Macrophage Migration Inhibitory Factor Inhibition Is Deleterious for High-Fat Diet-Induced Cardiac Dysfunction

Aurore Palud#, Camille Marciniak#, David Montaigne, Xavier Marechal, Caroline Ballot, Sidi Mohamed Hassoun, Brigitte Decoster, Remi Neviere, Steve Lancel*

EA4484, Physiology Department, Lille 2 University, Lille, France

Abstract

Aims: Development of metabolic syndrome is associated with impaired cardiac performance, mitochondrial dysfunction and pro-inflammatory cytokine increase, such as the macrophage migration inhibitory factor MIF. Depending on conditions, MIF may exert both beneficial and deleterious effects on the myocardium. Therefore, we tested whether pharmacological inhibition of MIF prevented or worsened metabolic syndrome-induced myocardial dysfunction.

Methods and Results: C57BL/6J mice were fed for ten weeks with 60% fat-enriched diet (HFD) or normal diet (ND). MIF inhibition was obtained by injecting mice twice a week with ISO-1, for three consecutive weeks. Then, triglycerides, cholesterol, fat mass, glucose intolerance, insulin resistance, ex vivo cardiac contractility, animal energetic substrate utilization assessed by indirect calorimetry and mitochondrial respiration and biogenesis were evaluated. HFD led to fat mass increase, dyslipidemia, glucose intolerance and insulin resistance. ISO-1 did not alter these parameters. However, MIF inhibition was responsible for HFD-induced cardiac dysfunction worsening. Mouse capacity to increase oxygen consumption in response to exercise was reduced in HFD compared to ND, and further diminished in ISO-1-treated HFD group. Mitochondrial respiration was reduced in HFD mice, treated or not with ISO-1. Compared to ND, mitochondrial biogenesis signaling was upregulated in the HFD as demonstrated by mitochondrial DNA amount and PGC-1α expression. However, this increase in biogenesis was blocked by ISO-1 treatment.

Conclusion: MIF inhibition achieved by ISO-1 was responsible for a reduction in HFD-induced mitochondrial biogenesis signaling that could explain major cardiac dysfunction observed in HFD mice treated with MIF inhibitor.

Introduction

A cluster of metabolic disorders that predispose to cardiovascular diseases characterizes metabolic syndrome (MetS) [1]. Its prevalence is constantly rising and already ~25% Americans and ~15% Europeans are affected by MetS [2–4]. Patients with MetS have abdominal obesity in combination with two other metabolic abnormalities such as hypertriglyceridemia, low HDL cholesterol levels, raised blood pressure, glucose intolerance or type 2 diabetes mellitus [1]. Additional factors such as genetics, hormonal changes, and a proinflammatory state also contribute to the development of MetS.

Among the proinflammatory cytokines, several clinical studies established that macrophage migration inhibitory factor (MIF) levels positively correlated to body mass index, plasma free fatty acids and impaired glucose tolerance [5,6]. In addition, elevated circulating MIF levels conferred to women, not to men, higher risks to develop type 2 diabetes and cardiovascular pathologies [7–9]. Thus, it is believed that MIF may be a key player in the development of cardiovascular disease associated with MetS although the underlying mechanisms are not fully understood.

MIF is a homotrimeric protein containing tautomerase activity [10]. It has been identified as a product of monocytes/macrophages [11], adipocytes [12], β pancreatic cells [13] or cardiomyocytes [14]. This pleiotropic cytokine participates to pathogenesis of inflammatory diseases including atherosclerosis [15], arthritis [15] and sepsis [16]. MIF contributes to regulation of glucose metabolism and is involved in development of insulin resistance [17]. MIF also directly targets cardiovascular system. Indeed, while MIF depresses myocardial function in a context of systemic inflammation [14], it exerts protective effects on the heart in circumstances of ischemia-reperfusion injury [18]. This beneficial impact of MIF has been attributed to changes in cardiac metabolism, i.e. enhanced cardiac glucose uptake [18].
As MIF may contribute to the pathogenesis of MetS-associated cardiomyopathy and modify cardiac metabolism, we tested the effects of MIF inhibition in mice fed with a fat-enriched diet. First, we sought whether induction of MetS in mice changed cardiac MIF expression along with myocardial dysfunction. Then, we tested the effects of pharmacological inhibition of MIF with ISO-1, a competitive inhibitor of tautomerase activity, on MetS-induced myocardial dysfunction. Finally, we determined whether MIF inhibition could modulate myocardial function through changes in cardiac glucose utilization and mitochondrial function.

Materials and Methods

Ethics Statement
All experiments were carried out in accordance with our national guidelines and approved by the “direction départementale de la cohésion sociale et de la protection des populations – Nord - Pas-de-Calais – Lille” (Permit Number 59-350206).

Animals
Female C57BL/6J mice at 4 weeks of age were obtained from Charles River Laboratory (L’Arbresle, France). Mice had free access to tap water and were fed with either a standard chow diet (normal diet ND) or a high-fat diet (HFD, D12492 SSNII, Soest, Germany) in which 60% calories were from lard fat. Animals were kept under 12 h light/dark cycles.

ISO-1 Treatment
MIF inhibition was achieved by ISO-1 (Merck Chemicals, Darmstadt, Germany), a pharmacological compound binding to the tautomerase active site of MIF [19] and that has been widely used in various in vivo and in vitro experimental models [20]. After 10 weeks of feeding, mice received ISO-1 injections [21] (20 mg.kg⁻¹ total body weight, intraperitoneally) twice a week or the equivalent amount of DMSO used as solvent. After three weeks of treatment, mice were subjected to cervical dislocation.

Plasma Analyses
Mice were fasted for six hours. Then, after tail incision, 100 µL of blood was withdrawn and collected into EDTA-coated capillary tubes. Plasma was obtained after a centrifugation performed at 1,000 g, at 4°C, for 15 min. Samples were aliquoted and stored at −80°C. Plasma true triglycerides were quantified by the use of the triglyceride determination kit from Sigma Aldrich (St Quentin Fallavier, France). Total cholesterol was measured with the cholesterol RTU kit, according manufacturer’s instructions (Biomerieux, Marcy-L’Etoile, France).

MIF Activity
MIF tautomerase activity assay was performed as previously described [22]. Briefly, 1 µL of plasma was added in 499 µL of assay buffer (1 mM EDTA and 10 mM NaH₂PO₄, pH 6.4), and kept at room temperature for 2 min. Then, 500 µL of freshly prepared substrate solution (assay buffer containing 0.6 mM L-dihydroxyphenylalanine methyl ester and 20 mM Na m-periodate) were added into the cuvette, mixed and read at 475 nm using a spectrophotometer (Biomate 5, Thermo electron corp., USA). Glucose levels were measured using a glucose meter (Nova biochemical, Waltham, MA).

Materials and Methods

Indirect Calorimetry
Mice were placed into a hermetic chamber connected to an indirect calorimetry system (Oxymax, Columbus Instruments, Columbus, OH). Animals had free access to tap water and food. Oxygen consumption (VO₂) and CO₂ production (VCO₂) were recorded over 3 consecutive days. Respiratory quotient (RQ) was calculated as RQ = VCO₂/VO₂ ratio. Relative cumulative frequency curve analysis was used to determine substrate flexibility, as previously described [23,24].

In another set of experiments, mice were subjected to an exercise stress test, as reported elsewhere [25]. Briefly, mice were placed on a treadmill enclosed into a metabolic chamber that was connected to the Oxymax apparatus. After a 30 min stabilization period, mice started running at 10 m.min⁻¹ on a 0% incline and speed was incremented by 4 m.min⁻¹ every 3 min until exhaustion. Exhaustion was reached when mice remained on the treadmill for 5 sec. Basal VO₂ and maximal VO₂ (VO₂max) obtained during exercise were measured, allowing the calculation of ΔVO₂ = VO₂max-VO₂.

Myocardial Function
Freshly excised mouse hearts were immediately mounted onto a Langendorf® apparatus, perfused at constant coronary flow (2.5 mL.min⁻¹) with Krebs-Henseleit bicarbonate buffer and paced at 10 Hz. A latex balloon, inserted into the left ventricle, was filled with an aqueous solution to achieve a left ventricular end-diastolic pressure of 6-8 mmHg. The balloon was linked to a pressure transducer connected to MI118 bridge amplifier that fed into a Powerlab 8 SP high-performance data acquisition system (ADInstruments Ltd. by Phymep, Paris, France). Isoluminal contraction was appreciated by measuring left ventricular developed pressure (LVDP) and its first derivatives. Data were obtained with rising concentrations of the β-adrenergic receptor agonist isoproterenol (0, 1, 10 and 100 nM). Vasoractivity was evaluated by perfusing sodium nitroprusside at different concentrations (0, 2, 20 and 200 µM).

Mitochondrial Function
Freshly excised heart was rinsed, minced and homogenized into a mitochondrial extraction buffer (in mM: sucrose 300, TES 5, EGTA 0.2, pH 7.2) as previously described [26]. After centrifugations, mitochondrial pellets were suspended into mitochondrial respiration media Mitomexc2 [27]. Mitochondria (50 µg) were placed into oxygraph chambers (O2K, Oroboros Instruments, Innsbruck, Austria) filled with 2 mL of Mitomexc2, at 25°C. L-glutamate (10 mM) and L-malate (2 mM) were added in the first chamber, palmitoyl-L-carnitine (5 µM) and L-malate (2 mM) were added into the second chamber. After signal stabilization, ADP (2.5 mM) was injected and oxygen consumption was measured.

Oral Glucose Tolerance Test (OGTT)
In order to assess glucose tolerance, animals were fasted for six hours. Then, mice received by gavage a glucose solution (2 mg.g⁻¹ total body weight). Tail blood samples were taken before glucose administration and 10, 20, 30, 60 and 120 min after glucose ingestion. Glucose levels were measured using a glucose meter (Nova biomedical, Waltham, MA).

Insulin Tolerance Test (ITT)
Insulin resistance was tested by injecting fasted mice intraperitoneally with insulin (0.5 U.kg⁻¹ total body weight). Blood glucose concentration was measured before injection and 15, 30 and 60 min later.

Indirect Calorimetry
Mice were placed into a hermetic chamber connected to an indirect calorimetry system (Oxymax, Columbus Instruments, Columbus, OH). Animals had free access to tap water and food. Oxygen consumption (VO₂) and CO₂ production (VCO₂) were recorded over 3 consecutive days. Respiratory quotient (RQ) was calculated as RQ = VCO₂/VO₂ ratio. Relative cumulative frequency curve analysis was used to determine substrate flexibility, as previously described [23,24].

In another set of experiments, mice were subjected to an exercise stress test, as reported elsewhere [25]. Briefly, mice were placed on a treadmill enclosed into a metabolic chamber that was connected to the Oxymax apparatus. After a 30 min stabilization period, mice started running at 10 m.min⁻¹ on a 0% incline and speed was incremented by 4 m.min⁻¹ every 3 min until exhaustion. Exhaustion was reached when mice remained on the treadmill for 5 sec. Basal VO₂ and maximal VO₂ (VO₂max) obtained during exercise were measured, allowing the calculation of ΔVO₂ = VO₂max-VO₂.

Myocardial Function
Freshly excised mouse hearts were immediately mounted onto a Langendorf® apparatus, perfused at constant coronary flow (2.5 mL.min⁻¹) with Krebs-Henseleit bicarbonate buffer and paced at 10 Hz. A latex balloon, inserted into the left ventricle, was filled with an aqueous solution to achieve a left ventricular end-diastolic pressure of 6-8 mmHg. The balloon was linked to a pressure transducer connected to MI118 bridge amplifier that fed into a Powerlab 8 SP high-performance data acquisition system (ADInstruments Ltd. by Phymep, Paris, France). Isoluminal contraction was appreciated by measuring left ventricular developed pressure (LVDP) and its first derivatives. Data were obtained with rising concentrations of the β-adrenergic receptor agonist isoproterenol (0, 1, 10 and 100 nM). Vasoractivity was evaluated by perfusing sodium nitroprusside at different concentrations (0, 2, 20 and 200 µM).

Mitochondrial Function
Freshly excised heart was rinsed, minced and homogenized into a mitochondrial extraction buffer (in mM: sucrose 300, TES 5, EGTA 0.2, pH 7.2) as previously described [26]. After centrifugations, mitochondrial pellets were suspended into mitochondrial respiration media Mitomexc2 [27]. Mitochondria (50 µg) were placed into oxygraph chambers (O2K, Oroboros Instruments, Innsbruck, Austria) filled with 2 mL of Mitomexc2, at 25°C. L-glutamate (10 mM) and L-malate (2 mM) were added in the first chamber, palmitoyl-L-carnitine (5 µM) and L-malate (2 mM) were added into the second chamber. After signal stabilization, ADP (2.5 mM) was injected and oxygen consumption was measured.
Ratio substrates+ADP/substrates without ADP was calculated as a surrogate of mitochondrial coupling. At the end of the experiment, exogenous cytochrome c was added in order to test mitochondrial membrane intactness.

Fructose-2,6-biphosphate Content Measurement
Fructose-2,6-biphosphate (Fru-2,6-P$_2$) content has been evaluated as previously described by Hue et al. [28]. Briefly, flash frozen tissue was homogenized in 50 mM NaOH, heat up to 80°C for 10 min and cooled down. A solution containing acetic acid 25 mM and HEPES 20 mM was added until pH reached 8.0. After centrifugation at 15,000 u for 15 min at 4°C, supernatant was collected to measure its Fru-2,6-P$_2$ content. Samples were added in a buffer containing: Tris 200 mM, pH 8.0, MgCl$_2$ 100 mM, NADH 0.15 mM, aldolase 10 U, triose phosphate isomerase 10 U, glycerol-3-phosphate dehydrogenase (GDH) 10 U, phosphofructokinase : PFK, fructose-6-phosphate 5 mM. Reaction was initiated by adding 0.5 mM pyrophosphate. GDH-dependent NADH oxidation was followed by measuring absorbance at 340 nm for 5 min.

Western-blotting
Whole cardiac tissue extracts were prepared as described previously [25]. Briefly, flash frozen hearts were homogenized in RIPA buffer (in mM: Tris 10; NaCl 140; EDTA 5; Triton X-100 1%; deoxycholate 1%; SDS 0.1%, pH 7.4; with antiprotease cocktail and antiphosphatases Na$_3$VO$_4$ 1 mM; NaF 20 mM) using a glass tissue grinder. After centrifugation, supernatants were collected and stored at −80°C until use. Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with the following antibodies: anti-MIF (AbCam, Cambridge, UK), anti-GAPDH (Cell Signaling, Beverly, MA, USA). Blots were developed with ECL Plus reagent (GE Healthcare, Templemars, France). Protein concentration was determined using the Bradford assay.

DNA and RNA Extractions
Total DNAs were extracted and purified by the use of QIAamp DNA Mini kit (Qiagen, Courtaboef, France), as stated by the manufacturer. Briefly, hearts were homogenized in the supplied tissue lysis buffer containing proteinase K. Then, samples were treated with RNase A (Life Technologies SAS, Saint Aubin, France) and placed onto Qiagen Mini Spin columns. After washes, elution of DNA was achieved by adding 100 μL of AE buffer.

After tissue homogenization in Trizol reagent (Life Technologies SAS), total RNAs were purified with the PureLink Micro-to-Midi total RNA purification kit (Life Technologies SAS) according to manufacturer’s instructions. cDNA were prepared using transcriptor first strand cDNA synthesis kit (Roche Applied Science, Meylan, France).

Real-time PCR and RT-PCR
Real-time PCRs and RT-PCRs were performed using Eppendorf Realplex S2 (Eppendorf, Le Pecq, France) and Mesa Blue qPCR Master Mix Plus for SYBR assay (Eurogentec, Angers, France). PCR primers used for determination of mitochondrial DNA copy numbers were Ppia (nuclear-encoded gene), mtCOII (mtDNA-encoded gene) and Nad1 (mtDNA-encoded gene) and are presented Table S1. Primers used for RT-qPCR were against Mif, Slc2a1, Pfkp, Pdk3 and Pglycer (Table S2). β-actin was used as an internal control (Table S2). Realplex software was used to quantity differences in gene expression. Results are expressed as fold expression.

Statistical Analysis
Data are presented as means ± S.E.M. Statistical analysis was carried out using two-tailed unpaired t-test when comparing two groups and ANOVA when comparing three groups or more. Analysis was performed on GraphPad Prism software 5.0. Differences were considered significant when p<0.05.

Results
HFD Induced MIF Expression Increase
First, we evaluated plasma tautomerase activity, which is mainly related to circulating MIF concentration. As shown in Figure 1A, tautomization rate of L-dopachrome methyl ester into the dextrogyre isomer was doubled in plasma of HFD fed mice. As MIF is secreted by the heart to act as an autocrine/paracrine cytokine, we measured both Mif mRNA (Figure 1B) and protein (Figure 1C) in cardiac tissue. HFD induced elevation in cardiac MIF synthesis at both levels (Figures 1B and 1C).

MIF Inhibition Worsened HFD-induced Cardiac Dysfunction
Next, we tested the effects of the pharmacological MIF inhibitor ISO-1 on isolated hearts. Changes in LVDP were observed with no differences in coronary perfusion pressure (Figure 2A). LVDP (Figure 2B) as well as its first derivatives ±dP/dt (Figures 2C and 2D) were reduced in the HFD compared to ND mice. Addition of isoproterenol in the perfusion buffer exerted marked positive inotropic effects on hearts isolated from ND mice but not on hearts obtained from HFD mice (Figures 2B-2D). MIF inhibitor dramatically worsened HFD-caused cardiac dysfunction even in presence of the β-adrenergic receptor agonist (Figures 2B-2D). ISO-1 injection in ND mice did not alter LVDP (Figure 2B) and its first derivatives (Figures 2C-2D).

MIF Inhibition had no Effects on Lipid and Carbohydrate Utilization
We evaluated whether ISO-1 was responsible for an aggravation of metabolic parameters triggered by the high-fat regimen. HFD induced massive adipose tissue accumulation in the abdomen (Figure 3A) and in a lower extent in the subcutaneous compartment (Figure 3B), resulting in an elevation of the abdominal/subcutaneous fat mass ratio (Figure 3C). ISO-1 had no effects on fat mass amount and distribution (Figures 3A-3C). Similarly, both HFD and ISO-1-treated HFD mice had significant elevation of plasma true triglycerides and cholesterol compared to ND mice (Figures 3 D and 3E). In addition, ISO-1 did not reverse elevation in fasting blood glucose found in HFD mice (Figure 3F). Compared to ND mice, HFD or ISO-1-injected HFD mice were both intolerant to glucose and resistant to insulin, as suggested by the oral glucose tolerance (Figure 3G) and the insulin tolerance (Figure 3H) tests, respectively.

MIF Inhibition did not Change HFD-induced Preferential Lipid Utilization as Substrate
Indirect calorimetry revealed that ND mice preferentially burned lipids during the light period and switched to glucose consumption during the dark phase, as suggested by the RQ oscillating from 0.7 to 1, respectively (Figure 4A). Thus, ND mice displayed broad substrate flexibility (Figure 4B). On the contrary, RQ of HFD mice remained close to 0.7 during light and dark periods, indicative of preferential lipid utilization (Figure 4A). HFD mice lost the phasic substrate flexibility observed in the ND
MIF Inhibition Worsens Diabetic Heart Dysfunction

Indirect Calorimetry Revealed that MIF Inhibition was Associated with Changes in Oxygen Consumption

Before exercise, compared to ND mice, HFD animals had higher oxygen consumption VO2 (Figure 5A) and a reduced CO2 production (Figure 5B). HFD mice treated with ISO-1 consumed even more oxygen than HFD mice (Figure 5A). These results were consistent with RQ observed during 24 hour-cycles (Figure 4A).

We next subjected mice to maximal physical exercise to test their maximal aerobic capacity as an indication of global mitochondrial function. When subjected to the treadmill test, elevation of VO2 was about 48% in the ND group while it increased by only 21% in the HFD group (Figure 5A), resulting in a significant reduction in ΔVO2 between ND and HFD mice (Figure 5A). At the exercise peak, VO2 max was not significantly different between HFD and HFD+ISO-1 mice (Figure 5C). As a consequence, ISO-1-treated HFD mice had diminished ΔVO2 as compared to HFD mice (Figure 5D).

MIF Inhibition did not Improve Mitochondrial Function but Reduced Biogenesis

We explored oxidative phosphorylation on isolated cardiac mitochondria with either glutamate/malate or palmitoylcarnitine/malate as substrates for respiratory chain complexes. In both conditions, in absence of ADP, no differences were observed between groups (Figures 6A and 6B). However, ADP-driven respiration was almost halved in HFD and HFD+ISO-1 compared to ND mitochondria (Figures 6A and 6B).

Mitochondrial biogenesis was evaluated by quantitative PCR targeting mtCOII and Nd1, two mitochondrial-encoded genes, and by measuring Pgc-1α transcript expression. Mitochondrial DNA copy number was significantly higher in the HFD group compared to ND mice as mtCOII and Nd1 were increased (Figure 6C). On the contrary, Nd1 and mtCOII in HFD+ISO-1 group were notably reduced compared to HFD (Figure 6C). Similar results were obtained for Pgc-1α expression (Figure 6D).

Discussion

The causative link between MIF secretion and metabolic disorders has still to be demonstrated. Here, we induced metabolic disorders in mice that resembles to human MetS by the use of high-fat regimen. We also found that, in female mice, circulating level of MIF was increased, probably due to a massive release by adipocytes [12]. Such variations have similarly been reported in male animals [29]. Interestingly, we detected that heart increased its endogenous production of MIF. While this autocrine/endocrine function has been previously illustrated in sepsis [14] and ischemia/reperfusion injury [18], the overall cardiac effects of MIF are challenging as both protective and deleterious consequences have been reported [18,30–32].

To test whether MIF participates to the HFD-induced cardiac dysfunction, we treated mice with ISO-1, an isoxazoline. This compound binds to MIF active site, hence inhibiting MIF tautomerase activity, which is required for part of its biological activity [33]. Although such a pharmacological approach may also have ‘off-target’ effects on MIF-sensitive metabolic organs such as liver, skeletal muscles or adipocytes [17], we observed that ISO-1 had no effects on cardiac function in ND mice but deteriorated contractile parameters in HFD animals. Consistent results have been recently reported in a type 1 diabetes mellitus model. For example, Tong et al. [34] found that cardiomyocytes isolated from streptozotocin-treated MIF knockout mice exhibited exacerbated contractile dysfunction compared to streptozotocin-treated wild-type mice. Thus, increased MIF production induced by HFD may be adaptive and exert cardioprotective effects, either directly or indirectly.

The importance of contractile dysfunction of heart isolated from ISO-1-treated HFD animals was striking because mice were still able to support sustained exercise running test. This may show some
limitations of the Langendorff evaluation that does not take into account parameters influencing hemodynamic performance in vivo such as neuroendocrine regulation and circulating substrates that can be consumed by the heart. To explain marked deleterious effects of MIF inhibition on cardiac function, we measured metabolic parameters such as circulating triglycerides, cholesterol or insulin that may affect the myocardium energetics and contractile function. Contrary to other reports [35,36], we did not detect any MIF inhibition-induced improvement of fasting blood glucose, glucose intolerance, insulin resistance or dyslipidemia. Of note, it should be pointed out that we injected ISO-1 when mice already had features of MetS [25] while most studies using pharmacological inhibitors [37], blocking antibodies [37] or genetically engineered mice [35] were performed in a prophylactic manner.

As measured circulating parameters were not affected by ISO-1, cardiac MIF production in response to HFD may represent a protective and adaptive response to maintain cardiac function. This contention is supported by the findings of Miller et al. that attributed beneficial effects of MIF in the ischemia/reperfusion injured heart to activation of the cardioprotective AMPK pathway and increased glucose uptake [18]. In the present study, endogenous cardiac energetic substrate utilization did not seem to be affected by MIF inhibition as the amount of Fru-2,6-P2 or genes involved in glycolysis remained similar in both HFD and HFD+ISO-1 animals. Consistently, indirect calorimetry revealed that both HFD and HFD+ISO-1 groups had a similar RQ, at around 0.7, meaning that a higher proportion of fat is oxidized at the expense of carbohydrates. Beside availability of heart fuels, cardiac energy production also relies on mitochondria number and efficiency. As previously reported [25], cardiac mitochondrial respiration from HFD mice was reduced either in presence of Krebs cycle or β-oxidation -related substrates. ISO-1 did not deteriorate respiration of isolated mitochondria, suggesting that intrinsic mitochondrial function was similar in both HFD groups. However, indirect calorimetry experiments revealed that basal oxygen consumption (in the amount of which the heart is likely the principal oxygen-consuming organ) was increased in HFD+ISO-1 mice compared to HFD mice. Taken together, these results prompted us to study cardiac mitochondrial mass, whereas we could not rule out changes in other oxygen-consuming tissues such

Figure 2. ISO-1 amplified HFD-induced cardiac dysfunction. (A) Coronary perfusion pressure (CPP) measured in presence of rising concentration of sodium nitroprusside (SNP) on isolated heart preparations obtained from ND, ISO-1-injected ND mice (ND+ISO-1), HFD and ISO-1-treated HFD mice (HFD+ISO-1). (B) Left ventricular developed pressure (LVDP), (C) +dP/dt max and (D) –dP/dt min measured on ND, ND+ISO-1, HFD, HFD+ISO-1 hearts, in presence of the β-adrenergic receptor agonist isoproterenol. Data are means ± SEM of 5 experiments. *p<0.05 vs. ND; †p<0.05 vs. HFD.

doi:10.1371/journal.pone.0058718.g002
as skeletal muscles. Specifically, we studied mitochondrial population by measuring mtDNA copy number and Pgc-1α expression as mitochondrial biogenesis hallmarks [38]. Interestingly, ISO-1 abrogated HFD-induced increases in mtDNA copy number and Pgc-1α expression. Thus, although the understanding of underlying molecular mechanisms warrants further studies, cardiac MIF

Figure 3. ISO-1 treatment did not modify metabolic syndrome-associated parameters. (A) Abdominal fat mass, (B) subcutaneous fat mass, (C) abdominal to subcutaneous fat mass ratio, (D) plasma true triglyceride levels, (E) plasma cholesterol concentration and (F) fasting blood glucose. Data are means ± SEM, n = 5; *p<0.05 vs. ND. (G) Oral glucose tolerance test and (H) insulin tolerance test performed on ND, HFD and HFD+ISO-1 mice. n = 5–10 in each group. *p<0.05 vs. ND.

doi:10.1371/journal.pone.0058718.g003
expression in response to HFD may somehow participate to mitochondrial biogenesis and improve energy production.

Beside the mitochondrial hypothesis, other mechanisms, including calcium mishandling and reactive oxygen species (ROS) production, may explain why ISO-1 was deleterious for HFD hearts. Calcium mishandling is a well-known feature of diabetes-related cardiomyopathy [39]. For instance, reduced sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA) activity has been described in different models [40,41]. In the present study, isoproterenol failed to increase cardiac force in both HFD and HFD+ISO-1 mice, suggesting disturbances in calcium homeostasis. Thus, regarding the dramatic reductions in cardiac force, contractility and relaxation parameters ($\pm$ dP/dt) observed in the HFD+ISO-1 group, it can be hypothesized that MIF inhibition had worsened calcium properties in cardiac cells. However, opposite data on the role of MIF on calcium homeostasis has been published. On the first hand, calcium amplitude currents through L-type channels are reduced in presence of recombinant MIF in atrial cells [42]. On the other hand, further altered calcium transients have been observed in adult cardiomyocytes isolated from streptozotocin-treated MIF knockout mice compared to streptozotocin-treated wild-type mice [34]. Eventually, apart from its tautomerase activity, MIF bears antioxidant properties. Typically, Koga et al. [30] reported that MIF was able to reduce oxidative stress in the post-ischemic heart. In our model of HFD, massive amount of circulating lipids may lead to myocardial oxidative stress due to lipotoxicity. Thus, HFD-induced cardiac MIF expression may represent a protective response towards increased oxidative stress. Nevertheless, a recent study showed that MIF itself was able to induce ROS production and subsequently autophagy in hepatocarcinoma cells [43].

**Figure 4. ISO-1 did not change animal energy balance and cardiac glucose utilization pathway.** (A) Respiratory quotient, calculated as $V_{CO2}/V_{O2}$ ratio, over three consecutive days. Shaded areas between 8 pm and 8 am indicate dark periods. (B) Relative cumulative frequency (RCF) of respiratory quotient distribution. Experiments were performed on three mice in each group. Presented data are means only. (C) Fructose-2,6-biphosphate (Fru-2,6-P$_2$) measured in cardiac lysates. (D) Cardiac expression levels of Slc2a1, Pfkp and Pdk3 mRNAs. n = 4–5; *p≤0.05 vs. ND. doi:10.1371/journal.pone.0058718.g004

**Figure 5. ISO-1 reduced aerobic capacity.** (A) Basal $V_{O2}$ and (B) $V_{CO2}$ measured by indirect calorimetry. (C) Maximal $V_{O2}$ ($V_{O2}$max) obtained from mice subjected to treadmill exercise. (D) Difference between $V_{O2}$ and $V_{O2}$max ($\Delta V_{O2}$) in ND, HFD and ISO-1-treated HFD mice. n = 6–10, *p≤0.05 vs. ND, †p≤0.05 vs. HFD. doi:10.1371/journal.pone.0058718.g005
In conclusion, MIF inhibition by ISO-1 worsened HFD-induced cardiac dysfunction without any effects on metabolic parameters. However, ISO-1 injections in HFD mice led to a reduced mitochondrial biogenesis signaling that may, in turn, lead to an insufficient ATP supply required for cardiac contraction.

Supporting Information

Table S1 Primer sequences used for qPCR.

Table S2 Primer sequences for RT-qPCR.

References

1. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, et al. (2009) Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Vol. 120. 1640–1645. doi:10.1161/CIRCULATIONAHA.109.192644.

2. Ford ES, Giles WH, Dietz WH (2002) Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 287: 356–359.

3. Balkau B, Charles M-A, Drivsholm T, Borch-Johnsen K, Wareham N, et al. (2002) Frequency of the WHO metabolic syndrome in European cohorts, and an alternative definition of an insulin resistance syndrome. Diabetes Metab 28: 364–376.

4. Grundy SM (2008) Metabolic syndrome pandemic. Arterioscler Thromb Vasc Biol 28: 629–636. doi:10.1161/ATVBAHA.107.151092.

5. Dandona P, Aljada A, Ghanim H, Mohanty P, Tripathy C, et al. (2004) Increased plasma concentration of macrophage migration inhibitory factor (MIF) and MIF mRNA in mononuclear cells in the obese and the suppressive...
action of metformin. J Clin Endocrinol Metab 89: 5043–5047. doi:10.2353/jcem.2004.03416.

6. Herder C, Klooß N, Baumert J, Müller M, Klyuschinova N, et al. (2008) Effect of macrophage migration inhibitory factor (MIF) gene variants and MIF serum concentrations on the risk of type 2 diabetes: results from the MONICA/KORA Augsburg Case-Cohort Study, 1984–2002. Diabetologia 51: 276–284. doi:10.1007/s00125-007-0800-3.

7. Kim HR, Lee SA, Kim HJ, Kong MH, Kim YR, et al. (2011) Elevated levels of macrophage migration inhibitory factor in women with metabolic syndrome. Horm Metab Res 43: 642–645. doi:10.1055/s-0031-1283130.

8. Makino A, Nakamura T, Hiranu M, Sato K, Sano K, et al. (2010) High plasma levels of macrophage migration inhibitory factor are associated with adverse long-term outcome in patients with stable coronary artery disease and impaired glucose tolerance or type 2 diabetes mellitus. Atherosclerosis 213: 573–578. doi:10.1016/j.atherosclerosis.2010.09.004.

9. Herder C, Ilig T, Baumert J, Müller M, Klooß N, et al. (2008) Macrophage migration inhibitory factor (MIF) and risk for coronary heart disease: results from the MONICA/KORA Augsburg case-cohort study, 1984–2002. Atherosclerosis 200: 380–388. doi:10.1016/j.atherosclerosis.2007.12.023.

10. Suzuki M, Sugimoto H, Nakagawa A, Tanaka I, Niizuhara J, et al. (1996) Crystal structure of the macrophage migration inhibitory factor from rat liver. Nat Struct Biol 3: 259–266.

11. Bucala R (1994) Identification of MIF as a new putative hormone and macrophage cytokine and its role in endotoxic shock. ImmunoL Lett 45: 23–26.

12. Skurk T, Herder C, Kraft I, Müller-Scholze S, Hauner H, et al. (2005) Effect of hyperglycemia on plasma levels of macrophage migration inhibitory factor. Diabetologia 48: 1464–1467. doi:10.1007/s00125-005-1940-y.

13. Bucala R, Chernik NL, Mackay CR, Kopp PB, Layton KA, et al. (1991) Macrophage migration inhibitory factor. Proc Natl Acad Sci USA 88: 3483–3487.

14. Skurk T, Herder C, Kraft I, Müller-Scholze S, Hauner H, et al. (2005) Production and release of macrophage migration inhibitory factor from human adipocytes. Endocrinology 146: 1006–1011. doi:10.1210/en.2004-0924.

15. Waber G, Calandra T, Roduit R, Haefliger JA, Bonny C, et al. (1997) Insulin secretion is regulated by the glucose-dependent production of islet beta cell macrophage migration inhibitory factor. Proc Natl Acad Sci USA 94: 4762–4767.

16. Garnero LB, Willis MS, Carlson DL, Dimaio JM, White MD, et al. (2003) Macrophage migration inhibitory factor is a cardiac-derived myocardial depressant factor. Am J Physiol Heart Circ Physiol 285: H2500–H2509. doi:10.1152/ajpheart.00432.2003.

17. Morand EF, Leher I, Muñoz S, Haussmann J, Schürmann J, et al. (1987) Macrophage migration inhibitory factor: a new cytokine linking innate and adaptive immunity. J Immunol 138: 1793–1799.

18. Calandra T, Roger T (2003) Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3: 791–800. doi:10.1038/nri1027.

19. Toso C, Emaamaeja MA, Merani S, Shapiro AMJ (2008) The role of macrophage migration inhibitory factor on glucose metabolism and diabetes. Diabetologia 51: 1937–1946. doi:10.1007/s00125-008-0693-5.

20. Miller EJ, Li J, Leng L, McDonald C, Atsumi T, et al. (2008) Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the macrophage. Proc Natl Acad Sci USA 105: 17333–17338. doi:10.1073/pnas.0804230105.

21. Tong C, Morrison A, Yan X, Zhao P, Yeung ED, et al. (2010) Macrophage migration inhibitory factor deficiency augments cardiac dysfunction in Type 1 diabetic murine cardiomyocytes. J Diabetes 2: 267–274. doi:10.1530/JDS.109.017533.

22. Sanchez-Zamora Y, Terrazas LL, Vilches-Flores A, Leal E, Juárez I, et al. (2010) Macrophage migration inhibitory factor is a therapeutic target in treatment of non-insulin-dependent diabetes mellitus. FASEB J 24: 2583–2590. doi:10.1096/fj.09-147066.

23. Cvetkovic I, Al-Abed Y, Miljkovic D, Maksimovic-Ivanic D, Roth J, et al. (2005) Critical role of macrophage migration inhibitory factor activity in experimental autoimmune diabetes. Endocrinology 146: 2942–2951. doi:10.1210/en.2004-1393.

24. Scarpo C, Pagnotta R (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 88: 611–638. doi:10.1152/physrev.00025.2007.

25. Boudina S, Abel ED (2007) Diabetic cardiomyopathy revisited. Circulation 115: 3215–3223. doi:10.1161/CIRCULATIONAHA.106.679957.

26. Fiaccadori E, Bondioli M, Basso D, Gagliardini E, Gualdi P, et al. (2009) Decreased cardiac sarcoplasmic reticulum Ca²⁺-ATPase activity contributes to cardiac dysfunction in streptozotocin-induced diabetic rats. J Physiol Biochem 65: 1–8.

27. Volpi E, Montagna W, Marzari E, Porta C, Pigozzo G, et al. (2007) Sarcoplasmic reticulum Ca²⁺-ATPase activity and skeletal muscle in patients with type 1 diabetes: role of circulating cytokines. J Clin Endocrinol Metab 92: 4454–4461. doi:10.1210/jc.2007-0407.

28. Panza JA, Taddei S, Ghiadoni L, Chiarchiaro M, Conti F, et al. (2010) Endothelial progenitor cells in patients with type 1 diabetes: effect of insulin treatment. J Clin Endocrinol Metab 95: 1960–1964. doi:10.1210/jc.2010-0398.

29. Kameda Y, Ikegami H, Chikahisa M, Takayama N, Endoh Y, et al. (2010) Decreased cardiac sarcoplasmic reticulum Ca²⁺-ATPase activity and skeletal muscle from patients with type 1 diabetes. J Clin Endocrinol Metab 95: 1965–1969. doi:10.1210/jc.2009-1974.