cGMP-dependent Protein Kinase Type I Inhibits TAB1-p38 Mitogen-activated Protein Kinase Apoptosis Signaling in Cardiac Myocytes*

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Cardiac myocyte apoptosis during ischemia and reperfusion (I/R) is tightly controlled by a complex network of stress-responsive signaling pathways. One pro-apoptotic pathway involves the interaction of the scaffold protein TAB1 with p38 mitogen-activated protein kinase (p38 MAPK) leading to the autophosphorylation and activation of p38 MAPK. Conversely, NO and its second messenger cGMP protect cardiac myocytes from apoptosis during I/R. We provide evidence that the cGMP target cGMP-dependent protein kinase type I (PKG I) interferes with TAB1-p38 MAPK signaling to protect cardiac myocytes from I/R injury. In isolated neonatal cardiac myocytes, activation of PKG I inhibited the interaction of TAB1 with p38 MAPK, p38 MAPK phosphorylation, and apoptosis induced by simulated I/R. During I/R in vivo, mice with a cardiac myocyte-restricted deletion of PKG I displayed a more pronounced interaction of TAB1 with p38 MAPK and a stronger phosphorylation of p38 MAPK in the myocardial area at risk during reperfusion and more apoptotic cardiac myocytes in the infarct border zone as compared with wild-type littermates. Notably, adenoviral expression of a constitutively active PKG I mutant truncated at the N terminus (PKG1-ΔN1–92) did not inhibit p38 MAPK phosphorylation and apoptosis induced by simulated I/R in vitro, indicating that the N terminus of PKG I is required. As shown by co-immunoprecipitation experiments in HEK293 cells, cGMP-activated PKG I, but not constitutively active PKG I-ΔN1–92 or PKG I mutants carrying point mutations in the N-terminal leucine-isoleucine zipper, interacted with p38 MAPK, and prevented the binding of TAB1 to p38 MAPK. Together, our data identify a novel interaction between the cGMP target PKG I and the TAB1-p38 MAPK signaling pathway that serves as a defense mechanism against myocardial I/R injury.

Acute myocardial infarction is usually caused by coronary thrombosis that leads to critical tissue ischemia and cardiac myocyte death. Although coronary reperfusion is essential for myocardial salvage, it may at first exacerbate cellular damage sustained during the ischemic period, a phenomenon known as reperfusion injury (1).

Cardiac myocyte death during reperfusion primarily reflects apoptosis, an energy-dependent process that is tightly controlled by a network of interdependent signaling pathways, including, for example, phosphoinositide 3-OH kinase and Akt, protein kinase C isoforms, and mitogen-activated protein kinases (MAPK)² (2). The present study reveals a link between two apoptosis signaling intermediates in cardiac myocytes, p38 MAPK and the NO/cGMP downstream target cGMP-dependent protein kinase type I (PKG I).

p38 MAPK has emerged as a central regulator of apoptosis in cardiac myocytes that can have both pro- and anti-apoptotic effects depending on the cell death-inducing stimulus and upstream signaling events leading to its activation (2–6). In cultured neonatal cardiac myocytes and in isolated perfused hearts, p38 MAPK is weakly activated during simulated ischemia and markedly activated during subsequent reperfusion (7, 8). p38 MAPK activation in cardiac myocytes during ischemia and reperfusion (I/R) occurs independent of the upstream kinases mitogen-activated protein kinase kinase 3 (MKK3) and MKK6 and is mediated instead by an interaction of p38 MAPK with the scaffold protein TAB1 (TAK1 (transforming growth factor-β-activated protein kinase 1)-binding protein 1), which promotes p38 MAPK autophosphorylation (9, 10). Pharmacological inhibition of p38 MAPK protects cardiac myocytes from apoptosis during simulated I/R in vitro, indicating that p38 MAPK functions as a pro-apoptotic signaling effector in this setting (7, 8). Studies in genetically modified mice support this concept: in one study, transgenic overexpression of a dominant-negative mutant of p38α MAPK, the predominant isoform of p38 MAPK in the mammalian heart, reduced cardiac myocyte apoptosis and myocardial infarct sizes during I/R (11). In another report, mice with a heterozygous null mutation of

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² The abbreviations used are: MAPK, mitogen-activated protein kinase; AAR, area at risk; I/R, ischemia and reperfusion; LAD, left anterior descending coronary artery; MKK, MAPK kinase; MAPKAPK-2, MAPK-activated protein kinase 2; PKG I, cGMP-dependent protein kinase type I; NOS, nitric-oxide synthase; 8-pCPT-cGMP, 8-para-chlorophenylthio-cGMP; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; DMEM, Dulbecco’s modified Eagle’s medium; VASP, vasodilator-stimulated phosphoprotein; TUNEL, TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling.
p38α MAPK were found to be less susceptible to cardiac myocyte apoptosis during I/R (12).

NO, which is produced by all three NO synthase (NOS) isoforms in the heart, is another important modulator of cardiac myocyte apoptosis during I/R (13, 14). Although toxic concentrations of NO can promote cardiac myocyte apoptosis via the formation of nitrosative stress (15, 16), a large body of evidence indicates that NO, when produced endogenously or supplied exogenously in lower concentrations, represents an important defense mechanism against I/R injury (14). For example, pharmacological inhibition of NOS has been shown to promote apoptosis in neonatal cardiac myocytes and in isolated hearts during simulated I/R (17, 18). Consistent with an anti-apoptotic role of NO, genetic deletion of NOS2 or NOS3 promotes apoptosis (19, 20), whereas overexpression of NOS2 or NOS3 suppresses cardiac myocyte apoptosis during I/R in vivo (21, 22). The signaling pathways whereby NO protects the heart from I/R injury are highly complex (14). The protective effects appear to be mediated, in part, by soluble guanylyl cyclase activation and cGMP formation, although the mechanisms downstream from cGMP remain incompletely understood (23–25).

We and others have previously identified PKG I as an important cGMP target that promotes NO and cGMP effects on contractility, hypertrophy, and gene expression in cardiac myocytes (26–30). PKG I belongs to the serine/threonine kinase family and is composed of three functional domains; the N-terminal domain, which comprises a leucine-isoleucine zipper motif; the regulatory domain that encompasses tandem cGMP-binding sites; and the C-terminal catalytic domain, which contains the MgATP- and peptide-binding pockets (31). The N terminus (the first 90–100 amino acid residues) of PKG I is encoded by two alternatively spliced exons, thus giving rise to PKG Iα, which is predominantly expressed in cardiac myocytes (29, 32), and PKG Iβ. The N terminus is required for homodimerization, interaction with other proteins and subcellular microdomains and autoinhibition of enzymatic activity (31, 33, 34). Upon cGMP binding, PKG I undergoes a conformational change that releases the inhibition of the catalytic center by the N terminus to allow phosphorylation of substrate proteins (31).

In the present study, we provide evidence that cGMP activation of PKG I protects cardiac myocytes from apoptosis during I/R injury in vitro and in vivo, at least in part, by inhibiting p38 MAPK activation. This inhibition involves the binding of cGMP-activated PKG I, via its N terminus, to p38 MAPK, thereby interfering with TAB1-mediated p38 MAPK autophosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The PKG-selective cGMP analog 8-pCPT-cGMP was purchased from BioLog, the NO donor S-nitroso-N-acetyl-d,L-penicillamine (SNAP) was from Biotrend, and the p38 MAPK inhibitor SB203580 was from Calbiochem.

**Cell Culture, Adenoviral Infection, and Plasmid Transfection**—Ventricular cardiac myocytes were isolated from 1–3-day-old Sprague-Dawley rats (Charles River) by Percoll density gradient centrifugation and plated in DMEM/medium 199 (4:1), supplemented with 10% horse serum, 5% fetal calf serum, 2 mmol/liter glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin in gelatin-coated culture dishes (Nunc) at a density of 4 × 10⁴ cells/cm² (29). The next morning, the medium was replaced by DMEM/medium 199 supplemented with glutamine and antibiotics only (maintenance medium). Cardiac myocytes were infected with replication-deficient adenoviruses (1 × 10⁴ viral particles/cell, unless otherwise indicated), as described (28). For plasmid transfection, HEK293 cells were plated in DMEM, supplemented with 5% fetal calf serum and 2 mmol/liter glutamine in 6-cm plates at a density of 80–90%. HEK293 cells were transfected with 3 μg of DNA of various expression plasmids using Lipofectamine 2000 (Invitrogen). After 24 h, the medium was replaced with serum-free DMEM.

**Recombinant Adenoviruses**—The replication-deficient adenovirus encoding human PKG Iα (Ad.PKG Iα) has been described previously (35). For constructing PKG I-ΔN1–92, a PKG I mutant lacking the N-terminal 92 amino acids, a Kozak consensus sequence, and a new ATG codon were introduced by PCR upstream of Val93 of human PKG Iβ (36), using the following primer pair: 5'-'TCCCCCGGGAATTCACTGTGAGC- CCTGCCTTTACCCCTAC-3' and 5'- CCTGGACCACATG-TACACACAC-3'. PKG I-ΔN1–92 cDNA was introduced into a replication-deficient adenovirus using the AdEasy XL adenoviral vector system (Stratagene) and the Adeno-X virus purification kit (BD Bioscience). Similarly, replication-deficient adenoviruses encoding human TAB1 and TAB1β were generated using TAB1 and TAB1β cDNAs that were kindly provided by Dr. Jiahui Han (The Scripps Research Institute, La Jolla, CA) (9, 37). Replication-deficient adenoviruses encoding constitutively active MKK3 (Ad.MKK3bE) or MKK6 (Ad.MKK6bE) mutants were kindly provided by Dr. Yibin Wang (University of California, Los Angeles, CA) (38).

**Expression Plasmids**—PKG Iα and PKG I-ΔN1–92 cDNAs were cloned into the expression vector pcDNA3 (Invitrogen). pcDNA3-PKG Iα was used for site-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene) to create three distinct leucine-isoleucine zipper mutants, LZ₁A₂A (Leu13 to Ala and Ile19 to Ala), LZ₁B (Leu26 to Pro) and LZ₄A₅A (Ile53 to Ala and Leu40 to Ala) (33), and a control mutant, LZ₁L (Leu12 to Leu). All of the zipper mutants were confirmed by DNA sequencing. pcDNA3-FLAG-p38α MAPK, pcDNA3-TAB1, and pcDNA3-TAB1β expression plasmids were provided by Dr. Jiahui Han (9).

**Simulated I/R in Vitro**—Cardiac myocytes were exposed to simulated I/R, as described (39, 40). In brief, the cells were switched from maintenance medium to a buffer containing (in mmol/liter) 137 NaCl, 12 KCl, 0.5 MgCl₂, 0.9 CaCl₂, 4 HEPES, 10 2-deoxy-glucose, and 20 sodium lactate (pH 6.2) and were incubated at 37 °C in a hypoxia chamber (Modular Incubator Chamber-101; Billups-Rothenberg) flushed with 5% CO₂ and 95% N₂ (simulated ischemia). Control cells were cultured in a buffer containing (in mmol/liter) 137 NaCl, 3.8 KCl, 0.5 MgCl₂, 0.9 CaCl₂, 4 HEPES, 10 glucose, and 20 pyruvate (pH 7.4) and incubated at 37 °C in an atmosphere containing 5% CO₂ and 95% room air (normoxia). After 60 min, cardiac myocytes were switched back to maintenance medium and kept in 5% CO₂ and 95% room air at 37 °C (simulated reperfusion).
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Assessment of Apoptotic Cell Death after Simulated I/R in Vitro—Cardiac myocyte apoptosis was assessed by a cell death detection enzyme-linked immunosorbent assay (Roche Applied Science), which measures the formation of histone-associated DNA fragments (29), and by TUNEL using the ApopTag fluorescein detection kit from Chemicon. After nuclear counter-staining with Hoechst 33258, the number of TUNEL-positive cardiac myocytes was determined by fluorescence microscopy (Leica DM4000 DB); the area at risk (AAR) and infarct sizes were determined 24 h after reperfusion by Evans blue and 2,3,5-triphenyl-tetrazolium chloride staining and planimetry (40). Apoptotic cardiac myocytes in the infarct border zone were quantified in paraformaldehyde-fixed tissue sections by TUNEL assay (Chemicon) and Hoechst 33258 staining; cardiac myocytes were identified by anti-sarcomeric α-actinin immunoblotting and expressed as the percentage of all Hoechst-positive nuclei. In each experiment, approximately 400–500 nuclei were examined per condition.

Immunoblotting and Immunoprecipitation—Standard immunoblotting techniques were employed using primary antibodies against p38 MAPK, phospho-p38 MAPK, phospho-MK3/6, MAPK-activated protein kinase 2 (MAPKAPK-2), and phospho-MAPKAPK-2 (all from Cell Signaling), TAB1 (kindly provided by Dr. Jun Ninomiya-Tsuji, North Carolina State University, Raleigh, NC) (41), PKG I (42), and Ser239-phosphorylated VASP (Sigma), followed by immunoblotting using anti-PKG I or anti-TAB1 antibodies. Myocardial tissue and cultured cardiac myocyte lysates were incubated with the anti-TAB1 antibody or with an anti-p38 MAPK antibody (BIOSOURCE), respectively. Thereafter, 30 μl of protein A/G-agarose (Roche Applied Science) were added, followed by immunoblotting with the anti-p38 MAPK or anti-PKG I antibodies, respectively.

Assessment of Cardiac Myocyte Hypertrophy—Atrial natriuretic peptide and B-type natriuretic peptide mRNA expression levels were assessed by Northern blotting and normalized against 18 S rRNA expression, as described (30, 44). Cardiac myocyte size was determined by phase contrast microscopy and computerized planimetry (Quantimet 500MC, Leica) (29); approximately 100–150 cells were analyzed per experiment and condition.

In Vivo I/R Model—Generation and breeding of cardiac myocyte-restricted C57BL/6 PKG I knockout mice has been described (26). 8–12-week-old male mice were subjected to transient left anterior descending coronary artery (LAD) ligation, as described (40). In brief, the mice were anesthetized and ventilated with enflurane (3%). A left thoracotomy was performed, and the LAD was ligated over a PE-10 polyethylene tube (ischemia); after 1 h, blood flow was re-established by removal of the tube (reperfusion). Additional mice underwent a sham operation where the suture around the LAD was not tied into a knot. The area at risk (AAR) and infarct sizes were determined 24 h after reperfusion by Evans blue and 2,3,5-triphenyl-tetrazolium chloride staining and planimetry (40). Apoptotic cardiac myocytes in the infarct border zone were quantified in paraformaldehyde-fixed tissue sections by TUNEL assay (Chemicon) and Hoechst 33258 staining; cardiac myocytes were identified by anti-sarcomeric α-actinin immunostaining (Sigma) (40). The number of TUNEL-positive cardiac myocyte nuclei were determined in n = 4 transversal sections/heart and expressed as the percentage of all Hoechst-positive nuclei. Ten to 15 high power fields (400 × magnification) were examined per left ventricular tissue section. All of the animal procedures were approved by our local state authorities.

Statistical Analysis—The data are presented as the means ± S.E. n refers to the number of mice or number of independent cardiac myocyte preparations. Immunoblotting and immunoprecipitation data are representative of three or more inde-
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RESULTS

cGMP-activated PKG I Protects Cardiac Myocytes from Apoptosis during Simulated I/R—To start exploring whether activation of PKG I can protect cardiac myocytes from apoptosis during I/R, we employed a well-established cell culture model of simulated I/R. As shown by TUNEL and Hoechst staining and histone enzyme-linked immunosorbent assay, simulated ischemia for 60 min followed by reperfusion for 60 min promoted apoptosis in neonatal cardiac myocytes (Fig. 1, A–C). We have previously analyzed the time course of the appearance of apoptosis in our cell culture model (40) and have observed that cardiac myocytes undergo apoptosis primarily at the time of reperfusion, which is consistent with other data in the literature (45). Treatment of cardiac myocytes with the NO donor SNAP (250 μmol/liter) diminished the number of TUNELpos cells and the formation of histone-associated DNA fragments after simulated I/R (Fig. 1, A–C). Because SNAP, via cGMP formation, has been shown to activate PKG I in neonatal cardiac myocytes (28, 29), we next investigated whether cGMP-activated PKG I can promote anti-apoptotic effects during simulated I/R. Indeed, the protective effects of SNAP were mimicked by 8-pCPT-cGMP (500 μmol/liter), a cell membrane-permeable, cGMP analog acting selectively on PKG I in cardiac myocyte and other cell types (29, 46, 47) (Fig. 1, A–C). Intriguingly, adenoviral expression of a constitutively active PKG I mutant (Fig. 1D), lacking the autoinhibitory N terminus (PKG I-ΔN1–92), did not protect cardiac myocytes from I/R-induced apoptosis (Fig. 1, A–C), indicating that the N terminus of PKG I is required for the protective effects. As shown in Fig. 1E, activation of endogenous PKG I by 8-pCPT-cGMP and adenoviral expression of PKG I-ΔN1–92 both promoted Ser239 and Ser157 phosphorylation of the established PKG I substrate vasodilator-stimulated phosphoprotein (VASP) in cardiac myocytes under normoxic conditions and during simulated I/R, confirming that PKG I-ΔN1–92 is constitutively active in our experimental setting.

cGMP-activated PKG I Inhibits p38 MAPK Phosphorylation during Simulated I/R—Consistent with previous data (7, 8), the p38 MAPK catalytic site inhibitor SB203580 (10 μmol/liter) protected cardiac myocytes from I/R-induced apoptosis (Fig. 1, A–C). Based on this observation and previous reports pointing toward a critical involvement of p38 MAPK in I/R-induced cardiac myocyte apoptosis (see Introduction), we postulated that cGMP-activated PKG I may promote its protective effects (at least in part) by inhibiting p38 MAPK. As shown in Fig. 2 (A and B), 60 min of simulated ischemia followed by 10 min of reperfusion promoted a strong phosphorylation of p38 MAPK in cardiac myocytes. Analysis of the time course indicated that p38 MAPK phosphorylation occurred at the time of reperfusion but not during ischemia in our model system (not shown). p38 MAPK phosphorylation after I/R was significantly attenuated by the NO donor SNAP (Fig. 2, A and B). Similarly, 8-pCPT-cGMP-activation of endogenous PKG I diminished I/R-induced p38 MAPK phosphorylation (Fig. 2, A and C). The suppression of I/R-induced p38 MAPK phosphorylation by SNAP- and 8-pCPT-cGMP-activation of endogenous PKG I diminished I/R-induced p38 MAPK phosphorylation (Fig. 2, A and C). The suppression of I/R-induced p38 MAPK phosphorylation by SNAP- and 8-pCPT-cGMP-activation of endogenous PKG I was maximal and not further enhanced by infection of cardiac myocytes with Ad.PKG I (Fig. 2, A–C). Based on this observation, we explored whethercGMP-activated PKG I can inhibit p38 MAPK phosphorylation during Simulated I/R. Cardiac myocytes were kept under normoxic conditions (N) or were subjected to simulated ischemia (60 min) followed by reperfusion (10 min) (I/R), in the presence or absence of SNAP (250 μmol/liter, unless otherwise indicated) or 8-pCPT-cGMP (500 μmol/liter). Where shown, cardiac myocytes were infected with Ad.PKG I or Ad.PKG I-ΔN1–92 prior to I/R. Expression and phosphorylation status of p38 MAPK were analyzed by immunoblotting. Representative blots are shown in A. Data from n = 7–10 experiments are summarized in B and C. *p < 0.01 versus normoxia; #p < 0.01 versus I/R alone (B). The suppression of I/R-induced p38 MAPK phosphorylation by SNAP- and 8-pCPT-cGMP-activation of endogenous PKG I was maximal and not further enhanced by infection of cardiac myocytes with Ad.PKG I (C).

FIGURE 2. cGMP-activated PKG I inhibits p38 MAPK phosphorylation during I/R. Cardiac myocytes were kept under normoxic conditions (N) or were subjected to simulated ischemia (60 min) followed by reperfusion (10 min) (I/R), in the presence or absence of SNAP (250 μmol/liter, unless otherwise indicated) or 8-pCPT-cGMP (500 μmol/liter). Where shown, cardiac myocytes were infected with Ad.PKG I or Ad.PKG I-ΔN1–92 prior to I/R. Expression and phosphorylation status of p38 MAPK were analyzed by immunoblotting. Representative blots are shown in A. Data from n = 7–10 experiments are summarized in B and C. *p < 0.01 versus normoxia; #p < 0.01 versus I/R alone (B). The suppression of I/R-induced p38 MAPK phosphorylation by SNAP- and 8-pCPT-cGMP-activation of endogenous PKG I was maximal and not further enhanced by infection of cardiac myocytes with Ad.PKG I (C).
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PKG I contains an N-terminal leucine-isoleucine zipper motif between amino acids 12 and 40. To examine whether the leucine-isoleucine zipper is involved in the binding of PKG Iα to p38α MAPK, nonsense mutations were introduced by site-directed mutagenesis into the leucine-isoleucine zipper of PKG Iα to replace specific leucine and isoleucine residues by alanine or proline (Fig. 3B). Co-transfection of HEK293 cells with FLAG-p38α MAPK and the PKG Iα leucine-isoleucine zipper mutants, followed by anti-FLAG immunoprecipitation and anti-PKG I immunoblotting, indicated that the leucine-isoleucine zipper mutations virtually abolish the capacity of 8-pCPT-cGMP-activated PKG Iα to interact with p38α MAPK (Fig. 3C, left panels). As expected, PKG Iα-LZ11, a control mutant that was obtained by site-directed mutagenesis using a nonmutated primer, interacted with p38α MAPK after 8-pCPT-cGMP activation similar to wild-type PKG Iα (Fig. 3C, right panels). It appears therefore that the N-terminal leucine-isoleucine zipper of PKG Iα is required for the interaction with p38α MAPK. To assess whether endogenous PKG I and endogenous p38 MAPK interact in cardiac myocytes and during simulated I/R, cardiac myocyte lysates were subjected to simulated ischemia (60 min) followed by reperfusion (10 min) (I/R) in the presence or absence of 8-pCPT-cGMP. Anti-p38 MAPK immunoprecipitation followed by anti-PKG I immunoblotting was performed; equal loading was assessed by anti-p38 MAPK immunoblotting.

PKG I Inhibition of TAB1-p38 MAPK Apoptosis Signaling—The PKK kinases MKK3 and MKK6 are known to activate p38 MAPK in cardiac myocytes (11, 38). Specifically, expression of a constitutively active MKK3 mutant (MKK3bE) has been shown to activate p38α MAPK and to promote apoptosis in cardiac myocytes (38). However, phosphorylation of p38 MAPK following adenoviral expression of MKK3bE was not inhibited by 8-pCPT-cGMP activation of endogenous PKG Iα (Fig. 4A). Moreover, 8-pCPT-cGMP failed to protect cardiac myocytes against MKK3E-induced apoptosis (Fig. 4, A–C) and p38 MAPK phosphorylation (Fig. 2, A and B) during I/R. Expression of a constitutively active MKK6 mutant (MKK6bE) has been shown to stimulate hypertrophy and fetal gene expression in cardiac myocytes via p38β MAPK (38). In agreement with these data, adenoviral expression of MKK6bE promoted p38 MAPK phos-}

FIGURE 3. Interaction of cGMP-activated PKG Iα via its leucine-isoleucine zipper with p38 MAPK. HEK293 cells were transfected with FLAG-p38α MAPK, and PKG Iα or PKG I-ΔN1–92 plasmid expression vectors, and stimulated with 8-pCPT-cGMP (500 μmol/liter), as indicated (A). Anti-FLAG immunoprecipitation (IP) followed by anti-PKG I immunoblotting (IB) was performed; equal loading was assessed by anti-p38 MAPK immunoblotting. A diagram outlining the domain structure of wild-type PKG Iα and different leucine-isoleucine zipper mutants is shown in B. Specific leucine and isoleucine residues were mutated to alanine, proline, or leucine, as indicated. HEK293 cells in C were transfected with FLAG-p38α MAPK and wild-type PKG Iα or PKG I-ΔN1–92 plasmid expression vectors, and stimulated with 8-pCPT-cGMP, as indicated. Anti-FLAG immunoprecipitation followed by anti-PKG I immunoblotting was performed; equal loading was assessed by anti-p38 MAPK immunoblotting. Cardiac myocytes in D were kept under normoxic conditions (N) or subjected to simulated ischemia (60 min) followed by reperfusion (10 min) (I/R) in the presence or absence of 8-pCPT-cGMP. Anti-p38 MAPK immunoprecipitation followed by anti-PKG I immunoblotting was performed; equal loading was assessed by anti-p38 MAPK immunoblotting.

A and B). More importantly, adenoviral expression of wild-type PKG Iα did not significantly enhance the inhibitory effects of SNAP and 8-pCPT-cGMP, indicating that endogenous PKG Iα already promotes nearly maximal effects (Fig. 2). Similar to the data shown in Fig. 1, adenoviral expression of constitutively active PKG I-ΔN1–92 did not inhibit p38 MAPK phosphorylation during I/R (Fig. 2, A and B), indicating that the N terminus of PKG I is required for p38 MAPK inhibition.

cGMP-activated PKG I Interacts with p38 MAPK via Its N-terminal Leucine-Isoleucine Zipper—Given the apparent importance of the N terminus of PKG I for the inhibition of p38 MAPK phosphorylation during I/R (Fig. 2) and its established role in mediating interactions between PKG I and other proteins (33, 34), we postulated that PKG I may bind to p38 MAPK. To start exploring the molecular nature of such a potential interaction, HEK293 cells were transfected with pcDNA3 plasmid vectors driving the expression of FLAG-p38α MAPK and PKG Iα or PKG I-ΔN1–92 under the control of the cytomegalovirus promoter. Anti-FLAG immunoprecipitation followed by anti-PKG I immunoblotting revealed a physical interaction between p38α MAPK and 8-pCPT-cGMP-activated PKG Iα (Fig. 3A). PKG Iα did not interact with p38α MAPK in HEK293 cells in the absence of 8-pCPT-cGMP (Fig. 3A). The PKG I-ΔN1–92 mutant did not interact with p38α MAPK in the presence or absence of 8-pCPT-cGMP, indicating that the N terminus of PKG I is required for p38α MAPK binding (Fig. 3A).
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phorylation (Fig. 4B), increases in atrial natriuretic peptide and B-type natriuretic peptide mRNA expression levels (Fig. 4E), and increases in cell size in cardiac myocytes (Fig. 4F). However, p38 MAPK phosphorylation, fetal gene expression, and hypertrophy in MKK6bE expressing cardiac myocytes were not significantly affected by 8-pCPT-cGMP (Fig. 4, B, E, and F). Together, these data indicate that cGMP-activated PKG I does not inhibit MKK3- and MKK6-induced p38 MAPK phosphorylation and downstream signaling events in cardiac myocytes.

cGMP-activated PKG I Inhibits TAB1-p38 MAPK Apoptosis Signaling—Recently, a MKK3- and MKK6-independent mode of p38 MAPK activation has been described that involves the interaction of p38 MAPK with the scaffold protein TAB1 (9). Because TAB1 promotes p38 MAPK autophosphorylation and apoptosis during I/R in cardiac myocytes (10), we speculated that cGMP-activated PKG I may protect cardiac myocytes from I/R injury by inhibiting TAB1-induced p38 MAPK activation. Consistent with previous data (10, 48), adenoviral expression of TAB1 in cardiac myocytes promoted p38 MAPK phosphorylation independent of MKK3 or MKK6 phosphorylation (Fig. 5A) and promoted apoptotic cell death (Fig. 5, C and D). TAB1-induced p38 MAPK phosphorylation and apoptosis were significantly reduced by 8-pCPT-cGMP (Fig. 5, B–D), thus providing evidence that cGMP-activated PKG I interferes with TAB1-p38 MAPK apoptosis signaling in cardiac myocytes. Importantly, adenoviral expression of the constitutively active, truncated PKG I-ΔN1–92 mutant did not protect TAB1-overexpressing cardiac myocytes from apoptosis (Fig. 5, C and D). To examine whether cGMP-activated PKG I interferes with the physical interaction between TAB1 and p38 MAPK and to explore whether the N terminus of PKG I is important in this process, HEK293 cells were transfected with FLAG-p38 MAPK, TAB1, and PKG Iα or PKG I-ΔN1–92 expression plasmids (Fig. 5E). As predicted from earlier data (9), anti-FLAG immunoprecipitation followed by anti-TAB1 immunoblotting revealed a physical interaction between TAB1 and p38α MAPK (Fig. 5E). Notably, co-expression and 8-pCPT-cGMP activation of PKG Iα prevented this interaction (Fig. 5E), whereas expression of PKG I-ΔN1–92 did not affect the interaction of TAB1 with p38α MAPK in the presence or absence of 8-pCPT-cGMP (Fig. 5E). Together, these data indicate that cGMP-activated PKG I, via its N terminus, prevents TAB1 binding to p38α MAPK, thereby interfering with p38 MAPK autophosphorylation. To explore whether endogenous, cGMP-activated PKG I interferes with the interaction between endogenous TAB1 and p38 MAPK during I/R, cardiac myocyte lysates were subjected to transient coronary artery ligation followed by anti-TAB1 immunoprecipitation followed by anti-p38 MAPK immunoblotting (Fig. 5F). In agreement with a previous report (10), interaction of TAB1 with p38 MAPK was enhanced during simulated I/R in cardiac myocytes (Fig. 5F). Consistent with our observations in HEK293 cells (Fig. 5E), 8-pCPT-cGMP activation of PKG I reduced TAB1 binding to p38 MAPK during I/R (Fig. 5F).

Greater Infarct Sizes after I/R Injury in Conditional PKG I Knock-out Mice—To assess the in vivo relevance of these findings, mice with a cardiac myocyte-restricted knock-out of PKG I were subjected to transient coronary artery ligation followed by reperfusion. Use of conditional PKG I-deficient mice (Fig. 6A) provided us with the opportunity to explore the role of PKG I in cardiac myocytes while avoiding confounding influences of PKG I deletion in other cell types, including vascular smooth muscle cells and platelets. Homozygous PKG I-floxed (PKG I floxed/floxed) mice were
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Gene targeting was used to generate cardiac myocytes containing one wild-type allele and one PKG I null allele. The resulting progeny, MLC2a-Cre^+/−; PKG I^+/−, were subjected to a sham operation or subjected to simulated ischemia (60 min) and reperfusion (10 min) in vivo. It has recently been shown that TAB1-activated p38 MAPK, unlike MKK3- or MKK6-activated p38 MAPK, does not phosphorylate the established downstream target MAPAPK-2 (48). Confirming these data, adeno-viral expression of MKK3E or MKK6E, but not TAB1, promoted phosphorylation of MAPAPK-2 in cardiac myocytes in vitro (Fig. 7C). Similarly, p38 MAPK phosphorylation in the anterolateral left ventricular wall during ischemia or I/R alone.

Enhanced TAB1-p38 MAPK Signaling during I/R Injury in Conditional PKG I Knock-out Mice—After a sham operation, phosphorylation levels of p38 MAPK in the anterolateral left ventricular wall increased to a similar extent in control and PKG I-KO mice (Fig. 7A). After 1 h of coronary ischemia, p38 MAPK phosphorylation levels in the anterolateral left ventricular wall increased to a similar extent in control and PKG I-KO mice (Fig. 7A). After 1 h of ischemia followed by 24 h of reperfusion, phosphorylation levels of p38 MAPK in the anterolateral left ventricular wall had returned toward base line in control mice but remained markedly increased in PKG I-KO mice (Fig. 7A). Enhanced p38 MAPK phosphorylation during reperfusion in conditional PKG I-KO mice was associated with an enhanced interaction of TAB1 with p38 MAPK (Fig. 7B), indicating that PKG I in cardiac myocytes inhibits TAB1 interaction with p38 MAPK (Fig. 7B) and p38 MAPK phosphorylation (Fig. 7A) during reperfusion in vivo. It has recently been shown that TAB1-activated p38 MAPK, unlike MKK3- or MKK6-activated p38 MAPK, does not phosphorylate the established downstream target MAPAPK-2 (48). Confirming these data, adeno-viral expression of MKK3E or MKK6E, but not TAB1, promoted phosphorylation of MAPAPK-2 in cardiac myocytes in vitro (Fig. 7C). Similarly, p38 MAPK phosphorylation in the anterolateral left ventricular wall during ischemia or I/R in vivo in control and PKG I-KO mice (Fig. 7A, fourth, fifth, sixth, and ninth lanes) was not accompanied by enhanced MAPAPK-2 phosphorylation (Fig. 7D, fourth, fifth, sixth, and ninth lanes), supporting the notion that p38 MAPK phosphorylation during I/R is mediated by TAB1 but not MKK3 or MKK6.
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FIGURE 6. Greater infarct sizes after I/R in conditional PKG I knock-out mice. The wild-type (+) PKG I locus, the floxed allele, and the null (−) allele (which is obtained after Cre-mediated recombination of the floxed allele) are shown in A. Filled triangles represent loxP sequences; the open box represents a thymidine kinase-neo fusion gene (tk-neo). BamHI, EcoRI, HindIII, Ncol, and Nhel restriction sites are indicated. MLC2a-Cre 
PKG I+/floxed mice served as control 1, PKG I−/floxed mice served as control 2, and MLC2a-Cre 
PKG I−/floxed mice served as conditional PKG I knock-out (PKG I-KO) mice. Control and PKG I-KO mice underwent coronary artery ligation for 1 h followed by reperfusion for 24 h (I/R). AAR and infarct sizes were determined by Evans blue and 2,3,5-triphenyl-tetrazolium chloride staining. Typical left ventricular cross-sections are shown in B. The area of the myocardium not stained with Evans blue represents the AAR, infarcted areas appear pallid (highlighted with pink (highlighted with green stripes). Data from n = 9–16 mice per genotype are summarized in C. The AAR and infarcted area (MI) were expressed as the percentage of the left ventricular cross-sectional area (LV); MI was also calculated as the percentage of AAR. *, p < 0.01 versus both controls. Apoptotic cardiac myocytes were detected by TUNEL and Hoechst and anti-actinin staining in the infarct border zone after I/R. Exemplary sections (overlay) and data from n = 4 animals/genotype are shown in D. *, p < 0.01 versus both controls.

DISCUSSION

The present study shows that the NO/cGMP downstream target PKG I protects cardiac myocytes from I/R-induced apoptosis in vitro and in vivo, at least in part, by interfering with the pro-apoptotic TAB1-p38 MAPK signaling pathway. Binding of cGMP-activated PKG I to p38 MAPK and inhibition of TAB1-induced p38 MAPK autophosphorylation appears to be the molecular mechanism of p38 MAPK inhibition by PKG I.

Two lines of evidence support the conclusion that PKG I protects cardiac myocytes from I/R-induced apoptosis. First of all, 8-pCPT-cGMP, a cell membrane-permeable cGMP analog acting selectively on PKG I in cardiac myocytes and other cell types (29, 46, 47), protected cardiac myocytes from apoptosis in a cell culture model of simulated I/R. Furthermore, mice with a cardiac myocyte-restricted knock-out of PKG I developed greater infarct sizes and displayed more TUNEL-positive cardiac myocytes in the infarct border zone after transient coronary artery ligation. Larger infarct sizes in conditional PKG I knock-out mice were associated with a prolonged phosphorylation of p38 MAPK during reperfusion, indicating that PKG I in cardiac myocytes is required to limit p38 MAPK signaling in vivo. In line with this concept, the anti-apoptotic effects of the NO donor SNAP and 8-pCPT-cGMP, which are both known to activate PKG I in cardiac myocytes (29), were associated with an inhibition of p38 MAPK phosphorylation during simulated I/R in vitro, suggesting that the cytoprotective effects PKG I are related, at least in part, to an inhibition of pro-apoptotic p38 MAPK signaling.

Notably, the inhibitory effects of SNAP or 8-pCPT-cGMP on I/R-induced p38 MAPK phosphorylation in vitro could not be enhanced by adenoviral expression of PKG I, indicating that endogenous PKG I is expressed at sufficient levels and at the necessary intracellular microdomains to promote maximal inhibitory effects on p38 MAPK phosphorylation after NO/cGMP activation. This is in contrast to previous reports showing that the anti-hypertrophic effects of SNAP and 8-pCPT-cGMP in cardiac myocytes can be further enhanced by overexpression of PKG I (28–30).

When exploring the molecular mechanism of p38 MAPK inhibition by cGMP-activated PKG I, we first noted that adenoviral expression of the constitutively active PKG I-ΔN1–92 mutant, lacking the N-terminal autoinhibitory domain, did not mimic the p38 MAPK-inhibitory and anti-apoptotic effects of 8-pCPT-cGMP-activated endogenous PKG I during I/R. This observation indicated that the N terminus of PKG I is involved in p38 MAPK inhibition and suppression of apoptosis. As revealed by co-immunoprecipitation, endogenous PKG I interacted with endogenous p38 MAPK in cultured cardiac myocytes, an interaction that was further enhanced during I/R and by 8-pCPT-cGMP stimulation. Additional studies in HEK293 cells indicated that the N-terminal leucine-isoleucine zipper of PKG Iα is required for binding of cGMP-activated PKG Iα to p38α MAPK. Previous reports have highlighted the importance of the leucine-isoleucine zipper of PKG I for mediating interactions with other proteins. For example, PKG Iα binds to the myosin-binding subunit of myosin phosphatase via its leucine-isoleucine zipper, thereby leading to myosin phosphatase activation and smooth muscle cell relaxation (33). Moreover, PKG Iα interacts with troponin T via its leucine-isoleucine zipper, which brings PKG Iα in close proximity to its phosphorylation target troponin I (34).

p38 MAPKs are phosphorylated and activated by the
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FIGURE 7. Enhanced TAB1-p38 MAPK signaling after I/R in conditional PKG I knock-out mice. Expression and phosphorylation levels of p38 MAPK (A) and MAPKAPK-2 (B) were determined by immunoblotting (IB) in myocardial tissue lysates obtained from the anteroapical left ventricular wall (corresponding to the AAR) after sham LAD ligation, myocardial ischemia (1 h of LAD ligation), or after myocardial I/R (1 h of LAD ligation, followed by 24 h of reperfusion). In B, tissue lysates from the anteroapical left ventricular wall after I/R were analyzed by anti-TAB1 immunoprecipitation (IP) followed by anti-p38 MAPK immunoblotting; equal loading was assessed by anti-TAB1 immunoblotting. Cardiac myocytes in C were infected with Ad.TAB1, Ad.MKK3bE, or Ad.MKK6bE (all viruses: 2.5 × 10^6 viral particles/cell), as indicated. Expression and phosphorylation levels of MAPKAPK-2 and p38 MAPK were analyzed by immunoblotting 24 h after infection.

upstream kinases MKK3 and MKK6 (49). In cardiac myocytes, overexpression of constitutively active MKK3 promotes p38 MAPK activation and apoptosis (38). MKK3, however, appears not to be required for p38 MAPK activation during I/R, because p38 MAPK phosphorylation and infarct sizes are not diminished in MKK3 knock-out mice subjected to I/R (10). It has been proposed instead that p38 MAPK activation during I/R is induced by the scaffold protein TAB1 (10), which has been identified by yeast two-hybrid screening as a p38 MAPK-interacting protein (9). TAB1 binding to p38α MAPK promotes p38α MAPK autophosphorylation and activation in many cell types, including cardiac myocytes (9, 10, 50). The involvement of TAB1 or MKKs in the activation of p38 MAPK in cardiac myocytes depends on the upstream stimulus: p38 MAPK activation during I/R is mediated through TAB1, whereas tumor necrosis factor-α activation of p38 MAPK depends on MKK3 (10). Consistent with these earlier data, adenoviral expression of TAB1 promoted p38 MAPK phosphorylation and apoptosis in cardiac myocytes in our study. 8-pCPT-cGMP activation of endogenous PKG I significantly reduced these TAB1-mediated effects. cGMP-activated PKG I did not inhibit signaling via MKK3-p38 MAPK or MKK6-p38 MAPK, indicating that PKG I selectively targets the TAB1-p38 MAPK pathway.

Concerning the mechanism by which cGMP-activated PKG I interferes with TAB1-p38 MAPK signaling, several lines of evidence indicate that cGMP-activated PKG I prevents the binding of TAB1 to p38 MAPK during I/R: 1) cGMP-activated PKG Iα prevented the binding of TAB1 to p38α MAPK in HEK293 cells; 2) the enhanced interaction of TAB1 with p38 MAPK during simulated I/R in cardiac myocytes was reduced by 8-pCPT-cGMP-activation of endogenous PKG I; and 3) greater infarct sizes after I/R in conditional PKG I knock-out mice were associated with an enhanced interaction of TAB1 with p38 MAPK and increased p38 MAPK phosphorylation levels in the area at risk during reperfusion. The N terminus of PKG I appears to be required for inhibiting the TAB1-p38 MAPK pathway, because the constitutively active PKG I mutant truncated at the N terminus did not inhibit TAB1-induced apoptosis in isolated cardiac myocytes and did not inhibit the binding of TAB1 to p38α MAPK in HEK293 cells.

TAB1 binds to and activates TAK1, a MAPK kinase that can phosphorylate and activate MKK3 and MKK6 (49, 51, 52), raising the possibility that TAB1 mediates p38 MAPK phosphorylation in cardiac myocytes via MKK3 and MKK6 and that cGMP-activated PKG I interferes with this cascade upstream of MKK3 or MKK6. Several lines of evidence argue against this concept, however: 1) adenoviral expression of TAB1 did not promote phosphorylation of MKK3 or MKK6 in cardiac myocytes; 2) phosphorylation of MAPKAPK-2, a substrate of MKK3- and MKK6-activated p38 MAPK but not TAB1-activated p38 MAPK (48), was not detected in the area at risk after I/R in wild-type or conditional PKG I knock-out mice; and 3) adenoviral expression of TAB1β, a TAB1 splice variant that is capable of promoting p38α MAPK autophosphorylation but lacking the TAK1 binding domain (37), promoted phosphorylation of p38 MAPK and apoptosis (TUNEL) in cardiac myocytes, effects that could be inhibited with 8-pCPT-cGMP (not shown). Moreover, we also observed that TAB1β interacts with p38α MAPK in HEK293 cells and that this interaction could be prevented by expression and 8-pCPT-cGMP activation of PKG Iα (not shown).

Although our study highlights one mechanism whereby NO and cGMP can protect the heart from I/R injury, additional mechanisms need to be considered to fully appreciate the complexity of NO and cGMP signaling during myocardial I/R (reviewed in Ref. 14). It should be noted in this regard that NO may reduce I/R injury by promoting salutary effects in the non-cardiac myocyte compartment (e.g. by reducing leukocyte infiltration) (20, 53). Moreover, NO has been shown to protect cardiac myocytes from I/R injury by inducing the expression of cyclooxygenase-2, thus promoting the formation of cytoprotective prostanooids (21), and by activating mitochondrial K_ATP channels (54), possibly via cGMP-PKG I-dependent mechanisms (25).

In conclusion, the present study identifies PKG I as an endogenous cellular defense mechanism that protects cardiac myocytes from I/R injury by interfering with the TAB1-p38 MAPK signaling cascade. Future studies should explore whether and how PKG I interacts with additional signaling mediators to modulate cardiac myocyte apoptosis during I/R and whether the protective effects of PKG I can be exploited therapeutically.
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