Upregulation of TRPM7 Channels by Angiotensin II Triggers Phenotypic Switching of Vascular Smooth Muscle Cells of Ascending Aorta

Zheng Zhang,* Mi Wang,* Xiao-Han Fan, Jing-Hui Chen, Yong-Yuan Guan, Yong-Bo Tang

Rationale: Angiotensin II (Ang II) has pleiotropic effects on vascular smooth muscle cells (VSMCs). It has been demonstrated to promote the proliferative phenotype of VSMCs in mouse ascending aorta, but the underlying mechanisms remain incompletely understood.

Objective: The present study was designed to explore whether the Ca\(^{2+}\)-permeable transient receptor potential melastatin 7 (TRPM7) channel is involved in Ang II–induced phenotype switching of ascending aortic VSMCs and to dissect the molecular mechanisms by which TRPM7 modulates VSMC phenotype.

Methods and Results: As revealed by current recording, Ang II infusion increased TRPM7 whole-cell currents in ascending aortic VSMCs. The increase in TRPM7 currents was found to result from enhanced expression of TRPM7 protein rather than elevated single-channel activity (open probability and slope conductance) and/or reduced Mg\(^{2+}\)-mediated channel block. Mechanistically, Ang II elevated TRPM7 expression via Ang II type 1 receptor–mediated ERK1/2 signaling. As indicated by the expression levels of VSMC differentiation marker genes, phenotypic switching of ascending aorta occurred during Ang II infusion. Meanwhile, ERK1/2-Elk-1 signaling pathway known to suppress VSMC differentiation was activated in Ang II–infused ascending aorta. Knockdown of TRPM7 with small interfering RNA established a causative role of TRPM7 in Ang II–induced phenotypic change and promotion of cell proliferation. Moreover, TRPM7 was shown to be required for Pyk2-ERK1/2-Elk-1 pathway activation by Ang II, which potentiated TRPM7 channel function and thus activated the Ca\(^{2+}\)-sensitive kinase Pyk2. Finally, TRPM7 knockdown attenuated Ang II–induced displacement of myocardin from SM22 promoter, but the effects could be reversed by expression of constitutively active c-Src.

Conclusions: Our data establish that upregulation of TRPM7 channels by Ang II contributes to the development of the proliferative phenotype of ascending aortic VSMCs, and TRPM7 channel suppresses VSMC gene expression via Ca\(^{2+}\) influx-mediated activation of Pyk2-ERK1/2-Elk-1 pathway. (Circ Res. 2012; 111:1137-1146.)

Key Words: angiotensin II  ■  TRPM7  ■  phenotypic modulation  ■  smooth muscle  ■  calcium signaling  ■  hyperplasia  ■  ion channels  ■  vascular smooth muscle

Vascular smooth muscle cells (VSMCs), the major constituent of blood vessels, play an essential role in the regulation of blood pressure, blood vessel tone, and blood flow distribution. Unlike the terminally differentiated skeletal or cardiac myocytes, VSMCs retain remarkable phenotypic plasticity in response to a wide variety of stimuli. Although the plasticity is indispensable for normal vascular development, dysregulated irreversible change in VSMC phenotype is associated with a number of cardiovascular diseases, including atherosclerosis, aneurysm, and hypertension.\(^1\)\(^2\) The phenotypic modulation (or phenotypic switching) of VSMCs is a multifaceted process, and the underlying mechanisms remain incompletely defined. VSMCs express a repertoire of Ca\(^{2+}\)-permeable ion channels, some of which have been implicated in phenotypic modulation of VSMCs. For instance, maintenance of the differentiated state of VSMCs requires voltage-gated Ca\(^{2+}\) channels;\(^3\) Ca\(^{2+}\)-permeable TRPC6 channels underlie pulmonary arterial hypertension by promoting proliferation of pulmonary VSMCs;\(^4\) the store-operated Ca\(^{2+}\) channel—Orai1—is involved in neointimal...
formation in the animal model of vascular injury. All the evidence converges to highlight the importance of finely tuned Ca\(^{2+}\) signaling in VSMC homeostasis. Transient receptor potential melastatin 7 (TRPM7), a member of TRP melastatin subfamily, is a Ca\(^{2+}\)-permeable nonselective cation channel whose expression has been detected in VSMCs. Through Ca\(^{2+}\) signals, TRPM7 channels participate in many physiological and pathophysiological processes, including directed cell migration, cell adhesion, anoxic neuronal death, and transdifferentiation of cardiac fibroblast. Moreover, TRPM7 is essential for embryonic development, as evidenced by genetic ablation leading to embryonic lethality. However, the functional role of TRPM7-mediated Ca\(^{2+}\) signaling in the phenotypic switching of VSMCs has not been explored.

VSMCs change their phenotype in response to various environmental cues, of which angiotensin II (Ang II) has been extensively studied. Ang II exerts multiple effects on vascular smooth muscle, including contraction of arteries under normal circumstances. Nevertheless, in certain forms of hypertension, persistent elevation of Ang II level gives rise to vascular remodeling, in which growth of VSMCs plays a pivotal role. The majority of the studies both in vitro and in vivo have demonstrated the hypertrophic effects of Ang II in rat thoracic aortic VSMCs, which are most widely used. Nonetheless, Owens et al recently reported an interesting observation that Ang II acts on mouse aorta in a region-specific manner; it promotes hyperplasia of the ascending aorta while inducing hypertrophy of the other aortic regions. Inhibitor of differentiation 3 has been proposed to account for the differential effects, but it is unclear whether other molecules are also involved. In the present study, we attempted to explore whether the Ca\(^{2+}\)-permeable ion channel—TRPM7—is involved in Ang II–mediated phenotypic modulation of ascending aortic VSMCs and whether TRPM7-mediated Ca\(^{2+}\) signal is behind the phenotypic change.

**Methods**

For detailed experimental procedures, please see the Online Data Supplement.

RNA Extraction and Quantitative Real-Time PCR

Trizol reagents (Invitrogen) were used to extract RNA from cultured cells or tissues. Real-time PCR was carried out according to QuantiTect SYBR Green PCR Kit protocol (Qiagen) on the ABI fast real-time PCR system (Applied Biosystems). Gene expression was normalized versus GAPDH.

**Animal Model**

All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China. At the age of 8 to 10 weeks, healthy C57BL/6 male mice were implanted with Alzet mini-osmotic pumps (Model 2004, Durect Corp) loaded with saline (0.9% NaCl) or Ang II (Sigma cat No. A9525, 1000 ng/kg per minute) for 4 weeks. Losartan (20 mg/kg, Sigma) or RDEA119 (35 mg/kg, Selleckchem) was administered by oral gavage for 4 weeks.

**Recording of TRPM7 Currents**

Native TRPM7 currents were recorded as previously described. For whole-cell recording, voltage ramps of 100-ms duration from −100 mV to +100 mV were applied at an interval of 2 seconds to cells held at 0 mV. The external solution for whole-cell recording contained (in mmol/L): 145 NaCl, 5 KCl, 2 CaCl\(_2\), 10 HEPES, 10 glucose, pH 7.4; the internal solution was composed of (in mmol/L): 145 Cs-methanesulfonate, 8 NaCl, 10 Cs-EGTA, 10 HEPES, pH 7.2 adjusted with CsOH. Single-channel TRPM7 currents under inside-out configuration were acquired at 10 kHz and digitally filtered off-line at 1 kHz. The internal solution used contained (in mmol/L): 140 Na-methanesulfonate, 8 NaCl, 5 EGTA, 5 EDTA, 10 HEPES, 10 glucose, pH 7.4; the external solution contained (in mmol/L): 140 Cs-methanesulfonate, 8 NaCl, 5 EGTA, 5 EDTA, 10 HEPES, pH 7.2.

**Data Analysis**

All data are expressed as means±SEM. The n value denotes the number of independent experiments unless otherwise stated. Statistical analysis was determined by an unpaired 2-tailed Student t test or 1-way ANOVA followed by the Bonferroni multiple comparison post hoc test with a 95% confidence interval. Probability values of <0.05 were considered statistically significant.

**Results**

**Ang II Increases TRPM7 Channel Expression In Vivo**

Whereas TRPM7 protein has been detected in VSMCs, its electrophysiological properties have not been characterized in VSMCs from the ascending aorta. In isolated ascending aortic VSMCs, a robust TRPM7-like current was readily recorded. The characteristic current-voltage relationship with small inward currents and strong outward currents resembles that of heterologously expressed TRPM7. The currents were inhibited by extracellular application of 100 μmol/L 2-APB, potentiated by removal of extracellular divalent ions, and inhibited by intracellular high Mg\(^{2+}\) levels (Online Figure I); all of these properties are similar to those of the recombinant TRPM7. Furthermore, the single-channel conductance of ≈33 pS was close to that of overexpressed TRPM7 channel. Having characterized the native TRPM7 currents in VSMCs, we explored whether TRPM7 channel in aortic VSMCs is regulated by in vivo Ang II stimulation. The mouse model of Ang II infusion was established (Online Figure II A) and TRPM7 currents were measured in freshly dispersed cells. Strikingly, whole-cell TRPM7 currents progressively increased after Ang II infusion, compared with the corresponding saline-infused controls (Figure 1A and 1B). Of interest, cell capacitance, an indicator of cell size, remained unchanged...
in Ang II–treated cells, thus indicating there was no significant VSMC hypertrophy (Figure 1C); this line of evidence is in line with Owens et al’s observation that ascending aorta underwent hyperplasia rather than hypertrophy. Generally, the increase in whole-cell currents can be attributed to elevated single-channel activity, increased protein abundance, and/or altered channel regulation. Single-channel measurement revealed that the single-channel properties, including open probability and slope conductance, were indistinguishable in VSMCs from control or Ang II–infused mice (Figure 1D, 1E, and 1F). Of note, the Mg^2+ sensitivity of TRPM7 channel from both groups, revealed by the dose-response curves for Mg^2+–mediated TRPM7 inhibition, was similar (Figure 1G). Collectively, the increase in macroscopic TRPM7 currents may be caused solely by elevated protein expression. The inference was substantiated by western blot analysis of TRPM7 protein (Figure 1H and Online Figure IID). Given the relative effects of Ang II on different aortic regions, we also measured TRPM7 currents in VSMCs harvested from thoracic aorta at week 4 of Ang II infusion and found that TRPM7 currents were increased as well but to a lesser extent (Online Figure IIB). Besides, VSMC capacitance in the Ang II group was elevated significantly, indicating a hypertrophy of thoracic aortic VSMCs (saline, 11.3±0.32pF; Ang II, 14.9±0.21pF; *P<0.05; Online Figure IIC).

Upregulation of TRPM7 by Ang II Type 1 Receptor–Elicited Extracellular Signal-Regulated Kinase Signaling
To understand how Ang II enhances TRPM7 protein expression, a set of in vitro experiments was performed. As displayed in Figure 2A, Ang II increased TRPM7 expression in a dose-dependent manner. Ang II treatment also led to a ~2.3-fold rise in TRPM7 currents (Figure 2B and 2C). These results are in line with the in vivo findings. Ang II exerts its biological effects by binding to its cognate receptors, including Ang II type 1 receptor (AT1) and AT2. It was found that the AT1 blocker (losartan) effectively suppressed, whereas AT2 blocker (PD123319) slightly increased, TRPM7 transcripts (*P<0.05, Figure 2D). These results suggest that Ang II increased TRPM7 expression exclusively through AT1 receptor and that AT2 receptor signaling may act to attenuate AT1 signaling. AT1-elicited activation of mitogen-activated protein kinase (MAPK) pathway has been demonstrated to regulate the expression of a wide range of genes. To test the possibility of Ang II–elicited MAPK signaling also controlling TRPM7 expression, U0126, SB203580, and SP600125 were used to block MAPK extracellular signal-regulated kinase (ERK) kinase1/2 (MEK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK), respectively. The results showed that only U0126 offset Ang II–induced rise in TRPM7 mRNA levels (Figure 2E). The data obtained with the pharmacological blockers were strengthened by the application of specific small interfering RNA (siRNA) against AT1 and

Figure 1. Ang II infusion increases TRPM7 channel expression in vivo. A and B, Average density of whole-cell TRPM7 currents in freshly isolated ascending aortic VSMCs measured at 1 week, 2 weeks, 3 weeks, and 4 weeks of saline (n=7–9 cells per time point) or Ang II infusion (n=8–10 cells per time point). In B, Ctrl (control) denotes 1-week saline-infused cell. C, C, Average cell capacitance of ascending aortic VSMCs (saline, n=9 cells for each time point; Ang II, n=10–11 cells for each time point). D through F, Excised single-channel measurement under inside-out configuration at 4 weeks of saline (n=6 patches) or Ang II infusion (n=9 patches). Cells were held at various voltages as indicated. Dashed line denotes the closed state of TRPM7 single channel. Single-channel open probability was analyzed at 80 mV over the span of 90 seconds. Slope conductance of TRPM7 single channels fitted with linear regression is shown in F, G, Dose-response curves for Mg^2+–mediated inhibition of TRPM7 currents recorded in ascending aortic VSMCs from saline-infused or Ang II–infused mice at week 4. Each plot was averaged from 5 to 6 cells for both groups. H, Protein levels of TRPM7 over the course of Ang II infusion. Mice infused with saline for 4 weeks served as control (n=4).
ERK1/2 (Figure 2F). Taken together, TRPM7 expression was increased as a result of AT1-mediated ERK1/2 signaling. To find out whether the in vitro findings also hold true in vivo, we treated Ang II–infused mice with losartan or RDEA119—a potent and specific MEK1/2 inhibitor.24,25 As Figure 2G shows, both losartan and RDEA119 were able to normalize TRPM7 currents in ascending aortic VSMCs.

**Ang II Infusion Changes the Cellular Phenotype of Ascending Aorta**

Decreased expression of VSMC differentiation marker genes and increased proliferation rate are 2 cardinal features of VSMC phenotypic switching.1,26 A transition from a contractile phenotype to a proliferative phenotype. Analysis of the phenotypic markers of differentiated VSMCs (SM–α-actin, SM-MHC, SM-22α) and proliferation indicator (Ki-67) revealed phenotypic switching of ascending aorta during Ang II infusion, as indicated by downregulated VSMC differentiation markers and upregulated Ki-67 (Figure 3A and Online Figure II). As shown in Figure 3C, 3D, and 3E, the mRNA levels of neither Myocdn, SRF, nor Elk-1 were altered, suggesting no change in expression at the transcription level.

The data were obtained from the ascending aorta of mice treated with the indicated compounds in G. VSMCs were cultured in serum-free medium for 24 hours before Ang II treatment (A through F). A, Western blot analysis of TRPM7 protein in cultured cells treated with varying concentrations of Ang II for 48 hours. B and C, TRPM7 current recording after the cells were subjected to Ang II treatment (0.5 μmol/L) for 48 hours. The typical TRPM7 currents are shown in B, where the data were plotted from 10 cells and 12 cells for control (Ctrl) and Ang II groups, respectively. D, Real-time quantitative PCR of TRPM7 mRNA under the conditions as indicated. Losartan (AT1 blocker, 10 μmol/L) or PD123319 (AT2 blocker, 10 μmol/L) was administered 1 hour before Ang II treatment (0.5 μmol/L) for 24 hours (n=5). E, TRPM7 mRNA levels in cells treated with the blockers of MAPK signal transduction. Cells were pretreated for 1 hour with U0126 (MEK1/2 inhibitor, 10 μmol/L), SB203580 (p38 MAPK inhibitor, 10 μmol/L), or SP600125 (JNK inhibitor, 10 μmol/L), followed by Ang II exposure (0.5 μmol/L) for 24 hours (n=5). F, TRPM7 mRNA levels in cells treated with specific siRNA against AT1 and ERK1/2. The cells were treated with Ang II (0.5 μmol/L) for 24 hours. The plots are the average data from 5 experiments. G, TRPM7 currents in ascending aortic VSMCs harvested from the animals treated with vehicle (placebo), AT1 blocker (losartan), or MEK1/2 blocker (RDEA119) for 4 weeks. The plot represents 7, 10, 12, and 11 cells for Ctrl, placebo, losartan, and RDEA119, respectively.

ERK1/2 (Figure 2F). Taken together, TRPM7 expression was increased as a result of AT1-mediated ERK1/2 signaling. To find out whether the in vitro findings also hold true in vivo, we treated Ang II–infused mice with losartan or RDEA119—a potent and specific MEK1/2 inhibitor.24,25 As Figure 2G shows, both losartan and RDEA119 were able to normalize TRPM7 currents in ascending aortic VSMCs.

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Moreover, we looked into the signaling mediated by Pyk2, the Ca\(^{2+}\)-sensitive proline-rich tyrosine kinase,\(^{27,28}\) and its downstream kinases MEK1/2-ERK1/2, which has been linked to PDGF-mediated inhibition of VSMC gene expression.\(^{28}\) It was found that the activity of Pyk2-MEK1/2-ERK1/2 was significantly increased by Ang II infusion (Figure 3G and Online Figure IVD). Despite the differential relationship between them by means of TRPM7 silencing with siRNA. A pair of TRPM7-targeting siRNA sequences (siRNA1 and siRNA2) was evaluated and siRNA1 was used in the study due to a slightly better efficiency (Online Figure IVA through C). Furthermore, Ang II was demonstrated to be able to modulate the phenotype of ascending aorta, as indicated by reduced VSMC gene expression (SM-α-actin, SM-MHC, SM-22α, calponin). It did not affect the expression levels of the transcriptional regulators of VSMC differentiation (Myocdn, SRF, Elk-1) and Pyk2. TRPM7 was shown to be required for Ang II–triggered phenotypic modulation of ascending aortic VSMCs because deficiency of TRPM7 largely restored VSMC gene expression in Ang II–treated cells (Figure 4A). Meanwhile, cell phenotype analysis in thoracic aortic VSMCs showed that Ang II increased and TRPM7 knockdown nearly normalized VSMC gene expression (SM-α-actin, SM-MHC, and SM-22α; Online Figure IVD). Despite the differential effects of Ang II on VSMC genes, suppression of TRPM7 normalized VSMC gene expression in both ascending and thoracic aortic VSMCs.

We subsequently evaluated if TRPM7 promotes Ang II–induced proliferation of aortic VSMCs. In cultured VSMCs harvested from ascending aorta, Ang II induced significant VSMC proliferation, which was dramatically inhibited on TRPM7 knockdown, as indicated by cell count and BrdU incorporation (Figure 4B and 4C). Also, Ang II treatment facilitated cell cycle transition from quiescent G\(_0\)/G\(_1\) to synthetic S phase (Figure 4D). However, cell cycle distribution of thoracic aortic VSMCs did not change in the presence of Ang II, and TRPM7 suppression exerted no effects (Online Figure IVE).

### Pyk2 Activation by TRPM7-Mediated Ca\(^{2+}\) Influx Controls VSMC Gene Expression

In vivo experiments showed that the signaling pathway Pyk2-ERK1/2 and its downstream target Elk-1 were activated during Ang II infusion (Figure 3), but whether TRPM7 is required for pathway activation must be validated. We treated cultured ascending aortic VSMCs with negative or specific TRPM7 siRNA and then examined the pathway after Ang II treatment. As in Figure 5A, Ang II treatment led to activation of Pyk2 and ERK1/2, which in turn phosphorylates Elk-1; no change in Myocdn and SRF protein levels was observed. Intriguingly, TRPM7 knockdown considerably inhibited the activation of the pathway Pyk2–ERK1/2–Elk-1, suggesting a causal link between TRPM7 and Ang II–mediated activation of Pyk2 (Figure 5A and Online Figure VA). Nonetheless, 2 questions arise: (1) does acute application of Ang II influence TRPM7 channel activity, and (2) does TRPM7 opening activate Pyk2? Using a relatively physiological recording condition (see Figure 5B and 5C), we found that TRPM7 currents were significantly potentiated by 30% in response to Ang II. Besides, knockdown of TRPM7 with siRNA in cultured ascending aortic VSMCs led to significantly reduced Ca\(^{2+}\) entry in response to Ang II, indicating the contribution of TRPM7 to Ang II–induced Ca\(^{2+}\) influx (Online Figure VC and D). The evidence collectively suggests that Ang II–mediated TRPM7 opening and resultant Ca\(^{2+}\) entry contributed to Pyk2 activation. To examine if TRPM7-mediated Ca\(^{2+}\) influx activates Pyk2, cells were first subjected to nominally divalent-free solution and then 20 mmol/L Ca\(^{2+}\)-containing
solution was applied, as in Ca²⁺ imaging experiment. The results showed that Pyk2 was activated when Ca²⁺ was re-added to DVF-treated cells and that Pyk2 activation was inhibited in cells with reduced TRPM7 (Figure 5D and Online Figure VE). In conclusion, TRPM7 opening induced Ca²⁺ influx, and Pyk2 activation ensued.

Next, we attempted to investigate whether Ang II alters the transcriptionsal program that controls VSMC gene expression and whether TRPM7-Pyk2–ERK1/2–Elk-1 signaling is involved in Ang II–elicited suppression of VSMC gene transcription. As detected by coimmunoprecipitation, Ang II stimulation enhanced the association of Elk-1 with SRF, accompanied by a decline in Myocdn–SRF association (Figure 5E). In addition, as revealed by ChIP assay (Figure 5F and 5G), association of Elk-1 with SRF-binding sites (CArG box sequence) in the promoter of SM-22 was increased in response to Ang II, which was accompanied by a decreased occupancy by Myocdn of the same sites. These data suggest a signal-dependent competitive exchange of Elk-1 for Myocdn on SRF in the promoter region of SMC gene in the presence of Ang II in ascending aortic VSMCs. Notably, these effects exerted by Ang II on VSMC gene transcription was reversed by TRPM7 knockdown, and SM-22 mRNA levels were determined. As shown in Figure 5H, SM-22 mRNA levels were nearly normalized by siRNA treatment against the pathway. Moreover, reconstitution of the whole pathway by introducing constitutively active mutants of c-Src, ERK1, Elk-1 to TRPM7-silenced VSMCs abrogated the effects of TRPM7 suppression on SM-22 expression, suggesting that ERK1/Elk-1 is downstream of TRPM7 and regulates the marker gene expression of ascending aortic VSMCs (Figure 5I). Transfection of wild-type c-Src, ERK1, and Elk-1 did not change SM-22 expression (Online Figure VF). Together, TRPM7-Pyk2–ERK1/2–Elk-1 signaling plays a key role in transcriptionally controlling the expression of VSMC marker gene.

**Discussion**

The present study investigated the phenotypic modulation of VSMCs by TRPM7, a member of TRPM channel family, which is constitutively active and brings Ca²⁺ into cells under physiological conditions. Our data demonstrated that Ang II upregulated TRPM7 channels in ascending aortic VSMCs and that TRPM7-mediated Ca²⁺ signals activated Pyk2–ERK1/2–Elk-1 signaling pathway and thus suppressed VSMC gene expression. Many studies have demonstrated the hypertrophic effects of Ang II on the commonly cultured rat
thoracic aortic VSMCs, whereas a lesser number have revealed proliferation. The discrepancy in growth response in in vitro experiments is thought to result from cell culture conditions, including cell type, cell passage, cell confluence, and cytokine milieu. Of interest, Owens et al defined the region-specific effects of Ang II infusion on mouse aortic media. Chronic administration of Ang II promotes uniform medial thickening of the whole aorta, but the underlying cause differs in a region-specific fashion. VSMCs hyperplasia occurs in the ascending aorta, whereas hypertrophy of VSMCs is evident in all other aortic parts (thoracic, abdominal, suprarenal, infrarenal). In vitro experiments reveal that Ang II potently stimulates cell proliferation in ascending aorta–derived VSMCs but has no effects on other aortic VSMCs of different origin. Consistent with Owens et al’s finding, our cell cycle assays showed that Ang II significantly facilitated...
cell cycle progression of ascending aortic VSMCs from G₁ to S phase but barely affected that of thoracic VSMCs. Suppression of TRPM7 considerably inhibited both cell proliferation rate and cell cycle progression, indicating the proproliferative role of TRPM7 in ascending aorta VSMCs. Indeed, TRPM7 channel has been implicated in cell proliferation in many other cell types, including chicken DT-40 B cells,⁶ tumor cells,⁹ and cardiac fibroblasts.¹⁴ Strikingly, a novel difference between ascending and nonascending aortic VSMCs was uncovered in our study: VSMC gene expression was reduced by Ang II in ascending aortic VSMCs but an enhancement was observed in the cells from thoracic aorta. Despite the opposite reactions to Ang II, TRPM7 suppression normalized VSMC gene expression in both cells. This indicates that block of TRPM7 in vivo would be beneficial to all aortic regions in the setting of chronic Ang II stimulation. Presently, the pathological significance of VSMC proliferation in the ascending aorta remains unknown, but Ang II–related diseases such as heart failure could be aggravated by expanded ascending aorta because of increased afterload. The diverse responses to Ang II may be attributable to the heterogeneity in embryonic origin of aortic VSMC;⁶,³⁷ VSMCs in ascending aorta are derived from neural crest, whereas thoracic aortic VSMCs are from somite lineage.³⁶,³⁸

Ang II has been known to regulate the expression and/or activity of many ion channels. For example, it downregulates Ca²⁺-activated Cl⁻ channel TMEM16A in cerebral VSMCs³⁹ and large conductance Ca²⁺-activated K⁺ channels during hypertension.⁴⁰ In the present study, we identified TRPM7 as a target of Ang II. Based on our findings, we propose that Ang II ameliorates TRPM7-mediated Ca²⁺ signals and the downstream pathway Pyk2–ERK1/2–Elk-1 and hence promotes the proliferative phenotype of ascending aorta. Nevertheless, it should be pointed out that Ang II might also act on other Ca²⁺-permeable channels (possibly TRPC channel and Orai channel), which have not been explored in ascending aortic VSMCs, to fulfill its proproliferative role. In the context of Ang II–triggered proliferation of ascending aorta, TRPM7 may be a unique ion channel target of Ang II, since (1) it is a constitutively active Ca²⁺-permeable channel abundantly expressed in ascending aortic VSMCs, and (2) its proliferation-promoting actions have been well documented in many cell types. Our results revealed that TRPM7 expression was elevated due to AT1 receptor–mediated ERK1/2 activation. Interestingly, in vivo administration of either AT1 receptor blocker or ERK1/2 blocker abolished Ang II–induced increase in TRPM7 function. Together with our finding that TRPM7 in ascending aortic VSMCs is required for ERK1/2 signaling, these data suggest that AT1 receptor and TRPM7 channels probably form a positive feedback loop through ERK1/2 signaling; AT1-mediated activation of ERK1/2 increases TRPM7 expression, which in turn aggravates ERK1/2 signaling through Pyk2 activation. This feedback loop may have pathological implications, but it must be verified in vivo. Additionally, we found that TRPM7 protein in ascending aortic VSMCs was elevated during Ang II infusion to a much larger extent, compared with thoracic aortic VSMCs. Despite the consistency in TRPM7 upregulation, the difference in fold increase may play a role in determining the relative effects of Ang II in specific aortic parts, because the strength and duration of Ca²⁺ signal is known to preferentially activate signaling pathways.⁴¹,⁴²

Although it is generally believed that Ang II is a contractile agonist, the evidence from Owens et al’s and ours strongly indicates that Ang II acts on ascending aortic VSMCs as a mitogenic agonist, which, exemplified by platelet-derived growth factor BB (PDGF), substantially suppresses VSMC differentiation.⁴³ It has been defined that PDGF suppresses VSMC gene expression by activating ERK1/2 and consequently phosphorylating the ternary complex factor—Elk-1.²⁹ Phosphorylated Elk-1 competes for SRF with myocardin, which is a master regulator of VSMC differentiation gene expression.²⁸,³⁰ Based on the similarity between Ang II and PDGF with regard to the effects on ascending aortic VSMCs, it is plausible to hypothesize that these 2 vasoactive factors may use similar mechanisms to regulate VSMC gene expression. The hypothesis was corroborated in our study. Ang II stimulation gave rise to activation of ERK1/2 and phosphorylation of Elk-1. As a result, myocardin was displaced from SRF. Knockdown of TRPM7 considerably ameliorated ERK1/2 activation as well as Elk-1 phosphorylation. Remarkably, the dynamic competition for SRF binding between phosphorylated Elk-1 and myocardin was regulated by TRPM7 channel, as revealed by TRPM7 knockdown experiment. In delineating how TRPM7 contributes to activation of ERK1/2–Elk-1 pathway, we focused on Pyk2¹⁰ because it serves as an upstream kinase of ERK1/2 signaling and importantly is sensitive to cytosolic Ca²⁺. The involvement of Pyk2 in Ang II signaling is well documented in rat thoracic aortic VSMCs,⁴⁴,⁴⁵ but the Ca²⁺ source for Pyk2 activation remains incompletely defined. In the present study, we demonstrated that TRPM7 opening provided Ca²⁺ to activate Pyk2 in ascending aortic VSMCs. More importantly, activation of Pyk2 by TRPM7-mediated Ca²⁺ signals potentiated ERK1/2 signaling and thus resulted in suppression of VSMC gene expression. Pyk2 has been shown to relay TRPM2 channel-mediated Ca²⁺ signals to NF-xB activation in monocytes⁴⁶ and participate in TRPC channel–mediated neuronal survival.⁴⁷ Our description of TRPM7 activating Pyk2 in VSMCs is the first, to the best of our knowledge.

The phenotype of VSMCs is modulated by many environmental cues, and, in the present study, Ang II was demonstrated to be highly proproliferative in ascending aortic VSMCs. Mechanistically, Ang II upregulates the expression of the Ca²⁺-permeable TRPM7 channels and augments TRPM7-mediated Ca²⁺ signals, which in turn activate Pyk2–ERK1/2–Elk-1 and reprogram VSMCs to suppress differentiation and promote proliferation. Elk-1 phosphorylation due to TRPM7-Pyk2-ERK1/2 is capable of coordinating the 2 aspects of VSMC phenotypic modulation. On one hand, phospho-Elk-1 displaces myocardin from SRF on the promoters of VSMC differentiation genes and hence suppresses VSMC gene expression. On the other hand, it binds to SRF on the promoter of proto-oncogenic genes, such as c-fos, to increase the proliferation rate. Based on our data, we propose that the signal transduction by TRPM7-Pyk2–ERK1/2–Elk-1 may represent an important mechanistic basis for Ang
II–induced phenotypic switching of ascending aortic VSMCs. Although our in vivo data clearly showed that Pyk2–ERK1/2–Elk-1 pathway as well as TRPM7 function was enhanced by Ang II infusion, the decisive link between TRPM7 and the pathway activation awaits validation in vivo with the use of VSMC-specific TRPM7 deletion. Furthermore, it is still unclear whether TRPM7 channel is involved in VSMC phenotypic change induced by other agonists, such as PDGF and TGF-β.

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**Disclosures**
None.

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**TRPM7 Modulates VSMC Phenotype**

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**Novelty and Significance**

**What Is Known?**

- Ang II infusion induces a proliferative phenotype in the cells of the mouse ascending aorta.
- Ca2+ signaling plays a critical role in modulating VSMC phenotype.
- TRPM7, a ubiquitously expressed Ca2+-permeable cation channel, is required for cell proliferation in many cell types.

**What New Information Does This Article Contribute?**

- Ang II infusion increases TRPM7 channel expression in mouse ascending aortic VSMCs; Ang II upregulates TRPM7 via AT1-ERK1/2 signaling.
- TRPM7 is involved in Ang II–triggered phenotypic switching of ascending aortic VSMCs in vitro.

- TRPM7 channel–mediated Ca2+ influx activates Pyk2–ERK1/2–Elk-1 signaling pathway and thus promoting the proliferative phenotype.

Chronic Ang II stimulation produces multiple deleterious effects in VSMCs that could contribute to the etiology of several cardiovascular diseases. Ang II induces a differentiated-to-proliferative switch of cells in the ascending aorta but the contribution of perturbations in intracellular Ca2+ to this process is unclear. In the present study, we demonstrate a critical role for the Ca2+-permeable channel, TRPM7, in phenotypic switching induced by Ang II and link TRPM7–mediated Ca2+ influx to the activation of the Ca2+-sensitive kinase Pyk2. Our data underscore the importance of Ca2+ homeostasis in VSMC biology and indicate that TRPM7 could be a therapeutic target for mitigating Ang II–induced proliferation of vascular smooth muscle cells.
Upregulation of TRPM7 Channels by Angiotensin II Triggers Phenotypic Switching of Vascular Smooth Muscle Cells of Ascending Aorta
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Expanded methods and materials

RNA extraction and quantitative real-time PCR
Trizol reagents (Invitrogen) were used to extract RNA from cultured cells or tissues. Total RNA (1µg) was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad). Real time PCR was carried out according to QuantiTect SYBR Green PCR Kit protocol (Qiagen) on ABI fast real-time PCR system (Applied Biosystems, USA). Sequences of the gene-specific primers derived from Primerbank (http://pga.mgh.harvard.edu/primerbank)1 are available upon request. Specificity of the primers was verified by dissociation curves and gene expression was normalized versus GAPDH.

Animal model and blood pressure measurement
Animals were supplied by the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). All experimental procedures were performed in accordance with the policies of the Sun Yat-Sen University Animal Care and Use Committee and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China. At the age of 8-10 weeks, healthy C57BL/6 male mice were anesthetized with 2% sodium pentobarbital (0.2ml/100g, intraperitoneal injection), followed by implantation of Alzet mini-osmotic pumps (Model 2004, Durect Corp) loaded with saline (0.9% NaCl) or Ang II (Sigma cat# A9525, 1000ng/kg/min) for 4 weeks. At 1w, 2w, 3w, 4w of minipump infusion, mice were sacrificed for current measurement or protein detection. Systolic blood pressure was measured in conscious mice weekly using the non-invasive tail cuff system (ADInstruments, USA). To explore the effects of losartan and RDEA119 on TRPM7 currents, mice implanted with Ang II minipumps were administered daily with vehicle, losartan (20mg/kg, Sigma) or RDEA119 (35mg/kg, Selleckchem) by oral gavage for 4 weeks. Losartan was reconstituted in PBS while RDEA119 was reconstituted in 10% 2-hydroxypropyl-beta-cyclodextrin (Sigma) dissolved in PBS. 10% 2-hydroxypropyl-beta-cyclodextrin dissolved in PBS was administered as vehicle (Placebo).

Fresh isolation of mouse aortic VSMCs and primary cell culture
Fresh isolation was carried out as described2. Briefly, mice were anesthetized with pentobarbital, and aorta were quickly removed and carefully cleaned of connective tissues in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) containing 25mM HEPES, pH 7.4 and 1mg/ml bovine serum albumin (BSA). Afterwards, ascending aorta (above the heart to left subclavian artery) or thoracic aorta (left subclavian artery to last intercostal artery) was separately subjected to digestion in medium described above supplemented with 200 units/ml collagenase type-III, 0.1 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor at 37°C for 30 min and then in medium containing 130 units/ml collagenase type-III, 0.1 mg/ml elastase and 0.5 mg/ml

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soybean trypsin inhibitor at 37°C for 45 min. After that, the medial tissue was transferred to fresh digestion medium containing 130 units/ml collagenase type-III, 0.1 mg/ml elastase and 0.5 mg/ml soybean trypsin inhibitor, minced finely with scissors and further digested at 37°C for an additional 1 h. The suspended cells were collected by centrifugation at 200g for 3 min and then seeded for primary culture or single cell experiments. Prior to confluence, the freshly seeded cells were maintained in DMEM with 20% FBS (Gibco), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen) in a 37°C incubator with 5% CO₂. After the 1st subculture, cells were cultured in DMEM containing 10% FBS plus 100 U/ml penicillin/streptomycin. Passages 8 through 12 of primarily cultured VSMCs were used for experiments.

**Recording of TRPM7 currents**

Native TRPM7 currents were recorded as previously described³,⁴. Single-channel and whole-cell currents were measured at room temperature with the use of Axopatch 200B patch clamp amplifier (Axon Instrument, USA). The currents were sampled at 10 kHz and filtered at 2 kHz. Patch pipettes with a resistance of 3-5 MΩ when filled with internal solutions were pulled from borosilicate glass and fire-polished. For whole-cell recording, voltage ramps of 100ms duration from -100mV to +100mV were applied at an interval of 2s to cells held at 0mV. The external solution for whole cell recording contained (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 10 HEPES, 10 Glucose, pH 7.4, while the internal solution was composed of (in mM): 145 Cs-methanesulfonate, 8 NaCl, 10 Cs-EGTA, 10 HEPES, pH 7.2 adjusted with CsOH. Single-channel TRPM7 currents under inside-out configuration were acquired at 10 kHz and digitally filtered off-line at 1 kHz. The internal solution used contained (in mM): 140 Na-methanesulfonate, 8 NaCl, 5 EGTA, 5 EDTA, 10 HEPES, 10 Glucose, pH 7.4, and the external solution contained (in mM): 140 Cs-methanesulfonate, 8 NaCl, 5 EGTA, 5 EDTA, 10 HEPES, pH 7.2.

**Western blot**

Total proteins were prepared from cultured aortic VSMCs or pooled ascending aorta from Ang II-infused mice or their corresponding controls (4-5 ascending aorta from each group were used as 1 sample), and western blotting was performed as previously described⁵-⁷. Briefly, ascending aorta were carefully cleaned of connective tissues in ice-cold PBS and then finely minced in ice-cold lysis buffer: Tris-HCl 50mM, NaCl 150mM, NaN₃ 0.02%, Nonidet P-40 1%, sodium dodecyl sulfate 0.1%, sodium deoxycholate 0.5% and 1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). The lysis process lasted for 30 minutes. Primarily cultured cells were rinsed with PBS twice and then harvested and lysed with the abovementioned lysis buffer. Protein was quantified by BCA assay (Beyotime, China) and separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked at room temperature for 1h in 5% non-fat milk dissolved in TBST (in mM: NaCl 150, Tris 50, Tween 0.1%, pH 7.5), and incubated with specific primary antibodies for 2h at room temperature and 4°C overnight, and finally with the appropriate secondary peroxidase-conjugated antibodies for 1.5 h at room temperature. Bands were detected by chemiluminescence assay (Beyotime, China) and quantified using ImageJ program (version 1.4.3). Rabbit anti-phospho-Pyk2 (Tyr402), rabbit anti-Pyk2, rabbit anti-phospho-MEK1/2 (Ser217/221), rabbit anti-MEK1/2, rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) and rabbit anti-ERK1/2 antibodies were purchased from Cell Signaling Technology. Rabbit anti-phospho-Elk-1 (Ser383), rabbit anti-Elk-1 and rabbit anti-myocardin antibodies were from Santa Cruz Biotechnology. All the other antibodies were obtained from Abcam.

**Cell proliferation assays**

Cell proliferation was assessed by cell counts and 5-bromo-2’-deoxyuridine (BrdU) incorporation as previously described⁵-⁷. VSMCs were trypsinized and seeded into 6-well plates at a density of 5×10⁵ cells/well in complete growth media. After 24 hours, siRNA transfection was performed in
serum-free media for 8 hours and then the transfection complex was replaced with serum-free media for 24 hours to render the cells quiescent. Afterwards, the cells with or without siRNA transfection were treated with Ang II for 48 hours. Cell number was determined with a hemocytometer. BrdU incorporation was performed to assess DNA synthesis. BrdU (10mM) was added to the media 24 hours before Ang II treatment terminated. The cells were then fixed and treated with anti-BrdU primary antibody for 1h at room temperature. Unbound antibodies were carefully washed away. After that, the cells were incubated with horseradish peroxidase-conjugated goat anti-IgG for 30 min, and then 100 mmol/L 3, 3', 5, 5'- tetramethylbensidine was added as the substrate for horseradish peroxidase. Incorporation was measured at 450 and 540 nm on an Elisa microplate reader (BIO-TEK Synergy HT, USA).

**Ratiometric Ca^{2+} imaging**
Freshly isolated VSMCs from saline- or Ang II-infused mice were seeded on glass coverslips and loaded with 2µM Fura-2 AM (Molecular Probes, Invitrogen Life Technologies) for 30 min at 37°C in the dark in HEPES-buffered saline solution (HBSS) containing (mM): 140 NaCl, 4.7 KCl, 1.2 MgCl2, 1.3 CaCl2, 1.2 KH2PO4, 20 HEPES, 10 Glucose, pH 7.4. Intracellular Ca^{2+} levels, indicated by the ratio of two fluorescence intensities (F340/F380), were measured at a rate of 1 Hz with a confocal laser scanning system (Olympus, Tokyo, Japan) equipped with an Olympus FV500 inverted microscope with a 20x 1.4 NA objective. Cells were bathed in nominally Ca^{2+}, Mg^{2+}-free solution (in mM: 145 NaCl, 5 KCl, 10 HEPES, 10 Glucose, pH 7.4) for 5 minutes, and then Ca^{2+} influx was determined by perfusing the cells with external solution containing 20mM Ca^{2+} (in mM: 145 NaCl, 5 KCl, 10 HEPES, 20 CaCl2, 10 Glucose, pH 7.4).

**siRNA and plasmid transfection**
The siRNA duplexes against TRPM7 (siRNA1, AACCGGAGGTCAGGTCGAAAT, corresponding to the coding region 1630-1650 of murine TRPM7 mRNA; siRNA2, CCTGATGAGGTTGTCACAGAG, corresponding to the coding region 4680-4700 of murine TRPM7 mRNA; GenBank accession number NM_021450) synthesized by Invitrogen was transiently transfected with Hiperfect Transfection Reagent (Qiagen) according to the manufacturer’s instructions as previously described. A nonsilencing RNA (Qiagen) was used as negative control. siRNA strand with a final concentration of 40 nM and Hiperfect Transfection Reagent were diluted in serum- and antibiotics-free DMEM. The mixture of siRNA/Hiperfect was kept at room temperature for 10 min to form the transfection complexes. The complexes were then added to VSMCs and were swirled gently to ensure uniform distribution. After incubation for 8h at 37°C, transfection complexes were replaced with complete growth media for the assessment of TRPM7 knockdown efficiency at 48h with whole-cell current measurement. For measurement of VSMC differentiation marker genes, cells were trypsinized and seeded into 6-well plates at a density of 5×10^5 cells/well in complete growth media. After 24 hours, cells were maintained in serum-free media for consecutive 72 hours. On day 2 of serum starvation, siRNA duplexes diluted in serum-free media was added to cell culture media for 8 hours and then replaced with normal serum-free media. At the end of 72-hour-long growth arrest, cells were treated with Ang II for 24 hours, followed by quantification of mRNA with real-time PCR. The same strategy of serum-starvation and TRPM7 suppression was used for signaling pathway analysis in cultured VSMCs. Specific siRNA sequences against AT1, Pyk2, ERK1, ERK2 and Elk-1 were purchased from Qiagen and transfected as described for TRPM7. In experiments that required the use of the constitutively active mutants of c-Src, ERK1 and Elk-1 (obtained from Addgene), plasmid vectors were co-transfected with siRNA duplex using Lipofactamine 2000 (Invitrogen) according to the manufacturer’s instructions.
Cell cycle analysis with flow cytometry
Phases of cell cycle were evaluated by flow cytometry as previously described\textsuperscript{5, 7}. After appropriate treatment, cells were collected by centrifugation at 200g for 5 min at 4°C. Pellets were rinsed twice with ice-cold phosphate-buffered-saline (PBS) and fixed in 70% ethanol for at least 12h. Samples were then stained with propidium iodide (PI). DNA content was analyzed by flow cytometry (EPICS XL, Beckman Coulter, Florida, USA) to determine distribution of each phase in cell cycle.

Co-immunoprecipitation assay
For co-immunoprecipitation, 10×10\textsuperscript{6} cells were used. Whole-cell extracts were prepared using the lysis buffer (10mM Tris-HCl pH 8, 420mM NaCl, 1mM EDTA, 0.5% NP-40, with protease inhibitors). The cell lysate was immunoprecipitated at 4°C overnight with protein A agarose beads (Invitrogen) prebound with anti-myocardin or anti-Elk-1 antibodies. Immunoprecipitates were washed 4 times with wash buffer (10mM Tris-HCl pH 8, 100mM NaCl, 1mM EDTA, 0.5% NP-40 and 0.5% Triton X-100), boiled in 1× sample loading buffer and separated by 10% SDS–PAGE. The separated protein was then transferred to PVDF membrane and immunoblotted with anti-SRF antibody.

Chromatin immunoprecipitation (ChIP)
Chromatin immunoprecipitation was carried out as described\textsuperscript{8}. VSMCs (10×10\textsuperscript{6} cells) were fixed in PBS with 1% formaldehyde at 37°C for 10 minutes and then lysed in solution containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1 plus protease inhibitors. The lysate was then sonicated for 20 minutes in a cold water bath to achieve an average DNA size of 1,000 base pairs. Input was saved and lysate was diluted in immunoprecipitation buffer (1% Triton, 2mM EDTA, 150mM NaCl, 20 mM Tris-HCl, pH 8.1) and incubated overnight with Protein A Dynabeads (Invitrogen) that were prebound overnight at 4°C with anti-myocardin or anti-Elk-1 antibodies (Santa Cruz). Beads were washed 5 times with RIPA buffer (50mM HEPES, 1mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5M LiCl, pH 7.6) and twice with TE, and then incubated in solution (1% SDS, 0.1M NaCHO\textsubscript{3}) at room temperature for 30 minutes. To reverse crosslinks, both the input and the immunoprecipitate were subjected to 6-hour heating in a 65°C water bath. DNA was purified with a QIAquick spin kit (Qiagen) and qPCR was performed in triplicate with QuantiTect SYBR PCR kits (Qiagen) with the primers corresponding to the CArG-containing region (GGTCCCTGCCCATAAAAAGGTCTT; TGCCCATGGAAGTCTGCTTGG) of SM22 promoter\textsuperscript{9}. The primers used as controls were from the exon 5 region of SM22 gene (TCCTGGAGTAAGCGGAG; CAGGGTTCTGGGAATGCTAA).

Data analysis
All data are expressed as mean±S.E.M. N value denotes the number of independent experiments unless otherwise stated. Statistical analysis was determined by an unpaired 2-tailed Student t test or 1-way ANOVA followed by the Bonferroni multiple comparison post hoc test with a 95% confidence interval. P values of <0.05 were considered as statistically significant (* p<0.01, † p<0.05, n.s, not significant).

References
Online Figure I. Characterization of endogenous TRPM7 currents in freshly isolated VSMCs from mouse ascending aorta. A-B, application of 100µM 2-APB as indicated (n=6). The typical current traces are shown in B. C-D, current recording without (Ctrl, n=6) or with (n=4) 3 mM free Mg$^{2+}$ in the pipette solution. E-F, application of DVF solution, which is Ca$^{2+}$-free, Mg$^{2+}$-free with 2 mM EDTA added (n=5). Please note the drastic increase in inward currents when DVF was applied.
**Online Figure II.** A, measurement of systolic blood pressure at 0, 1, 2, 3, 4 weeks of saline or Ang II infusion. B-C, measurement of TRPM7 currents (B, n=8-9 cells for each plot) and cell capacitance (C, n=9-10 cells) in freshly harvested VSMCs from mouse thoracic aorta at week 4 of Ang II infusion. The data for ascending aortic VSMCs are the same as the 4w data in Fig. 1A, 1C. D, the densitometric analysis of TRPM7 protein expression during Ang II infusion as in Fig. 1H.
Online Figure III. A-B, densitometric analysis of SMC differentiation marker genes and Ki-67 expression over the course of Ang II infusion, as in Fig. 3A. C, Fold change of protein as in Fig. 3B. D, Fold change of signaling molecules as in Fig. 3G. E, ratiometric Ca^{2+} imaging in cultured ascending aortic VSMCs treated with none (Ctrl), negative (Neg) or TRPM7 siRNA. 4 independent experiments were run. Ca^{2+} signals (F) were normalized versus the initial Ca^{2+} signal (F_0) as F/F_0.
Online Figure IV. A-B, TRPM7 current measurement in cultured ascending aortic VSMCs transfected with none (Ctrl, n=8), negative siRNA (Neg, n=10), TRPM7-specific siRNA1 (n=14) or siRNA2 (n=12). The representative TRPM7 currents under the indicated conditions were displayed (B). C, analysis of TRPM7 protein expression in cultured ascending aortic VSMCs treated with negative control (Neg) or specific TRPM7-targeting siRNA (siRNA1 & siRNA2); n=4. D, relative mRNA expression of SMC differentiation genes in cultured thoracic aortic VSMCs with various treatments. Cultured VSMCs were deprived from serum for 72 hours and then treated with or without Ang II (0.5µM) for 24 hours. E, distribution of cell cycle when cultured thoracic aortic VSMCs were treated as indicated. VSMCs were serum-starved for 24 hours before 48-hour-long Ang II treatment (0.5µM).
Online Figure V. A, densitometric analysis of proteins as in Fig. 5A. B, Ang II (0.5µM)-induced Ca^{2+} signals (340/380 ratio) in cultured ascending aortic VSMCs treated with negative or specific TRPM7 siRNA1 (n=6). C-D, TRPM7 current recording in cultured ascending aortic VSMCs treated with TRPM7 siRNA (Ctrl, n=8; Ang II, n=9) or negative siRNA (Ctrl, n=5). E, densitometric analysis for Fig. 5D. F, mRNA levels of SM-22 in cultured ascending aortic VSMCs under the indicated conditions (wt, wild type; n=4).