Streptozocin- and Alloxan-Induced H_2O_2 Generation and DNA Fragmentation in Pancreatic Islets

H_2O_2 as Mediator for DNA Fragmentation

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Streptozocin (STZ) and alloxan (ALX) exhibit the most potent diabetogenicity and are used for induction of experimental diabetes mellitus. An understanding of the mechanisms of action of the typical diabetogenic agents is important for elucidating the causes of diabetes. Okamoto proposed a model in which DNA fragmentation plays an important role in the development of diabetes. DNA fragmentation supposedly results from the accumulation of superoxide or hydroxyl radicals. However, direct evidence for this accumulation is lacking. With isolated rat pancreatic islets in vitro, we demonstrated that STZ and ALX stimulated H_2O_2 generation and caused DNA fragmentation. Addition of STZ or ALX resulted in an increase in H_2O_2 generation. On DNA analysis, when incubated without STZ or ALX, DNA sedimented as a single peak; when incubated with STZ or ALX, DNA sedimented slower as a broad peak and was fragmented. Graded doses of STZ and ALX stimulated H_2O_2 generation and induced DNA fragmentation; their effects on H_2O_2 generation and DNA fragmentation were evident at a concentration of 0.1 mM and were maximal at 1 mM. Administration of STZ or ALX to rats in vivo stimulated H_2O_2 generation and caused DNA fragmentation in pancreatic islets. H_2O_2 itself also induced DNA fragmentation. These findings may support Okamoto's proposal that STZ and ALX induce diabetes through the following biochemical events: STZ and ALX→H_2O_2 generation→DNA fragmentation→β-cell destruction. This study may constitute the first demonstration of STZ- and ALX-stimulated H_2O_2 generation, which probably acts as a mediator of STZ- and ALX-induced DNA fragmentation.
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The removal of the insulin-producing pancreatic β-cells of islets induces diabetes mellitus. In 1890, von Mering and Minkowski (1) produced this form of diabetes by surgical removal of the pancreases of dogs. Subsequently, alloxan (ALX; 2) and streptozocin (STZ; 3) were found to be toxic to β-cells and thus induced diabetes in animals. Since then, ALX and STZ have been widely used for induction of experimental diabetes mellitus. An understanding of the mechanisms of action of the typical diabetogenic agents is important for elucidating the causes of diabetes. Thus, many mechanisms have been considered over the last 40 yr for the specific β-cell toxicity of ALX and STZ. From the studies of Yamamoto et al. (4) and Uchigata et al. (5) and from their own study, Okamoto et al. (6) have gathered evidence and proposed a model that has been increasingly accepted and is now termed *Okamoto's model* (7). Central to this model is the fragmentation of nuclear DNA of pancreatic β-cells. DNA fragmentation seems to be important for the development of diabetes and is supposed to result from the accumulation of superoxide or hydroxyl radicals especially in the case of ALX (6). However, direct evidence for this accumulation is lacking. With rat pancreatic islets, we present evidence that STZ and ALX stimulate H_2O_2 generation, which induces DNA fragmentation.

RESEARCH DESIGN AND METHODS

Pancreatic islets were obtained from male Wistar rats weighing 180–210 g by collagenase (Wako, Osaka, Japan) digestion as described by Lacy and Kostianovsky (8).

Experiments were conducted with male Wistar rats weighing 180–210 g that were fed ad libitum. STZ or ALX was dissolved in acetate (pH 4) just before use and injected via the tail vein of ether-anesthetized rats at a dose of 65 mg/kg; this dose yielded 100% diabetogenesis in rats in our system (50 mg/kg STZ and 40 mg/kg ALX have been shown to yield 100% diabetogenesis in rats; 9).

H_2O_2 generation was recorded continuously as described previously (10). This method utilizes the medium H_2O_2 in the presence of exogenously added peroxidase to convert homovanillic acid to a fluorescent metabolite. The washed islets were suspended in Krebs-Ringer bicarbonate–20 mM HEPES buffer (pH 7.4) with 0.5% bovine serum albumin (BSA). The islet suspension (100 islets/0.5 ml) was transferred to a thermostatic quartz cuvette (37°C) for measurement of H_2O_2 generation; the cuvette contained horseradish peroxidase (type II, final concn 0.1 mg/ml) and homovanillic acid (final concn 0.44 mM). H_2O_2 generation was measured in a Hitachi F-4000 fluorometer (Tokyo). Fluorescence was recorded with excitation and emission wavelengths of 315 and 425 nm, respectively. The islet suspensions were stirred continuously. After reaching steady state, STZ or ALX was added to the cuvette with a microsyringe. The rates of STZ- or ALX-stimulated H_2O_2 generation were obtained from the slopes after reaching steady state, calculated as the differences between STZ- or ALX-stimulated H_2O_2 generation and basal (expressed as nmol · islet^{-1} · min^{-1}). Standard curves were produced from incubations in which the islets were replaced by known amounts of H_2O_2.

Density-gradient analysis of islet DNA was performed as previously stated (6). Batches of 100 islets were incubated at 37°C in 200 μl Krebs-Ringer bicarbonate medium (pH 7.4) containing 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM monosodium glutamate, 2 mg/ml BSA, and 2.8 mM glucose in an atmosphere of 95% O_2/5% CO_2. After a 5-min preincubation, STZ, ALX, or H_2O_2 was added to the medium,

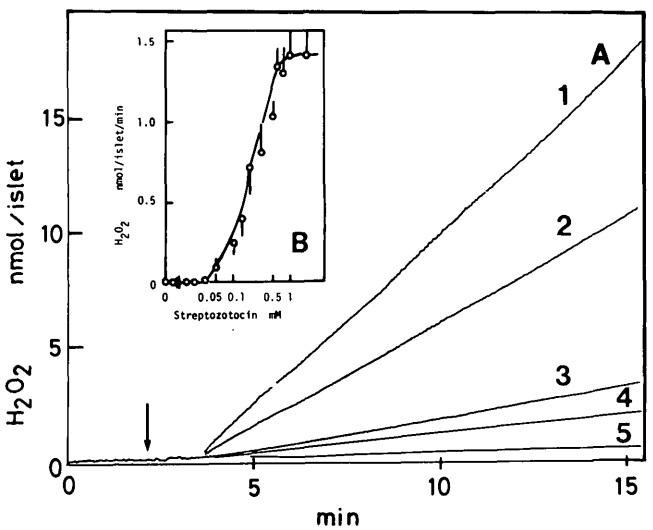

FIG. 1. Effect of streptozocin (STZ) on H_2O_2 generation in vitro. *A*: time course of STZ-stimulated H_2O_2 generation. H_2O_2 generation was recorded continuously. Addition of 1 (*1*), 0.3 (*2*), 0.1 (*3*), and 0.05 mM (*4*) STZ (*arrow*) to pancreatic islet suspension resulted in linear increase in H_2O_2 generation with 1.5-min initial lag. When STZ was not added, basal H_2O_2 generation increased slightly (*5*). Rates of H_2O_2 generation were obtained from linear slopes after reaching steady states. Amounts of STZ-stimulated H_2O_2 generation were differences between STZ stimulated (*1–4*) and basal (*5*). *B*: effects of graded doses of STZ on H_2O_2 generation. Rates of STZ-stimulated H_2O_2 generation were obtained from linear slopes after reaching steady state and calculated as differences between those of STZ-stimulated H_2O_2 generation and basal (nmol · islet^{-1} · min^{-1}). Graded doses of STZ stimulated H_2O_2 generation; effect of STZ on H_2O_2 generation was evident at concentration of 0.1 mM ($P < 0.05$) and maximal at 1 mM. Values are means ± SE of 5–7 determinations.

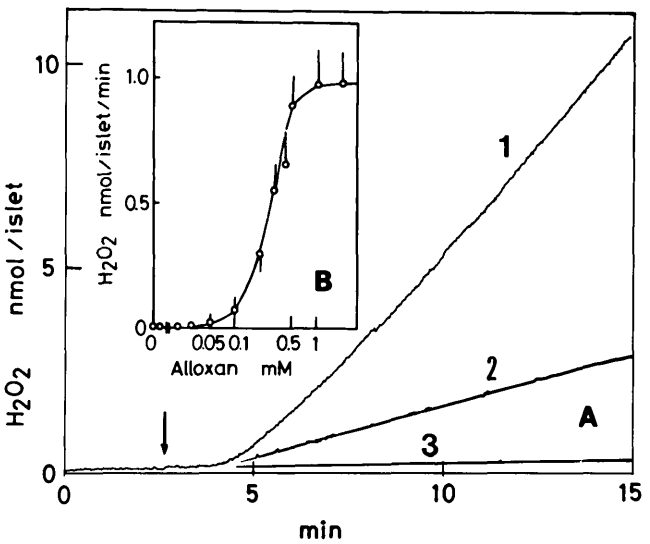

FIG. 2. Effect of alloxan (ALX) on H_2O_2 generation in vitro. *A*: time course of ALX-stimulated H_2O_2 generation. H_2O_2 generation was recorded continuously. Addition of 1 (*1*) and 0.2 mM (*2*) ALX (*arrow*) to pancreatic islet suspension resulted in linear increase in H_2O_2 generation with 1.5-min initial lag. When ALX was not added, basal H_2O_2 generation increased slightly (*3*). Amounts of ALX-stimulated H_2O_2 generation were differences between ALX-stimulated H_2O_2 generation (*1,2*) and basal (*3*). *B*: effects of graded doses of ALX on H_2O_2 generation. Rates of ALX-stimulated H_2O_2 generation were calculated as in Fig. 1. Graded doses of ALX stimulated H_2O_2 generation; effect of ALX on H_2O_2 generation was evident at concentration of 0.1 mM ($P < 0.05$) and maximal at 1 mM. Values are means ± SE of 5–7 determinations.

and the islets were incubated for the indicated periods at 37°C. After incubation, saline-washed islets were suspended in 50 μl of cold saline and immediately layered over 0.5 ml of lysis solution (1 N NaOH, 0.01 M EDTA, 1% [vol/vol] Triton X-100) that had been just layered over 5 ml of a 5–20% (wt/vol) linear sucrose gradient containing 0.3 N NaOH, 0.7 M NaCl, and 0.01 M EDTA. On the bottom of each gradient was a 1-ml 80% (wt/vol) sucrose shelf. The loaded gradients were placed in the dark at room temperature for 30 min. Then the gradients were centrifuged at 50,000 rpm at 20°C for 200 min in a Beckman swing rotor. After centrifugation, fractions of 20 drops were collected from the gradients. DNA in each fraction was precipitated by adding 2 ml of 20% cold trichloroacetic acid with 200 μg BSA as carrier. The precipitate was washed three times with cold trichloroacetic acid and was then assayed for DNA content by a fluorometric method (11).

Horseradish peroxidase, homovanillic acid, ALX, and STZ were obtained from Sigma (St. Louis, MO). All other chemicals were of the highest purity available commercially. Experiments were conducted at least five times. Typical data and final concentrations of STZ, ALX, and H_2O_2 are shown in RESULTS and the figures. Data were statistically analyzed with analysis of variance (ANOVA) or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Addition of STZ or ALX to islet suspension resulted in a gradual increase in H_2O_2 generation with initial 1.5-min lag. The STZ- or ALX-stimulated H_2O_2 generation increased linearly up to 60 min (data not shown). The rates of STZ- or

ALX-stimulated H_2O_2 generation were obtained from the linear slopes after reaching a steady state and calculated as the differences between those of STZ- or ALX-stimulated H_2O_2 generation (Fig. 1*A*, *1–4*; Fig. 2*A*, *1* and *2*) and basal H_2O_2 generation (Fig. 1*A5*; Fig. 2*A3*). Graded doses of STZ and ALX stimulated H_2O_2 generation (Figs. 1*B* and 2*B*); the effects of STZ and ALX on the rates of H_2O_2 generation were evident at a concentration of 0.1 mM and maximal at 1 mM. STZ and ALX failed to stimulate H_2O_2 generation in the absence of islets or in the broken cells.

Islets were incubated with STZ or ALX for 7–20 min, and velocity sedimentation of DNA was examined in an alkaline sucrose density gradient (Fig. 3). DNA of islets incubated without STZ for 20 min was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA sediments (Fig. 3*A*). However, after only a 7-min incubation with 1 mM STZ, a considerable amount of DNA sedimented as a broad peak in the middle of the gradient with a concomitant decrease in undamaged DNA (Fig. 3*B*). After incubation with 1 mM STZ for 20 min, the DNA was almost completely fragmented (Fig. 3*C*); the islet DNA sedimented slower as a broad peak, indicating that STZ induces islet DNA fragmentation. ALX also induced DNA fragmentation. Graded doses of STZ and ALX induced DNA fragmentation, and their effects were observed at a concentration of 0.1 mM and were maximal at 1 mM (Fig. 3*D*; data not shown).

STZ (65 mg/kg) or ALX (65 mg/kg) was injected intravenously into rats. Twenty minutes after STZ or ALX injection, the islets were isolated from the pancreas, and H_2O_2 generation was measured (Fig.4). Injection of STZ or ALX increased the rates of H_2O_2 generation; the rate of STZ- or ALX-stimulated H_2O_2 generation was higher than the control rate. Twenty minutes after STZ or ALX injection, the islets were also isolated from the pancreas, and the velocity sedi-

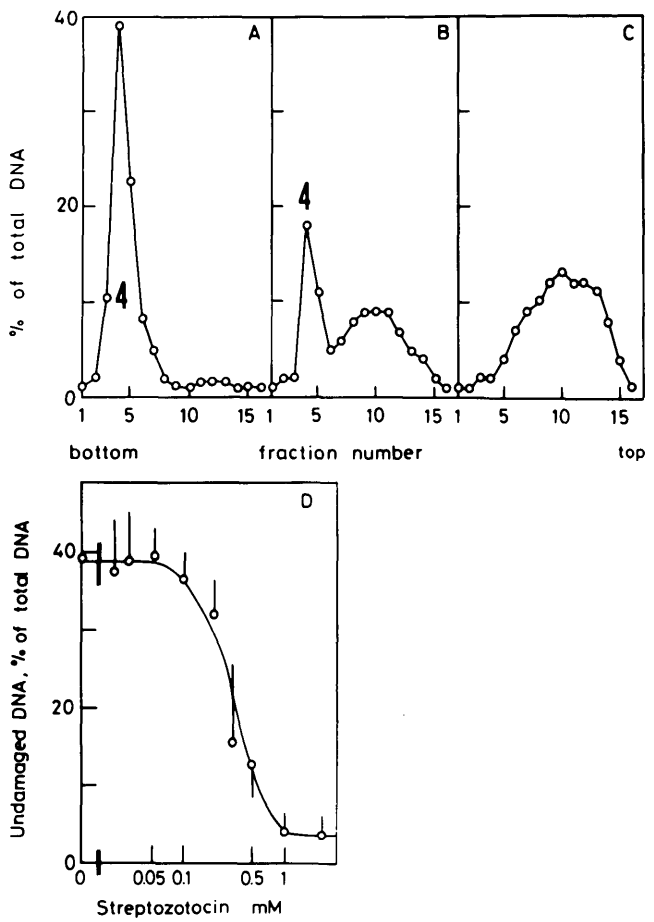

FIG. 3. Effect of streptozocin (STZ) on DNA fragmentation in vitro. *A–C*: effect of STZ on DNA fragmentation (velocity sedimentation of DNA). Islets were incubated with STZ for 7–20 min, and velocity sedimentation of DNA was examined in alkaline sucrose density gradient. DNA of islets incubated without STZ for 20 min was recovered as single peak near bottom of gradient (*4*, fraction 4), position at which undamaged DNA sediments (*A*). However, after 7-min incubation with 1 mM STZ, considerable amount of DNA sedimented as broad peak in middle of gradient with concomitant decrease in undamaged DNA (fraction 4; *B*). After incubation with 1 mM STZ for 20 min, DNA sedimented slower as broad peak and was almost completely fragmented (*C*). Identical results were obtained when islets were incubated with 1 mM ALX instead of 1 mM STZ (data not shown). *D*: effect of 20-min exposure to graded doses of STZ on undamaged DNA contents. Effects of graded doses of STZ or ALX (data not shown) on DNA fragmentation were studied as in *C*. DNA in fraction 4 was considered undamaged. Graded doses of STZ and ALX decreased amounts of undamaged DNA contents in fraction 4; effects were evident at concentration of 0.1 mM ($P < 0.05$) and maximal at 1 mM. Values are means ± SE of 5–7 determinations.

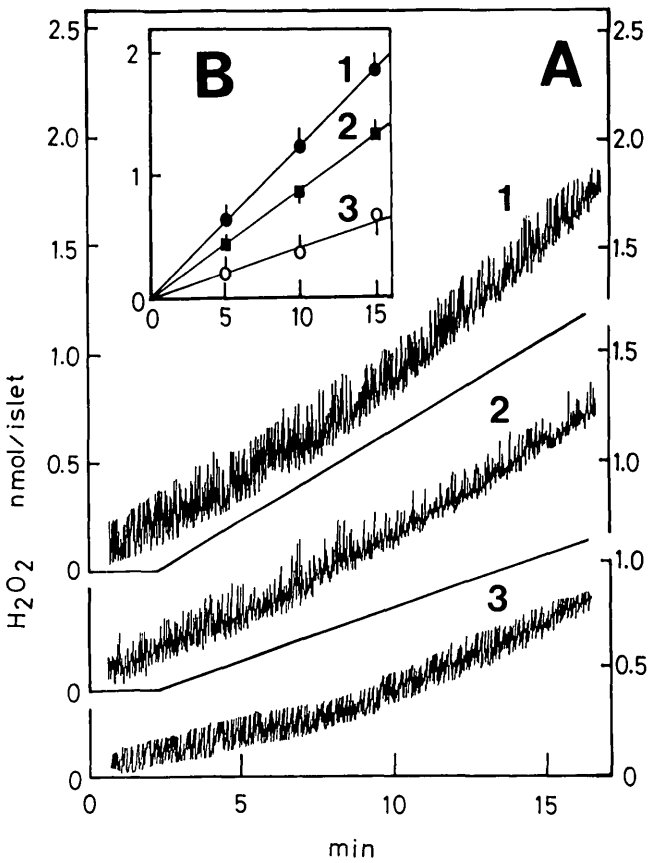

FIG. 4. Effects of administration of streptozocin (STZ) or alloxan (ALX) in vivo on H_2O_2 generation. *A*, *B*: 65 mg/kg i.v. STZ (*1*) or 65 mg/kg i.v. ALX (*2*) was injected into rats. Twenty minutes after STZ or ALX injection, islets were isolated from pancreas, and H_2O_2 generation was measured. Injection of STZ (*1*) or ALX (*2*) increased rate of H_2O_2 generation, which was higher than control rate (*3*). One typical experiment is shown in *A*, and data from 5–7 different experiments are shown in *B*. Values are means ± SE of 5–7 determinations; *1* or *2* is significantly different from *3* at 10 min ($P < 0.05$).

mentation of islet DNA in the alkaline sucrose gradient was examined (Fig. 5). The DNA of islets of rats not injected with STZ or ALX was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA sediments (Fig. 5*A*). However, injection of STZ or ALX induced DNA fragmentation, and a considerable amount of DNA sedimented as a broad peak in the middle of the gradient with a concomitant decrease in undamaged DNA (Fig. 5, *B* and *C*).

Islets were incubated with graded doses of H_2O_2 for 20 min, and velocity sedimentation of DNA was examined in an alkaline sucrose density gradient (Table 1). The DNA of islets incubated without H_2O_2 for 20 min was recovered as a single peak near the bottom of the gradient, the position at which undamaged DNA sediments. However, after incubation with 500 μM H_2O_2 for 20 min, the DNA was almost completely fragmented; the islet DNA sedimented slower as a broad peak, indicating that H_2O_2 induces islet DNA fragmentation. Graded doses of H_2O_2 induced DNA fragmentation (Table 1); the effect of H_2O_2 on DNA fragmentation was evident at a concentration of 30 μM and maximal at 500 μM.

TABLE 1
Effect of H_2O_2 on DNA fragmentation in vitro (velocity sedimentation of DNA)

H_2O_2 concentration (μM)	DNA content in fraction 4 (% of total DNA)
0	39 ± 5
1	38 ± 7
10	39 ± 6
20	34 ± 5
30	30 ± 4
50	27 ± 4
80	14 ± 5
100	8 ± 3
200	6 ± 2
500	4 ± 1
1000	4 ± 1

Values are means ± SE of 5–7 determinations. Islets were incubated without or with graded doses of H_2O_2 for 20 min, and velocity sedimentation of DNA was examined in an alkaline sucrose density gradient (Fig. 3). DNA of islets incubated without H_2O_2 for 20 min was recovered as a single peak near bottom of gradient. However, after incubation with H_2O_2 for 20 min, peak of DNA became lower, and DNA sedimented slower as a broad peak; after incubation with 500 μM H_2O_2 for 20 min, DNA sedimented slower as a broad peak and was almost completely fragmented. Effects of 20-min exposure to graded doses of H_2O_2 on DNA fragmentation were studied (Fig. 3*D*). Fraction 4, undamaged DNA. Graded doses of H_2O_2 decreased amounts of undamaged DNA contents in fraction 4; effect was evident at concentration of 30 μM ($P < 0.05$) and maximal at 500 μM.

DISCUSSION

STZ and ALX are diabetogenic (2) and induce DNA fragmentation in isolated rat pancreas islets in vitro and in rats in vivo to cause diabetes mellitus (4–6,12). However, precise mechanisms for the STZ- and ALX-induced DNA fragmentation are not known. We demonstrated that STZ and ALX stimulated H_2O_2 generation, which induced DNA fragmentation. These findings may support Okamoto's proposal that STZ and ALX induce diabetes mellitus through the following biochemical events: STZ and ALX→H_2O_2 generation→DNA fragmentation→β-cell destruction.

Catalase has been shown to protect against ALX-induced islet DNA fragmentation (6), indicating that H_2O_2 generation might mediate the action of ALX. In this study, we demonstrated that ALX stimulated H_2O_2 generation, which caused DNA fragmentation. When ALX is injected into rats, it accumulates in the islets of Langerhans (13). We demonstrated that ALX stimulated H_2O_2 generation and DNA fragmentation in vitro and in vivo; H_2O_2 generation and DNA fragmentation may play some role in the development of diabetes mellitus.

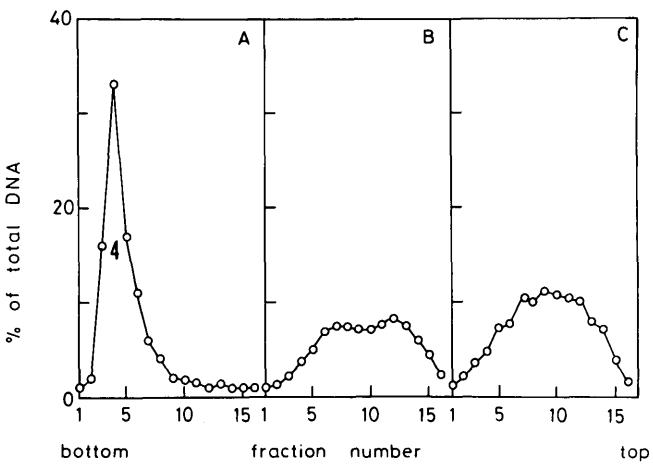

FIG. 5. Effects of administration of streptozocin (STZ) or alloxan (ALX) in vivo on DNA fragmentation. STZ or ALX (65 mg/kg i.v.) was injected into rats. Twenty minutes after STZ (*B*) or ALX (*C*) injection, islets were isolated from pancreas, and velocity sedimentation of islet DNA in alkaline sucrose gradient was examined. DNA of rat islets, not injected with STZ or ALX, was recovered as single peak near bottom of gradient (*4*, fraction 4), position at which undamaged DNA sediments (*A*). However, injection of STZ (*B*) or ALX (*C*) induced DNA fragmentation, and considerable amount of DNA sedimented as broad peak in middle of gradient with concomitant decrease in undamaged DNA.

Another drug that has been used to induce diabetes in experimental animals is STZ, a nitrosourea compound produced by *Streptomyces achomogenes*. STZ has been thought to damage DNA directly by a chemical reaction of its nitroso group with the DNA rather than by increasing oxygen radical formation. However, we found that STZ itself stimulated H_2O_2 generation. When STZ was injected, it accumulated in the islets (14). STZ stimulated H_2O_2 generation and DNA fragmentation in vitro and in vivo. STZ-induced H_2O_2 generation may play some role in DNA fragmentation and the development of diabetes mellitus.

STZ, ALX, and H_2O_2 induced DNA fragmentation (Fig. 3; Table 1). STZ and ALX produced 100–1000 μM H_2O_2/10 min, and 100–1000 μM H_2O_2 induced DNA fragmentation. Thus, it is reasonable to propose that H_2O_2 generation may act as a mediator for STZ- and ALX-induced DNA fragmentation, which induces diabetes mellitus (4–7). The H_2O_2-induced DNA fragmentation is not specific for pancreatic islets and has been reported in other cells (15). STZ and ALX are structurally different but have a common pathway to induce H_2O_2 generation and DNA fragmentation to inhibit β-cell function. This seems to be of special importance in understanding the pathogenesis of insulin-dependent diabetes mellitus.

Generation of H_2O_2 appears to be a natural process and is of universal occurrence in various cells. However, there have been no reports to show generation of H_2O_2 in pan-

creatic islets. Although direct evidence was lacking for the presence of H_2O_2 in the β-cells, Heikkila et al. (16) presented data to support the view that oxygen radicals, including H_2O_2, play a primary role in the diabetogenic action of ALX. We demonstrated the presence of H_2O_2 generation in pancreatic islets. STZ and ALX stimulate H_2O_2 generation. H_2O_2 is believed to be produced mainly via O_2^-. Thus, we studied the effects of STZ and ALX on O_2^- production and found that they had no effects on O_2^- production (unpublished observations). Several enzymes produce H_2O_2 without the O_2^- intermediate. These include glycolate oxidase, D-amino acid oxidase, and urate oxidase. We plan to study the effects of STZ and ALX on these enzymes. However, we do not know how H_2O_2 is generated or how STZ and ALX stimulate H_2O_2 generation. Further studies will be required to answer these questions.

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