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Up-regulation of intermediate calcium-activated potassium channels (IKCa), counterbalances the impaired endothelium-dependent vasodilation in SHRSP

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Abstract

Endothelial dysfunction has been linked to a decrease in nitric oxide (NO) bioavailability and attenuated endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation. The small (SK_{Ca}) and intermediate (IK_{Ca}) calcium-activated potassium channels play a key role in endothelium-dependent relaxation. Since the repressor element 1-silencing transcription factor (REST) negatively regulates IK_{Ca} expression, we hypothesized that augmented REST and decreased IK_{Ca} expression contributes to impaired endothelium-dependent vasodilation associated with hypertension. Acetylcholine (ACh) responses were slightly decreased in small mesenteric arteries from male stroke-prone spontaneously hypertensive rats (SHRSP) vs. arteries from Wistar Kyoto (WKY) rats. Incubation with L-NAME (100μM) and indomethacin (100μM) greatly impaired ACh responses in vessels from SHRSP. Iberiotoxin (0.1μM), selective inhibitor of large-conductance K_{Ca} (BK_{Ca}) channels, did not modify EDHF-mediated vasodilation in SHRSP or WKY. UCL-1684 (0.1μM), selective inhibitor of SKCa channels almost abolished EDHF-mediated vasodilation in WKY, and decreased relaxation in SHRSP. TRAM-34 (10μM) and charybdotoxin (0.1μM), both IKCa inhibitors, produced a small decrease of EDHF relaxation in WKY, but completely abrogated EDHF vasodilation in SHRSP. EDHF-mediated relaxant responses were completely abolished in both groups by simultaneous treatment with UCL-1684 and TRAM-34 or charybdotoxin. Relaxation to SK_{Ca}/IK_{Ca} channels agonist NS-309 was decreased in SHRSP arteries. Expression of SK_{Ca} was decreased, whereas IK_{Ca} was increased in SHRSP mesenteric arteries. REST expression was reduced in arteries from SHRSP. Vessels incubated with TRAM-34 (10μM) for 24 hours, displayed reduced REST expression, and no differences in IK_{Ca}. In conclusion, IK_{Ca} channels upregulation, via decreased REST, seems to compensate deficient activity of SK_{Ca} channels in the vasculature of spontaneously hypertensive rats.

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Keywords

small calcium-activated potassium channels (SK_{Ca}); intermediate calcium-activated potassium channels (IK_{Ca}); repressor element 1-silencing transcription factor (REST)

INTRODUCTION

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing vasodilator factors, such as prostacyclin (PGI_2), nitric oxide (NO), and a yet unidentified endothelium-derived hyperpolarizing factor (EDHF) (1). Because EDHF appears to decline with advancing age and to be targeted in diseases such as hypertension and diabetes, knowledge of the ionic mechanisms underlying EDHF actions would be expected to improve understanding of the nature of EDHF and how vascular tone is regulated during health or disease conditions (2).

Extensive studies have been performed in order to better understand EDHF actions. Accordingly, different hypotheses try to explain the mechanisms that mediate EDHF-induced vasodilatation. One of these hypothesis suggest that EDHF leads to endothelial hyperpolarization via activation of Ca^{2+}-activated K^+ channels (K_{Ca}). Hyperpolarization of the vascular smooth muscle cells can be elicited via myo-endothelial gap junctions to result in hyperpolarization of the smooth muscle cells (3–6), and reduced Ca^{2+} influx via voltage-operated Ca^{2+} channels (7–9).

Ca^{2+}-activated K^+ (K_{Ca}) channels are especially important in EDHF-mediated relaxation and hyperpolarization in resistance-sized arteries but the role of these channels during hypertension is far from clear (10–14). Changes in expression or activity of K_{Ca} channels may therefore be a fundamental event contributing to the progression of arterial dysfunction during hypertension. It was previously shown that small-mesenteric arteries from angiotensin II hypertensive rats maintain EDHF-like responses, despite reduced expression of intermediate K_{Ca} (IK_{Ca}) and small K_{Ca} (SK_{Ca}), (15). Whether these changes in K_{Ca} channel function and expression during hypertension occur at the level of resistance-sized arteries, and whether they are needed to regulate vascular tone and hence local blood flow, is not completely understood and requires further studies (14).

Repressor element 1-silencing transcriptional factor (REST, also known as NRSF) is a transcription factor that regulates IK_{Ca} channel gene expression (16–18). The IK_{Ca} channel gene sequence is encoded by the *KCNN4* sequence and REST has been shown to regulate this sequence. Cheong and colleagues (2005) showed that this transcription factor modulates potassium channel expression in vascular smooth muscle cells (17). Therefore, alterations in REST regulation can have an important impact in the development of vascular disease by K_{Ca} channels alterations. Therefore, we hypothesized that increased REST expression during hypertension negatively modulates IK_{Ca} channel expression, contributing to reduced endothelium-dependent vasodilation in hypertension.

METHODS

Animals

Five month-old male stroke-prone spontaneously hypertensive rats (SHRSP) were obtained from the breeding colony at the Medical College of Georgia. Age-matched male Wistar-Kyoto (WKY) rats were purchased from Harlan (Indianapolis IN). Rats were maintained on a 12-hour light dark cycle, housed two per cage and allowed access to normal chow and water *ad libitum*. Systolic blood pressure (SBP) was measured in non-anesthetized animals by tail cuff

using a RTBP1001 blood pressure system (Kent Scientific Corporation, Connecticut, MA, USA). All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education.

Preparation and study of mesenteric arteries

After euthanasia, the mesentery was rapidly excised and placed in an ice-cold physiological salt solution (PSS) containing: 130 mM NaCl, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4.7H_2O$, 1.6 mM $CaCl_2.2H_2O$, and 0.026 mM EDTA. Second-order mesenteric arteries were carefully dissected and mounted as ring preparations on two stainless steel wires (≅200 mm in length with internal diameter ≅ 100 to 150 μm) in an isometric Mulvany-Halpern small-vessel myograph (Danish MyoTech, Aarhus, Denmark). One wire was attached to a force transducer and the other to a micrometer. Both dissection and mounting of the vessels were carried out in cold (4°C) PSS. The segments were adjusted to maintain a passive force of 3.5 mN. Vessels were equilibrated for 45 min in PSS at 37°C. Arterial integrity was assessed first by stimulation of vessels with KCl (120 mM) and, after washing and a new stabilization period, by contracting the segments with phenylephrine (10μM) followed by stimulation with acetylcholine (ACh; 10μM).

After being washed, the arterial rings were contracted with the thromboxane analog 9,11-dideoxy-9_α,11_α-methanoepoxyprostaglandin $F_{2\alpha}$ (U-46619, 0.3μM). Cumulative concentration-response curves to ACh were performed under control conditions and in the combined presence of indomethacin (inhibitor of cyclooxygenase, 10 μM) and N^ω-nitro-$_L$-arginine methyl ester [L-NAME, inhibitor of NO synthase, 100μM]. To study the role of K_{Ca} channels in EDHF-mediated relaxations, ACh-induced vasorelaxation was determined in the presence of L-NAME and indomethacin. This procedure rules out any potential interference of NO and prostaglandins on EDHF-mediated responses (19). The effects of IK_{Ca} inhibitors (TRAM-34, 10μM and charybdotoxin, 0.1μM), a SK_{Ca} inhibitor (UCL-1684, 0.1μM) and a BK_{Ca} inhibitor (Iberiotoxin, 0.1μM) on ACh-induced relaxations were determined. These concentrations were chosen based on previous studies from our laboratory (14).

Western blotting

Mesenteric arteries from hypertensive and control rats were isolated, cleaned from fat, dissected and frozen in liquid nitrogen. Proteins (40 μg) extracted from the mesenteric bed were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween (0.1%) for 1 hour at 24°C. Membranes were then incubated with antibodies overnight at 4°C. Antibodies were as follows: anti-potassium channel $K_{Ca}2.3$ (also known as SK3) and $K_{Ca}3.1$ (also known as IK1); (1:200, Sigma-Aldrich, MO, USA); REST/NRSF (1:500, Abcam) and β-actin (1:1000, Sigma-Aldrich, MO, USA). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results were normalized by β-actin expression and expressed as percentage of control.

Data analyses and statistics

Experimental values were calculated relative to the maximal changes from the contraction produced by U-46619 in each segment. Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies and maximum response are expressed as negative logarithm of the molar concentration of agonist producing 50% of the maximum response (pD_2) and maximum effect elicited by the agonist (E_{max}), respectively. Data are expressed as means ± SEM (n), where n is the number of experiments performed. Statistical analysis of the

concentration-response curves was performed by using two-way analysis of variance (ANOVA) for comparison between the groups. Western blot data were analyzed by one-sample t test. Values of $P<0.05$ were considered a statistically significant difference.

Drugs

L-Phenylephrine hydrochloride, ACh, L-NAME, iberiotoxin, indomethacin, sodium nitroprusside and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) were all purchased from Sigma-Aldrich. 9,11-Dideoxy-9_{α},11_{α}-methanoepoxyprostaglandin $F_{2\alpha}$ (U-46619) was purchased from Calbiochem (San Diego, CA) and 6,12,19,20,25,26-Hexahydro-5,27:13,18:21,24- trietheno-11,7-metheno-7H-dibenzo [b,n] [1,5,12,16] tetraazacyclotricosine-5,13-diium ditrifluoroacetate (UCL-1684) was from Tocris (Ellisville, MI). Indomethacin was dissolved in ethanol, and TRAM-34 and UCL-1684 were dissolved in DMSO. All other stock solutions were prepared by using PSS. Control solutions containing vehicle levels of ethanol and DMSO were also used through the experimental protocol.

RESULTS

Systolic blood pressure and body weight of the rats

At 24 weeks, SHRSP displayed higher systolic blood pressure (mmHg), in comparison with WKY rats (211±7.6, n=15 vs. 119±1.8, n=15; respectively). Body weight of SHRSP was significantly decreased (296 ±6.1g; n=15) when compared with WKY rats (345±5.2g; n=15),

Endothelium-dependent and EDHF-mediated relaxation in small-mesenteric arteries

Concentration-response curves to ACh were performed in vessels contracted with U-46619 (0.3μM) to evaluate endothelium-dependent relaxation. ACh-induced relaxation was slightly impaired in small mesenteric vessels from SHRSP vs. those from normotensive rats [E_{max} 87.7 ±4% vs. 102.2±1.4%, respectively, ($p<0.05$)], as shown at Figure 1A.

EDHF-mediated relaxation is particularly apparent when NO and prostaglandins production are blocked. Therefore, simultaneous inhibition of prostaglandins and NO production (by indomethacin; 10μM and L-NAME; 100μM, respectively) were performed in order to assess EDHF-mediated relaxation. At this condition, ACh-induced relaxation was greatly diminished in SHRSP, but not in WKY vessels [E_{max} 38.45±1.7% vs. 100.6±0.6%, respectively, ($p<0.05$), Figure 1B]. These results suggest impairment of EDHF production in vessels from hypertensive animals.

It has been shown that relaxations observed in the presence of NO-synthase and cyclooxygenase inhibitors are not necessarily attributed to EDHF-mediated responses, but may result of residual NO release (20,21). To rule out this hypothesis, ACh-concentration response curve were performed in arteries from WKY and SHRSP, incubated with L-NAME (100μM) and indomethacin (10μM), in the presence or absence of Carboxy-PTIO (100μM), a NO scavenger. Addition of Carboxy-PTIO did not result in greater inhibition of ACh-induced relaxation in WKY [E_{max} 96.9±7.5% vs 89.1±4.1% (without Carboxy_PTIO)] or SHRSP [E_{max} 42.5±5.5% vs. 38.4±4.7% (without Carboxy_PTIO)]. These results show that residual NO does not seem to contribute to the differences observed in ACh responses in small-mesenteric arteries from WKY and SHRSP rats in the presence of L-NAME and indomethacin, reinforcing that relaxation is attributed to EDHF-mediated effects.

Contribution of K_{Ca} channels to EDHF-mediated relaxation

As previously discussed, K_{Ca} channels are especially important for EDHF-mediated relaxation and hyperpolarization in resistance arteries. Accordingly, the effects of K_{Ca} channel blockade on EDHF-mediated actions were always studied on arteries treated with both indomethacin

(10μM) and L-NAME (100μM). The contribution of different subtypes of K_{Ca} channels, BK_{Ca}, SK_{Ca} and IK_{Ca} to ACh-induced relaxation was determined by using selective inhibitors. The effects of these inhibitors on pD_2 and E_{max} values for ACh are summarized at Table 1.

Iberiotoxin (0.1μM), a BK_{Ca} channel inhibitor, did not significantly change EDHF-mediated relaxation either in arteries from SHRSP or WKY rats (Figure 2), suggesting a minor contribution of BK_{Ca} channel to EDHF-mediated relaxation.

UCL-1684 (0.1μM), a SK_{Ca} channel inhibitor, greatly impaired EDHF-induced relaxation in WKY small arteries [82.8% of inhibition of E_{max}, ($p<0.05$), Figure 3A], as well as reduced the relaxation in SHRSP arteries [61.9% inhibition of E_{max}, ($p<0.05$), Figure 3B]. These data suggest that the SK_{Ca} channels mediate EDHF-induced relaxation in arteries from both normotensive and hypertensive rats.

The blockade of IK_{Ca} channel with TRAM-34 (10μM) significantly inhibited EDHF-mediated relaxation in arteries from SHRSP [80.5% inhibition of E_{max}, ($p<0.05$), Figure 4B], but not in WKY (18.9% inhibition of E_{max} – Figure 4A). Inhibition of IK_{Ca} channel with charybdotoxin (0.1μM) produced similar results. Charybdotoxin significantly inhibited EDHF-mediated relaxation in arteries from SHRSP [78.9% inhibition of E_{max}, ($p<0.05$), Figure 4D], but not in WKY (5.3% inhibition of E_{max} – Figure 4C). These findings suggest that the IKCa channels greatly contribute to EDHF-mediated relaxation in small arteries from SHRSP, but play a minor role in arteries from WKY.

Simultaneous inhibition of IK_{Ca} and SK_{Ca} completely abolished EDHF-induced relaxation in WKY [91.9% inhibition of E_{max}, ($p<0.05$), Figure 5A], and SHRSP [88% inhibition E_{max}, ($p<0.05$), Figure 5B].

Concentration-response curves to NS309, a SK_{Ca} and IK_{Ca} channels opener, were also performed. The response to NS309 was decreased in arteries from SHRSP, when compared to those in control arteries [E_{max} 77.9±3.2 vs. 103.1±3.1%, respectively, ($p<0.05$), Figure 6]. NS309-induced relaxation was abolished with concomitant incubation of UCL-1684 (0.1μM) and charybdotoxin (0.1μM) – (data not shown).

Responses to NS1619 (a BK_{Ca} channel opener) were similar in vessels from SHRSP and WKY rats (E_{max} 96.1±2.1, n=5 and 92.8±1.4, n=5; respectively) and were fully blocked by incubation with iberiotoxin (0.1μM).

Expression of K_{Ca} channel isoforms in small- mesenteric arteries

The functional studies performed in small-mesenteric arteries from normotensive and hypertensive animals indicated that EDHF-mediated relaxation has different profiles and is mediated by different subtypes of K_{Ca} channel. Analysis of protein expression of BK, SK3 (a SKCa isoform) and IK1 (also known as IK_{Ca}) channels were performed to better characterize possible differences in the expression of the K_{Ca} channels isoforms, between WKY and SHRSP arteries. SK3 isoform was chosen because this isoform is present in the vascular endothelium (22).

No differences were found in BK protein levels between the groups (Figure 7B). IK1 protein expression was increased in mesenteric arteries from SHRSP, when compared to WKY arteries (Figure 7B). On the other hand, protein expression levels of SK3 in mesenteric arteries from SHRSP were reduced compared with those in WKY values (Figure 7B). Although IK_{Ca} channel was increased in arteries of SHRSP, it seems that this up-regulation is not sufficient to restore impaired EDHF-mediated relaxation in SHRSP.

Modulation of IK_{Ca} expression by REST

Because REST was shown to negatively regulate IK_{Ca} channel expression (17), we evaluated protein levels for this transcription factor. Anti-REST antibody resulted in a predicted band size of 121kDa and an additional band was observed at 50kDa position. Mesenteric arteries from SHRSP displayed reduced REST expression, when compared with arteries from WKY (Figure 8A–B).

We took advantage from a pharmacological approach to inhibit IK_{Ca} channel, and determined whether blockade of these channels could lead to changes in REST or IK1 expression. After 24 hours of incubation with TRAM-34 (10μM) or vehicle (DMSO), proteins were extracted from mesenteric vessels from normotensive and hypertensive rats and expression of both IK1 and REST was determined. REST expression was drastically reduced after TRAM-34 incubation, both in arteries from WKY and SHRSP (Figure 8A–B). The difference in IK1 expression between arteries from normotensive and hypertensive animals was abolished after 24 hours of IK_{Ca} channel blockade. In order to control for changes in protein expression due to the 24 hour-incubation period, we compared IK1 and REST expression in freshly harvested mesenteric arteries and in arteries incubated for 24 hours with DMSO. No significant differences were found in IK1 and REST expression between freshly isolated and incubated arteries from control or hypertensive animals, indicating that changes in protein expression between WKY and SHRSP vessels are not due to the incubation procedure. Collectively, these results demonstrate that modifications in the activity of IK_{Ca} channel resulted in changes of REST and IK_{Ca} channel expression, reinforcing that REST negatively modulates IK_{Ca} channel in vascular cells.

DISCUSSION

The endothelium is a major regulator of vascular tone, releasing vasoactive substances such as EDHF, NO, cyclooxygenase metabolites, endothelin-1 and other endothelium-derived constrictor factors (23). In most models of experimental hypertension, the endothelium-dependent relaxation is impaired. However, this endothelial dysfunction presents different characteristics depending on the experimental model studied (24). Konishi and Su first showed that endothelium-dependent relaxation is impaired in arteries from SHR (23). Similar results have been reported in the mesenteric artery from SHRSP (25,26). Although ACh-induced relaxation was slightly impaired in small mesenteric vessels from SHRSP animals, in the present study we observed pronounced impairment in EDHF-mediated responses.

The IK_{Ca} and SK_{Ca} channels occur in abundance in endothelial cells and their activation results in EDHF-like hyperpolarization of these cells (10–15,27,28). The resulting endothelial hyperpolarization spreads via myoendothelial gap junctions to result in the EDHF-induced hyperpolarization and relaxation of the smooth muscle (2). EDHFs-mediated responses exhibit exquisite sensitivity to the combination of charybdotoxin and apamin (29)? showing the importance of both SK_{Ca} and IK_{Ca}, respectively, to EDHFs-mediated relaxation. Accordingly, experiments conducted in a transgenic mouse ($SK3^{T/T}$), in which SK3 expression levels are suppressed with dietary doxycycline, demonstrated that decreased SK3 expression results in a pronounced and reversible elevation of blood pressure, around 30mmHg (30). In addition, K (Ca)$3.1^{(-/-)}$ [or $IK1^{(-/-)}$] mice display reduced endothelial and smooth muscle hyperpolarization in response to acetylcholine and significant increase in arterial blood pressure, around 30 mmHg (31). Furthermore, $IK1^{(-/-)}/SK^{(T/T)}$ mice treated with dietary doxycycline, which results in combined IK1/SK3 deficiency, display impaired acetylcholine-induced EDHF-mediated relaxation dilation in conduit arteries and in resistance arteries as well as elevated arterial blood pressure (32). These results indicate that the endothelial SK_{Ca} and IK_{Ca} are fundamental regulators of blood pressure and it seems likely that EDHF, through vascular dependent mechanisms, can influence blood pressure levels.

We have examined the relative contributions of SK_{Ca}, IK_{Ca} and BK_{Ca} to EDHF-mediated response, in SHRSP small mesenteric arteries. Our results demonstrate that the mechanisms underlying EDHF-mediated responses of small mesenteric arteries from SHRSP and WKY rats are distinct. EDHF-mediated relaxation in small mesenteric arteries from WKY rats was prevented by the blockade of SK_{Ca} channels, with UCL-1684.

In SHRSP, although SK_{Ca} contributes to EDHF-meditate relaxation, this was mainly mediated by IK_{Ca} channels. These results are in agreement with a previous report showing that EDHF-mediated relaxation is impaired in SHRSP and that K_{Ca} channels have a differential contribution in hypertensive and normotensive animals (26). In addition, the response to NS309 was decreased in arteries from SHRSP, further reinforcing that SK_{Ca} and IK_{Ca} function is impaired in mesenteric arteries from SHRSP. Unfortunately, no selective agonist for these channels, SK_{Ca} and IK_{Ca}, are available and although NS309-induced relaxation seems to be abolished in conditions where endothelium is removed (33,34), it is important to consider that NS309 has been shown also to inhibit L-type voltage-dependent Ca^{2+}-channel (35).

In agreement with our functional data, a differential expression of SK_{Ca} and IK_{Ca} channels was observed between the groups. Small arteries from SHRSP, when compared with WKY arteries, showed decreased levels of SK_{Ca} channel, whereas IK_{Ca} channel expression was increased.

Induction of IK_{Ca} plays an important role in the vasculature. The gene encoding IK_{Ca}, KCNN4, contains a functional REST binding site that is repressed by REST (17). REST has been considered a dynamic factor in smooth muscle cells. Cheong et al (2006) showed that REST gene expression declines when the cells proliferate in culture. Furthermore, they showed that loss of REST is a switch enabling KCNN4 expression (16). Indeed, some reports speculate that REST may be a common factor helping to suppress vascular diseases (16). Here, we showed for the first time that REST expression is closely associated with IK_{Ca} expression in arteries from hypertensive animals

Given that IK_{Ca} channels are important modulators of EDHF-induced relaxation in small mesenteric vessels from SHRSP, we have hypothesized that REST modulation of IK_{Ca} channel expression is decreased in hypertension, as a compensatory mechanism in response to impaired EDHF-mediated relaxation. Our main finding was that REST is down-regulated whereas IK_{Ca} channels are over expressed in arteries from SHRSP, when compared with WKY. Furthermore, we showed that the blockade of IK_{Ca} channels, by using TRAM-34 for 24 hours, induces vascular down-regulation of REST expression, and also abolishes the differences in IK_{Ca} channels expression between arteries from SHRSP and WKY. It is possible that continued inhibition of IK_{Ca} channels with TRAM-34 resulted in a compensatory tentative of the vascular tissue to restore this channel function. We speculate that this phenomenon, decreased REST expression and consequently, augmented IK_{Ca} channels expression, is a compensatory mechanism in the vasculature, to counter balance impaired EDHF-mediated relaxation.

It has been previously shown that TRAM-34 treatment modulates IK1 and REST expression. Tharp and colleagues (2008) demonstrated that coronary balloon injury robustly increased IK1 and decreased REST expression 2 hours postangioplasty. Interestingly, they showed that TRAM-34 delivered via balloon catheter blocked not only IK1 upregulation, but also the suppression of REST, which is in close opposition to the results presented here. However, one needs to consider that a different experimental model (an *in vivo* model for coronary injury), different TRAM-34 concentration and treatment duration may partially explain the differential results. In addition, our results point to the important observation that IK1 activity is able to modulate REST expression. REST expression probably is modulated by various factors, but the lack of information on REST expression regulation precludes further speculations.

Although K_{Ca} channels seem to play a major role in EDHFs-mediated relaxation (29), endothelial cell hyperpolarization can also result from endothelial to vascular smooth muscle cells interactions through myo-endothelial gap junctions (36,37), as well as activation of inwardly K^+ channels and Na^+-K^+-ATPase (38,39). Other important point is that alterations in the vessel structure and vascular remodeliling, as a consequence of prolonged hypertension, could contribute to impaired EDHF-response in small-mesenteric arteries from SHRSP. It seems likely that increases in the number of SMC in the media may have an impact on EDHF ability to hyperpolarize the smooth muscle (40). These are important points that also need to be further evaluated in order to completely understand EDHF-dependent mechanisms involved in vascular relaxation during physiological and pathophysiological conditions.

In conclusion, we have shown that small-mesenteric arteries from SHRSP display significant impairment in EDHF-mediated relaxation. In addition, SK_{Ca} channels importantly contribute to this phenomenon both in normotensive and hypertensive animals, but IK_{Ca} channel plays a greater and important role in the EDHF-mediated relaxation in arteries from SHRSP. Protein levels of SK_{Ca} and IK_{Ca} channels were altered in arteries from hypertensive and REST seems to be a negative modulatory mechanism, controlling the augmented levels of IK_{Ca} channels in the SHRSP vasculature.

LIST OF ABBREVIATIONS

ACh, acetylcholine
BK_{Ca}, large-conductance calcium-activated potassium channels
EDHF, endothelium-derived hyperpolarizing factor
EET, epoxyeicosatrienoic acid
E_{max}, maximum effect elicited by the agonist
IK_{Ca}, intermediate calcium-activated potassium channels
K_{Ca}, calcium-activated potassium channels
K_{IR}, inward rectifier K^+ channels
L-NAME, N^{ω}-nitro-$_L$-arginine methyl ester
NO, nitric oxide
pD_2, negative logarithm of the molar concentration of agonist producing 50% of the maximum response
PGI_2, prostacyclin
PSS, physiological salt solution
REST, repressor element 1-silencing transcription factor
SHRSP, stroke-prone spontaneously hypertensive rats
SK_{Ca}, small calcium-activated potassium channels
U-46619, 9,11-dideoxy-9α, 11α-methanoepoxyprostaglandin $F_{2\alpha}$
WKY, Wistar Kyoto

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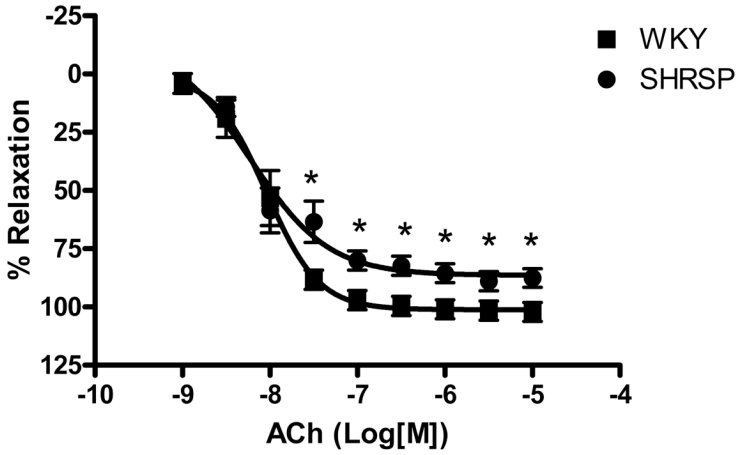

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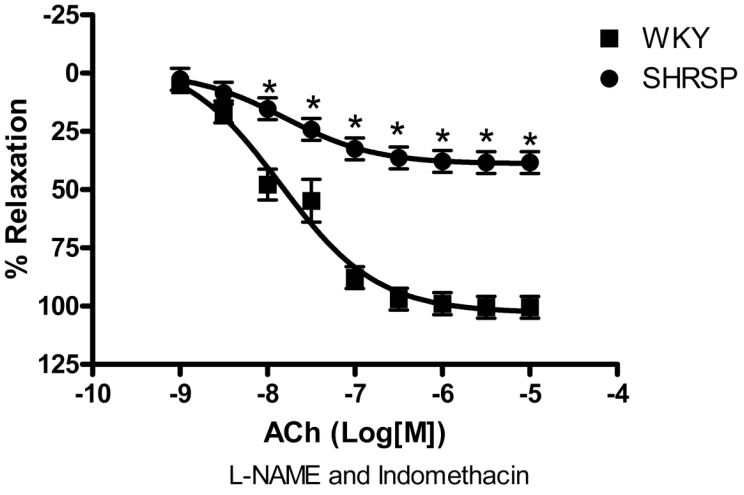


Figure 1. EDHF-mediated relaxation is impaired in SHRSP small mesenteric arteries

Concentration-response curves to ACh (1nM to 100μM) in U-46619-contracted (0.3μM) second-order mesenteric arteries from (■) WKY and (●) SHRSP, in the absence (A) or in the presence (B) of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue, which was taken as 100%. Results are presented as mean ± SEM of n=8 in each experimental group. *, $P<0.05$ compared with WKY.

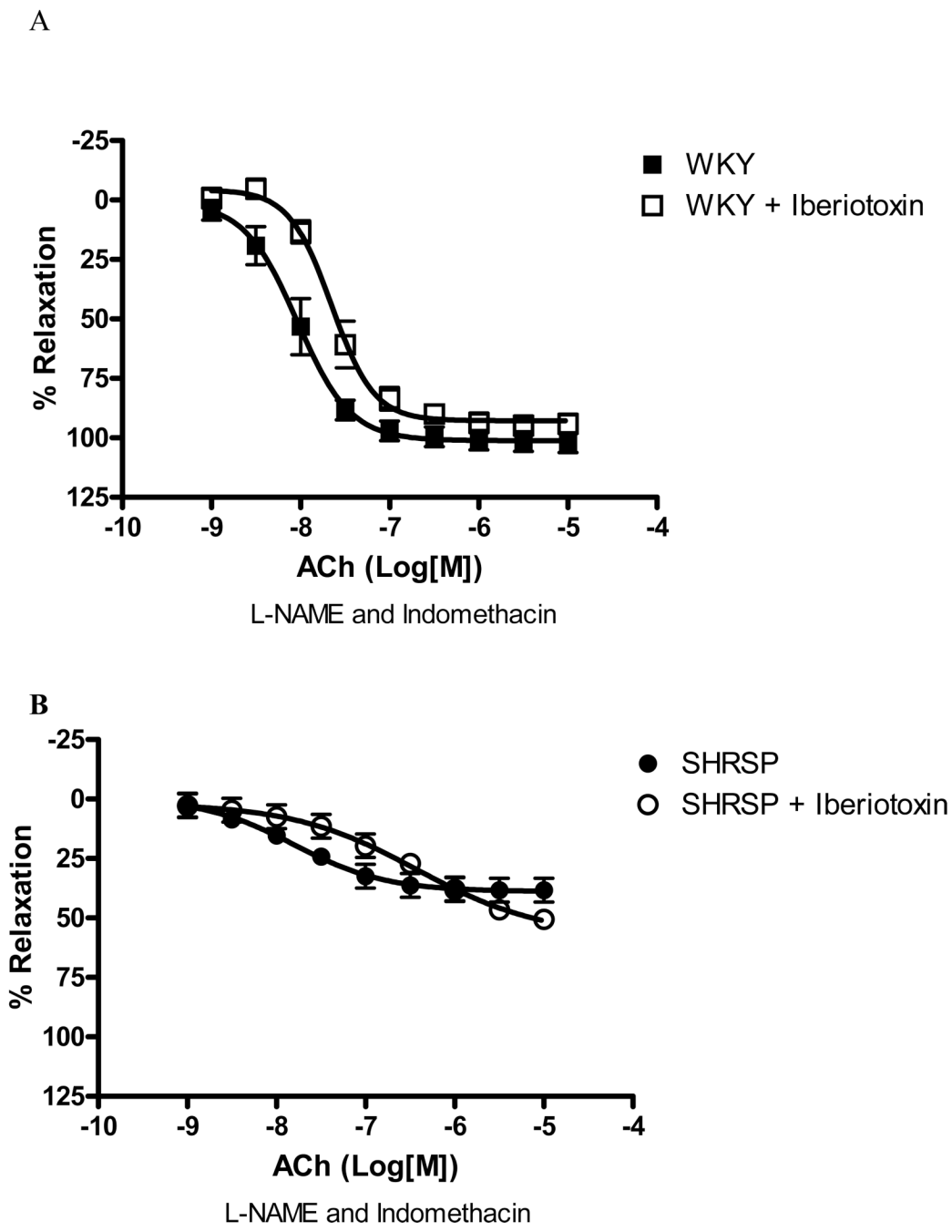
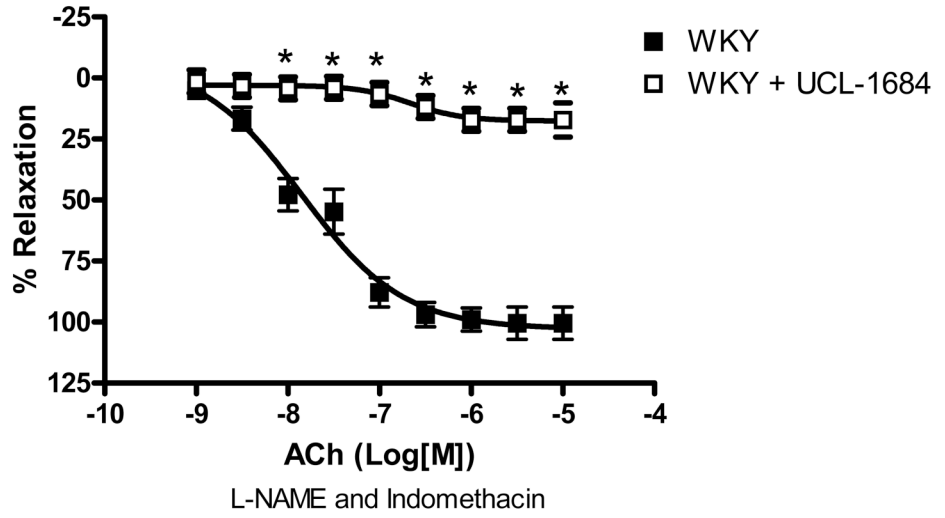


Figure 2. Inhibition of BK_{Ca} does not affect EDHF-induced relaxation in SHRSP or WKY small mesenteric arteries

Concentration-response curves to ACh in U-46619-contracted (0.3μM) second-order mesenteric arteries from **(A)** WKY and **(B)** SHRSP in the absence (closed symbols) or presence (open symbols) of iberiotoxin (0.1μM). All the curves were performed in the presence of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue. Results are presented as mean ± SEM of n=5 in each experimental group.

3A



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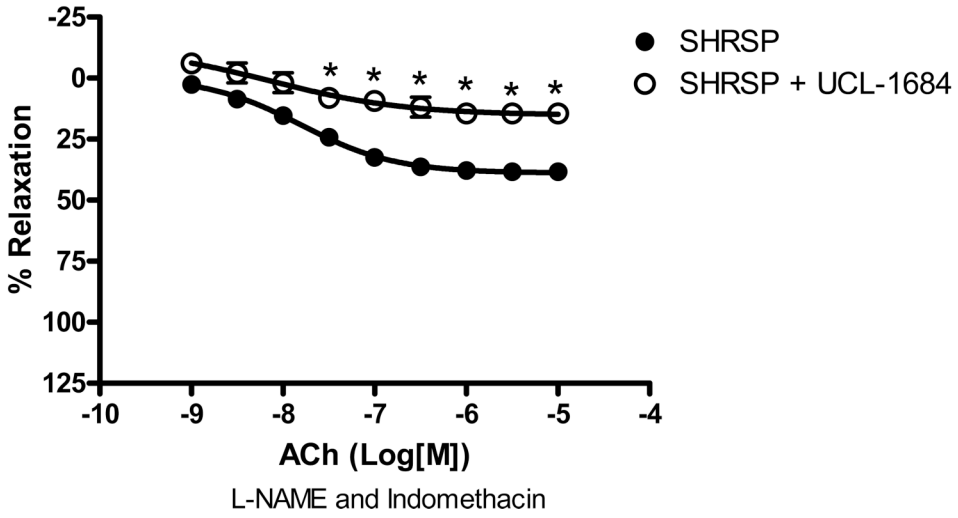

Figure 3. SK_{Ca} inhibition affects EDHF-induced relaxation in SHRSP and WKY small mesenteric arteries

Concentration-response curves to ACh in U-46619-contracted (0.3μM) second-order mesenteric arteries from **((A)** WKY and **(B)** SHRSP in the absence (closed symbols) or presence (open symbols) of UCL-1684 (0.1μM). All the curves were performed in the presence of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue. Results are presented as mean ± SEM of n=5 in each experimental group. *, $P<0.05$ compared with respective control (vehicle).

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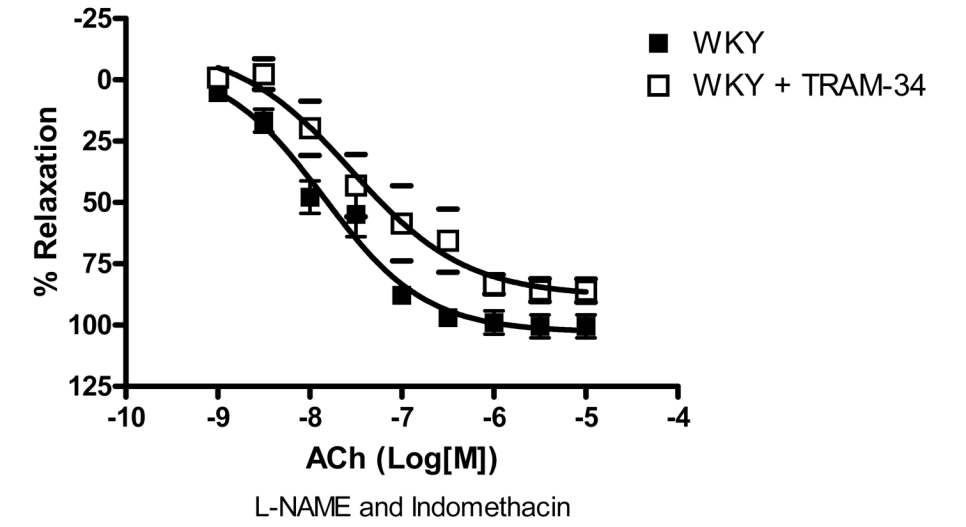

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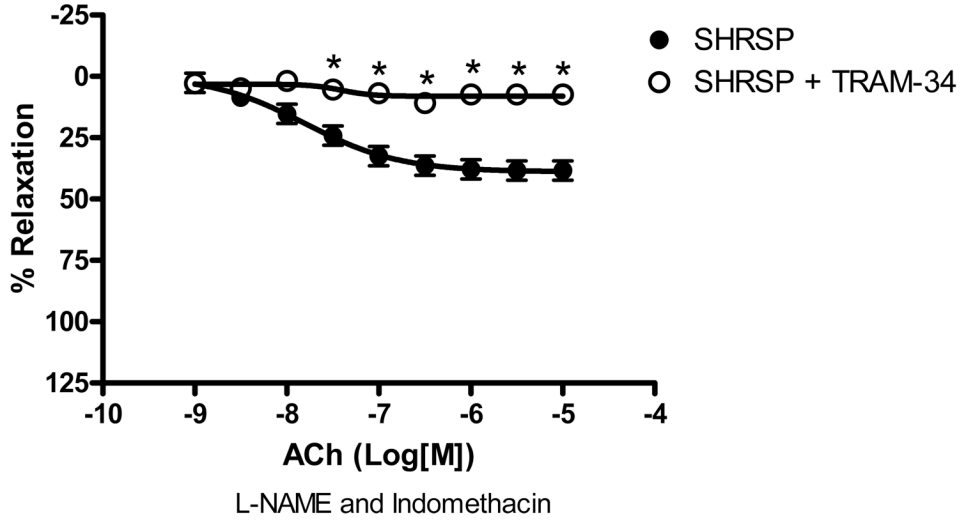

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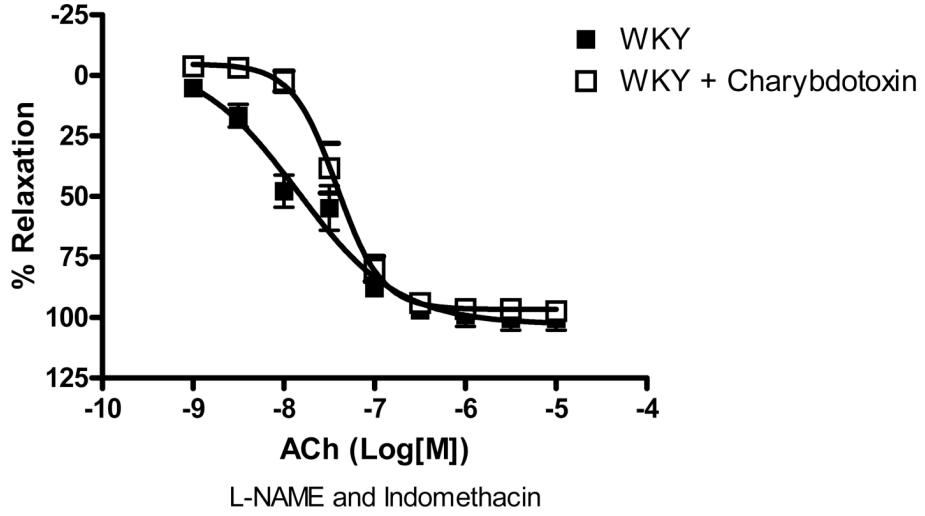

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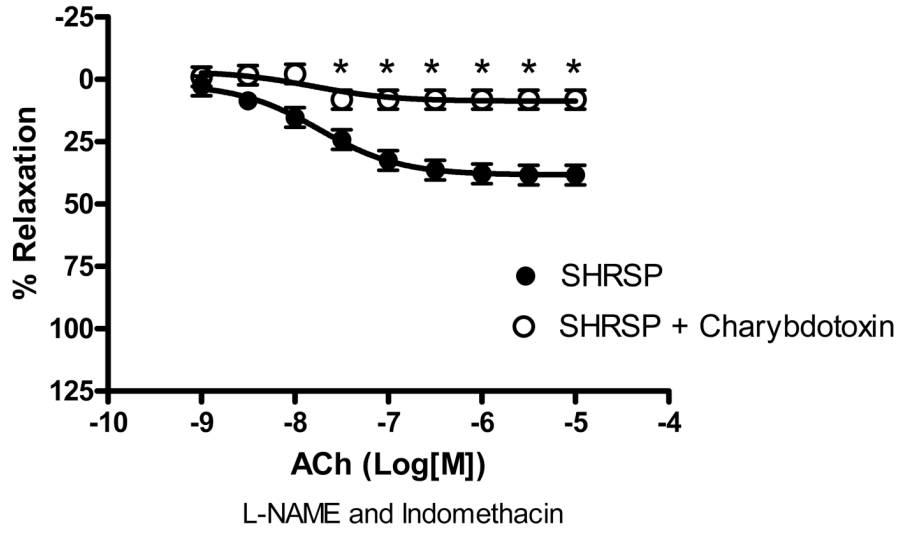

Figure 4. IK_{Ca} inhibition affects EDHF-induced relaxation in SHRSP, but not WKY small mesenteric arteries

Concentration-response curves to ACh in U-46619-contracted (0.3μM) second-order mesenteric arteries from **(A, C)** WKY and **(B, D)** SHRSP in the absence (closed symbols) or presence (open symbols) of (A, B) TRAM-34 (10μM) or (**C, D**) charybdotoxin (0.1μM). All the curves were performed in the presence of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue. Results are presented as mean ± SEM of n=5 in each experimental group. *, $P<0.05$ compared with respective control (vehicle).

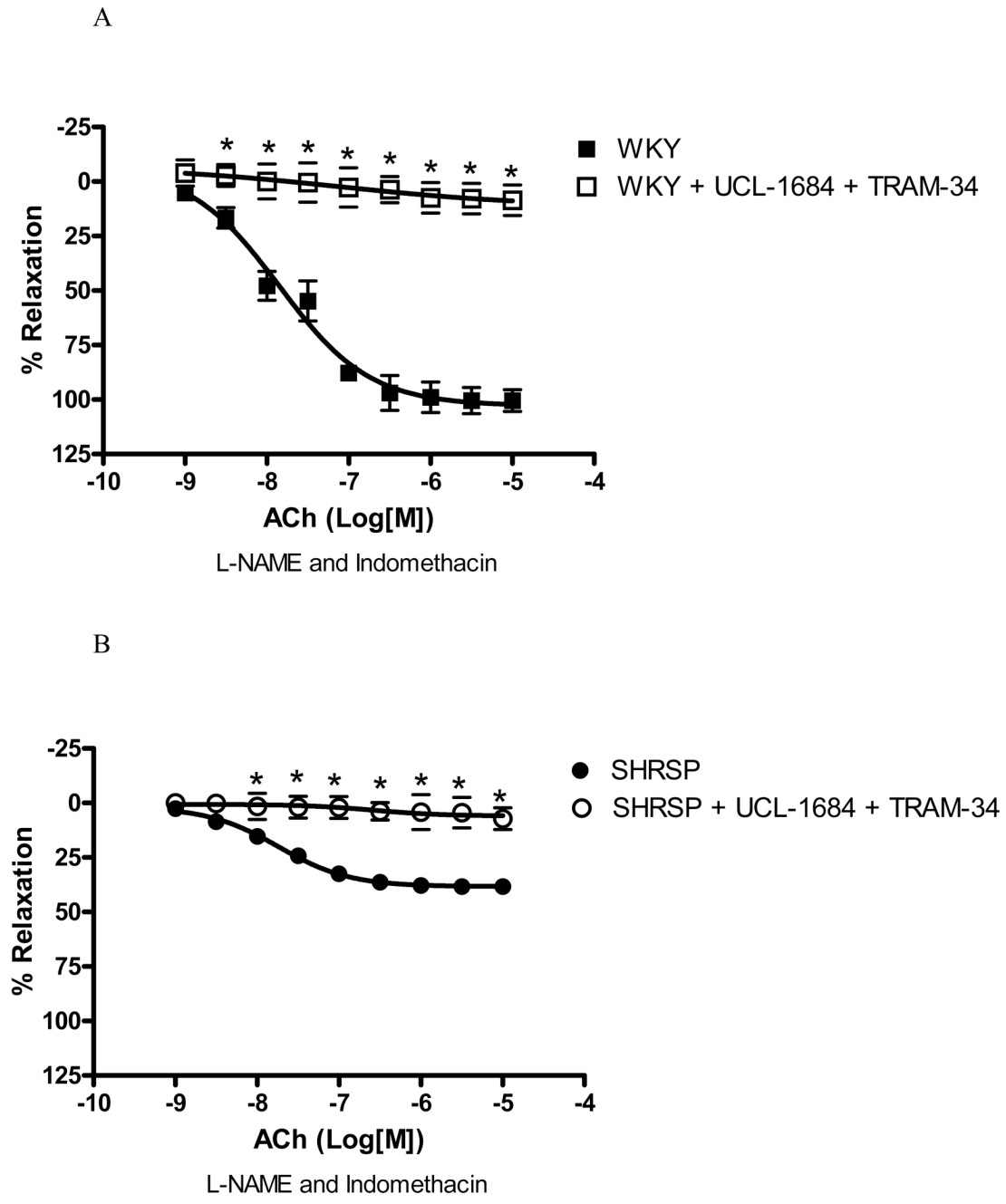

Figure 5. EDHF-induced relaxation is completely abolished after simultaneous inhibition of IK_{Ca} and SK_{Ca}

Concentration-response curves to ACh in U-46619-contracted (0.3μM) second-order mesenteric arteries from **(A)** WKY and **(B)** SHRSP in the absence (closed symbols) or presence (open symbols) of UCL-1684 (0.1μM) and TRAM-34 (10μM). All the curves were performed in the presence of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue. Results are presented as mean ± SEM of n=5 in each experimental group. *, $P<0.05$ compared with respective control (vehicle).

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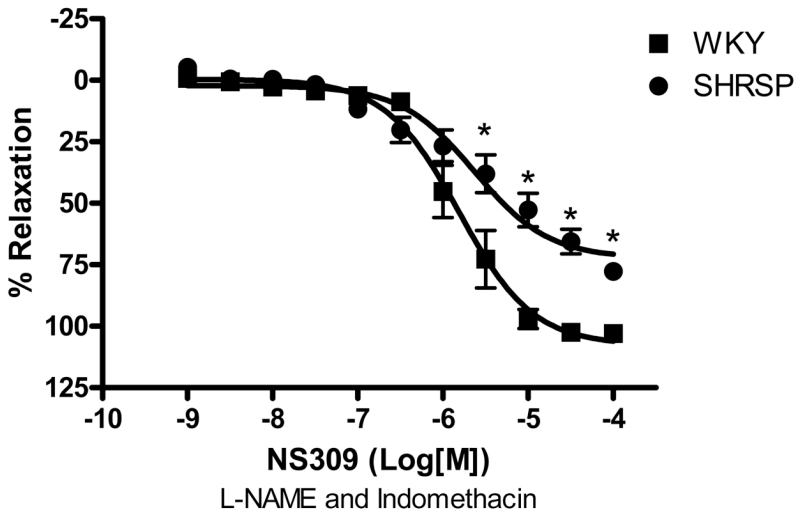

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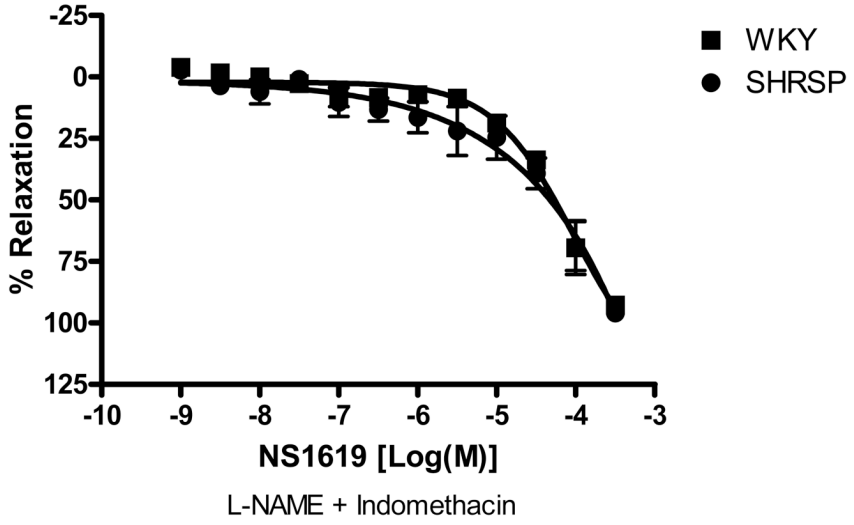

Figure 6. Relaxation-response to IK_{Ca} and SK_{Ca}, but not BK_{Ca}, channels opener is impaired in SHRSP small mesenteric arteries

Concentration-response curves to (**A**) NS309 (1nM to 100μM) and (**B**) NS1619 in U-46619-contracted (0.3μM) second-order mesenteric arteries from WKY (■) and SHRSP (●). All the curves were performed in the presence of L-NAME (100μM) and indomethacin (10μM). Experimental values of the relaxation induced by NS-309 were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue. Results are presented as mean ± SEM of n=5 in each experimental group. * $P<0.05$ vs. WKY.

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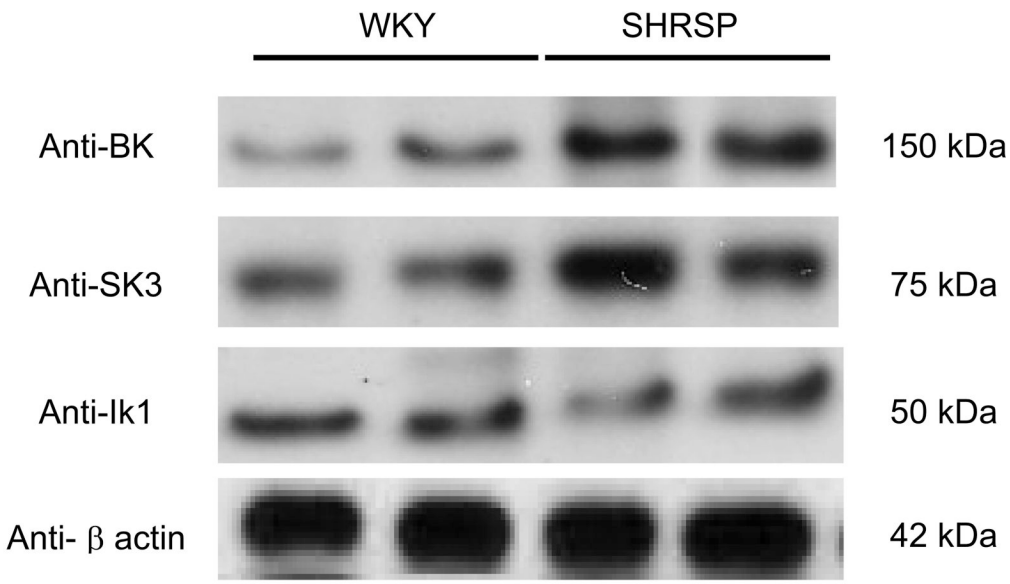

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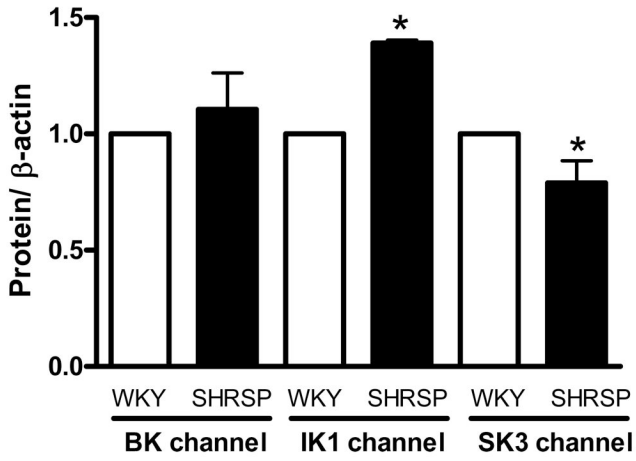

Figure 7. IK_{Ca} and SK_{Ca} channel expression is altered in mesenteric arteries from SHRSP

On the top, **(A)** representative immunoblots for BK, SK3, IK1 and β-actin expression in WKY and SHRSP mesenteric arteries. On the bottom, corresponding bar graphs demonstrating the **(B)** BK, **(C)** IK1 and **(D)** SK3 channel expression. Values, expressed in arbitrary units, are mean ± SEM of n=5 experiments and were normalized by β-actin protein expression. * P<0.05 vs. WKY.

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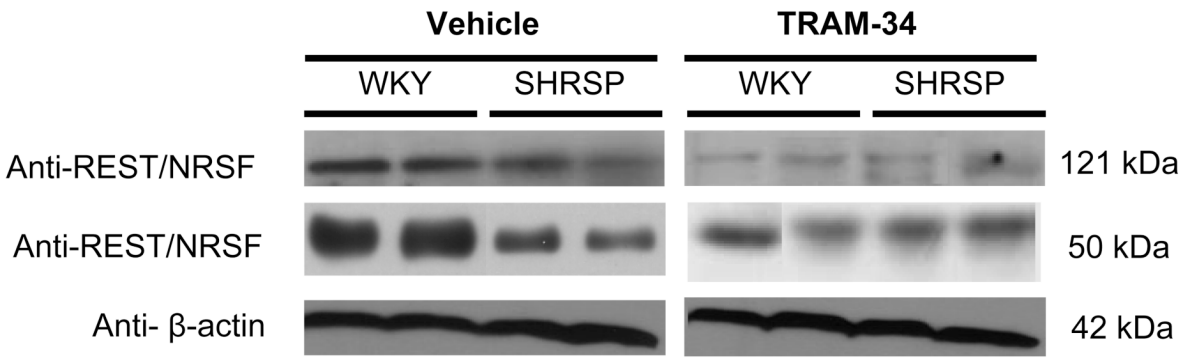

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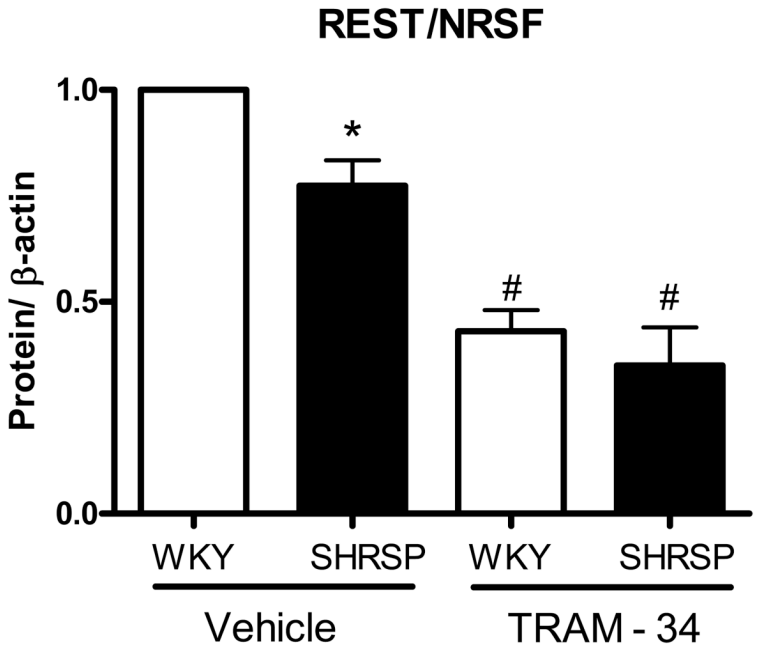

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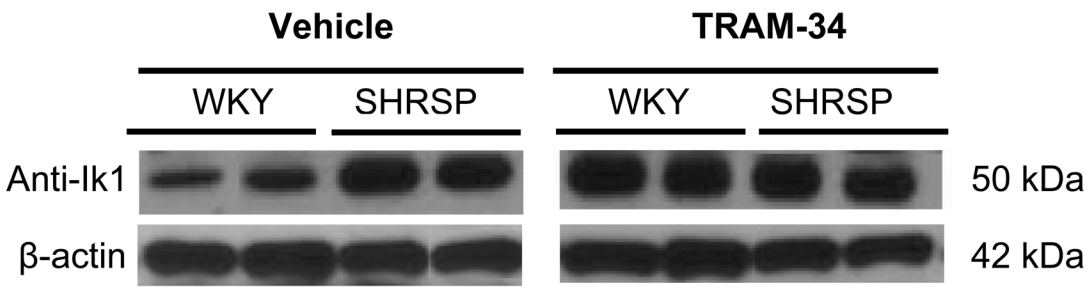

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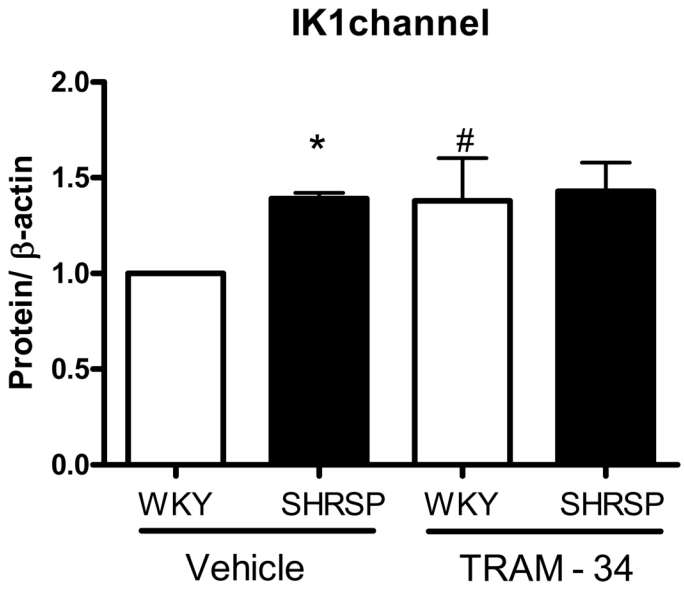

Figure 8. REST expression in mesenteric arteries from SHRSP and WKY

(A) Representative immunoblots for REST, and β-actin expression in WKY and SHRSP mesenteric arteries, after vehicle or TRAM-34 (10μM, 24 hours) incubation and **(B)** corresponding bar graphs demonstrating REST expression. **(C)** Representative immunoblots for IK1 and β-actin expression in WKY and SHRSP mesenteric arteries, after vehicle or TRAM-34 (10μM, 24 hours) incubation and **(D)** corresponding bar graphs demonstrating IK1 expression. Values, expressed in arbitrary units, are mean ± SEM of n=5 experiments and were normalized by β-actin protein expression. * P<0.05 vs. WKY, # P<0.05 vs. vehicle SHRSP.

Table 1

pD_2 and E_{max} values for ACh-induced dilation in arteries from WKY and SHRSP

pD_2 values are −log EC_{50} and Emax values represent the percentage of relaxation of U-46419 Experimental values of the relaxation induced by ACh were calculated relative to the maximal changes from the contraction produced by U-46619 in each tissue, which was taken as 100%. Results are presented as mean ± SEM of n=8 in each experimental group.

	WKY		SHRSP	
	pD_2	E_{max}	pD_2	E_{max}
Vehicle	8.0±0.01	102.2±1.4	8.2±0.11*	87.7±5.8*
L-NAME + Indomethacin	7.8±0.08*	100.6±0.6	7.8±0.22	38.4±1.6*†
Iberiotoxin L-NAME + Indomethacin	7.6±0.01	94.2±1.8	6.4±0.02*†	50.7±3.6*‡
UCL-1684 L-NAME + Indomethacin	6.7±0.03‡	17.3±2.4‡	8.1±0.15*‡	14.6±2.4‡
TRAM-34 L-NAME + Indomethacin	7.5±0.07	86.0±2.7‡	7.4±0.12*‡	7.5±0.5*‡
Charybdotoxin L-NAME + Indomethacin	7.4±0.04	97.4±0.9‡	7.8±0.31*‡	7.4±0.01‡
UCL-1684 + TRAM-34 L-NAME + Indomethacin	7.1±0.02‡	8.6±1.5‡	6.6±0.15*‡	7.3±1.1‡

*P<0.05 vs. WKY

†P<0.05 vs. respective vehicle-treated

‡P<0.05 vs. respective L-NAME and indomethacin-treated.