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CTRP3 improves the insulin sensitivity of 3T3-L1 adipocytes by inhibiting inflammation and ameliorating insulin signalling transduction

Białko CTRP3 zwiększa wrażliwość na insulinę adipocytów 3T3-L1 przez hamowanie procesu zapalnego i poprawę przekazywania sygnału insulinowego

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Abstract

Introduction: C1q/TNF-related Protein-3 (CTRP3) is a novel adipokine with multiple effects such as lowering glucose levels, inhibiting glyconeogenesis in the liver, and increasing angiogenesis and anti-inflammation. But little is known about the effects of CTRP3 on insulin resistance in adipose tissue. This study aims to investigate the effects and mechanisms of CTRP3 on the insulin sensitivity of 3T3-L1 adipocytes.

Material and methods: Insulin resistant 3T3-L1 adipocytes were induced by palmic acid cultivation. Such adipocytes were treated with recombinant CTRP3 protein at different concentrations (0, 10, 50, 1,250 ng/mL for 12 hours, and at a concentration of 250 ng/mL for differing times (2, 6, 12, and 24h). Another group was pre-treated with wortmannin, the special inhibitor of phosphatidylinositol-4,5- bisphosphate 3-kinase (PI3K), for 20 minutes before the treatment with 250 ng/mL CTRP3. The glucose consumption, the glucose uptake, the expression and release of tumour necrosis factor α (TNF-α) and interleukin-6(IL-6) in supernatant, and the protein relative expression of PI3K and protein kinase B (PKB)(ser437) were detected.

Results: Compared to the control group, glucose consumption in the CTRP3 intervention group at concentrations of 10, 50, 250, and 1,250 ng/mL was increased by 22.1%, 42.9%, 76.6% and 80.5% respectively (all $P < 0.01$); the glucose uptake was increased by 39.0%, 68.0%, 108.0% and 111.0% respectively (all $P < 0.01$); the content of TNF-α in the culture media of CTRP3 (10, 50, 250 ng/mL) intervention group was decreased by 7.6% ($P > 0.05$), 13.0% ($P < 0.05$) and 17.4% ($P < 0.01$) respectively; the content of IL-6 was decreased by 7.1%, 12.4% and 17.1% respectively (all $P < 0.01$); the protein relative expression of PI3K was increased by 0.63-, 1.00- and 1.36-fold respectively (all $P < 0.01$), and PKB(ser437) increased by 0.65-, 1.61- and 1.93-fold respectively (all $P < 0.01$); the mRNA relative expression of GLUT-4 was increased by 23.0%, 47.0% and 62.0% respectively (all $P < 0.01$). After the treatment with wortmannin, glucose consumption, glucose uptake, PI3K and PKB(ser437) protein relative expression, as well as GLUT-4 mRNA relative expression, was decreased by 53.2%, 44.7%, 43.4%, 56.1 and 30.9% respectively (all $P < 0.01$).

Conclusions: CTRP3 could improve insulin sensitivity of insulin resistant 3T3-L1 adipocytes by decreasing inflammation and ameliorating insulin signalling transduction, indicating that CTRP3 may be a new target for the prevention and cure of insulin resistance and type 2 diabetes. **(Endokrynol Pol 2014; 65 (4): 252–258)**

Key words: *C1q/TNF related protein 3, insulin resistance, 3T3-L1 adipocytes, inflammation, insulin signalling transduction*

Streszczenie

Wstęp: Białko związane z C1q/TNF typu 3 (CTRP3, *C1q/TNF-related Protein-3*) jest nowo odkrytą adipokiną o wielorakim działaniu obejmującym obniżenie stężenia glukozy we krwi, hamowanie glukoneogenezy w wątrobie, pobudzanie angiogenezy i działanie przeciwzapalne, Niewiele jednak wiadomo na temat wpływu CTRP3 na insulinooporność komórek tłuszczowych. Badanie to przeprowadzono w celu oceny mechanizmów działania tej adipokiny i jej wpływu na wrażliwość na insulinę adipocytów 3T3-L1.

Materiał i metody: Insulinooporne adipocyty 3T3-L1 uzyskano poprzez dodanie do hodowli tych komórek kwasu palmitynowego. Następnie adipocyty te poddano działaniu rekombinowanego białka CTRP3 w różnych stężeniach (0, 10, 50, 1250 ng/ml przez 12 godzin oraz w stężeniu 250 ng/ml przez różny czas (2, 6, 12, 24 godz.). Inną grupę hodowli komórkowych przed dodaniem CTRP3 w stężeniu 250 ng/ml inkubowano wstępnie z wortmaniną, inhibitorem kinazy fosfatydyloinozytolu-4,5 (PI3K, *phosphatidylinositol-4,5- bisphosphate 3-kinase*) przez 20 minut. Określono zużycie glukozy, wychwyt glukozy, ekspresję i uwalnianie czynnika martwicy nowotworów typu alfa (TNF-α, *tumor necrosis factor* α) i interleukiny 6 (IL-6, *interleukin-6*,) w supernatancie oraz ekspresję PI3K i kinazy białkowej B (PKB, *protein kinase B*) (ser437).

Wyniki: Zużycie glukozy w hodowlach poddanych działaniu CTRP3 w stężeniach 10, 50, 250, 1250 ng/ml było większe niż w hodowli kontrolnej odpowiednio o 22,1%, 42,9%, 76,6% i 80,5% (dla wszystkich porównań $p < 0,01$). Wychwyt glukozy był większy o 39,0%, 68,0%, 108,0% i 111,0% (dla wszystkich porównań $p < 0,01$) Zawartości TNF-α w medium hodowli komórkowej z dodatkiem CTRP3 (10, 50, 250 ng/ml) były mniejsze odpowiednio o 7,6% (p > 0,05), 13,0% ($p < 0,05$) i 17,4% ($p < 0,01$), a zawartości IL-6 były mniejsze o odpowiednio 7,1%, 12,4% i 17,1% (dla wszystkich porównań $p < 0,01$). Związana z białkami ekspresja PI3K stanowiła odpowiednio 0,63-, 1,00- i 1,36-krotność wartości uzyskanej w hodowli kontrolnej (dla wszystkich porównań $p < 0,01$), a ekspresja PKB(ser437) stanowiła odpowiednio 0,65-, 1,61- i 1,93-krotność (dla wszystkich porównań $p < 0,01$); Względna ekspresja mRNA GLUT-4 była większa odpowiednio o 23,0%, 47,0% i 62,0% (dla wszystkich porównań $p < 0,01$). W hodowlach poddanych wstępnie działaniu wortmaniny zużycie glukozy,

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wychwyt glukozy, ekspresja PI3K i PKB(ser437) oraz ekspresja mRNA GLUT-4 były mniejsze odpowiednio o 53,2%, 44,7%, 43,4%, 56,1% i 30,9% (dla wszystkich porównań $p < 0,01$).
Wnioski: Białko CTRP3 może powodować zwiększenie wrażliwości na insulinę insulinoopornych adipocytów 3T3-L1 przez hamowanie procesu zapalnego i poprawę przewodzenia sygnałów insulinowych, co wskazuje, że białko to może być nowym celem w zapobieganiu i leczeniu insulinooporności i cukrzycy typu 2. **(Endokrynol Pol 2014; 65 (4): 253–258)**

Słowa kluczowe: *białko związane z C1q/TNF typu 3; insulinooporność; adipocyty 3T3-L1; zapalenie; przekazywanie sygnałów insulinowych*

Introduction

Adipose tissue is the largest endocrine organ and secretes many bioactive molecules which are called adipokines, such as leptin, adiponectin, resistin, retinol-binding protein 4, omentin, and vaspin. Adipokines, the levels of which are often dysregulated in conditions of obesity and/or diabetes, play important roles in insulin sensitivity and energy balance [1]. Much research has implicated adiponectin as a major insulin-sensitising adipokine as well as an important biomarker and therapeutic target for obesity-associated metabolic disorders [2]. However, the fact that there is only mild metabolic dysfunction in adiponectin knock-out mice indicates that there might be additional molecules with similar functions to adiponectin [3]. It was as a byproduct of discovering such metabolic regulators that led to the identification of C1q/TNF-related proteins 1–15 (CTRP 1 to 15), which together with adiponectin belong to the C1q/TNF superfamily [4].

CTRP3 plays many roles in regulating inflammation and metabolism. Studies *in vitro* have indicated that recombinant human CTRP3 could reduce the secretion of proinflammatory cytokine IL-6 and TNF-α in response to lipopolysaccharide stimulation in primary monocytes derived from healthy humans [5]. Studies *in vivo* have indicated that a modest three-fold elevation of plasma CTRP3 levels by recombinant protein administration in normal and insulin resistant ob/ob mice is sufficient to lower glucose levels which may be mediated by hepatic PKB activation and suppression of the expression of the gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [6]. A recent study showed that CTRP3 attenuated diet-induced hepatic steatosis by regulating triglyceride metabolism [7], indicating that CTRP3 was a very important regulator of glucose metabolism as well as lipid metabolism. Apart from this, previous studies also suggested that CTRP3 could be a novel antiapoptotic, proangiogenic, and cardioprotective adipokine, the expression of which is significantly inhibited after myocardial infarction [8].

Insulin resistance (IR) is the main and triggering factor in the pathogenesis of type 2 diabetes mellitus (T2DM). In obesity, there is chronic and low grade inflammation involved in the pathogenesis of T2DM, hypertension, atherosclerosis, fatty liver, cancer, asthma, and sleep apnoea [9]. Such inflammation starts mainly in adipose tissue with elevated macrophage infiltration and expression of proinflammatory cytokines. Inflammation induces IR through several mechanisms, the most important of which is the inhibition of insulin signalling transduction [10]. As mentioned above, CTRP3 has beneficial effects on the metabolism of glucose and fat as well as chronic low grade inflammation, yet the effect of CTRP3 on IR is largely unknown. So the present study aimed to investigate the effects and mechanisms of CTRP3 on the insulin sensitivity of 3T3-L1 adipocytes with IR.

Material and methods

Adipocyte cell culture

3T3-L1 preadipocytes (ATCC) were cultured at a 5% CO^2 atmosphere at 37 °C in DMEM (Gibco) supplemented with 10% newborn calf serum (Sigma) and penicillin/streptomycin (GIBCO). At confluence, the cells were differentiated into adipocytes by treating them with DMEM/F12/glutamate-medium supplemented with 0.5 mmol/L 3-isobutyl-methyl-xanthine (IBMX), 0.25 μmol/L corticosterone, 1 μmol/L insulin, 200 μmmol/L ascorbate, 2 μg/mL transferrin, 1 μmmol/L biotin, 17 μmmol/L panthothenate, and 300 mg/L Pedersen-fetuin for five days. Thereafter, the cells were exposed to DMEM/F12/glutamate medium with 1nmol/L insulin until they reached the fully differentiated phenotype.

Insulin resistant adipocytes construction and assessment

The differentiated adipocytes were incubated with Krebs-Ringer phosphate buffer (KRP) (pH 7.6) containing 10 nm glucose, 1 mm palmitic acid PA), and 1% BSA at 37°. Control cells were treated identically except that BSA without fatty acid was added to the KRP buffer. The duration of incubation was overnight for 3T3-L1 adipocytes. The incubation buffer was removed and cells were resuspended for another hour at 37°, with KRP (pH 7.0) containing 1 mm pyruvate and 1% BSA without fatty acid. Cells were then washed twice with KRP (pH 7.6) containing 1% BSA without fatty acid and resuspended in appropriate buffers for experimentation. To evaluate insulin resistance, the glucose consumption and glucose uptake were detected. Adipocytes treated with PA showed signifi-

cantly lower glucose consumption and glucose uptake than untreated cells, and were considered to be insulin resistant. These insulin-resistant cells were used for the subsequent experiments.

Intervention

The insulin resistant adipocytes were given recombinant CTRP3 protein (Jiahui, China) at different concentrations (0, 10, 50, 250, and 1,250 ng/mL) for 12h, together with DMEM containing 25 mmol/L glucose. To investigate the role of insulin signal molecule PI3K in the effects of CTRP3 on insulin resistant adipocytes, 100 nmol/L wortmannin, the special inhibitor of PI3K, pre-treated the cells for 20 min before the treatment with 250 ng/mL CTRP3 in another group.

Detection

Glucose consumption

Glucose level in the cell culture media was determined by glucose oxidase method (Beihua Kangtai,China) and glucose consumption was calculated as (25-glucose level) mmol/L.

Glucose uptake assay

Differentiated 3T3-L1 cells were induced with various concentrations of CTRP3 in the presence or absence of 10 nmol/L insulin for 30 min. After induction, cells were washed with KRP buffer and glucose uptake was initiated by the addition of 0.5 ml KRP buffer with 0.5 μCi 2-deoxy-d-[^{3}H]-glucose (Kailong, China). Glucose uptake was terminated after 10 min by washing the cells with ice-cold PBS buffer three times. Cells were solubilised with 1ml NaOH (0.1mmol/L) for two hours and were counted by a liquid scintillation counter after being kept in the dark in scintillation fluid for 12h. Nonspecific uptake, determined in the presence of 10 μmol/L cytochalasin b, was subtracted from all values.

Inflammation factors secretion

The concentration of TNF-α and IL-6 in the cell culture media was measured by Enzyme Linked Immunosorbent Assay (Zhongzhi, China). Each sample was measured in duplicate.

Real-time-PCR Analysis (RT-PCR)

RNAs were isolated from 3T3-L1 adipocytes using TRIzol (Promega) and reverse transcribed using Superscript II RNase H-reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR primers (Saibaisheng, China) used in the study were as follows: (1) IL-6: Sense 5-AGT TGC CTT CTT GGG ACT GA-3; Anti-sense: 5-CAG AAT TGC CAT TGC ACA AC-3; (2) TNF-α: Sense 5-ACG GCA TGG ATC TCA AAG AC-3; Anti-sense: CGG CAG AGA CCA CCT TGA ACT-3; (3) GLUT-4: Sense 5-CCC CGC TGG AAT GAG GTT TTT GAG GTG AT-3; Anti-sense: CAG ACA GGG GCC GAA GAT TGG GAG ACA GT-3; (4)β-actin: Sense 5-ACA CCC GCC ACC AGT TCG C-3; Anti-sense 5-TCT CCC CCT CAT CAC CCA CAT-3.

Western blot analysis

The cultured cells were washed twice with PBS, harvested gently with a cell scraper, centrifuged, and resuspended in 100 μl PBS. Whole cell proteins were extracted from 3T3-L1 adipocyte cells using lysis buffer (20 mmol/L MOPS, pH 7.0, 2 mmol/L EGTA, 5 mmol/L EDTA, 30 mmol/L sodium fluoride, 40 mmol/L ß-glycerophosphate, 10 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 3 mmol/L benzamidine, 5 μmol/L pepstatin A, 10 μmol/L leupeptin, 0.5% nonidet P-40, and 0.5% Triton X-100). Samples were boiled for 5 min in SDS loading buffer and equal amounts (25–50 μg per sample) of protein extracts were then separated by 8–12% of SDS-PAGE and electrotransferred onto PVDF membrane (Bio-Rad). Membranes were blocked with 5% non-fat skimmed milk in Tris-buffered saline/0.1% Tween20 (TBS-T) for 1 h, and then incubated with affinity-purified goat polyclonal primary antibodies were used at the following working dilutions: PI3K (1:1,000 dilution); beta-actin: (1: 1,000 dilution) and PKB(Ser473): (1:1,000 dilution). Appropriate secondary antibodies conjugated to horseradish peroxidase were incubated with respective membranes for 1 h at room temperature. Following five intermittent washes with 1 × TBS-T, the membranes was processed for autoradiography using enhanced chemiluminescence (ECL, Pierce Chemical). The results were quantified by densitometric analysis using the Image-Quant software. All Western blot experiments were performed in triplicate.

Statistical analysis

Data was expressed as the mean ± SE and evaluated statistically using One-way ANOVA with SPSS (version 19.0) software. A value of $P < 0.05$ was considered to be statistically significant.

Results

Insulin resistant adipocytes assessment

Compared to the normal group, the glucose consumption and glucose uptake of the insulin resistant group was decreased by 50.6% [(17.34 ± 1.20) *vs.* (8.56 ± 0.73) mmol/L, $P < 0.01$] and 57.9% ($P < 0.01$) respectively, indicating that the insulin resistant 3T3-L1 adipocytes model was conducted successfully.

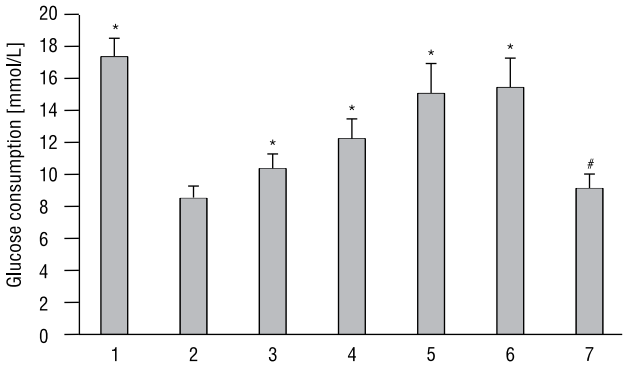

Figure 1. *Impacts of CTRP3 on the glucose consumption of insulin resistant 3T3-L1 adipocytes (mean ± SE).*
*1–7: Normal group, Insulin resistance control group, 10 ng/mL CTRP3 intervention group, 50 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 intervention group, 1,250 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 + wortmannin intervention group vs. Insulin resistance control group *P < 0.01; vs. 250 ng/mL CTRP3 intervention group #P < 0.01*

Rycina 1. *Wpływ białka CTRP3 na zużycie glukozy przez insulinooporne adipocyty 3T3-L1 (średnia ± SE)*

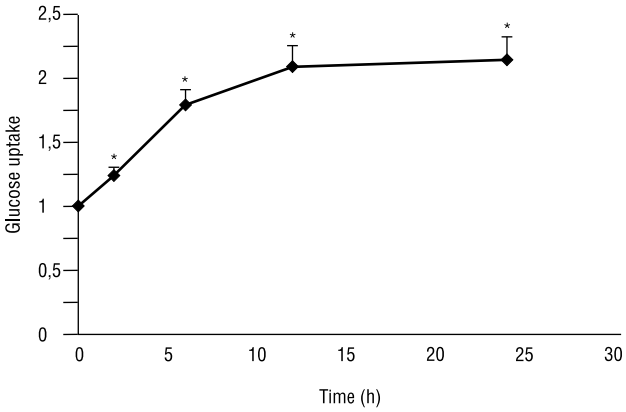

Figure 2. *The time-effect relationship of CTRP3 on glucose uptake ratio of insulin resistant 3T3-L1 adipocytes (mean ± SE). * vs. Time 0 P < 0.01.*

Rycina 2. *Zależność efektu od czasu ekspozycji na działanie białka CTRP3 w ocenie współczynnika wychwytu glukozy insulinoopornych adipocytów 3T3-L1 (średnia ± SE)*

Table I. ***The dose-effect relationship of CTRP3 on glucose uptake of insulin resistant 3T3-L1 adipocytes (mean ± SE)***
Tabela I. ***Zależność efektu od dawki białka CTRP3 w ocenie wychwytu glukozy insulinoopornych adipocytów 3T3-L1 (średnia ± SE)***

CTRP3 content [ng/mL]	0	10	50	250	1,250	250$^{\Delta}$
Glucose uptake (n = 9)	1	1.39 ± 0.11*	1.68 ± 0.13*	2.08 ± 0.19*	2.11 ± 0.18*	1.15 ± 0.08$^{\#}$

$^{\Delta}$pretreated with 100 nmol/L wortmannin for 20 min. *vs.* CTRP3 content 0 $^{*}P < 0.01$, *vs.* CTRP3 content 250 $^{\#}P < 0.01$

Impacts of CTRP3 on the glucose consumption of insulin resistant 3T3-L1 adipocytes

With the increasing concentrations of CTRP3 (10, 50, 250, and 1,250 ng/mL), the glucose consumption was increased by 22.1%, 42.9%, 76.6% and 80.5% respectively (all $P < 0.01$) compared to that of the control group. The differences of glucose consumption among 10 ng/mL, 50 ng/mL and 250 ng/mL CTRP3 intervention groups were also significant ($P < 0.05$ or $P < 0.01$). There was no significant difference in glucose consumption between the 1,250 ng/mL and the 250 ng/mL CTRP3 intervention groups ($P > 0.05$), indicating that 250 ng/mL might be the optimal concentration of CTRP3 in promoting insulin resistant 3T3-L1 adipocytes to utilise glucose (Fig. 1).

Impacts of CTRP3 on the glucose uptake of insulin resistant 3T3-L1 adipocytes

The dose-effect relationship of CTRP3 on glucose uptake

Compared to the control group, the glucose uptake in the CTRP3 intervention group at the concentrations of 10, 50, 250, and 1,250 ng/mL was increased by 39.0%, 68.0%, 108.0% and 111.0% respectively (all $P < 0.01$). The differences of glucose uptake among the CTRP3 intervention groups at the concentrations of 10 ng/mL, 50 ng/mL and 250 ng/mL were also significant (all $P < 0.01$). There was no significant difference in glucose uptake between the 1,250 ng/mL and the 250 ng/mL CTRP3 intervention groups ($F = 0.343$, $P = 0.735$), indicating that 250 ng/mL might be the optimal concentration of CTRP3 in improving insulin resistance in insulin resistant 3T3-L1 adipocytes (Table I).

The time-effect relationship of CTRP3 on glucose uptake

To investigate the time-effect relationship of CTRP3 on glucose uptake in insulin resistant adipocytes, this research detected the glucose uptake in the 250 ng/mL CTRP3 intervention group at different times (0, 2, 6, 12, and 24h). Compared to 0h, the glucose uptake at 2h, 6h, 12h and 24h was increased by 23.0%, 79.0%, 109.0% and 114.0% respectively (all $P < 0.01$). With the intervention duration extended from 2h to 12h, the glucose uptake was also increased accordingly. There was no significant difference in glucose uptake at 12h and 24h ($P > 0.05$), indicating that the effects of CTRP3 intervention at the concentration of 250 ng/mL for 12h on glucose uptake in insulin resistant 3T3-L1 adipocytes might be the maximal (Fig. 2).

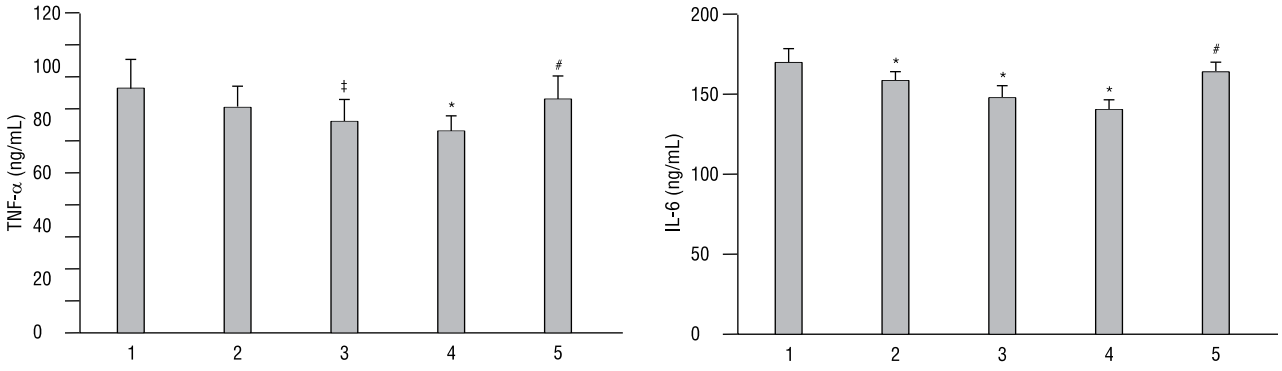

Figure 3. *Impacts of CTRP3 on the release of TNF-α and IL-6 of insulin resistant 3T3-L1 adipocytes (mean ± SE). 1–5: Control group, 10 ng/mL CTRP3 intervention group, 50 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 + wortmannin intervention group. vs. control group *P < 0.01, ‡P < 0.05; vs. 250 ng/mL CTRP3 intervention group #P < 0.01*

Rycina 3. *Wpływ białka CTRP3 na uwalnianie TNF-α i IL-6 przez insulinooporne adipocyty 3T3-L1 (średnia ± SE)*

Impacts of CTRP3 on the expression and release of inflammation factors of insulin resistant 3T3-L1 adipocytes

Compared to the control group, the content of TNF-α in the culture media of CTRP3 (10, 50, 250 ng/mL, for 12h) intervention group was decreased by 7.6% ($F = 1.625$, $P = 0.124$), 13.0% ($P < 0.05$) and 17.4% ($P < 0.01$) respectively; the content of IL-6 was decreased by 7.1%, 12.4% and 17.1% respectively (all $P < 0.01$); the mRNA relative expression of TNF-α was decreased by 8.0%, 16.0% and 26.0% respectively (all $P < 0.01$ or $P < 0.05$); and the mRNA relative expression of IL-6 was decreased by 9.0%, 11.0% and 19.0% respectively (all $P < 0.01$ or $P < 0.05$) (Figs. 3 and 4).

Impacts of CTRP3 on the expression of insulin signal molecules of insulin resistant 3T3-L1 adipocytes

Compared to the control group, with increases to the content of CTRP3 (10, 50, 250 ng/mL) treated for 12h, the protein relative expression of PI3K was increased by 0.63-, 1.00- and 1.36-fold respectively (all $P < 0.01$), and PKB(ser437) increased by 0.65-, 1.61- and 1.93-fold respectively (all $P < 0.01$). The difference among the CTRP3 intervention groups was also significant (all $P < 0.01$) (Fig. 5).

Impacts of CTRP3 on the expression of GLUT-4 of insulin resistant 3T3-L1 adipocytes

Compared to the control group, with increases to the content of CTRP3 (10, 50, 250ng/mL) treated for 12h, the mRNA relative expression of GLUT-4 was increased by 23.0%, 47.0% and 62.0% respectively (all $P < 0.01$),which may be the direct reason for the increasing glucose uptake with CTRP3 intervention (Fig. 4).

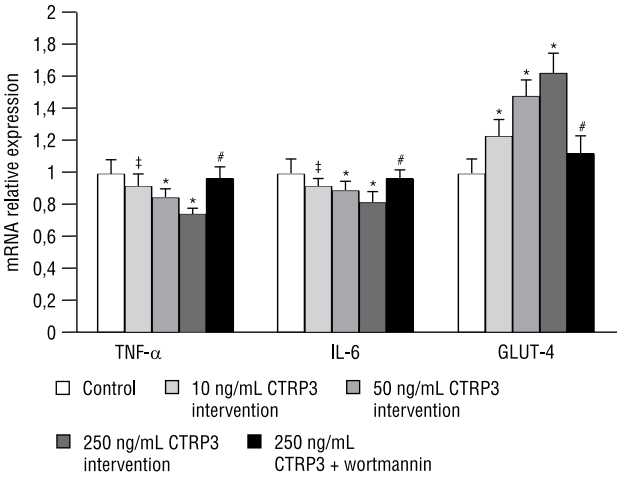

Figure 4. *Impacts of CTRP3 on the relative expression of TNF-α, IL-6 and GLUT-4 mRNA of insulin resistant 3T3-L1 adipocytes (mean ± SE). vs. control group * P < 0.01, ‡P < 0.05; vs. 250 ng/mL CTRP3 intervention group # P < 0.01*

Rycina 4. *Wpływ białka CTRP3 na względną zmianę ekspresji TNF-α, IL-6 i mRNA GLUT-4 w insulinoopornych adipocytach 3T3-L1 (średnia ± SE)*

Impacts of wortmannin on the glucose consumption, glucose uptake and insulin signal transduction of insulin resistant 3T3-L1 adipocytes with 250 ng/mL CTRP3 intervention

Compared to the insulin resistant 3T3-L1 adipocytes treated with 250 ng/mL CTRP3, the glucose consumption, glucose uptake, PI3K protein relative expression, PKB (ser437) protein relative expression and GLUT-4 mRNA relative expression in the 250 ng/mL CTRP3 treated insulin resistant 3T3-L1 adipocytes pretreated with wortmannin was decreased by 53.2%, 44.7%, 43.4%, 56.1 and 30.9% respectively (all $P < 0.01$), indicating that CTRP3 may increase insulin sensitivity of

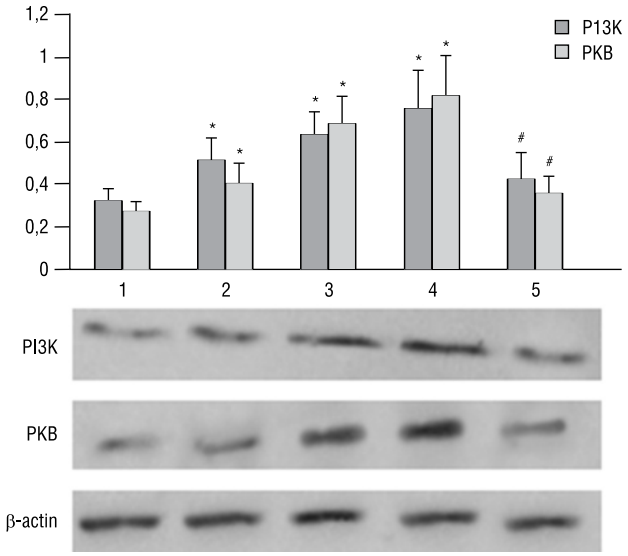

Figure 5. *Impacts of CTRP3 on the relative expression of PI3K and PKB protein of insulin resistant 3T3-L1 adipocytes (mean ± SE). 1–5: Control group, 10 ng/mL CTRP3 intervention group, 50 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 intervention group, 250 ng/mL CTRP3 +wortmannin intervention group. vs. control group * P < 0.01, ‡P < 0.05; vs. 250 ng/mL CTRP3 intervention group # P < 0.01*

Rycina 5. *Wpływ białka CTRP3 na względną zmianę ekspresji PI3K i PKB w insulinoopornych adipocytach 3T3-L1 (średnia ± SE)*

insulin resistant 3T3-L1 adipocytes through PI3K signal transduction (Table I, Figs. 1, 3, 4, 5).

Discussion

CTRP3, a paralogue of adiponectin, is a member of the C1q and tumour necrosis factor (TNF)-related protein (CTRP) superfamily. It is expressed at high levels in adipose tissue and has recently emerged as a novel adipokine. Recombinant CTRP3 reduced glucose output in cultured rat hepatoma cells by suppressing gluconeogenic genes, significantly inhibited LPS-induced IL-6 and TNF-a secretion in THP-1 cells, and reduced NF-kB p65 activity [4–8]. Clinical research indicated that circulating CTRP3 concentration had a significant association with cardiometabolic risk factors, such as obesity, glucose levels, lipid parameters, eGFR, and adiponectin levels [11]. These results suggested that CTRP3 may have anti-diabetic and anti-inflammatory properties. Chronic low-grade inflammation was thought to be the key in the pathogenesis of insulin resistance, T2DM and cardiovascular disease (CVD) [12]. As CTRP3 can inhibit inflammation, it is possible for CTRP3 to improve insulin resistance. But there no report has addressed this issue. So this study aimed to investigate the effects and mechanisms of CTRP3 on the insulin resistance of insulin resistant 3T3-L1 adipocytes.

Our research found that treatment with CTRP3 significantly improved the glucose consumption and glucose uptake in insulin resistant 3T3-L1 adipocytes, suggesting that CTPR3 may increase the insulin sensitivity of insulin resistant adipocytes. It also showed that the optimal concentration of CTRP3 intervention was 250 ng/mL, and the optimal time was 12h. To the best of our knowledge this is the first report into the impacts of CTRP3 on insulin sensitivity, and the optimal concentration as well as duration of CTRP3 treatment on insulin resistant adipocytes.

Elevated serum-free fatty acid (FFA) levels induce insulin resistance in animals and humans. To use FFA to construct an insulin resistant model either *in vivo* or *in vitro* is a classic and universal method [13]. In this research, we used palmitic acid incubation to construct insulin resistant 3T3-L1 adipocytes model according to a previous report [13]. We found that compared to normal adipocytes, the glucose consumption of palmitic acid incubated adipocytes was decreased by 50.6% [(17.34 ± 1.20) *vs.* (8.56 ± 0.73) mmol/L, $P < 0.01$] , indicating that the palmitic acid incubated 3T3-L1 adipocytes could be considered as insulin resistant adipocytes.

From previous studies, it is well established that when there is an expansion of adipose tissue, there is a sustained inflammatory response accompanied by adipokine dysregulation, which leads to chronic subclinical inflammation as well as insulin resistance [14]. One of the mechanisms leading to insulin resistance is chronic low-grade inflammation that involves a number of protagonists such as inflammatory cytokines, lipids and their metabolites, reactive oxygen species (ROS) and endoplasmic reticulum stress [15]. As mentioned above, CTRP-3 inhibits LPS-induced IL-6 and TNF-α release in human monocytes [5]. This research found that under the treatment with CTRP3 for 12h (10, 50, 250 ng/mL), the release of TNF-α was decreased by 7.6% ($F = 1.625$, $P = 0.124$), 13.0% ($P < 0.05$) and 17.4% ($P < 0.01$) respectively; the release of IL-6 was decreased by 7.1%, 12.4% and 17.1% respectively (all $P < 0.01$); and the mRNA relative expression of IL-6 and TNF-α had the same tendency. This indicates that CTRP3 could also inhibit inflammation in insulin resistant adipocytes, by which means CTRP3 increases insulin sensitivity of insulin resistant adipocytes.

Insulin resistance is a pathological condition that arises when insulin signalling is impaired, forcing pancreatic β-cells to produce more insulin in order to compensate for body demands and to maintain glucose homeostasis [16]. Insulin signalling involves a complex signalling cascade downstream of the insulin receptor. This signalling cascade branches into two main pathways. The first is the PI3K/PKB pathway which is

largely responsible for insulin action on glucose uptake, as well as other metabolic actions of insulin, including the suppression of gluconeogenesis. The second pathway is the Ras-mitogen-activated protein kinase (MAPK) pathway which mediates gene expression, but also interacts with the PI3K/PKB pathway to control cell growth and differentiation [17]. This article mainly focused on the PK3K/PKB signalling, and found that as the content of CTRP3 (10, 50, and 250 ng/mL) treatment increased, the protein relative expression of PI3K was increased by 0.63-, 1.00- and 1.36-fold respectively (all $P < 0.01$), and PKB(ser437) increased by 0.65-, 1.61- and 1.93- folds- respectively (all $P < 0.01$), the mRNA relative expression of GLUT-4 was increased by 23.0%, 47.0% and 62.0% respectively (all $P < 0.01$), which may be the direct reason for the increased glucose uptake with CTRP3 intervention.

To determine the role of insulin signal molecule PI3K in the effects of CTRP3 on insulin resistant adipocytes, we also used wortmannin, the special inhibitor of PI3K, to pretreat adipocytes before the treatment with 250 ng/mL CTRP3. We found that the glucose consumption, glucose uptake, PI3K protein relative expression, PKB(ser437) protein relative expression and GLUT-4 mRNA relative expression in the adipocytes pretreated with wortmannin was decreased by 53.2%, 44.7%, 43.4%, 56.1 and 30.9% respectively (all $P < 0.01$), indicating that CTRP3 may increase insulin sensitivity of insulin resistant 3T3-L1 adipocytes through PI3K signal transduction.

Insulin and inflammation signalling are intertwined. Jun N-terminal kinase-1 (JNK1) and inhibitor of kB kinase (IKKβ) are inflammatory signalling pathways which promote insulin resistance. Activation of either pathway leads to serine phosphorylation of insulin receptor substrate protein-1 (IRS-1), causing attenuation of insulin action [18]. This article found that CTRP3 decreased the expression of inflammation factors of insulin resistant 3T3-L1 adipocytes and increased the expression of insulin signal molecules. So it is possible that the effects of CTRP3 on insulin signalling in 3T3-L1 adipocytes may be just an indirect result following on from the inhibition of inflammation.

CTRP3 also regulates the expression and secretion of other adipokines, such as adiponectin. A dose of 10 ng/mL CTRP3 increased adiponectin secretion in 3T3-L1 adipocytes significantly [19]. As we all know, adiponectin is the most highly expressed and intensely studied adipokine and has insulin-sensitising, anti-inflammatory and antiatherogenic properties [20]. So the modulation of the expression of other adipokines may be another mechanism for CTRP3 in increasing insulin sensitivity of 3T3-L1 adipocytes.

To sum up, this study found that CTRP3 may improve insulin sensitivity of insulin resistant 3T3-L1 adipocytes by decreasing inflammation and ameliorating insulin signalling, indicating that CTRP3 could be a new target for the prevention and cure of insulin resistance and T2DM. But this study could not answer how CTRP3 decreases inflammation and improves insulin signalling, whether the impact is direct or indirect, and whether the effects still exist *in vivo*.

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