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Nitric Oxide-cGMP-Protein Kinase G Pathway Contributes to Cardioprotective Effects of ATP-Sensitive K^+ Channels in Rat Hearts

Dang Van Cuong*, Nari Kim*, Hee Cheol Cho, Euiyong Kim, and Jin Han

Department of Physiology & Biophysics, Molecular Cell Physiology Research Group, Biohealth Products Research Center, College of Medicine, Inje University, Busan 614–735, Korea

Ischemic preconditioning (IPC) has been accepted as a heart protection phenomenon against ischemia and reperfusion (I/R) injury. The activation of ATP-sensitive potassium (K_{ATP}) channels and the release of myocardial nitric oxide (NO) induced by IPC were demonstrated as the triggers or mediators of IPC. A common action mechanism of NO is a direct or indirect increase in tissue cGMP content. Furthermore, cGMP has also been shown to contribute cardiac protective effect to reduce heart I/R-induced infarction. The present investigation tested the hypothesis that K_{ATP} channels attenuate DNA strand breaks and oxidative damage in an *in vitro* model of I/R utilizing rat ventricular myocytes. We estimated DNA strand breaks and oxidative damage by mean of single cell gel electrophoresis with endonuclease III cutting sites (comet assay). In the I/R model, the level of DNA damage increased massively. Preconditioning with a single 5-min anoxia, diazoxide (100 μM), SNAP (300 μM) and 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) (100 μM) followed by 15 min reoxygenation reduced DNA damage level against subsequent 30 min anoxia and 60 min reoxygenation. These protective effects were blocked by the concomitant presence of glibenclamide (50 μM), 5-hydroxydecanoate (5-HD) (100 μM) and 8-(4-Chlorophenylthio)-guanosine-3',5'-cyclic monophosphate, Rp-isomer (Rp-8-pCPT-cGMP) (100 μM). These results suggest that NO-cGMP-protein kinase G (PKG) pathway contributes to cardioprotective effect of K_{ATP} channels in rat ventricular myocytes.

Key Words: Nitric oxide, ATP-sensitive K^+ channel, Oxidative damage, Ischemic preconditioning

INTRODUCTION

Cardiovascular disease is a major cause of disability and mortality in which ischemic heart disease remains the largest causes. A reduction in coronary blood flow can be sufficiently prolonged to result in serve damage to the myocardium which leads to cellular injury and eventually to cellular death due to apoptosis and or necrosis.

Ischemic preconditioning (IPC), in which short-terms occlusion then reperfusion of a coronary artery followed by a long-term occlusion, can blunt subsequent lethal injury of the heart (Murry et al, 1986). ATP-sensitive K^+ (K_{ATP}) channels, both sarcolemmal ($sarcK_{ATP}$) and mitochondrial ($mitoK_{ATP}$) channels, are thought to play important roles in the phenomenon of IPC in the heart as the end effectors (Dos Santos et al, 2002; Peart et al, 2002). The activation of these channels may improve recovery of regional contractile function of stunned myocardium by shortening action potential duration and attenuating membrane depolarization, thus decreasing contractility and preserving energy during ischemia (Gross et al, 2002; Ichinose et al, 2003).

Additionally, other studies indicated the increased nitric oxide (NO) production after ischemia and reperfusion (I/R) might be involved in inhibition of myocardial apoptotic pathways (Moncada et al, 1991; Csonka et al, 1999; Buchwalow et al, 2001). NO works in several mechanisms such as stimulating soluble guanylate cyclase (GC) leading to the production of cGMP and activation of protein kinase G (PKG) or direct K_{ATP} channels serine-threonin residue phosphorylation and activation (Han et al, 2002). A common action mechanism of NO is a direct or indirect increase in tissue cGMP content. The natural cardioprotection mechanism in which individual and or relational NO, cGMP, PKG and K_{ATP} channels (Vila-Petroff et al, 1999; Taimor et al, 2000) reduce oxidative damage induced by I/R is still largely unknown. Therefore, in the present study we determined the hypothesis that NO-cGMP-PKG pathways contribute to cardioprotective effect of $sarcK_{ATP}$ and $mitoK_{ATP}$ channels in rat hearts. The results gave strong evidence that both $sarcK_{ATP}$ and $mitoK_{ATP}$ channels protect I/R-induced cardiomyocytes significantly by reducing DNA strand breaks and oxidative-damages. This also was the first *in vitro* study of

Corresponding to: Jin Han, Department of Physiology & Biophysics, Molecular Cell Physiology Research Group, College of Medicine, Inje University, Busan 614-735, Korea. (Tel) 82-51-890-6714, (Fax) 82-51-894-4500, (E-mail) phyhanj@ijnc.inje.ac.kr
*These two authors contributed equally to this work.

ABBREVIATIONS: IPC, iscehmic preconditioning; I/R, ischemic reperfusion; PKG, protein kinase G; $sarcK_{ATP}$, sarcolemmal ATP-sensitive K^+ channel; $mitoK_{ATP}$ channel, mitochondrial ATP-sensitive K^+ channel.

I/R-induced DNA damage in rat hearts.

METHODS

Cardiomyocytes isolation

The cardiomyocytes were isolated from Spargue Dawley rats weighing 280±40 g as described previously (Han et al, 2002; Han et al, 2003). Briefly, the rats were anesthetized by intraperitoneal injection of pentobarbital sodium (10 mg/ml, 1 ml/1 kg body wt) and heparin (300 IU/ml). The heart was rapidly removed via thoracotomy and the aorta was cannulated. A dissected heart then was mounted on a Langendorff apparatus and perfused retrogradely with oxygenated normal Tyrode solution for 5~6 min and then was perfused with normal Ca^{++} free Tyrode solution for 5~6 min, followed by 15~25 min perfusion with Ca^{++} free Tyrode solution containing 0.01% collagenase. The heart was washed by Kraft-Bruhe (KB) solution for 5 min and removed from cannula, the atria were discarded, the ventricular walls and septum were cut into small 4~6 pieces, gently agitated in a small beaker with KB solution to obtain single cells. Isolated ventricular cells were applied into 6-wells plates and incubated at 37°C, 95% O_2−5% CO_2 for 45 min for stabilization, then were used immediately as experimental protocols.

Experimental protocols

The experimental protocols are depicted in Fig. 1A. The stable cells in multi-well plates were divided into 9 groups: preconditioning by ischemic solution (IPC, group 1), diazoxide (100 μM, group 2), s-nitroso-n-acetylpenicillamine (SNAP 300 μM, group 3) and the potent membrane permeable activator of PKG (8-pCPT-cGMP 100 μM, group 4); ischemic preconditioning with the presence of 50 μM glibenclamide (group 5), 5-hydroxydecanoate (5-HD 100 μM, group 6), the potent membrane permeable inhibitor of PKG (Rp-8-pCPT-cGMP 100 μM, group 7), non-preconditioning (I/R, group 8) and control (group 9). Except control, all other groups were subjected to 30 min in ischemic solution and 60 min oxygenated in normal Tyrode solution. Processes were kept in incubator at 37°C. Ischemic solution was nitrogenated and Tyrode solution were oxygenated for 45 min before use.

Comet assay

At the end of experimental procedures mentioned above, the cells were collected by centrifugation at 600 Xg for 3 min then mixed in LMAgarose 1% in PBS solution and placed on standard agarose pre-coated slides. The embedded cells were overnight lysed in lysis buffer to eliminate nuclear membranes, proteins, and all non-nuclei components. After that, samples were subjected to enzymatic digestion by endonuclease III for 45 min at 37°C. Then slides were placed in an ice-cold electrophoresis chamber containing electrophoresis solution for 40 min to allow DNA unwinding (pH>12.5). The electrophoresis was subsequently conducted for 40 min at constant 25V. At the end of electrophoresis, the cells were washed by neutralizing solution, stained with propidium iodide (5 μg/mL). The stained cells were kept in dark for 1 hour then analyzed under fluorescent microscope. Five types of comet, from class I (undamaged, no discernible head) to class V (almost DNA in tail, insignificant head) were distinguished (Fig. 1B). Comets were visual scored and imaged by *IMAGE Pro Plus*™ (Version 2.0, Media Cybernetics L.B, Silver Spring, MD, USA). The edge of slides was not scored to avoid atypical comet. 200~400 comets were scored for each slide (Collins et al, 2002, McKelvey-Martin et al, 1993; Singh et al 1988). The lysis solution contained: 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% Tritron X-100, pH=10 adjusted by NaOH. The electrophoresis solution contains: 0.3 M NaOH, 1 mM Na_2EDTA, pH>12.5. The components of neutralizing solution was 0.4 M Tris-Cl (pH=7.5).

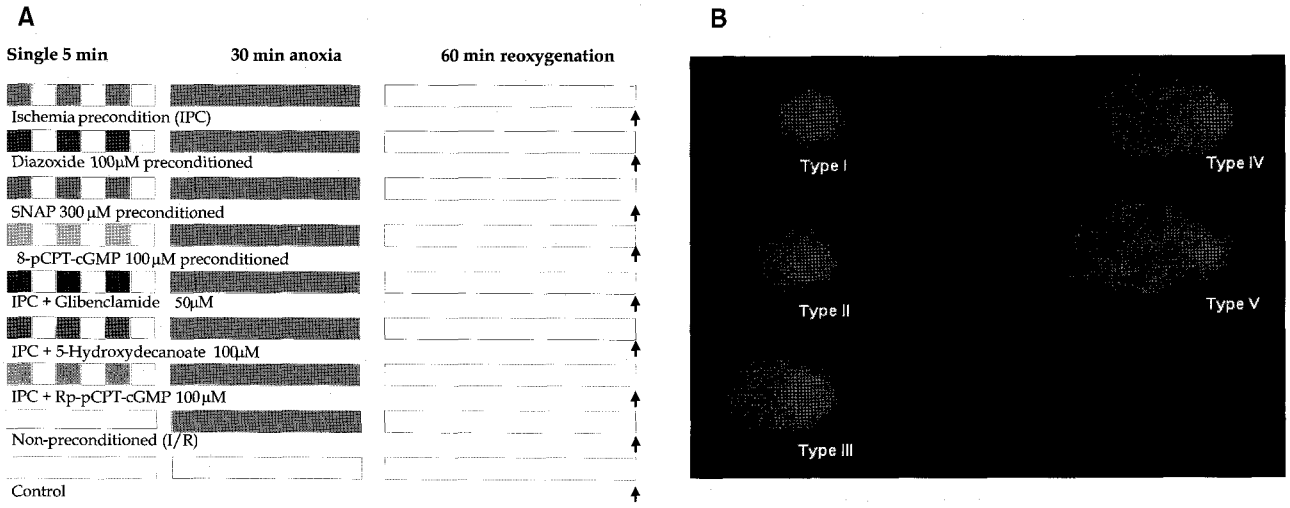

Fig. 1. (A) Experimental protocols. Cells subject to comet assay analysis were performed at the times indicated by arrowheads. All processes were kept at 37°C. (B) Imitation of DNA damage classification in comet assay (DNA strand beaks and oxidative damage were alkaline un-winded and cleaved by Endonuclease III): from type I: DNA undamaged, no discernible head, to type V: almost DNA in tail, insignificant head.

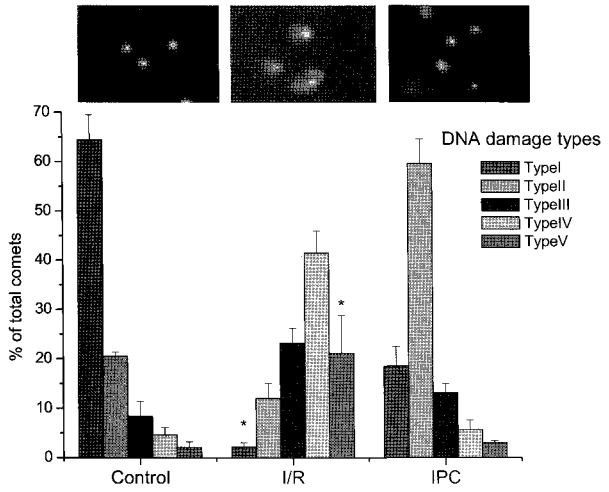

Fig. 2. The images showed the DNA damage as comet tail in Control, I/R and IPC. The percentage of each damage type representing random counting of 200~400 cells, classified into 5 types. I/R significantly increased DNA damage, especially type IV and V vs. Control; Anoxic preconditioning prior to I/R period showed protective function against I/R injury. * $p<0.05$ compare with respective damage types in Control.

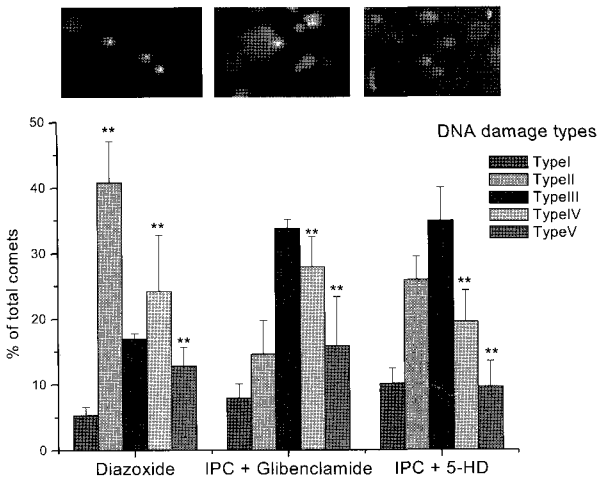

Fig. 3. Comet images scored in each treatment protocol. Glibenclamide and 5-HD abolished the effects of anoxic preconditioning. Direct activation of mitoK_{ATP} channels by diazoxide without anoxic preconditioning decreased injury. **$p<0.05$ compare with respective damage types in I/R (Data of I/R are the same in Fig. 2).

Drugs and solutions

The normal Tyrode solution contained (in mM): 143.0 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5.0 HEPES, pH= 7.4, oxygenated 45 min before used. The modified KB solution contained (in mM): 70.0 KOH, 50.0 L-glutamate, 40.0 KCl, 20.0 KH_2PO_4, 20.0 taurine, 3.0 $MgCl_2$. 10.0 HEPES, 0.5 EGTA, 10.0 glucose, pH=7.4. The ischemic solution was prepared as the following compositions (in mM): 143.0 NaCl, 8.0 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10.0 2-deoxy d-glucose, 5 HEPES, pH=7.4, deoxygenated by bubbling with 95% N_2 – 5% CO_2 for 45 min before used.

SNAP was obtained from EMD Biosciences (Darmstadt, Germany); 8-pCPT-cGMP and Rp-8-pCPT-cGMP purchased from Biog Life Science Institute (Bremen, Germany); 5-HD from Biomol Research Labs Inc. (Plymouth Meeting, PA, USA); LMAgarose from Invitrogen (Carlsbad, CA, USA); standard agarose from USB (Cleveland, Ohio, USA); Endonuclease III from New England Biolabs Inc. (Beverly, MA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA). Glibenclamide was dissolved in DMSO which in its final concentration did not exceed 0.1% that could not affect the K_{ATP} channels activity.

Data analysis

Data are shown as mean±SEM. A p value of less than 0.05 considered indicating statistically significant.

RESULTS

DNA damage increased during ischemia and reperfusion (I/R)

DNA damage type I was 64.5±5.1% of total comets in Control but decreased into 2.2±0.9% in I/R sample. This indicated that I/R induced oxidative damage significantly. DNA damage type V in I/R was 21.1±7.6%, much higher in comparison with control 2.1±1.1% (*, $p<0.05$) (Fig. 2).

Ischemia preconditioning reduced I/R-induced DNA damages

Even IPC increased DNA damage at type II compare with control, but the DNA damage in type IV and type V were only 5.7±1.9% and 3.1±0.3% compare with I/R 41.5±4.5% and 21.1±7.6%, respectively. This suggests that IPC can protect the ventricular myocytes against I/R-induced oxidative stress (Fig. 2).

Role of K_{ATP} channels on cardioprotection by reducing DNA damage level

Compare with non-precondition (I/R), preconditioning by diazoxide before I/R periods significantly reduced DNA damage type IV and V: 24.2±8.6% and 12.8±2.9% (**, $p<$ 0.05). This protection was blocked with the presence of both sarcolemmal or mitochondrial K_{ATP} channels inhibitors (glibenclamide and 5-HD). Blocking mitoK_{ATP} channels slightly increased damage type IV 19.5±4.8% and type V 9.6±3.9%, compare with blocking sarcK_{ATP} channels: 27.9±4.6% and 15.5±7.5%, respectively. It may demonstrate that both sarc-K_{ATP} and mitoK_{ATP} play protection function against I/R injury but mitoK_{ATP} channels seem to be more effective (Fig. 3).

NO donor can mimic IPC to decrease DNA oxidative damage

In the samples that were preconditioned by chemical NO donor (SNAP), even it increased DNA damage at type II but decreased damage type IV 14±7.2% and type V 7.9± 4.5%, compare with I/R (**, $p<0.05$) (Fig. 4A).

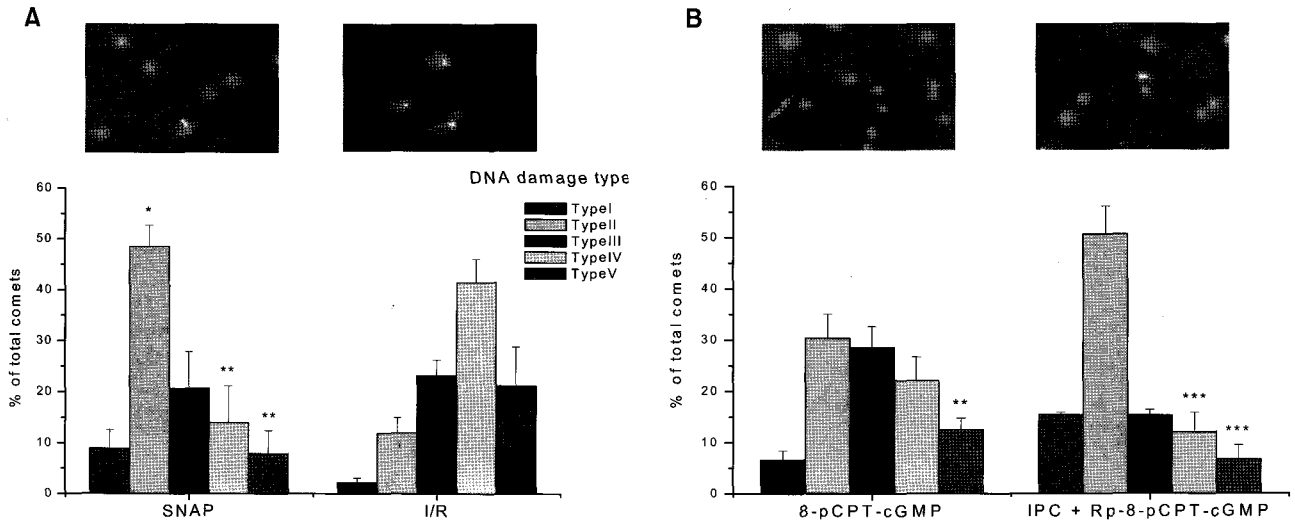

Fig. 4. Precondition by SNAP reduced DNA damage, type V was much lower than I/R (A) The activation of PKG by 8-pCPT-cGMP (100 μM) helped to increase damage at lower level type II 30.3±4.7% in comparison with I/R 12±3.0% and decreased damage type V 12.5±2.3% vs. 21.1±7.6% in I/R. Anoxic precondition with the presence of PKG inhibitor increased DNA damage, especially type IV and V compare with IPC. **p<0.05 compare with respective DNA damage types in I/R, ***p<0.05 compare with respective DNA damage types in IPC. The data of I/R shown in Fig. 2 is represented for comparison.

PKG activation decreased I/R-induced DNA damage

Direct activation of PKG by 8-pCPT-cGMP also decreased DNA damage, especially type V 12.5±2.3%, much lower than in I/R. Anoxic precondition with the presence of PKG inhibitor (Rp-8-pCPT-cGMP) increased DNA damage type IV and V: 12±3.9% and 6.7±2.8%, higher in comparison with IPC (***, $p<0.05$) (Fig. 4B).

DISCUSSION

The reoxygenated reperfusion episode after prolonged ischemia has been suggested as the main cause that leads to organs injury, cardiac infarction, apoptosis or necrosis and cell death. Recent studies demonstrated the cardioprotective function of NO, PKG and K_{ATP} channels during I/R (Fryer et al, 2001; Maejima et al, 2003; Martin et al, 2003), but the precise mechanisms are still remained a debate. In this study, by using single cardiac myocytes model, we measured the DNA strand breaks and oxidative damage to test the NO-cGMP-PKG-K_{ATP} channels signaling pathways in protection mechanism against I/R injury. There have been some papers measured DNA damages induced by I/R (Narula et al, 1996; Garlid et al, 1997), but this is the first study using comet technique, a sensitive method to visualize molecular damages in isolated rat ventricular myocytes. The damage includes single and double strands DNA breaks, DNA-products link breaks and endonuclease III cutting sites.

It has been known that activation of several kinases figures prominently in the signal transduction cascade of IPC in which K_{ATP} channels as effectors. K_{ATP} channel agonists mimic IPC in the absence of anoxic preconditioning. It seems now much more likely that mitoK_{ATP} channels are the dominant players (Carroll et al, 2001; Hanley et al, 2002). It was demonstrated that diazoxide decreased the rate of cell death and change the mitochondrial redox state (Hanley et al, 2002; Ichinose et al, 2003). Garlid and coworkers (1997) showed prior administration of diazoxide at a mitochondrial specific concentration conferred strong protection against I/R heart injury, and that this protection was abolished by either glibenclamide or 5-HD. The findings of this study were suitable with that: I/R-induced DNA damage decreased by mitoK_{ATP} channel opener and increased by both sarcK_{ATP} and mitoK_{ATP} channels inhibitor. DNA damage was increased in IPC with the presence of either glibenclamide or 5-HD. This indicated its role insights into cardioprotection mechanism. I/R have been suggested to activate mitochondrial transition pore (MTP), cause mitochondria dysfunction and release cytochrom c, thus leading to apoptosis and necrosis pathways. Activation of mitoK_{ATP} channels can inhibit MTP during I/R episode, so effect to matrix volume resulting in rupture of mitochondria (Crompton et al, 1999; Hausenloy et al, 2003). By using patch clamp technique, our previously studies in rabbit cardiomyocytes implicated that NO activates sarcK_{ATP} channels via cGMP-PKG pathways, thus may play cardioprotective function against I/R injury (Han et al, 2002). By these results, we suggest that both sarcK_{ATP} and mitoK_{ATP} channels plays roles in cardioprotective mechanism.

NO have been described to activate soluble guanylate cyclase (GC), leading to production of cGMP and activation of PKG (Vila-Petroff et al, 1999). Direct activation of PKG also leads to reactive oxygen species (ROS) production, this effects is dependence on mitoK_{ATP} channels opening. Several studies showed the role of cGMP against I/R-induced heart injury. Oldenburg and coworkers (2003) indicated NO, CG, cGMP and PKG are key players in opening mito-K_{ATP} channels and in reactive oxygen/nitrogen species production in rabbit cardiomyocytes. Our results also gave clear evidence that the preconditioning by both chemicals NO donor SNAP (300 μM) or PKG activator 8-pCPT-cGMP (100 μM) significantly decreased DNA damage. The activa-

tion of K_{ATP} channels, directly or via NO-cGMP-PKG pathways, reduced I/R injury, specially decreased DNA damage type IV and V. In addition, the inhibition of K_{ATP} channels by 5-HD increased DNA damage level higher in comparison with by glibenclamide, indicating that mitoK_{ATP} seems to play more important roles to protect the heart against I/R injury.

NO was also suggested to mimic IPC phenomenon in cardioprotective effect, involves cardiomyocytes contractile function and energy production. Cardiac NO is produced in acidic environment in a reaction catalyzed by NO synthase. Depending on its flow and concentration, NO may play as two edges of a sword: anti-apoptosis and pro-apoptosis. NO can combine with ROS to form reactive nitric oxide $NO^{\cdot}$ and peroxynitrite $ONOO^{-}$, cytotoxic reagents that cause cells damage (Beckman et al, 1996; Lefer et al, 2000; Carroll et al, 2001). In the other hand, preconditioning by NO donor prior a prolonged ischemia reduced heart injury during reperfusion. In fact, the natural mechanism in which NO plays its cardioprotection function is still unclear. Our results gave the evidence that preconditioning by SNAP at 300 μM in concentration reduced I/R-induced damages. By patch clamp technique, we previously demonstrated NO trigger to cGMP, thus activate PKG, leading to K_{ATP} channels opening. This result also supported that hypothesis. Furthermore, it gave the evidence of mitoK_{ATP} channels role in IPC phenomenon: reduced oxidative damage. By this way, mitochondria respiratory complex may be activated during ischemia episode and K_{ATP} channels play a role in balance reactive oxygen/nitrogen species during oxygenated reperfusion phase. Speculation is as that matrix alkalinization normally accompany K^+ uptake, due to imbalance between uptake K^+ and anionic equivalents, which driven by proton ejection by electron transport leading to profound alkalinization. Although there is uptake of phosphate and other substrate anions, the cytosolic concentration of these anions are far lower than that of K^+, and the imbalance results in higher matrix pH. (Radi et al, 1994)

In conclusion, our results gave the evidence of DNA strand breaks and oxidative damage induced by I/R and the individually effect of NO-cGMP-PKG-K_{ATP} channels cardioprotection mechanism. It supports to the NO-cGMP-PKG pathways that contribute to mitoK_{ATP} channels cardioprotective role. It may be a valuable reference for the strategy of pharmaceutical treatment against ischemic heart disease.

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