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Redox regulation of macrophage migration inhibitory factor expression in rat neurons

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ABSTRACT

Macrophage migration inhibitory factor (MIF) expression is induced by angiotensin II (Ang II) in normal rat neurons and serves a negative regulatory role by blunting the chronotropic actions of this peptide. The aim here was to determine whether hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) that is a key intracellular mediator of the neuronal actions of Ang II, is a trigger for MIF production in neurons. Thus, we tested the effects of H_2O_2 on MIF expression in primary neuronal cultures from newborn normotensive (Wistar Kyoto [WKY] or Sprague–Dawley [SD]) rat brain, cells that respond to Ang II by increasing MIF levels. Treatment of WKY or SD rat neuronal cultures with a non-cytotoxic concentration of H_2O_2 elicited a significant, time-dependent increase in MIF mRNA and protein levels. Glucose oxidase, which produces H_2O_2 via oxidation of glucose in the cell-culture medium, elicited a similar increase in neuronal MIF mRNA levels. The stimulatory action of H_2O_2 was not apparent in neuronal cultures from spontaneously hypertensive rats (SHR), cells that fail to express increased MIF in response to Ang II. Finally, preincubation of SD rat cultures with either polyethylene glycol-catalase or actinomycin D abolished the H_2O_2-induced increase in MIF, suggesting that this ROS is acting intracellularly to increase transcription of the MIF gene. These results suggest the presence of a redox regulatory mechanism for induction of MIF in normotensive rat neurons.

Introduction

Macrophage migration inhibitory factor (MIF) is a small, highly conserved protein with nearly ubiquitous tissue distribution that was originally described as a cytokine in the 1960s. Recently, it has become apparent that MIF and its functions are far more complicated than originally thought. It is now known that MIF has many, sometimes seemingly disparate, biological and cellular functions. Physiologically, MIF plays modulatory roles in the immune, endocrine, and nervous systems [1]. Pathologically, MIF contributes to a wide range of inflammatory disorders and plays a pivotal role in tumor biology [2,3].

The functions of MIF can be divided into two basic categories. Much of the traditional literature focuses on the pro-inflammatory, cytokine-like function of MIF. However, in recent years, it has been discovered that MIF has two enzymatic activities, one of which is a thiol-protein oxidoreductase (TPOR) activity that lies between amino acid residues 57 and 60. Accordingly, it has recently been suggested that MIF be re-classified as not only a cytokine, but also a thioredoxin (Trx) family member due to the fact that it contains this TPOR motif, an identifying characteristic of Trx proteins [4]. Members of the Trx family are essential to maintaining redox balance in the intracellular environment by scavenging of reactive oxygen species (ROS), and may also influence the activity of other proteins by reducing critical cysteines [5]. Whether MIF acts as a pro-inflammatory cytokine or an antioxidant protein seems to depend on the concentration of MIF in the tissue, with relatively low concentrations acting as a pro-inflammatory mediator and higher concentrations serving an antioxidant function [6].

MIF is relevant to the field of blood pressure regulation due to its ability to serve as a negative regulator of the central actions of angiotensin II (Ang II). *In vitro*, Ang II up-regulates MIF in neurons cultured from normotensive rat hypothalamus and brainstem [7], and increased intracellular levels of MIF protein exert a negative regulatory action over the neuronal chronotropic effects of Ang II via its type 1 receptor (AT1R) [8]. *In vivo*, CNS injections of Ang II in normotensive rats increase MIF expression in the paraventricular nucleus (PVN) of the hypothalamus, an area that has a key role in regulating sympathetic outflow and hypothalamus/pituitary axis activity. Increased MIF within PVN sympathetic regulatory neurons blunts the AT1R-mediated increases in discharge of these cells elicited by Ang II and the increases in blood pressure produced by CNS-injected Ang II [9]. Thus, it is of major interest to understand the intracellular mechanisms that control MIF expression in normal rat neurons. It is established that Ang II, acting via

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the AT1R, induces ROS production in neurons by activating NADPH oxidase [10]. This leads to superoxide production, which is metabolized to hydrogen peroxide (H_2O_2) by superoxide dismutases [11]. Many studies have established that H_2O_2 is a product of Ang II signaling in many cell types, including neurons [12]. Furthermore, it has been demonstrated that H_2O_2 can cause induction of MIF expression in peripheral tissues [13]. Therefore in the present study the aim was to determine whether H_2O_2, a key intracellular mediator of the neuronal actions of Ang II, elicits MIF production in neurons from normotensive rats.

Methods

Animals. Newborn SD or WKY rats or SHR, derived from our breeding colony, were used for the production of neuronal cultures. Adult breeder rats were purchased from Charles River Farms (Wilmington, MA). All experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Cell cultures. Neuronal cultures (~90% neuronal, 10% glial) were prepared from the forebrain of newborn rats as described previously [7]. Cultures were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% plasma-derived horse serum (PDHS; Sigma) for a further 12–16 days before use. Glial cultures were derived from the neuronal cultures by killing the neurons by brief exposure (5 min) to 100 mM KCl, and then re-culturing the surviving glial cells in DMEM containing 10% fetal bovine serum (FBS; Sigma) to ~80% confluency. The DMEM/FBS medium was then replaced with DMEM/10% PDHS and glial cultures were grown for at least a further 7 days to parallel the feeding/utilization schedule of the corresponding neuronal cultures.

Analysis of MIF mRNA. cDNA was produced from neuronal cultures using a Cells-to-cDNA II kit (Ambion). Levels of MIF mRNA were quantified by real-time RT-PCR as described previously [8]. Data were normalized to 18S rRNA.

Analysis of MIF protein. Neuronal cultures underwent lysis in Laemmli Sample Buffer (Bio-Rad) and 10 μg of total protein was loaded on the gel. Transfer to a PVDF membrane (Bio-Rad) was performed at 75 V for 1.5 h in Towbin Buffer. Western blots were carried out using a rat MIF antibody (Torrey Pines Biolabs) and analyses of MIF protein levels (normalized against β-actin) were performed as detailed previously [9].

Determination of cytotoxicity. Neuronal cultures were treated as indicated and medium was collected from each well. Replicates were pooled, and then samples were centrifuged at 4 °C and 5000 rpm for 5 min to pellet any particulate matter or unattached cells. Supernatant was then utilized (50 μL) according to the protocol provided with the CytoTox96 kit (Promega).

Determination of protein carbonyl content. Protein carbonyl content was determined using the Protein Carbonyl Assay Kit from Cayman Chemical.

Cell treatments. A stock solution of 30% H_2O_2 in water was diluted 100-fold into the culture medium to the final dose indicated. PEG-catalase (Sigma) and Glucose Oxidase (Calbiochem) were each dissolved in sterile DPBS then diluted 100-fold into the culture medium to the final dose indicated. Actinomycin D (Act D; Sigma) was dissolved in DMSO to give a concentration of 1 mM. It was then diluted 500-fold into the culture medium to a final concentration of 1 μM.

Data analysis. Results are expressed as means ± 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

Hydrogen peroxide stimulates an increase in MIF levels in neurons cultured from normotensive rats

In the first set of experiments we determined whether H_2O_2 can induce expression of MIF mRNA in primary neuronal cultures from normotensive rats. Incubation of primary neuronal cultures prepared from newborn SD or WKY rats with H_2O_2 (30 μM; 1, 2 or 3 h.) resulted in time-dependent increases in MIF mRNA levels in cells from both strains (Fig. 1A and B). Importantly, this concentration of H_2O_2 had no cytotoxic action on the cultured cells. For exam-

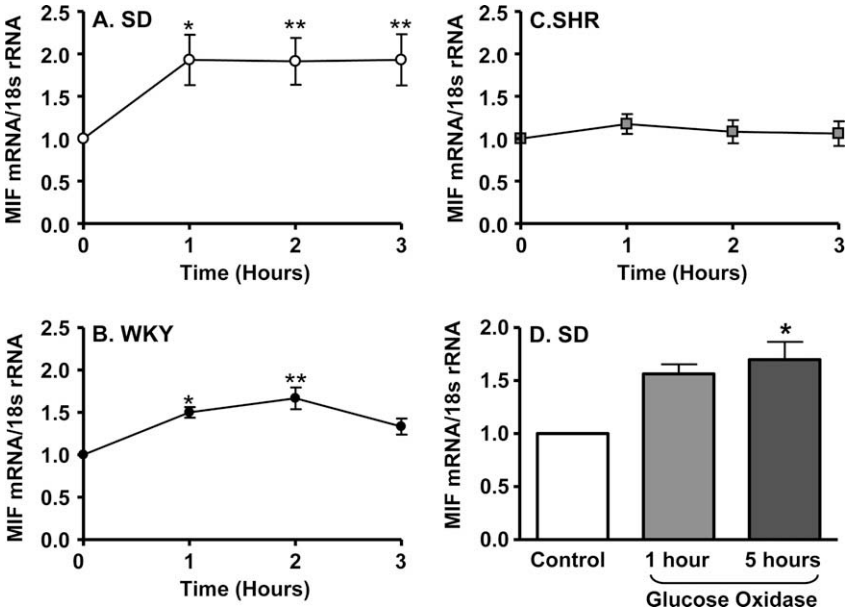

Fig. 1. Hydrogen peroxide increases MIF mRNA levels in primary neuronal cultures from normotensive rats. (A) SD rat, (B) WKY rat or (C) SHR neuronal cultures were incubated with either vehicle (H_2O) or 30 μM H_2O_2 for 1, 2, or 3 h. (D) SD rat neuronal cultures were incubated with either vehicle (DPBS) or 0.5 mU glucose oxidase for 1 and 5 h. These incubations were followed by analysis of MIF mRNA levels as described in Materials and methods. Data are means ± SEM (n = 7, 5, 6 and 4 expts for panels A, B, C and D, respectively) of the ratio of MIF mRNA to 18S rRNA at each time point. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

ple, in the cultures treated with 30 μM H_2O_2 the cell survival rate at 1 and 3 h was 100 ± 2.00% and 98 ± 0.75% (n = 4 expts), respectively. Note that in cultures treated with a cytotoxic concentration of H_2O_2 (100 μM), as a positive "killing" control, the cell survival rate at 1 and 3 h was 95 ± 4.57% and 63 ± 4.30%* (n = 4 expts), respectively (*P < 0.001 vs. control). Interestingly, in contrast to its effects in SD and WKY rat neuronal cultures, incubation of SHR neuronal cultures with H_2O_2 under the same conditions failed to alter MIF mRNA levels throughout the 3 h time period observed (Fig. 1C). To support the above findings obtained with a bolus application of a H_2O_2 solution to the neuronal cultures, primary neuronal SD rat cultures were incubated with glucose oxidase, which produces H_2O_2 in a more chronic manner via oxidation of glucose in the cell-culture medium. Treatment of neuronal cultures with 0.5 mU glucose oxidase for 1 or 5 h resulted in a time-dependent increase in MIF mRNA levels (Fig. 1D), consistent with the stimulatory action of H_2O_2 on MIF mRNA in SD rat neuronal cultures. Incubation of SD rat neuronal cultures with H_2O_2 (30 μM; 1, 2 or 3 h) also produced a time-dependant increase in MIF protein, an effect that was significant by 3 h (Fig. 2A). As might be predicted, the H_2O_2-induced increase in MIF mRNA preceded the increase in MIF protein levels stimulated by this ROS. As the neuronal cultures used here contain a small (<10%) number of glial cells, we investigated whether H_2O_2-induced increases in MIF mRNA were restricted to neurons or if the effects could also be observed in glial cells. Incubation of SD rat glial cultures that were devoid of neurons with 1, 10, 30, or 50 μM H_2O_2 for 1 h elicited no significant changes in MIF mRNA levels in glial cultures (Fig. 2B). Collectively, these results indicate that MIF mRNA and protein expression can be regulated by ROS in primary neurons from normotensive rats, and that this form of regulation is absent in neurons cultured from SHR.

Hydrogen peroxide increases MIF mRNA expression in primary neurons through a specific intracellular action

At high concentrations, ROS can cause a generalized oxidative stress response in cells. Thus, we investigated whether the stimulatory effect of H_2O_2 on MIF mRNA levels in neurons is a specific intracellular action of H_2O_2, rather than a non-specific response to oxidative stress.

First, the effects of PEG-catalase on H_2O_2-stimulated increases in MIF mRNA levels in neurons were determined. Catalase metabolizes H_2O_2 into water and molecular oxygen, and the PEG-conjugation of the enzyme not only renders it cell permeant, but also stable once inside the cell [14]. Pre-treatment of SD neuronal cultures with PEG-catalase for 24 h prevents the H_2O_2-stimulated increases in MIF mRNA and protein levels, indicating that the exogenously-applied H_2O_2 acts intracellularly to increase MIF levels (Fig. 3A/B).

Furthermore, we sought to determine whether a 30 μM dose of H_2O_2 represents a state of oxidative stress to the neuronal cultures by measuring protein carbonyl content, an indicator of intracellular oxidative stress. Treatment of SD rat neuronal cultures with 30 μM H_2O_2 for 1 h (i.e. the earliest time point of significant MIF induction in the presence of H_2O_2) did not significantly alter the protein carbonyl concentration when compared with control neurons (Fig. 4A). Collectively, these results suggest that the effects of H_2O_2 on MIF mRNA levels in primary neurons are a specific, intracellular signaling action of this ROS and not the result of a generalized oxidative stress response.

In a final set of experiments we determined the effects of actinomycin D (Act D), a general inhibitor of mRNA synthesis, on H_2O_2-stimulated increases in MIF mRNA levels. Treatment of SD rat neuronal cultures with 1 μM Act D revealed that MIF mRNA is stable in neurons for at least 6 h, with half-life of degradation not yet reached by 8 h (data not shown). The stability of MIF mRNA in the SD rat neuronal cultures is similar to that reported previously in other cell types [15]. Pre-treatment of neuronal cultures with 1 μM Act D for 1 h completely abolished the increase in MIF mRNA levels elicited by incubation of cultures with 30 μM H_2O_2 for 1 h (Fig. 4B). This result suggests that the effects of H_2O_2 on MIF mRNA levels in neurons are primarily due to increased MIF mRNA synthesis.

Discussion

This study represents the first demonstration that ROS can regulate the expression of MIF in CNS neurons. Furthermore, we have shown that this effect of H_2O_2 involves intracellular events that are specific to neurons, and the data suggest that the increase in MIF involves *de novo* transcription. Finally, the observation that H_2O_2 fails to elicit an increase in MIF in neurons cultured from SHR, in contrast to their normotensive controls, provides support for the contention that the MIF gene responds in a specific and regulated fashion to redox signaling.

In this study, H_2O_2 was selected as the ROS donor for many reasons. First, the aim was to study a ROS that is downstream of Ang II and the AT1R in neurons. It has already been established that Ang II

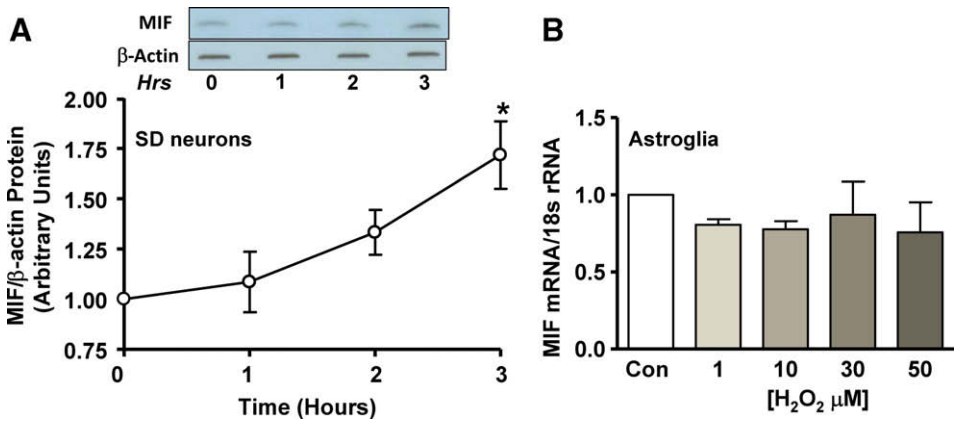

Fig. 2. Contrasting effects of hydrogen peroxide on MIF levels in primary neuronal and glial cultures. (A) SD rat neuronal cultures were incubated with either vehicle (H_2O) or 30 μM H_2O_2 for 1, 2, or 3 h, followed by Western blot analysis of MIF protein as described in Materials and methods. Top: Representative Western blot showing the effects of H_2O_2 on 12.5 kDa MIF protein levels. Also shown are the corresponding levels of 42 kDa β-actin protein Bottom: Data are means ± SEM (n = 3 expts) of the relative levels of MIF protein at each time point, normalized against β-actin. *P < 0.01 vs. control. (B) SD rat glial cultures were incubated with either vehicle (H_2O) or 1–50 μM H_2O_2 for 1 h, followed by analysis of MIF mRNA levels as described in the Materials and methods. Bar graphs are means ± SEM (n = 3 expts) of the ratio of MIF mRNA to 18S rRNA at each concentration. Statistical analysis showed no significance at any time point observed.

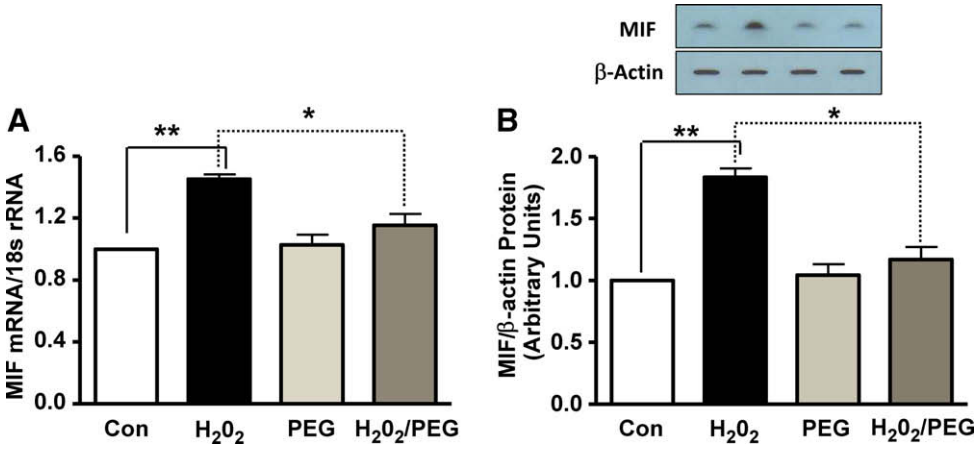

Fig. 3. PEG-catalase inhibits the hydrogen peroxide induced increase in MIF levels in primary neuronal cultures. (A) SD rat neuronal cultures were incubated with either vehicle (DPBS) or 100 U of PEG-catalase (PEG) for 24 h. Media were replaced with fresh conditioned medium taken from age-matched, untreated neuronal cultures. Cultures were then incubated with either control vehicle (H_2O) or 30 μM H_2O_2 for 1 h followed by analysis of MIF mRNA levels as described in Materials and methods. Bar graphs are means ± SEM (n = 6 expts) of the ratio of MIF mRNA to 18S rRNA under each treatment condition. $^*P < 0.01$ vs. H_2O_2; $^{**}P < 0.001$ vs. control. (B) SD rat neuronal cultures were incubated with PEG-catalase and H_2O_2 as in (A). Top: Representative Western blot showing the effects of H_2O_2 on 12.5 kDa MIF protein levels under each treatment condition. Bottom: Bar graphs are means ± SEM (n = 3 expts) of the ratio of MIF protein to 42 kDa β-actin protein under each treatment situation. $^*P < 0.01$ vs. H_2O_2; $^{**}P < 0.01$ vs. control.

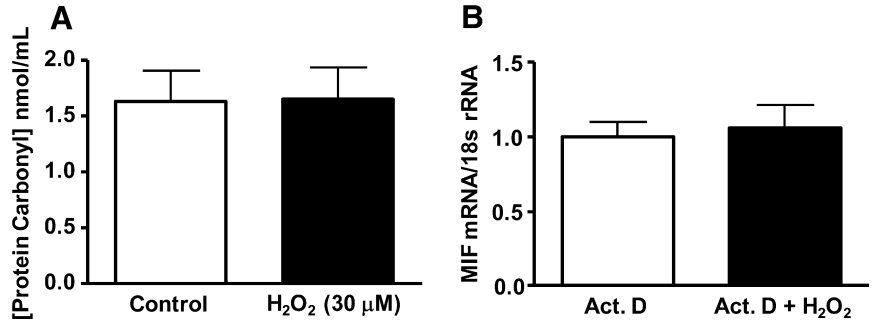

Fig. 4. Specificity of hydrogen peroxide induced increase in MIF mRNA expression in primary neuronal cultures. (A) SD rat neuronal cultures were incubated with either vehicle (H_2O) or 30 μM H_2O_2 for 1 h. Cell lysates were collected and assayed for protein carbonyl content as indicated in Materials and methods. Bar graphs are means ± SEM (n = 10 expts). (B) SD rat neuronal cultures were incubated with actinomycin D (1 μM) for 1 h. The cultures were then incubated with either vehicle (H_2O) or 30 μM H_2O_2 for 1 h followed by analysis of MIF mRNA levels as described in Materials and methods. Bar graphs are means ± SEM (n = 5 expts) of the ratio of MIF mRNA to 18S rRNA.

is capable of producing intracellular H_2O_2 in many cell types, including neurons, and that this H_2O_2 has significant physiological effects (e.g. influencing sympathetic activity in the brain) [12]. Second, it is readily cell permeant, with exogenously-applied H_2O_2 establishing equilibrium across the cell membrane within minutes [16]. Finally, it is relatively stable since, while it is a ROS, it is not a free-radical. While the concentration of H_2O_2 [30 μM] that was required to induce MIF expression is relatively high, the cytotoxicity, protein carbonyl, and PEG-catalase experiments support the notion that this ROS is functioning as a signaling agent in our neuronal cultures, rather than a mediator of cell death and/or oxidative stress. The necessity of a relatively high concentration of H_2O_2 is likely due to the presence of a small population of glia (astrocytes, oligodendrocytes, microglia) within the neuronal cultures. These cells all have the capacity to detoxify H_2O_2 [17], and so it is possible that a portion of the exogenous H_2O_2 is rapidly degraded when added to the medium of the neuronal cultures, and, consequently, the neurons are not exposed to doses high enough to create oxidative stress in the intracellular environment. Moreover, the knowledge that a bolus application of H_2O_2 can be rapidly detoxified by many of the cell types in our neuronal cultures prompted us to perform the experiments utilizing glucose oxidase, which represents a more chronic means of administering H_2O_2, to confirm that this ROS can induce MIF expression.

The results raise important questions as to the mechanism by which H_2O_2 is inducing MIF expression in CNS neurons. It is now widely recognized that H_2O_2, like nitric oxide, may be a readily-diffusible small molecule that acts as a signaling agent. Indeed, in prokaryotes and yeast, systems that sense and signal in response to H_2O_2 are well-characterized [11]. In higher mammals, many of the signaling pathways affected by ROS are still under investigation. Nevertheless, it is becoming clear that several kinase pathways are modulated by ROS and the activity of many transcription factors is subject to redox regulation [18–20]. For example, transcription factors such as AP-1, SP-1, CREB, and NFκB are sensitive to redox regulation [21–23], and binding sites for these transcription factors have been identified in the promoter of the human MIF gene [24]. Furthermore, SP-1 and CREB are important transcriptional regulators of the MIF gene [25]. Experiments to determine if these transcription factors may be the mediators of redox regulation of the MIF gene in neuronal cell lines are ongoing.

This study is also significant because, as we have established in prior reports, MIF is up-regulated in neurons in response to Ang II signaling via the AT1R [7–9]. MIF then serves, either directly or indirectly, as a negative regulator of the chronotropic actions of Ang II in neurons that lie along key sympathetic and neuroendocrine pathways in the brain such as the PVN [8–9]. Intriguingly, a

recent publication has shown that H_2O_2 produced in the PVN in response to Ang II may play a role in regulating sympathetic activity [12]. Accordingly, it is tempting to visualize a feed-back loop such that Ang II causes H_2O_2 production in the PVN, which stimulates MIF production [9], subsequently feeding back to decrease the sensitivity of the neuron to Ang II and, perhaps, influencing the central sympathetic and/or neuroendocrine actions of Ang II.

We believe that MIF may act in this regard by scavenging ROS, as do some other proteins that contain TPOR motifs (e.g. Trx, peroxiredoxins) [5], but the exact mechanism is still under investigation.

A further interesting point is the failure of H_2O_2 to induce MIF expression in SHR neuronal cultures. This finding provides further evidence that the effect of H_2O_2 on neuronal MIF expression is a specific signaling event, rather than a non-selective oxidative stress-mediated mechanism. Aside from this, we previously demonstrated that Ang II does not induce MIF expression in SHR neurons in culture [26] and in PVN neurons of SHR [27], and this lack of MIF induction may contribute to the hyper-sensitivity of these neurons to Ang II, and perhaps even the development and/or maintenance of hypertension in these animals. This idea is borne out by recent findings that viral-mediated over-expression of MIF in the PVN of young SHRs attenuates the development of hypertension [27]. Future studies will include investigating why Ang II and H_2O_2 fail to elicit MIF expression in SHR neurons.

In conclusion, this study establishes that MIF expression in neurons can be regulated by ROS. It serves as a basis for further studies on whether H_2O_2 is a mediator of Ang II-induced MIF expression in normal rat neurons, and whether H_2O_2 signaling is "broken" in SHR neurons and, hence, leads to an inability of Ang II to induce MIF expression.

Acknowledgment

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