Increased Transient Receptor Potential Canonical Type 3 Channels in Vasculature From Hypertensive Rats

Daoyan Liu, Dachun Yang, Hongbo He, Xiaoping Chen, Tingbing Cao, Xiaoli Feng, Liqun Ma, Zhidan Luo, Lijuan Wang, Zhencheng Yan, Zhiming Zhu, Martin Tepel

Abstract—We tested the hypothesis that transient receptor potential canonical type 3 (TRPC3) channels are increased in vascular smooth muscle cells and aortic tissue from spontaneously hypertensive rats (SHR) compared with normotensive Wistar Kyoto rats. Expression of TRPC3 was analyzed by immunohistochemistry and Western blotting. TRPC3 gene knockdown was performed by specific small interfering RNA and TRPC3 overexpression using the pAdEasy-1 system. Cytosolic calcium was measured using fluorescence spectrophotometry and vasoconstriction of aortic rings using a force transducer. In SHR, the expression of TRPC3 channel protein was significantly higher in aortic rings (1.48±0.05 versus 1.00±0.06; each n=6; P<0.01) and vascular smooth muscle cells (1.28±0.08 versus 1.00±0.03; each n=6; P<0.05) compared with Wistar Kyoto rats. Knockdown of TRPC3 gene expression by specific small interfering RNA significantly reduced the angiotensin II–induced calcium influx by 30±3% (n=6; P<0.01), whereas TRPC3 overexpression significantly increased it by 55±3% (n=6; P<0.01). The angiotensin II–induced calcium increase was significantly enhanced in vascular smooth muscle cells from SHR compared with Wistar Kyoto rats, even in the presence of the calcium channel blocker amlodipine. Angiotensin II significantly elevated the TRPC3 channel protein expression in vascular smooth muscle cells from SHR from 1.28±0.08 to 1.61±0.08 (each n=6; P<0.01). Angiotensin II–induced TRPC3 expression was prevented by telmisartan. Administration of telmisartan to SHR for 4 weeks significantly reduced blood pressure, angiotensin II–induced vasoconstriction, and TRPC3 channel protein expression in aortic tissue. TRPC3 expression was not significantly reduced after reduction of blood pressure in SHR using amlodipine. In conclusion, we give experimental evidence that increased TRPC3 channel protein expression in the vasculature is important for elevated blood pressure. (Hypertension. 2009;53:70-76.)

Key Words: transient receptor potential canonical channel type 3 • calcium • spontaneously hypertensive rats

Transient receptor potential canonical (TRPC) are non-selective cation channels that had been identified in several tissues, including heart and vascular smooth muscle cells (VSMC). TRPC type 3 (TRPC3) channels play an important role in several cardiovascular diseases and probably hypertension.1-10 An increased TRPC3 expression could be demonstrated in peripheral monocytes from spontaneously hypertensive rats (SHR) compared with normotensive Wistar Kyoto rats (WKY).5,6 Dietrich et al reported that TRPC6 knockout mice showed elevated TRPC3 channel expression, increased vasoconstriction, and increased blood pressure.7 Increased angiotensin II–induced vasoconstriction and proliferation of VSMC, as well as augmented angiotensin II–induced intracellular signal transduction pathways and calcium influx, have frequently been observed in primary hypertension.6,11-15 Part of the action of angiotensin II may occur through TRPC channels. Although data from the literature gave evidence that calcium influx through TRPC3 channels may contribute to vasoconstriction and hypertension, this has not been tested yet using VSMC from SHR. In the present study, we tested the hypothesis that calcium influx through TRPC3 is increased in VSMC and aortic tissue from SHR compared with WKY.

Methods
A detailed description of the Methods can be found in an online supplement available at http://hyper.ahajournals.org.

Animals (SHR and WKY), interventions and hemodynamic measurements, preparation of aortic rings and vasoconstriction,16,17 culture of VSMC, measurements of cytosolic calcium concentration,18,19 RNA isolation and RT-PCR, and immunoblottings and immunohistochemistry had been described previously by our group. All experiments were performed as approved by the animal care and use committee. The TRP channel blocker SKF-96365 (Merck Biosciences; final concentration 10 μmol/L) was used.20,22 Changes of cytosolic calcium were reported as described.23 Antibodies were...
Statistical Analysis

All results are expressed as the mean±SEM of ≥3 independent experiments. Data were compared using 2-tailed Student t test or ANOVA and Tukey’s multiple comparisons post hoc test as appropriate. Two-sided P values <0.05 were considered significant. Where error bars do not appear on the figures, error was within the symbol size.

Results

Identification of TRPC in the Vasculature

First, we used RT-PCR to show the presence of TRPC3 transcripts in vasculature from adult normotensive WKY, 5-week-old prehypertensive SHR (preSHR), and adult SHR. Calculating the TRPC3/GAPDH ratio indicated that TRPC3 transcripts were significantly more expressed in vasculature from adult SHR compared with adult WKY or preSHR (each P<0.05; Figure 1A).

Second, we identified TRPC3 channel proteins in VSMC from SHR and normotensive WKY using specific antibodies and immunohistochemistry (Figure 1B).

Increased Expression of TRPC3 Channel Protein in Aortic Tissue From SHR

Systolic blood pressure was 196±6 mm Hg in adult SHR and 109±4 mm Hg in adult WKY (each n=6; P<0.05). Body weight was 329±13 g in SHR and 326±11 g in WKY. The expression of TRPC3 channel protein was significantly elevated in aortic tissue (1.48±0.05 versus 1.00±0.06; each n=6; P<0.01; Figure 2A) from SHR compared with WKY. In contrast, the expression of angiotensin II type 1 receptor (AT1R) protein was not significantly different in aortic rings from SHR and WKY (1.13±0.03 versus 1.00±0.06; each n=6; P=NS; Figure 2B).

Increased Expression of TRPC3 Channel Protein in VSMC From SHR

The expression of TRPC3 channel protein was significantly elevated in VSMC from SHR compared with WKY (1.28±0.08 versus 1.00±0.03; each n=6; P<0.05; Figure 2C). In contrast, the expression of AT1R protein was not significantly different in VSMC from SHR and WKY (1.14±0.06 versus 1.00±0.05; each n=6; P>0.05; Figure 2D). In additional experiments, we established that TRPC3 channel protein expression was not affected by cell culture by comparing TRPC3 channel protein expression in freshly isolated cells and cultured VSMC from the third and tenth passage. As shown in Figure 2E for WKY (freshly isolated cells 1.05±0.12; third passage 1.09±0.05; tenth passage 1.06±0.06; each n=3; P=NS) and Figure 2F for SHR (freshly isolated cells 1.22±0.10; third passage 1.27±0.07; tenth passage 1.21±0.11; each n=3; P=NS), TRPC3 channel protein expression was not affected by cell culture.

As indicated in Figure 2G and 2H, both systolic blood pressure and the expression of TRPC3 channel protein in aortic tissue from adult SHR were significantly higher compared with 5-week-old preSHR or with adult WKY.

Now, we investigated whether the increased TRPC3 channel protein expression in SHR was associated with enhanced angiotensin II–induced elevation of blood pressure, vasoconstriction, and calcium increase in SHR. The intravenous injection of angiotensin II at a rate of 24 μg/kg per hour for 60 minutes increased mean arterial blood pressure in rats. The angiotensin II–induced elevation of arterial blood pressure was significantly higher in SHR compared with WKY (53±3 mm Hg versus 22±4 mm Hg; each n=6; P<0.05).

Furthermore, angiotensin II–induced aortic constriction was significantly higher in SHR compared with WKY (82±3% of maximal potassium contraction versus 54±6% of maximum potassium contraction; each n=6; P<0.01). Additional experiments showed that compared with control conditions (100±8%; n=12), the administration of calcium channel blocker amiodipine significantly reduced the angiotensin II–induced aortic constriction to 72±7% (n=9; P<0.05), whereas...
the administration of TRP cation channel blocker SKF-96365 significantly reduced it to 18/1100 [P<0.05 by ANOVA and Tukey’s multiple comparisons post hoc test; Figure 3A]). As indicated in Figure 3A, the angiotensin II–induced aortic contraction was also significantly higher in SHR compared with WKY in the presence of amlodipine but not in the presence of SKF-96365.

Baseline cytosolic calcium concentration was similar in VSMC from SHR and WKY (103±12 versus 99±9 nmol/L, respectively; each n=6; P=NS).

The angiotensin II–induced calcium increase was significantly enhanced in VSMC from SHR compared with WKY (increase of cytosolic calcium 229±10 versus 115±13 nmol/L, respectively; P<0.01). In the presence of calcium channel blocker amlodipine, the angiotensin II–induced calcium influx was still significantly higher in VSMC from SHR compared with WKY (170±10 versus 130±12 nmol/L, respectively; P<0.01). In contrast, in the presence of the TRP cation channel blocker SKF-96365, the angiotensin II–calcium influx was similar in VSMC from SHR and WKY (126±13 versus 113±10 nmol/L, respectively; P=NS by ANOVA and Tukey’s multiple comparisons post hoc test; Figure 3B).

Using TRPC3 channel protein overexpression, we confirmed that an increased TRPC3 channel protein expression in VSMC caused elevated angiotensin II–induced calcium influx, whereas TRPC3 gene knockdown in VSMC reduced TRPC3 expression and caused reduced angiotensin II–induced

![Figure 2. Increased expression of TRPC3 channel protein in aortic tissue and VSMC from SHR. Representative Western blotting and summary data of TRPC3 channel protein or AT1R protein expression in aortic tissue (A and B) or VSMC (C and D) from WKY (open bars) and SHR (filled bars). Data are mean±SEM of n=6 independent experiments. **P<0.01. Expression of TRPC3 channel protein was compared in freshly isolated cells (freshly) and cultured VSMC from WKY (E) and SHR (F), the third and tenth passage, respectively. Data are mean±SEM of n=3 independent experiments. Systolic blood pressure (G) and expression of TRPC3 channel protein in aortic tissue (H) from adult normotensive WKY (open bars), 5-week-old preSHR (hatched bars), and adult SHR (filled bars). Data are mean±SEM of n=3 independent experiments. *P<0.05 and Tukey’s multiple comparisons post hoc test.](http://hyper.ahajournals.org/)

**Figure 2.** Increased expression of TRPC3 channel protein in aortic tissue and VSMC from SHR. Representative Western blotting and summary data of TRPC3 channel protein or AT1R protein expression in aortic tissue (A and B) or VSMC (C and D) from WKY (open bars) and SHR (filled bars). Data are mean±SEM of n=6 independent experiments. **P<0.01. Expression of TRPC3 channel protein was compared in freshly isolated cells (freshly) and cultured VSMC from WKY (E) and SHR (F), the third and tenth passage, respectively. Data are mean±SEM of n=3 independent experiments. Systolic blood pressure (G) and expression of TRPC3 channel protein in aortic tissue (H) from adult normotensive WKY (open bars), 5-week-old preSHR (hatched bars), and adult SHR (filled bars). Data are mean±SEM of n=3 independent experiments. *P<0.05 by ANOVA and Tukey’s multiple comparisons post hoc test.
calcium influx. Transfecting TRPC3 gene into cultured VSMC from SHR significantly increased TRPC3 channel protein expression from 100±4% to 167±4% (n=6; P<0.01; Figure 4A) and hence significantly elevated the angiotensin II–induced calcium increase by 55±4% (n=6; P<0.01; Figure 4B).

Specific small interfering RNA against TRPC3 significantly reduced TRPC3 channel protein expression in cultured VSMC from SHR from 100±11% to 49±4% (n=3; P<0.05; Figure 4C), whereas TRPC6 channel protein expression was not affected (100±18% versus 99±16%; P=NS; Figure 4D). The reduction of TRPC3 channel protein expression in VSMC significantly reduced the angiotensin II–induced calcium increase by 36±3% (n=5; P<0.05; Figure 4E).

Next, we determined whether angiotensin directly increases the expression of TRPC3 channel protein in VSMC. To this end, VSMC from SHR or WKY were cultured in the continuous presence of 100 nmol/L angiotensin II throughout culturing. The administration of angiotensin II significantly elevated the expression of TRPC3 channel protein in VSMC from SHR from 1.28±0.08 to 1.61±0.08 (each n=6; P<0.01) but only slightly changed TRPC3 channel protein expression in VSMC from WKY from 1.00±0.03 to 1.11±0.07 (each n=6; P=NS; Figure 5A). As indicated in Figure 5B, the administration of the AT1R antagonist telmisartan prevented the angiotensin II–induced TRPC3 channel protein expression in VSMC from SHR (0.74±0.05 versus 1.61±0.08; each n=6; P<0.01).

These data indicated that angiotensin II increased the expression of TRPC3 channel protein in VSMC from SHR in vitro, and this effect could be blocked by the AT1R antagonist telmisartan. Now, we investigated whether the effects of telmisartan on TRPC3 channel protein expression could also be observed in vivo. To support the hypothesis that blocking of angiotensin II but not reduction of blood pressure per se affects TRPC3 channel protein expression in VSMC, we also used the calcium channel blocker amlo- dipine in SHR. SHR were randomly allocated to placebo, telmisartan (5 mg/kg per day), or amlo- dipine (10 mg/kg per day), which was administered for 4 weeks. Compared with placebo (201±5 mm Hg; n=6), the administration of telmisartan significantly reduced blood pressure to 124±8 mm Hg (n=6), whereas amlo- dipine significantly reduced blood pressure to 116±5 mmHg (n=5; each P<0.01 compared with placebo by ANOVA and Tukey’s multiple comparisons post hoc test; Figure 6A). Furthermore, the angiotensin II–induced vasoconstriction of aortic rings was significantly lower in the telmisartan group compared with the placebo group (30±8% versus 82±3%; each n=6; P<0.01). Most important, compared with placebo, telmisartan significantly reduced TRPC3 channel protein expression from 1.00±0.07 to 0.32±0.20 (each n=3; P<0.05 by ANOVA and Tukey’s multiple comparisons post hoc test), whereas TRPC3 channel protein expression was unchanged in the amloidipine group (1.30±0.15; n=3; P=NS; Figure 6B).

**Discussion**

This report is the first to give experimental evidence for the importance of increased TRPC3 channel protein expression in the vasculature for elevated blood pressure in SHR. Foremost, for the first time, our present study showed an increased TRPC3 channel protein expression in the vasculature from adult SHR compared with adult WKY. In line with these findings, we observed increased TRPC3 transcripts in the vasculature from SHR compared with WKY. Furthermore, we showed that TRPC3 channel protein expression in the vasculature from 5-week-old prehypertensive SHR was similar to normotensive WKY. Second, for the first time, this study reported that TRPC3 channel proteins are regulated by angiotensin II in VSMC from SHR. Third, for the first time, this study showed that the long-term administration of telmisartan (but not amloidipine) to hypertensive rats reduces TRPC3 channel protein expression, confirming the importance of TRPC3 channel protein regulation by angiotensin II in vivo as well. However, additional electrophysiological data are needed to support these results.

The increased expression of TRPC3 was associated with enhanced angiotensin II–induced elevation of blood pressure, enhanced angiotensin II–induced vasoconstriction, and enhanced angiotensin II–induced calcium influx in VSMC from SHR. The hyper-responsiveness of vessels and VSMC from SHR after angiotensin II stimulation has been demonstrated clearly by several groups.6,12–15 Our present findings support previous results from several groups showing an increased
angiotensin II–induced calcium influx in VSMC from SHR. Although major calcium influx in VSMC is mediated by voltage-gated calcium channels, TRPC channels are important signal transducers for agonist-mediated vascular contractility. It should be noted that the increased angiotensin II–induced calcium influx in primary hypertension could also be observed in the presence of the calcium channel blocker amlodipine. On the other hand, in the presence of the nonselective TRP cation channel blocker SKF-96365, the angiotensin II–induced calcium influx was similar in VSMC from SHR and WKY. Furthermore, in SHR, the angiotensin II–induced calcium influx was considerably more reduced in the presence of SKF-96365 compared with amlodipine, supporting an important role of calcium influx through TRP channels in SHR. Use of SKF-96365 has been validated to block TRP cation channels by several investigators.

We gave experimental evidence that TRPC3 channels are directly involved in angiotensin II–induced calcium influx.

**Figure 4.** Effect of TRPC3 upregulation or TRPC3 gene knockdown on angiotensin II–induced calcium increase in VSMC from SHR. Expression of TRPC3 channel protein (A) and angiotensin II (Ang II)–induced calcium influx (B) in VSMC from SHR under control conditions (open bars) and after transfection with TRPC3 (dotted bars). Data are mean±SEM of n=6 independent experiments. **P<0.01 compared with control. Expression of TRPC3 channel protein (C) or TRPC6 channel protein (D) and Ang II–induced calcium influx (E) in VSMC under control conditions (open bars) and after TRPC3 gene knockdown using specific small interfering RNA against TRPC3 (dotted bars). Data are mean±SEM of n=5 independent experiments. *P<0.05 compared with control.

**Figure 5.** Angiotensin II (Ang II) increases TRPC3 channel protein in VSMC from SHR. A, Representative Western blotting and summary data of TRPC3 channel protein expression in VSMC from SHR (filled bars) and WKY (open bars) under resting conditions (Basal) and after administration of 100 nmol/L AngII. Data are mean±SEM of n=6 independent experiments. **P<0.01 for the comparison SHR versus WKY under resting conditions. B, Representative Western blotting and summary data of TRPC3 channel protein expression in VSMC from SHR under control conditions (Cont) or after administration of 100 nmol/L Ang II in the absence and presence of telmisartan (Telmi). Data are mean±SEM of n=6 independent experiments. **P<0.01 compared with Cont; ###P<0.01 compared with Ang II alone.
TRPC3 overexpression in VSMC increased the angiotensin II–induced calcium influx, whereas TRPC3 gene knockdown reduced angiotensin II–induced calcium influx in VSMC. In accordance with these results, Kaznacheyeva et al recently showed that downregulation of TRPC3 reduces calcium influx after depletion of intracellular stores in A431 cells.29 Second, the administration of angiotensin II significantly elevated the expression of TRPC3 in VSMC from SHR but not from WKY, supporting the proliferative action of angiotensin II in hypertension. Third, the administration of the AT1R antagonist telmisartan prevented the angiotensin II–induced elevation of TRPC3 expression in cultured VSMC from SHR in vitro. Long-term administration of telmisartan significantly reduced blood pressure, vasoconstriction, and most important, TRPC3 expression, in aortic tissue in vivo. On the other hand, we showed that the reduction of blood pressure using amlodipine did not significantly change TRPC3 expression in vasculature. In line with these results, Kogata et al reported that long-term treatment with telmisartan but not amlodipine improved the acetylcholine-induced vessel relaxation in hypertensive rats, although blood pressure reduction was similar in both groups.30 The observed reduction of TRPC3 after administration of telmisartan but not amlodipine may also explain in part the protective effects of AT1 antagonists in hypertension beyond blood pressure reduction as indicated by recent literature. The AT1 receptor antagonists irbesartan and candesartan but not amlodipine treatment showed beneficial effects in diabetic apolipoprotein E–null mouse or monkeys.31–32

**Perspectives**

We observed an increased TRPC3 cation channel expression in vasculature from SHR compared with WKY, which was associated with increased contraction, an increased angiotensin II–induced TRPC3 expression, and finally, the reduction of TRPC3 expression after administration of telmisartan but not amlodipine in vivo. These results add to a considerable body of evidence that TRP channels are involved in vascular mechanisms.33 Furthermore, the present results point to the relevance of TRPC3 in the pathogenesis of primary hypertension. As indicated by recent literature, expression of several TRP channel subtypes may be divergent and redundant in cardiovascular diseases. Moreover, TRP channels display diverse properties, localization, and regulation as a result of their assembly into distinct homomeric and heteromeric channel complexes.34 However, TRP channels will be fascinating targets to elucidate novel pathogenic mechanisms in hypertension, and TRP channels will be new therapeutic targets for cure of hypertension.

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**Disclosures**

None.

**References**

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Increased transient receptor potential canonical type 3 channels in vasculature from hypertensive rats

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Methods

Animals, interventions and hemodynamic measurements

Male 3-month-old SHR and WKY were housed under a 12h/12h day/night cycle. Some experiments were performed in 5-weeks-old prehypertensive SHR. Rats were obtained from Charles Rivers Laboratories and fed with standard chow and had water ad libitum. All experiments were performed as approved by the Animal Care and Use Committee. Hemodynamic measurements were done as described in the literature. Rats were anesthetized with pentobarbital sodium (60mg/kg IP). While rats were breathing spontaneously, the right carotid artery was cannulated for measurements of arterial blood pressure with a pressure transducer (model MLT 1030, Power Lab, Australia). The left jugular vein was cannulated for IV infusion of angiotensin II (24µg/kg/h). Infusion with angiotensin II was performed for 60 minutes.

The effect of long term administration of the angiotensin II receptor 1 (AT1R) antagonist telmisartan or the calcium channel blocker amlodipine on blood pressure and TRPC3 expression was investigated in SHR. SHR were randomly allocated to the groups. Telmisartan (5mg/kg/day), amlodipine (10mg/kg/day), or placebo was given by gavage for 4 weeks.

Systolic blood pressure which was measured by tail-cuff plethysmography and body weights were quantified every week. At the end of 4 weeks, the rats were killed by decapitation and aorta was used for measurements.

Preparation of aortic rings and vasoconstriction

The thoracic aorta was dissected, carefully freed from connective tissue, and placed in Krebs solution, containing (in mmol/L) NaCl 120, CaCl2 2.5, KCl 5.6, MgSO4 1.2, NaHCO3 25, NaH2PO4 1.2, D-glucose 5.5, equilibrated with 95% O2/5% CO2, pH 7.4 at 37°C. After careful removal of endothelial layer isometric force of the aortic rings was measured.
according to established methods using a force transducer connected with a polygraph (model AD Instrument, Power Lab) as described by our group. The aortic rings were equilibrated for 60 minutes. The resting tension was set to 2 g. Angiotensin II (100 nmol/L) was added and the contractions of the rings were measured. Data were given relative to maximum potassium contraction.

Culture of vascular smooth muscle cells

VSMC were obtained from thoracic aortas and cultured by tissue explant method as described. VSMC were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 50 U/mL penicillin G, and 50 µg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were made quiescent by incubation of 95%-confluent cell cultures in serum-free DMEM/F12 (1:1) containing 15 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4, 0.1% bovine serum albumin, and 5 µg/mL transferrin for 48 h. In additional experiments VSMC from SHR or WKY were cultured in the continuous presence of 100 nmol/L angiotensin II. To verify that cultured cells were VSMCs, immunocytochemical localization of smooth muscle-specific α-actin was performed using monoclonal antibodies ASM-1 raised against smooth muscle α-actin. A viability of VSMC more than 95% was observed by trypan blue exclusion.

Measurement of cytosolic calcium concentration

Cytosolic calcium concentration was measured in cultured VSMC using the fluorescent dye technique as previously described by our group. VSMC were loaded with 2 µmol/L of the calcium-sensitive, cell permeable, intracellular fluorescence dye fura2/AM (Sigma-Aldrich, USA) at room temperature for 60 minutes and washed to remove extraneous dye. Fluorescence was measured at 510 nm emission with excitation wavelengths of 340 nm and 380 nm, and
fluorescence excitation ratio was calculated. Additional experiments were performed in the presence of the calcium channel blocker amlodipine or 1-[β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF-96365; Merck Biosciences; final concentration, 10µmol/L), which has been reported to block non-selective TRP cation channels by several investigators. As indicated by recent literature data on angiotensin II-induced calcium increase are expressed as differences in cytosolic calcium from baseline in nmol/L.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was isolated from aortic tissue of adult normotensive Wistar Kyoto rats (WKY), 5-weeks-old prehypertensive spontaneously hypertensive rats (preSHR), and adult spontaneously hypertensive rats (SHR) using TRIzol reagent (Invitrogen, USA). RNA was used to synthesize first-strand cDNA using the Reverse Transcription System (Promega A3500, USA). 2.5µg total mRNA was reverse-transcribed with a reverse transcription mixture consisting of oligo dT and 15 U AMV reverse transcriptase at 42°C for 60 min, followed by heating to 95°C for 5 min. 3µl of 1:3 diluted single-stranded cDNA was added to 25µl PCR mix and amplified using 2xTaq Plus PCR MasterMix (Tiangen Biotech CO, PR China). PCR was started with denaturation at 95°C for 5 min, then 34 cycles were performed under the following conditions: denaturation at 95°C for 40s, annealing at 60°C for 30s, and extension at 72°C for 40s. The sense and antisense primers for coding regions of TRPC3 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were the following:

TRPC3 (NM_021771) Forward 5'-CTGGCACAATATGTGGGCAATA-3';
Reverse 5'-GCGTTCGGATGAGAAGGTAGG-3';
GAPDH (NM_017008) Forward 5'-ACGGCAATTTCAACGGCAGTCA-3';
Reverse 5'-TGGGGGCATCGGCAG AAGG-3'.

The PCR products were size fractionated on 1.5% agarose gels, and DNA was visualized by ethidium bromide staining using an imaging analyzer (GelDoc 2000, BioRad, Germany). The expected product sizes were 442bp for TRPC3 and 231bp for GAPDH, respectively.

Western blotting

Immunoblottings of TRPC3 and AT1R were performed using standard techniques. Quiescent VSMC grown on culture plates were exposed to Hanks balanced salt solution for control or angiotensin II at a final concentration of 100nmol/L for 24 hours VSMC were homogenized in high-salt buffer containing NaCl 600mmol/L, MOPS 40mmol/L, DTT 1mmol/L, leupeptin 1µg/mL, aprotinin 1µg/mL, phenylmethylsulfonyl fluoride 50mmol/L. Cells were scraped off, transferred to Eppendorf tubes, and sonicated for 5 seconds. The protein supernatant was separated by centrifugation, and protein concentrations were determined with Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA). Proteins were separated by using 10% sodium dodecyl sulfate polyacrylamide gel and transferred to Hybond-ECL nitrocellulose membranes (NEN Life Science Products, Boston, USA) at 100V for 1 hour. Membranes were blocked with blocking buffer containing tromethamine hydrochloride–buffered saline and 0.1% polysorbate-20 with 5% wt/vol nonfat dry milk and incubated for 8 hours at 4°C. Membranes were incubated with primary rabbit anti-TRPC3 antibody (alomone Labs, Jerusalem, Israel), which had been used by several investigators, or rabbit polyclonal IgG anti-AT1-Receptor (Santa Cruz Biotechnology, USA) diluted 1:1000 for 8 hours at 4°C. After washing membranes were incubated with secondary antibodies (goat anti-rabbit horseradish peroxidease) diluted 1:2000 for 1 hour at room temperature, and washed extensively. Each sample was processed three times. The data for TRPC expression in SHR were normalized to the expression in WKY.

Knockdown of TRPC3 gene expression using small interfering RNA
RNA interference for the down-regulation of a specific gene in living cells by small interfering RNA (siRNA) was performed. VSMCs were transfected with siRNA specific for TRPC3 for 24 hours using a silencer siRNA transfection kit (Ambion, Austin, USA) or with negative control siRNA. Briefly, VSMCs were resuspended in advanced Dubecoo’s modified Eagle’s medium containing 10% fetal bovine serum and incubated with siPORT amine (Ambion) and 1µL chemically synthesized siRNA (final concentration, 20nmol/L; Ambion) specific for the TRPC3 channel. The target sequence for TRPC3 was 5’– GGUUAACCUCUCACUCAAtt–3’ (sense) and 5’– UGAGUGAAGAGGUUUAACCtg–3’ (antisense), respectively.

Over-expression of TRPC3 using pAdEasy-1 system

The cDNA encoding human TRPC3 was amplified by RT-PCR from TRPC3pcDNA3. Recombined shuttle plasmid pAdTrack-CMV-TRPC3 was constructed by linking the pAdTrack-CMV with the TRPC3 and was sequenced. For generation of recombinant adenoviral plasmids, pAdTrack-CMV-TRPC3 was linearized with PmeI, and was transformed into electrocompetent E. coli BJ5183 cells which contained adenoviral backbone vectors (pAdEasy-1). Clones showing inserts were screened by restriction endonuclease digestions. For production of adenoviruses in HEK293 cells, recombinant adenoviral plasmid DNA, digested with PacI and purified by gel extraction kit, were used for transfection of HEK293 cells. Transfected cells were monitored for green fluorescent protein (GFP) expression and collected 7–10 days after transfection by scraping cells off flasks and pelleting them along with any floating cells in the culture. After three cycles of freezing in a methanoly dry ice bath and rapid thawing at 37°C, viral lysate was used to infect VSMC in flasks.

Immunohistochemistry
Cultured VSMC were fixed with 10% formalin for 10 minutes at room temperature, washed with phosphate buffered saline (PBS) and then permeabilized with PBS containing 0.5% Triton X-100 for 20 minutes at room temperature. VSMC were incubated with PBS containing with 5% bovine serum and 0.1% Triton X-100 for 1 hour at room temperature. Then VSMC were incubated with rabbit anti-TRPC3 antibody (1:200, alomone) over night at 4°C. VSMC were then washed and incubated with antibodies conjugated to a fluorescent probe (FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA), 1:200 for 30 minutes at room temperature. In control experiments, the primary antibodies were pre-incubated for 12 h at 4°C with antigenic peptide (1:25). After removing the unbound secondary antibodies by washing the preparations with PBS, imaging was performed using the fluorescence microscope (NiKon, TE2000).

Statistical analysis
All results are expressed as the mean±SEM of at least 3 independent experiments. Data were compared using two-tailed Student’s t-test or ANOVA and Tukey’s multiple comparisons post hoc test as appropriate. Two-sided p values less than 0.05 were considered significant. Where error bars do not appear on the figure, error was within the symbol size.

References


