Title: Downregulation of Endothelial Transient Receptor Potential Vanilloid Type 4 Channel Underlines Impaired Endothelial Nitric Oxide-Mediated Relaxation in the Mesenteric Arteries of Hypertensive Rats

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Short title:

Downregulation of endothelial TRPV4 impairs vasorelaxation

Summary

The endothelium contributes to the maintenance of vasodilator tone by releasing endothelium-derived relaxing factors, including nitric oxide (NO). In hypertension, endothelial nitric oxide synthase (eNOS) produces less NO and could be one of the contributing factors to the increased peripheral vascular resistance. Agonist-induced Ca^{2+} entry is essential for the activation of eNOS. The transient receptor potential vanilloid type 4 (TRPV4) channel, a Ca^{2+}-permeant cation channel, is expressed in the endothelial cells and involved in the regulation of vascular tone. The present study aimed to investigate the role of TRPV4 channel in endothelium-dependent NO-mediated relaxation of the resistance artery in hypertensive rats. Using a wire myograph, relaxation response to the TRPV4 activator, 4α-phorbol-12,13-didecanoate (4αPDD) was assessed in mesenteric arteries obtained from Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). Compared to WKY, SHR demonstrated a significantly attenuated 4αPDD-induced endothelium-dependent NO-mediated relaxation. Immunohistochemical analysis revealed positive staining for TRPV4 in the endothelium of mesenteric artery sections in both WKY and SHR. Furthermore, TRPV4 mRNA and protein expressions in SHR were significantly lower than their expression levels in WKY rats. We conclude that 4αPDD-induced endothelium-dependent NO-mediated vasorelaxation is reduced in SHR and downregulation of TRPV4 could be one of the contributing mechanisms.

Keywords: TRPV4, hypertension, endothelium, nitric oxide, spontaneously hypertensive rats.

Introduction

An increase in the peripheral vascular resistance is a characteristic finding of essential hypertension in humans and genetic strains of spontaneously hypertensive rats (SHRs) (Tobia *et al.* 1974, Thomas *et al.* 1990, Mayet and Hughes 2003, Hall *et al.* 2013). It is well established that the interplay between endothelial cells and the underlying vascular smooth muscle cells plays a crucial role in determining peripheral vascular resistance, which may increase as a result of either enhanced contractility or impaired relaxation of the vascular smooth muscle cells of the resistance arteries (Giles *et al.* 2012, Kang 2014).

The endothelium is a continuous monolayer of epithelial cells lining the blood vessels. The endothelial cells regulate the vascular tone by releasing a number of endothelium-derived relaxing factors, including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2), as well as through the generation of endothelium-dependent hyperpolarization (EDH) mainly via the activation of the small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}) located on endothelial cells. The generated EDHs are electrically transmitted to the adjacent vascular smooth muscle cells via the myoepithelial gap junctions (Félétou and Vanhoutte 2006, Bratz *et al.* 2008, Edwards *et al.* 2010, Goto *et al.* 2018, Loh *et al.* 2018). An increase in endothelial cytosolic calcium concentration is essential, at least in part, for the generation of these vasorelaxing factors (Loh *et al.* 2018). Various receptor agonists such as acetylcholine (ACh) and bradykinin, as well as mechanical stimuli (e.g., shear stress), can rapidly increase endothelial intracellular Ca^{2+} concentration $[Ca^{+2}]_i$ (Kaneto *et al.* 2010), which subsequently leads to the synthesis and release of the endothelial vasorelaxing factors (Hill *et al.* 2001). In endothelium, the rise of cytosolic calcium concentration enhances the formation of calcium-calmodulin complexes, which in turn will activate the calmodulin-binding domain of the endothelial nitric oxide synthase (eNOS), and causes NO production. Moreover, the increase in hemodynamic shear stress in the blood vessel will induce the phosphorylation of the eNOS and hence its activation with the resultant NO production. Subsequently, NO will diffuse into adjacent vascular smooth muscles to stimulate the activity of the components down its signaling cascade such as soluble guanylyl cyclase (sGC), cyclic guanosine monophosphate (cGMP), and protein kinase G (PKG), hence resulting in vasodilation. The production of the NO can further stimulate the Kca channels and voltage-activated K^+ channels (Kv) through the sGC-independent pathway (Quillon *et al.* 2015, Loh *et al.* 2018).

Several studies have reported the impairment of endothelial function in various models of hypertension (Fujii *et al.* 1992, Hilgers and Webb 2007, Dal-Ros *et al.* 2009, Giles *et al.* 2012). Endothelial dysfunction in hypertension has been suggested to be due to reduced activity and expression of endothelial SK_{Ca} and IK_{Ca} channels, which are known to be the major contributors to EDH-mediated effect (Weston *et al.* 2010, Seki *et al.* 2017) or reduced NO-dependent relaxation (Yang and Kaye 2006). eNOS was found to produce less nitric oxide in hypertensive rats than it did in cells from normotensive rats (Hayakawa and Raij 1998). This low NO production could be attributed to either uncoupling of eNOS, which shifts it from NO production to reactive oxygen species (ROS) production (Landmesser *et al.* 2003), or insufficient increase in the cytosolic calcium concentration that is necessary for eNOS activation (Mendoza *et al.* 2010, Loh *et al.* 2018).

Recent studies have introduced the importance of Transient Receptor Potential Vanilloid 4 (TRPV4) channels in the regulation of vascular tone by providing a potential Ca^{2+} entry channel in the vascular endothelial cells for the activation of vasodilator mechanisms in conduit and resistance arteries. Thus the intracellular calcium increase, mediated by TRPV4 channels, triggers both NO- and/or EDH-dependent vasodilatation, an effect that seems to be dependent on the studied vascular bed (Filosa *et al.* 2013, Nishijima *et al.* 2014). Interestingly, in several cell types including endothelial and smooth muscle cells, calcium handling proteins are located in caveolae. TRPV4 was shown to be also expressed as a heterotetramer with TRPC1 and, that alone or in complex with TRPC1, it is able to interact with the structural caveolar protein caveolin-1 and this interaction is functionally important for the TRPV4-mediated calcium signaling and subsequent vasodilation (Filosa *et al.* 2013).

TRPV4 was initially reported as an osmo- or mechano-sensor (Liedtke *et al.* 2000, Strotmann *et al.* 2000) that can be activated by diverse chemical stimuli, including the synthetic phorbol ester 4α-phorbol 12,13-didecanoate (4α-PDD), GSK1016790A (Watanabe *et al.* 2002a) (Watanabe *et al.* 2002a, Willette *et al.* 2008), as well as moderate warmth (>27°C) (Güler *et al.* 2002, Watanabe *et al.* 2002b) and inhibited by HC-067047 (Everaerts *et al.* 2010). TRPV4 is widely expressed throughout the body, including esophageal (Mihara *et al.* 2011), gastric (Mihara *et al.* 2016) and urinary bladder (Everaerts *et al.* 2010) epithelia as well as skin keratinocytes (Sokabe *et al.* 2010), hippocampal neurons (Shibasaki *et al.* 2007) and endothelial cells (Watanabe *et al.* 2002b), where it contributes to numerous physiological processes, such as regulation of cell volume, vascular tone, vascular permeability, mechanosensation, proliferation, angiogenesis, secretion, apoptosis and cell death (Yao and Garland 2005, Mihara *et al.* 2011, 2016, Persson 2015).

Recently, downregulation of endothelial TRPV4 was shown to contribute to the impairment of EDH-mediated relaxation in the mesenteric arteries of stroke-prone hypertensive rats (Seki *et al.* 2017). However, the potential role of endothelial TRPV4 in mediating NO-dependent relaxation in hypertension is rather unknown. Therefore, in the present study, we investigated whether a dysfunctional TRPV4 is underlying any possible impairment of NO-dependent relaxation in the mesenteric artery of spontaneously hypertensive rats (SHRs).

Materials and Methods:

Animals

Ten to twelve-week-old male spontaneously hypertensive rats (SHRs) and age-matched normotensive Wistar-Kyoto (WKY) rats were used in this study. Rats were housed in a controlled environment (12 h light–12 h dark cycle; room temperature, 22–24°C; 50–60% relative humidity) and received standard rat rodent chow and water *ad libitum*. All procedures were performed in accordance with Sultan Qaboos University (SQU) Animal Ethics Committee regulations and SQU Guidelines for Care and Use of Laboratory Animals.

Determination of blood pressure

Systolic (SBP) and diastolic (DBP) blood pressure was measured non-invasively by tail-cuff plethysmography using BP-2000 Blood Pressure Analysis System^{TM} (Visitech Systems, USA). First, the selected animals were acclimated to the equipment once per day for two days before actual testing began. Then, the mean of three measurements of blood pressure was recorded.

Vessel preparation and isometric tension recording

Rats were anesthetized with an intraperitoneal injection of ketamine (140mg/Kg) and xylazine (40mg/Kg) mixture. Mesenteric tissues were excised and placed in cold, oxygenated physiological solution containing (in mM) 119 NaCl, 4.7 KCl, 1.18 KH_2PO4, 1.17 $MgSO_4$, 25 $NaHCO_3$, 5.5 glucose, 10 HEPES and 1.6 $CaCl_2$ (pH 7.4 adjusted with NaOH). Second order branches from the mesenteric artery were cleaned of connective and adipose tissues and cut into segments of 2 mm length. According to the experimental requirement, the endothelium was either kept intact or removed by gentle rubbing of the lumen with a fine hair. Then arterial rings were threaded on two thin wires (40µm in diameter) and mounted in a four-chamber wire myograph DMT (Danish Myo Technology A/S, Aarhus, Denmark) containing a physiological solution that was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2.

After the normalization procedure, arteries were left to equilibrate for 60 min at 37 °C before subsequent evaluation. At the end of this period, the tension created by the arterial segments was considered as the resting (basal) tension, and no further mechanical adjustment was made during experimentation. Isometric responses were filtered and recorded using a PowerLab acquisition system and LabChart 8 software (AD Instruments, Bella Vista NSW, Australia).

Experimental protocol

The resting tension of vessels was measured 60 min after mounting the vessels at 37 °C. After that, arteries were contracted twice with 60 mM KCl to test their viability, then KCl was washed out, and vessels were contracted with 4 µM phenylephrine (PE) and then relaxed with 1 µM ACh. Segments showing relaxation of more than 80% for WKY and 50% for SHR were considered as endothelium-intact (endo+), whereas those with a relaxation of less than 5%, in both strains, were considered as endothelium denuded (endo–) (Bratz *et al.* 2008). Vessels with relaxation between 5% and 80% for WKY and 5% to 50% for SHR were excluded. Thereafter, tissues were washed twice to remove PE and ACh. Then the preparations were re-contracted with PE (4 µM), and after the contraction reached a steady state, the response to 5 μM 4αPDD (selective TRPV4 agonist) was determined (Ma *et al.* 2013). To determine whether the 4αPDD-induced response is mediated by TRPV4 stimulation, the effect of 4αPDD was examined on arterial preparations pre-incubated for 30 min with the selective TRPV4 antagonist, HC067047 (1 μM) (Everaerts *et al.* 2010) before being contracted with PE (4 μM). Additionally, to explore whether NO is involved in mediating the 4αPDD-induced response, the effect of 4αPDD was studied in preparations pre-incubated with L-NAME (100 μM) for 30 min.

The concentrations of the different agonists and antagonists were selected according to previous studies (Hwa *et al.* 1994, Bubolz *et al.* 2012, Ma *et al.* 2013, Albarwani *et al.* 2015, Zhang *et al.* 2016, Seki *et al.* 2017).

Immunostaining

For section preparation, rats were anesthetized with intraperitoneal injection of ketamine (140mg/Kg) and xylazine (40mg/Kg) mixture. Then the thoracic cavity was cut open, and the hearts were perfused by intracardiac infusion of 0.1M Phosphate-buffered saline (PBS). The small intestines were removed and spread open in dissecting dish containing 4% paraformaldehyde (PFA). Six second-order mesenteric arterial segments were removed from each rat and fixed in 4% PFA for 1 hour at 4°C. PFA-fixed arterial preparations were washed 3 times in 0.1M PBS (15 minutes each). Then the mesenteric arterial segments were incubated in PBS-sucrose (PBS containing 20% sucrose) overnight at 4°C. Next day, arteries were embedded in Optimal Cutting Temperature (OCT) compound, and 10-μm-thick cross sections were collected onto glass slides, and air dried at room temperature for 1 hour. Four immunostaining, arterial section were cleared with PBS-T 0.3% (PBS plus 0.3% TritonX-100) (3 × 5 min). To reduce non-specific antibody binding, tissues were pre-incubated in 10%

hydrogen peroxide (H_2O_2 in PBS) for 1 hour at room temperature before adding primary antibody. Then preparations were washed 3 times with PBS–T 0.3% (5 min each).

To study the localization of TRPV4 in the mesenteric artery wall, preparations were incubated with the primary antibody against TRPV4 that was diluted with PBS-T 0.3% containing 3% bovine serum albumin (BSA). In order to detect best immunofluorescence signal, serial dilutions of the primary antibody were used in a pilot study as follows: 1:100, 1:200, 1:300, 1:400, 1:500 and 1:1000. The dilution of 1:400 was used for the subsequent experiments because it gave the best signal with least background staining. After overnight incubation with primary antibody at 4°C, preparations were washed 3 times by PBS-T 0.3% (5 min each). Then, the secondary antibody (1:500) was added and incubated for 1 hour at room temperature. After being washed 3 times in PBS-T 0.3% (5 min each), the sections were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:1000) for 5 min to detect the nuclei in the sections. Finally, the slides were mounted using AquaMountant™. The stained sections were visualized using fluorescence microscopy, and pictures were captured with a camera (Olympus, DP71, Japan).

RNA extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

Second order mesenteric arteries were quickly isolated from anesthetized rats and put in sterile tubes, and immediately snap frozen in liquid Nitrogen. Total RNA was extracted using TRIzol reagent. cDNA was synthesized from 1μg of total RNA using the iScript™ kit and following the protocol provided by BIO-RAD.

Quantitative Real-time amplifications were performed for TRPV4 and GAPDH by using TaqMan® Fast Advanced Master Mix (Applied Biosystems) detection protocol. They were run in duplicate on 96-well plates using (7500 Fast Real-Time PCR System, Applied Biosystems). The total volume of reactions was 20 μl containing: 2 μl cDNA template, 1 μl primer, 10 μl TaqMan® Fast Advanced Master Mix (Applied Biosystems) and 7 μl RNase/DNase-free sterile water. Negative controls were done in duplicate for each master mix. The RT-PCR reaction started with an initial incubation at 50˚C for 2 minutes, then a hold for 20 seconds at 95˚C, followed by 40 cycles of amplification with denaturation at 95˚C for 3 seconds, annealing and extension at 60˚C for 30 seconds.

Western immunoblotting

Western immunoblotting was performed according to (Albarwani *et al.* 2016) with some modification. Briefly, the frozen arteries were thawed and homogenized in RIPA Lysis buffer (25 mmol/L Tris-HCl [pH 7.6], 150 mmol/L NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS]) supplemented with a protease inhibitor cocktail (Roche Applied Science, UK) and centrifuged at 500g for 10 min at 4ºC. The concentration of the extracted protein was measured employing BCA protein assay kit (Thermofisher, UK) using bovine serum albumin as standard. Equal amounts of protein samples (20 µg) were loaded, and protein was allowed to be separated in 7.5% SDS-PAGE gel for one hour. Then, proteins were transferred from the gel onto a nitrocellulose membrane using semi-dry transfer (Trans-Blot Turbo, Bio-Rad, USA) method. The membrane was blocked with 10% of nonfat dry milk. The protein of interest was detected by probing the membrane with anti-TRPV4 (1:500, Abcam, UK) overnight at 4°C or anti-GAPDH (1:5000, ABGENT, USA) for two hours at room temperature. After washing, the membrane was incubated with secondary antibodies, conjugated to horseradish peroxidase (1:5000, Thermo Scientific, UK) for 1 hour at room temperature. Then the membrane was processed for chemiluminescent detection using Super Signal West Dura substrate (Thermo Scientific, UK) using G:Box Chemi XR5 (Syngene, UK). All reactions were performed in duplicate, and GAPDH was used as an internal control.

Drugs and solutions

Acetylcholine (ACh), phenylephrine (PE), N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), 4α-Phorbol 12,13-didecanoate (4αPDD), HC067047, 4% paraformaldehyde, TritonX-100 and H_2O_2 were purchased from Sigma Chemicals (Germany). ACh, PE, and L-NAME solutions were prepared in distilled water. The 4αPDD solution was reconstituted in absolute ethanol and HC067047 in dimethyl sulfoxide (DMSO). Their concentrations as indicated in the text are the final concentrations in the chamber solutions. Final concentrations of the vehicle were less than 0.1%. PBS (0.1M) was prepared according to the following composition (mM): NaCl, 137; KCl, 2.7; Na_2HPO_4, 10.1; KH_2PO_4, 1.8; $CaCl_2.2H_2O$, 0.9, $MgCl_2.6H_2O$, 0.5, and aerated with 95% O_2 and 5% CO_2. Optimal Cutting Temperature compound was purchased from VWR chemicals (UK). DAPI (ab228549), BSA (ab64009), primary (Rabbit polyclonal to TRPV4; ab39260) and secondary antibodies (Goat Anti-Rabbit IgG Alexa Fluor® 594; ab150080) were purchased from Abcam company (UK) and were diluted using PBS. The iScript™ kit that was used for cDNA synthesis was purchased from Bio-Rad company (USA), and TaqMan® Master Mix was purchased from Applied Biosystems (USA). TaqMan® gene expression assay for TRPV4 and GAPDH were purchased from ThermoFisher Scientific Company (Cat. # 4331182, Rn00576745_m1 and Cat. # 4331182, Rn01775763_g1, respectively).

Analysis and statistics

The PE-induced contraction of arterial rings was expressed as the percentage to the respective resting tension, and the extent of the relaxation response to different agonists was expressed as the percentage reversal of PE-induced contraction (Albarwani *et al.* 2015). The relative change in gene expression was studied by calculating the fold change (Sukumaran *et al.*

2013). The immunoblot band densities were analyzed by using ImageJ software (NIH, USA) and the optical density of each sample was normalized to GAPDH of the respective sample.

The collected data were analyzed using SPSS 23.0 and results were expressed as mean ± S.E.M. with *n* refers to the number of rats. To determine the level of significance, statistical comparisons were performed using the unpaired (independent) Student's *t*-test and the differences were considered statistically significant when $p < 0.05$.

Results

Blood pressure of SHR and WKY rats

As expected, the blood pressure in SHR was significantly higher than WKY rats (189±11/115±8 mmHg vs. 144±7/83±5 mmHg; n=5-6; $p < 0.01$ for SBP and $p < 0.05$ for DBP).

Basal tensions and responses to PE

At the end of the equilibration period, the resting tensions of the SHR mesenteric arteries were slightly higher compared to arteries obtained from WKY rats (both endothelium-intact). However, the difference was not significant (3.34 ± 0.46 mN; n=6 vs. 2.48 ± 0.32 mN; n=5; $p = 0.1755$). Similarly, the PE-induced contractions in SHR were higher, though not significant, in comparison to WKY rats (326.80 ± 44.25% vs. 231.69 ± 44.99%; n = 5-6; $p = 0.1690$) (Fig. 1a). Incubation of arteries with L-NAME or HC067047 did not significantly affect the basal tension or PE-induced contractions (data not shown).

Endothelium-dependent vasodilation

The endothelium-dependent relaxation to ACh (1 μM) was significantly lower in mesenteric arteries of SHR compared with those of WKY rats (58.63 ± 4.20%; vs. 98.95 ± 5.53%; n = 5-6: $p < 0.01$ (Fig. 1b). These ACh-mediated relaxations were abolished by removal of the endothelium in both strains (data not shown).

TRPV4-mediated vasodilation in WKY rats

Stimulation of arterial rings obtained from WKY rats pre-contracted with PE (4 μM) with the specific TRPV4 agonist, 4αPDD (5 μM), induced a relaxation response that was significantly inhibited by the selective TRPV4 antagonist HC067047 (1 μM) (85.98 ± 4.18% vs. 18.30 ± 2.86%; n= 5, $p < 0.01$) (Fig. 2). To determine whether the TRPV4-induced relaxation is endothelium-dependent and mediated by endothelial NO, we next studied the effect of endothelium removal and L-NAME (100 μM), separately, on TRPV4-induced relaxation response in the mesenteric artery. As shown in Figure 2, there was a significant impairment of the TRPV4-induced relaxation in both endothelium-denuded (19.93 ± 1.50%; n = 5, $p < 0.01$) and L-NAME-treated vessels (28.18 ± 3.09%; n = 5, $p < 0.01$) (Fig. 2).

TRPV4-mediated response in SHR compared to WKY rats

In order to compare the functional expression of TRPV4 in SHR and WKY rats, relaxation responses to 4αPDD (5 μM) in SHR were compared to those in WKY rats. As shown in Figure 2, the TRPV4-mediated relaxation was significantly lower in SHR compared to WKY rats (85.98 ± 4.18% vs. 43.99 ± 6.12%; n = 5-6; $p < 0.01$). This significantly lower TRPV4-mediated relaxation was significantly reduced by HC067047 (1 μM) (11.76 ± 4.18%; n = 6; $p < 0.01$), endothelium denudation (8.64 ± 1.50%; n = 6; $p < 0.01$) and L-NAME (100 μM) (17.50 ± 3.40%; n = 6; $p < 0.01$) (Fig. 2).

Expression of TRPV4 in the mesenteric arteries of SHR and WKY rats

Using immunohistochemical analysis, a positive immunofluorescence signal was visualized for TRPV4 protein in the endothelial layer of mesenteric arteries obtained from both WKY and SHR strains (Fig. 3). To exclude the possibility of a pseudo-positive reaction for the fluorescence signal, a blank control test without TRPV4 antibody was performed and a negative result was confirmed (data not shown). A weak background staining was found in the underlying smooth muscle cells of mesenteric arteries from all four groups similar to the staining found in the negative control test. Nuclei were stained using DAPI to show the outline of the blood vessel wall (Fig. 3).

Relative expression of TRPV4 mRNA and protein in the mesenteric arteries of SHR and WKY rats

Real-time PCR study was done to assess the level of TRPV4 expression in the mesenteric artery of WKY and SHR strains. TRPV4 mRNA expression was significantly lower in SHR compared to WKY rats (0.67 ± 0.34 RU vs. 2.34 ± 0.15 RU; n = 5; $p < 0.05$) (Fig. 4A). Western immunoblotting revealed a significantly lower TRPV4 protein expression in SHR mesenteric arteries compared to WKY rats (0.312 ± 0.065 RU vs. 0.821 ± 0.079 RU; n = 5; $p < 0.05$) (Fig. 4B).

Discussion

The main findings of the present study are as follows: (1) activation of TRPV4 with the specific agonist, 4αPDD, induced an endothelium-dependent relaxation of second-order mesenteric arterial rings pre-contracted with PE and this 4αPDD-induced relaxation was markedly inhibited by blockade of TRPV4 channels by a selective antagonist HC067047, denudation of the endothelium and by the NOS inhibitor, L-NAME; (2) the TRPV4-mediated relaxation was significantly lower in SHR compared to WKY rats; (3) TRPV4 channels were predominantly expressed in the endothelial layer of small mesenteric arteries obtained from WKY and SHR rats, (4) expression of TRPV4 mRNA and protein were significantly lower in SHR compared to WKY rats.

In agreement with the results of previous studies, ACh induced a relaxation response in rat mesenteric arterial ring preparations that was almost completely abolished by endothelium denudation (Lacy *et al.* 2000, Chen and Qian 2015). The relaxation response to ACh was significantly lower in the mesenteric resistance arteries of SHR compared to WKY rats, which is consistent with previous studies (Tesfamariam and Halpern 1988, Fujii *et al.* 1992, Bennett *et al.* 1996, Shimokawa *et al.* 1996, Goto *et al.* 2004, Seki *et al.* 2017).

Stimulation of the pre-contracted mesenteric artery preparations with the selective TRPV4 agonist, 4αPDD, induced a relaxation response that was markedly inhibited by the selective TRPV4 antagonist, HC067047, confirming that the observed 4αPDD-induced relaxation response is mainly due to stimulation of TRPV4 and is consistent with results from previous studies on rat (Ma *et al.* 2013, Zhang *et al.* 2016) and mouse mesenteric arteries (Zhang *et al.* 2009, Mendoza *et al.* 2010). These results are further supported by Mendoza's group who demonstrated that GSK1016790A, another TRPV4 agonist, induced a relaxation response in mouse mesenteric artery which was absent in TRPV4-knockout ($TRPV4^{-/-}$) mice. Moreover, the flow-mediated dilation and vessel relaxation detected in mesenteric arteries of wild-type mice were significantly reduced in $TRPV4^{-/-}$ mice (Mendoza *et al.* 2010).

Our data also reveal that 4αPDD-induced relaxation is primarily endothelium-dependent since denudation of the endothelium significantly inhibited this relaxation, confirming that the response requires an intact endothelium. This is supported by previous findings where 4αPDD failed to induce cationic current in endothelial cells of TRPV4$^{-/-}$ mice carotid artery (Hartmannsgruber *et al.* 2007) and was able to induce a relaxation response in endothelium-intact, but not in endothelium-denuded rat and mouse mesenteric arteries (Zhang *et al.* 2009, 2016, Mendoza *et al.* 2010), while endothelium-independent relaxations in both, wild-type and TRPV4$^{-/-}$ mice, were nearly the same (Mendoza *et al.* 2010). The observed 4αPDD-induced relaxation in endothelium-denuded preparations of both strains could be due to the activation of vascular smooth muscle TRPV4 (Yang *et al.* 2006, Earley *et al.* 2009, Gao and Wang 2010, Baylie and Brayden 2011), and the significantly lower response seen in SHR could be attributable to the alteration in the function and expression of the smooth muscle TRPV4 in this strain compared to WKY rats.

To explain the observed significant dependence of 4αPDD-induced relaxation on stimulation of endothelial TRPV4 channels, immunohistochemical analysis was employed and it revealed that TRPV4 channels are mainly expressed on the endothelium of mesenteric arteries in both WKY and SHR, and in agreement with previous studies on mouse mesenteric and aortic arteries (Zhang *et al.* 2009, Mendoza *et al.* 2010), and rat mesenteric, pulmonary and carotid arteries (Loot *et al.* 2008, Willette *et al.* 2008, Sukumaran *et al.* 2013, Peixoto-Neves *et al.* 2015). Furthermore, the immunofluorescent signal for TRPV4 protein that was seen in the endothelial cells of wild-type mesenteric and carotid arteries was not seen in those of TRPV4$^{-/-}$ mice (Hartmannsgruber *et al.* 2007, Mendoza *et al.* 2010). Sukumaran's group measured the relative mRNA and protein expression of TRPV4 in endothelium-intact and endothelium-denuded pulmonary artery, and they reported that mRNA and protein expression of TRPV4 in endothelium-intact were significantly higher than in endothelium-denuded (Sukumaran *et al.* 2013), which provide further evidence for the predominance of endothelial expression of TRPV4.

To explore whether stimulation of TRPV4 by 4αPDD will release NO to mediate the endothelium-dependent relaxation response, we blocked the enzyme nitric oxide synthase by L-NAME. After an incubation period with L-NAME for 30 min, 4αPDD-induced relaxation was significantly inhibited in mesenteric arteries precontracted with PE indicating that NO is involved in mediating 4αPDD-induced relaxation. Therefore, NO seems to be a major mediator of TRPV4 relaxing effect in the current study. Stimulation of TRPV4 by 4αPDD may, therefore, increases endothelial $[Ca^{+2}]_i$, which in turn stimulates the enzyme nitric oxide synthase that is crucial for NO synthesis in endothelial cells (Félétou and Vanhoutte 2006, Loh *et al.* 2018).

The amplitude of relaxation induced by TRPV4 stimulation in SHR was significantly lower when compared to WKY. Our data clearly illustrate the existence of a difference in the functional expression of TRPV4 between WKY and SHR. Recently, and in agreement with our results, Seki's group showed that GSK1016790A (TRPV4 agonist) induced a TRPV4-mediated relaxation response in mesenteric arterial rings obtained from WKY rats and this response was markedly impaired in stroke-prone spontaneously hypertensive rats (SHRSPs). However, they attributed the TRPV4-induced relaxation to be mainly mediated by the activation of endothelial IK_{Ca} & SK_{Ca} with minimal or no role of NO, which is dissimilar to our results (Seki *et al.* 2017). The reason for this disagreement is not apparent. However, several reasons might be behind this difference. For instance the strain of rat (SHRSP), the age (20 weeks) or the size of the mesenteric artery (main branch of the superior mesenteric artery) they used. Simonsen's group had shown that endothelium-dependent relaxation of the rat superior mesenteric artery is directly correlated to the endogenous release of NO (Simonsen *et al.* 1999). Further, it was shown that the relative importance of NO and EDHF in endothelium-dependent relaxation in rat mesenteric vascular bed is dependent on vessel size where NO is a major player in proximal larger mesenteric artery, whereas EDHF plays a more important role in the relaxation of smaller more distal branches (Hwa *et al.* 1994, Tomioka *et al.* 1999, Hilgers *et al.* 2006). Nitric oxide synthase inhibition with L-NAME was shown to attenuate EDHF-mediated relaxation induced by GSK1016790A (TRPV4 agonist) in rat pulmonary artery (Addison *et al.* 2016). This could provide an explanation for the apparent discrepancy between our findings and Seki's group results; i.e., lower level of NO produced by the endothelium will lead to less EDHF-mediated relaxation (Seki *et al.* 2017). Seki's group had also shown that the impaired EDH-mediated responses in the superior mesenteric arteries of SHRSP are attributable to downregulation of TRPV4 and SK_{Ca} channels (Seki *et al.* 2017). SK_{Ca} deficient mice exhibited impaired NO-mediated dilatation to ACh and shear stress stimulation (Brähler *et al.* 2009, Kerr *et al.* 2012). Taking all the aforementioned explanations together, the interplay between NO- and EDH-dependent vasorelaxing mechanisms is well established, and it provides further explanation of our results in light of Seki's group findings.

To find out whether the decrease in the amplitude of 4αPDD-induced relaxation in SHR was due to downregulation or dysfunction of TRPV4 that is expressed on the endothelium, we performed RT-PCR. This later showed significantly lower level of TRPV4 mRNA expression in the mesenteric artery of SHR compared to its expression level in the WKY. These results were in correlation with the immunoblotting results, where TRPV4 protein expression was shown to be significantly lower in SHR mesenteric arteries compared to WKY rats, and clearly explain the significantly lower relaxation response to 4αPDD observed in SHR compared to WKY. Our data agree with the recent study where TRPV4 was shown to be downregulated in mesenteric arteries of SHRSPs (Seki *et al.* 2017).

In conclusion, our results suggest that TRPV4 is expressed primarily in the endothelium of rat mesenteric artery and its activation causes endothelium-dependent relaxation mediated by NO. Downregulation of TRPV4 causes attenuation of this endothelium-dependent NO-mediated vasorelaxation in hypertensive rats. Impairment of TRPV4 functional activity, as part of endothelium dysfunction, will represent a target for novel pharmacological or gene-based treatment of human hypertension.

Conflict of interest

The authors declare that there is no conflict of interest associated with this study.

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Figure 1. Phenylephrine (PE)-induced contractions and acetylcholine (ACh)-induced relaxations in mesenteric arteries obtained from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). (A) PE 4 μM evoked contractions that were slightly higher, though not significant, in artery segments from SHR compared to WKY controls. (B) ACh-evoked relaxations were significantly lower in PE-precontracted mesenteric artery segments from SHR compared to WKY controls. ****** $P < 0.01$ vs. WKY controls (n=5-6).

Figure 2. 4αPDD-induced relaxations in mesenteric artery segments obtained from spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) controls. Artery segments were precontracted with 4 μM PE. 4αPDD-induced relaxations were significantly lower in segments from SHR than those from WKY controls. The 4αPDD-induced relaxations were significantly blocked by HC067047, endothelium denudation and L-NAME in both WKY and SHR. ***** $P < 0.05$, ****** $P < 0.01$ vs. WKY, $^{††}P < 0.01$ vs. WKY control (WKY 4αPDD) and **‡‡** $P < 0.01$ vs. SHR control (SHR 4αPDD) (n=5-6).

Figure 3. TRPV4 immunoreactivity in Wistar-Kyoto (WKY) and spontaneously hypertensive rat (SHR) mesenteric artery. TRPV4 immunoreactivity was observed in WKY and SHR mesenteric artery endothelial layers. Scale bar indicates 100 μm. DAPI: sections were stained with 4',6-diamidino-2-phenylindole; TRPV4/DAPI: both images (with TRPV4 staining and with DAPI staining) were merged.

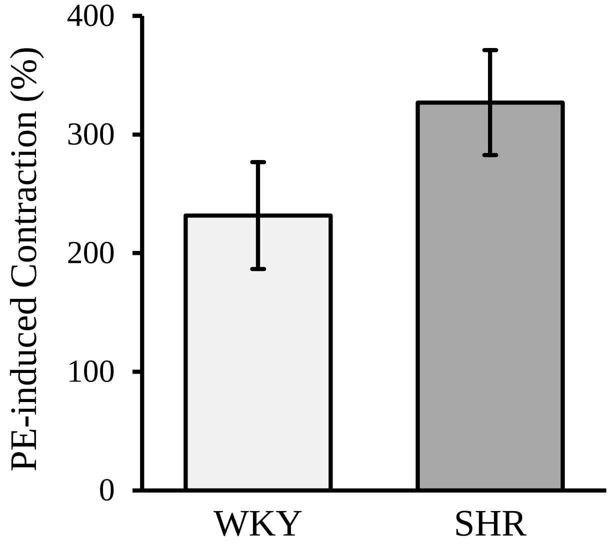
Figure 4. Relative expression of TRPV4 mRNA (A) and protein (B) in endothelium-intact mesenteric arteries of Wistar-Kyoto (WKY) rats and, spontaneously hypertensive rats (SHRs).

Data were shown as mean ± S.E.M. in relative units (RU) after normalization to GADPH.

*$p < 0.05$ vs. WKY (n=5).

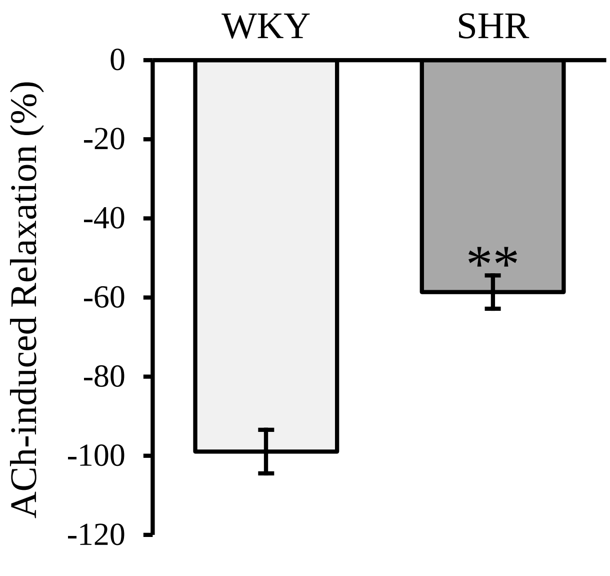
Figure 1

A



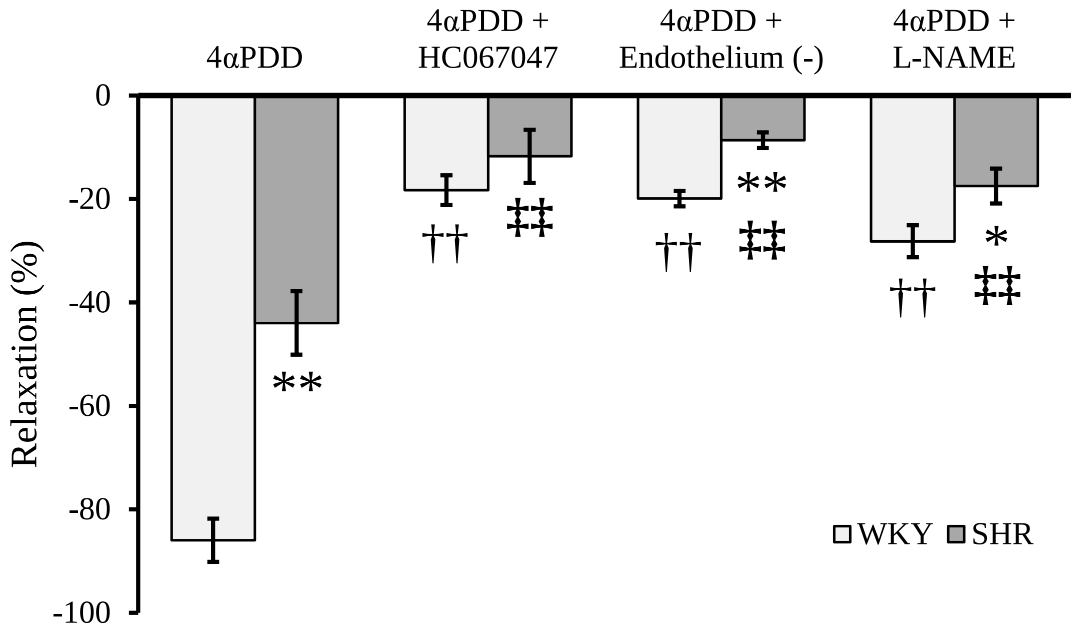
PE-induced Contraction (%)
400
300
200
100
0
WKY
SHR

B



WKY
SHR
0
-20
-40
-60
-80
-100
-120
ACh-induced Relaxation (%)
**

Figure 2



4αPDD
4αPDD + HC067047
4αPDD + Endothelium (-)
4αPDD + L-NAME
0
-20
-40
-60
-80
-100
Relaxation (%)
**
††
‡‡
††
**
‡‡
††
*
‡‡
WKY
SHR

Figure 3

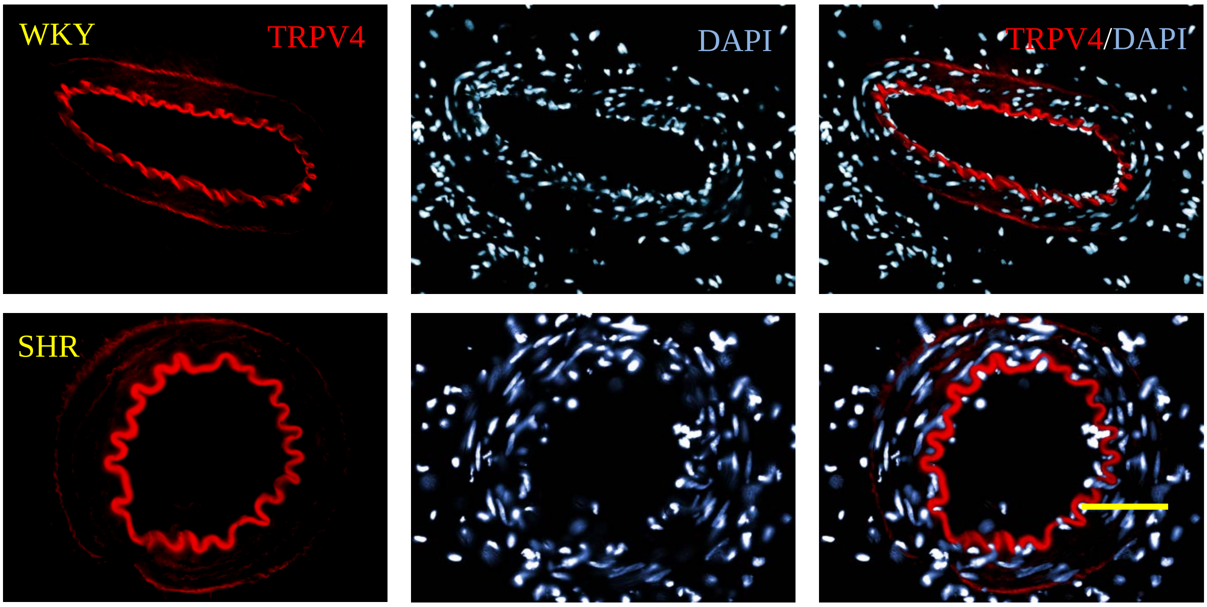
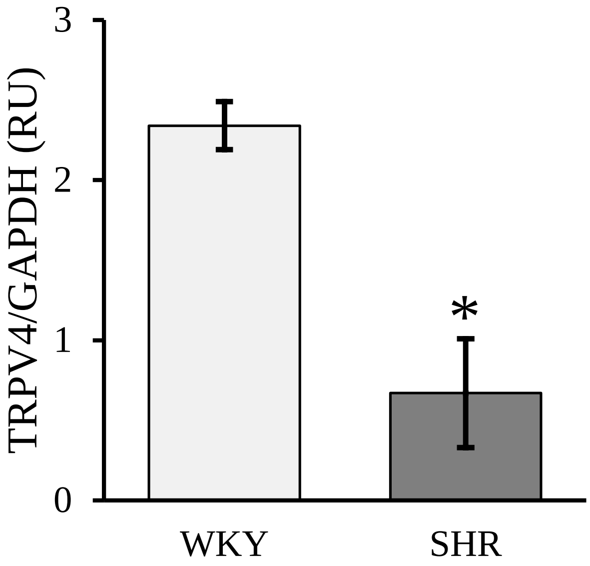


Figure 4

A. RT-PCR



B. Western Blot