Dietary Zinc Supplementation Inhibits NFκB Activation and Protects Against Chemically Induced Diabetes in CD1 Mice

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Zinc status in patients with Type I diabetes is significantly lower than healthy controls. Whether zinc supplementation can prevent the onset of Type I diabetes is unknown. Recent studies have suggested that the generation of reactive oxygen species (ROS) is a cause of β cell death leading to Type I diabetes. In addition, we found that activation of NFκB (a ROS-sensitive transcription factor that regulates immune responses) may be the key cellular process that bridges oxidative stress and the death of β cells. Zinc is a known antioxidant in the immune system. Therefore, this study is designed to test whether an increase in dietary zinc can prevent the onset of Type I diabetes by blocking NFκB activation in the pancreas. The results show that high zinc intake significantly reduced the severity of Type I diabetes (based on hyperglycemia, insulin level, and islet morphology) in alloxan and streptozotocin-induced diabetic models. Zinc supplementation also inhibited NFκB activation and decreased the expression of inducible NO synthase, a downstream target gene of NFκB. It is concluded that zinc supplementation can significantly inhibit the development of Type I diabetes. The ability of zinc to modulate NFκB activation in the diabetogenic pathway may be the key mechanism for zinc's protective effect. Inhibition of the NFκB pathway may prove to be an important criterion for choosing nutritional strategies for Type I diabetes prevention. [E.B.M. 2001, Vol 226:103–111]

Key words: reactive oxygen species; free radicals; zinc; NFκB; diabetes; alloxan; streptozotocin

Type I diabetes is a devastating disease that occurs most often in children or young adults. This disease is characterized by the profound destruction of the β cells of the islets of Langerhans in the pancreas, resulting in the inability to produce insulin. Without insulin, severe disturbance in glucose metabolism results, causing intracellular starvation and a dramatic elevation in blood glucose levels or hyperglycemia. Patients are dependent on exogenous insulin therapy for life. Even with insulin therapy, numerous life-threatening complications can result in these patients, such as cardiovascular disease, neuropathy, retinopathy, and kidney failure.

Although it is known that the pancreatic islets cells are destroyed in Type I diabetes, it is unclear the exact mechanism which causes their death. There is increasing evidence that excess free radical production may contribute to the death of the β cells (1–4). The pancreatic islets also lack antioxidant protection (5, 6), rendering them especially susceptible to damage by free radicals produced during inflammatory and immune processes. The overall objective of the present study is to examine the effect of the antioxidant nutrient, zinc, in the development of Type I diabetes and explore the cellular mechanisms by which zinc exerts its effects.

The efficacy of antioxidant supplementation in preventing or delaying the onset of Type I diabetes in pre-diabetic patients (7–10) and animals (11–15) has been tested. Zinc supplementation shows extreme promise in defending the islet cells from oxidative damage because of its antioxidant capabilities and its unique relation to carbohydrate metabolism, insulin, and immune function (16–20). In addition, decreases in zinc status have been documented in both Type I diabetic patients and in animal models for the disease (21–25). Zinc deficiency is also associated with an impairment in glucose tolerance (26, 27) and an increased sensitivity to diabetogenic agents. It is possible that compromised zinc status in diabetics may predispose these individuals to external triggers for Type I diabetes.

In our laboratory, we have shown that free radical induced activation of NFκB may be a key cellular signal in initiating the sequence of events leading to β cell destruction (28). NFκB is a redox-sensitive transcription factor that controls the expression of genes important for immune and inflammatory responses (29, 30). Whether zinc supplemen-

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tation can be beneficial for the prevention or amelioration of Type I diabetes by reducing the activation of NFκB is not known.

The aims of this study, therefore, were to investigate the effect of dietary zinc supplementation on NFκB activation in *in vivo* models for Type I diabetes and to test the efficacy of dietary zinc supplementation in protecting against the development of Type I diabetes.

Materials and Methods

Animals. Weanling male CD-1 mice (Harlan) weighing 20–25 g were used in all experiments. All animals were housed in individual cages in a temperature-controlled environment (22 ± 2°C) with light period between 0600 hr and 1800 hr. Mice were randomly allocated to one of 3 zinc groups; normal (50 parts per million zinc) or high zinc (500 or 1000 ppm zinc). All animals were fed diets based on a modified AIN93G rodent diet (31) with additional zinc as zinc carbonate (Dyets, Bethlehem, PA). Animals were allowed free access to the diet with the exception of fasting periods for blood glucose determination. Animal protocol was approved by the OSU Institutional Laboratory Animal Care and Use Committee.

Serum and Pancreatic Zinc and Copper Levels. Serum and pancreatic zinc and copper levels were assessed according the method by Luterotti *et al.* (32). Briefly, serum or pancreatic homogenates were digested in 2 *N* hydrochloric acid for 24 hr at room temperature. Samples were then centrifuged at 7000*g* for 25 min, and the supernatant was used for direct measurement of metal concentrations using an atomic absorption spectrometer (Model AA-5, Varian Techtron, Australia).

Effect of Zinc Supplementation on Alloxan and STZ-Induced Diabetes. To examine the effect of zinc on the development of alloxan- and STZ-induced diabetes, mice were fed zinc diets for 2 weeks prior to alloxan or STZ injections. Alloxan was administered i.v. (50 mg/kg) to induce diabetes. For STZ-induced diabetes, multiple low doses of STZ, a commonly used method to induce diabetes were employed (33). STZ (40 mg/kg) dissolved in citrate buffer (pH 4.0), was immediately injected (i.p.) into mice (n = 5) for 5 consecutive days. Control animals received sham saline injection. Blood was obtained from the intra-orbital sinus following an 8-hr fast using a 10-μl capillary tube. Glucose concentrations were measured using the ONE-TOUCH™ II complete blood glucose monitoring kit. To minimize the effects of diurnal fluctuations, blood samples were collected between the hours of 9 AM and 10 AM each day.

Hyperglycemia was monitored for 9 days after injections. On Day 9, plasma insulin levels were assessed using ELISA (CrystalChem, Chicago, IL).

Histology. Pancreas from each group were removed on Day 10 and immediately immersion-fixed in neutral buffered formaldehyde (4%) for 24 hr. Tissues were then paraffin embedded using standard histological techniques. Serial sections were cut (5 μ*M*) and stained with hematoxylin and eosin (H&E) for pathology studies. Adjacent sections were used for immunocytochemical studies as detailed below.

Immunocytochemistry. Immunocytochemical labeling of insulin was performed as previously described (34). Briefly, paraffin-embedded pancreatic sections were de-waxed in xylene. Antigen retrieval was performed on these sections by heating the sections in a microwave in a citrate buffer (pH 6.0) for 5 min. The sections were then incubated with polyclonal anti-insulin antibody (1:400, Dako, Carpinteria, CA) at 4°C overnight. Cellular localization of insulin was visualized using biotinylated secondary antibody and the conventional avidin–biotin–peroxidase method with diaminobenzidine as the substrate.

Determination of NFκB Activation *In Vivo*. For NFκB activation analysis, mice were fed for 2 weeks on respective zinc diets and were then injected with 50 mg/kg alloxan or 50 mg/kg STZ (i.v.) (Sigma, St. Louis, MO). Control animals received saline injections. All mice were sacrificed 30 min after injection and pancreas was immediately removed. NFκB activation was determined using an electrophoretic mobility shift assay (EMSA). Crude nuclear extracts were prepared from pancreatic tissue as described by Deryckere and Gannon (35). All gel-shift assays were performed with 5 pooled pancreas samples, and repeated with a separate group of 5 animals twice (a total of 15 animals). Due to the small size of the pancreas, we have found it necessary to pool samples to isolate enough nucleuses for assaying binding *in vivo*. Double-stranded synthetic oligonucleotides probes for NFκB (5′-AGTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI) were end-labeled using [γ-^{32}P] (Amersham, Piscataway, NJ) and T4 polynucleotide kinase (Promega, Madison, WI). Binding reactions containing equal amounts of protein (~10 μg) and labeled oligonucleotide probes were performed for 20 min at room temperature in binding buffer (4% glycerol, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, pH 8.0, 0.5 m*M* DTT, 50 m*M* NaCl, 10 m*M* Tris, 50 μg/ml poly [dI-dC]. Specific binding was confirmed using 100–400-fold excess unlabeled NFκB oligonucleotide as a specific competitor. Protein–DNA complexes were separated using 6% non-denaturing polyacrylamide gel electrophoresis followed by radiography to detect the level of retardation produced by binding to NFκB probe.

Inducible Nitric Oxide Synthase (iNOS) and Metallothionein (MT) Protein Expression. iNOS and MT protein expression in the pancreas in was determined by Western blot analysis. Pancreatic homogenates containing equal protein were mixed with an equal volume of sample buffer (125 m*M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, bromphenol blue) and boiled for 5 min. SDS electrophoresis was carried out under standard conditions (36). Protein was transferred from the SDS gel to nitrocellulose membranes at 50 mA overnight. iNOS blots

were blocked with in 5% non-fat dry milk for 1 hr at 37°C. Blots were incubated for 3 hr at 37°C with rabbit anti-mouse antibody (Alexis Biochemical, San Diego, CA) at a dilution of 1:2000. The blots were washed 5 times with PBS+0.2% Tween 20 and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit antisera (Sigma, St. Louis, MO) at a dilution of 1:4000, for 1 hr at 37°C. iNOS was detected by enhanced chemiluminescence using Hyperfilm and ECL reagents (Amersham, Piscataway, NJ) at 1:2000. For MT, the primary antibody used was a mouse anti-human MT antibody (Zymed, San Francisco, CA) at a dilution of 1:2000. For secondary antibody, blots incubated with horseradish peroxidase-conjugated donkey anti-mouse antisera (Sigma, St. Louis, CA) at a dilution of 1:4000.

Statistics. One-way analysis of variance (ANOVA) was performed to assess the differences between control and zinc treatments. Difference in means among treatments was tested by using Duncan's test. Level of significance was evaluated at $P < 0.05$.

Results

To establish the influence of our diets on zinc status, serum and pancreatic zinc determinations were taken following a 2-week feeding period of 50, 100, 500, or 1000 ppm zinc. There was also a concern that high zinc supplementation may compromise copper status, thus we concurrently measured serum and pancreatic copper levels. Figure 1 shows both serum and pancreatic zinc and copper levels following the 2-week feeding period with each zinc diet. A significant enhancement in serum and pancreatic zinc could only be seen in animals fed a diet containing 500 ppm and higher. The diet containing 50 ppm zinc closely approximates the level of zinc recommended by the 93AIN guidelines. Supplementation with high amounts of zinc did not appear to compromise copper status in these mice. No significant change in both serum and pancreatic copper levels were found with high zinc supplementation. Zinc supplementation also did not affect body weight gain or food intake in these mice (data not shown). Since zinc status was only increased at levels of 500 ppm and higher, we chose to use 500 and 1000 ppm zinc levels for our diabetic intervention studies. To examine the effect of zinc supplementation on the development of Type I diabetes, we examined several indices of diabetes development including hyperglycemia, serum, and pancreatic insulin and islet cell morphology. Figure 2 illustrates the ability of zinc to inhibit both alloxan and STZ-induced hyperglycemia. In Fig. 2A, we can see that injection with alloxan (50 mg/kg i.v.) causes a significant elevation in fasting blood glucose levels in mice fed the normal 50 ppm zinc diet compared to saline injected controls. Alloxan treated animals supplemented with 1000 ppm zinc show a marked reduction in blood glucose levels compared to un-supplemented animals fed 50 ppm zinc. Reduced hyperglycemia was only seen in the initial days in animals supplemented with 500 ppm zinc. By Day 9, no

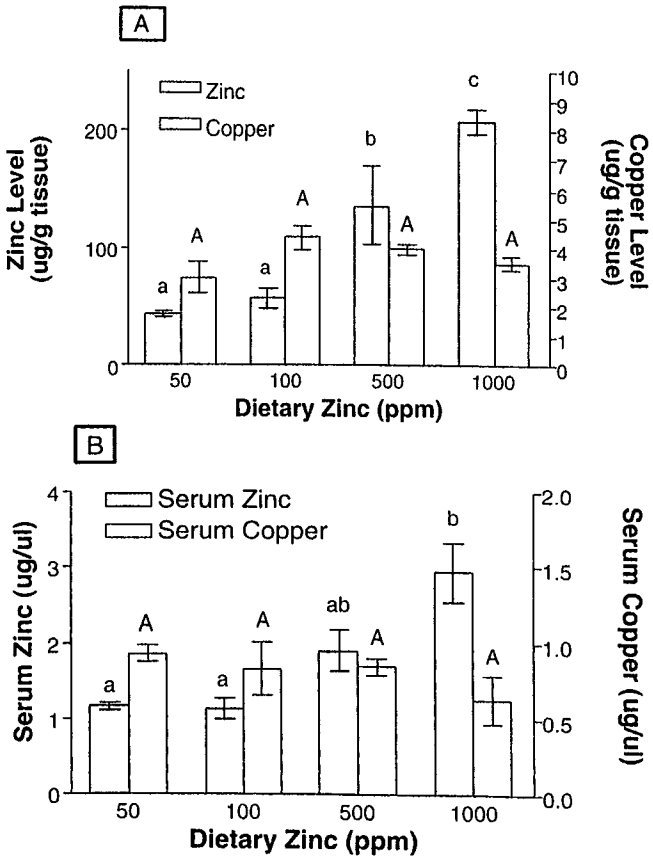

Figure 1. Serum and pancreatic zinc and copper levels with dietary zinc supplementation. Zinc and copper levels in (A) serum and (B) pancreas following 2-week feeding period with diets containing 50 (normal), 100, 500, and 1000 ppm zinc. Values are expressed as mean ± SE. Mean values were compared at each day using one-way ANOVA. Level of significance was evaluated using Duncan's test at $P < 0.05$.

difference in blood glucose levels was detected between 500 ppm supplemented and 50 ppm unsupplemented animals. Figure 2B shows that zinc supplementation had a similar protective effect in STZ-induced diabetes. Multiple low dose injections of STZ caused marked hyperglycemia in un-supplemented animals fed 50 ppm zinc. Supplementation with 500 ppm zinc had little effect on reducing STZ-induced hyperglycemia. On the other hand, supplementation with 1000 ppm zinc significantly reduced fasting blood glucose levels. Thus, high zinc supplementation does appear to have a profound effect on both alloxan and STZ-induced hyperglycemia.

To determine the effect of zinc supplementation on pancreatic islet cell death and islet cell functioning, histology, immunocytochemistry, and serum insulin measurements were performed. Figure 3 shows representative islets from mice fed 50, 500, or 1000 ppm zinc following alloxan or STZ injections and stained with H&E. Control is an islet from saline injected mice. In Fig. 3, panel A represents a normal healthy islet cell control. Panel B is a representative islet from un-supplemented animals (50 ppm zinc) injected with alloxan. Apparent coagulative necrosis was found in

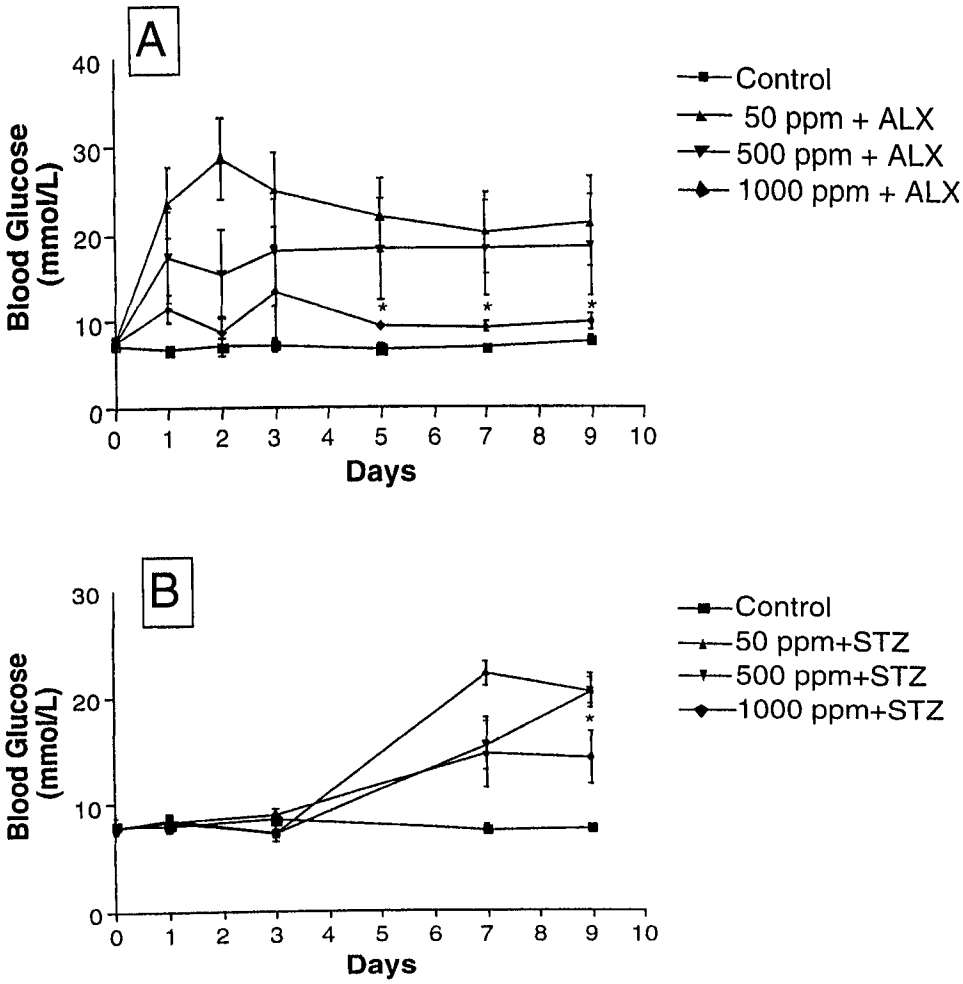

Figure 2. High zinc supplementation reduces alloxan and STZ-induced hyperglycemia. Mice (n = 5 at each dose) were injected with alloxan (A) (50 mg/kg i.v.) or STZ (B) (40 mg/kg i.p. for 5 days). Controls (•) were injected with saline only. Fasting blood glucose measurements following alloxan or STZ exposure were taken in animals fed 50 ppm (▲), 500 ppm (▼), and 1000 ppm (◆). Day 1 is first day after alloxan or last STZ injection. Mean values were compared at each day using one-way ANOVA. Level of significance was evaluated using Duncan's test at $P < 0.05$. "*" above data point denotes a significant difference from the 50 ppm group.

these animals (on average, for each of the randomly sampled 20 islets, over 80% of the islet was necrotic). In mice supplemented with 500 ppm zinc (panel C), the necrotic area in an islet was reduced to about 40–50%. In panel D, animals supplemented with 1000 ppm zinc showed a marked reduction in necrotic areas in the islets (only 10–20% area of the islet was necrotic). In STZ treated animals, microphotographs of representative islets show that STZ causes severe islet cell death with concomitant infiltration of the islets by lymphocytes (Fig. 3B). Supplementation with 500 ppm of zinc reduced the area of necrosis and the amount of infiltrating lymphocytes (Fig. 3C). With 1000 ppm of zinc, only small area of cellular necrosis was found, and the infiltration of lymphocytes was substantially attenuated (Fig. 3D). Thus, a significant reduction in both islet cell death and islet infiltration is apparent with zinc supplementation in STZ-induced diabetes.

To examine β cell function, both serum insulin and immunocytochemical staining for insulin in islet cells were performed. Figure 4 depicts serum insulin levels in zinc-supplemented animals following alloxan or STZ exposure. Animals injected with saline were used as controls. Animals injected with either alloxan or STZ show a decline in circulating serum insulin, suggesting that pancreatic β cell function is severely compromised. Supplementation only at the 1000 ppm zinc level caused a significant increase of insulin levels and restoration of β cell function. Immunocytochemical staining also showed that zinc supplementation protected islet cells from alloxan and STZ-induced loss of insulin (Fig. 5). Cells stained brown indicate insulin-producing cells. The stain intensity indicates the amount of insulin present in the cells. In panels A, a representative control islet shows staining of insulin for almost all of the islet cells (96.8% of cells were stained in dark brown). In un-supplemented animals (50 ppm zinc), the loss of insulin production is reflected in the loss of insulin-staining cells in the islets of both alloxan-treated (33.8% of cells were insulin positive) and STZ-treated mice (13% insulin-positive cells, panel B). With zinc supplementation there was a dose-dependent restoration of insulin production (panels C and D) in both models. The most dramatic change is in the 1000 ppm zinc supplemented groups (panel D). In these groups, the staining patterns for insulin were restored close to the levels of controls (83% insulin-positive cells in STZ-treated animals, and 84% insulin-positive cells in the islets of alloxan-treated animals. Taken together, zinc supplementa-

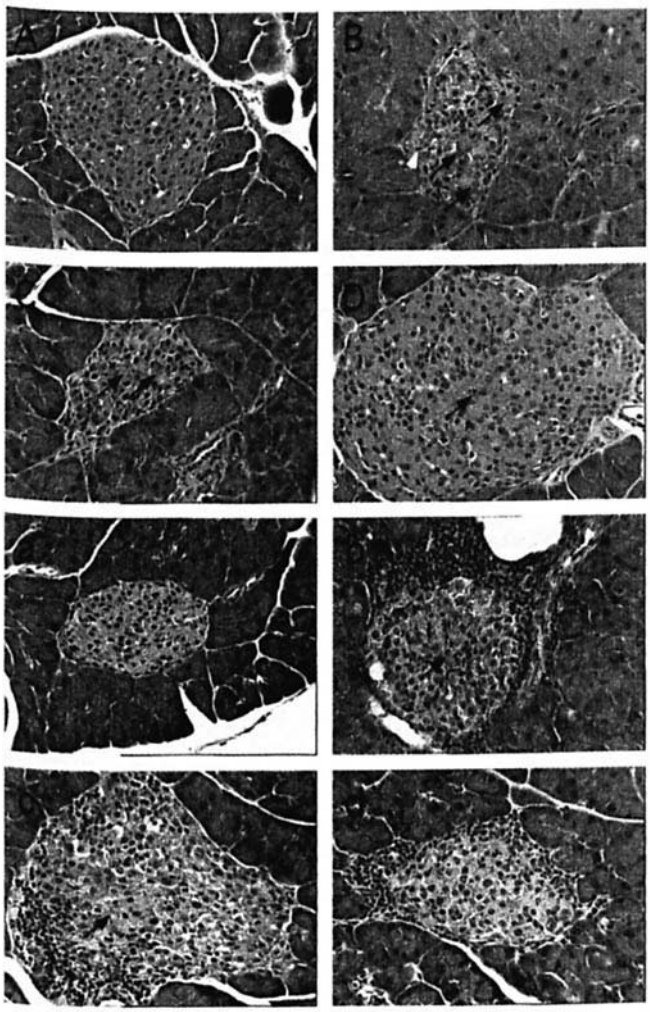

Figure 3. Islet cell pathology in zinc supplemented mice following alloxan and STZ exposure. On day 9, pancreas were removed, fixed, and paraffin embedded. Conventional H&E stains were performed to assess islet cell pathology. In panel 1, animals were injected with alloxan (ALX, 50 mg/kg i.v.): (A) control islet (saline injected); (B) 50 ppm + ALX; (C) 500 ppm + ALX; (D) 1000 ppm + ALX. Arrows point to obvious areas of cellular necrosis. In panel 2, animals were injected with low multiple doses of streptozotocin (STZ, 40 mg/kg i.p. for 5 days): (a) control islet (saline injected); (b) 50 ppm + STZ; (c) 500 ppm + STZ; (d) 1000 ppm + STZ.

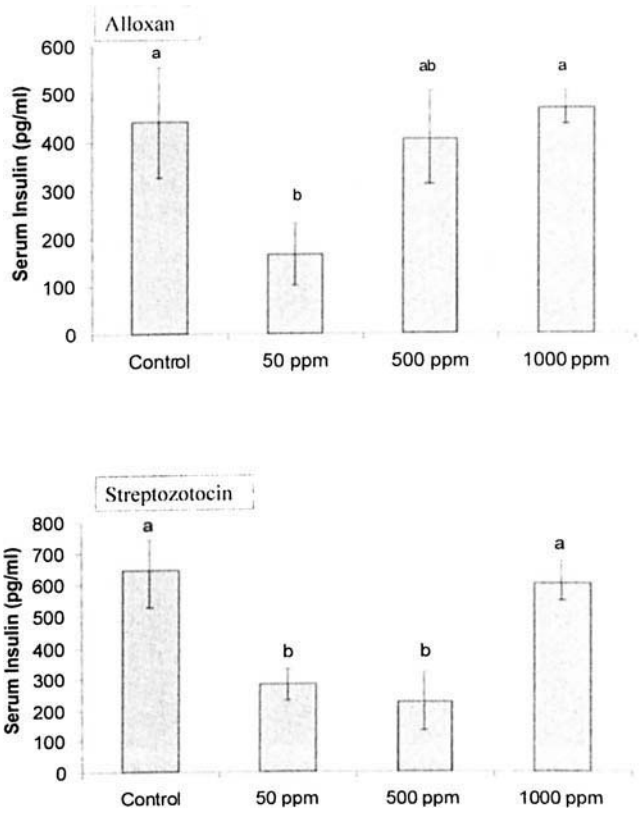

Figure 4. Serum insulin levels of zinc supplemented mice following alloxan or STZ exposure. Plasma insulin levels were assessed 9 days after alloxan injection (50 mg/kg i.v.) or STZ (40 mg/kg i.p. for 5 days) in mice fed 50 (normal zinc level), 500, and 1000 ppm zinc diets. Control animals received saline injection and were fed 50 ppm zinc diet (normal diet). Values are expressed as mean ± SE. Mean values were compared using one-way ANOVA. Level of significance was evaluated using Duncan's test at $P < 0.05$. Bars with the same letter are not statistically different.

tion prevented hyperglycemia, and islet cell death, restored insulin production in the two distinct models for Type I diabetes.

To determine the effect of dietary zinc supplementation on pancreatic NFκB activation, electromobility shift assays (EMSA) were performed. Figure 6 shows the EMSA of pancreatic extracts from zinc-supplemented animals 30 min after an alloxan injection. No NFκB activation was detectable (lanes 3–5) in the pancreas of animals that were supplemented with zinc alone. Significant activation of NFκB in the pancreas of mice injected with alloxan was detected. Animals that were injected with alloxan and fed the normal dietary zinc level (50 ppm) also exhibited significant activation of NFκB (lane 5). In animals that were fed higher levels of zinc the alloxan-induced NFκB activation (lanes 6 and 7) was significantly inhibited. Animals supplemented with 1000 ppm zinc completely inhibited the activation of NFκB (lane 7). These findings demonstrate that zinc supplementation was able to effectively inhibit alloxan-induced NFκB activation.

A similar inhibitory effect was seen with zinc supplementation in the STZ-induced diabetic model (Fig. 7). Lane 1 shows no activation of NFκB in the pancreas of animals injected with saline. Significant activation of NFκB was induced in the pancreas of animals that were injected with STZ but fed the normal 50 ppm zinc diet (lane 2). In contrast, animals that were fed the 1000 ppm zinc diet showed a significant inhibition of this activation. Thus, high zinc supplementation also effectively inhibited STZ-induced NFκB activation.

To verify the effect of zinc supplementation on the NFκB-initiated transactivation, we assessed the expression of NFκB-responsive genes. One protein that is downstream of NFκB is the inducible form of nitric oxide synthase (iNOS). Figure 8 shows the result of the Western blot for iNOS following STZ injection in unsupplemented and zinc-supplemented animals. A significant increase in iNOS ex-

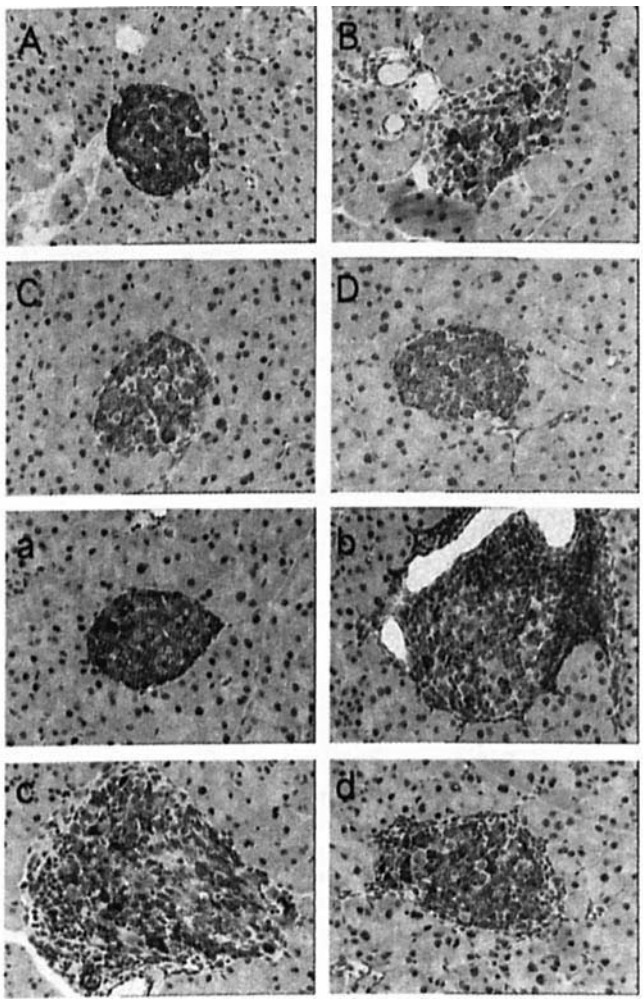

Figure 5. Immunocytochemical stains for insulin in zinc supplemented mice following alloxan and STZ exposure. On day 9, pancreas were removed, fixed, and paraffin embedded. Tissue sections were stained with hematoxylin and the immunohistochemically stained for insulin specifically. Cells stained brown indicate insulin-producing cells. The stain intensity indicates the amount of insulin present in the cells. In panel 1, animals were injected with alloxan (ALX, 50 mg/kg i.v.): (A) control islet (saline injected); (B) 50 ppm + ALX; (C) 500 ppm + ALX; (D) 1000 ppm + ALX. In panel 2, animals were injected with low multiple doses of streptozotocin (STZ, 40 mg/kg i.p. for 5 days): (a) control islet (saline injected); (b) 50 ppm + STZ; (c) 500 ppm + STZ; (d) 1000 ppm + STZ.

pression is apparent with STZ injections in the pancreas of unsupplemented animals (50 ppm). Mice supplemented with 500 ppm zinc prior to STZ exposure also showed significant iNOS protein expression. In contrast, mice supplemented with 1000 ppm zinc prior to STZ exposure show a significant reduction in iNOS expression. Thus, high zinc supplementation effectively inhibited iNOS induction with STZ. These results are consistent with the effect of zinc supplementation on NFκB translocation. Thus, high zinc supplementation inhibits both alloxan- and STZ-induced NFκB activation and NFκB-controlled gene expression.

To determine if metallothionein (MT) plays a role in the antioxidant defense in pancreas of zinc-supplemented groups, pancreatic MT expression was determined by West-

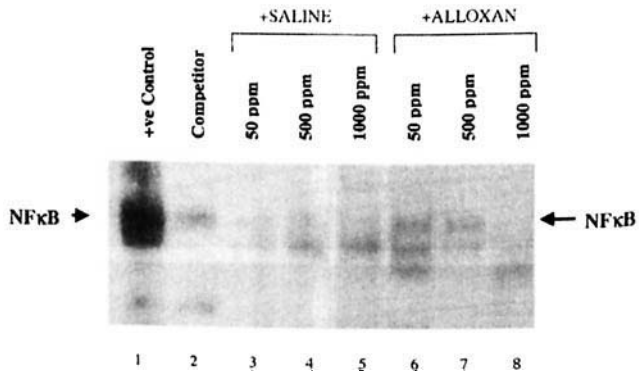

Figure 6. High zinc supplementation inhibits alloxan-induced NFκB activation in the pancreas. +ve control were HeLa nuclear extracts. Competitor reactions include cold specific NFκB oligonucleotides. Samples in lanes 3–5 received only sham saline injections. Alloxan treated groups received alloxan (50 mg/kg i.v.) (lanes 6–8). Pancreatic tissue was removed from mice 30 min following alloxan or saline injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This figure is a representative of 2 individual experiments.

ern blot. Figure 9 illustrated that pancreatic MT is induced in mice supplemented with 1000 ppm zinc only. There is no dose-dependent response of MT induction in the pancreas.

Discussion

In the present study, we demonstrated that dietary supplementation with zinc effectively protected against the development of Type I diabetes in two distinct animal models. Zinc supplementation prevented islet cell death and mitigated the infiltration of immune cells to the islets. We

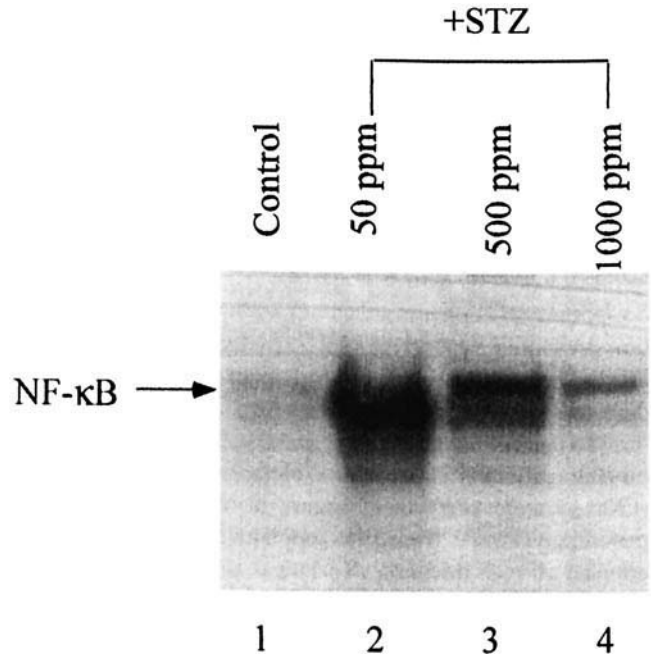

Figure 7. High zinc supplementation inhibits STZ-induced NFκB activation in the pancreas. Control (lane 1) received only sham saline injections. STZ-treated groups received STZ (50 mg/kg i.v.) (lanes 2–4). Pancreatic tissue was removed from mice 30 min following STZ or saline injection. Nuclear extracts were prepared from 5 pooled pancreas samples from each group. This figure is a representative of 2 individual experiments.

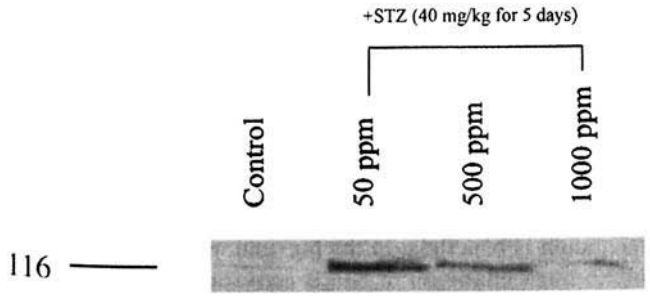

Figure 8. High zinc supplementation inhibits STZ-induced iNOS expression in the pancreas. iNOS expression was determined by Western blot analysis in mice injected with STZ and fed 50, 500, or 1000 ppm zinc. Control animals were injected with saline. SDS–PAGE was performed as outlined in Methods. Each sample is contains 5 pooled pancreases from each group. Results are representative of 2 individual blots.

also demonstrated that zinc supplementation modulates the activation of the redox-sensitive transcription factor NFκB and consequently modulates downstream expression of iNOS *in vivo*. These results are consistent with previous studies that have shown a protective effect of zinc in animal models for Type I diabetes (12). In addition, the current study provides important insights into the molecular mechanisms by which zinc may exert its effects.

There are several mechanisms by which zinc supplementation may prevent islet cell death. Firstly, zinc is known to have several unique antioxidant properties. It acts to stabilize membrane structure and protects sulfhydryl groups from oxidation. Zinc also may compete with iron (Fe), a potent inducer of toxic Fenton reactions, which result in the production of hydroxyl radicals. Zinc is also an essential cofactor in antioxidant enzyme copper/zinc superoxide dismutase (CuZnSOD), one of the first-line defense enzymes in scavenging reactive oxygen species. Transgenic animals that over-express CuZnSOD are also protected against developing alloxan and STZ-induced diabetes (37, 38). However, we have found that high zinc supplementation does not significantly affect CuZnSOD activity in the pancreas (data not shown). Yang and Cherian have also

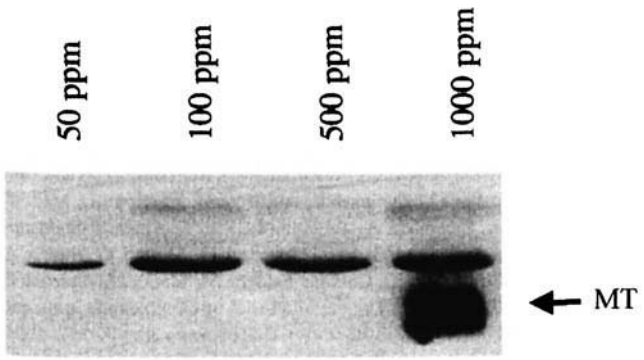

Figure 9. Pancreatic metallothionein (MT) expression is induced with high zinc supplementation. MT protein expression in the pancreas was determined by western blot analysis following 2-week dietary treatment with 50, 100, 500, and 1000 ppm zinc diets. SDS–PAGE was performed as outlined in Methods. Results are representative of 4 individual blots.

demonstrated that STZ-induced lipid peroxidation can be improved with zinc without any changes in SOD activity (39).

Zinc may also exert its antioxidant activity through the induction of MT. MT is a small molecular weight, cysteine-rich protein with significant antioxidant activity (40). We show a significant induction of pancreatic MT expression when animals are fed a diet containing 1000 ppm zinc (Fig. 9). Although pancreatic zinc is clearly elevated with 500 ppm zinc in the diet (Fig. 1A), little induction is seen at lower supplementation levels (100 and 500 ppm). It is possible the observed the effects of zinc supplementation was mediated by the production of MT at high concentration of zinc supplementation. Apostolova *et al.* (41), however, has shown that zinc pretreatment (1 mg/kg) had a protective effect in MT-null mice but no effect in non-transgenic mice. Therefore, zinc may exert its protective effect independent of MT. This is also observed in our study that zinc supplementation at 500 ppm reduced the severity of hyperglycemia (Fig. 2) without induction of pancreatic MT (Fig. 9). Whether the production MT contributed to the protective effects of zinc seen in the present study remains to be elucidated.

The results of the present study clearly demonstrate that zinc supplementation can significant influence the development of Type I diabetes. This is consistent with the view that inadequate nutrition, low zinc status in particular, and or inherent low antioxidant nutrient status may predispose individuals to developing the disease. Indeed, inadequate zinc intake has been reported in Type I diabetes children (42). However, before recommendations for supplementation can be made, issues of dose and safety need to be addressed. In these studies, zinc supplementation at 1000 ppm zinc level most dramatically prevented symptoms of hyperglycemia, cell death and loss of insulin in both alloxan and STZ-induced diabetes. Thus, very high doses of zinc (20 times that normal level) are required to prevent the development of the Type I diabetes. In the human, this level of supplementation may be difficult, because issues of compliance, gastrointestinal absorption, and toxicity will come into play. However, if the key mechanisms by which zinc exerts its protective effect can be identified, combining zinc with other dietary supplements may result in successful strategies for preventing Type I diabetes.

Two distinct models for Type I diabetes were tested in the present study. Alloxan is thought to produce free radicals in its metabolism (43, 44), although the precise mechanisms explaining the selective cytotoxicity to the islet cells are still unknown. STZ also produces free radicals in its metabolism, but STZ destroys the islet cells by a different mechanism. Unlike alloxan, STZ induces pronounced immune and inflammatory processes prior to cell death. Following the injection of multiple low doses of STZ, pancreatic islets show obvious insulitis with infiltrating lymphocytes and macrophages, architectural distortion and β cell

death (33). Evidence for apoptosis as the mode of β cell death induced by STZ has been reported (45).

It is remarkable that zinc supplementation showed strong protective effects in these two very different models. It is possible that zinc may have exerted its effects through different mechanisms in each model, or zinc may affect a common essential pathway in both disease models. It is known that both alloxan and STZ specifically activate pancreatic NFκB (11, 46). Supplementation with zinc in the present study inhibited NFκB activation and protected islet cell death in both alloxan and STZ models. It is possible that zinc-induced suppression of the NFκB activation is crucial for its protective effects in both of these models. Recently, there has been an increased interest in understanding how free radicals act as cellular messengers in disease pathways. In particular, the activation of NFκB has become the target in several disease models (47–49). Because NFκB activation is exquisitely sensitive to oxidative stress, responds to and amplifies inflammatory responses, it can be envisioned that NFκB activation serves an essential cellular process leading to the pathogenesis of Type I diabetes in both of our models. Indeed, we observed and increase in activation of NFκB in the pancreas of in both alloxan- and STZ-treated animals. In addition, the ability of zinc to protect against the development of the Type I diabetes correlated with its ability to inhibit NFκB activation in both models. These results suggest that suppression of NFκB activation may be an important mechanism for the protective effects of zinc.

Supplementation with zinc also inhibited iNOS protein expression. The induction of iNOS has been implicated in mediating β cell death in Type I diabetes (4, 50). iNOS is a downstream target of NFκB activation. Thus, the increase in iNOS expression can be used as indirect evidence for the transactivation of NFκB. Again, our data show that the protective effects of zinc correlated with its ability to block NFκB and downstream iNOS expression.

To further confirm the role of zinc in NFκB activation and the events leading to inflammatory and immune responses in Type I diabetes, many other downstream target such as cytokines, chemokines, and adhesion molecules still need to be examined. Additional studies investigating β cell apoptosis also needs to be examined. It is possible that zinc may have profound influences on NFκB and activation of cell death via apoptotic pathways. However, NFκB is generally thought to be an anti-apoptotic factor. Thus, it is curious how NFκB activation correlates with an increase in β cell apoptosis. However, cellular apoptosis is also a key mechanism in controlling immune responses. It is possible that activation of NFκB may limit apoptosis of immune cells, causing amplified immune and inflammatory responses, which in turn causes β cell death. We demonstrated in the present study that zinc inhibits NFκB activation and significantly limits infiltration of immune cells in the STZ-induced model. Thus, zinc and NFκB may exert its effects by altering immune responses, not by directly altering cell death pathways in the β cell itself. Regardless, the present results are consistent with the hypothesis that free radical-induced NFκB activation plays a central role in the signaling events leading to the destruction of the pancreatic β cells Further analysis of the role of NFκB activation in the pathogenesis of Type I diabetes is being pursued by our lab currently.

In summary, the present results show that zinc supplementation is beneficial in the prevention of diabetes, possibly through inhibition of NFκB activation. If NFκB proves to be the central pathway leading to β cell destruction, then the detection of NFκB activation can be used for screening potential preventive agents. It is becoming increasing clear that the role of free radicals and antioxidants in disease processes is far more complex than previously thought. Not all antioxidants act the same, thus the use of additional criteria, e.g., NFκB activation, may significantly improve the strategies for finding effective preventative agents.

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