Myocardial Preconditioning against Ischemia-Reperfusion Injury is Abolished in Zucker Obese Rats with Insulin Resistance

Prasad V.G. Katakam\textsuperscript{1}, James E. Jordan\textsuperscript{2,1}, James A. Snipes\textsuperscript{1}, Christina D. Tulbert,\textsuperscript{1} Allison W. Miller\textsuperscript{1} and David W. Busija\textsuperscript{1}.

Department of Physiology and Pharmacology\textsuperscript{1}, Department of Cardiothoracic Surgery\textsuperscript{2}, Wake Forest University Health Sciences, Winston-Salem, North Carolina 27157

\textbf{Running Title: Impaired Myocardial Preconditioning in Zucker Obese Rats}

\textbf{Correspondence:} Prasad V.G. Katakam, M.D., Ph.D.

\texttt{Dept of Physiology and Pharmacology,}
\texttt{Wake Forest University Health Sciences}
\texttt{Hanes 1050, Medical Center Blvd, Winston-Salem, NC 27157}
\texttt{336-713-4367 (phone)}
\texttt{336-716-0504 (fax)}
\texttt{pkatakam@wfubmc.edu}

Number Text Pages: 31
Number of Tables: 1
Number of Figures: 4
ABSTRACT

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 versus 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=NS). 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 K\textsubscript{ATP} antagonist, 5-hydroxydecanoic acid, inhibited ischemic and diazoxide-induced preconditioning in ZL hearts while it had no effect on ZO hearts. Diazoxide elicited reduced depolarization of isolated mitochondria from ZO hearts compared to ZL (73±9% in ZL versus 39±9% in ZO, p<0.05). Diazoxide also failed to enhance superoxide generation in isolated mitochondria from ZO compared to ZL. Electronmicrographs of ZO hearts revealed 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. Thus, obesity accompanied by IR is associated with the inability to precondition against ischemic cardiac injury that is mediated by enhanced mitochondrial oxidative stress and impaired activation of mitochondrial K\textsubscript{ATP}.

Key Words: Zucker Obese, Insulin Resistance, Preconditioning, Ischemia/Reperfusion Injury
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). Based on the American Diabetes Association (1) and Adult Treatment Panel III (19) criteria approximately a third of the healthy population (13) may be insulin resistant. Without necessary dietary and lifestyle modifications, the IR syndrome progresses to type II 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 versus their non-diabetic counterparts (5, 9, 20, 30, 32). Yet, 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 insulin resistance and type 2 diabetes with Zucker lean rats (ZL) as controls (Fa/fa, heterozygous for the mutation) (2). 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 to ZL controls (26, 36, 38, 47). Furthermore, we have observed greater myocardial damage in ZO rats following myocardial ischemia/reperfusion injury (MI/R) compared to their ZL controls (25). Based on 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. In order to determine the effects of IR
associated with obesity on cardiovascular function, we studied the ZO rats at the age of 10-12 weeks 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 weeks of age and housed in the animal care facility. The animals received standard rat chow and tap water.

Myocardial Ischemia and Reperfusion Studies. The rats aged 10-12 weeks were randomized to receive one of the following treatments (Fig. 1): 1) Index ischemia of 30 minutes followed by 4 hour reperfusion, 2) normal saline as placebo, 3) ischemic preconditioning (IPC, 5 minute ischemia followed by 5 minute reperfusion), 4) 5-hydroxydecanoic acid (5-HD, 10 mg/kg i.v.) + IPC, 5) Diazoxide (10 mg/kg i.v.) or 6) Diazoxide + 5-HD. Treatments 2-6 were followed by index ischemia/reperfusion. 5-HD has been proposed as a specific blocker of mitoKATP channels and has been used in numerous studies to block preconditioning against MI/R injury (10, 16, 41). The rats were anesthetized with sodium pentobarbital (induction: 60 mg/kg i.p; maintenance: 25 mg/kg i.v, 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 treatments, index ischemia was initiated by tightening the suture around the coronary artery and then reperfusion was achieved by loosening the suture. Subsequently, infarct size was determined by differential staining and triphenyltetrazolium chloride (TTC) as described previously, with minor modifications (25) and expressed as: area of necrosis/area at risk x100.
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): mannitol 225, sucrose 75, MOPS 5, EGTA 0.5, taurine 2, 0.2% BSA (pH 7.25). The homogenate was centrifuged twice at 1,000g for 5 minutes (4 °C) and the supernatant was then centrifuged at 10,000g for 10 minutes (4 °C). After washing, the pellet was re-suspended in buffer containing (in mmol/L): mannitol 225, sucrose 25, MOPS 5, EGTA 1, KH$_2$PO$_4$ 5, and taurine 2 supplemented with 0.2% BSA (pH = 7.4), placed on ice and used within 3 hours. Mitochondrial isolation was carried out from a matched pair of ZO and ZL hearts simultaneously.

Electron Microscopy. As previously reported (39), an isolated mitochondrial pellet or a finely chopped heart tissue was fixed in 2.5% glutaraldehyde. It is noted that the hearts were not subjected to ischemia/reperfusion injury prior to EM studies. After washing in 0.1 M Millonig phosphate buffer, the samples were postfixed in 2% osmium tetroxide followed by dehydration in a graded series of ethanol. Next, the samples were infiltrated in a propylene oxide and spurr resin before embedding and curing. Ultrathin sections were cut, placed on copper grids and stained with uranyl acetate and lead citrate. Electron microscopy was performed using 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-d-lysine coated glass bottom culture dishes were studied using an inverted microscope connected to a Zeiss LSM-510 laser scanning confocal system with a Zeiss C-Apochromat
63x/NA 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 using the Zeiss software.

Mitochondrial membrane potential was monitored using the tetramethylrhodamine ethyl ester (TMRE, 100 nmol/L) and reactive oxygen species were assessed using dihydroethidium (HEt, 5 µmol/L) (Molecular Probes, Eugene, OR) as previously described (4, 39). TMRE fluorescent images (excitation λ = 543 nm, emission λ > 560 nm) were recorded after application of vehicle or diazoxide (100 or 500 µmol/L) or carbonyl cyanide 3-chlorophenyhydrazone (CCCP, 1 µmol/L). CCCP is an ionophore that dissipates the mitochondrial membrane potential by non-ion channel mechanism leading to depolarization of the mitochondria. In contrast, K⁺ channel activators such as NS1619 (K_{Ca} activator) or diazoxide (mitoK_{ATP} 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 mitoK_{ATP} channels. The mitochondrial depolarization was expressed as the % decrease in average TMRE fluorescence from the average steady state fluorescence prior to the 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 λ = 488 nm and emission λ > 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 using 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 \( \mu \text{mol/L} \)) and 5 \( \mu \text{mol/L} \) 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. The data was expressed as % of the average fluorescence of untreated ZL mitochondria.

**Immunoblot quantification of MnSOD.** Equal amounts of protein from mitochondrial lysate samples were separated by 4-20% SDS-PAGE, transferred onto a polyvinylidene 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 using enhanced chemiluminescence and the densities of the immunobands were quantitated.

**Real time PCR quantification of MnSOD.** The tissue was homogenized and total cellular RNA was extracted using an SVtotal RNA isolation system (Promega). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR primers and probes were designed using the Primer Express program, with the forward and reserve primers located in different exons and the probe spanning an intron-exon boundary. PCR products were detected using probes labeled with reporter day FAM (6-carboxy-fluorescein) at the 5' end and quencher dye TAMRA (6-carboxy-
tetramethyl-rhodamine) at the 3' end. The $2^{-\Delta\Delta C_t}$ method described by Livak and Schmittgen (29) was used to analyze the results. In brief, the $C_t$ (threshold cycle) value of a gene was subtracted from the $C_t$ value of a reference housekeeping gene (GAPDH) to standardize for the amounts of RNA template and efficiencies of reverse transcription. The resulting change in $C_t$ values was then converted to a linear form using $2^{-\Delta C_t}$ and used in subsequent statistical analysis.

**Data Analysis.** All values are expressed as mean ± S.E.M. Analysis of variance with a Newman-Keuls post hoc test was used to determine whether any significant differences existed among groups for area at risk or infarct size. Similarly, Tukeys post hoc test was used for TMRE and HEt fluorescence data. The criteria for significance were $p<0.05$. 
RESULTS

Effect of IPC and diazoxide on Infarct Size. All groups studied had a similar area of the left ventricle made ischemic following occlusion of the left coronary artery (area at risk) with no significant difference among the groups ($p=NS$) (Table 1). In the saline/placebo groups, the area of necrosis was significantly greater in the ZO rats ($57.3 \pm 3\%$, $n=7$) versus ZL rats ($39.2 \pm 3.2\%$, $n=9; p<0.05$) (Figure 1). Ischemic preconditioning decreased the area of necrosis in ZL rats ($12.7 \pm 2\%$, $n=9; p<0.05$) while it had no significant effect on ZO rats ($47.2 \pm 4.3\%$, $n=8; p=NS$). Administration of 5-HD prior to ischemic preconditioning partially inhibited the protective effect of IPC in ZL rats ($27.4 \pm 3.9\%$, $n=12, p<0.05$ versus IPC alone) while it had no effect on IPC in ZO rats ($51.9 \pm 3.5\%$, $n=4, p<0.05$ versus IPC alone). Administration of diazoxide prior to the induction of the index ischemia reduced the area of necrosis in ZL rats ($16.3 \pm 6.7\%$, $n=5, p<0.05$ versus untreated group). In contrast, diazoxide administration had no effect on area of necrosis in ZO rats ($52.5 \pm 5.8\%$, $n=7, p=NS$ versus untreated). Administration of 5-HD to diazoxide treated-ZL rats prior to the induction of the index ischemia reversed the protection afforded by diazoxide ($41.9 \pm 2.5\%$, $n=7, p<0.05$ versus diazoxide alone). In contrast, 5-HD had no effect on area of necrosis in diazoxide treated ZO rats ($45 \pm 2.8\%$, $n=6, p=NS$ versus diazoxide alone).

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

**Effect of diazoxide on mitochondrial membrane potential.** 500 µmol/L diazoxide decreased the TMRE fluorescence in isolated mitochondria from ZL hearts indicating depolarization of mitochondria. However, the ‘% decrease in TMRE fluorescence’ following diazoxide treatment was significantly less in mitochondria from ZO hearts compared to ZL hearts (73±9% in ZL mitochondria versus 39±9% in ZO mitochondria; n=5 rats each, p<0.05) (Figure 3A). CCCP elicited similar decrease in TMRE fluorescence in ZL (50±3%) and ZO (49±4%; n=5 each, p=NS) mitochondria.

**Effect of diazoxide on mitochondrial ROS production.** Following 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±30) compared to ZL (101±4; n=3 rats each, p<0.05). 500 µmol/L diazoxide increased the HEt fluorescence in mitochondria from ZL (209±25; p<0.05) compared to HEt fluorescence in the absence of diazoxide. However, the mitochondria from ZO hearts did not show enhancement in HEt fluorescence following diazoxide application (145±36; p=NS) (Figure 3B) Similar observations were made by measuring HEt fluorescence in a fluorescence plate reader where it
was possible to simultaneously study several hundred mitochondria. HEt fluorescence was significantly elevated in ZO mitochondria at baseline compared to ZL mitochondria. Normalized HEt fluorescence was 170±37% in ZO mitochondria compared to 101±1% in ZL. Diazoxide (100 µmol/L) increased HEt fluorescence in mitochondria to 123±5% in ZL (n=4; p<0.05) while it failed to elicit any response in ZO (170±38%, n=4; p=NS).

**Expression of MnSOD.** Immunoblots identifying the MnSOD showed increased immunoband intensity in ZO mitochondrial protein (168±3.3 pixels, n=6; p<0.05) compared to ZL (133±9.6 pixels, n=6) (Figure 4). However, the mRNA transcripts of MnSOD in the heart showed identical expression in ZO and ZL \(2^{-\Delta\Delta Ct}\) in arbitrary units: 1.1±0.3, n=5, in ZL versus 1.1±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 following MI/R injury compared to ZL rats. 2) Ischemic and diazoxide induced preconditioning failed to confer cardioprotection in ZO rats compared to ZL rats. 3) Inhibition of mitoK\textsubscript{ATP} channels abolished both diazoxide induced and ischemic preconditioning in ZL hearts while it had no effect on ZO hearts. 4) Diazoxide elicited significantly diminished membrane depolarization and ROS generation in isolated mitochondria from ZO hearts compared to ZL. 5) Mitochondria from ZO hearts exhibit enhanced ROS production as well as 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 following MI/R injury compared to ZL rats (25). Recently, this observation was confirmed in Zucker diabetic fatty (ZDF) rats, which are genetically identical to 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 ZDF rats (27). Since 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. Ischemic preconditioning is a mechanism observed in almost all vital organs in which a short period of ischemia followed by reperfusion affords protection from subsequent I/R injury. Several pharmacological agents such as diazoxide and anesthetics also induce preconditioning against ischemic injury. Since ischemic 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 preconditioning with IPC and diazoxide reduced the infarct size in ZL hearts while 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, 18, 21, 23, 41). Mitochondrial depolarization via opening of mitoK\textsubscript{ATP} is one such important mechanism known to mediate preconditioning (6, 18). Inhibition of mitoK\textsubscript{ATP} channels in ZL hearts with 5-HD abolished the protection conferred by both IPC and diazoxide suggesting that opening of mitoK\textsubscript{ATP} is critical to preconditioning. However, in ZO hearts, 5-HD had no effect on infarct size following IPC suggesting impaired activation of mitoK\textsubscript{ATP} channels. Therefore, we evaluated the opening of mitoK\textsubscript{ATP} channels in isolated mitochondria in response to diazoxide, a prototype mitoK\textsubscript{ATP} opener.

Diazoxide has been used extensively to study pharmacological preconditioning where it appears to confer cardioprotection by activating mitoK\textsubscript{ATP} channels (6, 17). In isolated mitochondria from ZL hearts, diazoxide induced depolarization of mitochondria indicating the opening of mitoK\textsubscript{ATP} channels. In contrast, diazoxide elicited diminished depolarization of mitochondria from ZO hearts. Thus, the mitoK\textsubscript{ATP} 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 mitoK\textsubscript{ATP} 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/ion channels leading to altered diazoxide binding/signaling in ZO hearts. Furthermore, the inability of diazoxide to increase ROS levels in mitochondria from ZO hearts may eliminate an essential preconditioning stimulus.

Since 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 in the isolated mitochondrial preparations from ZO obese hearts although mRNA levels were unchanged compared to 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 $K_{\text{ATP}}$ channel antagonist (21). 5-HD is a well accepted inhibitor of ischemic and pharmacologic preconditioning (10, 16, 41) and thus we believe that the preconditioning we observed is a viable mechanism for myocardial protection. Moreover, there is no general agreement concerning the role of mito$K_{\text{ATP}}$ channels in membrane depolarization and the specific ion channels mediating preconditioning (IPC and diazoxide). Similarly, conflicting reports exist regarding the molecular structure of mito$K_{\text{ATP}}$ and the contribution of inward rectifier potassium channels ($K_{\text{ir}}$) (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 while acknowledging possible limitations. 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 did not depolarize or enhance ROS production in response to diazoxide (22).
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 mitoK\textsubscript{ATP} by direct modification of channel proteins or alteration of membrane properties by lipid peroxidation. This could lead to diminished depolarization in response to diazoxide/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 the hearts to precondition against ischemic injury. It appears that increased mitochondrial oxidative stress in IR underlies the altered activation of mitoK\textsubscript{ATP} channels and critical protective signaling pathways leading to the inability to precondition the heart against ischemia/reperfusion injury.
ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health (NIH) grants HL-30260, HL-50587, HL-46558, DK 62372, HL-77731, HL-65380, HL-66074 and AHA grant 0270114N. The authors appreciate the technical guidance with electron microscopy provided by Kenneth Grant of Department of Pathology, Wake Forest University Health Sciences.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Infarct size expressed as a percentage of the area at risk. (*) indicates $p<0.05$ compared with ZL-placebo treated.

**Figure 2.** Transmission electron micrographs (EM) showing the ultrastructural features of mitochondria. Panel A, isolated heart mitochondria (25000X); Panels B and C, microsections of hearts from ZO and ZL rats. Panel B: cross sections of hearts in low (3300X) and high (19500X) magnification. Mitochondria from ZO hearts exhibit vacuolation (V) and disorganized cristae (C). Panel C: longitudinal sections of hearts (5500X) from ZL and ZO rats. Focal areas of widely dispersed mitochondria were observed in ZO hearts compared to the tightly packed mitochondria in ZL hearts.

**Figure 3.** Membrane potential measured by TMRE fluorescence and ROS measured by HEt fluorescence. A, representative images of TMRE loaded mitochondria from ZL and ZO (top) and corresponding differential interference contrast (DIC) images merged with TMRE fluorescence (bottom) are shown. Diazoxide induced depolarization of ZL mitochondria, indicated by decrease in TMRE fluorescence. B, representative images of HEt treated mitochondria from ZL and ZO (top) and corresponding DIC images merged with HEt fluorescence (bottom) are shown. Diazoxide enhanced HEt fluorescence in ZL mitochondria, while ZO mitochondria were unaffected. C, representative images of HEt treated sections of ZL (left) and ZO (right) hearts and corresponding DIC images merged with HEt fluorescence (bottom) are shown. ZO hearts displayed enhanced HEt fluorescence compared to ZL hearts.
Figure 4. Immunoblots of Mn SOD were performed with the lysates of isolated mitochondria from ZL (left) and ZO (right) hearts. ZO mitochondria showed a modest increase in Mn SOD protein.
Table 1. Area at Risk (% of Left Ventricle)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index Ischemia</td>
<td>18.5±1 (n=9)</td>
<td>25.1±2.4 (n=7)</td>
</tr>
<tr>
<td>IPC</td>
<td>21.6±1 (n=9)</td>
<td>21.9±1.8 (n=8)</td>
</tr>
<tr>
<td>5-HD + IPC</td>
<td>19.8±1.2 (n=12)</td>
<td>27.1±4 (n=4)</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>23±1 (n=5)</td>
<td>25.8±2.6 (n=7)</td>
</tr>
<tr>
<td>5-HD + Diazoxide</td>
<td>22.7±2 (n=7)</td>
<td>25.3±3.5 (n=6)</td>
</tr>
</tbody>
</table>
Figure 1

Infarct Size (% area at risk)

Lean

Obese

Placebo
IPC
5HD
Diazoxide
Diazoxide+5HD

*
Figure 2

Lean
A. Isolated Mitochondria

Obese

B. Heart-Cross Section
C. Heart Longitudinal Section
Figure 3

A  
TMRE Fluorescence in Mitochondria

B  
HEt Fluorescence in Mitochondria