British Journal of Pharmacology (2007) 151, 610–617
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Crocetin attenuates palmitate-induced insulin insensitivity and disordered tumor necrosis factor-α and adiponectin expression in rat adipocytes

L Xi[1], Z Qian[1], G Xu[2], C Zhou[1] and S Sun[1]

[1]*Department of Pharmacology, China Pharmaceutical University, Nanjing, PR China and* [2]*Center for New Drug Research and Development, College of Life Science, Nanjing Normal University, Nanjing, PR China*

Background and purpose: A number of studies have implicated adipocyte-derived factors in the development of insulin resistance. Intracellular redox status has been reported to play a significant role in the modulation of insulin action. This study was designed to investigate the potential of crocetin, a potent antioxidant, to protect adipocytes against the induction of insulin insensitivity and disordered expression of tumor necrosis factor (TNF)-α and adiponectin *in vitro*.
Experimental approach: We used palmitate to induce insulin resistance in freshly isolated rat adipocytes, and observed the effect of crocetin, N-acetylcysteine, diphenyleneiodonium, rotenone and oxypurinol. Insulin sensitivity was measured using 2-deoxy-D-[1-^{3}H]-glucose uptake assay. Levels of glucose transporter 4, TNF-α and adiponectin were evaluated by immunoblot analysis, and levels of mRNA for TNF-α and adiponectin by reverse transcription-polymerase chain reaction analysis. Intracellular production of reactive oxygen species (ROS) was determined spectrofluorometrically using 2′,7′-dichlorofluorescin diacetate.
Key results: Palmitate induced a 45% decrease in insulin-stimulated glucose uptake in adipocytes. The mRNA and protein expression of TNF-α were enhanced by 64% and 59% respectively whereas the mRNA and protein expression of adiponectin were reduced by 43% and 36% respectively by palmitate treatment. These changes were accompanied by a 54% increase in intracellular ROS levels. Crocetin, N-acetylcysteine and diphenyleneiodonium were found to attenuate these abnormalities.
Conclusions and Implications: Crocetin blocked the impaired insulin-stimulated glucose uptake and disordered TNF-α and adiponectin expression induced by palmitate in rat adipocytes. Inactivation of NADPH oxidase may account for these observations.
British Journal of Pharmacology (2007) **151,** 610–617; doi:10.1038/sj.bjp.0707276; published online 30 April 2007

Keywords: crocetin; palmitate; insulin resistance; TNF-α; adiponectin; oxidative stress; NADPH oxidase; adipocyte

Abbreviations: BSA, bovine serum albumin; DPI, diphenyleneiodonium; FFAs, free fatty acids; GLUT4, glucose transporter 4; KRBH, Krebs–Ringer bicarbonate HEPES; MMLV, Moloney murine leukaemia virus; NAC, *N*-acetylcysteine; PKC, protein kinase C; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumour necrosis factor-α

Introduction

Insulin resistance, characterized by lowered responses of body tissues to insulin action, is a major public health problem closely associated with obesity, dyslipidemia, hypertension and type II diabetes mellitus (Reaven and Laws, 1994). The molecular mechanisms underlying the development of insulin resistance and its related diseases remain unclear but are thought to involve a number of adipocyte-derived factors (Arner, 2003; Lau *et al*., 2005). As a primary energy-storing organ, adipocytes accumulate triglycerides and provide energy in the form of free fatty acids (FFAs), which is accepted as a crucial link between obesity and insulin resistance. Adipocytes also produce numerous hormones and cytokines that participate in a variety of physiological functions, among which tumour necrosis factor (TNF-α) and adiponectin, as well as some other adipokines, have been considered as important modulators of insulin sensitivity (Borden, 1997). Although expressed at high level in other cells, the inflammatory cytokine TNF-α produced from adipocytes has received much attention because of its insulin resistance-

Correspondence: Professor Z Qian, Department of Pharmacology, China Pharmaceutical University, PO Box 46, 24 Tongjia Xiang, Nanjing 210009, PR China.
E-mail: zhiyu.qian@yahoo.com
Received 13 February 2007; revised 26 March 2007; accepted 27 March 2007; published online 30 April 2007

inducing property (Hotamisligil *et al.*, 1993). Adiponectin is an adipocyte-specific circulating protein possessing insulin-sensitizing, anti-inflammatory and other beneficial effects (Bays *et al.*, 2004).

Adipocyte dysfunction characterized by excessive release of FFAs and disordered expression/secretion of adipokines is implicated in the progression of insulin resistance (Arner, 2003; Bays *et al.*, 2004; Evans *et al.*, 2004), but the underlying mechanisms are seldom known. Growing evidence suggests an important role of intracellular redox status in the modulation of insulin action, and increased oxidative stress in accumulated fat is recognized as an early instigator of insulin resistance syndrome (Evans *et al.*, 2004; Furukawa *et al.*, 2004). In cultured adipocytes, FFAs lead to dysregulated production of adipokines owing to increased cellular oxidative stress (Furukawa *et al.*, 2004) and chronic exposure to oxidative stress impairs insulin signalling (Rudich *et al.*, 1998). The use of antioxidants has been proposed as a potential new approach for the treatment of insulin resistance and associated diseases (Caballero, 1993; Tsuda *et al.*, 2003; Evans *et al.*, 2004; Vasdev *et al.*, 2004). Crocetin is a unique carotenoid found in the dried stigma of *Crocus sativus* Linne and the fruit of *Gardenia jaminoides* Ellis. This compound is a potent antioxidant possessing multiple pharmacological properties including anti-inflammatory, anti-atherosclerotic and anticarcinogenic activities (Giaccio, 2004). Our laboratory has recently demonstrated that crocetin has a beneficial effect on insulin sensitivity in fructose-fed rats (Xi *et al.*, 2007).

In the present study, we demonstrate that crocetin may protect against impaired insulin-stimulated glucose uptake and disordered expression of adiponectin and TNF-α induced by palmitate, a saturated fatty acid, in rat adipocytes, probably via its potent antioxidant effects.

Methods

Isolation and treatment of rat adipocytes

Male Wistar rats from Slac Laboratory Animal Company (Shanghai, China) were housed in a temperature- and light-controlled (12 h light/dark cycles) room, with free access to water and standard laboratory chow. All procedures were performed in accordance with the institutional guidelines for animal care of China Pharmaceutical University and approved by the local animal research committee.

The epididymal adipocytes were isolated by collagenase digestion based on the method of Rodbell (1964) with minor modification. Briefly, the animals at the body weight of 180–200 g were killed by decapitation after an overnight fast. The epididymal fat pads were removed, and washed with 0.9% NaCl solution (37°C) under sterile conditions. The minced adipose tissues were digested into cells for 90 min at 37°C in Krebs–Ringer bicarbonate HEPES (KRBH) buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 20 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) supplemented with 0.5% bovine serum albumin (BSA, Fraction V), 1 mM pyruvate and 0.1% type IV collagenase. The cell suspension was filtered through nylon mesh (250 μm), and washed with KRBH buffer containing 0.5% BSA and 1 mM pyruvate three times. The harvested cells were resuspended to give $1-1.5 \times 10^6$ cells ml^{-1} in KRBH buffer containing 0.5% BSA and 1 mM pyruvate at 37°C, in 5% CO_2/95% O_2.

Table 1 The inhibitors of different enzymes involved in ROS production, which were used in this study

Agents	*Type of action*
N-acetylcysteine	General antioxidant
Diphenyleneiodonium	Specific inhibitor of NADPH oxidase
Rotenone	Specific inhibitor of mitochondrial electron transport chain complex I
Oxypurinol	Specific inhibitor of xanthine oxidase

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

After stabilization (incubation at 37°C for 1 h; 5% CO_2/95% O_2), the cells ($2–3 \times 10^5$ per tube; 2 ml, sterile polypropylene) were treated with control solutions (fatty acid-free BSA and vehicle; see below), palmitate (300 μM), palmitate (300 μM) plus high-concentration crocetin (50 μM) and low-concentration crocetin (5 μM), respectively, for 10 h at 37°C, in 5% CO_2/95% O_2. The incubation period is based on preliminary experiments in our laboratory indicating that insulin-stimulated glucose uptake was significantly decreased (by approximately 50%) by palmitate at this time point. Other experimental groups, used to examine the possible role of oxidative stress and different enzymes in this cell model, were treated under the same conditions, with palmitate plus either *N*-acetylcysteine (NAC, 10 mM), diphenyleneiodonium (DPI, 20 μM), rotenone (100 μM) or oxypurinol (100 μM); these are different types of inhibitors of the enzymes that catalyse the production of reactive oxygen species (ROS) (Table 1).

The palmitate solution used for incubation with the adipocytes was prepared by conjugating the fatty acid with fatty acid-free BSA according to the method of (Cousin *et al.*, 2001). Control cells were treated with identical concentration of fatty acid-free BSA without palmitate. Crocetin was dissolved in 0.1% dimethyl sulphoxide in KRBH buffer and other groups were treated with identical concentrations of dimethyl sulphoxide.

Glucose uptake assay

Glucose uptake assay was performed using radiolabeled 2-deoxyglucose. Briefly, the adipocytes were incubated with or without insulin (10 nM) in KRBH buffer containing 0.5% BSA and 1 mM pyruvate at 37°C for 30 min. Then 2-deoxy-D-[1–^{3}H]glucose in KRBH buffer was added (final concentration 0.1 mM) to the cells at 37°C for 5 min. The reaction 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 cell layer was measured using a Wallac Guardian 1414 liquid scintillation counter (PerkinElmer Life and Analytical Sciences Inc., Boston, MA, USA) in OptiPhase HiSafe 3 liquid scintillation cocktail (Wallac, Turku, Finland). Nonspecific glucose uptake was simultaneously determined in the presence of cytochalasin B.

Plasma membrane preparation and GLUT4 translocation assay
The plasma membrane fraction of adipocytes was prepared based on a previously published method (McKeel and Jarett, 1970) with minor modification. Briefly, after treatment with or without 10 nM insulin for 30 min, the adipocytes were homogenized in a buffer containing 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM Tris-HCl (pH 7.4) in the presence of protease inhibitors. The homogenate was centrifuged at 12 000 *g* for 15 min. After washing, the pellet was resuspended and centrifuged at 800 *g* for 10 min. After centrifugation of the supernatant at 12 000 *g* for 20 min, the pellet was resuspended and centrifuged at 110 000 *g* for 60 min on a linear sucrose gradient with the density ranging from 1.05 to 1.25 g ml^{-1}. The plasma membrane band was removed and centrifuged at 45 000 *g* for 30 min. All these centrifugations were carried out at 4°C. The final pellet was resuspended to give a concentration of 1 mg protein ml^{-1}. The plasma membrane extract was subjected to immunoblot analysis as described below.

Immunoblot analysis
The adipocytes were solubilized in cold lysis buffer (50 mM HEPES, 50 mM NaCl, 1% Triton X-100 and 10 mM EDTA) containing freshly added protease inhibitors (25 μg ml^{-1} aprotinin, 25 μg ml^{-1} leupeptin and 1 mM phenylmethylsulphonyl fluoride). For immunoblot analysis, samples of total cell lysates or plasma membrane extracts were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% resolving gel with 6% stacking gel. Proteins were blotted onto polyvinylidine difluoride membranes by electrotransfer. After blocking with 5% dried milk in PBS at 4°C overnight, membranes were incubated with specific antibodies against glucose transporter 4 (GLUT4), TNF-α or adiponectin in PBS containing 1% dried milk. Subsequently, membranes were incubated with horseradish peroxidase-linked secondary antibodies for enhanced chemiluminescent detection (Amersham, Sweden). The density of the bands was scanned using a Bio-Rad Molecular Imager.

Protein determination
The concentrations of protein in different samples were determined with a bicinchoninic acid protein assay kit (Jiangcheng, Nanjing, China), using BSA as the standard.

Reverse transcription-polymerase chain reaction analysis
Total cellular RNA (2.0 μg), extracted using Trizol reagent (Gibco BRL, Burlington, Canada), was incubated with Oligo dT(18) primer (0.5 μg μl^{-1}) at 70°C for 5 min, and reverse transcribed to cDNA in a mixture containing 5 × Moloney murine leukaemia virus (MMLV) reaction buffer, 40 U RNase inhibitor (Toyobo, Osaka, Japan), 0.52 mM deoxynucleoside triphosphate (dNTP) Mix (Bio-Rad, Hercules, CA, USA), and 200 U MMLV reverse transcriptase (Toyobo). Polymerase chain reaction (PCR) was performed in a mixture containing 2.5 μl 10 × PCR buffer, 2 μl reverse transcribed template solution, 1.5 mM $MgCl_2$, 0.5 μM of each sense and antisense primers (Sangon, Shanghai, China), 0.2 mM dNTP Mix (Bio-Rad) and 1 U iTaq DNA polymerase (Bio-Rad). Preliminary experiments were conducted with different cycles to find the optimal conditions for PCR amplification of each gene. The specific primer sequences were: β-actin (GenBank No.NM_031144) 5′-GAG AAG ATT TGG CAC CAC AC-3′ (sense) and 5′-CAT CAC AAT GCC AGT GGT AC-3′ (antisense), GLUT4 (GenBank No. NM_012751) 5′-GTG TGG CTG TGC CAT CTT GA-3′ (sense) and 5′-AGG AAG GTG AAG ATG AAG AAG CC-3′ (antisense), TNF-α (GenBank No.X66539) 5′-CTC GAG TGA CAA GCC CGT AG-3′ (sense) and 5′-TTG ACC TCA GCG CTG AGC AG-3′ (antisense), and adiponectin (GenBank No. NM_1447445′-TCC TGG TCA CAA TGG GAT ACC-3′ (sense) and 5′-ATC TCC TGG GTC ACC CTT AGG-3′ (antisense). iTaq DNA polymerase was activated by a 94°C incubation step for 5 min. After initial denaturation, reactions were performed as follows: for β-actin detection, 56°C for 50 s, 72°C for 40 s, 30 cycles (213 bp); for GLUT4, 58°C for 40 s, 72°C for 40 s, 35 cycles (318 bp); for TNF-α detection, 55°C for 50 s, 72°C for 40 s, 30 cycles (386 bp); for adiponectin detection, 56°C for 40 s, 72°C for 40 s, 28 cycles (109 bp). A final extension step at 72°C for 7 min was performed. The products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The density of the bands was measured using a Bio-Rad Gel Documentation system. Relative quantitation for the PCR products was calculated after normalization to the amount of β-actin mRNA level.

Determination of ROS production
Intracellular ROS production was measured using 2′,7′-dichlorofluorescein diacetate as described previously (Moini *et al*., 2002). Briefly, the cells were loaded with 20 μM 2′,7′-dichlorofluorescein diacetate for 30 min. The chemical is oxidized intracellularly by ROS to the fluorescent form of the dye 2′,7′-dichlorofluorescein. After the medium was removed, the cells were sonicated and then the fluorescence of 2′,7′-dichlorofluorescein was measured with a spectrofluorometer at 485-nm excitation and 525-nm emission. Based on the previous studies in our laboratory, crocetin has no effect on fluorescence with both excitation and emission wavelength for ROS determination. The data were expressed in arbitrary units and normalized to the protein content of adipocytes for determination.

Data analysis
All data were expressed as mean ± s.d. unless otherwise stated. The differences among groups were analysed by analysis of variance. *P*-values less than 0.05 were considered to be statistically significant.

Chemicals
Crocetin (> 98%, high-performance liquid chromatography) were prepared from the fruit of *Gardenia jaminoides* Ellis in our laboratory (Xi *et al*., 2005). BSA was purchased from Roche (Mannheim, Germany). 2-Deoxy-D-[1–^{3}H]glucose (5.4 Ci mmol^{-1}) was purchased from Atom High-tech (Beijing, China). Polyclonal antibodies against GLUT4, adiponectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and other antibodies from Boster

Bioengineering (Wuhan, China). Other chemicals were of analytical or molecular biology grade and obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

Results

Insulin-stimulated 2-deoxyglucose uptake, GLUT4 translocation, intracellular GLUT4 protein and mRNA expression

As shown in Figure 1, palmitate treatment (300 μM, 10 h) induced a significant inhibition of insulin-stimulated 2-deoxyglucose uptake by adipocytes, indicating that the cells became resistant to insulin. This inhibition was reversed by adding crocetin at either 50 μM or 5 μM, NAC or DPI, but rotenone and oxypurinol did not show any reversal of the palmitate-induced inhibition (Figure 1). There was no significant difference in basal glucose uptake among all groups. Basal and insulin-stimulated GLUT4 translocation (Figure 2a), intracellular GLUT4 protein expression (Figure 2b) and mRNA expression (Figure 4) were comparable among all groups.

Intracellular protein and mRNA expression of TNF-α and adiponectin

Figures 3 and 4 demonstrate that both protein and mRNA expression of TNF-α were significantly enhanced, while those of adiponectin were reduced in palmitate-treated adipocytes. This abnormal expression of TNF-α and adiponectin was corrected by adding crocetin at either 50 μM or 5 μM, NAC or DPI, but rotenone and oxypurinol were ineffective in reversing palmitate-induced changes (Figure 4)

Intracellular ROS production

A significant elevation in intracellular ROS level was observed in palmitate-treated adipocytes (Figure 5) and this

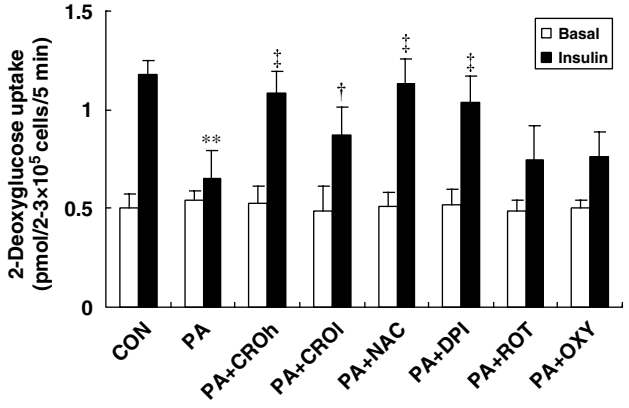

Figure 1 Insulin-stimulated 2-deoxyglucose uptake in control (CON) and treated adipocytes. Treatments were with palmitate alone (PA) or palmitate with crocetin (50 μM, PA + CROh; 5 μM, PA + CROI), *N*-acetylcysteine (PA + NAC), diphenyleneiodonium (PA + DPI), rotenone (PA + ROT) or oxypurinol (PA + OXY). 2-Deoxy-D-[1–^{3}H]glucose was added (0.1 mM) at 37°C for 5 min after incubation of adipocytes (2–3 × 10^5) with 10 nM insulin at 37°C for 30 min, and then 2-deoxy-D-[1–^{3}H]glucose uptake was measured. Data are expressed as mean ± s.d. of three independent experiments ($n = 6$). **$P < 0.01$ vs CON; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs PA.

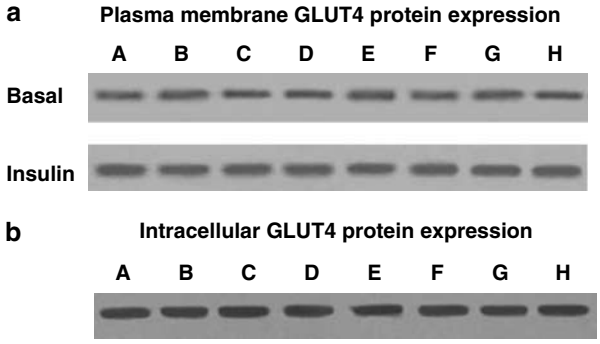

Figure 2 GLUT4 translocation (**a**) and intracellular GLUT4 protein expression (**b**) in control (CON) and treated adipocytes. Treatments were with palmitate alone (PA) or palmitate with crocetin (50 μM, PA + CROh; 5 μM, PA + CROI), *N*-acetylcysteine (PA + NAC), diphenyleneiodonium (PA + DPI), rotenone (PA + ROT) or oxypurinol (PA + OXY). (**a**) After treatment with or without 10 nM insulin for 30 min, plasma membrane extracts were prepared and GLUT4 protein expression was examined by immunoblot analysis. (**b**) Total cell lysates were prepared and GLUT4 protein expression was examined by immunoblot analysis. Representative results of an immunoblot analysis are shown. A = CON; B = PA; C = PA + CROh; D = PA + CROI; E = PA + NAC; F = PA + DPI; G = PA + ROT; H = PA + OXY.

was controlled by DPI (a specific nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor), NAC (a general antioxidant) and two concentrations of crocetin. Rotenone and oxypurinol, however, did not exhibit any inhibitory effects on the increased intracellular ROS production, induced by palmitate. These results suggest that NADPH oxidase may constitute the major source of enhanced oxidative stress in palmitate-treated adipocytes.

Discussion and Conclusions

Much attention has focused on the potential role of adipocyte dysfunction in the onset and development of insulin resistance (Bergman and Mittelman, 1998). Although traditionally recognized as an energy-storing organ, the adipocyte has been clearly established as an active endocrine and paracrine organ, which secretes various adipokines. Adipocyte-derived factors, including FFAs and adipokines, are closely related to the alteration of insulin sensitivity (Arner, 2003). We have recently demonstrated that crocetin can enhance insulin sensitivity and favourably regulate TNF-α and adiponectin expression in white adipose tissues in fructose-fed rats (Xi *et al*., 2007). This study employed palmitate (a saturated fatty acid)-treated rat adipocytes to investigate the effect of crocetin on impaired insulin action, as well as disordered expression of TNF-α and adiponectin, and explored the possible cellular mechanisms of action of crocetin.

It is well known that elevated plasma levels of FFAs can cause inhibition of insulin-stimulated glucose disposal and insulin signalling *in vivo* (Shulman, 2000; Belfort *et al*., 2005). However, the effect of FFAs (especially palmitate) on insulin action in adipocytes is presently not well understood as previous studies have yielded contradictory results (Joost and

Steinfelder, 1985; Hardy *et al*., 1991; Hunnicutt *et al*., 1994; Van Epps-Fung *et al*., 1997; Lundgren and Eriksson, 2004). These discrepancies may be due to differences in cell types or experimental conditions including incubation periods and concentrations of FFAs in these *in vitro* studies. It is noteworthy that washout of FFAs completely reverses their inhibitory effect on insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Nguyen *et al*., 2005), indicating that

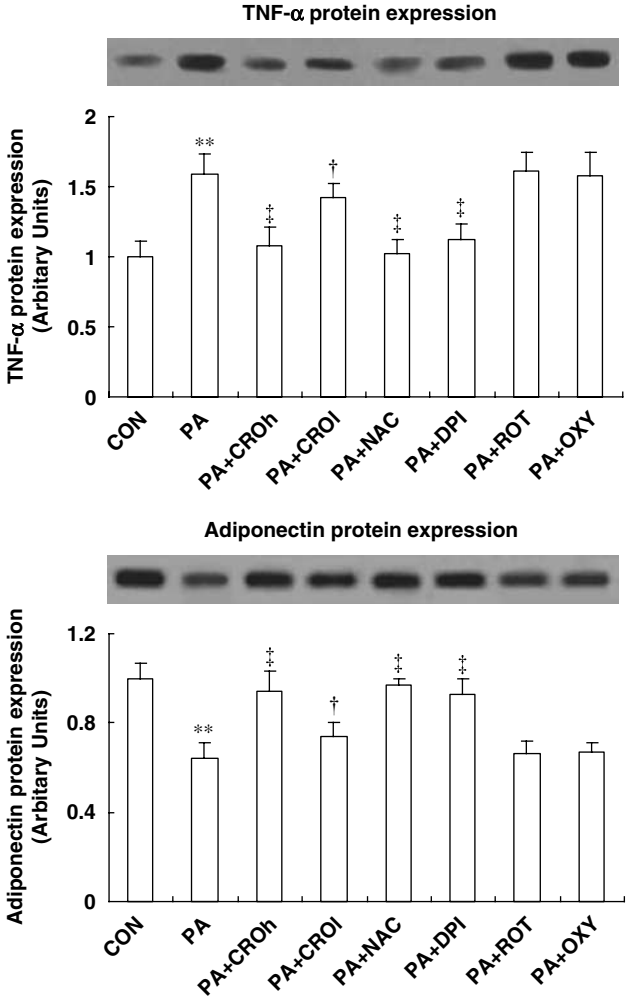

Figure 3 TNF-α and adiponectin protein expression in control (CON) and treated adipocytes. Treatments were with palmitate alone (PA) or palmitate with crocetin (50 μM, PA + CROh; 5 μM, PA + CROI), *N*-acetylcysteine (PA + NAC), diphenyleneiodonium (PA + DPI), rotenone (PA + ROT) or oxypurinol (PA + OXY). Total cell lysates were prepared and the protein expression of TNF-α and adiponectin were examined by immunoblot analysis. Representative results of an immunoblot analysis are shown. Data are expressed as mean ± s.d. of three independent experiments ($n = 6$). $^{**}P < 0.01$ vs CON; $^{\ddagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs PA.

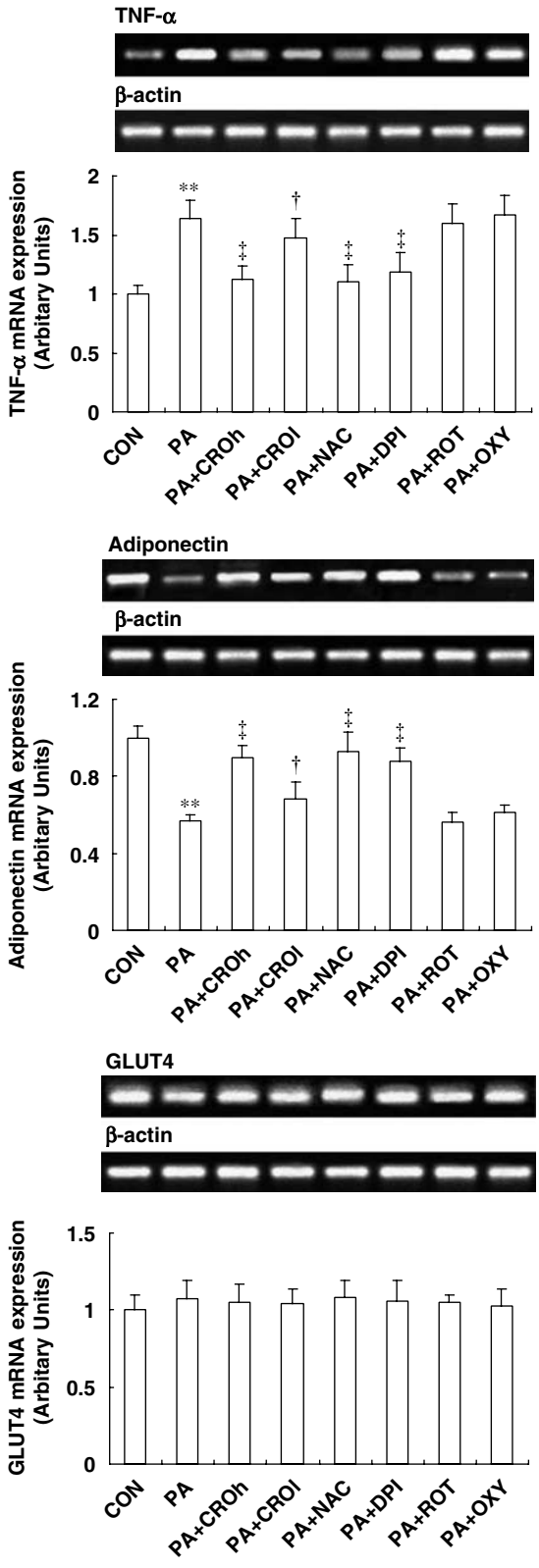

Figure 4 TNF-α, adiponectin and GLUT4 mRNA expression in control (CON) and treated adipocytes. Treatments were with palmitate alone (PA) or palmitate with crocetin (50 μM, PA + CROh; 5 μM, PA + CROI), *N*-acetylcysteine (PA + NAC), diphenyleneiodonium (PA + DPI), rotenone (PA + ROT) or oxypurinol (PA + OXY). Total cellular RNA was extracted and the expression of mRNA for β-actin, TNF-α, adiponectin and GLUT4 were examined by RT-PCR analysis. The mRNA expression was calculated after normalization to β-actin mRNA expression. The representative results of RT-PCR analysis are shown. Data are expressed as mean ± s.d. of three independent experiments ($n = 6$). $^{**}P < 0.01$ vs CON; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs PA. RT-PCR, reverse transcription-polymerase chain reaction.

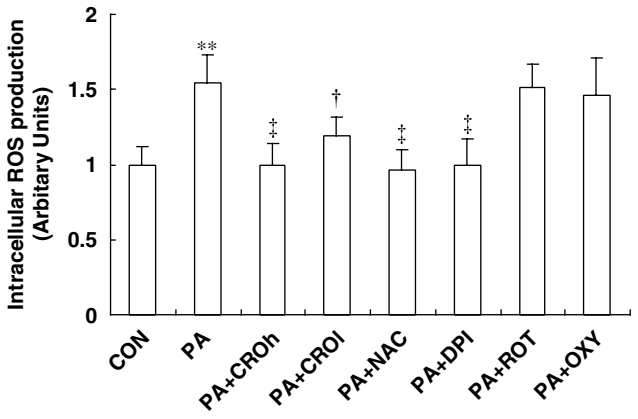

Figure 5 ROS production in control (CON) and treated adipocytes. Treatments were with palmitate alone (PA) or palmitate with crocetin (50 μM, PA + CROh; 5 μM, PA + CROl), *N*-acetylcysteine (PA + NAC), diphenyleneiodonium (PA + DPI), rotenone (PA + ROT) or oxypurinol (PA + OXY). Cells were loaded with 20 μM 2′,7′-dichlorofluorescein diacetate. Intracellular fluorescence was measured with a spectrofluorometer at 485 nm excitation and 525 nm emission. The results are normalized to control. Data are expressed as mean ± s.d. of three independent experiments ($n = 6$). $^{**}P < 0.01$ vs CON; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs PA.

experimental design may directly affect results. Our study used palmitate-treated cells for further analysis without washing and the results showed that chronic exposure of adipocytes to palmitate decreased insulin-stimulated glucose uptake. The uptake of glucose into insulin-sensitive tissues is mediated by a family of structurally related carrier proteins, of which GLUT4 has been established as the predominant glucose transporter (Zierath *et al*., 1996; Charron *et al*., 1999). Much evidence has demonstrated that the decrease in the activity and the expression at the mRNA or protein level of GLUT4 is an important molecular mechanism of insulin resistance (Zierath *et al*., 1996; Charron *et al*., 1999; Pessin *et al*., 1999). In this study, palmitate treatment did not evidently affect GLUT4 translocation or expression in adipocytes. These data were consistent with previous reports suggesting that palmitate leads to insulin resistance in adipocytes by inhibiting GLUT4 activation instead of GLUT4 translocation (Van Epps-Fung *et al*., 1997).

As well, adipocyte dysfunction is involved in the progress of insulin resistance by altered production of adipokines. TNF-α, an inflammatory cytokine produced by adipocytes, directly inhibits insulin signalling cascade by increasing serine phosphorylation of insulin receptor substrate-1 and thus reduces insulin receptor kinase activity and induces insulin resistance (Hotamisligil *et al*., 1996). Adiponectin, a circulating protein specifically produced from adipocytes, enhances insulin sensitivity and suppresses inflammation and a low adiponectin level is correlated with whole-body insulin insensitivity and inflammatory states (Arner, 2003). It is already known that FFAs affect the expression of adipokines (Ajuwon and Spurlock, 2005; Suganami *et al*., 2005). The present study found that both mRNA and protein expression of TNF-α were enhanced, while those of adiponectin were reduced, in palmitate-treated cells. Crocetin, as well as NAC and DPI, showed positive regulatory effects on the abnormal expression of these two adipokines, owing to palmitate.

Although much remains unknown about how FFAs induce insulin resistance, researchers have recognized that oxidative stress may constitute an underlying factor associated with insulin resistance and related diseases (Najib and Sanchez-Margalet 2001; Haber *et al*., 2003; Urakawa *et al*., 2003; Evans *et al*., 2004; Esposito *et al*., 2006). Recent reports have described increased oxidative stress in white adipocytes both in insulin-resistant animal models and FFA (linoleic acid, oleic acid and arachidonic acid)-treated adipocytes, and have also indicated that mitochondrial oxidative stress can lead to impaired insulin action and dysregulated production of adipokines (Rudich *et al*., 1998; Furukawa *et al*., 2004; Ajuwon and Spurlock, 2005; Delbosc *et al*., 2005; Song *et al*., 2005), further suggesting a close association between increased oxidative stress in adipocytes and insulin resistance. We observed increased production of ROS in palmitate-treated cells, which was inhibited by incubation with crocetin, NAC and DPI, suggesting that oxidative stress may also be involved in palmitate-induced insulin resistance in adipocytes. It has been reported that FFAs increase NADPH oxidase activity in cultured vascular cells and 3T3-L1 adipocytes (Inoguchi *et al*., 2000; Furukawa *et al*., 2004). Activation of NADPH oxidase is proposed as a fundamental step in mediating inflammation in the metabolic syndrome (Ferder *et al*., 2006). Furthermore, NADPH oxidase has recently found to be involved in the development of insulin resistance and cardiovascular complications in the fructose-fed rat model (Delbosc *et al*., 2005). However, there is lack of information on whether inhibition of NADPH oxidase activity can result in an improvement of insulin sensitivity in FFAs-treated adipocytes. Our study revealed that the negative effects induced by palmitate on rat adipocytes were attenuated by concomitant treatment with the specific NADPH oxidase inhibitor, DPI, in adipocytes, but not by selective inhibitors of other types of enzymes involved in ROS generation (see Table 1). The findings suggest that palmitate exerts its influence in this *in vitro* model mainly via an increased activity of NADPH oxidase and that NADPH oxidase may be a potential important therapeutic target for pharmacological intervention in insulin resistance and related abnormalities.

The potential mechanisms for the association between elevated oxidative stress, reduced insulin sensitivity and disordered expression of adipokines need further investigation. It has been demonstrated that inhibition of protein kinase C (PKC) activity partially restores glucose disposal and insulin signal transduction in muscle (Cortright *et al*., 2000; Itani *et al*., 2000). A previous study has indicated that PKC is able to activate NADPH oxidase and thus induce ROS production in vascular (endothelial and smooth muscle) cells (Inoguchi *et al*., 2000). Furthermore, TNF-α expression has recently found to be triggered by PKC in skeletal muscle cells (Jové *et al*., 2006). Therefore, it is worth assessing the role of the PKC pathway in impaired insulin action and enhanced TNF-α expression induced by FFAs in adipocytes. Based on our current findings and previous studies, some possible mechanisms involved in the effect of crocetin on palmitate-induced insulin resistance and disordered

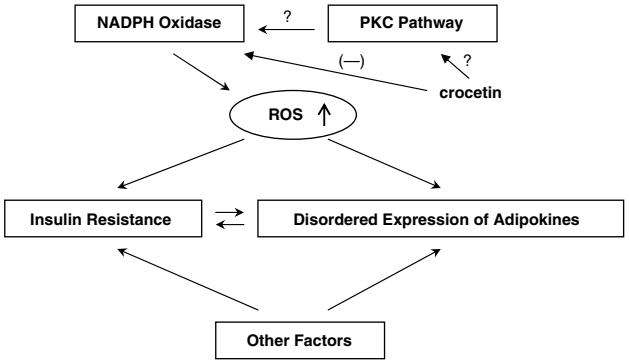

Figure 6 Some of the possible mechanisms underlying the effect of crocetin on palmitate-induced insulin resistance and disordered expression of adipokines in isolated rat adipocytes. Based on the current study, crocetin may inhibit intracellular ROS production and thus attenuate insulin insensitivity owing to inactivation of NADPH oxidase in this cell model. Further studies are needed to investigate whether PKC is a potential activator of NADPH oxidase, as well as an important target of crocetin for pharmacological intervention in the development of insulin resistance. NADPH, nicotinamide adenine dinucleotide phosphate; PKC, protein kinase C.

expression of adipokines in rat adipocytes are illustrated in Figure 6.

In conclusion, the current study provides evidence that crocetin has the ability to protect against impaired insulin-stimulated glucose uptake and dysregulated expression of TNF-α and adiponectin induced by palmitate in rat adipocytes. In addition to supporting previous reports indicating that palmitate possesses insulin resistance-inducing properties in adipocytes, we have demonstrated that oxidative stress, mainly via increased activity of NADPH oxidase, may be involved in the possible mechanisms by which palmitate exerts its influence on insulin action in adipocytes. The antioxidant effects of crocetin including inactivation of NADPH oxidase may, at least in part, explain the ability of this compound to prevent insulin insensitivity in this cell model.

Acknowledgements

We express our thanks to Professor Yifan Li (Nanjing Medical University) for his technical assistance in radiolabeled 2-deoxyglucose uptake assay.

Conflict of interest

The authors state no conflict of interest.

References

Ajuwon KM, Spurlock ME (2005). Palmitate activates the NF-κB transcription factor and induces IL-6 and TNFα expression in 3T3-L1 adipocytes. *J Nutr* **135**: 1841–1846.

Arner P (2003). The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* **14**: 137–145.

Bays H, Mandarino L, DeFronzo RA (2004). 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* **89**: 463–478.

Belfort R, Mandarino L, Kashyap S, Wirfel K, Pratipanawatr T, Berria R *et al.* (2005). Dose-response effect of elevated plasma free fatty acid on insulin signaling. *Diabetes* **54**: 1640–1648.

Bergman RN, Mittelman SD (1998). Central role of the adipocyte in insulin resistance. *J Basic Clin Physiol Pharmacol* **9**: 205–221.

Borden G (1997). Role of fatty acids in the pathogenesis of insulin resistance and NIDDM. *Diabetes* **46**: 3–10.

Caballero B (1993). Vitamin E improves the action of insulin. *Nutr Rev* **51**: 339–340.

Cortright RN, Azevedo Jr JL, Zhou Q, Sinha M, Pories WJ, Itani SI *et al.* (2000). Protein kinase C modulates insulin action in human skeletal muscle. *Am J Physiol Endocrinol Metab* **278**: E553–E562.

Charron MJ, Katz EB, Olson AL (1999). GLUT4 gene regulation and manipulation. *J Biol Chem* **274**: 3253–3256.

Cousin SP, Hugl SR, Wrede CE, Kajio H, Myers Jr MG, Rhodes CJ (2001). Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic β-cell line INS-1. *Endocrinology* **142**: 229–240.

Delbosc S, Paizanis E, Magous R, Araiz C, Dimo T, Cristol JP *et al.* (2005). Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis* **179**: 43–49.

Esposito K, Ciotola M, Schisano B, Misso L, Giannetti G, Ceriello A *et al.* (2006). Oxidative stress in the metabolic syndrome. *J Endocrinol Invest* **29**: 791–795.

Evans JL, Youngren JF, Goldfine ID (2004). Effective treatments for insulin resistance: trim the fat and douse the fire. *Trends Endocrinol Metab* **15**: 425–431.

Ferder L, Inserra F, Martinez-Maldonado M (2006). Inflammation and the metabolic syndrome: role of angiotensin II and oxidative stress. *Curr Hypertens Rep* **8**: 191–198.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y *et al.* (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* **114**: 1752–1761.

Giaccio M (2004). Crocetin from saffron: an active component of an ancient spice. *Crit Rev Food Sci Nutr* **44**: 155–172.

Haber CA, Lam TKT, Yu Z, Gupta N, Goh T, Bogdanovic E *et al.* (2003). *N*-acetylcysteine and taurine prevent hyperglycemia-induced insulin resistance *in vivo*: possible role of oxidative stress. *Am J Physiol Endocrinol Metab* **285**: E744–E753.

Hardy RW, Ladenson JH, Henriksen EJ, Holloszy JO, McDonald JM (1991). Palmitate stimulates glucose transport in rat adipocytes by a mechanism involving translocation of the insulin sensitive glucose transporter (GLUT4). *Biochem Biophys Res Commun* **177**: 343–349.

Hotamisligil GS, Shargil NS, Spiegelman BM (1993). Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance. *Science* **259**: 87–91.

Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity-induced insulin resistance. *Science* **271**: 665–668.

Hunnicutt JW, Hardy RW, Williford J, McDonald JM (1994). Saturated fatty acid induced insulin resistance in rat adipocytes. *Diabetes* **43**: 540–545.

Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M *et al.* (2000). High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* **49**: 1939–1945.

Itani SI, Zhou Q, Pories WJ, Macdonald KG, Dohm GL (2000). Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes* **49**: 1353–1358.

Joost HG, Steinfelder HJ (1985). Insulin-like stimulation of glucose transport in isolated adipocytes by fatty acids. *Biochem Biophys Res Commun* **128**: 1358–1363.

Jové M, Planavila A, Sánchez RM, Merlos M, Laguna JC, Vázquez-Carrera M (2006). Palmitate induces tumor necrosis factor-α

expression in C2C12 skeletal muscle cells by a mechanism involving protein kinase C and nuclear factor-κB activation. *Endocrinology* **147**: 552–561.

Lau DCW, Dhillon B, Yan H, Szmitko PE, Verma S (2005). Adipokines: molecular links between obesity and atherosclerosis. *Am J Physiol Heart Circ Physiol* **288**: H2031–H2041.

Lundgren M, Eriksson JW (2004). No *in vitro* effects of fatty acids on glucose uptake, lipolysis or insulin signaling in rat adipocytes. *Horm Metab Res* **36**: 203–209.

McKeel DW, Jarett L (1970). Preparation and characterization of a plasma membrane fraction from isolated fat cells. *J Cell Biol* **44**: 417–432.

Moini H, Tirosh O, Park YC, Cho KJ, Packer L (2002). R-α-lipoic acid action on cell redox status, the insulin receptor, and glucose uptake in 3T3-L1 adipocytes. *Arch Biochem Biophys* **397**: 384–391.

Najib S, Sanchez-Margalet V (2001). Homocysteine thiolactone inhibits insulin signaling, and glutathione has a protective effect. *J Mol Endocrinol* **27**: 85–91.

Nguyen MTA, Satoh H, Favelyukis S, Babendure JL, Imamura T, Sbodio JI *et al*. (2005). JNK and tumor necrosis factor-α mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. *J Biol Chem* **280**: 35361–35371.

Pessin JE, Thurmond DC, Elmendorf JS, Coker KJ, Okada S (1999). Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. *J Biol Chem* **274**: 2593–2596.

Reaven GM, Laws A (1994). Insulin resistance, compensatory hyperinsulinemia, and coronary heart disease. *Diabetologia* **37**: 948–952.

Rodbell M (1964). Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* **239**: 375–380.

Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N (1998). Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes* **47**: 1562–1569.

Shulman GI (2000). Cellular mechanisms of insulin resistance. *J Clin Invest* **106**: 171–176.

Song D, Hutchings S, Pang CCY (2005). Chronic *N*-acetylcysteine prevents fructose-induced insulin resistance and hypertension in rats. *Eur J Pharmacol* **508**: 205–210.

Suganami T, Nishida J, Ogawa Y (2005). A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor α. *Arterioscler Thromb Vasc Biol* **25**: 2062–2068.

Tsuda T, Horio F, Uchida K, Aoki H, Osawa T (2003). Dietary cyanidin 3-*O*-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in Mice. *J Nutr* **133**: 2125–2130.

Urakawa H, Katsuki A, Sumida Y, Gabazza EC, Murashima S, Morioka K *et al*. (2003). Oxidative stress is associated with adiposity and insulin resistance in men. *J Clin Endocrinol Metab* **88**: 4673–4676.

Van Epps-Fung M, Williford J, Wells A, Hardy RW (1997). Fatty acid-induced insulin resistance in adipocytes. *Endocrinology* **138**: 4338–4345.

Vasdev S, Longerich L, Gill V (2004). Prevention of fructose-induced hypertension by dietary vitamins. *Clin Biochem* **37**: 1–9.

Xi L, Qian Z, Sheng X, Wen N, Zhang Y (2005). Crocetin prevents dexamethasone-induced insulin resistance in rats. *Planta Med* **71**: 917–922.

Xi L, Qian Z, Xu G, Zheng S, Sun S, Wen N *et al*. (2007). Beneficial impact of crocetin, a carotenoid from saffron, on insulin sensitivity in fructose-fed rats. *J Nutr Biochem* **18**: 64–72.

Zierath JR, Houseknecht KL, Kahn BB (1996). Glucose transporters and diabetes. *Semin Cell Dev Biol* **7**: 295–307.