Attenuation of Ca\textsuperscript{2+} homeostasis, oxidative stress, and mitochondrial dysfunctions in diabetic rat heart: insulin therapy or aerobic exercise?

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Submitted 15 October 2014; accepted in final form 30 April 2015

Diabetic cardiomyopathy leads initially to diastolic dysfunction, which frequently progresses to heart failure and sudden death (12). Cardiac systolic and diastolic dysfunctions are associated with impaired intracellular calcium (Ca\textsuperscript{2+}) homeostasis (16, 23, 43, 48) attributable to decreased expression and/or activity of Ca\textsuperscript{2+} regulatory proteins (10, 16, 23, 43, 47, 48).

Heart failure induced by diabetes is also associated with increased reactive oxygen species (ROS) (19) as well as with NADPH oxidase (Nox) activation by glycolyzed proteins and mitochondrial dysfunction (47). Mitochondrial dysfunctions in diabetic hearts are related with increased expression of mitochondrial uncoupling protein-3 (UCP-3) (8, 19, 44), impaired respiratory capacity, altered expression of respiratory chain complexes (8) and Ca\textsuperscript{2+} uptake, higher susceptibility to mitochondrial permeability transition pore (MPTP) opening, and elevated apoptotic signaling molecules (8, 26). Recently, it has been demonstrated that in diabetic hearts the expression of phosphorylated Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) and Nox was augmented, and its activation by impaired Ca\textsuperscript{2+} metabolism increases ROS production (32).

Aerobic exercise and insulin replacement are strategies to manage diabetes (41, 46). Endurance exercise training was shown to improve cardiomyocyte Ca\textsuperscript{2+} cycling and restore its intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) transient and hence contractile function in diabetic rats (41). Endurance exercise is also known to protect the rat myocardium against oxidative stress (22). The stimulation of Ca\textsuperscript{2+} uptake by insulin replacement is involved in the regulation of heart metabolism and transporter activities (34). However, the effects of combined endurance exercise training and insulin treatment on cardiac oxidative stress and heart mitochondrial function of diabetic rats are poorly understood. This study sought to examine the effects of swimming training combined with insulin treatment on cardiac oxidative stress and mitochondrial dysfunctions in streptozotocin (STZ)-induced diabetic rats.

**MATERIALS AND METHODS**

**Experimental animals.** Male Wistar rats (30 days; 80.2 ± 1.8 g) had free access to chow and water, were housed at 22 ± 2°C on a 12-h:12-h light/dark cycle, and were separated into control sedentary (CS), control exercised (CE), diabetic sedentary (DS), diabetic exercised (DE), diabetic sedentary insulin (DSI), and diabetic exercised insulin (DEI) groups. Experiments were approved by the Ethics Committee of the Federal University of Viçosa (protocol number 51/2011).

**Induction of diabetes and insulin treatment.** Diabetic groups received an intraperitoneal injection [60 mg/kg of body wt (BW)] of STZ (Sigma, St. Louis, MO) diluted in sodium citrate buffer (0.1 M, pH 4.5), and control groups received the buffer. Seven days later, animals with fasting blood glucose (BG) above 300 mg/dl were

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considered diabetic. Animals from DEI and DSI received a daily dose of human insulin (1–4 U/day).

Exercise training protocol. Animals from CE, DE, and DEI groups were subjected to a swimming training program (5 days/wk for 8 wk) [Adapted from Gomes et al. (13)]. For the first week, the animals exercised with no load for 10–50 min/day, and exercise duration was increased by 10 min/day. In the second week, the animals exercised with a load corresponding to 1% of BW, and the exercise duration was increased by 10 min/day up to a total of 90 min of continuous swimming in one session. From the third week on, the load was increased weekly (1% of BW/wk) up to a load of 5% of BW on the eighth week.

Resting heart rate assessment. Resting heart rate (RHR) was obtained from electrocardiogram. Animals were placed inside a chamber for anesthesia (isoflurane 2% and oxygen 100%) at a constant flow of 1 l/min. The electrocardiogram (DII) was acquired using the data acquisition system PowerLab (AD Instruments, São Paulo, SP, Brazil), and data were analyzed using the program Lab Chart Pro (AD Instruments). Heart rate was obtained by average of five consecutive cardiac cycles.

Echocardiographic examinations. Animals were anesthetized with isoﬂurane via mask (isoflurane 3% and oxygen 100%) at a constant flow of 1 l/min. Cardiac contraction and relaxation were assessed noninvasively by transthoracic echocardiography using parasternal long- and short-axis images. Two-dimensional, M-mode echocardiographic images and color-guided pulsed-wave Doppler images were obtained by standard echocardiographic techniques (11) (MyLab 30; ESAOTE, Genova, Italy). The systolic function was assessed using the ejection fraction (EF) and fractional shortening (FS), whereas the diastolic function was assessed using the mitral flow data [i.e., early filling wave (peak E); late filling wave (peak A) and E/A ratio].

Isolation of cardiomycocytes and mitochondria from left ventricle. Two days after the last exercise training session, the rats were euthanized by cervical dislocation and their hearts were removed. Left ventricular (LV) myocytes were enzymatically isolated [Adapted from Carneiro-Júnior et al. (9)] using 1 mg/ml of collagenase type II (Worthington, Lakewood, NJ) and 0.1 mg/ml of protease (Sigma). LV mitochondria were isolated by standard differential centrifugation (40).

\[\text{[Ca}^{2+}]_\text{i}\text{, measurements. [Ca}^{2+}]_\text{i}\text{, transients in cardiomycocytes were evaluated as described previously (35) using 5 }\mu\text{M Fluor 4-AM (Molecular Probes, Eugene, OR). A Meta LSM 510 scanning system (Carl Zeiss, Jena, Germany) with an }\times63\text{ oil-immersion objective was used for confocal fluorescence imaging (488/510 nm ex/em). Digital image processing was performed using routines custom written in the MatLab platform. The amplitude of the [Ca}^{2+}]_\text{i}\text{, transient, measured as fluorescence ratio (F/F0), with fluorescence intensity (F) normalized to the minimal intensity measured between 1-Hz contractions at diastolic phase (F0), the time to peak, and the time from the peak to half resting level of the [Ca}^{2+}]_\text{i}\text{, transient were determined.}

Heart redox state. Reduced glutathione (GSH) and oxidized glutathione (GSSG) and protein carbonyl levels were assessed in LV tissue (50 mg/ml in cold 0.1 M Tris·HCl buffer, pH 7.4) by the fluorometric ortho-phthalaldehyde method (20). Protein carbonyl was detected spectrophotometrically by derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (39, 40). Superoxide dismutase (SOD) content in LV tissue was determined spectrophotometrically by the tetrazolium salt formation using a Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI).

Mitochondrial standard incubation procedure and assays. Mitochondria were energized with 5 mM glutamate and malate in an incubation medium containing of 125 mM sucrose, 65 mM KCl, 2 mM K3HPO4, and 10 mM HEPES-KOH, pH 7.4, at 30°C. Mitochondrial respiration was monitored using a temperature-controlled computer-interfaced Clark-type oxygen electrode (Oxytherm; Hansatech Instruments, Kings Lyn, Norfolk, UK). Hydrogen peroxide production was monitored spectrophotometrically using 1 μM Amplex red (Molecular Probes) and 1 UI/ml horseradish peroxidase (49); these assays were performed in the presence of 0.1 mM EGTA. Ca2+ influx and efflux were monitored spectrophotometrically using 150 nM Calcium Green 5N (Molecular Probes) (36). Ca2+ flux and H2O2 production were monitored in a Model F-4500 Hitachi fluorescence spectrophotometer at the 506/531 and 563/587 (ex/em) wavelength pair, respectively.

Analysis of mRNA expression. RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). For real-time PCR analysis, RNA was reverse transcribed using the Reverse Transcriptase IMPROM II (Promega, Madison, WI) and used in quantitative PCR reactions containing EVA-green fluorescent dye (Bio-Rad, Hercules, CA). Relative expression of mRNAs was determined after normalization by β-actin using the ΔΔCt method (4). Quantitative PCR was performed using Eppendorf Realplex4 Mastercyteter Instrument (Eppendorf, Hamburg, Germany). Primers for Nox-4, β-actin, and UCP-2 were described as in Table 1.

Statistical analysis. Normal distribution of the data was determined by the Shapiro-Wilk test. Nonnormally distributed variables were log-transformed before statistical analyses. Comparisons between groups were performed by using the factorial analysis two (sedentary × exercised) by three (control × diabetes × insulin), followed by Tukey’s post hoc test (SAS version 9.3; SAS, Cary, NC). Statistical significance was defined at P ≤ 0.05. Results are presented as means ± SE.

RESULTS

General characteristics of rats. The initial BW levels were no different among the groups (Table 2). STZ augmented the final BW levels to ~500 mg/dl during the experimental period (factor effect: P < 0.05). Insulin treatment reduced the final plasma glucose (factor effect: P < 0.05) in diabetic rats by ~35% (control: 88.70 ± 24.35; diabetes: 510.70 ± 24.30; insulin: 327.51 ± 24.34, in mg/dl). However, neither exercise training effect nor interaction between factors was observed (P > 0.05).

The initial BW was not different among groups; however, STZ-injected animals gained less BW (factor effect: P < 0.05) than controls until the end of the experimental period (control: 318.90 ± 12.39 g; diabetes: 194.80 ± 12.38 g; insulin: 216.71 ± 12.37 g). Neither insulin treatment nor exercise training alone altered significantly the BW gain in diabetic rats (factor effect: P < 0.05). Interaction between factors was observed (factor effect: P < 0.05). For example,

Table 1. Forward and reverse sequences of primers used in the real time RT-PCR assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox-4</td>
<td>5′TTCTGGACCTTTTGTGCTCATACG3′</td>
<td>5′GCATGACATCTGAGGGATGATT3′</td>
</tr>
<tr>
<td>UCP</td>
<td>5′ATGTGCTAAAAGTCGCCCTC3′</td>
<td>5′GATTTGCGCAGCACATGCG3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′GAGTTTCTTCAATGAGTCGCG3′</td>
<td>5′GTGCGATGCTAGTACATGG3′</td>
</tr>
</tbody>
</table>

UCP-2, uncoupling protein-2; Nox-4, NADPH oxidase-4.
Table 2. General characteristics of animals in the experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>CS (n = 7)</th>
<th>DS (n = 7)</th>
<th>DSI (n = 7)</th>
<th>CE (n = 7)</th>
<th>DE (n = 7)</th>
<th>DEI (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW, g</td>
<td>22.50 ± 7.98</td>
<td>18.50 ± 6.54</td>
<td>18.00 ± 7.43</td>
<td>22.00 ± 6.45</td>
<td>20.00 ± 7.25</td>
<td>19.50 ± 6.87</td>
</tr>
<tr>
<td>Initial BG, mg/dl</td>
<td>100.00 ± 5.00</td>
<td>105.00 ± 6.00</td>
<td>102.00 ± 5.50</td>
<td>107.00 ± 5.50</td>
<td>104.00 ± 6.00</td>
<td>103.00 ± 5.50</td>
</tr>
<tr>
<td>Initial RHR, beats/min</td>
<td>300.00 ± 10.00</td>
<td>320.00 ± 15.00</td>
<td>315.00 ± 12.00</td>
<td>305.00 ± 13.00</td>
<td>310.00 ± 14.00</td>
<td>308.00 ± 12.00</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE of 10 animals in each group. CS, control sedentary; DS, diabetic sedentary; DSI, diabetic sedentary with insulin; CE, control exercised; DE, diabetic exercised; DEI, diabetic exercised with insulin.

Table 3. Left ventricular systolic and diastolic functions measured by resting echocardiography at the end of the experimental period

<table>
<thead>
<tr>
<th>Group</th>
<th>CS (n = 7)</th>
<th>DS (n = 7)</th>
<th>DSI (n = 7)</th>
<th>CE (n = 7)</th>
<th>DE (n = 7)</th>
<th>DEI (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejection fraction, %</td>
<td>77.33 ± 3.00</td>
<td>78.33 ± 3.00</td>
<td>77.00 ± 3.00</td>
<td>78.00 ± 3.00</td>
<td>79.00 ± 3.00</td>
<td>78.50 ± 3.00</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>41.00 ± 1.50</td>
<td>42.00 ± 1.50</td>
<td>41.00 ± 1.50</td>
<td>42.00 ± 1.50</td>
<td>43.00 ± 1.50</td>
<td>42.50 ± 1.50</td>
</tr>
<tr>
<td>Peak E, m/s</td>
<td>0.049 ± 0.010</td>
<td>0.051 ± 0.011</td>
<td>0.048 ± 0.010</td>
<td>0.050 ± 0.011</td>
<td>0.052 ± 0.012</td>
<td>0.051 ± 0.011</td>
</tr>
<tr>
<td>Peak A, m/s</td>
<td>0.316 ± 0.032</td>
<td>0.318 ± 0.033</td>
<td>0.314 ± 0.032</td>
<td>0.316 ± 0.033</td>
<td>0.318 ± 0.034</td>
<td>0.316 ± 0.033</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.66 ± 0.15</td>
<td>1.67 ± 0.15</td>
<td>1.65 ± 0.15</td>
<td>1.66 ± 0.15</td>
<td>1.67 ± 0.15</td>
<td>1.66 ± 0.15</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE of n (number of animals) in each group. Peak E, early filling wave; Peak A, late filling wave. *Different from CS; †different from CE; ‡different from DE; §different from DSI.
An additional indication of low SOD content in hearts of our diabetic sedentary rats was verified by the GSH/GSSG because GSH is consumed by glutathione peroxidase to degrade \( \text{H}_2\text{O}_2 \)-generating GSSG and H$_2$O. We found a slight increase (factor effect: \( P < 0.05 \); control: 1.77 ± 0.07; diabetes: 2.30 ± 0.11; insulin: 1.89 ± 0.16) in GSH/GSSG in diabetic hearts (Fig. 2C), indicating low productions of \( \text{H}_2\text{O}_2 \) despite the apparent increase of \( \text{O}_2^- \) production by elevated Nox-4 expression. Neither exercise training nor insulin treatment had an independent effect on GSH/GSSG (factor effect: \( P > 0.05 \)); nevertheless there was interaction between factors (\( P < 0.05 \)). Hearts from DEI group showed lower GSH/GSSG than those from DE and DS groups.

The oxidative damage, as a consequence of an unbalanced production/inactivation of \( \text{O}_2^- \), was verified by the content of carbonyl proteins (Fig. 2D). Proteins of diabetic rat hearts were more oxidized, an effect that was reversed by insulin treatment (factor effect: \( P < 0.05 \); control: 0.40 ± 0.07 mmol/mg; diabetes: 0.84 ± 0.10 mmol/mg; insulin: 0.37 ± 0.07 mmol/mg). Exercise training had no independent effect (factor effect: \( P > 0.05 \)); however, interaction between factors was observed (\( P < 0.05 \)). Hearts from DEI group showed lower carbonyl content than those from DS and DE groups. Furthermore, the modulation of carbonyl contents promoted by diabetes and treatments (insulin or exercise) follow the profile found for Nox-4 expression, suggesting linked effects.

**Mitochondrial function.** Diabetes did not affect (factor effect: \( P > 0.05 \)) the respiration rates (Table 4) in the phosphorylating state (state III) but increased the rates in the resting state (state IV). Diabetes decreased (factor effect: \( P < 0.05 \)) the respiratory control ratios (control: 10.05 ± 0.58; diabetes: 7.39 ± 0.60; insulin: 7.94 ± 0.36), indicating a weakness of the coupling between respiration and phosphorylation. Neither swimming training nor insulin treatment of diabetic rats restored these respiratory parameters (factor effect: \( P > 0.05 \)), and no interaction between factors was observed for such parameters (\( P > 0.05 \)). However, mitochondria from DE group exhibited lower \( \text{O}_2 \) consumption in state IV than those from DS, DSI, and DE groups.

In addition, LV of diabetic rats also presented an augmentation (factor effect: \( P < 0.05 \)) in the expression of UCP-2 (control: 0.77 ± 0.09 UCP/\( \beta \)-actin; diabetes: 1.83 ± 0.22 UCP/\( \beta \)-actin; insulin: 1.13 ± 0.19 UCP/\( \beta \)-actin) (Fig. 3). These proteins located at the inner membrane can dissipate the proton gradient built by respiratory chain, promoting a mild uncoupling of the oxidative phosphorylation. UCP-2 mRNA was partially restored by insulin treatment as an independent factor. Likewise, exercise training reduced (factor effect: \( P < 0.05 \)) the expression of UCP-2 (sedentary: 1.60 ± 0.21 UCP/\( \beta \)-actin; exercised: 0.94 ± 0.11 UCP/\( \beta \)-actin) as an independent factor as well as in diabetic rats when combined with insulin treatment (interaction: \( P < 0.05 \)). For example, LV from DEI group exhibited lower UCP-2 mRNA expression than those from DS, DSI, and DE groups.

The release of \( \text{H}_2\text{O}_2 \) in isolated heart mitochondria was monitored (Fig. 4). The representative experiment (Fig. 4A) and average data (Fig. 4B) show that heart mitochondria from sedentary diabetic rats release lower amounts (factor effect: \( P < 0.05 \)) of \( \text{H}_2\text{O}_2 \) compared with those from controls (control: 0.67 ± 0.02 nmol·mg$^{-1}$·min$^{-1}$; diabetes: 0.41 ± 0.01 nmol·mg$^{-1}$·min$^{-1}$; insulin: 0.52 ± 0.04 nmol·mg$^{-1}$·min$^{-1}$).

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**Fig. 1. Intracellular global Ca$^{2+}$ transient in isolated left ventricular myocytes.** A: amplitude of transient. B: time to peak. C: time from peak transient to half resting value. F/F0, fluorescence ratio [fluorescence intensity (F) normalized to the minimal intensity measured between 1-Hz contractions at diastolic phase (F0)]. CS, control sedentary; DS, diabetic sedentary; DSI, diabetic sedentary with insulin; CE, control exercised; DE, diabetic exercised; DEI, diabetic exercised with insulin. *Different from CS; † different from CE; ‡ different from DE; ‡⁄ different from DS; ‡⁄⁄ different from DSI; n = 87–103 cells per group.

**Fig. 2.** Mitochondria from diabetic rats exhibited lower O$_2$ consumption in state IV than those from DS and DE groups. The release of H$_2$O$_2$ in isolated heart mitochondria was monitored (Fig. 4). The representative experiment (Fig. 4A) and average data (Fig. 4B) show that heart mitochondria from sedentary diabetic rats release lower amounts (factor effect: \( P < 0.05 \)) of H$_2$O$_2$ compared with those from controls (control: 0.67 ± 0.02 nmol·mg$^{-1}$·min$^{-1}$; diabetes: 0.41 ± 0.01 nmol·mg$^{-1}$·min$^{-1}$; insulin: 0.52 ± 0.04 nmol·mg$^{-1}$·min$^{-1}$).
Neither exercise training nor insulin treatment alone affected the reduced mitochondrial H$_2$O$_2$ release (factor effect: $P > 0.05$). However, there was interaction between factors ($P < 0.05$). Swimming training associated with insulin reversed the reduced H$_2$O$_2$ release in heart mitochondria from diabetic rats because heart mitochondria from DEI group presented higher H$_2$O$_2$ values compared with those of DS, DSI, and DE groups.

Diabetes slightly increased the capacity of Ca$^{2+}$ uptake (Fig. 5A) in isolated heart mitochondria (control: 180.00 ± 12.00 nmol/mg; diabetes: 209.33 ± 20.77 nmol/mg; insulin: 147.27 ± 15.55 nmol/mg), which was reduced below control levels by insulin treatment (factor effect: $P < 0.05$). Exercise training had no independent effect (factor effect: $P < 0.05$); nevertheless interaction between factors was observed ($P < 0.05$). Heart mitochondria from DS group showed higher capacity of Ca$^{2+}$ uptake than those from DE and DSI, and mitochondria from DEI group exhibited higher capacity of Ca$^{2+}$ uptake compared with those from DSI group. It indicates that exercise training either alone or in combination with insulin restored the Ca$^{2+}$ uptake to control levels in diabetic animals.

The Ca$^{2+}$ retention capacity was monitored to check the susceptibility to MPTP opening in isolated heart mitochondria (Fig. 5B). Traces of the external Ca$^{2+}$ concentration dynamics in response to sequential additions of Ca$^{2+}$ show that heart mitochondria isolated from DS rats did not sustain the pre-loaded Ca$^{2+}$ (after 6 additions of 20 nmol Ca$^{2+}$), in contrast to those isolated from CS rats, indicating an increased susceptibility to Ca$^{2+}$-induced inner membrane permeabilization (Fig. 5B). This condition was only reversed by exercise training combined with insulin treatment (DEI group). Surprisingly, both swimming training and insulin treatment separately further reduced the capacity of Ca$^{2+}$ retention in heart mitochondria of diabetic rats. Cyclosporin A, a classical inhibitor of MPTP opening, totally prevented the release of accumulated Ca$^{2+}$ in heart mitochondria of DS, DE, or DEI groups and partially prevented it in heart mitochondria of DSI (Fig. 5C). Together, these results indicate that diabetes induces high Ca$^{2+}$ uptake and thus increases the susceptibility to MPTP opening in heart mitochondria. Higher Ca$^{2+}$ uptake and the susceptibility to MPTP opening, as well as the respiratory, UCP-2 expression, and H$_2$O$_2$ release patterns, were reversed by insulin treatment combined with exercise training.

**DISCUSSION**

We examined the effects of swimming training and insulin therapy, either alone or in combination, on the intracellular
Ca\textsuperscript{2+} homeostasis, oxidative stress, and mitochondrial functions in diabetic rat hearts. Our data showed that endurance training associated with insulin treatment was more effective in attenuating intracellular Ca\textsuperscript{2+} homeostasis disruptions, cardiac oxidative stress, and mitochondrial dysfunctions caused by STZ-induced diabetes in rat hearts.

Here, diabetes impaired the in vivo cardiac systolic and diastolic functions as it reduced the EF and FS and increased peak E/peak A ratio in rats. These dysfunctions were also evident at the cellular level inasmuch as diabetes reduced the amplitude and prolonged the time to half-decay of the [Ca\textsuperscript{2+}], transient in LV myocytes. These disruptions in experimental diabetes have been shown previously and are related to dysfunctions in the cellular Ca\textsuperscript{2+}-regulatory proteins (23). Regarding the systolic dysfunction, the Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) is not uniform in cardiomyocytes of diabetic rodents, which is attributable to reductions in the amount of functional ryanodine receptor 2 (6), reduced Ca\textsuperscript{2+} channel activities (23), and depressed sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2) expression or activity and hence intra-SR Ca\textsuperscript{2+} stores (23). This would help to explain the observed reduced [Ca\textsuperscript{2+}], transient amplitude. As for the diastolic dysfunction, the prolonged Ca\textsuperscript{2+} transient decay observed here is consistent with a prolonged cytosolic Ca\textsuperscript{2+} removal via decreased SERCA2 expression or activity in diabetic hearts (23). It is noteworthy that an increased activity of sodium-calcium exchanger (NCX) may contribute to reduced SR Ca\textsuperscript{2+} load in diabetic myocytes although the relative contribution of NCX to cytosolic Ca\textsuperscript{2+} removal in rats is small (~7%), and in diabetic hearts increased or unchanged NCX activity or decreased NCX expression was reported (10). Nevertheless, as we did not measure either the expression or the activity of these proteins, their role in these results found in this study should be taken with caution.

Despite the fact that cytosolic removal of Ca\textsuperscript{2+} into mitochondria in rats is small (~1%) and seems not to impact on the [Ca\textsuperscript{2+}], transient (5), we observed an increased capacity of mitochondrial Ca\textsuperscript{2+} uptake in mitochondria isolated from diabetic hearts. Mitochondria are sensitive to Ca\textsuperscript{2+} concentration, which is important for the modulation of mitochondrial metabolism. These organelles may act as a Ca\textsuperscript{2+}-buffering system removing and modulating the local Ca\textsuperscript{2+} concentration. Calcium is transported through the inner membrane matrix by two mechanisms, a uniporter (MCU) and a rapid uptake mode (RaM), that are dependent on the electrochemical gradient for Ca\textsuperscript{2+}. Calcium flux rates through the MCU are fast, equivalent to fast-gated pores but slower than most channels. Calcium uptake through the RaM occurs very rapidly, faster than MCU and at the beginning of Ca\textsuperscript{2+} pulse. The localization of mitochondria close to Ca\textsuperscript{2+} release sites of the endoplasmic reticulum or SR or even near plasma membrane Ca\textsuperscript{2+} channels also facilitates mitochondrial Ca\textsuperscript{2+} handling (see Ref. 15 for review). Furthermore, in different cell types, an increased peak of mitochondrial Ca\textsuperscript{2+} concentration in response to Ca\textsuperscript{2+}-mobilizing stimuli has been described to be increased by UCP-2 and UCP-3 overexpression (45). In fact, we found elevated UCP-2 expression and indications of increased UCP activity, such as enhanced resting respiration rate and reduced mitochondrial H\textsubscript{2}O\textsubscript{2} release in diabetic hearts. Although the role of UCPs in the heart is controversial and incompletely understood, mild uncoupling promoted by UCP decreases ROS formation by accelerating electron transport rates through the respiratory chain, thus decreasing the lifetime of intermediates capable of donating electrons toward superoxide radical formation (42). This process could represent a mechanism to protect the cell against oxidative damage. Anyway, our findings agree with previous works (18) showing that mitochondria per se, apart from the insulin-deficient diabetic profile, remain free from ROS production despite considerable evidence suggesting that diabetes is associated with oxidative tissue damage in the heart of diabetic rodents (2). However, mitochondrial uncoupling may augment oxygen consumption without proportionately increasing mitochondrial ATP production. The resulting energy deficit may explain the lack of increase in cardiac systolic and diastolic functions, resulting in reduced cardiac efficiency, as found in hearts of db/db mice (7).

Our results showed that cardiac oxidative damage could be related to upregulation of Nox-4 expression, as shown previously by Maalouf and coworkers (27) and also found in LV
dysfunctions such as heart failure progression and aging (1). Nox-4 is localized in perinuclear organelles, including mitochondria (1), and is supposed to be constitutively active, not requiring cytosolic factors for its activation. Therefore, its expression levels determine the amount of O$_2^-$ production in the cells. Oxidative damage is also favored when O$_2^-$ production is associated with an impaired antioxidant system, as demonstrated here by diminished SOD in hearts of diabetic rats. Reductions in SOD content and/or activity have been shown previously in STZ-induced diabetic cardiomyopathy (28). Thus oxidative damage in LV of diabetic rats could be a consequence of high production and low inactivation of O$_2^-$ promoted by high Nox-4 expression and low SOD content. We also suggest that increased Nox-4 activity could also sensitize MPTP opening in the heart mitochondria of STZ-induced diabetic rats, inasmuch as Nox-4 activity leads to cysteine oxidation of mitochondrial proteins, including components of the MPTP complex and mitochondrial damage, as reported previously (1). This sensibility associated with an excessive mitochondrial Ca$^{2+}$ load could trigger MPTP opening, following dissipation of the inner mitochondrial membrane potential and swelling.

It is possible that the impaired intracellular Ca$^{2+}$ homeostasis in our diabetic rat hearts has increased oxidative stress by activating CaMKII. Nishio et al. (30) demonstrated an augmented [Ca$^{2+}$], in cardiomyocytes exposed to high glucose concentrations attributable to increased sodium-hydrogen exchanger expression and activity, which activated NCX in reverse mode. High glucose also upregulated the phosphorylated CaMKII expression that was suppressed by inhibiting NCX in reverse mode. In addition, a CaMKII inhibitor attenuated the ROS level in these myocytes. In STZ-induced diabetic rat hearts, they observed upregulation of ROS level and components of NADPH oxidase, p47phox, and p67phox, which were attenuated by a CaMKII inhibitor. In fact, our diabetic rat hearts exhibited augmented expression Nox-4, the major catalytic component of Nox.

More importantly, our results showed that 8 wk of combined swimming training and insulin therapy is more effective in restoring intracellular Ca$^{2+}$ homeostasis as well as cardiac oxidative stress and mitochondrial dysfunctions. Regarding the Ca$^{2+}$ homeostasis, both exercise training and insulin treatment either alone or in combination restored the time course and the amplitude of the [Ca$^{2+}$], transient in LV myocytes of diabetic rats. Although we did not measure Ca$^{2+}$-handling proteins, exercise training has been shown to improve SR Ca$^{2+}$ re-estimation via increases in SERCA2a and phospholamban expression and/or activity along with augmentations of Ca$^{2+}$ efflux via NCX in diabetic rat hearts (24, 31). It is noteworthy that SR Ca$^{2+}$-induced Ca$^{2+}$ release is insulin dependent, as insulin regulates the cardiac function by stimulating $I_{\text{CaL}}$. Insulin also interacts with SERCA2 via insulin receptor substrate, indicating that insulin receptor substrate proteins bind to the SERCA2 in an insulin-regulated fashion (3).

Our echocardiographic data show that diabetic rats exercised and treated with insulin had their systolic function partially restored despite no recovery of diastolic function. On the subject of cardiac oxidative stress, the combined treatments normalized Nox-4 expression and reduced the content of carbonyl proteins. Among the factors known for Nox modulation in diabetic heart (see Ref. 44 for review), exogenous insulin replacement can act to control BG levels (27) and thereby the intracellular Ca$^{2+}$ homeostasis (32), as described above, drastically downregulating Nox-4 expression; exercise training can act to increase the levels of protein kinase C (17), reestablishing Nox-4 expression to the control levels.

As for the mitochondrial STZ-induced dysfunctions, the combination of insulin with exercise training was able to reduce UCP-2 expression, Ca$^{2+}$ uptake, O$_2$ consumption, and MPTP opening susceptibility and to increase H$_2$O$_2$ release. Insulin itself is known to improve the activities of complexes I, II, and/or IV after 4 wk, as shown by others (38). It is probably related to the levels of mRNA for the peroxisome proliferator-activated receptor $\gamma$ coactivator 1$\alpha$ that upregulate...
nuclear genes required for mitochondrial biogenesis (31). Long-term treadmill running (14 wk) prevented the elevation of proteins involved in MPTP pore formation and apoptotic signaling in hearts of diabetic rats (26). Nevertheless, in our 8-wk treatment, endurance exercise training or insulin alone restored the mitochondrial Ca^{2+} uptake only.

Finally, we observed that diabetes reduced the RHR in rats, as shown elsewhere (21). This can be explained in part by the reduction in the expression of β-adrenergic receptors (β1 and β2) in diabetic rats (29). As expected, Bradycardia was normalized in sedentary and exercised animals by insulin, as demonstrated previously (37). Insulin exerts positive inotropic and chronotropic effects on the myocardium (25). Along with the impaired growth of the animals induced by STZ, by the end of the experiment, diabetic rats exhibited lower HW. In rats with diabetes, in addition to insulin, the secretion of hormones such as the growth hormone, glucagon, pancreatic polypeptide, and, consequently, growth factor similar to that of insulin, was malnourished in sedentary and exercised diabetic animals. The increased HW/BW ratio in both sedentary and exercised diabetic animals. The increased HW/BW ratio in 8-wk diabetic rats treated with insulin reflects the recovery of growth in the heart of these animals, inasmuch as their final HW did not change.

The combination of an 8-wk swimming training program with daily insulin replacement was more effective than isolated treatments in attenuating oxidative stress, Ca^{2+} homeostasis disruptions, and mitochondrial dysfunctions in the hearts of rats with STZ-induced type 1 diabetes.

ACKNOWLEDGMENTS

The confocal experiments were performed in the facilities of the Núcleo de Microscopia e Microanalise at the Federal University of Viçosa (UFV). We acknowledge Ieda M. R. Prado for technical support.

GRANTS

This study was funded by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, CDS APAQ 01171/11) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 2010/17259-9). A. J. Natali is a CNPq fellow. M. F. da Silva was a recipient of a doctoral scholarship from FAPEMIG.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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