Endotoxin-Induced Myocardial Dysfunction
Effects of Macrophage Migration Inhibitory Factor Neutralization

Frederic Chagnon, Christine N. Metz, Richard Bucala, Olivier Lesur

Abstract—The pathophysiology of sepsis-induced myocardial dysfunction still remains controversial. Macrophage migration inhibitory factor (MIF) has recently been identified as a cardiac-derived myocardial depressant factor in septic shock. Putative mechanisms by which MIF affects cardiac function are unknown. In an investigation of possible mechanisms of action, a rat model of endotoxin toxicity was designed using intraperitoneal (IP) injection of lipopolysaccharides (LPS) with or without coinfusion of neutralizing anti-MIF or isotypic-matched antibodies. Echocardiographic evaluation revealed that MIF neutralization reversed endotoxin-induced myocardial dysfunction at 24 hours after injection. RNase protection assay (RPA) and Western blot established that MIF neutralization prevented LPS-induced mRNA expression and production of heart-derived inflammatory paracrine and autocrine cytokines such as IL-1s and IL-6. Moreover, MIF immunoneutralization increased heart Bcl-2/Bax protein ratio and suppressed endotoxin-induced release of mitochondrial cytochrome-c, as demonstrated by Western blotting. Inhibition of mitochondrial loss of cytochrome-c decreased in heart caspase-3 activity at 6 and 24 hours after injection. MIF neutralization also restored the LPS-induced deficient nuclear translocation of phospho-Akt and consequently the expression of the heart survival nuclear factor GATA-4. The restoration of the translocation/expression of survival factors by MIF inhibition resulted in lowered endotoxin-induced DNA fragmentation at 24 hours, a hallmark of downstream cardiomyocyte apoptosis. Our data indicate that early inactivation of MIF significantly reverses the imbalance of proapoptotic to prosurvival pathways and reduces acute inflammation of the heart thereby improving myocardial dysfunction induced by endotoxin. (Circ Res. 2005;96:1095-1102.)

Key Words: sepsis ■ myocardial dysfunction ■ migration inhibitory factor neutralization ■ lipopolysaccharides

Severe sepsis and septic shock are leading and growing causes of morbidity and mortality in hospitalized patients, and contribute to an incidence of almost 250 cases per 100,000 population per year in the United States. These conditions account for the deaths of 150,000 Americans per year, and more than 75% involve cardiovascular failure with severe, sustained, and sometimes refractory hypotension. Over the last decade, it has become clear that myocardial dysfunction is linked to cardiovascular failure in human and experimental septic shock. Bacteria or endotoxins (lipopolysaccharides [LPS]) contribute to and initiate a sequence of cellular events that lead to decreased contractile efficiency and left ventricular enlargement and dysfunction.

The sequelae of systemic infection can produce reversible or irreversible damage to cardiomyocytes, such as impairment of intracellular calcium homeostasis, alterations of excitation-contraction coupling, and enhanced programmed cell death (or apoptosis). Apoptosis is a new culprit for cardiac failure and sepsis-induced heart dysfunction that is being increasingly targeted for potential therapies. Among the triggering molecules for this dysfunction are inflammatory mediators, including cytokines such as tumor necrosis factor-α (TNF-α), which is abundantly produced in acute phase sepsis. However, anti–TNF-α therapies have failed to improve the mortality of patients with severe sepsis and shock, suggesting the involvement and commitment of other important mediators in this pathogenesis.

Macrophage migration inhibitory factor (MIF) is a neutrophil and inflammatory mediator, mainly involved in immune homeostasis. Elevated concentrations of circulating MIF are found in animals and patients during septic shock. Moreover, deletion of the MIF gene or MIF protein immunoneutralization in animal models protects from lethal endotoxemia and septic shock. A recent study has demonstrated that MIF is constitutively expressed by the myocardium, is released in response to LPS challenge, and is a myocardial depressant factor in a mouse model of endotoxin (LPS)-induced cardiac dysfunction. However, the precise
mechanism by which MIF affects cardiac function still remains unknown.

It has been postulated that MIF is a major triggering molecule of LPS-induced heart dysfunction, leading to enhanced cardiac-derived inflammatory mediator expression and to increased apoptosis. Hence, it is hypothesized that neutralizing MIF (1) can prevent LPS-induced myocardial dysfunction, (2) is correlated with a reduction in cardiac inflammatory mediator expression together with decreased apoptosis signaling and rate, and (3) can reduce LPS-induced cardiac apoptosis by a mitochondrial-dependent; p53-independent pathway with restoration of phosphorylated Akt (pAkt) and GATA4 nuclear translocation. The present study was aimed at verifying the contribution of MIF in myocardial dysfunction as well as addressing possible mechanisms by which MIF neutralization improves sepsis outcome using a rat model of endotoxin challenge.

Materials and Methods

For complete Materials and Methods, please see online data supplement at http://circres.ahajournals.org.

Experimental Animal Model

Pathogen-free male Wistar rats (350 g) were purchased from Charles River Laboratories Inc (Wilmington, Mass) and received care in compliance with The Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care (1993, CCAC, 2nd ed.). This protocol was approved by our institution’s Ethics Committee for animal care and experimentation. Rats were given an intraperitoneal (I/P) injection of 1 mL phosphate-buffered saline (PBS) or 10 mg/kg of Escherichia coli 055:B5 LPS (Sigma) in 1 mL PBS. Concomitantly, mouse monoclonal anti-MIF (IgG1, III.D.9) or isotypic-matched (IgG1, HB49) control antibodies originating from comparable clones as those described by Calandra et al17 were injected (10 mg/kg in 1 mL PBS I/P). These anti-MIF antibodies had been previously shown to neutralize murine MIF bioactivity in vivo and in vitro.18 Note that rat MIF differs from mouse MIF by a single amino acid residue (mouse: Asn 54, rat Ser 54) that does not influence immunoreactivity with anti-MIF clone III.D.9).19 At 6 hours or 24 hours after injection, the hearts were removed under isoflurane anesthesia, snap frozen, and stored at −80°C.

Echocardiographic Study

Transathoracic echocardiography was performed under isoflurane anesthesia (2%) before as well as 24 hours after injection. All measurements were performed using a s12 HP 5 to 12-mHz high resolution small footprint transducer at a 60-Hz acquisition rate (Hewlett-Packard Sonos 5500) (see online data supplement). All echocardiograms were performed by the same qualified technician, and quality control was ensured by members of the Cardiology Department according to actual recommendations.20

RNA Extraction and RNase Protection Assay

Heart samples were thawed on ice, and total RNA was extracted with TRizol (Invitrogen) according to the manufacturer’s protocol. RNase protection assay, with the multiprobe template set rCK-2, was performed as described in the standard PharMingen RNase protection kit protocol (see online data supplement). The intensity of each band was analyzed and normalized to values of the housekeeping gene L32, to quantify expression.

Western Blotting

Standard Western blot analyses were performed to determine protein expression (see online data supplement). Forty to 100 μg of total proteins and 10 to 20 μg of mitochondrial or nuclear proteins were separated on a 10% or 15% SDS polyacrylamide gel under reducing conditions and transferred onto nitrocellulose membranes. Membranes were incubated overnight at 4°C with: polyclonal rabbit anti–IL-6 (1:1000; PeproTech, Rocky Hill, NJ), rabbit anti–IL-1β, anti–TNF-α (1:1000; Biosource, Camarillo, Calif), rabbit anti–Bcl-2 (1:200; Calbiochem, Cambridge, Mass), mouse anti-Bax; anti–TNFR1 (1:100 to 200); goat anti-actin (1:3000); rabbit anti–GATA-4 (1:500; Santa Cruz Biotechnology, Santa Cruz, Calif), anti–Fas (1:1000; B&D Biosciences, Mississauga, Ontario, Canada), rabbit anti–phospho-Akt (1:1000); anti–cytochrome-c (1:2000); anti–phospho-p53 (1:1000); anti–p53 (1:1000; Cell Signaling Technology, Beverly, Mass) or followed by appropriate species specific anti-IgG-HRP antibodies. Bands were revealed using ECL reagents (see online data supplement).

Immunofluorescence

Briefly, slices of heart (see online data supplement) were incubated with rabbit anticleaved caspase-3 (1:50; Cell Signaling Technology, Beverly, Mass), goat antimyosin heavy chain (1:10; Santa Cruz Biotechnology), and 1.5% mouse serum. Specific amplification was obtained by further incubation with mouse anti-rabbit IgG biotin and QDot 605 streptavidin conjugates (Quantum Dot, Hayward, Calif) for cleaved caspase-3, mouse anti-goat IgG FITC (Santa Cruz Biotechnology) for myosin heavy chain, and 2% mouse serum. Sections were analyzed using an Axiostar 2 fluorescence microscope (Carl Zeiss, Inc, Thornwood, NY) at a magnification of 1000×.

Caspase-3 Activity

Frozen heart samples were ground into and resuspended in ice-cold lysis buffer. Homogenates were centrifuged, and 100 μg of proteins in the supernatants were diluted with assay buffer and incubated at 37°C with the colorimetric substrates (Biomol, Plymouth, Pa). Ac-DEVD-pNA (200 μmol/L) in 96-well microtiter plates (see online data supplement). Cleavage of the p-nitroaniline (pNA) dye from the peptide substrate was determined by measuring the absorbance at 405 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, Calif). Results were expressed in mDO/hr/milligram protein.

DNA Fragmentation Detection

A Nucleosome ELISA kit (Calbiochem) was used according to manufacturer’s instructions for quantification of DNA fragmentation. Briefly, small pieces (40 to 50 mg) of hearts were homogenized in the provided lysis buffer and the supernatants were frozen at −20°C and further analyzed by ELISA. Results were expressed as a ratio of nucleosome units relative to total protein concentration (see online data supplement).

Statistical Analysis

Results are expressed as mean±SD. A repeated measures one-way analysis of variance was used to evaluate within-group differences. Inter-group difference was tested using a two-way analysis of variance (repeated time measurements with treatment as independent variable) and the Kruskal–Wallis test (nonparametric ANOVA). Correlations between data were obtained using a Spearman Rank correlation test for nonparametric parameters. P<0.05 was considered as the threshold for significance.

Results

Endotoxin (LPS)-Induced Myocardial Dysfunction Is Reversed by MIF Neutralization

To validate our experimental model, serial in vivo echocardiographies (M-mode) were performed before (baseline) and 24 hours after the experimental challenge (Figure 1a and 1b). This allowed to document that I/P injection of LPS together with the isotype Ab (further denoted as control) induced an almost 20% decrease in left ventricular ejection fraction

DNA Fractionation Detection
(LVEF) and a 110% increase in LVESv at 24 hours after challenge (Figure 2a and 2b; \( P < 0.05 \) versus baseline/normal). MIF neutralization by anti-MIF Ab in parallel to I/P challenge significantly reversed the LPS-induced fall in LVEF (Figure 2a; \( P < 0.05 \) versus control) and reduced the LPS-induced rise in LVESv by \( 80\% \) (Figure 2b; \( P < 0.05 \) control). Earlier 6-hour time-point assessment revealed more variable LPS-induced LVEF decreases (10% to 15%) with marginal impact of MIF neutralization. Respective controls injected with PBS plus isotype Ab or plus anti-MIF Ab at similar concentrations did not modify echocardiographic parameters.

MIF Neutralization Inhibits IL-6, IL-1\( \alpha/\beta \), and TNF-\( \alpha \) Myocardial Production Induced by LPS Challenge

RNase protection assays were performed to evaluate the compartmentalized impact of I/P LPS challenge on heart tissue. Results demonstrated respective increases of 818% and 378% in IL-1\( \alpha \) and IL-6 mRNA expressions at 24 hours after challenge (Figure 3a through 3c; \( P < 0.05 \) versus normal). MIF neutralization downregulated this LPS-induced expression of IL-1\( \alpha \) and IL-6 mRNA to \( 82\% \) and \( 21\% \), respectively, and similar modulations of IL-1\( \beta \) and IL-1ra mRNA expressions (Figure 3a through 3c; \( P < 0.05 \) versus control). Modulation of IL-1\( \beta \) and IL-6 mRNA expressions was paralleled by myocardial protein levels (Figure 3d). On the other hand, LPS challenge induced a transient rise in TNF-\( \alpha \) transcript expression (1712%, 400%, 341%, and 142% versus normal at 1, 3, 6, and 24 hours, respectively) but a more sustained protein overexpression, which is also inhibited by MIF neutralization at 24 hours after challenge (Figure 3d).

MIF Neutralization Reduces LPS-Induced Cardiac Cell Apoptosis

To assess whether LPS challenge induces cardiac cell apoptosis, immunofluorescence staining of rat hearts was performed, revealing active (cleaved) caspase-3, mostly nuclear in distribution, in myosin heavy chain–expressing cells at 24 hours after challenge (Figure 4a). Whole heart caspase-3 enzymatic activity was also measured and showed levels \( \approx 3.1\)-fold greater in LPS plus isotype Ab–challenged rats at both 6 and 24 hours after challenge (Figure 4b; \( P < 0.05 \) versus normal). This rise in enzyme activity was correlated with a drop in LVEF level (data from delta 24 hours-0 hour, \( n=50, r=0.519, P=0.001 \)). Hearts from rats receiving LPS plus anti-MIF Ab exhibited only a 1.3-fold induction of caspase-3 activity (Figure 4b; \( P < 0.05 \) versus control), suggesting MIF neutralization is partly protective of caspase-3 activation.
To determine whether this level of caspase-3 activation led to end-stage (downstream) apoptosis or if survival pathway(s) aborted the completion of the apoptotic process, DNA fragmentation was quantified in whole hearts. Nucleosome ELISA revealed a 322% increase in oligonucleosomal DNA fragmentation in hearts from LPS plus isotype Ab-treated rats at 24 hours after challenge (Figure 4c; \( P < 0.05 \) versus normal), and MIF neutralization reduced this LPS-induced DNA fragmentation by more than 2-fold (Figure 4c; \( P < 0.05 \) versus control).

MIF Neutralization Affects the Mitochondrial Apoptotic Pathway

To establish which of the main upstream apoptotic pathways is preferentially committed during the LPS-induced activation of caspase-3, the putative involvement of the mitochondrial pathway was evaluated by measuring mitochondrial and cytoplasmic cytochrome-c contents in LPS-challenged rat hearts at 6 hours after injection. Cytochrome-c levels were significantly lower in the mitochondrial fraction of heart extracts from LPS plus isotype Ab-challenged animals in comparison to normal. MIF neutralization restored the mitochondrial pool in rat heart extracts exposed to LPS (Figure 5). The other upstream Fas-TNF Receptor apoptotic pathway remained unaffected in this setting (data not shown).

MIF Neutralization Induces Bcl-2 Dominance in LPS-challenged Heart and Restores GATA-4 and pAkt Nuclear Translocations

To determine which molecular cascade is inhibited by MIF neutralization during LPS-induced heart apoptosis, mitochondria-associated prosurvival Bcl-2 and proapoptotic Bax were measured as representative modulating proteins. The Bcl-2/Bax ratio remained stable in heart extracts from rats injected with LPS plus isotype Ab as well as in normal, whereas MIF neutralization revealed an almost 2-fold increase at 6 hours after challenge (Figure 6; \( P < 0.05 \) versus control).

The transcription factor GATA-4 has previously been identified as a specific myocardial survival factor that induces the transcription and expression of Bcl-2, which, together with phospho-Akt, is also associated with cell survival. The presence of these mediators was investigated in heart nuclear extracts at 6
hours after LPS injection. LPS challenge induced more than a 2-fold drop in GATA-4 and the near disappearance of pAkt nuclear translocation, respectively, whereas MIF neutralization restored their presence (Figure 7a and 7b). These events were p53-independent (or at least phosphorylated p53-independent; Figure 7c), which is consistent with the known involvement of p53 in several MIF-mediated apoptotic processes.21–22

Discussion
The present study demonstrates that MIF neutralization reverses endotoxin-induced myocardial dysfunction in an experimental rat model. Moreover, MIF neutralization prevented LPS-induced mRNA and protein expressions of heart-derived inflammatory paracrine and autocrine cytokines such as IL-1s, IL-6, and TNF-α, which can be potentially harmful as myocardial depressants. Consequently, this study revealed that MIF blockade significantly inhibited endotoxin-induced cardiomyocyte apoptosis, leading to a proposed mechanism by which MIF mediates endotoxin-induced myocardial dysfunction. Under the proposed mechanism, MIF blockade induces (1) an increase in Bcl-2/Bax ratio that inhibits the release of mitochondrial cytochrome-c, (2) an inhibition in the loss of mitochondrial cytochrome-c which prevents caspase-3 activation, (3) a restoration of the nuclear translocation of survival factors GATA-4 and pAkt, and (4) a reduction in DNA fragmentation, which is a hallmark of downstream apoptosis. Overall, inhibition of MIF partially reverses the imbalance of proapoptotic to prosurvival path-

Figure 4. Effect of anti-MIF antibody on LPS-induced cardiomyocyte apoptosis. A, Immunofluorescence imaging of a heart section from a 24-hour LPS postchallenged rat. Left, Active caspase-3 labeled with Qdot605 (red, indicated by white arrows), myosin heavy chain labeled with FITC (green), and nuclear labeled with DAPI (blue). Right, a negative control (secondary antibodies without the primary antibodies) with background staining. Magnification 1000×. B, Measurement of heart caspase-3 activity. Bar chart represents the percent increase in caspase-3 activity (mean±SD) for the 6-hour (gray bars) and 24-hour (black bars) postchallenged rats as compared with normal group. C, Nucleosome determination of cardiac DNA fragmentation. Bar chart represents the percent increase in DNA fragmentation (mean±SD) for the 24-hour postchallenged rats as compared with normal group. *$P<0.05$ vs normal. †$P<0.05$ vs control (isotype Ab group).

Figure 5. Effect of anti-MIF antibody on LPS-induced release of heart mitochondrial cytochrome-c. Western blot of heart mitochondrial cytochrome-c from control, LPS+isotype control Ab, and LPS+anti-MIF Ab in 6 hour postchallenged rats. Bar chart represents cytochrome-c densitometry (mean±SD) of the mitochondrial fraction. *$P<0.05$ vs normal. †$P<0.05$ vs control (isotypic Ab group).

Figure 6. Effect of anti-MIF antibody on myocardial prosurvival and proapoptotic proteins. Representative Western blots of cardiac Bcl-2 and Bax from LPS+isotype Ab and LPS+anti-MIF Ab 24-hour postchallenged rats. Bar chart represents the densitometry of the cardiac Bcl-2-to-Bax protein ratio (mean±SD). *$P<0.05$ vs control (LPS+isotype Ab group).
Contributive Factor

Cardiocirculatory Shock of Which MIF Is a

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Contributive Factor

Despite growing evidence, myocardial dysfunction remains a debated concept in the pathophysiology of septic shock. Many reports describing myocardial dysfunction are from experimental models, but data from patients in septic shock or from normal volunteers after endotoxin challenge have been obtained as well. Hearts that become dysfunctional after LPS challenge or sepsis insult demonstrate diffuse and reversible decreases in ejection fraction with enlargement of ventricular diameter/volumes. Recognized factors that influence this dysfunction include cytokines such as TNF-α, IL-1β, IL-6, and more recently MIF. Other inflammatory mediators also include nitric oxide, PAF, sphingosine, and other lipid metabolites. These incriminating factors are generally considered to be paracrine circulating molecules, however cardiac tissues also have been reported to synthesize TNF-α, IL-1β, and IL-6. Moreover, increased local production of cytokines has been strongly implicated in myocardial dysfunction observed during several pathological conditions. Saito et al have demonstrated that LPS-induced IL-6 transcripts in mice hearts were mainly but not exclusively expressed by nonmyocardial cells. However it is not clear whether other LPS-upregulated cytokine mRNAs originate from resident and infiltrating inflammatory cells. Nevertheless, increased synthesis and autocrine secretion of inflammatory cytokines may have local consequences on cardiac contractile protein mRNA expression. For instance, IL-1β activates whereas IL-6 represses contractile protein genes. Whether this autocrine production of cytokine, in addition to cytokines produced as a consequence of the systemic inflammatory response, contributes importantly to the loss of myocardial contractility during sepsis, is unknown. On the other hand, MIF neutralization downregulated most of LPS-induced mRNA and protein upregulation for several heart cytokines including IL-1α/β, IL-6, and even TNF-α after 24 hours. This adds to the IL-1β-dependent suppression pathway described by Saito et al on cardiac IL-6—derived production and to the protection toward ventricular dysfunction conferred by CD14-deficient mice with blunted myocardial IL-1β and TNF-α. As partly observed by Garner et al in a mouse model, MIF blockade can reverse LPS-induced altered LVEF (~20%) and systolic ventricular enlargement as assessed by echocardiography. Several studies have shown that MIF mice are resistant to the lethal effects of high doses of LPS and that anti-MIF antibodies protect normal mice from lethal peritonitis induced by cecal ligature and puncture or E. coli infusion. Moreover, Calandra et al reported that anti-MIF treatment protected TNF-α knockout mice from lethal peritonitis, providing evidence of an intrinsic contribution of MIF to the pathogenesis of sepsis, independent of TNF-α. MIF is known to upregulate the expression of Toll-like receptor 4 (TLR4) on macrophages, thus promoting the recognition of endotoxin-expressing bacterial pathogens, in addition to sustaining proinflammatory function by inhibiting p53-dependent apoptosis as well as counter-regulating the immunosuppressive effects of glucocorticoids on immune cells. MIF is constitutively expressed by a broad spectrum of cells and tissues, including immune cells, the anterior pituitary gland, the lung, the kidney, and skeletal muscle. Recently, Garner et al demonstrated that MIF also is constitutively expressed by the myocardium and released in response to LPS, although its impact on cardiomyocyte apoptosis is unknown.

Heart Apoptosis and MIF are Intricately Involved in Sepsis-Induced Myocardial Dysfunction

Myocardial apoptosis has recently been recognized as a significant contributor to cardiac failure as well as in endotoxin-induced heart dysfunction. Caspase-3 activation and downstream Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) staining were reported to correlate with cardiac performance ex vivo.
and in vivo,10–11 as well as with myofibrillar protein cleavage in vitro.38 Thus, there is evidence that cardiomyocyte apoptosis is a genuine potential participant in sepsis-induced heart dysfunction, a concept further supported by the present study using both upstream (caspase-3, cytochrome-c) and downstream (oligonucleosomal fractions) markers. The low absolute levels of cardiomyocyte apoptosis observed in heart failure as well as in sepsis-induced heart dysfunction10–11,35–38 is intriguing and suggests that rescue pathways may be involved. However, broad-spectrum caspase inhibitors significantly restore cardiac performance, at least in experimental set-ups, and this clearly correlates with improved heart function.11 An important observation in our model is that MIF neutralization significantly reversed heart apoptosis by reducing caspase-3 as well as DNA fragmentation. This is the first instance in which MIF is described as a potential apoptosis inducer in the heart, over and above its role in preventing apoptosis in inflammatory and immune cells.22,39

Mechanistic Studies Reveal that the Mitochondrial Pathways Are Preferentially Targeted by MIF Neutralization

A cornerstone of the protection conferred by anti-MIF treatment appears to be the change in Bcl-2/Bax ratio. Indeed, as previously demonstrated, Bcl-2 protein expression declined in LPS-challenged cardiomyocytes.10,40 Specifically, the half-life of Bcl-2 protein was shorter in response to inflammatory mediator.38 On the other hand, caspases have the capacity to cleave and consequently downregulate Bcl-2 family members.22,39 Thus, MIF neutralization upregulates heart Bcl-2/Bax ratio in a sepsis context by 2 dependent processes: (1) recovery of LPS-induced deficient GATA-4 expression leading to increased Bcl-2 transcription and production, and (2) a rise in Bcl-2 expression that maintains mitochondrial integrity and prevents cytochrome-c release resulting in less LPS-induced caspase-3 activity for cleaving Bcl-2. The zinc finger transcription factor GATA-4 is a key regulator of heart development and an essential survival factor for postnatal cardiomyocytes. The critical role of GATA-4 as a survival factor may be explained, in part, by its function as an upstream activator of the Bcl-2 gene family and hence mitochondrial function and integrity.41 Recently, a series of experiments have probed whether the functional relationship between GATA-4 factor and apoptosis could be extended to cardiac myocytes. Kim et al42 have demonstrated that apoptotic stimuli such as anthracyclines can suppress GATA-4 activity in freshly isolated adult rat cardiac myocytes and cultured HL-1 mouse cardiac muscle cells, because of decreased GATA-4 protein and mRNA expression levels.

A second point is that activated phospho-Akt translocation to the nucleus is restored by MIF blockade. Akt-associated pathways and substrate proteins leading to antiapoptotic effects and cellular survival are complex, but recent studies suggest that biologically relevant targets of phospho-Akt action are localized in the nucleus.32,36,43 The present study did reveal a downregulation of nuclear phospho-Akt in LPS-challenged rat hearts with subsequent recovery to normal levels after MIF blockade. On the other hand, total phospho-Akt did not fluctuate significantly, indicating that the effects remained at the nuclear level. In fact, Morisco et al44 reported that Akt, one of the major downstream targets of phosphatidylinositol 3-kinase (PI3-kinase), positively regulates transcriptional activity of GATA-4 in cardiomyocytes by phosphorylation and subsequent inactivation of glycogen synthase kinase 3β (GSK-3β). Furthermore, GSK-3β negatively regulated the transcriptional activity of GATA-4 through translocation of GATA-4 from the nucleus to the cytoplasm. This could explain why GATA-4 modulation follows the same pattern as nuclear phosphor-Akt among the various modulators assessed in the present study. In addition, it is notable that Akt overexpression in mice improves cardiac contractility in vivo.45

Interestingly, protection of heart apoptosis occurs independently of p53 commitment. Indeed, MIF is released from cells of the innate immune system in bacterial infectious diseases on Toll-like receptor activation, and it contributes to the accumulation of inflammatory cells by inhibition of apoptosis.39 Specifically, MIF sustains macrophage function by negatively regulating the p53-dependent apoptosis.22–23 However, our model demonstrated an absence of p53 activation (phosphorylation) in LPS-challenged rat hearts. This suggests that endotoxin induces cardiomyocyte apoptosis in a p53-independent manner. Consequently, MIF neutralization does not potentiate LPS-induced cardiomyocyte apoptosis by activating p53 but rather stimulates p53-dependent apoptosis of inflammatory cells, thereby reducing inflammation-induced cardiomyocyte apoptosis.

In summary, MIF is a significant contributor to endotoxin-induced myocardial dysfunction and is committed in cardiac apoptosis by a mitochondrial preferential pathway. MIF neutralization improves cardiomyocyte survival and function by increasing Bcl-2 dominance with restoration of GATA-4 and pAkt nuclear translocation.

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References

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METHODS

Experimental animal model

Pathogen-free male Wistar rats (350 g) were purchased from Charles River laboratories Inc (Wilmington, MA) and received care in compliance with The Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care (1993, CCAC, 2nd ed.). The following protocol was approved by our institution’s Ethics Committee for animal care and experimentation. Rats were given an intraperitoneal (I/P) injection of 1 ml phosphate-buffered saline (PBS) or 10 mg/kg of Escherichia coli 055:B5 LPS (Sigma) in 1 ml PBS. Concomitantly, mouse monoclonal anti-MIF (IgG1, III.D.9 ) or isotypic matched (IgG1, HB49 ) control antibodies originating from comparable clones as those described by Calandra et al\(^1\), were injected (10 mg/kg in 1ml PBS) intraperitoneally. These anti-MIF antibodies had been previously shown to neutralize murine MIF bioactivity in vivo and in vitro.\(^1\) Note that rat MIF differs from mouse MIF by a single amino acid residue (mouse: Asn 54, rat: Ser 54) that does not influence immunoreactivity with anti-MIF clone III.D.9\(^2\). Animals were exposed to 12 hr light/dark cycles and given free access to food and water. At 6 hrs or 24 hrs post-injection, the hearts were removed under isoflurane anesthesia, snap frozen in liquid nitrogen, and stored at -80°C.

Echocardiographic study

Transthoracic echocardiography were performed under isoflurane anesthesia (2%) prior to injection as well as 24hrs post-injection. All measurements were performed using a s12 HP 5-12mHz high resolution small footprint transducer with a 60-Hz acquisition rate (Hewlett-Packard Sonos 5500). Anesthetized animals were positioned in lateral decubitus and parameters recorded on 2-D triggered digitalized M-mode tracing from the short axis of the left ventricle (LV) at the level of papillary muscles, as described by others. All echocardiographs were performed on three consecutive cardiac cycles and ECG-gated. The left ventricular diameter during the end of systole (LVESd), left ventricular diameter during the end of diastole (LVEDd) and the fractional shortening were established by the following formula \([\frac{LVEDd-LVESd}{LVESd}] \times 100\) and according to the standards for the standards for M-mode measurements set up by the American Society of Echocardiography. Computed fractional shortening allowed LV mass estimation using the cube formula and the left ventricular volume during systole (LVESv) was established together with the left ventricular volume during diastole (LVEDv). All echocardiographs were performed by the same qualified technician (with intra-observer
variability below 15%) and quality control was ensured by members of the Cardiology Department according to actual recommendations³.

RNA extraction and RNase Protection Assay

Heart pieces were thawed on ice, and total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer’s protocol. We carried out RNA quantification by reading the optical density of samples at 260 nm using a Beckman spectrophotometer, and RNA quality was monitored by simultaneous optical density reading at 280 nm. RNase protection assay were performed as described in the standard PharMingen RNase protection protocol (San Diego, CA). Briefly, the multiprobe template set rCK-2 (PharMingen) was used to synthesize $[\alpha-^{32}\text{P}]$UTP (NEN Life Science Products, Boston, MA)-labeled probes in the presence of GACU pool using a T7 RNA polymerase. Probes were hybridized overnight with 10 µg of target RNA, followed by RNase digestion and proteinase K treatment. Samples were chloroform-extracted, ethanol-precipitated in the presence of ammonium acetate, and loaded on an acrylamide-urea sequencing gel in 0.53 M Tris-borate-EDTA buffer. After electrophoresis at 50 W for 1-2 hrs, the gel was adsorbed to filter paper and dried under vacuum. The dried gel blot was exposed to a phospho screen for phospho imagery analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The intensity of each band was analyzed and normalized to the values of the housekeeping gene L32, with normalized values used to quantify expression.

Protein extraction

Heart pieces were thawed and homogenized on ice in a lysis buffer containing 50 mM Tris, 10 mM EDTA, 5 mM EGTA, 10 mM NaF, 10 mM NaPP, 0.5 mM Na$_3$VO$_4$, 1 tablet Mini Complete (protease inhibitor mix, Roche) /10 ml, 1 mM PMSF, and 1% Nonidet P-40, for total proteins extraction. Homogenates were incubated 30 min on ice and sonicated. Homogenates were centrifuged 15 min at 10,000x g (4°C), and the supernatant fractions were collected. A hundred mgs of heart were homogeneized in 0.8 ml of ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 tablet Mini Complete (protease inhibitor mix, Roche)/10 ml, 50 mM NaF, and 1 mM Na$_3$VO$_4$), for nuclear and mitochondrial proteins. After being centrifuged for 30 sec at 2,000x rpm at 4°C, the supernatants were incubated on ice for 20 min, vortexed for 30 sec after the addition of 50 µl 10% Nonidet P-40, and then centrifuged for 1 min at 10,000x rpm at 4°C. The pelllets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM
EGTA, 1 mM DTT, 0.5 mM PMSF, 1 tablet Mini Complete (protease inhibitor mix, Roche)/10 ml, 50 mM NaF, and 1 mM Na$_3$VO$_4$), and collected as mitochondrial and nuclear fraction. Supernatants were centrifuged for 60 min at 50,000x rpm at 4°C and the resulting supernatant fractions were collected as cytoplasmic extracts. Protein content was determined by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA).

**Western blotting**

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solution and 15 min in biotin solution (Avidin/Biotin Blocking kit; Vector Laboratories). Slices were recovered with polyclonal rabbit anti-cleaved caspase-3 (1:50; Cell Signaling Technology, Beverly, MA), polyclonal goat anti-myosin heavy chain (1:10; Santa Cruz Biotechnology) and 1.5% mouse serum. Specific amplification was obtained by further incubation with monoclonal mouse anti-rabbit IgG biotin conjugate (1:1500; Sigma) followed by QDot 605 streptavidin conjugate (Quantum Dot, Hayward, CA) for cleaved caspase-3, mouse anti-goat IgG FITC (1:50; Santa Cruz Biotechnology) for myosin heavy chain, and 2% mouse serum. Slides were mounted in Vectashield Mounting Medium (Vector Laboratories) after being washed in PBS. Nuclear contrast was obtained by 4',6'-diamidino-2-phenylindole (DAPI) staining (2 µg/ml; Sigma). Slices were analyzed using an Axioskop 2 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) at a magnification of 1000x. Photomicrographs were captured using a SPOT-3 color digital camera (Diagnostic Instruments Inc, St. Sterling Height, MI). The images were processed using SPOT software.

**Caspase-3 activity**

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**DNA fragmentation detection**

A Nucleosome ELISA kit (Calbiochem) was used according to manufacturer instructions for the quantification of DNA fragmentation. Briefly, small pieces (40-50 mg) of hearts were homogenized in the provided lysis buffer, incubated for 30 min on ice, followed by centrifugation at 1,500x g for 10 min at 4°C. The supernatants were frozen for at least 18 hrs at -20°C. Following determination of protein content, supernatants were analyzed by ELISA. Results were expressed as a ratio of nucleosomes units relative to total protein concentration.
REFERENCES


METHODS

Experimental animal model

Pathogen-free male Wistar rats (350 g) were purchased from Charles River laboratories Inc (Wilmington, MA) and received care in compliance with The Guide to the Care and Use of Experimental Animals from the Canadian Council of Animal Care (1993, CCAC, 2nd ed.). The following protocol was approved by our institution’s Ethics Committee for animal care and experimentation. Rats were given an intraperitoneal (I/P) injection of 1 ml phosphate-buffered saline (PBS) or 10 mg/kg of *Escherichia coli* 055:B5 LPS (Sigma) in 1 ml PBS. Concomitantly, mouse monoclonal anti-MIF (IgG1, III.D.9) or isotypic matched (IgG1, HB49) control antibodies originating from comparable clones as those described by Calandra et al \(^1\), were injected (10 mg/kg in 1ml PBS) intraperitoneally. These anti-MIF antibodies had been previously shown to neutralize murine MIF bioactivity *in vivo* and *in vitro* \(^1\). Note that rat MIF differs from mouse MIF by a single amino acid residue (mouse: Asn 54, rat: Ser 54) that does not influence immunoreactivity with anti-MIF clone III.D.9\(^2\). Animals were exposed to 12 hr light/dark cycles and given free access to food and water. At 6 hrs or 24 hrs post-injection, the hearts were removed under isoflurane anesthesia, snap frozen in liquid nitrogen, and stored at -80°C.

Echocardiographic study

Transthoracic echocardiography were performed under isoflurane anesthesia (2%) prior to injection as well as 24hrs post-injection. All measurements were performed using a s12 HP 5-12mHz high resolution small footprint transducer with a 60-Hz acquisition rate (Hewlett-Packard Sonos 5500). Anesthetized animals were positioned in lateral decubitus and parameters recorded on 2-D triggered digitalized M-mode tracing from the short axis of the left ventricule (LV) at the level of papillary muscles, as described by others. All echocardiographs were performed on three consecutive cardiac cycles and ECG-gated. The left ventricular diameter during the end of systole (LVESd), left ventricular diameter during the end of diastole (LVEDd) and the fractional shortening were established by the following formula \((\frac{LVEDd-LVESd}{LVESd} \times 100)\) and according to the standards for the standards for M-mode measurements set up by the American Society of Echocardiography. Computed fractional shortening allowed LV mass estimation using the cube formula and the left ventricular volume during systole (LVESv) was established together with the left ventricular volume during diastole (LVEDv). All echocardiographs were performed by the same qualified technician (with intra-observer
variability below 15%) and quality control was ensured by members of the Cardiology Department according to actual recommendations.

**RNA extraction and RNase Protection Assay**

Heart pieces were thawed on ice, and total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer’s protocol. We carried out RNA quantification by reading the optical density of samples at 260 nm using a Beckman spectrophotometer, and RNA quality was monitored by simultaneous optical density reading at 280 nm. RNase protection assay were performed as described in the standard PharMingen RNase protection protocol (San Diego, CA). Briefly, the multiprobe template set rCK-2 (PharMingen) was used to synthesize $[\alpha ^{32}P]$UTP (NEN Life Science Products, Boston, MA)-labeled probes in the presence of GACU pool using a T7 RNA polymerase. Probes were hybridized overnight with 10 µg of target RNA, followed by RNase digestion and proteinase K treatment. Samples were chloroform-extracted, ethanol-precipitated in the presence of ammonium acetate, and loaded on an acrylamide-urea sequencing gel in 0.53 M Tris-borate-EDTA buffer. After electrophoresis at 50 W for 1-2 hrs, the gel was adsorbed to filter paper and dried under vacuum. The dried gel blot was exposed to a phospho screen for phospho imagery analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The intensity of each band was analyzed and normalized to the values of the housekeeping gene L32, with normalized values used to quantify expression.

**Protein extraction**

Heart pieces were thawed and homogenized on ice in a lysis buffer containing 50 mM Tris, 10 mM EDTA, 5 mM EGTA, 10 mM NaF, 10 mM NaPP, 0.5 mM Na$_3$VO$_4$, 1 tablet Mini Complete (protease inhibitor mix, Roche) /10 ml, 1 mM PMSF, and 1% Nonidet P-40, for total proteins extraction. Homogenates were incubated 30 min on ice and sonicated. Homogenates were centrifuged 15 min at 10,000x g (4°C), and the supernatant fractions were collected. A hundred mgs of heart were homogenized in 0.8 ml of ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 tablet Mini Complete (protease inhibitor mix, Roche)/10 ml, 50 mM NaF, and 1 mM Na$_3$VO$_4$), for nuclear and mitochondrial proteins. After being centrifuged for 30 sec at 2,000x rpm at 4°C, the supernatants were incubated on ice for 20 min, vortexed for 30 sec after the addition of 50 µl 10% Nonidet P-40, and then centrifuged for 1 min at 10,000x rpm at 4°C. The pellets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM
EGTA, 1 mM DTT, 0.5 mM PMSF, 1 tablet Mini Complete (protease inhibitor mix, Roche)/10 ml, 50 mM NaF, and 1 mM Na$_3$VO$_4$), and collected as mitochondrial and nuclear fraction. Supernatants were centrifuged for 60 min at 50,000x rpm at 4°C and the resulting supernatant fractions were collected as cytoplasmic extracts. Protein content was determined by the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA).

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