Zinc as a Paracrine Effector in Pancreatic Islet Cell Death

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Because of a huge amount of Zn^{2+} in secretory granules of pancreatic islet β-cells, Zn^{2+} released in certain conditions might affect the function or survival of islet cells. We studied potential paracrine effects of endogenous Zn^{2+} on β-cell death. Zn^{2+} induced insulinoma/islet cell death in a dose-dependent manner. Chelation of released endogenous Zn^{2+} by CaEDTA significantly decreased streptozotocin (STZ)-induced islet cell death in an in vitro culture system simulating in vivo circumstances but not in the conventional culture system. Zn^{2+} chelation in vivo by continuous CaEDTA infusion significantly decreased the incidence of diabetes after STZ administration. *N*-(6-methoxy-quinolyl)-para-toluenesulfonamide staining revealed that Zn^{2+} was densely deposited in degenerating islet cells 24 h after STZ treatment, which was decreased by CaEDTA infusion. We show here that Zn^{2+} is not a passive element for insulin storage but an active participant in islet cell death in certain conditions, which in time might contribute to the development of diabetes in aged people. *Diabetes* 49:367–372, 2000

Certain central neurons and pancreatic islet β-cells contain a substantial amount of chelable zinc ion (Zn^{2+}) (1,2). Neuronal Zn^{2+} is co-secreted with neurotransmitters after excitation and might be involved in the modulation of ion channel activity (3,4). Recently, reports indicating a role of endogenous Zn^{2+} in neuronal death were published. First, in vitro treatment with Zn^{2+} induced neuronal cell death (5,6). Second, Zn^{2+}, highly concentrated in synaptic vesicles of neurons (7), was shown to be translocated to degenerating postsynaptic neurons after cerebral ischemia or prolonged seizure (8,9). Finally, administration of a Zn^{2+} chelator abrogated ischemic neuronal injury (10). These findings suggest that Zn^{2+} in synaptic vesicles may be causally related to neuronal death. Similar phenomena may occur in pancreatic β-cells. Islet β-cells contain even more Zn^{2+} than do neurons. Zn^{2+} in β-cells, involved in the formation of insoluble insulin hexamer in secretory granules (11,12), is also co-secreted with insulin after stimulation with secretagogues (13). Zn^{2+} released in certain conditions may have an impact on pancreatic β-cells if significant local concentration is achieved. The current work was carried out to explore this possibility, focusing on the role of Zn^{2+} in islet cell death.

RESEARCH DESIGN AND METHODS

Cell culture. MIN6N8 cells, SV40-transformed insulinoma cells (14) (kindly provided by Prof. Miyazaki, Osaka University, Osaka, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, Rockville, MD)–15% fetal calf serum (FCS). The effect of Zn^{2+} was examined by culturing cells in the presence of $ZnSO_4$. The presence of $ZnSO_4$ up to 1 mmol/l did not significantly affect pH of the culture medium. In experiments studying the effect of Zn^{2+} without binding to proteins or amino acids, control salt solution (CSS) (120 mmol/l NaCl, 5.4 mmol/l KCl, 0.8 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, 15 mmol/l glucose, 25 mmol/l Tris-HCl, pH 7.4) (5) was used instead of culture medium. CaEDTA (Sigma, St. Louis, MO) was used to chelate Zn^{2+} and to study specific effects of Zn^{2+}, while ZnEDTA (Sigma) was used as a control reagent (10,15). In some experiments, 3×10^6 insulinoma cells in 30 µl DMEM–15% FCS were incubated in each well of V-shaped 96-well plates to achieve a cell:medium ratio of about 1:5–10 and to simulate in vivo circumstances (2). *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketon (z-VAD.fmk) (Enzyme Systems, Livermore, CA), a broad-spectrum inhibitor of multiple caspases, was added to the wells in some experiments.

Isolation of human islet cells. Human pancreatic islets were obtained from brain-dead patients by a modification of the automated method for human islet isolation (16). Briefly, 350 ml of Hanks' buffered salt solution (HBSS) containing 9.1 µmol/l collagenase solution (Liberase; Boehringer-Mannheim, Mannheim, Germany) was injected through the pancreatic duct after cannulation. The pancreas was loaded onto a stainless steel digestion chamber, and islets were separated during a continuous digestion process for 30–45 min. During the recirculation phase (flow rate, 85 ml/min), intrachamber temperature was increased at a rate of 2°C per min by flushing the solution through a stainless steel coil immersed in a water bath (50°C). The chamber containing the distended pancreas was gently agitated, and samples were taken every 2 min to monitor digestion. After 20–30 min of recirculation, digestion was stopped by diluting in 400 ml/min ice-cold HBSS. In this phase, the digested tissue was rapidly collected in sterile bottles containing 200 ml FCS. Purification of the islets was carried out over discontinuous Euro-Ficoll gradients (1.108, 1.096, 1.037, and HBSS). Purified islets were then washed twice, evaluated for purity, and counted after dithizone (Sigma) staining. To make single islet cells, islets were resuspended in 3 ml warm 53.7 µmol/l trypsin–3 mmol/l EDTA. After incubation at 37°C for 5 min and pipetting for 2–3 min, islets became invisible. Cells were then washed with warm RPMI–10% FCS at 1,500 rpm for 5 min. Single islet cells were frozen in liquid nitrogen until use. At the time of assays, single islet cells were thawed and 1.4×10^5 cells in RPMI–10% FCS were plated in each well of 96-well plates. Viability of the islet cells after thawing was >90%, as judged by trypan blue staining and acridine orange/propidium iodide staining. Informed consent was obtained from the family members of the patients.

Assays. Fifty microliters of 12.1 mmol/l 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) solution was added to each well containing 200 µl of culture medium or buffer. Precipitated crystals were dissolved in DMSO

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CSS, control salt solution; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' buffered salt solution; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ppm, parts per million; STZ, streptozotocin; TSQ, *N*-(6-methoxy-quinolyl)-para-toluenesulfonamide; z-VAD.fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketon.

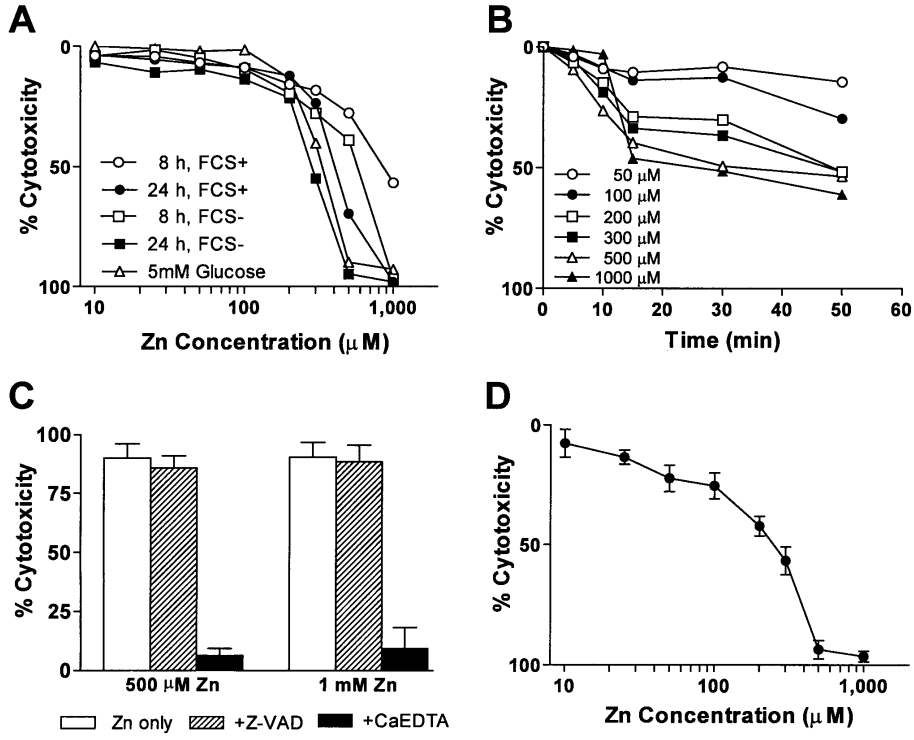

FIG. 1. Insulinoma or human islet cell death by exogenous Zn^{2+} measured with MTT assays. *A*: Zn^{2+} treatment for 8–24 h induced insulinoma cell death in DMEM–15% FCS. In the absence of FCS, insulinoma cell death was more pronounced at concentrations >200 μmol/l. Cytotoxicity measured in 5 mmol/l glucose–DMEM was essentially similar to that in DMEM (25 mmol/l glucose). *B*: In CSS buffer without Zn^{2+}-binding protein or amino acids, Zn^{2+} rapidly induced insulinoma cell death in 15 min. *C*: CaEDTA, a Zn^{2+} chelator, abrogated insulinoma cell death by Zn^{2+} treatment for 24 h in DMEM–15% FCS. z-VAD.fmk, a broad-spectrum caspase inhibitor, did not significantly affect Zn^{2+}-induced insulinoma cell death. *D*: Zn^{2+} induced death of single human islet cells as well. All values are mean ± SD from three independent experiments done in triplicate. Error bars were omitted in *A* and *B* for clarity.

(Sigma), and absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay reader. In the case of high-density culture as in Fig. 2*B*, absorbance was measured after 20-fold dilution. DMEM (25 mmol/l glucose) was used for MTT assays using MIN6N8 cells because MIN6N6 cells have been adapted to DMEM (14). In a control experiment, DMEM containing 5 mmol/l glucose (GibcoBRL) was used to rule out the effect of a high glucose concentration on the relative cytotoxicity measured by MTT assays.

Measurement of Zn^{2+} content. MIN6N8 cells were plated in 6-well plates, and Zn^{2+} release was studied after reaching confluency (~10^6 cells/well). Total cellular Zn^{2+} was measured after complete lysis of insulinoma cells in 33.2 mmol/l nonylphenoxy polyethoxy ethanol-40. Zn^{2+} concentration in the culture supernatant was measured using an inductively coupled plasma–mass spectrometry (Fison Instrument, Birmingham, U.K.) by plotting the intensity of m/z 66 in the samples against that of the standard solutions. Zn^{2+} concentration in sample blank was 122.4 nmol/l (n = 3). This was subtracted from the Zn^{2+} concentrations in the samples.

In vivo administration of Zn^{2+} chelator. Streptozotocin (STZ) (Sigma), 245.1 μmol/kg, (65 mg/kg) was administered intraperitoneally to Sprague-Dawley rats after overnight fasting. CaEDTA (10 mmol · m^{-2} · day^{-1}) in Hartman solution (Samsung Medical Center, Seoul, Korea) was started 30 min before STZ administration and continued for 24 h. Rats were immobilized in a restrainer during Ca(Zn)EDTA infusion. One hour after STZ administration, the infusion fluid was changed to Hartman–280 mmol/l glucose solution (Samsung Medical Center) containing CaEDTA, which was infused until 1 h before blood glucose measurement. Blood glucose was measured with a One Touch II blood glucose meter (LifeScan, Miltitas, CA) using the glucose oxidase method.

Staining. Cryostat sections of the fresh pancreata of 7-μm thickness were immersed in 4.5 μmol/l *N*-(6-methoxy-quinolyl)-para-toluenesulfonamide (TSQ) (Molecular Probes, Eugene, OR) –140 mmol/l sodium barbital–140 mmol/l sodium acetate buffer, pH 10.0 for 90 s before fluorescent microscopy (9). Immunohistochemistry and/or hematoxylin staining were performed on adjacent cryostat sections to correlate the changes in Zn^{2+} staining pattern with morphological changes. For immunohistochemistry, sections were incubated with appropriate dilution of anti-insulin antibody (DAKO, Carpinteria, CA) after blocking with goat serum. Incubation with biotinylated anti-guinea pig immunoglobulin G, and then with avidin-biotin-peroxidase complex (Vector, Burlingame, CA) followed. Diaminobenzidine was used as a color substrate.

Statistical analyses. Student's t test was used to compare mean values between the two groups. The binomial test was used to compare the incidences of diabetes between the two groups. P values <0.05 were regarded as statistically significant. All values were expressed as mean ± SD.

RESULTS

Islet (insulinoma) cell death by Zn^{2+}. MTT assays showed that Zn^{2+} induced the death of MIN6N8 insulinoma cells in a dose-dependent manner. Zn^{2+} treatment for 8–24 h in DMEM–15% FCS induced significant cell death (Fig. 1*A*). When similar studies were performed without FCS to study the effect of Zn^{2+} in the absence of protein binding (17), Zn^{2+} induced a similar cytotoxicity. However, cell death was more pronounced at concentrations >200 μmol/l (Fig. 1*A*). Cytotoxicity was also measured in 5 mmol/l glucose–DMEM because a high glucose concentration in DMEM might have affected MTT assays, which reflect metabolic activity of cells. However, MTT assays showed that relative cytotoxicity induced by Zn^{2+} in 5 mmol/l glucose–DMEM was essentially similar to that in DMEM, excluding such a possibility (Fig. 1*A*). In CSS, without amino acids such as cysteine and histidine that have a high unsaturated Zn^{2+}-binding capacity (17), Zn^{2+}-induced cytotoxicity was much more pronounced. Incubation with 200 μmol/l Zn^{2+} for only 10–15 min exerted a significant cytotoxicity on insulinoma cells (Fig. 1*B*), indicating that unbound Zn^{2+} is responsible for most of the insulinoma cell death. Insulinoma cell death after incubation in

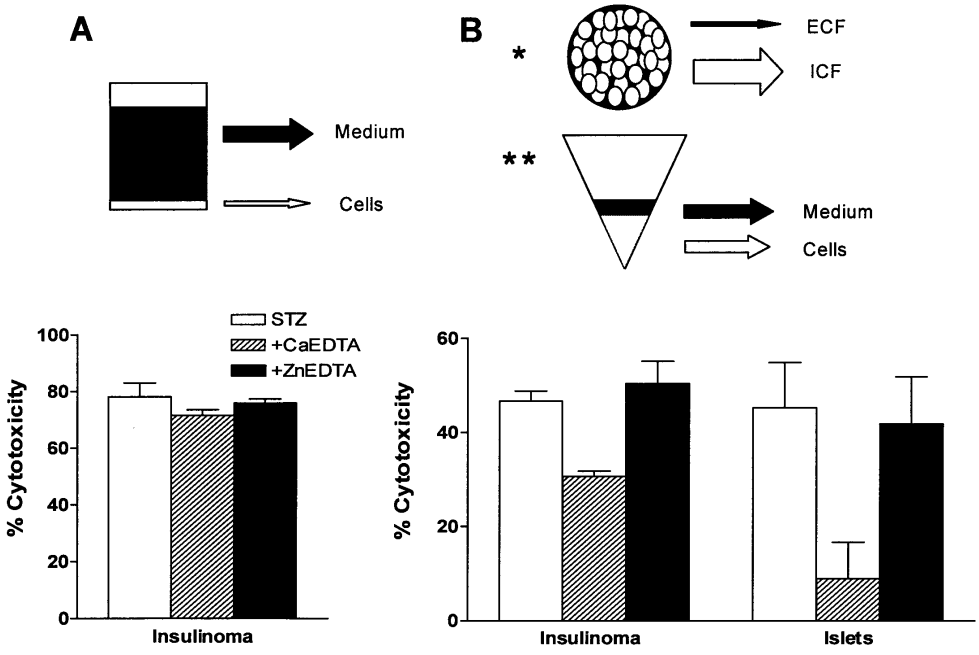

FIG. 2. Effect of Zn^{2+} chelation on STZ-induced insulinoma or islet cell death. *A*: In the conventional culture, Zn^{2+} chelation by CaEDTA did not affect STZ-induced insulinoma cell death. *B*: In a high-density culture (**) to simulate in vivo circumstances (*), Zn^{2+} chelation by CaEDTA significantly decreased STZ-induced death of insulinoma cells and human islets. ZnEDTA, a control agent, did not affect STZ-induced cell death in any condition. All values are mean ± SD from 3 or 4 independent experiments done in triplicate.

Zn^{2+}–DMEM–15% FCS for 24 h was almost completely abrogated by 5 mmol/l CaEDTA, a Zn^{2+} chelator, indicating that Zn^{2+} was directly responsible for the cytotoxicity (Fig. 1*C*). We also employed single islet cells as target cells because insulinoma cells and primary islet cells might have different susceptibility to Zn^{2+}. However, treatment of single human islet cells with Zn^{2+} for 8 h exerted a cytotoxicity similar to that on insulinoma cells (Fig. 1*D*). Because MTT assays measure metabolic activity of the cells rather than direct cytotoxicity, trypan blue exclusion assays were carried out after treatment of insulinoma cells with Zn^{2+} for 24 h in DMEM–15% FCS. The result was almost identical to that of MTT assays (data not shown). The effect of Zn^{2+} on the viability of insulinoma cells was irreversible, in that changing to new medium without Zn^{2+} after treatment with Zn^{2+} for 24 h did not affect MTT assay results (data not shown).

To see whether Zn^{2+}-induced death of insulinoma cells has characteristics of apoptosis, we studied possible DNA fragmentation after Zn^{2+} treatment. However, DNA laddering pattern was not observed (data not shown). Furthermore, z-VAD.fmk did not inhibit Zn^{2+}-induced cytotoxicity on insulinoma cells, suggesting that Zn^{2+}-induced insulinoma cell death is not a classical caspase-dependent apoptosis (Fig. 1*C*). In control experiments, z-VAD.fmk effectively inhibited apoptosis of Jurkat cells by anti-Fas antibody (data not shown).

High Zn^{2+} content in islet cells. Next, we measured Zn^{2+} content in islet cells. Total Zn^{2+} from 10^6 cells (~1 mg) was 426.7 ± 30.6 pmol (27.9 ± 2.0 ng, mean ± SD, $n = 3$) (27.9 ± 2.0 parts per million [ppm]), which is higher than that in hippocampal neurons (16 ppm) (1,2) and implies the possibility that released endogenous Zn^{2+} may reach a high ambient concentration and affect islet β-cells in certain conditions.

Effects of in vitro Zn^{2+} chelation. To study whether endogenous Zn^{2+} could indeed impose islet cell damage and act as a paracrine death effector, the STZ treatment model was used because STZ could induce Zn^{2+} release after synchronous islet cell damage. The effect of Zn^{2+} chelation by CaEDTA on STZ-induced insulinoma cell death was then examined. We chose a 15 mmol/l STZ concentration and a 6-h incubation period because they were optimal for a high-density culture. However, the addition of 5 mmol/l CaEDTA did not significantly decrease insulinoma cell death after incubation in 15 mmol/l STZ–DMEM–15% FCS for 6 h (78.2 ± 4.8% cytotoxicity with STZ alone vs. 71.6 ± 1.9% cytotoxicity with CaEDTA plus STZ; $P > 0.05$) (Fig. 2*A*). Because our failure to decrease STZ-induced cell death by CaEDTA in the conventional culture could have been due to a low cell:medium ratio (about 1:600) that allowed instantaneous dilution of released Zn^{2+} into culture medium (Fig. 2*A*), we increased the cell:medium ratio to the highest possible level to simulate in vivo circumstances (intracellular fluid:extracellular fluid volume ratio, 85:15) (2) (Fig. 2*B*). We plated 3×10^6 insulinoma cells in 30 µl DMEM–15% FCS into each well of V-shaped 96-well plates to achieve a cell:medium ratio of ~1:5–10 and studied the effects of CaEDTA on STZ-induced insulinoma cell death (Fig. 2*B*). In this high-density culture, cells became unhealthy after prolonged incubation (>16 h) due to numerous cells without sufficient culture fluid (data not shown). Thus, we chose an incubation period of 6 h instead of 24 h. To achieve sufficient cell death in 6 h, the STZ concentration of 15 mmol/l was chosen, because STZ treatment of <15 mmol/l concentration for 6 h did not induce sufficient cytotoxicity (10.7% cytotoxicity with 5 mmol/l STZ). In this condition, 5 mmol/l CaEDTA significantly decreased STZ-induced cell death from 46.7 ± 2.1% (with 15 mmol/l STZ alone) to 30.6 ± 1.1% consistently in four independent experiments (34.5% decrease on average; $P < 0.001$) (Fig. 2*B*). In control experiments, 5 mmol/l ZnEDTA that did not chelate Zn^{2+} did not inhibit STZ-induced cell death (50.4 ±

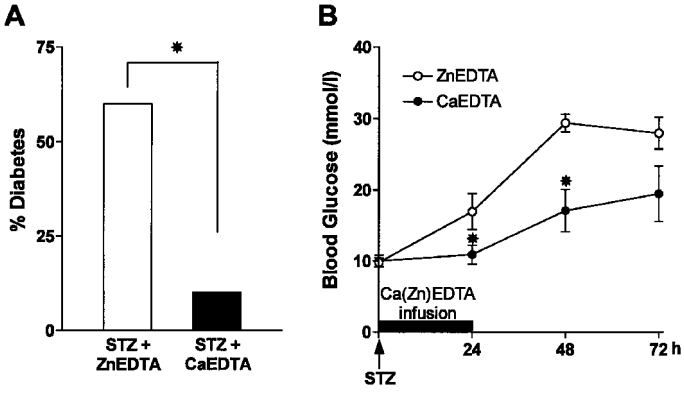

FIG. 3. Inhibition of STZ-induced diabetes by Zn^{2+} chelation in vivo. *A*: Intravenous CaEDTA infusion for 24 h significantly decreased the incidence of diabetes after STZ treatment, from 60% (ZnEDTA-infused control rats) to 10%. *B*: Blood glucose levels 24 h after STZ were significantly lower in CaEDTA-infused rats than in the control rats. In CaEDTA-infused rats, mean glucose level 24 h after STZ administration was not significantly higher than the pretreatment level. After cessation of CaEDTA infusion, blood glucose level increased. However, it did not reach that in the control rats (*$P < 0.05$).

4.8% cytotoxicity with ZnEDTA plus STZ) (Fig. 2*B*). CaEDTA alone had negligible effects on insulinoma cells (–6.2 ± 14.4% cytotoxicity).

Essentially the same study was conducted using pancreatic islets. When 800 pancreatic islets corresponding to 2.0×10^6 islet cells (18) were suspended in 30 µl culture medium and treated with 15 mmol/l STZ, 45.2 ± 9.6% cytotoxicity was observed. In the presence of 5 mmol/l CaEDTA, surprisingly only 8.9 ± 7.8% cytotoxicity was observed consistently in three independent experiments (80.3% decrease in cytotoxicty on average), suggesting that Zn^{2+} is indeed an important paracrine effector in pancreatic islet cell death in vitro ($P < 0.01$). ZnEDTA did not affect STZ-induced islet death (41.8 ± 10.0% with ZnEDTA plus STZ) (Fig. 2*B*).

Effects of in vivo Zn^{2+} chelation. To see if similar phenomena could occur in vivo, we next studied the effects of Zn^{2+} chelation on STZ-induced diabetes. When Sprague-Dawley rats were treated with STZ and CaEDTA was infused for 24 h to chelate released Zn^{2+}, only 1 of 10 rats became diabetic (>16.7 mmol/l) 24 h after STZ injection, whereas 6 of 10 control rats became diabetic with infusion of ZnEDTA (10 vs. 60%, $P < 0.05$) (Fig. 3*A*). The 60% diabetes incidence at 24 h in our control experiments (STZ + ZnEDTA) was not significantly lower than that in other control rats to which STZ alone was administered (7 of 10, 70% at 24 h after STZ) ($P > 0.1$), indicating that STZ was properly given and absorbed regardless of Ca(Zn)EDTA infusion. Mean blood glucose level 24 h after STZ administration was significantly lower in CaEDTA-infused rats compared with control rats (10.9 ± 4.2 vs. 17.0 ± 8.0 mmol/l, $n = 10$) ($P < 0.05$) (Fig. 3*B*). In CaEDTA-infused rats, mean glucose level 24 h after STZ administration was not significantly higher than the pretreatment level (10.0 ± 2.7 mmol/l, $n = 10$) ($P > 0.1$). After cessation of CaEDTA infusion, the glucose level rose to the diabetic range, suggesting that the presence of Zn^{2+} determines whether islet cells will be significantly destroyed by STZ or not. However, the glucose level in CaEDTA-infused rats did not reach that in control rats, underscoring the effect of Zn^{2+} chelation during the first 24 h after STZ administration (Fig. 3*B*).

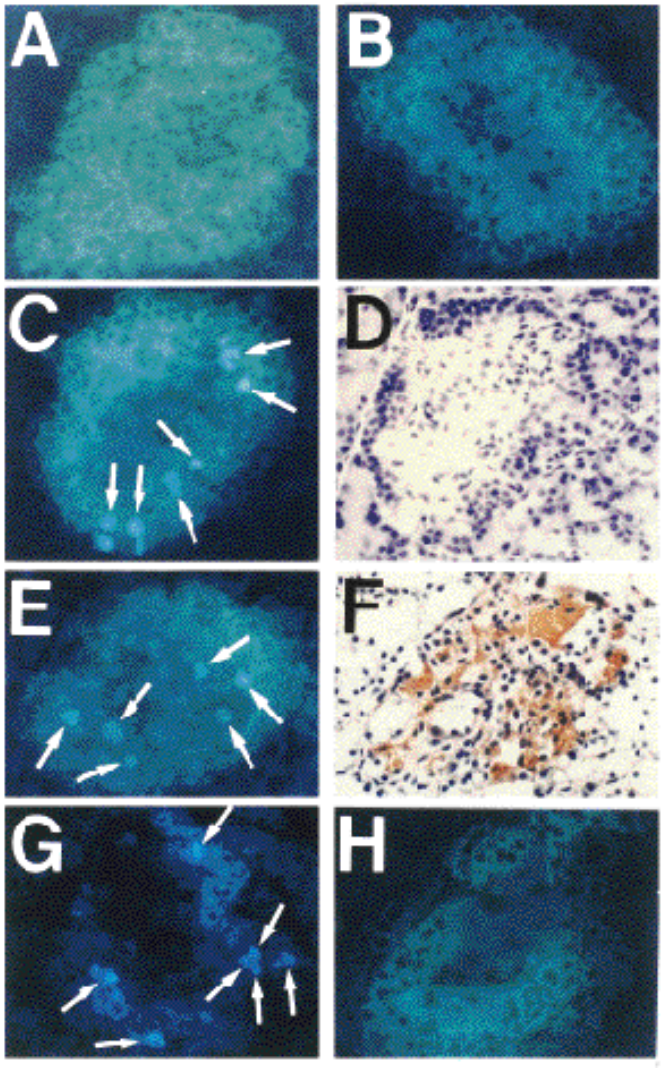

FIG. 4. Inhibition of STZ-induced Zn^{2+} translocation by CaEDTA detected with TSQ staining. *A*: Diffuse Zn^{2+} fluorescence with distinct nuclear shadow was observed in pancreatic islets of untreated rats. *B*: Four hours after STZ treatment, rarefaction of Zn^{2+} fluorescence was seen in the central area of each islet, indicating early islet cell damage. *C* and *E*: Twenty-four hours after STZ administration, rarefaction of Zn^{2+} fluorescence became more severe, and several cells with dense fluorescence but without nuclear shadow (arrows) were scattered in the pancreatic islets, indicating deposition of Zn^{2+} in islet cells and their death. Hematoxylin staining (*D*) and insulin immunostaining (*F*) on adjacent cryostat sections showed that the Zn^{2+}-negative core observed in *C* and *E* was composed of necrotic cell debris/cell-free space, and Zn^{2+} was deposited in the mantle area of each islet, probably on both β- and non–β-cells. Infusion of CaEDTA for 24 h decreased the number of cells with dense Zn^{2+} deposition after STZ treatment (*H*) compared with the control rats with ZnEDTA infusion (*G*). The number of viable cells with diffuse cellular fluorescence and distinct nuclear shadow was increased by CaEDTA infusion (*H*) compared with the control rats (*G*).

At 48 h after STZ treatment, 9 of 10 control rats (90%) with ZnEDTA infusion became diabetic, which was not different from the rate in other control rats to which STZ alone was given (9 of 10 mice, 90%), again indicating that STZ was properly given and absorbed regardless of Ca(Zn)EDTA infusion.

Translocation of Zn^{2+} after STZ administration. Because the in vitro and in vivo results suggest that Zn^{2+} released from damaged islet cells exerts cytotoxicity on adja-

cent islet cells, thus amplifying islet cell damage, we traced the movement of Zn^{2+} after STZ treatment. TSQ staining revealed diffuse Zn^{2+} fluorescence with distinct nuclear shadow in the whole pancreatic islets of untreated rats (Fig. 4*A*). Four hours after STZ treatment, fluorescence became faint in the central area of each islet, indicating Zn^{2+} release from damaged islet cells (Fig. 4*B*). Twenty-four hours after STZ administration, rarefaction of Zn^{2+} fluorescence became more severe, and some cells showed dense Zn^{2+} fluorescence without nuclear shadow, suggesting that released Zn^{2+} was deposited in some islet cells and probably induced their death (Fig. 4*C* and *E*). Hematoxylin staining (Fig. 4*D*) and insulin immunostaining (Fig. 4*F*) on cryostat sections adjacent to Fig. 4*C* and *E*, respectively, revealed that the Zn^{2+}-negative core was composed of necrotic cell debris/cell-free space and that Zn^{2+} was deposited in the mantle region of islets, probably on both β- and non–β-cells. Insulin immunoreactivity was observed not only in residual β-cells but also in some part of cell-free space, indicating that acellular debris contained insulin or its fragments (Fig. 4*F*). We finally studied the effects of CaEDTA on the Zn^{2+} deposition after STZ administration. A central dark area without Zn^{2+} fluorescence, several cells with dense Zn^{2+} deposition, and a few viable cells with distinct nuclear shadow were seen in pancreatic islets of ZnEDTA-infused rats 24 h after STZ treatment (Fig. 4*G*), similar to the rats treated with STZ alone. The number of cells with dense Zn^{2+} deposition 24 h after STZ administration was decreased by CaEDTA infusion, while the number of viable cells with distinct nuclear shadow and diffuse cellular fluorescence was increased (Fig. 4*H*), suggesting that Zn^{2+} chelation protected islet cell death after STZ administration.

DISCUSSION

First, we demonstrated that Zn^{2+} induced cytotoxicity on both insulinoma cells and islet cells in vitro. Unbound Zn^{2+} appears to be responsible for most of the insulinoma cell death, because the effect of Zn^{2+} was much more pronounced in a salt solution without Zn^{2+}-binding proteins or amino acids (17), similar to previous reports employing neuronal cells (5). The direct role of Zn^{2+} in insulinoma cell death was confirmed by an almost complete inhibition of their death by CaEDTA. CaEDTA is used as a Zn^{2+} chelator because EDTA has a much higher affinity for Zn^{2+} than for Ca^{2+}, and EDTA releases Ca^{2+} to chelate Zn^{2+} (log stability constants for EDTA-metal complexes at pH 7.0 are 5.4 for Mg^{2+}, 7.3 for Ca^{2+}, 10.9 for Fe^{2+}, 13.1 for Zn^{2+}, and 15.5 for Cu^{2+}) (10,15).

Zn^{2+} content in islet cells was higher than that in hippocampal neurons. Because islet cells contain such a high Zn^{2+} content and islet cell damage could occur at 100 μmol/l Zn^{2+} concentration in protein-free solution within a relatively short time (Fig. 1*B*), the chances of islet cells being damaged by their own Zn^{2+} could be substantial. Significance of Zn^{2+} concentration in the static culture is hard to extrapolate to in vivo circumstances where constant washout of released Zn^{2+} takes place. However, if 20% of total cellular Zn^{2+} is extruded instantaneously and intracellular fluid:extracellular fluid volume ratio of 85:15 is assumed (2), Zn^{2+} concentration will be 475 μmol/l, which could be high enough to damage islet cells, particularly in interstitial fluid without Zn^{2+}-binding protein. Furthermore, local concentration of Zn^{2+} could be much higher than the calculated values because of the close geographic relationship between individual cells in pancreatic islets.

In our experiments to study the role of endogenous Zn^{2+} on islet cell death, we observed significant inhibition of STZ-induced insulinoma or islet cell death by CaEDTA only when the culture conditions simulated in vivo circumstances. Because CaEDTA did not affect STZ-induced insulinoma cell death in the conventional culture (Fig. 2*A*), the effect of CaEDTA does not involve its possible chemical interaction with STZ. The effect of CaEDTA is not due to its possible chelation of iron, which has a higher affinity for EDTA than Ca^{2+} (15), because EDTA chelation of iron does not inhibit radical generation through Fenton reaction (19,20). However, the possibility that the capture of Fe ion by EDTA might be involved in the protection of islet cells from free radical injury cannot be completely excluded because of the complexity of FeEDTA effects on free radical reactions. CaEDTA effect is also not related to Cu^{2+} because even control ZnEDTA will bind Cu^{2+} that has a higher affinity for EDTA than Zn^{2+} (15). CaEDTA will not bind Mg^{2+} that has a lower affinity for EDTA than Ca^{2+} (15). Released Ca^{2+} from CaEDTA (<0.5 mmol/l) would not affect the viability of insulinoma cells in the presence of 2 mmol/l basal Ca^{2+} or more in the culture medium or in vivo (21). Similar degrees of cytotoxicity on insulinoma cells and human islet cells in the high-density culture by 15 mmol/l STZ, despite a higher content of secretory granules and Zn^{2+} in islet cells compared with insulinoma cells, seem to be due to a high Zn^{2+} concentration in the culture supernatant after STZ treatment of insulinoma cells reaching a plateau Zn^{2+} concentration. Indeed, cytotoxicity after treatment with 1 mmol/l Zn^{2+} for 6 h in high-density culture was ~50% for both insulinoma cells and islet cells (data not shown) instead of almost 100% killing observed in conventional culture (Fig. 1*A*). This indicates that 50% killing of insulinoma cells is almost the maximal degree of cell killing in high-density culture, and the release of more Zn^{2+} from islets than from insulinoma cells might not increase cytotoxicity. A lower cytotoxicity by 15 mmol/l STZ or 1 mmol/l Zn^{2+} in high-density culture (~50%) compared with that in conventional culture may be explained by a lower penetration of nutrients and reagents into each cell along with a lower amount of STZ per cell compared with conventional culture.

The effect of endogenous Zn^{2+} on islet cell death was also demonstrated in vivo. A significant decrease in the incidence of diabetes after STZ administration by CaEDTA indicates that Zn^{2+} chelation inhibits STZ-induced islet cell death in vivo as well as in vitro and that islet cell death, which we believe is due to STZ, is at least partly due to endogenous Zn^{2+} released from islet cells. However, Zn^{2+} is not related to the initial β-cell injury by STZ; STZ itself is responsible for the initial islet cell death. Zn^{2+} released after the initial injury would damage adjacent β-cells and probably some α-cells. Thus, mostly β-cells would be damaged and perhaps some α-cells, although we did not quantitate the damage of β-cells and α-cells. Because we used intact whole human pancreatic islets for most of our experiments, particularly in high-density culture, to maintain their physiological shape, we could not tell whether the Zn^{2+} effect observed in this study was specific for β-cells or not.

Finally, direct visualization of Zn^{2+} movement using a fluorescent Zn^{2+}-chelating dye supports the role of endogenous Zn^{2+} in islet cell death. TSQ staining demonstrated that Zn^{2+} released after STZ treatment was translocated to other islet cells, amplifying islet cell death, similar to the translocation of Zn^{2+} to postsynaptic neuronal cell body in ischemic brain

injury that causes neuronal cell death (8,9). The initial rarefaction of Zn^{2+} fluorescence at 4 h after STZ treatment could be due to massive release of granular Zn^{2+} together with insulin and/or release of cytosolic Zn^{2+} after early islet cell damage (22). Amelioration of the STZ-induced changes in TSQ staining pattern by CaEDTA strongly indicates that CaEDTA protected islet cells from Zn^{2+}-induced damage. These results are consistent with the inhibition of diabetes by Zn^{2+} chelation observed in this study and also with previous reports showing protection of neurons from ischemic injury by CaEDTA (10). In this study, pancreatic islets from three different species were used due to technical reasons, and susceptibility of islet cells to STZ might be different among different species. However, islet cells from all three species were susceptible to STZ, and the effect of Zn^{2+} chelation on STZ-induced islet cell death observed in all three species tested might strengthen, rather than weaken, our contention that Zn^{2+} amplifies islet cell damage as a paracrine effector.

In conclusion, our findings *1*) of Zn^{2+}-induced islet cell death; *2*) that a huge amount of Zn^{2+} was present in islet cells; *3*) that chelation of released Zn^{2+} inhibited islet cell death in vitro and diabetes in vivo; and *4*) that endogenous Zn^{2+} translocated to other islet cells, probably leading to their death, all suggest that Zn^{2+} in pancreatic islets is not a passive element for stabilization of insulin but an active participant in certain pathological conditions. Zn^{2+}, as a necessary component for insulin storage, could be harmful to its host (islet) cells, as is glutamate or Zn^{2+} to neuronal cells. Even though acute synchronous damage to islet cells would hardly occur in physiological conditions and islets are not easily damaged by Zn^{2+} co-secreted with insulin, the chances of islet cell damage on a minute scale even in physiological conditions might not be negligible in the long run. Relatively synchronous damage to islet β-cells may occur in type 1 diabetes, and paracrine Zn^{2+} may contribute to its rapid progression by providing supportive toxicity to islet cells. For type 2 diabetes, small islet cell damages might accumulate over a long period of time in the later stage of decreasing β-cell mass and eventually lead to grossly impaired insulin secretion and diabetes, particularly in subjects with insulin resistance and hyperinsulinemia. The vulnerability of pancreatic islet β-cells to their own Zn^{2+} could be related to the high incidence of diabetes in humans, particularly in obese or elderly subjects.

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