Biochemical Pharmacology, Vol. 42, No. 12, pp. 2275–2282, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EXPOSURE OF PANCREATIC ISLETS TO DIFFERENT ALKYLATING AGENTS DECREASES MITOCHONDRIAL DNA CONTENT BUT ONLY STREPTOZOTOCIN INDUCES LONG-LASTING FUNCTIONAL IMPAIRMENT OF B-CELLS

DÉCIO L. EIZIRIK,* STELLAN SANDLER, GUNNAR AHNSTRÖM† and MICHAEL WELSH
Department of Medical Cell Biology, Uppsala University, Uppsala and †Department of Radiobiology, University of Stockholm, Stockholm, Sweden

(*Received* 3 *June* 1991; *accepted* 19 *August* 1991)

Abstract—Pancreatic B-cells exposed *in vivo* or *in vitro* to streptozotocin (SZ), the *N*-nitrosourea derivative of glucosamide, present a long-lasting impairment in the production and release of insulin while other cell functions are better preserved. This functional impairment is associated with a defective mitochondrial function. To further study the mechanisms behind SZ actions, mouse pancreatic islets were exposed *in vitro* to SZ (1.5 mM) or to different concentrations of methyl methanesulfonate (MMS; 2, 4 and 6 mM). The effect of the aglucone moiety of SZ, nitroso-*N*-methylurea (NMU; 2, 4 and 6 mM) was also tested. Islets were either studied immediately after exposure to the drugs (day 0) or after six days in culture following toxin treatment (day 6). On day 0 the islets showed a decrease in the NAD + NADH content, decreased glucose oxidation rates and an impaired insulin release in response to glucose. Six days after exposure to SZ there was still impaired glucose oxidation and insulin release, and decreased islet insulin mRNA and insulin content, but the NAD + NADH content was again similar to the control group. On the other hand, islets which survived for 6 days in culture following exposure to either MMS or NMU were able to regain normal B-cell function. The mouse islets exposed to SZ, NMU and MMS showed on day 6 a 30–40% decrease in the content of the mitochondrial DNA encoded cytochrome *b* mRNA and a 60–70% decrease in total mitochondrial DNA, as evaluated by dot and Southern blot analysis. Only SZ decreased the insulin mRNA content whereas both MMS and NMU decreased the glucagon mRNA content. As a whole, the data obtained indicate that SZ, NMU and MMS induce damage to the mitochondrial genome, and this may contribute to the B-cell dysfunction observed after SZ treatment. It is conceivable that the glucose moiety of SZ may direct the methylation to other intracellular sites besides the mitochondrial DNA, thus explaining the different functional responses of islets following exposure to SZ and NMU.

There are several experimental and epidemiological observations suggesting that environmental factors may play a role in the etiology of insulin-dependent diabetes mellitus [1]. Among these factors, chemical toxins seem to be of relevance. There are several different possible mechanisms by which these agents could induce pancreatic B-cell impairment and/or destruction. It has been suggested recently that non-lethal doses of streptozotocin (SZ‡), the *N*-nitrosourea derivative of glucosamide [2], alter the ability of the pancreatic B-cells to produce insulin. Thus, B-cells exposed *in vivo* or *in vitro* to the toxin present a long-lasting impairment in then ability to produce and release insulin while other vital functions of the cells are much less affected [3–5]. This SZ-induced functional impairment has been associated with defective substrate catabolism at the mitochondrial level [6], decreased ATP generation [4] and a decrease in the mitochondrial encoded cytochrome *b* mRNA [7, 8]. Interestingly, the capacity to induce long-lasting functional impairment to the B-cells seems to be unique to SZ. Indeed, following exposure to alloxan, interleukin-1 or heat shock the surviving B-cells present a complete functional recovery after one week in culture (for a review see Ref. 9).

During decomposition of SZ, highly reactive carbonium ions are formed which cause alkylation of DNA bases, including alkylation of the exocyclic oxygen atom of guanine, forming O^6-alkylguanine residues [10, 11]. It has been suggested that the DNA alkylation is followed by excision DNA repair which induces DNA strand breaks and activation of the nuclear enzyme poly(ADP-ribose) synthetase, thus generating a critical decrease in the cellular content of the substrate NAD leading to decreased cell function, and eventually cell death [12, 13]. Although this sequence of events can explain the acute effects of SZ on the B-cells, the mechanisms responsible for the long-lasting suppressive effects of SZ remain to be elucidated. The initial event of DNA damage described above could provide an explanation for this phenomenon since DNA repair processes, especially those at the mitochondrial level, may not be entirely complete.

In order to further clarify this issue, mouse pancreatic islets were exposed *in vitro* to SZ or to

* Corresponding author: Décio L. Eizirik, Department of Medical Cell Biology, Biomedicum, P.O. Box 571, S-751 23 Uppsala-Sweden. Tel. (46) 0-18-174925; FAX (46) 0-18-556401.

‡ Abbreviations: SZ, streptozotocin; NMU, nitroso-*N*-methylurea; MMS, methyl methanesulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; KRBH, Krebs–Ringer bicarbonate buffer plus Hepes.

different concentrations of MMS, or NMU, the aglucone moiety of SZ. The main alkylation products induced in DNA by MMS and NMU are N-7 methylguanine and N-3 methyladenine. In addition, NMU gives a high yield of O^6-methylguanine as well as methylated phosphate groups [14–16]. Glucose-induced insulin release, glucose oxidation and the NAD + NADH content were determined immediately after islet exposure to the different drugs (day 0), or after 6 days in tissue culture (day 6). Furthermore, on day 6 the content of insulin, glucagon, cytochrome *b* mRNA and total mitochondrial DNA was evaluated in the different experimental groups.

MATERIAL AND METHODS

Chemicals. The chemicals were purchased from the following sources: culture medium RPMI 1640 and donor calf serum from Flow Laboratories (Irvine, U.K.); bovine serum albumin from Miles Laboratories (Slough, U.K.); alcohol dehydrogenase, Hepes and MNU from the Sigma Chemical Co. (St Louis, MO, U.S.A.); MMS from Eastman Kodak Company (Rochester, NY, U.S.A.); oligonucleotide labelling kit, [^{32}P]dCTP, EcoRI, Hyperfilm and [6-^{14}C]glucose (55 mCi/mM) from Amersham International (Amersham, U.K.). SZ (lot 1458F) was a generous gift from Dr A. Y. Chang, Upjohn Co. (Kalamazoo, MI, U.S.A.). All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany).

Tissue culture and test agent treatment. Male NMRI mice (Anticimex, Sollentuna, Sweden) which had been starved overnight were used. Islets were isolated from collagenase-digested pancreata with the aid of Ficoll gradients [17] and subsequently picked by means of a braking pipette. The islets were maintained free-floating for 5–6 days at 37° [18] before exposure to NMU, MMS or SZ. The culture medium was RPMI 1640 containing 10% calf serum, benzylpenicillin (60 mg/mL), streptomycin (0.1 mg) and 11.1 mM glucose. The culture medium was changed every 48 hr. The islets were then exposed *in vitro* to different concentrations of NMU, MMS or SZ for 15 min at 37°, according to a previously described protocol [3]. Control islets were exposed only to the vehicle for SZ (citrate buffer, 10 mM, pH 4.5). Immediately thereafter the islets were used either for functional studies (day 0, see below) or transferred to culture medium RPMI 1640 + 10% calf serum, and maintained in culture for 6 days before further studies were performed.

Insulin release, glucose oxidation and NAD + NADH content. For the insulin release determinations triplicate groups of 10 islets each were transferred to sealed glass vials containing 0.25 mL Krebs–Ringer bicarbonate buffer [19] supplemented with 2 mg/mL bovine serum albumin and 10 mM Hepes (KRBH). During the first hour of incubation the KRBH was supplemented with 1.7 mM glucose. The medium was then removed gently and replaced by KRBH containing 16.7 mM glucose, and the incubation was continued for a second hour. The insulin concentration in the incubation medium was measured by radioimmunoassay [20]. After the insulin release experiments, the islets were pooled and disrupted ultrasonically in 0.2 mL redistilled water. An aliquot of the homogenate was mixed with acid ethanol and the insulin was extracted overnight at 4°. DNA was measured by fluorophotometry in another fraction of the water homogenate [21, 22].

For the determination of [6-^{14}C]glucose oxidation, triplicate groups of 10 islets were transferred to glass vials containing 100 μL KRBH and non-radioactive glucose to a final concentration of 16.7 mM. Islet glucose oxidation was subsequently measured as described in detail elsewhere [23]. In these and the experiments described above the mean of the triplicate observations was considered as one observation.

For the NAD + NADH measurements groups of 30 islets were transferred to plastic tubes containing 100 μL KRBH and incubated for 60 min at 37° in air + 5% CO_2; NAD + NADH content was determined as described previously [4].

Dot-, Northern- and Southern-blot analysis. For dot blot analysis, RNA was isolated from 100 islets in each experimental group by the sodium dodecyl sulphate phenol method followed by denaturation with glyoxal and application to Genescreen filters™ (NEN, Boston, MA, U.S.A.) by a suction apparatus. The blot was first hybridized with [^{32}P]dCTP labelled pMTal probe in the presence of 50% formamide and 1% SDS at 42° [24, 25] and washed 3 × 30 min in 15 mM NaCl, 1.5 mM sodium citrate at 50°. The MTal DNA contains sequences coding for the mitochondrial cytochrome *b* gene [26] and was labelled by oligonucleotide-prime labelling. The blots were washed at 75° in 1.5 mM NaCl and rehybridized to pRI-7, containing sequences coding for the rat insulin I gene [27], or to pshglu, containing sequences coding for the Syrian hamster glucagon gene [28].

For Northern blot analysis, RNA was prepared from 1000 islets in each experimental group by the guanidine isothiocyanate method and 20 μg total RNA were denatured with formaldehyde [29]. This was followed by electrophoresis on a 1% agarose/formaldehyde gel. The gel was stained with ethidium bromide and photographed, and RNA was transferred to a Genescreen filter™. The blot was hybridized after UV-crosslinking to ^{32}P-labelled pMTal.

For Southern blot analysis total DNA was prepared from 200 islets in each experimental group by dissolving them in 4 M guanidium isothiocyanate, as described previously [30]. After repeated precipitations with ethanol, 5 μg DNA was digested with restriction endonuclease EcoRI, electrophoresed on a 0.7% agarose gel and transferred onto a Genescreen™ filter [31]. The blot was first hybridized with [^{32}P]dCTP labelled mouse mitochondrial DNA probe in the presence of 50% formamide and 1% SDS at 42° [24]. The mouse mitochondrial DNA probe was generated by a polymerase chain reaction between the nucleotides 15926 and 1478 [32], and was labelled by oligonucleotide-prime labelling after purification on agarose gels, and electroelution. This probe contains

Table 1. Effects of MMS, NMU and SZ on islet recovery, islet DNA content and islet insulin content 6 days after exposure (day 6)

Drugs (mM)		Islet recovery (%)	DNA content (ng/10 islets)	Insulin content (ng/10 islets)	(ng/ng DNA)
MMS	0	93 ± 1	252 ± 14	562 ± 43	2.20 ± 0.26
	2	93 ± 1	227 ± 33	507 ± 91	2.38 ± 0.61
	4	62 ± 8†	211 ± 27	527 ± 83	2.74 ± 0.54
NMU	0	96 ± 1	194 ± 17	498 ± 51	2.66 ± 0.11
	2	93 ± 2	186 ± 14	544 ± 56	3.06 ± 0.19
	4	88 ± 3*	154 ± 7*	468 ± 18	3.13 ± 0.14
	6	55 ± 7‡	118 ± 14†	316 ± 42*	2.68 ± 0.16
SZ	0	89 ± 5	221 ± 16	728 ± 81	3.20 ± 0.17
	1.5	53 ± 2†	152 ± 13	292 ± 56†	1.95 ± 0.41†

Pancreatic islets obtained from NMRI mice were exposed for 15 min at 37° to MMS, NMU or SZ at the concentrations given in the first column. The islets were either examined immediately (data not shown) or after 6 days in culture. The recovery of islets on day 6 was expressed as the percentage of islets remaining in culture on day 6 as compared to day 0. The results are means ± SEM of 5–6 (MMS and SZ) or 7–8 (NMU) experiments. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ when compared to respective controls, using paired t-test.

sequences coding for $tRNA^{Pro}$, $tRNA^{Phe}$, control region and part of 12S RNA. The blots were washed 3 × 30 min at 50° in 15 mM NaCl + 1.5 mM sodium citrate + 0.1% SDS. In some experiments, the filter was washed at 75° in 1.5 mM NaCl after autoradiography and then rehybridized to MTal.

Statistical analysis. Data were computed as the means ± SEM and compared using Student's t-test for paired samples.

RESULTS

Exposure of pancreatic islets to increasing concentrations of MMS and NMU induced a decrease in the islet recovery after 6 days in culture (Table 1). At 6 mM MMS (data not shown) it was possible to recover just 28% of the islets, precluding further studies at that drug concentration. Treatment of the islets with 1.5 mM SZ induced a similar decrease in islet recovery as observed after treatment with 4 mM MMS or 6 mM NMU. While treatment with MMS did not decrease the DNA content of the surviving islets, exposure to 4 and 6 mM NMU induced a 20–40% decrease in the DNA values. SZ also decreased the DNA content but this decrease did not reach statistical significance ($P < 0.1$, paired t-test). Exposure to MMS did not affect the islet insulin content. Following NMU exposure there was a significant decrease in insulin content only at 6 mM but this difference was not evident when the data were corrected per DNA content. On the other hand, SZ induced a more than 50% decrease in insulin content which was also evident when the data were corrected per DNA. In none of the different experimental groups was there any significant difference in DNA or insulin content immediately after drug exposure, as compared to the corresponding controls (day 0, data not shown).

Exposure of the mouse islets to the different test agents did not affect the basal insulin release at 1.7 mM glucose on either day 0 or 6 (data not shown). The mean basal insulin release of the pooled control groups was 0.8 ± 0.1 ng/10 islets × 1 hr on day 0 (N = 18) and 1.3 ± 0.2 ng/10 islets × 1 hr on day 6 (N = 18). However, there was a clear decrease in glucose-stimulated insulin release (16.7 mM glucose) immediately after exposure to MMS, NMU and SZ (Table 2), both when the results were expressed per islets and per DNA. Following 6 days in culture the islets exposed to MMS were able to recover a normal insulin release in response to glucose. In the NMU groups there was still an impaired insulin release but this difference was probably due to smaller islets and disappeared following correction of the values per DNA. The SZ-treated islets presented a similar 50–70% decrease in insulin release on both day 0 and 6, and this difference was not affected by recalculating the data per DNA. The different experimental series shown in Tables 1 and 2 were performed at different time points which can probably explain the variations in islet size and glucose-induced insulin release observed between the different control groups. Thus, all comparisons described above were performed using only the controls studied in parallel with the experimental groups.

The oxidation of D-[6-^{14}C]glucose at 16.7 mM glucose in the different experimental groups (Fig. 1) followed a similar pattern to that observed for the glucose-stimulated insulin release. Thus, there was an acute reduction in the glucose oxidation rate following exposure to MMS, NMU (4 and 6 mM) and SZ which was maintained after 6 days in the SZ and NMU groups (4 and 6 mM), but not in the islets treated with MMS. Due to technical reasons, it was not feasible to retrieve the islets for DNA determinations following the measurements of glucose oxidation. However, correcting the oxidation values for the DNA values obtained from the islets used in the insulin release experiments, the differences observed on day 6 were maintained in the SZ-treated but not in the NMU treated islets (data not shown).

Table 2. Effects of MMS, NMU and SZ on islet glucose-stimulated insulin release immediately (day 0) and 6 days after exposure (day 6)

Drugs (mM)		Insulin release at 16.7 mM glucose			
		Day 0		Day 6	
		(ng/10 islets × 1 hr)	(ng/μg DNA)	(ng/10 islets × 1 hr)	(ng/μg DNA)
MMS	0	16.4 ± 2.8	70 ± 11	13.4 ± 2.6	53 ± 10
	2	8.2 ± 1.4†	40 ± 8*	13.5 ± 2.2	69 ± 17
	4	3.5 ± 0.5†	16 ± 2†	13.0 ± 2.0	65 ± 13
	6	2.2 ± 0.4†	12 ± 3†	—	—
NMU	0	20.9 ± 3.7	110 ± 20	18.5 ± 2.4	96 ± 8
	2	13.1 ± 3.9	65 ± 14*	17.9 ± 4.8	94 ± 20
	4	4.6 ± 0.7†	22 ± 5†	14.2 ± 2.0*	102 ± 10
	6	1.9 ± 0.3†	11 ± 3†	11.1 ± 1.9*	100 ± 14
SZ	0	22.8 ± 3.3	135 ± 15	19.6 ± 3.3	88 ± 10
	1.5	6.4 ± 1.8†	33 ± 8†	7.4 ± 1.5*	46 ± 7†

The groups of islets were isolated and maintained in culture as described in the legend to Table 1. Insulin release experiments were performed by initially incubating the islets in triplicate groups of 10 in KRBH containing 1.7 mM glucose (data not shown). After 60 min the medium was removed and the islets incubated for another 60 min in medium containing 16.7 mM glucose. The results are means ± SEM of 5–7 experiments. *$P < 0.05$; †$P < 0.01$ when compared to the respective controls, using paired t-test.

Immediately after exposure to MMS, NMU or SZ there was a severe decrease in islet NAD + NADH content (Fig. 2). However, after 6 days in culture the NAD + NADH content was restored to a value similar to that observed in the control group. Correction per DNA of the data obtained on day 6 did not modify the above described observations (data not shown).

Six days after exposure of the mouse islets to MMS, NMU and SZ there was a 30–40% decrease in the content of cytochrome b mRNA (Table 3). The SZ-induced reduction in cytochrome b mRNA was confirmed by Northern blot analysis (results not shown). SZ, but not MMS or NMU, induced a 30% decrease in the islet insulin mRNA content (Table 3). On the other hand, both MMS and NMU, but not SZ, induced a marked decrease in the content of islet glucagon mRNA. Southern blot analysis of mitochondrial DNA, with the use of a probe able to recognize several regions of the mitochondrial genome, showed that the three tested agents induced a similar and severe decrease in mitochondrial DNA (Table 3 and Fig. 3). In line with these observations, mouse islets exposed to SZ also showed a significant decrease in cytochrome b DNA, to 54 ± 9% of the values observed in the respective control islets (data corrected per DNA content), as assessed by densitometric analysis after hybridization with pMTal ($N = 3$; $P < 0.05$). Similar observations were made with islets from rats. Thus, rat islets exposed to 0.55 mM SZ and maintained in culture for 6 days also presented a decrease in cytochrome b DNA content, with values of 45 ± 1% of the values observed in the respective controls ($N = 3$; $P < 0.001$; data corrected per DNA content and assessed by densitometric analysis).

DISCUSSION

Exposure of the islets to the three different alkylating agents, SZ, NMU and MMS, induced a similar initial response. Thus, there was an acute decrease in NAD + NADH content, a decrease in glucose oxidation and an impaired insulin release. These results are similar to data obtained in previous studies dealing with the short-term actions of SZ and NMU on pancreatic islets [4, 33, 34]. To our knowledge, the effects of MMS on pancreatic islets has not been studied previously, but the observed decrease in NAD + NADH content is similar to observations made in other cell types, reflecting poly(ADP-ribose) synthetase activation [15]. Thus, as proposed by Okamoto [13], it is conceivable that the three agents induced DNA alkylation, DNA strand breaks and activation of poly(ADP-ribose) synthetase, resulting in NAD + NADH depletion and impairment in the Krebs cycle, as assessed by the D-[6-^{14}C]glucose oxidation. Since substrate metabolism and ATP generation at the mitochondrial level are crucial for the glucose-induced insulin release [35], an impaired mitochondrial function could explain the observed decrease in insulin release.

Six days after exposure to 1.5 mM SZ the islets still presented an impaired glucose oxidation and insulin release, and a decreased insulin mRNA and insulin content. Similar observations were made previously in islets exposed to 1.1, 2.2 and 4.4 mM SZ [3, 4, 6]. On the other hand, islets exposed to both MMS and NMU were able to regain a normal function after 6 days in culture, at least according to the data corrected per DNA. These findings may allow us to exclude some possible causes of the SZ-induced long-lasting impairment in B-cell function. The first is a role for NAD + NADH depletion. Indeed, six days after SZ exposure the NAD + NADH levels were similar between control and SZ-treated islets. It could be argued that the acute SZ-induced NAD + NADH depletion

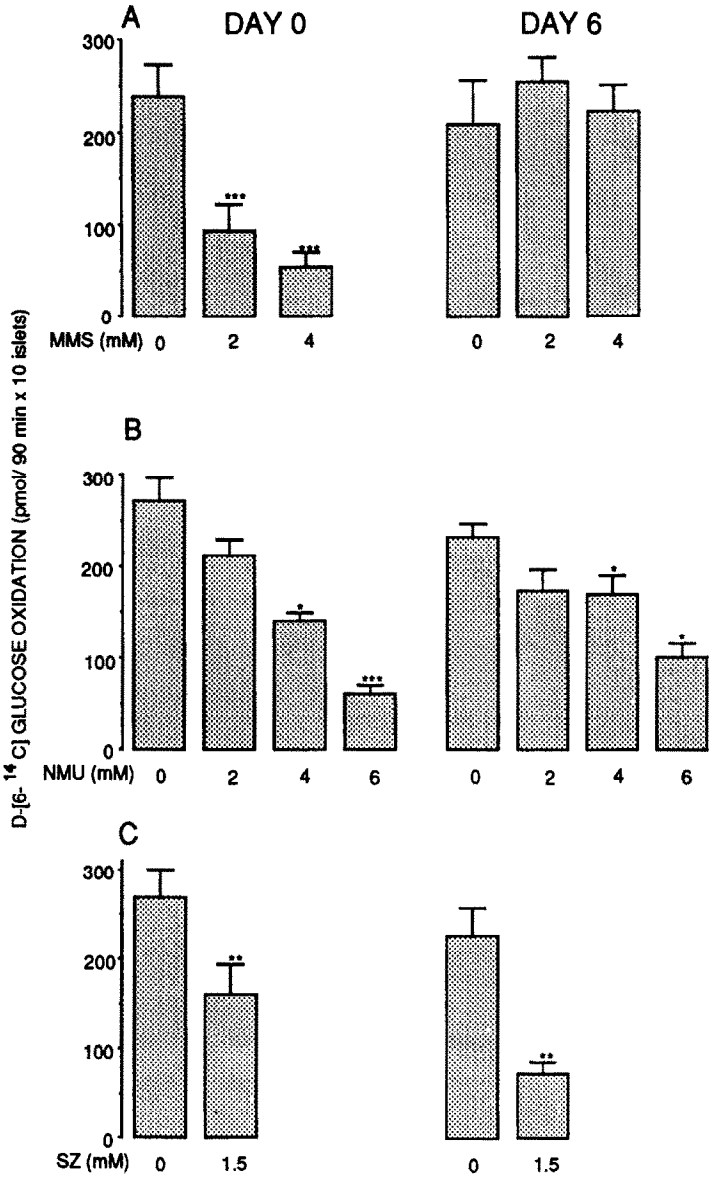

Fig. 1. Effects of different concentrations of MMS (A), NMU (B) and SZ (C) on islet D-[6-^{14}C]glucose oxidation immediately and 6 days after exposure. Values are given as means ± SEM of 5–6 experiments, each performed in triplicate. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ when compared to respective controls, using paired t-test.

somehow induced a long-lasting impairment in the islet metabolic machinery, despite the absence of prolonged NAD + NADH depletion. However, the observations that high concentrations of MMS and NMU induced similar or even more severe NAD + NADH depletion then observed after SZ exposure, without inducing long-lasting functional impairment, rule out this possibility.

Another possibility is related to SZ-induced alkylation of the O^6-position of guanine [10]. Indeed, particular attention has been focused on alkylation at this position, since O^6-alkylguanine residues in DNA can persist for long periods in some tissues and have been shown to be correlated with mutagenesis and tumor induction [36, 37]. DNA methylation at specific sites of the genome (e.g. promotor regions) is also causally related to gene inactivation [38], and it could be envisaged that SZ long-term deleterious actions are related to DNA methylation, especially at the exocyclic atom of guanine. The observation that MMS, an alkylating agent which induces similar amounts of N-7 methylguanine and N-3 methyladenine as methylnitrosoureas but induces less alkylation at the O^6-position of guanine [14], could not induce long-lasting suppression of islet function is in line with this hypothesis. However, there is another important difference between SZ and MMS to be considered,

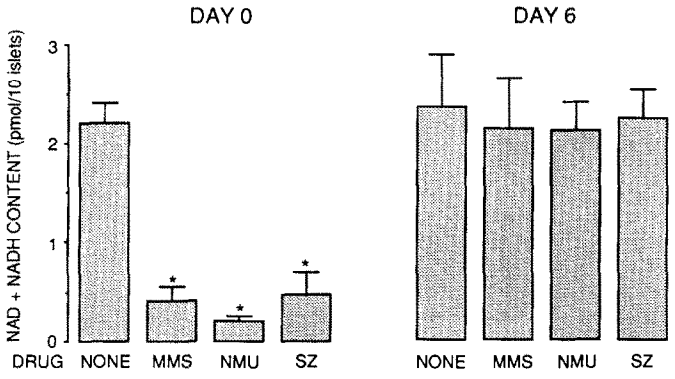

Fig. 2. Effects of MMS (4 mM), NMU (6 mM) and SZ (1.5 mM) on islet NAD + NADH content immediately and 6 days after exposure. Values are given as means ± SEM of 7–8 experiments. *P < 0.001 when compared to respective controls, using paired *t*-test.

Table 3. Effects of MMS (4 mM), NMU (6 mM) and SZ (1.5 mM) on islet content of cytochrome *b* mRNA, insulin mRNA, glucagon mRNA and mitochondrial DNA on day 6

mRNA or DNA	MMS	NMU	SZ
	(% of the respective controls)		
Cytochrome *b* mRNA	74 ± 4*	62 ± 11*	71 ± 9*
Insulin mRNA	104 ± 16	107 ± 41	67 ± 1‡
Glucagon mRNA	29 ± 5†	30 ± 13*	74 ± 17
Mitochondrial DNA	42 ± 14*	30 ± 2‡	30 ± 7†

The groups of islets were isolated and maintained in culture as described in the legend to Table 1. The content of the different mRNAs was determined by dot blot analysis and of mitochondrial DNA by Southern blot analysis, as described in Material and Methods. The blots were evaluated by densitometric scanning and, after normalization for islet DNA content, the values were expressed as percentages of the respective control groups. The results are means ± SEM of 3–5 experiments. *P < 0.05; †P < 0.01 and ‡P < 0.001 when compared to respective controls, using paired *t*-test.

namely that SZ possesses a glucose moiety which is probably related to the specificity of the effects of the drug to the pancreatic B-cells [1]. This specificity was confirmed by the current data which show that SZ affected to a larger extent the islet insulin mRNA content, as compared to glucagon mRNA content. To address this problem, NMU, the *N*-nitrosourea derivative of SZ without glucose was also tested. As already mentioned, NMU could not induce long-lasting damage to the surviving B-cells. This suggests that the key issue for understanding the action of SZ is not only the purine position that will be alkylated but the possibility that the alkylating agent, i.e. *N*-nitrosourea, when coupled to glucose will be targeted to specific intracellular sites of the B-cells related to glucose-induced insulin release [39].

Our previous studies suggested that chronic impairment in mitochondrial function was a consequence of SZ exposure [3, 4, 6]. Nitrosourea scan alkylate both nuclear and mitochondrial DNA [40, 41], and the findings that SZ treatment induced a clear decrease in the mitochondrial encoded cytochrome *b* mRNA, cytochrome *b* DNA and mitochondrial DNA were in good agreement with a SZ-mediated damage to the mitochondrial DNA. Furthermore, in accordance with the above described "targeting hypothesis", mitochondrial DNA could be a primary target of the *N*-nitrosourea coupled to glucose, i.e. SZ. The observations that NMU and MMS also decreased the cell content of mitochondrial DNA without inducing a long-lasting impairment in cell function suggest that either damage to mitochondrial DNA is not the complete explanation for the actions of SZ or that the effects of MMS and NMU are mainly confined to non-B-cell populations. Indeed, when the long-term effects of these drugs on glucagon mRNA were tested, MMS and NMU but not SZ induced a marked decrease in the content of this messenger, suggesting targeting of these compounds to peripheral A-cells. The volume fraction of B-cells in mouse islets maintained in culture for 6–7 days, under similar conditions as used in the present experiments, is around 75% [8] and it is conceivable that severe damage to the remaining 25% non-B cell population by NMU and MMS can affect measurements of total islet mitochondrial mRNA and DNA. The reasons behind this apparently preferential effect of MMS and NMU

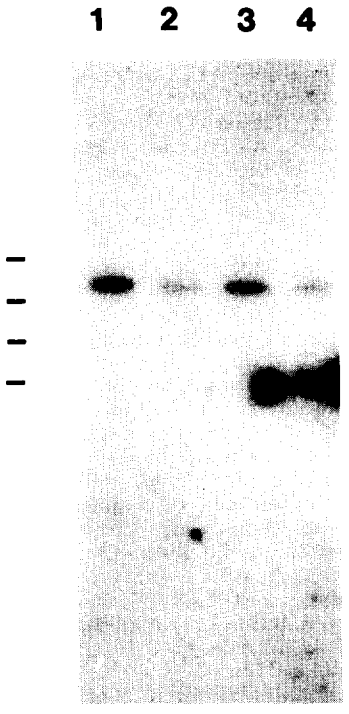

Fig. 3. Southern blot analysis of mouse islets six days after exposure to SZ (1.5 mM), MMS (4 mM) or NMU (6 mM). DNA was digested with EcoRI and hybridized to the mitochondrial DNA probe. The lines indicate the positions 23, 9.4, 6.6 and 4.4 kb (top to bottom). The experiment is representative of three to four separate experiments (see densitometric quantification in Table 3). Lane 1, controls; lane 2, SZ; lane 3, MMS; lane 4, NMU.

on A-cells remains to be clarified. One possibility is that A-cells located in the periphery of the islets could be slightly more exposed to the drugs than the B-cells located in the islet core.

It has been shown that SZ treatment also induces a chronic impairment in the expression of two genes coded at the nuclear level, i.e. the insulin and the adenine nucleotide translocator genes [3, 42]. Although these findings could be secondary to an impaired substrate metabolism due to mitochondrial damage, they raise the possibility that long-lasting nuclear DNA damage could be a simultaneous consequence of SZ exposure. The possibility of SZ-induced damage to other cell components, besides the mitochondrial genome, could perhaps explain the observed discrepancies between the effects of SZ and the other alkylating agents. Finally, other SZ actions independent of genomic damage cannot be excluded, like activation of the xanthine oxidase system and generation of oxygen free radicals [43].

As a whole, the present data suggest that further clarification of the molecular mechanisms behind SZ-induced B-cell damage demands new experiments aimed at an improved characterization of the mechanisms behind preferential genetic damage to and repair of the B-cells [44].

Acknowledgements—The technical assistance of Ing-Britt Hallgren, Eva Forsbeck, Astrid Nordin and Sigrun Svanholm is gratefully acknowledged. We thank Dr K. Koike for providing the pMTal probe. This work was supported by grants from the Juvenile Diabetes Foundation International, the Swedish Medical Research Council (12X-109; 12X-8273; 12X-9237; 12X-9886), the Swedish Diabetes Association, the Swedish Society of Medicine, the Nordic Insulin Fund, the Hoechst Diabetes Foundation, the Aage-Louis Hansen Mindefond, the Family Ernfors Fund, the Torsten and Ragnar Södeberg Foundation and the Magnus Bergvalls Stiftelse.

REFERENCES

1. Rayfield EJ and Ishimura K, Environmental factors and insulin-dependent diabetes mellitus. *Diabetes Metab Rev* **3**: 925–957, 1987.
2. Herr RR, Jahnke HK and Argoudelis AD, The structure of streptozotocin. *J Am Chem Soc* **89**: 4808–4809, 1967.
3. Eizirik DL, Sandler S, Welsh N and Hellerström C, Preferential reduction of insulin production in mouse pancreatic islets maintained in culture after streptozotocin exposure. *Endocrinology* **122**: 1242–1249, 1988.
4. Strandell E, Eizirik DL, Korsgren O and Sandler S, Functional characteristics of cultured mouse pancreatic islets following exposure to different streptozotocin concentrations. *Mol Cell Endocrinol* **59**: 83–91, 1988.
5. Eizirik DL, Sandler S, Korsgren O, Jansson L and Andersson A, Persistent impairment of the insulin response to glucose both *in vivo* and *in vitro* after streptozotocin exposure: studies with grafted pancreatic islets and islets maintained in culture. *Acta Endocrinol (Kbh)* **121**: 849–856, 1989.
6. Eizirik DL, Sandler S, Sener A and Malaisse WJ, Defective catabolism of D-glucose and L-glutamine in mouse pancreatic islets maintained in culture after streptozotocin exposure. *Endocrinology* **123**: 1001–1007, 1988.
7. Svensson C, Welsh N, Krawetz S, Svanholm S, Hellerström C and Welsh M, Expression of genes involved in the oxidative metabolism of pancreatic B cells under different functional status. *Diabetologia* **32**: 544A, 1990.
8. Moreira JE, Hand AR, Borg LAH, Sandler S, Welsh M, Welsh N and Eizirik DL, Decrease of insulin-containing secretory granules and mitochondrial gene expression in mouse pancreatic islets maintained in culture following streptozotocin exposure. *Virchows Arch B (Zellpathol)* **60**: 337–344, 1991.
9. Eizirik DL and Sandler S, Function and metabolism of pancreatic B-cells maintained in culture following experimentally induced damage. *Pharmacol Toxicol* **65**: 163–168, 1989.
10. Bennet RA and Pegg AE, Alkylation of DNA in rat tissues following administration of streptozotocin. *Cancer Res* **41**: 2786–2790, 1981.
11. LeDoux SP, Woodley SE, Patton NJ and Wilson GL, Mechanisms of nitrosourea-induced B-cell damage. Alterations in DNA. *Diabetes* **35**: 866–872, 1986.
12. Yamamoto H, Uchigata Y and Okamoto H, Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature* **294**: 284–286, 1981.
13. Okamoto H, Regulation of proinsulin synthesis in pancreatic islets and a new aspect to insulin-dependent diabetes mellitus. *Mol Cell Biochem* **37**: 43–61, 1981.
14. Beranek DT, Weiss CC and Swenson DH, A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography. *Carcinogenesis* **1**: 595–606, 1980.
15. Wielckens K, George E, Pless T and Hilz H, Stimulation

of poly(ADP-ribosyl)ation during Ehrlich ascites tumor cell "starvation" and suppression of concomitant DNA fragmentation by benzamide. *J Biol Chem* **258**: 4098–4104, 1983.
16. Ahnström G and Ljungman M, Effects of 3-aminobenzamide on the rejoining of DNA-strand breaks in mammalian cells exposed to methyl methanesulfonate; role of poly(ADP-ribose) polymerase. *Mutat Res* **194**: 17–22, 1988.
17. Lernmark Å, Nathans A and Steiner DF, Preparation and characterization of plasma membrane-enriched fractions from pancreatic islets. *J Cell Biol* **17**: 606–623, 1976.
18. Andersson A, Isolated mouse pancreatic islets in culture: effects of serum and different culture media on the insulin production of the islets. *Diabetologia* **14**: 397–404, 1978.
19. Krebs HA and Henseleit K, Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe Seylers Z Physiol Chem* **210**: 33–66, 1932.
20. Heding LG, Determination of total serum insulin (IRI) in insulin-treated patients. *Diabetologia* **8**: 260–266, 1972.
21. Kissane JM and Robins E, The fluorimetric measurements of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J Biol Chem* **233**: 184–188, 1958.
22. Hinegardner RT, An improved fluorimetric assay for DNA. *Anal Biochem* **39**: 197–201, 1971.
23. Andersson A and Sandler S, Viability tests of cryopreserved endocrine pancreatic cells. *Cryobiology* **20**: 161–168, 1983.
24. Thomas PS, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* **77**: 5201–5205, 1980.
25. White BA and Bancroft FC, Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell of tissue samples. *J Biol Chem* **257**: 8569–8572, 1982.
26. Koike K, Kobayashi M, Yaginuma K, Taira M, Yoshida E and Imai M, Nucleotide sequence and evolution of the rat mitochondrial cytochrome *b* gene containing the ochre termination codon. *Gene* **20**: 177–185, 1982.
27. Chan SJ, Noyes BE, Agarwal KL and Steiner DF, Construction and selection of recombinant plasmids containing full-length complementary DNAs corresponding to rat insulins I and II. *Proc Natl Acad Sci USA* **76**: 5036–5040, 1979.
28. Bell GI, Santerre RF and Mullenbach GT, Hamster preproglucagon contains the sequence of glucagon and two related peptides. *Nature* **302**: 716–718, 1983.
29. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982.
30. Welsh N, Öberg C, Hellerström C and Welsh M, Liposome mediated *in vitro* transfection of pancreatic islet cells. *Biomed Biochim Acta* **49**: 1157–1164, 1990.
31. Southern EM, Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**: 503–517, 1975.
32. Anderson S, Bankier AT, Barrell BG, de Bruijin MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R and Young IG, Sequence and organization of the human mitochondrial genome. *Nature* **290**: 457–465, 1981.
33. Gunnarson R, Berne C and Hellerström C, Cytotoxic effects of streptozotocin and *N*-nitrosmethylurea on the pancreatic B cells with special regard to the role of nicotinamide-adenine dinucleotide. *Biochem J* **140**: 487–494, 1974.
34. Sandler S, Welsh M and Andersson A, Streptozotocin-induced impairment in islet B-cell metabolism and its prevention by a hydroxyl radical scavenger and inhibitors of poly(ADP-ribose) synthetase. *Acta Pharmacol Toxicol* **53**: 392–400, 1983.
35. Malaisse WJ, Metabolic factors influencing synthesis and secretion of pancreatic islet hormones. In: *The Endocrine Pancreas* (Ed. Samols E), pp. 73–92. Raven Press, New York, 1991.
36. Saffhill R, Margison GP and O'Connor PJ, Mechanisms of carcinogenesis induced by alkylating agents. *Biochim Biophys Acta* **823**: 111–145, 1985.
37. Swenberg JA and Fennell TR, DNA damage and repair in mouse liver. *Arch Toxicol* **10** (Suppl): 162–171, 1987.
38. Doerfler W, DNA methylation and gene activity. *Annu Rev Biochem* **52**: 93–124, 1983.
39. LeDoux SP, Hall CR, Forbes PM, Patton NJ and Wilson GL, Mechanisms of nicotinamide protection from alloxan and streptozotocin toxicity. *Diabetes* **37**: 1015–1019, 1988.
40. Wunderlicht V, Schüt M, Böttger M and Graffi A, Preferential alkylation of mitochondrial deoxyribonucleic acid by *N*-methyl-*N*-nitrosourea. *Biochem J* **118**: 99–109, 1970.
41. Wilkinson R, Hawks A and Pegg AE, Methylation of rat liver mitochondrial deoxyribonucleic acid by chemical carcinogens and associated alterations in physical properties. *Chem Biol Interact* **9**: 157–167, 1975.
42. Welsh N, Svensson C and Welsh M, Content of adenine nucleotide translocator mRNA in insulin producing cells of different functional states. *Diabetes* **38**: 1377–1380, 1989.
43. Nukatsuka M, Sakurai H, Yoshimura Y, Nishida M and Kawada J, Enhancement by streptozotocin of O_2^- radical generation by the xanthine oxidase system of pancreatic β-cells. *FEBS Lett* **239**: 295–298, 1988.
44. LeDoux SP, Patton NJ, Nelson JW, Bohr VA and Wilson GL, Preferential DNA repair of alkali-labile sites within the active insulin gene. *J Biol Chem* **265**: 14875–14880, 1990.