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HYPOTENSIVE AND SYMPATHOINHIBITORY RESPONSES TO SELECTIVE CENTRAL AT2 RECEPTOR STIMULATION IN SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract

The type 2 angiotensin (AT_2) receptor has been suggested to counterbalance the type 1 angiotensin (AT_1) receptor in the central regulation of blood pressure and sympathetic tone. We here investigated the blood pressure responses to stimulation of central AT_2 receptors by the selective agonist Compound 21 in conscious SHR and normotensive WKY rats. We also assessed the impact on norepinephrine plasma levels, autonomic function, spontaneous baroreflex sensitivity, and the possible involvement of the nitric oxide-pathway and the AT_1 receptor.

Chronic intracerebroventricular Compound 21 infusion lowered blood pressure and norepinephrine plasma levels in both rat strains. The nighttime hypotensive effect was greater in SHR compared to WKY. Compound 21 improved spontaneous baroreflex sensitivity more in SHR than in WKY. These effects were abolished by co-administration of AT_2 receptor antagonist PD123319 or nitric oxide-synthase inhibitor L-NAME. Central AT_1 receptor blockade did not enhance the hypotensive response to Compound 21.

Chronic selective stimulation of central AT_2 receptors lowers blood pressure through sympatho-inhibition, and improves spontaneous baroreflex sensitivity more in SHR than in WKY. These responses appear to require a functioning central nitric oxide-pathway, but are not modified by central AT_1 receptor blockade.

Collectively, the data demonstrate specific beneficial effects of stimulation of central AT_2 receptors in hypertension associated with increased sympathetic tone and suggest that central AT_2

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Author contribution

SB co-designed the study protocols, performed experiments, analysed data, wrote the manuscript and obtained funding. IS, RDW and AGD initiated the study project, obtained the necessary funding, co-designed the study protocols, supervised the analysis and co-authored and edited the manuscript.

receptors may represent a potential new therapeutic target for the treatment of neurogenic hypertension.

Keywords

Angiotensin II Type 2 Receptor; Compound 21; blood pressure; hypertension; central nervous system; Angiotensin II Type 1 Receptor

Introduction

Angiotensin II (AngII), the key player in the renin-angiotensin-system (RAS), mediates its effects mainly via the type 1 (AT_1R) and type 2 angiotensin receptor (AT_2R) [1]. AT_1R are widely distributed throughout the body and mediate the classical cardiovascular effects of AngII, such as vasoconstriction, sodium retention, promotion of inflammatory responses, vascular smooth muscle cell proliferation and cardiac hypertrophy [1]. Current evidence suggests that the AT_2R plays a counter-regulatory role opposing the AT_1R-mediated actions by promoting vasodilation, natriuresis and anti-inflammatory, anti-proliferative and anti-fibrotic responses [2]. Activation of the so-called protective arm of the RAS through stimulation of the AT_2R has shown therapeutic potential in protecting against myocardial and brain injury [3]. Although AT_2R stimulation can cause vasodilation *ex vivo*, peripheral AT_2R stimulation does not translate into a significant antihypertensive effect *in vivo*, probably due to the dominating AT_1R mediated vasoconstrictive tone [4].

The key role of the brain RAS, and in particular of the AT_1R, in the regulation of blood pressure and sympathetic tone is well established [5,6]. It is well known that brain AngII, acting through AT_1R, increases mean arterial pressure (MAP) and sympathetic nerve activity, but the possible role(s) of the central AT_2R in cardiovascular regulation remains incompletely understood. Recent evidence suggests that the AT_2R may also have a role in blood pressure regulation through sympatho-modulation [7,8]. Early investigations showed that intracerebroventricular (icv) injection of AngII evoked a larger increase in blood pressure in AT_2R knockout mice compared to wild type mice, linking the central AT_2R to blood pressure regulation and suggesting a counter-regulatory role for brain AT_2R [9,10]. In addition, overexpression of AT_2R in the rostral ventrolateral medulla (RVLM), a primary brainstem nucleus related to the control of sympathetic outflow, reduced blood pressure and urinary norepinephrine (NE) excretion in normal Sprague-Dawley rats [11].

The availability of the non-peptide AT_2R agonist Compound 21 (C21) [12,13] offers the possibility to selectively and specifically investigate AT_2R-mediated effects. C21 was reported to have cardio-, cerebro- and nephroprotective as well as anti-inflammatory effects. Its effect on vascular tone is complex and depends on experimental conditions [13]. We are aware of only one previous study, in conscious normotensive Sprague-Dawley rats, using central administration of C21 to investigate the effect of selective brain AT_2R stimulation on blood pressure [14]. Central infusion of C21 in this rat strain decreased blood pressure and nighttime urinary NE excretion, suggesting a central inhibitory influence of C21 on sympathetic outflow [14]. In previous studies in our lab we were unable to detect direct blood pressure lowering effects following intravenous bolus injection or infusion of different

doses of C21, even during AT_1R blockade [15], indicating a lack of consistent blood pressure lowering effect after peripheral C21 administration. Currently, it is unknown whether central AT_2R stimulation decreases blood pressure and sympathetic tone in the hypertensive setting. In the present study, we first aimed to confirm that *in vivo* chronic central stimulation of AT_2R by C21 reduces blood pressure in Wistar Kyoto rats (WKY), another normotensive rat strain. Our main objective was to investigate the responses evoked by chronic icv infusion of C21 in Spontaneously Hypertensive Rats (SHR), a model of neurogenic hypertension. We also explored the potential mechanism(s) underlying the impact of C21 on blood pressure by investigating the effects of C21 on autonomic function and spontaneous baroreflex sensitivity (SBRS). As nitric oxide (NO) generated within the central nervous system (CNS) is known to interact with the brain RAS, including the AT_2R, to modulate the sympathetic nerve activity and blood pressure, we also determined the possible role of the NO-pathway in the responses evoked by central AT_2R activation by C21 [6,8,16–20].

Methods

Animals

Male WKY and SHR rats (Charles River Laboratories, USA), 14 weeks of age, were housed individually in a temperature (range 68–79°F) and humidity-controlled (range 30–70%) facility under a 12-h light/dark cycle, maintained on normal rat diet with free access to tap water. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Boston University School of Medicine Institutional Animal Care and Use Committee.

Surgical Procedures

Telemetry Probe Implantation: A radiotelemetry device (PA-C40, DSI, MN, USA) was implanted into the abdominal aorta via the left femoral artery under ketamine anaesthesia (30 mg/kg intraperitoneally (ip) ketamine; 3 mg/kg ip xylazine).

Icv cannula and osmotic minipump placement: Following telemetry implantation and surgical recovery (5–7 days) animals were anaesthetized (30 mg/kg ip ketamine in combination with 3 mg/kg ip xylazine) and stereotaxically implanted with a stainless steel cannula into the right lateral cerebral ventricle (Plastics One, VA, USA), which was connected via silastic tubing to an osmotic minipump (model 2004; Durect Corporation, CA, USA) for icv infusion [21].

Experimental protocols

Responses to icv infusion of the selective AT_2R agonist C21 were assessed in WKY and SHR. Following completion of all surgical procedures and surgical recovery (5–7days) baseline blood pressure was recorded by telemetry on scheduled sampling 10-s every 10-min interval over a 7-day period, in WKY and SHR. The mean of these values for every 24-hour period was calculated to obtain a mean value for every day of the protocol. For the daytime and nighttime measurements, the mean values for respectively the 12-hour light cycle and dark cycle were calculated. In order to compare different treatments, changes in

MAP were calculated as the change in blood pressure compared to day 7 of the baseline period. After 7 consecutive days of baseline measurements in animals receiving an icv saline vehicle infusion, rats were randomly assigned (n=6–8 per group) into treatment groups. WKY were followed up for an additional 7-day treatment period and SHR for a 14-day treatment period as their blood pressure continued to increase over the whole 14-day period in contrast with WKY. All compounds were dissolved in isotonic saline and infused icv at a rate of 0.25μl/h. Comparisons between WKY and SHR were done on the same treatment days. The 500ng dose of C21 was selected based on a previous study showing a hypotensive response in normotensive Sprague-Dawley rats [14]. In addition, lower doses were also investigated in order to detect the minimal effective dose of 20ng C21. Co-infusion treatments with AT_1R-blocker losartan (10μg/h) and NO-synthase inhibitor L-NAME (50μg/h) were given through the same osmotic minipump. The protocol is given in Figure S1 (see Online Supplement). Treatment groups and their doses are depicted in Table 1.

Spontaneous baroreceptor reflex sensitivity (SBRS) was calculated using the sequence method [22]. This was done during the baseline period on day 2 and 7 for WKY and SHR, and during the icv treatment period on day 9 and 14 for WKY, and on day 9, 14 and 21 for SHR (HemoLab Software Ver. 16.0). A time-dependent analysis was done. Radiotelemetry data were collected, stored, and analyzed using Dataquest A.R.T. 4.33 software (DSI, MN, USA). In order to further investigate possible changes in autonomic nervous system activity evoked by C21-infusion, acute systemic atropine and propranolol challenges were performed. Following a 30-minute continuous measurement of baseline heart rate (HR) and MAP via radiotelemetry ip bolus atropine (1mg/kg) or propranolol (2mg/kg) was administered and peak changes in HR and MAP were recorded. These studies were conducted for WKY on day 14, for SHR on day 21.

In certain groups, at the end of the protocol (day 15 for WKY, day 22 for SHR) plasma was collected and stored for subsequent measurement of plasma norepinephrine (NE) concentrations. After conscious decapitation, trunk blood was collected in EDTA tubes, immediately centrifuged for 15 minutes at 1000×g at 4°C and the supernatant was stored at −80°C. The harvesting was done in the morning at the same time point for all animals, synchronized with the previous SBRS measurements.

In the saline-vehicle control experiments and the experiments involving losartan infusion, after measurement of baseline water intake for 30 minutes, animals received an icv injection of angiotensin II (100ng) and the dipsogenic response was recorded over 30 minutes [23] to confirm the blockade of the central AT_1R.

After completing the above-described protocol, rats from the saline-vehicle control group and the C21 (20ng/h) group were housed in individual metabolic cages for a 24h period (model 18cv, Fenco, Cataumet, MA, USA) with external food containers and water bottles, for WKY on day 14, for SHR on day 21. Metabolic cages were equipped with a double-fine mesh screen that allowed separation of food and faeces contamination from urine that was collected in vials that contained a layer of mineral oil to prevent urine evaporation. Measurements were made for food and water intake, and urine output during a 24h period enabling calculation of sodium and water balance [24].

Plasma NE concentration was determined by ELISA (IBL, MN, USA). For all groups, the ELISA was performed in duplicate and a mean of the results was taken. The plasma samples were not pooled from animals, each sample is from 1 individual rat. Urinary sodium content was determined by flame photometry (model 943; Instrumentation Laboratories, MA, USA) and urinary osmolality was measured by vapour pressure osmometer (Vapro 5500, Wescor Inc., Logan, UT, USA). Free water clearance and 24h sodium excretion were calculated. Urinary sodium excretion (meq/24h) equals 24h urine output (ml) times urinary sodium concentration (meq/liter). Free water clearance (CH20) was calculated as a difference between the rate of urine volume (ml) per 24h and the osmolar clearance [24].

Drugs

Losartan, PD123319 and Angiotensin II were purchased from Sigma-Aldrich Co. (St. Louis, USA). L-NAME was purchased from Santa Cruz Biotechnology (Dallas, USA). C21 was provided by Vicore Pharma AB (Göteborg, Sweden). Doses were selected based on previous studies [14,25,26].

Statistical Analysis

Data are expressed as mean ± SEM. Data were analyzed using Student's t-tests, ANOVA and appropriate post hoc analyses. Differences occurring between treatment groups (e.g., C21 vs. control) were assessed by a two-way repeated measures ANOVA, followed by a Bonferroni post hoc test, to compare variations among the groups. Alpha was set at 0.05. All calculations and graphs were obtained by using GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA).

Results

AT_2R-mediated blood pressure and heart rate responses to icv C21

WKY—Mean blood1 pressure and heart rate values (average over 24 hours) in WKY during the 7-day period of baseline measurements were 110±2 mmHg and 360±4 bpm, these values remain constant. In the control group, during saline vehicle infusion over the 14-day study period, these values remained constant (Figure 1A and S2A).

Icv infusion of C21 during the 7-day treatment period at doses of 20ng/h and 500ng/h significantly lowered MAP by −6.1±0.6 and −5.6±0.9 mmHg compared to pretreatment at day 7, and by −6.8±0.7 and −6.3±0.5 mmHg compared to saline-vehicle control group values at day 14 ($p<0.05$) (Figure 1A and Figure S3A). Lower doses of C21 ((2ng/h), (10ng/h)) did not alter MAP (Figure S3B).

Co-infusion of the AT_2R antagonist PD123319 with C21 (20ng/h and 500ng/h) abolished the C21 evoked decrease in MAP (Figure 1A, S2A and S3A). PD123319 alone did not significantly change MAP (Figure S3C). HR did not change in any of these experimental groups (Figure S4A).

SHR—MAP increased progressively in SHR over the 7-day baseline period from day 0 onwards, with however some variation of the magnitude of the blood pressure increase

between individual animals. We therefore considered in each group the average MAP measured at day 7, i.e. immediately before infusion of test compounds versus saline vehicle, as a baseline. These baseline values for MAP and HR were 153±5 mmHg and 355±4 bpm. The MAP increased from baseline progressively during the 14-day period in the saline vehicle control SHR by +11.7±2.9 mmHg to a value of 164±5 mmHg ($p<0.01$) (Figure 2A and S5A).

Icv infusion of C21 for a 14-day period at doses of 20ng/h and 500ng/h prevented this spontaneous blood pressure increase and reduced MAP from baseline values recorded on day 7 of saline infusion by −6.1±1.6 and −3.8±1.5 mmHg respectively. After 7 days of C21-infusion, at day 14 of the protocol, the difference in MAP compared to the saline-vehicle control group was −9.0±1.7 and −5.3±1.4 mmHg respectively and the decrease in MAP was highly significant after 14 days of C21-infusion, at day 21 of the protocol, −18.0±2.0 and −15.5±1.6 mmHg respectively ($p<0.001$) (Figure 2A and S6A). The hypotensive effect of icv C21 infusion was abolished by PD123319 co-infusion ($p<0.001$ vs C21 alone) (Figure 2A, S5A and S6A). PD123319 alone did not significantly change MAP compared to the saline control group (Figure S6C). No significant HR changes were observed in these SHR experiments (Figure S7A). The magnitude of the hypotensive response (24h average) after 7-days infusion of C21 (20ng/h) (compared to saline for the same period) tended to be slightly greater in SHR than in WKY, but the difference did not reach statistical significance. However, after 14 days infusion in SHR, the blood pressure lowering effect was more pronounced and significantly greater than observed after 7 days in both, WKY and SHR ($p<0.001$).

Nighttime versus daytime blood pressure—We performed an additional analysis of the day versus nighttime blood pressures. In both strains, nighttime blood pressure was significantly higher than daytime blood pressure both under saline infusion (Night vs. Day MAP (mmHg): WKY +5.0±0.3; SHR +5.8±0.5; $p<0.001$) and during C21-infusion (20ng/h) (Night vs. Day MAP (mmHg): WKY +4.9±0.3; SHR +5.4±0.5; $p<0.001$). The magnitude of the blood pressure reduction induced by 7-day C21 infusion was similar in the two strains during daytime (C21 evoked peak change in MAP (mmHg): WKY −5.5±0.6, SHR −5.4±1.5). However, during nighttime, icv C21 for 7 days reduced blood pressure significantly more in SHR than in WKY from baseline values (C21 evoked peak change in nighttime MAP (mmHg): WKY −8.2±0.8 vs. SHR −12.6±1.9; $p<0.01$). In SHR, the magnitude of the nighttime hypotensive responses to icv C21 for 7 or 14 days was significantly greater than during daytime both after 7 ($p<0.05$) and 14 ($p<0.001$) days; a similar but statistically not significant trend was seen in WKY (Table 2).

Effect of icv C21-infusion on AT_2R-mediated changes in autonomic and renal function

Norepinephrine plasma levels—In control icv vehicle saline-infused rats, plasma NE levels were significantly lower in WKY (217.0±19.4pg/mL) than in SHR (317.2±14.7pg/mL; $p<0.05$). C21-infusion (20ng/h) significantly decreased NE plasma concentration versus saline infusion in WKY to 180.1±13.6pg/mL ($p<0.05$) and in SHR to 262.9±19.8pg/mL ($p<0.05$). In both strains, PD alone had no effect on NE (n=3, data not

shown) but abolished the decreases in NE plasma concentration evoked by icv C21-infusion (Figure 3).

Autonomic function—C21-infusion had no effect on the increase in HR evoked by ip bolus atropine in either WKY (vehicle +119.7±11.0 vs C21 +114.0±12.5 bpm; NS) or in SHR (vehicle +102.2±11.3 vs C21 +107.1±5.4 bpm; NS). The bradycardic response to ip bolus propranolol was significantly attenuated in the C21-treated animals (WKY: vehicle −49.7±3.0 vs C21 −25.0±3.2 bpm; $p<0.05$; SHR: vehicle −68.2±2.4 vs C21 −36.3±4.1 bpm; $p<0.05$). PD123319 co-infusion abolished this effect of C21 (Figure 4).

SBRS—SBRS, as measured on day 2 and day 7 of the baseline period under saline vehicle infusion, was significantly impaired in the SHR compared to WKY (SBRS (ms/mmHg) saline infusion: WKY D2 2.6±0.3, D7 2.5±0.4 vs SHR D2 2.0±0.1, D7 1.8±0.2; both $p<0.05$) (Figure 5A and 5E).

C21-infusion (20ng/h) immediately and significantly increased SBRS in both strains; this effect was maintained throughout the infusion period (SBRS (ms/mmHg) C21 20ng/h infusion; WKY D9 3.6±0.3, D14 3.7±0.4; both $p<0.01$ vs baseline; SHR D9 3.2±0.2, D14 3.4±0.2, D21 3.7±0.1; all $p<0.01$ vs baseline) (Figure 5A and 5E). The improvement in SBRS on D14 was more pronounced in SHR than in WKY (84 vs 46%; $p<0.001$). A higher concentration C21-infusion (500ng/h) also increased significantly SBRS (SBRS (ms/mmHg) C21 500 ng/h infusion: WKY D9 3.9±0.4, D14 3.8±0.5; both $p<0.01$; SHR D9 3.0±0.6, D14 3.0±0.5, D21 3.2±0.5; all $p<0.01$) (Figure S8). The C21-induced increase in SBRS was abolished by co-infusion of PD123319 in both strains (Figure 5B and 5F), whereas infusion of PD123319 alone had no significant effect on SBRS.

Fluid and Electrolyte Homeostasis—C21-infusion did not alter sodium balance or free water clearance in WKY or SHR. We observed no difference in sodium excretion (WKY: vehicle −0.67±0.2 vs C21 −0.77±0.2 mEq/day; NS; SHR: vehicle −0.54±0.1 vs C21 −0.47±0.1; NS) or free water clearance (WKY: vehicle −24.3±4.5 vs C21 −25.3±4.2 ml/day; NS; SHR: vehicle −26.5±4.2 vs C21 −28.1±4.2; NS) following C21-infusion (Figure S9).

Impact of icv AT_1R and NO on AT_2R-mediated physiological effects

WKY—Co-infusion of the AT_1R antagonist losartan, in a dose (10μg/h) that abolished the dipsogenic response to icv injection of AngII seen in control WKY and SHR (Figure S10), with C21 (20 and 500ng/h) did not further enhance the MAP lowering effect seen with C21 alone (20 and 500ng/h) (Figure 1B, S2B and S3D).

L-NAME infusion alone significantly increased MAP from baseline values by +11.8±1.7 mmHg, ($p<0.001$) (Figure 1C and S2C) and decreased HR from baseline by −24.4±4.7 bpm ($p<0.05$) (Figure S4B) without altering SBRS (Figure 5D). Co-infusion of L-NAME and C21 abolished the hypotensive effect of C21, evoking an increase in MAP from baseline values by +8.4±0.9 mmHg ($p<0.01$ vs C21 alone) (Figure 1C and S2C) and also abolished C21-induced increases in SBRS (Figure 5C).

SHR—As in the WKY co-infusion of the AT_1R antagonist losartan (10μg/h) with C21 (20 and 500ng/h) did not further enhance the MAP lowering effect seen with C21 alone (20 and 500ng/h) in SHR (Figure 2B, S5B and S6B).

L-NAME infusion alone significantly increased MAP from baseline by +38.7±2.5 mmHg ($p<0.05$) (Figure 2C and S5C) and tended to reduce HR. L-NAME (50μg/h) co-infusion blocked the effect of C21 (20ng/h) and increased MAP from baseline by +37.1±3.2 mmHg ($p<0.05$ vs C21 alone) (Figure 2C and S5C) and significantly reduced HR from baseline by −16.0±2.8 bpm ($p<0.05$) (Figure S7B). Although MAP was still significantly different ($p<0.05$) from day 10 until day 13 between the groups C21 20ng+L-NAME and L-NAME alone, MAP and HR with co-infusion of C21+L-NAME were not different from the corresponding values with L-NAME infusion alone at D21. C21-induced increases in SBRS were again abolished by co-infusion of L-NAME (Figure 5G); icv infusion of L-NAME alone did not alter SBRS (Figure 5H).

Discussion

The major novel finding of the present study is that central chronic stimulation of the AT_2 receptor by the selective non-peptide AT_2R agonist Compound 21 evoked a sustained decrease in blood pressure not only in normotensive but also in spontaneously hypertensive rats *in vivo*, and that this hypotensive response is associated with sympatho-inhibition and increased spontaneous baroreflex sensitivity. These data further demonstrate there is a differential response to C21 between the SHR and WKY rat in multiple parameters.

It is well established that brain AngII induces tonic sympatho-excitatory effects resulting in blood pressure increases through stimulation of central AT_1R. However, the possible role of brain AT_2R in blood pressure control is less well understood, although current evidence suggests that AT_2R in the RVLM may mediate sympatho-inhibitory effects [6,11]. In the current experiments, we observed that chronic icv infusion of C21, at doses of 20 or 500 ng/h, consistently lowered blood pressure and plasma NE concentrations in normotensive WKY. Recent *in vitro* experiments suggested that C21 may, like most other drugs, induce unspecific effects in concentrations above 1μM [27]. However, the central blood pressure lowering and sympatholytic effects observed in the present study were observed at much lower concentrations of C21 (0.04μmol/L). Moreover, these effects were both abolished by concomitant infusion of PD123319 administered in a dose known to selectively block the AT_2R [28] and not reaching the high concentration at which it would be expected to also block the AT_1R, confirming that these responses were probably AT_2R-mediated.

The results obtained in WKY validate and extend earlier findings conducted in male Sprague-Dawley rats [14] that AT_2R activation lowers blood pressure in normotensive rat phenotypes. These authors also reported a reduction in nighttime urinary NE excretion, supporting our finding of a sympatho-inhibitory response to chronic central AT_2R stimulation. It is of interest to note that, whereas the effects of C21 were abolished by PD123319, indicating that exogenous stimulation of brain AT_2R in normotensive rats results in sympatho-inhibition, chronic infusion of PD123319 alone had no effect on MAP nor NE

levels, suggesting that endogenous activation of brain AT_2R does not appear to contribute significantly to the control of blood pressure and sympathetic tone under basal conditions.

The most important novel observations of the present study relate to the experiments conducted in SHR. To our knowledge this is the first study investigating responses to chronic central AT_2R stimulation th.rough icv infusion of C21 in an *in vivo* conscious animal model of hypertension. As expected, and in line with the available literature [29], blood pressure progressively increased by almost 12 mmHg in the control SHR followed for a 21-day period of saline-vehicle infusion. Icv infusion of C21 from day 8 till day 21 completely prevented this progressive blood pressure increase and further lowered MAP to below the baseline levels on day 7. The magnitude of this response was significantly greater than the response seen in either SHR or WKY after 7 days of infusion. As expected, nighttime blood pressure was significantly higher than daytime blood pressure in both strains. Interestingly, whereas the magnitude of the blood pressure reduction induced by C21 was similar in the two strains during daytime, in SHR, the nighttime hypotensive response to C21 was significantly greater than during daytime. Moreover, during nighttime, icv C21 for 7 days reduced blood pressure significantly more in SHR than in WKY, suggesting that brain AT_2R may be involved in blood pressure control in particular during nighttime in SHR.

The marked hypotensive response in a model of hypertension induced by specific and selective stimulation of brain AT_2R observed in the present study is in sharp contrast to the lack of effect on blood pressure by stimulation of peripheral AT_2R [4]. Indeed, although evidence of AT_2R mediated vasodilation is available *ex vivo, in vivo* studies on the possible blood pressure lowering effect of peripheral AT_2R stimulation in hypertensive animal models have yielded conflicting results, and hypotensive responses were either not detectable or only during co-administration of an AT_1 antagonist at low dose [30,31]. This lack of significant antihypertensive effect has resulted in the conclusion that non-peptide AT_2R agonists would not become a new class of antihypertensive drugs. The present study, however, suggests that centrally acting AT_2R agonists may have significant blood pressure lowering effects provided they can cross the blood-brain barrier.

Our observation that C21 mediated stimulation of central AT_2R prevented the spontaneous blood pressure increase in SHR is in line with the recent demonstration by Blanch et al that increased expression of AT_2R in the solitary-vagal complex, a brainstem region important in the control of blood pressure, attenuates the increase in arterial pressure observed in a rat model with 2-kidney 1-clip renovascular hypertension [32].

In line with previous evidence indicating that SHR have a higher sympathetic tone compared to normotensive control rats [33], baseline plasma NE concentrations were higher in SHR compared to WKY. As observed in the WKY, the hypotensive response to infusion of C21 in SHR was also associated with a significant reduction of the plasma concentrations of NE, measured as a surrogate marker of sympathetic tone. Again, both responses were abolished by concomitant infusion of PD123319, confirming the direct involvement of the AT_2R in these responses. As in the WKY experiments, infusion of PD123319 alone had no significant effect on MAP or plasma NE concentrations. These results suggest that

exogenous stimulation of brain AT_2R prevents the progressive increase in blood pressure in SHR through a sympatho-inhibitory action.

In order to further investigate possible changes in autonomic nervous system activity evoked by C21-infusion, acute systemic atropine and propranolol challenges were performed in both, SHR and WKY. In both strains, the peak tachycardic responses to an ip bolus injection atropine were unaltered by icv C21-infusion, suggesting that exogenous AT_2R stimulation did not alter parasympathetic tone. However, the peak bradycardic responses to propranolol were significantly reduced by C21-infusion, in SHR as well as in WKY, and PD123319 abolished this effect. This is in line with the hypothesis of a C21 induced AT_2R-mediated decrease in central sympathetic outflow, without a relevant effect on parasympathetic activity.

Another important finding of our study is that exogenous brain AT_2R stimulation improved spontaneous baroreceptor reflex sensitivity in both WKY and SHR. Baroreflex dysfunction is an important hallmark of hypertension [34] that is closely related to sympathetic hyperactivity and activation of the circulating and local RAS [35]. Reduction in baroreflex sensitivity is considered an independent marker of the risk of mortality and major adverse cardiovascular events in hypertensive patients [36]. SHR are known to exhibit impaired baroreceptor reflex function [37]. Accordingly, in the present study, SBRS measurements were impaired at baseline and in control experiments with saline-vehicle infusion in SHR compared to WKY. Icv infusion of C21 improved the SBRS in both strains rapidly after the start of the infusion and this was maintained throughout the whole experiment, but the effect in SHR was significantly greater than in WKY. This C21 induced improvement in spontaneous baroreceptor reflex sensitivity was abolished by concomitant infusion of the selective AT_2R antagonist PD123319 confirming that this effect is mediated through this receptor. It is tempting to speculate that the prevention of the progression of hypertension in SHR induced by central AT_2R stimulation observed in the present study is, in part, related to this restoration of baroreceptor function. These results also confirm the recent observation that increased expression of AT_2R in the solitary-vagal complex restores baroreflex function and sympathetic modulation of arterial pressure to normal values in rats with 2-kidney 1-clip renovascular hypertension, another rat model characterized by baroreceptor impairment and increased sympathetic tone [32].

The possible in vivo role of peripheral AT_2R stimulation in renal sodium handling has been extensively studied [2]. AT_2R stimulation by intravenous C21-infusion has been reported to promote natriuresis by a direct effect on tubular function [38–40]. On the other hand, in AT_2-null mice, no changes in natriuresis were seen, except under additional AngII-infusion, where an antinatriuretic hypersensitivity was observed [9]. Regarding a possible role for central AT_2R, a transient increase in urine excretion was reported in normotensive rats with overexpression of AT_2R in the RVLM [11]. We therefore also investigated whether chronic icv infusion would have an effect on sodium balance and on free water clearance, but we could not detect any effect of C21 on fluid and electrolyte handling in WKY nor SHR. This negative result is not necessarily in contradiction with the observation of Gao et al, as the increase in urine excretion that they observed was transient and short lasting compared with the prolonged overexpression of RVLM AT_2R that they generated, and the measurements of

sodium balance and free water clearance performed in the present study were made at the end of the C21 treatment periods (after 7 days for WKY and 14 days for SHR). Further, the same group did not report the effect of C21-infusion on renal parameters in their subsequent study in Sprague-Dawley rats [14].

The interplay between NO and different components of the RAS, including the AT_2R has been previously reported [16,41,42]. We therefore also evaluated whether the responses to icv C21 can be affected by central inhibition of NO-synthase. In line with the previous observation in normotensive Sprague-Dawley rats [14], we demonstrated that in both WKY and SHR the depressor responses to central AT_2R stimulation are blocked by icv infusion of the NO-synthase-inhibitor L-NAME, although we cannot completely exclude that the lack of detectable hypotensive response to C21 under L-NAME infusion might at least in part be related to the very pronounced blood pressure increase induced by L-NAME itself.

Further, although the hypotensive response to C21 was abolished after 14-days of L-NAME infusion, it was only partly reversed by L-NAME during the first days of co-infusion. Therefore other possible mechanisms might also be involved such as direct crosstalk or downregulation of AT_1 receptors. AT_2R have been shown upon activation to heterodimerize with AT_1R, reducing their cell surface [43] expression. AT_2R-AT_1R heterodimerization may also affect intracellular AT_1R signalling [44,45] resulting in a functional inhibition of the latter and adding to the complexity of AT_2R physiopathology. Nevertheless, as AT_2R antagonists like PD123319 have also been reported to also antagonize Mas receptor mediated effects [46], other effects not directly linked to AT_2R stimulation, or an interaction with the Mas-related G-protein-coupled receptor cannot be entirely excluded. However, we also showed that co-infusion of L-NAME abolished the improvement in baroreceptor sensitivity induced by central AT_2R stimulation by C21, indicating that these effects of AT_2R stimulation require a functional central NO pathway. These results are in line with a recent study in anaesthetized Wistar rats suggesting a facilitatory role for AT_2R in high pressure baroreflex regulation which is NO-dependent [16]. Icv infusion of L-NAME alone significantly increased MAP and reduced HR (the latter probably through activation of the high pressure baroreflex), both in WKY and SHR, supporting current evidence of the important role for endogenous NO in the brain in the central control of blood pressure [42]. However, no differences were detected when L-NAME infused alone was compared with combined icv infusion of L-NAME with C21.

We also addressed the possibility of a contribution of the AT_1R in the central AT_2R-mediated responses in the present study based on the hypothesis that stimulation of both receptors could result in opposing responses. Peripheral AT_1R blockade to functionally antagonize the AT_1R has been required to unmask AT_2R-mediated vasodilation in SHR [30,31]. Therefore we also investigated the centrally mediated C21 responses when the counterbalancing actions of AT_1R were blocked with losartan given at a dose known to have no hypotensive effect by itself after central administration [47] but sufficient to block the dipsogenic effect of exogenous AngII. In contrast with the findings reported for the peripheral vasculature, the hypotensive response to chronic icv C21-infusion is not enhanced by additional central AT_1R blockade, in either WKY or in SHR. In our studies the response

to C21 co-infusion at two different doses (20ng/h and 500ng/h) with or without losartan did not differ.

In conclusion, the results of the present study provide the first evidence that chronic selective stimulation of the central AT_2 receptor by the selective non-peptide AT_2R agonist Compound 21 induces a vasodepressor response in hypertensive rats, preventing the progressive blood pressure increase normally observed in this animal model. This hypotensive response is associated with lowered NE plasma levels, suggesting a decrease in sympathetic tone, and improvement of the SBRS. The improvement in baroreceptor reflex sensitivity and the hypotensive effect during nighttime is more pronounced in SHR than in WKY. In addition this study shows that these brain AT_2R-mediated responses require a functioning central NO-pathway, but are independent of the presence of functioning central AT_1R. The modulation of blood pressure regulation and correction of impaired sympatho-regulation that is potentially achievable by balancing the actions of central inhibitory AT_2R versus excitatory AT_1R effects through central AT_2R stimulation could open new therapeutic opportunities for diseases characterized by sympatho-excitation. However, it should be stressed that although many other studies also reported beneficial responses to AT_2R stimulation, the opposite has also been reported in pathological conditions or ageing animals and depending on the location of the receptor (endothelium or smooth muscle cell), as well as its capacity to heterodimerize with AT_1 receptors [48].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Perspectives

Collectively, the data from our study support a role of the AT_2R as an important element in the beneficial arm of the RAS. We provide the first evidence that chronic selective central AT_2R stimulation attenuates hypertension and improves autonomic dysfunction and impaired baroreflex sensitivity in SHR. In contrast to peripheral AT_2R receptors for which there is now abundant evidence that their stimulation does not result in consistent blood pressure lowering effects, the present study suggests that centrally acting AT_2R agonists may have significant blood pressure lowering effects provided they can cross the blood-brain barrier. Further research into a better understanding of the location, age and disease-dependent roles of the AT_2 receptor is warranted before AT_2-receptor agonists can be brought to the clinic.

Summary Statement

This *in vivo* study demonstrates that chronic selective central AT_2R stimulation by C21 results in a NO-dependent hypotensive effect through sympatho-inhibition and improved spontaneous baroreflex sensitivity. These effects are more pronounced in spontaneously hypertensive rats compared to normotensive rats.

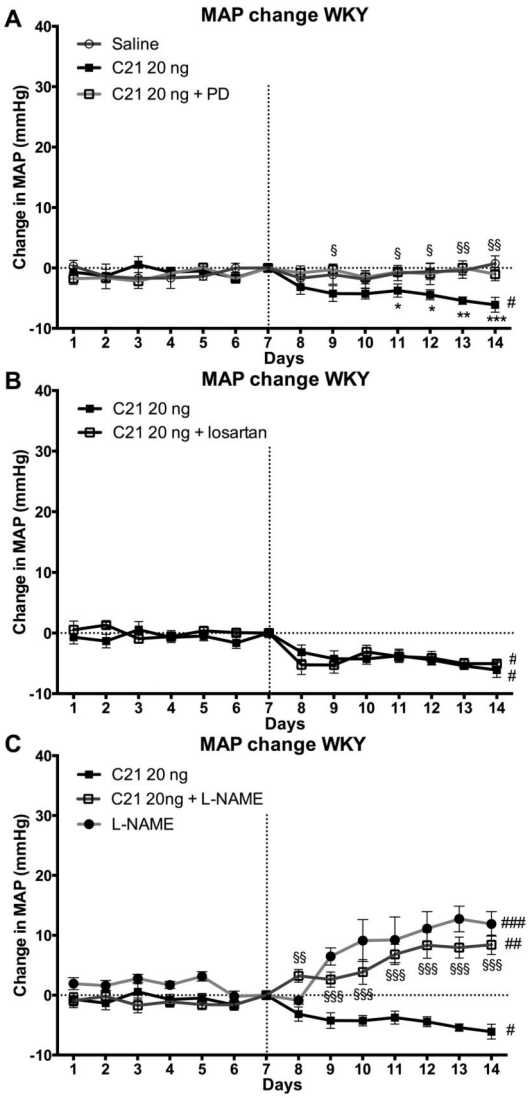

Fig. 1.

Change in mean arterial blood pressure (MAP) in Wistar Kyoto rats (WKY). Data are shown as mean±SEM (n=6–8 per group). §P<0.05, §§P<0.01, §§§P<0.001 compared to C21 20ng; *P<0.05, **P<0.01, ***P<0.001 compared to saline-vehicle control (multiple comparisons 2-way ANOVA). #P<0.05, ##P<0.01; ###P<0.001 compared to baseline values on day 7 (ANOVA for repeated measures).

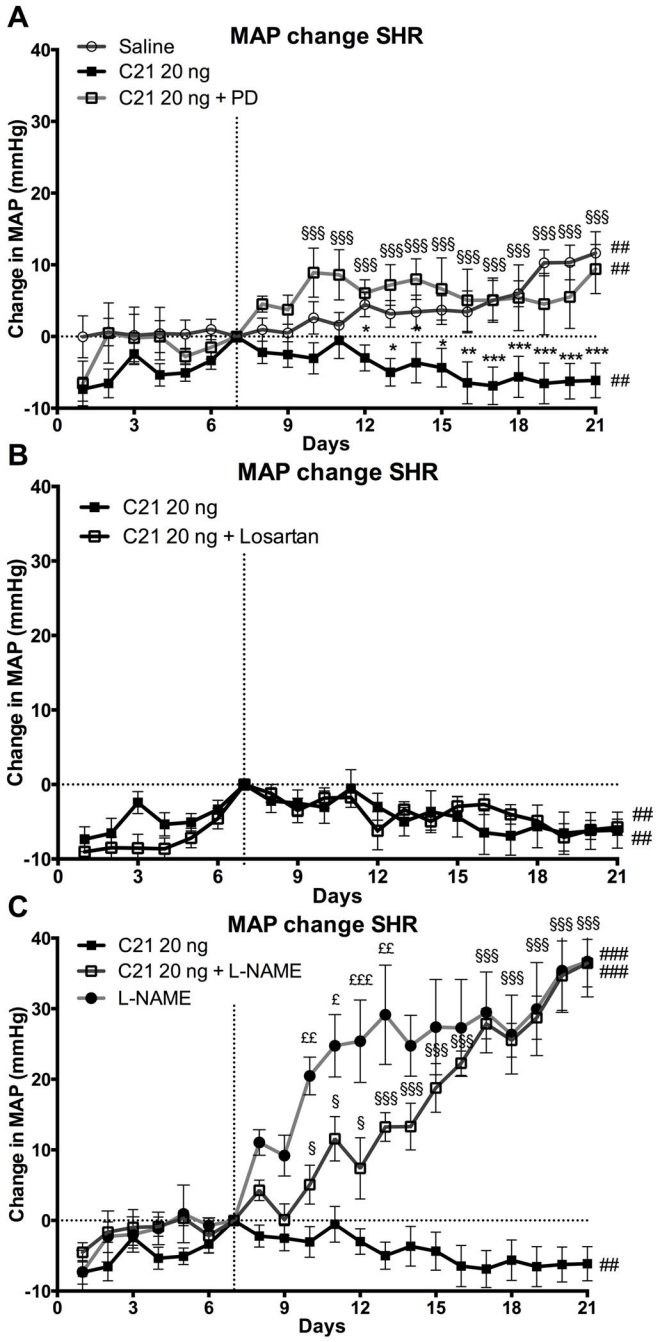

Fig. 2.

Change in mean arterial blood pressure (MAP) in Spontaneously Hypertensive rats (SHR). Data are shown as mean±SEM (n=6–8 per group). §P<0.05, §§§P<0.001 compared to C21 20ng; *P<0.05, **P<0.01, ***P<0.001 compared to saline-vehicle control; £P<0.05, £ £P<0.01, £££P<0.001 compared to C21 20ng + L-NAME (multiple comparisons 2-way ANOVA). ##P<0.01, ###P<0.001 compared to baseline values on day 7 (ANOVA for repeated measures).

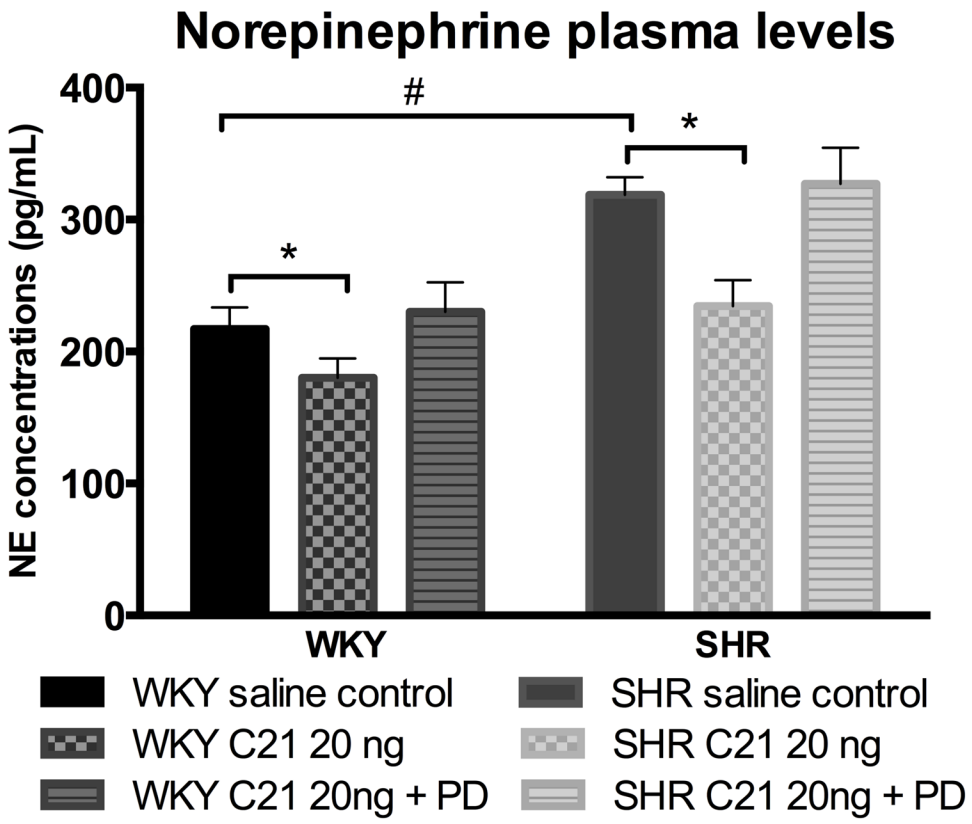

Fig. 3.

Norepinephrine (NE) plasma levels in Wistar Kyoto rats (WKY) and Spontaneously Hypertensive rats (SHR). Data are shown as mean±SEM (n=6–8 per group). *P<0.05 compared to corresponding saline control. #P<0.05 comparing saline control in SHR to corresponding saline control in WKY.

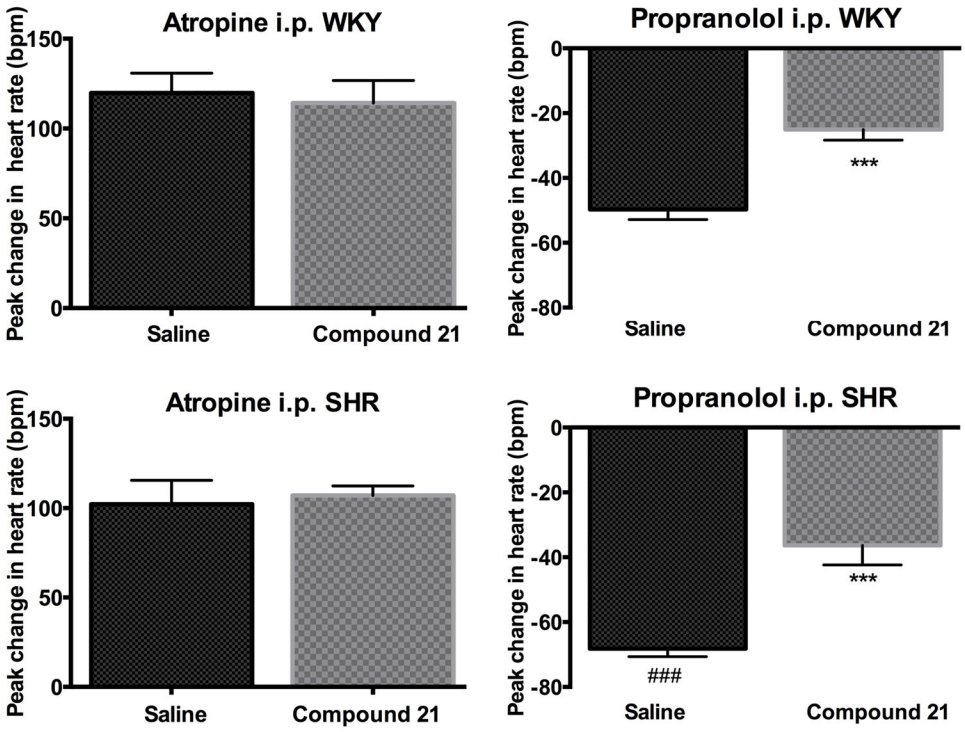

Fig. 4.

Peak change in heart rate (HR) in Wistar Kyoto rats (WKY) (upper panels) and Spontaneously Hypertensive rats (SHR) (lower panels) after ip injection of atropine and propranolol at the end of C21-infusion. Data are shown as mean±SEM (n=6–8 per group). ***P<0.001 compared to corresponding saline control. ###P<0.001 comparing saline control in SHR to corresponding saline control in WKY on D7.

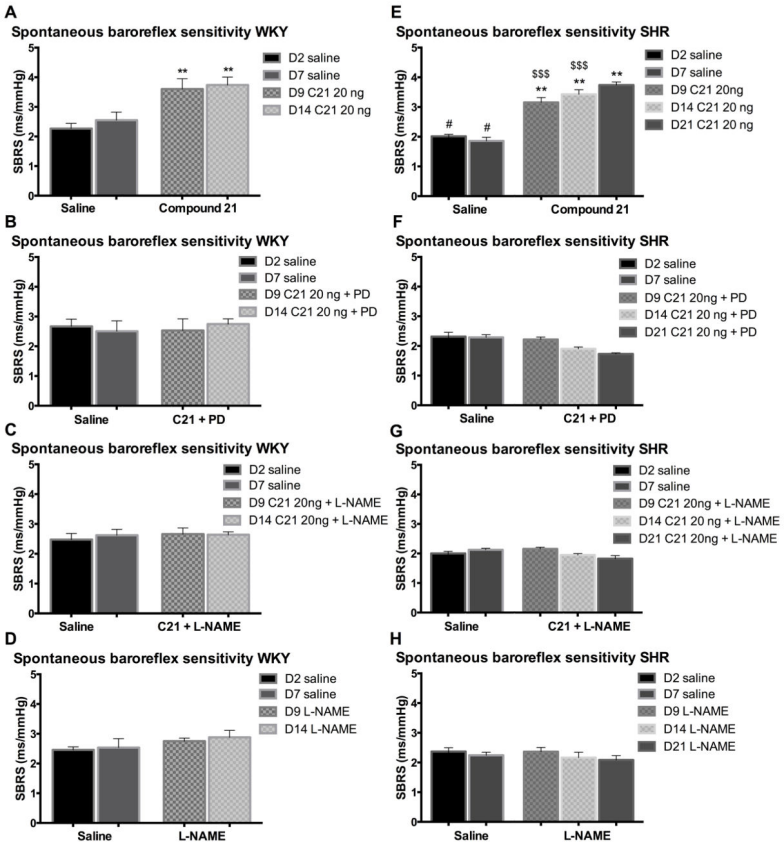

Fig. 5.

Spontaneous baroreflex sensitivity (SBRS) in Wistar Kyoto rats (WKY) (panels A,B,C,D) and Spontaneously Hypertensive rats (SHR) (panels E, F, G, H). Data are shown as mean ±SEM (n=6–8 per group). **P<0.01 compared to corresponding saline control, D2 vs D9, D7 vs D14, D7 vs D21. #P<0.05 comparing saline control in SHR to corresponding saline control in WKY. $$$P<0.001 comparing change in SBRS in SHR to corresponding change in WKY.

Table 1

Overview of the treatment groups in WKY and SHR.

WKY	**SHR**
saline vehicle control	saline vehicle control
C21 (2ng/h)	
C21 (10ng/h)	
C21 (20ng/h)	C21 (20ng/h)
C21 (500ng/h)	C21 (500ng/h)
C21 (500ng/h) + PD123319 (500ng/h)	C21 (500ng/h) + PD123319 (500ng/h)
C21 (20ng/h) + PD123319 (20ng/h)	C21 (20ng/h) + PD123319 (20ng/h)
PD123319 (20ng/h)	PD123319 (20ng/h)
C21 (500ng/h) + losartan (10μg/h)	C21 (500ng/h) + losartan (10μg/h)
C21 (20ng/h) + losartan (10μg/h)	C21 (20ng/h) + losartan (10μg/h)
C21 (20ng/h) + L-NAME (50μg/h)	C21 (20ng/h) + L-NAME (50μg/h)
L-NAME (50μg/h)	L-NAME (50μg/h)

Table 2

Change in mean arterial blood pressure (MAP; mmHg) in Wistar Kyoto rats (WKY) (left) and Spontaneously Hypertensive rats (SHR) (right). Data are shown as mean±SEM (n=6–8 per group). D: day.

WKY			**SHR**					
D14	**C21 icv**	**Saline icv**	**D14**	**C21 icv**	**Saline icv**	**D21**	**C21 icv**	**Saline icv**
24h	−6.8±0.7 *	+0.7±0.1	24h	−9.0±1.7 #	+3.9±1.1	24h	−18.0±2.0 ###	+11.6±2.1
Night	−8.2±0.8 **	+0.8±0.1	Night	−12.6±1.9 # $$	+5.2±0.4	Night	−24.8±2.2 ###	+14.6±1.5
Day	−5.5±0.6 *	+1.1±0.2	Day	−5.4±1.5 #	+3.8±0.3	Day	−11.3±1.8 ###	+8.0±0.8

*P<0.05,

**P<0.01 vs WKY saline control,

#P<0.05,

###P<0.001 vs SHR saline control,

$$P<0.01 vs WKY C21.