Cardioprotection during Diabetes

The Role of Mitochondrial DNA

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ABSTRACT

Background: Diabetes alters mitochondrial bioenergetics and consequently disrupts cardioprotective signaling. The authors investigated whether mitochondrial DNA (mtDNA) modulates anesthetic preconditioning (APC) and cardiac susceptibility to ischemia–reperfusion injury by using two strains of rats, both sharing nuclear genome of type 2 diabetes mellitus (T2DN) rats and having distinct mitochondrial genomes of Wistar and fawn-hooded hypertensive (FHH) rat strains (T2DNmtWistar and T2DNmtFHH, respectively).

Methods: Myocardial infarct size was measured in Wistar, T2DNmtWistar, and T2DNmtFHH rats with or without APC (1.4% isoﬂurane) in the presence or absence of antioxidant N-acetylcysteine. Flavoprotein fluorescence intensity, a marker of mitochondrial redox state, 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence intensity, a marker of reactive oxygen species generation, and mitochondrial permeability transition pore opening were assessed in isolated rat ventricular cardiomyocytes with or without isoﬂurane (0.5 mmol/l).

Results: Myocardial infarct size was decreased by APC in Wistar and T2DNmtWistar rats (to 42 ± 6%, n = 8; and 44 ± 7%, n = 8; of risk area, respectively) compared with their respective controls (60 ± 3%, n = 6; and 59 ± 9%, n = 7), but not in T2DNmtFHH rats (60 ± 2%, n = 8). N-acetylcysteine applied during isoﬂurane treatment restored APC in T2DNmtFHH (39 ± 6%, n = 7; and 38 ± 5%, n = 7; 150 and 75 mg/kg N-acetylcysteine, respectively), but abolished protection in control rats (54 ± 8%, n = 6). Similar to the data on infarct size, APC delayed mitochondrial permeability transition pore opening in T2DNmtWistar but not in T2DNmtFHH cardiomyocytes. Isoﬂurane increased flavoprotein and 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence intensity in all rat strains, with the greatest effect in T2DNmtFHH cardiomyocytes.

Conclusion: Differences in the mitochondrial genome modulate isoﬂurane-induced generation of reactive oxygen species which translates into differential susceptibility to APC and ischemia–reperfusion injury in diabetic rats. (Anesthesiology 2014; 120:870-9)

Evidence for definitive associations between mitochondrial DNA (mtDNA) mutations and the development of diabetes in humans is inconclusive; however, type 2 diabetes is clearly associated with dysfunction in mitochondrial biogenesis and reactive oxygen species (ROS) production. A recent analysis of the effect of the mitochondrial genome on diabetes and its complications revealed that certain European mtDNA haplogroups were associated with the development of complications of diabetes; whereas, haplogroups did not seem to play a role in the onset of diabetes. Diabetes has been shown to substantially increase the risk of cardiovascular death, and mitochondria play a central role as effectors of myocardial ischemia and reperfusion (I/R) injury and participate in cardioprotective signaling. Until now, the role of the mitochondrial genome to modulate susceptibility to I/R injury during diabetes has not been explored, in part, because of the lack of suitable animal models with mtDNA variations. Thus, the current investigation examined the hypothesis that the mitochondrial genome modulates the efficacy of anesthetic preconditioning (APC) with isoﬂurane through alterations in mitochondrial ROS production using a novel model of type 2 diabetes in rats with mtDNA variations.
Materials and Methods

Animal Model of Type 2 Diabetes
In brief, the type 2 diabetic (T2DN) models were developed by crossbreeding male Goto Kakizaki rats with female fawn-hooded hypertensive (FHH) rats.6 The Goto Kakizaki rat is a spontaneously diabetic nonobese Wistar strain with defective insulin secretion and impaired glucose tolerance, bearing Wistar mtDNA.7,8 The FHH rat is a spontaneous model of renal failure with early systemic, pulmonary, and glomerular hypertension, proteinuria, albuminuria, and development of focal and segmental glomerulosclerosis, bearing FHH mtDNA.9,10 A crossbreeding strategy was used to develop diabetic rats with mtDNA from Wistar (T2DNmtWistar) or FHH (T2DNmtFHH) rats, as previously described.11

Twelve- to 14-week-old male Wistar, T2DNmtWistar, and T2DNmtFHH rats11 were obtained from The Human and Molecular Genetics Center (Medical College of Wisconsin, Milwaukee, WI), housed in pairs, and fed a standard Purina 5001 diet (American Institute of Nutrition diet AIN-76A; Teklad, Madison, WI). Tap water was provided ad libitum. Baseline fasting glucose levels were increased in both diabetic strains, but there were no differences between T2DNmtWistar and T2DNmtFHH rats at 12 weeks of age.11 All experimental procedures used in this study were approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the Guide for the Care and Use of Laboratory Animals.

Myocardial I/R Injury In Vivo
Adult male Wistar or T2DN rats (weighing 300 to 360 g) were anesthetized with thiobutabarbital sodium (Inactin; Sigma-Aldrich, St. Louis, MO; 150 mg/kg, intraperitoneal), and additional doses of thiobutabarbital sodium (15 mg/kg) were given during the experiment (1 h after the initial dose) as needed to maintain anesthesia (movement to noxious stimulation). Body temperature was maintained between 36.8° and 37.5°C. The animals were instrumented for the measurement of systemic hemodynamics as previously described.12 In brief, heparin-filled catheters were inserted into the right jugular vein and the right carotid artery for administration of fluid and measurements of arterial blood pressure, respectively. A tracheotomy was performed, and the trachea was cannulated. Rats were ventilated with positive end-expiratory pressure by using an air-and-oxygen mixture. A thoracotomy was performed in the fifth intercostal space, and the pericardium was opened. A 6-0 prolene ligature was placed around the proximal left coronary artery and vein in the area immediately below the left atrial appendage. The ends of the suture were threaded through a propylene tube to form a snare. Coronary artery occlusion was produced by clamping the snare onto epicardial surface of the heart with a hemostat and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperemic response. Euthanasia was performed by rapidly extracting the heart. Determination of area at risk and myocardial infarct size was done, as described previously.13

Myocardial infarct size was measured in Wistar, T2DNmtWistar, and T2DNmtFHH rats subjected to 30 min of left coronary artery occlusion and 2 h of reperfusion with or without APC (isoflurane 1.0 minimum alveolar concentration) and in the presence or absence of the ROS scavenger N-acetylcysteine (NAC; 75 or 150 mg/kg in 0.9% saline intravenous; Sigma-Aldrich) in six separate experimental groups (figs. 1 and 2).

Isolation of Rat Ventricular Cardiomyocytes
Rats were anesthetized with thiobutabarbital sodium (Inactin; Sigma-Aldrich, 150 mg/kg, intraperitoneal) and hearts were rapidly extracted. Cardiomyocytes were isolated from the hearts of adult male rats by enzymatic dissociation with collagenase type II (Invitrogen, Carlsburg, CA) and protease type XIV (Sigma-Aldrich), as reported previously.14 After isolation, myocytes were resuspended and stored in Tyrode solution (NaCl 132 mmol/l, HEPES 10 mmol/l, glucose 5 mmol/l, KCl 5 mmol/l, CaCl2 1 mmol/l, and MgCl2 1.2 mmol/l; adjusted to pH 7.4) at room temperature. Cells were allowed to recover from isolation stress for 1 h, and experiments were conducted within 5 h of isolation. Only rod-shaped, quiescent cells with distinct cross-striations and preserved membrane integrity were used for the experiments. The appropriate volume of isoflurane was dispersed in experimental solution by sonication and delivered to the recording chamber from airtight glass syringes. At the end of each experiment, samples were taken from the outflow of the recording chamber, and experimental isoflurane concentrations were analyzed by gas chromatography (Gas chromatograph GC-8A; Shimadzu, Kyoto, Japan). The mean isoflurane concentration was 0.5±0.06 mmol/l, which is equivalent to 1 minimum alveolar concentration.

Laser Scanning Confocal Microscopy and Image Processing
Isolated cardiomyocytes were placed in a polycarbonate recording chamber (Warner Instruments, Hamden, CT) on a confocal microscope stage, and cells were allowed to settle and spontaneously attach to the bottom of the recording chamber for 10 min. Cells were imaged using an inverted laser-scanning confocal microscope (Nikon Eclipse TE2000-U, Nikon, Tokyo, Japan) with a 60×/1.4 oil-immersion objective (Nikon). Settings of the confocal microscope were consistent in all experimental groups. Fluorescent images were acquired using the EZ-C1 2.10 software (Nikon), and data were analyzed off-line with the MetaMorph 6.2 software (Universal Imaging, West Chester, PA). Results were expressed as percent change in fluorescence intensity relative to baseline (F/F0; where baseline = 100%).

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Mitochondrial redox state was assessed by monitoring native fluorescence intensity of mitochondrial flavoproteins. Isolated cardiomyocytes were superfused with Tyrode solution in the recording chamber and flavoprotein fluorescence (FPF) intensity was recorded by laser-scanning confocal microscopy at room temperature for 24 min, before and after isoflurane exposure for 10 min (fig. 3). The FPF was acquired at the excitation (argon laser) and emission wavelengths of 488 and 500 to 550 nm, respectively. For the purpose of statistical analyses, the mean fluorescence intensity recorded before (baseline) and during isoflurane exposure was compared.

Formation of ROS in isolated cardiomyocytes was monitored by using the ROS-sensitive indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (Invitrogen), which yields fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein upon de-esterification and oxidation by ROS. Isolated myocytes were loaded with 2 μM of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester (Invitrogen), which yields fluorescent 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein diacetate, acetyl ester for 20 min, followed by 7-min washout. Cells were superfused with Tyrode solution at room temperature, and fluorescence intensity from individual cells was monitored by confocal microscopy over 32 min, with isoflurane added at the indicated time (fig. 4). Fluorescence of 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein was excited at the 488 nm wavelength of an argon laser, and emission was collected at 500 to 550 nm by a photomultiplier tube and digitized. Neutral-density filters 4 and 8 (ND4; ND8) were used to minimize dye bleaching. The
intensity of baseline fluorescence was compared with the mean fluorescence intensity during isoflurane application.

**Mitochondrial Permeability Transition Pore Opening Measurements in Cardiomyocytes**

Mitochondrial permeability transition pore (mPTP) opening was induced with oxidative stress which was generated by photoexcitation of the fluorescent dye tetramethylrhodamine ethyl ester (TMRE; 100 nmol/l; Invitrogen) in a selected narrowly focused region of cardiomyocytes.\(^{15–18}\) Opening of the mPTP was determined by collapse of the mitochondrial membrane potential, indicated by a rapid and complete dissipation of TMRE fluorescence. TMRE fluorescence intensity was acquired using the confocal microscope at excitation (green HeNe laser) and emission wavelengths of 543 and 570 to 610 nm, respectively. After loading of TMRE for 25 min, the dye was washed out, and a 50 × 50 μm recording region of cardiomyocytes was subjected to intensive laser scanning at 3.5-s intervals (fig. 5). The ND4 filter was adjusted to minimize dye bleaching. The arbitrary mPTP opening time was determined as the time to loss of average TMRE fluorescence intensity from the recorded region (excluding nucleus) by half between initial and residual fluorescence intensity. All the confocal microscope settings, laser-scanning intensity, and initial TMRE fluorescence intensity were identical in all experimental groups.

**Statistical Analysis**

Data were expressed as mean ± SD. Comparison of several means was performed using one-way (infarct size) or two-way (hemodynamics) ANOVA, when appropriate, and the post hoc test used was the Newman–Keuls test. Hemodynamic data were analyzed with repeated measures. For cell experiments, between-group comparisons were performed by one-way ANOVA with the Bonferroni test for post hoc analysis. Changes within and between the groups were considered statistically significant if P value was less than 0.05 (two-tailed). Statistical analysis was performed by using NCSS 2007 software (Statistical Solutions, Cork, Ireland).

**Results**

**Systemic Hemodynamics**

Five experiments were excluded from the analysis as a result of technical problems with instrumentation or intractable

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**Fig. 3.** Native fluorescence of flavoproteins (FPs) depicted as an indicator of mitochondrial redox state in cardiomyocytes. (A) Experimental protocol. (B) Representative confocal microscopy images of FP fluorescence in cardiomyocytes before (Baseline), during (ISO), and after (Washout) the addition of 0.5 mmol/l isoflurane. (C) Data summary of baseline fluorescence intensity indicates no significant difference between the strains (P > 0.05). (D) Summarized data of FP fluorescence during ISO exposure and washout expressed as change from the baseline (100%). Isoflurane induced oxidation of mitochondrial FPs in cardiomyocytes isolated from type 2 diabetes mellitus (T2DN\(^{mTyr}FHH\)) rats to a larger extend than in Wistar and T2DN\(^{mTyr}Wistar\) cardiomyocytes, respectively. Data are expressed as mean ± SD, n = 6. *P < 0.05 versus baseline; #P < 0.05 versus Wistar and versus T2DN\(^{mTyr}Wistar\), a.u. = arbitrary units; FHH = fawn-hooded hypertensive; ISO = isoflurane; mt = mitochondrial; TYR = Tyrode solution.
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ventricular fibrillation during coronary artery occlusion and reperfusion (1, T2DN mtWistar; 1, T2DN mtFHH; 1, T2DN mtWistar + NAC; 1, T2DN mtFHH + NAC; and 1, T2DN mtFHH + LD NAC). There were no significant differences in baseline systemic hemodynamics among groups (table 1). APC produced similar decreases ($P < 0.05$) in heart rate (HR) and mean arterial pressure (MAP) in each rat strain. NAC alone also decreased HR and MAP in each experimental group. APC produced greater decreases in HR and MAP in NAC-treated as compared with untreated diabetic rats. Decreases in HR persisted during coronary artery occlusion in NAC-treated rats; however, there was no relationship between HR and myocardial infarct size in any experimental group (data not shown). MAP returned toward baseline values in NAC-treated rats during coronary artery occlusion although statistically significant decreases in MAP persisted in NAC-treated diabetic rats. HR recovered to baseline values after 2 h of reperfusion in Wistar rats, but this did not consistently occur in diabetic rats. MAP was decreased to a similar extent after 2 h of reperfusion in all experimental groups. As previously demonstrated, baseline blood glucose concentrations were variable, but similar, in T2DN mtWistar (218 ± 76; n = 13) and T2DN mtFHH (241 ± 105 mg/dl; n = 28) rats.

Mitochondrial Genome Defines Myocardial Susceptibility to I/R Injury

Body weight, left ventricle weight, and area at risk expressed as a percentage of left ventricle weight were similar among groups (table 2). APC significantly ($P < 0.05$) reduced myocardial infarct size (fig. 2) in both Wistar (42 ± 6% of the left ventricle area at risk, n = 8) and T2DN mtWistar rats (44 ± 7%, n = 8), as compared with control experiments (60 ± 3%, n = 6). In contrast, APC did not decrease infarct size in T2DN mtFHH rats (60 ± 2%, n = 8). The ROS scavenger NAC (150 mg/kg) alone decreased infarct size in T2DN mtFHH (44 ± 5%, n = 7) and T2DN mtWistar rats (41 ± 9%, n = 7), and also restored APC in T2DN mtFHH rats (39 ± 6%, n = 7). NAC blocked infarct size reduction during APC in non-diabetic Wistar rats (54 ± 8%, n = 6). To establish that the beneficial effect of NAC was not merely additive to APC, additional experiments were completed at a lower NAC dose (75 mg/kg) that alone had no effect on infarct size in T2DN mtFHH rats (53 ± 6%, n = 6). Low-dose NAC was equally effective to restore APC cardioprotection in T2DN mtFHH rats (38 ± 5%, n = 7).

Mitochondrial Genome Alters Mitochondrial Redox Response to Isoflurane

The mitochondrial redox state was assessed by monitoring FPF in isolated cardiomyocytes before and after the administration of isoflurane (fig. 3). Baseline FPF did not differ significantly among Wistar, T2DN mtWistar, and T2DN mtFHH cardiomyocytes (fig. 3C). Isoflurane caused an increase in FPF intensity in cardiomyocytes from all three strains, indicating mitochondrial oxidation (fig. 3D); however, isoflurane-induced
increases were significantly (P < 0.05) greater in T2DN-mtFHH (203 ± 29% of baseline, n = 6) as compared with Wistar (148 ± 22%, n = 13) or T2DNmtWistar (146 ± 29%, n = 6) cardiomyocytes. The decline in FPF to baseline values during washout was delayed in the T2DNmtFHH versus Wistar and T2DNmtWistar cardiomyocytes (fig. 3D).

Mitochondrial Genome Affects Isoflurane-induced ROS Generation
T2DNmtWistar and T2DNmtFHH cardiomyocytes exhibited greater (P < 0.05) ROS formation (higher 5-(and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence intensity) at baseline as compared with that observed in cells harvested from Wistar rats (fig. 4); however, there were no differences in ROS generation between diabetic strains at baseline. Isoflurane caused an increase in ROS generation in cardiomyocytes from all three strains. After 24 min of isoflurane exposure, increases in ROS were greatest in T2DN mtFHH (167 ± 61% of baseline, n = 14) followed by T2DN mtWistar (107 ± 28%, n = 17), and the least in Wistar (22 ± 14%, n = 19) cardiomyocytes (fig. 4D).

Mitochondrial Genome Affects APC-induced Delay in mPTP Opening during Oxidative Stress
Mitochondrial permeability transition pore opening was assessed in isolated cardiomyocytes by determining the time to mitochondrial membrane potential collapse in the presence of oxidative stress (fig. 5). APC significantly (P < 0.05) delayed mPTP opening time in T2DNmtWistar, but not in T2DN mtFHH cardiomyocytes (fig. 5, C and D).

Discussion
Alterations in mitochondrial genome clearly contribute to the extent of cardiomyocyte death after I/R through the generation of excessive quantities of ROS, calcium overload, and opening of the mPTP. Conversely, exposure to volatile anesthetic agents before ischemia (APC) decreased myocardial injury at the time of reperfusion and this effect was, in part, attributable to mitochondria and attenuation of excessive ROS formation at reperfusion. Preconditioning by isoflurane seems to trigger myocardial protection through an action on mitochondria and subsequent stimulation of prosurvival signaling pathways. In contrast, hyperglycemia and diabetes abolished the cardioprotective effects of APC, increased oxidative stress, enhanced mPTP opening through modulation of the mitochondrial electron transport chain (ETC), and abrogated mitochondria-dependent

Fig. 5. Opening of the mitochondrial permeability transition pore (mPTP; measured with the mitochondrial potentiometric dye tetramethylrhodamine [TMRE; 100 nmol/l]) after photostimulation-generated oxidative stress and assessed as rapid and complete mitochondrial depolarization in cardiomyocytes. (A) Experimental protocol. (B) Representative confocal microscopy images of TMRE fluorescence from a 50 μm² region of cardiomyocytes showing mPTP opening in individual mitochondria. (C) Arbitrary mPTP opening time expressed as

Fig. 5. (Continued) time required to decrease baseline TMRE fluorescence intensity by half (arrows). (D) Anesthetic preconditioning (APC) extended the arbitrary mPTP opening time in Wistar, type 2 diabetes mellitus (T2DNmtWistar), but not in T2DN mtFHH cardiomyocytes. * P < 0.05 versus T2DN mtFHH. a.u. = arbitrary units; FHH = fawn-hooded hypertensive; ISO = isoflurane; mt = mitochondrial; TYR = Tyrode solution.
cardioprotection. Thus, mitochondria and mitochondrial ROS production seem to function as crucial determinants of susceptibility to myocardial infarction and they are also modulated by volatile anesthetics and diabetes.

The current findings that alterations in mtDNA modulated vulnerability to I/R injury during diabetes and APC confirm and extend previous evidence from our laboratory. Differences in mitochondrial ROS production seemed to underlie strain-related differences in diabetic and nondiabetic animals. Diabetes has been shown to be associated with an increased myocardial ROS production; however, a specific link between mtDNA and susceptibility to myocardial infarction during diabetes has not previously been investigated. Although rats from both diabetic strains exhibited increased glucose levels compared with Wistar rats, only T2DNmWistar but not T2DNmFHH rats were protected by APC. The difference in APC response between the two strains was also not explained by differences in baseline cardiac function, as previous characterization demonstrated similar cardiovascular phenotype at 12 weeks of age.

The efficacy of APC to produce protection in T2DNmFHH rats was restored by the ROS scavenger NAC, at a dose that had no effect on infarct size alone. In contrast, NAC abolished APC in normal Wistar rats, confirming that small quantities of ROS are required to induce cardioprotective signal transduction during APC, whereas, excessive ROS during reperfusion contributed to I/R injury.

In vitro ROS production was greater in cardiomyocytes harvested from T2DN compared with that in Wistar rats, consistent with evidence implicating oxidative stress in the pathogenesis of myocardial injury in diabetes. Isoflurane exposure triggered ROS generation during APC via interaction with complex I of the ETC; and was greatest in cardiomyocytes from T2DNmFHH rats. T2DNmFHH rats share a similar nuclear genome

### Table 1. Systemic Hemodynamics

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Values are mean ± SD.
* Significantly (P < 0.05) different from baseline; † Significantly (P < 0.05) different from respective Wistar (Con) value; ‡ Significantly (P < 0.05) different from respective T2DNmWistar value; § Significantly (P < 0.05) different from respective T2DNmFHH value.

APC = anesthetic preconditioning; Con = control; FHH = fawn-hooded hypertensive; HR = heart rate; LD = low dose (NAC, 75 mg/kg); MAP = mean arterial pressure; mt = mitochondrial; N = number of animals; NAC = N-acetylcysteine (150 mg/kg); OCC = coronary artery occlusion; T2DN = type 2 diabetes mellitus.
with diabetic T2DNmtWistar rats, but express the mitochondrial genome from FHH rats. Stimulated ROS production was less in T2DNmtWistar as compared with T2DNmtFHH rats, but was lowest in nondiabetic Wistar rats. The mtDNA from T2DNmtFHH rats differs at several locations from that of T2DNmtWistar and Wistar rats. Amino acid modifications in ND2 and ND4, subunits of complex I of the ETC\textsuperscript{11,30} are present in T2DNmtFHH rats, and activity of complex I is mildly reduced in cardiac mitochondria from T2DNmtFHH compared with T2DNmtWistar rats. APC-induced generation of ROS has been shown to be dependent on isoflurane actions on complex I,\textsuperscript{21} and amino acid alterations in this ETC protein may be responsible for the more pronounced effect of isoflurane on ROS generation during APC in T2DNmtFHH versus T2DNmtWistar cardiomyocytes. In contrast to the predominant mtDNA differences between diabetic strains, the results may have suggested that nuclear genome effects contributed to the relatively greater ROS production in diabetic versus Wistar cardiomyocytes. Thus, isoflurane-induced ROS formation is regulated in a complex manner that depends on both mitochondrial and nuclear genome products, and is sensitive to acute antioxidant treatment.

Cardiomyocyte oxidation in response to isoflurane was also indicated by the redox state of flavoproteins and was increased in T2DNmtFHH cardiomyocytes, in parallel with pronounced ROS generation. Interestingly, isoflurane-induced flavoprotein oxidation was similar in Wistar and T2DNmtWistar cardiomyocytes. These findings suggested that isoflurane-induced differences in flavoprotein redox state were primarily related to alterations of mtDNA-coded ETC proteins; and are in agreement with previous findings that isoflurane actions on ETC complexes are direct.\textsuperscript{29} Alterations in ROS production measured with 5- (and-6)-chloromethyl-2',7'-dichlorofluorescein fluorescence are sensitive to ROS produced from multiple sites within cardiomyocytes, including extramitochondrial nicotinamide adenine dinucleotide phosphate oxidase which is encoded solely by nuclear and not mitochondrial DNA. In contrast, flavoprotein oxidation reflects the function of ETC proteins that are encoded partly by mitochondrial DNA and are indirectly affected by ROS production. Thus, the finding that flavoprotein oxidation was increased in T2DNmtFHH rats and that these rats were not sensitive to APC supports the contention that mutations in the ETC mediated by the mitochondrial genome are critical determinants of sensitivity of myocardium to protection by APC. Increased oxidation of flavoproteins is likely to be a compensatory response of the electron transfer rate during mitochondrial depolarization and uncoupling caused by isoflurane.\textsuperscript{18,31} Although mitochondrial K⁺ channels were previously suggested to mediate mitochondrial depolarization by APC and compensatory flavoprotein oxidation,\textsuperscript{27,32} more recent evidence implied direct effects of isoflurane on the ETC\textsuperscript{29} as an underlying mechanism for flavoprotein oxidation.

The opening of the mPTP plays a critical role to mediate cardiomyocyte cell death during I/R injury\textsuperscript{33,34} and mitochondria from diabetic hearts have been shown to be sensitized to mPTP opening.\textsuperscript{35} Experimental evidence also suggests that delay or prevention of mPTP opening may represent an end-effector of volatile anesthetic-induced cardioprotection.\textsuperscript{36–38} Similar to the findings in vivo, APC was effective to delay mPTP opening in T2DNmtWistar and Wistar cardiomyocytes,\textsuperscript{17,18} but had no effect in cardiomyocytes harvested from T2DNmtFHH rats. Taken together, the results indicated that mtDNA may importantly modulate the response to I/R injury in diabetes.

Although, HR was decreased by NAC during coronary artery occlusion in both T2DNmtWistar and T2DNmtFHH rats, infarct size was not related to HR or MAP in any experimental group. Thus, it is unlikely that differences in hemodynamics among the groups contributed to the observed results. In vivo experiments were performed to elucidate detailed intracellular mechanisms contributing to ROS and mPTP

### Table 2. Area at Risk for Infarction

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>BW (g)</th>
<th>LVW (g)</th>
<th>AARLV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar (Con)</td>
<td>6</td>
<td>322 ± 18</td>
<td>0.61 ± 0.05</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>Wistar + APC</td>
<td>8</td>
<td>314 ± 27</td>
<td>0.59 ± 0.11</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>Wistar + NAC</td>
<td>6</td>
<td>335 ± 13</td>
<td>0.64 ± 0.05</td>
<td>32 ± 10</td>
</tr>
<tr>
<td>Wistar + APC + NAC</td>
<td>6</td>
<td>358 ± 21</td>
<td>0.66 ± 0.08</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>T2DNmtWistar</td>
<td>7</td>
<td>315 ± 37</td>
<td>0.66 ± 0.04</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>T2DNmtWistar + APC</td>
<td>8</td>
<td>298 ± 48</td>
<td>0.61 ± 0.08</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>T2DNmtWistar + NAC</td>
<td>7</td>
<td>302 ± 30</td>
<td>0.59 ± 0.08</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>T2DNmtWistar + APC + NAC</td>
<td>7</td>
<td>225 ± 17</td>
<td>0.62 ± 0.04</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>T2DNmtFHH</td>
<td>7</td>
<td>261 ± 70</td>
<td>0.52 ± 0.1</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>T2DNmtFHH + APC</td>
<td>8</td>
<td>270 ± 65</td>
<td>0.59 ± 0.13</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>T2DNmtFHH + NAC</td>
<td>7</td>
<td>315 ± 13</td>
<td>0.59 ± 0.05</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>T2DNmtFHH + LD NAC</td>
<td>6</td>
<td>326 ± 5</td>
<td>0.63 ± 0.03</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>T2DNmtFHH + APC + NAC</td>
<td>7</td>
<td>317 ± 19</td>
<td>0.60 ± 0.02</td>
<td>44 ± 5</td>
</tr>
<tr>
<td>T2DNmtFHH + APC + LD NAC</td>
<td>7</td>
<td>314 ± 15</td>
<td>0.61 ± 0.06</td>
<td>40 ± 3</td>
</tr>
</tbody>
</table>

Values are mean ± SD.
AARLV = area at risk as a percentage of left ventricle weight; APC = anesthetic preconditioning; BW = body weight; Con = control; FHH = fawn-hooded hypertensive; LD = low dose (NAC, 75 mg/kg); LVW = left ventricle weight; mt = mitochondrial; N = number of animals; NAC = N-acetylcysteine (150 mg/kg); T2DN = type 2 diabetes mellitus.
opening in diabetic cardiomyocytes after APC. The genetic strategy used to investigate the role of mtDNA during APC and diabetes avoids the potential confounding effects of cardi- 
specific transgene manipulation; however, ubiquitous 
mtDNA alterations expressed in cells other than cardiomyo-
cytes could have contributed to the observed results.

In conclusion, the major finding of the study was that dif-
fferences in the mitochondrial genome in the two diabetic rat 
strains (T2DN\textsuperscript{mtWire} vs. T2DN\textsuperscript{mtFHH}) conferred a differential 
sensitivity to APC and susceptibility to I/R injury. This 
 altered sensitivity most likely occurred because of increased 
ROS production after I/R in the rats bearing the FHH mito-
chondrial genome. Mitochondrial ROS production and 
redox state were central in the signaling pathway modulated 
by diabetes and APC. The results also suggested that mito-
chondrial proteins could be a therapeutic target for interven-
tion during diabetes to decrease the risk of myocardial injury.

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Competing Interests
The authors declare no competing interests.

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