Mechanisms of Nitrosourea-Induced β-Cell Damage

Activation of Poly(ADP-Ribose) Synthetase and Cellular Distribution

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It has been hypothesized that the critical step in streptozocin (STZ)-induced β-cell toxicity is the overactivation of the nuclear enzyme poly(ADP-ribose) synthetase resulting from DNA strand breaks. Overactivation of this enzyme leads to a lethal depletion of its substrate, NAD, in the β-cell. However, recently it has been shown that a lethal concentration of STZ and a nontoxic concentration of its nitrosoamide moiety methylnitrosourea (MNU) damage β-cell DNA to the same extent and cause comparable amounts of DNA strand breaks. This study was performed to determine whether STZ and MNU activate poly(ADP-ribose) synthetase to the same extent. Monolayer cultures of islet cells from neonatal rats were exposed to concentrations of MNU and STZ of 10^{-3} to 10^{-2} M. The results show that both chemicals caused comparable activation of the enzyme at all concentrations tested. These data demonstrate that activation of poly(ADP-ribose) synthetase alone is not the critical step in STZ-induced β-cell toxicity. Based on this finding, it appeared possible that STZ may be selectively sequestered into some critical site in the β-cell other than the nucleus. Therefore, studies were initiated with ^{14}C-labeled STZ and MNU to determine whether STZ might be distributed in the β-cell differently than MNU. Total cellular DNA and protein from both RINr (clone 38) and islet cell monolayers were separated on hydroxylapatite columns after exposure to ^{14}C-labeled chemicals. The amount of label incorporated into each fraction was determined by liquid scintillation spectrometry, and the ratio of label incorporated in protein to that in DNA was determined. The results showed that RINr cells, which are resistant to the toxic effects of STZ, have similar protein-to-DNA ratios for MNU (0.32 ± 0.04) and STZ (0.39 ± 0.16) at an equimolar concentration. However, in β-cells the ratio was approximately three times greater for STZ (0.60 ± 0.17) compared with that for MNU (0.21 ± 0.17). These data demonstrate that in β-cells, which are very sensitive to the toxic effects of STZ, many more of the reactive carbonium ions bind to protein. This finding indicates that in β-cells, STZ is sequestered differently than MNU. *Diabetes* 37:213–16, 1988

Streptozocin (STZ) is a naturally occurring nitrosoamide that has been used extensively to produce diabetes in experimental models. Although this chemical has been studied for many years, the exact mechanisms by which it exerts its toxic effects have yet to be fully elucidated. One proposed site for the action of STZ has been at the level of nuclear DNA; it has been suggested that STZ spontaneously decomposes to form carbonium ions that alkylate DNA bases. As part of the process to repair some of these DNA adducts, the nuclear enzyme poly(ADP-ribose) synthetase becomes activated to such an extent that cellular levels of its substrate NAD become critically depleted (1,2). However, this hypothesis recently has been called into question by the finding that a lethal concentration of STZ and a nonlethal concentration of its nitrosoamide moiety methylnitrosourea (MNU) alkylate the DNA of β-cells at the N^7 position of guanine to the same extent and cause comparable amounts of DNA strand breaks (3). This finding suggests that other factors, in addition to the activation of poly(ADP-ribose) synthetase, contribute to the specific toxicity of STZ to β-cells. If indeed other factors are required, it would be expected that both MNU and STZ would activate poly(ADP-ribose) synthetase to the same extent. Our first set of experiments were initiated to test this assumption. The β-cell cultures were exposed to equimolar concentrations of MNU or STZ, and the activation of this nuclear enzyme was assessed.

Because STZ contains glucose in its structure, it is possible that the β-cell may uniquely recognize and transport

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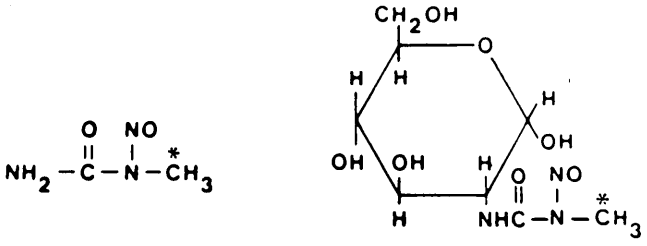

FIG. 1. Structure of methylnitrosourea (*left*) and streptozocin (*right*) showing location of ^{14}C-labeled carbon (*asterisk*).

this toxin to some critical compartment. Our second set of experiments were initiated to determine whether STZ may be sequestered differently in β-cells. Both RINr cells and normal β-cells were exposed to either ^{14}C-labeled MNU or STZ and the content of label in the protein and DNA of these cells was determined.

MATERIALS AND METHODS

Chemicals. STZ was kindly supplied by A. Chang (Upjohn, Kalamazoo, MI); MNU was purchased from Sigma (St. Louis, MO); ^{14}C-labeled MNU and ^{14}C-labeled NAD were purchased from Amersham (Arlington Heights, IL); and ^{14}C-labeled STZ was purchased from Research Triangle Institute (Research Triangle Park, NC). Both radioactive chemicals were synthesized so that the label was placed on the 3′ carbon of the *N*-nitroso moiety (Fig. 1). This is the carbon that forms the carbonium ion for alkylating reactions.

Pancreatic β-cell cultures. The techniques used to prepare monolayer cultures from the pancreases of neonatal rats have been described previously (4,5). After dissociation, islet cells were plated into 60-mm culture dishes, treated with 4 μg/ml iodoacetic acid to remove the contaminating fibroblastoid cells, and maintained in medium 199 supplemented with 10% fetal bovine serum, 16.5 mM glucose, and 1 mg/dl gentamicin. The RINr clone 38 cells were obtained from W. Chick (Univ. of Massachusetts Medical School, Worcester, MA). These cells were plated into 60-mm culture dishes and maintained in medium 199 supplemented with 10% horse serum and 1 mg/dl gentamicin. Cultures were used 3 days after plating; the RINr cells were nearly confluent at this time.

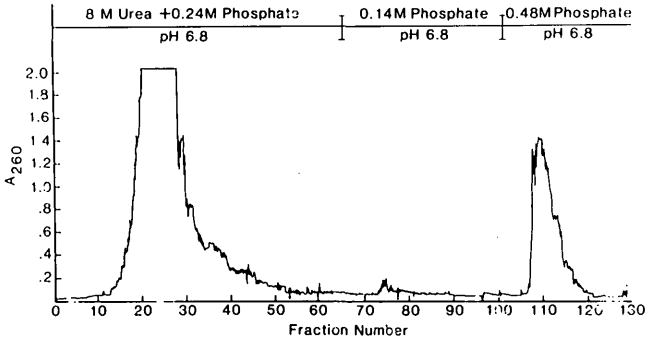

FIG. 2. Separation of DNA from protein by column chromatography. Columns of hydroxylapatite, which had been equilibrated with 0.24 M sodium phosphate buffer containing 8 M urea, pH 6.8, were loaded with sheared-cell lysate from either RINr cells or β-cells treated with ^{14}C-labeled STZ or methylnitrosourea. Representative absorbance tracing of eluate from column is shown. Column initially was washed with 0.24 M sodium phosphate buffer containing 8 M urea, pH 6.8, to remove protein, lipid, and RNA (1st peak). Next, column was washed with 0.14 M sodium phosphate buffer, pH 6.8, to remove urea. Finally, DNA was eluted from column with 0.48 M sodium phosphate buffer, pH 6.8 (2nd peak).

Drug preparation and exposure. STZ and MNU were dissolved in citrate buffer (pH 4.5), and desired concentrations were obtained by serial dilution in Hanks' balanced salt solution (HBSS). Before drug exposure, the culture medium was removed and cells were washed with HBSS. Exposure to the drugs was for 60 min. The ^{14}C-labeled STZ had a specific activity of 1.30 mCi/mM, and ^{14}C-labeled MNU had a specific activity of 61 mCi/mM. To bring the ^{14}C-labeled MNU to the same specific activity as the ^{14}C-labeled STZ, the labeled MNU was solubilized with 20.22 mg cold MNU in 4 ml ether under a dry argon atmosphere. The MNU was then aliquoted into vials, and the ether was evaporated by heating (34°C) under argon. Finally, the vials were sealed under argon and stored at −70°C until used.

DNA and protein isolation. DNA and protein were isolated with the hydroxylapatite technique described previously (3). With this technique, the cellular protein can be easily separated from the DNA (Fig. 2). The protein fraction was collected and dialyzed for 36 h to remove the salt and then lyophilized. The lyophilized protein was resuspended in 1 ml water, and a 0.1-ml aliquot was removed for protein determination by the method of Lowry et al. (6). The remainder of the protein was lyophilized in a scintillation vial, and scintillation fluid was added. The DNA fraction was collected and dialyzed for 36 h to remove the salt and was then lyophilized. The DNA was resuspended in 1 ml water, and the concentration of DNA was determined with the 260:280 absorbance ratio. The sample then was lyophilized in a scintillation vial, and scintillation fluid was added. Both samples were counted in a Beckman model LS6800 (Fullerton, CA) liquid scintillation counter. Treatment of the protein sample with RNase did not appreciably diminish the counts in this sample (data not shown), indicating that almost all of the label was bound to protein. It has been shown that STZ will not alkylate lipids appreciably (7).

Poly(ADP-ribose) synthetase activity. Poly(ADP-ribose) synthetase activity was determined with the procedures described by Benjamin and Gill (8).

RESULTS

To determine the effects of exposure of β-cells to either MNU or STZ on the activity of poly(ADP-ribose) synthetase, β-cell cultures were treated with 1, 2, 5, or 10 mM concentrations of either toxin, and the activity of the enzyme was assayed. Previous work has shown that STZ is toxic to cultured β-cells at a concentration of 1 mM, whereas MNU is not lethal at this concentration and is only mildly toxic at 10 mM (3,5). The results of these studies show that there is no statistically significant difference in the activation of this enzyme by nonlethal concentrations of MNU and equimolar lethal concentrations of STZ (Fig. 3).

To assess the cellular distribution of MNU and STZ, cultures of RINr or normal β-cells were exposed to drug labeled with ^{14}C at the 3′ carbon of the nitrosoamide for 1 h. The cells then were removed from the culture vessel by light trypsinization and lysed, and the protein was separated from

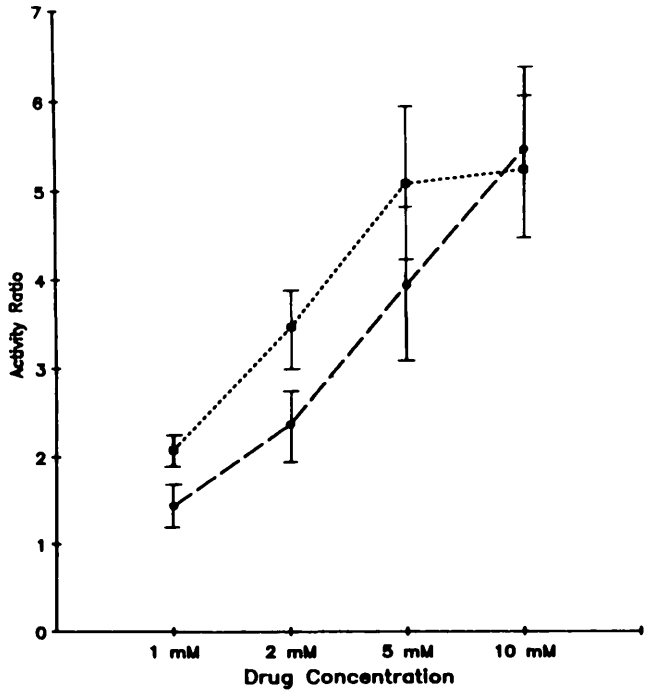

FIG. 3. Effects of STZ (*dashed line*) and methylnitrosourea (*dotted line*) on activity of poly(ADP-ribose) synthetase in monolayer cultures of rat islet cells. Cultures were exposed to various concentrations of STZ or methylnitrosourea, and activity of poly(ADP-ribose) synthetase was determined by method of Benjamin and Gill (8). Data are expressed as ratio of activity in drug-treated cultures to that in control cultures. Values are means ± SE; *n* = 4.

the DNA on a hydroxylapatite column. The amount of label incorporated in each fraction was determined by liquid scintillation spectrometry. In RINr cells, more carbonium ions from MNU reacted with both protein and DNA than those from STZ (Table 1). However, the proportion of label in the protein fraction compared with that in the DNA fraction was virtually the same for both chemicals. Conversely, in β-cells, more carbonium ions from STZ reacted with protein and DNA than those from MNU. In contrast to the findings with RINr cells, three times more label was incorporated into the protein fraction of β-cells exposed to STZ than in β-cells exposed to MNU.

DISCUSSION

The results of this study show that MNU and STZ, at equimolar concentrations, activate poly(ADP-ribose) synthetase to the same extent. This finding supports our previous work that demonstrated that MNU and STZ, at an equimolar concentration, where STZ was toxic and MNU was not, alkylated the N^7 position of guanine to the same extent and produced comparable DNA strand breaks as part of the excision repair process to remove these lesions (3). Therefore, the amount of reduction in NAD resulting from the activation of poly(ADP-ribose) synthetase would be that seen after exposure to MNU (~50% of control after treatment with 1 mM MNU). The remaining drop in NAD concentration resulting from exposure to STZ (13% of control after treatment with 1 mM STZ) must be due to other factors.

The experiments with the labeled nitrosoureas demonstrate that STZ can have different effects in β-cells than MNU. The results from the determination of incorporation of label into cellular protein and DNA support the findings of other investigators (9), which show that more STZ than MNU is taken into β-cells. However, note that the amount of incorporation of label from MNU into protein and DNA may be somewhat reduced by spontaneous decomposition of this chemical during the dilution process necessary for final preparation. This dilution step was not required for the labeled STZ because it was possible to purchase this chemical in a final usable form. As a result of this decomposition, the results should not be used to compare exactly the absolute amounts of label in the DNA or protein from cells exposed to STZ with those exposed to MNU. The more valid measurement for direct comparison between the two chemicals is the protein-to-DNA ratio. The finding that STZ is sequestered in such a manner that a smaller proportion of carbonium ions from this chemical alkylate DNA in β-cells appears to explain why STZ and MNU activate poly(ADP-ribose) synthetase to the same extent, even though more STZ is taken into the cell.

Although the proteins that are specifically alkylated by STZ have not been identified, it seems reasonable to speculate that they may be related to the glucose-sensing mechanism of the β-cell. Several lines of evidence support this notion. First, it is the glucose moiety in the structure of the STZ molecule that conveys its unique properties, because both MNU and STZ are identical except for glucose. Second, RINr cells in this study were found to sequester MNU and STZ in a similar fashion. These cells have previously been found to be nonresponsive to glucose-stimulated insulin release and insensitive to STZ toxicity (10).

Based on these findings, it appears likely that the lethal drop in NAD concentrations after exposure of β-cells to STZ is related in some fashion to the glucose-sensing mechanism

TABLE 1
Binding of carbonium ions from ^{14}C-labeled STZ or MNU to protein or DNA in RINr cells and β-cells

	n	Drug (1 mM)	Protein (pM/mg)	DNA (pM/mg)	Protein/DNA
RINr cells	6	STZ	4.2 ± 1.2	15.9 ± 4.4	0.39 ± 0.16
	10	MNU	12.2 ± 2.0	50.8 ± 4.0	0.32 ± 0.04
β-Cells	5	STZ	30.6 ± 8.8	52.9 ± 8.1	0.60 ± 0.17
	10	MNU	5.3 ± 1.4	30.4 ± 5.8	0.21 ± 0.04

Values are means ± SE. Cultures were exposed to ^{14}C-labeled drug for 1 h. Cells were then removed from culture vessel by light trypsinization, lysed, and protein was separated from DNA on a hydroxylapatite column. Amount of label incorporated in each fraction was determined by liquid scintillation spectrometry. STZ, streptozocin; MNU, methylnitrosourea.

of these cells. To ascertain how the glucose-sensing mechanism could be tied to the critical depletion of NAD, it is important to consider that NAD concentrations represent an equilibrium between the breakdown of NAD and its resynthesis. In the β-cell, NAD is probably synthesized by an ATP-requiring two-step reaction utilizing nicotinamide as a precursor (11). If ATP levels in the β-cell are depleted, as might result if glycolysis were blocked, then this synthetic process would in turn be impaired, and the equilibrium between breakdown and resynthesis would be shifted toward breakdown. A depletion of ATP has been reported in islets treated with STZ (12). It is important to note that in that study, MNU did not significantly lower ATP levels.

As a result of our study, we think a new hypothesis for the action of STZ is warranted. We propose that on entering the β-cell, STZ alkylates not only DNA but key components necessary for the generation of ATP (e.g., glycolytic or mitochondrial enzymes). As part of the process to repair the DNA lesions, the enzyme poly(ADP-ribose) synthetase is activated with NAD as a substrate. The fall of NAD levels initiated by this process is that seen after treatment with MNU. However, when β-cells are treated with STZ, concomitant with the activation of poly(ADP-ribose) synthetase, there is a drop in ATP formation, possibly due to alkylation of vital enzymes. This fall in ATP generation would impair the resynthesis of NAD, causing the levels of this key cellular component to drop below critical levels. Therefore, it is the combination of two critical processes occurring simultaneously that allows STZ to selectively intoxicate β-cells. Clearly, additional studies that specifically identify nonnuclear sites of alkylation are warranted to prove this hypothesis.

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