, ≠$P<0.01$ in comparison to iNOS transfected cells.

3.4. Effect of media from eNOS- or iNOS-transfected VSMC on endothelial cell proliferation

Conditioned media from HASMC cultures induced the proliferation of HUVEC (Fig. 7). Media harvested from HASMC transfected either with iNOS or with eNOS exerted a stronger mitogenic response than conditioned medium from non-transfected cells ($P<0.05$). The effect of the conditioned medium was significantly attenuated when the medium was preincubated with anti-VEGF antibodies (Fig. 7), whereas non-immune IgG1 did not show any influence (data not shown). This indicates that VEGF was mediating the increased proliferative effect of conditioned medium derived from NOS-transfected cells.

4. Discussion

The salient findings of the present study are that: (1) Vascular smooth muscle cells transfected with the endothelial or inducible isoform of nitric oxide synthase generate NO, an effect which is associated with an increase in the generation of VEGF by these cells; (2) The effect of the transfection to enhance VEGF synthesis is antagonized by the NOS inhibitor, L-NAME, but not by its inactive enantiomer D-NAME; (3) In the presence of a NOS inhibitor, the NO precursor L-arginine restores VEGF production; (4) Moreover, by comparison to transiently transfected cells, stable transfected cells produced more NO, an effect which was associated with a proportionately greater increase in VEGF synthesis; (5) Additionally, we demonstrated that NO-induced VEGF was active, which is important in view of the findings of Xiong and colleagues, discussed below. Taken together, these studies indicate that NO enhances angiogenesis in part by stimulating the synthesis of VEGF.

Although there is a growing body of evidence that NO has angiogenic effects, in part mediated by VEGF, there is not unanimity of opinion in this regard. Indeed there are some reports of an inhibitory effect of NO-donors on the generation of VEGF. Specifically, the NO donor sodium nitroprusside (SNP) inhibits VEGF synthesis in vascular smooth muscle cells [18] and in retinal epithelial cells [17]. By contrast, other NO-donors, such as SIN-1, SNAP, DETA or GSNO have been reported to stimulate VEGF synthesis from a variety of cultured cells ([23–27] and the

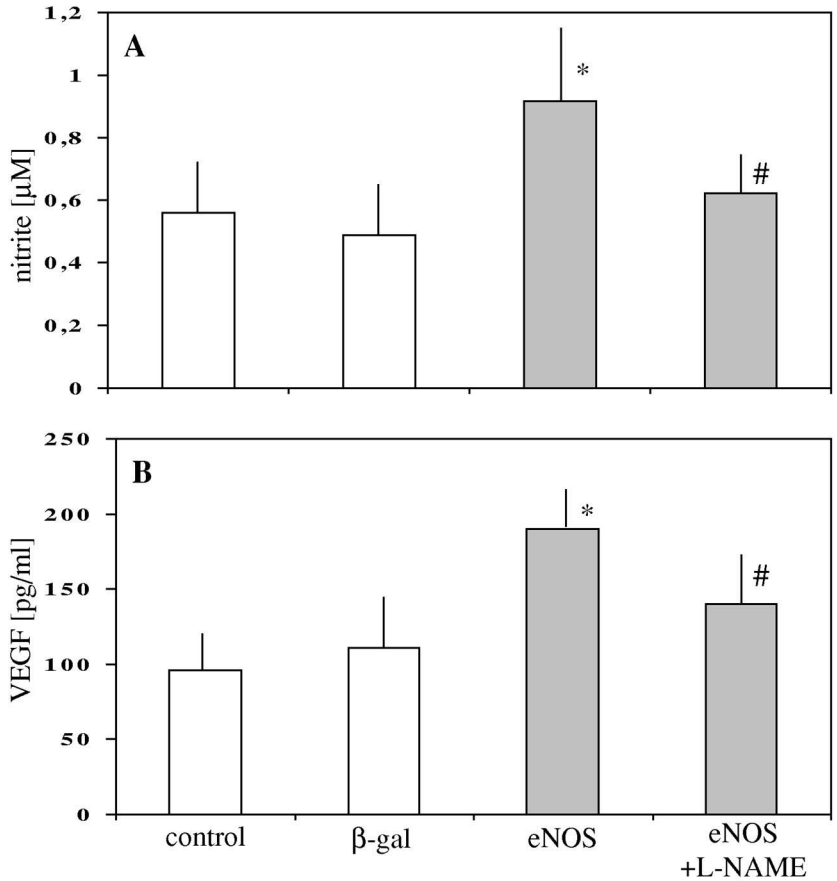

Fig. 5. (A) Effect of transfection of human VSMC with eNOS on the generation of nitrites after 24 h incubation (fluorimetric method). (B) Effect of transfection of rat VSMC with eNOS on the generation of VEGF after 24 h incubation (ELISA). One of three similar experiments made in triplicates. *$P<0.05$ in comparison to control, intact cells, ≠$P<0.05$ in comparison to eNOS transfected cells.

current study). Because NO chemistry is highly redox-sensitive, these discrepancies may be due to subtle differences in the cellular environment in which the assays were performed [27]. Furthermore, the incongruent effects of SNP may be due to the fact that, apart from donating NO, SNP fragments into ferrocyanide, ferricyanide, iron ions, and cyanide, each of which may influence a variety of biological functions [28]. We have observed that the effect of SNP to inhibit VEGF synthesis is NO-independent, as it is mimicked by potassium ferri-or ferrocyanide [14]. Additionally, cytotoxicity induced in vitro by high doses of SNP could contribute to the discrepancies observed with this compound [14,27].

We have previously shown that cytokine-induced increases in VEGF generation by rat VSMC is dependent on the induction of iNOS [14]. VEGF synthesis is also enhanced in cells transiently transfected with an eNOS plasmid [29]. As further proof that NO is responsible for the upregulation of VEGF synthesis, we find a direct relationship between the amount of NO produced by a cell, and the amount of VEGF that is synthesized. In the stably transfected cells, NO production is greater; so too is VEGF production. Furthermore, the effect of the NOS transfection is antagonized by the NOS inhibitor L-NAME. The inhibition is reversed by co-administration of the NO precursor, L-arginine. These studies provide further evidence that VEGF production is regulated by NO. Our findings are concordant with observation that exercise-induced VEGF generation in rat skeletal muscle is NO-dependent and strongly attenuated after treatment of the animals with L-NAME [30]. Similarly, nitric oxide released from NO-donors or from acetylcholine-stimulated endothelial cells augments the expression of VEGF mRNA in resting skeletal muscles [31]. The NO-mediated upregulation of VEGF is not restricted to a particular cell type, since it has also been observed in RAW macrophages [16], keratinocytes [15,32], hepatocytes [23], and in mesangial cells [25]. Furthermore, NO-dependent enhancement in expression of VEGF mRNA has been demonstrated in several tumors [27]. Ambs et al. [33] showed that transfection of human lung carcinoma cells with nitric oxide synthase resulted in increase in VEGF mRNA and an augmented secretion of VEGF protein. As in our studies, this effect was reduced by NOS inhibition [33]. The same authors showed that tumors engineered to express iNOS, contained more small blood vessels than control counterparts, and synthesized more VEGF [34].

The potential mechanism underlying the NO-induced

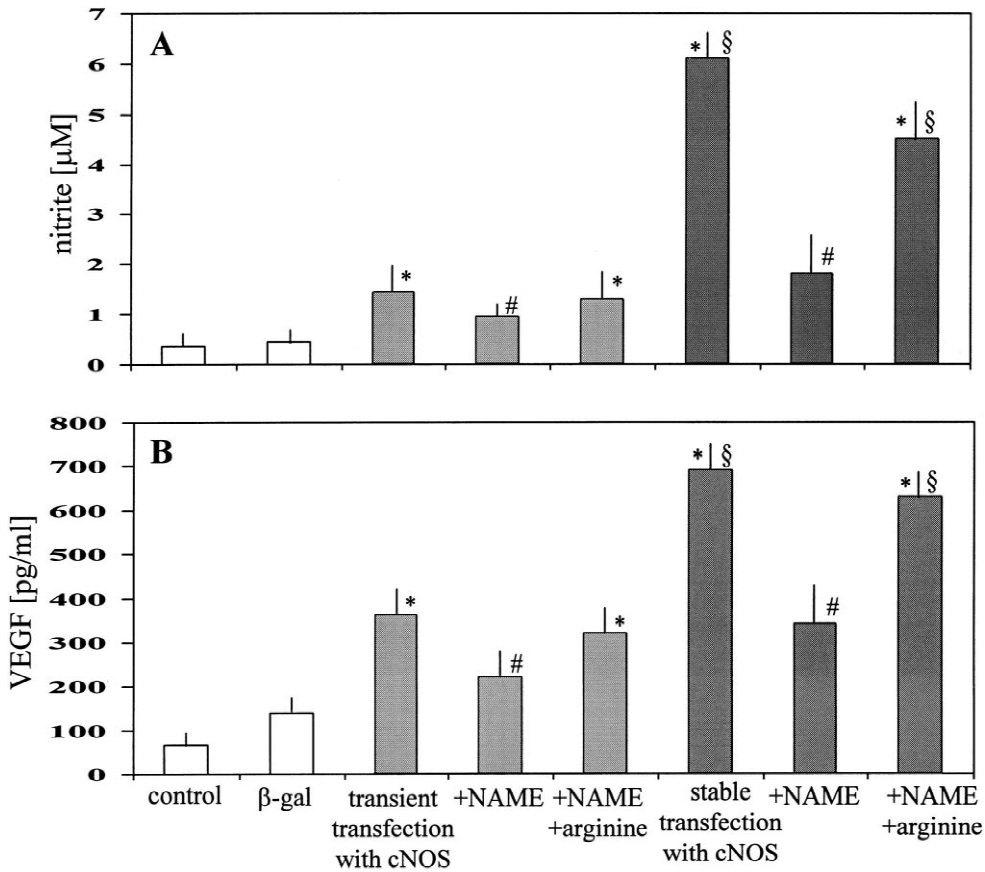

Fig. 6. (A) Effect of transient or stable transfection of rat VSMC with eNOS on the generation of nitrites after 48 h incubation (fluorimetric method). (B) Effect of transient or stable transfection of rat VSMC with eNOS on the generation of VEGF after 48 h incubation (ELISA). Numbers of cells were not significantly different in all groups ($P>0.1$). One of five similar experiments made in triplicates. *$P<0.01$ in comparison to β-galactosidase transfected cells, #$P<0.05$ in comparison to eNOS transfected cells, §$P<0.001$ in comparison to cells transiently transfected with eNOS.

augmentation of VEGF expression in VSMC is not completely understood. The VEGF promoter contains many regulatory sequences including consensus binding sites for SP-1, AP-1, and AP-2 transcription factors, as well as for hypoxia-inducible factor-1 (HIF-1) [35,36]. The NO donor SNP attenuated VEGF synthesis through inhibition of HIF-1 or AP-1 [18,37]; however, as noted above, SNP has actions independent of its release of NO. It has been also reported that NO upregulates AP-1 binding activity in VSMC [38]. Furthermore, new data using tumor cells, has revealed that NO stabilizes HIF-1α subunit in normoxic conditions, leading to higher HIF-1 binding capacity and increased transcriptional activity of the VEGF promoter [27]. In the current study, we have clearly shown that NO-donors or NOS transfection increases transcriptional activity of the VEGF promoter in VSMC. In tumor cells, NO prolongs the half-life of VEGF mRNA [24]. Thus it is possible that in VSMC the overall response to NO may be due to a combination of transcriptional activation and mRNA stabilization. Additionally, NO can influence gene expression by direct control of iron regulatory proteins (IRP) [39]. Thereby NO is capable of depleting intracellular iron storage, imitating the effect of iron-chelators [40]. Iron depletion is known to activate VEGF expression [41]. One can speculate that the possible influence of NO on the iron metabolism might contribute to the observed VEGF increase.

Xiong et al. showed that VEGF can be produced both in active and inactive forms. Post-translational ADP-ribosylation of VEGF may reduce its angiogenic capacity [16]. Therefore it is important to assay not only the VEGF concentration but also its mitogenic potency. In this study, we showed that VEGF produced by VSMC transfected with iNOS or eNOS was biologically active. Specifically conditioned media harvested from the transfected cells increased endothelial cell proliferation. Noteworthy, this effect was significantly reduced after incubation of the media with VEGF-inactivating antibodies, confirming that the mitogenic potency of NOS-transfected cells was actually VEGF-dependent. NO-dependent upregulation of VEGF expression may contribute to the acceleration of wound-healing by L-arginine [32], and the effect of L-arginine to augment angiogenesis in the ischemic limb [9]. NO upregulation of VEGF expression may also be involved in

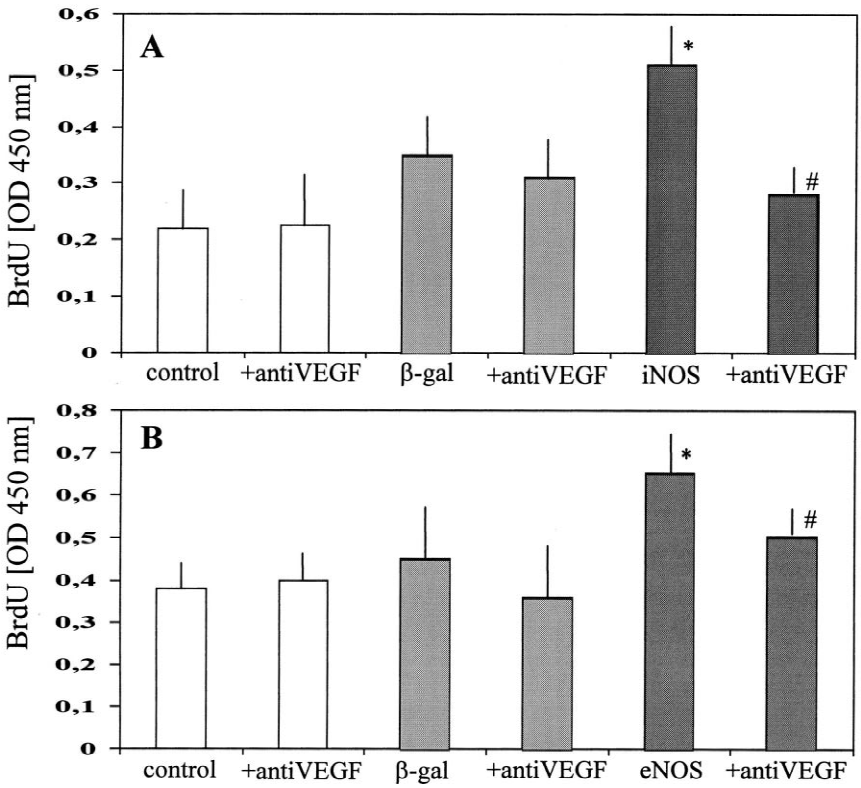

Fig. 7. Effect of conditioned media harvested from human vascular smooth muscle cells transfected with iNOS (A) or eNOS (B) on the proliferation of HUVEC after 48 h incubation (BrdU incorporation assay). One of four similar experiments made in triplicates. *$P<0.05$ in comparison to β-galactosidase transfected cells, #$P<0.05$ in comparison to iNOS or eNOS transfected cells.

the effect of *S*-nitrosylated albumin to enhance re-endothelialization after denudation in a rabbit model of vascular injury [42].

Nitric oxide is an important regulator of endothelial cell growth and angiogenesis. Inhibition of NO synthesis blocks vessel formation in the cornea micropocket assay [43], reduces flow in tumor associated neovasculature [44], delay healing of gastric ulcers [45], and retards closing of excisional wounds [46]. NO appears to be a downstream mediator of VEGF-induced endothelial cell proliferation and migration [3,8,12,13]. Our results demonstrate that NO is also an upstream promoter of VEGF expression. We found that the low concentration of NO obtained after transfection with eNOS or iNOS genes, as well as the high amounts released by NO-donors or produced after cytokine-mediated iNOS induction [14] enhance the VEGF production. Thus we suppose that NO can be an important modulator of VEGF synthesis under physiological conditions, e.g. in the vessel wall, when small amounts of NO are generated, and in pathological situations, e.g. in inflammatory diseases, when large amounts of NO are produced. We propose the existence of a paracrine loop between endothelial cells, producing NO, and the vascular smooth muscle cells generating VEGF. Consistent with this model are the results of Reynolds and colleagues, who also found a reciprocal relationship between NO and VEGF during neoangiogenesis in the developing corpus luteum [47].

In conclusion, we showed that VSMC transfected with iNOS or eNOS genes generate NO, and produce greater amounts of biologically active VEGF. The effect of NOS transfection is blocked by NOS inhibitors, and is restored by the NO precursor L-arginine. Our results indicate the existence of positive feedback between NO and VEGF, and establishes a pro-angiogenic interaction between endothelium and perivascular cells.

Acknowledgements

We thank Ms Ewa Piatkowska, Ms Anna Smolira and Mr Robert Smolira for technical assistance. The research was supported in part by grants from the Polish State Committee for Scientific Research (KBN grant Nos. 4 P05A 108 174 and P05A 131 14, awarded to A.J. and J.D., respectively) and by a grant from the National Heart, Lung and Blood Institute (R01 HL-58638 awarded to J.P.C.). Dr Dulak is the recipient of a fellowship from the Austrian Society of Cardiology. Dr Cooke is an Established Investigator of the American Heart Association.

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