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Regional Differences in Mitochondrial Anti-oxidant State during Ischemic Preconditioning in Rat Heart

Vu Thi Thu, Dang Van Cuong, Nari Kim, Jae Boum Youm, Mohamad Warda, Won Sun Park, Jae Hong Ko, Euiyong Kim, and Jin Han

Mitochondrial Signaling Laboratory Mitochondria Research Group, Department of Physiology and Biophysics, Cardiovascular and Metabolic Diseases Research Center, Biohealth Products Research Center, Inje University College of Medicine, Busan 614-735, Korea

Ischemic preconditioning (IPC) is known to protect the heart against ischemia/reperfusion (IR)-induced injuries, and regional differences in the mitochondrial antioxidant state during IR or IPC may promote the death or survival of viable and infarcted cardiac tissues under oxidative stress. To date, however, the interplay between the mitochondrial antioxidant enzyme system and the level of reactive oxygen species (ROS) in the body has not yet been resolved. In the present study, we examined the effects of IR- and IPC-induced oxidative stresses on mitochondrial function in viable and infarcted cardiac tissues. Our results showed that the mitochondria from viable areas in the IR-induced group were swollen and fused, whereas those in the infarcted area were heavily damaged. IPC protected the mitochondria, thus reducing cardiac injury. We also found that the activity of the mitochondrial antioxidant enzyme system, which includes manganese superoxide dismutase (Mn-SOD), was enhanced in the viable areas compared to the infarcted areas in proportion with decreasing levels of ROS and mitochondrial DNA (mtDNA) damage. These changes were also present between the IPC and IR groups. Regional differences in Mn-SOD expression were shown to be related to a reduction in mtDNA damage as well as to the release of mitochondrial cytochrome c (Cyt c). To the best of our knowledge, this might be the first study to explore the regional mitochondrial changes during IPC. The present findings are expected to help elucidate the molecular mechanism involved in IPC and helpful in the development of new clinical strategies against ischemic heart disease.

Key Words: DNA damage, Ischemic preconditioning, Mitochondria, Oxidative stress, Superoxide

INTRODUCTION

Cardiovascular disease is a major cause of morbidity and mortality among different populations (Li & Jackson, 2002). Ischemic heart disease is an important risk factor for cardiac death (Hanley & Daut, 2005). Oxidative stress, resulting from the increased production of reactive oxygen species (ROS) in the cardiac tissues, has been implicated in the development of IR-induced injuries, which can lead to irreversible cellular damage and death (Maulik et al, 1998; Dhalla et al, 2000; Kumar et al, 2002; Faraci & Didion, 2004; Hanley & Daut, 2005; Hool, 2006). Nitric oxide (NO) reacts with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (Giordano, 2005), an active species with widespread effects, including oxidative modifications of lipids and proteins, DNA damage, mitochondrial injury, and tissue apoptosis and necrosis (Wang et al, 1999; Dhalla et al, 2000; Liu et al, 2001; Li et al, 2004; Hanley & Daut, 2005; Kim et al, 2006).

The heart contains several endogenous antioxidant systems, including the critical antioxidant enzyme Mn-SOD, which regulates the level of ROS and facilitates the formation of hydrogen peroxide (H_2O_2) from O_2^- (Friedman et al, 2004) and decreased Mn-SOD activity due to hypoxia greatly influences the steady-state concentration of mitochondrial O_2^- (Li & Jackson, 2002). Recent studies indicate that treatment with antioxidants reduces morbidity and mortality after myocardial infarction (Wang et al, 1999; Guo et al, 2001; Qin et al, 2003; Friedman et al, 2004; Hinerfeld et al, 2004; Hool, 2006). Disease states associated with vascular oxidative stress are characterized by altered vascular expression of Mn-SOD (Faraci & Didion, 2004). Overexpression of Mn-SOD in transgenic mice decreases the incidence of IR-induced injuries, as reflected by improved cardiac performance and decreases lactate dehydrogenase release (Chen et al, 1998). Furthermore, decrease in infarct size has been seen following treatment with SOD and catalase (Jolly et al, 1984). It was reported that Cyt c and Mn-SOD are lost from mitochondria during periods of ischemia (Jin et al, 2005; Qian et al, 2005;

Corresponding to: Jin Han, Mitochondrial Signaling Laboratory Mitochondria Research Group, Department of Physiology and Biophysics, Cardiovascular and Metabolic Diseases Research Center, Biohealth Products Research Center, Inje University College of Medicine, 633-165, Gaegeum- dong, Busanjin-gu, Busan 614-735, Korea. (Tel) 82-51-890-6727, (Fax) 82-51-894-5714, (E-mail) phyhanj@inje.ac.kr

ABBREVIATIONS: IR, ischemia/reperfusion; IPC, ischemic preconditioning; ROS, reactive oxygen species; mtDNA, mitochondrial DNA.

Lundberg & Szweda, 2006) and this process continues even after reperfusion, because of the presence of ROS/reactive nitrogen species (Wang et al, 1999; Beresewicz et al, 2004; Kim et al, 2006).

The number of ROS produced during reperfusion after ischemia can be reduced by IPC (Beresewicz et al, 2004; Friedman et al, 2004; Hanley & Daut, 2005; Jin et al, 2005). It has been proposed that low levels of ROS can trigger IPC by switching on cardioprotective mechanisms (Hanley & Daut, 2005). IPC improves the recovery of contractile function, reduces the infarct size, and protects the myocardium from subsequent ischemic insults (Murry et al, 1986, 1990; Shiki & Hearse, 1987; Cohen et al, 1991; Liu et al, 2001; Kato & Foex, 2002; Yellon & Downey, 2003; Zhao et al, 2003). However, the mechanisms by which IPC protects the myocardium against IR-induced injuries are not fully understood (Liu et al, 2001; Hanley & Daut, 2005; Kim et al, 2006). Moreover, the relationship between regional differences in the level of mitochondrial damage and Mn-SOD expression during O_2^--induced oxidative stress with IR and IPC has not yet been fully explained. In this study, we examined how oxidative stress affected mitochondrial function in viable and infarcted areas, evaluated mitochondrial levels of Mn-SOD expression and Cyt c release, recorded the dynamic changes in mitochondrial O_2^- during IPC and IR. These results are expected to contribute to better understanding of the mechanism involved in IPC, thus positively impacting the novel therapeutic intervention against cardiovascular disorders.

METHODS

Isolation of rat hearts

Sprague-Dawley rats (280~350 g each) were used in the study. Rats were first anesthetized with sodium pentobarbital (1 mg/kg body weight) and heparin (300 IU/ml/kg body weight). After opening the chest, the aorta was cannulated. Hearts were then rapidly excised, hung on a noncirculating apparatus (Langendorff system), and perfused with normal Tyrode's solution at 37°C for 10 to 20 min to remove the blood. Tyrode's solution was equilibrated with 95% O_2 and 5% CO_2 for 45 min prior to experiment. The isolated hearts were then subjected to experimental protocols or used to isolate individual cardiomyocytes, mtDNA, and mitochondrial proteins.

Isolation of single cardiomyocytes

Heart was mounted as described above. After a stabilization period (perfusion for 20 min with normal Tyrode's solution), the heart was perfused first with Ca^{2+}-free Tyrode's solution for 15 min followed by 0.01% collagenase in Ca^{2+}-free Tyrode's solution. The heart was then washed with an oxygenated Kraft-Brühe (KB) solution (pH 7.4) for 15 min. The atria were discarded, and the left ventricular wall and septum were cut into small pieces, followed by agitation in KB solution to obtain single cardiomyocytes (Cuong et al, 2005). The isolated cardiomyocytes were kept in KB solution at 4°C until use.

Experimental protocols

Experimental procedures are described in Fig. 1. Rats were randomly divided into three groups: control, IR, and IPC. In the control group, hearts were continuously perfused with normal Tyrode's solution for 110 min. In the IR group, hearts were perfused first with normal Tyrode's solution for 30 min, then with ischemic solution for 30 min, and finally reoxygenated for 50 min with normal Tyrode's solution. In the IPC group, hearts were subjected to twice 5 min perfusion with ischemic solution and normal Tyrode's solution prior to 30 min of ischemic treatment and 50 min of reoxygenation. Unlike the single cardiomyocytes preparations, all of hearts were then further perfused with 1% TTC (2,3,5-triphenyl tetrazolium chloride) in normal Tyrode's solution for 20 min.

TTC staining and viable/infarcted area analysis

Following treatments, hearts were stained with normal Tyrode's solution containing 1% TTC for 20 min. To calculate the infarct size and to image the infarcted area, each heart was sliced into six sections from apex to base. In viable myocardium, TTC was converted by lactic acid dehydrogenase isoenzymes into a brick-red formazan pigment, whilst infarct tissue remained unstained because of the absence of this enzyme activity (Wang et al, 1999; Zhao et al, 2003; Kim et al, 2006). The stained slices were scanned using a HP scanner, and the size of the infarct was analyzed using ImageJ software (version IJ 134; NIH, Bethesda, MD, USA). The infarcted and viable tissues were then dissected for further experiments. All procedures were carried out either on ice or in a cold room to protect against any biopolymer degradation.

Electron microscopy

Electron microscopy was used to qualitatively assess the ultrastructural changes in the mitochondria of the IR and IPC groups relative to the control. Thus, viable and infarcted tissues from control, IR, and IPC rat hearts were separated, fixed in a 2.5% glutaraldehyde in PBS solution at 4°C for 2~4 h, and then with 1% Osmium Tetroxide in PBS for 2 h. The tissues were then washed, dehydrated, embedded and sectioned (0.5~1 μm). After that, further

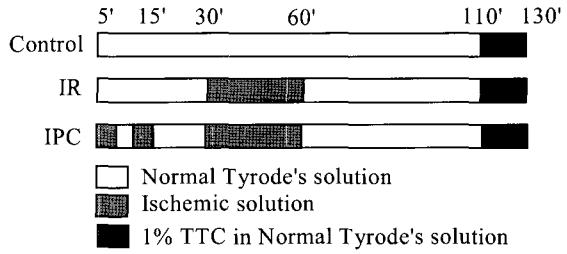

Fig. 1. Experimental procedures. Eighteen isolated rat hearts were randomly divided into three equal groups: control, IR and IPC. All hearts were stabilized by perfusion with normal Tyrode's solution for 20 min prior to the application of one of the protocols. Except for the control group, all hearts were perfused with an ischemic solution for 30 min (gray bar). The IR group was perfused for 30 min with the ischemic solution alone. In contrast, the IPC hearts were continuously perfused with ischemic and normal Tyrode's solution. This brief IR cycle was repeated before the 30-min ischemic treatment. After 110 min, all hearts were perfused with normal Tyrode's solution plus 1% TTC (black dotted bar).

ultra-section (60~90 nm) was done. The slices were double stained with uranyl acetate and lead citrate and imaged using a JEM-1200 EX2 electron microscope (Jeol, Japan). Film was developed, and pictures were scanned by a HP ScanJet 3500C.

Measurement of mitochondrial superoxide production

Fluorescence MitoSOX Red was used to measure the level of mitochondrial O_2^- in the cardiomyocytes. MitoSOX Red is a novel fluorogenic dye that is highly selective for mitochondrial O_2^-. Thus, cardiomyocytes were incubated with 5 μM MitoSOX Red for 30 min at 37°C and then washed twice with KB solution. The cells were then placed in a perfusion chamber adapted for fluorescence microscopy. Fluorescence was detected every 20 s using an Axiovert200 confocal microscope (Carl Zeiss, Jena, Germany) with a suitable filter set for excitation and emission at 510 nm and 580 nm, respectively. The images were analyzed using LSM-510 META software (Carl Zeiss).

Measurement of mtDNA fragmentation

Mitochondrial DNA was obtained using an mtDNA Extractor CT Kit (Wako Chemicals Inc., Osaka, Japan). Samples were homogenized in ice-cold homogenization buffer, and homogenates were centrifuged at 1,000×g at 4°C for 15 min. The supernatant was collected and further centrifuged at 10,000×g at 4°C for 15 min, and solutions I, II, and III were added to the pellet as per the manufacturer's instructions. The mtDNA was precipitated by the addition of NaI and isopropanol, and the concentration was measured using a spectrophotometer (ND-1000; NanoDrop, Wilmington, DE, USA).

To visualize the extent of fragmentation, 1 μg of mtDNA was loaded onto an EtBr-containing 1% agarose gel and run in 0.5× TAE buffer. To detect oxidative stress-induced damage, mtDNA was incubated with EcoRI (Promega, Madison, WI, USA) and Endonuclease III (EndoIII; New England Biolabs, Ipswich, MA, USA) at 37°C for 1 h. The digested fragments were resolved by electrophoresis, and the gels were photographed and analyzed using a LAS3000 apparatus (Fuji Film, Miyagi, Japan).

Expression of Mn-SOD and Cyt c by Western blotting

Hearts were treated as described in the experimental protocols, and the mitochondria were then isolated and purified using a discontinuous Percoll gradient method (Cuong et al, 2005; Kim et al, 2006). A mitochondrial protein extract was prepared by sonication using a Sonics Viabra Cell Apparatus (Sonics & Material, Inc., Newtown, CT, USA). The protein concentration was measured by the Bradford method using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and 40 μg of each extract was subjected to 12~16% SDS-PAGE. The proteins were then transferred to PDVF membranes, blocked for 2 h in 1% casein PBS buffer (pH 7.5~8.0), and probed with primary goat polyclonal anti-Mn-SOD and anti-Cyt c antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1 : 500 dilution for 2 h at room temperature. The membranes were then washed twice and incubated with secondary anti-goat IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at a dilution of 1 : 1,000 for 2 h at room temperature. After washing the membranes three times, the immunoreactive bands were detected using an ECL Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ, USA) and imaged by a LAS 3000 Image Reader (Fuji Film) (Kim et al, 2005). Three immunoblot experiments were performed for each group.

Statistical analysis

All data are presented as means±SEM. Student's *t*-test was used between the groups, and $p<0.05$ was considered to be significant.

RESULTS

IR increased the infarct size and induced mitochondrial swelling

Images of the TTC-stained cross-sectioned tissues are shown in Fig. 2A. The smallest infarcted area occurred in

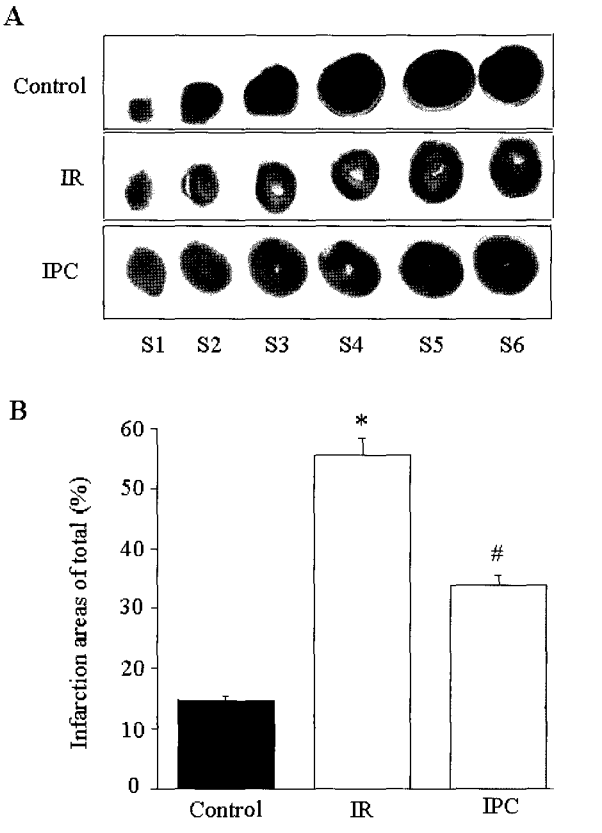

Fig. 2. Infarction size. TTC was converted by lactate dehydrogenase isoenzymes in the viable myocardium to form a red formazan pigment that stained the tissues brick-red. The infarcted areas remained pale in color. The infarction size is expressed as the percentage of the areas at risk. (A) Images of sectioned heart slices (slices 1~6) from the CT, IR, and IPC groups. (B) Mean value of infarction size calculated from total 6 slices each of heart from CT, IR and IPC group. The values are presented as means±SEM. *: $p<0.05$ compared to the control group, #: $p<0.05$ compared to the IR group.

the control group (8.54±0.92%, n=6), while the largest infarcted area was seen in the IR group (55.62±6.94%, n=6). IPC reduced the infarct size (33.83±4.21%, n=6) compared to the IR group (Fig. 2B).

As shown in Fig. 3, an electron microscopy revealed regional morphological differences in the mitochondria. The mitochondria from the control group were round and healthy, with an electron-dense matrix and tightly packed cristae. In comparison, however, the mitochondria in the viable areas of the IR tissues exhibited generalized swelling, and some fusion was observed. In the infarcted areas, the mitochondria of the IR group had disorganized cristae and disrupted membranes (Fig. 3B and D). The mitochondria sampled from the infarcted and viable areas of the IPC samples were indistinguishable from those of the control samples, with tightly packed cristae and electron-dense matrices; however, the increased intermitochondrial space and electron lucency of the IPC samples indicated an overall reduction in the number of mitochondria or cellular swelling. The mitochondria from the viable and infarcted regions of the IPC rat hearts are presented in Fig. 3C and Fig. 3E, respectively.

Mitochondrial DNA fragmentation

A significant increase of mtDNA damage was detected in both the viable and infarcted regions of the IR tissues compared to those in the IPC and control rat hearts. The severity of the damage in the IR group was evidenced by the increased smear length and brightness of the mtDNA on electrophoresis. The damage in the IR group was also shown by treatment of the mtDNA with EcoRI and EndoIII, which resulted in greater numbers of bands and the accumulation of DNA at the ends of the lanes by electrophoresis. The level of mtDNA fragmentation in the IPC group was similar to that in the control group. In fact, the

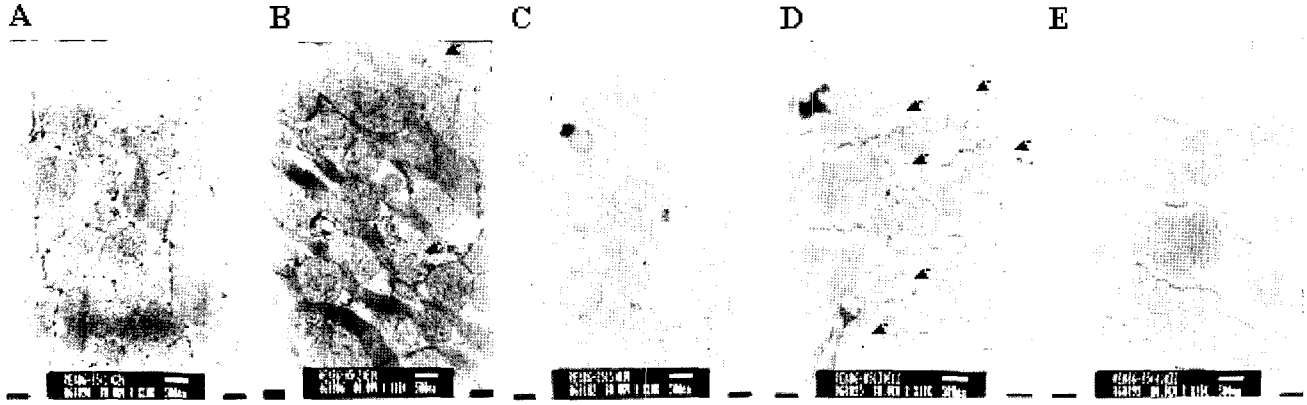

Fig. 3. Images of myocardium. The micrographs illustrate representative changes in different regions of the control, IR, and IPC rat hearts taken at a magnification of ×10 K. (A) The mitochondria in the viable region of the control tissues were round, with an electron-dense matrix and tightly packed cristae. Mitochondria in both the viable (B) and infarcted regions of the IR samples (D) were swollen, with disorganized cristae and disrupted mitochondrial membranes (black arrows). Mitochondria in both the viable (C) and infarcted regions (D) of the IPC samples were round and exhibited tightly packed cristae. Electron lucency and separation of mitochondria were also observed in the viable IPC tissues.

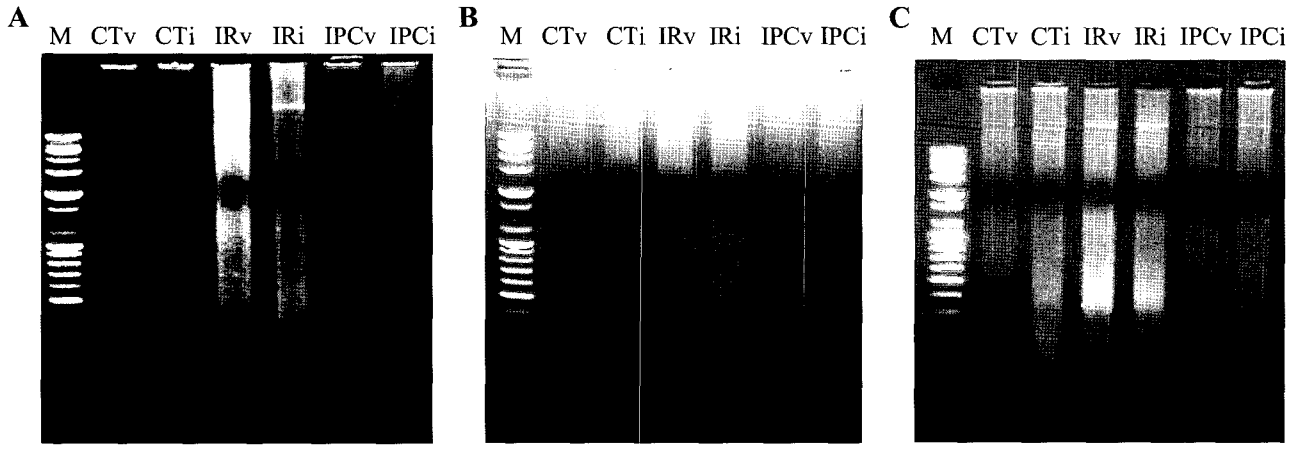

Fig. 4. mtDNA fragmentation. mtDNA from the viable and infarcted areas of the control, IR, and IPC rat hearts (n=6 for each group) was left un-treated or treated with restriction enzymes (EcoRI and EndoIII) for 1 h at 37°C. mtDNA damage was examined by 1% agarose gel electrophoresis. (A) No enzyme treatment, (B) EcoRI treatment, (C) EndoIII treatment. M: 100 bp DNA marker, CT: control, IR: ischemia/reperfusion, IPC: ischemic preconditioning, v: viable area, i: infarcted area.

IPC samples had well preserved mtDNA in the viable regions and attenuated damage in the infarcted regions. The fragmentation observed in the control, IR, and IPC groups is described in Fig. 4.

Expression of mitochondrial Mn-SOD and Cyt c

Compared to the control, the level of Mn-SOD expression in the IR and IPC groups was higher in the infarcted areas than in the viable areas (Fig. 5). Western blotting showed that the infarcted region of the control sample had a reduced Mn-SOD content relative to the viable region (79.1 ±4.99%). In comparison, however, the level of Mn-SOD in the IR group was significantly lower in both the viable (63.9 ±7.33%) and infarcted regions (65.99±10.39%). On the other hand, IPC increased the level of Mn-SOD in the viable (69.64±8.85%) and infarcted regions (88.17±9.54%), compared to IR.

The levels of mitochondrial Cyt c expression in different regions of the control, IR, and IPC rat heart samples are shown in Fig. 6. Similar to Mn-SOD, the level of Cyt c in the infarcted region of the control group was lower (81.33± 7.76%) than in the viable region. In contrast to the control, the Cyt c content in the infarcted tissues was higher than in the viable tissues for the IR and IPC rat hearts. The Cyt c was significantly decreased in both the viable (70.71± 4.12%) and infarcted tissues (74.54±0.89%) of the IR group. In contrast, the levels of Cyt c in the viable and infarcted regions of the IPC samples were increased to 82.42±5.42% and 85.19±4.51%, respectively, compared to the IR samples.

Mitochondrial superoxide production

Our results indicate that an increase in mitochondrial O_2^- production occurred during the period of IR compared to the control. The O_2^- level was stable during IPC, and then decreased during the 15-min reperfusion period. It was subsequently recovered and increased slowly during the ischemic period. Thereafter, the O_2^- level was stable, although slightly attenuated until the end of the IPC experimental period. As shown in Fig. 7, within the same period, the fluorescent intensity increased from 132±6.63% to 195.9±9.81% in the IR group and from 115.87±3.25% to 130.85±2.69% in the IPC group, indicating changes in the level of MitoSOX Red.

DISCUSSION

Oxidative stress promotes cardiomyocyte death via apoptosis or necrosis (Wei & Lee, 1998; Kumar et al, 2002). Increased formation of ROS is generally associated with oxidative stress and subsequent cardiovascular tissue injury (Dhalla et al, 2000). Elevated levels of ROS-induced

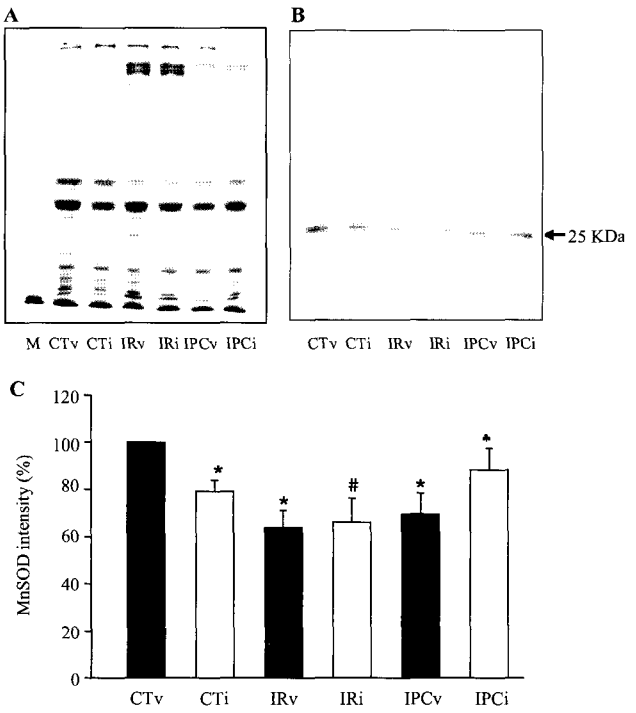

Fig. 5. Detection of Mn-SOD in mitochondria by Western blotting. SDS-PAGE (14%) for separation of the mitochondrial proteins (A), the intensity of 25-kDa immunoreactive band corresponding to Mn-SOD (B), and a histogram of Mn-SOD showing regional changes from the control (CT), ischemia/reperfusion (IR), and ischemic preconditioning (IPC) rat heart samples (C). v: viable region, i: infarcted region. *: $p<0.05$ as compared with the CTv.

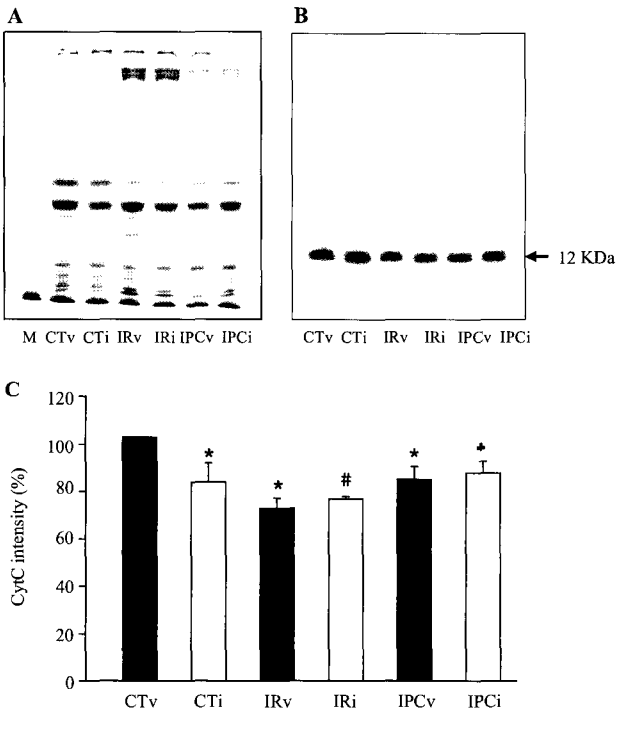

Fig. 6. Detection of mitochondrial Cyt c by Western blotting. SDS-PAGE (16%) for separation of mitochondrial proteins (A), the intensity of 12-kDa immunoreactive band that corresponds to Cyt c (B), and a histogram of Cyt c showing regional changes from the control (CT), ischemia reperfusion (IR), and ischemic preconditioning (IPC) rat heart samples (C). v: viable region, i: infarcted region. *: $p<0.05$ as compared with the CTv, #: $p<0.05$ as compared with the CTi.

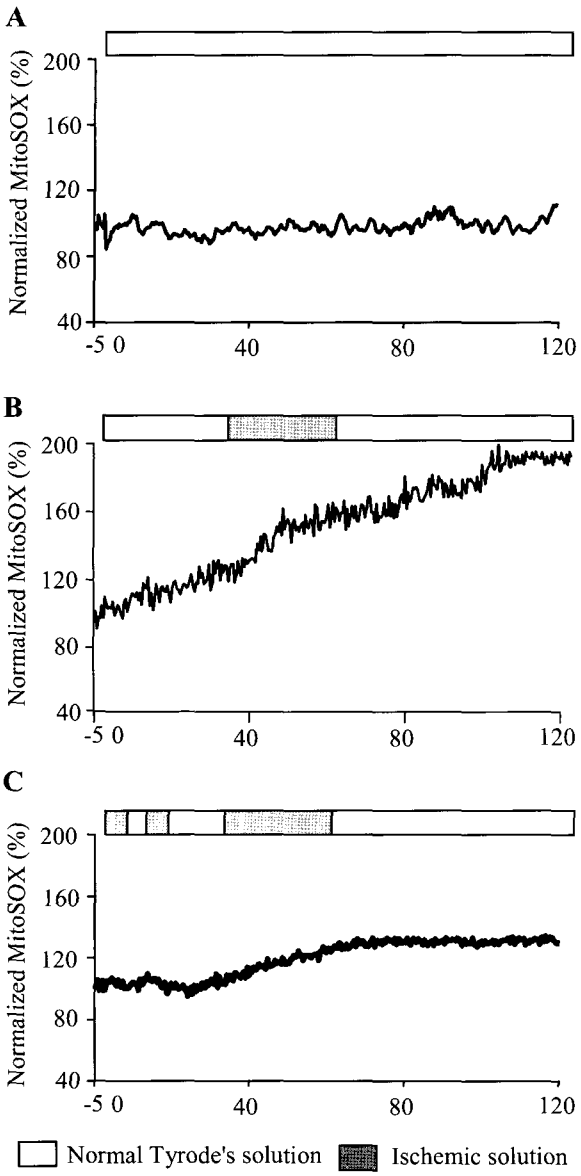

Fig. 7. Mitochondrial superoxide production. Individual cardiomyocytes were stained with MitoSOX Red. The cells were then placed in a perfusion chamber, and fluorescence was detected every 20 seconds (Ex/Em: 510/580 nm) at magnifications of ×200 and ×400 by laser scanning confocal microscopy during periods of perfusion. (A, B, and C) The changes in fluorescence for the control, IR, and IPC groups.

oxidative stress are implicated in the development of IR-related injuries (Faraci & Didion, 2004; Hanley & Daut, 2005; Hool, 2006), including damage to cell membranes, proteins, and lipids; double-stranded breaks in DNA; and oxidization of the bases in DNA (Liu et al, 2001; Hanley & Daut, 2005; Kim et al, 2006). In the early 1980s, it was discovered that increased oxidative damage and large deletions or duplications of mtDNA are associated with increased age (Wei & Lee, 1998). Furthermore, DNA damage and mutations caused by IR have been used as indicators of apoptotic cell death (Aikawa et al, 1997; Turner et al, 1998; Guo et al, 2001; Kumar et al, 2002).

The present results are consistent with those in previous reports (Turner et al, 1998; Wei & Lee, 1998; Wang et al, 1999; Guo et al, 2001; Kumar et al, 2002; Hamilton et al, 2004; Hanley & Daut, 2005; Kim et al, 2006). We found that the infarct size and level of mitochondrial damage were highest in the IR group and attenuated in the IPC group. In addition, the increase in DNA damage and decrease in the number of intact mitochondria corresponded with an elevated level of mitochondrial O_2^-. A significant increase in mitochondrial O_2^- production was observed in the IR group. Such a result was not obtained in the IPC group, which received brief and repeated cycles of IR. Different levels of mtDNA damage were detected in the regional control, IR, and IPC rat hearts. The severely oxidized and broken mtDNA observed in the IR group might have been due to the elevated level of O_2^-. Changes in the size of the mtDNA and/or an increase in the number of oxidized nucleotides, recognized by restriction enzymes, were observed under the conditions of elevated O_2^-. The level of mtDNA damage in the IPC group was higher in the infarcted tissues than in the viable regions, similar with the control. Therefore, we propose that regional IPC may mediate the protective effect of IPC against ischemic injuries in rat hearts under oxidative stress.

Furthermore, oxidative stress in cardiomyocytes might be due to an increase in the formation of ROS and/or a decrease in the antioxidant reserve (Dhalla et al, 2000). The balance between the formation and elimination of ROS by endogenous antioxidant mechanisms plays a critical role in preserving cardiac function (Jin et al, 2005; Hanley & Daut, 2005). Mn-SOD may serve as a putative stabilizer of myocardial subcellular and contractile functions during O_2^--induced oxidative stress (Faraci & Didion, 2004; Hamilton et al, 2004; Hanley & Daut, 2005). Animal studies have indicated that antioxidant treatment reduces morbidity and mortality after myocardial infarction (Wang et al, 1999; Guo et al, 2001; Friedman et al, 2004; Hool, 2006). Steady-state levels of O_2^- are dependent on the rate of O_2^- production and the level of Mn-SOD activity. A reduction in the mitochondrial level of Mn-SOD accompanied by an increase in O_2^- production has been observed in myocardial injury (Wei & Lee, 1998; Jin et al, 2005). In addition, an increase in mitochondrial Mn-SOD and a reduction in cytosolic Mn-SOD during IPC were detected in fresh and/or freeze-thawed samples (Jin et al, 2005). However, in contrast, few studies assessed directly the changes in specific antioxidant enzymes during IR-induced oxidative stress in rat hearts.

In this study, we investigated whether mitochondrial damage is related to the antioxidant status in regional heart tissues. Our data, which showed the mitochondrial expression of Mn-SOD in viable and infarcted regions of control, IR, and IPC rat hearts, are in good agreement with previous results (Faraci & Didion, 2004; Jin et al, 2005). The changes observed in Mn-SOD expression mirrored the changes in O_2^- production under insufficient oxygen conditions (i.e., in the IR and IPC rat hearts). Compared to IR, IPC reduced the level of IR-induced injuries and resulted in increased Mn-SOD and Cyt c mitochondrial expression. Taken together with the changes in mitochondrial morphology, decreased expression of Mn-SOD and Cyt c in the mitochondria strongly point to the increase of mitochondrial damage: our findings are consistent with previous results (Jin et al, 2005; Lundberg & Szweda, 2006), which showed a rapid release of Mn-SOD and Cyt

c into the cytosol following mitochondrial membrane disruption, indicating apoptotic cell death.

To the best of our knowledge, this study might be for the first time investigates the changes in mitochondrial oxidative stress and antioxidan correlation from separated regions of control, IR, and IPC rat hearts. Based on our analyses of mtDNA damage and mitochondrial morphology, we suggest that Mn-SOD activity may be inhibited in infarcted tissues, but enhanced in viable tissues. It is highly likely that the damaged mitochondria in the viable tissues of the IPC rat hearts were quickly destroyed, leaving intact mitochondria and contributing to the survival of cardiomyocytes under oxidative stress. In this study, the mitochondrial changes corresponded to an increase in O_2^-, which may explain the mechanism of injury during IR-induced oxidative stress in cardiovascular disease as well as the protective role of IPC against ischemic injuries.

In conclusion, as for the first time examination of regional changes in rat heart mitochondria, present results are expected to contribute to our understanding about the molecular mechanism of IR-induced oxidative stress as well as the protective role of ischemic preconditioning mechanism against ischemic heart disease. Nevertheless, to further evaluate the cardioprotective mechanism of IPC in greater detail, additional approaches such as DNA sequencing and proteomics should be exploited to examine the mitochondrial response to reactive oxygen species, especially mitochondrial superoxide.

ACKNOWLEDGEMENT

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REFERENCES

Aikawa R, Komuro I, Yamazaki T, Zou Y, Kudoh S, Tanaka M, Shiojima I, Hiroi Y, Yazaki Y. Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats. *J Clin Invest* 100: 1813–1821, 1997

Beresewicz A, Maczewski MC, Duda M. Effect of classic preconditioning and diazoxide on endothelial function and O_2^- and NO generation in the post-ischemic guinea-pig heart. *Cardiovas Res* 63: 118–129, 2004

Chen EP, Bittner HB, Davis RD, Van Trigt P, Folz RJ. Physiologic effects of extracellular superoxide dismutase transgene overexpression on myocardial function after ischemia and reperfusion injury. *J Thorac Cardiovasc Surg* 115: 450–458, 1998

Cohen MV, Liu GS, Downey JM. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* 84: 341–349, 1991

Cuong DV, Kim NR, Joo H, Youm YB, Chung JY, Lee YS, Park WS, Kim EY, Park YS. Subunit composition of ATP-sensitive potassium channels in mitochondria of rat hearts. *Mitochondrion* 5: 121–133, 2005

Dhalla NS, Temsah RM, Nettican T. Role of oxidative stress in cardiovascular diseases. *J Hypertens* 18: 655–673, 2000

Faraci FM, Didion SP. Vascular protection: superoxide dismutase isoform in the vessel wall. *Arterioscler Thromb Vasc Biol* 24: 1367–1373, 2004

Friedman JS, Lopez MF, Fleming MD, Rivera A, Martin FM, Welsh ML, Boyd A, Doctrow SR, Burakoff SJ. SOD2-deficiency anemia: protein oxidation and altered protein expression reveal targets of damage, stress response, and antioxidant responsiveness. *Blood* 104: 2565–2573, 2004

Giordano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest* 115: 500–508, 2005

Guo ZM, Yang H, Hamilton ML, VanRemmen H, Richardson A. Effects of age and food restriction on oxidative DNA damage and antioxidant enzyme activities in the mouse aorta. *Mech of Ageing and Dev* 122: 1771–1786, 2001

Hamilton CA, Miller WH, Al-Benna S, Brosnan MJ, Drummond RD, Mcbride MW, Dominiczak AF. Strategies to reduce oxidative stress in cardiovascular disease. *Clin Sci* 106: 219–234, 2004

Hanley PJ, Daut J. KATP channels and preconditioning: a re-examination of the role of mitochondrial KATP channels and an overview of alternative mechanisms. *J Mol Cell Cardiol* 39: 17–50, 2005

Hinerfeld D, Traini MD, Veinberger RP, Cochran B, Doctrow SR, Harry J, Mellov S. Endogenous mitochondrial oxidative stress: neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice. *J Neurochem* 88: 657–667, 2004

Hool LC. Reactive oxygen species in cardiac signaling: from mitochondria to plasma membrane ion channel. *Clin Exp Pharmacol Physiol* 33: 146–151, 2006

Jin ZQ, Zhou HZ, Cecchini G, Gray MO, Karliner JS. Mn-SOD in mouse heart: acute responses to ischemic preconditioning and ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 288: 2986–2994, 2005

Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR. Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide and catalase. *Circ Res* 54: 277–285, 1984

Kato R, Foex P. Myocardial protection by anesthetic agents against ischemia-reperfusion injury: an update for anesthesiologists. *Can J Anesth* 49: 777–791, 2002

Kim HJ, Kim NR, Joo H, Youm JB, Park YS, Warda M, Kang SH, Thu VT, Khoa TM, Jin Han. Differential activation of Ras/Raf/MAPK pathway between heart and cerebral artery in isoproterenol-induced cardiac hypertrophy. *Korean J Physiol Pharmacol* 9: 299–304, 2005

Kim NR, Lee YS, Kim HK, Joo H, Youm JB, Park WS, Warda M, Cuong DV, Han J. Potential biomarkers for ischemic heart damage identified in mitochondrial proteins by comparative proteomics. *Proteomics* 6: 1237–1249, 2006

Kumar D, Lou H, Singal PK. Oxidative stress and apoptosis in heart dysfunction. *Herz* 27: 662–668, 2002

Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia reoxygenation injury. *Am J Physiol Cell Physiol* 282: 227–241, 2002

Li F, Hayes JK, Wong KC. Gene therapy: a novel method for the treatment of myocardial ischemia and reperfusion injury mini review. *Stroke* 35: 2676–2679, 2004

Liu H, McPherson BC, Yao Z. Preconditioning attenuates apoptosis and necrosis: role of protein kinase C_ε and -γ isoforms. *Am J Physiol Heart Circ Physiol* 281: 404–410, 2001

Lundberg KC, Szweda LI. Preconditioning prevents loss in mitochondrial function and release of cytochrome *c* during prolonged cardiac ischemia/reperfusion. *Arch Biochem Biophys* 453: 128–132, 2006

Maulik N, Yoshida T, Das DK. Oxidative stress developed during the reperfusion of ischemic myocardium induces apoptosis. *Free Radic Biol Med* 24: 869–875, 1998

Murry CE, Richard VJ, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124–1136, 1986

Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slow energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 66: 913–931, 1990

Qian LP, Zhu SS, Cao JL, Zeng YM. Isoflurane preconditioning protects against ischemia-reperfusion injury partly by attenuating cytochrome c release from subsarcolemmal mitochondria in isolated rat hearts. *Acta Pharmacol Sin* 26: 813–820, 2005

Qin F, Shite J, Liang CS. Antioxidants attenuate myocyte apoptosis and improve cardiac function in CHF: association with changes in MAPK pathways. *Am J Physiol Heart Circ Physiol* 285: 822–832, 2003

Shiki K, Hearse DJ. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am J Physiol* 22: 1470–1476, 1987

Turner NA, Xia F, Azhar G, Zhang X, Liu L, Wei JY. Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells. *J Mol Cell Cardiol* 30: 1789–1801, 1998

Wang D, Yang XP, Liu YH, Carretero OA, LaPointe MC. Reduction of myocardial infarct size by inhibition of inducible nitric oxide synthase. *Am J Hypertens* 12: 174–182, 1999

Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Proc Soc Exp Biol Med* 217: 53–63, 1998

Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev* 83: 1113–1151, 2003

Zhao Z, Corvera S, Halkos ME, Kerendi F. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 285: 579–588, 2003