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Inhibition of Insulin Biosynthesis by Alloxan, Streptozotocin, and *N*-Nitrosomethylurea

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SUMMARY

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Isolated mouse pancreatic islets were used to evaluate the effect of a single intravenous injection of alloxan (100 mg/kg body weight), streptozotocin (200 mg/kg), or *N*-nitrosomethylurea (78 mg/kg) on glucose-stimulated biosynthesis of insulin in the B-cell. The animals were killed 10 min after injection of the drugs, and the rate of insulin synthesis was estimated by measurements of the incorporation of [^{3}H]leucine into proinsulin and insulin. Alloxan completely blocked insulin biosynthesis, whereas with streptozotocin and *N*-nitrosomethylurea the inhibition was only partial. Nicotinamide restored insulin biosynthesis after the latter drugs but was completely without effect on alloxan-induced inhibition.

INTRODUCTION

The insulin-producing pancreatic B-cell seems to have a unique susceptibility to some cytotoxic drugs (1). Two of these agents, alloxan and streptozotocin, have been widely used to produce diabetes in experimental animals. Although extensively studied, the mechanisms behind the toxic actions of these drugs on the pancreatic B-cell remain obscure. The injurious effects of alloxan can be prevented *in vivo* by a prior injection of glucose to the experimental animal or *in vitro* by previous exposure of islets to a high extracellular glucose concentration (2–5). This protection by glucose against alloxan diabetes may depend on the D-glucose α-anomer (6).

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Although streptozotocin diabetes cannot be prevented by glucose (4, 7) nicotinamide administered prior to streptozotocin prevents diabetes and, if administered sufficiently early after streptozotocin, may even reverse the injury (7–10).

Attempts have been made to define the toxic effects of alloxan and streptozotocin on islet metabolism even before any signs of morphological injury appear. Thus, shortly after administration, both drugs were found to inhibit glucose-stimulated insulin secretion and to decrease islet glucose oxidation (5, 11–20). In addition, streptozotocin has been reported to affect insulin biosynthesis in rat islets *in vitro* (21). Streptozotocin markedly reduced islet NAD content, and it was suggested that NAD depletion of B-cells might be the primary toxic effect of this drug (19, 22–24).

The present study is an attempt to characterize further the B-cytotoxic actions of

alloxan and streptozotocin by studies of their effects on insulin biosynthesis in the B-cell.The aglycone of streptozotocin, *N*-nitrosomethylurea, was included in the study since it has recently been shown to be B-cytotoxic in different animal species (19, 25).

MATERIALS AND METHODS

Reagents. Alloxan (monohydrate) was obtained from Nutritional Biochemicals Corporation; streptozotocin, from Upjohn Company; and *N*-nitrosomethylurea, from Pfaltz and Bauer, New York. Crude collagenase was purchased from Worthington Biochemical Corporation; bovine plasma albumin (fraction V), from Armour Pharmaceutical Company; guinea pig anti-insulin (bovine) serum, from Miles Laboratories; and Sephadex G-50 and Sepharose 4B (activated with cyanogen bromide), from Pharmacia Fine Chemicals. L-(4,5-^{3}H)Leucine (specific activity given below) and (^{125}I)insulin (bovine) were from The Radiochemical Centre, Amersham. Soluene 350 and Instagel were from Packard Instrument Company. Other reagents used were of analytical grade.

Animals. Male mice about 12 weeks old, from a stock originating from the Naval Medical Research Institute, Bethesda, Md. (NMRI mice; Anticimex, Sollentuna, Sweden), were used. The animals were fed a commercial pellet food (Anticemex 210) with the following composition by weight (except for trace elements and vitamins): crude protein, 20.8%; crude fat, 3.7%; nitrogen-free extractable substances, 53.8%; fiber, 5.8%; ash, 7.4%; water content, 8.3%. The animals were fasted overnight before the experiments, but were allowed free access to water.

Administration of drugs. All drugs were injected as freshly prepared solutions into a tail vein. The doses were 100 mg/kg for alloxan, 200 mg/kg for streptozotocin, and 78 mg/kg for *N*-nitrosomethylurea. This dose of *N*-nitrosomethylurea was equimolar to that of streptozotocin. Alloxan was dissolved in redistilled water, and the other two drugs in acidified saline (0.154 M NaCl adjusted to pH 4.5 with 0.05 M citric acid). The drug concentrations were adjusted so that the volume injected was 2 μl/g of body weight. Controls received the same amount of saline alone.

Isolation of pancreatic islets. Two mice were used in a single experiment and were killed by decapitation 10 min after administration of the drug. The pancreases were immediately excised, and the islets of Langerhans were kept in Hanks' balanced salt solution (with 2.2 mM glucose) using a collagenase method as described by Howell and Taylor (26). In some experiments, described below, 10 mM nicotinamide was added to Hanks' solution.

Incubation of isolated islets. In each experiment four to six groups of 10 islets each were incubated for 120 min at 37° in 100 μl of a bicarbonate buffer (27) supplemented with 10 μCi of L-[4,5-^{3}H]leucine (specific activity, 46 Ci/mmole), 20 μg/ml of each of the amino acids in the mixture described by Eagle (28) except for leucine, 0.2% (w/v) albumin, and either 3.3 or 16.7 mM glucose.

In some experiments 10 mM nicotinamide was added to the incubation medium. All incubations were performed in small glass vials (29). After the incubation period the islets were quickly washed twice in Hanks' solution containing an excess of unlabeled leucine (10 mM) and homogenized by sonication in 200 μl of redistilled water. Carrier insulin (see below) was added when the islet homogenates were subjected to gel chromatography.

Gel chromatography of islet homogenates. Each islet homogenate was supplemented with 0.5 mg of crystalline bovine insulin and 0.5 mg of bovine plasma albumin. Proteins were precipitated by addition of 200 μl of 10% (w/v) trichloracetic acid, centrifuged, and washed twice with 5% trichloracetic acid. Extraction with acid–ethanol was avoided because the total radioactivity incorporated into islet proteins was to be estimated (30). The washed precipitate was dissolved in 3 M acetic acid and subjected to gel chromatography at 4° on a Sephadex G-50 (fine) column (67 × 1 cm inner diameter). The column was calibrated with blue dextran 2000, bovine plasma albumin, horse heart cytochrome *c*, bovine insulin, and bovine glucagon.

The optical density of the eluate at 280 nm was recorded continuously by an absorptiometer (Uvicord II, LKB-Produkter, Bromma, Sweden). A 0.8-ml sample of each fraction (0.9 ml) was mixed with Instagel and counted in a liquid scintillation spectrometer (Packard model 3380).

Quantitative measurements of insulin biosynthesis. Proinsulin and insulin in the islet homogenates were determined by an immunoadsorption assay (31, 32). Duplicate homogenate samples, 3.3 μl each, were incubated with insulin antiserum linked to Sepharose 4B (33).[1] The binding capacity of the antiserum was 1.5 units/ml, and about 90% of the activity remained after coupling to Sepharose. The antiserum was shown to react with both proinsulin and insulin present in the islet homogenates which were chromatographed. The reaction between the Sepharose-linked antibodies and the proinsulin and insulin in the homogenates was allowed to proceed during a 2-hr incubation at room temperature in 0.04 M sodium phosphate buffer, pH 7.4, with 0.1 M NaCl and 0.2% (w/v) albumin. The tubes were continuously rotated (20 rpm) in order to keep the Sepharose beads in suspension. Nonspecific binding was estimated by simultaneous incubations of samples with Sepharose-linked serum from nonimmunized guinea pigs. As shown in separate experiments, the amount of added antiserum exceeded by about 3-fold the amount of antibodies which could completely adsorb an added tracer dose of [^{125}I]insulin (bovine). This dose of Sepharose-linked antiserum was sufficient to adsorb about 90% of the proinsulin in the islet homogenates, as indicated by the corresponding reduction of radioactive proinsulin in the supernatant when this was extracted and gel-chromatographed as described above.

After incubation of an islet homogenate with antiserum the Sepharose beads were centrifuged and washed three times with the same sodium phosphate buffer without albumin. The radioactivity on the Sepharose beads was subsequently eluted with Soluene 350, and after addition of scintillator the radioactivity was counted in a liquid scintillation spectrometer (Packard model 3380). The counting efficiency was 33%. Radioactivity adsorbed to the normal guinea pig serum was subtracted (32). In each experiment two islet samples were incubated with either 3.3 or 16.7 mM glucose and subsequently subjected to immunoadsorption. The mean value of these two samples was used in the statistical calculations. Total islet protein synthesis was estimated by precipitation of duplicate samples (6.6 μl each) from the islet homogenates with 5% (w/v) trichloracetic acid. The precipitates were washed twice and dissolved in Soluene 350, and radioactivity was determined as described above. The quenching was the same as after elution of radioactivity from the Sepharose beads.

To exclude the possibility that significant amounts of newly synthesized proinsulin-insulin were released, the content of ^{3}H-labeled hormone was measured in the medium. It was found that in all experimental groups the release of proinsulin-insulin was less than 10% of that within the islets. Only the incorporation of [^{3}H]leucine into the proinsulin-insulin of islet homogenates was therefore measured.

RESULTS

Qualitative observations. Gel chromatographs of islet homogenates from control mice showed two discrete peaks of radioactivity, one of which migrated with the void volume proteins and the other in a region between the void volume and the marker insulin (Fig. 1). The labeled material in the latter region had an approximate molecular weight of 9000 and was bound to an excess of insulin antibodies (see above), indicating that it represented proinsulin. Likewise, labeled material migrating with the insulin marker reacted with insulin antibodies and, although this protein did not form a distinct peak, it represented a substantial amount of radioactivity. It was therefore concluded that both newly synthesized proinsulin and insulin were present in the islet eluates. Figure 1 also shows that administration of alloxan considerably decreased the labeling both in

[1] C. Berne, personal communication.

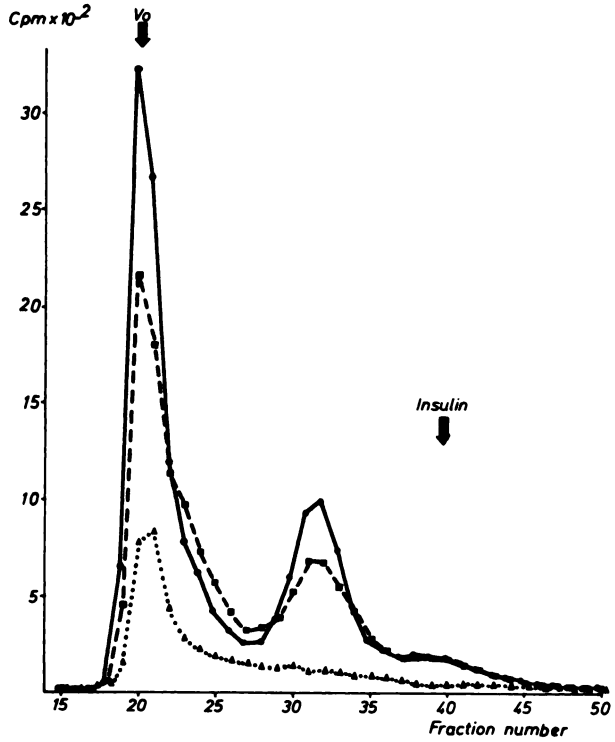

FIG. 1. *Gel filtration profiles of* (^{3}H)*leucine incorporation into proteins of isolated islets obtained after injection of the animals with either alloxan* (▲···▲), *streptozotocin* (■- - -■) *or saline* (●——●)

Islets were isolated from mice killed 10 min after injection of the drug and incubated for 2 hours with (^{3}H)leucine in 16.7 mM glucose. The figure shows the elution patterns of islet proteins obtained with gel filtration on a Sephadex G-50 (Fine) column. The profile plotted for each experimental group represents the mean of three separate experiments.

the proinsulin and insulin regions and in the void volume. Streptozotocin produced a general decrease of incorporation of radioactivity, although to a lesser degree than after alloxan. The elution pattern for *N*-nitrosomethylurea treatment was the same as for streptozotocin.

Quantitative measurements. An increase in glucose concentration from 3.3 to 16.7 mM stimulated the incorporation of labeled leucine into proinsulin-insulin about 10-fold whereas the corresponding stimulation for all the trichloracetic acid-precipitable proteins was only 3-fold (Table 1). The amount of label in proinsulin-insulin, calculated as a percentage of the radioactivity incorporated into the trichloracetic acid-precipitable proteins at the same time, increased from 8% to 25%.

After alloxan treatment the radioactivity adsorbed to the antibody-coated Sepharose beads did not differ from the blank values irrespective of the glucose concentration in the incubation medium (Table 1). The radioactivity incorporated into the trichloracetic acid-precipitable proteins at 3.3 mM glucose was in the same range as observed in the control groups. There was, however, no significant stimulation of the total islet protein synthesis with the high glucose concentration.

Administration of streptozotocin decreased the radioactivity in the proinsulin-insulin pool although there was still a significant stimulation of insulin biosynthesis by glucose. As with alloxan, the labeling of trichloracetic acid-precipitable proteins at 3.3 mM glucose remained unaffected by streptozotocin and the biosynthesis of such proteins was not stimulated

by increasing the glucose concentration. The effect of *N*-nitrosomethylurea on islet protein biosynthesis was similar to that of streptozotocin, although less pronounced.

Nicotinamide is known to protect against the diabetogenic effects of both streptozotocin and *N*-nitrosomethylurea (19). The extent to which insulin biosynthesis could be restored by addition of nicotinamide to the media *in vitro* during isolation and incubation of the pancreatic islets was therefore evaluated. There was no effect of this drug after alloxan (Table 2). By contrast, the biosynthesis of both insulin

TABLE 1

Radioactivity incorporated into proinsulin-insulin and islet trichloracetic acid-precipitable proteins

Treatment	No. of experiments	Glucose	Proinsulin-insulin (I)	Trichloracetic acid-precipitable proteins (II)	(I/II) × 100
		mM	*cpm* × 10^{-3}/*islet*/2 *hr* (± *SEM*)		%
Saline	7	3.3	3.0 ± 0.6	38.1 ± 6.4	8 ± 2
		16.7	31.3 ± 6.8[a]	120.3 ± 17.2[b]	25 ± 4[a]
Alloxan	5	3.3	—[c]	29.0 ± 4.5	—[c]
		16.7	—[c]	36.0 ± 9.3	—[c]
Streptozotocin	7	3.3	1.4 ± 0.4[d]	33.3 ± 6.0	5 ± 1
		16.7	7.8 ± 0.9[a, e]	47.1 ± 3.2[e]	17 ± 2[a]
N-Nitrosomethylurea	7	3.3	2.6 ± 0.8	37.8 ± 13.9	7 ± 2
		16.7	16.4 ± 1.8[a, d]	73.2 ± 6.6[d]	25 ± 2[a]

[a] $p < 0.01$ in comparison with incorporation at 3.3 mM glucose.
[b] $p < 0.05$ in comparison with incorporation at 3.3 mM glucose.
[c] Incorporation did not differ from blank values.
[d] $p < 0.05$ in comparison with saline-treated control.
[e] $p < 0.01$ in comparison with saline-treated control.

TABLE 2

Radioactivity incorporated into proinsulin-insulin and islet trichloracetic acid-precipitable proteins in the presence of 10 mM nicotinamide

Treatment	No. of experiments	Glucose	Proinsulin-insulin (I)	Trichloracetic acid-precipitable proteins (II)	(I/II) × 100
		mM	*cpm* × 10^{-3}/*islet*/2 *hr* (± *SEM*)		%
Saline	8	3.3	3.8 ± 0.6	31.6 ± 4.1	12 ± 2
		16.7	24.9 ± 3.6[a]	79.3 ± 9.3[a]	32 ± 2
Alloxan	5	3.3	—[b]	24.7 ± 4.9	—[b]
		16.7	—[b]	37.1 ± 6.6[c]	—[b]
Streptozotocin	7	3.3	3.9 ± 1.1	31.1 ± 4.4	13 ± 4
		16.7	23.9 ± 6.6[a]	81.1 ± 12.9	28 ± 4[d]
N-Nitrosomethylurea	7	3.3	1.9 ± 0.4	20.0 ± 3.1	12 ± 3
		16.7	19.3 ± 3.2[a]	85.4 ± 19.6[a]	24 ± 2

[a] $p < 0.01$ in comparison with incorporation at 3.3 mM glucose.
[b] Incorporation did not differ from blank values.
[c] $p < 0.01$ in comparison with saline-treated controls.
[d] $p < 0.05$ in comparison with incorporation at 3.3 mM glucose.

and trichloracetic acid-precipitable proteins proceeded at a normal rate after streptozotocin and *N*-nitrosomethylurea in the presence of nicotinamide.

DISCUSSION

The present results demonstrate a marked inhibitory effect of the B-cytotoxic drugs on the biosynthesis of insulin. Alloxan appeared most potent in this respect, since it virtually abolished the incorporation of radioactivity into proinsulin and insulin, whereas streptozotocin and *N*-nitrosomethylurea only partially suppressed the incorporation. It was also clear that a high glucose concentration in the incubation medium could not overcome the alloxan-induced noxious effects on the B-cell. This was in marked contrast to the observation that nicotinamide was able to suppress entirely the inhibitory effects of both streptozotocin and *N*-nitrosomethylurea on insulin biosynthesis. Nevertheless, the apparent difference in toxicity between alloxan and streptozotocin so far must be regarded as tentative, since only one concentration of each drug and only one time of exposure were evaluated.

The present data confirm previous observations (30)[1, 2] indicating that glucose has a stimulatory action not only on the synthesis of insulin but also on islet protein synthesis in general. The total islet protein synthesis appeared, however, to be less affected by the B-cytotoxic drugs, at least at a low glucose concentration. The residual protein synthesis after alloxan treatment may in part reflect protein synthesis in the non-B-cell portion of the islets, e.g., A_1- and A_2-cells, endothelium, and connective tissue.

The marked depression of the glucose-stimulated insulin synthesis presently observed after alloxan, streptozotocin, and *N*-nitrosomethylurea may be either primary or secondary effects of the drugs. In view of the marked effects of the B-cytotoxic agents on islet oxidative metabolism mentioned above, it seems most plausible to interpret the diminished synthesis of insulin in response to glucose as secondary to this metabolic deterioration. The reversal of the functional disturbance after streptozotocin and *N*-nitrosomethylurea by nicotinamide suggests that maintenance of normal B-cell NAD concentrations may be essential for insulin biosynthesis. Parry and Taylor (34), however, reported no inhibition of insulin biosynthesis in rabbit islets by the glutamine analogue azaserine, which has been found to reduce NAD in rat islets (35). The failure of azaserine to reduce protein synthesis in rabbit islets could reflect species differences in the action of this compound. This raises the additional question to what extent the drugs are organ-specific in reducing the rate of protein synthesis. *N*-Nitrosomethylurea has recently been shown to reduce protein synthesis in several organs and tissues, e.g., liver and muscle (36), but nothing is known so far about the general effects of alloxan and streptozotocin on protein synthesis.

In conclusion, the present study shows that a brief exposure of the B-cell *in vivo* to alloxan, streptozotocin, or *N*-nitrosomethylurea induces a blockade in glucose-stimulated insulin biosynthesis. It is of interest in this context that defective insulin biosynthesis in response to glucose has recently been observed in spontaneously diabetic mice homozygous for the mutation "diabetes" (gene symbol *db*).[2] In contrast with the present situation, the reduced insulin biosynthesis in diabetic mice was not associated with a decrease in islet glucose metabolism.

[2] R. Gunnarsson, unpublished observations.

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