MACROPHAGE MIGRATION INHIBITORY FACTOR AND THIOREDOXIN IN THE BRAIN: IMPLICATIONS FOR HYPERTENSION

By

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To my husband, Brad. Your constant support and encouragement have made this possible.

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I must begin by acknowledging my husband. He has stood by me and held me up through the most difficult of times. Without his constant humor and perspective, there would often have been little laughter through the tears.

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By

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Previous studies from our laboratory have established that macrophage migration inhibitory factor serves as a negative regulator of the neuronal chronotropic actions of angiotensin II in normotensive rats, but not in spontaneously hypertensive rats. Furthermore, hydrogen peroxide is a known effector of angiotensin II signaling in paraventricular hypothalamic neurons and is an established inducer of macrophage migration inhibitory factor expression in the periphery. Therefore, for the first study contained in this dissertation, we hypothesized that hydrogen peroxide may be able to induce macrophage migration inhibitory factor expression in neurons, and we sought to ascertain whether normotensive and spontaneously hypertensive neurons respond differentially to hydrogen peroxide stimulation with regard to expression of this small protein.

We examined the effects of hydrogen peroxide (30 μmol/L) on macrophage migration inhibitory factor expression in neuronal cultures from normotensive (Wistar Kyoto and Sprague Dawley) and spontaneously hypertensive newborn rats. The data indicate that hydrogen peroxide induces macrophage migration inhibitory factor expression in neurons cultured from normotensive rats, but not spontaneously hypertensive rats. Lactate dehydrogenase and protein carbonyl assays suggest that 30 μmol/L hydrogen peroxide is neither cytotoxic to the neurons,

nor does it cause oxidative stress. Studies with polyethylene glycol-catalase and actinomycin D suggest that the hydrogen peroxide is acting intracellularly to increase transcription of the macrophage migration inhibitory factor gene. We conclude that macrophage migration inhibitory factor expression is regulated differentially in normotensive and spontaneously hypertensive rat neurons in response to hydrogen peroxide signaling.

Furthermore, oxidative stress has become an exciting field of study with regard to neurogenic hypertension. However, it remains to be determined if oxidative stress in the brain contributes to the development of or is a consequence of this disease. The second study contained in this dissertation examines the expression and cellular localization of two important antioxidant proteins, macrophage migration inhibitory factor and thioredoxin, in the hypothalamic paraventricular nucleus of spontaneously hypertensive rats and their normotensive controls, Wistar Kyoto rats. Importantly, these studies were performed in newborn (i.e., not yet hypertensive) rat brains. Our studies establish dysregulation of expression of these two proteins in the brain of newborn rats that are destined to be hypertensive and associates them with oxidative stress that occurs well before the onset of hypertension.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Characteristics of Macrophage Migration Inhibitory Factor

Macrophage migration inhibitory factor (MIF) (Figure 1-1A) is a small (12.5 kDa), highly conserved protein with nearly ubiquitous tissue distribution that was originally described in 1966 as a soluble factor that was expressed by T cells in delayed type hypersensitivity and inhibited the random migration of macrophages.[1-3] The factor behind this activity was not cloned and identified as MIF until 1989,[1] and, when MIF was originally characterized, it was designated a lymphokine (later commuted to the more modern and inclusive term "cytokine"). Structurally, MIF does not have any notable domains[4] (though it does have some enzymatic motifs that will be discussed in detail later), and it is still not clear whether the MIF homotrimer, the entity identified in all of the structural studies, is the functionally relevant form. On the contrary, several studies suggest that the dimer or the monomer may be responsible for the actions of MIF in vivo.[5-7]

Today, determining a functional niche for MIF is far more complicated than it was 40 years ago. It has been suggested that MIF is a cytokine, a chemokine, a hormone, and an enzyme, and the arguments for all of these designations are compelling.[8, 9] Clearly MIF has many, sometimes seemingly disparate, biological and cellular functions. Physiologically, MIF plays modulatory roles in the immune, endocrine, and nervous systems.[10] Pathologically, when dysregulated, MIF contributes to a wide range of inflammatory disorders and plays a pivotal role in tumor biology.[11, 12]

MIF seems to be somewhat of a "moon-lighting" protein in that its functions can be divided into two basic categories. It is not yet clear, but we hypothesize that this may depend on whether it is secreted to initiate signal transduction (cytokine-like) or remains in the intracellular

space (enzymatic, perhaps antioxidant). Much of the traditional literature focuses on the pro-inflammatory, cytokine-like function of MIF. MIF is considered to be rather an orphan cytokine, or perhaps the first discovered member of a new cytokine family, since it bears no similarity to other cytokine families,[1] nor does its receptor, a complex consisting of CD74 and CD44. CD74 is necessary for MIF binding, and CD44 appears to responsible for initiating intracellular signaling cascades.[13]

Yet, clearly MIF can function as a pro-inflammatory mediator, and the overwhelming majority of the literature supports this notion. It can be secreted, presumably via a leaderless pathway,[14] by many types of immune, endocrine, and parenchymal cells,[15, 16] and it is well-established that secreted MIF is a critical player in inflammatory conditions such as sepsis, rheumatoid arthritis, asthma, cystic fibrosis, atherosclerosis, and glomerulonephritis — to name only a few.[17-20] It should be noted that MIF appears to be acting as a chemokine, signaling via chemokine receptors rather than it's recently-identified (and assumed to be canonical) CD74/CD44 receptor complex, in the instance of atherosclerosis.[9]

However, MIF is distinct from other traditional cytokines in many interesting ways. Evidence suggests that it is constitutively expressed and exists in pre-formed pools in the cytosol of many immune and non-immune cells.[15, 21] In contrast, traditional cytokines are usually produced *de novo*, with a lag time for transcription and translation, when induced.[22] Furthermore, as previously mentioned, the MIF peptide does not appear to contain a leader sequence, exists as an entity free of secretory vesicles in the cytoplasm of cells, and is most likely secreted in a regulated fashion by a non-traditional route, rather than going through a traditional secretory pathway. Exactly how MIF is secreted is not yet fully understood, but the limited evidence available suggests it is most likely via a transporter belonging to the ATP-binding cassette

(ABC) family.[14] MIF is also unique in that its secretion is induced by glucocorticoids, and it plays a critical role as a circulating counter-regulator of the immunosuppressive effects of glucocorticoids. MIF is able to promote inflammatory responses despite physiological levels of serum glucocorticoids, and it has been suggested that permitting inflammatory responses when appropriate in the face of glucocorticoids - which have many homeostatic functions, aside from their immunosuppressive actions - is precisely the intended purpose and physiological role of secreted MIF. Concordant with this suggested anti-glucocorticoid role, under normal conditions glucocorticoid-induced MIF secretion follows a bell-shaped dose-response curve, with MIF secretion peaking at physiological glucocorticoid levels, and recent studies suggest that MIF fluctuates in a circadian rhythm that is correlated to serum glucocorticoid levels in rodents.[23]

Aside from its classification as a cytokine, in recent years, a revolutionary group of investigators have discovered that MIF is also an enzyme, exhibiting a thiol-protein oxidoreductase (TPOR) activity that lies between amino acid residues 57 and 60.[24] Accordingly, it has recently been suggested that MIF be re-classified as not only a cytokine, but also a member of the thioredoxin (Trx) family of antioxidant proteins due to the fact that it contains this TPOR motif, an identifying characteristic of Trx family members.[25] However, it should be noted that there are cysteine-dependent, redox-active enzymes that are not considered members of the Trx family, as well. Peroxiredoxins, enzymes that contribute to the regulation of cellular signaling by scavenging and, therefore, controlling the intracellular levels of hydrogen peroxide — an important signaling molecule — at the expense of reducing equivalents donated by Trx, are one such example.[26] Should future investigations and further clarification of the functions and characteristics of MIF (which has yet to be crystallized as a monomer or homodimer) determine that it is not a Trx-family member, it may be possible that MIF, like peroxiredoxin 1, is the

canonical member of a new family of TPOR-dependent enzymes (and, perhaps, these enzymes could be assigned to a larger family designated simply as the TPOR family, as was suggested by Thiele and Bernhagen in their recent review on the enzymatic activities of MIF). This would hardly be surprising, given the fact that researchers working on the secreted, cytokine-like function of MIF have proposed that the same could be said of MIF as a cytokine. Efforts to assign it to a cytokine family have failed, yet the evidence clearly shows that it can serve this function.

Nonetheless, assuming the proposition that MIF belongs in the Trx family is correct, as one would expect, there is limited evidence that the production of MIF can be influenced by the redox status of the cell.[25] Because of this proposed dichotomous nature of MIF, many authors have come to refer to it affectionately as a "cytozyme" or "redoxkine". Members of the thioredoxin family are essential to maintaining redox balance in the intracellular environment by scavenging of reactive oxygen species (ROS), act as electron donors for reducing enzymes, and they may also influence signaling pathways and the activity of other proteins by reducing critical cysteines, as will be discussed in detail later; and the available evidence, though tentative compared to the body of literature concerning these functions of Trx, is ever-mounting and suggests that MIF is no exception in this regard.[25, 27]

To reconcile these seemingly disparate functions of MIF, great minds in the MIF field suggested several years ago that perhaps whether MIF acts as a pro-inflammatory cytokine or an antioxidant protein may 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.[28] While the circumstances that determine whether MIF fulfills its cytokine or TPOR-based enzymatic function remain a mystery, a growing body of evidence indicates that

the simplest explanation is that something as simple as localization may be the determining factor. Perhaps, when MIF is needed as a pro-inflammatory mediator, it is secreted and signals via its canonical or chemokine receptors. To further complicate matters, it has been suggested that, even when secreted MIF is acting in its cytokine capacity, the TPOR motif of MIF might be involved in mediating its association with the CD74 portion of its receptor complex.[25] Conversely, in the absence of a stimulus that would promote secretion, MIF remains in the cytoplasm of the originating cell, and studies from many laboratories, including ours, suggest that it then acts as a TPOR protein and probable member of the Trx family, influencing redox-regulated targets and perhaps even scavenging ROS.

Introduction to the Thioredoxin Family of Proteins

Thioredoxin (Trx) (Figure 1-1B) was first discovered in the 1960s — coincidentally, around the same time as MIF — as a hydrogen donor for deoxyribonucleotide synthesis in E. coli.[29] Contrary to the long history in prokaryotes, interest in the eukaryotic Trxs is a relatively new matter, taking shape over the past couple of decades. The following sections will focus on mammalian Trxs, specifically Trx1, referred to here after simply as "Trx". As the characteristics of Trx are discussed, I will point out ways in which Trx and MIF are alike — to support the argument that MIF may be a Trx family member — but also ways in which MIF and Trx may be different in order to present a balanced perspective. Interestingly, in some ways, MIF exhibits characteristics more similar to glutaredoxin (Grx), the other major member of the Trx family. When appropriate, similarities between MIF and Grx will also be discussed.

Before launching into a detailed discussion of Trx and its many interesting features, in the interest of perspective, a brief review of Trx systems in mammals is warranted. There are two distinct isoforms of Trx in mammals. Trx1 is usually found in the cytoplasm of cells, [27] but can be induced to localize to the nucleus or be exported from the cell under the correct stimuli.[30, 31]

Trx2 is a mitochondrial protein.[32] The active site of Trxs becomes oxidized, forming a disulfide bond between the active site cysteines, over the course of catalysis (Figure 1-2). They are reduced, returning to the active form, (usually at the expense of NADPH) by Trx reductases (TrxR), large selenocysteine enzymes with active sites consisting of the amino acid sequence Gly-Cys-SeCys-Gly. There are 3 types of TrxRs: TrxR1 (cytoplasmic), TrxR2 (mitochondrial), and the testis-specific thioredoxin glutathione reductase.[27] The thioredixon 1 gene (TXN1) is well-characterized and contains two overlapping promoters exhibiting elements for basal and inducible regulation of Trx expression. Interestingly, Sp1 seems to be important in the basal regulation of the Trx gene,[33] and a recent publication has shown the same to be true for MIF.[34] An important inducible cis element of the Trx gene that, so far, appears to be lacking in the regulation of the MIF gene is the antioxidant response element (ARE), which is particularly well-studied because it is responsible for the induction of Trx under conditions of oxidative stress.[30, 35]

Glutaredoxins (Grxs) are the other major well-known members of the Trx family. They are further-removed from the purposes of this discussion than Trx, so will be reviewed in much more brevity. They share some functions with Trx enzymes, but also have some independent functions. Mammalian cells contain 3 Grx isoforms: the dithiol-mechanism Grxs known as Grx1 (cytosolic) and Grx2 (mitochrondrial), and the monothiol-mechanism Grx5 (named as such due to homology to yeast Grx5, appears to be mitochondrial). Oxidized Grxs are reduced by glutathione, which is then reduced by glutaredoxin reductases at the expense of NADPH. Grx2 is interesting in that it can also be reduced by TrxRs.[27]

Characteristics and Functions of Thioredixon 1: A Comparative Study

Trx family members have a characteristic CXXC motif (Figure 1-1), with X being any amino acid, which is responsible for the redox enzymatic function of these proteins. Trx's active

site contains a Cys-Gly-Pro-Cys motif that was identified when the protein was first sequenced in 1968.[36] MIF follows this rule; as mentioned above it shares this identifying characteristic, exhibiting a Cys-Ala-Leu-Cys motif in its active site. Importantly, MIF also shares some additional conserved residues with other Trx family members: a phenylalanine that is 5 to 7 residues upstream of the N-terminal Cys of the CALC motif and a leucine/valine (a leucine in the case of MIF) an additional 2 to 3 residues in the N-terminal direction.[25]

Though the structure of the MIF monomer is vaguely similar to the Trx monomer, one important characteristic of the Trx family that MIF is lacking is the "thioredoxin fold" structural motif, consisting of 4 beta sheets and 3 alpha helices in most family members. Trx contains this basic thioredoxin fold, plus an additional beta sheet and alpha helix at the N-terminus.[37] In contrast, MIF is structurally more like bacterial tautomerases and human D-dopachrome tautomerase, and MIF has a tautomerase enzymatic motif at its N-terminus, in-depth discussion of which is beyond the scope of these studies. The importance of the tautomerase activity of MIF is still unclear and remains controversial, as an in vivo substrate has yet to be identified. Furthermore, there is little sequence homology between MIF and tautomerases, despite their mild structural similarities.[25] However, it is worth noting that MIF is certainly far from the only Trx-like protein that has been discovered but may not fit perfectly into the "Trx mold". There are many, usually tissue- or organelle-specific, proteins that contain CXXC motifs but deviate somewhat from the classical Trx characteristics, yet are still considered to be Trx family members; some of which are redox active, some of which are not.[27]

Nonetheless, there are hundreds of available solved Trx structures, and they reveal some important characteristics of reduced and oxidized Trx. Trx undergoes some conformational changes upon reduction that involve hydrogen bonds in the active site. These changes can affect

the binding of Trx to other proteins.[38, 39] It has been argued that MIF may share this feature of altered conformational states (and, perhaps, altered binding activity) depending on its redox status.[25, 40] Biochemical studies of MIF indicate that none of its 3 cysteines form an intermolecular disulfide bond, but that the cysteines of the CALC motif form an intramolecular bond under the correct conditions (as would be expected of a 2-Cys mechanism Trx protein).[24] Despite the available evidence to the contrary, the limited crystal structures of the MIF homotrimer that are available seem to indicate some spatial constraints on the location of the two cysteines of the CALC motif, with one lying at the N-terminus of a beta sheet and the other located in the preceding loop, which would make an intermolecular disulfide unlikely. However, as Thiele and Bernhagen pointed out in their excellent review on the TPOR activities of MIF, it bears noting that the crystal structures currently available for MIF were obtained from solutions containing MIF at unnaturally high concentrations under reducing conditions. It is possible that this encouraged aggregation of MIF and pushed the equilibrium of the various oligomers towards the homotrimer species.[25] Interestingly, it would be appropriate to mention here that, like MIF, Trx can form dimers (though this claim is controversial and the physiological consequences are unknown)[41, 42] and that Grx2 (a Trx family member) can utilize iron-sulfur clusters to form a bridge that effectively "homodimerizes" the molecule, rendering it enzymatically inactive.[43] It may, therefore, be possible that the formation of oligomers of these redox active enzymes could serve as regulators of their redox activity, and perhaps even sensors of oxidative stress. Despite the conclusions drawn from the 3D structures of the MIF homotrimer, biochemical solution studies on MIF utilized MIF at more physiological concentrations and re-folded MIF under oxidizing conditions, and the resulting MIF was biologically and redox active. From this, the authors concluded that perhaps MIF, like Trx, undergoes major conformational changes during

catalysis that would be more permissive of intramolecular disulfides and have not yet been detected by the limited crystal structures of MIF that are currently available.[25]

Furthermore, regarding interesting regulatory and structural characteristics of Trx, this enzyme has at least 3 cysteines that lie outside the active site and are not involved directly in catalysis: Cys62, Cys69, and Cys73. Cys62 and 69 can form an intramolecular disulfide bond that is not reducible by TrxR and seems to decrease the rate of reduction of the active site by TrxR, which may regulate recycling of Trx to the reduced state.[44, 45] Cys73 also seems to be important in regulating the functions of Trx. It can undergo reversible s-nitrosylation, s-glutathionylation, or disulfide formation. The s-nitrosylation appears to be important in Trx's ability to modulate caspase 3 activity.[46] The glutathionylation of Cys73 renders the enzyme inactive.[47] Most intriguingly, Cys73 can form an intramolecular disulfide bond with the Cys73 of another Trx monomer, leading to the formation of a Trx homodimer.[48] Dimerization, as previously mentioned, is reminiscent of MIF, though the function of dimerization of either molecule remains a mystery.

Though little is known about and intense investigation still surrounds the possible mechanisms of MIF catalysis and recycling, the mechanisms governing the catalysis and reduction of Trx and most of its other family members are well-understood. Trx utilizes its active site cysteines to catalyze the reduction of disulfides via a dithiol mechanism (in contrast to the monothiol mechanism that can be utilized by Grxs). The initial binding of Trx to its targets is governed by a hydrophobic area around the active site. Next, the thiolate of the N-terminal cysteine attacks the target disulfide, which results in a mixed disulfide intermediate. This mixed disulfide between Trx and its target is then reduced by the C-terminal thiolate of the Trx active

site. The result is a disulfide bond between the cysteines of the Trx CXXC motif, which, as mentioned previously, is reduced by TrxR at the expense of NADPH.

Finally, regarding the functions of the Trx molecule, it is most well known as a redox enzyme, but Trx also has functions that are presumed to be non-redox related, or at least are not involved in what is classically thought of as "redox regulation of cellular function". We now know that Trx, like MIF, is a multi-functional molecule, serving at times in many capacities: thiol redox control of transcription factors and enzymes, as an electron donor for many reductive enzymes and peroxiredoxins, as a ROS scavenger and absolutely crucial antioxidant. Other, more recently identified functions of Trx include a cytokine/chemokine-like function (when secreted, interestingly similar to MIF in this regard) and roles in the regulation of protein folding, apoptosis (like MIF, which negatively regulates p53-mediated apoptosis), and NO metabolism.[27]

Macrophage Migration Inhibitory Factor and Thioredoxin in the Brain

It has been demonstrated that MIF is present in the cell bodies and processes of both central and peripheral neurons. In 1998, Bacher *et al*. assessed MIF distribution in the brain of male sprague dawley rats utilizing various techniques such as immunohistochemistry and *in situ* hybridization. They demonstrated the presence of MIF mRNA and protein in neurons of the cortex, hippocampus, cerebellum, pons, and hypothalamus. The authors also reported a diffuse MIF signal localizing to glial cells throughout the brain.[49]

Several laboratories, including our own, have investigated the possible functions of CNS-localized MIF. The aforementioned study showed that MIF can play an inflammatory role in the CNS when its expression and secretion is induced upon central administration of LPS. However, Bucala points out that the high constitutive expression levels of MIF in neurons argue for other, non-inflammatory, physiological functions in the brain.[10] Nishio *et al.* found that MIF may play a role in regeneration of peripheral nerves.[50] Another group has suggested that MIF participates

in detoxifying products of catecholamine metabolism, perhaps serving a neuroprotective function.[51] Also, the glucocorticoid-antagonism function of secreted MIF may be important in protecting hippocampal neurons from glucocorticoid-induced atrophy in situations when these steroids are elevated, such as chronic stress.[10, 49] MIF may also have important functions in modulating the release of cytokines and nitric oxide in the brain.[52, 53] Finally, as will be discussed later in detail, MIF can serve as a negative regulator of the central chronotropic actions of angiotensin II (Ang II).[54]

The localization and functions of Trx in the brain are better understood than is the case for MIF. This is due to the Trx system's status as one of the most important antioxidant defense systems in neurons, which are highly metabolically active – causing them greater exposure to ROS than many other cell types. Moreover, neurons usually exhibit lower levels of other important antioxidants, such as glutathione (GSH), than other tissues. Hence, the Trx system may play a larger role in antioxidant defense in the CNS than it does systemically.[55] Regarding basal expression, Trx is found in neurons throughout the brain, while being mostly absent from glial cells. A notable exception is glial cells of white matter.[27]

However, under conditions of acute stress, such as ischemia, glial cells are the main source of induced Trx expression and secretion, consistent with their supportive role towards neurons.[56, 57] Experiments in culture and *in vivo* show that secreted Trx can have neuroprotective effects. Infusion of Trx, systemically, protects the brain from ischemic events in rodents.[58] Furthermore, when Trx secretion is induced from astrocytomas in cultures, the resulting conditioned medium can be utilized to promote the surivival of neuronal cultures in the absence of serum.[27] Trx may even be an indirect neurotrophic factor, mediating downstream effects of nerve growth factor (NGF).[59] However, dysregulation of the Trx system has been implicated in several

neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and CNS malignancies.[27] Finally, as has been indicated for MIF and will be discussed in detail later, studies from our laboratory suggest that an intracellular function of Trx may be to negatively regulate the central chronotropic actions of Ang II.[60]

The Brain Renin-Angiotensin System

The brain renin-angiotensin system (RAS) is of interest and particularly important due to its implications in the development of essential hypertension. Essential hypertension, also known as primary hypertension, is often simply defined as persistent high blood pressure with no known cause, and sometimes with the added caveat that no preexisting renal disease is present. Kaplan goes further to describe primary hypertension as "the elevation of blood pressure seen in younger people which has a genetic foundation and is shaped by many environmental factors", while making the distinction that the common form of hypertension seen in the elderly is usually isolated systolic hypertension, which "reflects a stiffness of the proximal, capacitance vessels". He further adds that essential hypertension usually reflects an "increase in cardiac output and functional (i.e., reversible) constriction of peripheral resistance vessels."[61] Despite the fact that decades of research into the pathogenesis of this disease have not yet revealed the exact etiology, it is widely held that the brain plays a critical role in the development and maintenance of essential hypertension. Specifically, it is commonly believed that dysregulation of sympathetic outflow and alterations in baroreflex function are the primary avenues by which the CNS contributes to essential hypertension.[62] Much of the evidence for the involvement of the CNS in the pathogenesis of this disease derives from the most-studied animal model of essential hypertension, the spontaneously hypertensive rat (SHR). However, before the evidence concerning this model is presented, a modest review of the components of the RAS is required.

All aspects of the RAS (Figure 1-3) have been identified in the brain, but they have not all been localized to the same cell. Accordingly, it is still not understood exactly how Ang II, the octapeptide mediating the pressor effects of the brain RAS, is generated in the brain and, hence, the subject remains controversial. Nonetheless, there is available evidence suggesting that it is, indeed, generated behind the blood-brain barrier.[63, 64] The RAS is a cascade that originates with the peptide angiotensinogen, which is cleaved by renin. The resulting peptide, angiotensin I, is cleaved by angiotensin converting enzyme (ACE), to the pressor peptide angiotensin II (Ang II). Ang II can then be cleaved into smaller peptides that have, just recently, been identified as having independent functions, but the details are beyond the scope of this discussion.

Once Ang II is generated, it then goes on to bind to either of its cognate receptors, the type I (AT1R) or type II (AT2R) receptor, both of which are G-protein coupled 7 transmembrane-spanning receptors. The AT1R mediates the pressor effects of Ang II and is the predominant isoform expressed in the brain. The contribution of the AT2R to blood pressure regulation remains unknown, though, in a broad sense, the actions of the two receptor sub-types are thought to be antithetical in nature. Significantly, functional studies utilizing receptor blockade strategies indicate the presence of the AT1R in several cardiovascular regulatory regions of the brain, including the paraventricular nucleus (PVN) of the hypothalamus. The PVN is thought to play a critical role in integrating relevant afferent and humoral signals and adjusting endocrine and autonomic responses accordingly.[62]

Studies on the over-activity of the brain RAS in SHR, the most commonly-used model of neurogenic hypertension, abound and are reviewed in excellent detail by Veerasingham and Raizada.[62] Therefore, they will be only briefly touched upon here. In pre-hypertensive animals, Tamura *et. al.* have observed increased brain angiotensinogen in SHR.[65] Ruiz *et. al.*

demonstrated higher levels of renin-like activity in areas important to cardiovascular control such as the NTS and the hypothalamus in SHR compared to control Wistar Kyoto (WKY) rats.[66] Ang II content was found to be increased in the PVN of SHR.[67] Many studies have demonstrated greater Ang II binding and increased AT1R mRNA in most of the areas of the brain thought to be important to fluid balance, control of the cardiovascular system, and/or sympathetic tone such as the SFO, MnPO, PVN, NTS, and RVLM.[68-70]

Studies observing the action of centrally-applied Ang II have concluded, as would be expected when taking all of these observations into account, a greater pressor response in SHR compared to WKY. Studies using pharmacological inhibitors of the RAS have provide further evidence that overactivity of the RAS is involved in hypertension in SHR. For example, losartan, an AT1R inhibitor, injected into the lateral ventricle decreases blood pressure in SHR, but not in normotensive animals. Studies utilizing antisense gene targeting that reduce levels of angiotensinogen and the AT1R decrease BP in SHR as well, but not in WKY. Finally, transgenic studies over-expressing parts of the RAS in the brains of normotensive animals show that hyperactivity of the brain RAS in normotensive animals is sufficient to cause hypertension in these models. Considering all of these points, Veerasingham and Raizada conclude that the increase in brain RAS activity precedes or parallels the development of hypertension in SHR. It is not known exactly how all of these increases in brain RAS activity contribute to hypertension, but it is currently commonly held that they result in increased sympathetic vasomotor tone.[62]

Reactive Oxygen Species, Neuronal Function, and Hypertension

Dr. Robin Davisson, one of the leaders in the neuronal ROS field, has recently published an extensive and excellent review on the neuropathogenesis of hypertension and oxidative stress,[71] therefore the concepts will be only briefly reviewed here. For reference, the proposed pathway for ROS generation in the brain is briefly illustrated in Figure 1-4.

Recently, the fields of redox signaling and neurogenic hypertension have been speedily merging, and the collision has produced several significant studies establishing a firm argument for the involvement of ROS in physiological Ang-II neuronal signaling and oxidative stress in the pathogenesis of hypertension. For example, the Davisson lab has published a study demonstrating that the ability of peripheral Ang II acting on the subfornical organ (SFO), a circumventricular organ that is well-established in the central pressor actions of Ang II and which sends efferents to the PVN,[72] to induce hypertension is mediated by intracellular superoxide.[73] This group has further determined that scavenging of superoxide in the SFO abrogates physiological responses to intracerebroventricular (ICV) Ang II in mice.[74] Other labs have substantiated these studies utilizing ICV tempol, a superoxide dismutase (SOD) mimetic that scavenges superoxide, to prevent ICV Ang II-induced increases in blood pressure and sympathetic activity.[75, 76] On the molecular level, *in vitro* studies have conclusively shown that Ang II regulates neuronal firing by a pathway involving superoxide generation and modulation of potassium currents.[77] Finally, a recent provocative study from our laboratory has indicated oxidative stress in the development of hypertension by showing that over-expression of MIF in the PVN can attenuate the development of high blood pressure in SHR, and this ability of MIF depends on the TPOR motif and, therefore presumably, MIFs catalytic redox activity.[78]

Likewise, advances in understanding the mechanisms by which ROS affect neuronal behavior are steadily progressing. Davisson names at least three ways that ROS can modulate neuronal activity: regulating ion channels, affecting transcription factor activity, and modulating intracellular nitric oxide (NO) levels.[71] Ang II-induced ROS have been shown to affect both calcium and potassium channels in neurons. Specifically, superoxide may open calcium channels directly, stimulating L-type calcium current, and close potassium channels, inhibiting delayed

rectifier potassium current[77, 79, 80] Ang II-derived ROS are also implicated in MAP kinase-mediated neuronal activation, though the details of how MAPKs act in this regard are yet undiscovered.[81]

ROS are also implicated in the modulation of gene expression in neurons by activating various transcription factors. One such example is activator protein 1 (AP-1), a dimer of c-jun and c-fos. The available evidence so far indicates that Ang II-mediated activation of AP-1 via ROS depends upon the MAPK family. The likely MAPK candidates for redox-sensitive Ang II-mediated activation of AP-1 are JNK and FRK.[82-84] Another significant example of a redox-sensitive transcription factor regulated by Ang II-induced ROS is nuclear factor κB (NFκB). Like AP-1, NFκB binds to DNA as a dimer, the most common consisting of p50 and p65.[82] Also similar to AP-1 is the likelihood that Ang II-based redox regulation of NFκB is mediated by redox-responsive MAPKs, most likely JNK and p38.[82] Some very thought-provoking features of NFκB redox-sensitive regulation in the periphery include activation by increased levels of oxidized Trx[82] and Ang II-induced hydrogen peroxide.[85] Furthermore, a significant recent study showed that peripheral inhibition of NFκB in young SHR prevents the development of hypertension.[86] Davisson suggests the intriguing hypothesis that redox-dependent modulation of NFκB in cardiovascular control regions of the brain could also be involved in hypertension, given that many aspects of Ang II signaling in the periphery are mimicked in neurons.[71]

Finally, ROS can affect neuronal behavior by interacting with nitric oxide (NO). Though the role of NO with regard to sympathetic outflow is controversial, it is generally thought to be sympathoinhibitory. Ang II-induced superoxide decreases NO availability, directly, by reacting with NO to form peroxiynitrite and indirectly, by down-regulating neuronal nitric oxide synthase (nNOS) in cardiovascular control regions such as the PVN.[87, 88] Davisson concludes, therefore,

that these studies clearly demonstrate that impairment of NO availability is at least one of the mechanisms through which Ang II increases sympathetic drive.[71]

Macrophage Migration Inhibitory Factor and Thioredoxin as Negative Regulators of Angiotensin II in the Central Nervous System

MIF is of keen interest to our lab and the field of blood pressure regulation due to its ability to serve as a negative regulator of the neuronal actions of angiotensin II (Ang II). Specifically, Ang II up-regulates MIF in neurons cultured from normotensive rat hypothalamus and brainstem,[89] and increased intracellular levels of MIF protein exert a negative regulatory action over the neuronal chronotropic effects of Ang II.[54] Similar interactions between MIF and Ang II are observed in the rat brain *in vivo*. For example, CNS injection of Ang II increases MIF expression in the paraventricular nucleus (PVN) of the hypothalamus, an area that has a key role in regulating sympathetic outflow and hypothalamus/pituitary (HPA) axis activity. The increased levels of intracellular MIF in PVN sympathetic regulatory neurons serve to blunt the increases in discharge of these cells elicited by Ang II and the increases in blood pressure produced by CNS-injected Ang II.[90] Furthermore, it is apparent that MIF's ability to negatively regulate the actions of Ang II is mediated by its TPOR activity and, possibly, via scavenging of ROS (Figure 1-5).[54, 90]

These findings became even more important when considering the fact that Ang II fails to increase MIF expression in neurons cultured from the hypothalamus of spontaneously hypertensive rats (SHR), or in the PVN of SHR.[91] In addition, experiments from our laboratory indicate that neurons in the PVN of SHR are devoid of immunoreactive MIF.[78] However, intracellular application of exogenous MIF into SHR hypothalamic neurons in culture can depress the neuronal chronotropic action of Ang II in these cells, an effect mediated by the TPOR activity of MIF. Thus, while MIF has the potential to depress the chronotropic action of

Ang II in SHR neurons, it is unlikely that this regulatory mechanism occurs since Ang II does not up-regulate this protein in SHR neurons.[91] By extrapolation, it is possible that a lack of this MIF regulatory mechanism contributes to the hyper-responsiveness to Ang II in the PVN of SHR and the consequent high blood pressure observed in these animals. This idea is borne out by studies from our laboratory which indicate that long-term viral-mediated over expression of MIF in the PVN of young SHR attenuates the development of high blood pressure in these animals.[78]

Based on the above, it is of major interest to understand the intracellular mechanisms that control MIF expression in normal rat neurons, and to identify the defects that are responsible for a lack of MIF expression in SHR neurons. To this end, we have been investigating possible mechanisms for inducible expression of MIF in CNS neurons, which will be explored in detail in the following chapters.

Finally, our hypothesis that MIF acts via it's TPOR activity to negatively regulate Ang II's effects on neuronal currents lead us to investigate if other TPOR-containing proteins, namely Trx, could also exert a negative-regulatory influence over the central actions of Ang II. Given that ROS are now identified as important mediators of Ang II actions in neurons and that MIF's TPOR activity appears to be a negative regulator of Ang II, we believe that TPOR-containing proteins may, perhaps, represent a general mechanism whereby Ang II sensitivity can be modulated in neurons. Indeed, in a recent study, we found that Trx, like MIF, is increased in neurons in response to Ang II signaling. Furthermore, like MIF, Trx is able to prevent Ang II-stimulated increases in delayed-rectifier potassium current, and this activity of Trx is dependent on the action of its TPOR motif.[60] Taking all of the above interactions between MIF, Trx, and Ang II into consideration, we have also endeavored to ascertain the levels and distribution of these important TPOR-containing proteins in areas pertinent to sympathetic activity of pre-

hypertensive SHR and WKY brains and to correlate this data with the oxidative status of these tissues. The results and implications of these studies follow.

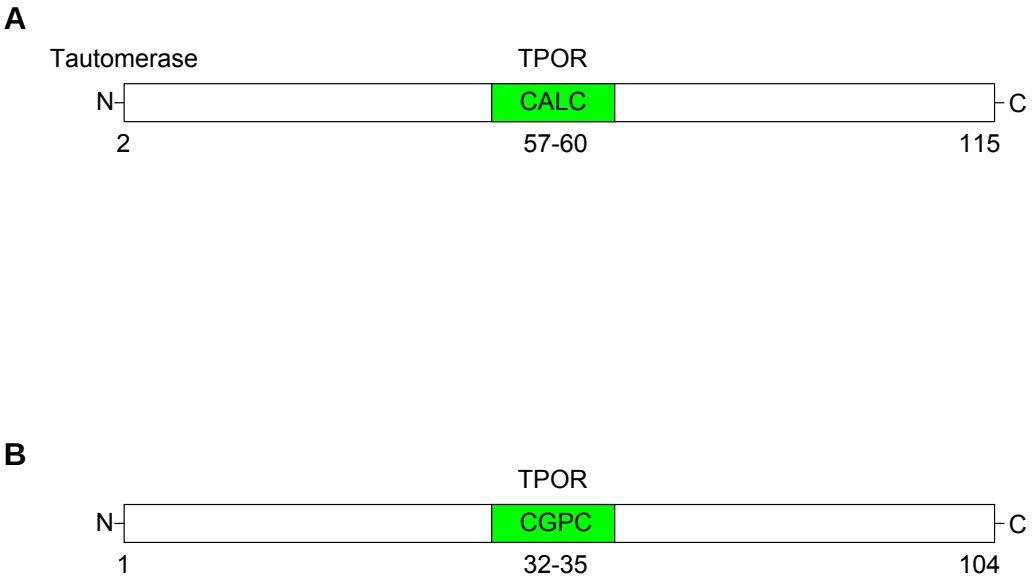

Figure 1-1. Features of A) macrophage migration inhibitory factor and B) thioredoxin. The TPOR motif of each protein is marked in green. N indicates the N-terminus of the peptide, C indicates the C-terminus. Note that the MIF peptide begins at amino acid 2, as the N-terminal proline is cleaved off during post-translational processing. The N-terminal tautomerase domain of MIF is also noted.

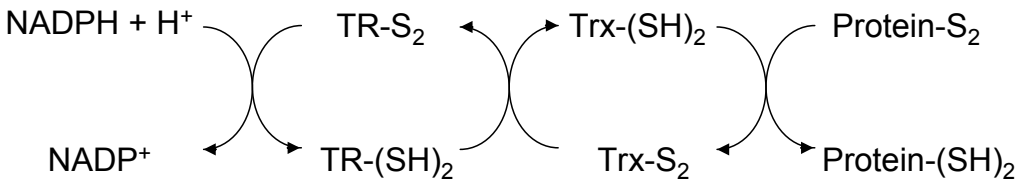

Figure 1-2. Catalytic mechanism of the thioredoxin system. Arrows indicate the direction of each reaction. The catalytic mechanism of MIF is not known, but may be similar in nature. TR = thioredoxin reductase, Trx = thioredoxin, $(SH)_2$ = thiols (reduced form), S_2 = disulfide (oxidized form).

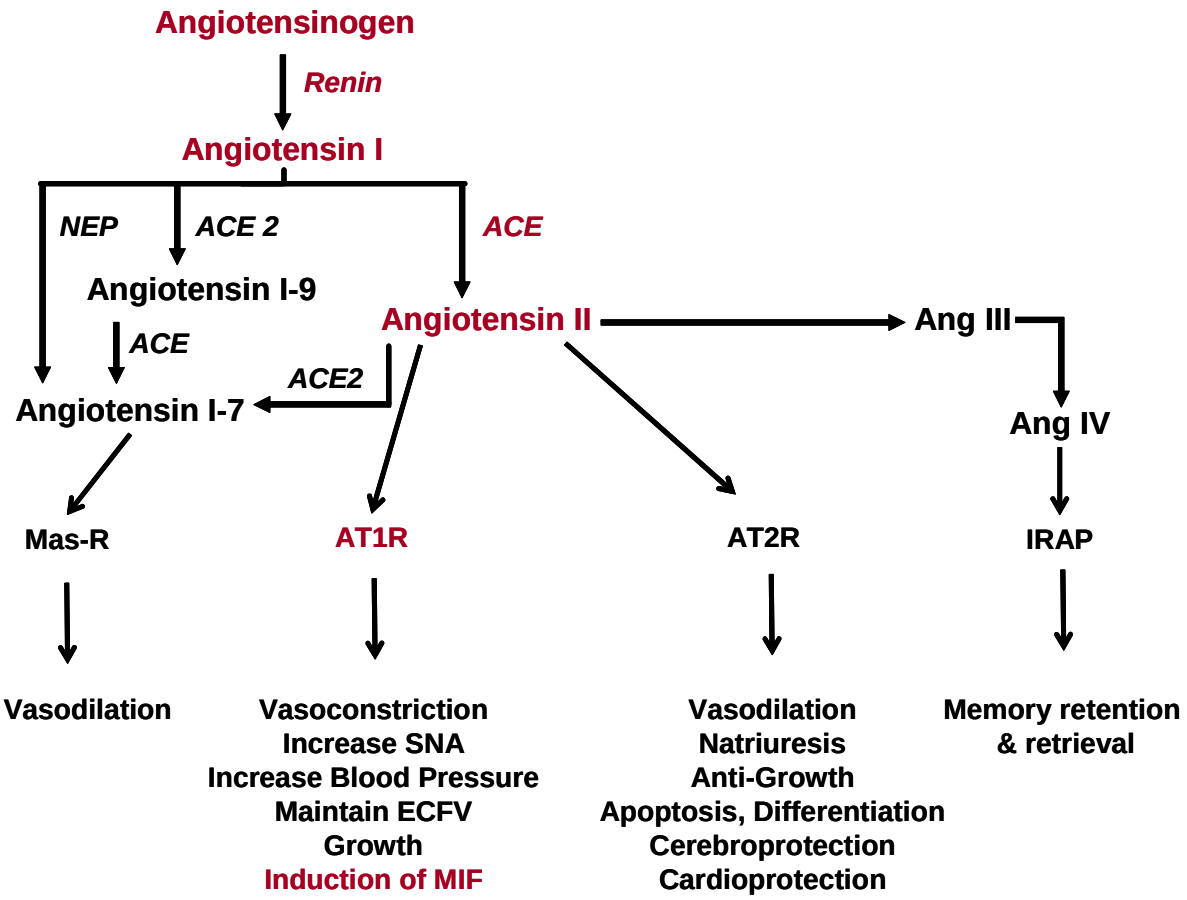

Figure 1-3. The renin-angiotensin system. Simplified schematic of the components of the RAS. The components relevant to this work are depicted in red.

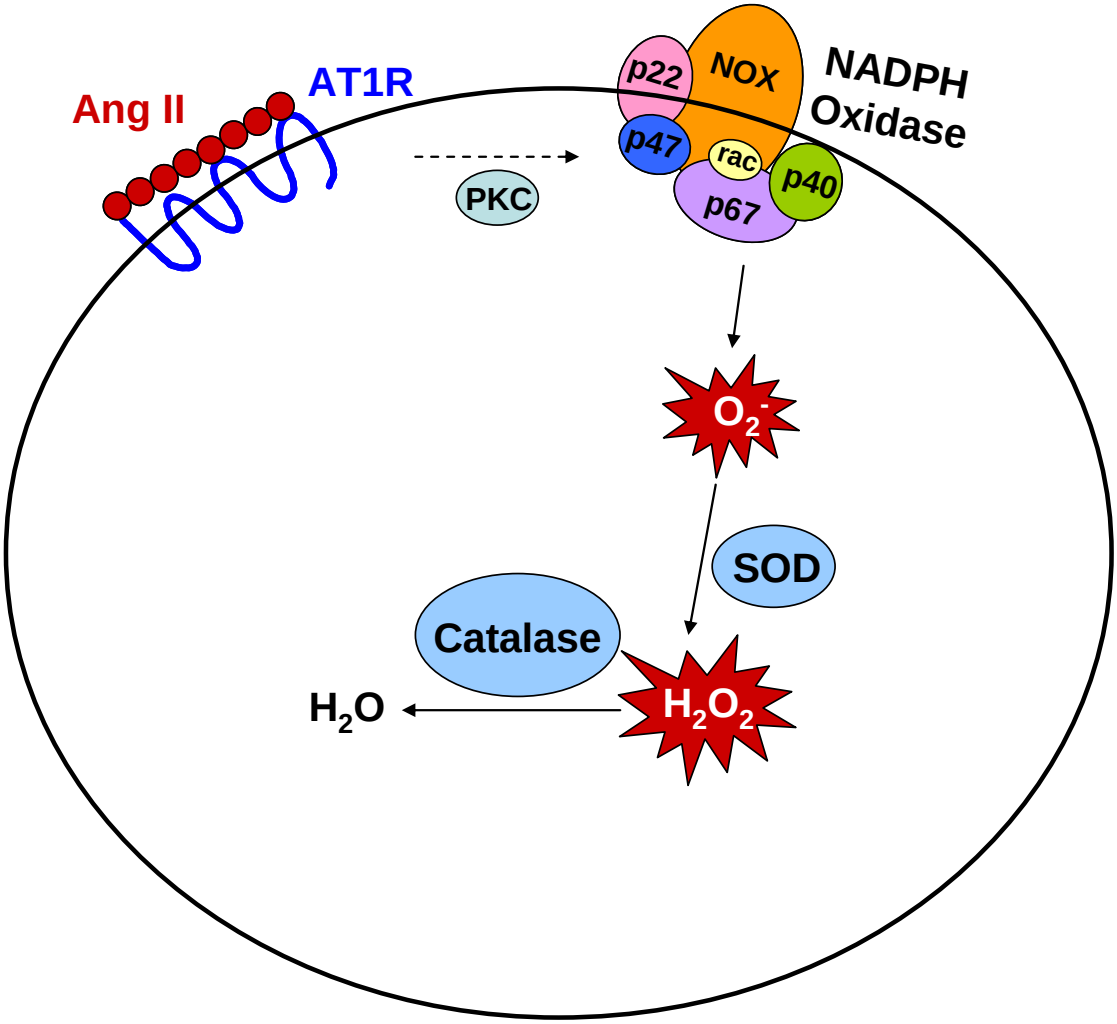

Figure 1-4. Angiotensin II-induced reactive oxygen species production. Ang II stimulates the AT1R, which initiates intracellular signaling events, such as protein kinase C (PKC) activation, ultimately leading to the assembly and activation of the NADPH oxidase complex at the cell membrane. NADPH oxidase generates superoxide, which is metabolized into hydrogen peroxide by superoxide dismutase (SOD). Catalase is one of several enzymes that can detoxify peroxide after it has served its signaling purpose.

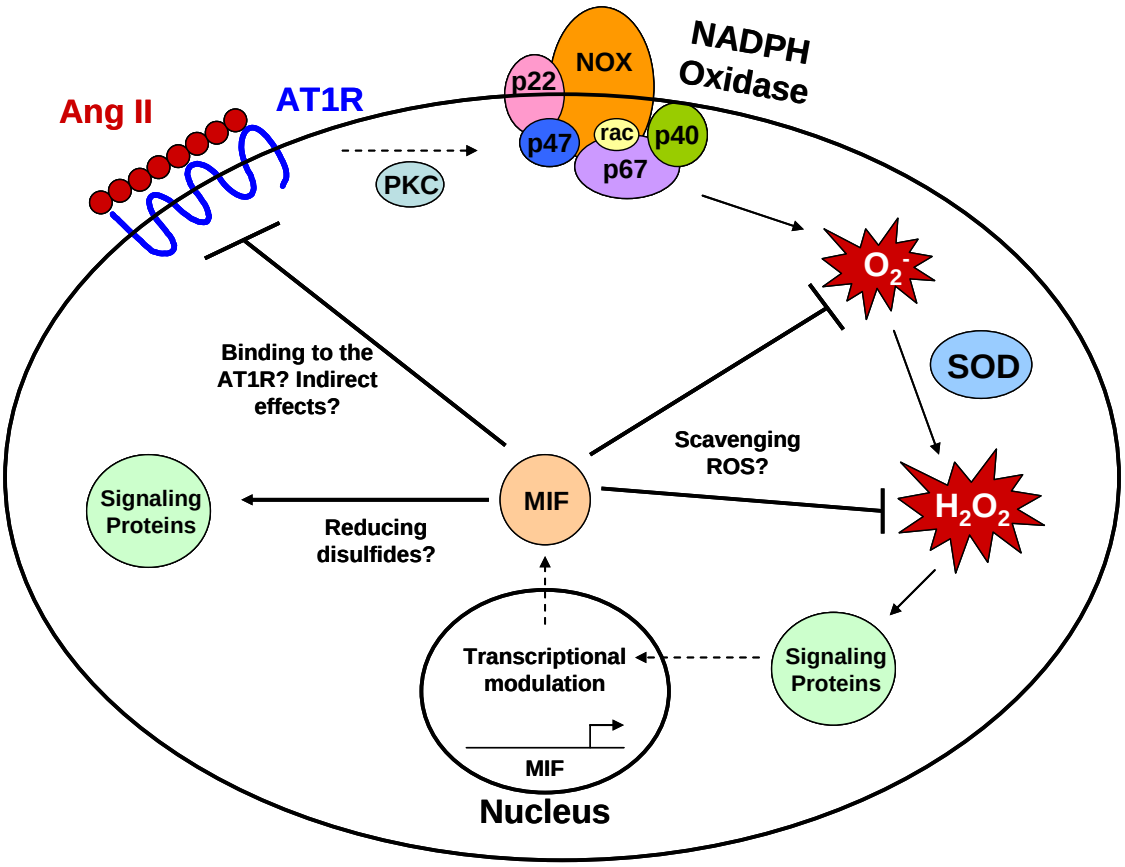

Figure 1-5. Model of possible interactions between angiotensin II signaling and macrophage migration inhibitory factor. Proposed negative regulatory mechanisms of MIF are shown. MIF may negatively regulate the actions of Ang II directly through scavenging of ROS second messengers or binding to the intracellular face of the AT1R, leading to desensitization. It is also possible that MIF may indirectly affect the receptor or Ang II signaling mediators by interacting with other signaling and regulatory proteins.

CHAPTER 2
INDUCIBLE EXPRESSION OF MACROPHAGE MIGRATION INHIBITORY FACTOR IN NEURONS

Introduction

Chapter 1 discusses the importance of understanding the mechanisms that control inducible expression of MIF due to its recently-established role as a negative feed-back regulator of the chronotropic actions of Ang II in neurons. The observation that MIF expression can be induced in response to Ang II signaling in neurons, and that it is not in neurons of pre-hypertensive SHR, indicates that the central actions of MIF could possibly be implicated in the development of hypertension and poses some important questions. What downstream-mediators of Ang II and the AT1R might be inducing MIF expression in CNS neurons? Furthermore, as established in chapter 1, it appears that the AT1R is uncoupled from the control of MIF expression in SHR neurons. Therefore, it follows to inquire whether these potential mediators perform the same in normotensive and SHR rat neurons.

Our first goal in these studies was to establish a candidate downstream mediator of Ang II that induces MIF expression in neurons. Accordingly, it is now well known that Ang II, acting via the AT1R, induces ROS production in neurons by activating NADPH oxidase.[92] This leads to superoxide production which should be metabolized to hydrogen peroxide (H_2O_2) by superoxide dismutases.[93] As expected, many studies have concluded that H_2O_2 is a product of Ang II signaling in many cell types, including neurons.[94] Furthermore, it has been demonstrated that H_2O_2 can cause induction of MIF expression in peripheral tissues.[95] Finally, it has been shown, recently, that the human MIF promoter is regulated by Sp1 and CREB,[34] two redox-sensitive transcription factors,[96, 97] indicating that MIF expression might be sensitive to the redox environment of the cell. Therefore, we hypothesized that H_2O_2 may be, at least in part, responsible for inducible expression of MIF in CNS neurons in response to Ang II signaling.

Furthermore, we sought to determine whether H_2O_2 possesses the same abilities with respect to inducing MIF expression in normotensive rat and SHR neurons.

In the present study, our aim was to determine whether H_2O_2 can cause up-regulation of MIF expression in CNS neurons and whether MIF is differentially regulated by ROS in neurons cultured from SHR and WKY rats. Together, the data presented here demonstrate that H_2O_2 can cause inducible expression of MIF in CNS neurons cultured from normotensive, but not SHR, newborn rats.

Materials and Methods

Animals. For our experiments, we utilized adult Sprague-Dawley (SD), Wistar Kyoto (WKY), and Spontaneously Hypertensive (SHR) rats as breeders to produce rat pups that were used for the production of neuronal cultures. The 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.

Materials. Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Grand Island, NY). Plasma-derived horse serum (PDHS), Fetal Bovine Serum (FBS), 30% stabilized H_2O_2 solution, PEG-catalase, Actinomycin D, B-actin monoclonal antibody, and secondary HRP-conjugated antibodies were obtained from Sigma (St. Louis, MO). Cells-to-cDNA II kits were purchased from Ambion (Austin, TX). Primers for MIF and 18S for real-time RT-PCR were obtained from Applied Biosystems (Foster City, CA). Glucose Oxidase was purchased from Calbiochem (San Diego, CA). CytoTox96 Non-Radioactive Cytotoxicity Assays were purchased from Promega (Madison, WI). Rat MIF antibody was purchased from Torrey Pines Biolabs (East Orange, NJ).

Preparation of neuronal cultures and tissues. Neuronal cultures were prepared from the hypothalamus and cortex of newborn rats as described previously.[98] Cultures were grown in DMEM containing 10% PDHS for a further 12–16 days before use.

Preparation of glial cultures. Glial cultures were derived from the aforementioned neuronal cultures (which are ~90% neuronal, 10% astrocytes). Neurons were killed by incubating the dishes in 100 mM KCl at room temperature for 5 minutes. The KCl solution was replaced with DMEM containing 10% FBS to encourage cell proliferation and cultures were monitored until ~80% confluency. The DMEM/FBS medium was then replaced with DMEM/PDHS medium and glial cultures were cultured for at least a further 7 days to parallel the feeding/utilization schedule of the corresponding neuronal cultures.

Analysis of MIF mRNA in cultures. cDNA was produced from neuronal cultures with the Cells-to-cDNA II kit, which allows reverse transcription without RNA purification. Levels of MIF mRNA were quantified by real-time RT-PCR as described previously[54]. Data were normalized to 18S rRNA.

Analysis of MIF protein in cultures. Neuronal cultures were lysed in Laemmli Sample Buffer (Biorad, Hercules, CA) and 10 ug of total protein was loaded on the gel. Transfer to a PVDF membrane (Biorad, Hercules, CA) was performed at 75 V for 1.5 hours in Towbin Buffer. Western blots were carried out and analyzed as detailed previously.[89]

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 degrees Celsius and 5000 rpm for 5 minutes to pellet any particulate matter or unattached cells. Supernatant was then utilized (50 uL) according to the protocol provided with the CytoTox96 kit.

Determination of Protein Carbonyl Content. Neuronal cultures (12 wells of a standard 24-well plate, equal to approximately 6 million cells) were treated as indicated, and protein extracts were prepared in MES buffer as instructed. Protein carbonyl content was determined according to kit protocol utilizing the Protein Carbonyl Assay Kit from Cayman Chemical (Ann Arbor, Michigan).

Cell Treatments. For experiments involving application of H_2O_2 , a stock solution of H_2O_2 in water was diluted 100-fold into the culture medium to the final dose indicated.

For experiments involving PEG-Catalase and Glucose Oxidase, the enzyme was dissolved in sterile DPBS then diluted 100-fold into the culture medium to the final dose indicated.

For experiments involving Actinomycin D (Act D), the Act D was dissolved in DMSO to give a concentration of 1 mmol/L. It was then diluted 1000-fold into the culture medium to give a final dose of 1 μmol/L.

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

Hydrogen Peroxide Stimulates an Increase in Macrophage Migration Inhibitory Factor in Neurons Cultured from Normotensive Rats, but not Spontaneously Hypertensive Rats.

Our first goal was to establish whether H_2O_2 contributes to inducible expression of MIF mRNA in primary neuronal cultures from normotensive rats and SHR. To test this idea, primary neuronal cultures prepared from newborn SD and WKY rats and SHR were incubated with H_2O_2 (30 μmol/L; 1, 2 or 3 hr.), a cell-permeant source of ROS. This was followed by analysis of MIF mRNA expression via real time RT-PCR in neurons from all three genetic strains and analysis of MIF protein levels in SD rat neurons. The results indicate that H_2O_2 elicits an increase in MIF

mRNA that is statistically significant within one hour in neurons from both of the normotensive rat strains (Figure2-1, A and B). Conversely, the same dose of H_2O_2 was unable to elicit any significant increase in neurons cultured from SHR throughout the 3 hour time period observed (Figure2-1C). As expected, H_2O_2 also caused a time-dependant increase in MIF protein in SD rat cultures (Figure 2-2).

To confirm these results, primary neuronal SD rat cultures were also incubated with glucose oxidase. Glucose oxidase produces H_2O_2 via oxidation of glucose in the cell-culture medium. Primary neuronal cultures were treated with 0.5 mU glucose oxidase for 1 and 5 hours. The results show that glucose oxidase-derived H_2O_2 produces an increase in MIF mRNA levels that is significant by 5 hours (Figure 2-3).

Finally, because 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. To this end, glial cultures that were devoid of neurons were incubated with 1, 10, 30, or 50 μmol/L H_2O_2 for one hour, the time point at which the increase in MIF reaches significance in the corresponding neuronal cultures, and MIF levels ascertained by real time RT-PCR. The results demonstrate that H_2O_2 is unable to stimulate an increase in MIF mRNA levels in glial cultures (Figure 2-4). Later time points were briefly investigated in pilot studies, and also showed now effect (data not shown). Collectively, these results confirm our hypotheses that MIF mRNA and protein expression can be regulated by ROS in primary neurons.

Hydrogen Peroxide Increases Macrophage Migration Inhibitory Factor mRNA Expression in Primary Neurons Through a Specific Intracellular Action.

Because ROS at high concentrations can cause a generalized stress response in cells, we sought to determine if 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, in order to determine if the effects of H_2O_2 on MIF mRNA levels in neurons were due to an intracellular action of H_2O_2, we utilized PEG-catalase as an intracellular inhibitor of H_2O_2 signaling. Catalase is an enzyme of very high activity that quickly and efficiently metabolizes H_2O_2 into water and molecular oxygen. The PEG-conjugation of the enzyme not only renders it cell-permeant, but also particularly stable once inside the cell.[16] Neuronal cultures were pre-treated with PEG-catalase for 24 hours. The medium was then removed and replaced with fresh conditioned medium, and the cells were stimulated with H_2O_2 (30 μmol/L) for one hour. The results show that scavenging the exogenously applied H_2O_2 in the intracellular environment prevents an increase in MIF mRNA levels, indicating that the exogenously applied H_2O_2 acts intracellularly to increase MIF levels (Figure 2-5).

Next, we investigated whether the dose of H_2O_2 used for our studies was cytotoxic to the neuronal cultures. Primary SD rat neurons were incubated with H_2O_2 (30 μmol/L) for 1 and 3 hours, followed by analysis of lactate dehydrogenase (LDH) within the cell culture medium. The LDH assay measures the activity of LDH released into the medium by dying or dead cells. The results indicate that the dose of H_2O_2 utilized for all experiments in this study is not cytotoxic to primary neuronal cultures. However, higher doses of H_2O_2 (e.g. 100 μmol/L, utilized as a positive "killing" control) are, indeed, cytotoxic to the neuronal cultures (Table 2-1).

Furthermore, we sought to determine whether a 30 μmol/L dose of H_2O_2 represents a state of oxidative stress to our neuronal cultures by measuring protein carbonyl content, an accepted

indicator of intracellular oxidative stress. Primary SD neuronal cultures were treated with 30 or 100 (utilized as a positive control) μmol/L H_2O_2 for 1 hour (i.e., the earliest time point of significant MIF induction in the presence of H_2O_2) and protein carbonyl content measured. The data indicate that protein carbonyl content is not significantly different in control versus 30 μmol/L H_2O_2-treated neurons (Figure 2-6). Collectively, these results demonstrate that the effects of H_2O_2 on MIF mRNA levels in primary neurons are a specific, intracellular signaling action of H_2O_2 and not the result of a generalized oxidative stress response.

The Increase in Macrophage Migration Inhibitory Factor Levels Observed in the Presence of Hydrogen Peroxide Involves Increased Synthesis of Macrophage Migration Inhibitory Factor mRNA.

Since real time RT-PCR measures steady-state mRNA levels, and steady-state mRNA levels represent the summation of both synthesis and degradation, we investigated whether the increase observed in steady-state MIF mRNA levels in the presence of H_2O_2 was due to an increase in transcription. To this end, we utilized actinomycin D (Act D), a general inhibitor of mRNA synthesis. Treatment of neuronal cultures with 1 μmol/L Act D reveals that MIF mRNA is stable in neurons for at least 6 hours, with half-life not yet reached by 8 hours (data not shown). This observed stability of MIF mRNA in our experimental conditions is similar to that reported previously in other cell types.[99] Neuronal cultures were pretreated with 1 μmol/L Act D for one hour and then stimulated with 30 μmol/L H_2O_2 for 1 hour. MIF mRNA levels were then ascertained by real time RT-PCR. The data indicate that inhibiting transcription concomitantly with H_2O_2 stimulation prevents an increase in MIF mRNA levels (Figure 2-7). 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

To our knowledge, 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 hydrogen peroxide 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, we selected hydrogen peroxide as our ROS donor for many reasons. First, we were aiming to study a reactive oxygen species that is downstream of Ang II and the AT1R in neurons, and it has already been established that Ang II 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).[94] Second, it is readily cell permeant, with exogenously-applied H_2O_2 establishing equilibrium across the cell membrane on average within minutes.[100] Finally, in the realm of possibilities of ROS donors, it is relatively stable since, while it is a ROS, it is not a free-radical. Simply stated, addition of H_2O_2 to the culture medium is the easiest and most reliable way to manipulate the intracellular redox environment, and it was desirable to manipulate intracellular ROS levels in the absence of the myriad, confounding possible signaling actions for these proof-of-principle studies.

Interestingly, the LDH, protein carbonyl, and PEG-catalase experiments (Table 2-1, Figure 2-6, and Figure 2-5, respectively) support the notion that exogenously-applied H_2O_2 functions as a signaling agent in our neuronal cultures, rather than a mediator of cell death and/or oxidative stress. This signaling function would be only natural for intracellular ROS produced in response to a physiological ligand (i.e., Ang II), but it may seem unusual for the exogenous dosage

utilized, 30 μmol/L, considering that many in vitro studies conclude that this dosage, added exogenously to cell lines in culture, should result in an approximately 3 μmol/L intracellular concentration at equilibrium. This intracellular concentration might constitute an environment of oxidative stress, since *in vitro* studies suggest that the highest observed intracellular concentration of H_2O_2 generated for signaling purposes in mammalian cells is 0.7 μmol/L.[93] However, there are some important mitigating factors to consider in the context of our neuronal cultures. Our cultures consist of primary cells, which evidence suggests produce less endogenous H_2O_2 than transformed cell lines. Therefore, it has been suggested the application of more exogenous H_2O_2 is required to oxidatively stress primary cells than transformed cells in culture.[93] Furthermore, our cultures, while mostly neuronal in nature, always contain a small portion of glial cells. Neurons, microglia, astrocytes, and oligodendrocytes all have the capacity to detoxify H_2O_2 with varying efficiencies. Astrocytes and oligodendrocytes, in particular, are remarkably efficient at detoxifying extracellular peroxide. *In vitro* studies reveal that H_2O_2 added to the medium of astrocyte cultures has a half-life of only a few minutes. Oligodendrocytes follow at a slightly slower rate, though their over-all capacity to detoxify H_2O_2 exceeds that of astrocytes.[101] Therefore, it is highly possible that the exogenous H_2O_2 is rapidly detoxified when added to the medium of our 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 cultures prompted us to perform the experiments utilizing glucose oxidase, which represents a more chronic means of administering exogenous H_2O_2, to confirm our results.

Our results raise some 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.[93] In higher mammals, redox signaling is emerging as a very important and complicated field, and 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.[102-104] For example, it has already been demonstrated that the MAP kinase, p38, is sensitive to Ang II-based ROS signaling in vascular smooth muscle cells and neurons.[81, 105] Further, it has been shown in many settings that transcription factors such as AP-1, SP-1, CREB, and NFκB are sensitive to redox regulation,[106-108] and binding sites for these transcription factors have been identified in the promoter of the human MIF gene.[109] Furthermore, a recent publication examining the constitutive and inducible expression of the MIF promoter has demonstrated, for the first time, that SP-1 and CREB are important transcriptional regulators of the MIF gene.[34] Experiments to determine if these transcription factors may be the mediators of redox regulation of the MIF gene in neuronal cultures are planned for the near future in our laboratory.

This study is provocative and physiologically significant because, as we have established in prior reports, MIF is up-regulated in neurons in response to Ang II signaling via the AT1R. 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.[54, 89, 90] Our studies strongly suggest that MIF may act in this regard by scavenging ROS,[54] as do some other proteins that contain TPOR motifs (e.g. thioredoxin, peroxiredoxins),[27] but the exact mechanism is still under investigation. In this way, we speculate

that MIF may serve as a way for neurons to regulate their sensitivity to Ang II, especially Ang II-based ROS production and their downstream effects.

Furthermore, we have previously shown that Ang II does not induce MIF expression in PVN neurons of SHR, in contrast to their normotensive controls, and we have hypothesized that this lack of MIF induction may contribute to the hyper-sensitivity of these neurons to Ang II,[91] and perhaps even the development and/or maintenance of hypertension in these animals. This idea is born out by current studies in our laboratory showing that viral-mediated over-expression of MIF in the PVN of young SHRs attenuates the development of hypertension.[78] We believe that in SHR the AT1R is uncoupled from the signaling pathway that induces MIF in normotensive animals. Therefore, in the present study, we have sought to investigate signaling pathways that are downstream of the AT1R in neurons, namely ROS signaling. Indeed, we have found that our initial hypothesis regarding ROS and MIF expression is correct. This information serves as a strong basis on which to build further investigation into the actions of ROS in normotensive animals regarding Ang II signaling and MIF expression.

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.[94] 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, 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. Exactly how MIF is providing this negative feedback is still unknown and remains the subject of intense investigation in our laboratory.

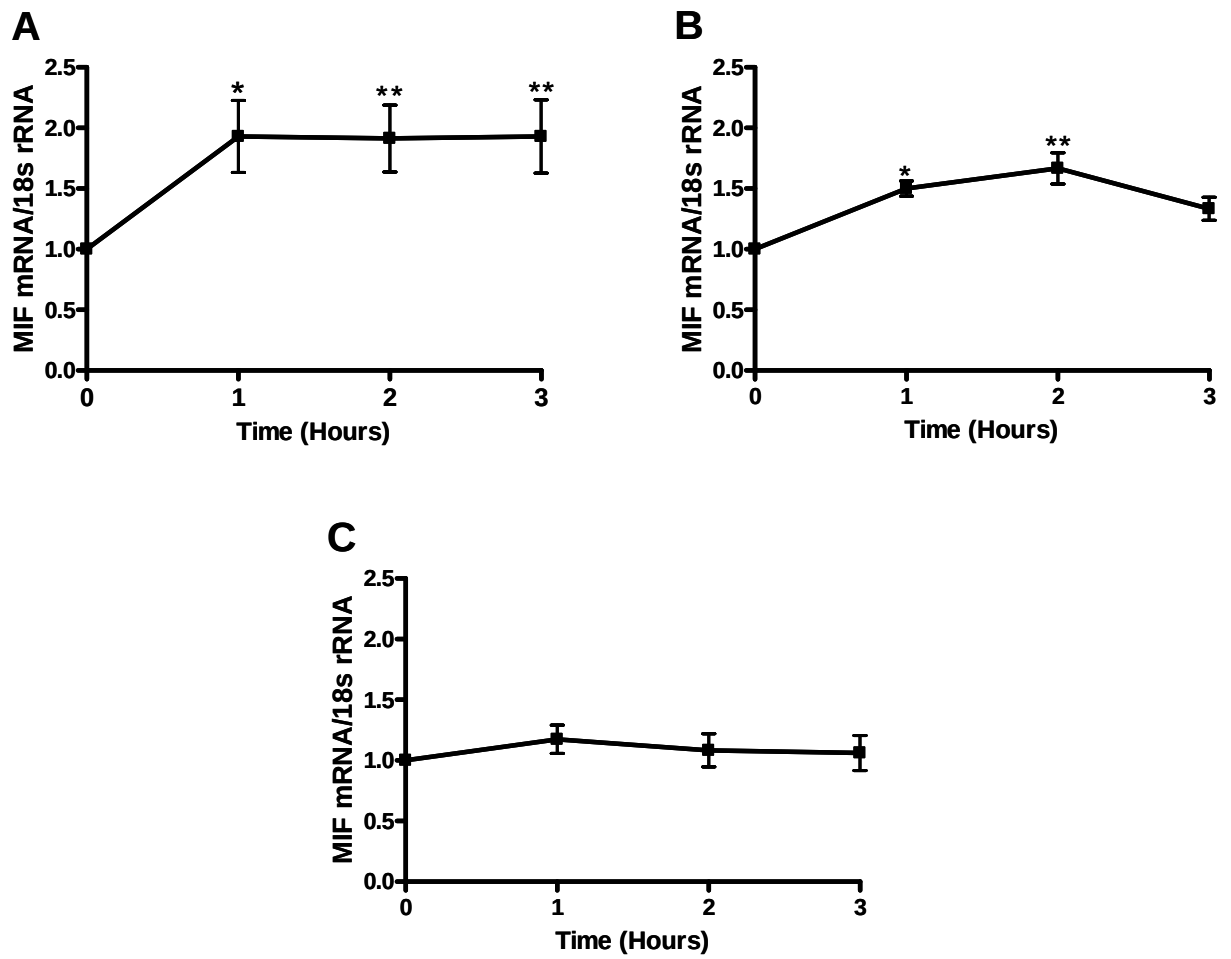

Figure 2-1. Hydrogen peroxide increases macrophage migration inhibitory factor mRNA levels in primary neuronal cultures from normotensive rats, but not spontaneously hypertensive rats. A) SD, B) WKY, and C) SHR neuronal cultures were incubated with either vehicle (H_2O) or 30 μmol/L H_2O_2 for 1, 2, or 3 hours, followed by analysis of MIF mRNA levels as described in the materials and methods. Means ± SE (n = 7 for SD, n = 5 for WKY, n = 6 for SHR) of the ratio of MIF mRNA to 18S rRNA at each time point are shown. *P < 0.05 vs. control, **P < 0.01 vs. control

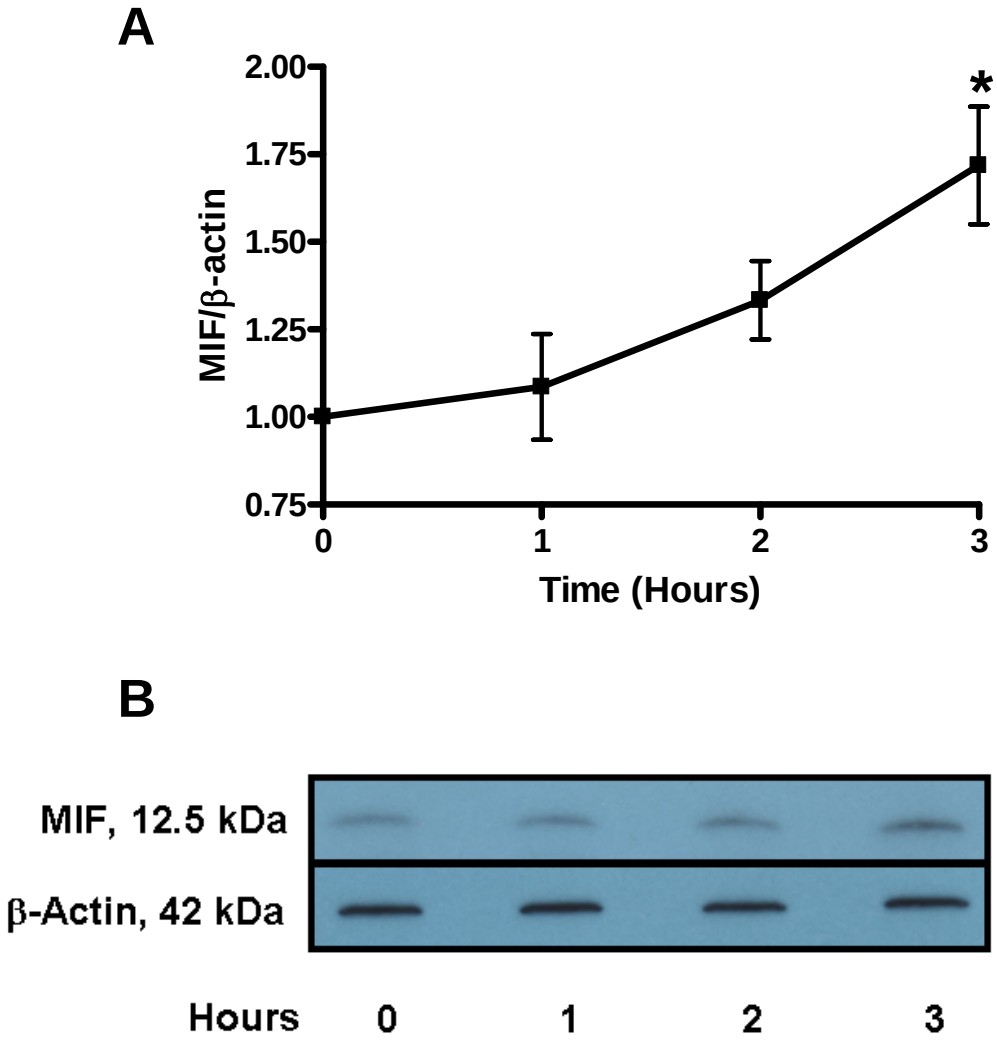

Figure 2-2. Hydrogen peroxide increases macrophage migration inhibitory factor protein levels in primary neuronal cultures. SD rat neuronal cultures were incubated with either vehicle (H_2O) or 30 μmol/L H_2O_2 for 1, 2, or 3 hours, followed by Western Blot analysis of MIF protein as described in the materials and methods. A) Data are means ± SE (n = 3) of the ratio of MIF protein to ß-actin at each time point. *P < 0.01 vs. control. B) Representative Western Blot showing the effects of hydrogen peroxide on MIF levels.

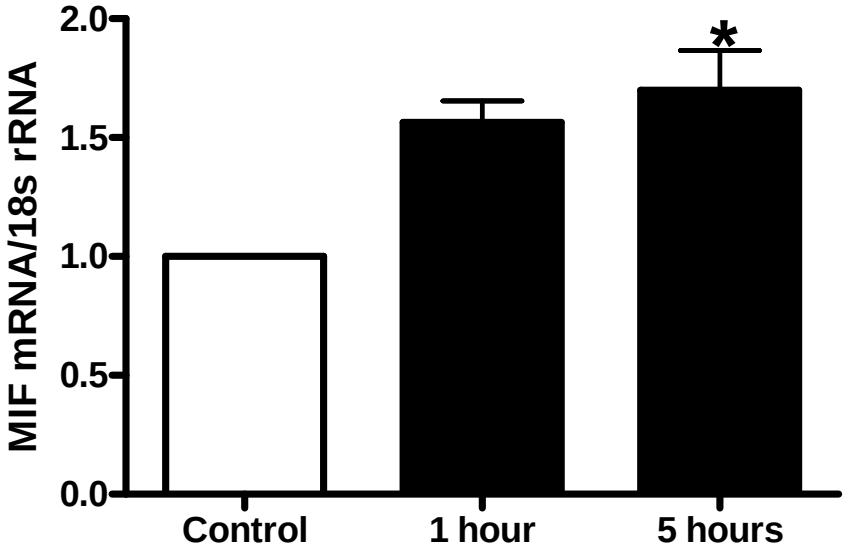

Figure 2-3. Glucose oxidase increases macrophage migration inhibitory factor mRNA levels in primary neuronal cultures. SD rat neuronal cultures were incubated with either vehicle (DPBS) or 0.5 mU glucose oxidase for 1 and 5 hours, followed by analysis of MIF mRNA levels as described in the materials and methods. Bar graphs shown here are means ± SE (n = 4) of the ratio of MIF mRNA to 18S rRNA at each time point. *P < 0.05 vs. control.

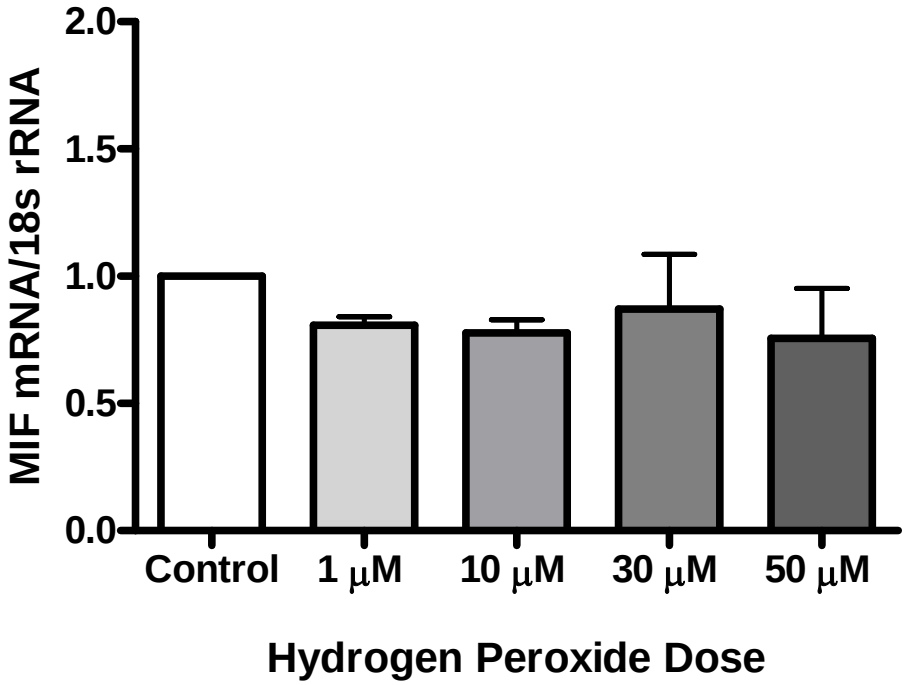

Figure 2-4. Hydrogen peroxide does not increase macrophage migration inhibitory factor mRNA expression in primary glial cell cultures. SD rat glial cultures were incubated with either vehicle (H_2O) or 1-50 μmol/L H_2O_2 for 1hour, followed by analysis of MIF mRNA levels as described in materials and methods. Bar graphs shown here are means ± SE (n = 3) of the ratio of MIF mRNA to 18S rRNA at each concentration. Statistical analysis showed no significance at any time point observed.

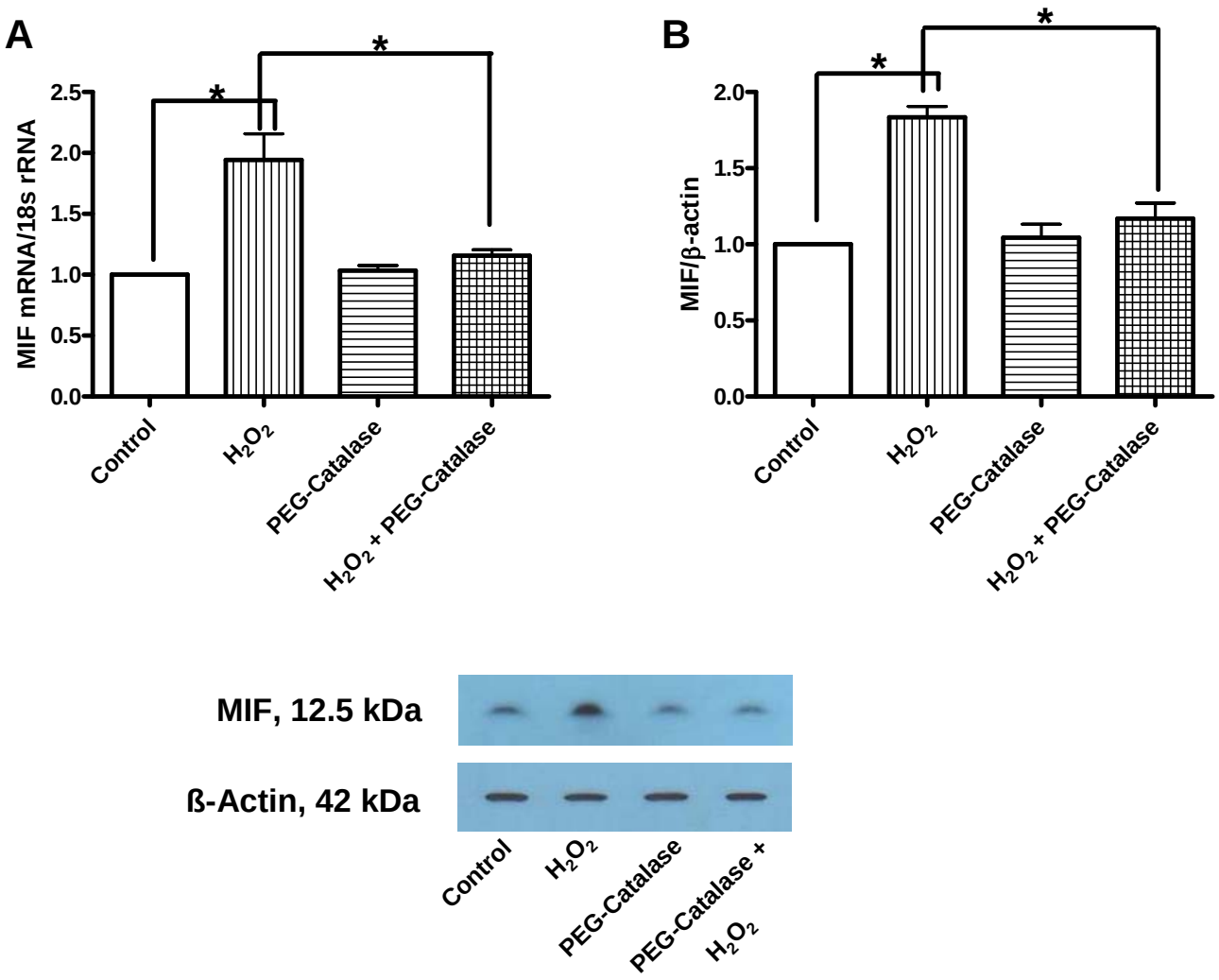

Figure 2-5. Hydrogen peroxide acts intracellularly to elicit an increase in macrophage migration inhibitory factor expression in primary neuronal cultures. SD rat neuronal cultures were incubated with either vehicle (DPBS) or 100 U of PEG-catalase for 24 hours. Medium was changed to fresh conditioned medium taken from age-matched, naïve neuronal cultures. Cultures were then incubated with either vehicle (H2O) or 30 µmol/L H2O2 for 1 hour followed by analysis of MIF mRNA and protein levels as described in materials and methods. A) Bar graphs shown are means ± SE (n = 6) of the ratio of MIF mRNA to 18S rRNA under each treatment condition. *P < 0.001. B) Bar graphs shown are means ± SE (n = 3) of the ratio of MIF protein to ß-actin protein under each treatment condition. *P < 0.01. Western blot shown in lower panel is representative of the 3 experiments quantified in B.

Table 2-1. Hydrogen peroxide (30 µmol/L) does not elicit cytotoxic effects in primary neuronal cultures. SD rat neuronal cultures were incubated with either vehicle (H2O) or 30 µmol/L H2O2, or 100 µmol/L H2O2 for 1 or 3 hours. Culture medium was collected and subjected to an LDH cytotoxicity assay as detailed in the materials and methods. Means ± SE (n = 4) at each time point are shown. *P < 0.001 vs. control.

	30 µmol/L Hydrogen Peroxide	**100 µmol/L Hydrogen Peroxide**
% Survival at 1 hour	100 ± 2.00	95 ± 4.57
% Survival at 3 hours	98 ± 0.75	*63 ± 4.30

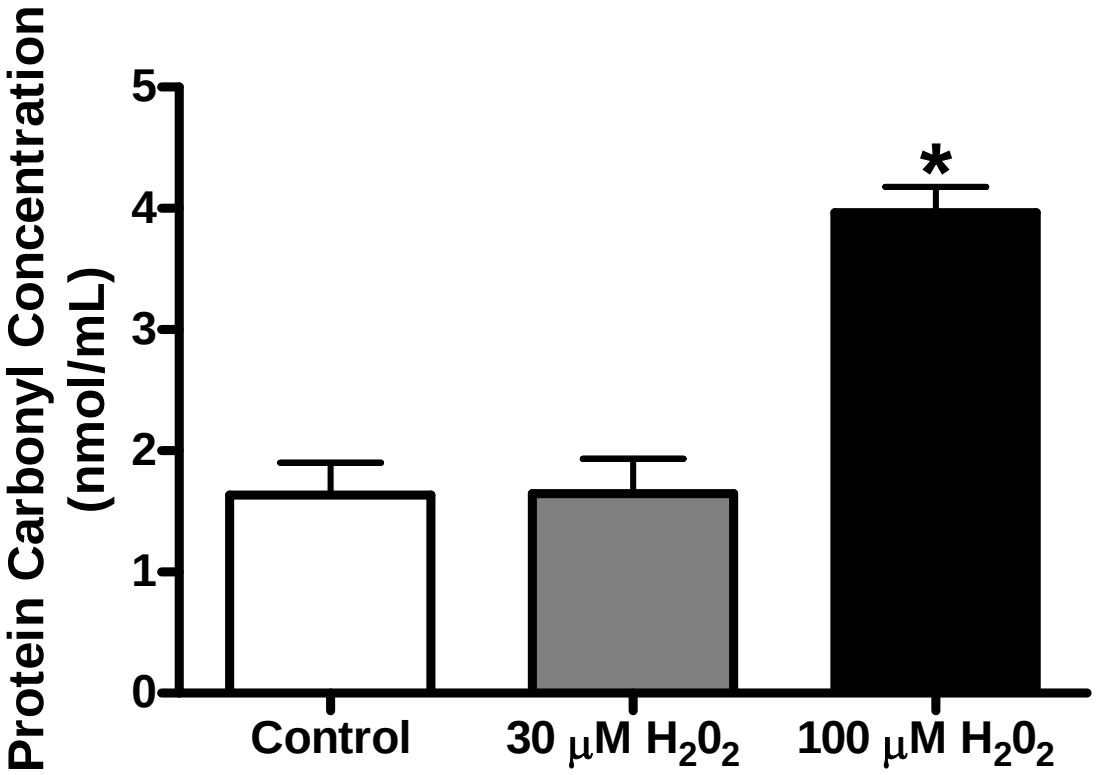

Figure 2-6. 30 μmol/L hydrogen peroxide does not alter protein carbonyl formation. SD rat neuronal cultures were incubated with either vehicle (H_2O), 30, or 100 μmol/L H_2O_2 for 1 hour. Cell lysates were collected and assayed for protein carbonyl content as indicated in materials and methods**.** Bar graphs shown here are means ± SE (n = 10 for control and 30 μmol/L H_2O_2, n = 3 for 100 μmol/L H_2O_2). *P < 0.01.

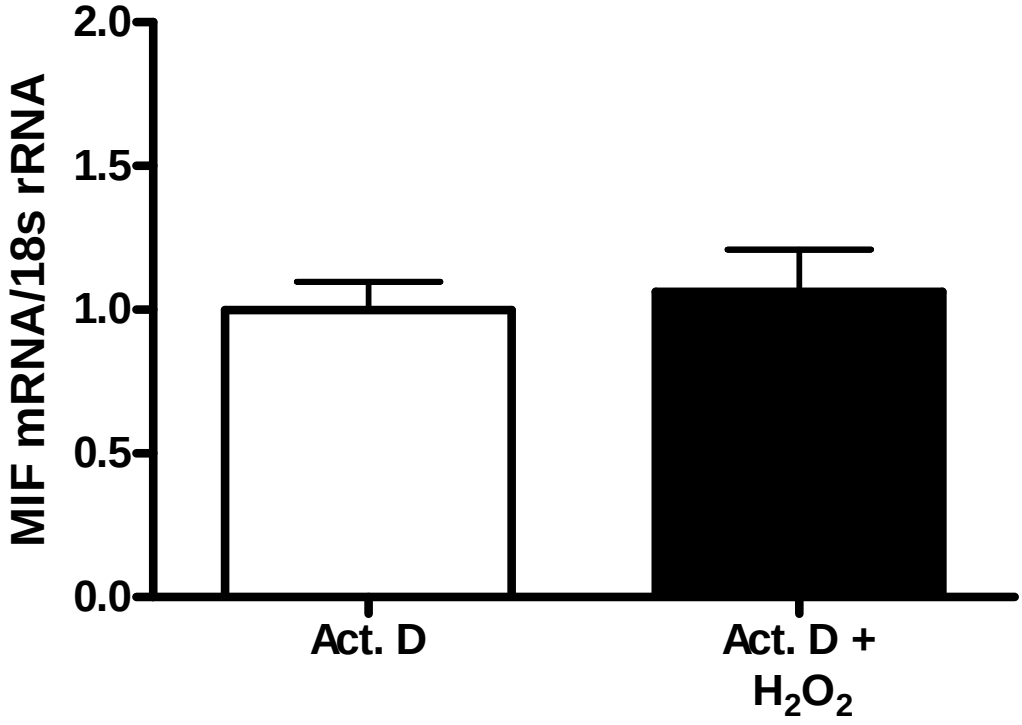

Figure 2-7. Hydrogen peroxide-induced increases in macrophage migration inhibitory factor expression involve a transcriptional event. SD rat neuronal cultures were incubated with Actinomycin D (1 μmol/L) for 1 hour. The cultures were then incubated with either vehicle (H2O) or 30 μmol/L H2O2 for 1 hour followed by analysis of MIF mRNA levels as described in materials and methods. Bar graphs shown here are means ± SE (n = 5) of the ratio of MIF mRNA to 18S rRNA.

CHAPTER 3
MACROPHAGE MIGRATION INHIBITORY FACTOR AND THIOREDOXIN IN THE BRAIN AND OXIDATIVE STRESS

Introduction

Recently, interest in the relationship between neurogenic hypertension and oxidative stress has been gaining momentum. Studies in adult SHR rats have demonstrated that oxidative stress is critically involved in the neurogenic hypertension observed in this model and in the related stroke-prone spontaneously hypertensive rat (SHRSP) model.[110-112] However, these studies have mostly focused on the role of oxidative stress in the adult SHR. Studies examining oxidative stress in any area of the brains of pre-hypertensive (i.e., newborn or very young) animals are lacking.

Coincidentally, and in agreement with the aforementioned studies, a recent publication from our laboratory has shown that the localization of MIF is dysregulated in adult SHR brains. Specifically, though absolute levels of MIF mRNA and protein appear normal in adult SHR, immunostaining reveals that MIF expression *is lacking in neurons of the PVN*. Furthermore, this powerful study determined that the lack of basal MIF expression in neurons is most likely involved in the development of hypertension in SHR, since long-term, viral-mediated over-expression of MIF in the PVN of young SHRs with mildly elevated blood pressure was able to significantly attenuate the development of robust hypertension in these animals over the 12 week period studied. Notably, *the ability of MIF over-expression in the PVN to attenuate the development of hypertension in SHR appears to be redox-dependent*, since a mutated form of MIF carrying a substitution in the TPOR motif was unable to recapitulate the effects of wild-type MIF.[78] This finding is not surprising, given that oxidative stress has already been observed in adult SHR brains and the evidence is ever-mounting that MIF is a Trx family member, sensitive to and most likely participating in redox homeostasis.

Merging together the studies concerning 1) oxidative stress and neurogenic hypertension in adult SHR and 2) our studies on the role of neuronal MIF in the development of hypertension in SHR leads us to ask some important questions. We already know that absolute MIF levels do not differ between adult SHR and WKY PVNs. Instead, the cellular distribution of MIF seems to be the major difference, and this difference contributes to the disparate blood pressures normally observed in these two genetic strains.[78] Accordingly, are MIF and/or Trx expression and distribution dysregulated in the brains of pre-hypertensive, newborn SHRs? The results of studies in chapter 1 demonstrating disparate inducible expression prompted us to sequence the putatitive MIF promoter and gene in SHRs and WKYs, revealing SNP's located 5' to transcriptional start, suggesting to us that examination of basal expression may be in order (Figure 3-1). However, a more detailed analysis of the MIF promoter in rats is necessary to begin to understand the implications of any possible mutations. Also, our studies do suggest that MIF is lacking specifically in neurons of adult SHRs and that this lack contributes to the development of hypertension in these animals. However, it has not yet been established when the distribution of MIF becomes abnormal in these animals (i.e., pre- or post-hypertension).

Furthermore, like their adult counterparts, do newborn SHRs exhibit oxidative stress in cardiovascular-relevant regions of the brain? Again, we do not yet know if the oxidative stress observed in adult animals is the cause or a consequence of their hypertension. Therefore, it is important to establish the situation with regard to MIF and Trx in pre-hypertensive animals. If MIF and/or Trx are, indeed, lacking or redistributed in newborns, then it follows that oxidative stress may be present long before hypertension becomes evident. Establishing the timing of the development of oxidative stress in the brains of these animals with respect to the progression of hypertension would be a considerable contribution to this exciting new field of research.

To begin addressing these questions, the comparative studies in this chapter will aim to 1) determine relative quantities of MIF and Trx in the hypothalamus and brainstem of newborn (i.e., pre-hypertensive) SHR and WKY rats, 2) determine the cellular localization of MIF and Trx in the PVN of newborn SHR and WKY rats, and 3) ascertain relative levels of oxidative stress in the hypothalamus and brainstem of newborn SHR versus WKY. Our decision to examine the hypothalamus and brainstem is based on the fact that they are rich in cardiovascular-relevant nuclei such as the PVN, the RVLM, and the NTS. Trx is being examined along with its relative MIF due to the similarities between the two molecules concerning regulation of neuronal firing (i.e., effects on delayed rectifier current), and ability to negatively regulate the central actions of Ang II.[60, 113] Furthermore, Trx is more established as an important regulator of the intracellular redox environment, so a correlation between Trx dysregulation and oxidative stress is even more likely than is the case for MIF.

Materials and Methods

Analysis of MIF and Trx mRNA. Relevant brain areas were dissected from newborn rat brains and placed in RNAlater (Ambion, Austin, TX) at 4 degrees celsius overnight. They were then placed at -20 degrees celcius until processing. RNA was isolated from the tissues utilizing the RNAeasy minikit (Qiagen, Valencia, CA). cDNA was produced from the RNA samples utilizing the iScript cDNA synthesis kit (Biorad, Hercules, CA). Real time was performed using commercially available primers and universal taqman 2x PCR master mix (Applied Biosystems, Foster City, CA). Results are expressed as a ratio of MIF or Trx mRNA to 18s rRNA.

Analysis of MIF and Trx protein. Relevant brain regions were dissected from newborn rat brains and placed in cold boiling buffer consisting of 4% SDS, 0.25 M Tris HCl (pH 6.8), 10% glycerol, and 2% ß-mercaptoethanol. Tissues were homogenized in this buffer by sonication, followed by boiling at 100 degrees celcius for 3 minutes. The lysates were

centrifuged at 14,000 RPM and 4 degrees celcius for 5 minutes. Supernatants were collected and utilized for western blotting. Concentrations were determined by Bradford Assay. 10 ug of total protein was loaded onto a 15% gel for SDS-PAGE, then transferred to a PVDF membrane (Biorad, Hercules, CA) at 75 V for 1.5 hours. After a brief rinse in PBS-T (PBS containing 0.05% Tween-20), membranes were blocked in PBS-T containing 10% non-fat milk for at least one hour at room temperature. Primary antibodies were applied overnight at 4 degrees celcius. MIF (Torrey Pines, East Orange, NJ) and Trx (Chemicon International, Temecula, CA) polyclonal antibodies were diluted in PBS-T/1% milk. MIF antibody was diluted at 1:1000, Trx antibody at 1:10,000. Beta-actin monoclonal antibody (Sigma, St. Louis, MO) was diluted at a concentration of 1:100,000 in PBS-T/5% milk. The next day, membranes were washed in PBS-T for 1 x 10 minutes and 4 x 5 minutes, then incubated in secondary antibody at a 1:30,000 dilution (MIF), 1:50,000 dilution (Trx), or 1:90,000 (beta-actin) in 2% milk/PBS-T for one hour at room temperature. Secondary HRP-conjugated antibodies (anti-mouse and anti-rabbit) were purchased from Sigma (St. Louis, MO). Chemiluminescence detection was carried out according to instructions with the Western Lighting kit from PerkinElmer (Boston, MA).

Immunostaining of brain sections for MIF and Trx. Brains were placed in Formaldehyde Fresh (Fisher, Waltham, MA) overnight for fixation. They were then transferred to 70% ethanol and left overnight for partial dehydration and further fixation. The brains were then subjected to dehydration and impregnation with paraffin. After imbedding in paraffin, 5 micron sections were cut and subjected to immunostaining. Following rehydration, slides were rinsed and incubated in TBS-T (TBS containing 0.05% tween) for at least 5 minutes, followed by a blocking step consisting of TBS-T and 1.5% horse serum for at least 1 hour (blocking serum was “matched” to secondary antibodies, which are donkey). Excess blocking solution was wiped

from the slides and primary antibodies were applied for an overnight, 4 degrees celcius incubation. MIF and Trx antibodies (same as described in "analysis of MIF and Trx protein" section) were used at a 1:200 dilution, NeuN and GFAP antibody (Chemicon International, Temecula, CA) at a 1:400 dilution. Primary and secondary antibodies were diluted in PBS containing 10% goat serum. The next day, after a brief rinse in TBS-T, slides were rinsed for 2 x 10 minutes. Secondary antibody (donkey anti-mouse and donkey anti-rabbit) was applied for 1 hour. Alexa Flour 488 and Alexa Flour 594 (Invitrogen, Grand Island, NY) were utilized at a dilution of 1:500. After a brief rinse in TBS-T, slides were rinsed for 2 x 10 minutes, then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed and photographed on an Olympus microscope.

Measurement of protein carbonyl content. Hypothalamus and brainstem were dissected from newborn rat brains and homogenized in 1 ml of ice cold 50 mM MES buffer, pH 6.7, containing 1 mM EDTA (Sigma, St. Louis, MO) by sonication. Lysates were then centrifuged at 10,000 g for 15 minutes at 4 degrees celsius. Supernatant was collected and frozen at -80 degrees celcius until utilization. Assay was performed exactly according to the protocol provided with the Protein Carbonyl Assay kit (Cayman Chemical, Ann Arbor, Michigan).

Results

Macrophage Migration Inhibitory Factor and Thioredoxin Expression is Lacking in Spontaneously Hypertensive Rat Hypothalamus.

Our first goal was to establish the situation with respect to the expression of MIF and Trx in the hypothalamus and brainstem of SHR and WKY newborn (i.e., pre-hypertensive) rats. To determine MIF and Trx mRNA expression, newborn rat brains were dissected, and relevant regions were homogenized and subjected to RNA extraction. The RNA was then reverse transcribed, and the resulting cDNA utilized for real-time PCR as described in materials and

methods. The results (Figure 3-2A) show that there is a statistically significant lack of MIF and Trx mRNA in the hypothalamus of newborn SHRs compared to their WKY controls.

In contrast, when subjected to the same measurement, there was not a statistically significant decrease in the brainstems of SHRs compared to WKY. However, there was a trend toward a slight decrease (Figure 3-2B).

Our second goal concerning MIF and Trx expression was to determine whether the protein levels paralleled the mRNA data (i.e., whether the protein is significantly decreased in the hypothalamus of SHR). Newborn hypothalamus and brainstem were subjected to protein extraction as described in the materials and methods, and western blotting was utilized to determine relative protein levels. The results indicate that MIF and Trx protein is significantly reduced in the hypothalamus, which parallels the mRNA data (Figure 3-3, A and B). In contrast to the mRNA data, Trx protein appeared to be reduced in the brainstem (Figure 3-4). To further clarify the significance of the lack of these proteins, oxidative stress in these two tissues was measured next.

Newborn Spontaneously Hypertensive Rats Exhibit More Oxidative Stress in the Hypothalamus and Brainstem than Wistar Kyoto Rats.

An important aim of this study was to investigate whether SHR newborns exhibit oxidative stress in cardiovascular-relevant regions of the brain, similar to their adult counterparts. To this end, we utilized a protein carbonyl kit (as described in materials and methods) to assess protein carbonyl levels, one of the several accepted indicators of intracellular oxidative stress.[114] Protein was extracted from newborn SHR and WKY hypothalamus and brainstem and subjected to this colorimetric assay. The results indicate that SHR have a statistically significant greater level of protein carbonyl content in the hypothalamus than WKY (Figure 3-5A). SHR also exhibit a higher level of protein carbonyl concentration in the brainstem, though the difference is not as

great or significant (Figure 3-5B). Since the hypothalamus, in contrast to the brainstem, appears to exhibit greater levels of oxidative stress in SHR, our further studies focused on the hypothalamus.

Macrophage Migration Inhibitory Factor is Absent from Paraventricular Nucleus Neurons of Spontaneously Hypertensive Rats.

Since MIF distribution appears to be altered (i.e., lacking in neurons) in the PVN of adult SHR, we were eager to determine whether MIF and Trx were also lacking in neurons in the pre-hypertensitve newborns. Newborn SHR and WKY brains were formalin-fixed and paraffin-embedded, and 5 micron sections of the PVN were then subjected to Trx or MIF immunostaining. Sections were also stained with NeuN, to identify neurons, or GFAP, to identify astrocytes. The results indicate that, similar to what is found in adult animals, MIF is lacking in neurons of newborn SHR PVN compared to WKY. Similar to what we have found in adults, MIF is mostly localized to astrocytes in the SHR PVN (Figure 3-6). In contrast, Trx localization was similar between WKY and SHR PVN (Figure 3-7).

Discussion

To our knowledge, the studies in this chapter represent the first demonstration of oxidative stress in newborn SHR hypothalamus. Furthermore, these studies are the first to show reduced expression of MIF and Trx expression in newborn SHR hypothalamus, which contains the important sympathetic and neuroendocrine-regulating nucleus, the PVN. Finally, the observations contained herein demonstrate that the lack of neuronal MIF previously seen in adult PVN can also be found in newborn (i.e., pre-hypertensive) SHR. In contract, cellular localization of Trx was found to be similar between SHR and WKY PVNs.

Our findings that MIF and Trx are lacking in the hypothalamus of newborn SHR are significant for several reasons. Trx is a well-established redox-homeostasis enzyme that is

essential for maintaining a proper redox balance in neurons. Further, the evidence is steadily mounting that MIF has important redox balancing and/or signaling roles in the brain. A lack of both of these enzymes is suggestive of oxidative stress or altered redox signaling. Indeed, the results of our protein carbonyl studies suggest an association between the lower levels of these TPOR enzymes and a dysregulated redox balance in the hypothalamus. Based on the observations made here, we cannot conclude decisively that the lack of these proteins is solely responsible for the altered redox environment observed in SHR hypothalamus. However, combining them with our previous study supplementing MIF in the PVN of pre-hypertensive SHR and preventing the development of robust hypertension in a TPOR-dependent fashion[78] is certainly suggestive that the lack of these TPOR enzymes in the hypothalamus leads to oxidative stress or some other form of redox signaling dysfunction and, consequently, contributes to the development of hypertension in these animals. One important experiment that is yet to be performed, and would further support this proposed cause and effect relationship, would be to over-express MIF or Trx in the PVN of pre-hypertensive SHR and determine whether the attenuation of hypertension is accompanied by a reduction in oxidative stress in the relevant brain areas. These studies would also further clarify whether MIF and Trx are redundant or have slightly divergent functions in this brain area.

One important and confounding question these studies raise is: why are these antioxidant enzymes paradoxically not up-regulated in the face of oxidative stress? We are not the first group to observe a lack of Trx induction in the presence of oxidative stress. Tanito et. al. reported in 2004 that hypertensive SHR and severely hypertensive SPSHR exhibited a lack of Trx in the heart, vasculature, and kidney that correlated to the severity of their hypertension.[115] Furthermore, they reported dysfunctional Trx induction in response to Ang II signaling in

peripheral blood mononuclear cells of hypertensive rats. The authors concluded, therefore, that a genetic mechanism is the likely responsible for the lack of Trx that they observed. Similarly, the results of the studies contained in chapter 1 showing a dysfunctional induction of MIF in response to hydrogen peroxide treatment in SHR neurons prompted us to sequence the putative MIF promoter and MIF gene in SHR and WKY. Several single-nucleotide polymorphisms were identified (Figure 3-1), but an in-depth study is required to determine if they are important in basal or inducible MIF expression. Likewise, a sequencing study of the Trx promoter and gene in SHR and WKY is certainly warranted and necessary to determine if genetic alterations are responsible for the lack of Trx in newborn SHR hypothalamus and the apparently dysfunctional inducible expression (i.e., lack of induction in an oxidative stress situation) of this protein.

Our findings concerning an abnormal absence of MIF in neurons of the PVN in newborn SHR further support an argument for genetic alterations in hypertensive animals and our recently developing model of dysregulated TPOR enzymes in the brain as a cause, rather than an effect, of hypertension. If the lack of MIF could only be observed in adult, hypertensive rats, then this would suggest an epigenetic alteration that is a *consequence* of high blood pressure. Yet, our studies presented here argue that this is not the case, since we have found the same phenomenon in newly-born SHRs that have not yet experienced elevated blood pressure. However, antioxidant enzymes from several families, including glutathione peroxidase, superoxide dismutase (SOD), and catalase, have been reported to exhibit abnormally low activity in SHR in several tissues.[116, 117] The fact that SHR exhibit such a wide-spread dysfunction in antioxidant systems could suggest a catastrophic failure in global oxidative signaling and redox regulation. In addition to sequencing and promoter studies, perhaps we should consider that the mutation is not to be found in the promoters of these antioxidant enzymes but in the promoter of an up-

stream, common mediator(s) that control(s) expression of redox-balancing proteins and systems. Accordingly, a prime candidate that should be investigated in future studies is the transcription factor Nrf2, which binds to the antioxidant reponse element (ARE) and increases expression of many antioxidant enzymes.[118-120] An intriguing study by Zhu *et. al*. showed that Nrf2-deficient mice have dysregulated basal and inducible expression of several antioxidant and detoxifying enzymes and small molecule antioxidants, including SOD, glutathione, glutathione S-transferase, glutathione reductase, and catalase in cardiac fibroblasts.[121] Though not examined in Zhu's study, other publications have shown that thioredoxin expression is clearly regulated by Nrf2, as well.[122, 123] Experiments have not yet been done to determine whether Nrf2 also regulates MIF expression. Disappointingly, no groups have yet studied blood pressure regulation or sympathetic nerve activity in Nrf2-deficient mice. Nevertheless, studies such as the aforementioned make it easy to imagine that perhaps an indirect global regulator of redox balance such as Nrf2 is playing a role in the lack of TPOR enzymes we are observing in newborn SHR brains. Clearly, extensive further study involving genetics, epigenetics, and signal transduction is needed before it can be determined exactly what sort of abnormality is responsible for the lack of MIF and Trx in the hypothalamus of SHR.

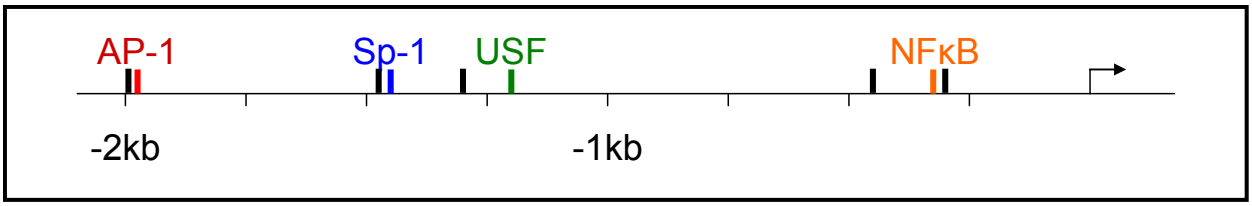

Figure 3-1. Single nucleotide polymorphisms of the putative macrophage migration inhibitory factor promoter in spontaneously hypertensive rats. Those identified in color are potential binding sites for the transcription factors indicated. Note that all 4 are redox sensitive transcription factors.

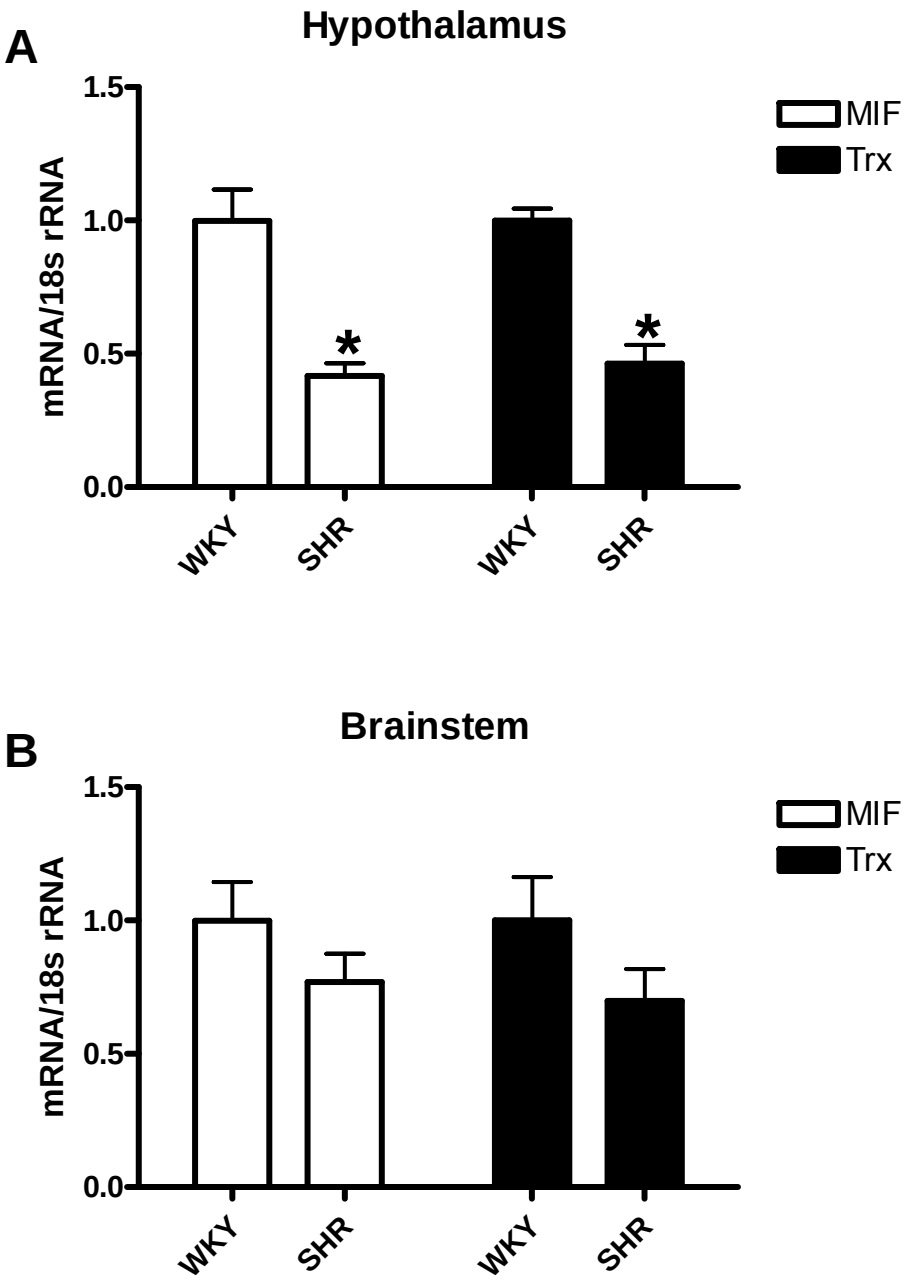

Figure 3-2. Macrophage migration inhibitory factor and thioredoxin mRNA expression is reduced in spontaneously hypertensive rat hypothalamus. mRNA was isolated from the A) hypothalamus and B) brainstem of newborn WKY and SHR and relative expression levels were ascertained by reverse transcription and real-time PCR as described in materials and methods. Means ± SE (n = 10 for each tissue) of the ratio of MIF or Trx mRNA to 18S rRNA are shown. *P < 0.0001 vs. WKY.

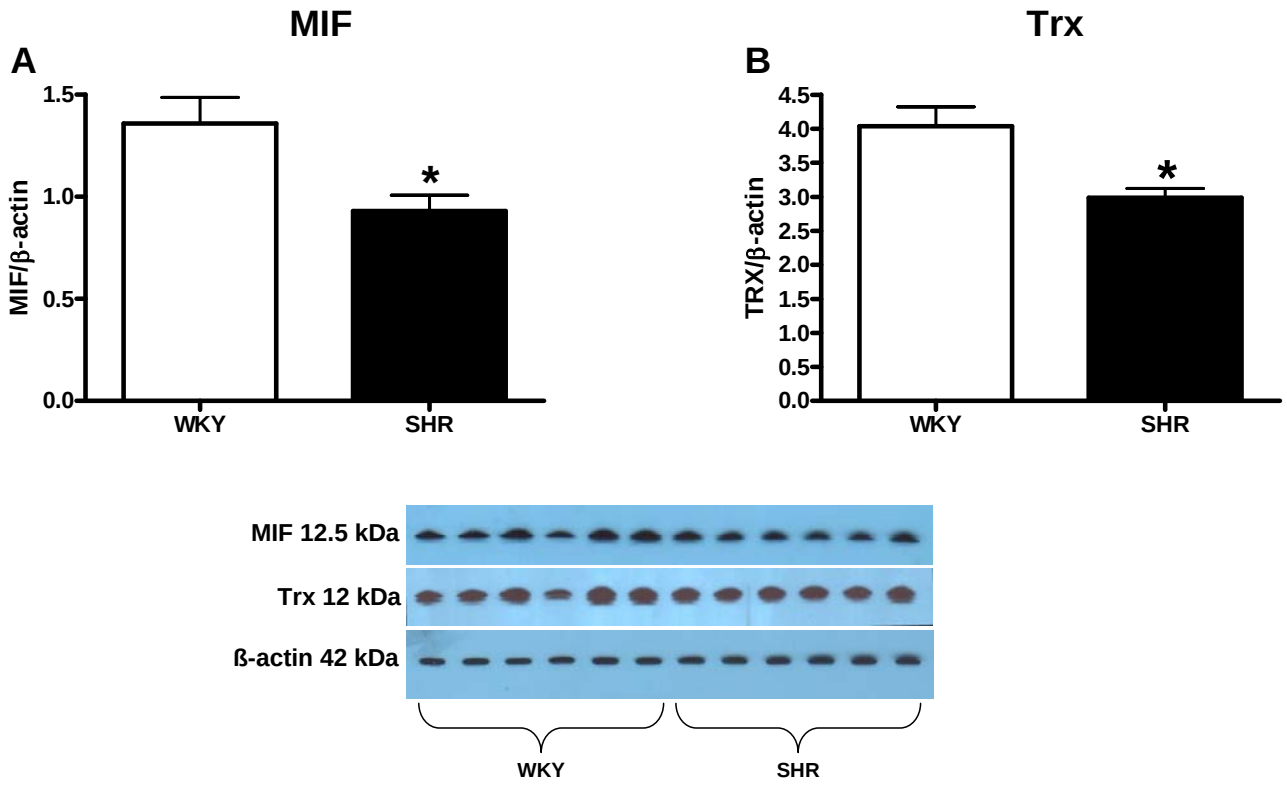

Figure 3-3. Macrophage migration inhibitory factor and thioredoxin protein expression is lower in newborn spontaneously hypertensive rat hypothalamus. Total protein was extracted from hypothalamus of newborn WKY and SHR and relative expression was determined by western blotting as described in materials and methods. Means ± SE (n = 6 for each tissue) of the ratio of A) MIF or B) Trx protein to β-actin are shown. Bottom panel contains representative western blots. *P < 0.05 vs. WKY.

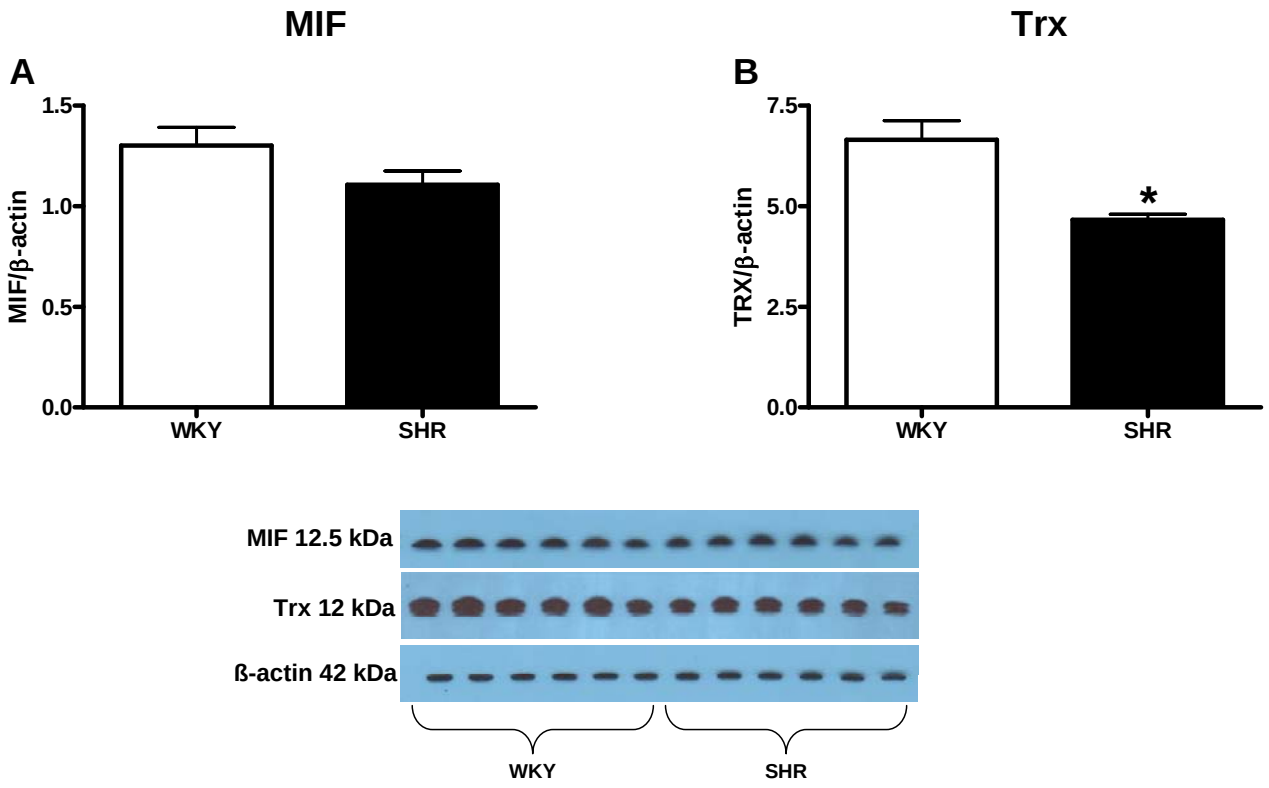

Figure 3-4. Macrophage migration inhibitory factor and thioredoxin protein in newborn brainstem**.** Total protein was extracted from the brainstem of newborn WKY and SHR and relative expression was determined by western blotting as described in materials and methods. Means ± SE (n = 6 for each tissue) of the ratio of A) MIF or B) Trx protein to β-actin are shown. Bottom panel contains representative western blots *P < 0.05 vs. WKY.

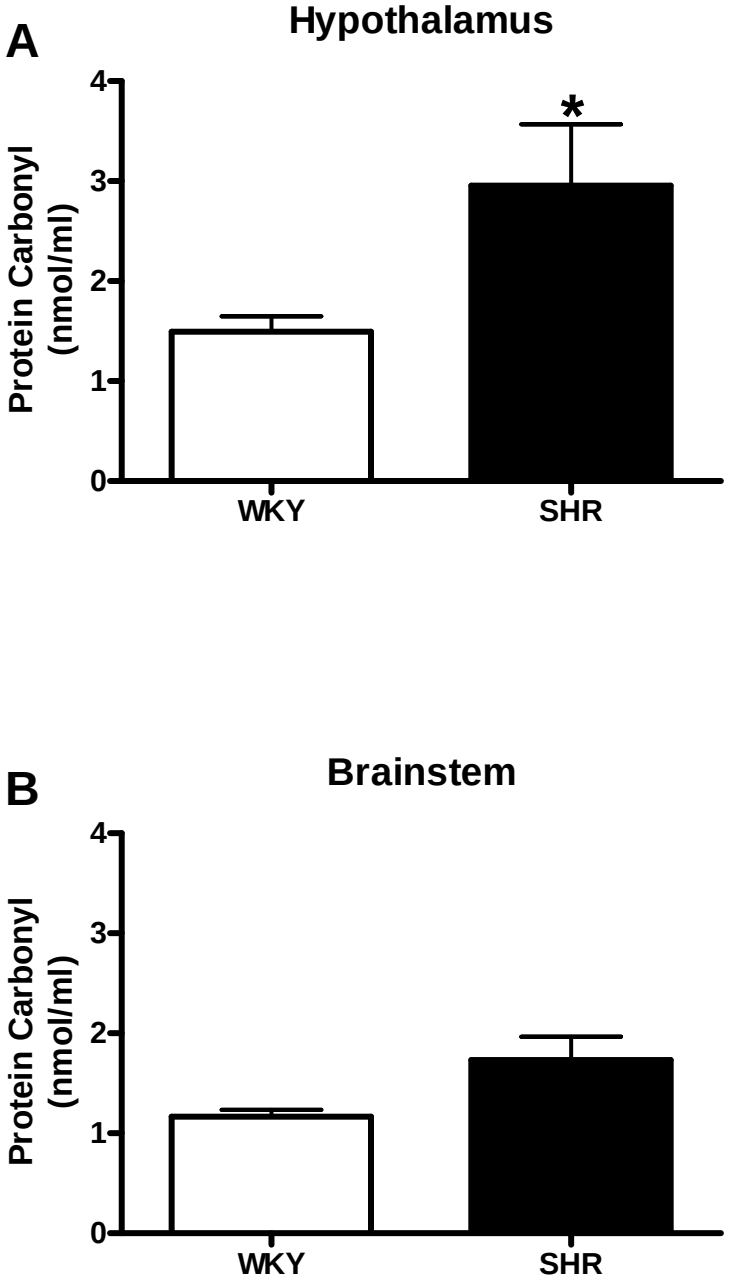

Figure 3-5. Protein carbonyl concentration is greater in spontaneously hypertensive rat hypothalamus than in Wistar Kyoto. Total protein was extracted from A) hypothalamus and B) brainstem of newborn WKY and SHR and carbonyl levels measured as described in materials and methods. Means ± SE (n = 6 for WKY, n = 6 for SHR) of the protein carbonyl concentration of each tissue measured are shown. *P < 0.05 vs. WKY.

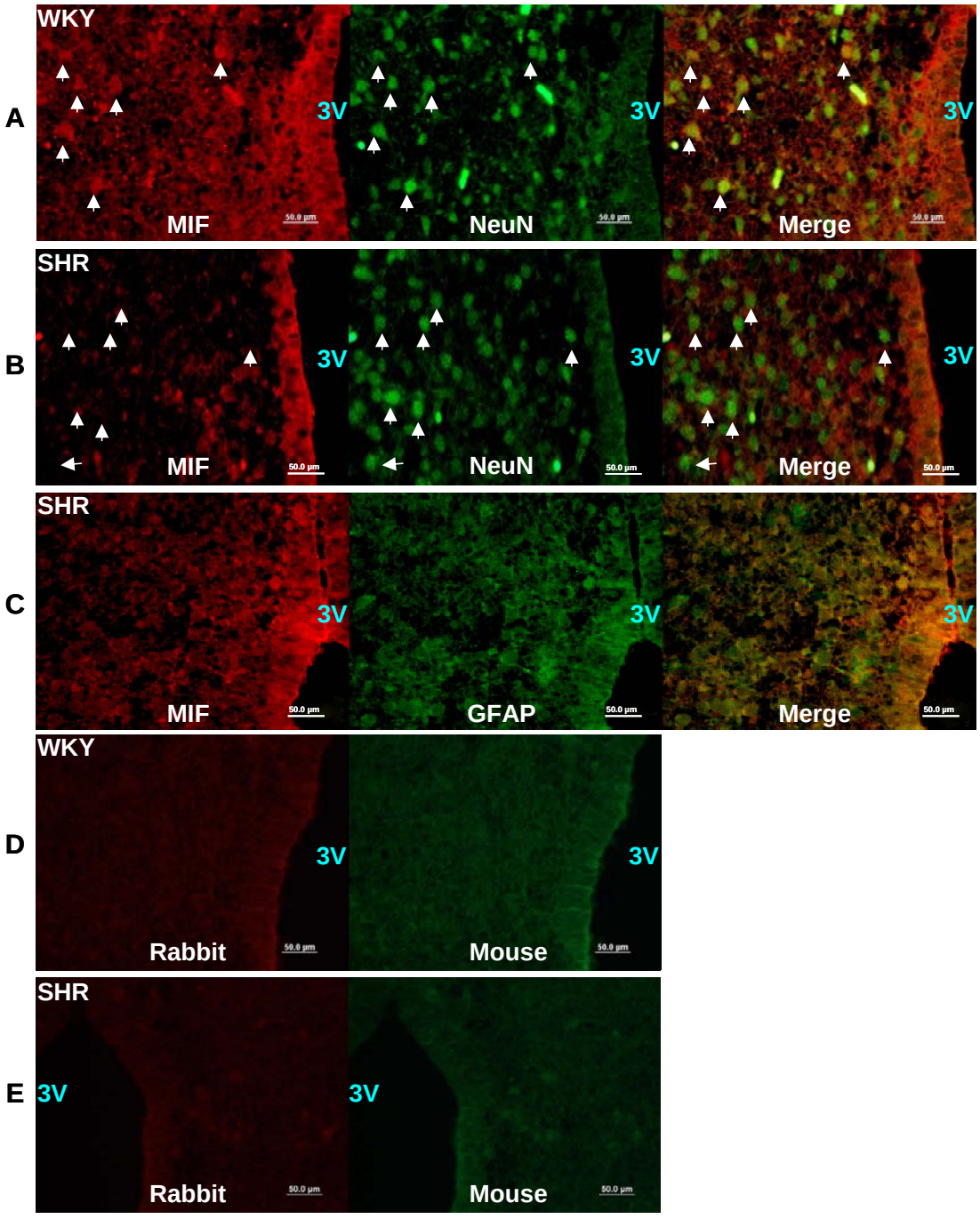

Figure 3-6. Spontaneously hypertensive rat paraventricular nucleus neurons contain less macrophage migration inhibitory factor. Brains from newborn A) WKY and B and C) SHR rats were subjected to immunostaining for MIF, NeuN (a neuronal marker), and GFAP (an astroglial marker). Bars in lower right represent a scale of 50 microns. Areas of interest are marked with arrows. D and E) are Ig controls. Sections pictured are representative of 4 animals from each genetic rat strain.

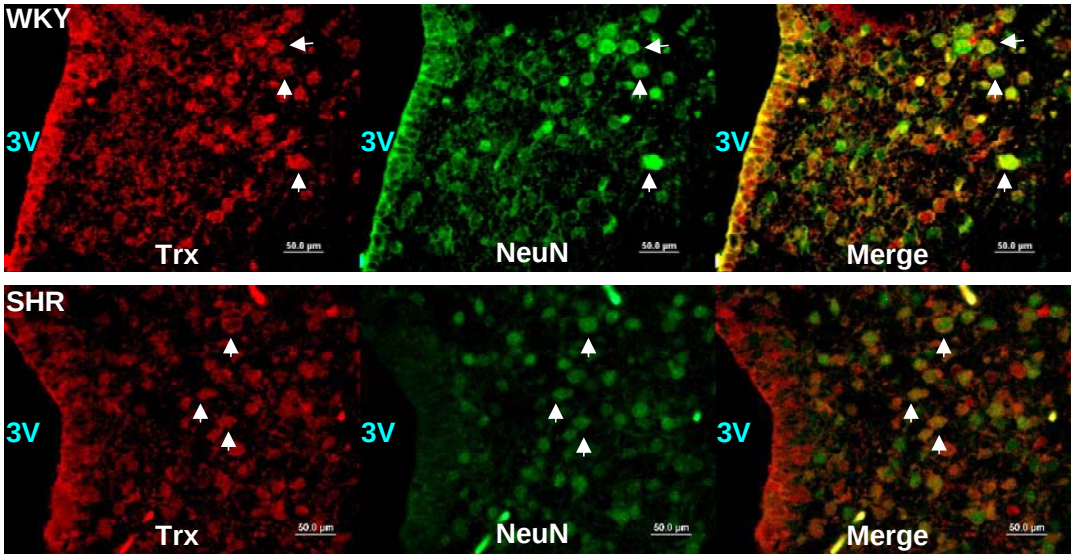

Figure 3-7. Thioredoxin cellular distribution is normal in spontaneously hypertensive rat paraventricular nucleus. Brains were removed from newborn A) WKY and B) SHR rats, fixed, and paraffin-embedded as described in materials and methods. 5 micron sections of the hypothalamus were subjected to immunostaining for Trx and NeuN (a neuronal marker). Bars in lower right represent a scale of 50 microns. Selected areas of co-localization are marked with arrows. For controls, refer to D and E of Figure 3-6. Sections pictured are representative of 4 animals from each genetic rat strain.

CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

Over the past several years, many laboratories, including our own, have made substantial contributions to our understanding of how ROS signaling and oxidative stress contribute to neural control of blood pressure in the physiological state and hypertension, respectively. Progress is being steadily made in this field, but there is still much we do not understand about redox signaling in the brain and its contribution to blood pressure regulation. The studies contained in this dissertation are important because they contribute small steps toward understanding the larger picture with regard to redox homeostasis in the brain and neurogenic hypertension.

From the studies reported in chapter 1, we have been able to make advances in understanding possible pathways that regulate inducible expression of MIF in neurons. Further, by concluding that MIF expression can be sensitive to the intracellular redox environment of the cell, we have contributed greatly to the paradigm shift that is presently occurring in categorizing MIF as a TPOR enzyme and potential member of the Trx family. Finally, these studies provide direction, in the form of ROS signaling, as a basis for future studies examining the inducible expression of MIF in normotensive animals in response to Ang II stimulation of neurons. Also, the knowledge that ROS do not signal appropriately in SHR neurons, at least with regard to MIF expression, lends further direction to future studies that endeavor to determine why the AT1R is uncoupled from inducible MIF expression in SHR.

The studies contained in chapter 2 present compelling evidence that oxidative imbalance is present in SHR hypothalamus prior to hypertension and that a lack of MIF and Trx may be at least partly responsible. At the very least, there is an association between reduced levels of these important antioxidant enzymes and markers of oxidative stress in the hypothalamus of SHR.

Further, a very telling and important finding of these studies is the lack of MIF in neurons of the PVN of pre-hypertensive SHR, indicating that this loss of MIF is not a consequence of developing or advanced hypertension, but perhaps it may be a contributor to the cause. This likelihood becomes even more compelling when coupled with our recent studies on MIF supplementation in the PVN and reduction of blood pressure in SHR. Given the fact that, due to the efforts of our laboratory, MIF — and, to a lesser extent, Trx — is now a known negative regulator of the central actions of Ang II, and that it is well-established that SHR suffer over-activity of and hypersensitivity to the brain RAS, we can now see a much clearer picture of how a lack of MIF and/or Trx may lead to enhanced ROS signaling and/or oxidative stress in important cardiovascular brain nuclei and how this may contribute to the development of hypertension in SHR. Many hypertensive patients exhibit increased sympathetic nervous activity,[124] and there is a constant influx of new evidence showing that ROS are primary effectors through which Ang II mediates its central actions, including effects on the sympathetic nervous system.[71] Therefore, dysfunction of important redox-regulating systems in the brain is becoming a more likely contributor to the complex story of hypertension all the time. Our results in the preceding chapters pave the way for a number of exciting new studies and inspire many important questions, which will be explored and discussed in the following sections.

Inducible Expression of Macrophage Migration Inhibitory Factor in Neurons and Angiotensin II

A very critical question that remains unanswered by our studies is whether hydrogen peroxide is, indeed, the down-stream mediator that induces MIF expression in neurons of normotensive animals. In order to answer this question, a suitable model will have to be established. Areas of the brain that are rich in AT1R should be dissected and cultured together in order to receive a robust induction of MIF when stimulated with Ang II. In the past, we usually

accomplished this by culturing the hypothalamus with the brainstem isolated from newborns. However, in recent months, our laboratory has been developing a technique for culturing PVN neurons isolated from adult rats, a very technically challenging feat. The use of these neurons as a model for studying Ang II and AT1R signaling would be even more desirable. Regardless of whether newborn or adult neuronal cultures are utilized, the experiments detailed in chapter 1 involving PEG-catalase should be repeated with these cultures, except Ang II stimulation should be used in the place of hydrogen peroxide application. If one could interrupt Ang II-mediated up-regulation of MIF expression in the cultures with PEG-catalase treatment, this would provide the necessary evidence needed to determine that Ang II-mediated ROS signaling, specifically hydrogen peroxide, is the pathway through which Ang II induces MIF in neurons. It would also be interesting to repeat the aforementioned experiments utilizing PEG-SOD, in order to show conclusively that superoxide is the source of the intracellular hydrogen peroxide, as we surmise.

These studies could, theoretically, be extrapolated to *in vivo* studies utilizing viral-mediated over expression of SOD and catalase in pertinent brain areas, followed by Ang II application and study of MIF expression. Other labs have successfully used SOD and tempol in the brain to interrupt Ang II signaling,[74-76] so this seems to be a very plausible way of determining whether Ang II-induced ROS signaling is the mechanism by which MIF expression is up-regulated in the PVN *in vivo*.

Mechanisms of Redox Regulation of Macrophage Migration Inhibitory Factor Expression

Another important question that remains to be answered is that of the down-stream mediators that are sensing and responding to changes in the intracellular redox environment and, ultimately, altering the expression of MIF. The most sensible way to initially embark on these studies would be to clone the putative MIF promoter from normotensive animals, make strategic deletions and recombine with a reporter system (such as luciferase). The constructs would then

be transfected into a neuronal cell line (such as SH-SY5Y, known to be Ang II-sensitive)[125, 126] and stimulated with hydrogen peroxide and/or Ang II to investigate which areas of the MIF promoter are redox-sensitive. As we have seen in the discussion of chapter 1, many transcription factors (TFs) are sensitive to redox-signaling, so identifying the cis-acting elements of the MIF promoter that are responsible for redox-sensitive increases in MIF transcription would be the first step to identifying which redox-regulated signaling pathways are inducing MIF up-regulation. Candidate signaling pathways could then be determined in a "back-tracking" manner using the abundant information available concerning which TFs mediate effects of particular pathways.

Once areas of the MIF promoter are identified that mediate induction of MIF both in response to Ang II and hydrogen peroxide, the focus can be placed on these sequences and which TFs may bind there. The cells can then be stimulated with Ang II or hydrogen peroxide and ChIP assays at the native MIF promoter performed for the relevant TFs to ascertain if, indeed, their binding at the MIF promoter is increased under stimulated conditions. The final piece of evidence needed to confirm which TFs are mediating the effects of ROS at the MIF promoter are mutational studies. The MIF promoter-luciferase recombinant constructs could be mutated via PCR to ablate the relevant TF-binding sites and tested for their sensitivity to hydrogen peroxide and Ang II stimulation. ChIP assays should also be performed in Ang II and hydrogen peroxide-treated primary neurons in order to confirm that the effects seen are not specific to the particular cell line selected for the studies.

Once the promoter studies have begun to identify TFs and candidate signaling pathways that may be regulating Ang II- and ROS-induced MIF expression in neurons, these pathways can be further clarified utilizing pharmacological interventions and the aforementioned recombinant

reporter constructs. Furthermore, the results of these studies will serve as a basis for comparison and may help identify the defects in Ang II signaling with respect to MIF induction in SHR.

Dysregulation of Macrophage Migration Inhibitory Factor and Thioredoxin Expression in the Hypothalamus of Spontaneously Hypertensive Rats

The studies contained in chapter 3 highlight the importance of determining the cause of the lower expression levels of MIF and Trx in the newborn SHR hypothalamus. As the future directions concerning this endeavor were previously explored in the discussion of chapter 3, they will not be recapitulated here.

One major question that remains to be answered is that concerning the loss of MIF in neurons of the PVN in SHR, which can be observed in both pre- and post-hypertensive animals. There are several possibilities that must be further explored. For example, is the MIF gene simply not transcribed in these cells? Is MIF being transcribed, but not translated properly? If MIF is being transcribed and translated in neurons, is it then being abnormally degraded? If this is not the case, perhaps MIF is being improperly secreted from neurons so that its presence cannot be detected in these cells by immunostaining? The most reasonable way to begin addressing this issue is to determine whether MIF mRNA can be detected in these neurons. This would determine if the root cause most likely lies in a loss of MIF transcription. In this instance, the application of single-cell RT-PCR, a technique we have recently developed in our laboratory, would yield the most specific information. Application of other commonly-used techniques is complicated by the fact that PVN and hypothalamic cultures consist of a heterogenous population of cells, so determining the situation specifically in neurons is rather difficult. Cells could be cultured from the PVN of SHR and WKY and neurons screened for the presence of MIF using single-cell RT-PCR. Another technique that would be useful for detecting MIF mRNA and confirming the single-cell RT-PCR results is *in situ* hybridization. As the

immunostaining data indicates, we should be able to easily detect MIF mRNA in neurons cultured from WKY. If MIF mRNA cannot be detected in the SHR cultures, this would argue strongly that the problem is a transcriptional issue. However, we cannot rule out the possibility of a stability problem with MIF mRNA in these cells. Obviously, further experiments would be needed to clarify the situation. Addressing this question would be a time-consuming and difficult undertaking, but the information obtained would merit the investment.

Contrary to observations in newborn hypothalamus, absolute MIF levels are unchanged in the heterogenous population of cells in the PVN of adult SHR.[78] One possible explanation for this difference is that the situation in the PVN may be different from that in the rest of the hypothalamus. Another possibility is that MIF is upregulated in glial cells of adult SHR PVN to somehow compensate for the loss of MIF in neurons. Investigating this possibility will be very difficult, for reasons relating to the heterogeneity of cells already mentioned, but the most practical way to begin addressing it would be to culture the PVN of SHR and WKY, enrich for astroglia as previously described,[127] and ascertain basal levels of MIF in these primary glial cultures, comparing mRNA and protein between WKY and SHR via real time RT-PCR and ELISA, respectively. Increased levels of MIF in the SHR cultures would suggest that MIF is upregulated in astroglia in SHR and this is why absolute levels appear normal in the PVN, even when expression is lost in neurons.

Oxidative stress in the Hypothalamus of Spontaneously Hypertensive Rats

The studies in chapter 3 indicate that oxidative stress is present in the hypothalamus of newborn SHRs. The redox environment represents a summation of oxidant-producing systems and antioxidant systems. Therefore, oxidative stress could result from either a loss of antioxidant function and/or an increase in ROS producing systems. Our studies indicate that there may be a lack of antioxidant enzymes, such as those of the Trx family, in SHR hypothalamus. By

extrapolation, it would be useful to investigate whether there is a lack of other important antioxidant systems, such as the glutathione system in this tissue. Conversely, the expression and activity of oxidant-producing systems should also be investigated. For example, NADPH oxidase is currently considered to be one of the most important regulated enzymatic sources of ROS in neurons.[71, 128] It would be prudent to investigate whether this system is up-regulated in the brain of SHR, which could contribute to an imbalanced redox environment.

Physiological Implications

Ultimately, the goal of future studies should be to investigate the physiological implications of improper redox signaling and redox-regulated cellular functions in hypertension. Previous studies from our lab have already determined that neuronal MIF supplementation can have a significant effect on the development of hypertension in SHR over time. However, one very important outcome that remains to be determined is whether MIF supplementation in PVN neurons of SHR and the resulting reduction in blood pressure are accompanied by a reduction in oxidative stress in this tissue. Since it has been demonstrated in several instances that ROS can affect neuronal function by modulating neuronal firing,[71] another important outcome to measure in the context of MIF supplementation in SHR PVN would be sympathetic drive. Previous experiments published by our laboratory[78] should be repeated and these important parameters determined to further complete the picture of how a lack of MIF contributes to the neuropathogenesis of hypertension.

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BIOGRAPHICAL SKETCH

Rachael Harrison was born in 1980 in Jacksonville, FL. She has resided in Florida all of her life and has wanted to become a scientist since she was a very small child. Rachael's career aspirations ranged from marine biology to being a science teacher through her junior high school and high school years. Rachael began to think seriously about a career in biomedical research in her later high school years, and pursued a degree in molecular biology and microbiology (with an emphasis on human health and disease) from the college of Health and Public Affairs at the University of Central Florida, where she graduated with honors and went on to graduate school at the University of Florida, College of Medicine. Rachael joined the Physiology and Functional Genomics department under the supervision of Dr. Colin Sumners in fall 2004, two years after she entered graduate school in 2002. In Dr. Sumners' laboratory, her research has focused on redox biology and neurogenic hypertension. She will receive her doctorate in August of 2008 and go on to a career in cancer research at the Sunnybrook Health Sciences Center in Toronto, Ontario.