MAP Kinase–Independent Signaling in Angiotensin II Regulation of Neuromodulation in SHR Neurons

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Abstract—Angiotensin II (Ang II), via its interaction with the angiotensin type 1 (AT\(_1\)) receptor subtype, causes enhanced stimulation of norepinephrine (NE) neuromodulation. This involves increased transcription of NE transporter, tyrosine hydroxylase, and dopamine \(\beta\)-hydroxylase genes in Wistar-Kyoto rat (WKY) brain neurons. AT\(_1\) receptor–mediated regulation of certain signaling events (such as activation of the Ras-Raf-1–mitogen activated protein (MAP) kinase signaling pathway, nuclear translocation of transcription factors such as Fos and Jun, and the interactions of these factors with AP-1 binding sites) is involved in this NE neuromodulation (Lu et al. J Cell Biol. 1996;135:1609–1617). The aim of this study was to compare the signal transduction mechanism of Ang II regulation of NE neuromodulation in WKY and spontaneously hypertensive rat (SHR) brain neurons, in view of the fact that AT\(_1\) receptor expression and Ang II stimulation of NE neuromodulation are higher in SHR neurons compared with WKY neurons. Despite this hyperactivity, Ang II stimulation of Ras, Raf-1, and MAP kinase activities was comparable between the neurons from WKY and SHR. Similarly, central injections of Ang II caused a comparable stimulation of MAP kinase in the hypothalamic and brain stem areas of adult WKY and SHR. Inhibition of MAP kinase by either an MAP kinase kinase inhibitor (PD98059) or an MAP kinase antisense oligonucleotide completely attenuated the stimulatory effects of Ang II on \[^{3}H\]-NE uptake, NE transporter mRNA, and tyrosine hydroxylase mRNA levels in WKY neurons. These treatments resulted in only 43% to 50% inhibition of \[^{3}H\]-NE uptake and NE transporter and tyrosine hydroxylase mRNAs in SHR neurons. Thus, Ang II stimulation of NE neuromodulation was completely blocked by MAP kinase inhibition in WKY neurons and only partially blocked in the SHR neurons. These observations suggest the presence of an additional signal transduction pathway involved in NE neuromodulation in SHR neurons that is independent of the MAP kinase pathway. (Hypertension. 1998;32:473-481.)

Key Words: angiotensin ■ intracellular signaling ■ MAP kinase ■ neurons ■ norepinephrine ■ rats, inbred SHR

Brain angiotensin II (Ang II) plays a key role in the central control of blood pressure (BP). This action is initiated by the interaction of Ang II with the Ang II subtype 1 (AT\(_1\)) receptor that is localized in the cardioregulatory-relevant areas of the brain.\(^1\)–\(^6\) The physiological mechanisms of the regulation of Ang II of BP control implicate the stimulation of sympathetic pathways involving catecholamines, the dampening of baroreflexes, and the release of vasopressin.\(^1\)–\(^9\) The significance of the brain angiotensin system and the AT\(_1\) receptor in the control of BP is further underscored by studies with the spontaneously hypertensive rat (SHR, a genetic model of human essential hypertension) and with the reintransgenic rat model of hypertension.\(^10,11\) For example, studies with SHR demonstrate that this model expresses a hyperactive brain angiotensin system as a result of an increased AT\(_1\) receptor gene expression.\(^6,12,13\) In addition, Ang II–mediated regulation of catecholamine turnover has also been heightened in the SHR.\(^5,9,14,16\) Similar hyperactivity of the brain angiotensin system has recently been reported for the reintransgenic rat.\(^17\) Finally, interruption in the hyperactivity of this system by either pharmacological or genetic means in the SHR normalizes BP, further confirming the involvement of brain angiotensin in the development and establishment of high BP and hypertension.\(^6,18–20\)

Despite excellent physiological studies defining the involvement of catecholamines and vasopressin in Ang II–mediated control of BP, limited information is available about the cellular and molecular mechanisms of these physiological actions. Our group has been exceedingly interested in this aspect and had established neuronal cells in primary culture from the hypothalamus/brain stem areas of 1-day-old rats as an in vitro model to study the cellular mechanism of Ang II actions and the molecular basis of a hyperactive brain angiotensin system in SHR.\(^4\) These studies have revealed that a hyperactive brain angiotensin system expressed in vivo could be maintained in neuronal cell cultures.\(^4\) We have established, with the use of these neuronal cultures, that the interaction of Ang II with the AT\(_1\) receptor initiates a cascade of signaling events leading to a transcriptional increase in the norepinephrine (NE) transporter activity (NET system) and tyrosine hydroxylase (TH).\(^5,21,22\) These neuromodulatory actions have been likened to AT\(_1\) receptor–mediated increases in NE uptake, synthesis, and release.\(^6,8,9\) Studies have also established that the Ras-Raf-1–mitogen-activated protein...
(MAP) kinase signal transduction pathway is involved in the NE neuromodulatory actions of Ang II.\textsuperscript{23,24} The signaling pathway activates transcription factors relevant to AP-1 binding sites (Fos, Jun, serum response factor), which are proposed to interact with the promoter regions of the NET, TH, and dopamine \( \beta \)-hydroxylase (D\( \beta \)H) genes and to stimulate their transcription.\textsuperscript{24–26} In view of this recently uncovered signaling mechanism and our previous observations that AT\( _{1} \) receptor gene expression in SHR neurons is higher compared with that in Wistar-Kyoto rat (WKY) neurons,\textsuperscript{13} we decided to compare the AT\( _{1} \) receptor-mediated signal transduction mechanism between the 2 strains of neurons. We hypothesized that the Ras-Raf-1–MAP kinase pathway would be stimulated by AT\( _{1} \) receptor activation in parallel with its increased expression of these receptors in the SHR neurons. This, we argued, would be consistent with the increased NE neuromodulatory action of Ang II in these neurons compared with in WKY neurons. Our data, however, do not support this hypothesis. However, they show that the Ras-Raf-1–MAP kinase signaling pathway is responsible only for partial activation of NET and TH activities in the SHR neurons. They suggest the presence of an MAP kinase–independent mechanism of AT\( _{1} \) receptor regulation of NE neuromodulation in the neurons from the SHR.

Methods

Materials

One-day-old WKY and SHR were obtained from our breeding colony, which originated from Harlan Sprague-Dawley (Indianapolis, Ind). Blood pressure for breeder WKY was an average of 117 \( \pm \) 5 mm Hg, and it was 205 \( \pm \) 8 mm Hg for SHR. Dulbecco’s modified Eagle’s medium (DMEM), plasma-derived horse serum (PDHS), and 1 \( \times \) crystalized trypsin were from Central Biomedia. Phosphate-free DMEM was purchased from Life Technologies. [\( ^{3} \)P]Orthophosphate (1 mCi=37 MBq), [\( ^{32} \)P]ATP (3000 Ci/mmol), [\( ^{3} \)P]dCTP (3000 Ci/mmol), and chemiluminescence assay reagents were from DuPont/NEN. Nitrocellulose membranes were from Micron Separations Inc. Ang II and polyethyleneimine-cellulose thin-layer plates were purchased from Sigma Chemical Co. Losartan potassium was a gift from Dupont/Merck. PD123319 was from RBI, PD98059 was from Calbiochem, and the polymerase chain reaction (PCR) kit containing Taq DNA polymerase was purchased from Perkin Elmer Cetus. Superscript RNA H\textsubscript{\textregistered} reverse transcriptase (RT), and deoxynucleotide mixture were from Life Technologies. Oligo(dTs) was from Promega, and Dynal beads and other reagents for poly(A\textsuperscript{+}) RNA isolation were from Dynal Inc. Anti-Ras, anti-Raf-1, and anti-MAP kinase [anti-ERK2 (C-14)] polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. All other reagents were purchased from Fisher Scientific and were the highest quality available.

Primers for NET, TH, \( \beta \)-actin, and MAP kinase sense and antisense oligonucleotides (SON and AON) were synthesized in the DNA synthesis facility of the Interdisciplinary Center for Biotechnology Research, University of Florida (Gainesville). The sequences of these primers and oligonucleotides have been published previously.\textsuperscript{21–25}

Protocol

Hypothalamus–Brain Stem Neuronal Cells in Primary Culture

Hypothalamus–brain stem areas of 1-day-old WKY and SHR brains were dissected, and brain cells were dissociated by trypsin.\textsuperscript{12,13} They were plated onto poly-l-lysine–precoated 35-mm tissue culture dishes (3 \( \times \)10\textsuperscript{5} cells per dish) or 100-mm dishes (2 \( \times \)10\textsuperscript{6} cells per dish) in DMEM containing 10% PDHS; neuronal cultures were established as described previously.\textsuperscript{12,13} They were allowed to grow for 15 days before experiments. These cultures contain 85% to 90% neuronal cells and 10% to 15% astroglial cells. They have been used extensively by us as

Figure 1. Effects of Ang II on NET and TH systems in WKY and SHR neurons. Neuronal cultures of WKY and SHR brains were preincubated with 100 nmol/L Ang II for 4 hours at 37°C. Levels of specific [\( ^{3} \)H]–NE uptake and NET mRNA (A) as well as TH activity and TH mRNA (B) were measured as described in Methods. Data are mean \( \pm \) SE (3 experiments). *Significantly different from control (\( P<0.01 \)); \( \bullet \) Ang II stimulation in SHR neurons was significantly different from that in WKY neurons (\( P<0.01 \), Ang II–treated WKY vs SHR).

in vitro model to study hyperactivity of the brain AT\( _{1} \) receptor functions and its interaction with catecholamines in the SHR brain.\textsuperscript{21,22}

Measurement of Ras, Raf-1, and MAP Kinase Activities

Analysis of Ras Activation

The ratio of GTP-Ras over GDP-Ras was used to determine the levels of activated Ras by Ang II in neuronal cultures as described previously.\textsuperscript{21,22} Briefly, neuronal cultures of WKY and SHR brains were established in 100-mm culture dishes and prelabeled with [\( ^{3} \)P]orthophosphate (2 mCi per dish) in phosphate-free DMEM for 1 hour at 37°C in parallel. After treatment with Ang II, cultures were rinsed with ice-cold PBS (pH 7.4, suspended in the immunoprecipitation buffer (50 mmol/L Tris-HCl [pH 7.4], 20 mmol/L MgCl\textsubscript{2}, 150 mmol/L NaCl, 0.5% Nonidet P-40, and 10 \( \mu \)g/ml aprotinin) for 10 minutes and centrifuged at 3000 g for 5 minutes. The supernatant was used to immunoprecipitate Ras with the use of anti-Ras polyclonal antibody agarose conjugated as described previously.\textsuperscript{21,22} The immunoprecipitate was washed twice with the immunoprecipitation buffer and twice with the washing buffer (50 mmol/L Tris-HCl [pH 7.4], 20 mmol/L MgCl\textsubscript{2}, and 150 mmol/L NaCl) and was suspended in 20 \( \mu \)L of 20 mmol/L Tris-HCl (pH 7.4), 30 mmol/L EDTA, 2% SDS, 0.5 mmol/L GDP, and 0.5 mmol/L GTP. The suspension was heated at 65°C for 5 minutes and centrifuged. The supernatant was spotted onto a polyethyleneimine-cellulose thin-layer plate and developed with 0.75 mol/L KH\textsubscript{2}PO\textsubscript{4} (pH 3.4) followed by autoradiography. The radioactivity was quantitated with a UVP Image 5000 system (Ultra Violet Products Ltd). The percentage of Ras-GTP complex relative to the total amount of Ras was calculated, and the data were presented as percentage of activated Ras.\textsuperscript{21,22}

Measurement of Raf-1 Kinase Activation

Raf-1 kinase activation by Ang II was analyzed as previously described.\textsuperscript{24,25} Briefly, neuronal cells after Ang II treatment were

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lysed for 10 minutes in the lysis buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 10% glycerol, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mmol/L EDTA, 1 mmol/L PMSF, 10 μmol/L aprotinin, and 100 μmol/L sodium orthovanadate). The lysates were centrifuged at 10,000 g for 5 minutes, and supernatants were used for immunoprecipitation with anti-Raf-1 antibody at 4°C for 1 hour.24,29,30 Immune precipitates were resuspended in 20 μL of reaction buffer (20 mmol/L HEPES [pH 7.4], 1 mmol/L NaCl, and 5 mmol/L MgCl₂) containing 10 μCi [γ-32P]ATP (3000 Ci/mol). After 10 minutes at 24°C, reactions were stopped by the addition of Laemmli’s sample buffer, and proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. Quantitation of phosphorylated Raf-1 was performed by determination of observed density of radioactive bands of ~74 kDa on a UVP Image 5000 system.

Measurement of MAP Kinase Activity

Neuronal cell lysates were prepared in the lysis buffer (25 mmol/L Tris-HCl [pH 7.4], 25 mmol/L NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 0.5 mmol/L EGTA, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 0.8 μg/mL leupeptin), and lysates were immunoprecipitated with anti-ERK2 antibody agarose conjugated overnight at 4°C.23,31 Immune precipitates were electrophoresed on a 10% SDS-PAGE gel containing 0.5 mg/mL myelin basic protein. After electrophoresis, SDS was removed by washing the gel with 2 changes of 20% 2-propanol in 50 mmol/L Tris-HCl (pH 8.0) for 1 hour and then with 2 changes of 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L 2-mercaptoethanol for 1 hour. The enzyme was denatured by incubating the gel with 2 changes of 6 mol/L guanidine hydrochloride for 1 hour and then renatured with 3 changes of 50 mmol/L Tris HCl (pH 8.0) containing 0.04% Tween 40 and 5 mmol/L 2-mercaptoethanol overnight at 4°C. The gel was then incubated with 40 μL HEPES (pH 8.0) containing 2 mmol/L DTT, 10 mmol/L MgCl₂, 0.5 mmol/L EGTA, 40 μmol/L ATP, and 10 μCi [γ-32P]ATP (3000 Ci/mol), washed with a 5% trichloracetic acid solution containing 1% sodium pyrophosphate, dried, and subjected to autoradiography.23,31

Figure 2. Ang II stimulation of Ras activity in WKY and SHR neurons. Neuronal cultures were incubated with 100 nmol/L Ang II for indicated time periods (A) or with indicated angiotensin receptor subtype-specific antagonists (B). Levels of GTP-Ras/GDP-Ras plus GDP-Ras were determined as described in Methods. Los indicates losartan 10 μmol/L; PD, PD123319 10 μmol/L. Representative autoradiograms are shown at top. Data are mean±SE (3 experiments). *Significantly different from control (P<0.05).

Measurement of MAP Kinase Immunoreactivity by Immunoblotting

Immunoblotting was used to identify the presence of p42 and p44 proteins (subtypes of MAP kinase) in neuronal cultures. Briefly, cell-free lysates were prepared as described above for MAP kinase activity. Neuronal cell lysates were prepared in the lysis buffer (25 mmol/L Tris-HCl [pH 7.4], 25 mmol/L NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 0.5 mmol/L EGTA, 1 mmol/L PMSF, 10 μg/mL aprotinin, and 0.8 μg/mL leupeptin), and lysates were immunoprecipitated with anti-ERK2 antibody agarose conjugated overnight at 4°C.23,31 Immune precipitates were electrophoresed on a 10% SDS-PAGE gel containing 0.5 mg/mL myelin basic protein. After electrophoresis, SDS was removed by washing the gel with 2 changes of 20% 2-propanol in 50 mmol/L Tris-HCl (pH 8.0) for 1 hour and then with 2 changes of 50 mmol/L Tris-HCl (pH 8.0) containing 5 mmol/L 2-mercaptoethanol for 1 hour. The enzyme was denatured by incubating the gel with 2 changes of 6 mol/L guanidine hydrochloride for 1 hour and then renatured with 3 changes of 50 mmol/L Tris HCl (pH 8.0) containing 0.04% Tween 40 and 5 mmol/L 2-mercaptoethanol overnight at 4°C. The gel was then incubated with 40 μL HEPES (pH 8.0) containing 2 mmol/L DTT, 10 mmol/L MgCl₂, 0.5 mmol/L EGTA, 40 μmol/L ATP, and 10 μCi [γ-32P]ATP (3000 Ci/mol), washed with a 5% trichloracetic acid solution containing 1% sodium pyrophosphate, dried, and subjected to autoradiography.23,31

Figure 3. Activation of Raf-1 by Ang II in WKY and SHR neurons. Neuronal cultures were incubated with 100 nmol/L Ang II for indicated time periods. Cells were lysed, Raf-1 immunoreactivity was immunoprecipitated, and autophosphorylation of Raf-1 was carried out as described in Methods. Top, Representative autoradiogram. Bottom, Data from 3 experiments (mean±SE). *Significantly different from zero time (P<0.05).
activation. Lysates were electrophoresed on 10% SDS-PAGE gels, and proteins were transferred to nitrocellulose membranes. Membranes were blocked by using 5% nonfat dry milk in TBST (20 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, and 0.05% Tween 20) for 1 hour followed by incubation for 1 hour at room temperature with rabbit anti-erk-I-III antibody. Protein-bound antibody was detected by incubation of the membrane with horseradish peroxidase–labeled second antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence assay reagents. The bands recognized by the primary antibody were visualized by autoradiography.

**Measurement of MAP Kinase Immunoreactivity In Vivo by Immunoblotting**

Adult male WKY and SHR (100±6 [n=6] mm Hg and 190±10 [n=6] mm Hg, respectively) were housed singularly in stainless steel cages with Purina rat chow pellets and tap water ad libitum. Rats were fitted with an indwelling cannula (10 mm long, 23-gauge stainless steel) stereotaxically aimed to end in or just above the lumen of the right lateral ventricle and firmly fixed to the skull with jeweler’s screws and dental acrylic as described previously. Surgery was performed with rats under ketamine and xylazine (50 and 5 mg/kg IP) anesthesia; rats were allowed to recover for 1 week before use in the experiments. Injections were made through an 11-mm, 30-gauge injector needle attached to a 35-mm syringe. Five microliters of either PBS or PBS containing 10 ng Ang II was injected into each rat. After 0, 5, and 15 minutes, rats were killed. Hypothalami and brain stems were dissected and homogenized in the lysis buffer; homogenates were subjected to immunoblotting for the measurement of MAP kinase immunoreactivity as described above.

**Measurements of NET, TH, and β-Actin mRNA Levels**

The mRNA levels for NET, TH, and β-actin were measured by RT-PCR as described previously. Bands corresponding to [32P]-labeled PCR products on the x-ray film were scanned with the use of the UVP Imagestore 5000 system, and the density of each PCR product was quantified by the SW 5000 gel analysis program.
Measurement of \[^{3}H\]-NE Uptake and TH Activity

Neuronal cultures were grown in 100-mm-diameter tissue culture dishes and treated with Ang II for indicated time periods. Specific \[^{3}H\]-NE uptake and TH activity were measured as described previously. 21-22

Treatment of Neuronal Cultures With Various Inhibitors

MAP kinase depletion was accomplished by treatment of neuronal cultures with specific MAP kinase AON as described previously. 23 Cultures grown in 35-mm dishes were pretreated with 1 \(\mu\)mol/L AON or SON for 24 hours at 37°C. This was followed by incubation with Ang II for 4 hours and analysis of NET and TH activities and mRNA levels. Cultures were also pretreated with 50 \(\mu\)mol/L PD98059 for 30 minutes before experiments. This condition has been shown previously to cause a 90% inhibition of MAP kinase activity. 21 All inhibitors were prepared in PBS (pH 7.4).

Experimental Groups and Data Analysis

Each experiment for the effect of Ang II on the activation of Ras, Raf-1, and MAP kinase was conducted in triplicate culture dishes, and cells in each dish were derived from multiple brains of 1-day-old rats. WKY and SHR brain neurons were used in parallel for each experiment, and both samples were run on the same gel to minimize experimental variation. Each experiment was repeated at least 3 times unless indicated otherwise. \[^{3}H\]-NE uptake and TH activities were determined with the use of the same number of cultures in triplicate dishes. For the analysis of mRNA levels, triplicate culture dishes were used for each data point, and poly(A\(^+\)) RNA was pooled. Each experiment was repeated 3 times unless indicated otherwise.

Densities of PCR bands were quantified, and data were presented as relative absorbance of the mean±SE, derived from normalization with \(\beta\)-actin for equal loading. 21-23 Comparisons between the control and experimental groups were made using 1-way ANOVA and Dunnett’s tests with the use of statistical software.

Results

Comparison of Ang II Stimulation of Ras-Raf-I–MAP Kinase Activation in WKY and SHR Neurons

Our first objective was to compare the effects of Ang II on NET and TH activities between WKY and SHR, since neuronal cultures from the SHR brain express 2- to 4-fold higher levels of AT\(_1\) receptors. 6,13 Incubation with 100 nmol/L Ang II caused a 4- to 5-fold increase in \[^{3}H\]-NE uptake and a 3- to 5-fold increase in NET mRNA levels in WKY neurons (Figure 1A). Similarly, Ang II stimulated both TH activity (2.5-fold) and TH mRNA levels (5-fold) in WKY neurons (Figure 1B). Although Ang II caused a significant stimulation of \[^{3}H\]-NE uptake, TH activity, NET mRNA, and TH mRNA levels in both strains, the levels of stimulation of these activities were \(\approx\)2-fold higher in the SHR neurons compared with the WKY neurons (Figure 1). This heightened NE neuromodulatory action of Ang II in the SHR neurons seemed to be specific, since Ang II stimulation of \[^{3}H\]-NE uptake and NET and TH mRNA levels was comparable between the neuronal cultures of WKY neurons and those from the Sprague-Dawley rat neurons (data not shown).

The AT\(_1\) receptor stimulation initiates a cascade of signaling events involving activation of the Ras-Raf-1–MAP kinase pathway in WKY neurons. Stimulation of MAP kinase is intimately linked with the NE neuromodulatory effect of Ang II in normotensive rat brain neurons. 6,23 Thus, our next objective was to compare the AT\(_1\) receptor–mediated activation of this signaling pathway between WKY and SHR neurons. We hypothesized that because AT\(_1\) receptors and AT\(_1\) receptor–mediated NE neuromodulation are increased in the SHR neurons, a parallel increase in the signaling pathway would be observed in the SHR neurons.

Ang II caused activation of Ras, as judged by an increase in the ratio of GTP-Ras to GDP-Ras in both WKY and SHR neurons (Figure 2A). The activation was optimal (3-fold) within 5 minutes, followed by a gradual decrease reaching basal levels within 30 to 60 minutes in neurons of both strains. Interestingly, both the level of stimulation and the time course were comparable between the 2 strains of neurons despite a higher level of AT\(_1\) receptors and AT\(_1\) receptor stimulation of the NET and TH systems in the SHR. Stimulation of Ras activity was completely attenuated by 10 \(\mu\)mol/L losartan in both WKY and SHR neurons (Figure 2B). PD123319 at 10 \(\mu\)mol/L showed little but significant inhibition of Ras activity. These findings indicate that Ang II stimulation of Ras activity is primarily mediated via activation of AT\(_1\) receptor subtype. However, a role of AT\(_2\) receptors in this activation cannot be ruled out, since PD123319 shows some degree of inhibition.

Ang II also caused a time-dependent increase in the activity of Raf-1 in a transient fashion (Figure 3). A maximal stimulation of \(\approx\)3-fold was observed with 100 nmol/L Ang II.
in 5 minutes in both WKY and SHR neurons. Activation of Raf-1 by Ang II was also comparable between WKY and SHR neurons. The effect of Ang II on the activation of MAP kinase was compared between WKY and SHR neurons. Ang II caused a time-dependent, transient increase in MAP kinase activity, and a 4-fold stimulation was comparable in WKY and SHR neurons (Figure 4A). Activation of MAP kinase was also detected using a gel mobility shift assay. Figure 4B shows that Ang II stimulated the phosphorylation of the predominantly p42 subtype of MAP kinase, and this stimulation was nearly identical between WKY and SHR neurons.

Adult WKY and SHR were cannulated and injected with Ang II to determine whether MAP kinase stimulation occurs in hypothalamic and brain stem areas under in vivo conditions. The level of phosphorylated MAP kinase was used as a measure of its activity. The phosphorylated p42 subtype of MAP kinase was predominant in both hypothalamus and brain stem (data not shown), whose levels were comparable. In addition, the basal levels of phosphorylated p42 subtype were also comparable between WKY and SHR (Figure 5). Ang II caused a time-dependent stimulation of phosphorylated p42 subtype in both brain areas of WKY and SHR. This stimulation was 3-fold in 15 minutes. No difference in the degree of stimulation between either the hypothalamus and brain stem (data not shown) or WKY and SHR was observed (Figure 5). This was consistent with the neuronal cell culture data (Figure 4).

### Role of MAP Kinase in Ang II–Induced NE Neuromodulation in WKY and SHR Neurons

Our next objective was to study the role of MAP kinase activation on AT1 receptor–mediated, heightened NE neuromodulatory actions of Ang II in the SHR neurons with the use of MAP kinase kinase inhibitor, PD98059, on Ang II stimulation of NET mRNA (A), [3H]-NE uptake (B), and TH mRNA (C) in WKY and SHR neurons. Neuronal cultures, established in 35-mm culture dishes, were incubated in the absence or presence of 100 nmol/L Ang II without or with 50 μmol/L PD98059 for 4 hours at 37°C. Total poly(A+) RNA was isolated and subjected to RT-PCR for the measurements of NET (A) and TH (C) mRNA levels. Whole cells attached to culture dishes were used to measure [3H]-NE uptake (B) as described in Methods. Top, A and C, Representative autoradiograms. Bottom, A and C, Data are mean±SE (3 experiments). *Significantly different from respective control (P<0.05). **Significantly different from SHR control (lane 5) and Ang II treatment (lane 6). Data in panel B are mean±SE (3 experiments). P<0.05, lane 2 vs lane 6.
of PD98059, a selective MAP kinase kinase inhibitor in neurons and in other systems.\textsuperscript{31,32} Preincubation of 50 \(\mu\)mol/L PD98095 caused a complete inhibition of Ang II–stimulated phosphorylation of p42 subtype of MAP kinase in the neurons of both WKY and SHR (Figure 6). An identical treatment of WKY neurons with PD98095 exhibited a complete attenuation of stimulatory effects of Ang II on NET mRNA (Figure 7A) and \([3\text{H}]\)-NE uptake (Figure 7B). PD98059 treatment also attenuated Ang II stimulation of NET mRNA and \([3\text{H}]\)-NE uptake in the SHR neurons (Figure 7A and 7B). However, the inhibition was only \(\approx 43\%\) and interestingly brought the levels of NET mRNA and \([3\text{H}]\)-NE uptake activity to Ang II–stimulated levels in WKY neurons. A similar observation was found when the effect of PD98059 on Ang II stimulation of TH mRNA was compared between WKY and SHR neurons (Figure 7C). PD98059 completely attenuated Ang II stimulation of TH mRNA in WKY neurons, whereas the attenuation was only 45% in the SHR neurons. This differential effect of MAP kinase on Ang II stimulation of NET and TH in the SHR neurons could not be due to a differential inhibition of MAP kinase, since PD98095 caused a comparable inhibition of MAP kinase in both WKY and SHR neurons (Figure 6).

AON to MAP kinase was used to further confirm the specificity of these differences between WKY and SHR neurons. MAP kinase AON has been used previously by us to selectively deplete neurons of MAP kinase, which causes attenuation of Ang II stimulation of \(c\)-\(fos\), NET, and TH mRNAs in WKY neurons.\textsuperscript{22,23} It also inhibits basal levels of MAP kinase comparably in WKY and SHR neurons (Figure 6). Pretreatment of WKY neurons with MAP kinase AON caused a 95% decrease in the ability of Ang II to stimulate NET and TH mRNAs (Figure 8). Although this treatment also caused a significant attenuation of Ang II stimulation of NET and TH mRNA levels in SHR neurons, the inhibition was only 50% (Figure 8). These observations indicate that the significant level of Ang II–induced NE neuromodulation in SHR neurons is MAP kinase–independent.

The effect of inhibitors of other protein kinases and intracellular signaling pathways on Ang II stimulation of NE neuromodulation was studied to determine the identity of a possible alternate pathway that could account for this MAP kinase–independent NE neuromodulatory action of Ang II in the SHR neurons. It has been suggested that the calcineurin-mediated activation of NFAT transcription factor may be involved in the activation of AP-1 binding activity.\textsuperscript{33,34} Because AP-1 activation on the promoter of NET, TH, and \(D\beta H\) is proposed to be the mechanism of Ang II regulation of these genes,\textsuperscript{6,25,26} we studied the effect of cyclosporin A, an inhibitor of calcineurin activation. Figure 9 shows that cyclosporin A, under conditions where it completely inhibits the calcineurin system, failed to influence Ang II stimulation of \([3\text{H}]\)-NE uptake in either WKY and SHR neurons. Similarly, treatment of WKY neurons with H89 (protein kinase A inhibitor), lavendustin (protein tyrosine kinase inhibitor), KN93 (CAM kinase II inhibitor), cyclosporin A, and trifluoperazine (calmodulin inhibitor) had little effect on Ang II stimulation of NET mRNA (Table). However, protein kinase C inhibitor (bisindolylmaleimide) caused a 70% inhibition of Ang II stimulation of NET mRNA (Table). The effects of these inhibitors on Ang II stimulation of NET mRNA in the SHR neurons were similar to those observed in the WKY neurons. These observations indicate that calcineurin, protein kinase A, and protein tyrosine kinase do not play a major role.
The involvement of protein kinase C in Ang II stimulation of NE neuromodulation is interesting and may be associated with the activation of Raf-1.

Discussion

The most significant finding of this study is the observation that inhibition of MAP kinase does not result in complete attenuation of AT₁ receptor–mediated NE neuromodulatory actions of Ang II in the SHR neuron. This suggests that an additional intracellular signaling pathway must exist to account for a heightened Ang II stimulation of the NE system in this strain of neurons. The identity of such a pathway remains speculative and is currently under investigation.

A hypertensive brain angiotensin system has been demonstrated in SHR.\(^4,6\) Associated with this hyperactivity is an increase in the levels of AT₁ receptor subtypes and AT₁ mRNA in the cardioregulatory-relevant brain areas of the SHR, both in vitro and in vivo.\(^4,13,35\) In vitro studies have also established that the AT₁ receptor–mediated stimulation of NE turnover, synthesis, and uptake is increased in proportion to the increase in the AT₁ receptors in SHR neurons.\(^4,6\) Despite both increased AT₁ receptors and a heightened Ang II–mediated NE neuromodulation in SHR neurons, the Ras-Raf–MAP kinase signal transduction mechanism appears to be comparable in the SHR and WKY neurons.

MAP kinase is a key enzyme in Ang II action because its inhibition or depletion completely attenuates AT₁ receptor–mediated NE neuromodulation in the WKY neuron. However, this kinase does not appear to be completely responsible for Ang II stimulation of NE and TH activities in SHR neurons. The following evidence supports this: (1) MAP kinase AON causes attenuation of NET and TH mRNA by only \(\approx 50\%\) in SHR compared with their complete inhibition in WKY neuron; (2) an MAP kinase inhibitor, PD98059, exerts an identical differential effect in SHR neurons as observed with MAP kinase AON treatment; and (3) the levels of AT₁ receptor–mediated activation of Ras, Raf-1, and MAP kinase are comparable between the 2 strains of neurons. Thus, despite a 2- to 3-fold increase in the numbers of AT₁ receptors and a parallel stimulation of NE neuromodulation, the Ras-Raf–MAP kinase signaling pathway remains comparable between the neurons of WKY and SHR. Interestingly, these observations are in contrast to the observations of Ang II stimulation of MAP kinase in vascular smooth muscle cells (VSMCs) of SHR.\(^6\) The involvement of MAP kinase in the regulation by Ang II of hypertrophic and hyperplastic effects in VSMCs of WKY and SHR has been proposed.\(^6\) Lucchesi et al\(^6\) have shown that although Ang II–mediated activation of MAP kinase was comparable between strains, its inactivation was more rapid in SHR VSMCs than in WKY VSMCs. In addition, SHR MAP kinase showed a greater dependence on Ca\(^{2+}\) mobilization. In contrast, in the SHR neurons there was neither increased stimulation by Ang II nor a delay in the inactivation of this enzyme (Figure 4A and 4B). The reason for these differences between the 2 cell types is speculative at the present time but may be related to 2 distinct proposed roles of MAP kinase, trophic in VSMCs and neuromodulatory in the neurons.

An important question that arises from our study concerns the nature and identity of the proposed alternate signaling pathway for SHR neurons. There is no concrete data to propose the involvement of known signaling kinases; however, we have been able to eliminate certain kinases. For example, the calcineurin/NFAT signaling pathway, as well as protein kinase A and protein tyrosine kinase, does not appear to be involved in Ang II regulation of NE neuromodulation. The possibility of the involvement of phosphatidylinositol-3

### Effects of Protein Kinase Inhibitors on Ang II Stimulation of NET mRNA Levels in WKY Neurons

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<thead>
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<th>Treatment</th>
<th>Concentration, μmol/L</th>
<th>NET mRNA Levels, Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>...</td>
<td>1</td>
</tr>
<tr>
<td>Ang II</td>
<td>0.1</td>
<td>3.80±0.07</td>
</tr>
<tr>
<td>H89</td>
<td>20.0</td>
<td>0.96±0.02</td>
</tr>
<tr>
<td>H89+Ang II</td>
<td>20.0</td>
<td>3.62±0.11</td>
</tr>
<tr>
<td>Lavendustin A</td>
<td>4.0</td>
<td>1.01±0.08</td>
</tr>
<tr>
<td>Lavendustin A+Ang II</td>
<td>4.0</td>
<td>3.70±0.12</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1.0</td>
<td>0.98±0.04</td>
</tr>
<tr>
<td>Cyclosporin A+Ang II</td>
<td>1.0</td>
<td>3.91±0.13</td>
</tr>
<tr>
<td>KN93</td>
<td>5.0</td>
<td>1.02±0.04</td>
</tr>
<tr>
<td>KN93+Ang II</td>
<td>5.0</td>
<td>3.72±0.11</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>10.0</td>
<td>0.92±0.03</td>
</tr>
<tr>
<td>Trifluoperazine+Ang II</td>
<td>10.0</td>
<td>3.65±0.12</td>
</tr>
<tr>
<td>Bisindolylmaleimide</td>
<td>1.0</td>
<td>0.94±0.04</td>
</tr>
<tr>
<td>Bisindolylmaleimide+Ang II</td>
<td>1.0</td>
<td>1.78±0.08</td>
</tr>
</tbody>
</table>

Neuronal cultures were treated with indicated concentrations of drugs for 4 hours at 37°C in the absence or presence of 100 nmol/L Ang II. NET mRNA levels were measured as described in Methods.
kinase (PI-3 kinase), however, cannot be ruled out at the present time. This is particularly important in view of recent reports that PI-3 kinase could be linked to functional aspects of other G protein–coupled receptors and that Ang II stimulates this enzyme in VSMCs.\textsuperscript{37,38} The role of protein kinase C is also intriguing, since its inhibition attenuates Ang II stimulation of NET mRNA in the neurons of both WKY and SHR.\textsuperscript{21} Thus, it is reasonable to propose that the alternate signaling pathway in the SHR may involve protein kinase C. Further studies are needed to support this view.

Acknowledgments

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MAP Kinase–Independent Signaling in Angiotensin II Regulation of Neuromodulation in SHR Neurons
Hong Yang and Mohan K. Raizada

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