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Cyclic GMP-dependent Protein Kinase Iα Attenuates Necrosis and Apoptosis Following Ischemia/Reoxygenation in Adult Cardiomyocyte*

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Cyclic GMP-dependent protein kinases protein kinase G (PKG) Iα and PKGIβ are major mediators of cGMP signaling in the cardiovascular system. PKGIα is present in the heart, although its role in protection against ischemia/reperfusion injury is not known. We investigated the direct effect of PKGIα against necrosis and apoptosis following simulated ischemia (SI) and reoxygenation (RO) in cardiomyocytes. Adult rat cardiomyocytes were infected with adenoviral vectors containing hPKGIα or catalytically inactive mutant hPKGIαK390A. After 24 h, the cells were subjected to 90 min of SI and 2 h RO for necrosis (trypan blue exclusion and lactate dehydrogenase release) or 18 h RO for apoptosis studies. To evaluate the role of K_{ATP} channels, subgroups of cells were treated with 5-hydroxydecanoate (100 μM), HMR1098 (30 μM), or glibenclamide (50 μM), the respective blockers of mitochondrial, sarcolemmal, or both types of K_{ATP} channels prior to SI. The necrosis observed in 33.7 ± 1.6% of total myocytes in the SI-RO control group was reduced to 18.6 ± 0.8% by PKGIα (mean ± S.E., $n = 7$, $p < 0.001$). The apoptosis observed in 17.9 ± 1.3% of total myocytes in the SI-RO control group was reduced to 6.0 ± 0.6% by PKGIα (mean ± S.E., $n = 7$, $p < 0.001$). In addition, PKGIα inhibited the activation of caspase-3 after SI-RO in myocytes. Myocytes infected with the inactive PKGIαK390A mutant showed no protection. PKGIα enhanced phosphorylation of Akt, ERK1/2, and JNK, increased Bcl-2, inducible nitric-oxide synthase, endothelial nitric-oxide synthase, and decreased Bax expression. 5-Hydroxydecanoate and glibenclamide abolished PKGIα-mediated protection against necrosis and apoptosis. However, HMR1098, had no effect. A scavenger of reactive oxygen species, as well as inhibitors of phosphatidylinositol 3-kinase, ERK, JNK1, and NOS, also blocked PKGIα-mediated protection against necrosis and apoptosis. These results show that opening of mitochondrial K_{ATP} channels and generation of reactive oxygen species, in association with phosphorylation of Akt, ERK, and JNK, and increased expression of NOS and Bcl-2, play an essential role in the protective effect of PKGIα.

Apoptotic cell death in cardiac myocytes is well recognized to be responsible for myocardial infarction following ischemia/reperfusion injury (1), hypertrophy (2), and development of heart failure (3). Preconditioning is a cardioprotective phenomenon whereby repeated brief episodes of ischemia protect the myocardium from more prolonged periods of ischemia and future myocardial infarction as well as stunning (4). A number of receptors and intracellular signaling pathways have been identified that play an essential role in the cardioprotective effect of preconditioning. Endogenously released agents including adenosine, norepinephrine, opioids, free radicals, and bradykinin are involved in preconditioning (5, 6). Several pharmacological interventions mimic this preconditioning-like cardioprotective effect (6, 7). In recent years, there has been considerable interest in the role of the NO-cGMP-protein kinase G (PKG)[2] pathway in protection of the heart against ischemia/reperfusion injury (8). Inhibition of cGMP-specific phosphodiesterase 5A with sildenafil citrate (Viagra) induced protective effects against ischemia/reperfusion injury in the intact heart and adult cardiomyocytes (9–13). Conceptually, sildenafil inhibits the enzymatic hydrolysis of cGMP, which in turn maintains the tissue accumulation of cGMP, leading to downstream protective mechanisms involving PKG activation and opening of mitochondrial ATP-sensitive potassium ($mitoK_{ATP}$) channels (6). It has been shown that sildenafil induces preconditioning through NO generated from endothelial and/or inducible nitric-oxide synthase (eNOS/iNOS) and via activation of protein kinase C and opening of the $mitoK_{ATP}$ channels (9, 10, 12). Phosphodiesterase 5A inhibition also attenuated cell death resulting from necrosis and apoptosis by increasing the Bcl-2/Bax ratio through NO signaling (13).

A recent study also showed that phosphodiesterase 5A inhibition by sildenafil generates a potent anti-hypertrophic effect by enhancing PKGI activity, without increasing total cGMP in mice (14). Adenoviral gene transfer of PKGIβ selectively enhanced anti-hypertrophic effects of NO, without increasing apoptosis (15). Qin

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[2] The abbreviations used are: PKG, protein kinase G; SI, simulated ischemia; RO, reoxygenation; 5-HD, 5-hydroxydecanoate; Glib, glibenclamide; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NOS, nitric-oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; ROS, reactive oxygen species; PI, phosphatidylinositol; MAPK, mitogen-activated protein kinase; MEKK, MAPK/ERK kinase kinase; MPG, *N*-(2 mercaptopropionyl)glycine; L-NAME, L-nitro-amino-methyl-ester; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end label.

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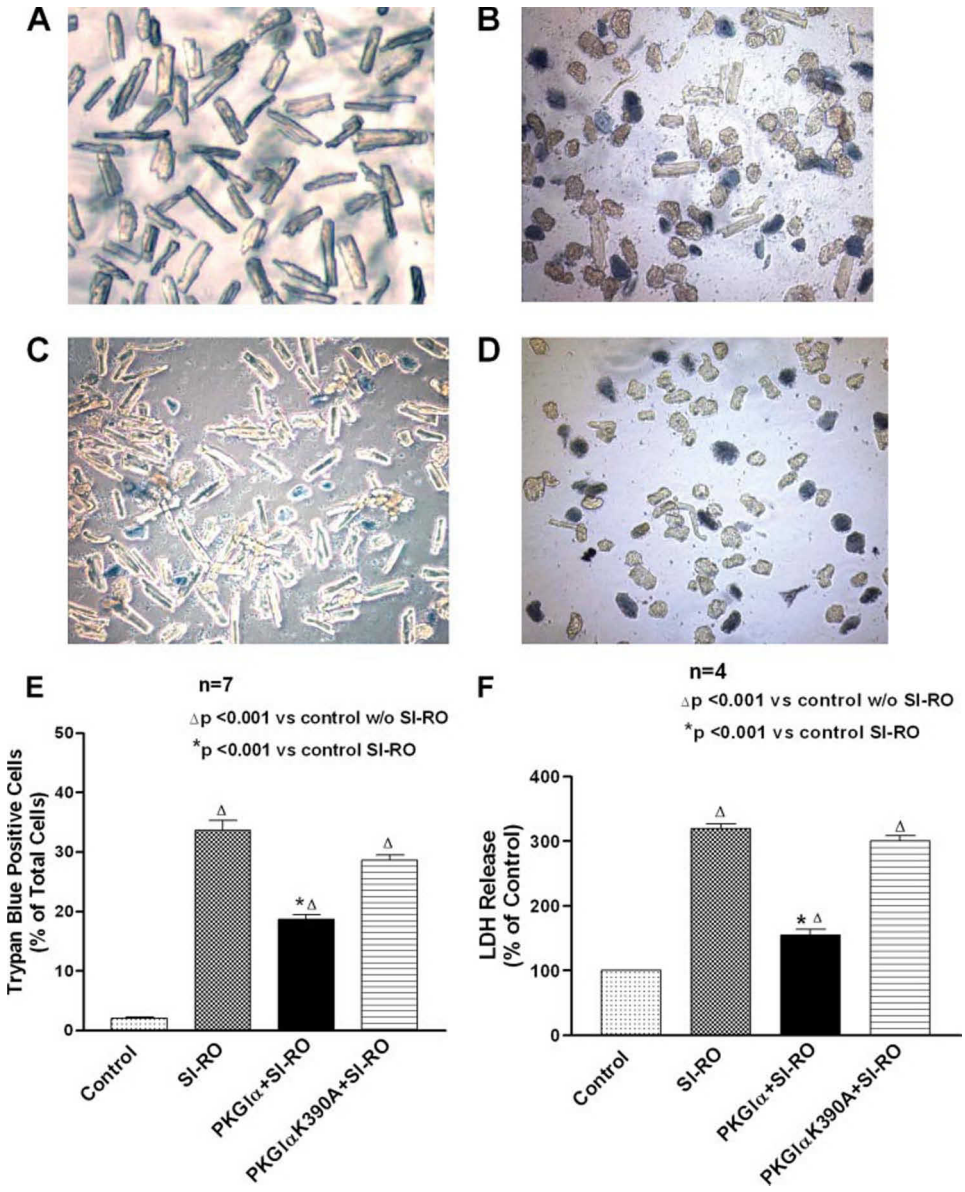

FIGURE 1. **Representative images of cardiomyocytes and PKGIα-induced protection against necrosis.** *A*, normal isolated rat cardiomyocytes. *B*, cardiomyocytes subjected to 90 min of SI and 2 h of RO. Cell necrosis is shown by trypan blue-positive cardiomyocytes. *C*, cardiomyocytes overexpressing PKGIα show fewer trypan blue-positive cells as compare with control SI-RO cardiomyocytes. *D*, cardiomyocytes overexpressing catalytically inactive mutant of PKGIα subjected to SI-RO. *E* and *F*, quantitative results of anti-necrotic protection. The trypan blue-positive cells (*E*) ($n = 7$) and the release of lactate dehydrogenase (*F*) ($n = 4$) into the culture medium were reduced in PKGIα-overexpressing cardiomyocytes, *, $p < 0.001$ *versus* SI-RO; Δ, $p < 0.001$ *versus* control without SI-RO.

et al. (16) showed that exogenous NO triggers preconditioning effect by stimulation of guanylyl cyclase to make cGMP, activation of PKG, opening of mitoK$_{ATP}$ channels, and production of reactive oxygen species (ROS). Peptide blockers of PKG inhibited the ROS generation by pharmacological preconditioning agents such as acetylcholine and bradykinin in cardiomyocytes (17). Nevertheless, the direct role of PKGIα and the downstream signaling pathways that lead to protection of cardiomyocytes against apoptosis after ischemia/reoxygenation injury remain to be elucidated.

PKGI has a number of effects that may be relevant to its regulation of apoptosis. PKGI can modulate gene expression in a variety of cell types by either stimulating or inhibiting extracellular signal-regulated kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) (18, 19). PKGI has been shown to suppress cell proliferation by inhibiting the Ras/MAPK pathway in baby hamster kidney cells transfected with PKGIβ (20). PKGI also induced the expression of MAPK phosphatase-1, which reverses activation of the MAPK pathway (20). PKGIα induced ERK1/2 by activating MAPK/ERK kinase in smooth muscle cells (21) but activated p38 MAPK in fibroblasts (22). In human colon cancer cells, PKG activated the MEKK1-SEK1-JNK1 pathway, by directly phosphorylating and activating MEKK1 (23).

Although PKG effects on cell survival/proliferation pathways have been observed in various cells types, the mechanism of PKG attenuation of ischemia/reoxygenation injury in the heart is not clear. The present study was designed to examine whether the expression of PKGIα protects cardiomyocytes from necrosis and apoptosis following simulated ischemia and reoxygenation. We also examined whether opening of mitoK$_{ATP}$ channel, ROS generation and activation of multiple signaling pathways including Akt, NOS, and MAPKs are essential to PKG-mediated cellular protection. Our findings define mechanisms by which PKGIα inhibits necrosis and apoptosis signaling pathways to protect cardiomyocytes from ischemia/reperfusion injury.

EXPERIMENTAL PROCEDURES

Isolation of Ventricular Cardiomyocytes and Adenoviral Infection—Adult male Wistar rats (300 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Ventricular cardiomyocytes were isolated using an enzymatic technique as previously reported (13). The freshly isolated cardiomyocytes were plated with Medium 199 containing 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 5 mM glucose, 0.1 μM insulin, and 1% penicillin-streptomycin. After 1 h of plating, the myocytes were infected with adenoviral vectors containing hPKGIα (Ad.PKGIα) (24) or catalytically inactive hPKGIαK390A (25) in serum-free growth medium for 24 h. The cells were routinely infected with the viruses at a concentration of 1×10^3 particles/cell.

Simulated Ischemia/Reoxygenation Protocol—After 24 h of adenoviral infection, the cells were subjected to simulated ischemia (SI) for 90 min by replacing the cell medium with an "ischemia buffer" that contained 118 mM NaCl, 24 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 2.5 mM $CaCl_2$-$2H_2O$, 1.2 mM $MgCl_2$, 20 mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH adjusted to 6.2) as reported previously (13). The cells were incubated at 37 °C in tri-gas incubator adjusting 1–2% O_2 and 5% CO_2 during the entire SI period. RO was accomplished by replacing the ischemic buffer with normal cell medium under normoxic conditions. Cell necrosis and apoptosis were assessed after 2 or 18 h of reoxygenation, respectively.

Evaluation of Cell Viability and Apoptosis—Trypan blue exclusion assay and lactate dehydrogenase release into the medium were used to assess cell necrosis (13). Cardiomyocyte apoptosis was analyzed by TUNEL staining as reported previously (13).

Detection of Activated Caspase 3—Activated caspase was detected using the CaspaTagTM Caspase 3,7 *in situ* assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions (13).

Inhibitor Studies—Each experiment was started with a change of medium in the wells. To evaluate the involvement of K_{ATP} channels in PKG1α-mediated protection, Ad.PKGIα-infected cells were treated for 30 min before SI-RO with 5-hydroxydecanoate (5-HD, 100 μM), HMR1098 (30 μM), or glibenclamide (Glib, 50 μM), the respective blockers of mitochondrial, sarcolemmal, or sarcolemmal/mitoK_{ATP} channels. To test the effect of a ROS scavenger on PKG-induced protection, a subgroup of cells were treated with *N*-(2 mercaptopropionyl)glycine (MPG, 1 mM) for 30 min prior to SI-RO. Other subgroups of cells were treated similarly with the NOS inhibitor L-nitro-amino-methyl-ester (L-NAME, 100 μM), PI 3-kinase inhibitors wortmannin (100 nM) and LY-294002 (10 μM), the ERK inhibitor PD-98059 (25 μM), and the JNK inhibitor SP600125 (10 μM).

Western Blot Analysis—Western blots were performed as described previously (13). The blots were incubated with rabbit polyclonal primary antibody or mouse monoclonal antibody at a dilution of 1:1000 for each of the respective proteins, *i.e.* PKGIα, nNOS, iNOS, eNOS, Bcl-2, Bax, pAkt, Akt, pERK, ERK1, pp38, p38, pJNK, JNK, and actin (all purchased from Santa Cruz) for 2 h. The membranes were then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; Amersham Biosciences) for 1 h. The blots were developed using a chemiluminescent system, and the bands were scanned and quantified by densitometric analysis.

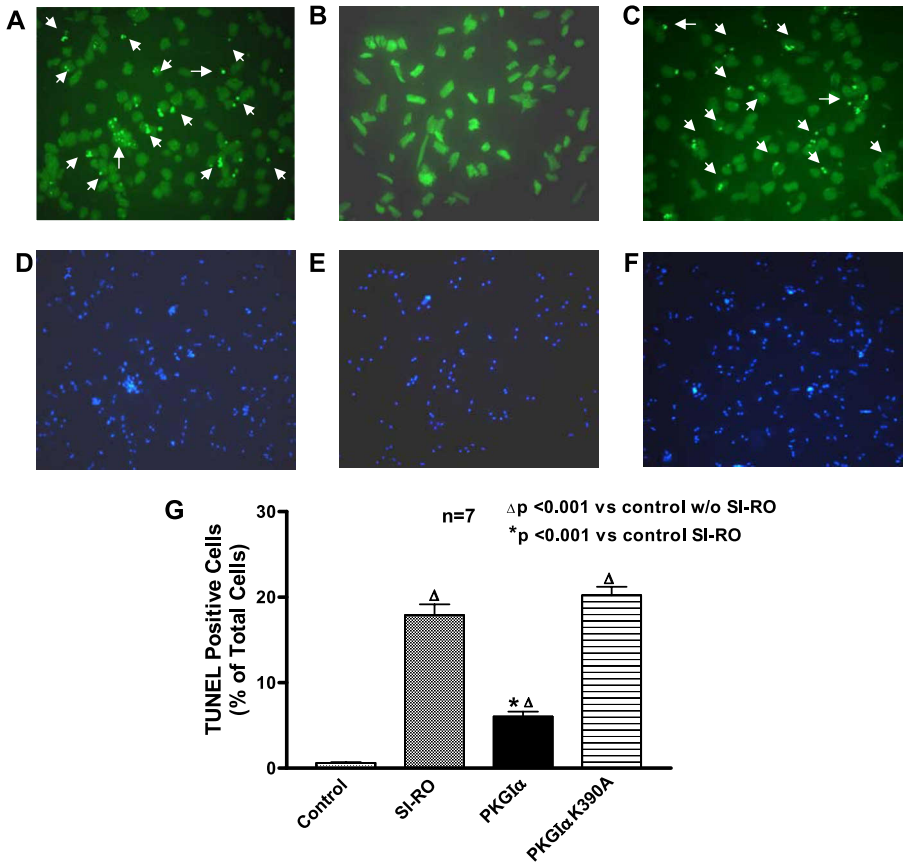

FIGURE 2. **PKGIα inhibits apoptosis in cardiomyocytes.** Apoptotic nuclei were observed using TUNEL assay after 90 min of SI and 18 h of RO in cardiomyocytes with no adenoviral transfection (*A* and *D*), with overexpression of PKGIα (*B* and *E*) or catalytically inactive PKGIαK390A (*C* and *F*). *A–C*, TUNEL-positive myocyte nuclei (stained in *green* fluorescent color); *D–F*, total nuclei (4′,6-diamidino-2-phenylindole staining). Note that PKGIα overexpression protects cardiomyocytes from apoptotic cell death following SI-RO in comparison with control SI-RO-treated cardiomyocytes. *G*, bar diagram shows quantitative data of TUNEL-positive cells from seven independent experiments. *, $p < 0.001$ *versus* SI-RO; Δ, $p < 0.001$ *versus* control without SI-RO. *H–P*, caspase 3 activity was detected by using CaspaTag reagent and a fluorescence microscope. The *red* fluorescent signal is a direct measure of activated caspase 3 in the cell (*red*, *left panels*) and nuclei stained by Hoechst (*blue*, *right panels*). *H* and *L*, control cardiomyocytes; *I* and *M*, cardiomyocytes subjected to 90 min of SI and 18 h of RO; *J* and *N*, PKGIα-overexpressing cardiomyocytes exposed to SI-RO; *K* and *O*, cardiomyocytes expressing catalytically inactive PKGIαK390A. Note that PKGIα-overexpressing cardiomyocytes show a negligible amount of red fluorescence signal as compared with the control SI-RO or PKGIαK390A expressing cardiomyocytes. *P*, bar diagram showing quantitative data of active caspase-positive cells from seven independent experiments *, $p < 0.001$ *versus* SI-RO; Δ, $p < 0.001$ *versus* control without SI-RO.

Data Analysis and Statistics—The data are presented as the means ± S.E. The difference between the groups was analyzed respectively with an unpaired *t* test or one-way analysis of variance followed by Student-Newman-Keul post-hoc test. $p < 0.001$ was considered to be statistically significant.

RESULTS

PKGIα Overexpression Inhibits Necrosis and Apoptosis—Fig. 1*A* shows a typical preparation of our isolated adult rat cardiomyocytes. At least 90% of the cardiomyocytes had rod-

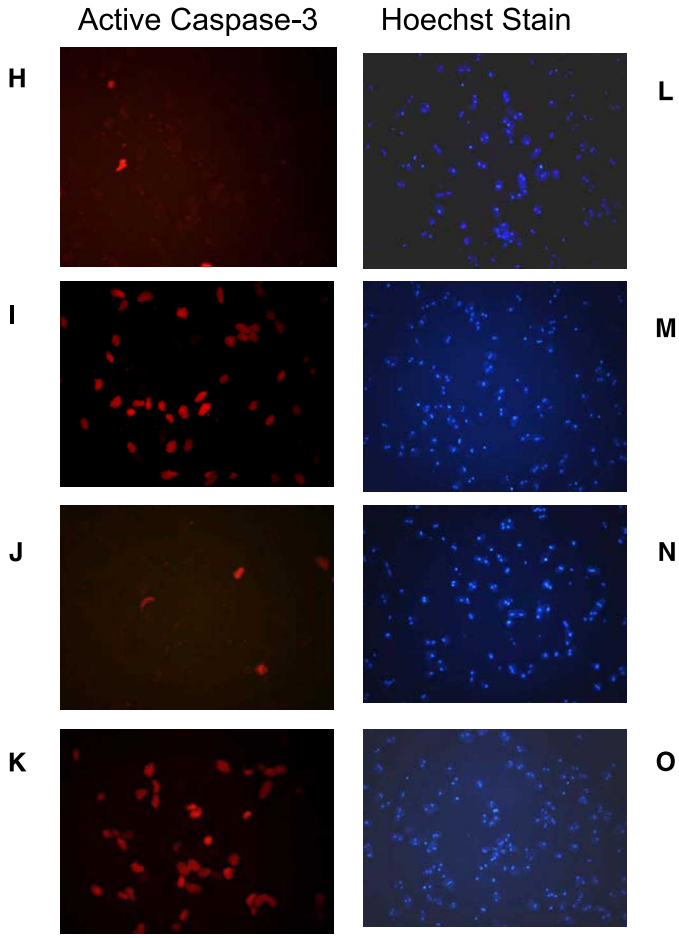

FIGURE 2—*continued*

shaped morphology. After SI (90 min)-RO (2 h), the percentage of necrotic, trypan blue-positive cardiomyocytes increased to 33.7 ± 1.6 as compared with non-SI-RO controls (2.0 ± 0.2) ($n = 7$; $p < 0.001$; Fig. 1, *A*, *B*, and *E*). PKGIα expression in cardiomyocytes exposed to SI-RO reduced trypan blue-positive cardiomyocytes ($n = 7$; $p < 0.001$ *versus* SI-RO alone; Fig. 1, *C* and *E*). The overexpression of catalytically inactive PKGIαK390A failed to protect cells (Fig. 1, *D* and *E*). Similarly, PKGIα overexpression attenuated the release of lactate dehydrogenase as compared with SI-RO alone, whereas cardiomyocytes overexpressing PKGIαK390A did not (Fig. 1*F*).

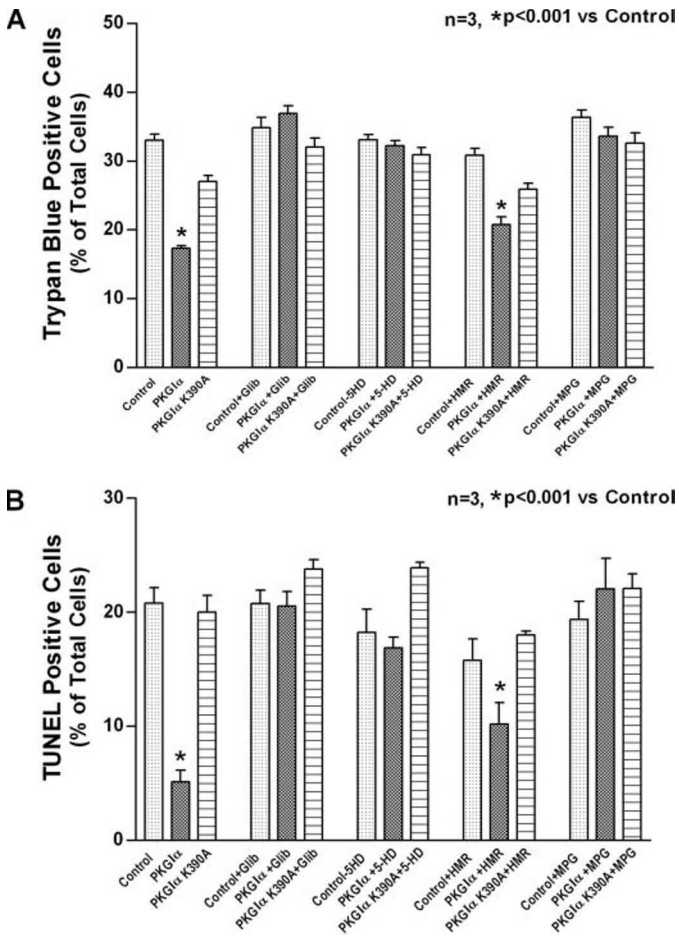

FIGURE 3. **Effect of K_{ATP} channel blockers and ROS scavenger on PKGIα-induced cardiomyocyte protection.** 24 h after infection with PKGIα, cardiomyocytes were treated with 5-HD (100 μM), HMR1098 (*HMR*, 30 μM), or Glib (50 μM) for 30 min before SI-RO. Another subgroup of cells was treated with MPG (1 mM) for 30 min before SI-RO. *A*, cell necrosis as determined by trypan blue-positive cells after 90 min of SI and 2 h of RO; *B*, apoptotic nuclei identified using TUNEL assay after 90 min of SI and 18 h of RO. Note that the protection against necrosis and apoptosis by PKGIα was abolished by 5-HD, Glib, and MPG.

Apoptosis was not detectable after 90 min of SI and 2 h of RO. With an extended RO period of 18 h, TUNEL-positive cells increased as compared with the control group ($p < 0.001$ *versus* control, $n = 7$; Fig. 2, *A* and *G*). PKGIα overexpression reduced the TUNEL-positive cells ($n = 7$; $p < 0.001$ *versus* SI-RO group; Fig. 2, *B* and *G*), whereas PKGIαK390A failed to do so (Fig. 2, *C* and *G*). Representative staining of total nuclei with 4′,6-diamidino-2-phenylindole is shown for SI-RO control (Fig. 2*D*) and cells overexpressing PKGIα (Fig. 2*E*) or PKGIαK390A (Fig. 2*F*). Likewise, the red fluorescence of active caspase-positive cells increased in cardiomyocytes following SI and 18 h of RO (Fig. 2*I*) as compared with the control group (Fig. 2*H*), however clearly reduced by PKGIα overexpression (Fig. 2*J*) but not by PKGIαK390A (Fig. 2*K*). Representative panels (Fig. 2, *L–O*) show that 4′,6-diamidino-2-phenylindole staining of total nuclei was more or less similar in three groups, suggesting that the observed changes in caspase-3 staining represented true differences in apoptosis, not the number of nuclei. Quantitative measurements showed that the active caspase-positive cardiomyo-

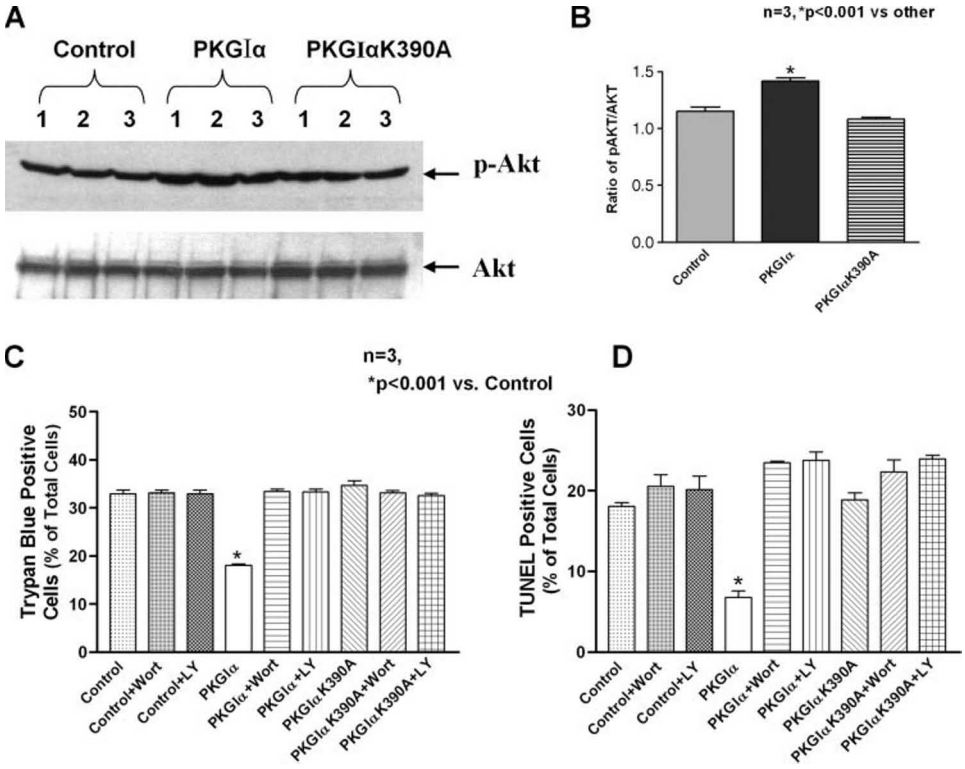

FIGURE 4. **Role of Akt phosphorylation in PKGIα-induced cardiomyocyte protection.** *A*, representative Western blots showing expression of phosphorylated Akt and total Akt after 24 h of infection with PKGIα or PKGIαK390A. *B*, bar diagram showing average ratio of p-Akt to total Akt. PKGIα expression increased significantly compared with control (without infection) and PKGIαK390A expression. Treatment with wortmannin (*Wort*, 100 nM) or LY-294002 (*LY*, 10 μM) before SI-RO abolished PKG-dependent protection. *C*, cell necrosis is determined by trypan blue-positive cells after 90 min of SI and 2 h of RO. *D*, apoptotic nuclei identified using TUNEL assay after 90 min of SI and 18 h of RO. Note that wortmannin and LY-294002 abolished PKGIα-induced protection against necrosis and apoptosis. Wortmannin and LY-294002 had no effect on control or PKGIαK390A-overexpressing cardiomyocytes exposed to SI-RO.

cytes are significantly higher in SI-RO-treated control (25.6 ± 0.6%) and PKGIαK390A cardiomyocytes (26.3 ± 0.7%) as compared with the cells overexpressing PKG1α (7.2 ± 0.5%) ($p < 0.001$, $n = 7$; Fig. 2*P*). Nonischemic group showed only 1.3 ± 0.1% ($n = 7$) of the caspase-positive cardiomyocytes.

Effect of K_{ATP} Channel Blockers and a ROS Scavenger on PKGIα-induced Protection—Both Glib and 5-HD abolished PKGIα protection of cardiomyocytes exposed to SI-RO ($p < 0.001$ *versus* PKGIα; $n = 3$; Fig. 3*A*). Similarly, Glib and 5-HD blocked PKGIα protection against apoptosis, as demonstrated by an increase in TUNEL-positive cells ($p < 0.001$ *versus* PKGIα; $n = 3$; Fig. 3*B*). HMR1098 failed to block the protective effect of PKGIα against necrosis (Fig. 3*A*) as well as apoptosis (Fig. 3*B*) ($p > 0.05$ *versus* PKGIα; $n = 3$), ruling out a role of sarcolemmal K_{ATP} channels in PKGIα-induced protection. Glib, 5-HD, and HMR1098 had no effect on necrotic or apoptotic cell death after SI-RO in control or PKGIαK390A-expressing cardiomyocytes, thereby ruling out any potential PKG-independent effects of these agents. MPG abolished protective effects of PKGIα, *i.e.* increased the percentage of trypan blue-positive cells (Fig. 3*A*) and TUNEL-positive nuclei (Fig. 3*B*) after SI-RO, suggesting a role for ROS in PKGI-dependent cardiomyocyte protection.

PKGIα Increases Phosphorylation of Akt—Because phosphorylation of Akt at Ser^{473} is required for its full activation, we used an antibody that specifically recognized Akt phosphorylated at Ser^{473}. PKGIα overexpression increased the phosphorylation of Akt as compared with control (without infection) and PKGIαK390A in cardiomyocytes (Fig. 4, *A* and *B*). No further increases in Akt phosphorylation were observed when cardiomyocytes were subjected to SI and 15 or 30 min of reoxygenation following transfection with PKGIα (data not shown). Wortmannin and LY-294002 increased the percentage of trypan blue-positive cardiomyocytes from 18.1 ± 0.3 (PKGIα overexpression group) to 33.5 ± 0.6 and 34.7 ± 0.9, respectively ($n = 3$; $p < 0.001$; Fig. 4*C*). The number of TUNEL-positive nuclei was also increased from 6.8 ± 0.8 (in PKGIα overexpression group) to 23.5 ± 0.2 and 23.8 ± 1.0 by wortmannin and LY-294002, respectively ($n = 3$; $p < 0.001$; Fig. 4*D*). Wortmannin and LY-294002 had no effect on necrosis or apoptosis in control or PKGIαK390A-overexpressing cardiomyocytes exposed to SI-RO.

Effect of PKGIα on Expression of NOS, Bcl-2, and Bax—Adenoviral transfection with PKGIα and PKGIαK390A augmented the expression of PKGIα protein after 24 h (Fig. 5*A*). PKGIα overexpression clearly increased iNOS, eNOS, and Bcl-2 expression as compared with the control cells or cells infected with PKGIαK390A (Fig. 5, *A* and *B*). However, Bax was significantly decreased with overexpression of PKGIα as compared with control and PKGIαK390A-overexpressing cells (Fig. 5, *A* and *B*). No increase in nNOS was observed after PKGIα or PKGIαK390A overexpression as compared with control cells (Fig. 5, *A* and *B*). Similar results were obtained when cardiomyocytes were subjected to SI and 15 or 30 min of reoxygenation following transfection with PKGIα (data not shown).

To demonstrate the effect of NOS expression on PKGIα-induced cardiomyocyte protection, we treated cells with the nonselective NOS inhibitor L-NAME before SI-RO. As shown in Fig. 6*A*, L-NAME increased the percentage of trypan blue-positive cardiomyocytes from 18.1 ± 0.3 (PKGIα-overexpressing group) to 31.8 ± 1.2 ($n = 3$; $p < 0.001$). The number of TUNEL-positive nuclei also increased from 6.8 ± 0.8 in PKGIα-overexpressing cardiomyocytes to 21.5 ± 1.1 following pretreatment with L-NAME ($n = 3$; $p < 0.001$; Fig. 6*B*). L-NAME had no effect on necrosis and apo-

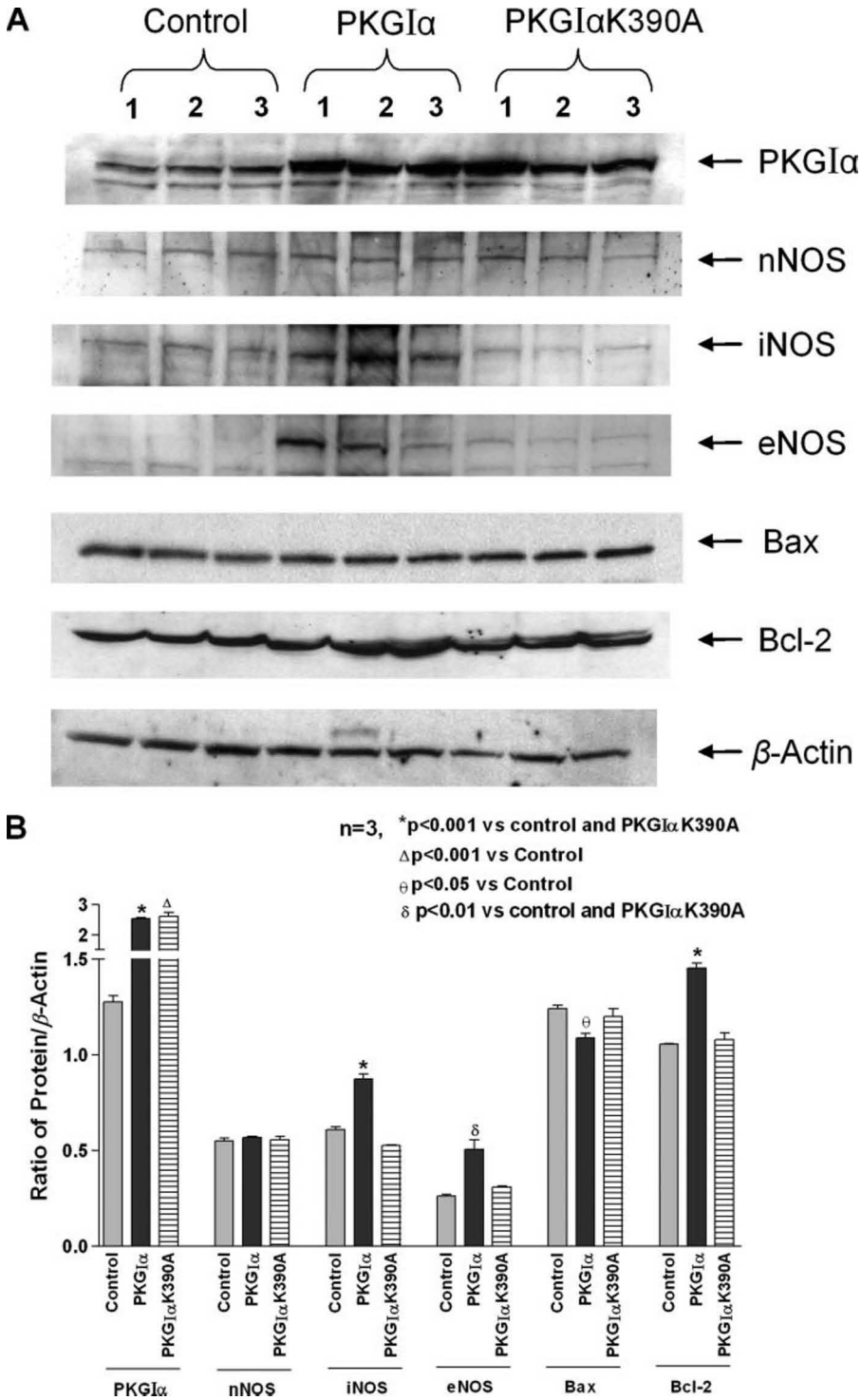

FIGURE 5. **Effect of PKGIα on expression of NOS isoforms, Bax, and Bcl-2.** *A*, representative Western blots show expression levels of PKGIα, nNOS, iNOS, eNOS, Bax, Bcl-2, and β-actin 24 h after infection with PKGIα or PKGIαK390A. *B*, bar diagrams showing density of bands after normalization to β-actin in the same samples. Note that iNOS, eNOS, and Bcl-2 proteins were significantly increased, but Bax was decreased by PKGIα overexpression.

ptosis in control and PKGIαK390A-overexpressing cardiomyocytes exposed to SI-RO, ruling out any PKG-independent effect of the drug. These results suggest that the protective effect of PKGIα is mediated by the NOS-dependent pathway.

Effect of PKGIα on Phosphorylation of MAPKs—ERK1 and JNK phosphorylation were increased in cardiomyocytes overexpressing PKGIα as compared with control and PKGIαK390A-overexpressing cells (Fig. 7). However, p38 phosphorylation was not enhanced in cells overexpressing PKGIα. The expression of total ERK1, JNK, and p38 was not altered with PKGIα or PKGIαK390A. Similar to Akt, no further increases in ERK1 and JNK phosphorylation was observed when cardiomyocytes were subjected to SI and 15 or 30 min of reoxygenation following transfection with PKGIα (data not shown). To determine the relationship between PKGIα-induced cytoprotection and activation of JNK and ERK, cardiomyocytes were treated with inhibitors of MAPKs. As shown in Fig. 8*A*, PD-98059 and SP600125 increased the percentage of trypan blue-positive cardiomyocytes from 19.6 ± 0.4 in PKGIα-overexpressing cardiomyocytes to 29.5 ± 1.3 and 33.2 ± 1.8, respectively ($n = 3, p < 0.001$). In addition, PD-98059 and SP600125 increased TUNEL-positive nuclei from 6.6 ± 0.4 in PKGIα-overexpressing cardiomyocytes to 15.4 ± 1.0 and 15.8 ± 0.6, respectively ($n = 3$; $p < 0.001$; Fig. 8*B*). These results suggest that phosphorylation of ERK and JNK were involved in PKGIα-induced protection against SI-RO injury in cardiomyocytes.

DISCUSSION

PKG is a serine/threonine protein kinase and is one of the major intracellular receptors for cGMP. There are two types of PKG present in eukaryotic cells: type I and type II. The N terminus of PKG type I is encoded by two alternatively spliced exons that produce Iα and Iβ isoforms (21, 26, 27). PKG is present in high concentrations in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular end plate, and the kidney vasculature (18). Low levels have been identified in vascular endothelium, granulocytes, chondrocytes, and osteoclasts. The PKGIα isozyme is mainly found in lung, heart, platelets, and cerebellum. The Iβ

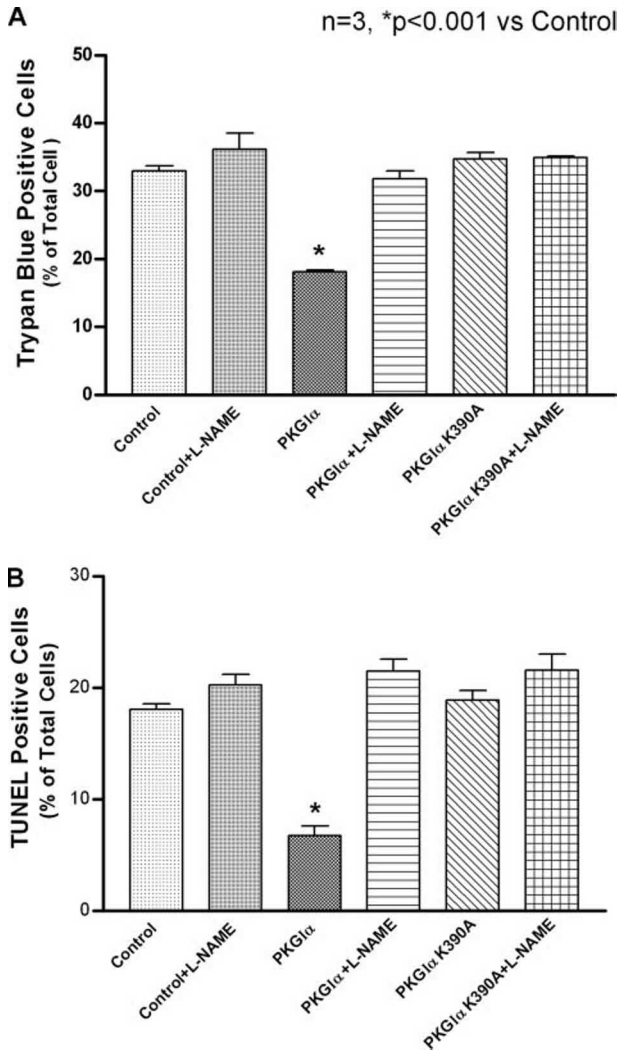

FIGURE 6. **NOS inhibitor blocks PKGIα-induced cardiomyocyte protection.** PKGIα-overexpressing cardiomyocytes were treated with 100 μM of the nonselective NOS inhibitor L-NAME for 30 min before SI-RO. *A*, cell necrosis is determined by trypan blue-positive cells after 90 min of SI and 2 h of RO. *B*, apoptotic nuclei identified using TUNEL assay after 90 min of SI and 18 h of RO. Note that L-NAME blocked cardiomyocyte protection by PKG ($n = 3$; $p < 0.001$ *versus* control). L-NAME had no effect on necrosis or apoptosis in control and PKGIαK390A-expressing cardiomyocytes exposed to SI-RO.

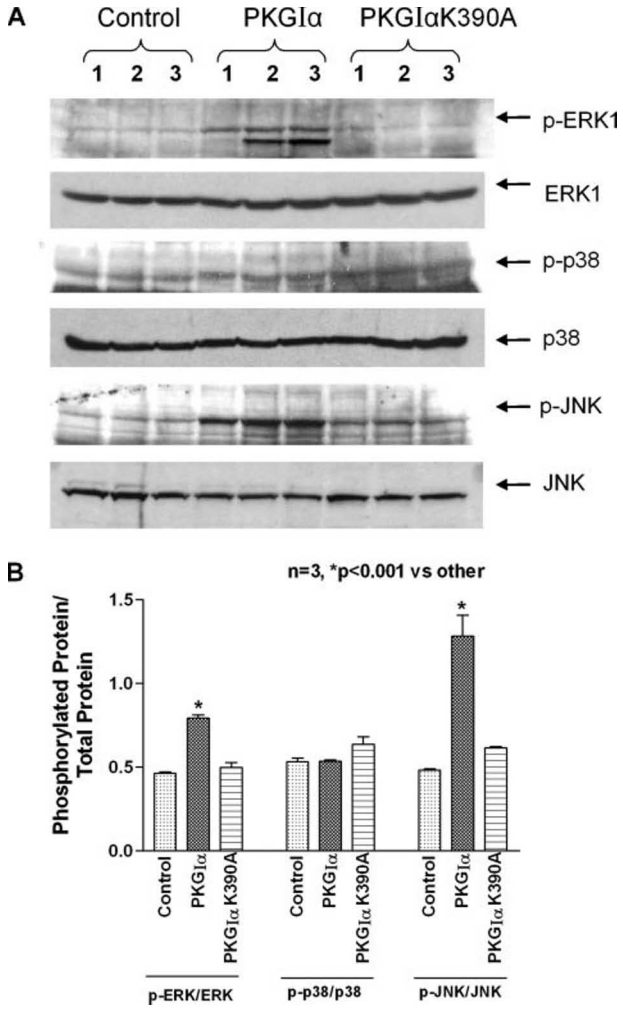

FIGURE 7. **PKGIα induces phosphorylation of ERK and JNK.** *A*, representative Western blots showing expression levels of phospho-ERK, total ERK, phospho-p38, total p38, phospho-JNK, and total JNK in PKGIα- or PKGIαK390A-expressing cardiomyocytes. *B*, bar diagram showing the ratio of phospho-ERK to total ERK, phospho-p38 to total p38, and phospho-JNK to total JNK expression. Note that phospho-ERK and phospho-JNK were increased with PKG1α expression ($n = 3$; $p < 0.001$ *versus* control).

form is highly expressed with Iα in smooth muscle, including uterus, vessels, intestine, and trachea (18, 28). The activation of PKG phosphorylates many intracellular proteins and regulates important physiological functions such as relaxation of vascular smooth muscle, inhibition of cell differentiation and proliferation, and inhibition of platelet aggregation and apoptosis (18, 29).

Several studies have demonstrated that generation of NO from iNOS or eNOS and opening of the mitoK$_{ATP}$ channel plays an important role in ischemic as well as pharmacological preconditioning in the heart (30). Opening the mitoK$_{ATP}$ channel partially compensates the membrane potential, which enables additional protons to be pumped out to form a H^+ electrochemical gradient for both ATP synthesis and Ca^{2+} transport (31). Sarcolemmal channels are the premier sensors of the energy state of the myocyte and, when activated, shorten or interrupt the action potential (32). In times of metabolic stress, decreasing cellular electrical excitability could protect cells against injury. Han *et al.* (8) first suggested that K$_{ATP}$ channels are regulated by a PKG signaling pathway that induces ischemic preconditioning in cardiomyocytes. In this study, NO donors and PKG activators facilitated pinacidil-induced K$_{ATP}$ channel activities in a concentration-dependent manner, and a selective PKG inhibitor abrogated these effects. However, the effect of PKGIα and associated downstream signaling pathways in protection against SI-RO injury independent of confounding factors associated with preconditioning stimuli or drugs has never been investigated. Therefore, we used adenoviral transfection of PKGIα to evaluate its direct effect on protection of adult cardiomyocytes. Our results show that PKGIα induced protection of cardiomyocytes against SI-RO injury that was mediated by opening of mitoK$_{ATP}$ channels, because protection was abrogated by glibenclamide and 5-HD (Fig. 3). The protective effect of PKGIα was not inhibited by HMR1098, the selective blocker of sarcolemmal K$_{ATP}$ channel, thereby further supporting the role of mitoK$_{ATP}$ channels in PKGIα-dependent protection.

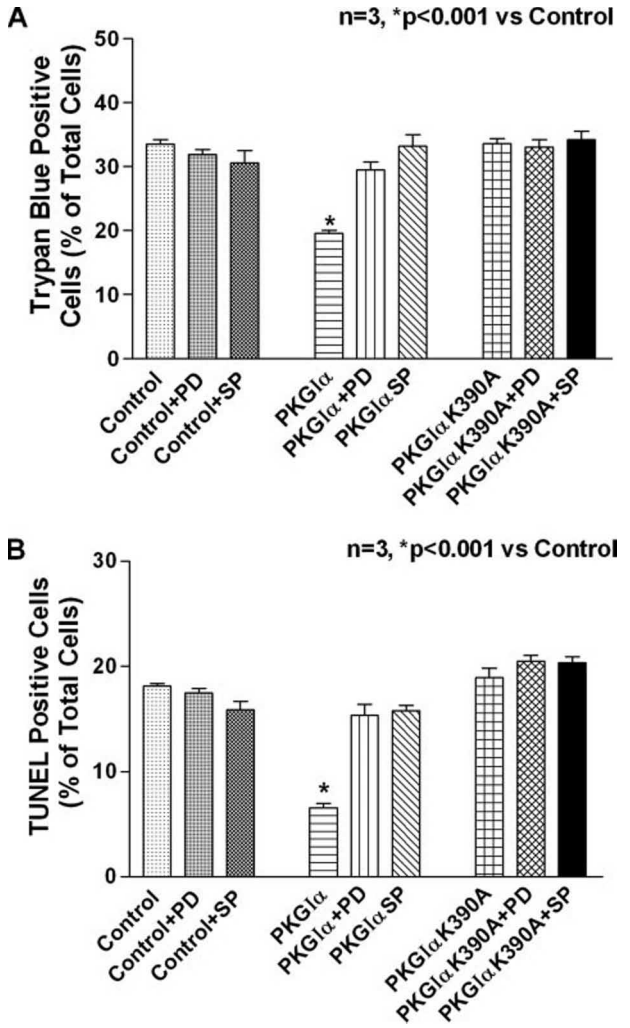

FIGURE 8. **PKGIα-induced cardiomyocyte protection is abolished by MAPK inhibitors.** PKGIα expressing cardiomyocytes were pretreated with the ERK inhibitor, PD-98059 (*PD*, 25 μM), or the JNK inhibitor, SP600125 (*SP*, 10 μM), for 30 min before SI-RO. *A*, cell necrosis is determined by trypan blue-positive cells after 90 min of SI and 2 h of RO. *B*, apoptotic nuclei identified using TUNEL assay after 90 min of SI and 18 h of RO. Note that the inhibitors of ERK and JNK abolished PKGIα-induced protection ($n = 3$; $p < 0.001$ *versus* control).

Our results also showed that PKGIα-induced protection against SI-RO was associated with enhanced phosphorylation of Akt, ERK1/2, and JNK. The inhibitors of PI 3-kinase (Fig. 4, *C* and *D*), ERK, or JNK1 (Fig. 8) abolished PKGIα-dependent protection against necrosis and apoptosis. These results suggest that PKGIα-dependent myocyte protection relies upon multiple and possibly interrelated pathways involving activation of PI 3-kinase, Akt, ERK, and JNK1. PKGIα also increased the expression of iNOS, eNOS, and Bcl-2 and decreased expression of Bax (Fig. 5). The nonselective NOS inhibitor L-NAME significantly blocked PKG-dependent cardiomyocyte protection against SI-RO (Fig. 6). Previous studies have shown that PI 3-kinase plays an important role in preconditioning of the heart against ischemia/reperfusion injury (33). Many growth factors and hormones have been shown to exert their cellular function, including the activation of eNOS via the PI 3-kinase/Akt signaling pathway (34). The pharmacological activation of the anti-apoptotic pro-survival kinase signaling cascades including PI 3-kinase-Akt and ERK by administration of growth factors at the time of reperfusion conferred cardioprotection by limiting both the apoptotic and necrotic components of cell death (35). Also, it has been shown that PI 3-kinase-Akt is activated and protects against ischemia/reperfusion injury by recruitment of downstream anti-apoptotic pathways of cell survival (36). These include phosphorylation and inactivation of pro-apoptotic proteins such as BAD (37), Bax (38, 39), and caspases (40, 41), as well as phosphorylation and activation of eNOS (42). In neuronal cells, NO has been shown to suppress apoptosis by inducing PKG activation, which induces phosphorylation of Akt and Bad (43). In the present study, PKGIα caused increased expression of Bcl-2, decreased expression of Bax, and activity of caspase-3, which may be mediated by the activation of PI 3-kinase-Akt.

Signaling pathways including the activation of protein kinase C and MAPKs (12, 44), synthesis of iNOS, generation of NO, and opening of mitoK$_{ATP}$ channels have also been implicated in cardioprotection (45–47). PI 3-kinase activates Akt through phosphoinositide-dependent kinases (PDK-1 and -2), which in turn activate eNOS (42, 48–51). The NO generation from eNOS stimulates guanylyl cyclase, which then activates PKG. MAPKs are important mediators of signal transduction from the cell surface to the nucleus, thereby regulating several cellular responses (21). Three distinct MAPKs have been identified in mammalian cells: ERK, JNK, and p38 kinase. ERK is involved in myocardial ischemic preconditioning (51–55) and antiapoptotic pathways (56). Our results show a significant increase of phosphorylation of ERK1 and JNK, but not p38, after overexpression of PKGIα as compared with the nontreated control and PKGIαK390A-overexpressing cardiomyocytes (Fig. 7). Previous studies have also shown that PKG activated the JNK1 pathways via phosphorylation of MEKK1 in human colon cancer cells (23) and in cultured rat aortic smooth muscle cells (21). In the present study, we showed that ERK and JNK inhibitors completely abolished the protection against necrosis and apoptosis (Fig. 8), suggesting an essential role for these two kinases in PKGIα-induced protection.

Our data show that MPG, a putative antioxidant blocked, the protective effect of PKGIα against necrosis and apoptosis following SI-RO (Fig. 3). These results suggest a possible role for redox signaling in the protective pathway(s) triggered by PKGIα. The exact molecular target(s) of ROS by PKGIα is not clear from the present study. However it could possibly include activation of MAPKs, PI 3-kinase, and opening of mitoK$_{ATP}$ channels. It has been shown that the release of ROS following the opening of mitoK$_{ATP}$ channel trigger cardioprotection (57). It was suggested that opening of these channels allows potassium to enter the mitochondrial inner matrix, which then causes generation and release of ROS from the respiratory chain (58). ROS then acts as second messengers to activate a downstream pathway of protective kinases, including protein kinase C and possibly p38 MAPK, that finally converge on the cardioprotective end effector. The G_i-coupled receptors, including those binding bradykinin and acetylcholine, have also been shown to mediate protection by opening mitoK$_{ATP}$ channels with the generation of ROS that act as second messengers to activate protein kinase C (17).

In summary, this study provides the first direct evidence that adenoviral expression of PKGIα in the absence of any stimulus

(such as preconditioning or drugs) promotes anti-necrotic and anti-apoptotic effects following ischemia-reoxygenation injury in cardiomyocytes. Our data also show that opening of mitoK$_{ATP}$ channels; generation of ROS; phosphorylation of Akt, ERK, and JNK; increased expression of iNOS, eNOS, and Bcl-2; and decrease of Bax play an essential role in the protective effect of PKGIα. Future studies are needed to further define interactions of the several signaling pathways that eventually lead to PKGIα-dependent attenuation of necrosis and apoptosis in cardiomyocytes. Strategies for enhancing PKGIα preconditioning-like effects should be examined for their potential to expand therapeutic options for protecting the heart against ischemia/reperfusion injury.

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