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Adenovirus-mediated Gene Transfer of cGMP-dependent Protein Kinase Increases the Sensitivity of Cultured Vascular Smooth Muscle Cells to the Antiproliferative and Pro-apoptotic Effects of Nitric Oxide/cGMP*

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Studies *in vitro* have underestimated the importance of cGMP-dependent protein kinase (PKG) in the modulation of vascular smooth muscle cell (SMC) proliferation and apoptosis *in vivo*. This is attributable, in part, to a rapid decline in PKG levels as vascular SMC are passaged in culture. We used a recombinant adenovirus encoding PKG (Ad.PKG) to augment kinase activity in cultured rat pulmonary artery SMC (RPaSMC). Incubation of Ad.PKG-infected RPaSMC (multiplicity of infection = 200) with 8-Br-cGMP decreased serum-stimulated DNA synthesis by 85% and cell proliferation at day 5 by 74%. The effect of 8-Br-cGMP on DNA synthesis in Ad.PKG-infected RPaSMC was blocked by KT5823 (PKG inhibitor), but not by KT5720 (cAMP-dependent protein kinase inhibitor). A nitric oxide (NO) donor compound, *S*-nitrosoglutathione, at concentrations as low as 100 nM, inhibited DNA synthesis in Ad.PKG-infected RPaSMC, but not in uninfected cells or in cells infected with a control adenovirus. In addition, 8-Br-cGMP and *S*-nitrosoglutathione induced apoptosis in serum-deprived RPaSMC infected with Ad.PKG, but not in uninfected cells or in cells infected with a control adenovirus. These results demonstrate that modulation of PKG levels in vascular SMC can alter the sensitivity of these cells to NO and cGMP. Moreover, these observations suggest an important role for PKG in the regulation of vascular SMC proliferation and apoptosis by NO and cGMP.

The endothelium plays a pivotal role in the regulation of vascular tone, the prevention of thrombosis, and the modulation of adhesive interactions between inflammatory cells and the vessel wall. The endothelium modulates the functions of the subjacent vascular smooth muscle, in part, by producing active effector molecules, including angiotensin II, heparinoids, and nitric oxide (NO)[1] (1, 2). Endothelial dysfunction is a shared process in the pathogenesis of vascular disorders, including atherosclerosis, neointima formation after angioplasty, and vascular remodeling associated with pulmonary or systemic hypertension (3). This dysfunction is associated with an alteration of the balance between cell growth and apoptosis and with dysregulation of cell-cell as well as cell-matrix interactions (1).

In addition to its role as an endothelium-derived relaxing factor (4), NO regulates platelet adhesion and aggregation (5, 6), leukocyte recruitment and activation (7), and cytokine-induced endothelial cell activation (8) as well as vascular smooth muscle cell (SMC) apoptosis (9–11), proliferation (12–14), and migration (15, 16). NO acts, in part, by stimulating soluble guanylate cyclase to produce the intracellular second messenger cGMP (17). cGMP activates cGMP-dependent protein kinase (PKG), leading to many of the effects of NO (18). The two isoforms of PKG detected in vascular smooth muscle (Iα and Iβ) share substrate-binding/catalytic domains, but differ in cGMP affinity (19, 20).

The effects of NO on vascular SMC proliferation have been extensively investigated. Although several studies established that NO decreases proliferation of vascular SMC stimulated with various mitogens (12–14, 21–26), the molecular mechanisms responsible for this effect are incompletely characterized. In most studies, the antiproliferative effect of NO on SMC was mediated via a cGMP-dependent mechanism (12–14, 21–23). Recently, Yu *et al.* (21) observed that cGMP-elevating agents decreased DNA synthesis and proliferation of epidermal growth factor-stimulated vascular SMC. The effects of cGMP on both DNA synthesis and cell proliferation were inhibited by the selective PKG inhibitor KT5823 (21). These results differ from those of Cornwell *et al.* (24), who reported that NO and cGMP inhibited proliferation of cultured rat aortic SMC (RASMC) via a mechanism mediated by cAMP-dependent protein kinase (PKA) and not by PKG.

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[1] The abbreviations used are: NO, nitric oxide; SMC, smooth muscle cell(s); PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; RASMC, rat aortic smooth muscle cell(s); RPaSMC, rat pulmonary artery smooth muscle cell(s); GSNO, *S*-nitrosoglutathione; pfu, plaque-forming unit(s); m.o.i., multiplicity(ies) of infection; PBS, phosphate-buffered saline; cAMP-S, adenosine cyclic 3′:5′-phosphorothioate.

PKG Modulates Vascular SMC Proliferation and Apoptosis

Similarly, the role of NO in the induction of the apoptotic cell death program has been extensively studied (9–11, 27–31). NO appears to be a bifunctional modulator of cell death capable of either inhibiting or stimulating apoptosis depending on the cell type and the concentration of NO applied. Several investigators reported that high concentrations of NO donor compounds induce apoptosis in vascular SMC (9–11, 27). However, experiments designed to determine the role of cGMP in the activation of programmed SMC death yielded conflicting results (9–11, 27).

Because PKG expression rapidly decreases as vascular SMC are passaged in culture (32, 33), the contribution of PKG to the modulation of vascular SMC proliferation and apoptosis by NO donor compounds and cGMP analogues is likely to be underestimated. To further investigate the role of PKG in the regulation of the balance between SMC proliferation and apoptotic SMC death, we used adenovirus-mediated gene transfer to increase PKG activity in cultured rat pulmonary artery SMC (RPaSMC). We report that augmentation of PKG activity in RPaSMC, using adenovirus-mediated gene transfer, inhibited proliferation induced by serum supplementation and increased apoptosis associated with serum deprivation. Moreover, PKG gene transfer increased the sensitivity of vascular SMC to the antiproliferative and pro-apoptotic effects of NO and cGMP.

EXPERIMENTAL PROCEDURES

These investigations were approved by the Subcommittee for Research Animal Studies at the Massachusetts General Hospital.

Reagents

All chemicals were purchased from Sigma, except *S*-nitrosoglutathione (GSNO), which was obtained from Alexis Corp. (San Diego, CA), and the protein kinase inhibitors KT5823 and KT5720, which were purchased from Calbiochem.

Cloning of a Human PKG cDNA

A polymerase chain reaction was performed to amplify a PKG cDNA from reverse-transcribed rat brain mRNA using degenerate oligonucleotides corresponding to amino acids 1–4 and 105–110 shared by bovine and human PKG-Iβ isoforms: 5′-CCGAATTCAGGAGCAT-GGGCACCYTGCG-3′ and 5′-CCGGATCCTTTATRAGATCCTTGGA-3′ (34–36). The polymerase chain reaction product was ligated into pUC18, and its nucleotide sequence was determined. To obtain a human PKG-Iβ cDNA, a λgt11 cDNA library prepared from human placenta (CLONTECH) was screened using a ^{32}P-labeled *Eco*RI-*Bam*HI restriction fragment of the rat PKG-Iβ cDNA. One cDNA insert from a hybridizing bacteriophage contained sequences identical to nucleotides 22–900 of the sequence for human PKG-Iβ (36). This cDNA was used to re-screen the library, resulting in the isolation of a second cDNA insert containing sequences identical to amino acids 430–3740 of the human PKG-Iβ sequence. The two inserts were ligated at *Nco*I to produce a human PKG-Iβ cDNA specifying the full-length protein. The PKG-Iβ cDNA was subcloned into pRc/RSV (Invitrogen, San Diego, CA).

Construction of E1-deleted Recombinant Adenoviruses

The *Eco*RI-*Bgl*II restriction fragment of pRc/RSV.PKG was ligated into the *Not*I and *Bam*HI sites of the vector pAd.RSV₄ (kindly provided by Dr. B. Davidson, University of Iowa), which contains the Rous sarcoma virus long terminal repeat as a promoter and the SV40 polyadenylation signal, using a linker encoding the FLAG epitope (DYKD-DDDK) behind a translation start site (5′-GGCCGCATGGACTACAAA-GACGATGACGACAA-3′ and 5′-AATTTTGTCGTCATCGTCTTTGTA-GTCCATGC-3′) (37). The resulting plasmid vector (pAd.RSV₄.PKG) was cotransfected into 293 cells with pJM17 (generously provided by Dr. F. Graham, McMaster University) (38). Homologous recombinants containing the PKG-Iβ cDNA substituted for E1 were detected using RNA blot hybridization. The recombinant adenovirus Ad.PKG was expanded, purified, and titered in 293 cells as described previously (39). Two additional recombinant adenoviruses were used as controls: Ad.GFP, which specifies the EGFP mutant of the green fluorescent protein (40), and Ad.βgal, which specifies a nuclear-targeted form of β-galactosidase (generously provided by Dr. D. Dichek, Gladstone Institute for Cardiovascular Diseases, San Francisco, CA) (41). The titer of viral stocks used for these studies was 2 × 10¹⁰ plaque-forming units

(pfu)/ml (Ad.PKG), 1 × 10¹⁰ pfu/ml (Ad.GFP), and 4 × 10¹⁰ pfu/ml (Ad.βgal), with particle/pfu ratios of 35:1, 45:1, and 38:1, respectively. Contamination of viral stocks with replication-competent adenoviruses was excluded by the absence of cytopathic effect after infection of a monolayer of A549 lung carcinoma cells with viral stocks (20 pfu/cell) and by the failure to amplify a 200-base pair fragment of the E1 region using polymerase chain reaction.

Preparation of Rat Pulmonary Artery Smooth Muscle Cells

Primary cultures of RPaSMC were prepared from explants of endothelium- and adventitia-stripped pulmonary arteries of adult Sprague-Dawley rats as described previously (42). RPaSMC were maintained in RPMI 1640 medium supplemented with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA), penicillin, and streptomycin. Cells were used between passages 3 and 10.

Detection of PKG Transgene Expression

Immunoblotting—RPaSMC were plated at a concentration of 5 × 10⁵ cells/dish in 60-mm culture dishes and infected with Ad.PKG using multiplicities of infection (m.o.i.) ranging from 50 to 500 pfu/cell for 2 h. After removal of the virus suspension, cells were incubated for 48 h and then harvested in PEM buffer containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 5 μg/ml aprotinin), 2 mM EDTA, 15 mM 2-mercaptoethanol, and 20 mM KH₂PO₄, pH 6.8. The cell suspension was homogenized and centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants of centrifuged cell extracts (15 μg) were fractionated by 8% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters (Micron Separations, Westborough, MA). Filters were blocked at room temperature for 1 h in phosphate-buffered saline, pH 7.4, containing 5% nonfat dry milk (PBS/milk) and then incubated for 1 h at room temperature with an anti-FLAG M2 mouse monoclonal antibody (Eastman Kodak Co.) diluted in PBS/milk at a concentration of 10 μg/ml. After washing, the blots were incubated with a horseradish peroxidase-conjugated anti-mouse IgG (Bio-Rad) diluted 1:5000 in PBS/milk. Proteins were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposure to x-ray film.

Immunocytochemistry—RPaSMC were plated on four-well chamber slides (Nalge Nunc International, Naperville, IL) and infected with Ad.PKG (m.o.i. = 50–500 pfu/cell). The virus suspension was removed 2 h after infection, and cells were incubated in RPMI 1640 medium with 0.1% NuSerum. After 1, 2, 3, 5, and 7 days, cells were fixed in 4% paraformaldehyde containing 5% sucrose for 20 min at room temperature. Cells were washed in PBS, treated with 1% SDS in PBS, washed in PBS, and incubated with 0.1% Triton X-100 in PBS. After washing in PBS, cells were incubated with the anti-FLAG M2 antibody in PBS (6 μg/ml) containing 1% bovine serum albumin (1 h at room temperature) and subsequently with Cy3-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in PBS to a final concentration of 1 μg/ml. Slides were examined immediately after preparation using a fluorescence photomicroscope. Specificity of the immunostaining was ascertained using uninfected cells and cells infected with Ad.βgal as well as by incubation without the primary antibody. The percentage of cells expressing the transgene at 1, 2, 3, 5, and 7 days after infection was determined.

Measuring PKG Enzyme Activity in SMC

To assess the biological activity of the transgene product, PKG enzyme activity was measured in RPaSMC infected with Ad.PKG using methods described previously (43). Briefly, 5 × 10⁶ cells were infected with Ad.PKG (m.o.i. = 50–500 pfu/cell). After 48 h, cells were harvested and homogenized in 0.3 ml of PEM buffer before centrifugation at 10,000 × *g* for 10 min at 4 °C. After determination of the protein concentration using a Bradford protein assay (Bio-Rad), the supernatants (10 μl) of centrifuged cell extracts were assayed in a 50-μl reaction mixture containing 20 μM Tris, pH 7.4, 200 μM ATP, a custom-synthesized peptide RKISASEF (136 μg/ml) (Genosys Biotechnologies, Inc., The Woodlands, TX) (44), 20 mM MgCl₂, 100 μM 3-isobutyl-1-methylxanthine, 1 μM (R_p)-cAMP-S (Biolog Life Science Institute, La Jolla, CA), and 30,000 cpm/μl [γ-³²P]ATP. Assays were conducted in the absence or presence of 10 μM cGMP at 30 °C for 10 min and were terminated by transferring samples onto phosphocellulose P-81 paper (Whatman) and washing in 75 mM phosphoric acid. Papers were then washed once in ethanol and dried. Radiolabeled peptide bound to the phosphocellulose paper was quantitated by liquid scintillation spectrometry. PKG enzyme activity is expressed as nanomoles of peptide phosphorylated per min/mg of cell extract protein.

Measurement of [³H]Thymidine Incorporation

RPaSMC were plated in 96-well plates (10^3 cells/well) and infected with Ad.PKG or Ad.GFP (m.o.i. = 50–200 pfu/cell). After 2 h, the viral suspension was removed, and cells were incubated in RPMI 1640 medium containing 0.1% NuSerum for 48 h to achieve a quiescent state. The culture medium was subsequently replaced with RPMI 1640 medium containing 0.1 or 4% NuSerum supplemented with 8-Br-cGMP, GSNO, 8-Br-cAMP, KT5823, or KT5720 or combinations of these reagents. After 24 h, the media and reagents were replaced in each well, and [³H]thymidine (NEN Life Science Products) was added to a final concentration of 1 μCi/ml. After 12 h, cells were dissociated from the culture dishes using trypsin, and radiolabeled DNA was transferred to micro-glass-fiber filters (Cambridge Technology, Inc., Watertown, MA) using a PHD cell harvester (Cambridge Technology Inc.). Filters were then washed and dried, and [³H]thymidine-labeled DNA was quantitated by liquid scintillation spectrometry. For each experiment, 8–12 wells were used for each experimental condition, and [³H]thymidine incorporation was measured in duplicate for each well.

Measurement of the Cell Proliferation Rate

RPaSMC were plated in six-well plates (2×10^4 cells/well) and infected with Ad.PKG or Ad.GFP (m.o.i. = 200 pfu/cell). The virus suspension was removed 2 h after infection, and cells were growth-arrested in RPMI 1640 medium with 0.1% NuSerum for 48 h. Cells were then incubated in RPMI 1640 medium containing 0.1 or 4% NuSerum in the absence or presence of 1 mM 8-Br-cGMP. For each experimental condition, cells from three wells were harvested with trypsin and counted with a hemocytometer at days 0, 1, 2, 3, 4, and 5. Cell viability was assessed by trypan blue exclusion, and the number of cells floating in the culture medium was determined daily.

Nuclear Staining of Apoptotic Cells

The DNA-binding dye Hoechst H33258 was used to define nuclear chromatin morphology as a quantitative index of apoptosis within the cell culture system. RPaSMC were plated on four-well chamber slides (2×10^4 cells/well) and infected with Ad.PKG or Ad.GFP (m.o.i. = 200 pfu/cell). The virus suspension was removed 2 h after infection, and cells were incubated in RPMI 1640 medium containing 0.1% NuSerum during 48 h. The culture medium was replaced and supplemented with 8-Br-cGMP, GSNO, or KT5823 or combinations of these reagents. After 24 h, cells were fixed in 4% paraformaldehyde containing 5% sucrose, washed in PBS, incubated in 95% ethanol (3 min), and stained with H33258 (1 μg/ml) before examination by fluorescence microscopy. Apoptosis was defined by the presence of nuclear chromatin condensation or nuclear fragmentation. To quantify apoptosis, 300 nuclei from each of five random microscopic fields were analyzed by an observer blinded as to the treatment group. The percentage of apoptotic nuclei was calculated as the ratio of apoptotic nuclei to total nuclei.

Microscale Analysis of DNA Fragmentation

To analyze the pattern of DNA fragmentation, RPaSMC were cultured in 10-cm dishes (1×10^6 cells/plate), infected with Ad.PKG or Ad.GFP (m.o.i. = 200 pfu/cell), and incubated under low serum conditions. Forty-eight h after infection, the cells were rinsed with PBS and incubated in RPMI 1640 medium containing 0.1% NuSerum in the absence or presence of 8-Br-cGMP (1 mM) for 24 h. Subsequently, adherent cells were detached from the culture dishes with trypsin and pooled with cells floating in the supernatant. Cells were pelleted by centrifugation, washed in PBS, and incubated for 4 h at 37 °C in lysis buffer containing 100 mM Tris, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 0.1 mg/ml proteinase K. The genomic DNA was precipitated with 700 μl of isopropyl alcohol, resuspended in Tris/EDTA buffer, and extracted with phenol and chloroform before precipitation in the presence of 3 M sodium acetate and ethanol. After treatment with RNase (12.5 μg/ml for 1 h at 37 °C), DNA was quantitated by measuring the absorbance at 260 and 280 nm. DNA samples (1 μg) were subsequently labeled using [α-³²P]dCTP and Exo⁻ Klenow polymerase (Stratagene, La Jolla, CA) and fractionated by electrophoresis on 2% agarose gels. Gels were dried and subjected to autoradiography for 2 h at −70 °C.

Statistical Analysis

Each experiment was repeated a minimum of three times. Data are presented as the means ± S.D. Statistical analysis was performed by analysis of variance followed by Bonferroni's *t* test for post hoc analysis. To compare the rate of proliferation of vascular SMC for each treatment group, logarithmic transformation of cell counts (n) was performed, and the slopes of the linear relationship fitting $\log(n)$ as a function of time
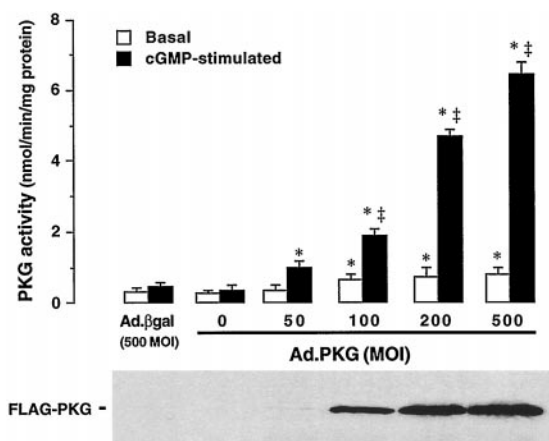

FIG. 1. **Ad.PKG infection increases PKG levels and activity in cultured RPaSMC.** Levels and enzyme activity of the PKG transgene product were measured in uninfected RPaSMC and in RPaSMC infected with increasing concentrations of Ad.PKG (m.o.i. = 50, 100, 200, and 500 pfu/cell) and with Ad.βgal (m.o.i. = 500 pfu/cell). Forty-eight h after infection, proteins in the soluble fraction of cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The 80-kDa PKG transgene product, tagged with the FLAG epitope, was detected using a monoclonal antibody directed against FLAG. PKG enzyme activity was determined by measuring the ability of cell extracts to phosphorylate the synthetic peptide RKISASEF in the presence of the PKA inhibitor (R_p)-cAMP-S. Basal and stimulated PKG enzyme activities were measured by incubating cell extracts in the absence or presence of 10 μM cGMP, respectively. Transduction of RPaSMC with Ad.PKG increased PKG protein levels and enzyme activity in a m.o.i.-dependent manner. *, $p < 0.05$, differed from control cells incubated with or without cGMP; ‡, $p < 0.05$, cGMP-stimulated enzyme activity differed from basal enzyme activity.

were compared using analysis of covariance. A value of $p < 0.05$ was considered significant.

RESULTS

Ad.PKG Infection Increases PKG Activity in Vascular SMC in Culture—To augment PKG enzyme activity in vascular SMC in culture, we used a replication-deficient recombinant adenovirus encoding the human PKG-Iβ isoform. A FLAG epitope was encoded at the amino terminus to facilitate transgene detection. To determine whether increased levels of PKG protein could be achieved in cultured SMC using adenovirus-mediated gene transfer, RPaSMC were infected with increasing concentrations of Ad.PKG (m.o.i. = 50, 100, 200, and 500 pfu/cell), and the expression of the recombinant PKG protein was measured by immunoblotting. Forty-eight h after infection, expression of PKG was detectable in cell extracts from RPaSMC infected at m.o.i. = 50 pfu/cell and increased in a m.o.i.-dependent manner (Fig. 1). The recombinant protein was detectable as early as 24 h, peaked at 48 h, and persisted for 7 days after infection (data not shown).

To confirm that the FLAG peptide did not interfere with the biological activity of the transgene product, PKG activity was measured in cell extracts from RPaSMC infected with Ad.PKG (m.o.i. = 0–500 pfu/cell). As shown in Fig. 1, adenovirus-mediated PKG gene transfer at m.o.i. = 100 resulted in a significant increase in basal PKG activity. cGMP-stimulated activity was markedly increased in cell extracts from RPaSMC infected at m.o.i. ≥ 50. Transduction at m.o.i. = 200 resulted in a cGMP-stimulated PKG activity of ∼4.7 nmol/min/mg of protein, which is similar to the activity measured in extracts of freshly isolated RASMC (45) as well as in intact rat pulmonary arteries (data not shown). Infection of SMC with a control adenovirus (Ad.βgal) did not affect PKG activity.

To identify the m.o.i. required to infect the majority of vascular SMC in culture, RPaSMC were incubated with Ad.PKG
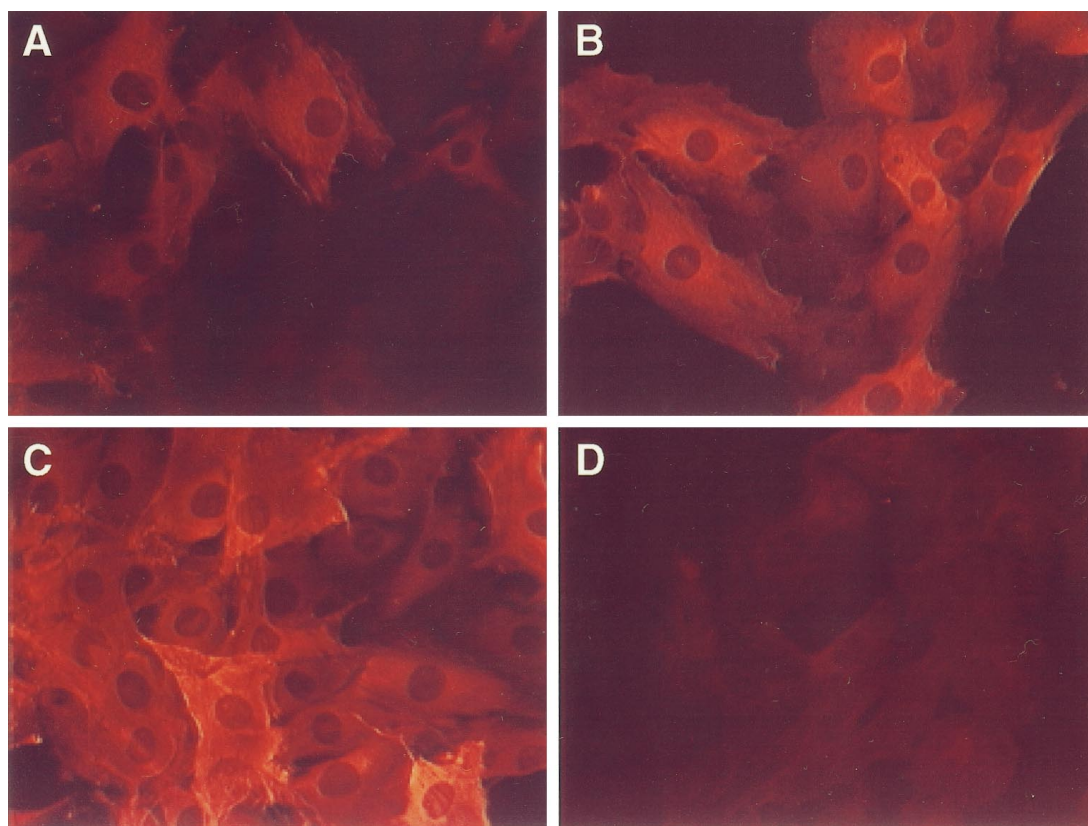
PKG Modulates Vascular SMC Proliferation and Apoptosis

FIG. 2. **Ad.PKG infection efficiently transduces PKG into cultured RPaSMC.** RPaSMC were plated on four-well chamber slides and infected with Ad.PKG (m.o.i. = 50 (*A*), 100 (*B*), and 200 (*C*) pfu/cell) or with Ad.βgal (m.o.i. = 200 pfu/cell (*D*)). Forty-eight h after infection, cells were fixed and sequentially incubated with the anti-FLAG M2 antibody and a Cy3-conjugated secondary antibody. Cells were examined immediately after preparation using an epifluorescence photomicroscope. Infection of RPaSMC with Ad.PKG (m.o.i. = 200 pfu/cell) infected 100% of the cells in culture.

at various m.o.i., and 48 h later, the percentage of cells containing PKG was determined by immunocytochemical techniques using an anti-FLAG monoclonal antibody and a Cy3-conjugated secondary antibody. FLAG immunoreactivity was detected in 30, 50, and 100% of RPaSMC infected with Ad.PKG at 50, 100 and 200 pfu/cell, respectively, but not in RPaSMC infected with Ad.βgal (Fig. 2). There was no evidence of cytopathic effect in vascular SMC infected with Ad.PKG at any m.o.i. tested.

PKG Expression Decreases DNA Synthesis in Vascular SMC—To determine the effect of PKG on DNA synthesis in vascular SMC, RPaSMC were infected with Ad.PKG (m.o.i. = 0–500 pfu/cell) and stimulated to undergo DNA replication with 4% NuSerum. Compared with cells maintained in 0.1% NuSerum, incubation with 4% NuSerum resulted in an 8–10-fold increase in DNA synthesis in RPaSMC, as assessed by [^{3}H]thymidine incorporation (data not shown). Adenovirus-mediated gene transfer of PKG decreased [^{3}H]thymidine incorporation in a m.o.i.-dependent fashion (Fig. 3). The maximum antiproliferative effect was obtained in RPaSMC infected at m.o.i. = 200 pfu/cell, and transduction of RPaSMC with 500 pfu/cell did not further decrease [^{3}H]thymidine incorporation despite increased cGMP-stimulated PKG activity. Exposure to 8-Br-cGMP, a membrane-permeable cGMP analogue, markedly potentiated the antimitogenic effect of Ad.PKG infection. Transduction of RPaSMC at m.o.i. = 100 pfu/cell significantly decreased DNA synthesis in the presence, but not in the absence, of 1 mM 8-Br-cGMP. The 8-Br-cGMP-induced reduction in [^{3}H]thymidine incorporation was significantly greater in RPaSMC transduced with Ad.PKG (m.o.i. = 200 pfu/cell) than in uninfected cells.

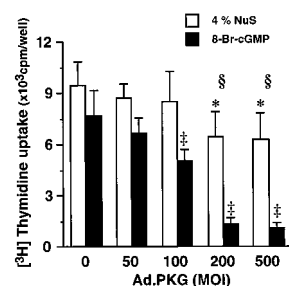To evaluate the effect of Ad.PKG infection (m.o.i. = 200

FIG. 3. **PKG activation decreases DNA synthesis in RPaSMC.** RPaSMC were plated in 96-well plates (10^3 cells/well) and infected with Ad.PKG (0, 50, 100, 200, and 500 pfu/cell). Cells were incubated in RPMI 1640 medium containing 0.1% NuSerum (*NuS*) for 48 h. The culture medium was then replaced with RPMI 1640 medium containing 4% NuSerum in the absence (*open bars*) or presence (*closed bars*) of 1 mM 8-Br-cGMP. After 24 h, the medium was replaced in each well (with supplements), and [^{3}H]thymidine was added to a final concentration of 1 μCi/ml. Incorporation of [^{3}H]thymidine into DNA was determined 12 h later. Data are expressed as the means ± S.D. (*n* = 24). Adenovirus-mediated PKG gene transfer decreased DNA synthesis in serum-stimulated RPaSMC. *, $p < 0.05$ *versus* uninfected cells; ‡, $p < 0.05$ *versus* uninfected cells treated with 8-Br-cGMP; §, $p < 0.05$, cGMP-induced reduction in DNA synthesis differed from that in uninfected cells.

pfu/cell) on the sensitivity of RPaSMC to the antiproliferative effects of 8-Br-cGMP, [^{3}H]thymidine incorporation was measured in cells incubated in the presence of 0, 10^{-5}, 10^{-4}, and 10^{-3} M 8-Br-cGMP. As shown in Fig. 4, incubation of Ad.PKG-infected cells with as little as 10^{-4} M 8-Br-cGMP significantly inhibited DNA synthesis as compared with Ad.PKG infected cells incubated without 8-Br-cGMP. Infection with Ad.PKG also increased the sensitivity of RPaSMC to the antiprolifera-
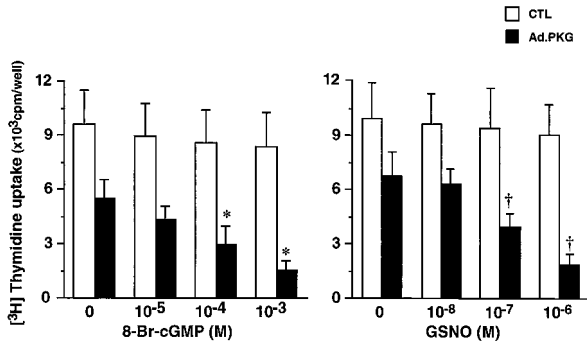
FIG. 4. **PKG increases the sensitivity of vascular SMC to the antimitogenic effects of NO and cGMP.** Incorporation of [³H]thymidine was measured in uninfected RPaSMC (*open bars*) or in RPaSMC transduced with Ad.PKG (m.o.i. = 200 pfu/cell; *closed bars*). RPaSMC were plated in 96-well plates (10³ cells/well), infected, and incubated in RPMI 1640 medium containing 0.1% NuSerum for 48 h. The culture medium was then replaced with RPMI 1640 medium containing 4% NuSerum without or with 8-Br-cGMP (10^{-5}, 10^{-4}, and 10^{-3} M; *left panel*) or GSNO (10^{-8}, 10^{-7}, and 10^{-6} M; *right panel*). After 24 h, the medium was replaced in each well (with supplements), and [³H]thymidine was added to a final concentration of 1 μCi/ml. Incorporation of [³H]thymidine into DNA was determined 12 h later. Data are expressed as the means ± S.D. ($n = 36$). PKG gene transfer into RPaSMC potentiated the antimitogenic effects of 8-Br-cGMP and GSNO. *, $p < 0.05$ *versus* NuSerum without 8-Br-cGMP; †, $p < 0.05$ *versus* NuSerum without GSNO. *CTL*, control.

tive effects of NO: incubation of Ad.PKG-infected cells with as little as 10^{-7} M GSNO (a NO donor compound) decreased [³H]thymidine incorporation as compared with Ad.PKG-infected cells incubated without GSNO. In contrast, concentrations of 8-Br-cGMP and GSNO up to 1 mM and 10^{-6} M, respectively, did not alter DNA synthesis in uninfected RPaSMC (Fig. 4) or in RPaSMC infected with a control recombinant adenovirus (Ad.GFP, m.o.i. = 200 pfu/cell) (Fig. 5). These results suggest that the effects of Ad.PKG infection on DNA synthesis were transgene-specific. Moreover, adenovirus-mediated PKG gene transfer increased the sensitivity of vascular SMC to the antiproliferative effects of NO and cGMP.

Previous studies suggested that the antiproliferative effect of NO was mediated via PKA (21). To evaluate the relative roles of cyclic nucleotide-dependent protein kinases in adenovirus-transduced vascular SMC, we used protein kinase inhibitors that are selective for PKG and PKA: KT5823 and KT5720, respectively. Incubation of uninfected vascular SMC with 0.25 μM KT5823 induced a 16% decrease in serum-stimulated [³H]thymidine incorporation (data not shown). KT5720 did not significantly alter DNA synthesis at concentrations between 0.05 and 1 μM. Preincubation with 0.25 μM KT5823 reversed the antimitogenic effects of 1 mM 8-Br-cGMP and 10^{-6} M GSNO on Ad.PKG-infected RPaSMC (Fig. 5). In uninfected cells, 0.5 μM KT5720 blocked the antiproliferative effect of 1 mM 8-Br-cAMP, a membrane-permeable cAMP analogue (Fig. 6). However, KT5720 did not affect the antimitogenic activity of 8-Br-cGMP in Ad.PKG-infected cells (Fig. 6). Taken together, these results suggest that the antimitogenic effects of cGMP-elevating agents on Ad.PKG-infected cells are PKA-independent.

PKG Activation Decreases Vascular SMC Proliferation—To determine whether PKG-induced inhibition of DNA synthesis was associated with a decrease in the rate of cell proliferation, we counted uninfected RPaSMC and RPaSMC infected with Ad.PKG or Ad.GFP that were incubated in the presence of serum with or without 8-Br-cGMP (Fig. 7). Uninfected and adenovirus-infected RPaSMC were incubated in 0.1% NuSerum for 48 h, at which time the number of cells did not differ among groups. Thereafter, the culture medium was replaced and supplemented with 4% NuSerum with or without 1
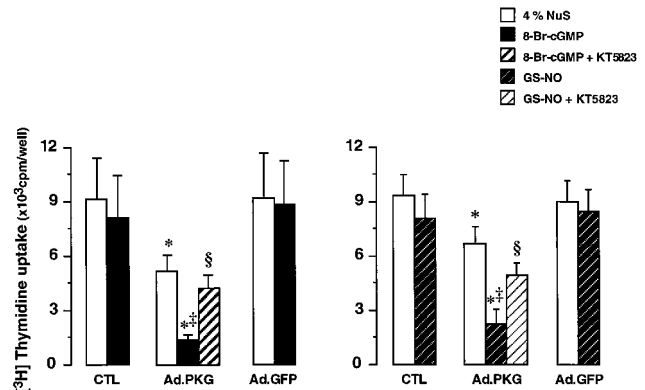

FIG. 5. **KT5823 blocks the inhibitory effect of NO/cGMP on DNA synthesis in Ad.PKG-infected vascular SMC.** RPaSMC plated in 96-well plates (10³ cells/well) were incubated in the absence of adenovirus (uninfected) or in the presence of Ad.PKG or Ad.GFP (m.o.i. = 200 pfu/cell) for 2 h. After 48 h of incubation in RPMI 1640 medium with 0.1% NuSerum (*NuS*), the culture medium was replaced with RPMI 1640 medium containing 4% NuSerum. In experiments presented in the *left panel*, the medium was supplemented with 1 mM 8-Br-cGMP with or without 0.25 μM KT5823. In experiments presented in the *right panel*, 1 μM GSNO with or without 0.25 μM KT5823 was added to the culture medium. After 24 h, the medium was replaced in each well (with supplements), and [³H]thymidine (1 μCi/ml) was added. [³H]Thymidine uptake was measured after 12 h. Data are expressed as the means ± S.D. ($n = 36$). 8-Br-cGMP and GSNO decreased DNA synthesis in Ad.PKG-infected RPaSMC, but not in uninfected or Ad.GFP-infected RPaSMC. The inhibitory effect of 8-Br-cGMP or GSNO was attenuated by KT5823. *, $p < 0.05$ *versus* uninfected cells; ‡, $p < 0.05$ *versus* Ad.PKG-infected cells not treated with 8-Br-cGMP or GSNO; §, $p < 0.05$ *versus* Ad.PKG-infected cells treated with 8-Br-cGMP or GSNO. *CTL*, control.
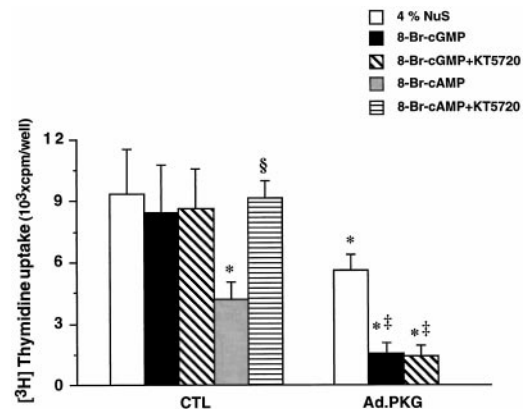

FIG. 6. **PKA inhibition does not block the inhibitory effect of cGMP on DNA synthesis in Ad.PKG-infected vascular SMC.** Uninfected RPaSMC plated in 96-well plates (10³ cells/well) were incubated in RPMI 1640 medium containing 0.1% NuSerum (*NuS*) for 48 h. The culture medium was then replaced with RPMI 1640 medium containing 4% NuSerum with and without 1 mM 8-Br-cGMP, 1 mM 8-Br-cAMP, and the cyclic nucleotides combined with 0.5 μM KT5720. Additional RPaSMC were infected with Ad.PKG (m.o.i. = 200 pfu/cell) and then incubated in RPMI 1640 medium containing 0.1% NuSerum for 48 h. Infected cells were incubated with and without 8-Br-cGMP in the presence or absence of 0.5 μM KT5720. Twenty-four h after initiation of growth stimulation, the medium was replaced in each well (with supplements); [³H]thymidine (1 μCi/ml) was added; and incorporation of [³H]thymidine was measured 12 h later. Data are expressed as the means ± S.D. ($n = 36$). The PKA inhibitor KT5720 did not block the antimitogenic effect of 8-Br-cGMP in Ad.PKG-infected RPaSMC. *, $p < 0.05$ *versus* uninfected and untreated cells; ‡, $p < 0.05$ *versus* Ad.PKG-infected cells not treated with 8-Br-cGMP; §, $p < 0.05$ *versus* uninfected cells treated with 8-Br-cAMP. *CTL*, control.

mM 8-Br-cGMP. Cells were counted daily for 5 days, and the rate of proliferation was assessed by comparing the slope of the linear relationships fitting logarithmic transformation of cell
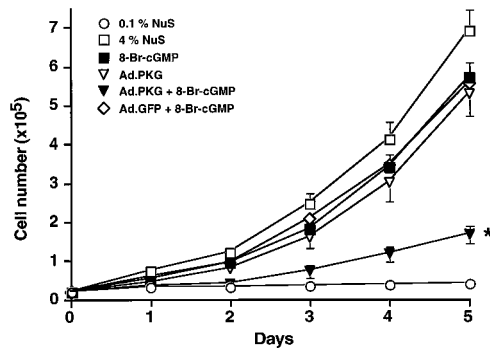
FIG. 7. **PKG activation decreases proliferation of RPaSMC.** RPaSMC were plated in six-well plates (2×10^4 cells/well). RPaSMC cultures were infected with Ad.PKG (*Ad.GK*) or Ad.GFP (m.o.i. = 200 pfu/cell), whereas others were uninfected. After 2 h, the virus suspension was removed, and cells were incubated in RPMI 1640 medium containing 0.1% NuSerum (*NuS*) for 48 h. Subsequently, the culture medium was replaced with RPMI 1640 medium containing 0.1 or 4% NuSerum in the absence or presence of 1 mM 8-Br-cGMP. Media and reagents were replaced every 24 h. Cells were dissociated from the culture dish with trypsin and counted daily for 5 days ($n = 3$ for each condition). The rate of proliferation was assessed by comparing the slope of the linear relationships fitting logarithmic transformation of cell counts as a function of time in each treatment group. Data are expressed as the means $\pm$ S.D. The rate of proliferation of Ad.GFP-infected cells incubated in the absence (data not shown) or presence of 8-Br-cGMP did not differ from that of uninfected RPaSMC. Incubation with 8-Br-cGMP significantly decreased the rate of proliferation of RPaSMC infected with Ad.PKG. *, $p < 0.05$ *versus* all other groups.

counts as a function of time in each treatment group. The rate of proliferation did not differ for uninfected RPaSMC and RPaSMC infected with Ad.PKG or Ad.GFP. Incubation of uninfected or Ad.GFP-infected RPaSMC with 8-Br-cGMP did not alter the rate of cell proliferation. However, 8-Br-cGMP markedly inhibited the proliferation of RPaSMC infected with Ad.PKG (Fig. 7). To confirm that the antiproliferative effect of PKG did not result from cell toxicity, viability was assessed by trypan blue exclusion and was found to be >95% in all groups. Moreover, the number of cells floating in the culture medium determined daily for 5 days after serum stimulation was found to be <1% and did not differ among groups. These data demonstrate that adenovirus-mediated PKG gene transfer increases the sensitivity of vascular SMC to the antiproliferative effects of cGMP.

PKG Activation Induces Vascular SMC Apoptosis—To test the hypothesis that PKG activation induces apoptosis of vascular SMC, RPaSMC infected with Ad.PKG or Ad.GFP and uninfected cells were incubated in culture medium containing 0.1% NuSerum in the absence or presence of 1 mM 8-Br-cGMP. Fig. 8 (*A* and *B*) shows the nuclear morphology of uninfected and Ad.PKG-infected RPaSMC, respectively, incubated with 8-Br-cGMP for 24 h. The nuclear morphology of uninfected RPaSMC incubated with 8-Br-cGMP was homogeneous. In contrast, the nuclear morphology of Ad.PKG-infected cells incubated with 8-Br-cGMP was not homogeneous, with many cells having condensed and coalesced nuclei (Fig. 8*B*, *inset*). To confirm that PKG activation induced apoptotic cell death, DNA was extracted from adenovirus-infected RPaSMC for assessment of internucleosomal DNA fragmentation by gel electrophoresis. Incubation of Ad.PKG-infected RPaSMC with 1 mM 8-Br-cGMP for 24 h resulted in a pattern of DNA fragmentation characteristic of apoptosis (Fig. 8*C*). In contrast, analysis of radiolabeled genomic DNA by gel electrophoresis did not reveal DNA fragmentation in uninfected RPaSMC incubated with 1 mM 8-Br-cGMP (Fig. 8*C*) or in Ad.GFP-infected cells in the presence or absence of 8-Br-cGMP (data not shown).

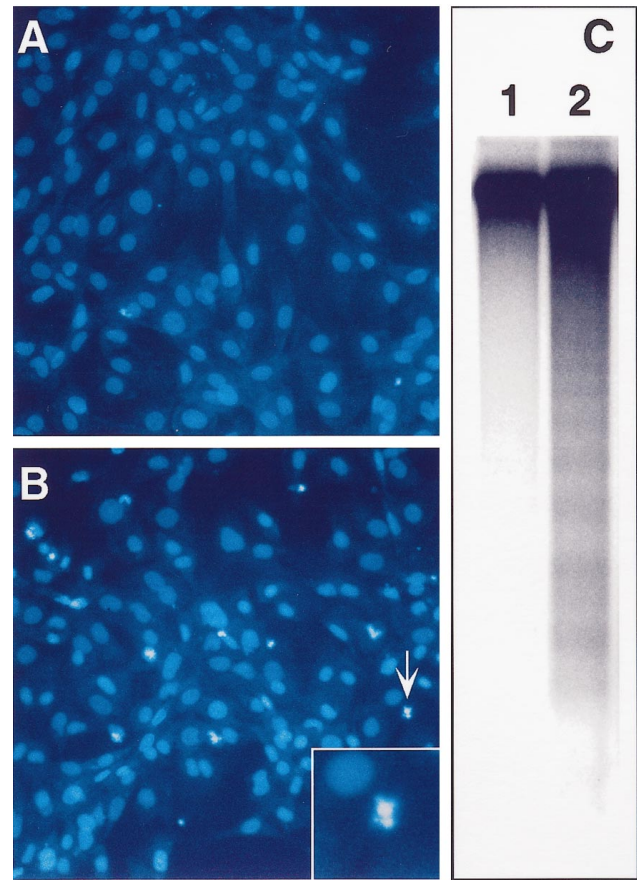To quantitate the impact of adenovirus-mediated gene trans-

FIG. 8. **8-Br-cGMP induces apoptosis in vascular smooth muscle cells infected with Ad.PKG.** Uninfected RPaSMC (*A*) or RPaSMC infected with Ad.PKG (m.o.i. = 200 pfu/cell; *B*) were incubated in RPMI 1640 medium containing 0.1% NuSerum. After 48 h, the culture medium was replaced with RPMI 1640 medium containing 0.1% NuSerum supplemented with 1 mM 8-Br-cGMP, and the cells were incubated for an additional 24 h. Thereafter, cells were fixed, stained with the DNA-binding dye Hoechst H33258 (1 μg/ml), and examined by fluorescence microscopy. Cells undergoing apoptosis were identified by their characteristic condensed and coalesced nuclei (*B*, *inset*). Incubation with 8-Br-cGMP increased the percentage of apoptotic nuclei in RPaSMC infected with Ad.PKG, but not in uninfected cells. In *C*, uninfected RPaSMC or RPaSMC infected with Ad.PKG (m.o.i. = 200 pfu/cell) were incubated in RPMI 1640 medium containing 0.1% NuSerum. After 48 h, the culture medium was replaced with RPMI 1640 medium containing 0.1% NuSerum supplemented with 1 mM 8-Br-cGMP, and the cells were incubated for an additional 24 h. Genomic DNA was extracted, radiolabeled, and separated by gel electrophoresis before autoradiography. Incubation with 8-Br-cGMP resulted in a pattern of DNA fragmentation characteristic of apoptosis in RPaSMC infected with Ad.PKG, but not in uninfected cells.

fer on vascular SMC apoptosis, the percentage of cells with apoptotic nuclei was determined by an investigator blinded as to the treatment group, with an intra-assay variability of 4%. Ad.PKG-infected cells incubated in the absence of 8-Br-cGMP exhibited condensed nuclei more frequently than uninfected cells. However, the percentage of apoptotic nuclei did not differ between Ad.PKG- and Ad.GFP-infected RPaSMC incubated in the absence of 8-Br-cGMP, suggesting that adenovirus infection itself was associated with a modest increase in cells undergoing apoptosis (Fig. 9). Incubation with 8-Br-cGMP for 24 h markedly increased the frequency with which Ad.PKG-infected RPaSMC underwent apoptosis, but did not increase apoptosis in uninfected or Ad.GFP-infected RPaSMC. To confirm that the pro-apoptotic effect of cGMP was mediated by PKG, RPaSMC infected with Ad.PKG were incubated under low serum conditions in the presence of 0.25 μM KT5823 and 1 mM 8-Br-cGMP. The PKG inhibitor blocked the pro-apoptotic effect of cGMP in
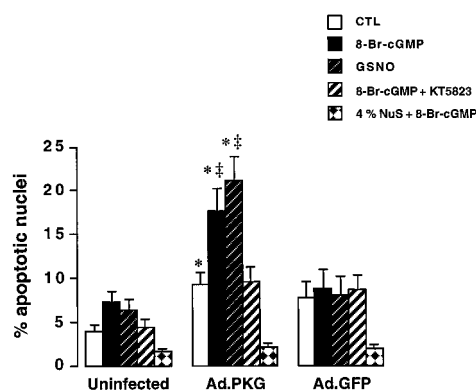
FIG. 9. **Quantitative analysis of apoptosis.** RPaSMC plated on four-well chamber slides were uninfected or infected with Ad.PKG or Ad.GFP (m.o.i. = 200 pfu/cell) and incubated in RPMI 1640 medium containing 0.1% NuSerum (*NuS*). After 48 h, the culture medium was replaced with RPMI 1640 medium containing 0.1% NuSerum supplemented with 1 mM 8-Br-cGMP, 1 mM 8-Br-cGMP + 0.25 μM KT5823, or 1 μM GSNO. Cells were incubated for 24 h before staining with the DNA-binding dye Hoechst H33258 (1 μg/ml). Vascular SMC were then examined by fluorescence microscopy, and the nuclear morphology of 300 nuclei from each of five random microscopic fields was analyzed by an observer blinded as to the treatment group. The percentage of apoptotic nuclei is defined as the ratio of apoptotic nuclei to total nuclei. In the absence of 8-Br-cGMP or GSNO, the apoptotic index of Ad.GFP-infected cells did not differ from that of Ad.PKG-infected or uninfected cells. Infection with Ad.PKG increased the sensitivity of vascular SMC to the pro-apoptotic effects of NO and cGMP. *, $p < 0.05$ *versus* uninfected and untreated cells; ‡, $p < 0.05$ *versus* Ad.PKG-infected cells not treated with 8-Br-cGMP or GSNO. *CTL*, control.

RPaSMC transduced with Ad.PKG, but did not significantly alter the percentage of apoptotic nuclei in uninfected cells or cells infected with Ad.GFP.

To test whether infection with Ad.PKG increased the sensitivity of vascular SMC to the pro-apoptotic effect of NO, uninfected RPaSMC and Ad.PKG- or Ad.GFP-infected RPaSMC were incubated in the presence of GSNO, and the percentage of apoptotic nuclei was determined. In uninfected cells, the percentage of apoptotic nuclei did not increase significantly after treatment with 1 μM GSNO. In contrast, after incubation with 1 μM GSNO for 24 h, ~22% of the nuclei from Ad.PKG-infected RPaSMC exhibited morphological features characteristic of apoptosis (Fig. 9). Finally, incubation of RPaSMC in RPMI 1640 medium containing 4% NuSerum markedly inhibited apoptosis in all treatment groups (data not shown), suggesting that apoptosis did not contribute to the antiproliferative effect of PKG that we observed in Ad.PKG-infected cells treated with GSNO or 8-Br-cGMP. Taken together, these results suggest that PKG activation can induce apoptosis in serum-deprived vascular SMC and that adenovirus-mediated PKG gene transfer increases the sensitivity of vascular SMC to the pro-apoptotic effects of NO and cGMP.

DISCUSSION

Modulation of SMC proliferation and apoptosis is a prominent feature of the vascular remodeling associated with endothelial dysfunction and arterial injury (1). Although it is well established that NO (synthesized endogenously or delivered exogenously) inhibits vascular remodeling (22, 46–49), it is uncertain whether this effect is mediated by a direct action of NO on vascular SMC or whether NO acts indirectly by reducing the release of growth modulatory factors from other cell types (such as platelets and leukocytes). At least *in vitro*, NO regulates many functions of vascular SMC, including SMC proliferation (12–14, 21–26), migration (15, 16), apoptosis (9–11, 27), and extracellular matrix formation (50). However, the concentrations of NO required to elicit these effects are typically

higher than those likely to be achieved *in vivo* (12–14, 21, 22). Moreover, in some studies, the effects of NO on vascular SMC functions appear to be cGMP- and PKG-independent (25, 32, 33). Cornwell and co-workers (32, 33) observed that levels of PKG decline rapidly as vascular SMC are passaged in culture. It is therefore likely that studies of the effects of NO on vascular SMC in culture have underestimated the contributions of cGMP and PKG *in vivo*.

To investigate the role of PKG in vascular SMC proliferation and apoptosis, we used adenovirus-mediated gene transfer to increase PKG activity in cultured RPaSMC. In a recently reported study, Gambaryan *et al.* (51) used a similar approach to investigate the role of PKGs in the modulation of renin secretion by juxtaglomerular cells. We found that restoration of PKG activity in RPaSMC decreased serum-stimulated DNA synthesis and cell proliferation. The inhibitory effect of PKG gene transfer in 8-Br-cGMP-treated RPaSMC was blocked by the PKG-selective inhibitor KT5823, but not by the PKA-selective inhibitor KT5720. These findings are supported by those of Yu *et al.* (21), who observed that inhibition of endogenous PKG in vascular smooth muscle cells attenuated the antiproliferative effects of NO and cGMP. Moreover, our finding suggests that, in SMC containing abundant PKG, cGMP inhibits proliferation via PKG rather than via PKA.

Our results differ from the recently published observations of Boerth *et al.* (52), who concluded that PKG does not play a critical role in regulating vascular SMC proliferation. These investigators studied RASMC stably transfected with the PKG-Iα isoform or the constitutively active PKG catalytic domain (which is shared by both isoforms): platelet-derived growth factor-stimulated proliferation did not differ in control-transfected RASMC, PKG-Iα-transfected RASMC incubated with 8-(*p*-chlorophenylthio)-cGMP, and RASMC transfected with the PKG catalytic domain. In addition, the investigators observed that infection of RASMC with recombinant adenoviruses specifying PKG increased kinase activity; however, the effect of adenovirus-mediated PKG gene transfer on cell proliferation was not reported. There are several potential explanations for the apparent discrepancy between our observations and those of Boerth *et al.* First, we used RPaSMC rather than RASMC, and there may be differences in PKG sensitivity between SMC derived from different blood vessels. This possibility is unlikely because we also observed that augmentation of PKG activity in RASMC using adenovirus-mediated gene transfer decreased serum-stimulated DNA synthesis and increased apoptosis associated with serum deprivation (data not shown). A second possible explanation for the differing results may be attributable to differences in the mitogenic stimuli used in the two studies (platelet-derived growth factor *versus* serum). A third potential explanation for these conflicting results is that isolation of stably transfected SMC requires multiple cell divisions and selection for cell growth, potentially resulting in dedifferentiation and loss of the ability to respond to the antiproliferative effects of PKG activation. In contrast, adenovirus-mediated PKG gene transfer permitted us to study the impact of PKG activation on SMC functions in early passage cells without selection.

In this study, adenovirus-mediated augmentation of PKG activity induced apoptosis in serum-deprived RPaSMC. These findings are consistent with those of Pollman *et al.* (9), who reported that endogenous PKG has an important role in mediating vascular SMC apoptosis induced by high concentrations of NO. In contrast, other investigators reported that the pro-apoptotic effect of NO on vascular SMC was cGMP-independent (10, 11, 27). Nishio *et al.* (10) observed that NO donor compounds, but not dibutyryl cGMP, triggered vascular SMC

apoptosis and that inhibition of the Na^+/H^+ antiporter suppressed NO-induced apoptosis. Zhao *et al.* (27) reported that high concentrations of sodium nitroprusside (0.5–1 mM) induced vascular SMC apoptosis, but cGMP analogues did not. They observed that NO induced apoptosis by decreasing intracellular levels of reduced glutathione and increasing expression of the p53 tumor suppressor gene. Our observations suggest that the differences in the impact of cGMP on apoptosis of cultured vascular SMC described in these reports may be attributable to differences in PKG levels. Moreover, our results suggest that, in cells containing abundant PKG, this kinase is an important mediator of vascular cell apoptosis induced by NO and cGMP.

A striking finding of this study is that adenovirus-mediated PKG gene transfer increased the sensitivity of RPaSMC to the antiproliferative and pro-apoptotic effects of NO: activation of the PKG transgene with submicromolar concentrations of GSNO significantly decreased vascular SMC proliferation, and similar low concentrations of GSNO induced apoptosis of vascular SMC. The amounts of NO generated by these concentrations of GSNO are comparable to those endogenously generated by endothelial cells (53, 54) and to those required to dilate intact blood vessels (55). An important consideration is that levels of PKG achieved in adenovirus-infected RPaSMC are similar to those found in freshly isolated vascular SMC (45) and in extracts prepared from rat pulmonary or carotid arteries (data not shown). The regulatory role of endothelial NO production in vascular SMC proliferation and apoptosis has been uncertain because the concentrations of NO required to modulate these functions in cultured cells are greater than those achievable *in vivo*. Our observations in Ad.PKG-infected RPaSMC suggest that physiologically relevant levels of NO, such as those produced by vascular endothelial cells, can modulate proliferation and apoptosis in vascular smooth muscle cells containing abundant PKG (such as those in intact blood vessels). This hypothesis is further supported by the observation that vascular smooth muscle cells *in vivo* contain high levels of the PKG-Iα isoform (18), which is ~10-fold more sensitive to cGMP than the PKG-Iβ isoform used in our studies (19, 20).

The observations that increasing the PKG levels in RPaSMC modulates the sensitivity of these cells to the antiproliferative and pro-apoptotic effects of NO and cGMP, together with the findings that, in stably transfected RASMC, PKG modulates cell migration (52) and extracellular matrix synthesis (56), suggest that changes in PKG levels can modulate the ability of NO to regulate important vascular cell functions. Changes in PKG levels have been reported in platelet-derived growth factor-stimulated vascular SMC (57), in bovine aortic SMC exposed to agents that increase cAMP or cGMP (58), and in lungs and aorta of isosorbide-treated rats (58). In preliminary studies of animal models of vascular injury associated with balloon angioplasty (59), decreased PKG levels were observed in the smooth muscle cells of the neointima as compared with those of the media. Yan and Hansson (60) observed, in the rat carotid artery model of vascular injury, that cultured cells derived from the neointima are less sensitive to the antiproliferative effects of NO than cells derived from the media. It is therefore possible that decreased PKG levels may contribute to the resistance of neointimal cells to the antiproliferative effect of NO.

In summary, we used adenovirus-mediated gene transfer techniques to restore PKG activity in vascular SMC. Our results establish the importance of PKG in mediating the antiproliferative effects of NO and suggest that *in vivo* endothelium-derived NO synthesis directly inhibits the proliferation of the subjacent vascular SMC. We also confirmed the role of the cGMP-PKG pathway in the activation of apoptotic cell death programs in response to NO. These findings may have important implications for the design of novel strategies to augment the efficacy of NO in the treatment of disorders associated with endothelial dysfunction and vascular injury.

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Adenovirus-mediated Gene Transfer of cGMP-dependent Protein Kinase Increases the Sensitivity of Cultured Vascular Smooth Muscle Cells to the Antiproliferative and Pro-apoptotic Effects of Nitric Oxide/cGMP

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