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TRANSLATIONAL PHYSIOLOGY

Angiotensin-(1–7) attenuates the chronotropic response to angiotensin II via stimulation of PTEN in the spontaneously hypertensive rat neurons

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Modgil A, Zhang Q, Pingili A, Singh N, Yao F, Ge J, Guo L, Xuan C, O'Rourke ST, Sun C. Angiotensin-(1–7) attenuates the chronotropic response to angiotensin II via stimulation of PTEN in the spontaneously hypertensive rat neurons. *Am J Physiol Heart Circ Physiol* 302: H1116–H1122, 2012. First published December 23, 2011; doi:10.1152/ajpheart.00832.2011.—Several studies have focused on the beneficial effects of peripheral angiotensin-(1–7) [Ang-(1–7)] in the regulation of cardiovascular function, showing its counterregulatory effect against the actions of angiotensin II (ANG II). However, its actions in the central nervous system are not completely understood. In the present study, we investigated the intracellular mechanisms underlying the action of ANG-(1–7) using the patch-clamp technique in neurons cultured from the hypothalamus of neonatal spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats. Superfusion of neurons with ANG II (100 nM) significantly increased neuronal firing in both strains of rats, and this chronotropic effect of ANG II was significantly enhanced in prehypertensive SHR neurons compared with WKY rat neurons. The enhanced chronotropic effect of ANG II was attenuated by a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, LY 294002 (10 μM). Superfusion of neurons with ANG-(1–7) (100 nM) did not alter the neuronal firing rate in either SHR or WKY neurons; however, it significantly attenuated the chronotropic action of ANG II exclusively in prehypertensive SHR neurons. This counterregulatory effect of ANG-(1–7) on ANG II action in prehypertensive SHR neurons was attenuated by cotreatment with either A-779, a Mas receptor antagonist, or bisperoxovanadium, a phosphatase and tensin homologue deleted on chromosome ten (PTEN) inhibitor. In addition, incubation of WKY and prehypertensive SHR neurons with ANG-(1–7) significantly increased PTEN activity. The data demonstrate that ANG-(1–7) counterregulates the chronotropic action of ANG II via a PTEN-dependent signaling pathway in prehypertensive SHR neurons.

Mas receptor; angiotensin II; phosphatidylinositol 3-kinase; phosphatase and tensin homologue deleted on chromosome ten

IT IS WELL ESTABLISHED THAT the brain renin angiotensin system (RAS) exerts regulatory influences in the control of blood pressure (BP) and plays an important role in the development and establishment of hypertension (1). Brain angiotensin (ANG) II is one of the most well-studied peptides and represents the major effector hormone of this system. Hyperactivity of this hormone system is linked to hypertension and other cardiovascular diseases (41). The contributions of ANG II to the central nervous system control of BP are manifested via alterations in the electrical activity of neurons at specific cardiovascular regulatory regions of the hypothalamus, which also receive many inputs from brainstem sites such as the rostral ventrolateral medulla (RVLM) and nucleus tractus solitarius, with subsequent activation of hypothalamic sites, such as the paraventricular nucleus (17). Previous studies (19) demonstrated that these actions of ANG II are amplified in the cardiovascular regulatory regions of spontaneously hypertensive rats (SHRs) and that interruption of brain AT_1-receptor function by pharmacological or genetic means lowers BP in these animals. Our previous in vitro studies (34, 35) also demonstrate that the chronotropic action of ANG II is enhanced in neurons cultured from the SHR hypothalamus. The enhanced chronotropic action of ANG II in SHR neurons is mediated by phosphatidylinositol 3-kinase (PI3-kinase; Refs. 34, 35). Thus it is essential to identify the neuronal factors that regulate the actions of ANG II or target intracellular signaling molecules of the ANG II pathway in SHR neurons.

Several peptides have been identified in the RAS. One of the most interesting members of the RAS is the heptapeptide ANG-(1–7). In the classical RAS, ANG-(1–7) was considered to be an inactive metabolic breakdown product of ANG II. This view has been challenged with the discovery of angiotensin-converting enzyme 2 (ACE2; Refs. 11, 25, 37), which cleaves ANG II to ANG-(1–7), and the G-protein-coupled receptor, Mas, which has been recognized as the first binding site for ANG-(1–7) (28). In addition, a large body of evidence has proven several beneficial effects of this peptide in the cardiovascular system, which is often opposite to the effects elicited by ANG II (13, 27, 28). This discovery provides a new axis to the brain RAS, comprising ACE2, Mas receptor, and ANG-(1–7), counterregulating the classical ACE/AT_1-receptor/ANG II axis through generation of ANG-(1–7). The dramatic beneficial effects of ANG-(1–7) in the peripheral cardiovascular system, which occur via counterregulating ANG II actions, have been well studied (13, 27, 28). Meanwhile, ANG-(1–7) and its Mas receptor are widely expressed in hypothalamic region (3, 7). The role of this peptide in central control of BP and in the pathogenesis of neurogenic hypertension has been studied by several research groups using different techniques.

It has been reported that ANG-(1–7) acts as an important neuromodulator, increases sensitivity of baroreflexes, and prevents norepinephrine release in SHRs (6, 28). More interestingly, viral vector-mediated overexpression of ACE2, an enzyme responsible for converting ANG II to ANG-(1–7), in the RVLM prevents the development of hypertension in SHRs (37). In addition, central-specifically overexpression of ACE2

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significantly prevents ANG II-induced hypertension in mice (12). Höcht et al. (18) reported that intrahypothalamic injection of ANG II induced a significantly greater pressor response in SHR and that the enhanced pressor response to ANG II was reduced by coadministration with ANG-(1–7). This study indicates that ANG-(1–7) acts as an antagonist on ANG II pressor response and counterregulates the cardiovascular effect of ANG II in hypothalamus of SHR. However, the cellular mechanism(s) underlying the central action of ANG-(1–7) are still not clear.

In this study, we examined the direct effect of ANG-(1–7) on neuronal activity in neurons cultured from the hypothalamus of SHR and WKY rats. We also investigated interactions between ANG II and ANG-(1–7) in the control of neuronal activity, as well as the intracellular cross-talk of signaling pathways between these two neuronal regulatory peptides in both SHR and WKY rats.

METHODS

Animals. Twelve-week-old male SHR and WKY rats were obtained from Charles River Farms (Wilmington, MA). These rats were used as breeders to produce a constant supply of newborn SHR and WKY rat pups to prepare neuronal cultures. Rats were housed at 25 ± 2°C on a 12:12-h light-dark cycle and provided with food and water ad libitum. All animal protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee (Protocol A0741).

Preparation of neuronal cultures. Neuronal cocultures were prepared from the hypothalamic block of newborn SHR and WKY rats as described previously (39). Trypsin (375 U/ml)-and DNase I (496 U/ml)-dissociated cells were resuspended in DMEM containing 10% plasma-derived horse serum (PHDS) and plated on poly-L-lysine-precoated 35-mm Nunc plastic tissue culture dishes. After the cells were grown for 3 days at 37°C in a humidified incubator with 95% air-5% CO_2, they were exposed to 1 μM cytosine-β-D-arabinofuranoside for 2 days in fresh DMEM containing 10% PDHS. Then, cytosine-β-D-arabinofuranoside was removed and the cells were incubated with fresh DMEM (containing 10% PDHS) for a further 9–12 days before use. At the time of use, cultures consisted of 90% neurons and 10% astrocyte glia, as determined by immunofluorescent staining with antibodies against neurofilament proteins and glial fibrillary acidic protein (32).

Electrophysiological recordings. Spontaneous action potentials (APs) were recorded from WKY and SHR neurons at room temperature using the whole cell patch-clamp technique in current-clamp mode as described in our previous publication (39). Cells were bathed in Tyrode solution containing the following (in mmol/l): 140 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 2.0 $MgCl_2$, 0.3 NaH_2PO_4, 10 HEPES, and 10 dextrose, pH adjusted to 7.4 with NaOH. The patch electrodes (resistance from 3 to 4 MΩ) were filled with an internal pipette solution containing the following (in mmol/l): 140 KCl, 4 $MgCl_2$, 4 ATP, 0.1 guanidine-5′-triphosphate, 10 dextrose, and 10 HEPES, pH adjusted to 7.2 with KOH. Neuronal firing rate was measured as the number of fully developed APs (depolarization beyond 0 mV) per second (Hz).

PTEN activity assay. PTEN activity in extracts from neuronal cultures was measured using a phosphate detection kit (Biomol Research Laboratories, Plymouth Meeting, PA) as described previously (30). Neuronal cell lysates were prepared in ice-cold phosphatase lysis buffer, containing the following: 50 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, 1% NP-40, 1 mM EDTA, 1 mM PMSF, and a mixture of protease inhibitors (Roche Applied Science), sonicated for 10 s, and briefly centrifuged at 2,000 *g* for 5 min. NaF, a protein serine/threonine phosphatase inhibitor, was included in the lysis buffer to preserve the protein phosphorylation state. PTEN belongs to the protein tyrosine phosphatase family; thus NaF did not influence the PTEN assay. Lysates were subsequently precleared with protein G-agarose beads for 1 h. Cell lysates were immunoprecipitated with a specific antibody against PTEN (Santa Cruz Biotechnology, Santa Cruz, CA) using protein G-agarose beads. The beads were washed three times and mixed with 100 μl of PTEN buffer containing 25 mM Tris·Cl pH 7.4, 140 mM NaCl, 2.7 mM KCl, and 10 mM DTT. PTEN activity was measured using a phosphate detection kit (Biomol Research Laboratories). Before proceeding, the enzyme preparations and reaction solutions were checked to be free of contaminating phosphate by addition of 100 μl of Malachite Green to each solution in the microtiter and the absorbance was measured at 620 nm. The PTEN solution was added first, followed by addition of water soluble phosphatidylinositol-3,4,5-trisphosphate (PIP3) substrate (200 μM) to start the reaction. The plate was sealed and placed on a shaker for 30 s. The reactions were further incubated at 37°C for 15 min. The reaction was stopped by addition of Malachite Green solution (100 μl/well). The plate was further incubated on a plate shaker for 15 min at room temperature to develop color. The beads were separated by centrifugation, and the supernatant was placed in 96-well microtiter plates. The absorbance was read at 620 nm. Free phosphate was determined in each sample by interpolation from the standard curve.

Data analysis. Results are expressed as means ± SE. Statistical significance was evaluated with the use of one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P < 0.05$.

RESULTS

ANG-(1–7) attenuates the enhanced chronotropic response to ANG II in SHR neurons. We first examined the effect of ANG-(1–7) and ANG II on neuronal firing rate in neurons cultured from the hypothalamus of SHR and WKY rats using the whole cell patch-clamp configuration in current-clamp mode. Superfusion of neurons with ANG-(1–7) (100 nM) did not significantly alter the neuronal firing rate in either SHR [0.57 ± 0.12 vs. 0.72 ± 0.16 Hz before and after ANG-(1–7) treatment; $n = 7$, 8 cells; $P > 0.05$] or WKY rat neurons [0.47 ± 0.09 vs. 0.55 ± 0.12 Hz before and after ANG-(1–7) treatment; $n = 7$, 8 cells; $P > 0.05$]. In contrast, ANG II significantly increased neuronal firing rate in both SHR and WKY neurons and the ANG II-induced increases in neuronal firing were enhanced in SHR neurons compared with WKY rat neurons (Fig. 1). Superfusion of neurons with ANG-(1–7) did not significantly alter the basal neuronal firing; however, it completely blocked the enhanced chronotropic action of ANG II in SHR neurons (Fig. 1, *B* and *C*). In WKY neurons, coadministration of ANG-(1–7) did not alter the ANG II-induced increase in neuronal firing rate (Fig. 1*C*). Similar to ANG-(1–7), the PI3-kinase inhibitor LY-294002 (10 μM, a concentration established in previous studies; Refs. 34, 38) did not alter the chronotropic action of ANG II in WKY rat neurons but abolished the enhanced chronotropic action of ANG II in SHR neurons (Fig. 1*D*). These studies demonstrate that the chronotropic action of ANG II is enhanced in SHR neurons compared with WKY rat neurons and that ANG-(1–7) attenuates the enhanced chronotropic action of ANG II in SHR neurons. This inhibitory action of ANG-(1–7) was mimicked by the PI3-kinase inhibitor LY-294002, indicating that they may share the same intracellular signaling pathway by inhibiting PI3-kinase activity.

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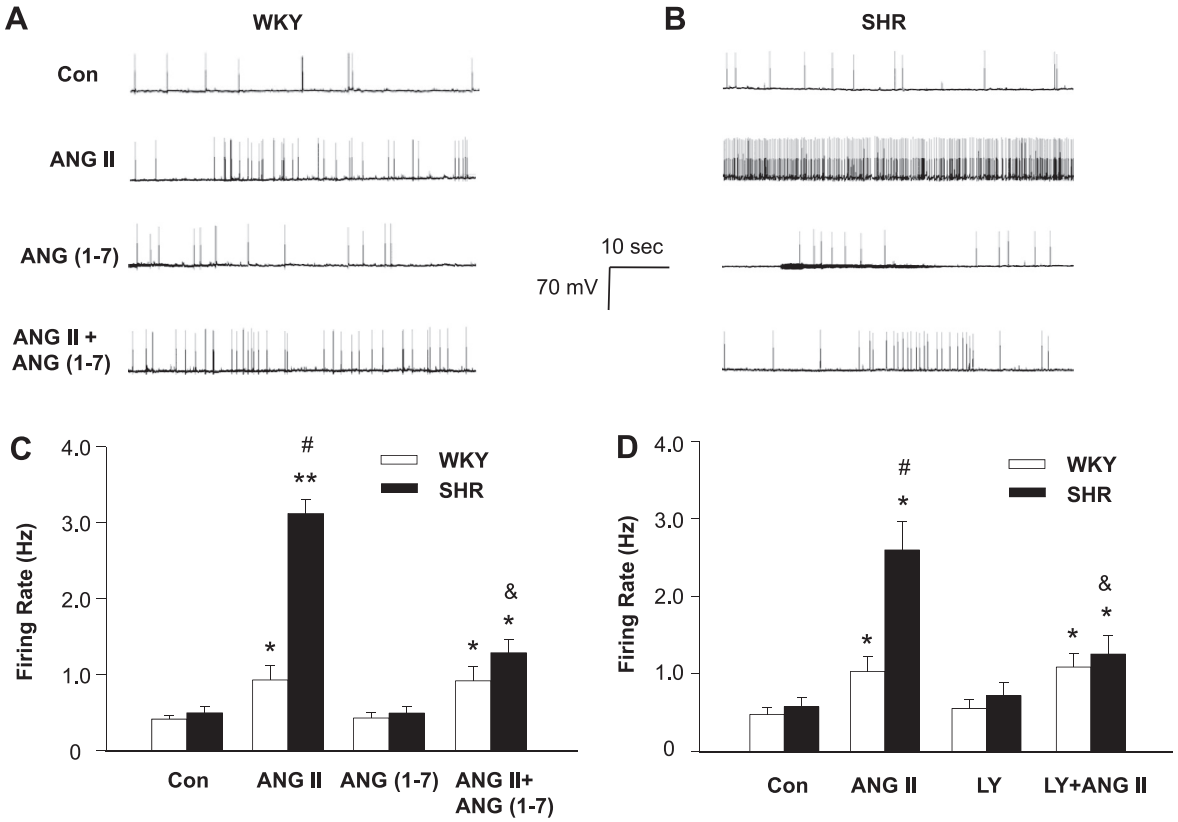

Fig. 1. Effect of angiotensin(1–7) [ANG-(1–7)] and LY-294002 (LY) on the chronotropic action of ANG II in spontaneously hypertensive (SHR) and Wistar Kyoto (WKY) rat neurons. Firing rate was recorded from neurons under each treatment condition. *A*: representative tracings showing the action potentials (APs) recorded from a single WKY rat neuron under the following sequential treatment conditions: superfusion with control (Con) solution (PBS), ANG II (100 nM), and washout of ANG II, and then neurons were superfused with 100 nM ANG-(1–7); this was followed by superfusion of ANG II + ANG-(1–7). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the chronotropic action of ANG II in SHR and WKY rat neurons. Data are means ± SE from 6 neurons for each group. $^{*}P < 0.05$, compared with respective control recordings. $^{**}P < 0.01$, compared with respective control. $^{\#}P < 0.01$, compared recordings between SHR and WKY rat neurons under the same condition. $^{\&}P < 0.05$, compared recording between ANG II and ANG II + ANG-(1–7) in the same strain rat neuron. *D*: bar graphs summarizing the effect of LY (10 μM), a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, on the chronotropic action of ANG II in SHR and WKY neurons. Neurons were treated under the following treatment conditions: control, ANG II, washout of ANG II, and superfusion with LY, followed by superfusion with LY + ANG II. Data are means ± SE from 6–9 neurons. $^{*}P < 0.05$, compared with respective control recordings. $^{\#}P < 0.01$, compared recording between SHR and WKY rat neurons under the same conditions. $^{\&}P < 0.05$, compared recording between ANG II and ANG II + LY in the same strain rat neuron.

Mas receptor antagonist blocks the counterregulatory effect of ANG-(1–7) on ANG II action in SHR neurons. The G-protein-coupled receptor Mas is a functional receptor for ANG-(1–7). Previous studies have described that most of the peripheral actions of ANG-(1–7) are mediated through Mas (28) and Mas receptors are found to be highly expressed in the cardiovascular regulatory areas of the brain (3, 27). Therefore, we examined the effect of a Mas receptor antagonist, A-779, on the ability of ANG-(1–7) to oppose the excitatory action of ANG II in SHR and WKY rat neurons. The results are presented in Fig. 2 and demonstrate that superfusion of neurons with A-779 alone did not alter the basal firing rate in both SHR and WKY rat neurons. When coadministered with ANG-(1–7), A-779 (10 μM) blocked the inhibitory effect of ANG-(1–7) on the enhanced chronotropic action of ANG II in SHR neurons (Fig. 2, *B* and *C*). These results suggest that the counterregulatory effect of ANG-(1–7) on ANG II action in SHR neurons is mediated by Mas receptors.

PTEN inhibitor abolishes the counterregulatory effect of ANG-(1–7) on ANG II action in SHR neurons. Further experiments focused on possible mechanisms for the counterregulatory effect of ANG-(1–7) on the chronotropic action of ANG II in SHR neurons. We (34) demonstrated previously that the enhanced chronotropic effect of ANG II in SHR neurons is mediated by PI3-kinase, which is confirmed in the present study (Fig. 1*D*). It is well known that PTEN dephosphorylates PIP3, thus antagonizing PI3-kinase activity. In this regard, we examined the hypothesis that PTEN is involved in the counterregulatory effect of ANG-(1–7) on the chronotropic action of ANG II in SHR neurons. Treatment of SHR neurons with the PTEN inhibitor bisperoxovanadium (BPV; 10 μM, a concentration shown previously to be effective; Ref. 2) abolished the counterregulatory effect of ANG-(1–7) on the ANG II action (Fig. 3). However, BPV alone did not alter basal neuronal activity. These results support our hypothesis that ANG-(1–7) attenuates the enhanced chronotropic action of ANG II via stimulation of PTEN activity in SHR neurons.

ANG-(1–7) stimulates PTEN activity in SHR and WKY rat neurons. Next, we examined the direct effect of ANG-(1–7) on PTEN activity in SHR and WKY rat neurons. PTEN activity was examined using a PTEN detection kit, as described in the METHODS, in neuronal cultures treated with control (PBS),

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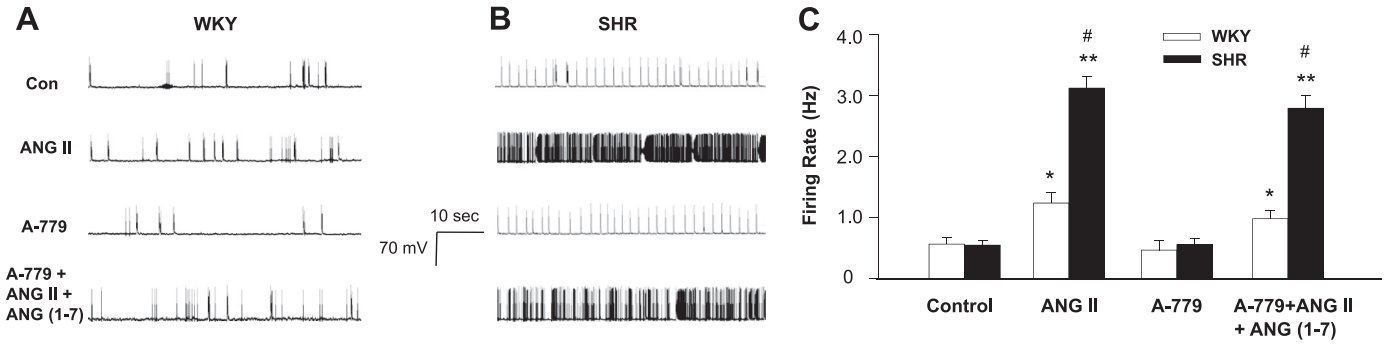

Fig. 2. Effect of blockade of Mas receptor on the counterregulatory action of ANG-(1–7) against ANG II. *A*: representative tracings showing the APs recorded from a single WKY rat neuron under the following sequential treatment conditions: basal firing rate (Con), superfusion with 100 nM ANG II, washout of ANG II, and superfusion with the Mas receptor antagonist A-779 (10 μM); this was followed by superfusion with A-779 + ANG II + ANG-(1–7) (100 nM). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of ANG-(1–7) on the chronotropic action of ANG II in SHR and WKY rat neurons in each treatment situation described in *A*. Data are means ± SE from 7–9 neurons in each group. $^{*}P < 0.05$, compared with respective control. $^{**}P < 0.01$, compared with respective control. $^{\#}P < 0.01$, compared between SHR and WKY rat neurons under the same condition.

ANG-(1–7) (100 nM), ANG-(1–7) plus A-779 (10 μM), or ANG-(1–7) plus BPV (10 μM) for 10 min. The results are presented in Fig. 4, indicating that ANG-(1–7) treatment significantly increased the PTEN activity in SHR (from 74.1 ± 6.8 to 144.4 ± 14.9; $n = 4$; $P < 0.01$) and in WKY rat neurons (from 71.5 ± 6.5 to 133.4 ± 13.1; $n = 4$; $P < 0.01$). This stimulatory effect of ANG-(1–7) on PTEN was completely abolished by the Mas receptor antagonist A-779, indicating this stimulatory effect of ANG-(1–7) on PTEN is mediated by a Mas-dependent mechanism. Treatment of neurons with ANG II (100 nM) alone did not alter PTEN activity in neurons from either SHR or WKY rats (Fig. 4).

DISCUSSION

The present study examined the interaction between ANG-(1–7) and ANG II as the major active peptides of the brain RAS in SHR and WKY rat neurons. The results demonstrated that ANG-(1–7) increases PTEN activity via stimulation of Mas receptors and abolishes the enhanced chronotropic effect of ANG II in the SHR neurons (Fig. 5), indicating that ANG-(1–7) counterregulates the chronotropic action of ANG II by a PTEN-dependent mechanism. This conclusion is supported by the following lines of evidence: *1*) superfusion of neurons with ANG-(1–7) significantly abolished the enhanced chronotropic action of ANG II in SHR neurons; *2*) the counterregulatory effect of ANG-(1–7) on the ANG II action was diminished by a Mas-receptor antagonist or a PTEN inhibitor; and *3*) treatment of neurons with ANG-(1–7) significantly increased PTEN activity and this ANG-(1–7)-induced stimulatory effect on PTEN was blocked by cotreatment with a Mas-receptor antagonist.

It is well known that the hypothalamus and RVLM contain cardiovascular regulatory neurons that control sympathetic nervous system activity (10). Significant increases in the discharge rate and differences in electrophysiological properties of hypothalamic neurons in SHR compared with WKY have been reported (33–35), indicating that this region may play a role in the genesis of the hypertension seen in the SHR. Another important cardiovascular regulatory brain area is the RVLM, which contains presynaptic neurons of sympathetic nerves. ANG II microinjected into the RVLM induces significant increases in BP and HR in both SHR and WKY rats, and this pressor response evoked by ANG II is also enhanced in the SHR (31). This phenomenon is explained by our in vitro study showing that ANG II evoked an enhanced chronotropic response in neurons cultured from SHR compared

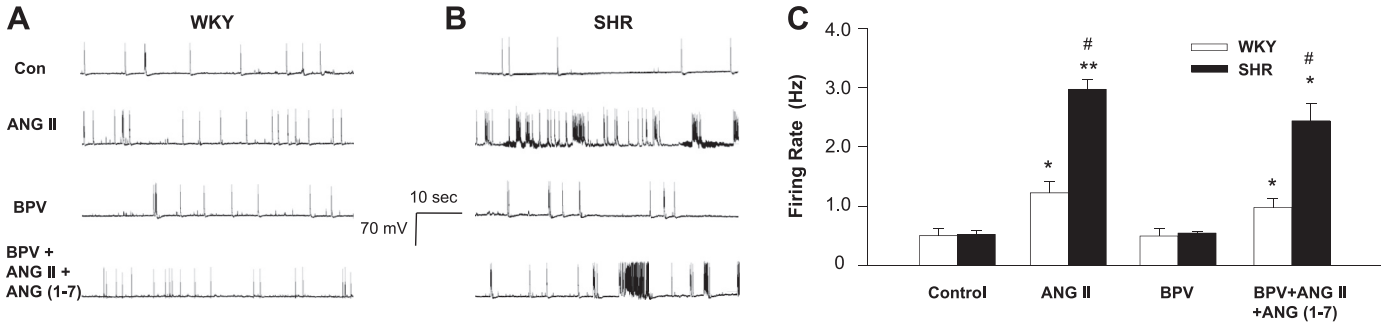

Fig. 3. Effect of phosphatase and tensin homologue deleted on chromosome ten (PTEN) inhibition on the counterregulatory action of ANG-(1–7) against ANG II. *A*: representative tracings showing the APs recorded from a single WKY rat neuron under the following sequential treatment conditions: basal firing rate (Con), superfusion with 100 nM ANG II, washout of ANG II, and superfusion with the PTEN inhibitor bisperoxovanadium (BPV; 10 μM); this was followed by superfusion with BPV + ANG II + ANG-(1–7) (100 nM). *B*: representative tracings showing the APs recorded from a single SHR neuron under the same sequential treatment conditions as in *A*. *C*: bar graphs summarizing the effect of BPV on the inhibitory action of ANG-(1–7) in SHR and WKY rat neurons in each treatment situation described in *A*. Data are means ± SE from 7–9 neurons. $^{*}P < 0.05$, compared with respective control. $^{**}P < 0.01$, compared with respective control. $^{\#}P < 0.01$, compared between SHR and WKY rat neurons under the same conditions.

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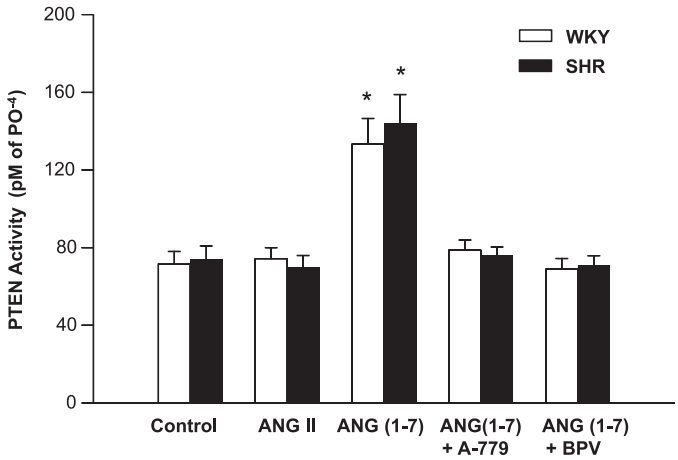

Fig. 4. Effect of ANG II, ANG-(1–7), A-779, and BPV on PTEN activity in SHR and WKY rat neurons. PTEN activity was measured using immunoprecipitation, followed by biochemical phosphatase assay, as described in the METHODS. Neurons were incubated with ANG II (100 nM) or ANG-(1–7) (100 nM) in the absence or presence of A-779 (10 μM) or BPV (10 μM) for 10 min at 37°C. Data are means ± SE derived from 4 experiments and ≥7 dishes in each experiment. $*P < 0.05$, compared with respective control.

with WKY rats. Blockade of PI3-kinase attenuates this action of ANG II exclusively in SHR neurons, suggesting that the increased PI3-kinase activity in the cardiovascular regulatory regions of SHR brain may be a unique signaling pathway that contributes to the hyperactivity of ANG II observed in these rats. This speculation is supported by a previous study (29) showing that wortmannin, a PI3-kinase inhibitor, microinjected into the RVLM lowers BP in SHR but not in WKY rats.

ACE2, an enzyme that cleaves ANG II to ANG-(1–7) (11, 25, 37), is present in brain cardiovascular regulatory regions. Overexpression of this enzyme in the RVLM did not alter the BP in WKY rats; however, it induced a long-term decrease in BP in SHR (37). This effect of ACE2 could be mediated by lowering ANG II levels or by increasing ANG-(1–7) levels in this brain area. Several studies (18, 25, 40) have focused on the in vivo central effect of ANG-(1–7) in the regulation of cardiovascular function. To clarify and further dissect the functional role of ANG-(1–7), we observed the direct effect of ANG-(1–7) on neuronal activity on neurons cultured from the hypothalamus of SHR and WKY rats. The present study demonstrates that ANG-(1–7) did not alter neuronal firing rate in either SHR or WKY rat neurons. However, coadministration of ANG-(1–7) with ANG II significantly attenuated the chronotropic response evoked by ANG II in SHR neurons, indicating that ANG-(1–7) may counterregulate ANG II actions in the central control of BP exclusively in SHR. The results are consistent with other in vivo observations indicating that the opposing effect of ANG-(1–7) on responses to ANG II is enhanced in animal models of hypertension both in the central nervous system and in the peripheral vasculature (18, 27). Höcht et al. (18) have reported that intrahypothalamic injection of ANG II induced a significantly greater pressor response in SHR compared with WKY rats and that the coadministration of ANG-(1–7) with ANG II reduced the pressor response to ANG II in SHR but not in WKY rats. Similarly, Benter et al. (4) have demonstrated that ANG-(1–7) attenuates ANG II-induced vasoconstriction in SHR but not in WKY rats. However, several other studies (14, 31) have demonstrated that microinjection of ANG-(1–7) increases BP and the pressor response to ANG-(1–7) is similar to that evoked by ANG II. This discrepancy may be due to differences in animal species, the doses of ANG-(1–7) used in the experiments, or other experimental conditions. For example, a low dose of ANG-(1–7) (50 ng) in the hypothalamus did not alter BP in either SHR or WKY rats. In contrast, a high dose of ANG-(1–7) (250 ng) significantly increases BP in SHR and this pressor effect of ANG-(1–7) is blocked by irbesartan, indicating that the pressor effect of ANG-(1–7) is mediated by an AT_1-receptor-dependent mechanism (18).

One question raised in this study centers on the cellular mechanism(s) underlying the ANG-(1–7)-induced counterregulatory effect of ANG II action in SHR. To unravel these intracellular signaling pathways, we hypothesized that ANG-(1–7) stimulates PTEN, which downregulates the enhanced PI3-kinase by dephosphorylating PIP3, the direct product of PI3-kinase. The present study provides direct evidence for this hypothesis. Incubation of neurons with ANG-(1–7) significantly increases PTEN activity via stimulation of Mas receptor in both WKY and SHR neurons. In addition, we also observed that the counterregulatory effect of ANG-(1–7) on the ANG II action is abolished by the PTEN inhibitor BPV in SHR neurons. Another concern in the current study is the role of PTEN in normotensive rat brain neurons because ANG-(1–7) stimulates this enzyme in both WKY and SHR. Current observations demonstrated that inhibition of PI3-kinase in WKY rat neurons has no effect on neuronal activity or on the chronotropic action of ANG II. These results suggest that neuronal activity in WKY rat neurons is not linked to the PI3-kinase signaling pathway. Thus the stimulatory action of ANG-(1–7) on PTEN activity in WKY rat neurons would not alter the chronotropic effect of ANG II. However, PTEN activation results in many cellular effects that are mediated by PDK1, Akt/PKB, and rac1/cdc42 (5). In neurons, these signaling molecules are involved in the regulation of gene expression and dendrite outgrowth (8, 22, 24). Thus ANG-(1–7)-induced PTEN acti-

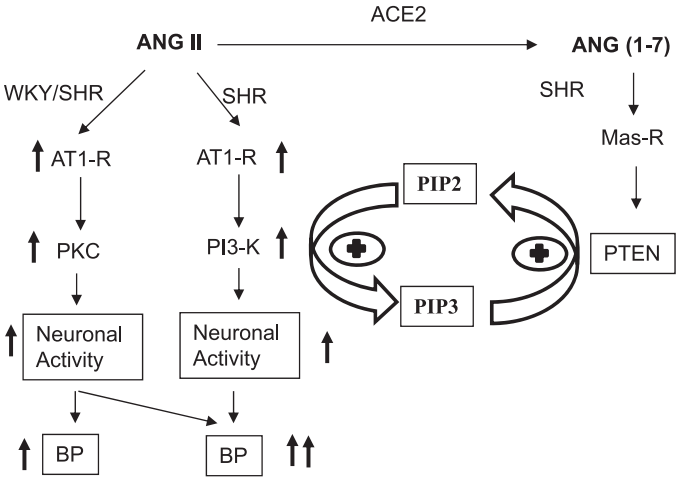

Fig. 5. Diagram summarizing proposed signaling pathways for the counterregulatory effect of ANG-(1–7) on ANG II action in SHR and WKY rat neurons. PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, soluble phosphatidylinositol-3,4,5-trisphosphate; Mas-R, Mas receptor. ACE2, angiotensin-converting enzyme 2; BP, blood pressure; AT1-R, ANG II type 1 receptor.

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vation in WKY rats may be linked to other cellular effects such as neurogenesis and apoptosis. Although there is no current evidence in the present study in favor or against this hypothesis, the possibility cannot be discounted. We believe, based on the evidence, that in SHR neurons the ANG II-induced PI3-kinase activity leads to phosphorylation of its downstream target, Akt/PKB (38). Previous studies (9) have concluded that PTEN is involved in dephosphorylation of p-Akt through inhibition of PIP3 production. Both Akt and PIP3 regulate the activity of ion channels or channel-associated proteins, which are involved in neuronal action potential generation and neuronal activity (21, 23). Further in vitro and in vivo studies will be necessary to validate the relevance of the proposed brain PTEN activity in BP control in SHR. Nonetheless, these observations suggest that stimulation of PTEN alters ANG II action on SHR brain neuronal activity and that the enzyme could be an important therapeutic target for the control of neurogenic hypertension.

The potential mechanisms by which ANG-(1–7) stimulates PTEN activity remain to be clarified. PTEN protein has multiple domains that may harbor the basic residues essential for its translocation to the phospholipid membrane and for its activation. The C terminal of PTEN protein contains a cluster of serine and threonine phosphorylation sites that may regulate its stability, activity, and recruitment to the membrane. Phosphorylation by protein kinases or dephosphorylation by phosphatases on these domains could regulate PTEN activity (36). More interestingly, the actions of ANG-(1–7) have been shown to be mediated by Src homology protein SHP-2 phosphatase, tyrosine phosphatase SHP-1, and mitogen-activated protein kinase phosphatase (15, 16, 26). Whether those phosphatases are involved in PTEN activation in neurons is still unknown. Another possible signaling mechanism underlying ANG-(1–7)-induced PTEN activation could be mediated by reactive oxygen species (ROS). The catalytic domain of PTEN can form a disulfide bond between Cys124 and Cys 71 in the enzyme active site. Free oxygen radicals could induce disulfide bonding within the active site of PTEN, rendering it inactive (20). It has been reported that reduced ROS and increased nitric oxide are involved in the action of ANG-(1–7) in different tissues (40). However, the role of ROS in the ANG-(1–7)-induced PTEN activation in neurons is not currently known and is the focus of ongoing investigation.

In addition, several limitations of the present study should be pointed out. In this study, neurons were cultured from the hypothalamus of SHR neonates. Neonatal SHRs are not yet hypertensive, and their BP is comparable to that of WKY rats at this stage of development. The SHR is a genetically hypertensive animal model, carrying hypertension-related genes that cause them to develop hypertension. Thus they are prehypertensive rats, showing some abnormalities that may or may not contribute to the development of hypertension later in life. Using these neuronal cultures from prehypertensive SHRs, we observed that ANG-(1–7) has a counterregulatory effect on ANG II action via stimulation of PTEN activity. However, future studies will be necessary to confirm whether or not this phenomenon is related to the development of hypertension in the adult SHRs. Another limitation of the current study is that the neurons used were cultured from the hypothalamus, which contains a heterogeneous population of neurons. As such, we cannot be certain which of the neurons used in the current study connect to sympathetic nerves related to the regulation of cardiovascular function. Nevertheless, a key advantage of this cellular model is that it allows us to study the effects of ANG-(1–7) on individual neurons in a manner that minimizes potential confounding factors related to inputs from other neuronal networks. Improved retrograde labeling techniques, which could be used to identify the presympathetic neurons but are technically difficult to perform in neonatal rats at present, may be useful in overcoming this limitation in future studies.

In conclusion, we demonstrated direct cross-talk between signaling pathways underlying the actions of ANG-(1–7) and ANG II in neurons cultured from the hypothalamus of SHR and WKY rats, indicating the central role of these peptides. ANG II stimulated neuronal activity, which is enhanced in SHR neurons, and coadministration of ANG-(1–7) attenuates the ANG II-induced-chronotropic effect exclusively in SHR neurons. The in vitro observations suggest that ANG-(1–7) stimulates PTEN activity via a Mas receptor, leading to a counterregulatory effect on ANG II action in SHR neurons.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.M., Q.Z., A.P., N.S., F.Y., J.G., L.G., and C.X. performed experiments; A.M. analyzed data; A.M. prepared figures; S.T.O. and C.S. conception and design of research; S.T.O. and C.S. edited and revised manuscript; C.S. interpreted results of experiments; C.S. drafted manuscript; C.S. approved final version of manuscript.

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