Upregulation of AT$_1$R and iNOS in the Rostral Ventrolateral Medulla (RVLM) Is Essential for the Sympathetic Hyperactivity and Hypertension in the 2K-1C Wistar Rat Model

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BACKGROUND
We hypothesized that upregulation of angiotensin type 1 receptor (AT$_1$R) and inducible nitric oxide (NO) synthase (iNOS) within the rostral ventrolateral medulla (RVLM) could contribute to two-kidney, one-clip (2K-1C) hypertension.

METHODS
The experiments were performed in male Wistar rats, 6 weeks after the renal surgery. The animals were divided into control (SHAM, n = 18) and hypertensive groups (2K-1C, n = 18). Bilateral tissue punches were taken from sections containing the RVLM to perform iNOS gene expression analyses by the real-time PCR technique, and AT$_1$R and iNOS protein expression analyses by western blotting. In addition, we injected losartan (1 nmol), an AT$_1$R antagonist, and aminoguanidine (250 pmol), an iNOS inhibitor, bilaterally into the RVLM to analyze the mean arterial pressure (MAP) and renal sympathetic nerve activity (rSNA).

RESULTS
iNOS mRNA expression levels were greater ($P < 0.05$) in the 2K-1C group compared to the SHAM group. Furthermore, the AT$_1$R and iNOS protein expression were significantly increased in the RVLM of 2K-1C rats compared to SHAM rats. Injection of losartan into the RVLM reduced the MAP (11%) and rSNA (18%) only in the 2K-1C rats, whereas injection of aminoguanidine in the same region decreased the MAP (31%) and rSNA (34%) in hypertensive rats.

CONCLUSIONS
The present study suggests that upregulation of AT$_1$R and iNOS in the RVLM is important in the maintenance of high blood pressure and renal sympathetic activation in 2K-1C hypertension.

Keywords: angiotensin receptors; blood pressure; genes; Goldblatt hypertension; hypertension; nitric oxide; rats; RVLM; sympathetic nervous system

constitutively expressed, but iNOS is expressed only during pathophysiological states such as hypertension, heart failure, endotoxin shock, and in aging. Recent study suggested that NO, specifically nNOS, mediates sympathetic cardiovascular responses through its action in the RVLM. Interactions between NO and Ang II have been observed in the central nervous system; however, the role of iNOS within the RVLM in the regulation of sympathetic outflow is not well established in hypertension.

Therefore, the first aim of the present study was to examine whether AT1R and iNOS are upregulated within the RVLM— the major region involved in the central control of sympathetic vasomotor tone—in the 2K-1C hypertensive model. For this purpose, iNOS mRNA, and AT1R and iNOS protein expression were quantified in the RVLM of 2K-1C hypertensive rats. Furthermore, to test the functional significance of these molecular results, we performed injection of losartan (antagonist of the AT1R receptor) and aminoguanidine (specific inhibitor of iNOS) into the RVLM in renovascular hypertensive rats.

**METHODS**

All experimental procedures were conducted according to the National Institutes of Health guidelines for the use and care of animals, and the study protocol was approved by the Ethics in Research Committee of the Federal University of Sao Paulo School of Medicine (process no. 0662/04). Male Wistar rats (n = 36, 150–180 g) were obtained from the animal care facility of the University. The animals were housed in cages in groups of 5–6 rats with respect to their group (SHAM or 2K-1C), with free access to rat chow and tap water. Animals were maintained in a temperature-controlled environment (23°C) on a 12-h light/dark cycle. The drugs (urethane (ethyl carbamate), hexamethonium, and aminoguanidine: Sigma Chemical, St Louis, MO and losartan: Fluka Analytical, Shanghai, China) were all dissolved in saline (pH 7–7.4).

**Experimental protocols.** The study was divided into five independent series of experiments. In the first series, the iNOS mRNA expression levels in the RVLM were quantified by real-time PCR. In the second series, western blotting was used to quantify the AT1R and iNOS protein expression levels in the RVLM. In these series, the groups were divided into control (SHAM) (n = 5) and 2K-1C rats (n = 5). Series three to five were performed in urethane-anesthetized rats. In a third series of experiments, saline (100 nl) was bilaterally injected into the RVLM in two different groups of animals: (i) SHAM + saline (n = 5); and (ii) 2K-1C + saline (n = 5). This series served as a control for series four and five. In a fourth series of experiments, losartan (1 mmol/100 nl) was bilaterally injected into the RVLM in two different groups of rats: (i) SHAM + losartan (n = 4); (ii) 2K-1C + losartan (n = 5). Finally, in a fifth series of experiments, aminoguanidine (250 pmol/100 nl) was bilaterally injected into the RVLM in two different groups of rats: (i) SHAM + aminoguanidine (n = 4); (ii) 2K-1C + aminoguanidine (n = 3). The cardiovascular parameters analyzed in the last three series of experiments were the mean arterial pressure (MAP), heart rate, and renal sympathetic nerve activity (rRNA).

**Hypertensive animals.** To obtain hypertensive animals, male Wistar rats (150–180 g) were anesthetized with ketamine and xylazine (40 and 20 mg/kg, IP, respectively), and the left renal artery was partially obstructed with a 0.2 mm wide silver clip. The control animals (SHAM) were subjected to the same surgical procedure but without partial renal artery occlusion. All experiments were performed 6 weeks after the renal surgery.

**Extraction of RNA from RVLM.** Rats were euthanized, the brainstem quickly removed, and bilateral punches of RVLM were obtained. Histology was then performed to confirm the localization of the punches (Figure 1b).

**Quantitative real-time PCR.** The RVLM region was homogenized in 10 μl of 10% Triton and RNAsin (Invitrogen, Carlsbad, CA). To avoid contamination with genomic DNA, the RNA samples were treated with DNase (RQI RNase-free DNase; Promega, Madison, WI). Genomic contamination was excluded by performing PCR with samples that were not treated with reverse transcriptase. The following primer sequences were used: β-actin, forward (CCT-CTA-TGC-CAA-CAC-AGT-GC), reverse (ACA-TCT-GCT-GGA-AGG-TGG-AC); iNOS, forward (AGG-TGT-TCA-GGG-TGC-TCC-AC), reverse (AGT-TCA-GCT-TGG-CGG-CCA-CC). The sizes of the PCR products amplified with the primers were as follows: β-actin, 191 bp; and iNOS, 114 bp. Real-time amplification was carried out using a RotorGene 600 (Uniscience, Sydney, Australia).

**Western blotting.** The total protein present in the RVLM samples was subjected to electrophoresis in 8% SDS-PAGE gels (Bio-Rad, Hercules, CA). Separated proteins were transferred onto a nitrocellulose membrane (0.20 mm). The blots were then blocked with 5% nonfat dry milk for 6 h, followed by incubation with anti-AT1 Dy800 (1:100,000), anti-iNOS (1:1,200), and anti-β-actin (1:1,000) at 4°C for 18 h. Blots were visualized using the secondary antibody at a fluorescence emission of 700 or 800 nm. The band intensity obtained by western blotting was measured using the Odyssey system (Li-COR Biosciences, Lincoln, NE). The antibodies used were anti-AT1 (Proteimax Biotecnologia, Cotia, Brazil), anti-iNOS (GeneTex, Irvine, CA), and anti-β-actin (Proteimax Biotecnologia). Indeed, the second antibody employed was anti-rabbit IgG with a fluorescence at 700 nm at a dilution of 1:10,000.

**Surgical instrumentation for the acquisition of cardiovascular parameters.** Six weeks after the renal surgery, rats were anesthetized with ketamine and xylazine (40 and 20 mg/kg, IP, respectively) and fitted with femoral venous and arterial catheters for drug injection and arterial pressure recording, respectively. After 1 day, the MAP and heart rate were first recorded in conscious rats, and then the rats were slowly anesthetized with urethane (1.2–1.4 g/kg, IP). These procedures were performed in the fifth to fourth series of experiments.
Injection procedures. In the third, fourth, and fifth series of experiments, urethane-anesthetized rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Saline (100 nl), losartan (1 nmol/100 nl), or aminoguanidine (250 pmol/100 nl) were bilaterally injected into the RVLM. An interval of 10–20 s separated the injections between sides in the RVLM. At the end of the experiments, 100 nl of Evans blue dye (2%) was injected into the RVLM, and histology was subsequently performed to confirm injection sites. Figure 1a shows a representative histological coronal view of the dye distribution within the RVLM region.

Analysis of the sympathetic nerve activity. For the rSNA recording, the left renal nerve was retroperitoneally exposed, placed on bipolar silver electrodes, and covered with paraffin oil. The signal from the renal nerve was displayed on an oscilloscope (TDS 220; Tektronix, Beaverton, OR), and the nerve activity was amplified (gain 20K, NeuroLog; Digitimer, Welwyn Garden City, UK), filtered by a band-pass filter (50–1,000 Hz), and collected for display and later analysis using a PowerLab data acquisition system (ADInstruments, Bella Vista, Australia). At the end of the experiments, the background noise level was determined following hexamethonium bromide (30 mg/kg, IV) administration. The rSNA was rectified online, integrated from the raw data obtained for each heart period, and expressed as volts per second. Additionally, the neural activity was analyzed offline using the appropriate software (Spike Histogram; ADInstruments). The responses of rSNA to the various stimuli are expressed as the percentage of change compared to the basal value obtained immediately before each test. For this purpose, the raw nerve signal was passed through a spike discriminator (PowerLab) to remove background noise, and then the total nerve activity expressed in spikes/s was computed from the time at which it changed from the basal value to when it returned to the basal value. The basal rSNA is expressed as spikes/s over a period of 60 s. The mean value obtained was compared to the mean value determined before each test. Only experiments in which the level of background noise was confirmed at the end of the experiments following hexamethonium and terminal anesthesia are included in this report. The clip and rSNA were performed in the same side (left), however not in the same place. The clip was positioned in the left renal artery close to the kidney, and the electrode to record rSNA was positioned at the junction of the aorta and the left renal artery. Considering that the surgery for the clip implantation was performed very carefully to avoid any damage in the renal nerve, therefore, we believe that the integrity of nerve was not affected after clipping.

Statistical analyses. Results are presented as means ± s.e. Data were evaluated by one-way analysis of variance, followed by the Student Newman–Keuls method and the paired Student’s t-test when appropriate. The level of statistical significance was defined as *P* < 0.05. Statistical evaluation was carried out using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

RESULTS

Analysis of the gene and protein expression levels of AT1R and iNOS in the RVLM of 2K-1C and SHAM rats

According to the western blotting results, AT1R protein expression was increased in the RVLM of the 2K-1C rats when compared to SHAM rats (2K-1C, 133 ± 30 vs. SHAM, 100 ± 7%, *P* < 0.05) (Figure 2b). This data corroborate with the levels of AT1R gene expression in the RVLM shown previously in our laboratory8 (Figure 2a).

Concerning iNOS, the gene expression was significantly higher in 2K-1C rats (58.3 ± 16.5 arbitrary units) compared to SHAM rats (1.0 ± 0.1 arbitrary units) (*P* < 0.02) in the RVLM, as shown in Figure 2c. The increase was also observed for the protein expression, with values of 209 ± 26% and 100 ± 7% in the 2K-1C and SHAM rats in the RVLM, respectively (*P* < 0.04) (Figure 2d).

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**Figure 1** | Representative histological coronal view of the brain stem. Typical injection site in the RVLM as evaluated by (a) 100 nl of Evans blue diffusion, (b) bilateral punches, and (c) a schematic representation. CST, corticospinal tract; ION, inferior olivary nucleus; NA, nucleus ambiguous; NTS, nucleus of the tractus solitarii; RVLM, rostral ventrolateral medulla; STN, spinal trigeminal nucleus.
original contributions

AT1R Receptor and iNOS in the RVLM

Effects of losartan injection into the RVLM of urethane-anesthetized rats

Six weeks after the renal surgery, the 2K-1C group displayed a significant increase in MAP and rSNA, as compared to the SHAM group (2K-1C, 193 ± 8 mm Hg; SHAM, 115 ± 5 mm Hg and 2K-1C, 152 ± 4 spikes/s; SHAM 115 ± 5 spikes/s, respectively, *P* < 0.05). Heart rate and body weight did not differ between the groups.

In hypertensive rats, losartan decreased MAP by 6 ± 4% (from 180 ± 8 to 168 ± 8 mm Hg) in 50 min, with a further gradual reduction until 30 min after the injection (11 ± 6%, from 180 ± 8 to 162 ± 8 mm Hg, *P* < 0.05), as shown in Figure 3a. The reduction in MAP was accompanied by a significant decrease in rSNA, with a maximal reduction during the 30 min (−18 ± 2%, *P* < 0.05) following losartan injection in the 2K-1C animals (Figure 3b). In contrast, MAP and rSNA remained unchanged in SHAM animals. Figure 3c,d show the representative responses to bilateral losartan injection into the RVLM of the SHAM or 2K-1C rats.

Effects of aminoguanidine injection into the RVLM of urethane-anesthetized rats

In the 2K-1C group, a higher reduction in MAP was observed 45 min following aminoguanidine injection (from 188 ± 8 to 128 ± 15 mm Hg, *P* < 0.05), with a gradual return to the basal condition by ~60 min after the injection (146 ± 9 mm Hg) (Figure 4a). The aminoguanidine injected into the RVLM significantly reduced the rSNA only at 45 min after injection (34 ± 8%). In contrast, MAP and rSNA remained unchanged in SHAM rats (Figure 4b). Figure 4c,d show the representative responses to aminoguanidine injection into the RVLM of SHAM or 2K-1C rats.

DISCUSSION

In the present study, we showed that AT1R protein levels and iNOS at both the gene and protein levels are upregulated in the RVLM of 2K-1C hypertensive animals. Additionally, we observed that losartan and aminoguanidine administration into the RVLM decreased MAP and rSNA only in hypertensive rats. Taken together, the results suggest that Ang II (via AT1R) and NO (produced by iNOS) in the RVLM are involved in the maintenance of arterial hypertension and sympathoexcitation in the 2K-1C rats.

It has been suggested that the increase in peripheral sympathetic activity present in 2K-1C model could be due to changes in the central nervous system triggered by an increase in circulating Ang II. Katholi *et al*., in 1982,17 showed that renal denervation of the nonclipped kidney produced no change in systolic blood pressure, whereas renal denervation of the clipped kidney resulted in a significant decrease in systolic blood pressure. This data indicate that the depressor effect of clipped kidney denervation in the 2K-1C model is associated with a decrease in sympathetic nervous system activity in the absence of changes in renin–angiotensin system activity.17

One mechanism by which circulating Ang II exerts its effects centrally is by binding to its receptors on neurons in specialized brain regions that lack a blood–brain barrier, known as
circumventricular organs. Densely populated with fenestrated capillaries for high permeability, the circumventricular organs are localized around the third and fourth ventricles and are known to be pivotal in mediating the increases in blood pressure. Neurons in these regions, such as in the subfornical organ and organum vasculosum of the lamina terminalis, may send their signal to other downstream cardiovascular control regions of the brain, such as the RVLM, which additionally activates several neurohumoral pathways.

It is well known that Ang II plays a key role in the central cardiovascular control through the activation of AT1R in the RVLM. In the present study, we discovered the protein upregulation of AT1R in the RVLM in the 2K-1C hypertension model. Similar results have been observed in other experimental models, such as in experimental myocardial infarction; 19 Likewise, this model also presents a sympathetic hyperactivity. Accordingly, we observed a slow and gradual decrease in MAP following losartan administration into the RVLM of hypertensive animals only. The maximum response was observed 30 min later. This slow depressing response can be explained by the mechanism of action of Ang II in the RVLM. In vitro studies of the neurons present in the RVLM region demonstrated that C1 cells possess AT1R, whose activation leads to a reduction of the resting potassium conductance. This effect increases the discharge rate and excitability of these cells, which is likely to contribute to the sympathoexcitation observed when Ang II is injected into the RVLM in vivo.20 Moreover, in the present study, blocking of the AT1R also promoted a gradual sympathetic inhibition in hypertensive animals.

Different experimental models have demonstrated that Ang II may act through the AT1R pathway in the RVLM, promoting hypertension, as occurs in spontaneously hypertensive rats21 and deoxycorticosterone acetate-salt22 hypertension in transgenic rats harboring the mouse renin Ren-2 gene (TGR (mREN2) 27).23 Thus, our aim was to investigate whether a similar mechanism occurs in the 2K-1C hypertension model because it is an angiotensin–renin-dependent model.

In 2001, Allen showed that the administration of candesartan (AT1R antagonist) into the RVLM produces hypotension and a 20% reduction in lumbar SNA in spontaneously hypertensive rat.24 Additionally, further studies performed by the same author showed that transfection of the AT1R adeno-virus into the RVLM results in arterial hypertension, which is maintained for 3–4 days. This is an interesting finding, showing for the first time that a chronic increase in AT1RA activity in the RVLM promotes an increase in blood pressure over a period of days.7

Our data suggest an important role of the activation of the central angiotensin–renin system in the maintenance of hypertension in the 2K-1C model and corroborate previous reports from the literature. In 1992, Nishimura et al. showed increases of mRNA angiotensinogen levels in several brain regions, such as in the circumventricular organs; in 1999, Allen et al. showed that the administration of candesartan into the RVLM produced a profound reduction in renal SNA and blood pressure; and in 2001, Allen et al. showed that injection of losartan into the RVLM resulted in a slow and gradual hypotensive response in hypertensive rats.20 These findings are consistent with the current study, which demonstrates the importance of the activation of the AT1R pathway in the RVLM in the maintenance of hypertension in the 2K-1C model.
as the medulla oblongata and hypothalamus, during the stable phase of 2K-1C hypertension. Moreover, there is evidence that intracerebroventricular infusion of AT₁R receptor antisense prevents 2K-1C hypertension by the inhibition of receptor synthesis. It is possible that the described prevention of hypertension is related to the antisense actions on RVLM.

It is known that losartan binds with high affinity and specificity to the AT₁R with a slow dissociation rate, and it is 30,000-fold more selective for the AT₁R than for the AT₂R. Pharmacologically, AT₁R antagonists block the pressor and functional responses of Ang II both in vitro and in vivo. In contrast to AT₁R, the functions of AT₂R regarding the central regulation are not well understood. However, the patch clamp data from the cultured individual neurons of the hypothalamus and brainstem clearly demonstrated an increase in the potassium current induced by AT₁R, an effect contrary to that of the AT₁R on neuronal channel function. The functions and intracellular signaling pathways of the AT₁R in most peripheral tissues and organs are also opposite to that of the AT₁R. For example, stimulating AT₂R induces vasodilation, stimulates NO production and hypotension. Taken together, these data led us to suggest that the blockade of AT₁R leaves Ang II available for AT₁R in the RVLM and this may be critical to maintain sympathetic tone in normal conditions. However, further integrative and molecular studies are necessary for a better understanding of the balance between AT₁R and AT₂R expression in hypertension.

Another important finding of the present study was that both gene and protein iNOS expression were increased in the RVLM of 2K-1C hypertensive animals. Furthermore, the iNOS inhibition in the RVLM promoted hypotension and sympathetic inhibition only in hypertensive animals. Thus, either the AT₁R receptor and/or NO produced by iNOS plays a key role in the maintenance of 2K-1C hypertension and sympathoexcitation. However, we have thus far obtained no information regarding which cell types are involved in the upregulation of AT₁R and iNOS within the RVLM. The role of glial cells and the phenotypes of neurons and endothelial cells must be addressed in future work.

The iNOS can be induced by a variety of factors involved in inflammation such as LPS and cytokines in macrophages, endothelial cells, hepatocytes, and neutrophils. Previous experiments of our laboratory showed that the gene expression of interleukin-1β is upregulated in the RVLM of 2K-1C animal (data not shown). However, further experiments are needed to explain this mechanism.

The effects of NO in blood pressure regulation are controversial. Some studies showed that the NO in the RVLM reduces blood pressure by inhibiting the sympathetic nervous system, however, conflicting results have also been reported. Moreover, there is evidence that NO can cause biphasic responses depending on the administered dose. However, differences between these results may be related to different approaches; for example, most experiments were performed in anesthetized rats and evaluated the acute effects of NO donors, and sometimes the blockers administered were not specific for NO.

In 2005, Kimura et al. showed that adenovirus vectors encoding iNOS transfected into the RVLM in Wistar-Kyoto rats increase blood pressure via activation of the sympathetic nervous system. Aminoguanidine administration into the RVLM after transfection resulted in a reduction in blood pressure and in sympathetic activity. Additionally, tempol (SOD mimetic) administration promoted hypotension and
sympathetic inhibition, with a consequent reduction in the production of superoxide and lipid peroxidation in the same animals. The results thus showed that upregulation of iNOS in the RVLM elicits hypertension by activating the sympathetic nervous system, and these effects might be mediated by an increase in oxidative stress in the RVLM because NO may be inhibitory or excitatory for neurons in the RVLM region according to its bioavailability.

Previous studies from our laboratory have shown that vitamin C (antioxidant) and tempol administration into the RVLM promote depressor effects, such as rRNA reduction in 2K-1C hypertensive animals. Thus, we cannot exclude the possibility that the increased production of NO resulting from iNOS activation promotes a greater interaction with superoxide. Superoxide anions react rapidly with NO, forming peroxynitrite and decreasing the bioavailability of NO. Therefore, an increase in the superoxide anion levels in the RVLM might decrease the bioavailability of NO in these regions, leading to an increase in the neuronal firing. This mechanism might contribute to the increase in sympathetic nerve activity and hypertension.

Our results corroborate a recent study in spontaneously hypertensive rat. The authors of that study demonstrated that upregulation of iNOS in the RVLM contributes to the increase in blood pressure in this model and that blocking of the iNOS upregulation of iNOS in the RVLM contributes to the increase in sympathetic outflow and resulting in blood pressure changes. An interesting recent work showed that an enrichment of AT1R receptors in the RVLM is associated with increased sympathetic drive in heart failure, and a similar phenomenon is described in the present study in renovascular arterial hypertension. Therefore, the present study provides new insights regarding the central mechanism underlying the actions of Ang II and NO in conditions characterized by increased sympathetic activity, such as arterial hypertension and other chronic cardiovascular diseases.

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AT1R Receptor and iNOS in the RVLM


