The potential mechanism of the diabetogenic action of streptozotocin: inhibition of pancreatic β-cell *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase

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Streptozotocin (STZ), an analogue of GlcNAc, inhibits purified rat spleen *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase (O-GlcNAcase), the enzyme that removes *O*-GlcNAc from protein. We have shown previously that STZ increases pancreatic islet O-linked protein glycosylation. In light of these data, we investigated the possibility further that STZ causes β-cell death by inhibiting O-GlcNAcase. In isolated islets, the time course and dose curve of STZ-induced O-glycosylation correlated with β-cell toxicity. STZ inhibition of rat islet O-GlcNAcase activity also paralleled that of its β-cell toxicity, with significant inhibition occurring at a concentration of 1 mM. In contrast, STZ inhibition of rat brain O-GlcNAcase and β-TC3 insulinoma cell O-GlcNAcase was significantly right-shifted compared with islets, with STZ only significantly inhibiting activity at a concentration of 5 mM, the same concentration required for β-TC3 cell toxicity. In comparison, *N*-methyl-*N*-nitrosourea, the nitric oxide-donating portion of STZ, did not cause increased islet O-glycosylation, β-cell toxicity or inhibition of β-cell O-GlcNAcase. Enhanced STZ sensitivity of islet O-GlcNAcase compared with O-GlcNAcase from other tissues or an insulinoma cell line suggests why actual islet β-cells are particularly sensitive to STZ. Confirming this idea, STZ-induced islet β-cell toxicity was completely blocked by GlcNAc, which also prevented STZ-induced O-GlcNAcase inhibition, but was not even partially blocked by glucose, glucosamine or GalNAc. Together, these data demonstrate that STZ's inhibition of β-cell O-GlcNAcase is the mechanism that accounts for its diabetogenic toxicity.

Key words: islet, O-GlcNAcase, O-glycosylation.

INTRODUCTION

For several decades, the β-cell-specific toxin streptozotocin (STZ), an analogue of GlcNAc (Figure 1), has been used to create animal models of diabetes, despite an incomplete understanding of how STZ actually causes β-cell death [1]. The ability of STZ to act as a NO donor has led many investigators to postulate that NO is involved [2–6], but the diabetogenic effect of STZ *in vivo* cannot be readily duplicated with *N*-methyl-*N*-nitrosourea (MNU, the portion of STZ that actually donates NO) [7–11]. Recently, STZ has been shown to inhibit the enzyme *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase (O-GlcNAcase), which removes *O*-GlcNAc from protein, and is thus the final enzyme in the pathway of O-glycosylation in the β-cell [12,13].

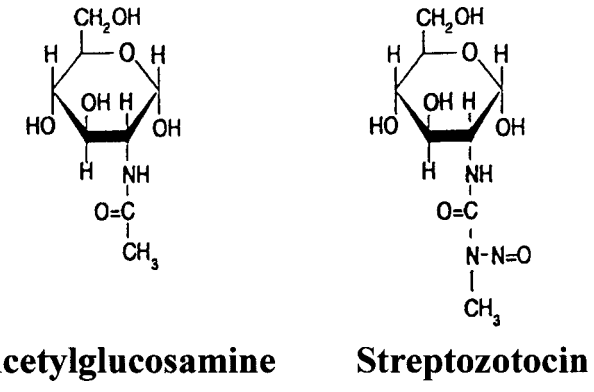

Figure 1 The chemical structures of GlcNAc and STZ

In STZ, a nitrosourea group corresponds to the acetate present in GlcNAc.

The pathway involving β-cell O-glycosylation of proteins starts with the conversion of glucose to glucose 6-phosphate and then to fructose 6-phosphate. The enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT) converts fructose 6-phosphate to glucosamine 6-phosphate [14–16], providing the UDP-GlcNAc, which is necessary for protein glycosylation [17]. In the β-cell, O-glycosylation of cytosolic and nuclear proteins occurs with O-linkage of the monosaccharide GlcNAc to proteins at serine and threonine residues [18–27]. Pancreatic β-cells have been proposed to be selectively sensitive to STZ because the enzyme responsible for transferring *O*-GlcNAc to proteins, O-GlcNAc transferase (OGT) [28,29], is expressed at higher levels in the β-cell than in any other cell [12,13,30], although this view has been challenged by at least one group [31].

We have shown previously that STZ causes increased O-glycosylation in isolated islets [32,33]. In light of these data, we have investigated the possibility that STZ's inhibition of islet O-GlcNAcase is the mechanism that accounts for its diabetogenic toxicity.

MATERIALS AND METHODS

Islet isolation

In a typical experiment, islets were isolated aseptically from three or four male Sprague–Dawley rats as described previously [32]. Islets were isolated using Hanks balanced salt solution (HBSS)

Abbreviations used: STZ, streptozotocin; MNU, *N*-methyl-*N*-nitrosourea; OGT, O-GlcNAc transferase; O-GlcNAcase, *O*-GlcNAc-selective *N*-acetyl-β-D-glucosaminidase; HBSS, Hanks balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; H&E, haematoxylin and eosin.

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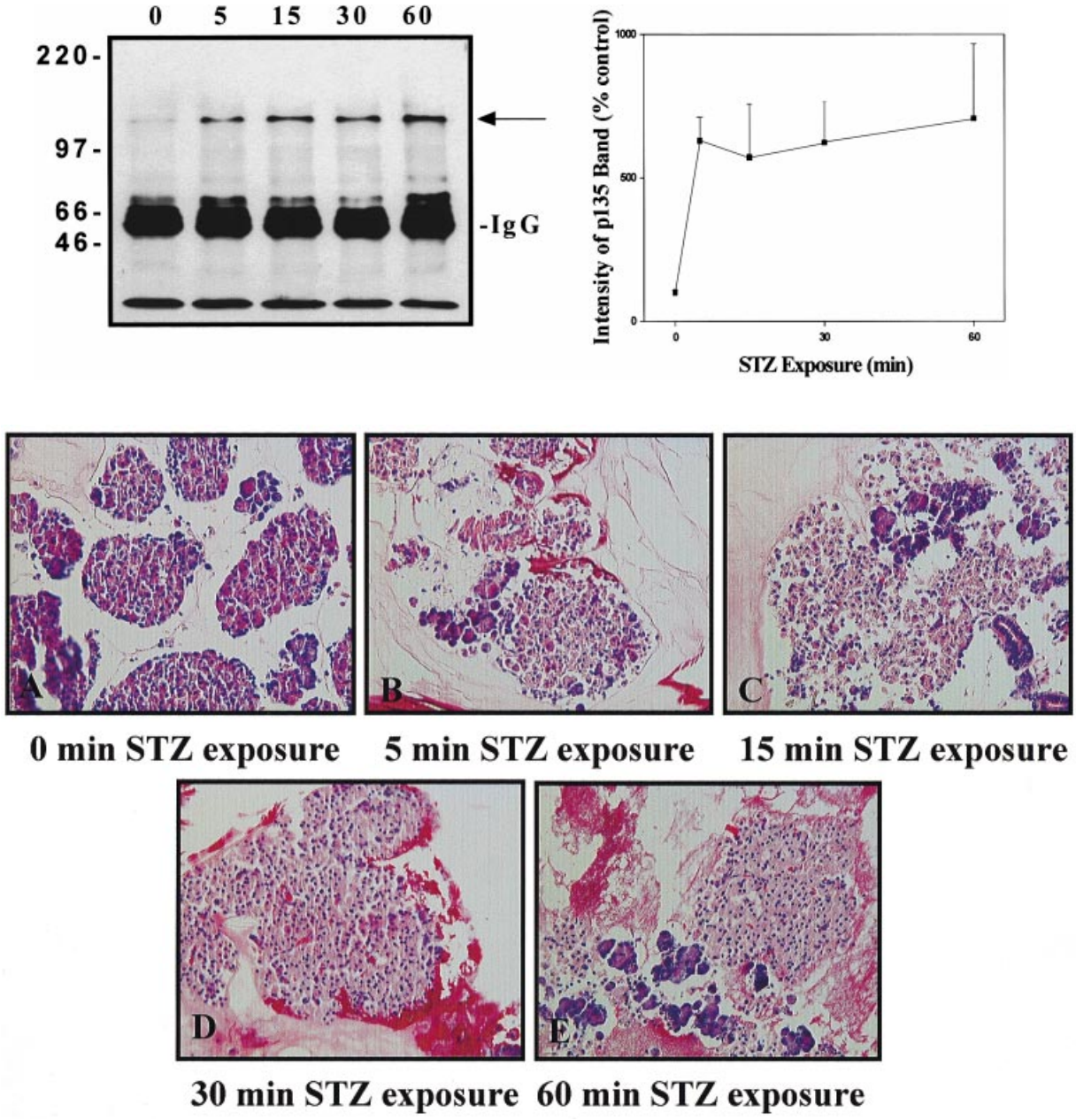

Figure 2 Time course of STZ-induced O-glycosylation and histological β-cell toxicity

(Top-left panel) Islets (100 per condition) were incubated in 3 mM glucose + 5 mM STZ for 0–60 min. Following incubation with STZ, supernatant was removed from the tubes and islets were incubated with 3 mM glucose. Total incubation time for each tube was 90 min. At the end of the experiment, supernatant was removed from the tubes and O-glycosylated proteins were immunoprecipitated with RL2 antibody, separated and transferred on to nitrocellulose for RL2 Western blotting. (Top-right panel) Data corresponding to the top-left panel in which the intensity of the p135 band is shown as the mean ± S.E.M. from four independent sets of observations from two separate experiments (two independent sets of observations per experiment). (Bottom panels) Islets (100 per condition) were incubated in 3 mM glucose + 5 mM STZ for 0–60 min. After incubation with STZ, supernatant was removed, and islets were incubated with 3 mM glucose for an additional 5.5 h. At the end of the experiment, supernatant was removed, and islets were fixed in formalin. Islets were placed into cell blocks, which were sectioned and stained with H&E. Results shown are from one experiment representative of two separate experiments.

and Ficoll supplemented with 5.5 mM glucose, 1 mM L-glutamine, 25 units/ml penicillin and 25 units/ml streptomycin. During surgery, the common bile duct of each pancreas was cannulated, and the pancreas was inflated with 10–20 ml of HBSS. The distended pancreas was excised, and lymph nodes, fat, blood vessels and bile ducts were removed under a stereo dissecting microscope. Tissue was chopped extensively, rinsed five or six times with HBSS, and digested with collagenase P (3 mg/ml tissue) at 37 °C for 3–4 min. Digested tissue was rinsed three or four times with HBSS, and islets were purified on a discontinuous Ficoll gradient consisting of 27 %, 23 %, 20.5 % and 11 % Ficoll in 25 mM Hepes/HBSS buffer. Islets were harvested and washed once with HBSS and six times in the appropriate incubation buffer. This isolation procedure typically provided 400–500 islets per rat, which were used as described below.

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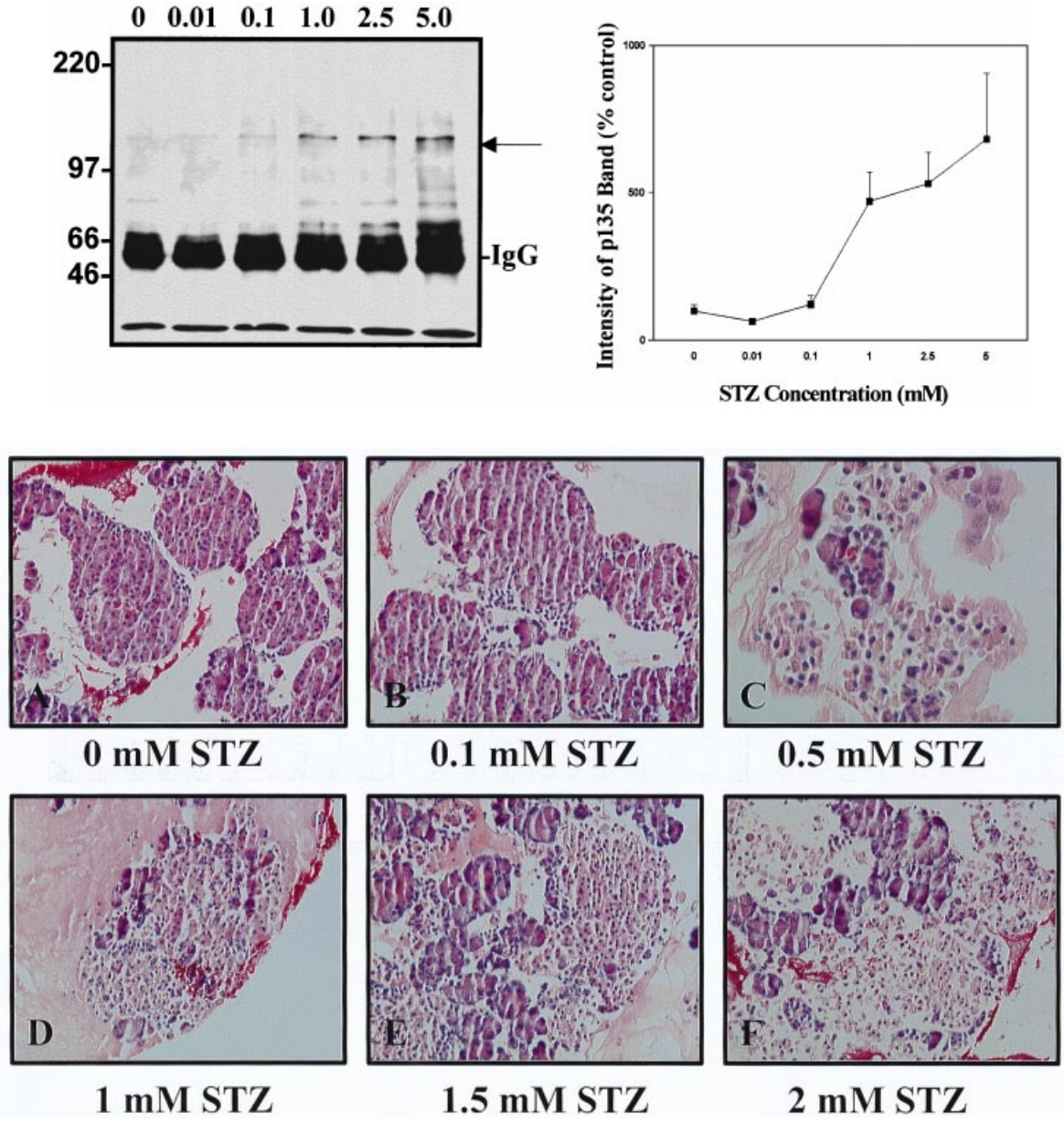

Figure 3 Dose curve of STZ-induced O-glycosylation and β-cell toxicity

(Top-left panel) Islets were pre-incubated in 3 mM glucose supplemented with 0–5 mM STZ for 60 min. Following pre-incubation, supernatant was removed and islets were incubated with 3 mM glucose for an additional 30 min. At the end of the experiment, O-glycosylated proteins were analysed as described in Figure 2. (Top-right panel) Data corresponding to the top-left panel in which the intensity of the p135 band is shown as the mean $\pm$ S.E.M. from four independent sets of observations from two separate experiments (two independent sets of observations per experiment). (Bottom panels) Isolated islets (100 per condition) were incubated in 3 mM glucose + 0–2 mM STZ for 30 min. Supernatant was removed from the tubes, and islets were incubated with 3 mM glucose for an additional 5.5 h. At the end of the experiment, islets were processed as in Figure 2. Results shown are from one experiment representative of two separate ones.

Incubation of islets for insulin secretion and protein O-glycosylation

Freshly isolated islets were washed six times with Krebs/Hepes buffer (25 mM Hepes, pH 7.4/115 mM NaCl/24 mM $NaHCO_3$/5 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$) supplemented with 0.1 % BSA, 3 mM glucose and 1 mM L-glutamine. Islets (100 per condition) were placed into siliconized 16 mm $\times$ 100 mm round-bottomed screw-cap tubes. Pre-incubation and all subsequent incubations were performed at 37 °C under an atmosphere of 95 % O_2/5 % CO_2. After pre-incubation buffer was aspirated, islets were incubated with Krebs/Hepes buffer supplemented with the appropriate compound. At the end of the experiment, supernatants were removed from the tubes for insulin RIA, and islets were processed as described below [32,33]. For data analysis, insulin secretion in the control sample was set at 100 %, and all other results were expressed as a percentage of the control.

Processing of islets for immunoprecipitation of O-glycosylated proteins

At the end of the experiment, all supernatant was removed from the tubes. Ice-cold lysis buffer (1 ml; 50 mM Hepes, pH 7.4/150 mM NaCl/1 % Triton X-100/5 mM EDTA/5 mM EGTA/

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20 mM NaF/20 mM $Na_4P_2O_7$/1 mM $NaVO_4$/1 mg/ml bacitracin/1 mM PMSF/1 μg/ml leupeptin/1 μg/ml aprotinin) was added to each tube. Borosilicate tubes containing islets were vortexed for 30 s, placed on ice for 30 min, and vortexed for an additional minute before transfer to 1.5 ml conical screw-cap Eppendorf tubes. All subsequent immunoprecipitation steps were performed at 4 °C. Samples were centrifuged at 10000 ***g*** for 15 min. The supernatant was transferred to a second 1.5 ml conical Eppendorf tube, and O-glycosylated proteins were immunoprecipitated for 2 h on a rocker with 1 μl of mouse monoclonal RL2 antibody, expressed from a hybridoma (RL2 antibody is also available commercially from Affinity Bioreagents, Golden, CO, U.S.A.). This antibody selectively binds O-glycosylated protein [12,13,32,33], and immunoprecipitation of O-glycosylated proteins by RL2 is specifically blocked by GlcNAc [32]. After 2 h, 20 μl of Protein A trisacryl beads (Pierce) pre-adsorbed with 20 μg of rabbit anti-mouse antibody (Sigma) were added to the tubes and the incubation was continued overnight. At the end of the incubation, beads were washed once with wash buffer 1 (150 mM NaCl/10 mM Hepes, pH 7.4/1 % Triton X-100/0.1 % SDS) and once with wash buffer 2 (10 mM Hepes, pH 7.4/1 % Triton X-100/0.1 % SDS). After the final washing step, 25 μl of 2 × sample buffer (100 mM Tris, pH 6.8/4 % SDS/20 % glycerol/20 μg/l Bromophenol Blue) was added to each tube. Samples were vortexed for 30 s, boiled for 5 min, vortexed for an additional 30 s and stored at −20 °C prior to subsequent analysis.

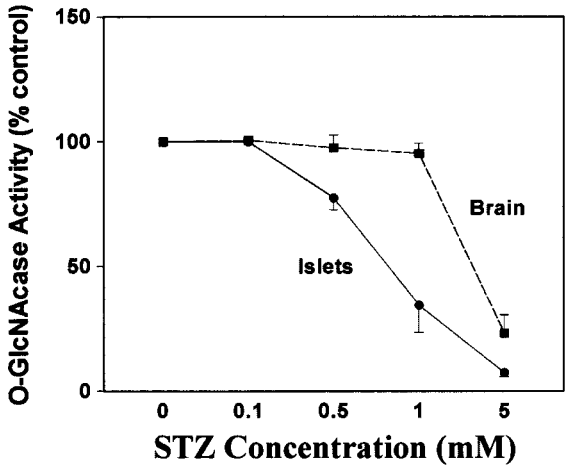

Figure 4 Dose curves of STZ inhibition of rat islet versus rat brain O-GlcNAcase

Isolated rat islets or rat brain were homogenized and centrifuged at 55000 ***g*** to obtain O-GlcNAcase-rich cytosol, which was incubated with 0–5 mM STZ for 30 min. Afterwards, samples were incubated with 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide for 1 h. The reaction was stopped with the addition of 0.5 M sodium carbonate, and colour was measured spectrophotometrically at 400 nm. Data are shown as the means ± S.E.M. from two independent sets of observations from two separate experiments (one independent set of observations per experiment).

Western blotting

Samples were loaded on to SDS/polyacrylamide gels (7.5 %). Coloured molecular-mass markers (Amersham) were run on each gel. Proteins were separated for 1 h at 175 V at room temperature using a Bio-Rad Mini-PROTEAN II dual-slab cell. Proteins were transferred to ECL nitrocellulose paper (Amersham) for 1.5 h (100 V, 4 °C). Nitrocellulose blots were blocked for 1 h at room temperature in blocking buffer (5 % BSA in 10 mM Tris, pH 7.4/150 mM NaCl/0.1 % sodium azide/0.05 % Tween 20). After blocking, the blots were probed with RL2 antibody (1:1000 dilution in blocking buffer) for 1 h at room temperature. Blots were washed six times (5 min each) with TBST (10 mM Tris, pH 7.4/150 mM NaCl/0.05 % Tween 20). After washing, blots were probed with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) at a 1:1500 dilution in TBST for 1 h at room temperature. Blots were washed again and developed with ECL reagent (Amersham). After air-drying, blots were exposed to Bio-Max X-ray film (Kodak). For each experiment, the intensity of the control sample was set at 100 % and all other results were expressed as a percentage of the control.

Incubation of islets for histological analysis

Freshly isolated islets were washed six times in complete Dulbecco's modified Eagle's medium (DMEM) containing 3 mM glucose, 1 mM L-glutamine and 10 % fetal calf serum. Islets (100 per condition) were placed into siliconized 16 mm × 100 mm round-bottomed screw-cap tubes. Pre-incubation and all subsequent incubations were performed at 37 °C under an atmosphere of 95 % O_2/5 % CO_2. After pre-incubation with DMEM supplemented with the appropriate compound(s), supernatant was aspirated, and islets were incubated with DMEM supplemented with the appropriate compound(s). At the end of the experiment, supernatant was removed, and islets were fixed in 1 ml of buffered formalin for 30 min. Following fixation, islets were placed into cell blocks [34], which were stained with 10 μl of tissue stain for 5 min, and washed twice with 1 ml of buffered formalin. Afterwards, complete cell blocks were fixed in formalin in tissue cassettes and embedded into paraffin for histological sectioning. After sectioning, slides were stained with haematoxylin and eosin (H&E) to assess islet morphology microscopically, and images were captured using a digital camera. In selected experiments, in addition to H&E staining, immunohistochemical staining was performed with M30 cytoDEATH antibody (Roche/Boehringer Mannheim, Indianapolis, IN, U.S.A.), which specifically detects a caspase-cleavage product that is an early marker of apoptosis in epithelial-derived cells, including those of the exocrine and endocrine pancreas [35–37].

β-TC3 cell culture and incubation for histological analysis

β-TC3 cells were obtained from the University of Pennsylvania Diabetes Center (Philadelphia, PA, U.S.A.) courtesy of Dr D. Hanahan (University of California, San Francisco, CA, U.S.A.). The cells were cultured in 10 cm dishes in the presence of RPMI 1640 medium supplemented with 10 % fetal bovine serum, 75 μg/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine as described previously [32]. Cells were trypsinized and subcloned weekly. Media were changed twice weekly and on the day prior to an experiment, and cells were used between passages 38 and 60. In each experiment, pre-incubation and all subsequent incubations were performed in 10 cm dishes at 37 °C under an atmosphere of 95 % air/5 % CO_2. After incubation with DMEM supplemented with the appropriate compound(s), cells in dishes were photographed using a digital camera.

Measurement of O-GlcNAcase activity

Isolated islets were washed six times with Krebs/Hepes buffer supplemented with 0.1 % BSA, 3 mM glucose and 1 mM L-glutamine. Islets were then washed twice with ice-cold PBS

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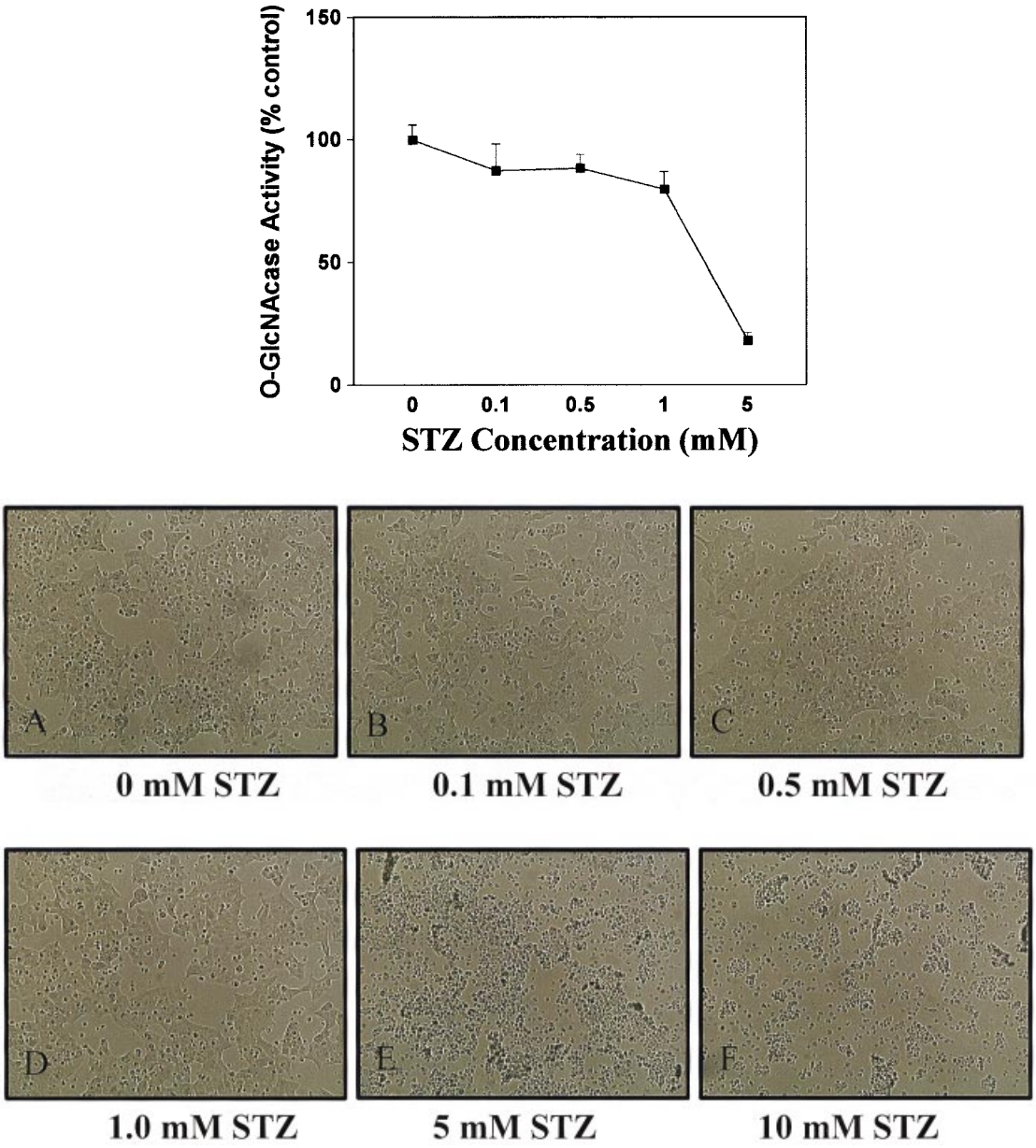

Figure 5 Dose curve of STZ inhibition of β-TC3 O-GlcNAcase and STZ-induced toxicity

(Top panel) β-TC3 insulinoma cells were homogenized and centrifuged at 55000 ***g*** to obtain O-GlcNAcase-rich cytosol, which was incubated with 0–5 mM STZ for 30 min. Following incubation with STZ, O-GlcNAcase activity was measured as in Figure 4. Data are shown as the means $\pm$ S.E.M. from four independent sets of observations from two separate experiments (two independent sets of observations per experiment). (Bottom panels) β-TC3 insulinoma cells grown in 10 cm dishes in monolayers were incubated in 0–10 mM STZ for 6 h. Afterwards, cells were examined microscopically for morphological changes. Results shown are from one experiment representative of two separate ones.

supplemented with 0.1% BSA and placed in a homogenizing tube. Supernatant was removed, and islets were homogenized on ice in 20 mM Na_2HPO_4, pH 7.7, containing 10 mM $MgCl_2$, 15 mM mercaptoethanol and 1 mM PMSF. In the case of rat brain or β-TC3 cells, homogenization followed two washes with ice-cold PBS supplemented with 0.1% BSA. After homogenization, samples were centrifuged at 55000 ***g*** for 30 min to obtain O-GlcNAcase-rich cytosol, which was incubated first with 0–5 mM STZ for 30 min and then with 50 mM sodium cacodylate, pH 7.0, containing 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide for 1 h. At the end of the incubation, the reaction was stopped with the addition of 0.5 M sodium carbonate. Samples were placed on ice, vortexed and colour was measured spectrophotometrically at 400 nm with non-specific activity blanks routinely subtracted. Using this method, one measurement of O-GlcNAcase activity could be performed for each 400–500 islets processed. Because of the large number of islets required for this assay, in selected experiments β-TC3 cells or rat brain were used as a source of O-GlcNAcase activity. In each experiment performed, control samples containing no STZ were considered to have 100% activity, and all values were expressed as a percentage of the control.

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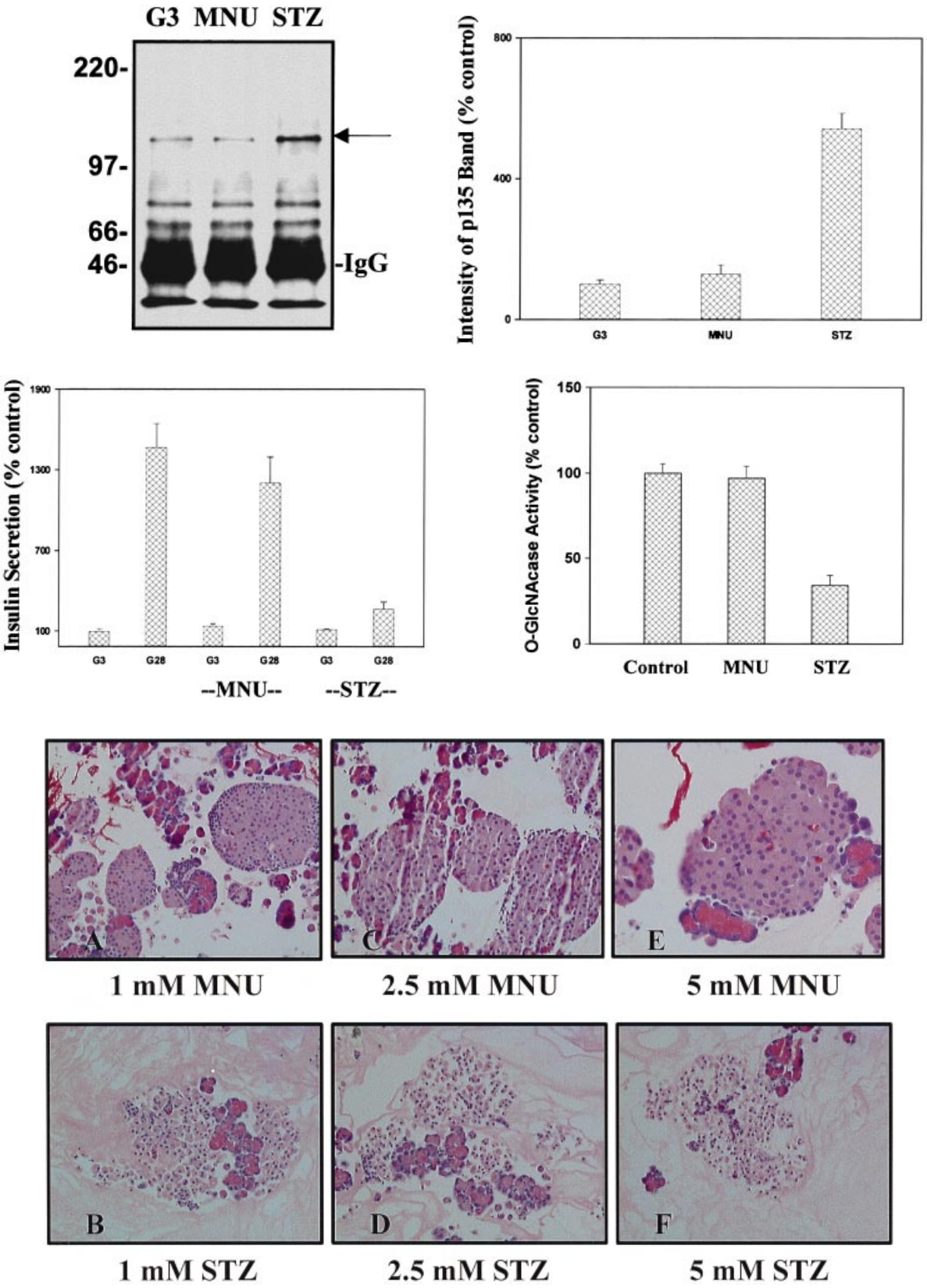

Figure 6 MNU, the NO-donating portion of STZ, does not cause increased islet O-glycosylation, β-cell toxicity or inhibition of β-cell O-GlcNAcase

(Top-left panel) Following a 30 min pre-incubation in 3 mM glucose, islets (100 per condition) were incubated in 3 mM glucose (G3), 3 mM glucose + 5 mM MNU or 3 mM glucose + 5 mM STZ for 60 min. Afterwards, O-glycosylated proteins were analysed as in Figure 2. (Top-right panel) Data corresponding to the top-left panel in which the intensity of the p135 band is shown as the mean ± S.E.M. from eight independent sets of observations from two separate experiments (four independent sets of observations per experiment). (Middle-left panel) Islets (25 per condition) were pre-incubated for 30 min in 3 mM glucose, 3 mM glucose + 5 mM STZ or 3 mM glucose + 5 mM MNU for 30 min. Afterwards, islets were incubated for 60 min in either 3 mM glucose

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Data analysis

Films and slides were photographed using a digital camera. Intensities of bands were quantitated using the program NIH Image. Results are expressed as means ± S.E.M., using the Windows-compatible version 2.98 of the program FigP (Biosoft, St. Louis, MO, U.S.A.). Statistical analysis was performed using the same program. Data were analysed by ANOVA followed by multiple comparisons between the means using the least-significant-difference test. A probability of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

To determine the time course of STZ-induced O-glycosylation, islets were exposed to 3 mM glucose + 5 mM STZ for 0–60 min. Islets were then incubated in 3 mM glucose to bring the total incubation time to 90 min to allow O-glycosylated protein to accumulate. Figure 2 (top-left panel) shows results from a representative experiment. Immunoprecipitation and subsequent Western blotting with RL2 antibody demonstrated that O-glycosylation of a 135 kDa protein (p135) was increased significantly after islets were exposed to STZ for as little as 5 min (628 ± 84 %) compared with the 3 mM glucose control ($P < 0.05$ versus control; Figure 2, top-right panel). Exposure to STZ for longer periods of time also resulted in significantly increased O-glycosylation compared with the control but not compared with O-glycosylation caused by a 5 min STZ exposure, suggesting that the effect of STZ on O-glycosylation is maximal and irreversible after 5 min.

As Figure 2 (bottom panels) demonstrates, the time course of STZ exposure required for increased O-glycosylation corresponded with that required for histological β-cell toxicity. After 6 h following a 5–60 min STZ treatment, large numbers of pyknotic nuclei become apparent in the islets, and cytoplasmic degeneration was widespread. Additional changes noted included loss of nuclei, absence of cohesion of individual cells in the islets, and loss of cytoplasmic eosinophilia. Immunohistochemical staining for apoptosis of islets 6 h after a 30 min exposure to STZ showed no significantly increased apoptosis compared with control non-STZ-treated islets, confirming that the mechanism of high-dose STZ-induced β-cell death *in vitro* involves necrosis rather than apoptosis, consistent with previous reports [38,39].

To determine the dose curve of STZ on O-glycosylation, isolated islets were exposed to 0–5 mM STZ for 60 min. Following exposure to STZ, islets were returned to 3 mM glucose for an additional 30 min to allow O-glycosylated proteins to accumulate. Figure 3 (top-left panel) shows results from a representative experiment in which O-glycosylation was significantly increased after islets were exposed to 1 mM STZ (471 ± 99 %) compared with the 3 mM glucose control ($P < 0.05$; Figure 3, top-right panel). Exposure to higher concentrations of STZ also resulted in significant increases in O-glycosylation compared with the 3 mM glucose control, with the maximal effect being a 684 ± 223 % increase observed at a concentration of 5 mM STZ ($P < 0.05$ versus control). Figure 3 (bottom panels) demonstrates that the dose curve of STZ exposure required for increased O-glycosylation corresponded with that required for histological β-cell toxicity, with similar histological toxicity to that in Figure 2 (bottom panels) noted at a dose of 0.5–1.0 mM STZ.

We next performed a dose curve for STZ inhibition of islet O-GlcNAcase. As Figure 4 shows, STZ inhibited islet enzyme activity by 23 ± 5 % at a concentration of 0.5 mM ($P < 0.05$ versus 0 mM STZ control) and by 66 ± 11 % at a concentration of 1 mM ($P < 0.05$ versus 0 mM STZ control). This dose curve of STZ inhibition of islet O-GlcNAcase paralleled that of STZ-induced β-cell toxicity observed *in vitro* (Figure 2, top-right panel). Figure 4 also shows that STZ failed to inhibit brain O-GlcNAcase activity at a concentration of 1 mM and only significantly inhibited activity at 5 mM, similar to what has been reported previously for the dose curve of STZ inhibition of purified rat spleen O-GlcNAcase [12].

In the case of an insulinoma cell line (β-TC3 cells) the dose curve of STZ inhibition of O-GlcNAcase corresponded to that of brain O-GlcNAcase (Figure 5, top panel). Significant inhibition occurred only at a concentration of 5 mM STZ (82 ± 3 %, $P < 0.05$ versus 0 mM STZ control). This dose curve corresponded with that of STZ-induced histological toxicity to the β-TC3 cells (Figure 5, bottom panels), suggesting that O-GlcNAcase inhibition correlates with toxicity in clonal β-TC3 cells as well as in actual islets.

We next investigated whether the above effects of STZ could also be caused by its NO-donating, non-diabetogenic aglucone moiety MNU. To determine if increased O-glycosylation could be caused by MNU, islets were incubated for 60 min in 3 mM glucose, 3 mM glucose + 5 mM STZ or 3 mM glucose + 5 mM MNU. Afterwards, O-glycosylated proteins were analysed as in Figure 2. As shown in Figure 6 (top panels), STZ increased p135 O-glycosylation 542 ± 45 % compared with 3 mM glucose alone ($P < 0.05$), while MNU was without significant effect.

To examine the effect of STZ and MNU on subsequent glucose-induced insulin secretion, isolated islets were pre-incubated for 30 min in 3 mM glucose, 3 mM glucose + 5 mM STZ or 3 mM glucose + 5 mM MNU. Islets were then stimulated with either 3 or 28 mM glucose, and insulin release measured (Figure 6, middle-left panel). Exposure to 28 mM glucose caused a 1470 ± 27 % increase in insulin secretion compared with 3 mM glucose control ($P < 0.05$). Pre-incubation with STZ caused a drastic reduction in 28 mM glucose-induced insulin secretion (265 ± 52 % compared with the control, $P < 0.05$), whereas MNU caused no significant inhibition insulin release.

When isolated islets were pre-incubated for 30 min in 3 mM glucose, 3 mM glucose + 1–5 mM STZ or 3 mM glucose + 1–5 mM MNU, and then incubated for 5.5 h in 3 mM glucose, only STZ caused β-cell death (Figure 6, bottom panels). Similar to the insulin-secretion data, islets exposed to 5 mM MNU exhibited no adverse histological changes compared with the control. In contrast, islets exposed to 1 mM STZ exhibited pyknotic nuclei, cytoplasmic degeneration, loss of some nuclei and decreased cytoplasmic eosinophilia, with similar changes noted after exposure to 2.5 mM and 5 mM STZ (Figure 6, bottom panels). Consistent with its inability to cause histological β-cell toxicity *in vitro*, MNU also failed to inhibit β-cell O-GlcNAcase *in vitro*. As Figure 6 (middle-right panel) shows, 5 mM MNU caused no

(G3) or 28 mM glucose (G28). At the end of the experiment, supernatants were removed from the tubes for insulin RIA. Results are shown as the means ± S.E.M. from eight independent sets of observations from two separate experiments (four independent sets of observations per experiment). (Bottom panels) Islets (100 per condition) were pre-incubated for 30 min in either 3 mM glucose + 1–5 mM STZ or 3 mM glucose + 1–5 mM MNU for 30 min. Afterward, islets were incubated for 5.5 h in 3 mM glucose. At the end of the experiment, islets were processed as in Figure 2 (bottom panels). Data shown are from one experiment representative of two separate experiments. (Middle-right panel) β-TC3 cells were homogenized and centrifuged to obtain O-GlcNAcase-rich cytosol, which was incubated in the presence or absence of 5 mM STZ or 5 mM MNU for 30 min. Afterwards, O-GlcNAcase activity was measured as in Figure 4. Data are shown as the means ± S.E.M. from four independent sets of observations from two separate experiments (two independent sets of observations per experiment).

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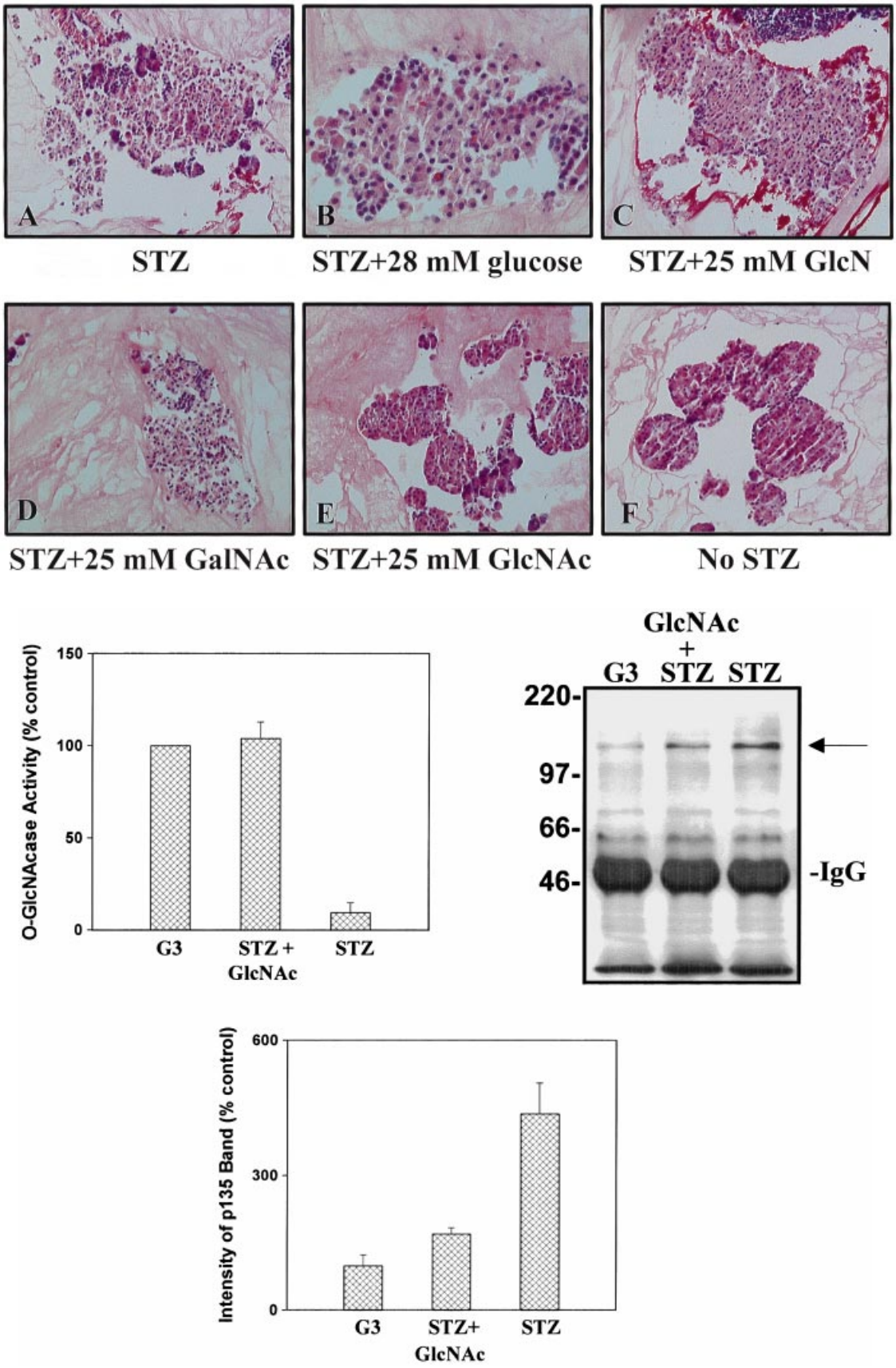

Figure 7 STZ induced β-cell death *in vitro* is blocked by GlcNAc but not by glucose, glucosamine or GalNAc

(Top panels) Islets were incubated for 30 min in 1 mM STZ in the presence of 3 mM glucose (STZ), 28 mM glucose, 3 mM glucose + 25 mM glucosamine (GlcN), 3 mM glucose + 25 mM GalNAc or 3 mM glucose + 25 mM GlcNAc. Afterwards, islets were incubated in 3 mM glucose for an additional 5.5 h. At the end of the experiment, islets were processed as in Figure 2 (bottom panels).

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significant inhibition of β-cell O-GlcNAcase activity, whereas 5 mM STZ inhibited activity by $66 \pm 6\%$ compared with the control ($P < 0.05$).

Data shown in Figures 1–5 demonstrated that β-cell toxicity corresponded to O-GlcNAcase inhibition and subsequent accumulation of O-glycosylated protein. In light of these data, we investigated the relationship between O-GlcNAcase inhibition and subsequent β-cell toxicity further. Isolated islets were incubated for 30 min with STZ in the presence of 3 mM glucose, 28 mM glucose, 3 mM glucose + 25 mM glucosamine, 3 mM glucose + 25 mM GalNAc or 3 mM glucose + 25 mM GlcNAc. Afterwards, islets were incubated for an additional 5.5 h in 3 mM glucose and then examined for histological signs of β-cell toxicity. Figure 7 (top panels) shows the results from this series of experiments. STZ toxicity was completely blocked by GlcNAc but was not even partially blocked by glucose, glucosamine or GalNAc, each of which failed to prevent histological toxicity characterized by pyknotic nuclei, cytoplasmic degeneration, decreased eosinophilia and the disappearance of some nuclei.

Figure 7 (middle-left panel) confirms that the addition of GlcNAc to STZ completely blocks the ability of STZ to inhibit islet O-GlcNAcase activity. In the presence of GlcNAc STZ caused no significant inhibition of O-GlcNAcase activity, whereas in the absence of GlcNAc STZ reduced O-GlcNAcase activity to $10 \pm 5\%$ of the control ($P < 0.05$). In order to determine if GlcNAc could also prevent STZ-induced irreversible O-glycosylation, islets were pre-incubated for 30 min in 3 mM glucose (G3), 3 mM glucose + 1 mM STZ or 3 mM glucose + 25 mM GlcNAc + 1 mM STZ. Islets were then washed three times in 3 mM glucose to remove any excess GlcNAc and incubated for an additional 60 min in 3 mM glucose to allow any increased reversible O-glycosylation caused by GlcNAc itself to diminish, before analysing O-glycosylated proteins. As Figure 7 (middle-right and bottom panels) demonstrates, GlcNAc largely blocked the ability of STZ to cause irreversibly increased O-glycosylation, consistent with its ability to protect O-GlcNAcase activity from STZ. When GlcNAc and STZ were added together for 30 min, removed and islets were allowed to incubate for an additional hour in 3 mM glucose, O-glycosylation was increased only $169 \pm 13\%$ compared with the control. In contrast, in the absence of GlcNAc, a 30 min exposure to STZ followed by removal of STZ and incubation of the islets in 3 mM glucose irreversibly increased O-glycosylation by $436 \pm 67\%$ compared with the control ($P < 0.05$). Together, the data in Figure 7 demonstrate that O-GlcNAcase inhibition is the mechanism by which STZ causes β-cell toxicity and death.

DISCUSSION

The above results demonstrate that STZ increases pancreatic islet O-linked protein glycosylation in a dose-dependent, irreversible fashion. The time course and dose curve of histological STZ toxicity in islets both correlated with those of STZ-induced O-glycosylation. Additionally, STZ significantly inhibited rat islet O-GlcNAcase activity in a dose-dependent manner that correlated with its histological β-cell toxicity, suggesting that accumulation of O-glycosylated protein is a result of O-GlcNAcase inhibition. In contrast, the dose curves of STZ inhibition of rat brain or β-TC3-cell O-GlcNAcase activities were markedly right-shifted compared with that of rat islets. In the case of β-TC3 insulinoma cells, this inhibition paralleled STZ-induced toxicity. The effects of STZ on O-glycosylation, β-cell toxicity and O-GlcNAcase activity were not observed with MNU, the NO-donating portion of STZ. Confirming the idea that STZ-induced β-cell toxicity is due to O-GlcNAcase inhibition in isolated islets, STZ toxicity was blocked completely with GlcNAc (which blocked STZ-induced O-GlcNAcase inhibition) but was not even partially blocked by glucose, glucosamine or GalNAc.

For several decades, a dose of 50–100 mg of STZ/kg administered to a rat has been known to cause β-cell death within 8 h and the subsequent development of diabetes [7–11]. As shown in Figure 1, STZ resembles GlcNAc and contains a nitrosourea group that can release a molecule of NO [1]. In light of the fact that the O-GlcNAcase enzyme contains a free thiol group in its active site [40], one possibility may be that STZ acts as a suicide substrate by S-nitrosylating the enzyme [41–44].

The mechanism by which STZ causes diabetes has never been well understood, but it has recently been proposed that pancreatic β-cells are selectively sensitive to STZ, because they contain much more OGT (the enzyme responsible for attaching *O*-GlcNAc to proteins) than any other cell type [12,13,30], although one group has challenged this view [31]. Our current data suggest that in addition to the high level of OGT expression in pancreatic β-cells, normal β-cells also contain an O-GlcNAcase that is more sensitive to inhibition by STZ than O-GlcNAcase from other tissues or even from an insulinoma cell line. This combination of high OGT expression and an STZ-sensitive O-GlcNAcase appears to render β-cells selectively sensitive to this toxin. These observations also explain why STZ toxicity *in vivo* is exacerbated by administration of glucose after STZ treatment as well as why it is ameliorated by administration of non-metabolizable analogues of glucose after STZ treatment [13,45–47]. It is unclear at this time why β-cells should express O-GlcNAcase with heightened sensitivity to STZ, but possibilities include a different isoform of the enzyme or selective post-translational processing. Addressing these and other possibilities, however, will probably have to wait until the O-GlcNAcase enzyme is cloned.

What is clear from our data, though, is that STZ toxicity can be blocked completely by the co-administration of GlcNAc, which negates the ability of STZ to inhibit O-GlcNAcase and cause irreversibly increased O-glycosylation, presumably by providing substrate protection for the enzyme. Therefore, in experiments where GlcNAc and STZ were added simultaneously, it appears that GlcNAc protects O-GlcNAcase from acute and irreversible STZ inhibition. In these experiments STZ and GlcNAc were added together for a short period of time and then removed from the islets, thus allowing resumption of the normal

Of all compounds tested, only GlcNAc prevented STZ-induced β-cell death. Data shown are from one experiment representative of two separate experiments. (Middle-left panel) To determine if GlcNAc prevented STZ inhibition of islet O-GlcNAcase, islets were incubated for 30 min in 3 mM glucose (G3), 3 mM glucose + 1 mM STZ (STZ) or 3 mM glucose + 25 mM GlcNAc + 1 mM STZ (GlcNAc + STZ). Afterward, islets were washed three times in Krebs/Hepes buffer and O-GlcNacase activity was measured as in Figure 4. Data are shown as the means $\pm$ S.E.M. from two independent sets of observations from two separate experiments (one independent set of observations per experiment). (Middle-right panel) To determine if GlcNAc could also prevent STZ-induced irreversible O-glycosylation, islets were pre-incubated for 30 min in 3 mM glucose, 3 mM glucose + 1 mM STZ or 3 mM glucose + 25 mM GlcNAc + 1 mM STZ. Islets were then washed three times in 3 mM glucose to remove any excess GlcNAc and incubated for an additional hour in 3 mM glucose in order to allow any increased reversible O-glycosylation to diminish. At the end of the experiment, O-glycosylated proteins were analysed as in Figure 2. (Bottom panel) Data corresponding to the middle-right panel in which the intensity of the p135 band is shown as the mean $\pm$ S.E.M. from six independent sets of observations from two separate experiments (four independent sets of observations in one experiment and two in the other).

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cyclical removal of *O*-GlcNAc from β-cell intracellular proteins. Hence β-cells did not exhibit STZ-induced histological toxicity because GlcNAc prevented STZ inhibition of O-GlcNAcase and any O-glycosylation caused by GlcNAc itself was reversible since O-GlcNAcase activity was preserved. The fact that islet O-GlcNAcase is selectively sensitive to STZ and that pancreatic β-cells can be protected completely from STZ toxicity *in vitro* with GlcNAc and not even be partially protected with glucose, glucosamine or GalNAc suggests clearly that O-GlcNAcase inhibition accounts for the diabetogenic toxicity of STZ.

It is necessary, however, to reconcile this idea with our previous observations that glucose stimulates increased islet O-glycosylation [32,33], as well as the observation that transgenic mice with impaired β-cell glucosamine synthesis are resistant to STZ + glucose yet develop diabetes in response to STZ + glucosamine [13]. An important difference between glucose-induced O-glycosylation and STZ-induced O-glycosylation is that the former is reversible both *in vitro* [32] and *in vivo* [13], whereas STZ-induced O-glycosylation is irreversible both *in vitro* and *in vivo* [32,33]. Therefore it appears that increased O-glycosylation may be significantly more toxic to β-cells when it is irreversible. In the case of transgenic mice with impaired β-cell glucosamine synthesis, low-dose STZ + glucose is unlikely to cause diabetes due to the fact that decreased substrate is available for O-glycosylation. Thus even if O-GlcNAcase is inhibited irreversibly, there is insufficient O-glycosylation to cause β-cell toxicity. In contrast, with the addition of glucosamine to STZ, O-GlcNAcase is inhibited and there is ample substrate provided for irreversible O-glycosylation to occur, which in turn results in β-cell toxicity. At the present time, it is unclear why irreversible O-glycosylation itself is toxic to β-cells. This is an important and logical next question that will have to be addressed.

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REFERENCES

1 Herr, R. R., Jahnke, J. K. and Argoudelis, A. D. (1967) The structure of streptozotocin. J. Am. Chem. Soc. **89**, 4808–4809

2 Kroncke, 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-nitroso-urea streptozotozin contributes to islet cell DNA damage. Biol. Chem. **376**, 179–185

3 Eizirik, D. L., Sandier, S., Ahnstrom, G. and Welsh, M. (1991) Exposure of pancreatic islets to different alkylating agents decreases mitochondrial DNA content, but only streptozotocin induces long-lasting functional impairment of β-cells. Biochem. Pharmacol. **42**, 2275–2282

4 Turk, J., Corbett, J. A., Ramanadham, S., Bohrer, A. and McDaniel, M. L. (1993) Biochemical evidence for nitric oxide formation from streptozotocin in isolated pancreatic islets. Biochem. Biophys. Res. Commun. **197**, 1458–1464

5 Standell, E., Decio, E. L., Korsgren, O. and Sandler, S. (1988) Functional characteristics of cultured mouse pancreatic islets following exposure to different streptozotocin concentrations. Mol. Cell. Endocrinol. **59**, 83–91

6 Decio, E. L., Sandler, S., Welsh, N. and Hellerstrom, C. (1988) Preferential reduction of insulin production in mouse pancreatic islets maintained in culture after streptozotocin exposure. Endocrinology **122**, 1242–1249

7 Wilson, G. L. and Leiter, E. H. (1990) Streptozotocin interactions with pancreatic β-cells and the induction of insulin-dependent diabetes. Curr. Top. Microbiol. Immunol. **156**, 27–54

8 Voss, C., Herrmann, I., Hartmann, K., Zuhlke, H., Besch, W. and Keilacker, H. (1988) Diabetogenic effects of N-nitrosomethylurea in rats. Exp. Clin. Endocrinol. **92**, 25–31

9 Golden, P., Baird, L., Malaisse, W. J., Malaisse-Lagae, F. and Walker, M. M. (1976) Effect of 1-methyl-1-nitrosourea and streptozotocin on glucose-induced insulin secretion by isolated islets of Langerhans. Diabetologia **12**, 207–209

10 LeDoux, S. P., Woodley, S. E., Patton, N. J. and Wilson, G. L. (1986) Mechanisms of nitrosourea-induced β-cell damage. Diabetes **35**, 866–872

11 Mossman, B. T., Ireland, C. M., Filipak, M., LeDoux, S. and Wilson, G. L. (1986) Comparitive interactions of streptozotocin and chlorozotocin with DNA of an insulin secreting cell line. Diabetologia **29**, 186–191

12 Roos, M. D., Wen, X., Kaihong, S., Clark, J. A., Xiaoyong, Y., Chin, E., Paterson, A. J. and Kudlow, J. E. (1998) Streptozotocin, an analog of N-acetylglucosamine, blocks the removal of O-GlcNAc from intracellular proteins. Proc. Assoc. Am. Physicians **110**, 1–11

13 Liu, K., Paterson, A. J., Chin, E. and Kudlow, J. E. (2000) Glucose stimulates protein modification by O-linked GlcNAc in pancreatic β-cells: linkage of O-linked GlcNAc to β-cell death. Proc. Natl. Acad. Sci. U.S.A. **97**, 2820–2825

14 McKnight, G. U., Mudd, S. L., Mathewes, S. L., Traxinger, R. R., Marshall, S., Sheppard, P. O. and O'Hara, P. J. (1992) Molecular cloning, CDNA sequence, and bacterial expression of human glutamine fructose-6-phosphate amidotransferase. J. Biol. Chem. **267**, 25208–25212

15 Sayeski, P. P., Wang, D., Su, K., Han, I.-O. and Kudlow, J. E. (1997) Cloning and partial characterization of the mouse glutamine:fructose-6-phosphate amidotransferase (GFAT) gene promoter. Nucleic Acids Res. **25**, 1458–1466

16 Sayeski, P. P., Paterson, A. J. and Kudlow, J. E. (1994) The murine glutamine:fructose-6-phosphate amidotransferase-encoding cDNA sequence. Gene **140**, 289–290

17 Kornfeld, R. (1967) Studies on L-glutamine D-fructose 6-phosphate amidotransferase: feedback inhibition by uridine diphosphate-N-acetylglucosamine. J. Biol. Chem. **242**, 3135–3141

18 Hart, G. W. (1997) Dynamic O-GlcNAcylation of nuclear and cytoskeletal proteins. Annu. Rev. Biochem. **66**, 315–335

19 Starr, C. M. and Hanover, J. A. (1990) Glycosylation of nuclear pore protein p62. Reticulocyte lysate catalyzes O-linked N-acetylglucosamine addition *in vitro*. J. Biol. Chem. **265**, 6868–6873

20 Jackson, S. P. and Tjian, R. (1988) O-Glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell **55**, 125–133

21 Reason, A. J., Morris, M. L., Panico, M., Marais, R., Treisman, R. H., Haltiwanger, R. S., Hart, G. W., Kelly, W. G. and Dell, A. (1992) Localization of O-GlcNAc modification on the serum response transcription factor. J. Biol. Chem. **267**, 16911–16921

22 Chou, T. Y., Hart, G. W. and Dang, C. V. (1995) c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. J. Biol. Chem. **270**, 18961–18965

23 Jiang, M. S. and Hart, G. W. (1997) A sub-population of estrogen receptors are modified by O-linked N-acetylglucosamine. J. Biol. Chem. **272**, 2421–2428

24 Han, I.-O. and Kudlow, J. E. (1997) Reduced O-glycosylation of Spl is associated with increased proteasome susceptibility. Mol. Cell. Biol. **17**, 2550–2558

25 Roos, M. D., Su, K., Baker, J. R. and Kudlow, J. E. (1997) O-Glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. Mol. Cell. Biol. **17**, 6472–6480

26 Kadonaga, J. T., Courey, A. J., Ladika, J. and Tjian, R. (1988) Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science **242**, 1566–1570

27 Gill, G., Pascal, E., Tseng, Z. H. and Tjian, R. (1994) A glutamine-rich hydrophobic patch in transcriptional factor Sp1 contacts the dTAFII110 component of the *Drosophila* TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. U.S.A. **91**, 192–196

28 Kreppel, L. K., Blomberg, M. A. and Hart, G. W. (1997) Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J. Biol. Chem. **272**, 9308–9315

29 Lubas, W. A., Frank, D. W., Krause, M. and Hanover, J. A. (1997) O-Linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats. J. Biol. Chem. **272**, 9316–9324

30 Hanover, J. A., Lai, Z., Le, G., Lubas, W. A. and Sato, S. M. (1999) Elevated O-linked N-acetylglucosamine metabolism in pancreatic beta-cells. Arch. Biochem. Biophys. **367**, 51–60

31 Akimoto, Y., Kreppel, L. K., Hirano, H. and Hart, G. W. (1999) Localization of the O-linked N-acetylglucosamine transferase in rat pancreas. Diabetes **48**, 2407–2413

32 Konrad, R. J., Janowski, K. M. and Kudlow, J. E. (2000) Glucose and streptozotocin stimulate p135 O-glycosylation in panreatic islets. Biochem. Biophys. Res. Commun. **267**, 26–32

33 Konrad, R. J., Liu, K. and Kudlow, J. E. (2000) A modified method of islet isolation preserves the ability of β-cells to regulate O-linked protein glycosylation in response to glucose and streptozotocin. Arch. Biochem. Biophys. **381**, 92–98

34 Yang, G. C. H., Wan, L. S., Papellas, J. and Waisman, J. (1997) Compact cell blocks. Acta Cytologica **42**, 703–706

35 Kusama, K., Jiang, Y., Toguchi, M., Ohno, J., Shikata, H., Sakashita, H. and Sakagami, H. (2000) Use of the monoclonal antibody M30 for detecting HSG cell apoptosis. Anticancer Res. **20**, 151–154

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36 MacFarlane, M., Merrrison, W., Dinsdale, D. and Cohen, G. M. (2000) Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. J. Cell. Biol. **148**, 1239–1254
37 Leers, M. P., Kolgen, W., Bjorkland, V., Bergman, T., Tribbick, G., Persson, B., Bjorkland, P., Ramaekers, F. C., Bjorkland, B., Nap, M., Jornvall, H. and Schutte, B. (1999) Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during apoptosis. J. Pathol. **187**, 567–572
38 Saini, K. S., Thompson, C., Winterford, C. M., Walker, N. I. and Cameron, D. P. (1996) Streptozotocin at low doses induces apoptosis and at high doses causes necrosis in a murine pancreatic β-cell line. Biochem. Mol. Biol. Int. **39**, 1229–1236
39 Hoorens, A. and Pipeleers, D. (1999) Nicotinamide protects human β-cells against chemically-induced necrosis, but not against cytokine-induced apoptosis. Diabetologia **42**, 55–59
40 Dong, D. L. and Hart, G. W. (1994) Purification and characterization of an O-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J. Biol. Chem. **269**, 19321–19330
41 Melino, G., Bernassola, F., Knight, R. A., Corasaniti, M. T. and Finazzi-Argo, G. N. (1997) S-Nitrosylation regulates apoptosis. Nature (London) **388**, 432–433
42 Su, X. D., Taddei, N., Stefani, M., Ramponi, G. and Nordlund, P. (1994) The crystal structure of a low molecular weight phosphotyrosine protein phosphatase. Nature (London) **370**, 575–578
43 Caselli, A., Marzocchini, R., Camici, G., Manao, G., Moneti, G., Pieraccini, G. and Ramponi, G. (1998) The inactivation mechanism of low molecular weight phosphotyrosine phosphatase by hydrogen peroxide. J. Biol. Chem. **273**, 32554–32560
44 Caselli, A., Camici, C., Manao, G., Moneti, G., Pazzagli, L., Cappugi, G. and Ramponi, G. (1994) Nitric oxide causes inactivation of the low molecular weight phosphotyrosine protein phosphatase. J. Biol. Chem. **269**, 24878–24882
45 Rossini, A. A., Williams, R. M., Appel, M. C. and Like, A. A. (1978) Complete protection from low-dose streptozotocin-diabetes in mice. Nature (London) **276**, 182–183
46 Rossini, A. A., Like, A. A., Dulin, W. E. and Cahill, G. F. (1977) Pancreatic β-cell toxicity by streptozotocin anomers. Diabetes **26**, 1120–1124
47 Ganda, O. P., Rossini, A. A., Like, A. A., Dulin, W. E. and Cahill, G. F. (1976) Studies on streptozotocin diabetes. Diabetes **25**, 595–603

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