Overexpression of Metallothionein in Pancreatic β-Cells Reduces Streptozotocin-Induced DNA Damage and Diabetes

Hainan Chen,1 Edward C. Carlson,2 Lori Pellet,3 Jon T. Moritz,3 and Paul N. Epstein1

The release of reactive oxygen species (ROS) has been proposed as a cause of streptozotocin (STZ)-induced β-cell damage. This initiates a destructive cascade, consisting of DNA damage, excess activation of the DNA repair enzyme poly(ADP-ribose) polymerase, and depletion of cellular NAD+. Metallothionein (MT) is an inducible antioxidant protein that has been shown to protect DNA from chemical damage in several cell types. Therefore, we examined whether overexpression of MT could protect β-cell DNA and thereby prevent STZ-induced diabetes. Two lines of transgenic mice were produced with up to a 30-fold elevation in β-cell MT. Cultured islets from control mice and MT transgenic mice were exposed to STZ. MT was found to decrease STZ-induced islet disruption, DNA breakage, and depletion of NAD+. To assess in vivo protection, transgenic and control mice were injected with STZ. Transgenic mice had significantly reduced hyperglycemia. Ultrastructural examination of islets from STZ-treated mice showed that MT prevented degranulation and cell death. These results demonstrate that MT can reduce diabetes and confirm the DNA damage mechanism of STZ-induced β-cell death. Diabetes 50:2040–2046, 2001

Pancreatic β-cells (1) are extremely vulnerable to damage caused by reactive oxygen species (ROS). A striking example of β-cell vulnerability is the severe damage done by streptozotocin (STZ). This natural toxin has several destructive actions, including DNA methylation (2), protein modification (3), and ROS generation (4). STZ enters the cytoplasm via GLUT2, which is the β-cell’s glucose transporter (5). The presence of this transporter may account for part of the specific vulnerability of the β-cell. However, GLUT2 is also present in the liver and kidney, tissues that are relatively resistant to STZ damage. The much greater sensitivity of the β-cell is probably due to its very low level of antioxidant enzyme expression and activity (6–8), which leaves it unable to inactivate ROS. It has been demonstrated in vivo that overexpression of the antiapoptotic, antioxidant protein thioredoxin (9), as well as the antioxidant protein catalase (10), protects the β-cell from STZ. However, it is not possible to markedly induce these proteins in vivo. Therefore, we are exploring the potential of another antioxidant protein that is highly inducible, metallothionein (MT).

MTs are a family of low–molecular weight cysteine-rich proteins that bind heavy metals with high affinity. MT appears to play important roles in zinc homeostasis and ROS protection. Both functions are due to the presence of abundant cysteine residues. MT protects against many agents known to act through ROS, including hydrogen peroxide, radiation, glutathione depletors, adriamycin, and xanthine oxidase (11,12). MT is distinct among ROS scavengers in the breadth of its protection, in that it scavenges nitric oxide radicals (13), superoxide radicals (14), and hydroxyl radicals (15), which are the most destructive ROS species (11). MT scavenging of hydroxyl radicals is especially potent; the rate constant of MT for reaction with hydroxyl radical (11) is >100-fold higher than that of glutathione (16), and MT is 50 times more effective than glutathione in protecting DNA from hydroxyl radicals on a molar basis (17). Probably due to its antioxidant activity, MT can protect DNA against the damage produced by anticancer agents (18) and hydrogen peroxide (19). The association between cellular MT levels and sensitivity to DNA damage by hydrogen peroxide has been demonstrated in cells with increased MT levels, produced by zinc induction, and in cells with decreased MT levels, produced by MT antisense expression (19).

MT I and II genes are inducible in many tissues. Pancreatic islets exposed to zinc or STZ, either in vivo (20) or in vitro (21), demonstrate significantly increased MT content, as assayed by increased cadmium binding in islet extracts. As demonstrated by Minami et al. (22), in vivo exposure to another diabetogenic agent, alloxan, induces MT in pancreatic exocrine tissue, but the authors could not show an increase in pancreatic endocrine cells, as assessed by MT immunohistochemistry. Investigators have taken advantage of the ability of zinc to induce MT in order to assess its potential to reduce STZ-induced diabetes in mice (20) and rats (23). However, the logical inference that the protection they observed was a result of induction of MT in pancreatic β-cells may have been confounded by additional actions of zinc. Many proteins other than MT are affected by zinc (24), and zinc induces MT in cells outside

From the 1Department of Pediatrics, University of Louisville, Louisville, Kentucky; the 2Department of Anatomy and Cell Biology, University of North Dakota, Grand Forks, North Dakota; and the 3Department of Pharmacology, Physiology and Therapeutics, University of North Dakota, Grand Forks, North Dakota.

Address correspondence and reprint requests to Paul N. Epstein, Department of Pediatrics, University of Louisville, Louisville, KY 40202. E-mail: paul.epstein@louisville.edu.

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BSA, bovine serum albumin; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; KRBB, Krebs-Ringer bicarbonate buffer; MT, metallothionein; ROS, reactive oxygen species; STZ, streptozotocin.
of the pancreas that could alter the sensitivity of the islet. Finally, zinc itself has important antioxidant properties (25,26). Several recent studies have established that STZ activates a deleterious cascade of events, beginning with DNA cleavage, that lead to β-cell death (27). Extensive DNA damage by STZ activates the repair enzymepoly-(ADP-ribose) polymerase that utilizes and depletes cellular NAD⁺ (28,29). Because MT is an antioxidant that has a recognized ability to protect DNA, we proposed that MT could reduce β-cell damage produced by STZ. Our results confirm this proposition, using a highly specific genetic approach to increase β-cell MT.

RESEARCH DESIGN AND METHODS

Chemicals. STZ, collagenase (type V), Histopaque 1077, and metallothionein were obtained from Sigma (St. Louis, MO); Hank’s balanced salt solution (HBSS), RPMI-1640 medium, and fetal bovine serum (FBS) were supplied by Gibco BRL (Rockville, MD). Proteinase K was purchased from Qiagen (Valencia, CA), and 100X Cadmium was purchased from Amersham (Piscataway, NJ). Rat insulin standard was bought from Linco (St. Charles, MO). Rabbit antiserum to guinea pig insulin was purchased from Bio Genex (San Ramon, CA). Mouse anti-horse MT antibody was supplied by Dako (Carpinteria, CA). For construction of the transgene, enzymes were obtained from New England Biolabs (Beverly, MA).

Generation and identification of transgenic mice. A β-cell transgene for the overexpression of MT was designated HMT and constructed utilizing the plasmid INS.HBS kindly provided by Dr. Timothy Stewart (Genentech, CA). This plasmid contained the human insulin promoter and first intron, followed by the human MT cDNA fragment containing all introns and exons of the human MT II gene. This plasmid was designated with the insulin promoter, utilizing the BglII and HincII sites of INS.HBS. Before ligation, the NcoI and BamHI sites were blunt-ended with Klenow polymerase. After microinjection, the 4,100-bp HMT transgene was removed from plasmid sequences by cutting with HindIII and EcoRI.

FVB mice obtained from the University of North Dakota Biomedical Research Center were used to produce transgenic lines containing the HMT transgene. Standard procedures were used for producing transgenic animals. A second transgene containing a cDNA for the enzyme tyrosinase was coinjected with the HMT transgene. The enzyme tyrosinase, which produces melanin, is expressed in the pigment cells of the pancreas that could alter the sensitivity of the islet.

Electron microscopy. Isolated pancreatic islets were centrifuged to a soft pellet, fixed in Karnovsky’s fixative (35) at 4°C overnight, and the remainder of the sonicate was digested with an equal volume of 200 μg/ml proteinase K in 100 mM Tris HCl, pH 8.5, 5 mM EDTA, and 0.2% SDS at 55°C for 2 h and used for RNA/DNA quantification with picogreen using a kit from Molecular Probes. Insulin was measured with the coated tube radioimmunoassay kit from Diagnostic Products using rat insulin standards.

In vitro STZ treatment. In vitro STZ treatment was performed in 24-well tissue culture plates. Overnight incubated islets were handpicked and digested into 2 ml RPMI-1640 medium. Within 1 min before use, STZ was dissolved in 4°C 0.1 M NaOH to a concentration of 150 μM. The islets were then sonicated in 0.1 M NaOH for 30 s. Fifty microliters of the solution was used to extract islet insulin with 100 μl of ethanolic 75% (vol/vol) at 4°C overnight. The remainder of the sonicate was washed in HBSS and disrupted in 250 μl precooled 0.1 mol/l bicine (pH 7.8) by freeze/thaw, followed by sonication. The sample was centrifuged at 16,000g for 10 min, and 100 μl of the supernatant was applied for NAD⁺ determination by an alcohol dehydrogenase cycling method (33).

Briefly, 100 μl of supernatant was reacted with 100 μl freshly prepared reagent mix (to obtain final concentration of 0.1 mol/l bicine [pH 7.5], 0.5 mol/1 ethanol, 4.17 mM EDTA-Na₂, 0.83 mg/ml BSA, 0.42 mmol/l 3-[4,5-dimethyl-thiazol-2-yl]-diphenyltetrazolium bromide [MTT], 1.66 mmol/l phenazine ethosulfate, and 1.25 units/ml alcohol dehydrogenase). The reaction was carried out on a 96-well microplate. Color was developed in the dark at room temperature for 45 min, and the absorbance was read at 560 nm. Sample NAD⁺ concentrations were calculated from an NAD⁺ standard curve. Values were expressed per microgram of islet protein in the sonicate, as measured with the Pierce BCA kit.

In vivo STZ treatment. A total of 12 HMT-2 mice, 16 HMT-1 mice, and 26 control mice received a single intraperitoneal dose (220 mg/kg body wt) of freshly dissolved STZ in 0.1 mol/l sodium citrate (pH 4.5). Mice were between 13 and 17 weeks of age, and each group contained the same proportion of males and females. For the 6 days following STZ administration, blood samples were taken from a tail clip, and glucose values were measured with a Fast Take Glucometer (Lifescan).

Electron microscopy. Isolated pancreatic islets were centrifuged to a soft pellet and prepared for transmission electron microscopy by techniques previously described (34). In brief, fixation was carried out for 4–2 h in cold (4°C) Karnovsky’s (35) fixative (pH 7.4). The pellets were then rinsed in 0.2 mol/l sodium cacodylate buffer, postfixed in 1% OsO₄ at 4°C (90 min), and rinsed with distilled water. Dehydration was carried out in graded ethanol and propylene oxide. Pellets were embedded in Epon/Araldite and cured for 48 h at 55°C. Epoxy blocks were thin-sectioned (silver-gray interference color) with a Diatome diamond knife. Sections were mounted on 200-mesh copper grids and stained with lead citrate and uranyl acetate (4% in 1% aqueous uranyl acetate).

Data analysis. Data are presented as means ± SE. Statistical significance for two-group analysis was performed by Student’s t test (two-tailed) and for three-group analysis was performed by analysis of variance and Dunnett’s post hoc (two-tailed) test. Computations were done using the statistical program from SPSS (version 10.0).
RESULTS

MT levels and β-cell function in MT-overexpressing mice.

Two independent transgenic lines, designated HMT-1 and HMT-2, were produced in our study. As shown in Fig. 1, islets of lines HMT-1 and HMT-2 contained 30- and 4-fold higher levels of MT, respectively, than islets of FVB. Islet-specific expression of the transgene was confirmed in line HMT-1 by immunohistochemical staining with MT-specific antibodies (Fig. 2). Figure 2 also demonstrates that insulin staining and islet morphology appeared normal in transgenic islets. Similar results were obtained with line HMT-2 (data not shown). Because several transgenes have produced unintended β-cell dysfunction (36–38), we also characterized HMT-1 and HMT-2 islets with respect to insulin content, DNA content, and glucose-stimulated insulin secretion (Table 1). Essentially, the same results were obtained from control and transgenic islets. We also performed intraperitoneal glucose tolerance tests (data not shown) that revealed no distinctions between transgenic and control animals. These results demonstrated that MT overexpression was not harmful to the β-cell.

MT protects cultured islets from STZ.

Transgenic line HMT-1 was selected for analysis of in vitro sensitivity to STZ because of its higher level of MT expression. Islets were cultured in STZ as described in RESEARCH DESIGN AND METHODS. Visual observation revealed obvious reproducible differences in the sensitivity of transgenic and control islets (Fig. 3). Control islets were visibly damaged at the lowest concentration of STZ (0.25 mmol/l) and generally disintegrated into single cells at 0.5 mmol/l. Transgenic islets did not exhibit a similar degree of damage until the STZ concentration was raised to 1 mmol/l. DNA fragmentation initiates β-cell destruction by STZ. To evaluate

![FIG. 1. MT levels in isolated islets of FVB, HMT-1, and HMT-2 transgenic mice. The transgene resulted in a multifold increase of MT production. Data were calculated from four or more assays using ≥100 islets each. *P < 0.05 vs. FVB.](image)

![FIG. 2. Staining of FVB and HMT-1 islets with antibodies to MT and insulin. The transgene markedly increased MT staining but did not appear to alter insulin staining. A (FVB) and B (HMT-1) show MT staining. C (FVB) and D (HMT-1) show insulin staining. Magnification ×200.](image)

![FIG. 3. Photomicrographs of FVB and HMT-1 islets after STZ treatment in vitro. In HMT-1 islets, MT overexpression reduced the disruption to morphology. The dose of STZ is shown on the left. Similar results were obtained in seven independent experiments. Magnification ×200.](image)

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<th>Characteristics of control and transgenic islets</th>
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Data are means ± SE. Results were calculated from at least nine assays. No significant differences were found between groups.
whether MT reduced this damage, islet DNA was examined as shown in Fig. 4. Decrease in the strength of the band running above 48 kb was taken as the primary indicator of STZ-induced DNA damage. MT clearly protected this high–molecular weight band, particularly at the lower doses of STZ. In control islets, the high–molecular weight DNA declined at concentrations between 0.25 and 0.5 mmol/l STZ. In islets overexpressing MT, this degree of degradation did not occur until a concentration of 1.0 mmol/l STZ was reached. DNA protection by MT was found in seven consecutive experiments. These results demonstrated that MT increases the threshold for DNA damage. However, this protection was abrogated by approximately doubling the level of STZ. In no instance did we see a DNA ladder characteristic of apoptosis. DNA damage by STZ occurs at largely random sites (2,39), due to chemical attack rather than regularly spaced sites produced by enzymatic cleavage, as seen in apoptosis.

STZ-induced depletion of cellular NAD$^+$ is thought to be a consequence of DNA damage. Therefore, we anticipated that MT would protect cellular NAD$^+$ content. This was tested in islets exposed to STZ under the same conditions used for DNA damage analysis. As shown in Fig. 5, MT overexpression right-shifted the dose response curve for depletion of NAD$^+$. Because it is the lack of NAD$^+$ that ultimately causes the downfall of the β-cell, this result indicates that MT overexpression can prevent β-cell death and diabetes.

**MT reduces STZ-induced hyperglycemia.** Transgenic and control mice received a single injection of STZ as described in Research Design and Methods. Blood glucose values, followed for the next 6 days (Fig. 6), indicated that the onset of diabetes was delayed by the transgene, particularly in HMT-1 mice. In control animals, blood glucose values >400 mg/dl usually occurred 2 days after the administration of STZ. A similar degree of diabetes required 3 days in HMT-2 mice and did not occur during the 6-day observation period in HMT-1 mice.

**MT reduces STZ-induced β-cell degranulation and necrosis.** Morphological observations of HMT-1 and FVB islets were made on at least two preparations of each type from untreated mice and three preparations of each type from STZ-treated mice. Untreated HMT-1 islets appeared normal and were indistinguishable from FVB islets (Fig. 7A and B). Most of the islet cross-sectional area was occupied by cells, as similarly shown in published reports of insulin-producing β-cells (40). They contained prominent Golgi complexes, extensive rough endoplasmic reticulum, and numerous granules. The latter exhibited moderately dense spherical cores surrounded by a clear halo that is characteristic of insulin secretory granules. After STZ treatment, islet preparations from FVB mice administered STZ showed markedly different morphological features (Fig. 7C). In these preparations, many cells putatively identified as β-cells exhibited several features of necrosis, including vacuolization, moth-eaten mitochondria, dilated cisternae of rough endoplasmic reticulum,
and electron-lucent cytoplasm. Other apparent β-cells demonstrated various stages of degranulation but otherwise exhibited normal cytological features. Overall, β-cells in these preparations showed fewer granules than their untreated counterparts, and many were morphologically disorganized or frankly necrotic. On the contrary, non-β endocrine cells were intact, highly granulated, and in all respects indistinguishable from the same cell types in untreated control islets. In contrast, islets isolated from STZ-treated HMT-1 transgenic mice showed no evidence of necrosis, and though some β-cells were moderately degranulated, vacuolization and biomembrane discontinuities were not seen (Fig. 7D).

**DISCUSSION**

In the present study, we produced two transgenic lines that overexpressed MT in pancreatic islets. The increase in MT levels had no detrimental effect on normal function but did decrease the sensitivity of the β-cell to STZ. In vitro
assays demonstrated that elevated MT increased resistance to DNA damage and to depletion of NAD⁺. In vivo exposure to STZ showed that transgenic mice were more resistant to hyperglycemia than control mice and that transgenic islets suffered markedly less morphological damage than control islets. These results confirm that MT by itself provides significant benefit when expressed specifically in the islet.

The protective value of MT in the β-cell was anticipated because MT is an antioxidant protein (11), β-cell toxins induce it (20–22), and MT has demonstrated efficacy against DNA damage in other cell types (18). These facts led several laboratories to examine whether increased levels of MT could produce a more resistant β-cell. Initial results were very promising. Ohly et al. (21) showed that, after STZ treatment in vitro, induction of MT with zinc pretreatment helped preserve islet function and prevented diabetes induced by multiple low doses of STZ treatment in vivo (20). However, zinc has actions other than MT induction (24,25). A particularly relevant example of zinc actions that are independent of MT is shown in the report by Apostolova et al. (41), who found that, in mice lacking functional MT I and II genes, zinc treatment reduces several actions of STZ, including hypoinsulinemia and elevated blood glucose. It should be noted that the effect of zinc on diabetes was not determined in those experiments because the dose of STZ used was insufficient to produce diabetes. Our experiments used a far more specific approach than zinc pretreatment to elevate MT. We definitively confirmed the original suggestion that MT can protect β-cells.

MT overexpression reduced DNA cleavage. Protection of DNA implies a nuclear site of action for MT. However, MT is generally considered to be a cytoplasmic protein. Close inspection of our immunohistochemistry with MT antibodies (Fig. 2) showed that nuclei were less intensely stained than cytoplasm, similar to that observed with insulin staining. This is consistent with the electron microscopic localization of overexpressed MT in cardiomyocytes recently reported by Zhou and Kang (42), who found most MT in the cytoplasm, with lower levels in the nucleus. Our results imply that relatively low concentrations of MT in the nucleus can protect DNA. Alternatively, cytoplasmic MT can intercept reactive species before they enter the nucleus.

MT is a highly inducible protein. Pretreatment with agents such as endotoxins, glucocorticoids, and heavy metals can increase expression by over 10-fold in some cell types (43). This induction is similar to the 4- and 30-fold increases we obtained with our transgene. Because even the lower level of overexpression provided significant protection to the β-cell, induction of the endogenous MT genes may provide an alternative strategy for producing a more robust β-cell, without the need to insert foreign genes.

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