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Mif deficiency promotes adiposity in fructose-fed mice

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Abstract

The macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine involved in inflammation, regulation of energy metabolism and glucocorticoid action. Chronic low-grade inflammation may be caused by fructose intake, contributing to visceral adipose tissue (VAT) dysfunction. Since MIF is a known antagonist of glucocorticoid signaling, and deregulated glucocorticoid signaling can contribute to lipid metabolism disturbances, we hypothesized that altered MIF signaling might underlie fructose-induced adiposity through glucocorticoid action. We analyzed physiological and biochemical parameters, adipose tissue histology, insulin sensitivity and lipid metabolism in WT and MIF^{-/-} C57Bl/6J mice consuming 20% fructose solution for 9 weeks. Glucocorticoid prereceptor metabolism and glucocorticoid receptor (GR) protein level were examined in VAT, together with the expression of glucocorticoid-target genes involved in lipid metabolism. The expression of adipogenic and lipogenic transcriptional regulators peroxisome proliferator-activated receptor gamma (PPARG) and sterol regulatory element-binding protein 1c (SREBP1c) was also assessed. Results showed disturbed insulin sensitivity in all MIF^{-/-} mice, regardless of the diet. Mice on fructose diet had increased energy intake, but increased visceral adiposity and enlarged adipocytes were observed only in fructose-fed MIF^{-/-} mice. Increased VAT corticosterone level and 11 beta-hydroxysteroid dehydrogenase type 1, hexose-6-phosphate dehydrogenase and GR protein levels were observed in the same animals, together with induced expression of examined lipogenic genes and accumulation of PPARG and SREBP1c. In conclusion, the results showed that dietary fructose was associated with increased visceral adiposity through activation of GR-regulated lipogenic genes, but only in the absence of MIF, which set the state of hyperinsulinemia and insulin resistance.

Key Words

- ▶ macrophage migration inhibitory factor
- ▶ insulin resistance
- ▶ fructose
- ▶ glucocorticoids
- ▶ visceral adipose tissue
- ▶ lipid metabolism

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Introduction

The rate of fructose consumption has been increasing worldwide and it is considered to be a contributing factor to the rising prevalence of obesity and metabolic disturbances. There are evidence from human and animal studies supporting the ability of high-fructose diet to upregulate lipid metabolism leading to dyslipidemia, insulin resistance and type 2 diabetes (T2D) (Basciano *et al.* 2005,

Tappy & Le 2010). Excess fructose intake can also contribute to intra-abdominal adiposity and dysfunction of the adipose tissue, resulting in the infiltration of immune cells and development of chronic low-grade inflammation (Monteiro & Azevedo 2010).

Macrophage migration inhibitory factor (MIF) is a pleiotropic ubiquitously expressed cytokine that

regulates proinflammatory response and is associated with numerous inflammatory and autoimmune diseases including T2D and obesity (Yabunaka *et al.* 2000, Church *et al.* 2005, Cvetkovic *et al.* 2005). The elevation of MIF plasma concentration and MIF mRNA level found in obese subjects and animal models was positively associated with elevated plasma free fatty acid (FFA) concentrations and increased BMI (Skurk *et al.* 2005, Finucane *et al.* 2012, Veličković *et al.* 2013). A growing body of evidence indicates that MIF has a role in energy metabolism in the adipose tissue and liver, since its expression in these tissues is regulated by glucose and insulin (Sakaue *et al.* 1999, Morrison & Kleemann 2015). *Mif* deletion may contribute to the development of systemic insulin resistance through deregulation of glucose metabolism (Atsumi *et al.* 2007, Saksida *et al.* 2013, Nikolic *et al.* 2013a). However, the data are still inconclusive, since studies are reporting that *Mif*-knockout mice display improved insulin sensitivity and glucose tolerance (Verschuren *et al.* 2009, Kleemann & Bucala 2010).

Although MIF synthesis and secretion are upregulated by the glucocorticoid hormones (GCs), MIF acts as the counter-regulator of GC anti-inflammatory action to control the magnitude of the immune response (Calandra & Bucala 1997). Systemic hypercortisolemia and overexpression of enzymes converting inactive forms of GCs to active ones, 11-beta-hydroxysteroid dehydrogenase type-1 (11BHSD1) and hexose-6-phosphate dehydrogenase (H6PDH), were previously associated with increased accumulation of visceral adipose tissue (VAT) (Seckl *et al.* 2004, Mariniello *et al.* 2006). GCs affect carbohydrate and lipid metabolism through hormone-dependent transcription regulator, glucocorticoid receptor (GR) (Peckett *et al.* 2011). Depending on nutritional and energy state, GCs can dually affect lipid metabolism. These effects can be opposite to insulin action, since GR directly regulates transcription of lipolytic enzymes, adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (Lee *et al.* 2014). On the other hand, when insulin levels are high, GCs and insulin synergistically increase expression of lipogenic genes, including lipoprotein lipase (*Lpl*), fatty acid synthase (*Fas*), acetyl-CoA carboxylase (*Acc*) and phosphoenolpyruvate carboxykinase (*Pepck*) (Chakravarty *et al.* 2005, Campbell *et al.* 2011, Peckett *et al.* 2011). Besides GR, a number of insulin-regulated transcriptional factors are engaged in lipid metabolism and adipose tissue development. The examples are sterol regulatory element-binding protein 1c (SREBP1c), responsible for triglyceride biosynthesis, as well as

PPARG involved in adipocyte differentiation (Rosen & Spiegelman 2001, Eberlé *et al.* 2004).

Circulating MIF levels and intracellular GCs are shown to be elevated in obese subjects; however, their role and interaction in changes of lipid metabolism and insulin resistance induced by high-fructose diet remain elusive. The aim of this study was to investigate whether genetic deletion of *Mif* results in GC-mediated disturbances of glucose and lipid metabolism in VAT of male mice fed with 20% fructose-enriched diet. Glucose metabolism was assessed through analysis of systemic insulin sensitivity, while lipid metabolism was analyzed at the level of visceral adiposity, VAT histology and the expression of GC-target genes involved in lipogenesis. Besides GR signaling in VAT, the expression of PPARG and SREBP1c, as key transcriptional regulators involved in adipogenesis and lipogenesis, was also examined.

Materials and methods

Animals and treatment

The generation of homozygous *Mif* gene-deficient (*MIF*^{-/-}) mice on C57BL/6J background has been described elsewhere (Fingerle-Rowson *et al.* 2003). Breeder mice, both *MIF*^{-/-} (on a C57BL/6J background) and WT C57BL/6J mice were a kind gift from Dr Christine Metz (Laboratory of Medicinal Biochemistry, The Feinstein Institute for Medical Research, North Shore LIJ Health System, NY) to Dr Stanislava Stosic-Grujicic from the Institute for Biological Research 'Siniša Stanković'. All animals were bred under standard pathogen-free laboratory conditions in the Animal Facility of the Institute. During 9 weeks, mice were kept in a temperature-controlled room (22±2°C) with a 12-h light/darkness cycle (lights on at 07:00h). The 2-month-old animals were divided into four experimental groups (*n*=12 animals per group): WT and *MIF*^{-/-} mice had standard diet (commercial chow and drinking water available *ad libitum*), while WT and *MIF*^{-/-} mice on fructose-rich diet had *ad libitum* access to commercial chow and to 20% fructose solution instead of drinking water (WT FrD and *MIF*^{-/-} FrD, respectively). The detailed composition of the diet was described previously (Veličković *et al.* 2013). The animals were housed 4 per cage, and daily food and fluid intake per cage were measured during 9 weeks. Body mass was measured weekly. Daily energy intake for mice on standard diet was calculated as food weight (g) per cage×11 kJ, while energy intake for mice on fructose diet was calculated as sum of calories ingested as food and fructose solution

(food weight (g) per cage $\times$ 11 kJ + fructose intake (mL) per cage $\times$ 3.44 kJ). All animal procedures were in compliance with the EEC Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research 'Siniša Stanković', University of Belgrade.

Intraperitoneal glucose (IP-GTT) and insulin tolerance tests (IP-ITT)

IP-GTT was performed 3 days before the end of the treatment. Mice were fasted overnight and glucose challenge was given intraperitoneally (2 g/kg) without anesthesia in order to avoid the effect of anesthetic on glucose level and kinetics of glucose disposal. In order to determine glucose concentration, blood samples were taken from the tip of the tail at 0, 15, 30, 60, 90 and 120 min after injection. For IP-ITT, mice were fasted for 4 h prior to the test. Blood glucose was determined at 0, 15, 30, 60, 90 and 120 min after the insulin injection (0.75 IU/kg). The blood glucose concentration was measured using AccuChek Active strips (F. Hoffmann-La Roche AG). The area under the concentration vs time curve (AUC glucose 0–120 min, mmol/L vs min) was calculated by the trapezoidal rule.

Blood sample collection and determination of plasma parameters

Mice were killed by rapid decapitation after overnight fasting. Trunk blood was collected into EDTA-containing tubes. Blood glucose and triglycerides were measured by Accutrend strips (F. Hoffmann-La Roche AG).

Plasma was prepared by low-speed centrifugation (1600g/10 min) and stored at -20°C for subsequent processing. FFAs were determined by the Semi-auto Chemistry Analyzer (Rayto 1904C) using Randox NEFA kit (Randox Laboratories Ltd, Crumlin, UK).

Leptin concentrations were determined by Mouse/Rat Leptin Quantikine ELISA kit according to manufacturer's instructions (R&D Systems). Absorbance at 450 nm (reference 570 nm) was measured spectrophotometrically (Multiskan Spectrum, Thermo, Finland). Leptin concentrations were determined using regression analysis curve-fitting method (Prism, GraphPad Software) and given as pg/mL. The assay sensitivity was 22 pg/mL, while intra-assay and inter-assay CVs were 3.8 and 5.0%, respectively.

Insulin concentration was determined by RIA method, using mouse insulin standards (INEP, Belgrade, Serbia).

The assay sensitivity was 0.6 mIU/L and intra-assay coefficient of variation was 5.24 %.

Isolation of visceral adipose tissue

VAT was excised, washed by 0.9% NaCl solution, weighed and frozen in liquid nitrogen. Excised VAT consisted of gonadal, retroperitoneal and perirenal depots, representing the pooled fat of the intra-abdominal cavity, which was previously correlated with development of insulin resistance and metabolic syndrome, especially in male rodents (Wajchenberg 2000, Bjørndal *et al.* 2011).

For histological analysis, part of the gonadal VAT was excised and fixed in 10% neutral formalin for 24 h, dehydrated in ethanol gradient, cleared in xylene and embedded in paraffin. Gonadal fat pads were chosen for the histological analysis not only because they are the largest and most readily accessible fat pads in mice (Chusyd *et al.* 2016), but also because they are the primary storage depot in C57BL/6J mice, expanding initially in diet-induced obesity (van Beek *et al.* 2015).

Histological analysis of VAT

VAT blocks were sectioned at 10 μm thickness and stained with hematoxylin-eosin. Morphometric analysis was carried on workstation comprising microscope (Olympus) equipped with a charge-coupled device video camera (PixelINK, Ottawa, Canada). The system was controlled by the newCAST software package (Visiopharm Integrator System, version 3.2.7.0, Visiopharm, Denmark). Images were analyzed by Adiposoft software (Galarraga *et al.* 2012). The cell diameter and sectional area were measured using three images per section (20 $\times$ magnification), three sections per animal and five animals per group.

Preparation of cytoplasmic fraction and determination of VAT corticosterone concentrations

For the preparation of cytoplasmic fraction from VAT, tissue was homogenized (w/v=1:1) in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.2, 10% glycerol, 50 mM NaCl, 1 mM EDTA- Na_2 , 1 mM EGTA- Na_2 , 2 mM DTT, protease and phosphatase inhibitors). The cell lysate was filtered through gauze and centrifuged at low speed (2000g, 15 min, 4°C). The resulting supernatant was centrifuged (10,000g, 25 min, 4°C), recentrifuged (150,000g, 90 min, 4°C , Beckman ultracentrifuge) and the final supernatant was used as cytoplasmic fraction. Lowry method was used for the determination of protein concentration (Lowry *et al.* 1951).

VAT corticosterone (CORT) was assessed using Corticosterone High Sensitivity EIA kit according to manufacturer's instructions (Immunodiagnostic Systems LTD, Tyne and Wear, UK). Absorbance at 450 nm (reference 650 nm) was measured spectrophotometrically (Multiskan Spectrum, Thermo, Finland). CORT concentrations were determined using four parameter logistic (4PL) curve-fitting method (Prism, GraphPad Software) and given as ng/mg of protein. The assay sensitivity was 0.17 ng/mL, while intra-assay and inter-assay CVs were 5.9 and 8.9%, respectively.

RNA extraction and reverse transcription

VAT total RNA was isolated using TRIzol (Ambion, Life Technologies). Samples were centrifuged at 12,000g for 15 min at 4°C after homogenization. RNA was precipitated with isopropanol. Qualitative and quantitative evaluation of the isolated RNA was performed on 2% agarose gel and spectrophotometrically (OD 260/280 > 1.8 was considered satisfactory). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions and the cDNAs were stored at -70°C until use.

Real-time PCR

The expression of genes was analyzed using Power SYBR Green PCR Master Mix. Specific primers (Applied Biosystems) were used to amplify *Hsl* selectively: F 5'-GGC TCA CAG TTA CCA TCT CAC C-3', R 5'-GAG TAC CTT GCT GTC CTG TCC-3'; *Atgl*: F 5'-AAC ACC AGC ATC CAG TTC AA-3', R 5'-GGT TCA GTA GGC CAT TCC TC-3'; *Pepck*: F 5'-AAC TGT TGG CTG GCT CTC-3', R 5'-GAA CCT GGC GTT GAA TGC-3'; *Lpl*: F 5'-TTC CAG CCA GGA TGC AAC A-3', R 5'-GGT CCA CGT CTC CGA GTC C-3'; *Acc*: F 5'-GAG AGG GGT CAA GTC CTT CC-3', R 5'-CTG CTG CCG TCA TAA GAC AA-3'; *Fas*: F 5'-TTG CTG GCA CTA CAG AAT GC-3', R 5'-AAC AGC CTC AGA GCG ACA AT-3'. Normalization of cDNA was performed using previously validated (Djordjevic *et al.* 2012) hypoxanthine guanine phosphoribosyl transferase (*Hprt*) as endogenous control (F 5'-TCC TCC TCA GAC CGC TTT T-3', R 5'-CCT GGT TCA TCA TCG CTA ATC-3'). All reactions were performed in triplicate in 25 µL volume containing 20 ng of cDNA template using QuantStudio 3 (Applied Biosystems). Thermal cycling conditions were 2-min incubation at 50°C, 10 min at 95°C followed by 60 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analyses were performed at the end of every experiment to confirm the formation

of a single PCR product. Relative quantification of gene expression was performed using the comparative $2^{-\Delta\Delta Ct}$ method, where ΔCt represents the difference between Ct value of target gene and the endogenous control. The results were analyzed by QuantStudio Design and Analysis Software v1.4.0 (Applied Biosystems) with a confidence level of 95% ($P \leq 0.05$).

Sample preparation

For the preparation of VAT total protein fraction, TRIzol was used according to the manufacturer's instruction. After aqueous phase separation for RNA precipitation, the remaining organic phase was centrifuged at 2000g for 5 min at 4°C after adding ethanol. Protein fraction was precipitated from phenol-ethanol supernatant, using acetone and centrifuged at 12,000g for 10 min at 4°C. The protein pellets were dispersed in 0.3M guanidine hydrochloride in 95% ethanol with 2.5% glycerol by sonication on ice and washed in 0.3M guanidine hydrochloride in 95% ethanol with 2.5% glycerol. After sedimenting the protein by centrifugation at 8000g for 5 min at 4°C, pellets were dispersed in the lysis buffer containing 2.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 50 mM DTT. Samples were stored at -70°C until use.

Western blot analysis

Proteins concentration was measured using Lowry method (Lowry *et al.* 1951) and 60 µg of proteins were subjected to electrophoresis on 7.5% SDS-PAGE. After transfer to PVDF membrane (Immobilon-FL membrane, Millipore), using a Transblot system (Bio-Rad Laboratories), the unbound sites were blocked (1 h with 2% non-fat dry milk) and the membranes were probed with specific primary rabbit polyclonal antibodies for anti-phospho-p38 Tyr 182 (1:750) (sc-7975-R, Santa Cruz Biotechnology), anti-p38 MAPK (1:500) (sc-535, Santa Cruz Biotechnology), anti-GR (1:500) (sc-8992, Santa Cruz Biotechnology), anti-11BHSD1 (1:1000) (ab393364, Abcam), anti-H6PDH (1:1000) (sc-67394, Santa Cruz Biotechnology), anti-SREBP1c (1:500) (sc-366, Santa Cruz Biotechnology) and mouse monoclonal anti-PPARG (1:1000) (sc-7273, Santa Cruz Biotechnology). Monoclonal mouse anti-B-actin antibody (1:10,000) (AC-15, Sigma-Aldrich) was used as a loading control. Blots were developed with secondary ECL anti-rabbit IgG HRP-linked whole antibody (1:10,000) (Amersham Pharmacia Biotech) or with anti-mouse IgG HRP-linked whole antibody (1:20,000) (ab97046, Abcam). Signal was developed using enhanced chemiluminescence

(ECL) and densitometry of protein bands on X-ray films (Kodak, Rochester, USA) was performed by Image J software (NIH).

Statistical analyses

The data are presented as means $\pm$ S.E.M. To determine the effects of *Mif* deficiency and fructose diet, as well as their interaction, two-way ANOVA followed by *post hoc* Tukey test was used. A probability level less than 0.05 was considered statistically significant. Statistical analyses were performed by Statistica 10 Software (Stat Soft. Inc., Tulsa, USA).

Results

Physiological measurements

No difference in food intake was observed between WT and MIF^{-/-} mice on standard diet; however, both groups on fructose diet showed a typical decrease in food intake, paralleled with increased liquid intake (Table 1, $P < 0.001$). As expected, all mice on fructose diet had increased energy intake compared to both WT and MIF^{-/-} animals ($P < 0.001$).

Despite increased energy intake, body mass and weight gain of fructose-fed mice were not significantly different from the animals on the standard diet (Fig. 1A). Interestingly, mass of VAT was higher in *Mif*-deficient mice in comparison to WT animals (Fig. 1B, $P < 0.05$), while fructose-fed MIF^{-/-} mice had significantly more VAT than both WT ($P < 0.001$) and WT mice consuming fructose ($P < 0.01$). However, VAT-to-body mass ratio, taken as an index of visceral adiposity, was significantly increased only in *Mif*-deficient mice on fructose diet in comparison to WT groups on standard and fructose diet (Fig. 1C, $P < 0.001$ and $P < 0.01$, respectively), as well as to MIF^{-/-} mice on standard diet ($P < 0.05$).

Measurements of plasma triglycerides, FFA, leptin and insulin sensitivity parameters

Both plasma triglycerides and FFA were the same in all experimental groups (see Table 1). Leptin concentrations were significantly higher in MIF^{-/-} mice, regardless of the diet, when compared to the WT animals ($P < 0.05$).

As shown in Table 2, MIF^{-/-} mice had significantly higher fasting glucose than WT animals ($P < 0.05$), while in fructose-fed MIF^{-/-} mice glucose level was higher

Table 1 Physiological and biochemical parameters: food, liquid and energy intake, triglycerides and free fatty acids (FFAs).

	WT	WT FrD	MIF ^{-/-}	MIF ^{-/-} FrD	Two-way ANOVA		
					Fructose	<i>Mif</i> deficiency	Interaction
Food intake (g/day/cage)	15.96 $\pm$ 0.60	9.74 $\pm$ 0.45 ^b	16.69 $\pm$ 0.30	11.20 $\pm$ 0.18 ^{b,f}	<0.001	<0.05	NS
Liquid intake (mL/day/cage)	21.26 $\pm$ 0.83	30.32 $\pm$ 0.83 ^b	20.83 $\pm$ 0.40	25.13 $\pm$ 1.01 ^{a,d,e}	<0.001	<0.01	<0.01
Energy intake (kJ/day/cage)	172.25 $\pm$ 5.75	216.40 $\pm$ 6.32 ^b	183.63 $\pm$ 3.30	209.88 $\pm$ 3.52 ^{b,e}	<0.001	NS	NS
Triglycerides (mmol/L)	0.93 $\pm$ 0.04	1.16 $\pm$ 0.08	1.21 $\pm$ 0.09	1.24 $\pm$ 0.10	NS	NS	NS
FFA (mmol/L)	1.83 $\pm$ 0.12	1.78 $\pm$ 0.11	2.03 $\pm$ 0.11	1.94 $\pm$ 0.11	NS	NS	NS
Leptin (pg/mL)	320.44 $\pm$ 79.36	295.31 $\pm$ 44.99	2595.47 $\pm$ 482.88 ^b	1625.06 $\pm$ 443.41 ^{a,c}	NS	<0.001	NS

The data are presented as means $\pm$ S.E.M. ($n = 3$ for food, liquid and energy intake and $n = 12$ animals per group for triglycerides, FFA and leptin). A value of $P < 0.05$ was considered statistically significant. Significant between-groups differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows: ^a $P < 0.05$; ^b $P < 0.001$, all groups vs WT; ^c $P < 0.001$, MIF^{-/-} FrD vs WT FrD; ^d $P < 0.05$; ^e $P < 0.001$, MIF^{-/-} FrD vs MIF^{-/-} FrD; fructose diet; NS, not significant.

Figure 1

Physiological and biochemical parameters. Body mass, VAT mass and histological analysis: Data for body mass and weight gain (A), VAT mass (B) and VAT/body mass ratio (C) are presented as mean $\pm$ s.e.m. ($n = 12$ animals per group). Representative hematoxylin-eosin staining of VAT sections from C57BL/6J mice (WT) (D), WT mice on fructose diet (WT FrD) (E), mice with genetically deleted *Mif* (MIF^{-/-}) (F) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD) (G) (magnification $\times 20$, bar = 100 μ m). Morphometric data on adipocyte cell diameter (H) and area (I) are presented as mean $\pm$ s.e.m. (100 adipocytes per section, three sections per animal and five animals per group). A value of $P < 0.05$ was considered statistically significant. Two-way ANOVA showed significant effect of fructose on VAT mass ($P < 0.01$), VAT/body mass ratio ($P < 0.05$), as well as on the adipocyte cell diameter and area ($P < 0.01$). *Mif* deficiency also had significant effect on both VAT and VAT/body mass ratio ($P < 0.001$), as well as on the adipocyte cell diameter ($P < 0.01$) and area ($P < 0.05$). Significant between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all groups vs WT; # $P < 0.05$, ## $P < 0.01$, MIF^{-/-} FrD vs WT FrD; † $P < 0.05$, MIF^{-/-} FrD vs MIF^{-/-}. Intraperitoneal tests of glucose and insulin tolerance IP-GTT (J) and IP-ITT (K): After determination of fasting glucose concentrations, animals were challenged with intraperitoneal injection of glucose (2 g/kg) or insulin (0.75 IU/kg). Blood glucose was measured 15, 30, 60, 90, and 120 min after injection, and glucose concentration vs time was plotted for C57BL/6J mice (WT), WT mice on fructose diet (WT FrD), mice with genetically deleted *Mif* (MIF^{-/-}) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD). A value of $P < 0.05$ was considered statistically significant. Data are presented as mean $\pm$ s.e.m. ($n = 12$ animals per group). Significant between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all groups vs WT; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, MIF^{-/-} FrD vs WT FrD; † $P < 0.05$, MIF^{-/-} FrD vs MIF^{-/-}.

only in comparison to WT mice consuming fructose ($P < 0.01$). Increased insulin was present in MIF^{-/-} mice in comparison to WT ($P < 0.05$), while MIF^{-/-} mice on fructose diet had higher insulin level than WT mice on both standard ($P < 0.01$) and fructose diet ($P < 0.05$).

Insulin sensitivity was estimated by IP-GTT and IP-ITT tests and the results showed that both fructose diet and *Mif* deficiency contributed to its disturbance (Table 2). Namely, MIF^{-/-} mice showed worse profiles for both GTT and ITT AUC values compared to WT animals

Table 2 Insulin sensitivity parameters: fasting glucose, insulin, IP-GTT glucose AUC and IP-ITT glucose AUC.

	WT	WT FrD	MIF ^{-/-}	MIF ^{-/-} FrD	Two-way ANOVA	
					Fructose	Mif deficiency Interaction
Glucose (mmol/L)	5.04 ± 0.24	4.53 ± 0.29	5.92 ± 0.13 ^a	5.52 ± 0.19 ^e	<0.05	<0.001 NS
Insulin (mmol/L)	1.68 ± 0.16	2.94 ± 0.53	6.28 ± 0.76 ^a	8.09 ± 3.38 ^{b,d}	<0.01	<0.05 NS
IP-GTT glucose AUC	1603.13 ± 38.79	1906.31 ± 73.43	2199.00 ± 213.68 ^c	2626.50 ± 72.96 ^{c,f,g}	<0.01	<0.001 NS
IP-ITT glucose AUC	9182.4 ± 403.0	8899.6 ± 426.6	10,945.3 ± 303.6 ^a	11,259.7 ± 586.1 ^{a,f}	NS	<0.001 NS

The data are presented as means ± S.E.M. (*n* = 12 animals per group). A value of *P* < 0.05 was considered statistically significant. Significant between-groups differences obtained from two-way ANOVA followed by *post hoc* Tukey test are given as follows: ^a*P* < 0.05; ^b*P* < 0.01; ^c*P* < 0.001, all groups vs WT; ^d*P* < 0.05; ^e*P* < 0.01; ^f*P* < 0.001, MIF^{-/-} FrD vs WT FrD; ^g*P* < 0.05, MIF^{-/-} FrD vs MIF^{-/-}.

FrD, fructose diet; NS, not significant.

(Fig. 1J and K, *P* < 0.001). While fructose feeding worsened glucose tolerance in both genotypes (Fig. 1J and Table 2, *P* < 0.001), it had no impact on ITT AUC values beyond the effect of *Mif* deficiency (Fig. 1K and Table 2).

Histological and morphometrical analysis of VAT

VAT histological analysis revealed large adipocytes in MIF^{-/-} mice on fructose diet (Fig. 1D, E, F, G, H and I). Morphometrical analysis showed that both adipocyte cell diameter and area (Fig. 1H and I) were significantly increased in fructose-fed MIF^{-/-} mice in comparison to both WT mice on standard (*P* < 0.01) and fructose diet (*P* < 0.05), as well as in comparison to *Mif*-deficient mice on standard diet (*P* < 0.05).

Signaling pathway of p38 kinase in VAT

Elevation of total p38 levels were observed in both fructose-fed groups, as well as in standard-fed MIF^{-/-} mice vs WT animals (Fig. 2, *P* < 0.001). Also, MIF^{-/-} animals on fructose diet had elevated total p38 protein level compared to the WT on the same dietary regimen (*P* < 0.001), while the level of this protein was decreased in comparison to MIF^{-/-} animals on standard diet (*P* < 0.001). On the other hand, a significant increase of phosphorylated p38, as well as the ratio of phosphorylated to total p38 level, was observed in all fructose-fed animals (Fig. 2, *P* < 0.05), which indicates the impact of fructose diet, without the effect of the genotype.

Glucocorticoid prereceptor metabolism and signaling

VAT glucocorticoid signaling was examined at the level of prereceptor metabolism (protein levels of 11BHSD1 and H6PDH enzymes and intracellular level of CORT) and at GR protein level. As shown in Fig. 3A and B, an increase of 11BHSD1 and H6PDH was observed in MIF^{-/-} animals on fructose diet in respect to WT (*P* < 0.001 and *P* < 0.01, respectively) and MIF^{-/-} mice on standard diet (*P* < 0.01).

In line with enhanced prereceptor metabolism, increased VAT CORT (Fig. 3C) was observed in fructose-fed MIF^{-/-} mice in comparison to both normally fed and fructose-fed WT mice (*P* < 0.05), as well as in comparison to standard-fed MIF^{-/-} animals (*P* < 0.01). In addition, GR protein level was elevated only in fructose-fed MIF^{-/-} mice compared to WT group (Fig. 3D, *P* < 0.05).

Lipid metabolism in VAT

The results showed changes in the expression of GR-target genes involved in VAT lipid metabolism in fructose-fed

Figure 2

Phosphorylated and total p38 kinase and their ratio in the VAT. Representative Western blots and relative quantification of total and phospho-p38 kinase and their ratio in the total protein extract of VAT from C57BL/6J mice (WT), WT mice on fructose diet (WT FrD), mice with genetically deleted *Mif* (MIF^{-/-}) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD). Immunoreactivities of total and phosphorylated p38 are normalized to Beta-actin as loading control and the data are presented as mean $\pm$ S.E.M. ($n = 12$ animals per group). A value of $P < 0.05$ was considered statistically significant. Two-way ANOVA showed that total p38 protein level was significantly affected by *Mif* deficiency ($P < 0.001$) and its interaction with fructose diet ($P < 0.001$). Phosphorylation and activity of p38 was significantly affected only by fructose ($P < 0.001$). Significant between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, *** $P < 0.001$, all groups vs WT; ### $P < 0.001$, MIF^{-/-} FrD vs WT FrD; † $P < 0.05$, †† $P < 0.001$, MIF^{-/-} FrD vs MIF^{-/-}.

MIF^{-/-} mice (Fig. 4). Namely, the increase of *Fas* was detected in VAT of these animals in comparison to WT animals irrespective of the diet ($P < 0.01$), and also in comparison to MIF^{-/-} mice on standard diet ($P < 0.05$). *Acc* mRNA level was also increased in MIF^{-/-} mice in comparison to WT ($P < 0.001$), fructose-fed WT ($P < 0.01$) and normally fed MIF^{-/-} ($P < 0.05$) mice. *Pepck* mRNA level was significantly increased in *Mif*-deficient mice on fructose diet in comparison to all other experimental groups ($P < 0.001$). However, mRNA level for the main

lipolytic enzyme *Atgl* was also significantly higher in fructose-fed MIF^{-/-} mice compared to both WT groups (standard diet: $P < 0.001$; fructose diet: $P < 0.01$), as well as compared to normally fed MIF^{-/-} ($P < 0.01$). Interestingly, the level of *Hsl* mRNA was unchanged (Fig. 4). In addition, *Lpl* mRNA was increased in fructose-fed WT mice, and in both MIF^{-/-} mice on standard and fructose diet in comparison to the WT animals ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively), but also in MIF^{-/-} mice compared to fructose-fed MIF^{-/-} animals ($P < 0.01$).

Figure 3

Glucocorticoid prereceptor metabolism and signaling. Representative Western blots and relative quantification of 11BHSD1 (A), H6PDH (B) and GR (D) protein levels and CORT concentration (C) in total protein extract of VAT from C57BL/6J mice (WT), WT mice on fructose diet (WT FrD), mice with genetically deleted *Mif* (MIF^{-/-}) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD). Data are presented as mean $\pm$ S.E.M. ($n = 12$ animals per group). A value of $P < 0.05$ was considered statistically significant. Two-way ANOVA showed significant effects of fructose ($P < 0.01$) and *Mif* deficiency ($P < 0.05$) for both 11BHSD1 and H6PDH protein level, while GR was affected only by fructose treatment ($P < 0.01$). CORT concentration in VAT was affected by fructose diet ($P < 0.05$), as well as by its interaction with *Mif* deficiency ($P < 0.05$). Significant between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all groups vs WT; # $P < 0.05$, MIF^{-/-} FrD vs WT FrD; †† $P < 0.01$, MIF^{-/-} FrD vs MIF^{-/-}.

Figure 4

Relative levels of mRNA for genes involved in VAT lipid metabolism. Relative quantification *Hsl*, *Atgl*, *Pepck*, *Lpl*, *Fas* and *Acc* mRNA level, normalized to *Hprt1* housekeeping gene, in the VAT of C57BL/6j mice (WT), WT mice on fructose diet (WT FrD), mice with genetically deleted *Mif* (MIF^{-/-}) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD). Data are presented as mean $\pm$ S.E.M. ($n = 12$ animals per group) of the triplicate analysis of RNA samples. A value of $P < 0.05$ was considered statistically significant. According to two-way ANOVA the effect of fructose was detected for *Atgl* ($P < 0.001$), *Pepck* ($P < 0.001$), *Fas* ($P < 0.05$) and *Acc* ($P < 0.05$) mRNA levels. *Mif* deficiency significantly affected expression of *Atgl* ($P < 0.01$), *Pepck* ($P < 0.001$), *Lpl* ($P < 0.001$), *Fas* ($P < 0.01$) and *Acc* ($P < 0.01$). Interaction between fructose and the absence of MIF was detected for *Atgl* ($P < 0.01$), *Pepck* ($P < 0.001$), *Lpl* ($P < 0.001$), *Fas* ($P < 0.05$) and *Acc* ($P < 0.05$) mRNA levels. Significant between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, all groups vs WT; ## $P < 0.01$, ### $P < 0.001$, MIF^{-/-} FrD vs WT FrD; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$, MIF^{-/-} FrD vs MIF^{-/-}.

Protein levels of key transcriptional regulators involved in adipogenesis and lipogenesis, PPARG and SREBP1c, were determined in VAT total protein extract (Fig. 5). PPARG was increased in MIF^{-/-} mice on fructose diet, as compared to WT and MIF^{-/-} animals ($P < 0.05$), while SREBP1c protein level was significantly increased in

the same group of animals compared to WT ($P < 0.001$) and both fructose-fed WT and MIF^{-/-} ($P < 0.01$).

Discussion

Many studies have indicated the importance of MIF for glucose homeostasis, however, its role in metabolic disturbances is less clear. In our study, *Mif*-deficient mice were hyperglycemic, hyperinsulinemic and had impaired insulin sensitivity. On the other hand, energy-rich fructose diet in MIF^{-/-} mice resulted in increased visceral adiposity, underlain by stimulated expression of lipogenic genes and the activation of key transcriptional regulators of VAT adipogenesis and lipogenesis, PPARG and SREBP1c. These metabolic perturbations in *Mif*-deficient mice might be a result of enhanced VAT glucocorticoid signaling in the hyperinsulinemic setting. The deregulated glucocorticoid signaling in the absence of MIF possibly contributes to the stimulation of lipogenic program under the conditions of caloric overload induced by high-fructose diet.

Increasing evidence suggests that in obesity, MIF is released by the adipose tissue and that it is also involved in inflammatory and metabolic processes underlying the development of T2D. Namely, it was already shown that MIF influences glucose metabolism at several levels, including secretion of insulin from pancreas (Waeber et al. 1997), modulation of skeletal muscle and adipose tissue glycolysis, as well as adipocyte insulin resistance mediated by TNFA (Toso et al. 2008). In our study the hallmarks of systemic insulin resistance were observed in all MIF^{-/-} mice, regardless of the diet regimen and

Figure 5

Transcriptional regulators of VAT lipid metabolism. Representative Western blots and relative quantification of PPARG (A) and SREBP1c (B) total protein levels in the VAT of C57BL/6j mice (WT), WT mice on fructose diet (WT FrD), mice with genetically deleted *Mif* (MIF^{-/-}) and MIF^{-/-} mice on fructose diet (MIF^{-/-} FrD). Beta-actin was used as a loading control. Data are presented as mean $\pm$ S.E.M. ($n = 12$ animals per group). A value of $P < 0.05$ was considered statistically significant. Two-way ANOVA showed significant effects of fructose diet on both PPARG ($P < 0.01$) and SREBP1c ($P < 0.001$), while the deficiency of *Mif* affected only SREBP1c protein level ($P < 0.01$). Between-group differences from *post hoc* Tukey test are given as follows: * $P < 0.05$, *** $P < 0.001$, all groups vs WT; ## $P < 0.01$, MIF^{-/-} FrD vs WT FrD; † $P < 0.05$, †† $P < 0.01$, MIF^{-/-} FrD vs MIF^{-/-}.

adiposity. Namely, all *Mif*-deficient mice exhibited hyperglycemia, hyperinsulinemia and impaired glucose and insulin tolerance. However, other authors revealed different results on the role of MIF in the regulation of glucose homeostasis. Verschuren *et al.* (2009) reported unaltered adiposity, decreased fasting insulin and glucose levels and improved glucose tolerance and insulin sensitivity in *Mif*-deficient LDLR^{-/-} mice. In consistence with this, Finucane *et al.* (2014) demonstrated that obese MIF^{-/-} mice are partially protected from insulin resistance evoked by high-fat diet, since both GTT and ITT AUC values were significantly lower in these mice compared to the WT animals. On the other hand, there are studies showing that MIF^{-/-} mice exhibit disturbed glucose homeostasis and signs of obesity. Serre-Beinier *et al.* (2010) showed that the absence of MIF led to impaired glucose tolerance, hyperinsulinemia and increased body fat mass in mature mice compared to the WT. Interestingly, Nikolic *et al.* (2013b) demonstrated that *Mif* deficiency evokes hyperglycemia and blunted glucose tolerance, but the animals had normal insulin levels and remain responsive to exogenous insulin administration. In addition, the same group of authors showed that insulin resistance appears in MIF^{-/-} mice, but after the introduction of high-fat diet (Saksida *et al.* 2012). These contradictory findings point to the complexity of MIF's role in glucose and energy metabolism, which could clearly be diet and age dependent. Also, human studies suggest that *Mif* gene polymorphism contributes to abdominal obesity in a sex-specific manner, since it is related to higher T2D risk in men alone (Coban *et al.* 2015). Our results confirmed the presence of glucose intolerance in MIF^{-/-} mice, which was further aggravated by fructose diet. Furthermore, we have also observed impaired insulin sensitivity in these animals, but it was not additionally affected by fructose diet. One of the possible reasons for such a discrepancy in the results from different studies could be found in the age of experimental animals. It was already shown that mature adult mice, like those used in the present study, are more prone to insulin resistance and obesity than the young ones (Serre-Beinier *et al.* 2010).

Besides regulating glucose metabolism, MIF may also be linked to disturbed energy metabolism under caloric overload (Finucane *et al.* 2014, Kim *et al.* 2015). Thus one study reported that *Mif* deficiency in C57BL/6 mice kept on high-fat diet has adverse effects on glucose tolerance and promotes weight gain, while adipose tissue inflammation was reduced in the absence of MIF (Heinrichs *et al.* 2014). The present study demonstrated increased visceral adiposity and adipocyte hypertrophy only in MIF^{-/-} mice

on fructose diet, which points out the importance of MIF in the development of obesity induced by fructose feeding. Possible mechanism underlying this finding could be found in plasma leptin levels. Taking into account that our *Mif*-deficient mice had higher insulin levels and that increased insulin levels directly correlate with plasma leptin levels (Askari *et al.* 2010) and expanding adipose tissue (Makki *et al.* 2013), it is possible that increased VAT mass in MIF^{-/-} animals contributes to the elevation of circulatory leptin levels. Indeed, in the present study both standard-fed and fructose-fed *Mif*-deficient mice had increased leptin levels compared to the WT ones, which is in accordance with the study by Saksida *et al.* (2012) that also showed increased leptin levels in high-fat fed MIF^{-/-} mice compared to the WT ones, at the time when weight gain between the two strains was observed.

The other important hormonal factors interfering with MIF activity are the GCs. It was previously established that their physiological concentrations could increase MIF expression and secretion, but there are also evidence showing *vice versa*. Namely, MIF can limit GCs effects, most likely through p38 phosphorylation, while the absence of endogenous MIF is associated with increased sensitivity to GCs through decreased phosphorylation of p38 MAPK (Calandra *et al.* 1995, Aeberli *et al.* 2006a,b). Proinflammatory p38 kinase is activated by oxidative stress and inflammation in 3T3-L1 cells and human adipocytes (Merkel *et al.* 2002, Chuang *et al.* 2010, Vazquez Prieto *et al.* 2015, Cheng *et al.* 2017), and fructose diet is known to enhance the production of proinflammatory cytokines and oxidative stress mediators in rodent visceral adipose tissue (Kovačević *et al.* 2016, Pektas *et al.* 2016). Our results showed that fructose diet indeed led to the elevation in both total and phospho p38 protein levels in WT and *Mif*-deficient mice. However, p38 activity (represented as the ratio of phospho-p38 to total p38) was increased only in fructose-fed mice, which implies that increase of p38 activation is independent of *Mif* deficiency.

Indeed, VAT CORT was elevated in these animals, most likely as a result of enhanced glucocorticoid prereceptor metabolism, as revealed by elevated 11BHSD1 and H6PDH protein levels in VAT of fructose-fed MIF^{-/-} mice. These results are in accordance with human and animal studies showing that fructose can elevate 11BHSD1 gene expression in VAT (London & Castonguay 2011), which can further increase visceral adiposity and have deleterious metabolic effects (Walker 2006, Atsumi *et al.* 2007, Nikolic *et al.* 2013a). As expected, increased VAT CORT and glucocorticoid prereceptor metabolism were accompanied with elevated GR protein level in VAT of

fructose-fed MIF^{-/-} mice. However, Nikolic *et al.* (2013b) reported lower GR expression in the adipose tissue and elevated plasma CORT, while Conboy *et al.* (2011) found unchanged plasma CORT in *Mif*-deficient mice. Although there is evidence that elevated glucocorticoids in *Mif*-deficient mice are involved in the impaired glucose tolerance and immunosuppression (Nikolic *et al.* 2013b), their effects on VAT lipid metabolism are still largely unknown.

In the adipose tissue, GCs can modulate both lipogenesis and lipolysis (Wang *et al.* 2012). However, direction of these effects on adipose tissue lipid storage crucially depends on the nutritional and/or hormonal conditions (Kleemann & Bucala 2010, Peckett *et al.* 2011, Saksida *et al.* 2013). GCs can increase the expression of numerous genes involved in lipogenesis and fat deposition, acting synergistically with supraphysiological insulin levels (Campbell *et al.* 2011). In the present study, the main resultant of fructose consumption combined with *Mif* deficiency was the activation of glucocorticoid-regulated enzymes involved in VAT *de novo* lipogenesis. Namely, the significant increase of *Fas* and *Acc* mRNA levels was observed in VAT of fructose-fed MIF^{-/-} mice, which are both critically involved in *de novo* lipogenesis (Strable & Ntambi 2010). They are under direct transcriptional control of SREBP1c (Mandard & Kersten 2006), which is known to be regulated by both GCs and insulin (Kim *et al.* 1998, Foretz *et al.* 1999, Ayala-Summano *et al.* 2013). Indeed, SREBP1c was significantly increased in the VAT of fructose-fed MIF^{-/-} mice, which was consistent with the observed hypertrophic state of adipocytes. Yet, another transcriptional regulator involved in adipogenesis, PPARG, was also significantly increased in the VAT of the same animals. Previous studies, investigating the role of MIF in adipogenesis, showed that MIF^{-/-} cells had elevated PPARG expression during adipocyte differentiation (Atsumi *et al.* 2007). However, in our study, significant effect on PPARG protein level was observed only when MIF^{-/-} mice were fed with fructose, while it was not changed either in MIF^{-/-} mice on standard diet or in the fructose-fed WT mice, which implies that fructose-rich diet generates both adipogenesis and hypertrophy only in the absence of *Mif* and in the presence of enhanced glucocorticoid signaling. On the other hand, in the fructose-fed MIF^{-/-} mice, glucocorticoid-mediated lipolysis was not observed. Although *Atgl* expression was increased, it was not accompanied with increased plasma FFA release, possibly due to increased *Pepck* and *Lpl* expression, known to be involved in FFA re-esterification in the adipose tissue (Merkel *et al.* 2002). These results

shed new light on the role of MIF in the diet-induced obesity, where *Mif* deficiency promotes visceral adiposity under the conditions of energy overload.

In conclusion, the present study showed that *Mif* deficiency is important for the development of glucose and insulin resistance. The metabolic stress, in the form of energy-rich fructose diet, led to visceral adiposity and adipocyte hypertrophy in *Mif*-deficient mice, concomitantly with stimulated adipose tissue lipogenesis. We assume that these harmful effects of *Mif* deficiency might be conducted through the enhanced glucocorticoid prereceptor metabolism and glucocorticoid-facilitated lipogenesis in the VAT, a proposal deserving further exploration. In that case, *Mif* deficiency might be a crucial factor for glucocorticoid-mediated adiposity in response to fructose-enriched diet, providing a setting for hyperinsulinemia and insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Ljupka Gligorovska conducted the experiments, analyzed the data and wrote the paper. Biljana Bursać provided experimental help, Sanja Kovačević performed histological analyses. Nataša Veličković and Gordana Matić revised the paper before submission. Ana Djordjević designed the study and revised the paper before submission.

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