METHODS

A novel high-throughput assay for respiration in isolated brain microvessels reveals impaired mitochondrial function in the aged mice

Venkata N. Sure · Siva S. V. P. Sakamuri · Jared A. Sperling · Wesley R. Evans ·
Ivan Merdzo · Ricardo Mostany · Walter L. Murfee · David W. Busija ·
Prasad V. G. Katakam

Received: 2 July 2018 / Accepted: 27 July 2018 / Published online: 3 August 2018
© American Aging Association 2018

Abstract Cerebral blood flow (CBF) is uniquely regulated by the anatomical design of the cerebral vasculature as well as through neurovascular coupling. The process of directing the CBF to meet the energy demands of neuronal activity is referred to as neurovascular coupling. Microvasculature in the brain constitutes the critical component of the neurovascular coupling. Mitochondria provide the majority of ATP to meet the high-energy demand of the brain. Impairment of mitochondrial function plays a central role in several age-related diseases such as hypertension, ischemic brain injury, Alzheimer's disease, and Parkinson disease. Interestingly, microvessels and small arteries of the brain have been the focus of the studies implicating the vascular mechanisms in several age-related neurological diseases. However, the role of

microvascular mitochondrial dysfunction in age-related diseases remains unexplored. To date, high-throughput assay for measuring mitochondrial respiration in microvessels is lacking. The current study presents a novel method to measure mitochondrial respiratory parameters in freshly isolated microvessels from mouse brain ex vivo using Seahorse XFe24 Analyzer. We validated the method by demonstrating impairments of mitochondrial respiration in cerebral microvessels isolated from old mice compared to the young mice. Thus, application of mitochondrial respiration studies in microvessels will help identify novel vascular mechanisms underlying a variety of age-related neurological diseases.

Keywords Mouse brain microvessels · Oxygen consumption rate (OCR) · Aging · XFe24 analyzer

V. N. Sure · S. S. V. P. Sakamuri · J. A. Sperling ·
W. R. Evans · I. Merdzo · R. Mostany · D. W. Busija ·
P. V. G. Katakam
Department of Pharmacology, Tulane University School of
Medicine, New Orleans, LA 70112, USA

W. R. Evans · R. Mostany · D. W. Busija ·
P. V. G. Katakam (✉)
Tulane Brain Institute, Tulane University, 1430 Tulane Avenue;
Room 3554C, 8683, New Orleans, LA 70112, USA
e-mail: pkatakam@tulane.edu

I. Merdzo
Department of Pharmacology, University of Mostar School of
Medicine, Mostar, Bosnia and Herzegovina

W. L. Murfee
J. Crayton Pruitt Family Department of Biomedical Engineering,
University of Florida, Gainesville, FL 32611, USA

Introduction

The brain is one of the highest energy demanding organs in the human body with the third highest metabolic rate after the heart and kidney (Elia's Ki values 440 for heart and kidneys, 240 for brain and 200 for liver) (Wang et al. 2010). Glucose is the obligatory energy substrate for the adult human brain except under special circumstances such as during development, starvation (Nehlig 2004), and intense physical activity (van Hall et al. 2009; Belanger et al. 2011) when the brain can use other energy substrates such as glycogen, ketone bodies, and lactate. Even though the human brain is only 2% of the

body's mass, its metabolic rate accounts for approximately 20% of oxygen and 25% of glucose consumed by the whole body (Wang et al. 2010; Belanger et al. 2011). A continuous supply of oxygen and glucose is maintained by cerebral blood flow (CBF) to neural tissue through the complex web of blood vessels in the brain's vascular system (Williams and Leggett 1989). A stable blood supply to the brain is achieved through regulation of cerebral vasculature by both the local and systemic mechanisms. The cerebral vasculature is endowed with neurovascular coupling that regulates CBF to local meet energy demand by coordinating the interaction of neurons, glia, and vascular cells (Girouard and Iadecola 2006). Neurons and glia initiate signals for the vasodilation process, whereas vascular cells such as endothelial cells, smooth muscle cells, and pericytes carry out controlled vasodilation to increase CBF to provide more substrates for active part of brain (Girouard and Iadecola 2006).

Interestingly, several age-related pathological conditions including hypertension (Girouard and Iadecola 2006; Csiszar et al. 2017; Tucsek et al. 2017), Alzheimer's disease (AD) (Farkas and Luiten 2001; Tarantini et al. 2017a), and ischemic stroke (Iadecola 1993) are accompanied by impaired neurovascular coupling (Ungvari et al. 2010a). Disruptions in neurovascular coupling not only reduce delivery of energy substrates but also impair the removal of deleterious by-products of cerebral metabolism which ultimately contributes to brain dysfunction in age-related cerebrovascular diseases (Girouard and Iadecola 2006; Tarantini et al. 2017b). Previous studies have shown a strong correlation between decreased total cerebral blood flow and brain perfusion with aging (Girouard and Iadecola 2006; Farkas and Luiten 2001). Similarly, cerebrovascular structure and function are profoundly altered in AD (Farkas and Luiten 2001). Recent studies have shown that cerebrovascular dysfunction precedes the onset of cognitive impairment, and vascular impairment has been proposed as a causative mechanism of the dementia (Iadecola 1993; de la Torre 1994). Ischemic stroke is also characterized by cerebrovascular dysfunction. It has been shown in both and focal and global ischemia models that with the onset of reperfusion, the cerebral circulation experiences a state of vasoparalysis due to the impairment of neurovascular coupling (Kunz and Iadecola 2009). Several studies have demonstrated that age-dependent decreases in CBF and reduced metabolic support for neuronal signaling may contribute to the neurodegenerative changes found in

aging animals and humans (Ungvari et al. 2010a; Sonntag et al. 1997). However, the exact mechanisms mediating the age-dependent decline in CBF are poorly understood.

Mitochondria play a key role in a variety of cellular processes. Primarily, mitochondria are the main bioenergetic organelle involved in ATP production, oxidative phosphorylation, and oxygen sensing. Additionally, mitochondria are important for redox and ion homeostasis (Grimmig et al. 2017). They play an important role in cellular signaling and regulation of apoptosis (Dai et al. 2012; Ungvari et al. 2010b). Mitochondrial dysfunction has been proposed to play a central role in cellular senescence and aging-related diseases such as AD (Grimmig et al. 2017; Castellani et al. 2002; Devi et al. 2006; Reddy and Beal 2008; Deepa et al. 2017) and ischemic brain injury (Starkov et al. 2004; Chan 2005; Sure and Katakam 2016; Yu et al. 2017). It has been shown that cerebral microvascular endothelial dysfunction and increased oxidative stress impair neurovascular coupling contributing to the age-related decline of higher cortical functions (Tarantini et al. 2018). There is increasing evidence supporting the importance of mitochondrial function in a range of age-related cellular impairments, but their role in cerebral microvasculature and neurovascular uncoupling remains unexplored (Dai et al. 2012; Springo et al. 2015). Thus, gaining the insights into mitochondrial bioenergetics in the microvasculature of the aging brain may help identify new therapeutic targets.

Many techniques exist to measure mitochondrial respiration in cultured cells and isolated mitochondria from different organs. However, there are no high-throughput techniques available to measure mitochondrial respiration in isolated brain microvessels to date. Previously, the Clark electrode (Clark 1956) and many oxygen electrode systems have been employed to measure oxygen consumption rate (OCR) in biological samples (Clark and Sachs 1968; Jung et al. 1999; Pasarica et al. 2009; Grassi et al. 1996). More recently, Seahorse Bioscience introduced the "mito stress test" using the Seahorse Extracellular Flux Analyzer as a complementary method for the assessment of mitochondrial parameters in adherent cell culture (Katakam et al. 2016; Katakam et al. 2014; Wu et al. 2007; Zhang et al. 2012), pancreatic islets (Navarro et al. 2018) and isolated mitochondria (Sakamuri et al. 2018). Agilent Seahorse XF Analyzer is a sensitive, robust, high-throughput, less labor intensive, and automated system compared to traditional Clark-type oxygen

electrode and Oroboros Oxygraph-2 k systems (Oroboros O2k) (Horan et al. 2012). Furthermore, Agilent Seahorse XF system can do internal calibration with background correction and can measure OCR, ECAR, and CO₂ parallelly (Horan et al. 2012). Agilent Seahorse XF Analyzers can simultaneously measure OCR which is an indicator of mitochondrial respiration and extracellular acidification rate (ECAR) which gives information about glycolysis. The analyzer makes use of a disposable sensor cartridge with two fluorophores for measuring oxygen and pH in a transient microchamber formed by lowering sensor cartridge at the bottom of the well in the microculture plate at regular intervals (Ferrick et al. 2008). For the first time, we have developed a high-throughput assay for real-time assessment of mitochondrial respiration in freshly isolated microvessels from the mouse brain using Seahorse XFe24 Analyzer (Fig. 1). OCR measurements in the isolated brain microvessels using electron transport chain modulating agents such as oligomycin, FCCP, and antimycin A/rotenone allow for the determination of changes in the mitochondrial respiratory parameters such as basal respiration, ATP production, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial respiration. Here, we validated our novel protocol for measuring mitochondrial respiration by comparing the mitochondrial respiratory parameters of microvessels isolated from young and old mouse brains.

Our studies showed decreased bioenergetic profile in brain microvessels isolated from aged mice compared to

the microvessels isolated from young mice as shown in Fig. 2. A detailed analysis of OCR measurements has shown that brain microvessels of aged animals showed significantly decreased basal respiration, maximal respiration, spare respiratory capacity, proton leak, and non-mitochondrial respiration compared to the brain microvessels from young animals as shown in Fig. 3. These results are consistent with the previous reports of mitochondrial dysfunction in several age-related vascular diseases (Marzetti et al. 2013; Payne and Chinnery 1847; Izzo et al. 2018). Furthermore, the findings from the experiments demonstrate the sensitivity of the novel assay by identifying the microvascular mitochondrial impairments even at the early stage of aging in 18-month-old mice.

Materials and equipment

Chemicals and equipment Heparin sodium injection, USP 5000 U/mL (NDC 25021-402-10), Fisherbrand™ FH10 Peristaltic Tubing Pump (13-310-651, Fisher Scientific, Hampton, NH), Butterfly Needle 21G (134331, Praxidienst Medical Supplies, Longuich, Germany), Dulbecco's phosphate-buffered saline (DPBS 1X, 14190-144, Gibco, Waltham, MA), Dextran 60–90 kD (14495, Affymetrix, USB, OH), bovine serum albumin fraction V, heat shock (9048-46-8, Roche Diagnostics, Mannheim, Germany), Wheaton 15-mL Tissue grinder (357544, Wheaton, NJ), Centrifuge 5804R (Eppendorf, Hamburg, Germany), Centrifuge 5430 R (Eppendorf,

Fig. 1 Schematic representation of the Agilent Seahorse XF Mito Stress assay. The Agilent SeahorseXFe24 analyzer measures OCR at basal and after injection of oligomycin, FCCP, and antimycin A/rotenone for three measurement cycles at each step. Key parameters of mitochondrial function such as basal respiration, ATP

production, proton leak, maximal respiration, spare respiratory capacity, and nonmitochondrial respiration can be interpreted as shown in the figure. Time points of different drug injections are shown with arrows

Fig. 2 Bioenergetic profile of microvessels isolated from young and old mouse brains. Seahorse mito stress assay was performed to measure OCR in freshly isolated microvessels from young and old mouse following sequential injection of oligomycin, FCCP, and antimycin A/rotenone to determine key parameters of mitochondrial respiration. Microvessels from aged mice brains have shown decreased mitochondrial respiration compared to microvessels from young mice brains

Hamburg, Germany), non-sterile single-tip cotton swab with wood handle, Falcon™ Cell Strainers 40 μ m Nylon (08-771-1, Fisher Scientific, Hampton, NH), pluriStrainer® 200 μ m, (43-50200-50, pluriSelect Life Science, Leipzig, Germany), Pierce BCA Protein Assay Kit (23227, Thermo Fisher Scientific, Waltham, MA), Seahorse XFe24 Analyze (Agilent Technologies, Santa Clara, CA), XFe24 extracellular flux assay kit (100777-004, Agilent Technologies, Santa Clara, CA), Seahorse XF24 cell culture microplates (Agilent Technologies, Santa Clara, CA), Seahorse XF calibrant (100840-000, Agilent Technologies, Santa Clara, CA), Seahorse XF assay medium modified DMEM (102365-100, Agilent Technologies, Santa Clara, CA), sodium pyruvate (P8574 Sigma-Aldrich, MO), D-(+)-glucose (G7528, Sigma-Aldrich, MO), GlutaMAX™-I (100X) (35050-061, Gibco, Waltham, MA), oligomycin, FCCP (103015-100, Agilent Technologies, Santa Clara, CA), antimycin A (A8674, Sigma-Aldrich, MO), rotenone (R8875, Sigma-Aldrich, MO).

Primary antibodies Polyclonal rabbit anti-von Willebrand factor (vWF) antibody (F3520, Sigma-Aldrich, St. Louis, MO), monoclonal mouse anti-zonula occludens-1 (ZO-1) antibody (33-9100, Thermo Fisher Scientific, Carlsbad, CA), monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (G3893, Sigma-Aldrich, St. Louis, MO), polyclonal rabbit anti-microtubule-associated protein-2

(MAP-2) antibody (SC-20172 Santa Cruz Biotechnology, Dallas, TX).

Secondary antibodies Goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488 (A11029, Thermo Fisher Scientific, Carlsbad, CA), goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 546 (A11035, A11029, Thermo Fisher Scientific, Carlsbad, CA).

Isolation of microvessels from mouse brain

All experimental protocols were approved by the Institution of Animal Care and Use Committee (IACUC) of Tulane University School of Medicine in accordance with the National Institute of Health (NIH) guidelines for the animal care and use. The Department of Comparative Medicine of Tulane University provided the animal care. We used 3 months (young) and 18 months (old) wild type (C57BL/6J strain) male mice purchased from Jackson Laboratory (Bar Harbor, ME). The rationale for using 18-month-old mice was to highlight the sensitivity of the novel assay we described here in detecting the microvascular mitochondrial impairments even at the early stage of aging. Isolation of microvessels from mouse brains protocol has been modified from previously published protocols (Silbergeld and Ali-Osman 1991; Yousif et al. 2007; Stirone et al. 2005; Merdzo et al. 2016, 2017).

Method of isolation

- 1) Inject each mouse with 50 μ L (250 USP units) of intraperitoneal heparin. Anesthetize the mouse 15 min after heparin injection using isoflurane (VETOne, ID).
- 2) Perform an intracardiac perfusion using DPBS (approximately 20 mL for 5–10 min until the organs turn pale) to remove blood from the vessels.
- 3) After perfusion, cut the mouse head and section the skin with scissors from the neck to the nose and peel off the skin. Wash the head with DPBS to remove the small hair on the skull.
- 4) Cut the skull into two parts on the top using scissors and pull the broken skull away to expose the brain. Use a spatula to lift the brain from the bottom and place it in ice cold DPBS on ice (be

Fig. 3 Effect of age on mitochondrial respiration in microvessels. OCR measurements showed that microvessels from aged mice brain showed significant decrease in **a** basal respiration, **c** maximal respiration, **d** spare respiratory capacity, **e** proton leak, and **f** Non-mitochondrial respiration. There is no significant difference in **b** ATP production between microvessels isolated from young and old mice even though there is a trend of reduced ATP production in

aged brain microvessels. All data are expressed as mean $\pm$ standard error of the mean (mean $\pm$ SEM) and analyzed using a *t* test to compare two groups. *n* = 8–12 wells, microvessels separated from seven to eight mice each group. A *p* value of < 0.05 was considered statistically significant. GraphPad Prism 5 software was used for statistical analysis. * *p* < 0.05 ** *p* < 0.01

quick in removing the brain and transferring it into ice cold DPBS).

- 5) Place the 15-mL glass homogenizer with 5 mL DPBS on ice. Place the brain on filter paper and remove the hindbrain and olfactory lobes. Open the cerebral hemispheres using curved forceps and remove the white matter as much as possible. Then, roll the cortical hemispheres on the filter paper to remove large surface vessels. (This step is critical for microvessel yield and recommended to be performed on ice.)
- 6) Transfer the cerebral tissue to the homogenizer with 5 mL ice cold DPBS on the ice and homogenize using gentle strokes without generating bubbles until tissue is thoroughly homogenized (Depending on the force you use, it takes approximately 15–17 strokes.).

Note: Use 2 to 2.5 brains per one 15 mL homogenizer with 5 mL DPBS.

- 7) After homogenization, transfer the homogenate into a new 50-mL conical tube. Rinse the glass rod and homogenizer with an additional 5 mL of DPBS to collect additional homogenate on the glass rod and in the homogenizer and transfer it to the tube with 5 mL homogenate. At the end of

this step, each tube should have 10 mL of homogenate.

- 8) Centrifuge the homogenate at $3234\times g$ (max speed) for 15 min using a tabletop centrifuge (5804R, Eppendorf, Hamburg, Germany).
- 9) After centrifugation, remove the supernatant carefully and resuspend the pellet in 10 mL of 17.5% Dextran.
- 10) Filter the resuspension through a 200- μ m filter using a 5-mL pipette to remove unhomogenized tissue pieces or hair (Based on your interest in the microvessels size range, you can also use 300 or 100 μ m mesh.).

Note: Microvessels are very fragile and sensitive. Quality and yield depends on gentle pipetting during resuspension or filtration.

- 11) Centrifuge the filtrate at $7917\times g$ (max speed) for 15 min using the centrifuge (5430 R, Eppendorf, Hamburg, Germany)
- 12) After the centrifugation, a dense white myelin plug at the surface of supernatant and microvessels pellet at the bottom can be seen. Go through steps 13 to 15 or directly to step 16.
- 13) Pour the supernatant with myelin plug into a new tube and shake the tube by hand a few times to mix

the homogenate. Centrifuge the suspension at 7917×g (max speed) for 15 min.

- 14) During centrifugation, clean the tube with microvessels pellet using Kim wipes rolled over Q-tips to remove myelin on the walls. Resuspend the pellet in 5 mL of 17.5% Dextran.
- 15) After the centrifugation of the new tube with myelin plug suspension, discard the supernatant with myelin plug and resuspend the microvessels pellet in 5 mL of 17.5% Dextran. Combine the two tubes with 5 mL suspension each and centrifuge at 7917×g (max speed) for 15 min.

Note: Steps 13 to 15 are optional to improve microvessels yield. After first centrifugation with 17.5% Dextran, myelin plug still might have some microvessels. Resuspending the myelin plug with 17.5% dextran and centrifuging in the new tube will increase microvessel yield especially while isolating the microvessels from rat brain.

- 16) Discard the myelin plug and supernatant and clean the walls carefully with Kim Wipes rolled over Q-tips to remove any remaining myelin.
- 17) The pellet containing the vessels remains attached at the bottom of the tube.
- 18) Gently resuspend the pellet in 1 mL of 2% BSA solution and add an additional 1 mL of 2% BSA. Carefully resuspend the vessels.
- 19) Filter the microvessels suspension through a 40-μm filter into an empty 50 mL tube. Repeat the step with the extra 1 mL resuspension.

Note: Make sure not to wet the filter walls during filtration. It will be hard to get a good yield as wetting the walls makes many of the vessels to stick to the walls and it will be difficult to wash them off the filter.

- 20) After filtration, flip the filter and place it over a new 50-mL conical tube. Use 2% BSA to wash the vessels off the filter into the new tube. Use enough force to wash off vessels from the filter but do not use excess force. It takes approximately 10 mL (use 1 mL each time) to wash off all the vessels from the filter.
- 21) Centrifuge 10 mL of microvessels suspension in 2% BSA at 7917×g for 20 min.

Measurement of oxygen consumption rate using seahorse XFe 24 bio analyzer

Day before the assay

1. Open the Agilent Seahorse XF24 flux assay kit and place the sensor cartridge upside down next to the utility plate
2. Add 1 mL of XFe24 calibration buffer to each well of XFe24 sensor cartridge utility plate
3. Place the sensor cartridge back onto the utility plate by submerging the sensors in XF Calibrant buffer
4. Incubate the plate overnight in a non-CO₂ incubator at 37 °C
5. Wrap the sensor cartridge utility plate tightly with parafilm to prevent evaporation of calibrant during the overnight incubation in a non-CO₂ incubator. (Optional)

On the day of experiment

Preparation of seahorse assay media

During the last spin of microvessels isolation, prepare seahorse mito stress assay media by adding 450 mg of D-glucose (25 mmol/L), 2 mL of GlutaMax (4 mmol/L), and 110 mg of sodium pyruvate (10 mmol/L) to 100 mL of XFe Assay media. Keep the media in water bath at 37 °C for 15 to 20 min and then adjust the pH to 7.42 using 0.1 N NaOH solution. Keep the assay media at 37 °C until ready to use.

Seahorse assay

1. After the final centrifugation, remove the supernatant and gently resuspend the microvessels pellet in 2 mL of warm and pH adjusted assay media. Split the 2 mL of microvessels re-suspension (microvessels from two mouse brains) into two 2-mL tubes
 2. Centrifuge the tubes (each tube has 1 mL of suspension from approximately 1 brain) at 800×g for 10 min
- Note: During the centrifugation, prepare drug solutions of desired concentrations in assay media. Make stock solutions of oligomycin, FCCP, antimycin/rotenone as follows.

Note: Oligomycin and FCCP stock solutions were made from Agilent seahorse mito stress kit and

antimycin and rotenone stocks were made from drugs bought from Sigma-Aldrich. Oligomycin—100 $\mu\text{mol/L}$ (in assay media). FCCP—100 $\mu\text{mol/L}$ (in assay media). Antimycin A/rotenone—10 mmol/L (in 95% alcohol)

3. Decant the supernatant and resuspend the pellet into 100 μL of assay media by gentle pipetting
4. Add 50 μL of microvessels resuspension into each well of the seahorse micro culture plate (approximately 0.5 brain to each well). Centrifuge the plate at $1500\times g$ for 12–15 min at 4 $^{\circ}\text{C}$
5. During the centrifugation, dilute the drug solutions to the desired concentrations as shown in Table 1 (oligomycin 20.88 $\mu\text{mol/L}$, FCCP 12.44 $\mu\text{mol/L}$, antimycin/rotenone 97.77 $\mu\text{mol/L}$) with assay media and load them in different injection ports of the XFe24 sensor cartridge as follows
 - Port A: oligomycin (30 μL);
 - Port B: FCCP (30 μL);
 - Port C: antimycin A and rotenone (30 μL).
6. Design the assay protocol (as shown in the Table 2) on seahorse XFe 24 analyzer's wave software and load the sensor cartridge plate with drug solutions in the ports for calibration
7. After the centrifugation of seahorse micro culture plate (from step 4), add 150 μL of assay media very carefully. Try to preserve the microvessels attachment to the bottom of the well
8. Incubate the plate in a non- CO_2 incubator until the calibration is done (At least 10 min). Exchange the calibration plate with the culture plate with microvessels and run the assay
9. After the experiment, take out the plate and transfer the content of each well to 2 mL tube and centrifuge them at high speed. Decant the supernatant and add 50 to 75 μL of NP-40 lysis buffer to each tube and determine protein concentration using PierceTM

Table 2 Experimental design to program the mix, wait measure, and injection protocol for Seahorse XFe24 mito stress assay on isolated microvessels from mouse brain

Command	Time	Compound
Equilibration		
Basal		
Number of cycles	3	
Mix	3 min	
Wait	2 min	
Measure	3 min	
Inject port A		Oligomycin
Number of cycles	3	
Mix	3 min	
Wait	2 min	
Measure	3 min	
Inject port B		FCCP
Number of cycles	3	
Mix	3 min	
Wait	2 min	
Measure	3 min	
Inject port C		Antimycin A/rotenone
Number of cycles	3	
Mix	3 min	
Wait	2 min	
Measure	3 min	

BCA Protein Assay Kit. Normalize the data with protein concentration

Seahorse data analysis

1. Export data from seahorse XFe24 analyzer's Wave 2.4.0. software to excel file

Table 1 Preparation of the drug solutions to load sensor cartridge ports for Seahorse XFe 24 analyzer. Starting volume of the well is 200 μL

Drug	Stock solution		Assay medium	Total	Volume to be added	Drug concentrations	
	Concentration	Volume (μL)	Volume (μL)	Volume (μL)	To port (μL)	In port ($\mu\text{mol/L}$)	In well ($\mu\text{mol/L}$)
Oligomycin	100 $\mu\text{mol/L}$	470	1780	2250	30	20.88	2.72
FCCP	100 $\mu\text{mol/L}$	280	1980	2250	30	12.44	1.44
Antimycin A (A) + rotenone (R)	10 mmol/L	22	2206	2250	30	97.77	10
	10 mmol/L	22					

2. Check for blank values if there are any false positive OCR values and deselect them before exporting data to excel sheet
3. Normalize the OCR values with the protein concentration and calculate the mean value of normalized control basal OCR values. Use this normalized control mean value to normalize the OCR values of other wells and express them as percentage of control
4. After normalization, calculate the various components of mitochondrial respiration such as basal respiration (basal OCR–OCR values after injecting A/R), maximal respiration (highest OCR values of OCR after FCCP injection–OCR values after injecting A/R), spare respiratory capacity (maximal

respiration–basal respiration), ATP production (basal respiration–OCR values after injecting oligomycin), proton leak (OCR values after injecting oligomycin–OCR values after injecting A/R), and non-mitochondrial respiration (OCR values after injecting A/R) as shown in the Fig. 1

Immuno-labeling of microvessels

1. Decant the 2% BSA solution and resuspend microvessels in 300 μL $1\times$ DPBS
2. Transfer 100 μL of suspension to 0.2-mL PCR tubes using a low binding tip. Make sure not to leave microvessels inside the tip while transferring

Fig. 4 Characterization of isolated mouse brain microvessels. Brain microvessels were immunolabeled. **a** Endothelial cell marker: vWF (red color). **b** Blood-brain barrier (BBB) marker: ZO-1 (green color). **c** Neuronal marker: MAP-2 (red color). **d** Glial cell marker: GFAP (green color). Blue: Nucleus stained by DAPI. Negative controls were shown for each antibody. Bright field images were shown for each slide. Negative control (without

primary antibodies) samples showed no fluorescence. Image were taken at $\times 40$ using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Carlsbad, CA). Scale bar in the figure is 100 μm . Mouse brain microvessels are positive for endothelial cell, BBB markers and negative for neuronal cell and astroglial cell markers as shown in the figure (color figure online)

3. Use a mini centrifuge to spin down the microvessels to the bottom of the tube at each step (2000 g for 5 min)
4. Spin down the microvessels resuspended in DPBS and decant the supernatant and incubate the microvessels with 200 μ L of fixing solution (4% para formaldehyde in DPBS) at room temperature (RT) for 20 min to fix microvessels
5. Wash the microvessels three times with DPBS at RT. (5 min each time followed by 5 min spin at 2000 $\times$ g)
6. After three washes, add blocking solution (2% BSA solution in DPBS) and incubate for 1 h at RT (Gently mix the suspension using pipette for every 10 to 15 min to make sure that all microvessels in the suspension have access to the blocking solution)
7. Spin down the microvessels, decant the blocking solution, and resuspend the microvessels in primary antibody solution (diluted in 1%BSA in DPBS) and incubate overnight at 4 $^{\circ}$ C
8. Wash the microvessels three times with DPBS, 5 min each time. Incubate the vessels with secondary antibodies (diluted in 1% BSA in DPBS) for 2 h at RT
9. After three washes with DPBS at RT, resuspend the microvessels in 50 μ L of DPBS
10. Transfer the microvessels to a glass slide and remove as much as liquid possible. Apply the mounting medium with DAPI onto the microvessels and place the coverslip
11. Let it dry overnight at RT in dark. Take fluorescent images using confocal microscope or any fluorescent microscope

Note: As shown in the Fig. 4a–d, immunolabeling of isolated microvessels from mice brains using fluorescent antibodies against von Willebrand factor (vWF), Zonula occludens-1 (ZO-1), microtubule-associated protein-2 (MAP-2), and glial fibrillary acidic protein (GFAP) has shown that microvessels were positive for endothelial cell marker and tight junction marker, whereas it is negative for neuronal and astroglial cell markers.

Acknowledgments We thank Ms. Sufen Zheng for her technical help for the studies.

Sources of funding This research project was supported by the National Institutes of Health: National Institute of Neurological Disorders and Stroke and National Institute of General Medical

Sciences (NS094834—PV Katakam), National Heart, Lung and Blood Institute (HL-077731 and HL093554—DW Busija), National Institute on Aging (R01AG047296—R Mostany, R01AG049821—WL Murfee); American Heart Association (National Center Scientist Development Grant, 14SDG20490359—PV Katakam, Greatersoutheast Affiliate Predoctoral Fellowship Grant, 16PRE27790122—V.N. Sure), Louisiana Board of Regents grants (Endowed Chairs for Eminent Scholars program; DW Busija, RCS, LEQSF(2016-19)-RD-A-24—R Mostany), and COBRE on Aging and Regenerative Medicine (P20GM103629—R Mostany). The content does not necessarily represent the official views of the NIH and is solely the responsibility of the authors.

Compliance with ethical standards All experimental protocols were approved by the Institution of Animal Care and Use Committee (IACUC) of Tulane University School of Medicine in accordance with the National institute of Health (NIH) guidelines for the animal care and use.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Belanger M, Allaman I, Magistretti PJ (2011) Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell Metab* 14:724–738
- Castellani R, Hirai K, Aliev G, Drew KL, Nunomura A, Takeda A, Cash AD, Obrenovich ME, Perry G, Smith MA (2002) Role of mitochondrial dysfunction in Alzheimer's disease. *J Neurosci Res* 70:357–360
- Chan PH (2005) Mitochondrial dysfunction and oxidative stress as determinants of cell death/survival in stroke. *Ann N Y Acad Sci* 1042:203–209
- Clark LC (1956) Monitor and control of blood and tissue oxygen tensions. *Trans Am Soc Artif Intern Organs* 2:41–48
- Clark LC Jr, Sachs G (1968) Bioelectrodes for tissue metabolism. *Ann N Y Acad Sci* 148:133–153
- Csiszar A, Tarantini S, Fulop GA et al (2017) Hypertension impairs neurovascular coupling and promotes microvascular injury: role in exacerbation of Alzheimer's disease. *Geroscience* 39:359–372
- Dai DF, Rabinovitch PS, Ungvari Z (2012) Mitochondria and cardiovascular aging. *Circ Res* 110:1109–1124
- de la Torre JC (1994) Impaired brain microcirculation may trigger Alzheimer's disease. *Neurosci Biobehav Rev* 18:397–401
- Deepa SS, Bhaskaran S, Espinoza S, Brooks SV, McArdle A, Jackson MJ, van Remmen H, Richardson A (2017) A new mouse model of frailty: the Cu/Zn superoxide dismutase knockout mouse. *Geroscience* 39:187–198
- Devi L, Prabhu BM, Galati DF, Avadhani NG, Anandatheerthavarada HK (2006) Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *J Neurosci* 26:9057–9068

- Farkas E, Luiten PG (2001) Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol* 64:575–611
- Ferrick DA, Neilson A, Beeson C (2008) Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov Today* 13:268–274
- Girouard H, Iadecola C (2006) Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. *J Appl Physiol* (1985) 100:328–335
- Grassi B, Poole DC, Richardson RS, Knight DR, Erickson BK, Wagner PD (1996) Muscle O₂ uptake kinetics in humans: implications for metabolic control. *J Appl Physiol* (1985) 80: 988–998
- Grimmig B, Kim SH, Nash K, Bickford PC, Douglas SR (2017) Neuroprotective mechanisms of astaxanthin: a potential therapeutic role in preserving cognitive function in age and neurodegeneration. *Geroscience* 39:19–32
- Horan MP, Pichaud N, Ballard JW (2012) Review: quantifying mitochondrial dysfunction in complex diseases of aging. *J Gerontol A Biol Sci Med Sci* 67:1022–1035
- Iadecola C (1993) Regulation of the cerebral microcirculation during neural activity: is nitric oxide the missing link? *Trends Neurosci* 16:206–214
- Izzo C, Carrizzo A, Alfano A, Virtuoso N, Capunzo M, Calabrese M, de Simone E, Sciarretta S, Frati G, Olivetti M, Damato A, Ambrosio M, de Caro F, Remondelli P, Vecchione C (2018) The impact of aging on cardio and cerebrovascular diseases. *Int J Mol Sci* 19
- Jung SK, Gorski W, Aspinwall CA, Kauri LM, Kennedy RT (1999) Oxygen microsensor and its application to single cells and mouse pancreatic islets. *Anal Chem* 71:3642–3649
- Katakam PV, Gordon AO, Sure VN, Rutkai I, Busija DW (2014) Diversity of mitochondria-dependent dilator mechanisms in vascular smooth muscle of cerebral arteries from normal and insulin-resistant rats. *Am J Physiol Heart Circ Physiol* 307: H493–H503
- Katakam PV, Dutta S, Sure VN et al (2016) Depolarization of mitochondria in neurons promotes activation of nitric oxide synthase and generation of nitric oxide. *Am J Physiol Heart Circ Physiol* 310:H1097–H1106
- Kunz A, Iadecola C (2009) Cerebral vascular dysregulation in the ischemic brain. *Handb Clin Neurol* 92:283–305
- Marzetti E, Csiszar A, Dutta D, Balagopal G, Calvani R, Leeuwenburgh C (2013) Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. *Am J Physiol Heart Circ Physiol* 305:H459–H476
- Merdzo I, Rutkai I, Tokes T, Sure VN, Katakam PV, Busija DW (2016) The mitochondrial function of the cerebral vasculature in insulin-resistant Zucker obese rats. *Am J Physiol Heart Circ Physiol* 310:H830–H838
- Merdzo I, Rutkai I, Sure VN, McNulty CA, Katakam PV, Busija DW (2017) Impaired mitochondrial respiration in large cerebral arteries of rats with type 2 diabetes. *J Vasc Res* 54:1–12
- Navarro G, Allard C, Morford JJ, Xu W, Liu S, Molinas AJR, Butcher SM, Fine NHF, Blandino-Rosano M, Sure VN, Yu S, Zhang R, Münzberg H, Jacobson DA, Katakam PV, Hodson DJ, Bernal-Mizrachi E, Zsombok A, Mauvais-Jarvis F (2018) Androgen excess in pancreatic beta cells and neurons predisposes female mice to type 2 diabetes. *JCI Insight* 3
- Nehlig A (2004) Brain uptake and metabolism of ketone bodies in animal models. *Prostaglandins Leukot Essent Fat Acids* 70: 265–275
- Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR (2009) Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* 58:718–725
- Payne BA, Chinnery PF (1847) Mitochondrial dysfunction in aging: much progress but many unresolved questions. *Biochim Biophys Acta* 2015:1347–1353
- Reddy PH, Beal MF (2008) Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends Mol Med* 14:45–53
- Sakamuri S, Sperling JA, Sure VN et al (2018) Measurement of respiratory function in isolated cardiac mitochondria using Seahorse XFe24 Analyzer: applications for aging research. *Geroscience* 40:347–356
- Silbergeld DL, Ali-Osman F (1991) Isolation and characterization of microvessels from normal brain and brain tumors. *J Neuro-Oncol* 11:49–55
- Sonntag WE, Lynch CD, Cooney PT, Hutchins PM (1997) Decreases in cerebral microvasculature with age are associated with the decline in growth hormone and insulin-like growth factor 1. *Endocrinology* 138:3515–3520
- Springo Z, Tarantini S, Toth P, Tucsek Z, Koller A, Sonntag WE, Csiszar A, Ungvari Z (2015) Aging exacerbates pressure-induced mitochondrial oxidative stress in mouse cerebral arteries. *J Gerontol A Biol Sci Med Sci* 70:1355–1359
- Starkov AA, Chinopoulos C, Fiskum G (2004) Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. *Cell Calcium* 36:257–264
- Stirone C, Duckles SP, Krause DN, Procaccio V (2005) Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. *Mol Pharmacol* 68:959–965
- Sure VN, Katakam PV (2016) Janus face of thrombospondin-4: impairs small artery vasodilation but protects against cardiac hypertrophy and aortic aneurysm formation. *Am J Physiol Heart Circ Physiol* 310:H1383–H1384
- Tarantini S, Fulop GA, Kiss T, Farkas E, Zölei-Szénási D, Galvan V, Toth P, Csiszar A, Ungvari Z, Yabluchanskiy A (2017a) Demonstration of impaired neurovascular coupling responses in TG2576 mouse model of Alzheimer's disease using functional laser speckle contrast imaging. *Geroscience* 39:465–473
- Tarantini S, Yabluchanskiy A, Fulop GA et al (2017b) Pharmacologically induced impairment of neurovascular coupling responses alters gait coordination in mice. *Geroscience* 39:601–614
- Tarantini S, Valcarcel-Ares NM, Yabluchanskiy A, Fulop GA, Hertelendy P, Gautam T, Farkas E, Perz A, Rabinovitch PS, Sonntag WE, Csiszar A, Ungvari Z (2018) Treatment with the mitochondrial-targeted antioxidant peptide SS-31 rescues neurovascular coupling responses and cerebrovascular endothelial function and improves cognition in aged mice. *Aging Cell* 17
- Tucsek Z, Noa Valcarcel-Ares M, Tarantini S, Yabluchanskiy A, Fülöp G, Gautam T, Orock A, Csiszar A, Deak F, Ungvari Z (2017) Hypertension-induced synapse loss and impairment in synaptic plasticity in the mouse hippocampus mimics the

- aging phenotype: implications for the pathogenesis of vascular cognitive impairment. *Geroscience* 39:385–406
- Ungvari Z, Kaley G, de Cabo R, Sonntag WE, Csizsar A (2010a) Mechanisms of vascular aging: new perspectives. *J Gerontol A Biol Sci Med Sci* 65:1028–1041
- Ungvari Z, Sonntag WE, Csizsar A (2010b) Mitochondria and aging in the vascular system. *J Mol Med (Berl)* 88:1021–1027
- van Hall G, Stromstad M, Rasmussen P et al (2009) Blood lactate is an important energy source for the human brain. *J Cereb Blood Flow Metab* 29:1121–1129
- Wang Z, Ying Z, Bosy-Westphal A, Zhang J, Schautz B, Later W, Heymsfield SB, Müller MJ (2010) Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *Am J Clin Nutr* 92:1369–1377
- Williams LR, Leggett RW (1989) Reference values for resting blood flow to organs of man. *Clin Phys Physiol Meas* 10: 187–217
- Wu M, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA (2007) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol* 292:C125–C136
- Yousif S, Marie-Claire C, Roux F, Scherrmann JM, Decleves X (2007) Expression of drug transporters at the blood-brain barrier using an optimized isolated rat brain microvessel strategy. *Brain Res* 1134:1–11
- Yu H, Yang C, Chen S, Huang Y, Liu C, Liu J, Yin W (2017) Comparison of the glycopattern alterations of mitochondrial proteins in cerebral cortex between rat Alzheimer's disease and the cerebral ischemia model. *Sci Rep* 7:39948
- Zhang J, Nuebel E, Wisidagama DR et al (2012) Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat Protoc* 7: 1068–1085