Streptozotocin, but Not Alloxan, Induces DNA Repair Synthesis in Mouse Pancreatic Islets in Vitro

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Summary. In the present investigation, the abilities of streptozotocin and alloxan to induce DNA repair synthesis in isolated mouse pancreatic islets have been compared using an autoradiographic technique. Streptozotocin exposure in vitro induced a dose-dependent DNA repair synthesis, whereas no such effect was observed after alloxan treatment. The hydroxyl radical scavenger dimethyl urea and the poly(ADP-ribose) synthetase inhibitors nicotinamide and theophylline reduced

the streptozotocin-induced DNA repair. The results suggest that the initial events in streptozotocin-induced B cell injury are DNA damage and repair and that alloxan exerts its major cytotoxic effect by a different mechanism.

Key words: Alloxan, DNA repair, dimethyl urea, pancreatic islets, poly(ADP-ribose)synthetase, streptozotocin.

The precise mechanisms of the diabetogenic action of streptozotocin (STZ) and alloxan are not yet fully understood. It has been suggested recently that the primary effect of both these drugs on the endocrine pancreatic B cell is primarily induction of lesions of the DNA strand [1]. The injury induces DNA repair involving the activation of polyadenosine diphosphoribose (poly(ADP-rib))synthetase which uses cytosolic NAD as a substrate. It is thought that the lowered concentrations of NAD following this sequence of events may be the final step in the destruction of the B cell [1–3]. This hypothesis is further corroborated by the protective effect of compounds that inhibit nuclear poly(ADP-rib)synthetase [4–7] against the depression of insulin biosynthesis caused by STZ and alloxan in vitro [3].

In the present study, we have used an autoradiographic technique to compare the abilities of STZ and alloxan to induce DNA repair synthesis in isolated pancreatic islets. In addition, the protective effects of both poly(ADP-rib)synthetase inhibitors and the hydroxyl radical scavenger dimethyl urea against STZ-induced DNA repair were evaluated.

Materials and Methods

Chemicals

Streptozotocin (lot 60, 273-5 U9889) was a gift from Dr. W.E. Dulin, Upjohn, Kalamazoo, Michigan, USA. Alloxan (2,4,5,6-tetraoxypy-

rimidine) monohydrate and dimethyl urea were supplied by Sigma Chemicals, St. Louis, Missouri, USA. Collagenase was from Worthington Biochemicals, Freehold, New Jersey, USA and bovine albumin (fraction V) from Miles Laboratories, Slough, Bucks, UK. ³H-thymidine (spec. act. 5 Ci/mmol) was obtained from Amersham International, Bucks, UK. Emulsion Kodak NTB-2, developer Kodak D-19 and fixative F-24 were obtained from Kodak, Sollentuna, Sweden. Other chemicals of analytical grade were from E. Merck, Darmstadt, FRG.

Islet Isolation and Incubation

Islets of Langerhans were isolated by collagenase digestion [8] from overnight starved NMRI mice (Anticimex AB, Sollentuna, Sweden). Batches of 150 islets were pre-incubated in a Krebs-Ringer bicarbonate buffered medium [9] containing Hepes (10 mmol/l) and albumin (2 mg/ml) subsequently referred to as KRBH, for 30 min at 37 °C in a humidified atmosphere of 5% CO₂ in air. In experiments involving STZ, KRBH (1 ml) supplemented with glucose (5.6 mmol/l) was used and KRBH (2 ml) containing either 2.8 or 16.7 mmol/l glucose was used in incubations with alloxan. Agents with a potentially protective effect against STZ were included in the media from the beginning of the pre-incubation period.

STZ was dissolved in cold citric acid buffer (10 mmol/l, pH 4.5) and alloxan in KRBH containing glucose (2.8 mmol/l) < 1 min before addition to the incubation media. In control experiments buffer alone was added to the media. The incubations were continued for a further 30 min at 20 °C in air and subsequently terminated by rinsing the islets in the appropriate KRBH medium.

Islets were then incubated in 5 ml KRBH with glucose (5.6 mmol/l) and ³H-thymidine (10 µCi/ml) for 120 min at 37 °C in a humidified atmosphere of 5% CO₂ in air for determination of DNA repair synthesis. After incubation the islets were washed in non-radioactive medium and fixed in Bouin's solution.

Fig. 1. Autoradiographic nuclear grain count distribution of islets exposed to streptozotocin. After exposure to STZ, the islets were incubated with ^3H -thymidine and autoradiographed as described in Materials and Methods. The number of silver grains overlying each islet cell nucleus was scored in at least 2000 cells in each group. The numbers of nuclei with a certain grain count is expressed as a percentage of the total number of counted cells

Table 1. DNA repair synthesis of islets of Langerhans after exposure to streptozotocin in vitro

	Streptozotocin (mmol/l)				
	0	0.55	1.1	2.2	4.4
Nuclear grain count	1.67 ± 0.07	2.19 ± 0.20^a	3.43 ± 0.32^b	4.39 ± 0.30^b	3.29 ± 0.26^b

The values are given as mean $\pm$ SEM for five preparations. Significance of difference between control and STZ treated islets ^a $p < 0.05$; ^b $p < 0.001$

Histological Examination and Autoradiography

The fixed islet specimens were embedded in paraffin, sectioned at 7 μm and mounted on glass slides. The slides were dipped in Kodak NTB-2 emulsion, carefully desiccated and exposed for 9 weeks at 4°C. Slides were then developed in Kodak D-19 at 20°C for 6 min, fixed in Kodak F-24 for 10 min and finally stained with haematoxylin.

To estimate the islet cell DNA repair synthesis, the nuclear grain count (i.e. the number of silver grains directly overlying each islet cell nucleus) was counted in at least 200 islet cells in each preparation. The distribution of nuclear grain counts and the mean nuclear grain count was calculated, the latter being used as an index of the DNA repair synthesis.

The grain count per unit area not overlying the tissue sections (i.e. the background grain count) was similar in all experimental groups. Furthermore, the background grain count did not differ from the grain count per unit area of the emulsion overlying the islet sections in the control groups, which had been incubated in the absence of STZ and alloxan. The few islet cells undergoing DNA replication showed heavily labelled nuclei and were easily distinguished from the rest of the islet cells. The replicatory activity was generally low ($< 0.5\%$) both in the islets exposed to STZ or alloxan and in the controls.

In addition, the degree of islet fragmentation and islet cell pycnosis was estimated in each histological preparation. Islet sections with pycnotic nuclei and fragmented tissue architecture had a grain count per unit area similar to the background grain count, irrespective of the grain count obtained from apparently viable cells in the same preparation. The pycnotic portions of the islet sections were therefore excluded from the estimations of mean nuclear grain counts.

Results

Effects of Streptozotocin on Islet DNA Repair

Exposure of isolated islets to STZ induced a dose-dependent DNA repair synthesis, as indicated by an increased silver grain count (Table 1). The histogram describing the distribution of grain counts (Fig. 1) of individual nuclei showed a single discrete peak in both control and STZ-treated islets with a marked shift towards higher counts in the latter group. The highest rate of DNA repair synthesis was found in islets exposed to 2.2 mmol/l STZ (Table 1). At this concentration, small areas of islet nuclear pycnosis occasionally could be observed, a finding not present at the lower concentrations of STZ or in the control groups. At 4.4 mmol/l STZ, all experimental preparations showed nuclear pycnosis, in some sections up to 25% of the cells.

Effects of Alloxan on Islet DNA Repair

Alloxan exposure did not induce DNA repair synthesis in isolated islets (Table 2). Over the alloxan concentration range 0.5–2.0 mmol/l there was no increase in the nuclear grain count. At 4.0 mmol/l alloxan, all the islet cell nuclei were pycnotic and the tissue had begun to disintegrate. At the lower alloxan concentrations there appeared to be a dose-dependent increase of nuclear pycnosis which comprised at the most 50% of the islets cell nuclei.

Effects of Poly(ADP-ribose)synthetase Inhibitors and Dimethyl Urea on Streptozotocin-Induced DNA Repair

The poly(ADP-rib)synthetase inhibitors nicotinamide (6.1 mmol/l) and theophylline (5 mmol/l) both reduced STZ induced DNA repair (Table 3). At the concentrations used nicotinamide reduced DNA repair to levels similar to those observed in the control group, whereas theophylline did not cause a notable inhibition. Dimethyl urea (68 mmol/l) also caused a partial inhibition of STZ-induced DNA repair.

Table 2. DNA repair synthesis of islets of Langerhans after exposure to alloxan in vitro

	Alloxan (mmol/l)					
	0	0.5	1.0	2.0	4.0	2.0
Glucose (mmol/l)	2.8	2.8	2.8	2.8	2.8	16.7
Nuclear grain count	1.26 ± 0.07(6)	1.37 ± 0.09(6)	1.49 ± 0.12(4)	1.35 ± 0.13(4)	—(6) ^a	1.26 ± 0.07(6)

Values for nuclear grain count are given as mean ± SEM with number of observations in parentheses.

^a Nuclear grain count not possible due to pycnosis

Table 3. DNA repair synthesis of islets of Langerhans after exposure to streptozotocin and protective agents in vitro

	Streptozotocin (mmol/l)				
	0	2.2	2.2	2.2	2.2
Protective agent (mmol/l)	0	0	nicotinamide (6.1)	theophylline (5.0)	dimethyl urea (68)
Nuclear grain count	1.89 ± 0.15	4.99 ± 0.22 ^b	2.41 ± 0.25 ^e	3.54 ± 0.32 ^{a,d}	3.83 ± 0.32 ^{b,c}

Values are given as mean ± SEM for five observations. Significance of difference between control and STZ exposed islets

^a $p < 0.01$; ^b $p < 0.001$ and between islets treated with STZ only and those treated with STZ and a potentially protective agent ^c $p < 0.05$;

^d $p < 0.01$; ^e $p < 0.001$

Discussion

There are several advantages to the use of autoradiographic techniques in the estimation of DNA repair synthesis [10]. Firstly, both the background grain count and the grain count of the control tissue can be compared to establish that there is no significant repair during control conditions. Secondly, during the microscopical examination of the autoradiographs the few cells undergoing DNA replication can be identified easily and excluded from the calculations of mean grain counts, provided that the correct exposure time and development conditions are chosen. This procedure increases the sensitivity of the method thus making it possible to detect low levels of DNA repair. Indeed, if the ³H-thymidine incorporation were measured after STZ treatment in trichloroacetic acid precipitable islet material, most of the isotope recovered would be derived from replicating cells (Swenne and Sandler, unpublished results). Finally, the morphological examination allows a general assessment of the viability of the tissue to be made.

The distribution of grain counts following STZ-treatment suggests that the majority of islet cells were affected by the drug. The present demonstration of an active DNA repair process in combination with the previously observed DNA fragmentation and poly(ADP-rib) formation after islet STZ exposure in vitro [2, 3] strongly supports the view that STZ may act primarily by damaging the islet DNA. In further support of this hypothesis both nicotinamide and theophylline completely or partially inhibited STZ-induced DNA repair. Interestingly, the degree of inhibition correlated well with the degree of metabolic deterioration in the presence of these drugs as observed in other experiments [11]. Furthermore, it was found that the hydroxyl radical

scavenger dimethyl urea offered partial protection against STZ-induced DNA repair. A free radical mediated B-cell cytotoxicity of STZ has indeed been suggested [12–15] although free radical scavengers do not always protect the B cell against STZ-induced injuries [3, 16].

It was not possible to demonstrate an induction of DNA repair by alloxan in isolated mouse islets. From the present data it cannot be excluded that alloxan itself might affect the uptake of ³H-thymidine, which could also be the case after exposing islets to dimethyl urea or nicotinamide. Previous observations, however, indicate that theophylline is without effect on ³H-uptake [17]. Alternatively, alloxan might damage the DNA repair mechanism itself, resulting in an apparently lowered DNA repair. The morphological findings, nevertheless, suggest that alloxan had a prompt and pronounced effect on islet cell viability which could indicate that the crucial site of B cell cytotoxicity of alloxan is different from that of STZ. In support of this view, we have failed to observe a protective effect of nicotinamide against the alloxan-induced depression of glucose stimulated proinsulin and insulin biosynthesis and islet oxygen uptake in vitro [18]. Furthermore, nicotinamide which protects against STZ diabetogenicity in vivo [19, 20] was ineffective in preventing hyperglycaemia when administered similarly in conjunction with alloxan [18]. There is, indeed, evidence to suggest that the primary site of alloxan action may be on either the B cell mitochondria [21, 22] or the cell membrane [23, 24]. Although, poly(ADP-rib)synthetase activation has been demonstrated in islets after alloxan exposure [3] it does not appear to be the major event leading to B cell destruction.

The increased understanding of the molecular mechanism for STZ-induced B cell damage has thus far not accounted for the remarkable specificity of the drug

for the B cell. A preferential uptake of the drug due to the glucose moiety of the molecule has been put forward to explain the B-cell cytotoxicity [25–27]. An especially high sensitivity of the B cell to NAD depletion is also a possible explanation but one which requires further experimental support. In the case of alloxan, a selective uptake in conjunction with low intracellular concentrations of glutathione peroxidase, an enzyme scavenger of oxygen radicals, may explain the high B cell susceptibility [28, 29].

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