THE GERONTOLOGICAL SOCIETY OF AMERICA®

Journals of Gerontology: Biological Sciences
cite as: *J Gerontol A Biol Sci Med Sci*, 2017, Vol. 72, No. 12, 1627–1637
doi:10.1093/gerona/glx079
Advance Access publication May 16, 2017

OXFORD

Original Article

Impact of Aging on Calcium Signaling and Membrane Potential in Endothelium of Resistance Arteries: A Role for Mitochondria

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Received: December 9, 2016; Editorial Decision Date: April 16, 2017

Decision Editor: Rafael de Cabo, PhD

Abstract

Impaired blood flow to peripheral tissues during advanced age is associated with endothelial dysfunction and diminished bioavailability of nitric oxide (NO). However, it is unknown whether aging impacts coupling between intracellular calcium ($[Ca^{2+}]_i$) signaling and small- and intermediate K^+ channel (SK_{Ca}/IK_{Ca}) activity during endothelium-derived hyperpolarization (EDH), a signaling pathway integral to dilation of the resistance vasculature. To address the potential impact of aging on EDH, Fura-2 photometry and intracellular recording were applied to evaluate $[Ca^{2+}]_i$ and membrane potential of intact endothelial tubes (width, 60 μm; length, 1–3 mm) freshly isolated from superior epigastric arteries of young (4–6 mo) and old (24–26 mo) male C57BL/6 mice. In response to acetylcholine, intracellular release of Ca^{2+} from the endoplasmic reticulum (ER) was enhanced with aging. Further, treatment with the mitochondrial uncoupler FCCP evoked a significant increase of $[Ca^{2+}]_i$ with membrane hyperpolarization in an SK_{Ca}/IK_{Ca}-dependent manner in the endothelium of old but not young mice. We conclude that the ability of resistance artery endothelium to release Ca^{2+} from intracellular stores (ie, ER and mitochondria) and hyperpolarize V_m via SK_{Ca}/IK_{Ca} activation is augmented as compensation for reduced NO bioavailability during advanced age.

Keywords: Artery—Cardiovascular—Endothelial Cell—Mitochondria—Biology of Aging

Gerontologists predict that individuals of advanced age (≥65 years) will compose approximately 25% of the United States population by 2030 (1). Aging is the key risk factor for cardiovascular disease (2,3) with endothelial dysfunction a prime indicator (4,5). This disorder is typically manifested by impaired vasodilation to acetylcholine (ACh) (6,7) or the restriction of blood flow during physical activity (6,8). Nevertheless, recent findings indicate that aging may be beneficial to endothelial cell (EC) survival (9). In the present study, our goal was to determine whether, and if so, how the endothelium of resistance arteries may adapt to maintain its role in effecting vasodilation during advanced age.

During the local control of tissue blood flow, vasodilation is coordinated along the branches of resistance networks, including the proximal feed arteries and the arteriolar networks they supply (10). An integral mechanism of vasodilation in the resistance vasculature entails endothelium-derived hyperpolarization, "EDH" (11,12). Thus, in response to a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the opening of small- and intermediate- calcium-activated K^+ channels (SK_{Ca}/IK_{Ca}) in ECs generates hyperpolarization, which spreads from cell to cell through gap junctions between ECs and into surrounding smooth muscle cells (SMCs) through myoendothelial gap junctions (13,14). In resistance arteries, the endothelium serves as the principal cellular pathway for electrical signal transmission (15–18) which can be modulated by affecting gap junctions coupling neighboring cells or altering the activity of ion channels expressed in the plasma membrane (11,19). In contrast to the lack of selective pharmacological interventions for gap junctions (20,21), SK_{Ca}/IK_{Ca} are effective targets for pharmacological treatment of endothelial dysfunction (22–25). Whereas advanced age diminishes blood flow to key organs and tissue in association with a decrease in the bioavailability of nitric oxide (NO) (26,27), little is known of how the aging process impacts coupling between $[Ca^{2+}]_i$ signaling and SK_{Ca}/IK_{Ca} activity underlying EDH. Because these signaling events effect

vasodilation in the small resistance arteries that control tissue blood flow (28,29), greater insight is needed to understand how EDH may be affected during advanced age, where tissue perfusion may be compromised.

The principal organelles that govern the intracellular storage, sequestration, and release of $[Ca^{2+}]_i$ are the endoplasmic reticulum (ER) and mitochondrion (30–32). Muscarinic receptor (M_3) stimulation (eg, by ACh) increases the availability of cytoplasmic inositol trisphosphate (IP_3), thereby triggering a rise in $[Ca^{2+}]_i$ via the activation of inositol 1,4, trisphosphate receptors (IP_3Rs) in the ER (12,33). The rise in $[Ca^{2+}]_i$ is sustained by the influx of extracellular Ca^{2+} through open transient receptor potential channels (33–35) and this response is enhanced during advanced age (9). Because ECs rely primarily on glycolysis rather than oxidative phosphorylation for generating adenosine triphosphate (ATP) (36,37), key roles for endothelial mitochondria entail the regulation of $[Ca^{2+}]_i$ and the production of reactive oxygen species (31,38). Moreover, the role of mitochondria in buffering $[Ca^{2+}]_i$ can increase with advanced age, as shown for intestinal SMCs (39) and peripheral sympathetic neurons (40). In turn, release of Ca^{2+} from mitochondria effects membrane hyperpolarization via SK_{Ca}/IK_{Ca} activation and these ion channels are highly expressed in the endothelium of resistance arteries (41). The proton ionophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) dissipates the inner mitochondria membrane potential (≈ −180 mV relative to cytosol) with consequences of releasing mitochondrial Ca^{2+} (42–47) leading to activation of K_{Ca} channels (46). Further, the ability of FCCP to uncouple mitochondria and depolarize the inner mitochondrial membrane potential ($\Delta\Psi_{mt}$; 42–44) may also reflect the physiological actions of uncoupling proteins (48) and mitochondrial K^+ channels (49,50). Thus, FCCP may be used as a tool to evaluate the role of mitochondria as a Ca^{2+} source for governing membrane potential (V_m) of ECs. However, the respective roles of ER and mitochondria in shaping $[Ca^{2+}]_i$ signaling and activating endothelial SK_{Ca}/IK_{Ca} to evoke hyperpolarization has received little attention.

The effect of aging and associated diseases (eg, obesity, type II diabetes, hypertension) on SK_{Ca}/IK_{Ca} function in generating EDH-dependent vasodilation during aging lacks consensus. Some have suggested a loss of SK_{Ca}/IK_{Ca}-dependent vasodilation (25,51–53), whereas others have observed enhanced function of SK_{Ca}/IK_{Ca} in both intact arteries (54–60) and freshly isolated endothelium (41). The latter finding supports the hypothesis that an increase in EDH signaling serves as a compensatory mechanism in response to diminished NO bioavailability for endothelium-dependent vasodilation (61,62). By eliminating external influences (eg, perivascular nerves, SMCs, blood flow, hormones), the study of intact, freshly isolated endothelium from resistance arteries provides a powerful experimental approach to evaluate how aging affects the intrinsic ability of the ER and mitochondria within ECs to intrinsically activate SK_{Ca}/IK_{Ca} and to thereby engage in transducing $[Ca^{2+}]_i$ dynamics into changes in V_m.

In accord with impaired tissue blood flow during cardiovascular aging (27,61,63), we questioned how EDH as a vasodilator signal was affected by advanced age. A specific aim of this study was to resolve the interplay between mitochondria-associated $[Ca^{2+}]_i$ signaling and hyperpolarization in endothelium freshly isolated from resistance arteries of mouse skeletal muscle with advanced age. Using mice at ages that correspond to humans in their early 20's and mid-60s (64), we tested the hypothesis that enhanced SK_{Ca}/IK_{Ca} activation effected through increased $[Ca^{2+}]_i$ signaling following mitochondrial uncoupling is integral to EDH during advanced age.

Methods

Animal Care and Use

All animal care and experimental procedures were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Research Council's Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Experiments were performed on young (4–6 months; n = 31) and old (24–26 months; n = 43) male C57BL/6 mice obtained from the National Institute on Aging (NIA) colonies at Charles River Laboratories (Wilmington, MA). Prior to use in experiments, mice were housed at the University Missouri for at least one week on a 12 hours: 12 hours light: dark cycle at approximately 23°C with fresh tap water and standard chow available ad libitum. On the morning of an experiment, a mouse was anesthetized using pentobarbital sodium (60 mg/kg, intraperitoneal injection) and abdominal fur was removed by shaving. Following removal of tissues for experiments, the anesthetized mouse was killed by exsanguination.

Surgery and Microdissection

A ventral midline incision was made through the skin from the sternum to the pubis to expose the abdominal musculature. While viewing through a stereo microscope (SMZ800, Nikon; Tokyo, Japan), fat and connective tissue superficial to the sternum were removed to expose the proximal end of superior epigastric artery (SEA) bilaterally; each SEA was ligated together with its adjacent vein (6-0 silk suture; Ethicon; Somerville, NJ) to maintain blood in the lumen and thereby facilitate visualization during dissection. The abdominal musculature was removed from the mouse and each half was pinned onto transparent silicone rubber (Sylgard 184, Dow Corning; Midland, MI) in physiological salt solution (PSS) maintained at 4°C. Each artery was dissected from its proximal end to the first branch point (segment length: ~2 cm) and then cannulated at one end to flush blood the vessel lumen with PSS. Cannulae (tip outer diameter (OD), 50–80 μm) were made from heat-polished borosilicate glass capillaries (G150T-4, Warner Instruments; Hamden, CT).

Solutions

Control PSS contained the following: (in mmol/L): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10 glucose. For "0 $[Ca^{2+}]_o$", $CaCl_2$ was eliminated and EGTA (10^{-3} M) was added to sequester any free Ca^{2+} in the PSS (65). The pH of all solutions was adjusted to 7.4 using NaOH prior to use. During vessel dissection, $CaCl_2$ was absent from the PSS to relax SMCs. During dissociation of SMCs to obtain endothelial tubes (hereafter referred to as "tubes", which are effectively collapsed under these conditions), PSS contained 0.62 mg/mL papain (≥6 units), 1.5 mg/mL collagenase (≥15 units), 1.0 mg/mL dithioerythritol, 0.1% bovine serum albumin (USB Corp.; Cleveland, OH), and 0.1 mmol/L $CaCl_2$. Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

Endothelial Tube Isolation and Superfusion

Endothelial tubes were prepared as described (11,66). Briefly, each SEA was cut into segments 3–5 mm long, incubated in dissociation PSS for 30 minutes at 37°C, then transferred to a tissue chamber (RC-27N, Warner) containing dissociation PSS at room temperature. To dissociate SMCs, a vessel segment was gently triturated using aspiration and ejection from a micropipette during visual inspection at 200X. Dissociation pipettes were prepared from borosilicate

glass capillary tubes [1.0 mm OD/0.58 mm ID; World Precision Instruments (WPI), Sarasota, FL] that were pulled (P-97; Sutter Instruments; Novato, CA) and heat-polished at one end (tip internal diameter: 80–120 μm). Following dissociation, the tissue chamber containing an endothelial tube (width: ~60 μm, length: ≤1 mm) was secured to an aluminum platform (width: 14.5 cm, length: 24 cm, thickness: 0.4 cm). A micromanipulator (DT3-100, Siskiyou Corp.; Grants Pass, OR) mounted at each end of the platform held a blunt ended heat-polished micropipette (OD, 60–100 μm) that was used to position and secure the tube against the bottom (coverslip) of the tissue chamber. The tube was superfused at approximately 4 mL/min with control PSS; flow through the chamber was parallel to the axis of the tube. The aluminum platform was mounted on an inverted microscope (Eclipse TS100, Nikon) positioned on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA). Throughout experiments, temperature of the chamber was maintained at 32°C using an in-line heater (SH-27B, Warner) and heating platform (PH6, Warner) coupled to a temperature controller (TC-344B, Warner). These preparations are stable for about 5 hours (33,66) and the present experiments were typically completed within 2 hours.

Dye Tracking of the ER and Mitochondria

Mitochondria and ER were labeled simultaneously in endothelial tubes using MitoTracker Deep Red (Excitation: 644/Emission: 665) and ER Tracker Green (Excitation: 504/Emission: 511), respectively, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). Briefly, Mitotracker Deep Red (10^{-7} M) and ER tracker (10^{-7} M) were applied to freshly isolated tubes for 30 minutes at 37°C before images were captured using a 63× glycerol immersion objective (numerical aperture = 1.3) on a Leica SP5 confocal microscope using LAS Software (Leica Microsystems, Wetzlar, Germany).

Ca^{2+} Photometry

Ca^{2+} photometry was performed using an IonOptix system (Milford, MA) as described (33,34,67). Briefly, prior to loading Fura-2 dye, the endothelial tube was maintained at room temperature for 10 minutes, whereas autofluorescence was recorded at 510 nm during alternate excitation at 340 and 380 nm (10 Hz). Fura-2 AM dye (5 μM; F14185, Life Technologies, Eugene, OR) was loaded for 20 minutes followed by 20 minutes of washout to allow for intracellular de-esterification. Temperature was raised to 32°C during the final 10 minutes. Autofluorescence during excitation at 340 and 380 nm (average values over 30 s acquisition) were subtracted from respective recordings at 510 nm. The imaging window was 140 μm × 50 μm using a 40X objective (Nikon S Fluor; numerical aperture, 0.90) and encompassed about 50 ECs (34).

Intracellular Recording and Current Microinjection

Microelectrodes were pulled (P-97; Sutter) from glass capillary tubes (GC100F-10, Warner) and backfilled with 2 mol/L KCl (tip resistance, ~150 MΩ). Membrane potential of ECs was measured using an Axoclamp 2B electrometer (Molecular Devices; Sunnyvale, CA) coupled to a function generator (CFG253, Tektronix; Beaverton, OR) and an IE-210 amplifier (Warner). An Ag/AgCl pellet placed in effluent PSS served as a reference electrode. Amplifier outputs were connected to an analog-to-digital converter (Digidata 1322A, Molecular Devices) and data were recorded at 1,000 Hz on a Dell personal computer using Axoscope 10.1 software (Molecular Devices). Individual ECs were penetrated along the midline of the endothelial tube while viewing at 400X magnification (11,34,41). For simultaneous Ca^{2+} photometry and electrophysiology, the photometric window for acquiring Ca^{2+} measurements was positioned adjacent to the recording electrode (34).

Pharmacology

To examine the effect of ER Ca^{2+} release and Ca^{2+} influx underlying hyperpolarization with advancing age, 3×10^{-6} M ACh was applied for 2 minutes, which we have shown to evoke maximum hyperpolarization and peak $[Ca^{2+}]_i$ in preparations of SEA endothelium identical to those studied here (9,33,41). As the role of mitochondrial Ca^{2+} release and consequential changes in V_m evoked by the mitochondrial uncoupler FCCP have not been tested in the intact endothelium, we evaluated full concentration-response relationships for $[Ca^{2+}]_i$ and V_m in response to a cumulative increase in [FCCP], from 10^{-8} M to 10^{-5} M. Responses were allowed to stabilize for 3 minutes at each [FCCP]. Based on these data, ensuing experiments were performed using 3×10^{-7} M (submaximal) and 10^{-6} M (maximal) [FCCP] as these concentrations revealed differences in $[Ca^{2+}]_i$ and V_m responses between young vs. old mice. Values for summary data were collected during the peak of F_{340}/F_{380} and V_m responses. During exposure to 3×10^{-7} M FCCP, 3×10^{-7} M apamin + 10^{-7} M charybdotoxin were applied to block SK_{Ca} and IK_{Ca}, respectively, to evaluate the role of respective ion channels in EC hyperpolarization.

Data Analysis

Analyses included: (a) Fura-2 fluorescence emission collected at 510 nm and expressed as the ratio during excitation at 340 nm and 380 nm (F_{340}/F_{380}); (b) change in F_{340}/F_{380} ratio ($\Delta F_{340}/F_{380}$) = peak response F_{340}/F_{380} – preceding baseline F_{340}/F_{380}; (c) resting baseline V_m (mV); (d) change in V_m (ΔV_m) = peak response V_m – preceding baseline V_m. Summary data reflect values averaged over 10 seconds during stable recordings. Statistical analyses (GraphPad Software, Inc.; La Jolla, CA) included paired and unpaired Student's *t*-tests, one-way and two-way repeated measures analysis of variance, with Tukey and Bonferroni post-hoc comparisons, respectively. Differences were accepted as statistically significant with $p < .05$. Summary data are presented as means ± standard error. Values for *n* reflects the number of independent endothelial tubes each studied from a different mouse for a given experimental protocol.

Results

Both ER and mitochondria are present in the endothelium of young and old mice (see Supplementary Figure 1). How Ca^{2+} and electrical signaling are shaped by respective organelles to initiate EDH is unknown, particularly in the context of advancing age. In the present experiments, we tested how Ca^{2+} release from the ER or from the mitochondria affected V_m through SK_{Ca}/IK_{Ca} activation in endothelium of resistance arteries from young and old mice.

Aging Enhances ER-Dependent $[Ca^{2+}]_i$ Increases and Hyperpolarization

As illustrated in Figures 1A and 1B (left panels), from resting baseline in the presence of 2 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), ACh evoked an initial "peak" rise in F_{340}/F_{380} attributable to release of Ca^{2+} from the ER followed by a sustained "plateau" attributable to Ca^{2+} influx (35,65). Resting values and peak F_{340}/F_{380} responses to ACh were similar between age groups (Figures 1C and

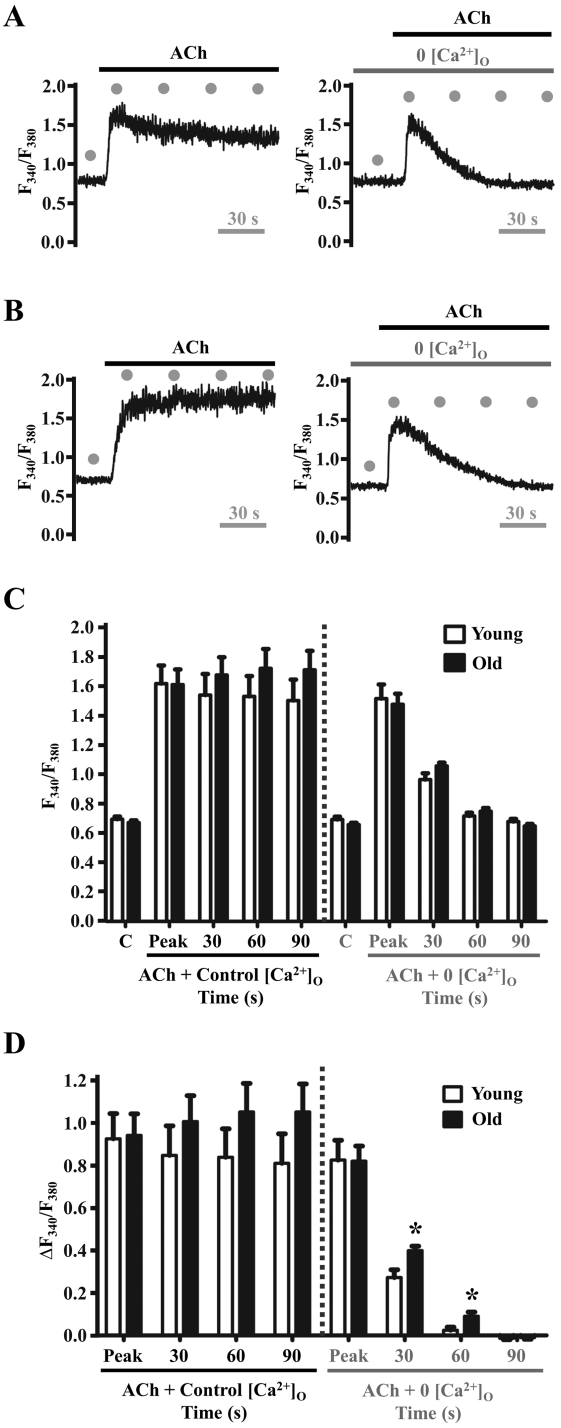

Figure 1. Aging enhances ER-dependent $[Ca^{2+}]_i$ increases in response to ACh. Fura-2 recordings of $[Ca^{2+}]_i$ in response to 3 × 10^{-6} M ACh in the presence of 2 × 10^{-3} M $[Ca^{2+}]_o$ (Control, "**C**") and with 0 $[Ca^{2+}]_o$ in A, Young and B, Old endothelium. C, Summary data for F_{340}/F_{380} and D, $\Delta F_{340}/F_{380}$ at designated time points (gray dots; refer to traces in A and B). Note initial peak of $[Ca^{2+}]_i$ transients (reflecting Ca^{2+} release from ER) followed by sustained plateau (reflecting Ca^{2+} entry through the plasma membrane) during 90 s following peak response. Note diminished plateau during 0 $[Ca^{2+}]_o$ to eliminate Ca^{2+} entry (**A** and **B**, right vs. left panels). Peak $[Ca^{2+}]_i$ responses were similar across groups irrespective of $[Ca^{2+}]_o$ (**C** and **D**). However, in Old endothelium, increases in $[Ca^{2+}]_i$ remain elevated at 30 s and 60 s with 0 $[Ca^{2+}]_o$ (D). *p < .05, old vs. young, n = 10 per group. ACh = acetylcholine; ER = endoplasmic reticulum.

D). Pretreatment with 0 $[Ca^{2+}]_o$ PSS containing EGTA (10^{-3} M) for 1 minute prior to ACh application eliminated the plateau phase leaving only the initial peak phase of the $[Ca^{2+}]_i$ response (Figures 1A and B, right panels). A trend for greater plateau $[Ca^{2+}]_i$ was observed in old vs. young endothelium under control conditions and was statistically significant at 30 seconds and 60 seconds (≥1.5-fold for $\Delta F_{340}/F_{380}$) following the peak phase of the $[Ca^{2+}]_i$ response to ACh during removal of extracellular Ca^{2+} (Figure 1D). The peak phase of either age group was not altered significantly during 0 $[Ca^{2+}]_o$ PSS (Figures 1C and D). The kinetics of V_m in response to ACh mirrored those of $[Ca^{2+}]_i$ (Figures 2A and B, left panels). Thus, hyperpolarization exhibited both a peak phase and a plateau phase under control conditions, whereas V_m returned rapidly to resting levels during 0 $[Ca^{2+}]_o$ despite sustained exposure to ACh (Figures 2A and B, right panels). However, under control conditions, V_m at rest and in response to ACh were significantly greater in old vs. young endothelium (p < .05) and this difference between age groups persisted during the intermediate period (30 s and 60 s) of the plateau phase during 0 $[Ca^{2+}]_o$ PSS (Figure 2C). Though not consistently reaching statistical significance, similar differences between age groups were apparent for the changes in V_m from respective control baselines (Figure 2D). These data illustrate that the rise in $[Ca^{2+}]_i$ and hyperpolarization to ACh are enhanced during aging.

Aging Enhances $[Ca^{2+}]_i$ and Membrane Hyperpolarization Responses to Mitochondrial Uncoupling

Whether aging affects mitochondrial Ca^{2+} release and V_m in microvascular endothelium have not been determined. Thus, concentration-response relationships to FCCP (10^{-8} M to 10^{-5} M) for $[Ca^{2+}]_i$ (Figure 3) and V_m (Figure 4) were determined for young and old endothelium. FCCP is a proton ionophore that collapses the inner mitochondrial membrane potential (typically −180 mV in reference to the cytoplasm) which reduces the affinity for Ca^{2+} ions within the mitochondrial matrix (42–45). As a result, a slow increase in $[Ca^{2+}]_i$ ensues (as compared to rapid $[Ca^{2+}]_i$ increases on M_3 receptor stimulation; compare Figures 1 and 3). Increases in $[Ca^{2+}]_i$ occurred during treatment with 3 × 10^{-7} M FCCP and were similar in young (Figure 3A, left and Figures 3C and D) vs. old (Figure 3B, left and Figures 3C and D). However, the magnitude of $[Ca^{2+}]_i$ increased further during 10^{-6} M FCCP in old (Figure 3B, right and Figures 3C and D) but not in young (Figure 3A, right and Figures 3C and D). Thus, peak $[Ca^{2+}]_i$ reached a higher maximum in old vs. young endothelium in response to FCCP, suggesting a greater capacity for mitochondrial Ca^{2+} release in old. Net changes in V_m to FCCP were variable in young, being mild hyperpolarization (ΔV_m < −10 mV) during 3 × 10^{-7} M (Figure 4A, left and Figures 4C and D) with a significant depolarization (>+10 mV) in response to 10^{-6} M (Figure 4A, right and Figures 4C and D). In old, significant hyperpolarization of V_m (>−10 mV) occurred to both 3 × 10^{-7} M and 10^{-6} M FCCP (Figures 4B–D). Altogether, peak $[Ca^{2+}]_i$ increases to FCCP were higher in old and matched by hyperpolarization, whereas peak $[Ca^{2+}]_i$ increases in young were lower in magnitude and typically corresponded to depolarization. Thus, $[Ca^{2+}]_i$ responses to FCCP are greater in old vs. young endothelium, with membrane hyperpolarization corresponding to mitochondrial uncoupling during aging.

Because FCCP evoked hyperpolarization in Old but not Young endothelium and the activation of SK_{Ca}/IK_{Ca} channels is integral to EC hyperpolarization (14,62,68), we tested whether the effect of FCCP was susceptible to the actions of the SK_{Ca}/IK_{Ca} channel

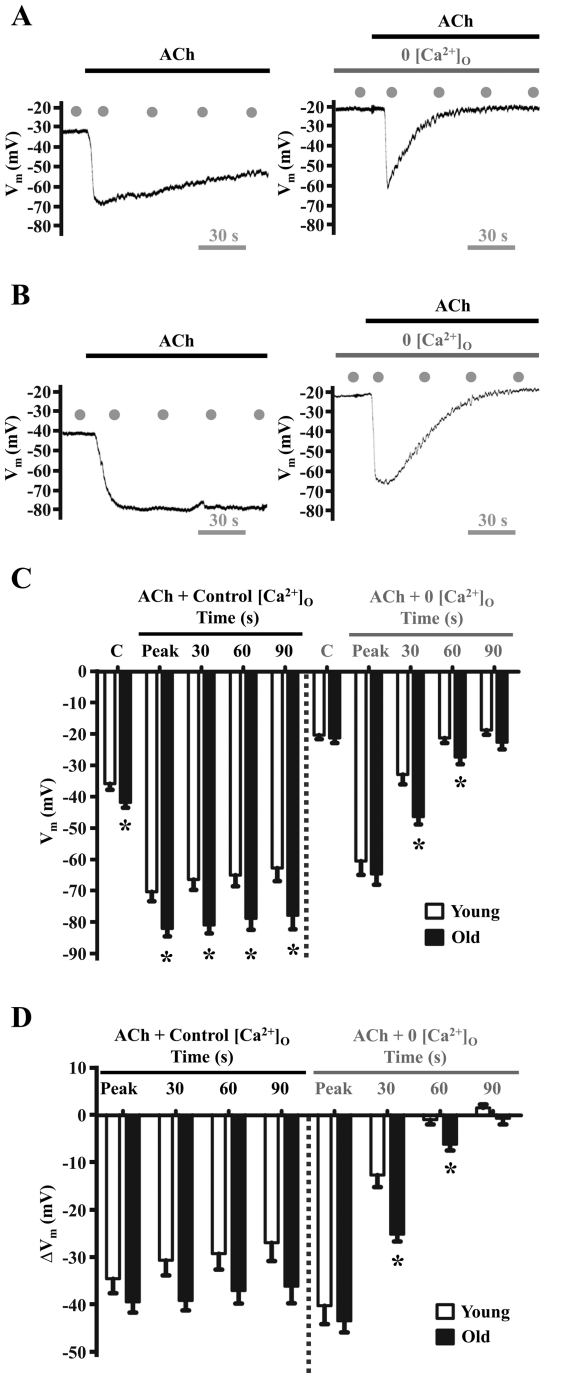

Figure 2. Aging enhances hyperpolarization in response to ACh. V_m recordings (obtained simultaneously with Fura-2 data in Figure 2) in response to 3 × 10^{-6} M ACh in the presence of 2 × 10^{-3} M $[Ca^{2+}]_o$ (Control, "**C**") and with 0 $[Ca^{2+}]_o$ in A, Young and B, Old endothelium. Summary data indicate C, V_m and D, ΔV_m (treatment – control) for respective age groups. Note initial "peak" of hyperpolarization followed by sustained "plateau" through 90 s (**A** and **B**, left panels). Defined time points indicated by gray dots in panels A and B. Note diminished plateau during 0 $[Ca^{2+}]_o$ to eliminate Ca^{2+} entry (A and B, right vs. left panels)]. Peak V_m responses were greater ($p < .05$) in old vs. young in the presence of 2 × 10^{-3} M $[Ca^{2+}]_o$ (panels C and D). Further, V_m remained hyperpolarized (*$p < .05$) in old vs. young at 30 s and 60 s during 0 $[Ca^{2+}]_o$, reflecting sustained internal release of Ca^{2+} (panel D). Traces for Figures 1A and 2A (Young) and Figures 1B and 2B (Old) represent simultaneous $[Ca^{2+}]_i$ and electrical measurements. *$p < .05$, old vs. young, $n = 10$ per group. ACh = acetylcholine.

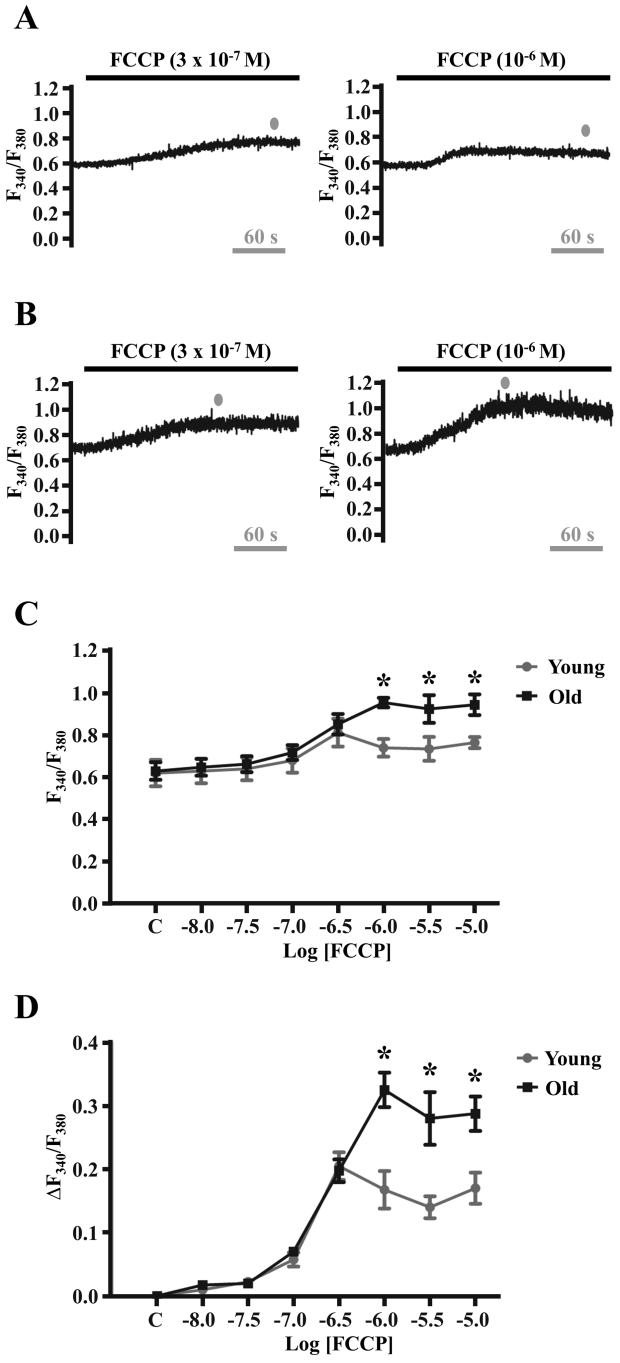

Figure 3. Aging elevates $[Ca^{2+}]_i$ during mitochondrial uncoupling. Fura-2 recordings during 3 × 10^{-7} M and 10^{-6} M FCCP in **A**, young and **B**, old endothelium. Note that the F_{340}/F_{380} ratio did not change from 3 × 10^{-7} M to 10^{-6} M FCCP in young endothelium, whereas it increased in old endothelium. Summary data indicate **C**, peak F_{340}/F_{380} ratio and **D**, responses relative to control ("**C**") during exposure to 10^{-8} to 10^{-5} M FCCP. As the traces for Figures 3A and 4A (young) and Figures 3B and 4B (old) represent simultaneous $[Ca^{2+}]_i$ and electrical measurements, the gray dots in A and B indicate peak $[Ca^{2+}]_i$ responses that coincide with peak V_m responses in the traces shown for Figures 4A and 4B. *$p < .05$ for young vs. old ($n = 4$ per group). Additional experiments (n = 12–22 total) were performed for comparisons for 3 × 10^{-7} M to 10^{-6} M FCCP treatments between young and old [(3 × 10^{-7} M: young, $\Delta F_{340}/F_{380}$: 0.25 ± 0.02, n = 14 and old, $\Delta F_{340}/F_{380}$: 0.23 ± 0.02, n = 12) and (10^{-6} M: young, $\Delta F_{340}/F_{380}$: 0.23 ± 0.02, n = 12 and old: $\Delta F_{340}/F_{380}$: 0.42 ± 0.04, n = 22; $p < .05$ vs. young)]. FCCP = carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone.

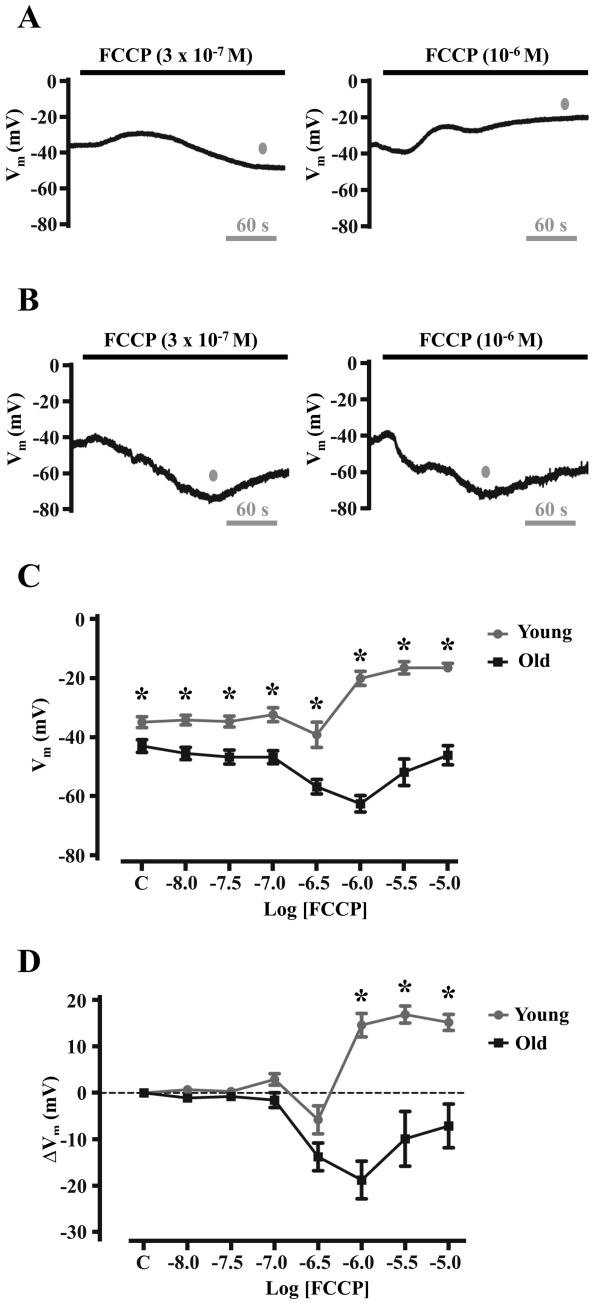

Figure 4. Aging reverses V_m responses of endothelium to mitochondrial uncoupling. (**A**, **B**) V_m recordings during 3 × 10^{-7} M and 10^{-6} M FCCP in (A) young and (B) old endothelial tubes. Note that mild hyperpolarization (<–10 mV) shifts to significant depolarization (>+10 mV) from 3 × 10^{-7} M to 10^{-6} M in young, whereas hyperpolarization responses are sustained in Old. Summary data for peak V_m (**C**) and changes in V_m (**D**) relative to control ("**C**") in response to 10^{-8} to 10^{-5} M FCCP. As the traces for Figures 3A and 4A (young) and Figures 3B and 4B (old) represent simultaneous $[Ca^{2+}]_i$ and electrical measurements, the gray dots in A and B indicate peak V_m responses that coincide with peak $[Ca^{2+}]_i$ responses in the traces shown for Figures 3A and 3B. Endothelial tubes from young reach a maximal depolarization ($\Delta V_m \approx 15$ mV), whereas those from old reach a maximal hyperpolarization ($\Delta V_m \approx -20$ mV) at the same [FCCP] of 10^{-6} M. *$p < .05$ for young ($n = 10$) vs. old ($n = 8$). Additional experiments ($n = 20$–22 total) were performed for comparisons for 3 × 10^{-7} M to 10^{-6} M FCCP treatments between young and old [(3 × 10^{-7} M: young, ΔV_m: –5 ± 3 mV, $n = 20$ and old, ΔV_m: –13 ± 2 mV, $n = 21$; $p < .05$ vs. young) and (10^{-6} M: young, ΔV_m: 14 ± 2 mV, $n = 22$ and old: ΔV_m: –19 ± 3 mV, $n = 22$; $p < .05$ vs. young)]. FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

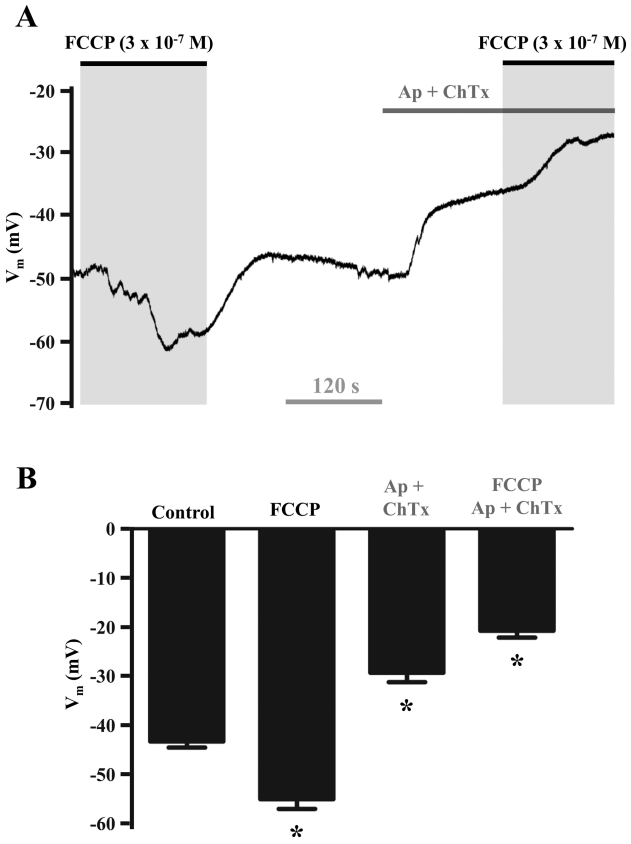

Figure 5. Blockade of SK_{Ca}/IK_{Ca} inhibits hyperpolarization in response to FCCP in endothelium of old mice. (**A**) Representative continuous V_m recording during 3 × 10^{-7} M FCCP in the absence and presence of SK_{Ca}/IK_{Ca} blockade (3 × 10^{-7} M apamin + 10^{-7} M charybdotoxin; Ap + ChTx). (**B**) Summary data illustrating that SK_{Ca}/IK_{Ca} blockade reverses hyperpolarization to depolarization during mitochondrial uncoupling with FCCP. Compared to control V_m, FCCP evoked hyperpolarization ($\Delta V_m \approx -15$ mV). Treatment with Ap + ChTx alone produced ≈10 mV depolarization; in the presence of FCCP, Ap + ChTx depolarized cells by ≈ 25 mV vs. control. *$p < .05$ ($n = 4$). FCCP = carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; SK_{Ca}/IK_{Ca}, small- and intermediate K^+ channel.

blockers apamin and charybdotoxin in the endothelium from old mice. As illustrated in Figure 5, blockade of SK_{Ca}/IK_{Ca} channels in old endothelium converted the response to FCCP (3 × 10^{-7} M) from hyperpolarization to depolarization. Thus, when SK_{Ca} and IK_{Ca} were prevented from opening in old endothelium, the V_m response to FCCP approximated that in young endothelium. This difference between age groups suggests that SK_{Ca}/IK_{Ca} channel activation responds to $[Ca^{2+}]_i$ increases following mitochondrial uncoupling as an adaptation to advanced age.

Discussion

In accord with impaired tissue blood flow with cardiovascular aging (27,61,63), this study investigated whether advanced age affected EDH signaling and whether mitochondrial regulation of $[Ca^{2+}]_i$ was involved. A specific aim was to resolve the interplay between mitochondria-associated $[Ca^{2+}]_i$ signaling and membrane hyperpolarization in native intact endothelium freshly isolated from resistance arteries of mouse skeletal muscle at ages that correspond to humans in their early 20's and mid-60s (64). We tested the hypothesis that enhanced SK_{Ca}/IK_{Ca} activation effected through increased $[Ca^{2+}]_i$

signaling following mitochondrial uncoupling is integral to EDH during advanced age. The present data indicate that aging enhances $[Ca^{2+}]_i$ signaling and membrane hyperpolarization of ECs in response to ACh, the "gold standard" stimulus for evoking EDH (12,14,34). A key finding is that, in old but not young endothelium, mitochondrial uncoupling with FCCP evoked robust $[Ca^{2+}]_i$ increases and hyperpolarized V_m. In contrast, FCCP stimulation of young endothelium resulted in depolarization during the rise in $[Ca^{2+}]_i$. Further, block of SK_{Ca}/IK_{Ca} channels with apamin and charybdotoxin in old endothelium reversed FCCP responses from hyperpolarization to depolarization similar to that observed in young endothelium. Our findings collectively demonstrate that cardiovascular aging enhances $[Ca^{2+}]_i$ signaling and SK_{Ca}/IK_{Ca} channel activity that underlies vasodilation through EDH and that Ca^{2+} release from mitochondria may be integral to this adaptation.

EDH With Aging: ER and Mitochondria-Mediated Ca^{2+} Release

In response to the classic endothelium-dependent vasodilator ACh, the duration of elevated $[Ca^{2+}]_i$ governed the duration of V_m hyperpolarization (Figures 1 and 2). These data illustrate the integral role of $[Ca^{2+}]_i$ (ie, via IP_3R-mediated Ca^{2+} signaling from the ER) to initiate membrane hyperpolarization of the endothelium in response to a physiological agonist (68). The ability of resistance artery endothelium to release Ca^{2+} from the ER in response to ACh (Figure 1) and hyperpolarize V_m (Figure 2) via SK_{Ca}/IK_{Ca} activation was enhanced in old vs. young endothelium. These findings are consistent with our previous studies (9,41) using the same experimental model and age groups studied here.

Mitochondria can sequester Ca^{2+} to upregulate the activity of citric acid cycle proteins involved in oxidative phosphorylation, potentially increasing the rate of respiratory chain activity and production of hydrogen peroxide (69). As ECs primarily depend on glycolysis for the generation of ATP (36,37), it has been proposed that mitochondria of vascular endothelium play a prominent role in regulating $[Ca^{2+}]_i$ (31,38). Indeed, the mitochondrium is a potent Ca^{2+} buffering organelle equipped with Ca^{2+} uniporters, Na^+/Ca^{2+} exchangers, and a negative $\Delta\Psi_{mt}$ (≈−180 mV) that attract cations (70). Further, mitochondrial Ca^{2+} signaling can increase during advanced age (39,40) and may thereby provide an alternative source of Ca^{2+} for activating SK_{Ca}/IK_{Ca} channels in ECs. We therefore reasoned that dissipation of $\Delta\Psi_{mt}$ with FCCP would trigger release of mitochondrial Ca^{2+} into the cytosol and thereby activate K_{Ca} channels to evoke hyperpolarization. Indeed, in old but not young endothelium, robust $[Ca^{2+}]_i$ increases (Figure 3) and membrane hyperpolarization (Figure 4) due to SK_{Ca}/IK_{Ca} activation (Figure 5) were observed in response to FCCP. Further, blocking SK_{Ca}/IK_{Ca} channels in old endothelium prior to FCCP exposure approximated the depolarization to FCCP observed in young endothelium under control conditions (compare young data in Figure 4 with old in Figure 5).

Experimental Advantages and Limitations: Simultaneous Ca^{2+} Photometry and Intracellular V_m Recordings

An advantage of using Ca^{2+} photometry is the ability to obtain data from the same ECs throughout protocols of at least 5 minutes duration (see Figures 1–4). In this manner, Fura-2 photometry concomitant with intracellular recording provides insight with respect to the relationship between $[Ca^{2+}]_i$ and SK_{Ca}/IK_{Ca} channel activation during membrane hyperpolarization. However, a limitation of Ca^{2+} photometry is that it reflects an average $[Ca^{2+}]_i$ for ECs within the recording window, whereas intracellular recording using sharp microelectrodes reflects the difference in electrochemical driving force between the cytoplasm and the extracellular fluid. A consequence of this limitation is the inability to detect subcellular events occurring in subcellular signaling microdomains within the vicinity of individual ion channels (71). Nevertheless, the present findings provide a foundation for future studies using techniques that enable such higher resolution.

Use of FCCP to Uncouple Mitochondria and Mobilize Mitochondrial Ca^{2+}

As an agent that generally uncouples mitochondria and depolarizes $\Delta\Psi_{mt}$ (42–44), FCCP may reflect the broader physiological and pathological functions of native mitochondrial uncoupling proteins (48) and mitochondrial K^+ channels (49,50). In this manner, FCCP also targets mitochondrial Ca^{2+} buffering (43,46,47,72,73) and thereby activates K_{Ca} channels (46). However, depolarization to micromolar concentrations FCCP in young endothelium (see Figure 4) may also reflect nonselective actions of H^+ and/or Na^+ influx into the cell interior across the plasma membrane (43). In old endothelium, the possibility of such depolarization to FCCP may be "masked" by relatively large $[Ca^{2+}]_i$ increases vs. young and the predominant effect of SK_{Ca}/IK_{Ca} activation and hyperpolarization (compare Figures 4 and 5). Earlier findings concluded that FCCP may also promote depletion of ER Ca^{2+} based on reasoning that mitochondrial production of ATP is reduced, thereby impairing the ability of the smooth ER Ca^{2+} ATPase (commonly referred to as SERCA) pumps to refill the ER with Ca^{2+} (74). However, those findings were based on a low affinity divalent cation dye (ie, Furaptra) (74) that is nonselective for both Ca^{2+} vs. Mg^{2+} and ER vs. mitochondria (75). In addition, because ECs rely primarily on glycolysis for generating ATP (36,37), glucose was maintained at 10^{-2} M in the PSS used for superfusion throughout experiments. Importantly, the kinetics of $[Ca^{2+}]_i$ responses to FCCP found here (ie, stable peak within 3 min) do not align with the earlier study [(74); which required ≥5 minutes for a response with no apparent stability]. Further, the present experiments used freshly isolated endothelium (within 1 hour of being in the animal), whereas previous studies have evaluated mitochondrial Ca^{2+} buffering in isolated ECs or cultured ECs having altered (ie, "cobblestone") morphology (76) and ion channel expression (77). Such conditions alter the spatial and functional relationships between ER, mitochondria and the plasma membrane that are inherent to native endothelium. In accord with the present findings, we posit that increased mitochondrial Ca^{2+} release, and an overall increase in the release of Ca^{2+} from internal stores, promotes EDH to help maintain vasodilation—and thereby tissue blood flow—during advanced age.

SK_{Ca}/IK_{Ca} Function With Aging and Vascular Disease

How SK_{Ca}/IK_{Ca} function conveys EDH-dependent vasodilation during aging has lacked a consensus. Some studies suggest a loss of SK_{Ca}/IK_{Ca}-dependent vasodilation (51,53,78), whereas others have found enhanced SK_{Ca}/IK_{Ca} function (41,55,57). Difficulty in interpretation may be attributed to factors confounding EC function such as perivascular nerves, SMCs and the flow of blood (carrying hormones, etc.). In addition, complementary signaling pathways include $[Ca^{2+}]_i$ activation of endothelial NO synthase (eNOS) and large conductance calcium-activated K^+ channels (BK_{Ca}) on SMCs, which can hyperpolarize ECs through myoendothelial coupling (79). These vasodilator pathways can also be governed by endothelial

mitochondria and reactive oxygen species (70,71). Indeed, BK_{Ca} function is increased with aging in SMCs of the mouse SEA (the source of endothelial tubes used within the current study) (80). Discrepancies between studies may also reflect a lack of awareness for structural changes (eg, arterial stiffening and smooth muscle hypertrophy) of the vasculature that may occur in conjunction with altered ion channel activity during aging (53,80,81). Thus, a key aspect of the experimental design used here was to eliminate other influences to evaluate the intrinsic ability of SK_{Ca}/IK_{Ca} to engage in the transduction of $[Ca^{2+}]_i$ signals to hyperpolarization in the endothelium isolated from resistance arteries of young and old mice. The present findings are consistent with our previous reports indicating that the function of underlying components of EDH signaling (ie, $[Ca^{2+}]_i$ and SK_{Ca}/IK_{Ca} function) is enhanced during aging (9,41).

SK_{Ca}/IK_{Ca} as Pharmacological Targets: Increasing NO Bioavailability Through Hyperpolarization-Induced Ca^{2+} Entry

Through pharmacological intervention, SK_{Ca}/IK_{Ca} can be activated without elevating $[Ca^{2+}]_i$ (22–25,82,83). Thus, in the face of impaired vascular NO production with aging, endothelial hyperpolarization via direct SK_{Ca}/IK_{Ca} activation can promote Ca^{2+} influx into the cell according to its electrochemical gradient (34) and thereby increase eNOS activation to generate NO (23,84), particularly with concomitant activation of Ca^{2+}-permeant channels in the plasma membrane (34). The activation of SK_{Ca}/IK_{Ca} may also serve as a feed-forward mechanism, whereby the increase in Ca^{2+} influx amplifies hyperpolarization via SK_{Ca}/IK_{Ca} activation (34,85). In such manner, the direct activation of SK_{Ca}/IK_{Ca} can be used effectively to promote vasodilation and tissue blood flow (86,87) during cardiovascular aging (27,61,63).

Enhanced Endothelial Ca^{2+} Mobilization and Activation of SK_{Ca}/IK_{Ca} Channels During Aging: A Potential Compensatory Mechanism for Reduced NO Bioavailability

The current study offers new perspective with regard to ER and mitochondrial mobilization of intracellular Ca^{2+} to activate endothelial SK_{Ca}/IK_{Ca}. In particular, the release of Ca^{2+} from endothelial mitochondria can activate SK_{Ca}/IK_{Ca} channels to generate hyperpolarization and may thereby compensate for decreased bioavailability of NO in old age (26,27,62). However, only 10 to 15 mV of hyperpolarization from resting V_m (ie, −30 to −40 mV) is sufficient for maximal dilation of arteries and arterioles (16,18). Overactivation of SK_{Ca}/IK_{Ca} to the extent where V_m >−60 mV can cause "leaky" membranes, whereby the initiation and longitudinal spread of hyperpolarization along electrically coupled ECs is short-circuited (11,41). Thus, a method for preserving endothelial function during old age may be to restrict Ca^{2+} overload of mitochondria and limit the production of reactive oxygen species (69,88) to prevent overactivation of SK_{Ca}/IK_{Ca} channels (41). Nevertheless, the ability of endothelial mitochondria to affect SK_{Ca}/IK_{Ca} channel function via increases in $[Ca^{2+}]_i$ and reactive oxygen species may be harnessed to promote vasodilation and maintain blood flow during healthy aging to compensate for reductions in NO bioavailability (89). Thus, a balanced therapeutic approach is implied.

Summary and Conclusions

With advancing age, individuals 65 years or older will comprise approximately 25% of the US population by 2030 (1). This demographic typically manifests morbidity and mortality in the form of cardiovascular disease (2,3) with the hallmark of endothelial dysfunction (4,5) underlying impaired tissue blood flow. Our goal in the present study was to determine whether, and if so, how the endothelium of resistance arteries may adapt to maintain its role in effecting vasodilation during advanced age. We focused on the relationship between Ca^{2+} release from internal stores and the activation of SK_{Ca}/IK_{Ca} channels in native endothelium freshly isolated from skeletal muscle of young and old mice with an emphasis on the role of mitochondria. Whereas the increases in both $[Ca^{2+}]_i$ and membrane potential in response to ACh (ie, muscarinic receptor activation) were sustained with advanced age, uncoupling mitochondria in the endothelium of old mice enhanced $[Ca^{2+}]_i$ responses and augmented membrane hyperpolarization through activation of SK_{Ca}/IK_{Ca} channels. Thus, despite elevated oxidative signaling (9,41,61) and diminished endothelium-dependent dilation with advancing age (26,27), the ability of endothelium to mediate vasodilation via the EDH electrical signaling axis is preserved. In such manner, mitochondrial $[Ca^{2+}]_i$ signaling may effectively govern SK_{Ca}/IK_{Ca} activity during advanced age. With the integrity of EDH maintained via mitochondria versus the ER, targeting specific intracellular Ca^{2+} stores that govern SK_{Ca}/IK_{Ca} activity offers the potential for advancing therapeutic strategies designed to protect and restore tissue blood flow and oxygen delivery.

Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

Funding

This work was supported by National Institutes of Health grants K99/R00-AG047198 (EJB), R01-HL086483 (SSS), and R37-HL041026 (SSS). The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgments

Rebecca Shaw provided excellent technical assistance obtaining the images in Supplementary Figure 1.

Conflict of Interest

None.

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