Transient Receptor Potential Vanilloid Type 4–Deficient Mice Exhibit Impaired Endothelium-Dependent Relaxation Induced by Acetylcholine In Vitro and In Vivo

David X. Zhang, Suelhem A. Mendoza, Aaron H. Bubolz, Atsuko Mizuno, Zhi-Dong Ge, Rongshan Li, David C. Warltier, Makoto Suzuki, David D. Gutterman

Abstract—Agonist-induced Ca\(^{2+}\) entry is important for the synthesis and release of vasoactive factors in endothelial cells. The transient receptor potential vanilloid type 4 (TRPV4) channel, a Ca\(^{2+}\)-permeable cation channel, is expressed in endothelial cells and involved in the regulation of vascular tone. Here we investigated the role of TRPV4 channels in acetylcholine-induced vasodilation in vitro and in vivo using the TRPV4 knockout mouse model. The expression of TRPV4 mRNA and protein was detected in both conduit and resistance arteries from wild-type mice. In small mesenteric arteries from wild-type mice, the TRPV4 activator 4α-phorbol-12,13-didecanoate increased endothelial [Ca\(^{2+}\)]\(_i\) in situ, which was reversed by the TRPV4 blocker ruthenium red. In wild-type animals, acetylcholine dilated small mesenteric arteries that involved both NO and endothelium-derived hyperpolarizing factors. In TRPV4-deficient mice, the NO component of the relaxation was attenuated and the endothelium-derived hyperpolarizing factor component was largely eliminated. Compared with their wild-type littermates, TRPV4-deficient mice demonstrated a blunted endothelial Ca\(^{2+}\) response to acetylcholine in mesenteric arteries and reduced NO release in carotid arteries. Acetylcholine (5 mg/kg, IV) decreased blood pressure by 37.0±6.2 mm Hg in wild-type animals but only 16.6±2.7 mm Hg in knockout mice. We conclude that acetylcholine-induced endothelium-dependent vasodilation is reduced both in vitro and in vivo in TRPV4 knockout mice. These findings may provide novel insight into mechanisms of Ca\(^{2+}\) entry evoked by chemical agonists in endothelial cells. (Hypertension. 2009;53:532-538.)

Key Words: transient receptor potential ■ endothelium ■ endothelium-derived factors ■ NO ■ calcium

A variety of agonists such as acetylcholine, bradykinin, and even mechanical stimuli induce a rapid increase in endothelial Ca\(^{2+}\), leading to the synthesis and release of relaxing factors, including NO, prostacyclin, and endothelium-derived hyperpolarizing factors (EDHFs).\(^1\) In endothelial and other mammalian cells, the Ca\(^{2+}\) increase is usually a consequence of Ca\(^{2+}\) release from intracellular stores of the endoplasmic reticulum and Ca\(^{2+}\) influx through Ca\(^{2+}\)-permeable cation channels in the plasma membrane via store-operated or receptor-operated mechanisms.\(^2\) The influx of Ca\(^{2+}\) from the extracellular space contributes to the sustained increase of the cytosolic Ca\(^{2+}\) concentration. Despite the importance of calcium entry in the synthesis of endothelial relaxing factors, the proximate cause of this critical signaling event remains elusive.

The discovery of transient receptor potential (TRP) channels provides new insights into potential mechanisms of Ca\(^{2+}\) entry in endothelial cells. TRP channel–mediated Ca\(^{2+}\) entry has been implicated in diverse responses, including changes in vascular permeability, angiogenesis, vascular remodeling, and vasorelaxation.\(^3,4\) Of many subtypes of TRP channels expressed in endothelial cells, TRP vanilloid type 4 (TRPV4) channels have received increasing attention. These channels are widely expressed in vascular endothelial cells of several species and activated by both chemical and physical stimuli, including hypotonic cell swelling,\(^5,6\) moderate heating (>27°C),\(^7,8\) shear stress,\(^9\) and the synthetic phorbol-derivative 4α-phorbol-12,13-didecanoate (4α-PDD),\(^10\) as well as arachidonic acid and its metabolites.\(^11,12\) The TRPV4 channel has also been implicated in the release of endothelium-derived relaxing factors and regulation of vascular tone.\(^13-16\)

Study of TRP channels in endothelial cells has been challenging because of the lack of specific channel blockers and coexpression of multiple TRP channels in the endothelium. Recently, 2 lines of TRPV4-deficient mice have been generated and found to exhibit phenotypic changes in several body systems, such as altered regulation of systemic tonicity, defects in the alveolar barrier, deficits in renal tubular K\(^{+}\)...
secretion, and blunted arterial shear response.\textsuperscript{15–20} Using this knockout mouse model, the present study examined the role of TRPV4 channels in agonist-induced endothelial Ca\textsuperscript{2+} signaling and endothelium-dependent vasodilation. Both in vitro and in vivo vascular responses were examined.

**Methods**

An expanded Methods section is available in the online data supplement at http://hyper.ahajournals.org.

**Animals**

Fifty-two male TRPV4 knockout (TRPV4\textsuperscript{−/−}) and 60 male wild-type (WT) C57BL/6J mice at 2 to 4 months of age were used in this study. All of the experiments were conducted in accordance with the Institutional Animals Care and Use Committee guidelines.

**RNA Extraction and RT-PCR**

Total RNA from vascular tissues was extracted with TRIzol, and cDNA was synthesized, followed by PCR amplification of TRPV4 and platelet/endothelial cell adhesion molecule 1 fragments using gene-specific primers.

**Western Blot Analysis**

Protein samples (20 \(\mu\)g) were subjected to 10% SDS-PAGE, and membranes were blotted with a polyclonal antibody against TRPV4 (1:1000 dilution; MBL International), followed by peroxidase-conjugated secondary antibodies. To ensure equal protein loading, the blots were reprobed with a polyclonal anti-endothelial NO synthase antibody (1:1000 dilution; BD Transduction Laboratories).

**Immunohistochemistry**

Frozen tissue sections were incubated with a polyclonal antibody against TRPV4 (1:100 dilution; Alomone Laboratories), followed by a goat antirabbit IgG conjugated with Alexafluor 568. Images were captured using a regular fluorescence microscope.

**Measurement of Intracellular Ca\textsuperscript{2+}**

Endothelial intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) was measured in situ in freshly isolated mesenteric arteries using Fura-2, as we described previously.\textsuperscript{21}

**Measurement of Endothelial NO**

The fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein or diaminofluorescein-FM (DAF-FM) diacetate was used to measure endothelial NO in situ in freshly isolated carotid arteries.\textsuperscript{21}

**Isometric Tension Recording**

Small mesenteric arteries (first-order branch from superior mesenteric artery, \(\sim 200 \mu\)m) were dissected and mounted in a wire myograph, as described previously.\textsuperscript{22}

**Measurement of Vascular Responses In Vivo**

TRPV4\textsuperscript{−/−} and WT mice were anesthetized with 12% urethane (1.2 g/kg body weight, IP) or ketamine/xylazine (50 mg/kg/10 mg/kg, IP). The right common carotid artery was cannulated for measurement of arterial blood pressure and the tail vein for drug administration. Heart rate was monitored by ECG at the V6 position. All of the drugs were given as a single IV bolus, including acetylcholine.
Data Analysis

Data are presented as means±SEMs. Significant differences between mean values were evaluated by Student t test or ANOVA followed by the Student’s-Newman-Keuls multiple comparison test. A value of P<0.05 was considered statistically significant.

Results

TRPV4 Expression in Conduit and Resistance Arteries

The loss of the TRPV4 gene in TRPV4−/− mice was confirmed by genotyping with PCR amplification of genomic DNA (Figure 1A). TRPV4 transcripts and proteins were detected in aorta and carotid and mesenteric arteries of WT but not TRPV4−/− mice (Figures 1B and 1C). The TRPV4 antibody detected 2 bands of ≈95 and ≈110 kDa in WT mice. The 95-kDa band is in good agreement with the calculated molecular weight of the unprocessed TRPV4 protein (98 kDa). The 110-kDa band presumably represents the glycosylated form of the TRPV4 protein.23 Immunohistochemical analysis revealed a strong staining for TRPV4 in the endothelium of WT aortic sections (Figure 1D). There was much less immunofluorescence in underlying smooth muscles. Hematoxylin and eosin staining confirmed an intact vascular structure of tissue sections from WT and TRPV4−/− mice.

We examined the TRPV4-mediated Ca2+ response in the endothelium in situ of isolated mesenteric arteries. As shown in Figure 2, infusion of 4α-PDD (1 μmol/L), a specific TRPV4 channel opener, elicited a rapid increase in [Ca2+]i in endothelial cells of WT mice (Δ[Ca2+]i; 105.8±13.2 nmol/mL). This response was rapidly reversed by the addition of ruthenium red (10 μmol/L), a TRPV4 channel blocker (Δ[Ca2+]i; 30.4±3.3 nmol/mL). Preincubation of arteries with ruthenium red also prevented endothelial Ca2+ response to 4α-PDD, whereas ruthenium red itself had no significant effect on basal [Ca2+]i in WT or TRPV4−/− mice (data not shown). 4α-PDD did not induce significant Ca2+ influx in mesenteric arteries of TRPV4−/− mice (Δ[Ca2+]i; 11.2±1.0 nmol/mL). Removal of the endothelium at the end of experiments abolished the fluorescence, confirming that the measured fluorescence is specific to endothelial cells.

TRPV4 in Agonist-Induced Vasodilation In Vitro

In mouse mesenteric arteries, acetylcholine elicited concentration-dependent relaxations (maximal dilation: 93.3±2.2%; −logEC50: 7.7±0.1; Figure 3A). Pretreatment of arteries with a NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (l-NAME), markedly inhibited acetylcholine-induced relaxations (maximal dilation: 34.8±4.7%; −logEC50: 6.7±0.3). The addition of the cyclooxygenase inhibitor indomethacin had no further effect (maximal dilation: 40.8±4.7%; −logEC50: 6.3±0.5). The residual dilation in the presence of l-NAME and indomethacin was abolished by high K+ (maximal dilation: 5.3±9.3%). These results confirm the involvement of both NO and K+ channels (or EDHF) in acetylcholine-induced relaxations.

Compared with WT mice, acetylcholine-induced vasodilation was significantly reduced in TRPV4−/− mice (maximal dilation: 49.9±8.3% versus 88.1±3.5% for WT; −logEC50: 6.6±0.3 versus 7.2±0.1 for WT; Figure 3B). l-NAME largely eliminated acetylcholine-induced vasodilation in TRPV4−/− animals (maximal dilation of 9.0±2.3%). Endothelium-independent dilation to papaverine was similar in TRPV4−/− and WT mice (maximal dilation: 94.8±1.1% and 98.8±0.6%, respectively; Figure 3C). Furthermore, there was no difference in contractile responses to U46619 or high K+ between those animals (data not shown). 4α-PDD (1 μmol/L) also induced marked relaxations of intact but not denuded mesenteric arteries, with maximal relaxations of 87.9±5.0% and 1.0±2.5%, respectively (n=4 vessels from 4 mice).

Figure 2. TRPV4-mediated Ca2+ responses in endothelial cells in situ of mouse mesenteric arteries. A, Representative images of endothelial Ca2+ measured with the fluorescence Ca2+ indicator Fura-2. Mesenteric arteries of WT and TRPV4−/− (KO) mice were isolated and cannulated, and 4α-PDD (1 μmol/L), a specific TRPV4 agonist, was infused into the lumen of arteries, followed by addition of the TRPV4 blocker ruthenium red (RuR; 10 μmol/L) into the bath. The endothelium was removed [EC(−)] at the end of experiments to confirm that the fluorescence is specific to endothelial cells. B, Representative traces of Ca2+ response to 4α-PDD and/or ruthenium red in WT and TRPV4−/− mice. C, Summarized data of endothelial [Ca2+]i increase. *P<0.05 vs 4α-PDD in WT (n=5 to 7 mice, 20 to 30 endothelial cells per each vessel).
Blood Pressure Response to Agonists

Resting arterial pressures and heart rates were similar in TRPV4<sup>−/−</sup> and WT mice, with mean values of 82.5±3.5 versus 92.3±8.3 mm Hg (P=0.2; n=14 and 10, respectively), and 497±28 versus 499±31 bpm (P value not significant), respectively. The systolic and diastolic blood pressures in TRPV4<sup>−/−</sup> and WT mice were 69.3±4.8 and 99.5±2.9 mm Hg and 75.1±5.6 and 112.6±11.9 mm Hg (P value not significant for both), respectively. Intravenous acetylcholine acutely reduced blood pressure in WT mice (mean change: 37.0±6.2 mm Hg 3 to 5 minutes after acetylcholine injection; Figure 4A). This response was significantly blunted in TRPV4<sup>−/−</sup> mice (mean reduction: 16.6±2.7 mm Hg). Acetylcholine produced a similar drop in heart rate in TRPV4<sup>−/−</sup> and WT mice (mean changes: 216±48 and 224±61 bpm, respectively; Figure 4B). When animals were matched for baseline blood pressure (>80 mm Hg), the acetylcholine-induced reduction in blood pressure was also significantly lower in TRPV4<sup>−/−</sup> versus WT animals, with mean decreases of 19.1±4.0 and 38.0±7.4, respectively. Baseline MAPs were found to be 91.0±3.3 and 102±5.3 mm Hg for matched TRPV4<sup>−/−</sup> and WT mice, respectively (P=0.1; n=8 for both).

4α-PDD transiently lowered blood pressure in WT but not in TRPV4<sup>−/−</sup> mice, with mean arterial blood pressure changes of 18.8±7.1 and −3.8±1.7 mm Hg, respectively (Figure 4C). Phenylephrine caused similar blood pressure increases in TRPV4<sup>−/−</sup> and WT mice (mean changes: 31.5±3.7 and 38.5±3.8 mm Hg, respectively). Nitroprusside similarly reduced blood pressure in TRPV4<sup>−/−</sup> and WT animals (mean changes: 55.5±7.2 and 61.7±5.5 mm Hg, respectively; n=6 to 8).

TRPV4 in Acetylcholine-Induced Ca<sup>2+</sup> and NO Increase

Acetylcholine induced a rapid increase in endothelial [Ca<sup>2+</sup>], of mesenteric arteries from WT mice, with [Ca<sup>2+</sup>] changes of 47.8±4.4 and 31.2±4.4 nmol/L at peak and 1 minute after peak, respectively (Figure 5). Compared with WT controls, the Ca<sup>2+</sup> response was more transient and of less magnitude in TRPV4<sup>−/−</sup> mice, with [Ca<sup>2+</sup>] changes of 17.9±1.3 and 5.9±0.3 nmol/L at peak and 1 minute after peak, respectively. This is consistent with a role for TRPV4 in endothelial Ca<sup>2+</sup> entry during the plateau phase of Ca<sup>2+</sup> response.

We also measured NO production in vascular tissues of TRPV4<sup>−/−</sup> and WT mice using DAF fluorescence assay. The
Acetylcholine-induced Ca\(^{2+}\) responses in endothelial cells in situ of mesenteric arteries from WT and TRPV4\(^{-/-}\) (KO) mice. A, Representative traces of endothelial Ca\(^{2+}\) measured with the fluorescence Ca\(^{2+}\) indicator Fura-2. Mesenteric arteries of WT and TRPV4\(^{-/-}\) mice were isolated and cannulated, and acetylcholine (1 \(\mu\)mol/L) was added into the bath. B, Summary of endothelial Ca\(^{2+}\) increase at 0 and 1 minute after peak response to acetylcholine. *P<0.05 vs WT (n=5 to 7 mice, 20 to 30 endothelial cells per each vessel).

Discussion

Using a TRPV\(^{-/-}\) mouse model, we provide several lines of evidence supporting TRPV4 channels as novel mediators of agonist-induced, endothelium-dependent vasodilation. For the first time, we found that TRPV4 channels are expressed in both resistance and conduit arteries of mice, and activation of these channels increases endothelial Ca\(^{2+}\) leading to vasodilation in resistance vascular beds. Acetylcholine-induced vasodilator responses in vitro and in vivo are markedly reduced in TRPV4\(^{-/-}\) mice, which is accompanied by blunted Ca\(^{2+}\) and NO responses in endothelial cells. These new findings further extend the functional roles of endothelial TRPV4 channels in the regulation of vascular tone and endothelial Ca\(^{2+}\) signaling.

Consistent with the results of previous studies,\(^{24–26}\) we found that both NO and EDHF contribute to endothelium-dependent relaxation induced by acetylcholine in mouse mesenteric arteries. Compared with WT mice, the l-NAME–sensitive component of acetylcholine-induced relaxation was reduced in TRPV4\(^{-/-}\) mice, whereas the K\(^{+}\)-sensitive relaxation was virtually abolished, indicating that the TRPV4 channel is involved in both NO- and EDHF-dependent vasodilation. The involvement of TRPV4 channels in NO-mediated dilatation was also supported by the observation that acetylcholine-induced NO production was significantly reduced in TRPV4\(^{-/-}\) mice versus WT control mice.
in vascular endothelial cells of TRPV4−/− mice. These results are generally in agreement with previous reports that activation of TRPV4 channels induces NO- and EDHF-dependent vasodilation in rat carotid and gracilis arteries, as well as rat cerebral arteries.13,14

An agonist-induced increase in [Ca2+]i is critical in the synthesis of relaxing factors such as NO and EDHF in endothelial cells.1 However, the [Ca2+]i threshold is higher for EDHF-dependent dilation than for NO-dependent responses.27 Therefore, reduction in endothelial Ca2+ would have a greater effect on EDHF-mediated than NO-mediated relaxation. This may partially explain our findings that TRPV4−/− affected the K+-sensitive relaxation more than the NO-mediated relaxation in small mesenteric arteries, a resistance vascular bed where EDHF-mediated dilation is more prominent. TRPV4 activation and resulting Ca2+ influx may also selectively elicit the generation of EDHF and/or NO through specific signaling systems located in subcellular domains. TRPV4 channels have been shown to form a Ca2+ signaling complex with ryanodine receptors and large-conductance Ca2+-activated K+ channels in vascular smooth muscle cells.28 A recent study has also reported a close association of Ca2+ influx and EDHF-mediated relaxation in the caveolar microdomain of endothelial cells.26

In contrast to blood pressure changes, acetylcholine induced similar drops in the heart rate in TRPV4−/− animals compared with WT control animals, indicating that TRPV4 plays a minimal role in the control of heart rate in these animals. A recent study has also reported that TRPV4 agonists have no significant effect on rate or contractility in the isolated, buffer-perfused rat heart.29

Acetylcholine-induced Ca2+ increase (plateau phase) was reduced but not eliminated in TRPV4−/− mice, indicating that other Ca2+ entry pathways may coexist in vascular endothelial cells. Other TRP channels including TRPC (canonical) and TRPM (Melastatin) subfamilies have been found in endothelial cells.3 Several TRPC channels have been proposed as store-operated Ca2+ channels in response to agonist stimulation.4 A previous study indicates that agonist-induced endothelial Ca2+ current and vasodilatation is reduced in the aorta from TRPC4−/− mice.30 In another recent study, Fleming et al31 reported that bradykinin induces translocation of TRPC6 to the cell membrane and TRP channel-mediated Ca2+ influx in human endothelial cells. Future studies are required to determine whether these TRP channels contribute to the remaining Ca2+ entry in endothelial cells of TRPV4−/− animals.

Immunohistochemical analysis of mouse aorta revealed that TRPV4 channels are mainly expressed in the endothelium. However, a TRPV4 channel has also been found in vascular smooth muscle cells of rat aortic, cerebral, and pulmonary arteries.14,28,32,33 We also found evidence for the TRPV4 protein in human and bovine coronary vascular smooth muscle but in much smaller amounts than in endothelial cells (unpublished observations). Therefore, expression of TRPV4 channels in vascular smooth muscle cells may depend on species and vascular beds. However, denuded mouse mesenteric arteries do not dilate to 4α-PDD; thus, we conclude that any TRPV4 channels in vascular smooth muscle do not contribute to the observations made in this study.

Baseline blood pressure in TRPV4−/− mice was not statistically higher than in their WT controls, as might be expected from reduced release of endothelial relaxing factors. In contrast, a trend toward lower blood pressure was observed in TRPV4−/− animals. These results are consistent with those of a previous study in unanesthetized animals.34 Although not further explored in the current study, the absence of baseline blood pressure change in TRPV4−/− mice could reflect compensatory pressure homeostatic mechanisms that minimize blood pressure changes observed in TRPV4−/− mice. Alternatively, compared with the mesenteric circulation examined in this study, TRPV4 expression and function might be different in other vascular beds. A conditional TRPV4 knockout specific to endothelial cells would help to address this possibility in future studies.

Perspectives

TRPV4 channels are expressed in endothelial cells of various species and vascular beds. Given the complex expression pattern of TRP channels and lack of specific channel blockers, TRPV4−/− mice provide a good model to study molecular and functional properties of endothelial TRPV4 channels in its native cellular environment. Our data suggest that TRPV4 channels, known to be involved in vascular mechanotransduction, are also involved in chemical agonist-induced increases in endothelial Ca2+ and endothelium-dependent vasodilation. However, the cellular mechanisms responsible for TRPV4 activation, ie, via receptor or store-operated mechanism, remain to be determined. Because activation of TRPV4 by channel agonists reduces blood pressure, the endothelial TRPV4 channel might serve as a novel pharmacological target for the treatment of hypertension.13 It will also be of interest to determine whether TRPV4-mediated endothelial responses are altered in other cardiovascular diseases, eg, atherosclerosis, where pharmacological manipulation of channel function might have beneficial therapeutic effects.

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Disclosures

None.

References


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Trpv4-Deficient Mice Exhibit Impaired Endothelium-Dependent Relaxation Induced by Acetylcholine in Vitro and in Vivo

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Short title: TRPV4 and Endothelium-Dependent Relaxation

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Expanded Materials and Methods

Animals. Fifty-two male TRPV4 knockout (TRPV4<sup>-/-</sup>) (1) and sixty male wild-type (WT) C57BL/6J mice were used in this study. The animals were maintained in our animal facility and used at 2-4 months of age. The study was approved by the institutional review committee. All experiments were conducted in accordance with the Institutional Animals Care and Use Committee guidelines. Genotyping was performed by polymerase chain reaction (PCR) with the following primers: TRPV4 (forward 5'-TGT TCG GGG TGG TTT GGC CAG GAT AT-3' and reverse 5'- GCT GAA CCA AAG GAC ACT TGC ATA G-3' ; a 796-bp product in WT and no signal in TRPV4<sup>-/-</sup>), and knockout neomycin cassette (forward 5'- GCT GCA TAC GCT TGA TCC GGC TAC-3' reverse 5'- TAA AGC ACG AGG AAG CGG TCA GCC-3'; a 366-bp product in TRPV4<sup>-/-</sup>).

RNA extraction and RT-PCR. Total RNA from vascular tissues was extracted with TRIzol, and cDNA was synthesized using iScript Reverse Transcriptase Kit (BioRad). Specific TRPV4 and PECAM-1 fragments were amplified by PCR using the following primers: TRPV4 (forward 5'-GAT GGA GGA GAA AGG TCG TG -3' and reverse 5'- GAG AAC TGT CTC CAG GTT GC -3', for a 734-bp fragment [NM_022017]) (2), PECAM-1 (forward 5'-GCA AGA AGC AGG AAG GAC AG-3' and reverse 5'-TGA CAA CCA CCG CAA GAC AG-3' and reverse 5'-TGA CAA CCA CCG CAA GAC AG-3', for a 138-bp fragment [NM_001032378]).

Western blot analysis. Vascular tissues were homogenized in ice-cold lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% NP40) supplemented with a protease inhibitor cocktail (Roche), and centrifuged at 1000 g for 10 min. Protein samples (20 μg) were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blotted with a polyclonal antibody against TRPV4 (1:1000 dilution, MBL International), followed by peroxidase-conjugated secondary antibodies. To ensure equal protein loading, the blots were reprobed with a polyclonal anti-endothelial NO synthase (eNOS) antibody (1:1000 dilution, BD Transduction Laboratories).

Immunohistochemistry. Small segments of mouse vessels were embedded and frozen in OTC compound and cut into 10 μm sections. Tissue sections were washed in phosphate-buffered saline (PBS), and blocked with 5% normal goat serum in PBS containing 0.3% Triton X-100. Sections were incubated with a polyclonal antibody against TRPV4 (1:1000 dilution, Alomone Labs) for 30 min at room temperature, followed by a goat anti-rabbit IgG conjugated with Alexafluor 568 for 1 hour. After several washes with PBS, images were immediately captured using a regular fluorescence microscope.

Measurement of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>). Endothelial [Ca<sup>2+</sup>] was measured in situ in freshly isolated mesenteric arteries using a modified method as we described previously (3). Vessels were cannulated and Fura-2 AM (10 μmol/L) infused into the lumen for 30 min under low pressure (20 cmH<sub>2</sub>O). Fluorescence images were captured and analyzed using an image system consisting of an inverted epifluorescence microscope (Nikon)
TE200) with a 20× fluor objective, a high-speed wavelength switcher (Lambda DG-4 from Sutter Instrument Company), a PC-controlled digital CCD camera (Hamamatsu C4742-95) and Metafluor software (Universal Imaging). Fluorescence was measured every 3 seconds at an excitation of 340 and 380 nm and emission of 510 nm. Fura-2 signals were calibrated and intracellular Ca²⁺ concentration ([Ca²⁺]) calculated as described previously (4). Experiments were performed at 37°C in physiological salt solution (PSS) composed of (in mmol/L) NaCl 123, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 21, EDTA 0.026, and glucose 11.0, pH 7.4 bubbled with 21% O₂/5% CO₂. 4α-PDD (1 μmol/L) was administered via intraluminal perfusion, and ruthenium red (10 μmol/L) and acetylcholine (1 μmol/L) were added into the bath.

**Measurement of endothelial NO.** The fluorescent NO indicator 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) was used to measure endothelial NO in situ in freshly isolated carotid arteries (3). An arterial segment was cut open along its longitudinal axis and pinned onto a Sylgard-coated dish with lumen side upward. The arterial segment was incubated with DAF-FM DA (10 μM) at room temperature for 30 min in a modified Hanks' buffered saline solution (HBSS) containing (in mmol/L) 137 NaCl, 5.4 KCl, 1.3 CaCl₂, 0.5 MgCl₂, 0.4 MgSO₄, 4.2 NaHCO₃, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 5.5 glucose, 10 HEPES, pH 7.4. The NO fluorescence was measured at 490-nm excitation and 510- to 560-nm emission using the same imaging system for Ca²⁺ measurement. To help locate the endothelial layer, endothelial Ca²⁺ was simultaneously measured by adding Fura-2 AM (10 μM) into the loading buffer (5). Results were expressed as integrated fluorescence intensity within the area observed.

**Isometric tension recording.** Small mesenteric arteries (1st-order branch from superior mesenteric artery, ~200 μm) were dissected in cold HEPES buffer containing (in mmol/L): NaCl 150, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 6, and HEPES 10, pH 7.4. Arterial segments were mounted in a 4-chamber wire myograph (model 610M, Danish Myo Technology A/S), and maintained at 37°C in PSS gassed with 21% O₂/5% CO₂ as previously described (6). Arteries were stretched to a resting tension of 1 millinewton (mN) and stimulated 2-3 times with the potassium-substituted PSS (K-PSS; 145 mM K⁺) for 3 min at 10 min intervals. Arteries were then contracted with submaximal concentrations of U46619 (50-300 nmol/L) to 50-75% of their maximum KCl challenge. Cumulative concentration responses to acetylcholine (10⁻¹⁰-10⁻⁶ mol/L) or papaverine (10⁻⁶-10⁻⁴ mol/L) were performed. Responses were repeated after pretreatment with a NOS inhibitor, N⁷ nitro-L-arginine methyl ester (L-NAME, 100 μmol/L) and/or a cyclooxygenase inhibitor, indomethacin (10 μmol/L) for 30 min. Vasodilator responses are expressed as % maximal relaxation relative to U46619 preconstriction with 100% representing basal tension. To assess sensitivities of acetylcholine in different treatment or animal groups, −logEC50 (negative logarithm of the molar concentration of a vasodilator that produces 50% of the maximal dilation) was calculated.

**Measurement of vascular responses in vivo.** TRPV4⁻/⁻ and WT mice were anesthetized with 12% urethane (1.2 g/kg body weight, ip) or ketamine/xylazine (50 mg/kg/10 mg/kg, ip). The mice were intubated with a polyethylene-60 tube, and ventilated with room air supplemented with 100% oxygen using a mouse ventilator. The
right common carotid artery was cannulated for measurement of arterial blood pressure, and the tail vein for drug administration. Heart rate was monitored by ECG at V6 position. After stabilization for 20 min, baseline arterial blood pressure and heart rate were recorded for 10 min. All drugs were given as a single iv bolus, including acetylcholine (15 µg/kg), 4α-PDD (1 µg/kg), phenylephrine (1 mg/kg), sodium nitroprusside (5 mg/kg). The arterial blood pressure and heart rate were continuously recorded for up to 20 min after drug administration. Blood pressure responses to these agents were reported as mean arterial blood pressure (MAP). Animals with baseline MAP < 60 mmHg were excluded from the statistical analysis: 3/17 and 2/12 for TRPV4−/− and WT, respectively.

Materials and Solutions. U46619 was obtained from Cayman Chemical Company, and ruthenium red from Calbiochem. All other chemicals were purchased from Sigma. Stock solutions were made in distilled water, except U46619 (ethanol), 4α-PDD (DMSO), and indomethacin (0.2 mol/L Na2CO3).

Data analysis. Data are presented as mean ± SEM. Significant differences between mean values were evaluated by Student t test or ANOVA followed by the Student-Newman-Keuls multiple comparison test. A value of p<0.05 was considered statistically significant.

References