The mitochondrial function of the cerebral vasculature in insulin-resistant Zucker obese rats

Ivan Merdzo, Ibolya Rutkai, Tunde Tokes, Venkata N. L. R. Sure, Prasad V. G. Katakam, and David W. Busija

Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana

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Merdzo I, Rutkai I, Tokes T, Sure VN, Katakam PV, Busija DW. The mitochondrial function of the cerebral vasculature in insulin-resistant Zucker obese rats. Am J Physiol Heart Circ Physiol 310: H830–H838, 2016. First published February 12, 2016; doi:10.1152/ajpheart.00964.2015.—Little is known about mitochondrial functioning in the cerebral vasculature during insulin resistance (IR). We examined mitochondrial respiration in isolated cerebral arteries of male Zucker obese (ZO) rats and phenotypically normal Zucker lean (ZL) rats using the Seahorse XF24 analyzer. We investigated mitochondrial morphology in cerebral blood vessels as well as mitochondrial and nonmitochondrial protein expression levels in cerebral arteries and microvessels. We also measured reactive oxygen species (ROS) levels in cerebral microvessels. Under basal conditions, the mitochondrial respiration components (nonmitochondrial respiration, basal respiration, ATP production, proton leak, and spare respiratory capacity) showed similar levels among the ZL and ZO groups with the exception of maximal respiration, which was higher in the ZO group. We examined the role of nitric oxide by measuring mitochondrial respiration following inhibition of nitric oxide synthase with Nω-nitro-l-arginine methyl ester (L-NAME) and mitochondrial activation after administration of dazoxide (DZ). Both ZL and ZO groups showed similar responses to these stimuli with minor variations. L-NAME significantly increased the proton leak, and DZ decreased nonmitochondrial respiration in the ZL group. Other components were not affected. Mitochondrial morphology and distribution within vascular smooth muscle and endothelium as well as mitochondrial protein levels were similar in the arteries and microvessels of both groups. Endothelial nitric oxide synthase (eNOS) and ROS levels were increased in cerebral microvessels of the ZO. Our study suggests that mitochondrial function is not significantly altered in the cerebral vasculature of young ZO rats, but increased ROS production might be due to increased eNOS in the cerebral microcirculation during IR.

middle cerebral arteries; insulin resistance; mitochondria; mitochondrial respiration; reactive oxygen species; cerebral vessels; cerebral microvessels

NEW & NOTEWORTHY

This is the first study that has directly measured mitochondrial respiration in the cerebral arteries of insulin-resistant rats. Our results show that severe mitochondrial dysfunction is not necessary for the development of IR and that oxidative stress plays a significant role in vascular dysfunction during IR.

INSULIN RESISTANCE (IR), with the contributing factors of central obesity, genetic factors, and a sedentary lifestyle, is a primary feature in the development of the metabolic syndrome (1). Impaired insulin metabolism can lead to various metabolic and vasculature abnormalities resulting in clinical consequences, such as hypertension, atherosclerosis, dyslipidemia, diabetes, cerebrovascular dysfunction, and/or stroke (2, 32). Although the incidence of IR is growing dramatically, the mechanisms of its pathological effects on cerebral blood vessels as well as the underlying etiology of IR have not been completely elucidated. Impaired mitochondrial function and mitochondrial content have been suggested as underlying causes of IR (31, 39), whereas other reports have not found altered mitochondrial respiratory capacity (18, 33) or mitochondrial content (18, 26) in insulin-resistant humans. Our laboratory has shown that mitochondrial-dependent vasodilatory responses of major cerebral arteries are diminished in Zucker obese (ZO) rats (27, 28). On the other hand, we have also shown that local cortical microcirculatory responses to pharmacological blood flow stimulators, such as bradykinin, N-methyl-d-aspartate (NMDA), bicuculline, and physiologically induced hypercapnia, were not significantly altered in insulin-resistant ZO rats compared with phenotypically normal, Zucker lean (ZL) controls (24).

Therefore, we further investigated mitochondrial energetics in the cerebral vasculature during IR using young ZO rats with normal levels of glucose and blood pressure but with elevated plasma insulin levels (16, 17). We hypothesized that mitochondrial respiration would be reduced in large cerebral arteries of young insulin-resistant ZO rats as would mitochondrial and nonmitochondrial protein expression associated with mitochondrial function. We also examined the effects of the nonselective nitric oxide (NO) synthase (NOS) inhibitor Nω-nitro-l-arginine methyl ester (L-NAME) and dazoxide (DZ), a mitochondrial ATP-sensitive potassium (mitoKATP) channel opener, on mitochondrial respiration. L-NAME was used to test the role of NO, and DZ was used because of its known depolarizing effect on mitochondria (47). We used freshly isolated cerebral vessels to determine levels of mitochondrial and related nonmitochondrial proteins. In freshly isolated cerebral microvessels of ZO and ZL rats, we measured and compared superoxide (O$_2^-$) levels to determine oxidative...
stress levels in the cerebral microcirculation during IR. Finally, we assessed for the first time the morphology of large cerebral arteries and microvessels using electron microscopy to determine whether there are any differences between ZO and ZL rats.

**MATERIALS AND METHODS**

The procedures and protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University School of Medicine and comply with the National Institutes of Health (NIH) guidelines. Animal care was provided by the Department of Comparative Medicine. Animals were given standard food and tap water ad libitum. We used 10–12-wk-old male ZO and ZL rats from Charles River Laboratories (Wilmington, MA) (n = 34 each).

ZO rat model. ZO rats have been widely used as a model of IR. These rats have a leptin receptor mutation (fa/fa, homozygous for the mutation), which leads to hyperphagia with subsequent obesity and IR. ZL rats (Fa/fa, heterozygous for the mutation) were used as genetically appropriate controls (6). We and other laboratories have shown that the metabolic profile of ZO rats is similar to the human condition (16, 17, 19, 36). At 10–12 wk of age, ZO rats develop hyperinsulinemia, hypercholesterolemia, hypertriglyceridemia, and impaired glucose tolerance but retain normal levels of glucose and arterial blood pressure (27).

Isolation of cerebral arteries and microvessels. Animals were deeply anesthetized using 5% isoflurane (VetOne, Boise, ID) and decapitated. Brains were removed immediately and placed in ice-cold PBS. All of the following procedures were carried out on ice or at 4°C. Anterior, middle cerebral, and basilar arteries were isolated, cleaned, and used for mitochondrial respiration measurements and for Western blotting. Brain microvessel isolation was then performed as previously described (42, 43, 49) with minor modifications. Cerebellum, brain stem, choroid plexus, and large superficial arteries and meninges were all removed. The remaining cortex was homogenized using a hand-held glass homogenizer and centrifuged at 1,000 g for 10 min. A three-step process yielded the final microvessels for the experiments: 1) the supernatant was discarded, and the pellet was resuspended in 10 ml of 17.5% dextran (64–76 kDa, Sigma-Aldrich, St. Louis, MO), passed through 300-μm nylon mesh, and then centrifuged at 4,400 g for 15 min; 2) the microvessel pellet was transferred to a clean tube, and the supernatant was resuspended and centrifuged as before to increase the yield of microvessels; and 3) the two resulting microvessel pellets were collected, resuspended, and centrifuged again, as described previously. The resulting microvessel pellet was collected in 1% BSA and passed through 70-μm nylon mesh. Captured microvessels were washed with ice-cold PBS, collected, and used for Western blotting and determination of superoxide levels. Purity of the microvessels was constantly monitored and evaluated via immunohistochemistry (Fig. 1).

**Immunohistochemical evaluation of the microvessel samples.** Immunohistochemistry experiments were done essentially as described by Toth et al. (44). Microvessel pellets were embedded in the Tissue-Tek optimal cutting temperature compound (Sakura Finitek, Torrance, CA), and 10-μm-thick cryostat sections were prepared and fixed in acetone. After being blocked with normal goat serum (Sigma-Aldrich), samples were stained with antibodies specific for different cells that could be present in the sample to evaluate the purity of the microvessel sample. The antibodies used in these experiments were antifibrillary acidic protein (GFAP, 10x DAPI, 10x vWF, 10x Merge, 10x Abcam, Cambridge, MA), as an endothelial cell marker. After incubation with primary antibodies, sections were washed with PBS and incubated with adequate, fluorescence-conjugated, secondary antibodies. Sections were then visualized via fluorescence microscopy (Fig. 1).

**Electron microscopy.** For characterization of large arteries, rats were euthanized with anesthesia and perfused with a PBS solution containing 2% glutaraldehyde and 3% formaldehyde. Arteries were removed and kept in the perfusion solution for 1 h. The microvessel pellets were fixed in a PBS solution containing 2% glutaraldehyde and 3% formaldehyde. Microvessels were cleaned, and used for mitochondrial respiration measurements and for Western blotting. Brain microvessel isolation was then performed as previously described (42, 43, 49) with minor modifications. Cerebellum, brain stem, choroid plexus, and large superficial arteries and meninges were all removed. The remaining cortex was homogenized using a hand-held glass homogenizer and centrifuged at 1,000 g for 10 min. A three-step process yielded the final microvessels for the experiments: 1) the supernatant was discarded, and the pellet was resuspended in 10 ml of 17.5% dextran (64–76 kDa, Sigma-Aldrich, St. Louis, MO), passed through 300-μm nylon mesh, and then centrifuged at 4,400 g for 15 min; 2) the microvessel pellet was transferred to a clean tube, and the supernatant was resuspended and centrifuged as before to increase the yield of microvessels; and 3) the two resulting microvessel pellets were collected, resuspended, and centrifuged again, as described previously. The resulting microvessel pellet was collected in 1% BSA and passed through 70-μm nylon mesh. Captured microvessels were washed with ice-cold PBS, collected, and used for Western blotting and determination of superoxide levels. Purity of the microvessels was constantly monitored and evaluated via immunohistochemistry (Fig. 1).

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3% formaldehyde. Arteries and microvessels were postfixed in 1% osmium tetroxide and embedded in Spurr’s resin. Ultrathin sections (80–90 nm) were mounted on formvar-coated copper grids (200 mesh), air dried, and stained with uranyl acetate and lead citrate (at 7 min and 7 min, respectively). The sections were put on grids and viewed at a magnification of ×11,000 using a FEI Tecnai BioTwin 120 keV TEM with a digital imaging setup (Wake Forest University Health Sciences, Winston-Salem, NC) (Fig. 2).

Mitochondrial respiration measurement. The Seahorse Bioscience XFe24 extracellular flux analyzer was used to measure mitochondrial oxygen consumption rate (OCR) as an indicator of mitochondrial respiration (5, 20, 22). For these measurements, we used freshly isolated cerebral arteries as described previously (40). Briefly, arteries were placed into wells of the XF24 islet capture microplate (no. 101122-100; Seahorse Bioscience, Billerica, MA), and wells were filled with 525 μl XF assay medium (no. 102365-100, Seahorse Bioscience) containing 5.0 mM/l glucose and 2 mM/l pyruvate and maintained at 37°C for 20 min in a non-CO2 incubator before the measurement. Oxygen and hydrogen ion-sensitive fluorophores of the Seahorse XFe24 analyzer then repeatedly measured the oxygen and hydrogen concentration in the medium surrounding the arteries, thus calculating OCR. The assay protocols consisted of three cycles of baseline measurements followed by five cycles for each treatment (Fig. 3). To test the effect of DZ on mitochondrial respiration, plates were loaded with 250 μM/l DZ or an equivalent amount of dimethyl sulfoxide (DMSO) as vehicle. The plates were then exposed sequentially to 2 μM/l oligomycin, 1 μM/l carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, and 1.5 mM/l antimycin plus 1.5 mM/l rotenone to evaluate different components of mitochondrial respiration. The same approach was used to test the effect of L-NAME at 100 μM/l using deionized water as a control treatment (untreated group). We had four sample groups each of ZO and ZL rats: 1) untreated group, 2) L-NAME-treated group, 3) DMSO-treated group, and 4) DZ-treated group. The OCR data were normalized to arterial protein concentration as determined by Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) and expressed in picomoles per microgram per minute. After data normalization, a series of calculations were performed using OCR data to provide information about specific

Fig. 2. Electron microscopy of middle cerebral arteries and microvessels from Zucker obese (ZO) and lean (ZL) rats. The basic features of mitochondrial morphology and location are similar in arteries from ZO (A) and ZL rats (B) and conform in general terms to the Western blot and oxygen consumption rate (OCR) data. Representative sections show heavy investment of mitochondria in cerebral vascular endothelium and vascular smooth muscle (VSM) in both types of rats. To illustrate the spatial arrangement of mitochondria especially in the VSM cells, we used a longitudinal cross section of the artery from an insulin-resistant (IR) rat (A) and a cross-sectional view in the middle cerebral artery from the Zucker lean rat (B). Mitochondria are usually present singly and dispersed throughout in the endothelial cells. Mitochondria in VSM are much larger and of different configurations than in endothelium. Typically, mitochondria in VSM are relatively large and numerous and are present in fields or clusters with sarcoplasmic reticulum (SR) and are not dispersed evenly throughout the VSM. This characteristic can be seen clearly by comparing the longitudinal and cross sections of the arteries. Representative sections of microvessels from ZO (C) and ZL (D) rats are shown. As described in MATERIALS AND METHODS, harvested microvessels are composed of arterioles and capillaries (not shown) with little contamination of nonvascular cells except for occasional erythrocytes. Similar to arteries, the VSM of the arterioles contains extensive mitochondrial fields intermixed with SR in both strains of rats. In the arterioles, the mitochondria in VSM retain relatively normal configuration, and the mitochondria in endothelium also appear intact in ZO and ZL rats. However, the mitochondria in the endothelium of the capillaries in both strains are often swollen in appearance or are difficult to visualize (images not shown), suggesting that mitochondrial morphology is better preserved during the harvesting procedure in the more muscular blood vessels. Magnification was ×11,000 in each section. IEL, internal elastic lamina; M, mitochondrion or mitochondria; EEL, external elastic lamina.
Antimycin+rotenone

NaCl, 4.69 KCl, 1.87 CaCl$_2$, 1.20 MgSO$_4$, 25 NaHCO$_3$, 1.03 modified Krebs-Hepes (KH) buffer containing (in mmol/l) were dissolved under nitrogen gas bubbling in ice-cold 2,2,5,5-tetramethyl-pyrrolidine (CMH), as described previously (28).

Freshly isolated microvessels were used to measure ROS (ROS), specifically superoxide, in the cerebral microvessels of ZO and ZL rats. Electron spin resonance studies. We used electron spin resonance (ESR) spectroscopy to measure the levels of reactive oxygen species (ROS), specifically superoxide, in the cerebral microvessels of ZO and ZL rats. Freshly isolated microvessels were used to measure ROS production using the spin probe 1-hydroxy-3-methoxyxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH), as described previously (28). Deferoxamine (25 μmol/l) and diethylthiocarbamate (DETC; 2.5 μmol/l) were dissolved under nitrogen gas bubbling in ice-cold modified Krebs-Hepes (KH) buffer containing (in mmol/l) 99.01 NaCl, 4.69 KCl, 1.87 CaCl$_2$, 1.20 MgSO$_4$, 25 NaHCO$_3$, 1.03 K$_2$HPO$_4$, 20 sodium-HEPES, and 11.1 d-glucose, at pH 7.35. Microvessel samples were placed in a microtube containing 300 μl of freshly made 200 μmol/l CMH solution of KH buffer containing DETC and deferoxamine. Samples were incubated at 37°C for 60 min. Afterward, the needle end of a 1-ml syringe barrel was cut, the plunger was retracted from the cut end, and microvessels with CMH containing KH buffer were placed into the syringe barrel and frozen in liquid nitrogen. When the sample was ready to be measured, the syringe was warmed gently between the palms of the hands, and the syringe plunger was used to push the frozen column of microvessels out of the syringe barrel directly into a finger Dewar (Noxygen Science Transfer & Diagnostics, Elzach, Germany) containing liquid nitrogen. Subsequently, the ESR spectra were obtained by placing the finger Dewar into the measuring cavity of a benchtop X-band EMX series ESR spectrometer (Bruker Biospin, Karlsruhe, Germany) using a high-sensitivity SHQ microwave cavity. Time-dependent formation of ROS was determined using the following ESR settings: center field, 1.99 g; microwave power, 20 mW; modulation amplitude, 2 G; sweep time, 10 s; number of scans, 10; field sweep, 60 G. The amplitude measurements of the ESR spectra were normalized to microvessel protein concentrations and expressed in arbitrary units; n represents the number of experiments that include microvessels isolated from two rat brains.

Western blot analysis. Proteins were harvested as described previously (28, 40). Briefly, cerebral arteries and microvessels were homogenized in ice-cold NP40 lysis buffer (Invitrogen, Frederick, MD) supplemented with proteinase inhibitor cocktail (cat. no. P8340, Sigma-Aldrich) and phosphatase inhibitor cocktail (cat. No. P2850, Sigma-Aldrich). Samples were centrifuged, and the supernatant was used for further analysis. The protein concentration was determined using Pierce BCA protein assay (Thermo Scientific). Protein samples were separated by gel electrophoresis on a 4–20% SDS-PAGE gradient gel, and proteins were transferred onto a polyvinylidene difluoride membrane. Membranes were blocked with casein blocking buffer (no. 92742020; Li-Cor, Lincoln, NE) for 60 min at room temperature. Membranes were then washed with Tris-buffered saline and 0.1% Tween-20 (TBST) (Sigma-Aldrich) and incubated overnight at 4°C with primary antibodies in casein-blocking buffer. The following primary antibodies for mitochondrial proteins were used: anti-Complex II Fp subunit I at 1:1,000 dilution (70 KDa, no. 459200; Invitrogen); anti-Complex III Subunit I core at 1:1,000 dilution (53 KDa, no. 459140; Invitrogen); ATP synthase Complex V subunit α at 1:500 dilution (50 KDa, no. 459240; Invitrogen); anti-voltage-depencence anion channel (VDAC) at 1:1,000 dilution to detect the endogenous levels of total VDAC (32 KDa, no. 486685; Cell Signaling Technology, Danvers, MA); and total dynamin-related protein-1 (DRP-1) at 1:1,000 dilution (no. 611112; BD Transduction, San Jose, CA). Antibodies for primary nonmitochondrial proteins were anti-manganese superoxide dismutase (MnSOD) aa114-220 at 1:5,000 (25 KDa, no. 611581; BD Transduction Laboratories); total endothelial nitric oxide synthase (eNOS) at 1:500 dilution (140 KDa, no. 610297; BD Transduction Laboratories); and the loading control β-actin at 1:5,000 dilution (42 KDa, no. A5411; Sigma-Aldrich). After incubation with the primary antibody, membranes were washed and incubated for 90 min at room temperature in 1% BSA-TBST with secondary goat anti-rabbit IgG at 1:2,500 dilution (no. 7074S, Cell Signaling Technology) or goat anti-mouse IgG at 1:5,000 dilution (no. 7076P2, Cell Signaling Technology). The final reaction was visualized using chemiluminescence (LumiGLO, Gaithersburg, MD) and autoradiography. Immunebands were scanned, and the optical density of each band was quantified and normalized to the intensity of the corresponding β-actin band using ImageJ software.

Data analysis and statistics. Results were expressed as means ± SE; n indicates the number of independent measurements. Data were analyzed using unpaired t-test and one-way ANOVA with Tukey’s post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Electron microscopy. The basic features of mitochondrial morphology and location are similar in middle cerebral arteries from ZO and ZL rats (Fig. 2, A and B, respectively) and microvessels from ZO and ZL rats (Fig. 2, C and D, respectively). Representative sections of middle cerebral arteries show heavy investment of mitochondria in cerebral vascular endothelium and vascular smooth muscle (VSM) in both types of rats. Usually, mitochondria are present singly and dispersed throughout the endothelial cells. However, mitochondria in VSM are much larger and display different configurations than in endothelium. Additionally, mitochondria in VSM are relatively large and numerous and characteristically are present in fields or clusters together with sarcoplasmic reticulum (SR) and are not dispersed evenly throughout the VSM. Harvested microvessels, prepared as described in MATERIALS AND METHODS, are composed of arterioles and capillaries with little contamination of nonvascular cells except for occasional erythrocytes. Similar to arteries, the VSM of arterioles contain extensive mitochondrial fields intermixed with SR in both strains of rats. In the arterioles, the VSM mitochondria retain relatively normal configuration, and the endothelial mitochondria also ap-
Fig. 4. Mitochondrial respiration profile of major cerebral arteries from ZO and ZL rats. A: effects of 1-NAME treatment on mitochondrial respiration in cerebral arteries of ZL and ZO rats. B: effects of treatment with diazoxide (DZ) on mitochondrial respiration of ZL and ZO groups. Data are expressed as means ± SE; n = 16–17 per group.

Mitochondrial respiration measurements. Oxygen consumption rates for different segments of mitochondrial and nonmitochondrial respiration are shown in Figs. 4 and 5. Maximal respiration was significantly higher in the untreated ZO group (375 ± 6.5 pM/μg protein per min) compared with the untreated ZL group (334.6 ± 6.66 pM/μg protein per min; n = 15, P < 0.05) (Fig. 5D). Other components of mitochondrial (basal respiration, ATP production, spare capacity, and proton leak) and nonmitochondrial respiration did not show a statistically significant difference among the untreated groups (Fig. 5, A–F). The 1-NAME treatment significantly increased the proton leak in the ZL group (65.24 ± 8.5 pM/μg protein per min; 94.94 ± 9.4 pM/μg protein per min; n = 15, P < 0.05) but not in the ZO group [68.95 ± 7.4 pM/μg protein per min; 61.43 ± 9.45 pM/μg protein per min; n = 15, P = not significant (NS)] (Fig. 5F). The 1-NAME treatment had no significant effect on the other components of mitochondrial and nonmitochondrial respiration (Fig. 5, A–F). In the DMSO-treated group, basal respiration (173.6 ± 12.7 pM/μg protein per min; 134.5 ± 11.03 pM/μg protein per min; n = 17, P < 0.05) and proton leak (122 ± 11.07 pM/μg protein per min;
86.5 ± 10.47 pM/μg protein per min; n = 17, P < 0.05) were significantly increased in ZO compared with ZL rats (Fig. 5, H and L). Similar to observations from the untreated group, DMSO had no significant effect on other mitochondrial and nonmitochondrial respiration components of the ZL and ZO groups (Fig. 5, G–L). Treatment with DZ significantly decreased nonmitochondrial respiration in the ZL (73.15 ± 6.89 pM/μg protein per min; 53.24 ± 5.81 pM/μg protein per min; n = 16, P < 0.05) but not in the ZO groups (67.48 ± 6.43 pM/μg protein per min; 65.43 ± 12.85 pM/μg protein per min; n = 16, P = NS) (Fig. 5G). The DZ treatment did not significantly alter other mitochondrial respiration components in either ZL or ZO groups (Fig. 5, H–L).

**Protein expression.** Levels of mitochondrial proteins (Complex II, Complex III, Complex V, VDAC, DRP-1, and MnSOD) were not significantly different in cerebral arteries and microvessels of ZO compared with ZL (Figs. 6 and 7). Levels of eNOS were significantly higher in microvessels of ZO compared with ZL (132.7 ± 20.84%; 76.67 ± 10.54%, n = 8, P < 0.05).

**ROS production in cerebral microvessels.** A characteristic ESR signal with characteristic spectrum (28) was detected in microvessels incubated with CMH. The magnitude of this signal (normalized arbitrary units) was significantly increased in the freshly isolated cerebral microvessels of the ZO (1,397.33 ± 317.86) compared with the ZL group (560.6 ± 63.43, n = 7 each, P < 0.05) (Fig. 8).

**DISCUSSION**

We have characterized for the first time the mitochondrial fine structure, mitochondrial protein mass, and functional mitochondrial dynamics of cerebral arteries and microvessels of ZL and ZO rats. The major findings of this study are the following: 1) mitochondrial morphological profiles in endothelium and VSM are similar in ZL and ZO arteries and extend to the level of arterioles and capillaries; 2) differences in mitochondrial respiration between ZL and ZO cerebral arteries are modest; 3) levels of mitochondrial proteins were similar between ZL and ZO groups both for major arteries and for microvessels; 4) the eNOS levels were significantly higher in the cerebral microvessels of the ZO compared with the ZL group; and 5) the mitochondrial ROS production was significantly increased in ZO cerebral microvessels compared with ZL. Collectively, these findings indicate that subtle differences, which might set the stage for more extensive changes during prolonged IR, are present in mitochondrial dynamics in the early stages of the metabolic syndrome.

Although the fine structure of large cerebral arteries in normal rats has been examined previously (41), we are unaware of any examination of mitochondrial profiles in cerebral arteries or microvessels of IR rats (11, 30, 40). Similar to our previous studies in male, non-IR rats (40), we found that mitochondria in endothelial cells are smaller and more oval in appearance than mitochondria in VSM and that mitochondria are dispersed throughout the endothelium. In contrast, mitochondria are normally localized in discrete fields running down the longitudinal axis rather than evenly distributed in VSM cells in both normal ZO and ZL rats. Additionally, mitochondria in these fields are interspersed with SR, cellular structures associated with calcium release and storage (11, 13, 14). We and others have shown that mitochondrial activation in VSM of large cerebral arteries leads to the generation of calcium sparks from the SR, which promotes vasorelaxation (11, 30, 47). A new finding is that the mitochondrial profile seen in large surface arteries is also found in parenchymal arterioles in our microvessel preparation. Thus it seems likely that the func-

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**Fig. 6.** Mitochondrial and nonmitochondrial protein expression in major cerebral arteries of ZL and ZO rats. Representative Western blots and summary data: Complex II, 70 kDa (A); Complex III, 53 kDa (B); Complex V, 50 kDa (C); voltage-dependence anion channel (VDAC), 32 kDa (D); dynamin-related protein-1 (DRP-1), 80 kDa (E); and manganese superoxide dismutase (MnSOD), 24 kDa (F). L = lean, O = obese. Data are expressed as means ± SE (n = 8–12 per group).

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tional relationship between mitochondria and SR in VSM is present at all levels of the precapillary cerebral circulation. Nonetheless, we have found that the mitochondrial-SR relationship, with respect to mitochondrial depolarization and the generation of calcium sparks to diazoxide and BMS-191095, are reduced in large cerebral arteries of IR rats (15, 16, 27). Additionally, because we could rapidly correct vascular dysfunction in cerebral arteries from young, IR animals with a ROS scavenger or a PKC inhibitor, it appears that obvious and irreversible changes in vascular structures have not yet occurred at this stage of the metabolic syndrome. The only difference in vascular morphology relates to the presence of a large number of vacuoles in endothelial cells of the microvessels from the ZO animals, which might be related to enhanced superoxide anion generation.

Different aspects of mitochondrial (basal respiration, ATP production, spare respiratory capacity, and proton leak) and nonmitochondrial respiration showed similar results in untreated ZL and ZO cerebral arteries except for maximal respiration, which was significantly higher in the ZO compared with the ZL group. Although there are opposing reports regarding the correlation between mitochondrial dysfunction and IR (18, 26, 31, 33, 39), our data are similar to that found by other laboratories regarding ex vivo measurements of mitochondrial respiration in different samples (3, 4, 8, 12, 23). Our previous data showed impaired vasodilation of cerebral arteries from ZO rats mediated by mitochondrial depolarization via DZ (27). Therefore, we examined mitochondrial respiration in cerebral arteries of ZO rats. Our data suggest that mitochondrial respiration is not significantly altered in cerebral arteries of young ZO rats. Increased maximal mitochondrial respiration levels support this claim, suggesting that the mechanisms necessary to increase respiration are preserved in young ZO rats and possibly are more functional than in the ZL group.

We also treated arteries with the nonselective NOS inhibitor, L-NAME, to examine the influence of NO in the mitochondrial respiration of these arteries. NO is an inhibitor of the mitochondrial respiratory chain, and as such it binds to cytochrome oxidase at the oxygen-binding site, causing reversible inhibition of cytochrome oxidase, thus reducing ATP production. It can also act through production of reactive nitrogen species, causing slow and weak but irreversible inhibition of different mitochondrial components (7). Therefore, we would have expected an increase in mitochondrial respiration after the inhibition of NOS, as has been reported previously (40). However, there was no significant effect of L-NAME treatment on the components of the mitochondrial respiration in the ZL and ZO groups with the exception of significantly increased proton leak in the ZL group. During conditions with normal oxygen levels, very little NO reaches cytochrome oxidase in mitochondria because it is scavenged by oxymyoglobin for nitrate and metmyoglobin production (7). Morley and Mattammal (34)
showed that levels of NOS are significantly lower in ZO compared with ZL rats. Therefore, we can also project that there are certain variabilities in NOS and NO levels between ZL rats and other typically used strains. In contrast, we reported increased eNOS levels in cerebral arteries from ZO rats (16, 27). However, despite these increased levels, NO bioavailability was found to be diminished (27, 29). We went on to demonstrate that eNOS in ZO arteries partly exists in an uncoupled state, contributing to increased ROS levels (29).

Our laboratory previously demonstrated the importance of mitoKATP channels in preconditioning and regulation of vascular tone (9, 10, 28, 30, 41). In the present study, treatment with DZ did not significantly affect any of the segments of mitochondrial respiration in either the ZL or ZO group. This finding is consistent with some of the previous findings (37, 40). However, DZ treatment significantly reduced nonmitochondrial respiration but only in the ZL group, which suggests the existence of differences in other segments of cellular respiration in these groups. MitoKATP channel openers may have different effects on mitochondrial respiration under different energetic conditions (38). This could explain the different tendencies in response to DZ, although without statistical significance. During this experiment, we also found increased proton leak and basal respiration of vehicle (DMSO)-treated ZO compared with ZL samples. Considering the multiple possible effects of DMSO, such as inhibition of inflammation, reduction in free radical formation, and increase in membrane transport (25), we suggest that any of these effects may be more noticeable in ZO than in ZL arteries.

Expression of mitochondrial (Complex II, Complex III, Complex V, VDAC, MnSOD, and DRP-1) proteins was not significantly different among the major cerebral artery groups. This finding is consistent with mitochondrial respiration measurements and corresponds with studies that found no correlation between IR and mitochondrial dysfunction (3, 4, 18, 23, 26, 33). Mitochondrial protein expression levels were similar in microvessel samples of the ZL and ZO groups.

We also measured ROS production in cerebral microvessels and found that superoxide levels were significantly higher in ZO compared with ZL rats. Higher eNOS levels and ROS production in ZO rats were observed previously in major cerebral arteries in which they were considered to be a possible compensatory mechanism to increased oxidative stress (16, 27); however, it is also feasible that uncoupled eNOS is the source of elevated superoxide levels (45, 48). Furthermore, the presence of relatively larger amounts of vacuoles in the endothelial cells is consistent with enhanced superoxide levels in microvessels from the ZO rats.

Type 2 diabetes and metabolic syndrome in general are enormous public health problems. With tens of millions of people affected by type 2 diabetes, it represents a heavy burden for both patients as well as the health system (21). Considering the complications of type 2 diabetes, attributable to increased oxidative stress such as diabetic neuropathy (35, 46) in which the ROS levels are increased in the initial stage of the disease, early antioxidative treatment could be beneficial in slowing the disease, specifically its complications.

There were a few limitations to our study. We did not have a sufficient microvessel mass for reproducible determinations of OCR, but we hope to overcome this limitation in future studies. In addition, we performed our experiments on young, male ZO and ZL rats and thus do not know how sex, aging, or a greater expression of the metabolic syndrome affects mitochondrial dynamics of large cerebral arteries and microvessels. Fisher-Wellman et al. (18) argued that mitochondrial dysfunction needs to be presented early in development to be considered a possible cause of IR. Also, the initial NO levels in the examined cerebral arteries were unknown, and therefore the interpretation of NOS inhibition is limited. Possible changes in mitochondrial function that may occur later in the development of the metabolic syndrome and their eventual influence on cerebral vasculature are yet to be investigated. Even though our present study and some of the previous studies from our laboratory show that uncoupled eNOS may be the source of increased ROS production, additional tests are required to fully understand underlying mechanisms.

In summary, we have for the first time directly measured mitochondrial respiration and determined levels of mitochondrial proteins in the large cerebral arteries and microvessels of insulin-resistant rats. Our results show that severe mitochondrial dysfunction is not present in the early stages of IR and that oxidative stress, possibly arising from uncoupling eNOS, might play a significant role in vascular dysfunction during IR.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: I.M. and D.W.B. conception and design of research; I.M., I.R., T.T., V.N.S., and P.V.G.K. performed experiments; I.M. and D.W.B. prepared figures; I.M. and D.W.B. analyzed data; I.M. and D.W.B. interpreted results of experiments; I.M. and D.W.B. drafted manuscript; I.M., I.R., T.T., V.N.S., P.V.G.K., and D.W.B. edited and revised manuscript; I.M., I.R., T.T., V.N.S., P.V.G.K., and D.W.B. approved final version of manuscript.

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