

K_{ATP} Channel Openers Protect Rat Islets Against the Toxic Effect of Streptozotocin

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We examined the influence of two K_{ATP} channel openers, diazoxide and an analog (NNC 55-0118), on experimental β -cell damage induced by streptozotocin (STZ; 0.5 mmol/l). Rat pancreatic islets were exposed to diazoxide or NNC 55-0118 for 30 min and were further incubated for 30 min after the addition of STZ. The islets were then washed and cultured for 24 h. Islets exposed to STZ alone showed extensive morphological damage, reduced glucose oxidation, low insulin content, and severely impaired glucose-stimulated insulin secretion and proinsulin biosynthesis. Islets treated with STZ in the presence of the channel openers (0.03–0.30 mmol/l) showed dose-dependent preservation of the morphology and improved glucose oxidation rates, insulin content, and secretion. NNC 55-0118 was capable of fully counteracting the STZ impairment, whereas diazoxide had a less protective effect. NNC 55-0118 did not counteract STZ-induced depression of islet NAD levels when examined 2 h after STZ exposure, which suggests that the mechanism of action by NNC 55-0118 is not through an inhibition of poly(ADP-ribose) polymerase. The results illustrate that K_{ATP} channel openers can protect insulin-producing cells against toxic damage, an effect that may be of use in subjects with ongoing insulinitis. *Diabetes* 49:1131–1136, 2000

In human autoimmune diabetes, the insulin-producing β -cells are generally thought to be destroyed by cytotoxic T-cells that are driven by an antigen-specific process (1,2). In experiments with isolated islets, we have found that glucose increases the expression of β -cell autoantigens (3) and that diazoxide lowers the antigen amount (4). Against this background, we conducted a clinical trial and observed that a 3-month supplementary treatment with diazoxide had a beneficial effect on residual β -cell function in patients with new-onset type 1 diabetes (5). “ β -cell rest” may thus protect islet cells from autoimmune destruction (6) and may help to explain the remission phenomenon often noted subsequent to the initiation of insulin treatment.

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ANOVA, analysis of variance; BSA, bovine serum albumin; EC₅₀, 50% effective concentration; FCS, fetal calf serum; KRBH, Krebs-Ringer bicarbonate buffer with HEPES; PARP, poly(ADP-ribose) polymerase; STZ, streptozotocin; TCA, trichloroacetic acid.

Diazoxide and other K_{ATP} channel openers such as cromakalim and pinacidil have been used in experimental studies of the ischemic heart, and beneficial cardioprotective effects have been observed (7–9). Although the exact mechanism of this phenomenon is not understood, an opening of mitochondrial K⁺ channels seems to be involved that results in dissipation of the inner mitochondrial membrane potential. This leads to net oxidation of the mitochondria with an apparent reduction in energy wastage (7,9,10).

Diazoxide is known to act on K_{ATP} channels in the plasma membrane of β -cells (11). It hyperpolarizes the membrane and inhibits the energy-consuming process of insulin secretion (12). Recently, exposure of β -cells to diazoxide has also been found to engage mitochondrial K_{ATP} channels (13). In the present study, we examined the influence of diazoxide and NNC 55-0118, a new K_{ATP} channel opener analog, on experimental β -cell damage induced by streptozotocin (STZ), an agent known to cause energy depletion and β -cell death (14).

RESEARCH DESIGN AND METHODS

Islet isolation, culture, and experimental design. Pancreases from Sprague-Dawley rats were collagenase digested, and islets were collected with a braking pipette as previously described (15). Islets were precultured free floating in RPMI-1640 medium (Sigma, St. Louis, MO) with 10% (vol/vol) fetal calf serum (FCS; Sigma) and 11 mmol/l glucose for 3 days in air (5% CO₂) at 37°C before the experiments. The medium was changed twice during preculture. Islets were then transferred to sterile petri dishes containing 1 ml Krebs-Ringer bicarbonate buffer with HEPES (KRBH) medium with 2 mg/ml bovine serum albumin (BSA) and 5.6 mmol/l glucose.

Stock solutions of 100 mg/ml diazoxide (Sigma) and NNC 55-0118 (Novo Nordisk, Copenhagen, Denmark) dissolved in dimethyl sulfoxide were prepared and added to a final concentration of 0.03 or 0.3 mmol/l in the petri dishes. Islets were incubated in 5% CO₂ at 37°C for 30 min with or without diazoxide or NNC 55-0118, and then dry STZ powder (gift from Pharmacia-Upjohn, Kalamazoo, MI) was dissolved in 0.9% NaCl to a concentration of 50 mmol/l. A total of 10 μ l of the freshly prepared solution was then added to the petri dishes to give a final concentration of 0.5 mmol/l STZ. Previous studies have shown that this process will not affect the pH of the incubation medium (data not shown). The incubation continued for another 30 min and was terminated by the addition of 1 ml cold KRBH. The islets were then washed twice in KRBH and studied for morphology and insulin secretion (0 h point), or they were incubated for 2 or 24 h in RPMI-1640 medium with 10% FCS and 11 mmol/l glucose before morphological and biochemical examinations.

Morphology and islet recovery. Approximately 100 islets per condition were carefully transferred to a glass tube and spun down at 800 rpm for 1 min. The medium was removed, and ~200 μ l was left before the fixation with 8 ml of Bouin's medium followed by dehydration in ethanol. The pellets were embedded in paraffin, cut into 5- μ m sections, and stained for insulin (guinea pig anti-insulin, 1:100 dilution, Dako, Älvsjö, Sweden) using the peroxidase-antiperoxidase method. For estimation of islet recovery, 30 islets from each condition were transferred to petri dishes as described above, and the remaining islets were counted after 24 h.

Insulin secretion and islet insulin contents. Triplicates of 5 islets were transferred to 200 μ l KRBH with 2 mg/ml BSA and 16.7 mmol/l glucose and were incubated for 60 min in 5% CO₂ at 37°C. Islets from each condition were then pooled and sonicated in 200 μ l redistilled water. A 50- μ l aliquot of the homogenate was mixed with 125 μ l acid ethanol (0.18 mol/l HCl in 95%

ethanol), and insulin was extracted overnight. Insulin concentration in the sonicate and in the culture medium was determined with radioimmunoassay as previously described (16).

Proinsulin biosynthesis and total protein biosynthesis. For each condition, duplicate samples of 20 islets were transferred to multiwell plates containing 100 μ l KRBH with L-[4,5- 3 H]leucine (50 μ Ci/ml; Amersham, Amersham, U.K.), 2 mg/ml BSA, and 16.7 mmol/l glucose and were incubated for 120 min in 5% CO_2 at 37°C. Islets were then washed in Hanks' solution supplemented with 10 mmol/l nonradioactive leucine and were subsequently sonicated in 200 μ l redistilled water. A 50- μ l fraction of the aqueous homogenate was incubated for 90 min with insulin antibodies coupled to sepharose beads to separate proinsulin from other labeled proteins (17). Total protein biosynthesis was obtained by precipitating the labeled proteins with trichloroacetic acid (TCA). The antibody-bound radioactivity and TCA-precipitable radioactivity were determined in a liquid scintillation counter.

Glucose oxidation. Groups of 10 islets were transferred to glass vials with 100 μ l KRBH supplemented with D-[U- 14 C]glucose and nonradioactive glucose to a final concentration of 16.7 mmol/l glucose. Triplicate samples were used. The vials were suspended in scintillation flasks, were gassed with 5% CO_2 , and were sealed airtight. The flasks were then shaken for 90 min at 37°C. Metabolism was stopped by injection of 100 μ l of 0.05 mmol/l antimycin A into the center vial. Immediately thereafter, 250 μ l hyamine hydroxide was injected into the outer flask. CO_2 was released from the incubation medium by injecting 100 μ l of 0.4 mol/l Na_2HPO_4 solution (pH 6.0) into the center vial. To allow

the CO_2 to be trapped by the hyamine hydroxide, the vials were incubated for another 120 min at 37°C. Scintillation fluid was then added to each flask, and the radioactivity was counted in a liquid scintillation counter.

NAD content. Islet NAD content was determined according to a method described earlier (18). Briefly, 50 islets per condition were lysed in 500 μ l of 1 mol/l perchloric acid and thereafter neutralized in an excess of 1 mol/l N,N-diethylmethylamine in $CHCl_3$. The water phase was used for analysis by high-performance liquid chromatography.

Statistics. Data are means \pm SE. A mean was calculated from each duplicate or triplicate group and then was considered to be 1 separate observation. Furthermore, every observation represented different islet donors. Student's paired *t* test and analysis of variance (ANOVA) were used when appropriate.

RESULTS

Islet recovery and morphology. The islets exposed to STZ for 30 min showed variable morphological signs of functional change and damage including degranulation and characteristics of cell death when examined at later time points. Occasionally, in areas of the islets harboring intact β -cells, a reduced insulin stain could be seen already at the 2-h time point (Fig. 1A). Moreover, apparently pyknotic cell nuclei and some β -cells had lost discernable cell boundaries after the STZ treat-

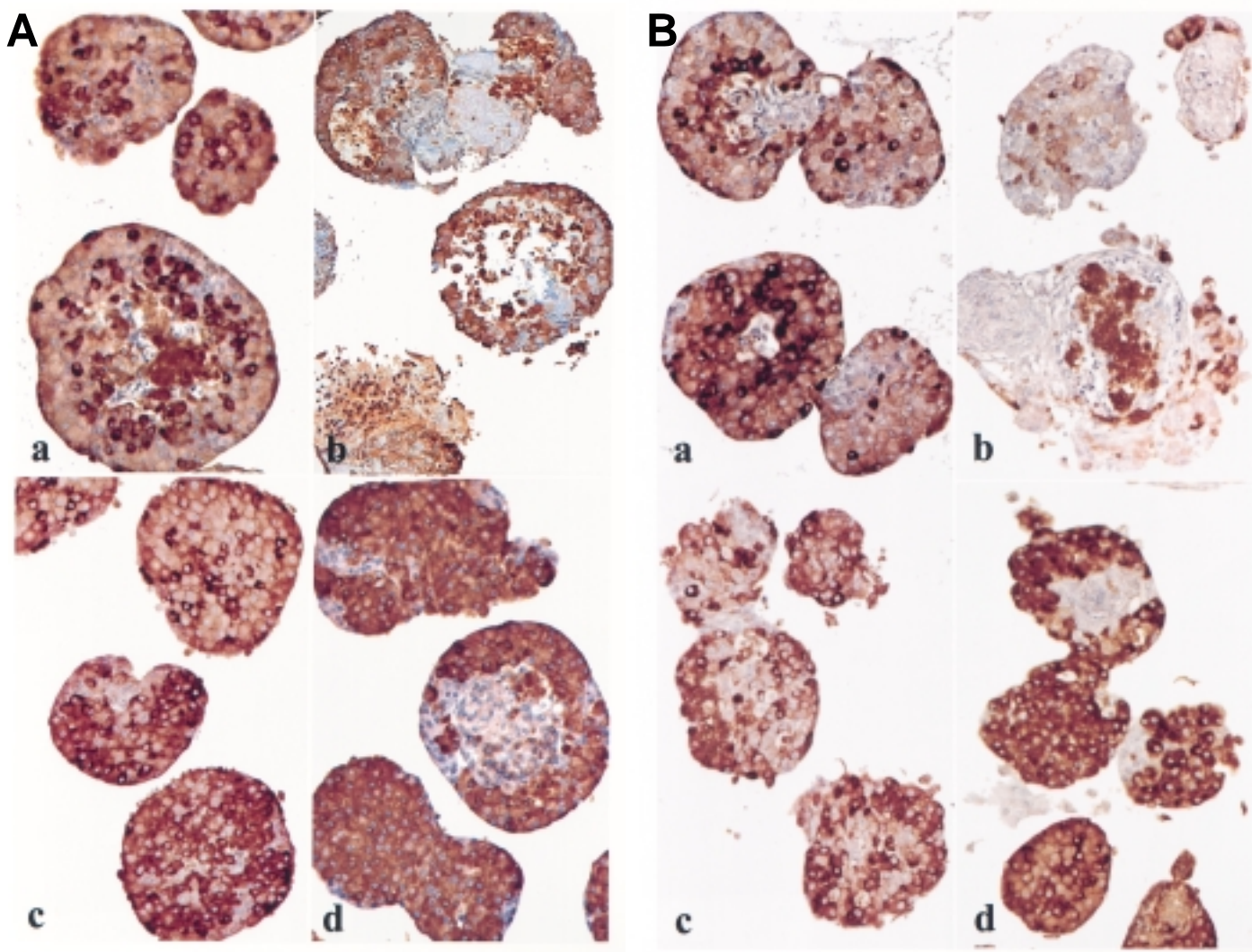


FIG. 1. Morphology and insulin staining of rat islets incubated for 60 min in KRBH medium containing 5.6 mmol/l glucose. Islets were incubated in medium alone (*a*), with 0.5 mmol/l STZ during the latter 30 min (*b*), and in 0.3 mmol/l diazoxide (*c*) or 0.3 mmol/l NNC 55-0118 with 0.5 mmol/l STZ (*d*) during the latter 30 min. Islets were examined after subsequent culture in RPMI-1640 medium with 10% FCS and 11 mmol/l glucose for 2 h (*A*) and 24 h (*B*). Unstained areas in the center of some islets correspond with nonviable cells, which most likely reflect insufficient diffusion of medium during culture.

TABLE 1

Characteristics of rat islets after 24 h of recovery after 30 min of exposure to STZ in the presence or absence of K_{ATP} channel openers

Pretreatment	Medium	0.3 mmol/l diazoxide	0.3 mmol/l NNC 55-0118	Medium + 0.5 mmol/l STZ	0.3 mmol/l diazoxide + 0.5 mmol/l STZ	0.3 mmol/l NNC 55-0118 + 0.5 mmol/l STZ
Islets recovered (%)	96 ± 2 (5)	94 ± 2.3 (5)	94 ± 4 (5)	59 ± 3.3 (5)*	71 ± 5 (5)*	98 ± 1.7 (5)†
Proinsulin biosynthesis (dpm · 20 islets ⁻¹ · 90 min ⁻¹)	1,078 ± 82 (8)	1,300 ± 156 (8)	1,268 ± 135 (8)	380 ± 40 (8)*	533 ± 71 (8)*	1,149 ± 86 (8)†
Total protein biosynthesis (dpm · 20 islets ⁻¹ · 90 min ⁻¹)	4,730 ± 462 (8)	6,023 ± 864 (8)	5,039 ± 626 (8)	2,445 ± 190 (8)‡	3,437 ± 315 (8)	5,186 ± 250 (8)†
Proinsulin/total protein biosynthesis × 100 (%)	23.2 ± 1.2 (8)	22.0 ± 0.8 (8)	23.5 ± 0.7 (8)	15.3 ± 0.7 (8)*	15.8 ± 1.5 (8)*	22.8 ± 1.5 (8)
Glucose oxidation (pmol · 10 islets ⁻¹ · 90 min ⁻¹)	235 ± 37.6 (7)	277 ± 51.4 (7)	202 ± 43.2 (7)	38.4 ± 17.2 (7)‡	58.9 ± 20.5 (7)‡	250 ± 63.8 (7)§

Data are means ± SE (*n* experiments). **P* < 0.001 vs. control (medium); †*P* < 0.001 vs. STZ group; ‡*P* < 0.01 vs. control (medium); §*P* < 0.01 vs. STZ group by ANOVA.

ment. No signs of recovery were found, but further destruction and disintegration of islets were evident at 24 h (Fig. 1B).

At 24 h, an average of 59 ± 3.5% of the STZ islets were recovered when counted in a stereomicroscope. The islet loss was less in the groups of islets treated with 0.3 mmol/l diazoxide plus STZ, and it was not detectable in the 0.3 mmol/l NNC 55-0118 plus STZ condition (98 ± 1.7%; *P* < 0.01 vs. STZ alone) (Table 1).

Islets incubated with diazoxide plus STZ or NNC 55-0118 plus STZ appeared morphologically intact at the 0-h time point (data not shown). At 2 h, the shape of the islets showed some irregularity (Fig. 1A), and this was more apparent at 24 h (Fig. 1B), particularly with the islets incubated in the lower concentration (0.03 mmol/l) of the channel openers (data not shown). NNC 55-0118 was more potent than diazoxide in preserving the morphology (Fig. 1B). The numerous pyknotic nuclei, as seen in the STZ group, were not found in the

0.3 mmol/l diazoxide plus STZ or 0.3 mmol/l NNC 55-0118 plus STZ islet incubations.

Functional characteristics. STZ exposure caused an ~40% loss of islets when estimated after 24 h (Table 1). This loss was counteracted by 0.3 mmol/l NNC 55-0118 but not by diazoxide. Addition of 0.03–0.30 mmol/l diazoxide or NNC 55-0118 alone did not affect the number of islets retrieved (Table 1) or the insulin content when examined at the 24-h time point (Fig. 2A). Islets exposed to STZ had a reduced insulin content, which was partially restored by 0.3 mmol/l diazoxide and normalized by 0.3 mmol/l NNC 55-0118. The islets recovered 24 h after STZ treatment had an ~50% reduced glucose-stimulated insulin release (Fig. 2B). This was to a minor extent restored by 0.3 mmol/l diazoxide and was fully maintained by 0.3 mmol/l NNC 55-0118. Exposure to diazoxide or NNC 55-0118 alone had no effect on insulin secretion when studied 24 h after withdrawal of the agents. Inhibitory effects of dia-

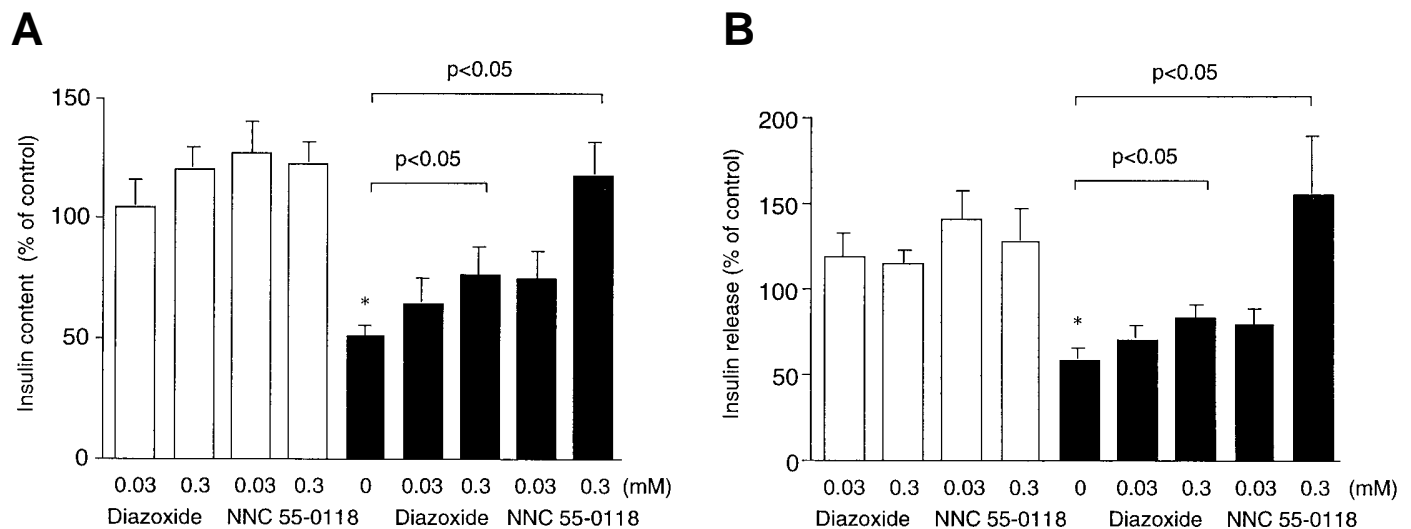


FIG. 2. Islet insulin content (A) and insulin release (B) after 24 h of recovery after a 60-min incubation with diazoxide or NNC 55-0118 with (■) or without (□) 0.5 mmol/l STZ during the latter 30 min. Triplicates of 5 islets were used for each condition and were incubated for 60 min in KRBBH with 2 mg/ml BSA and 16.7 mmol/l glucose. Insulin content in the medium and in 15 pooled islets sonicated in redistilled water was determined by radioimmunoassay. Insulin release and insulin content in control islets were 17.8 ± 2.9 ng · 5 islets⁻¹ · 60 min⁻¹ and 185 ± 14.9 ng · 5 islets⁻¹, respectively. A total of 7 experiments were performed with diazoxide, and 6 experiments were performed with NNC 55-0118. Results are a percentage of control islets. **P* < 0.05 vs. control islets.

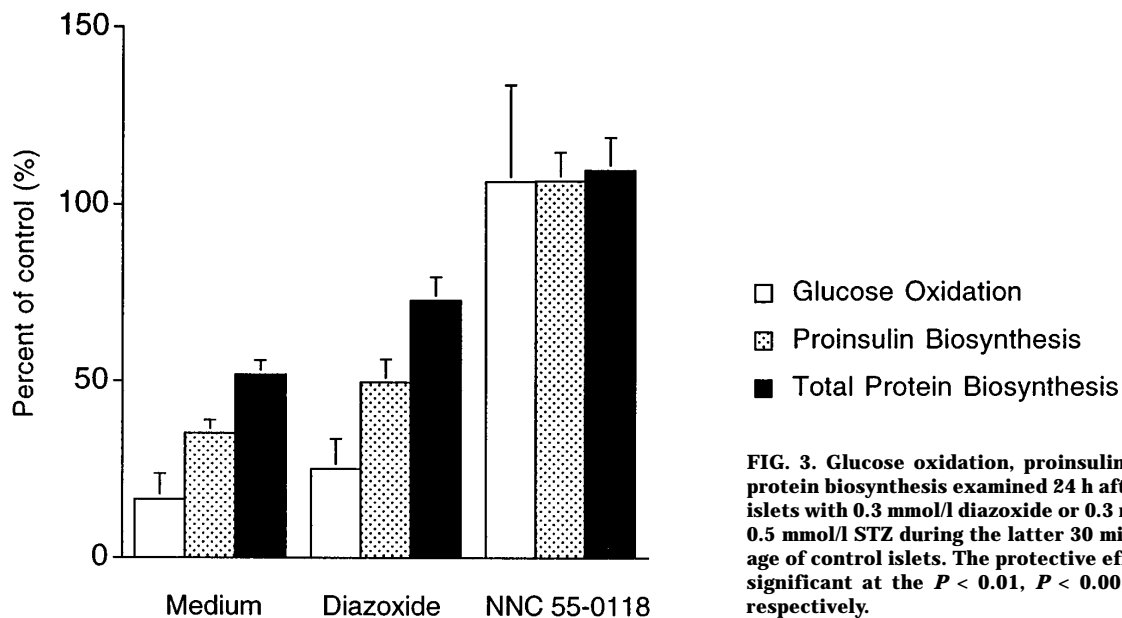


FIG. 3. Glucose oxidation, proinsulin biosynthesis, and total protein biosynthesis examined 24 h after 60-min incubations of islets with 0.3 mmol/l diazoxide or 0.3 mmol/l NNC 55-0118 with 0.5 mmol/l STZ during the latter 30 min. Results are a percentage of control islets. The protective effects of NNC 55-0118 are significant at the $P < 0.01$, $P < 0.001$, and $P < 0.001$ levels, respectively.

zoxide and NNC 55-0118 on glucose-stimulated insulin release were apparent at 0 and 2 h (data not shown).

In glucose oxidation experiments performed 2 h after exposure to K_{ATP} channel openers and STZ, a significant difference was found between islets treated with STZ (33 ± 16.8 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$ [$n = 8$]) and 0.3 mmol/l NNC 55-0118 plus STZ (104 ± 28.5 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$) ($P = 0.035$). A difference was also evident between normal control (237 ± 39.0 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$) and NNC 55-0118 plus STZ islets ($P < 0.001$). Diazoxide plus STZ islets (33 ± 15.2 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$) were not significantly different from islets treated with STZ only ($P = 0.983$, 1-way ANOVA; $n = 8$). Also at the 24-h time point, the islet glucose oxidation rate was markedly impaired by STZ. This was prevented by 0.3 mmol/l NNC 55-0118 (Fig. 3). In another experiment, we aimed to examine whether NNC 55-0118 acutely influenced the islet glucose oxidation rate. When present for 90 min at 16.7 mmol/l glucose in KRBH, 0.3 mmol/l NNC 55-0118 did not significantly affect the glucose oxidation rate (control: 268 ± 22.9 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$; 0.3 mmol/l NNC 55-0118: 219 ± 21.5 pmol \cdot 10 islets $^{-1}$ \cdot 90 min $^{-1}$; $P > 0.05$ [$n = 8$]).

The STZ treatment lowered the rates of total protein and proinsulin biosynthesis. The latter was relatively more inhibited, which led to a decline in the fraction proinsulin biosynthesis of the total protein biosynthesis (Table 1). This indicates a preferential β -cell toxic effect of STZ. Incubation with diazoxide did not alter the effect of STZ on proinsulin and total protein biosynthesis. In the islets treated with 0.3 mmol/l NNC 55-0118 plus STZ, the proinsulin and total protein biosynthesis rates were not different from control islets (Fig. 3).

To investigate whether the mechanism of action by NNC 55-0118 may depend on an inhibition of poly(ADP-ribose) polymerase (PARP) activity, we assessed islet NAD contents 2 h after STZ treatment (Fig. 4). In these experiments, STZ caused a $>50\%$ decrease in the islet NAD content. This decline was not affected by 0.3 mmol/l NNC 55-0118.

DISCUSSION

In the present study, we found that two K_{ATP} channel openers, diazoxide and NNC 55-0118, can protect β -cells against the

toxic action of STZ. The toxin has a half-life of 5–15 min (19) and is taken up via GLUT2 expressed in β -cells and hepatocytes (20). The toxin has a glucose moiety and a reactive methyl group and is known to induce a rapid depletion of NAD in the islets (21). NAD is essential for redox reactions involved in the generation of ATP via glycolysis and mitochondrial oxidative phosphorylation. An important part of STZ toxicity is thought to occur through methylation of DNA, which leads to strand breaks and activates PARP for DNA repair (22). The enzyme catalyzes the formation of poly(ADP-ribose) and lowers ATP production by consuming NAD. The recent observations on PARP-deficient mice (23) lend strong support to such a toxic action of STZ.

Concerning the morphological effects of STZ, that in vivo administration of STZ causes extensive damage is well known (24). Nuclear pyknosis at 1 h affecting several nuclei of β -cells in rat pancreatic islets was observed in a study by Junod et al. (25). In another study, 1 h after the administration of STZ, secre-

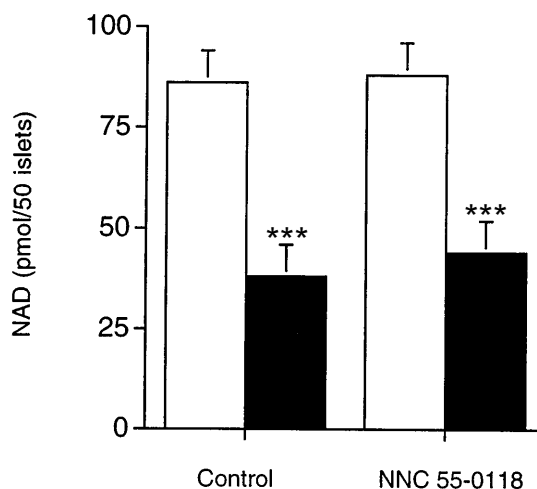


FIG. 4. Islet NAD content 2 h after exposure to no STZ (\square) or 0.5 mmol/l STZ (\blacksquare). Data are means \pm SE for 8 experiments. *** $P < 0.001$ vs. corresponding islets not exposed to STZ using Student's paired t test.

tory granules became fewer compared with those of normal nuclei (electron microscopic observation), and the stainability of most pancreatic β -cells to aldehyde-fuchsin was remarkably decreased compared with those in the normal pancreas (26). In the present study, islets treated with STZ for 30 min showed subsequently variable signs of toxic damage. In some islets, the central areas had a necrotic appearance with a lack of discernable cell boundaries but showed insulin staining, which indicates that plasma membranes had been destroyed. The areas of the islets harboring intact β -cells showed occasionally reduced insulin staining compared with control islets. This finding agrees with the *in vivo* observations reported by others (25,26).

One could argue that the protective effect of the K_{ATP} channel openers may be a reduction in the uptake of STZ or a direct inhibitory action of PARP activity. We have found, however, that the amount of GLUT2 in the plasma membrane seems to be upregulated by diazoxide (27). As expected, the STZ treatment caused a reduction in the islet levels of NAD. However, NNC 55-0118 did not influence this decline, which argues against the compound acting as a PARP inhibitor.

In this investigation, NNC 55-0118 appeared to be ~10-fold more potent than diazoxide in protecting islets against STZ damage. We have observed a similar difference in potency regarding inhibition of glucose-stimulated insulin secretion in rat islets (50% effective concentration [EC₅₀], 20 μ mol/l diazoxide and 3 μ mol/l NNC 55-0118, respectively; unpublished data). The present protective effects of the K_{ATP} channel openers were seen at concentrations known to influence mitochondrial K_{ATP} channel activity. In a study by Grimmsmann and Rustenbeck (13), rat islet mitochondrial membrane potential measured with rhodamine 123 was affected by diazoxide in the 0.1–1.0 mmol/l range. Studies have also been conducted on Naval Medical Research Institute mouse islet cell plasma membrane and mitochondrial membrane potential using the fluorescent probes DiBAC4[3] and rhodamine 123. The estimated EC₅₀ values for diazoxide were 54 μ mol/l for plasma membrane potential and 1 mmol/l for mitochondrial membrane potential, whereas the corresponding values for NNC 55-0118 were estimated to be 0.6 μ mol/l for plasma membrane potential and 200 μ mol/l for mitochondrial effects (P.O.G. Arkhammar, personal communication).

Mitochondrial ATP is thought to be involved with mitochondrial volume control and energetics. Opening mitochondrial K_{ATP} channels results in a dissipation of the inner mitochondrial membrane potential (7), which leads to uncoupling of the oxidative phosphorylation and net oxidation of the mitochondria (7,9,10). Observations have suggested that mitochondrial K_{ATP} channel activation may counteract ATP wastage and preserve cellular functions possibly through activation of endogenous mitochondrial ATPase inhibitor (IF₁), or blocking the Ca²⁺ uniporter of the mitochondrion or changing the glycolytic pathways during ischemia (28). In experiments with perfused rat hearts, the cardioprotective K_{ATP} channel opener BMS-180448 was found to preserve mitochondrial integrity as well as the efficiency of oxygen utilization (29). In neurons, hypoxia has been found to activate K_{ATP} channels (30) and to protect against oxidative injury induced by exposure to FeSO₄ and amyloid β -peptide (31). Opening K_{ATP} channels can also counteract neurotoxic damage by cyanide (32), a compound known to stop electron transfer and ATP production by paralyzing cytochrome oxidase in the mitochondrion.

The mechanism by which K_{ATP} channel openers preserve insulin-producing β -cells from damage by STZ remains to be clarified. The present data indicate that it may involve actions at the level of the mitochondria. K_{ATP} channel openers may possibly protect against other forms of toxic impairment such as that caused by cytokines altering the mitochondrial function (33). If so, our findings will have clinical implications and suggest a potential role of K_{ATP} channel openers in the management of individuals with or at risk for developing type 1 diabetes.

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