Protection by Superoxide Dismutase, Catalase, and Poly(ADP-ribose) Synthetase Inhibitors against Alloxan- and Streptozotocin-induced Islet DNA Strand Breaks and against the Inhibition of Proinsulin Synthesis*

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We have shown previously that alloxan and streptozotocin, two major diabeticogenic agents, cause DNA strand breaks in rat pancreatic islets and stimulate nuclear poly(ADP-ribose) synthetase, thereby depleting intracellular NAD level and inhibiting proinsulin synthesis (Okamoto, H. (1981) Mol. Cell. Biochem. 37, 43–61; Yamamoto, H., Uchigata, Y., and Okamoto, H. (1981) Nature 284, 284–286). In the present study, superoxide dismutase and catalase, scavengers of radical oxygens, were found to protect against islet DNA strand breaks and inhibition of proinsulin synthesis induced by alloxan. The radical scavengers did not affect islet DNA strand breaks or inhibition of proinsulin synthesis induced by streptozotocin. On the other hand, compounds that inhibit islet nuclear poly(ADP-ribose) synthetase were found to protect against alloxan- as well as streptozotocin-induced inhibition of proinsulin synthesis. The poly(ADP-ribose) synthetase inhibitors were ineffective in protection against DNA strand breaks induced by the agents. These results may provide an important clue for elucidating the prevention of insulin-dependent diabetes as well as for understanding the causes of diabetes.

Chemical compounds that selectively damage pancreatic β-cells constitute a class of diabeticogenic agents. Among such compounds, alloxan and streptozotocin exhibit the most potent diabeticogenicity, and have been widely used for induction of experimental diabetes (1–3). To understand the mechanisms of action of the typical diabeticogenic agents seems to be of great importance for elucidating not only the causes of insulin-dependent diabetes but also its prevention.

Alloxan and streptozotocin have been known to inhibit proinsulin synthesis in pancreatic islets (4, 5). With isolated islets of rats, we have recently clarified the mechanism through which the diabeticogenic compounds inhibit islet proinsulin synthesis; alloxan and streptozotocin cause DNA strand breaks to stimulate nuclear poly(ADP-ribose) synthetase, thereby depleting intracellular NAD level and inhibiting proinsulin synthesis (6, 7). More recently, we also found that islet DNA strand breaks are actually induced in vivo by administration of diabeticogenic doses of alloxan or streptozotocin to rats (8). Therefore, it is reasonable to assume that alloxan- or streptozotocin-induced inhibition of proinsulin synthesis can be protected by either preventing islet DNA strand breaks or inhibiting islet nuclear poly(ADP-ribose) synthetase.

Here we present evidence that superoxide dismutase and catalase, scavengers of radical oxygens, protect against alloxan-induced islet DNA strand breaks, thereby preventing the inhibition of proinsulin synthesis. Islet nuclear poly(ADP-ribose) synthetase inhibitors were shown to protect against inhibition of proinsulin synthesis induced by either alloxan or streptozotocin without affecting the DNA breaks. The possible mechanism by which alloxan or streptozotocin damages islet DNA is also discussed.

MATERIALS AND METHODS

Chemicals—Alloxan monohydrate was purchased from Wako Pure Chemical Industries, Osaka, Japan; streptozotocin, superoxide dismutase (from bovine blood), and catalase (from bovine liver, thymol free) were from Sigma, ~[3,4,5-3H]leucine (112 Ci/mmol) was from New England Nuclear, and nicotinamide [U-14C]adenine dinucleotide (14C-NAD) (286 mCi/mmol) was from Radiochemical Centre. Benzamide, 3-aminobenzamide, 3-nitrobenzamide, and 3-methoxybenzamide were generous gifts from Dr. H. Nakano in Research Laboratories, Chugai Pharmaceutical Co., Ltd., Tokyo. Calf thymus histone H1 was kindly supplied by Prof. Y. Nishizuka, Department of Biochemistry, Kobe University School of Medicine, Kobe.

Isolation of Pancreatic Islets—Pancreatic islets of Langerhans were isolated by the method of Okamoto et al. (9) from male Wistar rats, weighing 200 to 250 g, which were fed ad libitum.

Alkaline Sucrose Density Gradient Analysis of Islet DNA—Batches of 100 islets were preincubated at 37 °C for 30 to 60 s in 200 μl of Krebs-Ringer bicarbonate medium (pH 7.4) containing 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mg/ml of bovine serum albumin, 2.8 mM glucose in an atmosphere of 95% O2, 5% CO2 (9, 10) with or without the addition of superoxide dismutase, catalase, reduced glutathione (GSH), or inhibitors of islet poly(ADP-ribose) synthetase. Then alloxan or streptozotocin was added to the medium, and the islets were incubated for 20 min at 37 °C. Superoxide dismutase was dissolved in 0.05 M potassium phosphate buffer (pH 7.4), and catalase in 0.05 M sodium phosphate buffer (pH 7.4). Alloxan or streptozotocin was dissolved in doubly distilled water just before addition because of their relatively short half-lives (1, 2). After incubation, saline-washed islets were suspended in 50 μl of cold saline and immediately layered over 0.5 ml of lysis solution (1.0 M NaOH, 0.01 M EDTA, 1% (w/v) Triton X-100) that had just been layered over 14.8 ml of a 5 to 29% (w/v) linear sucrose gradient containing 0.3 mM NaOH, 0.7 mM NaCl, 0.01 M EDTA. On the bottom of each gradient was a 1-ml 80% (w/v) sucrose shelf. The loaded gradients were placed in the dark at room temperature for 30 min. Then the gradients were centrifuged at 26,000 rpm at 20 °C for 200 min in a Beckman SW 27.1 rotor. After centrifugation, fractions of 33 drops were collected from the gradient. DNA in each fraction was precipi-
tated by adding 2 ml of 20% cold trichloroacetic acid with 200 μg of bovine serum albumin as carrier. The precipitate was washed three times with cold 5% trichloroacetic acid, once with cold 0.1 M potassium acetate in ethanol, twice with ethanol, and then assayed for DNA content by a fluorometric method described by Kissane and Robins (11).

**Determination of Proinsulin Synthesis in Islets—**Batches of 20 islets were preincubated at 37 °C for 30 to 60 s in 100 μl of the medium in the presence or absence of superoxide dismutase, catalase, GSH, or islet poly(ADP-ribose) synthetase inhibitors. After preincubation, islets were exposed to alloxan or streptozotocin for 5 min, and further incubated for 60 min with the addition of 100 μl of the medium containing 10 μCi of [3H]leucine, and 37.2 mM glucose. The final concentration of glucose was 20 mM, at which proinsulin comprises more than half of the total proteins synthesized de novo in islets and gives a major peak on SDS-polyacrylamide gel electrophoresis (12). Incubated islets were washed three times with cold Hank’s solution, sonicated in 200 μl of 60 mM Tris-phosphoric acid buffer (pH 6.8) containing 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and heated at 100 °C for 2 min. An 80-μl aliquot of the heated sample was submitted to SDS-polyacrylamide gel electrophoresis. The amount of proinsulin synthesized was calculated by summing the [3H] radioactivity incorporated in the proinsulin fractions (12). As shown in Fig. 1, both alloxan and streptozotocin inhibited islet proinsulin synthesis in a dose-dependent manner. Proinsulin synthesis was almost completely inhibited by 1.5 to 3.0 mM alloxan or 4.0 to 8.0 mM streptozotocin. Two mmol of streptozotocin and 0.5 mmol of alloxan were found to be about equivalent with regard to 50% inhibition of proinsulin synthesis isolated islets of rats. The molar equivalence was compatible with that estimated in the in vivo experiment using whole animals (13). In the present study, we employed 0.75 to 1.0 mM alloxan or 3 mM streptozotocin, and at these concentrations of the chemicals proinsulin synthesis is inhibited to 20 to 40% of the control (1 F).

**Assay of Islet Nuclear Poly(ADP-ribose) Synthetase—**About 5000 islets were homogenized with a Kontes Micro-Tissue-Grinder in 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, 3 mM CaCl2 (14). The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C in a Sorvall HB-4 rotor. The pellet was rehomogenized and centrifuged under the same condition. The sedimented nuclei were suspended in 500 μl of 50 mM Tris-HCl (pH 7.5) containing 30% glycerol, 1 mM EDTA, 0.5 mM EGTA (14). The suspension (islet nuclear fraction) contained 70 to 100 pl. The reaction was carried out at 25 °C for 4 h with 100 pl of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 mM EGTA (14). The homogenate was centrifuged under the same condition. The sedimented nuclei were suspended in 500 μl of 50 mM Tris-phosphoric acid buffer (pH 6.8) containing 30% glycerol, 1 mM EDTA, 0.5 mM EGTA (14). The suspension (islet nuclear fraction) contained 70 to 80 μl of islet nuclear DNA, which was measured by the fluorometric method (13). The assay mixture of poly(ADP-ribose) synthetase contained 100 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 0.2 mM NAD+, 1 mM dithiothreitol, 5.8 × 10−2 μCi of [3H]NAD, histone H1 (0.2 μg/μl), and the islet nuclear fraction consisting of about 1500 ng of DNA in a final volume of 100 μl. The reaction was carried out at 25 °C for 10 min and stopped by the addition of 1 ml of cold 25% trichloroacetic acid. Acid-insoluble materials were collected on Millipore filters and washed extensively with cold 25% trichloroacetic acid. Radioactivity in the filters was counted in a toluene-based scintillation solution.

**RESULTS**

**Effect of Radical Scavengers on Alloxan- or Streptozotocin-induced Islet DNA Strand Breaks—**Islets isolated from rat pancreas were incubated with alloxan or streptozotocin for 20 min in Krebs-Ringer bicarbonate medium, and velocity sedimentation of DNA was examined in an alkaline sucrose density gradient. DNA of islets incubated without the diabetogenic agents for 20 min was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA sediments (Fig. 2a). After incubation with 1 mM alloxan for 20 min, islet DNA sedimented slower as a broad peak in the middle of the gradient (Fig. 2b), indicating that alloxan generates islet DNA strand breaks as we have described previously (6, 8). When islets were incubated together with 1 mM alloxan and 2 × 105 units/ml of superoxide dismutase, a considerable amount of DNA was found to be recovered at the position of undamaged DNA, with a concomitant decrease in the amount of DNA which sedimented in the middle of the gradient (Fig. 2d), indicating that superoxide dismutase protects against islet DNA strand breaks induced by alloxan. Catalase (100 units/ml) also significantly protected against the DNA breaks (Fig. 2d). In co-existence with both superoxide dismutase and catalase, the DNA breaks were almost completely abolished (Fig. 2d). These results show that alloxan yields radical oxygen to break islet DNA.

It has been recently suggested that reduction of alloxan to dialauric acid leads to generation of radical oxygen (15). Accordingly, we examined whether or not a reductant enhances alloxan-induced islet DNA strand breaks. As shown in Fig. 2e, co-existence of reduced glutathione (GSH) resulted in more extensive DNA fragmentation than that induced by alloxan alone. This result may also strongly indicate that alloxan-induced islet DNA strand breaks are mediated by the formation of radical oxygens.

**Fig. 1.** Dose response to alloxan or streptozotocin of proinsulin synthesis in isolated pancreatic islets. Islets were exposed to 0 to 3 mM alloxan (○); or 0 to 8 mM streptozotocin (△) for 5 min at 37 °C in the presence of 2.8 mM glucose, and then incubated for 60 min in the presence of [3H]leucine and 20 mM glucose. Proinsulin synthesis was determined as described in the text. Each point indicates the percentage of the control without the agents. 100% corresponds to 3706 cpm/60 min/islet.

**Fig. 2.** Effect of radical scavengers or reduced glutathione (GSH) on alloxan- or streptozotocin-induced islet DNA strand breaks. DNA of islets incubated with the chemicals was analyzed by velocity sedimentation in an alkaline sucrose density gradient, as described under "Materials and Methods." Each point represents the percentage of total DNA recovered; recovery was between 85 and 100%. α, DNA of islets incubated without alloxan or streptozotocin (○); b, with 1 mM alloxan (●); e, with 3 mM streptozotocin (△); d, with 1 mM alloxan and 2 × 105 units/ml of superoxide dismutase (●), with 1 mM alloxan, 2 × 105 units/ml of catalase (×), with 1 mM alloxan, 2 × 105 units/ml of superoxide dismutase (●), with 1 mM alloxan and 100 units/ml of catalase (×), with 1 mM alloxan, 2 × 105 units/ml of superoxide dismutase, and 100 units/ml of catalase (●); e, with 1 mM alloxan and 1 mM GSH (●); f, with 3 mM streptozotocin and 2 × 105 units/ml of superoxide dismutase (△). Sedimentation was from left to right. Arrow indicates the position of a bacteriophage λ DNA (3.2 × 106 daltons, New England Biolabs).
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**FIG. 3 (left). Effect of superoxide dismutase and catalase on alloxan-induced inhibition of proinsulin synthesis.** Proinsulin synthesis was determined as described under "Materials and Methods." Each point indicates the percentage of the control without alloxan (2985 cpm of [3H]proinsulin synthesized/60 min/islet). e, 1 mM alloxan; m, 1 mM alloxan and 100 units/ml of catalase. The concentration of superoxide dismutase added is shown on the abscissa.

**FIG. 4 (right). Effect of GSH on alloxan-induced inhibition of proinsulin synthesis.** Each point indicates the percentage of the control without alloxan (3402 cpm/60 min/islet). e, with 0.5 mM alloxan; o, without alloxan. The concentration of GSH added is shown on the abscissa.

**FIG. 5. Inhibition of islet nuclear poly(ADP-ribose) synthetase.** Poly(ADP-ribose) synthetase activity was determined as described under "Materials and Methods." All activities were related to that of an equivalent quantity of islet nuclear fraction without inhibitor (57.3 pmol of poly(ADP-ribose) synthesized/10 min/pg of islet nuclear DNA; this value was approximately 5-fold higher than those assayed without the addition of histone H1 (6, 14)). Benzamide (O), 3-aminobenzamide (A), 3-nitrobenzamide (m), 3-methoxybenzamide (*), theophylline (C), IBMX (a), picolinamide (c), and nicotinamide (n).

As described previously (6, 8), 3 mM streptozotocin also significantly fragmented islet DNA (Fig. 2c). However, superoxide dismutase did not prevent the DNA strand breaks (Fig. 2f).

**Protection by Radical Scavengers against Alloxan-induced Inhibition of Proinsulin Synthesis—**That islet DNA strand breaks result in significant inhibition of proinsulin synthesis has been shown by our previous study (6, 7). We next examined whether or not the radical scavengers also protect against inhibition of proinsulin synthesis induced by alloxan. As shown in Fig. 3, 1 mM alloxan decreased proinsulin synthesis to 24% of the control. Superoxide dismutase (2 x 10^5 units/ml) and catalase (100 units/ml) resulted in more significant reversion of the alloxan-induced decrease (64% of the control), but benzamide did not affect the alloxan-induced decrease (data not shown).

**Radical scavengers per se did not affect islet proinsulin synthesis at the concentrations used.** On the other hand, streptozotocin-induced inhibition of proinsulin synthesis was unaffected by the scavengers (data not shown), as were the DNA breaks by the agent (Fig. 2f).

**Enhancement by GSH of Alloxan-induced Inhibition of Proinsulin Synthesis—**In the presence of GSH, alloxan caused more extensive fragmentation of islet DNA than did alloxan without GSH (Fig. 2e). We next examined the effect of GSH on alloxan-induced inhibition of proinsulin synthesis. When islets were incubated with 0.5 mM alloxan, proinsulin synthesis was decreased to 51% of the control, whereas in the presence of 0.5 mM GSH, proinsulin synthesis was decreased to 29% of the control (data not shown).

**FIG. 6. Effect of poly(ADP-ribose) synthetase inhibitors on alloxan- or streptozotocin-induced inhibition of proinsulin synthesis.** Islets were incubated in the presence of 0.75 mM alloxan (e to d) or 3 mM streptozotocin (e to h) after the pretreatment with benzamide (O), 3-aminobenzamide (A), 3-nitrobenzamide (m), 3-methoxybenzamide (*), theophylline (C), IBMX (a), picolinamide (c), and nicotinamide (n). Each point indicates the percentage of the control incubated without any addition in each experiment.
The co-existence of GSH proinsulin synthesis was decreased to less than 5% of the control (Fig. 4). Addition of GSH alone caused rather slight stimulation of proinsulin synthesis. Therefore, it may be conceivable that proinsulin synthesis in islets is more strongly inhibited under the condition which causes more extensive fragmentation of islet DNA. The potentiation of the alloxan actions by GSH is probably due to increased formation of radical oxygens as mentioned above.

Islet Nuclear Poly(ADP-ribose) Synthetase Inhibitors—Nicotinamide and picolinamide have been shown to be potent inhibitors of islet nuclear poly(ADP-ribose) synthetase (14). In addition, Fig. 5 shows the inhibitory effect on the enzyme activity of benzamide, 3-aminobenzamide, 3-nitrobenzamide, 3-methoxybenzamide, theophylline, and 3-isobutyl-1-methylxanthine. The concentrations at which these compounds cause 50% inhibition of the enzyme activity are as follows; benzamide, 4.5 μM; 3-aminobenzamide, 6.5 μM; 3-nitrobenzamide, 18 μM; 3-methoxybenzamide, 35 μM; theophylline, 65 μM; IBMX, 120 μM; picolinamide, 95 μM; nicotinamide, 120 μM. Benzamides were found to be the most potent inhibitors of islet poly(ADP-ribose) synthetase. The inhibitory ability of methylxanthines was similar to that of nicotinamide and picolinamide. These data are consistent with those obtained with non-islet cell nuclei (16-18).

Effect of Islet Nuclear Poly(ADP-ribose) Synthetase Inhibitors on Alloxan- or Streptozotocin-induced Inhibition of Proinsulin Synthesis—Pancreatic islets were incubated in the presence of alloxan or streptozotocin with or without the addition of the islet poly(ADP-ribose) synthetase inhibitors. With 0.2, 0.5, or 1.0 mM benzamide, alloxan-induced inhibition of proinsulin synthesis (32% of the control) was reversed to 48, 62, or 92%, respectively (Fig. 6a). Benzamide also protected against the streptozotocin-induced inhibition in a dose-dependent manner (Fig. 6e). Other islet poly(ADP-ribose) synthetase inhibitors also significantly reversed the inhibition of proinsulin synthesis induced by alloxan or streptozotocin (Fig. 6). The stronger inhibitors were found to protect against
Formation of radical oxygens. The streptozotocin-induced inhibition of proinsulin synthesis at the lower concentrations. The inhibitors per se essentially had no effect on islet synthesis of proinsulin (data not shown).

Effect of Poly(ADP-ribose) Synthetase Inhibitors on Alloxan- or Streptozotocin-Induced Islet DNA Strand Breaks—Poly(ADP-ribose) synthetase inhibitors were found to protect against inhibition of proinsulin synthesis by either alloxan or streptozotocin. We next looked to see if poly(ADP-ribose) synthetase inhibitors effect islet DNA strand breaks induced by alloxan or streptozotocin. As shown in Fig. 7, nicotinamide or 3-aminobenzamide did not affect the islet DNA breaks at all.

DISCUSSION

The present study has demonstrated that superoxide dismutase and catalase protect against alloxan-induced islet DNA strand breaks and also protect against the inhibition of proinsulin synthesis. It has been suggested that alloxan may work through the formation of the hydroxyl radical (OH·) (19–21) which is produced by the following interaction between superoxide (O2·−) and peroxide (H2O2) (22, 23):

O2·− + H2O2 → OH− + HO− + O2

Superoxide dismutase and catalase catalyze the removal of O2·− and H2O2, respectively (24, 25), and hence may reduce the formation of OH·. In this paper, we have shown that a combined administration of superoxide dismutase and catalase more effectively protects against alloxan-induced islet DNA breaks as well as the inhibition of proinsulin synthesis than does each scavenging enzyme alone. This strongly suggests that it is OH· rather than O2·− or H2O2 that attacks islet DNA (Fig. 8). Recently, Brawn and Fridovich also showed that hydroxyl radical breaks plasmid DNA (26). More recently, Grankvist et al. (27) reported that in vivo injections of superoxide dismutase to mice act prophylactically against alloxan-induced diabetes, although whether the enzyme interacts with the radical oxygens extracellularly or within islet cells remains to be elucidated. On the other hand, streptozotocin-induced islet DNA strand breaks were found to be unaffected by the radical scavengers. This is consistent with the recent result by Gold et al. that superoxide dismutase failed in protecting against the in vivo diabeticogenic action of streptozotocin (29). Therefore, in contrast to alloxan, it is unlikely that streptozotocin acts on islet DNA through the formation of radical oxygens. The streptozotocin-induced DNA breaks may probably be associated with the alkylating activity of the agent, as suggested with nitrosoureas (29, 30). The present study also showed that islet poly(ADP-ribose) synthetase inhibitors protect against both alloxan- and streptozotocin-induced inhibition of proinsulin synthesis but are ineffective in protection against DNA strand breaks induced by both agents. These findings may support our proposal (6, 7) that alloxan and streptozotocin induce diabetes through the following biochemical events: islet DNA strand breaks → stimulation of nuclear poly(ADP-ribose) synthetase → depletion of intracellular NAD → inhibition of proinsulin synthesis (Fig. 8).

The increase in islet poly(ADP-ribose) synthetase activity triggered by islet DNA strand breaks has been already shown to result in a significant depletion of islet NAD content (6–8).

This reduction in intracellular NAD to such a nonphysiological level may severely affect islet cell functions, including proinsulin synthesis. That depression of proinsulin synthesis may occur in consequence of reduction of islet NAD level is also shown by another of our experiments using rats maintained on a tryptophan-niacin-deficient diet. Islets isolated from these rats contained less NAD than those from the control and exhibited the diminished proinsulin synthetic ability.

The evidence presented here may have important implications for the prevention of insulin-dependent diabetes as well as for elucidation of its etiology (7).

REFERENCES


*Y. Yamamoto and H. Okamoto, unpublished data.*
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