Overexpression of a Constitutively Active Protein Kinase G Mutant Reduces Neointima Formation and In-Stent Restenosis

Peter Sinnaeve, MD, PhD; Jean-Daniel Chiche, MD; Hilde Gillijns; Natascha Van Pelt; Douglas Wirthlin, MD; Frans Van de Werf, MD, PhD; Desire Collen, MD, PhD; Kenneth D. Bloch, MD; Stefan Janssens, MD, PhD

Background—Neointima formation after arterial injury is associated with reduced vascular cyclic guanosine monophosphate (cGMP) and cGMP-dependent protein kinase (PKG), a major cGMP effector in vascular smooth muscle. We tested the effect of PKG overexpression on the neointimal response to vascular injury.

Methods and Results—Infection of cultured rat aortic smooth muscle cells (RASMCs) with an adenoviral vector specifying a cGMP-independent, constitutively active PKG mutant (AdPKGcat) reduced serum-induced migration by 33% and increased serum-deprivation–induced apoptosis 2-fold (P<0.05 for both). Infection with wild-type PKG (AdPKG), in the absence of cGMP, did not affect migration or apoptosis. Two weeks after balloon-injured rat carotid arteries were infected with 1 × 10^10 pfu AdPKGcat (n=12), AdPKG (n=8), or a control adenovirus (n=8), intima-to-media ratio was less in AdPKGcat-infected arteries than in AdPKG- or control adenovirus–infected vessels (0.26±0.06 versus 0.61±0.12 and 0.70±0.12, respectively, P<0.05 for both). Two weeks after intramural administration of 1.75×10^10 pfu AdPKGcat (n=8) or a control adenovirus (n=8) into porcine coronary arteries with in-stent restenosis, luminal diameter was greater in AdPKGcat-infected arteries than in control adenovirus-infected vessels (2.32±0.16 versus 1.81±0.13 mm, P=0.028), associated with reduced neointimal area (3.30±0.08 versus 3.99±0.12, respectively, P<0.05), and percent stenosis (45±6% versus 70±4%, P<0.05).

Conclusions—Expression of a constitutively active PKG reduces neointima formation after balloon injury in rats and reduces coronary in-stent restenosis in pigs. PKGcat gene transfer may be a promising strategy for vasculoproliferative disorders. (Circulation. 2002;105:2911-2916.)

Key Words: kinases ■ nitric oxide ■ apoptosis ■ restenosis

S trategies designed to increase vascular nitric oxide (NO) levels reduce neointima formation after balloon injury in rat carotid and porcine coronary arteries.1–3 Vascular NO concentrations can be increased by systemic or local administration of NO-donor compounds or the NO synthase substrate l-arginine or by NO synthase gene transfer. The mechanisms whereby NO modulates neointima formation remain unclear but may involve cyclic guanosine monophosphate (cGMP)–dependent or cGMP-independent effects in the vessel wall, as well as the interaction of circulating cells with the injured vascular wall.4 The cGMP-dependent effects of NO are mediated via a variety of cGMP targets, including the serine/threonine kinase cGMP-dependent protein kinase (PKG).5,6 PKG activation in vascular smooth muscle cells (VSMCs) can result in relaxation, apoptosis, and reduced proliferation and migration.7–12 Of the 2 PKG genes identified in the mammalian genome, PKG1 is expressed in VSMCs. PKGs have an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. Binding of cGMP by the regulatory domain leads to activation of the catalytic domain. Two PKG1 isoforms, α and β, are produced by alternative splicing of 5’ exons, and PKG1α is more sensitive to activation by cGMP than is PKG1β.13 Boerth and Lincoln14 described a constitutively active PKG1 mutant consisting of the catalytic domain without the regulatory domain (PKGcat). When rat aortic smooth muscle cells (RASMCs) were stably transfected with the cDNA encoding PKGcat, morphological alterations suggestive of a differentiated, contractile smooth muscle cell phenotype were observed.14 We previously reported that balloon injury decreases vascular expression of soluble guanylate cyclase (sGC), an NO
receptor responsible for cGMP synthesis. Moreover, adenovirus-mediated augmentation of sGC expression at the site of vascular injury enhanced the ability of an NO-donor compound to decrease neointima formation. In this report, we examined the impact of vascular injury on the cGMP target PKG. We observed that balloon injury decreased vascular PKG1 expression. We used adenovirus-mediated gene transfer to augment expression of wild-type PKG1β and PKGcat at the site of balloon injury in rat carotid arteries and found that PKGcat, but not PKG1β, could attenuate neointima formation. We also report that adenovirus-mediated PKGcat expression decreased neointima formation in a porcine model of coronary artery in-stent restenosis.

Methods

PKG Immunoreactivity and Enzyme Activity in Balloon-Injured Rat Carotid Arteries

All animal experiments were carried out according to institutional guidelines. Male Wistar rats (350 g; bred in our animal facility) anesthetized with pentobarbital (50 mg/kg IP) were subjected to balloon angioplasty of the right common carotid artery as described. Rats (bred in our own facility.) After various periods of time, paraffin-embedded carotid arteries were sectioned and stained with rabbit anti-human PKG1 antiserum, prepared as described. PKG enzyme activity was measured in carotid artery extracts in the presence and absence of 1 mmol/L cGMP, as described.

Production and Purification of Recombinant Adenoviruses

To construct a recombinant adenovirus specifying the catalytic domain of human PKG1 (AdPKGcat), a 2.3-kb Ndel/BglII restriction fragment of the PKG1-β cDNA11 was ligated into the NotI and BamHI sites of the vector pAdRSV4 by use of a linker encoding the FLAG epitope (DYKDDDDK) 3' to the translation start site (5'-GCCGGCATGGTACCTACAAAGGAGCTGAGCAGACAG-3' annealed to 5'-TACCTGTGCATCGTCTTTGTAGTCCATGC-3'). The transgene specifies amino acid residues 351 to 686 of human PKG1-β, which are identical to the amino acids encoding the catalytic domain of bovine PKG1α. Biological activity of recombinant PKGcat was confirmed by measuring PKG enzyme activity in AdPKGcat-infected 293 cells (data not shown). AdPKG, specifying FLAG-tagged 8-kDa wild-type PKG1β, was generated as described. AdRG5 is a control adenovirus expressing no transgene, and Adβgal expresses a nuclear-localizing variant of Escherichia coli β-galactosidase.

Adenovirus-Mediated PKG Gene Transfer in Cultured RASMCs

For detection of recombinant PKG and PKGcat in RASMCs, PKG and PKGcat expression was detected in AdPKG- or AdPKGcat-infected RASMCs (passages 6 to 10, multiplicity of infection [MOI] 500) by immunoblot techniques and an anti-FLAG monoclonal antibody (M5, Sigma).

Migration in Transduced RASMCs

RASMCs were incubated in DMEM containing 2% FBS for 6 hours in the absence or presence of AdPKG, AdPKGcat, or AdR55-infected RASMCs (MOI 500) followed by incubation in DMEM containing 0.1% FBS for 24 hours. The culture medium was then changed to DMEM containing 4% FBS with or without 8-Br-cGMP (1 mmol/L) in the presence or absence of the PKG-inhibitor KT5823 (0.25 μmol/L). After 12 hours, 5×10^5 cells were transferred to a Transwell migration chamber, and migrating cells were counted 6 hours later, as described. Migratory activities of adenovirus-infected and uninfected cells were compared. Each experiment was performed in duplicate and repeated 5 times.

Apoptosis in Transduced RASMCs

AdPKG-, AdPKGcat-, or AdR55-infected RASMCs (MOI 500) were incubated in 0.1% FBS for 48 hours, followed by incubation in serum-free medium for 12 hours with or without 8-Br-cGMP (1 mmol/L). DNA fragmentation was assessed, and the percentage of cells showing nuclear chromatin condensation after staining with the DNA-binding dye Hoechst H33258 was measured as described.

Adenovirus-Mediated PKGcat Gene Transfer Into Balloon-Injured Rat Carotid Arteries

Fourteen days after infection of balloon-injured carotid arteries with 10^10 pfu AdPKG (n=8), AdPKGcat (n=12), or AdR55 (n=8), quantitative morphometry was performed on 5-μm sections from the transduced segment by an investigator blinded to the experimental procedure, as described.

Adenovirus-Mediated PKGcat Gene Transfer Into Porcine Coronary Arteries

Juvenile Landrace pigs (20 to 25 kg; obtained from a local farm in Vertrijk, Belgium) were anesthetized (ketamine 10 mg/kg IV bolus followed by 10 mg · kg^-1 · h^-1 pentobarbital infusion) and heparinized and underwent overstretched balloon injury of the left anterior descending coronary artery (three 30-second inflations at 8 atm, balloon-to-artery ratio 1.2 to 1.3), followed by implantation of an 18×3.5-mm stent (Tristart, Guidant). Pigs were obtained from a local farm [Vertrijk, Belgium.]. Fourteen days later, the in-stent stenosis was dilated (three 30-second inflations at 8 atm), followed by in-stent delivery of 1.75×10^10 pfu (in 200 μL) AdPKGcat or Adβgal, via the Infiltrator catheter (IVT). In-stent restenosis was measured by quantitative coronary angiography (QCA) on day 0, on day 14 before and after gene transfer, and on day 28. Lumen area was measured offline on digital images by use of automatic edge detection (Siemens Quantaor QCA analysis software) and the 8F guiding catheter as reference diameter. Intracoronary ultrasound (ICUS; Endosonics Vision 5 64F/X) sequences were obtained with an automatic pull-back device on day 14 before gene transfer and on day 28. Vessel area delineated by the stent struts (stent area) and lumen area were assessed offline on 5 consecutive in-stent sections and on a proximal and distal reference section. Neointimal area was calculated by subtracting lumen area from vessel area. Animals were euthanized 14 days after gene transfer with a lethal injection of pentobarbital, and plastic-embedded coronary artery sections were prepared for histomorphometric analysis, including assessment of the relationship between neointima formation and vascular injury score, as described.

Statistical Methods

All values are expressed as mean±SEM. An unpaired Student’s t test was used to compare groups. To identify differences between multiple groups, ANOVA was performed, with a Tukey correction for multiple comparisons. Statistical significance was defined as P<0.05.

Results

Expression of Endogenous PKG in Injured Rat Carotid Arteries

Endogenous PKG1 expression was markedly less in medial smooth muscle cells 4 days after injury than in uninjured control segments. Two weeks after balloon injury, however, PKG expression in medial and neointimal cells returned toward levels found in uninjured vessels (Figure 1). Basal and cGMP-stimulated PKG enzyme activities were lower in
extracts from carotid arteries subjected to balloon injury 4 days previously (0.23±0.16 and 3.8±1.1 nmol·min⁻¹·mg protein⁻¹) than in uninjured arteries (0.79±0.23 and 8.9±1.2 nmol·min⁻¹·mg protein⁻¹; P<0.05 for both).

**Adenovirus-Mediated PKG and PKGcat Gene Transfer in Cultured RASMCs**

Recombinant PKG expression in AdPKG- or AdPKGcat-infected RASMCs was confirmed by immunoblot analysis using an antibody directed against the FLAG epitope at the amino-terminal end of both proteins (Figure 2A). No FLAG immunoreactivity was detected in uninfected control cells.

**PKG Activity Modulates VSMC Migration and Apoptosis**

Serum-stimulated migration of PKGcat-transduced RASMCs was 67±6% of that of uninjured cells (P<0.05), a reduction that was prevented by incubation with the specific PKG inhibitor KT5823 (Figure 2B). In contrast, infection with AdPKG or AdRR5 did not reduce RASMC migration. Addition of 8-Br-cGMP reduced migration in AdPKG-infected RASMCs to 65±4% of that observed in uninjured RASMCs (P<0.05), an effect that was also blocked by KT5823. 8-Br-cGMP did not reduce migration in uninfected RASMCs.

**Figure 1.** PKG1 immunoreactivity in uninjured and balloon-injured rat carotid arteries. PKG1 immunoreactivity (brown) was abundant in media of uninjured rat carotid arteries but markedly decreased in medial cells of carotid arteries 4 days after balloon injury. Two weeks after balloon injury, PKG1 expression was readily detectable in medial and neointimal cells, but at lower levels than in uninjured carotid arteries.

**Figure 2.** PKG gene transfer in cultured RASMCs. A, PKG1β (82 kDa) and PKG1 catalytic domain (42 kDa) transgene products were detected in extracts from AdPKG- and AdPKGcat-infected RASMCs, respectively, with immunoblot techniques and an antibody directed against FLAG epitopes on both proteins. B, Serum-stimulated migration was significantly reduced in AdPKGcat-infected RASMCs (P<0.05 vs uninfected and AdRR5-infected cells) and inhibitory effect was reversed in presence of PKG-inhibitor KT5823. Infection with AdPKG only reduced migration only in presence of 8-Br-cGMP (P<0.05 versus AdPKG-infected cells without 8-Br-cGMP), an effect that was prevented by KT5823. C, DNA fragmentation was detected in serum-deprived AdPKGcat-infected RASMCs but not in uninjured cells (Neg Control). DNA fragmentation was minimal in AdPKG-infected RASMCs but was increased by incubation with 8-Br-cGMP. D, An increased frequency of RASMCs with nuclear condensation characteristic of apoptosis was detected in AdPKGcat-infected RASMCs and in AdPKG-infected RASMCs but only after incubation with 8-Br-cGMP. 8-Br-cGMP did not induce apoptosis in AdRR5-infected RASMCs (P<0.05 vs AdRR5 and AdPKG).
To investigate the effect of PKGcat gene transfer on smooth muscle cell apoptosis, RASMCs were infected with AdPKGcat, AdPKG, or AdRR5 followed by serum deprivation. Intranucleosomal DNA fragmentation characteristic of apoptotic cell death was observed in AdPKGcat-infected cells (Figure 2C). Infection with AdPKG induced only minimal DNA fragmentation in the absence of 8-Br-cGMP, but marked DNA fragmentation was observed in cells incubated in the presence of 8-Br-cGMP. 8-Br-cGMP did not increase apoptosis in uninfected RASMCs.

The DNA-binding dye Hoechst H33258 was used to define nuclear chromatin morphology as a quantitative index of apoptosis. Cells infected with AdPKGcat showed condensed nuclei more frequently than AdRR5-infected cells (12±2% versus 6±1%, P<0.05, n=5; Figure 2D). Infection with AdPKG increased the frequency of apoptosis in RASMCs incubated in the presence of 8-Br-cGMP (9±1%, n=5, P<0.05) but not in cells incubated in its absence (6±2%, n=3). 8-Br-cGMP did not induce apoptosis in AdRR5-infected RASMCs (7±1%, n=5).

Adenovirus-Mediated PKG or PKGcat Gene Transfer in Balloon-Injured Rat Carotid Arteries

Fourteen days after balloon injury and infection with AdPKG, AdPKGcat, or AdRR5, the intima/media ratio was significantly lower in arteries infected with AdPKGcat than in AdRR5- or AdPKG-infected arteries (0.26±0.06 versus 0.70±0.12 and 0.61±0.11, respectively, P<0.05 for both; Figure 3). Total vessel area was unchanged in AdPKGcat-, AdRR5-, and AdPKG-infected arteries (0.39±0.01, 0.39±0.02, and 0.38±0.04 mm², respectively), suggesting that 14 days after transduction, PKGcat expression had no effect on arterial remodeling or vasodilation.

Discussion

Vascular injury profoundly modulates smooth muscle cell migration and apoptosis and alters both NO production and NO responsiveness in the vessel wall. In this study, we observed decreased expression of an important intracellular cGMP target, PKG1, in the vessel wall after balloon injury. To evaluate the effect of restored PKG function on VSMC functions in vitro and on neointima formation in the injured vessel wall, we constructed an adenoviral vector specifying a constitutively active PKG mutant, PKGcat. Infection of cultured RASMCs with this adenovirus decreased serum-stimulated migration and enhanced serum deprivation-induced apoptosis, both in a cGMP-independent manner. In vivo gene transfer with PKGcat reduced neointima formation after balloon injury in rat carotid arteries, whereas transduction of wild-type PKG in the absence of cGMP had no effect. Finally, we developed a new porcine model of in-stent restenosis and report a significant reduction of restenosis after infection with AdPKGcat, as demonstrated not only by postmortem morphometric analysis but also by on-line digital QCA and ICUS 14 days after catheter-mediated intramural gene transfer.

Modulation of downstream components of the NO/cGMP signal transduction cascade determines the vascular response to NO. In rats with hypertension and/or advanced age, reduced vascular sGC expression impaired NO-dependent vasodilatation.19 We15 and others20 observed that balloon injury decreased sGC expression in rat carotid arteries and
that restored sGC function in balloon-injured rat carotid arteries enabled a low concentration of NO donor compound to reduce neointima formation.\(^\text{15}\) The changes in sGC expression led us to investigate whether vascular injury also altered expression of cGMP effectors, including PKG1. We observed decreased PKG1 immunoreactivity and PKG enzyme activity in rat carotid arteries 4 days after balloon injury. Fourteen days after balloon injury, PKG1 immunoreactivity was evident in the neointima and media, but at lower levels than in uninjured arteries. These findings are similar to those reported by Anderson et al\(^{21}\) for balloon-injured porcine coronary arteries. In contrast, Monks et al\(^{22}\) found preserved PKG expression in rat carotid arteries 2 and 11 days after balloon injury. Differences between our findings and those of Monks et al may be attributable in part to the different time points after vascular injury at which PKG expression was measured.

NO and cGMP modulate VSMC proliferation,\(^{7,10,11}\) migration,\(^{9,23}\) and apoptosis,\(^{11}\) but the contribution of PKG1 to these effects is incompletely understood, in part because PKG1 expression is low in passaged RASMCs.\(^{24}\) We previously reported that restoration of PKG1 sensitized VSMCs to the antiproliferative and proapoptotic effects of NO and cGMP.\(^{11}\) We report here that PKG1 restoration also sensitizes the antiproliferative and proapoptotic effects of NO and cGMP.\(^{11}\) We report here that PKG1 restoration also sensitizes the antiproliferative and proapoptotic effects of NO and cGMP.\(^{11}\)

In conclusion, restoration of PKG expression by use of an adenovirus specifying a constitutively active form of the enzyme could contribute to neointima formation. Gene transfer with PKGcat at the time of rat carotid artery balloon injury reduced neointima formation, whereas gene transfer with wild-type PKG1β did not. Failure of PKG1β overexpression to inhibit neointima formation may be attributable to insufficient vascular cGMP levels caused by decreased sGC expression and decreased vascular NO production or, potentially, to differences in substrate specificities of PKGcat and PKG1β. It remains unknown, however, whether restoration of PKG expression in injured vascular tissues by use of the 1α isoform, which is activated by lower concentrations of cGMP than is the 1β isoform, is sufficient to attenuate neointima formation.

We also examined the impact of PKG gene transfer in a new experimental double-injury model directly applicable to clinical practice: the patient with in-stent stenosis. After in-stent neointima formation had been demonstrated by QCA and ICUS, gene transfer was performed with a catheter with multiple injection ports. Four days after infection, transgene expression was detected predominantly in neointimal cells, and to a lesser extent in medial and adventitial cells in the vicinity of the injector ports (Figure 4a). Semiquantitative analysis indicated that 25% to 40% of the vascular cells at sites of maximal gene transfer were transduced (data not shown). Compared with control adenovirus, infection with PKGcat significantly reduced luminal renarrowing, as assessed by use of QCA, ICUS, and postmortem histomorphometry. Thus, restored PKG expression by use of catheter-based intramural gene transfer can attenuate progression of in-stent restenosis in the absence of NO-donor compounds and independent of local increases in vascular cGMP levels.

In conclusion, restoration of PKG expression by use of an adenovirus specifying a constitutively active form of the enzyme
Morphometric Parameters in Adenovirus-Infected Stented Porcine Coronary Arteries

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<th>ADPKGcat</th>
<th>ADβgal</th>
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<tr>
<td><strong>Histomorphometry</strong></td>
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<tr>
<td>Injury score, mean</td>
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<td>1.88±0.10</td>
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<td>Lumen area, mm²</td>
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<td>Vessel area, mm²</td>
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<td>NS</td>
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<tr>
<td>Neointima area/vessel area</td>
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<td>0.58±0.04</td>
<td>0.038</td>
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<tr>
<td>Percent stenosis, %</td>
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<td>70±4</td>
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<td><strong>QCA</strong></td>
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<tr>
<td>Mean luminal diameter day 14 before,† mm</td>
<td>2.23±0.09</td>
<td>2.36±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Mean luminal diameter day 14 after,† mm</td>
<td>2.99±0.09</td>
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<td>Mean luminal diameter day 28, mm</td>
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<td>1.81±0.13</td>
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<td>Mean luminal loss at day 28, mm</td>
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<td>-1.13±0.12</td>
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<tr>
<td><strong>ICUS</strong></td>
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All data are mean±SEM. *Before PTCA at day 14; †after PTCA and gene delivery at day 14.

limited neointima formation in balloon-injured rat carotid arteries and in a porcine model of in-stent coronary artery restenosis. Although the mechanisms by which PKG activation reduces neointima formation in vivo are unknown, in vitro studies suggest that the effects of PKG on vascular smooth muscle proliferation, migration, and apoptosis may have important roles. PKGcat gene transfer may be a promising strategy for the treatment of vasculoproliferative disorders.

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References


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