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N-Monomethyl-arginine and nicotinamide prevent streptozotocin-induced double strand DNA break formation in pancreatic rat islets

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Abstract. The impact of short term in vitro exposure to the diabetogenic drug streptozotocin on pancreatic islet glucose metabolism, insulin secretion, DNA fragmentation and cell viability, was studied. Streptozotocin impaired cell viability as well as insulin secretion and the oxidation of glucose. These effects were partially counteracted by inhibition of the inducible form of nitric oxide synthase with N-monomethyl-arginine and by scavenging oxygen free radicals with nicotinamide. Isolated islets underwent double strand DNA fragmentation after 24 h in culture. The degree of DNA breakdown was strongly enhanced by exposure of the islets to 0.55 mM streptozotocin for 30 min before culture. Prevention of streptozotocin-induced cleavage of islet DNA was obtained with N-monomethyl-arginine and nicotinamide. These data suggest that the generation of reactive oxygen and nitrogen species is involved in the deleterious action of streptozotocin on pancreatic islet tissue. A role for oxygen radicals generated during streptozotocin-induced islet cell damage as possible mediators of the expression of the inducible form of nitric oxide synthase and the scavenging action of nicotinamide on these radicals, is then proposed.
Key words. Pancreatic islets; DNA degradation; streptozotocin; nicotinamide; nitric oxide.

The sensitivity of pancreatic islet cells to the diabetogenic agent streptozotocin (STZ) has been used over the years as a model to study the mechanisms involved in islet β-cell damage and repair. New insights into the actions of STZ came from the finding that nitric oxide (NO) is released following spontaneous decomposition of the drug[1]. NO mediates actions of STZ on islet guanylate cyclase and aconitase[2]. On the other hand, the effects of STZ on islet cell survival are counteracted by nicotinamide, a scavenger of hydroxyl-free-radicals and NO and inhibitor of the DNA repairing enzyme poly(ADP-ribose) synthase[3–5]. Since NO is a mediator of DNA fragmentation and apoptotic cell death in several cell systems[6,7], the experiments reported in this paper were carried out to investigate the role of oxygen and nitrogen reactive species in streptozotocin-induced DNA damage and functional inhibition of islet cells. Pancreatic β-cells are able to express the inducible form of nitric oxide synthase (iNOS) in response to IL-1β as part of the cellular response to the oxidative stress induced by this inflammatory cytokine[8]. The possibility that the β-cell response to STZ could also involve the expression of the inducible form of nitric oxide synthase (iNOS) was tested by studying the reversibility of streptozotocin actions on islet function and DNA degradation by N-monomethyl-L-arginine (NMA), an inhibitor of iNOS.

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Materials and methods

Chemicals. Agarose was from FMC (Bio Products, USA); 100 bp DNA molecular weight marker from BRL; proteinase K, ribonuclease A, collagenase P and amphotericin B were from Boehringer-Mannheim (Germany); N-laurylsarcosine, N-monomethyl-L-arginine (NMA), streptotozin (STZ) and ethidium bromide were from Sigma (USA): fetal bovine serum, streptomycin, penicillin and RPMI 1640 from Flow Laboratories (Scotland); [α-^{32}P]-dCTP was from Dupont (USA); D-5[^{3}H]glucose, D-U[^{14}C]glucose and ^{125}I-insulin were from Amersham (UK).

Islet preparation and culture. Islets from male Wistar rats weighing 180–200 g were isolated by collagenase procedure[9]. Batches of 100 islets were then transferred to plastic tubes containing 1 ml of Hank's buffer supplemented with 10 mM Hepes and 1% BSA, and incubated for 20 min at 37 °C in a water bath. Streptozotocin (10 mM in citrate buffer pH 4.5) was dissolved in cold buffer within 1 min before use, and 10 µl of solution were added to obtain the required STZ concentration (0.55 mM). The islets were incubated with STZ for 30 min at 37 °C and the incubation was terminated by removal of the solution and addition of 3 ml of Hanks'

Table 1. Percentage of viable islet cells.

Treatment	Time (h)	
	0	24
Control	93.6 ± 2.1	94.5 ± 1.2
Control + 20 mM nicotinamide	92.5 ± 1.7	93.3 ± 0.5
Control + 0.2 mM NMA	94.1 ± 1.6	94.2 ± 1.3
STZ	82.1 ± 1.6*	83.2 ± 0.6*
STZ + 20 mM nicotinamide	86.2 ± 0.8*,**	87.6 ± 0.7*,**
STZ + 0.2 mM NMA	90.3 ± 1.5**	90.6 ± 1.2**

Cell viability was studied by trypan blue exclusion. Percentage ± SEM of viable cells in islet cells cultured in RPMI-1640 for 0 or 24 h after 30 min exposure to 0.55 mM STZ.
*$p < 0.01$ versus respective control group.
**$p < 0.01$ versus STZ-treated group.
Percentages are calculated from 6–8 determinations for each group.

Table 2. Insulin secretory response to 16.7 mM glucose of control and STZ-treated islets.

Treatment	Time (h)	
	0	24
	Insulin release; ng/10 islets/60 min	
Control	15.3 ± 2.1	13.5 ± 1.2
Control + 20 mM nicotinamide	16.7 ± 1.8	14.3 ± 0.8
Control + 0.2 mM NMA	16.2 ± 1.8	14.9 ± 1.7
STZ	5.2 ± 1.5*	6.4 ± 0.8*
STZ + 20 mM nicotinamide	6.2 ± 0.7*	9.2 ± 0.3*,**
STZ + 0.2 mM NMA	6.0 ± 0.3*	9.3 ± 0.5*,**

Insulin release was measured in batches of 10 islets in KRB buffer immediately after 30 min exposure to 0.55 mM STZ or buffer alone or following 24 h of culture in RPMI 1640 medium. Values are the mean ± SEM of 5–7 experiments.
*$p < 0.01$ vs control group.
**$p < 0.01$ vs STZ-treated group.

buffer. The islets were then transferred to RPMI-1640 medium containing 10% fetal calf serum, benzylpenicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (250 U/ml) and maintained in culture for 24 h at 37 °C in 95% air, 5% CO_2. Control islets were treated similarly with 10 µl citrate buffer.

Cell viability. Trypan blue exclusion was measured in islet cells cultured for 0 and 24 h after exposure to 0.55 mM STZ for 30 min. Islets were incubated with occasional trituration with a Pasteur pipette at 37 °C for 30 min in dissociation medium containing 0.05% trypsin and 0.1% EDTA. After two washes with cold phosphate buffered saline, dissociated cells were plated and allowed to recover in RPMI 1640 containing 10% FCS for 5 h, stained with 0.2% trypan blue, and the percentage of viable cells was counted.

Functional studies. Islet function was tested by measuring insulin responsiveness to glucose. Batches of 10 islets were incubated in quintuplicate in 1 ml of Krebs Ringer bicarbonate buffer (KRB) with 16.7 mM glucose for 1 h. Aliquots of this incubation medium were stored at −20 °C until immunoassay for insulin.

Islet glucose metabolism. Glucose usage through glycolysis was followed by the production of ^3H_2O from D-[5-^{3}H]glucose[10]. Batches of 10 islets were incubated in 100 µl modified KRB buffer containing 5 mM $NaHCO_3$, 10 mM Hepes (pH 7.4), 0.5% BSA, D-[5-^{3}H]glucose (final specific activity 3.5 and 0.6 Ci/mol), and unlabelled glucose (final concentration 2.75 and 16.7 mM, respectively). Incubations were performed at 37 °C for 90 min. ^3H_2O produced was separated from labelled glucose with Dowex 1 × 2 borate form. Glucose oxidation was determined as the formation of $^{14}CO_2$ from D-[U-^{14}-C]glucose[11]. Batches of 10 islets were incubated in 100 µl Krebs-Hepes buffer supplemented with 0.5% BSA and labelled glucose (final specific activity 1.05 and 0.34 Ci/mol) and nonradioactive glucose, final concentration 2.75 and 16.7 mM. Islets were incubated at 37 °C for 90 min. CO_2 was liberated from the incubation medium by addition of 100 µl 0.2 N HCl and subsequently trapped with 250 µl Hyamine.

DNA electrophoresis and detection of internucleosomal DNA fragmentation. DNA was extracted from islet cells as previously described for lymphocytes[12] with minor modifications. Islets were transferred to 0.3 ml Tris-EDTA buffer (50 mM Tris, 10 mM EDTA, pH 8.0) and supplemented with 0.5% (w/v) N-lauryl-sulphate and 0.5 mg/ml proteinase-K. After 1 h at 50 °C the incubation medium was supplemented with 0.5 mg/ml deoxyribonuclease-free ribonuclease-A, and further incubated at 50 °C for 1 h. DNA was purified by the phenol procedure, precipitated with ethanol at −60 °C and dissolved in Tris-EDTA buffer. Aliquots, 0.5–1 µg DNA, were radioactively labelled with [α-^{32}P]-dCTP by the polymerase reaction method previously described[13]. Samples were heated at 65 °C and supplemented with loading buffer (10 mM EDTA, pH 8, containing 0.25% Bromophenol blue, 1% low-gelling-temperature agarose) at a 1:5 (v/v) ratio. Electrophoresis was carried out in 1.8% agarose gel containing 0.5 µg/ml ethidium bromide, and the buffer used was 80 mM Tris/20 mM phosphate/2 mM EDTA, pH 8. Gels were dried under vacuum and subjected to autoradiography.

Statistical analysis. Statistical differences between groups were evaluated by Student's *t*-test.

Results and discussion

The cell viability data (table 1) show that the percentage of viable cells was 12% lower in the STZ-treated group than in the control group. The presence of nicotinamide partially restored viability to 87% whereas NMA restored viability up to 90%. Normal rat islets maintained the insulin secretory response to glucose when cultured for 24 h (table 2). Neither nicotinamide nor NMA modified the response to glucose in cultured control islets. Treatment with 0.55 mM STZ decreased by 66%

Table 3. Glucose metabolism (pmol · 90 min $^{-1}$ · islet $^{-1}$ in control and STZ-treated islets.

	Control	STZ	STZ+ 20 mM nicotinamide	STZ+ 0.2 mM NMA
Glucose utilization				
2.7 mM glucose	48.1 ± 1.2	45.3 ± 2.4	46.2 ± 1.2	43.7 ± 2.3
16.7 mM glucose	150.2 ± 1.5	145.6 ± 1.9	148.2 ± 2.1	152.3 ± 2.8
Glucose oxidation				
2.7 mM glucose	13.7 ± 0.6	12.5 ± 1.1	13.1 ± 0.4	13.5 ± 1.5
16.7 mM glucose	40.8 ± 0.9	19.3 ± 0.6*	26.2 ± 21*,**	30.2 ± 3.1*,**

Values are means ± SEM for 9–10 observations. Glucose metabolism was measured in islets cultured for 24 h after 30 min exposure to 0.55 mM STZ or buffer alone. In some groups, nicotinamide or NMA were present throughout the culture period.
*$p < 0.01$ vs control, 16.7 mM glucose group.
**$p < 0.01$ vs STZ, 16.7 mM glucose group.

the insulin secretory response to glucose. Culture of STZ-treated islets for 24 h led to 13% recovery of the insulin secretory response to glucose. These data are in agreement with those reported previously in the literature and indicate that short exposure of islets to STZ in vitro produces a stable suppression of the secretory response to glucose that last for several days[14]. The culture of STZ-treated islets in the presence of nicotinamide and NMA improved the secretory response to glucose 68% of the control islets.

There was no difference between control and STZ islets in the glucose usage at either 1.7 or 16.7 mM glucose (table 3). In addition, neither nicotinamide nor NMA modified the rate of glucose usage by cultured islets.

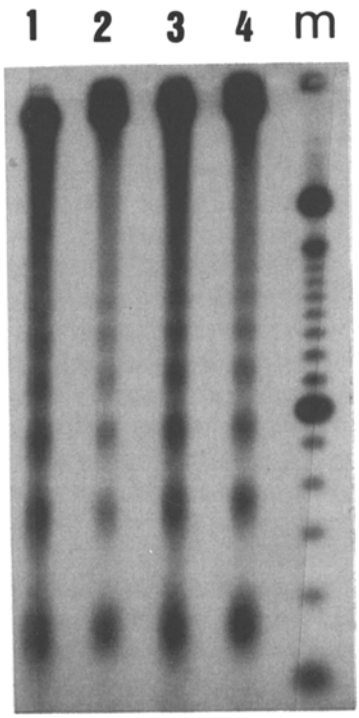

Figure 1. Molecular evidence of internucleosomal cleavage in cultured islets. Islets were cultured for 24 h. Extracted DNA was radiolabelled as described in 'Materials and methods' and run on agarose gels. *Lane 1)* Control, citrate-exposed islets. *Lane 2)* Islets cultured in the presence of 20 mM nicotinamide. *Lane 3)* Islets exposed to 0.55 mM streptozotocin prior to culture with 20 mM nicotinamide. *Lane 4)* Islets exposed to 0.55 mM streptozotocin prior to culture. *Lane M)* standard 100 bp DNA ladder marker. This autoradiograph is representative of three similiar experiments.

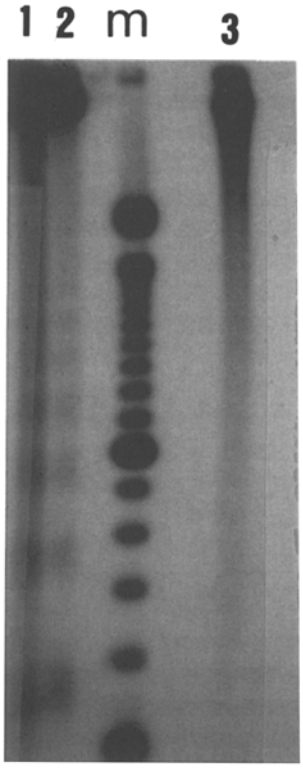

Figure 2. Effect of N-monomethylarginine on DNA breakdown. Islets were cultured for 24 h. Extracted DNA was radiolabelled as described in 'Materials and methods' and run on agarose gels. *Lane 1)* Islets exposed to 0.55 mM streptozotocin for 30 min prior to culture. *Lane 2)* Control islets. *Lane M)* Standard 100 bp DNA ladder marker. *Lane 3)* Islets cultured in the presence of 0.2 mM N-monomethyl arginine following 30 min exposure to 0.55 mM streptozotocin. This autoradiograph is representative of four similar experiments.

However, the oxidation of glucose was decreased by 53% in STZ islets at 16.7 mM glucose while the rate of glucose oxidation was unaffected at 2.7 mM glucose. Neither nicotinamide nor NMA modified glucose oxidation rates in control cultured islets, but both agents recovered glucose oxidation to 64% and 74% of the control islets at 16.7 mM glucose.

In summary, short exposure to STZ leads to stable alterations in insulin secretory functions and islet glucose oxidation. The finding that nicotinamide partially reverses these effects can be accounted for the recovery of the synthesis of proinsulin and also the prevention of the depletion of NAD[15]. It has recently been proposed that the Krebs cycle enzyme aconitase is inhibited by NO generated in islets following exposure to interleukin-1 β[8]. It is thus entirely possible that the effects of STZ treatment on glucose oxidation are mediated by NO since NMA counteracts this effect.

We next studied the possibility that, in addition to the above described impairment of islet function, STZ produces fragmentation in DNA. A clear pattern of DNA breakdown into oligonucleosomal fragments of multiples of 183 ± 6 bp was then observed when islets were cultured for 24 h (fig. 1, lane 1). It is worth noticing that no laddering was detected in freshly isolated islets (data not shown). The degree of spontaneous DNA cleavage was not altered by the presence of 20 mM nicotinamide in the culture medium. STZ significantly increased the breakdown of DNA, an effect which was blocked by 20 mM nicotinamide (fig. 1, lanes 3 and 4). 0.2 mM N-NMA also suppressed DNA breakdown induced by STZ (fig. 2).

The cleavage of DNA detected in cultured pancreatic islets could be the consequence of either the degradation of DNA from a small non β-cell population or/and the degradation of a small population of β-cells. Evidence showing apoptosis in a tumoral β-cell line supports the last contention[3]. STZ action in the β-cells has been associated with the generation of single strand breaks in DNA[15]. We found that STZ also induced double strand DNA breaks and this may be explained by assuming that internucleosomal DNA fragmentation in pancreatic islets is caused by frequent single-strand cuts[16]. STZ actions on islet DNA could be mediated by the formation of peroxynitrite since STZ also induces the formation of superoxide anion in islet cells[17,18]. Peroxynitrite will in turn decompose into NO_2 and the highly genotoxic hydroxyl radical. Nicotinamide might then prevent DNA from STZ-induced degradation by scavenging the hydroxyl radical[5]. These results agree with the recently described inhibition by nicotinamide of DNA fragmentation induced by cytokines in RINm5F cells[19], and can help in the understanding of the known effect of nicotinamide as a potent inducer of endocrine differentiation in cultured human fetal cells[20].

Interestingly, STZ and NO induce DNA damage and apoptosis in both islet cells and insulin-secreting tumor cells[21]. The inhibitory effect of NMA on STZ-induced cytotoxic and genotoxic effects supports the involvement of STZ in iNOS expression during β-cell injury. In this case, islet iNOS expression might be secondary to the generation of hydrogen peroxide[22]. The involvement of reactive oxygen species on STZ-induced iNOS expression in pancreatic islets and its relevance to the dysfunction and destruction of β-cells remains to be established.

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