RESEARCH PAPER

Renin activates PI3K-Akt-eNOS signalling through the angiotensin AT₁ and Mas receptors to modulate central blood pressure control in the nucleus tractus solitarii

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BACKGROUND AND PURPOSE

The renin-angiotensin system (RAS) is critical for the control of blood pressure by the CNS. Recently, direct renin inhibitors were approved as antihypertensive agents. However, the signalling mechanism of renin, which regulates blood pressure in the nucleus tractus solitarii (NTS) remains unclear. Here we have investigated the signalling pathways involved in renin-mediated blood pressure regulation, at the NTS.

EXPERIMENTAL APPROACH

Depressor responses to renin microinjected into the NTS of Wistar-Kyoto rats were elicited in the absence and presence of the endothelial nitric oxide synthase (eNOS)-specific inhibitor, N(5)-(-iminoethyl)-L-ornithine, Akt inhibitor IV and LY294002, a PI3K inhibitor and GP antagonist-2A [G_q inhibitor]. Lisinopril (angiotensin converting enzyme inhibitor), losartan, valsartan (angiotensin AT₁ receptor antagonists), D-Ala7-Ang-(1-7) (angiotensin-(1-7) receptor antagonist) were used to study the involvement of RAS on renin-induced depressor effects.

KEY RESULTS

Microinjection of renin into the NTS produced a prominent depressor effect and increased NO production. Pretreatment with G_q-PI3K-Akt-eNOS pathway-specific inhibitors significantly attenuated the depressor response evoked by renin. Immunoblotting and immunohistochemical studies further showed that inhibition of PI3K significantly blocked renin-induced eNOS-Ser¹¹⁷ and Akt-Ser⁴⁷³ phosphorylation *in situ*. In addition, pre-treatment of the NTS with RAS inhibitors attenuated the vasodepressor effects evoked by renin. Microinjection of renin also increased Ras activation in the NTS.

CONCLUSIONS AND IMPLICATIONS

Taken together, these results suggest renin modulated blood pressure at the NTS by AT₁ and Mas receptor-mediated activation of G_q and Ras to evoke PI3K-Akt-eNOS signalling.

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Abbreviations

7-NI, 7-nitroindazole; ACE, angiotensin converting enzyme; aCSF, artificial cerebrospinal fluid; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); DAB, diaminobenzidine; EGFR, epidermal growth factor receptor; eNOS, endothelium nitric oxide synthase; GPA-2A, GP Antagonist-2A; HR, heart rate; iNOS, inducible nitric oxide synthase; L-NAME, N-nitro-L-arginine methyl ester; L-NIO, N(5)-(-iminoethyl)-L-ornithine; MEK, MAP kinase-ERK kinase; nNOS, neuronal nitric oxide synthase; NTS, nucleus tractus solitarius; PBST, phosphate-buffered saline with Tween 20; PRR, (pro)renin receptor; RAS, renin-angiotensin system; RVLM, rostral ventrolateral medulla; SDS, sodium dodecyl sulfate; SFO, subfornical organ; SHR, spontaneously hypertensive rats; shRNA, short hairpin RNA; vinyl-L-NIO, N(5)-(1-imino-3-butenyl)-ornithine; WKY, Wistar-Kyoto rat

Introduction

Renin is a critical component of the renin-angiotensin system (RAS) and catalyzes the rate-limiting step of converting angiotensinogen to angiotensin I. Angiotensin I is converted to angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Dzau and his colleagues have demonstrated the expression of renin gene in mouse and rat brains. (Dzau *et al.*, 1986) Local synthesis of angiotensin, independent of angiotensin generation in the circulation, is now widely accepted, indicating that renin can function in tissue sites, including the brain. (Dampney, 1994; Davisson *et al.*, 2000) Taken together, these studies indicate that renin is expressed locally in the brain and may functionally regulate brain RAS through the generation of angiotensin peptides.

The RAS plays an important role in blood pressure regulation and cardiovascular disease risk profile. ACE inhibitors and antagonists of the angiotensin AT₁ receptor (receptor nomenclature follows Alexander *et al.*, 2011) are current antihypertensive therapies. In 2007, direct renin inhibitors were released as a new class of antihypertensive agents (Brown, 2008). Direct renin inhibitors can reduce renin activity, even in the presence of elevated renin activity induced by ACE inhibitors or AT₁ receptor antagonists. Direct renin inhibitors not only lower blood pressure but also have renoprotective effects and reduce the risk of heart failure (McMurray *et al.*, 2008; Parving *et al.*, 2008). These findings suggest that renin plays a critical role in blood pressure regulation. However, the role of renin itself and its mechanism of action in the brainstem remain unclear.

The nucleus tractus solitarius (NTS), which is located in the dorsal medulla of the brain stem, participates in the central control of blood pressure and sympathetic nerve activity. Our previous studies demonstrated that several neuromodulators are involved in cardiovascular control by the NTS, including Ang II (Mosqueda-Garcia *et al.*, 1990; Cheng *et al.*, 2010) and NO (Tseng *et al.*, 1996).

NO is synthesized by nitric oxide synthase (NOS) and there are three different types of NOS: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS; Calabrese *et al.*, 2007) *In vivo*, eNOS gene transfer experiments in the NTS caused hypotension and bradycardia (Sakai *et al.*, 2000; Hirooka *et al.*, 2003; Tai *et al.*, 2004) and eNOS and nNOS both participated in central cardiovascular regulation by the NTS. (Ho *et al.*, 2008; Chiang *et al.*, 2009; Cheng *et al.*, 2010; 2011) Nevertheless, the relationship between renin, NO syn-

thase activity and the corresponding modulation of blood pressure by the NTS has not been established.

In this study, we investigated whether microinjection of renin into the NTS might regulate blood pressure, and we determined which form of NOS could be activated by renin administration. In addition, we investigated which receptors and downstream signalling pathways were involved in renin-induced effects in the NTS. Our results suggest that the G_q-PI3K-Akt-eNOS signalling pathway was involved in the modulation of blood pressure by activation of AT₁ and Mas receptors in the NTS.

Methods

Animals

All animal care and experimental research protocols had been approved by the Research Animal Facility Committee of Kaohsiung Veterans General Hospital. Male Wistar-Kyoto (WKY) rats of 250 to 300 g were obtained from National Science Council Animal Facility and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). The rats were kept in individual cages in a room in which lighting was controlled (12 h on/12 h off), and the temperature was maintained at 23 to 24°C. The rats were allowed to acclimatise to the housing conditions for 1 week.

Intra-NTS microinjection

The preparation of animals for intra-NTS microinjection and the methods used to locate the NTS have been described previously (Tseng *et al.*, 1996). In the present study, each injection volume in the NTS was restricted to 60 nL. Rats were anaesthetized with urethane (1.0 g·kg⁻¹ i.p., supplemented with 300 mg·kg⁻¹ i.v. as necessary). A polyethylene catheter was placed in the femoral vein for drug administration. blood pressure was measured directly through a catheter placed in the femoral artery and connected to a pressure transducer (P23 ID, Gould Electronics, Eichstetten, Germany) and polygraph (RS3800, Gould Electronics). Heart rate (HR) was monitored continuously by a tachograph preamplifier (13-4615-65, Gould Electronics). Tracheostomy was performed to maintain airway patency during the experiment. For microinjection into brain stem nuclei, the rats were placed in a stereotaxic instrument (Kopf), with the head flexed downward at a 45° angle. The dorsal surface of the medulla was exposed by limited craniotomy, and the rats were rested for at least 1 h before experiments. Single-barrel glass catheter were

prepared (0.031-inch OD, 0.006 inch ID; Richland Glass Co, Vineland, NJ, USA) that had external tip diameters of 40 μm . The volume of microinjection (60 nL) was directly measured by the displacement of fluid meniscus in the micropipette barrel and confirmed visually under a microscope with a fine reticule (Tseng *et al.*, 1989; Dhar *et al.*, 2000). To verify that the needle tip of the glass electrode was exactly in the NTS, L-glutamate (0.154 nmol/60 nL) was microinjected. This would induce a characteristically abrupt fall in blood pressure ($\Delta\text{BP} \geq -35$ mmHg) and HR ($\Delta\text{HR} \geq -50$ bpm) if the needle tip was located precisely in the medial site of the intermediate one third of the NTS with the coordinates of anteroposterior, 0.0 mm; mediolateral, 0.5 mm; and vertical, 0.4 mm with the obex as reference (Tseng *et al.*, 1996).

The injected renin (2.4 fg, 24 fg, 240 fg and 2400 fg) was dissolved in artificial cerebrospinal fluid (aCSF; 142 mmol·L⁻¹ NaCl, 5 mmol·L⁻¹ KCl, 10 mmol·L⁻¹ glucose and 10 mmol·L⁻¹ HEPES, pH 7.4). Other compounds – L-NAME (33 nmol; non-selective NOS inhibitor, Huang *et al.*, 2004); L-NIO (6 nmol; inhibitor of eNOS, Ho *et al.*, 2008); vinyl-L-NIO (600 pmol; selective nNOS inhibitor Chiang *et al.*, 2009); lisinopril (2.4 fmol; ACE inhibitor); losartan (4 nmol; AT₁ receptor antagonist); D-Ala⁷-Ang-(1-7) (144 fmol; angiotensin-(1-7) (Ang-(1-7)) antagonist) and GPA-2A (1.98 pmol, G protein q subunit (Gq) inhibitor) were dissolved in aCSF. PD98059 (6 pmol; MEK inhibitor, Ho *et al.*, 2008); LY294002 (6 pmol; PI3K inhibitor, Huang *et al.*, 2004); valsartan (7.5 pmol; AT₁ receptor antagonist); gallein (240 fmol; G protein $\beta\gamma$ subunit (G $\beta\gamma$) inhibitor); 7-NI (66 pmol; nNOS inhibitor) and Akt inhibitor IV (375 fmol) were dissolved in DMSO, then diluted with aCSF.

Determination of NO in NTS

Renin and aCSF groups of rats (6 rats per group) were enrolled in the experiment. Rats were anesthetized with urethane (1.0 g kg⁻¹ i.p., supplemented with 300 mg kg⁻¹ i.v., if necessary). The NTS was dissected by micropunch (1-mm inner diameter) from a 1-mm thick brainstem slice at the level of the obex under a microscope. Total protein was prepared by homogenizing (BBX24 Bullet Blender homogenizer, Next Advance, Inc., Averill Park, NY, USA) the NTS tissue in lysis buffer (CellLytic MT Cell Lysis Reagent, Sigma-Aldrich, St. Louis, MO, USA), and incubating for 1 hour at 4 °C. Samples were then deproteinized using Microcon YM-30 centrifugal filter units (Millipore, Bedford, MA, USA). The amount of total NO (as nitrite and nitrate) in the samples was determined by a modification of the procedure based on the purge system of Sievers Nitric Oxide Analyzer (NOA 280i; Sievers Instruments, Boulder, CO, USA), which involves the use of chemiluminescence. Samples (10 μL) were injected into a reflux column containing 0.1 mol·L⁻¹ VCl₃ in 1 mol·L⁻¹ HCl at 90°C to reduce any nitrates and nitrites into NO. The NO was then combined with the O₃ produced by the analyzer to form NO₂. The resulting emission from the excited NO₂ was detected by a photomultiplier tube and recorded digitally (mV). The values were then interpolated to a standard curve of NaNO₃ concentrations determined concurrently. The measurements were made in triplicate for each sample. The levels of NO were expressed as concentrations of nitrate in each sample, corrected for the

total protein in the NTS samples from each rat (as μM nitrate per μg protein).

Immunoblotting analysis

The NTS of rats were removed after injection of renin or vehicle. Total protein was prepared by homogenizing the NTS for 1 h at 4°C in lysis buffer and proteinase inhibitor cocktail. Protein extracts (20 μg per sample assessed by bicinchoninic acid protein assay, Pierce Chemical Co., IL, USA) were subjected to 6% to 12.5% SDS-Tris glycine gel electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). The membranes were blocked and incubated at 4°C overnight with the appropriate antibody: rabbit anti-P-ERK1/2-Thr²⁰²/Tyr²⁰⁴ (1:1000; Cell Signaling Technology, Denver, MS, USA), rabbit anti-ERK1/2 (1:1000; Cell Signaling Technology), rabbit anti-P-Akt-Ser⁴⁷³ (1:1000; Cell Signaling Technology), rabbit anti-Akt (1:1000; Cell Signaling Technology), mouse anti-P-eNOS-Ser¹¹⁷⁷ (1:1000; BD Biosciences, San Jose, CA, USA) and mouse anti-eNOS (1:1000; BD Biosciences). Also, mouse anti-actin (1:10 000; Millipore), anti-P-nNOS-Ser¹⁴¹⁶ (1:1000; Abcam, Cambridge, UK), anti-nNOS (1:2000; Millipore), and anti-iNOS (1:1000; Millipore) were diluted in PBST with 5% bovine serum albumin. Horseradish peroxidase (HRP)-conjugated anti-mouse (1:10 000) or anti-rabbit antibody (1:10 000) was used as the secondary antibody at room temperature for 1 h.

Immunohistochemistry analysis

After perfusion with 0.9% normal saline, the rat brainstems were fixed immediately in 4% formaldehyde overnight and embedded in paraffin. The brainstems were sectioned coronally at a 5 μm thickness. The sections were deparaffinized, quenched in 3% H₂O₂/methanol, heated (microwave) in citrate buffer (10 mmol·L⁻¹, pH 6.0), blocked in 5% goat serum and incubated with an anti-P-eNOS-Ser¹¹⁷⁷ antibody overnight at 4°C. Next, the sections were incubated with biotinylated secondary antibodies (1:200; Vector Laboratories, Burlingame, CA, USA) for 1 h and in AB complex (1:100) for 30 min at room temperature. The sections were visualized with a DAB substrate kit (Vector Laboratories) and counterstained with haematoxylin. The sections were then photographed with a microscope mounted with a charge-coupled device camera.

Ras activation assay

Ras activation was measured using a Ras Activation ELISA Assay Kit (Millipore) following the manufacturer's instructions. The assay was initiated by the addition of recombinant Ras-binding domain of Raf-1 to the glutathione-coated ELISA plate via a glutathione-S-transferase/glutathione interaction, thus capturing the active Ras and allowing the inactive/GDP-bound Ras to be washed away. The captured, active Ras was detected and measured quantitatively through the addition of a monoclonal anti-Ras antibody that detects K-, H-, N-Ras isoforms from rat. A HRP-conjugated secondary antibody was then added for the detection. Following the addition of the chemiluminescent substrate, signals can be measured using a luminometer (Promega, Madison, WI, USA).

Data analysis

A paired Student's *t*-test was used for comparing blood pressure measurements and counting cells expressing P-eNOS-Ser¹¹⁷⁷. One-way ANOVA with Scheffe's *post hoc* test were applied to compare group differences. Differences with *P* < 0.05 were considered significant. All data are expressed as the means $\pm$ SEM.

Materials

Experimental drugs, such as urethane, Triton-X100, L-glutamate, heparin, human renin, lisinopril, losartan, N-nitro-L-arginine methyl ester (L-NAME), 7-nitroindazole (7-NI), LY294002, PD98059 and D-Ala7-Ang-(1-7), were obtained from Sigma-Aldrich (St. Louis, MO, USA). N(5)-(-iminoethyl)-L-ornithine (L-NIO), GP antagonist-2A (GPA-2A) and Akt inhibitor IV were obtained from Calbiochem (Darmstadt, Germany). N(5)-(1-imino-3-butenyl)-ornithine (vinyl-L-NIO) was obtained from ALEXIS (Lausen, Switzerland). Gallein and valsartan were obtained from Tocris (Bristol, UK).

Results

Renin induces systemic vasodepressor effect and NO release in the NTS

In this study, we utilized urethane-anaesthetized WKY rats to investigate the cardiovascular effects of microinjection of renin into the NTS. Tachyphylaxis was observed after repeated administration of similar doses of renin in the same site of the NTS under these experimental conditions (data not shown). Different concentrations of renin were then injected into the NTS of several rats. All the doses subsequently used (2.4 fg, 24 fg, 240 fg and 2400 fg) decreased mean blood pressure and heart rate (Supporting Information Figure S1). The response to renin (240 fg) unilaterally injected into the NTS was a fall in blood pressure (Figure 1A; mean values in Figure 1B, *n* = 6). aCSF was used as the drug vehicle and for control experiments. Interestingly, NO production in the NTS was significantly increased after the administration of renin.

Renin induces eNOS phosphorylation in the NTS

We further investigated which NOS isoform contributes to depressor effects and NO release induced by renin in the NTS of WKY rats. Pretreatment with the non-selective NOS inhibitor, L-NAME, attenuated the depressor effect of renin (Figure 2A, *n* = 6). Prior treatment with the selective eNOS inhibitor, L-NIO, also significantly attenuated the depressor effect of renin (Figure 2B, *n* = 6). In contrast, administration of the specific nNOS inhibitor, vinyl-L-NIO or 7-NI, did not diminish the depressor effect of renin in the NTS (Figure S2A and 2B, *n* = 6).

In addition, immunoblotting analyses of proteins extracted from the NTS demonstrated that treatment with renin did not increase nNOS-Ser¹⁴¹⁶ phosphorylation (Figure S2C, *n* = 6) or iNOS protein expression (Figure S2D, *P* > 0.05, *n* = 6) in the NTS. However, treatment with renin significantly increased the level of eNOS-Ser¹¹⁷⁷ phosphorylation (Figure 2C, *n* = 6) and (Figure 2D, *n* = 6) the proportion of P-

Figure 1

Systemic blood pressure (BP) and NO production in the NTS after administration of renin to the NTS. (A) Representative tracing show the depressor effect after unilateral microinjection of renin (240 fg) into the NTS. Artificial cerebrospinal fluid (aCSF) as the drug vehicle did not change blood pressure. Summary data (means $\pm$ SEM, *n* = 6) are shown in the graph. **P* < 0.05, significantly different from the aCSF group. (B) Levels of NO in samples of the NTS after microinjection of renin. The bar graph shows the NO concentration (as μ M nitrate per μ g of total protein). Pretreatment with renin significantly elevated NO levels in the NTS compared with aCSF. **P* < 0.05, significantly different from the aCSF group.

eNOS-Ser¹¹⁷⁷-positive cells in the NTS, compared with the corresponding values in the group treated with aCSF.

PI3K inhibitor attenuated renin-induced eNOS phosphorylation in the NTS

Our previous studies demonstrated that Akt and ERK could regulate NOS activity in the NTS (Huang *et al.*, 2004; Ho *et al.*, 2008). To test whether Akt or ERK may induce eNOS phosphorylation, the phosphorylation of Akt was determined by immunoblotting analyses with an antibody against

Figure 2

Microinjection of renin induces eNOS-Ser¹¹⁷⁷ phosphorylation in the NTS. (A) Representative tracing demonstrates that the depressor effect of renin was significantly attenuated by a non-selective NOS inhibitor, L-NAME (33 nmol). Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$ significantly different from the renin group. (B) Representative tracing demonstrates that the depressor effect of renin was significantly attenuated by the specific eNOS inhibitor, L-NIO (6 nmol). Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$, significantly different from the renin group. (C) The quantitative immunoblotting analysis demonstrates that renin treatment increased the level of P-eNOS-Ser¹¹⁷⁷ protein in the NTS. Densitometric analysis of P-eNOS-Ser¹¹⁷⁷ protein levels (means $\pm$ SEM, $n = 6$) after administration of aCSF or renin. $*P < 0.05$, significantly different from the aCSF group. (D) Immunohistochemical staining of the brainstem for P-eNOS-Ser¹¹⁷⁷ showed that injection of renin into the NTS induced P-eNOS-Ser¹¹⁷⁷ (c vs. d). Arrows indicate P-eNOS-Ser¹¹⁷⁷-positive cells. The scale bar represents 200 μ m. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. The percentage of P-eNOS-Ser¹¹⁷⁷-positive cells was determined by counting P-eNOS-Ser¹¹⁷⁷-expressing cells in each hemisphere of the NTS at 200 $\times$ magnification. These counts were divided by all of the cells in the same paraffin section. $*P < 0.05$, significantly different from the aCSF group.

P-Akt-Ser⁴⁷³. After administration of renin into the NTS, the phosphorylation level of Akt in the NTS was significantly elevated, compared with that after aCSF (Figure 3A, $n = 6$). However, there were no differences in ERK phosphorylation levels between the renin-treated and aCSF groups (Figure S3A, $n = 6$). Furthermore, pretreatment with the PI3K inhibitor, LY294002, attenuated renin-induced eNOS-Ser¹¹⁷⁷ phosphorylation in the NTS (Figure 3D, lane 2 and 3, $n = 6$).

We then used specific pharmacological inhibitors to confirm these results. Prior microinjection of the Akt-specific inhibitor, Akt inhibitor IV, significantly diminished the vasodepressor response evoked by renin (Figure 3B, $n = 6$). Pretreatment with LY294002 similarly attenuated the response to renin (Figure 3C, $n = 6$). In contrast, pretreatment of the NTS with the MEK inhibitor, PD98059, did not attenuate the depressor effect of renin (Figure S3B, $n = 6$).

Central administration of renin may regulate blood pressure through AT₁ and Mas receptors in the NTS

To assess the connection between renin and the downstream PI3K-Akt-eNOS signalling cascade in the NTS, we tested the effects of lisinopril (ACE inhibitor), losartan, valsartan (AT₁ receptor antagonists) and D-Ala7-Ang(1-7) [Ang-(1-7) antagonist] on blood pressure responses to renin, in the NTS. Pretreatment with lisinopril, losartan or valsartan all attenuated the depressor effect of renin (Figures 4A, 4B and 4C, $n = 6$). In addition, Figure 4D shows that injection of the Mas receptor antagonist, D-Ala7-Ang(1-7) also attenuated the depressor effect of renin ($n = 6$).

Renin may activate the PI3K-Akt-eNOS cascade via G_{α_q} and Ras signalling in the NTS

These results showed that renin could activate PI3K-Akt-eNOS signalling in the NTS. Previous studies have demonstrated that Ang II and Ang-(1-7) may activate PI3K and Akt through AT₁ receptors coupled to G_β (Quignard *et al.*, 2001), AT₁ receptors coupled to G_q (Dugourd *et al.*, 2003) or Mas receptors (Sampaio *et al.*, 2007). Therefore, we investigated the G-protein coupling involved in the effects of the PI3K-Akt-eNOS pathway within the NTS and its relevance to the response to renin. Pretreatment with the G_{βγ} inhibitor, gallein, did not diminish the depressor effect of renin in the NTS (Figure S4, $n = 6$). However, pre-treatment with the G_q inhibitor, GPA-2A, attenuated the depressor effect of renin at the NTS (Figure 5A, $n = 6$). We further assessed the involvement of Ras in the renin-induced depressor effect and found that administration of renin to the NTS enhanced Ras activation (Figure 5B, $n = 6$).

Discussion and conclusions

In the medulla of the brainstem, the nuclei have extensive connections to each other, and their major function is regulation of sympathetic nerve activity to modulate blood pressure. Ang II activates AT₁ and AT₂ receptors to modulate sympathetic nerve activity, in the brainstem but there is high

expression of only AT₁ receptors in the medial NTS (Lenkei *et al.*, 1997). Our present data show that microinjection of renin into the NTS of WKY rats induced a depressor effect and increased NO production (Figure 1). This renin-mediated depressor response was similar to the effect induced by Ang II in the NTS (Mosqueda-Garcia *et al.*, 1990; Fow *et al.*, 1994; Tan *et al.*, 2005). In addition, pre-treatment with an ACE inhibitor or AT₁ receptor antagonists, separately attenuated the depressor effect of renin, suggesting that renin induced depressor responses through Ang II and AT₁ receptors in the NTS (Figure 4). The Mas receptor is a G-protein-coupled receptor and is the endogenous receptor for Ang-(1-7) (Jackson *et al.*, 1988). Mas receptors can antagonize AT₁ receptor function by hetero-oligomeric interaction (Kostenis *et al.*, 2005) and unilateral injections of Ang-(1-7) into the NTS caused depressor and bradycardic effects (Campagnole-Santos *et al.*, 1989). In addition, the depressor effect evoked by Ang-(1-7) also involves a NO-mediated signalling (Alzamora *et al.*, 2002). Moreover, Ang-(1-7) up-regulated the PI3K-Akt-eNOS pathway to increase NO production, through Mas receptors (Sampaio *et al.*, 2007). However, we observed that pre-treatment with AT₁ receptor antagonists and a Ang-(1-7) antagonist both attenuated the depressor effect produced by renin (Figure 4). Thus, we would propose that both AT₁ and Mas receptors may contribute to the regulation of blood pressure by renin, at the NTS. The mechanisms connecting Mas receptors and Ras signalling in the NTS remain to be elucidated.

In the NTS, nNOS plays an important role in the modulation of blood pressure (Chiang *et al.*, 2009; Cheng *et al.*, 2010) and block of its expression in the NTS increased blood pressure (Maeda *et al.*, 1999). Also, overexpression of eNOS induced a depressor response (Sakai *et al.*, 2000; Hirooka *et al.*, 2003; Tai *et al.*, 2004). In Figure 2 and Figure S2, we found increased eNOS-Ser¹¹⁷⁷ phosphorylation levels in the NTS after administration of renin, whereas no activity or expression reductions were detected for nNOS or iNOS. Therefore, our results indicated that eNOS acts as a key factor in the production of NO in the NTS of WKY rats (Figure 1B). Because our previous study revealed that eNOS might play a key role in NTS cardiovascular function via the MAP kinase-ERK kinase (MEK)-ERK1/2 and eNOS signalling (Ho *et al.*, 2008), we decided to further investigate this possibility. Our present data indicated that renin might regulate blood pressure through AT₁ receptors. We propose that eNOS might act as a downstream target of the AT₁ and Mas receptors and may be involved in renin-induced NO production in the NTS, thereby regulating blood pressure.

It has been reported that Akt, AMP-activated protein kinase, PKA, protein phosphatase 2A, protein kinase G, Ca²⁺/calmodulin-dependent protein kinases and ERK can all phosphorylate eNOS at Ser¹¹⁷⁷ (Mount *et al.*, 2007; Ho *et al.*, 2008). Previously, we revealed the crucial role of the PI3K-Akt-eNOS pathway in regulating cardiovascular effects in the NTS (Huang *et al.*, 2004). In the present study, LY294002 inhibited eNOS-Ser¹¹⁷⁷ phosphorylation induced by renin (Figure 3D) suggesting that renin may phosphorylate eNOS via a PI3K-Akt cascade in the NTS. We tested the effect of the G_q inhibitor, GPA-2A and the G_{βγ} inhibitor, gallein, on the renin-induced depressor effect in the NTS to provide further evidence that renin may produce Ang II, which in turn

Figure 3

PI3K and Akt participate in renin-mediated eNOS-Ser¹¹⁷⁷ phosphorylation in the NTS. (A) The quantitative immunoblotting analysis demonstrates that renin treatment increased the level of P-Akt-Ser⁴⁷³ protein in the NTS. Densitometric analysis of P-Akt-Ser⁴⁷³ protein levels (means $\pm$ SEM, $n = 6$) after administration of aCSF or renin. * $P < 0.05$, significantly different from the aCSF group. (B) Representative tracings reveal the effects of BP by microinjection renin (240 fg) into the NTS pretreated with Akt inhibitor, Akt inhibitor IV (375 fmol). * $P < 0.05$, significantly different from the renin group. (C) The blood pressure of renin (240 fg) injection into the NTS after administration of the PI3K inhibitor, LY294002 (6 pmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by LY294002. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. * $P < 0.05$, significantly different from the renin group. (D) Immunoblotting analysis reveals that the P-eNOS-Ser¹¹⁷⁷ protein level was increased after renin administration in the NTS. Phosphorylation of eNOS-Ser¹¹⁷⁷ was reduced by pretreatment with LY294002. Densitometric analysis of P-eNOS-Ser¹¹⁷⁷ protein levels (means $\pm$ SEM, $n = 6$) after treatment with aCSF, renin or LY294002. * $P < 0.05$, significantly different from the aCSF group; # $P < 0.05$, significantly different from the renin group.

Figure 4

Angiotensin AT₁ and Mas receptors participate in renin-mediated depressor effects at the NTS. (A) Blood pressure response to renin (240 fg) injection into the NTS after administration of the ACE inhibitor, lisinopril (2.4 fmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by lisinopril. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$, significantly different from the renin group. (B) Blood pressure response to renin (240 fg) injection of into the NTS after administration of losartan (4 nmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by losartan. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$, significantly different from the renin group. (C) Blood pressure response to renin (240 fg) injection of into the NTS after administration of valsartan (7.5 pmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by valsartan. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$, significantly different from the renin group. (D) Blood pressure response to renin (240 fg) injection of into the NTS after administration of the angiotensin-(1-7) antagonist, D-Ala7-Ang-(1-7) (144 fmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by D-Ala7-Ang-(1-7). Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. $*P < 0.05$, significantly different from the renin group.

Figure 5

Microinjection of renin induces G_q -Ras signalling in the NTS. (A) Blood pressure response to renin (240 fg) injection into the NTS after administration of the G_q inhibitor, GPA-2A (1.98 pmol). Representative tracings demonstrate that the depressor effect of renin was significantly attenuated by GPA-2A. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. * $P < 0.05$, significantly different from the renin group. (B) Bar graph showing the activation ratio of Ras after microinjection of renin into the NTS. Note the significant increase in Ras activation after treatment with renin. * $P < 0.05$, significantly different from the aCSF group.

activates AT_1 receptor- G_q signalling, as described previously (Wong *et al.*, 2002). Ang II stimulated PI3K-Akt signalling via AT_1 receptors in the brain (Yang and Raizada, 1999) and chronic blockade of PI3K in the NTS of spontaneously hypertensive rat (SHR) resulted in hypertension (Zubcevic *et al.*, 2009). Furthermore, renin induced Ras activation in the NTS (Figure 5B) and similar results have been demonstrated in cardiac myocytes and vascular smooth muscle cells (Eguchi *et al.*, 1996; Sadoshima and Izumo, 1996). Activation of the epidermal growth factor receptors (EGFR) leads to signalling mediated by the PI3K-Akt and Ras-MAPK pathways. The EGFRs also can be transactivated by AT_1 receptors and lead to the activation of PI3K-Akt cascade or Ras-MAPK pathway (Eguchi *et al.*, 2001; Kippenberger *et al.*, 2005; Mifune *et al.*, 2005). Thus, further studies are needed to elucidate this possible mechanism in the NTS. However, we did not observe MEK-ERK signalling in the renin-mediated depressor response (Figure S2). Our data support the hypothesis that, in the NTS, renin activated the PI3K-Akt-eNOS signalling pathway, and consequently, the Ras pathway.

The (pro)renin receptor (PRR) consists of a 350-amino-acid protein with a single transmembrane domain that has comparable affinity for (pro)renin and renin (Nguyen *et al.*, 2002). The PRR is highly expressed in brain regions known to modulate the cardiovascular system, such as the cortex, hippocampus, subfornical organ and NTS (Shan *et al.*, 2008; Contrepas *et al.*, 2009). Levels of mRNA for PRR were 45% and 70% higher in the NTS and supraoptic nucleus of SHR,

compared to WKY rats (Shan *et al.*, 2010), indicating that PRR may contribute to central blood pressure regulation. In our study, inhibition of RAS activity attenuated renin-mediated depressor effects in the NTS (Figure 4 and Figure S3). Renin binds PRR with affinity in the nanomolar range (Nabi *et al.*, 2006) and in the present study, we have microinjected renin into NTS in the picomolar range. However, further studies are needed to establish the mechanisms connecting PRRs and their downstream signalling cascade in the NTS.

The study herein represents a key step in understanding how renin may participate in regulating the cardiovascular system through effects exerted in the NTS. We have utilized SensoLyte® 520 Rat Renin Assay Kit (ANASPEC, San Jose, CA, USA) to determine the concentration of renin in the NTS of WKY rats as 56 pM (data not shown). In Figure 1A, we microinjected 240 fg of renin into the NTS of WKY rats, equivalent to a concentration of 100 pM. However, the levels of angiotensin I, Ang II and Ang-(1-7), after administration of renin to the NTS are not known and these values should be determined. Another limitation of this study derives from the use of pharmacological inhibitors and their specificity. For instance, administration of losartan at higher doses induced non-specific effects in the RVLM (Averill *et al.*, 1994). Moreover, losartan may bind to non-Ang II binding sites and valsartan has approximately five-fold greater affinity than losartan, for the AT_1 receptor (de Gasparo and Whitebread, 1995). We used both these AT_1 receptor antagonists to assess the contribution of AT_1 receptors to the modulation

Figure 6

The proposed renin-induced signalling pathway in the NTS to regulate systemic blood pressure in WKY rats. Microinjection of renin stimulates the PI3K-Akt-eNOS cascade via activating AT₁ and Mas receptors, ultimately leading to elevated NO concentrations in the NTS and decreased blood pressure. ACE2, angiotensin converting enzyme 2; Agt, angiotensinogen; Ang I, angiotensin I.

of renin-induced depressor effect in this study (Figure 4B and C). We intend to utilize shRNA-based gene delivery to validate the role of central cardiovascular effects more precisely, which may help us overcome the limitations imposed in animals.

In conclusion, we propose that renin could mediate central control of blood pressure at the NTS, through Ras-PI3K-Akt signalling-regulated phosphorylation of eNOS (Figure 6). The present data further show that renin may modulate blood pressure via centrally located AT₁ and Mas receptors, and via G_q to activate Ras, Akt and eNOS. The RAS modulates sympathetic function in the NTS, where integration of autonomic control of the cardiovascular system occurs. Direct renin inhibitors have been approved for clinical use, however, the mechanisms by which direct renin inhibitors regulate blood pressure in the CNS have not been clarified. Our findings provide new insights into the mechanism(s) of central regulation of blood pressure by renin and may be of help in further development of direct renin inhibitors in therapy.

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Conflicts of interest

None.

References

- Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.
- Alzamora AC, Santos RAS, Campagnole-Santos MJ (2002). Hypotensive effect of ANG II and ANG-(1-7) at the caudal ventrolateral medulla involves different mechanisms. Am J Physiol Regul Integr Comp Physiol 283: R1187–R1195.
- Averill DB, Tsuchihashi T, Khosla MC, Ferrario CM (1994). Losartan, nonpeptide angiotensin II-type 1 (AT₁) receptor antagonist, attenuates pressor and sympathoexcitatory responses evoked by angiotensin II and L-glutamate in rostral ventrolateral medulla. Brain Res 665: 245–252.
- Brown MJ (2008). Aliskiren. Circulation 118: 773–784.
- Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM (2007). Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nat Rev Neurosci 8: 766–775.
- Campagnole-Santos MJ, Diz DI, Santos RA, Khosla MC, Brosnihan KB, Ferrario CM (1989). Cardiovascular effects of angiotensin-(1-7) injected into the dorsal medulla of rats. Am J Physiol Heart Circ Physiol 257: H324–H329.
- Cheng WH, Lu PJ, Ho WY, Tung CS, Cheng PW, Hsiao M *et al.* (2010). Angiotensin II inhibits neuronal nitric oxide synthase activation through the ERK1/2-RSK signaling pathway to modulate central control of blood pressure. Circ Res 106: 788–795.
- Cheng PW, Lu PJ, Chen SR, Ho WY, Cheng WH, Hong LZ *et al.* (2011). Central nicotinic acetylcholine receptor involved in Ca(2+)-calmodulin-endothelial nitric oxide synthase pathway modulated hypotensive effects. Br J Pharmacol 163: 1203–1213.
- Chiang HT, Cheng WH, Lu PJ, Huang HN, Lo WC, Tseng YC *et al.* (2009). Neuronal nitric oxide synthase activation is involved in insulin-mediated cardiovascular effects in the nucleus tractus solitarius of rats. Neuroscience 159: 727–734.
- Contrepas A, Walker J, Koulakoff A, Franek KJ, Qadri F, Giaume C *et al.* (2009). A role of the (pro)renin receptor in neuronal cell differentiation. Am J Physiol Regul Integr Comp Physiol 297: R250–R257.

- Dampney RA (1994). Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 74: 323–364.
- Davisson RL, Oliverio MI, Coffman TM, Sigmund CD (2000). Divergent functions of angiotensin II receptor isoforms in the brain. *J Clin Invest* 106: 103–106.
- Dhar S, Nagy F, McIntosh JM, Sapru HN (2000). Receptor subtypes mediating depressor responses to microinjections of nicotine into medial NTS of the rat. *Am J Physiol Regul Integr Comp Physiol* 279: R132–R140.
- Dugourd C, Gervais M, Corvol P, Monnot C (2003). Akt is a major downstream target of PI3-kinase involved in angiotensin II-induced proliferation. *Hypertension* 41: 882–890.
- Dzau VJ, Ingelfinger J, Pratt RE, Ellison KE (1986). Identification of renin and angiotensinogen messenger RNA sequences in mouse and rat brains. *Hypertension* 8: 544–548.
- Eguchi S, Matsumoto T, Motley ED, Utsunomiya H, Inagami T (1996). Identification of an essential signaling cascade for mitogen-activated protein kinase activation by angiotensin II in cultured rat vascular smooth muscle cells. Possible requirement of Gq-mediated p21ras activation coupled to a Ca²⁺/calmodulin-sensitive tyrosine kinase. *J Biol Chem* 271: 14169–14175.
- Eguchi S, Dempsey PJ, Frank GD, Motley ED, Inagami T (2001). Activation of MAPKs by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 276: 7957–7962.
- Fow JE, Averill DB, Barnes KL (1994). Mechanisms of angiotensin-induced hypotension and bradycardia in the medial solitary tract nucleus. *Am J Physiol* 267: H259–H266.
- de Gasparo M, Whitebread S (1995). Binding of valsartan to mammalian angiotensin AT1 receptors. *Regul Pept* 59: 303–311.
- Hirooka Y, Sakai K, Kishi T, Ito K, Shimokawa H, Takeshita A (2003). Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. *Hypertens Res* 26: 325–331.
- Ho WY, Lu PJ, Hsiao M, Hwang HR, Tseng YC, Yen MH *et al.* (2008). Adenosine modulates cardiovascular functions through activation of extracellular signal-regulated kinases 1 and 2 and endothelial nitric oxide synthase in the nucleus tractus solitarii of rats. *Circulation* 117: 773–780.
- Huang HN, Lu PJ, Lo WC, Lin CH, Hsiao M, Tseng CJ (2004). In situ Akt phosphorylation in the nucleus tractus solitarii is involved in central control of blood pressure and heart rate. *Circulation* 110: 2476–2483.
- Jackson TR, Blair LAC, Marshall J, Goedert M, Hanley MR (1988). The mas oncogene encodes an angiotensin receptor. *Nature* 335: 437–440.
- Kippenberger S, Loitsch S, Guschel M, Müller J, Knies Y, Kaufmann R *et al.* (2005). Mechanical stretch stimulates protein kinase B/Akt phosphorylation in epidermal cells via angiotensin II type 1 receptor and epidermal growth factor receptor. *J Biol Chem* 280: 3060–3067.
- Kostenis E, Milligan G, Christopoulos A, Sanchez-Ferrer CF, Heringer-Walther S, Sexton PM *et al.* (2005). G-protein-coupled receptor mas is a physiological antagonist of the angiotensin II type 1 receptor. *Circulation* 111: 1806–1813.
- Lenkei Z, Palkovits M, Corvol P, Llorens-Cortès C (1997). Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: a functional neuroanatomical review. *Front Neuroendocrinol* 18: 383–439.
- Maeda M, Hirano H, Kudo H, Doi Y, Higashi K, Fujimoto S (1999). Injection of antisense oligos to nNOS into nucleus tractus solitarii increases blood pressure. *Neuroreport* 10: 1957–1960.
- McMurray JJ, Pitt B, Latini R, Maggioni AP, Solomon SD, Keefe DL *et al.* (2008). Effects of the oral direct renin inhibitor aliskiren in patients with symptomatic heart failure. *Circ Heart Fail* 1: 17–24.
- Mifune M, Ohtsu H, Suzuki H, Nakashima H, Brailoiu E, Dun NJ *et al.* (2005). G protein coupling and second messenger generation are indispensable for metalloprotease-dependent, heparin-binding epidermal growth factor shedding through angiotensin II type-1 receptor. *J Biol Chem* 280: 26592–26599.
- Mosqueda-Garcia R, Tseng CJ, Appalsamy M, Robertson D (1990). Cardiovascular effects of microinjection of angiotensin II in the brainstem of renal hypertensive rats. *J Pharmacol Exp Ther* 255: 374–381.
- Mount PF, Kemp BE, Power DA (2007). Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J Mol Cell Cardiol* 42: 271–279.
- Nabi AH, Kageshima A, Uddin MN, Nakagawa T, Park EY, Suzuki F (2006). Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. *Int J Mol Med* 18: 483–488.
- Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD (2002). Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 109: 1417–1427.
- Parving HH, Persson F, Lewis JB, Lewis EJ, Hollenberg NK (2008). Aliskiren combined with losartan in type 2 diabetes and nephropathy. *N Engl J Med* 358: 2433–2446.
- Quignard JF, Mironneau J, Carricaburu V, Fournier B, Babich A, Nurnberg B *et al.* (2001). Phosphoinositide 3-kinase gamma mediates angiotensin II-induced stimulation of L-type calcium channels in vascular myocytes. *J Biol Chem* 276: 32545–32551.
- Sadoshima J, Izumo S (1996). The heterotrimeric Gq protein-coupled angiotensin II receptor activates p21 ras via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes. *EMBO J* 15: 775–787.
- Sakai K, Hirooka Y, Matsuo I, Eshima K, Shigematsu H, Shimokawa H *et al.* (2000). Overexpression of eNOS in NTS causes hypotension and bradycardia *in vivo*. *Hypertension* 36: 1023–1028.
- Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM (2007). Angiotensin-(1-7) through receptor mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185–192.
- Shan Z, Cuadra AE, Sumners C, Raizada MK (2008). Characterization of a functional (pro)renin receptor in rat brain neurons. *Exp Physiol* 93: 701–708.
- Shan Z, Shi P, Cuadra AE, Dong Y, Lamont GJ, Li Q *et al.* (2010). Involvement of the brain (pro)renin receptor in cardiovascular homeostasis. *Circ Res* 107: 934–938.
- Tai MH, Hsiao M, Chan JY, Lo WC, Wang FS, Liu GS *et al.* (2004). Gene delivery of endothelial nitric oxide synthase into nucleus tractus solitarii induces biphasic response in cardiovascular functions of hypertensive rats. *Am J Hypertens* 17: 63–70.
- Tan PS, Potas JR, Killinger S, Horiuchi J, Goodchild AK, Pilowsky PM *et al.* (2005). Angiotensin II evokes hypotension and renal sympathoinhibition from a highly restricted region in the nucleus tractus solitarii. *Brain Res* 1036: 70–76.

Tseng CJ, Mosqueda-Garcia R, Appalsamy M, Robertson D (1989). Cardiovascular effects of neuropeptide Y in rat brainstem nuclei. *Circ Res* 64: 55–61.

Tseng CJ, Liu HY, Lin HC, Ger LP, Tung CS, Yen MH (1996). Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 27: 36–42.

Wong LF, Polson JW, Murphy D, Paton JF, Kasparov S (2002). Genetic and pharmacological dissection of pathways involved in the angiotensin II-mediated depression of baroreflex function. *FASEB J* 16: 1595–1601.

Yang H, Raizada MK (1999). Role of phosphatidylinositol 3-kinase in angiotensin II regulation of norepinephrine neuromodulation in brain neurons of the spontaneously hypertensive rat. *J Neurosci* 19: 2413–2423.

Zubcevic J, Waki H, Diez-Freire C, Gampel A, Raizada MK, Paton JF (2009). Chronic blockade of phosphatidylinositol 3-kinase in the nucleus tractus solitarius is prohypertensive in the spontaneously hypertensive rat. *Hypertension* 53: 97–103.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 The bar graphs reveal hypotensive and bradycardic effects of unilateral administration of different doses of renin in the nucleus tractus solitarius (NTS). Both the mean blood pressure (MBP) and heart rate (HR) effects were significantly changed by renin. * $P < 0.05$ versus the 0 group.

Figure S2 (A) The cardiovascular effect of injection of renin (240 fg) into the NTS after administration of the selective nNOS inhibitor, vinyl-L-NIO (600 pmol). Representative tracings demonstrate that vinyl-L-NIO did not diminish the depressor effect of renin in the NTS. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph (B) The cardiovascular effect of injection of renin (240 fg) into the NTS after admin-

istration of the potent nNOS inhibitor, 7-NI (66 pmol). Representative tracings demonstrate that 7-NI did not diminish the depressor effect of renin in the NTS. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph. (C) Immunoblot depicts the levels of P-nNOS-Ser¹⁴¹⁶ protein in the NTS. Densitometric analysis of P-nNOS-Ser¹⁴¹⁶ protein levels (means $\pm$ SEM, $n = 6$) before and after treatment with renin. Note that there are no significant differences in nNOS-Ser¹⁴¹⁶ phosphorylation levels in the NTS between the aCSF and renin-treated groups. (D) Immunoblot shows iNOS protein levels (means $\pm$ SEM, $n = 6$) in the NTS. Note that there are no significant differences in iNOS protein levels in the NTS between aCSF and the renin-treated groups.

Figure S3 (A) Immunoblots depicting the levels of P-ERK1/2-Thr²⁰²/Tyr²⁰⁴ protein in the NTS. There were no significant differences in ERK1/2 phosphorylation levels in the NTS between the aCSF and renin-treated groups. Densitometric analysis shows P-ERK1/2-Thr²⁰²/Tyr²⁰⁴ protein levels (means $\pm$ SEM, $n = 6$) after treatment with renin. (B) The cardiovascular effect of injection of renin (240 fg) into the NTS after administration of the MEK inhibitor, PD98059 (10 pmol). Representative tracings demonstrate that PD98059 did not diminish the depressor effect of renin in the NTS. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph.

Figure S4 Blood pressure (BP) after injection of renin (240 fg) into the NTS following administration of the G $\beta\gamma$ inhibitor, gallein (240 fmol). Representative tracings demonstrate that the depressor effect of renin was not attenuated by gallein. Summary data (means $\pm$ SEM, $n = 6$) are shown in the graph.

Table S1 Cardiovascular response to microinjection of renin (240 fg) into the NTS in rats before and after microinjection of the pharmacological inhibitors

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