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Clioquinol attenuates zinc-dependent β-cell death and the onset of insulitis and hyperglycemia associated with experimental type I diabetes in mice

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Abstract

Zinc in the pancreas is co-released with insulin from β-cells reaching concentrations similar to those found in the vicinity of glutamatergic synapses. In the brain, the role of zinc in excitotoxic brain damage is well established. In contrast, its role in islet destruction during diabetes is poorly understood. We have studied the efficacy of zinc homeostatic proteins and an intracellular zinc chelator, clioquinol, in conferring resistance against zinc toxicity in pancreatic islets. We further assessed the ability of clioquinol to protect the islets in an experimental model of type I diabetes. Our results indicate that endogenous mechanisms for lowering $[Zn]_i$ are deficient in the insulinoma cell line, MIN6, and that permeation of Zn^{2+} triggered cell death. Application of the low affinity, intracellular zinc chelator, clioquinol, reduced Zn^{2+}-induced cell death by 80%. In addition, chelation of zinc ions by clioquinol *in vivo* prevented onset of multiple low dose streptozotocin-induced diabetes, and reduced the insulitis and hyperglycemia associated with this model. Furthermore, the glucose tolerance test (GTT) score of multiple low dose streptozotocin (MLD-STZ) mice pretreated with clioquinol was, statistically indistinguishable from that of untreated, control mice. Taken together, our results point to the potential utility of *in vivo* zinc chelation as a therapeutic strategy for treatment of idiopathic type I diabetes.

Keywords: Zinc homeostasis; Clioquinol; Zinc chelation; Diabetes; MLD-STZ

1. Introduction

Zinc's role in pancreatic islets as a structural component required for the formation of insoluble insulin hexamers is well documented (Dunn, 2005; Huang and Arvan, 1995; Zalewski et al., 1994). These Zn^{2+} ions are co-secreted with insulin after stimulation, and are released as free-Zn^{2+} into the extracellular space (Formby et al., 1984; Gee et al., 2002). The concentration of Zn^{2+} released from β-cells is of the same order of magnitude as that found in Zn^{2+}-enriched regions of the brain, where it has been linked to neuronal death in excitotoxic syndromes (Choi and Koh, 1998; Gee et al., 2002; Komatsu et al., 2005; Weiss et al., 2000). For this reason, it has been suggested that secretion and accumulation of free zinc may also play a role in the death of β-cells that precedes diabetes (Chang et al., 2003; Ishihara et al., 2003; Kim et al., 2000). Interestingly, L-type Ca^{2+} channels (LTCC), a major pathway for Zn^{2+} permeation, are abundantly expressed on islet cells and have now been shown to mediate intracellular Zn^{2+} permeation in β-cells (Gyulkhandanyan et al., 2006; Priel and Hershfinkel, 2006). Systemic chelation of Zn^{2+} by continuous perfusion of Ca-EDTA *in vivo* (Kim et al., 2000) has been shown to attenuate islet destruction and hyperglycemia induced by acute administration of streptozotocin (STZ). It has been further suggested that zinc-induced islet destruction is mediated by interference of zinc with glycolytic pathways in β-cells, leading to ATP depletion (Chang et al., 2003).

A major disadvantage of the acute streptozotocin model is that although it is effective in rapidly triggering islet cell death by acute oxidative stress, this is not the mechanism operating in the progressive autoimmune destruction of the islet, thought to underlie idiopathic type I diabetes (Strandell et al., 1988). Furthermore, while the releasable pool of zinc in the pancreas is potentially toxic, nutritional supplementation of zinc has proved to be effective in reducing the severity of diabetes in mice (Schott-Ohly et al., 2004). Hence, the physiological role or significance of zinc in diabetes is poorly understood.

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A number of studies have shown that chelation of zinc *in vivo* produces a protective effect in animal models of neurological disorders. For example, injection of the zinc chelator, Ca-EDTA, significantly reduced neuronal death resulting from experimentally-induced forebrain ischemia (Frederickson et al., 2005; Koh et al., 1996). Similarly, chelation of zinc using the membrane permeable zinc chelator, clioquinol (clioquinol), reduced the size and number of beta-amyloid plaques in a mouse model of Alzheimer's disease (Cherny et al., 2001). This chelator, formerly used as an antibiotic, has a relatively low affinity for zinc, with a short half-life of less than 3 h in tissue (Ogata et al., 1973; Toyokura et al., 1975). Several mechanisms for protecting against zinc toxicity have been described, most notable of these is the zinc transporter-1, ZnT-1, which by reducing zinc influx via the L-type calcium channel lowers intracellular Zn^{2+} accumulation (Segal et al., 2004); and the Na^{+}/Zn^{2+} exchanger, which transports Zn^{2+} out of the cell (Ohana et al., 2004). In the present study, we demonstrate that β-cells in the mouse pancreas are deficient in both ZnT-1 expression and Na^{+}/Zn^{2+} exchange activity, and therefore, lack key mechanisms to lower $[Zn^{2+}]_i$ (Ohana et al., 2004; Ohana et al., 2006; Segal et al., 2004). We have, in addition, addressed the posited role of zinc chelation (using clioquinol) in reducing damage to the islets of Langerhans following chronic administration of multiple low doses of streptozotocin (MLD-STZ). Chronic application of streptozotocin produces insulitis in mice, and is, therefore, considered a physiologically relevant model (Herold et al., 1995). Our results suggest that chelation of pancreatic zinc may be a useful intervention in preventing the onset of type I diabetes.

2. Materials and methods

2.1. Cell culture and fluorescent imaging

MIN6 (mouse insulinoma cell line) were grown in DMEM supplemented with fetal calf serum, penicillin streptomycin, L-glutamine and β-mercaptoethanol as described previously (Priel and Hershfinkel, 2006). Cells were seeded on uncoated glass coverslips for the zinc transport imaging experiment. Cells were loaded for 30 min with 5 μM Newport green-AM (Molecular Probes) or 5 μM Fura-2-AM (Tef-Labs) in 0.1% BSA in Ringer's solution. Following dye loading, cells were washed in 0.1% bovine serum albumin (BSA) in Ringer's solution, and the coverslips mounted in a chamber allowing superfusion of the cells. Newport green was excited at 480 nm, and imaged with a 510 nm long-pass filter; Fura-2 was excited at 340 nm/380 nm and imaged with a 505 nm long-pass filter. Fura-2 allows ratiometric imaging, and although it is considered a Ca^{2+}-sensitive probe, it has a 100-fold higher affinity to Zn^{2+} (Atar et al., 1995). Changes in intracellular Zn^{2+} can be easily distinguished from Ca^{2+} by the use of a Zn^{2+} intracellular chelator, such as TPEN (*N*,*N*,*N*′,*N*′-Tetrakis-(2-pyridylmethyl) ethylenediamine, 50 μM). Changes in cellular Zn^{2+} were acquired using digital imaging, and the results presented are the fluorescent signal (or ratio 340/380 nm for Fura-2) calibrated to a baseline value acquired at the beginning of the experiment, as previously described (Hershfinkel et al., 2001; Priel and Hershfinkel, 2006). All results shown are the means of four to six independent experiments, using 30–50 cells in each.

2.2. Cell viability assay

MIN6 cells, seeded on 96 well plates, were treated with Zn^{2+} (150 μM) in the presence or absence of high K^{+} (50 mM KCl replacing NaCl) for 5 min, and subsequently exposed to either clioquinol (100 μM) or clioquinol (100 μM)+EDTA (50 μM) or TPEN (20 μM) for 30 min at 37 °C. Lactate dehydrogenase (LDH) released into the extracellular medium 24 h after exposure was monitored colorimetrically as previously described (Aizenman et al., 2000) and normalized to cells treated with 2% Triton X-100 for half an hour (100% mortality). Viability was assessed using MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, colorimetric assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) according to the manufacturer's instructions. Each graph represents an average of at least three independent experiments. Statistical analysis was performed using unpaired Student's *t*-test, assuming unequal variance, comparing each treatment to zinc.

2.3. In vivo chelation studies

Six-week-old, male, ICR mice (Harlan labs, Israel) were injected (i.p.) daily for 5 consecutive days with clioquinol (30 mg/kg dissolved in dimethyl sulfoxide, DMSO, 200 μl) followed 6 h later by injection of freshly prepared streptozotocin (35 mg/kg, dissolved in citrate buffer, 100 mM pH 4.5). It is important to note that clioquinol was administered to fed mice, and food was provided for 2 additional hours following the injection. Streptozotocin administration was preceded by 4 h of fasting. Clioquinol was also administered one day before initiation of streptozotocin treatment (day 0) and in the days after completion of the streptozotocin treatment (day 6–8). Control animals were injected with either the same volumes of citrate buffer (instead of STZ) together with DMSO (instead of clioquinol) or with citrate buffer alone.

2.4. Glucose tolerance test (GTT) and blood glucose determination

All plasma glucose samples were collected from the tail vein of fed mice, and blood glucose determined using Accu-Chek Advantage glucometer (ROCHE). Groups of mice were subjected to glucose tolerance test (GTT) on day 10. Mice were fasted overnight and basal fasting glucose was measured. Additional blood samples were collected at 5, 10, 20, 30, 60 and 90 min subsequent to i.p. glucose (2 g/kg) injection.

2.5. Immunohistochemistry

Paraffin embedded sections were initially treated for 3 min in 1% trypsin at 37 °C followed by incubation in an anti-endogenous peroxidase solution containing 10% methanol, 3% H_2O_2 in dH_20 for 15 min. Pancreata from the *in vivo* studies