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RESEARCH PAPER

Crocetin improves the insulin resistance induced by high-fat diet in rats

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Background and purpose: The amelioration of insulin resistance by treatment with crocetin is closely related to the hypolipidaemic effect. The present study is designed to clarify the insulin-sensitizing mechanism of crocetin by elucidating the mechanism of regulation of lipid metabolism by crocetin.
Experimental approach: Rats given a high-fat diet were treated with crocetin for 6 weeks before hyperinsulinaemic–euglycaemic clamp. ^{14}C-palmitate was used as tracer to track the fate of non-esterified fatty acids or as substrate to measure β-oxidation rate. Triglyceride clearance in plasma and lipoprotein lipase activity in tissues were tested. Content of lipids in plasma and tissues was determined. Real-time PCR was used to assay the level of mRNA from genes involved in non-esterified fatty acid and triglyceride uptake and oxidation.
Key results: Crocetin prevented high-fat-diet induced insulin resistance (increased clamp glucose infusion rate), raised hepatic non-esterified fatty acid uptake and oxidation, accelerated triglyceride clearance in plasma, enhanced lipoprotein lipase activity in liver, and reduced the accumulation of detrimental lipids (DAG and long-chain acyl CoA) in liver and muscle. Genes involved in hepatic lipid metabolism which are regulated by peroxisome proliferator-activated receptor-α, were modulated to accelerate lipid uptake and oxidation.
Conclusions and implications: Through regulating genes involved in lipid metabolism, crocetin accelerated hepatic uptake and oxidation of non-esterified fatty acid and triglyceride, and reduced lipid availability to muscle, thus decreasing lipid accumulation in muscle and liver, and consequently improving sensitivity to insulin.
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Keywords: crocetin; fatty-acid clearance; insulin resistance; lipoprotein lipase; peroxisome proliferator-activated receptor-α

Abbreviations: ACO, acyl CoA oxidase; ACS, acyl CoA synthetase; ApoCIII, apolipoprotein CIII; CD36/FAT, CD36/fatty acid translocase; CPT-1, carnitine palmitoyl transferase-1; FATP, fatty acid transport protein; HL, hepatic lipase; LCACoA, long-chain acyl CoA; LDL, low-density lipoprotein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; PPAR, peroxisome proliferator-activated receptor; TG, triglyceride; VLDL, very-low-density lipoprotein

Introduction

Increased availability of circulating lipids (non-esterified fatty acid, NEFA and triglyceride, TG), leading to raised TG accumulation within skeletal muscle and liver, is strongly associated with the development of insulin resistance. It is known that excessive TG accumulated in those tissues will produce some harmful fatty acid-derived metabolites, such as DAG and long-chain acyl CoA (LCACoA). Although the effect of LCACoA remains controversial, it is generally believed that DAG mediates the negative effects on insulin signalling (Turinsky *et al*., 1990; Schmitz *et al*., 1997; Chavez and Summers, 2003).

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To ameliorate insulin resistance, much effort has been made to reduce the accumulation of detrimental lipids in muscle and liver, by decreasing the availability of TG and NEFA in the circulation. Agonists of the peroxisome proliferator-activated receptor-α (PPARα) are fairly successful in such attempts. PPARα is a transcription factor that has important effects on lipid homoeostasis via regulation of the expression of genes involved in lipid metabolism. It is predominantly expressed in the liver and regulates the transcription of genes involved in hepatic lipid uptake and oxidation, including fatty acid transport protein (FATP), CD36/fatty acid translocase (CD36/FAT), acyl CoA synthetase (ACS), acyl-CoA oxidase (ACO), carnitine palmitoyl transferase-1 (CPT-1) and lipoprotein lipase (LPL) (Escher and Wahli, 2000). Their activation is thought to cause sequestration of lipid into the liver for oxidation. These actions putatively reduce the amount of lipid available to skeletal

muscle, the largest insulin-sensitive organ in the body (Oakes *et al*., 1997; Guerre-Millo *et al*., 2000; Ye *et al*., 2001).

Our group has recently found that crocetin, a carotenoid extracted from *Gardenia jasminoides* Ellis, has the ability to alleviate insulin resistance induced by high-fructose diet in rats (Xi *et al*., 2007) and that this effect is accompanied by a reduction in plasma TG and NEFA. Some previous reports also confirmed that crocetin ameliorated hypertriglyceridaemia in rats and quails (Deng *et al*., 2004; He *et al*., 2007) induced by high-lipid diets. Thus, it is reasonable to presume that the reversal of insulin resistance by crocetin is closely related to its hypolipidaemic action, which may reduce lipid accumulation in insulin-sensitive tissues. So far, the mechanism(s) by which crocetin lowers plasma NEFA and TG and its significance in alleviating insulin resistance have not been clear. Here we adopted a rodent model characterized by both insulin resistance and dyslipidaemia to clarify this question. In the present study, we demonstrated that crocetin may protect against disordered lipid metabolism and the subsequent insulin resistance induced by high-fat diet in rat, probably via its modulation on some PPARα-related genes.

Methods

Preparation of crocetin

Crocetin was isolated from *Gardenia jasminoides* Ellis by our team. Briefly, comminuted dry fruit of *Gardenia jasminoides* Ellis was extracted by methanol–water. The extract was loaded onto a column of macroporous resin to eliminate saccharide and then was hydrolysed by acid. Crocetin was precipitated, collected and purified with ethanol–water. The purity of crocetin used in experiments is over 98% (assayed by HPLC) (chemical structure shown in Figure 1).

Experimental animals

All animal procedures were performed in accordance with the institutional guidelines for animal care of China Pharmaceutical University and approved by the local animal research committee. Male Wistar rats, aged 10–12 weeks from China Pharmaceutical University Animal Centre, were housed in a temperature-controlled ($22 \pm 1\,^{\circ}C$) environment with a 12-h light, 12-h dark cycle (lights on at 0600 h). After 1-week acclimatization period, 42 rats were assigned to three groups fed standard diet (normal group), high-fat diet (59% fat, 21% protein and 20% carbohydrate, expressed as a per cent of total dietary calories, control group) or high-fat diet supplemented with crocetin (crocetin group). Over the next 6 weeks, crocetin was administered in a daily dose of $50\,mg\,kg^{-1}$, for this dosage regimen significantly ameliorated hyperlipidaemia and increased insulin sensitivity (Deng *et al*., 2004; He *et al*., 2007; Xi *et al*., 2007). Nine to ten days before the experiment, the left carotid artery and right jugular vein of some rats were cannulated under halothane anaesthesia as previously described (Clark *et al*., 1990). Rats were handled daily to minimize stress and only those rats with fully recovered body weight were used for the final study.

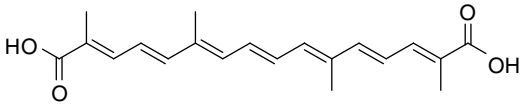

Figure 1 Chemical structure of crocetin.

Hyperinsulinaemic–euglycaemic clamping experiments

Conscious rats undergoing hyperinsulinaemic–euglycaemic clamp were infused with insulin (China Wanbang Biopharma, Xuzhou, Jiangsu, China) via the jugular cannula at a constant rate of $250\,mU\,kg^{-1}\,h^{-1}$ together with a variable rate of 30% glucose to maintain euglycaemia (Kraegen *et al*., 1983).

General tracer methodology

Arterial blood samples ($\sim 400\,\mu L$) were collected at baseline and directly before tracer administration. A CPT-1 inhibitor, etomoxir (Sigma-Aldrich Inc., Shanghai, China; $20\,\mu mol\,kg^{-1}$), was administered i.v. 20 min before tracer infusion to terminate the β-oxidation of ^{14}C-palmitate (Beijing Atom HighTech Co. Ltd, Beijing, China), so that the absolute magnitude of ^{14}C-palmitate taken up by tissue could be measured. The tracer, as the form of albumin-palmitate–^{14}C-palmitate complex (Oakes *et al*., 1999), was infused at a constant rate ($\sim 1.67 \times 10^4\,Bq\,min^{-1}$, $17\,\mu L\,min^{-1}$) into the jugular vein of conscious rats under postabsorptive (basal) conditions. To obtain plasma NEFA and ^{14}C-palmitate concentrations, 150 μL arterial blood samples were collected 10, 20, 40, 60, 80, 100 and 120 min after the start of tracer infusion. At 120 min, tracer infusion was stopped and additional blood samples were collected at 124, 128, 132 and 140 min. Plasma was rapidly separated in a refrigerated centrifuge, and a 25-μL aliquot was placed directly into 2 mL lipid extraction mixture (described below). After collection of the 140-min blood sample, rats were given a lethal dose of Na-thiobutabarbitol. Samples (~ 100 mg) of the following tissues were collected: liver and red gastrocnemius muscle ('skeletal muscle' hereafter), and epididymal fat was rapidly dissected, freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen and stored at $-70\,^{\circ}C$ awaiting analysis.

Calculations

Whole-body clearance of ^{14}C-palmitate (MCR*p*), tissue-clearance rates of ^{14}C-palmitate (K_f) and the rate of appearance of plasma NEFA (*Ra*) were calculated from tissue content and arterial plasma profile of radiolabelled tracer as previously described (Oakes *et al*., 1999, 2001). Because high-fat feeding of rats tends to increase the mass of both the liver and fat, NEFA clearance into these tissues was expressed per whole liver and adipose depot, respectively, to reflect their influence on whole-body uptake.

Determination of plasma and tissue tracer concentrations

The method of isolation of ^{14}C-palmitate from total ^{14}C plasma radioactivity has been described previously (Oakes

et al., 1999). Briefly, an initial acid lipid extraction, using a mixture of isopropanol/hexane/0.5 M H_2SO_4 (40:10:1), was followed by a polarity separation step, under alkaline conditions. The latter procedure predominantly partitioned esterified fatty acids into a hexane phase, and NEFAs in anionic form into an alcohol phase. Small corrections (<10%), based on separation of NEFA and esterified fatty acid standards, were applied for incomplete partitioning of tracer.

Tissue samples were homogenized in chloroform–methanol (2:1) using a glass hand-held homogenizer. An aliquot of this homogenate was taken to determine the total activity of the ^{14}C label.

Activities of ^{14}C in appropriate plasma and tissue fractions were measured on a Beckman LS 6000SC liquid-scintillation counter (Beckman Coulter Inc., Fullerton, CA, USA).

Plasma parameter measurements

Plasma NEFA concentration was determined using Cu-NEFA coextraction-based colorimetric kit (Nanjing Jiancheng Bioengineering Inc., Nanjing, China). Very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) were precipitated by phosphotungstic acid/Mg^{2+} reagent (Demacker *et al*., 1997), whereas LDL was selectively precipitated by polyvinylsulphate/polyethyleneglycol methyl ether (Demacker *et al*., 1984). The content of TG in plasma and corresponding supernatant containing high-density lipoprotein or high-density lipoprotein plus VLDL was determined enzymatically with commercial assay kits (GPO-PAP; Dongou Bioengineering Co. Ltd, Wenzhou, China), thus the content of TG in VLDL, LDL and high-density lipoprotein could be calculated. Plasma glucose was determined using a glucose analyser (GT-1640; ARKRAY Inc., Kyoto, Japan). Plasma insulin was determined by radioimmunoassay using commercial kits for the rat (Beijing Atom HighTech Co. Ltd).

Lipoprotein analysis

The distribution of lipoproteins in serum from individual rats was analysed by agarose–starch gel electrophoresis (Chalvardjian, 1970). Briefly, 10 μL of samples were separated by electrophoresis for about 40 min at a constant voltage of 140 V in a glycine-sodium barbitone buffer, pH 8.6. The wet gels were put into five volumes of 1.5% picric acid in 12% acetic acid for protein precipitation. After washing and drying, staining began in the mixed solution of Fat Red 7B and Oil Red O overnight. The gels were scanned using the Bio-Rad Gel-Doc XR system, and images were analysed by Quantity One (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Tissue lipid content

Triglyceride and DAG in liver and muscle were extracted and separated using the method of Kaluzny *et al*. (1985). Briefly, the lipid extract of tissue samples was separated on aminopropyl solid-phase extraction cartridges (LC-NH2, 500 mg; Supelco, Bellefonte, PA, USA) using five eluants in sequence: (1) chloroform–isopropanol 2:1; (2) hexane; (3) 1% diethyl ether, 10% methylene chloride in hexane; (4) 5% ethyl acetate in hexane; and (5) 15% ethyl acetate in hexane. Samples of TG and DAG, eluted by eluant (3) and (5), respectively, were evaporated to dryness under N_2 and reconstituted with isopropanol for assay using TG testing kit.

Long-chain acyl CoA in liver and muscle was extracted according to the method of Hegarty *et al*., 2004. Briefly, tissue samples (50 mg) were homogenized in 10% trichloroacetic acid and washed with diethyl ether and water in sequence. Then, LCACoA in tissue was hydrolysed by KOH in the presence of dithiothreitol. The liberated CoA was measured by HPLC (Hosokawa *et al*., 1989). LCACoA content was calculated using the peak area and comparison to CoA standards (Sigma-Aldrich, Shanghai, China).

β-oxidation and CPT-1 activity

For quantification of β-oxidation in liver and skeletal muscle homogenates, ^{14}C-labelled acid-soluble metabolites and CO_2 produced from ^{14}C-palmitate were counted as described previously (Doha *et al*., 2005).

Carnitine palmitoyl transferase-1 activity in liver and skeletal muscle was measured with isolated mitochondria with u.v. spectrophotometry described by Bieber *et al*. (1972). Proteins were determined by the procedure of Lowry *et al*. (1951), with BSA as standard.

Intravenous fat tolerance test and lipoprotein lipase/hepatic lipase activity test in tissues

According to the method of Chakrabarti *et al*. (2003), 20% intralipid was administered i.v. (5 mL kg^{-1}), and plasma TG levels were measured at 0, 1, 10, 30, 60 and 120 min after injection.

The enzyme extracts from liver, muscle and epididymal fat were prepared according to the method of Iverius and Lindqvist (1986), and the LPL/hepatic lipase (HL) activity was measured with commercial kits (Nanjing Jiancheng Bioengineering Inc.).

RNA isolation and real-time detection PCR

For the determination of mRNA expression levels of PPARα, FATP, CD36/FAT, ACS, L-CPT-1, ACO, LPL, apoCIII in liver, total RNA isolation, cDNA synthesis and relative quantification of target gene mRNA compared with the housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) mRNA was determined by real-time PCR as described previously (Duran-Sandoval *et al*., 2004; Achouri *et al*., 2005; Ringseis *et al*., 2007). Relative quantification was performed using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Relative-expression ratios are expressed as fold changes of mRNA abundance in the crocetin group compared with the control group.

Statistical analysis

All results are presented as means ± s.e.mean. Comparisons of two groups were performed by Student's *t*-test. Comparison of three groups was performed by one-way ANOVA incorporating a Fisher's protected least-significant-difference

post hoc test. Group sizes ranged from five to seven rats unless otherwise specified. $P<0.05$ was considered statistically significant for all comparisons.

Results

General parameters

Crocetin administration improved insulin action, which had been impaired by high-fat diets, shown as an increase in the glucose infusion rate necessary to maintain euglycaemia under hyperinsulinaemic conditions (Table 1). This beneficial effect of crocetin on insulin sensitivity was associated with a marked reduction in basal plasma insulin levels (Table 1). This last variable, a marker to judge insulin resistance, significantly increased in the control group. The normoglycaemic plasma glucose levels were unaffected (Table 1). As shown in Table 2, crocetin did not affect body weight, but did substantially decrease the ratio of fat mass to body weight. Although epididymal and mesenteric fat, classified as visceral fat, was significantly decreased, retroperitoneal fat and the subcutaneous fat, inguinal fat, did not change after crocetin treatment. The finding that the ratio of liver weight to body weight increased in the crocetin group but that the liver-weight size did not change suggests that the altered weight ratio was mostly due to the decreased weight of fat. Food intake showed no significant difference between control and crocetin groups (25.7 ± 1.5 vs $25.2 \pm 1.4\,g\,day^{-1}$ per rat; $P>0.05$), which excludes the possibility that crocetin reduces fat mass by inhibiting feeding.

Table 1 Effect of crocetin treatment on insulin sensitivity, plasma parameters and whole-body NEFA metabolism

	Normal	*Control*	*Crocetin*
GIR ($mg\,min^{-1}\,kg^{-1}$)	29.39 ± 3.52	$17.37 \pm 2.60^{\dagger\dagger}$	21.80 ± 3.05*
Insulin ($mU\,L^{-1}$)	25.56 ± 3.23	$36.94 \pm 4.89^{\dagger\dagger}$	28.99 ± 5.93*
Glucose (mM)	6.15 ± 1.42	7.61 ± 1.62	6.95 ± 1.59
TG (mM)	0.74 ± 0.13	$1.18 \pm 0.36^{\dagger}$	0.79 ± 0.11*
VLDL-TG (mM)	0.11 ± 0.06	$0.57 \pm 0.27^{\dagger\dagger}$	0.23 ± 0.07*
LDL-TG (mM)	0.17 ± 0.06	$0.32 \pm 0.11^{\dagger}$	0.37 ± 0.11
HDL-TG (mM)	0.46 ± 0.10	$0.28 \pm 0.10^{\dagger}$	0.19 ± 0.08
NEFA (mM)	0.37 ± 0.10	0.35 ± 0.09	0.29 ± 0.08
MCR*p*($mL\,min^{-1}$ per 100 g)	7.91 ± 0.73	$9.77 \pm 0.71^{\dagger\dagger}$	9.99 ± 0.99
*R*a ($\mu mol\,min^{-1}$)	9.31 ± 1.64	$12.79 \pm 1.76^{\dagger}$	10.48 ± 1.85

Abbreviations: GIR, glucose-infusion rate; HDL, high-density lipoprotein; LPL, lipoprotein lipase; MCR*p*, whole-body NEFA clearance; NEFA, non-esterified fatty acid; *R*a, rate of appearance of plasma NEFA; TG, triglyceride; VLDL, very-low-density lipoprotein.
Data are means ± s.e.mean. $n=6$–7 per group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs normal; *$P<0.05$ vs control (ANOVA).

Table 2 Effect of crocetin on the body weight and the weight ratio of liver and adipose tissue to body

	Normal	*Control*	*Crocetin*
Body weight (*g*)	391.9 ± 38.7	417.7 ± 49.5	400.6 ± 31.9
Tissue weight (% of body weight)			
Liver	3.78 ± 0.14	3.62 ± 0.23	3.91 ± 0.13*
Epididymal fat	1.14 ± 0.21	$1.95 \pm 0.34^{\dagger\dagger}$	1.51 ± 0.30*
Mesenteric fat	0.73 ± 0.18	$1.22 \pm 0.18^{\dagger\dagger}$	0.95 ± 0.18*
Retroperitoneal fat	2.01 ± 0.3	2.47 ± 0.53	1.98 ± 0.25
Inguinal fat	1.73 ± 0.47	2.15 ± 0.18	2.06 ± 0.40

Data are means ± s.e.mean. $n=6$–7 per group. $^{\dagger\dagger}P<0.01$ vs normal, *$P<0.05$ vs control (ANOVA).

Plasma lipids

In the crocetin group, circulating TG level was significantly reduced but plasma NEFA concentration had no obvious change compared with control rats (Table 1). By chemical precipitation, we found that crocetin significantly reduced the TG content in VLDL. To assess whether the decrease of VLDL-TG played any role in the reduction in plasma TG concentration, the overall relationships between VLDL-TG and plasma TG concentrations were tested. The result showed a significant positive relationship between VLDL-TG and plasma TG concentration ($r^2=0.8558$; $P<0.01$). Thus, in crocetin-treated rats, the reduction of TG content in VLDL accounted for the decrease in plasma TG level.

Tissue-specific and whole-body NEFA metabolism

The intrinsic ability of a tissue to take up a circulating substrate is described by the so-called tissue clearance. This parameter depends on factors such as substrate transporters in the plasma membrane and substrate sequestration due to intracellular enzyme activity. NEFA clearance (K_f) into liver, epididymal fat and skeletal muscle was measured in this study using the accumulation of fatty acid tracer, ^{14}C-palmitate, under the inhibition of CPT-1 by etomoxir. Crocetin-treated animals showed a substantially increased NEFA clearance into liver compared with untreated animals (Figure 2a), accompanied by a decreased tendency in NEFA clearance into muscle and fat (Figures 2b and c). This may explain why the whole-body NEFA clearance (MCR*p*) did not change after crocetin treatment (Table 1). Whole-body NEFA mobilization (*R*a) was estimated based on steady-state tracer dilution using a constant infusion of ^{14}C-palmitate. High-fat-fed rats showed substantially elevated *R*a than the rats fed with standard diet (Table 1). Crocetin treatment had no effect on *R*a, although there was a nonsignificant tendency for *R*a to be decreased in the crocetin group.

Triglyceride clearance

Rats on a high-fat diet, treated with crocetin, showed a significant enhancement (22%) in TG clearance (Figure 3) compared with control rats when challenged i.v. with a fat emulsion (20% intralipid).

Composition of serum lipoproteins

Separation of the different lipoprotein fractions by electrophoresis followed by lipostaining indicated a decrease in VLDL accompanied by a rise in LDL, for the ratio of LDL to VLDL was markedly increased by crocetin treatment (0.45 ± 0.11 vs 0.26 ± 0.02 in control group; $P<0.05$).

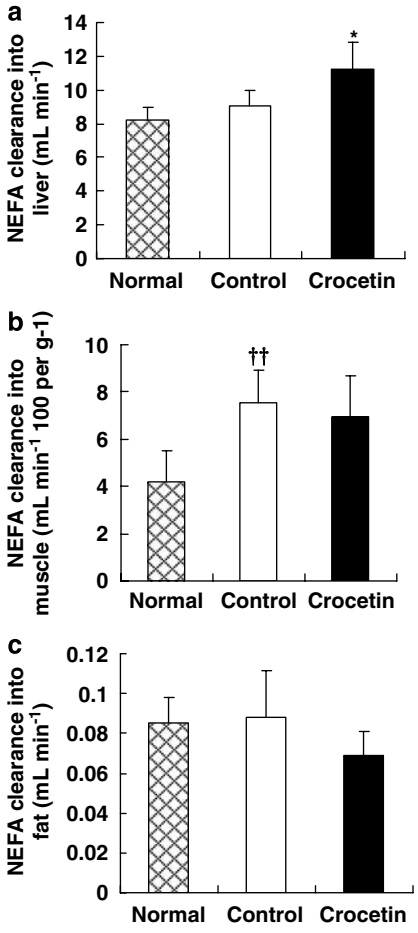

Figure 2 Effect of crocetin treatment of rats on a high-fat diet for 6 weeks on NEFA clearance (K_f) into (**a**) liver, (**b**) muscle and (**c**) fat. Normal, standard diet; control, high-fat diet alone; crocetin, high-fat diet + crocetin. Data are means ± s.e.mean. $n = 6$–7 per group. $^{††}P < 0.01$ vs normal; $^{*}P < 0.05$ vs control (ANOVA). NEFA, non-esterified fatty acid.

LPL/HL activity in tissues

Results *in vitro* of LPL activity in adipose and muscle and HL activity in liver showed no significant difference between crocetin and control groups, whereas a pronounced rise in hepatic LPL activity was observed after crocetin treatment, which may account for the increased LPL activity *in vivo* reflected by the i.v. fat tolerance test (Table 3).

β-*oxidation and CPT-1 activity in liver and muscle*

As seen in Table 4, rats on a high-fat diet showed no obvious change in NEFA β-oxidation rate or mitochondrial CPT-1 activity in liver and skeletal muscle compared with rats fed with standard diet. In the crocetin group, hepatic β-oxidation rate together with CPT-1 activity was significantly higher than that in control rats, but a similar increase was not observed in skeletal muscle.

Tissue lipid content

High-fat diet raised the hepatic (Figures 4a–c) and muscular (Figure 4d–f) content of TG, DAG and LCACoA. Crocetin

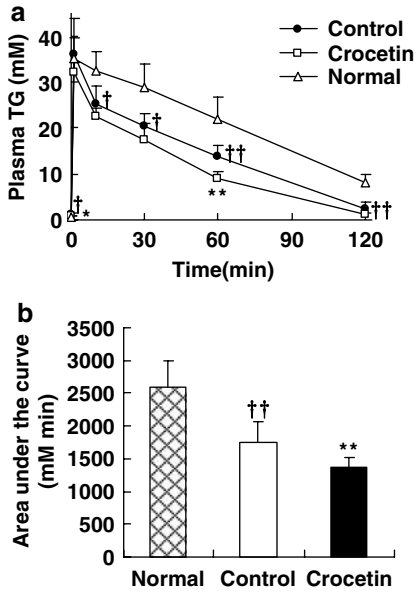

Figure 3 Effect of crocetin on plasma TG clearance in rats on a high-fat diet. (a) The concentration of TG in plasma at varied time; (b) The area under the cure, Animals fed with standard diet, high-fat diet or high-fat diet plus crocetin were injected with 20% intralipid, and then blood was collected at various times (as shown) for TG assay. Normal, standard diet; control, high-fat diet alone; crocetin, high-fat diet + crocetin. Data are mean ± s.e.mean. $n = 5$–6 per group. $^{†}P < 0.05$, $^{††}P < 0.01$ vs normal; $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (ANOVA). TG, triglyceride.

Table 3 Effect of crocetin on lipase (LPL and HL) activity in tissues

NEFA production ($\mu mol\,h^{-1}\,g^{-1}$)	*Normal*	*Control*	*Crocetin*
Liver-LPL	51.62 ± 7.75	59.78 ± 6.64	72.08 ± 5.58**
Liver-HL	62.40 ± 8.52	71.04 ± 15.98	82.89 ± 17.19
Muscle-LPL	42.77 ± 4.91	51.04 ± 5.73†	46.12 ± 11.30
Fat-LPL	48.56 ± 3.44	44.60 ± 2.18†	45.02 ± 6.61

Abbreviations: HL, hepatic lipase; LPL, lipoprotein lipase.
Data are means ± s.e.mean. $n = 6$–7 per group. $^{†}P < 0.05$ vs normal; $^{**}P < 0.01$ vs control (ANOVA).

Table 4 Effect of crocetin on (a) β-oxidation and (b) CPT-1 activity in liver and muscle

	Normal	*Control*	*Crocetin*
(a) β-oxidation			
Palmitate-oxidation rate ($\mu mol\,h^{-1}\,g^{-1}$)			
Liver	3.49 ± 0.54	3.25 ± 0.49	4.00 ± 0.59*
Muscle	0.77 ± 0.14	0.73 ± 0.17	0.80 ± 0.15
(b) CPT-1 activity			
CoA production ($nmol\,min^{-1}$ *per mg protein*)			
Liver	10.27 ± 1.08	9.85 ± 1.55	13.46 ± 2.11**
Muscle	4.11 ± 1.06	3.96 ± 1.10	4.50 ± 1.28

Abbreviations: CPT-1, carnitine palmitoyl transferase-1; NEFA, non-esterified fatty acid.
Data are means ± s.e.mean. $n = 6$–7 per group.
$^{*}P < 0.05$, $^{**}P < 0.01$ vs control (ANOVA).

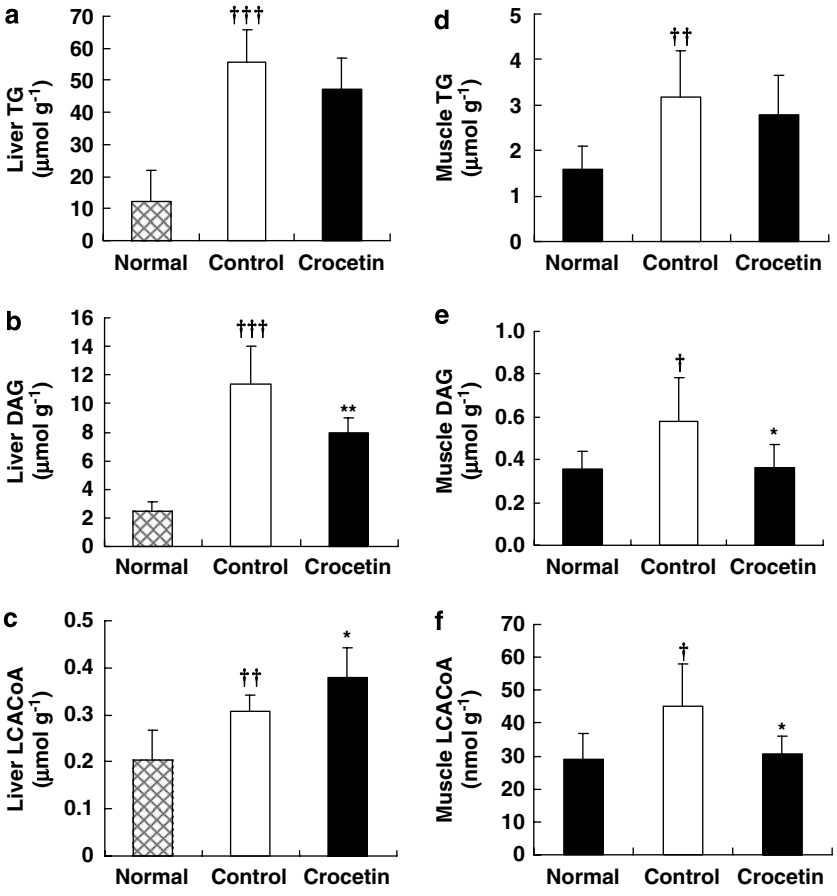

Figure 4 Tissue lipid contents of liver (**a**–**c**) and skeletal muscle (**d**–**f**). Normal, standard diet; control, high-fat diet alone; crocetin, high-fat diet + crocetin. Data are means ± s.e.mean. $n = 6$–9 per group. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs normal; $^{*}P<0.05$, $^{**}P<0.01$ vs control (ANOVA).

treatment normalized DAG and LCACoA levels but did not significantly lower TG in skeletal muscle (Figures 4d–f). In liver, the accumulation of DAG (Figure 4b) was reduced by crocetin but no obvious change was seen on TG content (Figure 4a). In contrast to the effects in skeletal muscle, crocetin increased the hepatic LCACoA content of high-fat-fed rats (Figure 4c).

Relative mRNA level in the liver of high-fat-fed rats

Relative mRNA levels of ACO, ACS, apoCIII, CD36/FAT, CPT-1, FATP and LPL in liver of crocetin-treated rats were altered significantly compared with control values ($P<0.05$; Figure 5). But PPARα expression did not show significant difference between two groups.

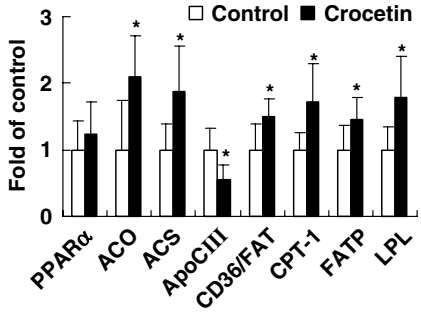

Figure 5 Effect of crocetin on hepatic abundance of mRNA from genes involved in oxidation and uptake of NEFA and TG, PPARα, ACO, ACS, apoCIII, CD36/FAT, CPT-1, FATP and LPL in rats on a high-fat diet. Control, high-fat diet alone; crocetin, high-fat diet + crocetin. Bars represent means ± s.e.mean and are expressed as fold changes of mRNA abundance in the crocetin group compared with the control group. $n = 5$–7 per group. $^{*}P<0.05$ vs control (*t*-test). ACO, acyl CoA oxidase; ACS, acyl CoA synthetase; ApoCIII, apolipoprotein CIII; CPT-1, carnitine palmitoyl transferase-1; FATP, fatty acid transport protein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; PPARα, peroxisome proliferator-activated receptor-α; TG, triglyceride.

Discussion and conclusions

In this study, we have shown, for the first time, that crocetin, a natural carotenoid, is an effective insulin-sensitizing agent in a non-genetic rat model of insulin resistance induced by high-fat diet. We have also provided evidence that the effect of crocetin in TG and NEFA metabolism plays a causative role in the amelioration of insulin resistance. The clearance of plasma TG and NEFA to liver was significantly elevated after crocetin treatment, and this impact may reduce the availability of lipids to skeletal muscle as the largest peripheral insulin-sensitive tissue.

The hypotriglyceridaemic action of crocetin may be explained by the increased plasma TG clearance, assayed by the i.v. fat tolerance test, which reflects the elevated total lipolytic activity of LPL and HL. LPL is the key enzyme in the hydrolysis of TGs in chylomicrons and VLDL (Applebaum-Bowden, 1995), and HL is known to be important in the final steps in the lipolytic conversion of VLDL to LDL by hydrolysis of the LDL TG (Connelly, 1999). The increased lipolytic activity in crocetin-treated rats induced more rapid stripping of TG from VLDL particles, leading to lowered TG content in VLDL and an accelerated conversion of VLDL to TG-poor remnants, LDL. Study on LPL/HL activity in tissues indicated that this increase in total lipolytic activity was mainly due to the elevation in hepatic LPL activity. Changes in LPL activity in different tissues determine, in part, not only plasma TG levels, but also the uptake of TG by different tissues (Jansen *et al*., 1998). Thus, despite the lack of direct kinetic evidence to demonstrate the turnover of plasma TG, it can be deduced that liver, with the increase in LPL activity after crocetin treatment, would take up more plasma TG.

In basal conditions, neither whole-body NEFA clearance nor whole-body NEFA mobilization were obviously changed by crocetin treatment, which helped to explain the non-significant variations in plasma NEFA level. However, tissue-specific clearance of NEFA was redistributed after crocetin treatment, that is NEFA clearance into liver significantly increased, accompanied by a tendency towards a decreased clearance rate in skeletal muscle and adipose tissue. This may suggest that liver had taken up the part of NEFA that should have been transported into skeletal muscle and adipose tissue by the increased capacity for NEFA uptake after crocetin treatment.

To illuminate the fate of TG and NEFA transported to liver, hepatic β-oxidation, as the formation of acid-soluble metabolites and CO_2 production, was measured, as well as the activity of CPT-1, the rate-limiting enzyme in NEFA mitochondrial β-oxidation. The elevation in these two parameters suggests that more of the NEFA and TG taken up into the liver in the crocetin group were subject to oxidation.

Elevation in hepatic β-oxidation may surpass the elevation in hepatic uptake of NEFA and TG, as crocetin reduced the hepatic DAG content and demonstrated a decreased trend in TG. Unexpectedly, LCACoA content in liver was increased by crocetin treatment. This increase could result from expanded peroxisome population as shown by elevated ACO expression (Moody *et al*., 1991). But as long as LCACoAs are confined within the peroxisomes, these lipid moieties might not be able to interfere with insulin signalling or carbohydrate metabolism in cytosol (Hegarty *et al*., 2004).

Although crocetin did not significantly reduce the accumulation of TG in skeletal muscle of high-fat-fed rats, a substantial decrement was observed in the content of DAG and LCACoA. Intramuscular TG is often used as a marker of insulin resistance, but there is no demonstration that TG *per se* interferes with insulin action. In contrast, DAG and LCACoA have been shown to be important molecules that interfere with various aspects of insulin signalling and carbohydrate metabolism and are likely to contribute to the detrimental effects of intramuscular lipid accumulation on insulin action (Shulman, 2000; Cooney *et al*., 2002). Besides, based on the primary importance of skeletal muscle in insulin-stimulated glucose disposal (it accounts for more than 80% of whole-body glucose disposal) (DeFronzo *et al*., 1981), the reduction of these lipid intermediates is believed to contribute greatly to the amelioration of whole-body insulin sensitivity. In fact, insulin resistance induced by high-fat diet is associated with an increase in NEFA clearance into skeletal muscle (Hegarty *et al*., 2002), but crocetin failed to correct this effect. As well, crocetin exerted no effect on muscular NEFA β-oxidation and LPL activity in skeletal muscle. Thus, the reduction of muscular lipid content by crocetin is presumably due to the lowered availability of TG. It may suggest that the main target organ of crocetin is liver, and the improvement of lipid profiles in skeletal muscle is a side effect of these hepatic effects of crocetin.

PPARα, once activated, would heterodimerize with the retinoid X receptor and bind to peroxisome proliferator response elements in the promoters of genes including LPL (Schoonjans *et al*., 1996), ACO (Kliewer *et al*., 1992), ACS (Schoonjans *et al*., 1995) and CPT-1 (Louet *et al*., 2001), the transcription of which would be activated by the complex. The gene for apoCIII is suppressed by PPARα activation due to competition on the same response element between PPARα–retinoid X receptor heterodimers and the liver-enriched transcriptional activator, hepatocyte nuclear factor-4 (Hertz *et al*., 1995). Although peroxisome proliferator response elements have not been identified in CD36/FAT or FATP genes to date, study on PPARα-null mouse indicates that PPARα has an obligatory role in induction of CD36/FAT and FATP mRNAs in liver (Motojima *et al*., 1998). On the basis of the organ selectivity of crocetin, only the hepatic expression of those PPARα-related genes was tested. The result showed that crocetin treatment increased hepatic mRNA level of LPL and reduced that of apoCIII, the apolipoprotein that inhibits the hydrolysing action of LPL, which is consistent with the elevated LPL activity in liver. The enhancement of hepatic NEFA uptake by crocetin fits well with the upregulation of FATP, CD36/FAT and ACS, the genes involved in NEFA transport (Martin *et al*., 1997; Motojima *et al*., 1998). The fact that crocetin raised mRNA level of hepatic CPT-1 may account for the increased β-oxidation in liver. Although crocetin did not directly enhance the expression of PPARα, it did increase the mRNA level of hepatic ACO, the well-known marker of PPARα activation. Furthermore, like typical PPARα activators, crocetin reduced the mass of visceral fat. All this evidence suggests that crocetin may act as a PPARα agonist.

In summary, crocetin enhanced the ability of the liver to take up and oxidize NEFA and TG by modulating the PPARα-related genes in liver, leading to the redistribution of tissue-specific NEFA clearance and depressed TG availability. Consequently, the hepatic and skeletal muscle accumulation of detrimental lipids decreased, with consequent improvement of insulin resistance induced by high-fat diets in rats.

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Conflict of interest

The authors state no conflict of interest.

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