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ORIGINAL PAPER

Interleukin-18 enhances glucose uptake in 3T3-L1 adipocytes

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Abstract In order to characterize the potential causative effects of interleukin-18 (IL-18) on insulin resistance, we measured glucose uptake in 3T3-L1 adipocytes treated with mouse recombinant IL-18. IL-18 surprisingly enhanced, rather than reduced insulin-mediated glucose uptake in adipocytes. Moreover IL-18 could counteract the glucose uptake suppression caused by tumor necrosis factor α in 3T3-L1 adipocytes. The mechanism dissection showed that the IL-18 upregulated phosphorylated Akt and down-regulated phosphorylated P38 MAPK. These findings indicated that the elevated serum IL-18 levels in obesity and diabetes might be a compensatory response to insulin resistance.

Keywords Interleukin-18 · Insulin resistance · 3T3-L1 adipocytes · Adipokines · p38 MAPK

Introduction

Interleukin-18 (IL-18) is now recognized as an important regulator of innate and acquired immune responses. It appears that IL-18 functions as a pleiotropic proinflammatory cytokine and plays an early role in the inflammatory cascade [1, 2]. In recent years, several reports have unveiled the association of IL-18 with obesity, insulin resistance, and type-2 diabetes [3–8]. Serum IL-18 levels were elevated in patients with diabetes [3, 4] and diabetic nephropathy [5]. Serum IL-18 was also increased in obese women, whereas declined as body weight lost [6]. Another research showed that IL-18 expression level in adipose tissue is increased in obesity but not affected by weight loss [7]. In a small group of women with polycystic ovarian syndrome (PCOS), IL-18 levels were found to positively correlate with visceral obesity and insulin resistance [9]. However, no correlation was found in obese women between IL-18 levels and insulin sensitivity in fasting state assessed by HOMA-IR [6]. Our previous work showed that serum IL-18 levels were significantly increased in PCOS women and firmly associated with insulin resistance displayed by euglycemic hyperinsulinemic clamp test [10]. In addition, a large study of non-diabetic population showed that elevated IL-18 levels were an independent risk predictor for the metabolic syndrome [11]. In type-2 diabetes, elevated plasma IL-18 was considered as a marker of insulin resistance [12]. However, it is still unclear whether elevated IL-18 is a pathogenic factor in insulin resistance since clinical studies could not elucidate the causative effect.

Interleukin-18 receptor, including IL-18Rα (previously named IL-18R1 and IL-1R-related protein, IL-1Rrp) and IL-18Rβ (also named IL18RAP), has been cloned and shown to be critical for IL-18 signaling [13, 14]. Our previous work has also identified IL-18 receptor 1 expression in human adipose tissue [15]. Thereafter we hypothesized IL-18 might play a role in mediating insulin resistance through its receptor in 3T3-L1 adipocytes. It has been reported that human adipose tissues could produce

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IL-18, which is a major source of systemic IL-18 concentrations [16]. The present study aims to explore the causative effect of IL-18 on insulin resistance.

Materials and methods

Reagents and antibodies

Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies, Incorporation. Isobutylmethylxanthine (IBMX), dexamethasone, wortmannin, cytochalasin B, [^{3}H] 2-deoxy-D-glucose were obtained from Sigma and Humulin R from Eli Lilly. Recombinant mouse IL-18 was obtained from R&D Systems. Polyclonal anti-Akt antibody, anti-phosphorylated Akt antibody, polyclonal anti-P38 MAPK antibody, anti-phosphorylated P38 MAPK (Thr180/Tyr182) antibody, anti-phosphorylated P44/42 MAPK (Thr202/Tyr204) antibody, anti-JNK antibody, and anti-phosphorylated SAPK/JNK (Thr183/Tyr185) antibody were purchased from Cell Signaling Technology, Incorporation. Anti-ERK antibody was obtained from Santa Cruz Biotechnology, Incorporation.

3T3-L1 Cell culture

The mouse fibroblast 3T3-L1 pre-adipocytes (CL-173) were purchased from the American Type Culture Collection. 3T3-L1 fibroblasts were cultured and differentiated into adipocytes as described by Seo et al. [17] with some modifications. In brief, cells were grown to confluence in DMEM supplemented with 25 mmol/l glucose and 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Two days later, 3T3-L1 cells were cultured in DMEM medium supplemented with 25 mmol/l glucose, 0.5 mmol/l IBMX, 1 μmol/l dexamethasone, 10 μg/ml insulin, and 10% FBS for 48 h and then in medium depleted of IBMX and dexamethasone for another 48 h. Thereafter, 3T3-L1 cells were maintained in DMEM supplemented with 25 mmol/l glucose and 10% FBS until 90–95% cells exhibited adipocyte phenotype. Mature 3T3-L1 cells were cultured in serum-free DMEM supplemented with 0.5% BSA for 6 h before tumor necrosis factor α (TNF-α) or IL-18 treatments. After this period, medium was changed and cells were harvested for 2-Deoxy-glucose uptake analysis or immunoblotting.

Reverse transcriptase-polymerase chain reaction

Based on our previous cDNA array assay, which showed IL-18Rα was expressed in adipocytes [15], we performed reverse transcriptase-polymerase chain reaction (RT-PCR) to detect IL-18, IL-18Rα, and IL-18Rβ transcripts in 3T3-L1 pre-adipocytes and adipocytes. For RT-PCR analysis, first-strand cDNAs were synthesized in 20 μl reaction system from 1 μg total RNA using oligo-dT (Promega). The PCR reaction mixture contained 1 μl cDNA, 0.5 μl 10 mM dNTP, 2 μl 10× PCR buffer, 5 U *Taq* plus DNA polymerase (Sangon), and 20 pmol intron-spanned primers. The sequences of primers used are as follows: mIL-18, 5′-ACAGCCTGTGTTCGA-GGATATG-3′ and 5′-TCTAT AAATCATGCAGCCTCGG-3′; mIL-18Rα, 5′-TCA-AGT CGGAAATGATCGTCG-3′ and 5′-GAGCTGTCCTCTTT CCTGATGC-3′; mIL-18Rβ, 5′-TTTGCTTGACCGAGAT GTGACC-3′ and 5′-GCCTGATCCACA CAGCAAGT TC-3′. PCR amplification was carried out by denaturation at 94°C for 30 s, annealing at 54–60°C for 30 s and extension at 72°C for 45 s for 30 cycles using MasterCycler gradient (Eppendorf).

[^{3}H]2-deoxy-D-glucose uptake in 3T3-L1 adipocytes

Glucose uptake in 3T3-L1 adipocytes was measured using a modified method described by Fletcher et al. [18]. The serum-starved cells were washed twice with Krebs-Ringer phosphate (KRP) buffer (pH 7.4). The cells were then incubated at 37°C for 30 min both in the presence and the absence of 10 or 100 nM insulin. About 1 ml KRP buffer containing 0.5 μCi [^{3}H]2-deoxy-D-glucose was added to each well for 15 min, and the cells were quickly washed thrice with ice-cold PBS containing 10 mM glucose. The treated cells were lysed in 0.1 N NaOH and subsequently solubilized in scintillation fluids (Triton X-100: methylbenzene was 1:2.5) overnight. [^{3}H]2-deoxy-D-glucose uptake was measured by scintillation counting. The non-specific glucose uptake was measured by subtracting values for [^{3}H]2-deoxy-D-glucose uptake in the presence of 10 μM cytochalasin B.

Immunoblotting and quantification

The cell lysates were extracted from IL-18 treated 3T3-L1 adipocytes with lysis buffer (RIPA, 1× PBS, 1% NP40, 5 mM EDTA, 0.5% SDS, 1 mM sodium orthovanadate, 1% PMSF, complete protease inhibitor cocktail and complete phosphatase inhibitors). Protein concentrations were determined by the Lowry method using DC Protein Assay Reagent (Bio-Rad Laboratories, CA, USA). Cell lysates were loaded onto SDS–PAGE and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Biosciences, NJ, USA). The membrane was blocked with 5% non-fat milk at RT for 1 h and

incubated with primary antibodies at 4°C overnight. Then the membrane was incubated with secondary antibodies at RT for 1 h. Immunoreactive bands were visualized using ECL western blotting protocol (Amersham Pharmacia). The densitometry analysis of the immunoreactive bands was performed using the LabWorks imaging and analysis software (UVP Bioimaging Systems).

Statistical analysis

Results are shown as means ± SD. Unpaired Student's *t*-tests were used to analyze the differences between various treatments. *P*-values <0.05 are considered significant and <0.01 are highly significant.

Results

IL-18 and IL-18R expression in 3T3-L1 adipocytes

The RT-PCR showed that IL-18 and IL-18 receptors (IL-18Rα and IL-18Rβ) were expressed in 3T3-L1 pre-adipocytes and adipocytes at mRNA level (Fig. 1). The amplified IL-18Rα and IL-18Rβ were further sequenced, which confirmed the authenticity of the amplified transcripts.

Insulin-mediated glucose uptake was enhanced by IL-18 in adipocytes

The effect of recombinant IL-18 on glucose transportation in 3T3-L1 adipocytes was evaluated using [^{3}H]2-deoxy-D-glucose uptake. IL-18 alone did not change basal D-glucose uptake in 3T3-L1 adipocytes at up to 300 ng/ml (Fig. 2a). However, D-glucose uptake was further increased by 23% by co-stimulation of insulin and IL-18 (100 ng/ml) compared to insulin alone ($P < 0.05$). When IL-18 concentrations were raised to 300 ng/ml, the glucose uptake was increased by 40% ($P < 0.001$) (Fig. 2b). These data clearly showed that IL-18 could significantly enhance insulin-stimulated glucose uptake in a dose-dependent manner in 3T3-L1 adipocytes. Furthermore, insulin-stimulated glucose uptake was enhanced by IL-18 in a time-

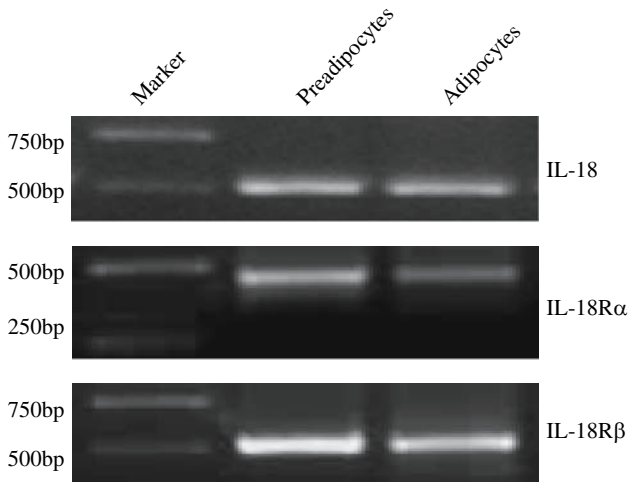

Fig. 1 IL-18 and its receptors were expressed in adipocytes. RT-PCR showed that IL-18 and its receptor subunits, IL-18Rα and IL-18Rβ were all expressed in 3T3-L1 pre-adipocytes and mature 3T3-L1 adipocytes. Marker was DL2000 ladder. The RT-PCR procedure was described in Sect. "Materials and methods"

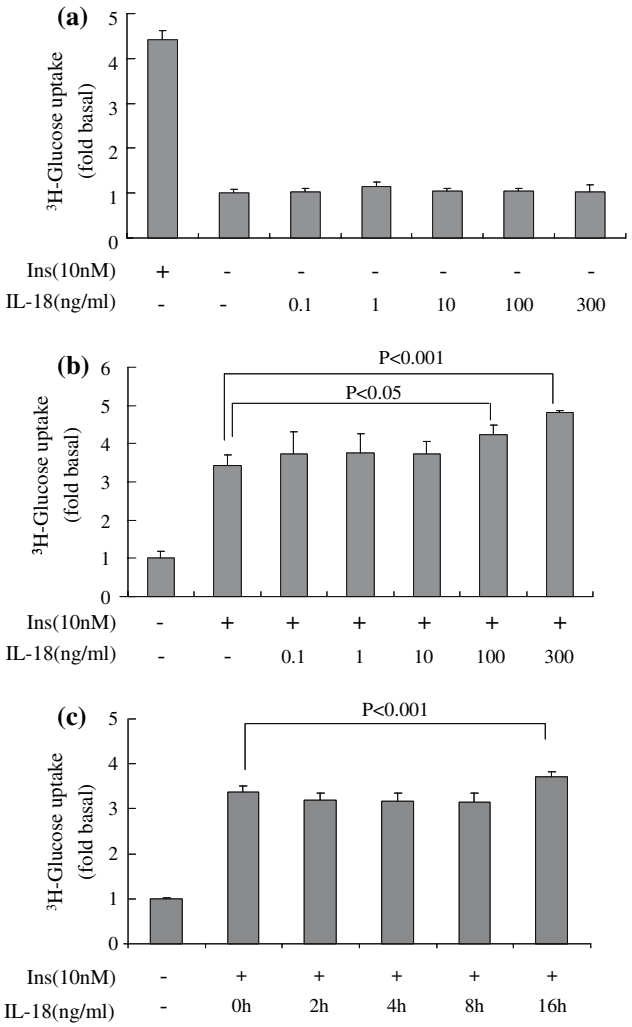

Fig. 2 Effect of IL-18 on basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. IL-18 alone did not alter glucose uptake in adipocytes even at 300 ng/ml (**a**). D-glucose uptake was increased by 2.4 folds with insulin treatment alone at 10 nM for 30 min ($P < 0.001$) and further increased by 40% with co-treatment of insulin and IL-18 (**b**). Insulin-stimulated glucose uptake was enhanced by IL-18 in a time-dependent manner with significant 21% increase detectable at 16 h of IL-18 treatment ($P < 0.01$) (**c**). Glucose uptake is indicated as folds of the basal in untreated cells. Results are means ± SD of six wells in each group. Each experiment was repeated three times

dependent manner with significant 21% increase detectable at 16 h of IL-18 (100 ng/ml) treatment ($P < 0.01$) (Fig. 2c).

The suppression of TNF-α mediated glucose uptake was improved by IL-18

In order to determine whether IL-18 could reverse the inhibition of glucose transport mediated by TNF-α, 3T3-L1 adipocytes were incubated with IL-18 alone or IL-18 plus insulin following treatment with TNF-α (10 ng/ml) for 3 days [19]. TNF-α could inhibit basal glucose uptake by 45% in 3T3-L1 adipocytes. IL-18 at 100 or 300 ng/ml alone could not reverse the suppression effect (Fig. 3a). These results demonstrated that IL-18 alone could not reverse basal glucose uptake suppression by TNF-α. However, IL-18 at 100 or 300 ng/ml plus insulin could enhance glucose uptake by 20.8 and 28.7%, respectively, in 3T3-L1 cells treated with TNF-α (both $P < 0.01$), which attenuated the insulin-stimulated glucose uptake by 60% ($P < 0.001$) (Fig. 3b). These results clearly showed that IL-18 could somehow reverse the inhibition of insulin-stimulated glucose uptake by TNF-α in 3T3-L1 adipocytes.

IL-18 upregulated phosphorylated Akt in adipocytes

In order to explore the signaling pathways activated by IL-18 in 3T3-L1 adipocytes, we first examined whether IL-18 enhanced the level of Ser473 phosphorylation of Akt. Immunoblotting showed that serine phosphorylation of Akt was significantly enchanced in the cells treated with insulin for 30 min, and addition of IL-18 (300 ng/ml) for 16 h could slightly but significantly increase Ser-473 phosphorylated Akt (Fig. 4).

We then determined which components of insulin-signaling cascades were affected by TNF-α and IL-18. Immunoblotting showed that Akt phosphorylation at Ser-473 stimulated by insulin was decreased in 3T3-L1 cells treated with TNF-α. IL-18 could significantly raise phosphorylated Akt decreased by TNF-α (Fig. 4), which was consistent with improvement of the effect of glucose uptake (Fig. 3b).

IL-18 downregulated phosphorylated p38 MAPK in adipocytes

p38, ERK, and JNK MAPKs were examined since they are all involved in insulin signaling and possibly in TNF-α-induced insulin resistance. Immunoblotting showed that

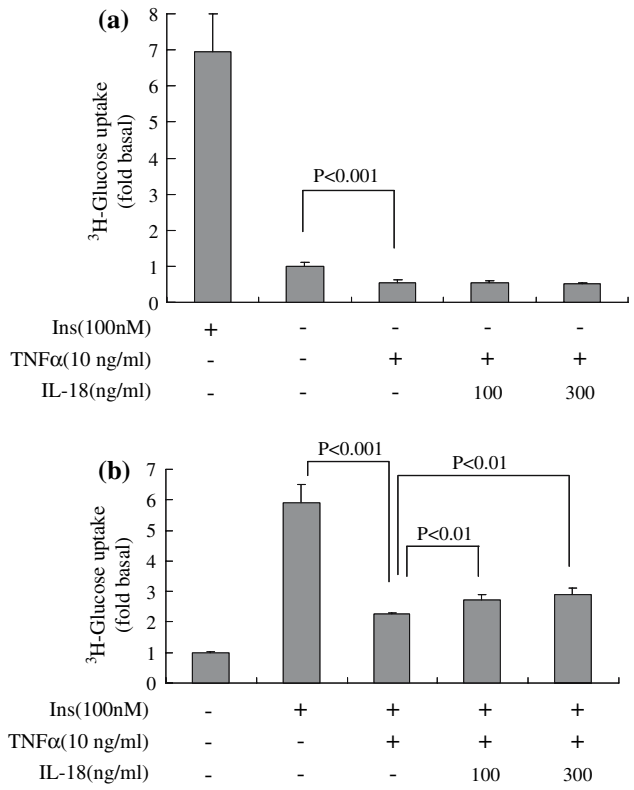

Fig. 3 IL-18 improved TNF-α mediated suppression of glucose uptake in 3T3-L1 adipocytes. TNF-α could inhibit basal glucose uptake by 45% in 3T3-L1 adipocytes. IL-18 at 100 or 300 ng/ml alone could not reverse the suppression effect (**a**). However, IL-18 at 100 or 300 ng/ml plus insulin could enhance glucose uptake by 20.8 and 28.7%, respectively, in 3T3-L1 cells treated with TNF-α (both $P < 0.01$), which attenuated the insulin-stimulated glucose uptake by 60% ($P < 0.001$) (**b**). Results are means ± SD of six wells in each group. Each experiment was repeated at least three times

TNF-α increased phosphorylated p38 MAPK, which was downregulated by insulin. Whereas IL-18 downregulated the phsophorylated p38 MAPK, which was increased by TNF-α (Fig. 5a). However, IL-18 could not alter phosphorylated JNK (Fig. 5b) or ERK (Fig. 5c) in TNF-α-treated 3T3-L1 adipocytes.

Discussion

Interleukin-18, originally described as an interferon γ-inducing factor, was cloned from the pancreas of animals with autoimmune type-1-like diabetes [20] and acts as a pleiotropic cytokine in both acquired and innate immunity. IL-18 responsiveness is mediated by IL-18R, a heterodimeric complex consisting of a ligand-binding α chain (IL-18Rα) and an associating β chain (IL-18Rβ). Our previous cDNA array showed that IL-18Rα was expressed in human adipose tissue and was further reported by others

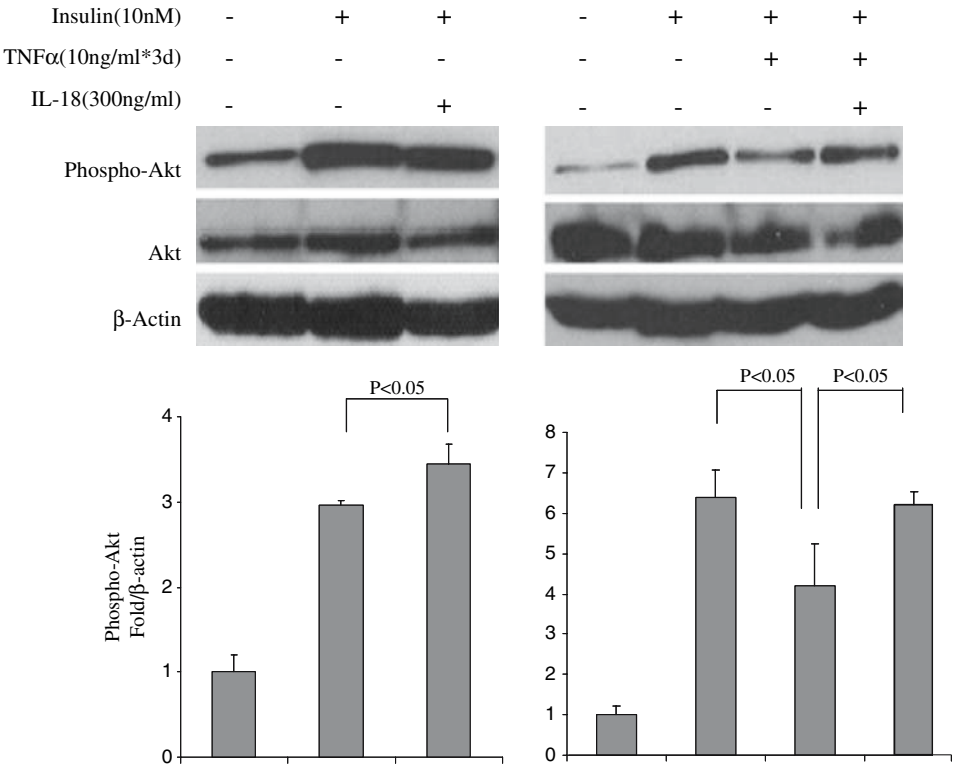

Fig. 4 IL-18 up-regulated phosphorylated Akt in TNF-α treated 3T3-L1 adipocytes Immunoblotting showed that Ser473 phosphorylation of Akt was significantly enchanced in 3T3-L1 adipocytes treated with insulin for 30 min, and addition of IL-18 (300 ng/ml) for 16 h could slightly but significantly increase Akt Ser-473 phosphorylation. Phosphorylated Akt stimulated by insulin was significantly inhibited by TNF-α, and the inhibition effect was ameliorated after treatment with 300 ng/ml IL-18 for 16 h. The densitometry analysis of the immunoreactive bands was performed using the LabWorks imaging and analysis software (UVP Bioimaging Systems). Results are means ± SD of three independent expriments

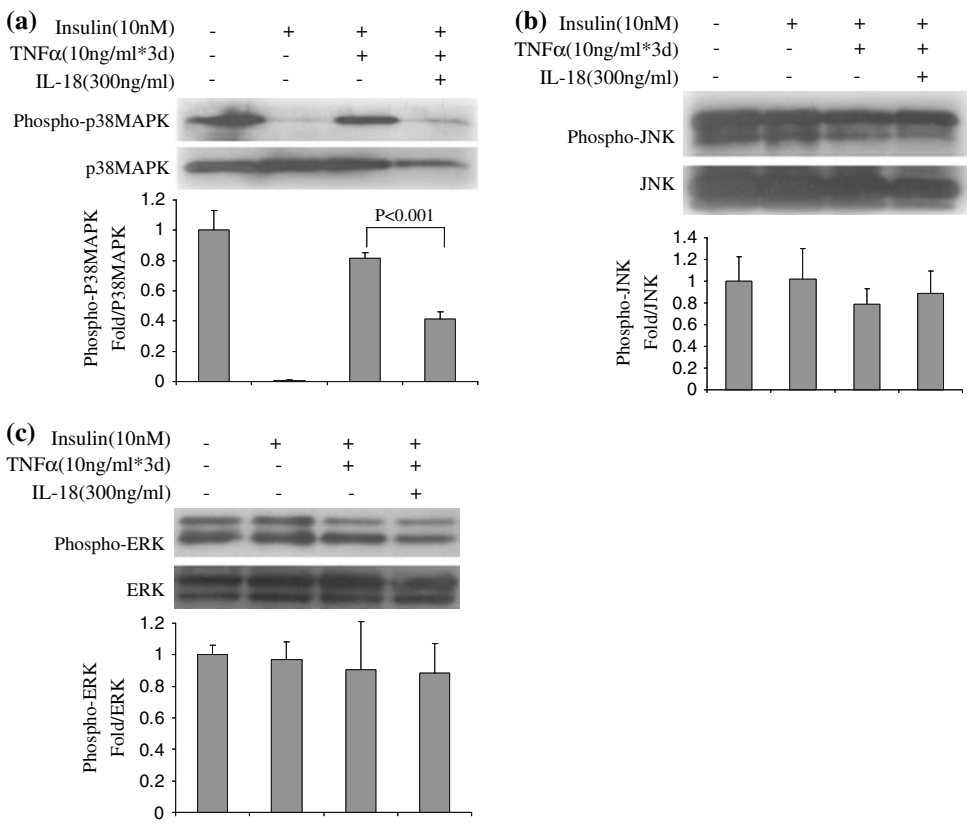

Fig. 5 The effects of IL-18 on P38, JNK, and ERK MAPKs involved in TNF-α-induced insulin resistance in 3T3-L1 adipocytes. Immunoblotting showed that IL-18 downregulated phosphorylated p38 MAPK, which was upregulated by TNF-α (**a**). IL-18 had no effect on phosphorylation of JNK (**b**) and ERK (**c**) in TNF-α-induced insulin resistant 3T3-L1 adipocytes. The experiment procedures were described in detail in Sect. "Materials and methods". The densitometry analysis of the immunoreactive bands was performed using the LabWorks imaging and analysis software (UVP Bioimaging Systems). Results are means ± SD of three independent expriments

[21]. In the present study, we also identified IL-18 and its two receptor subunits IL-18Rα and IL-18Rβ were expressed in 3T3-L1 adipocytes at mRNA level.

In the past few years, a correlation between serum IL-18 levels and obesity, type-2 diabetes, and PCOS was established from clinical studies. It has been speculated that elevated IL-18, like other inflammation factors such as IL-6 and TNF-α, might have a detrimental effect on obesity by inducing insulin resistance [16]. However, to our knowledge, we found that IL-18 significantly enhanced rather than suppressed insulin-mediated glucose uptake in 3T3-L1 adipocytes, which suggested that IL-18 did ameliorate insulin resistance. These results were supported by the recent in vivo research using IL-18 knockout models. IL-18 null mice had hyperphagia, obesity, and insulin resistance [22]. Furthermore, we found that IL-18 could improve TNF-α mediated glucose uptake suppression in adipocytes. The relationship between IL-18 and TNF-α remains undetermined although it has been reported that the serum concentrations of IL-18 and TNF-α were significantly higher in type-2 diabetes. Our present results demonstrated that elevated IL-18 may counteract the role of TNF-α in insulin resistance.

TNF-α is a mediator of insulin resistance in obesity and type-2 diabetes by impairing insulin signaling in adipocytes [23]. It has been known TNF-α could activate p38, ERK, and JNK MAPKs [24–27]. Our present results showed IL-18 increased phosphorylated Akt in TNF-α treated 3T3-L1 adipocytes, and while with insulin, reduced phosphorylated p38 MAPK, which was upregulated by TNF-α. P38 MAPK has a complicated role in regulation of glucose uptake as an acute effect of increasing insulin-stimulated glucose uptake and a chronic one of reducing GLUT4 protein levels [28]. A recent study showed that pharmacological inhibition of p38 MAPK resulted in glucose uptake improvement in insulin-resistant 3T3-L1 adipocytes [29]. Taken together, we proposed that IL-18 improved TNF-α mediated insulin resistance at least through both activating PI3K/Akt and inactivating p38 MAPK signaling pathways.

In conclusion, the elevated serum IL-18 levels in obesity and diabetes may be a compensatory response to counteract the state of insulin resistance mediated by other proinflammatory factor such as TNF-α. The present study would hopefully increase our knowledge of the complexity of proinflammatory factors involved in insulin resistance.

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