Zinc Has an Insulin-Like Effect on Glucose Transport Mediated by Phosphoinositol-3-Kinase and Akt in 3T3-L1 Fibroblasts and Adipocytes[1]

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ABSTRACT Zinc has insulin-like effects on cells, including promotion of both lipogenesis and glucose transport. The relationship between zinc and the stimulation of glucose transport is unclear. We hypothesize that zinc affects the insulin-signaling pathway. In this study, the effect of zinc on glucose transport and insulin signaling was examined in 3T3-L1-preadipocytes and -adipocytes. Treatment of cells with up to 200 μmol/L zinc significantly increased glucose transport ($P < 0.05$). The effect of zinc on adipocytes was greater than on preadipocytes, and the effect of zinc plus insulin was greater than that of either insulin or zinc alone. Cytochalasin D, which disrupts actin filaments, attenuated the increase of glucose transport induced by zinc or insulin ($P < 0.05$). At 100 nmol/L, wortmannin, the phosphoinositide (PI) 3-kinase inhibitor, decreased basal glucose transport and blocked zinc-stimulated glucose transport in both cell types ($P < 0.05$). H7, an inhibitor of protein kinase C, did not reduce basal glucose transport but decreased zinc-induced glucose transport ($P < 0.05$). Zinc increased tyrosine phosphorylation of the insulin receptor β subunit of both preadipocytes and adipocytes after 5–10 min of treatment ($P < 0.05$). Zinc at 200 μmol/L did not affect tyrosine phosphorylation of insulin receptor substrate (IRS)-1 or -2; further, there was no effect of zinc on the association of the p85 subunit of PI 3-kinase and IRS-1. Zinc significantly increased serine-473 phosphorylation of Akt in both preadipocytes and adipocytes ($P < 0.05$). The PI 3-kinase inhibitor, wortmannin, totally blocked the effect of zinc on Akt activation. Hence, it appears that zinc can induce an increase in glucose transport into cells and potentiate insulin-induced glucose transport, likely acting through the insulin-signaling pathway. J. Nutr. 131: 1414–1420, 2001.

KEY WORDS: • *zinc* • *insulin* • *insulin signaling* • *glucose transport.*

Insulin exerts many physiologic effects, including promoting glucose and amino acid transport, enhancing anabolic processes and decreasing catabolic processes. Among these, the promotion of glucose transport into target cells modulates glucose concentrations in the blood stream, and failure to enhance glucose transport into insulin-sensitive cells is a hallmark of diabetes (1). The first contribution to insulin-stimulated glucose transport is enhanced translocation of glucose transporters from intracellular compartments to the plasma membrane (2). The translocation of these glucose transporters is the final step in a complex process of vesicle trafficking. This translocation caused by insulin is believed to be carried out by a metabolic signaling pathway, which includes the insulin receptor (IR),[3] the insulin receptor substrate (IRS) proteins (3), phosphoinositide 3-kinase (PI 3-K), protein kinase C (PKC) isoforms (4–6) and Akt (or protein kinase B) (7). Recently, Akt was shown to promote glucose transporter (GLUT) 4 translocation, but not GLUT 1 (7). Sharma et al. (8) found that the translocation of GLUT4, caused by insulin, was independent of IRS-1 in 3T3-L1 adipocytes. Although tremendous advances have been made in delineating the insulin-signaling pathway, many details are still poorly understood.

Zinc is required for the function of many intracellular proteins, including enzymes, transcription factors and proteins involved in DNA replication. Zinc has an effect on epidermal growth factor (EGF)-stimulated intracellular signaling, stimulating tyrosine phosphorylation of the EGF receptor (9). Zinc also has been reported to have insulin-like effects as well. Zinc potentiates the mitogenic signaling of insulin (10) and activates extracellular-signal–regulated kinases 1 and 2 (11). Coulston and Dandona (12) first reported that zinc promoted lipogenesis of rat epididymal adipocytes, and this function is insulin-like. Also, May and Contoreggi (13) found that zinc had another insulin-like function, i.e., increasing glucose transport into rat epididymal adipocytes. Clinical research shows evidence of a correlation between zinc deficiency and diabetes (14). May and Contoreggi (13) proposed both direct effects of zinc on intracellular events and indirect effects

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[3] Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium: DPBS, Dulbecco's PBS; ECL, enhanced chemiluminesce; EGF, epidermal growth factor; FAK, focal adhesion kinase; FBS, fetal bovine serum; GLUT, glucose transporter; IBMX, 3-isobutyl-1-methyl-xanthine; IR-β, insulin receptor β subunit; IRS, insulin receptor substrate; MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C.

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related to the generation of H_2O_2. Since then, the intracellular signaling events have not been well investigated. To our knowledge, only one report exploring the relationship between zinc and insulin-signaling pathway exists (15). In that report, zinc stimulated glucose transport into rat adipocytes through a postinsulin receptor mechanism. Nevertheless, the relationship between zinc and the insulin-signaling pathway remains unclear. To determine which intracellular events are activated by zinc to stimulate glucose transport, we examined the effects of zinc on the members of insulin metabolic signaling pathway in 3T3-L1 preadipocytes and adipocytes.

MATERIALS AND METHODS

Materials. 3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD). Zinc chloride was from Fisher Scientific (Pittsburgh, PA). Cytochalasin B, cytochalasin D, 3-isobutyl-1-methyl-xanthine (IBMX), dexamethasone, Dulbecco's PBS (DPBS), wortmannin, H7 and 2-deoxy-D-glucose were from Sigma Chemical (St Louis, MO). Tritiated (1,2-^{3}H) 2-deoxy-D-glucose was from ICN (Costa Mesa, CA). Bovine insulin was from Gibco Life Technologies (Gaithersburg, MD). Protein A–coated agarose beads, anti-insulin receptor β (IR-β) subunit antibody, and anti-IRS-1 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IRS-2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Mouse anti-phosphotyrosine antibody PY 20 was from Transduction Laboratories (San Diego, CA). Anti-Akt and anti-phospho-Akt (Ser 473) were from New England Biolabs (Beverly, MA). The enhanced chemiluminescence (ECL) detection system was from Amersham Pharmacia (Buckinghamshire, UK).

Cell culture and induction of differentiation. Cells were grown in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS) and antibiotic-antimycotics in 5% CO_2/95% air at 37°C. To induce the differentiation of fibroblasts into adipocytes, confluent fibroblasts were incubated with DMEM plus 10% FBS, antibiotic-antimycotics, 1000 μg/L insulin, 0.5 mmol/L IBMX and 1 μmol/L dexamethasone for 3 d. Cells were then switched to DMEM plus 10% FBS, antibiotic-antimycotics and 1000 μg/L insulin for 3 d. After this treatment, cells were then switched to normal growth medium, and medium was changed at least every 3 d. After differentiation, at least 85% of the cells demonstrated the phenotype of adipocytes. Cell viability for all cellular treatments, including zinc and cell inhibitors, was monitored by trypan blue stain exclusion. No treatments used in this report affected cell viability.

Glucose transport assay. Cells were plated into gelatin-coated 24-well plates. The confluent fibroblasts and differentiated adipocytes were rinsed once with PBS and switched to serum-free medium for 3 h at 37°C in 5% CO_2/95% air. Cells were then rinsed three times with DPBS with 1.0 g/L bovine serum albumin (BSA) (pH 7.4). The cells were incubated in 250 μL DPBS with or without zinc or insulin for 30 min at 37°C. Cells were then washed three times with DPBS; then, 250 μL DPBS with 100 μmol/L 2-deoxy-D-glucose/2-deoxy-D-[1.2-^{3}H] glucose (final concentration of ^{3}H was 37 Bq/μL) was added into each well and incubated in the presence or absence of 20 μmol/L cytochalasin B at 37°C for 10 min. After incubation, the cells were quickly rinsed three times with ice-cold PBS (Sigma Chemical, pH 7.4). Cells were then solubilized with 0.4 mL 0.1% SDS. Radioactive 2-deoxy-glucose uptake was measured using liquid scintillation counting. Data were analyzed using ANOVA, with $P < 0.05$ set as an indicator of difference. When ANOVA indicated that significant differences existed, post-hoc differences between values were determined using Tukey's test.

In the experiments exploring the effects of protein kinase inhibitors, cells were incubated with an inhibitor for the last 30 min of a trial in DPBS (1.0 g/L BSA) at 37°C. To examine the role of actin filaments, cells were incubated with 2 μmol/L cytochalasin D in serum-free medium for 3 h; during the last 30 min, cells were incubated with 200 μmol/L $ZnCl_2$ or 14 nmol/L insulin. Cells were then washed 3 times with DPBS with 1.0 g/L BSA, and glucose uptake was measured as described above.

Immunoprecipitation. Cells were grown in 100-mm diameter dishes. Near-confluent 3T3-L1 fibroblasts and differentiated adipocytes were rinsed with and incubated in serum-free medium at 37°C for 6 h. Cells were then treated with 70 nmol/L insulin for 5 or 10 min, and 200 μmol/L $ZnCl_2$ for 5, 10 and 20 min at 37°C. Cells were then rinsed twice with ice-cold PBS and lysed in 200 μL lysis buffer [1% (v/v) Nonidet P-40, 50 mmol/L Tris, 100 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L sodium orthovandate, 10 mmol/L sodium pyrophosphate, 2.5 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/L aprotinin, 10 mg/L leupeptin and 10 mg/L pepstatin A, pH 7.4) on ice for 30 min. Cell lysates were then centrifuged at 13,000 $\times$ *g* at 4°C for 10 min, and the supernatant was incubated on a rotator with protein A-coated agarose beads (Santa Cruz Biotechnology) and a specific antibody at 4°C overnight. The immunoprecipitated complexes were washed 3 times with lysis buffer, resuspended in sample loading buffer, boiled for 5 min and subjected to electrophoresis and Western-blot analysis.

Western-blot analysis. Cell lysates or immunoprecipitated complexes were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked in TBS-0.1% Tween 20 containing 50 g/L nonfat dry milk for 1 h at room temperature. Detection of specific proteins was carried out by incubation with the primary antibody at 4°C overnight in TBS-0.1% Tween-20 containing 50 g/L nonfat dry milk or BSA. Blots were then incubated with horseradish peroxidase–labeled secondary antibody (1:2000). The immunolabeled bands were visualized on Kodak O-MAT film (Rochester, NY) with an ECL detection system using the manufacturer's suggested protocol.

RESULTS

Consistent with the results of previous studies, 14 and 70 nmol/L insulin increased ($P < 0.05$) glucose transport into 3T3-L1 fibroblasts (**Fig. 1**). Zinc chloride enhanced glucose transport ($P < 0.05$) in the absence of insulin. Insulin and $ZnCl_2$ also had a similar effect on the differentiated 3T3-L1 adipocytes; however, the effect on the differentiated adipocytes was greater than that on fibroblasts (**Fig. 2**). When zinc was included in the incubation medium, glucose uptake increased ($P < 0.05$) in a dose-dependent fashion with or without insulin in the medium. Other experiments showed that calcium and magnesium did not enhance glucose transport (data not shown).

When cytochalasin D was included in the cell culture, glucose uptake was inhibited ($P < 0.05$). However, in the presence of this inhibitor, zinc and insulin still significantly increased glucose uptake compared with the same fibroblasts and adipocytes treated with the inhibitor alone (**Fig. 3**). Treatment with wortmannin (**Fig. 4**) decreased ($P < 0.05$) basal glucose transport into 3T3-L1 fibroblasts and adipocytes. Wortmannin totally abolished ($P < 0.05$) the insulin- and $ZnCl_2$-induced increases in glucose transport. Unlike wortmannin, the PKC inhibitor H7 did not decrease basal glucose transport (**Fig. 5**), whereas it decreased ($P < 0.05$) glucose transport into 3T3-L1 adipocytes (but not fibroblasts) induced by $ZnCl_2$.

To help explain the results of transport tests and inhibitors, the phosphorylation state of several proteins in the insulin-signaling pathway were assayed. First, the effects of insulin and zinc on the tyrosine phosphorylation of the 95-kDa IR-β subunit were investigated (**Fig. 6**). Insulin increased tyrosine phosphorylation of the IR-β subunit on both 3T3-L1 fibroblasts and adipocytes. Treatment with $ZnCl_2$ also produced an insulin-like effect, but the extent was lower than that of insulin. Next in the pathway, IRS-1 and –2 were investigated. In the same experiments in which zinc was observed to stimulate phosphorylation of the IR-β subunit, IRS proteins were immunoprecipitated with the anti-phosphotyrosine PY-20 antibody and immunoblotted with anti-IRS antibodies. Neither

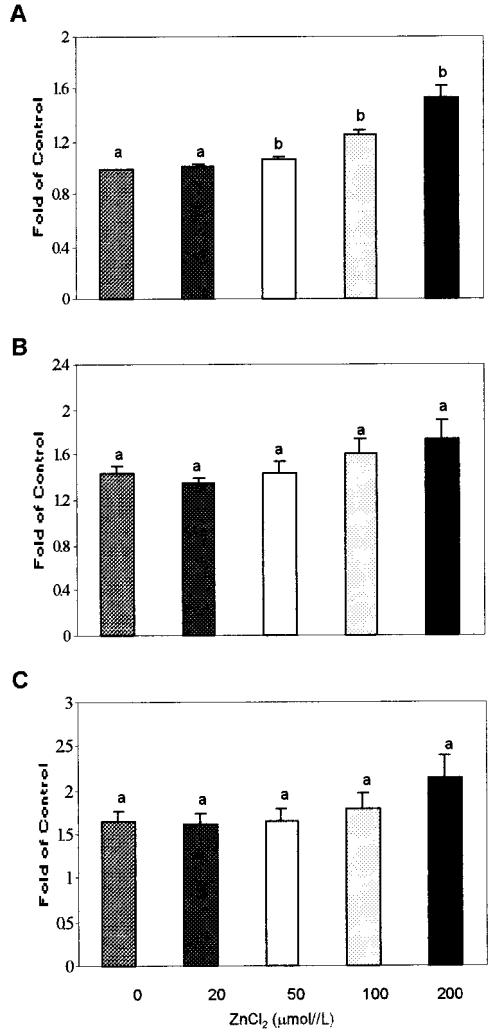

FIGURE 1 The effect of zinc on glucose uptake in 3T3-L1 fibroblasts in the absence or presence of insulin. Confluent cells in 24-well plates were incubated with $ZnCl_2$ in the presence and absence of insulin for 30 min, then assayed for [^{3}H]2-deoxyglucose uptake. (*A*) No insulin; (*B*) 14 nmol/L insulin; (*C*) 70 nmol/L insulin. Data are expressed as means ± SEM, $n = 4$. Means with different letters differ, $P < 0.05$.

IRS-1 nor IRS-2 increased tyrosine phosphorylation after zinc treatment (data not shown). Both IRS-1 and -2 were tested in five independent experiments; in every case, insulin stimulated IRS tyrosine phosphorylation, whereas zinc did not. Normally, the activation of PI 3-kinase caused by insulin is via the association of tyrosine-phosphorylated IRS proteins with the p85 subunit of PI 3-kinase. The IRS proteins were studied in a second way. After treatment with insulin and/or zinc, cell lysates were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with an antibody to the p85 subunit of PI 3-kinase. When PI 3-kinase is detected by immunoblot, an association of PI 3-kinase with IRS-1 is presumed. Only insulin caused this kind of association, whereas zinc had no effect. Five separate trials were conducted, with each trial including a variety of incubation times (data not shown). Although insulin treatment always resulted in PI 3-kinase being detected, zinc did not produce a similar result. We conclude that IRS-1 and IRS-2 may not be important in the increase in glucose transport induced by zinc, even when the IR phosphorylation was stimulated by the same treatment.

To determine whether the downstream factor Akt participates in the PI 3-kinase pathway in zinc-induced glucose transport, we explored the effect of zinc on the activity of Akt using immunoblot analysis with anti-phosphoAkt (Ser 473) antibody. After incubation with serum-free medium, cells were treated with insulin or zinc. Insulin treatment increased the phosphorylation on serine 473 of Akt in both preadipocytes and adipocytes. Zinc induced Ser 473 phosphorylation as well, and the extent of phosphorylation appeared to be similar whether zinc or insulin was included in the incubation (**Fig. 7**). To determine whether Akt phosphorylation can be carried out by other divalent cations, $CaCl_2$ and $MgCl_2$ were tested. When either 10 mmol/L $CaCl_2$ or 2 mmol/L $MgCl_2$ was tested, neither noticeably increased Ser 473 phosphorylation of Akt as had been observed for zinc. We do not rule out the possibility that Ca or Mg may affect Akt phosphorylation to some slight degree (**Fig. 8**). However, if there is some slight effect of Ca or Mg, it appears that it is much less than the effect of zinc. The phosphorylation of Akt is usually dependent on PI 3-kinase (16). To determine whether PI 3-kinase plays a critical

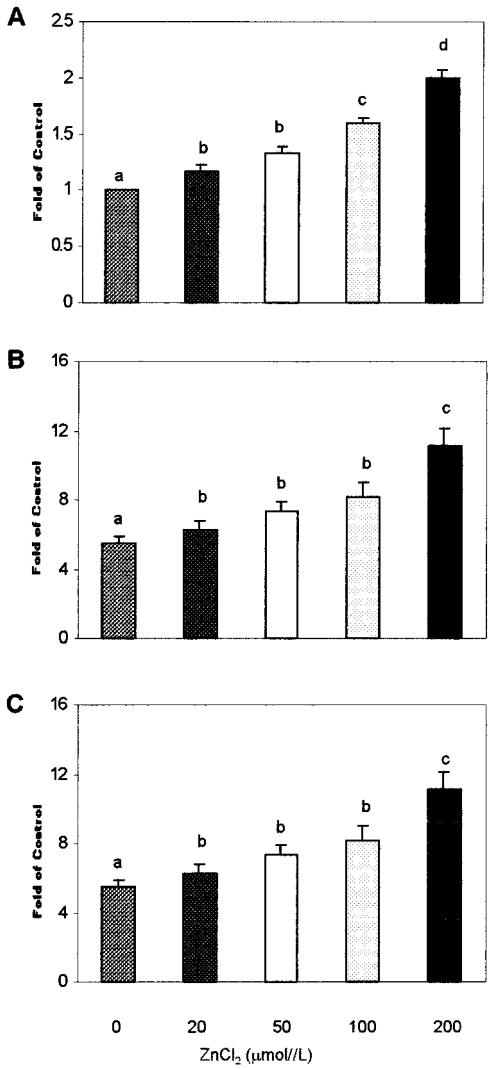

FIGURE 2 The effect of zinc on glucose uptake in 3T3-L1 adipocytes in the presence and absence of insulin. Confluent differentiated 3T3-L1 cells in 24-well plates were treated with $ZnCl_2$ in the presence and absence of insulin for 30 min, then assayed for [^{3}H]2-deoxyglucose uptake. (*A*) No insulin; (*B*) 14 nmol/L insulin; (*C*) 70 nmol/L insulin. Data are expressed as means ± SEM, $n = 4$. Means with different letters differ, $P < 0.05$.

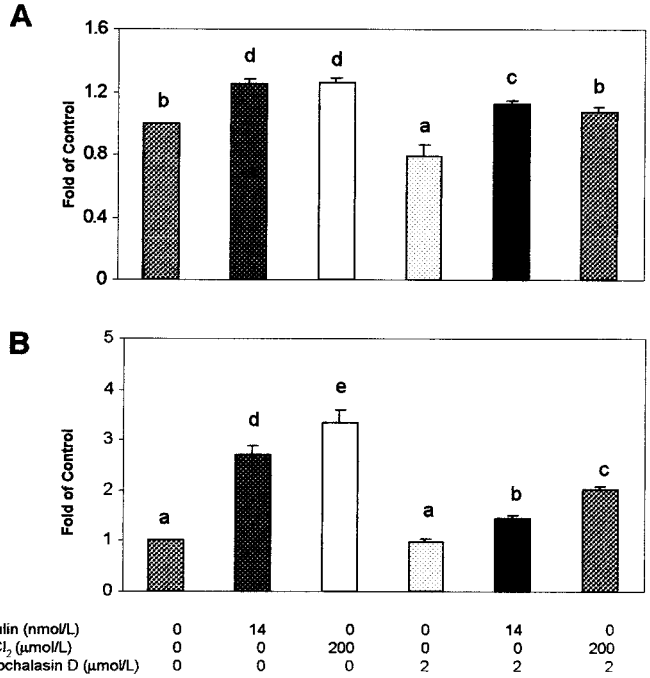

FIGURE 3 The effect of cytochalasin D on basal, zinc- and insulin-induced glucose transport in 3T3-L1 cells. Fibroblasts (*A*) and adipocytes (*B*) were pretreated with 2 μmol/L cytochalasin D in serum-free medium for 3 h, and during the last 30 min incubated with 14 nmol/L insulin or 200 μmol/L $ZnCl_2$, then assayed for [^{3}H]2-deoxyglucose uptake. Data are expressed as means ± SEM, $n = 4$. Means with different letters differ, $P < 0.05$.

role in zinc-potentiated Akt action, wortmannin was again used. Treatment with wortmannin alone did not affect the phosphorylation of Akt on serine 473 in either preadipocytes or adipocytes, whereas the inhibition of PI 3-kinase with 100 nmol/L wortmannin totally blocked the ability of zinc to increase the activity of Akt (**Fig. 9**). Therefore, the present data indicate that PI 3-kinase appears to be a key intracellular signaling mediator in the activation of Akt by zinc, rather than zinc acting on Akt directly.

DISCUSSION

It was reported recently that zinc promotes cell mitosis through the MAP kinase pathway (10). Perry et al. (17) and Fukamachi et al. (18) found that zinc suppresses apoptosis through inhibition of caspase-3 activity and an increase in the Bcl/Bax ratio, respectively. Thus, evidence is accumulating that zinc may be involved in certain intracellular signal transduction events. The present data show that zinc promotes glucose transport into cells through the PI 3-kinase signal transduction pathway. Because PI 3-kinase plays a critical role in insulin signaling, zinc may be exerting an insulin-like function by affecting phosphorylation or dephosphorylation of one or more components of the insulin pathway.

Zinc has insulin-like functions in lipogenesis (12), glucose transport (13,15) and leptin production in humans (19). 3T3-L1 fibroblasts predominantly express insulin-insensitive GLUT1, whereas 3T3-L1 adipocytes express more of the insulin-responsive GLUT4. Generally, most of the experiments presented in this report confirm that zinc has a greater effect on glucose transport in the 3T3-L1 adipocytes than in the preadipocyte fibroblast form. We consider it important to recognize that when we used a range of doses for zinc, the effect of zinc was dose dependent, and significant differences were observed between zinc-free and zinc concentrations as low as 20 or 50 μmol/L. Considering that normal serum concentration for zinc may be ~15 μmol/L, we consider significant effects seen at 20 or 50 μmol/L to be physiologic in nature rather than pharmacologic. Due to the nature of immunoblot experiments, it is more difficult to test a large number of conditions, including various zinc concentrations. Generally, zinc was tested at 0 or 200 μmol/L in these experiments. On the basis of the results of the glucose uptake experiments shown in Figures 1 and 2, we consider it likely that zinc-induced phosphorylation of Akt or the IR was responding to zinc in a similar manner, and dose-dependent effects of zinc on phosphorylation would be expected if dose-response studies on Akt and the IR were to be conducted.

The IR-β subunit is a tyrosine kinase and is activated through autophosphorylation when insulin binds to its receptor on the cell membrane (20). Like insulin, zinc also increases tyrosine phosphorylation of the IR-β subunit (Fig. 6). This result is consistent with the findings that zinc causes tyrosine phosphorylation of the EGF receptor, which is also a tyrosine kinase (9). In 1989, Ezaki (15) found that zinc did not increase tyrosine phosphorylation of the insulin receptor, which led to the conclusion that zinc affects glucose transport by a postinsulin receptor mechanism in rat adipocytes. However, Ezaki tested the effect of zinc on tyrosine phosphorylation of the IR only at 30 min. We examined and found an insulin-like effect at just 5 and 10 min after insulin treatment. Other data (not shown) indicate that phosphorylation of the IR returns to baseline at between 10 and 20 min. Although zinc enhances

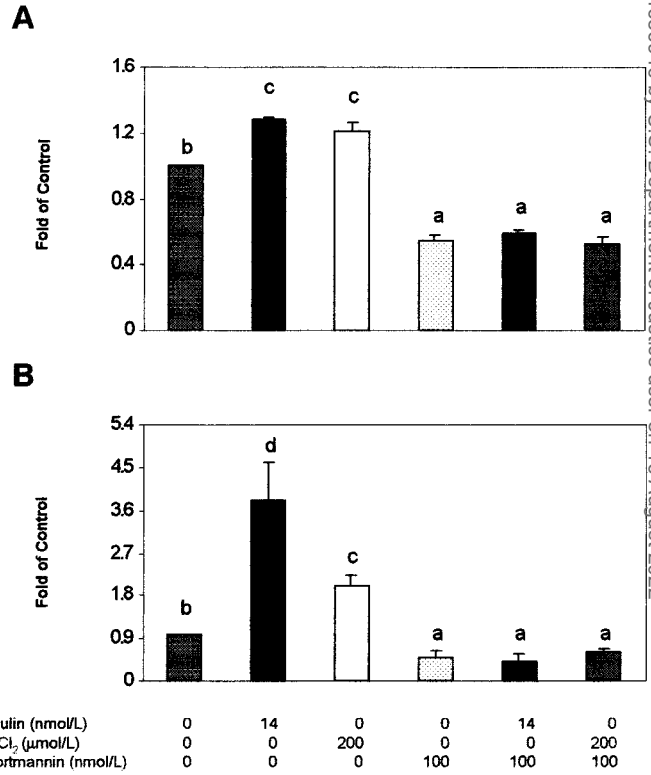

FIGURE 4 The effect of wortmannin on basal, zinc- and insulin-induced glucose transport in 3T3-L1 cells. Fibroblasts (*A*) and adipocytes (*B*) were pretreated with 100 nmol/L wortmannin for 30 min, incubated with 14 nmol/L insulin or 200 μmol/L $ZnCl_2$ for 30 min, then assayed for [^{3}H]2-deoxyglucose uptake. Data are expressed as means ± SEM, $n = 4$. Means with different letters differ, $P < 0.05$.

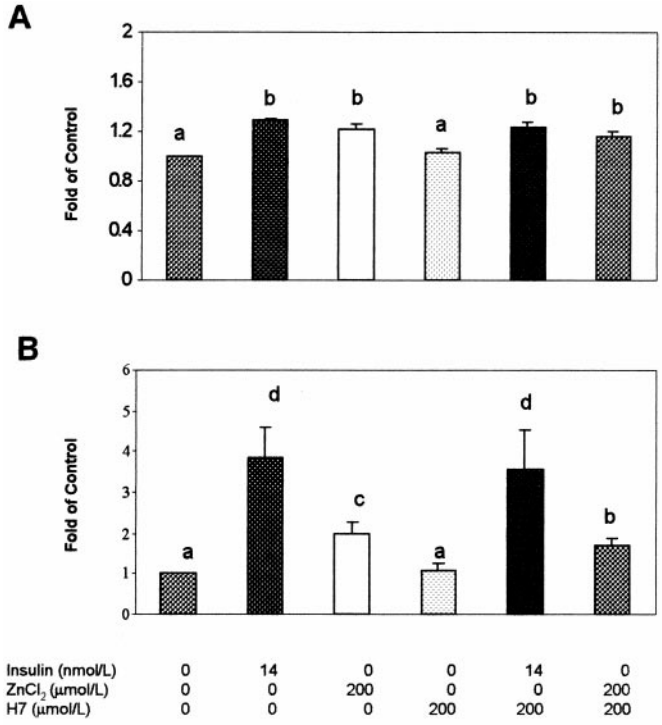

FIGURE 5 The effect of the protein kinase C (PKC) inhibitor H7 on basal, zinc- and insulin-induced glucose transport in 3T3-L1 cells. Fibroblasts (*A*) and adipocytes (*B*) were pretreated with 200 μmol/L H7 for 30 min, incubated with 14 nmol/L insulin or 200 μmol/L $ZnCl_2$ for 30 min, then assayed for [^{3}H]2-deoxyglucose uptake. Data are expressed as means ± SEM. Means with different letters differ, $P < 0.05$.

tyrosine phosphorylation of the IR, this is not conclusive evidence that this event is necessary for zinc-enhanced glucose transport.

The IRS-1 and -2 proteins play a critical role in insulin action. Homozygous IRS-1 gene knockout mice exhibit a mild insulin resistance, but do not develop diabetes (21). Disruption of the IRS-2 gene results in decreased insulin production, insulin resistance and diabetes (22). However, several reports suggest that IRS-1, -2 and -3 may not be required for GLUT4 translocation and insulin-stimulated glucose transport (8,23,24). We report here that zinc did not cause the association of IRS-1 and PI 3-kinase p85 subunit, even when glucose

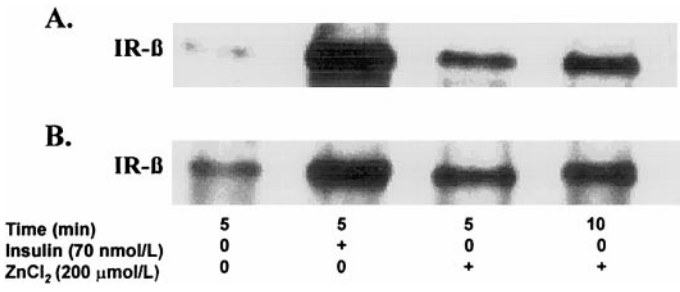

FIGURE 6 Tyrosine phosphorylation of insulin receptor β-subunit in cultured 3T3-L1 cells after treatment with insulin or zinc. Fibroblasts (*A*) and adipocytes (*B*) were incubated in serum-free medium and treated with 70 nmol/L insulin or 200 μmol/L $ZnCl_2$. The tyrosine phosphorylated insulin receptor β subunit was immunoprecipitated with PY 20 antibody and immunoblotted with anti-insulin receptor β subunit antibody. Figure shown is one of seven independent experiments. All seven experiments showed similar results.

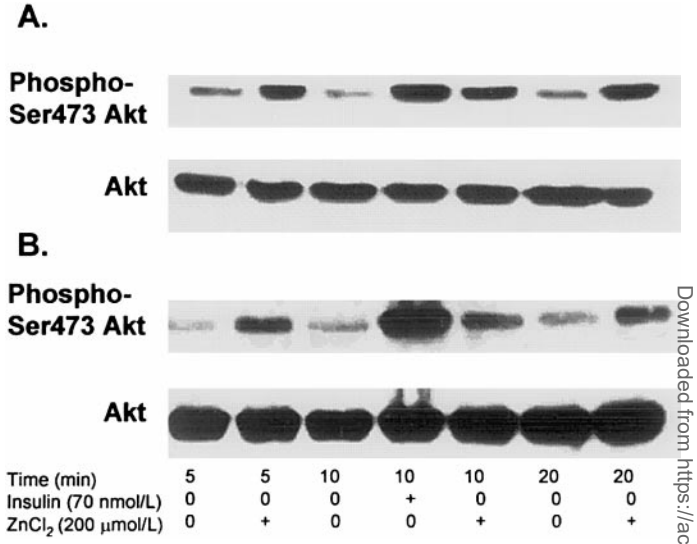

FIGURE 7 Phosphorylation of Akt at Ser 473 in cultured 3T3-L1 cells after treatment with insulin or zinc. Fibroblasts (*A*) and adipocytes (*B*) were incubated in serum-free medium and treated with 70 nmol/L insulin or 200 μmol/L $ZnCl_2$. Cell lysate was immunoblotted with anti-phospho-Akt (serine 473) antibody or anti-Akt antibody. Figure shown is one of four independent experiments. All four experiments showed similar results.

transport was enhanced (data not shown). We suggest that the interaction between the IR and IRS proteins may not be necessary for insulin- and zinc-induced glucose transport.

Actin filament rearrangement is important for GLUT4 translocation to the plasma membrane (25). Tsakiridis et al. (26) reported the recruitment of GLUT4, PI 3-kinase subunits and GLUT4-containing vesicles to a reorganized actin network. Although cytochalasin D does not have an effect on

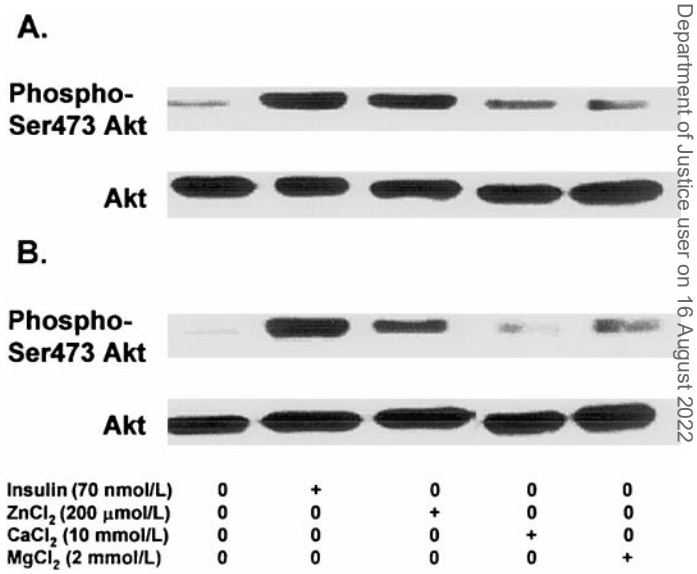

FIGURE 8 Effect of selected divalent cations on serine phosphorylation of Akt in cultured 3T3-L1 cells. Fibroblasts (*A*) and adipocytes (*B*) were incubated in serum-free medium and treated with insulin, $ZnCl_2$, $CaCl_2$ or $MgCl_2$ for 10 min. Cell lysate was immunoblotted with anti-phospho-Akt (serine 473) antibody or anti-Akt antibody. Figure shown here represents one of three independent experiments. All three experiments showed similar results.

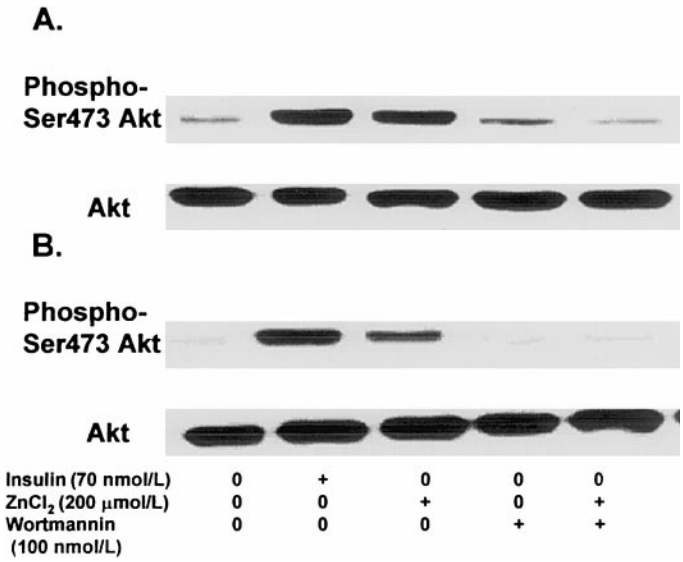

FIGURE 9 The effect of wortmannin on zinc-induced serine phosphorylation of Akt in cultured 3T3-L1 cells. Fibroblasts (*A*) and adipocytes (*B*) were incubated in serum-free medium and pretreated with 100 nmol/L wortmannin for 30 min, then incubated with insulin or $ZnCl_2$ for 10 min. Cell lysate was immunoblotted with anti-phospho-Akt (serine 473) antibody or anti-Akt antibody. Figure shown here represents one of three independent experiments. All three experiments showed similar results.

basal glucose transport, we showed that cytochalasin D inhibits zinc-induced glucose transport into cells (Fig. 3). Thus, the induction of transport caused by zinc is likely dependent on reorganization of the actin filament network, and that the last step of insulin- or zinc-induced glucose transport is a common one.

Phosphoinositide 3-kinase has been demonstrated to play a key role in insulin-stimulated glucose uptake (27). To investigate the role of PI 3-kinase in zinc-induced glucose transport, wortmannin, the potent mammalian PI 3-kinase inhibitor was used to block the phospholipid-mediated signaling pathway. Wortmannin can totally abrogate the effect of insulin on glucose transport into rat adipocytes (28). Expression of the dominant-negative PI 3-kinase p85 subunit mutant completely blocks insulin-induced GLUT4 translocation (1). Constitutively active PI 3-kinase has an effect on GLUT4 translocation similar to insulin's action in adipocytes (29). Consistent with this, our experiments showed that wortmannin decreases basal glucose transport of 3T3-L1 cells and also blocks insulin-induced glucose transport. Wortmannin inhibited zinc-induced glucose transport as well (Fig. 4). Because it has been reported that the 50% inhibitory concentration of wortmannin on mammalian PI 3-kinases is in the nanomolar range (30), and wortmannin concentrations >100 nmol/L may inhibit some isoforms of PI 4-kinases (31) and phospholipase A_2 (32), we used concentrations of wortmannin in this experiment that would specifically inhibit PI 3-kinase. This result suggests to us that the zinc effect requires PI 3-kinase to enhance glucose transport. Our result is consistent with the findings of Kim et al. (33) that zinc can activate PI 3-kinase. Regarding GLUT4 translocation, the specific subcellular site of PI 3-kinase activation is of importance. This is why insulin can stimulate GLUT4 translocation through targeting PI 3-kinase to GLUT4 vesicles, whereas platelet-derived growth factor activation of PI 3-kinase has a lesser effect on glucose transport (34). In the insulin pathway, it is most typically thought that PI 3-kinase is activated by the association of its p85 subunit and IRS proteins (35). However, IRS proteins are not necessary in insulin-induced glucose transport, probably because IRS proteins do not target PI 3-kinase to the proper intracellular location. For enhancement of glucose transport, the association with other intracellular factors likely activates PI 3-kinase. Imamura et al. (36) reported that Gq/α11 protein plays an important role in the activation of catalytic subunits of PI 3-kinase for insulin-induced glucose transport in 3T3-L1 adipocytes. Other evidence suggests ways that zinc may activate PI 3-kinase without IRS involvement, i.e., May and Contoreggi (13) observed that treatment of zinc (from 250 μmol/L to 1 mmol/L) causes the production of H_2O_2 in rat epididymal adipocytes, and H_2O_2 can activate focal adhesion kinase (FAK) (37). FAK can activate the PI 3-kinase-Akt pathway (38). We can advance at least one hypothesis for future testing, i.e., that PI 3-kinase is activated in part through the H_2O_2/FAK pathway and then delivered to GLUT4-containing vesicles. There is also the possibility of G-protein activation of PI 3-kinase after zinc treatment. Some proteins, including ARNO, bind to the product of PI 3-kinase, PI(3,4,5)-P_3, and regulate ARF6, which is involved in membrane ruffling and insulin-stimulated actin filament reorganization (39). Therefore, we suggest that like insulin, zinc mediates actin reorganization through PI 3-kinase, and finally stimulates glucose transport.

The PI 3-kinase downstream targets, PKC-β2, and atypical PKC family members PKC-λ and -ζ, are involved in insulin-stimulated GLUT4 translocation. Braiman et al. (4) found that the specific PKC-β2 inhibitor LY379196 blocks insulin-induced glucose transport in rat skeletal muscle, and wortmannin inhibits the activation of PKC-β2 by insulin. On the other hand, PKC-λ and PKC-ζ can be activated by insulin, and transfection studies with constitutively active and dominant-negative mutants demonstrate that these two PKC family members are involved in GLUT4 translocation in rat adipocytes (6,40). The general PKC inhibitor, H7, did not inhibit insulin-induced glucose transport in 3T3-L1 adipocytes (Fig. 5). This lack of inhibition may be due to the fact that although insulin acts mainly through PKC-λ and PKC-ζ, zinc partially induces glucose uptake through PKC β2.

Because there are at least two intermediaries downstream from PI 3-kinase involved in glucose transport, PKC proteins and Akt/protein kinase B (7), another hypothesis would suggest that Akt is responsible for the major contribution to zinc-induced glucose transport. Our study clearly demonstrates that zinc causes phosphorylation of Akt serine 473 in 3T3-L1 cells, and other divalent cations, such as calcium and magnesium, do not have a similar effect, if any at all (Figs. 7, 8). Consistent with our results, Kim et al. (33) also detected Akt serine 473 phosphorylation after treatment with 100 μmol/L zinc in Swiss 3T3 cells. We provide direct evidence that the activation of Akt caused by zinc is PI 3-kinase dependent (Fig. 9), and this mechanism of Akt activation is the same as that induced by insulin and other growth factors (7). Recently, accumulated evidence has shown the relationship between Akt and GLUT4 translocation. Constitutively active Akt stimulates glucose transport by the translocation of GLUT4 to plasma membrane (41). Kupriyanova and Kandror (42) demonstrated that Akt-2 associated with GLUT4-containing vesicles and phosphorylated vesicular proteins. The activation of Akt by zinc in this study suggests that Akt is important for zinc-stimulated glucose transport, but it will be necessary to examine the effect of a dominant-negative Akt mutant to confirm a role for Akt.

In conclusion, we have established that zinc exerts an

insulin-like effect in 3T3-L1 cells, which is more pronounced in the 3T3 L1 adipocytes. We measured this effect as a stimulation of glucose transport and demonstrated that zinc affects components of the insulin intracellular signaling pathway. Zinc stimulates the tyrosine phosphorylation of IR-β subunit and activates the PI 3-kinase pathway and Akt. IRS proteins appear not to participate in zinc's effect on glucose transport. From these results, we propose that zinc deficiency may indeed affect optimal function of the insulin-signaling pathway. How or whether zinc is involved in the development of insulin resistance is an important question; better understanding of the signaling pathway may ultimately help answer these questions.

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