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Kidney-Induced Hypertension Depends on Superoxide Signaling in the Rostral Ventrolateral Medulla

Elizabeth B. Oliveira-Sales, Débora S.A. Colombari, Robin L. Davisson, Sergey Kasparov, Aparecida E. Hirata, Ruy R. Campos, Julian F.R. Paton

Abstract—Reactive oxygen species in peripheral cardiovascular tissues are implicated in the pathogenesis of 2 kidney-1 clip hypertension. We recently identified an imbalance between reactive oxygen species generation and oxidant scavenging in the rostral ventrolateral medulla (RVLM) of 2 kidney-1 clip in rats. We tested whether enhanced superoxide signaling in RVLM of 2 kidney-1 clip rats contributes to the chronic hypertension via sympathetic activation in conscious rats. We enhanced superoxide scavenging in RVLM by overexpressing cytoplasmically targeted superoxide dismutase using an adenoviral vector (Ad-CMV-CuZnSOD) in Wistar rats (male, 150 to 180 g) in which the left renal artery was occluded partially 3 weeks earlier. Hypertension was documented using radiotelemetry recording of arterial pressure in conscious rats for 6 weeks. Renovascular hypertension elevated both serine phosphorylation of p47phox subunit of NADPH and superoxide levels in RVLM. The elevated superoxide levels were normalized by expression of CuZnSOD in RVLM. Moreover, the hypertension produced in the 2 kidney-1 clip rats was reversed 1 week after viral-mediated expression of CuZnSOD. This antihypertensive effect was maintained and associated with a decrease in the low-frequency spectra of systolic blood pressure variability, suggesting reduced sympathetic vasomotor tone. The expression of CuZnSOD was localized to RVLM neurons, of which some contained tyrosine hydroxylase. None of the above variables changed in control rats receiving Ad-CMV-eGFP in RVLM. In Goldblatt hypertension, superoxide signaling in the RVLM plays a major role in the generation of sympathetic vasomotor tone and the chronic sustained hypertension in this animal model. (*Hypertension*. 2010;56:290-296.)

Key Words: hypertension ■ renal ■ blood pressure ■ heart rate ■ brain ■ free radicals

Involvement of oxidative stress in the pathology of arterial hypertension was reported in various animal models, including the renovascular 2 kidney-1 clip (2K-1C),¹⁻³ the 1 kidney-1 clip,⁴ angiotensin II (Ang II)-induced hypertension,⁵⁻⁷ Dahl salt-sensitive (desoxycorticosterone acetate-salt) hypertension,⁸ spontaneously hypertensive rats (SHRs),^{9,10} and stroke-prone SHRs.^{11,12} In addition, oxidative stress also plays an important role in humans with renovascular hypertension¹³ and essential hypertension.¹⁴ From these studies it is clear that oxidative stress acts at the level of the vascular wall but also within the brain. The renin-angiotensin-aldosterone system plays a major role in the 2K-1C model.¹⁵ Ang II stimulates superoxide ($O_2^{\cdot-}$) radical generation by increasing the activity of NADPH oxidase within peripheral blood vessel walls.¹⁶⁻¹⁸

However, there is evidence that reactive oxygen species (ROS) in the brain are involved in neuronal signaling, contributing to sympathoexcitation and hypertension.^{12,19} In-

tracerebroventricular infusion of an NADPH oxidase inhibitor antagonizes both the increase in renal sympathetic nerve activity and pressor response induced by centrally administered Ang II.^{20,21} In the brain, virally mediated overexpression of superoxide dismutase (SOD), the enzyme responsible for $O_2^{\cdot-}$ breakdown, also abolishes the central pressor effect of Ang II.⁷

The 2K-1C rat model reflects renovascular hypertension.²² The seminal work of Johansson et al²³ indicated activation of the sympathetic nervous system in human renovascular hypertensives. In this regard, the rostral ventrolateral medulla (RVLM) is an important region for the maintenance of the hypertension.^{24,25} Because the RVLM is a key regulatory of sympathetic activity,²⁶ a change in ROS activity here may be an essential mechanism involved in generating excessive sympathetic activity in the 2K-1C model. We identified an imbalance between ROS generation and oxidant scavenging in the RVLM that promoted $O_2^{\cdot-}$ generation. In an acute

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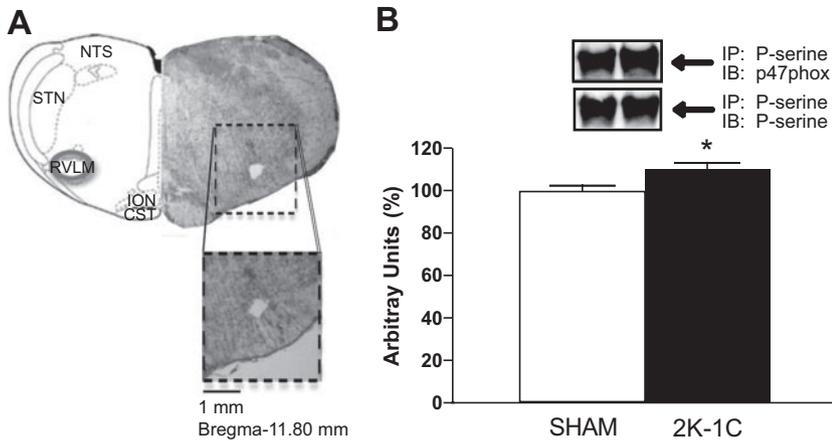


Figure 1. A, Schematic representation of the site of RVLM (left) and bilateral punches on right. CST indicates corticospinal tract; ION, inferior olivary nucleus; AN, ambiguus nucleus; NTS, nucleus of the tractus solitarius; STN, spinal trigeminal nucleus. B, Serine p47phox phosphorylation in sham (n=5) and 2K-1C (n=4) rats after 6 weeks of clipping. Protein was extracted and was used for immunoprecipitation (IP) with antiphosphoserine antibody. The aliquots were subjected to SDS-PAGE and immunoblotted (IB) with p47phox or antiphosphoserine antibody (loading control). Serine phosphorylation of p47phox is a key step for NADPH oxidase activation. Therefore, a significantly increase of serine phosphorylation of p47phox was detected in 2K-1C rats after 6 weeks of clipping. * $P < 0.05$ vs sham.

study, Tempol, a metal-independent SOD mimetic, was microinjected into the RVLM of anesthetized rats and produced a transient, dose-dependent decrease in blood pressure and renal sympathetic nerve activity in 2K-1C animals.³ However, it remains unknown whether long-term antioxidant treatment of the RVLM can affect renal-induced hypertension in awake animals. Thus, we hypothesized that there is a persistent increase in oxidative stress within the RVLM of the 2K-1C model that leads to increased neuronal excitability, excessive sympathetic activity, and chronic hypertension. Therefore, we examined whether increased chronic scavenging of $O_2^{\cdot-}$ in the RVLM of the 2K-1C model would revert the hypertension. To permit chronic $O_2^{\cdot-}$ scavenging we used an adenovirus to overexpress CuZnSOD⁷ in the RVLM of conscious 2K-1C rats.

Methods

The experiments of virus transfection, telemetry, and immunohistochemistry were performed in the University of Bristol and approved by United Kingdom Home Office Guidelines on Animals (Scientific Procedures) Act of 1986 (Personal Licence No. 30/8202). The Western blotting and dihydroethidium (DHE) protocol were 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=54, 150 to 180 g) were housed individually, allowed normal rat chow and drinking water ad libitum, and were kept on a 12-hour light/12-hour dark cycle. The study was divided into 3 independent series of experiments. Please see the online Data Supplement at <http://hyper.ahajournals.org> for details of the experimental protocol (Table S1).

Renovascular Hypertension Model

Rats were anesthetized with ketamine (60 mg/kg) and medetomidine (250 μ g/kg) intramuscularly, and the left renal artery was partially obstructed with a silver clip of 0.2 mm width¹⁵; this occlusion reduced renal blood flow significantly.²⁷ Control animals (sham) were submitted to the same surgical procedure without partial renal artery occlusion. Anesthesia was reversed with atipamezole (1 mg/kg).

Western Blotting

Please see the online Data Supplement for details of p47phox serine phosphorylation in the RVLM. And Figure 1A shows the histology of the RVLM punches.

Telemetric Recording of Arterial Pressure

The radiotransmitters (Data Sciences International; TA11PAC40) were implanted the same day of clipping to record arterial pressure

from the abdominal aorta as described previously.²⁸ A computer-based acquisition system, Hey Presto telemetry software (Mizuno software),^{29,30} was used for acquiring, displaying, storing, and analyzing the telemetry data. Please see the online Data Supplement for details.

In Vivo Gene Transfer into RVLM

Ad-CuZnSOD (1.2×10^8 plaque-forming units/ μ L), a recombinant E1-deleted adenoviral vector encoding human cytoplasmic SOD, was used as described previously.^{31,32} As a control, Ad-eGFP (1.57×10^9 plaque-forming units/ μ L) expressing enhanced green fluorescent protein (eGFP) was used.²⁸ Three weeks after renal artery clipping or sham surgery, animals were reanesthetized, and five 100-nL injections per side of viral suspension³³ (either Ad-CuZnSOD or Ad-eGFP) were made into the RVLM at separate sites spanning 12.0 to 12.4 mm caudal to the bregma to the lambda, 1.7 to 1.8 mm lateral to the midline, and 8.0 mm below the dorsal medullary surface (bite bar: -3.5 mm).³⁴ Each injection was made over 1 minute. The RVLM was initially mapped out with glutamate (indicate n=6) in the experimental rats before injection of the virus and identical coordinates used thereafter. Indeed, when the injection missed RVLM, rats were excluded from the analysis, but these animals did not show an attenuated pressor effect with viral transfection (data not shown) indicating site specificity. Viral injections were made at 3 weeks after clipping so that the expression of SOD was coincident with the rise in arterial pressure/changes in autonomic variables. Recordings of arterial pressure were made for a further 3 weeks. Note that viral expression is known to last for ≤ 4 weeks.⁷ There was no difference in the weight of rats between all of the groups at any time point in our study. Please see Figure S1 in the online Data Supplement, which shows the degree of spread.

Measurement of $O_2^{\cdot-}$ in the RVLM of 2K-1C Rats

The $O_2^{\cdot-}$ in the RVLM was analyzed 3 weeks after microinjection of either Ad-CuZnSOD or Ad-eGFP. Please see the online Data Supplement for details.

Validating CuZnSOD Transduction in the RVLM In Vivo

Double fluorescence immunohistochemistry was performed for CuZnSOD with either tyrosine hydroxylase (TH) or neuronal nuclei (NeuN) to confirm the presence of expression in RVLM neurons (please see the online Data Supplement for details).

Data Analysis

Results were presented as the mean \pm SEM. The telemetry data were evaluated using 2-way ANOVA followed by a Bonferroni or Tukey posttest with statistical software (Graphpad Prism 4.0). DHE fluorescence intensity was quantified using Leica software and differences analyzed using a 1-way ANOVA followed by the Tukey posttest. The level of statistical significance was defined as $P < 0.05$.

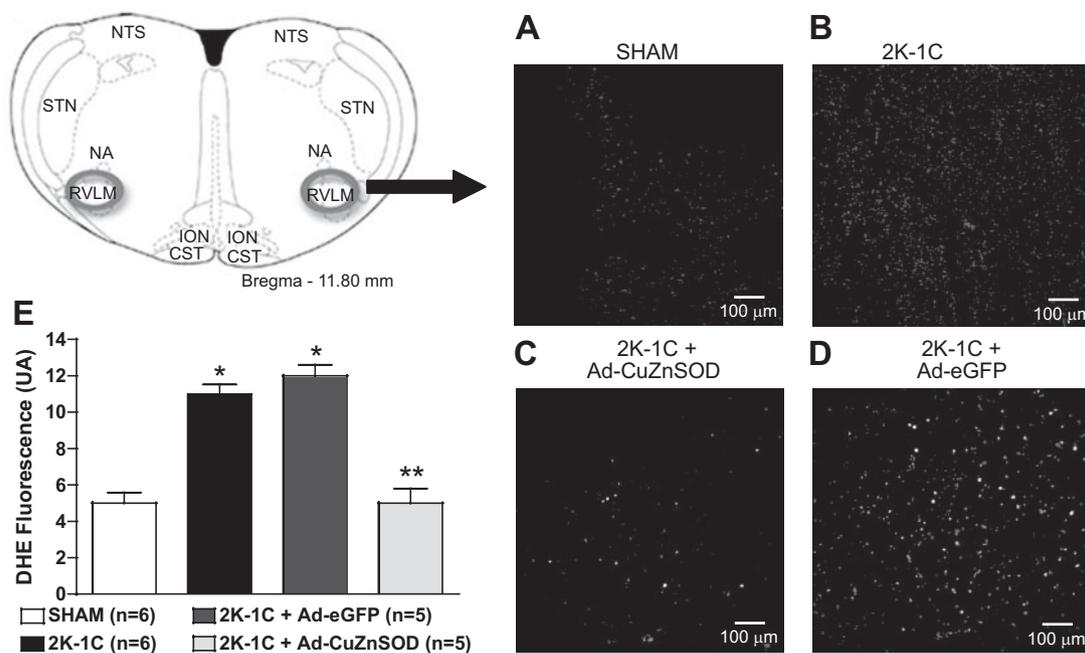


Figure 2. The brain stems of sham (A), 2K-1C (B), and 2K-1C animals receiving Ad-CuZnSOD (C) and Ad-eGFP (D) in the RVLM were incubated for 30 minutes in DHE dye, 1 mol/L as per Zimmerman et al.⁷ Four representative sections through the RVLM imaged with a laser confocal microscope show that, in RVLM tissue exposed to Ad-CuZnSOD, there is less $O_2^{\cdot-}$. Maximal DHE fluorescence was quantified and found to be significantly reduced in the presence of CuZnSOD (E). Tissues were studied 6 weeks after clipping and 3 weeks after gene transfer. * $P < 0.05$ vs sham and ** $P < 0.05$ vs 2K-1C.

Results

Activation of NADPH Oxidase in 2K-1C Rats

Serine phosphorylation of p47phox is a key step for NADPH oxidase activation. To evaluate NADPH oxidase activation in 2K-1C hypertension, an immunoprecipitation protocol was used. Punched-out RVLM tissues were immunoprecipitated with antiphosphoserine antibody and submitted to SDS-PAGE. The membranes were then immunoblotted with anti-p47phox antibody. A significant increase of serine phosphorylation of p47phox was detected in 2K-1C rats after 6 weeks of clipping (sham: $100 \pm 2.3\%$ versus 2K-1C: $110 \pm 3.1\%$; $P < 0.05$; Figure 1B).

$O_2^{\cdot-}$ Levels in the RVLM of Rats Transduced With Ad-CuZnSOD and Ad-eGFP

Figure 2 depicts the contrasting levels of $O_2^{\cdot-}$ in the RVLM of sham, 2K-1C, and 2K-1C rats transduced with Ad-CuZnSOD and Ad-eGFP. It is notable that, in the RVLM in which CuZnSOD had been overexpressed, there was considerably less DHE immunofluorescence compared with 2K-1C expressing Ad-eGFP; this was confined to the RVLM as sections rostral or caudal exhibited marked DHE immunofluorescence that was at the same level compared with control animals.

Ad-CuZnSOD and Ad-eGFP in the RVLM on Arterial Pressure and Systolic Blood Pressure Variability in Sham and 2K-1C Rats

The systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MBP) were initially recorded via radiotelemetry for 3 weeks before microinjection of either Ad-CuZnSOD or Ad-eGFP into the RVLM and

for a further 3 weeks after viral transfection. In all of the rat groups, baseline levels of SBP, DBP, and MBP were similar, as shown in Table S2. In the third week after renal artery clipping, SBP, DBP, and MBP were all elevated in 2K-1C rats compared with the sham group. Arterial pressure in the 2K-1C animals rats increased gradually and significantly for 2 weeks after clip placement but plateaued by the fifth week. Ad-CuZnSOD administration into the RVLM of 2K-1C or sham rats was performed at the end of the third week. SBP, DBP, and MBP in sham groups injected with either Ad-CuZnSOD or Ad-eGFP in the RVLM were unaffected (SBP: see Figure 3A; MBP and DBP: see Figure S2). In contrast, 1 week after transduction of the RVLM of 2K-1C rats with Ad-CuZnSOD, arterial pressure was reduced significantly (SBP: from 181 ± 8 to 140 ± 12 mm Hg, $P < 0.05$; DBP: from 142 ± 8 to 103 ± 3 mm Hg, $P < 0.05$; MBP: from 174 ± 13 to 115 ± 7 mm Hg, $P < 0.05$). These values remained unchanged for the remainder of the experimental period. These data suggest that virally mediated expression of CuZnSOD in the RVLM reversed the 2K-1C-induced hypertension. In contrast, arterial pressure remained elevated and was unchanged in the Ad-eGFP 2K-1C group (Figure 3).

Before the RVLM viral injection, the values of low-frequency power of the SBP (LF SBP) and very low frequency power of the SBP (VLF SBP) in the 2K-1C were not different from the control group (please see Table S3). In sham groups receiving microinjections of either Ad-CuZnSOD or Ad-eGFP into the RVLM, both LF SBP and VLF SBP were unaffected (Figure 3B and 3C).

We compared the LF SBP between 2K-1C+Ad-CuZnSOD and 2K-1C+Ad-eGFP groups. During the first week after viral injections, we found a significant drop in LF SBP in the

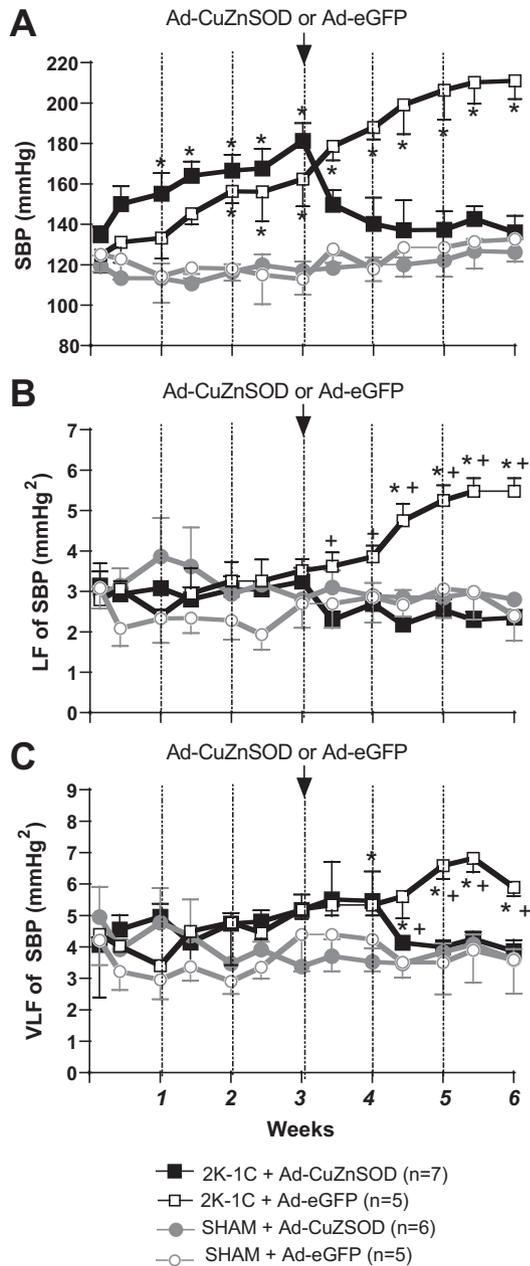


Figure 3. A, Summary of SBP recorded by radiotelemetry before and after microinjection of Ad-CuZnSOD and Ad-eGFP into the RVLM for 6 weeks. Values are expressed as the mean \pm SEM. These results showed that the blood pressure of hypertensive animals increased gradually and significantly over 2 weeks, reaching a plateau by the fifth week after clipping. A microinjection of Ad-CuZnSOD in the RVLM prevented 2K-1C hypertension. However, hypertension was observed in the Ad-eGFP group. B, Spectral parameters of low frequency power of the SBP (LF SBP) and (C) very low frequency power of the SBP (VLF SBP) calculated by Hey-Presto software in the Goldblatt model. Spectral analysis suggests that 2K-1C hypertension is associated with an increase in vasomotor sympathetic tone during the fourth week after renal artery clipping and is attenuated by Ad-CuZnSOD but not Ad-eGFP in the RVLM. * $P < 0.05$ vs sham. + $P < 0.05$ vs 2K-1C+CuZnSOD (2-way ANOVA followed by the Tukey posttest).

2K-1C+AdCuZnSOD group compared with the control animals that developed hypertension (2K-1C+Ad-eGFP; from 3.8 ± 0.2 to 2.6 ± 0.2 mm Hg²; $P < 0.0002$; Figure 3B). This drop in LF SBP coincided precisely with the start of the fall

in arterial pressure in the 2K-1C+CuZnSOD group and provides evidence that sympathetic activity is reduced as arterial pressure is lowered in these animals. The VLF SBP increased from 5.0 ± 0.4 to 6.4 ± 0.3 mm Hg² ($P < 0.05$) in the 2K-1C 2 weeks after Ad-eGFP viral transfection and persisted (Figure 3C). Comparing VLF SBP between the Ad-eGFP and Ad-CuZnSOD 2K-1C groups indicated that the latter showed a reduction 1 week after viral injection from 6.8 ± 0.3 to 3.9 ± 0.1 mm Hg² ($P < 0.05$) and, therefore, some what delayed relative to the start of the fall in arterial pressure in the Ad-CuZnSOD group.

Data from our spectral analysis did not show changes in respiratory frequency, suggesting no influence on respiratory rhythm-generating neurons. Please see this result in the online Data Supplement (Figure S3).

Location of RVLM Sites Transduced With Ad-CuZnSOD

Based on the localization of CuZnSOD immunoreactivity and eGFP expression, highly comparable regions were transduced with both viruses (Ad-CuZnSOD and Ad-eGFP) in 2K-1C and sham groups. Double staining with TH (Figure 4A) confirmed that $\approx 70 \pm 0.4\%$ of catecholaminergic C1 cells expressed SOD. Indeed, the double staining with NeuN (Figure 4B) confirmed the health of neurons. These were located bilaterally and found ventral to the compact division of the nucleus ambiguus and lateral to inferior olive and, therefore, in the RVLM.

Discussion

The present study reveals major advances in our understanding of chronic renovascular hypertension. In the Goldblatt model, we have found the following: (1) increased serine phosphorylation of the p47phox subunit of NADPH in the RVLM; (2) increased $O_2^{\cdot-}$ production in the RVLM; and (3) chronic reversal of the hypertension by raising the levels of SOD within the RVLM bilaterally, which is accompanied with a fall in low-frequency spectra of SBP. The latter persists for >3 weeks in conscious rats, which is coincident with the reported expression duration of CuZnSOD using our virus⁷ and is associated with reduced $O_2^{\cdot-}$ levels within the RVLM. We also show that the effective antioxidant in RVLM for renovascular hypertension targets cytoplasmic SOD³⁵ RVLM neurons, many of which contain TH. Our data support the notion that intracellular $O_2^{\cdot-}$ in RVLM neurons (including C1 cells) is a critical signaling mechanism underlying the development of raised sympathetic vasomotor tone and hypertension in the 2K-1C rat model.

Commonality of ROS Signaling in Brain for Numerous Hypertensive Animal Models

A previous study showed that microinjections of Tempol into the RVLM decreased blood pressure in a dose-dependent manner in stroke-prone SHR but not in Wistar Kyoto rats.¹² Furthermore, injection of adenovirus encoding the MnSOD gene into the RVLM of stroke-prone SHR decreased MBP and heart rate but had no effect on cardiovascular parameters in Wistar Kyoto rats.¹² However, until now it was not known whether a similar mechanism in RVLM mediated the arterial

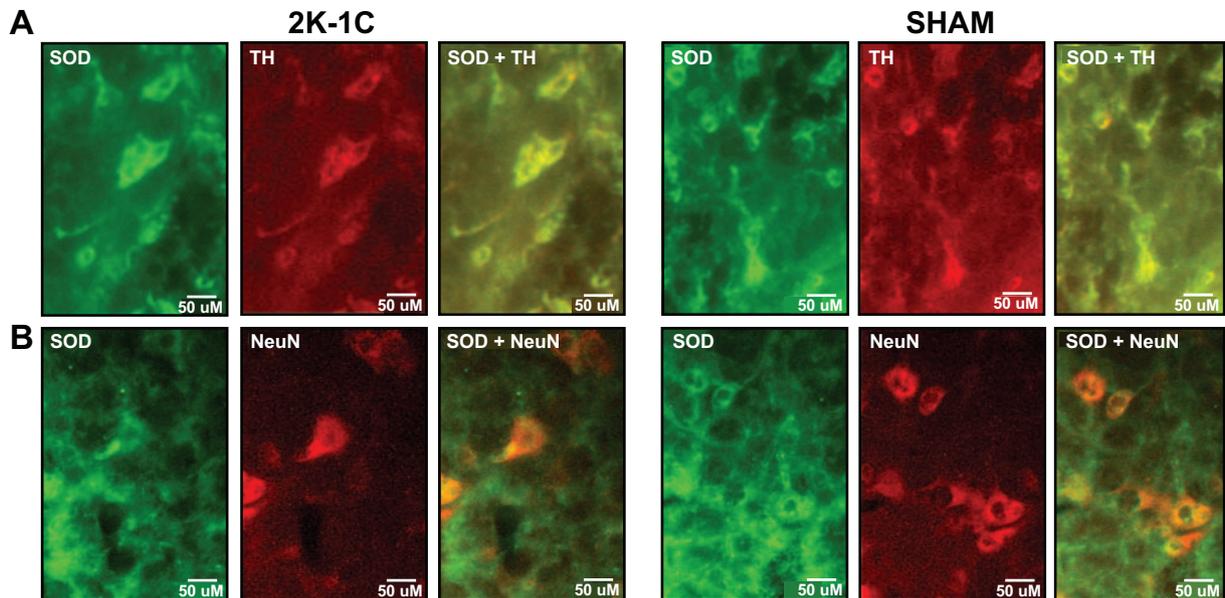


Figure 4. Cellular localization of Ad-CuZnSOD in the RVLM. A, Representative photomicrographs show immunofluorescence of SOD (left), TH (center), and merged images (SOD+TH; right) 3 weeks after microinjection of Ad-CuZnSOD into the RVLM in 2K-1C and sham rats. B, Photomicrographs show SOD and NeuN immunoreactivity in 2K-1C and sham rats. Costaining with NeuN and TH confirmed transfection of neurons, many of which were catecholaminergic (C1).

hypertension in the 2K-1C rat model. Elucidation of such data may be of great value in terms of identifying the commonality of mechanisms between different types of hypertension and, therefore, may greatly assist in generic approaches for treating this multifactorial-based disease.

Using intracerebroventricular administration of either Ad-MnSOD or Ad-CuZnSOD, Zimmerman et al⁶ reported prevention of centrally mediated Ang II-induced pressor responses. However, they also showed that Ad-ECSOD (targeted to the extracellular matrix) did not prevent development of Ang II-induced hypertension.^{7,21} These results suggest that Ang II infusion caused an increase in intracellular $O_2^{\cdot-}$ in the subfornical organ. The data from the present study indicate that $O_2^{\cdot-}$ signaling within the RVLM is an essential mechanism that contributes to 2K-1C hypertension, but whether this is also based on intracellular signaling is not known. Interestingly, there is evidence that Ang II levels are essential for increasing glutamatergic mechanisms driving RVLM neuronal excitability in the 2K-1C model 6 weeks after clipping³⁶; this coincides with raised levels of sympathetic drive in the 2K-1C model (see Figure 2). This is consistent with the finding that downregulation of gene expression and enzyme activity of the antioxidant CuZnSOD in the RVLM may underlie the augmented levels of radical $O_2^{\cdot-}$ in the RVLM, leading to oxidative stress, sympathoexcitation, and hypertension in the SHR.³⁷

Autonomic Mechanisms Underpinning 2K-1C Hypertension and Effect of Blocking $O_2^{\cdot-}$ Signaling in RVLM

It remains unclear how a renovascular insult (such as in the Goldblatt model) leads to excessive cellular production of $O_2^{\cdot-}$ in the RVLM. One possibility is that this is caused by raised circulating Ang II, but this remains to be tested. However, the present data indicate that the mechanism

involves serine phosphorylation of the p47phox subunit of NADPH in the RVLM, which is consistent with our previous finding of elevated mRNA expression of 2 NADPH oxidase subunits, p47phox and gp91phox, in the RVLM of the Goldblatt rat.² Intriguingly, Ang II type 1 receptor stimulation can both phosphorylate the p47phox subunit and activate the cytoplasmic subunits of NADPH that bind to their membrane subunits, resulting in the intracellular production of $O_2^{\cdot-}$.³⁸

Research over the last decade has revealed that $O_2^{\cdot-}$ is implicated in the regulation of neuronal excitability, as well as in neurotransmitter release.³⁹ Glutamatergic activity, for instance, is increased in the RVLM in 2K-1C. Bergamaschi et al²⁴ showed that microinjection of kynurenic acid into the RVLM produced a long-lasting decrease in arterial pressure in 2K-1C rats.

Previous studies showed that the sympathetic nervous system is involved in 2K-1C hypertension. Acute hexamethonium bromide administration in 2K-1C rats caused a greater decrease in arterial pressure and peripheral vascular resistance compared with controls from between 3 and 6 weeks after clipping.⁴⁰ This time frame is coincident with when we first detected raised low-frequency power in the SBP, indicative of elevated sympathetic outflow. These findings are in line with the reported increase in renal sympathetic nerve activity in the 2K-1C model.² McElroy and Zimmerman, in 1989,⁴¹ showed that the α 1-adrenergic receptor affinity for [3H]prazosin binding in hypertensive rabbits was significantly increased in the stenotic but not in the contralateral kidney at 2 weeks after clipping; however, the receptor affinity for both kidneys was significantly increased compared with those of the normotensive control group at 6 weeks of clipping. Taken together with the results from the present study, these findings support the notion that oxidative stress may be a cellular signaling mechanism driving RVLM

neuronal excitability, resulting in elevated sympathetic vasomotor tone.

The mechanisms by which the increase in ROS in the RVLM increased sympathetic nerve activity and blood pressure are not known. These responses might be mediated by an interaction between $O_2^{\cdot-}$ and NO. $O_2^{\cdot-}$ react rapidly with NO, forming peroxynitrite and thereby decreasing the bioavailability of NO.⁴² Note that NO in the RVLM causes hypotension and sympathoinhibition.⁴³ The increase in $O_2^{\cdot-}$ levels in the RVLM might decrease NO bioavailability in RVLM, contributing to the hypertension. However, we cannot rule out a possible role for hydrogen peroxide, which accumulates during $O_2^{\cdot-}$ scavenging.

The pressor response in the 2K-1C animals was associated with an increase in the low frequency of SBP, suggesting an increase in sympathetic vasoconstrictor action. There was also an increase in very low frequency, supporting enhanced neurohumoral-mediated vasoconstriction. These findings are consistent with those of Ponchon and Elghozi,⁴⁴ who showed that an increase in the LF SBP occurred after 6 weeks from renal artery clipping. They showed that a reduction of the slow fluctuations in LF SBP after combined blockade of the kallikrein-kinin and the renin-angiotensin systems suggested the contribution of these humoral systems to this LF SBP.⁴⁴ Other studies showed that increased LF oscillations of SBP occurred 2 weeks⁴⁵ or 6 weeks⁴⁶ after clipping. In the present study, transduction of the RVLM with adenovirus expressing CuZnSOD prevented the increased sympathetic vasomotor tone (ie, LF SBP) in 2K-1C, which persisted long term (3 week). Our interpretation is that reduction in sympathetic activity will have additional effects beyond reducing vasomotor tone, such as reducing force of contraction and decrease the level of circulating hormones from the adrenals and the kidney (eg, renin). Thus, chronic oxidative stress in RVLM can contribute to sympathetic hyperactivity in renovascular hypertension. Our data are consistent with a growing body of literature supporting the prosympathoexcitatory effects of $O_2^{\cdot-}$ in RVLM.^{47,48}

The present study also shows that chronic $O_2^{\cdot-}$ signaling in the RVLM reduced the tachycardia and prevented a reduction in the gain of the parasympathetic component of the cardiac baroreflex. Please see the online Dat Supplement for the details (Table S4 and Figure S4). We conclude that increased oxidative stress within the RVLM is a major mechanism driving sympathetic vasomotor tone and hypertension in a renovascular and Ang II-dependent experimental model of hypertension.

Perspectives

The RVLM contains many sympathetic premotor neurons involved in the maintenance of sympathetic vasomotor tone and, hence, blood pressure. Changes in neuronal excitability of RVLM neurons are probably a major mechanism involved in increased sympathetic drive in arterial hypertension. Others have shown that changes in neurotransmission in the RVLM contribute to elevated arterial pressure and activation of sympathetic activity. The present study indicates that oxidative stress within the RVLM is exacerbated and is responsible for altered sympathetic outflow in renovascular

arterial hypertension. Taken together with other evidence, changes in neuronal excitability in the RVLM are important mechanisms driving tonic sympathetic vasomotor tone in sympathoexcitatory diseases, such as arterial hypertension. Therefore, targeting oxidative stress in the RVLM becomes an obvious aspiration in the quest for combating neurogenic hypertension, whether of central or renal origin.

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Disclosures

None.

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ONLINE SUPPLEMENTS

Kidney-Induced Hypertension Depends On Superoxide Signaling In The Rostral Ventrolateral Medulla

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Methods

The study was divided into three independent series of experiments. Please see **Table S1** for details of experimental protocol.

Renovascular hypertension model

Male Wistar rats (150–180 g) were anesthetized with ketamine (60 mg/kg) and medetomidine (250 µg/kg) intramuscularly and the left renal artery was partially obstructed with a silver clip of 0.2 mm width.¹ The control animals (SHAM) were submitted to the same surgical procedure but without partial renal artery occlusion. Anesthesia was reversed with atipamezole (1 mg/kg).

Western blotting analysis

SHAM (n=5) and 2K-1C – 6 weeks after clipping (n=4) rats were anesthetized with ketamine and xylazine (40 and 20 mg/kg, IP, respectively) and decapitated. The brainstem was removed and frozen with dry ice, sections (200 µm) were cut by a cryostat -7 ± 1 °C, and the RVLM region was identified under a surgical microscope. Bilateral tissue punches were taken from sections containing the RVLM (six to eight punches in total, from three to four slices from each animal) with a sharpened needle (0.5 mm diameter). The RVLM was identified as the region extending caudally 600–800 µm from the caudal pole of the facial nucleus. This corresponded to 12.5–12.7 mm from the Bregma for the RVLM.² The RVLM region was bounded laterally by the spinal trigeminal tract, medially by the inferior olive and pyramids, and dorsally by the compact formation of the nucleus ambiguus. To evaluate p47phox serine phosphorylation, RVLM samples were used for immunoprecipitation with anti-phosphoserine antibody (Chemicon-Millipore, USA) and subjected to western blotting analysis using 1:1000 dilution of anti-p47 phox antibody (Upstate Biotechnology-Millipore, USA). For whole tissue extracts, similar sized aliquots were subjected to SDS-PAGE and immunoblotted with p47phox antibodies. Electrotransfer of proteins from the gel to nitrocellulose membrane was performed for 90 min at 120 V (constant). To reduce non-specific protein binding to the nitrocellulose membrane, the filter was preincubated overnight at 4 °C in a blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). The nitrocellulose membrane blots were incubated overnight at 4 °C with the specific antibodies described in the figure legends diluted in blocking buffer (3% nonfat dry

milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20). After that, the membranes were incubated with secondary antibody conjugated to horseradish diluted in blocking buffer (1% nonfat dry milk, 10 mM Tris, 150 mM NaCl and 0.02% Tween 20) for 60 min. To visualize the autoradiography, enhanced chemiluminescence reagents were used and the membranes were exposed to preflashed Kodak XAR film (Eastman Kodak, Rochester, NY). Quantitative analysis was done using Scion Image software (Frederick, MD, USA).

Radiotelemetry and Analysis

The blood pressure was monitored for 5 min every hour 24 h every day for 6 weeks. There was no qualitative difference between lights-on and lights-off periods, so we have presented 24h data. Using Fast Fourier transform (FFT) spectral variations in arterial pressure and heart rate were computed. The 3 frequency bands were: high frequency (HF), low frequency (LF) and very low frequency (VLF) at 0.75-3.0 Hz, 0.25-0.75 Hz and 0.01-0.25 Hz, respectively as described below.^{3,4} Changes in VLF of BP indicate changes in sympathetic outflow related to thermoregulation, hormonal activity or changes in blood flow to meet local metabolic demands⁵, while LF of BP reflects the level of sympathetic vasoconstrictor activity. HR was derived from the inter-pulse interval. The spontaneous baroreceptor reflex gain (sBRG) was calculated from a method employed by Oosting et al 1997⁶, which involves a sequence technique allowing alterations in arterial pressure to be divided by reflex changes in heart rate or pulse interval.

To evaluate baroreceptor reflex function, the gain was determined from spontaneous changes in BP and PI using a time-series method designed for the rat^{6,7,8}. First, to filter out respiration induced fluctuations moving averages of BP (either SBP or DBP) and the PI are calculated over 10 cardiac cycles. Second, from these moving average data, spontaneously occurring ramps of either decreasing or increasing BP of four beats or more are used to calculate baroreceptor reflex gain. Third, for each pair of BP and PI ramps, measurements are made at delays of three, four and five beats; this is based on the delay time from a change in BP to a reflex response in PI in the rat as described by Oosting et al. (1997)⁶. Fourth, from these three ramps, plots were made of the changes in PI versus BP to form slopes for each of the delays and averaged values of the slope are calculated for each of the delays. sBRS values quoted represent the mean value of the three values. Unlike Oosting et al. (1997)⁶, we only used values from the positive slopes thereby avoiding contamination of our baroreceptor reflex data with non-baroreceptor-mediated changes in PI as previously described.^{7,8} Therefore, the method yields predominantly estimates of the gain of the parasympathetic component of the cardiac baroreflex which correlate well with those obtained by pharmacological means. It should be noted that the delay of 3–5 beats is probably too short to take account of full sympathetic reactions. Therefore the sBRG index is mainly a measure of the parasympathetic reaction.

In Vivo Gene Transfer into RVLM

Three weeks after renal artery clipping or sham surgery, animals were re-anesthetized and five 100 nL injections per side of viral suspension (either Ad-CuZnSOD or Ad-eGFP) were made into the RVLM at separate sites spanning 12.0-12.4 mm caudal to the bregma to the λ , 1.7–1.8 mm lateral to the midline and 8.0 mm below the dorsal medullary surface (bite bar = -3.5 mm). **Figure S1** shows the degree of spread of microinjections.

Validating In Vivo Gene Expression

The double fluorescence immunohistochemistry was done for CuZnSOD with tyrosine hydroxylase or NeuN to confirm the presence of expression in RVLM neurons. On the final

day of AP recording, the animals were anesthetized with sodium pentobarbital (100 mg/kg IP) and perfused transcardially with 100 ml of 0.1 M phosphate buffered saline (PBS, pH 7.4) at room temperature followed by 300 ml of 4% (w/v) paraformaldehyde in 0.1 M PBS, as previously described.⁹ Brains were removed, stored, and cryoprotected in fixative containing 20% (w/v) sucrose overnight at 4°C. The following morning, brains were rapidly frozen in liquid nitrogen and four sets of coronal sections (30 µm) of the entire rostrocaudal axis of the forebrain were cut on a cryostat (Cryocut CM3050, Leica Microsystems, Milton Keynes, UK). The free-floating sections were collected in 24-well tissue culture plates containing PBS prior to being processed for immunohistochemical detection. To perform double-labelling fluorescence immunohistochemistry, free-floating RVLM sections were incubated for 15 min in a preblocking solution comprising 10% (v/v) normal rabbit serum (Sigma) and 0.3% (v/v) Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS (3x 10 min). Sections were then incubated with human CuZnSOD antibody (sheep anti-human IgG; The Binding Site Limited), diluted 1:100 associated either with mouse anti-Tyrosine Hydroxylase, (TH; Zymed Laboratories, Invitrogen) diluted 1:1000 or mouse anti-NeuN (anti-NeuN clone A60, biotin conjugated, Millipore) diluted 1:1000 in PBS containing 1% (v/v) of normal rabbit serum and 0.3% (v/v) Triton X-100 for 48 h at 4°C. After the primary antibody incubations, the sections were rinsed in PBS (3 x 10 min) prior to a 1-h incubation in PBS containing biotinylated rabbit anti-sheep IgG (Vector Laboratories, 1:500 dilution), 1% (v/v) of normal rabbit serum and 0.3% (v/v) Triton X-100 at room temperature. Following rinses in PBS (3 x 10 min), the sections were incubated for 1 h in the corresponding secondary labelled antibody (streptavidin-conjugated Alexa Fluor 488 (Invitrogen, 1:500 dilution) for anti-CuZnSOD, anti-mouse Alexa Fluor 594 (Invitrogen, 1:500 dilution) for TH and NeuN in PBS containing 1% (v/v) of normal rabbit serum and 0.3% (v/v) Triton X-100. Subsequent to further washes (3 x 5 min), sections were then mounted onto glass microscope slides with 0.5% (w/v) gelatin, allowed to air-dry for 10–15 min before being coverslipped using an antifade fluorescent mountant (VectorShield, Vector Laboratories). Images of the sections were acquired using a Leica DM IRB with C-Plan optics (Leica, Germany).

Measurement of superoxide in the RVLM of 2K-1C rats

The superoxide in the RVLM was analyzed three weeks after microinjection of either Ad-CuZnSOD or Ad-eGFP. The rats were anesthetized with ketamine and xylazine (40 and 20 mg/kg, IP, respectively), decapitated, the brainstem was quickly removed, and bilateral punches of RVLM were obtained. Two brainstem slices (300 µm thick) of each animal were incubated for precisely 30 min in dihydroethidium dye (DHE; 1 mol/L) and imaged with a laser confocal microscope (Leica SP) as per Zimmerman et al.⁹ The fluorescence intensity was compared quantitatively within the RVLM at identical time points after DHE incubation.

Results

Ad-CuZnSOD and Ad-eGFP in the RVLM on Arterial Pressure and SBP variability in SHAM and 2K-1C rats

The systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MBP) were initially recorded via radiotelemetry for three weeks before microinjection of either Ad-CuZnSOD or Ad-eGFP into the RVLM and for a further three weeks after viral transfection. In all rat groups, baseline levels of SBP, DBP and MBP were similar as shown **Table S2**. And DBP and MBP in SHAM groups injected with either Ad-CuZnSOD or Ad-eGFP in the RVLM were unaffected **Figure S2**. Before the RVLM viral injection, the values of low frequency power (LF SBP) and very low frequency power of the systolic blood pressure (VLF SBP) in the 2K-1C were not different from the control group (**Table S3**).

Ad-CuZnSOD and Ad-eGFP in the RVLM on respiratory rate in SHAM and 2K-1C rats

The respiratory rate was analyzed for three weeks before microinjection of either Ad-CuZnSOD or Ad-eGFP into the RVLM and for a further three weeks after viral transfection. In all rat groups, the respiratory rate was similar as shown **Figure S3**.

Ad-CuZnSOD and Ad-eGFP in the RVLM on heart rate and the gain of the parasympathetic component of the cardiac baroreflex in SHAM and 2K-1C rats

The baseline of heart rate (HR) and spontaneous baroreceptor reflex gain (sBRG (HR)) were not significantly different between animal groups prior to surgery (**Table S4**). In the SHAM groups injected with either Ad-CuZnSOD or Ad-eGFP in the RVLM, HR and sBRG (HR) were unaffected (**Figure S4**). The 2K-1C group presented a tachycardia that developed during the 4th week after surgery. Additionally, the sBRG (HR) decreased gradually until the 4th week after clipping at which time it plateaued. Interestingly, after Ad-CuZnSOD viral transfection of the RVLM in 2K-1C rats, there were no significant responses in either HR (394 ± 9 versus 388 ± 8 bpm) or sBRG (HR) (-1.7 ± 0.1 versus -2.4 ± 0.2 bpm/mmHg) (**Figure S4**). In contrast, two weeks after Ad-eGFP transduction of RVLM in 2K-1C rats, a tachycardia (from 415 ± 15 to 469 ± 6 bpm, $P < 0.05$) and a decrease in sBRG (HR) (from -1.8 ± 0.3 to -0.9 ± 0.09 bpm/mmHg, $P < 0.05$) was observed. This tachycardia increased during the first week after Ad-CuZnSOD viral transfection whereas the decreased baroreflex gain manifested gradually over two weeks and plateaued during the third week after transfection.

Discussion

Baroreflex changes after chronic blockade of superoxide generation in the RVLM of the Goldblatt rat

The present study also shows that chronic O₂⁻ signalling in the RVLM reduced the tachycardia and prevented a reduction in the gain of the parasympathetic component of the cardiac baroreflex. The mechanisms involved in normalizing of the cardiac baroreflex gain may be due to a reduction of sympathetic activity to the heart thereby allowing cardiac vagal transmission to become more effective. Levy, (1971)¹⁰ reported that non-linear interactions between the parasympathetic and sympathetic nervous system occur at the level of the heart whereby high levels of cardiac sympathetic activity can depress vagal bradycardia, for example. However, we cannot rule out the possibility of a central effect of CuZnSOD on neurons involved in baroreceptor reflex integration. Indeed, there is a relationship between oxidative stress and baroreflex impairment.¹¹

The mechanisms by which baroreflex sensitivity is reduced in 2K-1C hypertension are not known. In the classic study of Salgado & Krieger (1973)¹² arterial baroreceptors reset to operate at higher pressure levels in hypertension. Rapid (acute) resetting occurs within the first few minutes after elevation of arterial pressure, but is only partial because the increased threshold for baroreceptor activation represents only 25-50% of the arterial pressure increase. Therefore, complete resetting occurs when the increase in pressure threshold equals the increase in arterial pressure; in the rat this is present after 48 h hypertension. Therefore, we can not exclude that the increased sympathetic vasomotor tone in 2K-1C is in part mediated by baroreceptor reflex impairment. Furthermore, studies by Li et al, (1996)¹³ have shown that ROS generated in the carotid bulb of atherosclerotic rabbits reduce carotid sinus nerve responses to elevations of pressure and that this could be mimicked by exogenous administration of ROS and prevented by ROS scavenging. Therefore, increased RVLM neuronal excitability may, in part, be mediated by baroreceptor dysfunction increasing oxidative stress in the RVLM. However, the precise mechanisms by which ROS in the

RVLM alter baroreflex function occurs is not well established. Previous evidence supports the idea that reduced availability of NO in the cardiovascular neurons may be involved in the mechanism.¹⁴

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Table S1. Experimental Protocol

Series of experiments	Protocols
First series	The p47phox serine phosphorylation was quantified by immunoprecipitation in the RVLM of control (SHAM) (n=5) and 2K-1C rats – 6 weeks after clipping (n=4).
Second series	The superoxide was measurement in the RVLM of four groups: [i] SHAM (n=6), [ii] 2K-1C rats: 6 weeks after clipping (n=6), [ii] 2K-1C + Ad-CuZnSOD (n = 5); [iv] 2K-1C + Ad-eGFP (n = 5). The Ad-CMV-CuZnSOD (Ad-CuZnSOD) and the Ad-CMV-eGFP (Ad-eGFP) were bilaterally microinjected into the RVLM three weeks after renal artery clipping and the analyses were performed three weeks later.
Third series	The Ad-CuZnSOD was microinjected into the RVLM in two groups: [i] 2K-1C + Ad-CuZnSOD (n = 7); [ii] SHAM + Ad-CuZnSOD (n = 6). Additionally, the Ad-eGFP was used in two additional rat groups: [iii] 2K-1C + Ad-eGFP (n = 5); [iv] SHAM + Ad-eGFP (n = 5). The arterial pressure was recorded by telemetry system during 6 weeks. In the end of the last week the animals were sacrificed and immunohistochemistry was performed to evaluate the CuZnSOD transfection in the RVLM

Table S2. Summary of systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean blood pressure (MBP) in 2K-1C and SHAM groups

Pressure	Baseline		3 weeks		6 weeks	
	SHAM	2K-1C	SHAM	2K-1C	SHAM + eGFP	2K-1C + eGFP
SBP (mmHg)	122 ± 8	122 ± 9	113 ± 7	162 ± 9 ^{*+}	132 ± 11	211 ± 9 [*]
DBP (mmHg)	89 ± 6	97 ± 6	85 ± 11	130 ± 13 ^{*+}	102 ± 11	173 ± 9 [*]
MBP (mmHg)	101 ± 6	106 ± 8	94 ± 9	141 ± 13 ^{*+}	106 ± 7	188 ± 9 [*]

Values are MEAN ± SEM; SHAM and 2K1C groups.

^{*}*P*<0.05 compared with SHAM group –Baseline and SHAM – 3 weeks;

⁺*P* < 0,05 compared to 2K-1C – Baseline;

Table S3. Low frequency (LF SBP) and very low frequency power of systolic blood pressure (VLF SBP), high frequency power of pulse interval (HF PI) and ratio of LF power to HF power (LF/HF) in 2K-1C and SHAM rat groups

Spectral analysis	Baseline		3 weeks		6 weeks	
	SHAM	2K-1C	SHAM	2K-1C	SHAM + eGFP	2K-1C + eGFP
LF SBP (mmHg ²)	2.6 ± 0.8	2.8 ± 0.8	2.8 ± 0.6	3.4 ± 0.6	2.4 ± 0.6	5.4 ± 0.3*
VLF SBP (mmHg ²)	4 ± 0.8	4.7 ± 0.2	3.5 ± 0.6	5.1 ± 0.6	3.5 ± 1.0	5.8 ± 0.2*
HF (PI)	10.3±0.4	13.5±0.9	9.6±0.9	13.4±0.9*	10.1±1.9	8.3±0.01
LF/HF	0.24±0.03	0.20±0.04	0.29±0.05	0.21±0.02	0.24±0.01	0.31±0.03

Values are MEAN ± SEM; SHAM and 2K-1C groups.

* $P < 0.05$ compared with SHAM group – Baseline and SHAM – 3 weeks;

Table S4. Heart rate (HR) and spontaneous cardiac baroreceptor reflex gain (sBRG of HR) calculated by *Hey-Presto software* in 2K-1C and SHAM rat groups.

Cardiovascular Parameters	Baseline		3 weeks		6 weeks	
	SHAM	2K-1C	SHAM	2K-1C	SHAM + eGFP	2K-1C + eGFP
HR(bpm)	376 ± 9	406 ± 7	380 ± 15	415 ± 7	398 ± 11	469 ± 6*
sBRG (HR) (bpm/mmHg)	-2.4 ± 0.6	-2.4 ± 0.1	-2.7 ± 0.5	-1.8 ± 0.3	-2.4 ± 0,4	-0.9 ± 0,09*

Values are MEAN ± SEM; SHAM and 2K1C groups.

* $P < 0.05$ compared with SHAM group –Baseline and SHAM – 3 weeks;

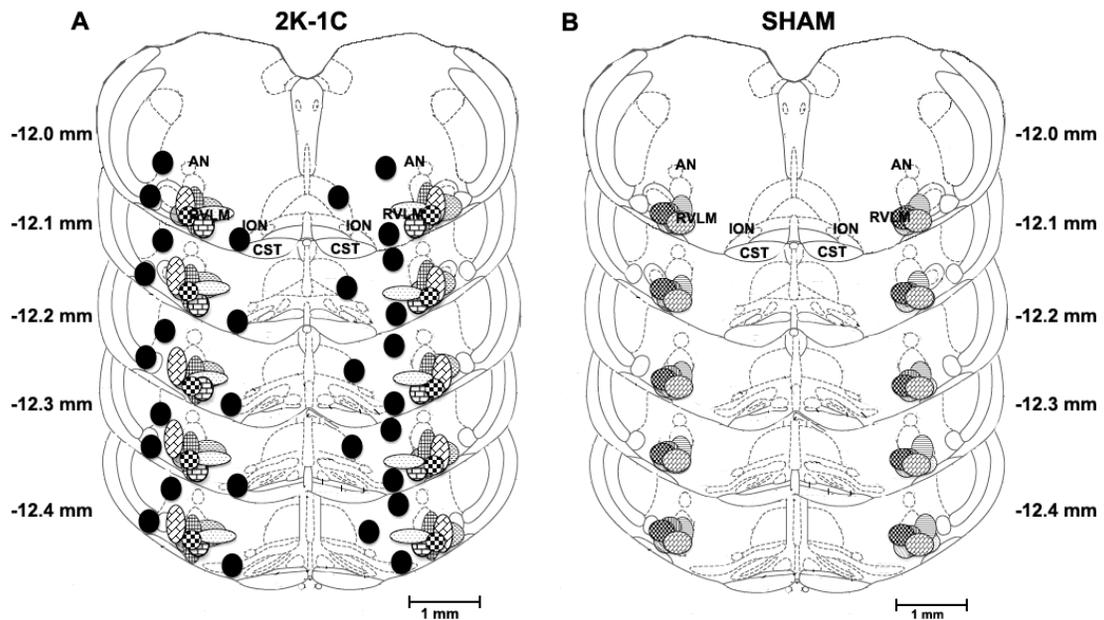


Figure S1: Schematic representation of consecutive coronal sections from the medulla oblongata showing the RVLM region. Hatched areas represent the spread of Ad-CuZnSOD injection (100 nL). A: overlap of bilateral injection sites from seven representative 2K-1C rats and four bilateral injection sites that were outside RVLM, which were ineffective at lowering blood pressure. B: overlap of bilateral injection sites from six representative SHAM rats. CST, corticospinal tract; ION, inferior olivary nucleus; AN, ambiguus nucleus; RVLM: rostral ventrolateral medulla.

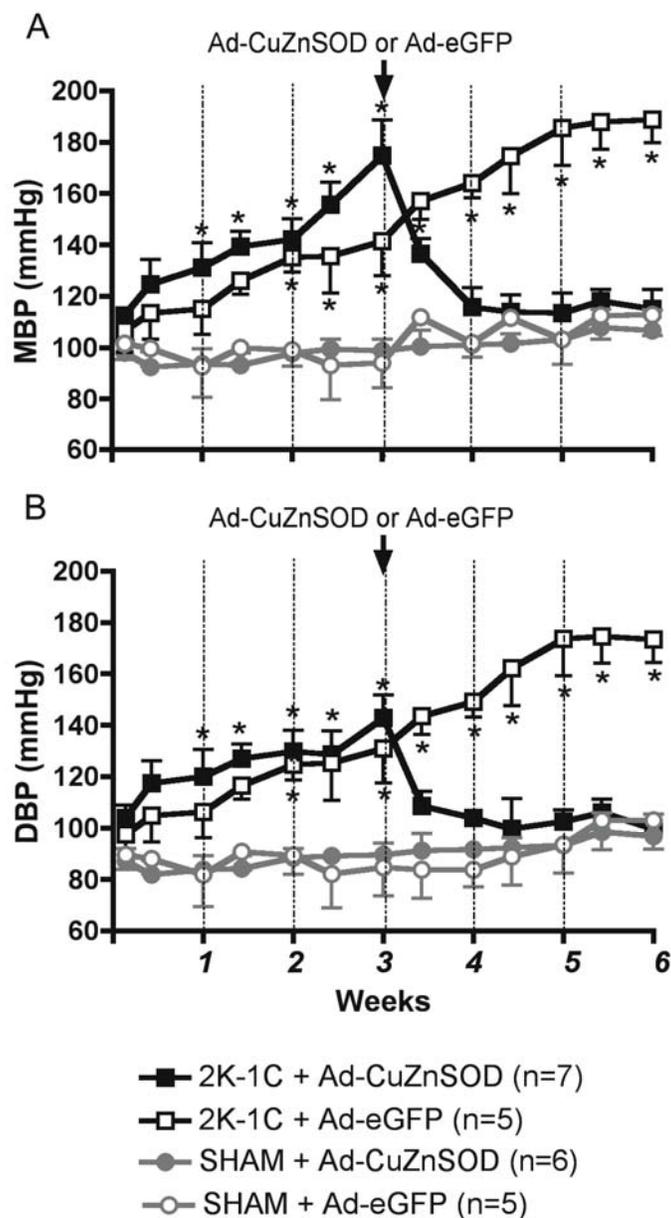


Figure S2: Increased scavenging of cytoplasmic O_2^- in the RVLM prevents Goldblatt hypertension. **[A]** Summary of mean blood pressure (MBP) and **[B]** diastolic blood pressure (DBP) recorded by radiotelemetry before and after microinjection of Ad-CuZnSOD and Ad-eGFP into the RVLM for 6 weeks. Values are expressed as the means \pm SEM. These results showed that the blood pressure of hypertensive animals increased gradually and significantly over 2 weeks reaching a plateau by the 5th week after clipping. A microinjection of Ad-CuZnSOD in the RVLM prevented 2K-1C hypertension. However, hypertension was observed in the Ad-eGFP group. * $P < 0.05$ compared to SHAM.

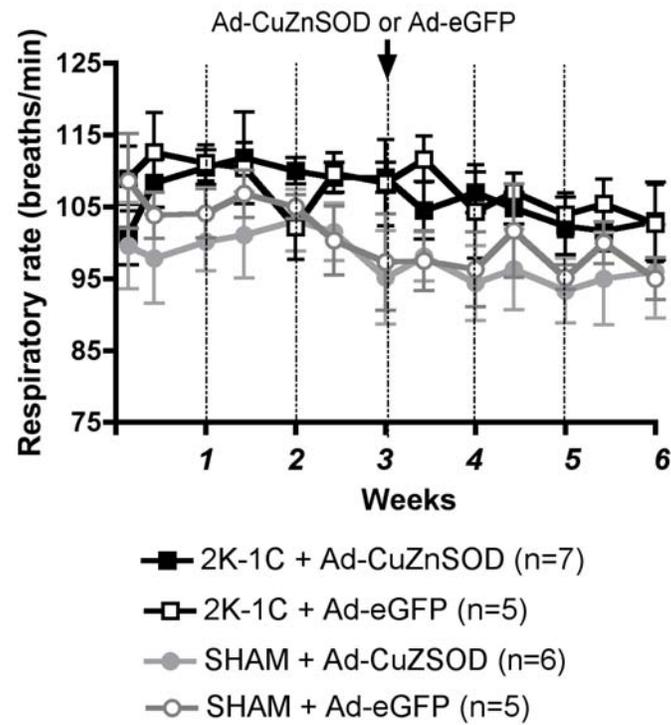


Figure S3: Respiratory rate in the Goldblatt model. Values are expressed as the means \pm SEM. There was no change in respiratory frequency suggesting no influence on respiratory rhythm generating neurons.

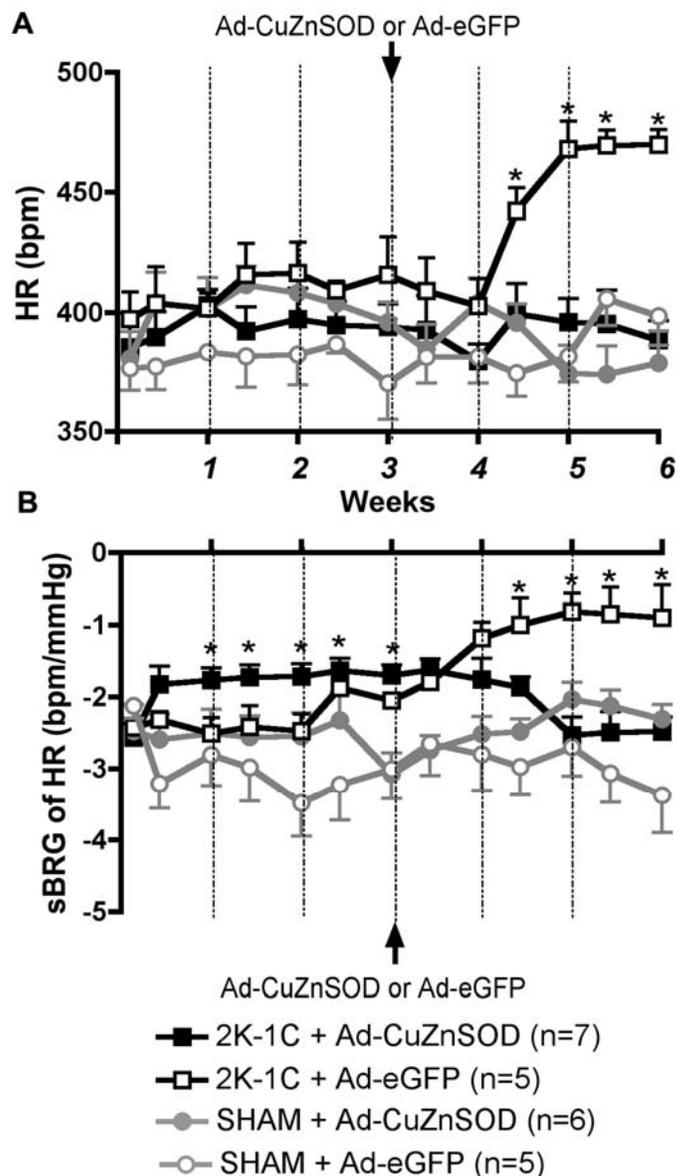


Figure S4: [A] Heart rate (HR) and [B] spontaneous cardiac baroreceptor reflex gain (sBRG of HR) in the Goldblatt model. Values are expressed as the means \pm SEM. In the Ad-eGFP 2K-1C group heart rate increased one week after viral transfection whereas the parasympathetic component of the cardiac baroreflex decreased gradually before plateauing 3 weeks after transfection. However, this tachycardia was blunted by Ad-CuZnSOD in the RVLM of 2K-1C rats whereas the gain of the parasympathetic component of the cardiac baroreflex remained equivalent to control. * $P < 0,05$ compared to SHAM.