Effect of *Coptidis Rhizoma* extracts in a water-based solution on insulin resistance in 3T3-L1 adipocytes

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**ABSTRACT**

The present study was designed to investigate effects and molecular mechanisms of *Coptidis Rhizoma* extracts (CRE) on the improvement of insulin resistance induced by tumor necrosis factor-α (TNF-α) in adipocytes. We examined whether CRE administration could directly influence the insulin resistance in 3T3-L1 adipocytes. Potential roles of CRE in glucose consumption, mRNA expression of peroxisome proliferators activated receptor (PPAR-γ), expression of insulin receptor substrate-1 (IRS-1) protein, and phosphorylation of IRS-1 Ser307 were also investigated in the present study. Our data demonstrated that TNF-α significantly reduced levels of glucose consumption and IRS-1 protein expression, while TNF-α increased the phosphorylation of IRS-1 Ser307 in adipocytes 24 h after the challenge, suggesting that TNF-α induced a condition with the occurrence of insulin resistance. Those alterations induced by TNF-α were prevented and the mRNA expression of PPAR-γ was up-regulated by the administration of CRE. Thus, our results indicate that CRE can be used to prevent from the TNF-α-induced insulin resistance through PPAR-γ pathways.

Insulin resistance is one of major pathophysiological abnormalities in patients with type II diabetes mellitus and obesity and plays an important role in the development of hyperglycemia of type II diabetes mellitus in skeletal muscle, adipose tissue, and liver. Previous reports have demonstrated that the primary component of *Coptidis Rhizoma*, berberine, could improve the insulin resistance through various pathways. However, it is difficult to apply berberine for the improvement of insulin resistance, since berberine is an alcohol extract of *Coptidis Rhizoma* and the extraction process is complicated. The present study applied the *Coptidis Rhizoma* extract (CRE) in a water-based solution to examine if CRE improves the insulin resistance. It has been reported that insulin resistance could be induced by tumor necrosis factor α (TNF-α) under various circumstances. In the present study, we intended to investigate effects of CRE on insulin signaling pathways in 3T3-L1 adipocytes with the insulin resistance induced by TNF-α treatment.

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MATERIALS AND METHODS

Materials. Coptis root was obtained from Sichuan, China. Water-soluble CRE was prepared as described previously (25). Briefly, 375 g Coptis roots were kept in 3,000 mL pure water for 12 h, heated for 2 h under a circumfluent condition, and then filtered. Filtered residues were again heated for 1 h under a circumfluent condition in 3,000 mL pure water, and then filtered again. The final filtered residues were heated for 0.5 h under a circumfluent condition in 2,250 mL pure water, and then filtered. The filtrates collected above were combined, evaporated to dryness by vacuum at room temperature, and then diluted with dimethyl sulfoxide (DMSO) at the concentrations indicated for the experiments. Dexamethasone, 3-isobutyl-1-methylxanthine, and fatty-acid-free bovine serum albumin (BSA) were obtained from Sigma (Shanghai, China). Insulin and oil red O were purchased from Novo Nordisk Co. (Oslo, Norway) and Shanghai Biochemical Reagent Co. (Shanghai, China), respectively; Dulbecco’s modified Eagle’s medium (DMEM), bovine calf serum, and antibiotic mixture (penicillin-streptomycin) were obtained from Gibco Co. (Shanghai, China), while fetal bovine serum (FBS) from Hyclone Co., (Shanghai, China). Real time PCR reagent kit was purchased from Takara Bio Inc. (Otsu, Japan). Anti-IRS-1, anti-human TNF-α, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Shanghai, China). Pioglitazone was from Takeda Pharmaceutical Co. (Osaka, Japan).

Cell culture. Murine 3T3-L1 preadipocytes (Shanghai Institute of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University) were cultured in 25 cm² flasks in DMEM with 25 mM glucose containing 10% fetal bovine serum (FBS), 60 μg/mL penicillin and 100 μg/mL streptomycin sulphate in a humified atmosphere of 5% CO₂ at 37°C. Murine 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (11). Briefly, cells were plated and grown for 2 days post-confluence in DMEM supplemented with 10% FBS. Differentiation to adipocytes was then induced by incubation in DMEM supplemented with 10% FBS, 1.7 μM insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (IDM) for 48 h. Then, the differentiated cells were cultured in the maintenance medium containing DMEM supplemented with 10% FBS. The maintenance medium was changed every 48 h until the cells were utilized for experimentation. Human TNF-α dissolved in PBS containing 0.2% BSA (growth factor-depleted) was added to the cell culture media when greater than 95% of the cells had the morphological and biochemical properties of adipocytes.

Experimental conditions. The fully differentiated 3T3-L1 adipocytes were cultured in serum-free DMEM containing 2% BSA for 12 h followed by culture in the medium containing 2% BSA for 24 h; 1) Control group, without 10 ng/mL TNF-α; 2) Model group, with 10 ng/mL TNF-α; 3) CRE intervention group, with 10 ng/mL TNF-α + CRE (10 μg/L, 30 μg/L, 60 μg/L or 100 μg/L); and 4) Pioglitazone group, with 10 ng/mL TNF-α + pioglitazone (10 μM dissolved in DMSO). The final concentration of DMSO was less than 0.20%, since DMSO less than 0.20% had no effects on cell morphology or metabolic activity.

Determination of intracellular lipid accumulation. Intracellular lipid accumulation was determined by oil red O staining. The oil red O working solution was prepared as described previously (3). 3T3-L1 adipocytes were harvested 8 days after differentiation, washed twice with PBS (pH 7.4) and then fixed with 10% neutral formalin for 20 min at room temperature. After removal of the 10% neutral formalin, 100% propylene glycol was added to each well for 3 min. Cells were decolorized with 60% propylene glycol before staining for 1 h with the oil red O working solution and then washed with water. The staining dye of cells was imaged with inverted microscope.

Determination of glucose concentration. The determination of glucose concentration was performed by an enzymatic/colorimetric method (GOD/POD) as described previously (22).

Quantitative realtime-PCR. Total RNAs were extracted using Trizol Reagent (Gibco) according to the manufacturer’s instruction. Total RNA was reversely transcribed by AMV reserve transcriptase (Takara Bio Inc.). For PPARγ detection, primers 5’-CAGTTGATTTTCTCCAGCATTT-3’ and 5’-CTT TGATCGCACCCTTGGTATT-3’ were designed with actin gene (5’-GTTCGTCCTCATACTGCTCA-3’ and 5’-TGCAACCTGTAAGGCGTTC-3’) as control. Real-time PCR was performed according to manufacturer’s instruction (Takara Bio Inc.): 30 s pre-denaturation at 95°C, 1 cycle; 10 s denaturation at 95°C; 20 s annealing at 56°C; and 20 s collection fluorescence at 72°C, 40 cycles. The products of real-
time quantitative PCR were then run on 1.5% agarose gel electrophoresis, and an equal-sized band was observed as predicted. Quantification of the gene expression was done with comparative CT method.

**Western blot analysis.** 3T3-L1 preadipocytes were grown and differentiated into adipocytes in 35-mm culture dishes for Western blots. Cells were washed twice with PBS and then lysed in lysis buffer (50 mM Tris-HCl at pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mM Na3VO4, 1 mM NaF). The cell lysates were then centrifuged (20,000 × g, 10 min, 4°C) and subjected to Western blot analysis. Protein concentrations were measured by BCA Protein Assay kit (Pierce, USA). The protein (30 μg) in 10 μL reducing sample buffer was boiled for 5 min, and resolved by SDS-PAGE for 2 h at 110 V. After the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, PVDF membrane was washed with 25 mL Tris-buffered saline (TBS) for 5 min at room temperature, incubated in 25 mL blocking buffer (TBS buffer (1×), 0.1% Tween-20 with 5% non-fat dry milk) for 2 h at room temperature, and then washed twice for 5 min each with 15 mL TBS/0.1% Tween-20 (TBS/T). Then, PVDF membrane was incubated with anti-IRS-1 antibody (1:500), anti-phosphorylated IRS-1 Ser307 antibody (1:500), or anti-actin antibody (1:1000) in 10 mL primary antibody dilution buffer with gentle agitation overnight at 4°C, and washed twice for 5 min each with 15 mL TBS/T. After these processes, the PVDF membrane was further incubated with HRP-conjugated secondary antibody (1:1000) in 10 mL blocking buffer with gentle agitation for 2 h at room temperature, and washed twice for 5 min each with 15 mL TBS/T. Immunoreactive bands were detected with the ECL kit (Pierce), and scanned on autoradiography films (Bio-Rad, Shanghai, China). Intensity of the immunoblot signal was analyzed quantitatively using Quantity One software (Bio-Rad).

**Data presentation.** Data are represented as the mean ± SD, at least three individual experiments. Statistical analysis was performed with ANOVA. A value of *P* < 0.05 was considered statistically significant.

**RESULTS**

**Differentiation of 3T3-L1 cells to adipocytes**

Fig. 1A shows 3T3-L1 cells without IDM treatment, while on 8-day after IDM treatment, over 95% of the cells were observed to accumulate lipid droplets in the cytoplasm (Fig. 1B) compared to group without IDM treatment (Fig. 1A), implying IDM treatment successfully induced adipocyte differentiation of 3T3-L1 cells. TNF-α treatment (Model in Fig. 1C) strikingly elevated the glucose level in the culture medium compared with that in control group (Control in Fig. 1C; *, *P < 0.01), indicating that TNF-α treatment down-regulated glucose consumption of differentiated 3T3-L1. This observation implies that TNF-α treatment successfully induced insulin resistance model in differentiated 3T3-L1 adipocytes. Treatment with pioglitazone (10 μM), a drug improving insulin resistance (3, 13), diminished TNF-α-induced elevation of glucose level in the culture medium (see Model and Pioglitazone in Fig. 1C; #, *P < 0.05), indicating that pioglitazone improved TNF-α-diminished glucose consumption, although the glucose level under a pioglitazone-treated condition was still higher than that in control (see Pioglitazone and Control in Fig. 1C; **, *P < 0.025). Similar to pioglitazone, treatment with CRE (10, 30, 60 or 100 μg/mL) also significantly diminished the TNF-α-induced elevation of glucose level in the culture medium (see Model and CRE intervention in Fig. 1C; ###, *P < 0.025) to a control level (no significant difference between CRE and Control in Fig. 1C). It is notable that CRE of 30 mg/mL had the most effective action on glucose consumption (Fig. 3C). Kudoh and his colleagues have reported that pioglitazone of 10 μM has the maximum effect on glucose uptake in 3T3-L1 adipocytes (13). These observations suggest that the CRE-induced improvement would be better than pioglitazone, although both CRE and pioglitazone improved glucose consumption diminished by TNF-α.

**mRNA expression of PPAR-γ**

We also measured mRNA expression of PPAR-γ, since PPAR-γ plays an important role in up-regulation of glucose uptake into adipocytes via an increase in insulin sensitivity (4). CRE (30 mg/L) treatment drastically increased mRNA expression of PPAR-γ in TNF-α-treated cells compared with that in cells treated with TNF-α alone (see CRE and Model in Fig. 2; *P < 0.05), which diminishes mRNA expression of PPAR-γ (10, 12). Pioglitazone also elevated TNF-α-diminished mRNA expression of PPAR-γ (see Pioglitazone and Model in Fig. 2; *P < 0.001). Although there was no significant difference between effects of CRE and pioglitazone on TNF-α-diminished mRNA expression of PPAR-γ, the observation shows tendency of better improve-
Next we studied protein expression of IRS-1, a key protein of insulin signaling. Protein expression of IRS-1 was significantly reduced after incubation with TNF-α compared with control group (see Model and Control in Figs. 3A and 3B; *, $P < 0.001$). The TNF-α-induced reduction was strikingly recovered by treatment with CRE at 30 mg/L, and 60 mg/L, but 100 mg/L (see Model and CRE in Figs. 3A and 3B; #, $P < 0.05$), although the level recovered by CRE treatment was still significantly lower than that in control (see Control and CRE in Fig. 3B; **, $P < 0.05$). Moreover, we measured phosphorylation levels of IRS-1 Ser307. TNF-α treatment significantly reduced phosphorylation of IRS-1 Ser307 compared with control group (see Control and Model in Figs. 3A and 3B; *, $P < 0.01$ compared with control; **, $P < 0.001$ compared with control; #, $P < 0.05$ compared with Model (TNF-α) group; ##, $P < 0.025$ compared with Model (TNF-α) group). There was no significant difference between glucose levels in the cultured medium in Control and CRE (TNF-α + CRE) group.

**Fig. 1** Oil red O stained 3T3-L1 preadipocytes without IDM treatment (A) and 3T3-L1 adipocytes on 8-day after IDM treatment (B) under inverted microscope (× 40). Glucose consumption in 3T3-L1 adipocytes on 8-day after IDM treatment (C) in Control, Model, Piog (10 μM), or CRE intervention groups was also shown. *, $P < 0.01$ compared with control; **, $P < 0.001$ compared with control; #, $P < 0.05$ compared with Model (TNF-α) group; ##, $P < 0.025$ compared with Model (TNF-α) group.

**Expression of IRS-1 and phosphorylated IRS-1**

We next studied protein expression of IRS-1, a key protein of insulin signaling. Protein expression of IRS-1 was significantly reduced after incubation with TNF-α compared with control group (see Model and Control in Figs. 3A and 3B; *, $P < 0.001$). The TNF-α-induced reduction was strikingly recovered by treatment with CRE at 30 mg/L, and 60 mg/L, but 100 mg/L (see Model and CRE in Figs. 3A and 3B; #, $P < 0.05$), although the level recovered by CRE treatment was still significantly lower than that in control (see Control and CRE in Fig. 3B; **, $P < 0.05$). Moreover, we measured phosphorylation levels of IRS-1 Ser307. TNF-α treatment significant-
CRE and insulin resistance

Increased the phosphorylation level of IRS-1 Ser307 (see Model and Control in Figs. 4A and 4B; *, P < 0.005). The TNF-α-induced elevation of IRS-1 Ser307 phosphorylation was significantly prevented by CRE treatment (see CRE and Model in Figs. 4A and 4B; #, P < 0.05), although the TNF-α-elevated phosphorylation level of IRS-1 Ser307 was still higher than control (see Control and CRE in Fig. 4B; **, P < 0.025).

DISCUSSION

The 3T3-L1 cell line is widely used as a model of adipocyte. The decreased adipocytic lipogenesis was considered as one of the mechanisms of proposed anti-obesity (16). In the present study, we found that CRE in water-based solution could improve TNF-α-induced insulin resistance in 3T3-L1 adipocytes. PPAR-γ is a member of the nuclear hormone receptor superfamily and functions as a transcriptional regulator of genes associated with adipogenesis, lipid metabolism, insulin sensitivity, energy expenditure, and insulin resistance (2, 17). Berberine, an alcohol extract of **Cortidis Rhizoma**, has been demonstrated to improve the insulin resistance through various pathways (7, 19, 20, 23). However, it is unfortunately difficult to apply berberine for the improvement of insulin resistance due to its characteristics as an alcohol extract. Therefore, we tried to apply **Coptidis Rhizoma** extract in a water-based solution, which is much easier applied than an alcohol extract, berberine. Our previous study (data not shown) funded by NSFC (National Natural Science Foundation of China) indicated that **Coptidis Rhizoma** extract in a water-based solution shows only a uni-peak in high-performance liquid chromatogram, implying a novel single substance. The present study shows that the **Coptidis Rhizoma** extract in a water-based solution can prevent the TNF-α-induced insulin resistance only in 3T3-L1 adipocytes. This means that: 1) the improving action of the **Coptidis Rhizoma** extract in a water-based solution on insulin resistance is not a direct pharmaco-

![Fig. 3](Image)

Expression of IRS-1 protein in Control, Model and CRE intervention groups: A) Western blotting, and B) Relative expression of IRS-1 protein to β-actin. *, P < 0.001 compared with Control group; **, P < 0.05 compared with Control group; #, P < 0.05 compared with Model (TNF-α) group. Control group, without drug treatment; Model group, TNF-α + vehicle treatment; CRE intervention group, TNF-α + CRE (30 mg/L, 60 mg/L, 100 mg/L) treatment.

![Fig. 4](Image)

mRNA expression of pIRS-1 protein (IRS-1 with Ser307 phosphorylation) in Control, Model and CRE intervention groups: A) Western blotting, and B) Relative expression of pIRS-1 protein to β-actin. *, P < 0.005 compared with Control group. **, P < 0.025 compared with control group. #, P < 0.05 compared with model (TNF-α) group. Control group, without drug treatment; Model group, TNF-α + vehicle treatment; CRE intervention group, TNF-α + CRE (30 mg/L, 60 mg/L, 100 mg/L) treatment.
logical action on glucose consumption, and 2) the *Coptidis Rhizoma* extract in a water-based solution would be expected to be applied for human. However, further *in vivo* studies are required to confirm if the *Coptidis Rhizoma* extract in a water-based solution can be applied for human need.

Adipose tissues have been known to produce and secrete PPAR-\(\gamma\), which plays roles in the early stage of adipocyte differentiation. Some studies have addressed the important role of PPAR-\(\gamma\) in the regulation of insulin sensitivity and glucose homeostasis (4). In this study, we found that CRE treatment improved TNF-\(\alpha\)-reduced expression of PPAR-\(\gamma\) mRNA, indicating that CRE may improve insulin resistance (glucose consumption) by affecting a PPAR-\(\gamma\)-mediated cascade.

The observations shown in the present study confirm that TNF-\(\alpha\) induces the formation of insulin resistance (reduction of glucose consumption), which was prevented by the treatment with CRE. We have also found that TNF-\(\alpha\) down-regulates the expression of IRS-1 proteins in those cells with insulin resistance (reduced glucose consumption). CRE at the concentration of 30 and 60 mg/L partly but not fully prevents TNF-\(\alpha\)-induced down-regulation of IRS-1 expression, improving the TNF-\(\alpha\)-induced reduction of glucose consumption. We propose that IRS-1 protein might be involved in the improving effects of CRE on insulin resistance in 3T3-L1 adipocytes. Phosphorylation of IRS-1 Ser307 precedes IRS-1 degradation, and phosphorylation of IRS-1 Ser307 has been recognized as an indicator molecule of insulin resistance (1, 8, 9, 15, 18, 21, 26). Therefore, we investigated the effect of CRE on phosphorylation of IRS-1 Ser307 with anti-phosphorylated IRS-1 Ser307 antibody by immunoblotting. As shown in Fig. 4, we found that TNF-\(\alpha\) increased phosphorylation of IRS-1 Ser307 in those cells associated with insulin resistance (reduced glucose consumption), while CRE improved both TNF-\(\alpha\)-induced insulin resistance and over-phosphorylation of IRS-1 Ser307 leading to elevation of IRS-1 expression keeping the insulin signal transduction cascade at normal levels. Thus, we indicate that CRE improves insulin resistance through the IRS-1-mediated signal pathway.

In conclusion, we found that CRE in a water-based solution could improve the TNF-\(\alpha\)-induced insulin resistance by elevating expression of PPAR-\(\gamma\) and IRS-1 via down-regulation of IRS-1 phosphorylation. Thus, the water-based CRE can be an alternative to treat metabolism disease of insulin resistance.

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REFERENCES

induced apoptosis and necrosis in pancreatic RINm5F cells. Life Sci 76, 917–929.