Dietary Zinc Reduction, Pyruvate Supplementation, or Zinc Transporter 5 Knockout Attenuates β-Cell Death in Nonobese Diabetic Mice, Islets, and Insulinoma Cells

Christian T. Sheline, Chunxiao Shi, Toshihiro Takata, Julia Zhu, Wenlan Zhang, P. Joshua Sheline, Ai-Li Cai, Ai-Li Cai, and Li Li

Abstract

Pancreatic zinc (Zn\(^{2+}\)) concentrations are linked to diabetes and pancreatic dysfunction, but Zn\(^{2+}\) is also required for insulin processing and packaging. Zn\(^{2+}\) released with insulin increases β-cell pancreatic death after streptozotocin toxin exposure in vitro and in vivo. Triosephosphate accumulation, caused by NAD\(^+\) loss and glycolytic enzyme dysfunction, occur in type-1 diabetics (T1DM) and animal models. We previously showed these mechanisms are also involved in Zn\(^{2+}\) neurotoxicity and are attenuated by nicotinamide- or pyruvate-induced restoration of NAD\(^+\) concentrations, Zn\(^{2+}\) restriction, or inhibition of Sir2 proteins. We tested the hypothesis that similar Zn\(^{2+}\)- and NAD\(^+\)-mediated mechanisms are involved in β-cell toxicity in models of ongoing T1DM using mouse insulinoma cells, islets, and nonobese diabetic (NOD) mice. Zn\(^{2+}\), streptozotocin, and cytokines caused NAD\(^+\) loss and death in insulinoma cells and islets, which were attenuated by Zn\(^{2+}\) restriction, pyruvate, nicotinamide, NAD\(^+\), and inhibitors of Sir2 proteins. We measured diabetes incidence and mortality in NOD mice and demonstrated that pyruvate supplementation, or genetic or dietary Zn\(^{2+}\) reduction, attenuated these measures. T-lymphocyte infiltration, punctate Zn\(^{2+}\) staining, and β-cell loss increased with time in islets of NOD mice. Dietary Zn\(^{2+}\) restriction or Zn\(^{2+}\) transporter 5 knockout reduced pancreatic Zn\(^{2+}\) staining and increased β-cell mass, glucose homeostasis, and survival in NOD mice, whereas Zn\(^{2+}\) supplementation had the opposite effects. Pancreatic Zn\(^{2+}\) reduction or NAD\(^+\) restoration (pyruvate or nicotinamide supplementation) are suggested as novel targets for attenuating T1DM.

Introduction

Type 1 diabetes (T1DM)\(^{11}\) is an autoimmune disease resulting from specific T-lymphocyte– and reactive oxygen species (ROS)-mediated destruction of the insulin-producing β-cells of the islets of Langerhans in the pancreas (1). It affects 1 in 300 people in the U.S. and is a major cause of mortality due to cardiovascular disease before 30 y of age (2). As such, the mechanisms of β-cell death in T1DM need to be better understood. Previous studies have shown the involvement of zinc (Zn\(^{2+}\)) toxicity in streptozotocin models of T1DM in vitro or in vivo (3–5). We have now examined more physiologic models of T1DM for the role of Zn\(^{2+}\) toxicity. We have tested the hypothesis that Zn\(^{2+}\) and NAD\(^{+}\)-mediated mechanisms are involved in β-cell toxicity in the mixed cytokine model of ongoing T1DM using mouse insulinoma cells and islets and in the nonobese diabetic (NOD) mouse.

NOD mouse model. The NOD mouse loses β-cells and glycemic control through autoimmune-mediated mechanisms accurately mimicking T1DM (1). Immune-mediated ROS generation results in oxidative injury, depolarization, and degranulation of β-cells, causing secretory granular Zn\(^{2+}\) release [reviewed in (6)]. Inhibition of the metabolic enzymes GAPDH and pyruvate dehydrogenase causes an increase in triosephosphates as occurs in T1DM patients, perhaps mediated by a reduced NAD\(^{+}\)-NADH

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\(^{3}\) Supplemental Figures 1 and 2 and Supplemental Tables 1–4 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

\(^{11}\) Abbreviations used: GD, glucose deprivation; HD, high-density culture; KO, knockout; MTT, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; [NAD\(^{+}\)]\(_{i}\), intracellular NAD concentration; Naph, 2-hydroxynaphthaldehyde; NOD, nonobese diabetic; RFU, relative fluorescence unit; ROS, reactive oxygen species; T1DM, type 1 diabetes; T2DM, type 2 diabetes; TPEN, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine; TSO, N-(6-methoxy-quinolyl)-para-toluenesulfonamide; WT, wildtype; [Zn\(^{2+}\)]\(_{i}\), intracellular zinc concentration; ZnR, reduced-zinc diet; ZnT, zinc transporter; ZP1, zinc transporter 1.

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ductions in NAD+:NADH, glucose oxidation, and glucose-induced loss of NAD+ concentrations through Sir2 proteins, resulting in the concentration ([Zn2+]i) is very high and within the pancreas Zn2+ is concentrated.

Streptozotocin. Streptozotocin is selectively toxic to pancreatic islets and a mouse β-cell insulinoma cell line (MIN6) causing reductions in NAD+·NADH, glucose oxidation, and glucose-induced insulin secretion (3,12). Streptozotocin induces Zn2+ release, alkylates DNA-activating poly-ADP ribose polymerase and NF-κB, and depletes NAD+ and ATP (3,13,14). Oxygen radical scavengers, nicotinamide, Zn2+ chelators, and pyruvate reduce streptozotocin-induced diabetes incidence [this study and (3–5,15)].

Pancrætic Zn2+. The pancreatic intracellular Zn2+ concentration ([Zn2+]i) is very high and within the pancreas Zn2+ is concentrated in secretory granules, where it allows insulin processing and hexahedral Zn2+/insulin crystal formation (3). Upon glucose induction and β-cell depolarization, insulin-bound and non-insulin-bound Zn2+ are released, both of which are cytotoxic to adjoining β-cells. This helps to explain the specificity of β-cell death (4,16). Because of this, dietary Zn2+ supplementation has become controversial. The reasoning for supplementation is that type-2 diabetics (T2DM) and the elderly tend to be Zn2+ deficient. Zn2+ supplementation has been proposed to attenuate diabetes incidence (17) and T2DM but not T1DM animal models were reported to be Zn2+ deficient [reviewed in (18)]. This bimodality of Zn2+ also occurs in neurons, where Zn2+ is required for cell survival and supplementation is beneficial if concentrations are reduced but is toxic under pathologic conditions where labile Zn2+ is in excess (19,20). Dietary Zn2+ changes have been shown to induce changes in Zn2+ transporters, depending on cell type, cellular localization, and the Zn2+ transporter [reviewed in (21)]. Intestinal expression of Zn2+ importers, ZIP4 in particular, has been shown to be upregulated by dietary restriction. Pancreatic acinar cell expression of the Zn2+ exporters ZnT1 and ZnT2 were shown to be reduced by dietary Zn2+ restriction (22). Under pathophysiologic, high-glucose, depolarizing conditions, the influx of Zn2+ into islets and β-cells is predominantly mediated by voltage-gated calcium channels rather than by transporters (23).

Zn2+ homeostasis in β-cells is genetically linked to T1DM and T2DM. Two slc30a family Zn2+ transporters (Zinc transporter (ZNT) 5 and ZNT8) are preferentially or specifically expressed in β-cells and are involved in physiologic pancreatic Zn2+ uptake into the Golgi and packaging into secretory granules (24,25). ZNT8 is a major epitope for autoantigen generation in T1DM, adversely affecting β-cell Zn2+ transport and increasing pancreatic autoimmune attack (26). A common single nucleotide polymorphism in ZNT8 is also linked to reduced susceptibility to T2DM (27). However, global and/or β-cell knockout of ZNT5 or ZNT8 are not clinically diabetic. This suggests redundancy in β-cell Zn2+ transport function (24,28,29).

In the studies described herein, we propose that diabetic immune-generated cytokines and ROS cause intracellular release of Zn2+ or reuptake of noninsulin-bound Zn2+ released exogenously by β-cell degranulation. This increased [Zn2+]i, potentiates loss of NAD+ concentrations through Sir2 proteins, resulting in glycolytic inhibition, β-cell death, and diabetes incidence in ongoing T1DM [Supplemental Fig. 1].

Materials and Methods

Cell culture and toxicity studies. Cultures of the mouse insulinoma cell line MIN6 (from Dr. John Corbett, while at Washington University) were maintained as described (3); where indicated, cultures were preloaded with 10 μmol/L ZnCl2 in the growth medium that did not affect cell number or viability. Then 35 μmol/L ZnCl2 in serum-free minimal essential medium or 300 μmol/L ZnCl2 in DMEM + 15% FBS was used for normal-density toxicities, and these conditions achieved similar levels of toxicity (Expt. 1). ZnCl2 (400 μmol/L) in HEPES buffered salt solution with or without 60 mmol/L KCl replacing NaCl and Ca2+ channel antagonists was present for 5 or 15 min in the [Zn2+]i measurements as described (30). For glucose deprivation (GD), normal-density cultures were washed 7 times in minimal essential medium lacking glucose and exposed in this medium plus normal growth additives with or without optimized concentrations of additional compounds for 24 h. The compounds used were: N2,N2'-retakish-[2-pyridylmethyl]-ethylenediamine (TPEN), a Zn2+-specific chelator; sirtuin and/or 2-hydroxynaphthaldehyde (Nap), sirtuin (Sir2 protein) inhibitors; nimbomide and mibepradil, specific L- and T-type calcium channel antagonists (10 μmol/L), respectively; and compounds that restored NAD+ concentrations (pyruvate, nicotinamide, and NAD+) at optimized concentrations (data not shown). High-density cultures (HDs) were used for streptozotocin and cytokine toxicities. MIN6 cells were collected, counted, resuspended in DMEM + 15% FBS, and plated at HD/low extracellular volume (5–10 × 105 cells/L) in 0.04 mL in V-shaped, 96-well plates (9). These HDs were plated in 7.5 mmol/L streptozotocin or a mixture of cytokines (250 μg/L IL-1β, 8 μg/L, TNFa, and 200 μg/L IFNy) with coexposure to the compounds tested. High cytokine concentrations were required due to the HDs and short exposure (6 h, Expt. 2). Cell viability was assayed at varying times later by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) staining (0.1% final) of individual wells of a tissue culture plate, the absorbance at 595 nm was then measured (n = 8–20 wells of cells from at least 3 independent experiments), and significance was tested using 1-way ANOVA (Expts. 1 and 2). Staining with propidium iodide (2.5 mg/L) for normal-density Zn2+ and GD toxicity studies followed by measurement of fluorescence gave comparable results (data not shown). HDs were only assayed with MTB, because a monolayer culture is required for propidium iodide staining.

Isolated islet generation. Islets were generated from C57Bl/6J mice (Jackson Labs) (31), recovered overnight, and handpicked into 24-well plates. Equivalent numbers of islets (~30) were put into each well in growth medium without phenol red. Alamar Blue (BioSource) was added (5%) to determine islet viability and basal fluorescence measured for islet content (excitation = 535 nm, emission = 595 nm). Islets were washed and exposed to 300 μmol/L Zn2+ for 20 h or a mixture of IL-1β, TNFa, and IFNy (10, 40, 50 μg/L) for 30 h (with or without 60 mmol/L pyruvate, nicotinamide, NAD+) in DMEM + 15% FBS. Higher concentrations of exogenous Zn2+ were necessary to induce death of islets, because 15% FBS is necessary for islet survival but binds a substantial quantity of Zn2+. Alamar Blue was re-added and fluorescence directly measured after 4 h (32). Death was normalized and expressed as a percentage of the complete death induced by 20 μmol/L A23187 (Ca2+ ionophore), and significance was tested using 1-way ANOVA (Expt. 3). NAD+ increased the basal Alamar Blue signal and therefore sham wash + NAD+ Alamar Blue fluorescence was used as 100% viability for the toxicity in the presence of NAD+. Determination of NAD+ concentrations. For the NAD+ measurements, 1.67 × 106 MIN6 cells or 50 islets were lysed by the addition of hot 75% ethanol/0.05 mol/L K2HPO4 after a 3-h exposure to 300 μmol/L Zn2+ or 15 mmol/L streptozotocin in DMEM + 15% FBS (MIN6 only) as previously described (10). We used the same exposure to 300 μmol/L Zn2+ in DMEM + 15% FBS for MIN6 cultures for comparison of NAD+ concentrations in MIN6 cultures to NAD+ concentrations in islets and significance was tested using 1-way ANOVA (Expt. 4).

65Zn2+ accumulation. These experiments were performed as described for neurons (30). MIN6 cultures were washed in HEPES solution containing the desired drugs and 65Zn2+ at 37°C for 5–15 min (2 mL/c, 1–5 Ci/g, DuPont NEN; total Zn2+ = 20 μmol/L) in the presence or absence of 60 mmol/L K+ and voltage-gated Ca2+ channel antagonists as indicated. A 5-min depolarizing exposure and a 15-min nondepolarizing exposure.
exposure caused equivalent accumulation of $^{65}$Zn$^{2+}$ and were used as the time points for the addition of antagonists. The stimulus solution was washed out 3 times with ice-cold quencher buffer, cells lysed in SDS and counted (Supplemental Table 1), and significance was tested using 1-way ANOVA (Expt. 5).

[Zn$^{2+}$], measurements. To monitor [Zn$^{2+}$], MIN6 cultures were washed and loaded with 5 μmol/L FluoZin-3-AM (excitation = 485 nm; emission = 530 nm) for 30 min. Cultures were then exposed as indicated and cellular fluorescence measured and compared with fluorescence in the presence of a Zn$^{2+}$ ionophore (delta max) compared with fluorescence in the presence of the Zn$^{2+}$ chelator TPEN (delta min). Calculations were performed exactly as described (30) and significance was tested using 1-way ANOVA (Expt. 6). FluoZin-3 is not influenced by the Ca$^{2+}$ or magnesium concentrations but weakly responds to other transition metals like lanthanum, mercury, or cadmium (33).

Colonial maintenance and trials. The NOD inbred mouse strain (Taconic) was maintained at Louisiana State University Health Sciences Center’s animal facility. Housing, killing, and anesthesia for all animals concurred with the institutional Animal Studies Committee guidelines and the Public Health Service Guide for the Care and Use of Laboratory Animals. Mice were killed by CO2 asphyxiation followed by thoracotomy. The animal handler and tissue processor were unaware of the treatment conditions. Treatment began on 11 groups of age-matched, female, NOD mice (higher disease penetrance in females) at 6 wk of age. Plastic cages and water bottles with no metal were used for the duration to minimize Zn$^{2+}$ contamination. The treatment groups tested were nonpurified diet (Harlan diet no. 2019) + saline injection, + 200 mg Zn$^{2+}$/L in the drinking water, + pyruvate (0.5 g/kg i.p. tri-weekly), and + Zn$^{2+}$ injections (100 μmol/kg/wk i.p. ZnSO$_4$). We also tested feeding a Zn$^{2+}$-deficient purified diet (TD.85419; 0.5–1.5 mg Zn$^{2+}$/kg diet, Harlan Teklad (34)) with 0, 1, 2, or 60 mg Zn$^{2+}$ (from ZnSO$_4$/kg diet supplied in purified (18 mOhm) water (Expt. 7). In addition, ZnT5 heterozygous knockout mice (24) were backcrossed to NOD mice for 10 generations followed by interbreeding to obtain ZnT5$^{−/−}$, ZnT5$^{+/−}$, and ZnT5$^{+/+}$ female mice in an NOD background, which were fed nonpurified diet (Expt. 8). Water and food ingestion and body weight were monitored weekly and did not significantly vary between groups (~5 mL of water and 5 g of food per mouse per day at 16 wk, decreasing to ~4 mL and 4 g at 32 wk). Blood glucose from fed mice was monitored every Monday afternoon starting at 10 wk; blood glucose from mice deprived of food for 6 h was determined periodically (glucose oxidase). These blood glucose samples gave qualitatively similar results. Mice acinetic with prodding or unable to eat and drink were killed and mortality was recorded; significance was tested using Kaplan-Meier estimates of the survival functions, with subsequent logrank tests and Sidak adjustment of $P$ values. The statistical analyses for survival time or time until diabetes developed were performed where the curves for each genotype or treatment were compared with wild-type littermates or the normal Zn$^{2+}$-containing diet. This was done using Kaplan-Meier estimates of the survival functions, with subsequent logrank tests and Sidak adjustment of $P$ values from multiple comparisons among the genotype or treatment conditions ($P < 0.05$) (35).

Reagents. Unless otherwise stated, all reagents were from Sigma Chemical.

Results. MIN6 [Zn$^{2+}$] concentrations increased after Zn$^{2+}$ and ROS exposure and were sensitive to voltage-gated Ca$^{2+}$ channel blockade. That toxic Zn$^{2+}$ enters β-cells first needed to be confirmed. Depolarization resulted in increased [Zn$^{2+}$], as measured by FluoZin3, which could be attenuated using voltage-gated calcium antagonists. The contribution of Zn$^{2+}$ uptake transporters was less evident in these acute uptake experiments, though the basal concentrations of both [Zn$^{2+}$], and $^{65}$Zn$^{2+}$ accumulation were partially dependent on temperature (data not shown). H$_2$O$_2$ also induced an increase in [Zn$^{2+}$], presumably by

**FIGURE 1** [Zn$^{2+}$], in MIN6 cultures exposed to Zn$^{2+}$, H$_2$O$_2$, and Ca$^{2+}$ channel antagonists (Expt. 6). MIN6 cultures were loaded with 5 μmol/L FluoZin3 for 30 min and the cells were washed (basal) and then exposed as indicated. The effects of a depolarizing Zn$^{2+}$ exposure on [Zn$^{2+}$], were determined, as was the antagonism of nimodipine. The effects of a nondepolarizing Zn$^{2+}$ exposure on [Zn$^{2+}$], was determined, as was the antagonism of mibefradil. The effects of 100 μmol/L H$_2$O$_2$ on [Zn$^{2+}$], was measured by Image J software from NIH. Four to 8 images were measured per animal and 6 animals/group. The mean intensities were calculated and compared between the treated and untreated groups by a Student’s $t$ test. Immunohistochemistry and hematoxylin/eosin staining were performed on adjacent postfixed sections.
intracellular release (Fig. 1). 65Zn2+ accumulation studies in MIN6 cultures confirmed these results using the additional Ca2+ channel antagonists Gd3+, Ni2+, or Ca2+ (Supplemental Table 1). These results agree with another report that studied [Zn2+]i in islets, dispersed islet cells, and insulinoma cell lines (23).

Pyruvate, nicotinamide, NAD+, and sirtuin inhibitors reduced Zn2+, streptozotocin, and cytokine toxicities. Compounds that attenuate Zn2+ neurotoxicity were tested in β-cells. MIN6 cultures were exposed to Zn2+ and GD under normal-density conditions or were exposed under high-density conditions to streptozotocin and cytokines as indicated; cell viability was determined by MTT staining (Fig. 2). High-density insulinoma culture conditions mimic the islet structure, allowing release of endogenous Zn2+ to attain toxic levels (3). Cytokine and streptozotocin toxicities were potentiated by preloading MIN6 cells with 10 μmol/L Zn2+, which increases [Zn2+]i to physiologic concentrations (15–20 μmol/L = [Zn2+] in plasma) (36,37). Zn2+ is present in the growth medium at 3–6 μmol/L, because it is present in FBS at 25–40 μmol/L (38). Optimized concentrations of compounds that reduce [Zn2+]i, (Zn2+ chelation and L- and T-type Ca2+ channel antagonists) or restore NAD+ (pyruvate, nicotinamide, and NAD+) attenuated Zn2+, cytokine, and streptozotocin-mediated deaths. Pyruvate was most effective against Zn2+ toxicity, whereas nicotinamide and NAD+ were most effective against streptozotocin toxicity. The partial efficacy of the Ca2+ channel antagonists (nimodipine and mibebradil) mimics the partial efficacy of the Zn2+ chelators (CaEDTA or TPEN) (3). This suggests that ~50% of these injuries are attributable to Zn2+ toxicity. In addition, optimized concentrations of the sirtuin pathway inhibitors sirtinol and Naph attenuated these injuries. The sirtuin pathway activator, resveratrol, or the NAD+ antagonist 3-AP potentiated these injuries. Pyruvate and lactate prevented GD-mediated death; however, lactate, which cannot regenerate NAD+, was ineffective against streptozotocin, Zn2+, and cytokine exposures. NAD+ and nicotinamide did not

TABLE 1 Islet viability after Zn2+ or mixed cytokine exposures treated with pyruvate, NAD+, and nicotinamide (Expt. 3)1

<table>
<thead>
<tr>
<th>Exposure condition</th>
<th>Islet viability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 5.7</td>
</tr>
<tr>
<td>300 μmol/L Zn2+</td>
<td>18.5 ± 5.4*</td>
</tr>
<tr>
<td>Zn2+ + 10 mmol/L pyruvate</td>
<td>103 ± 7.9*</td>
</tr>
<tr>
<td>Zn2+ + 10 mmol/L NAD+</td>
<td>108 ± 8.6*</td>
</tr>
<tr>
<td>Zn2+ + 10 mmol/L nicotinamide</td>
<td>40.1 ± 8.3*</td>
</tr>
<tr>
<td>Zn2+ + 10 mmol/L lactate</td>
<td>22.5 ± 5.8*</td>
</tr>
<tr>
<td>Mixed cytokines</td>
<td>10.1 ± 8.1*</td>
</tr>
<tr>
<td>Mixed cytokines + 10 mmol/L pyruvate</td>
<td>80.3 ± 8.9*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 5–6. *Different from control, P < 0.05; †different from Zn2+ exposure alone, P < 0.05; ‡different from mixed cytokine exposure alone, P < 0.05.
prevent GD-mediated death but did attenuate the toxin exposures. Mouse isolated islets were sensitive to Zn\(^{2+}\)- or cytokine-mediated death and pyruvate, nicotinamide, and NAD\(^{+}\) attenuated these islet injuries with efficacies similar to those reported for MIN6 cells (Table 1). Streptozotocin caused mouse islet membrane dissolution after 4.5 h, preventing determination of the toxicity of streptozotocin-released Zn\(^{2+}\). Control islets had 74\% dissolution, which is significantly different (\(n = 10\) wells).

In isolated islets and/or MIN6 cultures, pyruvate, nicotinamide, NAD\(^{+}\), and sirtinol attenuated the loss of NAD\(^{+}\) induced by Zn\(^{2+}\) and streptozotocin. The effects of \(\beta\)-cell toxicities on NAD\(^{+}\) concentrations were examined, because Zn\(^{2+}\)-neurotoxicity is mediated by the loss of NAD\(^{+}\). Both Zn\(^{2+}\) and streptozotocin exposure induced significant losses of NAD\(^{+}\) in MIN6 cells and Zn\(^{2+}\) induced significant NAD\(^{+}\) loss in isolated islets at 3 h which is before cell death (Table 2). Cytokines had a lesser effect on NAD\(^{+}\) concentrations (data not shown). Nicotinamide and NAD\(^{+}\) were the most effective at restoring NAD\(^{+}\) concentrations, followed by pyruvate and sirtinol, each of which had a significant effect on restoring NAD\(^{+}\) concentrations.

### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>NAD(^{+})</th>
<th>NAD(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (3 h)</td>
<td>3.64 ± 0.10</td>
<td>1.0 ± 0.06</td>
</tr>
<tr>
<td>300 (\mu)mol/L Zn(^{2+})</td>
<td>1.62 ± 0.22</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>Zn(^{2+}) + 10 mmol/L pyruvate</td>
<td>2.80 ± 0.24</td>
<td>0.80 ± 0.10</td>
</tr>
<tr>
<td>Zn(^{2+}) + 10 mmol/L nicotinamide</td>
<td>3.60 ± 0.24</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Zn(^{2+}) + 6 mmol/L NAD(^{+})</td>
<td>5.90 ± 0.50</td>
<td>2.35 ± 0.30</td>
</tr>
<tr>
<td>Zn(^{2+}) + 25 mmol/L sirtinol</td>
<td>2.20 ± 0.25</td>
<td>0.74 ± 0.09</td>
</tr>
<tr>
<td>Control (H, 3 h)</td>
<td>2.00 ± 0.39</td>
<td>NP</td>
</tr>
<tr>
<td>15 mmol/L streptozotocin</td>
<td>1.14 ± 0.24</td>
<td>NP</td>
</tr>
<tr>
<td>Streptozotocin + 10 mmol/L pyruvate</td>
<td>1.65 ± 0.09</td>
<td>NP</td>
</tr>
<tr>
<td>Streptozotocin + 10 mmol/L nicotinamide</td>
<td>2.87 ± 0.94</td>
<td>NP</td>
</tr>
<tr>
<td>Streptozotocin + 6 mmol/L NAD(^{+})</td>
<td>6.10 ± 1.70</td>
<td>NP</td>
</tr>
<tr>
<td>Streptozotocin + 25 mmol/L sirtinol</td>
<td>1.73 ± 0.15</td>
<td>NP</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, \(n = 6–7\). *Different from respective control concentrations; \#different from toxin exposure alone, \(P < 0.05\). HD, high-density culture; [NAD\(^{+}\)], intracellular NAD concentration; NP, not performed.

2 Quantification not possible due to streptozotocin mediated dissociation of islets.

Islet Zn\(^{2+}\) staining increased with age in NOD mice and was attenuated by reduced-zinc diet or knockout of ZnT5. Zn\(^{2+}\) staining precedes death induced by Zn\(^{2+}\) accumulation and was therefore tested in the islets of NOD mice. At 12, 15, 18, 21, and 24 wk, representative pancreatic sections were stained with hematoxylin/eosin, TSQ, ZP1, α-insulin, or anti-poly-ADP-ribose (Fig. 3; Table 3; Supplemental Fig. 2). Punctate Zn\(^{2+}\) staining increased in islets in conjunction with lymphocyte infiltration in an age-dependent fashion starting at 15 wk. Some islets (especially minus pyruvate treatment) appeared to be degranulated, suggesting ongoing injury. Islets that were densely infiltrated with lymphocytes had punctate Zn\(^{2+}\) staining and had lost insulin immunostaining (Supplemental Fig. 2). Anti-poly-ADP-ribose immunohistochemistry was not dramatically changed (data not shown). The reduced-zinc diet (ZnR) (2 mg Zn\(^{2+}\)/kg diet) or ZnT5 knockout (KO) in NOD decreased punctate and total pancreatic Zn\(^{2+}\) staining and increased insulin staining (Fig. 3; Table 3). Quantitation of the total Zn\(^{2+}\) staining of all the conditions shown (Fig. 3) gave the following results. The Zn\(^{2+}\) staining of pancreas from mice fed 60 mg Zn\(^{2+}\)/kg diet had 93.1 ± 19.2 relative fluorescence units (RFU)/mm\(^2\), whereas those from mice fed 2 mg Zn\(^{2+}\)/kg diet had 38.2 ± 11.2 RFU/mm\(^2\), which is significantly different. The Zn\(^{2+}\) staining of pancreas from ZnT5 WT in NOD mice had 99.4 ± 15 RFU/mm\(^2\), whereas those from ZnT5 KO in NOD mice had 7.8 ± 14 (\(n = 8–10\), which differs (\(P < 0.05\) by a Student’s t test).

### Figure 3

Pancreatic Zn\(^{2+}\) and insulin staining in NOD mice exposed to ZnR and ZnT5 KO (Expt. 12). Fresh frozen and dried or fixed sections of pancreas from NOD mice at 21 wk of age that were fed the Zn\(^{2+}\) diets indicated were stained with the Zn\(^{2+}\)-specific fluorescent dye ZP1 or with anti-insulin. ZnT5 KO and WT littermates were fed a normal Zn\(^{2+}\) diet or 60 mg Zn\(^{2+}\)/kg purified diet controls, primarily because the islet number increased (Fig. 3; Table 4; Supplemental Fig. 2). The insulin staining intensity also increased. Pyruvate treatment did not affect plasma cytokine concentrations, although a reduction was demonstrated for ZnR at 21 wk using a Bio-Rad multiplex. NOD mice without treatment and fed a normal Zn\(^{2+}\) diet had 295 ± 35 ng/L TNFα and 93.1 ± 5800 U/L IL-1β. NOD + pyruvate-treated mice had 230 ± 100 ng/L TNFα and 14,000 ± 5000 U/L IL-1β, which is not different. NOD + ZnR-treated mice had 200 ± 70 ng/L TNFα and 10,000 ± 5000 U/L IL-1β.
TABLE 3 Punctuate Zn²⁺ staining in aged NOD mice exposed to 60 or 2 mg/kg Zn²⁺ diet and ZnT5 KO (Expt. 11)

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>60 mg Zn²⁺/kg diet</th>
<th>60 mg Zn²⁺/kg diet + pyruvate</th>
<th>ZnT5⁻/⁻ in NOD</th>
<th>2 mg Zn²⁺/kg diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>98 ± 12</td>
<td>110 ± 19</td>
<td>NP</td>
<td>52 ± 11**</td>
</tr>
<tr>
<td>18</td>
<td>210 ± 25*</td>
<td>223 ± 28*</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>21</td>
<td>290 ± 37†</td>
<td>271 ± 30†</td>
<td>190 ± 21**</td>
<td>141 ± 25*</td>
</tr>
<tr>
<td>24</td>
<td>205 ± 38*</td>
<td>245 ± 26*</td>
<td>NP</td>
<td>NP</td>
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</tbody>
</table>

1 Values are means ± SEM, n = 5–6, of total Zn²⁺ puncta from 20 sections (per 10⁵ μm² of pancreas). *Different from 15-wk NOD, 60 mg Zn²⁺/kg diet mice, P < 0.05; † different from untreated, age-matched NOD mice, P < 0.05; ‡ different from 18-wk NOD, 60 mg Zn²⁺/kg diet mice, P < 0.05. NOD, nonobese diabetic; NP, not performed.

TABLE 4 β-Cell mass in aged NOD mice exposed to pyruvate, ZnR, and ZnT5 KO (Expt. 12)

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>60 mg Zn²⁺/kg diet (NOD)</th>
<th>NOD + pyruvate</th>
<th>ZnT5⁻/⁻ in NOD</th>
<th>2 mg Zn²⁺/kg diet (NOD)</th>
<th>260 mg ZnSO₄ (i.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>3.52 ± 0.3</td>
<td>4.10 ± 0.34</td>
<td>NP</td>
<td>3.67 ± 0.28</td>
<td>NP</td>
</tr>
<tr>
<td>21</td>
<td>1.00 ± 0.31†</td>
<td>3.49 ± 0.37†</td>
<td>2.9 ± 0.2*</td>
<td>2.03 ± 0.31**</td>
<td>0.50 ± 0.20*</td>
</tr>
<tr>
<td>24</td>
<td>0.37 ± 0.18*</td>
<td>2.67 ± 0.42*</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 5–6, of total β-cell mass from 20 sections (per 10⁵ μm² of pancreas). *Different from 15-wk NOD, 60 mg Zn²⁺/kg diet mice, P < 0.05; † different from untreated, 80 mg Zn²⁺/kg diet, age-matched NOD mice, P < 0.05. NOD, nonobese diabetic; NP, not performed.

Discussion

In this study, we have shown that: 1) Zn²⁺ entered insulinoma cells primarily through voltage-gated Ca²⁺ channels, increasing [Zn²⁺]i, and H₂O₂ also increased [Zn²⁺]; 2) blocking L- and T-type Ca²⁺ channels attenuated Zn²⁺ entry and Zn²⁺ or streptozotocin toxicity; 3) Zn²⁺ chelation, siroin inhibition, pyruvate, nicotinamide, and NAD⁺ prevented NAD⁺ loss and toxicity associated with Zn²⁺, streptozotocin, or cytokine exposures in insulinoma cultures or isolated islets; 4) punctate Zn²⁺ staining increased in the islets of aged NOD mice; 5) pyruvate attenuated the loss of β-cell mass, diabetes incidence, and death in this model, without changing lymphocyte infiltration, plasma cytokine concentrations, or Zn²⁺ staining; 6) ZnR and ZnT5 KO mice on an NOD background had decreased pancreatic Zn²⁺ staining, β-cell loss, diabetes incidence, and/or the immune response, and death in the NOD mouse model; and 7) oral or i.p. Zn²⁺ supplementation potentiated diabetes incidence and mortality.

Mechanisms of Zn²⁺ toxicity and amelioration by nicotinamide and pyruvate. In neurons, an increase in [Zn²⁺] causes a loss of NAD⁺ concentrations, resulting in a decrease in the NAD⁺: NADH ratio and inhibition of the ratio-sensitive enzymes GAPDH and pyruvate dehydrogenase, which causes triosephosphate accumulation. Pyruvate, nicotinamide, or exogenous NAD⁺ restore intracellular NAD⁺ concentrations ([NAD⁺]) and glycolytic flux and thereby attenuate death. Pyruvate is converted to lactate, regenerating NAD⁺ at the expense of NADH (9,10). A reduced Zn²⁺ diet, Zn²⁺ chelation, pyruvate, nicotinamide, or exogenous NAD⁺ prevent Zn²⁺ neurotoxicity in vitro or in vivo (where tested) after many injuries. These include: serum deprivation; Zn²⁺ or ROS exposure; global, focal, and retinal ischemias; hypoglycemia; head trauma; or target deprivation (9–11,20,39–41).

Here, we demonstrated a similar mechanism in β-cells. Pyruvate and lactate, but not nicotinamide or NAD⁺, can be used as the sole energy source in MIN6 cells. Pyruvate regenerates NAD⁺ in β-cells and is metabolized by their mitochondria (42). The inability of lactate and the ability of pyruvate, nicotinamide, or NAD⁺ to attenuate Zn²⁺, cytokine, and streptozotocin toxicities in insulinoma cultures, coupled with the diabetes incidence and mortality for female ZnT5⁻/⁻(WT)/NOD was identical to the parental NOD females. We performed a similar analysis of blood glucose, insulin and Zn²⁺ staining, and histology showing that ZnT5⁻/⁻(KO)/NOD mice had attenuated diabetes incidence, mortality, and Zn²⁺ staining while maintaining insulin staining compared with ZnT5⁻/⁻(WT)/NOD littermates (Figs. 3 and 5). The efficacy of ZnT5⁻/⁻/NOD heterozygous littermates was intermediate, demonstrating a gene dosage effect.
Studies showed that 2 mg Zn²⁺/kg diet can significantly decrease the Zn²⁺ concentration in the serum and brain and switching back to a Zn²⁺-adequate diet can restore Zn²⁺ concentrations (52,53). This ZnR diet, when used chronically, attenuated pancreatic Zn²⁺ staining and β-cell loss, whereas excess oral or i.p. Zn²⁺ potentiated diabetes incidence. These results, and those showing the efficacy of Zn²⁺ chelators against streptozotocin-induced diabetes (5), suggest that a diabetes-induced increase in β-cell Zn²⁺ accumulation is toxic. Chronic potentiation of toxicities by the NAD⁺ antagonist 3-actetylpyridine, showed that intracellular NAD⁺ concentrations are involved in the mechanism. Loss of NAD⁺ resulting in decreased glycolysis occurs in islets exposed to streptozotocin (12). The changes in [NAD⁺], were not always proportional to the changes in cell survival with each therapeutic compound, indicating that additional pathways were important for the therapeutic effects of pyruvate and sirtinol (Fig. 2A,C; Table 1). Pyruvate also inactivates H₂O₂ and activates mitochondria (43), and Sir2 proteins can mediate transcriptional modulation (44).

Nicotinamide can induce increased synthesis of NAD⁺ or decrease its degradation by NAD⁺-catabolizing enzymes (10). It is effective in the streptozotocin and NOD mouse models of T1DM (46), with partial therapeutic effects observed only if it is given to recently diagnosed T1DM patients (47). Both nicotinamide and NAD⁺ attenuated Zn²⁺, cytokine, or streptozotocin toxicities and restored [NAD⁺]. Pharmacologic, genetic, diabetes, or aging induced inhibition of the rate-limiting enzyme in NAD⁺ synthesis, nicotinamide phosphoribosyl transferase, in islets or in vivo also causes reduced NAD⁺ concentrations, reduced glucose tolerance, and reduced glucose-induced insulin secretion. These deficits could be restored by giving the product of the nicotinamide phosphoribosyl transferase enzyme (48,49). The protective effects of sirtinol and Naph and the antagonistic effects of resveratrol suggested that the NAD⁺-catabolic sirtuin pathway was involved.

Sirtuins are NAD⁺-dependent protein deacetylases that transfer modulatory acetyl groups from lysine residues of histones or transcription factors to NAD⁺, resulting in NAD⁺ catabolism and transcriptional regulation (for review, see (44)). Overexpression of SIRT1 in β-cells causes increased basal levels of glucose-induced insulin secretion (50). We showed that overexpression of SIRT1 in β-cells in vitro made them more susceptible to Zn²⁺, streptozotocin, and cytokine toxicity (51). We have implicated the sirtuin pathway in Zn²⁺ and ROS-mediated neurotoxicity (10,11). Sirtuins appeared to mediate part of the NAD⁺ loss after Zn²⁺ and streptozotocin exposures of MIN6 cells, because NAD⁺ concentrations were partially restored by sirtuin inhibition (Table 2). Transcriptional modulation may be the predominant mechanism of sirtuins against cytokines and Zn²⁺ neurotoxicity.

Previous studies showed that 2 mg Zn²⁺/kg diet can significantly decrease the Zn²⁺ concentration in the serum and brain and switching back to a Zn²⁺-adequate diet can restore Zn²⁺ concentrations (52,53). This ZnR diet, when used chronically, attenuated pancreatic Zn²⁺ staining and β-cell loss, whereas excess oral or i.p. Zn²⁺ potentiated diabetes incidence. These results, and those showing the efficacy of Zn²⁺ chelators against streptozotocin-induced diabetes (5), suggest that a diabetes-induced increase in β-cell Zn²⁺ accumulation is toxic.
Zn\(^{2+}\) deficiency can also attenuate the immune response, depending on cell type, concentration, and conditions [for review, see (54)]. We observed a reduction in lymphocyte infiltration and serum cytokine concentrations, suggesting this mechanism is involved in the beneficial effects of ZnR on diabetes. In contrast, oral Zn\(^{2+}\) supplementation studies previously suggested that Zn\(^{2+}\) may be beneficial against diabetic models. Short-term Zn\(^{2+}\) exposures may mediate beneficial effects in the periphery, rather than in the pancreas, through the insulin receptor or by induction of metallothionein II\(H\) preconditioning (55,56). That i.p. ZnSO\(_4\) supplementation had a dramatic detrimental effect only in NOD mice suggested that i.p. Zn\(^{2+}\) is more potent than dietary Zn\(^{2+}\) excess. Pancreatic Zn\(^{2+}\) concentrations are required for insulin packaging (1 mg Zn\(^{2+}\)/kg diet potentiated NOD diabetes incidence). This explains why the \(\beta\)-cell Zn\(^{2+}\) pool is one of the last labile, stainable Zn\(^{2+}\) pools to be depleted during ZnR [for review, see (57)]. However, 60 mg Zn\(^{2+}\)/kg diet increased NOD diabetes incidence compared with 2 mg Zn\(^{2+}\)/kg diet, demonstrating a sharp susceptibility curve for dietary Zn\(^{2+}\) and ongoing T1DM.

Physiologically, it is beneficial to have ~60 mg Zn\(^{2+}\)/kg diet to support the immune system, gut, and tissues that have cellular turnover [for review, see (54)]. However, pathophysiologically, in tissues that have an abundance of releasable Zn\(^{2+}\) and do not require Zn\(^{2+}\) for cellular turnover (pancreas, brain, eye), reducing dietary Zn\(^{2+}\) to just above that which is required for cell survival causes a reduction in the releasable labile Zn\(^{2+}\). Most Zn\(^{2+}\) in the body is tightly bound to proteins serving a structural or catalytic role, and this Zn\(^{2+}\) does not appreciably stain using dyes. We think the loss of labile, stainable, releasable Zn\(^{2+}\) reduces the Zn\(^{2+}\) concentration achieved in the susceptible tissues during pathological attack below that required for further toxicity (11,53,58).

Dietary Zn\(^{2+}\) deficiency occurs in many elderly adults (59), but T1DM affects primarily adolescents and young adults. Therefore, we suggest that ZnR can attenuate T1DM by reducing both the immune attack and the noninsulin-bound labile Zn\(^{2+}\) released from \(\beta\)-cells causing toxicity. ZnR may also be effective against other autoimmune diseases.

We wanted to genetically reduce pancreatic Zn\(^{2+}\) concentrations. The effects of ZnT5 KO on NOD-induced diabetes were to reduce diabetes incidence, mortality, and pancreatic Zn\(^{2+}\) staining. The effects of ZnT5 KO on the immune response are under study. ZNT5 is ubiquitously expressed in many cell types but is highly expressed in the pancreatic Golgi. Here, it is likely involved in maintaining Zn\(^{2+}\) concentrations sufficient to allow loading into newly synthesized proteins and granular packaging. ZnT5 KO mice do not develop diabetes spontaneously, though a percentage of male mice have sudden cardiac arrest and mortality. ZNT5 expression is ubiquitous but highest in pancreas (24). However, neither ZnT5 KO nor ZnT8 KO induces diabetes and both demonstrated a modest decrease in granular Zn\(^{2+}\) staining, perhaps reflecting a loss of labile Zn\(^{2+}\) (28,29). Therefore, multiple Zn\(^{2+}\) transporters maintain the Zn\(^{2+}\) concentrations necessary for sufficient insulin processing and packaging.

Our studies have implicated novel therapeutic targets for the prevention of ongoing diabetes: reduction of dietary Zn\(^{2+}\), partial Zn\(^{2+}\) chelation, modulation of Zn\(^{2+}\) transporters, inhibition of Ca\(^{2+}\) channels, maintenance of [NAD\(^{+}\)], and pyruvate supplementation. This is especially relevant, because pyruvate, Zn\(^{2+}\) chelation, and modulation of Zn\(^{2+}\) transporters also prevent the Zn\(^{2+}\)-dependent neuronal injury associated with hypoglycemia resulting from aggressive insulin therapy in animal models (41,60). Oral pyruvate is used routinely in foods and as a body-building supplement without symptoms and i.p. pyruvate has been used in multiple animal models of injuries without off-target effects (11,39,41). Pyruvate is not effective orally in attenuating injury; therefore, a pyruvate/insulin s.c. injection should be clinically tested.

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Literature Cited


