Lack of Macrophage Migration Inhibitory Factor Regulation Is Linked to the Increased Chronotropic Action of Angiotensin II in SHR Neurons

Chengwen Sun, Hongwei Li, Yongxin Gao, Tomokazu Matsuura, Patrick A. Upchurch, Mohan K. Raizada, Colin Sumners

Abstract—Macrophage migration inhibitory factor acts via its intrinsic thiol–protein oxidoreductase activity to negatively regulate the neuronal chronotropic actions of angiotensin II in normotensive rat neurons. Because the chronotropic action of angiotensin II is potentiated in spontaneously hypertensive rat neurons, we investigated whether this negative regulatory mechanism is absent in these rats. Angiotensin II (100 nM) elicited an ≈89% increase in neuronal firing in Wistar–Kyoto rat hypothalamus and brain stem cultured neurons and an increase in intracellular macrophage migration inhibitory factor levels in the same cells. The chronotropic action of angiotensin II was significantly greater (≈212% increase) in spontaneously hypertensive rat neurons, but angiotensin II failed to alter macrophage migration inhibitory factor expression in these cells. Intracellular application of recombinant macrophage migration inhibitor factor (0.8 nM) or its specific neuronal overexpression via Ad5-SYN-MIF (1×10^7 infectious units) significantly attenuated the chronotropic action of angiotensin II in spontaneously hypertensive rat neurons, similar to results from Wistar–Kyoto rat neurons. In contrast, C60S-macrophage migration inhibitory factor (0.8 nM), which lacks thiol–protein oxidoreductase activity, failed to alter the chronotropic action of angiotensin II in neurons from either rat strain. Thus, whereas macrophage migration inhibition factor has the potential to depress the chronotropic action of angiotensin II in spontaneously hypertensive rat neurons, it is unlikely that this regulatory mechanism occurs, because angiotensin II does not increase the expression of this protein. The lack of this regulatory mechanism may contribute to the increased chronotropic action of angiotensin II in spontaneously hypertensive rat neurons. (Hypertension. 2007;49:528-534.)

Key Words: hypothalamus ■ neuronal activity ■ hypertension ■ thiol–protein oxidoreductase ■ reactive oxygen species

The specific angiotensin II (Ang II) type 1 receptor (AT1R)–mediated actions of Ang II at hypothalamic and brain stem neurons play an important role in cardiovascular regulation and contribute to the pathogenesis of hypertension.1,2 These contributions of Ang II to the central nervous system (CNS) control of blood pressure are manifested via alterations in the electrical activity of neurons at specific circumventricular organs, with subsequent activation of hypothalamic and brain stem sites, such as the paraventricular nucleus (PVN), rostral ventrolateral medulla, and nucleus tractus solitarius.3 The result of these CNS actions of Ang II is the enhancement of sympathetic outflow, a blunting of the sensitivity of the baroreflex, and increased vasopressin secretion.3,4 Because these actions of Ang II are amplified in hypertension,5–8 it is essential to fully understand the intracellular mechanisms that regulate the AT1R-mediated effects of Ang II on the activity of CNS neurons. In this regard, much data now exist on the intracellular signals that mediate the neuronal chronotropic action of Ang II, including activation of a calcium-dependent protein kinase C (PKC) and calcium-calmodulin-dependent kinase II, generation of reactive oxygen species (ROS), and modulation of membrane K^+, Ca^{2+}, and nonselective cation currents.9–12 By comparison, there is little information on how these rapid neuronal actions of Ang II are counterregulated or turned off.

The interaction of ligands with G protein–coupled receptors initiates a series of intracellular signaling events, including activation of β-arrestins, that ultimately lead to desensitization and downregulation of the receptor.13 Because there is little evidence that these processes regulate the chronotropic actions of Ang II via AT1R in the CNS, our previous studies have focused on alternate mechanisms that may exist to counteract AT1R-mediated actions in neurons. To this end, we have studied macrophage migration inhibition factor (MIF) as a novel regulator of the CNS actions of Ang II via AT1R. MIF is a small (12.5 kDa) protein that is found in immune tissues and that has established roles in immune responses.14 MIF is also expressed in the CNS, including...
neurons within the hypothalamus.\textsuperscript{15} It has become apparent that MIF can exert intracellular enzymatic actions via its intrinsic tautomerase or thiol–protein oxidoreductase (TPOR) activities.\textsuperscript{16,17} Our studies have demonstrated that MIF is produced intracellularly in neurons cultured from the hypothalamus and brain stem of normotensive rats (Sprague-Dawley [SD] and Wistar–Kyoto [WKY]) and in rat PVN in response to Ang II (AT1R-mediated) stimulation.\textsuperscript{18,19} Furthermore, the increased levels of MIF act intracellularly to reduce the AT1R-mediated chronotropic actions of Ang II on SD and WKY rat hypothalamic neurons in culture and PVN neurons in brain slices from SD rats, via a TPOR-mediated mechanism.\textsuperscript{18,19} In addition, viral-mediated overexpression of MIF within PVN neurons of SD rats blunts the increase in blood pressure elicited by CNS injection of Ang II.\textsuperscript{19} Collectively, these studies suggest that MIF serves as a counter-regulator of the neuronal actions of Ang II, via AT1R, in normotensive rats.

Spontaneously hypertensive rats (SHRs) exhibit increased expression and activity of AT1R in a number of hypothalamic and brain stem regions,\textsuperscript{7,20} and interruption of brain AT1R function by pharmacological or genetic means lowers blood pressure in these animals.\textsuperscript{7–21} These data are supported by our in vitro studies indicating that AT1R expression and the chronotropic action of Ang II are enhanced in neurons cultured from the SHR hypothalamus and brain stem.\textsuperscript{9,22} There are various possible explanations for the hyperactivity of AT1R-mediated actions of Ang II in SHR neurons, including the following: (1) amplification of the neuronal intracellular signaling pathways that underlie Ang II actions; (2) specific intracellular mechanisms that do not exist in normotensive rat neurons are activated in SHR neurons; and (3) regulatory mechanisms that are present in normotensive rat neurons are lacking in SHR neurons. Considering our findings that MIF can serve as a negative regulator of Ang II actions in normotensive rat neurons, we examined whether this mechanism is present in SHR neurons. Here, we demonstrate that artificially elevated levels of intracellular MIF act via TPOR to depress the chronotropic action of Ang II in SHR hypothalamic neurons, similar to the regulatory effects seen in WKY rat neurons. However, our data also indicate that Ang II does not stimulate the increased expression of intracellular MIF in SHR neurons. Thus, an MIF-TPOR mechanism does not operate in SHR neurons, and, thus, a lack of this regulatory process may contribute to the enhanced chronotropic action of Ang II in these cells.

Methods

Experimental Animals

Female and male SHR and WKY rats were purchased from Charles River Farms. These rats were used as breeders to produce pups for the neuronal cultures and were housed in the animal care facility at the University of Florida Health Science Center, where they were provided with food and water ad libitum and kept under normal lighting conditions. This facility is approved by the American Association for the Accreditation of Laboratory Animal Care. The University of Florida Institutional Animal Care and Use Committee approved the experimental protocols conducted here (protocol E033).

Materials

Details of the materials used in this study are available in a data supplement available online at http://hyper.ahajournals.org.

Preparation of Neuronal Cultures

Neuronal cocultures were prepared from the hypothalamus and brain stem of newborn WKY rats and SHRs and grown either on Nunc 35-mm plastic dishes (electrophysiological, ELISA, and mRNA analyses) or on Nunc 12-well plates (receptor binding experiments) as described previously.\textsuperscript{18}

Analysis of MIF Protein

Intracellular and extracellular (media) MIF protein levels were analyzed via a Chemicon ELISA kit, as described by us previously.\textsuperscript{18}

Electrophysiological Recordings

Spontaneous action potentials from WKY rat and SHR neurons in culture were recorded using whole cell voltage clamp procedures in the current clamp mode. Intracellular application of recombinant MIF (rMIF), C60S-MIF, and MIF-neutralizing antibodies during the electrophysiological recordings were achieved by injection through the patch pipette. These procedures were exactly as described by us previously.\textsuperscript{18} Please see the data supplement for more details of these procedures.

Recombinant Adenoviral Constructs

The recombinant adenoviral vectors Ad5-SYN-MIF and Ad5-SYN-EGFP were constructed, purified, and titered as described previously.\textsuperscript{16} Cultured neurons from the SHR or WKY rat hypothalamus were transduced by the addition of the recombinant adenoviral vectors directly to the growth medium, and 3 days later the expression of MIF was confirmed by Western blots as detailed previously.\textsuperscript{18} Transduction of GFP was monitored by viewing green fluorescence using a Zeiss fluorescence microscope.

AT1R mRNA and AT1R Binding

The effects of Ad5-SYN-MIF and Ad5-SYN-EGFP on AT1R mRNA levels were examined using quantitative real-time PCR. Please see data supplement for details. AT1R-specific binding was assessed in WKY rat and SHR neuronal cultures, treated as above with viral vectors or PBS using \textsuperscript{125}I-(Sar\textsuperscript{1}-Ile\textsuperscript{8})-Ang II, as described previously.\textsuperscript{9}

Data Analysis

Results are expressed as mean±SEM. Statistical significance was evaluated with the use of a 1-way ANOVA, followed by a Newman–Keuls test to compare individual means. Differences were considered significant at \(P<0.05\).

Results

Ang II Fails to Increase MIF Expression in SHR Neurons

The chronotropic action of Ang II is significantly greater in SHR neurons in culture compared with their WKY rat controls,\textsuperscript{22} consistent with the enhanced chronotropic action of Ang II observed in the SHR brain in vivo.\textsuperscript{23} Because MIF has been identified as a potent regulator of Ang II’s chronotropic actions in normotensive rat neurons,\textsuperscript{18} our first objective was to compare the actions of Ang II on MIF expression in cocultures from WKY rats and SHRs. Elisa assays revealed that the basal levels of intracellular MIF were similar in neuronal cultures from each rat strain (Figure 1). Although incubation of WKY rat neuronal cultures with Ang II (100 nM, single application; 0.5 to 24 hours) produced a time-dependent increase in intracellular MIF levels, no such effect of Ang II was observed in SHR neuronal cultures after...
identical incubations (Figure 1). In addition, levels of MIF in the growth medium surrounding SHR neurons were not different between control and Ang II (100 nM; 5 hour) -treated cultures (61.5±10 and 69.5±8 ng/2 mL of medium, respectively; n=4 experiments). This excludes the possibility that Ang II is increasing MIF release from SHR neurons, thereby masking any effect on the intracellular levels of MIF.

MIF Negatively Regulates the Chronotropic Actions of Ang II in SHR Neurons

MIF, elevated in response to Ang II stimulation, provides an inhibitory influence over the chronotropic action of Ang II in normotensive rat neuronal cocultures. The above demonstration that Ang II fails to increase MIF expression in SHR neurons suggests that this protein may not serve as a negative regulator of Ang II’s chronotropic actions in these cells. Thus, we determined whether elevated levels of MIF still have the ability to suppress firing and Ang II’s chronotropic actions in SHR neurons, that is, whether the negative regulatory mechanism can operate when MIF is present.

In the first approach, neurons from WKY rats and SHRs were preincubated with a control solution (PBS) or Ang II (100 nM) for 0.5, 1, 3, 6, 12, or 24 hours; cells were lysed; and intracellular MIF levels (nanograms per milligram of protein) under each treatment condition. Data are mean±SEM from 5 experiments. *P<0.05 vs respective control (plotted at time 0).

In a second approach, we tested whether intracellular application of exogenous rMIF can attenuate the chronotropic action of Ang II in neurons from SHRs. Superfusion of WKY rat or SHR neurons with Ang II (100 nM, 5 minutes) produced respective increases in the neuronal firing rate of ≈89% and 212% (Figure 3), consistent with our previous data. Intracellular application of rMIF (0.8 nM), a concentration that does not alter basal neuronal firing, produced a significant blunting of the chronotropic effects of Ang II in both WKY rat and SHR neurons (Figure 3). Our previous studies indicated that this inhibitory action of MIF in normotensive rat neurons is mediated via a CALC motif at residues 57 to 60 that imparts TPOR activity, and we next studied whether this is the case in SHR neurons. In contrast to our data with wild-type rMIF, intracellular application of C60S-MIF (0.8 nM), a mutant protein that completely lacks TPOR activity, failed to inhibit the chronotropic action of Ang II (100 nM) in either SHR or WKY rat neurons (Figure 4).

Because the data shown in Figures 3 and 4 was obtained using different approaches to test the effects of MIF on Ang II–induced responses, thus, we examined whether overexpression of MIF within SHR neurons in culture modifies the chronotropic action of Ang II. For these experiments, SHR and WKY rat neurons were incubated with Ad-SYN-MIF (1×10⁷ infectious units [ifu] per dish) for 3 days. This viral vector produces selective overex-
pression of MIF within neurons, as detailed by us previously.\textsuperscript{18} Control cultures from each strain were incubated with either PBS or Ad-SYN-EGFP (1 × 10^7 ifu per dish) for 3 days. The transduction produced by these vectors was comparable in cultures from both strains, as evidenced by the localization of green fluorescence. Incubation of control (PBS-treated) WKY rat and SHR neurons with Ang II (100 nM) produced chronotropic effects that were not different from those obtained in the Ad-SYN-EGFP–treated cultures (Figure 5.). However, in the WKY rat and SHR neurons that had been

![Figure 3](image3.png)

**Figure 3.** rMIF decreases the chronotropic action of Ang II in WKY rat and SHR neurons. Recordings of action potentials from representative WKY rat and (A) and SHR (B) neurons under the following sequential treatment conditions: superfusion of control solution (PBS [Con]); superfusion of Ang II (100 nM); wash with superfusate solution; intracellular application of rMIF (0.8 nM); and intracellular application of rMIF plus superfusion of Ang II. Dashed lines indicate the 0 mV potential. C, Bar graphs are the percentage increase (over control) of firing rate produced by Ang II in the absence and presence of rMIF, in neurons form each rat strain. Data are mean±SEM from 7 neurons in each case. *P < 0.05 vs WKY rat neurons (Ang II–treated); ** P < 0.05 vs respective WKY rat and SHR neurons (Ang II only treatment).

![Figure 4](image4.png)

**Figure 4.** C60S-MIF does not modify the chronotropic action of Ang II in WKY rat and SHR neurons. Recordings of action potentials from representative WKY rat and (A) and SHR (B) neurons under the following sequential treatment conditions: superfusion of control solution (PBS [Con]); superfusion of Ang II (100 nM); wash with superfusate solution; intracellular application of C60S-MIF (0.8 nM); and intracellular application of C60S-MIF plus superfusion of Ang II. Dashed lines indicate the 0 mV potential. C, Bar graphs are the percent increase (over control) of firing rate produced by Ang II in the absence and presence of C60S-MIF, in neurons from each rat strain. Data are mean±SEM from 8 WKY rat and 6 SHR neurons. *P < 0.05 vs WKY rat neurons.
transduced with Ad-SYN-MIF and, thus, exhibit increased levels of MIF, the respective increases in firing rate produced by Ang II were severely blunted (Figure 5).

MIF Does Not Influence AT1R mRNA or Binding in WKY Rats and SHR Neurons

It is apparent that the inhibitory action of MIF over the neuronal chronotropic effects of Ang II involves the intrinsic TPOR activity of the MIF molecule. However, we have not established the locus of the MIF–TPOR activity in this inhibitory action. One possibility that must be considered is that MIF somehow influences AT1R expression or binding.

To investigate this idea, SHR and WKY rat neuronal cultures were incubated with PBS, Ad5-SYN-EGFP (5 × 10⁷ or 2 × 10⁸ ifu per dish), or Ad5-SYN-MIF (5 × 10⁷ or 2 × 10⁸ ifu per dish) for 3 days, followed by analysis of AT1R mRNA. The data in Figure 6A and 6B indicate that MIF overexpression does not alter the levels of AT1R mRNA in SHR or WKY rat neuronal cultures when compared with PBS- or Ad-SYN-EGFP–treated cultures. In addition, the levels of AT1R–specific binding in SHR neuronal cultures were not significantly altered by Ad-SYN-MIF. In Ad-SYN-MIF and Ad-SYN-EGFP (both 5 × 10⁷ ifu per well, for 3 days)–treated cultures, the respective levels of AT1R–specific binding were 8605 ± 158 and 9115 ± 193 counts per minute per well (n = 3 experiments). Ad-SYN-MIF was similarly ineffective in WKY rat neurons (H Li, unpublished data, 2006). Thus, it appears that the inhibitory effects of MIF on Ang II–mediated increases in neuronal firing cannot be explained by effects on the expression of the AT1R.

Discussion

The present findings demonstrate for the first time that the hyperactivity of Ang II in SHR neurons is in part a result of its failure to increase the neuronal levels of MIF, which is a counterregulator of Ang II’s chronotropic action in normotensive rat neurons. The fact that this MIF negative regulatory mechanism does not operate in SHR neurons raises a number of issues and questions. First and foremost, the reasons for the lack of effect of Ang II on MIF expression in SHR neurons are unknown and will remain so until the mechanisms that mediate Ang II’s stimulation of MIF expression in normotensive rat neurons are fully established. There are 2 likely possibilities. Ang II–induced increases in MIF involve activation of a calcium-dependent PKC but not calcium-calmodulin–dependent kinase II. Because the MIF gene promoter contains several sites (eg, activation protein-1 and cyclic AMP response element) that may be indirectly influenced by PKC, it is possible that PKC directly or indirectly modulates MIF gene expression. More recently, we have determined that ROS (specifically H₂O₂) can induce MIF expression in normotensive rat cultured neurons. Considering that the Ang II–induced neuronal chronotropic action is mediated via an AT1R/PKC/reduced nicotinamide-adenine dinucleotide phosphate oxidase/ROS (O₂⁻) mechanism in normotensive rat neurons, it is reasonable to suggest that H₂O₂, derived from O₂⁻, may be responsible...
for mediating the Ang II–induced increases in neuronal MIF expression. Thus, the failure of Ang II to increase MIF expression in SHR neurons may be because of a deficit in 1 or both of these mechanisms. Clearly, more studies are required to establish the exact mechanisms by which PKC and ROS may induce MIF expression (eg, involvement of transcription factors) in normotensive rat neurons before we can establish why Ang II does not increase MIF expression in SHR neurons.

One important issue concerns the in vivo implications of our findings. Our previous studies demonstrate that Ang II increases MIF expression in normotensive rat PVN and that selective neuronal overexpression of MIF at that site blunts the pressor action of centrally injected Ang II.21 However, Ang II does not increase MIF expression in the PVN of SHRs.26 Thus, it is possible that a lack of MIF regulation contributes to the enhanced pressor responsiveness of Ang II in SHRs.5,7 It is also tempting to speculate that the high blood pressure of SHRs may, in part, be because of a failure of MIF to regulate Ang II actions in the brains of these animals. Clearly this area requires more investigation and will be the subject of future studies in which we will elicit long-term (AAV2-mediated) overexpression of MIF within the PVN and determine the effects on basal and Ang II–induced blood pressure in SHRs.

Because MIF can regulate the rapid neuronal actions of Ang II, and a deficit of this MIF regulation may contribute to the hyperactivity of Ang II–induced chronotropic actions in SHR neurons, it is also important to understand the mechanisms by which MIF regulates Ang II–induced increases in neuronal firing. Our current and previous data indicate an involvement of the intrinsic TPOR activity mediated via cysteine residues at positions 57 and 60 of the MIF molecule.18 One potential consequence of an increase in TPOR activity is scavenging of ROS and blockade of oxidant-mediated intracellular actions.27,28 Because the neuronal chronotropic action of Ang II via AT1R involves generation of ROS,11 it is possible that MIF blunts the neuronal actions of Ang II via scavenging ROS. In support of this mechanism, we have demonstrated that elevated levels of MIF in hypothalamic neurons (via viral-mediated overexpression) abolish the Ang II–induced generation of ROS,18 but more data are required to establish a direct link between MIF generation and ROS scavenging in this system. Indeed, it can also be argued that MIF may act at a site upstream of ROS, such as AT1R or PKC, to disrupt Ang II–induced signaling. The former possibility appears not to be the case based on the failure of MIF overexpression to alter AT1R mRNA (Figure 6) or binding.

**Perspectives**

Ang II is a powerful CNS regulator of cardiovascular function, and there is much evidence that the central actions of Ang II are exacerbated in and contribute to hypertension. Thus, it is crucial to understand the cellular mechanisms that regulate the actions of Ang II on neuronal firing. Here, we have demonstrated that MIF, which inhibits Ang II’s neuronal chronotropic effects in normotensive rats, is unlikely to do so in SHRs. Although there is a complex interplay between many different factors to regulate Ang II function in the CNS, a lack of MIF’s regulatory actions likely contributes to the enhanced chronotropic responsiveness of Ang II in SHR neurons. By extrapolation, loss of MIF negative regulation in SHR neurons may contribute to the increased pressor responses elicited by Ang II in these animals.

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**Disclosures**

None.

**References**


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