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ORIGINAL ARTICLE

Mitofusin-2 ameliorates high-fat diet-induced insulin resistance in liver of rats

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Abstract

AIM: To investigate the effects of mitofusin-2 (MFN2) on insulin sensitivity and its potential targets in the liver of rats fed with a high-fat diet (HFD).

METHODS: Rats were fed with a control or HFD for 4 or 8 wk, and were then infected with a control or an MFN2 expressing adenovirus once a week for 3 wk starting from the 9th wk. Blood glucose (BG), plasma insulin and insulin sensitivity of rats were determined at end of the 4th and 8th wk, and after treatment with different amounts of MFN2 expressing adenovirus (10^8, 10^9 or 10^{10} vp/kg body weight). BG levels were measured by Accu-chek Active Meter. Plasma insulin levels were analyzed by using a Rat insulin enzyme-linked immunosorbent assay kit. Insulin resistance was evaluated by measuring the glucose infusion rate (GIR) using a hyperinsulinemic euglycemic clamp technique. The expression or phosphorylation levels of MFN2 and essential molecules in the insulin signaling pathway, such as insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), protein kinase beta (AKT2) and glucose transporter type 2 (GLUT2) was assayed by quantitative real-time polymerase chain reaction and Western-blotting.

RESULTS: After the end of 8 wk, the body weight of rats receiving the normal control diet (ND) and the HFD was not significantly different ($P > 0.05$). Compared with the ND group, GIR in the HFD group was significantly decreased ($P < 0.01$), while the levels of BG, triglycerides (TG), total cholesterol (TC) and insulin in the HFD group were significantly higher than those in the ND group ($P < 0.05$). Expression of MFN2 mRNA and protein in liver of rats was significantly down-regulated in the HFD group ($P < 0.01$) after 8 wk of HFD feeding. The expression of INSR, IRS2 and GLUT2 were down-regulated markedly ($P < 0.01$). Although there were no changes in PI3K-P85 and AKT2 expression, their phosphorylation levels were decreased significantly ($P < 0.01$). After intervention with MFN2 expressing adenovirus for 3 wk, the expression of MFN2 mRNA and protein levels were up-regulated ($P < 0.01$). There was no difference in body weight of rats between the groups. The levels of BG, TG, TC and insulin in rats were lower than those in the Ad group ($P < 0.05$), but GIR in rats infected with Ad-MFN2 was significantly increased ($P < 0.01$), compared with the Ad group. The expression of INSR, IRS2 and GLUT2 was increased, while phosphorylation levels of PI3K-P85 and AKT2 were increased ($P < 0.01$), compared with the Ad group.

CONCLUSION: HFDs induce insulin resistance, and this can be reversed by MFN2 over-expression targeting the insulin signaling pathway.

Key words: Mitofusin-2; High-fat diet; Insulin resistance; Insulin pathway; Liver

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INTRODUCTION

With obesity and diabetes reaching epidemic proportions worldwide, the role of insulin resistance and its consequences are attracting much attention. Insulin resistance is a critical mechanism of type 2 diabetes mellitus (T2DM) and of predisposing conditions for T2DM such as obesity and metabolic syndrome[1]. Chronic excess energy consumption has been shown to contribute to hyperinsulinemia and insulin resistance. The liver plays a critical role in energy metabolism and is a major insulin target organ responsible for glucose homeostasis[2]. In the liver, insulin acts through a complex signaling network and functions as an important regulator of carbohydrate and lipid homeostasis. Deficiency in insulin signaling may cause insulin resistance and subsequently lead to systemic insulin resistance and T2DM[3,4]. Specific members of the suppressor of cytokine signaling (SOCS) family of proteins are now thought to play a role in the development of insulin resistance owing to their ability to inhibit insulin signaling pathways. Work with hepatocyte-specific suppressor of cytokine signaling 3 (SOCS3)-deficient (L-SOCS3 cKO) mice, reveals that hepatic SOCS3 is a mediator of insulin resistance in the liver[5]. Therefore, it is of significance to identify the mechanism of insulin resistance and improve the function of the insulin signaling network for the cure of insulin resistance.

Mitochondria generate energy and play central roles in cell energy metabolism[6]. Studies have shown that insulin resistance states such as T2DM or obesity were correlated with a decrease in mitochondria number and function[7,8]. Fusion of mitochondria constitutes an important step in the regulation of mitochondrial morphology and function[9,10]. Mitofusin-2 (MFN2) encodes a mitochondrial membrane GTPase which participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network[11]. MFN2 plays a central role in mitochondrial metabolism and may be associated with metabolic diseases such as obesity and T2DM[12-14].

Previous studies revealed that a high-fat diet (HFD) induced insulin resistance[15] and MFN2 could play a important role in development of insulin resistance[12,16,17]. However, the role of MFN2, a key factor for mitochondrial function and energy metabolism, in liver insulin resistance and the insulin signaling pathway should be further elucidated. In this study, we established an HFD-induced insulin resistance model in rats and used an MFN2 expressing adenovirus to investigate the mechanism by which MFN2 ameliorates HFD-induced insulin resistance.

MATERIALS AND METHODS

Animal care and grouping

Male Wistar rats about 60-80 g (4 wk old) were housed in wire bottom cages to prevent coprophagia. The environment was controlled in terms of light (12:12-h light-dark cycle starting at 6:00 AM), humidity, and room temperature (20-23 ℃). Except for pretest overnight fasting and the immediate postoperative period, animals had free access to water and chow. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Centre for Gerontology and Geriatrics of Hebei Province in China.

Seven days after their arrival, rats were randomly divided into 9 groups (n = 6), as is schematically represented in Figure 1. In brief, rats were fed with a normal control diet (ND) or an HFD for 4 or 8 wk, and then some groups were infected with Ad-MFN2 or empty Ad adenovirus or PBS control once a week, for 3 wk. The HFD consisted of 59.8% fat, 20.1% protein, and 20.1% carbohydrate (kcal). The normal rodent chow diet contained 10.3% fat, 24.2% protein, and 65.5% carbohydrate (kcal). The MFN2 expressing adenovirus Ad-MFN2 and the empty control adenovirus Ad were obtained from Dr. Zhang, Hebei Medical University[18].

Blood samples were collected from the abdominal aorta. Blood glucose (BG) levels were measured by Accuchek Active Meter (ACCU-CHEK® Active; Roche, Germany) and insulin levels were analyzed using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem. Inc, United States). The liver tissue samples of rats were taken immediately and kept at -80 ℃ after being quickly frozen in liquid nitrogen.

Euglycemic hyperinsulinemic clamp

Hyperinsulinemic clamp studies were performed as previously described[19]. Rats were under general anesthesia (3% pelltobarbitalum natricum, 60 mg/kg, intraperitoneally), and catheters were inserted into the right jugular vein and the thoracic aorta of rats and exteriorized from the back of the neck subcutaneously. At the end, the catheters were flushed with isotonic saline containing heparin (50 U/mL). Rats were allowed to fully recover for a minimum of 3 d and only those that had lost less than 5% of their preoperative weights were used. Euglycemic-hyperinsulinemic clamp tests were performed on fasted, awake, and unrestrained animals. Insulin was infused at 4 mU/(kg·min) through the jugular vein catheter for 0-90 min. Glucose concentrations were clamped at euglycemic levels by a variable rate infusion of 30% glucose. BG levels were monitored with a glucometer (ACCU-CHEK® Active; Roche), and glucose infusion rates (GIR) were adjusted every 5-10 min as needed. A stable GIR was obtained within about 60 min after in-

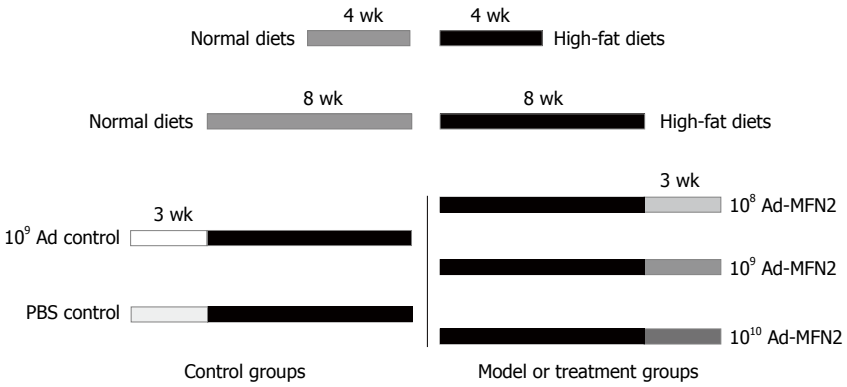

Figure 1 Schematic presentation of rat groups. Rats were fed with control or high-fat diets for 4 or 8 wk, and then some groups were infected with Ad-mitofusin 2 (MFN2) or empty Ad adenovirus or phosphate-buffered saline (PBS) control once a week for 3 wk.

Table 1 Primer sequences for quantitative polymerase chain reaction

Gene	bp	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank No.
GAPDH	120	TGAACGGGAAGCTCACTGG	GCTTCACCACCTTCTTGATGTC	NM_017008
MFN2	160	AGCGTCCTCTCCCTCTGACA	TTCCACACCACTCCTCCGAC	NM_130894
INSR	135	TTTGCCCAACCATCTGTAAG	GACCATCCAGGTAGAAGTTTCG	NM_017071.1
IRS2	81	TCTCTGGCAGTTCAGGTCG	AGTCCTCTGGGTAAGGGTTG	NM_001168633
PI3K	135	GCCTGCTCTGTAGTGGTAGATG	GGAGGTGTGTTGGTAATGTAGC	NM_013005.1
AKT2	79	CTGAGATGATGGAGGTAGCG	CCGAGGAGTTTGAGATAATCG	NM_017093.1
GLUT2	80	AGCACATACGACACCAGACG	CAGACAGAGACCAGAGCATAGTG	NM_012879
SOCS3	148	TCACCCACAGCAAGTTTCC	ACGGCACTCCAGTAGAATCC	NM_053565.1

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MFN2: Mitofusin-2; INSR: Insulin receptor; IRS2: Insulin receptor substrate 2; PI3K: Phosphoinositide-3-kinase; AKT2: Protein kinase beta; GLUT2: Glucose transporter type 2; SOCS3: Suppressor of cytokine signaling 3.

sulin infusion and maintained thereafter. At steady state, mean GIR was normalized to body weight.

Real-time polymerase chain reaction analysis

Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions. Equal amounts of RNA were used to synthesize first strand cDNA (Promega, United States), and quantitative real-time polymerase chain reaction (RT-PCR) was performed on an ABI PRISM 7300 PCR System (Applied Biosystems, United States) using Syber Green I GoTaq® qPCR Master Mix (Promega, United States). PCR was performed as: one cycle at 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 15 s, 58 ℃ for 20 s and 72 ℃ for 30 s. Then PCR products were analyzed by melting curve to confirm the specificity of amplification. The PCR primer sequences are shown in Table 1. mRNA expression of target genes was normalized to the internal reference gene glyceraldehyde 3-phosphate dehydrogenase. The relative expression of target genes was obtained using SDS v1.3.2 software linked with the PCR machine.

Western blotting

Protein samples were prepared with lysis buffer (10 mL/L Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, pH 8.0, 0.2 mmol/L Na_3VO_4, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 5 mL/L NP-40). Equal amounts of protein were separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and electrotransferred to polyvinylidene difluoride membranes, and were then blocked with 5 g/L bovine serum albumin for 2 h at room temperature. Membranes were incubated with appropriate diluted primary antibodies of MFN2, insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K-P85), p-PI3K-P85, AKT2, p-AKT2, glucose transporter type 2 (GLUT2) or β-actin (all from Santa Cruz or Cell Signaling Technology, United states) respectively overnight at 4 ℃, and then with the respective secondary antibody for 2 h. Proteins were detected with the enhanced chemiluminescence detection system. β-actin served as an internal control protein. The experiments were replicated three times.

Statistical analysis

Data were shown as mean ± SD. One-way analysis of variance was used to determine statistically significant differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

HFD decreased insulin sensitivity in rats

As shown in Figure 2, fasting BG and plasma insulin lev-

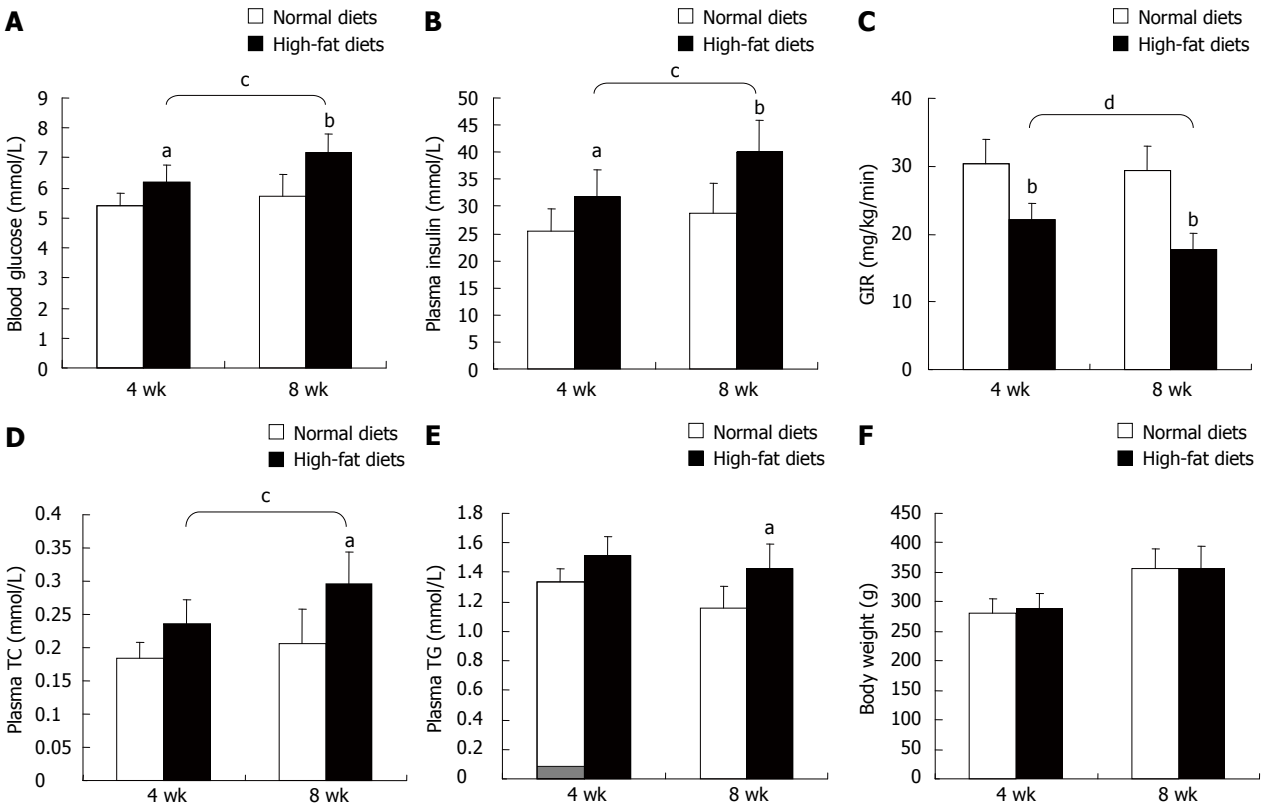

Figure 2 High-fat diets resulted in insulin resistance in rats (n = 6). Rats were fed with high-fat diets or normal control diets for 4 wk or 8 wk. A: The level of blood glucose; B: The level of plasma insulin. C: The values of glucose infusion rate (GIR) were used to assess insulin sensitivity of rats assayed by hyperinsulinemic euglycemic clamping; D: The level of plasma total cholesterol (TC); E: The level of plasma triglyceride (TG); F: The body weight. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* normal diets; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* 4 wk.

els of rats increased after 4 or 8 wk HFD feeding, while GIR, a more sensitive indicator for insulin sensitivity, decreased markedly. The levels of plasma total cholesterol (TC) and triglycerides (TG) were higher than those in the ND group at 4 and 8 wk. Greater changes in these values were seen at 8 wk than 4 wk, while the body weights of rats in each group were not significantly different at 4 or 8 wk.

HFD inhibited the expression of MFN2 and insulin signaling pathway factors and their phosphorylation levels

MFN2 is a key factor for energy metabolism, while the IRS2/PI3K cascade is the main insulin signaling pathway in hepatocytes, so we detected the expression of MFN2 and the IRS2/PI3K cascade pathway. As shown in Figure 3, the expression of MFN2, INSR, IRS2 and GLUT2 was down-regulated markedly by HFD both at 4 and 8 wk. While the mRNA and total protein expression of PI3K-P85 and AKT2 were not significantly changed (Figure 3A and B), their protein phosphorylation levels decreased markedly (Figure 3B). However, HFD seemed to have no effects on the expression of IRS1, PI3K-P110 and AKT1 or their phosphorylation (data not shown).

Over-expression of MFN2 ameliorated HFD induced insulin resistance in rats

In order to know the effect of MFN2 on insulin sensitivity, rats were fed with an HFD for 8 wk, and then were infected with different amounts of Ad-MFN2 (10^8, 10^9 or 10^{10} vp/kg body weight) or empty Ad adenovirus or PBS control for 3 wk. The results showed that MFN2 expression in the liver of rats increased dramatically after Ad-MFN2 infection (Figure 4A-C). At the same time, fasting BG, plasma insulin, TC and TG levels decreased (Figure 4D-G), while GIR increased (Figure 4H) markedly after infection with different amounts of Ad-MFN2. The body weight of rats in the two groups showed no significant difference (Figure 4I). The results indicated that MFN2 over-expression could neutralize the effects of HFD on insulin sensitivity.

Over-expression of MFN2 neutralized the inhibition of the insulin pathway by HFD in rats

Based on MFN2 over-expression in liver of rats, we detected changes in the insulin pathway by quantitative RT-PCR and Western-blot assays. The results showed that both mRNA and protein levels of INSR, IRS2 and GLUT2 were up-regulated markedly (Figure 5A and B). Though there were no changes in PI3K-P85 and AKT2 expression, their phosphorylation levels increased significantly (Figure 5B). After treated with MFN2 expressing adenovirus, the expression of SOCS3 was decreased (Figure 5C and D).The results suggested that MFN2-induced improvement in insulin sensitivity may be correlated with the promotion of the insulin signaling pathway.

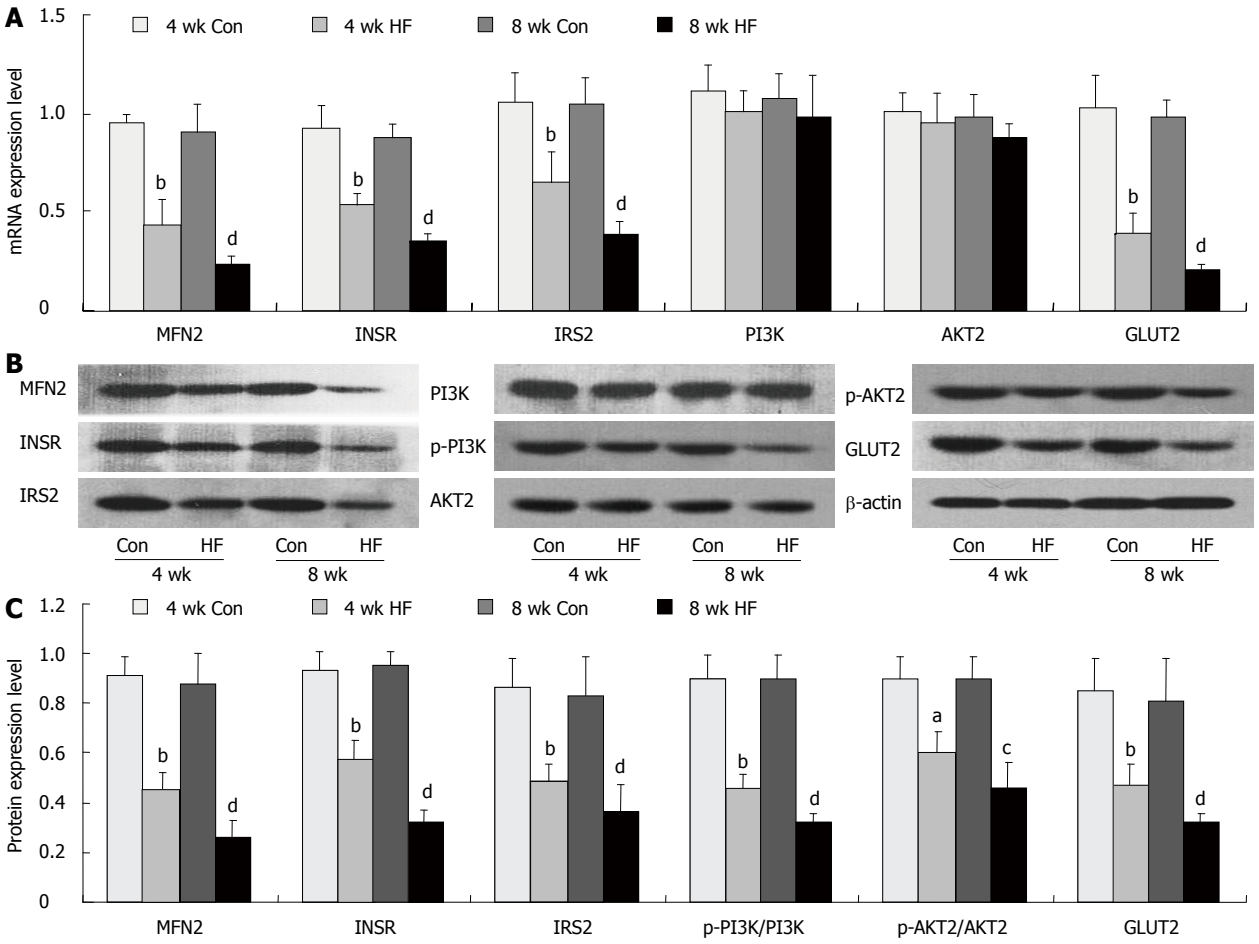

Figure 3 High-fat diets inhibited insulin pathway in liver of rats. Rats were fed with high-fat diets (HF) or normal control diets (Con) for 4 or 8 wk. A: The mRNA expression levels of mitofusin 2 (MFN2), insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), AKT2 and glucose transporter type 2 (GLUT2) were examined by quantitative real-time-polymerase chain reaction; B and C: The protein levels were detected by Western-blotting. $^{b}P < 0.01$ *vs* normal diets for 4 wk; $^{d}P < 0.01$ *vs* normal diets for 8 wk.

Over-expression of MFN2 alleviated hepatic steatosis

All of the tissue sections in the control and Ad groups exhibited diffuse hepatic steatosis under a light microscope. Hepatic steatosis was most obvious around the portal area and was accompanied by inflammatory cell infiltration. The liver HE staining of rats infected with Ad-MFN2 showed a lower cell volume and fat droplet accumulation (Figure 6).

DISCUSSION

HFDs induce dysfunction of energy metabolism and impaired insulin sensitivity[20]. Insulin resistance is not only the most important pathophysiological feature in many pre-diabetic states, but is also a key component of the metabolic syndrome, in which target cells fail to respond to normal levels of circulating insulin[21]. The liver is a vital organ for lipid metabolism and glycometabolism, and therefore one of the main organs in which insulin resistance occurs. Mitochondria are the power centres, and also the energy metabolism centres in cells, so mitochondrial dysfunction is the main reason for insulin resistance and is involved in the pathogenesis of T2DM[22]. The *MFN2* gene plays a central role in mitochondrial metabolism[23]; however, the role of MFN2 in insulin resistance and the insulin signaling pathway remains uncertain. Our study suggested that MFN2 expression and insulin sensitivity were inhibited by an HFD, while recovery of MFN2 expression could recover impaired insulin sensitivity, and may be associated with improvements of the insulin signaling pathway in liver.

An HFD enriched with lard, a significant contributor to the development of obesity and insulin resistance, has been widely used to induce an animal model of obesity with insulin resistance[24]. Previous studies have reported a long-term HFD could cause a marked increase in body weight. In this study, we confirmed that an HFD, for only 4 wk, could result in insulin resistance, which progressed further after 8 wk in the absence of major changes in total body weight. It may because an HFD produces altered fat distribution, leading to the accumulation of visceral fat[25,26]. At the same time, MFN2 expression was down-regulated dramatically, and the insulin signaling pathway was inhibited markedly in the liver of rats.

MFN proteins have been shown to regulate the biogenesis and maintenance of the mitochondrial netwo-

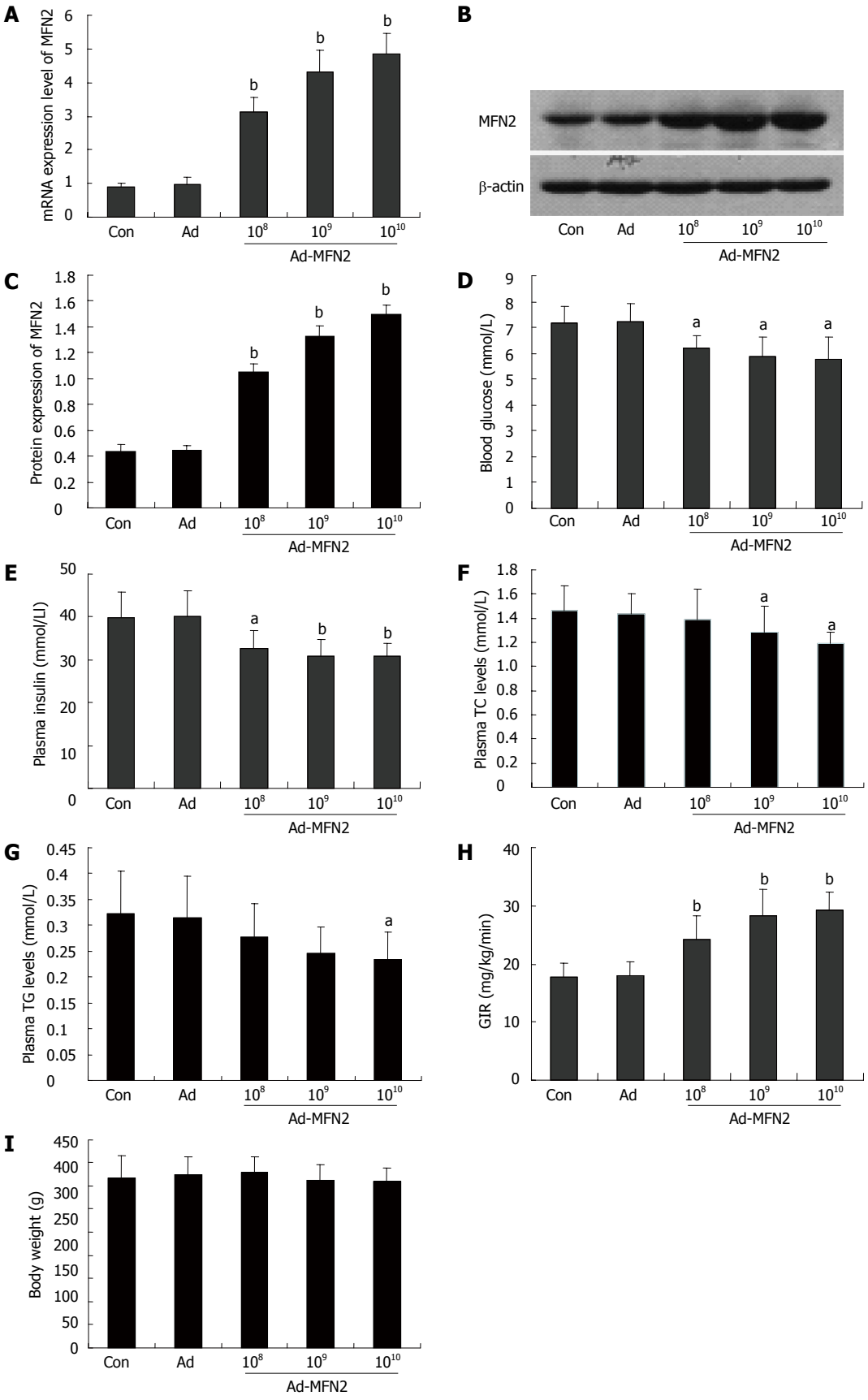

Figure 4 Mitofusin-2 over-expression improved insulin sensitivity of rats (n = 6). Rats were fed with high-fat diets for 8 wk, and then were infected with different amount of Ad-mitofusin-2 (MFN2) (10^8, 10^9 or 10^{10} vp/kg body weight) or empty Ad adenovirus or phosphate-buffered saline (PBS) control for 3 wk. MFN2 over-expression in liver of rats was confirmed by quantitative real-time-polymerase chain reaction (A) and Western-blotting (B and C). The levels of blood glucose (D), plasma insulin (E), triglycerides (TG) (G) plasma total cholesterol (TC) (F) and insulin sensitivity (H) levels were examined, respectively. The body weight of rats was measured (I). $^aP < 0.05$, $^bP < 0.01$ *vs* Ad. GIR: Glucose infusion rate.

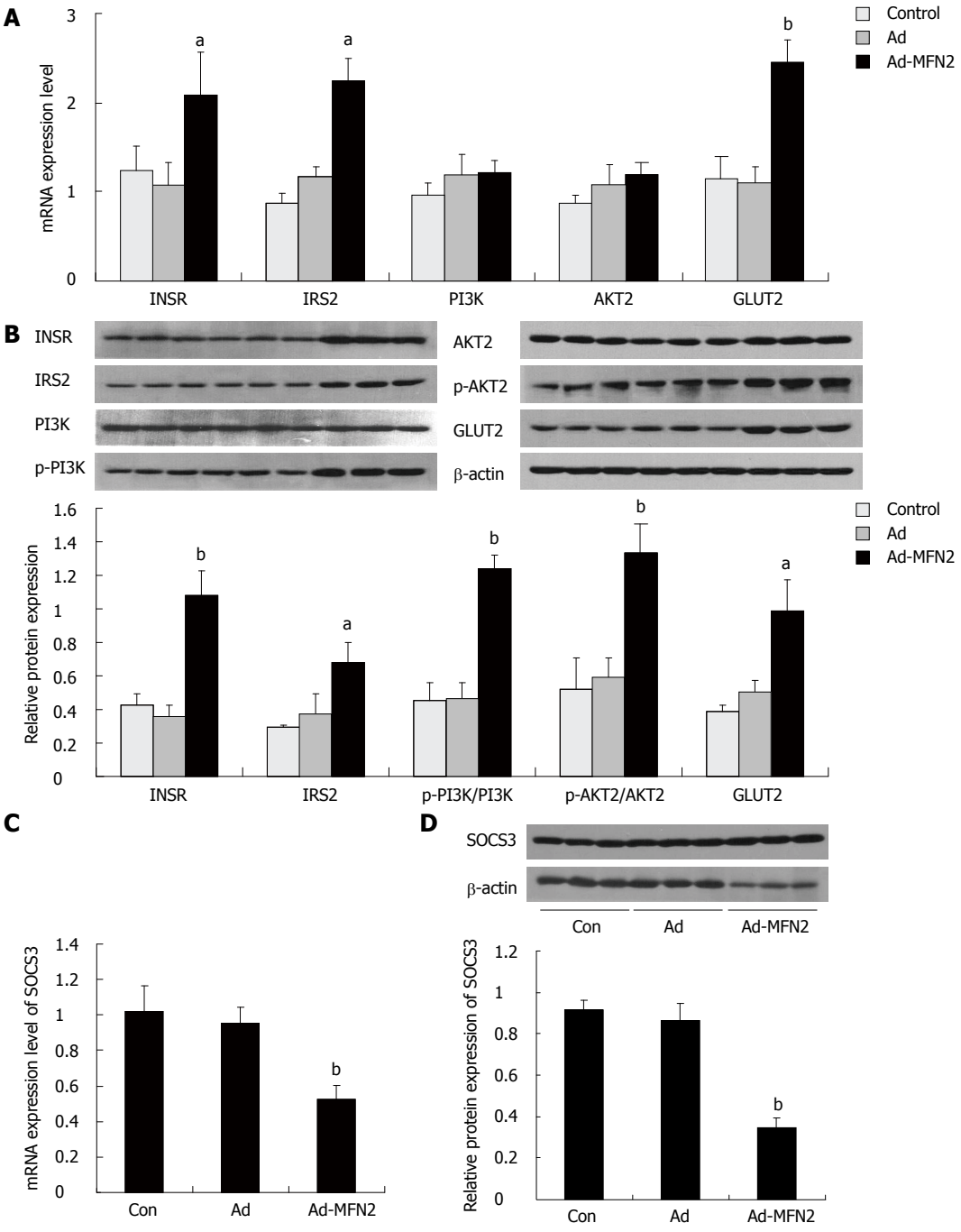

Figure 5 Mitofusin-2 over-expression up-regulated the expression of insulin pathway related genes in liver of rats. Rats were fed with high-fat diets for 8 wk, and then were infected with Ad-mitofusin-2 (MFN2) or empty Ad adenovirus or phosphate-buffered saline (PBS) control for 3 wk. The expression levels of insulin receptor (INSR), insulin receptor substrate 2 (IRS2), phosphoinositide-3-kinase (PI3K), AKT2 and glucose transporter type 2 (GLUT2) were determined by quantitative real-time-polymerase chain reaction (RT-PCR) (A) and Western-blot (B). The phosphorylation levels of PI3K and AKT2 were assayed by Western-blotting (B). The expression level of suppressor of cytokine signaling 3 (SOCS3) was determined by quantitative RT-PCR (C) and Western-blot (D). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Ad.

rk[27]. MFN protein deficiency could cause a failure in the mitochondrial architecture and decreases in oxidative capacity and glucose oxidation. MFN2 is a proliferation-inhibiting gene encoding a mitochondrial fusion protein that participates in the maintenance of the mitochondrial morphology and regulates mitochondrial metabolism and intracellular signaling[6]. A recent study found that liver MFN2 protein was significantly decreased, and fasting BG concentrations were increased in mice after interference with MFN2 protein expression[28]. Our data demonstrate that over-expression of MFN2 significantly restored insulin sensitivity and reduced the levels of BG and plasma insulin in rats, suggesting MFN2 as a potential therapeutic target in insulin resistance.

Most metabolic processes are regulated by insulin in muscle, adipocytes, and liver. A recent study indicated that mitochondrial protein down-regulation contributes to defects in insulin signaling in insulin resistance[29]. Insu-

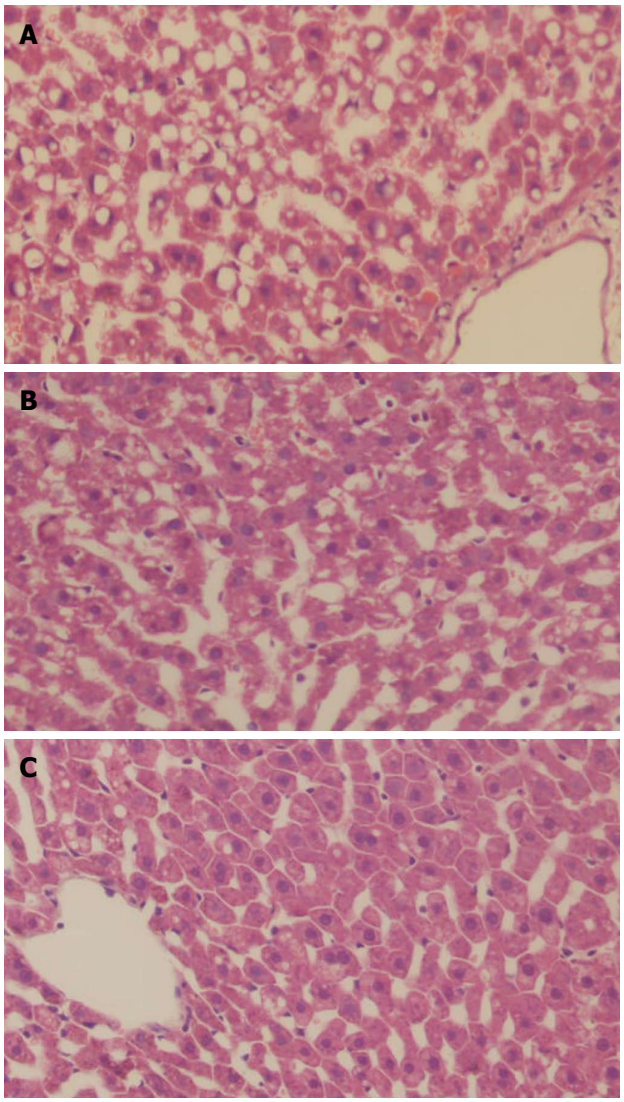

Figure 6 Over-expression of mitofusin-2 improves high-fat diets induced hepatic steatosis. Histological analysis of liver sections from rats fed with high-fat diets infected with phosphate-buffered saline control (A), empty Ad adenovirus (B) or Ad-mitofusin 2 (C) for 3 wk (HE, × 200).

lin acts through a complex signaling network including alternative or complementary pathways, with multiple molecular systems involved[30]. Abnormalities in the early stages of insulin signaling have been considered as an important component of many insulin-resistant states[31,32]. Our results showed that the expression of INRS, IRS2 and GLUT2 decreased; the phosphorylation of PI3K-P85 and AKT2 was also inhibited by HFD, but was restored markedly by recovery of MFN2 expression.

Hepatic expression of SOCS3 has been reported to be elevated in rodent models of obesity and insulin resistance[33,34]. SOCS3 was found to bind to phosphotyrosine 960 of the insulin receptor and prevent STAT5b activation by insulin[35]. In COS-7 cells, SOCS3 reduced IRS-2 phosphorylation and its subsequent association with p85, the regulatory subunit of PI3K[36]. In multiple cell lines, SOCS3 has been shown to bind IRS-2 and promote its ubiquitination and subsequent degradation[34,37]. The inhibition of SOCS3 expression restores IRS-1 and IRS-2 tyrosine phosphorylation, and IRS-1 and IRS-2 association with p85-PI3K and [Ser473] phosphorylation of Akt[38]. In our study, expression of SOCS3 in the liver of rats treated with MFN2 expressing adenovirus was decreased. MFN2 expression may improve insulin resistance by regulating the expression of SOCS3 in the liver of rats.

In conclusion, MFN2 could ameliorate insulin resistance induced by HFD by improvement of the insulin signaling pathway, and this may be a potential target for the treatment of insulin resistance and metabolic syndrome.

COMMENTS

Background

Insulin resistance is associated with numerous modern health problems, such as obesity, metabolic syndrome and type 2 diabetes mellitus. Mitofusin 2 (MFN2) regulates mitochondrial morphology and signaling, and is involved in the pathogenesis of insulin resistance and development of diabetes, but the exact mechanism is unclear.

Research frontiers

MFN2, a large transmembrane GTPase located in the outer mitochondrial membrane, promotes membrane fusion and is involved in the maintenance of the morphology of mitochondria. Recent studies show that *MFN2* gene expression is positively correlated with insulin resistance. Some metabolic diseases, such as type 2 diabetes show impaired MFN2 expression. MFN2 deficiency impairs insulin signaling in the liver and muscle. The research hotspot is the mechanism of MFN2 in improving insulin sensitivity.

Innovations and breakthroughs

MFN2, a large transmembrane GTPase located in the outer mitochondrial membrane, promotes membrane fusion and is involved in the maintenance of the morphology of mitochondria. Recent studies show that *MFN2* gene expression is positively correlated with insulin resistance and that MFN2 deficiency impairs insulin signaling in the liver and muscle.

Applications

The study results suggest that MFN2 over-expression improves insulin sensitivity, and may be used for preventing the development of diabetes in future.

Terminology

MFN2 encodes a mitochondrial membrane protein that participates in mitochondrial fusion and contributes to the maintenance and operation of the mitochondrial network. This protein is involved in the regulation of vascular smooth muscle cell proliferation, and it may play a role in the pathophysiology of obesity. Mutations in this gene cause Charcot-Marie-Tooth disease type 2A2, and hereditary motor and sensory neuropathy VI, which are both disorders of the peripheral nervous system. Defects in this gene have also been associated with early-onset stroke.

Peer review

The authors examined the expression of insulin signaling pathways and suppressor of cytokine signaling 3 (SOCS3) after infection with an MFN2 expressing adenovirus. It revealed that expression of insulin signaling pathways were increased and SOCS3 was inhibited in the liver of insulin resistant rats. Expression of MFN2 improves the insulin signaling pathway through the inhibition of SOCS3 expression. The results are interesting and may represent a molecular mechanism of MFN2 amelioration of insulin resistance.

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