Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 34 (2016) 126–135

Journal of Nutritional Biochemistry

Zinc stimulates glucose consumption by modulating the insulin signaling pathway in L6 myotubes: essential roles of Akt–GLUT4, GSK3β and mTOR–S6K1

Yuntang Wu[a,*,1], Huizi Lu[b,1], Huijun Yang[a], Chunlei Li[a], Qian Sang[a], Xinyan Liu[a], Yongzhe Liu[c], Yongming Wang[c], Zhong Sun[d,*]

[a]Department of Nutrition and Food Hygiene, School of Public Health, Tianjin Medical University, Tianjin, China
[b]Tanggu Centers for Disease Control and Prevention, Tianjin Binhai New Area,Tianjin, China
[c]Department of Toxicology, School of Public Health, Tianjin Medical University, Tianjin, China
[d]Department of Epidemiology and biostatistics, School of Public Health, Tianjin Medical University, Tianjin, China

Received 10 November 2015; received in revised form 10 May 2016; accepted 17 May 2016

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 GSK3β), 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 GSK3β phosphorylation. In contrast, zinc exerted insulin-mimetic effects in insulin-resistant L6 myotubes by upregulating Akt phosphorylation, GLUT4 translocation and GSK3β phosphorylation, and downregulating the expression of mTOR and S6K1. In conclusion, zinc might enhance glucose consumption by modulating insulin signaling pathways including Akt–GLUT4, GSK3β, mTOR and S6K1.

Keywords: Zinc; Glucose consumption; Insulin signaling pathway; L6 myotubes; Akt; mTOR; GSK3β

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 pathogeneses 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-3β 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 β 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; S6 K1, ribosomal protein S6 kinase, polypeptide 1; SDS, sodium dodecyl sulfate.

* Corresponding authors at: Department of Nutrition and Food Science and Department of Epidemiology and biostatistics, School of Public Health, Tianjin Medical University, 22 Qixiangtai Road, Heping District, Tianjin 300070, China. Tel.: +86 22 83336610; fax: +86 22 83336608.
E-mail address: Wuyuntang@tmu.edu.cn (Y. Wu).
[1] These two authors are co-first authors with equal contribution.

http://dx.doi.org/10.1016/j.jnutbio.2016.05.008
0955-2863/

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)-β 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), GSK3β, phospho-GSK3β (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 Zhongshanjinqiao 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 (1.5×10^6 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 μmol/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% β-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 ± 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 μmol/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 μmol/L) enhanced glucose consumption significantly ($P<.05$). Treatment with 20, 50 and 100 μmol/L zinc increased glucose consumption significantly and in a dose-dependent manner, compared with treatment with 10 μmol/L ($P<.05$). Co-incubation of zinc (20, 50 or 100 μmol/L) and insulin increased glucose consumption to a greater extent than incubation with insulin alone ($P<.05$). Furthermore, 50 and 100 μmol/L zinc had a stronger stimulating effect than 10 μmol/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 μmol/L zinc. Furthermore, the co-incubating cells with the combination of 100 μmol/L zinc and insulin showed a tendency to downregulate cell viability compared with 10–50 μmol/L zinc (data not shown). Therefore, only zinc concentrations ranging from 10 to 50 μmol/L were used in subsequent studies. In addition, different time points were used to