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Advanced age protects microvascular endothelium from aberrant Ca^{2+} influx and cell death induced by hydrogen peroxide

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Key points

- Calcium signalling in endothelial cells of resistance arteries is integral to blood flow regulation. Oxidative stress and endothelial dysfunction can prevail during advanced age and we questioned how calcium signalling may be affected.
- Intact endothelium was freshly isolated from superior epigastric arteries of Young (~4 months) and Old (~24 months) male C57BL/6 mice. Under resting conditions, with no difference in intracellular calcium levels, hydrogen peroxide (H_2O_2) availability was ~1/3 greater in endothelium of Old mice while vascular catalase activity was reduced by nearly half.
- Compared to Old, imposing oxidative stress (200 μM H_2O_2) for 20 min increased intracellular calcium to 4-fold greater levels in endothelium of Young in conjunction with twice the calcium influx. Prolonged (60 min) exposure to H_2O_2 induced 7-fold greater cell death in endothelium of Young.
- Microvascular adaptation to advanced age may protect endothelial cells during elevated oxidative stress to preserve functional viability of the intima.

Abstract Endothelial cell Ca^{2+} signalling is integral to blood flow control in the resistance vasculature yet little is known of how its regulation may be affected by advancing age. We tested the hypothesis that advanced age protects microvascular endothelium by attenuating aberrant Ca^{2+} signalling during oxidative stress. Intact endothelial tubes (width, ~60 μm; length, ~1000 μm) were isolated from superior epigastric arteries of Young (3–4 months) and Old (24–26 months) male C57BL/6 mice and loaded with Fura-2 dye to monitor $[Ca^{2+}]_i$. At rest there was no difference in $[Ca^{2+}]_i$ between age groups. Compared to Young, the $[Ca^{2+}]_i$ response to maximal stimulation with acetylcholine (3 μM, 2 min) was ~25% greater in Old, confirming signalling integrity with advanced age. Basal H_2O_2 availability was ~33% greater in Old while vascular catalase activity was reduced by half. Transient exposure to elevated H_2O_2 (200 μM, 20 min) progressively increased $[Ca^{2+}]_i$ to ~4-fold greater levels in endothelium of Young *versus* Old. With no difference between age groups at rest, Mn^{2+} quench of Fura-2 fluorescence revealed 2-fold greater Ca^{2+} influx in Young during elevated H_2O_2; this effect was attenuated by ~75% using ruthenium red (5 μM) as a broad-spectrum inhibitor of transient receptor potential channels. Prolonged exposure to H_2O_2 (200 μM, 60 min) induced ~7-fold greater cell death in endothelium of Young *versus* Old. Thus, microvascular endothelium can adapt to advanced age by reducing Ca^{2+} influx during elevated oxidative stress. Protection from cell death during oxidative stress will sustain endothelial integrity during ageing.

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Abbreviations DCFH, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; EDD, endothelial-dependent dilatation; EC, endothelial cell; EtBr, ethidium bromide; GPCR, G-protein coupled receptor; IK_{Ca}, intermediate conductance calcium-activated potassium channel; K_{Ca}, calcium-activated potassium channel; NO, nitric oxide; NOS, nitric oxide synthase; PSS, physiological salt solution; ROS, reactive oxygen species; RuR, ruthenium red; SK_{Ca}, small conductance calcium-activated potassium channel; SMC, smooth muscle cell; SNP, sodium nitroprusside; SEA, superior epigastric artery; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl; TRP, transient receptor potential.

Introduction

In the resistance vasculature, the role of the endothelium in governing tissue perfusion is driven by dynamic regulation of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. For example, elevations in endothelial cell (EC) $[Ca^{2+}]_i$ activate small- and intermediate-conductance K_{Ca} channels (SK_{Ca} and IK_{Ca}, respectively) along with production of nitric oxide (NO) and prostacyclin (Crutchley *et al.* 1983; Kruse *et al.* 1994; Campbell *et al.* 1996; Tran & Watanabe, 2006; Ledoux *et al.* 2008). These complementary signalling events serve to relax the surrounding smooth muscle cells (SMCs), with vasodilatation increasing tissue blood flow. With advanced age, blood flow regulation by the endothelium is characterized by greater levels of reactive oxygen species (ROS) and diminished bioavailability of NO (Taddei *et al.* 2001; Seals *et al.* 2011; Muller-Delp *et al.* 2012). In turn, these changes may impair EC function and compromise tissue perfusion (Feletou & Vanhoutte, 2006; Seals *et al.* 2011). These adverse effects of advanced age often coincide with a sedentary lifestyle and impaired functional work capacity (Dinenno *et al.* 1999, 2001; Donato *et al.* 2006). Of even greater concern, compromising the physiological integrity of the endothelium presages the advance of cardiovascular disease (Yeboah *et al.* 2007; Lind *et al.* 2011).

Superoxide generation occurs through mitochondrial respiration, substrate conversion via cytosolic and membrane-associated oxidases, and the uncoupling of nitric oxide synthase (NOS) (Drouin *et al.* 2007; Widlansky & Gutterman, 2011; Muller-Delp *et al.* 2012; Bachschmid *et al.* 2013). While able to react with NO and generate peroxynitrite (Fleming & Busse, 1999; van der Loo *et al.* 2000; Donato *et al.* 2007; Trott *et al.* 2009; Sindler *et al.* 2013), superoxide anions can be converted rapidly to the stable (and membrane-permeant) ROS hydrogen peroxide (H_2O_2) either spontaneously or via mitochondrial, cytosolic and membrane-associated superoxide dismutases (Widlansky & Gutterman, 2011; Muller-Delp *et al.* 2012; Bachschmid *et al.* 2013). While H_2O_2 can contribute to endothelial-dependent dilatation (Miura *et al.* 2003; Widlansky & Gutterman, 2011; Muller-Delp *et al.* 2012), high levels of H_2O_2 can elevate EC $[Ca^{2+}]_i$, impair normal function and initiate cell death (Volk *et al.* 1997; Hecquet *et al.* 2008; Sun *et al.* 2012). However, it is unknown how advanced age affects Ca^{2+} responses to H_2O_2 in endothelium of the resistance vasculature, or whether there may be associated changes in EC viability.

In the present study, we tested the hypothesis that advanced age protects microvascular endothelium by attenuating aberrant Ca^{2+} signalling during exposure to elevated H_2O_2. Endothelial tubes freshly isolated from skeletal muscle resistance arteries of Young (3–4 months) and Old (24–26 months) mice were exposed to H_2O_2 at a concentration (200 μM) shown to hyperpolarize ECs to their K^+ equilibrium potential within ∼20 min through activating K_{Ca} (Behringer *et al.* 2013). The present findings reveal that, with no difference in $[Ca^{2+}]_i$ under basal conditions, resting levels of H_2O_2 were higher in Old. Exposure to elevated H_2O_2 (200 μM) increased $[Ca^{2+}]_i$ to ∼4-fold higher levels and evoked cell death to a ∼7-fold greater extent in endothelium of Young compared to Old, suggesting that microvascular ECs adapt during advanced age to attenuate the rise in $[Ca^{2+}]_i$ and minimize cell death when subjected to an abrupt increase in oxidative stress.

Methods

Animal care and use

All procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri–Columbia. Experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (8th edn, 2011). Young (∼4 months) and Old (∼24 months) male C57BL/6 mice were obtained concurrently from the National Institute on Aging colonies (Charles River Laboratories, Wilmington, MA, USA). Prior to experiments, mice were acclimatized in the same room of the animal care facility at the University of Missouri School of Medicine for at least 1 week on a 12/12 h light–dark cycle at ∼23°C with fresh tap water and standard rodent chow available *ad libitum*. Young and Old mice were studied in random order under the same conditions within each experimental protocol. On the morning of an experiment, a mouse was anaesthetized with an intraperitoneal injection of

pentobarbital sodium (60 mg kg^{-1}) and abdominal fur was removed by shaving. The origin of each superior epigastric artery (SEA) was exposed near the sternum and ligated with 6-0 silk suture. The entire abdominal musculature was then excised and placed in 4°C dissection buffer (pH 7.4, 285–290 mOsm) composed of (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes, 10 glucose, 0.01 sodium nitroprusside (SNP) and 0.1% bovine serum albumin (BSA) (US Biochemical, Cleveland, OH, USA). Following tissue removal, the anaesthetized mouse was killed via cardiac excision or an overdose of pentobarbital via cardiac injection.

Isolation of endothelial tubes

Intact endothelial tubes were isolated from the SEA as described (Socha *et al.* 2011, 2012; Socha & Segal, 2013). Briefly, the abdominal musculature was transferred to a dissection chamber at 4°C and pinned onto a transparent silicone rubber block (Sylgard 184; Dow-Corning, Midland, MI, USA). While viewing through a stereomicroscope, each SEA was dissected from the site of ligation to the first major branch point (isolated segment length, 3–3.5 mm) and the lumen was flushed with chilled dissection buffer to remove residual blood. Each SEA was cut into 2–3 smaller segments (length, ~1–2 mm) and transferred into 12 × 75 mm culture tubes on ice containing 4 ml of dissection buffer. The culture tube was warmed to room temperature (~24°C) and vessel segments were transferred to a 12 × 75 mm culture tube containing 1 ml of preheated (37°C) dissociation buffer composed of (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes, 10 glucose, 2 $CaCl_2$, 0.1% BSA, 0.62 mg ml^{-1} papain, 1.5 mg ml^{-1} collagenase and 1.0 mg ml^{-1} dithioerythritol, pH 7.4. Vessel segments were incubated for 30 min at 37°C then transferred to enzyme-free dissociation buffer at room temperature. A vessel segment was transferred to a recording chamber (RC-27 N; Warner Instruments, Hamden, CT, USA). During visual inspection at 40× magnification, surrounding SMCs and adventitia were dissociated from the endothelium using gentle trituration through the heat-polished tip (internal diameter, ~120 μm) of a micropipette pulled (P-97; Sutter Instruments, Novato, CA, USA) from borosilicate glass capillary tubes (#1B100-4; World Precision Instruments, Sarasota, FL, USA). The freshly isolated endothelial tube was positioned at the centre of the chamber and aligned along the chamber axis in the direction of fluid flow. Each end was pressed gently against the chamber bottom (a 24 × 50 mm coverslip) using a blunt-tipped pinning pipette (heat-polished borosilicate glass; external diameter, ~100 μm) secured in three-axis micromanipulators (DT3-100; Siskiyou Design, Grants Pass, OR, USA). The endothelial tube was extended to approximate *in situ* length by slowly retracting each pinning pipette and superfused (3 ml min^{-1}) with physiological salt solution [PSS, 290–295 mOsm; pH 7.4, composed of (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 10 Hepes, 10 glucose and 2 $CaCl_2$].

Calcium photometry

The endothelial tube was equilibrated for ~15 min during superfusion with PSS at room temperature (~24°C) then incubated for 30 min without flow in Fura-2 AM dye (# F14185; Invitrogen, Carlsbad, CA, USA). The dye was dissolved in DMSO and diluted to 5 μM in PSS with final DMSO < 0.5% (Socha *et al.* 2011). Superfusion with PSS was resumed to wash out excess dye and allow dye within cells to de-esterify while temperature was raised to 32°C during 30 min of equilibration. Calcium photometry was performed with an IonOptix (Milford, MA, USA) system (Socha *et al.* 2011). Using a 20× objective (Nikon Fluor20; NA 0.75; Nikon Corporation, Tokyo, Japan), Fura-2 was excited alternately (250 Hz) at 340 and 380 nm with a 75 W Xe lamp while fluorescence emission was collected at 510 nm and expressed as the ratio F_{340}/F_{380}. Auto-fluorescence of the endothelium was recorded prior to dye loading and subtracted from respective recordings. The imaging window for collecting fluorescence emission was 320 μm long and adjusted to accommodate the width of each tube (range, 50–75 μm) while observing it on a video monitor using transmitted light from a halogen lamp (600 nm long pass filter) directed to a charge coupled device camera (LCL-902C; Watec, Orangeburg, NY, USA). To determine minimum and maximum F_{340}/F_{380} values in Young and Old mice, tubes were superfused with PSS containing 0 mM Ca^{2+} and 5 mM EGTA for 1 h followed by PSS containing 3 μM ionomycin and 10 mM Ca^{2+}, respectively (Socha *et al.* 2011). Following Fura-2 dye loading and equilibration, an index of basal $[Ca^{2+}]_i$ was determined by averaging the F_{340}/F_{380} ratio over 30 s under resting conditions. To evaluate $[Ca^{2+}]_i$ responses to G-protein coupled receptor (GPCR) activation, 3 μM ACh, which evokes peak responses for $[Ca^{2+}]_i$ and hyperpolarization (Behringer *et al.* 2012, 2013), was added to the superfusion solution for 2 min while the F_{340}/F_{380} ratio was recorded. Data collected during the final 30 s of the $[Ca^{2+}]_i$ response to ACh were averaged for analysis.

Dichlorofluorescein fluorescence

Endothelial tubes were secured in the recording chamber (see Calcium photometry above). To determine whether basal H_2O_2 availability in endothelium increases with advanced age, cells were loaded with the H_2O_2-sensitive fluorescent probe dichlorofluorescein (DCFH; 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; #C-400; Molecular Probes, Eugene, OR, USA).

For dye loading, DCFH was dissolved in DMSO and then diluted to 15 μM in PSS (pH 7.4; DMSO < 0.5%). The tube was immersed at room temperature (24°C) for 30 min without flow, then superfusion with PSS (3 ml min^{-1}) was restored to remove excess dye, the signal was allowed to stabilize (1–2 min) and initial baseline fluorescence (F_0) was determined. These experiments were maintained at room temperature because intracellular DCFH levels remained stable at 24°C for at least 30 min. In contrast, DCFH extrusion was pronounced within several min at 32°C (time controls not shown). Utilizing a 40× objective (NA = 0.9, Nikon) and recording from a 140 × 50 μm window, DCFH was excited at 460–500 nm while emission was recorded continuously at 510–560 (505 nm dichroic mirror; Chroma Technology Corp., Bellows Falls, VT, USA) for at least 30 min. Linear regression was performed on the first 10 min, which corresponded to the steepest rise in fluorescence.

Control experiments were performed to confirm the sensitivity of intracellular DCFH to H_2O_2. To inhibit the accumulation of DCFH fluorescence resulting from H_2O_2 produced endogenously, endothelial tubes were pre-incubated in PSS (pH 7.4) containing the superoxide dismutase mimetic TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl, 1 mM) + PEG-catalase (500 U ml^{-1}) to decompose intracellular H_2O_2 (Behringer *et al.* 2013). To estimate the [H_2O_2] required to increase DCFH fluorescence above its basal rate of accumulation, H_2O_2 was added to the superfusion solution (pH 7.4) at defined concentrations. Data were acquired using Ion Wizard software (IonOptix) and were analysed after subtraction of background fluorescence.

Catalase activity

To determine whether scavenging of H_2O_2 within the vessel wall is reduced with advanced age, catalase activity was measured using a colorimetric assay. Preliminary experiments determined the amount of vessel required. Thus, for each mouse, first- and second-order superior and inferior epigastric arteries were dissected bilaterally, flushed of blood, then pooled and cut into segments ~100 μm long. Each pooled sample (one per mouse) was placed in 100 μl lysis buffer consisting of: 50 mM Hepes, 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 2% glycerol and protease inhibitor cocktail (modified from Nystoriak *et al.* 2011). Samples were vortexed for 1 min, sonicated for 20 min, vortexed again for 1 min, sonicated for another 10 min, then centrifuged at 7000 *g* for 10 min at 4°C. The lysate supernatant was retained and used for assay. Protein concentrations for each sample were measured using the Micro BCA Assay (#23235; ThermoFisher, Waltham, MA, USA). A standard curve (0–200 μg μl^{-1} BSA) was prepared and protein concentrations were measured on a NanoDrop 2000c (ThermoFisher). The assay for catalase activity (#CAT100; Sigma-Aldrich, St Louis, MO, USA) was run at room temperature according to the manufacturer's instructions. Standard curves for H_2O_2 concentration were prepared fresh daily and each assay included positive and negative controls in addition to samples from Young and Old mice. Each reaction included 75 μl of lysate supernatant (containing 10–15 μg protein) and lasted 5 min. Assays were read at 520 nm in a quartz cuvette on the NanoDrop 2000c. Specific catalase activity is reported as μmol H_2O_2 consumed min^{-1} ml^{-1} (μg protein)$^{-1}$.

Hydrogen peroxide exposure

To determine whether advanced age impairs Ca^{2+} signalling in microvascular endothelium during acute oxidative stress, endothelial tubes were loaded with Fura-2 dye as described above to evaluate Ca^{2+} responses to H_2O_2. After warming to 32°C, the protocol consisted of an initial baseline recording, addition of ACh (3 μM) to the superfusion solution for 2 min, then washout and recovery to resting baseline (10 min) during superfusion with control PSS. In accordance with the protocol developed for earlier studies (Behringer *et al.* 2013), H_2O_2 (200 μM) was included in the superfusion solution (pH 7.4) for 20 min followed by 30 min washout and recovery with control PSS. Fura-2 emission was recorded for the final 10 s of each 5 min period during H_2O_2 exposure and throughout recovery with F_{340}/F_{380} averaged for each recording. In separate experiments to determine whether Ca^{2+} signals during H_2O_2 exposure were derived from intra- or extracellular sources, the 20 min exposure to H_2O_2 was performed using PSS that was nominally Ca^{2+}-free (i.e. prepared without $CaCl_2$). Superfusion with control PSS (2 mM Ca^{2+}) was restored during the 30 min recovery period with Fura-2 emission sampled as above. To determine whether advanced age affected EC viability during oxidative stress, the duration of exposure to H_2O_2 (200 μM) was extended to 60 min.

Manganese quench

Quenching of Fura-2 fluorescence by Mn^{2+} correlates with the rate of divalent cation flux across the plasma membrane, rendering this method an accurate assessment of Ca^{2+} influx (Potocnik & Hill, 2001; Tas *et al.* 2008). An endothelial tube was loaded with Fura-2 dye as described above and studied at 32°C. To obtain an index of Ca^{2+} influx at rest, the tube was superfused with PSS in which the $CaCl_2$ was replaced with $MnCl_2$ for 5 min, during which the peak rate of decrease in the Fura-2 signal was recorded at its isosbestic wavelength (360 nm). To determine how oxidative stress influenced Ca^{2+} influx, the

tube was superfused with 200 μM H_2O_2 in PSS containing 2 mM Ca^{2+} for 10 min, then with 200 μM H_2O_2 in nominally Ca^{2+}-free PSS for 10 min. As determined in preliminary experiments, this Ca^{2+}-free interval prior to Mn^{2+} addition prevented Ca^{2+} influx, thereby avoiding cytosolic Ca^{2+} accumulation and its binding to Fura-2 (which would limit Mn^{2+} binding sites for the quench assay). Ca^{2+}-free PSS containing 2 mM $MnCl_2$ was then added for 5 min during which the Fura-2 signal was recorded. This protocol resulted in measurements of H_2O_2-induced cation permeability coinciding with 20 min exposure to H_2O_2. Linear regression was performed on a 30 s interval during the steepest portion of the Fura-2 response during $MnCl_2$ exposure (i.e. during maximal Mn^{2+} influx) to determine the maximal rate of fluorescence quenching, thereby providing an index of the peak rate of cation influx across the plasma membrane.

Transient receptor potential (TRP) channel inhibition

To determine whether TRP channels were involved in $[Ca^{2+}]_i$ responses, the broad-spectrum TRP channel inhibitor ruthenium red (RuR; Ramsey *et al.* 2006) was dissolved directly into PSS (5 μM; pH 7.4) and included during superfusion with H_2O_2. Having determined that $[Ca^{2+}]_i$ responses to exogenous H_2O_2 in the endothelium of Young were far greater than in Old mice (see Results), endothelial tubes from Young mice were loaded with Fura-2 as described above. After recording baseline F_{340}/F_{380} values and control responses to 3 μM ACh, the endothelium was superfused for 20 min with PSS containing 200 μM H_2O_2 + 5 μM RuR followed by control PSS for 30 min of recovery. Fura-2 fluorescence emission was recorded for 10 s at the end of each 5 min period and averaged.

Cell viability assay

To determine whether EC death was responsible for the Fura-2 signal during the 20 min exposure to H_2O_2, a Live/Dead Viability/Cytotoxicity assay for mammalian cells (#L-3224; Life Technologies,; Carlsbad, CA, USA) was performed. Briefly, following isolation, tubes were superfused with 200 μM H_2O_2 for 20 min at 32°C as done for evaluating $[Ca^{2+}]_i$ responses. Following a 2 min wash with control PSS while cooling back to 24°C, the Live/Dead assay was performed at room temperature according to the manufacturer's recommendations. Thus, individual tubes were incubated for 30 min in PSS containing 2 μM calcein-AM (hydrolysed and retained in viable cells) and 4 μM ethidium bromide (EtBr; stains nuclei of dead and dying cells). The tube was washed again for 2 min in control PSS. As there was negligible EtBr staining following 20 min of H_2O_2 for either age group (see Results), additional experiments were performed in which the duration of H_2O_2 exposure was extended to 60 min prior to performing the Live/Dead assay. Images of each tube were acquired using a 40× oil immersion objective (NA = 1.25) on a Leica SP5 laser scanning confocal microscope equipped with LAS Software (Leica Microsystems, Buffalo Grove, IL, USA). Both dye molecules were excited with the 488 nm line of an argon laser with emission acquired at 500–535 nm (calcein) and 595–700 nm (EtBr). Transmitted light images were acquired concurrently. To quantify cell damage in each tube, the number of ECs stained with EtBr was expressed as a percentage of the total number of fluorescently labelled ECs [i.e. (EtBr-positive cells)/(EtBr-positive cells + calcein-positive cells)].

Data analysis and statistics

One EC tube was studied from each SEA. Unpaired Student's *t*-tests were used to evaluate differences between age groups in F_{340}/F_{380} values at rest, in response to ACh and during Mn^{+2} quench, during DCFH fluorescence and for catalase activity. Changes in F_{340}/F_{380} over time during H_2O_2 exposure were analysed using two-way ANOVA with Bonferroni post-tests. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Summary data are presented as means ± SEM. Differences were accepted as statistically significant at $P < 0.05$.

Results

Calcium photometry

Time controls confirmed that baseline F_{340}/F_{380} did not change significantly for at least 2 h in either Young or Old mice ($n = 4$ each). Thus, consistent with earlier findings (Socha *et al.* 2011), resting $[Ca^{2+}]_i$ remained stable at 32°C. To substantiate comparisons of F_{340}/F_{380} between Young and Old, minimum and maximum F_{340}/F_{380} values for the Ca^{2+} signal were determined as described previously for endothelial tubes (Socha *et al.* 2011). Thus, minimum F_{340}/F_{380} values were 0.61 ± 0.02 for Young ($n = 3$) and 0.64 ± 0.02 for Old ($n = 4$) with respective maximum F_{340}/F_{380} values of 5.13 ± 0.76 and 5.92 ± 1.35; neither value was significantly different between age groups ($P = 0.44$ and 0.61, respectively). With F_{340}/F_{380} as an index of $[Ca^{2+}]_i$, resting values were not different between endothelium of Young *versus* Old mice (Fig. 1*A*). In response to stimulation with ACh (3 μM), the mean steady-state $[Ca^{2+}]_i$ response was 25% higher in Old *versus* Young ($P < 0.05$) (Fig. 1*C*), confirming the integrity of signalling through muscarinic GPCRs in advanced age.

Hydrogen peroxide availability under basal conditions

Baseline raw fluorescence (F_0) units for DCFH at the onset of recordings were 474 ± 32 for Young and 402 ± 34 for Old (means ± SEM, $n = 10$ per age group) and not significantly different between age groups ($P = 0.15$). To provide an internal control for variability in dye loading and other factors that may influence F_0, these data are presented relative to F_0 (i.e. F/F_0). The rate of DCFH fluorescence accumulation was 33% greater in endothelium of Old *versus* Young ($P < 0.05$; Fig. 2*A*–*C*). In the presence of TEMPOL (1 mM) + PEG-catalase (500 U ml^{-1}) DCFH fluorescence did not change over time (Fig. 2*A*–*C*), confirming the applicability of this probe for detecting H_2O_2 in our experiments. In lysates of epigastric arteries, endogenous catalase activity was lower by nearly half in Old compared to Young ($P < 0.05$, Fig. 2*D*). Thus, microvascular endothelium of Old mice exhibits greater H_2O_2 availability under basal conditions in conjunction with diminished capacity of the vessel wall to remove H_2O_2 when compared to Young. To provide an index of H_2O_2 availability under basal conditions, H_2O_2 was added to the superfusion solution. In endothelial tubes of Young ($n = 3$), the slope of fluorescence accumulation was unaffected by 1 μM H_2O_2 but began to increase following addition of 5 μM H_2O_2. For reference, 200 μM H_2O_2 promptly increased DCFH fluorescence and saturated the signal within 2–3 min (data not shown).

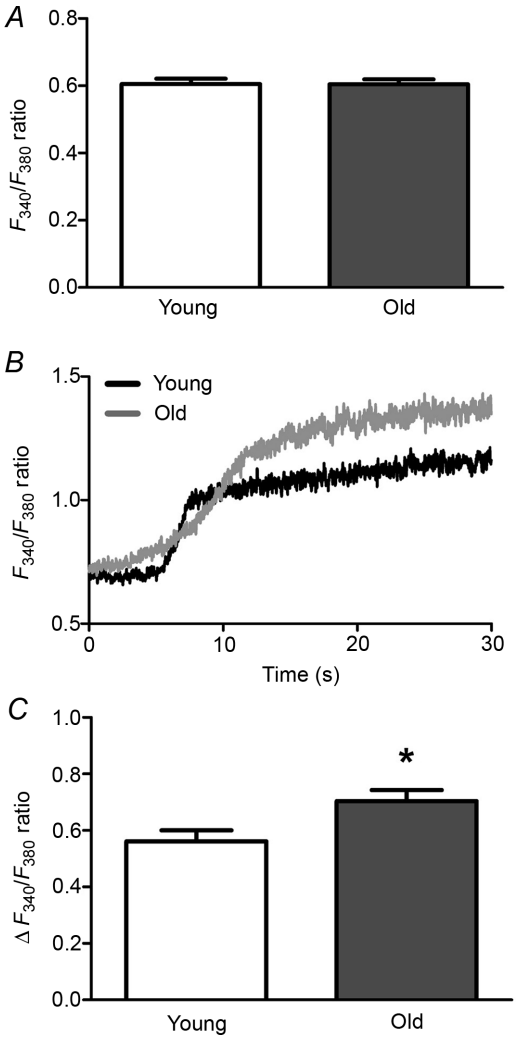

Figure 1. Enhanced $[Ca^{2+}]_i$ response to ACh in microvascular endothelium with advanced age
A, resting F_{340}/F_{380} ratios were not different between endothelium of Young *versus* Old mice indicating similar $[Ca^{2+}]_i$ at rest. *B*, representative F_{340}/F_{380} recordings from endothelium of Young and Old in response to 3 μM ACh (added at time 0). *C*, maximal change in $[Ca^{2+}]_i$ ($\Delta F_{340}/F_{380}$) of endothelium from Young and Old mice during sustained response to 3 μM ACh. Summary data are means ± SEM; $n = 33$ for Young, $n = 31$ for Old. *$P < 0.05$, Old *versus* Young.

Effect of H_2O_2 on endothelial Ca^{2+} and cellular integrity

Exposing the endothelium of Young mice to 200 μM H_2O_2 for 20 min progressively increased $[Ca^{2+}]_i$. After a delay of several min, the increase was significant by 20 min (Fig. 3*C*) and was nearly twice that observed in response to maximal stimulation with 3 μM ACh (Fig. 1*C*). During the ensuing washout of H_2O_2, this aberrant elevation in $[Ca^{2+}]_i$ persisted for the initial 5 min before recovering to control levels within 20–30 min (Fig. 3*A*). In endothelium of Old mice, exposure to 200 μM H_2O_2 also led to a rise in $[Ca^{2+}]_i$ with a time course similar to that for Young (Fig. 3*B*), although the $[Ca^{2+}]_i$ response was far less (~25%; $P < 0.05$) than observed for Young. In separate experiments, omitting Ca^{2+} from the superfusion solution abolished the response to H_2O_2 in both Young (Fig. 3*A*) and Old (Fig. 3*B*) when compared to responses in the presence of 2 mM $[Ca^{2+}]_o$. Within 5 min of reintroducing 2 mM Ca^{2+} during H_2O_2 washout, $[Ca^{2+}]_i$ increased in endothelium of Young (Fig. 3*A*) but not in Old (Fig. 3*B*), further indicating a persistent effect of H_2O_2 on Ca^{2+} entry.

The above data implicate a key role for extracellular Ca^{2+} in the $[Ca^{2+}]_i$ response during elevated H_2O_2. Thus, Ca^{2+} influx across the plasma membrane was evaluated through measuring Fura-2 quench by Mn^{+2} (Fig. 4). Consistent with the similarity in resting $[Ca^{2+}]_i$, there was no difference in the quench rate of Fura-2 fluorescence at rest between endothelium of Young *versus* Old mice (Fig. 4*A* and *B*). During exposure to 200 μM H_2O_2, the quench rate increased for endothelium of Young and Old; however, the rate was ~2-fold greater ($P < 0.05$) in Young (Fig. 4*C* and *D*). Given that TRP channels encompass redox-sensitive cation channels in the plasma membrane

of ECs (Poteser *et al.* 2006; Xu *et al.* 2008; Sun *et al.* 2012) and are integral to Ca^{2+} influx (Ramsey *et al.* 2006), we tested whether H_2O_2-induced Ca^{2+} influx was mediated by TRP channels. In endothelium of Young, inclusion of 5 μM RuR [a broad-spectrum blocker of TRP channels (Ramsey *et al.* 2006)] during exposure to 200 μM H_2O_2 abrogated the $[Ca^{2+}]_i$ response by ~70% (Fig. 5).

Elevated levels of H_2O_2 can damage ECs (Volk *et al.* 1997; Sun *et al.* 2012). To determine whether H_2O_2 exposure was associated with differences in cellular integrity between age groups, Live/Dead cell assays were performed. Following the 20 min exposure to 200 μM H_2O_2, cells remained viable in the endothelium of both age groups, as confirmed by their uptake and retention of calcein-AM and absence of staining with EtBr ($n = 3$ per age group; data not shown). Increasing the duration of H_2O_2 exposure to 60 min resulted in dead and dying cells within endothelial tubes of both age groups (Fig. 6*B* and *C*). Remarkably, there was a pronounced protective effect of advanced age. Thus, with 566 ± 139 cells counted per endothelial tube in Young ($n = 5$) and 616 ± 49 cells counted per endothelial tube in Old ($n = 5$), the percentage of ECs labelled with EtBr was ~7-fold greater ($P < 0.05$) in Young compared to Old (Fig. 6*E*). Consistent with the stability of Ca^{2+} and electrical responses in our previous studies (Socha *et al.* 2011; Behringer *et al.* 2012, 2013), time controls performed here confirmed that, in the absence of H_2O_2, there were no dead or dying cells in endothelial tubes of Young following 60 min under identical conditions ($n = 2$).

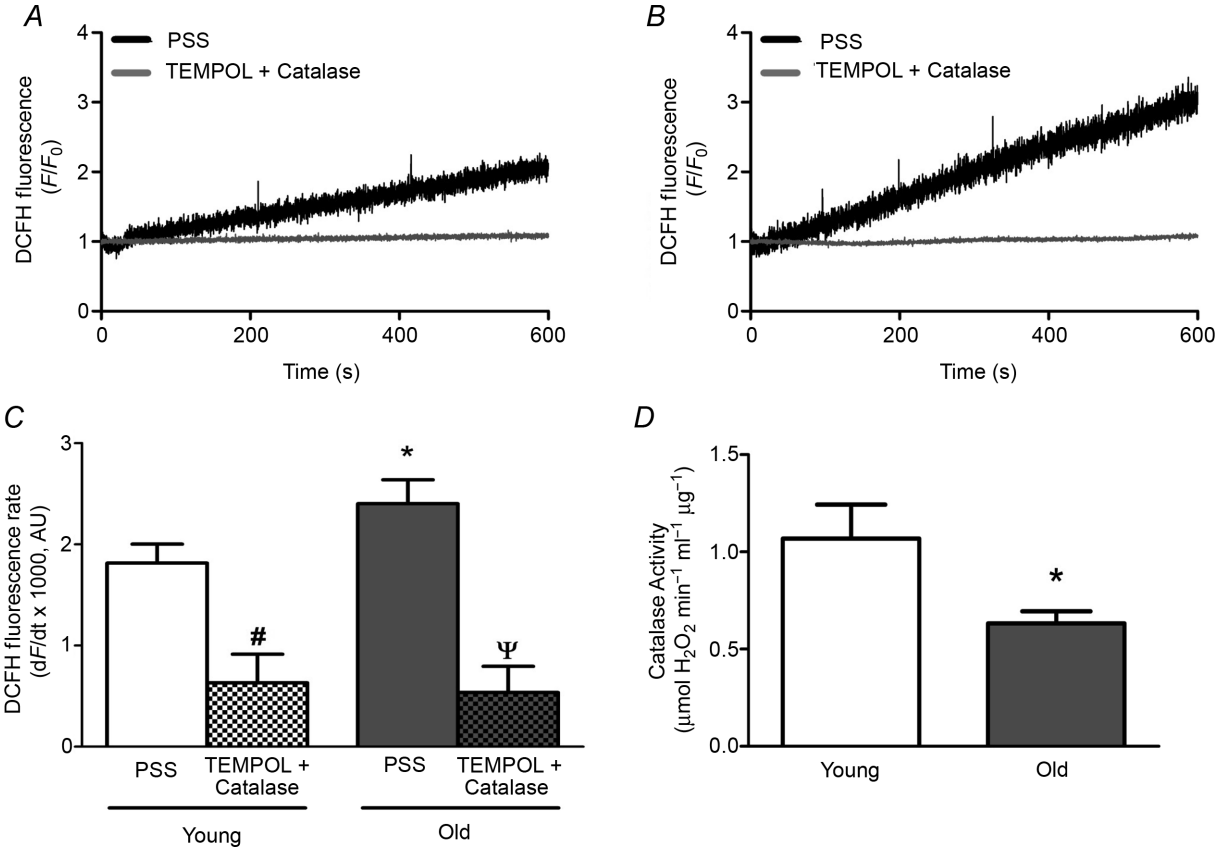

Figure 2. Enhanced H_2O_2 availability with diminished H_2O_2 decomposition during advanced age
A, representative recordings of DCFH in endothelium of Young mice illustrating progressive increase in fluorescence over 10 min during superfusion with PSS containing 2 mM Ca^{2+} that was prevented by treatment with TEMPOL (1 mM, superoxide dismutase mimetic) + PEG-catalase (500 U ml^{-1}, H_2O_2 scavenger). *B*, as in *A* for Old mice; note greater rate of fluorescence accumulation *versus* Young. *C*, summary data for rates of fluorescence accumulation in endothelium of Young and Old mice determined from recordings as represented by those in *A* and *B*. Rates of fluorescence accumulation were depressed by TEMPOL + PEG-catalase to values not different between Young and Old ($n = 10$ per age group with PSS; $n = 4$ per group for TEMPOL + PEG-catalase). $^*P < 0.05$, Old *versus* Young, $^{\#}P < 0.05$, TEMPOL + PEG-catalase *versus* PSS for Young; $^{\Psi}P < 0.05$, TEMPOL + PEG-catalase *versus* PSS for Old. *D*, catalase activity measured via H_2O_2 decomposition by lysates of epigastric arteries from Young and Old mice ($n = 5$ per age group). $^*P < 0.05$, Old *versus* Young. Summary data in *C* and *D* are means ± SEM.

Discussion

Calcium signalling is integral to endothelial function and vasomotor control. However, there is a paucity of information regarding the effects of advanced age on endothelial Ca^{2+} signalling during oxidative stress, particularly in the microcirculation. To address this gap in understanding, we evaluated $[Ca^{2+}]_i$ at rest, during muscarinic GPCR activation and in response to acute oxidative stress with H_2O_2 (200 μM) in endothelium freshly isolated from skeletal muscle resistance arteries of Young and Old mice. Resting $[Ca^{2+}]_i$ was not different between age groups, yet the peak steady-state $[Ca^{2+}]_i$ response to ACh (3 μM) was greater in endothelium of Old compared to Young. Furthermore, when compared to Young, the constitutive release of H_2O_2 under basal conditions was greater for endothelium of Old and was accompanied by lower vascular catalase activity. During oxidative stress imposed by exposure to H_2O_2 for 20 min, the endothelium of Young mice responded with an aberrant increase in $[Ca^{2+}]_i$ whereas the endothelium of Old mice did not. As indicated by its dependence on extracellular Ca^{2+}, this difference between age groups may reflect adaptation of microvascular endothelium during advanced age that attenuates influx of Ca^{2+} across the plasma membrane. Moreover, prolonged (60 min) exposure to H_2O_2 resulted in far greater cell death in endothelium of Young than in Old. These findings demonstrate a protective effect of advanced age on Ca^{2+} influx and cell viability in response to acute oxidative stress in native endothelium of resistance microvessels.

Endothelial dysfunction (e.g. impaired endothelial-dependent dilatation, EDD) accompanies advancing age in humans (Taddei *et al.* 2001; Seals *et al.* 2011) and is a risk factor for cardiovascular disease (Yeboah *et al.* 2007; Lind *et al.* 2011). In the peripheral circulation, the efficacy of endothelium-dependent vasodilators (e.g. ACh and methacholine; which act through muscarinic GPCRs to elevate $[Ca^{2+}]_i$) is diminished in elderly humans (e.g. > 60 years) compared to their younger (e.g. < 30 years) counterparts (Taddei *et al.* 2001). At the same time, endothelium-independent vasodilatation (e.g. to the NO donor SNP) was maintained (Taddei *et al.* 2001). Remarkably, little is known of how ageing may

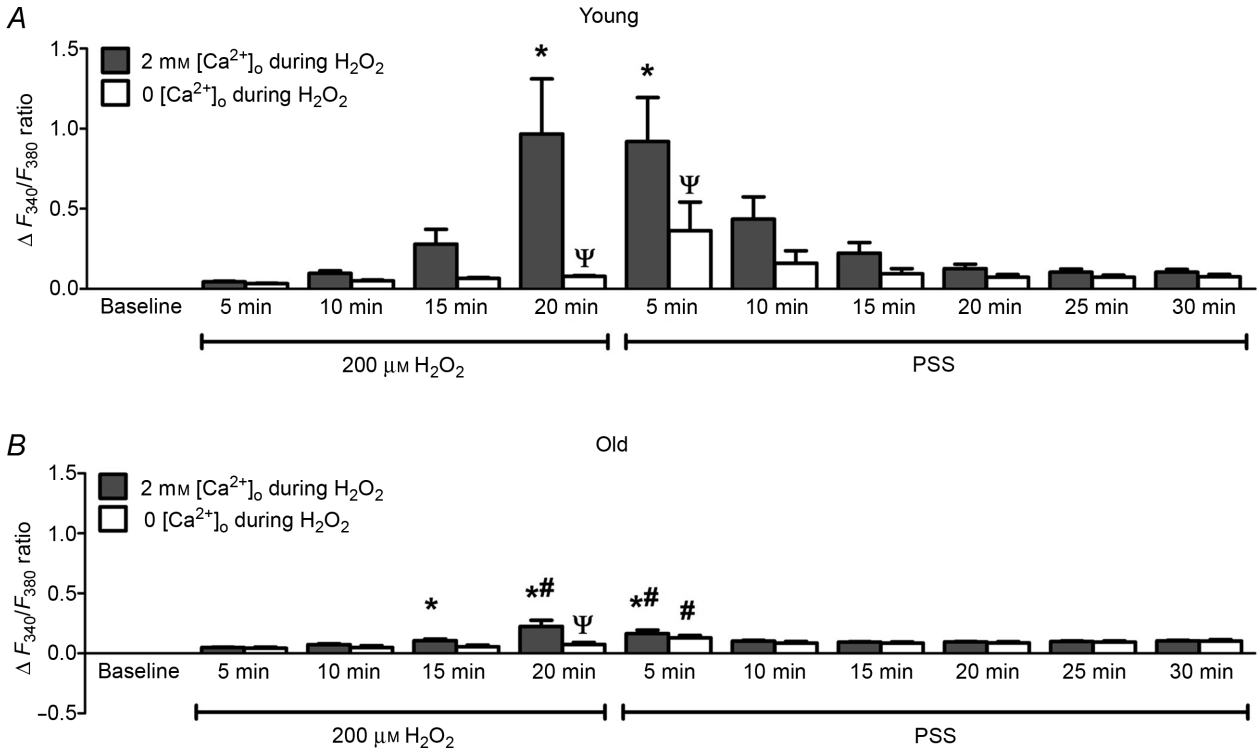

Figure 3. Reduced $[Ca^{2+}]_i$ response of microvascular endothelium to H_2O_2 with advanced age
A, exposing endothelium from Young mice to 200 μM H_2O_2 for 20 min progressively increased $[Ca^{2+}]_i$ ($\Delta F_{340}/F_{380}$). $[Ca^{2+}]_i$ recovered during 30 min washout of H_2O_2 with PSS containing 2 mM Ca^{2+}. Removing extracellular Ca^{2+} (0 $[Ca^{2+}]_o$) during H_2O_2 exposure prevented the rise in $[Ca^{2+}]_i$ in endothelial tubes of Young mice yet $[Ca^{2+}]_i$ increased within 5 min of H_2O_2 washout with PSS containing 2 mM Ca^{2+}. *B*, as in *A* for Old mice; note diminished $[Ca^{2+}]_i$ response compared to Young in the presence of 2 mM $[Ca^{2+}]_o$ and negligible $[Ca^{2+}]_i$ response in 0 $[Ca^{2+}]_o$. Summary data are means ± SEM. For both age groups $n = 9$ for 2 mM $[Ca^{2+}]_o$ during H_2O_2, and $n = 7$ for 0 $[Ca^{2+}]_o$ during H_2O_2. $^*P < 0.05$, *versus* Control. $^\#P < 0.05$, Old *versus* Young for given time and condition, $^\Psi P < 0.05$ for 0 $[Ca^{2+}]_o$ *versus* 2 mM $[Ca^{2+}]_o$ exposed to 200 μM H_2O_2.

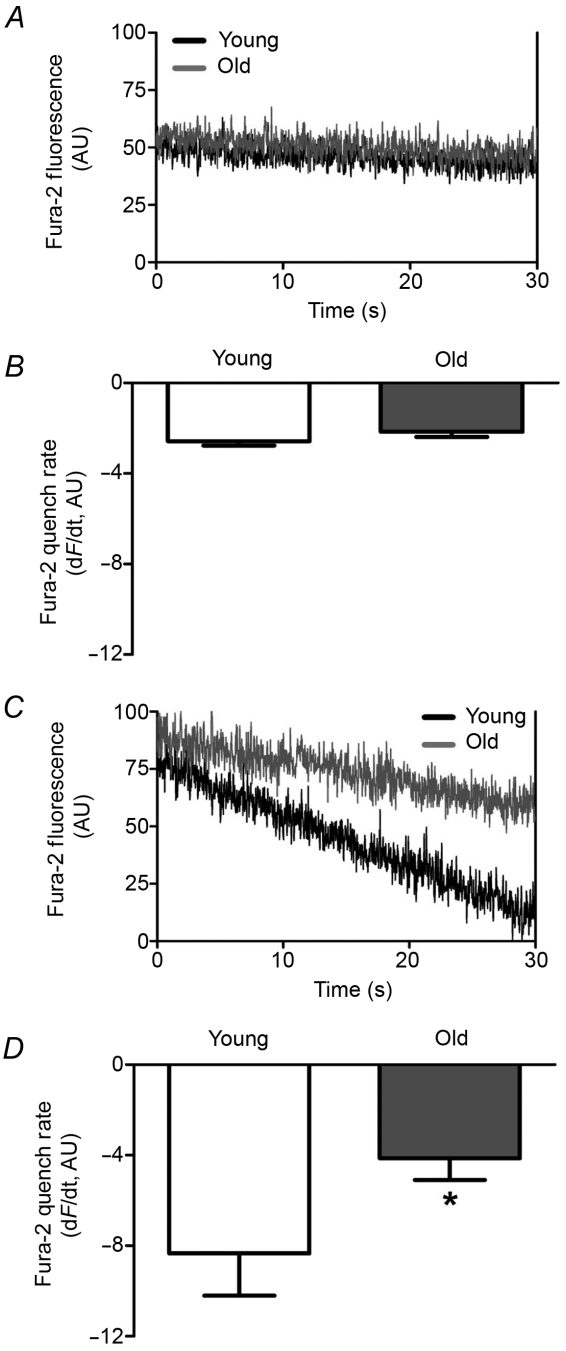

Figure 4. Enhanced Mn^{2+} quenching of Fura-2 fluorescence during H_2O_2 is reduced with advanced age
A, representative Fura-2 recording at isosbestic wavelength (360 nm) during superfusion with 2 mM $MnCl_2$ (replacing $CaCl_2$ in PSS) of endothelium from Young and Old mice at rest. Note similar rates of Fura-2 quench. *B*, summary data (means ± SEM) indicate no difference in Mn^{2+} influx between endothelium of Young ($n = 6$) and Old ($n = 8$) mice. *C*, as in *A* during exposure to 200 μM H_2O_2. Note increased rate of Fura-2 quench in both age groups compared to *A* with greater rate in Young *versus* Old. *D*, summary data (means ± SEM) indicate greater rate of Mn^{2+} influx in endothelium of Young ($n = 6$) versus Old ($n = 6$) during exposure to 200 μM H_2O_2. *$P < 0.05$, Old *versus* Young.

affect endothelial $[Ca^{2+}]_i$ responses to GPCR agonists or to acute oxidative stress. Therefore, we tested the hypothesis that advanced age impairs endothelial Ca^{2+} signalling. Under resting conditions, $[Ca^{2+}]_i$ was not different between endothelial tubes from Young *versus* Old mice (Fig. 1*A*). Through binding to muscarinic GPCRs, ACh initiates intracellular Ca^{2+} release from the endoplasmic reticulum with an ensuing prolonged phase of Ca^{2+} entry across the plasma membrane from the extracellular fluid (Tran & Watanabe, 2006; Socha *et al.* 2011). Our use of 3 μM ACh as a maximal criterion stimulus is based upon earlier studies of endothelial tubes, where ACh elevated $[Ca^{2+}]_i$ and evoked hyperpolarization through K_{Ca} activation in a concentration-dependent manner. Both $[Ca^{2+}]_i$ and hyperpolarization plateaued at ≥ 1 μM ACh (Behringer *et al.* 2012), with no difference in the magnitude of hyperpolarization between Young and Old (Behringer *et al.* 2013). During the prolonged phase studied here, elevations in $[Ca^{2+}]_i$ were ∼33% greater in endothelium of Old *versus* Young (Fig. 1*C*). This finding confirms the integrity of this signalling pathway while suggesting augmented Ca^{2+} entry with advanced age. Greater elevation of $[Ca^{2+}]_i$ during sustained muscarinic GPCR activation may help to compensate for age-related reductions in NO bioavailability (Muller-Delp, 2006; Seals *et al.* 2011). Thus, elevated $[Ca^{2+}]_i$ would promote the activation of endothelial NOS. However, elevated $[Ca^{2+}]_i$ can enter mitochondria, thereby increasing mitochondrial generation of ROS (Camello-Almaraz *et al.* 2006) and EC oxidative stress.

Augmented superoxide production and greater levels of oxidative stress have been demonstrated in ECs with ageing (Dhalla *et al.* 2000; Muller-Delp *et al.* 2012; Bachschmid *et al.* 2013). Elevation of ROS occurs as a consequence of greater synthesis, diminished removal or a combination thereof. Sources of superoxide (i.e. the precursor to H_2O_2) in ECs include NADPH oxidase, xanthine oxidase, mitochondria (i.e. respiratory enzymes) and uncoupled NOS (Lambeth, 2004; Forstermann, 2008; Touyz *et al.* 2011). In addition to constitutive ROS production, ECs are exposed to ROS released by surrounding cells (e.g. SMCs) and by cells carried in the bloodstream (Li & Shah, 2004; Feletou & Vanhoutte, 2006). Once generated, superoxide anions form H_2O_2 via the superoxide dismutase family of enzymes. Catalase then decomposes H_2O_2 by converting it to H_2O and O_2 to protect cells from oxidative damage. Levels of oxidative stress, as well as subsequent cellular responses, can vary between vascular beds (Grinnell *et al.* 2012). To determine whether oxidative stress was manifest under basal conditions within microvascular ECs during advanced age, we evaluated rates of H_2O_2 production using the H_2O_2-sensitive dye DCFH (Carter *et al.* 1994) after confirming its sensitivity to H_2O_2 under the conditions of our experiments. Our data illustrate

that the availability of H_2O_2 was significantly greater in the endothelium of Old mice under resting conditions (Fig. 2*C*). This finding helps to explain the enhanced activation of SK_{Ca} and IK_{Ca} observed for endothelial tubes of Old mice (Behringer *et al.* 2013). With no difference in $[Ca^{2+}]_i$ at rest (Fig. 1*A*), direct activation and/or enhanced sensitivity to Ca^{2+} of K_{Ca} is implied, as channel expression was not different between age groups (Behringer *et al.* 2013). Complementary experiments revealed that catalase activity was reduced by nearly half in epigastric arteries of Old mice (Fig. 2*D*), supporting higher levels of constitutive oxidative stress in microvascular ECs during advanced age.

Acute elevation of oxidative stress was imposed by superfusion of endothelial tubes with 200 μM H_2O_2 for 20 min. This concentration of H_2O_2 and duration of exposure were based upon finding that they effectively activated SK_{Ca} and IK_{Ca} in the same preparations studied here (Behringer *et al.* 2013). Moreover, the time course observed for the progression of hyperpolarization is consistent with that found here for the rise in $[Ca^{2+}]_i$; i.e. an initial delay upon exposure followed by a progressive increase during the 20 min exposure (Fig. 3). Observing that the effect of H_2O_2 on $[Ca^{2+}]_i$ persisted during the early period of washout suggests that, despite being membrane-permeant, the actions of H_2O_2 are not instantaneous and that they exert a residual effect following H_2O_2 removal. Compared to the gradual accumulation of DCFH fluorescence under basal conditions, the fluorescence signal saturated within 2–3 min upon exposure to 200 μM H_2O_2. Thus 200 μM H_2O_2 is far greater than levels present under basal conditions. As exposure to 5 μM H_2O_2 was found to increase DCFH fluorescence above basal accumulation, we suggest that this far lower concentration more closely approximates constitutive levels of H_2O_2 availability.

Regulation of EC $[Ca^{2+}]_i$ occurs though multiple signalling pathways which include stimulation of GPCRs with physiological agonists (Tran & Watanabe, 2006). Thus, ACh binding to muscarinic (M3) receptors generates inositol 1,4,5 trisphosphate, thereby stimulating releasing of Ca^{2+} from the endoplasmic reticulum followed by sustained influx through the plasma membrane (Tran & Watanabe, 2006). Free Ca^{2+} is then removed from the cytoplasm by ATPases in respective membranes. Indeed, the maintenance of $[Ca^{2+}]_i$ homeostasis is essential for EC function and survival, with aberrant levels of $[Ca^{2+}]_i$ leading to dysfunction and cell death (Orrenius *et al.* 2003; Berridge, 2005). Exposure to 200 μM H_2O_2 resulted in an ~4-fold greater increase in $[Ca^{2+}]_i$ for endothelium of Young (Fig. 3*A*) compared to Old (Fig. 3*B*). To evaluate the role of Ca^{2+} entry, extracellular Ca^{2+} was removed during H_2O_2 treatment, which effectively prevented the increase in $[Ca^{2+}]_i$. That H_2O_2 acted via augmenting Ca^{2+} entry was tested further by using Mn^{2+} influx to quench Fura-2 (Potocnik & Hill, 2001; Tas *et al.* 2008). Consistent with resting $[Ca^{2+}]_i$, we found no difference in the rate of Fura-2 fluorescence quenching between endothelium of Young *versus* Old at rest (Fig. 4*A* and *B*). However, exposure to 200 μM H_2O_2 evoked ~2-fold greater peak rates of Fura-2 fluorescence quenching for endothelium of Young *versus* Old (Fig. 4*C* and *D*). These findings indicate that elevated $[Ca^{2+}]_i$ in Young during H_2O_2 exposure can be explained by enhanced Ca^{2+} influx. In light of greater Ca^{2+} influx during ACh exposure in endothelium of Old *versus* Young mice, the corresponding reduction in Ca^{2+} influx during 200 μM H_2O_2 exposure in Old mice implies an adaptation that occurs in association with significantly higher basal levels of oxidative stress during advanced age.

TRP channels are Ca^{2+}-permeant, expressed in ECs and sensitive to oxidative stress (Poteser *et al.* 2006; Yoshida *et al.* 2006; Xu *et al.* 2008; Beech, 2013). In accordance with the far greater Ca^{2+} responses for endothelium of Young *versus* Old mice, we investigated whether TRP channels were involved in the H_2O_2-induced Ca^{2+} influx in endothelium of Young mice. Remarkably, inclusion of the broad-spectrum TRP channel antagonist RuR (Ramsey

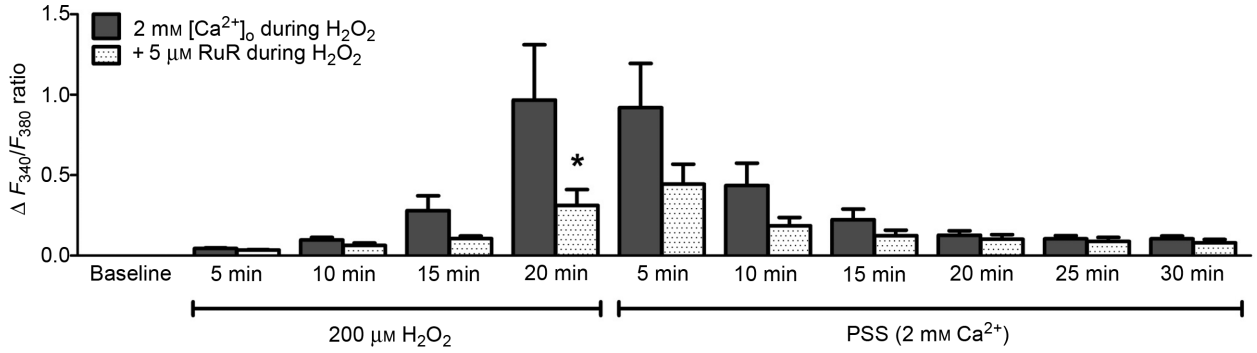

Figure 5. TRP channels contribute to Ca^{2+} influx induced by H_2O_2
In endothelium of Young mice, inclusion of 5 μM RuR to inhibit TRP channels reduced influx of Ca^{2+} during exposure to 200 μM H_2O_2 by ~75%. This effect was reversed within 5 min of washout of H_2O_2 with PSS. Summary data are means ± SEM; $n = 9$ for 2 mM $[Ca^{2+}]_o$ during H_2O_2 and $n = 5$ for 5 μM RuR during H_2O_2. *$P < 0.05$, Old *versus* Young.

et al. 2006) attenuated the H_2O_2-induced $[Ca^{2+}]_i$ rise (Fig. 5) to levels similar to those observed in the absence of $[Ca^{2+}]_o$ (Fig. 3). Multiple isoforms of TRP channels are redox sensitive through activation by oxidative or reductive agents. Thus, TRPC5 can be activated directly via sulfhydryl modification by NO and other oxidative agents (Yoshida *et al.* 2006) or by reducing agents such as thioredoxin (Xu *et al.* 2008). Furthermore, TRPV1 and TRPA1 can be activated by oxidizing agents through cysteine modification (Yoshida *et al.* 2006; Macpherson *et al.* 2007). As recently shown for endothelium of rat cerebral arteries, generation of H_2O_2 leads to lipid peroxidation products which activate 'TRPA1 sparklets' of Ca^{2+} influx (Sullivan *et al.* 2015). In addition to these homomeric channels, TRP channel protein isoforms can form redox-sensitive heteromeric channels. For

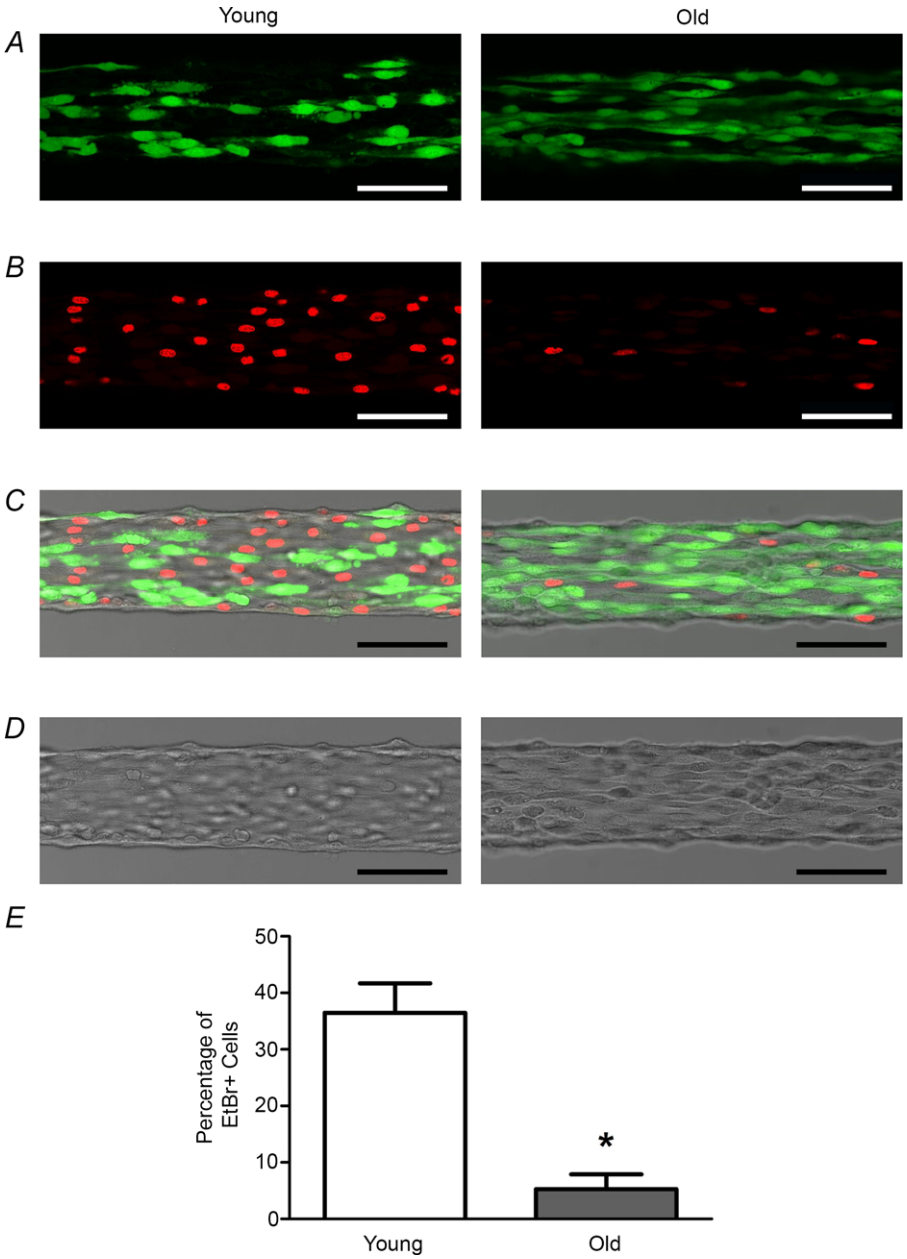

Figure 6. Advanced age abrogates cell death induced by prolonged exposure to H_2O_2
Following 60 min exposure to 200 μM H_2O_2, a Live/Dead cell assay was performed on endothelial tubes from Young (left) and Old (right) mice. *A*, living endothelial cells sequestered and retained calcein-AM (green), confirming their viability. *B*, dead and dying cells (which have nuclei stained red with EtBr) appear more frequently for endothelium of Young *versus* Old. *C*, overlay of images in *A* and *B*. *D*, brightfield images of endothelial tubes in *A*–*C*. *E*, the percentage of EtBr-positive cells is ~7-fold greater in endothelium of Young *versus* Old. Summary data are means ± SEM; $n = 5$ per age group. $^*P < 0.05$, Old *versus* Young. Scale bars = 50 μm.

example, association of TRPC3 and TRPC4 subunits have been found to form such channels in ECs (Poteser *et al.* 2006). The TRPM2 channel, which is robustly expressed in ECs, is activated by H_2O_2 and the resulting Ca^{2+} influx can lead to apoptosis (Hecquet *et al.* 2008; Sun *et al.* 2012). Owing to the possibility that the H_2O_2-induced Ca^{2+} influx could lead to cell death, we performed Live/Dead cell assays and found no evidence thereof following 20 min exposure to 200 μM H_2O_2. This outcome is in agreement with our earlier studies demonstrating that endothelial tubes remained viable (e.g. membrane potential was maintained) for this period under the same experimental conditions (Behringer *et al.* 2013). However, when the duration of exposure to 200 μM H_2O_2 was increased to 60 min, cell death was apparent in both Young and Old (Fig. 6). Remarkably, the prevalence of dead and dying cells (EtBr positive) observed in Young was ~7-fold greater (~36%) than that in Old (~5%). In turn, we suggest that the profound reduction in Ca^{2+} influx observed for endothelium of Old *versus* Young reflects a protective adaptation during advanced age that is associated with constitutively greater levels of H_2O_2 under basal conditions (Fig. 2).

The production of ROS within ECs can disrupt signalling pathways and lead to cytotoxicity (Dhalla *et al.* 2000). For example, superoxide can react with NO to form peroxynitrite and thereby reduce the bioavailability of NO while uncoupling NOS (Landmesser *et al.* 2003), contributing further to superoxide production. In diabetes, endothelial dysfunction induced by oxidative stress results from the activation of protein kinase C and an increase in glycation end-product formation (Brownlee, 2001). Additionally, NADPH oxidase-induced oxidative stress is a key step in the progression of hypertension (Laursen *et al.* 1997; Schnackenberg *et al.* 1998) while mounting evidence implicates ROS in the pathogenesis of atherosclerosis (Madamanchi & Runge, 2007). Furthermore, constitutively elevated H_2O_2 during advanced age impairs electrical signal transmission along the endothelium via activation of K_{Ca} channels, with 'leaky' membranes dissipating charge transfer from cell to cell (Behringer *et al.* 2013).

Scavenging ROS has been found to ameliorate endothelial dysfunction and promote blood flow to skeletal muscle in both humans (Kirby *et al.* 2009) and mice (Fleenor *et al.* 2012) of advanced age. Nevertheless, endothelial-derived ROS have increasingly been found to play a key role in cellular signalling pathways under physiological conditions (Ungvari *et al.* 2006; Thomas *et al.* 2008; Suvorava & Kojda, 2009; Wolin, 2009; Muller-Delp *et al.* 2012). Specifically, H_2O_2 has been identified as an ideal second messenger amongst ROS due to its relatively long half-life compared to superoxide or peroxynitrite. Furthermore, unlike the latter anions, which are membrane-impermeant and react in their vicinity of production, its lack of charge enables H_2O_2 to readily permeate cell membranes and thereby serve as an autacoid. Indeed, H_2O_2 has been shown to function as an endothelial-derived vasodilator. Thus, in mesenteric arteries of both humans and mice, endothelial-derived H_2O_2 can hyperpolarize and thereby relax SMCs (Matoba *et al.* 2000, 2002) to produce vasodilatation. In human coronary arterioles, H_2O_2 generated from mitochondria can regulate flow-mediated (i.e. endothelium-dependent) dilatation through activating large-conductance K_{Ca} channels of SMCs (Liu *et al.* 2003). Additionally, recent studies of first-order arterioles from rat soleus muscle indicate that with advanced age there is a shift in EDD from reliance on NO to a greater role for H_2O_2 (Sindler *et al.* 2013). Such a shift during ageing would imply an adaptation in the endothelium to reduce the cellular damage otherwise caused by H_2O_2, which is consistent with the present observations.

Summary and perspective

The present study has investigated Ca^{2+} signalling in endothelial tubes freshly isolated from resistance arteries of mouse skeletal muscle with respect to the effects of advanced age and acute oxidative stress induced by acute exposure to elevated H_2O_2. Utilization of this model enables studies of native intact microvascular endothelial function in the absence of confounding interactions with circulating blood, surrounding smooth muscle and adventitia. Our findings indicate that microvascular endothelium of Old mice has higher levels of H_2O_2 availability under basal conditions. With greater activation of K_{Ca} (Behringer *et al.* 2013) and no difference in $[Ca^{2+}]_i$, at rest in Old *versus* Young, the present data suggest that constitutive production of H_2O_2 activates K_{Ca} of native microvascular endothelium, consistent with the effects of H_2O_2 in human endothelial cells (Bychkov *et al.* 1999; Miura *et al.* 2003). Exposure to 200 μM H_2O_2 for 20 min evoked far greater $[Ca^{2+}]_i$ increases in the endothelium of Young *versus* Old mice with a time course similar to that observed for hyperpolarization (Behringer *et al.* 2013). This rise in $[Ca^{2+}]_i$ can be explained by greater Ca^{2+} influx across the plasma membrane and inhibiting this effect by blocking TRP channels further implicates the activation of membrane ion channels by H_2O_2. Remarkably, cell injury and death following prolonged (60 min) exposure to H_2O_2 were far greater in endothelium of Young mice compared to Old. Thus, constitutively elevated basal levels of oxidative stress during advanced age may lead to an adaptive response within the endothelium of resistance microvessels that limits aberrant Ca^{2+} influx and mitigates the cytotoxic effects of oxidative stress (Dhalla *et al.* 2000) in association with greater utilization of H_2O_2 as a mediator of EDD (Widlansky & Gutterman,

2011; Muller-Delp *et al.* 2012). Indeed, such protective adaptations to advanced age could explain why antioxidant therapy in humans has little beneficial effect and may instead act to increase patient morbidity and mortality (Yoshihara *et al.* 2010). Our findings collectively indicate a delicate balance between maintaining physiological levels of ROS during normal vascular function (e.g. 'healthy ageing') and pathological oxidative stress with associated endothelial dysfunction.

References

Bachschmid MM, Schildknecht S, Matsui R, Zee R, Haeussler D, Cohen RA, Pimental D & Loo B (2013). Vascular aging: chronic oxidative stress and impairment of redox signaling – consequences for vascular homeostasis and disease. *Ann Med* **45**, 17–36.

Beech DJ (2013). Characteristics of transient receptor potential canonical calcium-permeable channels and their relevance to vascular physiology and disease. *Circ J* **77**, 570–579.

Behringer EJ, Shaw RL, Westcott EB, Socha MJ & Segal SS (2013). Aging impairs electrical conduction along endothelium of resistance arteries through enhanced Ca^{2+}-activated K^+ channel activation. *Arterioscler Thromb Vasc Biol* **33**, 1892–1901.

Behringer EJ, Socha MJ, Polo-Parada L & Segal SS (2012). Electrical conduction along endothelial cell tubes from mouse feed arteries: confounding actions of glycyrrhetinic acid derivatives. *Br J Pharmacol* **166**, 774–787.

Berridge MJ (2005). Unlocking the secrets of cell signaling. *Annu Rev Physiol* **67**, 1–21.

Brownlee M (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813–820.

Bychkov R, Pieper K, Ried C, Milosheva M, Bychkov E, Luft FC & Haller H (1999). Hydrogen peroxide, potassium currents, and membrane potential in human endothelial cells. *Circulation* **99**, 1719–1725.

Camello-Almaraz C, Gomez-Pinilla PJ, Pozo MJ & Camello PJ (2006). Mitochondrial reactive oxygen species and Ca^{2+} signaling. *Am J Physiol Cell Physiol* **291**, C1082–1088.

Campbell WB, Gebremedhin D, Pratt PF & Harder DR (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* **78**, 415–423.

Carter WO, Narayanan PK & Robinson JP (1994). Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leukoc Biol* **55**, 253–258.

Crutchley DJ, Ryan JW, Ryan US & Fisher GH (1983). Bradykinin-induced release of prostacyclin and thromboxanes from bovine pulmonary artery endothelial cells. Studies with lower homologs and calcium antagonists. *Biochim Biophys Acta* **751**, 99–107.

Dhalla NS, Temsah RM & Netticadan T (2000). Role of oxidative stress in cardiovascular diseases. *J Hypertens* **18**, 655–673.

Dinenno FA, Jones PP, Seals DR & Tanaka H (1999). Limb blood flow and vascular conductance are reduced with age in healthy humans: relation to elevations in sympathetic nerve activity and declines in oxygen demand. *Circulation* **100**, 164–170.

Dinenno FA, Seals DR, DeSouza CA & Tanaka H (2001). Age-related decreases in basal limb blood flow in humans: time course, determinants and habitual exercise effects. *J Physiol* **531**, 573–579.

Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE & Seals DR (2007). Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-κB. *Circ Res* **100**, 1659–1666.

Donato AJ, Uberoi A, Wray DW, Nishiyama S, Lawrenson L & Richardson RS (2006). Differential effects of aging on limb blood flow in humans. *Am J Physiol Heart Circ Physiol* **290**, H272–278.

Drouin A, Thorin-Trescases N, Hamel E, Falck JR & Thorin E (2007). Endothelial nitric oxide synthase activation leads to dilatory H_2O_2 production in mouse cerebral arteries. *Cardiovasc Res* **73**, 73–81.

Feletou M & Vanhoutte PM (2006). Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *Am J Physiol Heart Circ Physiol* **291**, H985–1002.

Fleenor BS, Seals DR, Zigler ML & Sindler AL (2012). Superoxide-lowering therapy with TEMPOL reverses arterial dysfunction with aging in mice. *Aging Cell* **11**, 269–276.

Fleming I & Busse R (1999). NO: the primary EDRF. *J Mol Cell Cardiol* **31**, 5–14.

Forstermann U (2008). Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. *Nat Clin Pract Cardiovasc Med* **5**, 338–349.

Grinnell K, Duong H, Newton J, Rounds S, Choudhary G & Harrington EO (2012). Heterogeneity in apoptotic responses of microvascular endothelial cells to oxidative stress. *J Cell Physiol* **227**, 1899–1910.

Hecquet CM, Ahmmed GU, Vogel SM & Malik AB (2008). Role of TRPM2 channel in mediating H_2O_2-induced Ca^{2+} entry and endothelial hyperpermeability. *Circ Res* **102**, 347–355.

Kirby BS, Voyles WF, Simpson CB, Carlson RE, Schrage WG & Dinenno FA (2009). Endothelium-dependent vasodilatation and exercise hyperaemia in ageing humans: impact of acute ascorbic acid administration. *J Physiol* **587**, 1989–2003.

Kruse HJ, Grunberg B, Siess W & Weber PC (1994). Formation of biologically active autacoids is regulated by calcium influx in endothelial cells. *Arterioscler Thromb* **14**, 1821–1828.

Lambeth JD (2004). NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* **4**, 181–189.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE & Harrison DG (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* **111**, 1201–1209.

Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA & Harrison DG (1997). Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* **95**, 588–593.

Ledoux J, Bonev AD & Nelson MT (2008). Ca^{2+}-activated K^+ channels in murine endothelial cells: block by intracellular calcium and magnesium. *J Gen Physiol* **131**, 125–135.

Li JM & Shah AM (2004). Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am J Physiol Regul Integr Comp Physiol* **287**, R1014–1030.

Lind L, Berglund L, Larsson A & Sundstrom J (2011). Endothelial function in resistance and conduit arteries and 5-year risk of cardiovascular disease. *Circulation* **123**, 1545–1551.

Liu Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC & Gutterman DD (2003). Mitochondrial sources of H_2O_2 generation play a key role in flow-mediated dilation in human coronary resistance arteries. *Circ Res* **93**, 573–580.

Macpherson LJ, Dubin AE, Evans MJ, Marr F, Schultz PG, Cravatt BF & Patapoutian A (2007). Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature* **445**, 541–545.

Madamanchi NR & Runge MS (2007). Mitochondrial dysfunction in atherosclerosis. *Circ Res* **100**, 460–473.

Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, Mukai Y, Hirakawa Y & Takeshita A (2002). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun* **290**, 909–913.

Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H & Takeshita A (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* **106**, 1521–1530.

Miura H, Bosnjak JJ, Ning G, Saito T, Miura M & Gutterman DD (2003). Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res* **92**, e31–40.

Muller-Delp JM (2006). Aging-induced adaptations of microvascular reactivity. *Microcirculation* **13**, 301–314.

Muller-Delp JM, Gurovich AN, Christou DD & Leeuwenburgh C (2012). Redox balance in the aging microcirculation: new friends, new foes, and new clinical directions. *Microcirculation* **19**, 19–28.

Nystoriak MA, O'Connor KP, Sonkusare SK, Brayden JE, Nelson MT & Wellman GC (2011). Fundamental increase in pressure-dependent constriction of brain parenchymal arterioles from subarachnoid hemorrhage model rats due to membrane depolarization. *Am J Physiol Heart Circ Physiol* **300**, H803–812.

Orrenius S, Zhivotovsky B & Nicotera P (2003). Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* **4**, 552–565.

Poteser M, Graziani A, Rosker C, Eder P, Derler I, Kahr H, Zhu MX, Romanin C & Groschner K (2006). TRPC3 and TRPC4 associate to form a redox-sensitive cation channel. Evidence for expression of native TRPC3-TRPC4 heteromeric channels in endothelial cells. *J Biol Chem* **281**, 13588–13595.

Potocnik SJ & Hill MA (2001). Pharmacological evidence for capacitative Ca^{2+} entry in cannulated and pressurized skeletal muscle arterioles. *Br J Pharmacol* **134**, 247–256.

Ramsey IS, Delling M & Clapham DE (2006). An introduction to TRP channels. *Annu Rev Physiol* **68**, 619–647.

Schnackenberg CG, Welch WJ & Wilcox CS (1998). Normalization of blood pressure and renal vascular resistance in SHR with a membrane-permeable superoxide dismutase mimetic: role of nitric oxide. *Hypertension* **32**, 59–64.

Seals DR, Jablonski KL & Donato AJ (2011). Aging and vascular endothelial function in humans. *Clin Sci* **120**, 357–375.

Sindler AL, Reyes R, Chen B, Ghosh P, Gurovich AN, Kang LS, Cardounel AJ, Delp MD & Muller-Delp JM (2013). Age and exercise training alter signaling through reactive oxygen species in the endothelium of skeletal muscle arterioles. *J Appl Physiol* **114**, 681–693.

Socha MJ, Domeier TL, Behringer EJ & Segal SS (2012). Coordination of intercellular Ca^{2+} signaling in endothelial cell tubes of mouse resistance arteries. *Microcirculation* **19**, 757–770.

Socha MJ, Hakim CH, Jackson WF & Segal SS (2011). Temperature effects on morphological integrity and Ca^{2+} signaling in freshly isolated murine feed artery endothelial cell tubes. *Am J Physiol Heart Circ Physiol* **301**, H773–783.

Socha MJ & Segal SS (2013). Isolation of microvascular endothelial tubes from mouse resistance arteries. *J Vis Exp* **81**, e50759.

Sullivan MN, Gonzales AL, Pires PW, Bruhl A, Leo MD, Li W, Oulidi A, Boop FA, Feng Y, Jaggar JH, Welsh DG & Earley S (2015). Localized TRPA1 channel Ca^{2+} signals stimulated by reactive oxygen species promote cerebral artery dilation. *Sci Signal* **8**, ra2.

Sun L, Yau HY, Wong WY, Li RA, Huang Y & Yao X (2012). Role of TRPM2 in H_2O_2-induced cell apoptosis in endothelial cells. *PLoS One* **7**, e43186.

Suvorava T & Kojda G (2009). Reactive oxygen species as cardiovascular mediators: lessons from endothelial-specific protein overexpression mouse models. *Biochim Biophys Acta* **1787**, 802–810.

Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A & Salvetti A (2001). Age-related reduction of NO availability and oxidative stress in humans. *Hypertension* **38**, 274–279.

Tas PW, Stossel C & Roewer N (2008). Inhibition of the histamine-induced Ca^{2+} influx in primary human endothelial cells (HUVEC) by volatile anaesthetics. *Eur J Anaesthesiol* **25**, 976–985.

Thomas SR, Witting PK & Drummond GR (2008). Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* **10**, 1713–1765.

Touyz RM, Briones AM, Sedeek M, Burger D & Montezano AC (2011). NOX isoforms and reactive oxygen species in vascular health. *Mol Interv* **11**, 27–35.

Tran QK & Watanabe H (2006). Calcium signalling in the endothelium. *Handb Exp Pharmacol* **176**, 145–187.

Trott DW, Gunduz F, Laughlin MH & Woodman CR (2009). Exercise training reverses age-related decrements in endothelium-dependent dilation in skeletal muscle feed arteries. *J Appl Physiol* **106**, 1925–1934.

Ungvari Z, Wolin MS & Csiszar A (2006). Mechanosensitive production of reactive oxygen species in endothelial and smooth muscle cells: role in microvascular remodeling? *Antioxid Redox Signal* **8**, 1121–1129.

van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V & Luscher TF (2000). Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med* **192**, 1731–1744.

Volk T, Hensel M & Kox WJ (1997). Transient Ca^{2+} changes in endothelial cells induced by low doses of reactive oxygen species: role of hydrogen peroxide. *Mol Cell Biochem* **171**, 11–21.

Widlansky ME & Gutterman DD (2011). Regulation of endothelial function by mitochondrial reactive oxygen species. *Antioxidants Redox Signal* **15**, 1517–1530.

Wolin MS (2009). Reactive oxygen species and the control of vascular function. *Am J Physiol Heart Circ Physiol* **296**, H539–549.

Xu SZ, Sukumar P, Zeng F, Li J, Jairaman A, English A, Naylor J, Ciurtin C, Majeed Y, Milligan CJ, Bahnasi YM, Al-Shawaf E, Porter KE, Jiang LH, Emery P, Sivaprasadarao A & Beech DJ (2008). TRPC channel activation by extracellular thioredoxin. *Nature* **451**, 69–72.

Yeboah J, Crouse JR, Hsu FC, Burke GL & Herrington DM (2007). Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. *Circulation* **115**, 2390–2397.

Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, Tominaga M, Shimizu S, Sato Y & Mori Y (2006). Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol* **2**, 596–607.

Yoshihara D, Fujiwara N & Suzuki K (2010). Antioxidants: benefits and risks for long-term health. *Maturitas* **67**, 103–107.

Additional information

Competing interests

The authors declare no competing interests.

Author contributions

M.J.S. designed and performed the experiments using Fura-2 dye, analysed and interpreted the data, drafted the manuscript and prepared the figures. E.M.B. evaluated catalase activity, contributed to experimental design, data interpretation and edited the manuscript. R.L.S. evaluated H_2O_2 levels utilizing DCFH and edited the manuscript. E.J.B., T.L.D. and S.S.S contributed to experimental design, analysis, interpretation and presentation of data, and edited the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

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