Macrophage Migration Inhibitory Factor in the Paraventricular Nucleus Plays a Major Role in the Sympathoexcitatory Response to Salt

Eduardo Colombari, Debora S.A. Colombari, Hongwei Li, Peng Shi, Ying Dong, Nan Jiang, Mohan K. Raizada, Colin Sumners, David Murphy, Julian F.R. Paton

Abstract—Central hyperosmotic stimulation (HS) evokes increases in sympathetic nerve activity mediated by activation of angiotensin type 1 receptors in the hypothalamic paraventricular nucleus (PVN). Macrophage inhibition migration factor (MIF) is an intracellular inhibitory regulator of angiotensin type 1 receptor–mediated actions of angiotensin II within neurons of the PVN. MIF mediates its actions via its intrinsic thiol-protein oxidoreductase activity. We demonstrate that intracerebroventricular injection of hypertonic saline into Sprague-Dawley rats elicits a significant (≈112%) increase in MIF mRNA expression in the PVN. Next, we evaluated the effect of viral-mediated expression of either MIF or [C60S]-MIF (which lacks thiol-protein oxidoreductase activity) in the PVN on the sympathoexcitatory response evoked by HS. We used a decorticate, arterially perfused in situ preparation of male Wistar rats (60 to 80 g). HS was induced by raising perfusate osmolality from 290 to 380 milliosmoles for 40 seconds. Seven to 10 days before experiments, rats were injected bilaterally (500 nL per side) with 0.9% saline (control) or with adenoassociated virus to express MIF, [C60S]-MIF, or enhanced green fluorescent protein in the PVN. HS produced sympathoexcitation in both the 0.9% saline and enhanced green fluorescent protein groups (sympathetic nerve activity increase of +27±4% and +25±4%, respectively; P<0.05), an effect that was not observed in the MIF group (+4±5%). Conversely, the HS-induced increase in sympathetic nerve activity was potentiated in the [C60S]-MIF group (+45±6%; P<0.05). We propose that MIF acting within the PVN is a major counterregulator of HS-induced sympathoexcitation, an effect that depends on thiol-protein oxidoreductase activity. (Hypertension. 2010;56:956-963.)

Key Words: hypothalamus | gene transfer | sympathetic nerve activity | angiotensin type 1 receptors | macrophage migration inhibitory factor

The paraventricular nucleus (PVN) of the hypothalamus plays a crucial role in the regulation of cardiovascular function and body fluid homeostasis. Studies have shown that activation of PVN neurons increases blood pressure and sympathetic nerve activity in response to a hyperosmotic challenge, which is driven by the afferent inputs from the lamina terminalis in the forebrain. Two key structures have been well accepted as osmosensors in the central nervous system, the subfornical organ and organum vasculosum laminae terminalis (OVLT) located along the dorsal and ventral portions of the lamina terminalis, respectively. In respect to OVLT, it has been shown to possess osmosensi-

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ways,22–24 including glutamatergic25 and vasopressinergic pathways.19,26 Considering that hyperosmolality, angiotensin II (Ang II) and AT1R in the PVN exert profound stimulatory influences on sympathetic outflow and arterial blood pressure, it is, therefore, important to understand the mechanisms within this nucleus that mediate these responses. Our attempts to understand regulatory mechanisms led to the discovery of macrophage migration inhibitory factor (MIF) as a novel intracellular inhibitory regulator of AT1R-mediated actions of Ang II within PVN neurons. We demonstrated that MIF acts intracellularly to counterregulate the firing responses evoked by Ang II in PVN neurons cultured from normotensive rats.27,28 MIF elicits this inhibitory effect via its intrinsic thiol-protein oxidoreductase (TPOR) activity that is exerted by a C-A-L-C motif that exists at residues 57 to 60 of the MIF molecule.27,28 MIF is expressed in neurons in the PVN of normotensive rats, and intracerebroventricular (ICV) injection of Ang II increases MIF expression in this cardiovascular control center.28,29 In addition, viral-mediated increases in MIF levels in PVN neurons produce complete inhibition of the increase in sympathetic nerve activity elicited by hyperosmolar (high-salt) conditions. This inhibitory action of MIF involves its TPOR activity. These data identify MIF in the PVN as a major regulator of salt-induced increases in sympathetic outflow.

**Materials and Methods**

**Ethical Approval**

For the experiments that generated the data shown in Figures 1 and 2, we used a total of 15 male Sprague-Dawley rats (200 to 250 g),
Figure 2. Hyperosmotic stimulation induces MIF mRNA expression in the PVN via an AT1,R-dependent process. Rats were injected ICV with either 2 μL of losartan (Los; 2.1 nmol/μL) or 2 μL of isoosmotic (0.9%) saline. Fifteen minutes later, half of the rats from each group were injected ICV with 2 μL of isoosmotic (0.9%) saline, and the others were injected ICV with 2 μL of 2.0-mol/L NaCl. Three hours later brains were removed for analysis of MIF mRNA in the PVN. Data are mean±SEM of MIF mRNA levels normalized against 18S rRNA (n=5 to 10 per treatment condition). *P<0.05 (Student t test).

Figure 3. Increased MIF expression in the PVN decreases HS-evoked sympathoexcitation. Representative tracings showing changes in raw and integrated SNA during isoosmotic (290 mosmol/kg · water⁻¹) and hyperosmotic stimuli (HS; 380 mosmol/kg · water⁻¹; 40 seconds) in rats that had received bilateral injections of AAV2-CBA-eGFP, AAV2-CBA-MIF, or AAV2-CBA-[C60S]-MIF into the PVN 10 days earlier.

purchased from Charles River Farms (Wilmington, MA). All of these procedures were approved by the institutional animal care and use committee of the University of Florida. For the experiments that generated the data shown in Figures 3 to 6, we used a total of 42 male Wistar rats (60 to 120 g; postnatal age: 28 to 38 days), purchased from B&K. All of the procedures conformed to the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Bristol Ethical Review Committee.

Adenoassociated Viral Vector 2 Constructs

Three adenoassociated viral vectors (AAVs), AAV2-chicken β-actin promoter (CBA)-MIF, AAV2-CBA-[C60S]-MIF, and AAV2-CBA-enhanced green fluorescent protein (eGFP), were constructed and prepared exactly as detailed previously.29 These constructs contained expression cassettes flanked by the rAAV2 terminal repeats. Expressions of eGFP, MIF, and [C60S]-MIF were driven by a CBA with a human cytomegalovirus enhancer. Vector doses were expressed as genome copies.

Gene Transfer Into the PVN In Vivo

Juvenile (28- to 38-day–old) rats were anesthetized with ketamine (60 mg/kg) and medetomidine (250 mg/kg) by intramuscular injection and placed in a stereotaxic frame (David Kopf Instruments). The skull was leveled between bregma and lambda and bilateral injections (500 nL) of the recombinant AAV2-CBA-MIF (1.0×10⁸ genome copies per microliter), AAV2-CBA-C60S-MIF-eGFP (1.0×10⁸ genome copies per microliter), AAV2-CBA-eGFP (8.3×10⁶ genome copies per microliter), or control solution (0.9% saline) were performed into the PVN using the following stereotaxic coordinates: 0.8 mm caudal to bregma, 0.3 mm lateral to bregma, and 7.2 mm ventral to the dura mater. (Note that, in preliminary experiments, the appropriate PVN coordinates for this size of rat were determined.) Each injection was made over 1 minute. After surgery, anesthesia was reversed with an intramuscular injection of atipamezole (1 mg/kg). Animals were allowed to recover for 7 to 10 days before being studied using the decorticate-perfused in situ preparation.

Decorticate, Unanaesthetized, Arterially Perfused In Situ Rat Preparation

Experiments were performed at the University of Bristol. Male Wistar rats were prepared as described previously.19,30 Rats were placed under deep halothane anesthesia (5%) and assessed by a
of lidocaine to the chain, which was subtracted from the integrated signal during analysis.

**Osmotic Stimulus**
The osmolalities of iso-osmotic and hyperosmotic solutions were, respectively, 290 and 380 milliosmol (mosmol; kilograms \( \cdot \) water \(^{-1} \)), as measured by a freezing point depression osmometer (Camlab, Roebling Micro-osmometer). Hyperosmotic tonic Ringer solution was prepared by adjusting the final concentration of NaCl. The in situ preparation was perfused with isosmotic Ringer solution, and the osmotic stimulus was performed by perfusing the preparation for 40 seconds with hyperosmotic Ringer solution, contained in a separate reservoir and connected to the perfusion system via a 3-way tap.

**ICV Injections of Saline and Analysis of MIF mRNA**
Rats were anesthetized with a mixture of 4% isoflurane in pure \( O_2 \) (1 L/min) and placed in a Kopf stereotaxic frame. Anesthesia was maintained using an \( O_2 \)/isoflurane (2%) mixture delivered through a specialized nose cone for the duration of the injection procedure. Rats were injected into the right lateral cerebroventricle (ICV) with either 2 \( \mu \)L of losartan (2.1 nmol/\( \mu \)L) or isosmotic (0.9%) saline, followed 15 minutes later by ICV injections of 2 \( \mu \)L of iso-osmotic (0.9%) saline or 2.0 mol/L of NaCl at an infusion rate of 1 \( \mu \)L/min. All of the stereotaxic ICV injection procedures were as detailed previously. An analgesic agent (buprenorphine; 0.05 mg/kg SC) was administered to the rats before waking. Three hours later, rats were euthanized, brains were removed, and the PVN was isolated in the PVN. Differences were taken as significant at \( P < 0.05 \).

**Immunocytochemical Detection of Endogenous MIF in PVN Vasopressinergic Neurons**
Please see http://hyper.ahajournals.org for the online Data Supplement for text and Figures S2 and S3.

**Immunohistochemistry for Arginine Vasopressin, Neuron-Specific Nuclear Protein, and Virally Expressed MIF**
Please see the online Data Supplement for text and Figures S2 and S3 (http://hyper.ahajournals.org).

**Data Analysis**
All of the values are expressed as the mean ± SEM. One-way ANOVA followed by Student-Newman-Keuls post hoc or Student \( t \) test were used to assess differences between individual means. Differences were taken as significant at \( P < 0.05 \).

**Results**

**Colocalization of MIF and Arginine Vasopressin in the PVN**
In a previous study, we showed that endogenous MIF staining in the PVN of normotensive rats was localized to neurons primarily with lesser amounts in glia. Because of the importance of vasopressin and vasopressinergic neurons in cardiovascular control, we wished to determine whether endogenous MIF resided within this neuronal phenotype. The lower power fluorescence micrographs shown in Figure 1 (top) depict MIF (green) and arginine vasopressin (AVP) (red) immunofluorescence at 3 different levels of the PVN (−1.60 mm, −1.88 mm, and −2.12 mm relative to the bregma) and also where MIF and AVP overlap. The fluorescence micrographs shown in Figure 1 (middle) are a higher power view (from the inset shown at the top left) depicting MIF (green) and AVP (red) immunofluorescence at PVN level −1.60 mm relative to bregma. MIF and AVP colocalizations are shown in orange. Quantification of the colocalization of MIF and AVP in different parts of the PVN is shown in the bar graphs in Figure 1. The data in the left bar graph show the number of immunoreactive AVP cells counted at each of the 3 PVN levels and also the number that overlap with MIF-positive cells. The data in the right bar graph show the colocalization of AVP and MIF in various subregions of the PVN at each of the levels investigated. Collectively, these data indicate that the degree of colocalization is low within each of the PVN levels, and when it occurs it is in both magnocellular and parvocellular regions of the PVN.

**Induction of MIF mRNA Expression in the PVN by Central Hyperosmotic Stimulation**
Because the PVN mechanism for hyperosmolality-induced increases in sympathetic nervous system activity is Ang II/AT\(_R\)–dependent and MIF is a regulator of Ang II/AT\(_R\) actions in the PVN, \( \text{AT}_1\text{R} \) mediated20 we investigated whether hyperosmotic challenge could induce changes in endogenous MIF mRNA levels at this hypothalamic nucleus. The data in Figure 2 indicate that rats injected ICV with hypertonic NaCl (2 \( \mu \)L of 2.0 mol/L) displayed significantly greater levels of MIF mRNA in the PVN when compared with rats that underwent ICV injections of 0.9% saline. This stimulatory effect of hypertonic saline on MIF mRNA levels in the PVN was abolished by pretreatment of rats with losartan (2.1 nmol/\( \mu \)L; ICV; Figure 2).

**Increased Expression of MIF in the PVN Blunts the HS-Induced Sympathoexcitation**
As an initial step, we performed studies to confirm the dependency of HS-induced increases in SNA on AT\(_1\text{R}\) activation in the in situ preparation. Baseline SNA was recorded in rats perfused intra-arterially with isosmotic solution (290 mosmol/kg \( \cdot \) water \(^{-1} \)). After this, rats exposed to HS by perfusion for 40 seconds with a hyperosmotic solution (380 mosmol/kg \( \cdot \) water \(^{-1} \)) demonstrated a 23 ± 3% increase in SNA. Addition of the AT\(_1\text{R}\) antagonist losartan (20 \( \mu \)mol/L) to the perfusate for 10 minutes severely reduced the HS-induced sympathoexcitation to 5 ± 5% (\( P = 0.002 \)). Considering that the HS-induced sympathoexcitation depends on the PVN19 and is AT\(_1\text{R}\) mediated20 (please see Figure S1 at http://hyper.ahajournals.org), hyperosmotic challenge increases MIF expression in the PVN (Figure 2), and MIF counterregulates AT\(_1\text{R}\) actions with the PVN, \( \text{AT}_1\text{R} \) mediated20 we determined whether increased expression of MIF in this hypothalamic nucleus would modify the increase in SNA produced by acute HS. The left and right PVN of rats were microinjected with 0.9% saline, AAV2-CBA-MIF, or AAV2-CBA-eGFP, as described in the Methods section. AAV2-CBA-MIF and AAV2-CBA-eGFP produce expression of MIF and eGFP, respectively, in the PVN within 7 days. Seven to 10 days after the PVN injections, the decorticate-perfused in situ rat preparation was made. Baseline SNA in each group was established after intra-arterial perfusion of isosmotic Ringer solution (290 mosmol/kg \( \cdot \) water \(^{-1} \)). Intra-arterial infusion of
hyperosmotic solution (Ringer solution at 380 mosmol/kg water) for 40 seconds elicited sympathoexcitation in both the 0.9% saline and eGFP groups (increases of 27±4% and 25±4%, respectively; Figures 3 and 4). In contrast, HS for 40 seconds did not produce sympathoexcitation in the rats overexpressing MIF in the PVN (increased SNA of 4±5%; Figures 3 and 4A; P=0.008 versus saline). The area under the curve and the duration of the sympathoexcitation were reduced in the MIF-treated rats compared with the 0.9% saline (respectively, P=0.012 and P<0.001) and the eGFP groups (P=0.019 and P<0.001; Figure 4B and 4C). When MIF injections were located outside of the PVN (0.5 mm dorsal; see Figure 5A), there was no inhibition of the HS-induced sympathoexcitation, and a response similar to control was observed (data not shown), indicating a specific role of the PVN in this response. Finally, the sympathoexcitatory response to stimulating the peripheral chemoreflex (NaCN: 0.03%; 25 to 75 L) was similar between control and MIF-expressing rats indicating relative specificity of MIF in the PVN for HS-induced sympathoexcitation (data not shown).

The inhibitory effects of MIF on Ang II actions in the PVN are mediated by its intrinsic TPOR moiety.27,29 Because the sympathetic response to hyperosmotic stimulation was AT1R dependent, we next tested whether the inhibitory effect of MIF in the PVN on HS-induced sympathoexcitation was also

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**Figure 4.** HS-evoked sympathoexcitation is reduced by MIF and augmented by [C60S]-MIF expression in the PVN. Sympathoexcitation was measured as the percentage increase (peak response) from baseline over the 40-second period of HS (A), the total increase above baseline (B), and the duration of the response (C). Values in bar graphs are mean±SEM from rats that had received bilateral injections of saline, AAV2-CBA-eGFP, AAV2-CBA-MIF, or AAV2-CBA-[C60S]-MIF into the PVN 10 days earlier. (*P<0.05, 1-way ANOVA; A: F(3,20)=11.432; B: F(3,20)=24.891; C: F(3,20)=77.750; n=6 rats per group).

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**Figure 5.** Localization of MIF transduction in the PVN. A, The center of the injection sites for eGFP, MIF, and C60S-MIF within the PVN are indicated. Spread of transduction was between 400 to 500 µm in diameter. Injections of MIF that fell outside of the PVN were not effective in blocking the hyperosmolality-induced sympathoexcitation. Section levels are relative to the bregma. Abbreviations: PaDC indicates paraventricular hypothalamic nucleus, dorsal cap; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP, paraventricular hypothalamic nucleus, medial parvicellular part; PaV, paraventricular hypothalamic nucleus, medial magnocellular part; Pe, periventricular hypothalamic nucleus; Arc, arcuate hypothalamic nucleus; 3V, third ventricle. B, A representative example of MIF immunostaining in PVN induced by AAV2-CBA-MIF in vivo gene transfer.
attributed to its TPOR activity. An additional group of rats received injections of AAV2-CBA-[C60S]-MIF bilaterally into the PVN for expression of [C60S]-MIF as before.29 [C60S]-MIF, in which the cysteine at position 60 is replaced with serine, lacks TPOR activity.32 In contrast to MIF, the HS-induced sympathoexcitation was potentiated (45% versus 6%) in rats that expressed [C60S]-MIF (Figures 3 and 4A; P=0.023 versus saline). The area under the curve and the duration of the sympathoexcitation were both augmented significantly in the [C60S]-MIF group (Figure 4B and 4C; P<0.001 versus saline).

Localization of MIF Expression in the PVN
Schematic reconstruction of injection sites at different rostral-caudal levels of the PVN are shown in Figure 5A. As can be seen, the sites of injection of AAV2-CBA-MIF, AAV2-CBA-[C60S]-MIF, and AAV2-CBA-eGFP were, in most part, within the PVN (maximal spread was between 400 to 500 µm in diameter). We never saw transduction of circumventricular organs or ependymal cells. We recognize the difficulty of distinguishing between endogenous and virally expressed MIF, which is a limitation of our study. However, we did use a lower concentration of antibody to detect virally induced MIF that rarely detected endogenous protein (1:500 versus 1:200 dilution, respectively; please see Figure S2 at http://hyper.ahajournals.org). Figure 5 also shows sites of AAV2-CBA-MIF injection outside the PVN, which were ineffective. Figure 5B shows a fluorescence micrograph depicting bilateral expression of MIF immunoreactivity in the PVN, typically obtained after AAV2-CBA-MIF injection. Virally expressed MIF is largely found in neurons, as demonstrated by its coexpression with the specific neuronal marker neuron-specific nuclear protein (NeuN) (Figure 6A). Interestingly, the neuronal populations in which MIF is transduced in the PVN include vasopressinergic neurons (Figure 6B).

Discussion
The major findings of this study are as follows: (1) MIF is present in PVN vasopressinergic neurons; (2) hyperosmotic challenge increases MIF expression in the PVN, which is AT1R dependent; (3) increased MIF expression within the PVN of normotensive rats abolishes the sympathoexcitation produced by hyperosmotic stimulation; and (4) the actions of MIF appear to be mediated by its intrinsic TPOR activity. Collectively, these data provide the first indication that MIF,
present within neurons in the PVN, serves as a negative regulator of HS-induced sympathoexcitation. The demonstration that HS conditions also produce an increase in MIF mRNA expression in the PVN opens up the possibility that MIF is a major central nervous system regulator of sympathetic outflow in conditions of high salt load.

The osmotic stimulus used in our in situ experiments is higher than that used previously (see Reference 19). Here we used 380 mosmol/kg · water. This produced a robust and reproducible sympathoexcitation that was important for assessing accurately the magnitude by which MIF expression in PVN could sequester this evoked response. We acknowledge that the stimulus is high. However, this level of osmolality is unlikely to be seen by the preparation because of the transient nature of the stimulus and significant dilution that occurs within the perfusion circuit and preparation; this is discussed further in the online Data Supplement at http://hyper.ahajournals.org.

The mechanisms for hyperosmolality-induced increases in SNA from the PVN are Ang II/AT1R dependent, and the negative regulatory action of MIF quenches Ang II/AT1R-stimulated increases in neuronal discharge and elevations in arterial pressure. Findings from the latter study were consistent with the earlier in vitro observation that the Ang II-increased firing response of PVN neurons is blunted by MIF, which is likely to be activated by continual AT1R stimulation. Thus, an Ang II activity-dependent feedback mechanism may exist to protect against overstimulation by Ang II, which we presume operates in vivo. However, crucial further experiments should include determining whether HS-induced induction of endogenous MIF in the PVN can be prevented by AT1R blockade and provide tempering over HS-induced sympathoexcitation. Also, it now becomes important to determine the role that MIF plays in the PVN for chronic elevations of sympathetic nerve activity and arterial pressure observed during dehydration, for example.

We acknowledge that our study does not allow us to differentiate whether the MIF mechanism is sodium or osmotically sensitive. However, if endogenous MIF does serve as a “salt-sensitive” endogenous regulator of HS-induced sympathoexcitation, then one primary question concerns the nature of the mechanisms that mediate hyperosmolality-induced increases in MIF expression in the PVN. Osmolality-induced changes in SNA are mediated through osmosensitive neurons in the OVLT and subfornical organ, which subsequently transmit information concerning the osmotic status of the animal via efferent pathways to the PVN. Ang II, released either from these efferent pathways or generated locally within the PVN itself increases, via presynaptic and/or postsynaptic AT1R, the activity of PVN presympathetic neurons projecting to the intermediolateral nucleus or rostral ventrolateral medulla. Our current data confirm that the HS-induced sympathoexcitation is AT1R dependent. Considering that ICV injection of Ang II increases MIF expression in the PVN of normotensive rats, it is likely that the HS-induced MIF expression in the PVN is also Ang II/AT1R dependent. However, overexpression of MIF does not affect basal blood pressure in both normotensive (this study) and spontaneously hypertensive rats, suggesting a specific functional role related to salt-mediated sympathoexcitation.

The inhibitory action of MIF on HS-induced sympathoexcitation appears to involve its TPOR activity. This is because increased expression of the mutant protein [C60S]-MIF in the PVN failed to blunt the HS-induced increased in SNA (Figures 3 and 4). However, the [C60S]-MIF rats displayed an unexpected higher increase in sympathoexcitation in response to HS challenge compared with the eGFP group (Figures 3 and 4). The reasons for this action of [C60S]-MIF are not obvious but may involve a dominant-negative action of this mutant protein over endogenous MIF within the PVN; however, this remains speculative. If so, this would argue that PVN parvocellular neurons are under tonic control by MIF, which would be entirely consistent with the specific role as a mediator of increased sympathetic activity in response to salt loading.

The finding that a vasopressinergic descending pathway from PVN to intermediolateral nucleus contributes to sympathoexcitation elicited by hyperosmolality suggests that a possible location of MIF’s inhibitory actions in the PVN include the vasopressinergic neurons. Indeed, our data (Figure 1) illustrate that PVN vasopressinergic neurons express MIF endogenously. Moreover, Figure 6B demonstrates that the AAV2-CBA-MIF–injected rats display MIF transduction into AVP neurons in the PVN. Thus, it is likely that the inhibitory actions of MIF in the PVN on HS-induced sympathoexcitation involve, in part, the vasopressinergic neurons. However, whereas the CBA promoter that is used in the present study produces MIF expression primarily within neurons in the PVN, it does not discriminate between different neuronal phenotypes. Further development of promoters that would allow for transduction of MIF into specific neuron populations (eg, AVP) would help to tease out whether the actions of MIF in controlling sympathoexcitation in response to HS are limited to a specific neuronal phenotype, such as glutamatergic PVN neurons known to be activated by osmotic stimuli that project to the rostral ventrolateral medulla. Also, we cannot comment on whether AT1Rs involved are localized to the vasopressinergic neurons or nonvasopressinergic interneurons within PVN.

Perspectives
Our new findings indicate that MIF operates as a novel inhibitor within the PVN of osmolality-induced increases in sympathetic outflow and substantiates the developing notion that MIF is an important regulator of Ang II/AT1R–induced excitability within the PVN, especially to salt loading. Further phenotyping of the MIF containing neurons in the PVN in terms of their neurochemical content and connectivity is now needed. Subsequently, chronic studies to assess whether MIF expression in the PVN can prevent the hypertension associated with salt loading and dehydration in conscious animals would further validate the importance of this protein. Indeed, whether MIF plays a role in controlling other forms of hypertension, such as stress-related hypertension, would add further credence as to its wider applicability to hypertension. It also needs to be established where else in the brain MIF may exist to keep sympathetic nerve activity in check:
the nucleus tractus solitarii and rostral ventrolateral medulla are both likely sites. The significant finding described herein that the salt-induced sympathetic overdrive depended on the TPOR activity of MIF leads way to proof-of-principle studies testing whether the AAV2-CBA-C60S-MIF-eGFP vector can prevent hypertension development in a salt-sensitive rat model such as the Dahl. Accordingly, we propose that MIF might be exploitable as a novel target to control (salt-related) hypertension in humans.

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Disclosures
None.

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Macrophage migration inhibitory factor (MIF) in the paraventricular nucleus plays a major role in the sympathoexcitatory response to salt

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Methods
The following protocols were based on those used before (see supplemental references 1-4 below).

Immunocytochemical detection of endogenous MIF in PVN vasopressinergic neurons
Adult rats were anesthetized and euthanized following protocols approved by the University of Florida Institutional Animal Care and Use Committee. Brains were perfused transcardially in situ with 0.9% saline containing 4% formaldehyde and then cryoprotected in 30% sucrose for 1 week. The prepared brains were trimmed and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA). Forebrain blocks were stored at 80ºC until they were sectioned on a Leica CM 1850 cryostat. Fixed frozen sections (15 µm) were cut from the PVN and air dried overnight at room temperature. Slides were washed for 5 minutes to remove residual OCT. Sections were blocked in 5% goat serum for 30 mins and then incubated overnight at 4C in a mixture of 1:200 rabbit anti-MIF (Torrey Pines Biolabs, Inc, Houston, TX, USA) and 1:250 Guinea pig anti-AVP (Peninsula laboratories LLC, San Carlos, CA, USA). Slides were washed in buffer 3 times for 10 minutes and incubated for one hour at room temperature in combined secondary antibodies. (Alexa Fluor 594 goat anti- guinea pig and Alexa Fluor 488 goat anti-rabbit IgG, both at a 1:1000 dilution, Invitrogen, Carlsbad, CA). The sections were mounted in Vectashield and imaged using an Olympus BX41 fluorescence microscope. For the analyses of colocalization of MIF and AVP immunoreactivities, we focused on 3 levels of the PVN as described by Stocker et al., 2004. Relative to the bregma, these levels were -1.60mm, -1.88mm and -2.12mm. In addition, we utilized the same nomenclature as Stocker et al. to describe the subregions of the PVN at each of these levels: dp = dorsal parvocellular; mp = medial parvocellular; pm = posterior magnocellular; vp = ventrolateral parvocellular; lp = lateral parvocellular. Numbers of prominent green (MIF), red (AVP) and orange (MIF/AVP co-localized) cells in each subregion at each of the above 3 levels were counted. The data presented in Figure 1 are mean ± SEM of numbers of AVP or AVP/MIF positive cells.

Immunohistochemistry for AVP, NeuN and virally expressed MIF
After the experiments with the perfused in situ preparation, the brains were removed and left for 2 days in 4% paraformaldehyde in 0.1 M PBS at 4º C, followed by another 2 days in the cryoprotectant solution of 20% sucrose and 4% paraformaldehyde in 0.1 M PBS at 4ºC. The brains were then rapidly frozen over liquid nitrogen and coronal sections (30 µm) cut of the PVN using a cryostat (Leica Cryocut CM1900, Switzerland). The free-floating sections were collected in 24-well tissue culture plates containing PBS prior to being processed for immunohistochemical detection of MIF, vasopressin and/or NeuN as follows. Free-floating rat hypothalamic sections were incubated for 15 minutes in a blocking solution comprised of 10%
normal goat serum (NGS, Sigma, St. Louis, MO) and 0.3% Triton X-100 (Sigma) in 0.1 M PBS followed by rinses in PBS (3 × 10 minutes). One set of sections were incubated in rabbit anti-rat MIF primary antibody (1:500; Torrey Pines Biolabs) with mouse monoclonal anti-NeuN antibody (1:1000, from Chemicon International). A further set of sections were incubated with rabbit anti-rat MIF and monoclonal mouse anti-neurophysin II (1:200; vasopressin-derived; PS41) in PBS containing 1% NGS and 0.3% Triton X-100 for 24 hours at 4°C (anti-neurophysin II was kindly provided by Prof. H. Gainer, National Institutes of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, MD) as performed previously. After the primary antibody incubation the sections were rinsed in PBS (3 × 10 minutes) prior to 1-hour incubation in biotinylated goat anti-rabbit (1:500, Vector Laboratories), followed by another rinse in PBS (3 × 10 minutes). The sections were then incubated for 1 hour with Strept-avidin Alexa Fluor 488 conjugate and Alexa Fluor 594 goat anti-mouse (both 1:500, Molecular Probes). Following further rinses in PBS (3 × 5 minutes) sections were mounted onto slides in 0.5% gelatin and allowed to air-dry for 10-15 minutes before being cover slipped using an antifade fluorescent mounting solution (VectorShield, Vector Laboratories). The sections were visualized on a fluorescence microscope (Leica DM IRB with C-Plan optics; Leica, Germany) using the appropriate filter. The extent of virus spread were carefully examined and quantified. We observed that the dorsal-ventral, medio-lateral and rostral-caudal extent of the injection covers a sphere of approximately 400-500 μm diameter.

Discussion

Extended Discussion on the osmotic stimulus used in our in situ experiments: We used 380 mOsmol kg water⁻¹ as this produced a robust and reproducible sympathoexcitation that was important for assessing accurately the magnitude by which MIF expression in PVN could sequester this evoked response. We acknowledge that the stimulus is high. However, this level of osmolality is unlikely to be seen by the preparation. There is significant dilution of the hypertonic perfusate prior to entering the preparation. On making the switch there will be significant mixing of the hypertonic solution with the isotonic solution already in the perfusion circuit. The latter is around a metre in length and contains two ~18 ml bubble traps and a filter holder with an additional volume of ~15 ml; all the latter will contain isoosmotic perfusate providing dilution. The switch from the isoosmotic to the hypertonic perfusates is also transient so achieving osmotic equilibrium is unlikely. On switching back to isoosmotic perfusate the hyperosmotic solution will again be diluted. Further dilution will occur within the circulation and tissues of the preparation. Given the transient nature of the stimulus and its significant dilution, we do not believe that the brain is exposed to 380 mOsmol (kg water⁻¹). Moreover, the sympathetic hyperactivity observed was antagonised by losartan, suggesting that the response was specific and mediated by physiological mechanisms that sense high salt.
References


Online supplemental Figure S1

Online supplemental Figure 1 legend:
Sympathoexcitation evoked by HS is mediated by AT1R. Bar graphs are means ± SEM of the change in SNA produced by HS in the absence or presence of losartan (20 μM). (* = P<0.05, Paired t test; n=6).

Online Supplemental Figure S2
Online Supplemental Figure S3
Immunocytochemical preabsorption control for MIF staining

MIF staining - AAV2-CBA-MIF induced MIF expression

MIF Staining: MIF antibody pre-absorbed with rMIF

(10 μM)