Cyclic GMP-specific phosphodiesterase 5 regulates growth and apoptosis in pulmonary endothelial cells

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Zhu, Bing, Samuel Strada, and Troy Stevens. Cyclic GMP-specific phosphodiesterase 5 regulates growth and apoptosis in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol 289:L196–L206, 2005. First published March 25, 2005; doi:10.1152/ajplung.00433.2004.—Sustained increases in intracellular cGMP concentrations ([cGMP]i) inhibit cell growth and induce apoptosis. We now report that a cGMP-specific phosphodiesterase, PDE5, plays a dominant role in regulating [cGMP], transitions that inhibit cell growth and control susceptibility to apoptosis in pulmonary endothelium. Atrial natriuretic peptide (ANP) activates guanylyl cyclase A/B and induces a rapid [cGMP]i rise 2–5 min after its application, in both pulmonary arterial endothelial cells (PAECs) and pulmonary microvascular endothelial cells (PMVECs). However, increased [cGMP]i in PAECs is transient and decays within 10 min due to cytosolic PDE5 hydrolytic activity. Increased [cGMP]i in PMVECs is sustained for >3 h due to the absence of PDE5. Indeed, at any ANP concentration, the sustained (30 min) [cGMP]i rise is greater in PMVECs than in PAECs, unless PAECs are also treated with the PDE5 inhibitor zaprinast. Using RT-PCR, Western blot analysis, immunoprecipitation, and DEAE chromatography, we resolved the expression and activity of PDE5A1/A2 only in PAECs. Similarly, PDE5 expression was restricted to extra-alveolar endothelium in vivo. ANP induced growth inhibition and apoptosis in PMVECs, but similar effects were not seen in PAECs unless ANP treatment was combined with zaprinast. ANP blocked the VEGF-induced proliferation and migration in PMVECs. Collectively, these data suggest that PDE5-regulated [cGMP]i controls endothelial cell growth and apoptosis, representing a mechanism of heterogeneity between two endothelial phenotypes.

METHODS

PULMONARY ENDOTHELIAL CELLS form a semipermeable, dynamic barrier that restricts interstitial fluid accumulation important to optimize gas exchange (CO2 and O2). Transient intercellular gap formation and apoptosis both increase permeability that contributes to pulmonary edema, decreasing the efficiency of gas exchange. The mutually opposing actions of intracellular Ca2+ concentration ([Ca2+]i) and intracellular cAMP concentrations ([cAMP]i) control barrier function and are the focus of active investigations (2, 5, 14, 20, 35–37). However, signaling events that trigger endothelial cell apoptosis are still poorly understood.

Multiple cellular signaling pathways modulate the balance between proliferation and apoptosis in the endothelium. Hypoxia (11, 25, 28) and peptides like endothelins (28) stimulate the production of vascular endothelial growth factor (VEGF), and VEGF is an endothelial cell mitogen. In contrast, atrial natriuretic peptide (ANP), which is increased in heart failure (41) and hypoxia (1, 22), inhibits VEGF-mediated angiogenesis (28, 29) and induces apoptosis in endothelial cells (12), vascular smooth muscle cells (4, 13, 30), and cardiac myocytes (42).

Proapoptotic and antiangiogenic effects of ANP are presumed to require an increase in [cGMP], that occurs after its binding to natriuretic peptide-specific receptors, guanylyl cyclase (pGCs) (19). The receptor-activated [cGMP], transition is characterized by a large, transient rise that is followed by a smaller sustained increase; these phases appear to regulate discrete cell functions. Indeed, a sustained [cGMP]i rise is required to induce apoptosis, at least in cardiac myocytes and tumor cells (38, 42). cGMP-specific phosphodiesterases (PDEs) and most importantly PDE5 regulate both the magnitude and duration of the [cGMP], plateau, indicating a potential downstream mechanism that orchestrates life and death signals. If this idea is correct, then differential expression of PDE5 among cells would be an important determinant of their sensitivity to apoptosis. Functional consequences of differential PDE5 expression have not been rigorously examined. However, we now report that pulmonary artery endothelial cells (PAECs) express PDE5, whereas pulmonary microvascular endothelial cells (PMVECs) do not express PDE5. Such an absence of PDE5 in PMVECs prolongs [cGMP], signaling that inhibits proliferation and induces apoptosis. These findings indicate PDE5 expression is an important cause of heterogeneity among endothelial cell phenotypes that integrate life and death signals.

Reagents. [2,8-3H]cAMP and [8-3H]cGMP (Moravek Biochemicals, Brea, CA) were prepared as described previously (39). Rat ANP, VEGF, benzamidine, TLCK (tosyl-l-lysyl-chloro-ketone), aprotinin, pepstatin A, leupeptin, Dowex-1X8-400 resin, and snake venom were from Sigma (St Louis, MO). KT-5823, 8-bromo-cGMP, zaprinast, and other PDE isoform-selective inhibitors were purchased from CalBiochem (La Jolla, CA). pGC antagonist, A-71915, was obtained from EMD Biosciences (San Diego, CA). All compounds were dissolved in DMSO, and the final DMSO concentration was 0.5% as the vehicle control for all experiments. ANP, VEGF, and A-71915 were dissolved in sterilized 0.9% NaCl and stored at −70°C as stock aliquots until use. For cGMP stimulation experiments, ANP was added to the cell culture medium at the defined concentrations. For cell proliferation, ANP was added after cells were seeded, with repeated doses for every 24 h. For VEGF-stimulated proliferation, ANP was added to cells with the addition of VEGF. PDE5 inhibitors, zaprinast, and sildenafil, and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
at indicated concentrations were added 10 min before the addition of ANP in cGMP-stimulating and cell proliferation experiments.

Isolation and culture of rat pulmonary endothelial cells. PAECs and PMVECs were isolated and cultured in DMEM medium supplied with 10% FBS as described previously (35). A panel of specific cell surface markers was used routinely to characterize the phenotype of cultured cells (15). Cells used in all experiments were below passage 12.

[cGMP], measurements. The whole cell acid extracts in 0.2 N HCl/50% methanol were prepared as described (38). The amount of cGMP in acetylated samples were measured with enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI) and were expressed as picomoles of [cGMP]i generated per milligram total protein of the cells.

PDE assay in whole cell lysate. Preconfluent PAECs and PMVECs were collected in cold PBS. Cell pellets were resuspended in TME-PI-Triton buffer (20 mM Tris·HCl, 5 mM MgCl2, 0.5 mM EDTA, pH 7.4, with protease inhibitors: 10 μM TLCK, 2 μM leupeptin, 2 μM pepstatin A, 10 μM benzamidine, 0.02 μg/ml aprotinin/ml, supplemented with Triton X-100 at 0.8%) and homogenized on ice with a glass homogenizer. The homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant comprised over 99.5% of total PDE activity and was named as the whole cell lysate for PDE analysis. PDE activities in the supernatant were measured with 0.25 μM [3H]cAMP or [3H]cGMP as substrate (38). PDEs isomser-selective inhibitors (for details see Table 1) were dissolved in DMSO and added to the reaction mixture 5 min before [3H]cAMP or [3H]cGMP was added to start the assay. The final DMSO concentration in inhibitor treatment and vehicle control was 0.5%, by which concentration has been noted not to inhibit PDE activities (40, 44).

PDE fractionation with DEAE-Trisacryl M chromatography. Approximately 2 × 109 preconfluent PAECs were collected in cold PBS. Cell pellets were resuspended in 20 ml of TME-PI-Triton buffer and homogenized. The homogenate was centrifuged at 100,000 g for 60 min at 4°C. The supernatant fraction was diluted 1:4 with TME-PI buffer and applied to a DEAE-Trisacryl M column (10 × 1.5 cm). After washing the column with TME buffer, we eluted PDE activities with 135 ml of two successive linear NaCl (0–0.1, 0.1–0.5 M in TME buffer) gradients at a flow speed of 1.0 ml/min. Fractions were collected and assayed in every third fraction for cGMP and cAMP hydrolytic activities at 0.25 μM substrate concentration (38, 40). For further studies, fractions were pooled around each peak of PDE activity. The concentration for [3H]cAMP or [3H]cGMP was 0.25 μM for testing inhibitors, and the substrate concentrations varied from 0.1 to 20 μM for kinetic studies. For Western blot analysis, the pooled fractions were concentrated by methanol-chloroform precipitation and reconstituted in loading buffer before being applied to the gels (40).

RT-PCR. RT-PCR was performed with total RNA extracted from PMVECs and PAECs using a RNA isolation kit (Roche, Indianapolis, IN), and cDNA was prepared by First Strand RT-PCR kit (Stratagene, La Jolla, CA) with random primers. Primers for rat PDE5 (rPDE5) were: 5'-GATATTTCCAGTCATTTGGATGTCACAGCC-3' and 5'-TGAATATTGTGGTTTTGATGTCCCGTCCCC-3' to produce a 756-bp cDNA for the cGMP-binding site of all rPDE5A splice variants (A1/A2/A3). Forward primers specific to rPDE5A1 (5'-ACGATCACTGGGACCTTTACCTTCTC-3') or rPDE5A2 splice variants (5'-ATGGTGGCCTTTGGAGACAAAAC-3') produce a 352-bp or a 328-bp cDNA, respectively, with a common reverse primer (5'-GAGTCTTGAGACTGTGTCCTCCC-3'). To further compare PDE5A1 and 5A2 mRNA levels between PAECs and PMVECs vs. an internal control 18S RNA (488 bp), relative quantitative (RQ) RT-PCR was performed according to QuantumRNA 18S Internal Standards Comptimers techniques (Ambion, Austin, TX). Transcripts for rPDE5 as previous reports for PMVECs (40, 44) were detected in both PMVECs and PAECs as a native control by primers (forward 5'-ATGGCTCAGCAGAACCCGGAC-3' and reverse 5'-GATGTCGATTGTCCACATGAAAA-3') to produce a 287-bp cDNA in RQ RT-PCR.

Western blot analysis and immunoprecipitation. Whole cell extraction and immunoblotting were performed as described previously (38). Antibody to GST-fusion protein of the high-affinity cGMP-binding domain of human PDE5 (18) was used to recognize the PDE5A1/A2/A3 variants. The NH2-terminal peptides were used to make the specific antibodies for rPDE5A1 (CMERAGPSFGQQRQQQQQ) and A2 (CMLPFGDKTR). Antibody to PDE4 was prepared as previously described (40). PDE7A antibody and secondary antibodies conjugated with alkaline phosphatase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for PGC-A and -B were purchased from FabGennix (Shreveport, LA). For immunoprecipitation the whole cell extraction in TME-PI-Triton buffer with NaCl concentration was adjusted to 150 mM and incubated with PDE5 antibody and Ezview Red Protein A Affinity Gel (Sigma) at 4°C for 12 h. The adsorbed PDE5 was further used in Western blot experiments to test for expression of 5A1 and 5A2 splice variants, cGMP-PDE activity assays, and PDE activity assays after washing with the same incubation buffer four times. In Western blot, protein samples and adsorbed proteins on affinity gel were dissolved in 1× loading buffer before subjected to 7% precast Novex gels (Invitrogen, Carlsbad, CA) for electrophoresis (38, 40).

Immunohistochemistry. Sprague-Dawley rats weighing 250–350 g were anesthetized by pentobarbital sodium (Sigma), 50 mg/kg intraperitoneally. Our study and protocol was reviewed and approved by our ethics committee. Isolated rat lungs were perfusion fixed with 70% ethanol or directly fixed in 10% formalin. Lungs were paraffin embedded for serial sectioning. Sections (4–5 μm) were deparaffin and rehydrated with serial concentrations of alcohol washes and finally to water (15). Slides were blocked with 3% normal goat serum for 30 min, followed by staining for PDE5 alone or in combination with von Willebrand factor (vWF). For PDE5 only staining, the FITC- or Texas Red-conjugated goat anti-rabbit IgG and or FITC-conjugated donkey anti-sheep IgG (Santa Cruz Biotechnol-

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**Table 1. Specificity of isolated PDE activities in PAECs to PDE isomser-selective inhibitors**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PDE Isoform</th>
<th>IC50 for DEAE-Pooled Fractions, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinpocetine</td>
<td>1</td>
<td>Peak 1: 68.4 Peak 2: 45.8 Peak 3: 12.6</td>
</tr>
<tr>
<td>EHNA</td>
<td>&gt;100</td>
<td>&gt;44.9 &gt;44.9 &gt;44.9</td>
</tr>
<tr>
<td>Indolidin</td>
<td>2</td>
<td>&gt;100 &gt;100 &gt;100</td>
</tr>
<tr>
<td>Rolipram</td>
<td>4</td>
<td>&gt;100 &gt;100 &gt;100</td>
</tr>
<tr>
<td>Zantrapast</td>
<td>5, 6</td>
<td>0.119 57.3 68.6</td>
</tr>
<tr>
<td>IBMX</td>
<td>Mix</td>
<td>2.70 16.2 23.4</td>
</tr>
<tr>
<td>Candidates</td>
<td>PDE5</td>
<td>PDE7 PDE4</td>
</tr>
</tbody>
</table>

**Isolated phosphodusterase (PDE) activities in peak 1 (fraction 15–36), peak 2 (37–52), and peak 3 (55–79) from DEAE chromatography (refer to Fig. 4A) were used to test the sensitivity of the enzyme to isomser-selective inhibitors for PDE1–6 and the nonselective inhibitor EHMA. All PDE inhibitors were dissolved in DMSO at different series of concentrations to determine inhibition curves. The final concentration of the DMSO in PDE assay including vehicle controls was 0.5%, a concentration that has no significant effect on enzyme activity. IC50 values for each inhibitor were calculated by plot software from individual experiment of triplicate assay, which were repeated at least 2 times for the reproducive values. PAECs, pulmonary artery endothelial cells; EHMA, erythro-9-(2-hydroxy-3-nonyl)adenine.**
nology) was used and showed no specific immunoreactivity in these tissue sections.

Cell proliferation, DNA synthesis, DNA fragmentation, and migration assay. Cell proliferation was determined by the 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenylytetrazolium bromide (MTT) method as described (9). For PMVECs and PAECs cultured in DMEM with 10% FBS, 10⁵ cells were seeded in 35-mm dishes. ANP and/or zaprinast were added to cells every 24 h before the cell proliferation was determined. For VEGF-stimulated PMVEC growth, 2 × 10⁴ cells were seeded into 96-well plates with serum-free DMEM. VEGF and/or ANP were added 24 h after cell seeding and maintained in culture for 5 days. Cells were incubated with 50 μg/dish and 10 μg/well MTT for 3 h, and optical density at 570 nm (OD₅₇₀ nm) was measured after cell contents were dissolved in DMSO. The cell number in each dish or well was calibrated using an established standard curve for OD₅₇₀ nm, and cell number was counted under the same conditions. Doubling times for cell proliferation, EC₅₀ for VEGF stimulation, and Iₐ₅₀ for ANP inhibition were calculated using GraphPad Prism software.

Cell proliferation was confirmed using the bromodeoxyuridine (BrDU) incorporation ELISA assay (Roche) for measuring DNA synthesis rate. In brief, 5 × 10⁴ cells were seeded into 96-well plates with 10% FBS DMEM. ANP and/or zaprinast were added to cells with bromodeoxyuridine (BrDU, 10 μM), and BrDU incorporation (DNA synthesis rates) after 24-h culture was measured by anti-BrDU-POD-ELISA colorimetric development with OD at 450 nm. For VEGF-stimulated PMVEC DNA synthesis, 2 × 10⁴ cells were seeded into 96-well plates with serum-free DMEM. VEGF and/or ANP with BrDU were added 24 h after cell seeding and maintained in culture for BrDU incorporation for another 72 h, and BrDU incorporation was measured as described above.

DNA fragmentation for PAECs and PMVECs grown in 96-well plates was measured at OD₄₅₅–₄₉₀ nm using a DNA/histone-complex ELISA kit (Roche), as described (38). DNA fragmentation values were compared between cell types using the same cell number in each assay. ANP and/or zaprinast were added to cell culture medium 24 h before the test.

For VEGF-stimulated PMVEC migration, cells were grown to confluence on six-well plates in DMEM supplemented with 10% serum. Medium was serum restricted 24 h before the experiment. A scrape wound was created as previously described (29). VEGF (100 ng/ml) and/or ANP (10 nM) were added to the serum-free DMEM for another 96 h of cell culture. PMVEC migration was measured using a digital image system composed of an inverted microscope. The numbers of cells within the area (0.5 × 0.7 mm per measurement) adjacent to the wound were used for the migration index. Three measurements per well were taken.

Statistical analysis. Results of the dose-dependent cGMP elevation, proliferation, and inhibition experiments were analyzed by two-way ANOVA with post hoc multiple comparison by GraphPad Prism 3.1 software. All data represent means ± SE. P < 0.05 is considered statistically significant for the comparisons.

RESULTS

ANP-induced [cGMP]ᵢ accumulation in PAECs and PMVECs. Basal [cGMP]ᵢ (0.305 ± 0.083 pmol/mg total proteins) is ~2.5-fold higher in PMVECs than it is in PAECs (0.121 ± 0.042 pmol/mg total proteins). Specific binding of ANP to natriuretic peptide receptors increases [cGMP]ᵢ (19). A single ANP concentration (10 nM) induced a rapid [cGMP]ᵢ rise in both PAECs and PMVECs. The peak time was 2 min for PAECs and 5 min for PMVECs (Fig. 1). However, in PAECs, the ANP-initiated [cGMP]ᵢ rise declined rapidly to basal levels within 10 min, whereas in PMVECs the increase in [cGMP]ᵢ persisted for >30 min, requiring nearly 3 h to return to basal levels.

The peak ANP-induced [cGMP]ᵢ rise reflects the rate of pGC activation and cGMP synthesis. Dose-response curves showed maximal [cGMP]ᵢ was attained at 100–1,000 nM of ANP and was approximately four times higher in PAECs than in PMVECs (Fig. 2A). The EC₅₀ value was also higher in PAECs (5.73 nM) than in PMVECs (1.44 nM), indicating a greater maximal response but lower sensitivity or affinity for ANP-binding pGcs in PAECs. This result was supported by findings that a high-affinity ANP-specific pGC-A protein (125 kDa) was abundant in PAECs. In addition, a lower-affinity ANP-binding pGC-B protein was detected in PAECs and was not found in PMVECs (inset, Fig. 2A).

Although the transient [cGMP]ᵢ response to pGC-A/B activation was greater in PAECs, the duration of the ANP-stimulated [cGMP]ᵢ rise was prolonged in PMVECs. Indeed, at 30 min, the [cGMP]ᵢ response to any ANP concentration was higher in PMVECs than it was in PAECs; this difference was particularly prominent at high ANP concentrations (Fig. 2B). Moreover, a PDE5-selective inhibitor (zaprinast) enhanced the ANP-induced [cGMP]ᵢ accumulation in PMVECs at the 30-min time point but had no effect in PMVECs (Fig. 2C). These data suggest that a cGMP-specific hydrolytic activity attributable to PDE5 may exist in PAECs, but not in PMVECs, to limit the duration of [cGMP]ᵢ signaling.

Selective expression of cGMP-specific PDE5 in PAECs. To address whether PAECs express cGMP-PDE activity, direct PDE activity measurements were made using whole cell lysates from the two cell types. PAECs possessed cGMP-specific hydrolyzing activity, but PMVECs had only trace levels of activity (Fig. 3A), consistent with the finding in Figs. 1 and 2 and with a previous report for PMVECs (40). Indeed, the
cGMP-PDE activity was >30 times higher in PAECs than it was in PMVECs, whereas total cAMP-hydrolyzing activity was nearly identical between the two cell types (Inset, Fig. 3A).

The expression of PDE5 in PAECs was confirmed by RT-PCR and immunoblots. Products of PDE5A1 and A2 and all PDE5 cDNA with expected sizes were selectively amplified from PAECs but not from PMVECs (Fig. 3C). These data indicate PDE5A1 accounts for the principal cGMP-PDE activity present in PAECs, whereas it is downregulated in PMVECs.

Characterization of PDE isoforms by anion-exchange chromatography. A single cGMP-specific (peak 1) and two cAMP-specific (peaks 2 and 3) hydrolyzing activities were identified by DEAE fractionation (Fig. 4A and Table 1). The accumulated cGMP hydrolytic activity in peak 1 comprised >95% of total activity recovered from the DEAE column fractions; this activity was inhibited by PDE5-selective inhibitors but was not sensitive to other isoform inhibitors (Table 1), confirming the findings in whole cell lysates (refer to Fig. 3B). Kinetic
analysis for cGMP hydrolysis in peak 1 indicates apparent maximum velocity ($V_{\text{max}}$) and $K_m$ values of 2.50 nmol cGMP/18528 min/11002 mg protein/18528 and 0.592 M, respectively (Fig. 4B). The $V_{\text{max}}$ and $K_m$ values for this purified PAEC PDE5 activity are similar to that reported for PDE5 from other sources (3, 16, 34). Immunoblots using a PDE5-specific antibody demonstrated the presence of an 85-kDa protein in peak 1 but not in peaks 2 and 3 (inset, Fig. 4B), an identical molecular mass to the PDE5A1 isoform that was detected in Fig. 3D by immunoprecipitation. Activities for other cGMP-PDEs comprised 5% of total activity in PAECs, and only trace levels in PMVECs (see Fig. 3A); only trace levels of PDE3A/3B proteins were resolved by Western blot analysis in membrane fractions (data not shown).

The cAMP-specific PDE activity in peak 2 was rolipram insensitive but IBMX sensitive (Table 1). The cAMP-hydrolyzing activity in this peak was not affected by either 5 μM cGMP or exogenous calcium/calmodulin, indicating little or no contamination by PDE 1–3 isoforms. These data suggest existence of a cAMP-specific PDE7 isoform in peak 2, other than PDE4 (rolipram sensitive) or PDE8 (IBMX insensitive). This conclusion was further confirmed by the identification of two PDE7A isoforms, 55-kDa 7A1 (major) and 50-kDa 7A2 (minor) in Western blot analysis, and a kinetic curve (nonlinear) for cAMP hydrolysis (Fig. 4C). The cAMP-hydrolyzing activity of peak 3 is selectively inhibited by the PDE4 inhibitor rolipram (Table 1). The kinetic characteristics (Fig. 4D, $V_{\text{max}} = 2.52$ nmol cAMP·min$^{-1}$·mg protein$^{-1}$ and $K_m = 1.65$ μM) of this activity are typical of a PDE4 isozyme, which was further confirmed by RT-PCR (Fig. 3C, inset) and Western blot analysis (data not shown) with PDE4-specific primers and antibodies, respectively. In aggregate, these data indicate that PDE5 is the predominant cGMP-PDE in PAECs and, furthermore, that this enzyme is absent in PMVECs.

In vivo expression of PDE5 in rat pulmonary arterial endothelium. To confirm differential expression of PDE5 between PAECs and PMVECs was not an artifact of culture, its expression was examined in the intact lung by immunohistochemistry...
using PDE5 (all)-specific antibody. Positive immunoreactivity for PDE5 was identified in endothelium of large vessels but was not detected in capillaries (Fig. 5A). To confirm the PDE5 staining in large blood vessels is endothelial cell specific, vWF, a multimeric glycoprotein expressed in endothelial cells (8, 21), was costained with PDE5. vWF staining was observed in endothelium of both large blood vessels and capillaries (Fig. 5B).

PDE5 regulates ANP-induced growth inhibition. ANP inhibits proliferation in vascular endothelial cells (12) and smooth muscle cells (13), presumably due to increased \([cGMP]\). We therefore tested the direct effect of ANP on PAEC and PMVEC growth. In PMVECs, addition of 100 nM ANP at every 24-h interval induced a “delay” in serum-stimulated proliferation at 24–96 h (Table 2); however, the cell growth doubling time \(t_{1/2}\) at 15.9 h was not significantly changed. On the other hand, ANP changed neither PAEC proliferation nor the \(t_{1/2}\) at 16.3 h (Table 2). To inhibit PDE5, zaprinast \((10^{-6} M)\) was added to ANP-treated PAECs. PAEC proliferation was significantly inhibited by ANP + zaprinast during log-phase growth (Fig. 6A).

The effect of ANP on endothelial cell proliferation was also tested using BrdU incorporation. ANP \((100 \text{ nM})\) partially inhibited BrdU incorporation in PMVECs at 24 h (Fig. 6B; 76.7 ± 2.1% of control, \(P < 0.05\), from three separated experiments). In PAECs, ANP \((100 \text{ nM}, 92.5 ± 9.2\%\) of control, \(P > 0.05\), from three separate experiments) and zaprinast \((10 \mu M, 98.9 ± 0.1\%\) of control, \(P > 0.05\), from three separate experiments) did not directly affect BrdU incorporation. However, concurrent treatment with ANP \((100 \text{ nM})\) and zaprinast \((10 \mu M)\) decreased BrdU incorporation (Fig. 6B; 67.2 ± 9.0% of control, \(P < 0.05\), from three separate experiments). Another PDE5-selective inhibitor (38), E-4021 \((0.1 \mu M)\), showed a similar inhibition combined with ANP \((76 ± 7.0\%\) of control, \(P < 0.05\)). The cell-permeable PKG-selective inhibitor KT-5823 \((1 \mu M)\) reversed the ANP-inhibited BrdU incorporation in PMVECs \((86.9 ± 6.9\%\) of control, \(P < 0.05\) vs. ANP treatment), as did pGC antagonist (27) A-71915 \((100 \mu M; 97.0 ± 6.6\%\) of control, \(P < 0.05\) vs. ANP treatment). PKG activator 8-bromo-cGMP directly inhibited BrdU incorporation \((0.5 \mu M; 79.1 ± 9.2\%\) of control, \(P < 0.05\)).
ANP-induced apoptosis in PMVECs and PAECs. Sustained [cGMP] increases cause apoptosis in colon tumor cells (38) and ANP-treated cardiac myocytes (42). Because ANP induced a sustained [cGMP] rise in PMVECs and also in PAECs with PDE5 inhibition, we quantitated ANP and/or zaprinast-induced apoptosis in the two cell types under the same conditions for cell growth inhibition. With DNA fragmentation as the index for apoptosis, ANP-treated PMVECs exhibited a nearly two-fold higher rate of apoptosis at 24 h when compared with control cells (Fig. 6C). On the other hand, ANP alone had no direct apoptotic effect in PAECs, similar to our cell growth results. However, the rate of DNA fragmentation was doubled when ANP treatment was combined with zaprinast. A similar but lower apoptosis rate was also observed in 48–72 h of treatment of ANP in PMVECs and in PAECs in combination with zaprinast (data not shown). Interestingly, PMVECs appear to possess greater basal DNA fragmentation than do PAECs (Fig. 6C). These data suggest ANP-induced growth inhibition in PAECs and PMVECs is partially due to apoptosis that is caused by PDE5 downregulation or inhibition.

ANP-inhibited VEGF-stimulated cell proliferation and migration in PMVECs. VEGF promotes PMVEC growth and survival. Indeed, VEGF dose-dependently induced PMVEC growth in serum-free medium for 5 days (Fig. 7A, EC50 = 3.8 ng/ml). ANP abolished the VEGF-stimulated PMVEC proliferation but had no effect on the basal cell number in untreated PMVECs (Fig. 7B). The IC50 for ANP inhibition of cell growth is ~0.2 nM, ANP (10 nM) also abolished the VEGF-stimulated (EC50 = 1.7 ng/ml) BrdU incorporation in PMVECs (Fig. 7C). Furthermore, ANP significantly reduced VEGF-mediated cell migration in PMVECs, from 1,091 ± 50% of control to 324 ± 41% of control (P < 0.05, Fig. 7D). ANP itself did not affect the cell migration in serum-free control (112 ± 14%, P > 0.05).
Table 2. ANP delays cell proliferation in PMVECs, with less effect in PAECs

<table>
<thead>
<tr>
<th>Time, h</th>
<th>PMVECs, 10^5 cells</th>
<th>PAECs, 10^5 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANP−</td>
<td>ANP+</td>
</tr>
<tr>
<td>24</td>
<td>1.27±0.06</td>
<td>1.10±0.06</td>
</tr>
<tr>
<td>48</td>
<td>3.30±0.22</td>
<td>2.53±0.18*</td>
</tr>
<tr>
<td>60</td>
<td>5.88±0.47</td>
<td>3.41±0.26*</td>
</tr>
<tr>
<td>72</td>
<td>10.1±0.60</td>
<td>7.69±0.78*</td>
</tr>
<tr>
<td>96</td>
<td>15.8±0.30</td>
<td>13.8±0.72*</td>
</tr>
<tr>
<td>120</td>
<td>17.1±0.09</td>
<td>15.4±0.03*</td>
</tr>
<tr>
<td>144</td>
<td>17.3±0.68</td>
<td>16.2±0.66</td>
</tr>
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</table>

Pulmonary microvascular endothelial cells (PMVECs) and PAECs were seeded in 35-mm dishes with DMEM supplied with 10% FBS. Atrial natriuretic peptide (ANP, 100 nM, ANP+) was added to cells at 24-h intervals, and cell proliferation was determined by MTT method as described in MATERIALS AND METHODS. Cell proliferation rate was expressed as the number of cells in each dish; means ± SE, n = 3. *P < 0.05 as compared with ANP− controls. Doubling times for PMVECs and PAECs proliferation are 15.9 h and 16.3 h, respectively. ANP alone does not change the doubling time for both cells.

DISCUSSION

PDE5 is the predominant cGMP-related PDE in PAECs, whereas PDE5 is not appreciably expressed in PMVECs. Such heterogeneity in PDE5 expression and activity supports the emerging evidence that PAECs and PMVECs arise from different embryological origins and that they retain memories of their unique phenotypes even under similar culture conditions (6–8). The difference in endogenous PDE5 expression among lung macro- and microvascular endothelial cell phenotypes provides an opportunity to address important downstream signal transduction events and physiological role(s) of [cGMP] transitions (26).

PDE5 activity controls the sustained, [cGMP]i rise. Indeed, ANP activation of pGC accounts for the transient [cGMP]i accumulation that peaks 2–5 min after stimulation. Although the magnitude of this peak rise in [cGMP]i is due to the abundance and activity of pGC, both the length and magnitude of the [cGMP]i transition are controlled by PDE5 activity. PMVECs exhibit a sustained [cGMP]i rise in response to ANP that occurs because PDE5 is not expressed. On the other hand, a sustained [cGMP]i rise in PAECs, but, in combination, the rate of ANP−zaprinast. ANP (100 nM, solid bars) alone induced an increase of DNA fragmentation in PMVECs and PAECs. Apoptosis was detected by DNA fragmentation at 24 h, measuring the amount of endonuclease cleaved oligonucleosome in DNA/histone-complex at OD405–490 nm. ANP (100 nM, solid bars) alone inhibits BrdU incorporation in PMVECs compared with the ANP-free control (open bars). The same concentration of ANP or 10 μM zaprinast (+Zap) alone does not alter the DNA fragmentation in PMVECs and PAECs. Apoptosis was detected by DNA fragmentation at 24 h, measuring the amount of endonuclease cleaved oligonucleosome in DNA/histone-complex at OD405–490 nm.

**Fig. 6.** ANP-inhibited proliferation and -induced apoptosis requires PDE5 inhibition. A: ANP inhibited PAEC proliferation when combined with zaprinast. PAECs were treated with 10 μM zaprinast (solid bars) before 100 nM ANP were added. Cells were seeded in 35-mm dishes with DMEM supplied with 10% FBS, and proliferation was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) methods as described in Table 2. *P < 0.05 compared with ANP− control (open bars); means ± SE, n = 3. Doubling time for PMVECs was altered from 16.3 to 30.2 h by ANP + zaprinast. B: ANP inhibited DNA synthesis in PMVECs and PAECs. DNA synthesis rate was measured by bromodeoxyuridine (BrdU) incorporation at 24 h [optical density at 450 nm (OD450 nm)]. ANP (100 nM, solid bars) alone inhibits BrdU incorporation in PMVECs compared with the ANP-free control (open bars). The same concentration of ANP or 10 μM zaprinast (+Zap) alone does not alter the DNA synthesis in PMVECs and PAECs. Apoptosis was detected by DNA fragmentation at 24 h, measuring the amount of DNA fragmentation cleaved oligonucleosome in DNA/histone-complex at OD405–490 nm. ANP (100 nM, solid bars) alone induced DNA fragmentation in PMVECs and PAECs. The same concentration of ANP or 10 μM zaprinast (+Zap) alone does not alter the DNA fragmentation in PMVECs and PAECs. In combination, the index is increased. *P < 0.05 compared with corresponding ANP-free control; means ± SE, n = 4.
A sustained rise in [cGMP] has been implicated in cell growth inhibition. The mechanism of this action is incompletely understood, although increased [cGMP], appears to arrest cell cycle progression, e.g., G2/M arrest (23, 43, 45). Consistent with this possibility, the ANP-induced sustained [cGMP], rise delayed serum-stimulated cell proliferation in PMVECs and increased the t1/2 in PAECs treated with zaprinast. A sustained rise in [cGMP], has also been shown to induce apoptosis, in both cardiac myocytes and tumor cells (38, 42, 45). At least two cGMP/PKG phosphorylation-activated pathways are involved in PDE5 inhibitor-induced apoptosis in PMVECs and increased the t1/2 in PAECs treated with zaprinast. A sustained rise in [cGMP], has also been shown to induce apoptosis, in both cardiac myocytes and tumor cells (38, 42, 45). At least two cGMP/PKG phosphorylation-activated pathways are involved in PDE5 inhibitor-induced apoptosis in PMVECs and increased the t1/2 in PAECs treated with zaprinast.

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It is likely that the growth inhibitory actions of elevated [cGMP], are not ubiquitous among all growth factors, but specific to families of proangiogenic factors (27, 31). Serum-stimulated proliferation of PMVECs and PAECs is only partially inhibited by ANP. In contrast, prior studies indicate ANP inhibits VEGF-mediated angiogenesis through [cGMP]/PKG signaling pathways. ANP inhibits VEGF stimulation of proliferation, migration, and microvascular capillary tube formation, an effect that is reversed by the natriuretic peptide receptor-specific antagonist HS-142-1 and potentiated by the PKG activator 8-bromo-cGMP (24, 29). In addition, ANP inhibits endothelin- and hypoxia-induced VEGF production from vascular smooth muscle cells and subsequently reduces VEGF production.

**Fig. 7.** ANP blocks VEGF-induced cell proliferation in PMVECs. A: VEGF dose dependently increases PMVECs growth in serum-free culture (EC50 = 3.8 ng/ml). Cells were seeded in 96-well plates (2 x 10⁴ cells/well) in serum-free DMEM and cultured for 24 h before the addition of VEGF for another 5 days to measure cell proliferation. B and C: ANP inhibits VEGF-stimulated PMVEC proliferation in cell number and DNA synthesis. ANP (10 nM, solid bars) blocked 100 ng/ml VEGF-stimulated PMVEC proliferation (open bar at VEGF+) but did not affect cell proliferation in VEGF-free controls (open bar at VEGF−). PMVECs were cultured in the presence of VEGF and/or ANP for 5 days in cell number (MTT measurement) and for 72 h BrdU incorporation under serum-free conditions. The relative cell number and BrdU incorporation were normalized to VEGF-free control. *P < 0.05 compared with the VEGF+ control; means ± SE, n = 6. D: ANP inhibits VEGF-stimulated PMVEC migration. PMVECs were cultured and grown to confluence in six-well plates under serum-free conditions (control). After introduction of a scrape wound (indicated by the double dashed line), VEGF (100 ng/ml) and ANP (10 nmol/l) were added to the medium for another 96 h. The number of migrating cells was measured in 4 separate experiments for triplicate assay in each sample and was normalized to control groups (100 ± 10%); means ± SE. VEGF increased cell migration, and ANP inhibited cell migration. ANP itself did not influence the cell migration in serum-free control (112 ± 14%, P > 0.05 compared with control (CTL)).
released from smooth muscle cells that is related to the activation of natriuretic peptide receptor (28). Consistent with these earlier reports, we found that ANP inhibited VEGF-stimulated cell proliferation and migration within the concentration range that increases [cGMP]i (27). In conclusion, we have established that a stable phenotypic heterogeneity among macro- and microvascular endothelial cells is the expression of PDE5, which controls with temporal-heterogeneity among macro- and microvascular endothelial cells. Moreover, we have resolved that the absence of PDE5 activity interrupts VEGF-stimulated growth and increases apoptosis. Thus [cGMP], transitions mediate downstream survival cues evoked by VEGF family growth factors.

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GRANTS

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REFERENCES


