Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 18 (2007) 64–72
Journal of Nutritional Biochemistry

Beneficial impact of crocetin, a carotenoid from saffron, on insulin sensitivity in fructose-fed rats

Liang Xi[a], Zhiyu Qian[a,*], Guanglin Xu[b], Shuguo Zheng[a], Sai Sun[a], Na Wen[a], Liang Sheng[a] Yun Shi[a], Yabing Zhang[a]

[a]*Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, PR China*
[b]*College of Life Science, Nanjing Normal University, Nanjing 210097, PR China*

Received 29 December 2005; received in revised form 6 March 2006; accepted 23 March 2006

Abstract

Crocetin, a unique carotenoid with potent antioxidative and anti-inflammatory activities, is a major ingredient of saffron which is used as an important spice and food colorant in various parts of the world. In the present study, the effect of crocetin on insulin resistance and its related abnormalities induced by high-fructose diet were investigated in male Wistar rats. Compared to the control rats fed on normal laboratory diet, fructose-fed rats developed a series of pathological changes including insulin resistance, hyperinsulinemia, dyslipidemia and hypertension. Although having no evident effect on the body weight, fructose feeding caused a marked increase in the weight of epididymal white adipose tissue. Furthermore, a significant reduction in the expression of both protein and mRNA of adiponectin (an insulin-sensitizing adipocytokine) was observed, whereas those of tumor necrosis factor (TNF)-α and leptin were enhanced in epididymal white adipose tissue in fructose-fed rats. These disorders were effectively normalized in crocetin-treated rats. Crocetin was also demonstrated here to alleviate free fatty acid (FFA)-induced insulin insensitivity and dysregulated mRNA expression of adiponectin, TNF-α and leptin in primary cultured rat adipocytes. These findings suggest the possibility of crocetin treatment as a preventive strategy of insulin resistance and related diseases. The favorable impact on adiponectin, TNF-α and leptin expression in white adipose tissue may be involved in the improvement of insulin sensitivity observed in crocetin-treated rats.

Keywords: Crocetin; Insulin resistance; Adipose tissue; Adiponectin; TNF-α; Leptin

1. Introduction

Functional plant-based foods that contain bioactive components may provide desirable health benefits beyond basic nutrition and are practically useful for the prevention of chronic diseases such as cardiovascular diseases and cancer. Saffron, the world's highest priced spice, is collected from the dried stigmas of *Crocus sativus* L. which originated in the Middle Eastern region of the Eurasian continent [1]. This carotenoid-rich spice is commonly consumed in different parts of the world and also used as an herbal medicine. Among the major ingredients of saffron, crocetin, which is responsible for its coloring property, is a special carotenoid with multi-unsaturated conjugate olefin acid structure. The compound exhibits favorable effects in the prevention or treatment of a variety of diseases such as dyslipidemia, atherosclerosis, myocardial ischemia, hemorrhagic shock, cancer and arthritis [2]. Crocetin also showed obvious inhibitory effects on atherogenic factor-induced disorders in vascular endothelial cells, smooth muscle cells and monocyte-derived macrophages [3] (unpublished data). Recently, we have found that crocetin may prevent low-dose dexmethasone-induced insulin resistance in rats [4].

Insulin resistance is a remarkable and growing health problem tightly associated with obesity, dyslipidemia, hypertension and type 2 diabetes mellitus [5]. The development of insulin resistance is linked to both genetic and environmental factors. A key environmental element is diet

Abbreviations: CON, control; CRO, crocetin; CRO(H), high-dose crocetin; CRO(L), low-dose crocetin; FFA, free fatty acids; FRU, fructose; HDL-C, high-density lipoprotein cholesterol; KRBH, Krebs-Ringer bicarbonate HEPES buffer; LDL-C, low-density lipoprotein cholesterol; MMLV, Moloney murine leukemia virus; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

* Corresponding author. Tel.: +86 25 8327 1322; fax: +86 25 832 1355.
E-mail address: zhiyu.qian@yahoo.com (Z. Qian).

0955-2863/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2006.03.010

composition [6]. In this study, a well-established model in which insulin resistance was induced by feeding high-fructose diet was employed, and fructose-fed rats were characterized by hyperinsulinemia and dyslipidemia with normoglycemia.

Adipose tissue mass (especially visceral adipose) enlargement has been identified as a crucial factor responsible for insulin resistance [7]. As a primary energy-storing organ, adipose tissue accumulates triglycerides during nutrition excess and provides energy in the form of free fatty acids (FFAs), which may induce insulin resistance if produced excessively [8]. Besides, adipose tissue releases numerous bioactive molecules named adipocytokines that participate in a variety of physiological functions. For example, adiponectin is an important adipocyte-specific circulating protein that possesses insulin-sensitizing, anti-atherosclerotic and anti-inflammatory activities [9,10]. TNF-α is a central modulator of adipocyte metabolism which interferes in insulin-mediated biological processes. In addition to a direct inhibitory effect on insulin signaling cascade, TNF-α also raises FFA concentration by decreasing lipogenesis and increasing lipolysis [7,11]. Leptin, which is also chiefly secreted by adipocytes, has also been shown to affect insulin sensitivity [7,12]. Dysregulation of adipocytokines due to fat accumulation is implicated in the progress of insulin resistance, but the involved mechanisms are seldom known.

Oxidative stress caused by enhanced concentrations of FFA, glucose and inflammatory cytokines plays a major role in the development of insulin resistance, and increased oxidative stress in accumulated fat has been recognized as an early instigator of insulin-resistance syndrome [13,14]. In cultured adipocytes, elevated FFA increases oxidative stress, which leads to a dysregulated production of adipocytokines [13], and long-term oxidative stress impairs insulin signaling [15]. The use of antioxidants is proposed as a potential new approach for the treatment of insulin resistance and related diseases [13,14,16,17]. Besides, owing to the close relationship between insulin resistance and dyslipidemia [18], dietary fish oils and other lipid-regulating agents have been shown to be able to prevent adiposity and insulin resistance [19].

Based on these facts, it can be expected that crocetin has more favorable health-promoting effects. The current study adopted a fructose-fed rat model to investigate the effect of crocetin on insulin action. The results showed that fructose-induced insulin resistance and other accompanied abnormalities were significantly attenuated by crocetin. Since adipocytokine secretion and adipose-specific gene are proposed as potential important targets for management of insulin resistance by controlling their expression or actions [16], regulation of adipocytokine expression represents a candidate mechanism underlying the beneficial effect of crocetin on insulin sensitivity. Therefore, the influence of crocetin on the expression of protein and mRNA of adiponectin, TNF-α and leptin in epididymal white adipose tissue was investigated. Furthermore, the effect of crocetin on FFA-induced impairment of insulin sensitivity and disordered mRNA expression of adiponectin, TNF-α and leptin was observed in primary cultured adipocytes.

2. Methods and materials

2.1. Chemicals

Crocetin (>96%, HPLC) was purified from saffron in our laboratory. 2-Deoxy-D-[1-^{3}H]-glucose (5.4 Ci/mmol) was purchased from Atom High-Tech (Beijing, China). Other chemicals were obtained from Sigma or local manufacturers unless otherwise stated.

2.2. General protocol

Male Wistar rats (Slac Laboratory Animal, Shanghai, China) with a body weight of 120–150 g were housed at 24±2°C with a 12/12 h light–dark cycle. All rats were supplied with normal laboratory chow and water for 1 week. The experimental protocols were performed in accordance with the institutional guidelines for animal care of China Pharmaceutical University and approved by the local animal research committee.

After acclimation, the animals were randomly divided into the following groups consisting of 10 rats each: a control group (CON), a crocetin (40 mg/kg)-treated group (CRO), a fructose-fed group (FRU), two fructose-fed groups plus crocetin at a dose of 40 mg/kg [FRU+CRO(H)] and 20 mg/kg [FRU+CRO(L)], respectively. Fructose was supplied in drinking water at a concentration of 10% for 8 weeks, while the CON group received no supplemented fructose. Crocetin powder was mixed thoroughly into the powdered chow at a concentration of 0.02–0.08%, and crocetin-containing diet was given along with fructose supplement through the experiment. To ensure accurate dosing, food intake was measured for 5 days before the study and the dietary crocetin concentration was reset according to the changes in body weight and food consumption every 2 weeks during the study. Additional crocetin was given by oral gavage if the consumed diet did not match enough level of crocetin. Body weights were monitored twice weekly. The intake of food and fluid was recorded daily, using metabolic cages. Systolic blood pressure, heart rate, serum glucose and insulin were measured every 2 weeks from the beginning of the experiment (Week 0). Systolic blood pressure and heart rate were measured with the tail-cuff method, using a programmed sphygmomanometer (DeSci Biotech, Nanjing, China).

2.3. Biochemical analyses

Serum glucose, total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using a Beckman LX-20 automatic analyzer. Serum insulin was measured with a rat insulin radioimmunoassay kit (Linco Research, St. Charles, USA). Serum FFA was measured by a validated colorimetric method using a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.4. Adipocyte isolation

Epididymal adipocytes were isolated based on Rodbell's method [20] with minor modifications. Briefly, the animals were killed by decapitation. The epididymal fat pads were dissected and washed with 0.9% NaCl solution under sterile conditions. After mincing, the adipose tissues were digested into cells for 90 min at 37°C in Krebs-Ringer bicarbonate HEPES buffer (KRBH) (pH 7.4) supplemented with 0.5% bovine serum albumin (BSA), 1 mmol/L pyruvate and 0.1% type IV collagenase. The cell suspension was filtered through nylon mesh (250 μm) and washed with KRBH containing 0.5% BSA and 1 mmol/L pyruvate twice. The harvested cells were resuspended to adequate number in KRBH.

2.5. Glucose uptake assay

Glucose uptake assay was performed using radiolabeled 2-deoxy-D-glucose. Briefly, an aliquot of adipocytes suspension ($2–3\times10^5$ cells) was incubated with or without insulin (10 nmol/L) in KRBH containing 0.5% BSA and 1 mmol/L pyruvate at 37°C for 30 min. Then 2-deoxy-D-[1-^{3}H]-glucose in KRBH was added (final concentration, 0.1 mmol/L) at 37°C for 5 min. Uptake of 2-deoxyglucose was terminated by cold silicone oil and the cell layer was separated by centrifugation. The aqueous solution under the oil layer was abandoned, and the radioactivity of the adipocytes layer was measured using a Wallac Guardian 1414 liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) in OptiPhase HiSafe 3 liquid scintillation cocktail (Wallac, Turku, Finland).

2.6. RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from epididymal adipose tissue (100 mg) using Trizol Reagent (Gibco BRL Life Technologies, Burlington, ON, Canada). The concentration and purity of RNA extracted were checked by measuring the absorbance at 260 and 280 nm.

Total RNA (2.0 μg) was incubated with 2 μl Oligo dT(18) primer (0.5 μg/μl) at 70°C for 5 min, and reverse transcribed to cDNA in a reaction mixture of 5× Moloney murine leukemia virus (MMLV) reaction buffer, 40 U RNase inhibitor (Toyobo, Osaka, Japan), 0.52 mmol/L dNTP Mix (Bio-Rad Laboratories, Hercules, CA) and 200 U MMLV reverse transcriptase (Toyobo) at 37°C for 90 min. The mixture was heated at 95°C for 5 min to terminate the reaction. Polymerase chain reaction was performed in a mixture (25 μl) containing 2.5 μl 10× PCR buffer, 2 μl reverse transcribed template solution, 1.5 mmol/L $MgCl_2$, 0.5 μmol/L of each sense and antisense primers (Sangon Biological Engineering Technology and Services, Shanghai, China), 0.2 mmol/L dNTP Mix (Bio-Rad) and 1 U iTaq DNA polymerase (Bio-Rad). Preliminary experiments were conducted with different cycles to determine the optimal conditions for the PCR amplification for each gene. The specific primer sequences were as follows: β-actin (GenBank Accession No. NM_031144): 5′- GAG AAG ATT TGG CAC CAC AC-3′ (sense) and 5′-CAT CAC AAT GCC AGT GGT AC-3′ (antisense); adiponectin (GenBank Accession No. NM_144744): 5′-TCC TGG TCA CAA TGG GAT ACC-3′ (sense) and 5′-ATC TCC TGG GTC ACC CTT AGG-3′ (antisense); TNF-α (GenBank Accession No. X66539): 5′-CTC GAG TGA CAA GCC CGT AG-3′ (sense) and 5′-TTG ACC TCA GCG CTG AGC AG-3′ (antisense); leptin (GenBank Accession No. NM_013076): 5′-CAT TTC ACA CAC GCA GTC GG-3′ (sense) and 5′-AGC AGA TGG AGG AGG TCT CG-3′ (antisense). iTaq DNA polymerase was activated by a 94°C incubation step for 5 min; after an initial denaturation at 94°C for 30 s, reactions were performed as follows: for

Table 1
Physical and biochemical characteristics of the rats in the CON, CRO, FRU, FRU+CRO(H) and FRU+CRO(L) groups at the end of the experiment

	CON	CRO	FRU	FRU+CRO(H)	FRU+CRO(L)
Body weight (g)	315±17	320±12	314±15	316±14	317±23
Weight of epididymal fat pads (g)	4.97±0.26	5.10±0.28	6.18±0.54[a]	5.26±0.42[b]	5.51±0.52[c]
Relative weight of epididymal fat pads (g/100 g body weight)	1.58±0.04	1.60±0.10	1.97±0.11[a]	1.66±0.07[b]	1.74±0.12[b]
Food intake (g/rat per day)	19±2	19±1	14±1[a]	16±2[a]	16±1[a]
Fluid intake (ml/rat per day)	20±2	20±1	33±5[a]	36±7[a]	34±5[a]
Caloric intake (kJ/rat per day)	243±23	250±18	242±22	259±32	256±20
Systolic blood pressure (mmHg)	118±8	121±5	132±6[a]	124±6[b]	122±8[b]
Heart rate (beats/min)	348±12	352±11	374±13[a]	361±10[c]	371±8
Serum insulin (mU/L)	25.33±4.27	24.98±4.53	37.38±6.46[a]	25.20±3.13[b]	26.91±4.84[b]
Serum glucose (mmol/L)	6.57±0.80	6.49±0.64	6.81±0.87	6.54±1.47	6.56±1.20
Serum total cholesterol (mmol/L)	1.43±0.08	1.39±0.06	1.48±0.06	1.42±0.07	1.41±0.16
Serum triglyceride (mmol/L)	1.50±0.17	1.52±0.14	2.18±0.24[a]	1.42±0.35[b]	1.64±0.31[b]
Serum FFA (mmol/L)	0.21±0.04	0.20±0.05	0.30±0.08[d]	0.21±0.05[b]	0.23±0.04[c]
Serum LDL-C (mmol/L)	0.77±0.04	0.75±0.04	0.82±0.03[d]	0.75±0.03[b]	0.76±0.08
Serum HDL-C (mmol/L)	0.58±0.02	0.57±0.02	0.52±0.03[a]	0.57±0.03[b]	0.55±0.03[c]

Data are shown as mean±S.D. ($n=10$).
[a] $P<.01$ vs. CON.
[b] $P<.01$ vs. FRU.
[c] $P<.05$ vs. CON.
[d] $P<.05$ vs. FRU.

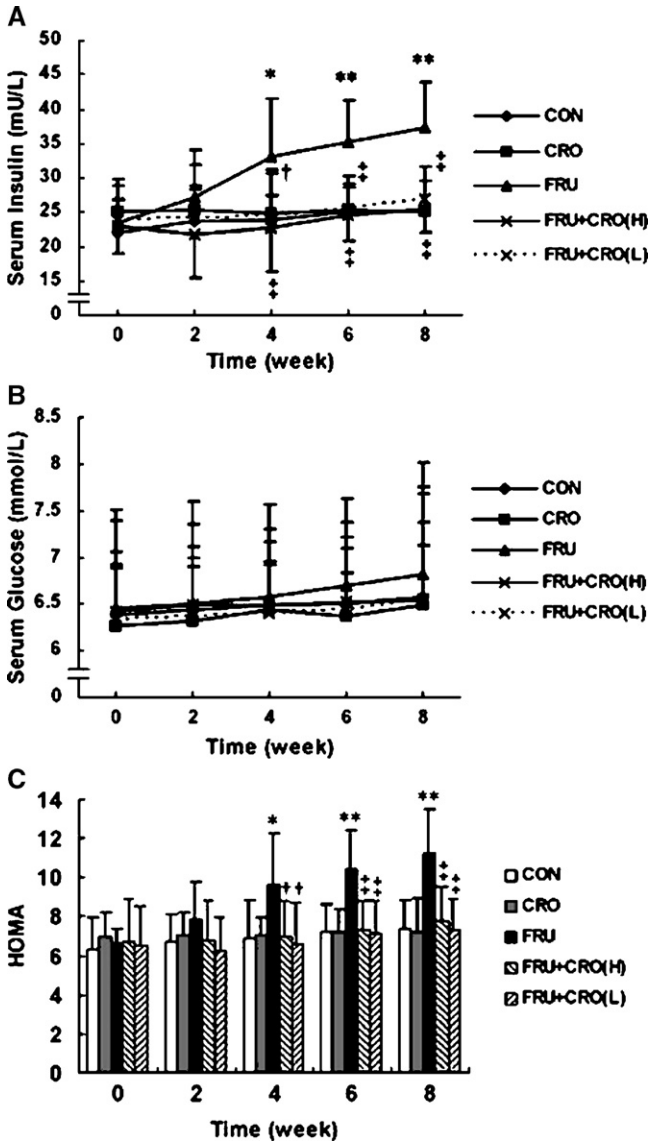

Fig. 1. Serum glucose level (A), serum insulin level (B) and HOMA value (serum insulin×glucose/22.5) (C) of the rats in the CON, CRO, FRU, FRU+CRO(H) and FRU+CRO(L) groups at Weeks 0, 2, 4, 6 and 8 during the experiment. Data are shown as mean±S.D. $n=10$. *$P<.05$, **$P<.01$ vs. CON; †$P<.05$, ‡$P<.01$ vs. FRU.

β-actin detection, 56°C for 50 s, 72°C for 40 s, 30 cycles (213 bp); for adiponectin detection, 56°C for 40 s, 72°C for 40 s, 28 cycles (109 bp); for TNF-α detection, 55°C for 50 s, 72°C for 40 s, 30 cycles (386 bp); for leptin detection, 58°C for 40 s, 72°C for 40 s, 30 cycles (201 bp). A final extension step at 72°C for 7 min was performed. The products were electrophoresed on 1.5% agarose gel. After the gel was stained with ethidium bromide, the relative density of the bands was measured using the Bio-Rad ChemiDoc XRS Gel Documentation system and Bio-Rad Quantity One 1-D analysis software. Relative quantitation for the PCR products was calculated after normalization to the amount of β-actin mRNA level.

2.7. Immunohistochemistry

Small pieces of epididymal adipose tissue were placed into 10% buffered formalin. The material was processed in paraffin wax, and 5-μm sections were obtained using a Leica RM2135 microtome (Wetzlar, Germany). The employed first antibodies were rabbit–antirat antibodies against adiponectin (1:100) (Santa Cruz Biotechnology, California, USA), TNF-α (1:100) and leptin (1:100) (Boster Bioengineering, Wuhan, China), respectively. Staining was

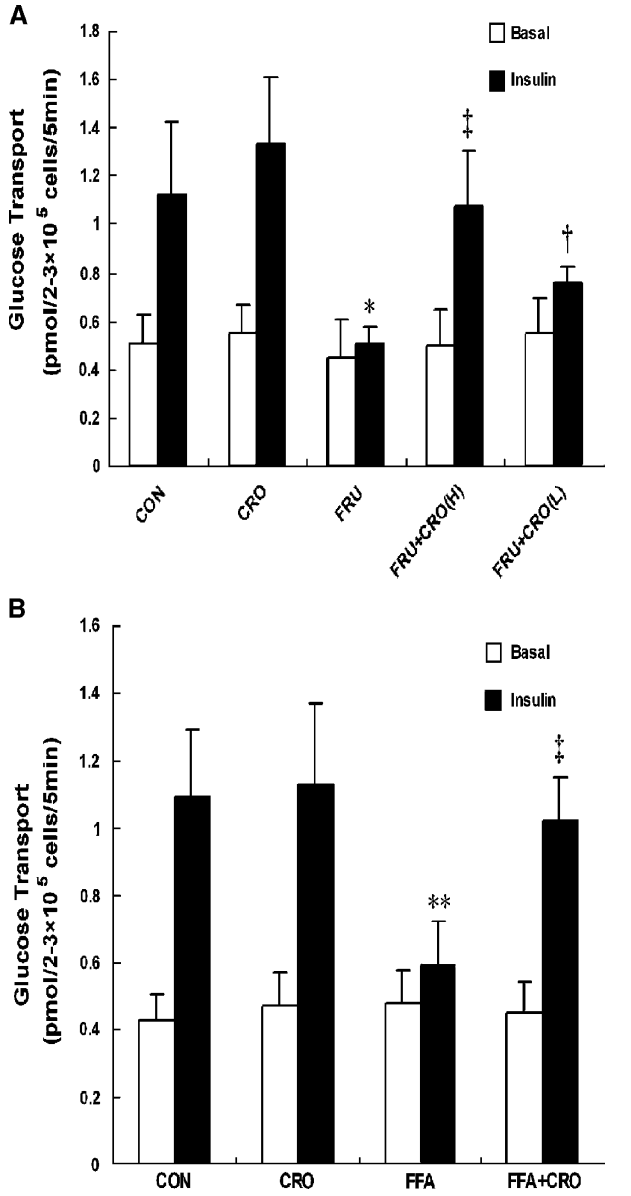

Fig. 2. Insulin-induced 2-deoxyglucose uptake by adipocytes from the rats in the CON, CRO, FRU, FRU+CRO(H) and FRU+CRO(L) groups (A) and by adipocytes in the CON, CRO, FFA, FFA+CRO groups in in vitro study (B). 2-Deoxy-D-[1-^{3}H]-glucose was added (final substrate concentration, 0.1 mmol/L) at 37°C for 5 min after incubation of adipocytes ($2–3\times10^5$) with 10 nmol/L insulin at 37°C for 30 min, and then 2-deoxy-D-[1-^{3}H]-glucose uptake was measured. Data are shown as mean±S.D. (A) $n=5$; (B) $n=3\times3$ independent experiments. *$P<.05$, **$P<.01$ vs. CON; †$P<.05$, ‡$P<.01$ vs. FRU.

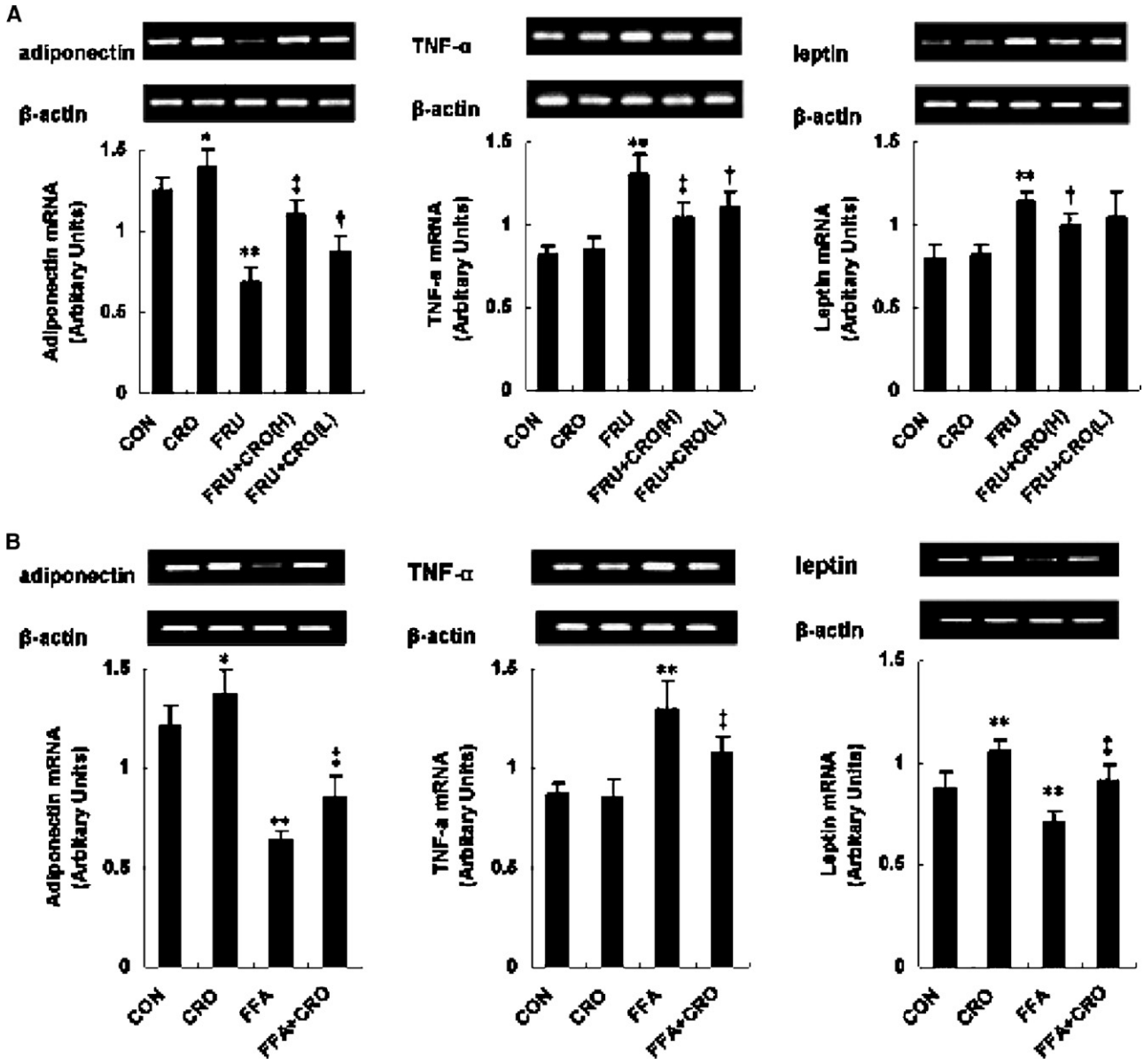

Fig. 3. The effect of crocetin on mRNA level of adiponectin, TNF-α and leptin in the epididymal white adipose tissue of the rats in the CON, CRO, FRU, FRU+CRO(H) and FRU+CRO(L) groups (A) and in adipocytes in the CON, CRO, FFA, FFA+CRO groups in in vitro study (B). The mRNA level was calculated after normalization to the amount of β-actin mRNA level. Total RNA (2.0 μg) was analyzed by RT-PCR. The representative agarose gel electrophoresis of RT-PCR products of adiponectin, TNF-α and leptin is shown. Data are shown as mean±S.D. (A) $n=6$; (B) $n=3\times3$ independent experiments. $^{**}P<.01$ vs. CON; †$P<.05$, ‡$P<.01$ vs. FRU.

carried out according to the instructions of Dako Envision System (for use with mouse and rabbit primary antibodies, Dako, Copenhagen, Denmark). The system contains peroxidase blocking reagent, labeled polymer (HRP), buffered substrate and liquid DAB chromogen. Two independent professional observers not aware of the different treatments made comments on the histological materials.

2.8. In vitro study

Adipocytes were isolated from epididymal fat pads of normal rats according to the previously described method. Then, the adipocytes were treated with control (CON), crocetin (50 μmol/L) (CRO), palmitic acid (300 μmol/L) (FFA), and palmitic acid (300 μmol/L) plus crocetin (50 μmol/L) (FFA+CRO) for 10 h. Glucose uptake assay was performed to evaluate the sensitivity of adipocytes to insulin. After the medium below the cell layer was removed, total RNA was extracted from the cultured cells and the mRNA expression was measured using RT-PCR as previously described.

2.9. Calculation and Statistical analysis

To estimate the degree of insulin resistance of the animals, the homeostasis model assessment (HOMA) was used as insulin-resistance index. The calculation was according to the following formula: [serum insulin (mU/L)×glucose

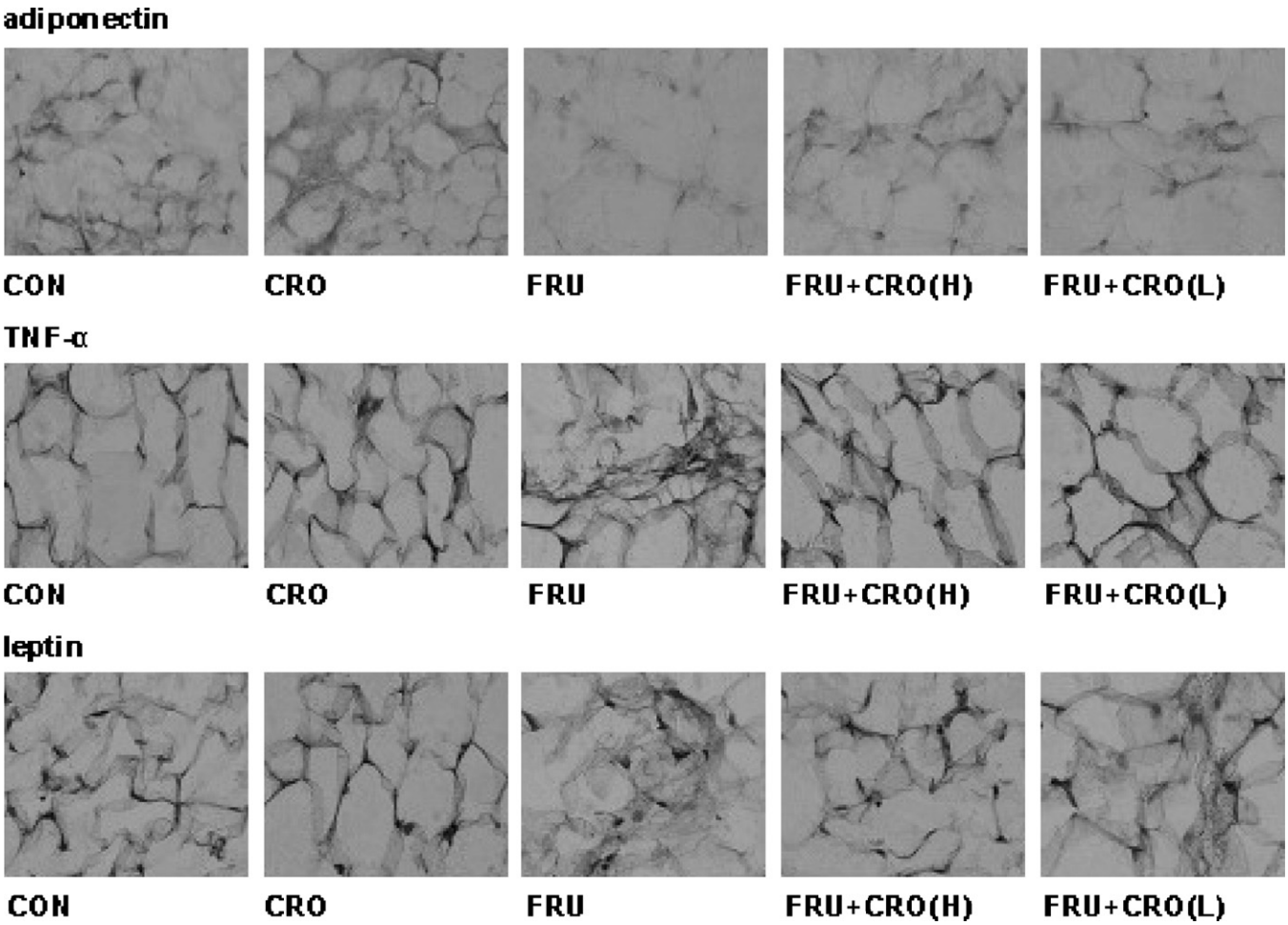

Fig. 4. The effects of crocetin on protein expression of adiponectin, TNF-α and leptin in the epididymal white adipose tissue of the rats in the CON, CRO, FRU, FRU+CRO(H) and FRU+CRO(L) groups as indicated by the representative results of immunohistochemical staining (×400). The distribution and expression intensity of leptin and TNF-α were significantly increased, whereas those of adiponectin were markedly decreased in the FRU group compared with the CON group. These changes in adiponectin, TNF-α and leptin protein expressions were prevented by crocetin. Crocetin itself also enhanced adiponectin protein expression.

(mmol/L)]/22.5. All data are expressed as mean±S.D. unless otherwise stated. The differences among groups were analyzed by analysis of variance. P value of less than .05 was considered statistically significant.

3. Results

There was no significant difference in body weight among groups at the end of the experiment. Daily fluid intake was significantly increased and daily food intake was contrastively reduced in the FRU, FRU+CRO(H) and FRU+CRO(L) groups, but the energy intake (expressed as kilojoules per day) was comparable in all groups throughout the experiment (Table 1). Compared with the CON group, the absolute and relative weights of epididymal fat pads were both significantly heavier in the FRU group. Crocetin suppressed the fructose supplement-induced increase in the weight of epididymal adipose tissue (Table 1).

The systolic blood pressure was significantly higher in the FRU group than in the CON group and was effectively controlled by crocetin (Table 1). Fructose feeding caused a significant increase in serum triglyceride, FFA and LDL-C levels and an evident reduction in serum HDL-C level. These disorders in serum lipid level were prevented by crocetin treatment except that low-dose crocetin had no obvious effect on serum LDL-C level (Table 1). Serum total cholesterol level was comparable in all groups.

During the experiment, serum insulin level in the FRU group was increased at Week 2, but this was not significantly different from the CON group (27.20±6.72 vs. 23.62±5.20 mU/L, P=.20). Serum insulin level in the FRU group was significantly higher than in the CON group from Week 4 (32.96±8.66 vs. 23.80±6.91 mU/L, P=.02) to Week 8, which was attenuated by crocetin (Fig. 1A). Serum glucose levels were similar among groups during the whole study (Fig. 1B). The development of insulin resistance, as reflected by higher HOMA value, in fructose-fed rats was prevented by crocetin (Fig. 1C). The isolated adipocytes from the FRU group clearly became insulin resistant based on the result of insulin-induced

2-deoxyglucose uptake assay (Fig. 2A). There was no difference in basal 2-deoxyglucose uptake into adipocytes among all groups.

After being normalized to β-actin, the relative mRNA level of adiponectin was significantly decreased, whereas TNF-α and leptin mRNA levels were enhanced in the FRU group compared with the CON group. Crocetin treatment significantly ameliorated the changes in mRNA levels of these adipoctokines, except that the effect of low-dose crocetin on leptin mRNA level was not significant (Fig. 3A). In the CRO group, an obvious increase in mRNA level of adiponectin was observed, whereas leptin and TNF-α mRNA expressions were not affected in the adipose tissue. These changes in mRNA expressions were consistent with the protein expressions of these adipocytokines as indicated by the results of immunohistochemical analysis (Fig. 4).

In vitro, incubation of primary cultured adipocytes with FFA induced a significant inhibition of insulin-stimulated glucose uptake. FFA also enhanced the mRNA expression of TNF-α and inhibited the mRNA expression of adiponectin and leptin in adipocytes. All the described disorders were alleviated by crocetin treatment (Figs. 2B and 3B). Crocetin itself did not enhance basal or insulin-stimulated glucose uptake, but, interestingly, it significantly up-regulated the mRNA expression of adiponectin and leptin in normal adipocytes.

4. Discussion

It is well known that additional fructose feeding leads to insulin resistance, hyperinsulinemia, dyslipidemia and hypertension in animal models [21]. In this study, insulin resistance was induced by feeding 10% fructose solution (equivalent to 48–57% fructose diet) in male Wistar rats [26]. The serum insulin level in the FRU group was not significantly higher than in the CON group within 2 weeks. This result is consistent with previous reports which indicate that 10% fructose feeding only leads to hypertriglyceridemia without inducing an insulin-resistant state in rats within a relatively short time [22,23]. During the whole experiment, the reduction in food intake in fructose-fed animals was probably compensated by the increased caloric intake from fructose solution as previously suggested [24]. Based on our results, fructose consumption had no effect on body weight but increased epididymal white adipose tissue weight in this rat model. The inhibition of the weight of epididymal fat pads by crocetin was not due to a restriction in diet consumption and energy intake since crocetin did not affect fructose consumption and calorie intake when compared to the FRU group.

Insulin resistance as a widespread feature of atherogenic diseases predisposes the affected individuals to various diseases including hypertension, dyslipidemia, cardiovascular problems and type 2 diabetes mellitus [3,25]. Lowering endogenous insulin levels is a key step to successful therapy directed at insulin resistance-related diseases [26]. Our experimental data clearly show that crocetin, especially at high dose, is capable of attenuating the development of insulin resistance, compensatory hyperinsulinemia, dyslipidemia and hypertension induced by fructose supplement. Low-dose crocetin also effectively managed these disorders, but had no obvious effect on elevated serum LDL-C level.

In recent years, the role of adipocyte dysfunction in the development of insulin resistance has attracted a great deal of attention. Adipose tissue is now recognized as the primary site responsible for insulin resistance [7,25,27]. Raised plasma FFA level is an important inductor of both peripheral and hepatic insulin resistance by inhibiting insulin signaling. Excessively released FFA also suppresses insulin secretion from pancreatic islet [7,9]. It has been suggested that the elevated FFA transport rate during fasting is due to an elevated rate of escape of FFA from esterification in adipose tissue rather than an impaired insulin action in obesity and type 2 diabetes mellitus [28]. Hypertriglyceridemia was secondary to increased output of FFA by adipose tissue, providing excess fatty acids to the liver for triglyceride synthesis [29]. So hypertriglyceridemia is also an important marker of insulin resistance [30]. Crocetin restored FFA metabolism disorders in fructose-fed rats, which may explain the biochemical and nutritional basis of its inhibitory action on the progress of insulin resistance.

Furthermore, it has been apparent that insulin sensitivity is regulated by adipocytokines, a wide group of bioactive proteins produced by adipose tissues. Substantial observations indicate an important role of adiponectin in the improvement of insulin resistance. A decrease in adiponectin release is causative for insulin resistance and atherosclerosis [31–33]. TNF-α interferes in several steps in insulin signaling cascade including reducing synthesis/translocation of insulin responsive glucose transporters, insulin receptor substrate 1 phosphorylation and tyrosine kinase activity of insulin receptor. This molecule is overproduced in adipose tissues in insulin-resistant rodents and humans [34,35]. Administration of TNF-α converting enzyme inhibitor has been demonstrated to enhance insulin sensitivity in fructose-fed rats [36]. As an inflammatory cytokine, TNF-α level is lowered in the liver by a similar compound of crocetin, trans-sodium crocetinate, during hemorrhagic shock [37]. Synthesis and circulating level of leptin are both strongly related to adiposity [21,38]. Leptin replacement therapy can reverse insulin resistance due to leptin gene mutations. Exogenous leptin administration may improve insulin resistance, and excess fructose consumption generally lowers circulating leptin concentration [9,21]. However, studies have also observed that there is an inverse relationship between plasma level or mRNA expression of leptin and insulin sensitivity [39,40]. Actually, high leptin concentration may reflect resistance of the body to the effects of this hormone [41]. The current study shows that leptin expression is significantly increased in white adipose tissue by fructose feeding.

Although dysregulated production of adipocytokines is known to participate in the pathogenesis of insulin

resistance, the mechanisms by which fat accumulation causes adipocytokines dysregulation are still elusive. Recently, increased oxidative stress is found to be an important culprit in the induction of insulin resistance, and consequently, reduction in oxidative stress in adipose tissue attenuates adipocytokine dysregulation and improves diabetes and hyperlipidemia [13]. Since crocetin possesses powerful antioxidant and anti-inflammatory properties, we focused on its effects on the expression of adiponectin, TNF-α and leptin in white adipose tissue. The reduction of adiponectin expression and the enhancement of TNF-α expression observed in fructose-fed rats were both suppressed by crocetin. Moreover, crocetin at higher dose significantly inhibited the abnormal increase of leptin expression in white adipose tissue. In primary cultured rat adipocytes, crocetin also prevented FFA-induced abnormalities in insulin action and mRNA expression of adiponectin, TNF-α and leptin. It is consistent with previous reports that this kind of fatty acid (palmitic acid) exerts a direct inhibitory effect on leptin mRNA expression in rat adipocytes in vitro [42]. In addition, crocetin itself increased the mRNA expression of adiponectin both in adipose tissue of normal rats and in primary cultured rat adipocytes, but only enhanced leptin mRNA expression in vitro. The discrepancy between in vivo and in vitro experimental results may reflect the fact that the production of these adipocytokines is affected by more complex factors in vivo. However, the circulating levels of adiponectin and leptin do not always correlate with their gene or protein expression in white adipose tissue [43]. Therefore, the changes in the plasma levels of these adipocytokines are undiscerned in this study. Similarly, we cannot know the changes in the secretion of these adipocytokines from isolated adipocytes in the situation in vitro. Future studies on these problems will further clarify the modes of action of crocetin involved in its impact on adipocytokines.

It is accepted that prevention is a more effective strategy than treatment for chronic diseases including insulin resistance and related diseases. Research has focused on identifying the active phytochemicals in functional plant foods, their biological outcomes and the mechanisms by which they function to provide health-promoting activities, which is likely to enhance the understanding of the nutritional effects of plant foods. In this study, crocetin, a special carotenoid contained in an ancient spice, saffron, exhibited a dramatic improvement in insulin resistance, hyperinsulinemia and other abnormalities in fructose-fed rats. Crocetin also shows a beneficial regulatory effect on the mRNA and protein expressions of adiponectin, TNF-α and leptin in the white adipose tissue of fructose-fed rats and in FFA-treated adipocytes, which provides further insight into the mechanisms by which crocetin exerts an insulin-sensitizing effect. However, the regulatory mechanism(s) underlying the effects of crocetin on the expression of these adipocytokines remains unclear. In addition, insulin resistance and adipocyte-derived factors including FFA, adiponectin, TNF-α and leptin are interrelated and interact with each other intricately. Therefore, the dynamic interactions between adipocytokines and insulin resistance should be another focus in future research.

Acknowledgments

We thank Biao Liu for the determination of the serum insulin level, Ms. Wang for performing the immunohistochemical staining and Prof. Li for technical assistance in 2-deoxyglucose uptake assay.

References

[1] Sampathu SR, Shivashankar S, Lewis YS. Saffron (*Crocus sativus* Linne): cultivation, processing, chemistry and standardization. Crit Rev Food Sci Nutr 1984;20:123–57.
[2] Giaccio M. Crocetin from saffron: an active component of an ancient spice. Crit Rev Food Sci Nutr 2004;44:155–72.
[3] Zheng S, Qian Z, Tang F, Sheng L. Suppression of vascular cell adhesion molecule-1 expression by crocetin contributes to attenuation of atherosclerosis in hypercholesterolemic rabbits. Biochem Pharmacol 2005;70:1192–9.
[4] Xi L, Qian Z, Shen X, Wen N, Zhang Y. Crocetin prevents dexamethasone-induced insulin resistance in rats. Planta Med 2005; 71:917–22.
[5] Reaven GM, Laws A. Insulin resistance, compensatory hyperinsulinemia, and coronary heart disease. Diabetologia 1994;37:948–52.
[6] Thresher JS, Podolin DA, Wei Y, Mazzeo RS, Pagliassotti MJ. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. Am J Physiol Regul Integr Comp Physiol 2000;279:R1334–40.
[7] Arner P. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. Trends Endocrinol Metab 2003;14: 137–45.
[8] Borden G. Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. Diabetes 1997;46:3–10.
[9] Bays H, Mandarino L, DeFronzo RA. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. J Clin Endocrinol Metab 2004; 89:63–478.
[10] Shimada K, Miyazaki T, Daida H. Adiponectin and atherosclerotic disease. Clin Chim Acta 2004;344:1–12.
[11] Ruana H, Lodish HF. Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-α. Cytokine Growth Factor Rev 2003;14:447–55.
[12] Pittas AG, Joseph NA, Greenberg AS. Adipocytokines and insulin resistance. J Clin Endocrinol Metab 2004;89:447–52.
[13] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, et al. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114:1752–61.
[14] Evans JL, Youngren JF, Goldfine ID. Effective treatments for insulin resistance: trim the fat and douse the fire. Trends Endocrinol Metab 2004;15:425–31.
[15] Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N. Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. Diabetes 1998;47:1562–9.
[16] Tsuda T, Ueno Y, Aoki H, Koda T, Horio F, Takahashi N, et al. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. Biochem Biophys Res Commun 2004;316:149–57.

[17] Vasdev S, Longerich L, Gill V. Prevention of fructose-induced hypertension by dietary vitamins. Clin Biochem 2004;37:1 – 9.
[18] Muller-Wieland D, Krone W. Disorders of lipid metabolism in insulin resistance (abstract). Herz 1995;20:33 – 46.
[19] Lombardo YB, Chicco AG. Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. J Nutr Biochem 2006;17:1 – 13.
[20] Rodbell M. Metabolism of isolated fat cells: I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 1964;239: 375 – 80.
[21] Elliott SS, Keim NL, Stern JS, Teff K, Havel PJ. Fructose, weight gain, and the insulin resistance syndrome. Am J Clin Nutr 2002;76: 911 – 22.
[22] Park J, Lemieux S, Lewis GF, Kuksis A, Steiner G. Chronic exogenous insulin and chronic carbohydrate supplementation increase de novo VLDL triglyceride fatty acid production in rats. J Lipid Res 1997;38:2529 – 36.
[23] Roglans N, Sanguino E, Peris C, Alegret M, Vázquez M, Adzet T, et al. Atorvastatin treatment induced peroxisome proliferator-activated receptor α expression and decreased plasma nonesterified fatty acids and liver triglyceride in fructose-fed rats. J Pharmacol Exp Ther 2002; 302:232 – 9.
[24] Dai S, McNeill JH. Fructose-induced hypertension in rats is concentration- and duration-dependent. J Pharmacol Toxicol Methods 1995;33:101 – 7.
[25] Hotamisligil GS. Molecular mechanisms of insulin resistance and the role of the adipocyte. Int J Obes Relat Metab Disord 2000;24:S23–7.
[26] Goldstein BJ. Insulin resistance as the core defect in type 2 diabetes mellitus. Am J Cardiol 2002;90:3G – 10G.
[27] Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, et al. Adipose-selective targeting of GLUT4 gene impairs insulin action in muscle and liver. Nature 2001;409:729 – 33.
[28] Riemens SC, Sluiter WJ, Dullaart RP. Enhanced escape of non-esterified fatty acids from tissue uptake: its role in impaired insulin-induced lowering of total rate of appearance in obesity and type II diabetes mellitus. Diabetologia 2000;43:416 – 26.
[29] Grundy SM. Hypertriglyceridemia, insulin resistance, and the metabolic syndrome. Am J Cardiol 1999;83:F25 – 9.
[30] Steiner G. Hyperinsulinaemia and hypertriglyceridaemia. J Intern Med 1994;736(Suppl):23 – 6.
[31] Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 2001;7:947 – 53.
[32] Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 2001; 7:941 – 6.
[33] Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, et al. Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem 2002;227:25863 – 6.
[34] Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. Science 1993;259:87 – 91.
[35] Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. TNF-α inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A 1994;91:4854 – 8.
[36] Togashi N, Ura N, Higashiura K, Murakami H, Shimamoto K. Effect of TNF-α-converting enzyme inhibitor on insulin resistance in fructose-fed rats. Hypertension 2002;39:578 – 80.
[37] Stennett AK, Gainer JL. TSC for hemorrhagic shock: effects on cytokines and blood pressure. Shock 2004;22:569 – 74.
[38] Frederich RC, Hamann A, Anderson S, Loellmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet induced resistance to leptin action. Nat Med 1995;1:1311 – 4.
[39] Segal KR, Landt M, Klein S. Relationship between insulin sensitivity and plasma leptin concentration in lean and obese men. Diabetes 1996;45:988 – 91.
[40] Juan CC, Au LC, Fang VS, Kang SF, Ko YH, Kuo SF, et al. Suppressed gene expression of adipocyte resistin in an insulin-resistant rat model probably by elevated free fatty acids. Biochem Biophys Res Commun 2001;289:1328 – 33.
[41] Lonnqvist F, Arner P, Nordfors L, Schalling M. Overexpression of the obese (ob) gene in adipose tissue of human obese subjects. Nat Med 1995;1:950 – 3.
[42] Rentsch J, Chiesi M. Regulation of ob gene mRNA levels in cultured adipocytes. FEBS Lett 1996;379:55 – 9.
[43] Rossi AS, Lombardo YB, Lacorte JM, Chicco AG, Rouault C, Slama G, et al. Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed, insulin-resistant rats. Am J Physiol Regul Integr Comp Physiol 2005;289:R486–94.