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The Phosphodiesterase 5 Inhibitor Sildenafil Stimulates Angiogenesis Through a Protein Kinase G/MAPK Pathway

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cGMP-degrading pathways have received little attention in the context of angiogenesis. In the present study we set out to determine whether cGMP-specific phosphodiesterase 5 (PDE5) inhibition affects new blood vessel growth. Incubation of chicken chorioallantoic membranes (CAMs) in vivo with sildenafil increased vascular length in a dose-dependent manner. Moreover, incubation of cultured endothelial cells (ECs) with the PDE5 inhibitor promoted proliferation, migration, and organization into tube-like structures. The effects of sildenafil on the angiogenesis-related properties of EC could be blocked by pre-treatment with the soluble guanylyl cyclase (sGC) inhibitor ODQ or the protein kinase G (PKG) I inhibitor DT-3. In addition, over-expression of sGC in EC led to an enhanced growth and migratory response to sildenafil. To study the signaling pathways implicated in the sildenafil-stimulated angiogenic responses we determined the phosphorylation status of mitogen-activated protein kinase (MAPK) members. Incubation of cells with sildenafil increased both extracellular signal regulated kinase 1/2 (ERK1/2) and p38 phosphorylation in a time-dependent manner. Inhibition of MEK by PD98059 and p38 with SB203580 blocked sildenafil-induced proliferation and migration, respectively, suggesting that these MAPK members are downstream of PDE5 and mediate the angiogenic effects of sildenafil. PDE5 inhibitors could, thus, be used in disease states where neo-vessel growth is desired.

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Angiogenesis is a tightly regulated process known to be essential for normal embryonic development that has also been implicated in physiological, as well as pathological, phenomena in fully developed organisms (Conway et al., 2001; Jain, 2003). In adults, endothelial cell (EC) turnover is low and revascularization is normally restricted to few tissues, such as healing wounds or the cycling ovary (Folkman and Shing, 1992; Carmeliet, 2003). Stimulation of new blood vessel growth has been proposed as a therapeutic approach to treat conditions that include ischemic heart disease, neurodegenerative disorders, and hair loss. On the other hand, an excessive and deregulated angiogenic response is thought to contribute to cancer, diabetic retinopathy, arthritis, and psoriasis (Carmeliet, 2003; Ferrara and Kerbel, 2005).

Among the soluble molecules that modulate the EC properties linked to angiogenesis are cyclic nucleotides. The intracellular levels of cAMP and cGMP reflect the balance between the rate of synthesis by cyclases and the rate of degradation by phosphodiesterases (PDEs) (Lucas et al., 2000; Essayan, 2001; Rybalkin et al., 2003). PDEs are currently classified into 11 distinct families, based on their different substrate specificities, mode of regulation, and sequence homology (Essayan, 2001; Rybalkin et al., 2003). Several PDEs including PDE1, PDE2, PDE3, PDE4, PDE5, and PDE7 are expressed in vascular ECs of different origin and affect their migration, proliferation, and/or apoptosis (Sadhu et al., 1999; Keravis and Lugnier Claire, 2000; Miro et al., 2000; Thompson et al., 2002; Favot et al., 2003; Netherton and Maurice, 2005). Some of the PDEs (PDE5, PDE6, PDE9) are cGMP-specific, some specifically degrade cAMP (PDE4 and PDE7), while others have dual function (e.g., PDE2) (Essayan, 2001). Inhibitors of cAMP PDE have been shown to exert a negative effect on EC properties associated with angiogenesis (Favot et al., 2003), while less is known about the effects of cGMP-specific PDE inhibitors on EC functions.

cGMP is formed in the vasculature through the action of two distinct forms of guanylyl cyclases (GCs), a membrane-bound form that is the receptor for natriuretic peptides and a soluble GC that is activated by nitric oxide (Lucas et al., 2000). Endogenous NO and NO-releasing activators of soluble guanylyl cyclase (sGC) enhance EC migration, growth, and organization into capillary-like structures in vitro, as well as angiogenesis in vivo (Ziche et al., 1994, 1997; Morbidelli et al., 1996, 2003; Papapetropoulos et al., 1997a,b; Murohara et al., 1998; Parenti et al., 1998; Fukumura et al., 2001; Zhang et al.,

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2003). In contrast to the angiogenic properties exhibited by activators of the NO/cGMP pathway, natriuretic peptides have been reported to inhibit EC proliferation and to reduce vascular endothelial growth factor (VEGF)-stimulated migration and angiogenesis-related signaling (Itoh et al., 1992; Pedram et al., 1997, 2001). To gain further understanding on the role of cGMP in the angiogenic process and to assess the potential for additional therapeutic applications for PDE5 inhibitors beyond erectile dysfunction and pulmonary hypertension, we ascertained the effects of sildenafil on angiogenesis. To this end, we determined the effects of sildenafil on neo-vascularization in the chicken chorioallantoic membrane and in angiogenesis-related properties of EC in vitro; we provide evidence that PDE5 inhibition stimulates EC proliferation, migration, and organization leading to increased neo-vessel formation.

Materials and Methods

Materials

Synthesis of DT-3 for initial screening experiments was performed by solid phase synthesis on a Rink Amide MBHA resin using the standard Fmoc/^{t}Bu chemistry (Carpino and Han, 1970; Bernatowitz et al., 1989). The product was initially purified by gel filtration and final purification was achieved by RP-HPLC. The verification of the peptide sequence was achieved by ES-MS. Larger quantities of the DT-3 and control AP peptides were obtained from W. M. Keck biotechnology resource center at Yale University School of Medicine. 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); leghorn fertilized eggs were obtained from Pindos (Iperos, Greece). SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Biotechnology (Rockford, IL); *DC* Protein assay kit, Tween 20, and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA); penicillin and streptomycin were purchased from Applichem (Darmstadt, Germany); amphotericin, gentamycin, and heparin were purchased from Biochrom AG (Berlin, Germany); sildenafil citrate was provided by Pfizer (New York, NY); extracellular signal regulated kinase 1/2 (ERK1/2) phospho-specific and total antibodies along with the secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA); the sGCβ1 antibody was obtained from Cayman Chemicals (Ann Arbor, MI); VEGF and the cGMP low pH kit were purchased from R&D (Minneapolis, MN). The cAMP EIA kit was purchased from Assay Designs (Ann Arbor, MI). All other reagents including bovine serum albumin, EDTA, ODQ, SB203580, PD098059, the sGCα1 antibody, and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

Methods

CAM angiogenesis assay. Fertilized White Leghorn chicken eggs were placed in an incubator as soon as embryogenesis started 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 sildenafil was added inside this restricted area. The indicated dose of sildenafil was added on the CAM as a solution of a final volume of 40 μl. After 48 h, CAMs were fixed in Carson's solution (saline-buffered formalin) and angiogenesis was evaluated using image analysis software. For the CAM experiments 35–50 eggs per group were analyzed (one image per egg covering most of the area within the O-ring was used for the statistical analysis).

Human umbilical vein endothelial cell (HUVEC) culture. HUVECs were isolated from two to four fresh cords and grown on 100-mm dishes in M199 supplemented with 15% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 5 U/ml sodium heparin, and 150–200 μg/ml EC growth supplement. Cells were used at the first or second passage. For in vitro EC experiments at least two different batches of pooled donors were used and measurements were performed on two separate occasions with different passage cells.

Cell proliferation. HUVECs were seeded in 24-well plates at 6×10^3 cells/cm^2 and incubated in M199 supplemented with 15% FBS medium for 24 h. To inhibit sGC, protein kinase G (PKG) I, MEK or p38, cells were exposed to 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM, 2 h), DT-3 (1 μM, 20 min), PD98059 (10 μM, 60 min) or SB203580 (10 μM, 30 min), prior to sildenafil treatment. Cells were treated with sildenafil (10 μM) and allowed to proliferate for 48 h. They were then trypsinized and cell number determined using a hemocytometer. It should be noted that incubation of cells for up to 48 h with 10 μM sildenafil did not affect their viability as judged by exclusion of the vital dye trypan blue (95.0 ± 1.3 vs. 91.3 ± 1.3% viability after 24 h and 89.3 ± 2.3 vs. 89.8 ± 2.4 after 48 h for control and sildenafil, respectively).

Matrigel in vitro tube-formation assay. The formation of tube-like structures by HUVEC was assessed on growth factor-reduced Matrigel. HUVECs were plated at 15,000 cells/well in 96-well plates, pre-coated with 45 μl of Matrigel in the presence or absence of sildenafil (0–1 μM). After 24 h of incubation, tube-like structure formation was quantified using image analysis software (one image per well was analyzed and used for the statistical analysis).

Cell migration. HUVECs were serum-starved overnight. After trypsinization, 1×10^5 cells were added to transwells (8 μM pore size) in 100 μl of serum-free medium containing 0.25% BSA. To inhibit sGC, PKG or mitogen-activated protein kinase (MAPK), cells were pre-treated with ODQ (10 μM, 2 h), DT-3 (1 μM, 20 min), SB203580 (10 μM, 30 min), and PD98059 (10 μM, 60 min). Sildenafil (10 μM unless otherwise indicated) or VEGF (50 ng/ml) were then added to the well containing the transwell inserts in 600 μl of medium. Inhibitors were added in both the upper and lower compartments of the transwell setup. HUVECs were allowed to migrate for 4 h after which non-migrated 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.

Western blotting. Proteins from HUVEC were extracted after homogenization in a lysis buffer containing 1% Triton-X, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 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). Samples were subjected to SDS–PAGE, transferred to a PVDF membrane, and incubated with the indicated primary antibodies. Following incubation with an appropriate secondary antibody immunoreactive proteins were detected using a chemiluminescent substrate.

Construction of adenoviral plasmids and production of adenoviruses. The adenoviral plasmids used in our study were constructed using standard methodology. Briefly, rat α1 and β1 cDNAs were subcloned into the pShuttle-CMV vector and recombined with pAdeasy-1 in BJ5183 cells. Recombinants were identified via restriction analysis and transfected into HEK cells (2×10^6) using the jetPEI reagent. Replication incompetent adenoviruses were then propagated in HEK cells and tittered using the cytopathic effect assay. HUVECs were infected with Ad-sGCα1 and Ad-sGCβ1 (20 MOI each) or Ad-GFP (40 MOI) as control. After 48 h of infection, the infected cells were used for cGMP determination, Western blot analysis or trypsinized and used in migration/proliferation assays.

Cyclic nucleotide measurements. To determine if sGC over-expression leads to increased cGMP, control or adenovirus-infected cells were washed twice with Hank's balanced salt solution and incubated for 15 min in the presence of the non-selective PDE inhibitor isobutylmethylxanthine (1 mM). To determine the ability of sildenafil to increase the levels of cyclic nucleotides, cells were exposed for up to 10 min to 10 μM sildenafil in the absence of any other PDE inhibitor. After the incubation, cells were lysed with 0.1 N HCl and cGMP or cAMP levels were analyzed in the extracts using commercially available enzyme immunoassay kits following the manufacturer's instructions.

Data analysis. Data are expressed as mean $\pm$ SEM of the indicated number of observations. Statistical comparisons between groups were performed using ANOVA or Student's *t*-test, as appropriate. Differences were considered significant when $P < 0.05$.

Results

Sildenafil promotes neo-vascularization in vivo

To ascertain if inhibition of PDE5 by sildenafil affects new blood vessel growth in vivo we utilized the CAM model. Treatment of CAMs with sildenafil resulted in a dose-dependent increase in vascular length (Fig. 1). To allow for comparisons with

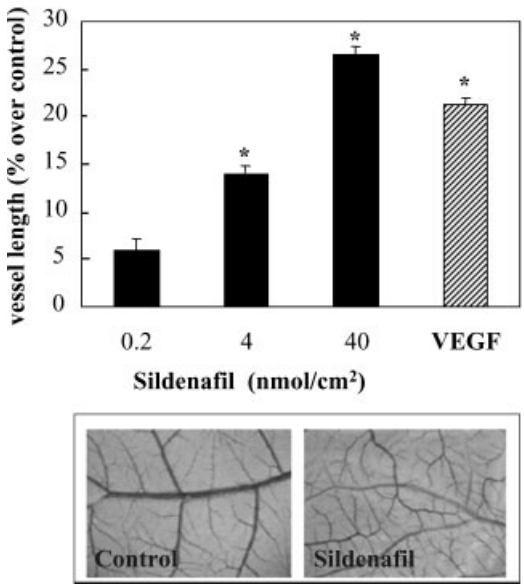

Fig. 1. Sildenafil promotes neo-vascularization in CAM. CAMs were exposed to the indicated dose of the sildenafil or VEGF (100 nmol) for 48 h; the total length of vessel network was determined using image analysis software. Representative photomicrographs of control and sildenafil-treated CAMs are shown (2.5× magnification). Data are expressed as mean ± SEM; n = 35–50; $^{*}P < 0.05$ versus control.

well-known angiogenic stimuli in the CAM, data obtained after exposure to 100 nmol VEGF are also shown in the same figure.

Sildenafil promotes angiogenesis-related properties of ECs

Treatment of EC with 10 μM sildenafil increased cGMP levels from undetectable to 4.5 ± 0.3 and 9.2 ± 0.6 pmol/well after 5 and 10 min, respectively (n = 4). In contrast, cAMP levels remained unaltered after exposure to the same concentration of sildenafil (0.3 ± 0.02, 0.4 ± 0.02, and 0.5 ± 0.1 pmol/well for control, 5 and 10 min of sildenafil; n = 3–4). To determine whether PDE5 inhibition affects EC properties essential for angiogenesis, we treated HUVEC with sildenafil. Such treatment stimulated EC proliferation in a concentration-dependent manner; some batches from pooled donors responded much more to sildenafil yielding an increase in cell number up to 250%; typically, a 60–80% increase in proliferation was observed (data not shown). Moreover, treatment with sildenafil resulted in a more robust migratory response than that produced by a saturating VEGF concentration (Fig. 2A). Concentrations of sildenafil as low as 10 nM were effective in promoting EC migration (21.5 ± 1.7 vs. 80.5 ± 4.9 cells migrated for control and sildenafil, n = 4; $P < 0.05$). Incubation of cells with sildenafil also resulted in increased capillary-like structure formation in cells grown on Matrigel (Fig. 2B).

Role of sGC and PKG in sildenafil-induced responses

To determine the contribution of sGC and PKG to the sildenafil-stimulated EC proliferation and migration, cells were pre-treated with ODQ or DT-3 prior to PDE5 inhibitor exposure. The cell-permeable PKGI peptide inhibitor DT-3 (Dostmann et al., 2000) blunted the sildenafil-stimulated growth and migratory responses (Fig. 3A,C), suggesting that activation of PKGI lies downstream of PDE5 and mediates the effects of sildenafil. It should be noted that a control peptide

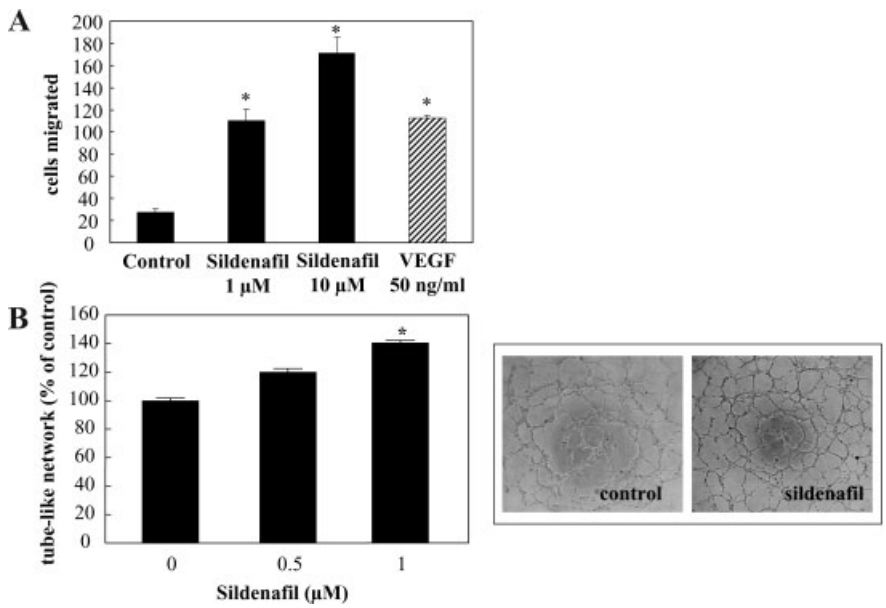

Fig. 2. Sildenafil induces EC migration and organization. A: ECs were allowed to migrate for 4 h in the presence of sildenafil (1 μM or 10 μM) or VEGF (50 ng/ml). Data are expressed as mean ± SEM; n = 6; $^{*}P < 0.05$ versus control. B: HUVECs were cultured on matrigel in the absence or presence of the indicated concentration of sildenafil for 24 h. Network-length was determined using image analysis software. Representative photomicrographs of control and sildenafil-treated cultures are shown (40× magnification). Data are expressed as mean ± SEM; n = 7; $^{*}P < 0.05$ versus control; the number 'n' is derived from observations made using independent wells with at least two different batches of pooled donors performed on two to three separate occasions with different passage cells.

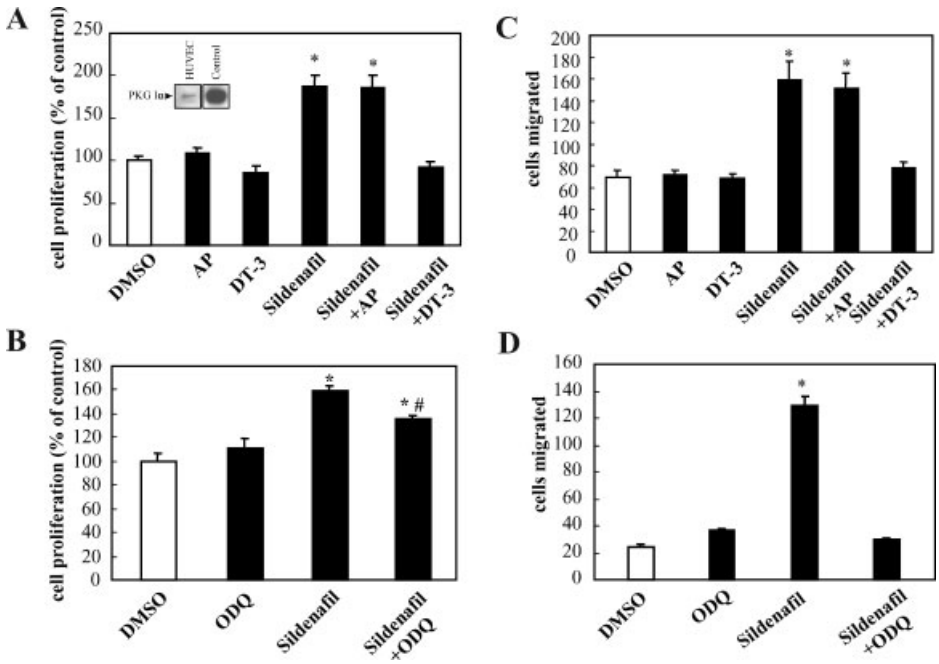

Fig. 3. Sildenafil-induced EC proliferation and migration is mediated by sGC/PKG. HUVECs were pre-treated with vehicle, a control peptide (AP), the PKGI inhibitor DT-3 (1 μM, 20 min; A and C) or ODQ (10 μM, 2 h; B and D) prior to stimulation with sildenafil (10 μM). Cells were allowed to proliferate in complete medium for 48 h (A and B) or migrate for 4 h (C and D) and were then counted. Data are expressed as mean ± SEM; n = 5–12; $^{*}P < 0.05$ versus vehicle (DMSO) and $^{\#}P < 0.05$ versus sildenafil. Inset in (A): Western blot analysis of lysates from HUVEC (lane 1) and COS transfected with PKGIα (lane 2; control) with a PKGIα antibody.

(AP) consisting of the Antennapedia internalization sequence did not affect the sildenafil responses. Moreover, ODQ significantly reduced the growth and migration brought about by sildenafil, indicating that sGC-derived cGMP contributes to the angiogenic responses observed in the presence of sildenafil (Fig. 3B,D).

Based on the observation that increased intracellular cGMP levels due to PDE5 inhibition promote EC growth and migration, we hypothesized that over-expression of sGC that generates cGMP would have similar effects on sildenafil. cGMP levels in cells infected with adenoviruses expressing sGCα1 and -β1 were significantly higher than those of uninfected or green fluorescent protein (GFP)-infected cells (Fig. 4A). Moreover, cells over-expressing sGC exhibited higher proliferation and migration rates (1.5- and 2-fold of control, respectively). EC transduced with sGC expressing adenoviruses also exhibited increased migration and growth responses to sildenafil (Fig. 4B,C).

Sildenafil-stimulated ERK1/2 and p38 activation is important for its angiogenic actions

To determine the mechanisms employed by sildenafil in exerting its angiogenic effects, we ascertained the effects of this PDE5 inhibitor on two MAPK members, namely ERK1/2 and p38. Exposure of HUVEC to sildenafil lead to a sustained increase in ERK1/2 phosphorylation that lasted at least 30 min; maximal activation of ERK1/2 was observed after 10 min (Fig. 5A). p38 phosphorylation followed different kinetics, as it was more short lived, peaking at 10–15 min and declining thereafter; moreover, sildenafil-induced p38 activation was of smaller magnitude as it increased by only 2.5- to 3-fold (Fig. 5B). To determine whether PKG is upstream of MAPK, phospho/total ERK1/2 and p38 levels were determined in the presence and absence of DT-3. As shown in Figure 5C inhibition of PKG blocked the sildenafil-induced phosphorylation of both MAPK members.

To test the functional relevance of sildenafil-induced MAPK activation with regard to EC growth and migration we used the pharmacological inhibitors of MEK and p38, PD98059 and SB203580. Inhibition of ERK1/2 activation reduced both basal and sildenafil-stimulated proliferation (Fig. 6A). In contrast, pre-treatment of cells with the p38 inhibitor SB203580 (10 μM) did not affect cell number (100 ± 3.2%, 204.5 ± 8.4%, 99.5 ± 1.8%, and 206.1 ± 8.9% of control, n = 4; for control, sildenafil, SB203580, and sildenafil + SB203580, respectively). On the other hand, inhibition of p38 abrogated the migratory response to sildenafil (Fig. 6B), while inhibition of MEK1/2 did not alter sildenafil-stimulated migration (30 ± 2.7, 139.7 ± 6.9, 28.6 ± 3.0, and 138.9 ± 7.1 cells migrated n = 4; for control, sildenafil, PD98059, and sildenafil+SB203580, respectively).

Discussion

Endogenously produced nitric oxide is known to stimulate new blood vessel formation. Neo-vascularization in response to tissue ischemia or angiogenic factors, such as VEGF, substance P, bradykinin is attenuated by NO synthase (NOS) inhibitors or targeted disruption of endothelial isoform of NOS (Ziche et al., 1994, 1997; Murohara et al., 1998; Fukumura et al., 2001). Moreover, pharmacological inhibition of NO production reduces the angiogenic properties of cultured EC (Papapetropoulos et al., 1997a,b; Ziche et al., 1997). Some of the effects of NO on EC proliferation and migration are blocked by sGC inhibition, suggesting that cGMP mediates the angiogenic actions of NO (Ziche et al., 1997; Parenti et al., 1998; Isenberg et al., 2005). To confirm and extend the association between cGMP and neo-vessel growth, we have evaluated the effects of the PDE5 inhibitor sildenafil in angiogenesis and

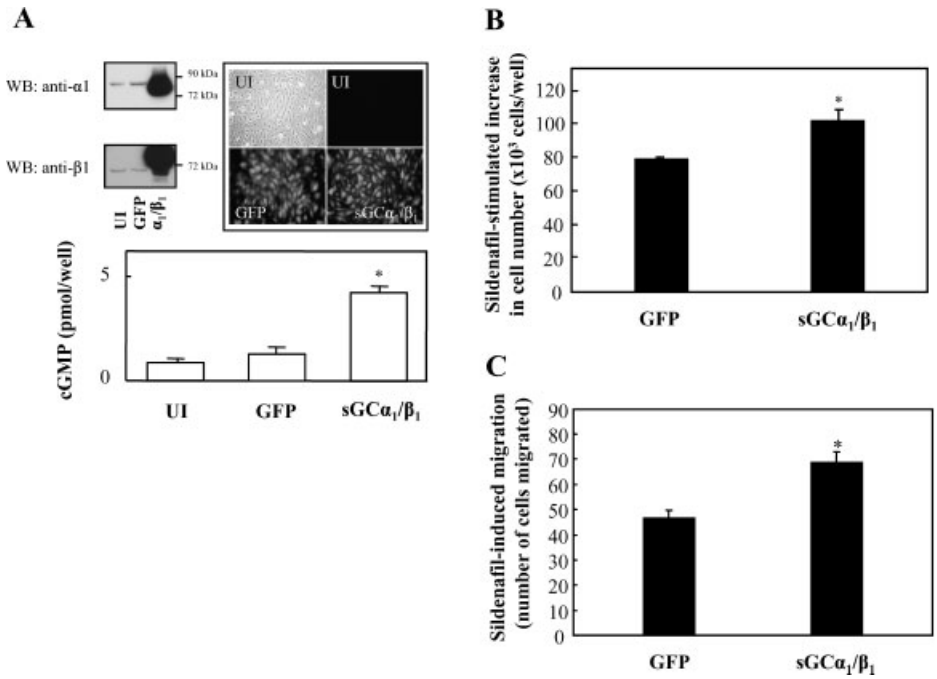

Fig. 4. Increased growth and migratory responses to sildenafil in EC over-expressing sGC. (A) Upper part: Representative photomicrographs and Western blots from HUVEC infected with GFP (40 MOI) or sGC-subunit expressing viruses (20 MOI each for sGCα1 and -β1 subunit). Lower part: cGMP levels in uninfected (UI), GFP- and sGCα1/β1-infected HUVEC. Data are expressed as mean ± SEM; n = 4; $^*P < 0.05$ versus GFP. B: After 48 h of infection, HUVECs were plated at 6×10^3 cells/cm^2 and allowed to proliferate in complete medium for 48 h in the absence or presence of sildenafil (10 μM). Cells were then trypsinized and counted using a hemocytometer. Data are expressed as mean ± SEM; n = 12; $^*P < 0.05$ versus GFP. C: Cells were infected as in (A) and after 48 h were trypsinized, placed in transwells, and allowed to migrate for 4 h in the absence or presence of sildenafil (10 μM). Data are expressed as mean ± SEM; n = 6; $^*P < 0.05$ versus GFP.

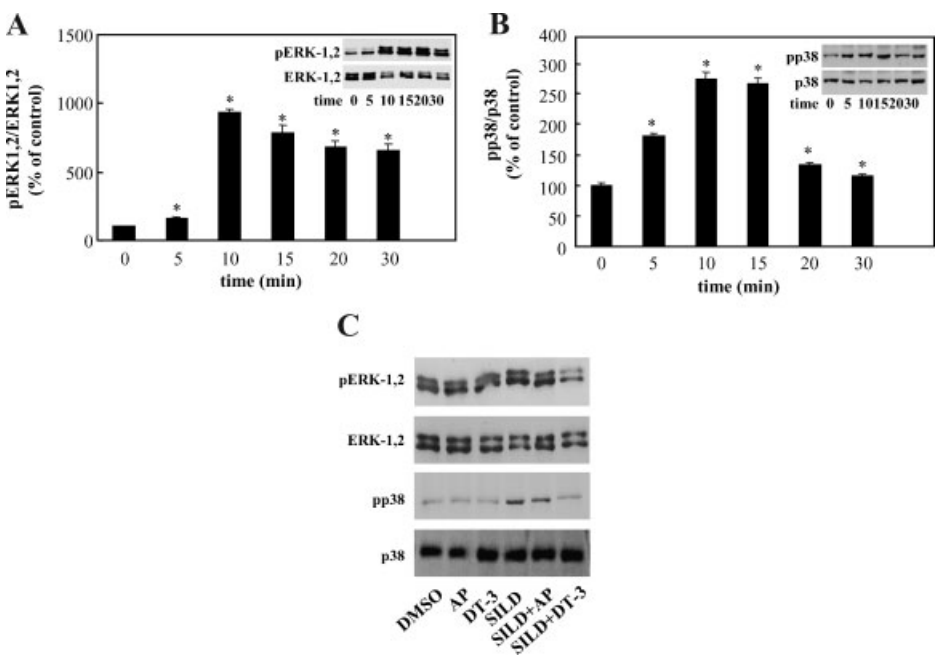

Fig. 5. Sildenafil activates ERK1/2 and p38 through PKG activation. A: HUVECs were serum-starved overnight and incubated with sildenafil (10 μM) for the indicated time. Lysates were prepared and analyzed by SDS–PAGE. Membranes were blotted with antibodies that recognize the activated (phosphorylated) or total ERK1/2 (A) or p38 (B). Representative blots and densitometric analysis are shown for both MAPK. C: Cells were incubated with the PKGI inhibitor DT-3 (1 μM, 20 min), exposed to sildenafil (10 μM) for 10 min and processed as in (A) and (B). Data are expressed as mean ± SEM; n = 3; $^*P < 0.05$ versus control.

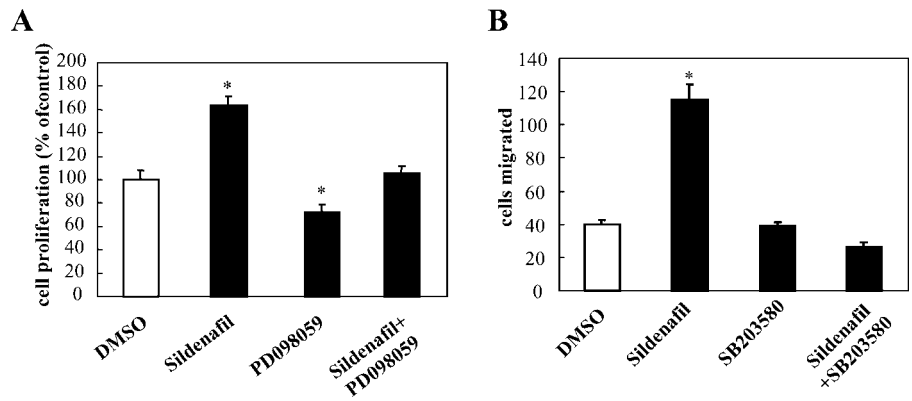

Fig. 6. MAPK activation mediates the angiogenesis-related properties of sildenafil. A: Cells were pre-treated with the MEK inhibitor PD098059 (10 μM) for 60 min; they were then exposed to sildenafil (10 μM) and allowed to proliferate for 48 h. B: Cells were pre-incubated with SB203580 (10 μM) for 30 min, trypsinized, placed in transwells, and allowed to migrate for 4 h in the absence or presence of sildenafil (10 μM). Data are expressed as mean ± SEM; n = 5–12; $^{*}P < 0.05$ versus vehicle (DMSO).

angiogenesis-related properties of EC and have investigated the mechanisms involved.

Treatment of CAMs with sildenafil increased vessel length in a dose-dependent manner. The magnitude of the observed angiogenic response for the maximal dose of sildenafil used is equal to or greater than that observed with the most efficacious neo-vascularization inducers ever tested in our laboratory in the CAM. Our findings that sildenafil promotes angiogenesis in the CAM are in line with the observations of Zhang et al. (2003), who reported that systemic administration of sildenafil increased angiogenesis in the ischemic boundary regions in a rat model of stroke. A pro-angiogenic action of sildenafil is further supported by observations that this PDE5 inhibitor promoted wound healing in dogs (Tas et al., 2003) and stimulated lung angiogenesis in rat pups exposed to hyperoxia (Ladha et al., 2005).

Several possibilities for the actions of sildenafil in the CAM exist including the following: sildenafil might be increasing angiogenesis by directly acting on EC or by modulating the behavior of supporting cells, like smooth muscle cells or pericytes; sildenafil might also modulate growth factor/growth factor receptors levels. To study whether sildenafil can directly stimulate angiogenesis-related properties of EC, we measured proliferation, migration, and organization in cultured human EC. Incubation of cells with sildenafil led to an increase in cell number, suggesting that PDE5 acts as an inhibitor of EC proliferation. This finding is in agreement with observations that endogenously produced NO or low concentrations of exogenously applied NO donor, as well as the cell-permeable cGMP analogue 8Br-cGMP, stimulate thymine incorporation and increase the proliferation of cultured EC (Ziche et al., 1994; Papapetropoulos et al., 1997b; Parenti et al., 1998; Isenberg et al., 2005). To determine the effects of cGMP-specific PDE5 inhibition on EC phenotype, we cultured HUVEC on matrigel and determined the effects of sildenafil on tube-like structure formation; such treatment resulted in a better-developed capillary-like network. Similar results have been reported by Zhang et al. using brain-derived and coronary EC (Zhang et al., 2003; Vidavalur et al., 2006).

We next sought to investigate the role of sGC and PKGI in the sildenafil-induced responses. Although it is widely accepted that HUVECs express sGC subunits and activity, it has previously been reported that this cells type, in contrast to other EC isolated from different vascular beads exhibits immunoreactivity for neither PKGI nor PKGII (Draijer et al., 1995). In the cells isolated and used in the present study, we were able to demonstrate expression of PKGIα. To determine whether PKGI activation mediates the effects of sildenafil we incubated cells with DT-3. DT-3, but not the control peptide (AP), abolished sildenafil-induced migration and proliferation. This finding is in line with the observation that PKGI−/− mice display reduced ischemia-induced angiogenesis, while transgenic mice over-expressing PKGI exhibit an enhanced response (Yamahara et al., 2003). Moreover, VEGF-stimulated proliferation was blocked by pharmacological inhibition of PKG (Hood and Granger, 1998) and adenovirus-mediated over-expression of PKGI in human EC increased migration (Kawasaki et al., 2003). The above-mentioned observations taken together are consistent with the hypothesis that increased intracellular levels of cGMP promote migration and proliferation by activating PKGI.

To ascertain the contribution of sGC in the sildenafil-induced proliferative and migratory responses, cells were pre-incubated with a cyclase inhibitor; ODQ attenuated sildenafil-stimulated proliferation and abolished sildenafil-triggered migration, suggesting that sGC represents a major source of cGMP regulating EC angiogenic behavior. The smaller effect of ODQ on sildenafil-induced proliferation, as compared to the abrogation of proliferation seen with DT-3 might be explained as follows: DT-3 completely inhibits PKG activation that is brought about by increases in cGMP generated by both sGC and pGC. The inability of the sGC-selective inhibitor ODQ to abolish proliferation indicates that non-sGC-derived cGMP plays an important role in the sildenafil-induced cell proliferation. The reason for the greater efficacy of ODQ in inhibiting sildenafil-induced migration might relate to the fact that migration experiments are performed in serum-free conditions. In the absence of serum (and therefore pGC ligands), the major agent affecting cGMP levels is endothelium-derived NO; ODQ blunts the increase in NO-stimulated cGMP production and prevents EC migration (to the same magnitude that the PKG inhibitor DT3 does). Of course, our data are open to alternative interpretations: EC migration might be equally supported by increases in the "membrane-associated" or cytosolic pools of cGMP, while increased proliferation might be better served by an increase in the cGMP that is compartmentalized to the cell membrane. In agreement to the observed effects of ODQ on sildenafil-stimulated EC properties related to angiogenesis, we and others have demonstrated that sGC inhibition blocks VEGF-stimulated cGMP synthesis, proliferation, and migration of EC

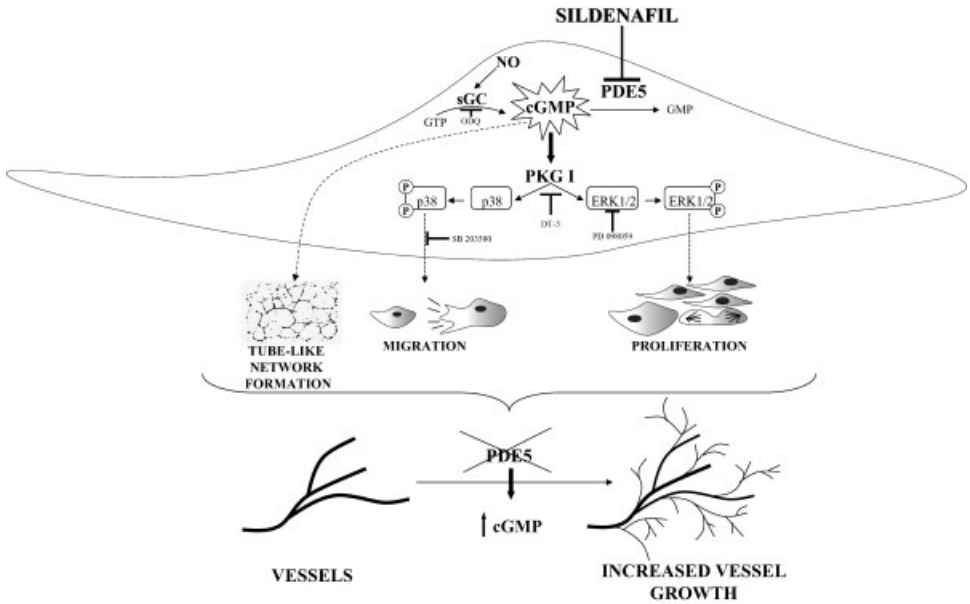

Fig. 7. Schematic diagram summarizing the proposed mechanisms of action of sindenafil-induced angiogenesis.

(Papapetropoulos et al., 1997b; Ziche et al., 1997; Parenti et al., 1998; Pyriochou et al., 2006), as well as sildenafil-induced tube-like structure formation on matrigel (Zhang et al., 2003). Our observation that sildenafil exerts augmented effects with respect to migration and proliferation in cells over-expressing sGC further supports the notion that sGC is crucial in mediating the sildenafil-induced responses.

ERK, together with c-Jun N-terminal kinase, and p38 form the MAPK family of proteins (Seger and Krebs, 1995). MAPK participates in diverse cellular functions including growth, differentiation, apoptosis, gene expression, and inflammation. In particular ERK1/2 has been shown to mediate the growth-stimulatory properties of many growth factors including VEGF (Zachary, 2003). NO donors and 8Br-cGMP promote phosphorylation and activation of ERK1/2 in EC and ODQ inhibits VEGF-stimulated ERK1/2 activity and proliferation (Parenti et al., 1998; Oliveira et al., 2003; Ridnour et al., 2005), suggesting that ERK1/2 is downstream of sGC in the VEGF signaling cascade. To determine if cGMP-specific PDE5 inhibition has an effect on MAPK signaling cascades, we determined the level of ERK1/2 and p38 activity using phospho-specific antibodies. Exposure of cells to sildenafil resulted in a sustained increase in ERK1/2 phosphorylation that was PKGI-sensitive. Inhibition of the upstream activator of ERK MEK with PD98059 reduced the ability of sildenafil to promote proliferation, providing evidence that ERK1/2 is functionally relevant and that ERK1/2 lies downstream of PDE5 in this pathway stimulating EC growth. Although the exact mechanisms mediating cGMP-induced ERK phosphorylation were not addressed in our study, evidence from the literature suggests that PKG phosphorylates and activates Raf-1 (Hood and Granger, 1998). In addition, 8Br-cGMP promotes Ras activation in intact cells, but not in an in vitro reconstituted system (Oliveira et al., 2003); this effect of 8Br-cGMP was postulated to be mediated through a cAMP/cGMP guanine nucleotide exchange factor. Similarly to what was observed with ERK1/2, inhibition of PDE5 led to the activation of another member of the MAPK family, p38. The effects of sildenafil on p38 phosphorylation were of smaller magnitude and duration compared to the effects observed with ERK1/2 and were also blocked by PKG inhibition. Interestingly, pharmacological inhibition of p38 abolished sildenafil-stimulated migration, pointing towards the existence of a functional link between rises in cGMP levels and p38-driven cell migration. Our observations are in line with findings that over-expression of MEK6, an upstream activator of p38, promotes EC migration and that VEGF-stimulated migration can be blocked by SB203580 (Zachary, 2003; McMullen et al., 2004, 2005).

Therapeutic applications of PDE5 inhibitors rely on their effects on smooth muscle; herein, we have provided proof of direct actions of sildenafil on EC that result in neo-vessel growth by modulating the activity of the cGMP/PKGI pathway (Fig. 7). The present findings, as well as evidence from the literature, suggest that NO produced by EC in response to angiogenic stimuli (VEGF, bradykinin, substance P) stimulates sGC activity and autocrine cGMP formation, leading to activation of PKGI that in turn promotes EC migration, proliferation, and organization. In this model, PDE5 would function to offset the angiogenic actions of NO-releasing growth factors by lowering cGMP levels. We conclude that PDE5 inhibitors might be useful in instances where neo-vascularization is desired, for example, during wound healing or in the course of collateral vessel formation in ischemic heart disease and critical limb ischemia.

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