

## Zinc stimulates glucose consumption by modulating the insulin signaling pathway in L6 myotubes: essential roles of Akt–GLUT4, GSK3 $\beta$ and mTOR–S6K1

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### Abstract

The present study was performed to evaluate the insulin-like effects of zinc in normal L6 myotubes as well as its ability to alleviate insulin resistance. Glucose consumption was measured in both normal and insulin-resistant L6 myotubes. Western blotting and immunofluorescence revealed that zinc exhibited insulin-like glucose transporting effects by activating key markers that are involved in the insulin signaling cascade (including Akt, GLUT4 and GSK3 $\beta$ ), and downregulating members of the insulin signaling feedback cascade such as mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase (S6K1). In normal L6 myotubes, zinc enhanced glucose consumption via a mechanism that might involve the activation of Akt phosphorylation, glucose transporter 4 (GLUT4) translocation and GSK3 $\beta$  phosphorylation. In contrast, zinc exerted insulin-mimetic effects in insulin-resistant L6 myotubes by upregulating Akt phosphorylation, GLUT4 translocation and GSK3 $\beta$  phosphorylation, and downregulating the expression of mTOR and S6K1. In conclusion, zinc might enhance glucose consumption by modulating insulin signaling pathways including Akt–GLUT4, GSK3 $\beta$ , mTOR and S6K1.

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**Keywords:** Zinc; Glucose consumption; Insulin signaling pathway; L6 myotubes; Akt; mTOR; GSK3 $\beta$

### 1. Introduction

Insulin resistance refers to the impaired response of insulin target tissues such as liver, adipose and skeletal muscle to physiological concentration of insulin. Insulin resistance contributes to hyperglycemia, which in turn worsens this pathological state, thereby resulting in a vicious circle. Insulin resistance is also associated with a number of diseases, including obesity, type 2 diabetes, cardiovascular disease and hypertension [1–4]. The diabetes epidemic has been increasing in recent years, and insulin resistance is one of the main pathogenesises of

type 2 diabetes. Therefore, it is of great significance to explore methods of attenuating insulin resistance [2,5].

It has long been a consensus that proper diet, exercise and drugs are cornerstones of the prevention and treatment of insulin resistance. However, a number of medications are associated with sometimes severe side effects and a high cost, which requires dietary supplementation. Zinc is an interesting candidate supplement since it is a component of insulin crystals and is also crucial for cell growth, mental development, immune function and metabolism. Furthermore, it functions as a second messenger and thereby plays a pivotal role in signal transduction pathways [6,7]. Previous *in vivo* studies showed that zinc exerted an insulin-like effect by lowering blood glucose levels [8], whereas zinc deficiency exacerbated insulin resistance. The mechanisms behind these effects might include inhibiting Akt by activating its negative regulators to downregulate Akt and GSK-3 $\beta$  phosphorylation, which ultimately increases the phosphorylation of glycogen synthase and disturbs lipid metabolism [9–11]. Zinc supplementation also exerted beneficial effects in animal models of insulin resistance [12]. *In vitro* studies also revealed insulin-mimetic effects of zinc, which activates the insulin receptor  $\beta$  to activate Akt and ERK and thereby lower glucose levels [6,7,12]. However, the results of population-based studies are controversial. For example, a cross-sectional study found that zinc levels were lower in the hair and

**Abbreviations:** AMPK, AMP-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GLUT4, glucose transporter 4; IR, insulin receptor; GS, glycogen synthase; HS, horse serum; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase pathway; S6K1, ribosomal protein S6 kinase, polypeptide 1; SDS, sodium dodecyl sulfate.

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blood but higher in the urine of diabetic patients compared with controls, suggesting decreased storage and increased urinary excretion of zinc in diabetic subjects [13,14]. Furthermore, the risk of developing insulin resistance can be reduced by zinc supplement [15]. However, other studies demonstrated that zinc treatment had no significant effect on insulin resistance [16]. Taken together, these data suggest that the potential of zinc in insulin resistance should be investigated further.

Skeletal muscle cells consume nearly 80% of ingested glucose; therefore, they have the highest level of insulin-stimulated glucose uptake and are the main site of insulin resistance [17]. Furthermore, defective insulin signaling plays a crucial role in insulin resistance in skeletal muscle. A previous study demonstrated that zinc exerts insulin-like effects by increasing the tyrosine phosphorylation of insulin receptor (IR)- $\beta$  and then activating the PI3K signaling pathway and stimulating Akt phosphorylation [18]. However, the effects of zinc on insulin signaling in normal and insulin-resistant skeletal muscle cells have not yet been investigated systematically. Therefore, the aim of the present study was to determine the effects of zinc on glucose consumption in both normal and insulin-resistant L6 myotubes, and then elucidate the mechanisms involved.

## 2. Materials and methods

### 2.1. Materials

Palmitic acid (PA) and insulin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Sijiqing Co. Ltd. (Hangzhou, China). Horse serum (HS) was purchased from HyClone (New Zealand, USA).  $ZnSO_4 \cdot 7H_2O$  was obtained from Dingguo Co. Ltd. (Beijing, China). Antibodies against Akt, phospho-Akt (Ser-473), GSK3 $\beta$ , phospho-GSK3 $\beta$  (Ser-9), GLUT4, mTOR, phospho-mTOR (Ser-2448), S6K1, phospho-S6K1 (Thr-389) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were supplied by Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH and horseradish peroxidase conjugated anti-rabbit IgG were purchased from Zhongshanjinjiao Co. Ltd. (Beijing, China).

### 2.2. Cell culture

Rat L6 myoblasts (Cell Bank, Chinese Academy of Sciences, Beijing, China) were maintained in DMEM supplemented with 10% FBS, 5.55 mmol/L glucose and 100 U/ml penicillin and streptomycin in 10-cm dishes ( $1.5 \times 10^6$  cells/dish). For differentiation, L6 myoblasts were transferred to differentiation medium containing 2% HS when cells reached 60%–70% in confluence, and the media were changed every other day. L6 myoblasts fused to form myotubes after 6–8 days of differentiation.

### 2.3. Fatty acid preparation and treatment

PA was prepared according to a previously published method. Briefly, fatty acids were diluted in ethanol to a final concentration of 100 mM. The mixture was then sonicated repeatedly on ice for 10-s bursts at 200 W until the mixture became a milky colored solution. The prepared fatty acid stocks were stored in the dark at 4 °C until use. Before use, the fatty acids were dissolved in growth medium or buffer that had been preheated to 60 °C.

### 2.4. Glucose consumption assay

Glucose consumption was measured in L6 myotubes cells grown in 96-well plates. L6 myotubes were washed twice with phosphate buffered saline (PBS) and starved in serum-free DMEM containing 5.55 mmol/L glucose for 24 h, and then they were incubated in the presence or absence of 0.4 mmol/L PA for 24 h to induce insulin resistance. The cells were then exposed to DMEM supplemented with 10 mmol/L glucose and different doses of zinc (0, 10, 20, 50 or 100  $\mu$ mol/L) in the presence or absence of insulin (100 nmol/L) for 3 h. Glucose consumption was then determined using the glucose oxidase method. Intracellular glucose was calculated by subtracting the glucose concentration in the medium from the total glucose concentration in blank wells.

### 2.5. Western blotting

Cells were lysed directly in sodium dodecyl sulfate (SDS) loading buffer after the indicated treatments, and sonicated twice for 9 s each at 200 W. The protein concentration was then measured using the BCA method from Beyotime Company

(Jiangsu, China). The lysates were heated for 5 min at 100 °C in loading buffer supplemented with 10%  $\beta$ -mercaptoethanol and separated using SDS-PAGE. The samples were then transferred to polyvinylidene fluoride membrane and blocked using 5% BSA solution (diluted in PBS) for 2 h. The membranes were incubated with the indicated monoclonal antibodies at 1:1000 dilutions at 4 °C overnight followed by HRP-conjugated secondary antibodies (1:10,000 dilution) for 1 h. Immunoblots were visualized using ECL (Bio-Rad) and quantified using Image J software. GAPDH was used as the internal control.

### 2.6. Immunofluorescence staining

Immunofluorescence was used to detect GLUT4 on the cell surface. L6 myotubes were seeded onto a glass-bottomed plate overnight. After treatment with zinc or insulin, the cells were placed on ice immediately and washed three times with ice-cold Krebs-Ringer HEPES buffer [KRBH; 120 mM NaCl, 25 mM HEPES, 4.6 mM KCl, 1 mM  $MgSO_4$ , 1.2 mM  $KH_2PO_4$  and 1.9 mM  $CaCl_2$  (pH 7.4)]. After blocking with 10% goat, the cells were incubated with anti-GLUT4 antibody at 4 °C overnight. They were then washed with ice-cold PBS for 10 min, incubated with FITC-conjugated goat anti-rabbit IgG (at 1:100 dilution) for 1 h, washed with ice-cold PBS, and then examined immediately using an Olympus FV500 confocal fluorescence microscope. Background absorbance was measured in samples incubated with FITC-conjugated anti-rabbit IgG alone (without primary antibody) and was subtracted from all measured values.

### 2.7. Statistical analyses

Statistical analyses were performed using SPSS 18.0. One-way analysis of variance (ANOVA) followed by LSD was used for comparisons between three or more treatment groups, and *t* test was applied to assess the difference between two treatment groups. Data are expressed as means  $\pm$  SD, and  $P < .05$  was used to define statistical significance.

## 3. Results

### 3.1. Zinc stimulates glucose consumption in normal and insulin-resistant L6 myotubes

We first assessed the role of zinc in glucose metabolism by examining its effects on glucose consumption. L6 myotubes were incubated with different doses of zinc in the presence or absence of insulin for 3 h, and glucose consumption was measured. As shown in Fig. 1A, treatment with insulin alone increased glucose consumption significantly in normal L6 myotubes ( $P < .05$ ). Different doses of zinc (ranging from 10 to 100  $\mu$ mol/L) also increased glucose consumption in the basal state ( $P < .05$ ). However, co-incubation with zinc and insulin did not enhance glucose consumption in L6 myotubes further compared with either zinc or insulin alone ( $P > .05$ ).

Glucose consumption in insulin-resistant L6 myotubes was upregulated by the addition of 100 nM insulin ( $P < .05$ ). In the basal state, treatment with different concentrations of zinc (20–100  $\mu$ mol/L) enhanced glucose consumption significantly ( $P < .05$ ). Treatment with 20, 50 and 100  $\mu$ mol/L zinc increased glucose consumption significantly and in a dose-dependent manner, compared with treatment with 10  $\mu$ mol/L ( $P < .05$ ). Co-incubation of zinc (20, 50 or 100  $\mu$ mol/L) and insulin increased glucose consumption to a greater extent than incubation with insulin alone ( $P < .05$ ). Furthermore, 50 and 100  $\mu$ mol/L zinc had a stronger stimulating effect than 10  $\mu$ mol/L zinc (Fig. 1B).

### 3.2. The effects of zinc on insulin signaling

Insulin exerts its effects by binding to the IR and thereby stimulating downstream signaling events that can lead to enhanced glucose consumption. In skeletal muscle, the PI3K/Akt pathway plays a pivotal role in these events; therefore, we next explored whether zinc-stimulated glucose consumption is mediated through insulin signaling pathways. The glucose consumption assays revealed that there is no significant difference in the additions of between 50 and 100  $\mu$ mol/L zinc. Furthermore, the co-incubating cells with the combination of 100  $\mu$ mol/L zinc and insulin showed a tendency to downregulate cell viability compared with 10–50  $\mu$ mol/L zinc (data not shown). Therefore, only zinc concentrations ranging from 10 to 50  $\mu$ mol/L were used in subsequent studies. In addition, different time points were used to

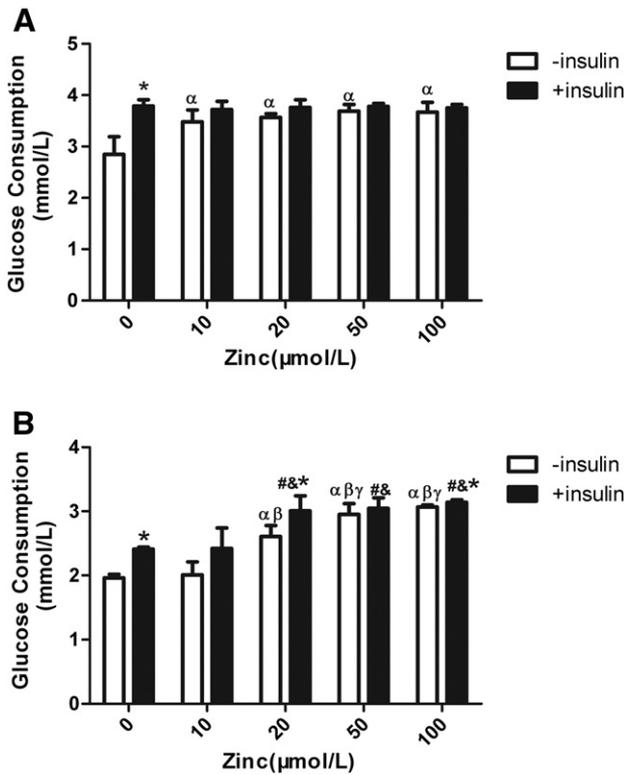


Fig. 1. The effects of zinc on glucose consumption in the presence or absence of insulin in normal L6 myotubes (A) and insulin-resistant L6 myotubes (B). L6 myotubes were treated with zinc in the absence or presence of 100 nM insulin for 3 h, and glucose consumption assays were performed. Data are presented as means ± SDs (n = 6 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \*P < .05 vs. the group adding the same dose of zinc alone; <sup>α</sup>P < .05 vs. 0 μmol/L zinc; <sup>β</sup>P < .05 vs. 10 μmol/L zinc; <sup>γ</sup>P < .05 vs. 20 μmol/L zinc; <sup>#</sup>P < .05 vs. 0 μmol/L zinc plus insulin; <sup>&</sup>P < .05 vs. 10 μmol/L zinc plus insulin.

examine glucose consumption and insulin signaling pathway molecules since we hypothesized that insulin activates the insulin signaling cascades in ~15–30 min after a meal in the normal state [19–21]; blood glucose levels then normalize after ~3 h [21–24]. Therefore, we used a 3-h time point to measure glucose consumption and a 15-min time point to investigate insulin signaling cascades in an attempt to replicate the actual conditions of the body’s reaction to postprandial blood glucose.

3.2.1. The effects of zinc on Akt phosphorylation and GLUT4 translocation

Insulin interacts with the insulin receptor at the cell membrane and stimulates its downstream signaling pathway, which results in the activation of its downstream effector Akt. The active form of Akt, which is phosphorylated at Ser-473, can promote the transmembrane activity of GLUT4. The transmembrane activity of GLUT4 contributes to glucose uptake and thus increases glucose consumption; therefore, we examined Akt activation and the translocation of GLUT4 to the cell membrane.

L6 myotubes were incubated with different doses of zinc in the presence or absence of insulin for 15 min. As shown in Fig. 2B, when normal L6 myotubes were incubated with insulin, the levels of pAkt Ser-473 in the basal state rose significantly (P < .05). Similarly, treatment with 20 and 50 μmol/L zinc increased pAkt Ser-473 levels compared with either control or 10 μmol/L zinc (P < .05). Zinc of 50 μmol/L induced a greater increase in pAkt levels than 10 μmol/L zinc in the presence or absence of insulin or insulin (P < .05). Finally, pAkt Ser-473 levels were upregulated by co-incubation with 10 μmol/L zinc and insulin compared with incubation with only 10 μmol/L zinc (P < .05).

The phosphorylation of Akt at Ser-473 was also increased significantly in insulin-resistant L6 myotubes after stimulation with insulin (P < .05). Treatment with zinc increased Akt phosphorylation in a dose-dependent manner (P < .05). Furthermore, co-treatment with 20 μmol/L zinc and insulin upregulated Akt phosphorylation more than insulin alone (P < .05) (Fig. 2C).

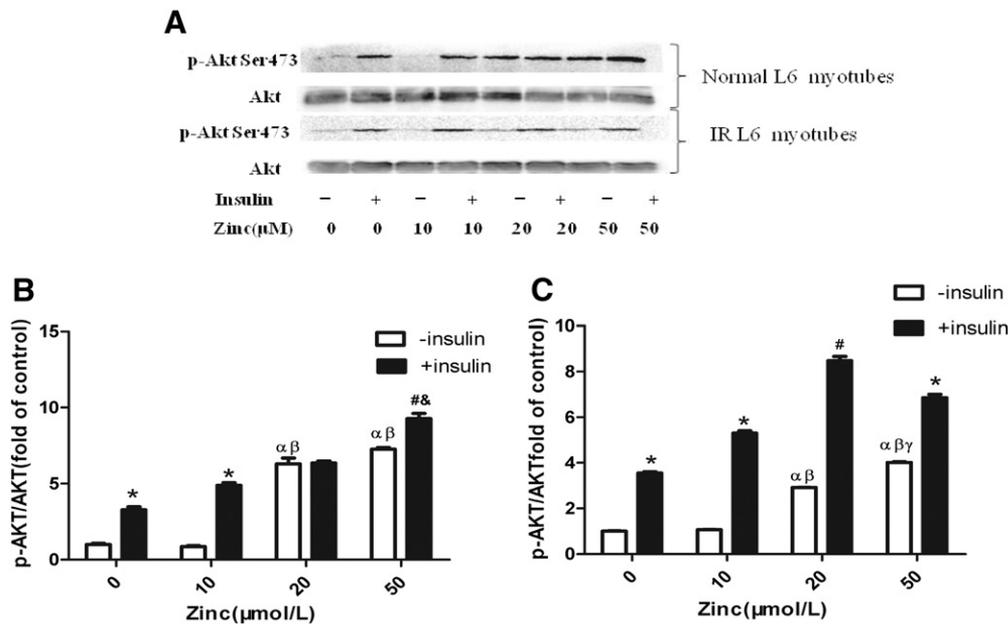


Fig. 2. The effects of zinc on Akt phosphorylation in the presence or absence of insulin in L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and Western blotting was performed. (B) Normal L6 myotubes. (C) Insulin-resistant L6 myotubes. Data are presented as means ± SD (n = 3 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \*P < .05 vs. the group adding the same dose of zinc alone; <sup>α</sup>P < .05 vs. 0 μmol/L zinc; <sup>β</sup>P < .05 vs. 10 μmol/L zinc; <sup>γ</sup>P < .05 vs. 20 μmol/L zinc; <sup>#</sup>P < .05 vs. 0 μmol/L zinc plus insulin; <sup>&</sup>P < .05 vs. 10 μmol/L zinc plus insulin.

GLUT4 translocation to the cell surface is the final step of the insulin-stimulated glucose uptake process, and the quantity of GLUT4 at the cell surface is the rate-limiting step of glucose disposal. As shown in Fig. 3A, treatment with insulin alone increased GLUT4 membrane translocation in normal L6 myotubes significantly. Zinc induced the translocation of GLUT4 in a dose-dependent manner in both the basal and insulin-stimulated states. Specifically, significant stimulation was observed after treatment with 20  $\mu\text{mol/L}$  zinc ( $P < .05$ ), and a maximal increase was observed with a dose of 50  $\mu\text{mol/L}$  ( $P < .05$ ). Meanwhile, co-incubation with different doses of zinc and insulin had a greater effect on GLUT4 translocation than zinc alone ( $P < .05$ ). Similar observations were made in insulin-resistant L6 myotubes (Fig. 3B).

3.2.2. The effects of zinc on GSK3 $\beta$  phosphorylation

AKT plays a critical role in glucose uptake, as well as glycogen synthesis. GSK3 $\beta$  is a downstream target of AKT and is inhibited when Ser-9 site is phosphorylated by AKT. Following the phosphorylation of Ser-9 of GSK3 $\beta$ , the activity of glycogen synthase is enhanced due to its dephosphorylation (dephosphorylated state is the active form of glycogen synthase). Subsequently, glycogen synthesis is increased, and blood glucose is decreased. Therefore, we next investigated

whether zinc-induced glucose consumption was accompanied by increased glycogen synthesis in L6 myotubes.

Incubation with insulin alone increased the phosphorylation of GSK3 $\beta$  Ser-9 significantly ( $P < .05$ ) in normal L6 myotubes. A similar effect was observed after treatment with 20 or 50  $\mu\text{mol/L}$  zinc ( $P < .05$ ). Furthermore, 20 or 50  $\mu\text{mol/L}$  zinc increased GSK3 $\beta$  phosphorylation to a greater extent than 10  $\mu\text{mol/L}$  zinc alone ( $P < .05$ ). Treatment with different concentrations of zinc and insulin together increased GSK3 $\beta$  Ser-9 phosphorylation significantly compared with insulin alone ( $P < .05$ ). Interestingly, GSK3 $\beta$  Ser-9 phosphorylation peaked when cells were incubated with insulin in combination with the lowest dose of zinc (10  $\mu\text{mol/L}$ , Fig. 4B;  $P < .05$  compared with incubation with 10  $\mu\text{mol/L}$  zinc alone).

As is shown in Fig. 4C, treatment with insulin or all concentrations of zinc alone showed a tendency to upregulate GSK3 $\beta$  Ser-9 phosphorylation in insulin-resistant L6 myotubes. However, statistical significance was only observed after treatment with 50  $\mu\text{mol/L}$  zinc ( $P < .05$ ). GSK3 $\beta$  Ser-9 phosphorylation was increased slightly, but this increase did not reach statistical significance when cells were incubated with lower doses of zinc (10 and 20  $\mu\text{mol/L}$ ) plus insulin compared with insulin alone ( $P > .05$ ). Surprisingly, the combination of 50  $\mu\text{mol/L}$  zinc and insulin produced a reversed effect compared with 50  $\mu\text{mol/L}$  zinc alone ( $P < .05$ ).

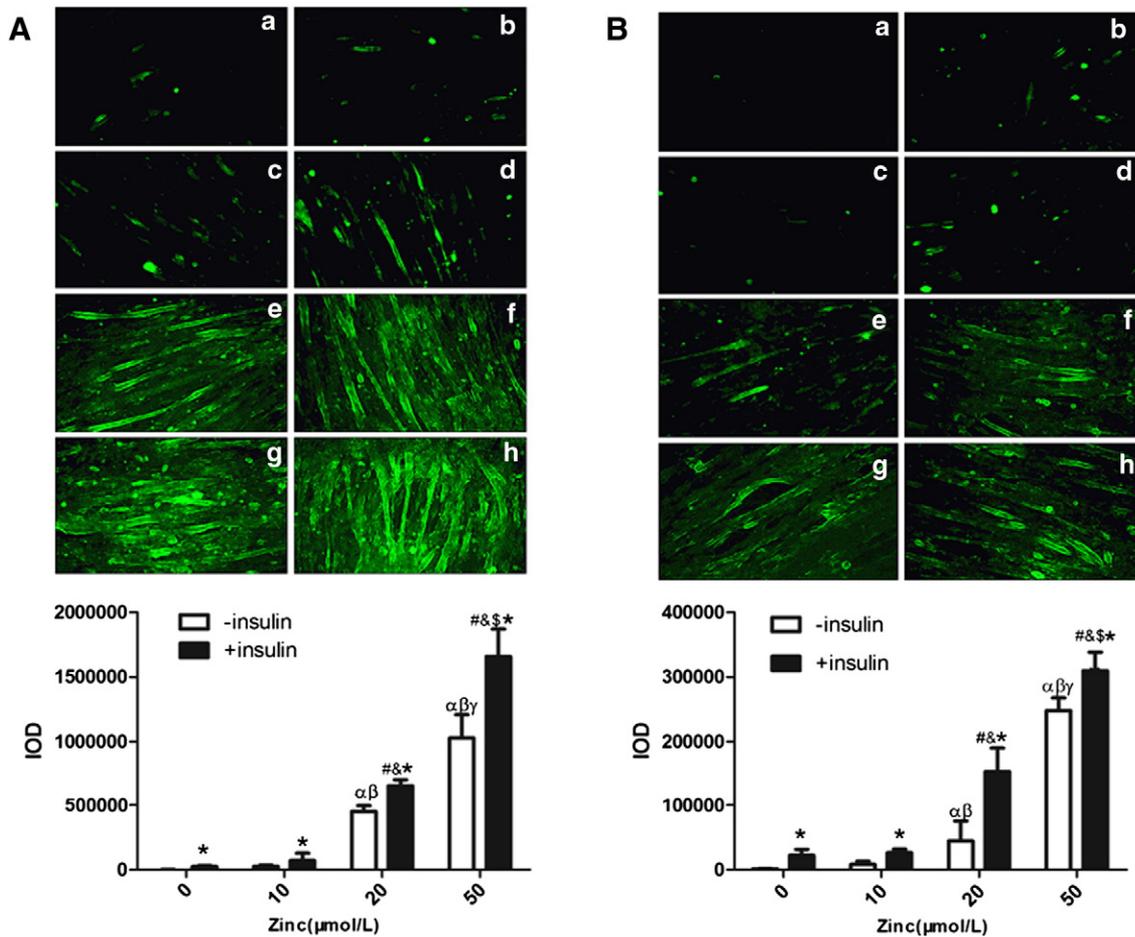


Fig. 3. The effects of zinc on cellular GLUT4 membrane localization. (A) L6 myotubes were incubated with or without 100 nM insulin in the presence of zinc (a, 0  $\mu\text{mol/L}$  zinc; b, 0  $\mu\text{mol/L}$  zinc plus insulin; c, 10  $\mu\text{mol/L}$  zinc; d, 10  $\mu\text{mol/L}$  zinc plus insulin; e, 20  $\mu\text{mol/L}$  zinc; f, 20  $\mu\text{mol/L}$  zinc plus insulin; g, 50  $\mu\text{mol/L}$  zinc; h, 50  $\mu\text{mol/L}$  zinc plus insulin) for 15 min. Immunofluorescence staining was then used to determine the subcellular localization of GLUT4. (B) L6 myotubes were treated with 0.4 mM PA to establish insulin-resistant cell model, then they were treated with different doses of zinc in the absence or presence of insulin as experiment A. Data are presented as means  $\pm$  SD ( $n = 6$  independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \* $P < .05$  vs. the group adding the same dose of zinc alone;  $^{\alpha}P < .05$  vs. 0  $\mu\text{mol/L}$  zinc;  $^{\beta}P < .05$  vs. 10  $\mu\text{mol/L}$  zinc;  $^{\gamma}P < .05$  vs. 20  $\mu\text{mol/L}$  zinc;  $^{\delta}P < .05$  vs. 0  $\mu\text{mol/L}$  zinc plus insulin;  $^{\epsilon}P < .05$  vs. 10  $\mu\text{mol/L}$  zinc plus insulin;  $^{\zeta}P < .05$  vs. 20  $\mu\text{mol/L}$  zinc plus insulin.

### 3.3. The effects of zinc on mTOR/S6K1

#### 3.3.1. The effects of zinc on mTOR

In the normal state, Akt promotes cell growth and protein synthesis by activating the mammalian target of rapamycin (mTOR) pathway. However, in the insulin-resistant state, mTOR levels increase because of the excess levels of nutritional factors and energy. mTOR plays an important role as a mediator of the crosstalk between nutritional signals and the insulin-mediated metabolic signals by downregulating the insulin receptor substrate (IRS) proteins. Therefore, we investigated the effects of zinc on mTOR to elucidate the signaling pathways involved in the effects of zinc on glucose consumption.

As shown in Fig. 5B, in normal L6 myotubes, mTOR expression was upregulated by all concentrations of zinc or insulin alone, although differences were not statistically significant. However, a significant increase in mTOR expression was observed when cells were co-treated with 50  $\mu\text{mol/L}$  zinc and insulin compared with insulin alone ( $P < .05$ ).

Treatment with 50  $\mu\text{mol/L}$  zinc inhibited mTOR expression in insulin-resistant L6 myotubes significantly ( $P < .05$ ). Moreover, incubation with different doses of zinc together with insulin decreased mTOR levels significantly compared with insulin alone ( $P < .05$ ) (Fig. 5C).

All doses of zinc increased mTOR phosphorylation in normal L6 myotubes; however, the effect was only significant in myotubes treated with 20  $\mu\text{mol/L}$  zinc ( $P < .05$ ). In the insulin-stimulated state, the effect also peaked after treatment with 20  $\mu\text{mol/L}$  zinc, but the increase in mTOR phosphorylation was not statistically significant (Fig. 6B).

In insulin-resistant L6 myotubes that had been incubated with 50  $\mu\text{mol/L}$  zinc, there was a trend toward reduced mTOR phosphorylation compared with control (Fig. 6C). Co-treatment with insulin and 20 or 50  $\mu\text{mol/L}$  zinc inhibited mTOR phosphorylation compared with insulin ( $P < .05$ ).

#### 3.3.2. The effects of zinc on S6K1

We next investigated the effects of zinc on the mTOR downstream target S6K1. No changes were observed in S6K1 expression or phosphorylation in normal L6 myotubes treated with zinc or insulin

(Figs. 7B and 8B). In insulin-resistant L6 myotubes, the expression of S6K1 was inhibited significantly in the presence of zinc in a dose-dependent manner ( $P < .05$ ); treatment with 50  $\mu\text{mol/L}$  zinc reduced S6K1 expression significantly compared with 10 or 20  $\mu\text{mol/L}$  ( $P < .05$ ). Incubating myotubes with the combination of 20 or 50  $\mu\text{mol/L}$  zinc and insulin inhibited S6K1 expression compared with incubating with insulin alone ( $P < .05$ ) (Fig. 7C).

S6K1 phosphorylation was reduced in insulin-resistant L6 myotubes that had been treated with the combination of 50  $\mu\text{mol/L}$  zinc and insulin compared with the combination of 10  $\mu\text{mol/L}$  zinc and insulin or insulin alone ( $P > .05$ ) (Fig. 8C).

## 4. Discussion

Type 2 diabetes mellitus is characterized by insulin resistance. The term “insulin resistance” is mainly account for diminished insulin-stimulated glucose utilization such as glucose consumption coupled with distorted insulin signaling pathway. Therefore, it plays a central role in ameliorating insulin resistance by upregulating glucose consumption and modulating the expression of key insulin signaling mediators [25].

Zinc is an essential trace element that plays a major role in the function of more than 300 enzymes. Several studies have revealed that it exerts insulin-mimetic effects by lowering blood glucose levels, and thereby has anti-insulin resistance properties [9,11,13,16,18,26]. Zinc levels in the blood are commonly 12–16  $\mu\text{mol/L}$  [27,28]. We referred to many articles and used 10–100  $\mu\text{mol/L}$  zinc in the current experiments because some tissues such as muscle and liver contain much higher zinc concentrations [29,30]. In addition, insulin-resistant myotubes might need more zinc because of their disrupted insulin signaling. The current study is consistent with previous studies, since the administration of 10–100  $\mu\text{mol/L}$  zinc increased basal glucose consumption in normal L6 myotubes. However, the combination of higher concentrations of zinc (20–100  $\mu\text{mol/L}$ ) and insulin stimulated both basal and glucose-stimulated glucose consumption in insulin-resistant L6

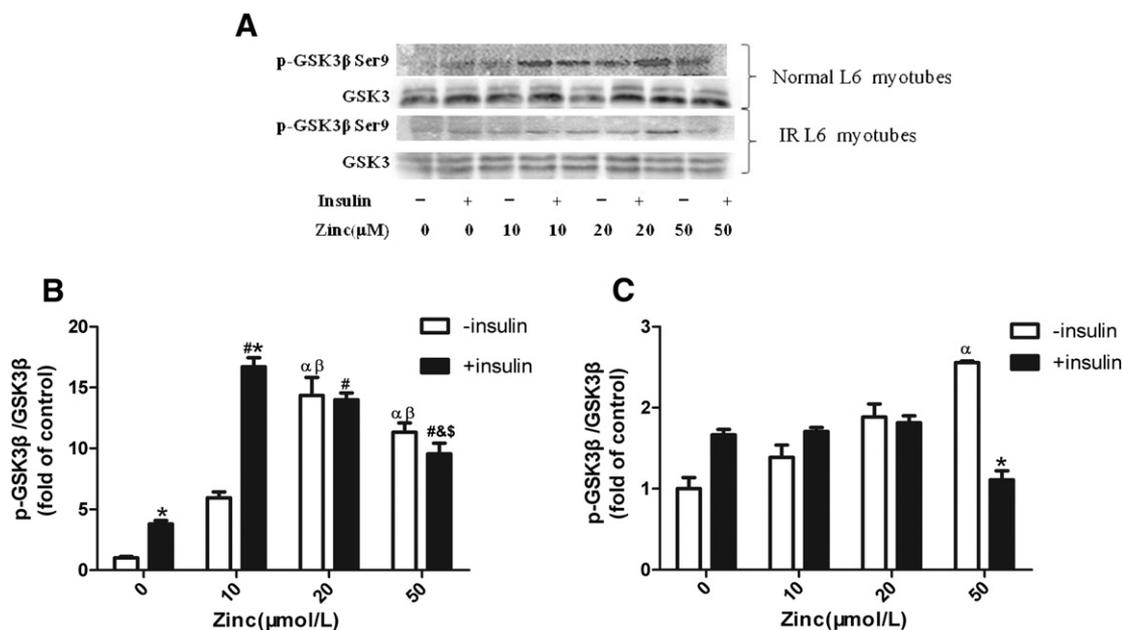


Fig. 4. The effects of zinc on GSK3 $\beta$  phosphorylation in the presence or absence of insulin by L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and were then analyzed using Western blotting. B refers to the results of the effects of zinc on normal L6 myotubes, C refers to the results of the effects of zinc on insulin-resistant L6 myotubes. Data are presented as means  $\pm$  SD ( $n = 3$  independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \* $P < .05$  vs. the group adding the same dose of zinc alone;  $\alpha P < .05$  vs. 0  $\mu\text{mol/L}$  zinc;  $\beta P < .05$  vs. 10  $\mu\text{mol/L}$  zinc; # $P < .05$  vs. 0  $\mu\text{mol/L}$  zinc plus insulin; & $P < .05$  vs. 10  $\mu\text{mol/L}$  zinc plus insulin; \$ $P < .05$  vs. 20  $\mu\text{mol/L}$  zinc plus insulin.

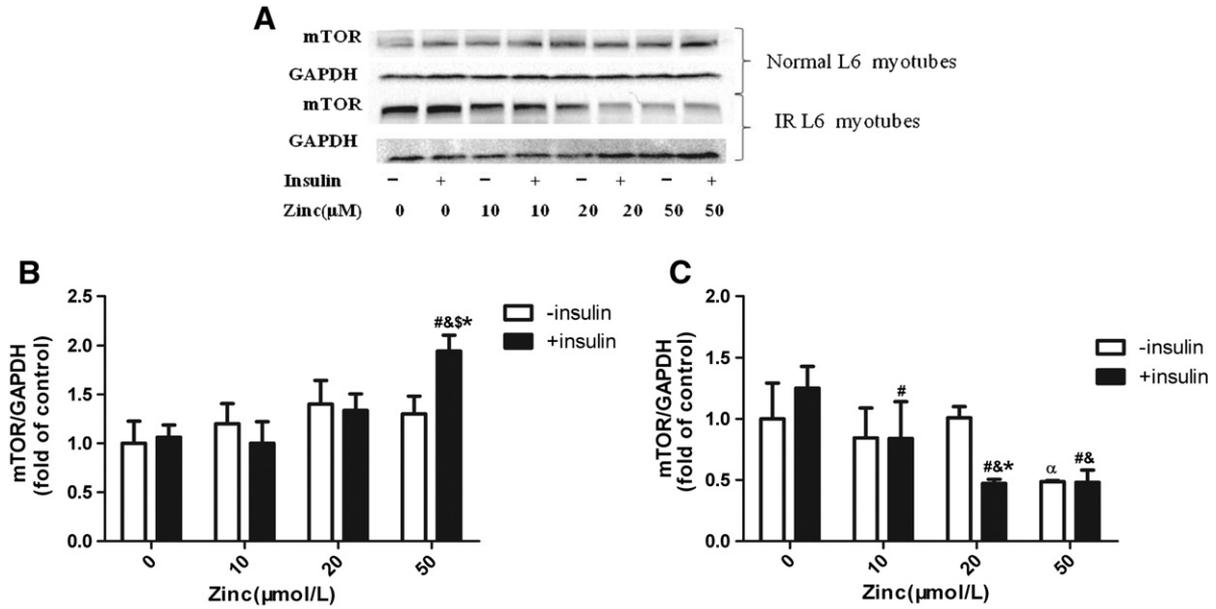


Fig. 5. The effects of zinc on mTOR expression in the presence or absence of insulin by L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and were then analyzed using Western blotting. (B) Normal L6 myotubes. (C) Insulin-resistant L6 myotubes. Data are presented as means ± SD (*n* = 3 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \**P* < .05 vs. the group adding the same dose of zinc alone; <sup>α</sup>*P* < .05 vs. 0 μmol/L zinc; <sup>#</sup>*P* < .05 vs. 0 μmol/L zinc plus insulin; <sup>&</sup>*P* < .05 vs. 10 μmol/L zinc plus insulin; <sup>\$</sup>*P* < .05 vs. 20 μmol/L zinc plus insulin.

myotubes. These data showed that incubation with insulin together with zinc did not enhance glucose consumption in normal myotubes compared with incubation with insulin alone. This suggests that zinc and insulin may act through the same downstream pathways. In normal myotubes, because of their normal insulin signaling, the addition of zinc did not produce an additive effect (cells incubated with insulin and zinc compared with cells incubated with insulin). In insulin-resistant L6 myotubes, because of the disrupted insulin

signaling, incubation with zinc and insulin could enhance glucose consumption compared with incubation with insulin alone.

Three pathways regulate blood glucose levels: the AMP-activated protein kinase (AMPK), phosphatidylinositol 3-kinase pathway (PI3K) and mitogen-activated protein kinase (MAPK) pathways. The AMPK pathway is regulated mainly by exercise, whereas the PI3K and MAPK pathways are mainly active when insulin binds to its receptor in peripheral tissues (adipose, liver, and skeletal muscle) to regulate

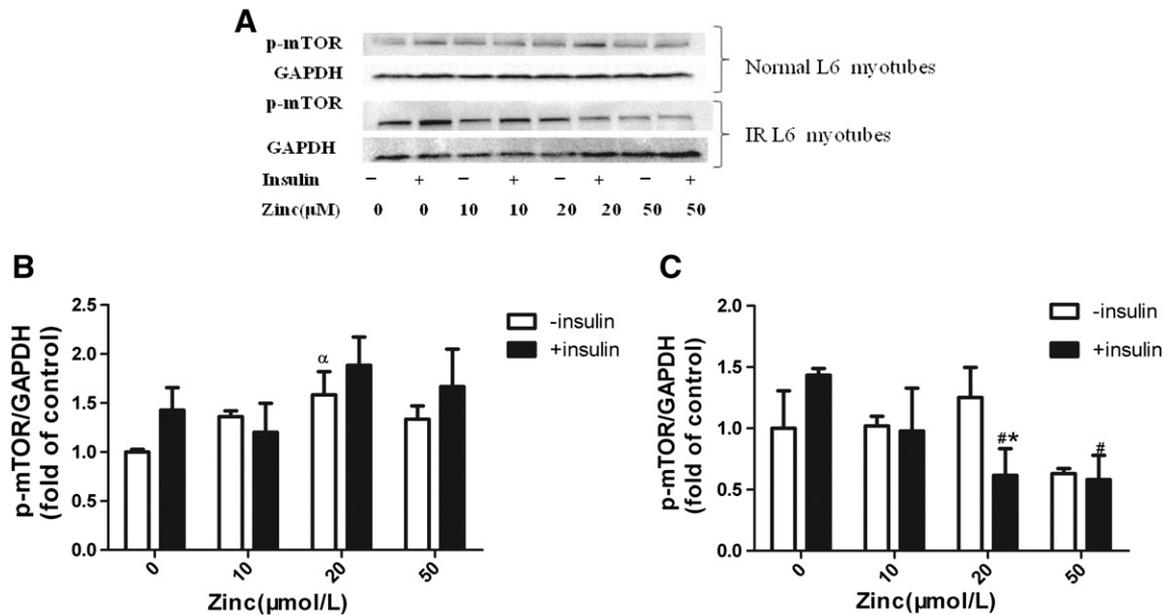


Fig. 6. The effects of zinc on mTOR phosphorylation in the presence or absence of insulin by L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and were then analyzed using Western blotting. (B) Normal L6 myotubes. (C) Insulin-resistant L6 myotubes. Data are presented as means ± SD (*n* = 3 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \**P* < .05 vs. the group adding the same dose of zinc alone; <sup>α</sup>*P* < .05 vs. 0 μmol/L zinc; <sup>\*\*</sup>*P* < .05 vs. 0 μmol/L zinc plus insulin; <sup>#</sup>*P* < .05 vs. 10 μmol/L zinc plus insulin.

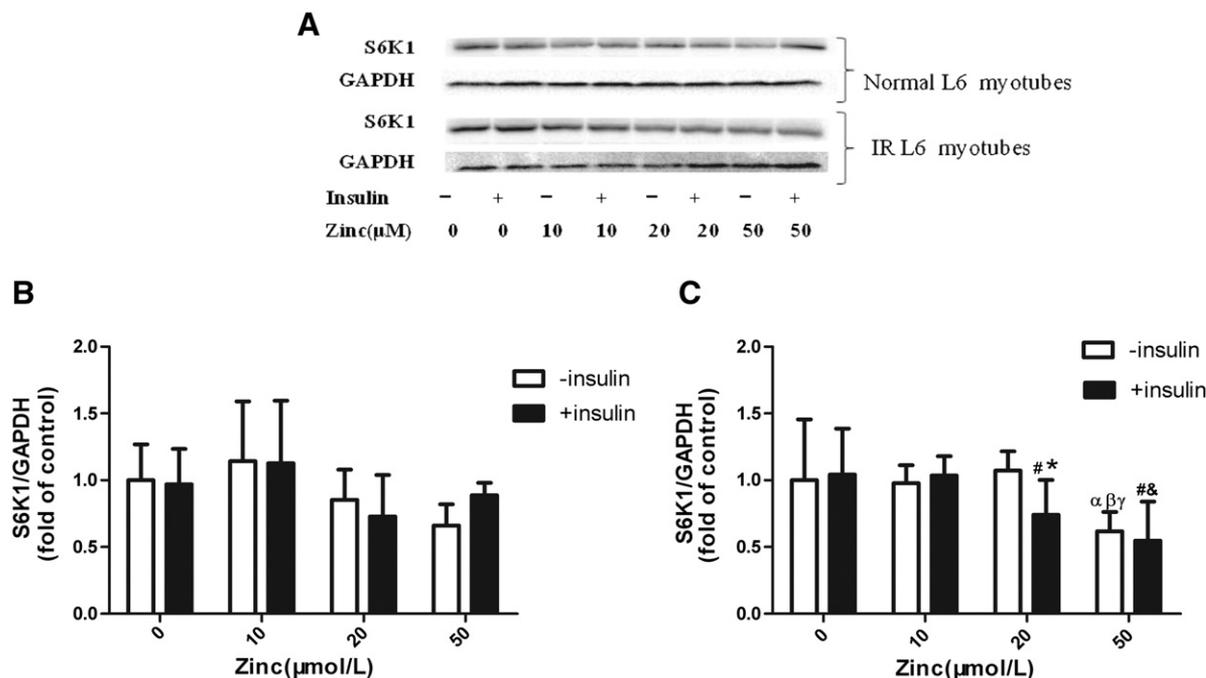


Fig. 7. The effects of zinc on S6K1 expression in the presence or absence of insulin by L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and were then analyzed using Western blotting. (B) Normal L6 myotubes. (C) Insulin-resistant L6 myotubes. Data are presented as means ± SD (n = 3 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. \*P < .05 vs. the group adding the same dose of zinc alone; <sup>α</sup>P < .05 vs. 0 μmol/L zinc; <sup>β</sup>P < .05 vs. 10 μmol/L zinc; <sup>γ</sup>P < .05 vs. 20 μmol/L zinc; <sup>#</sup>P < .05 vs. 0 μmol/L zinc plus insulin; <sup>&</sup>P < .05 vs. 10 μmol/L zinc plus insulin.

blood glucose. Among the three pathways, the PI3K/AKT pathway is of vital significance and has been extensively studied [3]. Active PI3K stimulates the phosphorylation and activation of its downstream molecule Akt, thus promoting the transmembrane activity of GLUT4 and thereby increasing glucose uptake [2,31]. The current study demonstrated that treatment with zinc exerted insulin-like effects by

upregulating the phosphorylation of Akt and the translocation of GLUT4 to the membrane of normal L6 myotubes in both the basal and insulin-stimulated states. Zinc also produced the same effects to attenuate insulin resistance in the insulin-resistant state.

The activation of Akt also plays a role in the transmission of insulin signals by stimulating the phosphorylation of Ser-9 of GSK-3 and

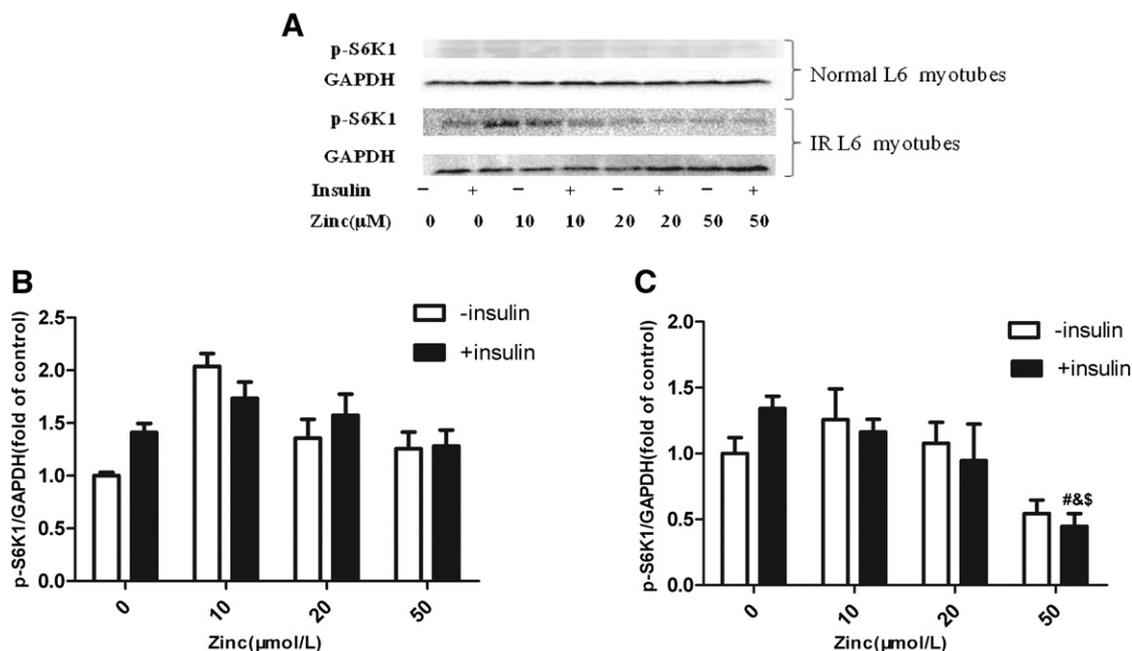


Fig. 8. The effects of zinc on S6K1 phosphorylation in the presence or absence of insulin by L6 myotubes. (A) L6 myotubes were incubated with zinc in the presence or absence of 100 nM insulin for 15 min, and were then analyzed using Western blotting. (B) Normal L6 myotubes. (C) Insulin-resistant L6 myotubes. Data are presented as means ± SD (n = 3 independent experiments) and were analyzed using one-way ANOVA followed by LSD tests. <sup>#</sup>P < .05 vs. 0 μmol/L zinc plus insulin; <sup>&</sup>P < .05 vs. 10 μmol/L zinc plus insulin.

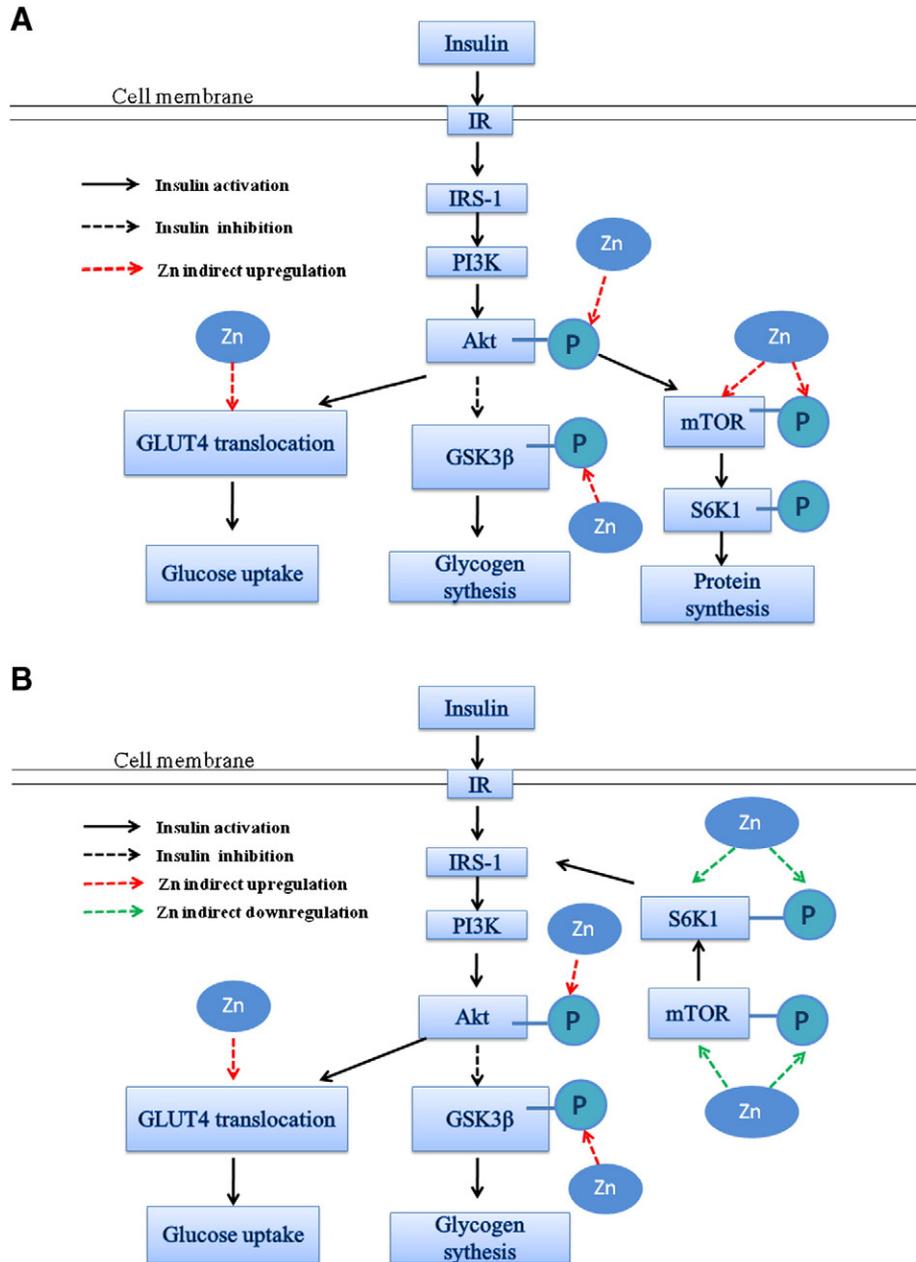


Fig. 9. The effects of zinc on insulin signaling in L6 myotubes. (A) Normal L6 myotubes. (B) Insulin-resistant L6 myotubes.

inhibiting its activity, thus activating glycogen synthase (GS) by dephosphorylation subsequently promoting glycogen synthesis and lowering blood glucose levels [32,33]. There are two isoforms of GSK-3: GSK-3 $\alpha$  and GSK-3 $\beta$ . In the current study, we focused on GSK-3 $\beta$  because it is the main isoform responsible for regulating GS in skeletal muscle. In the present study, treating normal L6 myotubes with zinc increased GSK-3 $\beta$  (Ser-9) phosphorylation in both the basal and insulin-stimulated states. In contrast, zinc only affected GSK-3 $\beta$  phosphorylation in the basal state under insulin-resistant conditions. Furthermore, the current study revealed a potential role for 50  $\mu\text{mol/L}$  zinc in basal GSK-3 $\beta$  (Ser9) phosphorylation in insulin-resistant L6 myotubes. To our surprise, 50  $\mu\text{mol/L}$  zinc in combination with insulin exerted lower effect in GSK-3 $\beta$  (Ser-9) phosphorylation than zinc alone. It is indicated that there existed optimal concentrations of zinc in combination with insulin by exhibiting the effect, showing that “high doses were not superior to low doses”; in addition, higher dose

of zinc with insulin might result in hyperglycemia and thereby produced a defected response to insulin. We also found that GSK3 $\beta$  phosphorylation did not mirror Akt phosphorylation or GLUT4 membrane translocation in the current study. This could be explained by previous studies showing that the function of GSK-3 $\beta$  can only be suppressed partially by the PI3K inhibitor wortmannin [34,35], suggesting that other signaling pathways, such as the MAPKAP-1/MAPK cascade or the p70 kinase/mTOR pathway [34,35], can also regulate GSK-3 $\beta$  phosphorylation.

The insulin signaling pathway feedback loop begins with the final nutrient and hormonal signals, such as blood glucose, blood amino acid, lipids and some hormones. These molecules send signals to PI3K and IRS-1 to play a central role in influencing insulin signaling. mTOR, a widely expressed serine/threonine protein kinase, regulates cellular metabolism, growth, proliferation and survival by activating its effector S6K1. It can also regulate the insulin signaling pathway by

sensing extracellular nutrients and hormonal signals [36–38]. This suggests that the mTOR/S6K1 complex plays a role in the crosstalk between the feedback loop and insulin signaling. Studies have shown that mTOR and S6K1 can be activated by Akt, and that they then promote protein synthesis in the normal state. In contrast, they are overactivated in insulin-resistant animal models; therefore, we aimed to investigate the effects of zinc on mTOR and S6K1 expression in both normal and insulin-resistant L6 myotubes to provide an in-depth exploration of the mechanisms involved.

Previous studies revealed that zinc promoted cell proliferation by upregulating the PI3K/Akt and mTOR pathways in embryonic stem cells [39,40]. The current results suggested that zinc did not significantly modify either S6K1 or p-S6K1 levels in normal L6 myotubes. However, 50  $\mu\text{mol/L}$  zinc significantly increased insulin-stimulated mTOR expression in L6 normal myotubes. Furthermore, 20  $\mu\text{M}$  zinc increased the phosphorylation of mTOR, suggesting that zinc might regulate the growth of normal cells by activating mTOR directly or stimulating mTOR via Akt activation.

Dyslipidemia and nutrient overload lead to overexpression of mTOR and S6K1, which phosphorylate IRS1; subsequently, the PI3K pathway is inhibited, leading to insulin resistance [41]. A number of previous studies demonstrated that mTOR/S6K1 might mediate insulin resistance and potentially type 2 diabetes [42–45]. In addition, the inhibitory effects of a high-fat diet on insulin signaling were diminished by the loss of mTOR and S6K1, and mTOR and S6K1 were highly elevated in insulin-resistant tissues such as liver, adipose and skeletal muscle [36,46]. Many previous studies have assessed the effects of exercise on ameliorating insulin resistance by regulating the mTOR/S6K1 pathway [47,48]. However, investigations into the effect of zinc on the mTOR/S6K1 pathway in the insulin resistant state are limited. The current study revealed that total mTOR levels decreased significantly in insulin-resistant L6 myotubes treated with 50  $\mu\text{mol/L}$  zinc in both the basal and insulin-stimulated states. However, 50  $\mu\text{mol/L}$  zinc decreased p-mTOR levels in only the insulin-stimulated state. Total S6K1 and phospho-S6K1 expression had a similar response to zinc supplementation, suggesting that zinc attenuated insulin resistance by downregulating mTOR/S6K1.

In conclusion, the present study showed that zinc induced glucose disposal, and also demonstrated that these effects are modulated via the Akt–GLUT4, GSK3 $\beta$  and mTOR–S6K1 pathways. Based on these findings, we hypothesize the possible mechanism of action of zinc on the insulin signaling in both normal and insulin-resistant L6 myotubes (Fig. 9), where zinc exhibits insulin-like effects. However, additional studies are needed to confirm our observations and better understand the role of zinc in insulin resistance.

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