Dietary Zinc Supplementation Attenuates Hyperglycemia in *db/db* Mice[1]

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Although zinc (Zn) deficiency has been associated with insulin resistance, and altered Zn metabolism (e.g., hyperzincuria, low-normal plasma Zn concentrations) may be present in diabetes, the potential effects of Zn on modulation of insulin action in Type II diabetes have not been established. The objective of this study was to compare the effects of dietary Zn deficiency and Zn supplementation on glycemic control in *db/db* mice. Weanling *db/db* mice and lean littermate controls were fed Zn-deficient (3 ppm Zn; dbZD and lnZD groups), Zn-adequate control (30 ppm Zn; dbC and lnC groups) or Zn-supplemented (300 ppm Zn; dbZS and lnZS groups) diets for 6 weeks. Mice were assessed for Zn status, serum and urinary indices of diabetes, and gastrocnemius insulin receptor concentration and tyrosine kinase activity. Fasting serum glucose concentrations were significantly lower in the dbZS group compared with the dbZD group (19.3 ± 2.9 and 27.9 ± 4.1 m*M*, respectively), whereas the dbC mice had an intermediate value. There was a negative correlation between femur Zn and serum glucose concentrations ($r = -0.59$ for lean mice, $P = 0.007$). The dbZS group had higher pancreatic Zn and lower circulating insulin concentrations than dbZC mice. Insulin-stimulated tyrosine kinase activity in gastrocnemius muscle was higher in the *db/db* genotype, and insulin receptor concentration was not altered. In summary, dietary Zn supplementation attenuated hyperglycemia and hyperinsulinemia in *db/db* mice, suggesting that the roles of Zn in pancreatic function and peripheral tissue glucose uptake need to be further investigated. [E.B.M. 2001, Vol 226:43–51]

Key words: zinc; hyperglycemic; insulin; tyrosine kinase; db/db mice

Insulin resistance, a primary characteristic of diabetes mellitus type II (DM-II), is defined as the decreased ability of insulin to act on peripheral tissues to stimulate glucose uptake and metabolism, and to inhibit hepatic glucose output (1). Insulin action is mediated by insulin binding to the extracellular α-subunit of its receptors, resulting in rapid autophosphorylation of intracellular tyrosine residues on the β-subunit and activation of the insulin receptor tyrosine kinase for phosphorylation of various intermediates of the insulin signal transduction cascade (1). Autophosphorylation and activation of the insulin receptor tyrosine kinase appears to be crucial for many of the biological effects of insulin (1). In humans with DM-II, decreased insulin-stimulated kinase activity has been reported in skeletal muscle (2, 3), adipocytes (4–6), hepatocytes (7), and erythrocytes (8); however, quantitatively, skeletal muscle is the most important site of insulin resistance (9).

Zinc (Zn) has been linked to insulin resistance in several studies. Zn-deficient rats are resistant to exogenous insulin injections (10) and have decreased glucose turnover during a euglycemic hyperinsulinemic glucose clamp (11). Zn-deficient rats have impaired glucose tolerance curves compared with pair-fed controls when glucose is administered by the intravenous or intraperitoneal route (10, 12, 13). Similarly, force-fed Zn-deficient rats (to control for reduced feed intake and altered eating patterns) have impaired glucose tolerance curves despite elevated blood insulin concentrations, normal glucagon concentrations, and normal pancreatic histology (14). These results suggest that pancreatic insulin secretion is not the limiting factor and indicate an involvement of peripheral insulin resistance. The abnormal glucose tolerance of Zn-deficient rats can be reversed after 1 week of Zn repletion without an increase in caloric intake (12), suggesting that Zn *per se* plays an important role in insulin action.

Several potential mechanisms have been suggested for Zn affecting insulin action, including a role for Zn in modulation of insulin receptor tyrosine kinase activity. Zn has been shown to enhance tyrosine kinase phosphorylation compared with other cations (15). In rat adipocytes, addition of Zn *in vitro* (100 μ*M*) increased autophosphorylation of the insulin receptor 2-fold and increased insulin-dependent phosphorylation of the insulin receptor substrate-1 5-fold (16). However, there is a lack of information on the effects of Zn nutrition on insulin receptor phosphorylation activity.

The purpose of the present investigation was to determine if Zn status plays a role in modulating insulin receptor tyrosine kinase activity in skeletal muscle in a genetic mouse model of DM-II. We chose *db/db* mice (mutation in leptin receptor gene) for our model of obesity and diabetes

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because their metabolic profile (i.e., hyperinsulinemia, hyperglycemia, hyperleptinemia, and obesity) more closely resembles DM-II than the hypoleptinemia of *ob/ob* mice (mutation in leptin gene) (17). Others have demonstrated that *db/db* mice fed a Zn-deficient diet (1 ppm Zn for 4 weeks) have elevated fasting blood glucose concentrations (18), and that *ob/ob* mice fed very high levels of Zn (1000 ppm Zn for 4 weeks) or given supplemental Zn in the drinking water have significantly improved hyperglycemia and hyperinsulinemia (19, 20); however, dietary Zn deficiency and supplementation have not been compared within the same animal model. To test our hypothesis that dietary Zn supplementation would attenuate diabetic signs, we felt that a preventative dietary approach should be initiated at the weanling age and continued for a period of 6 weeks to coincide with the period of peak hyperinsulinemia and hyperglycemia in *db/db* mice (21). The levels of Zn for the deficient and supplemented diets, 3 ppm and 300 ppm, respectively, were chosen to induce a marginal Zn deficiency in young mice and to minimize potential adverse effects of high dietary Zn on copper status (22). Thus, the objectives of the study were (1) to compare the effects of dietary Zn supplementation and Zn deficiency on hyperglycemia, hyperinsulinemia, and other diabetic parameters in *db/db* mice, and (2) to determine if dietary Zn supplementation or Zn deficiency modulates insulin receptor tyrosine kinase activity in gastrocnemius muscle from *db/db* mice.

Materials and Methods

Animals and Diet. Female 4-week-old weanling C57BL/KsJ (BL/Ks) homozygous diabetic (*db/db*) and heterozygous lean (*db/m* or "ln") control mice (Jackson Laboratory, Bar Harbor, ME) were fed laboratory chow for a 5-day adaptive period. The *db/db* and ln mice were then randomly assigned to diets with low (3 ppm), adequate (30 ppm), or supplemented (300 ppm) zinc for 6 weeks. The diet formulation based on the AIN-93G diet has been previously published (23). The mice were housed separately in stainless steel hanging cages and given free access to diet and to deionized water in polypropylene bottles with stainless steel sipper tubes. The mice were maintained in a controlled environment of 21°–23°C, 55% humidity, and 14:10-hr light:dark cycle. The mice were weighed weekly and were sacrificed at Week 6 after an overnight fast (12 hr) in polycarbonate metabolic cages (Nalgene, Fisher Scientific, Mississauga, ON) for the purpose of urine collection. The mice were given free access to water, but not feed, to ensure that urine samples were not contaminated by zinc from the diets. A protocol for animal care procedures was approved by the University of Manitoba Protocol Management and Review Committee.

Tissue Collection. Animals were sacrificed at Week 6 by CO_2 asphyxiation, and trunk blood was collected after decapitation; blood samples were stored on ice until centrifuged to obtain serum. The liver, pancreas, kidneys, auxiliary and inguinal region fat pads, gastrocnemius muscles, and hind quarters were excised and immediately frozen in liquid nitrogen. All tissue samples, urine, and serum were stored at −80°C until analyzed.

Biochemical Analyses. Glucose in the serum and urine was assessed using an enzymatic colorimetric kit (Procedure #315, Sigma, St. Louis, MO) based on the work of Trinder (24). Insulin in the serum was assayed using a sensitive rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO). Protein concentrations in the gastrocnemius muscle and urine were determined using the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford IL). Urinary creatinine was assayed by a colorimetric assay (Procedure #555, Sigma, St. Louis, MO) based on the method of Heinegard and Tiderstrom (25). The method of Folch (26) was used to quantitate total lipid content in the liver as previously described (23). After appropriate dilution of digested tissue samples (27), serum, and urine, Zn was analyzed by atomic absorption spectrophotometry (Varian Spectra AA-30 Spectrophotometer, Georgetown, ON), and femur calcium and phosphorus by emission spectrometry (Varian Liberty 200 ICP, Varian Canada, Georgetown, ON).

Insulin Receptor Concentration/Tyrosine Kinase Phosphorylation. Insulin receptor concentrations and tyrosine kinase activity were assessed in the gastrocnemius muscle, a primary site for glucose disposal. The procedure initially involves the isolation and immobilization of insulin receptors from total muscle homogenate by means of an anti-insulin receptor antibody bound to 96-well plates. Tyrosine kinase phosphorylation activity of the isolated receptors is determined by ^{32}P-ATP phosphorylation of an exogenous substrate (polyGLUTYR) under both basal and insulin-stimulated conditions. The isolated receptors are also used to determine the insulin receptor concentration per mg protein of muscle homogenate as the amount of ^{125}I-insulin bound is directly proportional to the concentration of receptors in the sample. These methods are adapted from Klein *et al.* (28) and Hamann *et al.* (29). All chemicals are reagent grade (Sigma Chemical Co., St. Louis, MO) unless otherwise indicated.

In preparation for insulin receptor immobilization, 50 µl IgG (Oncogene Science, Uniondale, NY, 2 µg/ml in 50 m*M* phosphate buffer) was pipetted onto 96-well easy-wash ELISA grade polystyrene plates (#25805–96, Corning Glass Works, Corning, NY). Plates were covered, incubated at 37°C for 2 hr, washed three times with deionized water, and inverted to blot dry. The plates were incubated with 50 µl of anti-Insulin Receptor antibody (AB-3, Oncogene Science, Uniondale, NY, 1 µg/ml), covered, and incubated overnight.

On Day 2, gastrocnemius muscle was homongenized (Potter-Elvejhem Homogenizer, Wiarton, ON) in ice-cold Buffer A (8 µl/mg muscle tissue; 1% Triton X-100, 2.5 m*M* phenylmethylsulfonylfluoride, 800 trypsin U/ml aprotinin, 8 m*M* EDTA, 2.5 mg/ml benzamidine, 2.5 µg/ml pepstatin, 2.5 µg/ml leupeptin, 160 m*M* NaF, 10 m*M* Na pyrophosphate, 0.2 m*M* Na vanadate, 2 m*M* dichloroacetic acid, and

20 m*M* HEPES, pH 7.4) (28). After 30 min at 4°C, samples were centrifuged at 15,000*g* for 20 min at 4°C to remove insoluble materials. In preparation for plating tissue lysate, the 96-well plates were washed three times with deionized water to remove excess antibody. Plates for the tyrosine kinase assay and the insulin binding assay were inverted to blot dry, and 40-μl aliquots of muscle lysate were pipetted into wells at 4°C. Insulin (final concentration of 10^{-7} *M*) was added to appropriate wells for tests of insulin-stimulated tyrosine kinase phosphorylation activity. Plates were covered and incubated overnight at 4°C.

Before initiating the tyrosine kinase procedure, wells containing lysate were washed five times with ice-cold Buffer B (0.05% Triton X-100, 50 m*M* NaCl, 1.2 m*M* KCl, 0.5 m*M* $CaCl_2$ H_20, 10 m*M* HEPES, 10% glycerol, 10% Na azide, and 0.5% bovine serum albumin) (29) and inverted to blot dry. The exogenous substrate, 20 μl polyGLUTYR [poly(glutamate:tyrosine 4:1) 4 mg/ml in water], was added to the washed plates, and the reaction was initiated with the addition of 20 μl/well reaction buffer (Buffer B with the addition of 5 m*M* $MnCl_2$, 10 m*M* $MgCl_2$, 0.5 μ*M* ATP, and ^{32}P ATP [3000 Ci/mmol (New England Nuclear, Dorval, PQ); 1,000,000 cpm/well]. After 15 min, the reaction was terminated by spotting 35-μL aliquots onto 2 × 2-cm squares of Whatman 3MM filter paper. Filter papers were allowed to dry for 5 min, washed in 10% trichloroacetic acid and 10 m*M* Na pyrophosphate three times for 15 min, immersed in 100% ethanol for 5 min, and dried before liquid scintillation counting (Beckman LS 6000TA, Beckman Instruments, Mississauga, ON). Radioactivity incorporated into substrate was corrected for blanks and for variation in protein concentration.

For the insulin binding assay, plates previously prepared with 40-μl aliquots of muscle lysate were washed 5 times in ice-cold buffer B and incubated overnight at 4°C with 40 μl/well Buffer B [with the addition of ^{125}I-insulin (2200 Ci/mmol, New England Nuclear, Dorval, PQ) 30,000 cpm/well] (29). Plates were washed 5 times with Buffer B to remove unbound insulin. The ^{125}I-insulin that had bound to the immobilized receptors was collected by adding 100 μl of a 10% solution of sodium dodecyl sulfate (SDS) twice for 15 min. Radioactivity was quantitated by gamma counting (Beckman Gamma 8000 Scientific Instruments, Irvine, CA). Samples were corrected for blanks and for variation in protein concentration.

Statistical Analysis. ANOVA (SAS 6.04, SAS Institute, Cary, NC) was used to determine significant differences between main effects (diet, genotype, and diet × genotype interaction). Duncan's multiple range test was used for means testing. Correlations were analyzed using Spearman's Correlation Coefficient. Differences were accepted as significant at $P < 0.05$.

Results

Body and Organ Weights. The effects of genotype [*db/db* (diabetic) or *db/m*(lean)] and dietary zinc treatment [ZD = zinc-deficient, C = zinc-adequate control or ZS = zinc-supplemented] on body weight are shown in Figure 1. At Week 0, the *db/db* mice weighed significantly more than the *db/m* lean (ln) mice (21.6 ± 0.5 vs. 15.9 ± 0.4 g, respectively). From Week 2 to Week 6, the dbC mice weighed significantly more than the dbZD and dbZS mice. At the end of the 6-week study, dbC mice weighed 33.8 ± 1.0 g compared with 29.0 ± 1.9 g for the dbZD group and 29.5 ± 1.0 g for the dbZS group. Throughout the study, the lean (*db/m*) mice weighed significantly less than the *db/db* mice, but there were no differences due to dietary zinc treatment in the lean mice.

The effects of genotype and dietary zinc treatment for 6 weeks on organ weights and liver lipid are shown in Table I. Fat pad weights were measured as an indication of obesity. The *db/db* mice had significantly higher fat pad weights and relative fat pad weights compared with the lean genotype. Dietary treatment did not influence fat pad weight in lean mice; the fat pad weight of the dbC group was significantly greater than the dbZD group but not different from the dbZS group. Although liver weights were significantly higher in *db/db* mice than lean mice (1.36 ± 0.04 vs. 0.94 ± 0.02 g, respectively), they were proportional to body weight as indicated by the similar relative liver weights among the treatment groups. The dbC and dbZS groups had significantly lower liver lipid concentrations (mg/g liver) than dbZD and the lean groups; however, when expressed as liver lipid content (mg/liver), there were no significant dif-

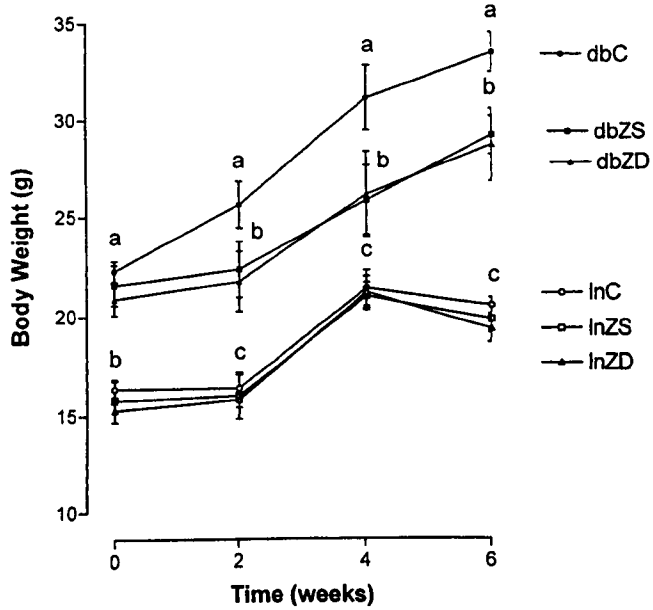

Figure 1. Effects of dietary zinc treatment on body weights of *db/db* and lean mice during a 6-week period. Data points are means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet (closed triangle), dbC = *db/db* control diet (closed circle), dbZS = *db/db* zinc-supplemented diet (closed square), lnZD = *db/m* zinc-deficient diet (open triangle), lnC = *db/m* control diet (open circle), lnZS = *db/m* zinc-supplemented diet (open square).

Table I. Effects of Dietary Zinc Treatment for 6 Weeks on Organ Weights and Liver Lipid in *db/db* and Lean Mice

	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Fat pad wt* (g)	4.64 ± 0.52^{a}	5.99 ± 0.42^{b}	$5.01 \pm 0.35^{a,b}$	1.01 ± 0.33^{c}	0.98 ± 0.12^{c}	1.16 ± 0.21^{c}
Relative fat pad wt (g/100 g body wt)	15.8 ± 1.1^{a}	17.7 ± 1.1^{a}	16.9 ± 0.8^{a}	4.9 ± 1.6^{b}	4.7 ± 0.6^{b}	5.7 ± 1.0^{b}
Liver wt (g)	1.28 ± 0.07^{a}	1.49 ± 0.07^{b}	1.32 ± 0.04^{a}	0.90 ± 0.04^{c}	0.97 ± 0.02^{c}	0.96 ± 0.07^{c}
Relative liver wt (g/100 g body wt)	4.5 ± 0.2	4.4 ± 0.3	4.5 ± 0.2	4.6 ± 0.1	4.7 ± 0.1	4.8 ± 0.2
Liver lipid concentration (mg/g)	$113.4 \pm 16.5^{a,b}$	96.5 ± 10.2^{b}	98.6 ± 9.7^{b}	139.1 ± 0.9^{a}	137.4 ± 1.0^{a}	142.4 ± 0.1^{a}
Liver lipid content (mg/liver)	141.3 ± 15.8	146.5 ± 21.8	130.2 ± 13.2	125.4 ± 15.5	133.1 ± 7.6	136.8 ± 14.5
Pancreas wt (mg dry wt)	64.2 ± 3.2^{a}	$53.9 \pm 5.0^{a,b}$	$52.3 \pm 5.6^{a,b}$	35.2 ± 3.6^{c}	$41.5 \pm 5.6^{b,c}$	$53.7 \pm 6.4^{a,b}$

Note. Values are means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Significant ($P < 0.05$) main effects for genotype: fat pad weight, relative fat pad weight, liver weight, liver lipid concentration, pancreas weight; for diet: liver weight; and for genotype x diet interaction: pancreas weight. Superscript letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet.
* Auxiliary and inguinal fat pads.

ferences. Pancreas dry weight did not differ among the *db/db* mice; however, lnZD mice had a significantly lower pancreas weight than lnZS mice.

Tissue Mineral Concentrations. The Zn concentration in the pancreas of *db/db* mice was significantly lower than lean mice (96.8 ± 5.3 vs. 119.4 ± 5.4 μg/g dry weight, respectively) (Fig. 2). The dbC group had a significantly lower pancreatic Zn concentration than the lnC group, and the marginal Zn-deficient diet did not further alter pancreas Zn concentration in either *db/db* or lean mice. Zn supplementation restored pancreatic Zn concentrations in *db/db* mice to lean values; the pancreatic Zn concentration of the dbZS group was not significantly different from lnZD, lnC, or lnZS mice. The higher pancreatic Zn concentration in the dbZS group was due to a higher Zn content, not differences in pancreas weight (pancreas dry weight was similar to *db/db* mice and the lnC group) (Table I).

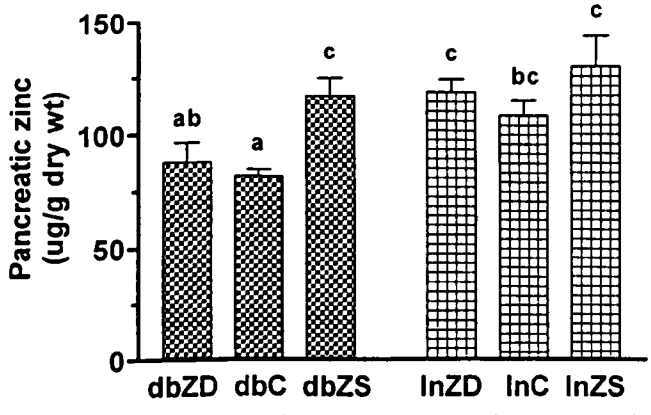

Figure 2. Effects of dietary zinc treatment for 6 weeks on pancreatic zinc concentration of *db/db* and lean mice. Columns represent means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Significant ($P < 0.05$) main effects were genotype and diet. Different lowercase letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet.

Femur Zn concentrations were used as an indicator of long-term Zn status and Zn nutriture (Table II).The dbZS group had significantly higher femur Zn concentrations than dbZD and dbC groups, and the lnZS group had significantly higher femur Zn concentrations than the lnZD group. The marginal Zn-deficient diet employed in this study resulted in dbZD and lnZD mice having 8% and 17% lower femur Zn concentrations than dbC and lnC mice, respectively, but this did not reach statistical significance. Genotype had a significant effect on femur weight (18.0 ± 0.4 vs 22.9 ± 0.4 mg for *db/db* and lean mice, respectively) but no significant effect on femur Zn concentrations (259 ± 12 vs 264 ± 11 μg/g dry weight for *db/db* and lean mice, respectively). However, femur calcium and phosphorus concentrations were significantly different in the diabetic versus lean genotype (221 ± 7 vs 241 ± 3 μg/g dry weight, respectively, for calcium; 112 ± 4 vs 127 ± 2 μg/g dry weight, respectively, for phosphorus). Both calcium and phosphorus concentrations in femur were lowest in the dbZD group and highest in the lnZD group. Dietary Zn treatment did not have a significant effect on femur calcium and phosphorus concentrations. Kidney Zn and liver copper concentrations were not significantly different among the groups.

Serum and Urine Indices of Diabetes. The *db/db* mice had significantly higher serum glucose concentrations compared with lean mice (23.1 ± 2.1 vs 10.2 + 1.0 m*M*, respectively) (Fig. 3a). Zn-supplemented *db/db* mice (dbZS) had a significantly lower serum glucose concentration than the dbZD group. This trend of lower serum glucose concentration with higher Zn in the diet was also observed in the lean mice but did not reach statistical significance. There

Table II. Effects of Dietary Zinc Treatment for 6 Weeks on Femur, Kidney, and Liver Mineral Concentrations in *db/db* and Lean Mice

	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Femur zinc (μg/g dry wt)	224 ± 12^{a}	243 ± 8^{a}	311 ± 19^{c}	219 ± 15^{a}	$263 \pm 17^{a,b}$	$304 \pm 11^{b,c}$
Femur calcium (μg/g dry wt)	211 ± 1^{b}	$224 \pm 10^{a,b}$	$229 \pm 16^{a,b}$	251 ± 9^{a}	$238 \pm 3^{a,b}$	$233 \pm 3^{a,b}$
Femur phosphorus (μg/g dry wt)	108 ± 5^{a}	$113 \pm 5^{a,b}$	$114 \pm 8^{a,b}$	132 ± 5^{c}	$125 \pm 1^{b,c}$	$123 \pm 1^{a,b,c}$
Femur weight (mg)	17.5 ± 0.9^{a}	18.6 ± 0.5^{a}	18.0 ± 0.5^{a}	22.3 ± 0.5^{b}	24.4 ± 0.6^{c}	22.3 ± 0.6^{b}
Kidney zinc (μg/g dry wt)	73.7 ± 8.9	127.1 ± 24.5	90.0 ± 11.9	87.0 ± 4.1	81.8 ± 6.5	76.3 ± 3.0
Liver copper (μg/g dry wt)	15.6 ± 0.9	15.8 ± 0.9	16.1 ± 0.6	14.6 ± 0.8	15.3 ± 0.4	16.5 ± 2.0

Note. Values are means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Significant ($P < 0.05$) main effects for genotype: femur calcium, femur phosphorus, femur weight, and for diet: femur zinc and femur weight. Superscript letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet.

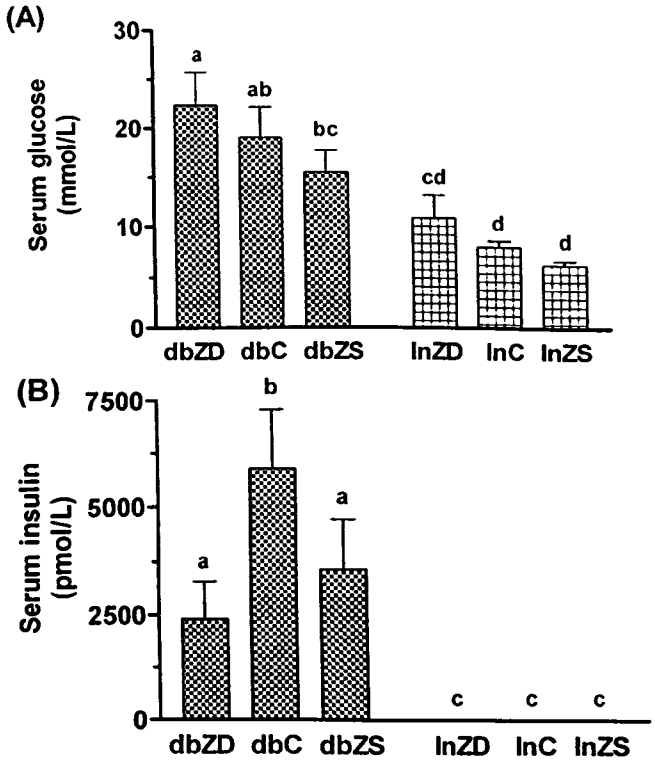

Figure 3. Effects of dietary zinc treatment for 6 weeks on (A) serum glucose concentration and (B) serum insulin concentration of *db/db* and lean mice. Columns represent means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Significant ($P < 0.05$) main effects were genotype: serum glucose and serum insulin, and diet: serum glucose. Different lowercase letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet. The values for serum insulin for the lean genotype were lnZD = 15 ± 2, lnC = 19 ± 2, and lnZS = 20 ± 3 p*M* and are not visible as bars on the graph.

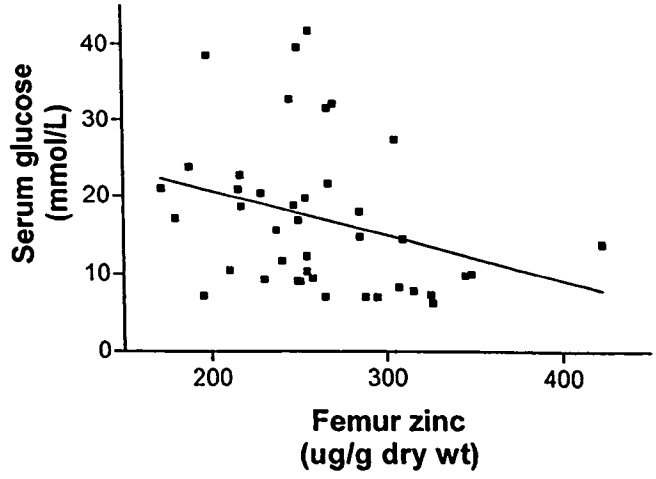

Figure 4. Scatter plot of femur zinc concentration versus serum glucose concentration. Data points are the values obtained from individual mice, $n = 41$; there was a significant negative correlation ($r = -0.38$, $P = 0.015$). Also, there was a significant negative correlation between serum glucose and femur Zn concentrations for lean mice ($r = -0.59$, $P = 0.007$, $n = 19$).

was a significant negative correlation between serum glucose and femur Zn concentrations for lean mice ($r = -0.59$, $P = 0.007$, $n = 19$) and for all mice in the study ($r = -0.38$, $P = 0.015$, $n = 41$; Fig. 4). Serum insulin concentrations were 100–275-fold higher in *db/db* vs lean mice (2853 ± 473 vs 18 ± 1 p*M*, respectively) (Fig. 3b). Serum insulin concentrations were significantly lower in both dbZD and dbZS mice compared with the dbC group.

There was a significant effect of genotype on urinary creatinine excretion (61.4 ± 0.8 vs 105.9 ± 10.7 μg/12 hr for *db/db* and lean mice, respectively); thus, the urinary data are reported per 12 hr and per mg creatinine (Table III). The *db/db* mice excreted approximately twice as much Zn as the lean mice over a 12-hr period, but they had significantly less urinary Zn excretion when expressed per mg creatinine (0.012 ± 0.004 and 1.055 ± 0.400 μg Zn/mg creatinine for *db/db* and lean mice, respectively). Urinary glucose excretion was significantly higher in *db/db* mice compared with lean mice. The dbZD group had significantly less urinary glucose excretion than the dbC group per 12 hr but not per mg creatinine. There were no differences in urinary protein excretion over 12 hr. The dbZD and dbZS groups excreted less protein than the dbZC mice when corrected for creatinine excretion.

Insulin Receptor Concentration and Tyrosine Kinase Phosphorylation. Insulin receptor concentrations were not significantly different due to genotype or diet

Table III. Effects of Dietary Zinc Treatment for 6 Weeks on Urinary Excretion of Creatinine, Zinc, Glucose, and Protein in *db/db* and Lean Mice

	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Urine creatinine (μg/12 hr)	$73 \pm 12^{a,b}$	40 ± 12^{a}	$77 \pm 10^{a,b}$	124 ± 27^{b}	115 ± 15^{b}	$78 \pm 19^{a,b}$
	$n = 6$	$n = 6$	$n = 4$	$n = 6$	$n = 7$	$n = 6$
Urine Zn (μg/12 hr)	0.76 ± 0.10^{a}	$0.74 \pm 0.14^{a,b}$	0.80 ± 0.22^{a}	$0.38 \pm 0.08^{b,c}$	$0.39 \pm 0.05^{b,c}$	0.35 ± 0.12^{c}
	$n = 7$	$n = 5$	$n = 5$	$n = 6$	$n = 7$	$n = 5$
Urine Zn (μg/mg creatinine)	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.41 ± 0.11	1.27 ± 0.55	1.53 ± 1.27
	$n = 6$	$n = 5$	$n = 5$	$n = 6$	$n = 7$	$n = 5$
Urine glucose (mg/12 hr)	$5.4 \pm 1.7^{a,b}$	25.6 ± 11.1^{c}	$18.9 \pm 3.9^{b,c}$	0.1 ± 0.0^{a}	0.1 ± 0.1^{a}	0.1 ± 0.0^{a}
	$n = 7$	$n = 7$	$n = 8$	$n = 6$	$n = 7$	$n = 5$
Urine glucose (mg/mg creatinine)	$96 \pm 36^{a,b}$	548 ± 346^{a}	$210 \pm 108^{a,b}$	1.2 ± 0.3^{b}	0.9 ± 0.4^{b}	2.1 ± 0.8^{b}
	$n = 6$	$n = 6$	$n = 4$	$n = 6$	$n = 7$	$n = 5$
Urine protein (mg/12 hr)	1.63 ± 0.31	3.28 ± 1.32	1.79 ± 0.43	3.51 ± 1.11	4.69 ± 0.83	3.17 ± 1.06
	$n = 6$	$n = 5$	$n = 7$	$n = 6$	$n = 7$	$n = 5$
Urine protein (mg/mg creatinine)	24.5 ± 5.1^{b}	86.2 ± 37.4^{a}	25.0 ± 5.5^{b}	26.4 ± 3.1^{b}	$39.3 \pm 2.9^{a,b}$	$42.8 \pm 5.1^{a,b}$
	$n = 5$	$n = 5$	$n = 5$	$n = 6$	$n = 7$	$n = 5$

Note. Values are means ± SEM. Significant ($P < 0.05$) main effects for genotype: urine creatinine (μg/12 hr), urine zinc (μg/12 hr, and μg/mg creatinine), urine glucose (mg/12 hr and mg/mg creatinine), and for diet: urine protein (mg/mg creatinine). Superscript letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet.

(Table IV). Basal and total insulin-stimulated tyrosine kinase activity in gastrocnemius muscle was not significantly different. However, when expressed as the difference between the basal and insulin-stimulated state, there was a significant genotype effect and significantly higher tyrosine kinase activity in the dbC group compared with the dbZS, lnC, and lnZS groups. The dbZD and lnZD groups had an intermediate tyrosine kinase activity in gastrocnemius muscle.

Discussion

The major finding of the present study was that dietary Zn supplementation attenuated fasting hyperglycemia whereas a marginally Zn-deficient diet exacerbated fasting hyperglycemia in *db/db* mice (Fig. 3). The *db/db* genotype (mutation in leptin receptor gene) is characterized by hyperglycemia, hyperinsulinemia, hyperleptinemia, and obesity. Dietary Zn supplementation of weanling *db/db* mice for 6 weeks (dbZS group) resulted in lower fasting serum glucose concentrations (19%), lower fasting serum insulin concentrations (60%), and lower body weight (13%) than *db/db* mice fed the Zn-adequate diet (dbC group); these results indicate an improvement in glycemic control and delayed progression of diabetes (i.e., less weight gain in young *db/db* mice). The dbZS mice had higher pancreatic Zn concentrations (44%) and lower circulating insulin concentrations (60%) than dbC mice, suggesting a possible association between Zn supplementation and improved pancreatic β-cell function and/or improved peripheral insulin sensitivity. Similar results have been reported in another obese diabetic animal model, the *ob/ob* mouse (hypoleptinemia due to mutation in leptin gene). Very high dietary Zn supplementation (1000 ppm for 4 weeks) of *ob/ob* mice attenuated fasting hyperglycemia and fasting hyperinsulinemia, elevated insulin content of pancreatic islets, and modulated the abnormally high insulin secretory response

Table IV. Effects of Dietary Zinc Treatment for 6 Weeks on Insulin Receptor Concentration, Basal and Insulin-Stimulated Tyrosine Kinase Phosphorylation In Gastrocnemius Muscle from *db/db* and Lean Mice

	dbZD	dbC	dbZS	lnZD	lnC	lnZS
Insulin receptor concentration (cpm/mg protein)	674 ± 89	746 ± 104	744 ± 80	848 ± 106	667 ± 129	457 ± 117
Basal tyrosine kinase phosphorylation (fmol/min/mg)	0.91 ± 0.24	0.88 ± 0.18	1.41 ± 0.24	1.43 ± 0.38	1.33 ± 0.25	1.09 ± 0.25
Total tyrosine kinase phosphorylation (fmol/min/mg)	2.50 ± 0.27	3.06 ± 0.43	2.32 ± 0.29	2.66 ± 0.35	2.00 ± 0.33	1.85 ± 0.33
Insulin-stimulated tyrosine kinase phosphorylation (fmol/min/mg)	$1.59 \pm 0.12^{a,b}$	2.18 ± 0.46^{a}	0.92 ± 0.22^{b}	$1.27 \pm 0.50^{a,b}$	0.92 ± 0.30^{b}	0.76 ± 0.16^{b}

Values are means ± SEM, $n = 7$ except for dbZS, lnZD, and lnZS where $n = 8$, 6, and 6, respectively. Significant ($P < 0.05$) main effects were genotype for insulin-stimulated tyrosine kinase phosphorylation. Superscript letters indicate significant differences ($P < 0.05$) between means. dbZD = *db/db* zinc-deficient diet, dbC = *db/db* control diet, dbZS = *db/db* zinc-supplemented diet, lnZD = *db/m* zinc-deficient diet, lnC = *db/m* control diet, lnZS = *db/m* zinc-supplemented diet.

of pancreatic islets to glucose *in vitro* (19). We used a lower level of Zn supplementation (300 ppm for 6 weeks) to minimize potential adverse effects on copper metabolism (22) and verified that liver copper concentrations were unchanged (Table II). Also, the dietary Zn supplementation did not affect growth in the lean genotype (Fig. 1) or Zn concentrations in the kidney (Table II).

In contrast to Zn supplementation, *db/db* mice fed the low-Zn diet (dbZD group) had higher serum fasting glucose (17%) and lower serum fasting insulin (63%) concentrations than *db/db* mice fed the Zn-adequate diet (dbC group). With a Zn deficiency study, there are concerns about how feed intake (anorexia) and meal patterns (length of fasting before blood collection) may be affecting serum concentrations of glucose and serum insulin. Unfortunately, we could not obtain accurate measurement of feed intake in this study due to the considerable spillage of powdered diet by mice on all dietary treatments. However, using a paste diet, we have found that feed intake was not different among weanling mice consuming 3 ppm, 30 ppm, or 300 ppm Zn diets for 6 weeks (unpublished data). If feed restriction were a factor, it would be expected to lower both serum fasting glucose and insulin (13), but in the present study, the fasting serum glucose concentration was highest in the dbZD group. A meal-feeding or intragastric force-feeding design would be required for strict control of the amount of feed consumption and the length of time between feed consumption and blood collection (14). Significantly higher fasting blood glucose concentrations in *db/db* mice fed a Zn-deficient diet (1 ppm Zn for 4 weeks) have also been reported by Southon *et al.* (18). These results suggest that low dietary Zn intake leads to a worsening of the diabetic condition that could be due to decreased glucose uptake by peripheral tissues and/or decreased insulin availability. In Zn-deficient rats, glucose intolerance (10, 12, 13) has been attributed to peripheral insulin resistance. For example, Zn-deficient rats are resistant to exogenous insulin injections (10) and have decreased glucose turnover during insulin infusion using the euglycemic hyperinsulinemia glucose clamp technique (11). The *db/db* mouse is characterized by hyperinsulinemia, and it is possible that low dietary Zn intake is potentiating glucose intolerance through increased peripheral insulin resistance. Furthermore, the dbZD mice in this study had elevated fasting hyperglycemia coupled with lower circulating fasting insulin concentrations (Fig. 3). Huber and Gershoff (30) reported that Zn-deficient rats have significantly lower circulating insulin but normal pancreatic insulin synthesis. Their proposal that low dietary Zn intake may impair insulin release and/or increase degradation of circulating insulin (30) may apply to the *db/db* model and could be experimentally tested in future studies.

In this study, pancreatic Zn concentrations were affected by both genotype and dietary Zn intake. Total pancreatic Zn includes Zn associated with both endocrine and exocrine portions of the organ, and with numerous metalloenzymes and metalloproteins. Insulin is stored as a crystalline structure with Zn atoms in the secretory vesicles of pancreatic β-cells (31), and it is believed that Zn is important in proinsulin hexamerization and potentially in its condensation into secretory vesicles (32). In addition, a large proportion of pancreatic Zn is associated with metallothionein, and pancreatic metallothionein is responsive to Zn injection and dietary Zn deficiency (33, 34). As previously reported in both *db/db* (18) and *ob/ob* (19, 35) mice, the diabetic genotype had a significantly lower pancreatic Zn concentration than their lean littermates. In the present study, pancreatic Zn concentrations in the *db/db* mice responded to dietary Zn supplementation. The pancreatic Zn concentration in the dbZS group was significantly higher than the dbZD and dbC groups and not significantly different from the lean mice (Fig. 2). Thus, the reduction of fasting hyperinsulinemia in the dbZS group may be linked in part to the higher pancreatic Zn concentrations. However, under the conditions of this study, the lower circulating insulin concentrations in the dbZD group were not associated with pancreatic Zn concentrations lower than the dbC group. This may reflect the fact that low dietary Zn intake (3 ppm Zn for 6 weeks) did not result in Zn deficiency based on femur Zn concentrations in *db/db* or lean mice, whereas the Zn-supplemented diet (300 ppm Zn for 6 weeks) significantly elevated femur Zn concentrations (Table II).

Hyperzincuria has been reported in the *db/db* mouse (36) and is a consistent finding in humans with diabetes (17), although its explanation remains obscure (17). In the present study, urinary Zn excretion per 12 hr was consistently 2-fold high in the *db/db* genotype, regardless of dietary Zn intake (Table III). When urinary Zn excretion was corrected for creatinine excretion, the *db/db* mice had substantially less urinary Zn excretion than lean mice. Despite these alterations in urinary Zn excretion, there was no significant effect of the *db/db* genotype on long-term Zn status as reflected by femur Zn concentrations (Table II). However, femur calcium and phosphorus concentrations were significantly lower in the *db/db* genotype, and this raises concerns for bone health in the diabetic state. The age of the mice during dietary intervention may have been a factor. In humans, there is a positive relationship between fat mass and bone mass in adults, but in growing children, body fat has a negative impact on bone mineral content and bone density (37). Urinary glucose excretion was significantly higher in the *db/db* genotype; however, it is unclear why the dbZD group did not excrete as much glucose per 12 hr as the dbC group and whether altered renal function is involved. Urinary creatinine excretion was originally employed in this study to act as a stable measurement against which urine glucose and protein could be quantitated. However, there were significant genotypic differences, and creatinine excretion, which tends to be proportional to body muscle mass, was significantly lower in the *db/db* mice. This may indicate that *db/db* mice have significantly lower muscle mass despite a significantly higher body weight and higher relative fat pad weight than lean mice (Table I).

Although it has been reported that Zn supplementation increased total carcass body fat in *ob/ob* mice or mice fed a high-fat diet (80% fat/20% protein) (38), there was no evidence in this study that dietary Zn supplementation adversely affects fat pad weight (Table I).

One possible explanation for the attenuation of hyperglycemia with dietary Zn supplementation is an improvement in glucose uptake due to increased insulin sensitivity. Because Zn has been shown to enhance tyrosine kinase phosphorylation in the insulin signal transduction using *in vitro* systems (15, 16), we evaluated whether dietary Zn manipulation modulates insulin receptor tyrosine kinase phosphorylation in gastrocnemius muscle from *db/db* mice. There were no differences in insulin receptor numbers, basal or total tyrosine phosphorylation due to genotype or diet (Table IV). However, insulin-stimulated tyrosine kinase phosphorylation in gastrocnemius muscle from dbC mice was significantly elevated compared with lnC mice, and dietary Zn supplementation (dbZS and lnZS groups) resulted in insulin-stimulated tyrosine kinase phosphorylation comparable to the lnC group (Table IV). Vicario *et al.* (39) had reported that insulin receptor tyrosine kinase activity of skeletal muscle from 24-week-old *db/db* mice was not different from lean littermates. Because hyperinsulinemia in *db/db* mice subsides at 16–24 weeks (21), we had hypothesized that there could be a difference in insulin-stimulated tyrosine kinase phosphorylation at an earlier age (10 weeks old in this study). However, the direction of change in the *db/db* mice was opposite to reports of decreased insulin-stimulated kinase activity in skeletal muscle biopsies from DM-II subjects (2, 3). A confounding factor in the present study may be differences in absolute muscle mass as reflected by less urinary creatinine excretion in *db/db* mice (Table III). Methodological differences may be a factor. In the present study, anti-insulin antibodies were used to obtain a partially purified insulin receptor preparation (28, 29) whereas the other studies (2, 3, 39) used wheat germ agglutinin affinity chromatography. Isolation of receptors using an anti-insulin receptor antibody is reported to lower background activity in kinase determinations, reduce the ratio of bound insulin receptor to insulin-like growth factor-1 receptor, and avoid selection of receptors based on their glycoslyation as does the lectin procedure (28). In this study, insulin-stimulated tyrosine kinase phosphorylation was highest in dbC mice, suggesting an adaptive response to the diabetic state. Furthermore, the attenuated hyperglycemia and hyperinsulinemia in Zn-supplemented *db/db* mice was associated with a reduction of insulin-stimulated tyrosine kinase phosphorylation to the level of lean mice, and the insulin-stimulated tyrosine kinase phosphorylation was not altered by Zn supplementation in lean mice. Although we do not have functional measurements of glucose uptake and insulin sensitivity in these mice, this study does suggest a potential role for dietary Zn modulation of insulin receptor signal transduction in muscle. In the clinical management of Type II diabetes mellitus, dietary modifications are often combined with oral agents. There has been considerable focus on the development of oral agents that enhance peripheral tissue insulin sensitivity; however, the potential interaction of oral agents and nutrients on insulin sensitivity has been relatively unexplored.

In summary, dietary Zn supplementation attenuated fasting hyperglycemia and hyperinsulinemia, and elevated pancreatic Zn concentrations in *db/db* mice. Feeding *db/db* mice a marginally Zn-deficient diet (3 ppm Zn) for 6 weeks exacerbated hyperglycemia and lowered circulating insulin despite no significant changes in Zn concentrations in either pancreas or femur (indicator of overall long-term Zn status). This situation may be analogous to the low-normal but not deficient Zn status in humans with diabetes (17). Overall, the results suggest a connection between Zn and glycemic control, and potential involvement of Zn in both β-cell and peripheral mechanisms of insulin action.

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