nature publishing group

Activation of Central Angiotensin Type 2 Receptors Suppresses Norepinephrine Excretion and Blood Pressure in Conscious Rats

Juan Gao[1], Hao Zhang[1], Khang D. Le[1], Jie Chao[1] and Lie Gao[1]

BACKGROUND

We have previously documented the finding that central angiotensin type 2 receptors (AT2R) negatively modulate sympathetic outflow and arterial blood pressure (BP). In this study, we investigated the effects of intracerebroventricular (icv) infusion of Compound 21 (C21), the first selective nonpeptide AT2R agonist, on norepinephrine (NE) excretion and BP in rats.

METHODS

C21 was infused icv for 7 days, using a micro-osmotic pump. Urinary NE concentration was measured using the NE enzyme immunoassay kit. BP was recorded by radiotelemetry. After 7 days, the rats were killed and three relevant samples from sympathetic brain regions and the cerebral cortex were obtained by micro-punching to measure neuronal nitric oxide synthase (nNOS) protein expression by western blot. In addition, the influence of C21 on neuronal potassium current (I_{Kv}) was determined by whole-cell patch-clamp in a neuron cell line, CATH.a.

RESULTS

(i) Icv treatment with C21 significantly decreased both the concentration and the amount of NE in night time urine, but had no effect on daytime urine. (ii) C21-treated rats exhibited a slight but significant decrease in BP. (iii) The effects of C21 on NE excretion and BP were abolished by use of the AT2R antagonist, PD123319, and nitric oxide synthase (NOS) inhibitor, *N*-omega-nitro-L-arginine methyl ester (L-NAME). (iv) C21 treatment significantly upregulated nNOS expression in the paraventricular nucleus of the hypothalamus (PVN) and rostral ventrolateral medulla (RVLM), but not in the nucleus of the solitary tract (NTS) and cerebral cortex. (v) In CATH.a neurons, C21 treatment significantly increased I_{Kv}, and this increase was completely abolished by PD123319 and L-NAME.

CONCLUSIONS

These results demonstrate a central inhibitory influence of C21 on sympathetic outflow by means of a nNOS-dependent mechanism that might be mediated by facilitating the neuronal potassium channel.

Keywords: *angiotensin type 2 receptor; blood pressure; central nervous system; hypertension; norepinephrine; potassium current*

American Journal of Hypertension, advance online publication 10 March 2011; doi:10.1038/ajh.2011.33

Even though it is widely accepted that angiotensin II (Ang II) primarily activates two receptor subtypes, the angiotensin type 1 receptor (AT1R) and angiotensin type 2 receptor (AT2R), our understanding of the functional significance of AT2R has not kept pace with that of AT1R, on which voluminous work has been done.[1,2] It has been well documented that AT1R mediates the majority of Ang II effects in both physiological and pathological conditions. These include the regulation of sympathetic outflow and cardiovascular function, water and electrolyte balance, thirst, and hormone secretion.[3,4] The AT2R, on the other hand, has long been viewed as having its major function during the early stages of development and growth of animals, in view of its ubiquitous expression at very high levels in the fetus and its rapid regression to low levels shortly after birth.[1,2] This limited view of the role of AT2R has dampened the enthusiasm to explore this receptor's function, especially in adult animals.

The results of some studies, however, consistently imply that the role of AT2R is of physiological significance. For example, *in vitro* experiments demonstrated that AT2R stimulation increases potassium current (I_{Kv}), thereby decreasing neuronal excitability.[5,6] Siragy *et al.*[7] reported that AT2R-null mice had slightly elevated systolic blood pressure (BP) as compared to wild-type control mice, and that infusion of a subpressor dose of Ang II induced no change in the BP in wild-type mice whereas it significantly increased the BP in AT2R knockout mice. Moreover, Li *et al.*[8] found that injection of Ang II into the cerebral ventricle evoked a larger increase in BP in AT2R knockout mice as compared to wild-type mice. These investigators further demonstrated that, in wild-type mice, central injection of Ang II plus the AT2R antagonist PD123319 initiated a greater pressor response than the one induced by Ang II alone. These results suggest that AT2R has a negative influence on neuronal function and cardiovascular activity, which opposes the stimulating effect of AT1R. Indeed, we recently

[1]Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, Nebraska, USA. Correspondence: Lie Gao (lgao@unmc.edu)

Received 30 September 2010; first decision 16 November 2010; accepted 9 January 2011.

found that AT2R protein expression was significantly downregulated in the rostral ventrolateral medulla (RVLM) of rats with chronic heart failure, and that this decrease in AT2R expression played a critical role in sympathoexcitation in this syndrome.[9] In conscious, normal rats, on the other hand, overexpression of AT2R in the RVLM evoked a transient but significant decrease in arterial blood pressure (AP) and excretion of norepinephrine (NE).[10] Interestingly, recent findings from our laboratory clearly demonstrate that, in the brainstem, liver, and kidney of adult rats, AT2R protein expression is significantly higher than in the corresponding tissues of fetuses and neonates,[11] thereby suggesting that AT2R may have a function not only in early life but also in adulthood.

Compound 21 (C21) is a recently created nonpeptide AT2R agonist which is metabolically stable, is effective when administered orally, and has potential therapeutic efficacy.[12] C21 has been shown to have beneficial effects in animal models of hypertension and myocardial infarction when administered systemically.[12,13] Moreover, C21-evoked dose-dependent vasorelaxation in isolated aortic and mesenteric vessels.[14] However, there have been no reports regarding its effects on the central nervous system, sympathetic outflow, and AP in animals. In this experiment, we hypothesized that central administration of C21 would have sympatho-inhibitory and depressor effects. We therefore determined the influences of central administration of C21 on NE excretion and AP in conscious normal rats.

METHODS

Animal experiments. In this experiment, 35 male Sprague–Dawley rats of weights ranging between 290 and 380 g were used. All the experimental procedures 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.

Under isoflurane anesthesia, the rats were implanted with radiotelemetry devices (model TA11PA-C40, Physiotel; Data Sciences International, St Paul, MN), which were secured in the inguinal area. The sensing catheter was inserted into the left femoral artery, against the direction of blood flow, for the purpose of measuring the values of pulsatile and mean arterial blood pressure (MAP).

The rats were randomly assigned to one of four groups to receive four different reagents. Each of the anesthetized rats was placed in a stereotaxic frame (Stoelting Instruments, Wood Dale, IL). The skull was exposed through a midline scalp incision. A small burr hole was made above the right cerebral ventricle (coordinates: 0.8 mm posterior to the bregma and 1.5 mm lateral from midline). ALZET Brain Infusion Kits and 1007D Micro-Osmotic Pumps (ALZET Osmotic Pumps; DURECT, Cupertino, CA) were used for the intracerebroventricular (icv) infusion of the appropriate reagent for 7 days. The four groups were: vehicle control group (artificial cerebrospinal fluid 1 µl/h), C21 (0.5 µg/µl/h) group, C21 (0.5 µg/µl/h) + PD123319 (0.5 µg/µl/h) group, and C21 (0.5 µg/µl/h) + *N*-omega-nitro-L-arginine methyl ester (L-NAME, 50 µg/µl/h) group. C21 was a gift from Vicore Pharma AB (Goteborg, Sweden). PD123319 and L-NAME were purchased from Sigma-Aldrich (St Louis, MO).

After the surgery, each of the rats was placed in a metabolism cage for the collection of urine, and at that time point the AP was also recorded. The urine was collected twice a day: at 8:00 AM (night urine) and at 8:00 PM (day urine), and frozen (−80 °C) until analysis for NE.

Urinary NE was measured using a Norepinephrine Enzyme Immunoassay kit (Labor Diagnostika Nord KG, Nordhorn, Germany). A 50-µl urine sample from each of the animals was diluted with 950 µl double-distilled H_2O to obtain a 20:1 dilution, from which 10 µl was used for NE measurements in accordance with the instructions provided by the company. Duplicate measurements were made for each sample. NE excretion was determined by multiplying the urinary NE concentration by the volume of urine collected over 12 h. The data are expressed as µg/12 h.

MAP was recorded 10 min/h for 24 h/d, using a macroprogram written for Chart (version 5.4.2) software (AD Instruments, Colorado Springs, CO).

Preparation of brain tissue samples and western blot analyses. After 7 days of icv infusion, the brains were removed, frozen on dry ice, blocked in the coronal plane, and sectioned at 100 µm thicknesses in a cryostat. The visual cortex, paraventricular hypothalamic nucleus (PVN), RVLM, and nucleus of the solitary tract (NTS) were punched out based on the technique of Palkovits and Brownstein.[15] The punch sites for each nucleus in each section were determined based on the description by Paxinos and Watson.[16] The coordinates of the punched brain regions were: PVN, 0.9–1.9 mm caudal to bregma, 0.0–0.6 mm lateral to midline, and 6.8–8.2 mm ventral to skull; NTS, 13.7–14.6 mm caudal to bregma, 0.4–1.2 mm lateral to midline, and 0.2–0.8 mm from the dorsal surface of the medulla; and RVLM, 11.8–12.7 mm posterior to bregma, 2.0–2.6 mm midline to lateral, and 2.8–3.6 mm from the dorsal surface of the medulla. Tissue samples were homogenized in RIPA buffer. Protein extracted from the homogenate was used to analyze neuronal nitric oxide synthase (nNOS) expression, using western blot. The samples were boiled for 5 min, followed by loading on a 7.5% SDS-PAGE gel (20 µg protein/30 µl/well) for electrophoresis using a Bio-Rad mini gel apparatus at 40 mA/gel for 45 min. The fractionized protein on the gel was transferred onto a polyvinyl difluoride (Millipore, Billerica, MA) and electrophoresed at 300 mA for 90 min. The membrane was probed with primary antibodies (nNOS rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA 1:1,000) and secondary antibody (goat anti-rabbit IgG-HRP; Santa Cruz Biotechnology, 1:2,500), and then treated with enhanced chemiluminescence substrate (Pierce, Rockford, IL) for 5 min at room temperature. The bands in the membrane were visualized and analyzed using UVP BioImaging Systems. After obtaining the nNOS blot density, the membrane was treated with Restore western Blot Stipping Buffer (Thermo Scientific, Rockford, IL) to

remove the nNOS signal, and then probed with GAPDH primary antibodies (GAPDH mouse monoclonal IgG, sc-32233; Santa Cruz Biotechnology, 1:1,000), using the same process as with the nNOS antibodies, to obtain the GAPDH blot densities. The final data reported are the nNOS band densities normalized to GAPDH.

Cell culture and patch-clamp experiments. CATH.a cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI-1640 containing 8% horse serum, 4% fetal bovine serum, and 1% penicillin–streptomycin, at 37 °C in a humidified atmosphere equilibrated with 5% CO_2. After incubation for 3–5 days, the cells were subcultured and differentiated with *N* 6,2′-O-dibutyryladenosine 3′,5′-monophosphate (1 mmol/l; Sigma, St Louis, MO). The differentiated CATH.a cells were then used in a series of experiments to determine the effect of C21 on delayed rectifier I_{Kv}.

The I_{Kv} was recorded by the whole-cell patch-clamp technique. Briefly, differentiated CATH.a cells were bathed in a solution containing (in mmol/l) NaCl 137, KCl 5.4, $CaCl_2$ 1.35, $MgSO_4$ 2, NaH_2PO_4 0.3, dextrose 10, HEPES 10, pH 7.4 (NaOH) with $CdCl_2$ (0.3 mmol/l) and TTX (1.5 µmol/l) added to block Ca^{2+} and Na^+ channels, respectively. The patch pipette microelectrodes were heat-polished before use, and had resistances of 3–4 MΩ when filled with an internal pipette solution containing (in mmol/l) KCl 130, $CaCl_2$ 0.25, $MgCl_2$ 2, ATP 1.0, GTP 0.1, EGTA 5, dextrose 8, and HEPES 10, pH 7.2 (KOH). Cell capacitance was calculated by integrating the area under the uncompensated capacitive transient evoked by a voltage step of 5 mV and dividing this area by the voltage step. All experiments were performed at room temperature (23–24 °C) using an Axopatch 200B amplifier and a Digitdata 1322A interface (Axon Instruments, Sunnyvale, CA). Cell capacitance was cancelled electronically, and the series resistance (<10 MΩ) was compensated by 75–80%. Currents were measured and analyzed using the pCLAMP 8.0 software system. Standard recording conditions for I_{Kv} were achieved by stepping up from a holding potential of −80 to +10 mV for 300 ms. I_{Kv} current was measured at 50 ms after the initiation of the test pulse. Current density was expressed as pA/pF.

Statistical analyses. All data are reported as the mean values ± s.e.m. A two-way analysis of variance followed by Student–Newman–Keuls was used for the statistical analysis of NE concentration, NE volume, and MAP, during both daytime and night time, from control and C21-treated rats. A paired *t*-test was used for statistical analysis of the difference in I_{Kv} between each of the C21 treatment groups and the control group. A one-way analysis of variance followed by Student–Newman–Keuls was used for the statistical analysis of all other data. Statistical analysis was carried out with the aid of the SigmaPlot software. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of C21 on NE excretion

Figure 1 shows the NE concentration (panel a) and NE volume (panel b) in daytime and night time urine in the control and C21-treated groups. **Figure 1a** shows that all the animals exhib-

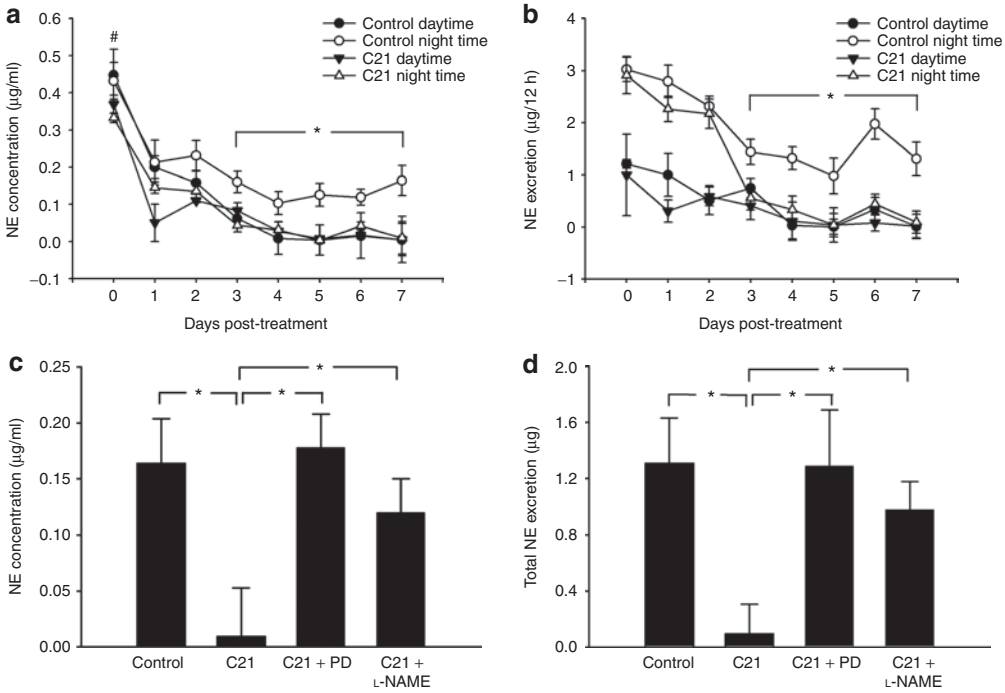

Figure 1 | Catecholamine secretion. (**a**, **b**) Norepinephrine (NE) concentration and NE volume in daytime and night time urine. #$P < 0.05$ when comparing values of day 1 and day 7; *$P < 0.05$ when comparing values for the daytime urine of control rats and the night time urine of Compound 21 (C21)-treated rats ($n = 8$ for the control group and $n = 10$ for the C21-treated group). At day 7, the C21-induced effects are abolished by an (**c**) angiotensin type 2 receptor antagonist and (**d**) nitric oxide synthase blocker. *$P < 0.05$. $n = 8$ for control group, 10 for C21 group, 9 for C21 + PD123319 group, and 8 for C21 + *N*-omega-nitro-L-arginine methyl ester (L-NAME) group.

ited significantly lower NE concentrations, in both daytime and night time urine, during the 7 days following the surgery than at baseline (day 0, surgery day). This was probably related to stress. From days 3 to 7, the control group exhibited a significantly higher NE concentration in night time urine than that in daytime urine. In contrast, the C21-treated group showed no significant difference in NE concentrations between night time and daytime urine. In other words, C21 prevented nocturnal increases in NE excretion. The NE concentration values in the night time urine of C21-treated rats were lower than night time values in the controls. In effect, therefore, the NE concentration levels in the C21-treated group, both night time and daytime, were similar to the daytime concentration values in the controls. Figure 1b shows 12-h NE volume in the control and C21-treated rats. As was seen with NE concentration values, the 12-h NE volume also showed a tendency toward reduction during the night time hours after C21 infusion.

In order to explore the potential mechanisms underlying the effect of C21 on night time NE excretion, we carried out additional experiments. C21 + PD123319 and C21 + L-NAME were infused icv in separate groups of rats. Figure 1c and d shows the night time NE concentration and volume values at day 7 after treatment. Both PD123319 and L-NAME completely abolished the C21-induced decrease in NE concentration and volume.

Effects of C21 on arterial blood pressure

Figure 2a shows the daytime and night time MAP values in controls and C21-treated rats. In both groups, there was a daily rhythmicity in values, with higher MAP at night time and lower MAP during the daytime hours. However, C21-treated rats exhibited lower MAP during both daytime and night time hours as compared to the control rats. Figure 2b shows the daytime and night time MAP at day 7 after treatment for the four groups: control, C21, C21 + PD123319, and C21 + L-NAME. Both PD123319 and L-NAME completely abolished the effect of C21 on MAP. In three different animals, we also observed the effects of PD123319 alone and L-NAME alone on MAP. PD123319 did not alter BP, but L-NAME evoked a slight, though nonsignificant, increase in BP.

Effects of C21 on nNOS expression

Figure 3 shows nNOS protein expression in the cortex, RVLM, PVN, and NTS of brain samples from control and C21-treated rats at day 7 after treatment. There was no difference in nNOS expression between control and C21-treated rats as regards the samples from the cortex and the NTS. However, the RVLM and PVN samples from C21-treated rats exhibited significantly higher nNOS protein expression levels than those from control rats.

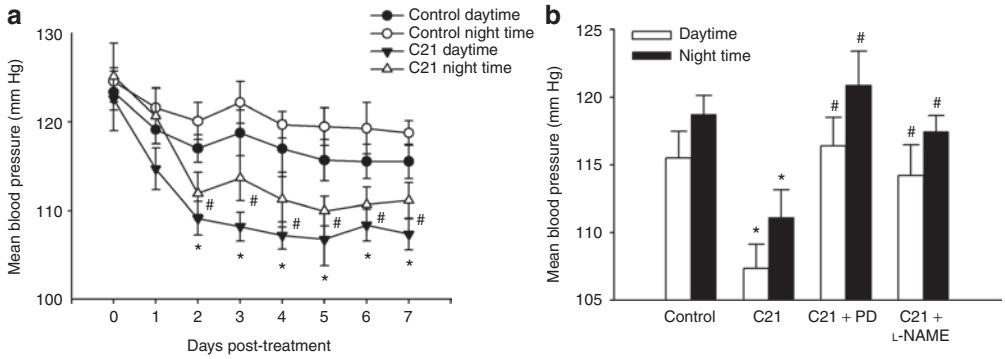

Figure 2 | Daytime and night time mean arterial pressure (MAP) values. (**a**) MAP in the control group ($n = 8$) and in Compound 21 (C21)-treated rats ($n = 10$). $^{*}P < 0.05$ as compared with daytime values in controls; $^{\#}P < 0.05$ as compared with night time values in controls. (**b**) MAP at day 7 after treatment, in control group ($n = 8$), C21 group ($n = 10$), C21 + PD123319 group ($n = 9$), and C21 + *N*-omega-nitro-L-arginine methyl ester (L-NAME) group ($n = 8$). $^{*}P < 0.05$ as compared with controls, $^{\#}P < 0.05$ as compared with C21-treated group.

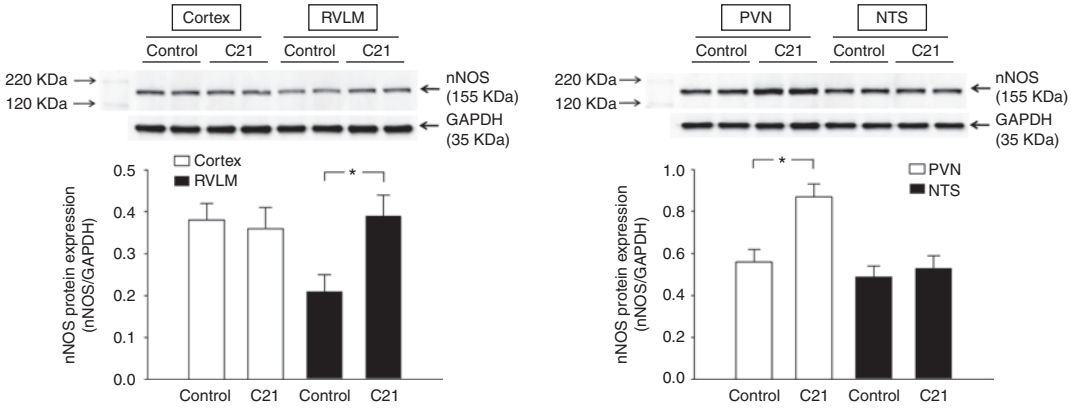

Figure 3 | Neuronal nitric oxide synthase (nNOS) protein expression in the cortex, rostral ventrolateral medulla (RVLM), paraventricular hypothalamic nucleus (PVN), and nucleus of the solitary tract (NTS) from controls ($n = 8$) and Compound 21 (C21)-treated rats ($n = 10$) at day 7 after treatment. $^{*}P < 0.05$.

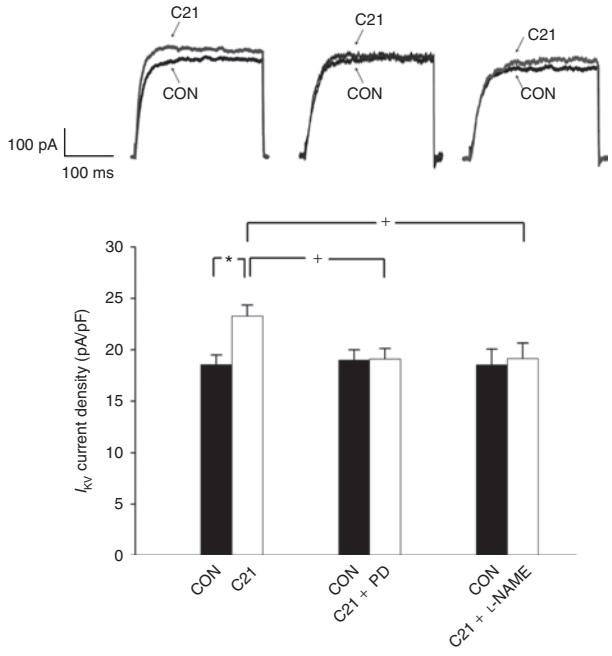

Figure 4 | Potassium current (I_{Kv}) in CATH.a cells, measured by whole-cell patch-clamp. Top: representative current tracings. Bottom: mean data of I_{Kv} densities. $^{*}P < 0.001$; $^{+}P < 0.05$. $n = 14$ for Compound 21 (C21) group, $n = 10$ for C21 + PD123319 group, and $n = 9$ for C21 + *N*-omega-nitro-L-arginine methyl ester (L-NAME) group. CON, control.

Effect of C21 on neuronal potassium current

The top panel of **Figure 4** shows representative current tracings of I_{kv}, while the bottom panel shows the group data. CATH.a neurons exhibited a small but significantly higher I_{Kv} after C21 treatment as compared to controls, suggesting that C21 facilitates potassium channel function. In addition, the AT2R antagonist, PD123319, and the NOS blocker, L-NAME, completely abolished the effects of C21 on I_{Kv}, demonstrating that the influence of C21 on potassium channel function was mediated by AT2R through the nitric oxide (NO) signaling pathway. PD123319 or L-NAME alone did not alter I_{Kv}, and washout of C21 completely reversed the effects on I_{Kv}.

DISCUSSION

Brain Ang II exhibits a marked influence on autonomic function and circulation activity. Activation of central AT1R evokes an increase in sympathetic nerve activity and BP,[17] but selective stimulation of AT2R in the RVLM evoked an effect opposite to that of AT1R.[9] In this experiment, we demonstrated that chronic icv infusion of C21, the first selective nonpeptide AT2R agonist, has a negative influence on NE excretion (a surrogate for sympathetic nerve activity) and on MAP in normal, conscious rats. We found that C21 treatment significantly decreased night time NE excretion and MAP. We further demonstrated that these inhibitory influences of C21 were completely abolished by PD123319, an AT2R antagonist, and L-NAME, a NOS inhibitor. Finally, we found that icv infusion of C21 significantly upregulated nNOS protein expression in the PVN and RVLM. These data strongly suggest a central suppressive effect of C21 on sympathetic drive and BP, mediated by AT2R through a nNOS/NO pathway. In addition, we also investigated the influence of C21 on potassium current in a neuronal cell line, the CATH.a; we found that C21 facilitates the neuronal potassium current, suggesting that C21 may have an inhibitory effect on neuronal excitability.

C21 is a newly created, orally effective, nonpeptide AT2R agonist, which permits selective stimulation of the AT2R under many experimental conditions.[12] Previous experiments have demonstrated the beneficial effects of this compound in hypertension and heart failure models. In spontaneously hypertensive rats, intravenous treatment with C21 significantly decreased MAP.[12] Also, intraperitoneal injection of C21 led to a pronounced improvement in systolic and diastolic cardiac function, coinciding with a smaller scar volume in rats with permanent coronary ligation, probably through an antiapoptotic mechanism.[13] It has recently been demonstrated that C21 evokes vasorelaxation in aortas of mice and spontaneously hypertensive rats, and in the mesenteric arteries of normotensive rats;[14] this finding provides further evidence, at organ level, for the hypotensive effect of C21. The data presented in this study are the first, to our knowledge, that demonstrate a central effect of C21 on sympathetic outflow and MAP. As expected, we further found that the C21-evoked decrease in NE excretion and MAP were abolished by PD123319, demonstrating that the AT2R is the exclusive mediator of the C21 effects. It is not clear why, in normal rats, central C21 treatment suppressed only night time NE excretion while exhibiting no effects on diurnal NE excretion. Being nocturnal rodents, rats are characterized by a higher locomotor activity and sympathetic tone at night time than during the daytime.[18] We therefore assumed that the already relatively low neuronal activity during the daytime may not be further suppressed by C21. However, Takekoshi *et al.*[19] demonstrated that activation of the AT2R can reduce tyrosine hydroxylase activity, and consequently NE biosynthesis; this biochemical observation lends support to our findings.

Patch-clamp and extracellular single unit discharge data have demonstrated that stimulation of AT2R facilitates neuronal potassium current and decreases neuronal spontaneous discharge. Kang *et al.*[20] reported that activation of AT2R increased neuronal I_{Kv} in neurons cultured from tissue specimens taken from the hypothalamus and brainstem of newborn rats. They further indicated that the third intracellular loop of the AT2R is a key component in the stimulation of neuronal I_{Kv} elicited by activation of this receptor.[21] Martens *et al.*[22] documented the finding that activation of AT2R by CGP42112 modulates whole-cell K^+ current in the hypothalamus and brainstem of rats by increasing their open probability. Matsuura *et al.*,[23] on the other hand, demonstrated an AT2R-mediated hyperpolarization and a decrease in the firing rate in bulbospinal RVLM neurons. These data imply that activation of AT2R facilitates potassium channel current and thereby suppresses neuronal excitability. Indeed, in our experiment we found that C21 significantly increased the neuronal potassium current in culture-grown neurons, and that this effect

was completely abolished by an AT2R antagonist. These data further imply that the facilitatory influence of C21 on neuronal potassium channels may be the mechanism underlying the central effects of C21 on NE excretion and arterial pressure that we observed in our study.

Matsubara[24] and Nouet[25] have suggested that there are at least three major transduction mechanisms responsible for AT2R-mediated intracellular signaling: (i) regulation of the NO-guanosine 3′,5′–cyclic monophosphate (NO-cGMP) system; (ii) stimulation of PLA2 with subsequent release of arachidonic acid; and (iii) activation of various protein phosphatases, causing protein dephosphorylation. Among these three pathways, the NO-cGMP pathway is expected to be potentially involved in the effects of AT2R on sympathetic outflow. An AT2R-coupled increase in NO generation has been found in neuronal cell lines PC12W[26] and NG-108–15.[27,28] On the other hand, it has been shown that NO regulates several types of K^+ channels, including ATP-dependent K^+ channels and Ca^{2+}-activated K^+ channels.[29] Han *et al.*[30] recently reported that, in neocortical neurons from mice, low concentrations of NO donor SNAP or an NO solution had the effect of enhancing whole cell delayed rectifier K^+-current (I_k) and left the fast inactivating A (I_A) current unchanged. In cell-attached experiments, a significant increase in channel open probability was observed when using low concentrations of SNAP or NO. The increase in channel activities by low concentrations of SNAP was abolished in the presence of either inhibitors of soluble guanylate cyclase or inhibitors of cGMP-dependent protein kinase G, thereby suggesting a link to the NO-cGMP signaling cascade. These results suggest that AT2R-induced activation of NO-cGMP might be responsible for the AT2R-evoked neuronal potassium current alteration. Indeed, in our experiment, we found that the L-NAME, a NOS blocker, completely abolished the C21-evoked inhibition of NE excretion and hypotension in the conscious rats, and facilitated potassium current in a neuronal cell line.

In order to determine which brain region(s) are involved in the C21 effects observed in this study, we measured nNOS protein expressions in three sympathetically relevant brain nuclei and in the cortex as a control area. We found that, nNOS expressions in the PVN and RVLM, but not in the NTS or cortex, of C21-treated rats were significantly higher than those in control rats. These data demonstrated that C21 upregulates nNOS protein expression, suggesting that activation of the NO pathway in PVN and RVLM is involved in the mechanism of suppression of NE excretion and BP by icv treatment with C21.

In summary, we found that direct central administration of C21 significantly decreases the night time NE excretion and BP in conscious, normal rats, and that these effects were completely abolished by an AT2R blocker and an NOS inhibitor. We further demonstrated that C21 increases potassium current in a neuronal cell line. These results suggest the existence of a central inhibitory influence of C21 on sympathetic outflow exclusively through AT2R, by means of an NO signaling pathway.

Acknowledgments: We acknowledge the expert technical assistance of Li Yu, Phyllis Anding, Johnnie F. Hackley, and Pamela Curry. We also thank Drs Mingqi Zheng and George J. Rozanski for their help with the patch-clamp experiments. We appreciate the suggestions from Dr Irving H. Zucker regarding the experiments, and his valuable inputs for the revision of the manuscript. The authors thank Vicore Pharma AB for their generous donation of Compound 21. This study was supported by a Scientist Development Grant from the American Heart Association 0635007N and NIH grants RO1HL093028 and P30HL101296.

Disclosure: The authors declared no conflict of interest.

1. Kaschina E, Unger T. Angiotensin AT1/AT2 receptors: regulation, signalling and function. *Blood Press* 2003; 12:70–88.
2. de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000; 52: 415–472.
3. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol, Cell Physiol* 2007; 292:C82–C97.
4. Touyz RM, Berry C. Recent advances in angiotensin II signaling. *Braz J Med Biol Res* 2002; 35:1001–1015.
5. Sumners C, Gelband CH. Neuronal ion channel signalling pathways: modulation by angiotensin II. *Cell Signal* 1998; 10:303–311.
6. Gelband CH, Sumners C, Lu D, Raizada MK. Angiotensin receptors and norepinephrine neuromodulation: implications of functional coupling. *Regul Pept* 1997; 72:139–145.
7. Siragy HM, Inagami T, Ichiki T, Carey RM. Sustained hypersensitivity to angiotensin II and its mechanism in mice lacking the subtype-2 (AT2) angiotensin receptor. *Proc Natl Acad Sci USA* 1999; 96:6506–6510.
8. Li Z, Iwai M, Wu L, Shiuchi T, Jinno T, Cui TX, Horiuchi M. Role of AT2 receptor in the brain in regulation of blood pressure and water intake. *Am J Physiol Heart Circ Physiol* 2003; 284:H116–H121.
9. Gao L, Wang WZ, Wang W, Zucker IH. Imbalance of angiotensin type 1 receptor and angiotensin II type 2 receptor in the rostral ventrolateral medulla: potential mechanism for sympathetic overactivity in heart failure. *Hypertension* 2008; 52:708–714.
10. Gao L, Wang W, Wang W, Li H, Sumners C, Zucker IH. Effects of angiotensin type 2 receptor overexpression in the rostral ventrolateral medulla on blood pressure and urine excretion in normal rats. *Hypertension* 2008; 51:521–527.
11. Yu L, Zheng M, Wang W, Rozanski GJ, Zucker IH, Gao L. Developmental changes in AT1 and AT2 receptor-protein expression in rats. *J Renin Angiotensin Aldosterone Syst* 2010; 11:214–221.
12. Wan Y, Wallinder C, Plouffe B, Beaudry H, Mahalingam AK, Wu X, Johansson B, Holm M, Botoros M, Karlén A, Pettersson A, Nyberg F, Fändriks L, Gallo-Payet N, Hallberg A, Alterman M. Design, synthesis, and biological evaluation of the first selective nonpeptide AT2 receptor agonist. *J Med Chem* 2004; 47:5995–6008.
13. Kaschina E, Grzesiak A, Li J, Foryst-Ludwig A, Timm M, Rompe F, Sommerfeld M, Kemnitz UR, Curato C, Namsolleck P, Tschöpe C, Hallberg A, Alterman M, Hucko T, Paetsch I, Dietrich T, Schnackenburg B, Graf K, Dahlöf B, Kintscher U, Unger T, Steckelings UM. Angiotensin II type 2 receptor stimulation: a novel option of therapeutic interference with the renin-angiotensin system in myocardial infarction? *Circulation* 2008; 118:2523–2532.
14. Bosnyak S, Welungoda IK, Hallberg A, Alterman M, Widdop RE, Jones ES. Stimulation of angiotensin AT2 receptors by the non-peptide agonist, Compound 21, evokes vasodepressor effects in conscious spontaneously hypertensive rats. *Br J Pharmacol* 2010; 159:709–716.
15. Palkovits M, Brownstein MJ. *Maps and Guide to Microdissection of the Rat Brain*. Elsevier Science Publishing Co., Inc.: New York, New York, 1988.
16. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. Compact 3rd edn. Academic Press, Inc.: San Diego, California, 1997.
17. Lu N, Helwig BG, Fels RJ, Parimi S, Kenney MJ. Central Tempol alters basal sympathetic nerve discharge and attenuates sympathetic excitation to central ANG II. *Am J Physiol Heart Circ Physiol* 2004; 287:H2626–H2633.
18. Bowers CW, Baldwin C, Zigmond RE. Sympathetic reinnervation of the pineal gland after postganglionic nerve lesion does not restore normal pineal function. *J Neurosci* 1984; 4:2010–2015.
19. Takekoshi K, Ishii K, Isobe K, Nanmoku T, Kawakami Y, Nakai T. Angiotensin-II subtype 2 receptor agonist (CGP-42112) inhibits catecholamine biosynthesis in cultured porcine adrenal medullary chromaffin cells. *Biochem Biophys Res Commun* 2000; 272:544–550.
20. Kang J, Sumners C, Posner P. Angiotensin II type 2 receptor-modulated changes in potassium currents in cultured neurons. *Am J Physiol* 1993; 265:C607–C616.

21. Kang J, Richards EM, Posner P, Sumners C. Modulation of the delayed rectifier K+ current in neurons by an angiotensin II type 2 receptor fragment. *Am J Physiol* 1995; 268:C278–C282.
22. Martens JR, Wang D, Sumners C, Posner P, Gelband CH. Angiotensin II type 2 receptor-mediated regulation of rat neuronal K+ channels. *Circ Res* 1996; 79: 302–309.
23. Matsuura T, Kumagai H, Onimaru H, Kawai A, Iigaya K, Onami T, Sakata K, Oshima N, Sugaya T, Saruta T. Electrophysiological properties of rostral ventrolateral medulla neurons in angiotensin II 1a receptor knockout mice. *Hypertension* 2005; 46:349–354.
24. Matsubara H. Pathophysiological role of angiotensin II type 2 receptor in cardiovascular and renal diseases. *Circ Res* 1998; 83:1182–1191.
25. Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. *Trends Endocrinol Metab* 2000; 11:1–6.
26. Zhao Y, Biermann T, Luther C, Unger T, Culman J, Gohlke P. Contribution of bradykinin and nitric oxide to AT2 receptor-mediated differentiation in PC12 W cells. *J Neurochem* 2003; 85:759–767.
27. Gendron L, Oligny JF, Payet MD, Gallo-Payet N. Cyclic AMP-independent involvement of Rap1/B-Raf in the angiotensin II AT2 receptor signaling pathway in NG108-15 cells. *J Biol Chem* 2003; 278:3606–3614.
28. Côté F, Laflamme L, Payet MD, Gallo-Payet N. Nitric oxide, a new second messenger involved in the action of angiotensin II on neuronal differentiation of NG108-15 cells. *Endocr Res* 1998; 24:403–407.
29. Prast H, Philippu A. Nitric oxide as modulator of neuronal function. *Prog Neurobiol* 2001; 64:51–68.
30. Han NL, Ye JS, Yu AC, Sheu FS. Differential mechanisms underlying the modulation of delayed-rectifier K+ channel in mouse neocortical neurons by nitric oxide. *J Neurophysiol* 2006; 95:2167–2178.