An Elevation in Physical Coupling of Type 1 Inositol 1,4,5-Trisphosphate (IP₃) Receptors to Transient Receptor Potential 3 (TRPC3) Channels Constricts Mesenteric Arteries in Genetic Hypertension

Adebowale Adebiyi, Candice M. Thomas-Gatewood, M. Dennis Leo, Michael W. Kidd, Zachary P. Neeb, Jonathan H. Jaggar

Abstract—Hypertension is associated with an elevation in agonist-induced vasoconstriction, but mechanisms involved require further investigation. Many vasoconstrictors bind to phospholipase C–coupled receptors, leading to an elevation in inositol 1,4,5-trisphosphate (IP₃) that activates sarcoplasmic reticulum IP₃ receptors. In cerebral artery myocytes, IP₃ receptors release sarcoplasmic reticulum Ca²⁺ and can physically couple to canonical transient receptor potential 3 (TRPC3) channels in a caveolin-1-containing macromolecular complex, leading to cation current activation that stimulates vasoconstriction. Here, we investigated mechanisms by which IP₃ receptors control vascular contractility in systemic arteries and IP₃R involvement in elevated agonist-induced vasoconstriction during hypertension. Total and plasma membrane-localized TRPC3 protein was ≈2.7- and 2-fold higher in mesenteric arteries of spontaneously hypertensive rats (SHRs) than in Wistar-Kyoto (WKY) rat controls, respectively. In contrast, IP₃R1, TRPC1, TRPC6, and caveolin-1 expression was similar. TRPC3 expression was also similar in arteries of pre-SHRs and WKY rats. Control, IP₃-induced and endothelin-1 (ET-1)-induced fluorescence resonance energy transfer between IP₃R1 and TRPC3 was higher in SHR than WKY myocytes. IP₃-induced cation current was ≈3-fold larger in SHR myocytes. Pyr3, a selective TRPC3 channel blocker, and calmodulin and IP₃, receptor binding domain peptide, an IP₃R-TRP physical coupling inhibitor, reduced IP₃-induced cation current and ET-1–induced vasoconstriction more in SHR than WKY myocytes and arteries. Thapsigargin, a sarcoplasmic reticulum Ca²⁺-ATPase blocker, did not alter ET-1–stimulated vasoconstriction in SHR or WKY arteries. These data indicate that ET-1 stimulates physical coupling of IP₃R1 to TRPC3 channels in mesenteric artery myocytes, leading to vasoconstriction. Furthermore, an elevation in IP₃R1 to TRPC3 channel molecular coupling augments ET-1–induced vasoconstriction during hypertension. (Hypertension. 2012;60:1213–1219.) ● Online Data Supplement

Key Words: hypertension • vasoconstriction • canonical transient receptor potential channels • inositol 1,4,5-trisphosphate receptors • endothelin-1

Hypertension is associated with arterial hypercontractility that alters vascular bed hemodynamics. Several mechanisms have been proposed to stimulate vasoconstriction in hypertension, including altered myocyte ion channel expression and function. An increase in myocyte voltage-dependent Ca²⁺ current density elevates intracellular Ca²⁺ concentration, leading to vasoconstriction in hypertension. In hypertensive animal models, a reduction in expression and function of the β1 subunit of the large conductance Ca²⁺-activated K⁺ (BKᵥ) channel increases vascular tone. Altered expression of several transient receptor potential (TRP) channels is also proposed to be associated with systemic and pulmonary hypertension. However, molecular mechanisms by which alterations in TRP channel expression result in vasoconstriction in hypertension are poorly understood.

Many vasoconstrictors bind to phospholipase C–coupled receptors, leading to an elevation in inositol 1,4,5-trisphosphate (IP₃) that activates endo/sarcoplasmic reticulum-localized IP₃ receptors (IP₃Rs). Three structurally and functionally distinct IP₃R isoforms, designated IP₃R1, IP₃R2, and IP₃R3, have been identified. IP₃R1 is the predominant molecular and functional isoform expressed in vascular myocytes. Agonist-induced IP₃R activation results in sarcoplasmic reticulum (SR) Ca²⁺ release in cerebral artery myocytes that partially contributes to constriction. Close spatial proximity of IP₃R1 to plasma membrane canonical transient receptor potential 3 (TRPC3) channels also permits IP₃ to promote binding of the IP₃R1 N terminus to the TRPC3 channel calmodulin and IP₃R binding (CIRB) domain. Isoform-selective physical coupling of IP₃R1 to...
TRPC3 channels activates a cation current ($I_{Ca}$) in cerebral artery myocytes.\textsuperscript{11,12} IP$_3$-induced $I_{Ca}$ activation leads to membrane depolarization, voltage-dependent Ca$^{2+}$ channel activation, an elevation in global intracellular Ca$^{2+}$ concentration, and vasoconstriction.\textsuperscript{13} This physical coupling mechanism is essential for IP$_3$-induced $I_{Ca}$ activation and a major contributor to agonist-induced constriction in cerebral arteries.\textsuperscript{11,12,14} In contrast, mechanisms by which IP$_3$Rs control systemic artery contractility are poorly understood, with contributions of SR Ca$^{2+}$ release and physical coupling of IP$_3$Rs to TRP channels unclear. Similarly, whether pathological alterations in IP$_3$R-mediated signaling contribute to elevated systemic vascular contractility during hypertension is poorly understood.

Here, we investigated physiological functions of IP$_3$Rs in myocytes of mesenteric arteries. We also tested the associated hypothesis that molecular and functional alterations in IP$_3$R signaling contribute to mesenteric artery vasoconstriction in hypertension. Our data indicate that IP$_3$ and endothelin-1 (ET-1) stimulate physical coupling of IP$_3$R1 to TRPC3 channels, leading to $I_{Ca}$ activation in mesenteric artery myocytes and vasoconstriction. Data also indicate that hypertension is associated with an elevation in IP$_3$-induced physical coupling of IP$_3$R1 to TRPC3 channels, leading to vasoconstriction. In contrast, ET-1 does not induce vasoconstriction by stimulating SR Ca$^{2+}$ release and physical coupling of IP$_3$Rs to TRP channels unclear. Similarly, whether pathological alterations in IP$_3$R-mediated signaling contribute to hypertension is poorly understood.

Hypertension is associated with an elevation in canonical transient receptor potential (TRPC) 3 but not TRPC1, TRPC6, inositol 1,4,5-trisphosphate receptor (IP$_3$R) 1, and caveolin-1 (cav-1) in Wistar-Kyoto (WKY) rat mesenteric arteries. A monoclonal mouse anti-IP$_3$R1 antibody coimmunoprecipitated IP$_3$R1 ($\approx 270$ kDa), TRPC3 ($\approx 90$ kDa), and cav-1 ($\approx 22$ kDa). Lysate supernatant ($\approx 40$ µg of protein) was used as the input control and mouse IgG as the negative control. Arteries pooled from $\approx 8$ rats were used in this experiment. IP indicates immunoprecipitation.

### Materials and Methods

Expanded Materials and Methods are available at the online-only Data Supplement.

## Results

**IP$_3$R1 Interacts With TRPC3 and Caveolin-1 in Mesenteric Arteries**

IP$_3$R1, TRPC3, and caveolin-1 (cav-1) are located in the same macromolecular complex and physically interact in cerebral artery myocytes.\textsuperscript{15} Whether these proteins structurally organize

![Figure 1](image1.png)

**Figure 1.** Inositol 1,4,5-trisphosphate receptor (IP$_3$R) 1, canonical transient receptor potential (TRPC) 3, and caveolin-1 (cav-1) are located in a macromolecular complex in rat mesenteric arteries. Monoclonal mouse anti-IP$_3$R1 antibody coimmunoprecipitated IP$_3$R1 ($\approx 270$ kDa), TRPC3 ($\approx 90$ kDa), and cav-1 ($\approx 22$ kDa). Lysate supernatant ($\approx 40$ µg of protein) was used as the input control and mouse IgG as the negative control. Arteries pooled from $\approx 8$ rats were used in this experiment. IP indicates immunoprecipitation.

**Hypertension Is Associated With an Elevation in TRPC3 but Not TRPC1, TRPC6, IP$_3$R1, or Cav-1 Protein in Mesenteric Arteries**

At 6 weeks of age, WKY and spontaneously hypertensive rat (SHR) blood pressures (tail cuff systolic, WKY versus SHR: $\approx 137$ versus $137$ mmHg) are similar.\textsuperscript{15} In contrast, at 12 weeks of age, WKY and SHR blood pressures ($\approx 151$ versus $217$ mmHg, respectively) are significantly different.\textsuperscript{15} Western blotting indicated that TRPC3 protein was $2.7$-fold higher in SHR (13-week) than in age-matched WKY rat mesenteric arteries (Figure 2A and 2G; Figure S1 in the online-only Data Supplement). In contrast, TRPC1, TRPC6,
Plasma Membrane-Localized TRPC3 Channel Protein Is Elevated in SHR Arteries

Arterial surface biotinylation was performed to examine cellular distribution of TRPC3 channels in mesenteric arteries. Biotinylation indicated that ≈99.6% and 98.8% of total TRPC3 protein was present in the arterial plasma membrane in SHR and WKY rats, respectively (Figure 3A and 3B).

Surface TRPC3 protein was ≈2-fold higher in SHR than in WKY arteries (Figure 3A and 3C). These data indicate that TRPC3 channels are predominantly membrane localized in mesenteric arteries. Furthermore, during hypertension the increase in TRPC3 channel expression directly translates to an elevation in plasma membrane TRPC3 protein.

Hypertension Is Associated With Increased Spatial Localization of TRPC3 Channels Nearby IP3R1 in Arterial Myocytes

Next, we investigated spatial proximity between arterial myocyte IP3R1 and TRPC3 in SHR and WKY mesenteric artery myocytes. Alexa 546- and 488-labeled secondary antibodies bound to primary antibodies targeting IP3R1 and TRPC3, respectively, produced a higher mean N-FRET of ≈23.7% in isolated WKY rat arterial myocytes (Figure 4A and 4B). In SHR myocytes, the same antibodies produced a higher mean N-FRET of ≈29.1% (Figure 4A and 4B). Bt-IP3 (2,3,6-Tri-O-Butyryl-myo-inositol 1,4,5-Trisphosphate-Hexakis[propionoxymethyl]) Ester, a membrane-permeant IP3 analogue, and ET-1, a vasoconstrictor, both increased mean N-FRET to ≈28% in WKY myocytes (Figure 4B). Bt-IP3 and ET-1 stimulated a larger increase in mean N-FRET to ≈34% and 35%, respectively, in SHR myocytes (Figure 4B). To test the hypothesis that hypertension is associated with an elevation in spatial localization of IP3R1 and TRPC3 channels in arterial myocytes, N-FRET was measured.
The functional significance of IP₃, R1 to TRPC3 channel physical coupling in mediating ET-1–induced vasoconstriction was
studied. 2-aminoethoxydiphenyl borate, an IP₃R and TRPC channel inhibitor, did not alter baseline arterial tension or depolarization-induced vasoconstriction (Figures S2 and S3). In contrast, 2-aminoethoxydiphenyl borate reduced ET-1–induced vasoconstriction, doing so more effectively in SHR than in WKY arteries (=34% versus 18% reduction, respectively; Figure 6C). Pyr3 and CIRBP-TAT did not alter baseline tension or depolarization-induced vasoconstriction, but both blockers inhibited ET-1–induced vasoconstriction (Figures S2 and S3; Figure 6C). Importantly, Pyr3 more effectively blocked ET-1–induced constriction in SHR than in WKY arteries (=83% versus 59% reduction, respectively; Figure 6C). CIRB-TAT also attenuated ET-1–induced constriction more in SHR than in WKY arteries (=57% versus 39% reduction, respectively; Figure 6C). Taken together, these data indicate that an elevation in plasma membrane TRPC3 channels and physical coupling of these channels to IP₃R1 in myocytes augment ET-1–induced vasoconstriction during hypertension.

Discussion

Here, we studied the functional significance of IP₃Rs and TRPC3 channels to agonist-induced vasoconstriction in mesenteric arteries and the contribution of pathological alterations in IP₃R-mediated signaling mechanisms during hypertension. Our data show for the first time that IP₃R1 is located in close proximity to TRPC3 and is contained within a macromolecular complex containing TRPC3 and cav-1 in mesenteric arteries. Hypertension is associated with an increase in both total and plasma membrane TRPC3 protein and IP₃R1 to TRPC3 spatial localization, leading to an elevation in IP₃R1-induced ICₐ in arterial myocytes. SR Ca²⁺ release does not contribute to ET-1–induced vasoconstriction in mesenteric arteries of either normotensive or hypertensive rats, suggesting that IP₃R activation stimulates vasoconstriction through alternate mechanisms. Our data indicate that physical coupling of IP₃R1 to TRPC3 channels contributes to ET-1–induced vasoconstriction in mesenteric arteries and that enhancement of this mechanism contributes to the elevation in ET-1–induced vasoconstriction during hypertension.

TRPC3 expression is altered in several cell types in hypertension. TRPC3 mRNA and protein were elevated in lung tissues and pulmonary artery myocytes from patients with idiopathic pulmonary hypertension. When compared with normotensive controls, TRPC3 protein was also higher in SHR and human monocytes, human renal arteriolar endothelium, kidney cortex of hypertensive Munich Wistar Frömter rats, and SHR aorta, mesenteric, carotid, and cerebral arteries. Our data indicating that TRPC3 protein is higher in mesenteric arteries of SHR than age-matched WKY rats are consistent with these results from other cell types. Mechanisms that elevate arterial myocyte TRPC3 channels in SHRs are unclear. Conceivably, the chronic elevation in blood pressure may stimulate transcriptional upregulation of TRPC3 channels. In addition, TRPC3 upregulation may be one mechanism that elevates systemic blood pressure. Functional and molecular alterations in IP₃R1 and cav-1 also occur during hypertension. Pulmonary hypertension was associated with an elevation in pulmonary artery IP₃R1 mRNA and protein, whereas IP₃R1 expression was lower in renal medulla of SHRs than WKY rats. Although cav-1 knockout mice develop pulmonary hypertension, cav-1 protein was lower in SHR than in WKY rat aorta. Data from this and our previous study indicate that IP₃R1, TRPC3, and cav-1 exist in a macromolecular signaling complex in both cerebral and mesenteric arteries. Cav-1 and IP₃R1 expressions were similar in SHR and WKY mesenteric arteries, suggesting that altered cav-1 and IP₃R1 expression may be tissue dependent in hypertension. TRPC3, TRPC1, and TRPC6 channels regulate membrane potential, intracellular Ca²⁺ concentration, and contractility in myocytes from a wide variety of different blood vessels. Lower carotid artery and higher mesenteric arteriole TRPC1 protein has been reported in SHRs. TRPC6 protein was also higher in mesenteric artery myocytes of Milan hypertensive rats and ouabain-induced hypertensive rats than in normotensive controls. Our data indicate that TRPC1 and TRPC6 protein is similar in SHR and WKY rat mesenteric arteries. These contradictory data may result from differences in the arterial bed or animal models of hypertension studied.

Hypertension is associated with pathological alterations in vascular myocyte contractility, morphology, gene expression, and proliferation. These changes may occur because of altered trafficking, cell surface expression, and spatial localization of signaling molecules, leading to dysregulation of physiological functions, including contractility. A proportion of TRPC3 protein is located intracellularly in a variety of cell types, including cerebral artery myocytes and cardiomyocytes. Data here show that essentially 100% of TRPC3 protein is located within the plasma membrane in mesenteric arteries, suggesting that TRPC3 distribution may differ depending on the cell type or anatomical origin of the vasculature. FRET experiments indicated that IP₃R1 and TRPC3 channels are located in close spatial proximity and that ET-1 and IP₃ increase spatial proximity of these proteins in mesenteric artery myocytes, consistent with data in cerebral artery myocytes. IP₃R1 and TRPC3 antibodies generated a higher FRET signal in SHR myocytes that could originate from an increase in the number of TRPC3 channels nearby IP₃R1 or from a reduction in the distance between TRPC3 and IP₃R1. Possibilities between which the FRET method cannot distinguish. Given that surface TRPC3 is higher and that IP₃R1 and TRPC3 are already in such close spatial proximity to permit physical coupling, it is highly likely that the higher FRET signal occurs, at least in part, because of a larger number of TRPC3 channels nearby IP₃R1.

ET-1 and uridine-5′-triphosphosphate activate an IP₃R1-dependent TRPC3-mediated ICₐ in cerebral artery myocytes. Uridine-5′-triphosphate-induced whole cell currents were also larger in SHR carotid artery myocytes, although signaling mechanisms involved were not determined. Here, data indicate that IP₃ stimulates physical coupling between IP₃R1 and TRPC3 channels, leading to ICₐ in mesenteric artery myocytes. IP₃-induced ICₐ density was larger in SHR than in WKY rat myocytes, likely attributable to an increased number of TRPC3 channels nearby IP₃R1 and an elevation in functional coupling. This conclusion is supported by data indicating that Pyr3 and CIRBP-TAT essentially abolished IP₃-induced ICₐ in both WKY and SHR myocytes. These data also indicate that hypertension is not associated with the induction of promiscuous coupling of IP₃R1 to...
TRP channels other than C3, a potential mechanism that may have also elevated I_{calc}.

Small arteries from hypertensive humans and rats generate higher spontaneous tone and constrict more in response to receptor agonists. Here, although ET-1 and membrane depolarization stimulated larger vasoconstriction in SHR arteries, ET-1 sensitivity was similar, consistent with previous studies in perfused mesenteric arteries. When combined with other findings in this study, these data suggest that ET-1 stimulates larger vasoconstriction, in part, through stimulating more effective IP_{3}R1-TRPC3 physical coupling in myocytes of hypertensive rats. This leads to larger I_{calc} membrane depolarization, voltage-dependent Ca^{2+} channel activation, and vasoconstriction. Our data also indicate that SR Ca^{2+} release does not contribute to ET-1–induced vasoconstriction in either SHR or WKY mesenteric arteries, supporting the concept that physical coupling to TRPC3 channels is a primary mechanism by which IP_{3}R stimulates vasoconstriction in mesenteric arteries. Data also show that IP_{3}Rs and TRPC3 channels do not contribute to depolarization-induced vasoconstriction and that 2-aminoethoxydiphenyl borate, Pyr3, and CIRBP-TAT do not block voltage-dependent Ca^{2+} channels, which elicit this response. Many vasoconstrictors, including angiotensin II and epinephrine, bind to phospholipase C–coupled receptors, leading to an elevation in intracellular IP_{3} in smooth muscle cells. Therefore, our study also raises the possibility that other agonists may induce vasoconstriction by stimulating IP_{3}R1 to TRPC3 coupling and that this mechanism may contribute to elevated contractility during hypertension.

In summary, data indicate that IP_{3}R1 stimulates vasoconstriction primarily through physical coupling to TRPC3 channels in mesenteric artery myocytes. We also demonstrate that genetic hypertension is associated with an increase in TRPC3 protein, TRPC3 channel cell surface expression, and enhanced physical coupling of TRPC3 to IP_{3}R1 channels. This pathological alteration elevates ET-1–induced vasoconstriction in hypertension.

**Perspectives**

An elevation in vascular resistance is a hallmark of hypertension. Identifying mechanisms that underlie vasoconstriction in hypertension is essential to develop novel therapies to induce vasodilation. We show that IP_{3}R1 physically and functionally couples to TRPC3 channels in mesenteric artery myocytes, similar to previous data in cerebral artery myocytes. We also identify a novel mechanism by which enhanced molecular and functional coupling of TRPC3 channels to IP_{3}R1 elevates arterial contractility in hypertension. An increase in TRPC3 channel protein, TRPC3 channel surface expression, and molecular localization of these channels nearby IP_{3}R1 enhances physical and functional coupling of IP_{3}R1 to TRPC3 in SHR mesenteric artery myocytes. This pathological alteration in IP_{3}R1 to TRPC3 coupling augments agonist and IP_{3}-induced I_{calc} in arterial myocytes and vasoconstriction in hypertension. Our study also provides evidence that interfering with physical and functional coupling between IP_{3}R1 and TRPC3 channels may be a novel therapeutic strategy to induce vasodilation in hypertension.

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**Disclosures**

None.

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by

Adebowale Adebiyi, Candice M. Thomas-Gatewood, M. Dennis Leo, Michael W. Kidd, Zachary P. Neeb, and Jonathan H. Jaggar*

from the

Department of Physiology
University of Tennessee Health Science Center
Memphis TN 38163
Materials and Methods

Tissue preparation and arterial myocyte isolation. Animal protocols used were reviewed and approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. Male (6 or 13 week old) Spontaneously Hypertensive Rats (SHR) and Wistar-Kyoto (WKY) rats were purchased from Taconic (Germantown, NY). Rats were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg bodyweight). The mesenteric arterial bed was removed from the abdominal cavity, and placed into oxygenated ice-cold (4°C) physiological saline solution (PSS) of the following composition (in mM): 112 NaCl, 4.8 KCl, 24 NaHCO3, 1.8 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 10 glucose, which was gassed with 21 % O2-5 % CO2-74 % N2 to pH 7.4. Third and fourth order mesenteric artery branches (150-250 μm in diameter) were dissected and cleaned of adventitial connective tissue. Myocytes were enzymatically dissociated from mesenteric arteries using a method similar to that previously described (1). Briefly, mesenteric arteries were placed into HEPES-buffered solution (in mM: 55 NaCl, 80 sodium glutamate, 5.6 KCl, 2 MgCl2, 10 HEPES, and 10 glucose; pH 7.3 with NaOH) containing 1 mg/ml papain, 1 mg/ml dithioerythreitol, and 1 mg/ml bovine serum albumin (BSA) for 25 min (at 37°C) and immediately transferred to isolation solution containing collagenase F (1 mg/ml), and H (0.5 mg/ml), 100 µM CaCl2 and 1 mg/ml BSA for 8 min (at 37°C). Arteries were subsequently washed in ice-cold isolation solution and triturated using a fire-polished glass pasteur pipette to yield single myocytes.

Co-immunoprecipitation (Co-IP). Co-IP was used to examine physical association between IP3R1, TRPC3, and cav-1 in mesenteric arteries, as previously described (2;3). Co-IP was performed using the Catch and Release co-IP kit (Millipore). Arteries collected from ~ 8 rats were used for each experiment. Protein lysate was harvested from mesenteric arteries using ice-cold immunoprecipitation lysis buffer (Pierce). Arterial lysate was incubated at 4 °C overnight with control mouse IgG or IP3R1 mouse monoclonal antibody (5 µg), affinity ligand and wash buffer in a spin column containing IP capture resin. Protein samples were subsequently eluted from the beads after several washes. Samples were then analyzed by Western blotting using specific antibodies against IP3R1, TRPC3, and cav-1.

Western blotting. Western Immunoblotting was performed as previously described (4;5). Briefly, mesenteric artery lysate protein concentrations were determined spectrophotometrically. Proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes using a Mini Trans Blot Cell (Bio-Rad, Hercules, CA). Membranes were then incubated with respective antibodies and developed using enhanced chemiluminescence (Thermo Scientific). Images were documented on Kodak In Vivo F Pro Imaging System (Carestream Molecular Imaging, Rochester, NY). Band intensity was determined using Quantity One software (BioRad).

Surface Biotinylation. Surface proteins were measured in intact mesenteric arteries using surface biotinylation technique (3;6). For each experiment, intact mesenteric arteries free of adipose and connective tissues were incubated for 1 h at room temperature in a 1 mg/ml mixture each of EZ-Link Sulfo-NHS-LC-LC-Biotin and EZ-Link Maleimide-PEG2-Biotin reagents (Thermo Scientific) in PBS pH 7.4. Unbound biotin was removed by quenching with PBS supplemented with 100 mmol/L glycine and excess removed by washing with PBS. Arteries were then homogenized in RIPA buffer (Sigma) and cellular debris removed by centrifugation. The total protein concentration of the supernatant was determined followed by pull down of biotinylated surface proteins using Avidin beads in spin columns (Thermo Scientific). Biotinylated proteins were eluted from Avidin beads by boiling in 1x SDS-buffer containing 2% 2-mercaptoethanol. Western blotting was used to quantify surface TRPC3 protein using rabbit polyclonal anti-TRPC3 (Abcam).

Immunofluorescence resonance energy transfer (immuno-FRET). ImmunoFRET was used to measure spatial proximity between IP3R1 and TRPC3 channels in mesenteric artery myocytes, as we have previously done (2;3). Freshly isolated mesenteric artery myocytes were allowed to adhere to poly-L-lysine coated coverslips. Cells were then fixed with 3.7% paraformaldehyde (PFA) in Phosphate-
Buffered Saline (PBS) for 15 min. PFA-fixed cells were then washed three times with PBS and permeabilized with 0.1% triton X-100 for 1 min at room temperature. Following 1 h incubation in PBS containing 5% BSA, myocytes were treated for 1 h at 37°C with primary antibodies: mouse monoclonal anti-IP₃R1 and polyclonal anti-TRPC3, each at a dilution of 1:100 in PBS containing 5% BSA. After a wash and block with PBS containing 5% BSA, myocytes were incubated for 1 h at 37°C with secondary antibodies: Alexa 546- and 488-labeled secondary antibodies bound to primary antibodies targeting IP₃R1 and TRPC3, respectively. Following Fluorescence images were acquired using a Zeiss LSM Pascal laser-scanning confocal microscope. Images were background-subtracted and N-FRET was calculated on a pixel-by-pixel basis for the entire image and in regions of interest (within the boundaries of the cell) using the Xia method (7) and Zeiss LSM FRET Macro tool version 2.5.

**Patch-Clamp Electrophysiology.** Patch-clamp electrophysiology was performed on isolated myocytes. Isolated myocytes were allowed to attach to a glass coverslip in the bottom of a chamber for ~ 15 min prior to experimentation. Membrane currents were measured using the patch-clamp technique (Axopatch 200B, Clampex 8.2). I_Ca was measured using the conventional whole cell patch-clamp configuration, as we have done previously.(2-5) Whole cell currents were measured by applying 940-ms voltage ramps between -120 and + 20 mV with a holding potential of -40 mV. Bath solution for conventional whole-cell experiments contained (in mmol/L): 140 NaCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 Hepes, and 10 glucose (pH 7.4). The pipette solution contained (in mmol/L): 140 CsCl, 10 HEPES, 10 glucose, 5 Mg-ATP, and 5 EGTA (with pH adjusted to 7.2 with CsOH), and 100 nmol/L free Ca²⁺. Current amplitude at -120 mV was analyzed offline using pClamp 9 (Molecular Devices).

**Wire myography.** Mesenteric artery segments (~250 µm diameter; ~ 1.5 mm long) were mounted on tungsten wires and equilibrated in physiological saline solution for ~ 45 min at 37°C in a multi-channel wire myograph system (DMT A/S Aarhus, Denmark). Arterial rings were subjected to a resting tension of 10 mN and allowed to equilibrate for ~ 30 min prior to experimentation. Myograph chambers were continuously gassed with 21 % O₂-5 % CO₂-74 % N₂. Data were acquired using LabChart software (ADInstruments).

**Antibodies and Chemicals.** Anti-TRPC3, -TRPC1, -cav-1, and -actin were purchased from Abcam. Anti-IP₃R1 and -TRPC6 were purchased from Alomone. Alexa 546 and 488 were purchased from Invitrogen. Unless otherwise stated, all reagents were purchased from Sigma Chemical (St. Louis, MO). Papain was purchased from Worthington Biochemical (Lakewood, NJ) and 2,3,6-Tri-O-Butyryl-myo-Inositol 1,4,5-Trisphosphate-Hexakis(propionoxymethyl) Ester (Bt-IP₃) was purchased from SiChem (Bremen, Germany). CIRBP-TAT was synthesized by Molecular Resource Center, UTHSC (Memphis, TN).

**Statistical Analysis.** Data are expressed as mean ± standard error of the mean. Statistical significance was calculated by using Student’s t-tests for paired or unpaired data or ANOVA followed by Student–Newman–Keuls test for multiple data sets. Ec₅₀ values were determined by non-linear regression analysis using GraphPad Prism software (GraphPad Software, San Diego, CA). P<0.05 was considered significant.
References


Results

Figure S1. Full Western blots illustrating TRPC3 protein expression in 6 and 13 week old SHR and WKY mesenteric arteries.

Figure S2. Mean data illustrating that thapsigargin (thapsi; 100 nM), 2-APB (50 µM), CIRBP-TAT (3 µM), and Pyr3 (1 µM) did not alter ($P>0.05$) baseline tension in SHR and WKY mesenteric arteries. N= (left to right) 8, 6, 6, 5, 13, 13, 6, and 7.

Figure S3. Mean data illustrating that 2-APB (50 µM), Pyr3 (1 µM) and CIRBP-TAT (3 µM) did not alter ($P>0.05$) depolarization (80 mM KCl)-induced constriction in SHR and WKY mesenteric arteries. N = (left to right) 6, 4, 4, 5, 4, and 4.