Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance

Prasad V. G. Katakam,1 James E. Jordan,1,2 James A. Snipes,1 Christina D. Tulbert,1 Allison W. Miller,1 and David W. Busija1

1Department of Physiology and Pharmacology and 2Department of Cardiothoracic Surgery, Wake Forest University Health Sciences, Winston-Salem, North Carolina

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Katakam PV, Jordan JE, Snipes JA, Tulbert CD, Miller AW, Busija DW. Myocardial preconditioning against ischemia-reperfusion injury is abolished in Zucker obese rats with insulin resistance. Am J Physiol Integr Comp Physiol 292: R920–R926, 2007. First published September 28, 2006; doi:10.1152/ajpregu.00520.2006.—Insulin resistance (IR) precedes the onset of Type 2 diabetes, but its impact on preconditioning against myocardial ischemia-reperfusion injury is unexplored. We examined the effects of diazoxide and ischemic preconditioning (IPC; 5-min ischemia and 5-min reperfusion) on ischemia (30 min)-reperfusion (240 min) injury in young IR Zucker obese (ZO) and lean (ZL) rats. ZO hearts developed larger infarcts than ZL hearts (infarct size: 57.3 ± 3% in ZO vs. 39.2 ± 3.2% in ZL; P < 0.05) and also failed to respond to cardioprotection by IPC or diazoxide (47.2 ± 4.3% and 52.5 ± 5.8%, respectively; P = not significant). In contrast, IPC and diazoxide treatment reduced the infarct size in ZL hearts (12.7 ± 2% and 16.3 ± 6.7%, respectively; P < 0.05). The mitochondrial ATP-activated potassium channel (KATP) antagonist 5-hydroxydecanoic acid inhibited IPC and diazoxide-induced preconditioning in ZL hearts, whereas it had no effect on ZO hearts. Diazoxide elicited reduced depolarization of isolated mitochondria from ZO hearts compared with ZL (73 ± 9% in ZL vs. 39 ± 9% in ZO; P < 0.05). Diazoxide also failed to enhance superoxide generation in isolated mitochondria from ZO compared with ZL hearts. Electron micrographs of ZO hearts revealed a decreased number of mitochondria accompanied by swelling, disorganized cristae, and vacuolation. Immunoblots of mitochondrial protein showed a modest increase in manganese superoxide dismutase in ZO hearts. Thus obesity accompanied by IR is associated with the inability to precondition against ischemic cardiac injury, which is mediated by enhanced mitochondrial oxidative stress and impaired activation of mitochondrial KATP.

mitochondrial KATP; obesity; Type 2 diabetes

INSULIN RESISTANCE (IR) is defined as a defect in the ability of insulin to stimulate glucose uptake and is characterized by impaired glucose tolerance and hyperinsulinemia (40). On the basis of the American Diabetes Association (12a) and Adult Treatment Panel III (19) criteria, approximately one-third of the healthy population (13) may be insulin resistant. Without necessary dietary and lifestyle modifications, the IR syndrome progresses to Type 2 diabetes mellitus (40). Both IR and Type 2 diabetes are known risk factors for the development of atherosclerosis and myocardial infarction (3, 20, 37). Several studies have shown that, in both humans and animals, those with diabetes have larger infarctions accompanied by decreased survival rates after myocardial infarction than their nondiabetic counterparts (5, 9, 20, 30, 32). However, the impact of IR that precedes diabetes on ischemic cardiac injury is not known.

Zucker obese (ZO) rats with leptin receptor mutation (fa/fa, homozygous for the mutation) have been widely used as models of IR and Type 2 diabetes, with Zucker lean (ZL) rats as controls (Fa/af, heterozygous for the mutation) (3). Previous data from our laboratory and others have shown that ZO rats develop IR with a metabolic profile very similar to the human condition (11, 12). We and others have also reported abnormal coronary vasoreactivity and cardiac function in ZO rats compared with ZL controls (26, 36, 38, 47). Furthermore, our group (25) has observed greater myocardial damage in ZO rats after myocardial ischemia-reperfusion injury (MI/R) than in the ZL controls (25). On the basis of the underlying metabolic derangements, coronary vascular dysfunction, and impaired tolerance to myocardial ischemia present in IR, we hypothesized that ZO rats will be resistant to the protection afforded by preconditioning against MI/R injury. Therefore, we examined the effects of ischemic and pharmacological preconditioning on MI/R in ZO rats. To determine the effects of IR associated with obesity on cardiovascular function, we studied the ZO rats at the age of 10–12 wk when they are euglycemic and largely normotensive (14, 15, 33, 45).

MATERIALS AND METHODS

The animal protocol was approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. All experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male ZO and ZL rats were obtained at 10 wk of age and housed in the animal care facility. The animals received standard rat chow and tap water.

MI/R studies. The rats aged 10–12 wk were randomized to receive one of the following treatments (Fig. 1): 1) index ischemia of 30 min followed by 4-h reperfusion, 2) normal saline as placebo, 3) ischemic preconditioning (IPC; 5-min ischemia followed by 5-min reperfusion), 4) 5-hydroxydecanoic acid (5-HD; 10 mg/kg iv) + IPC, 5) diazoxide (10 mg/kg iv), or 6) diazoxide + 5-HD. Treatments 2–6 were followed by index ischemia-reperfusion. 5-HD has been proposed as a specific blocker of mitochondrial ATP-activated potassium (KATP) channels and has been used in numerous studies to block preconditioning against MI/R injury (10, 16, 41). The rats were anesthetized with pentobarbital sodium (induction: 60 mg/kg ip; maintenance: 25 mg/kg iv, as needed) and were ventilated with 100% oxygen by a rodent ventilator adjusted to maintain exhaled CO2 between 3.5 and 4.5%. A left thoracotomy was performed, and a suture was placed around the left coronary artery. After appropriate

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treatments, index ischemia was initiated by tightening the suture around the coronary artery; reperfusion was then achieved by loosening the suture. Subsequently, infarct size was determined by differential staining and triphenyltetrazolium chloride, as described previously, with minor modifications (25), and is expressed as follows: area of necrosis/area at risk × 100.

Isolation of mitochondria. Heart mitochondria were isolated from animals that were not subjected to ischemia-reperfusion injury, using a previously described method (24). Briefly, hearts were homogenized in ice-cold isolation buffer containing (in mmol/l) 225 mannitol, 75 sucrose, 5 MOPS, 0.5 EGTA, and 2 taunine, with 0.2% BSA (pH 7.25). The homogenate was centrifuged twice at 1,000 g for 5 min (4°C), and the supernatant was then centrifuged at 10,000 g for 10 min (4°C). After the pellet was washed, it was resuspended in buffer containing (in mmol/l) 225 mannitol, 25 sucrose, 5 MOPS, 1 EGTA, 5 K2HPO4, and 2 taunine supplemented with 0.2% BSA (pH 7.4), placed on ice, and used within 3 h. Mitochondrial isolation was carried out simultaneously from a matched pair of ZO and ZL hearts.

Electron microscopy. As previously reported (39), an isolated mitochondrial pellet or a finely chopped heart tissue was fixed in 2.5% glutaraldehyde and 2% sucrose. After fixation, the tissue was washed in 0.1 M Millonig phosphate buffer, the samples were postfixed in 2% osmium tetroxide followed by dehydration in a propylene oxide and spurr resin before embedding and curing. Ultra-thin sections were cut, placed on copper grids, and stained with uranyl acetate and lead citrate. Electron microscopy was performed with a Philips TEM 400 transmission electron microscope.

Confocal microscopy. Sample mitochondria from ZO and ZL hearts were examined in blind studies (n = 5 each), one rat pair at a time and in an alternating sequence. Mitochondria placed in poly-lysine-coated glass-bottom culture dishes were studied with the use of an inverted microscope connected to a Zeiss LSM-510 laser-scanning confocal system with a Zeiss C-Apochromat ×63/numerical aperture 1.2 water-immersion objective. All experiments were performed at room temperature, and imaging conditions such as gain levels, confocal aperture size, and laser power were held constant. The average pixel intensity of mitochondria in each field (n = 30–50) was determined with Zeiss software.

Mitochondrial membrane potential was monitored with tetramethylrhodamine ethyl ester (TMRE; 100 nmol/l), and reactive oxygen species (ROS) were assessed with dihydroethidium (HEt; 5 μmol/l) (Molecular Probes, Eugene, OR) as previously described (4, 39). TMRE fluorescent images (excitation wavelength = 543 nm, emission wavelength of >560 nm) were recorded after application of vehicle or diazoxide (100 or 500 μmol/l) or 1 μmol/l CCCP. CCCP is an ionophore that dissipates the mitochondrial membrane potential by a non-ion channel mechanism leading to depolarization of the mitochondria. In contrast, K+ channel activators such as NS-1619 (calcium-activated potassium channel activator) or diazoxide (mitochondrial KATP channel activator) depolarize the mitochondria by opening K+ channels. Thus depolarization by CCCP was assessed in ZO and ZL mitochondria to compare the mitochondrial membrane potential and to evaluate the specificity of diazoxide activation of mitochondrial KATP channels. The mitochondrial depolarization was expressed as the percent decrease in average TMRE fluorescence from the average steady-state fluorescence before application of drugs. For measurement of ROS, mitochondria were treated with vehicle or diazoxide (100 or 500 μmol/l), and 5 min later, HEt was administered in the dark. HEt fluorescence images were obtained (excitation wavelength = 488 nm and emission wavelength of > 560 nm), and average peak intensity of HEt fluorescence was determined.

Fluorescent detection of mitochondrial ROS. To validate the HEt fluorescence measurements from confocal microscopy, additional experiments were performed with the use of a fluorescence spectrophotometer (BMG Labtechnologies, Durham, NC) at 37°C, with filter wavelengths of 510-nm excitation and 590-nm emission. Isolated mitochondrial samples (0.05–0.1 mg/ml) with and without diazoxide (100 and 500 μmol/l) and 5 μmol/l HEt were prepared on a 96-well plate. HEt fluorescence of mitochondria from ZL and ZO rats (on the same plate) was measured in relative fluorescence units once every 60 s for 45 cycles. Data were expressed as a percentage of the average fluorescence of untreated ZL mitochondria.

Immunoblot quantification of manganese superoxide dismutase. Equal amounts of protein from mitochondrial lysate samples were separated by 4–20% SDS-PAGE, transferred onto a polyvinylidine difluoride membrane, and blocked with 5% skim milk powder, Tris-buffered saline, and 0.1% Tween 20. Blots were incubated with monoclonal anti-manganese superoxide dismutase (MnSOD; 1:2,500; BD Transduction Laboratories). The membranes were then washed and incubated with anti-mouse IgG (1:10,000; Jackson Immuno Research) conjugated to horseradish peroxidase. The bound antibodies were visualized by enhanced chemiluminescence, and the densities of immunobands were quantitated.

Real-time PCR quantification of MnSOD. The tissue was homogenized, and total cellular RNA was extracted by an SVtotal RNA isolation system (Promega). Real-time RT-PCR was performed with the use of an ABI Prism 7700 sequence detection system (Applied Biosystems). PCR primers and probes were designed by the Primer Express program, with the forward and reverse primers located in different exons and the probe spanning an intron-exon boundary. PCR products were detected with probes labeled with fluorescent dye 6-carboxy-fluorescein at the 5’ end and quencher dye 6-carboxy-tetramethylrhodamine at the 3’ end. The 2-ΔΔCt method described by Livak and Schmittgen (29) was used to analyze the results. In brief, the threshold cycle (Ct) value of a gene was subtracted from the Ct value of a reference housekeeping gene (GAPDH) to standardize for the amounts of RNA template and efficiencies of reverse transcription. The resulting change in Ct values was then converted to a linear form by a non-ion channel mechanism leading to depolarization of the mitochondria. In contrast, K+ channel activators such as NS-1619 (calcium-activated potassium channel activator) or diazoxide (mitochondrial KATP channel activator) depolarize the mitochondria by opening K+ channels. Thus depolarization by CCCP was assessed in ZO and ZL mitochondria to compare the mitochondrial membrane potential and to evaluate the specificity of diazoxide activation of mitochondrial KATP channels. The mitochondrial depolarization was expressed as the percent decrease in average TMRE fluorescence from the average steady-state fluorescence before application of drugs. For measurement of ROS, mitochondria were treated with vehicle or diazoxide (100 or 500 μmol/l), and 5 min later, HEt was administered in the dark. HEt fluorescence images were obtained (excitation wavelength = 488 nm and emission wavelength of > 560 nm), and average peak intensity of HEt fluorescence was determined.

Effect of IPC and diazoxide on infarct size. All groups studied had a similar area of the left ventricle made ischemic after occlusion of the left coronary artery (area at risk), with no
significant differences among the groups \( [P = \text{not significant} \ (\text{NS})] \) (Table 1). In the saline (placebo) groups, the area of necrosis was significantly greater in the ZO rats \( (57.3 \pm 3\% \ n = 7) \) than in the ZL rats \( (39.2 \pm 3.2\% \ n = 9; \ P < 0.05) \) (Fig. 1). IPC decreased the area of necrosis in ZL rats \( (12.7 \pm 2\% \ n = 9; \ P < 0.05 \ \text{vs. IPC alone}) \), whereas it had no significant effect on ZO rats \( (47.2 \pm 4.3\% \ n = 8; \ P = \text{NS}) \). Administration of 5-HD before IPC partially inhibited the protective effect of IPC in ZL rats \( (27.4 \pm 3.9\% \ n = 12; \ P < 0.05 \ \text{vs. IPC alone}) \), whereas it had no effect on IPC in ZO rats \( (51.9 \pm 3.5\% \ n = 4; \ P < 0.05 \ \text{vs. IPC alone}) \). Administration of diazoxide before induction of the index ischemia reduced the area of necrosis in ZL rats \( (16.3 \pm 6.7\% \ n = 5; \ P < 0.05 \ \text{vs. untreated group}) \). In contrast, diazoxide administration had no effect on area of necrosis in ZO rats \( (52.5 \pm 5.8\% \ n = 7; \ P = \text{NS} \ \text{vs. untreated}) \). Administration of 5-HD to diazoxide-treated ZL rats before induction of the index ischemia reversed the protection afforded by diazoxide \( (41.9 \pm 2.5\% \ n = 7; \ P < 0.05 \ \text{vs. diazoxide alone}) \). In contrast, 5-HD had no effect on the area of necrosis in diazoxide-treated ZO rats \( (45 \pm 2.8\% \ n = 6; \ P = \text{NS} \ \text{vs. diazoxide alone}) \).

Morphological changes of mitochondria. The yield of mitochondrial proteins (mg/g heart wet wt) was consistent within each group of animals, suggesting minimal variation in the preparations of the mitochondrial fraction (data not shown). Electron micrograph images of isolated mitochondria established the presence of mitochondria in the preparations and validated our isolation procedures (Fig. 2A). Isolated mitochondria tend to be fragile during sample processing for electron microscopy. Therefore, we studied the morphology of in situ mitochondria in sections of the hearts. Electron micrograph images of randomly selected heart sections from ZO hearts displayed fewer and more widely dispersed mitochondria than images from ZL hearts. Also, focal morphological changes suggestive of mitochondrial stress, such as disorganized cristae and vacuolation were observed in ZO hearts (Fig. 2, B and C).

Effect of diazoxide on mitochondrial membrane potential. Diazoxide at 500 μmol/l decreased the TMRE fluorescence in isolated mitochondria from ZL hearts, indicating depolarization of mitochondria. However, the percent decrease in TMRE fluorescence after diazoxide treatment was significantly less in mitochondria from ZO hearts than from ZL hearts \( (73 \pm 9\% \ \text{in ZL mitochondria vs. } 39 \pm 9\% \ \text{in ZO mitochondria}) \), indicating a smaller percent decrease in TMRE fluorescence in ZL \( (50 \pm 3\% \ \text{and } 49 \pm 4\%; \ n = 5 \ each; \ P = \text{NS}) \) mitochondria.

Effect of diazoxide on mitochondrial ROS production. After application of HEt, isolated mitochondria exhibited fluorescence, indicating ROS generation. This baseline HEt fluorescence (relative fluorescence units) was elevated modestly in mitochondria from ZO \( (154 \pm 30) \) compared with ZL \( (101 \pm 4; \ n = 3 \ rats \ each; \ P < 0.05) \) hearts. Diazoxide at 500 μmol/l increased the HEt fluorescence in ZL mitochondria \( (209 \pm 25; \ P < 0.05) \) compared with HEt fluorescence in the absence of diazoxide. However, the ZO mitochondria did not show enhancement of HEt fluorescence after diazoxide application.
Fig. 3. Membrane potential measured by tetramethylrhodamine ethyl ester (TMRE) fluorescence and reactive oxygen species (ROS) measured by dihydroethidium (HEt) fluorescence. A: representative images of TMRE-loaded mitochondria from ZL and ZO hearts (top) and corresponding differential interference contrast (DIC) images merged with TMRE fluorescence (bottom). Diazoxide-induced depolarization of ZL mitochondria is indicated by a decrease in TMRE fluorescence. B: representative images of HEt-treated mitochondria from ZL and ZO hearts (top) and corresponding DIC images merged with HEt fluorescence (bottom). Diazoxide enhanced HEt fluorescence in ZL mitochondria, whereas ZO mitochondria were unaffected.
Fig. 4. Immunoblots of manganese superoxide dismutase (MnSOD) protein in the heart. Immunoblots identifying the Mn-SOD protein showed increased immunoband intensity in ZO mitochondria compared with ZL samples (Fig. 3B). Similar observations were made by measuring HEt fluorescence in a fluorescence plate reader in which it was possible to simultaneously study several hundred mitochondria. HEt fluorescence was significantly elevated in ZO mitochondria at baseline compared with ZL mitochondria. Normalized HEt fluorescence was $170 \pm 37\%$ in ZO compared with $101 \pm 1\%$ in ZL mitochondria. Diazoxide (100 $\mu$mol/l) increased HEt fluorescence in mitochondria to $123 \pm 5\%$ in ZO hearts ($n = 4$; $P < 0.05$), whereas it failed to elicit any response in ZO hearts ($170 \pm 38\%; n = 4$; $P = NS$). Expression of MnSOD. Immunoblots identifying the Mn-SOD protein showed increased immunoband intensity in ZO mitochondrial protein ($168 \pm 3.3$ pixels, $n = 6$; $P < 0.05$) compared with ZL samples ($133 \pm 9.6$ pixels, $n = 6$) (Fig. 4). However, the mRNA transcripts of MnSOD in the heart showed identical expression in ZO and ZL hearts ($2^{-\Delta\Delta Ct}$ in arbitrary units: $1.1 \pm 0.3 (n = 5)$ in ZL vs. $1.1 \pm 0.3 (n = 6)$ in ZO; $P = NS$).

**DISCUSSION**

The major findings of our study are as follows. 1) Hearts from ZO rats with IR sustain larger infarct sizes after MI/R injury than those from ZL rats. 2) IPC and diazoxide-induced preconditioning failed to confer cardioprotection in ZO rats compared with ZL rats. 3) Inhibition of mitochondrial K$_{ATP}$ channels abolished both diazoxide-induced preconditioning and IPC in ZL hearts, whereas it had no effect on hearts of ZO rats. 4) Diazoxide elicited significantly diminished membrane depolarization and ROS generation in isolated mitochondria from ZO hearts compared with ZL hearts. 5) Mitochondria from ZO hearts exhibit enhanced ROS production and morphological features suggestive of metabolic and oxidative stress.

Studies in our laboratory have demonstrated for the first time that hearts from IR ZO rats exhibited larger infarct area after MI/R injury than those from ZL rats (25). Recently, this observation was confirmed in Zucker diabetic fatty rats, which are genetically identical to the ZO rats used in our study, except that they develop severe hyperglycemia at an early age (27). Furthermore, these investigators showed that preconditioning does not occur in Zucker diabetic fatty rats (27). Because the ZO rats exhibit greater susceptibility to ischemic injury than ZL rats, we hypothesized that IR in the absence of hyperglycemia may also impair preconditioning against ischemic cardiac injury. IPC is a mechanism observed in almost all vital organs in which a short period of ischemia followed by reperfusion affords protection from subsequent ischemia-reperfusion injury. Several pharmacological agents such as diazoxide and anesthetics also induce preconditioning against ischemic injury. Because IPC and pharmacological preconditioning are known to share many mechanisms, we studied preconditioning by both approaches in ZO and ZL rats. Consistent with our hypothesis, we observed that IPC and diazoxide preconditioning reduced the infarct size in ZL hearts, whereas ZO rats failed to respond to preconditioning. Thus it appears that ZO hearts have lost their ability to precondition against ischemic injury.

It is widely accepted that mitochondria are the site of origin of critical signals that mediate IPC (6, 17, 21, 23, 41). Mitochondrial depolarization via opening of mitochondrial K$_{ATP}$ channels is one such important mechanism known to mediate preconditioning (6, 18). Inhibition of mitochondrial K$_{ATP}$ channels in ZL hearts with 5-HD abolished the protection conferred by both IPC and diazoxide, suggesting that opening of mitochondrial K$_{ATP}$ channels is critical to preconditioning. However, in ZO hearts, 5-HD had no effect on infarct size after IPC, suggesting impaired activation of mitochondrial K$_{ATP}$ channels. Therefore, we evaluated the opening of mitochondrial K$_{ATP}$ channels in isolated mitochondria in response to diazoxide, a prototype mitochondrial K$_{ATP}$ channel opener.

Diazoxide has been used extensively to study pharmacological preconditioning where it appears to confer cardioprotection by activating mitochondrial K$_{ATP}$ channels (6, 17). In isolated mitochondria from ZL hearts, diazoxide induced depolarization of mitochondria, indicating the opening of mitochondrial K$_{ATP}$ channels. In contrast, diazoxide elicited diminished depolarization of mitochondria from ZO hearts. Thus, mitochondrial K$_{ATP}$ channels from ZO hearts were resistant to activation by diazoxide. Interestingly, membrane depolarization in response to CCCP, an ionophore that dissipates the mitochondrial membrane potential and uncouples oxidative phosphorylation, was similar in mitochondria from both ZL and ZO hearts. This suggests that depolarization specifically mediated by mitochondrial K$_{ATP}$ channels was abnormal in mitochondria from ZO hearts. ROS have been implicated in adverse outcomes in cardiovascular disease. However, generation of ROS in the context of preconditioning has been shown to be beneficial. Enhanced generation of mitochondrial ROS in response to preconditioning has been proposed as a trigger for signaling pathways conferring cardioprotection (6, 16, 18, 21). In the present study, ROS production at baseline was elevated in mitochondria from ZO hearts, providing further evidence of a state of increased oxidative stress. Consistent with published reports (4, 22), diazoxide induced enhanced ROS generation in isolated mitochondria from ZL hearts. However, diazoxide failed to enhance ROS generation in mitochondria from ZO hearts. Thus it may be explained that increased oxidative stress at baseline causes oxidative modification of key mitochondrial enzymes and/or ion channels, leading to altered diazoxide binding and/or signaling in ZO hearts. Furthermore, the inability of diazoxide to increase ROS levels in mitochondria from ZO hearts may eliminate an essential preconditioning stimulus.

Because oxidative stress may also result from a deficiency of antioxidant mechanisms, we determined the expression of protein and mRNA of MnSOD, the primary antioxidant enzyme of mitochondria. Interestingly, we observed an increase in MnSOD protein and mRNA in ZO hearts compared with ZL samples (145 ± 36; $P = NS$) (Fig. 3B).
in MnSOD protein in the isolated mitochondrial preparations from ZO obese hearts, although mRNA levels were unchanged compared with ZL hearts. Oxidative stress has been reported to increase expression of antioxidant enzymes in several pathological models (7, 31, 42). Thus increased expression of MnSOD in ZO hearts may be an adaptation in response to oxidative stress. However, the modest increase in MnSOD in ZO hearts may account for some but not all of the reduced ROS levels with diazoxide, and it is likely that other factors are more important.

Several reports have described an association between mitochondrial oxidative injury and alterations in mitochondrial morphology (28, 34, 43). In ZO hearts, we observed a decrease in numbers of mitochondria and biogenesis, features linked to decreased capacity to generate ATP and mitochondrial stress. However, only a few scattered mitochondria with morphological correlates of increased oxidative stress, such as swelling, vacuolation, and disorganized cristae (8) were seen in ZO hearts. Thus, morphologically, ZO hearts exhibit relatively mild but definite evidence of mitochondrial dysfunction indicative of oxidative damage.

Recent reports have questioned the specificity of 5-HD as a mitochondrial K<sub>ATP</sub> channel antagonist (21). 5-HD is a well-accepted inhibitor of IPC and pharmacological preconditioning (10, 16, 41), and thus we believe that the preconditioning that we observed is a viable mechanism for myocardial protection. Moreover, there is no general agreement concerning the role of mitochondrial K<sub>ATP</sub> channels in membrane depolarization and the specific ion channels mediating preconditioning (IPC and diazoxide). Similarly, conflicting reports exist regarding the molecular structure of mitochondrial K<sub>ATP</sub> channels and the contribution of inward-rectifier potassium channels (44, 46). In the absence of ideal pharmacological probes of preconditioning, we used diazoxide, the most extensively studied agent (6, 10, 17, 34, 35) in preconditioning, although possible limitations are acknowledged. We are confident that impaired depolarization and ROS generation are two key mitochondrial defects that underlie the pathology associated with IR. Indeed, our findings in ZO rats are consistent with recent observations in patients with diabetes where isolated cardiac mitochondria from leptin-deficient ob/ob obese mice. J Endocrinol 188: 25–36, 2006.

Based on our observations, we conclude that an increased mitochondrial oxidative stress is central to the impaired preconditioning in ZO hearts. Oxidative free radicals could potentially alter the activation of mitochondrial K<sub>ATP</sub> channels by direct modification of channel proteins or alteration of membrane properties by lipid peroxidation. This could lead to diminished depolarization in response to diazoxide or ischemia and impaired preconditioning. Similarly, ROS can inhibit mitochondrial enzymes, including electron transport chain enzymes, leading to the inability to generate proton motive force across the inner mitochondrial membrane, decreased oxidative phosphorylation (ATP generation), and increased predisposition to ischemic injury.

The present study demonstrates for the first time that IR accompanying obesity impairs the ability of hearts to precondition against ischemic injury. It appears that increased mitochondrial oxidative stress in IR underlies the altered activation of mitochondrial K<sub>ATP</sub> channels and critical protective signaling pathways, leading to the inability to precondition the heart against ischemia-reperfusion injury.

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