Soluble Guanylyl Cyclase Activation Promotes Angiogenesis

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ABSTRACT

Soluble guanylyl cyclase (sGC) is a cGMP-generating enzyme carrying a heme prosthetic group that functions as a nitric oxide (NO) sensor. sGC is present in most cell types, including the vascular endothelium, where its biological functions remain largely unexplored. Herein, we have investigated the role of sGC in angiogenesis and angiogenesis-related properties of endothelial cells (EC). Initially, we determined that sGC was present and enzymatically active in the chicken chorioallantoic membrane (CAM) during the days of maximal angiogenesis. In the CAM, inhibition of endogenous sGC inhibited neovascularization, whereas activation promoted neovessel formation. Using zebrafish as a model for vascular development, we did not detect any effect on vasculogenesis upon sGC blockade, but we did observe an abnormal angiogenic response involving the cranial and intersegmental vessels, as well as the posterior cardinal vein. In vitro, pharmacological activation of sGC or adenovirus-mediated sGC gene transfer promoted EC proliferation and migration, whereas sGC inhibition blocked tube-like network formation. In addition, sGC inhibition blocked the migratory response to vascular EC growth factor. Cells infected with sGC-expressing adenoviruses exhibited increased extra-cellular signal-regulated kinase 1/2 and p38 MAPK activation that was sensitive to sGC inhibition by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, suggesting that these mitogen-activated protein kinases are downstream effectors of sGC in EC. A functional role for p38 in cGMP-stimulated migration was demonstrated using SB203580 [4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)]1H-imidazole; pharmacological inhibition of p38 attenuated BAY 41-2272 [5-cyclopropyl-2-[1-(2-fluoro-benzyl)]-1H-pyrazo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine] and sGC overexpression-induced EC mobilization. We conclude that sGC activation promotes the expression of angiogenesis-related properties by EC and that sGC might represent a novel target to modulate neovessel formation.

Angiogenesis, the formation of new blood vessels from pre-existing structures, is a highly orchestrated process that requires degradation of the extracellular matrix, proliferation, and migration of endothelial cells (EC) followed by organization of the EC into stable patent structures supported by mural cells (Folkman and Shing, 1992; Conway et al., 2001). In the adult, angiogenesis is tightly regulated with vessel growth limited to a few tissues; deregulated angiogenesis has been proposed to contribute to several disease processes, including tumor growth, psoriasis, arthritis, and diabetic retinopathy (Carmelit, 2003). During angiogenesis, EC integrate signals from various soluble and matrix-bound molecules to form new vessels (Bischoff, 1997; Conway et al., 2001). Among the endogenous mediators proposed to play an important role in neovascularization is the labile diatom molecule nitric oxide (NO) (Morbidelli et al., 2003). Exogenously applied NO donors have been shown to stimulate EC growth and migration in vitro (Ziche et al., 1994; Isenberg et al., 2005); moreover, endogenous NO mediates many of the effects of the prototype angiogenic factor, vascular endothelial growth factor (VEGF) (Papapetropoulos et al., 1997; 2001).

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ABBREVIATIONS: EC, endothelial cells; NO, nitric oxide; NOS, nitric-oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; BAY 41-2272, 5-cyclopropyl-2-[1-(2-fluoro-benzyl)]-1H-pyrazo[3,4-b]pyridin-3-yl]-pyrimidin-4-ylamine; CAM, chicken chorioallantoic membrane; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; hpf, hours post-fertilization; HUVEC, human umbilical vein endothelial cells; MAPK, mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; SNP, sodium nitroprusside; sGC, soluble guanylyl cyclase; l-NAME, Nω-nitro-l-arginine methyl ester; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)]1H-imidazole; DMSO, dimethyl sulfoxide; Ad, adenovirus; GFP, green fluorescent protein; m.o.i., multiplicity of infection; UTR, untranslated region; PD98059, 2′-amino-3′-methoxyflavone.
Ziche et al., 1997). In vivo, ischemia-induced angiogenesis was attenuated in endothelial nitric-oxide synthase (NOS) knockout animals, and VEGF-stimulated vessel formation in the cornea could be blocked by administration of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) (Ziche et al., 1997; Murohara et al., 1998). However, in spite of the wealth of information of the angiogenic effects of NO, the downstream pathways mediating its effects remain poorly characterized.

Soluble or NO-sensitive guanylyl cyclase is a ubiquitously expressed enzyme that acts as a “receptor” for NO (Hobbs, 1997). The low basal activity of sGC increases several hundred-fold upon activation with NO converting GTP to cGMP (Lucas et al., 2000). The most common form of the obligate sGC heterodimer is \(\alpha_1/\beta_1\) that is present in large amounts in smooth muscle, nerve cells, and platelets (Hobbs, 1997). In the vascular system, the biological role of sGC has been mostly studied in the context of smooth muscle tone and platelet aggregation (Lucas et al., 2000; Freibe and Koesling, 2003). We have previously shown that sGC subunit mRNA and catalytic activity are also present in endothelial cells from different vascular beds (Papapetropoulos et al., 1996); however, the physiological role of sGC in vascular endothelium remains for the most part unexplored. With respect to angiogenesis, some evidence for the involvement of sGC exists as NO-stimulated EC proliferation is cGMP-dependent, and a cell-permeable analog of cGMP promotes EC migration (Parenti et al., 1998; Kawasaki et al., 2003). The aim of the present study was to characterize the contribution of sGC in neovessel formation; to this end, we studied the effects of pharmacological activators and inhibitors of sGC in two in vivo models; moreover, we sought to determine whether alterations in sGC levels or activity affect the migratory and proliferative potential of EC and to elucidate the pathway(s) involved.

### Materials and Methods

#### Materials

Cell culture media and serum were obtained from Life Technologies Gibco-BRL (Paisley, UK). All cell culture plasticware was purchased from Corning-Costar Inc. (Corning, NY); cGMP enzyme immunoassay kits were from R&D Systems (Minneapolis, MN); SuperSignal West Pico chemiluminescent substrate was from Pierce (Rockford, IL); DC protein assay kit, Tween 20, and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA); jetPEI transfection reagent was obtained from Polyplus-transfection (Illkirch, France); penicillin and streptomycin were from GIBCO-BRL (Paisley, UK). HUVEC were isolated from fresh cords (Traver et al., 2003). Embryos were incubated with 5 or 10 \(\mu\)M ODQ in embryo water. Analyses shown here were carried out with 5 \(\mu\)M ODQ. Control embryos were incubated in 0.05% DMSO in embryo water. Embryos were fixed in 4% paraformaldehyde overnight and then imaged.

#### Luciferase Activity

African green monkey COSm6 cells (2 \(\times\) 10\(^5\) cells) were plated in six-well plates and grown overnight. Cells were then transfected using the jetPEI transfection reagent (3 \(\mu\)g of DNA and 6 \(\mu\)l of jetPEI per well). After 24 h, cells were collected, and approximately 1 \(\times\) 10\(^5\) cells were added inside each O-ring at 6, 9, and 12 days of embryogenesis. After a 48-h incubation, CAM tissues were removed and assayed for activity using the luciferase reporter assay system according to the manufacturer’s instructions.

#### Western Blotting

Proteins from tissues or cells were extracted after homogenization in a lysis buffer containing 1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA, and protease inhibitors (10 \(\mu\)g/ml aproatin, 10 \(\mu\)g/ml pepstatin, and 20 \(\mu\)M phenylmethylsulfonyl fluoride). Samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with the primary and secondary antibodies. Immunoreactive proteins were detected using a chemiluminescent substrate. Bands on autoradiographs were quantified using the Scion Image Release Beta 4.0.2 software. In the case of double bands, e.g., ERK1/2, the intensity from the two individual bands was calculated as one.

#### CAM Angiogenesis Assay

Fertilized White Leghorn chicken eggs were placed in an incubator and kept under constant humidity at 37°C. On day 4, a square window was opened in the shell and then sealed with adhesive tape. On day 9, an O-ring (1 cm\(^2\)) was placed on the surface of the CAM, and the tested substance (ODQ, BAY 41-2272, or zaprinast) was placed inside this restricted area. After 48 h, CAMs were fixed in Carson’s solution (saline-buffered formalin), and angiogenesis was evaluated using image analysis software as follows. CAM tissues were photographed using a digital camera attached to a stereoscope and then imported into the Scion Image software (Scion Image Release Beta 4.0.2 software; Scion Corporation, Frederick, MD). Photomicrographs were then transformed to binary files, thresholded, and skeletonized. Vessel length was measured in pixels and expressed as percentage of control.

#### Zebrasfish Lines and Pharmacological Treatment

Zebrasfish were raised under standard laboratory conditions at 28°C. We used embryos from an incross of double heterozygous carriers for the following transgenics: the panendothelial gfp line Tg[flk1:EGFP]\textsuperscript{py643} (Jin et al., 2005) and the erythrocyte-specific DsRed line Tg[gata1: DsRed] (Traver et al., 2003). Embryos were incubated with 5 or 10 \(\mu\)M ODQ in embryo water. Analyses shown here were carried out with 5 \(\mu\)M ODQ. Control embryos were incubated in 0.05% DMSO in embryo water. Embryos were fixed in 4% paraformaldehyde overnight and then imaged.

#### Luciferase Assay

CAM tissues were removed and washed in Hanks’ balanced salt solution. They were then incubated with the NOS inhibitor L-NAME for 10 min; tissues were then washed in Hanks’ balanced salt solution. They were then incubated with the primary and secondary antibodies. Immunoreactive proteins were detected using a chemiluminescent substrate. Bands on autoradiographs were quantified using the Scion Image Release Beta 4.0.2 software. In the case of double bands, e.g., ERK1/2, the intensity from the two individual bands was calculated as one.

#### cGMP Enzyme Immunoassay

CAM tissues were removed and washed in Hanks’ balanced salt solution. They were then incubated with the NOS inhibitor L-NAME for 10 min; tissues were then stimulated with sodium nitroprusside (100 \(\mu\)M) in the presence of the phosphodiesterase inhibitor isobutylmethylxanthine (1 mM) for 20 min. Media were then aspirated, and tissues were homogenized in 0.1 N HCl to extract cGMP. After 30 min, HCl extracts were collected, and cGMP was analyzed using a commercially available enzyme immunoassay kit following the manufacturer’s instructions.

#### Construction of Adenoviral Plasmids and Production of Adenoviruses

The adenoviral plasmids used in our study were constructed using standard methodological. In brief, rat \(\alpha_1\) and \(\beta_1\) cDNAs were subcloned into the pShuttle-cytomegalovirus vector and recombined with pAdEasy-1 in BJ5183 cells. Recombinants were identified via restriction analysis and transfected into human embryonic kidney cells (2 \(\times\) 10\(^5\)) using the jetPEI reagent. Replication incompetent adenoviruses were then propagated in human embryonic kidney cells and titered using the cytotoxic effect assay. Human umbilical vein endothelial cells (HUVEC) were infected with Ad-sGCa1 (20 m.o.i.) and Ad-sGC\textbeta1 (20 m.o.i. each) or Ad-GFP (40 m.o.i.) as control. After 20 to 48 h of infection, the infected cells were used for biochemical analyses or trypanized and used in migration, proliferation, and tube-formation assays.

#### HUVEC Cell Culture

HUVECs were isolated from fresh cords and grown on 100-mm dishes in M199 supplemented with 15% bovine calf serum, 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml gentamycin, 2.5 \(\mu\)g/ml amphotericin B, 5 U/ml sodium hepa-
rin, and 150 to 200 μg/ml endothelial cell growth supplement. HUVEC between passages 1 and 2 were used for all experiments.

**Matrigel in Vitro Tube-Formation Assay.** The formation of tube-like structures by HUVEC was assessed on growth factor-reduced Matrigel. HUVEC were plated at 15,000 cells/well on 96-well plates, precoated with 45 μl of Matrigel in the presence of ODQ (10 μM), BAY 41-2272 (1 μM), or DMSO as vehicle. After 24 h of incubation, tube formation was quantified. In brief, phase-contrast photomicrographs of endothelial cell cultures were recorded and imported into the freeware image analysis program Scion Image (Release Beta 4.0.2). Images were converted to a binary format, and the binary threshold was adjusted to obtain the best contrast of tubules. The images were then skeletonized, and total tubule length was measured in pixels. The area occupied by aggregates of cells was considered as noise. The total length calculated was then expressed as a percentage of control.

**Cell Proliferation.** HUVEC were seeded in 24-well plates at 6 × 10^4 cells/cm² and incubated in M199 supplemented with fetal bovine serum and endothelial cell growth supplement for 24 h. Cells were incubated with the indicated concentration of BAY 41-2272 (0.1 and 1 μM) and allowed to proliferate for 48 h. After this time, they were trypsinized, and cell number was determined using a hemocytometer.

**Cell Migration.** Cells were serum-starved overnight. After trypsinization, 1 × 10⁶ cells were added to Transwell inserts (8 μm pore size) (Corning Life Sciences, Acton, MA) in 600 μl of serum-free medium containing 0.25% bovine serum albumin. The test compound (1 mM 8-Br-cGMP, 1 μM BAY 41-2272, 50 ng/ml VEGF, ≥10 μM ODQ) or vehicle was added to the well containing the Transwell inserts. To inhibit p38, cells were preincubated with 4(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580) (10 μM) for 30 min. HUVEC were allowed to migrate for 4 h; 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.

**Data Analysis.** Data are expressed as means ± S.E.M. of the indicated number of observations. Statistical comparisons between groups were performed using ANOVA followed by a post hoc test or Student’s t test as appropriate. Differences were considered significant when p < 0.05.

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**Fig. 1.** sGC activation promotes angiogenesis in the CAM. A, Western blot analyses for the α₁ and β₁ sGC subunits in lysates of CAM tissue isolated at different days of development. B, α₁ promoter activity in the CAM. Luciferase activity was measured in the lysates of transfected COSm6 cells placed on the CAM for 2 days and harvested during different stages of development. Data are expressed as means ± S.E.M.; n = 11–19; *, p < 0.05 versus pGL3 (empty control vector); #, p < 0.05 versus Δ5’-UTR. 5’-UTR, mouse α₁ promoter containing the 5’-UTR region; Δ5’-UTR, 5’ upstream region without the UTR. C, sGC is catalytically active in the CAM. Tissues were removed at the indicated time after fertilization and incubated with L-NAME (100 μM) to inhibit NOS-induced cGMP formation; after 10 min they were stimulated with SNP (100 μM). Data are expressed as means ± S.E.M.; n = 8–16; *, p < 0.05 versus L-NAME. D, sGC inhibition attenuates angiogenesis. CAMs were treated with the indicated dose of ODQ for 48 h; the total length of vessel network was determined using image analysis software. A representative photomicrograph showing reduction in angiogenesis after ODQ treatment is shown. Data are expressed as means ± S.E.M.; n = 23–32; *, p < 0.05 versus control. E, cGMP-elevating agents increase neovascularization in CAM. CAMs were treated with the indicated dose of the NO-activator BAY 41-2272 or the phosphodiesterase 5 inhibitor zaprinast, and vessel length was determined. Data are expressed as means ± S.E.M.; n = 12–36; *, p < 0.05 versus control.
Results

Pharmacological Manipulation of sGC Activity Results in Altered Angiogenic Responses in Vivo. To determine whether sGC subunits are expressed in tissues exhibiting an active angiogenic response, we performed Western blot analysis of extracts from CAM tissue. In these experiments, both subunits of the most common sGC isoform (α1/β1) were detected (Fig. 1A); α1 and β1 levels were developmentally regulated, showing peak expression during days 9 through 12.

Using a heterologous system, we demonstrated that endogenous sGC protein levels correlate with α1 promoter activity (Fig. 1B). COSm6 cells were transfected with an empty luciferase vector (pGL3) or a vector in which luciferase was expressed under the control of the sGC α1 promoter (Vazquez-Padron et al., 2004); cells were then placed on the CAM. Growth factors and other mediators produced in CAM tissue diffuse and modulate α1 promoter activity in the COSm6 cells. In these experiments, we observed a time-dependent increase in luciferase activity that mirrored the protein expression of α1. Some of the regulatory sequences in the α1 promoter required for expression reside within the 5'-untranslated region (5'-UTR), because deletion of this region resulted in reduced luciferase activity at all of the time points tested. To determine whether the sGC present in the CAM is catalytically active, we measured cGMP accumulation in the presence and absence of the NO donor SNP. Indeed, stimulation of CAM tissues with SNP led to a robust increase in cGMP levels (Fig. 1C).

We next examined the impact of sGC inhibition in angiogenesis in the CAM. Incubation with the selective sGC inhibitor ODQ (Garthwaite et al., 1995) caused a dose-dependent inhibition of vascular length, suggesting that sGC is important in the formation of new vessels (Fig. 1D). On the other hand, treatment of CAMs with the NO-independent sGC activator BAY 41-2272 or the phosphodiesterase 5 inhibitor zaprinast stimulated angiogenesis as indicated by the increase in CAM vessel length (Fig. 1E). The increase in vessel length in the CAM in the presence of either BAY 41-2272 or zaprinast was approximately half of the response observed with 1 μg of VEGF (30 ± 9.1% increase over control).

To test whether sGC participates in endothelial cell formation and vascular development in vivo, we used zebrafish as a model organism (Supplementary Data). Zebrafish embryos were treated with different concentrations of ODQ in embryo water, and endothelial and erythrocyte birth and development were monitored in embryos that resulted from an in-cross of Tg(gata1:DsRed);Tg(flk1:EGFP)s843 double heterozygotes. When embryos were treated with 10 μM ODQ concentration from one cell stage, their development was arrested at gastrulation stage (data not shown). Inhibition of sGC using a lower ODQ concentration (5 μM) added at 10–24 hpf allowed normal formation and differentiation of EC; however, in these embryos, we observed that pericardial edema developed, thinner intersegmental vessels and cranial existed, and the posterior cardinal vein was disorganized (Fig. 2, compare B, D, and E with A and C). Furthermore, there was a significant reduction in gata1:DsRed-positive cells, which mark erythroid cells (Fig. 2, D and E; Supplemental Data). Finally, in embryos treated with ODQ at later stages
of development (48 hpf), erythrocytes seemed to clot in the posterior part, obstructing circulation (Fig. 2F); the above phenotypes were reversible upon removal of the sGC inhibitor. Our data, taken collectively, suggest that sGC is required for proper erythrocyte development and normal angiogenesis, at least in some vascular beds, in the zebrafish embryo.

**sGC Activation Promotes the Expression of an Angiogenic Phenotype in Cultured Endothelial Cells.** To determine whether sGC activation affects EC properties related to angiogenesis, we treated HUVEC with BAY 41-2272. Such treatment resulted in a concentration-dependent increase in EC proliferation (Fig. 3A). In addition, incubation of HUVEC with BAY 41-2272 or the cell-permeable analog of cGMP, 8-Br-cGMP, stimulated EC migration in vitro (Fig. 3B). In a different series of experiments, we observed that HUVEC grown on Matrigel in the presence of the sGC inhibitor ODQ formed fewer capillary-like structures (Fig. 3C). To test whether sGC is part of the signaling cascade activated by angiogenic factors, we employed vascular endothelial growth factor and measured EC migration in response to this growth factor in the presence and absence of ODQ. VEGF stimulated migration approximately 4-fold in an ODQ-sensitive manner, suggesting that sGC mediates some of the angiogenic actions of this prototype-angiogenic growth factor (Fig. 3D).

**Overexpression of sGC Promotes EC Proliferation, Migration, and Tube-Like Structure Formation.** We next examined EC properties associated with new blood vessel formation in cells transduced with adenoviruses to overexpress the sGC subunits. After infection, HUVEC expressed significantly higher α1 and β1 protein levels and sGC activity compared with uninfected or GFP-infected cells (data not shown). sGC-overexpressing cells exhibited higher proliferation rates than control cells (Fig. 4A). Similar results were obtained in migration assays where α1/β1-overexpressing cells showed a 2-fold greater basal migration rate compared with cells infected with a GFP-expressing adenovirus and exhibited an augmented migratory response to VEGF (Fig. 4B). Increased sGC expression also correlated with an increase in the ability of EC to form tube-like networks on Matrigel; the increase in network formation (40%) was similar to that seen with naive EC stimulated with BAY 41-2272 (Fig. 4C).

**Mechanisms of sGC-Triggered Angiogenic Responses.** We next sought to determine the pathways involved in sGC-regulated responses that are relevant to angiogenesis. Because MAPK members have been implicated in EC proliferation and migration, we tested whether sGC-overexpressing cells exhibit increased levels of ERK1/2 and p38 activation. Indeed, cells infected with viruses containing the sGC transgenes displayed a significantly higher pERK1/2/total ERK1/2 ratio, indicating that in these cells, ERK1/2 is activated (Fig. 5, A and B). Similar results were obtained for p38 (Fig. 5, A and B). Moreover, incubation of HUVEC with BAY 41-2272 stimulated ERK1/2 and p38 phosphorylation in a time-dependent manner (data not shown). Inhibition of sGC by ODQ resulted in a reduction in the phosphorylation of both MAPK members tested (ERK1/2 and p38) in cells infected with the α1 and β1 sGC subunits (Fig. 5, C and D); endogenous sGC inhibition by ODQ reduced only ERK1/2 activation in GFP-infected cells. Finally, to prove the functional relevance of p38 activation to sGC-triggered migration, we studied EC migration in the absence and presence of

![Fig. 3.](https://jpet.aspetjournals.org/)
Fig. 4. Adenovirus-mediated overexpression of sGC promotes EC proliferation, migration, and tube-like network formation. A, HUVEC were infected with GFP or sGC-subunit containing viruses. After 24 h, cells were plated at $6 \times 10^4$ cells/cm² and allowed to proliferate in complete medium for 48 h. Cells were then trypsinized and counted using a hemocytometer. Data are expressed as means ± S.E.M.; n = 8; *, p < 0.05 versus GFP. B, cells infected as in A, and after 24–48 h, cells were trypsinized, placed in Transwell inserts, and allowed to migrate for 4 h in the absence or presence of VEGF (50 ng/ml). Data are expressed as means ± S.E.M.; n = 6; #, p < 0.05 versus respective control, *, p < 0.05 versus respective GFP. C, uninfected cells or cells overexpressing GFP or sGC were placed on Matrigel and allowed to form networks. Alternatively, uninfected cells (hatched bars) were incubated with BAY 41-2272 (1 μM). After 24 h, cultures were photographed, and network area was quantified by image analysis software. Data are expressed as means ± S.E.M.; n = 7; *, p < 0.05 versus respective GFP or DMSO. D, representative photomicrograph of GFP- or sGC-infected cells grown on Matrigel.

Fig. 5. Increased sGC expression stimulates ERK1/2 and p38 cascades. Representative photomicrographs (B) and quantitation of pERK1/2/total ERK ratios and phospho-p38 (pp38)/p38 ratios (A) in naive, GFP-infected, and sGC-infected cells. Data are expressed as means ± S.E.M.; n = 4; *, p < 0.05 versus GFP. C and D, cells were treated for 2 h with ODQ (10 μM), lysates were then extracted, and blots were incubated with the indicated phosphospecific and total antibodies. Data are expressed as means ± S.E.M.; n = 4; *, p < 0.05 versus GFP control; #, p < 0.05 versus α1/β1. E, p38 inhibition attenuates BAY 41-2272 and sGC overexpression-induced EC migration. Cells were infected with a GFP- or a sGC-expressing virus. After 20 to 48 h, cells were incubated with the p38 inhibitor SB203580 (10 μM) for 1 h; they were then placed in a Transwell insert and allowed to migrate across a porous membrane for 4 h. Alternatively, naive cells (hatched bars) were stimulated with BAY 41-2272 (1 μM), with or without SB203580 pretreatment, and allowed to migrate for 4 h. Data are expressed as means ± S.E.M.; n = 5; *, p < 0.05 versus GFP control or DMSO; #, p < 0.05 versus control α1/β1; ¥, p < 0.05 versus BAY 41-2272.
p38 inhibitor SB203580. Pharmacological inhibition of p38 attenuated the migration of both uninfected cells stimulated with BAY 41-2272, as well as the migration brought about by increased sGC expression (Fig. 5E).

Discussion

Although present in substantial amounts in EC, our knowledge on the role of sGC in EC biology is limited. In the present study, we set out to determine the role of sGC in EC properties associated with angiogenesis and to evaluate the contribution of sGC in blood vessel growth. Initial experiments showed that α1 promoter activity, α3/β1 immunoreactivity, and sGC activity in the CAM were expressed in a manner that coincided with maximal angiogenic activity in this tissue (days 9–12) (Maragoudakis et al., 1988). Inhibition of endogenous sGC activity by ODQ resulted in an inhibition of angiogenesis, whereas incubation with the sGC activator BAY 41-2272 increased neoangiogenesis. In line with what was observed with the sGC activator BAY 41-2272, phosphodiesterase 5 inhibition by zaprinast led to an increase in vessel length in the CAM. It should be noted that conflicting results exist regarding the role of the endogenous sGC activator NO in the CAM; older studies (Pipili-Synetos et al., 1994) suggested that NO synthase inhibition stimulates angiogenesis, whereas more recent observations (Poltarchou and Papadimitriou, 2004) favor a proangiogenic role for NO in this system, in line with the angiogenesis-stimulating properties of NO in all other systems studied so far. The initially reported antiangiogenic actions of NO in the CAM might relate to the fact that, in the older studies, the material (coverslip) used to deliver the NO synthase inhibitor itself caused an inflammatory response, thereby making the use of cortisone acetate necessary and complicating data interpretation.

The molecular mechanisms that govern vascular development are highly conserved across species. Common growth and transcription factors participate in blood vessel formation in fish, avians, and mammals. For example, VEGF, a growth factor that signals through NO/cGMP, is critical for angioblast formation and differentiation into arterial endothelium in zebrafish (Nasevicius and Ekker, 2000). Likewise, EC are formed during development from angioblasts/endothelial precursors in response to VEGF-induced flk-1 activation in mice (Shalaby et al., 1995; Carmeliet et al., 1996). We took advantage of the fact that zebrafish embryos are free-living in the external environment, transparent during development, and that their vascular system develops rapidly to study the effects of sGC inhibition in vascular development. To visualize the appearance of EC in vivo, we used transgenic animals that expressed GFP under the control of the zebrafish flk-1 promoter. The GFP expression pattern is specific to the vasculature and recapitulates the endogenous expression of flk-1 (Liao et al., 1997). Incubation of embryos with ODQ starting at 10 hpf did not have an overt effect on vasculogenesis, because GFP-positive cells appeared normally and formed vessels. It should be noted that cGMP is detectable in zebrafish embryos at 8 hpf and that its levels double after 20 hpf (Holmqvist et al., 2004). Interestingly, embryos that had their sGC inhibited 10 to 24 hpf displayed fewer cranial vessels, thinner intersegmental vessels, and a disorganized posterior cardinal vein, indicative of an abnormal angiogenic response.

To evaluate the direct effects of sGC activation on EC properties that are important for neoangiogenesis, we used HUVEC, exposed them to BAY 41-2272, and determined their proliferation rate. BAY 41-2272 is a new NO-independent heme-dependent activator of sGC (Stasch et al., 2001). We chose to use this agent to enhance sGC activity and increase intracellular cGMP levels over NO-generating cGMP-activating agents, because we wanted to eliminate any cGMP-independent actions of NO donors, like S-nitrosylation, tyrosine nitration, interaction with heme and nonheme iron containing proteins or interaction with lipids and free radicals that could contribute to or modify the cGMP-mediated responses (Davis et al., 2001). Exposure of endothelial cells to BAY 41-2272 increased EC number in a concentration-dependent manner. Our observations are in line with those of Isenberg et al., who demonstrated that NO-donors, when used at low concentrations, promote EC growth in a cGMP-dependent manner, whereas higher concentrations have cGMP-independent inhibitory effects on proliferation (Isenberg et al., 2005).

Another property of EC that is important for angiogenesis is the ability of these cells to organize into patent capillary structures. Using Matrigel to drive network-like formation in vitro, we observed that EC incubated with ODQ engaged less in network formation. In agreement with these results, we have previously shown that inhibition of endogenous NO production attenuates VEGF- and transforming growth factor β1-induced capillary-like structure formation in three-dimensional collagen gels (Papapetropoulos et al., 1997). Finally, we tested whether activation of sGC or incubation with a cell-permeable analog of cGMP affects the ability of EC to migrate. Both BAY 41-2272 and 8-Br-cGMP promoted a 4-fold increase in EC migration in the absence of a growth factor, suggesting that sGC activation per se is sufficient to promote EC mobilization. Taken together, the data presented so far indicate that sGC plays an important role in all of the EC properties examined that are linked to angiogenesis. This conclusion is further strengthened by the observation that overexpression of sGC in EC using recombinant adenoviruses increased the proliferation rate, migratory ability, and organization of EC into network-like structures, mimicking the responses obtained using pharmacological activators of the enzyme.

VEGF is among the best characterized angiogenic factors (Ferrara et al., 2003; Zachary, 2003). Exposure of EC to VEGF increases NO production; the NO released then acts in a cGMP-dependent manner. Our observations are in line with the angiogenesis-stimulated responses (Parenti et al., 1998). To determine whether cGMP formation also mediates the action of VEGF with respect to migration, we treated EC with ODQ and determined the migratory response to VEGF. ODQ-treated cells exhibited a blunted migratory response, suggesting that sGC activation is required for this classic growth factor to transmit some of its angiogenic signals. Moreover, our observation that cells infected with sGC adenoviruses exhibit an increased migratory response to VEGF lends fur-
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Although the role of ERK1/2 in proliferation is well established, the biological significance of p38 in EC has only recently started to be investigated (Parenti et al., 1998; Zachary, 2003; McMullen et al., 2005). Activation of the p38 MAPK by angiogenic growth factors has been shown to occur in vitro and proposed to play a role in their ability to stimulate migration (McMullen et al., 2004). In addition, overexpression of mitogen-activated protein kinase kinase inhibitor 2′-amino-3′-methoxyflavone (PD98059) (Parenti et al., 1998).

In summary, we have shown that sGC contributes to several aspects of the angiogenic process by promoting EC proliferation, migration, and organization in network-like structures. Moreover, we have demonstrated that sGC inhibition leads to abnormal/reduced angiogenic responses in two different systems in vivo. These actions of sGC in EC correlate with activation of at least two members of the MAPK signaling cascade known to regulate angiogenic responses, ERK1/2 and p38. We conclude that stimulation of sGC and the concomitant increase in cGMP exert a permissive role on blood vessel formation.

Acknowledgments

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