Electrical dynamics of isolated cerebral and skeletal muscle endothelial tubes: Differential roles of G-protein-coupled receptors and K⁺ channels

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Abstract
Electrical dynamics of freshly isolated cerebral endothelium have not been determined independently of perivascular nerves and smooth muscle. We tested the hypothesis that endothelium of cerebral and skeletal muscle arteries differentially utilizes purinergic and muscarinic signaling pathways to activate endothelium-derived hyperpolarization. Changes in membrane potential (V_m) were recorded in intact endothelial tubes freshly isolated from posterior cerebral and superior epigastric arteries of male and female C57BL/6 mice (age: 3-8 months). V_m was measured in response to activation of purinergic (P2Y) and muscarinic (M₃) receptors in addition to small- and intermediate-conductance Ca²⁺-activated K⁺ (SKCa/IKCa) and inward rectifying K⁺ (KIR) channels using ATP (100 μmol·L⁻¹), acetylcholine (ACh; 10 μmol·L⁻¹), NS309 (0.01-10 μmol·L⁻¹), and 15 mmol·L⁻¹ KCl, respectively. Intercellular coupling was demonstrated via transfer of propidium iodide dye and electrical current (±0.5-3 nA) through gap junctions. With similarities observed across gender, peak hyperpolarization to ATP and ACh in skeletal muscle endothelial tubes was ~twofold and ~sevenfold higher, respectively, vs cerebral endothelial tubes, whereas responses to NS309 were similar (from resting V_m ~30 mV to maximum ~80 mV). Hyperpolarization (~8 mV) occurred during 15 mmol·L⁻¹ KCl treatment in cerebral but not skeletal muscle endothelial tubes. Despite weaker hyperpolarization during endothelial GPCR stimulation in cerebral vs skeletal muscle endothelium, the capability for robust SKCa/IKCa activity is preserved across brain and skeletal muscle. As vascular reactivity decreases with aging and cardiovascular disease, endothelial K⁺ channel activity may be calibrated to restore blood flow to respective organs regardless of gender.

Keywords
calcium-activated K⁺ channels, endothelium-derived hyperpolarization, muscarinic, purinergic

Abbreviations: [Ca²⁺]i, intracellular Ca²⁺ concentration; [Ca²⁺]o, extracellular Ca²⁺ concentration; [K⁺]o, extracellular K⁺ concentration; ACh, acetylcholine; ATP, adenosine triphosphate; EDH, endothelium-derived hyperpolarization; Eₜ, equilibrium potential for K⁺; GPCR, G-protein-coupled receptor; Kₐ channel, inward-rectifying K⁺ channel; M₃, muscarinic receptor type 3; NO, nitric oxide; P₂Y, purinergic receptor type 2Y; PCA, posterior cerebral artery; pEC5₀, negative log of the half-maximal concentration; PSS, physiological salt solution; SEA, superior epigastric artery; SKCa/IKCa channels, small- and intermediate-conductance Ca²⁺-activated K⁺ channels; V_m, membrane potential.

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INTRODUCTION

Endothelium-derived hyperpolarization (EDH) entails activation of small- and intermediate-conductance $Ca^{2+}$-activated $K^+$ (SKCa/IKCa) channels and increased plasma membrane potential ($V_m$). With the exception of capillaries, SKCa/IKCa channels play a substantial role in vascular resistance networks throughout the body and are thereby integral to tissue blood flow and oxygen delivery. As a “nonexcitable” tissue, the endothelium depends on elevated intracellular $Ca^{2+}$ ([Ca$^{2+}]_o$) to activate voltage-insensitive SKCa/IKCa channels to hyperpolarize cells while enhancing the electrochemical gradient by which extracellular $Ca^{2+}$ ions enter the cell interior. A parallel vasodilator pathway entails the production of nitric oxide (NO), also sensitive to increases in [Ca$^{2+}]_o$. Thus, via hyperpolarization-induced $Ca^{2+}$ entry, SKCa/IKCa channels have also been implicated in the enhanced production of NO while effectively serving as a feed-forward mechanism of the EDH pathway. Thus, the SKCa/IKCa channels have altogether emerged as important therapeutic targets for cardiovascular disease.

In order to understand native endothelial function independent of the added complexity of perivascular nerve and smooth muscle function, fresh skeletal muscle arterial endothelial tubes have been examined extensively for their fundamental $Ca^{2+}$ and electrical signaling dynamics. Despite our current understanding of EDH in peripheral skeletal muscle, whereby a dynamic increase (10- to 100-fold) in blood flow can take place from inactivity to maximal aerobic exercise, this identical examination of cerebral arterial endothelium underlying central blood flow distribution to the brain has not been examined. In the long-term, such investigation may provide new insight into healthy brain maturation during aging as well as the pathogenesis of chronic diseases (e.g., hemorrhagic stroke, dementia).

The goal of this study was to conduct a comparative analysis of electrical dynamics across cerebral and skeletal muscle endothelium in response to activation of classical G-protein-coupled receptors [GPCRs; purinergic type 2Y (P2Y), muscarinic type 3 (M3) receptors] and $K^+$ channels [SKCa/IKCa, inward rectifying $K^+$ (KIR) channels]. In turn, we addressed whether gender itself has an effect on the pharmacological regulation of endothelial $V_m$. We tested the central hypothesis that endothelial tubes isolated from cerebral and skeletal muscle arteries differentially utilize purinergic and muscarinic signaling pathways to activate EDH. Intact endothelium was freshly isolated from the posterior cerebral arteries (PCAs) and superior epigastric arteries (SEAs) of young adult C57BL/6 mice (age: 3-8 months).

2 MATERIALS AND METHODS

2.1 Animal care and use

All animal care use and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC; Protocol #8150028) of Loma Linda University and performed in accord with the National Research Council’s “Guide for the Care and Use of Laboratory Animals” (8th Edition, 2011) and the ARRIVE guidelines. Mice were housed on a 12:12 hour light-dark cycle at 22°C-24°C with fresh water and food available ad libitum. Animal housing was overseen by the Animal Care Facility at Loma Linda University. Mice were housed as 2-5 animals of the same gender per cage with enriched environments (e.g., toys in cages) and checked daily for fresh water and food, activity level, and physical appearance by experienced animal technicians. Routine visits were also performed by campus veterinarians available at all times.

Experiments were performed on C57BL/6 young mice (3-8 months old; male & female, n = 16 each) obtained from the National Institute on Aging colonies at Charles River Laboratories (Wilmington, MA, USA). All animals were allowed to acclimate to their housing environment for at least 1 week before experimentation and one mouse was studied per day. Animals were selected randomly with respect to gender and the arterial endothelium type (cerebral or skeletal muscle) examined.

2.2 Solutions

Physiological salt solution (PSS; composition in mmol·L$^{-1}$: 140 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose; pH 7.4, 295-300 mOsm] maintained the health of freshly isolated and intact endothelial tubes during all experiments. During blood vessel isolation, the PSS dissection buffer lacked CaCl$_2$ and contained filtered 0.1% BSA (Bovine Serum Albumin, A7906, Sigma). For dissociation of endothelium from smooth muscle, the PSS dissociation buffer contained a reduced $Ca^{2+}$ concentration (0.1 mmol·L$^{-1}$ CaCl$_2$), 0.1% BSA, and an enzyme cocktail (see below in Endothelial tube isolation and superfusion). For solutions with nominally zero extracellular $Ca^{2+}$ concentration ([Ca$^{2+}]_o$) or 15 mmol·L$^{-1}$ KCl, equimolar increases or decreases of NaCl maintained osmolarity. Reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or ThermoFisher Scientific (Pittsburgh, PA, USA) unless otherwise indicated; corresponding catalog numbers are shown in parentheses for nonstandard reagents or chemicals.

2.3 Surgery and microdissection

Each animal was anesthetized via inhalation of isoflurane followed by decapitation. The PCA and SEA were chosen as representative vascular endothelial study models for the brain and skeletal muscle, respectively. The electrical dynamics and structural integrity of respective isolated and intact endothelial tubes (width: ≥50 μm, length: ≥300 μm) facilitated measurements of intra- and intercellular
signaling along and among endothelial cells. In addition, studies of the rodent PCA\textsuperscript{21-23} and SEA\textsuperscript{12} are substantially represented in the literature and altogether encompass examination of fundamental endothelial signaling mechanisms, vascular development/aging, and pathology.

All dissection procedures required specimen magnification via stereomicroscopes (Stemi 2000 & 2000-C, Zeiss, NY, USA) and illumination provided by fiber optic light sources (Fostec: 8375, Schott, Mainz, Germany & KL200, Zeiss). Macrodissection procedures included removal of the brain and abdominal skeletal muscles for cerebral arteries and skeletal muscle arteries, respectively, using sharp dissection scissors [RS-5912, Roboz Surgical Instruments, Gaithersburg, MD, USA; 14393 & 15905, World Precision Instruments (or WPI), Sarasota, FL, USA] and forceps [coarse-tipped straight (RS-4960; Dumostar) & bone-cutting (RS-8480; Roboz)]. For microdissection to remove respective arteries, the brain or abdominal muscles were placed in chilled (4°C) PSS dissection buffer and secured using stainless steel pins (Living Systems Instrumentation, St. Albans City, VT, USA) in a glass petri dish (diameter: 9 cm, depth: 2.25 cm) containing a charcoal Sylgard coated bottom (depth: 0.75 cm) (Dow-Corning, Auburn, MI, USA). Microdissection instruments included Vannas style scissors [555640S (9.5 mm blades) & 143646 (3 mm blades), WPI; Moria MC52 (7 mm blades) & 15000-00 (3 mm blades), Fine Science Tools] and sharpened fine-tipped forceps (Dumont #5 & Dumont #55, FST). A customized stainless steel chamber apparatus containing a circular receptacle (diameter: 9.5 cm, depth: 2.5 cm) and surrounding cooling reservoir [diameter: 12.5 cm, depth: 9 cm; filled with a 1:1 coolant mixture of water and laboratory grade ethylene glycol as continuously cycled using a recirculating chiller (Isotemp 500LCU, ThermoFisher)] was used to maintain temperature of PSS in the dissection petri dish.

2.3.1 Cerebral arteries

While viewing through a stereomicroscope, an incision was made using only the tips of dissection scissors starting with the occipital bone and extending up through the nasal bone of the skull. The skull was carefully opened along the incision, whereas connective tissue was separated from the brain with an intact circle of Willis. The brain was then secured ventral side facing up in the chamber containing chilled dissection PSS. Posterior cerebral arteries (0.5-1 cm) from the circle of Willis were surgically removed free of the posterior communicating and the basilar arteries.

2.3.2 Skeletal muscle arteries

The general isolation procedure for the SEA has been previously described in detail [see\textsuperscript{13} for video illustration]. Briefly, a ventral midline incision was made from the sternum to the pubis. While viewing through a stereo microscope, fat and connective tissue superficial to the sternum were removed to expose the proximal ends of the SEA. The abdominal muscle corresponding to the visible SEA was then removed and pinned ventral side facing up in the dissection chamber containing chilled dissection PSS. The SEA segment (~2 cm) was then dissected free of the paired vein, surrounding tissue and fat cells.
2.4 | Endothelial tube isolation and superfusion

The apparatus for endothelial tube isolation included a modified Nikon inverted microscope (Ts2) equipped with phase contrast objectives (Ph1 DL; 10X & 20X) (Nikon Instruments Inc, Melville, NY, USA) and an aluminum stage (MXIX/AO; Siskiyou). An adjacent microsyringe pump controller [Micro4, World Precision Instruments (WPI), Sarasota, FL, USA] was used to isolate endothelium from surrounding adventitia and smooth muscle. The superfusion and experimental rig included a modified Nikon inverted microscope (Eclipse TS100) equipped with fluorescent objectives [20X (S-Fluor), and 40X (Plan Fluor)] and a manual aluminum stage (MXOP-5L, Siskiyou) mounted on a vibration isolation table (Micro-g; Technical Manufacturing, Peabody, MA, USA). For transferal between microscopes, a mobile tissue examination apparatus was used and included a Plexiglas superfusion chamber (RC-27; Warner Instruments, Camden, CT, USA) with a glass coverslip bottom (12-548-5M, Fisher; 2.4 × 5.0 cm) fastened into an anodized aluminum platform [PM6 or PH6 (diameter: 7.8 cm) & SA-NIK-AL (diameter: 10.8 cm), Warner Instruments] that, in turn, was secured into a compact aluminum stage (8090P, Siskiyou, Grants Pass, OR, USA; length: 23 cm, width: 20 cm, thickness: 0.76 cm). All glass capillary tubes used for trituration of partially digested arteries and mechanical stabilization of isolated endothelial tubes were prepared using an electronic puller (P-97 or P-1000, Sutter Instruments, Novato, CA, USA) and a microforge (MF-900; Narishige, East Meadow, NY, USA) for heat polishing. Procedures for enzymatic isolation, securing isolated EC tubes, solution delivery, and temperature control are detailed below. The time elapsed from live animal to preparation of isolated endothelium ready for experimentation was less than 2 hours.

Intact arterial segments were cut into segments (length, 1-3 mm) and placed in a PSS containing 0.1 mmol-L⁻¹ CaCl₂ and enzymes that dissociate intact endothelium from surrounding smooth muscle cells for a defined period of time (PCA: 10 minutes, SEA: 30 minutes) at 34°C. For PCs, enzyme solution contained the following: 0.31 mg·mL⁻¹ papain (P4762, Sigma), 0.5 mg·mL⁻¹ dithioerythritol (DB255, Sigma), 0.75 mg·mL⁻¹ collagenase (C8051, Sigma), and 0.13 mg·mL⁻¹ elastase (E7885, Sigma). As previously described,¹³ the SEA enzyme cocktail included 0.62 mg·mL⁻¹ papain, 1.0 mg·mL⁻¹ dithioerythritol, and 1.5 mg·mL⁻¹ collagenase. Following partial digestion, each arterial segment was gently triturated to remove adventitia and smooth muscle cells using borosilicate glass capillary tubes [1B100-4, World Precision Instruments (WPI), Sarasota, FL, USA] with an internal tip diameter of 80-120 μm. Before every experiment, confirmation of an endothelial tube containing only endothelial cells was verified at 400× magnification.

Following EC isolation, a micromanipulator (MX10, Siskiyou) at each end of the mobile tissue examination platform held a micropipette with a blunted, spherical end (heat polished, OD: 100-150 μm; prepared from thin wall borosilicate glass (G150T-6, Warner Instruments)) to secure the endothelial tube against the bottom of the superfusion chamber. Continuous delivery of PSS (containing 2 mmol·L⁻¹ CaCl₂) and respective drug solutions was ensured using a six-reservoir platform with valve controller (VC-6, Warner Instruments) and an inline flow control valve (FR-50, Warner Instruments). Temperature was continuously regulated using an inline heater (SH-27B, Warner Instruments) and heating platform (PM6 or PH6, Warner Instruments) coupled to a temperature controller (TC-344B, Warner Instruments). All experiments were performed during a flow rate of ~7-8 mL·min⁻¹ with PSS (pH: 7.4, 37°C) as consistent with laminar flow while matching flow speed to vacuum suction and completed within 2 hours.

2.5 | Intracellular recording and intercellular coupling

The Vₑ of isolated endothelial tubes was recorded with Axoclamp 2B and Axoclamp 900A amplifiers (Molecular Devices, Sunnyvale, CA, USA) using microelectrodes pulled (P-97 or P-1000, Sutter Instruments) from glass capillary tubes (GC100F-10, Warner Instruments). Microelectrodes were backfilled with 2 M KCl (tip resistance: ~150 MQ), whereas to test dye coupling, 0.1% propidium iodide (P4170, Sigma; mass, ~668 Da) dissolved in 2 mol·L⁻¹ KCl was used to backfill microelectrodes. Cells were penetrated with microelectrodes while viewing the endothelial tube at 400X magnification. An Ag/AgCl pellet placed in the effluent PSS served as a reference electrode. Amplifier outputs were connected to a data acquisition system (Digidata 1550A, Molecular Devices) and audible baseline monitors (BM-A-TM, Ampol US LLC, Sarasota, FL, USA). For dual simultaneous intracellular recordings,²⁴ a cell was designated as "Site 1" using one electrode connected to the Axoclamp 2B amplifier driven by a function generator (FG-8002; EZ Digital, Seoul, South Korea).
Acetylcholine (ACH) stimulation of hyperpolarization in isolated endothelium of skeletal muscle and cerebral arteries. All panels illustrate $V_m$ data before and during treatment with ACH (10 μmol L$^{-1}$; muscarinic receptor agonist and indirect SKCa/IKCa channel activator). **Cerebral endothelium:** Individual $V_m$ traces in (A) male and female (B) mice. (C) Summary data of $V_m$ before and during ACH treatment (peak hyperpolarization) in male and female endothelium. (D) As described in C for summary data of peak hyperpolarization and resting $V_m$. Data indicate $n = 9$ (male) and $n = 9$ (female). **Skeletal muscle endothelium:** (E), (F), (G), and (H) are as described for A, B, C, and D, respectively, corresponding to skeletal muscle endothelium. Data indicate $n = 6$ (male) and $n = 7$ (female). **Cerebral vs skeletal muscle endothelium:** With both male and female groups included in each group, (I) reflects combined C and G and (J) reflects combined D and H. Data indicate $n = 18$ (cerebral) and $n = 13$ (skeletal muscle). Note that while differences between genders are not apparent in either type of endothelium, peak hyperpolarization during ACH treatment is greater in skeletal muscle endothelium vs cerebral. *Significantly different from paired resting $V_m$. $^9$Significantly greater than ACH $V_m$ and $\Delta V_m$ of unpaired cerebral endothelial tubes.

Korea) and “Site 2” using another electrode connected to the Axoclamp 900A amplifier. For testing intercellular electrical coupling, current (±0.5-3 nA, ~20 second pulse duration) was delivered via the Axoclamp 2B amplifier at Site 1 against the direction of superfusion flow to Site 2 (distance: 250-500 μm).

Successful impalements were confirmed by sharp negative deflection of $V_m$, stable $V_m$ for >1 min, reversible depolarization ($\geq$20 mV) to 145 mmol L$^{-1}$ KCl, reversible hyperpolarization ($\geq$-20 mV) to 1 μmol L$^{-1}$ NS309 (direct SKCa/IKCa channel opener, Tocris, Bristol, UK) and/or correspondence between current injection with control PSS to baseline conditions was applied between each application of a pharmacological agent, whereby each application was ≥1 minute was allowed before application of another electrode to allow for records of stable peak $V_m$ responses. In the case of NS309 concentration-response determinations, washout of a pharmacological agent, whereby each application was ≥3 minutes as needed to allow for records of stable peak $V_m$ responses. In the case of NS309 concentration-response determinations, washout with control PSS to baseline conditions was applied between each concentration before the next concentration was added. ATP, ACH, and BaCl2 were prepared in PSS, whereas NS309 was dissolved in DMSO (final working concentration ≤1%). Pharmacological agents were added to the superfusion solution, thereby exposing the entire endothelial tube to each agent.

### 2.7 Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Differences between groups were accepted as statistically significant with $P < .05$. Summary data are presented as means ± SE, and $n$ represents the number of independent experiments using one endothelial tube from a different mouse for a given experimental protocol. Typically, each experimental procedure required ≥5 mice for a complete dataset in accord with statistical power analysis ($\alpha = 0.05$, power = 0.80). In the case of intracellular recordings, data analyses included (1) resting $V_m$ (mV) and (2) change in $V_m$ ($\Delta V_m$) = peak response $V_m$—preceding baseline $V_m$. Statistical analysis was performed using GraphPad Prism (version 6.07; GraphPad Software, La Jolla, CA, USA) and analyses included linear regression ($\Delta V_m$ at “Site 2” vs current injection at “Site 1”; $R^2 \geq 0.99$), curve fitting (concentration responses for pEC$_{50}$ determinations; sigmoidal “log (agonist) vs response with variable slope” fit, $R^2 \geq 0.99$), and unpaired (across gender and/or endothelial type) and paired (drug response vs resting $V_m$ in same endothelial tube) t-tests.

### 3 RESULTS

Endothelial tubes isolated from posterior cerebral and superior epigastric arteries were ~90-100 μm and ~60-70 μm in width, respectively, whereby arterial segments used for this study were typically between 0.5 and 1 mm in length. The mechanical stability of secured, isolated endothelium enabled continuous intracellular recordings of $V_m$ lasting typically up to 2 hours at 37°C. Resting $V_m$ was similar across cerebral (~30 ± 1 mV, $n = 19$) and skeletal muscle (~29 ± 2 mV, $n = 13$) endothelial tubes. The Results below qualitatively denote comparisons across respective types of endothelium and genders, whereby quantitative information (eg, $n$-values and means ± SE supporting general descriptions of observations is indicated in the Figures and Figure Legends.)

2.6 | Pharmacology

Adenosine 5′-triphosphate disodium salt hydrate (ATP, 100 μmol L$^{-1}$; Sigma, A2383) and acetylcholine chloride (ACH, 10 μmol L$^{-1}$; Sigma, A6625) were used independently to activate G-protein-coupled signaling through purinergic (P2Y type) or muscarinic (M$_2$ type) receptors, respectively, which, in arterial ECs, activate Ca$^{2+}$ release through IP$_3$ receptors and Ca$^{2+}$ influx via TRP channels to activate SKCa/IKCa channels.$^{122}$ NS309 (0.01-10 μmol L$^{-1}$; Tocris, Bristol, UK, 3895) was applied to directly evaluate the capacity for SKCa/IKCa channel-dependent hyperpolarization.$^{22,24}$ Extracellular KCl ([K$^+$]$_o$) elevated to 15 mmol L$^{-1}$ was used to examine the presence of hyperpolarization to K$_{IR}$ channel activation; a subset of experiments employed 15 mmol L$^{-1}$ KCl in combination with BaCl$_2$ (100 μmol L$^{-1}$; K$_{IR}$ channel blocker)$^{25,26}$

A stable resting $V_m$ of ≥1 minute was allowed before application of a pharmacological agent, whereby each application was ≥3 minutes as needed to allow for records of stable peak $V_m$ responses. In the case of NS309 concentration-response determinations, washout with control PSS to baseline conditions was applied between each
3.1 | Freshly isolated cerebral endothelium: Evidence of cell-to-cell coupling and electrical conduction

Cell-to-cell coupling through gap junctions has been observed in isolated endothelial tubes of skeletal muscle using visual and electrical approaches of microinjected propidium iodide dye and current injection via dual intracellular electrodes, respectively. As illustrated in Figure 1, the presence of dye transfer also occurs in cerebral endothelial tubes (Figure 1A), while using current injections of $\pm 0.5-3$ nA (Figure 1B), $\Delta V_m$ responses were linear throughout and reflected a $-10-11$ mV change per nA of current injected (250-500 $\mu$m from the site of current injection; Figure 1C). These data illustrate an intact cerebral endothelial tube system whereby cells are coupled through gap junctions. These observations demonstrating robust dye transfer and conduction amplitude measurements in cerebral endothelium are similar to skeletal muscle endothelium as shown in previous studies.

3.2 | Hyperpolarization of cerebral and skeletal muscle endothelium to indirect and direct activation of $SK_{Ca}/IK_{Ca}$ channels

Significant hyperpolarization of $V_m$ (≥5 mV) occurred in endothelial tubes of cerebral and skeletal muscle arteries in response to the classical endothelial GPCR agonists ATP (Figure 2) and ACh (Figure 3) as indirect activators of $SK_{Ca}/IK_{Ca}$ channels. As illustrated in Figure 2 (Panels A and B & E and F), hyperpolarization to ATP was typically transient (≤30 seconds) throughout individual $V_m$ recordings. With similar responses observed across genders for respective types of endothelial tubes (Figure 2C and D & G and H), peak hyperpolarization to ATP in skeletal muscle endothelial cells was ~twofold greater vs cerebral endothelial cells (Figures 2I and J). In skeletal muscle endothelial tubes only, hyperpolarization responses to ACh were sustained throughout duration of treatment (compare Figure 3 panels A and B with E and F). With similar responses observed across genders for respective types of endothelial tubes (Figure 3C and D & G and H), peak hyperpolarization to ACh in skeletal muscle endothelia was ~sevenfold greater vs cerebral endothelial tubes (Figures 3I and J).

Direct activation of $SK_{Ca}/IK_{Ca}$ channels with NS309 can fine tune $V_m$ from steady-state conditions in the absence of agonist exposure (or “resting” $V_m$) to the equilibrium potential for K+ ($\sim -90$ mV). Figures 4A & B illustrate sustained hyperpolarization responses in individual cerebral endothelial tubes to “low” (0.1 $\mu$mol-L$^{-1}$), “middle” (1 $\mu$mol-L$^{-1}$), and “high” (10 $\mu$mol-L$^{-1}$) concentrations of NS309 for males and females, respectively. Summary concentration response curves to NS309 (resting $V_m$ to maximum $V_m$ during 10 $\mu$mol-L$^{-1}$) indicate a similarity across genders with regard to respective pEC50s and maximal hyperpolarization (Figure 4C-F). Such experiments have been performed previously for male skeletal muscle endothelial tubes and thus, in this regard, comparisons are only indicated for effective concentrations of 1 $\mu$mol-L$^{-1}$ and 10 $\mu$mol-L$^{-1}$ NS309. Robust hyperpolarization to NS309 ($\Delta V_m > -20$ mV) for skeletal muscle endothelial tubes was preserved across genders (Figure 4G and H) as well as in comparison to cerebral endothelial tubes (Figure 4I and J).

3.3 | Hyperpolarization of cerebral endothelium to activation of $K_{IR}$ channels

Elevated $[K^+]_o$ (15 mmol-L$^{-1}$ KCl) stimulates activation of $K_{IR}$ channels in arteries of the brain and skeletal muscle. Significant hyperpolarization of $V_m$ (≥5 mV) occurred in response to 15 mmol-L$^{-1}$ KCl in endothelial tubes of cerebral vessels (Figure 5A-D) but not skeletal muscle (Figure 5E-H). With similar responses observed across genders for respective types of endothelium (Figure 5C and D & G and H), peak hyperpolarization to 15 mmol-L$^{-1}$ KCl in cerebral endothelial tubes averaged $\Delta V_m = 7-8$ mV. However, $\Delta V_m$ responses in skeletal muscle during 15 mmol-L$^{-1}$ KCl were indistinguishable from those observed in cerebral endothelial tubes during $K_{IR}$ blockade with a combined treatment using BaCl2 (100 $\mu$mol-L$^{-1}$) and 15 mmol-L$^{-1}$ KCl (Figures 5I-J).

4 | DISCUSSION

The endothelial layer of resistance arteries contributes to regulation of peripheral skeletal muscle and cerebrovascular smooth muscle tone and thereby modulates optimal blood flow ensuring a constant...
delivery of oxygen and nutrients to excitable tissues.\(^{30}\) Our understanding of endothelial function has been enhanced with the use of a skeletal muscle arterial endothelial tube study model containing cells coupled via gap junctions in the absence of perivascular nerves and smooth muscle. Using this study model, we have been able to resolve electrical signaling dynamics disparate from "excitable" cells such as neurons and cardiac myocytes\(^{31}\) while illuminating refined pharmacological approaches for controlling cardiovascular function.\(^{12}\)

With recognition of recent demonstrations of how the endothelium plays an important role in modulating moment to moment vascular tone and blood flow within the brain,\(^{3,32}\) we utilized the endothelial tube model to investigate purinergic and muscarinic receptor stimulation in mouse cerebral arteries relative to skeletal muscle arteries. To our knowledge, this study has comprehensively measured hyperpolarizing electrical dynamics in freshly isolated and intact cerebral arterial endothelial tubes (see Figure 1) of male and female mice for the first time. We found that although GPCR-triggered hyperpolarization responses in cerebral endothelium were modest relative to skeletal muscle, robust SK\(_{Ca}/IK_{Ca}\) and K\(_{IR}\) channel functions were present. We did not observe statistically significant differences across respective genders. Our findings are discussed below in the context of physiological integration of central vs peripheral distribution of blood flow with implications for treatment of cardio/cerebrovascular diseases.

4.1 | Physiological integration: Role of endothelial purinergic and muscarinic signaling

Along with noradrenaline, ATP can be released from perivascular sympathetic nerves that enmesh cerebral and skeletal muscle arteries.\(^{33}\) Actions of ATP are biphasic whereby activation of P2X receptors evokes constriction (alone or in combination of noradrenaline activation of smooth muscle \(\alpha_1\)-adrenoceptors) followed by smooth muscle relaxation via EDH resulting from activation of endothelial P2Y receptors.\(^{34,35}\) Although its effects are more straightforward for evoking endothelial M\(_3\) receptors and downstream hyperpolarization (vs ATP), the source of ACh is controversial and may include excess ACh "spillover" from neuromuscular junctions during skeletal muscle contraction,\(^{36}\) cholinergic sympathetic dilator nerves surrounding skeletal muscle blood vessels of experimental animal models,\(^{37}\) and/or the endothelium itself.\(^{28}\)

Regardless, we simplified our investigation here with examining EDH responses to ATP and ACh using freshly isolated endothelium and found that peak hyperpolarization was, respectively, twofold and sevenfold higher in skeletal muscle vs cerebral vascular endothelial tubes (Figures 2 and 3). These findings were not surprising as vascular resistance networks of skeletal muscle engage in "functional sympatholysis",\(^{39}\) a process that requires rapid endothelial activation and production of vasodilation to counteract the basal activation of smooth muscle \(\alpha_1\)-adrenoceptors along resistance networks in accord with metabolic demand.\(^{30}\) On the contrary, blood flow distribution to the brain is relatively constant throughout conditions of rest and exercise\(^{15}\) as the variable metabolic demands by neurons are typically met by alterations in cerebral blood flow governed by myogenic autoregulation that operates over a wide systolic pressure range of ~60-140 mmHg.\(^{40}\)

4.2 | Endothelial K\(_{Ca}\) and K\(_{IR}\) channels

The endothelial SK\(_{Ca}/IK_{Ca}\) channels have been recognized for their integral role during blood flow to the brain\(^{41}\) and skeletal muscle.\(^{12,30}\) Despite the differential hyperpolarization to ATP and ACh across organs, SK\(_{Ca}/IK_{Ca}\) channel activation and hyperpolarization to NS309 in cerebral endothelium (up to ~80 mV) was remarkably similar to skeletal muscle endothelium regardless of gender (Figure 4). These data indicate that the capacity of SK\(_{Ca}/IK_{Ca}\) channel activity is independent of hormonal or neurotransmitter activation of GPCRs. Similar to skeletal muscle blood flow, modulation of cerebral blood flow as influenced by endothelial SK\(_{Ca}/IK_{Ca}\) channels may be susceptible to posttranslational activity such as oxidative signaling.\(^{12}\)

Although studied primarily in smooth muscle, endothelial K\(_{IR}\) channels are present in the endothelium of rodent resistance arteries and arterioles.\(^{42}\) In agreement with recent studies,\(^{42,43}\) our findings here using isolated cerebral endothelium indicate consistent hyperpolarization responses to 15 mmol L\(^{-1}\) KCl (Figure 5A-D). Also, the apparent lack of endothelial K\(_{IR}\) function in SEAs (Figure 5E-H) is consistent with recent observations, whereby K\(_{IR}\) channels play a dominant role in the smooth muscle layer in young (3-6 month) mice despite detectable mRNA expression of endothelial K\(_{IR}\) in both cell layers.\(^{25}\) In contrast to skeletal muscle, K\(_{IR}\) channel currents have...
been observed in the endothelium but not in the smooth muscle of rodent mesenteric arteries. Thus, whereas both peripheral and central types of endothelium rely on SK<sub>Ca</sub>/IK<sub>Ca</sub> channels to generate vasodilatory hyperpolarization, only cerebral vessels may contain functional K<sub>IR</sub> channels in both smooth muscle and endothelium to sense [K]<sub>i</sub> as a metabolic byproduct to intimately match cerebral blood flow with neuronal metabolism. The dual presence of K<sub>IR</sub> channels in cerebral smooth muscle and endothelium produces a higher magnitude of vascular hyperpolarization (>20 mV vs <10 mV in this study using cerebral endothelium alone) as K<sup>+</sup> efflux through activated endothelial SK<sub>Ca</sub>/IK<sub>Ca</sub> channels raises [K]<sub>i</sub> to further stimulate smooth muscle K<sub>IR</sub> channels. Altogether, the presence and interaction of endothelial K<sub>IR</sub> and SK<sub>Ca</sub>/IK<sub>Ca</sub> channels should be considered for understanding and therapeutically addressing the loss of cerebrovascular autoregulation during disease conditions such as stroke.

4.3 Lack of gender-related differences in young mice

We prioritized the inclusion of both genders in this study as ≥80% of biomedical studies thus far have focused on males only and have thereby neglected 50% of the sample population. We did not find statistically significant differences across males and females for respective central and peripheral vascular endothelial systems. However, we do not exclude the possibility of gender-related differences emerging with advanced age and associated vascular diseases related to enhancement of oxidative signaling. Indeed, hypertension induced via global knockout of endothelial NO synthase and Cyclooxygenase-1 (components of parallel endothelial vasodilator pathways to EDH) has been found to have a minimal effect on vascular tone of mesenteric arteries and systolic blood pressure in female vs male mice. Although, these transgenic interventions were not specific to vascular endothelium and thus, there are several other confounding factors regarding relationships between central vs peripheral arteries and regulation of vascular tone via perivascular nerves vs smooth muscle vs endothelium. Regardless of previous efforts, our current electrophysiological findings suggest that endothelial function of central and peripheral arteries of young adult C57BL/6 mice is similar across males and females. Perhaps either gender can be used for future studies of endothelial function in young adult animals as consistent with the “reduction” component of the 3Rs (replacement, refinement, and reduction) of experimental animal use and care.

4.4 Use of the isolated endothelial tube model and lack of myoendothelial coupling

Coordination of vascular smooth muscle and endothelium to govern vascular tone and resistance has been demonstrated through mechanisms collectively known as “myoendothelial coupling”. An original investigation resolved the presence and structure of myoendothelial gap junctions that facilitate transmission of electrical signals between endothelium and smooth muscle in rat mesenteric arteries. Altogether, several studies over the past ~20 years have employed use of confocal and electron microscopic examination of isolated arteries (eg, of the gut, cremaster, and brain). As a result, an emerging working paradigm currently illustrates that endothelial projections through the internal elastic lamina contain a "microdomain" of spatially proximal myoendothelial gap junctions, IK<sub>Ca</sub> channels, transient receptor potential channels (eg, vanilloid class, TRPV4), and inositol 1,4,5-trisphosphate receptors (or IP<sub>3</sub>Rs). Either myoendothelial or endothelial gap junctions may contain connexins (or Cxns) 37, 40, and/or 43 with SK<sub>Ca</sub>/IK<sub>Ca</sub> channels primarily localized near endothelial gap junctions only. Regardless, as demonstrated by this study and others where such a microdomain is eliminated, components of endothelial [Ca<sup>2+</sup>]<sup>+</sup> regulation (influx and intracellular release), maintenance of V<sub>m</sub> hyperpolarization of V<sub>m</sub> (up to E<sub>K</sub>) via SK<sub>Ca</sub>/IK<sub>Ca</sub> activation, and intercellular coupling via gap junctions are retained. Although, we recognize that blood flow is not governed by either smooth muscle or endothelium alone. Indeed, the experimental strengths of the endothelial tube study model are also its physiological limitations with respect to the lack of perivascular nerves, smooth muscle, circulation of blood, and hormonal regulation. For balanced consideration among use of physiological study models, there are also significant limitations for experimental applications in vivo (eg, inflammation, artifacts of anesthesia) and ex vivo (eg, detachment from parenchyma, lack of blood flow) [reviewed for the brain in]. Our goal of this study was to address contrasting electrical dynamics of endothelium of cerebral and skeletal muscle resistance arteries. Our findings are complementary to previous intact PCA and SEA studies where myoendothelial communication was present. In turn, our data highlight use of freshly intact endothelium isolated from male and female mouse cerebral arteries for the first time.

5 SUMMARY AND CONCLUSIONS: TRANSLATIONAL PERSPECTIVE

The primary significance of this study is that despite weaker hyperpolarization in response to endothelial GPCR stimulation in cerebral vs skeletal muscle endothelium, the capability for robust SK<sub>Ca</sub>/IK<sub>Ca</sub> activity is preserved across brain and skeletal muscle regardless of gender. Furthermore, we are now confident that functionally active K<sub>IR</sub> channels are present in intact cerebral arterial endothelium, but lacking in skeletal muscle arterial endothelium. The activity of SK<sub>Ca</sub>/IK<sub>Ca</sub> channels underlies EDH and is amenable to “fine-tuning” with use of agents such as NS309 and SKA-31. While staying within physiological limits as previously discussed [eg, not exceeding a stable V<sub>m</sub> of ~60 mV], mild SK<sub>Ca</sub>/IK<sub>Ca</sub> channel activation may also serve as a feed-forward mechanism to enhance Ca<sup>2+</sup> entry to further amplify SK<sub>Ca</sub>/IK<sub>Ca</sub> channel activity and/or boost production of NO. As endothelial-derived relaxation decreases in old age and diseases such as hypertension, sleep apnea and obesity due to reduced nitric oxide signaling, SK<sub>Ca</sub>/IK<sub>Ca</sub> channel activity may be calibrated as needed to restore blood flow demands to respective organs. Altogether, these data provide fundamentally new insight.
for precision medicine, whereby selective treatments may be developed for key vascular biomarkers and their function across organ systems in order to promote health and prevent vascular disease.

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AUTHOR CONTRIBUTIONS

MAH and EJB designed and/or performed experiments in the laboratory of EJB. MAH, JNB, and EJB analyzed and interpreted data. MAH, JNB, and EJB edited the manuscript and/or prepared the figures. MAH, JNB, and EJB edited the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DISCLOSURES

The authors declare no conflicts of interest.

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