The soluble guanylyl cyclase inhibitor NS-2028 reduces vascular endothelial growth factor-induced angiogenesis and permeability

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Submitted 22 April 2009; accepted in final form 9 December 2009

Morbidelli L, Pyriochou A, Filippi S, Vasileiadis I, Roussos C, Zhou Z, Loutrari H, Waltenberger J, Stössel A, Giannis A, Ziche M, Papapetropoulos A. The soluble guanylyl cyclase inhibitor NS-2028 reduces vascular endothelial growth factor-induced angiogenesis and permeability. Am J Physiol Regul Integr Comp Physiol 298: R824–R832, 2010. First published December 23, 2009; doi:10.1152/ajpregu.00222.2009.—Nitric oxide (NO) is known to promote vascular endothelial growth factor (VEGF)-stimulated permeability and angiogenesis. However, effector molecules that operate downstream of NO in this pathway remain poorly characterized. Herein, we determined the effect of soluble guanylyl cyclase (sGC) inhibition on VEGF responses in vitro and in vivo. Treatment of endothelial cells (EC) with VEGF stimulated eNOS phosphorylation and cGMP accumulation; pretreatment with the sGC inhibitor 4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)(1,4)oxazin-1-one (NS-2028) blunted cGMP levels without affecting VEGF-receptor phosphorylation. Incubation of cells with NS-2028 blocked the mitogenic effects of VEGF. In addition, cells in which sGC was inhibited exhibited no migration and sprouting in response to VEGF. To study the mechanisms through which NS-2028 inhibits EC migration, we determined the effects of alterations in cGMP levels on p38 MAPK. Initially, we observed that inhibition of sGC attenuated VEGF-stimulated activation of p38. In contrast, the addition of 8-Br-cGMP to EC stimulated p38 phosphorylation. The addition of cGMP elevating agents (BAY 41-2272, DETA NO and YC-1) enhanced EC migration. To test whether sGC also mediated the angiogenic effects of VEGF in vivo, we used the rabbit cornea assay. Animals receiving NS-2028 orally displayed a reduced angiogenic response to VEGF. As increased vascular permeability occurs prior to new blood vessel formation, we determined the effect of NS-2028 in vascular leakage. Using a modified Miles assay, we observed that NS-2028 attenuated VEGF-induced permeability. Overall, we provide evidence that sGC mediates the angiogenic and permeability-promoting activities of VEGF, indicating the significance of sGC as a downstream effector of VEGF-triggered responses.

cGMP; vessels; sprouting; leakage; p38

Soluble guanylyl cyclase (sGC) is a ubiquitous enzyme that converts GTP to the second messenger molecule cGMP (12, 15). cGMP modifies the activity of protein kinases, phosphodiesterases, and ion channels to change the behavior of cells and tissues (21). sGC is a heterodimeric protein that consists of an alpha and a beta subunit; the holoenzyme also contains a molecule of heme that serves as a sensor for nitric oxide (15). Binding of nitric oxide (NO) to the heme moiety of sGC increases dramatically its catalytic activity (21). Both genetic and pharmacological approaches have proven the importance of sGC in reducing smooth muscle tone and inhibiting platelet aggregation (21, 22).

Although, many small molecules with sGC-stimulating activity have been identified and are extensively used in vitro and in vivo, there are only two chemically related sGC inhibitors, namely ODQ and NS-2028 (14, 27). Their mechanism of action is believed to involve oxidation of the heme moiety, thus, making sGC unresponsive to the action of NO. Between the two, ODQ is by far the most widely used inhibitor of sGC, but its use in vivo has been limited. To the best of our knowledge, NS-2028 has yet to be used in vivo. Lack of experimental evidence with sGC inhibitors in animal studies has limited our ability to determine the contribution of sGC in physiological and pathophysiological conditions in vivo.

Angiogenesis, the process of blood vessel formation from existing structures, is tightly regulated in adult organisms (3, 11). In fact, angiogenesis occurs only in a few physiological settings, such as those associated with wound healing and normal ovary cycling (4). Angiogenesis is a crucial adaptive response to exercise training and also critical for the survival of ischemic tissues, as it helps in collateral vessel formation (3). In contrast, excessive or deregulated angiogenic responses contribute to tumor growth, inflammation, and destruction of the retina in diabetes and are important for the pathogenesis of psoriasis and arthritis (4). Vascular endothelial growth factor (VEGF) is a key modulator of angiogenesis, promoting endothelial cell migration, proliferation, and survival (9). A number of studies have proposed that NO plays a permissive role for angiogenesis and that it mediates many of the angiogenic effects of VEGF in vitro and in vivo (13, 24, 28, 39, 40). Results from experiments with cultured EC suggest that some of the angiogenic actions of NO are dependent on cGMP formation (29, 30, 39), and GMP-elevating agents have the ability to enhance EC migration (30, 32). However, cGMP-independent angiogenic actions of VEGF and NO have also been described (6, 31). The aim of the present study was to determine which of the VEGF-triggered angiogenesis-related...
properties are sGC dependent and to investigate whether sGC contributes to VEGF-stimulated angiogenesis and permeability in vivo.

METHODS

Cell culture. Human umbilical vein endothelial cells (HUVEC) were isolated from 2–4 fresh umbilical cords and grown on culture dishes (Corning-Costar, Corning, NY) in M199 medium supplemented with 15% FCS (Life Technologies GIBCO-BRL, Paisley, UK), 50 U/ml penicillin and 50 μg/ml streptomycin (Applichem, Darmstadt, Germany), 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 5 U/ml sodium heparin (Biochrom AG, Berlin, Germany), and 150–200 μg/ml endothelial cell growth supplement (ECGS) made from bovine brain. HUVEC between passages 2 and 3 were used for all experiments. Starvation medium lacked serum and ECGS compared with complete medium. Postcapillary venular endothelial cells (CVEC) (33) were cultured on gelatin-coated dishes in DMEM, with 1,000 mg glucose/liter, containing 10% BCS (Hyclone, Logan, UT). Cells between passage 16 and 20 were used in the experiments. Porcine aortic endothelial cells (PAEC) overexpressing vascular endothelial growth factor receptor-2 (VEGFR-2) [PAEC-knase domain receptor (KDR)] (35) were maintained with Ham’s F-12 medium supplemented with 10% FCS and 500 μg/ml G418 sulfate antibiotic. PAEC cells were split weekly 1:3.

cGMP immunoassay. Confluent monolayers of HUVEC were washed twice with Hank’s balanced salt solution and incubated for 30 min with 0.1–10 μM NS-2058 or vehicle (DMSO) prior to being stimulated with VEGF (VEGF-A165; 20 ng/ml; Peprotech, London, UK) in the presence of the phosphodiesterase inhibitor isobutyl methylxanthine (1 mM; Sigma-Aldrich, St. Louis, MO). After 15 min, cells were lysed in 0.1 N HCl, and cGMP content was measured in the extracts using a commercially available enzyme immunoassay kit (Assay Designs; Ann Arbor, MI) following the manufacturer’s instructions. NS-2058 was synthesized on the basis of modifications of published methods (1).

Cell proliferation. HUVEC were seeded in 24-well plates at 6 × 10^5 cells/well and incubated for 24 h. Cells were then exposed to NS-2058 (10 μM) or vehicle in complete medium and allowed to proliferate for 48 h. Cell proliferation was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) method. In experiments aimed at evaluating DNA synthesis, subconfluent and serum-starved EC were treated with stimuli for 24 h, and bromodeoxyuridine (BrdU) uptake was assessed by a commercially available kit (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions; the number of stained cells was randomly counted at ×100 magnification. In these experiments, we chose to use CVEC instead of HUVEC, as serum starvation of the latter for 24 h leads to apoptosis. An additional advantage of using CVEC is that EC derived from postcapillary venules is a more relevant EC type to study in the context of angiogenic responses.

Caspase-3 activity assay. Caspase-3 activity was determined by measuring the proteolytic cleavage of the fluorogenic substrate Z-DEVD-AMC (Caspase-3 activity kit; Molecular Probes, Eugene, OR). Briefly, HUVEC were grown to confluence and then exposed to NS-2058 (10 μM). As a positive control, some groups of cells were treated with 100 μg/ml of the protein synthesis inhibitor cycloheximide (Sigma-Aldrich). After 24 or 48 h, both floating and adherent cells were collected and washed twice with PBS. Cells were then lysed in a buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.01% Triton X-100. The fluorescence of the cleaved reporter group was measured 30 min after the addition of 100 μM substrate at 380-nm excitation and 469-nm emission. Relative fluorescence unit data were normalized per milligram protein.

Cell migration. Cells were serum starved and subsequently treated with NS-2058 (10 μM), the p38 inhibitor SB203580 (3 μM), or vehicle for 30 min before trypsinization. After trypsinization, 1 × 10^5 cells were added to transwells (8-μM pore size; Corning Costar) in 100 μl of starvation medium. VEGF (20 ng/ml), bFGF (10 ng/ml; Peprotech, London, UK), DETA-NO (10 μM; Sigma-Aldrich), YC-1 (10 μM; Sigma-Aldrich), BAY 41-2272 (10 μM; Sigma-Aldrich), or the vehicle were added to the well containing the transwell inserts at 600 μl of volume. NS-2058 or SB203580 were added in both the upper and lower compartments of the transwell setup. HUVEC were allowed to migrate for 4 h at 37°C, and after this time, nonmigrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells were fixed in Carson’s solution for at least 30 min at room temperature and then stained in toluidine blue for 20 min at room temperature. Migrated cells were scored in eight random fields at ×200 magnification.

Scratch wound assay. CVEC were plated in 24-well plates at 1 × 10^5 cells/well and incubated for 24 h to become 90% confluent. Cell monolayers were scored vertically down the center of each well with a sterile tip. Each well was washed with PBS to remove detached cells. Fresh media containing the test substance with ARA C (2 μg/ml) to inhibit cell proliferation were added. Images of the wound in each well were acquired from time 0 for up to 24 h under phase contrast microscopy at the magnification of ×10. Results are expressed as a percentage change in wound area per time point.

Western blot analysis. After the indicated treatments, proteins were extracted after homogenization in a lysis buffer containing 1% Triton-X, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA, and protease inhibitors: 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 20 mM PMSF (all from Sigma-Aldrich). Samples were subjected to SDS-PAGE, transferred to an activated PVDF membrane, and incubated with the indicated primary Ab (anti-phospho-eNOS and eNOS antibodies, anti-phospho-tyrosine, phospho-p38, p38, phospho-ERK1/2, and ERK1/2 antibodies, obtained from Cell Signaling Technology, Beverly, MA; anti-KDR antibody, obtained from Upstate, Lake Placid, NY) and appropriate secondary antibodies (Cell Signaling Technology or Sigma-Aldrich). Immunoreactive proteins were detected using a chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). DC Protein assay kit and immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA).

Rat aorta ring assay. Aortas from Wistar rats (Charles River, Calco, Como, Italy) were isolated in sterile conditions. Rings 1–2 mm long were produced and included in a fibrin gel obtained by adding 400 μl of a bovine fibrinogen solution (3 mg/ml in M199 medium; Sigma-Aldrich) into each well of 48-multicwell plates. Gelation of the fibrinogen was induced with bovine thrombin (1.5 units/ml; Sigma-Aldrich). After 20 min, 400 μl of M199 medium was added with antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), amphotericin B (0.25 μg/ml) and 10% FCS were added to each well. After 24 h, the medium was removed, the gels were washed, and NS-2058 (10 μM) or vehicle (medium supplemented with 1% FCS and DMSO) was added for 30 min followed by treatment with VEGF or bFGF (50 ng/ml) or medium for 72 h. Quantitative evaluation of newly formed structures was carried out on day 3. At a magnification of 200, the area occupied by tubules was measured using an ocular grid (0.21 mm²). The result is expressed as the number of grid units (10).

Rabbit cornea in vivo angiogenesis assay. Experiments have been performed in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609). The effect of NS-2058 on the angiogenic activity of VEGF was assayed in vivo using the rabbit cornea assay, as described previously (40). Briefly, New Zealand White albino rabbits (Charles River, Calco, Como, Italy) were pretreated with NS-2058 (1 g/l) or vehicle (ethanol) provided in the drinking water for eight consecutive days and discontinued the day of pellet implantation. Water consumption was 300 ml/day. Slow-release pellets bearing VEGF (300 ng) were prepared in sterile conditions. Rabbits were anesthetized by sodium pentothal (30 mg/kg), and pellets were implanted in micro-
pockets surgically produced in the lower half of the eye in the transparent avascular corneal stroma. Subsequent daily observations of the implants were made for 15 days with a slit lamp stereomicroscope without anesthesia by a blinded observer. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3 or 4 days, and capillaries progressed to reach the implanted pellet. Angiogenic activity was expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency was scored by the number of newly formed vessels and by their growth rate. Data were expressed as angiogenesis score, calculated as vessel density × distance from limbus in millimeters as previously described (40).

Aortic ring vasorelaxant response. Rabbits treated with NS-2028 or vehicle for 8 days, as described above, were killed on day 9 after drug discontinuation by sodium pentothal overdose, and the thoracic aorta was cut into 3- to 3.5-mm-wide transverse rings. Each aortic ring was mounted in an organ bath by means of two triangle-shaped stainless-steel wires. The lower wire was fixed to the bottom of the chamber, while the upper wire was connected to the force transducer. The rings were suspended under a tension of 2 g, and isometric force of contraction was measured and recorded on a chart recorder. The preparations were allowed to equilibrate for at least 1 h in a gassed (95% O2, 5% CO2) Krebs solution (115 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.4 mM CaCl2, 1.2 mM MgSO4, 10 mM glucose) at 37°C. Cumulative dose-response curves for ACh (0.01–10 μM) were performed in preparations preconstricted with NA. The maximal force of contraction induced by NA was taken as 100%, and the relaxation induced by ACh was referred to this value.

Modified Miles assay. One hour prior to the measurement of vascular leakage, mice (C57/BL6) received 10 mg/kg NS-2028 ip or vehicle. Under anesthesia (ketamine/xylazine), mice were injected intravenously with Evans blue (30 mg/kg; Sigma-Aldrich). VEGF (300 ng in 15 μl) or saline was injected intradermally into the dorsal surface of the right and left ears, respectively. After 30 min, mice were euthanized, and the ears were removed, oven-dried at 55°C, and surface-ashed at 37°C. Cumulative dose-response curves for ACh (0.01–10 μM) were performed in preparations preconstricted with NA. The maximal force of contraction induced by NA was taken as 100%, and the relaxation induced by ACh was referred to this value.

RESULTS

sGC blockade inhibits the angiogenic phenotype of EC. In our first series of experiments, we examined the effect of sGC inhibition on VEGF-triggered angiogenesis-related properties of EC. In line with what is known about VEGF (28), incubation of cells with this growth factor stimulated eNOS phosphorylation on Ser 1177 (Fig. 1A). Phosphorylation of eNOS on this residue, along with the increase in intracellular Ca2+ brought about by VEGF, leads to eNOS activation that, in turn, activates sGC to increase cGMP levels (Fig. 1B). Pretreatment of HUVEC with NS-2028 inhibited VEGF-induced cGMP accumulation in a concentration-dependent manner. Concentrations of NS-2028 as low as 0.1 μM inhibited more than 90% of the VEGF response, while 10 μM of NS-2028 abolished VEGF-stimulated cGMP production (Fig. 1B). The highest NS-2028 concentration was used in all of the subsequent experiments in vitro, as we wanted to ensure complete blockade of VEGF-stimulated cGMP formation. We felt this was necessary, since the presence of 5–10% of normal sGC levels is sufficient to elicit maximal responses, at least in the context of vasodilation (22). To rule out a direct effect of NS-2028 on VEGF receptor phosphorylation and activation, cells were pretreated with the sGC inhibitor, and tyrosine phosphorylation (p-Tyr) of KDR (VEGFR2) was determined in cells overexpressing this VEGF receptor. NS-2028 exposure did not affect p-Tyr of KDR, while incubation with SU5614, a VEGF receptor kinase inhibitor, abolished VEGF-stimulated p-Tyr of KDR (Fig. 1C). The above data taken together confirm that disruption of VEGF signaling by NS-2028 occurs downstream of VEGF receptors.

Having shown that sGC is a downstream target of VEGF signaling, we sought to determine the effects of sGC inhibition on VEGF-stimulated migration, spouting, and growth. Initially, we determined whether inhibition of sGC affects proliferation. The number of cells present in HUVEC cultures grown in the presence of NS-2028 was ~25% lower compared with those grown in the presence of vehicle, underlining the significance of sGC in cell growth (Fig. 2A). To evaluate the effect of NS-2028 on VEGF-stimulated mitogenesis, we determined BrdU incorporation into DNA, a direct measure of cell prolif-

Fig. 1. Vascular endothelial growth factor (VEGF) signals through endothelial nitric oxide synthase (eNOS) and soluble guanylyl cyclase (sGC) in EC. A: cells were serum starved for 5 h and then treated with NS-2028 (10 μM) or vehicle for 30 min. They were then stimulated with VEGF (20 ng/ml) for the indicated times and lysed. Lysates were analyzed by SDS-PAGE. Membranes were blotted with antibodies that recognize the activated (phosphorylated) or total ENOS. A representative blot of at least three individual experiments is shown. B: cGMP levels were determined under baseline conditions (vehicle) or after a 15-min exposure to VEGF (20 ng/ml) in the presence of the indicated concentration of NS-2028; n = 4. *P < 0.05 vs. vehicle; #P < 0.05 vs. VEGF alone. C: porcine aortic endothelial cells (ECs) were stably transfected with the kinase domain receptor (KDR); serum-starved ECs were pretreated with NS-2028 (10 μM; 30 min) or SU5614 (10 μM; 30 min), and then stimulated with VEGF (50 ng/ml) for 15 min. Cell lysates were prepared and immunoblotted with an antibody against the phospho-tyrosine (p-Tyr) or KDR. The blot is representative of three individual experiments with similar results.
The motility of cells exposed to the NO-independent sGC blocked VEGF-stimulated cell migration (Fig. 4) and reduced C (MAPK; Fig. 4, cells to VEGF-activated p38 mitogen-activated protein kinase agreement with what is known in the literature, exposure of VEGF-stimulated properties of EC, focusing on migration. In mechanism involved in the inhibitory action of NS-2028 in the Eiting p38 MAPK activation.

VEGF.

the expression of the angiogenic phenotype in response to (Fig. 3, and the scratch wound assay, NS-2028 reduced VEGF-induced (Fig. 2, and 15 min of exposure. NS-2028 was capable of reducing the migration triggered by both a NO-dependent (DETA-NO) and (Fig. 4, A and B), suggesting that cGMP functions upstream of p38 to induce migration. To prove that elevations in intracellular cGMP can activate p38, cells were treated with the cell membrane-permeable analog of cGMP 8-Br-cGMP (Fig. 4F). Exposure of cells to 8-Br-cGMP led to a time-dependent increase in p38 and ERK1/2 phosphorylation that reached significance after 10 and 15 min of exposure. NS-2028 was capable of reducing the migration triggered by both a NO-dependent (DETA-NO) and a NO-independent (BAY 41-2272) activator of sGC (Fig. 4D). Overall, our results indicate that p38 is a downstream effector of sGC/cGMP in the signaling pathway of VEGF-induced migration of EC in vitro.

NS-2028 does not inhibit the angiogenic properties of bFGF. To test the specificity of action of NS-2028, we evaluated its ability to block VEGF-stimulated motility (Fig. 3), NS-2028 attenuated VEGF-enhanced p38 phosphorylation (Fig. 4, A and B), suggesting that sGC is important in VEGF-stimulated angiogenesis in vitro, we sought to determine whether NS-2028 administration could impair the process of VEGF-induced formation of new vessels in vivo. Rabbits receiving NS-2028 in the drinking water exhibited a significant reduction in the expression of the angiogenic phenotype in response to bFGF.

Fig. 2. sGC inhibition impairs VEGF-induced EC proliferation and sprouting. A: cells were treated with NS-2028 (10 μM) or DMSO (vehicle) and allowed to proliferate for 24 h. Cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; n = 3. *P < 0.05 vs. vehicle. B: BrdU uptake was evaluated in sparse and serum-starved postcapillary venular endothelial cells (CVEC) after 24-h incubation with test substances (20 ng/ml VEGF or 10 μM SNP) with or without pretreatment with 10 μM NS-2028. Data are reported as a number of positive cells counted at ×100 magnification; n = 3. *P < 0.05 vs. vehicle; #P < 0.05 vs. VEGF alone and $P < 0.05 vs. SNP alone. C: EC were treated with NS-2028 (10 μM) or the apoptosis inducer cycloheximide (CHX; 100 μg/ml) for the indicated time. Cell lysates were prepared, and caspase-3 activity was determined; n = 3. *P < 0.05 vs. vehicle. D: representative photomicrographs of EC sprouting from rat aorta rings under the different experimental conditions used. E: microvessel sprouting in rat aorta ring explants cultured in 3D-fibrin gels was evaluated under basal conditions (vehicle) or after treatment with VEGF (50 ng/ml) in the absence or presence of NS-2028 (10 μM); n = 4. *P < 0.05 vs. vehicle; #P < 0.05 vs. VEGF alone.

We next examined the effects of NS-2028 on VEGF-stimulated migration in using two different assays: a modified Boyden chamber and a scratch wound assay (Fig. 3). In the Boyden chamber assay, NS-2028 inhibited basal cell motility by 50% and abolished VEGF-induced sprout formation, we used an in vitro model. When rat aortic ring explants cultured in three-dimensional fibrin gels were treated with VEGF, microvessel formation increased three-fold (Fig. 2, D and E); VEGF-triggered sprouting was blocked by pretreatment with NS-2028.

We next studied the effects of NS-2028 on VEGF-stimulated migration in using two different assays: a modified Boyden chamber and a scratch wound assay (Fig. 3). In the Boyden chamber assay, NS-2028 inhibited basal cell motility by 50% and abolished VEGF-induced migration (Fig. 3, A and B). In the scratch wound assay, NS-2028 reduced VEGF-induced wound closure; this effect was prominent at the 24-h time point (Fig. 3, C and D). Our findings suggest that sGC is essential for the expression of the angiogenic phenotype in response to VEGF.

NS-2028 attenuates VEGF-induced EC migration by inhibiting p38 MAPK activation. We next investigated the signaling mechanism involved in the inhibitory action of NS-2028 in the VEGF-stimulated properties of EC, focusing on migration. In agreement with what is known in the literature, exposure of cells to VEGF-activated p38 mitogen-activated protein kinase (MAPK; Fig. 4, A and B). Inhibition of p38 with SB203580 blocked VEGF-stimulated cell migration (Fig. 4C) and reduced the motility of cells exposed to the NO-independent sGC activator YC-1 (Fig. 4E). Moreover, consistent with its ability to block VEGF-stimulated motility (Fig. 3), NS-2028 attenuated VEGF-enhanced p38 phosphorylation (Fig. 4, A and B), suggesting that cGMP functions upstream of p38 to induce migration. To prove that elevations in intracellular cGMP can activate p38, cells were treated with the cell membrane-permeable analog of cGMP 8-Br-cGMP (Fig. 4F). Exposure of cells to 8-Br-cGMP led to a time-dependent increase in p38 and ERK1/2 phosphorylation that reached significance after 10 and 15 min of exposure. NS-2028 was capable of reducing the migration triggered by both a NO-dependent (DETA-NO) and a NO-independent (BAY 41-2272) activator of sGC (Fig. 4D).

Overall, our results indicate that p38 is a downstream effector of sGC/cGMP in the signaling pathway of VEGF-induced migration of EC in vitro.

NS-2028 does not inhibit the angiogenic properties of bFGF. To test the specificity of action of NS-2028, we evaluated its ability to interfere with bFGF-induced migration and sprouting. Exposure of cells to bFGF stimulated their migration in the Boyden chamber assay and promoted wound closure in the scratch wound assay (Fig. 5, A, C, and D); both responses were unaffected by NS-2028 treatment. Similar results were obtained in the sprouting assay, in which microves- sel formation in response to bFGF was not inhibited by NS-2028 treatment (Fig. 5B).

NS-2028 inhibits VEGF-induced angiogenesis in vivo. Having established that sGC is important in VEGF-stimulated angiogenesis in vitro, we sought to determine whether NS-2028 administration could impair the process of VEGF-induced formation of new vessels in vivo. Rabbits receiving NS-2028 in the drinking water exhibited a significant reduction
of new vessel formation in the avascular rabbit cornea in response to VEGF pellet implants (Fig. 6, A and B). To our knowledge, this is the first report showing that NS-2028 is orally bioavailable. It is worth noting that NS-2028 administration was discontinued at the day of VEGF implantation for practical reasons (the amount of NS-2028 synthesized being limited) and, thus the contribution of sGC in VEGF-induced angiogenesis might be underestimated. To prove that in spite of early termination of NS-2028 treatment, the inhibitor was active after a week, the vasorelaxant responses to ACh were measured in NE preconstricted aortic rings isolated from rabbits. In these experiments, we could document that NS-2028-treated animals exhibited significantly reduced vasodilatory responses compared with vehicle-treated animals (Fig. 6C). In view of these results, we conclude that sGC is actively involved in VEGF-induced pathways associated with vessel formation.

NS-2028 blocks VEGF-induced vascular leakage in vivo. It is well known that VEGF increases microvascular permeability, which is directly associated with new vessel sprouting under both normal and pathophysiological conditions. To assess the effect of NS-2028 on the ability of VEGF to induce microvascular leakage, we used a modified Miles assay. Intradermal injection of VEGF in the mouse ear enhanced vascular permeability as indicated by Evan’s blue extravasation. However, pretreatment of animals with NS-2028 blunted the VEGF effect (Fig. 6D). Our data are consistent with the hypothesis that sGC is an important effector in the VEGF-mediated vascular permeability pathway.

DISCUSSION

The importance of NO production to vascular permeability and angiogenesis has been previously documented (38). Neovascularization in response to tissue ischemia, angiogenic factors, such as VEGF, or vasoactive substances, such as substance P, or bradykinin is attenuated by NOS inhibitors or targeted disruption of endothelial NOS (13, 23, 24, 39, 40). Pharmacological inhibition of NO production reduces the angiogenic properties of cultured EC (28, 29, 39), whereas exogenous application of NO donors at low concentration has opposite effects (17, 40). Interestingly, incubation of cells with high amounts of NO reduces cell growth and increases apoptosis (17). Several of the angiogenic actions of NO have been proposed to be sGC-mediated, and sGC activators promote endothelial growth, migration, and organization into capillary-like structures (29, 30). In addition, other cGMP-modifying agents [i.e., PDE5 inhibitors (32), natriuretic peptide receptor agonists (5, 36), and cGMP-dependent kinase activators (30, 36)] enhance the angiogenic responses of endothelial cells, suggesting that cGMP exerts important roles in blood vessel formation. On the other hand, NO donors (31) and VEGF-stimulated NO production (6, 31) have also been shown to promote endothelial cell migration and capillary formation through cGMP-independent pathways. More recently, the angiogenic actions of VEGF were demonstrated to occur via peroxynitrite production (8).

In spite of the wealth of information on the role of NO/cGMP pathways in angiogenesis presented above, the contribution of sGC to the angiogenic actions of VEGF, especially in vivo, remains elusive. To address this question, we have utilized the specific sGC inhibitor NS-2028 (27). We initially demonstrated that NS-2028 was able to blunt VEGF-stimulated cGMP production without affecting the ability of this growth factor to stimulate KDR (VEGF-R2) phosphorylation. Using two different in vitro assays, we established that NS-2028 attenuates VEGF-induced migration, suggesting that sGC is a downstream target of VEGF and an indispensable mediator of VEGF-stimulated EC motility. In addition, we observed that NS-2028 reduces EC number; since NS-2028 decreased VEGF-stimulated BrdU incorporation without promoting apoptosis, we propose that the decrease in cell number caused by NS-2028 results from inhibition of the mitogenic effects of
VEGF. It should be emphasized that the inhibitory effects of NS-2028 on angiogenesis were specific to VEGF-triggered pathways, as incubation of cells to this sGC inhibitor did not affect bFGF-induced migration or sprouting. This finding is in agreement with the observation that nitric oxide synthase participates in VEGF-triggered, but not bFGF-triggered, signaling(39).

To exert its angiogenic actions, VEGF recruits MAPK pathways (9, 37). We (29) and others (2) have shown that inhibition of NO production in EC, limits VEGF-stimulated ERK1/2 activation; however, a role of sGC in VEGF-stimulated angiogenesis functioning upstream of MAPK and provides the basis for testing NS-2028 in vivo to assess its effects on VEGF-triggered angiogenesis and permeability.

Although it has been shown that sGC inhibition leads to aberrant angiogenesis in zebrafish and treatment of cultured cells with ODQ reduces the formation of network-like structures on matrigel (30), the involvement of sGC in endothelial sprout formation in response to VEGF has not been determined. In our experiments, we observed that blockade of sGC activity limited VEGF-induced sprouting from isolated rat aortic rings, suggesting that sGC activation participates in all of the angiogenic properties of EC triggered by VEGF (growth, migration, and capillary morphogenesis). To extend our observations for the participation of sGC in VEGF-stimulated angiogenesis in vivo, we administered NS-2028 orally to rabbits and evaluated neovessel formation in their cornea. Indeed, NS-2028 inhibited the angiogenic response triggered by VEGF.
implants. These results strongly support that sGC significantly contributes to the angiogenic effect of VEGF not only in vitro and ex vivo, but most importantly in vivo. These actions of NS-2028 are in agreement with our findings that sGC inhibition by ODQ reduces developmental angiogenesis in the chicken chorioallantoic membrane (30). We feel that we should stress the fact that NS-2028 administration was discontinued on the day of VEGF implantation due to practical reasons (gram quantities of the drug needed, exceeding what was synthesized). Most likely, the magnitude of the antiangiogenic effects of NS-2028 is similar to that observed in the animal experiments.

Fig. 6. NS-2028 abrogates VEGF-induced angiogenesis and vascular leakage in vivo. NS-2028 (1 g/l) or vehicle was given in the drinking water of rabbits for 8 days. VEGF-bearing pellets were then implanted in the cornea, and angiogenesis was evaluated by stereomicroscopic examination, as described in METHODS (B). Data are expressed as mean ± SE; n = 4; *P < 0.05 vs. vehicle alone. C: representative pictures of scratch wound assay under basal conditions (vehicle) or after treatment with bFGF (10 ng/ml) in the absence or presence of NS-2028 (10 μM). Data are expressed as mean ± SE; n = 5; *P < 0.05 vs. vehicle. B: microvessel sprouting in rat aorta ring explants cultured in three-dimensional-fibrin gels was evaluated under basal conditions (vehicle) or after treatment with bFGF (50 ng/ml) in the absence or presence of NS-2028 (10 μM). Data are expressed as mean ± SE; n = 4; *P < 0.05 vs. vehicle alone. D: quantitative evaluation of the wound area during time and following the different experimental condition: vehicle (0.1% BCS), 10 μM NS-2028, bFGF 20 ng/ml, and (NS-2028 + bFGF); n = 4, *P < 0.05 vs. vehicle or NS-2028 alone; NS between bFGF and bFGF + NS-2028.

Fig. 5. sGC inhibition does not affect the in vitro angiogenic properties of bFGF A: HUVEC were pretreated with NS-2028 (10 μM, 30 min) or vehicle (DMSO), trypsinized, placed in transwells, and allowed to migrate for 4 h in the absence or presence of bFGF (10 ng/ml). Cells were then counted; n = 5; *P < 0.05 vs. vehicle. B: microvessel sprouting in rat aorta ring explants cultured in three-dimensional-fibrin gels was evaluated under basal conditions (vehicle) or after treatment with bFGF (50 ng/ml) in the absence or presence of NS-2028 (10 μM). Data are expressed as mean ± SE; n = 4; *P < 0.05 vs. vehicle alone. C: representative pictures of scratch wound assay under bFGF and bFGF + NS-2028 treatment at 0 and 24 h. Original magnification at ×100. D: quantitative evaluation of the wound area during time and following the different experimental condition: vehicle (0.1% BCS), 10 μM NS-2028, bFGF 20 ng/ml, and (NS-2028 + bFGF); n = 4, *P < 0.05 vs. vehicle or NS-2028 alone; NS between bFGF and bFGF + NS-2028.
genic action of NS-2028 would be greater had the drug administration been continued throughout the experiment. However, alternative explanations for the inability of NS-2028 to completely inhibit VEGF-triggered angiogenesis (in contrast to what was observed in vitro) exist. It is possible that cGMP-independent angiogenic pathways are mediating the effects of NO released by VEGF in the rabbit cornea. In line with this hypothesis, it has been shown that the angiogenic effects of VEGF-E (a VEGFR2-selective ligand) are NO dependent, but not mediated through cGMP and protein kinase G; the responses to VEGF-E were shown to involve phospholipase Cγ (6). In any case, and irrespective of the magnitude of inhibition of the VEGF responses achieved by NS-2028 in vivo, our results demonstrate that sGC is an important downstream effector in the VEGF angiogenic cascade.

Sprout formation and angiogenesis are preceded by an increase in vascular leakage (26). Endogenously produced NO has been reported to both limit and augment vascular permeability in inflamed tissues or in response to inflammatory mediators making the role of NO/cGMP pathways in permeability context dependent and complex. In most cases, inhibition of NO production has been shown to attenuate VEGF-stimulated permeability (13, 25). To examine whether NS-2028 in addition to blocking the angiogenic actions of VEGF also attenuates VEGF-induced vascular leakage, we used an established permeability ear test in mice. In these experiments, we observed that similar to what has been observed with NOS inhibitors, inhibition of sGC abolishes the VEGF-stimulated vascular permeability. It has been previously shown that vasoconstrictors do not limit the leakage of Evans blue from the skin vasculature after VEGF administration (25); therefore, we speculate a role for p38 in mediating VEGF-induced permeability were not identified in the course of our experiments, we speculate a role for p38 in this response, as p38 inhibitors have been shown to block VEGF-stimulated permeability in cultured EC (18, 19), and we showed herein that p38 is downstream of sGC in EC.

**Perspectives and Significance**

Using cell, organ, and animal experimental models, we have shown that the sGC inhibitor NS-2028 reduces VEGF-induced responses in vascular endothelium. Our data provide the first in vivo evidence that sGC is an integral element of the signaling pathway for neovascularization and vascular permeability in response to VEGF. The present findings, as well as evidence from the literature, suggest that NO, produced by EC in response to VEGF, activates endothelial cells in an autocrine manner. Following the stimulation of sGC, cGMP is formed and activates cGMP-dependent protein kinase cascades. This later kinase then activates members of the MAPK family (16) promoting neovascularization. As VEGF-inhibiting therapies have already proven to be effective in certain types of cancer and intraocular neovascular disorders (34), inhibitors of VEGF-triggered pathways might also exhibit therapeutic potential. The present results provide a rational basis for testing NS-2028 in diseases associated with increased VEGF levels that drive angiogenesis and vascular permeability.

**ACKNOWLEDGMENTS**

The technical work of Dr. Federica Finetti and Dr. Martina Monti, University of Siena, is greatly acknowledged.

**GRANTS**

This study was supported by grants from the Greek Secretariat of Research and Technology, the Greek Ministry of Education, the Thorax Foundation (Greece), and FP6 EU project EICOSANOX (LSHM-CT-2004-005033).3

**DISCLOSURES**

No conflicts of interest are declared by the authors.

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