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RESEARCH

Ang II-induced contraction is impaired in the aortas of renovascular hypertensive animal model

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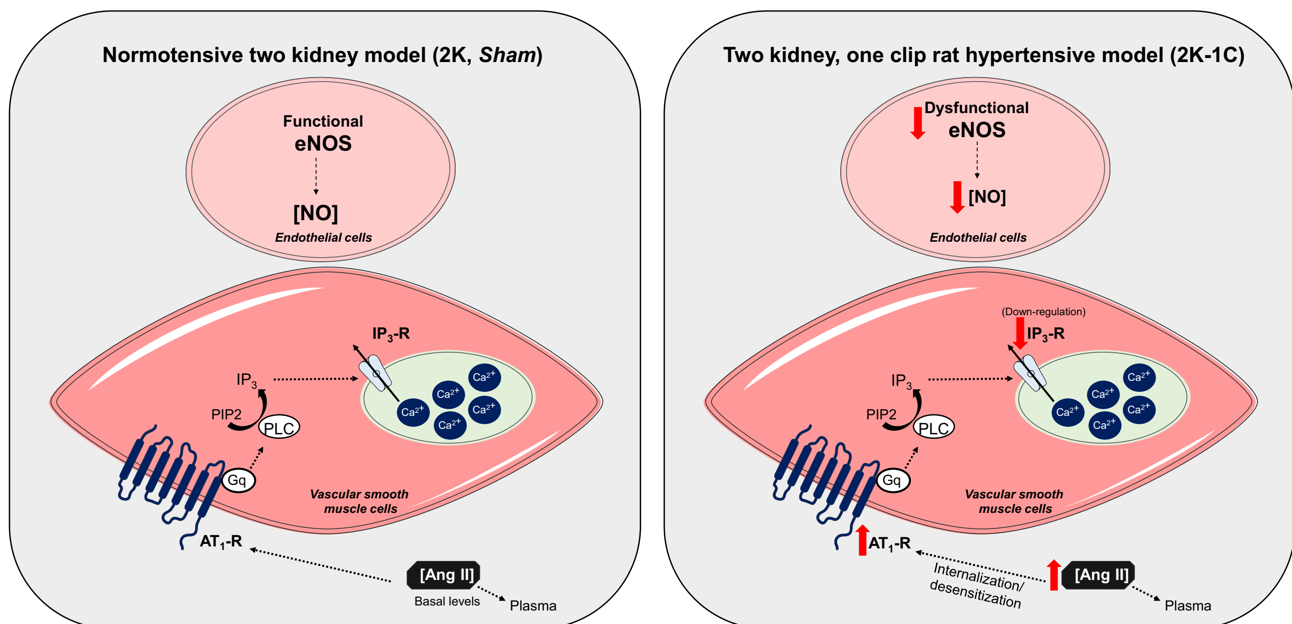
Graphical abstract



Abstract

Renin–angiotensin system plays a critical role in blood pressure control, and the abnormal activation of the AT_1 receptor contributes to the development of renovascular hypertension. This study aimed to evaluate the underlying cellular signaling for AT_1 receptor activation by Ang II and to compare this mechanism between aortas from 2K-1C and 2K rats. Effects of antagonists and inhibitors were investigated on Ang II-induced contractions in denuded or

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intact-endothelium aortas. The AT_1 receptor antagonist abolished Ang II-induced contraction in 2K-1C and 2K rat aortas, while AT_2 and Mas receptors antagonists had no effect. Endothelial nitric oxide synthase inhibition increased the maximal effect (Emax) of Ang II in 2K, which was not changed in 2K-1C aortas. It was associated with lower eNOS mRNA levels in 2K-1C. Endothelium removal increased the Emax of Ang II in 2K-1C and mainly in 2K rat aortas. Nox and COX inhibition did not alter Ang II-induced contraction in 2K and 2K-1C rat aortas. However, AT_1 expression was higher in 2K-1C compared to 2K rat aortic rings, whereas expression of phosphorylated (active) IP_3 receptors was lower in 2K-1C than in 2K rats. These results demonstrate that endothelium removal impairs Ang II-stimulated contraction in the aorta of 2K-1C rats, which is associated with the reduction of IP_3 receptor phosphorylation and activation. In addition, eNOS plays a critical role in Ang II-induced contraction in 2K rat aortas. It is possible that the high Ang II plasma levels could desensitize AT_1 receptor in 2K-1C rats, leading to impaired IP_3 receptors activation.

Keywords: angiotensin II; AT1 receptor; eNOS; IP3 receptor phosphorylation; renovascular hypertension

Introduction

Hypertension induces changes in the cardiovascular system, particularly in blood vessels, causing endothelial dysfunction (1). Consequently, a hypercontractile profile arises in vascular smooth muscle cells (VSMC) of renal hypertensive rats (2K-1C, two-kidney, one-clip model) (2).

Angiotensin II (Ang II) is a systemic peptide hormone produced by the renin–angiotensin–aldosterone system (RAAS), which participates in long-term blood pressure regulation (3). The effectiveness of RAAS blockers in reducing blood pressure demonstrates the close relationship between Ang II and hypertension (4).

Ang II produces its effects on blood vessels by binding to type 1 (AT_1) (5) and type 2 (AT_2) receptors (6). AT_1 receptors are essentially expressed in VSMC and mediate the physiological vasoconstrictor effects of Ang II (7). Meanwhile, AT_2 receptors are detected in vasculature, and their vasodilator effects involve different pathways (8). Under the action of angiotensin-converting enzyme 2 (ACE2), Ang II is converted to Ang-(1–7) (9), which interacts with Mas receptors and promotes vasodilation. Moreover, Ang-(1–7) can bind to AT_1 receptors, thus inhibiting the actions of Ang II (10).

The aforementioned receptors are linked to heterotrimeric G-proteins coupled receptors (GPCRs) like Gq/11, Gi, G_{12}, and G_{13}. AT_1 receptor activation elicits VSMC contraction by activation of phospholipase C beta (PLC-β) and formation of the second messenger inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, which is followed by calcium release from the sarcoplasmic reticulum by IP_3 receptors (11). Likewise, activated AT_1 receptors are linked to reactive oxygen species (ROS) production by NADPH oxidase (Nox) (12). In addition, the RAAS can be activated by reduced renal blood flow, which increases renin release and generates Ang II (13). Therefore, the renovascular hypertension model (14) is suitable for mimicking continuous physiological RAAS activation upon increments in Ang II levels.

Our lab and other research groups have shown worsened endothelial function (15, 16, 17), increased ROS production (18, 19), and cardiac and VSMC hypertrophy (20, 21) in the renovascular hypertension model. Moreover, Ang II stimulation can desensitize AT_1 receptors in cell culture within a short time (22), which is a typical phenomenon of GPCRs (23).

Because the RAAS is extensively activated in 2K-1C rats, we wondered whether the mechanisms of Ang II-induced contraction could be altered in blood vessels. We hypothesized that the Ang II-induced contractile response would be lower in 2K-1C rats due to constant AT_1 receptor activation by Ang II. Therefore, in this study, we aimed to evaluate the direct vasocontractile property of Ang II in 2K-1C rat aorta and the underlying cellular mechanisms involved in the possible alterations.

Methods

Animals

Experiments were performed on adult male Wistar and Wistar-Hannover rats (180–200 g) obtained from the Animal Facility on the Campus of the University of São Paulo in Ribeirão Preto. The rats were housed at controlled temperature (23.0 ± 2ºC), exposed to a daily 12 h light:12 h darkness cycle, and provided with food and water *ad libitum*. All experimental protocols complied with the guidelines of the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (License CEUA 17.1.617.60.0).

Induction of 2K-1C renovascular hypertension

The rats were anesthetized with tribromoethanol (2.5 mg kg^{-1}, i.p.). After midline laparotomy, a silver clip with an internal diameter of 0.20 mm was placed around the left renal artery of some rats; this group was named as 2K-1C hypertensive rats. Normotensive two-kidney

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(2K, Sham) rats were submitted to laparotomy only. Before and after surgery (laparotomy or clip implantation), all the rats had their blood pressure measured using the indirect tail-cuff method. For this study, 2K rats were included with systolic blood pressure ≤120 mm Hg and 2K-1C rats with systolic blood pressure ≥ 160 mm Hg. Some rats had their blood pressure measured weekly in order to demonstrate the development of renovascular hypertension, as shown in Fig. 1A.

Preparation of aortas and vascular reactivity experiments

Male Wistar and Wistar-Hannover rats were killed by decapitation under isoflurane anesthesia 6 weeks after surgery. The thoracic aorta was dissected of connective tissue, cut into 4-mm long rings, which were placed between two stainless-steel stirrups to measure tension in an isometric force transducer in presence of Krebs solution (pH 7.4, 37°C, 95% O_2 and 5% CO_2). The aortic rings were submitted to a basal tension of 1.5 g during 60 min for stabilization. Endothelial integrity was assessed by relaxation elicited by acetylcholine (ACh, 1 µmol/L) in the presence of contractile tone induced by phenylephrine (0.1 µmol/L). The endothelium was considered functional when relaxation was greater than 80% in 2K aortic rings and greater than 60% in 2K-1C aortic rings, thus being named as aortic rings with intact endothelium (E+). To investigate the role that Ang II plays in the smooth muscle layer, some aortic rings had their endothelium mechanically removed by rubbing the lumen to provide endothelium-free or denuded (E−) aortic rings with no degree of relaxation to ACh.

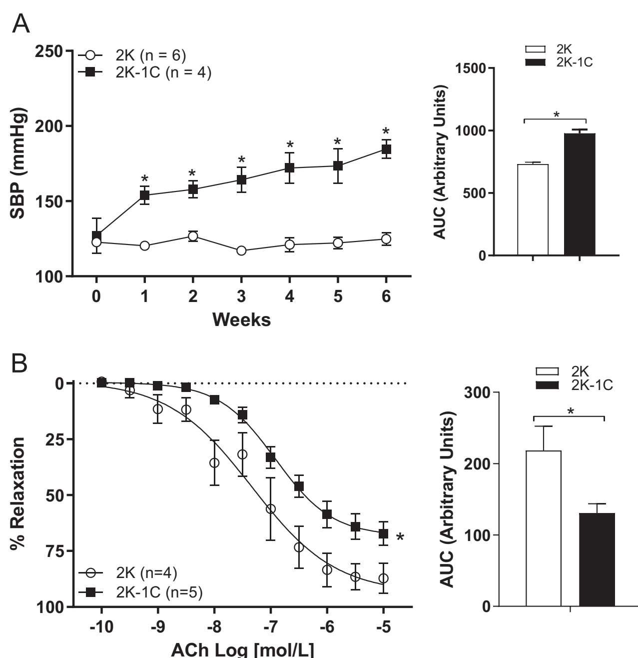

Figure 1

Systolic blood pressure (SBP, mm Hg) measurements in normotensive (2K) and hypertensive (2K-1C) rats, and ACh-induced relaxation. (A) Each point represents the mean ± S.E.M. of measurements carried out for 6 weeks after induction of renovascular hypertension (2K-1C) or Sham surgery (2K) (n = 4–6). *Difference in relation to 2K (P < 0.05). Student's t-test. (B) Concentration–effect curves were constructed in isolated aorta of 2K (n = 4) and 2K-1C (n = 5) rats pre-contracted with phenylephrine. *Difference in the maximum effect with P < 0.05. Student's t-test. In addition, the results of SBP and concentration–effect responses are expressed as area under the curve (AUC, in arbitrary units).

Next, concentration–effect curves to Ang II (0.1 nmol/L–0.1 µmol/L) were constructed for E+ and E− aortic rings. Some E+ aortic rings were incubated for 30 min in the absence (control) or in the presence of AT1 receptor antagonist (losartan, 1 µmol/L), AT2 receptor antagonist (PD123319, 1 µmol/L), Mas receptor antagonist (A779, 10 µmol/L), non-selective NOS inhibitor (L-NAME, 100 µmol/L), non-selective COX inhibitor (indomethacin, 10 µmol/L), or Nox inhibitor (apocynin, 100 µmol/L).

Real-time polymerase chain reaction

Total RNA was extracted from the E+ aortic ring samples using the SV total RNA isolation system (Promega Corporation, Madison, WI, USA); the manufacturer's instructions were followed. cDNA was synthesized from 10 µg of total RNA using the high capacity cDNA reverse transcription and stored at −20°C until use. Gene expression analysis of eNOS was performed by qRT-PCR, using Real-Time PCR instrument and kit (Taqman, Applied Biosystems, Waltham, MA, USA). All the primers showed efficiency higher than 90%. The results were analyzed as 2−(ΔΔCt) method. Specific sequences of the eNOS (forward: AGCATGAGGCCTTGGTATTG; reverse: CCCGACATTTCCATCAGC; Rn02132634_s1) and GAPDH (forward: TGACTCTACCCACGGCAAGTT; reverse TGATGGGTTTCCCGTTGATGA; Rn01775763_g1) primers.

Western blot

To determine the expression of AT1 and AT2 receptors, E+ aortas cleaned of blood and fat tissue, were used. To determine phosphorylated IP_3 (phospho-IP_3) receptors, total IP_3 receptors, and myosin light chain (MLC), E-aortic rings were employed. Each sample was homogenized in protein ice-cold lysis buffer RIPA in the presence of protease and phosphatase inhibitors. Protein concentrations in the samples were determined using the Bradford technique. The proteins were separated from the tissue (30 µg) on 8–12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% bovine serum albumin (BSA) at room temperature for 60 min. Then, the membranes were incubated with a primary antibody against AT1 receptor (sc-1173 – Santa Cruz Biotechnology, 1:500), AT2 receptor (sc-9040 – Santa Cruz Biotechnology, 1:500), IP3 receptor (8568S – Cell Signaling Technology,

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1:1000), phospho-IP3 receptor (3760S – Cell Signaling Technology, 1:1000), or MLC (8505S – Cell Signaling Technology, 1:1000) overnight. On the next day, the membranes were incubated with an HRP-conjugated secondary anti-rabbit (NA9340V – Abcam, 1:5000) body at room temperature for 60 min. Bands were detected by using autoradiography or a camera system that produces images of chemiluminescent components (Image Quant LAS 4000); α-tubulin or β-actin was used to normalize the results. The bands were quantified with the ImageJ Software (NIH Image).

Colorimetric Griess reaction

After decapitation, blood from both rat groups (2K and 2K-1C) was collected in a tube with heparin and submitted to centrifugation to separate the plasma. Then, 50 μL of plasma from groups 2K-1C and 2K rats were collected and added to 50 μL of Griess reagent (a 1:1 dilution of 1% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water and sulfanilamide 1% in 5% phosphoric acid) in a 96-well plate. The standard curve of sodium nitrite ranged from 3 μmol/L to 200 μmol/L for nitrite. Absorbance was read at 540 nm. Results were normalized to total protein concentration.

Angiotensin II levels

Plasma samples were used to evaluate circulating Ang II levels by ELISA; the angiotensin II kit (Sigma-Aldrich, RAB0010) was employed. The assay was performed according to the manufacturer's instructions, and the detection limits were 0.1–1000 pg/mL.

Statistical analysis

The pharmacological parameters efficacy (maximum effect, Emax) and potency (pD_2) obtained from the concentration–effect curves to Ang II were used to express the data. Emax was considered as the maximal amplitude response reached in the concentration–effect curves to the contractile agent. The concentration of the agent that produced half-amplitude of the maximum concentration was determined after logarithmic transformation of the normalized concentration–effect curves and it is reported as negative logarithm (pD_2) of the mean of individual values. In addition, the data were expressed as area under curve (AUC) in arbitrary units, which was calculated from the concentration–effect curves. As for *n*, it refers to the number of rats. The data are expressed as the mean ± standard error of the mean (s.e.m.). Statistical analyses were performed by using the software Prism 5.0 (GraphPad). Student's *t*-test or two-way ANOVA followed by Tukey *post hoc* test was applied when necessary to compare the results. Differences were considered statistically significant when $P < 0.05$.

Results

Blood pressure increases and endothelial function is impaired in 2K-1C rats

As depicted in Fig. 1A, blood pressure increased in 2K-1C rats 2 weeks after induction of hypertension and remained high until the last measurement. The values of the AUC, that in this case represents the magnitude of hypertension, was higher in 2K-1C. After 6 weeks, we tested the endothelial function by ACh-induced relaxation in some aortas from 2K and 2K-1C rats. Figure 1B shows that the Emax to ACh as well as AUC were decreased in 2K-1C, but the pD_2 values were similar to 2K rat aortas, which demonstrate impaired endothelial function in 2K-1C rat aortas. These results demonstrated that the surgery induced hypertension and impaired the vascular function.

Ang II-induced contraction depends on AT_1 receptors in endothelium-intact aortic rings

Based on Fig. 2A, the concentration–response curve induced by Ang II produced a low contraction in aortic rings with endothelium (E+) of 2K-1C and 2R rats. Although the profile of the concentration–effect curves shifted slightly to the right, Emax and pD_2 of 2K-1C (Emax: 0.23 ± 0.08 g; pD_2: 8.37 ± 0.14; $n = 7$) and 2K (Emax: 0.35 ± 0.10 g; pD_2: 8.52 ± 0.09; $n = 12$) rat aortas did not differ, as well as AUC values (2K: 0.54 ± 0.14; 2K-1C: 0.25 ± 0.05; arbitrary units). Next, we used selective antagonists to evaluate the role of AT_1, AT_2, and Mas receptor activation by Ang II. Figure 2B and C shows that losartan abolished the Ang II-induced contractile response in 2K (Emax from 0.30 ± 0.12 g to −0.002 ± 0.004 g) and in 2K-1C (Emax from 0.15 ± 0.03 g to −0.01 ± 0.01 g) rat aortas. In addition, losartan decreased the AUC values in 2K (0.01 ± 0.002, arbitrary units) and 2K-1C (0.02 ± 0.005, arbitrary units). On the other hand, the AT_2 receptor antagonist PD123319 did not change the Ang II-induced contractile response in 2K (Fig. 2D) or 2K-1C (Fig. 2E) rat aortas, neither changed the AUC values in both groups. The Mas receptor antagonist A779 did not change the Ang II-induced contractile response or AUC in 2K (Fig. 2F) or 2K-1C (Fig. 2G) rat aortas, either.

NO-Synthase inhibition does not counter-regulate Ang II-induced contraction in 2K-1C rat aorta

We evaluated how endothelial nitric oxide (NO) and ROS contributed to the Ang II-induced contractile response. According to Fig. 3A, the NOS inhibitor L-NAME increased Emax (from 0.38 ± 0.12 g; $n = 10$ to 1.01 ± 0.15 g; $n = 11$; $P = 0.0052$) and amplitude of AUC (from 0.54 ± 0.14 to 1.57 ± 0.24, arbitrary units; $P = 0.0037$) in 2K rat aorta, but the pD_2 values remained similar to the control (L-NAME pD_2: 8.48 ± 0.07; $n = 11$

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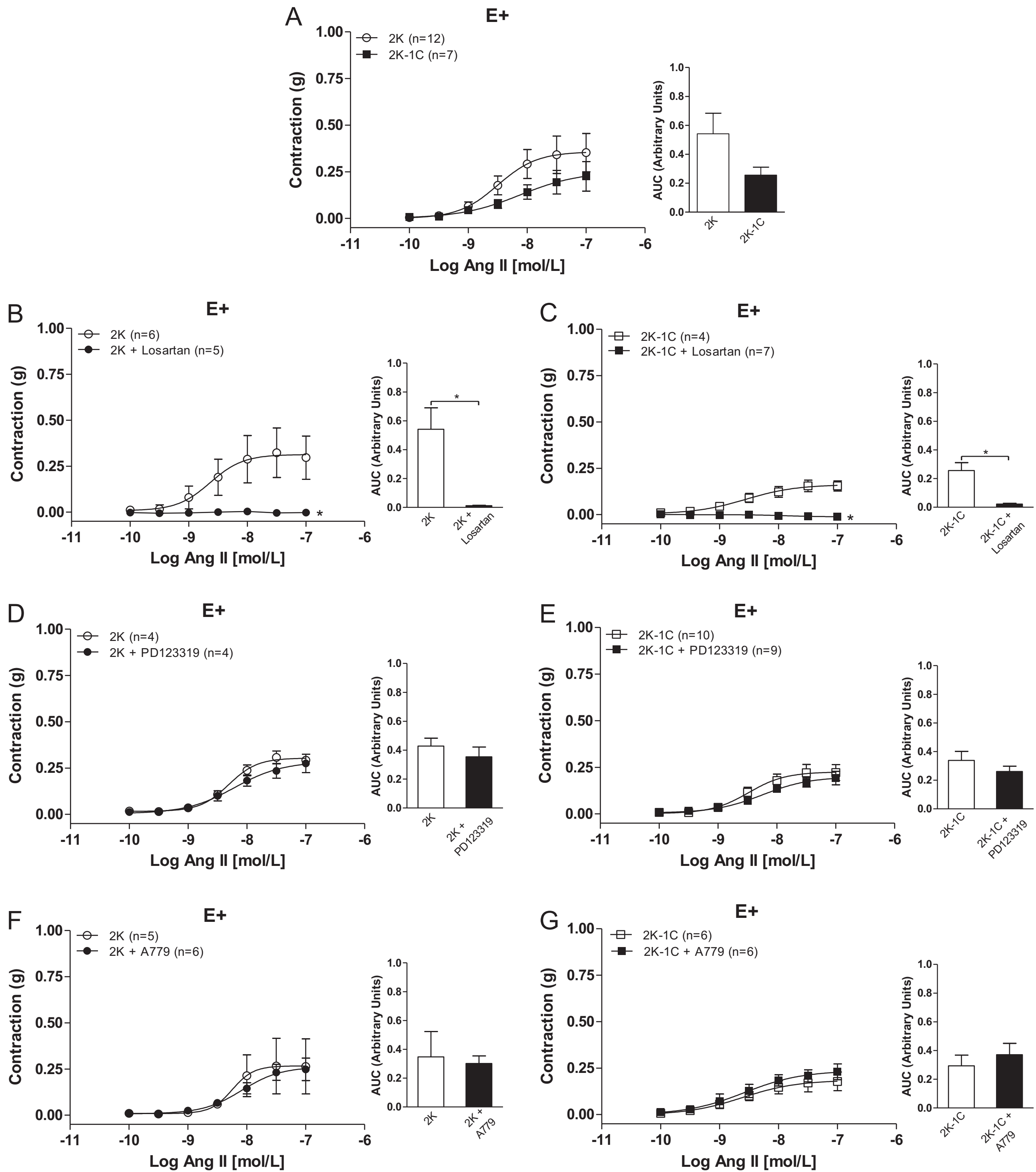

Figure 2

Ang II-induced contraction in endothelium-intact rat aortas. Concentration–effect curves to Ang II were constructed for endothelium-intact (E+) aortas in the (A) absence (control), (B, C) in the presence of AT_1 receptor antagonist (losartan, 10 μmol/L), (D, E) AT_2 receptor antagonist (PD123319, 1 μmol/L), or (F, G) Mas receptor antagonist (A779, 10 μmol/L) in normotensive (2K) and hypertensive (2K-1C) rat aortas. The results of concentration–effect responses are also represented by the area under the curve (AUC, in arbitrary units). Data represent the mean ± s.e.m. (n = 4–10). *Difference at the maximum effect of losartan vs control (P < 0.05); Student's t-test.

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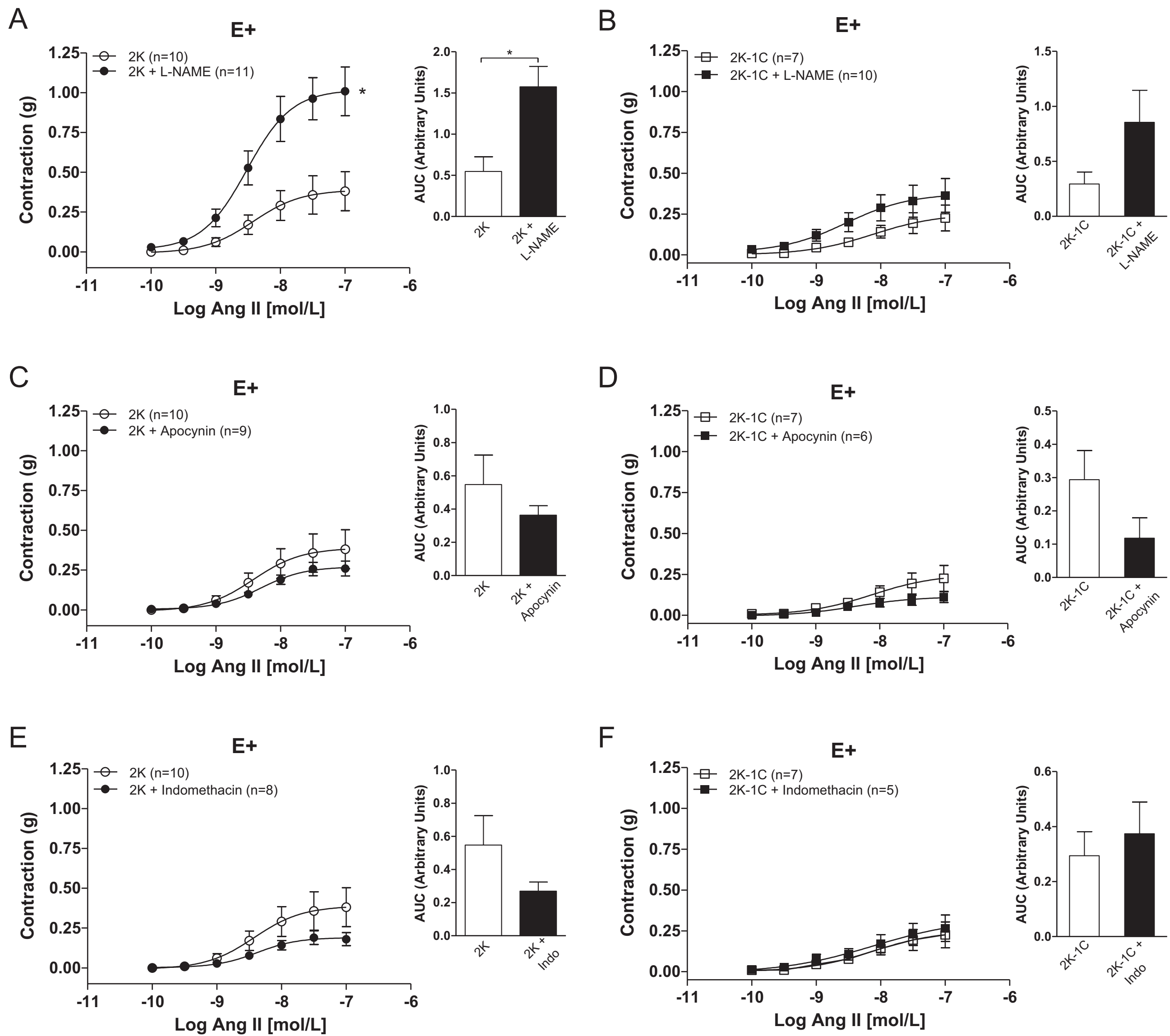

Figure 3

Endothelial enzymes contributing to Ang II-induced contractions. Concentration–effect curves to Ang II were constructed in the absence (open circle, open square) or in the presence of the enzyme inhibitors (closed circle, closed square) L-NAME (100 µmol/L) (A, B), apocynin (100 µmol/L) (C, D), or indomethacin (indo,10 µmol/L) (E, F) in endothelium-intact normotensive (2K) and hypertensive (2K-1C) rat aortic rings. The area under the curve (AUC, in arbitrary units) were calculated from the individual concentration–response curve plots. Data represent the mean ± s.e.m. (n = 5–11). *Difference at the maximum effect of L-NAME vs control (P < 0.05) by Student's t-test.

vs control pD_2: 8.47 ± 0.10; n=10). However, L-NAME did not change Ang II-induced contraction in 2K-1C rat aorta, Emax (L-NAME: 0.36 ± 0.14 g; n = 10 vs control: 0.23 ± 0.08 g; n=7), pD_2 (L-NAME: 8.50 ± 0.15; n=10 vs control: 8.37 ± 0.14; n=7) or AUC (L-NAME: 0.85 ± 0.29 vs control 0.29 ± 0.10, arbitrary units) values.

Among other effects, Ang II induces ROS production mediated by activation of Nox isoforms. Thus, we tested whether the Nox inhibitor apocynin could affect Ang II-induced contraction. As shown by the results obtained, apocynin did not alter Ang II-induced contraction in 2K (Fig. 3C) and 2K-1C (Fig. 3D) rat aortas (2K, Emax: 0.26 ± 0.05 g, pD_2: 8.31 ± 0.06, AUC: 0.36 ± 0.05; 2K-1C, Emax: 0.16 ± 0.03 g, pD_2: 8.18 ± 0.11, AUC: 0.11 ± 0.06). COX inhibition with indomethacin provided similar results (2K, Emax: 0.18 ± 0.04 g, pD_2: 8.32 ± 0.10, AUC: 0.26 ± 0.05; 2K-1C, Emax: 0.27 ± 0.08, pD_2: 7.98 ± 0.21, AUC: 0.37 ± 0.11).

eNOS mRNA quantification and NO_x levels are lower in 2K-1C than in 2K rat aortas

Particularly because NO produced by eNOS has a counter-regulatory effect on Ang II-induced contraction, we tested the RNA expression of eNOS in endothelium-

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intact 2K-1C and 2K rat aortas. As shown in the Fig. 4A, RNA was nearly 40% lower in 2K-1C as compared to the values obtained in 2K rat aorta. In addition, we demonstrate that NO_x levels are decreased in plasma from 2K-1C groups (0.1652 ± 0.01968, μmol/μg) compared to 2K (0.3342 ± 0.02687, μmol/μg) (Fig. 4B).

Ang II-induced contraction is counter-regulated by endothelial cells

To evaluate how endothelial factors and the activation of receptors expressed in VSMC affect Ang II-induced contraction, we used endothelium-intact and denuded aortas. As depicted in Fig. 5A, endothelium removal increased pD_2 (E−: 9.00 ± 0.07; n = 9 vs E+: 8.47 ± 0.10; n = 10), Emax (E−: 1.64 ± 0.14 g; n = 9 vs E+: 0.38 ± 0.12 g; n = 10) and AUC (E−: 3.10 ± 0.30 vs E+: 0.54 ± 0.17, arbitrary units) to Ang II in 2K rat aorta. In 2K-1C rat aorta, endothelium removal also increased Emax (E−: 0.74 ± 0.12 g; n = 11 vs E+: 0.23 ± 0.08 g; n = 7) and AUC (E−: 1.23 ± 0.19 vs E+: 0.29 ± 0.08, arbitrary units) to Ang II, but the values of pD_2 to Ang II remained unaltered (E−: 8.49 ± 0.13; n = 11 vs E+: 8.37 ± 0.14; n = 7) (Fig. 5B). We also observed a further reduction in Ang II-induced contraction in 2K-1C E− than in 2K E− aortic rings (Emax 2K: 1.64 ± 0.14 g; n = 9 vs Emax 2K-1C: 0.74 ± 0.12 g; n = 11), (pD_2 2K: 9.00 ± 0.07; n = 9 vs pD_2 2K-1C: 8.49 ± 0.13; n = 11), (AUC 2K: 3.10 ± 0.30 vs AUC 2K-1C: 1.23 ± 0.19, arbitrary units).

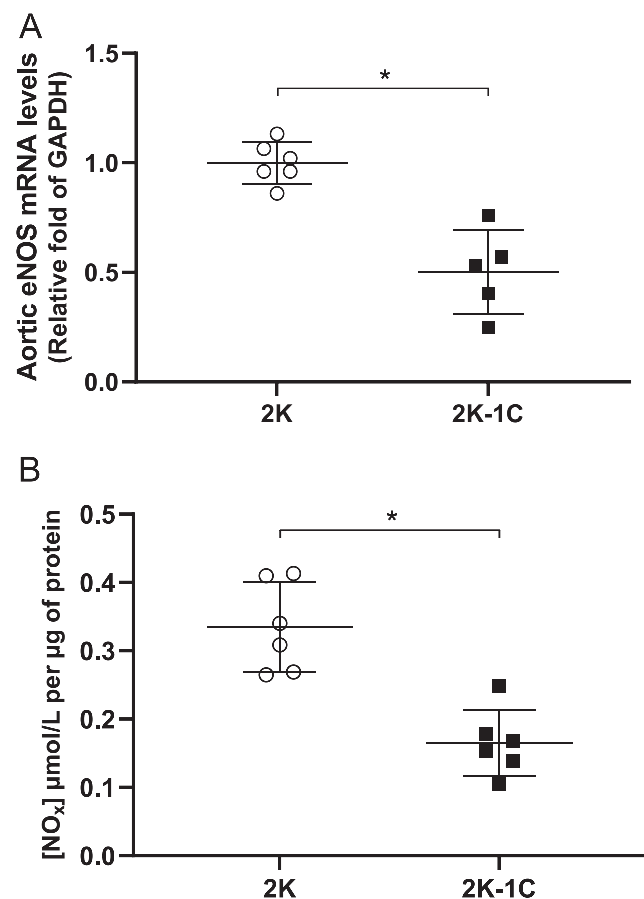

Figure 4

eNOS mRNA and NO levels. (A) eNOS mRNA expression levels measured in rat aorta homogenate through by qRT-PCR (ΔΔ C_t) and normalized to GAPDH. (B) NO levels measured in plasma from normotensive (2K) and hypertensive (2K-1C) rats. Data represent mean ± s.e.m. (n = 5–6). *Difference between 2K-1C vs 2K rats (P = 0.00030) by Student's t-test.

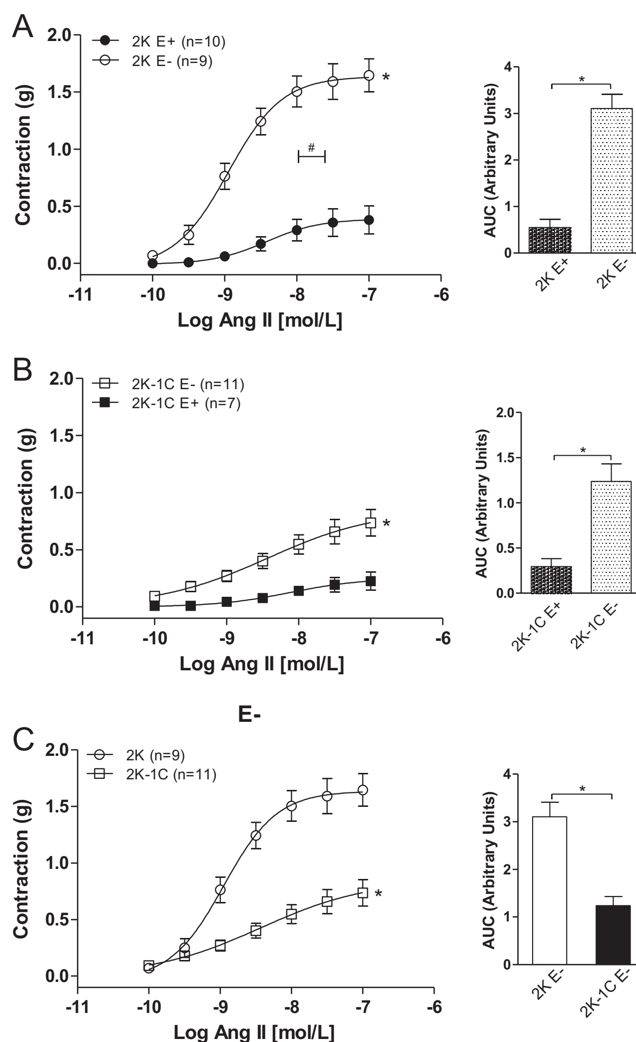

Figure 5

Effect of endothelium removal on Ang II-induced contraction Concentration–effect curves to Ang II were constructed for (A,B) endothelium-intact (E+) or (A,B,C) denuded (E−) normotensive (2K) and hypertensive (2K-1C) rat aortas and are expressed as area under the curve (AUC, in arbitrary units) from individual concentration–response curve plots. Data represent mean ± s.e.m. (n = 7–11). *Difference at the maximum effect between E+ vs E− or 2K-1C vs 2K (P < 0.05). #Difference at the pD_2 between E+ vs E− (P < 0.05) by Student's t-test.

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Expression of AT_1 , but not AT_2, in aorta and Ang II plasma levels are higher in 2K-1C than in 2K rats

Given that Ang II activates AT_1 and AT_2 receptors in VSMC, we evaluated their expression in 2K-1C and 2K rat aortas. Based on Fig. 6A, AT_1 receptor expression was higher in 2K-1C than in 2K rat aortas. However, 2K-1C and 2K rat aortas did not differ in terms of AT_2 receptor expression (Fig. 6B). Ang II plasma levels were also higher in 2K-1C than in 2K rat aortas (Fig. 6C).

KCl depolarization induces weaker contraction in 2K-1C than in 2K rat aortas

To evaluate contraction regardless to receptor activation, we induced contraction by depolarization with 120 mmol/L KCl before constructing the concentration–curve effect to Ang II for some E+ and E− aortic rings. Endothelium removal increased KCl-induced contraction in 2K (E+: 2.01 ± 0.14 g; n = 8 vs E−: 2.58 ± 0.21 g; n=7), but not in 2K-1C (E+: 1.62 ± 0.21 g; n = 7 vs E−: 1.39 ± 0.16 g; n = 9) rat aortic rings. In denuded aortas, the response was lower in 2K-1C than in 2K (1.39 ± 0.16 g; n = 9 vs 2.58 ± 0.21 g, respectively) (Fig. 7).

Basal IP_3 receptor phosphorylation is lower in 2K-1C than 2K rat aorta VSMC

We also evaluated the basal phospho-IP_3 receptor levels in E− aortic vessels. According to Fig. 8, phospho-IP_3 receptor expression was lower in 2K-1C than in 2K rat aortas (Fig. 8A), whereas 2K-1C and 2K rat aortas did not differ in terms of total IP_3 receptor (Fig. 8B) or myosin light chain enzyme expression (Fig. 8C).

Discussion

In this study, we investigated the underlying molecular mechanisms of Ang II-induced contraction in 2K and 2K-1C rat aortas. Intriguingly, our results showed that Ang II induced similar contraction in endothelium-intact 2K and 2K-1C rat aortic rings. Although the 2K-1C rat aorta presents dysfunctional eNOS, it does not seem to interfere with the contractile process induced by Ang II. However, endothelium removal increased Ang II-induced contraction in 2K compared to 2K-1C rat aortic rings, but AT_1 receptor expression was higher in 2K-1C rat aortic rings. In addition, 2K-1C rat displayed increased Ang II plasma levels, but lower basal expression of phosphorylated IP_3 receptors. These data demonstrate that although the 2K-1C rat aorta presents increased expression of the AT1 receptor, the contraction induced by Ang II was lower in the 2K-1C than in 2K rat aorta because the IP_3 receptor phosphorylation is reduced, that impairs the calcium release from the sarcoplasmic reticulum, which is necessary for the contraction.

In accordance to Navar *et al.* (24), Ang II concentrations are increased in the plasma of 2K-1C rats after unilateral renal artery stenosis. Ang II acts as the main effector peptide of the RAAS system and, through direct vascular effects, and then Ang II rapidly increases total peripheral resistance, to culminate in elevated blood pressure (25, 26). Based on our data, the systolic blood pressure increased progressively in 2K-1C rats, but it remained constant in 2K rats. Other research groups demonstrated that blood pressure begins to increase between the first and second weeks after the clip is implanted in 2K-1C rat renal artery (16, 27, 28, 29, 30), which supports our data. We also found endothelial dysfunction characterized by impaired endothelium-

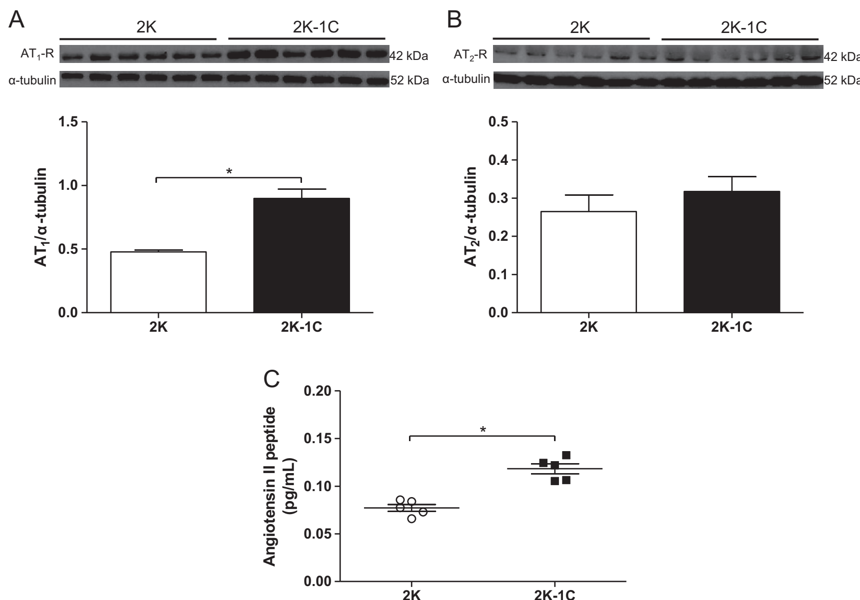

Figure 6

Basal AT_1 and AT_2 receptor expression in the aorta and Ang II plasma levels. (A) AT_1 and (B) AT_2 receptor expression in normotensive (2K) and hypertensive (2K-1C) rats. (C) ELISA detection of Ang II levels in 2K and 2K-1C rat plasma. Data represent mean ± s.e.m. (n = 5–6). *Difference between 2K-1C vs 2K (P < 0.05) by Student's t-test.

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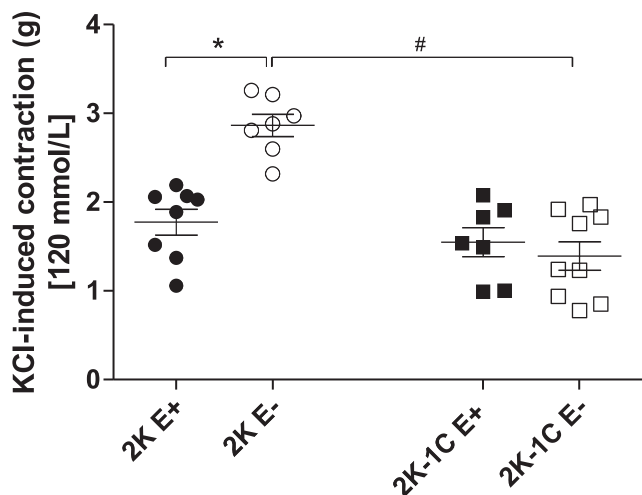

Figure 7

KCl-induced contraction in rat aorta. Contractile response was induced by KCl (120 mmol/L) in 2K and 2K-1C rat aortic rings. Data represent the mean ± S.E.M. (n = 7–9). *Difference at the maximum effect between E+ vs E− ($P < 0.05$). #Difference at the maximum effect between 2K-1C vs 2K ($P < 0.05$) by two-way ANOVA.

dependent relaxation, a known characteristic of hypertensive animals.

As observed in Fig. 2A, endothelium-intact 2K-1C and 2K aortic rings had similar low-grade concentration–effect curves to Ang II. Low-grade Ang II-induced contraction has been reported in normotensive rat aorta (31). Thus, Ang II induced weak contraction, which seems to be a characteristic of this peptide. In addition, Oliveira and collaborators (32) observed different results, where the contraction induced by Ang II was greater in the aortic rings of 2K-1C compared to 2K. However, the rats were used three weeks after surgery, which differs from our work.

We also evaluated the type of Ang II receptor that is involved in this response. To this end, we used losartan, a selective AT_1 receptor antagonist, and PD123319, a selective AT_2 receptor antagonist. Because Ang II can indirectly act through Mas receptors under ACE2 effects, we also used A779 to inhibit Mas receptors. Inhibition of AT_2 or Mas receptors did not change the profile of the concentration–effect curves to Ang II in 2K-1C or 2K rat aortic rings. In fact, only AT_1 receptor blockade abolished the Ang II vasoconstrictor response in both groups, which means that only AT_1 participated in the vasoconstrictor response in the presence of endothelium. Levy *et al.* (33) reported similar results.

Endothelial dysfunction associated with increased release of COX-pathway products (34) and elevated ROS production (16, 35) has been reported in 2K-1C rat vessels. This increase in vasoconstrictor substances and ROS impairs endothelium-dependent relaxation in aortic rings (20, 30, 35, 36) and mesenteric arteries (35, 37, 38, 39) of renovascular hypertension models.

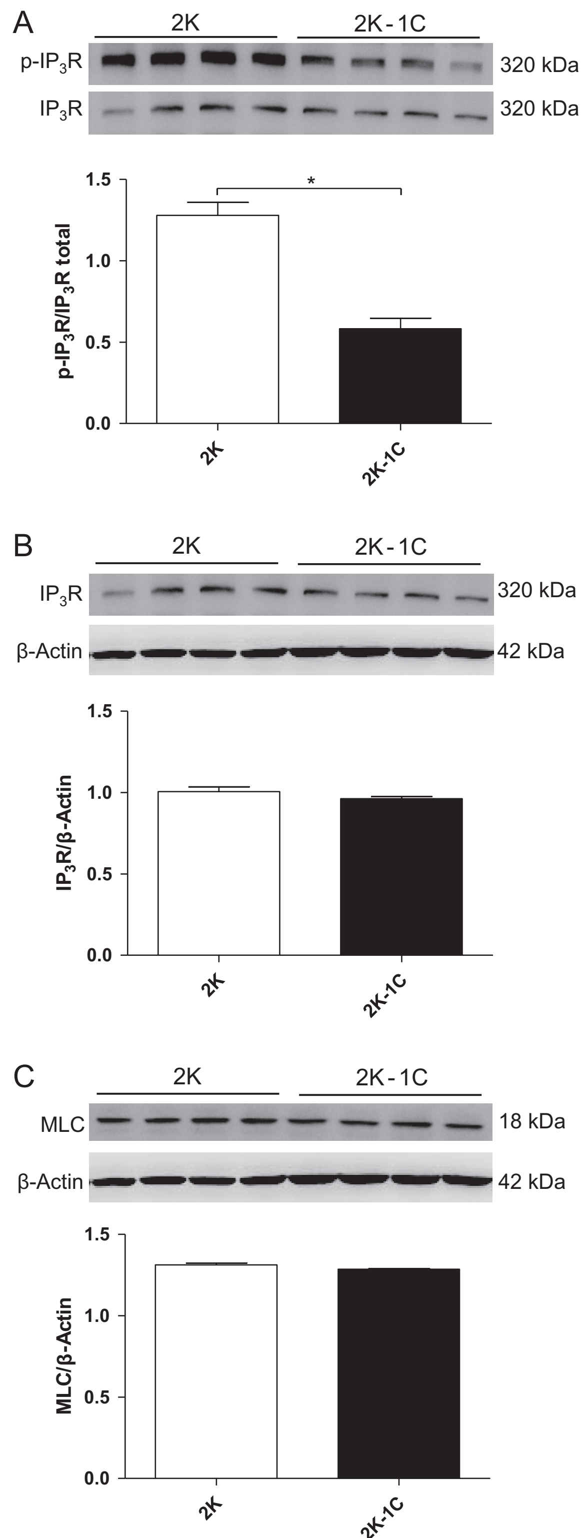

Figure 8

Basal phospho-IP_3 receptor, IP_3 receptor, and MLC expression in rat aorta. (A) phospho-IP_3 receptor, (B) IP_3 receptor, and (C) MLC expression were quantified in 2K and 2K-1C rat aorta VSMC. Data represent the mean ± S.E.M. (n = 4). *Difference between 2K-1C vs 2K ($P < 0.05$) by Student's t-test.

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Moreover, decreased eNOS expression has been reported in aortas (40, 41), which has been linked to lower NO levels (16, 35, 42) in 2K-1C rats. Therefore, we used pharmacological tools to evaluate these different pathways. NOS inhibition with L-NAME increased the vasoconstrictor effect of Ang II in 2K rat aortic rings because NOS is functionally active in 2K rat vessels. However, L-NAME shifted the concentration–effect curves slightly to the right without changing the contractile effect of Ang II in 2K-1C rat aortic rings, which could be associated with the decreased eNOS mRNA and NO levels observed in 2K-1C rat aortas compared to 2K rat aortas. Application of recombinant adenovirus expressing the eNOS functional gene to 2K-1C rats, to induce eNOS overexpression, can prevent the development of renovascular hypertension, confirming eNOS dysfunction in this model (43).

The increase in COX-pathway products (34) and ROS (16, 35) can modulate the vasoconstrictor effect of phenylephrine in 2K-1C rat aortic rings, as reported by our research group (44). However, COX-pathway inhibition with indomethacin or ROS inhibition with apocynin did not change the Ang II-induced vasoconstrictor response in 2K-1C and 2K rat aortic rings in the presence of endothelium. These results suggest that, in this case, COX-pathway products and ROS did not participate in the Ang II-induced contractile response.

Interestingly, the Ang II-induced maximum effect in denuded 2K rat aortic rings was higher than in 2K-1C rat aortic rings, despite increased AT_1 receptor expression in 2K-1C rat aorta. On the other hand, AT_2 receptor expression did not change in 2K-1C rat aorta compared to 2K rat aorta. As mentioned before by other groups (35, 45) and also confirmed here with our data showing that Ang II levels were higher in 2K-1C rat plasma than in 2K rat plasma.

Based on the literature, the time course of Ang II levels in 2K-1C model presents different stages after clipping of the renal artery. In the early phase (approximately 2 weeks after clipping), high blood pressure is induced by plasma renin activity and high levels of circulating Ang II levels. In the later phase (after 4 weeks of clipping), plasma renin activity and Ang II levels are reduced, but hypertension is maintained (45). It was demonstrated that Ang II levels induce upregulation of AT_1 receptor expression due to its role on the regulation of transcription factors NF-κB and Elk-1 in a neuronal cell model (46). In addition, Ang II levels regulate proximal tubule AT_1 receptor expression since Ang II increases AT_1 receptor mRNA at least in part by decreasing proximal tubule cAMP generation through a pertussis toxin-sensitive mechanism (47). Moreover, statin withdrawal in VSMC promotes an increase in Ang II levels and Ang II signaling due to rise AT_1 receptor density and its transcription (48). In this way, different studies have reported increased AT_1 receptor expression (29, 33) and high Ang II levels (33) in 2K-1C rat aorta, corroborating our data, also in renal tissue (49). These results suggest that high levels of Ang II are associated to upregulation of AT_1 receptor expression.

It is important to highlight that prolonged AT_1 receptor activation in aorta VSMC mediates AT_1 receptor internalization (50). In addition, overexpression of G protein-coupled receptor kinases (GRKs) 2, 3, or 5 augmented the agonist-induced AT_{1A}-receptor phosphorylation, indicating the role of one or several GRKs in the rapid agonist-induced desensitization of the AT_{1A}-receptor (51). Moreover, in glomerulosa cells, high concentrations of Ang II desensitize AT_1 receptor-mediated cellular responses in the long term (22). Therefore, the Ang II-induced maximum effect that is reduced in denuded aortic rings of 2K-1C can be explained by the fact that AT_1 receptors may act as a checkpoint for the regulation of direct Ang II effects on target tissues. Taken together, these results suggest that increased Ang II levels can regulate AT_1 receptor by its internalization (52) and/or desensitization (51), thus avoiding its hyper-activation.

Ang II binding to AT_1 receptors in VSMC stimulates phospholipase β (PLC-β), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), thus generating the second messenger IP_3 and diacylglycerol (53). IP_3 activates IP_3 receptors on the sarcoplasmic reticulum membrane, releasing the stored calcium (11, 53). Hence, we assume that some changes in calcium mobility through the membrane could take place or its storage in the sarcoplasmic reticulum could be altered in renovascular hypertension.

We verified that one single KCl (120 mmol/L) concentration contracted endothelium-intact 2K-1C and 2K rat aortic rings in a similar way, while KCl-stimulated contraction increased in denuded 2K rat aortic rings but not changed in denuded 2K-1C rat aortic rings. This result can be explained by the fact that aorta from 2K-1C demonstrates changes in the electric gradient (15), which could compromise the 2K-1C aortas in reaching the maximum response stimulated by a single dose of high concentration of KCl.

Other point to be considered is that high concentrations of extracellular KCl promote a contractile effect on VSMC through cell membrane depolarization, which is followed by activation of voltage-operated calcium channels and calcium release from the sarcoplasmic reticulum (54). Then, we analyzed IP_3 receptor phosphorylation. Interestingly, IP_3 receptor deletion results in lower smooth muscle contractile ability to different agonists as well as lower systolic blood pressure upon chronic Ang II infusion (55). Here, total IP_3 receptor expression or MLC in 2K-1C and 2K rat aortas did not change, but basal IP_3 receptor phosphorylation was reduced in 2K-1C rat VSMC. These results suggest that calcium release from the sarcoplasmic reticulum was decreased. It could be related to decreased contractile effect of Ang II seen in denuded 2K-1C rat aortic rings. How the constant stimulation of AT_1 receptors in

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hypertensive animals can induce lower IP_3 receptor phosphorylation levels or whether these alterations are produced by changes in other components of the classic PLCβ-IP_3-diacylglicerol pathway needs to be clarified in future studies.

Although our study brings new findings to the area of vascular biology, it has limitations, as it does not prove the internalization or desensitization of the AT1 receptor associated with high levels of Ang II as it was suggested in the discussion.

Conclusion

The maximum contraction induced by Ang II is similar in intact endothelium aortic rings from 2K and 2K-1C. On the other hand, the maximum contraction stimulated by Ang II was reduced in denuded aortic rings of 2K-1C, which is associated with the impairment of IP_3 receptor phosphorylation observed in VSMC of 2K-1C rats. Therefore, this result demonstrates the importance of the role of calcium released from the sarcoplasmic reticulum for the Ang II-induced contraction.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Author Contributions

BMF and LMB conceived and designed the experiments; BMF, SRP, TDP and MDG performed the experiments; BMF, SRP, TDP and MDG analyzed the data; LMB provided the drugs and all the necessary materials; BMF, SRP, and TDP discussed the data; BMF, SRP, TDP, and LMB contributed to manuscript drafting. All the authors contributed to the article and approved the submitted version.

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