Dynamics of Enhanced Mitochondrial Respiration in Female Compared with Male
Rat Cerebral Arteries

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Running Title: Sex and Mitochondrial Respiration

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Abstract

Objective: Mitochondrial respiration has never been directly examined in intact cerebral arteries. We tested the hypothesis that mitochondrial energetics of large cerebral arteries ex vivo are sex-dependent.

Approach and results: The Seahorse XFe24 analyzer was used to examine mitochondrial respiration in isolated cerebral arteries from adult male and female Sprague Dawley rats. We examined the role of nitric oxide (NO) on mitochondrial respiration under basal conditions, using L-NAME, and following pharmacological challenge using diazoxide (DZ), and also determined levels of mitochondrial and non-mitochondrial proteins using western blot, and vascular diameter responses to DZ. The components of mitochondrial respiration including basal respiration, ATP production, proton leak, maximal respiration, and spare respiratory capacity were elevated in females compared with males, but increased in both male and female arteries in the presence of the NOS inhibitor. Although acute DZ treatment had little effect on mitochondrial respiration of male arteries, it decreased the respiration in female arteries. Levels of mitochondrial proteins in Complexes I – V and the voltage-dependent anion channel protein were elevated in female compared with male cerebral arteries. The DZ induced vasodilation was greater in females than in males.

Conclusions: Our findings show that substantial sex-differences in mitochondrial respiratory dynamics exist in large cerebral arteries and may provide the mechanistic basis for observations that the female cerebral vasculature is more adaptable after injury.
Key words: ATP production, mitochondria, mitochondrial respiration, mitoK\textsubscript{ATP} channels, oxidative phosphorylation, VDAC, nitric oxide

**New and Noteworthy**

Mitochondrial respiration and protein mass are higher in female cerebral arteries. Mitochondrial depolarization by diazoxide only affected mitochondrial respiration in female arteries, but mitochondrial respiration increased dramatically in both sexes after nitric oxide synthase inhibition. New insights regarding the mechanisms of sex-related differences in health, and perhaps disease, are provided.

**INTRODUCTION**

Mitochondria are dual membrane, dynamic organelles that provide energy for ATP dependent processes via oxidative phosphorylation. (10) The inner mitochondrial membrane contains the respiratory chain complexes (Complex I – IV) and the ATP synthase, Complex V. Under physiological conditions the electron transport chain produces a proton gradient across the inner mitochondrial membrane leading to ATP production. (1) Individual respiratory chain proteins are encoded by either nuclear or mitochondrial genomes. (11, 20, 51) The outer membrane contains the voltage dependent anion channel (VDAC) and the proteins comprising this channel are nuclear encoded. (35) Intricate signaling as well as coordinating and transport mechanisms, which are not fully elucidated, ensure the proper composition and functioning of essential elements of the electron transport chain.

We and other laboratories have shown that mitochondria play an important role in physiological and pathophysiological functions of the cerebral circulation. (9, 49)
Under normal conditions, the mitochondrial dependent vasodilation of large cerebral arteries by diazoxide (DZ), which opens the ATP sensitive potassium (mitoK_{ATP}) channels located on the inner mitochondrial membrane, (23, 28) has endothelial and vascular smooth muscle (VSM) components that contribute to the overall, integrated vascular response in male rats. (12, 33, 58) Diseases such as insulin resistance and experimental stroke affect the expression of mitochondrial proteins as well as mitochondrial dependent vascular responses. (32, 49) In male rats and neonatal pigs, mitoK_{ATP} channel activation prior to transient global ischemia or transient middle cerebral artery occlusion reduces brain infarct volume, preserves the normal responsiveness of large cerebral arteries to vasoactive stimuli, and protects the blood-brain barrier. (15, 31, 34, 42)

Mitochondrial function has not been sufficiently examined in female cerebral arteries. Previous investigations have shown that estrogen enhances the expression of mitochondrial proteins and suppresses mitochondrial-derived oxidative stress while increasing the synthesis of substances such as nitric oxide (NO), which might affect mitochondrial respiratory rate in cerebral vascular cells. (2, 18, 22, 24) The suggestion that estrogen increases efficiency of mitochondrial respiration appears to be based on evaluations of mitochondrial protein levels of cerebral vascular cells rather than by direct measurements. Thus, there has been no specific research concerning whether mitochondrial respiration is different in male and female cerebral arteries. (39) Furthermore, whether mitochondrial function is affected by exogenous agents which depolarize mitochondria, such as DZ, or by inhibition of nitric oxide synthase (NOS) is also not known.
We have examined, for the first time, whether the mitochondrial energetics of large cerebral arteries are sex dependent and regulated via mitochondrial membrane potential and NO. Using large cerebral arteries, \textit{ex vivo}, from male and female rats, we determined: (1) the mitochondrial oxygen consumption rate (OCR) under basal conditions and after pharmacological challenge using a mitochondria-specific drug, DZ, in the presence and absence of the NOS inhibitor, Nω-Nitro-L-arginine methyl ester (L-NAME); (2) the levels of mitochondrial and non-mitochondrial proteins relevant to mitochondrial function using western blots; and (3) the vascular responses of isolated, pressurized cerebral arteries to DZ.

**MATERIALS AND METHODS**

**Animals**

Male and female, 8-10 week old Sprague Dawley rats (SD) from Charles River Laboratories (Wilmington, MA) (n = 38 and 40, respectively) were randomly assigned to the experimental protocols. Animals were housed and cared for according to the Institutional Animal Care and Use Committee (IACUC) guidelines of Tulane University in compliance with NIH guidelines. Animal care was provided by the Department of Comparative Medicine. Standard food and water were given \textit{ad libitum}. Under 5% isoflurane (VetOne, Boise, ID) induced anesthesia, rats were decapitated and brains were removed and transferred to 4°C, oxygenated (20% O₂, 5% CO₂, 75% N₂) Ca²⁺ Krebs solution (mmol/L: 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.0 glucose, and 24.0 NaHCO₃) at 7.4 pH. This mode of anesthesia was chosen to minimize animal discomfort as recommended by the IACUC. The anterior, middle
cerebral, and basilar arteries were isolated, removed, and cleaned for the experiments described below.

**Electron Microscopy**

Rats were euthanized with anesthesia and perfused with a PBS solution containing 2% of glutaraldehyde and 3% of formaldehyde. Arteries were removed and kept in the perfusion solution for 1 h and post-fixed in 1% osmium tetroxide and embedded in Epon812. Ultrathin sections (80-90 nm) were mounted on formvar-coated nickel grids (200 mesh), air dried, and stained with 4.7% uranyl acetate and lead citrate (at 10 min and 2 min, respectively). The sections were put on grids and viewed at a magnification of 11,000 X using a FEI Tecnai BioTwin 120 keV TEM with a digital imaging setup (Wake Forest University Health Sciences, Winston-Salem, NC).

**Measurement of mitochondrial function**

Arteries were transferred into an XF24 islet capture microplate (#101122-100, Seahorse Bioscience, Corporate US Headquarters, MA). The Seahorse Bioscience XFe24 extracellular flux analyzer measured mitochondrial OCR, an indicator of mitochondrial respiration. (8, 25, 29) The Seahorse XFe24 analyzer uses oxygen and hydrogen ion sensitive fluorophores for repeated measurements of oxygen and proton concentrations in the assay medium surrounding the arteries. The assay cartridge plate (#100867-100, Seahorse Bioscience, MA) was hydrated overnight using an XF calibration solution (#100867-000, Seahorse Bioscience, MA) at 37°C in a non-carbon dioxide incubator. Seahorse XF Assay medium (#102365-100, Seahorse Bioscience, MA), containing 5.0 mM/L glucose and 2.0 mM/L pyruvate at pH 7.4 and 37°C was used for the experiments. Isolated cerebral arteries were placed in the bottom of the islet
plate wells and covered with a screen to minimize movement during the assay. Wells were filled with 525 μl XF assay medium and maintained at 37°C in a non-CO₂ incubator for 20 min and then the islet plate was inserted into the instrument. Our assay protocol included three cycles for baseline measurements, and then five cycles for each treatment (Fig. 2). For the assay, plate wells were loaded with a final concentration of 250 μM/L DZ or an equivalent amount of vehicle (DMSO), and then exposed sequentially to 2 μM/L oligomycin, 1 μM/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), or 1.5 μM/L antimycin plus 1.5 μM/L rotenone in the presence and absence of the non-selective NOS inhibitor L-NAME at 100 μmol/L (Fig. 2). We had four groups for both males and females: (1) DMSO vehicle (Vehicle-only), (2) DZ only (DZ-only), (3) DMSO plus L-NAME (Vehicle + L-NAME), and (4) DZ plus L-NAME (DZ + L-NAME). We performed the following mathematical calculations using the raw OCR values after normalization for vascular protein mass. Non-mitochondrial respiration = the minimum value of the five OCR measurements after antimycin and rotenone injection. Basal respiration = the values for OCR measurements prior to the first injections minus non-mitochondrial respiration. Proton leak = OCR measurements after oligomycin injection prior to FCCP injection minus non-mitochondrial respiration. ATP production = basal respiration minus proton leak. Maximal respiration = OCR values after FCCP injection prior to antimycin/rotenone injection minus non-mitochondrial respiration. Spare respiratory capacity = maximal respiration minus basal respiration.

The protein concentration of each well was determined after each Seahorse experiment. The arteries from all islet plate wells were transferred into individual Eppendorf tubes containing 4°C NP40 lysis buffer (Invitrogen, Frederick, MD) with the
inhibitors proteinase and phosphatase, both at 5 μL/mL (Sigma Aldrich, St. Louis, MO).

Arteries were homogenized and centrifuged at 1,000 g for 10 min and the supernatant was used for the Bradford protein assay (Thermo Scientific, Rockford, IL) according to manufacturer instructions: the BCA reagent was diluted to 50:1, reagent A:B. We pipetted duplicates of the samples and BSA Standards with the following concentrations (mg/ml): 0, 25, 125, 250, 500, 750, 1000, 1500, and 2000 into a 96 well plate. The BCA reagents were added to the wells containing either samples or standards. Afterward the plate was incubated at 37°C for 30 min, and an uQuant (BioTek) spectrophotometer at 540 nm was used to read the absorbance of the samples and standards. The standard curve and interpolation was used to calculate the concentration of the artery samples.

For mitochondrial function, we expressed OCR data in pM/min/µg.

**Western blot**

For western blot analysis on isolated cerebral arteries, proteins were harvested as described above and separated by a 4% - 20% SDS-PAGE gradient gel and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). The arteries were not used for Seahorse experiments. Membranes were blocked with casein blocking buffer (# 92740200, Li-cor, Lincoln, NE) for 1 h at room temperature, then washed 3 times with Tris-buffered saline and 1% Tween-20 (TBST) (Sigma Aldrich, St. Louis, MO). The membranes were incubated overnight at 4°C with the primary antibodies in casein blocking buffer for the following mitochondrial proteins: Anti-Complex I MT-ND1 at 1:500 dilution (36 KDa; #Ab181848, Abcam, Cambridge, MA); Anti-Complex II Fp subunit I at 1:1000 dilution (70 KDa; #459200, Invitrogen, Frederick, MD); Anti-Complex III Subunit I core at 1:1000 dilution (53 KDa; #459140, Invitrogen, Frederick, MD); Anti-OxPhos
Complex IV Subunit I at 1:500 dilution (57 KDa; #Ab14705, Abcam, Cambridge, MA);
ATP synthase Complex V subunit alpha at 1:500 dilution (50 KDa; #459240, Invitrogen,
Frederick, MD); anti-VDAC at 1:1000 dilution, detects the endogenous levels of total
VDAC (32 KDa; #4866S, Cell Signaling Technology, Danvers, MA); and for non-
mitochondrial proteins: Anti-manganese superoxide dismutase (MnSOD) aa114-220 at
1:5000 (25 KDa; #611581, BD Transduction Laboratories, San Jose, CA); total
endothelial NOS (eNOS) at 1:500 dilution (140 KDa; #610297, BD Transduction
Laboratories, San Jose, CA) and its Ser\textsuperscript{1176} phosphorylated form (peNOS) at 1:500
dilution (140 KDa; #9571, Cell Signaling Technology, Danvers, MA); total neuronal NOS
(nNOS) at 1:1000 dilution (150 KDa; #610309, BD Transduction Laboratories, San
Jose, CA) and its Ser\textsuperscript{1417} phosphorylated form (pnNOS) at 1:1000 dilution (160 KDa;
#ab5583, Abcam, Cambridge, MA); and the loading control β-actin at 1:5000 dilution (42
KDa; #A5441, Sigma Aldrich, St. Louis, MO). The membranes were washed 3 times
and incubated with their respective horseradish peroxidase conjugated secondary anti
goat- rabbit IgG at 1:2500 dilution (#7074S, Cell Signaling Technology, Danvers, MA) or
mouse IgG at 1:5000 (#7076P2, Cell Signaling Technology, Danvers, MA) in 1% BSA-
TBST for 1 h at room temperature. Chemiluminescence (LumiGLO, Gaithersburg, MD)
and autoradiography were used to visualize the final reaction. The optical density of
each band was quantified and normalized to β-actin immunoband intensity using
ImageJ Software (% intensity).

**Isolated, pressurized artery technique**

The middle cerebral arteries (MCAs) were isolated in 4°C Ca\textsuperscript{2+} Krebs solution
and then cannulated on both ends using two glass micropipettes filled with Ca\textsuperscript{2+} Krebs
Intraluminal pressure and temperature (70 mmHg and 37°C, respectively) were maintained while changes in arteriolar diameter were continuously recorded using a video microscope (Nikon Eclipse TS100, Sony CCD camera, VDA-10 Living Systems Instrumentation Video Dimension Analyzer, LabView 2.0, and HP computer). The arteries developed spontaneous myogenic tone in response to 70 mmHg intraluminal pressure. Vascular responses to 100, 200, and 250 μmol/L of DZ (Sigma Aldrich, St. Louis, MO) were determined on MCAs with intact endothelium in the presence and absence of 100 μmol/L of L-NAME.

Data analysis and statistics
All data were expressed as mean ± SE and analyzed using one-way analysis of variance for repeated measures (ANOVA) and the Tukey post hoc test. A p < 0.05 was considered statistically significant. The abbreviation ‘n’ indicates the number of independent measurements for each protocol.

Results
Cerebral arteries are well supplied with mitochondria in both endothelium and VSM in adult cerebral arteries of both sexes (Fig. 1). Oxygen consumption rates for arteries during the various stages of the Seahorse protocol are presented in Figures 3 and 4. In general, OCR values for Vehicle-only treated arteries were higher in females than in males (Fig. 3A, Fig. 4) and were increased in both groups in the presence of L-NAME (Fig. 3B-C, Fig. 4). DZ-only treatment reduced the OCR of female arteries to male values, but it had little or no effect on male arteries (Fig. 3B-C, Fig. 4). Mitochondrial proteins (Fig. 5) and NOS (Fig. 6) were higher in female compared with male arteries and arterial dilation to DZ was greater in female arteries (Fig. 7).
Fine structure of cerebral arteries

Electron microscopy demonstrated that both endothelium and VSM of adult rats of both sexes are heavily invested with mitochondria (Fig. 1). Mitochondria are located singly or in small groupings across the endothelial cells, while in VSM, mitochondria are much larger in size and of different configurations. A characteristic feature in VSM from cerebral arteries is the clustering or grouping of large numbers of mitochondria which form extensive mitochondrial “fields” in both sexes. Higher magnification of the mitochondrial clusters or fields shows the close association of mitochondria and sarcoplasmic reticula (images not shown). Our techniques did not allow us to quantify and compare the numbers or total volume of mitochondria in cerebral arteries of male and female rats.

OCR in the Vehicle-only group

Oxygen consumption rate is much higher in female compared with male arteries and increased in both groups with the administration of L-NAME (Fig. 3). Calculated components and statistical comparisons of OCR are presented in Figure 4. Non-mitochondrial respiration was significantly greater in female (26.7 ± 3.8 pM/min/μg protein) than in male arteries (10.6 ± 2.2 pM/min/μg protein) in Vehicle-only groups (Fig. 4A). Inhibition of NOS (Vehicle + L-NAME) resulted in significantly higher non-mitochondrial respiration in both female and male groups (55.66 ± 5.7 pM/min/μg protein; 54.6 ± 10.2 pM/min/μg protein; respectively) compared with the measurements in Vehicle-only groups. In addition, L-NAME eliminated the difference between female and male groups that was present in Vehicle-only groups. Basal respiration was significantly higher in female arteries compared with male arteries (96.9 ± 15.2
Administration of L-NAME increased basal respiration in male (97.8 ± 14.4 pM/min/μg protein) but not in female (98.7 ± 8.8 pM/min/μg protein) arteries (Fig. 4B). **ATP production** was greater in female (33 ± 5.3 pM/min/μg protein) compared with male arteries (15.1 ± 4 pM/min/μg protein) in the Vehicle-only groups (Fig. 4C). Addition of L-NAME increased **ATP production** in male but not in female arteries, but values in Vehicle + L-NAME treated arteries were not significantly different for females and males (41 ± 6.1 pM/min/μg protein; 48.6 ± 8.6 pM/min/μg protein; respectively). In the Vehicle-only groups we found significantly higher **proton leak** values (Fig. 4D) in female (63.6 ± 10.5 pM/min/μg protein) compared with male arteries (21.2 ± 4.6 pM/min/μg protein). However, in the presence of L-NAME the **proton leak** increased in male but not in female arteries (43.2 ± 11.7 pM/min/μg protein; 57.8 ± 8.5 pM/min/μg protein; respectively). The **maximal respiration rate** (147.2 ± 21.6 pM/min/μg protein versus 62.8 ± 16 pM/min/μg protein) (Fig. 4E) and the **spare respiratory capacity** were significantly greater in female compared with male arteries in Vehicle-only groups (50.4 ± 8.4 pM/min/μg protein versus 26 ± 7.3 pM/min/μg protein, respectively) (Fig. 4F). In the presence of L-NAME **maximal respiration** rate (224.3 ± 25.8 versus 117.7 ± 16.7 pM/min/μg protein) and **spare respiratory capacity** (125.6 ± 20.2 versus 85.9 ± 9.7 pM/min/μg protein) were significantly increased in both female and male arteries compared with the Vehicle-only groups (Fig. 4E-F).
OCR in the DZ-only group

**ATP production** (18.6 ± 3.2 pM/min/μg protein; p<0.05) and **maximal respiration** (78.4 ± 13.7 pM/min/μg protein; p<0.05) were reduced from Vehicle-only values in female cerebral arteries by the administration of DZ (DZ-only group) (Fig. 4C, E). Although there was a tendency for DZ to decrease non-mitochondrial respiration, basal respiration, spare respiratory capacity, and proton leak, these differences were not statistically significant in female arteries (Fig. 4A, B, D, F). In contrast, administration of DZ-only to male arteries did not significantly decrease the non-mitochondrial respiration, basal respiration, maximal respiration, ATP production, spare respiratory capacity, or proton leak compared with Vehicle-only treated arteries (Fig. 4 A-E). Only spare respiratory capacity (Fig. 4F) showed a tendency to decrease upon DZ administration in the male arteries (p = 0.566). Treatment with DZ + L-NAME resulted in a significant increase in all mitochondrial respiratory parameters in the male arteries compared with DZ-only (non-mitochondrial respiration: 66.9 ± 10.7 pM/min/μg protein versus 14.3 ± 3.1 pM/min/μg protein; basal respiration: 119.3 ± 14.8 pM/min/μg protein versus 37.9 ± 11 pM/min/μg protein; ATP production: 41.8 ± 7.4 pM/min/μg protein versus 12.6 ± 3.5 pM/min/μg protein; proton leak: 77.5 ± 14 pM/min/μg protein versus 25.3 ± 7.8 pM/min/μg protein; maximal respiration: 227.9 ± 21.6 ± 16.7 pM/min/μg protein versus 49.47 ± 12.06 pM/min/μg protein; spare respiratory capacity: 108.5 ± 12 pM/min/μg protein versus 11.6 ± 1.6 pM/min/μg protein; respectively). NOS inhibition caused a significant increase only in the non-mitochondrial respiration in the female DZ + L-NAME group (49.5 ± 6.6 pM/min/μg protein) compared with the DZ-only treated group (19.3 ± 3.2 pM/min/μg protein).
Mitochondrial protein levels

All mitochondrial protein levels (Fig. 5A-F) except Complex III protein (84 ± 11.4 %; p = 0.08), were significantly increased in the female arteries, including the components of the respiratory chain: Complex I (71.6 ± 1.6 %), Complex II (89.8 ± 9%), Complex IV (29.7 ± 3.8%), Complex V (126.2 ± 6.4%), and the VDAC (97.8 ± 21.9%) when compared with male arteries (Complex I: 50.9 ± 3.8%, Complex II: 63.7 ± 6.8%, Complex IV: 20.2 ± 3%, Complex V: 107.2 ± 3.4%, VDAC: 46.6 ± 6.4%) (Fig. 5A-F).

Levels of MnSOD were similar in the female (240.7 ± 60%) and male (210.9 ± 47%) arteries (data not shown).

Non-mitochondrial protein levels

Levels of phosphorylated eNOS and the phosphorylated eNOS/total eNOS ratio were significantly higher in female (88.2 ± 11.6 and 2.2 ± 0.6%, respectively) compared with male arteries (43.7 ± 1 0 and 0.88 ± 0.2%, respectively) (Fig. 6A, 6C). However, values for total eNOS were similar in the female compared with the male group (59.1 ± 3.2 and 53.7 ± 1.3%, respectively) (Fig. 6B).

Neuronal NOS levels were elevated in the female (95.8 ± 6.8) compared with male (63.8 ± 5%) arteries (Fig. 6E). In addition, levels of phosphorylated nNOS were significantly higher in female (97.7 ± 17.2%) compared with male (33.4 ± 12%) arteries (Fig. 5D). Furthermore, the phosphorylated nNOS/total nNOS ratio was significantly higher in female (1.2 ± 0.2%) compared with male arteries (0.5 ± 0.2%) (Fig. 6F).

Cerebral vasoreactivity to DZ

There was no difference between the baseline diameters of female (266 ± 9 μm) and male (282 ± 16 μm) MCAs although the male MCAs tended to develop greater
myogenic tone (52 ± 2.3%; p>0.09) compared with females (38.6 ± 6.6%). Diazoxide-induced responses to 100 and 200 μmol/L were similar in both male (36.2 ± 7.5 and 40.9 ± 7.3, respectively) and female (35 ± 7.5 μm and 50.4 ± 8.4 μm, respectively) MCAs, whereas the response to 250 μmol/L was significantly increased in female (54 ± 6.5 μm) compared with male (30.1 ± 1.2 μm) arteries (Fig. 7A, 7C).

Inhibition of NOS increased the myogenic tone in both female and male groups but was greater in female (36.8 ± 5.2 μm) compared with male (23 ± 7 μm) arteries (Fig. 7B). Co-treatment of L-NAME with DZ to endothelium intact arteries resulted in similar reduced vasodilation to 100, 200, and 250 DZ μmol/L of DZ in both groups (male: 20.5 ± 8.3 μm, 17.1 ± 9.8 μm, and 17.5 ± 7.9 μm, respectively; female: 18.4 ± 7.3 μm, 30.7 ± 9.9 μm, 31.3 ± 9.4 μm, respectively) (Fig. 7B, 7C).

**Discussion**

This study demonstrates, for the first time, distinct sex-specific differences in the dynamics of mitochondrial respiration in freshly harvested cerebral arteries from adult rats as well as in levels of mitochondrial proteins and arterial dilator responses to DZ. There are many novel findings from our experiments. First, overall patterns as well as specific parameters of mitochondrial OCR were significantly greater in adult female than in male cerebral arteries. Second, the enhanced mitochondrial respiration in female cerebral arteries correlated with a greater level of mitochondrial proteins. Third, mitochondrial respiration after DZ administration was reduced in adult female but not in male arteries. Fourth, when comparing differences in baseline values of OCR, inhibition of NOS resulted in a greater effect on mitochondrial respiration in adult male than in female arteries. Fifth, dilator responses to DZ were greater in female arteries. The
combination of evidence from many different perspectives and methods shows that there is considerable sex-related diversity in mitochondrial mass, function, and dynamics in rat cerebral arteries.

We adapted the Seahorse XFe24 analyzer technology to allow the determination of mitochondrial OCR in freshly isolated cerebral arteries for the first time. Although cerebral capillaries have been reported to have a high content of mitochondria because of transport mechanisms associated with the blood brain barrier (38), we are unaware of a direct electron microscopy examination of mitochondria in endothelium and VSM of resistance arteries in male and female rats. While an extensive analysis is beyond the scope of the present study, electron microscopy showed that the endothelium and VSM heavily invested with mitochondria in male and female cerebral arteries. In particular, large clusters or fields of mitochondria are present in VSM while smaller, usually more isolated mitochondria are present in endothelium. Networks of interconnected mitochondria have been reported in cultured cerebral vascular endothelial cells, (9) but it is unclear whether mitochondrial syncytia occur in native cerebral vascular endothelium. Previously, in male cerebral arteries, we showed that the sarcoplasmic reticula, the cellular structures involved in calcium release and sequestration, are in close proximity to mitochondria in VSM and evidence supports the contention that the mitochondria-sarcoplasmic reticula interactions promote relaxation of VSM. (9)

Although untested, it appears that the similar mitochondrial morphology of female arteries would likewise support mitochondrial interactions with sarcoplasmic reticula in promoting relaxation in VSM.
Previous studies, based largely upon expression of mitochondrial proteins, have suggested differences in mitochondrial respiration between male and female arteries and in cerebral vascular endothelial cells treated with estrogen, but did not actually measure OCR. (39) In our current study when we measured OCR, we found significantly increased basal respiration in isolated female compared with male cerebral arteries and significantly increased non-mitochondrial respiration. Thus, elevated mitochondrial OCR in female arteries under basal conditions, as well for OCR following manipulation of the electron transport chain with oligomycin, FCCP, antimycin, and rotenone, are positively associated with increased mitochondrial mass. The majority of the mitochondrial complex proteins are encoded by nuclear DNA and only 13 of the proteins are mitochondrial DNA (mtDNA) encoded. (11, 20, 52) Therefore, we determined the expression of the components of the mtDNA encoded Complex I, Complex III, and Complex IV as well as the nuclear DNA encoded Complex II and Complex V subunits, and VDAC protein. All of the mitochondrial protein levels, except the Complex III subunit \( (p = 0.08) \), were significantly higher in female compared with male cerebral arteries from adult rats, corresponding to previous reports in the literature. (50) Increases in Complex proteins encoded by nuclear DNA as well as mtDNA indicate the operation of integrating mechanisms to ensure optimal function of increased mitochondrial mass in female arteries. These data might indicate the enhanced capability of the cell to survive during an energetic crisis. (14, 29)

Reactive oxygen species (ROS) such as superoxide anion \( (\text{O}_2^-) \) are produced as a by-product of oxidative phosphorylation under normal conditions and are important signaling agents for maintenance of cell function. (51, 54) Under physiological
conditions, the major sites of \( \text{O}_2^- \) production are Complex I and III, although Complex II has also been reported to contribute to the basal ROS levels. (21, 51, 54, 59) Complex I primarily releases \( \text{O}_2^- \) to the mitochondrial matrix (36, 37, 41) while Complex III apparently releases \( \text{O}_2^- \) to both the mitochondrial matrix and intermembrane space (59). Superoxide anion is rapidly converted to hydrogen-peroxide (\( \text{H}_2\text{O}_2 \)) by MnSOD and can exit the matrix via aquaporin-like channels and contribute to higher cytosolic ROS levels after passing through the VDAC in the outer mitochondrial membrane. (26-27, 52) We found significantly higher Complex I, II, III and VDAC protein levels as well as higher OCR in female cerebral arteries, and would therefore expect greater ROS availability in female arteries. Nonetheless, it has been reported that oxidative stress is lower in female compared to male arteries, probably due to higher levels of ROS scavengers, greater proton leak, and higher NO production. We found no difference in the expression level of the MnSOD protein between male and female arteries. Our results are similar to others who have reported a lack of substantial sex-dependent differences in MnSOD protein expression. (46, 53) However, this enzyme has an extremely high catalytic efficiency and therefore reactions between MnSOD and \( \text{O}_2^- \) are diffusion rather than rate limited. Alternatively, under certain conditions, estrogen activation of the MAP kinase and the NF-\( \kappa \)B pathway (6, 7) might reduce cellular ROS levels by increased production and/or activity of MnSOD. (43, 45, 57)

Increased OCR in female arteries corresponded with enhanced ATP production as well as a higher proton leak. Proton leak, via mitochondrial anion carrier proteins such as adenine nucleotide translocase, occurs normally during oxidative phosphorylation and thus protons can return to the matrix independently of ATP
synthase. Administration of oligomycin, which inhibits ATP synthase, allowed us to estimate the magnitude of proton leak. Prior findings indicated that increased proton leak is inversely linked to ROS release by mitochondria; therefore, increased proton leak provides an important mechanism to decrease the level of total cellular ROS derived from mitochondria.

We have shown significantly increased phosphorylated eNOS and nNOS levels in female compared with male arteries and these findings are similar to reports by other laboratories. (16, 17, 30, 44) To examine the role of NO in the mitochondrial respiration, we measured OCR in the presence of the NOS inhibitor, L-NAME. Similar to others, (40, 48, 56) we found that NOS inhibition caused an increase in the OCR in both groups, which was significant in males as well as in females. However, compared with OCR values in untreated arteries, the magnitude of the increase in males was greater than females after L-NAME treatment. This result shows that the dynamics of NO inhibition of mitochondrial respiration are different in male and female groups. Despite the increased NOS protein expression in females, the inhibitory effect of NO is lower in females compared with males on a percentage basis, a finding consistent with the results of others. (16, 39, 50) We propose that under physiological conditions the bioavailable NO has a greater inhibitory effect on the mitochondrial respiratory chain Complex proteins in males. Superoxide anion, produced normally by mitochondria, can also react with the NO produced by NOS under physiological conditions, and form peroxynitrite (ONOO\textsuperscript{−}). Peroxynitrite can interact with heme proteins and thereby inhibit Complex IV and decrease the activity of Complex I via S-nitrosation mediation. (13) Bolan et al (3, 5) have shown that ONOO\textsuperscript{−} irreversibly inhibits Complex IV via an
intracellular, time-dependent process without immediate effect. However, exogenous ONOO\(^-\) applied on isolated brain mitochondrial resulted in an irreversible loss of Complexes II and III. (4) Furthermore, several studies have reported that isolated heart mitochondria and cultured oligodendrocytes treated with ONOO\(^-\) and NO donors, respectively, caused a loss of Complex II activity. (19, 40, 48, 56) Thus, NO and O\(_2\)\(^-\) can singly or together alter mitochondrial respiration. On the other hand, our findings may represent the suppression of mitochondrial respiration by endogenous factors, including NO and ONOO\(^-\) in male arteries under baseline conditions, and thus a greater capacity to increase OCR following L-NAME administration.

We have shown that mitoK\(_{\text{ATP}}\) channels have an important role in pharmacological preconditioning as well as in changes in vascular tone (9-10, 32-33, 49). In our current study we found a significantly greater arterial dilation to DZ, at the concentration used to determine OCR, in the female compared with male arteries. In accordance with our vasoreactivity measurements, we found a greater effect of DZ on OCR values in the cerebral arteries of females compared with males under physiological conditions. Male MCAs developed a greater myogenic tone compared with female MCAs, but L-NAME given alone caused vasoconstriction which was greater in female compared with male, consistent with the literature. (16-18, 50) We also found similar vascular responses to DZ in females and males in the presence of L-NAME. When we explored the underlying mechanism of DZ in the presence of L-NAME application, we found a greater percent increase in OCR in males compared with females. However, the overall values of OCR in DZ + L-NAME treated arteries were similar. This information further supports our recent finding that endothelium plays a
key role in diazoxide induced vasodilation in MCAs and suggests that its effect may depend on the energetic condition of the mitochondria and the available substrate, as reported by others. (47) In addition, the effects of DZ on mitochondria, which could affect OCR, involve activation of the PI-3 kinase-Akt pathway, elevated production of ROS, uncoupling of the respiratory chain, increased intracellular levels of calcium, and augmented production of NO and prostaglandins. Whether these factors or other, yet unrecognized, factors caused the dramatic DZ-induced decrease in OCR in female arteries to values observed in male arteries as well as the different degrees of dilation to DZ in male and female arteries, is unknown. Furthermore, we speculate that effects of mitoK\textsubscript{ATP} channel opening on mitochondrial respiration might be dependent upon basal OCR.

There were a few limitations of our study. We did not separate the female rats based upon the stage of estrus cycle, which could have affected cerebral vascular protein levels, and therefore we may have missed minor variations in the amount of mitochondrial proteins as well as levels of mitochondrial respiration. For example, we did not see a significantly increased total eNOS protein expression in female compared with male arteries which is similar to work by Duckles and Krause et al. (16) Nevertheless, our experiments provide a baseline for future studies exploring the effect of estrogen using ovariectomized rats with placebo and estradiol treatment. On the other hand, studying mitochondrial respiration \textit{ex vivo} using freshly harvested cerebral arteries most closely mirrors the \textit{in vivo} situation and most previous studies of sex dependent differences in cerebral arteries have not considered estrus cycle status. In addition, we cannot say with certainty whether mitochondrial dynamics of large cerebral
arteries correspond to smaller arteries, capillaries, or veins. Lastly, regional and tissue
specific differences in mitochondrial dynamics can be examined in future studies.
Our overall findings have advanced knowledge and have further defined sex-
specific differences in mitochondrial function using a novel approach combined with
traditional methods on freshly isolated cerebral arteries. Our results provide new
insights regarding the underlying mechanisms of sex-related differences in health, and
perhaps, in disease.

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Legends to figures

Figure 1. Electron microscopy of male and female cerebral arteries.
Representative sections show heavy investment of mitochondria in cerebral vascular endothelium and VSM in female (A,B) and male (C,D) rats. Mitochondria are located singly or in groups across the endothelial cells. Mitochondria in VSM are much larger and of different configurations than in endothelial cells. Typically, mitochondria in VSM are particularly numerous and are characteristically present in clusters or fields. Our methods did not allow quantification and comparison of mitochondrial numbers or volume in female and male arteries. Nonetheless, the basic features of mitochondrial morphology and location are similar in arteries from male and female rats. Magnification was 11,000X in each section. VSM=vascular smooth muscle, E=endothelium, IEL=internal elastic lamina, M=mitochondrion or mitochondria.

Figure 2. Seahorse experimental design. Determinations of the various parameters of oxygen consumption rate (OCR), expressed as pM/min/μg protein, are shown schematically. The Roman numerals show five different measurement cycles and the red arrows demonstrate the injection of diazoxide/DMSO/L-NAME, oligomycin, FCCP, antimycin, and rotenone. The gray boxes represent the different parameters that were calculated to characterize mitochondrial bioenergetics in cerebral arteries: basal respiration, ATP production, proton leak, maximal respiration, non-mitochondrial respiration, and spare capacity.

Figure 3. Mitochondrial respiration profiles of cerebral arteries from male and female rats. Mitochondrial OCR in cerebral arteries is greater in female compared with male cerebral arteries (A) and affected by L-NAME and DZ (B,C). Statistical analyses of
the different components of OCR are presented in Figure 3. Data are expressed as mean ± SE. p < (Vehicle-only, male and female, n = 38 each; DZ-only, male and female, n = 42 each; L-NAME-only, male and female, n = 15 each; DZ + L-NAME, male and female, n = 15 each).

Figure 4. Components of OCR in male and female cerebral arteries. All mitochondrial bioenergetic parameters including: (A) non-mitochondrial respiration, (B) basal respiration, (C) ATP production, (D) proton leak, (E) maximal respiration, and (F) spare respiratory capacity were significantly increased in female compared with male cerebral arteries. Furthermore, DZ caused a significant decrease in ATP production and maximal respiration in female but not male arteries. While NOS inhibition resulted in a greater increase in the respiration in male compared with female arteries, final values were similar. Data are expressed as mean ± SE. Sample sizes are the same as in previous figure.* p < 0.05 (Vehicle-only, female vs. male), † p < 0.05 (DZ-only vs. Vehicle-only), # p < 0.05 (Vehicle in the absence of L-NAME vs. in the presence of L-NAME), ‡ p < 0.05 (DZ treated in the absence of L-NAME vs. in the presence of L-NAME).

Figure 5. Mitochondrial protein expression is higher in female than male cerebral arteries. Representative western blots and summary data: (A) 32 kDa VDAC, (B) 36 kDa Complex I, (C) 70 kDa Complex II, (D) 53 kDa Complex III, (E) 57 kDa Complex IV, and (F) 50 kDa Complex V. Histograms showing increased protein levels in female arteries compared with male. Data are expressed as mean ± SE. * p < 0.05 (female vs. male, n = 10 - 14 samples in each group).
Figure 6. Phosphorylations of eNOS and nNOS are higher in female compared with male cerebral arteries. Representative western blots and summary data: (A) 140 kDa phosphorylated eNOS (peNOS) and (B) total eNOS, (C) phosphorylated eNOS/total eNOS ratio, (D) 155 kDa phosphorylated nNOS (pnNOS), (E) total nNOS, and (F) phosphorylated nNOS/total nNOS ratio. Data are expressed as mean ± SE. * p < 0.05 (female vs. male, n = 10 - 14 samples in each group).

Figure 7. Dilator responses of male and female cerebral arteries to DZ. (A) Diazoxide caused a dose-dependent dilation which was significantly greater in female compared with male to the highest dose. (n = 5 measurements in each group, respectively). (B) Treatment with L-NAME (n = 5 measurements in each group) decreased the DZ induced dilation in all groups. (C) Original recordings of diameter measurement. Data are expressed as mean ± SE. * p < 0.05, female vs. male; # L-NAME vs. L-NAME + DZ.

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Figure 1.
Figure 3.
Figure 5.

A) 32 kDa: VDAC 
   42 kDa: β-actin

B) 36 kDa: Complex I
   42 kDa: β-actin

C) 70 kDa: Complex II
   42 kDa: β-actin

D) 53 kDa: Complex III
   42 kDa: β-actin

E) 57 kDa: Complex IV
   42 kDa: β-actin

F) 50 kDa: Complex V
   42 kDa: β-actin

* indicates a significant difference between male and female groups.
Figure 7.