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Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K⁺ channels in rat hearts

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Cuong, Dang Van, Nari Kim, Jae Boum Youm, Hyun Joo, Mohamad Warda, Jae-Wha Lee, Won Sun Park, Taeho Kim, Sunghyun Kang, Hyungkyu Kim, and Jin Han. Nitric oxide-cGMP-protein kinase G signaling pathway induces anoxic preconditioning through activation of ATP-sensitive K⁺ channels in rat hearts. *Am J Physiol Heart Circ Physiol* 290: H1808–H1817, 2006. First published December 9, 2005; doi:10.1152/ajpheart.00772.2005.—Nitric oxide (NO) plays an important role in anoxic preconditioning to protect the heart against ischemia-reperfusion injuries. The present work was performed to study better the NO-cGMP-protein kinase G (PKG) signaling pathway in the activation of both sarcolemmal and mitochondrial ATP-sensitive K⁺ (K_{ATP}) channels during anoxic preconditioning (APC) and final influence on reducing anoxia-reperfusion (A/R)-induced cardiac damage in rat hearts. The upstream regulating elements controlling NO-cGMP-PKG signal-induced K_{ATP} channel opening that leads to cardioprotection were investigated. The involvement of both inducible and endothelial NO synthases (iNOS and eNOS) in the progression of this signaling pathway was followed. Final cellular outcomes of ischemia-induced injury after different preconditioning in the form of lactate dehydrogenase release, DNA strand breaks, and malondialdehyde formation as indexes of cell injury and lipid peroxidation, respectively, were investigated. The lactate dehydrogenase and malondialdehyde values decreased in the groups that underwent preconditioning periods with specific mitochondrial K_{ATP} channels opener diazoxide (100 μM), nonspecific mitochondrial K_{ATP} channels opener pinacidil (50 μM), *S*-nitroso-*N*-acetylpenicillamine (SNAP, 300 μM), or β-phenyl-1,*N*²-etheno-8-bromoguanosine-3',5'-cyclicmonophosphorothioate, *Sp*-isomer (10 μM) before the A/R period. Preconditioning with SNAP significantly reduced the DNA damage. The effect was blocked by glibenclamide (50 μM), 5-hydroxydecanoate (100 μM), *N*^G-nitro-L-arginine methyl ester (200 μM), and β-phenyl-1,*N*²-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate, *Rp*-isomer (1 μM). The results suggest iNOS, rather than eNOS, as the major contributing NO synthase during APC treatment. Moreover, the PKG shows priority over NO as the upstream regulator of NO-cGMP-PKG signal-induced K_{ATP} channel opening that leads to cardioprotection during APC treatment.

guanosine 3',5'-cyclic monophosphate; adenosine 5'-triphosphate; oxidative damage

ISCHEMIC PRECONDITIONING, in which short-term occlusion and reperfusion of a coronary artery are followed by long-term occlusion, can reduce subsequent ischemia-induced injury to the heart (42). Nitric oxide (NO), protein kinase G (PKG), and

ATP-sensitive K⁺ (K_{ATP}) channels (both the sarcolemmal and mitochondrial subtypes) can mimic the effects of ischemic preconditioning in the heart, and mitochondrial K_{ATP} channels appear to be the end effectors (19, 20, 25). The activation of these channels may improve the recovery of regional contractility of myocardium by shortening the duration of action potentials and by attenuating membrane depolarization, both of which would decrease myocardial contractility and reduce energy expenditure during ischemia (6, 27).

NO formation is elevated after anoxia and reperfusion (A/R) episodes and may be involved in inhibiting myocardial apoptosis (12, 38, 40). NO operates via several mechanisms, including 1) the stimulation of soluble guanylate cyclase, which leads to the production of cGMP and the activation of PKG, and 2) the direct activation of K_{ATP} channels via the phosphorylation of serine-threonine residues (25, 57). Cardiac NO synthesis is catalyzed by endothelial, neuronal, and inducible NO synthases (eNOS, nNOS, and iNOS, respectively). Stress stimuli, such as those of the inflammatory response, may induce production of large amounts of NO in a process that is mediated by iNOS. Recent studies have demonstrated that the NO produced by iNOS can protect the heart and that this effect involves actions on K_{ATP} channels and contractile fibers (6, 49). However, the natural mechanism of cardioprotection by which NO, cGMP, PKG, and K_{ATP} channels reduce the oxidative damage induced by A/R is still largely unknown (46, 54, 57). In addition, the role of iNOS in NO production is a matter of conjecture (21, 61).

The results of our study expand on our previous findings (25, 26) concerning sarcolemmal K_{ATP} channels and provide evidence that both mitochondrial and sarcolemmal K_{ATP} channels protect ventricular myocytes from A/R-induced injury by reducing the amount of DNA damage. Thus mitochondrial K_{ATP} channels associated with NO/PKG signal transduction pathways could protect the heart against A/R-induced injury, and the anoxic preconditioning (APC)-induced synthesis of inducible NO may play a central protective role against oxidative damage.

MATERIALS AND METHODS

Heart Preparation

Male Sprague-Dawley rats (280 ± 20 g, mean ± SE) were used in all experiments. The investigation conforms to the *Guide for the Care*

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and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and was approved by the Institutional Animal Care and Use Committee of College of Medicine, Inje University. Heart slices and single cardiac myocytes were prepared as previously described (23, 25). Heart slices ($n = 251$) of 0.4- to 0.5-mm thickness were prepared by cross-sectioning with the use of Stadie-Riggs microtome (Thomas Scientific). The average weight of heart slices was 59.6 ± 2.9 mg. Slices were stored in ice-cold modified Cross-Taggart medium. The use of heart slices has an advantage over whole hearts in that the influence of global innervation in whole hearts is reduced and gap junction will be the mean contributor in cell-cell signaling and in heart slices. Single cardiac myocytes were stored in Kraftbrühe (KB) solution on ice and were used within 1 h. Before the experiment, both heart slices and myocytes were incubated in Tyrode solution at 37°C in 95% O_2 -5% CO_2 for 20 min for stabilization and then used as experimental protocols.

Experimental Protocols

Figure 1 shows the proposed role of K_{ATP} channels associated with NO/PKG signal transduction pathway leading to cardioprotection effects.

To understand better the cardioprotection role of mitochondrial K_{ATP} channels associated with NO/PKG signal transduction pathway during anoxic preconditioning, the study was performed on heart slices as depicted in Fig. 2.

Heart slices. Heart slices were randomly allocated to 34 groups. Heart slices in groups 4–34 were preconditioned three times at 5-min intervals with $50 \mu\text{M}$ pinacidil as nonspecific mitochondrial K_{ATP} channels opener (groups 15–19), $100 \mu\text{M}$ diazoxide as specific mitochondrial K_{ATP} channels opener (groups 20–24), $300 \mu\text{M}$ *S*-nitroso-*N*-acetylpenicillamine (SNAP; groups 25–29) as NO donor, $10 \mu\text{M}$ of the *Sp* isomer of β -phenyl-1, *N*²-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate (*Sp*-8-Br-PET-cGMPS; groups 30–34) as PKG activator, or anoxic preconditioning (APC), as a representative of ischemia preconditioning in vivo (groups 9–14). These preconditioning treatments were repeated in the presence of $50 \mu\text{M}$ glibenclamide, as nonspecific mitochondrial K_{ATP} channels blocker (groups 11, 16, 21, 26, and 31), $100 \mu\text{M}$ 5-hydroxydecanoate (5-HD) as specific mitochondrial K_{ATP} channels blocker (groups 12, 17, 22, 27, and 32), *N*^G-nitro-L-arginine methyl ester (L-NAME), a potent competitive inhibitor of NO synthesis (groups 13, 18, 23, 28, and 33), or the *Rp* isomer of β -phenyl-1, *N*²-etheno-8-bromo-

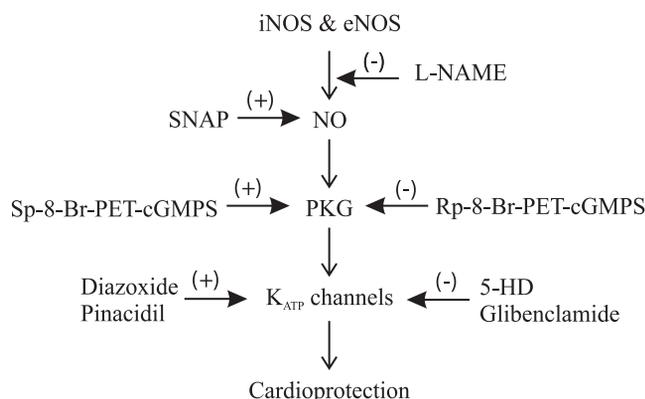


Fig. 1. Proposed signal transduction pathway that leads to cardioprotection. NO, nitric oxide; iNOS, inducible NO synthase; eNOS, endothelial NOS; SNAP, *S*-nitroso-*N*-acetylpenicillamine; *Sp*-8-Br-PET-cGMPS, *Sp* isomer of β -phenyl-1, *N*²-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate; *Rp*-8-Br-PET-cGMPS, *Rp*-isomer of β -phenyl-1, *N*²-etheno-8-bromoguanosine-3',5'-cyclic monophosphorothioate; 5-HD, 5-hydroxydecanoate; K_{ATP} , ATP-sensitive Ca^{2+} ; L-NAME, *N*^G-nitro-L-arginine methyl ester.

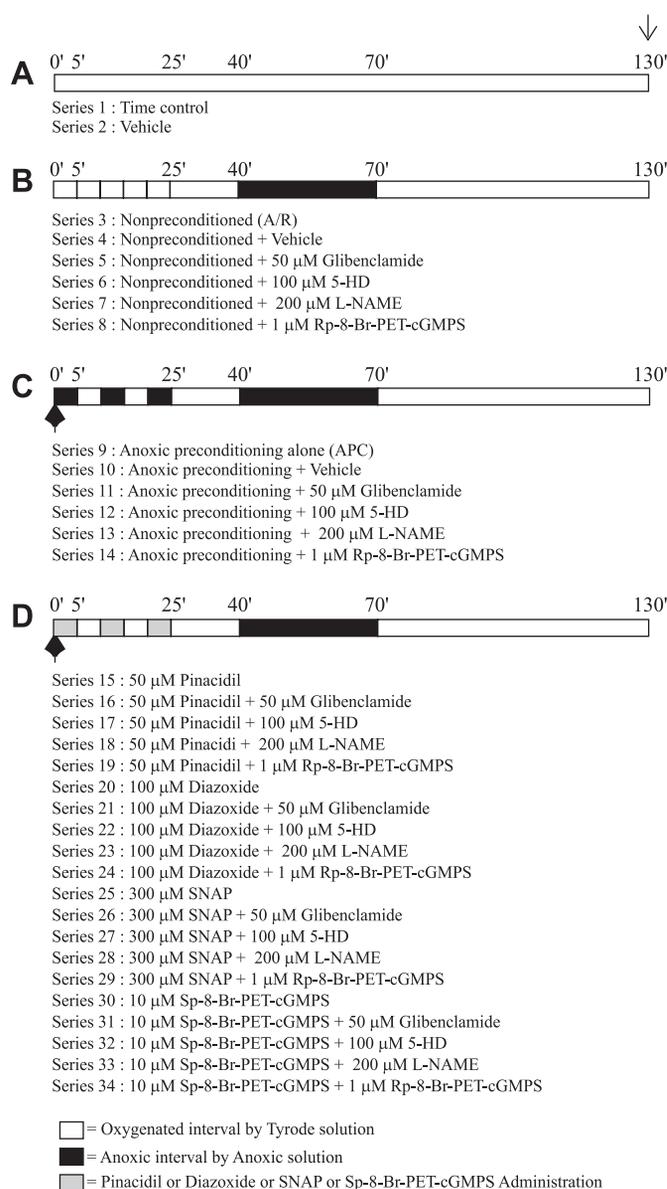


Fig. 2. Summary of experimental protocol. Heart slices were equilibrated in a bath for at least 45 min before application of one of the protocols. Excluding protocols for groups in A, all other protocols (B–D) involved anoxic perfusion with an anoxic solution for 30 min followed by reoxygenation with Tyrode solution for 60 min. Heart slices were incubated for 30 min in anoxic conditions alone (groups in B) or were pretreated with three 5-min periods of exposure to the anoxic solution (groups in C). In both groups (B and C), anoxic solution was given alone or in combination with $50 \mu\text{g}$ glibenclamide (Glib), $100 \mu\text{g}$ 5-HD, $200 \mu\text{g}$ L-NAME, or $1 \mu\text{g}$ Rp-8-Br-PET-cGMPS. Heart slices of groups in D were preconditioned as described above with $50 \mu\text{g}$ pinacidil (Pin), $100 \mu\text{g}$ diazoxide (Diaz), $300 \mu\text{g}$ SNAP, or $10 \mu\text{g}$ *Sp*-8-Br-PET-cGMPS with or without Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. A/R, anoxia-reperfusion.

guanosine-3',5'-cyclic monophosphorothioate (*Rp*-8-Br-PET-cGMPS) as PKG inhibitor (groups 14, 19, 24, 29, and 34). Control slices (groups 1–3) were either not preconditioned (group 1), exposed to vehicle without any drugs (group 2), or subjected to 30 min of anoxia (group 3). These control treatments were also repeated in the presence of glibenclamide, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS (groups 5–8, respectively).

Single cardiac myocytes. Cells from the same heart slice were used for the protocols in groups 1, 2, 3, 9, 11, 12, 26, 27 and 29.

The running temperature of all experiments was 37°C. The anoxic solution was nitrogenated, and Tyrode solution was oxygenated for 45 min before use. The lactate dehydrogenase (LDH) release, malondialdehyde (MDA) formation, and DNA damage were quantified at the end of a 60-min period of reoxygenation.

Measurement of LDH Release and MDA Content

Measurement of LDH release. LDH release was measured as previously described (20). Heart slices were homogenized in distilled water and centrifuged. After the pellet was discarded, LDH activity was measured in the supernatant and incubation medium by using an LDH assay kit (Asan Pharm; Kyunggee-do, Korea). Final values were expressed as a percentage of the total LDH released from cells.

Lipid peroxidation assay. Lipid peroxidation (quantified as MDA formation) was estimated by using thiobarbituric acid as previously described (23). The MDA content of the whole tissue homogenate was expressed in milligrams of protein. The protein content was measured by using Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Heart slices were collected and homogenized in ice-cold 1.15% KCl (5% wt/vol) solution. A 0.2-ml aliquot of the homogenate was mixed with 50 μ l of 8.1% sodium dodecyl sulfate (wt/vol) before being incubated for 10 min at room temperature. Acetic acid [375 μ l; 20% vol/vol (pH 3.5)] and 375 μ l of thiobarbituric acid (0.6% vol/vol) were added to the mixture. The mixture was then heated for 60 min in a boiling water bath. The samples were allowed to cool at room temperature. After adding *n*-butanol-pyridine solution (15:1; 1.25 ml), the mixture was vortexed before being centrifuged at 500 *g* for 5 min. The absorbance of the upper colored layer was measured at 535 nm and 520 nm with a spectrophotometer (U-2000; Hitachi) and was compared with the absorbance of freshly prepared 1,1,3,3-tetraethoxypropane standards. Final values were expressed as relative percentages of the values of the controls.

Western Blot Analysis of NOS Expression

Heart slices were suspended in extraction lysis buffer for 30 min, homogenized, and then centrifuged again (13,000 *g* for 30 min at 4°C) to remove debris. Protein concentrations were measured by using Bio-Rad Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (40 μ g) were run on a 7.5% SDS-PAGE gel. Western blotting was carried out by using rabbit polyclonal anti-iNOS and anti-eNOS primary antibodies (Santa Cruz Biotechnology) at a dilution of 1:300 each and mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1,000. Protein-bound antibodies were detected by using the enhanced chemiluminescence kit (ECL Western blotting detection reagents, Amersham Bioscience, Piscataway, NJ). Data acquisition of the results was performed by using SLA 3000 Image reader (Life Science). Immunoblot experiments were repeated four times.

Immunofluorescence

After experimental protocols were completed, whole hearts were immersed in -70°C isopropanol for 1 min and were then cross-sectioned into 10- μ m thicknesses by using a microtome. Slices were then fixed by PBS solution contained 4% formaldehyde for 20 min, washed three times by PBS and blocked in blocking solution for 1 h. The slices were then incubated with anti-iNOS (corresponding to peptide mapping at COOH terminus of iNOS of mouse origin) at 1:1,000 dilution for 2 h and then with rabbit anti-goat Alexa Fluor 488 secondary antibody (1:3,000 dilution) for 1 h. After they were washed three times with PBS, the slices were then simultaneously challenged in ethanol (70% for 1 h, 95% for 2 h, and 100% for 1 h) and xylene (1 h) and embedded with Permount. All processes were carried out in the dark. Images were taken with a LSM-510 META confocal laser scanning microscopy (Zeiss, Jena, Germany) and analyzed by using LSM-510 META software (Zeiss). Alexa Fluor-488 was detected by He-Ne light source, excitation at 488 nm, and emission at 510 nm.

Determination of NO Content in Heart Slices

NO level in heart slices was measured by using cell-permeant 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate dye that fluoresces on reacting with either NO or its derivatives. Heart slices were incubated in KB solution with 5 μ M DAF-FM for 30 min at room temperature. They were then washed two times and kept in the dark before being used in a perfusion chamber. The green fluorescence was excited at 488 nm and imaged through a 525-nm long-length filter. Images were taken with a laser scanning confocal microscope (LSM-510 META, Zeiss) and analyzed by using LSM-510 META software (Zeiss).

Quantification of DNA Damage by Comet Assay

At the end of each experiment, cells were collected by centrifugation (600 *g* for 3 min), suspended in LMAgarose (1% in PBS), and plated onto standard agarose-coated slides. The plated cells were lysed overnight in lysis buffer (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris, and 1% Triton X-100, and adjusted to pH 10 with NaOH) to eliminate nuclear membranes, proteins, and all nonnuclear components and were then subjected to enzymatic digestion by endonuclease III for 45 min at 37°C. Cells were incubated in ice-cold electrophoresis solution (0.3 M NaOH and 1 mM Na₂EDTA; pH > 12.5) for 40 min to allow DNA unwinding before electrophoresis. Slides were then placed in a cooling system-fitted electrophoresis chamber. Electrophoresis was carried out for 40 min at 25 V. After electrophoresis, the cells were washed in a neutralizing solution (0.4 M Tris, pH 7.5) and stained with propidium iodide (5 μ g/ml). The stained cells were kept in complete darkness for 1 h and then examined under a fluorescence microscope. DNA damage was classified according to comet head and tail lengths into five categories that ranged from type I (undamaged; no discernible head) to type V (highest level of DNA damage; insignificant head) (Fig. 8A). Comets were scored visually and were imaged by using Image-Pro Plus software (Media Cybernetics). Cells at the edges of the slides were excluded from the score to avoid including atypical comets. Between 200 and 400 comets were scored for each slide, and the assay was repeated five times (9, 10, 39, 51).

Drugs and Solutions

Normal Tyrode solution contained (in mM) 143.0 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5.5 glucose, and 5.0 HEPES (pH 7.4). The modified KB solution contained (in mM) 70.0 KOH, 50.0 L-glutamate, 40.0 KCl, 20.0 KH₂PO₄, 20.0 taurine, 3.0 MgCl₂, 10.0 HEPES, 0.5 EGTA, and 10.0 glucose (pH 7.4). The ischemic solution contained (in mM) 143.0 NaCl, 8.0 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 10.0 2-deoxy D-glucose, and 5 HEPES (pH 7.4); this solution was deoxygenated by bubbling with 95% N₂-5% CO₂ for 45 min before use. SNAP was obtained from Calbiochem-Novabiochem (La Jolla, CA). Rp-8-Br-PET-cGMPS was purchased from BioLog Life Science (Bremen, Germany). 5-HD was obtained from Biomol (Plymouth Meeting, PA). All other reagents were analytical grade from Sigma (St. Louis, MO). Glibenclamide, pinacidil, and diazoxide were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. The concentration of DMSO was kept below 0.1% of the solutions used during experiments.

The concentration of pinacidil at which K_{ATP} channel activity was half-maximal its value was estimated to be 30–50 μ M (26). On the basis of the data, 30–50 μ M pinacidil dose was used during viability and K_{ATP} channel experiments.

A number of investigators have shown that glibenclamide prevents effects related to ischemia (3, 17, 56, 60). In all of these reports, glibenclamide shows clear effects, although relatively high concentrations were needed (3–300 μ M) and often only partial restoration was achieved. This is surprising because it is known that the K_{ATP} channels in the pancreatic β -cells are inhibited by glibenclamide with high affinity (IC₅₀ = 4 nM) (62). For heart muscle cells, it has also

been reported that K_{ATP} channel opener-induced K_{ATP} channel currents are inhibited by glibenclamide in the nanomolar range (14, 22). Recently, we (26) found that K_{ATP} channels in inside-out patches (34) and pinacidil-activated K_{ATP} channels were completely blocked by 10–50 μM of glibenclamide. In addition, metabolic inhibition-activated channels were only partially blocked by 30 μM of glibenclamide (24). On the basis of these data, we used relatively high concentrations of glibenclamide (50 μM) to block the K_{ATP} channels in this study.

There is good evidence that diazoxide and 5-HD are more effective on mitochondrial rather than sarcolemmal K_{ATP} channels (16, 50). The concentration of diazoxide that we used (100 μM) has been reported to activate mitochondrial K_{ATP} channels but not sarcolemmal K_{ATP} channels in rabbit ventricular myocytes (36, 58). It is well known that 5-HD is an effective blocker of mitochondrial K_{ATP} channels at the concentrations of 50–500 μM (29, 32, 58). Because Hu et al. (29) argued that 5-HD at a concentration of 0.5 mM selectively blocks mitochondrial K_{ATP} channels without affecting sarcolemmal K_{ATP} channels of rabbit ventricular myocytes, the 100 μM 5-HD concentration used during viability and mitochondrial K_{ATP} channel experiments was an appropriate concentration.

By using NO donors [SNAP and sodium nitroprusside (SNP)], we performed careful concentration-response relationship experiments in the concentration range of 0.1 to 1,000 μM to demonstrate the minimal concentration of NO donors required to activate the channels (26). The NO donor SNAP appears to be a low-output NO donor with a dose of 1 mM to give an effective concentration of $0.46 \pm 0.2 \mu\text{M}$. It was also reported that in rat cardiac myocytes, 100 μM SNAP (with production of 14.7 μM NO) induced a large increase in cGMP. However, SNAP in concentration of 0.1–1 μM (with production NO in the range of about 0.1 to 0.5 μM) induced moderate increase in cGMP (57). This is favorably comparable with pathophysiological concentrations of NO found in cardiovascular tissue during normal or ischemic conditions (33, 55). SNP, another potent NO donor, in a concentration of 1,000 μM is thought to release NO to the same extent as that of 300 μM SNAP because of the amount of cGMP production (52). Therefore, we believe that our tissue responses to SNAP and SNP are relevant to pathophysiological tissue levels of either NO or cGMP.

We performed careful concentration-response relationships using both Rp-8-Br-PET-cGMPS in the concentration range of 0.001 to 10 μM and Sp-8-Br-PET-cGMPS in the concentration range of 0.01 to 100 μM (26). On the basis of the data, we used 1 μM Rp-8-Br-PET-cGMPS and 10 μM Sp-8-Br-PET-cGMPS during experiments.

To exclude cross-activation between protein kinase A (PKA) and PKG during measurements of the effects of PKG activation on K_{ATP} channel activity, we performed the experiments under conditions in which PKA was inhibited by the potent and selective PKA inhibitor Rp-8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphothioate, unless otherwise stated.

Statistical Analysis

All data are presented as means \pm SE. In the patch-clamp studies, the paired Student's *t*-test was used to compare the significant differences between data sets. In the heart slice studies, statistical analysis was performed by two-way repeated-measures ANOVA (Super-Anova, Abacus Concepts, Berkeley, CA) for time and treatment (experimental group) effects. If an overall significance between groups was found, comparison was done for the time point (the end of the 60-min reoxygenated period) by using either Student's unpaired *t*-test when two treatment groups were compared or one-way ANOVA followed by a post hoc Student-Newman-Keuls test. Tests were considered significant when $P < 0.05$.

RESULTS

K_{ATP} Channels Reduced LDH Release and MDA Formation via the NO-cGMP-PKG Signaling Pathway

To examine the possible interaction between K_{ATP} channels and the NO-cGMP-PKG signaling pathway in the effects of anoxic preconditioning on A/R injury, LDH release and lipid peroxidation (MDA formation) were evaluated after 60 min of anoxia in heart slices that were pretreated with anoxia, pinacidil, or diazoxide. LDH release and MDA formation in control heart slices were $10 \pm 3\%$ (as percentage of total LDH) and $220 \pm 120 \text{ pmol/mg protein}$, respectively (Figs. 3A and 6A). Vehicle alone did not affect LDH release or MDA formation under any experimental conditions. In contrast to control and vehicle-treated slices, LDH release and MDA formation were significantly elevated ($33 \pm 5\%$ and $950 \pm 150 \text{ pmol/mg protein}$, respectively) in heart slices that were not preconditioned. Glibenclamide (50 μM), 5-HD (100 μM), L-NAME (200 μM), and Rp-8-Br-PET-cGMPS (1 μM) had no effect on LDH release or MDA formation. As shown in Figs. 3B and 6A, pretreatment of myocytes with three 5-min periods of anoxia significantly reduced LDH release and MDA formation to $12 \pm 3\%$ and $320 \pm 90 \text{ pmol/mg protein}$, respectively. The beneficial effects of anoxic preconditioning were eliminated by glibenclamide, 5-HD, L-NAME, and Rp-8-Br-PET-cGMPS. Pretreatment with 50 μM pinacidil (Fig. 4A) and 100 μM diazoxide, as channel openers (Figs. 4B and 6B), 300 μM

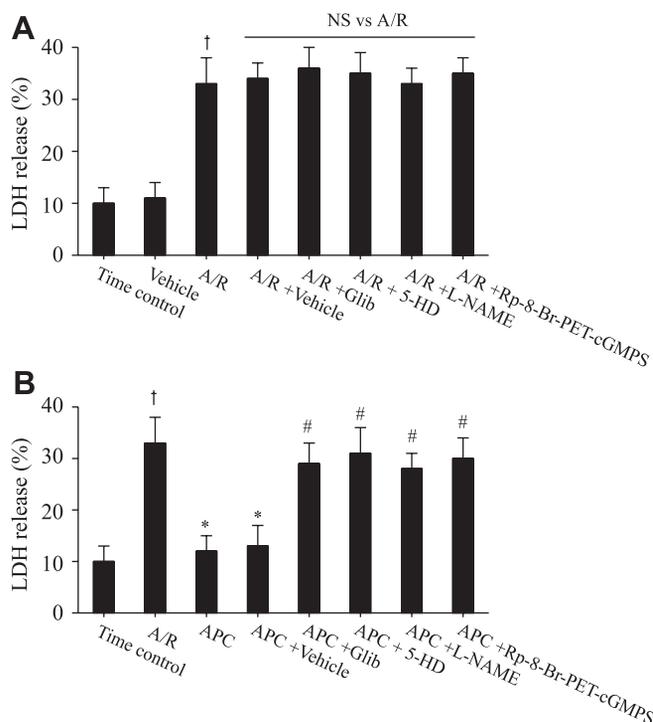


Fig. 3. Lactate dehydrogenase (LDH) release from cardiomyocytes. Heart slices were preconditioned by exposure to Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS before 30 min anoxia (A) or preconditioned together with three 5-min periods of exposure to the anoxic solution before 30 min of anoxic perfusion (B) [in μM : 50 Glib; 100 5-HD; 200 L-NAME; 1 Rp-8-Br-PET-cGMPS] (see Fig. 2). NS, differences are not statistically significant; A/R, anoxia-reperfusion. Data are presented as means \pm SE. † $P < 0.05$ compared with time control and vehicle; * $P < 0.05$ compared with A/R; # $P < 0.05$ compared with anoxic preconditioning (APC).

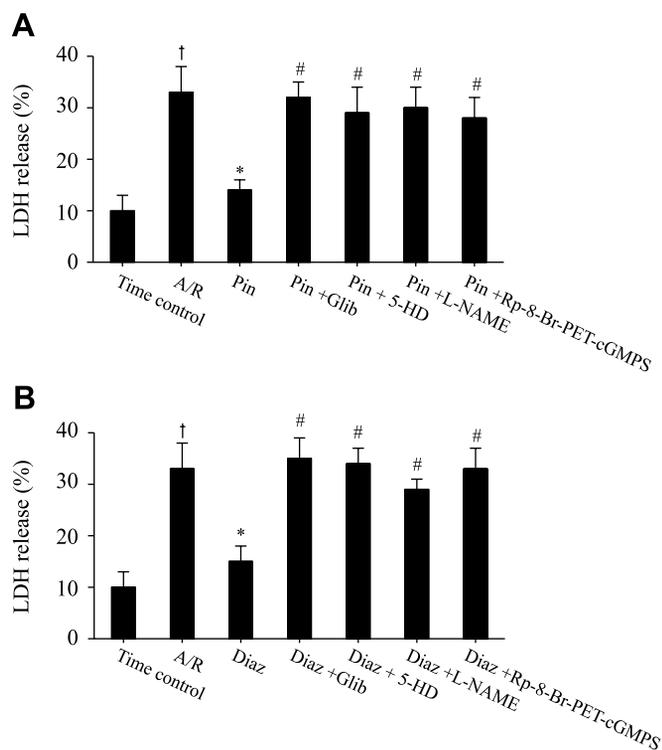


Fig. 4. Effects of Pin and Diaz on LDH release. *A*: heart slices were preconditioned as described in Fig. 2 in the presence of Pin alone or Pin + Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. *B*: effects of the same preconditioning protocol as in *A*, except in the presence of Diaz instead of Pin. Data are presented as means \pm SE. $\dagger P < 0.05$ compared with time control and vehicle; $*P < 0.05$ compared with A/R; $\#P < 0.05$ compared with APC.

SNAP (Figs. 5A and 6C), or 10 μ M Sp-8-Br-PET-cGMPS (Figs. 5B and 6D) also significantly reduced LDH release and MDA formation to levels that were indistinguishable from APC-exposed slices. This effect, however, was prevented by treatment with glibenclamide, 5-HD, or Rp-8-Br-PET-cGMPS. It is clear from Figs. 5 and 6 that there was marked decrease in LDH release as well as MDA formation when NO donor SNAP or PKG activator Sp-8-Br-PET-cGMPS was used in the study. This decrease however, was only noticed when L-NAME was used (Fig. 5, *A* and *B* and Fig. 6, *C* and *D*).

iNOS Expression and NO Production Increased During Anoxic Preconditioning

Both eNOS and iNOS were detected by Western blot analysis as single bands of ~ 145 kDa and ~ 130 kDa, respectively (Fig. 7A, *Aa* and *Ab*). Quantification of intensities of the immunoblot signals revealed that there was no significant difference between eNOS expression after different treatments. Moreover, iNOS expression was significantly higher in APC-treated heart slices than in control or A/R-treated heart slices (Fig. 7A*c*). To further confirm a significant iNOS expression in APC, immunofluorescence assay for iNOS was performed on rat heart slices. The expression of iNOS was more abundant in APC-treated heart slices than in control or A/R-treated heart slices (Fig. 7, *B* and *C*). To probe whether there is positive function between iNOS expression and NO production, we measured NO concentrations using NO-specific fluorescence dye DAF-FM diacetate in heart slices. Figure 7, *C* and *D*,

shows an increase in both iNOS expression and NO production in APC-treated heart slices compared with control or A/R-treated groups.

DNA Damage Increased During Anoxia and Reperfusion

DNA damage was investigated by alkaline single-cell microgel electrophoresis (Comet) assay (Fig. 8). The lowest level of DNA damage (type I) accounted for $64.4 \pm 5.1\%$ of total DNA damage in control samples. There was no difference between the control and vehicle-treated cells (data not shown). In contrast, type I DNA damage accounted for only $2.2 \pm 0.8\%$ of the DNA damage in A/R-treated samples, whereas there was significantly more high-level of DNA damage (type V) in A/R-treated cells than in the controls (21.2 ± 7.6 vs. $2.1 \pm 1.1\%$, respectively) (Fig. 8C). This was indicative of significant oxidative damage due to A/R.

Anoxic Preconditioning Reduced DNA Damage Caused by Anoxia and Reperfusion

Cells of the APC-treated group had higher levels of DNA damage than those of the control group. On the other hand, the amounts of type IV and type V DNA damage were substantially lower in APC-treated cells (5.7 ± 2 and $3.1 \pm 0.3\%$, respectively) than in A/R-treated cells (41.5 ± 4.5 and $21.1 \pm 7.6\%$ for types IV and V, respectively) (Fig. 8C). These data suggest that APC has a protective role on ventricular myocytes against A/R-induced oxidative stress.

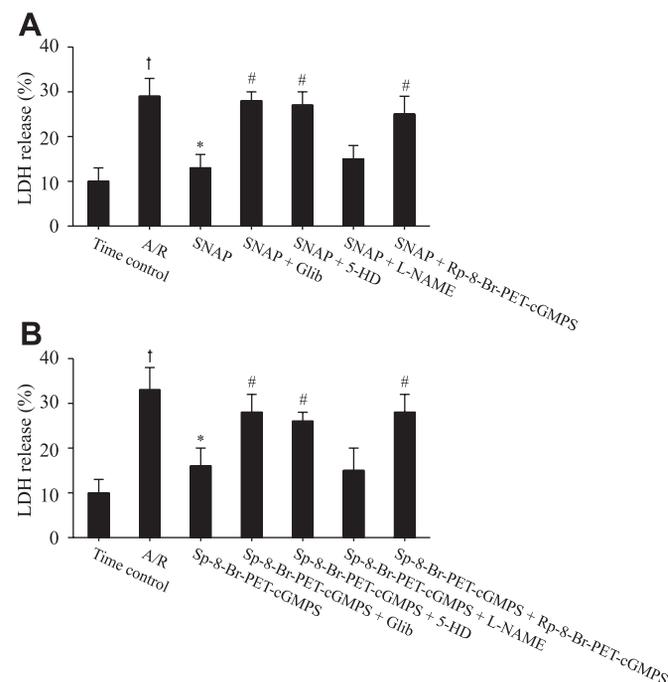


Fig. 5. Effect of SNAP and Sp-8-Br-PET-cGMPS on LDH release. *A*: heart slices were preconditioned as described in Fig. 2 in the presence of SNAP alone or SNAP + Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. *B*: effects of the same preconditioning protocol as in *A* except in the presence of Sp-8-Br-PET-cGMPS instead of SNAP. Data are presented as means \pm SE. $\dagger P < 0.05$ compared with time control and vehicle; $*P < 0.05$ compared with A/R; $\#P < 0.05$ compared with APC.

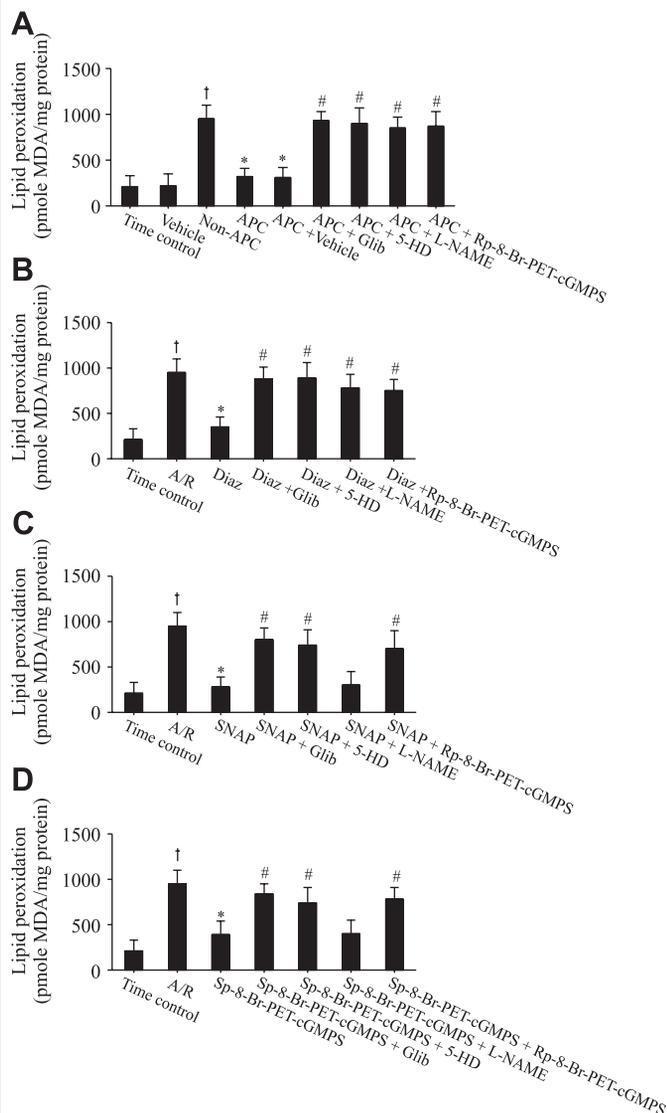


Fig. 6. Lipid peroxidation [malondialdehyde (MDA) formation] in cardiomyocytes. *A*: heart slices were preconditioned by exposure to anoxia (see Fig. 2) alone or in the presence of vehicle, Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. *B*: heart slices were preconditioned with Diaz alone or Diaz + Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. *C*: heart were preconditioned with SNAP alone or SNAP + Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. *D*: heart slices were preconditioned with Sp-8-Br-PET-cGMPS alone or Sp-8-Br-PET-cGMPS + Glib, 5-HD, L-NAME, or Rp-8-Br-PET-cGMPS. Values of control and A/R-treated cells in *B*, *C*, and *D* are the same as those in *A*. Data are presented as means \pm SE. †*P* < 0.05 compared with time control and vehicle; **P* < 0.05 compared with A/R; #*P* < 0.05 compared with APC.

NO Donor Mimicked Protective Effects of Anoxic Preconditioning

There was more type II DNA damage in myocytes that were preconditioned by exposure to the NO donor SNAP (300 μ M) as compared with the controls. However, there was less type IV and type V DNA damage (21.2 ± 7.6 and $7.9 \pm 4.5\%$, respectively) in these cells than in A/R-treated myocytes (41.5 ± 4.5 and $14 \pm 7.2\%$ for types IV and V, respectively) (Fig. 8C).

The aforementioned effects of SNAP were blocked in the presence of K_{ATP} channels inhibitors (glibenclamide and 5-HD) or a PKG inhibitor (Rp-8-Br-PET-cGMPS), which

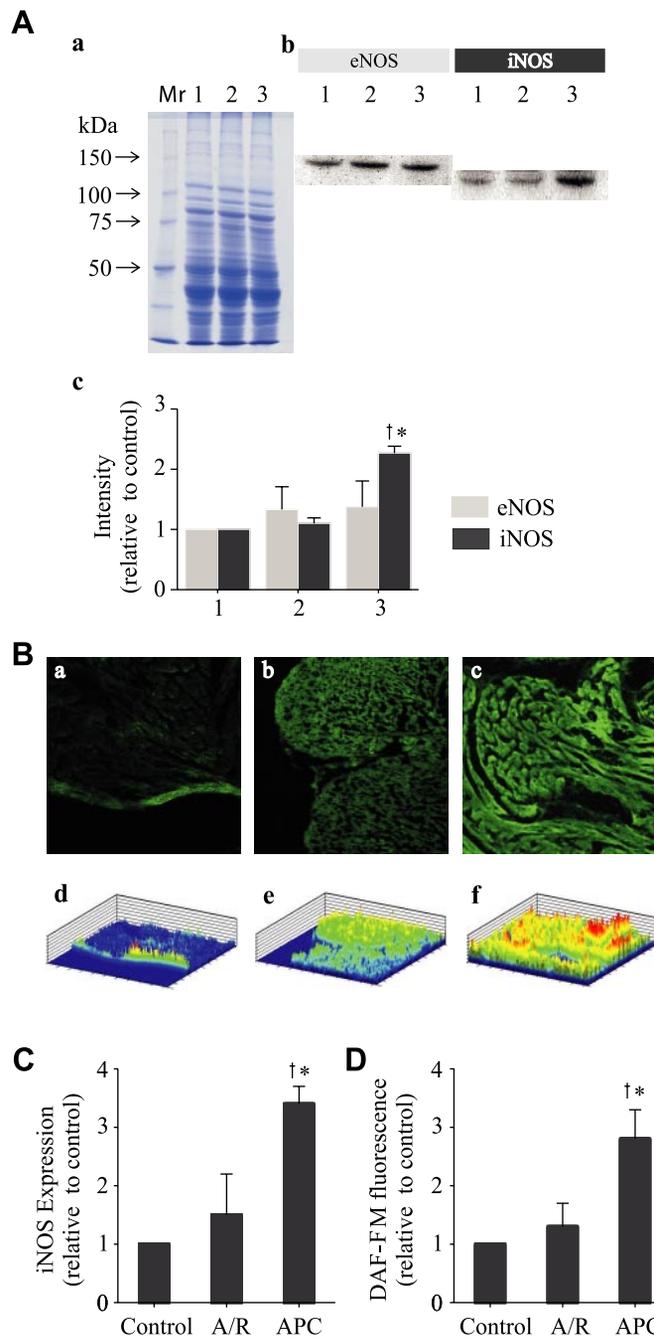


Fig. 7. Detection of eNOS and iNOS expression and NO in rat hearts. *A*: immunoblot data showing myocardial eNOS and iNOS protein expression: ~145- and ~130-kDa immunoreactive bands correspond to eNOS and iNOS, respectively (*A,a* and *A,b*). Values are shown as intensity of staining relative to control (*A,c*). Lanes 1–3 correspond to control, A/R-treated, and APC-treated heart tissues, respectively. *B*: fluorescence-based immunoassay of iNOS: heart slices labeled with Alexa Fluor 488 secondary antibody were brightly fluorescent in samples that were incubated with anti-iNOS antibody. iNOS immunofluorescence images and their pseudo three-dimensional images at control (*Ba* and *Bd*), A/R (*Bb* and *Be*), and APC (*Bc* and *Bf*). *C*: summarized data for iNOS immunofluorescence intensity: six heart slices were imaged in each group. *D*: summarized data for 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence intensity. Eight heart slices were imaged in each group. APC increased both iNOS expression and NO production. *M_r*, molecular weight marker. Data are presented as means \pm SE. †*P* < 0.05 compared with control; **P* < 0.05 compared with A/R.

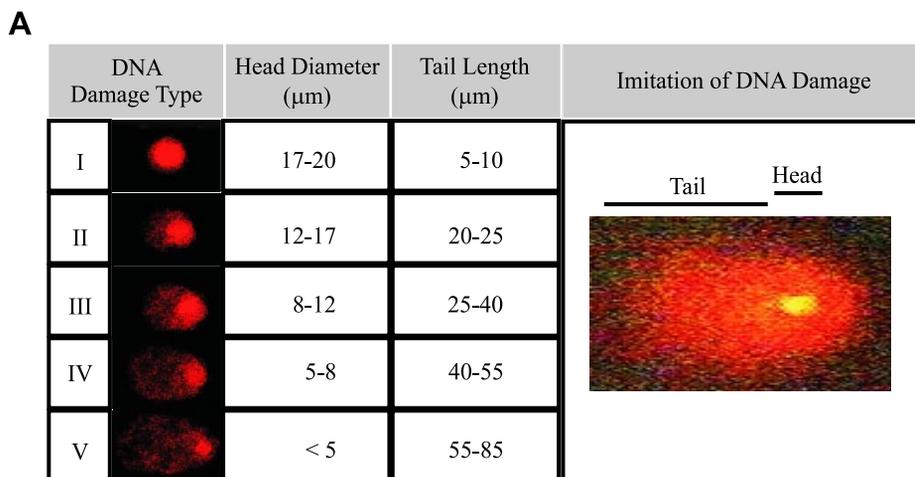
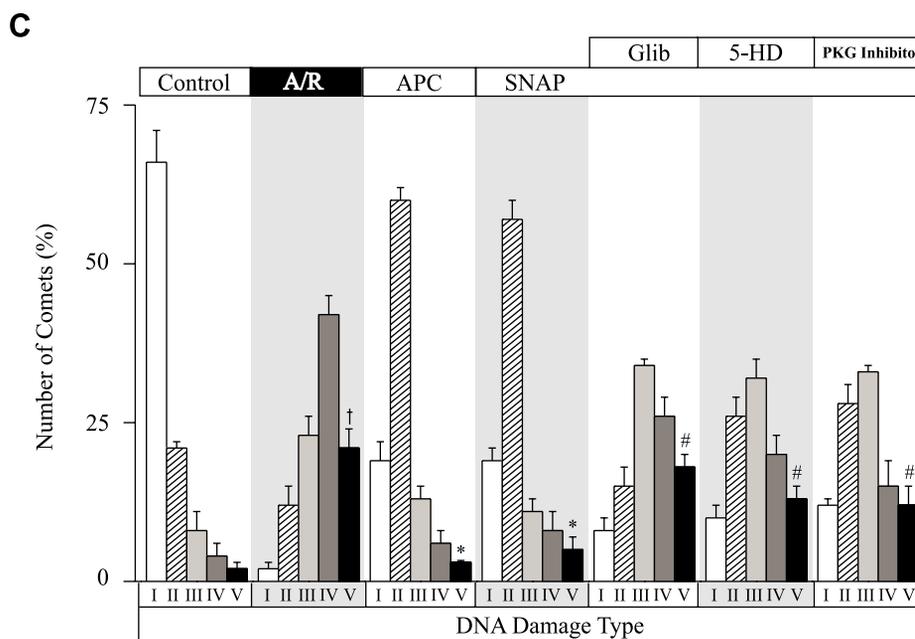
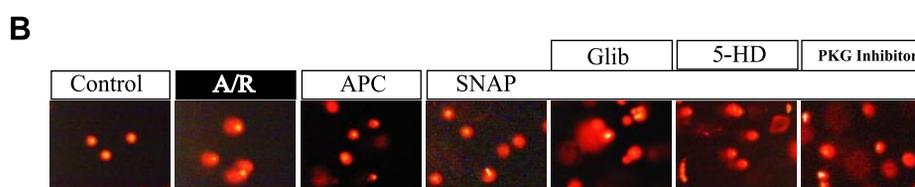


Fig. 8. Results of comet assay of DNA damage. DNA was stained with propidium iodide and examined under a fluorescence microscope. *A*: DNA damage was categorized into 5 levels (types I-V) based on comet head and tail length (see MATERIALS AND METHODS). Preconditioning of myocytes with SNAP + Glib and 5-HD increased A/R-induced DNA damage. A PKG inhibitor (Rp-8-Br-PET-cGMPS) also increased injury (*C*). *B*: propidium-iodide-stained DNA in each of the experimental conditions depicted in *C*. Values are means ± SE. †*P* < 0.05 compared with time control; **P* < 0.05 compared with A/R; #*P* < 0.05 compared with APC and SNAP.



resulted in higher levels of DNA damage in myocytes. When compared with APC, anoxic preconditioning in the presence of the PKG inhibitor Rp-8-Br-PET-cGMPS (1 μM) enhanced DNA damage of type IV (5.7 ± 2 vs. 12 ± 3.9% in the absence and presence of Rp-8-Br-PET-cGMPS) and type V (3.1 ± 0.3 vs. 6.6 ± 2.8% in the absence and presence of Rp-8-Br-PET-cGMPS) (Fig. 8C).

DISCUSSION

A massive increase in reactive oxygen species (ROS) production during the early stage of oxygenated reperfusion that follows prolonged ischemia has been suggested to form excessive amounts of free radical species. Free radicals are the main

cause of double-stranded DNA breaks and oxidization of DNA bases with consequent apoptosis or necrosis (2, 4, 18). APC (as well as several agents that mimic APC) reduces injury of the heart that is caused by A/R (16, 28). Although several recent studies have been carried out to elucidate the roles of NO, PKG, and K_{ATP} channels in the mechanism of cardioprotection during ischemia and reperfusion (I/R) (15, 23, 25, 38), many pathological features of I/R and the relationship between A/R (I/R in vitro) and APC remain unclear. In the present study, we used an in vitro model of I/R in rat hearts to quantify A/R-induced damage and assessed the roles of the NO/PKG signal transduction pathway and associated K_{ATP} channels in protecting myocytes against A/R-induced damage. Therefore, up-

stream regulation induced by either iNOS or eNOS and NO-cGMP-PKG signal-induced K_{ATP} channel opening were followed in the study.

Several laboratories have examined the DNA damage caused by A/R (18, 38, 48, 59). This study, however, considered for the first time the comet assay as a tool to assess the DNA damage in cardiac myocytes. The comet assay involves alkaline-induced unwinding of DNA and the subsequent digestion of DNA by endonuclease III to allow the detection of all DNA single- and double-strand breaks (9). This highly sensitive method of quantifying DNA damage currently in use to assess DNA damage in many cell types (31, 43, 53) is used in the present study to elucidate the role of K_{ATP} channels and components of the NO/PKG signal transduction pathway in protecting myocytes from ischemic injury.

The activation of several kinases appears to be prominent within the signal transduction cascade that mediates APC, and K_{ATP} channels are thought to be the effectors because agonists of K_{ATP} channels mimic the effects of APC. It now seems likely that mitochondrial K_{ATP} channels, rather than other types of K_{ATP} channels, are the dominant players in APC (8, 13, 46). It is well known that diazoxide, which specifically opens mitochondrial K_{ATP} channels at concentration of 100 μ M, induces also ROS production via succinate dehydrogenase inhibition at higher doses (>250 μ M) (7). Diazoxide concentration in the present study decreases the rate of cell death and alters the mitochondrial redox state (27, 30, 47). In addition, Garlid et al. (16) showed that prior administration of diazoxide at a mitochondrion-specific concentration (e.g., > 100 μ M) could confer strong protection against A/R-induced heart injury. This effect was abolished by treatment with either glibenclamide or 5-HD as K_{ATP} channels blockers. Our results support these findings (e.g., A/R-induced cardiac damage was decreased by treatment of myocytes with a mitochondrial K_{ATP} channel opener and was increased by the K_{ATP} channel inhibitors glibenclamide and 5-HD). The application of both the nonspecific mitochondrial K_{ATP} channel opener pinacidil and the blocker glibenclamide, which rendered the same final results as the application of specific openers and blockers on cardiac slices, clearly indicates that although the mitochondrial K_{ATP} channel has a positive impact on cardioprotection during APC, the involvement of other kinds of K_{ATP} channels cannot be ruled out. It is thought that preconditioning cells by activating mitochondrial K_{ATP} channels can inhibit the mitochondrial transition pore during A/R episodes. This would alter the volume of the mitochondrial matrix and result in the rupturing of mitochondria or the release of cytochrome *c* (11, 28), which in turn would lead to mitochondrial dysfunction and the initiation of apoptotic events. In our previous studies on rabbit cardiomyocytes, we implicated NO in activating sarcolemmal K_{ATP} channels via a cGMP-dependent mechanism (23, 25); this pathway contributes to the protective function played by these channels during A/R. The results of the present study extend our earlier observations (25, 26) in demonstrating that both sarcolemmal and mitochondrial K_{ATP} channels are involved in this protective function.

NO has been reported to activate soluble guanylate cyclase, which leads to the production of cGMP and the activation of PKG (54, 57). Direct activation of PKG also leads to the production of ROS, an event that depends on the opening of mitochondrial K_{ATP} channels. Oldenburg et al. (44) reported

that NO, guanylate cyclase, cGMP, and PKG are key players in the opening of mitochondrial K_{ATP} channels and in the consumption of ROS in rabbit cardiomyocytes.

The decrease in LDH release and MDA formation noticed when NO donor SNAP or PKG activator Sp-8-Br-PET-cGMPS were used together with the failure of L-NAME in preventing these effects in heart slices may suggest the priority of PKG over NO as an endogenous upstream regulating element controlling NO-cGMP-PKG signal-induced K_{ATP} channel opening that leads to cardioprotection. Therefore, the results of the present study indicate that preconditioning of myocytes by exposure to either a chemical NO donor (SNAP) or a PKG activator (Sp-8-Br-PET-cGMPS) significantly reduces the amount of damage to DNA as well as LDH release and MDA formation, either by direct activation of K_{ATP} channels or via the NO/PKG signal transduction pathway. The effects of the NO donor, the PKG activator, and K_{ATP} channels openers were blocked by glibenclamide and 5-HD. Moreover, these results were consistent with the outcome of the comet assay, which indicated that activation of the NO/PKG signal transduction pathway and K_{ATP} channels reduced DNA damage.

It has been suggested that NO may have pro- and antiapoptotic actions in cells depending on its concentration (2, 8, 35). The precise role of NO in cardioprotection due to APC is unclear. Small amounts of ROS are generated during ischemia, and the production of ROS increases substantially during the first few minutes of reperfusion (5). Because ROS can combine with NO to produce reactive NO and peroxynitrate ($ONOO^-$), high NO concentration may cause oxidative stress by inactivating mitochondrial respiratory complex and reducing ATP synthesis (41). A high level of iNOS expression was reported in the myocardium of failing hearts, suggesting its role as endogenous countercurrent contributor in competing heart failure progression (45).

The observed changes in the expression of eNOS and iNOS in the present study support the idea that NO has a role during A/R oxidative stress. Consistent with our results, the time courses of up- and downregulation of different kinds of NOS isoforms were recorded in rat skeletal muscle after 0.5 h of ischemic preconditioning (45). We found that whereas eNOS expression was unaffected by APC, both iNOS expression and NO production were elevated in APC-treated cells. eNOS has been proposed to interact with β -adrenoceptors and L-type Ca^{2+} channels to attenuate the influx of Ca^{2+} into cardiomyocytes (1, 37). eNOS is also thought to play a role in protecting cells against A/R-induced injury. Our results, however, do not support these hypotheses, because in the present study, eNOS expression in myocytes did not increase in response to A/R, irrespective of whether or not the cells were exposed to anoxic preconditioning before A/R. The high level of iNOS expression in APC-treated cells in the present work suggests that APC-induced iNOS expression may play an important role in maintaining mitochondrial K_{ATP} channels in an open state during A/R, thereby protecting myocytes against oxidative stress by regulating the mitochondrial respiratory chain and balancing the levels of ROS and reactive nitrogen species during reperfusion.

In conclusion, the results of the present study confirm that A/R causes oxidative damage and DNA strand breaks. In addition, NO, PKG, and K_{ATP} channels contribute in the mechanism that underlies cardioprotection due to APC. This



study indicates the involvement of NO-cGMP-PKG signaling pathway in cardioprotective function of mitochondrial K_{ATP} channels. The results indicate the priority of PKG over the NO as upstream regulator during this signal transduction pathway that protects against cardiac injury during APC treatment. Moreover, the results expose the role of iNOS, rather than eNOS, as the major contributing NO synthase during APC treatment. In this respect, our findings may be of considerable value as a new approach in the development and discovery of new multidisciplinary pharmaceutical targets that have better protection against ischemic heart disease.

GRANTS

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