Mechanisms of Nicotinamide and Thymidine Protection From Alloxan and Streptozocin Toxicity

SUSAN P. LEDOUX, CAROLYN R. HALL, PAM M. FORBES, NANCY J. PATTON, AND GLENN L. WILSON

A common mechanism has been proposed for the β-cell toxins alloxan (ALX) and streptozocin (STZ) involving the formation of single-strand breaks in DNA that lead to the overactivation of the enzyme poly(ADP-ribose) synthetase and the critical depletion of its substrate NAD. If the toxins act via this common mechanism, the poly(ADP-ribose) synthetase inhibitors nicotinamide and thymidine would be expected to affect the formation of DNA single-strand breaks in a similar fashion. To test the effects of these inhibitors, the formation of single-strand breaks in the DNA of insulin-secreting RINr cells was monitored by assessing changes in the supercoiling of nucleoids after exposure to STZ, ALX, or methylnitrosourea (MNU). With the inclusion of nicotinamide or thymidine and STZ or MNU, more single-strand breaks in RINr cell DNA were detected. These results would be expected if nicotinamide and thymidine acted through inhibition of poly(ADP-ribose) synthetase. However, when the inhibitors were used in combination with ALX, fewer single-strand breaks were present. This suggests a reduction in ALX-induced hydroxyl radicals available to interact with DNA. Because nicotinamide has been demonstrated to be a hydroxyl-radical scavenger, the ability of thymidine to scavenge hydroxyl radicals was investigated. Thymidine, like nicotinamide, was found to be a potent scavenger of hydroxyl radicals. Thus, the mechanisms by which nicotinamide and thymidine protect against the toxic effects of STZ or ALX appear different. These findings suggest that the actions of β-cell toxins are more complex than simply the overactivation of a single enzyme. *Diabetes* 37:1015–19, 1988

The β-cell toxins streptozocin (STZ) and alloxan (ALX) have been used extensively to produce animal models of diabetes mellitus. However, the mechanisms that impart this selective toxicity remain elusive. These chemical agents may act through a common pathway that is dependent on the formation of single-strand breaks in β-cell DNA. These breaks then activate the nuclear enzyme poly(ADP-ribose) synthetase in β-cells to such an extent that the stores of its substrate, NAD, become critically depleted (1,2). Much support for this hypothesis is based on the findings that poly(ADP-ribose) synthetase inhibitors, e.g., nicotinamide, protect the β-cells from the toxic effects of STZ and ALX (3). However, the mechanisms that allow for this protection may be more complex than the simple inhibition of a single enzyme pathway. Nicotinamide and other poly(ADP-ribose) synthetase inhibitors are capable of scavenging hydroxyl free radicals (4). Thus, these agents may protect β-cells by allowing less initial DNA damage to occur. A quantitative evaluation of the single-strand breaks that occur in DNA of cells exposed to varying concentrations of STZ or ALX in the presence of poly(ADP-ribose) synthetase inhibitors has not been reported. Herein, we used a nucleoid assay to examine the effects of the poly(ADP-ribose) synthetase inhibitors nicotinamide and thymidine on the formation of single-strand breaks in DNA from an insulin-secreting cell line (RINr cells) after exposure to ALX, STZ, or methylnitrosourea (MNU). MNU is the nitrosourea moiety of STZ. Previous studies have shown that MNU is considerably less toxic to β-cells than is STZ (5).

MATERIALS AND METHODS

Cell culture. A clonal isolate of rat insulinoma (RINr clone 38) cells was obtained from W. Chick (University of Massachusetts, Worcester, MA). These cells were plated in 60-mm culture dishes and maintained in medium 199 (Gibco, Grand Island, NY) supplemented with 5% horse serum (Hyclone, Logan, UT), glucose (16.5 mM), and gentamicin (1 mg/dl).

From the Department of Anatomy, University of South Alabama, College of Medicine, Mobile, Alabama.

Address correspondence and reprint requests to Dr. Glenn L. Wilson, Department of Anatomy, University of South Alabama, College of Medicine, Mobile, AL 36688.

Received for publication 19 November 1987 and accepted in revised form 22 January 1988.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/8/1015/356100/37-8-1015.pdf by guest on 12 September 2024

Drug preparation and exposure. STZ (provided by A.E. Chang, Upjohn, Kalamazoo, MI), MNU (Sigma, St. Louis, MO), and ALX (Kodak, Rochester, NY) were dissolved in citrate buffer (pH 4.2), and desired concentrations were obtained by serial dilution in Hanks' balanced salt solution (HBSS; Flow, McLean, VA). Before drug exposure, culture medium was removed and cells were washed with HBSS. After a 60-min exposure to the drugs, the cells were removed by gentle trypsinization.

Nucleoid assay. Nucleoids were prepared as described by Cook and Brazell (6), with some modifications. Gradients of 15–30% sucrose containing 1.95 mM NaCl, 0.01 M Tris, and 0.001 M EDTA (pH 8.0) were prepared in 4.6-ml polyallomer tubes with a Buchler density-gradient maker. A 0.3-ml aliquot of 80% sucrose was used as a cushion. Lysis solution (150 μl containing 2.6 M NaCl, 0.133 M EDTA, 2.6 mM Tris, and 0.34% Triton X-100) was layered gently onto the gradients. After gentle trypsinization, the cells were resuspended in 50 μl of ice-cold phosphate-buffered saline (0.01 M, pH 7.4). This suspension, which contained $\sim 10^5$ cells, was then layered gently on top of the lysing solution. After a 30-min lysis period in the dark, the gradients were centrifuged for 30 min at 25,000 rpm at 20°C with a Sw55 Ti rotor in a Beckman model L-2 ultracentrifuge. Gradients were pumped through a continuous-flow cell in a Pharmacia model UV-1 absorbance monitor, and absorbance was monitored at 254 nm. The migration ratio was then calculated as the distance migrated by damaged nucleoids relative to the distance migrated by undamaged, control nucleoids.

Assay for hydroxyl radical scavengers. The system for generating and detecting the hydroxyl radical was a modification of that described by Klein et al. (7) and by McCord and Day (8). A 1-ml volume of the reaction mixture contained the following: xanthine (40 μM; Sigma) and bovine milk xanthine oxidase (0.012 U/ml; Sigma) as the source of superoxide and H_2O_2, with 10 μM Fe-EDTA present to catalyze hydroxyl production. The radicals were trapped via reaction with 10 mM 1,1-dimethylurea, giving rise to the formation of formaldehyde. After incubation at 37°C for 30 min, the reactions were terminated by the addition of 0.5 ml of cold trichloroacetic acid (17.5% wt/vol). After centrifugation, 1.0 ml of supernatant was assayed for formaldehyde by the fluorometric method of Steffer and Netter (9). The abilities of the various compounds to scavenge the hydroxyl radical were determined by adding the compounds at the indicated concentrations to the initial reaction mixtures.

Statistical analysis. Results are expressed as means ± SE. Significant differences were assessed with an unpaired Student's *t* test. Only values with $P < .05$ were regarded as significant.

RESULTS

Changes in nucleoid sedimentation were used to assess DNA damage in RINr cells after exposure to STZ, MNU, and ALX. Nucleoids are structures that result when cells are lysed in the presence of a nonionic detergent and high salt concentrations. They resemble nuclei and contain all of the nuclear DNA and some of the RNA but are extremely depleted of protein. As nicks are made in this supercoiled DNA, a more relaxed and open structure results in a decreased rate of sedimentation of the nucleoids. Data are then expressed as migration ratios, which are calculated as the distance migrated by the damaged nucleoids relative to the distance migrated by undamaged control nucleoids. All three chemicals, STZ, MNU, and ALX, induce single-strand breaks in the DNA of RINr cells in a dose-dependent manner (Fig. 1).

Drug concentrations of STZ (1 mM), MNU (0.1 mM), and ALX (5 mM) that resulted in a migration ratio closest to 0.8 were used for the inhibitor studies. This allows maximum detection of any changes that occur in nucleoid migration in either direction. Neither 50 mM nicotinamide nor 50 mM thymidine alone caused any appreciable shift in the nucleoid migration ratio (Fig. 2). Therefore, at this concentration, neither inhibitor induced single-strand breaks in RINr cell DNA. However, when RINr cells were incubated with 1 mM STZ and 50 mM nicotinamide and nucleoids were prepared, the resulting migration ratios were less than those from cells incubated with 1 mM STZ alone, indicating that more breaks were present. An even greater decrease in migration ratios was noted when RINr cells were incubated with 1 mM STZ and 50 mM thymidine. Similar results were obtained with 0.1 mM MNU. The addition of 50 mM nicotinamide or 50 mM thymidine resulted in decreased migration ratios when compared with those obtained with 0.1 mM MNU alone. These findings show that inclusion of nicotinamide or thymidine during the exposure of RINr cells to STZ or MNU causes more single-strand breaks in the DNA of RINr cells to be present after 1 h.

Effects opposite to the results obtained with STZ and MNU were seen with ALX. The migration ratios of nucleoids prepared from RINr cells incubated with 5 mM ALX and 50 mM nicotinamide or 50 mM thymidine were greater than those from RINr cells incubated with 5 mM ALX alone. These findings demonstrate that inclusion of 50 mM nicotinamide or 50 mM thymidine resulted in fewer single-strand breaks in RINr cell DNA.

Nicotinamide has been demonstrated to be a hydroxyl-

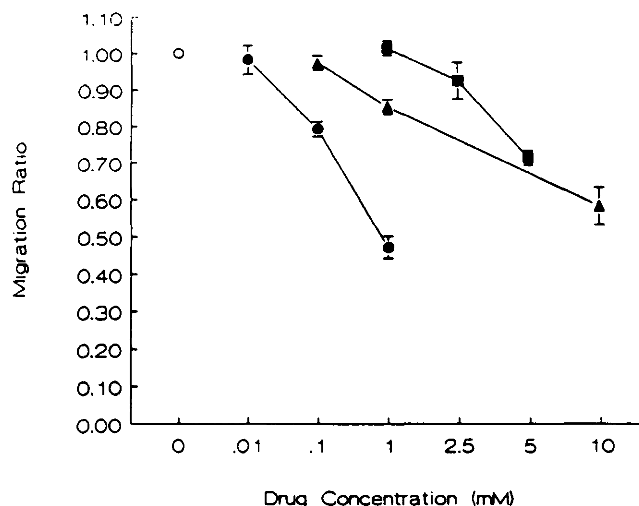

FIG. 1. Effects of streptozocin (STZ), methylnitrosourea (MNU), and alloxan (ALX) on sedimentation of nucleoids. RINr cells were exposed for 1 h to STZ (▲), MNU (●), or ALX (■). Control cultures (○) were exposed to Hanks' balanced salt solution with 1% citrate phosphate buffer (pH 4.5). Cells were then removed, nucleoids prepared, and sedimentation performed. Data are expressed as ratios of damaged nucleoid migration to undamaged control nucleoid migration. Values are means ± SE of 4 determinations per point.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/8/1015/356100/37-8-1015.pdf by guest on 12 September 2024

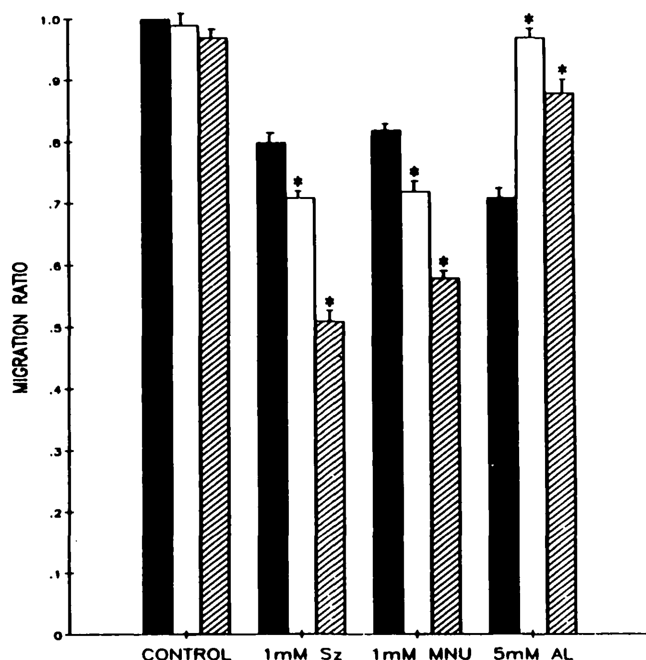

FIG. 2. Effects of nicotinamide (*open bars*) or thymidine (*hatched bars*) on sedimentation of nucleoids. RINr cells were exposed to streptozocin (Sz; 1 mM), methylnitrosourea (MNU; 0.1 mM), or alloxan (AL; 5 mM) alone (*solid bars*) or in combination with either 50 mM nicotinamide or 50 mM thymidine for 1 h. Control cultures were exposed to Hanks' balanced salt solution with 1% citrate buffer alone or in combination with nicotinamide or thymidine. Cells were then removed, nucleoids prepared, and sedimentation performed. Data are expressed as ratios of damaged nucleoid migration to undamaged control nucleoid migration. Values are means ± SE of 4 determinations per point. *$P < .05$.

radical scavenger (4). One possible explanation for the increased migration ratios observed with ALX and nicotinamide or thymidine is that the agents are acting to scavenge the hydroxyl radicals generated by ALX. The ability of thymidine to scavenge hydroxyl radicals was therefore investigated. Thymidine, like nicotinamide, can scavenge hydroxyl radicals in a dose-dependent manner (Table 1).

DISCUSSION

These studies were designed to quantitatively measure the effects of the poly(ADP-ribose) synthetase inhibitors nicotinamide and thymidine on DNA strand breaks after exposure of insulin-secreting cells to STZ, MNU, and ALX. With the nucleoid assay, we demonstrated that STZ, MNU, and ALX induce DNA strand breaks in a dose-dependent manner, and that the effects of nicotinamide and thymidine on the amount of DNA strand breaks present at 1 h varied, depending on which toxin was used. Because the nucleoid assay is performed at neutral pH, the strand breaks that are detected result from either direct breaks or from enzymatic breaks due to the multistep excision repair process. The strand breaks that were demonstrated with ALX were most likely due to direct breaks in DNA. In the spontaneous breakdown of ALX, oxygen-derived free radicals are generated (10,11). These free radicals are capable of causing direct breaks in DNA (12). With STZ and MNU, the breaks detected were probably due to the excision repair process. Whereas it is still not clear whether oxygen-derived free radicals are generated in the breakdown of STZ (13–15), there is direct evidence from our laboratory, with the use of islet cells (16), and other laboratories, with the use of the whole pancreas (17), that demonstrates that STZ spontaneously decomposes to form carbonium ions that alkylate DNA at specific nucleophilic sites. The repair of some of these modifications (e.g., alkylation at the N-7 position of guanine) leads to single-strand breaks in DNA. The damaged DNA is then removed, and new DNA is synthesized. The nicks are resealed, and the DNA is again supercoiled (18). It is important to stress that, in these experiments with the nucleoid assay, the number of single-strand breaks reflects the action of both incision and ligation processes measured concomitantly.

Nucleoids prepared from RINr cells exposed to STZ and nicotinamide or thymidine exhibited decreased migration ratios compared with nucleoids prepared from RINr cells exposed to STZ alone. These data showed that more single-strand breaks were present after 1 h when nicotinamide or thymidine were included. Similar results were obtained in RINr cells exposed to MNU and the inhibitors. These findings are in agreement with previous data that demonstrated increases in frequency of single-strand breaks, repair replication, and sister chromatid exchange in cells exposed to an alkylating agent and poly(ADP-ribose) synthetase inhibitors (19,20). From studies such as these, a role for poly(ADP-ribose) synthesis in DNA repair has been advocated. Currently, exactly what role poly(ADP-ribose) plays in DNA repair appears to be a controversial issue. Whether the mechanism is by a decreased rate of ligation (20) or by an increase in the activity of intracellular nuclease activity that causes additional DNA damage (19), the detectable result with the nucleoid assay would be an increase in the number of single-strand breaks. Therefore, the increase in the number of single-strand breaks seen in RINr cells exposed to STZ or MNU and nicotinamide or thymidine is consistent with the notions that, in this situation, nicotinamide and thymidine may be acting to inhibit poly(ADP-ribose) synthetase.

Nucleoids prepared from RINr cells that were exposed to ALX and either nicotinamide or thymidine exhibited greater migration ratios compared with nucleoids prepared from RINr cells exposed to ALX alone. These findings demonstrate that fewer single-strand breaks were formed in the presence of nicotinamide or thymidine. Thus, in this situation, nicotinamide and thymidine do not appear to be acting through the inhibition of poly(ADP-ribose) synthetase. A

TABLE 1
Comparison of hydroxyl-radical–scavenging properties of thymidine and nicotinamide to 1,1-dimethylurea

Scavenger	Decrease in formaldehyde formation (%)
Thymidine (mM)	
1	21 ± 5
10	72 ± 2
50	91 ± 3
Nicotinamide (mM)	
1	10 ± 7
10	21 ± 12
50	86 ± 8

Values are means ± SE of 4 experiments.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/8/1015/356100/37-8-1015.pdf by guest on 12 September 2024

more plausible explanation would be that this decrease in formation of single-strand breaks results from the fact that nicotinamide and thymidine are actually acting as scavengers of the oxygen-derived free radicals generated by ALX. This possibility is supported by the findings that thymidine, like nicotinamide, is indeed capable of scavenging the hydroxyl radical. Therefore, these studies suggest that nicotinamide and thymidine are capable of protecting β-cells not only by inhibiting poly(ADP-ribose) synthetase but also by a more direct action of oxygen-derived free-radical scavenging. That nicotinamide is a hydroxyl-radical scavenger may have potential importance in relation to spontaneous diabetes. It was recently shown that treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in nonobese diabetic (NOD) mice (21). This finding was interpreted to indicate that oxygen-derived free radicals may be involved in islet damage in this animal model of diabetes. Nomikos et al. (21) attributed the effects of nicotinamide to its ability to block poly(ADP-ribose) synthetase activity resulting from free-radical–induced DNA strand breaks. Therefore, Nomikos et al. feared that this therapy could promote tumor formation in the islet. However, our results would indicate that their fear may be unwarranted. Because nicotinamide can serve as a direct scavenger of the hydroxyl radical, it is likely that less DNA damage would result from the oxygen-derived free radicals produced due to the inflammation around the islet, and the potential for tumor formation would be actually lessened. Experiments measuring DNA strand breaks in transplanted islets of NOD mice treated with nicotinamide are clearly indicated.

It is well established that both STZ and ALX are selective β-cell toxins. The mechanisms that impart this selective toxicity remain controversial. Whereas the hypothesis that chemical agents act through a common mechanism is intriguing, certain lines of evidence, including the findings that numerous agents protect against ALX toxicity but not STZ (22) and that there are differences in the time course of β-cell necrosis after exposure to STZ and ALX (23), suggest that a more complex situation exists. Additionally, why a repair process that is normal in most other cell types is lethal in β-cells has not been explained. The results of these studies also suggest that the situation may be more complex than originally proposed, because nicotinamide and thymidine appear to be protecting β-cells from ALX toxicity by acting as free-radical scavengers rather than by inhibiting poly(ADP-ribose) synthetase. Additionally, recent studies from our laboratory demonstrated that a nonlethal concentration of the aglycone MNU caused comparable numbers of single-strand breaks in β-cell DNA compared with an equimolar yet lethal concentration of STZ (16). Thus, factors other than the simple induction of strand breaks in DNA must be operative for STZ to selectively destroy β-cells. Similarly, a recent report by Meglasson et al. (24) has shown that toxic mechanisms of ALX are also complex because, according to these investigators, this chemical can inactivate glucokinase as well as damage DNA. Interestingly, the inactivation of glucokinase does not appear to involve the generation of oxygen-derived free radicals. Based on these findings with ALX and our own studies with STZ and MNU (16), we feel a new concept for the action of β-cell toxins is appropriate. STZ and ALX critically interact with β-cells by a combination of nonspecific and specific toxic phenomena. Through the generation of hydroxyl radicals (ALX) and carbonium ions (STZ), these chemicals are able to produce nonspecific damage in β-cells, e.g., lesions in the nuclear DNA. However, they also are able to cause β-cell–specific damage via their ability to interact with the glucose-sensing mechanism of the β-cell. STZ accomplishes this by its glucose moiety and ALX by its unique structural conformation (25,26). This interaction with the glucose recognition system would allow the toxin to be sequestered differently in β-cells than in other cells and, therefore, cause β-cell–specific damage, e.g., the inactivation of glucokinase. It is the combination of the β-cell–specific and nonspecific damage that leads to the ultimate death of the cell. Inhibition of either β-cell–specific or nonspecific damage may be sufficient to allow the cell to repair the remainder of the damage and avoid the lethal effects of the toxin.

ACKNOWLEDGMENTS

We thank G. Amos and S. Sharma for assistance in the preparation of this manuscript.

This study was supported by NIH Grant ES-03456. G.L.W. is the recipient of NIH Research Career Development Award ES-00150.

REFERENCES

1. Okamoto H: Regulation of proinsulin synthesis in pancreatic islets and a new aspect to insulin-dependent diabetes. *Mol Cell Biochem* 37:43–61, 1981
2. Okamoto H: Molecular basis of experimental diabetes: degeneration, oncogenesis and regeneration of pancreatic β-cells of islets of Langerhans. *Bioessays* 2:15–21, 1985
3. Uchigata Y, Yamamoto H, Nagai H, Okamoto H: Effect of poly(ADP-ribose) synthetase inhibitor administration to rats before and after injection of alloxan and streptozotocin on islet proinsulin synthesis. *Diabetes* 32:316–18, 1983
4. Wilson GL, Patton NJ, McCord JM, Mullins DW, Mossman BT: Mechanisms of streptozotocin- and alloxan-induced damage in rat β cells. *Diabetologia* 27:587–91, 1984
5. Wilson GL, Mossman BT, Craighead JE: Use of beta-cells in culture to identify diabetogenic *N*-nitroso compounds. *In Vitro* 19:25–30, 1983
6. Cook PR, Brazell IA: Supercoils in human DNA. *J Cell Sci* 19:261–79, 1975
7. Klein SM, Cohen G, Cederbaum AI: The interaction of hydroxyl radicals with dimethylsulfoxide produces formaldehyde. *FEBS Lett* 116:220–22, 1980
8. McCord JM, Day ED Jr: Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. *FEBS Lett* 86:139–42, 1978
9. Steffer C, Netter KJ: On the mechanism of paraquat action on microsomal oxygen reduction and its relation to lipid peroxidation. *Toxicol Appl Pharmacol* 47:593–601, 1979
10. Heikkila RE, Winston B, Cohen G: Alloxan-induced diabetes: evidence for hydroxyl radical as a cytotoxic intermediate. *Biochem Pharmacol* 25:1085–92, 1976
11. Cohen G, Heikkila RE: The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J Biol Chem* 249:2447–52, 1974
12. Yamamoto H, Uchigata Y, Okamoto H: Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature* (Lond) 294:284–86, 1981
13. Asayama K, English D, Slonim AE, Burr IM: Chemiluminescence as an index of drug-induced free radical production in pancreatic islets. *Diabetes* 33:160–63, 1984
14. Robbins MJ, Sharp RA, Slonim AE, Burr IM: Protection against streptozotocin-induced diabetes by superoxide dismutase. *Diabetologia* 18:55–58, 1980
15. Sandler S, Andersson A: The partial protective effect of hydroxyl radical scavenger dimethyl urea on streptozotocin-induced diabetes in the mouse in vivo and in vitro. *Diabetologia* 23:374–78, 1982
16. LeDoux SP, Woodley SE, Patton NJ, Wilson GL: Mechanisms of nitrosourea-induced β-cell damage: alterations in DNA. *Diabetes* 35:866–72, 1986
17. Bennett RA, Pegg AE: Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res* 41:2786–90, 1981

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/8/1015/356100/37-8-1015.pdf by guest on 12 September 2024

18. Saffhill R, Margison GP, O'Connor PJ: Mechanisms of carcinogenesis induced by alkylating agents. *Biochim Biophys Acta* 823:111–45, 1985
19. Cleaver JE, Morgan WF: Poly(ADP-ribose) synthesis is involved in the toxic effects of alkylating agents but does not regulate DNA repair. *Mutat Res* 150:69–76, 1985
20. Shall S: ADP-ribose in DNA repair: a new component of DNA excision repair. *Adv Radiat Biol* 11:1–69, 1984
21. Nomikos IN, Prowse SJ, Carotenuto P, Lafferty KJ: Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. *Diabetes* 35:1302–304, 1986
22. Fischer LJ, Rickert DE: Pancreatic islet-cell toxicity. *CRC Crit Rev Toxicol* 3:231–63, 1975
23. Orci L, Amherdt M, Malaisse-Lagae F, Ravazzola M, Malaisse WJ, Perrelet A, Renold AE: Islet cell membrane alteration by diabetogenic drugs. *Lab Invest* 34:451–54, 1976
24. Meglasson MD, Burch PT, Berner DK, Najafi H, Matschinsky FM: Identification of glucokinase as an alloxan-sensitive glucose sensor of the pancreatic β-cell. *Diabetes* 35:1163–73, 1986
25. Weaver DC, McDaniel ML, Naber SP, Barry CD, Lacy PE: Alloxan stimulation and inhibition of insulin release from isolated rat islets of Langerhans. *Diabetes* 27:1205–14, 1978
26. Weaver DC, Barry CD, McDaniel ML, Marshall GR, Lace PE: Molecular requirements for recognition at a glucoreceptor for insulin release. *Mol Pharmacol* 16:361–68, 1979

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/8/1015/356100/37-8-1015.pdf by guest on 12 September 2024