Aldosterone Contributes to Sympathoexcitation in Renovascular Hypertension

Gisele S. Lincevicius,1 Caroline G. Shimoura,1 Erika E. Nishi,1 Juliana C. Perry,2 Dulce E. Casarini,3 Guiomar N. Gomes,1 Cássia T. Bergamaschi,1 and Ruy R. Campos1

BACKGROUND
Although angiotensin II (Ang II) is essential to the development of renovascular hypertension, aldosterone plays a role as well. Recent studies have demonstrated a cross-talk between Ang II type 1 and mineralocorticoid receptors in the brain and kidneys. However, the role of aldosterone in the autonomic and renal dysfunction of renovascular hypertension is not well understood.

AIM
The current study evaluated whether aldosterone contributes to cardiovascular and renal dysfunction in the 2 kidney-1 clip (2K1C) model.

METHODS
Mean arterial pressure (MAP) and baroreceptor reflex for control of the heart rate were evaluated in 2K1C treated or not treated with spironolactone (200 mg/kg/day, 7 days). Tonic and reflex control of renal sympathetic nerve activity (rSNA) were assessed in urethane-anaesthetized rats. Plasma renin activity (PRA), kidney renin protein expression, renal injury, and central AT1 receptor protein expression were assessed.

RESULTS
Spiro reduced MAP (198 ± 4 vs. 170 ± 9 mm Hg; P < 0.05), normalized rSNA (147 ± 9 vs. 96 ± 10 pp/s; P < 0.05), and increased renal baroreceptor reflex sensitivity in the 2K1C rats. Spiro reduced α-smooth muscle actin expression in the nonclipped kidney in the 2K1C group (5 ± 0.6 vs. 1.1 ± 0.2%; P < 0.05). There was no change in PRA; however, a decrease in renin protein expression in the nonclipped kidney was found in the 2K1C treated group (217 ± 30 vs. 160 ± 19%; P < 0.05). Spiro treatment decreased AT1 receptor in the central nervous system (CNS) only in 2K1C rats (138 ± 10 vs. 84 ± 12%; P < 0.05).

CONCLUSION
Aldosterone contributes to autonomic dysfunction and intrarenal injury in 2K1C, these effects are mediated by the CNS.

Keywords: baroreflex dysfunction; blood pressure; hypertension; renal injury; renin–angiotensin–aldosterone; renovascular hypertension; sympathoexcitation.

doi:10.1093/ajh/hpu300

Renovascular hypertension is a prevalent form of secondary hypertension, and its treatment remains challenging.1 The 2 kidney-1 clip (2K1C) model has been widely used to study renovascular hypertension experimentally. Renovascular hypertension is induced by partial unilateral occlusion of the renal artery, leading to renin secretion and, consequently, hyperactivation of the renin–angiotensin–aldosterone system (RAAS) and arterial hypertension.2 In the initial phase of the renovascular hypertension, RAAS activation is important to trigger blood pressure and other mechanisms involved in the cardiovascular and renal alterations. However, after this initial phase, the plasma renin comes back to normal and an increase in the sensitivity to angiotensin II (Ang II) appears to play an important role. In fact, in the intermediated phase of renovascular hypertension (5–6 weeks after clipping), the hypertension has a major neurogenic component and RAAS is upregulated.3,4 Ang II is believed to be the primary hormone in the pathophysiology of renovascular hypertension.5 Ang II acts on the Ang II type 1 receptor (AT1R) to induce a series of effects that impact the cardiovascular system, including increases in oxidative stress, baroreflex dysfunction, and sympathoexcitation.6,7 Indeed, knockout of central AT1R in brain areas related to cardiovascular control (e.g., rostral ventrolateral medulla (RVLM)) normalized blood pressure in 2K1C rats.3 Although Ang II is essential for the maintenance of high blood pressure in renovascular hypertension, aldosterone also plays a role. The stimulation of aldosterone secretion from the adrenal cortex by Ang II is well known. Recent studies have demonstrated powerful cross-talk between AT1R and mineralocorticoid receptor (MR).8–10 Indeed, the nongenomic and genomic effects of aldosterone are differentially dependent on the activity of 2 Ang II receptors, AT1R types a and b.8 In addition, the hypertensinogenic actions of Ang II are
potentiated by preconditioning with subpressor infusions of Ang II or aldosterone. However, the role of aldosterone in the autonomic and renal dysfunction of the 2K1C model is not well understood.

Aldosterone causes oxidative stress and cardiovascular and renal changes similar to those associated with RAAS activation. However, quantifying and differentiating the roles of Ang II and aldosterone has been difficult due to the fact that Ang II and aldosterone may act synergistically. It is known that Ang II leads to aldosterone secretion and aldosterone would decrease plasma renin and consequently decrease Ang II, whereas aldosterone is also able to stimulate the synthesis of renin and angiotensin-converting enzyme independent of Ang II or AT1 receptor. Furthermore, aldosterone increases the binding of Ang II to AT1R.

Several findings suggest that Ang II is not the only cause of cardiovascular and renal dysfunction in the 2K1C model. For example, blockade of the AT1R with losartan does not completely reverse the hypertension in the 2K1C model, and aldosterone may be secreted independently of AT1R. Considering these previous findings, we tested the hypothesis that aldosterone contributes to cardiovascular, autonomic, and renal dysfunction in renovascular hypertensive rats. To test this hypothesis, we studied 2K1C rats either treated with subchronic spironolactone or left untreated.

METHODS

All experimental protocols were approved by the Research Ethics Committee of the Federal University of São Paulo (process no. 0189/10). Male Wistar rats (150–180 g) were obtained from the central animal house of UNIFESP.

Experimental protocols

Four experimental groups were used: intact untreated rats (CT), intact rats that received spironolactone, rats with a renal clip (2K1C) that received no spironolactone, and rats with a renal clip that received spironolactone. To induce renovascular hypertension, a renal clip (0.2 mm gap) was placed on the left renal artery in rats anesthetized with ketamine and xylazine (40 and 20 mg/kg, intraperitoneal (i.p.); Vetbrands, Jacareí, São Paulo, Brazil), as previously described. Spironolactone (Tianjin Tianyao Pharmaceuticals, Tianjin, China) was dissolved in water and administered by oral gavage for 7 consecutive days in control rats or starting 5 weeks after renal clip placement in 2K1C, at a dose of 200 mg/kg/day. In the first experimental series, the metabolic, cardiovascular, and autonomic parameters were measured. After 5 days of spironolactone treatment, the rats were placed in metabolic cages for 24 hours, and water intake, urine volume, urinary sodium, and potassium content were measured (electrolyte analyzer ABL 800 Flex; Radiometer, Denmark). After 7 days of spironolactone treatment, the resting blood pressure, heart rate, and the cardiac baroreflex sensitivity were assessed in conscious rats. Then, the basal renal sympathetic nerve activity (rSNA) and the renal baroreflex sensitivity were measured in urethane-anesthetized rats.

In the second experimental series, after 1 week of treatment, the animals were euthanatized and plasma, kidneys, and brain were collected. In this experimental series, we evaluated plasma renin activity (PRA), renin protein expression in renal tissue, AT1R protein expression in the RVLM, and a renal injury marker.

Measurement of cardiac baroreflex sensitivity

Bradycardic and tachycardic reflex responses produced by bolus infusion of phenylephrine (1, 2, and 3 µg/kg, intravenous (i.v.)) and sodium nitroprusside (2, 5, and 7 µg/kg; i.v.) (Sigma-Aldrich, St Louis, MO) were measured. The cardiac baroreflex was evaluated by the mean index relating changes in heart rate to changes in mean arterial pressure (MAP) and expressed as beats per mm Hg.

Measurement of renal nerve activity and renal baroreflex sensitivity

Rats were slowly anesthetized with urethane (1.2–1.4 g/kg, i.v.; Sigma-Aldrich). A tracheotomy was performed to reduce airway resistance. The left renal nerve was exposed through a dorsal approach and placed on bipolar silver electrodes. The signal was amplified (2K) and filtered (100–1000 Hz band-pass filter) (Neurolog, Digitimer, UK), as previously described.

Baroreceptor control of rSNA was evaluated by intravenous infusion of vasoactive drugs. Phenylephrine (10 µg, 0.2 ml) or sodium nitroprusside (20 µg, 0.1 ml) was infused for 1 minute. The values of the MAP variations matching the reflex rSNA responses were plotted separately for each vasoactive drug to create linear regression curves of baroreceptor function for each group, and their slopes (spikes/second/mm Hg) were compared to evaluate changes in baroreflex sensitivity.

At the end of the experiments, the renal nerve background noise level was determined following hexamethonium bromide administration (30 mg/kg, i.v.; Sigma-Aldrich).

Measurement of PRA

The blood samples were collected in dry tubes and the plasma was used to measurement of renin activity. The PRA was measured by reverse phase high-performance liquid chromatography, as previously described.

Protein expression of renal renin and AT1R in the RVLM

About 20 µg of total renal protein and bilateral punches of RVLM were placed on 10% polyacrylamide gel and subjected to electrophoresis at 120 V for 90 minutes (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in a mini-protein Bio-Rad system (Bio-Rad Laboratories). Proteins were transferred to a nitrocellulose membrane at 230 mA for 2 hours. Nonspecific sites were blocked with 1% bovine serum albumin for 6 hours. Membranes were incubated overnight at 4 °C under agitation with anti-renin antibody (sc-133145, 1:500; Santa Cruz Biotechnology) or anti-AT1 receptor.
antibody (DY-800, 1:1,000; Proteimax, Brasil). Actin was used as a loading control and was measured with anti-actin antibody (1:5,000; Sigma-Aldrich). Proteins were labeled with anti-fluorescent secondary antibody (IRDye; Li-Cor Biosciences) and quantified using an infrared method (Odyssey Infrared System; Li-Cor Biosciences).

**Characterization of renal injury by immunohistochemistry**

The fixed renal tissue (Bouin’s liquid) was dehydrated, embedded in paraffin. About 5-µm sections were deparaffinized, hydrated, and subjected to peroxidase blockade. The sections were incubated overnight at 4 °C with anti-alpha smooth muscle actin antibody (α-SMA, 1:1,000; DAKO, Denmark). The reaction product was determined by 1-way analysis of variance, followed by Tukey’s post hoc test. In the healthy kidney, the expression of α-SMA was limited to arteries and arterioles; its presence in the interstitium and glomeruli suggests nephropathy.

**Table 1.** Effect of spironolactone treatment on water and electrolyte balance in intact control rats and in rats with a renal clip

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Spiro</th>
<th>2K1C</th>
<th>2K1C + Spiro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>334 ± 18 (6)</td>
<td>310 ± 14 (6)</td>
<td>288 ± 6 (6)</td>
<td>302 ± 19 (6)</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>32 ± 1 (11)</td>
<td>26 ± 2 (11)</td>
<td>47 ± 1 (14)</td>
<td>52 ± 3 (11)</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>14 ± 0.7 (9)</td>
<td>12 ± 0.4 (10)</td>
<td>37 ± 2 (12)</td>
<td>38 ± 4 (9)</td>
</tr>
<tr>
<td>Plasma [Na⁺] (mmol/l)</td>
<td>136 ± 1 (6)</td>
<td>137 ± 0.4 (6)</td>
<td>139 ± 1 (6)</td>
<td>137 ± 0.8 (6)</td>
</tr>
<tr>
<td>Na⁺ excretion (mmol/day)</td>
<td>1.8 ± 0.2 (6)</td>
<td>1.3 ± 0.06 (6)</td>
<td>1.5 ± 0.3 (6)</td>
<td>1.7 ± 0.1 (6)</td>
</tr>
<tr>
<td>Plasma [K⁺] (mmol/l)</td>
<td>5.3 ± 0.2 (6)</td>
<td>5.9 ± 0.2 (6)</td>
<td>4.8 ± 0.1 (6)</td>
<td>5.7 ± 0.2 (6)</td>
</tr>
<tr>
<td>K⁺ excretion (mmol/day)</td>
<td>5.8 ± 0.6 (6)</td>
<td>4.6 ± 0.2 (6)</td>
<td>5.4 ± 0.7 (6)</td>
<td>6.9 ± 0.1 (6)</td>
</tr>
</tbody>
</table>

Number of subjects are given within parenthesis.
Abbreviations: 2K1C, 2 kidney-1 clip; Spiro, spironolactone.
*Different from control.
+Different from 2K1C, 1-way ANOVA with Tukey’s post hoc test.

**Figure 1.** Effects of spironolactone treatment on resting blood pressure, HR, and rSNA. (a) MAP, *P = 0.0005 vs. control; **P = 0.0007 vs. 2K1C (Kruskal-Wallis with Mann-Whitney U-post hoc test). (b) HR. (c) rSNA, *P < 0.05 vs. control; **P < 0.05 vs. 2K1C (1-way ANOVA with Tukey’s post hoc test). Abbreviations: 2K1C, 2 kidney-1 clip; ANOVA, analysis of variance; CT, control; HR, heart rate; MAP, mean arterial pressure; rSNA, renal sympathetic nerve activity; SPIRO, spironolactone.
a renal clip (from 343 ± 14 bpm, n = 11, to 411 ± 14 bpm, n = 11; Figure 1b) but was not altered by spironolactone.

The renal clip increased resting rSNA (from 96 ± 11 pps, n = 6, to 147 ± 9 pps, n = 6). Spironolactone normalized resting rSNA in hypertensive rats (96 ± 10 pps, n = 8) but had no effect in normotensive rats (Figure 1c).

**Effects of spironolactone on baroreflex sensitivity**

Cardiac baroreflex sensitivity was reduced in rats with a renal clip (Figure 2a). Spironolactone did not change the cardiac baroreflex sensitivity in rats with a renal clip but increased the sensitivity in controls. Renal baroreflex sensitivity was reduced in rats with a renal clip. Spironolactone increased baroreflex sensitivity in both control and hypertensive rats (Figure 2b).

**Effects of spironolactone on activity renin plasma, renal renin, and α-SMA expression**

Six weeks after implantation of the renal clip, PRA was normal (Figure 3a). Spironolactone did not alter PRA in rats with a renal clip or in controls. The renin expression was increased in both clipped (CT = 100 ± 7, n = 6; 2K1C = 159 ± 4%, n = 6) and contralateral kidneys (CT = 100 ± 7, n = 6; 2K1C = 217 ± 30%, n = 5) in the hypertensive rats. Spironolactone did not change renin expression in the clipped kidney (Figure 3b) but reduced expression in the contralateral kidney of hypertensive rats (2K1C + Spiro = 160 ± 19%, n = 5 vs. 2K1C, respectively) (Figure 3c).

The renal clip increased the expression of α-actin in both the clipped (2.7 ± 0.2%, n = 4) (Figure 4a) and contralateral kidneys (5 ± 0.6%, n = 4) (Figure 4b, C1, and C2). Spironolactone reduced α-actin levels only in the contralateral kidney (1.1 ± 0.2%, n = 3 vs. 2K1C, respectively) (Figure 4b, C3, and C4). Spironolactone did not alter renal α-SMA levels in control rats. Figure 4c shows photomicrographs of α-SMA labeling in the contralateral kidney in all the experimental groups.

**Effects of spironolactone on AT1R protein expression in the RVLM**

AT1R protein expression was increased within the RVLM in 2K1C (38%, n = 8). Spironolactone treatment reduced the AT1R within the RVLM only in 2K1C (16%, n = 5) (Figure 5).

**DISCUSSION**

Although the 2K1C model is classically regarded as an Ang II-dependent model of hypertension, the present study showed that aldosterone powerfully contributes to the autonomic and renal dysfunction in this model. Our main findings were that mineralocorticoid blockade in the hypertensive animals (i) reduced blood pressure, (ii) normalized rSNA, (iii) improved the sensitivity of the renal baroreflex function, (iv) reduced renal renin protein expression, (v) reduced renal injury, and (vi) reduced AT1R within the RVLM. The autonomic dysfunctions were accompanied by intrarenal changes in hypertensive rats, and both were partially or totally reversed by using a MR antagonist.

In a previous study by our group, the blockade of the AT1R receptor failed to completely normalize the increase in blood pressure in 2K1C rats. The present study suggests that aldosterone contributes to the maintenance of hypertension, sympathoexcitation, and baroreceptor dysfunction in this model.

In this study, spironolactone reduced blood pressure, independent of changes in urine volume, urinary excretion of sodium and potassium, or plasma sodium. The 2K1C model is known to be independent of volume expansion and vasopressin secretion because the remaining intact kidney functions as an escape valve to normalize accumulating volumes and rising pressure. Thus, it is unlikely that spironolactone decreases blood pressure by volume-dependent mechanisms. Furthermore, considering that no changes in PRA were found after spironolactone administration in our study, the cardiovascular and renal actions of Ang II are preserved.

The depressor response induced by spironolactone may be associated with the reduction of sympathetic vasomotor activity. Indeed, spironolactone administration reduces sympathetic hyperactivity in humans and in rats. The increase in sympathetic nerve activity and induction of hypertension with the systemic infusion of aldosterone has been known for many years, and such effects are mediated by MR located within the central nervous system (CNS). For example, the intracerebroventricular infusion of aldosterone in rats increases blood pressure and rSNA, apparently by increasing oxidative stress. Furthermore, the progressive hypertension caused by a chronic increase in circulating aldosterone can be prevented by specific blockade.
Aldosterone and Sympathoexcitation

In addition, several studies have shown that the central or peripheral administration of mineralocorticoid antagonists such as spironolactone, eplerenone, and RU28318 prevents or reverses both the sympathoexcitation and the magnitude of hypertension and their complications in both humans and animals.16,27,28

In the present study, spironolactone normalized the rSNA in the 2K1C model. The mechanisms underlying the sympathoexcitation induced by aldosterone are unclear; however, we cannot exclude the possibility that aldosterone acts on brain receptors. We did not measure whether spironolactone reduced sympathetic activity to nonrenal tissues in 2K1C rats. However, mineralocorticoid blockade reduces muscular SNA in hypertensive patients27 and cardiac sympathetic innervation in rats after myocardial infarction.28 In the present study, spironolactone normalized rSNA but not MAP which suggests a specific or a preferential reduction of renal sympathetic activity, as previously described.29

The reduction in hypertension and sympathoexcitation following spironolactone treatment were associated with an improvement in the baroreflex sensitivity. The fact that acute and chronic peripheral aldosterone administration impairs baroreceptor responses in dogs30 and in humans31 is well known. In addition, microinjection of the mineralocorticoid antagonist eplerenone into the nucleus of the tractus solitarius improves the baroreflex sensitivity in sodium-loaded rats, and this effect was mediated by aldosterone-sensitive

of MR in the CNS.10 In addition, several studies have shown that the central or peripheral administration of mineralocorticoid antagonists such as spironolactone, eplerenone,
HSD2 neurons, which suggest a central action of aldosterone in the control of the baroreflex function. The present study supports these findings and suggests that a similar mechanism may occur in renovascular hypertension.

Lesion of anteroventral third ventricle (AV3V) attenuates the development and prevents the maintenance of hypertension in the 2K1C model, suggesting a strong neurogenic component in this model. It is already known that the CNS has all components of RAAS with local production of Ang II as well as aldosterone. Furthermore, circulating Aldo can access many brain regions, and evidence indicates that aldosterone may be synthesized de novo in the CNS, in the brain, increased mineralocorticoid levels may lead to generation of a hypertensinogenic viscous cycle by accelerating the activation of pressor components of the central RAAS.

We found previously that the activation of AT1R within the CNS is a major mechanism leading to sympathoexcitation and hypertension. In the present study, we described that spironolactone treatment was able to reduce AT1R expression protein in the brain. These results support our hypothesis that spironolactone effects are mediated by the CNS. It has been shown that Ang II and aldosterone may interact in the CNS and contribute to hypertension. In this way, there is an increase in sensitivity to Ang II in the intermediate phase of renovascular hypertension. It is possible that spironolactone induced changes in the RAAS in the CNS (specifically in the RVLM), contributing to peripheral effects, such as normalization in the rSNA, increase baroreflex sensitivity, and reduction in blood pressure. We suggest that spironolactone can penetrate blood–brain barrier and inhibit brain aldosterone effects and/or cross-talk between MR and AT1R.

PRA did not change in our 2K1C rats, but expression of renin protein increased in both kidneys, suggesting a hyperactive renal RAAS in 2K1C rats. It is important to emphasize that PRA, a clinical index used to determine systemic RAAS status, is distributed over a wide range in hypertensive subjects. This observation prompts the suggestion that alterations in tissue-specific RAAS, not detected by PRA, may underlie hypertension.

Furthermore, increased renin expression in the clipped kidney is most likely caused by reduced renal blood flow. Considering that spironolactone reduced renin expression in the nonclipped kidney without altering PRA, increased renin expression in the nonclipped kidney is dependent on aldosterone. Studies show that aldosterone stimulates the synthesis of renin and angiotensin-converting enzyme and enhances AT1R mRNA, as well as the binding Ang II to the AT1R. Therefore, the present study suggests that there is a difference in control of renin expression by aldosterone in the clipped and nonclipped kidney. It is important to point that the initial event caused by unilateral renal artery clipping (increased plasma Ang II) could play a role to trigger intrarenal events in the nonclipped kidney increasing the expression levels of renin. However, the exact mechanisms underlying that are not totally understood, our data are in line with other study in the 2K1C model. In phase studied, the hypertension has a strong neurogenic component and we suggested that tissue RAAS is upregulated. In fact, we found a significant increase in intrarenal renin protein expression in 2K1C. In the same way, Sadjadi et al. found increased intrarenal Ang II without any change in the plasma levels at the same phase of 2K1C hypertension we studied.

Finally, in the present study, we found that an aldosterone receptor antagonist reduced renal injury in the hypertensive animals. Previous studies indicate that elevated levels of aldosterone promote fibrosis, leading to renal and cardiovascular dysfunction. Therefore, the renoprotective effect of aldosterone blockade has been attributed to its antifibrotic activity in humans and rats. In fact, in this study, spironolactone reduced fibrosis in the unclipped kidney of 2K1C rats.

In addition, there is evidence that renin is an Ang II-independent profibrotic mediator. For instance, a recent study demonstrated that aliskiren, a direct renin inhibitor, markedly prevented renal end-organ damage due to its antifibrotic and nephroprotective effects in a mouse model of progressive renal fibrosis. In this study, we found that spironolactone reduced renin and fibrosis in the unclipped kidney, whereas there was no change in renin or fibrosis in the clipped kidney of hypertensive rats. This result suggests that the intrarenal renin may mediate renal fibrosis in renovascular hypertension.

In summary, this study demonstrated that spironolactone treatment reduced arterial pressure, normalized rSNA and reduced AT1R in the RVLM. Our results suggest that increased rSNA is a key event in cardiovascular changes in renovascular hypertension and that aldosterone has an important role in the control of rSNA. Furthermore, a different response in the renin expression was found between left and right kidneys after spironolactone treatment, which reduced the local renin expression in the nonclipped kidney, and this was associated with a reduction in renal injury. In conclusion, our results indicate that aldosterone has an important role in the sympathoexcitation, baroreceptor impairment, and renal dysfunction induced by renal artery stenosis.

LIMITATIONS OF STUDY

In the present study, changes in the brain and in the kidneys induced by renovascular hypertension are in part dependent on aldosterone. A limitation of the present study is that we have not measured all components of RAAS in the brain and kidneys that would improve our understanding regarding the mechanisms underlying the cross-talk between aldosterone and Ang II in renovascular hypertension. Furthermore, the exact mechanisms of cross-talk between the brain and the kidneys and relative importance of aldosterone and angiotensin II remain to be determined.

ACKNOWLEDGMENTS

We thank Dr Gus Schoorlemmer for his useful comments and suggestions; these have helped improve this paper. Financial support FAPESP (2010/04333-6; 2010/52180-4; 2013/22522-9); CAPES, and CNPq (472613/2013-8). D.E.C., C.T.B., and R.R.C. are recipients of CNPq fellowships.
DISCLOSURE

The authors declare no conflict of interest.

REFERENCES
