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ORIGINAL ARTICLE

Aliskiren and L-arginine treatments restore depressed baroreflex sensitivity and decrease oxidative stress in renovascular hypertension rats

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Renovascular hypertension is characterized by increased angiotensin II and oxidative stress, and by endothelial dysfunction. The purpose of this study was to test whether the administration of aliskiren (ALSK) and L-arginine (L-ARG) would restore impaired baroreflex sensitivity and reduce oxidative stress in a rat renovascular hypertension model. Hypertension was induced by clipping the left renal artery, and the following five groups were created: SHAM; two-kidney, 1-clip (2K1C); 2K1C plus ALSK (ALSK); 2K1C plus L-ARG (L-ARG); and 2K1C plus ALSK+L-ARG (ALSK+L-ARG). After 21 days of treatment, only the ALSK+L-ARG group was effective in normalizing the arterial pressure (108.8 ± 2.8 mm Hg). The L-ARG and ALSK+L-ARG groups did not show hypertrophy of the left ventricle. All the treatments restored the depressed baroreflex sensitivity to values found in the SHAM group. Acute administration of TEMPOL restored the depressed baroreflex sensitivity in the 2K1C group to values that resembled those presented by the other groups. All treatments were effective for an increase in the antioxidant pathway and reduction in the oxidative pathway. In conclusion, the treatment with ALSK or L-ARG reduced oxidative stress and restored reduced baroreflex sensitivity in renovascular hypertension. In addition, the treatments were able to normalize blood pressure and reverse left ventricular hypertrophy when used in combination.
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INTRODUCTION

One of the key mechanisms in controlling blood pressure in health and disease is the baroreflex. In pathological conditions, such as hypertension, there is an impairment of the autonomic control of blood pressure, resulting in changes in the baroreflex sensitivity.[1,2] Indeed, compelling evidence has shown that the baroreflex modulation of heart rate is impaired in animals and patients with renovascular hypertension.[3–5]

Importantly, in the two-kidney, one-clip (2K1C) model of renovascular hypertension, the renal artery stenosis caused by the clip reduces perfusion of the clipped kidney, promoting increases in plasma renin activity and circulating angiotensin II (Ang II) and increasing systolic blood pressure (SBP) because Ang II causes potent vasoconstriction, aldosterone secretion and sympathetic activation.[6,7] In addition, abundant evidence has suggested that an important mechanism by which Ang II influences blood pressure is via its ability to stimulate the production of ROS,[8,9] mainly superoxide anion,[8,10,11] by the activation of NADPH oxidase. Reactive oxygen species (ROS) have an important role in the development and maintenance of cardiovascular diseases, including hypertension,[12,13] atherosclerosis,[14] cardiac hypertrophy,[15,16] heart failure[16] and stroke.[17] In experimental models of 2K1C hypertension, increased vascular oxidative stress has an important role in the pathogenesis of renovascular hypertension and the enhancement of oxidation-sensitive mechanisms.[18] Ang II receptor blockers and β-blockers with antioxidant effects may inhibit ROS in the cardiovascular system and exhibit beneficial effects on oxidative stress.[19,20]

A previous study reported that oral L-arginine (L-ARG), a substrate of nitric oxide (NO) production, reduced blood pressure in the 2K1C hypertension model.[21,22] Senbel *et al.*[23] suggested that the protective effect resulted from the interaction between NO and ROS, and increased the NO bioavailability, because NO (synthesized from L-ARG) possibly acted as a superoxide radical scavenger. In addition, other studies have shown that treatment with aliskiren (ALSK), a direct renin inhibitor, reduced blood pressure and decreased oxidative stress.[24–26]

It is known that Ang II, acting through AT1 receptor, increases the sympathetic nerve activity, as well as the reduction of baroreflex gain is

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an important hallmark of hypertension, which is closely related to sympathetic hyperactivity and activation of the circulating and local renin angiotensin system.[4,6] The interplay between NO and different components of the RAS has been previously reported, including the effects of autonomic regulation of cardiovascular function.[27,28]

Therefore, in the present study, we tested the hypothesis that administration of ALSK or L-ARG would reduce oxidative stress and restore impaired baroreflex sensitivity in 2K1C hypertension.

METHODS

Animals and treatment

Male normotensive Wistar rats (150–170 g) were used for these studies. The animals were kept in cages with free access to both water and standard rat chow (Purina Labina, SP, Brazil) under controlled temperature (22–24 μC), humidity (60%) and light–dark cycle (12–12 h) conditions. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, revised 1996), and efforts were undertaken to minimize the animal's suffering. All the procedures were approved by the Institutional Ethical Committee for Animal Care and Use of the Federal University of Espırito Santo under protocol number 004/2010. The animals were randomly divided into one of the following groups ($n=8$): SHAM (normotensive control, vehicle saline); 2K1C (hypertension control, vehicle saline); ALSK (hypertension treated with ALSK, dose: 50 mg kg^{-1}); L-ARG (hypertension treated with L-ARG, dose: 10 mg kg^{-1}); and ALSK+L-ARG (hypertension treated with ALSK and L-ARG, doses: 50 and 10 mg kg^{-1}, respectively). All the treatments were performed by oral gavage for a total volume of 0.3 ml per day.

Surgical procedures

Renovascular hypertension was induced by the Goldblatt 2K1C method, as described in our previous reports.[21] Under i.p. anesthesia with ketamine (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}), a 0.20-mm internal diameter silver clip was placed through a flank incision around the left renal artery to induce renovascular hypertension. The SHAM rats underwent a similar procedure with manipulation of the left renal artery but without permanent application of the clip. The SBP of the tail artery was measured before the production of hypertension and 7 days after surgery in conscious rats using a non-invasive, computerized tail-cuff system. The criterion for hypertension in the present study was an SBP > 160 mm Hg. Only rats with SBP > 160 mm Hg 7 days after surgery were used in the experiments. The treatments were started 7 days after surgery and lasted for 3 weeks.

Direct measurements of blood pressure and heart rate recordings

After 4 weeks, the rats were anesthetized with ketamine and xylazine (75 and 10 mg kg^{1}, i.p., respectively), and polyethylene catheters inserted into the left femoral artery and vein. Both catheters were filled with heparinized saline, tunneled s.c., exteriorized and sutured to the dorsal surface of the neck. Twenty-four hours after the surgical procedures, experiments were performed on conscious rats. The blood pressure and heart rate were recorded using a pressure transducer connected to a computer running LabChart software (ADInstruments, Bella Vista, NSW, Australia).

Baroreflex sensitivity test

Following the baseline blood pressure and heart rate recordings, the baroreflex was activated using classical vasoactive drugs before and after the administration of phenylephrine (8 μg kg^{-1}, i.v.) and sodium nitroprusside (25 μg kg^{-1}, i.v.) randomly, given as intravenous bolus injections. After 10 min of stabilization, Tempol was administered (4-hydroxy-TEMPO 97%, Sigma, USA, 30 mg kg^{-1}, i.v.), a superoxide dismutase (SOD) mimetic agent, and 15 min later, new infusions of phenylephrine and sodium nitroprusside were administered. A 10-min interval was allowed between phenylephrine and sodium nitroprusside injections. Reflex changes in heart rate produced by vasoactive drug administration were quantified and plotted as changes in heart rate over changes in mean arterial pressure (ΔHR/ΔMAP), as described by Braga *et al*.[29] After the experiments, the animals were killed by decapitation. The heart was excised immediately and the left ventricle was used to determine weight/body weight ratios. The samples then remained for 24 h in an oven at 100 °C, and the dry weight of the ventricle was quantified (mg).

In another group of rats ($n=6$ per group), the heart was excised immediately and the left ventricle was used to evaluate the assay of advanced oxidation protein products, western blotting, catalase (CAT) and superoxide dismutase activity (SOD) and assay and detection of superoxide production so that there is no interference in the administration of TEMPOL used in baroreflex sensitivity protocol.

Assay of advanced oxidation protein products

Spectrophotometric determination of plasma and left ventricle advanced oxidation protein product (AOPP) levels was performed by the modification of Witko – Sarsat's method.[30] Samples were prepared in the following manner: 40 μl of the supernatant fraction of the homogenate or plasma was diluted 1:5 in PBS, and 10 μl of 1.16 M potassium iodide was then added, followed by the addition of 20 μl of acetic acid 2 min later. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 200 μl of PBS, 10 μl of KI and 20 μl of acetic acid. The chloramine-T absorbance at 340 nm was linear within the range of 5–100 μmol l^{-1}. AOPP concentrations were expressed as micromoles per litre of chloramine-T equivalents (μmol l^{-1} chloramine-T).

Western blotting analyses

The left ventricles were homogenized in lysis buffer containing (mmol l^{-1}) 150 NaCl, 50 Tris-HCl, 5 EDTA.2Na and 1 $MgCl_2$ plus protease inhibitor. The protein concentration was determined by the Lowry method and bovine serum albumin was used as the standard. Equal amounts of protein (50 μg) were separated by 10% SDS–PAGE. Proteins were transferred to polyvinylidene difluoride membranes incubated with mouse anti-rat monoclonal antibodies for CAT (1:2000), SOD-2 (1:1000), Gp91phox (1:1000) and rabbit anti-rat polyclonal antibodies for GAPDH (1:1000). After washing, the membranes were incubated with either an alkaline phosphatase-conjugated anti-mouse IgG (1:3000) or an anti-rabbit antibody (1:7000). The bands were visualized using a NBT/BCIP system (Invitrogen Corporation, Carlsbad, CA, USA) and were quantified using ImageJ software (National Institute of Health, NIH, Bethesda, MD, USA). The results were calculated using ratio of the density of specific proteins to the corresponding GAPDH.

Catalase and superoxide dismutase activity assay

CAT activity was measured in the supernatants, as described by Nelson and Kiesow.[31] In a cuvette, 2 ml of phosphate buffer (50 mM, pH 7.0) and 0.06 ml of homogenate of the left ventricle were mixed. The reaction was started by adding a substrate (250 μl of H2O2, 3 M), and the decrease in optical density was recorded at a wavelength of 240 nm every 15 s for 1 min. Experiments were performed in duplicate. CAT activity was expressed as ΔE min^{-1} mg^{-1} protein (ΔE representing the change in enzyme activity for 1 min).

SOD activity was determined in cardiac tissue using the method of Misra and Fradovich.[32] The reaction mixture consisted of 1.0 ml of carbonate buffer (0.2 M, pH 10.2), 0.8 ml of KCl (0.015 M), 0.1 ml of tissue and water for a final volume of 3.0 ml. The reaction was started by adding 0.2 ml of epinephrine (0.025 M). The change in absorbance was recorded at 480 nm at 15-s intervals for 1 min at 25 °C. A suitable control lacking enzyme preparation was run simultaneously. One unit of enzyme activity was defined as the amount of enzyme causing 50% inhibition of the auto-oxidation of epinephrine.

Detection of superoxide production (dihydroethidium fluorescence)

Unfixed frozen sections from the heart ($n=6$ per group) were cut into 8-μm-thick sections and were mounted on gelatine-coated glass slides. The samples were incubated with oxidative fluorescent dye dihydroethidium (2 μmol l^{-1}) in modified Krebs's solution (containing 20 mM HEPES), in a light-protected humidified chamber at 37 °C for 30 min, to detect superoxide. The intensity of fluorescence was detected at 585 nm and was quantified in the tissue sections using a confocal fluorescent microscope by an investigator blinded to the experimental protocol. Analysis of 15 fields per sample was performed.

Table 1 Effects of ALSK, L-ARG or ALSK+L-ARG treatment on the blood pressure and heart rate in 2K1C rats

	SBP (mm Hg)	*DPB (mm Hg)*	*MAP (mm Hg)*	*HR (b.p.m.)*
SHAM	112.09 ± 3.18	81.06 ± 1.6	105.62 ± 2.68	380.48 ± 13.72
2K1C	200.5 ± 5.36[a]	130.19 ± 7.15[a]	167.26 ± 5.85[a]	373.78 ± 26.02
ALSK	195.05 ± 9.03[a]	105.61 ± 9.68[b]	160.24 ± 10.26[a]	403.02 ± 23.05
L-ARG	152.19 ± 5.36[a,b]	103.62 ± 3.2[b]	135.23 ± 3.34[a,b,c]	357.4 ± 11.4
ALSK+L-ARG	123.9 ± 1.68[b,c,d]	87.29 ± 5.52[b]	108.87 ± 2.88[b,c,d]	372.48 ± 9.81

Abbreviations: 2K1C, two-kidney, one-clip; ALSK, aliskiren; ALSK+L-ARG, aliskiren plus L-arginine; DPB, diastolic blood pressure; HR, heart rate; L-ARG, L-arginine; MAP, mean arterial pressure; SPB, systolic blood pressure.
[a]$P<0.05$, when compared with SHAM.
[b]$P<0.05$, when compared with 2K1C.
[c]$P<0.05$, when compared with ALSK.
[d]$P<0.05$, when compared with L-ARG.
Data are expressed as mean ± s.e.m.

Table 2 Effects of ALSK, L-ARG or ALSK+L-ARG treatment on the left ventricle weight (mg g^{-1}) in 2K1C rats

	Dry weight (mg g^{-1})	*Wet weight (mg g^{-1})*
SHAM	2.23 ± 0.04	0.49 ± 0.08
2K1C	3.32 ± 0.16[a,b,c]	0.71 ± 0.06[a,b]
ALSK	3.26 ± 0.31[a,b,c]	0.64 ± 0.03[a,b]
L-ARG	2.47 ± 0.09	0.43 ± 0.01
ALSK+L-ARG	2.22 ± 0.15	0.59 ± 0.04

Abbreviations: 2K1C, two-kidney, one-clip; ALSK, aliskiren; ALSK+L-ARG, aliskiren plus L-arginine; L-ARG, L-arginine.
[a]$P<0.05$, when compared with SHAM.
[b]$P<0.05$, when compared with 2K1C.
[c]$P<0.05$, when compared with ALSK.
Data are expressed as mean ± s.e.m.

Statistical analyses

The results are expressed as the means ± s.e.m. The data were analyzed by one-way analysis of variance for repeated measures, followed by Fisher's *post hoc* test for multiple comparisons of the means. $P<0.05$ was considered statistically significant.

RESULTS

Effects of ALSK and L-ARG treatments on the development of 2K1C hypertension

The SBP and MAP were increased in the 2K1C group compared with those in the SHAM group (Table 1). After 21 days of treatment, only the ALSK+L-ARG group was effective in normalizing SBP and MAP. In addition, the L-ARG group showed reduced SBP and MAP compared with the 2K1C group; however, the ALSK group maintained high SBP and MAP compared with the SHAM group. In contrast, all the treatments reduced diastolic blood pressure compared with the 2K1C group. The heart rate was not different among the groups, as illustrated in Table 1.

The effects of ALSK and L-ARG treatments on the left ventricle

2K1C-induced hypertension promoted hypertrophy of the left ventricle compared with hearts from the SHAM group, and this difference was found in both of the weights, dry and wet (Table 2). In contrast, the L-ARG and ALSK+L-ARG groups showed similar values to the SHAM group, although the ALSK group values were not different from those of the 2K1C group, as illustrated in Table 2.

Effects of ALSK and L-ARG treatments on baroreflex sensitivity

The 2K1C group presented a reduction in baroreflex sensitivity after administration of phenylephrine and sodium nitroprusside (Figure 1a and b) compared with the SHAM group (-0.72 ± 0.12 *vs.* -1.91 ± 0.21b.p.m. and -1.03 ± 0.17 *vs.* -3.14 ± 0.26 mm Hg^{-1}, respectively, $P<0.05$) before the administration of TEMPOL. All the treatments restored the depressed baroreflex sensitivity to the values found in the SHAM group (ALSK: -2.7 ± 0.17, -2.85 ± 0.25; L-ARG: -2.07 ± 0.24, -2.99 ± 0.27 and ALSK+L-ARG: -2.19 ± 0.13, -2.52 ± 0.17 *vs.* SHAM: -1.91 ± 0.2 b.p.m., -3.14 ± 0.26 mm Hg^{-1}, respectively, $P<0.05$). Acute administration of TEMPOL, a well-known antioxidant, restored the depressed baroreflex sensitivity in the 2K1C group to values that resembled those presented by the other groups in both administrations (2K1C: -1.31 ± 0.25, -1.92 ± 0.32 *vs.* SHAM: -1.35 ± 0.17, -2.53 ± 0.33; ALSK: -1.54 ± 0.23, -1.75 ± 0.3; L-ARG: -1.78 ± 0.15, -2.28 ± 0.44 and ALSK+L-ARG: -1.38 ± 0.12 b.p.m., -1.81 ± 0.2 mm Hg^{-1}, respectively, $P<0.05$), as shown in Figure 1c and d.

Effects of ALSK and L-ARG treatments on advanced oxidation product levels

The AOPP levels in the plasma were significantly increased in the 2K1C group compared with those of the SHAM, ALSK and L-ARG groups (5.79 ± 0.67 *vs.* 3.79 ± 0.41; 3.96 ± 0.35; 4.26 ± 0.47 and 3.91 ± 0.36 μmol l^{-1} chloramine-T, respectively, $P<0.05$). Similar responses were found in left ventricle, with significant increases in the 2K1C group compared with the SHAM, ALSK and L-ARG groups (3.91 ± 0.36 *vs.* 1.26 ± 0.14; 1.21 ± 0.11; 1.37 ± 0.03 and 1.23 ± 0.13 μmol l^{-1} chloramine-T, respectively, $P<0.05$), as shown in Figure 2.

Expression of SOD-2, CAT and GP91phox in the heart

SOD-2 expression in the left ventricle was significantly decreased in the 2K1C group compared with that in the SHAM group and was increased in the ALSK, L-ARG and ALSK+L-ARG groups compared with that in the 2K1C group (Figure 3a). The CAT expression in the left ventricle was significantly decreased in the 2K1C group compared with that in the SHAM group and was increased in the ALSK, L-ARG and ALSK+L-ARG groups compared with that in the 2K1C and SHAM groups (Figure 3b). The gp91phox in the left ventricle was significantly increased in the 2K1C, ALSK, L-ARG and ALSK+L-ARG groups; however, the L-ARG and ALSK+L-ARG groups had significantly decreased gp91phox compared with the 2K1C and ALSK groups (Figure 3c).

CAT and SOD activities

CAT and SOD enzyme activities were significantly decreased in the left ventricles of the 2K1C group compared with those of the SHAM group. After treatment with ALSK+L-ARG, the enzyme activity of SOD was significantly increased. In addition, the enzyme activity of CAT increased in the ALSK, L-ARG and ALSK+L-ARG groups after treatment, as shown in Figure 4.

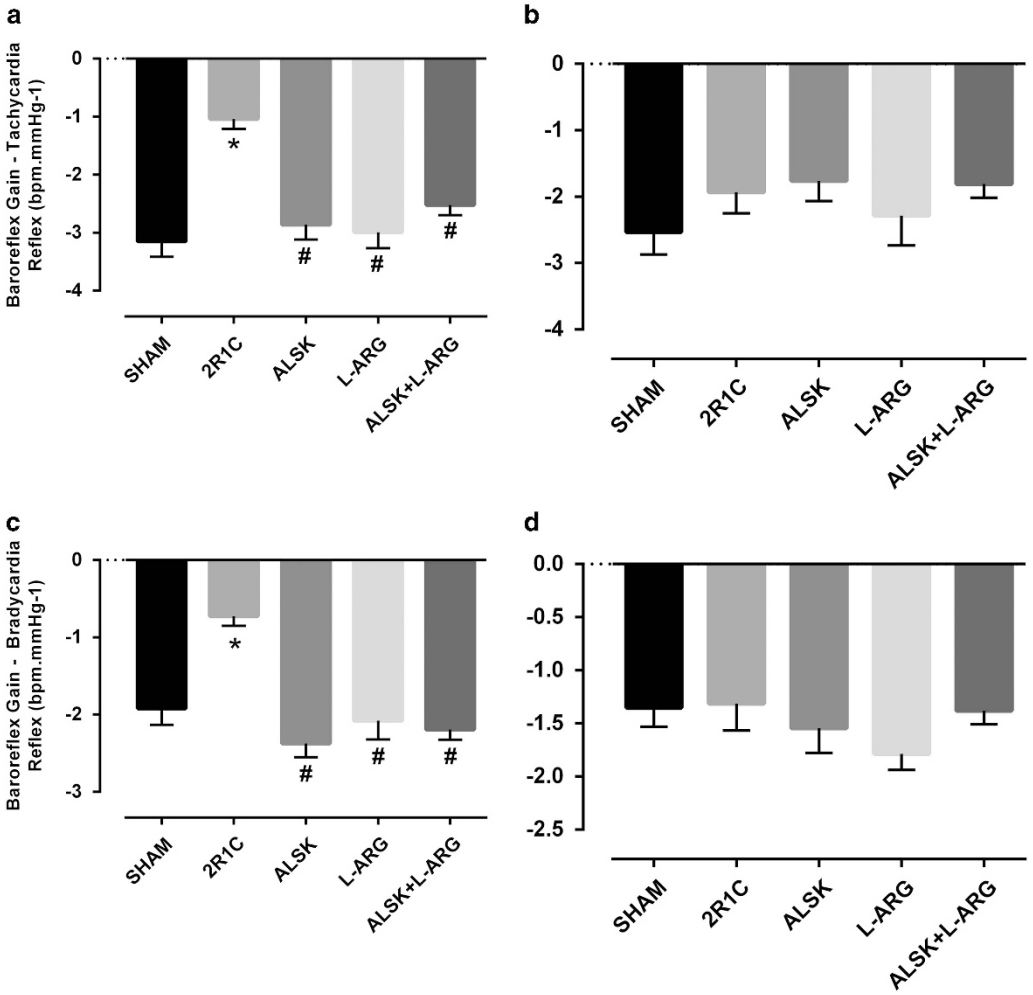

Figure 1 Effects of aliskiren (ALSK), L-arginine (L-ARG) or aliskiren plus L-arginine (ALSK+L-ARG) treatment on the parasympathetic and sympathetic components of the baroreflex before and after administration of TEMPOL in two-kidney, one-clip (2K1C) rats. Values for baroreflex sensitivity (b.p.m. and mm Hg^{-1}) determined by the modified Oxford method using intravenous injection of Sodium Nitroprusside (NPS) before administration of TEMPOL (**a**) and after administration of TEMPOL (**b**), and of Phe before administration of TEMPOL (**c**) and after administration of TEMPOL (**d**) of all the groups. $^{*}P<0.05$, when compared with the SHAM group and $^{\#}P<0.05$, when compared with the 2K1C group. Data are presented as mean ± s.e.m.

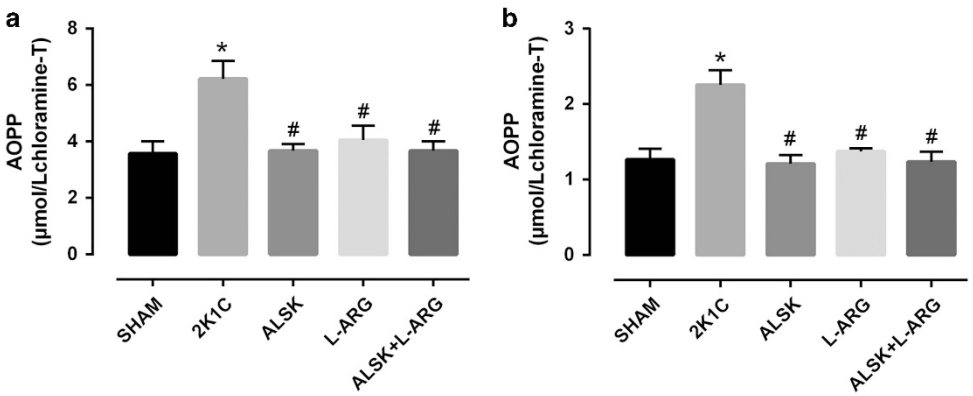

Figure 2 Effects of aliskiren (ALSK), L-arginine (L-ARG) or aliskiren plus L-arginine (ALSK+L-ARG) treatment on the advanced oxidation protein products (AOPP) levels in plasma (**a**) and left ventricle (**b**) in two-kidney, one-clip (2K1C) rats. $^{*}P<0.05$, when compared with the SHAM group and $^{\#}P<0.05$, when compared with the 2K1C group. Data are presented as mean ± s.e.m.

Analysis of oxidative stress by dihydroethidium fluorescence

Analysis of superoxide formation showed a significant increase in the fluorescence of the 2K1C, ALSK and L-ARG groups compared with that of the SHAM group. However, treatment with ALSK and L-ARG decreased these values compared with those of the 2K1C group. In addition, the ALSK+L-ARG

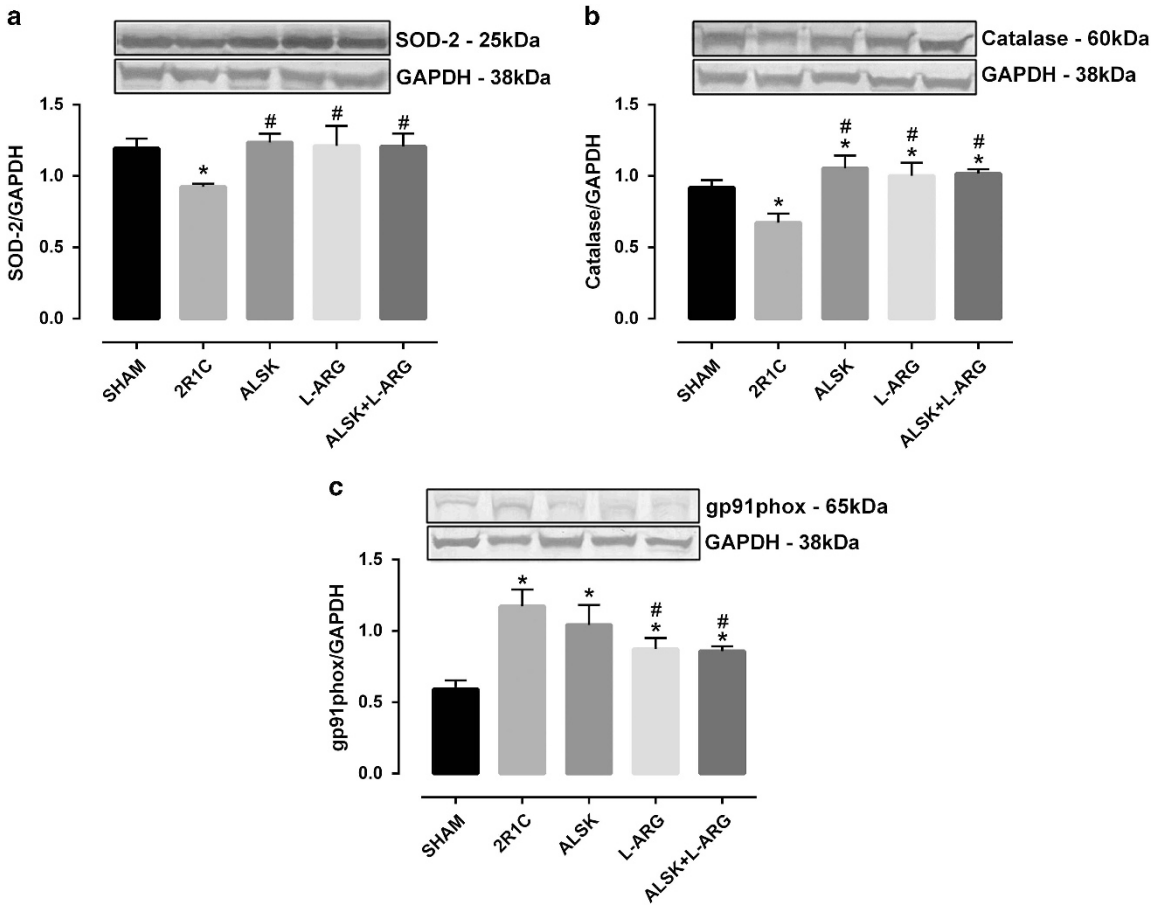

Figure 3 Effects of aliskiren (ALSK), L-arginine (L-ARG) or aliskiren plus L-arginine (ALSK+L-ARG) treatment on the densitometric analyses of western blots for superoxide dismutase (SOD)-2 (**a**), catalase (CAT) (**b**) and gp91phox (**c**) in two-kidney, one-clip (2K1C) rats. $^{*}P<0.05$, when compared with the SHAM group and $^{\#}P<0.05$, when compared with the 2K1C group. Data are presented as mean ± s.e.m.

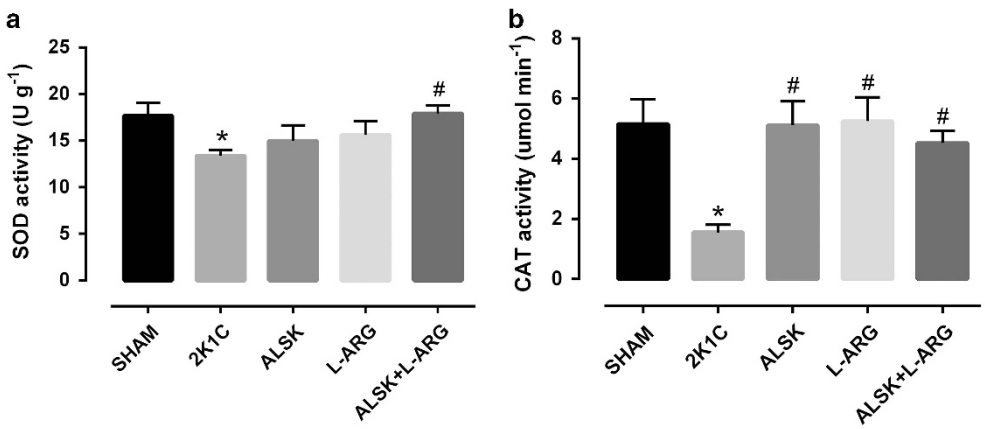

Figure 4 Effects of aliskiren (ALSK), L-arginine (L-ARG) or aliskiren plus L-arginine (ALSK+L-ARG) treatment on the enzymatic activity of catalase (CAT) (**a**) and superoxide dismutase (SOD) (**b**) in the left ventricle in two-kidney, one-clip (2K1C) rats. $^{*}P<0.05$, when compared with the SHAM group and $^{\#}P<0.05$, when compared with the 2K1C group. Data are presented as mean ± s.e.m.

group showed similar values to the SHAM group, as shown in Figure 5.

DISCUSSION

The main findings of the present study were that treatment with ALSK or L-ARG reduced oxidative stress and restored reduced baroreflex sensitivity in renovascular hypertension. In addition, the treatments were able to normalize blood pressure and reverse left ventricular hypertrophy when used in combination.

Renovascular hypertension is caused by increased generation of Ang II owing to increased renal renin release. Therefore, excess Ang II production via several different effector pathways is at least partially responsible for the establishment and development of hypertension and left ventricular hypertrophy,[9,15] and for reduced baroreflex

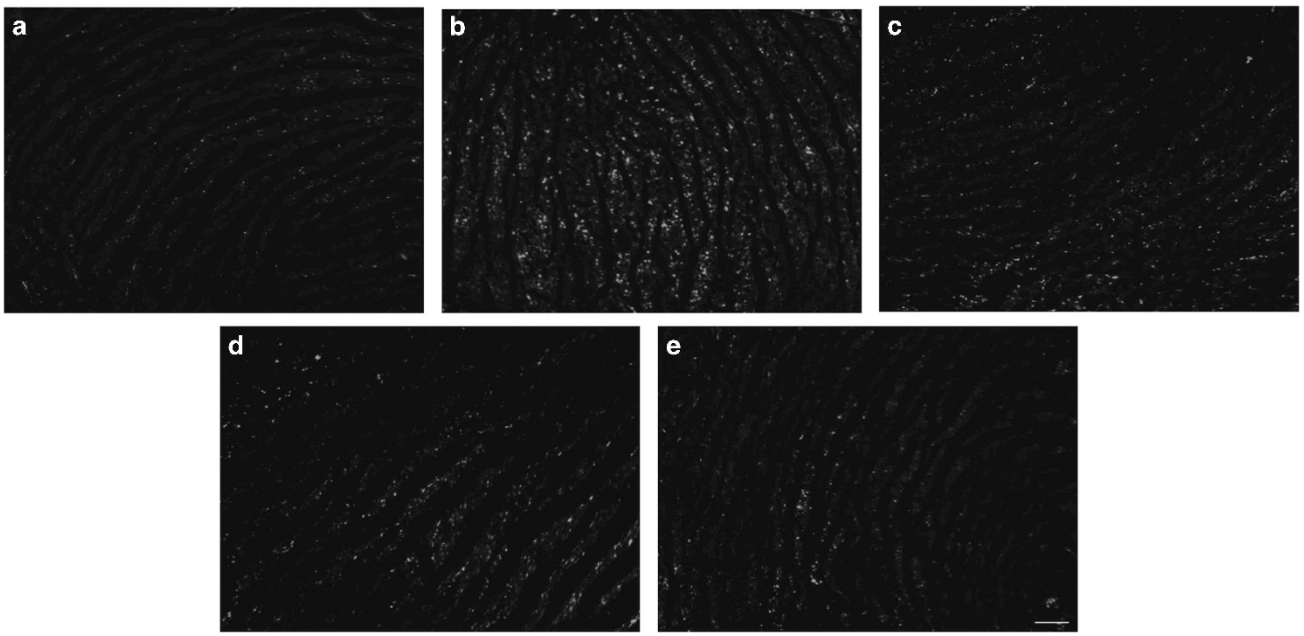

Figure 5 Effects of aliskiren (ALSK), L-arginine (L-ARG) or aliskiren plus L-arginine (ALSK+L-ARG) treatment on the superoxide formation in sections of cardiac tissue by the dihydroethidium fluorescence. Representative images of the SHAM (**a**), two-kidney, one-clip (2K1C) (**b**), ALSK (**c**), L-ARG (**d**) and ALSK +L-ARG (**e**) groups. Data are presented as mean ± s.e.m. $^{*}P<0.05$, when compared with the SHAM group and $^{\#}P<0.05$, when compared with the 2K1C group. Data are presented as mean ± s.e.m. Bar: 50 μm. A full color version of this figure is available at *Hypertension Research* online.

sensitivity.[3] The inhibition of renin with ALSK could therefore contribute to reducing the blood pressure levels in the rat model used in our study. However, we found that ALSK monotherapy did not reduce SBP or MAP. This result must be considered carefully because studies using the same dose found reductions in blood pressure only after 4 weeks of treatment,[26] and with lower hypertension level than in the present work. In contrast, treatment with higher doses showed that ALSK reduced heart rate and blood pressure.[33] Moreover, monotherapy with L-ARG was able to reduce, but not normalize SBP, as observed in this study and in previous studies from our laboratory.[21] However, the combination of these therapies normalized the blood pressure of hypertensive rats. The ALSK, L-ARG and ALSK+L-ARG treatments were able to reduce diastolic blood pressure after 21 days, demonstrating the importance of these therapies for controlling blood pressure.

Renovascular hypertension promoted left ventricular hypertrophy in the 2K1C group, which could be explained by an increase in Ang II, which in turn exerted an inotropic effect and promoted the proliferation and hypertrophy of cardiac fibroblasts, leading to myocyte hypertrophy.[34,35] ALSK treatment did not reduce cardiac hypertrophy. Other studies have suggested an explanation that direct blocking of renin reduces the ability to degrade angiotensinogen and to produce Ang I but does not inhibit the pro-fibrosis signal induced by the renin/pro-renin receptor.[24,36] In contrast, higher doses of ALSK, than those used in this study, could reduce myocyte apoptosis, revealing effective cardioprotection by ALSK.[33] In the heart, gp91phox has a key role. It has been previously demonstrated that the activation of AT1 receptor induces an enhancement in superoxide production by NADPH oxidase, causing hypertrophy by a mechanism dependent on Akt and Rac-1 in conjunction with gp91phox activation,[37,38] contributing to the permanence of hypertrophy in the ALSK group. However, the groups treated with L-ARG did not show left ventricular hypertrophy, suggesting that the progression of cardiac damage caused by renovascular hypertension was prevented.

The baroreflex is an autonomic reflex designed to buffer beat-to-beat fluctuations in arterial blood pressure.[27] In addition, Tsyrlin *et al.*[39] suggested that arterial baroreflex is involved in the long-term control of blood pressure, and another study showed that the deactivation of carotid body chemoreceptors does decrease blood pressure.[40] Further, the activity of the renal sympathetic nerves responsible for the regulation of sodium excretion by the kidney seems to be at least partly modulated by the long-term effects of the arterial baroreflex. Several studies have shown that the sensitivity of the baroreflex is diminished in several forms of hypertension.[41–43] Previously, Moyses *et al.*[3] demonstrated that with 7 days of the 2K1C hypertension model, the rats presented with hypertension and impaired the gain in baroreflex, emphasizing the importance of both treatments in restoring the damaged baroreflex caused by hypertension, considering that treatment was initiated after 7 days of renovascular hypertension. In addition, several studies have shown that oxidative stress is a possible cause of hypertension, based on a variety of mechanisms.[44–46] According to this study, the administration of antioxidants had no effect on baroreflex function in normotensive animals, as observed in other studies,[47,48] but improved the baroreflex in hypertensive rats. These data suggested that antioxidant therapy in the absence of oxidative stress had no influence on baroreflex sensitivity. Mutually, the results from the present study supported the insights that renovascular hypertension promotes oxidative stress, which reduces baroreflex sensitivity, and that treatment with ALSK or L-ARG could restore this sensitivity.

Although it is not possible to determine the precise mechanism by which therapy with ALSK or L-ARG exerted its positive influence on baroreflex function, recent evidence suggests that the improvement in baroreflex sensitivity observed in renovascular hypertension rats was caused by the improvement in autonomic function associated with a reduction in oxidative stress.[41] In addition, evidence from other animal studies has suggested that diminished baroreflex sensitivity was caused by endothelial dysfunction.[49] In particular, in

experimentally induced endothelial dysfunction, a decrease in prostacyclin and increase in thromboxane concentrations were associated with reduced baroreflex impulses from the carotid artery.[47] Moreover, experimental evidence has strongly suggested a direct suppressive influence of ROS on baroreceptors (that is, a peripheral site of action).[50]

Our previous results demonstrated that oral administration of ALSK and L-ARG normalized renal sympathetic nerve activity and SBP, suggesting that the Ang II and NO are involved in the enhanced sympathetic afferent reflex in renovascular hypertensive rats.[51] In addition, in a previous study we suggested that treatment with ALSK +L-arg was effective in releasing an endothelium-derived relaxation factor. Thus, the combination of drugs appeared to restore the endothelial dysfunction induced by the 2K1C model.[52] We already concluded that this new treatment proposal could reduce blood pressure levels, in addition to improving renal and cardiac function and sympathetic activity, and preventing endothelial dysfunction.[51,52] However, this report was the first to document the effectiveness of this treatment on baroreflex sensitivity in hypertensive rats.

The NO has been suggested to have an important role in autonomic and baroreflex control in humans and experimental animals.[53–55] Because reductions in NO bioavailability might be caused primarily by oxidative stress, it is possible that reduced bioavailability of NO might contribute to depressed levels of baroreflex sensitivity in renovascular hypertensive rats, secondary to increased levels of oxidative stress. Considering that we did measure NO bioavailability in the present study, we can only speculate that our treatment with L-ARG and ALSK might have increased baroreflex sensitivity, secondary to the increased bioavailability of NO.

We interpret the increased gp91phox expression as a likely indication that the production of ROS was increased, although dihydroethidium was increased with 2K1C. It has already been established that reductions in CAT and SOD promoted increased ROS; moreover, Ang II also affected antioxidant enzymes, promoting their reduction. Studies have demonstrated that the increase in antioxidant enzymes improved baroreflex sensitivity;[41,48] thus, we believe that treatment with L-ARG and ALSK increased the antioxidant enzymes protecting the endothelium from the action of ROS.

Oxidative stress is defined as an imbalance between pro and antioxidant systems that favor the former and causes cellular damage via an increase in ROS formation. NADPH oxidase is one of the main sources of superoxide production. This complex possesses two membrane-bound subunits (Gp91phox and p22phox), as well as more cytosolic subunits, which regulate and organize the complex in the membrane, thereby enhancing its activity and producing superoxide.[55] Hypertension is associated with increased vascular oxidative stress; however, debate persists regarding whether oxidative stress is a cause or a result of arterial hypertension

Considering that Ang II is an important and potent mechanism leading to the activation of NAD(P)H oxidase, the 2K1C Goldblatt model in rats, which is an Ang II-dependent model of experimental hypertension, has been used to investigate the relationships among Ang II, oxidative stress and hypertension.[9,41] Previous reports have suggested that baroreflex sensitivity is reduced during hypertension, and the mechanisms underlying its reduction involve ROS.[50]

The amount of oxidative stress was assessed by measuring the AOPP levels in plasma and cardiac tissue, and the level of endogenous antioxidant enzymes (SOD and CAT) and oxidant enzyme (gp91phox). The present study exhibited a significant increase in the AOPP levels, accompanied by significant reductions in the activity and expression of SOD and CAT, as well as increased gp91phox expression, in the cardiac tissue with 2K1C hypertension, in agreement with earlier studies.[8,9,41] These findings suggested that enhanced ROS could be one of the mechanisms through which 2K1C hypertension induced an increase in blood pressure, a reduction in sensitivity baroreflex and other functional and structural alterations of the target organs. Treatment with ALSK and L-ARG decreased AOPP levels, increased SOD expression, and CAT expression and activity. However, the SOD activity increased in only the group treated with the ALSK plus L-ARG. ROS production was demonstrably increased in the 2K1C group and decreased after treatment with ALSK or L-ARG, as demonstrated by dihydroethidium fluorescence. In addition, the association of treatments was able to normalize the values. We suggest that treatment with L-ARG was able to reduce the reactive species, and this route resulted in pressure control, as well as in heart protection, thus preventing hypertrophy.

In summary, we reported that treatment with ALSK or L-ARG restored baroreflex sensitivity in renovascular hypertensive rats. In addition, oxidative stress seemed to have an important role in the blunted baroreflex sensitivity observed in renovascular hypertension. The precise site of action where these treatments produced their beneficial effects of ameliorating baroreflex sensitivity is unknown. However, the increases in the expression and activity of antioxidant enzymes, as well as the reduction in the expression of oxidant enzymes and the decrease in AOPP levels, might have contributed to restoring the sensitivity baroreflex in renovascular hypertension rats.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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