Lisinopril Alters Contribution of Nitric Oxide and K_{Ca} Channels to Vasodilatation in Small Mesenteric Arteries of Spontaneously Hypertensive Rats

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Summary

To investigate lisinopril effect on the contribution of nitric oxide (NO) and K_{Ca} channels to acetylcholine (ACh)-induced relaxation in isolated mesenteric arteries of spontaneously hypertensive rats (SHRs). Third branch mesenteric arteries isolated from lisinopril treated SHR rats (20 mg/kg/day for ten weeks, SHR-T) or untreated (SHR-UT) or normotensive WKY rats were mounted on tension myograph and ACh concentration-response curves were obtained. Westernblotting of eNOS and K_{Ca} channels was performed. ACh-induced relaxations were similar in all groups while L-NMMA and indomethacin caused significant rightward shift only in SHR-T group. Apamin and TRAM-34 (SK_{Ca} and IK_{Ca} channels blockers, respectively) significantly attenuated ACh-induced maximal relaxation by similar magnitude in vessels from all three groups. In the presence of L-NMMA, indomethacin, apamin and TRAM-34 further attenuated ACh-induced relaxation only in SHR-T. Furthermore, lisinopril treatment increased expression of eNOS, SK_{Ca} and BK_{Ca} proteins. Lisinopril treatment increased expression of eNOS, SK_{Ca}, BK_{Ca} channel proteins and increased the contribution of NO to ACh-mediated relaxation. This increased role of NO was apparent only when EDHF component was blocked by inhibiting SK_{Ca} and IK_{Ca} channels. Such may suggest that in mesenteric arteries, non-EDHF component functions act as a reserve system to provide compensatory vasodilatation if (and when) hyperpolarization that is mediated by SK_{Ca} and IK_{Ca} channels is reduced.

Key words

Endothelium • Nitric oxide • Small mesenteric arteries • Lisinopril • K_{Ca} channels • ACE inhibition

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Introduction

The vascular smooth muscle cells (VSMC) relaxation is dependent on the endothelial lining of blood vessels (Rubanyi 1991). The vasodilatory effect of the endothelium is brought about through the actions of nitric oxide (NO) and prostacyclin (PGI_2) in addition to the more elusive endothelium derived hyperpolarizing factor (EDHF) (Nilius and Droogmans 2001).

The major component of EDHF is thought to be mediated by K^+ efflux through opening of endothelial calcium-activated K^+ channels of small (SK_{Ca}, $K_{Ca}2.3$) and intermediate (IK_{Ca}, $K_{Ca}3.1$) conductance (Edwards *et al*. 1998). Opening of these channels creates a cloud of K^+ that lies between the endothelial cells and VSMCs, which causes the activation of Na^+-K^+ pump together with the inward rectifier K^+ channels; an action that ultimately leads to hyperpolarization of VSMCs (Zaritsky *et al*. 2000, Edwards and Weston 2004), closure of its voltage-gated calcium channels and, in turn, relaxation of the VSM and vasodilatation. Besides, there is evidence that endothelial hyperpolarization may also spread to the underlying VSM *via* myoendothelial junctions (Sandow *et al*. 2002).

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The magnitude of contribution of each of these mechanisms in the eventual vasodilatation is not clear. Although NO has been recognised for many years as the primary endothelium-dependent vasodilator (Félétou and Vanhoutte 2006), more work has emerged suggesting different contributions of NO and/or other relaxing factors in different arterial trees. An important divergence from the primary role of NO in this respect, is the suggestion that NO is more important in large vessels; whereas in small arteries (which participate actively in the regulation of systemic peripheral resistance and thus blood pressure) EDHF is thought to be more important (Félétou and Vanhoutte 2006, Hilgers *et al*. 2006). This notion is of particular importance in appreciating the mechanism(s) underlying hypertension and its therapy.

Several studies have reported impairment of endothelial function in various models of hypertension (Fujii *et al*. 1992, Hilgers and Webb 2007, Dal-Ros *et al.* 2009). Endothelial dysfunction in hypertension has been suggested to be due to reduced NO-dependent relaxation (Yang and Kaye 2006), or reduced activity and expression of endothelium SK_{ca} and IK_{ca} channels, which are known to be the major contributor to EDHF-mediated effect (Weston *et al.* 2010). In transgenic mice, a reduced EDHF-mediated relaxation was associated with significant increase in blood pressure when SK_{Ca} (Taylor *et al*. 2003) and IK_{Ca} (Si *et al*. 2006), channels expression levels were suppressed or when both SK_{Ca} and IK_{Ca} genes were deleted (Brähler *et al.* 2009). On the other hand, pharmacological openers of SK_{Ca} and IK_{Ca} channels reduced blood pressure in mice (Sankaranarayanan *et al*. 2009) and in large animals like dogs (Damkjaer *et al.* 2012). These observations suggest that endothelial K^+ channels are important in regulating vascular tone and blood pressure, and therefore may represent therapeutic targets for the treatment of hypertension. This thought is supported by the noticed improvement of impaired endothelium function in humans reported to use different antihypertensive treatments (Neutel 2004, Deja *et al*. 2005).

In endothelial cells, angiotension converting enzyme inhibitors (ACEIs) increase expression and activity of eNOS and, in turn, increase NO bioavailability (Morawietz *et al.* 2006). Angiotensin II, through its action on endothelial AT1 receptors, was shown to activate superoxide anion generation *via* activation of membrane-bound NADH-/NADPH-oxidase, an effect that was inhibited by the ACEI lisinopril (Zhang *et al.* 1999). Furthermore, the activity of calcium-activated potassium channels involved in EDHF-mediated responses is decreased by the chronic action of superoxide anion (Kusama *et al.* 2005). However, no report, to the best of our knowledge, has investigated how the relative contribution of endothelial K_{Ca} channels and NO is altered with ACEIs treatment in hypertension. Thus, this study was designed to evaluate the contribution of both NO and of EDHF component that is mediated by K_{Ca} channels to ACh-induced relaxation in isolated small mesenteric arteries of spontaneously hypertensive rats (SHRs) and to ascertain if ten-weeks of the lisinopril treatment may modify this contribution.

Materials and Method

Experimental animals and treatment

All procedures were performed after approval of Sultan Qaboos University Animal Ethics Committee and in accordance to SQU Guidelines for Care and Use of Laboratory Animals. Male SHRs 10-12 weeks old and weighing 241±4.4 g were randomly assigned to two groups, the first (n=45) was treated with lisinopril for 10 weeks with 20 mg/kg/day by gavage (treated group, SHR-T); whereas the second group (n=45) was given daily tap water instead of lisinopril solution (untreated group, SHR-UT). WKY rats of same age (400±13 g) were used as controls (n=24). Rats were housed in Sultan Qaboos University Small Animal House Facility in a temperature-controlled room (22±2 °C) with a 12 h-12 h light-dark cycle, and received food and water *ad libitum*.

Measurement of blood pressure

The blood pressure of six from each SHR groups (UT and T) and five WKY rats was measured by telemetry (C50-PXT Implants, Data Science Int., St. Paul, MN, USA) which records systolic, diastolic blood pressure and heart rate in free moving animals through transmitters implanted in the abdominal aorta (Albarwani *et al.* 2013). Blood pressure data were collected weekly starting ten days after surgery and ten weeks thereafter.

Vessel preparation and isometric tension recording

At the end of the ten weeks treatment period, rats were sacrificed by an overdose of a mixture of ketamine (140 mg kg^{-1} i.m.) and xylazine (40 mg kg^{-1} i.m.). Mesentery were excised and placed in cold

physiological saline solution (PSS, 0-4 °C). Third-order branches of mesenteric arteries were isolated, cleaned free of fat and connective tissue, cut into short segments (2.0-2.3 cm in length), and mounted on an isometric wire myograph (Danish Myo Technology, Aarhus, Denmark). Arteries were then superfused with warm (37 °C) PSS of the following composition (mmol l^{-1}): 119 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 25 $NaHCO_3$, 5.5 glucose and 1.6 $CaCl_2$, pH 7.4 adjusted with NaOH. After the normalization procedure (Halpern *et al*. 1978), arteries were left to equilibrate for 45 min at 37 °C before subsequent evaluation. Throughout the experiments, arteries were continuously bubbled with 95 % O_2 and 5 % CO_2 mixture. Changes in isometric tension were recorded with Powerlab and Chart 7-pro software (ADInstruments, Australia).

Experimental protocol

The basal tension of vessels was measured 45 min after mounting the vessels at 37 °C. Thereafter, 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 relaxed with 1 μM acetylcholine (ACh). Endothelium was considered intact if arteries relaxed in response to ACh by more than 90 %.

To determine ACh-mediated relaxation, arteries were contracted with submaximal concentration of PE (4 μM), after the contraction reached a steady state, ACh was added to the bath in a cumulative concentration response manner from concentration of 1 nM to the final concentration of 10 μM. To determine the contribution of different endothelium vasoactive factors, vessels were incubated for 20 min with different inhibitors/blockers individually or in combination before contracting with 4 μM PE.

To examine the roles of NO and PGI_2, L-NMMA (100 μM) and indomethacin (10 μM) were used to inhibit eNOS and cyclooxygenase activity, respectively. To characterize the role of K^+ channels involved in ACh-mediated relaxation the following agents were used alone or in combination as appropriate: apamin (0.3 μM), TRAM-34 (10 μM) and Iberiotoxin (IbTx 0.1 μM) to block SK_{Ca}, IK_{Ca} and BK_{Ca} channels, respectively.

Western immunoblotting

Western immunoblotting was performed as described by Albarwani *et al.* (2010). Equal amounts of mesenteric artery proteins (20 μg) were loaded into adjacent lanes, separated by 10 % SDS-PAGE, and transferred onto nitrocellulose membrane. Membranes were blocked in 10 % skimmed milk for 2 h at room temperature and then incubated overnight at 8 °C with monoclonal anti-BK_{Ca}-α (1:200, BD Bioscience, USA) or monoclonal anti-eNOS (1:1,000, BD Bioscience, USA) or polyclonal anti-K_{Ca}3.1 (1:200, Sigma) or polyclonal anti-K_{Ca}2.3 (1:200, Santa Cruz Biotechnology). After washing, membranes were incubated for 2 h at 25 °C with the horseradish peroxidase-conjugated secondary antibodies (dilution 1:5,000, Santa Cruz Biotechnology). Immunoreactive bands corresponding to the molecular weight were detected by enhanced chemiluminescence (Advance ECL, Amersham, UK). Membranes were stripped (Restore™ Western Blot Stripping Buffer, Pierce, USA) and probed with β-actin antibody (1:500 Santa Cruz Biotechnology). Each protein sample was prepared from mesenteric arteries that were pooled from 3-4 rats. A total of 4 different samples were run for each animal group. Proteins were quantified using densitometry analysis normalized for loading differences to β-actin signal and expressed relative to SHR-UT density.

Analysis and statistics

Differences in tensions between PE-contraction and basal tension were considered as maximal tension (100 %) and relaxation to ACh was expressed as the percentage of relaxation from the maximal response induced by 4 μM PE. The concentrations of ACh that produced half maximal responses (EC50) were calculated using non-linear regression analysis (GraphPad Prism Software, San Diego, CA, USA). The EC50 values were expressed as the negative logarithm of the molar concentration (pEC50). All values are expressed as means ± SEM, n = represents number of vessels used in each experiment. Results were analyzed using two-way ANOVA for comparison between groups followed by Benferroni's post-test. Differences were considered statistically significant at $P<0.05$.

Chemicals

L-phenylephrine HCl, acetycholine, L-NMMA, indomethacin, apamin, IbTx, and TRAM-34 were obtained from Sigma, St. Louis, MO, USA. All stock solutions were prepared using PSS except indomethacin was dissolved in ethanol, and TRAM-34 in DMSO.

Results

Blood pressure

The average blood pressures (SBP/DBP) of rats at the beginning of experiments and after ten weeks were WKY 112±4/81±3 and 113±3.5/81.7±3.2, SHR-UT 165±4/112±8 and 177±6/118±7, SHR-T 165±4/118±4 and 84±6/51±7 mm Hg, respectively. The blood pressure of SHR-T at the end of ten weeks of treatment with lisinopril was significantly lower than that of SHR-UT and of WKY groups ($P<0.001$).

Vascular reactivity

Basal tensions and responses to KCl and PE

Basal tensions that were measured after arteries have gained its full tone, and contractions in responses to 4 μM PE or KCl (average of two contractions) were not significantly different among the three groups. Basal tensions were WKY 3.49±1.7 mN, SHR-UT 3.95±1.7 mN and SHR-T 4.13±1.4 mN and PE tensions were WKY 10.7±0.5 mN, SHR-UT 12.3±0.7 mN SHR-T 12.7±0.5 mN. Contractile responses to KCl were WKY 12.6±0.5 mN, SHR-UT 10.1±0.6 mN and SHR-T 10.8±0.4 mN. Incubation of arteries with L-NMMA, indomethacin or channel blockers did not significantly affect the basal tension or PE contraction. Number of arteries used to obtain results from WKY, SHR-UT, SHR-T were 28, 34, 40, respectively.

Effect of L-NMMA and indomethacin on ACh-induced relaxation

In arteries pre-contracted with 4 μM PE and in the absence of any blocker, ACh produced a concentration-dependent relaxation reaching a maximal value at 1 μM in WKY, SHR-UT and SHR-T rats. The pEC50 and the maximal relaxation were not significantly different in the three groups. This result suggests that the efficacy of ACh to induce relaxation was similar in all groups.

L-NMMA (100 μM) and indomethacin (10 μM) caused a significant rightward shift of the concentration-response curves in SHR-T (pEC50 ± SEM, 7.49±0.16 vs 6.95±0.07, n=12). On the other hand the shift was insignificantly different in arteries of SHR-UT (pEC50 ± SEM, 7.47±0.16 vs 7.27±0.06, n=15) and WKY (pEC50 ± SEM, 7.62±0.15 vs 7.41±0.06, n=12) (Fig. 1A,B and C). In all groups, the maximal relaxation in the presence of these inhibitors was similar suggesting that ACh-dependent non-NO, non-PGI_2 relaxation component(s) can still produce maximal response in the presence of inhibitors of NO and PGI_2 syntheses.

Effect of blocking endothelial SK_{Ca} and IK_{Ca} channels

Incubating arteries with specific blockers of SK_{Ca} (apamin 0.3 μM) and IK_{Ca} channels (TRAM-34 10 μM) significantly attenuated the maximal relaxation induced by highest ACh concentration used (10 μM) by similar magnitude in vessels from all three groups. Maximum relaxations were: 62±6.1 % (n=8), 63.7±5.4 % (n=12) and 68±4.6 % (n=8) for arteries from WKY, SHR-UT and SHR-T, respectively (Fig. 2A). Unlike the result obtained after inhibiting NO and PGI_2 syntheses, this result suggests that when SK_{Ca} and IK_{Ca} channels are blocked, ACh-dependent relaxation cannot reach maximal value.

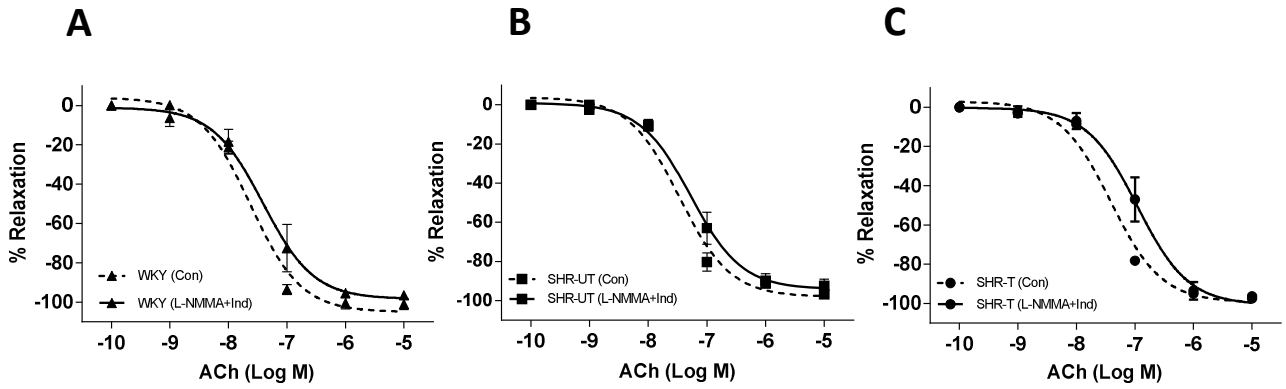

Fig. 1. Concentration-response curves of acetylcholine-relaxations in small mesenteric arteries after inhibiting eNOS and prostacyclin syntheses. Responses of isolated mesenteric arteries that were pre-contracted with 4 μM PE from WKY **(A)**, SHR-UT **(B)** and SHR-T **(C)** to cumulative concentrations of acetylcholine (ACh) before and after incubating the arteries with eNOS inhibitor (L-NMMA 100 μM) and with the cyclooxygenase inhibitor (indomethacin 10 μM, Ind). Values of relaxation were calculated as percentage of the PE-contraction. Con – responses to ACh in absence of any blocker/inhibitor.

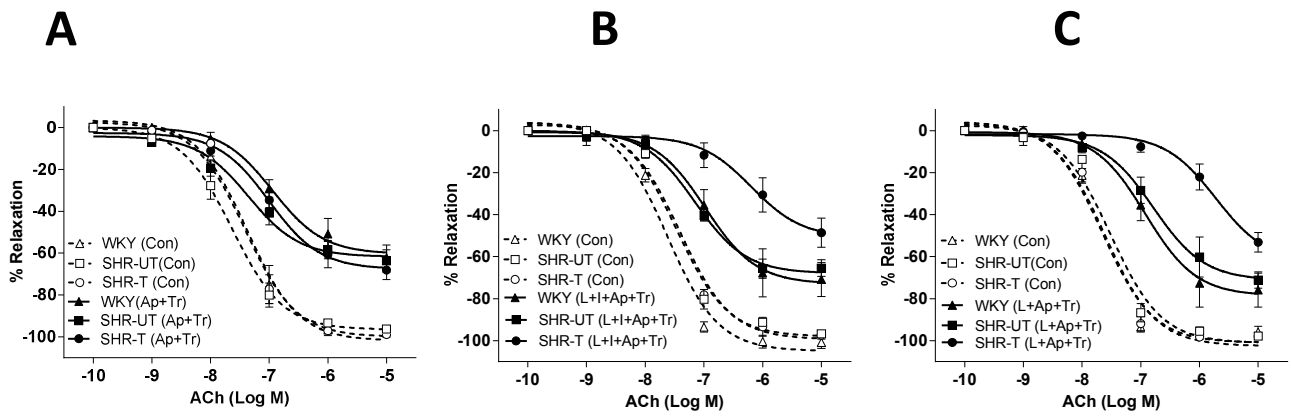

Fig. 2. Concentration-response curves of acetylcholine-relaxations in small mesenteric arteries in the presence of SK_{Ca} and IK_{Ca} channels blockers. Responses of isolated mesenteric arteries that were pre-contracted with 4 μM PE from WKY, SHR-UT and SHR-T to cumulative concentrations of acetylcholine (ACh) in the presence of SK_{Ca} channel blocker (apamin 0.3 μM, Ap) and IK_{Ca} channel blocker (TRAM-34 10 μM, Tr) **(A)**. **(B)** shows the effect of combination of apamin and TRAM-34 together with nitric oxide synthase inhibitor (L-NMMA 100 μM) and cyclooxygenase inhibitor (indomethacin 10 μM, Ind). **(C)** shows the results of experiments similar to those in Figure 2B but without addition of indomethacin. Con – responses to ACh in absence of any blocker/inhibitor.

Effect of the combined inhibition of eNOS and cyclooxygenase together with blocking endothelial SK_{Ca} and IK_{Ca} channels

A third set of experiment was performed in which SK_{Ca} and IK_{Ca} channels were blocked in the presence of L-NMMA (100 μM) and indomethacin (10 μM). Under these conditions, there was a significant rightward shift of the SHR-T curves compared to WKY and SHR-UT. The pEC50 (± SEM) of ACh-induced relaxation in the three groups were 7.57±0.22 (n=8), 7.15±0.07 (n=8), and 6.19±0.12 (n=8), for WKY, SHR-UT and SHR-T, respectively. Furthermore the relaxation induced by maximal ACh concentration used (10 μM) was significantly lower only in SHR-T (43.2±3.9 %) compared to when SK_{Ca} and IK_{Ca} channels were blocked alone (68.46±4.6 %), showing an extra 25 % average reduction in relaxation. The response of arteries from SHR-UT and WKY groups was not significantly different when compared with the response to this concentration of ACh in vessels subjected to SK_{Ca} and IK_{Ca} channels blockade only (WKY, 62±6.1 % vs 70.7±9.2 %) and SHR-UT (63.7±5.4 % vs 65±4.8 %) (Fig. 2B).

A fourth set of experiments was conducted to find out if blocking SK_{Ca} and IK_{Ca} channels would cause the same effect on the observed reduction in ACh-induced maximal relaxation in SHR-T group, if it were accompanied by inhibition of NO synthesis alone, or together with PGI_2 syntheses inhibition. Therefore, arteries were incubated with L-NMMA (100 μM), apamin (0.3 μM) and TRAM-34 (10 μM). Under these conditions the degree of inhibition was also significantly higher in SHR-T (n=8) compared to SHR-UT (n=6) and WKY (n=6). This result suggests that it is the combined inhibition of NO, SK_{Ca} and IK_{Ca} components that caused the extra reduction in ACh-induced relaxation to occur in SHR-T (Fig. 2C).

Effect of blocking BK_{Ca} channels

Figures 3A,B and C show ACh concentration-response curves obtained in the absence and presence of IbTx, a specific blocker of BK_{Ca} channels. In the presence of 100 nM IbTx, there was a significant rightward shift (pEC50 ± SEM) in arteries from WKY (7.60±0.15 to 7.39± 0.04, n=6) and SHR-T rats (7.5±0.12 to 7.01±0.06, n=8) without any changes to maximal relaxation.

When arteries were incubated with IbTx in the presence of L-NMMA, indomethacin, apamin and TRAM-34, ACh-concentration response curves obtained from all groups were shifted to the right, but, under these conditions, the maximal ACh-induced relaxations were further reduced by similar magnitude in arteries from WKY (from 74.9±11 % to 46.6±10 %, n=8), SHR-UT (from 65.5±4.0 % to 30.6±4.8 %, n=12) and SHR-T (from 48.6±7 % to 13.8±3.6 %, n=15) adding a further 30-35 % reduction in maximal relaxation, indicating that BK_{Ca} channels role in ACh-induced relaxations manifest when NO synthesis was inhibited and SK_{Ca} and IK_{Ca} channels were blocked (Fig. 3D,E and F).

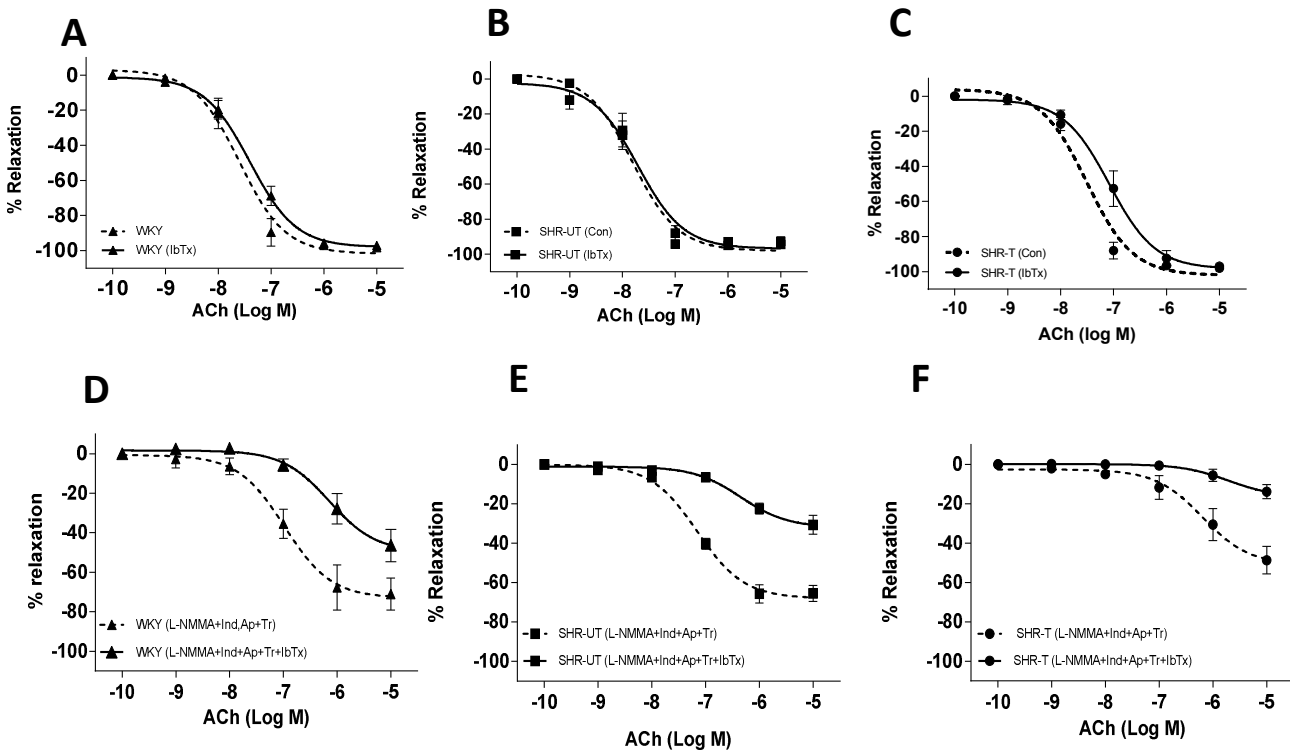

Fig. 3. Effect of BK_{Ca} channel blocker on ACh-concentration-response curves. Effect of BK_{Ca} channel blocker (iberiotoxin 0.1 μM, IbTx) on responses of isolated mesenteric arteries that were pre-contracted with 4 μM PE from WKY **(A)**, SHR-UT **(B)** and SHR-T**(C)** rats to cumulative concentrations of acetylcholine (ACh). Figures **D** (WKY), **E** (SHR-UT) and **F** (SHR-T) show the effect of IbTx (0.1 μM) in the presence of SK_{Ca} channel blocker (apamin 0.3 μM, Ap) and IK_{Ca} channel blocker (TRAM-34 10 μM, Tr) in addition to NO synthase inhibitor (L-NMMA 100 μM) and cyclooxygenase inhibitor (indomethacin 10 μM, Ind). Con – responses to ACh in absence of any blocker/inhibitor.

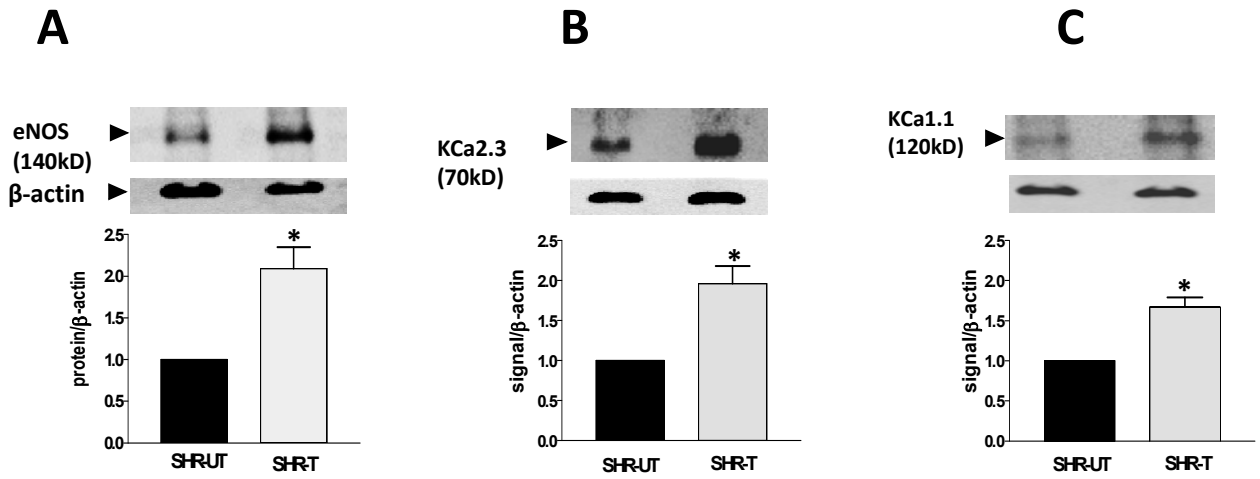

Fig. 4. Western immunoblots of eNOS, SK_{Ca} and BK_{Ca} channels proteins. Representative immunoblots of eNOS **(A)**, SK_{Ca} channel ($K_{Ca}2.3$, **B**) and BK_{Ca} channel ($K_{Ca}1.1$, **C**) and their representative β-actin signals from mesenteric arteries isolated from SHR-UT and SHR-T (lisinopril treated with 20 mg/kg/day for ten weeks). Lower panel shows average densitometric values each corresponds to the immunoblot protein above, normalized to β-actin densities from four Western blots. Bars represent means ± SEM, * significantly different from SHR-UT ($P<0.05$).

Western immunoblotting

Using specific antibodies, corresponding immunoreactive bands were detected by chemiluminescence in protein extracts from mesenteric arteries of all SHR-UT and SHR-T of rats. The upper panel of Figure 4 shows representative changes in immunoblots of eNOS (Fig. 4A), $K_{Ca}2.3$ (SK_{Ca}, Fig. 4B) and $K_{Ca}1.1$ (BK_{Ca}, Fig. 4C) proteins with its

corresponding β-actin signal. The relative amount of normalized protein signal to β-actin signal in four different membranes is shown in lower panel of each representative blot. Compared to SHR-UT, lisinopril treatment (SHR-T) consistently increased protein expression level of eNOS by 110±30 %, of $K_{Ca}2.3$ by 96±22 % and of $K_{Ca}1.1$ by 67±12 %. However, the expression level of $K_{Ca}3.1$ was not consistent on different runs ranging between no changes to small insignificant reduction (data not shown).

Discussion

Endothelial dysfunction is a common denominator to all types of hypertension regardless of its pathogenesis (Giles *et al.* 2012). Evidence is accumulating that EDHF-mediated vascular responses may also contribute to this dysfunction (Goto *et al*. 2004, Yang and Kaye 2006). EDHF-mediated responses are classically defined as endothelium-dependent relaxations that are resistant to eNOS and cyclooxygenase inhibitors and which require activation of endothelial K_{Ca} channels of which SK_{Ca} and IK_{Ca} have been identified to be the major player (Feletou and Vanhoutte 2009).

In our study, the concentration-response relation between ACh and vascular relaxation was not significantly different among the normotensive and hypertensive groups. Dissimilar to this result (Goto *et al*. 2004) reported that ACh caused less than 20 % relaxation in SHR compared to 90 % in WKY rats. The reason for this disagreement is not clear. However, one reason may be due to the difference in size of arteries they used (main branch of mesenteric artery) compared to smaller arteries (third branch) we used in our study. This regional heterogeneity of ACh-induced relaxation, based on the size of mesenteric arteries, has been clearly demonstrated by Hilgers *et al*. (2006). Besides, it was shown that small arteries from normotensive, angiotensin II-induced hypertension (Hilgers and Webb 2007) or from Dahl-sensitive hypertensive rats (Goto *et al.* 2012) exhibit similar overall relaxation to acetylcholine. Another reason might be related to the difference in the age of animals used, which was 48 weeks in their study and 22 weeks in our study.

In order to investigate the magnitude of the EDHF component, ACh-concentration response curves were performed in the presence of inhibitors of NO and PGI_2 syntheses. We found that the non-NO, non-PGI_2 component of ACh-induced relaxation was not significantly different among the three groups. Also of notice was the observation that maximal response to ACh could still be fully obtained, suggesting that the contribution of these two mediators, i.e. NO and PGI_2, to ACh-induced relaxation can be compensated by other vasodilator component(s) such as the EDHF.

A number of studies have demonstrated improved endothelial function with ACE inhibition (Goto *et al*. 2000, Ellis *et al*. 2009, Bondarenko *et al*. 2012) without specifying the role of IK_{Ca} and SK_{Ca} channels in this improvement. In our experiment, when ACh-concentration response curves were produced in the presence of SK_{Ca} and IK_{Ca} channels specific blockers, the relaxations were reduced by similar magnitude in all three groups of arteries and did not reach the maximal response obtained by ACh alone. In addition, when SK_{Ca} and IK_{Ca} channels were blocked together with NO and PGI_2 syntheses were inhibited, ACh-induced relaxation was further reduced by 25 % but only in arteries isolated from SHR-T. An effect that was reproduced in the absence of indomethacin, which may point out that, it is NO that may be of value in this respect. Such differential augmentation of ACh effect in SHR-T group in the presence of NO synthesis inhibitor and blockade of SK_{Ca} and IK_{Ca} channels may imply that lisinopril treatment enhanced the contribution of NO to ACh-induced relaxation, an effect that was only apparent when the predominant role of SK_{Ca} and IK_{Ca} component was suppressed. Of note is that this effect was independent of blood pressure level, as arteries from normotensive and untreated hypertensive rats showed similar and significantly less relaxation compared to arteries from SHR-T group.

We did not study the independent effects of SK_{Ca} and IK_{Ca} channels as our interest was to study the collective role of endothelium K_{Ca} channels to the endothelium-dependent relaxation as was suggested by Hinton and Langton (2003) who reported that, in order to completely eliminate the role of endothelial K^+ channels in EDHF responses, both SK_{Ca} and IK_{Ca} channels must be blocked simultaneously.

In our experiment, $K_{Ca}2.3$ (SK_{Ca}) protein intensity was doubled in arteries from lisinopril treated rats. Hence, one may expect to observe enhanced SK_{Ca} channel component and, therefore larger attenuation in ACh-induced relaxation in arteries from treated rats in the presence of apamin, since particularly SK_{Ca} channel has been shown to play a major role in ACh-induced relaxations in mesenteric arteries (Crane *et al*. 2003,

Hilgers and Webb 2007). However, such attenuation was only apparent when the channels were blocked and NO synthesis was inhibited concomitantly. As has been postulated above, the NO component may function as a reserve/buffer system to provide compensatory vasodilatation if hyperpolarization, that is mediated by SK_{Ca} and IK_{Ca} channels, is diminished, and vice versa. Therefore, when NO influence is increased in SHR-T animals, a reduced relaxation due to blockade of IK_{Ca} and SK_{Ca} channels alone may not manifest due to the overwhelming vasodilator effects of NO. An effect that can be justified by the increased expression of eNOS proteins that we observed in treated rats.

In our work, involvement of BK_{Ca} channels in ACh-induced relaxations was investigated in a separate set of experiments because it is still unclear yet if endothelial cells of mesenteric arteries express BK_{Ca} channel protein or these channels are present exclusively on VSMC (Grgic *et al*. 2009). The observed rightward shift of ACh-induced relaxation in arteries from SHR-T group, in the presence of BK_{Ca} channel blocker, suggests an increased contribution of these channels to the vasodilator effect of ACh. This effect was supported by the increase in BK_{Ca} channel protein ($K_{Ca}1.1$) expression in arteries from treated rats. However, these results cannot explain why there was no differential reduction in ACh-induced relaxation between SHR-UT and SHR-T groups despite the increased $BK_{Ca}1.1$ protein expression in arteries obtained from SHR-T. No likely explanation for this can be offered except that when endothelium hyperpolarizing signals (mainly SK_{Ca} and IK_{Ca} channels) are inhibited, it is expected that open probability of BK_{Ca} channels to be higher due to reduced hyperpolarization of VSMC. Therefore, under these conditions blocking BK_{Ca} channels would be expected to reduce relaxations as have been observed.

The residual ACh-dependent relaxation that persisted after blockade of all three types of K_{Ca} channels was significantly smaller in arteries from SHR-T compared to SHR-UT and WKY rats. The extent of EDHF contribution to ACh-induced vasodilatation varies among species, arterial tree, sex (Villar *et al.* 2008) and pathological conditions such as diabetes (Mauricio *et al.* 2013), atherosclerosis and hypertension (Dal-Ros *et al.* 2009). For example, it was reported that in small mesenteric arteries from WKY rats, myoendothelial junctions and epoxyeicosatrinoic acid (EETs) contribute more to EDHF-relaxation compared to SHR. Furthermore, treatment with enalapril increased EET contribution to ACh-induced relaxation (Ellis *et al.* 2009). Therefore, it is difficult to give a plausible explanation to the observed differences in the residual-relaxation noticed in the arteries of the three groups since there may be other EDHF components that have not been investigated in this study.

It is important to mention few limitations of this study that may reflect on our interpretations. First, the effects of SK_{Ca} or IK_{Ca} channels were not studied independent of one another. This is compounded by the inability to obtain reliable results from Western blotting on IK_{Ca} channel protein ($K_{Ca}3.1$) expression. Therefore, we cannot infer if the enhanced EDHF component by lisinopril treatment was due to increased contribution of SK_{Ca} channel alone or both SK_{Ca} and IK_{Ca} channels. Second, the tissue used in Western blotting was obtained from both the second and third branches of the mesentery arterial bed. That was necessary to yield sufficient protein quantity. Since heterogeneity in EDHF component has been established with different sizes of arteries (Hilgers *et al*. 2006), our results may be diluted or overemphasized by using mixed proteins from both vessel size. Third, since we have no evidence to ensure that L-NMMA has fully blocked NO synthesis then further blockade of relaxation by IbTx (in the presence of L-NMMA, indomethacin, apamin and TRAM-34) could possibly be due to residual NO that its synthesis was not fully inhibited by L-NMMA, thus, the remaining NO could activate the underlying BK_{Ca} channels on VSMC (Mistry and Garland 1998, Jeong *et al.* 2001). Therefore, treating arteries with IbTx could cause further reduction in ACh-induced relaxation because of removal of NO effects on BK_{Ca} channels. Ideally, EDHF-responses should be studied in the presence of NO scavengers in addition to inhibitors of NO and PGI_2 synthesis. Finally it is noteworthy to mention that lisinopril lowered blood pressure below the normal level (WKY level). This hypotensive effect cannot be excluded as one of the reasons that may contribute to the results obtained in this study.

In conclusion, our results suggest that both SK_{Ca} and IK_{Ca} channels and NO play significant role in small mesenteric arteries relaxation induced by ACh, though SK_{Ca} and IK_{Ca} channels have a predominate role. Lisinopril treatment (10 mg/kg/day for ten weeks) increased expression of eNOS, SK_{Ca}, BK_{Ca} channel proteins as well as the contribution of NO to ACh-mediated relaxation. An effect that manifested only when EDHF component was blocked by inhibiting SK_{Ca}

IK_{Ca} channels. Such may imply that non-EDHF component functions as a reserve system to provide compensatory vasodilatation if (and when) hyper-polarization, that is mediated by SK_{Ca} and IK_{Ca} channels, is reduced.

Conflict of Interest

There is no conflict of interest.

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