CMLS, Cell. Mol. Life Sci. 57 (2000) 158–164
1420-682X/00/010158-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

'Classical' and 'new' diabetogens—comparison of their effects on isolated rat pancreatic islets in vitro

E. Peschke[a,*], H. Ebelt[a], H.-J. Brömme[b] and D. Peschke[a]

[a]Institute of Anatomy and Cell Biology, Martin Luther University, Halle-Wittenberg, Grosse Steinstrasse 52, D-06097 Halle/Saale (Germany), Fax + 49 345 5571700
[b]Institute of Pathophysiology, Martin Luther University, Halle-Wittenberg (Germany)

Received 16 September 1999; received after revision 15 November 1999; accepted 26 November 1999

Abstract. This study compares functional and morphological alterations caused by application of alloxan, streptozotocin, xanthine oxidase/hypoxanthine (generation of reactive oxygen species), or S-nitroso-N-acetyl-D,L-penicillamine (SNAP, liberation of nitric oxide) to isolated rat pancreatic islets in vitro. In perifusion experiments, membrane leakage—detected by non-stimulated insulin release—was found after application of all drugs, but showed a substance-specific time pattern. Twenty-four hours after application of the classical diabetogens (alloxan or streptozotocin), potassium chloride- and glucose-stimulated insulin secretion were markedly reduced, while a persistent reduction was observed neither after exposure to xanthine oxidase/hypoxanthine, nor to SNAP. Morphological analysis of the islets revealed that nearly all β-cells were destroyed following alloxan or streptozotocin treatment, while the majority of β-cells were configured regularly after application of xanthine oxidase/hypoxanthine or SNAP. Necrotic cells found after xanthine oxidase/hypoxanthine usually differed in morphology from those observed after application of the classical diabetogens. While the former cells were characterised by swollen nuclei, the latter had shrunken nuclei with irregular condensed chromatin. Apoptosis was found only following nitric oxide exposure. Due to these differences, it seems unlikely that alloxan, streptozotocin, xanthine oxidase/hypoxanthine, and nitric oxide have a common major feature in their toxic action.

Key words. Alloxan; streptozotocin; xanthine oxidase/hypoxanthine; SNAP; reactive oxygen species.

For some decades, alloxan (ALX) and streptozotocin (STZ) have been used widely to induce diabetes mellitus in animals. Within the last few years, the hypothesis has been established that both compounds lead to a selective destruction of pancreatic β-cells through two characteristics: (i) rapidly accumulate into β-cells where they (ii) induce radical-generating reactions. β-Cells are very susceptible to oxidative changes because they possess only low antioxidative capacity [1–4]. The redox couple driven by ALX and its reduced derivative dialuric acid produces superoxide anions and hydrogen peroxide thereby consuming reduced glutathione and further weakening the cellular antioxidative defence system [2, 5–9]. During metabolisation of STZ, a variety of toxic intermediates are produced. Beside alkylating agents like methyl cations and methyl radicals [10, 11] it has been shown that reactive oxygen species (ROS) are produced by STZ as well [12, 13]. Additionally, STZ liberates nitric oxide (NO) which has been proposed to be one key intermediate of its toxicity [10, 14, 15].

In recent years, evidence has accumulated that ROS and NO contribute to the destruction of pancreatic islets in the pathogenesis of insulin-dependent diabetes mellitus [15–20]. Comparing data from the literature, it

* Corresponding author.

appears that ALX, STZ, ROS generated by xanthine oxidase/hypoxanthine (XO/HX), and NO liberated by different chemicals or cells seem to attack β-cells in a very similar way. All these diabetogens can lead to DNA strandbreaking, inhibition of various enzymes and membrane leakage [12, 13, 16, 18–28].

The aim of this study was to compare the functional and morphological alterations which are caused by ALX, STZ, XO/HX, or extracellularly liberated NO on isolated rat pancreatic islets in vitro.

Materials and methods

Chemicals. Collagenase was purchased from Serva (Heidelberg, Germany); tissue culture medium 199, ALX, STZ, XO (from micro-organisms) and HX were from Sigma (St. Louis, Mo.); S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was from Alexis Deutschland (Grünberg, Germany); 'Coat-A-Count' insulin radioimmunoassay was from Biermann (Bad Nauheim, Germany); proteinase K, biotin-16-dUTP, dATP, DNAse I, and TdT (terminale transferase) were from Boehringer (Mannheim, Germany); TdT buffer was from Life Technologies (Gaithersburg, Md.). All other chemicals (purity p.a.) were obtained either from Sigma or from Merck (Darmstadt, Germany).

Animals and islet preparation. For each experiment, the pancreatic islets of four Wistar rats (9–12 days old) of both sexes were isolated as described earlier [29]. In brief, rats were sacrificed by decapitation and the pancreata were removed. The organs were digested with 1 mg/ml collagenase dissolved in Hanks' solution containing 1 mg/ml bovine serum albumin (BSA). The islets were then separated manually from exocrine tissue.

Perifusion. Approximately 300 isolated islets supplemented with Sephadex G-10 were placed into a glass column of a perifusion system (detailed description in Csernus et al. [30]) and continuously perifused with culture medium supplemented with 2.22 g/l sodium hydrogen carbonate, 1.75 g/l BSA, 80 mg/l gentamycin, and 3 mmol/l glucose. After passing the cells, the medium was collected in fractions (intervals lasting 3 or 30 min). Insulin content was determined in duplicates by radio-immunoassay.

At the beginning of each perifusion, β-cell function was tested by a standard stimulation with potassium chloride (KCl) and glucose with a 30-min interval between the applications [30]. To determine the base line of insulin secretion, within the next 5 h (ten fractions), non-stimulated insulin secretion was recorded. Thereafter test compounds were applied. In the subsequent 24 h, the insulin content of the medium was determined without any additional change in the cells. Thereafter the standardisation procedure with KCl and glucose was repeated and histological preparation followed.

Morphological analysis. At the end of each experiment, the pancreatic islets were taken out of the perifusion system and fixed with paraformaldehyde for light microscopy or with Karnowsky's solution for electron microscopy. Light microscopic investigations were performed on paraffin sections (thickness: 7 μm). For electron microscopy, the islets were postfixed with osmium tetroxide and subsequently embedded in Durcupan following standard protocols. Ultra-thin sections were contrast-stained with uranyl acetate and lead citrate and examined and photographed using an electron microscope EM 900 (Zeiss, Oberkochen, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) assay. Deparaffinised sections were pretreated as described earlier [31]. Sections were then covered by TdT buffer containing 5 μmol/l biotin-16-dUTP and 100 μmol/l dATP for 15 min at 37 °C; after this, 50 U/ml TdT was added for 60 min at 37 °C. The reaction product was visualised with ABC-vectastain and diaminobenzidine. Positive controls were initially incubated with a solution of 10 mmol/l sodium acetate, 5 mmol/l $MgSO_4$ and 1000 U/l DNAse I for 60 min at 37 °C; for negative controls, TdT was not added to the TdT buffer mixture.

Results

Perifusion. Control groups (standardisation procedure only) showed no change over time in their pattern of insulin release (fig. 1). Insulin secretion following stimulation with KCl and glucose was in accordance with data obtained in earlier studies [30].

A 9-min application of either 2.0 mmol/l ALX, 2.0 mmol/l STZ, 75 mU/ml XO + 1.5 mmol/l HX, or 2.0 mmol/l SNAP resulted in an insulin release with a typical time pattern for each individual compound (figs. 1, 2).

At the end of the experiments (24 h after drug application), KCl- and glucose-stimulated insulin secretion were recorded. In comparison to hormone secretion at the beginning of the experiments, insulin release from control islets was $106 \pm 11.6\%$ after stimulation with KCl and $84 \pm 10.0\%$ after glucose stimulation. Corresponding values of the other groups were: ALX $54 \pm 13.7\%$ and $33 \pm 7.0\%$; STZ $64 \pm 1.4\%$ and $46.5 \pm 20.5\%$; XO/HX $127 \pm 14.5\%$ and $139 \pm 23.5\%$; SNAP $113 \pm 22.6\%$ and $111 \pm 29.5\%$, respectively (mean $\pm$ SE values of at least three experiments; fig. 3).

Morphology. Islets of control groups showed normal morphology in light and electron microscopy (fig. 4A). After application of ALX or STZ (corresponding to figs. 1–3), cytoplasm as well as nuclei of nearly all β-cells showed typical signs of degeneration. Separating cell membranes could not be identified over large segments, and cytoplasm consisted of many vesicular ele-

ments including still recognisable secretory granules. Nuclear chromatin was irregularly condensed, and the perinuclear space was dilated (fig. 4B).

Treatment with XO/HX led to remarkable alterations in only a few islet cells. While the majority of cells were configured regularly, some endocrine cells of all types showed vesicularly degenerated cytoplasm. Nuclei of these cells were either small with irregularly condensed chromatin, or large in size without chromatin aggregation (fig. 4C). In addition, a small number of cells with intact cytoplasm showed sharply demarcated chromatin abutting the nuclear envelope, indicating apoptosis.

When exposed to 2.0 mmol/l SNAP for 9 min, islets did not differ markedly from untreated controls. In contrast, prolonged application (60 min) led to a characteristic morphological appearance: many β-cells were characterised by regularly configured organelles and nuclei but showed single large vacuoles. These vacuoles were formed by dilatation of endoplasmic reticulum and the perinuclear space as seen in electron microscopy. Additionally, a considerable number of β-cells showed typical signs of apoptosis (fig. 4D).

TUNEL reaction. No TUNEL-positive staining of cells of control islets, nor of islets treated with ALX or STZ was detected. Islets treated with XO/HX showed a few cells with positively stained nuclei, especially at the islet periphery. In accordance with electron microscopy, TUNEL-positive cells were often found after SNAP treatment.

Discussion

Different alterations in β-cell function (enzyme activities, DNA strandbreaking, intracellular NAD^+ content; membrane permeability) were commonly found after application of ALX, STZ, XO/HX, as well as after liberation of NO. In particular, DNA strandbreaking leading to activation of poly(ADP-ribose) polymerase and, in consequence, to NAD^+ depletion has been proposed as a critical event in the toxicity of the classi-

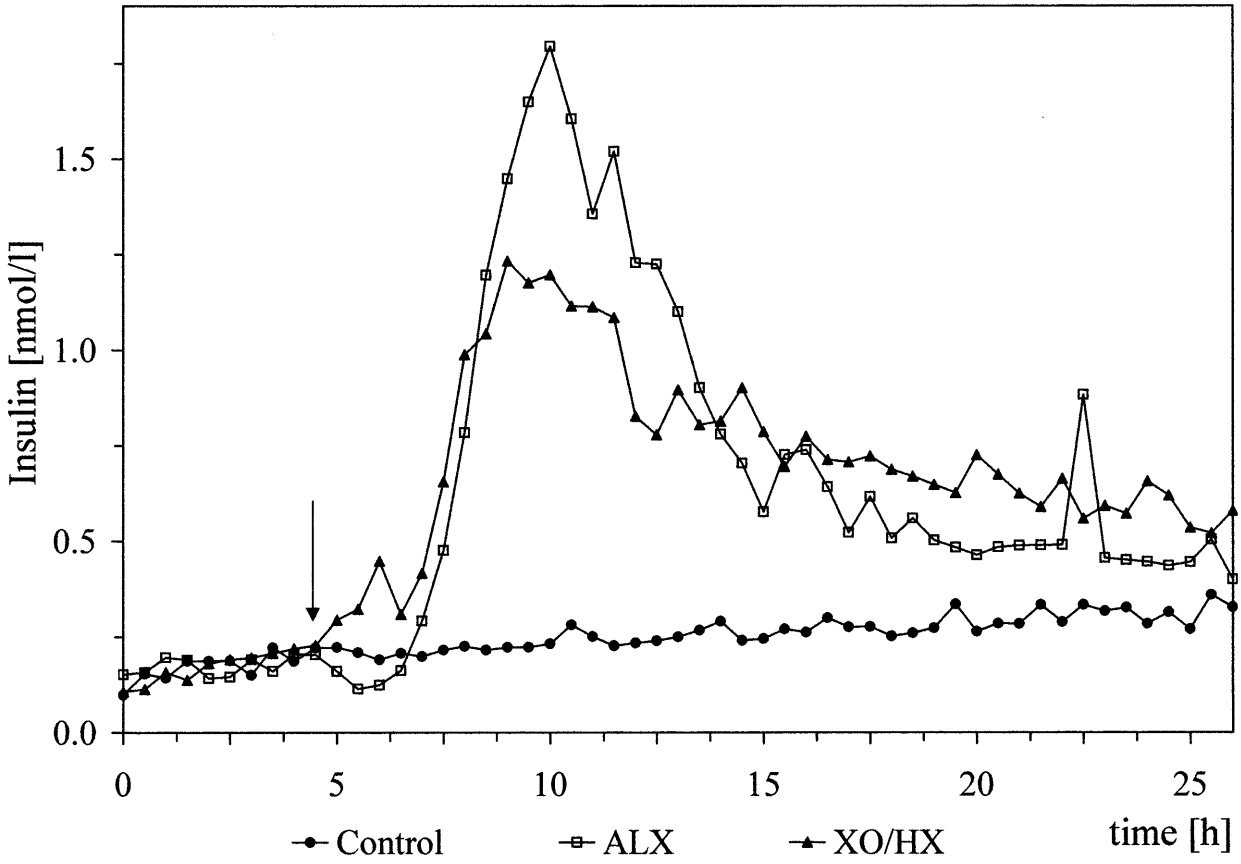

Figure 1. Insulin release from isolated rat pancreatic islets. Control, perifusion medium only; arrow, application of the test compounds for 9 min; ALX, 2.0 mmol/l alloxan; XO/HX, 75 mU/ml xanthine oxidase and 1.5 mmol/l hypoxanthine. Each curve shows a representative example of at least three experiments.

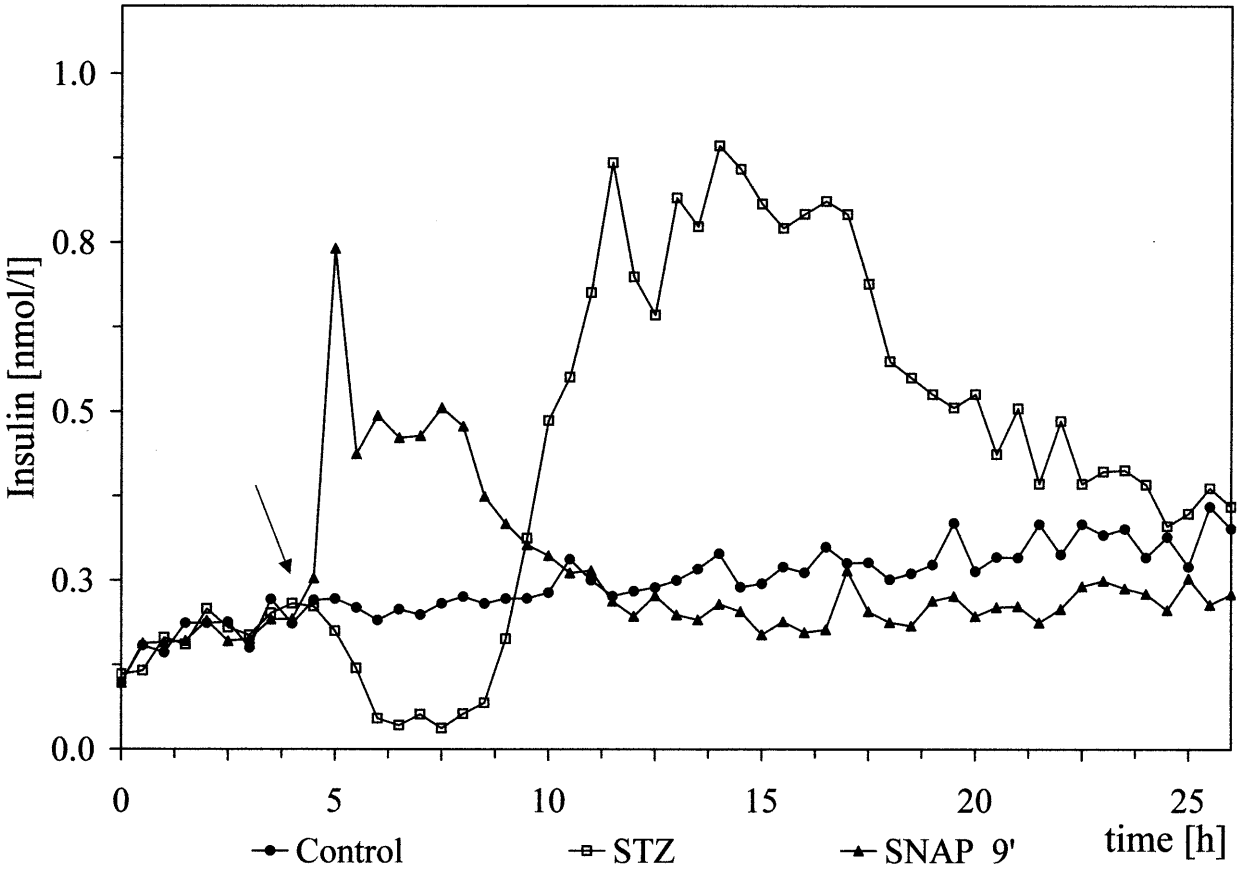

Figure 2. Insulin release from isolated rat pancreatic islets. Control, perifusion medium only; arrow, application of the test compounds for 9 min; STZ, 2.0 mmol/l streptozotocin; SNAP 9′, 2.0 mmol/l S-nitroso-N-acetyl-D,L-penicillamine. Each curve shows a representative example of three experiments.

cal diabetogens as well as ROS (generated by XO/HX) and NO [26, 27, 32]. Nevertheless, this study demonstrates that isolated rat pancreatic islets exposed to these drugs under an identical in vitro experimental protocol, clearly differ in crucial characteristics. Although all compounds caused disturbance of membrane integrity as detected by elevated insulin release in perifusion experiments, the time pattern of membrane leakage, the morphological appearance of the altered cells and their ability to recover from damage were quite different. Most β-cells exposed to ALX or STZ underwent necrosis as indicated by persistent inhibition of stimulated insulin secretion and by morphological appearance. In contrast, neither ROS generated by XO/HX, nor NO liberated from SNAP seemed to cause a critical enduring change in β-cells, although the concentrations and activities of the drugs used were sufficient to provoke membrane leakage (see figs. 1, 2) and exceeded the concentration used in the studies cited above.

In vivo application of ALX or STZ leads to a typical triphasic time pattern of glucose levels in animals: initial hyperglycaemia is followed by temporary hypoglycaemia, which is relieved by persistent long-term hyperglycaemia [33]. The time pattern of insulin release found in our in vitro experiments corresponded to this in vivo effect: after STZ treatment (and to a lesser extent also after ALX), there was an initial phase of reduced insulin release lasting several hours, followed by a temporarily elevated insulin release. Finally, insulin secretion in response to glucose stimulation was inhibited (figs. 2, 3). These results to our knowledge for the first time indicate that the initial hyperglycaemia is not only caused by extrapancreatic influences of the diabetogens but can be explained partly by direct inhibition of insulin release.

Morphological analysis of the islets showed different types of necrotic cells. Administration of the classical diabetogens ALX or STZ caused β-cell necrosis characterised by vesicularly degenerated cytoplasm and irregu-

lar condensation of nuclear chromatin, as described earlier [34–36]. After treatment with XO/HX, the few altered β-cells showed a similar cytoplasmic appearance but divergent nuclear characteristics. Apoptosis was frequently found only following NO exposure.

We reported previously that application of the hydroxyl radical scavenger melatonin could prevent the alterations caused by ALX, but failed to influence XO/HX toxicity [31]. With STZ and SNAP, melatonin also had no protective effect (data not shown).

As discussed before, cell lysis may lead to liberation of enzymes such as superoxide dismutase or catalase, thus influencing the radical cocktails generated outside cells in incubation experiments [31]. Normally, ALX and STZ come into contact with the intracellular milieu, while radical generation driven by XO/HX takes place outside. It seems imaginable that artificial liberation and accumulation of intracellular components—which does not take place in our perifusion system—may partly neutralise this difference, thereby artificially equalising divergent effects normally resulting from the different sites of action.

According to the present investigations, that the toxicity of ALX, STZ, XO/HX, and SNAP can be reduced to a single common feature seems unlikely. Additionally, the divergent effects observed following STZ and SNAP treatment do not support the hypothesis that STZ toxicity is mainly caused by liberation of NO.

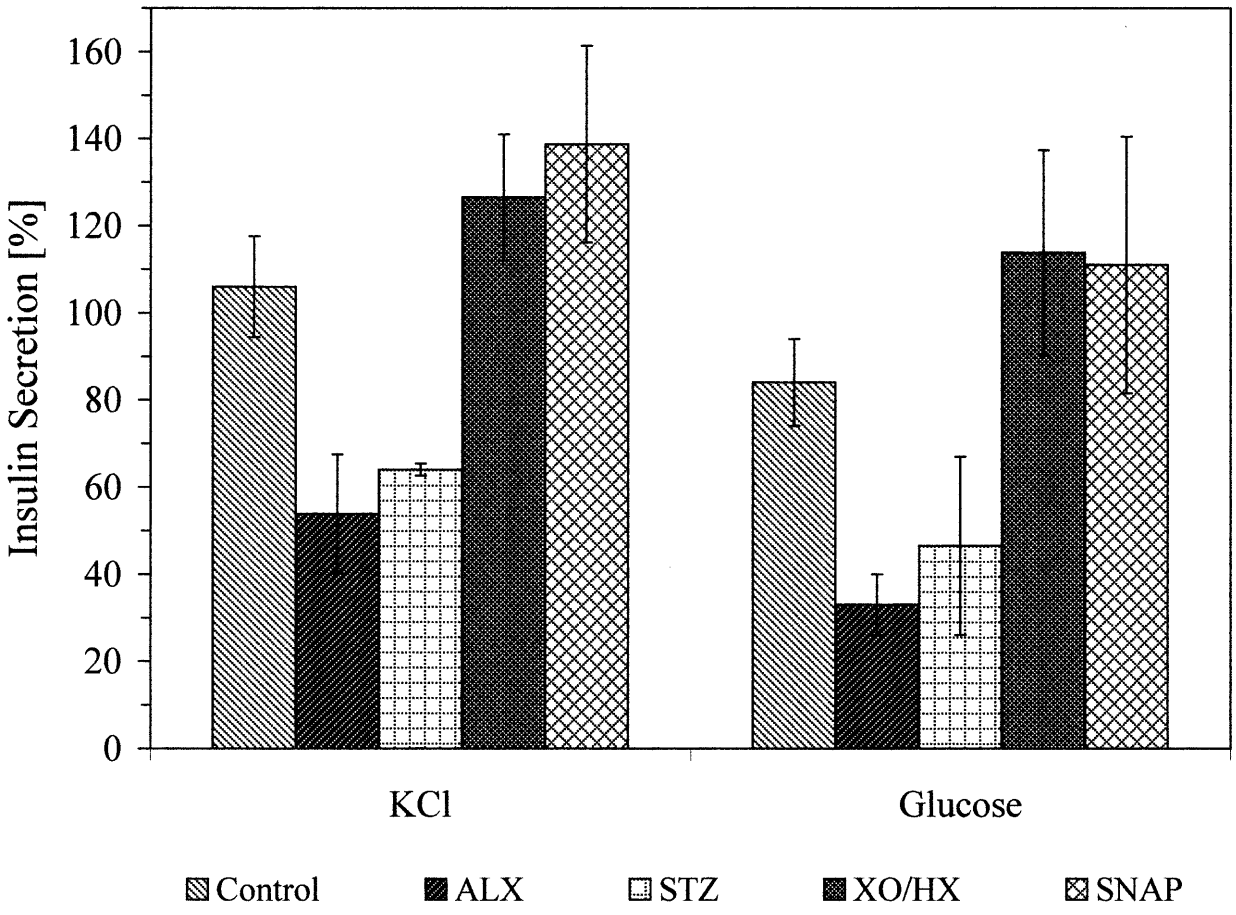

Figure 3. Insulin secretion from isolated rat pancreatic islets following standardised stimulation with KCl and glucose. Proportion of insulin release between the beginning and the end (24 h after administration of test compounds) of perifusion experiments. Control, perifusion medium only; ALX, application of 2.0 mmol/l alloxan for 9 min; STZ, application of 2.0 mmol/l streptozotocin for 9 min; XO/HX, application of 75 mU/ml xanthine oxidase and 1.5 mmol/l hypoxanthine for 9 min; SNAP, application of 2.0 mmol/l S-nitroso-N-acetyl-D,L-penicillamine for 60 min. Data corresponding to the end of the experiments are shown in figures 1 and 2. Mean $\pm$ SE values of three experiments.

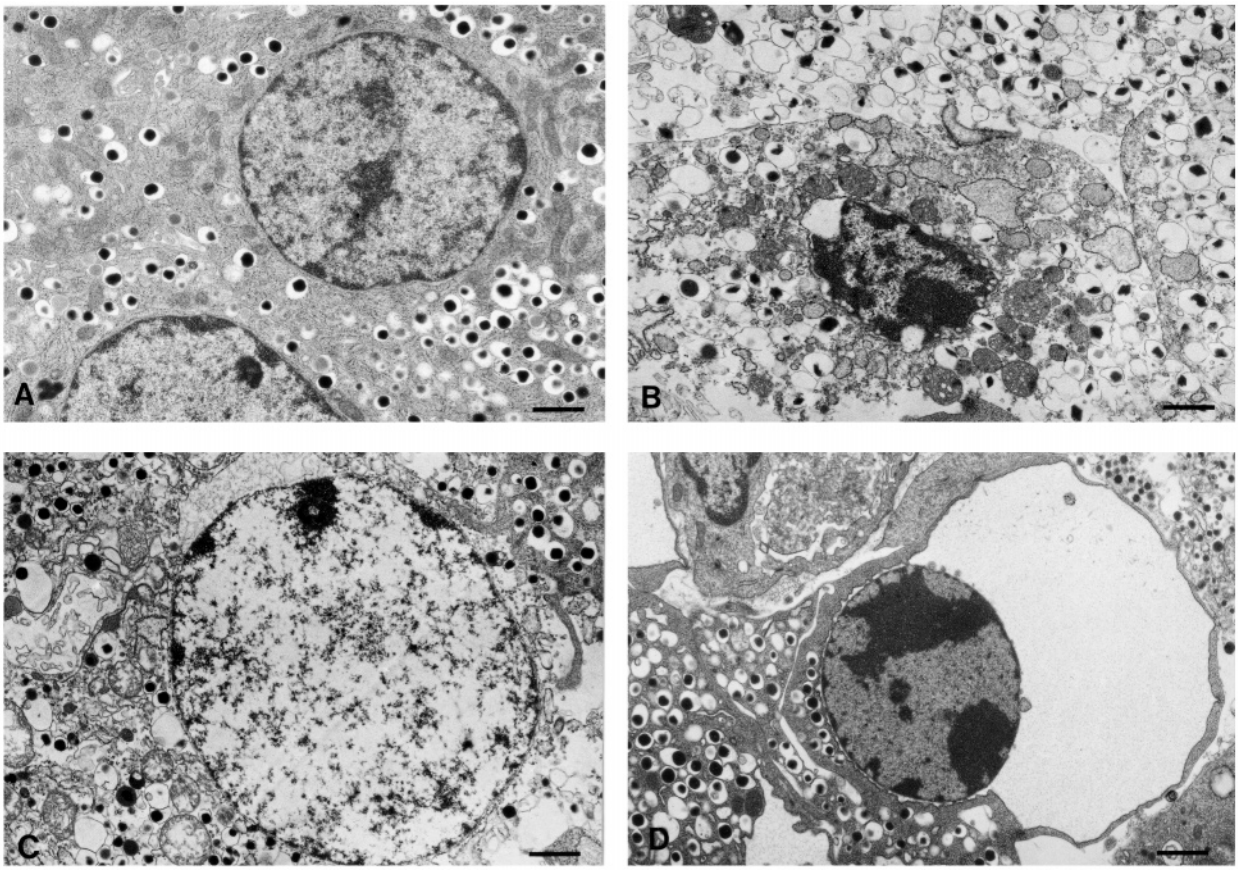

Figure 4. Electron microscopic photographs of β-cells from isolated rat pancreatic islets (bar, 1 μm). (*A*) Control. (*B*) Necrotic β-cell found after alloxan treatment (similarly damaged cells were also seen following application of streptozotocin). (*C*) Necrotic β-cell observed following treatment with xanthine oxidase/hypoxanthine. (*D*) Nucleus of apoptotic β-cell surrounded by a large intracellular vacuole, frequently found after exposure to S-nitroso-N-acetyl-D,L-penicillamine. For details see text.

Acknowledgements. The authors thank U. Engelhardt, R. Wohlrab for her skillful technical assistance, and S. Luginsland for proofreading the English of the manuscript.

1 Grankvist K., Marklund S. L. and Täljedal I. B. (1981) CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem. J. **199:** 393–398
2 Malaisse W. J., Malaisse-Lagae F., Sener A. and Pipeleers D. G. (1982) Determinants of the selective toxicity of alloxan to the pancreatic B cell. Proc. Natl. Acad. Sci. USA **79:** 927–930
3 Andersson M. and Grankvist K. (1995) Ascorbate-induced free radical toxicity to isolated islet cells. Int. J. Biochem. Cell. Biol. **27:** 493–498
4 Tiedge M., Lortz S., Drinkgern J. and Lenzen S. (1997) Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes **46:** 1733–1742
5 Gorus F. K., Malaisse W. J. and Pipeleers D. G. (1982) Selective uptake of alloxan by pancreatic B-cells. Biochem. J. **208:** 313–315
6 Munday R., Ludwig K. and Lenzen S. (1993) The relationship between the physicochemical properties and the biological effects of alloxan and several N-alkyl substituted alloxan derivatives. J. Endocrinol. **139:** 153–163
7 Zhang H. and Brunk U. T. (1993) Alloxan cytotoxicity is highly potentiated by plasma membrane- and lysosomal-associated iron – a study on a model system of cultured J-774 cells. Diabetologia **36:** 707–715
8 Brömme H. J., Ebelt H., Peschke D. and Peschke E. (1999) Alloxan acts as a pro-oxidant only under reducing conditions – influence of melatonin. Cell. Mol. Life Sci. **55:** 487–493
9 Jörns A., Munday R., Tiedge M. and Lenzen S. (1999) Effect of superoxide dismutase, catalase, chelating agents and free radical scavengers on the toxicity of alloxan to isolated pancreatic islets in vitro. Free Rad. Biol. Med. **26:** 1300–1304
10 Kröncke K. D., Fehsel K., Sommer A., Rodriguez M. L. and Kolb-Bachofen V. (1995) Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitrosourea streptozotocin contributes to islet cell DNA damage. Biol. Chem. **376:** 179–185
11 Murata M., Takahashi A., Saito I. and Kawanishi S. (1999) Site-specific DNA methylation and apoptosis: induction by diabetogenic streptozotocin. Biochem. Pharmacol. **57:** 881–887
12 Nukatsuka M., Sakurai H., Yoshimura Y., Nishida M. and Kawada J. (1988) Enhancement by streptozotocin of O_2^- radical generation by the xanthine oxidase system of pancreatic beta-cells. FEBS Lett. **239:** 295–298

13 Takasu N., Asawa T., Komiya I., Nagasawa Y. and Yamada T. (1991) Streptozotocin- and alloxan-induced H_2O_2 generation and DNA fragmentation in pancreatic islets. J. Biol. Chem. **266:** 2112–2114
14 Andrade J., Conde M., Sobrino F. and Bedoya F. J. (1993) Activation of peritoneal macrophages during the prediabetic phase in low-dose streptozotocin-treated mice. FEBS Lett. **327:** 32–34
15 Kaneto H., Fujii J., Seo H. G., Suzuki K., Matsuoka T. A., Nakamura M. et al. (1995) Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. Diabetes **44:** 733–738
16 Burkart V., Koike T., Brenner H. H. and Kolb H. (1992) Oxygen radicals generated by the enzyme xanthine oxidase lyse rat pancreatic islet cells in vitro. Diabetologia **35:** 1028–1034
17 Corbett J. A. and McDaniel M. L. (1992) Does nitric oxide mediate autoimmune destruction of β cells? Possible therapeutic interventions in IDDM. Diabetes **41:** 897–903
18 Delaney C. A., Tyrberg B., Bouwens L., Vaghef H., Hellman B. and Eizirik D. L. (1996) Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. FEBS Lett. **394:** 300–306
19 Tiedge M., Lortz S., Munday R. and Lenzen S. (1998) Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. Diabetes **47:** 1578–1585
20 Tiedge M., Lortz S., Munday R. and Lenzen S. (1999) Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. Diabetologia **42:** 849–855
21 Yoon J. I., Jun H. S. and Santamaria P. (1998) Cellular and molecular mechanisms for the initiation and progression of β cell destruction resulting from the collaboration between macrophages and T cells. Autoimmunity **27:** 109–122
22 Yamamoto H., Uchigata Y. and Okamoto H. (1981) DNA strand breaks in pancreatic islets by in vivo administration of alloxan or streptozotocin. Biochem. Biophys. Res. Commun. **103:** 1014–1020
23 Lenzen S. and Panten U. (1988) Alloxan: history and mechanism of action. Diabetologia **31:** 337–342
24 Fehsel K., Jalowy A., Qi S., Burkart V., Hartmann B. and Kolb H. (1993) Islet cell DNA is a target of inflammatory attack by nitric oxide. Diabetes **42:** 496–500
25 Heller B., Bürkle A., Radons J., Fengler E., Jalowy A., Müller M. et al. (1994) Analysis of oxygen radical toxicity in pancreatic islets at the single cell level. Biol. Chem. **375:** 597–602
26 Radons J., Heller B., Burkle A., Hartmann B., Rodriguez M. L., Kröncke K. D. et al. (1994) Nitric oxide toxicity in islet cells involves poly(ADP-ribose) polymerase activation and concomitant NAD^+ depletion. Biochem. Biophys. Res. Commun. **199:** 1270–1277
27 Heller B., Bürkle A., Radons J., Fengler E., Müller M., Burkart V. et al. (1997) DNA-damage and NAD^+-depletion are initial events in oxygen radical induced islet cell death. Adv. Exp. Med. Biol. **426:** 329–334
28 Steiner L., Kröncke K. D., Fehsel K. and Kolb-Bachofen V. (1997) Endothelial cells as cytotoxic effector cells: cytokine-activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide. Diabetologia **40:** 150–155
29 Peschke E., Peschke D., Hammer T. and Csernus V. (1997) Influence of melatonin and serotonin on glucose-stimulated insulin release from perifused rat pancreatic islets in vitro. J. Pineal Res. **23:** 156–163
30 Csernus V. J., Hammer T., Peschke D. and Peschke E. (1998) Dynamic insulin secretion from perifused rat pancreatic islets. Cell. Mol. Life Sci. **54:** 733–743
31 Ebelt H., Peschke D., Brömme H. J., Mörke W., Blume R. and Peschke E. (2000) Influence of melatonin on free radical induced changes in rat pancreatic beta-cells in vitro. J. Pineal Res. **28:** (in press)
32 Uchigata Y., Yamamoto H., Nagai H. and Okamoto H. (1983) Effect of poly(ADP-ribose) synthetase inhibitor administration to rats before and after injection of alloxan and streptozotocin on islet proinsulin synthesis. Diabetes **32:** 316–318
33 Boquist L., Boquist S. and Ericsson I. (1988) Structural beta-cell changes and transient hyperglycemia in mice treated with compounds inducing inhibited citric acid cycle enzyme activity. Diabetes **37:** 89–98
34 Boquist L. (1977) The endocrine pancreas in early alloxan diabetes. Acta Pathol. Microbiol. Scand. **85:** 219–229
35 Abdel-Rahman M. S., Elrakhawy F. I. and Iskander F. A. (1992) Protection of B cells against the effect of alloxan. Toxicol. Lett. **63:** 155–164
36 Jörns A., Munday R., Tiedge M. and Lenzen S. (1997) Comparative toxicity of alloxan, N-alkylalloxans and ninhydrin to isolated pancreatic islets in vitro. J. Endocrinol. **155:** 283–293