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Inhibition of glycogen synthase kinase-3β by bivalent zinc ions: insight into the insulin-mimetic action of zinc

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Abstract

Zinc is an important trace element found in most body tissues as bivalent cations and has essential roles in human health. The insulin-like effect of zinc cations raises the possibility that they inhibit glycogen synthase kinase-3β (GSK-3β), a serine/threonine protein kinase linked with insulin resistance and type 2 diabetes. Here we show that physiological concentrations of zinc ions directly inhibit GSK-3β in vitro in an uncompetitive manner. Treatment of HEK-293 cells with zinc enhanced glycogen synthase activity and increased the intracellular levels of β-catenin, providing evidence for inhibition of endogenous GSK-3β by zinc. Moreover, zinc ions enhanced glucose uptake 3-fold in isolated mouse adipocytes, an increase similar to activation with saturated concentrations of insulin. We propose that the in vivo insulin-mimetic actions of zinc are mediated via direct inhibition of endogenous GSK-3β. © 2002 Elsevier Science (USA). All rights reserved.

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Zinc is a transition metal found in most body tissues as bivalent cations. Zinc ions are essential supplement in human diet and their involvement in the regulation of numerous cellular processes is well documented (reviewed in [2,14,19]). Zinc deficiency may lead to impaired immune functions, developmental abnormalities, and alterations in carbohydrate metabolism [2]. Human plasma normally contain about 15 μM Zn^{2+} [3], most of it bound to proteins, but some tissues such as muscle and liver contain much higher concentrations [2,14,19]. Still, the molecular mechanism of zinc-induced cellular responses is not fully understood.

One of the remarkable in vivo features of zinc is its insulin-like function and its potential link with insulin resistance and type 2 diabetes. Zinc was shown to stimulate lipogenesis and glucose transport in adipocytes [7,12,20] and its supplementation in diet attenuated hyperglycemia in db/db mice [23]. In zinc-deficient rats, glucose tolerance was abnormal and could be reversed by repletion of the metal ions. Finally, lower serum zinc

concentrations were detected in type 2 diabetic patients compared to healthy subjects [1,4], suggesting that zinc deficiency may be linked with insulin resistance.

Glycogen synthase kinase-3 is a serine/threonine kinase that was originally discovered by its ability to phosphorylate and inhibit the enzyme glycogen synthase [6,27]. It was first cloned in 1990 and shown to exist as two isoforms, α and β, sharing 98% homology in their catalytic domain and similar biochemical (but not identical) properties [29]. In recent years, it was realized that GSK-3 is a key component of intracellular signaling, and that it differs from other known intracellular protein kinases by being constitutively active in resting cells, and inhibited upon cell activation by extracellular signals (reviewed in [13,15]). It was also shown that GSK-3 activity is negatively regulated by insulin [8,28]. Thus, GSK-3 inhibition might be essential for normal function of the insulin-activated signaling pathway. Further support came from studies showing that GSK-3β limits insulin receptor-mediated signaling via the phosphorylation of its substrate IRS-1 [10]. The involvement of abnormal GSK-3β activity in type 2 diabetes was demonstrated in fat tissue of obese diabetic

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mice [11] and in skeletal muscle of type 2 diabetes patients, in which GSK-3 α and β activities were significantly higher than in healthy subjects [21]. This could fit well with the notion that elevated activity of GSK-3 presumably contributed to impairment of insulin action.

Here we report that GSK-3β is inhibited by physiological concentrations of Zn^{2+} and that their addition to cells produces intracellular effects similar to those achieved by GSK-3β inhibition. We propose that at least some of the in vivo actions of zinc, in particular, those related to its insulin-mimetic action, are mediated via inhibition of endogenous GSK-3β.

Materials and methods

In vitro assays. Purified recombinant rabbit GSK-3β [9] was incubated with peptide substrates PGS-1 (YRRAAVPPSPSLSRHSS PSQS(p)EDEEE) together with $ZnCl_2$ at the indicated concentrations. The reaction mixture included 50 mM Tris–HCl, pH 7.3, 10 mM MgAc, [γ-^{32}P]ATP (100 μM), and 0.01% β-mercaptoethanol and was incubated for 10 min at 30 °C. Reactions were spotted on phosphocellulose paper (p81), washed with 100 mM phosphoric acid, and counted for radioactivity as described [9]. For the initial velocity experiments, the enzyme was incubated with various concentrations of the p9CREB peptide substrate [26] with different concentrations of $ZnCl_2$ as indicated. Autophosphorylation of GSK-3 was determined by incubation of purified recombinant GSK-3 with varying Zn^{2+} concentrations without substrate. ^{32}P intensity was measured by exposure of polyacrylamide gel to PhosphoImager SI (Molecular Dynamics). The effect of Zn^{2+} on cdc2 kinase (New England Bio-labs, MA, USA) was tested. Cdc2 (1 U) was incubated with indicated concentrations of Zn^{2+}, Histone H1 (Calbiochem, CA, USA) substrate (5 μg), and reaction mixture containing [γ-^{32}P]ATP (Amersham, NJ, USA). The reactions were boiled with SDS sample buffer, separated on gel electrophoresis, and autoradiographed.

β-catenin analysis. HEK 293 cells grown in 10 cm plates were treated with the indicated concentrations of $ZnCl_2$ for 3 h. The cells were collected and extracted with buffer G (20 mM Tris, pH 7.3, 10 mM β-glycerophosphate, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 5 mM NaPPi, 25 μg/mL leupeptin, 25 μg/mL aprotinin, 500 nM microcystine LR, and 1% Triton X-100). Cell extracts were subjected to gel electrophoresis, followed by immunoblot analysis with monoclonal antibodies directed against β-catenin or GSK-3β (Transduction Laboratories, KY, USA).

Glycogen synthase assays. Glycogen synthase activity was assayed according to the method of Thomas et al. [25] and based on the incorporation of uridine 5′-diphosphate[^{14}C]glucose (UDPG) into glycogen. Cell lysates were prepared as previously described and aliquots (15 μL) were incubated with 15 μL reaction mixture (66.6 mM Tris, pH 7.8, 32.5 mM KF, and 0.8 μCi/μL [^{14}C]UDPG (Amersham, NJ, USA, 400 μM)) and 13 mg/mL glycogen rabbit liver (Sigma, MO, USA) for 20 min at 30 °C as described [9]. The reactions were then spotted on ET31 (Whatmann) papers, washed with 66% ice-cold ethanol, and counted for radioactivity. Glycogen synthase assays were measured in the presence of 0.1 mM glucose-6-phosphate (G6P).

Glucose uptake assay. Mice adipocytes were isolated from epididymal fat pads by digestion with 0.8 mg/mL collagenase (Worthington Biochemicals, USA) as described previously [17]. Digested fat pads were passed through nylon mesh and cells were washed 3 times with Krebs-bicarbonate buffer (pH 7.4) containing 0.5% bovine serum albumin (Fraction V, Boehringer Mannheim, Germany), 10 mM Hepes (pH 7.3), and 200 nM adenosine. Cells were incubated with $ZnCl_2$ at the indicated concentration for 1 h, followed by addition of 2-deoxy-D-[^{3}H]glucose (Amersham, NJ, USA, 0.5 μCi/vial) for 10 min. The assays were terminated by centrifugation of cells through dinonylphthalate (ICN, CA, USA) and [^{3}H] was quantitated by liquid scintillation analyzer (Packard).

Results

We tested the ability of Zn^{2+} ions to directly inhibit GSK-3β in vitro. The purified enzyme was incubated with various concentrations of $ZnCl_2$ and its ability to phosphorylate PGS-1 peptide substrate was determined. As shown in Fig. 1A, Zn^{2+} inhibited purified GSK-3β at low concentrations with $IC_{50} = 15$ μM, similar to mean human serum physiological concentrations [3]. We next examined whether Zn^{2+} ions inhibit the autocatalytic activity of GSK-3β. Autophosphorylation of GSK-3β was determined in the presence of varying Zn^{2+} concentrations (Fig. 1B). The results demonstrated that Zn^{2+} ions interact directly with the enzyme and inhibit its catalytic activity. We also tested the inhibitory effect of two divalent transition metal ions Co^{2+} and Mn^{2+} that have ionic radii similar to that of zinc on GSK-3β activity. Neither Co^{2+} nor Mn^{2+} inhibited GSK-3β at concentrations of 10, 20, 50, and 100 μM (data not shown). Thus, the inhibition of GSK-3β by Zn^{2+} represents distinct interaction between the protein kinase and zinc cations. In addition, we examined whether Zn^{2+} inhibits cycline-dependent protein kinase-cdc2, which is closely related to GSK-3β. The enzyme was incubated with various concentrations of $ZnCl_2$ and the phosphorylation of Histone H1 substrate was determined (Fig. 2). Zn^{2+} did not inhibit cdc2, indicating that the inhibition of GSK-3β by this metal ion is unique.

The kinetic nature of Zn^{2+} inhibition was studied by measuring the initial velocity as the function of substrate concentration at several Zn^{2+} concentrations. Lineweaver–Burk plot analyses (Fig. 3) indicate that Zn^{2+} ions act as uncompetitive inhibitors of GSK-3β, suggesting that they interact with the enzyme at a site distinct from its substrate recognition site. This uncompetitve nature is similar to that previously reported for inhibition of GSK-3β by lithium cations [16]. We next examined if exposure of intact cells to Zn^{2+} can produce consequence similar to GSK-3β inhibition. We examined the impact of Zn^{2+} on two known physiological substrates of GSK-3β; glycogen synthase and β-catenin. Inhibition of GSK-3β should increase glycogen synthase activity and lead to up-regulation of cytoplasmic β-catenin [22,30]. As shown in Fig. 4A, treatment of HEK 293 cells with 20 μM Zn^{2+} for 2 h enhanced glycogen synthase activity 2-fold. In addition, treatment of HEK 293 cells with Zn^{2+} increased cytoplasmic β-catenin levels as determined by Western blot analysis (Fig. 4B). These results support the notion that Zn^{2+} ions inhibit endogenous GSK-3β.
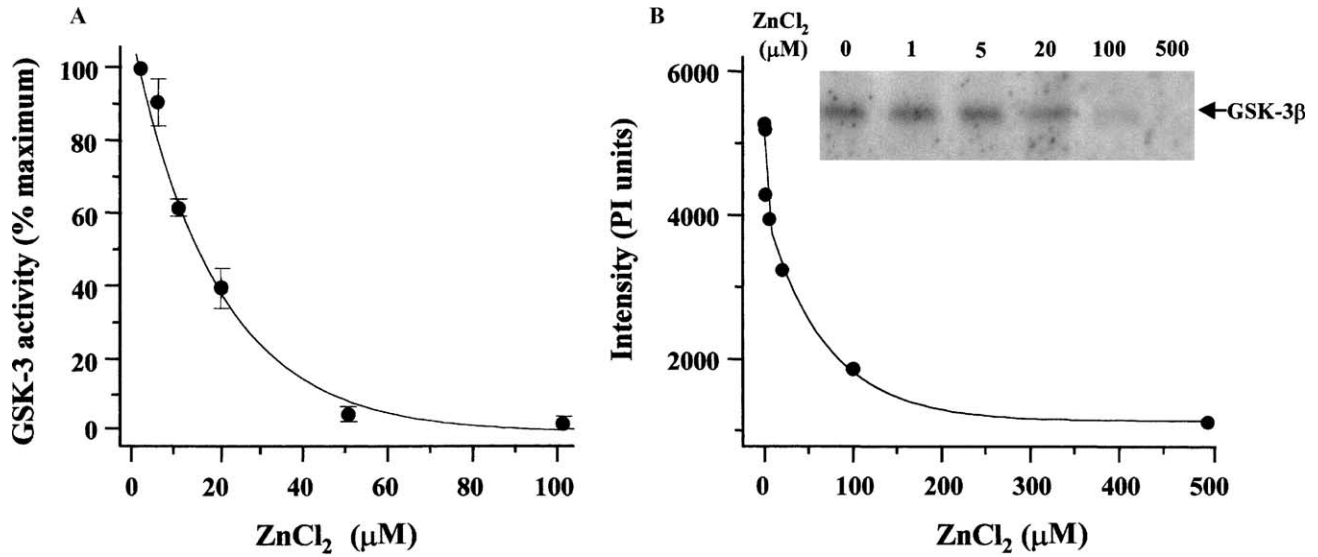
Fig. 1. Zinc ions inhibit GSK-3 activity. (A) The ability of purified GSK-3β to phosphorylate PGS-1 peptide substrate was measured in the presence of the indicated concentrations of Zn^{2+}. Results represent the percentage of maximum GSK-3β activity (designated 100%) in control incubation without zinc ions and are means of three independent experiments ± SEM, where each point was assayed in triplicate. (B) Autophosphorylation of purified GSK-3 was assayed in the presence of $ZnCl_2$ at indicated concentrations as described in Materials and methods. ^{32}P was measured by exposure of polyacrylamide gel to a PhosphoImager SI (Molecular Dynamics) and respective intensity units (PI units) are presented.
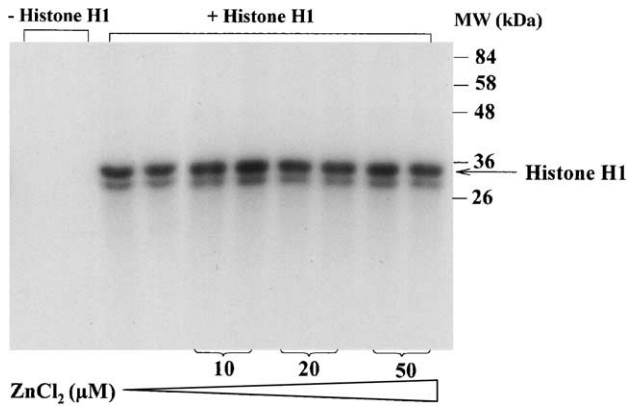

Fig. 2. Zinc does not inhibit cdc2 protein kinase. Cdc2 protein kinase activity was assayed with Histone H1 substrate with indicated concentration of $ZnCl_2$ as described in Materials and methods. Incorporation of ^{32}P into Histone H1 is presented.
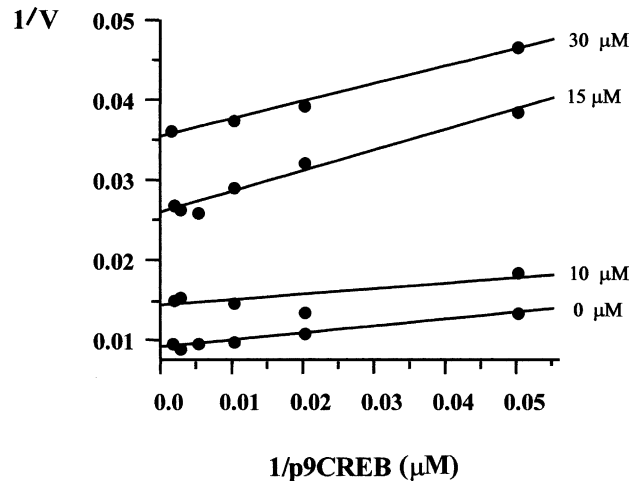

Fig. 3. Zinc is an uncompetitive inhibitor of GSK-3. Initial velocity of GSK-3β phosphorylation of p9CREB peptide was measured in the presence of $ZnCl_2$ at 0, 5, 15, and 30 μM. The data are shown as double-reciprocal plot. The experiments were repeated three times with similar results. Results represent one experiment where each point is a mean of a duplicated sample.

GSK-3β attenuates insulin signaling; thus, we hypothesized that Zn^{2+} should promote glucose uptake via its inhibitory effect on GSK-3β. It was previously shown that zinc activates glucose uptake in rat adipocytes, albeit, at relatively high non-physiological concentrations (1 mM) [12]. We examined the ability of Zn^{2+} to promote glucose uptake in mice adipocytes. Mice adipocytes were incubated with various concentrations of $ZnCl_2$ and glucose uptake was determined by the incorporation of 2-deoxy-D[^{3}H]glucose. As shown in Fig. 5, 10 μM Zn^{2+} increased hexose uptake 3-fold compared with non-treated cells. This activation was similar to that achieved by an optimal concentration of

insulin (10 nM, not shown). These studies also indicated that low concentrations of $ZnCl_2$ (10 μM) could activate glucose uptake in adipocytes. When we examined the effect of Zn^{2+} on glucose uptake in two cell lines, C6 rat glioma and GP8 rat brain endothelial cells, that express predominantly the glucose transporter-1 (GLUT1), we found that Zn^{2+} did not affect glucose transport in these cells (data not shown). These results suggested that Zn^{2+}-induced activation of glucose uptake in adipocytes
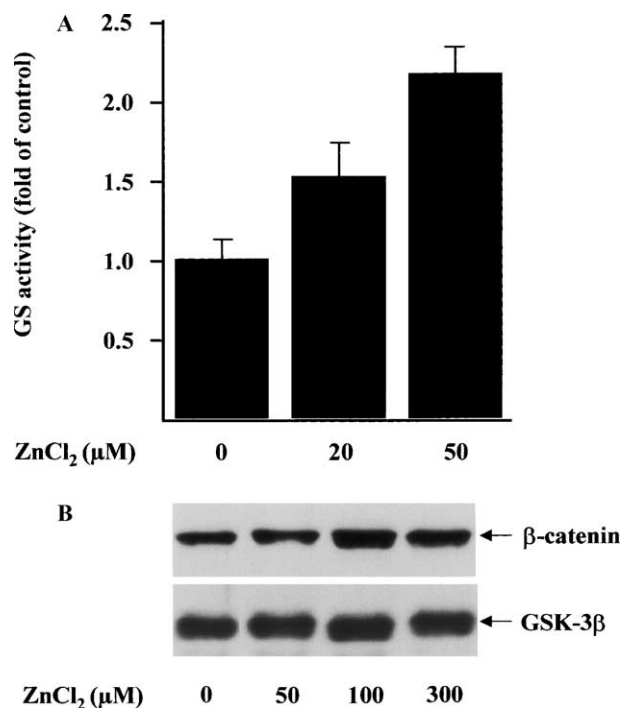
Fig. 4. Zinc inhibits endogenous GSK-3. (A) Glycogen synthase (GS) is activated by Zn^{2+}-HEK-293 cells were treated with Zn^{2+} for 2 h at 20 and 50 μM. Lysate supernatants were assayed for glycogen synthase activity as described in Materials and methods. The activity of glycogen synthase in non-treated cells was normalized to 1 U and the values for glycogen synthase activity observed in cells treated with zinc ions are presented as fold stimulation over the non-treated cells. Data are means of three independent experiments ± SEM where each point was assayed in duplicate. (B) β-Catenin levels are increased by Zn^{2+}-HEK293 cells were incubated with indicated concentrations of zinc ions for 3 h. Cytoplasmic cell extracts were subjected to gel electrophoresis, followed by immunoblot analysis with anti-β-catenin antibody. It is shown that β-catenin levels are increased in response to treatment with Zn^{2+} (upper panel). The membrane was stripped and re-probed with antibody against GSK-3β antibody (lower panel).
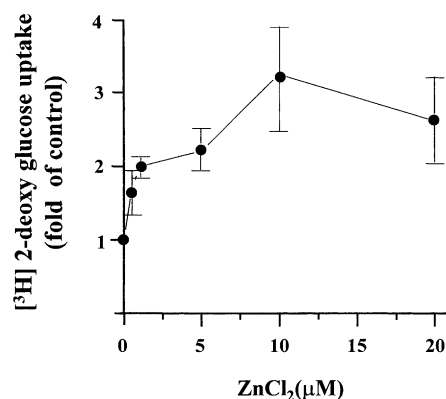

Fig. 5. Zinc ions mimic insulin action and increase glucose uptake. Adipocytes were isolated from mouse epididymal fat tissue and incubated with indicated concentrations of zinc ions for 1 h. Glucose uptake into cells was assayed with 2-deoxy-D[^{3}H]glucose as described in Materials and methods. The relative 2-deoxy-D[^{3}H]glucose incorporation observed in the control cells was normalized to 1 U and the values obtained for [^{3}H]2-deoxy glucose in zinc treated adipocytes are presented as fold activation over non-treated cells and are means of three independent experiments ± SEM, where each point was assayed in triplicate.

is most probably mediated through the insulin-sensitive transporter GLUT4.

Discussion

Zinc is involved in diverse cellular processes, both as an integral part of the active site of numerous enzymes (carboxypeptidases, metallopreteases, etc.) and as a regulatory factor. The insulin-mimetic function of zinc was demonstrated previously, though, the molecular target(s) modulated by zinc were not identified. In these studies, we undertook to investigate the role of zinc ions in the regulation of the protein kinase GSK-3β, a negative modulator of insulin action [10]. Here we show that zinc inhibits GSK-3β and suggest that its in vivo insulin-like effects are mediated via direct inhibition of endogenous GSK-3β.

The in vitro studies revealed that GSK-3β inhibition by physiological concentrations (∼15 μM) of Zn^{2+} is specific. In intact cells, zinc produced consequences similar to those achieved by inhibition of GSK-3β, namely, increased cytoplasmic β-catenine expression and activation of glycogen synthase, supporting the notion that it inhibits endogenous GSK-3β. The fact that zinc-induced glucose uptake gives further support for the insulin-mimetic action of Zn^{2+} and suggests that the inhibition of GSK-3β may be essential for insulin-induced glucose uptake. Our results correlate with studies done with lithium, another ion that is also an inhibitor of GSK-3β. Similar to Zn^{2+}, lithium ions were shown to increase β-catenin expression level, glycogen synthase activity, and glucose uptake in intact cells [5,24], suggesting a possible common mechanism for the insulin-mimetic action of these cations.

It is noteworthy that zinc deficiency, determined from its fasting serum concentrations, was reported for type 2 diabetes and for major depression patients [1,4,18]. The latter observation may be of particular clinical significance, since both lithium ions and valproic acid are widely used in the maintenance treatment of bipolar depression and shown to inhibit GSK-3β at therapeutic concentrations [16,31]. According to our present observations, it is plausible that inhibition of GSK-3β by dietary zinc could possibly allow clinical benefits similar to those achieved with lithium or valproic acid.

Altogether, the insulin-mimetic action of Zn^{2+} and the intriguing correlation between zinc deficiency in type 2 diabetes and in bipolar depression may be explained by its inhibitory action towards GSK-3β and may suggest a potential therapeutic value for dietary zinc supplementation in these chronic disorders.

Acknowledgments

References

[1] R.A. Anderson, A.M. Roussel, N. Zouari, S. Mahjoub, J.M. Matheau, A. Kerkeni, Potential antioxidant effects of zinc and chromium supplementation in people with type 2 diabetes mellitus, J. Am. Coll. Nutr. 20 (2001) 212–218.

[2] D. Beyersmann, H. Hasse, Functions of zinc in signaling, proliferation and differentionation of mammalian cells, Biometals 14 (2001) 331–341.

[3] K.H. Brown, Effect of infections on plasma zinc concentration and implications for zinc status assessment in low-income countries, Am. J. Clin. Nutr. 68 (Suppl. 2) (1988) 425S–429S.

[4] A.B. Chausmer, Zinc, insulin and diabetes, J. Am. Coll. Nutr. 17 (1998) 109–115.

[5] K. Cheng, S. Creacy, J. Larner, Insulin-like effect of lithium ion on isolated rat adipocytes stimulation of glycogenesis beyond glucose transport, Mol. Cell. Biochem. 56 (1983) 177–182.

[6] P. Cohen, Muscle glycogen synthase, in: P. Boyer, E.G. Krebs (Eds.), The Enzymes, vol. 17A, Academic Press, Orlando, FL, 1986, pp. 461–497.

[7] L. Coulston, P. Dandona, Insulin-like effect of zinc on adipocytes, Diabetes 29 (1980) 665–667.

[8] D.A. Cross, D.R. Alessi, J.R. Vandenheede, H.E. McDowell, H.S. Hundal, P. Cohen, The inhibition of glycogen synthase kinase-3 by insulin or insulin-like growth factor-1 in the rat skeletal muscle cell line L6 is blocked by wortmannin, but not by rapamycin: evidence that wortmannin blocks activation of the mitogen-activated protein kinase pathway in L6 cells between Ras and Raf, Biochem. J. 303 (1994) 21–26.

[9] H. Eldar-Finkelman, G.M. Agrast, O. Foord, E.H. Fischer, E.G. Krebs, Expression and Characterization of glycogen synthase kinase-3 mutants and their effect on glycogen synthase activity, Proc. Natl. Acad. Sci. USA 93 (1996) 10228–10233.

[10] H. Eldar-Finkelman, E.G. Krebs, Phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 impairs insulin action, Proc. Natl. Acad. Sci. 94 (1997) 9660–9664.

[11] H. Eldar-Finkelman, S.A. Schreyer, M.M. Shinohara, R.C. LeBoeuf, E.G. Krebs, Increased glycogen synthase kinase-3 activity in diabetes and obesity prone C57BL/6J mice, Diabetes 48 (1999) 1662–1666.

[12] O. Ezaki, IIb group metal ions (Zn^{2+}, Cd^{2+}, Hg^{2+}) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat adipocytes, J. Biol. Chem. 264 (1989) 16118–16222.

[13] S. Frame, P. Cohen, GSK-3 takes centre stage more than 20 years after its discovery, Biochem. J. 359 (2001) 1–16.

[14] L.A. Gaither, D.J. Eide, Eukaryotic zinc transporters and their regulation, Biometals 14 (2001) 251–270.

[15] C.A. Grimes, R.S. Jope, The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling, Prog. Neurobiol. 65 (2001) 391–426.

[16] P.S. Klein, D.A. Melton, A molecular mechanism for the effect of lithium on development, Proc. Natl. Acad. Sci. USA 93 (1996) 8455–8459.

[17] J.C. Lawrence, J.J. Guinovart, J. Larner, Activation of rat adipocyte glycogen synthase by insulin, J. Biol. Chem. 252 (1977) 444–450.

[18] M. Maes, N. De Vos, P. Demedts, A. Wauters, H. Neels, Lower serum zinc in major depression in relation to changes in serum acute phase proteins, J. Affect. Disord. 56 (1999) 189–194.

[19] W. Maret, Zinc biochemostry, physiology, and homeostasis-recent insights and current trends, Biometals 14 (2001) 187–190.

[20] J.M. May, C.S. Contoreggi, The mechanism of the insulin-like effects of ionic zinc, J. Biol. Chem. 257 (1982) 4362–4368.

[21] S.E. Nikoulina, T.P. Ciaraldi, S. Mudailar, P. Mohideen, L. Carter, R.R. Henry, Potential role of GSK-3 in skeletal muscle insulin resistance type 2 diabetes, Diabetes 49 (2000) 263–271.

[22] E. Siegfried, T.B. Chou, N. Perrimon, Wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate, Cell 71 (1992) 1167–1179.

[23] S.F. Simon, C.G. Taylor, Dietary zinc supplementation attenuates hyperglycemia in db/db mice, Exp. Biol. Med. 226 (2001) 43–51.

[24] V. Stambolic, L. Ruel, J.R. Woodgett, Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signaling in intact cells, Curr. Biol. 6 (1996) 1664–1668.

[25] J.A. Thomas, K.K. Schlender, J. Larner, A rapid filter paper assay for UDPglucose–glycogen glucosyltransferase, including an improved biosynthesis of UDP-14C-glucose, Anal. Biochem. 25 (1968) 486–499.

[26] M. Torres, H. Eldar-Finkelman, E.G. Krebs, R.T. Moon, Regulation of S6 ribosomal protein kinase p90RSK, glycogen synthase kinase-3 and beta-catenine in early Xenopus development, Mol. Cell. Biol. 19 (1999) 1427–1437.

[27] Y. Wang, P.J. Roach, Inactivation of rabbit muscle glycogen synthase by glycogen synthase kinase-3. Dominant role of the phosphorylation of Ser-640 (site-3a), J. Biol. Chem. 268 (1993) 23876–23880.

[28] G.I. Welsh, C.G. Proud, Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B, Biochem. J. 294 (1993) 625–629.

[29] J.R. Woodgett, Molecular cloning and expression of glycogen synthase kinase-3/factorA, EMBO J. 9 (1990) 2431–2438.

[30] C. Yost, M. Torres, J. Miller, E. Huang, D. Kimelman, R. Moon, The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase3, Genes Dev. 10 (1996) 1443–1454.

[31] P.X. Yuan, L.D. Huang, Y.M. Jiang, J.S. Gutkind, H.K. Manji, G. Chen, The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth, J. Biol. Chem. 276 (2001) 31674–31683.