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STREPTOZOTOCIN DIABETES—FURTHER STUDIES ON THE MECHANISM OF DEPRESSION OF NICOTINAMIDE ADENINE DINUCLEOTIDE CONCENTRATIONS IN MOUSE PANCREATIC ISLETS AND LIVER

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Abstract—NAD concentrations of individual isolated mouse pancreatic islets were significantly decreased 2 hr after the intravenous administration of a diabetogenic dose of streptozotocin. The decrease in NAD is prevented by pretreatment with nicotinamide. Isolated islets demonstrated a concentration-related reduction in NAD when incubated with streptozotocin *in vitro*. One hr after the intraperitoneal injection of ^{14}C-nicotinamide, streptozotocin-treated animals demonstrated a decrease in total radioactive material, and a decreased incorporation into NMN and NAD in islets and liver compared to buffer-treated controls. This was associated with an increase in total radio-active material and labeled nicotinamide in the whole blood of the streptozotocin-treated group. It is proposed that the diabetogenic action of streptozotocin is related to decrease in pancreatic beta cell pyridine nucleotide concentrations resulting from the combination of reduced tissue uptake of precursors and a decrease in synthesis of NAD.

STREPTOZOTOCIN [glucopyranose, 2-deoxy-2-(3-methyl-3-nitrosoureido)] is an antibiotic isolated from the cultures of *Streptomyces acromogenes*.[1] The compound can produce a permanent diabetes in rodents, dogs and monkeys[2–6] mediated through the selective destruction of the pancreatic beta cell, a toxicity that has been exploited clinically in the treatment of malignant insulinoma.[7] In previous reports we have demonstrated that the diabetogenic action of streptozotocin can be correlated with a depression of oxidized and reduced nicotinamide adenine dinucleotide (NAD and NADH) concentrations in rodent tissues.[3,8] Pharmacologic doses of nicotinamide were specific in preventing the decrease in pyridine nucleotides and protecting the beta cell from necrosis.[3,9] In this communication we demonstrate the effect of streptozotocin on NAD concentrations and synthesis in isolated islets of Langerhans and liver of the mouse.

METHODS

Male Swiss mice weighing between 20 and 25 g were used and maintained on a diet of Purina laboratory chow pellets and water *ad lib.* until time of sacrifice.

Streptozotocin (Upjohn lot No. MF 16235) was prepared immediately prior to use in 0·005 M citrate buffer, pH 4·5. Nicotinamide (Calbiochem) was dissolved in distilled water. The following standards were used for the identification of metabolites by

electrophoresis: nicotinamide adenine dinucleotide, NAD (Sigma); nicotinic acid (Calbiochem); nicotinuric acid (Calbiochem); nicotinamide mononucleotide, NMN (Sigma). Desamido-NAD was the kind gift of Dr. Nathan Kaplan. Phosphoribosyl-pyrophosphate (PRPP) and adenosine triphosphate (ATP) were obtained from Nutritional Biochemicals and Sigma respectively. Lactic acid dehydrogenase (LDH) from pig heart and alcohol dehydrogenase (ADH) from yeast were purchased from Boehringer, New York. Tritiated ethanol, spec. act., 25 mCi/m-mole, was a product of New England Nuclear, and sodium pyruvate, a product of Sigma. ^{14}C-nicotinamide, 60 mCi/m-mole (Amersham/Searle), was used in the studies of nicotinamide metabolism.

The procedure used for isolating islets of Langerhans was the following: after sacrifice by cervical dislocation, the pancreatic duct was injected and pancreatic parenchyma infiltrated with 1% collagenase (Worthington) in Hank's balanced salt solution (HBSS) with 1·0% albumin and 0·5% dextrose. The pancreases were subsequently removed, minced and agitated in the collagenase solution for 10 min at 37°. The tissue was then washed a minimum of four times with HBSS with albumin kept at 4°. Under a dissecting microscope, the individual islets were taken up in 5-μl pipets (Eppendorf) and serially transferred in HBSS until cleaned of contaminating exocrine cellular material. In an attempt to prevent a significant reduction of islet NAD during the isolation procedure, the HBSS isolation medium was kept at 4°. The individual islets were taken up in 5 μl HBSS and transferred to 1·5-ml polypropylene micro test tubes (Eppendorf). To extract NAD, the islets were ruptured with 5 μl of 0·1 M HCl and three cycles of freezing and thawing. Then water and HCl were removed by lyophilization. The pellet, reconstituted in 5 μl H_2O, was neutral. Five-μl aliquots of the final transfer solution were also taken up into micro test tubes and processed in the same manner to serve as blanks for the subsequent estimation of NAD. In the experiments *in vivo*, islets were isolated 2 hr after the intravenous administration of streptozotocin and compared with those of control animals, islets from animals given intraperitoneal nicotinamide, 500 mg/kg, 10 min prior to streptozotocin, or nicotinamide alone. In the studies *in vitro*, islets were incubated for 1 hr with graded concentrations of streptozotocin in 1·0-ml volumes of HBSS with 1·0% albumin (pH 7·4); the incubation vessels were gassed with O_2 and CO_2 (95:5). At the end of the incubation period, the islets were again isolated in a 5-μl volume for estimation of individual islet NAD content. Equal volumes of incubation medium were also taken to serve as blanks.

The concentration of NAD in individual islets was measured by a radiometric technique, the full details of which will be presented elsewhere. Briefly, NAD in the islet-extract was used as a cofactor for the dehydrogenation of tritiated ethanol by alcohol dehydrogenase, thereby producing tritiated NADH. In the presence of pyruvate and LDH, NAD^{3}H was reoxidized to NAD, permitting the reaction to cycle. After an 18-hr interval, tritiated lactate (and a minor amount of NAD^{3}H) was measured in the reaction mixture by evaporating the ^{3}H-ethanol and ^{3}H-acetaldehyde at 95° and counting the redissolved residue in a scintillation spectrometer (Beckman). NAD standards and appropriate blanks were simultaneously assayed using the same incubation medium as that used for islet samples, thus controlling for possible bacterial contamination.

In order to exclude a significant physical disruption of islets as an explanation for

change in NAD content, surface area measurements were carried out on representative islets of each of the treatment groups. Twenty islets from each treatment group were isolated and diameters measured in two perpendicular planes with a calibrated Zeiss eyepiece micrometer under a 2·5 × objective for estimation of surface area.

The metabolism of nicotinamide in mouse islets and liver was studied 2 hr after the administration *in vivo* of streptozotocin and compared to controls. One hr prior to sacrifice, the animals were given 10 μCi (0·17 μmole) of ^{14}C-nicotinamide intraperitoneally. After isolation, pools of 50 islets were placed in 1·5-ml polypropylene test tubes, centrifuged and the medium was removed by micropipets. The tissue was disrupted by the addition of 10 μl of 1 N HCl followed by freeze-thawing. The samples were then centrifuged for 2 min at 12,000 *g*. The supernatant was taken for separation of metabolites by electrophoresis and estimation of NAD content. Liver samples and volumes of whole blood were homogenized 1:3 (w/v or v/v) in 0·6 N perchloric acid, kept at 4°, and centrifuged for 5 min at 5000 *g*. The supernatant was neutralized with 2 N KOH and the resulting supernatant was assayed for NAD enzymatically, using alcohol dehydrogenase[10] or the radiometric technique described above. A 100-μl aliquot of the supernatant was lyophilized and reconstituted with 10 μl distilled water. Five-μl samples of the final islet and liver supernatants were spotted on to Whatman filter paper (WH-330) saturated with 0·1 M sodium phosphate buffer, pH 7·0, and over-spotted with a 5-μl sample containing known metabolites for subsequent identification. Separation was carried out using a Savant flat plate electrophoresis system (FP-30A) for 1·5 hr at 3000 V at 4°. After the paper was dried, the metabolite spots were identified by ultraviolet absorbance and counted in a scintillation spectrometer.

RESULTS

A diabetogenic dose of streptozotocin, 200 mg/kg, given intravenously 2 hr prior to sacrifice lowered the NAD content of mouse islets from the control mean of 0·78 pmole to 0·11 pmole (Table 1). Nicotinamide, 500 mg/kg, administered intraperi-

TABLE 1. EFFECT OF TREATMENT *in vivo* ON NAD CONTENT AND SURFACE AREA OF INDIVIDUAL MOUSE ISLETS OF LANGERHANS*

Treatment	Route	NAD (pmoles/islet)	N	Surface area (mm²)	N
Control	—	0·78 ± 0·05	21	0·0234 ± 0·0034	20
Streptozotocin (200 mg/kg)	i.v.	0·11 ± 0·02	18	0·0196 ± 0·0018	20
Nicotinamide (500 mg/kg) 10 min before Streptozotocin (200 mg/kg)	i.p. i.v.	0·97 ± 0·04	18	0·0232 ± 0·0018	20
Nicotinamide (500 mg/kg)	i.p.	1·04 ± 0·07	17	0·0263 ± 0·0023	20

* Values are expressed as the mean ± S.E.M. N = the number of determinations.

toneally 10 min prior to intravenous streptozotocin prevented the decrease in NAD content, 0·97 pmole. Nicotinamide given alone produced a mean NAD content of 1·04 pmoles which was significantly greater than control ($P < 0·05$), but not when compared with the nicotinamide–streptozotocin combination group. The mean islet NAD content of groups of 50 islets of mice treated with either intravenous citrate

buffer or streptozotocin, 200 mg/kg, for studies of nicotinamide metabolism demonstrated comparable results: 0·714 pmole/islet for control vs 0·169 pmole/islet for the streptozotocin-treated groups (Table 2).

TABLE 2. EFFECT OF STREPTOZOTOCIN TREATMENT ON THE INCORPORATION OF INTRAPERITONEAL (^{14}C)-NICOTINAMIDE* INTO NMN AND NAD OF 50 MOUSE ISLETS OF LANGERHANS

Treatment	(^{14}C)-nicotinamide (pCi/50 islets)	(^{14}C)-MNN (pCi/50 islets)	(^{14}C)-NAD (pCi/50 islets ± S.E.M.)	Total radioactive material (pCi/50 islets)	Mean NAD content/islet (pmoles)
Citrate buffer (0·005 M, pH 4·5 i.v.)	307	80	508 ± 59 (4)	948	0·714
Streptozotocin† (200 mg/kg., i.v.)	530	33	87 ± 12 (4)	756	0·169

* 0·17 μmol; spec. act., 60 mCi/m-mole, administered i.p. 1 hr prior to sacrifice.
† Two hr prior to sacrifice.

The mean surface area of islets isolated from mice receiving intravenous streptozotocin was decreased when compared to controls, 0·0196 and 0·0234 mm^2 respectively, but the reduction was not statistically significant (Table 1). The islets from the nicotinamide-treated group of mice, 0·0263 mm^2, were larger than those of the streptozotocin group ($P < 0·05$), but not the nicotinamide–streptozotocin combination, 0·0232.

Groups of islets incubated for 1 hr with streptozotocin demonstrated a concentration-related decrease in NAD content (Table 3). At 10^{-2} and 10^{-3} M streptozotocin,

TABLE 3. NAD CONTENT OF MOUSE ISLETS OF LANGERHANS INCUBATED *in vitro* WITH STREPTOZOTOCIN FOR 1 HR

Streptozotocin concn (M)	NAD (pmoles)	N
Control	0·75 ± 0·04	14
10^{-2}	0·35 ± 0·06	15
10^{-3}	0·57 ± 0·08	14
10^{-4}	0·66 ± 0·07	17

* Values are expressed as the mean ± S.E.M. N = the number of determinations.

the mean NAD was reduced to 0·35 and 0·57 pmoles/islet compared to 0·75 pmole/islet for controls. The effect of streptozocin treatment *in vivo* on the 1-hr incorporation of 10 μCi ^{14}C-nicotinamide into NMN and NAD extracted from groups of 50 isolated islets was compared to that observed with citrate buffer-treated controls (Table 2). At the 2-hr post-treatment period, streptozotocin had reduced the incorporation of counts into NMN and NAD by 59 and 83 per cent respectively. This was accompanied by a reduction of measured mean NAD content of 76 per cent in islets isolated from the streptozotocin group of animals. The specific activity of the incorporated ^{14}C-nicotinamide was 14 pCi/pmole of NAD and 10 pCi/pmole of NAD for the control

and streptozotocin-treated groups respectively. The concentration of all radioactive materials in islets was reduced from 948 pCi/50 islets in controls to 756 pCi/50 islets in the streptozotocin-treated group.

Incorporation of ^{14}C-nicotinamide into NMN and NAD by mouse liver was reduced by 61 and 63 per cent, respectively, in streptozotocin-treated animals compared to controls (Table 4). This corresponded to a decrease in measured NAD

TABLE 4. EFFECT OF STREPTOZOTOCIN TREATMENT ON THE INCORPORATION OF INTRAPERITONEAL (^{14}C)-NICOTINAMIDE* INTO METABOLITES OF MOUSE LIVER AND WHOLE BLOOD

	Liver				Whole blood			
	Control (nCi/g liver ± S.E.M.)	N	Streptozotocin† (nCi/g liver ± S.E.M.)	N	Control (nCi/ml blood ± S.E.M.	N	Streptozotocin† (nCi/ml blood ± S.E.M.)	N
Nicotinamide	499 ± 33	5	569 ± 11	5	52 ± 4	5	181 ± 18	5
NMN	64 ±	13	25 ± 3	14	—		—	
NAD	738 ± 54	11	275 ± 23	14	—		—	
Nicotinic acid	8 ± 1	5	5 ± 1	5	2 ± 0	5	3 ± 2	5
Desamido-NAD	55 ± 1	5	5 ± 2	5	—		—	
N_1-methylnicotinamide	12 ± 3	5	22 ± 2	5	4 ± 1	5	12 ± 1	5
Nicotinuric acid	52 ± 8	5	47 ± 3	5	—		—	5
Total radioactive material	1969 ± 79	5	1511 ± 29	5	238 ± 18	5	436 ± 29	5

* 0·17 μmole, spec. act., 60 mCi/m-mole i.p. 1 hr prior to sacrifice.
† Two hr after 200 mg/kg, i.v.

concentration from 0·984 μmole ± 0·05 (mean ± S.E.M.)/g wet wt for controls, to 0·349 μmole ± 0·12/g wet wt for the streptozotocin-treated group. The total concentration of radioactive material in the liver of streptozotocin-treated mice was lower than that in controls. This was associated with a greater than 2-fold increase in radioactive material in whole blood with a corresponding increase in nicotinamide and N_1-methylnicotinamide. The concentration of NAD in the acid-soluble fraction of whole blood was 1·324 nmoles ± 0·055 (mean ± S.E.M.)/ml for controls compared to 1·217 ± 0·108/ml for the streptozotocin-treated group.

DISCUSSION

In previous studies with streptozotocin and its cytotoxic moiety, 1-methyl-1-nitrosurea (MNU), it had been demonstrated that a single injection of these drugs could produce a rapid decrease in NAD and NADH concentrations in mouse and rat liver, with the nadir appearing at approximately 2 hr.[3,5,8] Reduction of liver pyridine nucleotides was also demonstrated for the nitrosamines, but not alloxan. A structure–activity relationship for NAD depression was proposed for the R—N—$(CH_2)_{1-2}$H side chain shared in common by this group of compounds.[8,11] Streptozotocin is unique for diabetogenic activity in animals, and the presence of glucose in its chemical structure which may facilitate its uptake into the pancreatic beta cell. Pharmacologic doses of nicotinamide, but not nicotinic acid, prevented the decrease in NAD and

diabetogenic activity of streptozotocin, but failed to produce the height of NAD concentrations obtainable when the vitamin was administered in the same dose alone.[3,5] These observations have led us to propose that depression of pyridine nucleotide concentrations was the principal biochemical mechanism for the destruction of the pancreatic beta cell by streptozotocin, mediated through a block in the utilization of nictotinamide for the synthesis of NAD and NADH. Decreases in rat pancreatic islet pyridine nucleotides have been demonstrated by Ho and Haskin,[12] and in the present study of the mouse islet.

A decrease in incorporation of radiolabeled nicotinamide into NMN and NAD, which roughly corresponded to the present reduction of measured NAD concentration, was demonstrated for both the liver and islets of streptozotocin-treated mice. The 2-hr post-treated time period was chosen for study because it represented the period of maximum liver NAD depression demonstrated in previous studies.[3] In the experiments reported here, there was some evidence of decreased total radioactive material in these tissues after streptozotocin treatment, but this does not appear to be sufficient to account for the entire reduction in incorporation into NAD. Whole blood of treated animals demonstrated a significant retention of nicotinamide coupled with an increase in its urinary metabolite, N_1-methylnicotinamide. There was no increase in blood NAD to suggest release of intact pyridine nucleotides from damaged hepatocytes after streptozotocin treatment.

Using ^{14}C-nicotinic acid, Chang[13] has reported a reduction in incorporation of this precursor into the NAD fraction of liver, and a significant decrease in total radioactive material in mouse liver only 2 hr after treatment with streptozotocin. This corresponded to the time period of maximum NAD measured spectrophotometrically. However, at both 1 and 6 hr after treatment, significant depressions of measured NAD were also recorded despite the finding of equal concentrations of radioactive material in both control and streptozotocin-treated whole liver and NAD fraction. It was suggested that streptozotocin depressed liver NAD concentrations by facilitating its removal and reducing tissue uptake of precursors.

Recent studies reported by Collins and Chaykin[14] suggest that nicotinic acid is converted to nicotinamide for the purpose of continuous NAD synthesis in the mouse through the operation of the Preiss–Handler pathway and NAD glycohydrolase.[15,16] A maximum incorporation of ^{14}C-nicotinic acid into liver NAD at 5 min was followed by a rapid reduction of label in this fraction. By 20 min, the majority of the radioactivity in liver was in the form of nicotinamide. In these studies nicotinic acid served as a rapid but transient precursor of liver pyridine nucleotide. In contrast, ^{14}C-nicotinamide, while producing a lower peak concentration into NAD than nicotinic acid, nevertheless made a sustained contribution lasting a minimum of 60 min, the length of the study. This is consistent with previous investigations which have demonstrated that, when administered in equal molar doses, nicotinamide is a more efficient precursor of NAD in the mouse than is nicotinic acid.[17,18]

The studies of nicotinic acid metabolism by Collins and Chaykin[14] in part explain the continued uptake of ^{14}C-nicotinic acid into liver NAD observed by Chang,[13] in contrast to the decreased incorporation of ^{14}C-nicotinamide found in the present study, in streptozotocin-treated mice. Chang[13] conducted his studies 10 min after the injection of ^{14}C-nicotinic acid and found an equal per cent incorporation into NAD for streptozotocin and control groups despite a measured decrease in NAD concentra-

tion in the former. This can be interpreted as demonstrating a failure of streptozotocin to inhibit the Preiss–Handler pathway, allowing rapid labeling of NAD to proceed; the limiting factor is the tissue uptake of ^{14}C-nicotinic acid. However, the subsequent steady accumulation of label into the nicotinamide fraction of liver in the streptozotocin-treated animals could be related to a partial inhibition in the nicotinamide pathway for NAD synthesis and thus is consistent with the results observed in the present study.

In previous investigations of the mechanism of NAD depression by MNU, both NAD glycohydrolase and NAD pyrophosphorylase were found to be essentially unchanged in activity.[11] The decreased incorporation of ^{14}C-nicotinamide into NMN observed in the present study suggested that the first step in the nicotinamide pathway was partially inhibited.

The importance of depression of pyridine nucleotides as the basis of the antitumor activity of streptozotocin against malignant insulinoma is implied, but awaits confirmation by direct study of these tissues.

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