Aging Impairs Electrical Conduction Along Endothelium of
Resistance Arteries Through Enhanced Ca\textsuperscript{2+}-Activated
K\textsuperscript{+} Channel Activation

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Objective—Intercellular conduction of electrical signals underlies spreading vasodilation of resistance arteries. Small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels of endothelial cells serve a dual function by initiating hyperpolarization and modulating electrical conduction. We tested the hypothesis that regulation of electrical signaling by small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels is altered with advancing age.

Approach and Results—Intact endothelial tubes (60 µm wide; 1–3 mm long) were freshly isolated from male C57BL/6 mouse (Young: 4–6 months; Intermediate: 12–14 months; Old: 24–26 months) superior epigastric arteries. Using dual intracellular microelectrodes, current was injected (±0.1–3 nA) at site 1 while recording membrane potential (V\textsubscript{m}) at site 2 (separation distance: 50–2000 µm). Across age groups, greatest differences were observed between Young and Old. Resting V\textsubscript{m} in Old (−38±1 mV) was more negative (P<0.05) than Young (−30±1 mV). Maximal hyperpolarization to both direct (NS309) and indirect (acetylcholine) activation of small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels was sustained (ΔV\textsubscript{m}=−40 mV) with age. The length constant (λ) for electrical conduction was reduced (P<0.05) from 1630±80 µm (Young) to 1320±80 µm (Old). Inhibiting small- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels with apamin+charybdotoxin or scavenging hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) with catalase improved electrical conduction (P<0.05) in Old. Exogenous H\textsubscript{2}O\textsubscript{2} (200 µmol/L) in Young evoked hyperpolarization and impaired electrical conduction; these effects were blocked by apamin+charybdotoxin.

Conclusions—Enhanced current loss through Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel activation impairs electrical conduction along the endothelium of resistance arteries with aging. Attenuating the spatial domain of electrical signaling will restrict the spread of vasodilation and thereby contribute to blood flow limitations associated with advanced age. Arterioscler Thromb Vase Biol. 2013;33:1892-1901.)

Key Words: endothelial cells ■ ion channels ■ oxidative stress

Aging is associated with endothelial dysfunction,\textsuperscript{1,2} a disorder characterized by impaired vasodilation in response to acetylcholine (ACh),\textsuperscript{3,4} muscular exercise,\textsuperscript{5} or heating the skin.\textsuperscript{6} As a stimulus that is well defined in its actions, ACh application triggers endothelium-dependent vasodilation by increasing the production of nitric oxide (NO) and activating small (K\textsubscript{Ca2.3}, KCN3) and intermediate (K\textsubscript{Ca3.1}, KCNN4)-conductance calcium-activated K\textsuperscript{+} channels of endothelial cells.\textsuperscript{7,8,9,10} The bioavailability of NO decreases with advancing age,\textsuperscript{11,12} and the function of endothelial SK\textsubscript{Ca2+}/IK\textsubscript{Ca} function has not been determined, particularly in light of impairments in blood flow that accompany advancing age.\textsuperscript{13,14,15} Cell-to-cell signaling through gap junctions is integral to endothelial function. Once initiated, hyperpolarization spreads rapidly along the endothelium and through myoendothelial junctions to relax smooth muscle cells (SMCs).\textsuperscript{16,17} By synchronizing vasomotor responses in resistance networks, the conduction of electrical signals along the endothelium serves to coordinate blood flow control along and among vessel branches.\textsuperscript{18,19} Nevertheless, the spatial domain of endothelial signaling has received little attention in the context of aging.

In previous studies, conducted vasodilation in response to ACh\textsuperscript{20} and ascending vasodilation in response to skeletal muscle contraction\textsuperscript{21} were decreased in Old (20 months) versus Young (3 months) male C57BL/6 mice. Although the mechanism underlying this functional deficit has remained undefined, altered cell-to-cell coupling through gap junctions\textsuperscript{22} could underlie impaired conduction. An alternative mechanism entails greater leakage of current through ion channels in plasma membranes, thereby precluding transmission of electrical signals along the endothelium.\textsuperscript{23} The activation of SK\textsubscript{Ca2+}/IK\textsubscript{Ca} initiates endothelial cell (EC) hyperpolarization and vasodilation.\textsuperscript{5,10,16,23} Recent findings have revealed a role for SK\textsubscript{Ca2+}/IK\textsubscript{Ca} activation in modulating the spread of electrical signals along the endothelium of resistance arteries.\textsuperscript{24} Thus, changes in SK\textsubscript{Ca2+}/IK\textsubscript{Ca} function with advancing age may alter the ability of electrical signals to travel along the endothelium and thereby affect vasomotor control.

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The present experiments were designed to define the ability of the endothelium to initiate and conduct electrical signals with advancing age. Using endothelial tubes freshly isolated from resistance arteries of skeletal muscle from Young (4–6 months), Intermediate (12–14 months), and Old (24–26 months) mice, we tested the hypothesis that the dual function of SKCa/IKCa to initiate and modulate electrical signaling along the endothelium is altered with aging. Findings reported here are the first to show that, by enhancing ion channel activation (particularly IKCa), aging promotes hyperpolarization of the endothelium while impairing its ability to conduct electrical signals. This increase in ion channel activation is attributable to oxidative stress manifested through the actions of H₂O₂.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Figures 1–7 illustrate Old versus Young mice. Key values for the Intermediate age group are stated in the text with their summary data shown in Figures II, III, and V in the online-only Data Supplement. Across experiments, resting \( V_m \) was more negative (\( P<0.05 \)) in Old (−38±1; \( n=28 \)) versus Young (−30±1; \( n=29 \)) or Intermediate (−31±1 mV; \( n=9 \)).

Expression of Connexins and SKCa/IKCa in Endothelial Tubes

The mRNA transcript expression for SKCa and IKCa (Figure 1A) and connexins (Cx37, Cx40, and Cx43; Figure 1A in the online-only Data Supplement) was similar between Young and Old. Fluorescence immunolabeling confirmed the presence of respective \( K_{Ca} \) (Figure 1B–1E) and connexin proteins (Figure 1B–1G in the online-only Data Supplement).

Endothelial Hyperpolarization to ACh is Sustained with Aging, Whereas Intercellular Transmission of Electrical Signals is Reduced

It is unknown whether the ability of ACh to initiate hyperpolarization in resistance artery endothelium is altered with advancing age. Thus, we determined whether hyperpolarization to ACh was affected by age. Figure 2A illustrates progressive hyperpolarization in response to cumulative [ACh] from 10⁻⁴ to 10⁻⁵ mol/L, with washout and recovery between exposures. Neither the pEC₅₀ for hyperpolarization (Young: 7.29±0.10; Intermediate: 7.48±0.11; Old: 7.16±0.11) nor the maximum response to ACh (\( \Delta V_m \); Young: −38±3 mV; Intermediate: −41±3 mV; Old: −39±1 mV; \( n=6 \) per group) differed between groups (Figure 2B and 2C; Figure IIA and IIB in the online-only Data Supplement).

To test the efficacy of cell-to-cell electrical coupling along the endothelium, current (±1–3 nA) was injected into 1 EC (site 1) while \( V_m \) was recorded from site 2 at constant separation distance (500 µm). Under control conditions, conduction amplitude (CA) for Old (5.9±0.5 mV/nA) was 66% of Young (8.9±0.7 mV/nA) and 36% of Intermediate (10.4±0.7 mV/nA; Figure 2D; Figure IIC in the online-only Data Supplement). ACh reduced CA in all groups in a concentration-dependent manner (Figure 2D; Figure IIC in the online-only Data Supplement). Because of their lower initial values for CA (Figure 2D), endothelial tubes from Old tended to maintain a higher fraction of control CA compared with Young during exposure to submaximal concentrations (<1 µmol/L) of ACh (Figure 2E). This difference between age groups was significant (\( P<0.05 \)) for Old versus Intermediate (Figure IID in the online-only Data Supplement).

Aging Decreases the Length Constant for Electrical Conduction

In light of electrical conduction along the endothelium being integral to conducted vasodilation²,24 and the finding that CA at a constant separation distance was impaired in Old during control conditions, we investigated whether aging would alter the effective distance of electrical conduction. As shown in Figure 3B and 3D, \( \Delta V_m \) at site 2 (\( \Delta V_{m2} \)) was related linearly (\( R^2=0.99 \)) to the amplitude and polarity of current injected at site 1 for Young and Old. The slope of the current–voltage relationship decreased with age and distance (Figure 3B versus 3D; Figure IIIA versus IIIB in the online-only Data Supplement). Nevertheless, CA at each distance was reduced (\( P<0.05 \)) for Old compared with Young.
Aging Decreases the Effect of SKCa/IKCa Activation on Electrical Conduction

Given that aging reduced CA and increased spatial decay, we tested whether the effect of direct SKCa/IKCa activation on electrical conduction would vary with age. NS309 (1 µmol/L) reduced CA at all distances (P<0.05) for endothelial tubes from Young and Old (Figure 3F) compared with Intermediate (Figure 3E; Table). The calculated length constant for electrical conduction (λ) was greater (P<0.05) in Young (1630±80 µm; n=12) and Intermediate (1900±90; n=8) compared with Old (1320±80 µm; n=9). When CA at each distance was normalized to respective local values, conduction efficiency was reduced (P<0.05) at 1500 to 2000 µm for Old versus Young (Figure 3F) or Intermediate (Figure IIID in the online-only Data Supplement). Increasing the amount of current injected for Old (by 40%) to achieve the same absolute CA at the local site as Young confirmed significantly greater spatial decay in Old (Table).

SKCa/IKCa Blockade Restores Electrical Conduction in Old

In light of evidence indicating greater SKCa/IKCa activity in Old, we hypothesized that blocking SKCa/IKCa activity in Old would enhance electrical conduction to a greater extent in endothelial tubes of Old versus Young. This was tested by injecting current

(Figure 3E; Table) or Intermediate (Figure IIIC in the online-only Data Supplement). The calculated length constant for electrical conduction (λ) was greater (P<0.05) in Young (1630±80 µm; n=12) and Intermediate (1900±90; n=8) compared with Old (1320±80 µm; n=9). When CA at each distance was normalized to respective local values, conduction efficiency was reduced (P<0.05) at 1500 to 2000 µm for Old versus Young (Figure 3F) or Intermediate (Figure IIID in the online-only Data Supplement). Increasing the amount of current injected for Old (by 40%) to achieve the same absolute CA at the local site as Young confirmed significantly greater spatial decay in Old (Table).
Figure 3. Electrical conduction along the endothelium of resistance arteries is impaired in Old vs Young mice. A. Membrane potential was recorded at site 2 ($V_{m2}$) located 500 μm from current injected at site 1 (±0.1–3 nA). Changes in $V_{m2}$ were related linearly to the amplitude and polarity of current injected at site 1. Note more negative $V_{m}$ and diminished (≈30%) $\Delta V_{m}$ responses in Old vs Young at each level of current injection. B. Summary data for experiments illustrated in A at 500 μm distance. Note lower ($P<0.05$) slope of Old vs Young (Young: 9.8±0.7 mV/nA [n=12]; Old: 6.6±0.6 mV/nA [n=9]). C. As in A with site 2 located 1500 μm from site 1. Note lower responses compared with A. D. As in B for site 2 at distance=1500 μm (Young: 5.0±0.3 mV/nA [n=12]; Old: 2.8±0.3 mV/nA [n=9]); the slope of respective current–voltage (I-V) relationships decreased as distance increased. E. Conduction amplitude (CA) vs distance. At each distance, values for Old were depressed relative to values for Young; data are in response to −1 nA current injection. F. Conduction efficiency=data from E normalized to respective CA at local (50 μm) site; note relatively greater decay with distance in Old. Calculated length constant for electrical conduction ($\lambda$) was greater ($P<0.05$) in Young (1630±80 μm; n=12) vs Old (1320±80 μm; n=9). *$P<0.05$, Young vs Old. Resting $V_{m}$ was more negative ($P<0.05$) in Old (−36±2 mV) vs Young (−28±2 mV). Summary data are means±SE.
and recording \(V_m\) continuously (at distance=500 µm) before and during exposure to apamin (Ap; 300 nmol/L) and charybdotoxin (ChTx; 100 nmol/L; Figure 5A and 5B). Blockade of \(SK_{Ca}/IK_{Ca}\) depolarized Old by 16±2 mV (to −24±2 mV; \(n=11\)), which was a greater \((P<0.05)\) effect than in Young (10±1 mV depolarization to −22±1 mV; \(n=11\)). During Ap+ChTx, CA increased more \((P<0.05)\) in Old (57±6%) versus Young (24±4%), thus CA in Old was restored to that of Young (Figure 5C). Expressing CA values as a fraction of control (Figure 5D) illustrated the relatively greater effect of \(SK_{Ca}/IK_{Ca}\) blockade on electrical conduction in Old versus Young. Experiments in which Ap or ChTx was applied individually indicated a prominent role for IK_{Ca} in dissipating injected current (Figure 5C and 5D).

Scavenging H\(_2\)O\(_2\) With Catalase Improves Electrical Conduction in Old

Aging and endothelial dysfunction have been attributed to heightened oxidative stress,\(^{11,25,26}\) including excess production of hydrogen peroxide (H\(_2\)O\(_2\)).\(^{11,27,28}\) As H\(_2\)O\(_2\) may alter the activity of \(K_{Ca}\),\(^{29,30}\) we tested whether scavenging H\(_2\)O\(_2\) with membrane-permeant catalase would improve electrical conduction. Catalase (500 U/mL) depolarized Young by 3±1 mV (to −24±2 mV; \(n=6\)) and Old by 9±1 mV (to −22±1 mV; \(n=7\)), which was a greater \((P<0.05)\) effect than in Young (10±1 mV depolarization to −22±1 mV; \(n=11\)). During Ap+ChTx, CA increased more \((P<0.05)\) in Old (57±6%) versus Young (24±4%), thus CA in Old was restored to that of Young (Figure 5C). Expressing CA values as a fraction of control (Figure 5D) illustrated the relatively greater effect of \(SK_{Ca}/IK_{Ca}\) blockade on electrical conduction in Old versus Young. Experiments in which Ap or ChTx was applied individually indicated a prominent role for IK_{Ca} in dissipating injected current (Figure 5C and 5D).

Inhibiting Endothelial NO Synthase Does Not Improve Electrical Conduction in Old

As the bioavailability of endothelium-derived NO decreases with advancing age,\(^{5,11}\) we tested whether uncoupled endothelial NO synthase was a source of H\(_2\)O\(_2\).\(^{31}\) Endothelial tubes from Old were treated with the inhibitor \(N^G\)-nitro-l-arginine methyl ester (100 µmol/L, 20 minutes). We found no significant effect on either resting \(V_m\) (control: −41±2 mV; \(N^G\)-nitro-l-arginine methyl ester: −40±3 mV; \(n=3\)) or CA (at 500 µm, control: 7.0±1.0 mV/nA; \(N^G\)-nitro-l-arginine methyl ester: 7.6±1.2 mV/nA; \(n=3\)).

**SK_{Ca}/IK_{Ca} Activation by H\(_2\)O\(_2\) Impairs Electrical Conduction**

In light of catalase restoring \(V_m\) and conduction of Old to approximate values of Young, we tested whether exogenous H\(_2\)O\(_2\) would hyperpolarize endothelial tubes and impair electrical conduction of Young. Addition of H\(_2\)O\(_2\) (200 µmol/L) hyperpolarized \(V_m\) progressively over time, approximating the equilibrium potential for K\(^+\) (\(E_K\), ~90 mV) after ~20 minutes (Figure 7A and 7B). CA decreased as hyperpolarization...
increased (Figure 7A and 7C). In separate experiments, inclusion of Ap+ChTx prevented changes in $V_m$ and CA during $H_2O_2$ exposure (Figure 7D and 7E; Figure VII and VIII in the online-only Data Supplement). On washout of Ap+ChTx, $H_2O_2$ evoked hyperpolarization and inhibited CA (Figure 7D and 7E; Figure VII and VIII in the online-only Data Supplement).

**Discussion**

Blood flow to skeletal muscle is attenuated with aging, but the underlying mechanisms have remained poorly defined. Evidence has pointed to a role for enhanced sympathetic neuroeffector signaling$^{14,15}$; however, little is known of changes that may occur within the vascular wall. Intrinsic to blood flow control in resistance networks is the conduction of electrical signals along the endothelium to coordinate SMC relaxation.$^{17,32,33}$ The present study has determined that the ability of the endothelium of skeletal muscle resistance arteries to conduct electrical signals is impaired with aging. The use of intact endothelial tubes freshly isolated from mouse superior epigastric arteries$^{22,34,35}$ enabled these changes to be resolved, independent of blood flow or surrounding cells. Remarkably, conduction along the endothelium of Old was restored to that of Young during selective blockade of SKCa/IKCa, particularly IKCa. Complementary experiments demonstrate that either direct (with NS309) or indirect (with ACh) activation of SKCa/IKCa in endothelial tubes of Young produced effects that mimicked the behavior of Old. In light of the association between aging and oxidative stress$^{11,25–27}$ and reports that $H_2O_2$ may alter the activity of $K_{Ca}$,$^{29,30}$ treating endothelial tubes of Old with catalase restored electrical conduction in a manner consistent with the effects of blocking $IK_{Ca}$ with ChTx. In a reciprocal manner, treating endothelial tubes of Young with $H_2O_2$ impaired electrical conduction, and this effect was also inhibited with SKCa/IKCa blockade. These data collectively support the hypothesis that, via the actions of $H_2O_2$, more $K_{Ca}$ channels are open under resting conditions in Old versus Young. In turn, the diminished resistance of cell membranes enables electrical signals to leak as they travel along the endothelium, reducing the spatial domain of electrical signaling.$^{22}$

**Impact of Age on Electrical Conduction: Effects of SKCa/IKCa Activation**

Endothelial dysfunction is characterized by impaired endothelium-dependent vasodilation.$^1$ Although such changes have been attributed to impaired NO bioavailability and signaling,$^{2,3}$ endothelium-dependent hyperpolarization initiated through SKCa/IKCa activation predominates as a signal (via myoendothelial coupling) for SMC relaxation in resistance vessels.$^{10,17,32,33}$ Alterations in SKCa/IKCa function have been associated with vascular disease$^{12,13}$; nevertheless, it has not been determined how these ion channels may be affected by aging. Nor has it been determined what consequences such changes may have on endothelial function, particularly in regard to the initiation and conduction of electrical signals. We show here that the ability of either direct (NS309; Figure 1VB in the online-only Data Supplement) or indirect (ACh; Figure 2C) SKCa/IKCa activation to produce hyperpolarization was preserved in endothelial tubes of Old. Despite a more negative resting $V_m$ in Old versus Young, the consistency of hyperpolarization to ACh indicates that the G-protein-coupled signaling events underlying SKCa/IKCa activation$^{9,10,12}$ were maintained in endothelial tubes of Old. This finding is
in contrast to reports that ACh-induced hyperpolarization of mesenteric arteries preconstricted with norepinephrine was greater in Young (1–8 months) versus Old (20–26 months) rats.

Others have reported diminished sensitivity for relaxation to SKCaIKCa activation (NS309) in saphenous arteries of 64-week versus 12-week male mice. However, a differential effect of NS309 was not apparent for hyperpolarization of endothelial tubes of Old versus Young (Figure IV in the online-only Data Supplement). Such differences between preparations illustrate the use of the endothelial tube as a model to evaluate properties intrinsic to the endothelium to avoid the influence of smooth muscle activation, which can alter endothelial function via signaling through myoendothelial gap junctions.

In accordance with the biophysical determinants of the electrical length constant \([\lambda = (r_m/\mu)^{1/2}]\), the ability of electrical signals to spread along the endothelium reflects the following: (1) the axial resistance to current flow between cells (ie, \(r_m\)), which is determined primarily by the patency of gap junctions; and (2) the leakiness of plasma membranes (ie, \(r_c\); the membrane resistance to current flow), which can be determined by the activation of ion channels (eg, \(SKCa/IKCa\)).

Respective signaling proteins are well expressed in endothelial tubes of both Old and Young (Figure I; Figure I in the online-only Data Supplement). Throughout our experiments, resting \(V_m\) was consistently 5 to 10 mV more negative in endothelial tubes of Old versus Young. These findings are consistent with the >6 mV more negative resting \(V_m\) of hippocampal pyramidal neurons from Old (>36 months) versus Young (2–3 months) rabbits, also attributed to enhanced Ca2+-activated K+ current in neurons of Old.

The linearity and stability of electrical responses (Figure 3B and 3D; Figure IIIA and IIIB in the online-only Data Supplement) enabled electrical recordings throughout a full range of current injections (±0.1–3 nA) at multiple distances (50–2000 μm). Under resting conditions, the \(\lambda\) we determined for endothelial tubes of Old was depressed by ≈20% compared with Young (Figure 3E and 3F; Figure III in the online-only Data Supplement). Our functional experiments illustrate that activating SKCaIKCa reduced \(\lambda\) (and CA) to a greater extent in Young (≈40%) compared with Old (≈25%; Figure 4). Furthermore, depolarization and augmentation of electrical conduction during selective blockade of SKCaIKCa with Ap+ChTx were significantly greater in Old versus Young and attributable primarily to actions on IKCa (Figure 5). We, therefore, suggest that, compared with Young (or Intermediate, which exhibited properties close to those of Young; Figure II and III in the online-only Data Supplement), the endothelium of Old has more IKCa open at rest, thereby allowing greater current leak and signal dissipation. In addition, the restoration of electrical conduction along endothelial tubes of Old to levels not different from Young on selective channel blockade (Figure 5C) indicates that gap junction patency was sufficient across age groups to maintain intercellular electrical coupling along the endothelium.

The endothelium can conduct depolarization as effectively as hyperpolarization (Figure 3A–3D; Figure IIIA and IIIB in the online-only Data Supplement). Such linearity of the current–voltage relationship indicates a negligible functional expression of voltage-sensitive ion channels (eg, large-conductance Ca2+-activated K+ channels) within EC plasma membranes. However, this behavior is not the case in intact feed arteries of hamster skeletal muscle, where depolarization conducted with less efficacy compared with hyperpolarization. This deviation from linearity can also be explained by myoendothelial coupling to SMCs, as the latter express voltage-gated K+ channels that increase conductance (ie, current leakage) on depolarization. Furthermore, given the ability of \(\alpha_1\)-adrenoreceptor activation on SMCs to activate SKCaIKCa of ECs via myoendothelial coupling, the impairment of conducted vasodilation along intact vessels can be depressed even further than the effects of aging on SKCaIKCa by an associated increase in sympathetic drive.
In turn, the present findings suggest how impaired ability of the endothelium to conduct electrical signals may underlie earlier in vivo observations in mouse skeletal muscle of decreased conducted vasodilation along arterioles and impaired ascending dilation of feed arteries with aging. Collectively, such effects may contribute to the restriction of blood flow and compromised oxygen delivery during physical activity in older (eg, >60 years) humans.

**Impact of Oxidative Stress on an Isolated Endothelial Syncytium**

Aging and endothelial dysfunction are associated with increased oxidative stress. Lower production of reactive oxygen species (eg, superoxide) was reported in aortae from long-living mice (Peromyscus leucopus) versus conventional house mice (Mus musculus). The reactive oxygen species signaling pathway begins with superoxide produced by mitochondria, nicotinamide adenine dinucleotide phosphate/xanthine oxidases, and uncoupled endothelial NO synthase. Excessive and highly reactive superoxide levels are converted to the stable intermediate H2O2 either spontaneously or through actions of superoxide dismutases. Catalase and glutathione peroxidase convert H2O2 into water, and these enzymes are expressed at higher levels in P. leucopus versus M. musculus, underscoring the ability to metabolize H2O2 as a determinant of maximum lifespan potential.

**Table. Spatial Decay of Electrical Conduction Is Greater in Old vs Young**

<table>
<thead>
<tr>
<th>Distance, μm</th>
<th>Young (−1 nA)</th>
<th>Old (−1 nA)</th>
<th>Old (−1.4 nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>−12.5±0.7</td>
<td>−9.0±0.7*</td>
<td>−12.5±0.9</td>
</tr>
<tr>
<td>500</td>
<td>−9.8±0.6</td>
<td>−6.8±0.6*</td>
<td>−9.1±0.8</td>
</tr>
<tr>
<td>1000</td>
<td>−7.4±0.4</td>
<td>−4.8±0.4*</td>
<td>−6.7±0.6</td>
</tr>
<tr>
<td>1500</td>
<td>−5.0±0.3</td>
<td>−2.7±0.3*</td>
<td>−3.8±0.5*</td>
</tr>
<tr>
<td>2000</td>
<td>−3.5±0.3</td>
<td>−1.5±0.4*</td>
<td>−2.0±0.5*</td>
</tr>
</tbody>
</table>

The standard current pulse microinjected at site 1 to evaluate a change in membrane potential at site 2 (ΔVm2) at distances of 50–2000 μm was −1 nA. The ΔVm2 response to −1 nA was reduced at all distances in Old (column 3) vs Young (column 2). To achieve the same ΔVm2 at the nearest distance (50 μm) required ~40% more current (−1.4 nA; column 4) in Old. Despite the same ΔVm2 at 50 μm, note progressively greater signal loss with distance in Old vs Young (compare column 2 with column 4). These data are complementary to Figure 3E and 3F.

*P<0.05 vs Young ΔVm2 responses to −1 nA at same distances (n=12 for Young and n=9 for Old).

**Figure 7. Endothelial hyperpolarization and loss of electrical conduction via small- and intermediate-conductance Ca2+-activated K+ channel (SKCa/IKCa) activation with H2O2. A, Representative recording of membrane potential responses at 500 μm (Vm2) from current injected at site 1 before and during H2O2 (200 μmol/L) exposure. Note progressive hyperpolarization and loss of Vm2 responses (with residual capacitance spikes). B, Summary data before (control) and during effect of H2O2 on resting Vm over 20 minutes. C, Summary data before (control) and during effect of H2O2 on conduction amplitude (CA; distance=500 μm) at times corresponding to those in B. D, Summary data for Vm before (control) and during apamin (Ap; 300 nmol/L) and charybdotoxin (ChTx; 100 nmol/L) during H2O2 with Ap+ChTx for 20 minutes (note lack of hyperpolarization), and after washout of Ap+ChTx with H2O2 still present (note hyperpolarization to ≈−80 mV). E, CA (distance=500 μm) at times corresponding to those in D. During H2O2 exposure, note maintenance of CA with Ap+ChTx present and loss of CA after their washout. *P<0.05 vs control; +P<0.05 vs preceding time point. Summary data are means±SE (n=6–8 per group). Data in B and C were obtained together in one set of experiments; data in D and E were obtained together in a separate set of experiments. All data in this Figure are based on continuous recordings from endothelial tubes of Young mice. See complementary data in Figure VIII in the online-only Data Supplement.
function. The physiological actions of H$_2$O$_2$ may thereby be manifested through its actions on the ion channels that are expressed in a given cell type (Figure 1). This reasoning is consistent with the actions of H$_2$O$_2$ on endothelial tubes from Young (Figure 7), where the consequences of activating $\text{SK}_{Ca}/\text{IK}_{Ca}$ (ie, hyperpolarization and impaired electrical conduction) were similar to the actions of either indirect (with ACh; Figure 2) or direct (with NS309; Figure 4) channel activation. In turn, these findings are consistent with the ability of catalase to restore resting $V_m$ and electrical conduction of Old to values not different from Young (Figure 6). The concentration of H$_2$O$_2$ used in the present study (200 µmol/L) is consistent with that used by others ($\pm$100–300 µmol/L) to evoke dilation of coronary arteries. Furthermore, the effects we observed for H$_2$O$_2$ (as well as aging) were sensitive to $\text{SK}_{Ca}/\text{IK}_{Ca}$ blockade with Ap+ChTx (Figure 7D and 7E; Figure VII and VIII in the online-only Data Supplement). Altogether, our study indicates that H$_2$O$_2$ activates $\text{SK}_{Ca}/\text{IK}_{Ca}$ of resistance artery endothelium. However, it should also be recognized that the effects of oxidative stress on the endothelium shown here may be masked in intact vessels by the presence of SMCs and their activation of large-conductance Ca$^{2+}$-activated K$^+$ channels in response to H$_2$O$_2$. As the inhibition of endothelial NO synthase had no effect (see Results section), key questions raised by the present findings point to resolving mitochondrial versus nonmitochondrial sources of H$_2$O$_2$ and direct versus indirect (ie, via increases in [Ca$^{2+}$]) or altered phosphorylation by protein kinase G-1-α$^6\gamma$ activation of $\text{K}_{Ca}$ by oxidative stress.

**Summary**

Our goal of this study was to determine the ability of the endothelium of resistance arteries from mouse skeletal muscle to initiate and conduct electrical signals with advancing age, independent from the prevailing influence of blood flow, SMCs, or other vasoactive stimuli. We focused on the roles of $\text{SK}_{Ca}/\text{IK}_{Ca}$ to initiate hyperpolarization and to modulate the transmission of electrical signaling. Our findings demonstrate that the function of $\text{SK}_{Ca}/\text{IK}_{Ca}$ to generate hyperpolarization was sustained with advancing age. However, more $\text{SK}_{Ca}/\text{IK}_{Ca}$ (particularly $\text{IK}_{Ca}$) were open at rest in the endothelium of Old animals, which resulted in a more negative resting $V_m$ and diminished electrical conduction, attributable to greater signal dissipation via charge loss through the plasma membrane. Scavenging H$_2$O$_2$ or blocking $\text{SK}_{Ca}/\text{IK}_{Ca}$ channels (particularly $\text{IK}_{Ca}$) depolarized the endothelium of Old and restored electrical conduction to values not different from the endothelium of Young. Conversely, exposing endothelial tubes of Young to H$_2$O$_2$ produced hyperpolarization and reduced electrical conduction, and these effects were also prevented by blocking $\text{IK}_{Ca}$ alone or together with $\text{SK}_{Ca}$. The present findings are the first to highlight the effect of aging on the role of $\text{K}_{Ca}$ channels in governing the initiation$^{21,20}$ and transmission of electrical signals$^{22}$ within vascular endothelium. Endothelial $\text{SK}_{Ca}/\text{IK}_{Ca}$ function thereby serves both to generate hyperpolarization underlying smooth muscle relaxation and to modulate the spread of vasodilation along resistance networks. With aging, attenuating the spatial domain of electrical signaling will restrict spreading vasodilation and thereby contribute to blood flow limitations.

**Sources of Funding**

This work was supported by the National Heart, Lung, and Blood Institute of the National Institutes of Health under award numbers R01-HL086483 (S.S. Segal), R37-HL041026 (S.S. Segal), F32-HL110701 (E.J. Behringer), and F32-HL107050 (M.J. Socha). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Disclosures**

None.

**References**

Aging is associated with endothelial dysfunction, a disorder contributing to restricted muscle blood flow and compromised oxygen delivery during physical activity. The endothelium is instrumental in coordinating dilation within resistance networks by conducting electrical signals (e.g., hyperpolarization via activation of small- and intermediate-conductance Ca2+-activated K+ channels; SKCa/IKCa) that dilate resistance arteries to increase peak tissue blood flow. How advancing age impacts electrical signals underlying vasodilation is unknown. Using endothelial tubes freshly isolated from mouse superficial epigastric arteries, we show that the initiation of hyperpolarization through SKCa/IKCa activation is sustained in old age, whereas the spread of electrical signals is impaired. This functional decrement in electrical conduction along the endothelium is explained by loss of current through SKCa/IKCa (particularly IKCa) in response to oxidative stress. Attenuating the spatial domain of electrical signaling will impair spreading dilation of resistance arteries and can thereby restrict tissue blood flow.

**Significance**

Aging with endothelial dysfunction, a disorder contributing to restricted muscle blood flow and compromised oxygen delivery during physical activity. The endothelium is instrumental in coordinating dilation within resistance networks by conducting electrical signals (e.g., hyperpolarization via activation of small- and intermediate-conductance Ca2+-activated K+ channels; SKCa/IKCa) that dilate resistance arteries to increase peak tissue blood flow. How advancing age impacts electrical signals underlying vasodilation is unknown. Using endothelial tubes freshly isolated from mouse superficial epigastric arteries, we show that the initiation of hyperpolarization through SKCa/IKCa activation is sustained in old age, whereas the spread of electrical signals is impaired. This functional decrement in electrical conduction along the endothelium is explained by loss of current through SKCa/IKCa (particularly IKCa) in response to oxidative stress. Attenuating the spatial domain of electrical signaling will impair spreading dilation of resistance arteries and can thereby restrict tissue blood flow.
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METHODS AND MATERIALS

Aging impairs electrical conduction along endothelium of resistance arteries through enhanced KCa channel activation

Erik J. Behringer\textsuperscript{1}, Rebecca L. Shaw\textsuperscript{1}, Erika B. Westcott\textsuperscript{1}, Matthew J. Socha\textsuperscript{1} and Steven S. Segal\textsuperscript{1,2}

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\textsuperscript{2}Dalton Cardiovascular Research Center, Columbia, MO 65211 USA

This Supplement contains:
Detailed Methods with References
Supplemental Table I
Methods and Materials

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). Mice were housed on a 12:12 h light-dark cycle at ~23°C with fresh tap water and standard chow available ad libitum. Experiments were performed on male C57BL/6 mice obtained from National Institute on Aging (NIA) colonies at Charles River Laboratories (Wilmington, MA, USA). Age groups were designated as: Young (3-6 months, n=44), Intermediate (12-14 months, n=9), and Old (24-26 months, n=39). In complementary experiments based upon our initial findings, Young male C57BL/6 mice bred at the University of Missouri (3-6 months, n=16) were used to test the effects of exogenous H₂O₂ on electrical signaling in endothelial tubes. Each mouse was anaesthetized using pentobarbital sodium (60 mg/kg, intraperitoneal injection) and abdominal fur was removed by shaving. Following surgical procedures, the mouse was killed with an overdose of pentobarbital via cardiac injection.

Solutions.
Physiological salt solution (control PSS) was used to superfuse endothelial tubes [(in mmol/L): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 Glucose]. During tissue dissection to obtain superior epigastric arteries, CaCl₂ was absent and 0.01 mmol/L sodium nitroprusside (SNP) was added to PSS (“dissection PSS”) to relax smooth muscle cells. During dissociation of smooth muscle cells to obtain endothelial tubes, PSS contained 0.62 mg/ml papain, 1.0 mg/ml dithioerythritol and 1.5 mg/ml collagenase and 0.1% Bovine Serum Albumin (USB Corp.; Cleveland, OH; USA) and SNP was replaced with 0.1 mmol/L CaCl₂ (“dissociation” PSS). All reagents were obtained from Sigma-Aldrich (St. Louis, MO; USA) unless indicated otherwise.

Surgery and microdissection.
A ventral midline incision through the skin was made 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 ends of superior epigastric arteries bilaterally. To maintain blood in the lumen and thereby facilitate visualization during dissection, each artery was ligated along with its adjacent vein using 6-0 silk suture (Ethicon; Somerville, NJ; USA). Abdominal muscles were removed bilaterally and placed in chilled (4 °C) dissection PSS. A muscle was pinned onto transparent silicone rubber (Sylgard 184, Dow Corning; Midland, MI; USA) and the vessel segment (length: ~2 cm) was dissected free from surrounding tissue. The vessel was cannulated at one end with a micropipette (tip outer diameter, 50-80 μm) made from heat-polished borosilicate glass capillaries (G150T-4, Warner Instruments; Hamden, CT; USA). The other end of the micropipette was connected to a static column (height, ~10 cm) of dissection PSS and residual blood was flushed from the vessel lumen.

Endothelial cell tube isolation and superfusion.
As described¹³, vessels were cut into segments (length, 3 - 5 mm) and placed into dissection PSS. The dissection PSS was replaced with dissociation PSS and incubated for 30 min at 34°C. Vessel segments were transferred to a 100 x 15 mm Petri dish and gently triturated to remove smooth muscle cells. For this purpose, vessel segments were drawn into and ejected from borosilicate glass capillary tubes [1.0 mm outer diameter (OD)/ 0.58 mm ID; World Precision Instruments (WPI), Sarasota, FL; USA] that were pulled (P-97; Sutter Instruments; Novato, CA; USA) and heat-polished at one end (tip internal diameter: 80-120 μm). Following dissociation of
smooth muscle cells (confirmed by visual inspection at 200X magnification), an endothelial tube (width: ~60 µm, length: 1-3 mm, individual endothelial cell length: ~35 µm) was transferred to a tissue chamber (RC-27N, Warner) secured on an aluminum platform (width: 14.5 cm, length: 24 cm, thickness: 0.4 cm) containing a micromanipulator (DT3-100, Siskiyou Corp.; Grants Pass, OR; USA) at each end that held a blunt fire-polished micropipette (OD, 60-100 µm) to secure the tube against the bottom (coverslip) of the tissue chamber. The entire preparation was secured on an inverted microscope (Eclipse TS100, Nikon) mounted on a vibration-isolated table (Technical Manufacturing Corp., Peabody, MA; USA) and superfused at 4 ml/min with PSS. Temperature was maintained at 32 ºC using an in-line heater (SH-27B, Warner) and heating platform (PH6, Warner) coupled to a temperature controller (TC-344B, Warner). Pharmacological agents were added to the superfusion PSS, thereby exposing the entire endothelial tube. Preliminary experiments ejecting blue dye from micropipettes positioned within the chamber confirmed that superfusion flow was laminar in the axial direction of endothelial tubes and that preparations were stable for at least 4 hours under these conditions.

**Real-time Polymerase Chain Reaction (qRT-PCR) and PCR.**

Endothelial tubes (~4-6 tubes/mouse, ~5,000 cells) were collected with great care to avoid contamination from smooth muscle cells. Total RNA was extracted using the RNAqueous Micro Kit from Ambion Inc. (Austin, TX) following the manufacturer’s instructions. RNA integrity was verified electrophoretically by SYBR safe gel stain from Molecular Probes (Eugene, OR) and by OD260/OD280 nm absorption ratio >1.95 using the Nanodrop from ThermoFisher. Reverse transcription was performed using the High Capacity cDNA Reverse Transcriptase Kit from Applied Biosystems (Foster City, CA), along with RNase Free DNase Set from 5Prime (Gaithersburg, PA) following the manufacturers’ instructions. Taqman primer assays specific to mouse Potassium intermediate/small conductance calcium-activated channel member 3 (KCNN3), Potassium intermediate/small conductance calcium-activated channel member 4 (KCNN4), Connexin37 (Gja4), Connexin40 (Gja5), Connexin43 (Gja1) and Glucuronidaseβ (Gusb) were purchased from Applied Biosystems (see Supplemental Table I). cDNA (2.5ng) was used as a template to perform RT-PCR for 40 cycles on the Applied Biosystems 7900 Fast Real Time PCR System. Taqman Fast Advanced Master Mix (Applied Biosystems) was utilized under the following temperature conditions as set by the manufacturer: 50 ºC for 2 min, 95 ºC 20 s, 95 ºC 1 s, 60 ºC 20 s. A minus RT reaction was also performed to confirm lack of contamination from genomic DNA. Linearity and efficiency of each gene were verified by creating standard curves plotting the critical threshold versus log of the dilution of cDNA. Data are reported (Fold change target gene/fold change Gusb) using the Pfaffl method of analysis. Outliers within relative expression data were identified using Grubb’s test (http://www.graphpad.com/quick calcs/Grubbs1.cfm) and excluded from statistical analysis.
### Supplemental Table I. Gene assay parameters and expression of SKCa/IKCa and connexins.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Gene Assaya</th>
<th>Standard curve C&lt;sub&gt;T&lt;/sub&gt; range&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Slope&lt;sup&gt;c&lt;/sup&gt;</th>
<th>r&lt;sup&gt;2d&lt;/sup&gt;</th>
<th>PCR efficiency&lt;sup&gt;e&lt;/sup&gt;</th>
<th>C&lt;sub&gt;T&lt;/sub&gt; for EC Young</th>
<th>C&lt;sub&gt;T&lt;/sub&gt; for EC Old</th>
</tr>
</thead>
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<tr>
<td>Kcn3 (SK)</td>
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<td>0.999</td>
<td>1.97</td>
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<tr>
<td>Kcn4 (IK)</td>
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<td>1.97</td>
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<td>Gja4 (Cx37)</td>
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<tr>
<td>Gja5 (Cx40)</td>
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<tr>
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<td>0.972</td>
<td>1.79</td>
<td>25.8±1.1</td>
<td>26.1±0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Applied Biosystems Taqman Inventoried Gene Expression Assay Number; <sup>b</sup> range of PCR crossing point (C<sub>T</sub>) values encompassed by standard curves used to estimate PCR efficiency; <sup>c</sup> slope of the regression line for PCR C<sub>T</sub> versus log of dilution value for 3-5 serial dilutions of cDNA for stated gene product; <sup>d</sup> coefficient of determination for linear regression; <sup>e</sup> PCR efficiency calculated as 10<sup>(-1/slope)</sup>. n=5 per group; summary data are means ± S.E.

**Immunohistochemistry.**

Immunofluorescence was modified from a previous study<sup>4</sup>. A CultureWell Multi Well Chambered Coverslip (GRACE Bio-Labs; Bend, Oregon) was placed over a Fisherbrand Superfrost Plus Microscope slide (Fisher Scientific; Pittsburgh, PA). Wells were pre-treated with Laminin at a concentration of 1mg/mL (Invitrogen; Carlsbad, CA) and dried at 34°C. Endothelial tubes were placed one per well, pinned and stretched to approximate their length when studied using intracellular recording. Tubes were fixed using 2% paraformaldehyde for 10 minutes and washed (3 x 5 minutes each) in PBS. Tubes were permeabilized and blocked in Phosphate-buffered saline (PBS)+0.1% Triton-X + 10% Normal Goat Serum for 1 hour at room temperature then washed (3 x 5 minutes) in PBS. Tubes were incubated overnight at 4°C with one of the following primary antibodies: rabbit polyclonal primary antibody for Cx37 (Cx37A11-A; Alpha Diagnostic, Owings Mills, MD), Cx40 (AB1726; Chemicon; Temecula, CA), KCNN3 (Abcam, Cambridge, MA; ab83737) and KCNN4 (Abcam; ab65985). Tubes were then washed (3 x 5 minutes) in PBS and incubated with a goat anti-rabbit IgG antibody conjugated with Alexa fluor 488 (Molecular Probes, Eugene, OR) in blocking buffer for 1 hr in the dark. Omission of the primary antibody was used to confirm the specificity of the immunofluorescence staining as well as control staining in other cell types. Following incubation, tubes were washed (3 x 5 minutes) in PBS and TO-PRO-3 iodide nuclear stain (Molecular Probes) was applied at 1 µmol/L concentration for 5 minutes. The tubes were then washed (3 x 5 minutes) with PBS, the MultiWell Coverslip was removed and ~20 µl of Vectashield Hard Set mounting media (Vector Laboratories, Inc.; Burlingame, CA) was added and sealed under a cover slip. Images from isolated tubes were obtained using a 63x glycerol immersion objective (numerical aperture = 1.3) on a Leica SP5 confocal microscope with LAS Software.
**Intracellular recording.**

Membrane potential ($V_m$) in endothelial tubes was recorded with an Axoclamp amplifier (2B; Molecular Devices; Sunnyvale, CA; USA) using microelectrodes pulled (P-97; Sutter) from glass capillary tubes (GC100F-10, Warner) and backfilled with 2 mol/L KCl (tip resistance, ~150 MΩ). An Ag/AgCl pellet was placed in effluent PSS to serve as a reference electrode. The output of the amplifier was connected to an analog-to-digital converter (Digidata 1322A, Molecular Devices; Sunnyvale, CA; USA) with data recorded at 1000 Hz on a Dell personal computer using Axoscope 10.1 software (Molecular Devices). For dual simultaneous intracellular recordings\textsuperscript{1,3}, a second amplifier (IE-210, Warner) was integrated into the data acquisition system. Current ($\pm 0.1$-3 nA, 2 s) was delivered using the Axoclamp electrometer driven by a function generator (CFG253, Tektronix; Beaverton, OR; USA). For current injection, an endothelial cell was penetrated at a site located ~150 µm from the downstream (with respect to the direction of PSS superfusion) end of where the endothelial tube was secured and referred to as “site 1”. A second endothelial cell was penetrated at a defined separation distance (50-2000 µm) from site 1 and referred to as “site 2”. Individual cells were penetrated along the midline of the endothelial tube and distances were defined with reference to a calibrated eyepiece reticle while viewing at 200X magnification. Successful impalements were indicated by sharp negative deflection of $V_m$, stable $V_m$ for $> 1$ min, hyperpolarization ($\geq 20$ mV) to 1 µmol/L NS309 ($\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ activator; Tocris; Bristol, UK), recovery to resting $V_m$ after NS309 washout and return to ~0 mV upon withdrawal from the cell. Correspondence between current injection at site 1 and $V_m$ responses at site 2 indicated simultaneous intracellular current injection and $V_m$ recording\textsuperscript{1,3}.

For determinations of the electrical length constant ($\lambda$), microelectrode impalement was maintained at site 1 while the microelectrode for recording $V_m$ at site 2 ($V_m2$) was repositioned\textsuperscript{1,3}. These recordings were typically initiated with site 2 at the greatest distance (2000 µm) and subsequent impalements progressed towards site 1. The ‘local’ responses were recorded with site 2 positioned at a distance of 50 µm (based on the length of individual ECs of ~35 µm). This precaution was taken to minimize electrical artifacts associated with trying to measure $V_m$ from the same electrode used for current injection and ensured that our $V_m2$ measurements were not from the same cell in which current was injected while recording as close to the signal origin (i.e., within one cell) as possible. For “standardized” evaluation of experimental interventions, separation distance between microelectrodes was maintained at 500 µm\textsuperscript{1,3}, which corresponds to the distance of ~15 ECs placed end-to-end. In all intracellular recording experiments, entire endothelial tubes were exposed to agents added to the superfusion solution and $V_m$ responses were confirmed to be the same between microelectrodes to ensure that the whole endothelial tube was isopotential before and after current injections.

**Pharmacology.**

The physiological agonist acetylcholine (ACh) chloride was used to indirectly activate $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels through $G_q$ protein-coupled muscarinic receptors\textsuperscript{8}. With hyperpolarization to ACh dependent upon intracellular $\text{Ca}^{2+}$ release, each exposure to ACh was followed by five minutes of superfusion with control PSS to allow full recovery of internal $\text{Ca}^{2+}$ stores through smooth endoplasmic reticulum $\text{Ca}^{2+}$ ATPase (SERCA) pumps. This precaution was taken to ensure stability over time (confirmed in preliminary experiments) and thereby obviate a potential decline in SERCA function in old animals\textsuperscript{9,10} which could otherwise impact intracellular release of $\text{Ca}^{2+}$ and activation of $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ during ACh concentration-response protocols. NS309 (Tocris; Bristol, UK) was used to evoke hyperpolarization of endothelial tubes by direct activation of $\text{SK}_{\text{Ca}}/\text{IK}_{\text{Ca}}$ channels\textsuperscript{11-13}. The NS309 was dissolved in DMSO and diluted to final working concentrations in PSS (DMSO $< 1\%$) on the day of an experiment. Apamin (Ap; Alomone; Jerusalem, Israel or Anaspec; Fremont, CA) and charybdotoxin (ChTx; Alomone or Anaspec) were dissolved in PSS and diluted into superfusion PSS in alone or in combination to block
SKCa/IKCa channels. To test for a role of reactive oxygen species on SKCa/IKCa function in governing Vm and electrical conduction, hydrogen peroxide (H2O2; Sigma-H1009, prepared in PSS) or polyethylene glycol (PEG)-catalase (Sigma-C4963, prepared in PSS) was added to the superfusion solution.

**Data analysis.**
For intracellular recordings, one endothelial tube was studied per mouse. Analyses included: 1) Resting Vm (mV) under Control conditions; 2) Change in Vm (Δ mV) = peak response Vm – preceding baseline Vm; 3) Conduction Amplitude (CA, mV/nA) = Vm at Site 2 / current injected at Site 1. With linearity of I-V relationships (R² ≥0.99) (see Figures 3B, 3D and Supplemental Figures IIIA, IIIB), a standard current of -1 nA was used for evaluating CA; 4) Fraction of Control CA = CA during treatment / preceding Control CA; 5) Conduction Efficiency = CA at each separation distance / CA at 50 μm (taken as the 'local' response); 6) Length constant (λ) = distance over which the electrical signal decayed to 37% (1/e) of the 'local' value. Linear regression (ΔVm at Site 2 versus current injection at Site 1), curve fitting (estimates of λ) were performed using GraphPad Prism (GraphPad Software, Inc.; La Jolla, CA; USA). Statistical analyses (GraphPad Prism) included repeated measures Analysis of Variance (one-way: within group, two way: within and between groups) with Tukey post-hoc comparisons and Student’s t-tests (paired: within experiment/group, unpaired: between separate experiments/groups). Differences between treatments were accepted as statistically significant with P < 0.05. Summary data are presented as means ± S.E.
References


Aging impairs electrical conduction along endothelium of resistance arteries through enhanced KCa channel activation

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²Dalton Cardiovascular Research Center, Columbia, MO 65211 USA

This Supplement contains:
Supplemental Tables II-III
Supplemental Figures I-VIII
Supplemental Table II. Spatial decay of electrical conduction increases during SK$_{Ca}$/IK$_{Ca}$ activation with 1 μmol/L NS309 in Young. The standard current pulse microinjected at site 1 to evaluate a change in membrane potential at site 2 ($\Delta V_m$) at distances of 50-2000 μm was -1 nA (Control, Column 2). Treatment with NS309 reduced $\Delta V_m$ at each distance (Column 3). To achieve the same $\Delta V_m$ at 50 μm in the presence of NS309 required ~90% greater current to be injected (~1.9 nA; Column 4) vs. Control. Despite the same local change in $V_m$ at 50 μm, note progressively greater signal loss at 500-2000 μm in the presence of NS309 vs. Control. These data are complementary to Figure 4C, D. *$P < 0.05$ vs. Control $\Delta V_m$ responses to -1 nA at the same distances ($n = 10$).

<table>
<thead>
<tr>
<th>Distance (μm)</th>
<th>-1 nA Control $\Delta V_m$ (mV)</th>
<th>-1 nA NS309 $\Delta V_m$ (mV)</th>
<th>-1.9 nA NS309 $\Delta V_m$ (mV)</th>
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<tr>
<td>50</td>
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<td>-12.7 ± 0.8</td>
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<td>-3.9 ± 0.3*</td>
<td>-7.5 ± 0.5*</td>
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<td>1000</td>
<td>-7.4 ± 0.4</td>
<td>-2.4 ± 0.2*</td>
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<tr>
<td>1500</td>
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<td>-1.3 ± 0.1*</td>
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<tr>
<td>2000</td>
<td>-3.5 ± 0.3</td>
<td>-0.6 ± 0.1*</td>
<td>-1.1 ± 0.3*</td>
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</tbody>
</table>
Supplemental Table III. Spatial decay of electrical conduction increases during SKCa/IKca activation with 1 μmol/L NS309 in Old. The standard current pulse microinjected at site 1 to evaluate a change in membrane potential at site 2 (ΔVm2) at distances of 50-2000 μm was -1 nA (Control, Column 2). Treatment with NS309 reduced ΔVm2 at each distance (Column 3). To achieve the same local ΔVm2 at 50 μm in the presence of NS309 required ~90% more current to be injected (-1.9 nA; Column 4) vs. Control. Despite the same local ΔVm2 at 50 μm, note progressively greater signal loss with increasing distance with NS309. These data are complementary to Figure 4E, F. Note consistently lower ΔVm2 responses to the same levels of current injection when compared to responses from Young in Supplemental Table III. *P < 0.05 vs. Control ΔVm2 responses to -1 nA at the same distances (n = 7).

<table>
<thead>
<tr>
<th>Distance (μm)</th>
<th>-1 nA</th>
<th>-1 nA</th>
<th>-1.9 nA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>NS309</td>
<td>NS309</td>
</tr>
<tr>
<td></td>
<td>ΔVm2 (mV)</td>
<td>ΔVm2 (mV)</td>
<td>ΔVm2 (mV)</td>
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<td>-5.2 ± 0.5*</td>
<td>-9.7 ± 0.8</td>
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<td>500</td>
<td>-7.1 ± 0.6</td>
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<td>-5.8 ± 0.6</td>
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Supplemental Figure I. Expression of connexins in resistance artery endothelium of Young and Old mice. The mRNA (qRT-PCR) and protein (immunohistochemistry) expression of Cx37, Cx40, and Cx43 were verified in Young and Old mice. A, Summary of abundance of mRNA (means ± S.E.) for Cx37, Cx40, and Cx43 relative to the expression of glucuronidase β (Gusb) in Young (n=5) and Old (n=5). B, Single confocal slice image of an isolated endothelial tube from a Young mouse indicating Cx37 in green and nuclei in blue. C, As in B for Old. D, As in B for Cx40 in Young. E, As in D for Old. F, As in B for Cx43 in Young. G, As in F for Old. Each image representative of at least three independent experiments. *P < 0.05, Young vs. Old.
Supplemental Figure II. Effects of ACh on membrane potential and electrical conduction in endothelial tubes of resistance arteries: Intermediate group included. For A-D, “C” on X-axis refers to Control values at rest. A, Effect of [ACh] on resting membrane potential ($V_m$). B, Effect of [ACh] on the change in $V_m$ ($\Delta V_m$) from Control. C, Effect of [ACh] on Conduction Amplitude [CA = change in $V_m$ (mV) per nA current injection] recorded 500 µm from site of -1nA current injection. D, Effect of [ACh] on CA relative to Control values in C (Fraction of Control CA=CA during respective [ACh]/Control CA). n = 6 per group. *P < 0.05, Young and Intermediate vs. Old. +P < 0.05, Young vs. Old. #P < 0.05, Intermediate vs. Old. Data for Young and Old reproduced from Figure 2. Summary data are means ± S.E.
Supplemental Figure III. Electrical conduction along endothelium of resistance arteries is impaired in Old vs. Young or Intermediate-aged mice. A, Membrane potential was recorded at site 2 (V_m2; distance= 500 µm) in response to current injected at site 1 (±0.1 to 3 nA). Changes in V_m were related linearly to the amplitude and polarity of current. Note lower slope of Old vs. Young and Intermediate. [(Young: 9.8±0.7 mV/nA (n=12), Intermediate: 10.1±0.6 (n=8), Old: 6.6±0.6 mV/nA (n=9)]. B, As in A with site 2 located 1500 µm from site 1; the slope of respective I-V relationships was decreased at greater distance [(Young: 5.0±0.3 (n=12), Intermediate: 6.1±0.5 (n=8), Old: 2.8±0.3 (n=9)]. C, Conduction Amplitude (to -1 nA) versus distance. Values for Old were depressed relative to Intermediate or Young. D, Conduction Efficiency = data from C normalized to respective CA at local (50 µm) site; note greatest relative decay with distance in Old. Calculated length constant for electrical conduction (λ) was greater (P < 0.05) in Young (1630±80 µm, n=12) and Intermediate (1900±90, n=8) versus Old (1320 ± 80 µm, n=9). *P < 0.05, Young and Intermediate vs. Old. Data for Young and Old reproduced from Figure 3. Summary data are means ± S.E.
Supplemental Figure IV. Effects of NS309 on membrane potential ($V_m$) and electrical conduction of endothelial tubes from Young and Old mice. A, Resting $V_m$ was consistently more negative in Old vs. Young through the range of [NS309]. B, There were no differences in the Change in $V_m$ ($\Delta V_m$) from Control between Young and Old through the range of [NS309]. C, Conduction Amplitude (for -1 nA current injection at 500 µm distance). The significant difference in CA under Control conditions was abolished by [NS309]. D, Effect of [NS309] on CA relative to control values in C (Fraction of Control CA=CA during respective [NS309]/Control CA). NS309 had a relatively greater effect on CA in Young versus Old at 0.3-1 µmol/L. *P < 0.05, Young vs. Old. Summary data are means ± S.E.; n = 4 per group. In each panel, “C” on X-axis indicates Control.
Supplemental Figure V. Impairment of electrical conduction during SK\textsubscript{Ca}/IK\textsubscript{Ca} activation along endothelium from Intermediate-aged mice. NS309 (1 µmol/L) reduced Conduction Amplitude (A) and Conduction Efficiency (B) in endothelial tubes from mice of Intermediate (12-14 months) age. *P < 0.05 vs. NS309 (n=8). Summary data are means ± S.E. These data are complementary to Figure 4.
Supplemental Figure VI. Effects of catalase on membrane potential and hyperpolarization of endothelial tubes from Young and Old mice. Membrane potential ($V_m$) was recorded continuously during Control conditions at rest, in response to ACh (3 µmol/L) and in response to NS309 (1 µmol/L) then respective measures were repeated after 20 minutes of exposure to catalase (500 U/ml). **A, C, and E:** peak $V_m$ during respective conditions; **B, D and F:** Corresponding changes in $V_m$ ($\Delta V_m$) from respective values in **A.** Summary: **A, B,** At rest, $V_m$ of Old (-43±1 mV) was ~8 mV more negative than Young (-35±2 mV); catalase depolarized Old by 11±2 mV (to -32±1 mV) with no significant effect on Young. For hyperpolarization to ACh, catalase had no significant effect on peak response $V_m$ for either Young or Old (**C**); however, the actual change in $V_m$ was greater in Old ($\Delta V_m = -47±2$ mV) vs. Young ($\Delta V_m = -33±5$ mV) following catalase treatment (**D**). For hyperpolarization to NS309, catalase reduced peak response $V_m$ in both Young (from -68±3 to -57±3 mV) and Old (from -75±4 to -67±2 mV; **E**). For the change in $V_m$, catalase had differential actions on responses to NS309 in Young vs. Old: It reduced the magnitude of hyperpolarization by 8±2 mV in Young (from -35±2 to -27±2 mV) and increased the magnitude of hyperpolarization by 4±1 mV in Old (from -32±3 to -36±3 mV) (**F**). *P<0.05, Young vs. Old; *P<0.05 vs. Young before catalase; #P<0.05 vs. Old before catalase.

Summary data are means ± S.E.; n = 4 per group.
Supplemental Figure VII. Blockade of SK$_{Ca}$/IK$_{Ca}$ maintains membrane potential and electrical conduction in endothelial tubes during H$_2$O$_2$ exposure. Representative recording in an endothelial tube from a Young mouse illustrating $V_{m2}$ responses (distance = 500 µm) with ±1 to 3 nA current injections at site 1 before and during the following treatments: Ap (300 nmol/L) + ChTx (100 nmol/L), addition of H$_2$O$_2$ during Ap + ChTx (20 min), and H$_2$O$_2$ following washout of Ap + ChTx. Note minimal change in baseline $V_m$ and sustained responses to current injection during H$_2$O$_2$ exposure in presence of Ap + ChTx (see Figure 7). Following washout of Ap + ChTx, sustained exposure to H$_2$O$_2$ hyperpolarized $V_m$ to ~-80 mV accompanied by loss of $V_{m2}$ responses to ±1-3 nA injected at site 1.
Supplemental Figure VIII. Effect of H$_2$O$_2$ on membrane potential and electrical conduction of endothelial tubes. A, Change in membrane potential ($\Delta V_m$) from Control (-30±1 mV) during H$_2$O$_2$ (200 µM) exposure for 20 minutes. B, Summary data for Fraction of Control Conduction Amplitude during exposure to H$_2$O$_2$; note ~90% reduction after 20 minutes. C, Summary data for $\Delta V_m$ from Control during Ap + ChTx alone (note ~10 mV depolarization); H$_2$O$_2$ with Ap + ChTx for 20 minutes, and after washout of Ap + ChTx with H$_2$O$_2$ alone. Note lack of hyperpolarization to H$_2$O$_2$ in the presence of Ap + ChTx (see representative recording in Supplemental Figure VII) and ~50 mV hyperpolarization to H$_2$O$_2$ following washout of Ap + ChTx. D, Summary data for Fraction of control CA at times corresponding to those in C. During H$_2$O$_2$ exposure, note increased responses with Ap + ChTx present and loss of conduction to H$_2$O$_2$ following their washout. * P<0.05 vs. Control, † P<0.05 vs. preceding time point. Summary data are means ± S.E.; n=6-8 per group. Data in A and B were obtained together in one set of experiments; Data in C and D were obtained together in another set of experiments. All data in this Figure are based upon continuous recordings from endothelial tubes of Young mice.