Enhanced Mitochondrial Transient Receptor Potential Channel, Canonical Type 3–Mediated Calcium Handling in the Vasculature From Hypertensive Rats

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Background—Mitochondrial Ca\(^{2+}\) homeostasis is fundamental to the regulation of mitochondrial reactive oxygen species (ROS) generation and adenosine triphosphate production. Recently, transient receptor potential channel, canonical type 3 (TRPC3), has been shown to localize to the mitochondria and to play a role in maintaining mitochondrial calcium homeostasis. Inhibition of TRPC3 attenuates vascular calcium influx in spontaneously hypertensive rats (SHRs). However, it remains elusive whether mitochondrial TRPC3 participates in hypertension by increasing mitochondrial calcium handling and ROS production.

Methods and Results—In this study we demonstrated increased TRPC3 expression in purified mitochondria in the vasculature from SHRs, which facilitates enhanced mitochondrial calcium uptake and ROS generation compared with Wistar-Kyoto rats. Furthermore, inhibition of TRPC3 by its specific inhibitor, Pyr3, significantly decreased the vascular mitochondrial ROS production and H\(_2\)O\(_2\) synthesis and increased adenosine triphosphate content. Administration of telmisartan can improve these abnormalities. This beneficial effect was associated with improvement of the mitochondrial respiratory function through recovering the activity of pyruvate dehydrogenase in the vasculature of SHRs. In vivo, chronic administration of telmisartan suppressed TRPC3-mediated excessive mitochondrial ROS generation and vasoconstriction in the vasculature of SHRs. More importantly, TRPC3 knockout mice exhibited significantly ameliorated hypertension through reduction of angiotensin II–induced mitochondrial ROS generation.

Conclusions—Together, we give experimental evidence for a potential mechanism by which enhanced TRPC3 activity at the cytoplasmic and mitochondrial levels contributes to redox signaling and calcium dysregulation in the vasculature from SHRs. Angiotensin II or telmisartan can regulate \([\text{Ca}^{2+}]_{\text{mito}}\), ROS production, and mitochondrial energy metabolism through targeting TRPC3. (J Am Heart Assoc. 2017;6:e005812. DOI: 10.1161/JAHA.117.005812.)

Key Words: mitochondria • pyruvate dehydrogenase • telmisartan • transient receptor potential channel, canonical type 3 • vasoconstriction
Clinical Perspective

What Is New?

- This study is the first to demonstrate that enhanced mitochondrial transient receptor potential channel, canonical 3 (TRPC3) expression in the vasculature from spontaneously hypertensive rats facilitates mitochondrial calcium entry and reactive oxygen species generation and impairs adenosine triphosphate synthesis.
- TRPC3 gene knockout ameliorates hypertension through reduction of angiotensin II–induced mitochondrial calcium overload and reactive oxygen species generation.
- Telmisartan administration inhibits TRPC3-mediated excessive mitochondrial reactive oxygen species generation and vasoconstriction in the vasculature of spontaneously hypertensive rats.

What Are the Clinical Implications?

- Telmisartan is widely used clinically as an antihypertensive drug. We demonstrate that angiotensin II and telmisartan may exert angiotensin receptor–independent effects by targeting TRPC3 in the vasculature.
- Telmisartan shows a promising and novel antihypertensive pathway through inhibition of vascular mitochondrial TRPC3-mediated redox signaling and calcium dysregulation.

membrane of mitochondria and to regulate mitochondrial Ca\(^{2+}\) uptake.\(^{14}\) Other groups have investigated that the basal \([\text{Ca}^{2+}]_{\text{mito}}\) levels were different in heart, kidney, and brain tissues from SHRs compared with WKY rats,\(^{15,16}\) but the differences and functions of \([\text{Ca}^{2+}]_{\text{mito}}\) in the vasculature of SHRs remain unclear.

Overproduction of ROS in the mitochondrial respiratory chain can progressively impair mitochondrial energy metabolism. Several studies have shown that mitochondria-produced ATP and ROS can regulate the functions of TRPCs,\(^{17,18}\) thus influencing intracellular \(\text{Ca}^{2+}\) signaling, vascular tone, and blood pressure (BP).\(^{19}\) Pyruvate dehydrogenase (PDH) was a key enzyme located in the mitochondrial matrix and functions in the metabolism of the Krebs cycle, and the activity of PDH can be regulated by \([\text{Ca}^{2+}]_{\text{mito}}\) and other vasoactive substances including angiotensin II in its subunit E1α.\(^{20,21}\)

Telmisartan is widely used in the treatment of hypertension.\(^{13}\) In general, angiotensin receptor blockers antagonize the action of angiotensin II by blocking vascular angiotensin II receptor subtype 1 (AT1R). Clinical trials have shown that the prognostic benefit of telmisartan is better in preventing cardiovascular events and hypertension than other angiotensin receptor blockers.\(^{22}\) We showed that angiotensin II upregulated TRPC3 expression levels and promoted cytosolic calcium influx in the vasculature from SHR, and this effect was inhibited by telmisartan.\(^{13}\) However, little is known about whether angiotensin II can regulate \([\text{Ca}^{2+}]_{\text{mito}}\) and ROS production in the mitochondrial respiratory chain and elevate blood pressure through targeting TRPC3 in the vascular smooth muscle cells (VSMCs) from SHRs. Therefore, we hypothesize that enhanced TRPC3-mediated mitochondrial Ca\(^{2+}\) uptake and ROS production involved elevated blood pressure in the vasculatures from SHRs compared with WKY rats.

Methods

Animals and Treatment

Eight-week-old male SHRs and normal control WKY rats were obtained from Charles River Laboratories (Wilmington, MA). The rats were maintained at a controlled temperature (21°C to 23°C) under a 12/12-hour light-dark cycle and with free access to food and water. After a 2-week adaptation period, 30 male SHRs and 30 age-matched WKY rats were randomly divided into normal diet (ND) and ND plus telmisartan (Telmi, 5 mg/kg per day) groups \((N=15 \text{ per group})\) and treated for 12 weeks. Every 4 weeks the tail-cuff blood pressure (BP) levels were measured. After 12 weeks of intervention, the rats were surgically implanted with telemetric transmitters to measure the 24-hour ambulatory BP levels. Then, the rats were euthanized, and tissue samples were isolated for further experiments.

Wild-type and \(\text{T}rpc3^-/-\) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Homozygotes, heterozygotes, and wild-type littermates were identified according to previously described methods.\(^{23}\) Briefly, 10 6-month-old \(\text{T}rpc3^-/-\) mice and 10 6-month-old wild-type littermates were surgically implanted with 14-day mini-osmotic pumps (Alzet Company, Cupertino, CA) filled with saline or angiotensin II (Sigma, St. Louis, MO) solutions randomly, each subgroup having 5 mice. The dose of angiotensin II was 0.7 mg/day per kilogram body weight. The tail-cuff BP was measured at baseline, as well as at 7 and 14 days after intervention. After intervention, the 24-hour ambulatory BP was measured, and then these animals were euthanized for other experiments. All of the experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Primary Aortic VSMC Culture and Treatment

The VSMC layer was isolated from aortas of WKY rats and SHRs by removing the intima and adventitia.\(^{11}\) The tissue blocks were then incubated with Dulbecco Modified Eagle Medium (DMEM; Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco), streptomycin (100 µg/mL), and penicillin (100 U/mL). Cultured VSMCs were plated and grown at 37°C in a humidified atmosphere of 95% air/5% CO\(_2\). Cultured VSMCs were used between passages 2 and 8.
MitoTEMPO was purchased from Santa Cruz Biotechnology (Dallas, TX). Telmisartan and Pyr3 were all purchased from Sigma-Aldrich (St. Louis, MO).

Small Interfering RNA and Plasmid Overexpression Experiments

RNA interference and overexpression experiments were conducted as previously described. Briefly, Lipofectamine 3000 was purchased from ThermoFisher Scientific (Waltham, MA), and rat TRPC3-specific small interfering RNA (siRNA) and negative control siRNA were purchased from Genecopoeia (Guangzhou, China). The pcDNA-TRPC3myc overexpression plasmid was kindly provided by Dr Craig Montell (Addgene plasmid 25902).

Tail-Cuff and 24-Hour Ambulatory BP Measurements

The tail-cuff BP measurement was conducted using a Softron BP-2010A as previously described. The 24-hour ambulatory BP measurement was conducted as previously described using surgically implanted telemetric transmitters (Data Sciences International, New Brighton, MN). In rats, the measurements were conducted after the animals had received a normal diet or a normal diet plus telmisartan for 12 weeks, whereas mice were assessed 14 days after the implantation of osmotic pumps. The implant catheter was placed into the abdominal aorta of rats or the descending carotid artery of mice. The animals were allowed to recover from the surgery for at least 7 days, and the 24-hour ambulatory systolic and diastolic BP were then measured by telemetry in conscious, unrestrained animals. We collected data once every minute and used the mean values of 24 hours for the analysis.

Measurement of Vascular Reactivity

The vascular reactivity was measured as previously described. Briefly, after the mice or rats were anesthetized with pentobarbital sodium, the mesenteric vascular bed was removed and placed in cold (4°C) Krebs solution (in mmol/L: 118 NaCl, 25 NaHCO3, 1.1 d-glucose, 4.7 KCl, 1.2 KH2PO4, 1.17 MgSO4, and 2.5 CaCl2, set to the pH of 7.4). The first branches of the mesenteric arteries (MAs) (for mice) and second branches of the MAs (for rats) were excised with the connective tissues, such as fat. Subsequently, 2-mm MA segments were mounted on a myograph (Danish Myo Technology, Aarhus, Denmark). After incubation in 95% O2 and 5% CO2 at 37°C, the MA segments were stretched to optimum baseline tension (1.8 mN for mouse MAs and 2.5 mN for rat MAs). The arteries were then equilibrated for 60 minutes before preconstriction with 60 mmol/L KCl. After several washouts, each ring was treated with different vasoactive substances (phenylephrine, U46619, acetylcholine, or nitroglycerin, all purchased from Sigma-Aldrich) or other substances (Pyr3, MitoTEMPO, etc). Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP; AD Instruments Pty Ltd, Castle Hill, Australia).

Evaluation of ROS Levels

The ROS levels were measured using a dihydroethidium (DHE) fluorescent probe for cytosolic ROS detection or MitoSOX Red (ThermoFisher Scientific, Waltham, MA) for mitochondrial ROS detection. DHE and MitoSOX staining and detection were conducted as previously described. The cellular H2O2 levels were detected using a Hydrogen Peroxide Assay Kit (Beyotime, Shanghai, China). Tissue sections were frozen in optimal cutting temperature compound and quickly sectioned, and the sections were then stained with DHE or MitoSOX dye according to the manufacturers’ protocols. To quantify the intensity of DHE and MitoSOX fluorescence, the plates were measured using a Fluoroskan Ascent Fluorometer (ThermoFisher, Helsinki, Finland). The ROS concentrations are expressed as a normalized value relative to the fluorescence intensity of the control cells. To visualize the staining, the sections or specimens were placed on an inverted fluorescence microscope (Nikon TE2000, Nikon Corporation, Tokyo, Japan).

ATP Content Measurement

The ATP levels in VSMCs were measured using a firefly luciferase-based ATP assay kit (Beyotime, Shanghai, China) as previously described. Briefly, treated VSMCs were lysed with a specific lysis buffer, followed by centrifugation at 12 000g for 5 minutes at 4°C. Subsequently, 100 μL of each supernatant was mixed with 100 μL of an ATP detection solution that had been diluted to the proper working concentration. Luminescence was measured using a Varioskan Flash instrument (ThermoFisher Scientific, Waltham, MA). The protein concentration of each treated group was also measured using a Bradford Protein Assay. The total ATP levels are expressed as nanomoles per milligram protein, and the ratios between different groups were calculated and presented.

High-Resolution Respirometry

Mitochondrial respiratory function was analyzed using a 2-channel titration injection respirometer (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) equipped with 2 chambers, as previously described. In general, VSMCs were harvested, resuspended in the MiR05 solution, and transferred separately to oxygraph chambers at a final cell density of ~1 x 10⁵ cells/mL. Routine respiration was assessed while respiration was stabilized. The plasma membrane was then
permeabilized with digitonin (10 μg per 10^6 cells), and Complex I-dependent oxidative phosphorylation (CI-OXPHOS) was measured after addition of glutamate (G, 5 mmol/L), malate (M, 2 mmol/L), and ADP (D, 5 mmol/L). The integrity of the outer mitochondrial membrane was assessed by adding cytochrome c (10 μmol/L). Subsequently, succinate (S, 10 mmol/L) was added to induce maximal oxidative phosphorylation (OXPHOS) capacity with convergent input through CI+II-OXPHOS. After uncoupling with 2-[(trifluoromethoxy)phenyl]hydrazinylidene]propanedinitrile (FCCP; injected stepwise up to 1-1.5 μmol/L) in the noncoupled state, CI-supported noncoupled respiration (CI+II-ETS) was measured. The addition of rotenone (0.5 μmol/L) allowed the determination of CI-ETS. Residual oxygen consumption was evaluated after the inhibition of CIII with antimycin A (2.5 μmol/L). CI-VETS was measured by adding the CIV substrates ascorbate (A, 2 mmol/L), malate (M, 2 mmol/L), and ADP (D, 5 mmol/L). The resultant data are presented as Fx/F0, the [Ca^{2+}]_c measurement,13 cells were digested and resuspended in Hank’s Balanced Salt Solution (in mmol/L: 136 NaCl, 5.4 KCl, 0.44 KH2PO4, 20 HEPES, 0.34 Na2HPO4, 5.6 d-glucose, 10 HEPES and 1 CaCl2, pH 7.4). For loading with Fura-2, the cells were incubated with 1 μmol/L Fura-2 AM and 0.025% F-127 (from Sigma-Aldrich) for 30 minutes at 37°C in the dark, washed 3 times with Ca2+-free Hank’s Balanced Salt Solution and resuspended in Ca2+-free Hank’s Balanced Salt Solution. Fluorescence was measured at baseline and after treatment at an emission of 510 nm using excitation wavelengths of 340 and 380 nm. The resultant data are presented as Fx/F0, where Fx is the ratio of fluorescence between excitation at 340 and 380 nm with emission at 510 nm, and F0 is the mean F340 nm/F380 nm value during the pretreatment period in each experiment. Changes in [Ca^{2+}]_c were deduced from the ratios of the transient increases in the fluorescence intensity at 340 and 380 nm.

For the [Ca^{2+}]_mito measurement,14 cells were digested and resuspended using an extracellular solution (in mmol/L: 120 NaCl, 6 KCl, 0.3 CaCl2, 2 MgCl2, 12 d-glucose, 12 sucrose, and 10 HEPES-free acid, pH 7.4). For loading with Rhod-2, cells were incubated with 5 μmol/L Rhod-2 AM and 0.025% F-127 for 30 minutes at 37°C in the dark and washed 3 times with an extracellular solution. Cells were permeabilized with 10 μmol/L digitonin for 5 minutes at 37°C in the intracellular solution (in mmol/L: 120 KCl, 10 NaCl, 1 K2HPO4, 20 HEPES, for 5 minutes at 4°C to remove intact cells and nuclei. The supernatant (cytosol fraction) was centrifuged twice (7000g, 10 minutes, 4°C), and the insoluble part was the crude mitochondrial fraction. To obtain the crude mitochondria fraction, the pellet was resuspended in starting buffer (in mmol/L: 225 mannitol, 75 sucrose and 30 Tris-HCl, pH 7.4) and centrifuged twice at 7000g and 4°C for 10 minutes. Then, crude mitochondrial fraction pelleted was resuspended in 2 mL of mitochondria-resuspending buffer (in mmol/L: 250 mannitol, 5 HEPES and 0.5 EGTA, pH 7.4) and loaded on Percoll medium (in mmol/L: 225 mannitol, 25 HEPES, 1 EGTA and 30% Percoll [vol/vol], pH 7.4). After centrifugation (95 000g, 30 minutes, 4°C), the mitochondrial fraction was isolated. Pure mitochondria were then obtained by centrifuging twice at 6300g for 10 minutes. The protein pellets were solubilized in SDS sample buffer and analyzed by Western blotting using antibodies against TRPC3, voltage-dependent anion-selective channel protein (as a mitochondrial control) and GAPDH (as a loading control).
2 Mg-ATP, 2 succinate and proteinase inhibitor mixtures, pH 7.2) and then washed 3 times with intracellular solution. Agonists (ATP or histamine) and Ca$^{2+}$ were added to the washing solution for treatment. The fluorescence was measured at an emission wavelength of 581 nm and an excitation wavelength of 552 nm at baseline and after treatment. The statistical analysis of the calcium measurements is graphically presented as the $R/R_0$ value, where $R$ is the emission values induced by excitation and $R_0$ is the mean value of $R$ during the pretreatment period in each experiment.

Statistical Analysis

The data are presented as the mean±SEM. For the comparisons of means between 2 groups, unpaired Student t test was used to examine the differences. For the comparisons of means among 3 or more groups, ANOVA followed by Bonferroni post hoc tests were used to examine the differences. For the analysis of wire myograph data, concentration-response curves were analyzed with a nonlinear regression followed by a 1-way ANOVA method to compare the $E_{\text{max}}$ as the maximal response and $pD_2$ as the negative logarithm of the drug concentration that produced 50% of $E_{\text{max}}$ among these groups. For the analysis of tail-cuff systolic BP changes in rats and angiotensin II–infused mice with repeated tests over time, a repeated-measures ANOVA method was used to compare the blood pressure changes over time. Meanwhile, for other comparisons with nonrepeated tests in animal experiments and all of the cellular experiments, unpaired Student t tests or 1-way ANOVA were used to examine differences between or among these groups. All the data analysis was conducted using GraphPad Prism (La Jolla, CA) or SPSS 19.0 (IBM Analytics, Armonk, NY). Two-sided $P$-value less than 0.05 was considered as statistically significant.

Results

Increased Mitochondrial TRPC3 Expression in the Vasculature From SHRs

First, we examined the expression levels of mitochondrial TRPC3 using purified mitochondria from primary cultured VSMCs from
WKY rats and SHRs. It showed that the expression levels of total and mitochondrial TRPC3 were significantly increased in VSMCs from SHRs compared with WKY rats but were significantly decreased on telmisartan treatment (Figure 1). These results indicated that increased vascular mitochondrial TRPC3 expression in SHRs was inhibited by telmisartan.

Enhanced Mitochondrial TRPC3-Mediated Calcium Handling in the Vasculature From SHRs

To assess the function of increased mitochondrial TRPC3 expression in the vasculature of SHRs, we examined the [Ca^{2+}]_{mito} changes in VSMCs. Specifically, ATP or histamine...
significantly increased the [Ca\textsuperscript{2+}]\textsubscript{mito} in VSMCs from SHRs compared with WKY rats, whereas the administration of telmisartan or TRPC3 inhibitor (Pyr3) significantly attenuated this effect (Figure 2A through 2D). Moreover, we used permeabilized VSMCs to exclude the contribution of plasma membrane TRPC3 to [Ca\textsuperscript{2+}]\textsubscript{mito}. The [Ca\textsuperscript{2+}]\textsubscript{mito} remained higher in SHRs, and this increase was significantly reduced by pretreatment with telmisartan or Pyr3 (Figure 2E and 2F). Furthermore, TRPC3 knockdown with a specific siRNA significantly reduced the [Ca\textsuperscript{2+}]\textsubscript{mito} in the permeabilized VSMCs from SHRs (Figure 2G and 2H), whereas the overexpression of TRPC3 with the pcDNA-TRPC3 plasmid significantly augmented the [Ca\textsuperscript{2+}]\textsubscript{mito} in WKY (Figure 2I and 2J). Taken together, these results suggest that increased mitochondrial TRPC3 expression regulated mitochondrial Ca\textsuperscript{2+} homeostasis in the vasculature of SHRs.

We further confirmed that increased mitochondrial TRPC3-mediated calcium uptake affected vascular tone. Specifically, mitoTEMPO or telmisartan treatment inhibited increases in store-operated calcium entry in the VSMCs of SHRs (Figure 3A and 3B), and the administration of mitoTEMPO or Pyr3 significantly suppressed the excessive vasoconstriction induced by either phenylephrine or U46619 in SHRs (Figure 3C and 3D). These findings suggested that increased TRPC3 activity at both the cytoplasmic and mitochondrial levels regulated Ca\textsuperscript{2+} homeostasis in the vasculature from SHRs. Inhibition of TRPC3 improved vascular tone in the setting of hypertension.

### Inhibition of TRPC3 Improved Mitochondrial Function and Reduced ROS Production in the Vasculature of SHRs

We then investigated the effect of telmisartan and Pyr3 treatment on mitochondrial ROS production and respiratory functions in VSMCs. Specifically, parameters of mitochondrial respiratory function, such as CI\textsubscript{OXPHOS}, CI+II\textsubscript{OXPHOS}, and CI+II\textsubscript{ETSS}, were significantly reduced in the VSMCs from SHRs compared with those from WKY rats, whereas telmisartan treatment significantly improved these parameters (Figure 4A). Furthermore, increased cellular and mitochondrial ROS production, enhanced H\textsubscript{2}O\textsubscript{2} production, but impaired ATP synthesis were identified in SHR compared with WKY. Both telmisartan and Pyr3 treatment significantly reversed these changes in mitochondrial parameters (Figure 4B through 4E). Specifically, the phosphorylation of the pyruvate dehydrogenase E1\textalpha subunit (phospho-PDHE1\textalpha) was significantly increased, but total PDHE1\textalpha was not changed in the VSMCs from SHRs compared with those from WKY rats. Both telmisartan and Pyr3 treatment significantly reduced the phospho-PDHE1\textalpha expression levels (Figure 4F). These results indicated that the inhibition of TRPC3 improved mitochondrial respiratory function.
function and reduced ROS production in the setting of hypertension.

**Chronic Telmisartan Treatment Attenuated the Vasoconstrictions Associated With the Inhibition of Mitochondrial TRPC3**

We further investigated the effect of TRPC3 inhibition on mitochondrial function and vascular tone in vivo. The total and mitochondrial TRPC3 expression levels were significantly increased in the aortas of SHRs compared with WKY rats, but both were attenuated by long-term telmisartan administration (Figure 5A, Figure S1). Moreover, chronic telmisartan treatment decreased cellular and mitochondrial ROS production in aortas of SHRs (Figure 5B). The enzyme activities of mitochondrial complex I, but not mitochondrial complex II, were significantly reduced in aortas of SHRs, and this decrease was attenuated by chronic telmisartan treatment (Figure 5C).

Chronic telmisartan treatment also significantly inhibited increases in vasoconstriction (Figure 5D) but only slightly improved endothelium-dependent relaxations and had no effect on endothelium-independent relaxations in SHRs (Figure S2A). Incubation with mitoTEMPO or Pyr3 only slightly improved the vascular constrictions in SHRs with
chronic telmisartan treatment, but without significant changes (Figure 5E). Furthermore, the administration of telmisartan consistently lowered blood pressure in SHRs (Figure S2B and S2C). Taken together, these results indicated that the inhibition of TRPC3 improved the vascular tone of SHRs both in vivo and in vitro.

**TRPC3 Knockout Ameliorated Angiotensin II–Induced Mitochondrial ROS Production and Hypertension**

Specifically, mitoTEMPO or Pyr3 administration significantly attenuated vasoconstriction induced by U46619 and PE in...
wild-type mice (Figure 6A). Meanwhile, Trpc3−/− significantly attenuated the enhanced mitochondrial Ca2+ uptake induced by angiotensin II infusion (Figure 6B and 6C). The total and mitochondrial ROS production on angiotensin II infusion was also decreased in the vasculature of Trpc3−/− mice compared with wild-type mice (Figure 6D). Additionally, angiotensin II significantly increased the phosphorylation of PDHE1α with no effect on the total PDHE1α expression levels in the aorta of wild-type mice, but this increase was partially ameliorated in Trpc3−/− mice (Figure 6E).

Furthermore, we used in vivo experiments to validate the role of TRPC3 in angiotensin II–induced hypertension and vasoconstriction. It showed that the angiotensin II–induced elevated tail-cuff systolic blood pressure, the 24-hour ambulatory blood pressure, as well as U46619- and PE-induced vasoconstriction were significantly attenuated in Trpc3−/− mice (Figure 7A through 7C). Moreover, the activation of RhoA/Rho kinase due to angiotensin II infusion was also partially inhibited in Trpc3−/− mice, and this effect was mediated by the inhibition of MYPT1 and MLC2 phosphorylation (Figure 7D through 7F). These data indicate that TRPC3 may act by regulating mitochondrial functions, thus influencing the redox state and RhoA/Rho kinase pathway, which modulates vascular tone and blood pressure in hypertension.

Discussion

This study is the first to provide important evidence showing that increased vascular mitochondrial TRPC3 function participates in abnormal mitochondrial Ca2+ homeostasis and enhances vascular tone in the setting of hypertension, and this regulation is associated with increased mitochondrial ROS production and impaired ATP synthesis. Moreover, long-term telmisartan administration improves mitochondrial function and decreases mitochondrial ROS production and vascular constriction by inhibiting mitochondrial TRPC3 in the vasculature from SHRs (Figure 8). Furthermore, a global knockout of TRPC3 attenuates angiotensin II–induced ROS production, suppresses vasoconstriction and lowers blood pressure in vivo. In our study, we demonstrated a novel mechanism by which enhanced mitochondrial TRPC3-mediated ROS production is involved in mitochondrial redox signaling and calcium dysregulation in the vasculature from a hypertensive model.

Mitochondrial Ca2+ is vital to mitochondrial function because it regulates the activities of Ca2+-sensitive enzymes in the Krebs cycle.31 The regulation of mitochondrial Ca2+ homeostasis is complicated, including a pro-uptake mitochondrial calcium uniporter, a prorelease sodium-calcium exchanger, a sodium-hydrogen exchanger, and a mitochondrial permeability transition pore.1,32 In this study, we provide evidence to show that elevated mitochondrial Ca2+ uptake is associated with enhanced mitochondrial Ca2+ uptake in hypertension and that telmisartan can decrease mitochondrial Ca2+ uptake by inhibiting TRPC3 in the vasculature from SHRs. Recently, Feng et al reported that 44% of total TRPC3 is localized to the inner membrane of mitochondria.14 We confirmed that mitochondrial TRPC3 expression was increased by 56% in the VSMCs from SHRs compared with WKY rats, whereas treatment with ATP enhanced the [Ca2+]mito in the permeabilized VSMCs from SHRs compared with WKY rats. Inhibition of TRPC3 attenuated these elevated [Ca2+]mito levels in the VSMCs from SHRs. Furthermore, TRPC3 overexpression enhanced [Ca2+]mito in permeabilized VSMCs from WKY rats. Therefore, targeting mitochondrial TRPC3 may modulate the mitochondrial Ca2+ homeostasis in the vasculature of hypertensive individuals.

Mitochondrial ROS plays a role in the regulation of vascular tone by influencing the functions of VSMCs and the endothelium.33,34 A recent study shows that mitochondrial ion channels/transporters act as sensors and regulators of cellular redox signaling.31 Under physiological conditions, Ca2+ diminishes ROS from both complexes I and III while enhancing ROS generation if these complexes are dysfunctional.35 However,
excessive mitochondrial Ca\(^{2+}\) uptake promotes ROS production.\(^{36}\) In this study, we provide evidence showing that the elevated mitochondrial TRPC3-mediated Ca\(^{2+}\) uptake is associated with increased mitochondrial ROS production in SHR. Chronic telmisartan administration decreased mitochondrial ROS levels by 56% in the VSMCs and by 55% in the aortas of SHRs through inhibiting TRPC3 and improving the enzyme activities of complexes in the mitochondrial respiratory chain. Generally, mitochondrial matrix Ca\(^{2+}\) can activate PDH at its E1\(\alpha\) subunit and promote the transformation of pyruvic acid to acetyl-CoA in the mitochondrial matrix.\(^{3}\) PDH is a key dehydrogenase, and its activity can also be blunted by

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**Figure 5.** The effects of chronic oral telmisartan intervention on mitochondrial and vascular function in the vasculature from SHRs. A, The TRPC3 expression levels in isolated mitochondria from the aortic tissues of WKY rats and SHRs, in the presence and absence of telmisartan treatment, as analyzed by Western blotting using voltage-dependent anion-selective channel protein as a mitochondrial control. Western blot bands were quantified and analyzed in the below panel. \(N=4\) per group. *\(P<0.05\) vs WKY-ND (indicates WKY rats with ND treatment); \#\(P<0.05\) vs SHR-ND. B, Representative images of DHE and MitoSOX staining. The relative fluorescence intensity of the aortas from WKY rats and SHRs were quantified in the presence and absence of telmisartan treatment. SHR-Telmi indicates SHRs after long-term administration of telmisartan. \(N=6\) per group. ***\(P<0.0001\) vs WKY-ND; \#\(P<0.0001\) vs SHR-ND. C, The effects of chronic telmisartan intervention on mitochondrial complex I and complex II activities of aorta isolated from WKY rats and SHRs. *\(P<0.05\) vs WKY; \#\(P<0.05\) vs SHR. D, The effects of chronic oral telmisartan intervention on U46619- and PE-induced vasoconstriction in MA from SHRs. \(N=6\) to 8 per group. *\(P<0.05\), **\(P<0.01\), ***\(P<0.0001\). E, The effects of mitoTEMPO and Pyr3 administration on U46619- and PE-induced vasoconstriction in the MAs of SHRs treated with telmisartan. Isolated MAs were preincubated with 50 \(\mu\)mol/L of mitoTEMPO or 10 \(\mu\)mol/L of TRPC3 inhibitor Pyr3 for 30 minutes before experiments. \(N=6\) to 8 per group. *\(P<0.05\), ***\(P<0.0001\). DHE indicates dihydroethidium; MA, mesenteric artery; MitoSOX, mitochondrial superoxide indicator; Pyr3, specific inhibitor of TRPC3; SHR, spontaneously hypertensive rats; Telmi, telmisartan; TRPC3, transient receptor potential channel, canonical type 3; VDAC, voltage-dependent anion-selective channel; WKY, Wistar-Kyoto.
Figure 6. The effects of TRPC3 on angiotensin II (Ang II)-induced vascular mitochondrial dysfunctions. A, The effects of Pyr3 and mitoTEMPO administration on vascular constrictions of MAs in Ang II-infused hypertensive mice. N = 6 to 8 per group. *P < 0.05, **P < 0.01, ***P < 0.0001. B and C, [Ca^{2+}]_{mito} changes in permeabilized primary VSMCs from WT and Trpc3^{−/−} mice, pretreated with 200 nmol/L Ang II and challenged with the cytosolic loading of 300 μmol/L (B) or 75 μmol/L (C) Ca^{2+}. N = 9 per group. ***P < 0.0001. D, Representative images of DHE- and MitoSOX-stained aortic sections from Ang II-infused WT and Trpc3^{−/−} mice. N = 7 per group. ***P < 0.0001. E, Immunoblotting of the expression levels of TRPC3, p-PDHE1α and t-PDHE1α in aortic tissues isolated from WT and Trpc3^{−/−} mice, using GAPDH as a loading control. Four Western blot bands in each group were quantified and analyzed in the right panels. *P < 0.05, ***P < 0.0001. DHE indicates dihydroethidium; MA, mesenteric artery; MitoSOX, mitochondrial superoxide indicator; Pyr3, specific inhibitor of TRPC3; TRPC3, transient receptor potential channel, canonical type 3; VSMC, vascular smooth muscle cells; WT, wild type.
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Figure 7. The effects of TRPC3 on angiotensin II (Ang II)–induced hypertension and vascular reactivity. A and B, The effects of TRPC3 on the tail-cuff SBP (A) and the 24-hour ambulatory blood pressure levels (B) in Ang II–infused hypertensive mice. N=5 per group. A, ***P<0.0001, **P<0.001, *P<0.01, vs corresponding tail-cuff SBP levels at baseline via repeated measures ANOVA within each group; **P<0.01 vs WT-Ang II (indicates WT mice with Ang II infusion) at the same time point. B, **P<0.01, ***P<0.001. C, The effects of TRPC3 on vascular constrictions in Ang II–infused hypertensive mice. N=7 to 8 per group. *P<0.05, **P<0.01, ***P<0.001. D and E, The expression levels of phosphorylated and total MYPT1 and MLC2 in aortic tissues from these groups were detected using Western blot (D). E, Quantification of Western blot bands presented as the amount of phosphorylated/total levels. N=4 per group. *P<0.05, **P<0.01, ***P<0.001. F, Representative images of the immunofluorescence staining of TRPC3, p-MLC2 and DAPI using aortic sections from Ang II–infused mice. Quantification of the intensities in each graph was presented in the right 2 panels. N=6 per group. **P<0.01, ***P<0.001. DAPI, 4',6-diamidino-2-phenylindole; DBP, diastolic blood pressure; MLC2, myosin light chain 2; MYPT1, myosin phosphatase targeting protein; SBP, systolic blood pressure; transient receptor potential channel, canonical type 3; WT, wild type.

angiotensin II through increasing the vascular PDH phosphorylation. However, the regulation of PDH activities in the vasculature in hypertension is poorly understood. In this study, we demonstrated that the phosphorylation of PDHE1α was significantly increased by 101%, but total PDHE1α was not changed in the VSMCs from SHRs compared with WKY rats. Moreover, parameters of mitochondrial respiratory functions and the enzyme activity of mitochondrial Complex I were...
impaired in the vasculature from SHRs. Furthermore, mitochondrial TRPC3 inversely correlated with the activities of PDH in the vasculature of SHRs and angiotensin II–infused mice, which may be partially attributable to TRPC3-mediated vascular mitochondrial redox signaling and calcium dysregulation. PDHE1α phosphorylation is mediated by redox-sensitive PDH kinase, a kinase that could be activated by mitochondrial ROS. Therefore, reduction of mitochondrial ROS in the VSMCs from TRPC3/C0 mice can be responsible for reduced PDH kinase activity and recovery of PDH activity, increased mitochondrial Complex IOXPHOS levels, and ATP synthesis.

Angiotensin II may exert AT1R-independent effect by targeting TRPC3 in the vasculature. Although the role of AT1R-NADPH oxidase by modulating the cellular and mitochondrial ROS productions in cardiovascular diseases has been investigated, recent studies have shown that TRPC3 participates in RAS-related cardiovascular complications. Angiotensin II–induced vasomotion was associated with increased TRPC3 expression in the mesenteric arteries from SHRs. TRPC3 is involved in atherosclerosis through regulation of vascular cell adhesion molecule-1 and phospho-IκBα expression in apoE−/− mice, and the inhibition of TRPC3 has been shown to reduce pathological cardiac hypertrophy. We recently showed that long-term taurine supplementation improved vascular tone by increasing plasma H2S content and targeting TRPC3-mediated Ca2+ influx in SHRs. Other groups reported that TRPC3 influenced contractility and peripheral vascular resistance in hypertension. In the present study, we provided experimental evidence that angiotensin II can regulate \([\text{Ca}^{2+}]_{\text{mito}}\), ROS production, and mitochondrial energy metabolism through targeting TRPC3. Specifically, the angiotensin II–induced activation of the RhoA/Rho kinase pathway was significantly attenuated in Trpc3/C0 mice. We have shown that the expression levels of AT1R in the vasculatures of WKY rats and SHRs did not significantly differ, which was in agreement with other reports. There were studies that indicated that
mitochondrial angiotensin receptors existed in many other models.48,49 Therefore, we proposed that angiotensin II may exert an AT1R-independent effect by targeting TRPC3.

Telmisartan as a first line antihypertensive drug is commonly used clinically.22 We showed before that telmisartan improved the insulin resistance and body weight by activating peroxisome proliferator-activated receptor-δ.50,51 Telmisartan has been proven to promote phosphorylation of endothelial nitric oxide synthase and improve mitochondrial function in VSMCs in a peroxisome proliferator-activated receptor-γ-dependent manner.52,53 Moreover, a recent study showed that telmisartan modulates the balance between oxidative stress and antioxidant properties, which improves cardioprotection, but the mechanism is unclear.54 In this study, the long-term administration of telmisartan inhibited mitochondrial Ca²⁺ uptake, reduced ROS production, and improved vascular tone through inhibition of vascular mitochondrial TRPC3 in vivo. However, the effect of telmisartan in improving mitochondrial functions may attributed to several pathways, which include reduced NADPH oxidase activity,52 reduced inflammation,55,56 and diminished Ca²⁺-induced mitochondrial oxidative stress.

In summary, we tested the hypothesis that increased TRPC3-mediated mitochondrial Ca²⁺ uptake in VSMCs and aortic tissues of SHRs compared with WKY rats. TRPC3 knockout significantly reduced angiotensin II–mediated ROS production and vasoconstriction. Telmisartan shows promise in the treatment of hypertension through inhibition of TRPC3 and improvement of vascular mitochondrial redox signaling and calcium dysregulation.

Study Limitations
The limitation of this study is that it cannot rule out the plasma membrane TRPC3 channel which also contributes to the regulation of calcium handling and ROS generation.57 Further experiments are needed to distinguish plasma membrane and mitochondrial TRPC3–mediated effects. In addition, they should also examine how TRPC3 is integrated into mitochondria and whether this process is genetically altered in the vasculature of hypertensive rats.

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Disclosures
None.

References


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Figure S2

A

B

C

Relaxation (% of Phe tone)

Nitroglycerin (log mol/L)

WKY-ND

SHR-ND

SHR-Telmi

WKY-ND

SHR-ND

SHR-Telmi

Tail-off SBP (mmHg)

Time (week)

24-hour Ambulatory SBP (mmHg)

Time of day

24-hour Ambulatory DBP (mmHg)

Time of day
Supplemental Figure Legends:

**Figure S1.** The effect of chronic telmisartan treatment on TRPC3 expression levels. **A,** Representative images of the immunofluorescence staining of TRPC3, α-smooth actin and DAPI using aortic sections from WKY rats and SHR, subjected to or forgoing telmisartan intervention. Quantification of the intensities in each graph was presented in the right panel. N=6 per group. *P<0.05, ***P<0.0001. **B,** Expression levels of TRPC3 in aortic tissues from these groups were detected using western blot. Quantification of western blot bands was presented as the amount of target protein / GAPDH levels. N=4 per group. **P<0.01, ***P<0.0001.

**Figure S2.** The effect of chronic oral telmisartan intervention on blood pressure (BP) levels in SHR. **A,** Effects of telmisartan treatment on acetylcholine (ACh)- and nitroglycerin-induced relaxation in mesenteric arteries (MAs) from WKY rats and SHR. N=8~12 per group. Concentration-response curves were analyzed by non-linear regression followed by Student’s t-test or one-way ANOVA for comparisons between two groups or among more than two groups, respectively. *P<0.05 vs 10^-7mol/L Ach-induced relaxation of SHR MAs; ***P<0.0001 vs SHR. **B,** Effects of 12-week telmisartan intervention on the tail-cuff systolic blood pressure (SBP) levels of SHR. N=10 per group. ***P<0.0001 vs WKY at baseline, #P<0.0001 vs SHR-Telmi at baseline using repeated measures ANOVA, †P<0.0001 vs SHR at the same time point. **C,** Effects of telmisartan intervention on the 24-hour ambulatory BP levels in SHR. N=5 per group. **P<0.01, ***P<0.0001.
Enhanced Mitochondrial Transient Receptor Potential Channel, Canonical Type 3–Mediated Calcium Handling in the Vasculature From Hypertensive Rats
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