Macrophage migration inhibitory factor antagonizes pressure overload-induced cardiac hypertrophy

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1Center for Heart and Lung Research, The Feinstein Institute for Medical Research, Manhasset, New York; 2The Elmezzi Graduate School of Molecular Medicine, North Shore-Long Island Jewish Health System, Manhasset, New York; and 3Pediatric Cardiology, Cohen Children’s Medical Center of New York, New Hyde Park, New York

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Koga K, Kenessey A, Ojamaa K. Macrophage migration inhibitory factor antagonizes pressure overload-induced cardiac hypertrophy. Am J Physiol Heart Circ Physiol 304: H282–H293, 2013. First published November 9, 2012; doi:10.1152/ajpheart.00595.2012.—Macrophage migration inhibitory factor (MIF) functions as a proinflammatory cytokine when secreted from the cell, but it also exhibits antioxidant properties by virtue of its intrinsic oxidoreductase activity. Since increased production of ROS is implicated in the development of left ventricular hypertrophy, we hypothesized that the redox activity of MIF protects the myocardium when exposed to hemodynamic stress. In a mouse model of myocardial hypertrophy induced by transverse aortic coarctation (TAC) for 10 days, we showed that growth of the MIF-deficient heart was significantly greater by 32% compared with wild-type (WT) TAC hearts and that fibrosis was increased by fourfold (2.62 ± 0.2% vs. 0.6 ± 0.1%). Circulating MIF was increased in TAC animals, and expression of MIF receptor, CD74, was increased in the hypertrophic myocardium. Gene expression analysis showed a 10-fold increase (P < 0.01) in ROS-generating mitochondrial NADPH oxidase and 2- to 3-fold reductions (P < 0.01) in mitochondrial SOD2 and mitochondrial aconitase activities, indicating enhanced oxidative injury in the hypertrophied MIF-deficient ventricle. Hypertrophic signaling pathways showed that phosphorylation of cytosolic glycogen synthase kinase-3α was greater (P < 0.05) at baseline in MIF-deficient hearts than in WT hearts and remained elevated after 10-day TAC. In the hemodynamically stressed MIF-deficient heart, nuclear p21cip1 increased sevenfold (P < 0.01), and the cytosolic increase of phospho-p21cip1 was significantly greater than in WT TAC hearts. We conclude that MIF antagonizes myocardial hypertrophy and fibrosis in response to hemodynamic stress by maintaining a redox homeostatic phenotype and attenuating stress-induced activation of hypertrophic signaling pathways.

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Mean heart rates for WT and KO mice were 52.7 beats/min, respectively. There were no statistical differences in these measurements between groups. Data obtained from 21 WT sham, 27 TAC groups, with mean pressures of 26.6 mmHg for 9-wk-old male WT and MIF KO mice were 22.6 ± 0.3 and 21.2 ± 0.2 g, respectively. Cardiac hypertrophy was produced by coarctation of the transverse thoracic aorta [transverse aortic coarctation (TAC)], as previously published (1). Briefly, mice were intubated and anesthetized using isoflurane; respiration was controlled by mechanical ventilation (Harvard Apparatus, Boston, MA). The left thorax was opened at the second intercostal space to expose the aortic arch, and the transverse aorta between the left common carotid artery and innominate artery was constricted using a suture tied around a 27-gauge needle. Sham surgery (sham animals) was similarly performed for this study, were bred and maintained in The Feinstein Institute. Wild-type (WT) C57BL/6 male mice were purchased from Taconic Farms (Albany, NY). Mean body weights before surgery of 9-wk-old male WT and MIF KO mice were 22.6 ± 0.3 and 21.2 ± 0.2 g, respectively. Cardiac hypertrophy was produced by coarctation of the transverse thoracic aorta [transverse aortic coarctation (TAC)], as previously published (1). Briefly, mice were intubated and anesthetized using isoflurane; respiration was controlled by mechanical ventilation (Harvard Apparatus, Boston, MA). The left thorax was opened at the second intercostal space to expose the aortic arch, and the transverse aorta between the left common carotid artery and innominate artery was constricted using a suture tied around a 27-gauge needle. Sham surgery (sham animals) was similarly performed for this study, were bred and maintained in The Feinstein Institute.

Materials and Methods

Animal protocols. All animals used in the experiments were treated in accordance with National Institutes of Health (NIH) guidelines for the use and care of laboratory animals (NIH Pub. No. 85-23), and all study protocols were approved by the Animal Care and Use Committee of The Feinstein Institute. MIF-deficient [mif−/− knockout (KO)] mice were originally developed as described by Bozza et al. (4) and, for this study, were bred and maintained in The Feinstein Institute. Mortality was <1% over this time period and was similar for both groups. Body weight gains after 10- and 28-day TAC were not statistically different between WT and MIF KO mice (0.62 ± 0.11 and 0.52 ± 0.09 g after 10-day TAC; 1.48 ± 0.13 and 1.41 ± 0.14 g after 28-day TAC, respectively). Thus, heart weight-to-body weight ratios reflected cardiac hypertrophy and not changes in body weight. Arterial pressure gradients across the constriction were measured using pressure catheters (1.4-Fr, Millar Instruments, Houston, TX) inserted into the left and right carotid arteries, and data were collected and analyzed using a PowerLab 8/30 acquisition system (AD Instruments, Colorado Springs, CO). Pressure gradients were similar in TAC groups, with mean pressures of 26 ± 3 and 22 ± 8 mmHg for WT and MIF KO animals, respectively. Mean ± SE right and left carotid artery pressures were 89.5 ± 7.8 and 65.5 ± 6.9 mmHg for WT mice and 88.7 ± 4.9 and 70.8 ± 4.8 mmHg for MIF KO mice. Mean heart rates for WT and KO mice were 527 ± 19 and 563 ± 25 beats/min, respectively. There were no statistical differences in these measurements between groups. Data obtained from 21 WT sham, 27 WT 10-day TAC, 20 MIF KO sham, and 26 MIF KO 10-day TAC mice were used for the various analyses reported in this study.

Analysis of inflammatory cytokines. Arterial blood samples were collected into heparinized tubes at the end of the experimental period before removals of the anterior thoracic aorta. Hearts were formalin fixed and paraffin embedded. Four longitudinal sections (NIH) and expressed as a fraction of the total LV (free wall plus septum) area. To measure the infiltration of inflammatory granulocytes into the heart after 10-day TAC, paraffin-embedded tissue slides were deparaffinized and stained for collagen using Masson’s trichrome staining procedures (American MasterTech, Lodi, CA). LV sections were visualized microscopically (Zeiss Axiosvert 200M), images were captured, and areas of fibrosis were quantified using ImageJ software (NIH) and expressed as a fraction of the total LV (free wall plus septum) area. To measure the infiltration of inflammatory granulocytes into the heart after 10-day TAC, paraffin-embedded tissue slides were deparaffinized and stained for esterase activity using the substrate naphthol AS-D chloroacetate, essentially as described by the manufacturer (Sigma-Aldrich, St. Louis, MO).

Measurement of oxidative enzyme activities. Activities of SOD and catalase in mitochondrial and cytoplasmic fractions and cytosolic glutathione peroxidase (GPx) were measured in extracted LV tissue.
using commercially available assay kits following the manufacturer’s recommendations (Cayman Chemical, Ann Arbor, MI).

**Culture of neonatal rat ventricular myocytes and adeno viral transduction.** Ventricular myocytes were isolated from hearts of 2-day-old rats by collagenase digestion as we have previously described (19). Myocytes were plated at \(1.5 \times 10^4\) cells/cm\(^2\) on collagen-coated 60-mm dishes, cultured for the first 20 h in DMEM-F-12 medium containing 10% FBS, l-glutamine, cytosine \(\beta\)-d-arabinofuranoside (10 \(\mu\)M), and antibiotics, and then transduced with adenovirus (75 multiplicity of infection) expressing WT MIF (Ad-MIF) or Ad-LacZ, as we have previously described (22). To study the oxidoreductase function of MIF, this enzyme activity was rendered inactive by mutating Cys\(^{60}\) to Ser\(^{60}\) at the thiol protein oxidoreductase site using site-directed mutagenesis as recommended by the manufacturer (Stratagene, La Jolla, CA). Recombinant adenovirus was constructed, which expressed redox inactive MIF and human recombinant green fluorescent protein (GFP) from a dicistronic gene (Ad-C60S). Forty-eight hours after viral transduction, >90% of the cardiomyocytes expressed exogenous protein, as determined by GFP fluorescence imaging. Neonatal rat ventricular myocytes (NRVMs) were then exposed to 16 h of hypoxia (<1% O\(_2\)) or to 16 h of hypoxia followed by 1 h of reoxygenation before being harvested. In some experiments, transduced NRVMs were treated with the reducing agent N-mercaptopyrroline (2-MPG; 400 \(\mu\)M), and antibiotics, and then transduced with adenovirus expressing reducing agent expressing WT MIF (Ad-MIF) or Ad-LacZ, as we have previously described (22). To study the oxidoreductase function of MIF, this enzyme activity was rendered inactive by mutating Cys\(^{60}\) to Ser\(^{60}\) at the thiol protein oxidoreductase site using site-directed mutagenesis as recommended by the manufacturer (Stratagene, La Jolla, CA). Recombinant adenovirus was constructed, which expressed redox inactive MIF and human recombinant green fluorescent protein (GFP) from a dicistronic gene (Ad-C60S).

Measurement of apoptosis and oxidative stress in cultured cardiomyocytes. Cell fractionation of cytoplasmic and nuclear proteins for immunoblot analysis was done as previously described (19). Cleaved caspase 3 (Cell Signaling Technology) was quantified by immunoblot analysis and normalized to GAPDH as a measure of cell apoptosis. Additionally, apoptosis was quantified using a commercially available ELISA kit (Roche, Nutley, NJ) that detected cytoplasmic mono- and oligonucleosomes using anti-histone antibodies. Concentrations of GSH and GSSG were determined by the established glutathione reductase/5,5′-dithiobis(2-nitrobenzoic acid) recycling procedure using a commercially available kit (Cayman Chemical), as we have previously described (22).

Statistical analysis. Data are presented as means ± SE. Sample numbers per group are indicated in the results and figures. Statistical analysis was performed using a paired Student’s t-test or one-way ANOVA followed by Holm-Sidak post hoc analysis. \(P\) values of <0.05 were considered statistically significant. Statistical analysis used SigmaStat 3.1 (Systat Software, Richmond, CA).

**RESULTS**

**Augmented hypertrophic responses to pressure overload in MIF KO hearts.** To determine whether endogenous MIF plays a role in regulating cardiomyocyte growth, MIF KO and WT mice were subjected to TAC for 10 or 28 days. Hearts that were deficient in MIF were 32% larger (\(P < 0.01\)) than WT hearts after 10 days of pressure overload, and this difference persisted to 28 days (Fig. 1A). Furthermore, after 10-day TAC, LV fibrosis as measured by collagen staining was 4.4-fold higher (\(P < 0.001\)) in the MIF-deficient ventricle compared with the WT ventricle (Fig. 1B). To determine whether there was inflammation in the hypertrophied hearts, we stained tissue sections for esterase activity, which would indicate infiltration of cells of the granulocytic lineage. We failed to observe any positive cells after 10-day TAC in either WT or MIF KO hearts (data not shown).

MIF has been shown to be released from the heart and other tissues in response to oxidative stress (36, 49); therefore, we measured its content in LV tissue and in the blood of TAC animals. MIF was detected in the plasma of WT 10-day TAC mice and remained elevated after 28-day TAC (\(P < 0.01\)), with no significant reduction in tissue MIF levels (Fig. 1C). Secreted MIF can function in an autocrine/paracrine manner by binding to CD74 cell surface receptors, which we found to be significantly elevated in hypertrophied ventricles of both WT and MIF KO mice after 10-day TAC (Fig. 1D). Previously published studies have shown that extracellular recombinant MIF phosphorylates AMP kinase (36) and thus confers protection of the postischemic myocardium (45). In the present study, pressure overload-induced hypertrophy had no effect on AMP kinase activation in MIF KO or WT hearts (data not shown).

Inflammatory response in pressure overload-induced myocardial hypertrophy. To determine whether the complete absence of MIF changed the inflammatory response to pressure overload, we measured plasma concentrations of seven cytokines/chemokines, including IL-1\(\beta\), IL-12 p70, IFN-\(\gamma\), IL-6, KC/GRO, IL-10, and TNF-\(\alpha\). We found no significant responses in any cytokine in either WT or MIF KO mice after 10 or 28 days of pressure overload compared with sham control mice. However, there was a trend for IL-1\(\beta\) to increase in both WT and MIF KO mice after 10-day TAC (0.93 ± 0.01, 0.96 ± 0.10, 0.34 ± 0.03, and 0.65 ± 0.28 pg/ml in WT sham, WT TAC, MIF KO sham, and MIF KO TAC mice, respectively). Additionally, the anti-inflammatory cytokine IL-10 showed a non-significant decrease in 10-day TAC animals (154.0 ± 4.1, 79.4 ± 67.6, 180.0 ± 27.0, and 137.7 ± 20.3 pg/ml in WT sham, WT TAC, MIF KO sham, and MIF KO TAC mice, respectively).

Differential induction of oxidative stress genes in the hypertrophied MIF-deficient myocardium. We (22) have previously shown that oxidative stress is greater in the MIF-deficient postischemic myocardium. Therefore, we investigated the response of stress/antioxidant genes in hypertrophied hearts after 10-day TAC to determine whether MIF deficiency leads to an enhanced response to cellular stress. Of the 84 oxidative stress genes measured in the PCR array, the expression of 20 genes was significantly altered in response to pressure overload in either MIF KO or WT hearts compared with WT sham mice. In general, genes encoding proteins with antioxidant function were reduced, whereas those that promoted oxidative stress were increased. The fold regulation and \(P\) values of these genes are shown in Table 1. Expression of two stress genes was significantly greater at baseline (sham) in MIF KO compared with WT mice, namely, thioredoxin reductase and xin actin-binding repeat containing 1 (Xirp1), an F-actin-binding protein recently shown to be upregulated in injured muscle, possibly involved in cellular repair (42, 43). Notably, Xirp1 expression was increased with pressure overload in either mouse strain. The expression of some genes, such as vimentin, was upregulated similarly in MIF KO and WT hearts in response to pressure overload, whereas others were differentially regulated. These are shown in Table 2. Of particular note was the 4-fold difference in the expression of the ROS-generating mitochondrial oxidase NOX4 in response to pressure overload, which was increased 3-fold in WT hearts and 10-fold in MIF KO hearts (Fig. 2B). Immunoblot analysis of NOX4 protein confirmed the fourfold difference in expression between MIF
KO and WT TAC hearts (Fig. 2A). However, NOX4 protein but not mRNA was significantly higher even at baseline (sham) in MIF KO hearts compared with WT hearts. In contrast, the significant increase in NOX4 mRNA was not observed in NOX4 protein in WT hearts in response to TAC (Fig. 2A).

To further assess the regulatory role of MIF in maintaining redox homeostasis, we measured the protein content of mitochondrial SOD2 and UCP3 and the enzymatic activities of SOD2, GPX, and aconitase, a mitochondrial enzyme sensitive to oxidative damage. SOD2 protein was significantly decreased in hypertrophied MIF KO hearts compared with WT hearts. In contrast, the SOD2 mRNA was significantly higher even at baseline (sham) but not mRNA was significantly higher at 10- and 28-day TAC (n = 4–5 mice/group). **P < 0.001 vs. the respective sham-operated [sham (sh)] group. Statistical P values between wild-type (WT) TAC and MIF KO TAC groups are shown. B: representative histological sections of 10-day TAC left ventricles (LVs) showing Masson’s trichrome staining of collagen, with a 4-fold increase in fibrosis of MIF KO hearts. C, top: representative immunoblot of MIF in LV homogenates and plasma of WT mice after 10 and 28 days of TAC and in sham control mice. Bottom: graph showing a quantitation of the Western blot of plasma MIF, showing elevated levels after 10- and 28-day TAC (n = 4–5 mice/group). **P < 0.001 vs. the WT sham group. D, top: representative Western blot of putative MIF receptor CD74 in LV homogenates of WT and MIF KO hearts after 10-day TAC. Bottom: graph showing quantitation of CD74 normalized to GAPDH, showing increased expression in TAC ventricles (n = 4 mice/group). *P < 0.01 vs. the sham group.

Altered expression of mitochondrial UCP3, SOD2, glutathione-S-transferase (GST)-ζ1, and NOX4 in hearts of MIF KO hearts would be expected to increase oxidative stress. Thus, as an indicator of oxidative stress, we measured mitochondrial aconitase activity because its iron-sulfur cluster is inactivated by superoxide, as we and others (6, 22) have previously shown in the postsischemic heart. Aconitase activity was significantly lower in hypertrophied MIF KO hearts compared with WT hearts (Fig. 2F). Although aconitase activity was measurable in cytosolic fractions, there were no differences in activity among the groups; similarly, total cytosolic GPX activity was not different among the experimental groups (48.6 ± 4.6, 44.7 ± 2.5, 46.8 ± 7.5, and 53.7 ± 7.1 nmol·min⁻¹·mg⁻¹ for WT sham, WT TAC, MIF KO sham, and MIF KO TAC mice, respectively).

Altered intracellular signaling in MIF-deficient hypertrophic cardiac growth. To determine the molecular basis by which pressure overload-induced cardiac hypertrophy was markedly increased with a deficiency of MIF, we examined known growth regulatory pathways (12, 55) and cell cycle

Fig. 1. Pressure overload-induced hypertrophic growth is augmented in the macrophage migration inhibitory factor (MIF)-deficient [knockout (KO)] heart. A: pressure overload-induced cardiac hypertrophy was produced by coarctation of the transverse aortic (TAC) for either 10 days (10d) or 28 days (28d). Cardiac growth is expressed as the ratio of ventricular (heart) weight to body weight. Values are means ± SE; n = 4–5 mice/group. *P < 0.01 vs. the respective sham-operated [sham (sh)] group. Statistical P values between wild-type (WT) TAC and MIF KO TAC groups are shown. B: representative histological sections of 10-day TAC left ventricles (LVs) showing Masson’s trichrome staining of collagen, with a 4-fold increase in fibrosis of MIF KO hearts. C, top: representative immunoblot of MIF in LV homogenates and plasma of WT mice after 10 and 28 days of TAC and in sham control mice. Bottom: graph showing a quantitation of the Western blot of plasma MIF, showing elevated levels after 10- and 28-day TAC (n = 4–5 mice/group). **P < 0.001 vs. the WT sham group. D, top: representative Western blot of putative MIF receptor CD74 in LV homogenates of WT and MIF KO hearts after 10-day TAC. Bottom: graph showing quantitation of CD74 normalized to GAPDH, showing increased expression in TAC ventricles (n = 4 mice/group). *P < 0.01 vs. the sham group.

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Table 1. Oxidative gene expression in response to pressure overload in WT and MIF KO mice

<table>
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<tr>
<th>Gene</th>
<th>Expression level (WT TAC)</th>
<th>Expression level (MIF KO Sham)</th>
<th>Expression level (MIF KO TAC)</th>
<th>P value</th>
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<td>Xipr1</td>
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Levels of expression of mRNAs for each experimental group are expressed as fold up- or downregulation compared with the wild-type (WT) sham-operated (sham) group. Negative values indicate fold downregulation; positive values indicate fold upregulation. TAC, transverse aortic coarctation; MIF, macrophage migration inhibitory factor; KO, knockout; Als2, amyotrophic lateral sclerosis 2; Xipr1, xin actin-binding repeat containing 1; Ercc2, excision repair; Fancc, Fanconi anemia; Gab1, growth factor receptor-bound protein 2-associated protein 1; Gpx, glutathione peroxidase; Gstk1, glutathione-S-transferase-x1; Nox4, NADPH oxidase 4; Nud15, nucleoside diphosphate-linked moiety X; Park7, Parkinson disease 7; Prdx5, peroxiredoxin 5; Prdx6-rs1, peroxiredoxin 6-related sequence 1; Serpinb1b, serine (cys) peptidase inhibitor; Slc41a3, solute carrier family 41, member 3; SOD2, mitochondrial superoxide dismutase; Txnip, thioredoxin-interacting protein; Txnrd, thioredoxin reductase; Ucp3, uncoupling protein 3 (a mitochondrial proton carrier); Vim, vimentin. *Only P values of genes that were expressed significantly differently from the WT sham group are shown.

Table 2. Altered expression of oxidative stress genes in response to pressure overload in MIF KO hearts compared with WT hearts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Regulation (MIF KO vs. WT)</th>
<th>P value</th>
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</thead>
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<td>Ercc2</td>
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<tr>
<td>Fancc</td>
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For gene abbreviations, see Table 1. Fold regulation represents increased or decreased gene expression in MIF KO hearts compared with WT hearts after 10-day TAC. Negative values represent fold downregulation; positive values represent upregulation. Student’s t-test was used to determine significance between group means.
GSK-3 has been shown to be inactivated in the hypertrophic myocardium when phosphorylated by upstream kinases such as Akt. However, the function of the two GSK-3 isoforms differs, with GSK-3β phosphorylation in response to pressure overload leading to fibrosis, increased hypertrophy, and LV dysfunction, whereas phosphorylation of GSK-3α appears to act in a compensatory fashion (33). To address the role of GSK-3 isoforms in the hypertrophic growth of MIF KO hearts, we measured the phosphorylation of cytosolic and nuclear GSK-3 isoforms. The most remarkable changes were observed in the GSK-3α isoform, with significant increases in p-GSK-3α in the cytosolic fraction of MIF KO hearts (Fig. 5A). Phosphorylation of nuclear GSK-3α was also increased, albeit not significantly, with a decrease in total GSK-3α protein content in MIF KO hearts (Fig. 5B). Although p-GSK-α expression was significantly different between WT and MIF KO hearts, the difference between WT and MIF KO hypertrophied hearts did not reach statistical significance. We also observed a trend toward increased phosphorylation of GSK-3β in both cytosolic and nuclear fractions of MIF KO hearts and in the cytosol of WT TAC hearts (Fig. 5, C and D).

Overexpression of WT MIF but not MIF redox mutant resuces cardiomyocytes after oxidative/reductive stress. An essential function of this pleiotropic factor is its oxidoreductase activity, which we and others (22, 29, 41) have shown to be important in maintaining cellular redox homeostasis in the heart and other tissues. To study the importance of MIF oxidoreductase activity in response to cellular stress, we transduced primary NRVMs with an adenovirus expressing a mutant MIF protein lacking redox activity (Ad-C60S). To elicit an oxidative-reductive stress response, cells were exposed to a reduced oxygen environment (hypoxia) or hypoxia followed by

**Fig. 2.** Altered expression of mitochondrial oxidative-reductive stress enzymes in the MIF-deficient heart. A, *top*: Western blot showing NADPH oxidase (NOX4) protein in representative LV homogenates of WT and MIF KO mice after 10-day TAC. *Bottom*, bar graph showing the quantitation of NOX4 normalized to GAPDH (*n* = 4 mice/group). *P* < 0.01 vs. the WT sham group; **P* < 0.01 vs. all groups. B: NOX4 mRNA determined by quantitative PCR (*n* = 3 mice/group). *P* < 0.01 vs. the respective sham group; **P* < 0.05 vs. the WT TAC group. C, *top*: representative immunoblot of mitochondrial SOD in WT and MIF KO hearts after 10-day TAC. *Bottom*, bar graph showing the quantitation of SOD2 normalized to GAPDH (*n* = 4 mice/group). *P* < 0.01 vs. the WT sham group. D, *top*: immunoblot of uncoupling protein-3 (UCP3) in representative LV homogenates from WT and MIF KO mice. *Bottom*, bar graph showing quantitation of UCP3 normalized to GAPDH (*n* = 4 mice/group). *P* < 0.01 vs. all groups; *P* = 0.06 vs. the WT sham group. E: SOD activity measured in purified mitochondrial fractions of the LV from WT and MIF KO hearts after 10-day TAC (*n* = 4 mice/group). **P* < 0.01 vs. all groups. F: aconitase activity in purified mitochondrial fractions of the LV from WT and MIF KO hearts (*n* = 4 mice/group). *P* < 0.01 vs. the WT sham group; **P* = 0.06 vs. the WT TAC group.
results supported a protective effect of WT MIF overexpression on cardiomyocytes exposed to hypoxic stress (Fig. 6B). Intracellular oxidative stress, as measured by GSH-to-GSSG ratios, showed that MIF overexpression significantly improved the intracellular redox environment in cells exposed to hypoxia (Fig. 6C). This protective antioxidative effect of MIF on cell survival could be mimicked by treatment of cardiomyocytes with a reducing agent, 2-MPG, during exposure to hypoxia and reoxygenation (Fig. 6D). These data further support a potentially important oxidoreductive role of MIF in the heart during stress conditions, including hypertrophic stress, which is known to promote ROS production.

**DISCUSSION**

The seminal results in the present study support our primary hypothesis that MIF functions to decrease oxidative/reductive stress and thus attenuates myocardial hypertrophy in response to hemodynamic load. Using an MIF gene deletion mouse model, we have shown that deficiency of MIF resulted in significantly greater cardiac hypertrophy with increased fibrosis in response to pressure overload compared with WT hearts. In WT mice, MIF protein was detected in the circulation of 10- and 28-day TAC animals, with increases in myocardial expression of the MIF receptor, CD74, in both WT and MIF KO hearts. Several recent studies (36, 45) have supported a cardioprotective effect of MIF on the postsischemic myocardium, which signals through the CD74 receptor, resulting in AMP kinase activation and suppression of the JNK pathway. However, in the present study, neither AMP kinase nor JNK phosphorylation was significantly altered in response to hemo-
dynamic stress in either WT or MIF KO hearts. An MIF homolog, D-dopachrome tautomerase (D-DT), which binds CD74 cell surface receptor with high affinity and initiates proinflammatory responses but lacks oxidoreductase activity, has been recently reported (35). Thus, D-DT cannot compensate for the loss of MIF redox activity in MIF KO mice and is unlikely to account for the augmented hypertrophic response observed in MIF KO TAC hearts since our data showed that expression of CD74 was induced to a similar extent in both WT and MIF KO hearts in response to pressure overload. It follows, therefore, that the intracellular oxidoreductase activity of MIF likely plays a key role in attenuating stress in response to hypertrophic stimuli. However, we cannot rule out a role for the increased circulating MIF that may activate receptor-initiated intracellular signaling mechanisms that participate in the myocardial response to pressure overload.

MIF has been shown to be a regulator of innate immunity that promotes proinflammatory functions of immune cells (8). Since circulating MIF was elevated in TAC animals and expression of its cell surface receptor, CD74, was increased in the heart, it was reasonable to ask whether its proinflammatory function played a role in the hypertrophic response. However, we failed to observe any differences in activation of known MIF receptor-mediated intracellular signaling pathways between WT and MIF KO mice. Furthermore, systemic inflammation was not evident in this hemodynamic stress model,
although we cannot rule out this possibility at a time point earlier than 10 days after coarctation.

To determine how MIF deficiency altered the oxidative stress response of the myocardium to pressure overload, we studied the expression of 84 stress-specific genes. Of the 20 genes whose expression was altered in hypertrophied hearts, 11 genes were differentially regulated in MIF KO compared with WT hearts, producing a phenotype indicative of oxidative stress. Particularly striking was the 10-fold increase in NOX4 mRNA in MIF KO TAC hearts compared with the 3-fold increase in WT TAC hearts, which was similar to that previously reported in WT mice (1, 7). Protein analysis showed similar increases in NOX4 in MIF KO hypertrophied hearts, but, in contrast to the mRNA data, NOX4 protein was significantly higher even at baseline before TAC. Ago et al. (1) have recently shown that NOX4 is expressed primarily in the mitochondria and that mitochondrial proteins containing iron-sulfur clusters, such as adenine nucleotide translocator-1, citrate synthase, and aconitase, could be oxidized by $O_2^{-}/H_2O_2$ generated by NOX4. These authors also reported that cysteine residues of many mitochondrial proteins were oxidized to a greater extent in transgenic NOX4 mice than in WT mice. In support of an
increased oxidative environment as a result of NOX4 expression in the hypertrophied myocardium, we found that mitochondrial aconitase activity was significantly reduced in MIF KO but not WT TAC hearts. Further evidence supporting an essential role of NOX4 in O$_2^-$ production in mitochondria derives from a study (23) in which increased O$_2^-$ production induced by pressure overload was abolished in cardiac-specific NOX4 KO mice. To maintain intracellular redox homeostasis, mitochondrial MnSOD converts reactive O$_2^-$ to H$_2$O$_2$, which serves as a mechanism to protect mitochondrial proteins from oxidation (30). We found that mitochondrial SOD activity was significantly reduced in MIF KO hearts but not WT hearts when subjected to pressure overload, further supporting a role for MIF in redox homeostasis.

There were concurrent decreases in other antioxidant defense proteins in MIF KO hypertrophied hearts, including mitochondrial UCP3, GST-κ1, and cytosolic peroxiredoxin-5. Mitochondrial UCP3 has been shown to reduce excessive production of ROS (31) and to prevent lipid peroxidation and reduce damage to mitochondrial proteins (3, 5). UCP3 has also been reported to be upregulated in hearts exposed to ischemic preconditioning, resulting in attenuated infarct size and improved functional outcomes (34). GSTs encompass a family of enzymes that conjugate glutathione to various endogenous molecules; the κ-class GSTs with peroxidase activity are localized to mitochondria and peroxisomes and probably function to detoxify lipid peroxides generated during oxidative stress (38). Taken together, the reduced expression of antioxidant proteins, UCP3 and GST-κ1, in the MIF KO heart subjected to TAC would further stress the myocardium, in which ROS generated by increased mitochondrial NOX4 would promote protein and lipid oxidation and myocyte dysfunction. When we examined the fold changes in the expression of redox-related genes, the magnitude of change was greater in MIF KO TAC hearts than in WT TAC hearts, with 18 versus 8 genes differentially regulated, respectively. Expression of only 2 of 84 genes was significantly different at baseline (sham) between WT and MIF KO mice. Thus, the marked hypertrophy of the MIF KO heart in response to pressure overload is, in large part, a result of increased oxidative stress due to the loss of MIF redox function and to altered signaling pathways leading to the phosphorylation and inhibition of GSK-3 activity, which removes its negative constraint on cardiac hypertrophy (9, 48). Thus, in basal unstimulated conditions, GSK-3α is active serine/threonine kinases that function to limit cardiac growth by phosphorylating positive mediators of hypertrophy. The two isoforms, GSK-3α and GSK-3β, have distinct functions, with GSK-3α functioning to protect against TAC-induced hypertrophy, as shown by a study (58) using GSK-3α KO mice. Similar to the effects of deletion of GSK-3α, we observed a significant increase in the phosphorylation of cytosolic GSK-3α in MIF KO hearts after 10-day TAC. However, we could not show a significant difference in cytosolic p-GSK-3α between WT and MIF KO TAC hearts that could account for the differences in the hypertrophic response to pressure overload. The effect of increased p-GSK-3α at baseline in the MIF KO heart is presently unclear, but indicates that additional mechanisms mediating hypertrophic response to stress may be playing a role in the MIF KO heart. There remains some controversy regarding the role of GSK-3α in cardiac hypertrophy, as evidenced by results from a constitutively active GSK-3α S21A knockin mouse model in which pressure overload promoted hypertrophy, fibrosis, and myocyte apoptosis with a preferential localization of GSK-3α in the nucleus (33). We observed a significant decrease of total nonphosphorylated GSK-3α in the nucleus, with no significant change in its phosphorylation status. Many of the observed changes in GSK-3 show a trend toward differential expression in the MIF KO heart; thus, their significance in the hypertrophic response may be meaningful only when considered in context with other molecular changes.

In conclusion, the present data support an important role for the intrinsic oxidoreductase activity of MIF to maintain redox homeostasis within the cell, such that loss of this activity in the heart results in augmented cardiac hypertrophy in response to hemodynamic stress. Furthermore, increased circulating MIF in the hemodynamically stressed animal may provide benefits through currently unknown mechanisms. The increase in ROS-generating enzymes, such as NOX4, and the reduction of antioxidant mitochondrial proteins, such as UCP3 and GST-1κ, in the MIF-deficient heart may be secondary mechanisms by which the MIF-deficient heart fails to cope with cellular stress. Thus, targeting MIF’s redox activity, similar to that of other intrinsic antioxidants, such as thioredoxin, may provide benefits for patients with cardiomyopathy, pathological hypertrophy or heart failure.

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