Suppression of Hypoxia-Associated Vascular Endothelial Growth Factor Gene Expression by Nitric Oxide via cGMP

Neil Ghiso,1 Richard M. Roban,1 Shiro Amano,1,2 Rebecca Garland,1 and Anthony P. Adamis1,2

PURPOSE. To investigate the suppressive effect of nitric oxide (NO) on vascular endothelial growth factor (VEGF) gene expression and to elucidate its mechanism of action.

METHODS. Immortalized human retinal epithelial (RPE) cells, H-ras-transfected murine capillary endothelial cells, and nuclear factor-κB (NF-κB) RelA knockout 3T3 fibroblasts had VEGF gene expression stimulated by hypoxia, TPA (phorbol ester 12-O-tetradecanoylphorbol-13 acetate), and ras-transfection. The dose response and time course of inhibition of VEGF gene expression by NO were characterized by northern blot analysis, ribonuclease protection assay, and enzyme-linked immunsorbent assay. The effects of NF-κB and cGMP in the NO-induced suppression of VEGF gene expression were quantitated. cGMP production was inhibited by LY 83583 (6-anilino-5,8-quino-linedione), a specific inhibitor of guanylate cyclase production, and cGMP accumulation was quantitated by immunoassay. RelA knockout 3T3 fibroblasts were used to assess the contribution of NF-κB to the downregulation of VEGF by NO.

RESULTS. The NO donor sodium nitroprusside (SNP) decreased hypoxia-induced VEGF gene expression in a dose- and time-dependent manner. One hundred fifty micromolar SNP completely suppressed hypoxia-induced VEGF mRNA levels for at least 24 hours. Constitutive VEGF expression was not altered by SNP. The SNP-mediated decreases in VEGF expression were associated with increases in intracellular cGMP and were blocked by LY 83583. Sodium nitroprusside was able to decrease hypoxia-induced VEGF mRNA increases in fibroblasts deficient in the RelA subunit of NF-κB. Nitric oxide was also effective at suppressing increased VEGF expression secondary to mutant ras and TPA.

CONCLUSIONS. These data indicate that NO decreases hypoxia-induced VEGF via a cGMP-dependent mechanism and suggest that NO may serve as an endogenous inhibitor of both hypoxia- and non-hypoxia-enhanced VEGF expression in vivo. (Invest Ophthalmol Vis Sci. 1999;40:1033–1039)
ing the characteristics and mechanisms of NO regulation on hypoxia-induced VEGF expression.

**METHODS**

**Materials**

A 404-bp *NcoI/BgIII* fragment from the human VEGF<sub>121</sub> cDNA, a 575-bp fragment from the coding region of the mouse VEGF cDNA, and a 400-bp fragment from the 3′ untranslated region (UTR) of the human β-actin gene were used to probe northern blots. Radiolabeled probes were synthesized using a [α-32P]-dCTP (New England Nuclear, Boston, MA). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO). Sodium nitroprusside, 12-O-tetradecanoylphorbol-13 acetate (TPA), and LY 83583 were purchased from Sigma Chemical (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma Chemical (St. Louis, MO).

**Cell Lines and Culture Conditions**

Human retinal pigment epithelial (RPE) cells were immortalized with SV40 large T-antigen as described previously. This cell line has accurately predicted the in vivo regulation of retinal VEGF gene expression in previous studies. The RPE cells were grown in DMEM supplemented with 10% FCS and 1% GPS. Murine pancreatic islet capillary endothelial cell lines transfected with SV40 large T alone (MSI) or SV40 large T and oncogenic H-ras (SVR) were created as described previously and were grown in DMEM + 5% FCS and 1% GPS. The NF-κB subunit RelA +/- and RelA -/- 3T3 fibroblasts were generated as described previously and grown in DMEM + 10% bovine calf serum and 1% GPS. Unless otherwise indicated, all experiments were performed on all blots within the linear range of quantitation, and the VEGF signal was normalized to the corresponding actin signal for each lane using an IS-1000 Digital Imaging System with version 1.97 software (Alpha Inotech, Torrence, CA). Each northern blot result was repeated at least twice. When repeat experiments were done on the same gel, statistical testing was performed.

**RNase Protection Assay**

The entire coding region of the human VEGF<sub>121</sub> cDNA was used to generate α-32P-labeled antisense riboprobe according to the manufacturer’s protocols (Ambion, Austin, TX). All samples were simultaneously hybridized with an 18S riboprobe (Ambion) to normalize for variations in loading and recovery of RNA. RNase protection assays were performed essentially as described previously except that organic extraction was replaced with precipitation of the protected fragments from a chaotropic solution. Protected fragments were separated on gels of 5% acrylamide, 8 M urea, and 1 X Tris-buffered EDTA and quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**VEGF Protein Assay**

Secreted VEGF protein was assayed by sandwich enzyme-linked immunosorbent assay using the Quantikine human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN). Cell-conditioned medium was incubated with polyclonal anti-VEGF antibody bound to a 96-well microtiter plate. Enzyme-linked antibody was added, and a colorimetric assay was used to determine the amount of VEGF present according to the manufacturer’s instructions. VEGF concentrations were quantified by interpolation from a standard curve and normalized to the total protein.

**cGMP Assay**

Subconfluent RPE cells in 35-mm wells were exposed to serum-free medium for 30 minutes, with or without LY 83583. The RPE cells were incubated with the indicated amount of SNP for the final 10 minutes of incubation with LY 83583. The media were removed, and the cells were harvested by ice-cold 65% EtOH, lyophilized, and resuspended in the assay buffer of the cGMP enzyme immunoassay kit (Amersham, Piscataway, NJ). Intracellular samples competed with known quantities of peroxidase-labeled cGMP to bind to antibody in assay plates. The amount of bound peroxidase-labeled cGMP was quantified by immunoassay using 450 nm optical density measurements. The cGMP levels in the samples were determined by comparing optical density measurements to those of known doses of cGMP.
VEGF Suppression by NO 1035

RESULTS

Hypoxic Stimulation of VEGF Expression Inhibited by SNP-Donated NO

Because hypoxia is a physiologically relevant stimulus for VEGF expression,12 the effects of the NO-donor SNP on hypoxia-induced VEGF in RPE-conditioned media were examined. VEGF protein levels increased twofold in hypoxic versus normoxic RPE-conditioned media. Increasing concentrations of SNP blocked the hypoxia-induced VEGF increases in a dose-dependent manner. Hypoxia increased VEGF mRNA levels approximately fivefold in RPE cells, and a similar dose-dependent decrease with SNP was observed (Fig. 1A). One hundred fifty micromolar SNP completely suppressed the hypoxic induction of VEGF mRNA. The same concentration of SNP decreased VEGF protein levels less dramatically. The disparity is most likely due to the 12 hours required for the mRNA levels to decrease when exposed to 150 μM SNP (Fig. 2).

Another potential reason is that some of the secreted VEGF may remain cell-associated, as has been previously shown.29 No clear significant dose-dependent decrease in constitutive VEGF mRNA levels by SNP was observed. Sodium nitroprusside concentrations greater than 10 mM induced cell injury manifesting as rounded and floating cells and RNA degradation. No toxic changes were seen at the lower doses that suppressed VEGF gene expression.

Time Course of SNP Reversal of VEGF Hypoxic Upregulation

Hypoxia-induced increases in RPE VEGF mRNA reach steady state by 24 hours and remain constant for at least 6 days (N. Ghiso and A. P. Adamis, unpublished observations). The time course of SNP action on steady state hypoxia-induced VEGF mRNA increases was determined by exposing RPE cells that had been preincubated under hypoxic conditions for 24 hours to 150 μM SNP. Reoxygenation and free radical formation were avoided through use of a glove chamber, which allowed culture manipulation without reoxygenation. Reoxygenation and free radicals have been shown to be potent stimulators of VEGF gene expression.11 Cells placed in continuous hypoxia exhibited a nearly threefold induction of VEGF mRNA relative to normoxia (Fig. 2). Sodium nitroprusside-induced decreases in VEGF mRNA were noted as early as 4 hours and reached a nadir by 12 hours, remaining constant to 24 hours (Fig. 2).

Inhibition by SNP of VEGF Upregulation by Phorbol Ester

Phorbol ester increases VEGF expression.8 To test the blocking effects of SNP-donated NO on VEGF induced by a nonhypoxic mechanism, 12-O-tetradecanoylphorbol-13 acetate (TPA, final concentration 100 nM) was added to RPE cell cultures preincubated for 16 hours with and without 150 μM SNP. Total RNA was collected 2 hours after TPA was added. SNP completely suppressed the TPA-induced increases in VEGF mRNA levels (Fig. 3A).

FIGURE 1. Sodium nitroprusside-donated NO blocks hypoxic-induced VEGF expression in RPE cells. (A) RPE-conditioned media were assayed for secreted VEGF after 24 hours of normoxia (21% O2) or hypoxia (3% O2) and increasing doses of SNP. VEGF was normalized to total secreted protein, which remained unchanged with NO exposure (data not shown). Sodium nitroprusside significantly blocked hypoxic induction of VEGF (bars 2 through 7) but had no significant effect on the normoxic expression of VEGF (data not shown). The bars marked with the same symbols (*, **, ***) have statistically significant differences between them (Student’s t test). (B) RPE VEGF mRNA on a representative northern blot was normalized to β-actin after 24 hours of normoxia or hypoxia and increasing doses of SNP. Sodium nitroprusside blocked the hypoxic induction of VEGF (lanes 4, 5, 6) in a dose-dependent manner. Constitutive VEGF levels were not affected in a dose-dependent manner (lanes 1, 2, 3).

FIGURE 2. Time course of SNP effect on hypoxia-induced VEGF expression. RPE cells placed in a hypoxic airtight glove box permitted experimental manipulation without exposure to normoxia. Hypoxic VEGF mRNA levels in this representative blot were increased threefold over normoxic cells. Sodium nitroprusside (150 μM) added at time 0 decreased VEGF mRNA levels at the indicated time intervals.
Inhibition by SNP of VEGF Upregulation Secondary to ras Transformation

Activation of the ras oncogene\(^1\) has been shown to increase VEGF expression. Transfection of murine endothelial cells with oncogenic H-ras\(^2\) resulted in approximately a fourfold increase in VEGF expression, as previously described.\(^3\)

The effect of SNP-donated NO was tested on ras-stimulated VEGF by incubating SVR cells with increasing concentrations of SNP. A dose-dependent decrease in VEGF expression was observed with 250 \(\mu\)M SNP, reducing VEGF mRNA levels to those of non-ras-transfected cells (Fig. 3B).

Involvement of cGMP in SNP-Mediated VEGF Suppression

Nitric oxide can increase cGMP in many cell types in a few minutes, and cGMP mediates many of the downstream effects
FIGURE 4. Sodium nitroprusside’s action on VEGF depends on cGMP. (A) RPE cells tested for cGMP levels after exposure to increasing doses of SNP. Ten minutes of 150 μM SNP increased cGMP levels over twofold (bars 1, 2, 3). Production of cGMP was blocked when the cells were pretreated for 20 minutes with the guanylate cyclase inhibitor LY 83583. (B) VEGF protein was increased after 24 hours of hypoxia (bars 1, 2), which could be blocked by SNP added at time 0 (bars 2, 3). The SNP-induced decreases were reversed by a 20-minute pretreatment with LY 83583 (bars 4, 5, 6). *P < 0.05 between bars 2 and 5 and bars 2 and 6, where n = 4 per condition (Bonferroni-Dunn adjustment of the ANOVA).

DISCUSSION

The NO donor SNP potently suppressed hypoxia-induced VEGF gene expression in a dose-dependent and sustained fashion in an RPE cell line used to research VEGF behavior in the retina. The effects of SNP on VEGF expression were also observed in transformed cells with high VEGF levels secondary to mutant H-ras or phorbol ester. Taken together with the previous observation that different NO donors can decrease protein kinase C- and tyrosine kinase pp60src-induced VEGF expression, these data suggest that NO either works on a pathway common to multiple VEGF stimuli or that NO exerts its effects through multiple pathways. Tsurumi and colleagues showed that NO blocked protein kinase C- and tyrosine kinase pp60src-induced VEGF increases by interfering with the binding of the transcription factor AP-1 to the VEGF promoter. However, previous work in the RPE cell line that we used has shown that the hypoxia-induced VEGF increases are primarily due to increased stability of the VEGF mRNA transcript. This indirectly suggests that the NO-induced decrease of VEGF protein was mediated through cGMP, which is consistent with the hypothesis that NO acts through guanylate cyclase to increase cGMP levels. The role of cGMP in mediating the NO effects was tested with the guanylate cyclase inhibitor LY 83583. Increasing doses of SNP (50 μM and 150 μM) added to RPE cells for 10 minutes increased intracellular cGMP levels 2.5-fold. This induction of cGMP was completely blocked by the addition of 2.0 μM LY 83583 20 minutes before the addition of SNP (Fig. 4A). However, the inhibition of cGMP levels by LY 83583 only blocked up to 59% of the NO-induced decreases in hypoxic RPE VEGF gene expression (Fig. 4B).

NF-κB/RelA Subunit: Not Involved in the SNP Blockade of Hypoxia-Induced VEGF Upregulation

Nitric oxide is known to inhibit NF-κB activity by decreasing DNA binding56 and by increasing IκBα stabilization.55 NF-κB is a dimeric family of transcription factors (reviewed in Ref. 39), the most common form being a heterodimer of RelA and p50. We tested the possible role of NF-κB in the SNP-mediated suppression of VEGF in 3T3 RelA knockouts. Wild-type and RelA −/− 3T3 fibroblasts were exposed to hypoxia with and without 150 μM SNP. Both wild-type and RelA −/− cells showed a nearly threefold induction of VEGF secondary to hypoxia (Fig. 5), although the constitutive VEGF levels in the RelA −/− cells were lower. The hypoxic induction of VEGF mRNA was completely blocked by 150 μM SNP in both cell types.

Figure 5. Sodium nitroprusside’s action is independent of NF-κB/RelA. 3T3 RelA +/+ (wild-type) and RelA −/− cells had VEGF mRNA levels quantitated after 16 hours of normoxia or hypoxia and the addition of SNP (150 μM). Hypoxia-induced increases in VEGF mRNA by northern blot were of the same fold of magnitude in both the RelA +/+ and RelA −/− cells (lanes 1, 2 and lanes 4, 5, respectively). The addition of 150 μM SNP blocks the induction in both cell lines (lanes 2 versus 3, lanes 4 versus 5, respectively). P < 0.01 between lanes 4 and 6 (Student's t-test, n = 3).
creases in RPE VEGF occur through a reduction in the half-life of the VEGF transcript in hypoxia. As such, the data are consistent with NO affecting multiple pathways. This conclusion is further supported by the data showing that LY 83583 completely blocked NO-induced cGMP production but only partially suppressed the effect of NO on VEGF expression.

General NO effects are known to be mediated primarily through cGMP and/or NF-κB. The data from these studies suggest that intracellular cGMP, but not NF-κB, mediates the in vitro VEGF-lowering effects of SNP. Increased intracellular cGMP levels correlate with decreased VEGF levels, and use of the specific inhibitor of soluble guanylate cyclase, LY 83583, lowered intracellular cGMP and partially blocked NO’s effect on VEGF protein levels in RPE cells. Interestingly, the effect of NO on cGMP took 10 minutes, whereas the effect on steady state VEGF levels required 4 to 8 hours, a time delay potentially consistent with subsequent signaling steps, and/or mRNA turnover rates. The effect of NO on the hypoxia-induced increases in VEGF expression did not depend on NF-κB/RelA. Murine fibroblasts deficient in the RelA component of NF-κB increased VEGF mRNA expression under hypoxic conditions to the same degree as did the wild-type cells. Moreover, SNP was able to completely abrogate the hypoxia-induced VEGF increases in these NF-κB/RelA-deficient cells.

Hypoxia is a relevant inducer of VEGF expression in tumors and the retina. Nitric oxide is present in the normal retina, and NO levels are increased during retinal ischemia and inflammatory disorders. Thus, the inhibition of VEGF expression by NO may have significant biological relevance. Moreover, it is well documented that VEGF increases NO production in endothelial cells. Thus, it is possible that a reciprocal regulation between VEGF and NO exists in the retina, much like that recently demonstrated in the coronary arteries by Tsurumi and colleagues. Ischemia may directly increase VEGF expression and NO concurrently, with their simultaneous expression preventing the unchecked upregulation of VEGF.

Because NO not only prevents, but also reduces, high VEGF levels, NO may serve an additional valuable clinical role in the future. Clinically approved drugs like SNP and nitroglycerin may prove useful in the treatment of ischemic retinal disease, much as they already do in ischemic heart disease. Exogenous NO may be used to augment endogenous NO to dilate the vasculature and to increase retinal blood flow. In addition, the NO may serve to check the increased expression of VEGF and the pathological consequences that accompany it in the eye. However, the extrapolation of the current results to the in vivo state must be made with caution. In vivo confirmation is ultimately needed. The potential uses for NO deserve further study.

Acknowledgment

The 404-bp NcoI/HindII fragment of the human VEGF cDNA was generously provided by Herbert Weich. The RelA-1 cells were generously provided by Amer Beg.

References


