Published in final edited form as:

Sci Signal. ; 8(358): ra2. doi:10.1126/scisignal.2005659.

Localized TRPA1 channel Ca^{2+} signals stimulated by reactive oxygen species promote cerebral artery dilation

Michelle N. Sullivan¹, Albert L. Gonzales², Paulo W. Pires³, Allison Bruhl¹, M. Dennis Leo⁴, Wencheng Li¹, Agathe Oulidi³, Frederick A. Boop⁵, Yumei Feng¹, Jonathan H. Jaggar⁴, Donald G. Welsh⁶, and Scott Earley^{3,*}

¹Vascular Physiology Research Group, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA

²Department of Pharmacology, University of Vermont School of Medicine, Burlington, VT 05405, USA

³Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557–0318, USA

⁴Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA

⁵Department of Neurosurgery, University of Tennessee Health Science Center, Memphis, TN 38163, USA

⁶Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Abstract

Reactive oxygen species (ROS) can have divergent effects in cerebral and peripheral circulations. We found that Ca^{2+} -permeable transient receptor potential ankyrin 1 (TRPA1) channels were present and colocalized with NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase 2 (NOX2), a major source of ROS, in the endothelium of cerebral arteries but not in other vascular beds. We recorded and characterized ROS-triggered Ca^{2+} signals representing Ca^{2+} influx through single TRPA1 channels, which we called “TRPA1 sparklets.” TRPA1 sparklet activity was low under basal conditions but was stimulated by NOX-generated ROS. Ca^{2+} entry during a single TRPA1 sparklet was twice that of a TRPV4 sparklet and ~200 times that of an L-type Ca^{2+} channel sparklet. TRPA1 sparklets representing the simultaneous opening of two TRPA1 channels were more common in endothelial cells than in human embryonic kidney (HEK) 293 cells expressing TRPA1. The NOX-induced TRPA1 sparklets activated intermediate-conductance, Ca^{2+} -sensitive K^{+} channels, resulting in smooth muscle

*Corresponding author. searley@medicine.nevada.edu.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/8/358/ra2/DC1

Author contributions: S.E. conceived and designed the study. All authors performed experiments and analyzed data. M.N.S. and S.E. wrote the initial draft of the manuscript and prepared the figures. A.L.G., J.H.J., and D.G.W. provided comments on the manuscript and figures.

Competing interests: The authors declare that they have no competing interests.

hyperpolarization and vasodilation. NOX-induced activation of TRPA1 sparklets and vasodilation required generation of hydrogen peroxide and lipid-peroxidizing hydroxyl radicals as intermediates. 4-Hydroxy-nonenal, a metabolite of lipid peroxidation, also increased TRPA1 sparklet frequency and dilated cerebral arteries. These data suggest that in the cerebral circulation, lipid peroxidation metabolites generated by ROS activate Ca^{2+} influx through TRPA1 channels in the endothelium of cerebral arteries to cause dilation.

INTRODUCTION

Regulation of the cerebral circulation differs from that of the rest of the body to meet the metabolic demands and specific anatomical constraints of the brain. One example of this disparity is the observation that reactive oxygen species (ROS) such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) primarily cause vasodilation in the cerebral circulation and vasoconstriction in peripheral arteries (1). The NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase (NOX) family, consisting of five isoforms (NOX1 to NOX5), is a major source of ROS in the vasculature (2, 3). Although the generation of ROS by NOX is much greater in healthy cerebral arteries than in other vascular beds (4), the molecular mechanism responsible for translating these higher amounts of ROS into a qualitatively different vascular response is not known.

Potential candidate mediators of the vascular actions of ROS include Ca^{2+} -permeable members of the transient receptor potential (TRP) channel family, several of which are present in the vasculature and can be regulated by ROS and ROS-derived products (5–8), including the ankyrin TRP (TRPA) channel TRPA1. Initially characterized as a detector of noxious electrophilic substances in nociceptive and sensory neurons (9, 10), TRPA1 has since been shown to be present in mast cells, enterochromaffin cells, epithelial cells, and other tissues, suggesting a broader biological role for this channel. TRPA1 is present in the endothelium and mediates vasodilation of cerebral arteries in response to allyl isothiocyanate (AITC) (11), a pungent compound found in mustard oil. Endogenous regulators of TRPA1 activity in the endothelium are currently unknown. In vagal and sensory nerves, hypoxic and hyperoxic conditions can increase TRPA1 activity (12). Additional evidence indicates that TRPA1 in neurons can be activated by oxidative modification of cysteines in its cytoplasmic N terminus by ROS, including H_2O_2 and O_2^- (7, 13). TRPA1 is also activated by compounds produced by peroxidation of $\omega 6$ polyunsaturated fatty acids in the plasma membrane, such as 4-hydroxy-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE), and 4-hydroxy-hexenal (7, 14). 4-HNE and related substances are produced by hydroxyl radicals ($\text{OH}\bullet$) formed during degradation of H_2O_2 (15), suggesting that the oxidant and redox signaling mechanisms acting on TRPA1 could be linked by the lipid peroxidation process. This proposed signaling cascade has not been studied in vascular endothelium, and its effects on TRPA1 activity, endothelial function, and vasomotor responses have not been characterized.

Elementary Ca^{2+} influx events through single TRPV4 channels have been optically recorded from endothelial cells using total internal reflection fluorescence (TIRF) and confocal microscopy (16, 17). These events, termed “TRPV4 sparklets,” are fundamental signals

underlying endothelium-dependent dilation of mesenteric arteries (16). In theory, all Ca^{2+} -permeable TRP channels with sufficient conductance are capable of being detected optically as sparklets, with amplitude, frequency, and spatial spread reflecting the unitary conductance, Ca^{2+} permeability, and gating kinetics of the channel.

Previous studies are consistent with the possibility that TRPA1 channels are critical sensors of cellular oxidant and redox status. However, little is currently known about the relationship between ROS generation and TRPA1 channel activity in the endothelium. Moreover, it is not clear how the potential of these channels to serve as ROS sensors in the vasculature could account for differences in the effects of ROS between the peripheral and cerebral circulations. Here, we investigated how NOX-generated ROS could provoke vasodilation through activation of TRPA1 channels and sought to determine how this response differed between cerebral and peripheral arteries. We showed that TRPA1 colocalizes with NOX isoform 2 (NOX2) in the endothelium of cerebral arteries but not in other vascular beds. We further developed a method for recording and characterizing TRPA1 sparklets, demonstrating the presence of large-amplitude TRPA1 sparklets with unique properties in cerebral artery endothelial cells. Finally, we demonstrated that ROS generated by NOX increased TRPA1 sparklet frequency and dilated cerebral arteries through a process that requires the peroxidation of membrane lipids. Together, these data indicate that Ca^{2+} influx through TRPA1 channels elicits endothelium-dependent vasodilation in response to the generation of lipid peroxidation products, and this response is specific to the cerebral vasculature.

RESULTS

TRPA1 colocalizes with NOX2 in the endothelium of cerebral arteries but not in other vascular beds

We detected *TRPA1* mRNA by reverse transcription polymerase chain reaction (RT-PCR) in rat cerebral arteries but not in renal, coronary, or mesenteric arteries (Fig. 1A). To confirm this unusual distribution pattern, we mounted arteries en face and immunolabeled the endothelium with an antibody against TRPA1. TRPA1 protein was detected in rat cerebral endothelia, where it was abundant in perinuclear regions; it was also present within fenestrations of the internal elastic lamina (IEL), sites of close contact between the endothelial and smooth muscle cell plasma membranes known as myoendothelial projections (Fig. 1B, arrows, inset). TRPA1 immunolabeling was not detected in coronary, mesenteric, or renal arterial endothelium (Fig. 1B), suggesting that this channel was selectively present in the cerebral endothelia. Arteries from human subjects displayed the same TRPA1 distribution pattern. *TRPA1* mRNA was detected by RT-PCR in whole human cerebral arteries but not in isolated smooth muscle cells (Fig. 1C), and TRPA1 protein was detected by Western blotting in tissue homogenates of human cerebral arteries (Fig. 1D). *TRPA1* message was not detected by RT-PCR in RNA isolated from human coronary, renal glomerular, or neonatal dermal artery endothelial cells, but was found in endothelial cells from human cerebral arteries (Fig. 1E). These findings demonstrate that the presence of TRPA1 in the endothelium is a distinctive feature of the cerebral circulation.

To test our proposal that ROS and/or ROS-derived products are endogenous agonists of TRPA1 in the endothelium, we first examined cerebral arteries for the presence of NOX isoforms. NOX5 is not present in rats or mice, and NOX3 is found only in the inner ear (18); however, the other three isoforms are present in the rodent vasculature (19, 20). We detected three NOX isoforms—NOX1, NOX2, and NOX4—in the endothelium of cerebral arteries by immunolabeling (Fig. 1F and fig. S1). All three isoforms were present in perinuclear regions and IEL fenestrations, but NOX2 and NOX4 were more abundant in myoendothelial projections compared with NOX1 (Fig. 1F, insets, and fig. S2).

We used in situ proximity ligation assays (PLAs) (21) to investigate whether NOX2 and NOX4 colocalize with TRPA1 in the endothelium. PLAs for TRPA1:NOX2 produced abundant red puncta, indicating significant colocalization (< 40 nm) in cerebral artery endothelial cells (Fig. 1G). PLAs for TRPA1:NOX4 revealed few red puncta (Fig. 1G). Few puncta (about one to two per cell) were present when primary antibodies were omitted. We also used PLAs to assess TRPA1 and NOX colocalization in intact cerebral arteries (Fig. 1H). These assays revealed the presence of puncta throughout the endothelium, including in IEL fenestrations, in arteries probed with antibodies against NOX2 and TRPA1 (Fig. 1H and fig. S3). Our data indicate that TRPA1:NOX2 complexes are present in cerebral artery endothelial cells within myoendothelial junctions and in other regions. Coimmunoprecipitation for NOX2 and TRPA1 failed to detect direct interaction between the two proteins (fig. S4A). These data demonstrate that NOX2 and TRPA1 selectively colocalize but do not physically interact in the cerebral artery endothelium, suggesting that metabolites generated by NOX2 could regulate TRPA1 activity in this tissue.

ROS stimulate TRPA1 sparklet activity in cerebral artery endothelial cells

We studied changes in TRPA1 activity in cerebral artery endothelial cells in response to ROS by using TIRF microscopy to record subcellular Ca^{2+} signals representing Ca^{2+} influx through single TRPA1 channels, which we called TRPA1 sparklets. To initially characterize TRPA1 sparklets, we treated primary cerebral artery endothelial cells loaded with the Ca^{2+} indicator dye Fluo-4 AM with the TRPA1 agonist AITC. Very few events were seen under basal conditions, but AITC induced a concentration-dependent increase in sparklet frequency (Fig. 2, A and B, and movie S1). The half-maximal effective concentration (EC_{50}) for sparklet activation was $4.4 \mu\text{M}$ (Fig. 2B), similar to the previously reported EC_{50} for AITC-induced dilation of cerebral arteries (EC_{50} , $16.4 \mu\text{M}$) (11). The increase in sparklet frequency stimulated by AITC was not affected by depletion of intracellular stores with cyclopiazonic acid (fig. S5A) but was absent when cells were bathed in Ca^{2+} -free solution (fig. S5B), indicating that these signals are generated by influx of Ca^{2+} . The selective TRPA1 antagonist HC-030031 (22) blocked AITC-induced increases in sparklet frequency (Fig. 2C), confirming that the subcellular Ca^{2+} influx events activated by AITC were bona fide TRPA1 sparklets. Single-site analysis indicated that the total number of active TRPA1 sparklet sites increased from 1.4 ± 0.7 sites to 6.1 ± 1.8 sites after the addition of AITC (Fig. 2D), suggesting that AITC recruited previously inactive TRPA1 channels and did not increase the frequency of basally active sites. Further, these data showed that only about four to eight TRPA1 sparklet sites per cell were active during maximal stimulation.

We compared TRPA1 sparklets recorded from cerebral artery endothelial cells to those recorded from human embryonic kidney (HEK) 293 cells transfected with a TRPA1-GFP (green fluorescent protein) fusion protein. AITC caused an increase in TRPA1 sparklet frequency in transfected HEK cells, which was inhibited by the TRPA1 blocker HC-030031 and absent in untransfected HEK cells (fig. S6, A and B). Modal duration, attack time, decay time, and spatial spread of TRPA1 sparklets recorded from TRPA1-GFP-transfected HEK cells were essentially identical to those recorded from cerebral artery endothelial cells (table S1). Duration histograms indicated that the most frequently occurring TRPA1 sparklets lasted less than 200 ms and fit single exponential functions in endothelial cells ($\tau = 408$ ms; Fig. 2E) and transfected HEK cells ($\tau = 210$ ms; fig. S6C). Spatial spread distribution was also similar between TRPA1 sparklets recorded from endothelial cells and HEK cells, with most events having an area of less than $1 \mu\text{m}^2$ (Fig. 2E and fig. S6C).

A multiple Gaussian fit of a histogram of TRPA1 sparklet amplitudes recorded from transfected HEK cells (fig. S6C) indicated three distinct peaks of $F/F_0 = 1.13$, 1.26, and 1.39. These data suggest that the unitary TRPA1 sparklet amplitude is $F/F_0 = 0.13$, and the peaks represent the opening of one, two, or three individual TRPA1 channels, with the most frequently occurring TRPA1 sparklets ($F/F_0 = 1.13$) signifying the opening of a single TRPA1 channel. Identical results were obtained in endothelial cells ($F/F_0 = 1.13$, 1.26, 1.39), consistent with a unitary TRPA1 sparklet amplitude of $F/F_0 = 0.13$ in these native cells (Fig. 2E). This is also apparent from plots of fluorescence intensity over time for regions of interest (ROIs) with active TRPA1 sparklet sites, where three distinct amplitudes are seen (Fig. 2F and fig. S6D). Unlike TRPA1 sparklets in HEK cells, the most commonly occurring TRPA1 sparklets recorded from endothelial cells had an amplitude of $F/F_0 = 1.26$ ($F/F_0 = 0.26$), indicating that simultaneous opening of two TRPA1 channels at the same active site occurred much more frequently than expected. These data suggest that native TRPA1 channels exhibit binary coupled gating more frequently in cerebral artery endothelial cells compared with cloned TRPA1 channels expressed in HEK cells.

Colocalization of NOX2 and TRPA1 (Fig. 1H) in the cerebral artery endothelium supports the concept that NOX-derived ROS could regulate TRPA1 channel activity in this tissue. To test this hypothesis, NOX activity was stimulated by administration of NADPH (23). We found that enhanced NOX activity increased cerebral artery TRPA1 sparklet frequency in a concentration-dependent manner (Fig. 2, G and H) and that the TRPA1 blocker HC-030031 abolished this response (Fig. 2I). These findings indicate that NOX-derived ROS stimulate TRPA1 sparklet activity in cerebral artery endothelial cells, suggesting that TRPA1 is a ROS sensor in this tissue.

ROS generated by NOX dilate cerebral arteries by activating TRPA1

The effects of ROS-induced increases in TRPA1 sparklet frequency on vessel diameter were studied using intact cerebral arteries that were pressurized to physiological values (80 mmHg) to allow spontaneous myogenic tone to develop. Our data showed that the addition of NADPH to increase generation of ROS by NOX caused concentration-dependent vasodilation (Fig. 3, A and B). This response was abolished by the TRPA1 blocker

HC-030031 (Fig. 3, A and C), indicating that NOX-derived ROS bring about dilation of cerebral arteries by increasing the frequency of TRPA1 sparklets.

One mechanism by which the endothelium can promote arterial dilation is by releasing diffusible substances such as nitric oxide (NO) or prostacyclin (PGI₂). We found that NADPH-induced dilation of cerebral arteries was not altered by blocking NO and PGI₂ synthesis with L-N^G-nitroarginine (L-NNA) and indomethacin, respectively (Fig. 3, D and E), indicating that these pathways are not involved in the response. The endothelium can also cause dilation by direct electrotonic spread of endothelial cell membrane hyperpolarization through myoendothelial gap junctions to underlying vascular smooth muscle cells. Small-conductance and intermediate-conductance Ca²⁺-activated K⁺ (IK) channels are implicated in this form of endothelium-dependent vasodilation, and functional IK channels are present in cerebral artery endothelial cells at myoendothelial junctions (11, 24). Our data showed that intraluminal administration of the selective IK channel blocker TRAM34 nearly abolished NADPH-induced vasodilation (Fig. 3, F and G), suggesting that TRPA1 sparklets act through IK channels in the endothelium to dilate cerebral arteries.

To determine if increases in TRPA1 sparklet activity were associated with smooth muscle cell hyperpolarization, we recorded the membrane potential of arterial myocytes from intact, pressurized (80 mmHg) cerebral arteries using intracellular microelectrodes. We found that administration of NADPH to stimulate ROS generation hyperpolarized the membranes of smooth muscle cells by about -8 mV (Fig. 3, H and I). Blocking TRPA1 channels with HC-030031 abolished NADPH-induced membrane potential hyperpolarization (Fig. 3, H and I), demonstrating that NOX-derived ROS hyperpolarize smooth muscle cells in pressurized cerebral arteries by activating TRPA1. TRPA1 channels are not present in smooth muscle cells in cerebral arteries (Fig. 1C) (11, 25), suggesting that this response is mediated by the endothelium. Together, our findings suggest that Ca²⁺ influx through TRPA1 channels in the endothelium activates nearby IK channels to initiate K⁺ efflux. The ensuing hyperpolarization of the endothelial cell plasma membrane is conducted to underlying smooth muscle to hyperpolarize and relax that tissue, resulting in arterial dilation.

ROS-derived lipid peroxidation metabolites stimulate TRPA1 sparklets and dilate cerebral arteries

NOX-derived ROS could stimulate endothelial cell TRPA1 activity directly or through generation of lipid peroxidation products (Fig. 4A). We found that the NOX inhibitor apocynin attenuated NADPH-induced increases in TRPA1 sparklet frequency (Fig. 4B) and inhibited vasodilation in response to NADPH (Fig. 4C). These findings were supported by experiments showing that NADPH-induced increases in TRPA1 sparklet frequency and NADPH-induced dilation were attenuated by the NOX2 inhibitory peptide gp91ds-tat (26) but not by a scrambled control peptide (scr. gp91ds-tat) (Fig. 4, B and D). These data confirm that NADPH increases the generation of ROS by a NOX isoform (probably NOX2), resulting in increased TRPA1 sparklet frequency and vasodilation.

When present on the plasma membrane, NOX produces O₂⁻ in the extracellular space, which is rapidly dismutated to H₂O₂ by spontaneous or superoxide dismutase-catalyzed

reactions (Fig. 4A). We investigated whether H_2O_2 downstream of NOX was involved in the activation of TRPA1 by using catalase, a membrane-impermeable enzyme that rapidly degrades H_2O_2 . Catalase prevented NADPH-induced increases in TRPA1 sparklet frequency (Fig. 4B), indicating that extracellular generation of H_2O_2 is required for this response. Administration of NADPH in the presence of catalase constricted intact cerebral arteries (Fig. 4E), indicating that H_2O_2 is required for the vasodilatory response. Our data indicate that increases in endothelial cell TRPA1 sparklet activity and cerebral artery dilation in response to ROS generated by NOX require the generation of extracellular H_2O_2 .

In the presence of iron (Fe^{2+}), H_2O_2 is degraded to $OH^\bullet$ through the Fenton reaction. $OH^\bullet$ are highly unstable and rapidly react with polyunsaturated fatty acids in the plasma membrane to generate lipid peroxidation products such as 4-HNE. To distinguish between the direct effects of H_2O_2 on TRPA1 activity and by-products generated by $OH^\bullet$, we chelated iron with deferoxamine to inhibit the Fenton reaction and diminish the formation of $OH^\bullet$ and lipid peroxidation (15). Deferoxamine blocked NADPH-induced increases in TRPA1 sparklet frequency (Fig. 4B) and blunted NADPH-induced dilation of intact cerebral arteries (Fig. 4F), suggesting that generation of $OH^\bullet$ and lipid peroxidation are necessary for NOX-induced increases in TRPA1 sparklet activity and cerebral artery dilation.

These findings are consistent with the possibility that compounds generated by ROS-dependent peroxidation of polyunsaturated fatty acids such as 4-HNE and related compounds could serve as endogenous agonists of TRPA1 channels in cerebral arteries. These substances react with cysteine, histidine, and lysine residues to form stable protein adducts that are recognized by specific antibodies (27). To determine if lipid peroxidation metabolites are present, we probed intact cerebral arteries, mounted en face, with an antibody that binds to 4-HNE-modified proteins. These experiments revealed that 4-HNE-modified proteins were abundant in the cerebral artery endothelium (Fig. 5A), with immunolabeling present in perinuclear regions and within IEL fenestrations (Fig. 5A, arrows, inset) where NOX2, NOX4, and TRPA1 channels are also present. Coimmunoprecipitation experiments indicated association of TRPA1 and 4-HNE in cerebral arteries (fig. S4B).

To test our hypothesis that lipid peroxide metabolites activate TRPA1 channels in the endothelium, we examined the effects of endogenous administration of these substances on TRPA1 sparklet activity and cerebral artery diameter. Our findings show that exogenous administration of 4-HNE induced a concentration-dependent increase in TRPA1 sparklet frequency in cerebral artery endothelial cells (EC_{50} , 64.8 ± 50.4 nM) (Fig. 5C), which was blocked by the TRPA1 inhibitor HC-030031 (Fig. 5D). 4-HNE (Fig. 5E) also dilated intact cerebral arteries. 4-HNE-induced vasodilation was concentration-dependent and was blocked by HC-030031 (Fig. 5, F and G). 4-HNE stimulated TRPA1 sparklet activity at a much lower concentration than that required to cause vasodilation, possibly because the compound reacts with other cells in the isolated vessel preparation, reducing availability to the endothelium. Our findings indicate that the lipid peroxidation product 4-HNE can stimulate TRPA1 sparklets in the endothelium and dilate cerebral arteries.

ROS-derived lipid peroxidation products fail to dilate cerebral arteries from endothelial cell-specific *TRPA1*-knockout mice

We used endothelial cell-specific *TRPA1* knockout (*eTRPA1*^{-/-}) mice to further investigate the involvement of TRPA1 channels in ROS-induced dilation in the cerebral circulation. TRPA1 immunolabeling was detected in the endothelium of cerebral arteries from mice but not in the coronary, renal, or mesenteric arterial beds (fig. S7). TRPA1 was not detected in Western blots of whole cerebral arteries from *eTRPA1*^{-/-} mice (Fig. 6A), and immunolabeling studies demonstrated that TRPA1 was present in dorsal root ganglion (DRG) neurons but not in the endothelium of cerebral arteries of *eTRPA1*^{-/-} mice (Fig. 6B). AITC failed to increase the frequency of TRPA1 sparklets in cerebral artery endothelial cells from *eTRPA1*^{-/-} mice (Fig. 6C). Passive diameter, myogenic tone, and vasoconstriction in response to KCl did not differ between cerebral arteries from control or *eTRPA1*^{-/-} mice (table S2). However, administration of 4-HNE or NADPH failed to dilate intact pressurized cerebral arteries from *eTRPA1*^{-/-} mice (Fig. 6, D and E), demonstrating the critical involvement of TRPA1 channels in this response.

DISCUSSION

The brain requires continuous perfusion to provide a constant supply of oxygen and nutrients, but the enclosing skull tightly limits vascular distension, presenting unique challenges to the cerebral circulation. Among other factors, endothelial control of arterial diameter is critical for precise regulation of global blood flow to the brain and for matching regional flow to metabolic demand. The ability of the endothelium to rapidly detect changes in local oxidant and redox status and effect appropriate vasomotor responses is centrally important for this function but is poorly understood. The results of the current study provide strong evidence that TRPA1 channels in the endothelium sense ROS generated by NOX. The resulting increase in Ca²⁺ influx through TRPA1 channels causes vasodilation. This pathway appears to be present only in cerebral arteries, identifying a fundamental distinction in function between blood vessels in the brain and those in the periphery.

Our results show that elementary Ca²⁺ signals arising from the influx of Ca²⁺ through single TRPA1 channels (TRPA1 sparklets) cause endothelium-dependent dilation of cerebral vessels in response to ROS generation. Our data indicate that TRPA1 sparklets are very large Ca²⁺ influx events, with a unitary amplitude about twice that of a TRPV4 sparklet reported in our previous publication (17) ($F/F_0 = 0.13$ versus 0.06), consistent with the larger single-channel conductance and greater Ca²⁺ permeability of TRPA1 (10, 28–30). The signal mass of a TRPV4 sparklet was estimated to be ~100 times that of an L-type Ca²⁺ channel sparklet (31), suggesting that TRPA1 sparklets are at least ~200 times greater than L-type Ca²⁺ channel sparklets. We also showed that the most frequently occurring TRPA1 sparklets recorded from endothelial cells have amplitudes consistent with the simultaneous opening of two TRPA1 channels, doubling the amount of Ca²⁺ entering during a typical event. This coupled gating arrangement supports the concept that TRPA1 channels are present in a tight binary structure in the endothelial cell plasma membrane and that the opening of one of the channels in this pair triggers the adjacent channel, perhaps through binding of incoming Ca²⁺ to a Ca²⁺-sensing EF-hand domain on the N terminus (9).

Coupled gating of TRPA1 has not been previously described, and we showed that it occurred less frequently in HEK cells expressing TRPA1, suggesting that formation of binary structures is not an inherent property of the channel but may be mediated by adaptor or scaffolding proteins selectively expressed by the endothelium. It is possible that TRPA1 channels could also couple with Ca^{2+} -sensitive channels, such as TRPV4, but our TRPA1 sparklet data provide no evidence for such an arrangement. Our data also showed that only a few (about four to eight) TRPA1 sparklet sites are active per cell under conditions sufficient to induce maximal dilation of cerebral arteries. These findings support a scheme in which a large amount of Ca^{2+} entering the cell during individual TRPA1 sparklet events is sufficient to allow these few active sites to generate very high local Ca^{2+} concentration in subcellular domains, particularly within the confined space of myoendothelial projections where TRPA1 and IK channels are present (11). We propose that local increases in Ca^{2+} created by TRPA1 sparklets activate nearby IK channels in myoendothelial projections, either directly or indirectly through Ca^{2+} -induced Ca^{2+} release from inositol trisphosphate receptors (32). The resulting efflux of K^{+} hyperpolarizes the endothelial cell membrane, and the electrotonic spread of this influence through myoendothelial gap junctions subsequently hyperpolarizes the underlying smooth muscle to cause vasodilation (Fig. 6F) (33, 34).

ROS are involved in the control of cerebral endothelial cell function, vascular reactivity, and blood flow. NOX is a major ROS generator in the cerebral circulation (35, 36), and NOX abundance and basal O_2^{-} production are up to 120-fold higher in cerebral arteries than in the aorta and carotid, mesenteric, and renal arteries (4). Our data demonstrated that ROS generated by NOX stimulates TRPA1 activity in the cerebral endothelium. We also showed that NOX2 colocalizes with TRPA1 and that NOX-induced TRPA1 activation and vasodilation are blocked by the gp91ds-tat peptide, which is thought to be a specific inhibitor of NOX2 (26). These data provide evidence that generation of ROS by NOX2 increases TRPA1 activity in the cerebral artery endothelium. Our findings also indicate that NOX-derived O_2^{-} does not directly activate TRPA1 in the endothelium but requires the generation of H_2O_2 and $\text{OH}^{\bullet}$ intermediates. Immunolabeling studies presented here indicated that NOX2-, TRPA1-, and 4-HNE-modified proteins are abundant within IEL fenestrations. This arrangement suggests that localized generation of lipid peroxidation products within myoendothelial projections activates TRPA1 sparklets to elicit vasodilation, a concept supported by our data showing that 4-HNE increased TRPA1 sparklet activity in endothelial cells and dilated cerebral arteries. Together, these findings provide support for a signaling cascade in which O_2^{-} generated by NOX2 is converted to H_2O_2 and then to $\text{OH}^{\bullet}$, leading to peroxidation of membrane lipids. The resulting metabolites activate TRPA1 channels in the endothelium. Ca^{2+} influx through TRPA1 channels activates IK channels to hyperpolarize the endothelial and smooth muscle cell plasma membrane, resulting in arterial dilation (Fig. 6F).

Collectively, our data demonstrate that TRPA1 channels are central to a ROS-sensing signaling pathway unique to the cerebral circulation that causes endothelium-dependent vasodilation. The effects of NOX-derived ROS were investigated here, but it is also possible that other sources of ROS, such as mitochondrial respiration, could influence TRPA1 activity or that NOX-derived ROS could influence other ROS-sensitive channels, such as TRPM2 or TRPM7. NOX activity and ROS production are increased in cerebral arteries

during hypertension and other pathological conditions (37), suggesting that TRPA1 channels and the pathway we described here may provide some protection against cerebrovascular disease.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (300 to 400 g; Harlan) were deeply anesthetized with pentobarbital sodium (50 mg, intraperitoneal) and euthanized by exsanguination. Male and female mice (10 to 12 weeks old) were deeply anesthetized by isoflurane inhalation (3%) and euthanized by cervical dislocation followed by decapitation. Brains were isolated and placed in ice-cold Mops-buffered saline. Cerebral and cerebellar arteries were isolated from the brain, cleaned of connective tissue, and stored in Mops-buffered saline. The University of Nevada School of Medicine and Colorado State University Institutional Animal Care and Use Committees approved all animal procedures.

Generation of *eTRPA1*^{-/-} mice

eTRPA1^{-/-} mice were created by crossing mice homozygous for loxP-flanked TRPA1 S5/S6 transmembrane domain construct ("floxed TRPA1"; The Jackson Laboratory, 008650 B. 129-TRPA1^{tm2K_yK_w>/J}) and mice hemizygous for *Cre recombinase* under the control of the receptor tyrosine kinase *Tek* (Tie2) promoter/enhancer [The Jackson Laboratory, B6.Cg-*Tg(Tek-cre)*^{Ywa/J}]. *Tek* imparts consistent *Cre* expression exclusively in the endothelium during development and adulthood. F1 progeny positive for *Cre* were mated with homozygous floxed TRPA1 mice. F2 progeny were genotyped by PCR with genomic DNA obtained from ear or tail biopsies, and those positive for *Cre* and homozygous for floxed TRPA1 were crossed with homozygous floxed TRPA1 mice. *eTRPA1*^{-/-} mice are viable and fertile. Control mice were homozygous for floxed TRPA1 but negative for *Cre*.

RNA isolation and RT-PCR

For rat samples, total RNA was extracted and purified from the left main and septal coronary, renal interlobar, cerebral, cerebellar, and first- to fourth-order mesenteric arteries. For human samples, total RNA was isolated from human cerebral arteries or isolated smooth muscle cells, as well as primary human microvascular endothelial cells from neonate dermis and human brain microvascular endothelial cells; total RNA from human renal glomerular endothelial cells (4005) and human cardiac microvascular endothelial cells (6005) was obtained from ScienCell Research Laboratories. First-strand cDNA was synthesized, and PCR was performed using primer sets specific for rat *TRPA1*, rat *eNOS*, human *TRPA1*, or human *eNOS*. PCRs always included a template-free negative control. Approval to use the human tissues and cells was granted by the University of Calgary Institutional Review Board.

Immunohistochemistry

Immunohistochemistry was performed on arteries in the en face preparation as previously described (11). Briefly, arteries were isolated, cleaned of connective tissue, and cut open longitudinally. Tissue was fixed with 4% formaldehyde, then permeabilized and blocked

with a phosphate-buffered saline (PBS) solution containing 1% Triton X-100 and 2% bovine serum albumin (BSA). Arteries were incubated with primary antibody overnight at 4°C, then washed and incubated with a Texas red–conjugated secondary antibody for 2 hours at room temperature. Tissue was washed and mounted on a slide with UltraCruz Mounting Medium (Santa Cruz Biotechnology, sc-24941), which contains DAPI nuclear stain. Fluorescence images were obtained using a FluoView 1000 laser-scanning confocal microscope (Olympus).

Western blotting for human TRPA1

Human brain samples were obtained in accordance with the guidelines of the Declaration of Helsinki after obtaining approval from the University of Tennessee Health and Science Center review board and receiving written informed consent. A temporal lobe sample was obtained from an adolescent male who underwent a lobectomy and had no history of hypertension or stroke. The cerebral tissue was immediately placed in chilled Dulbecco's modified Eagle's medium for transportation. Human cerebral arteries were dissected from the sample within 1 to 2 hours of surgery and placed in ice-cold physiologic saline solution (PSS). Arteries were then transferred to chilled radioimmunoprecipitation assay (RIPA) buffer for lysis, after which SDS was added and samples were heated in a boiling water bath for 3 min. After protein estimation, samples were run on an SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, blocked with 5% milk, and incubated overnight with anti-TRPA1 antibody. The lower part of the same blot was cut and probed independently for actin. Membranes were washed with tris-buffered saline–Tween 20 buffer and incubated with secondary antibody for 1 hour. Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Western blotting for mouse TRPA1

Cerebral and cerebellar arteries from each mouse were isolated and snap-frozen in liquid nitrogen. Fifty microliters of RIPA lysis buffer (Pierce) containing a protease inhibitor cocktail (Calbiochem) was added to each artery sample and homogenized by sonication (20 × 2-s pulses) and mechanical disruption by a Fisher Scientific TissueMiser (10 s) on ice. Samples were then centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a new tube. Protein concentration for each sample was determined using a bicinchoninic acid (BCA) protein assay (Pierce). Ten nanograms of each protein sample was added to SDS sample buffer and heated at 70°C for 10 min. Immediately after denaturation, proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk, 1% BSA in PBS containing 0.1% Tween and 0.02% sodium azide (PBS-TA) for 30 min at room temperature on a rocker and then exposed to a rabbit anti-TRPA1 antibody (1:500, Alomone Labs) in 5% milk, 1% BSA (PBS-TA) overnight at room temperature on a rocker. The membrane was then washed with PBS-T 3 × 5 min and exposed to a goat anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:10,000; Invitrogen) in 5% milk, 1% BSA (PBS-T) for 2 hours at room temperature on a rocker. The membrane was then washed 5 × 5 min with PBS-T, incubated in SuperSignal ECL substrate (Pierce) for 1 to 3 min, and imaged. Protein amount was quantified using ImageJ software.

Coimmunoprecipitation

Cerebral and cerebellar arteries from each mouse were snap-frozen in liquid nitrogen. Fifty microliters of IP Lysis Buffer (Pierce) containing a protease inhibitor cocktail (Calbiochem) was added to each artery sample and homogenized using a Fisher Scientific TissueMiser (10 s) on ice. Samples were then centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a new tube. Protein concentration for each sample was determined using a BCA protein assay (Pierce). Coimmunoprecipitation was performed using a Dynabeads Protein G Immunoprecipitation Kit (Invitrogen) according to the manufacturer's protocol. Fifty microliters of Dynabeads was added to a tube and placed on the DynaMag-2 magnet to separate the beads from the solution. The solution was removed, and 200 μ l of antibody binding and washing buffer containing 2 μ g of rabbit anti-TRPA1 antibody (Alomone Labs) or goat anti-NOX2 antibody (Santa Cruz Biotechnology) was added to the beads. Tubes were incubated at room temperature on a rocker to allow antibodies to bind the beads for 10 min. The tube was then placed on the magnet to separate the bead-antibody complexes from the solution and unbound antibodies. Bead-antibody complexes were then washed with 200 μ l of antibody binding and washing buffer and again separated from the supernatant. Ten micrograms of protein was diluted in 100 μ l of immunoprecipitation buffer and added to the tube containing the bead-antibody complexes and incubated at room temperature for 10 min on a rocker. The tube was then placed on the magnet, and the supernatant was transferred to a clean tube for later use. The bead-antibody-antigen complexes were then washed three times with 200 μ l of washing buffer, resuspended in 100 μ l of washing buffer, transferred to a new tube, and placed on the magnet to remove the supernatant. The bead-antibody-antigen complexes were resuspended in 20 μ l of elution buffer, 7.5 μ l of 4 $\times$ SDS sample buffer, and 2.5 μ l of double-distilled water (ddH₂O). The supernatant and 10 μ g of input protein samples were diluted in 7.5 μ l of 4 $\times$ SDS sample buffer and ddH₂O to a total volume of 30 μ l. All three samples (pull-down, supernatant, and input) were heated at 70°C for 10 min. Samples were immediately used for SDS-PAGE and Western blotting, using a similar method as described above with an anti-TRPA1 antibody (1:500, Alomone Labs) or an anti-4-HNE antibody (1:1000, Abcam).

Cerebral artery endothelial cell isolation

Basilar arteries were cut into three segments. Each segment was then pinned onto a Silgard block, cut open longitudinally, and placed intima side down onto 35-mm microwell MatTek dishes coated with Matrigel containing 1 drop of supplemented medium. Tissue was incubated at 37°C, 6% CO₂ for 4 to 5 hours to allow adherence, and then additional medium was added. The medium was changed every 2 to 3 days. After 1 week, tissue was removed from culture, and migrated endothelial cells were allowed to proliferate. Endothelial cells were identified by their typical cobblestone-like appearance. RT-PCR and immunocytochemistry were used to confirm the presence of *eNOS* and *TRPA1* mRNA in the isolated cells.

TRPA1 sparklet recording and analysis

TIRF microscopy was performed essentially as previously described (17). Briefly, TIRFM recordings (3-ms exposure time) were acquired using a through-the-lens TIRF system built

around an inverted Olympus IX-70 microscope equipped with an Olympus PlanApo 60× oil-immersion lens (numeral aperture, 1.45) and an Andor iXon charge-coupled device camera. Cells in a physiological Hepes-buffered solution were loaded with Fluo-4 AM for 20 min at 37°C, 6% CO₂ in the dark and imaged. All experiments were performed at room temperature (22° to 25°C). Endothelial cells were superfused with Hepes-buffered solution containing AITC (30 μM), NADPH (10 μM), HC-030031 (10 μM), apocynin (30 μM), gp91ds-tat (1 μM), scr. gp91ds-tat (1 μM), catalase (500 U/ml), deferoxamine (100 μM), or 4-HNE (300 nM). Each recording was 1500 frames and ~30 to 60 s in length.

TIRF image data were processed using a custom algorithm implemented as a plugin (LC_Pro) for ImageJ software essentially as previously described (17). Location (x, y), amplitude, duration, attack time, decay time, and spatial spread were then calculated for each event. Fluorescence was calculated as F , the difference between peak fluorescence (F), and the local minimum fluorescence within each ROI. Duration was defined as the time interval at 50% maximum peak fluorescence. Attack and decay time were determined by the time interval from 50% peak-to-peak fluorescence (attack) or from peak fluorescence to 50% peak (decay). Spatial spread was calculated as the area of the maximum best-fit ellipse at 95% of the peak fluorescence of an event.

Isolated vessel experiments

Isolated vessel experiments were performed as previously described (11). Briefly, after being transferred to a vessel chamber (Living Systems), arterial segments were cannulated on a glass micropipette, secured with monofilament thread, pressurized to 20 mmHg with PSS, and superfused with warmed (37°C), aerated PSS to equilibrate for 15 min. Inner diameter was continuously monitored using video microscopy and edge-detection software (IonOptix). Viability of the tissue was assessed by exposing pressurized arteries (20 mmHg) to isotonic PSS containing 60 mM KCl. Arteries were allowed to equilibrate for an additional 15 min, then pressurized to 80 mmHg (rat) or 60 mmHg (mouse) and allowed to develop stable myogenic tone. A change in diameter in response to NADPH (10 μM), HC-030031 (10 μM), LNNA (300 μM), indomethacin (10 μM), TRAM-34 (1 μM), apocynin (30 μM), gp91ds-tat (1 μM), scr. gp91ds-tat (1 μM), catalase (750 U/ml), deferoxamine (100 μM), or 4-HNE (10 μM) was recorded. Passive diameter was determined by superfusing vessels with Ca²⁺-free PSS (no added Ca²⁺, 3 mM EGTA). Percent myogenic tone was calculated as the difference in active and passive diameter at 80 mmHg divided by the passive diameter and multiplied by 100. Percent dilation was calculated as the change in myogenic tone between baseline and treatment.

Proximity ligation assay

Colocalization of TRPA1 with either NOX2 or NOX4 in primary rat cerebral artery endothelial cells or intact, en face rat cerebral arteries was studied using an in situ PLA detection kit (Duolink, Olink Biosciences Inc.) (21), essentially as previously described (38). Cells and vessels were fixed with 4% formaldehyde for 10 or 20 min, respectively, at room temperature followed by a 2-hour fixation at 4°C. After being washed with PBS, cells or vessels were permeabilized with cold methanol (−80°C) or 1% Triton X-100, respectively, and incubated overnight in a 2% BSA blocking solution containing primary antibodies.

After incubation with primary antibodies, cells or vessels were washed in blocking solution followed by three 10-min washes with 5 ml of Duolink In Situ Wash Buffer A. Cells and vessels were incubated in a humidified chamber at 37°C for 1 hour with secondary anti-rabbit PLUS and anti-goat MINUS PLA probes and then washed three times (5 min each) in 5 ml of Wash Buffer A at room temperature. Samples were incubated in ligation-ligase solution for 30 min at 37°C in a humidified chamber and then washed three times (2 min each) in 5 ml of Wash Buffer A at room temperature. Last, samples were incubated in Amplification-Polymerase solution for 100 min at 37°C in a humidified chamber and then washed twice (2 min each) in 5 ml of Duolink In Situ Wash Buffer B. Cells were further washed in 1% Wash Buffer B for 1 min and mounted using Duolink In Situ Mounting Medium containing DAPI nuclear stain. Fluorescence images were obtained using a spinning disc confocal microscope (Andor) and a 100× oil-immersion objective. Positive signals (bright red puncta) were only generated when the two PLA probes were in close proximity (<40 nm). Excitation of fluorescent puncta was achieved at 543 nm, and autofluorescence of the cytosol was illuminated at 488 nm. Images were analyzed with Volocity imaging software (v6.0, Perkin-Elmer Inc.). Negative control experiments were performed by omitting primary antibodies or PLA probes; no positive signals were detected under these conditions. The density of positive puncta per cell was determined using an automated object-finding protocol in Volocity.

Smooth muscle cell membrane potential

Smooth muscle cell membrane potential recordings were performed in isolated, pressurized cerebral arteries as previously described (11). Briefly, cerebral arteries were isolated and pressurized to 80 mmHg. Smooth muscle cells were impaled through the adventitia with glass intracellular microelectrodes (tip resistance, 100 to 200 megohms). A WPI Intra 767 amplifier was used for recording membrane potential (E_m). Analog output from the amplifier was recorded using IonWizard software (sample frequency, 20 Hz). Criteria for acceptance of E_m recordings were (i) an abrupt negative deflection of potential as the microelectrode was advanced into a cell, (ii) stable membrane potential for at least 1 min, and (iii) an abrupt change in potential to ~0 mV after the electrode was retracted from the cell. Changes in smooth muscle membrane potential in response to NADPH (10 μ M) and HC-030031 (10 μ M) were assessed.

Data analysis and statistics

All data are means $\pm$ SE. Statistical analyses were performed, and graphs were constructed using SigmaPlot v11.0. Unpaired or paired t tests were used to compare two groups. Multiple groups were compared using one-way or two-way analysis of variance followed by a Student-Newman-Keuls post hoc test to ascertain statistical differences. A value of P 0.05 was considered statistically significant for all experiments. Histograms were constructed and fit to multiple Gaussian functions using OriginPro v8.5, and SigmaPlot was used to create the figures. Concentration-response curves were made by fitting data to a four-parameter logistic equation using SigmaPlot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. Hill-Eubanks for critical comments on the manuscript and editorial assistance.

Funding: This work was supported by the NIH [grants HL091905 (to S.E.), F31HL094145 (to A.L.G.), and HL067061, HL110347, and HL094378 (to J.H.J.)], an American Heart Association Postdoctoral Fellowship (to M.D.L.), an American Heart Association Scientist Development Grant (11SDG7360050 to Y.F.), an operating grant from the Canadian Institute of Health Research (to D.G.W.), and a Monfort Excellence Award from the Monfort Family Foundation (to S.E.).

REFERENCES AND NOTES

1. Paravicini TM, Sobey CG. Cerebral vascular effects of reactive oxygen species: Recent evidence for a role of NADPH-oxidase. *Clin Exp Pharmacol Physiol*. 2003; 30:855–859. [PubMed: 14678250]
2. Babior BM, Kipnes RS, Curnutte JT. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J Clin Invest*. 1973; 52:741–744. [PubMed: 4346473]
3. Miller AA, Drummond GR, Sobey CG. Reactive oxygen species in the cerebral circulation: Are they all bad? *Antioxid Redox Signal*. 2006; 8:1113–1120. [PubMed: 16910759]
4. Miller AA, Drummond GR, Schmidt HH, Sobey CG. NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res*. 2005; 97:1055–1062. [PubMed: 16210546]
5. Weissmann N, Sydykov A, Kalwa H, Storch U, Fuchs B, Mederos y Schnitzler M, Brandes RP, Grimminger F, Meissner M, Freichel M, Offermanns S, Veit F, Pak O, Krause KH, Schermuly RT, Brewer AC, Schmidt HH, Seeger W, Shah AM, Gudermann T, Ghofrani HA, Dietrich A. Activation of TRPC6 channels is essential for lung ischaemia–reperfusion induced oedema in mice. *Nat Commun*. 2012; 3:649. [PubMed: 22337127]
6. Zhang L, Papadopoulos P, Hamel E. Endothelial TRPV4 channels mediate dilation of cerebral arteries: Impairment and recovery in cerebrovascular pathologies related to Alzheimer's disease. *Br J Pharmacol*. 2013; 170:661–670. [PubMed: 23889563]
7. Andersson DA, Gentry C, Moss S, Bevan S. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci*. 2008; 28:2485–2494. [PubMed: 18322093]
8. Takahashi N, Kozai D, Kobayashi R, Ebert M, Mori Y. Roles of TRPM2 in oxidative stress. *Cell Calcium*. 2011; 50:279–287. [PubMed: 21616534]
9. Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, Meng ID, Julius D. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*. 2004; 427:260–265. [PubMed: 14712238]
10. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*. 2003; 112:819–829. [PubMed: 12654248]
11. Earley S, Gonzales AL, Crnich R. Endothelium-dependent cerebral artery dilation mediated by TRPA1 and Ca²⁺-activated K⁺ channels. *Circ Res*. 2009; 104:987–994. [PubMed: 19299646]
12. Takahashi N, Kuwaki T, Kiyonaka S, Numata T, Kozai D, Mizuno Y, Yamamoto S, Naito S, Knevels E, Carmeliet P, Oga T, Kaneko S, Suga S, Nokami T, Yoshida J, Mori Y. TRPA1 underlies a sensing mechanism for O₂. *Nat Chem Biol*. 2011; 7:701–711. [PubMed: 21873995]
13. Sawada Y, Hosokawa H, Matsumura K, Kobayashi S. Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. *Eur J Neurosci*. 2008; 27:1131–1142. [PubMed: 18364033]
14. Uchida K. 4-Hydroxy-2-nonenal: A product and mediator of oxidative stress. *Prog Lipid Res*. 2003; 42:318–343. [PubMed: 12689622]

15. Gutteridge JM, Richmond R, Halliwell B. Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem J.* 1979; 184:469–472. [PubMed: 230833]
16. Sonkusare SK, Bonev AD, Ledoux J, Liedtke W, Kotlikoff MI, Heppner TJ, Hill-Eubanks DC, Nelson MT. Elementary Ca^{2+} signals through endothelial TRPV4 channels regulate vascular function. *Science.* 2012; 336:597–601. [PubMed: 22556255]
17. Sullivan MN, Francis M, Pitts NL, Taylor MS, Earley S. Optical recording reveals novel properties of GSK1016790A-induced vanilloid transient receptor potential channel TRPV4 activity in primary human endothelial cells. *Mol Pharmacol.* 2012; 82:464–472. [PubMed: 22689561]
18. Mukherjee D, Jajoo S, Sheehan K, Kaur T, Sheth S, Bunch J, Perro C, Rybak LP, Ramkumar V. NOX3 NADPH oxidase couples transient receptor potential vanilloid 1 to signal transducer and activator of transcription 1-mediated inflammation and hearing loss. *Antioxid Redox Signal.* 2011; 14:999–1010. [PubMed: 20712533]
19. Görlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, Busse R. A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ Res.* 2000; 87:26–32. [PubMed: 10884368]
20. Ago T, Kitazono T, Kuroda J, Kumai Y, Kamouchi M, Ooboshi H, Wakisaka M, Kawahara T, Rokutan K, Ibayashi S, Iida M. NAD(P)H oxidases in rat basilar arterial endothelial cells. *Stroke.* 2005; 36:1040–1046. [PubMed: 15845888]
21. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gústafsdóttir SM, Ostman A, Landegren U. Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol.* 2002; 20:473–477. [PubMed: 11981560]
22. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM, Fanger CM. TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci USA.* 2007; 104:13525–13530. [PubMed: 17686976]
23. Didion SP, Faraci FM. Effects of NADH and NADPH on superoxide levels and cerebral vascular tone. *Am J Physiol Heart Circ Physiol.* 2002; 282:H688–H695. [PubMed: 11788419]
24. Hannah RM, Dunn KM, Bonev AD, Nelson MT. Endothelial SK_{Ca} and IK_{Ca} channels regulate brain parenchymal arteriolar diameter and cortical cerebral blood flow. *J Cereb Blood Flow Metab.* 2011; 31:1175–1186. [PubMed: 21179072]
25. Earley S, Gonzales AL, Garcia ZI. A dietary agonist of transient receptor potential cation channel V3 elicits endothelium-dependent vasodilation. *Mol Pharmacol.* 2010; 77:612–620. [PubMed: 20086034]
26. Rey FE, Cifuentes ME, Kiarash A, Quinn MT, Pagano PJ. Novel competitive inhibitor of NAD(P)H oxidase assembly attenuates vascular O_2^- and systolic blood pressure in mice. *Circ Res.* 2001; 89:408–414. [PubMed: 11532901]
27. Uchida K, Szweda LI, Chae HZ, Stadtman ER. Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. *Proc Natl Acad Sci USA.* 1993; 90:8742–8746. [PubMed: 8378358]
28. Nagata K, Duggan A, Kumar G, García-Añoveros J. Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *J Neurosci.* 2005; 25:4052–4061. [PubMed: 15843607]
29. Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J Biol Chem.* 2002; 277:47044–47051. [PubMed: 12354759]
30. Strotmann R, Schultz G, Plant TD. Ca^{2+} -dependent potentiation of the non-selective cation channel TRPV4 is mediated by a C-terminal calmodulin binding site. *J Biol Chem.* 2003; 278:26541–26549. [PubMed: 12724311]
31. Mercado J, Baylie R, Navedo MF, Yuan C, Scott JD, Nelson MT, Brayden JE, Santana LF. Local control of TRPV4 channels by AKAP150-targeted PKC in arterial smooth muscle. *J Gen Physiol.* 2014; 143:559–575. [PubMed: 24778429]
32. Qian X, Francis M, Solodushko V, Earley S, Taylor MS. Recruitment of dynamic endothelial Ca^{2+} signals by the TRPA1 channel activator AITC in rat cerebral arteries. *Microcirculation.* 2013; 20:138–148. [PubMed: 22928941]

33. Coleman HA, Tare M, Parkington HC. EDHF is not K^+ but may be due to spread of current from the endothelium in guinea pig arterioles. *Am J Physiol Heart Circ Physiol*. 2001; 280:H2478–H2483. [PubMed: 11356601]
34. Coleman HA, Tare M, Parkington HC. K^+ currents underlying the action of endothelium-derived hyperpolarizing factor in guinea-pig, rat and human blood vessels. *J Physiol*. 2001; 531:359–373. [PubMed: 11230509]
35. Touyz RM, Briones AM. Reactive oxygen species and vascular biology: Implications in human hypertension. *Hypertens Res*. 2011; 34:5–14. [PubMed: 20981034]
36. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. *Physiol Rev*. 2007; 87:245–313. [PubMed: 17237347]
37. Paravicini TM, Chrissobolis S, Drummond GR, Sobey CG. Increased NADPH-oxidase activity and Nox4 expression during chronic hypertension is associated with enhanced cerebral vasodilatation to NADPH in vivo. *Stroke*. 2004; 35:584–589. [PubMed: 14739416]
38. Gonzales AL, Yang Y, Sullivan MN, Sanders L, Dabertrand F, Hill-Eubanks DC, Nelson MT, Earley S. A $PLC\gamma 1$ -dependent, force-sensitive signaling network in the myogenic constriction of cerebral arteries. *Sci Signal*. 2014; 7:ra49. [PubMed: 24866019]

Fig. 1. TRPA1 colocalizes with NOX2 in the endothelium of cerebral arteries but not in other vascular beds

(A) RT-PCR for *TRPA1* (A1) and *eNOS* (+) mRNA in rat coronary (C), renal (R), mesenteric (M), and cerebral (CA) arteries. NTC, no complementary DNA (cDNA) template control ($n = 2$ rats). (B) Top: Immunolabeling for TRPA1 in coronary, renal, mesenteric, and cerebral arteries; scale bar, 10 μ m. Autofluorescence of the IEL is green. TRPA1 protein (red) was only detected in the endothelium of cerebral arteries and was present within myoendothelial projections (arrows, inset; scale bar, 5 μ m). Bottom: Immunolabeling was

not detected when the primary antibody was omitted. *n* = 3 rats. **(C)** RT-PCR for *TRPA1* mRNA in whole human cerebral arteries (CA) and smooth muscle cells isolated from human cerebral arteries (SMC) (*n* = 3 independent biological replicates). **(D)** Western blot for TRPA1 in human cerebral arteries (CA) (*n* = 2 independent biological replicates). **(E)** RT-PCR detection of human *TRPA1* (A1) and *eNOS* (+) mRNA in primary coronary (C), renal (R), dermal (D), and cerebral artery (CA) endothelial cells. *TRPA1* mRNA was detected only in CA. Representative of three independent experiments. **(F)** Top: Cerebral arteries immunolabeled for NOX1 (left), NOX2 (middle), or NOX4 (right) (red); scale bars, 10 μ m. NOX2 and NOX4 are more abundant within myoendothelial projections compared with NOX1 (arrows, insets; bars, 5 μ m). Endothelial cell nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), and IEL autofluorescence is green. Bottom: No primary antibody control. *n* = 3 rats per group. **(G)** PLA experiments for TRPA1:NOX4 (top) and TRPA1:NOX2 (bottom) in cerebral artery endothelial cells. Puncta corresponding to positive PLA results are red, cellular autofluorescence is green, and DAPI-stained nuclei are blue. Puncta density is summarized at the right (*n* = 4 cells per group, 3 rats); **P* = 0.05 versus TRPA1:NOX4. **(H)** PLA for TRPA1:NOX4 (top) and TRPA1:NOX2 (bottom). TRPA1:NOX2 puncta within IEL fenestrations are indicated by arrows. *, an example magnified and shown in cross section in the insets. Puncta density is summarized at the right (*n* = 8 to 14 vessels per group, 3 rats); **P* = 0.05 compared with TRPA1:NOX4.

Fig. 2. ROS stimulate TRPA1 sparklets in cerebral artery endothelial cells

(A) Time-lapse image of an AITC-induced TRPA1 sparklet recorded from a cerebral artery endothelial cell; scale bar, 8 μm . (B) AITC induces a concentration-dependent increase in TRPA1 sparklet frequency in cerebral artery endothelial cells ($n = 5$ to 52 cells per concentration, 4 independent cell isolations). (C) Summary data showing that HC-030031 inhibits AITC-induced increases in TRPA1 sparklet frequency in cerebral artery endothelial cells ($n = 10$ to 24 cells, 3 independent cell isolations); $*P < 0.05$ compared to control at baseline. (D) Active TRPA1 sparklet sites per cell before and after administration of AITC ($n = 10$ cells, 5 rats). (E) Amplitude (left), duration (middle), and spatial spread (right) histograms for TRPA1 sparklets ($n = 762$ total events, 43 independent experiments). (F) Representative recordings of change in fluorescence (F/F_0) within an ROI on primary cerebral artery endothelial cells stimulated by AITC. Dotted lines indicate the opening of one, two, or three TRPA1 channels. (G) Time-lapse image of a TRPA1 sparklet stimulated by NADPH; scale bar, 8 μm . (H) The NOX substrate NADPH induced a concentration-dependent increase in TRPA1 sparklet frequency ($n = 9$ to 22 cells per concentration, 4 independent cell isolations). (I) Summary data indicating that HC-030031 inhibits NADPH-induced increases in TRPA1 sparklet frequency ($n = 12$ to 28 cells per group, 3 independent cell isolations); $*P < 0.05$ compared with baseline, control.

Fig. 3. ROS generated by NOX dilate cerebral arteries by activating TRPA1

(A) Representative recordings of the intraluminal diameter of an intact, pressurized cerebral artery over time. Introduction of NADPH to the bathing solution induced vasodilation, which was nearly abolished by the TRPA1 blocker HC-030031. (B) NADPH-induced vasodilation is concentration-dependent ($n = 3$ vessels per concentration, 3 rats). (C) Summary data indicating that NADPH-induced dilation is attenuated by HC-030031 ($n = 5$ vessels, 3 rats); $*P < 0.05$ compared to control. (D and E) Representative recordings (D) and summary data (E) indicating that the NO synthase inhibitor L-NNA and the cyclooxygenase inhibitor indomethacin do not affect NADPH-induced vasodilation ($n = 3$ vessels, 3 rats). (F and G) Representative recordings (F) and summary data (G) showing that NADPH-induced vasodilation (left) is inhibited when the IK channel blocker TRAM34 is present in the lumen (right) ($n = 5$ vessels, 3 rats); $*P < 0.05$ compared with control. (H) Representative recordings of smooth muscle cell membrane potential (E_m) in a pressurized cerebral artery.

Smooth muscle cells were hyperpolarized by NADPH. NADPH-induced hyperpolarization was blocked by HC-030031. (I) Summary data ($n = 4$ vessels, 4 rats); $*P < 0.05$ compared with vehicle, control; $\#P < 0.05$ compared with vehicle, NADPH.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. ROS-derived lipid peroxidation metabolites stimulate TRPA1 sparklets and dilate cerebral arteries

(A) Proposed pathway and pharmacological interventions for activation of TRPA1 by NOX-generated ROS metabolites. (B) NADPH-induced increases in TRPA1 sparklet frequency in endothelial cells at baseline ($n = 27$ to 60 cells per group, 5 independent cell isolations) were attenuated by inhibition of NOX with apocynin ($n = 8$ to 10 cells per group, 3 independent cell isolations) and gp91ds-tat ($n = 7$ to 9 cells per group, 3 independent cell isolations) compared to vehicle and a scrambled peptide (scr. gp91ds-tat), respectively; H_2O_2 degradation with extracellular catalase ($n = 9$ to 11 cells per group, 3 independent cell isolations); and iron chelation with deferoxamine ($n = 8$ to 12 cells per group, 3 independent cell isolations); $*P < 0.05$ compared with baseline, control. (C to F) Representative traces and summary data indicating inhibition of NADPH-induced vasodilation by apocynin ($n = 5$

vessels, 3 rats) (C), gp91ds-tat ($n = 5$ vessels, 3 rats) (D), catalase ($n = 5$ vessels, 3 rats) (E), and deferoxamine ($n = 5$ vessels, 3 rats) (F); $*P < 0.05$ compared with vehicle control or scr. gp91ds-tat.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Lipid peroxidation products activate TRPA1 sparklets in endothelial cells and dilate cerebral arteries

(A) Cerebral artery mounted en face and immunolabeled for 4-HNE–modified proteins (red, top). 4-HNE labeling was present in perinuclear regions and within IEL fenestrations (arrows, inset; scale bar, 5 μ m). Immunolabeling was not detected in the absence of primary antibody (bottom); scale bar, 10 μ m. Endothelial cell nuclei are stained with DAPI (blue), and autofluorescence of the IEL is green ($n = 3$ rats). (B) Time-lapse image of a 4-HNE–induced TRPA1 sparklet; scale bar, 8 μ m. (C) 4-HNE–induced increases in TRPA1 sparklet frequency are concentration-dependent ($n = 9$ to 11 cells per concentration, 3 rats). (D) 4-HNE–induced increases in TRPA1 sparklet frequency are abolished by the TRPA1 blocker HC-030031 ($n = 19$ to 31 cells, 4 rats); * $P < 0.05$ compared with baseline, control. (E) Representative recording of 4-HNE–induced vasodilation of a pressurized cerebral artery. 4-HNE–induced dilation (left) was inhibited by HC-030031 (right). (F) Concentration-response curve for 4-HNE–induced dilation in cerebral arteries ($n = 3$ to 5 vessels per group, 5 rats). (G) Summary data indicating that 4-HNE–induced dilation is abolished by HC-030031 ($n = 5$ vessels, 4 rats); * $P < 0.05$ compared with control.

Fig. 6. ROS-derived lipid peroxidation products fail to dilate cerebral arteries from endothelial cell-specific *TRPA1*-knockout mice

(A) Western blot for TRPA1 protein (138 kD) in cerebral arteries from control and *eTRPA1*^{-/-} mice (*n* = 3 mice per group). (B) TRPA1 immunolabeling in the cerebral endothelium (top) and DRG neurons (bottom) from control and *eTRPA1*^{-/-} mice (*n* = 3 mice per group). (C) Summary data indicating the effect of AITC on the frequency of sparklets recorded from endothelial cells isolated from control compared with *eTRPA1*^{-/-} mice (*n* = 20 cells per group, 3 independent cell isolations per group). (D and E) 4-HNE (D) and NADPH (E) dilate cerebral arteries from control but not *eTRPA1*^{-/-} mice (*n* = 5 vessels per group, 3 mice per group); **P* < 0.05 compared with control. (F) Proposed signaling pathway: In endothelial cells (EC), NOX generates O₂⁻, which is rapidly dismutated to H₂O₂. In the presence of iron, H₂O₂ undergoes the Fenton reaction to yield OH•. Oxidation of membrane lipids by OH• generates lipid peroxidation products (LPP), which activate TRPA1 sparklets through binary-coupled TRPA1 channels. Ca²⁺ domains created by TRPA1 sparklets stimulate outward K⁺ currents through IK channels to hyperpolarize the EC plasma membrane (*E_m*). Electrotonic spread of EC hyperpolarization through myoendothelial gap junctions (MEGJ) causes smooth muscle cell (SMC) hyperpolarization and vasodilation.