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Advanced age decreases local calcium signaling in endothelium of mouse mesenteric arteries in vivo

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Boerman EM, Everhart JE, Segal SS. Advanced age decreases local calcium signaling in endothelium of mouse mesenteric arteries in vivo. Am J Physiol Heart Circ Physiol 310: H1091–H1096, 2016. First published March 4, 2016; doi:10.1152/ajpheart.00038.2016.—Aging is associated with vascular dysfunction that impairs tissue perfusion, physical activity, and the quality of life. Calcium signaling in endothelial cells (ECs) is integral to vasomotor control, exemplified by localized Ca2+ signals within EC projections through holes in the internal elastic lamina (IEL). Within these microdomains, endothelium-derived hyperpolarization is integral to smooth muscle cell (SMC) relaxation via coupling through myoendothelial gap junctions. However, the effects of aging on local EC Ca2+ signals (and thereby signaling between ECs and SMCs) remain unclear, and these events have not been investigated in vivo. Furthermore, it is unknown whether aging affects either the number or the size of IEL holes. In the present study, we tested the hypothesis that local EC Ca2+ signaling is impaired with advanced age along with a reduction in IEL holes. In anesthetized mice expressing a Ca2+-sensitive fluorescent protein (GCaMP2) selectively in ECs, our findings illustrate that for mesenteric arteries controlling splanchnic blood flow in anesthetized mice, spontaneous Ca2+ signals in ECs was reduced by ~85% in old (24–26 mo) vs. young (3–6 mo) animals. At the same time, the number (and total area) of holes per square millimeter of IEL was reduced by ~40%. We suggest that diminished signaling between ECs and SMCs contributes to dysfunction of resistance arteries with advanced age.

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NEW & NOTEWORTHY

In endothelial cells of mesenteric arteries controlling splanchnic blood flow in anesthetized mice, spontaneous Ca2+ signals occur far less frequently with advanced age. The internal elastic lamina also has fewer holes enabling direct contact between endothelial and smooth muscle cells. These changes with advanced age can contribute to vascular dysfunction.

ENDOTHELIUM-DEPENDENT VASODILATION is governed by a rise of intracellular Ca2+ concentration ([Ca2+]i), which stimulates the production of autacoids (e.g., nitric oxide, prostacyclin) and initiates endothelium-derived hyperpolarization (EDH). Whereas relaxation of smooth muscle cells (SMCs) via autacoids is manifest in conduit arteries, EDH prevails in resistance arteries and arterioles (6, 13). With advanced age, these respective signaling pathways, and therefore vasodilation, are impaired (2, 7, 17, 27). In vitro preparations of resistance arteries have revealed localized Ca2+ signals in endothelial cells (ECs) that arise from both intracellular (14) and extracellular (21, 30) sources. These discrete Ca2+ events occur spontaneously as well as through complementary signaling pathways (32), thereby activating nearby small- and intermediate-conductance Ca2+-activated K+ channels (KCa2.3 and KCa3.1, respectively). The ensuing hyperpolarization is transmitted to SMCs through gap junctions located at endothelial projections through holes (i.e., fenestrae) in the internal elastic lamina (IEL) that enable direct physical contact with SMCs (8, 11, 13, 23, 32). Thus signaling through gap junctions localized to myoendothelial junctions (MEJs) provides a unique intercellular signaling microdomain whereby local increases in EC [Ca2+]i lead to SMC relaxation and vasodilation (14, 30, 34).

Previous studies of local Ca2+ signals in ECs of isolated vessel preparations have relied upon the addition of fluorescent Ca2+-sensitive indicators, opening the vessel to expose the endothelium, or combinations thereof (14, 15, 19, 25, 30, 34). Whereas intravital microscopy has been used to image global [Ca2+]i signaling of arteriolar ECs in vivo (1, 31), these techniques have not been applied to resolve local Ca2+ signals of resistance arteries controlling tissue blood flow. Advancing age is associated with impaired vascular function attributed primarily to the endothelium (7, 16, 27); however, the effect of aging on EC Ca2+ signaling at MEJs is unknown. Thus a key goal of the present study was to adapt our intravital preparation of mesenteric arteries (MAs) (35) for confocal imaging, using mice that express the fluorescent Ca2+-inducer GCaMP2 selectively in ECs (31) to evaluate the effect of advanced age on spontaneous EC Ca2+ signals during blood flow control. While the number and size of IEL holes vary with vessel caliber and location (22, 23), the effect of aging on IEL holes is unknown. Therefore, the second goal of this study was to determine whether (and if so, how) the number and size of IEL holes differ between MAs of young and old mice. We tested the hypothesis that local EC Ca2+ signaling is impaired with...
advanced age along with a reduction in IEL holes that enable MEJ formation.

**METHODS**

**Ethical approval and animal use.** All procedures were approved by the Institutional Animal Care and Use Committee of The University of Missouri and performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Intravital experiments were performed on young (3–6 mo) and old (24–26 mo) Tg (RP24-25504-GCaMP2)1Mik (GCaMP2) mice bred on a C57BL/6J background at The University of Missouri and genotyped at weaning. Males and females positive for the GCaMP2 transgene were studied under identical conditions, with order randomized across age and sex. Experiments examining the IEL were performed on young (3–6 mo) and old (24–28 mo) C57BL/6J mice bred at colonies maintained by the National Institute of Aging at Charles River Laboratories (Wilmington, MA). Experimental protocols included MAs from three to seven mice of each age group, typically utilizing at least two vessel segments (studied independently) per mouse. Each mouse was anesthetized with pentobarbital sodium (60 mg/kg, ip injection), and abdominal fur was removed by shaving. After experimental procedures, the anesthetized mouse was euthanized with an overdose of pentobarbital via intracardiac injection followed by cervical dislocation.

**Surgical preparation for studying mesenteric arteries in vivo.** After initial injection of pentobarbital, the mouse was given supplemental injections (15 mg/kg) as needed to maintain stable anesthesia as confirmed by lack of withdrawal to toe or tail pinch. The mouse was positioned on a heated aluminum plate to maintain esophageal temperature at 37°C. A midline laparotomy was performed to exteriorize the intestine in vivo (35). Confocal imaging detected spontaneous events with frequency 0.19 ± 0.10 Hz and amplitude (F/Fo) 1.26 ± 0.47 for Ca2+ imaging, we carefully dissected periarterial fat away from the intestine in vivo (35). Confocal imaging detected spontaneous events with frequency 0.19 ± 0.10 Hz and amplitude (F/Fo) 1.26 ± 0.47 for Ca2+ imaging. After surgical preparation for studying mesenteric arteries in vivo, we carefully dissected periarterial fat away from the intestine or its vascular supply. The intestine was pinned en face (endothelium exposed) to a 12-well plate coated with Sylgard 184 with pins made from tungsten wire (diameter = 25 µm; Goodfellow, Huntingdon, UK). Arteries were fixed in 4% paraformaldehyde for 20 min, washed in phosphate-buffered saline (PBS; Sigma catalog no. P5368), incubated in Alexa Fluor 633 hydrazide (10–6 M; catalog no. A-30634, Life Technologies, Carlsbad, CA) for 60 min to label the IEL, and then washed with PBS and mounted on slides with ProLong Gold (Life Technologies) and a coverslip. Slides were imaged with a Leica SP8 confocal laser-scanning microscope (Leica Microsystems, Buffalo Grove, IL). Images of fluorescent staining of the IEL were acquired with a HCX PL APO ×60 glycerol immersion objective (numerical aperture 1.3; Leica) and an ×8 line average. For all slide sets, young and old MAs were imaged with similar laser power and gain settings. Two MA segments from each of four mice per age group were used for analysis of IEL holes. For each vessel 3–5 images were acquired, for a total of 30 images from young MAs and 34 from old MAs. Holes in the IEL were counted and analyzed with a custom macro in ImageJ. Each image (3,750 µm2) was adjusted to increase contrast and then converted to binary form with the “threshold” command such that only the holes remained black. The “Particle Analyzer” function was then used to count and measure holes that fell within defined size and circularity ranges. The resulting data were exported to a spreadsheet for analyses.

**Chemicals and reagents.** All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Solutions were prepared fresh for each day’s experiments.

**RESULTS**

**In vivo EC Ca2+ signaling events in old and young MAs.** To determine whether advanced age is associated with impaired local EC Ca2+ signaling in MAs, we exposed the mesentery in anesthetized mice to observe the vascular supply of the small intestine in vivo (35). Confocal imaging detected spontaneous local Ca2+ signaling in ECs of MA segments. For recordings of 10-s duration in a FOV that contained ~15 ECs, local Ca2+ signaling events occurred in young MAs in 4.3 ± 0.5 active sites and exhibited the following properties (Fig. 1): frequency: 0.31 ± 0.03 Hz; amplitude (F/Fo): 1.31 ± 0.01; full duration at half-maximal amplitude (FDHM): 0.11 ± 0.01 s. In old MAs, local Ca2+ signaling events occurred in 0.6 ± 0.2 sites (P < 0.05 vs. young MAs). Each of the properties of local Ca2+ signals was also altered significantly in old vs. young MAs, with frequency 0.19 ± 0.03 Hz, amplitude 1.61 ± 0.16, and FDHM 0.08 ± 0.01 s (Fig. 1). Thus advanced age was associated with a ~85% reduction in the number of active Ca2+ signaling sites in ECs, a ~40% reduction in the frequency of these localized signaling events, a ~20% increase in their amplitude, and a ~25% reduction in duration.

**Effect of aging on IEL holes.** Compared with MAs from young mice (Fig. 2A), MAs from old mice (Fig. 2B) had ~40% fewer IEL holes per square millimeter (young: 14.1 ± 0.8, old: 8.2 ± 0.4; P < 0.05; Fig. 2C). However, the average area of individual holes did not change significantly with age (young: 8.2 ± 0.4, old: 8.7 ± 0.7; P = 0.17; Fig. 2C). Analyses of EC Ca2+ signals were performed with SparkAn software provided by Dr. Adrian Boney (University of Vermont). A 10 × 10-pixel region of interest (ROI; 6.25 × 6.25 µm) was placed over each site exhibiting a Ca2+ event during the 10-s recording period applying a boxcar average of 3 frames. Events with amplitudes > 1.2 F/Fo, where F = signal and Fo = baseline fluorescence) were analyzed, and these data were imported into Microsoft Excel. Statistical analyses were performed with GraphPad Prism 5 (La Jolla, CA). Differences were accepted as statistically significant with P ≤ 0.05.

**Fluorescence staining and imaging the IEL.** Second-order MAs from young and old mice were isolated, cut open longitudinally, and pinned en face (endothelium exposed) to a 12-well plate coated with Sylgard 184 with pins made from tungsten wire (diameter = 25 µm; Goodfellow, Huntingdon, UK). Arteries were fixed in 4% paraformaldehyde for 20 min, washed in phosphate-buffered saline (PBS; Sigma catalog no. P5368), incubated in Alexa Fluor 633 hydrazide (10–6 M; catalog no. A-30634, Life Technologies, Carlsbad, CA) for 60 min to label the IEL, and then washed with PBS and mounted on slides with ProLong Gold (Life Technologies) and a coverslip. Slides were imaged with a Leica SP8 confocal laser-scanning microscope (Leica Microsystems, Buffalo Grove, IL). Images of fluorescent staining of the IEL were acquired with a HCX PL APO ×60 glycerol immersion objective (numerical aperture 1.3; Leica) and an ×8 line average. For all slide sets, young and old MAs were imaged with similar laser power and gain settings. Two MA segments from each of four mice per age group were used for analysis of IEL holes. For each vessel 3–5 images were acquired, for a total of 30 images from young MAs and 34 from old MAs. Holes in the IEL were counted and analyzed with a custom macro in ImageJ. Each image (3,750 µm2) was adjusted to increase contrast and then converted to binary form with the “threshold” command such that only the holes remained black. The “Particle Analyzer” function was then used to count and measure holes that fell within defined size and circularity ranges. The resulting data were exported to a spreadsheet for analyses.

**Chemicals and reagents.** All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Solutions were prepared fresh for each day’s experiments.
1.8 ± 0.1 μm², old: 2.0 ± 0.1 μm²; Fig. 2D), nor did distribution of hole sizes change significantly between ages (Fig. 2F). Thus with advanced age the percentage of total IEL area occupied by holes also decreased by 40% in old vs. young MAs (young: 2.6 ± 0.3%, old: 1.6 ± 0.1%; P < 0.05; Fig. 2E).

**DISCUSSION**

This is the first study to utilize high-speed confocal microscopy to image local Ca²⁺ signals of MAs in vivo. The presence of spontaneous localized Ca²⁺ pulsars from inositol 1,4,5-trisphosphate (IP₃) receptors (14) and sparklets from transient receptor potential vanilloid 4 (TRPV4) channels (30) associated with MEJs. Local EC Ca²⁺ signals are also integral to the regulation of vasomotor function through the activation of TRPA1 (21) and KCa3.1 (14) channels, which are both concentrated in EC projections through IEL holes. Because such local events can be amplified into larger Ca²⁺ signals within ECs to evoke vasodilation (30), their diminution with advanced age is functionally relevant in the context of impaired endothelium-dependent vasodilation and restricted blood flow in the aging vascular supply (7, 17).

The dramatic reduction in number of sites for local EC Ca²⁺ signaling events in MAs from old mice (Fig. 1C) is a major finding of this study and likely reflects a reduction in endothelial projections as well as the number of IEL holes. Nevertheless, the properties of Ca²⁺ signaling events were also altered by advanced age. Thus the frequency as well as the duration of individual Ca²⁺ events were decreased significantly in old vs. young ECs, while their amplitude increased (Fig. 1, D–F). These changes in Ca²⁺ signal morphology with advanced age likely reflect changes in calcium content within the endoplasmic reticulum together with the expression and localization of relevant receptors and ion channels. Such changes having been identified here, further studies are required to determine the cellular and molecular adaptations in the signaling pathways underlying these adaptations, particularly in light of their effects on vasomotor control.

This study is the also the first to investigate the effect of aging on IEL holes in resistance arteries. Our results in MAs highlight that while the relative distribution of IEL hole sizes was not altered with advanced age, their density and total area were markedly lower in MAs of old mice compared with MAs of young mice (Fig. 2). A study of pig thoracic aortas (9) found no age-related changes in the size of IEL holes at 6–9 mo compared with those at 6–8 wk. However, the 6–9 mo age group does not reflect changes in IEL structure with advanced age, as it approximates human adolescents. In contrast, mice at 24–26 mo approximate humans at 65–70 yr. Furthermore,
MEJs are more prevalent in small resistance arteries compared with larger conduit arteries (22, 23). Thus an aging-related decline in myoendothelial communication may be of more functional significance in resistance vessels, which rely on EDH-mediated vasodilation to a greater extent than conduit arteries, where endothelium-derived autacoids act as a primary mediator of SMC relaxation (24, 28, 33). The decrease in IEL holes and total hole area seen here with advanced age in mouse MAs is consistent with findings in MAs of hypertensive rats, where vascular and EC dysfunction were associated with smaller holes in the IEL compared with normotensive rats (10). Hypertensive stroke-prone rats also had smaller IEL holes in carotid arteries vs. normotensive control rats (3). We therefore propose that the age-related decrease in IEL holes contributes to vascular dysfunction via decreased myoendothelial signaling between ECs and SMCs at MEJs in MAs of old mice.

In light of myoendothelial communication during vasomotor control, a decline in both local EC Ca\(^{2+}\) signals and IEL holes may also impact the effects of sympathetic nerve activity, which increases with advanced age (26). The release of noradrenaline from perivascular sympathetic nerves acts on \(\alpha_1\)-adrenoreceptors of SMCs, leading to formation of IP\(_3\) and (via Ca\(^{2+}\) release from the sarcoplasmic reticulum) a global rise in SMC \([\text{Ca}^{2+}]_i\) to evoke vasoconstriction (20). In turn, diffusion of these second messengers through MEJs (11, 12, 14) evokes local \([\text{Ca}^{2+}]_i\) signals in endothelial projections, which provide negative feedback to SMCs through the activation of \(K_{\text{Ca}}\) channels (12, 19, 30, 34) as well as endothelial nitric oxide synthase (eNOS), which are both enriched at MEJs (18, 34). In this manner, bidirectional signaling between SMCs and ECs is integral to the physiological regulation of vascular resistance and blood flow control. With
advancing age, the increase in sympathetic nerve activity may help to compensate for the desensitization of adrenoceptors observed in old MAs (35). Nevertheless, the reduction in spontaneous Ca\(^{2+}\) signaling (Fig. 1) and loss of IEL holes (Fig. 2) would attenuate negative feedback from ECs to SMCs, thereby elevating vascular resistance and restricting tissue blood flow. Furthermore, loss of local EC Ca\(^{2+}\) signaling is consistent with a reduction in eNOS activity in rats (4, 5) as well as humans (29) with advanced age. Also contributing to endothelial dysfunction with advanced age are a reduction in eNOS cofactor availability and an increase in reactive oxygen species that effectively reduce nitric oxide bioavailability (16, 27). In accord with the present findings, we propose that the reduction in spontaneous local Ca\(^{2+}\) signals with advanced age reflects impaired myoendothelial communication in association with a reduction in IEL holes.

It should be recognized that the occurrence of IEL holes need not be proportional to the occurrence of MEJs, as there are far more IEL holes than there are endothelial projections through them at any given time (23). However, this association has only been studied in young animals, where IEL holes are likely to remain relatively consistent. On the basis of the present findings, we suggest that the significant reduction in the number and total area of IEL holes with advanced age reduces the probability for MEJ formation and can thereby impair both the initiation of local EC Ca\(^{2+}\) signals and the bidirectional signaling between ECs and SMCs, including EDH and its role in modulating vascular smooth constriction. A limitation of the present study is that neither the source of local EC Ca\(^{2+}\) events nor their approximation to IEL holes containing MEJs was resolved definitively in vivo. Further studies are also needed to determine whether the observed age-related changes in IEL hole numbers impact MEJ formation and signaling events that have been localized to this microdomain (14, 19, 30, 34). Nevertheless, the reduction in both spontaneous EC Ca\(^{2+}\) signaling events and IEL holes reported here for MAs of old vs. young mice suggests that vascular dysfunction with advanced age is associated with a decrease in the capacity for myoendothelial signaling in resistance arteries.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). 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.

AUTHOR CONTRIBUTIONS

Author contributions: E.M.B. and S.S.S. conception and design of research; E.M.B. and J.E.E. performed experiments; E.M.B., J.E.E., and S.S.S. analyzed data; E.M.B. and S.S.S. interpreted results of experiments; E.M.B. prepared figures; E.M.B. drafted manuscript; E.M.B. and S.S.S. edited and revised manuscript; E.M.B., J.E.E., and S.S.S. approved final version of manuscript.

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