A Dietary Agonist of Transient Receptor Potential Cation Channel V3 Elicits Endothelium-Dependent Vasodilation[S]

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ABSTRACT

The Mediterranean diet may be responsible for lower cardiovascular disease rates in Southern versus Northern European countries. Oregano is used abundantly in Mediterranean cooking, but potential cardiovascular benefits have not been investigated. Carvacrol, present in oregano, activates the transient receptor potential (TRP) cation channels TRPA1 and TRPV3. We hypothesized that chemosensing of this dietary molecule by TRP channels in the endothelium promotes arterial relaxation. TRPA1 and TRPV3 were detected in the endothelium of intact arteries. Carvacrol causes concentration-dependent increases in the intracellular $[Ca^{2+}]$ of native cerebral artery endothelial cells and is more potent ($EC_{50} = 34$ μM) than the TRPA1 agonist allyl isothiocyanate ($EC_{50} = 400$ μM) or the TRPV3 agonist eugenol ($EC_{50} = 2.3$ mM). Carvacrol also activates TRPV3-like cation currents in cerebral artery endothelial cells. Carvacrol elicits vasodilation of intact cerebral arteries ($EC_{50} = 4.1$ μM) that is accompanied by smooth muscle hyperpolarization and a decrease in the intracellular $[Ca^{2+}]$ of arterial myocytes. Endothelium disruption inhibits carvacrol-induced vasodilation, but block of nitric-oxide synthase and cyclooxygenase activity does not alter the response. Vasodilation in response to carvacrol is inhibited when blockers of Ca^{2+}-activated K^+ channels are present in the lumen or when the inwardly rectifying K^+ channel blocker $BaCl_2$ is present in the superfusion bath. Carvacrol-induced dilation is not diminished by a TRPA1 antagonist but is inhibited by the TRPV blocker ruthenium red. Our findings show that oregano can relax arteries by activating TRPV3 channels in the endothelium. This effect may account for some of the cardioprotective effects of the Mediterranean diet.

Chemical stimuli activate a subset of the transient receptor potential (TRP) cation channels. Chemosensitive TRP channels in sensory neurons are responsible for the perception of flavors and temperatures of certain foods and spices (Xu et al., 2006). For example, the vanilloid TRP channel TRPV1 produces a sensation of heat when activated by capsaicin, a compound present in hot chili peppers (Caterina et al., 1997), whereas activation of the melastatin TRP channel TRPM8 is responsible for the cooling sensation of menthol (McKemy et al., 2002; Peier et al., 2002a). Perception of pungent compounds such as allicin, abundant in garlic, and allyl isothiocyanate (AITC), present in mustard oil, results

from activation of the ankyrin TRP channel TRPA1 (Story et al., 2003; Jordt et al., 2004). TRPV3 channels (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002) are stimulated by substances such as vanillin, thymol (from thyme), and eugenol (derived from cloves) (Xu et al., 2006). It is interesting that chemosensitive TRP channels are present in tissues outside of the sensory nervous system (Yang et al., 2006; Ueda et al., 2009), suggesting that local detection of environmental compounds can regulate cellular responses to chemical stimuli. Non-neuronal chemosensing may be particularly important in tissues exposed to food- or blood-borne substances, such as the gastrointestinal epithelium and the vascular endothelium. The endothelium, in particular, is situated to rapidly modify arterial function in response to TRP channel agonists that enter the blood stream as dietary molecules.

In addition to their use as flavorings, foods such as garlic and mustard oil have traditionally been used by some cultures to diminish the likelihood of cardiovascular disease (Singh et al., 1997), suggesting that allicin and AITC could

ABBREVIATIONS: TRP, transient receptor potential; TRPV, vanilloid transient receptor potential; TRPA, ankyrin transient receptor potential; AITC, allyl isothiocyanate; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel; eNOS, endothelial nitric-oxide synthase; IEL, internal elastic lamina; L-NNA, N^{ω}-nitro-L-arginine; COX, cyclooxygenase; K_{IR}, inwardly rectifying K^+ channel; RuR, ruthenium red; NOS, nitric-oxide synthase; PSS, physiological saline solution; AM, acetoxymethyl ester; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HC-030031, 2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-*N*-(4-isopropylphenyl)acetamide.

act through TRPA1 to improve vascular health by promoting relaxation of resistance arteries. Consistent with this hypothesis, our recent work shows that activation of TRPA1 with AITC causes endothelium-dependent dilation of cerebral arteries (Earley et al., 2009). The goal of the current study is to expand this concept and identify other dietary molecules that act through chemosensitive TRP channels in the endothelium to influence vascular function.

Carvacrol, a monoterpenoid phenol compound found in high (up to 65% of mass) concentrations in the essential oil of oregano (Sarer et al., 1982), is an agonist for both TRPA1 and TRPV3 (Xu et al., 2006). We became interested in this compound because oregano is part of the cardioprotective Mediterranean diet (Dontas et al., 2007; Lairon, 2007), and the effects of carvacrol on vascular function have not been reported previously. Using intact cerebral and cerebellar arteries and freshly isolated endothelial cells from these vessels, we find that carvacrol is a potent vasodilator, acting through TRPV3 channels present in the endothelium. These findings suggest that activation of TRPV3 channels in the endothelium may improve vascular function by promoting arterial relaxation. Furthermore, our results provide a novel mechanistic basis for investigating the cardioprotective benefits of a diet that includes reasonable amounts of oregano.

Materials and Methods

Animals. Male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN) 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 of Colorado State University (Fort Collins, CO). Brains were isolated in ice-cold MOPS-buffered saline (3 mM MOPS, pH 7.4, 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM KH_2PO_4, 0.02 mM EDTA, 2 mM pyruvate, 5 mM 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 before further manipulation.

RNA Isolation and RT-PCR. Vessels were cut into ~2-mm segments and placed in a cell isolation solution containing 60 mM NaCl, 80 mM sodium glutamate, 5 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. Arterial segments were initially incubated at room temperature in 1 mg/ml papain (Worthington Biochemicals, Freehold, NJ), 1 mg/ml dithioerythritol, and 0.5 mM $CaCl_2$ for 30 min, followed by 25 min incubation at 37°C in 2 mg/ml type II collagenase (Worthington Biochemicals) and 0.5 mM $CaCl_2$. Digested segments were washed three times in ice-cold cell isolation solution and triturated to release endothelial and smooth muscle cells. After enzymatic dispersal, total RNA was extracted (RNeasy Protect Mini Kit), and first-strand cDNA was synthesized using an Omniscript Reverse Transcriptase kit (both from QIAGEN, Valencia, CA). RT-PCR was performed using primer sets specific for TRPA1, TRPV3, endothelial nitric-oxide synthase (eNOS) and smooth muscle α-actin (QIAGEN). PCR products were resolved on agarose gels. PCR reactions always included a template-free negative control and were performed using RNA isolated from at least three animals.

Immunohistochemistry. Superior cerebellar arteries were cut lengthwise and were mounted on Silgard blocks with the endothelium exposed. The tissue was fixed with 4% formaldehyde for 15 min, permeabilized with 0.2% Triton X-100, blocked with 2% bovine serum albumin, and incubated with primary rabbit polyclonal antibodies (anti-TRPA1, 1:500; anti-TRPV3, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Arteries were subsequently washed and incubated with fluorescent secondary anti-rabbit antibody (Texas Red, Santa Cruz Biotechnology) for 1 h at room temper-

ature. Immunofluorescence was detected using a Zeiss LSM 510 Meta laser scanning confocal microscope (excitation, 543 nm; emission, 610 nm; Carl Zeiss Inc., Thornwood, NY). Autofluorescence of the internal elastic lamina was assessed at excitation and emission wavelengths of 488 and 510 nm, respectively. For three-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.

Isolated Vessel Experiments. For experiments examining the effects of carvacrol on vasodilatory responses and changes in smooth muscle intracellular $[Ca^{2+}]$, posterior cerebral or superior cerebellar arteries isolated from rats were loaded with fura-2-AM (10 μM at room temperature for 60 min in the presence of 0.05% Pluronic acid) and transferred to a vessel chamber (Living Systems Instrumentation, Burlington, VT). These procedures preferentially load vascular smooth muscle cells. 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 mm Hg with physiological saline solution (PSS) (119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 0.2 mM KH_2PO_4, 10.6 mM glucose, and 1.1 mM EDTA) and superfused (5 ml/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O_2, 6% CO_2, balance N_2). After a 15-min equilibration period, intraluminal pressure was slowly increased to 110 mm Hg, vessels were stretched to remove bends, and pressure was reduced to 10 mm Hg for an additional 15-min equilibration period. Inner diameter was continuously monitored using video microscopy and edge-detection software (IonOptix, Milton, MA). Arteries were intermittently excited with UV light at 340 and 380 nm (10 Hz). Deep red wavelength emissions were recorded using a photomultiplier tube and expressed as the ratio of emissions at 340 nm excitation to those at 380 nm excitation (340:380 ratio). To assess viability of the preparation, arteries pressurized to 10 mm Hg were exposed to isotonic PSS containing 60 mM KCl. To assess the effects of carvacrol on vasomotor responsiveness, arteries were pressurized to 70 mm Hg, and spontaneous myogenic tone was allowed to develop before agonist administration. To determine the maximum (i.e., passive) diameter, intraluminal pressure was maintained at 70 mm Hg, and vessels were superfused with Ca^{2+}-free PSS (119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 0.2 mM KH_2PO_4, 10.6 mM glucose, 1.1 mM EDTA, 3 mM EGTA, and 0.01 mM diltiazem). The values for percentage vasodilation and percentage change in luminal diameter were calculated as the difference in diameter before and after agonist administration normalized to the maximal diameter; change in vessel wall with $[Ca^{2+}]$ was calculated as the difference in the 340:380 ratio recorded before and after administration of carvacrol normalized to the 340:380 ratio recorded under Ca^{2+}-free conditions.

Ca^{2+} Imaging Experiments. Cells from enzymatically dispersed cerebral arteries were loaded with the ratiometric Ca^{2+} indicator dye fura-2-AM (10 μM for 30 min at room temperature) in the presence of Pluronic acid (0.05%). Fura-loaded cells were allowed to adhere to the bottom of an imaging chamber for approximately 20 min at room temperature and were then continuously superfused with HEPES-buffered saline solution (134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4). Endothelial cells were identified by cobblestone-like morphology and were selected as regions of interest. Cells were alternately excited at 340 or 380 nm at a frequency of 10 Hz (IonOptix Hyperswitch), and images were captured at a frame rate of 60 Hz using a charge-coupled device camera (DAGE-MTI, Michigan City, IN). Images were continuously recorded under baseline conditions and after TRP channel agonist administration. Background-subtracted fluorescence emission at 340 and 380 nM were calculated and continuously recorded (Ionwizard 5.0.8; IonOptix). To calibrate Ca^{2+} responses, cells were treated with the Ca^{2+} ionophore ionomycin (1 μM) to determine maximal responses and with Ca^{2+}-free HEPES-buffered saline solution (134

mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 3 mM EGTA, 10 mM glucose, and 10 mM HEPES, pH 7.4) to determine unbound responses.

Intracellular $[Ca^{2+}]$ was calculated by the equation (Grynkiewicz et al., 1985) $[Ca^{2+}] = K_d \cdot [(R - R_{min})/(R_{max} - R) \cdot Sf2/Sb2]$, where K_d is the dissociation constant for Ca^{2+} for fura-2, R_{max} is the ratio of fluorescence emissions at 340 nm versus 380 nm at saturating Ca^{2+} levels, R_{min} is the ratio of fluorescence emissions at 340 nm versus 380 nm in the absence of Ca^{2+}, R is the ratio of fluorescence emissions at 340 nm versus 380 nm, Sf2 is the emissions during 380 nm excitation under Ca^{2+}-free conditions, and Sb2 is the emissions during 380 nm excitation under saturating Ca^{2+} conditions. The Ca^{2+} K_d of fura-2 for our Ca^{2+} imaging system was empirically determined in vitro using Ca^{2+} standards (Invitrogen, Carlsbad, CA).

Patch-Clamp Electrophysiology. Carvacrol-activated currents were recorded from human cerebral artery microvascular endothelial cells (Cell Systems Corporation, Kirkland, WA). Cells were cultured as recommend by the supplier and were used between passages 4 and 7. Endothelial cells were trypsinized and allowed to adhere to glass coverslips for 3 to 4 h at 37°C. Coverslips were transferred to a recording chamber (Warner Instruments, Hamden, CT) and voltage-clamped in the conventional whole-cell configuration. Whole-cell currents were recorded using an AxoPatch 200B amplifier equipped with an Axon CV-203BU head stage (Molecular Devices, Sunnyvale, CA). Recording electrodes (resistance 1–3 MΩ) were pulled, polished, and coated with wax to reduce capacitance. The pipette solution contained 125 mM cesium, 120 mM methane sulfonate, 8 mM NaCl, 4.1 mM $CaCl_2$, 10 mM EGTA, 2 mM Mg^{2+}-ATP, and 20 mM HEPES, pH adjusted to 7.2 with CsOH. The extracellular solution contained 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.4. The calculated reversal membrane potential for the total monovalent cations for these solutions is 2.5 and -58.5 mV for monovalent anions. Clampex and Clampfit versions 10.2 (Molecular Devices) were used for data acquisition and analysis, respectively. Voltage ramps (400 ms) from -100 to $+100$ mV were applied every 4 s from a holding potential of 0 mV.

Calculations and Statistics. All data are mean $\pm$ S.E. Values of n refer to the number of arteries for isolated vessel experiments or the number of cells for Ca^{2+} imaging and patch-clamp experiments. Experiments examining changes in endothelial cell Ca^{2+} in response to AITC, eugenol, or carvacrol stimulation or investigating the effects of endothelium disruption, apamin, and TRAM34, $BaCl_2$, HC-030031, and ruthenium red on carvacrol-induced vasodilation were performed as repeated measures, and Student's paired t test was used to detect differences. Differences in carvacrol-induced vasodilation during nitric-oxide synthase (NOS) and cyclooxygenase (COX) inhibition were examined using one-way repeated measures analysis of variance. A level of $P \leq 0.05$ was accepted as statistically significant for all experiments.

Results

TRPA1 and TRPV3 Channels Are Present in the Endothelium of Cerebral Arteries. RT-PCR experiments using RNA from rat cerebral and cerebellar arteries show that mRNAs encoding TRPA1 and TRPV3 are present in these tissues (Fig. 1A). Expression of eNOS and smooth muscle α-actin was also detected in these samples, demonstrating that RNA was recovered from both endothelial cells and smooth muscle cells. To determine the location of these channels within the arterial wall, superior cerebellar arteries were cut longitudinally and pinned to Silgard blocks with the endothelium side face-up and were immunostained using antibodies against TRPA1 and TRPV3. Consistent with a prior report from our laboratory (Earley et al., 2009), TRPA1 expression was present in the endothelium and was concen-

trated in projections of the endothelial cell membrane that span the internal elastic lamina (IEL) that are sites of myoendothelial junctions (Fig. 1B). TRPV3 immunostaining was also present in the endothelium of these vessels but was uniform and was not concentrated in IEL-spanning endothelial cell membrane projections (Fig. 1B). Removal of the endothelium abolished detection of both TRPA1 (Earley et al., 2009) and TRPV3 (Supplemental Fig. 1), suggesting that expression of these channels is confined to endothelial cells.

Carvacrol Stimulates Endothelial Cell Ca^{2+} Influx. TRPA1 and TRPV3 channels are permeable to Ca^{2+} ions. Ratiometric Ca^{2+} imaging experiments were performed to determine whether agonists for TRPA1 and TRPV3 channels elicit Ca^{2+} influx in freshly isolated native cerebral artery endothelial cells. Representative data and images for these experiments are shown in Supplemental Fig. 2. We found that the TRPA1 agonists AITC (Supplemental Fig. 3, A and B) and allicin, present in diluted garlic oil (Supplemental Fig. 3C), caused concentration-dependent increases in endothelial cell $[Ca^{2+}]$. The EC_{50} of AITC for endothelial cell Ca^{2+} influx was $\sim$400 μM. Eugenol, a compound found in cloves, is an agonist for TRPV3 channels (Xu et al., 2006). Administration of eugenol to freshly dispersed cerebral artery endothelial cells also caused an increase in endothelial cell $[Ca^{2+}]$ (Supplemental Fig. 3, D and E), although the compound was much less potent than AITC (EC_{50} for eugenol = 2.3 mM).

Administration of the combined TRPA1 and TRPV3 agonist carvacrol to isolated endothelial cells caused a robust increase in intracellular $[Ca^{2+}]$ (Fig. 2, A and B). Increases in
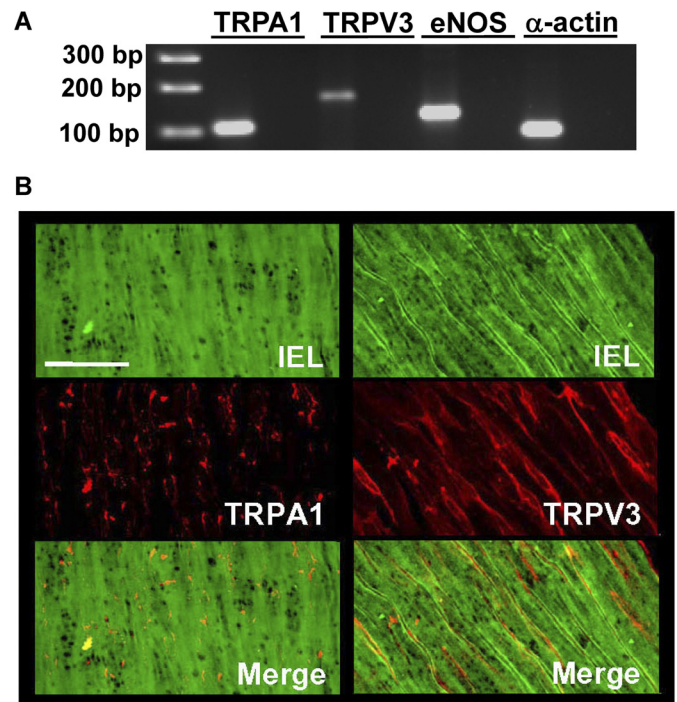

Fig. 1. TRPV3 channels are present in the endothelium of cerebral and cerebellar arteries. A, RT-PCR demonstrating expression of TRPA1, TRPV3, eNOS, and smooth muscle α-actin in RNA isolated from cerebral and cerebellar arteries. The second lane for each PCR reaction is the no-template control. B, confocal images of superior cerebellar arteries that were cut longitudinally and pinned to Silgard blocks with the luminal surface facing up. Top, the autofluorescence of the IEL; middle, immunostaining for either TRPA1 or TRPV3; bottom, a merged image of the two. Images are representative of tissue from at least three animals. Scale bar, 50 μm.

endothelial cell $[Ca^{2+}]$ were stimulated by concentrations of carvacrol as low as 30 μM and were maximal at 100 μM (Fig. 2B). The EC_{50} for the carvacrol-induced increase in endothelial cell $[Ca^{2+}]$ was 34 μM. Carvacrol-induced increases in intracellular Ca^{2+} were absent when Ca^{2+} was not present in the bathing solution (Supplemental Fig. 4), demonstrating that carvacrol elicits Ca^{2+} influx. Carvacrol did not cause Ca^{2+} influx in isolated cerebral artery smooth muscle cells (Supplemental Fig. 5).

Our findings demonstrate that carvacrol elicits greater changes in endothelial cell $[Ca^{2+}]$ and is more potent compared with AITC and eugenol. The magnitude of the increase in endothelial cell $[Ca^{2+}]$ versus baseline that was induced by carvacrol (100 μM) is 5.8 $\pm$ 1.2-fold ($n = 14$). Carvacrol-induced increases in endothelial cell $[Ca^{2+}]$ are greater than AITC-induced increases (1000 μM; 2.2 $\pm$ 0.3-fold, $n = 24$) and eugenol (3000 μM; 3.2 $\pm$ 0.3-fold, $n = 15$) (Fig. 2C). The order of EC_{50} values is carvacrol < AITC < eugenol (Fig. 2D).

Carvacrol Stimulates TRPV3 Cation Currents in Cerebral Artery Endothelial Cells. Patch-clamp electrophysiology was used to further investigate the effects of carvacrol on cation influx in endothelial cells. Carvacrol activates both TRPA1 and TRPV3 channels. However, TRPA1 channels desensitize in response to repeated application of carvacrol, whereas TRPV3 channels become sensitized to this agonist in response to recurring administration (Xu et al., 2006). We used these properties to determine which channel is activated by carvacrol in endothelial cells. Endothelial cells were patch-clamped in the conventional whole-cell configuration using intracellular and extracellular solutions that are symmetrical for total cation concentration. Mean endothelial cell capacitance was 20.0 $\pm$ 1.4 pF ($n = 9$). Under these conditions, initial application of carvacrol (100 μM) activated a dually rectifying current that reversed near 0 mV. This current returned to baseline when carvacrol was washed from the

recording bath and increased in magnitude in response to subsequent administrations (Fig. 3, A and B). These properties are consistent with those reported for TRPV3. We used pharmacological inhibitors of TRP channel activity to further characterize the carvacrol-induced current. HC-030031 is a selective blocker of TRPA1 currents with an IC_{50} of 0.7 $\pm$ 0.1 μM. At the concentration used for this study (3 μM), HC-030031 does not block TRPV1, TRPV3, or TRPV4 channels (McNamara et al., 2007). Selective TRPV3 blockers are not currently available, but TRPV1-V4 channels are blocked by the organic polycationic compound ruthenium red (RuR). HC-030031 did not alter carvacrol-induced currents, whereas RuR (10 μM) blocked ~50% of the carvacrol-induced current (Fig. 3C). These data indicate that functional TRPV3 channels are present in cerebral artery endothelial cells.

Carvacrol Causes Endothelium-Dependent Vasodilation of Cerebral and Cerebellar Arteries. Isolated posterior cerebral and superior cerebellar arteries were used to examine the effects of carvacrol on vasomotor responsiveness. Vessels were loaded with the ratiometric Ca^{2+} indicator dye fura-2-AM, pressurized to 70 mm Hg, and allowed to develop spontaneous myogenic tone. The addition of carvacrol to the superfusion bath caused concentration-dependent ($EC_{50} = 4.1$ μM) vasodilation (Fig. 4, A and B). Carvacrol-induced dilation was associated with a corresponding decrease in vessel wall $[Ca^{2+}]$, representative of smooth muscle intracellular $[Ca^{2+}]$ (Fig. 4A). Vessel wall $[Ca^{2+}]$ was significantly lower than baseline when arteries were treated with 10 μM carvacrol (340:380 ratio = 1.877 $\pm$ 0.076 before carvacrol versus 1.823 $\pm$ 0.072 after; $P < 0.05$, $n = 5$). The vasodilatory response was both reversible and reproducible. Arteries treated with carvacrol redeveloped myogenic tone after the drug was washed out of the superfusion bath, and dilation in response to a second application did not differ from the first (Supplemental Fig. 6). All subsequent experi-
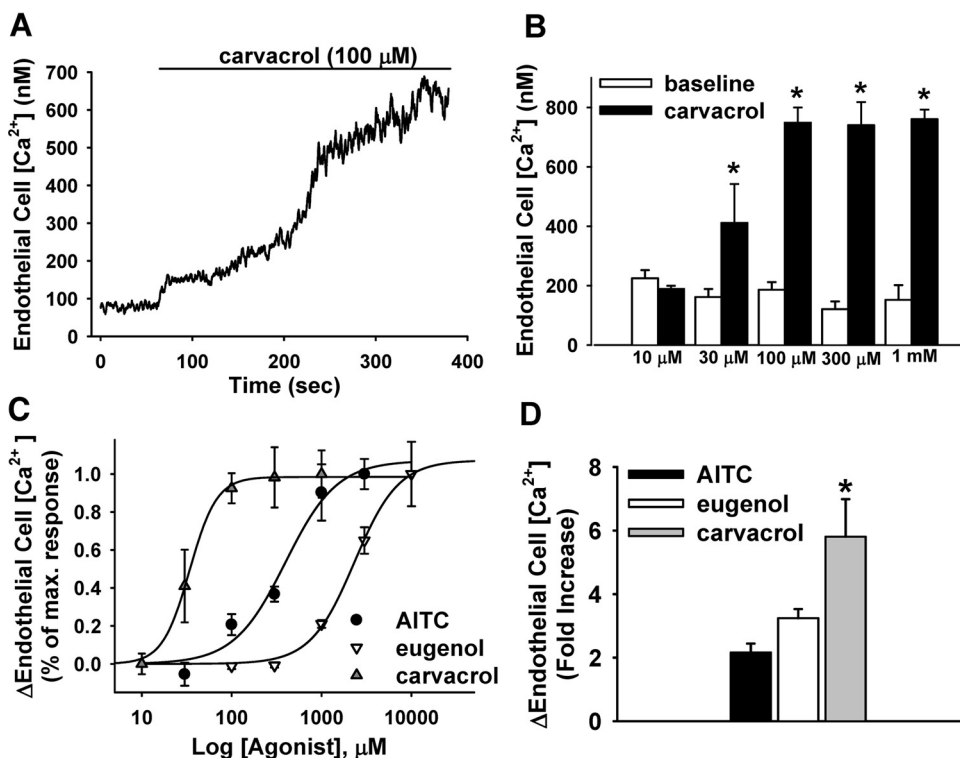

Fig. 2. Carvacrol elicits robust increases in intracellular $[Ca^{2+}]$ in native endothelial cells. A, representative recording of increases in endothelial cell $[Ca^{2+}]$ in response to carvacrol (100 μM). B, endothelial cell $[Ca^{2+}]$ before and after administration of carvacrol. $*$, $P \geq 0.05$ versus baseline; $n = 3$ to 15 cell per group. C, fold increase in endothelial intracellular $[Ca^{2+}]$ in response to AITC (1000 μM; $n = 24$), eugenol (3000 μM; $n = 15$), or carvacrol (100 μM, $n = 14$). $*$, $P \leq 0.05$ versus AITC and eugenol. D, change in endothelial cell $[Ca^{2+}]$ as a function of agonist concentration for AITC, eugenol, and carvacrol. Data are normalized to maximum response. EC_{50} values: carvacrol, 34 μM; AITC, 400 μM; and eugenol, 2.3 mM.

ments were performed in paired fashion and were analyzed using unpaired t tests or repeated-measures analysis of variance, as appropriate.

Carvacrol-induced arterial dilation was recorded before and after the endothelium was damaged by passing air through the lumen. Carvacrol-induced dilation was nearly abolished after disruption of the endothelium, demonstrating that functional endothelial cells are critical for this response (Fig. 4, C and

D). In some arteries, transient constriction to carvacrol was observed after disruption of the endothelium (Fig. 4C). These findings suggest that carvacrol-induced increases in endothelial cell $[Ca^{2+}]$ are responsible for arterial dilation. However, the EC_{50} for carvacrol-induced vasodilation of endothelium-intact arteries (4.1 μM) is approximately an order of magnitude less than that of carvacrol-induced endothelial cell Ca^{2+} influx (34 μM). It is possible that this disparity results from
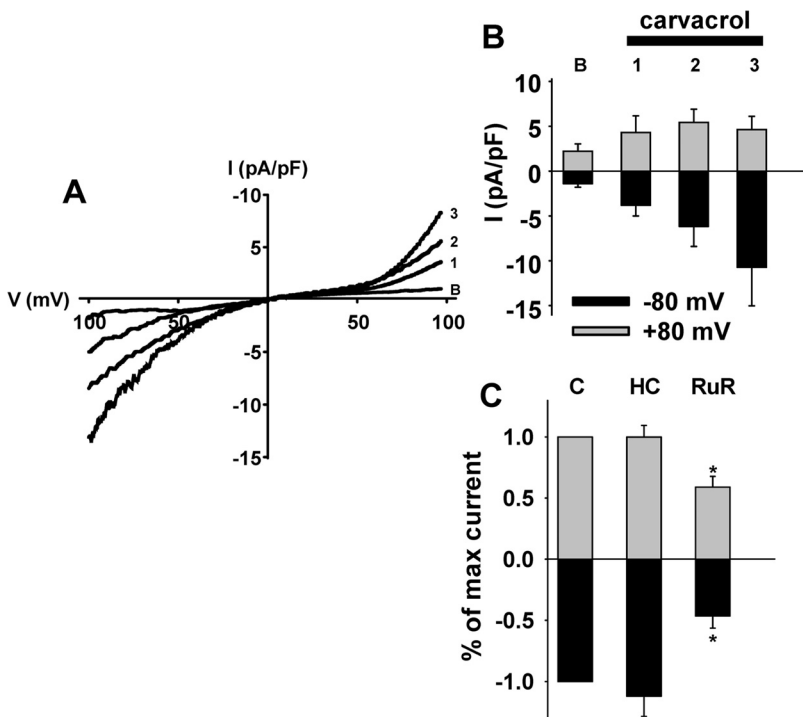

Fig. 3. Carvacrol stimulates TRPV3 cation currents in cerebral artery endothelial cells A, representative whole-cell currents recorded from human microvascular cerebral artery endothelial cells during voltage ramps from −100 to +100 mV. Current density is shown for cells under baseline conditions (B) and after first (1), second (2), and third (3) application of carvacrol (100 μM). B, summary data demonstrating sensitization of the carvacrol-induced current ($n = 6$). C, summary data showing the effects of the TRPA1 antagonist HC-030031 (HC, 3 μM) and the TRPV1–4 blocker RuR (10 μM) on carvacrol-induced currents recorded from cerebral artery endothelial cells; $n = 5$ for HC-030031, $n = 4$ for ruthenium red. Current magnitude was normalized to peak carvacrol-induced currents. ∗, $P \leq 0.05$ versus control (C).
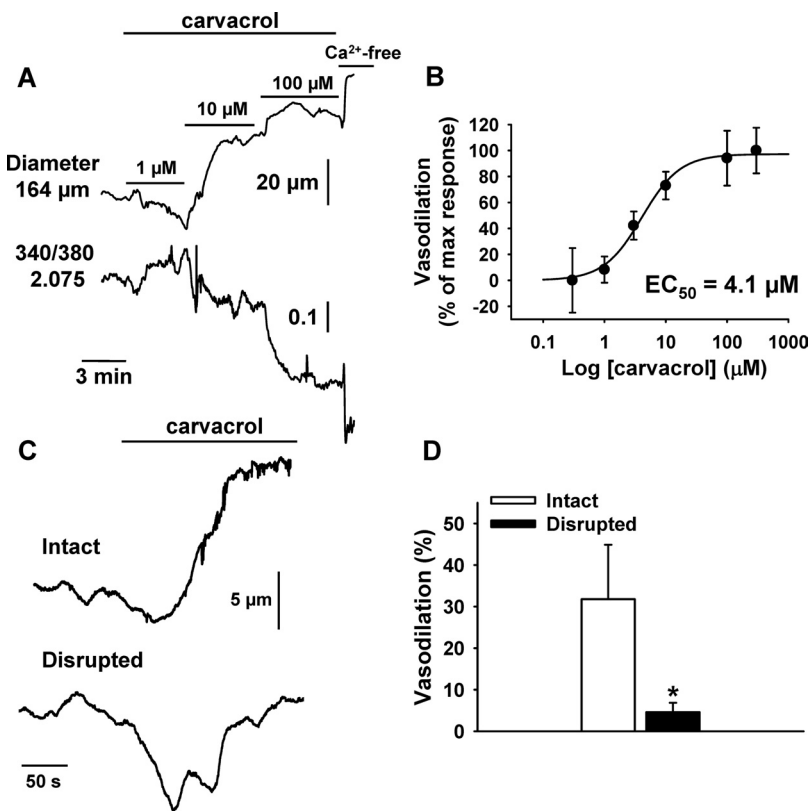
Fig. 4. Carvacrol causes endothelium-dependent dilation of cerebral and cerebellar arteries. A, representative recording of simultaneous changes in luminal diameter and vessel wall $[Ca^{2+}]$ (expressed as the 340:380 ratio) in response to increasing concentrations of carvacrol. B, vasodilation normalized to the maximal response as a function of carvacrol concentration; $EC_{50} = 4.1$ μM. C, representative recording of carvacrol-induced dilation before and after the endothelium was disrupted by passage of air and distilled water through the lumen. D, effects of endothelial disruption on carvacrol-induced dilation. ∗, $P \leq 0.05$ versus endothelium-intact; $n = 5$.

temperature differences between the Ca²⁺-imaging experiments (performed at room temperature, ~20°C) and isolated vessel experiments (performed at 37°C). Consistent with this possibility, TRPV3-dependent cation currents and Ca²⁺ influx are reportedly significantly greater at 37°C compared with 20°C (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002). Thus, it is likely that carvacrol-induced increases in endothelial intracellular [Ca²⁺] are responsible for arterial dilation.

Increases in endothelial cell [Ca²⁺] augment the production of endothelial-derived vasodilatory factors such as nitric oxide and prostacyclin. Blockade of NOS with N^{ω}-nitro-L-arginine (L-NNA, 300 μM) or combined blockade of the NOS and COX pathways (L-NNA, 300 μM and indomethacin, 10 μM) tended to elevate myogenic tone (myogenic tone = $20.5 \pm 6.9\%$ under basal condition, $34.2 \pm 3.0\%$ after L-NNA treatment, and $43.0 \pm 10.0\%$ after treatment with L-NNA

and indomethacin, $n = 4$), but these differences did not reach statistical significance for this sample size. NOS and NOS + COX inhibition did not alter carvacrol-induced vasodilation (Supplemental Fig. 7). Although these findings do not rule out the possibility that carvacrol administration increases the production of nitric oxide or prostacyclin, these results show that carvacrol can cause arterial dilation by a pathway that is independent of NOS and COX activity.

Increases in endothelial cell [Ca²⁺] can also induce vasodilation by activating Ca²⁺-activated K⁺ channels (Coleman et al., 2001a,b; Taylor et al., 2003). The resulting hyperpolarization of the endothelial cell membrane in turn hyperpolarizes underlying smooth muscle by a mechanism involving myoendothelial gap junctions (Fukuta et al., 1999; Sandow and Hill, 2000). Consistent with this mechanism, we find that carvacrol-induced arterial dilation is associated with a
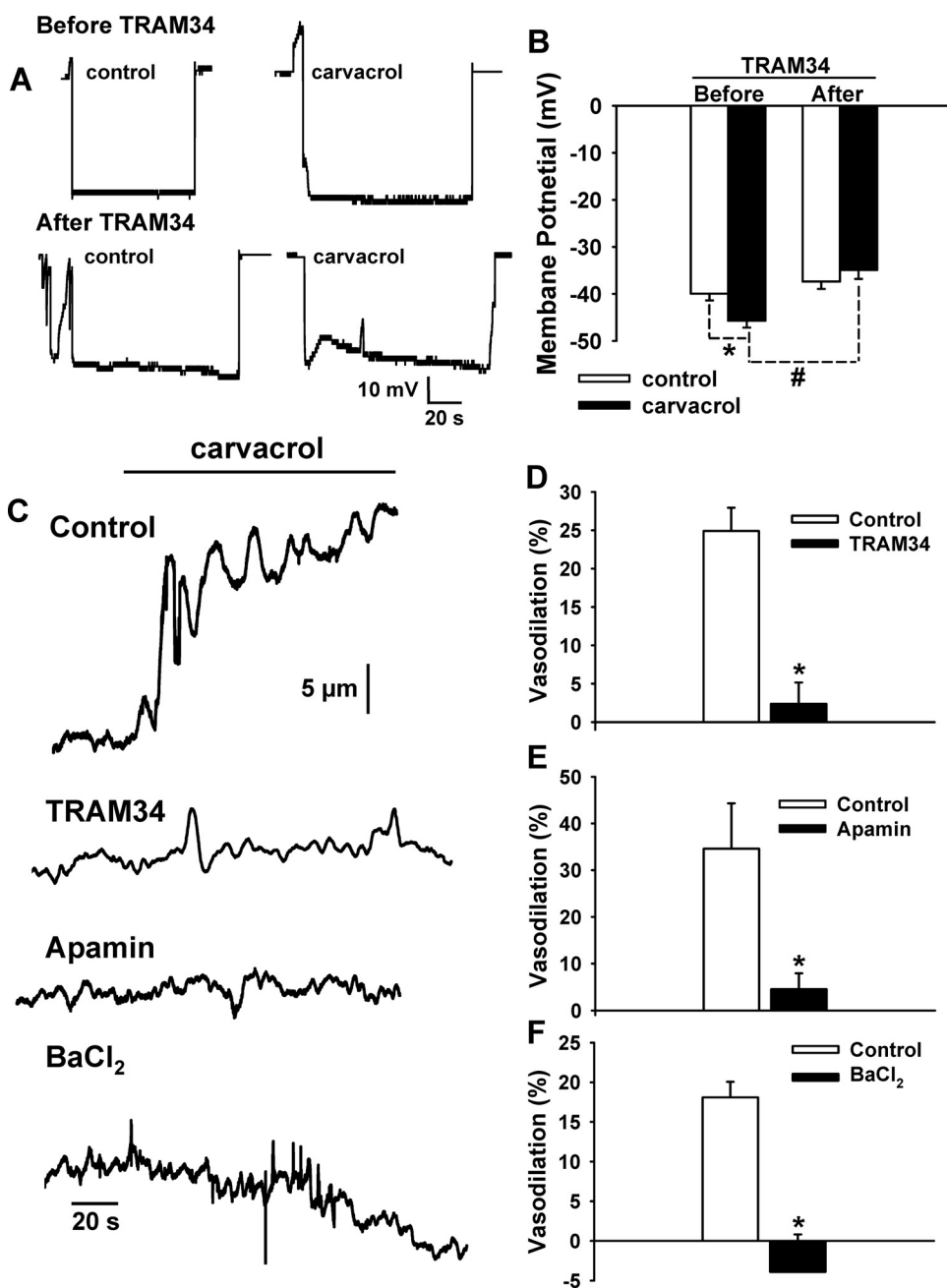

Fig. 5. Carvacrol-induced smooth muscle cell hyperpolarization and vasodilation requires IK_{Ca}, SK_{Ca}, and K_{IR} channel activity. A, representative recordings of smooth muscle membrane potential in isolated arteries pressured to 70 mm Hg under control conditions and in the presence of carvacrol (10 μM) before and after TRAM34 (1 μM) was introduced into the lumen. B, effects of luminal administration of TRAM34 (1 μM) on carvacrol-induced smooth muscle hyperpolarization. ∗, $P \leq 0.05$ versus control; #, $P \leq 0.05$ versus carvacrol-treated in the presence of TRAM34, $n = 5$. C, representative recordings of carvacrol (10 μM)-induced dilation under control conditions, when TRAM34 (1 μM) or apamin (1 μM) is present in the lumen, or when BaCl₂ (30 μM) is present in the superfusion bath. D, effects of luminal administration of TRAM34 (1 μM) on carvacrol-induced dilation. ∗, $P \leq 0.05$ versus control, $n = 6$. E, effects of luminal administration of apamin (1 μM) on carvacrol-induced dilation. ∗, $P \leq 0.05$ versus control, $n = 7$. F, effects of BaCl₂ (30 μM) in the superfusion bath on carvacrol-induced dilation. ∗, $P \leq 0.05$ versus control, $n = 5$.

decrease in vessel wall $[Ca^{2+}]$, suggesting that carvacrol hyperpolarizes the smooth muscle cell plasma membrane, resulting in diminished Ca^{2+} influx via voltage-dependent Ca^{2+} channels. To further examine this possibility, the effects of carvacrol on the membrane potential of smooth muscle cells in posterior cerebral and superior cerebellar arteries pressured to 70 mm Hg was recorded using intracellular microelectrodes. We find that smooth muscle cell membrane potential is hyperpolarized after carvacrol (10 μM) administration (-40.0 ± 1.4 versus -45.7 ± 1.5 mV; $n = 5$) (Fig. 5, A and B). At these membrane potentials, the voltage sensitivity of cerebral artery smooth muscle in vessels pressurized to 60 mm Hg is estimated to be 7.5 μm/mV (Knot and Nelson, 1998); small changes in membrane potential can cause large changes in arterial diameter. Carvacrol administration does not alter smooth muscle membrane potential when TRAM34 (1 μM), an inhibitor of intermediate conductance (IK_{Ca}) Ca^{2+}-activated K^+ channels, is present in the arterial lumen (-37.3 ± 1.5 versus -34.9 ± 1.9 mV; $n = 5$) (Fig. 5, A and B). TRAM34 also blocks vasodilation in response to carvacrol (10 μM) (Fig. 5, C and D), demonstrating that IK_{Ca} channel activity is required for both smooth muscle cell hyperpolarization and arterial dilation. Resting membrane potential is not altered by treatment with TRAM34 under control conditions or basal myogenic tone However, myogenic tone tends to be elevated after luminal administration of apamin (1 μM), a blocker of small-conductance (SK_{Ca}) Ca^{2+}-activated K^+ channels (myogenic tone = $20.3 \pm 4.6\%$ before apamin versus $29.1 \pm 4.5\%$ after; $n = 7$, $P = 0.07$, unpaired t test). Apamin also significantly attenuates carvacrol-induced dilation (Fig. 4, C and E). These findings demonstrate that carvacrol elicits arterial dilation by hyperpolarizing the sarcolemma via a pathway that requires the activity of both IK_{Ca} and SK_{Ca} channels.

Inwardly rectifying K^+ (K_{IR}) channel activity is associated with smooth muscle cell hyperpolarization and vasodilation after activation of endothelial cell IK_{Ca} and SK_{Ca} channels. These channels are activated by membrane hyperpolarization and can amplify a smooth muscle hyperpolarizing stimulus generated in the endothelium (Smith et al., 2008). To examine a potential role for K_{IR} channels in carvacrol-induced dilation, the response was recorded before and after

$BaCl_2$ (30 μM), a selective blocker of K_{IR} channels in cerebral artery smooth muscle cells (Knot et al., 1996), was added to the superfusion bath solution. This treatment increased basal myogenic tone (myogenic tone = $24.5 \pm 4.81\%$ before $BaCl_2$ versus $31.0 \pm 3.9\%$ after, $n = 5$). In addition, we found that in the presence of $BaCl_2$, carvacrol-induced dilation is absent, and in some arteries, slight vasoconstriction occurred in response to carvacrol administration (Fig. 5, C and F), indicating that K_{IR} activity is required for the vasodilatory response.

TRPV3 Activity Is Sufficient for Carvacrol-Induced Dilation. The respective roles of TRPA1 and TRPV3 channels in carvacrol-induced vasodilation were examined using a pharmacological approach. When present in the arterial lumen, the TRPA1 inhibitor HC-030031 blocks vasodilation in response to AITC (Earley et al., 2009). In agreement with our previous report (Earley et al., 2009), we found that myogenic tone was significantly elevated when HC-030031 (3 μM) is present in the lumen of pressurized cerebral arteries (myogenic tone = $19.2 \pm 4.2\%$ before versus $26.5 \pm 4.9\%$ after, $n = 5$). However, pretreatment with HC-030031 does not alter carvacrol-induced vasodilation (Fig. 6, A and B), demonstrating that carvacrol can cause arterial dilation in the absence of TRPA1 activity. Basal myogenic tone is elevated when the TRPV1–4 blocker RuR (10 μM) is present in the arterial lumen (myogenic tone = $17.7 \pm 3.9\%$ before versus $24.4 \pm 3.0\%$ after, $n = 6$). In addition, carvacrol-induced dilation is significantly attenuated in the presence of RuR (Fig. 6, A and C), suggesting that TRPV channel activity is required for the response. Functional TRPV3 channels are present in the endothelium (Fig. 3), and carvacrol does not activate TRPV2 or TRPV4 channels in patch-clamp experiments (Xu et al., 2006). Furthermore, TRPV1, TRPV5, and TRPV6 channels are not detected in total RNA extracted from cerebral arteries (Inoue et al., 2006). Therefore, we conclude that carvacrol causes arterial dilation by activating Ca^{2+} influx through TRPV3 channels in the endothelium.

Discussion

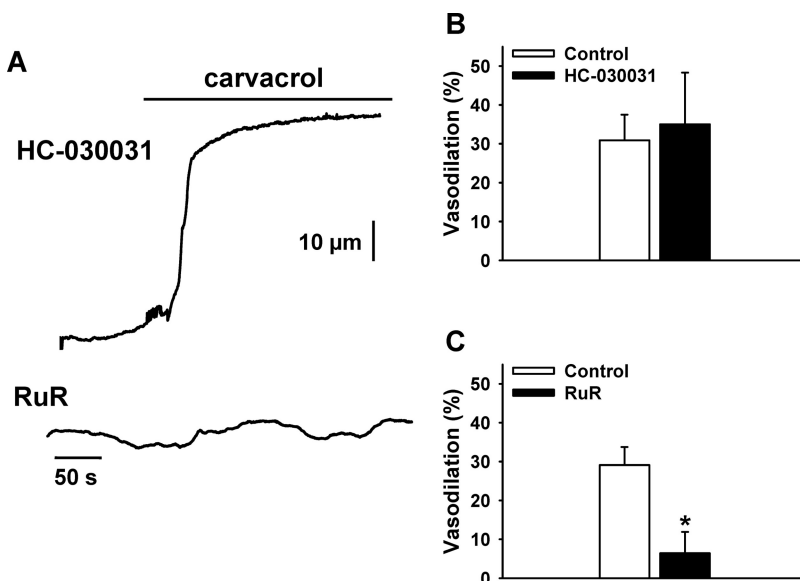Our results demonstrate that carvacrol, a dietary agonist of TRPA1 and TRPV3 channels, is a potent endothelium-depen-

Fig. 6. Carvacrol-induced dilation requires TRPV but not TRPA1 activity. A, representative recordings of carvacrol (10 μM)-induced dilation after the TRPA1 blocker HC-030031 (3 μM) or the TRPV blocker RuR (10 μM) was introduced to the lumen. B, effects of luminal administration of HC-030031 (3 μM) on carvacrol-induced dilation, $n = 5$. There were no significant differences. C, effects of luminal administration of RuR (10 μM) on carvacrol-induced dilation. *, $P \leq 0.05$ versus control, $n = 5$.

dent dilator of cerebral arteries. We propose that carvacrol causes Ca^{2+} influx via TRPV3 channels in the endothelium. Increases in endothelial intracellular $[Ca^{2+}]$ activate IK_{Ca} and SK_{Ca} channels, hyperpolarizing the plasma membrane of endothelial cells and underlying smooth muscle. K_{IR} channels in smooth muscle cells amplify this initial hyperpolarization, ultimately resulting in vasodilation. The major findings that support this conclusion are as follows: 1) functional TRPV3 channels are present in the endothelium of cerebral and cerebellar arteries; 2) carvacrol causes a robust increase in endothelial cell $[Ca^{2+}]$; 3) carvacrol is a potent endothelium-dependent dilator of isolated cerebral and cerebellar arteries; 4) vasodilation in response to carvacrol is associated with hyperpolarization of the smooth muscle cell plasma membrane; 5) carvacrol-induced vasodilation is independent of NOS and COX activity but is inhibited by blockade of IK_{Ca}, SK_{Ca}, and K_{IR} channels; and 6) carvacrol-induced dilation is not altered by inhibition of TRPA1 channels but is attenuated by the blockade of TRPV channels with RuR. This is the first study to demonstrate that functional TRPV3 channels are present in endothelial cells and contribute to endothelium-dependent vasodilation. Furthermore, our results suggest that dietary intake of carvacrol could have cardioprotective benefits.

The endothelium controls functions that are vital for normal vascular health, including the regulation of vascular wall permeability and production of antithrombotic and vasoactive factors. Changes in the intracellular $[Ca^{2+}]$ influence all of these critical properties. Endothelial cell Ca^{2+} handling is not fully understood, and a recent report of dynamic, spatially restricted signaling events (Ledoux et al., 2008) in this tissue demonstrates complexity. Prior studies show that TRP channels, including TRPC4 (Freichel et al., 2001), TRPV4 (Köhler et al., 2006; Marrelli et al., 2007; Vriens et al., 2005;), and TRPA1 (Earley et al., 2009), are involved in the regulation of endothelial cell $[Ca^{2+}]$ and function. Our results demonstrate that TRPV3 is also a Ca^{2+} influx channel in native cerebral artery endothelial cells and is an important regulator of vascular tone. Consistent with this physiological role, TRPV3 is modestly selectivity for Ca^{2+} versus Na^+ ions ($\sim$10:1) and has a single-channel conductance of 170 pS, larger than other TRPV channels. TRPV3 was first identified as a "warm" temperature-sensitive channel (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002). TRPV3 knockout mice display impaired thermosensation (Moqrich et al., 2005), but the cardiovascular phenotype of these mice has not been reported. TRPV3 is present in numerous tissues, including skeletal muscle, gastrointestinal tract, skin, tongue, dorsal root ganglion, spinal cord, and brain (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002). In addition, low-level expression of TRPV3 mRNA has been detected in rat aorta and intralobar pulmonary artery (Yang et al., 2006), although the function of the channel in these vascular beds has not been reported. In cerebral arteries, we find that activation of TRPV3 causes endothelium-dependent vasodilation, suggesting that the channel is an important contributor to vascular function and health.

Endothelial dysfunction, leading to diminished production of vasodilatory and anticlotting factors, is commonly associated with cardiovascular diseases such as hypertension and atherosclerosis. Endothelial cells line the lumen of all vessels and are directly exposed to molecules circulating in the blood.

Our prior work shows that TRPA1 channels promote vasodilation in response to AITC (Earley et al., 2009), a compound derived from mustard oil, whereas the current results show that TRPV3 channels promote arterial dilation in response to carvacrol, a major component of oregano. These findings demonstrate that TRP channels present in the endothelium can act as receptors for dietary molecules involved in the regulation of vascular tone. We find that carvacrol is acting through TRPV3 and not TRPA1, probably indicating that TRPV3 has a higher affinity for the compound. In addition, we found that carvacrol is much more potent in provoking Ca^{2+} entry in the endothelium than either AITC or eugenol. It is interesting that our results show that carvacrol causes increases in endothelial cell $[Ca^{2+}]$ and vasodilation of cerebral arteries at concentrations much lower than that reported for activation of TRPV3 currents in *Xenopus laevis* oocytes expressing the channel (Vogt-Eisele et al., 2007). The reason for this difference in sensitivity is not clear, but substances, including arachidonic acid and other unsaturated fatty acids (Hu et al., 2006) and 2-aminoethoxydiphenyl borate (Chung et al., 2004), have been shown to potentiate TRPV3 currents. It is possible that endogenous factors present in the endothelium act in a similar manner to sensitize TRPV3 channels and promote Ca^{2+} influx. This apparent enhanced sensitivity to carvacrol in the endothelium may have important implications. Carvacrol composes up to 65% of the total mass of the essential oil of oregano (Sarer et al., 1982), and if it is efficiently absorbed by the digestive tract, our results suggest that consumption of less than 10 mg is sufficient to provoke a vasodilatory response. Thus, our findings are consistent with the conclusion that diets including reasonable amount of oregano could improve vascular health by promoting endothelium-dependent arterial relaxation and reduction of vascular resistance and systemic blood pressure. In addition, our study suggests that TRPV3 activators may be useful in treating cardiovascular diseases involving endothelial dysfunction.

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