Effects of Angiotensin Type 2 Receptor Overexpression in the Rostral Ventrolateral Medulla on Blood Pressure and Urine Excretion in Normal Rats

Lie Gao, Weizhong Wang, Wei Wang, Hongwei Li, Colin Sumners, Irving H. Zucker

Abstract—Central angiotensin II plays a critical role in the regulation of cardiovascular function and autonomic activity, in part, via angiotensin type 1 receptors in the rostral ventrolateral medulla (RVLM). Increasing evidence indicates that angiotensin II can also act on angiotensin type 2 receptors (AT2Rs) to exert antagonistic effects. In the current study we determined the effects of overexpression of AT2R in the RVLM on sodium and water excretion and on blood pressure in conscious rats. The overexpression of AT2R was induced by bilateral microinjection of the AT2R adenovirus (Ad5-SYN-AT2R-IRE5-EGFP, 2.5×10^6 injection units in 0.5 μL; Ad5-SYN-EGFP as the control, 2.5×10^6 injection units in 0.5 μL) into the RVLM of rats. Immunofluorescence staining showed that microinjection of AT2R adenovirus into the RVLM evoked local overexpression. Significant overexpression of AT2R in the RVLM began at 24 hours and was sustained up to 12 days after microinjection. Overexpression of AT2R in the RVLM significantly decreased the nocturnal arterial blood pressure and increased the 24-hour urine excretion at days 2, 3, and 4 after gene delivery compared with the control rats. These alterations were abolished by the microinfusion of captopril into the RVLM and were enhanced by angiotensin II infusion. Overexpression of AT2R in the RVLM also significantly decreased the urine concentration of noradrenaline and 24-hour noradrenaline excretion (1.1±0.5 μg in control rats and 2.4±0.5 μg in AT2R rats; P<0.05). These results suggest that overexpression of AT2R in the RVLM induced a diuresis that may be mediated, in part, by sympathoinhibition. (Hypertension. 2008;51[part 2]:521-527.)

Key Words: angiotensin II type 2 receptor □ rostral ventrolateral medulla □ arterial blood pressure □ urine excretion □ noradrenaline excretion

At least 2 distinct subtypes of angiotensin receptors, the angiotensin type 1 receptor (AT1R) and type 2 receptor (AT2R), have been identified in the mammalian brain. It is well known that angiotensin II (Ang II) has a significant influence on cardiovascular function and sympathetic nervous activity via activation of central AT1R. Microinjection of Ang II into a lateral cerebral ventricle, paraventricular nucleus, rostral ventrolateral medulla (RVLM), and nucleus of the tractus solitarius elicits an increase in blood pressure and sympathetic nervous activity. These effects of Ang II are abolished by pretreatment with the AT1R antagonist losartan. On the other hand, data from a whole-cell patch-clamp recording and extracellular single unit discharge recording document that stimulation of AT1R significantly inhibits the neuronal potassium channel current9,10 by increasing channel open probability,11 the effects of central AT1R activation on blood pressure and sympathetic nerve activity in intact animals are largely unknown. The RVLM is an important region of the brain in maintaining tonic sympathetic nerve activity. By direct projections to the sympathetic preganglionic neurons of the spinal cord and by receiving inputs from other sympathetic-related central nuclei, the RVLM acts as a crucial relay station and integrative center to transfer central signals from more rostral structures to peripheral sympathetic nerves. In both anesthetized12-14 and conscious animals,15 bilateral inactivation or ablation of neurons in the RVLM leads to a profound fall in arterial pressure and sympathetic nerve activity. Therefore, in the present study, we investigated the effect of AT1R gene transfer into the RVLM on blood pressure and urine excretion in conscious rats.

Methods
Male Sprague-Dawley rats weighing between 274 and 365 g were used in these experiments. All of the experiments were approved by the institutional animal care and use committee of the University of...
Nebraska Medical Center and were carried out under the guidelines of the American Physiological Society and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**AT₂R Gene Transfer into the RVLM**

An adenoviral vector containing the rat AT₂R gene (Ad5-SYN-AT2R-RES-EGFP, with Ad5-SYN-EGFP as the control virus), driven by the neuron-specific synapsin promoter, was used to induce the overexpression of the AT₂R protein in the RVLM. Under anesthesia, rats were placed in a stereotaxic frame (Stoelting Instruments), and the skull was exposed through an incision on the midline of the scalp. Two small holes on the skull above the RVLM were made bilaterally according to the following coordinates: 12.5-mm posterior to the bregma and 2.0-mm lateral from midline. The tip of 0.5-µL Hamilton microsyringe was inserted into the RVLM 10.0 mm below the dorsal surface of skull. The AT₂R adenovirus (0.5 µL of 2.5 × 10⁵ infection units) was microinjected bilaterally and sequentially into the RVLM over a 25-minute period with a microinjection device.

**Chronic Infusion of Reagents Into the RVLM**

After the above microinjection, a cannula (made from a 30-gauge needle) connected to a 2-week osmotic minipump (0.25 µL/h; 1002 Alzet Osmotic Pumps) was implanted bilaterally into the RVLM and fixed on the skull by dental cement to chronically infuse Ang II or the Ang Ⅱ-converting enzyme inhibitor, captopril. The cannula used in this experiment evoked no obvious mechanical damage in the RVLM. We did not observe any significant alteration in mean arterial pressure (MAP) or heart rate in the control rats after inserting the cannula into the RVLM.

**Telemetric Recording of Arterial Pressure in the Conscious State**

A radiotelemetry device (model TA11PA-C40, Physiotel, Data Sciences International) was implanted for the measurement of arterial pressure and heart rate in the conscious state. Under ketamine (100 mg/kg, IP) anesthesia, a central groin incision was made, and a radiotelemetry device was secured to the inguinal area. The sensing catheter of this device was inserted into the left femoral artery against blood flow. The signals received by the device were processed and digitized as radiofrequency data, which were recorded and stored in a computer using the Dataquest IV system (Data Sciences International). In the conscious state, arterial blood pressure was recorded 10 min/h for 24 h/d during the entire experimental period, using a macro written for the Chart (version 5.4.2) software (ADInstruments, Inc.).

**Measurement of Urine Flow**

Rats were placed in a metabolic cage to measure water and food intake, urine and feces excretion, and body weight on a daily basis. The urine was collected under mineral oil and frozen (−80°C) until it was used for the measurement of noradrenaline and electrolyte concentrations.

**Measurement of Urinary Noradrenaline Excretion**

This measurement was done using a Noradrenaline Enzyme Immunoassay kit (Labor Diagnostika Nord GmbH & Co KG). The range of urinary noradrenaline concentration measurable by this kit is between 0.1 and 1000.0 ng/mL. According to our preliminary data, the concentration of urinary noradrenaline is ~30 to 500 ng/mL in the most diluted and concentrated urine of rats, respectively.

**Immunofluorescence Staining**

The rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. The brain was removed, and coronal sections (20 µm) were made in a cryostat. The sections were washed with PBS, followed by incubation with primary antibodies (rabbit polyclonal IgG anti-AT₂R) overnight at 4°C. On the following day, the sections were washed and incubated with secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG, Molecular Probes; at 1:250 in the same diluent used for the primary antibodies) for 2 hours at room temperature. The sections were rinsed 3 times in PBS and mounted in Vectashield. The stained sections were photographed with a confocal laser-scanning microscope.

**Preparation of RVLM and Western Blot Analysis of AT₂R Protein**

The brains were removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned at 100-µm thickness in a cryostat. The RVLM was punched and homogenized in radioimmunoprecipitation assay buffer. The protein extraction from homogenates was used to analyze the AT₂R expression by Western blot. The concentration of protein extracted was measured using a protein assay kit (Pierce) and adjusted to the same with equal volumes of 2X 4% SDS sample buffer. The samples were boiled for 5 minutes, followed by loading on the 7.5% SDS-PAGE gel (10 µg of protein, 30 µL per well) for electrophoresis using a Bio-Rad minigel apparatus at 40 mA per each gel for 45 minutes. The fractionized protein on the gel was electrochemically transferred onto the polyvinylidene fluoride membrane (Millipore) at 300 mA for 90 minutes. The membrane was probed with primary antibody (AT₂R rabbit polyclonal antibody, Santa Cruz, 1:1000) and secondary antibody (goat anti-rabbit IgG horseradish peroxidase, Santa Cruz, 1:2500) and then treated with enhanced chemiluminescence substrate (Pierce) for 5 minutes at room temperature. The bands in the membrane were visualized and analyzed using UVP BiolImaging Systems.

**Statistical Analyses**

All of the data are described as the mean ± SEM. A 1-way or 2-way ANOVA was used followed by either the Newman-Keuls or Bonferroni posthoc analysis where appropriate. Statistical analysis was done with the aid of the SAS software. P < 0.05 was considered statistically significant.

**Results**

**Overexpression of AT₂R in the RVLM by Gene Transfer**

Figure 1 shows immunofluorescence images of AT₂R expression in the RVLM at the seventh day after microinjection of the AT₂R adenovirus. The top panels show a high-magnification view of AT₂R expression within single neurons of the RVLM. The bottom panels show views of a strong immunoreactive signal in the RVLM after transfection.

**Time Course of AT₂R Protein Expression in the RVLM After Gene Transfer**

We determined the time course of AT₂R expression in the RVLM after adenoviral transfection at days 1 through 12, as shown in Figure 2. The top panel is a representative Western blot showing AT₂R protein levels in the RVLM at various time points after gene transfer. The bottom panel shows the quantified group data. Compared with the control rats, AT₂R gene transfer into the RVLM induced a significant increase in AT₂R protein expression within 24 hours (ratio of AT₂R/GAPDH: 0.1 ± 0.1 in control and 0.4 ± 0.1 at 24 hours after gene transfer; n = 4; P < 0.05). At day 6 after gene transfer, AT₂R protein reached its peak expression (0.9 ± 0.1) and was sustained for ≤12 days (0.9 ± 0.4).
Effect of Gene Transfer on Urine Flow and Other Physiological Parameters

There were no notable differences in body weight, food intake, or feces excretion between the AT₂R-transfected rats and the control rats. However, we observed a marked difference in urine excretion and water intake between these groups. As can be seen in Figure 3, there was a significant increase in urine volume in AT₂R adenoviral-transfected rats at days 2, 3, and 4 compared with the baseline urine volume. In contrast, the urine excretion was significantly decreased in the control adenoviral-transfected rats at day 2 compared with baseline. These alterations in urine flow were abolished and enhanced by the microinfusion of captopril or Ang II into the RVLM, respectively. In these 6 groups of rats, the alteration of water intake was similar in time to the change in the urine excretion. There was no difference in the 24-hour sodium excretion (data not shown).

Effect of Gene Transfer on Arterial Pressure

In addition to the increase in urine excretion described above, we also observed changes in arterial blood pressure in the conscious state during the duration of the experiment. The top panels of Figure 4 show that, during days 2 to 4 after gene transfer, arterial pressure exhibited a daily rhythm in the control adenoviral-transferred rats with a higher blood pressure at night (10:00PM to 8:00AM) compared with that during daytime (8:00 AM to 10:00 PM). However, in the rat with AT₂R adenoviral transfection, arterial pressure was continuously lower, without an obvious circadian rhythm. The blood pressure, therefore, was lower in rats with AT₂R overexpression compared with control rats, at least during the nighttime period. The bottom panel of the Figure 4 is the group data showing the average blood pressure during day and night times in the control- and AT₂R-transfected rats. Nighttime MAP in control rats was significantly higher than the daytime average blood pressure and the nighttime blood pressure of the AT₂R rats. These data strongly suggest that overexpression of the AT₂R in the RVLM reduces arterial pressure.

In AT₂R-transfected rats receiving chronic RVLM microinfusion of captopril or Ang II, arterial pressure exhibited no daily rhythm. We, therefore, averaged 24-hour MAP as the representative arterial pressure for each day. These data are shown in Figure 5. RVLM microinfusion of captopril completely abolished the difference in arterial pressure between the control virus and AT₂R viral-transfected rats. In contrast, RVLM microinfusion of Ang II evoked hypertension in the control viral-transfected rats but hypotension in the AT₂R viral-transfected rats.

Figure 1. Immunofluorescence images showing overexpression of AT₂R protein in the RVLM of a rat after 7 days of AT₂R gene transfer (bottom). The top panels show higher-power magnification of individual neurons where AT₂R protein is shown by the green fluorescence in the membrane of these cells. The red signal represents cell nuclei. Scale bars=20 μm in top panels and 1 mm in bottom panels.

Figure 2. Western blot showing the time course of AT₂R protein expression in the RVLM after gene transfer. The top panel shows representative Western blots, and the bottom panel is the group data. *P<0.05 and **P<0.01 vs the control group. n=4 in each group.

Figure 3. Group data showing the alterations in urine excretion before and after gene transfer into the RVLM. GFP indicates rats with over expression of GFP in the RVLM; AT₂R, rats with overexpression of AT₂R in the RVLM; Cap, rats receiving the microinfusion of captopril into the RVLM; Ang II, rats receiving the microinfusion of Ang II into the RVLM. *P<0.05 vs the urine excretion before gene transfer. n=6 to 7 per group.

Figure 4. Top panels: Arterial pressures during day (8:00 AM to 10:00 PM) and night (10:00PM to 8:00AM) in control- and AT₂R-transfected rats. Bottom panel: Bar graph of the average blood pressure during day and night in the control- and AT₂R-transfected rats. Nighttime MAP in control rats was significantly higher than the daytime average blood pressure and the nighttime blood pressure of the AT₂R rats. These data strongly suggest that overexpression of the AT₂R in the RVLM reduces arterial pressure.

Figure 5. Bar graph showing the 24-hour MAP in the control- and AT₂R-transfected rats. RVLM microinfusion of captopril completely abolished the difference in arterial pressure between the control virus and AT₂R viral-transfected rats. In contrast, RVLM microinfusion of Ang II evoked hypertension in the control viral-transfected rats but hypotension in the AT₂R viral-transfected rats.
Effect of Gene Transfer on Noradrenaline Excretion

Figure 6 shows the 24-hour noradrenaline excretion at days 2, 3, and 4 after RVLM gene transfer. Urine flow was significantly increased in the AT2R gene-transfected rats compared with the control rats. The middle panel shows the urinary noradrenaline concentration. Noradrenaline concentration was significantly lower in the AT2R gene-transfected rats compared with control rats. The bottom panel shows the 24-hour noradrenaline excretion. Noradrenaline excretion was significantly decreased in the AT2R gene-transfected rats compared with the control rats.

Discussion

The major findings of the present study are that overexpression of AT2R in the RVLM evoked a significant increase in urine excretion, a decrease in arterial blood pressure, and a reduction in noradrenaline excretion in conscious rats. These results provide the first evidence that AT2Rs in the RVLM are capable of playing a role in the regulation of cardiovascular function, sympathetic nerve activity, and perhaps renal function.

The AT2R recombinant adenoviral vector Ad5-SYN-AT2R-IRES-EGFP, constructed by Li et al., contains the AT2R gene and an IRES-linked EGFP reporter gene driven by the neuron-specific synapsin II gene promoter. Therefore the AT2R gene is specifically overexpressed in neurons. It has been demonstrated that microinjection of this vector into the rat paraventricular nucleus evoked an area of specific overexpression of AT2R in neurons, which lasted \( \geq 30 \) days. By microinjection of this vector into the RVLM, we successfully induced an overexpression of AT2R, demonstrating sustained expression \( \leq 12 \) days (Figures 1 and 2).
An interesting finding in the present experiments was an increase in urine excretion in rats with overexpression of AT2R in the RVLM. This increase was transient compared with the prolonged overexpression of AT2R (Figures 2 and 3). In control rats, overexpression of green fluorescent protein (GFP) in the RVLM evoked a decrease in the urine excretion at day 2 after gene transfer. We, therefore, speculate that the stress of recent surgery may have elevated Ang II levels, which were capable of stimulating the overexpressed AT2R. It is possible that after ≈5 days, stress-induced Ang II may have subsided so that the stimulation of the AT2R was reduced. In control rats, just the opposite may take place. That is, elevated endogenous Ang II would stimulate the more dominant AT1R in the RVLM to decrease urine excretion. Indeed, microinfusion of the angiotensin-converting enzyme inhibitor captopril into the RVLM to inhibit endogenous production of Ang II completely abolished the increase in urine excretion in both control and AT2R gene-transfected rats (Figure 3). In addition, microinfusion of exogenous Ang II into the RVLM augmented and sustained the increase in urine excretion.

Another major finding of this experiment is that overexpression of AT2R in the RVLM prevents the nocturnal increase in arterial blood pressure (Figure 4). In control rats, the arterial blood pressure exhibited a daily rhythm with a higher blood pressure at night and lower blood pressure during the day at days 2, 3, and 4 after gene transfer. However, in rats with AT2R gene transfer, arterial blood pressure was continuously lower, without an obvious circadian rhythm. These data suggest that overexpression of the AT2R in the RVLM reduces arterial blood pressure, at least at night. It is well established that cardiovascular parameters change with a 24-hour cycle. For example, blood pressure, heart rate, cardiac output, and stroke volume are higher in the active phase than that in the rest phase. The circadian rhythm for arterial pressure in our control rats was similar to that reported previously. Values for blood pressure were consistently higher during the dark period than during the light period. Although several investigators have studied the cardiovascular circadian rhythms in humans and in animals, it has not yet been determined whether the daily rhythm of blood pressure is controlled by an endogenous circadian clock or depends solely on rest-activity cycles. However, it has been suggested that the circadian pattern of blood pressure is influenced by the renin-angiotensin system. In TG(mRne2)27 transgenic rats, the circadian blood pressure pattern was reversed. On the other hand, systemic Ang II infusion also reversed the blood pressure circadian rhythm. Interestingly, the circadian expression pattern of AT1R mRNA expression in the suprachiasmatic nuclei of the anterior hypothalamus in rats has been reported recently. Moreover, the plasma concentration of Ang II also exhibits a circadian variation. Rittig et al reported that plasma Ang II showed a marked circadian rhythm in normal children with a nocturnal increase. In the current experiment, we found that, in both the AT2R and GFP gene-transfected rats, microinfusion of Ang II or captopril into the RVLM prevented the circadian rhythms for arterial blood pressure. On the other hand, microinfusion of Ang II into the RVLM evoked a long-lasting hypertension in the GFP-transfected rats and a hypotension in the AT2R-transfected rats (Figure 5). These results suggest that, in control rats, Ang II stimulating the dominant AT1R in the RVLM contributes to sympathoexcitation and an increase in arterial blood pressure. In contrast, in the AT2R gene-transfected rats, Ang II activating the now overexpressed AT2R in the RVLM decreases arterial blood pressure. Indeed, electrophysiological experiments have demonstrated that stimulation of AT1R significantly inhibits the neuronal potassium channel current and increases the neuronal spontane-
ous firing rate in the RVLM.4,5 On the other hand, activation of AT2R significantly increased the neuronal voltage-gated potassium channel current10,11 and resulted in a decrease in the firing rate of presympathetic RVLM neurons.31

The exact mechanism underlying the increase in urine flow and relative hypotension in rats with AT2R overexpression in the RVLM is not clear. However, the lower arterial blood pressures in the AT2R-overexpressed rats with or without RVLM infusion of Ang II imply a relatively lower sympathoexcitatory tone compared with their control partners. Indeed, we measured noradrenaline excretion at the days 2, 3, and 4 after gene transfer and found that both the noradrenaline concentration and the total 24-hour noradrenaline excretion were lower in the AT2R-transfected rats compared with control rats (Figure 6). We, therefore, conclude that overexpression of AT2R protein in the RVLM may result in a reduction in sympathetic outflow and a decrease in the arterial blood pressure, contributing to an increase in urine volume. We speculate that sympathoinhibition may result in renal vasodilation, thus, contributing to the observed diuresis. However, because electrical stimulation of the C1 area increases plasma vasopressin32 and lesion of C1 cells reduces stress-induced vasopressin release,33 we, therefore, cannot rule out the possibility that inhibition of C1 cells by AT2R overexpression could have caused a reduction in vasopressin secretion relative to the control rats and contributed to the increase in urine flow.

Perspectives

The present study provides insights into the effects of overexpression of AT2R in the RVLM on urine excretion, arterial blood pressure, and noradrenaline excretion in conscious rats. Our data demonstrated that overexpression of AT2R in the RVLM of normal rats significantly increased urine excretion, decreased arterial blood pressure, and reduced noradrenaline excretion. These results suggest that activation of AT2R in the RVLM inhibits sympathetic nerve activity and induces a diuresis. This is the first evidence to our knowledge showing the involvement of central neuronal AT2R in the regulation of these parameters. In recently acquired preliminary data, we demonstrated that there was markedly lower AT2R protein expression in the RVLM of heart-failure rats. Interestingly, similar to our finding in heart-failure rats, Peng and Phillips34 have reported a down-regulation of AT2R mRNA expression in the brain stem and hypothalamus of cold-induced hypertension rats, an animal model with the same characteristics of sympathoexcitation as the heart failure model. These data imply that the downregulation of central AT2R, especially in the RVLM, is a potential contributor of pathological sympathoexcitation in such diseases as heart failure and hypertension. Strategies to target the AT2R in the central nervous system may prove to be beneficial in the treatment of sympathoexcitatory states.

Acknowledgments

We acknowledge the expert technical assistance of Pamela Curry, Johnnie F. Hackley, Phyllis Anding, and Li Yu.

Sources of Funding

This study was supported by a Scientist Development Grant from the American Heart Association National Center (award No. 0635007N) and National Institutes of Health grants PO-1-HL-62222 and RO-1-HL-38690.

Disclosures

None.

References


Effects of Angiotensin Type 2 Receptor Overexpression in the Rostral Ventrolateral Medulla on Blood Pressure and Urine Excretion in Normal Rats
Lie Gao, Weizhong Wang, Wei Wang, Hongwei Li, Colin Sumners and Irving H. Zucker

Hypertension. 2008;51:521-527; originally published online December 17, 2007;
doi: 10.1161/HYPERTENSIONAHA.107.101717

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/51/2/521

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial
Office. Once the online version of the published article for which permission is being requested is located,
click Request Permissions in the middle column of the Web page under Services. Further information about
this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/