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Contrasting effects of aliskiren *versus* losartan on hypertensive vascular remodeling

Alisson Martins-Oliveira [a], Michele M. Castro [a], Diogo M.M. Oliveira [a], Elen Rizzi [a], Carla S. Ceron [a], Danielle Guimaraes [a], Rosana I. Reis [b], Claudio M. Costa-Neto [b], Dulce E. Casarini [c], Amanda A. Ribeiro [c], Raquel F. Gerlach [d], Jose E. Tanus-Santos [a,*]

[a] Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil
[b] Department of Biochemistry and Immunology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil
[c] Department of Medicine, Nephrology Division, Federal University of Sao Paulo, Brazil
[d] Department of Morphology, Estomatology and Physiology, Dental School of Ribeirao Preto, University of Sao Paulo, Brazil

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ABSTRACT

Background: Hyperactivation of the renin–angiotensin system contributes to hypertension-induced upregulation of vascular matrix metalloproteinases (MMPs) and remodeling, especially in the two kidney, one clip (2K1C) hypertension model. We hypothesized that the AT_1R antagonist losartan or the renin inhibitor aliskiren, given at doses allowing similar antihypertensive effects, could prevent *in vivo* vascular MMPs upregulation and remodeling, and collagen/elastin deposition found in 2K1C hypertension by preventing the activation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and transforming growth factor-β_1 (TGF-β_1). We also hypothesized that aliskiren could enhance the effects of losartan.
Methods: 2K1C rats were treated with aliskiren (50 $mg.kg^{-1}.day^{-1}$), or losartan (10 $mg.kg^{-1}.day^{-1}$), or both by gavage during 4 weeks.
Results: Aliskiren, losartan, or both drugs exerted similar antihypertensive effects when compared with 2K-1C rats treated with water. Aliskiren reduced plasma renin activity in both sham and 2K-1C rats. Losartan alone or combined with aliskiren, but not aliskiren alone, abolished 2K1C-induced aortic hypertrophy and hyperplasia, and prevented the increases in aortic collagen/elastin content, MMP-2 levels, gelatinolytic activity, and expression of phospho-ERK 1/2 and TGF-β_1. No significant differences were found in the aortic expression of the (pro)renin receptor.
Conclusions: These findings show that although losartan and aliskiren exerted similar antihypertensive effects, only losartan prevented the activation of vascular profibrotic mechanisms and MMP upregulation associated with vascular remodeling in 2K1C hypertension. Our findings also suggest that aliskiren does not enhance the protective effects exerted by losartan.

1. Introduction

Hyperactivation of the renin–angiotensin system (RAS) contributes to hypertension and promotes cardiovascular remodeling [1]. Mounting evidence indicates that a proteolytic imbalance involving upregulated matrix metalloproteinases (MMPs), especially MMP-2 [2], plays a major role in these alterations, especially in the two kidney, one clip (2K1C) hypertension model [3,4]. Indeed, angiotensin II increases MMP-2 expression and activity [5,6] via activation of angiotensin II type 1 receptors (AT_1R) [1,7,8] and therefore promotes cardiovascular remodeling [2]. Moreover, increased renin and prorenin formation activates the (pro)renin receptor (PRR) [9], which is widely expressed in renal mesangial and in vascular smooth muscle cells (VSMCs) [10,11]. Importantly, PRR activation exerts dual molecular functions by stimulating signaling pathways that are both dependent and independent of angiotensin II (ANG II) generation [12]. The last one elicits intracellular signaling that upregulates profibrotic pathways including extracellular regulated kinase 1/2 (ERK 1/2) and transforming growth factor-β_1 (TGF-β_1) [13,14], which contribute to hypertensive cardiovascular remodeling.

While antihypertensive drugs interfering with the RAS exert beneficial effects against the cardiovascular remodeling associated with hypertension [1], it is not known whether AT_1R antagonists affect profibrotic pathways and vascular MMP upregulation in hypertensive animals or humans. Moreover, although aliskiren (a direct renin inhibitor) decreases angiotensin II levels and improves cardiac remodeling [15], its effects on hypertension-induced vascular MMP upregulation and remodeling are not known. While compelling results suggest that aliskiren enhances the protective effects of AT_1R antagonists [16,17], the effects of this drug combination on the increases in vascular MMPs and remodeling associated with hypertension are not known.

* Corresponding author at: Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirao Preto, SP, Brazil. Tel.: +55 16 3602 3163; fax: +55 16 3602 0220.
E-mail addresses: tanus@fmrp.usp.br, tanussantos@yahoo.com (J.E. Tanus-Santos).

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In this study, we hypothesized that the AT_1R antagonist losartan or the renin inhibitor aliskiren prevents *in vivo* vascular MMPs upregulation and remodeling, and elastin/collagen deposition found in 2K1C hypertension [4,18] by preventing the activation of profibrotic pathways involving ERK 1/2 and TGF-β_1. Because increased transmural pressure is sensed by integrins [19], which transform signals into adaptive vascular remodeling, thus affecting vascular MMP activities [20], we compared the effects of losartan and aliskiren given at doses that allowed very similar antihypertensive effects in pilot studies. Moreover, we hypothesized that aliskiren could enhance the effects of losartan, as previously suggested [16].

2. Methods

2.1. Animals and treatments

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, and the animals were handled according to the guiding principles published by the National Institutes of Health. Male Wistar rats (180–200 g) were maintained on 12-h light/dark cycle at 25 °C with free access to rat chow and water.

2K1C hypertension was induced by clipping the left renal artery with a silver clip (0.2 mm). Sham-operated rats underwent the same surgical procedure (under general anesthesia with ketamine 100 mg/kg and xylazine 10 mg/kg i.p.), except for the placement of the renal artery clip. The systolic blood pressure (SBP) was measured by tail-cuff plethysmography weekly, and the rats were considered hypertensive when SBP > 160 mm Hg two weeks after the surgery.

Animals were randomly assigned to one of eight groups: 2K1C and Sham groups that received tap water; 2K1C and Sham groups that received losartan (Cozaar®; Merck Sharp & Dohme) at 10 mg/kg per day [8]; 2K1C and Sham groups that received aliskiren at 50 mg/kg [21] (Rasilez®; Novartis) per day; and 2K1C and Sham groups that received the combination of aliskiren 50 mg/kg + losartan 10 mg/kg per day. The treatments were started two weeks after 2K1C hypertension was induced and maintained for additional four weeks. All treatments were given daily by oral gavage and distilled water was used as vehicle.

2.2. Plasma renin activity assay

Blood samples were collected in tubes containing 5.0 mmol/L of EDTA. Blood samples were centrifuged at 2000 ×*g* for 30 min at 4 °C. Plasma renin activity was determined by assessing the generation of angiotensin I by reversed phase HPLC after incubation of plasma with a synthetic tetradecapeptide substrate at 37 °C, as previously described [22].

2.3. Morphometric analysis of the vascular wall

At the end of the experiments, animals were anesthetized and killed by decapitation. The thoracic aorta was harvested, cleaned of connective tissue, and immediately fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, and embedded in paraffin blocks. Four micrometer thick slices were stained with hematoxylin and eosin. Media cross-sectional area (CSA), media to lumen diameter (*M/L*) and the number of VSMC were quantified as previously described [23]. Sirus red and Orceine staining were used to assess aortic collagen and elastin contents using ImageJ Program (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011) [4].

2.4. Measurement of aortic MMP-2 levels

Aortic tissues were homogenized in buffer containing 20 mmol/L Tris–HCl, pH 7.4, 1 mmol/L 1,10-phenanthroline, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L N-ethylmaleimide (NEM), and 10 mmol/L $CaCl_2$. Tissue extracts were normalized for protein concentration and gelatin zymography was performed as previously described [24]. The forms of MMP-2 were identified as bands at 75, 72 and 64 kDa [24].

2.5. In-situ zymography and immunofluorescence for MMP-2

MMPs activity was measured in the media and intima of frozen thoracic aorta using DQ Gelatin as the fluorogenic substrate (E12055, Molecular Probes, Oregon, USA). Proteolytic activity was detected as bright green fluorescence, indicating substrate breakdown and MMP enzymatic activity. Aortic MMPs activity was also co-localized with aortic MMP-2 levels by immunofluorescence. After DQ gelatin, tissue sections were incubated with MMP-2 primary mouse anti-human monoclonal antibody (1:1000; Chemicon) for 1 h in dark humidified chambers. Red fluorescence was visualized by adding the rhodamine conjugated as secondary antibody (1:200; Chemicon) for 1 h. Sections were examined with fluorescent microscopy (Leica Imaging Systems Ltd., Cambridge, England) and the image was captured at X400. MMP activity and MMP-2 levels were evaluated by using ImageJ Program.

2.6. Western blotting

Aortas from 2K1C and sham rats were homogenized on ice-cold RIPA buffer. Forty micrograms of protein extracts were subject to SDS-PAGE using a 12% polyacrylamide gel. The proteins were then transferred onto nitrocellulose membranes and blocked with TBST (NaCl 100 mmol/L; Tris–Cl 100 mmol/L; Tween 0.1%) containing 5% bovine serum albumin. The membranes were incubated overnight at 4 °C with anti-MMP-2 (1:1000, Chemicon), anti-TGF-β1 (1:1000, Chemicon), anti-phosphorylated-ERK 1/2 (anti-p-ERK 1/2; 1:2000, Santa Cruz Biotechnology) or anti-prorenin receptor (anti-PRR; 1:250, Abcam) antibodies. Anti-β-actin (1:10,000, Millipore) was used as a loading control. The antibodies were washed in TBST and incubated with horseradish peroxidase (HRP)-secondary goat anti-mouse antibody (1:2000, Millipore) or HRP-secondary goat anti-rabbit antibody (1:1000, Millipore) for 1 h. Immunolabeled proteins were visualized using chemiluminescence ECL (Millipore) and registered by ImageQuant 350 detection system (GE Healthcare). The signal intensities were quantified using ImageJ Program (NIH — National Institute of Health).

2.7. Statistical analysis

Results are expressed as means ± S.E.M. Comparisons between groups were assessed by two-way or one way ANOVA followed by the Bonferroni test. A probability value < 0.05 was considered significant.

3. Results

3.1. Effects of aliskiren and losartan on SBP levels and plasma renin activity

We found similar baseline SBP in all experimental groups and no effects on SBP in the Sham groups (Fig. 1). While SBP increased progressively in 2K1C rats treated with water (208 ± 2 mm Hg), treatment of 2K1C rats with aliskiren, losartan, or both drugs exerted similar antihypertensive effects, especially after 4 weeks of treatment (SBP = 157 ± 2 mm Hg, 158 ± 2 mm Hg, 146 ± 2 mm Hg, respectively; Fig. 1, P < 0.05). Similar body weight gain was found in the eight

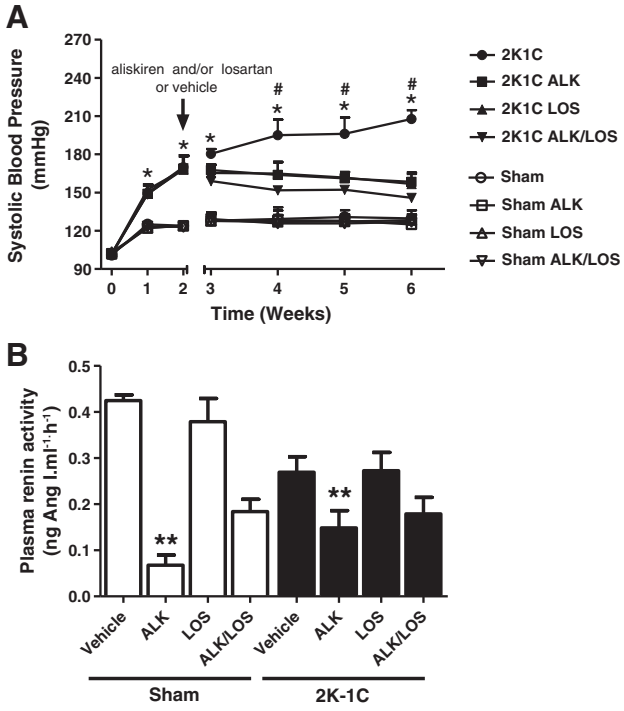

Fig. 1. Systolic blood pressure (mm Hg) measured by tail-cuff method (Panel A) and plasma renin activity (Panel B) in all experimental groups at the end of six weeks of antihypertensive treatment. Data are shown as mean ± S.E.M (n = 11–15 per group). *P < 0.05 for the 2K1C groups *versus* the Sham groups. **P < 0.05 versus the Sham + vehicle or versus 2K-1C + vehicle. #P < 0.05 for the 2K1C group *versus* the other 2K1C groups.