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J Immunol (2007) 179 (8): 5399–5406.

<https://doi.org/10.4049/jimmunol.179.8.5399>

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The Proinflammatory Cytokine Macrophage Migration Inhibitory Factor Regulates Glucose Metabolism during Systemic Inflammation¹

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Inflammation provokes significant abnormalities in host metabolism that result from the systemic release of cytokines. An early response of the host is hyperglycemia and resistance to the action of insulin, which progresses over time to increased glucose uptake in peripheral tissue. Although the cytokine TNF- α has been shown to exert certain catabolic effects, recent studies suggest that the metabolic actions of TNF- α occur by the downstream regulation of additional mediators, such as macrophage migration inhibitory factor (MIF). We investigated the glycemic responses of endotoxemic mice genetically deficient in MIF (MIF^{-/-}). In contrast to wild-type mice, MIF^{-/-} mice exhibit normal blood glucose and lactate responses following the administration of endotoxin, or TNF- α . MIF^{-/-} mice also show markedly increased glucose uptake into white adipose tissue in vivo in the endotoxemic state. Treatment of adipocytes with MIF, or anti-MIF mAb, modulates insulin-mediated glucose transport and insulin receptor signal transduction; these effects include the phosphorylation of insulin receptor substrate-1, its association with the p85 regulatory subunit of PI3K, and the downstream phosphorylation of Akt. Genetic MIF deficiency also promotes adipogenesis, which is in accord with a downstream role for MIF in the action of TNF- α . These studies support an important role for MIF in host glucose metabolism during sepsis. *The Journal of Immunology*, 2007, 179: 5399–5406.

Severe infection, trauma, or tissue invasion provoke metabolic abnormalities that contribute significantly to morbidity and mortality (1, 2). An early response of the host is hyperglycemia and resistance to the action of insulin. Tissue glycolysis continues despite decreased perfusion, and lactate production increases because of the incomplete oxidation of glycolytic end products. These metabolic derangements produce a catabolic state that compromises host immunity and tissue repair, and if unresolved, produce cachexia, progressive organ dysfunction, and death (1).

The development of a catabolic state in the setting of infection results from a combination of stress-induced hormonal responses and the production of proinflammatory mediators (1, 2). One postulated role for the cytokine TNF- α is to effect a redistribution of energy stores from adipose to peripheral tissue (1, 3). TNF- α has been shown to induce the synthesis of the allosteric stimulator of glycolysis, fructose 2,6-bisphosphate, leading to a depletion of

plasma glucose and an accompanying elevation of glucose uptake into peripheral tissue. Indeed, TNF- α was cloned as the mediator “cachectin” because of its systemic effects on host metabolism, and an accumulation of data support a role for TNF- α in the development of insulin resistance (4). TNF- α also mediates insulin resistance and lipolysis in adipocytes, thus preventing the uptake and use of circulating glucose into this insulin sensitive tissue.

Studies in endotoxemic mice suggest that certain of the metabolic actions of TNF- α may be mediated by the coordinate or downstream expression of macrophage migration inhibitory factor (MIF)³ (5). MIF is expressed by many cell types, is released early in the activation response from preformed intracellular pools (5, 6), and its circulating levels correlate with the clinical severity of sepsis (7, 8). Notably, the immunoneutralization of MIF in endotoxemic mice normalizes glucose levels, prevents liver glycogen depletion, and decreases levels of fructose 2,6-bisphosphate in muscle. These effects also were observed in TNF- α ^{-/-} mice treated with anti-MIF, thus verifying the intrinsic action of MIF in this inflammation-induced, catabolic response of liver and muscle (5).

The finding that cultured adipocytes secrete MIF in response to TNF- α (9) prompted us to investigate more closely MIF’s action in glucose metabolism in adipocytes during endotoxemia. In the present report, we present evidence that the action of TNF- α on adipose tissue during the systemic inflammatory response can be explained in large part by the autocrine/paracrine action of MIF.

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Received for publication August 6, 2007. Accepted for publication August 9, 2007.

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¹ This work was supported by Grants AI42310 (to R.B.), AR050498 (to R.B.), U24 DK-59635 (to J.K.K.), and ADA 1-04RA-47 (to J.K.K.) from the National Institutes of Health.

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³ Abbreviations used in this paper: MIF, macrophage migration inhibitory factor; IRS-1, insulin receptor substrate 1; MEF, murine embryonic fibroblast; PPAR, peroxisome proliferator-activated receptor; 2-[¹⁴C]DG, 2-deoxy-D-[1-¹⁴C]glucose; WT, wild type.

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Materials and Methods

Materials

Neutralizing anti-MIF mAb (NIH-III.D9) was previously described (7). Abs directed against the insulin receptor β subunit (C-19), TNFR-1, and cyclin D1 were obtained from Santa Cruz Biotechnology, and Abs against Akt and phospho-Akt (Ser⁴⁷³) were obtained from Cell Signaling Technology. Abs directed against phosphotyrosine, insulin receptor substrate 1 (IRS-1), and the p85 regulatory subunit of the PI3K were purchased from Upstate Biotechnology. Recombinant mouse TNF- α and the mouse TNF- α ELISA kit were purchased from R&D Systems. MIF was measured by a murine-specific capture ELISA (7). Recombinant murine MIF was purified from an *Escherichia coli* expression system by FPLC and C8 chromatography to remove contaminating endotoxin (10), and contained <1 μ g of endotoxin/gram of protein. *E. coli* LPS O111:B4 was obtained from Sigma-Aldrich, and troglitazone was a gift from Sankey Company.

In vivo glucose and lactate metabolism in endotoxemic MIF^{-/-} and MIF^{+/+} mice

MIF^{-/-} mice (11) were bred onto a pure BALB/c background (generation N10). BALB/c mice (wild type (WT), MIF^{+/+}) were obtained from Charles River Breeding Laboratories. Male mice (age 6–8 wk) were used, and all mice showed an equivalent increase in body weight with age. At least 4 days before in vivo experiments, the mice were anesthetized and surgery was performed to establish an indwelling catheter in the right internal jugular vein (12). Mice were fasted overnight (18 h) before experiments but had free access to water. At the indicated times, blood was collected from tail vessels to measure glucose levels. All studies were approved by the Yale Institutional Animal Care and Use Committee.

Organ-specific glucose metabolism in vivo was determined in awake mice using a modification of hyperinsulinemic-euglycemic clamp experiments as previously described (12). Briefly, a sublethal dose of LPS (16.6 μ g/g) was i.p. injected into age-matched MIF^{+/+} and MIF^{-/-} mice ($n = 7$ –8 for each group). At 30 min postinjection, a blood sample (40 μ l) was collected for glucose and insulin measurements. This was followed by a continuous infusion of 3-[³H]glucose (0.1 μ Ci/min) for 2 h to estimate whole body glucose turnover. To determine basal glucose uptake in individual organs, 2-deoxy-D-[1-¹⁴C]glucose (2-[¹⁴C]DG, 10 μ Ci) was i.v. administered as a bolus at 2 h postinjection. Blood samples were taken at 5-min intervals during the remaining 30 min for the measurement of plasma [³H]glucose and 2-[¹⁴C]DG concentrations. At the end of experiments (2.5 h postinjection with LPS), mice were anesthetized with sodium pentobarbital injection, and tissue samples (gastrocnemius, epididymal white adipose tissue, and intrascapular brown adipose tissue) were taken for biochemical analysis.

Biochemical analysis and calculations

Plasma glucose concentration was determined by the glucose oxidase method using the Beckman Glucose Analyzer 2, and plasma insulin concentration was measured by radioimmunoassay (Linco Research). Plasma concentrations of 3-[³H]glucose and 2-[¹⁴C]DG were determined after deproteinization of plasma samples as previously described (12). For the determination of tissue 2-[¹⁴C]DG-6-phosphate content, tissue samples were homogenized and the supernatants subjected to ion-exchange chromatography to separate 2-[¹⁴C]DG-6-phosphate from 2-[¹⁴C]DG.

The rates of basal whole body glucose turnover and hepatic glucose production were determined as the ratio of the [³H]glucose infusion rate to the specific activity of plasma glucose during the final 30 min of the experiments. Glucose uptake in individual tissues was calculated from the plasma 2-[¹⁴C]DG profile, which was fitted with a double exponential or linear curve using mathematical modeling MLAB software (Civilized Software) and tissue 2-[¹⁴C]DG-6-phosphate content (12).

Immunoblotting studies

Cells were lysed in ice-cold buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, and 50 mM NaF) that included a protease inhibitor mixture (Roche), and disrupted by aspiration through a 21-gauge needle. After incubation on ice for 30 min and microcentrifugation for 10 min, the supernatants were collected and equal amounts of cellular proteins were fractionated on 10% SDS gels, transferred to polyvinylidene difluoride membranes, and analyzed by Western blotting.

Immunoprecipitation

Equal amounts of cell lysates were incubated with anti-p85 Ab, and the samples were mixed by rotation overnight at 4°C. A total of 20 μ l of

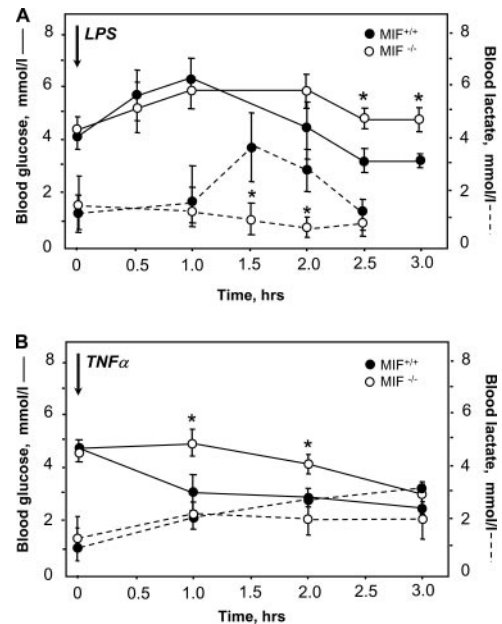


FIGURE 1. Glycemic and plasma lactate responses to endotoxemia. *A*, Blood glucose (solid line) and lactate (dotted line) levels in MIF^{-/-} and WT (MIF^{+/+}) mice after injection of sublethal LPS (16.6 μ g/g). *B*, Blood glucose (solid line) and lactate (dotted line) levels in MIF^{-/-} and MIF^{+/+} mice after a single i.p. injection of TNF- α (160 μ g/kg). Results are the calculated mean glucose level \pm SD for four mice per time point. *, $p < 0.05$ for the MIF^{-/-} vs the MIF^{+/+} mice. For the blood glucose response in the MIF^{+/+} mice, the 2.5-h and 3-h values differed significantly from the 0.5-h and 1-h values ($p < 0.05$). For the blood lactate response in the MIF^{+/+} mice, the 1.5-h and 2-h values differed significantly from the 1-h and 2.5-h values ($p < 0.05$). The experiments shown are representative of three independent studies.

protein A/G coupled-beads were then added for an additional 1 h of mixing at 4°C, and the pellets were collected by centrifugation and washed twice with 1 ml of ice-cold lysis. The pellets finally were resuspended in 50 μ l of 2 \times sample buffer, boiled for 5 min, and analyzed by SDS-PAGE and Western blotting.

Glucose transport assay

Glucose uptake into cells was measured by an established protocol (13). Briefly, differentiated 3T3-L1 adipocytes were washed twice with transport solution (140 mM NaCl, 20 mM HEPES/Na, 5 mM KCl, 2.5 mM MgSO₄, and 1 mM CaCl₂) and incubated for 10 min with 0.5 μ Ci/ml of 2-deoxy-[³H]glucose. The cells then were washed three times and lysed with 50 mM NaOH. The lysates were collected and radioactivity was measured. Cytochalasin B (10 μ M) was used for the determination of noncarrier-mediated deoxyglucose uptake.

Preparation of murine adipocytes

Mouse primary adipocytes were prepared from the epididymal fat pads of MIF^{-/-} and MIF^{+/+} mice. Adipose tissue was removed, minced, and digested with 1 mg/ml collagenase in DMEM and 1% fatty acid-free BSA (14). Cells were filtered through nylon mesh, washed, and incubated overnight with or without TNF- α .

For differentiation studies, murine embryonic fibroblasts (MEF) from MIF^{-/-} and MIF^{+/+} were prepared from day 14.5 embryos (15). The cells were cultured in DMEM/10% FBS in either 6-well plates or in chamber slides. Adipocyte differentiation of primary murine fibroblasts was induced by DMEM/10% FBS with 0.5 mM isobutylmethylxanthine, 1.0 μ M dexamethasone, and 10 μ g/ml insulin for 48 h. The medium then was replaced with DMEM supplemented with 10% FBS (16). Adipocyte differentiation in different experiments was induced 48 h after confluence by addition of the differentiation medium indicated in *Results*. The differentiation medium was replaced every 48 h.

Oil red O staining

Cultured cells were washed with PBS and fixed with buffered formalin, and cytoplasmic lipid accumulation was analyzed by staining with Oil Red O

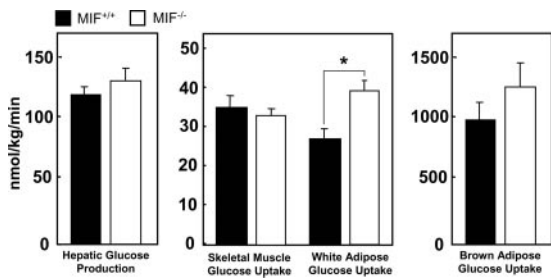


FIGURE 2. In vivo glucose metabolism in endotoxemic MIF^{-/-} and MIF^{+/+} mice. Seven to eight mice per group were pretreated with LPS, and organ-specific glucose metabolism was measured using labeled glucose in awake mice as described in the *Materials and Methods*. *, $p < 0.03$.

(16). The excess stain was removed and the cells were washed several times with water before microscopic examination and color quantification by NIH Image Analysis software.

Statistical analysis

Data are presented as mean \pm SD. The statistical comparisons between groups were conducted using Student's *t* test. Values for $p < 0.05$ were considered significant.

Results

MIF^{-/-} mice have an altered glycemic and plasma lactate response to endotoxin

Gram-negative endotoxin (LPS) is a powerful stimulus for the release of TNF- α and other proinflammatory cytokines, and LPS induces an acute hypoglycemic response when administered to ex-

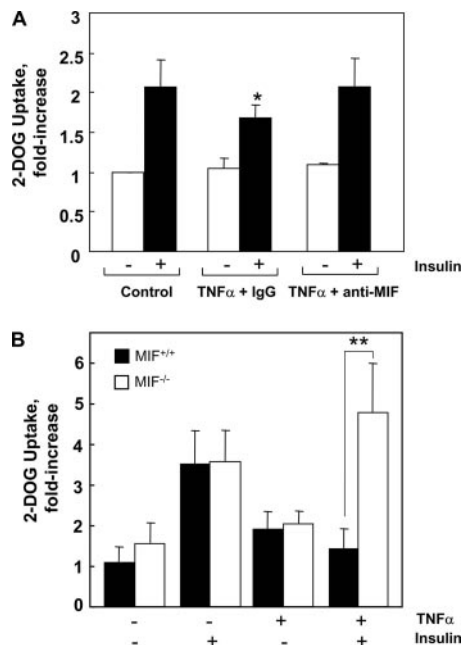


FIGURE 3. TNF- α has stimulatory action in glucose uptake into adipocytes. *A*, MIF neutralization reverses TNF- α -mediated reduction in adipocyte glucose transport. Cultured WT adipocytes were treated with TNF- α (20 ng/ml) together with an anti-MIF mAb or isotype control (each at 80 μ g/ml). Basal and insulin-stimulated (100 nM, 5 min) uptake of 2-deoxyglucose (2-DOG) were performed as described in *Materials and Methods*. *B*, Primary mouse adipocytes were prepared from MIF^{-/-} and MIF^{+/+} mice and incubated overnight with or without TNF- α (20 ng/ml) for glucose transport studies. Data are means \pm SD ($n = 4$ cultures). Data shown are representative of three independent experiments. *, $p < 0.05$ vs control; **, $p < 0.005$.

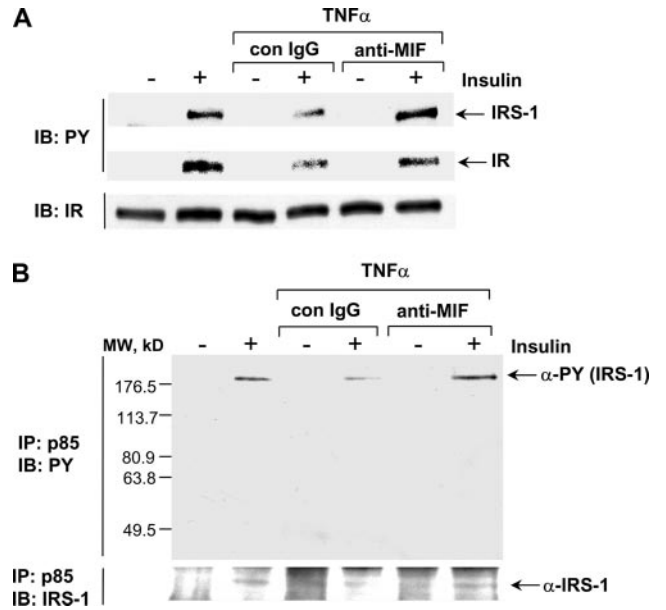
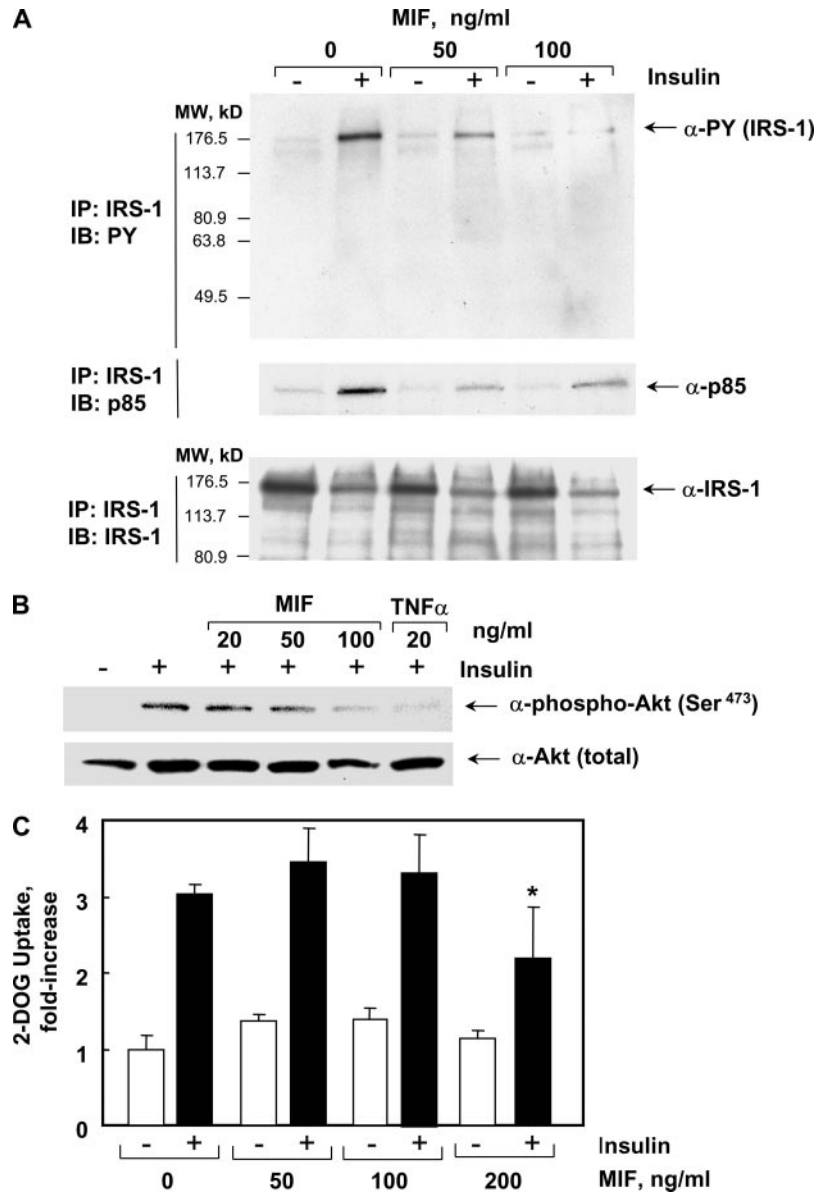


FIGURE 4. TNF- α action on the signal transduction events of the insulin receptor. *A*, Anti-MIF reduces TNF- α inhibition of the phosphorylation of IRS-1. 3T3-L1 adipocytes were stimulated with 20 ng/ml TNF- α for 6 h together with anti-MIF mAb or an isotypic control (IgG1) (each at 80 μ g/ml) followed by stimulation with insulin (100 nM, 5 min). Cells were lysed, and Western blot analysis was performed with an anti-phosphotyrosine Ab (α -PY). The blots were stripped and reprobed with an insulin receptor-specific Ab (α -IRS-1). *B*, Anti-MIF mAb reduces the TNF- α -mediated decrease in the association of IRS-1 and PI3K subunit, p85. Cultured 3T3-L1 adipocytes were treated with 20 ng/ml TNF- α for 6 h together with anti-MIF mAb or an isotype control (each at 80 μ g/ml), followed by stimulation with 100 nM insulin for 5 min. Whole cell lysates were immunoprecipitated with an anti-p85 Ab followed by Western blot analysis with an anti-phosphotyrosine Ab (α -PY). The blots were stripped and reprobed with an anti-IRS-1 Ab (α -IRS-1).

perimental animals or human subjects (17). Prior studies using a neutralizing Ab approach demonstrated that anti-MIF inhibits the characteristic development of hypoglycemia in LPS-injected mice (5). We sought to verify these findings by administering a sublethal dose of LPS (16.6 μ g/g) to MIF^{-/-} mice and WT controls (MIF^{+/+}), and sampling their blood for measuring plasma glucose and lactate levels. MIF^{-/-} mice showed a preservation of their glycemic and plasma lactate responses when compared with the WT mice (Fig. 1A). These differences were transient but most evident for lactate at 1.5 h and glucose at 2.5 h, which reflects the time necessary for systemic production of proinflammatory cytokines such as TNF- α and MIF (18). Plasma glucose levels were markedly lower (at 2.5 and 3 h), and plasma lactate levels were correspondingly higher (at 1.5 and 2 h) in the MIF^{+/+} mice than in the MIF^{-/-} mice. Although MIF has been shown to influence β -cell function (19), we observed no significant changes in plasma insulin levels, either at baseline (data not shown) or after LPS (2.5 h: MIF^{+/+}, 79 ± 12 pM, MIF^{-/-}, 69 ± 11 pM, $p = \text{NS}$; $n = 5$ mice per group). We also measured serum corticosterone, which may mediate increased glycemia as part of a systemic stress response. Notably, MIF^{-/-} mice showed a decrease in circulating corticosterone levels when compared with MIF^{+/+} mice, suggesting that glucocorticoids did not account for the increase in blood sugar during MIF deficiency (2.5 h: MIF^{+/+}, 0.42 ± 0.07 μ M, MIF^{-/-}, 0.16 ± 0.04 μ M, $p < 0.05$). There also were no differences in MIF^{+/+} vs MIF^{-/-} mice in circulating adiponectin, TNF- α , or

FIGURE 5. MIF-induced action on insulin signal transduction in adipocytes. **A**, MIF inhibits the insulin-induced tyrosine phosphorylation of IRS-1. Adipocytes were treated with increasing concentrations of recombinant MIF followed by insulin (100 nM) for 5 min. Immunoprecipitates were prepared from whole cell lysates using an anti-IRS-1 Ab followed by blotting analysis with anti-phosphotyrosine Ab (α -PY) (see *Materials and Methods*). The membrane was stripped and then reprobed with an anti-p85 Ab (α -p85) or an anti-IRS-1 Ab (α -IRS-1). **B**, MIF inhibits insulin-mediated phosphorylation of Akt. Cultured adipocytes were pretreated with MIF followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser⁴⁷³ phospho-Akt (α -phospho-Akt) and total Akt (α -Akt). **C**, MIF decreases insulin-mediated 2-deoxyglucose (2-DOG) uptake in cultured adipocytes. Basal and insulin-stimulated uptake of 2-deoxyglucose into adipocytes was analyzed as described in *Materials and Methods*. Results were obtained in triplicate wells. Data shown (mean \pm SD) are representative of three independently performed experiments. *, $p < 0.05$ vs control.



IL-6 levels, either at baseline (data not shown) or 2.5 h after LPS (adiponectin: MIF^{+/+}, 3.9 \pm 0.3 ng/ml, MIF^{-/-}, 3.5 \pm 0.3 ng/ml, $p = \text{NS}$; TNF- α : MIF^{+/+}, 57.0 \pm 13.3 ng/ml, MIF^{-/-}, 69.6 \pm 15.3 ng/ml, $p = \text{NS}$; and IL-6: MIF^{+/+}, 310 \pm 100 ng/ml, MIF^{-/-}, 340 \pm 80 ng/ml, $p = \text{NS}$). These genetically based results support an important role for MIF in the dysregulation of carbohydrate metabolism that occurs during endotoxemia.

Certain aspects of the metabolic effects of LPS can be recapitulated by the administration of TNF- α (4). To provide evidence for an effector role for MIF in the action of TNF- α , we next examined circulating glucose levels in MIF^{-/-} and WT mice treated with a single, i.p. dose of TNF- α (160 μ g/kg). TNF- α caused a marked reduction of blood glucose level in the WT mice, which is in agreement with prior reports (5, 17), and this effect occurred at least 1 h earlier than in mice administered LPS, which is in accord with the time delay necessary for TNF- α transcription, translation, and secretion. By contrast, MIF^{-/-} mice treated with TNF- α were euglycemic in the acute phase (≤ 2 h), but then showed a reduction in blood glucose levels after 2 h (Fig. 1B). These data are in agreement with a prior report that anti-MIF may prevent TNF- α -induced hypo-

glycemia (5). The acute phase changes also are consistent with experiments supporting an MIF release response by cells and tissues exposed to TNF- α (5), and the reduction in blood glucose after 2 h may reflect a TNF- α -mediated induction of additional, downstream mediators (1).

Glucose uptake into adipose tissue is increased in endotoxemic, MIF^{-/-} mice

To better assess the impact of MIF deficiency on glucose metabolism during endotoxemia, we measured organ-specific glucose uptake in awake mice at 2.5 h following sublethal treatment with LPS. Hepatic glucose production and glucose uptake into skeletal muscle (gastrocnemius) and adipose tissues were determined using continuous infusion of 3-[³H]glucose and a bolus injection of 2-[¹⁴C]DG as a nonmetabolizable glucose analog. As shown in Fig. 2, MIF deficiency was associated with a significant and selective increase in glucose uptake into white adipose tissue. In contrast, glucose uptake in skeletal muscle and brown adipose tissue, and hepatic glucose production were not significantly affected under these experimental conditions.

The inhibitory action of TNF- α on insulin-mediated glucose transport is reversed by MIF immunoneutralization or genetic deficiency

The preceding observations, together with the report that TNF- α -treated adipocytes secrete MIF (9), prompted us to examine the role of MIF in mediating the effect of TNF- α on adipocyte glucose metabolism. We verified that TNF- α (2 ng/ml) induced MIF secretion from cultured adipocytes, and we observed a diminution in MIF release at very high TNF- α concentrations (≥ 20 ng/ml), which is in agreement with prior observations in TNF- α -treated monocytes/macrophages (6 and data not shown).

TNF- α inhibits the stimulatory action of insulin on glucose uptake into adipocytes (20). We treated adipocytes with TNF- α and measured insulin-stimulated uptake of 2-deoxyglucose in the presence of anti-MIF mAb or an isotypic, IgG1 control. The insulin-stimulated uptake of 2-deoxyglucose was significantly greater upon MIF immunoneutralization (Fig. 3A). The inhibitory action of MIF upon 2-deoxyglucose uptake was verified in studies performed in primary adipocytes isolated from MIF^{-/-} mice. As shown in Fig. 3B, adipocytes genetically deficient in MIF showed a significant increase in 2-deoxyglucose uptake despite treatment with TNF- α . These data support a model whereby TNF- α inhibits insulin signal transduction via the autocrine/paracrine secretion of MIF. Such a mechanism of TNF- α action also is consistent with studies in differentiated myotubes, where TNF- α was found to induce MIF release, leading to a downstream, autocrine/paracrine response (5).

Immunoneutralization of MIF reverses TNF- α -mediated insulin resistance in cultured adipocytes

A well-characterized action of TNF- α with respect to glucose transport is inhibition of insulin signal transduction leading to insulin resistance (21). We followed a standard protocol for inducing insulin resistance in vitro and treated adipocytes with TNF- α together with a neutralizing anti-MIF mAb or isotype control, followed by stimulation with insulin for 5 min. As expected, insulin induced the rapid tyrosine phosphorylation of the insulin receptor and the IRS-1, and these events were inhibited by TNF- α pretreatment (Fig. 4A). The addition of anti-MIF mAb, but not control Ab, significantly reduced the action of TNF- α on the tyrosine phosphorylation of the insulin receptor and IRS-1.

The enzyme PI3K is a downstream mediator of insulin signal transduction, and it plays an important role in the insulin-dependent translocation of GLUT4 (22). PI3K is comprised of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit with Src homology 2 domains that bind to the tyrosine-phosphorylated isoform of IRS-1. The functional significance of the TNF- α -dependent reduction in IRS-1 tyrosine phosphorylation can be monitored by coimmunoprecipitation of the IRS-1 complex containing the p85 regulatory subunit of PI3K (23). We examined the effect of MIF neutralization on the TNF- α -mediated decrease in the association of IRS-1 and PI3K (p85). As shown in Fig. 4B, the insulin-induced association of IRS-1 with PI3K (p85 subunit) was reduced by TNF- α treatment, and anti-MIF inhibited this specific action of TNF- α on adipocytes. These data, taken together, are consistent with a role for MIF in mediating the action of TNF- α on the proximal signal transduction events induced by insulin receptor ligation.

Recombinant MIF inhibits insulin signal transduction in adipocytes

To provide a more direct assessment of MIF action on adipocytes, we treated cells with different concentrations of MIF before stim-

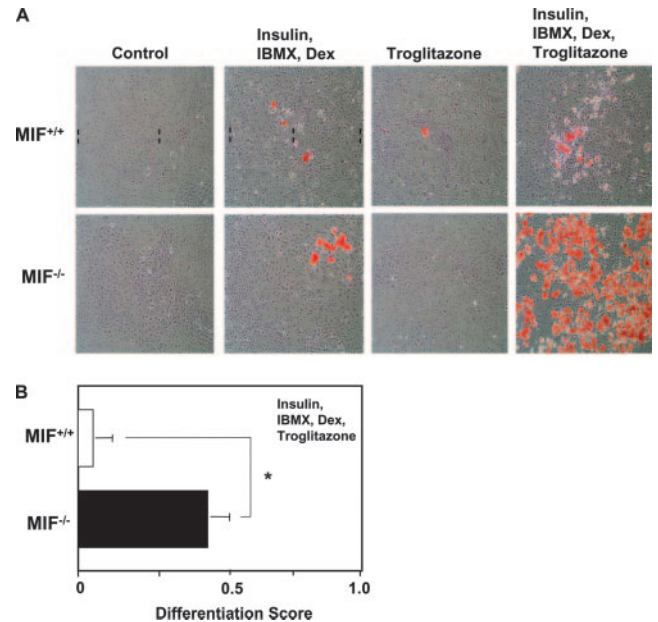


FIGURE 6. MIF deficiency enhances adipocyte differentiation. *A*, MEFs were prepared from MIF^{+/+} or MIF^{-/-} mice, and differentiation into adipocytes induced as shown. Differentiation was assessed visually by staining with Oil red O at a magnification of $\times 100$. *B*, Quantification of color intensity by NIH Image Analyzer software is shown. Data are representative of four experiments each performed with independently derived fibroblast cell lines. *, $p < 0.001$.

ulation by insulin. As shown in Fig. 5A, exogenously added MIF decreased the tyrosine phosphorylation of IRS-1 as well as the insulin-induced association of IRS-1 with the p85 regulatory subunit of PI3K. This effect occurred in a dose-dependent fashion, and at concentrations of MIF that are within the range for those reported in patients with sepsis or severe inflammation (7, 8). The serine-threonine kinase Akt is a downstream target of PI3K (24) that is recruited to the membrane by PI3K-generated phospholipids. Akt undergoes phosphorylation and activation, and provides signals for the synthesis of new glucose transporters and enhanced glucose uptake. We examined the effect of exogenously added MIF on insulin-mediated, Ser⁴⁷³ phosphorylation of Akt in adipocytes. Cells were treated with MIF followed by insulin (100 nM) for 10 min. As shown in Fig. 5B, MIF inhibited the insulin-induced phosphorylation of Akt.

Next, we investigated the action of MIF on basal and insulin-mediated glucose uptake. The addition of MIF to adipocytes did not cause an appreciable change in basal 2-deoxyglucose uptake (Fig. 5C). A decrease in insulin-stimulated glucose uptake was observed in this cultured cell system at 200 ng/ml MIF. These data differ from those reported in differentiated myotubes, where MIF was found to augment basal glucose uptake and not to influence insulin-mediated glucose uptake (5). This difference in response likely reflects the selective use of insulin-sensitive vs insensitive glucose transporters in these two differentiated cell types (25).

Genetic deficiency in MIF increases adipogenesis

We next examined the influence of MIF in adipogenesis, which is known to be inhibited by TNF- α (26). We prepared embryonic fibroblasts from MIF^{+/+} and MIF^{-/-} mice and subjected them to defined adipocyte differentiation protocols (27). We found that although MIF^{-/-} cells responded similarly to MIF^{+/+} cells when exposed to a standard differentiation medium (insulin, isobutylmethylxanthine, and dexamethasone) or to the peroxisome proliferator-activated receptor (PPAR) γ agonist, troglitazone, the

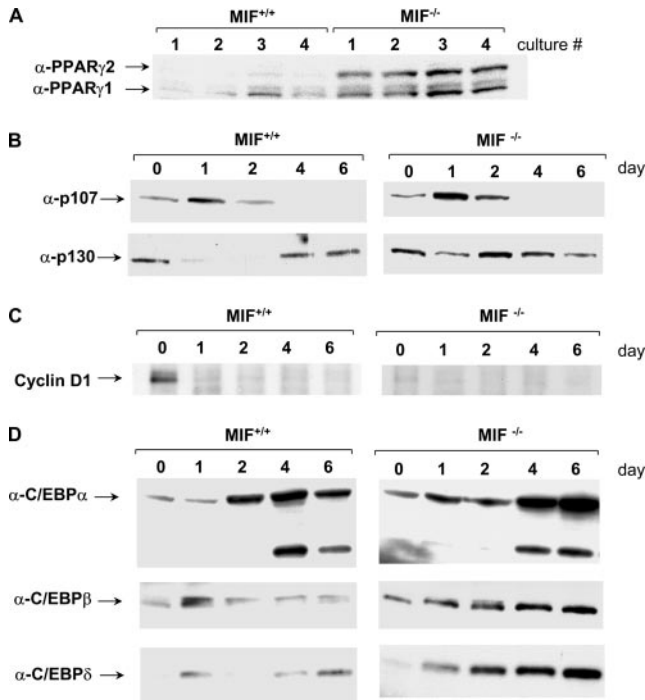


FIGURE 7. Impact of MIF deficiency on signaling pathways associated with adipogenesis. *A*, The expression of PPAR γ proteins after induction of adipogenesis. Cells were grown in standard differentiation medium in the presence of troglitazone and harvested at day 9. The Western blot analysis of lysates from cultures of four different MEF cell lines (lanes 1–4) is shown. *B*, The expression of the p107 and p130 transcriptional regulatory proteins during adipocyte differentiation. MIF^{+/+} and MIF^{-/-} MEFs were grown in standard differentiation medium in the presence of troglitazone and harvested at the indicated days for Western blot analysis with anti-p107 (α -p107) or anti-p130 (α -p130) Ab. *C*, The expression of cyclin D1 protein during adipocyte differentiation. MIF^{+/+} and MIF^{-/-} MEFs were grown in standard differentiation medium in the presence of troglitazone and analyzed by Western blotting with anti-cyclin D1 Ab. *D*, The expression of C/EBP α , C/EBP β , and C/EBP δ proteins during adipogenesis. Cells were grown as described, harvested on the indicated days, and Western blot analyzed with the C/EBP-specific Abs shown. The blot for C/EBP α shows both the p42 and the p30 isoforms.

MIF^{-/-} cells were significantly more sensitive to the combination of standard differentiation medium plus troglitazone (Fig. 6). Adipocyte differentiation is known to be linked to the transcriptional activation of the PPAR γ , which is a target for the differentiation agent, troglitazone. We observed higher expression of the PPAR γ 1 and PPAR γ 2 isoforms during differentiation of the MIF^{-/-} cells vs the MIF^{+/+} cells (Fig. 7A). Fibroblasts exposed to adipogenic factors enter a stage of postconfluent, cell division that is associated with a switch in the expression of the p130:p107 transcriptional regulatory proteins (28). We analyzed the expression of p130:p107 during adipocyte differentiation and found a similar pattern of expression in both the MIF^{-/-} and MIF^{+/+} cells (Fig. 7B). The expression of cyclin D1 and the subsequent activation of cyclin-dependent kinases has been shown to inhibit adipocyte differentiation (38). We analyzed cyclin D1 levels by western blotting and observed the expected down-regulation of this protein after the addition of differentiation medium to MIF^{+/+} cells. By contrast, the expression of cyclin D1 was significantly reduced at baseline in MIF^{-/-} cells, which appears consonant with the increased sensitivity of these cells to adipocyte differentiation (Fig. 7C).

Finally, we examined the expression of C/EBP α , C/EBP β , and C/EBP δ , which are transcriptionally activated during adipogenesis.

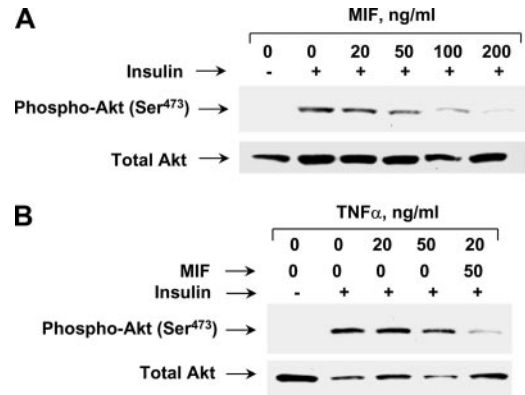


FIGURE 8. The effect of MIF or TNF- α on the insulin-mediated phosphorylation of Akt in MIF^{-/-} adipocytes. *A*, Cultured adipocytes were pretreated with MIF for 6 h followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser⁴⁷³ phospho-Akt and total Akt. *B*, Cultured adipocytes were pretreated with TNF- α , or TNF- α plus MIF, for 6 h followed by insulin (100 nM) for 10 min. Total cell lysates were separated by SDS-PAGE and blotted with Abs against Ser⁴⁷³ phospho-Akt and total Akt.

C/EBP β and C/EBP δ are induced early and activate the expression of PPAR γ , and C/EBP α is induced later in time (29). MIF deficiency was associated with an up-regulation of C/EBP α , C/EBP β , and C/EBP δ during the six-day period of adipocyte differentiation (Fig. 7D). MIF-deficient cells thus show an increased ability to differentiate into adipocytes in response to PPAR γ ligands, and this effect may result from a constitutive reduction in baseline cyclin D1 levels and an enhancement in cellular C/EBP α , C/EBP β , and C/EBP δ content.

The effect of MIF or TNF- α on insulin-mediated phosphorylation of Akt in MIF^{-/-} adipocytes

To further evaluate the functional phenotype of MIF^{-/-} adipocytes, we examined the effect of MIF or TNF- α on insulin-mediated signal transduction in these cells. MIF^{-/-} adipocytes were exposed to MIF or TNF- α and the insulin induced phosphorylation of Akt was analyzed by western blotting. As shown in Fig. 8, MIF inhibits the insulin-mediated phosphorylation of Akt, whereas TNF- α showed no significant effect. We also examined the presence of TNFRs during the differentiation of both MIF^{-/-} and MIF^{+/+} adipocytes, because a reduction in the TNFR could offer an explanation for the TNF insensitivity of MIF^{-/-} cells. The expression of TNFR-1 protein was induced in equivalent levels in MIF^{-/-} and MIF^{+/+} cells, and no differences were noted in the expression of the TNFR-2 isoform (Fig. 9 and data not shown).

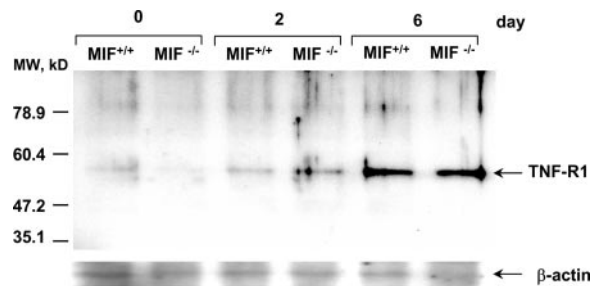


FIGURE 9. The expression of TNFR (TNF-R1) during adipocyte differentiation of MIF^{+/+} and MIF^{-/-} MEFs. Total cell lysates were separated by SDS-PAGE and blotted with Abs against TNFR-1 Ab. The blot was stripped and reprobed with anti- β -actin Ab.

Discussion

Investigations of the metabolic dysregulation that accompanies severe infection or tissue invasion led in the 1980s to the definition of the mediator “cachectin”, which was later determined to be structurally identical with TNF- α (30). Among the metabolic changes noted in these studies were insulin resistance and alterations in glucose and lipid homeostasis that affected the rate of substrate production and use by tissues. There has been an accumulation of data supporting the importance of TNF- α in insulin resistance, both in acute disease and in conditions such as diabetes and obesity (3, 20, 21, 31). The importance of metabolic homeostasis to clinical outcome also has been highlighted by observations of decreased mortality in critically ill patients treated with insulin therapy (32).

MIF is an established mediator of sepsis lethality (7), and it is secreted by activated immune cells (6), by the anterior pituitary (10), and by the β -cells of the pancreatic islets, where it is a positive, autocrine regulator of insulin release (19). In a recent study, MIF was described to be released from cultured myotubes stimulated with TNF- α and then to act in an autocrine/paracrine manner to stimulate muscle glucose catabolism (5). In the present study, MIF $^{-/-}$ mice showed a near normalization in glucose metabolism in response to endotoxin, which induces a transient (<2 h) alteration in circulating glucose and lactate levels. Moreover, TNF- α itself did not influence blood glucose in the setting of genetic MIF deficiency. An intrinsic role of MIF in the glycemic response during endotoxemia was verified by in vivo experiments using labeled glucose in awake mice, which showed increased glucose uptake into white adipose tissue in mice genetically deficient in MIF. Anti-MIF mAb also prevented TNF- α inhibition of insulin-mediated glucose transport in adipocytes, which is in accord with a modulating effect of MIF on the action of TNF- α at the level of insulin signal transduction. The precise mechanism for the inhibitory effect of MIF on insulin signal transduction events may involve inhibition of Akt phosphorylation, which together with PI3K is necessary for the serine phosphorylation of the IRS-1 protein (23, 33).

Genetic MIF deficiency also promotes adipogenesis in a defined model of adipocyte differentiation requiring PPAR γ agonism, and this effect is consistent with an inhibitory action of TNF- α on adipocyte differentiation (34). Among the mechanisms by which MIF may mediate this inhibition it is notable that the E2F family regulates the differentiation of adipocytes. E2F1 induces PPAR γ transcription during clonal expansion (35), and Petrenko et al. (36) recently showed that the expression of E2F1 is up-regulated in MIF $^{-/-}$ cells. An additional pathway may relate to the defect in cyclin D1 activity that has been described in fibroblasts deficient in MIF (37). Fu et al. (38) have reported that cyclin D1 inhibits PPAR γ -mediated adipogenesis via an action on histone deacetylase. MIF $^{-/-}$ cells showed reduced levels of cyclin D1, which is in agreement with the enhanced adipogenic potential of these cells. MIF also showed an inhibitory action on C/EBP δ . The C/EBP δ transcription factor is induced by the dexamethasone (39), and this action is likely consistent with the glucocorticoid counter-regulating properties of MIF (18, 40).

TNF- α did not affect the insulin-mediated phosphorylation of Akt in MIF $^{-/-}$ adipocytes, further confirming the role for MIF as a downstream effector of TNF- α . This effect was not due to reduced expression of TNFR-1 by these cells, which may be suggested by a prior report (42). It is known, however, that the action of TNF- α on insulin signaling is reduced upon PPAR γ activation (41). Although we cannot rule out a role for MIF in affecting PPAR γ activation, the PPAR γ ligand troglitazone by itself did not show a differential effect on adipocyte development from MIF $^{+/+}$

vs MIF $^{-/-}$ cells. Further studies will be necessary to better define the signaling pathways influenced by the interaction of TNF- α and MIF in this model system.

In conclusion, these studies support the concept that TNF- α mediates insulin resistance in adipocytes by the downstream, autocrine/paracrine action of MIF on key steps in the insulin signal transduction pathway. The present studies nevertheless do not signify that all of the metabolic actions of TNF- α are necessarily attributed to MIF. A broader question regards the potential action of MIF in carbohydrate and lipid homeostasis in physiologic settings outside of severe inflammation. Patients who have type 2 diabetes have increased circulating levels of MIF (43, 44), and the recent discovery of functional alleles in the human *MIF* gene (45), prompt consideration of the role of MIF in the pathogenesis of insulin resistance that occurs more commonly in diabetes, aging, and obesity.

Disclosures

Drs. Leng, Metz, Mitchell, and Bucala are coinventors on patents describing the potential therapeutic value of inhibiting MIF.

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