The traditional view of adipose tissue was as a passive reservoir for energy storage. The discovery and subsequent study of several molecules secreted from adipose tissue unveiled their physiological and metabolic functions and transformed our understanding of this tissue to that of a large active endocrine organ [1]. Adipose tissue is now known to express and secrete numerous adipokines, which not only regulate metabolism and chronic inflammation associated with metabolic syndrome, but also seem to function as a fundamental link between the adipose tissue and a wide array of tissues underlying physiological functions and/or metabolic disorders [2].

Many studies have implicated adiponectin as a major insulin-sensitizing adipokine as well as an important biomarker and therapeutic target for obesity-associated metabolic disorders [3]. Leptin increases energy expenditure and reduces food intake. A physiological increase in serum leptin levels has been shown to inhibit insulin secretion [4]. Recently more adipokines, such as visfatin and apelin, have been discovered and studied. Both of them improve insulin sensitivity and maintain glucose homeostasis in rodents and humans. Visfatin has been shown to play an important role in pancreatic beta-cell function by acting as an intra- and extracellular NAD biosynthetic enzyme and regulating glucose-stimulated insulin secretion [5]. Apelin increases the expression of uncoupling proteins (UCP) 1 and 3, increases energy expenditure and decreases respiratory quotient, resulting in increased fat oxidation [6]. C1q and tumor necrosis factor (TNF) -related...
protein 3 (CTRP3), together with adiponectin, belong to the CTRP superfamily. It is expressed at high levels in adipose tissue and has recently emerged as a novel adipokine [7]. Recombinant CTRP3 reduced glucose output in cultured rat hepatoma cells by suppressing gluconeogenic genes [8], and significantly inhibited LPS-induced IL-6 and TNF-α secretion in THP-1 cells, and reduced NF-kB p65 activity [9]. 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 [10]. These results suggested that CTRP3 may have anti-diabetic and anti-inflammatory properties.

CTRP3 has been reported to increase the expression of adiponectin in adipocytes independent of peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling pathway [11]. For adipokines to exert the important effects on the metabolism as mentioned above, it is possible that it is effected through the regulation of the expression of other adipokines. But such effects of CTRP3 are largely unknown. This study is thus aimed to investigate the impact of CTRP3 on the expression and secretion of adiponectin, leptin, visfatin, and apelin in 3T3-L1 adipocytes. The effect of insulin resistance on their impact was also investigated.

Materials and Methods

Adipocyte cell culture

3T3-L1 preadipocytes (American Type Culture Collection, Manassas, VA, USA) were cultured at a 5% CO₂ atmosphere at 37°C in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% newborn calf serum (Sigma, St. Louis, MO, USA) and penicillin/streptomycin (Gibco, Grand Island, NY, USA). 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 μmol/L ascorbate, 2 μg/mL transferrin, 1 μmol/L biotin, 17 μmol/L panthothenate, and 300 mg/L Pedersen-fetuin for 5 days. Thereafter, the cells were exposed to DMEM/F12/glutamate medium with 1 nmol/L insulin until they reached the fully differentiated phenotype [11].

Insulin resistant adipocytes construction

The differentiated adipocytes were incubated with Krebs-Ringer phosphate buffer (KRP) (pH 7.6) containing 10 nmol/L glucose, 1 mmol/L palmitic acid (PA), and 1% BSA at 37°C. Control cells were treated identically except that BSA without fatty acid was added to the KRP buffer. After overnight incubation, the incubation buffer was removed and cells were resuspended for another hour at 37°C, with KRP (pH 7.0) containing 1 mmol/L pyruvate and 1% BSA without fatty acid. Cells were then washed two times with KRP (pH 7.6) containing 1% BSA without fatty acid and resuspended in appropriate buffers for experimentation.

Recombinant CTRP3 generation and purification

Recombinant CTRP-3 was generated and purified according to a former study [9] in a company (Jiahui, Wuhan, China). pcDNA3.1 expression construction encoding a C-terminal FLAG-tagged mouse CTRP3 was used in transient transfection. FreeStyle™293-F cells (Invitrogen, Carlsbad, CA, USA) were cultured in FreeStyle™293 expression medium and transfection was performed using 293fectin™ (Invitrogen, Carlsbad, CA, USA). Four days later, the supernatants were collected and purified by an anti-FLAG affinity gel (Sigma, St. Louis, MO, USA). Purified protein was dialyzed against 20 mmol/L HEPES buffer.

Intervention

3T3-L1 adipocytes were treated with different concentrations (0, 10, 50, 250, 1250 ng/mL) of recombinant CTRP3 protein for 12 h, and with 250 ng/mL CTRP3 for different times (0, 6, 12, 24, 48 h), together with DMEM containing 25 mmol/L glucose. To investigate the impact of insulin resistance on the effect of CTRP3 on the expression of adipokines, this study also compared the expression of adipokines between normal adipocytes and insulin resistant adipocytes under the same treatment with 250 ng/mL CTRP3 for 12 h. To investigate the role of AMP activated protein kinase (AMPK) signaling in the modulation of CTRP3 on the expression of adiponectin in adipocytes, another group was pretreated with 10 μmol/L Compound C (Calbiochem, San Diego, CA, USA), a special inhibitor of AMPK, for 1 h before it was treated with 250 ng/mL CTRP3 for 12 h.

Detection

Glucose consumption

Glucose level in the cell culture media was determined by glucose oxidase method (Beihua Kangtai, Beijing, China) and glucose consumption was calcu-
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Biotechnology, Inc. (Dallas, TX, USA). 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 were processed for autoradiography using enhanced chemiluminescence (ECL, Pierce Chemical, Rockfield, IL, USA). The results were quantified by densitometric analysis using the ImageQuant software. All Western blot experiments were performed in triplicate.

Statistical analysis

Data were expressed as the mean ± SE and evaluated statistically using One-way ANOVA with SPSS (version 19.0) software. Further comparisons between two groups were performed by SNK-q test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Impact of CTRP3 on the secretion of adipokines in 3T3-L1 adipocytes

The dose effect of CTRP3 on adipokine secretion

To investigate the dose effect of CTRP3 on adipokine secretion in 3T3-L1 adipocytes, this study detected the adipokine secretion under treatment with different concentrations (0, 10, 50, 250, 1250 ng/mL) CTRP3 for 12 h. Compared with normal control group, with the increase in CTRP3 concentration, the secretion of adiponectin, leptin, visfatin, and apelin were all increased accordingly, which was significant under the treatment with 250 ng/mL and 1250 ng/mL CTRP3 (all $P < 0.01$). The differences of adipokine secretion between 50 ng/mL and 250 ng/mL CTRP3 intervention group were also significant (all $P < 0.05$ or $P < 0.01$). There were no significant differences in adipokine secretion between 1250 ng/mL and 250 ng/mL CTRP3 intervention group (all $P > 0.05$), indicating that 250 ng/mL may be the optimal concentration for CTRP3 in regulating adipokine secretion in 3T3-L1 adipocytes under treatment for 12 h. (Fig. 1)

The time effect of CTRP3 on adipokine secretion

To investigate the time effect of CTRP3 on adipokine secretion in 3T3-L1 adipocytes, this study detected the adipokine secretion under treatment with different concentrations (0, 10, 50, 250, 1250 ng/mL) CTRP3 for 12 h. With the increase in the duration of CTRP3 treatment, the secretion of adiponectin, leptin, visfatin, and apelin were all increased accordingly and significantly at 12 h, 24 h and 48 h in comparison with that of 0 h.
Li et al. all increased accordingly and significantly under the treatment with 50 ng/mL and 250 ng/mL CTRP3 (all \(P<0.01\)). The differences of the gene relative expression between 50 ng/mL and 250 ng/mL CTRP3 intervention group were also significant (all \(P<0.01\)) (Fig. 3).

Impact of insulin resistance on the effects of CTRP3 on the expression and secretion of adipokines in 3T3-L1 adipocytes

To investigate the impact of insulin resistance on the effects of CTRP3 on the expression and secretion of adipokines in 3T3-L1 adipocytes, this study also compared the secretion and gene expression of adipokines between normal and insulin resistant 3T3-L1 adipocytes under the same treatment with 250 ng/mL CTRP3 for 12 h. Compared with normal group, the glucose consumption of insulin resistant group was decreased by 50.6\% \((17.34\pm1.20) \text{ vs } (8.56\pm0.73) \text{ mmol/L, } P<0.01\), indicating that the insulin resistant 3T3-L1 adipocyte model was conducted successfully. Compared with

(all \(P<0.01\)). The differences of adipokine secretion between 6h and 12h intervention group were also significant (all \(P<0.01\)), and so were between 12h and 24h intervention group (all \(P<0.05\)). There were no significant differences in adipokine secretion between 24h and 48h intervention group (all \(P>0.05\)). The results indicated that 12 h may be the effective time and 24 h may be the optimal time for 250 ng/mL CTRP3 treatment in modulating adipokine secretion in 3T3-L1 adipocytes (Fig. 2).

Impact of CTRP3 on gene expression of adipokines in 3T3-L1 adipocytes

To investigate the impact of CTRP3 on gene expression of adipokines in 3T3-L1 adipocytes, this study detected the relative expression of adipokines mRNA under treatment with different concentrations of CTRP3 \((0, 10, 50, 250 \text{ ng/mL})\) for 12 h. With the increase in CTRP3 concentration, the relative expression of adiponectin, leptin, visfatin, and apelin mRNA were all increased accordingly and significantly under the treatment with 50 ng/mL and 250 ng/mL CTRP3 (all \(P<0.01\)). The differences of the gene relative expression between 50 ng/mL and 250 ng/mL CTRP3 intervention group were also significant (all \(P<0.01\)) (Fig. 3).

Impact of insulin resistance on the effects of CTRP3 on the expression and secretion of adipokines in 3T3-L1 adipocytes

To investigate the impact of insulin resistance on the effects of CTRP3 on the expression and secretion of adipokines in 3T3-L1 adipocytes, this study also compared the secretion and gene expression of adipokines between normal and insulin resistant 3T3-L1 adipocytes under the same treatment with 250 ng/mL CTRP3 for 12 h. Compared with normal group, the glucose consumption of insulin resistant group was decreased by 50.6\% \((17.34\pm1.20) \text{ vs } (8.56\pm0.73) \text{ mmol/L, } P<0.01\), indicating that the insulin resistant 3T3-L1 adipocyte model was conducted successfully. Compared with
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Fig. 2  Time effects of CTRP3 on secretion of adipokines in 3T3-L1 adipocytes (mean ± SE)
3T3-L1 adipocytes were treated with 250 ng/mL CTRP3 for different times (0, 6, 12, 24, 48h). CTRP3: C1q/TNF related protein 3. vs 0h * P<0.01.

Fig. 3  Impact of CTRP3 on gene expression of adipokines in 3T3-L1 adipocytes (mean ± SE)
3T3-L1 adipocytes were treated with different concentrations (0, 10, 50, 250 ng/mL) of CTRP3 for 12 h. NC, normal control. CTRP3: C1q/TNF related protein 3. vs NC group * P<0.01.
the normal adipocytes treated with CTRP3, the secretions of adiponectin, leptin, visfatin, and apelin in insulin resistant adipocytes treated with CTRP3 were decreased by 28.6%, 21.0%, 24.5% and 17.9%, respectively ($q=6.42, 6.13, 5.70, 4.34$, all $P<0.01$), and gene expressions were decreased by 21.6%, 17.2%, 15.6%, and 18.9%, respectively ($q=9.59, 7.56, 6.74, 8.24$, all $P<0.01$) (Fig. 4).

**Role of AMPK signaling in the modulation of CTRP3 on the expression of adiponectin in 3T3-L1 adipocytes**

To investigate the role of AMPK signaling in the modulation of CTRP3 on the expression of adiponectin in 3T3-L1 adipocytes, this study used Compound C, a special inhibitor of AMPK, to pre-treat 3T3-L1 adipocytes for 1 h before the treatment with 250 ng/mL CTRP3 for 12 h. Compared with the adipocytes treated with CTRP3 only, the relative expression of AMPK (thr172) in adipocytes pre-treated with Compound C was decreased by 57.3% ($q=31.18$, $P<0.01$), the mRNA relative expression and protein release of adiponectin were decreased by 26.1% ($q=12.05$, $P<0.01$) and 54.1% ($q=12.08$, $P<0.01$), respectively, indicating that AMPK signaling may participate in the mod-

![Fig. 4](image-url)
CTRP3 modulates adipokines

CTRP3 modulates adipokines

Discussion

Obesity plays a causative role in the pathogenesis of the metabolic syndrome. Most adipokines with pro-inflammatory properties are overproduced with increasing adiposity, while some adipokines with anti-inflammatory or insulin-sensitizing properties, like adiponectin, are decreased [12]. This dysregulation of adipokine production may promote obesity-linked metabolic disorders. Many researches have shown that adiponectin may be a major insulin-sensitizing adipokine as well as an important biomarker and therapeutic target for obesity-associated metabolic disorders. However, the only mild metabolic dysfunctions in adiponectin knockout mice indicated that there might be additional molecules with similar functions as adiponectin [13].

CTRP3, a paralog of adiponectin, has many roles in regulating inflammation and metabolism as well as the expression of other adipokines, such as adiponectin, resistin, and leptin [14]. Studies in vivo indicate that a modest 3-fold elevation of plasma CTRP3 levels by

Fig. 5 Role of AMPK signaling in the modulation of CTRP3 on the expression of adiponectin in 3T3-L1 adipocytes (mean ± SE)

A, Adiponectin secretion in 3T3-L1 adipocytes; B, Adiponectin gene expression in 3T3-L1 adipocytes; C, AMPK and AMPK(thr172) phosphorylation in 3T3-L1 adipocytes; D, Comparison of the protein expression of AMPK(thr172) phosphorylation in 3T3-L1 adipocytes

3T3-L1 adipocytes were pre-treated with or without 10 μmol/L Compound C for 1 h, followed with or without 250 ng/mL CTRP3 for 12 h. NC, normal control. CTRP3: C1q/TNF related protein 3. CTRP3+C: CTRP3+Compound C. vs NC group * P<0.01. vs CTRP3 group # P<0.01.
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 [8]. Studies \textit{in vitro} show that CTRP3 inhibits monocyte chemoattractant protein-1 (MCP-1) release in adipocytes, whereas small interfering RNA-mediated knockdown of CTRP-3 upregulates MCP-1 release, reduces lipid droplet size, and decreases intracellular triglyceride concentration in adipocytes, causing a dedifferentiation into a more proinflammatory and immature phenotype [9]. Wölfing \textit{et al.} [11] reported that CTRP3 (1, 10, 50, and 250 ng/mL) in higher doses stimulated the secretion of adiponectin and resistin from murine adipocytes independent of peroxisome proliferator-activated receptor-\( \gamma \) (PPAR-\( \gamma \)) signaling. But the effects of CTRP3 on other adipokines, such as visfatin and apelin, and the dose effect and time effect of CTRP3 on such adipokines, are still largely unknown.

This study reported the dose effect and time effect of CTRP3 on the expression and secretion of adiponectin, leptin, visfatin, and apelin in 3T3-L1 adipocytes for the first time. To do this, this study detected the adipokine secretion under treatment with different concentrations (0, 10, 50, 250, 1250 ng/mL) CTRP3 for 12 h and the treatment with 250 ng/mL CTRP3 for different times (0, 12, 24, 48 h). Our results indicated that 250 ng/mL may be the optimal concentration for CTRP3 in regulating adipokine secretion in 3T3-L1 adipocytes under treatment for 12 h, and 12 h may be the effective time and 24 h be the optimal time for 250 ng/mL CTRP3 treatment. A previous study indicated that 10ng/mL CTPR3 increased the secretion of adiponectin significantly [11], while in this study CTRP3 at the same concentration did not increase the secretion of adiponectin significantly. The possible reason may be the different intervention duration, which was 24 h in the former research and 12 h in our study. The former study detected the effects of CTRP3 on the secretion of adiponectin and resistin, while our study also studied the effects of CTRP3 on the expression of leptin, visfatin, and apelin. The former study also found that human adipocytes from lean individuals secreted higher amounts of adiponectin and leptin compared with adipocytes of individuals with a preobesity BMI (25–30 kg/m\(^2\)), and CTRP3 failed to stimulate adiponectin or leptin secretion from human adipocytes, irrespective of BMI value. Whether these effects are also present in other races will require further studies to identify with any accuracy.

Consistent with the changes of adipokine secretion, with the increase in CTRP3 concentration (0, 10, 50, 250ng/mL), the relative expression of adiponectin, leptin, visfatin, and apelin mRNA were all increased accordingly. The expression of the adipokine mRNA was increased significantly under treatment with 50 ng/mL CTRP3 for 12 h, while the secretion of adipokines under the same condition was not increased significantly. This phenomenon may be explained by the fact that the duration of the intervention was too small for adipocytes to increase the expression and secretion of the protein under the treatment with 50 ng/mL CTRP3 for 12 h.

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 [15]. But the impact of insulin resistance on the effects of adipokines is unclear. This study thus investigated the impact of insulin resistance on the effects of CTRP3 on the expression and secretion of adipokines in 3T3-L1 adipocytes for the first time through comparing the secretion and gene expression of adipokines between normal 3T3-L1 adipocytes and insulin resistant 3T3-L1 adipocytes under the same treatment with 250 ng/mL CTRP3 for 12 h. We found that the secretion and gene expression of adipokines in insulin resistant 3T3-L1 adipocytes were all decreased significantly in comparison with that of normal 3T3-L1 adipocytes, indicating that insulin resistance may inhibit the up-regulating effects of CTRP3 on the expression and secretion of adiponectin, leptin, visfatin, and apelin.

Adiponectin is mainly synthesized by adipocytes, and many studies have shown that PPAR-\( \gamma \) appears to be a master regulator of its expression [16]. But a former study showed that CTRP3 increased the expression of adiponectin independent of PPAR-\( \gamma \) signaling [11]. Adiponectin exists in cells and the plasma in three major forms: trimers, hexamers, and the high-molecular weight (HMW) forms, and the HMW form has been shown to be the most bioactive [17-18]. Recently an adiponectin interactive protein DsbA-L (disulfide bond-A oxidoreductase-like protein) that promotes adiponectin multimerization was identified [19]. Activation of AMPK by AICAR markedly enhanced the protein levels of DsbA-L and adiponectin in 3T3-L1 adipocytes
A recent study showed that resveratrol promoted the multimerization and cellular levels of adiponectin in 3T3-L1 adipocytes via the AMPK signaling pathways. These results indicate that AMPK signaling may have important roles in modulating the expression and multimerization of adiponectin.

To investigate the role of AMPK signaling in the modulation of CTRP3 on the expression of adiponectin, this study used Compound C, a special inhibitor of AMPK, to pre-treat 3T3-L1 adipocytes for 1 h before the treatment with 250 ng/mL CTRP3 for 12 h. Together with the expression of AMPK (thr172), the relative mRNA expression and protein release of adiponectin in adipocytes pre-treated with Compound C were all decreased significantly in comparison with that in adipocytes treated with CTRP3 only, indicating that AMPK signaling may participate in the modulation of CTRP3 on the expression of adiponectin in 3T3-L1 adipocytes. To our knowledge, this is the first report on this issue.

On the whole, this study found that CTRP3 increased the gene expression and protein secretion of adiponectin, leptin, visfatin, and apelin, and insulin resistance inhibited the effects, indicating that there are complex interactions taking place among the adipokines and insulin resistance, and that CTRP3 may be a new target to cure and prevent insulin resistance and type 2 diabetes. This research also showed that CTRP3 up-regulated the expression of adiponectin in 3T3-L1 adipocytes via AMPK signaling pathway. But how CTRP3 activates AMPK signaling and how AMPK up-regulates the expression of adiponectin, and whether there are other signaling pathways involved in the effects of CTRP3 are still unknown.

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Disclosure

None of the authors have any potential conflicts on interest associated with this research.

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