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Protective Role of Superoxide Dismutase against Diabetogenic Drugs

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ABSTRACT   Copper-zinc superoxide dismutase (SOD) is present in relatively high concentrations in the β-cells of human islets. The activity of the extracted enzyme is partially inhibited upon incubation with the diabetogenic drugs alloxan, streptozotocin, or Vacor. The role of this enzyme in protecting β-cells against chemically induced diabetes was further investigated.

Incubation of intact canine islets with alloxan (0.2 mg/ml) and 4 mM glucose decreased the insulin secretory response by 87% during subsequent exposure to 28 mM glucose. Concomitantly the SOD-specific activity (units of enzyme activity per milligram immunoreactive SOD) decreased 50% in alloxan-exposed islets. When islets were protected from alloxan toxicity by including 28 mM glucose with alloxan, the insulin secretory response and SOD specific activity remained identical to controls. Thus, SOD specific activity correlates with maintenance of β-cell function.

To test the effectiveness of SOD against streptozotocin in vitro, canine islets were incubated 10 min with or without streptozotocin (0.1 mg/ml) with 4 mM glucose; their functional integrity was tested subsequently as the insulin secretory response to 28 mM glucose. Exposure to streptozotocin alone decreased the response by 70%; inclusion of SOD (1.5 mg/ml) before and during exposure to streptozotocin completely prevented this effect. Cyanide-inactivated SOD was not effective.

The potential of SOD to prevent streptozotocin-induced diabetes was tested in rats in vivo. SOD injected 10 s or 50 min before streptozotocin prevented or significantly attenuated diabetes. Injection of SOD and streptozotocin simultaneously was much less effective, and cyanide-inactivated SOD was ineffective. No protection was afforded by injection of SOD 12 or 24 h before streptozotocin.

Our results support hypotheses that (*a*) oxygen radicals mediate the β-cell toxicity of both alloxan and streptozotocin, and (*b*) β-cells may be particularly vulnerable to oxygen radical damage.

INTRODUCTION

Streptozotocin and alloxan are widely used in studies of experimental diabetes because these agents destroy the pancreatic β-cells with relative selectivity. Recent evidence indicates that the generation of superoxide radicals may mediate the cytotoxic effects of these drugs on β-cells although this has only been clearly demonstrated for alloxan (1).

Superoxide dismutase (superoxide oxidoreductase, E.C. 1.15.1.1., SOD)[1] is a widely distributed enzyme that scavenges superoxide radicals. In eukaryotic cells CuZnSOD is primarily located in the cytosol and MnSOD in the mitochondria (2). We have previously reported that the islets of Langerhans are relatively rich in CuZnSOD (3) and observed selective localization of the enzyme in the human β-cell (4).

The diabetogenic drugs alloxan, streptozotocin, and Vacor cause partial inhibition of the biological activity of CuZnSOD, but not MnSOD, in vitro and in vivo. The in vitro inhibition was shown with the purified erythrocyte enzyme as well as with intact erythrocytes or the enzyme extracted from islets. The in vivo inhibition was relatively tissue specific and was observed in erythrocytes and retinae of the rat after injection of streptozotocin or alloxan but not in a number of other tissues studied (5). No information is available concerning the in vivo effects of diabetogenic agents on the SOD activity of the β-cell.

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[1] *Abbreviations used in this paper:* ANOVA, analysis of variance; CNSOD, cyanide-inactivated superoxide dismutase; GTT, glucose tolerance test; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SOD, superoxide dismutase.

The above experiments suggested that the diabetogenic drugs may exert their cytotoxic effects in part by inhibiting SOD in β-cells. Administration of a glucose load before alloxan is known to prevent its diabetogenic effect (6). However, injection of glucose before alloxan prevented diabetes but not the alloxan-induced inhibition of erythrocyte SOD (5). Evidently, if SOD inhibition plays a role in the diabetogenic effect of alloxan, glucose would have to protect β-cell SOD from alloxan.

Several studies indicate that incubation of isolated pancreatic islets with chemical scavengers of oxygen radicals, including SOD, protects the islets against the toxic effects of alloxan (7). No such information is available concerning the effects of streptozotocin in vitro. Robbins et al. (8) reported that pretreatment of rats with intravenous SOD protected against streptozotocin-induced diabetes; however, a recent report by Gold et al. (9) questioned these observations. The latter authors concluded that SOD therapy was ineffective in protecting mice against streptozotocin diabetes or rat islets against streptozotocin toxicity in vitro.

The fact that β-cells contain much more SOD than α-cells or cells of the exocrine pancreas (4) suggested that this enzyme may play an important role in β-cell homeostasis. Thus, further studies concerning islet SOD and its interaction with the diabetogenic drugs seemed warranted. In this report the following questions are addressed. Does alloxan inhibit SOD in the intact islet? If so, is this inhibition prevented by high concentrations of ambient glucose? Does SOD protect β-cells against the toxic effects of streptozotocin in vitro and/or in vivo? If SOD administration protects β-cells, does it exert its effect by interacting with the diabetogenic agents before they reach the cell, by protecting cell components from oxygen radicals generated outside the cell, or by entering the β-cell?

METHODS

Reagents

Purified canine and bovine erythrocyte SOD, alloxan monohydrate, collagenase (type V), cyanuric acid, and amylase assay kits were obtained from Sigma Chemical Co. (St. Louis, MO). Streptozotocin was kindly supplied by Dr. William Dulin, Upjohn Co. (Kalamazoo, MI). Tissue culture medium 199, goat antiserum to rabbit globulin, and fetal bovine serum were obtained from Grand Island Biological Company (Grand Island, NY). Pyrogallol and sodium diethylenepentaminetetraacetic acid were obtained from Fisher Scientific Co., Pittsburgh, PA. Methoxypolyethylene glycol (Carbowax 2000) was the gift of Union Carbide Corp., S. Plainfield, NJ. Carrier-free [^{125}I]sodium iodide was obtained from Amersham Corp. (Arlington Heights, IL) and lactoperoxidase-glucose oxidase enzymobeads from Bio-Rad Laboratories, Richmond, CA. Aprotonin (Trasylol) was purchased from Mobay Chemicals (New York).

Superoxide dismutase derivatizations

Polyethylene glycol-derivatized SOD (PEG-SOD) was prepared by the procedure of Abuchowski et al. (10) by cyanuric acid coupling of SOD to methoxypolyethylene glycol (Carbowax 2000). Purity of the product was determined by gel electrophoresis on 7.5% polyacrylamide (R_f = 0.10–0.18). PEG-SOD enzyme activity was 30–40% of that of the native SOD. Cyanide-inactivated SOD (CNSOD) was prepared by reacting SOD with KCN (10 M excess) for 20 min. Absence of SOD activity was confirmed by enzyme assay. Free cyanide was removed by dialysis against distilled water (14 changes). SOD derivatives were lyophilized and stored at $-70°C$ until use.

Isolation and maintenance of pancreatic islets of Langerhans

Islets were prepared from freshly killed mongrel dogs as previously described (11–13). Islet preparations were allowed to incubate at 37°C in tissue culture medium 199 with 15% fetal bovine serum under 95% air/5% CO_2 for 16–18 h with two to three medium changes. This method results in relative enrichment of islets because much of the exocrine tissue undergoes autolysis and islet harvesting is facilitated (14). The purity of a representative islet preparation was evaluated by the insulin/amylase ratio as described by Matas et al. after overnight culture in Medium 199 (glucose 5.5 mM) and was found to be 67 μg insulin per milligram amylase (11). Viability of islets was evaluated by their response to increased glucose concentration with release of insulin (Tables I and II).

SOD assays

Activity assay. Enzymatic activity was determined by spectrophotometric assay based upon the ability of SOD to inhibit the autooxidation of pyrogallol in basic solution (15). Intraassay variability was 15±3% (n = 30) while interassay variability was 7.8±2% (n = 3). One unit of activity is the amount of enzyme that inhibits pyrogallol autooxidation by 50%.

For presentation of data, enzyme activity measurements were converted to the more widely used "xanthine oxidase" units (1 pyrogallol U = 0.333 xanthine oxidase U = 100 ng purified bovine erythrocyte SOD) (15).

Radioimmunoassay. Immunoreactive SOD was determined by competitive binding radioimmunoassay using an antibody specific for canine CuZnSOD. Rabbit antiserum to canine erythrocyte SOD was characterized on double diffusion Ouchterlony plates as described (3). Purity of antigen (98%) was established by a single protein band on polyacrylamide gels (7.5%) and by the specific activity (enzyme activity units per milligram) of the protein. Iodination was modified after the method of Marchalonis (16) by lactoperoxidase coupled to insoluble beads, which yielded ^{125}I-SOD of sp act 67–160 μCi/mg. Free iodide was separated from ^{125}I-SOD by gel filtration over Sephadex G-25. The product was identified as ^{125}I-SOD by isoelectric focusing on polyacrylamide gels (7.5%, pH 4–6) and staining for SOD enzymatic activity (17). Serial 5 mm gel slices were counted; radioactivity was associated exclusively with the SOD band. ^{125}I-SOD was 95–100% precipitable with either trichloroacetic acid (10 g/100 ml) or anticanine SOD antiserum/goat antirabbit γ-globulin.
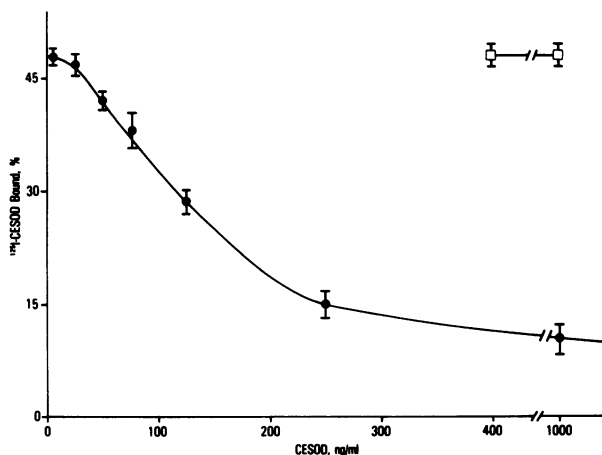
FIGURE 1 Standard radioimmunoassay for canine erythrocyte superoxide dismutase (CESOD). Canine SOD concentration, ng/ml (● —— ●); rabbit SOD concentration, ng/ml (□ —— □). Measurements are expressed as mean±SEM, n = 6. Total cpm per tube = 10,000.

In the standard assay, 33 ng ^{125}I-SOD and 100 μl rabbit anticanine SOD (1:2,000) were incubated with 50 μl unlabeled SOD (1 ng/ml to 1 μg/ml) in a total volume of 500 μl in 0.3 M borate buffer containing 5 g/100 ml bovine serum albumin, pH 8.6, at 4°C for 24 h. Immune complexes were precipitated by incubating at 4°C for 24 h with 100 μl goat antirabbit globulin (diluted 1:6 in 5% bovine serum albumin/borate). Samples were centrifuged (4,500 g, 30 min.), and the precipitate was counted in a Beckman 4000 γ-counter (Beckman Instruments, Inc., Fullerton, CA). Precipitates of blanks prepared without SOD antiserum contained 15–22% of total precipitable activity in the presence of antibody and were subtracted from all assay samples. All measurements were made in triplicate at two dilutions.

A representative curve is shown in Fig. 1; the assay is linear between 5 ng/ml and 100 ng/ml. Specificity for canine SOD was demonstrated by absence of crossreactivity with rabbit CuZnSOD. Serial dilutions were assayed for SOD enzymatic activity and immunoreactivity; specific activity (units enzyme activity per milligram immunoreactive SOD) was 97% of the expected specific activity of the purified erythrocyte enzyme.

The effect of alloxan on the radioimmunoassay was tested both by the addition of the drug (1 fM–1 M) to the standard radioimmunoassay system and by incubating SOD (0.49 μM) with the drug (40 mM) at 25°C for 15 min before radioimmunoassay and bioassay.

In vitro studies

Canine islets were rinsed thoroughly with phosphate-buffered saline (PBS) and allowed to sediment. All experiments were carried out at 37°C under incubator conditions, using ~100 islets/dish in 3 ml PBS containing 2 mg/ml BSA, 100 U/ml Trasylol, and other additions as indicated.

Alloxan effect on specific activity of canine islet SOD; protection by D-glucose. Islets were preincubated for 10 min with either low (4 mM) or high (28 mM) D-glucose. Alloxan (0.2 mg/ml) was added for 5 min to some samples.

After washing with 50 vol PBS (X3) the islets were exposed either to PBS-high glucose to stimulate insulin secretion or to PBS-low glucose to allow identification of samples releasing insulin secondary to alloxan damage. After 1 h the supernatant was decanted, and insulin in the medium was measured by radioimmunoassay with a procedure (18) modified from Morgan and Lazarow (19). The islets were sonicated in 1 ml water at 50 W for 1 min at 4°C. Insoluble cell components were removed by centrifugation at 500 g for 15 min. The supernatant was frozen at −70°C for subsequent assay of SOD enzymatic activity and immunoreactivity.

SOD protection against streptozotocin. Islets were preincubated for 10 min with or without SOD (1.5 mg/ml) or CNSOD (1.5 mg/ml), then streptozotocin (0.1 mg/ml) was added to some dishes for 10 additional min. Islets were washed in 50 vol PBS and resuspended in PBS-low glucose or PBS-high glucose. After 1 h, islets were removed and insulin in the medium was assayed.

In vivo experiments

Rats. Male Wistar rats (Charles River Breeding Laboratories, Inc., Wilmington, MA), 175–225 g, were fasted for 18 h and anesthetized with methoxyflurane before injection via the tail vein. Bovine erythrocyte SOD was administered in 100 mM sodium acetate/acetic acid buffer, pH 5.2, 105 U(=35 μg)/g body wt, 40 mg/ml. In one series one half this dose was used. PEG-SOD was assayed enzymatically and the quantity of PEG-SOD increased to deliver identical activity per gram body weight to that delivered with the native enzyme. Gram-equivalent doses of CNSOD (35 μg/g body wt) were substituted for native SOD. Streptozotocin (50 mg/kg body wt, 42 mg/ml) was solubilized immediately before injection in 100 mM sodium acetate/acetic acid buffer, pH 5.2. In one series, streptozotocin (42 mg/ml) was incubated for 15 min at 37°C in 100 mM sodium acetate/acetic acid buffer, pH 5.2, with and without SOD (40 mg/ml). Experimental animals received native or derivatized SOD at various time intervals before streptozotocin (10 s–24 h) or streptozotocin incubated with SOD. Controls were injected with one of the following: vehicle, SOD, streptozotocin, or streptozotocin incubated in vehicle.

5 d after injection, food was withheld from 4 p.m. until 10 a.m. the next morning, when glucose tolerance tests (GTT) were performed. Freely flowing blood was collected from cut tails. After obtaining a base-line sample (0 min), glucose (1 g/kg body wt, 0.25 g/ml H_2O) was injected i.p., and blood samples were taken at 15, 30, and 60 min. Animals were maintained under light methoxyflurane anesthesia for the duration of GTT. Plasma glucose was analyzed immediately by the Beckman glucose oxidase/oxygen system (Beckman Instruments, Inc., Fullerton, CA). In some experiments random nonfasting plasma glucose concentration was determined on blood samples obtained by cardiac puncture. All animals were monitored for glycosuria with TesTape (Eli Lilly & Co., Indianapolis, IN). Rats injected with streptozotocin alone had 3–4$^+$ glycosuria and random, nonfasting plasma glucose levels exceeding 300 mg/100 ml (mean 410±45, n = 5).

Nonfasting plasma glucose levels were not measured before GTT in the experiments shown in Figs. 2a and b. After an 18-h fast, the mean plasma glucose of all rats injected with streptozotocin alone was 94±7.2 mg/100 ml (n = 24) vs. 62±7 (n = 9) in controls $P < 0.02$). In view of 3–4$^+$ glycosuria and previous experience with rats injected under identical conditions with 50 mg/kg streptozotocin i.v., gly-
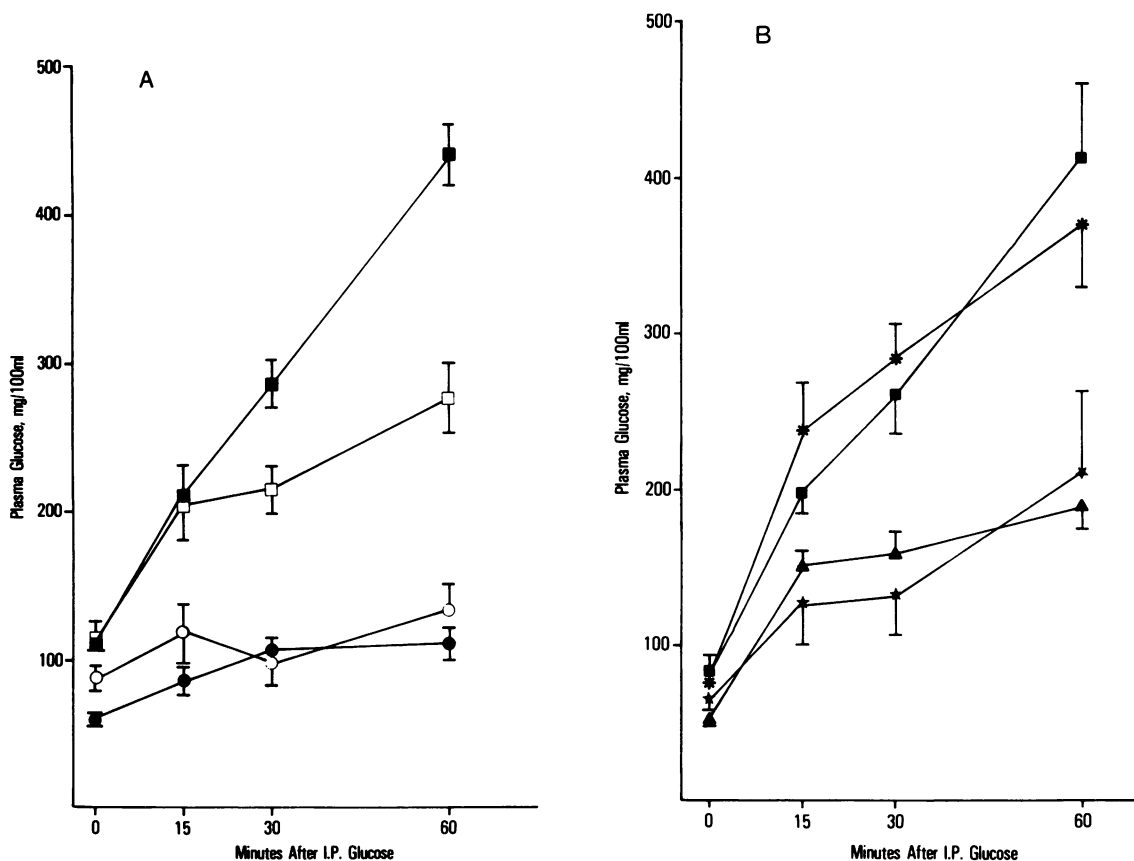
FIGURE 2 Effect of SOD treatment on streptozotocin-induced diabetes as assessed by glucose tolerance tests. Male Wistar rats, 175–225 g, were injected i.v. with agents dissolved in 0.1 M sodium acetate/acetic acid buffer, pH 5.2. GTT were performed 5 d later after an 18-h fast by i.p. injection of 1 g/kg glucose. In A, streptozotocin was incubated with or without SOD at 37°C for 15 min before injection. Rats were treated with: diluent (● —— ●), n = 9; SOD, 105 U/g (○ —— ○), n = 5; streptozotocin, 50 mg/kg (■ —— ■), n = 10; and SOD + streptozotocin (□ —— □), n = 8. In B, streptozotocin and SOD were dissolved immediately before injection, and administered sequentially. Rats were injected 10 s before streptozotocin with CNSOD, 35 µg/g (* —— *), n = 10; or with SOD, 105 U/g = 35 µg/g (★ —— ★), n = 9. SOD was injected 50 min before streptozotocin (▲ —— ▲), n = 7. Streptozotocin alone (50 mg/kg) was injected in one series (■ —— ■), n = 14. Comparing the sum of all glucose values (0–60 min) for each animal (ANOVA) streptozotocin-injected rats were significantly protected against diabetes when injected with SOD either simultaneously with streptozotocin ($P < 0.019$) or 10 s ($P < 0.0007$) or 50 min before streptozotocin ($P < 0.0015$). When each time point in the glucose tolerance tests was analyzed separately, no significant differences in fasting plasma glucose values were observed between rats injected with streptozotocin alone vs. any of the groups treated with streptozotocin and SOD. Each of the SOD treatment regimens resulted in significantly lower mean plasma glucose values 30 and 60 min after the glucose load as compared with controls treated with streptozotocin alone ($P < 0.02$–$P < 0.0001$).

cemia was probably between 250 and 500 mg/100 ml in the fed state. Decreases in glycemia of this magnitude after similar periods of food withdrawal have been documented in rats with moderate (nonketotic) diabetes (20, 21).

Mice. Male CD-1 mice (Charles River Breeding Laboratories, Inc.), 30–35 g, were fasted overnight. Bovine erythrocyte SOD (1,000 U/g body wt in 0.9 g/ml sodium chloride, 200 mg/ml) was injected into the tail vein or i.p. either immediately or 15 min before i.p. streptozotocin injection

(140 mg/kg, 20 mg/ml in 100 mM sodium acetate/acetic acid buffer, pH 5.2). Mice were tested for glycosuria and glycemia 5 d after streptozotocin.

Statistical analysis

Nonparametric analysis was performed on GTT results, defining diabetes as any plasma glucose exceeding 250 mg/

100 ml during GTT; Fisher's exact test was used (22). Parametric analysis of GTT results was performed using the sum of all glucose values (0–60 min) for a given animal; one way analysis of variance (ANOVA) was used (23). The significance of difference between multiple groups of islets with regard to insulin secretion, SOD enzyme specific activity, and radioimmunoassayable SOD content were also analyzed by ANOVA. Student's t test was used where appropriate.

RESULTS

Effect of alloxan on enzymatic vs. radioimmunoassayable activity of purified canine erythrocyte SOD. The enzymatic activity and immunoreactivity of purified erythrocyte SOD were parallel upon serial dilution. Alloxan (1 fM–1 M) had no effect on the radioimmunoassay of SOD and thus interference by the presence of free alloxan with specific and/or nonspecific binding of immune complexes was eliminated; nonspecific binding was 97±3% of control and total binding was 95±0.8% of control (n = 16). When SOD was incubated with 40 mM alloxan, enzymatic activity per milligram protein was inhibited 50% (3), resulting in a decreased ratio of enzymatic activity vs. radioimmunoassayable activity at all SOD dilutions tested. The mean specific activity of canine erythrocyte SOD was 2,866±551 U/mg for the native enzyme, and 1,288±181 U/mg for alloxan treated SOD, (n = 3, P < 0.025).

Alloxan-induced inhibition of superoxide dismutase from intact islets and protection by D-glucose. Incubation of intact canine islets with alloxan in the

presence of 4 mM D-glucose resulted in inhibition of insulin release upon subsequent challenge with 28 mM D-glucose, as described (6). The specific activity of SOD in the extracts of islets so treated was decreased by 40–60% (P < 0.001). Increasing glucose to 28 mM during incubation with alloxan prevented the alloxan-induced inhibition of insulin release as well as the decline in specific activity of SOD (Table I). Immunoreactive SOD content (nanograms per islet) was not affected by alloxan; neither the biological activity nor the immunoreactivity of SOD were altered by varying the glucose concentration in the medium.

SOD protection against streptozotocin toxicity in isolated canine islets. Incubation of isolated islets with streptozotocin resulted in inhibition of insulin release in response to a glucose stimulus (9). Islets that were exposed to SOD (1.5 mg/ml) for 10 min before addition of streptozotocin responded to glucose challenge as well as control islets not exposed to streptozotocin (P < 0.001, Table II). CNSOD did not protect the islets against streptozotocin.

In vivo protection against streptozotocin by superoxide dismutases. All rats injected with streptozotocin alone developed diabetes as defined by 4+ glycosuria and plasma glucose exceeding 250 mg/100 ml 30–60 min after injection of glucose (Table III; Fig. 2). Rats treated with vehicle or SOD alone had plasma glucose < 250 mg/100 ml during GTT. Intravenous injection of SOD 50 min before streptozotocin protected against the diabetogenic effect. Injection of

TABLE I
Alloxan-induced Inhibition of Superoxide Dismutase from Intact Islets and Protection by Glucose

Group	Pretreatment, glucose	Alloxan	Glucose challenge	Insulin response	SOD sp. act.	Normalized sp. act.*	Immunoreactive SOD
	mM		*mM*	*ng/100 islets/h*	*U/mg*	*%*	*ng/islet*
1	4	−	4	4.27±2.4	2028±332	95±15	43±7
2	4	−	28	23.8±3.7	2270±532	98±16	52±6
3	28	−	4	5.5±2.1	2347±332	104±9	46±3
4	28	−	28	28.7±9.1	2074±301	101±17	63±8
5	4	+	4	3.6±1.5	788±186	37±3	60±5
6	4	+	28	2.8±1.5	1367±251	59±7	52±8
7	28	+	4	5.5±2.2	2209±521	103±21	53±8
8	28	+	28	30.0±4.9	2539±543	115±18	42±4

Islets (100/dish) were preincubated 10 min with glucose; alloxan was then added to some dishes for an additional 10 min. Following washing the islets were incubated for 1 h with 4 or 28 mM glucose. Insulin released into the medium was measured, as well as the biological and immunoreactive SOD content of the islets. SOD specific activity = enzyme activity, U/radioimmunoassayable SOD, mg. Insulin secretion and SOD determinations are expressed as mean±SEM; n = 6–7 observations in three separate experiments.

* Since there was some variability in SOD specific activity between experiments, normalized specific activities were calculated; 100% is defined as the mean SOD specific activity of all islets in a given experiment which were not exposed to alloxan. ANOVA of SOD sp act indicates that there is no difference between groups 5 and 6; or between the remaining groups. Group 5 is different from all other groups except 6 (P < 0.005). When groups 5 and 6 are treated as one population and compared to all observations in groups 1–4 or in 7–8 the mean of group Σ5–6 is different from that of Σ1–4, or Σ7–8, P < 0.001 Student's t test.

SOD immediately (10 s) before streptozotocin resulted in a similar level of protection; the glucose tolerance of these rats was significantly improved ($P < 0.0007$) over that of rats receiving streptozotocin alone but was significantly impaired as compared with controls receiving no streptozotocin ($P < 0.01$). While i.v. injection of SOD 50 min before streptozotocin appeared to be slightly more effective than the rapid sequential administration, neither parametric nor nonparametric analysis demonstrated a significant difference between these two groups. SOD injected 12 or 24 h before streptozotocin failed to protect against diabetes. Intravenous injection of one half the standard dose of SOD 10 s before streptozotocin was also ineffective.

Cyanide-inactivated SOD injected immediately before streptozotocin had no protective effect. Preincubation of streptozotocin with SOD prior to i.v. injection offered minimal protection and was less effective than SOD injection 10 s ($P < 0.02$) or 50 min ($P < 0.03$) before streptozotocin. Incubation of streptozotocin alone in vehicle for 15 min at 37°C did not decrease its diabetogenic effect in these experiments.

PEG-SOD was ineffective in protecting against streptozotocin diabetes when injected 50 min, 6 h, or 12 h prior to streptozotocin. GTT were not performed on rats receiving PEG-SOD because all developed overt diabetes as defined by random nonfasting plasma glucose concentration of 400 mg/100 ml or greater and 4$^+$ glycosuria.

GTT correlated with glycosuria as assessed by TesTape. Rats that were protected by SOD showed 0–

TABLE II
Superoxide Dismutase Protection against Streptozotocin
Toxicity in Isolated Canine Islets

Pretreatment	Streptozotocin	Glucose stimulus	Insulin release
	0.1 mg/ml	mM	ng/100 islet/h
None	−	4	6.7±1.6 (6)
None	−	28	19.8±1.0 (9)
None	+	4	6.6±2.1 (6)
None	+	28	4.7±0.6 (9)
SOD	+	4	6.6±2.4 (6)
SOD	+	28	19.5±1.2 (9)
SOD	−	28	21.3±7.5 (3)
CNSOD	−	28	21.0±7.3 (3)
CNSOD	+	28	5.1±0.9 (3)

100 islets/dish were preincubated with 4 mM glucose for 10 min with or without SOD (1.5 mg/ml) or cyanide-inactivated SOD (1.5 mg/ml). Streptozotocin was added to some dishes for an additional 10 min; then the islets were washed and incubated for 1 h with 4 or 28 mM glucose and insulin released into the medium measured. Values are means±SEM; the number of observations is shown in parentheses.

TABLE III
In Vivo Protection of Rats against Streptozotocin
by Superoxide Dismutases

Injections		Time from SOD to Sz injection	Diabetic animals
			%
Enzyme	Sz		
SOD, 105 U/g	−		0, n = 4
−	+		100, n = 14
−	+•		100, n = 10
SOD, 105 U/g	+•	0 min	75, n = 8
SOD, 52 U/g	+	10 s	75, n = 4
SOD, 105 U/g	+	10 s	33, n = 9
SOD, 105 U/g	+	50 min	0, n = 7
SOD, 105 U/g	+	12 h	100‡, n = 6
SOD, 105 U/g	+	24 h	100‡, n = 6
CNSOD, 35 µg/g	+	10 s	100, n = 10
PEG-SOD, 105 U/g	+	50 min	100‡, n = 4
PEG-SOD, 105 U/g	+	6 h	100‡, n = 2
PEG-SOD, 105 U/g	+	12 h	100‡, n = 2

SOD, CNSOD, or PEG-SOD were administered i.v. at the times indicated before i.v. injection of streptozotocin (Sz, 50 mg/kg). Rats not receiving one of the agents were injected i.v. with diluent. Streptozotocin was dissolved immediately before injection except as designated by • where streptozotocin or a streptozotocin/SOD mixture was incubated 15 min at 37°C in 100 mM sodium acetate/acetic acid buffer, pH 5.2, before injection. In this analysis except as noted by ‡, rats were classified as diabetic if their plasma glucose exceeded 250 mg/100 ml 30 or 60 min after i.p. injection of 1 g/kg glucose following an 18-h fast on day 5 after streptozotocin injection.
‡ GTT was not performed. Animals had nonfasting plasma glucose values exceeding 400 mg/100 ml and 4$^+$ glucosuria.

1$^+$ glycosuria while unprotected streptozotocin injected rats showed 3–4$^+$ glycosuria.

Neither intravenous nor intraperitoneal SOD altered the course of acute streptozotocin diabetes in CD-1 mice ($n = 8$). All animals injected with streptozotocin had 3–4$^+$ glycosuria whether or not they received SOD.

DISCUSSION

We have previously shown that alloxan inhibits the enzymatic activity of purified erythrocyte SOD and that of SOD extracted from rat and canine islets (3). The present results demonstrate that when alloxan damages intact islets, it concomitantly inhibits SOD activity. The decrease in the biological activity of SOD after incubation of intact islets with alloxan was of the same magnitude as the maximal inhibition observed when purified erythrocyte SOD or SOD extracted from islets was incubated with increasing concentrations of the drug (3). Alloxan did not diminish immunoreactive

SOD, suggesting that normal antigenicity was retained. Because the enzyme is localized to the cytosol, alloxan or a metabolite or alloxan-generated oxygen species must have entered the cell. Hydrogen peroxide, a known inactivator of SOD (24), is known to be produced in the metabolism of alloxan (1) and may mediate this effect. It is noteworthy that inhibition of SOD activity, which can be achieved with H_2O_2 (22) or with alloxan, streptozotocin, or Vacor (3), is ~50%.

When the islets were protected from alloxan toxicity by high glucose in the medium, SOD specific activity was unaffected by alloxan. The mechanism of glucose protection is controversial. There is evidence suggesting that glucose may compete with alloxan for a membrane-associated receptor (6), an intracellular receptor (25), or that glucose may provide protective reducing equivalents inside the cell (26). We have previously shown that in a cell-free system glucose does not protect SOD from alloxan inhibition (3). Thus, the protection of SOD by glucose in intact islets would not represent a direct effect of glucose upon SOD-alloxan interaction. Our data cannot discriminate between the above hypotheses concerning the effect of glucose; however, the fact that SOD specific activity correlated with maintenance of β-cell function supports the hypothesis that inhibition of islet SOD is one of the deleterious effects whereby alloxan damages β-cells. Because the kinetics of SOD inhibition have not been characterized, it is possible that the measured 50% inhibition of enzyme activity represents an even greater loss of biological protection in the cell if, e.g., alloxan exposure decreases the affinity of the enzyme for its substrate.

In vitro, Fischer and Hamburger (27) have shown that SOD can protect beta cells against alloxan, and suggested a relationship between oxygen radical generation and alloxan toxicity. The fact that SOD completely protected islets in vitro against streptozotocin toxicity suggests that this agent may also generate oxygen radicals. In our in vitro experiments (Table II) SOD was removed before testing the islets' capacity to release insulin in response to glucose; thus the protective effect was not an artifact of increased protein concentration in the medium inhibiting insulin degradation. The failure of CNSOD to protect against streptozotocin is evidence of the specificity of the protective effect of SOD enzyme activity. Gold et al. (9) recently reported that SOD at one-tenth the dose used in the experiments reported here failed to protect rat islets in vitro against streptozotocin. However, these investigators have since observed partial protection in their system.[2] Thus, the discrepancy between these

results may represent species, i.e., dog vs. rat, or SOD dose differences.

Problems associated with the canine islet preparation have been discussed by Scharp et al. (12). Misidentification of pancreatic acinar fragments of size and shape similar to islets can lead to significant acinar contamination and overestimation of islet number. The basal and stimulated insulin secretion rates per islet reported here are lower than those observed by Downing et al. (13) in canine islets; however the ratios of basal/stimulated secretion (0.33) were identical in the two studies. The criterion of insulin/amylase ratio has been proposed (11) for the evaluation of the purity of islet preparations; reported ratios of 70–100 μg insulin per milligram amylase (11) are similar to our determination of 67 μg insulin per milligram amylase in a representative preparation.

There has been recent controversy concerning the ability of SOD to protect against streptozotocin-induced diabetes. Our in vivo experiments in rats confirm the observation of Robbins et al. (8). The differences between the experiments of Robbins et al. and Gold et al. (9) are best explained by species differences because in our hands large doses of SOD ($\times$10 greater per gram body weight than the protective dose in rats) failed to protect against streptozotocin-induced diabetes in the CD-1 mouse.

Our experiments show that the protective effect of SOD is linked to enzyme activity; cyanide-inactivated enzyme was ineffective. Protection is unlikely due to interaction or binding of streptozotocin and SOD in the circulation because preincubation of the enzyme with the drug offered much less protection than sequential injection of SOD followed by streptozotocin. Note that preincubation of streptozotocin alone at pH 5.2 did not result in significant loss of diabetogenic activity in these experiments. Rapid sequential injection of SOD followed by streptozotocin significantly improved glucose tolerance as compared with streptozotocin alone: 66% of rats so treated were partially protected from diabetes.

Injection of SOD 50 min before streptozotocin was equal or better in protective efficacy to rapid sequential injection. Because the plasma $t_{1/2}$ of the enzyme is 6 min (28), the amount of SOD remaining in the circulation after 50 min should be negligible. Thus, the persistence of the protective effect suggests that the enzyme induces prolonged metabolic changes in the β-cell or, more likely, that the enzyme enters cells and intracellular $t_{1/2}$ is longer than that in plasma. Petkau et al. (29) and Kelly et al. (30) have reported uptake of SOD into blood cells, marrow cells, hepatocytes, and kidney cells as demonstrated by the use of ^{125}I-SOD or radioimmunoassay of species specific

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[2] Grodsky, G. M. Personal communication.

SOD. Although we have no information concerning the $t_{1/2}$ of intracellular SOD, no protection was observed when SOD was injected 12 h or longer before streptozotocin.

The half life of PEG-SOD in plasma has been reported to be 35 h (28). We hoped that treatment of rats with PEG-SOD might offer continuous protection with once or twice daily injections. However, in our hands PEG-SOD was completely ineffective in protecting at 50 min, 6 h, or 12 h before streptozotocin. Since unit-equivalent doses of SOD activity were delivered, it appears likely that our PEG-SOD did not gain access to the intracellular space.

While this manuscript was in preparation, Grankvist et al. (31) reported attenuation of diabetes in mice treated with PEG-SOD 12 h before the i.v. injection of alloxan. There are several differences between Grankvist's studies and ours (e.g., rat vs. mouse, streptozotocin vs. alloxan, relative dose of diabetogenic agent). A most likely discrepancy may be in the preparation of the PEG derivative, as Grankvist observed a complex disappearance curve of PEG-SOD from serum, with an apparent $t_{1/2}$ of 6 h, as contrasted with 35 h reported by McCord and Wong (28). The derivatives may be heterogeneous and the molecular size or structure may determine their efficacy. The success of Grankvist's experiments suggest that long acting derivatives of SOD may indeed prove useful in the prophylaxis of experimental chemically induced diabetes and may be worthy of testing in other animal models of diabetes.

SOD may play a role in the pathogenesis of insulin-dependent diabetes. Known etiological factors include viral, autoimmune, and/or toxic insults (32). Recent work by Pottathil et al. (33) indicates that intracellular SOD is linked to interferon-mediated antiviral competence. Furthermore, T cell-generated lymphokines may induce oxygen radical production in target cells (34). SOD has been suggested to be required for activity of suppressor T lymphocytes (35). Thus, the effectiveness of exogenously administered SOD in protecting β-cells against insult in vitro and in vivo may reflect exquisite vulnerability of these cells to oxygen radical damage and/or a critical dependence on oxygen radical scavengers.

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