Endothelium-Dependent Cerebral Artery Dilation Mediated by TRPA1 and Ca\(^{2+}\)-Activated K\(^{+}\) Channels

Scott Earley, Albert L. Gonzales, Rachael Crnich

Abstract—Although it is well established that changes in endothelial intracellular [Ca\(^{2+}\)] regulate endothelium-dependent vasodilatory pathways, the molecular identities of the ion channels responsible for Ca\(^{2+}\) influx in these cells are not clearly defined. The sole member of the ankyrin (A) transient receptor potential (TRP) subfamily, TRPA1, is a Ca\(^{2+}\)-permeable nonselective cation channel activated by electrophilic compounds such as acrolein (tear gas), allicin (garlic), and allyl isothiocyanate (AITC) (mustard oil). The present study examines the hypothesis that Ca\(^{2+}\) influx via TRPA1 causes endothelium-dependent vasodilation. The effects of TRPA1 activity on vascular tone were examined using isolated, pressurized cerebral arteries. AITC induced concentration-dependent dilation of pressurized vessels with myogenic tone that was accompanied by a corresponding decrease in smooth muscle intracellular [Ca\(^{2+}\)]. AITC-induced dilation was attenuated by disruption of the endothelium and when the TRPA1 channel blocker HC-030031 was present in the arterial lumen. TRPA1 channels were found to be present in native endothelial cells, localized to endothelial cell membrane projections proximal to vascular smooth muscle cells. AITC-induced dilation was insensitive to nitric oxide synthase or cyclooxygenase inhibition but was blocked by luminal administration of the small and intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) channel blockers apamin and TRAM34. BaCl\(_2\), a blocker of inwardly rectifying K\(^{+}\) channels, also inhibited AITC-induced dilation. AITC-induced smooth muscle cell hyperpolarization was blocked by apamin and TRAM34. We conclude that Ca\(^{2+}\) influx via endothelial TRPA1 channels elicits vasodilation of cerebral arteries by a mechanism involving endothelial cell Ca\(^{2+}\)-activated K\(^{+}\) channels and inwardly rectifying K\(^{+}\) channels in arterial myocytes. (Circ Res. 2009;104:987-994.)

Key Words: TRP channels • EDHF • AITC • inwardly rectifying K\(^{+}\) channels

Changes in endothelial intracellular [Ca\(^{2+}\)] regulate endothelium-dependent vasodilation through diverse signaling pathways. Production of potent vasoactive substances such as nitric oxide (NO)\(^1\) and prostacyclin (PGI\(_2\)) are stimulated by increases in intracellular [Ca\(^{2+}\)]. In addition, small (K\(_{Ca2.3}\)) and intermediate (K\(_{Ca3.1}\)) conductance Ca\(^{2+}\)-activated K\(^{+}\) channels hyperpolarize the endothelial cell plasma membrane and influence smooth muscle excitability, vascular tone, and arterial blood pressure by direct electric communication via myoendothelial gap junctions\(^3,4\) or release of K\(^{+}\) ions.\(^5\) Despite the impact of endothelial cell Ca\(^{2+}\) mobilization and dynamics on critical aspects of vascular function, the molecular identities and regulation of Ca\(^{2+}\) entry channels present in these cells are poorly understood. Further characterization of the ion channels responsible for endothelial cell Ca\(^{2+}\) influx is expected to provide critical insight into the nature of endothelium-dependent vasodilation.

Ca\(^{2+}\)-permeable ion channels belonging to the transient receptor potential (TRP) superfamily\(^6\) are present in vascular endothelial cells and likely play a major role in Ca\(^{2+}\)-dependent signaling processes.\(^7-9\) The sole member of the ankyrin (A) TRP subfamily, TRPA1,\(^10\) is a Ca\(^{2+}\)-permeable nonselective cation channel that is activated by electrophilic compounds such as acrolein (an active component of tear gas), allicin (found in garlic), and allyl isothiocyanate (AITC) (derived from mustard oil).\(^11,12\) Unsaturated aldehydes produced endogenously in response to oxidative stress, such as 4-hydroxy-2-nonenal (4-HNE),\(^13,14\) 4-oxo-nonenal (4-ONE),\(^13\) and 4-hydroxyhexenal (4-HHE),\(^13\) also activate TRPA1. TRPA1 is expressed by a subset of nociceptive sensory neurons and mediates inflammatory pain in response to stimuli such as topical administration of chemical irritants\(^15\) and inhalation of cigarette smoke.\(^16\) TRPA1 is also present in nonneuronal tissues such as basal urothelial cells\(^17\) and prostate epithelial cells,\(^18\) although the functional significance of the channel in these tissues is not known.

The effects of TRPA1 activity on endothelium-dependent dilation of cerebral resistance arteries was investigated. We find that endothelial cell TRPA1 channels mediate vasodilation by a novel pathway involving Ca\(^{2+}\)-activated K\(^{+}\) channels in endothelial cells and inwardly rectifying K\(^{+}\) (K\(_{IR}\)) channels in arterial myocytes.

Materials and Methods
Cerebral and cerebellar arteries used for these studies were isolated from male Sprague–Dawley rats (250 to 350 g; Harlan, Indianapolis, Ind). All animal use procedures were in accordance with institutional...
guidelines and approved by the Institutional Animal Care and Use Committee of Colorado State University.

For isolated vessel experiments, arteries were cannulated, pressurized with physiological saline solution, and superfused with aerated physiological saline solution at 37°C. To monitor changes in vessel wall [Ca\textsuperscript{2+}] (representative of smooth muscle intracellular [Ca\textsuperscript{2+}]), arteries were loaded with the ratiometric Ca\textsuperscript{2+} indicator dye fura-2/AM from the abluminal surface. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix). Pressurized vessels were intermittently excited with UV light at 340 and 380 nm (10 Hz), and deep red wavelength emissions were recorded using a photomultiplier tube and expressed as the ratio of emissions during 340 nm excitation versus 380 nm excitation (340/380 ratio). For some experiments, endothelial cell function was disrupted by passage of air and distilled water through the vessel lumen. AITC-induced dilation was found to be very reproducible (Figure I in the online data supplement, available at http://circres.ahajournals.org), allowing all experiments examining the effects of endothelial disruption, NO synthase (NOS) and cyclooxygenase (COX) inhibition, or block of K\textsuperscript{+} channels on vasodilation to be performed and analyzed using a paired design. Smooth muscle cell membrane potential was recorded in pressurized (70 mm Hg) cerebral arteries with intracellular microelectrodes using previously described methods.\textsuperscript{19,20}

To assess TRPA1 mRNA expression in vascular tissue, enzymatically dispersed native endothelial cells were visually identified using phase-contrast microscopy and were collected using a micro-manipulator controlled pipette. Total RNA was isolated from these cells and RT-PCR was performed using TRPA1-specific primers yielding a product of 500 bp. RT-PCR was performed using RNA isolated from 3 animals.

Immunostaining for TRPA1 was performed using intact cerebral arteries with the endothelium exposed by cutting the vessel lengthwise and pinning the tissue to a Silgard block. Fixed tissue was probed with anti-TRPA1 (Santa Cruz Biotechnology: 1:1000), anti-K\textsubscript{Ca3.1}, and/or anti-K\textsubscript{Ca2.3} (both Alomone, 1:1000) overnight at 4°C. Arteries were probed with fluorescent secondary antibodies (Texas Red, Santa Cruz Biotechnology, 1:500; or Alexa 633, Molecular Probes, 1:500), and immunofluorescence was detected using a Zeiss LSM 510 Meta laser scanning confocal microscope. Immunofluorescence was not detected in tissues probed with secondary antibodies alone.

An expanded Materials and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Results

TRPA1 Agonists Dilate Cerebral Arteries by an Endothelium-Dependent Mechanism

To investigate the effects of TRPA1 channel activity on vasomotor tone of cerebral arteries, changes in luminal diameter and vessel wall [Ca\textsuperscript{2+}] were recorded when the TRPA1 agonist AITC was present in the bathing solution. For these experiments, fura-2/AM was administered to the abluminal surface, preferentially loading vascular smooth muscle cells.\textsuperscript{22} Arteries were pressurized to 70 mm Hg, and spontaneous myogenic tone was allowed to develop. AITC administration resulted in robust, persistent vasodilation that was accompanied by a corresponding decrease in vessel wall [Ca\textsuperscript{2+}] (Figure 1A). Arteries contracted to their original diameter when AITC was removed from the bathing solution (Online Figure I), and subsequent AITC administration elicited equivalent dilator responses (Online Figure I), demonstrating that TRPA1-mediated vasodilation is reversible and reproducible. AITC elicited statistically significant vasodilation and reduction in vessel wall [Ca\textsuperscript{2+}] at concentrations as low as 3 \textmu mol/L, and induced maximal changes in luminal diameter at a concentration of 30 to 100 \textmu mol/L (Figure 1B and 1C). The half-maximal effective concentration (EC\textsubscript{50}) for vasodilation was 16.4 \textmu mol/L (Figure 1D). These findings show that activation of TRPA1 channels causes dilation of preconstricted cerebral arteries by reducing smooth muscle intracellular [Ca\textsuperscript{2+}]. During myogenic constriction, voltage-dependent Ca\textsuperscript{2+} channels are the primary Ca\textsuperscript{2+} influx pathway in cerebral artery myocytes.\textsuperscript{22} Thus, these findings suggest that TRPA1 activity elicits dilation by hyperpolarizing the membrane potential of vascular smooth muscle cells.

Activation of TRPA1 channels in any of the cell types within the vascular wall, including smooth muscle, endothelial cells, or perivascular nerve terminals, could be responsible for AITC-induced vasodilation. To determine whether the endothelium contributes to TRPA1-dependent vasodilation, endothelial cell function was disrupted by briefly perfusing isolated cerebral arteries with distilled water followed by air. Vasodilation and changes in vessel wall [Ca\textsuperscript{2+}] in response to AITC were recorded before and after endothelial cell disruption. Damage to the endothelium resulted in impaired AITC-induced vasodilation (Figure 2A and 2B), demonstrating that endothelial cells are involved in arterial dilation associated
with TRPA1 channel activation. In addition, AITC administration did not cause significant changes in vessel wall [Ca$^{2+}$] in endothelium-disrupted arteries, suggesting that this agonist has no direct effect on vascular smooth muscle cells.

A recently described blocker of TRPA1 channels, HC-030031,23 was used to further probe the role of TRPA1 channels in endothelium-dependent vasodilation. The half-maximal inhibitory concentration (IC$_{50}$) of HC-030031 for AITC-induced TRPA1 currents in patch clamp studies is reportedly 0.7 ± 0.1 µmol/L.23 HC-030031 is selective for TRPA1 channels and does not inhibit TRPV1, TRPV3, TRPV4, hERG, or NaV1.2 channel activity in the concentration range used for the current study (IC$_{50}$ for these channels >10 to 20 µmol/L).23 To block endothelial cell TRPA1 channels, HC-030031 (3 µmol/L) was administered to the lumen of isolated cerebral arteries. In paired experiments, AITC-induced dilation was impaired in the presence of luminal HC-030031 (3 µmol/L) (Figure 2D), demonstrating that activation of TRPA1 channels in vascular endothelial cells causes vasodilation of cerebral arteries. Interestingly, luminal administration of HC-030031 also caused a small, yet statistically significant increase in myogenic tone (Online Figure II). These data suggest that endothelial TRPA1 channels support a tonic vasodilatory influence that is independent of exogenous TRPA1 activators.

TRPA1 Channels Are Present in Cerebral Artery Endothelial Cells

Endothelial cell damage (Figure 2B) and luminal administration of the TRPA1 blocker HC-030031 inhibits AITC-induced vasodilation. A, Vasodilation in response to AITC (100 µmol/L) before and after disruption of endothelial cell function. B, Summary data of the effects of endothelium disruption on AITC-induced vasodilation (n=3). *P<0.05 vs control. C, Vasodilation in response to AITC (100 µmol/L) before and after luminal administration of the TRPA1 blocker HC-030031 (3 µmol/L). D, Summary data for the effects of luminal HC-030031 on AITC-induced vasodilation (n=5). *P<0.05 vs control.

Figure 2. Endothelial cell TRPA1 channels mediate AITC-induced vasodilation. A, Vasodilation in response to AITC (100 µmol/L) before and after disruption of endothelial cell function. B, Summary data of the effects of endothelium disruption on AITC-induced vasodilation (n=3). *P<0.05 vs control. C, Vasodilation in response to AITC (100 µmol/L) before and after luminal administration of the TRPA1 blocker HC-030031 (3 µmol/L). D, Summary data for the effects of luminal HC-030031 on AITC-induced vasodilation (n=5). *P<0.05 vs control.

Figure 3. TRPA1 is present in endothelial cells isolated from rat cerebral arteries. A, RT-PCR for TRPA1 using total RNA from freshly isolated rat cerebral artery endothelial cells (EC). NT indicates no template control; -RT, no reverse transcriptase control. Data are representative of RNA isolated from 3 rats. B through H, Localization of TRPA1 and KCa3.1 channels in cerebral artery endothelial cell membrane projections. Images show immunostaining for TRPA1 (red) (C through E) and KCa3.1 (red) (F through H). The level of the IEL is shown in green. Black holes (arrow) in the IEL indicate endothelial cell membrane projections in the direction of vascular smooth muscle cells (C and F). Superimposed images demonstrate that TRPA1 channels and KCa3.1 channels are abundant in the holes in the IEL (E and H). Bar=20 µm. I, Z-stack image showing projection of TRPA1 immunostaining though the IEL. Bar=10 µm. J through L, Coimmunostaining for TRPA1 and KCa3.1 in cerebral artery endothelial cell membrane projections. The level of the IEL is shown in green (J). Black holes (arrow) in the IEL indicate endothelial cell membrane projections in the direction of vascular smooth muscle cells. J, Immunostaining for TRPA1 (red). K, Immunostaining for KCa3.1 (blue). L, Superimposed images demonstrate that TRPA1 channels and KCa3.1 channels colocalize in black holes in the IEL. Bar=25 µm. All immunostaining data are representative of arteries isolated from at least 3 animals.
TRPA1-Mediated Vasodilation Requires Endothelial KCa and Smooth Muscle KIR Channels

Stimulation of TRPA1 activity causes endothelium-dependent vasodilation. Pharmacological inhibition of the NOS and COX pathways was used to examine their contribution to TRPA1-induced vasodilation. Superfusion of arteries with the NOS inhibitor N-nitro-L-arginine (L-NNA) (300 µmol/L, 20 minutes) or a combination of L-NNA and the COX inhibitor indomethacin (10 µmol/L, 15 minutes) caused an increase in basal myogenic tone (myogenic tone: 19.4±4.4% under control condition; 25.0±7.6% in the presence of L-NNA; 27.4±10.0% in the presence of L-NNA+indomethacin; n=5). AITC-induced vasodilation was not diminished by NOS or COX inhibition (Online Figure V), demonstrating that activation of TRPA1 channels in the endothelium causes vasodilation by a pathway that is independent of NO and PGI2 production. These data do not directly address the effects of AITC on NOS and COX activity.

Ca2+-activated K+ channels are present in vascular endothelial cells, and activation of these channels can cause vasodilation.34,26 To determine whether TRPA1-dependent Ca2+ influx elicits dilation by activating these channels, the KCa3.1 inhibitor TRAM34 (1 µmol/L) or a combination of TRAM34 and the KCa blocker apamin (1 µmol/L) was administered to the lumen of cerebral arteries that had been depolarized by the IEL (Figure 3H). TRAM34 and the SKCa blocker apamin (1 µmol/L) significantly decreased AITC-induced vasodilation (n=5). *P<0.05 vs control. D, Representative recording of vasodilation in response to AITC (10 µmol/L) in the presence of the KCa blocker BaCl2 (30 µmol/L). E, Summary of the effects of BaCl2 on AITC-induced vasodilation (n=5). *P<0.05 vs control.

in response to a stimulus that hyperpolarizes vascular smooth muscle cells. These data show that Ca2+ influx via endothelial TRPA1 stimulates SKCa/KCa3.1 channels, causing K+ efflux and vasodilation. Selective activation of SKCa/KCa3.1 versus NOS and COX pathways in response to AITC is in agreement with the hypothesis that stimulation of TRPA1 channels does not globally increase intracellular [Ca2+] but instead results in localized elevation of intracellular [Ca2+] in subcellular microdomains containing KCa channels. Consistent with this possibility, TRPA1 and KCa3.1 channels are colocalized in endothelial cell membrane domains spanning the IEL (Figure 3I through 3L). Consistent with this possibility, TRPA1 immunostaining projects though the IEL (Figure 3H). TRAM34 immunostaining was not present following mechanical disruption of the endothelium (Online Figure IV). These findings demonstrate that TRPA1 channels are present in the endothelium and are abundant in trans-IEL membrane domains with elevated levels of KCa3.1 channels.
mV) causes K⁺ efflux, which hyperpolarizes the sarcolemma, resulting in myocyte relaxation and vasodilation.³⁰ Low (micromolar) concentrations of Ba²⁺ ions are effective at selectively blocking KIR channels in smooth muscle cells.³⁰ Extracellular BaCl₂ (30 μmol/L) essentially abolished AITC-induced vasodilation (Figure 4D and 4E), demonstrating that KIR channel activity is required for cerebral artery dilation in response to TRPA1 activation. These experiments also show that pinacidil-induced vasodilation was not blocked by BaCl₂ (Figure 4D; 96.5 ± 1% of Ca²⁺-free diameter, n = 4), indicating that inhibition of KIR channels does not impair KATP-induced myocyte hyperpolarization and arterial dilation.

TRPA1 Channel Activation Hyperpolarizes Cerebral Artery Myocytes

The effects of TRPA1 channel activation on smooth muscle cell membrane potential were directly assessed using intracellular microelectrodes. The resting membrane potential of arterial myocytes in cerebral arteries pressurized to 70 mm Hg under control conditions is 37.0 ± 1.0 mV (n = 7) (Figure 5A and 5D), whereas in the presence of AITC (30 μmol/L), smooth muscle membrane potential is 44.6 ± 2.1 mV (n = 7) (Figure 5B and 5D). These data demonstrate that TRPA1 channel activity hyperpolarizes the sarcolemma. To investigate a potential role for SKCa/KCa3.1 channels in AITC-induced hyperpolarization, experiments were performed when apamin and TRAM34 (1 μmol/L each) were present in the arterial lumen. This treatment had no significant effect on membrane potential or myogenic tone under control conditions. In addition, smooth muscle cell membrane potential did not differ when recordings were obtained under control conditions (37.3 ± 1.8 mV, n = 7), following luminal administration of apamin and TRAM34 (33.6 ± 2.6 mV, n = 5) and when apamin and TRAM34-treated arteries were superfused with AITC (30 μmol/L) (~36.7 ± 1.8 mV, n = 7; Figure 5C and 5E). These findings show that activation of TRPA1 in the endothelium hyperpolarizes smooth muscle cells by a mechanism that requires SKCa and KCa3.1 channel activity (Figure 6).

Discussion

This study examined the functional consequences of TRPA1-mediated endothelial cell Ca²⁺ influx on the vasomotor activity of cerebral arteries. The major findings are: (1) the TRPA1 agonist AITC causes concentration-dependent dilation of cerebral arteries that is associated with a decrease in smooth muscle intracellular [Ca²⁺]; (2) TRPA1 agonist-dependent dilation is impaired by disruption of the endothelium and by luminal administration of a TRPA1 blocking compound; (3) TRPA1 channels are present in the vascular endothelium and are localized to membrane projections that approach underlying smooth muscle cells; (4) vasodilation resulting from stimulation of TRPA1 activity is independent of NOS and COX activity but is attenuated by blockade of...
SKCa and KCa3.1 channels; (5) inhibition of KIR channel activity abolishes TRPA1-dependent vasodilation; and (6) activation of TRPA1 causes smooth muscle cell hyperpolarization by a mechanism that requires the activity of SKCa and KCa3.1 channels. The findings of this study demonstrate that endothelial TRPA1 channels are part of a vasodilatory signaling complex that includes KCa channels in endothelial cells and KIR channels in cerebral artery myocytes (Figure 6).

Endothelium-dependent dilation of cerebral arteries following stimulation of TRPA1 channels is not altered by blockade of NOS and COX, but is sensitive to inhibition of small- and intermediate-conductance Ca2+-activated K+ channels. In addition, activation of TRPA1 results in smooth muscle cell hyperpolarization that is sensitive to KCa channel blockade. These properties are hallmarks of “endothelium-derived hyperpolarizing factor” (EDHF)-induced dilation. Despite considerable effort, the identity of the underlying molecular nature of EDHF remains elusive. Compelling evidence in support of a number of mechanisms, including cytochrome P450 epoxygenase products, K+ ions, hydrogen peroxide, and gap junctional communication, has been reported. The present findings demonstrate that endothelial TRPA1 channels promote EDHF-type vasodilation in cerebral arteries. These observations are in agreement with those of earlier studies demonstrating that dilation of pulmonary and mesenteric arteries in response to TRPA1 agonist allicin is independent of NOS and COX activity. However, in contrast to findings presented here demonstrating a critical role for the vascular endothelium, Bautista et al showed that allicin-induced relaxation of mesenteric arteries was attenuated by pretreatment with a calcitonin gene-related peptide (CGRP) antagonist. Bautista et al conclude that activation of TRPA1 channels in perivascular sensory nerve endings causes vasodilation of mesenteric arteries by stimulating release of CGRP. A potential role for the endothelium in mesenteric artery relaxation in response to TRPA1 stimulation was not investigated. The present findings show that disruption of the endothelium or luminal administration of a TRPA1 blocking compound eliminates approximately 75% of AITC-induced cerebral artery dilation (Figure 2B and 2D), clearly demonstrating that endothelial cells mediate changes in vascular tone in response to TRPA1 activity in this vascular bed. Transient vasodilation in response to AITC following disruption of the endothelium (Figure 2A) or in the presence of apamin and TRAM34 (Figure 4A) may result from release of CGRP or other endothelium-independent mechanisms. Although it is possible that stimulation of TRPA1 channels present in perivascular nerve endings mediates a component of the response, the endothelium appears to play a major role in steady-state TRPA1-dependent vasodilation in the cerebral vasculature.

The findings of this study provide new insight into the relationship between endothelial cell Ca2+ dynamics and endothelium-dependent vasodilation. The TRPA1 vasodilatory response is attenuated by blockade of Ca2+-activated K+ channels (Figure 4A through 4C) but not by inhibition of the NOS/COX pathways. Because all of these vasodilatory mechanisms are activated by increases in endothelial cell [Ca2+], the present findings suggest that spatially distinct Ca2+ signals regulate particular endothelium-dependent vasodilatory pathways. This conclusion is consistent with recent work by Ledoux et al, demonstrating that localized Ca2+ release events (“Ca2+ pulsars”) exist in native endothelial cells and are dependent on Ca2+ release from inositol trisphosphate (IP3) receptors located on the endoplasmic reticulum. These dynamic Ca2+ events occur in structures that project from endothelial cells through the IEL to underlying vascular smooth muscle cells. Ledoux et al also report that endoplasmic reticulum elements, IP3 receptors, and KCa3.1 channels colocalize with myoendothelial membrane projections in mesenteric arteries. The present study demonstrates that TRPA1 channels and KCa3.1 channels are associated with these endothelial cell membrane projections in cerebral vessels. These findings are consistent with the hypothesis that TRPA1 channels and KCa3.1 channels form a vasodilatory signaling complex in endothelial cell membrane projections proximal to arterial myocytes. In this proposed pathway, localized TRPA1 Ca2+ signals can either directly stimulate KCa channel activity or can be amplified by causing a Ca2+-dependent increase in the open probability of IP3 receptors located in myoendothelial membrane projections, which serves to elevate the frequency of Ca2+ pulsars and activate KCa channels. A combination of the SKCa channel blocker apamin and the KCa3.1 channel blocker TRAM34 appears to be more effective in blocking TRPA1-induced vasodilation compared with TRAM34 alone. The most abundant SKCa channel in cerebral artery smooth muscle, is critical for endothelium-dependent vasodilation and regulation of endothelial cell membrane potential. This channel is absent from myoendothelial membrane projections that span the IEL (Online Figure III). It is not clear from the present studies whether SKCa channels are directly involved in TRPA1-dependent responses or whether the effects of apamin reflect a tonic hyperpolarizing effect of SKCa channel activity on the endothelial cell membrane. KCa channel activity hyperpolarizes the endothelial cell plasma membrane and serves to hyperpolarize underlying smooth muscle by charge transfer mediated by myoendothelial gap junctions present at the tip of the endothelial cell membrane projection spanning the IEL (Figure 6).

AITC-induced dilation was blocked by Ba2+, demonstrating that KIR channel activity is required for vasodilation in response to activation of TRPA1 channels. KIR channels are prominent in smooth muscle cells and may be present in the endothelium of some vascular beds. Although the present study does not rule out the possibility that endothelial KIR channels contribute to TRPA1-mediated vasodilation, prior reports provide a strong conceptual framework supporting an important role for smooth muscle KIR activity in vasodilatory responses. For example, release of K+ from KCa channels into the interstitial space between the endothelium and underlying vascular smooth muscle cells could increase [K+] in a nominal 3 mmol/L to 8 to 12 mmol/L, sufficient to activate smooth muscle KIR channels and account for TRPA1-dependent vasodilation. Biophysical properties of KIR channels suggest another mechanism that could explain involvement of this channel in TRPA1-mediated vasodilation. KIR channels composed of KIR2.1 and 2.2 subunits have an
unusual current–voltage relationship known as “negative slope conductance.”27,28 Over a limited voltage range, outward $K^+$ currents increase as membrane potential becomes more hyperpolarized. This effect is apparent for membrane potentials that are physiologically relevant for vascular smooth muscle cells. Message encoding $K_{Ca}$ and $K_{IR}$ channels appear to facilitate endothelium-dependent vasodilation in these vessels.29 Furthermore, using both computational models and empirical studies, Smith et al demonstrate that the negative slope conductance of $K_{Ca}$ channels serves to amplify endothelium-derived hyperpolarizing stimuli.29 $K_{IR}$-dependent amplification of gap junction-mediated change and temporal relationships of ion channels and $Ca^{2+}$ dependent amplification of gap junction-mediated change vasodilation resulting from TRPA1 activity. This proposed signaling pathway highlights the importance of spatial and temporal relationships of ion channels and $Ca^{2+}$ signals in endothelial and smooth muscle cells.

Although the electrophilic agonist AITC is commonly used to stimulate TRPA1 activity, recent reports suggest that TRPA1 channels may also be activated by endogenously occurring substances. 4-HNE, 4-ONE, and 4-HHE are lipid peroxidation products that potently activate TRPA1 channels expressed by HEK cells and cultured sensory neurons.13,14,38 Inhibition of TRPA1 activity in isolated cerebral arteries results in a small, yet statistically significant, increase of the production of prostacyclin by the vascular endothelium.1982;79:495–499. Furthermore, using both computational models and empirical studies, Smith et al demonstrate that the negative slope conductance of $K_{Ca}$ channels serves to amplify endothelium-derived hyperpolarizing stimuli.29 $K_{IR}$-dependent amplification of gap junction-mediated change and temporal relationships of ion channels and $Ca^{2+}$ signals in endothelial and smooth muscle cells.

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Disclosures

None.

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Online Figure I: AITC-Induced Cerebral Artery Dilation is Reversible and Reproducible. A: Representative recording of vasodilation of an isolated cerebral artery pre-constricted with intraluminal pressure (70 mmHg) in response to repeated administration of AITC (30 µM). B: Summary data for vasodilation in response to repeated administration of AITC (30 µM). n=4.
Online Figure II: TRPA1 Inhibition Increases Myogenic Tone.
Myogenic tone (at 70 mmHg) before and after luminal administration of HC-030031 (3 µM). n=5. * P≤0.05 vs. Control.
Online Figure III: Localization of K\textsubscript{Ca}2.3 channels in cerebral artery endothelial cells. Images show immunostaining for K\textsubscript{Ca}2.3 (yellow). The level of the internal elastic lamina (IEL) is shown in green (A). The superimposed image demonstrates that K\textsubscript{Ca}2.3 channels do not localize to holes in the IEL (C). Bar = 50 µm. Immunostaining data are representative of arteries isolated from three animals.
Online Figure IV: Immunostaining for TRPA1 is Absent following Removal of the Endothelium. Cerebral arteries were cut lengthwise to expose the endothelium and pinned to a Silgard block. The endothelium was gently removed using a cotton swab and the tissue was fixed, immunostained, and imaged using the same procedures used for the images shown in Figure 3. The level of the internal elastic lamina (IEL) is shown in green. TRPA1 Immunostaining for TRPA1 was not visible. This image is representative of tissue obtained from three animals.
Online Figure V: AITC-Induced Vasodilation is Independent of NOS and COX Activity. Vasodilation in response to AITC (100 µM) under control conditions, in the presence of the NOS inhibitor N-nitro-L-arginine (L-NNA, 300 µM), or in the presence of L-NNA (300 µM) and the COX inhibitor indomethacin (10 µM), n=5.
EXPANDED MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250-350 g; Harlan, Indianapolis, Indiana, USA) were used for these studies. Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and euthanized by exsanguination according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Colorado State University. Brains were isolated in ice-cold MOPS-buffered saline (in mM): 3 MOPS (pH 7.4), 145 NaCl, 5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1 KH₂PO₄, 0.02 EDTA, 2 pyruvate, 5 glucose and 1% bovine serum albumin. Cerebral and cerebellar arteries were dissected from the brain, cleaned of connective tissue, and stored in MOPS-buffered saline on ice prior to further manipulation.

Isolated Vessel Experiments

For experiments examining the effects of TRPA1 agonists on vasodilatory responses and changes in smooth muscle intracellular [Ca²⁺], cerebral arteries isolated from rats were loaded with fura-2AM (10 µM, room temperature, 60 minutes, in the presence of 0.05% pluronic acid) and transferred to a vessel chamber (Living Systems, Inc.). The proximal end of the vessel was cannulated with a glass micropipette and secured with monofilament thread. Blood was gently rinsed from the lumen, and the distal end of the vessel was cannulated and secured. Arteries were pressurized to 10 mmHg with physiological saline solution (PSS) (in mM): 119 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2
MgSO₄, 24 NaHCO₃, 0.2 KH₂PO₄, 10.6 glucose, 1.1 ethylenediaminetetraacetic acid (EDTA) and superfused (5 mL/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O₂, 6% CO₂, balance N₂). Following a 15-minute equilibration period, intraluminal pressure was slowly increased to 110 mmHg, vessels were stretched to remove bends, and pressure was reduced to 10 mmHg for an additional 15-minute equilibration period. Inner diameter was continuously monitored using video microscopy and edge-detection software (Ionoptix). Arteries were intermittently excited with UV light at 340 and 380 nm (10 Hz) and deep red wavelength emissions were recorded using a photomultiplier tube and expressed as the ratio of emissions during 340 nm excitation vs 380 nm excitation (340/380 ratio). To assess viability of the preparation, arteries pressurized to 10 mmHg were exposed to isotonic PSS containing 60 mM KCl. To assess the effects of TRPA1 activation on vasomotor responsiveness, arteries were pressurized to 70 mmHg and spontaneous myogenic tone was allowed to develop prior to AITC administration. Vessels dilated in response to AITC. The time of onset of maximum vasodilation in response to 30 µM AITC was 79.6±9.3 sec (n=11) after the AITC containing solution first started flowing into the tissue bath. Myogenic tone recovered to original levels 284.4±77 sec (n=4) after AITC-free solution began flowing in the tissue bath. To determine maximum (i.e. passive) diameter, intraluminal pressure was maintained at 70 mmHg and vessels were superfused with Ca²⁺-free PSS (in mM): 119 NaCl, 4.7 KCl, 1.2 MgSO₄, 24 NaHCO₃, 0.2 KH₂PO₄, 10.6 glucose, 1.1 EDTA, 3 EGTA, and 0.01 diltiazem. “% Vasodilation” and “% Change in Luminal Diameter” was
calculated as the difference in diameter before and after agonist administration normalized to the maximal diameter. For some experiments, endothelial cell function was disrupted by passage of air and distilled water through the vessel lumen. The efficacy of endothelium disruption was confirmed by loss of vasodilation in response to luminal administration of the endothelium-dependent vasodilator UTP.

Cerebral Artery Endothelial Cell Preparation

To isolate endothelial cells, vessels were cut into ~2-mm segments and placed in cell isolation solution containing (in mM): 60 NaCl, 80 Na-glutamate, 5 KCl, 2 MgCl₂, 10 glucose, and 10 HEPES; (pH 7.4). Arterial segments were initially incubated at room temperature in 1 mg/mL papain (Worthington), 1 mg/mL dithioerythritol, and 0.5 mM CaCl₂ for 30 min, followed by 25 min incubation at 37 °C in 2 mg/mL type II collagenase (Worthington) and 0.5 mM CaCl₂. Digested segments were washed three times in ice-cold cell isolation solution and triturated to release endothelial and smooth muscle cells. Cells were stored on ice in isolation solution for use the same day.

RNA Isolation and RT-PCR

Following enzymatic dispersal, endothelial cells were visualized using phase-contrast microscopy and isolated by hand using a micromanipulator-derived pipette. Total RNA was extracted (RNeasy Protect mini kit; Qiagen, Inc.) and first strand cDNA was synthesized using a Omniscript Reverse
Transcriptase kit (Qiagen, Inc). RT-PCR using primers for eNOS and smooth muscle alpha actin (both from Qiagen, Inc.) was performed to establish cell type specificity of the RNA preps. RT-PCR was performed using TRPA1 specific primers (forward: 5'-AATGAGGCTCTGGAAGCAG-3' reverse: 5'-GAGCACAAGGCCAATACAT-3'). PCR products were resolved on agarose gels. These primers yield a PCR product of 500 bp and span an intron-exon boundary to minimize amplification of genomic DNA. The nucleotide sequence of the PCR product was consistent with the published sequence of TRPA1 cDNA. All PCR reactions included a template-free control and RT-PCR was performed using RNA extracted from at least three animals.

**Immunohistochemistry**

Cerebral arteries were cut lengthwise and were mounted on Sligard blocks with the endothelium exposed. The tissue was fixed with 4% formaldehyde for 15 minutes, permeabilized with 0.2 % Triton-X 100, blocked with 2 % bovine serum albumin and incubated with anti-TRPA1 (Santa Cruz, 1:1000)\(^1\), anti-K\(_{Ca}\)3.1 (Alomone, 1:1000) and/or anti-K\(_{Ca}\)2.3 (Alomone, 1:1000) overnight at 4°C. Arteries were probed with fluorescent secondary antibodies (Texas Red, Santa Cruz, 1:500 or Alexa 633, Molecular Probes, 1:500) for 1 hour at room temperature. Immunofluorescence was detected using a Zeiss LSM 510 Meta laser scanning confocal microscope (excitation 543 nm; emission 610 nm). Autofluorescence of the internal elastic lamina was assessed at excitation and emission wavelengths of 488 nm and 510 nm, respectively. For 3-dimensional
volume rendering, images were recorded in z-stacks at 1 μm increments from the base of the endothelium to the surface of the subintimal smooth muscle, and reconstructed using Zeiss LSM Image Browser.

Smooth Muscle Cell Membrane Potential

For measurement of smooth muscle cell membrane potential, cerebral arteries were isolated and pressurized (70 mmHg), and vascular smooth muscle cells were impaled through the adventia with glass intracellular microelectrodes (tip resistance 100-200 MΩ). A WPI Intra 767 amplifier was used for recording membrane potential. Analog output from the amplifier recorded using IonOptix Software (sample frequency 60 Hz). Criteria for acceptance of membrane potential recordings were: 1) an abrupt negative deflection of potential as the microelectrode was advanced into a cell; 2) stable membrane potential for at least 30 sec; and 3) an abrupt change in potential to approximately 0 mV after the electrode was retracted from the cell. Vascular smooth muscle cell membrane potential was recorded before and after administration of AITC (30 μM), and in a second set of experiments, under control conditions, after apamin (1 μM) and TRAM34 (1 μM) were administered to the arterial lumen, and in the presence of AITC following treatment with apamin and TRAM34.

Calculations and Statistics

All data are mean ± SE. Values of n refer to number of arteries for isolated vessel experiments or number of cells for membrane potential experiments.
Statistical methods conform to the recommendations of the editors of *Circulation Research*. Experiments examining changes in endothelial cell Ca$^{2+}$ in response to TRPA1 stimulation or investigating the effects of endothelium disruption, HC-030031, apamin and TRAM34, and BaCl$_2$ on AITC-induced vasodilation were performed as repeated measures and Student’s unpaired t-test was used to detect differences. Student’s paired t-test was used to test for differences in smooth muscle membrane potential. One-way ANOVA was used to compare smooth muscle membrane potentials between control vessels, apamin and TRAM34 vessels, and apamin and TRAM34-treated vessels in the presence of AITC. One-way repeated measures ANOVA was used to test for differences in AITC-induced vasodilation during NOS and COX inhibition. A level of $P \leq 0.05$ was accepted as statistically significant for all experiments.

**LITERATURE CITED**
