Nerve Growth Factor Increases in Pancreatic β Cells After Streptozotocin-Induced Damage in Rats

MARIA ELENA LARRIETA,* PAZ VITAL,* ADRIANA MENDOZA-RODRÍGUEZ,† MARCO CERBÓN,† AND MARCIA HIRIART*,[1]

Department of Biophysics, Instituto de Fisiología Celular; and †Department of Biology, Facultad de Química, Universidad Nacional Autonóma de México, UNAM, Circuito Exterior, Ciudad Universitaria, D. F. 04510, Mexico

We investigated short-term *in vivo* and *in vitro* effects of streptozotocin (STZ) on pancreatic β cells. Male Wistar rats were treated with 75 mg/kg STZ, and, after 4 hrs blood glucose and insulin were measured and islet cells were isolated, cultured for 16 hrs, and challenged with 5.6 and 15.6 m*M* glucose. Treated rats showed hyperglycemia (14 m*M*) and a 70% decrease in serum insulin levels as compared with controls. Although insulin secretion by isolated β cells from STZ-treated rats was reduced by more than 80%, in both glucose concentrations, nerve growth factor (NGF) secretion by the same cells increased 10-fold. Moreover, *NGF* messenger RNA (mRNA) expression increased by 30% as compared with controls. Similar results were obtained in an *in vitro* model of islet cells, in which cells were exposed directly to STZ for 1, 2, and 4 hrs and then challenged for 3 hrs with the same glucose concentrations. Our data strongly suggest that an early increase in NGF production and secretion by β cells could be an endogenous protective response to maintain cell survival and that diabetes mellitus may occur when this mechanism is surpassed. Exp Biol Med 231:396–402, 2006

Key words: insulin secretion; diabetes mellitus; ELISA; *NGF* mRNA; TUNEL

Introduction

Type I diabetes mellitus is a multifactorial autoimmune condition that results from an immune destruction of the insulin-producing pancreatic β cells. The susceptibility to suffer this condition is determined by genetic and environmental factors. Several studies have shown the importance of cytokines, such as tumor necrosis factor-α and different interleukins, in the initiation and outcome of this disease (1).

Nerve growth factor (NGF) plays an important role in the development and survival of peripheral, sympathetic, and sensory neurons. NGF also has trophic actions in cells of the endocrine and immune systems (2).

We previously demonstrated that pancreatic β cells synthesize and secrete NGF (3), which, by autocrine communication, increases insulin secretion (4), and that, acting in concert with insulin, NGF increases the survival of β cells (5).

It has been recently suggested that NGF participates as a cytokine in acute inflammatory responses and that NGF levels are higher in certain autoimmune and allergic diseases (6, 7).

Thus, it is possible that heightened pancreatic NGF secretion is part of an initial stage of the acute response of the pancreas to damage. In this study, we investigated early responses of pancreatic NGF to damage caused by streptozotocin (STZ) in rat β cells.

Materials and Methods

Reagents were obtained from the following sources: STZ, bovine serum albumin (BSA; fraction V), Ficoll, trypsin, Triton X-100, sodium citrate, trypan blue, and poly-L-lysine from Sigma-Aldrich (St. Louis, MO); collagenase type IV from Worthington (Freehold, NJ); fetal bovine serum (FBS) from Equitech-bio (Ingram, TX); RPMI-1640, penicillin G, streptomycin, fungizone, Hank's balanced salt solution (HBSS), and TRIzol reagent from GIBCO (Grand Island, NY).

Animals and STZ Treatment. Animals were

This work was supported by grants 211800 and 203903 from the Dirección General de Asuntos del Personal Académico (DGEP), Universidad Nacional Autónoma de México (UNAM), and grant D39822-Q from the Consejo Nacional de Ciencia y Tecnología (CONACyT). M.E.L and P.V. were recipients of scholarship grants from CONACyT and DGEP, UNAM.

[1] To whom correspondence should be addressed at Department of Biophysics, Instituto de Fisiología Celular, UNAM; Ciudad Universitaria, AP 70-253 Coyoacán, México D. F. 04510, México. E-mail: mhiriart@ifc.unam.mx

Received May 23, 2005.
Accepted December 15, 2005.

1535-3702/06/2314-0396$15.00

handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH 85–23, revised 1985). All methods used in this study were approved by the Internal Council and the Animal Care Committee of the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México.

Adult male Wistar rats (250–300 g) were obtained from local animal facilities, maintained in a 12:12-hr light:dark cycle (0700–1900 hrs), and had free access to tap water and food throughout the experiments, except during fasting.

Rats received a single intraperitoneal injection of STZ (75 mg/kg body weight) freshly dissolved in 0.1 m*M* citrate buffer, pH 4.5. Control rats received an equivalent volume of buffer solution.

Animals referred as STZ-4h were rats treated with STZ, left without food for 4 hrs, and sacrificed to obtain the islets. Isolation of β cells to measure insulin and NGF secretion and *NGF* messenger RNA (mRNA) was performed 4 hrs after STZ injection because after 4 hrs, it was not possible to obtain enough islets in good condition to be assayed for hormone secretion.

Blood Extraction and Glucose Measurement. Blood extraction was carried out in STZ-4h rats and once a week during the 4 weeks after STZ injection, always at 1100 hrs. Rats were fasted for 4 hrs (from 0700 to 1100 hrs) and anesthetized with ether before the blood extraction by intraorbital retrobulbar plexus puncture. Blood samples were collected into polypropylene tubes and centrifuged at 10,000 *g* at 4°C for 10 mins, sera were separated and stored at –20°C for insulin assay. Blood glucose concentration was measured in a drop of blood from the capillary tube used in the collection of blood using a standard glucometer (Precision QID, MediSense, Inc., Abbott Laboratories Company, Mexico DF, Mexico); only STZ-4h rats with blood glucose concentrations greater than 13.5 m*M* were included in the study.

Body weight, blood glucose, and serum insulin were measured 1 day before STZ injection, and at 4 hrs, and 1, 2, 3, and 4 weeks after STZ treatment.

Pancreatic Islets and Isolated β Cells Culture. Pancreatic β cells were obtained, according to the technique previously described (8), from pancreases from six STZ-4h rats or respective controls that were obtained and pooled per condition; or islet cells of six pooled pancreases from rats without treatment that were then treated with STZ. Briefly, pancreatic islets were isolated and separated from the acinar tissue by collagenase digestion and a Ficoll gradient centrifugation. Clean islets were then handpicked; dissociation of the cells was achieved by incubating the islets in a shaker bath for 10 mins at 37°C in calcium-free Spinner salt solution, with 15.6 m*M* glucose, 0.5% BSA, and 0.01% trypsin, followed by mechanical disruption.

Groups of 50,000 isolated β cells were seeded on glass coverslips previously treated with poly-L-lysine and cultured for 16 hrs at 37°C in RPMI-1640 supplemented with 10% FBS, 200 U/ml penicillin G, 200 mg/ml streptomycin, and 0.05 mg/ml fungizone to recover from the isolation.

Isolated rat β cells were cultured in control conditions or with 1 m*M* STZ for 1, 2, or 4 hrs (9, 10) in RPMI-1640 supplemented with 0.1% FBS, 200 U/ml penicillin G, 200 mg/ml streptomycin, and 0.05 mg/ml fungizone. In preliminary experiments, we cultured β cells with 1 and 5 m*M* STZ and analyzed insulin secretion; we did not find significant differences between the two concentrations, therefore, we use the lowest concentration for the current experiments.

All of the cultures were performed in duplicate, and at least three independent experiments were performed.

Insulin and NGF Secretion Assays. To measure hormone secretion, β cells isolated from both rat models were first preincubated for 30 mins at 37°C in HBSS (5.6 m*M* glucose) with 0.1% BSA. This solution was then replaced with fresh HBSS containing 5.6 or 15.6 m*M* glucose, and β cells were incubated during 3 hrs at 37°C. At the end of the incubation period, supernatants were collected and supplemented with Complete Mini protease inhibitor cocktail (Roche Molecular Biochemical, Mannheim, Germany) for subsequent insulin and NGF determinations.

The quantitative determinations of insulin in rat serum and β cell supernatants were performed in duplicate, using an Ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA; ALPCO Diagnostics, Windham, NH), as instructed by the manufacturer. Absorbances were measured at 450 nm using an ELISA reader (Bio-Rad, Laboratories, Richmond, CA). All determinations were performed in duplicate.

NGF secreted by isolated β cells was measured in duplicate *via* a quantitative two-site enzyme immunoassay using a NGF E_{max} Immunoassay System (Promega, Madison, WI), as instructed by the manufacturer. Absorbance from colorimetric reactions of horseradish peroxidase and 3,3′,5,5′-tetrametilbencidine was determined *via* ELISA reader and converted to picomolar NGF protein level by normalizing the data to the standard curve. The manufacturer indicates that serum NGF levels cannot be detected using this ELISA kit.

Measurements of *Insulin* and *NGF* mRNA. Total RNA was extracted, using the TRIzol reagent, from pancreatic islets of control and STZ-4h rats treated with 1 m*M* STZ.

Reverse transcriptase (RT) polymerase chain reaction (PCR) was carried out with 400 ng of total RNA for *insulin*, and *NGF* mRNA detection was performed following the protocol of the manufacturer (Applied Biosystems, Foster City, CA). A parallel reaction was carried out in the same mRNA sample, using the *β-actin* housekeeping gene, for quantitative purposes.

All oligonucleotide primers were synthesized and used to prime the amplification of the complementary DNA template. Based on the published sequences of *NGF*, *insulin*, and *β-actin*, we chose the 5′-GGCATGCTGGACC-

Table 1. Changes in Blood Glucose Concentration and Body Weight of Control and STZ-Treated Rats[a]

	Blood glucose (m*M*)		Body weight (g)	
	Control	STZ	Control	STZ
0 hr	7.5 ± 0.1	7.5 ± 0.1	300 ± 11	283 ± 14
4 hrs	8.2 ± 0.2	13.9 ± 0.5*,**	300 ± 11	283 ± 14
1 week	7.7 ± 0.3	21.0 ± 0.5*,**,***	331 ± 12	263 ± 11***
2 weeks	8.0 ± 0.3	22.3 ± 0.6*,**,***	356 ± 11*	244 ± 11**,***
3 weeks	7.6 ± 0.3	23.4 ± 0.8*,**,***	380 ± 11*	239 ± 10**,***
4 weeks	8.4 ± 0.3	24.9 ± 0.6*,**,***	415 ± 13*	238 ± 13**,***

[a] Data represent mean ± SE; $n = 16$ in each group.
In blood glucose levels: *$P < 0.0001$ with respect to 0-hr STZ; **$P < 0.0001$ with respect to control in each point; ‡$P < 0.0001$ with respect to STZ-4h treatment.
In body weight: *$P < 0.02$ with respect to control at 0 hr; **$P < 0.02$ with respect to STZ at 0 weeks; ‡$P < 0.02$ with respect to control in each week.

CAAGCTC-3′ sequence for the sense primer and the 5′-GCGCTTGCTCCGGTGAGTCC-3′ for the antisense primer for *NGF* mRNA detection, the 5′-AAGAGCCATCAGCAAGC-3′ sequence for the sense primer and the 5′-GAGCAGATGCTGGTGCAGC-3′ for the antisense primer for *insulin* mRNA detection, and the 5′-GGGTCAGAAGGATTCCTATG-3′ sequence for the sense primer and the 5′-GGTCTCAAACATGATCTGGG-3′ sequence for the antisense primer for *β-actin* mRNA amplification. Twenty-five cycles of amplification were performed with an annealing temperature of 59°C for *NGF* and 57°C for *insulin* and *β-actin*. The amplified material was submitted to a 1% agarose gel electrophoresis, and products were visualized by ethidium bromide staining and processed for densitometric analysis using Scion Image Beta 4.02 (Scion Corp., Frederick, MD).

Viability and Cell Death Measurements. The viability of the β cells was measured by trypan blue exclusion. After the secretion assay, STZ-treated β cells were incubated with 0.04% trypan blue in isotonic Krebs-Ringer buffer solution for 10 mins. Stained cells (dead cells) and nonstained cells (living cells) were counted, at least 300 cells per experimental condition, and the survival percentage was calculated.

Apoptosis Measurement. Apoptotic β cells were detected using the Tdt-mediated dUTP nick-end labeling (TUNEL) method, following the instructions of the manufacturer (Roche Molecular Biochemical). Briefly, after the secretion assay, STZ-treated β cells were fixed for 30 mins in 0.4% paraformaldehyde in 0.1 *M* phosphate-buffered saline (PBS; pH 7.4). Then, cells were perforated with 0.1% triton in sodium citrate solution during 2 mins on ice, washed three times with PBS, and incubated for 60 mins with the TUNEL reaction mixture at 37°C in a humid chamber protected from light. Positive cells were counted under a Nikon Axiophot inverted microscope connected to a fluorescence lamp; at least 300 cells per experimental condition were counted. Results were expressed as the percentage of apoptotic cells.

Statistical Analysis. Data are presented as mean ± SEM. Multiple comparisons between the different groups were performed by one-way analysis of variance (ANOVA) and Fisher's partial least-squares difference test to calculate the statistical difference between the groups (Statview 4.57.0.0, Abacus Concepts Inc., Berkeley, CA).

Results

Blood Glucose Concentration Increased After STZ Treatment. The control mean basal (4 hrs of fasting) blood glucose concentration was 7.5 ± 0.1 m*M* before STZ administration, and this concentration was maintained throughout the experiment in control rats. However, this parameter increased to 13.9 ± 0.5 m*M* in STZ-4h rats. This result suggests that, during this time, STZ treatment causes severe damage to β cells, which is reflected by hyperglycemia. One week after STZ treatment, the mean basal blood glucose concentration increased to 21 ± 0.5 m*M* and remained constant for the next 4 weeks (Table 1).

Body Weight Loss Caused by STZ Treatment. Body weight of control rats increased gradually, nearly 9% per week. In contrast, STZ-treated rats lost weight, by nearly 7% by the first week and by 15% between the second and fourth weeks, although they ate more than control rats. Furthermore, mean body weight of STZ-treated rats was approximately 37% lower than the controls after the second week of the study (Table 1). These data indicate that STZ-treated rats did not grow normally, which would be expected, because their insulin levels were very low.

Decreased Serum Insulin Levels After STZ Treatment. Four hours after STZ injection, insulin serum levels were 70% lower as compared with their initial control levels. Figure 1 shows that, in the following weeks, these levels continued to decrease. This observation can be correlated to the hyperglycemia observed at the same points (Table 1).

***In Vivo* STZ Treatment Increased NGF but Decreased Insulin Secretion by Isolated β Cells.** Insulin and NGF secretion of β cells isolated of control and STZ-4h rats was measured in response to a 3-hr challenge with 5.6 m*M* (basal) or 15.6 m*M* (high) glucose. As shown

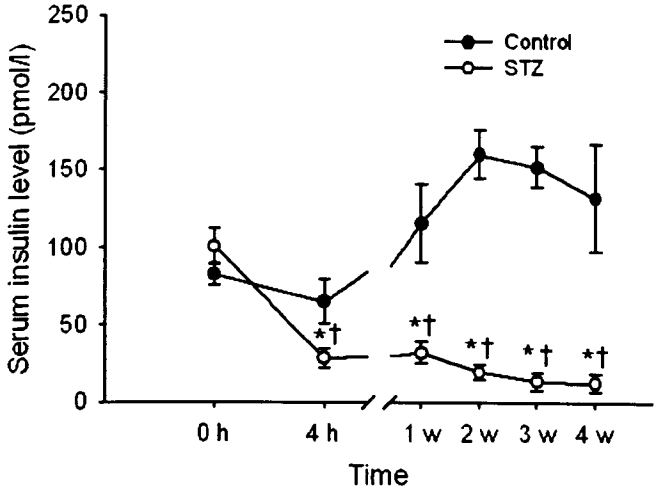

Figure 1. Effect of STZ on β cells decreased serum insulin levels. Data represent mean ± SEM. *$P < 0.05$ with respect to 0 hr; †$P < 0.05$ with respect to control rats at each point; $n = 8$ per group.

in Figure 2A, insulin secretion in control cells in response to high glucose was 3-fold higher than the basal glucose concentration. In contrast, insulin secretion in STZ-4h β cells was nearly 85% lower in both glucose concentrations as compared with controls. Interestingly, STZ-4h β cells secreted 10-fold more NGF than the controls in both glucose concentrations (Fig. 2B).

***In Vivo* STZ Treatment Decreased *Insulin* mRNA Levels but Increased *NGF* mRNA Levels.** *Insulin* mRNA expression in STZ-4h pancreatic islets was 55% lower compared with controls (Fig. 3). In contrast, mean *NGF* mRNA expression in the STZ-4h islets was 30% higher than in controls (Fig. 3B).

The decrease in *insulin* mRNA expression could partially explain the fall in serum insulin level, because these measures were taken from the same animals.

***In Vitro* STZ Treatment Increased NGF but not Insulin Secretion by β Cells.** Insulin and NGF secretion was also measured in β cells treated *in vitro* with 1 m*M* STZ for 1, 2, or 4 hrs. After this incubation, β cells were incubated for 3 hrs in HBSS with basal or high glucose concentrations to measure insulin and NGF secretion (see Materials and Methods). Figure 4 shows insulin and NGF secretion.

Insulin secretion in basal glucose concentration was similar in control and STZ-treated β cells. In contrast, insulin secretion in response to high glucose, in STZ-treated β cells treated for different periods, was 1.3-fold lower than in control cells (Fig. 4A). Only one control bar for each glucose concentration is shown because there were no differences between periods.

Interestingly, NGF secretion by STZ-treated β cells, increased in both basal- and high-glucose concentrations, as compared with controls, in all STZ-treatment periods studied (Fig. 4B). Maximal NGF secretion was observed after a 1-hr incubation with STZ.

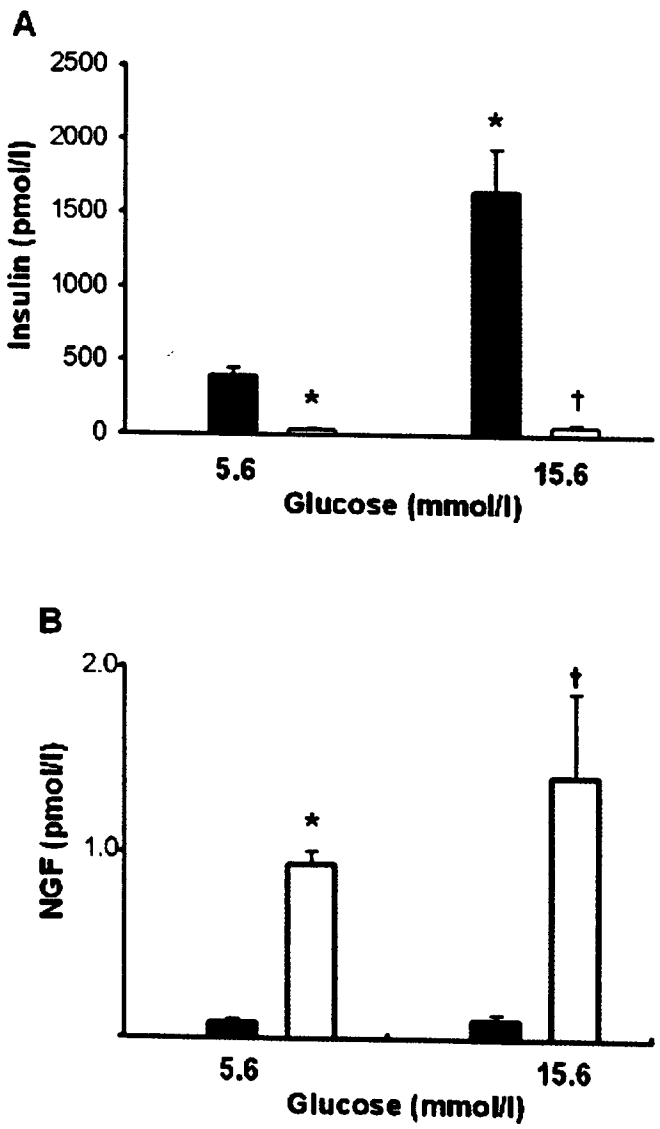

Figure 2. Insulin and NGF secretion of isolated β cells. (A) Concentration of insulin secreted by β cells from control (black bars) or STZ-4h rats (white bars). (B) Concentration of secreted NGF from control (black bars) or STZ-4h rats (white bars). Data represent mean ± SE of three independent experiments, each performed in duplicate (see Materials and Methods). *$P < 0.002$ compared with controls in 5.6 m*M* glucose; †$P < 0.002$ compared with controls in 15.6 m*M* glucose.

***In Vitro* STZ Treatment Decreased Survival and Increased Apoptosis.** Survival and apoptosis were measured in control and 1-, 2-, or 4-hr STZ-treated β cells, in basal- and high-glucose concentrations. Survival percentage of STZ-treated cells was nearly 60%, compared with 80%–90% for controls; we found no differences between groups in basal- and high-glucose concentrations. Figure 5 shows that most of the observed cell death occurred by an apoptotic mechanism, even in controls. Apoptosis percentage increased in a time-dependent manner with STZ exposure. Apoptosis percentages in control cells did not vary between incubation periods.

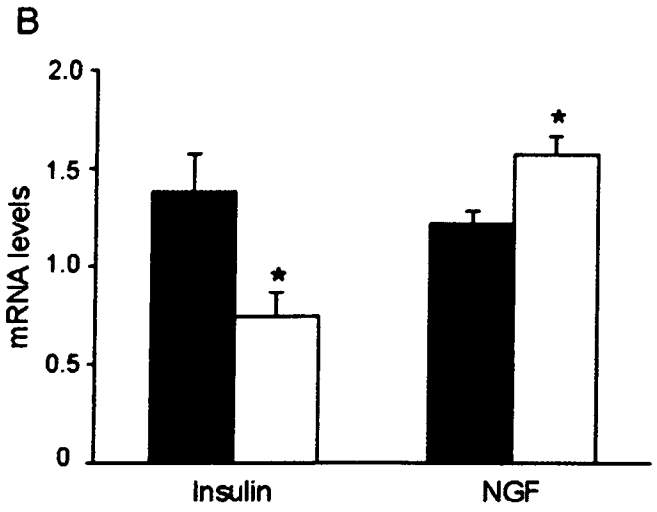

Figure 3. Insulin and *NGF* mRNA expression in pancreatic islets. (A) RT-PCR products of pooled islets of six control rats (C, black bars) and six STZ-4h rats (white bars). (B) mRNA *insulin* and *NGF* levels normalized to *β-actin* levels. Data represent mean ± SE of four independent experiments. $^*P < 0.05$ compared with control rats.

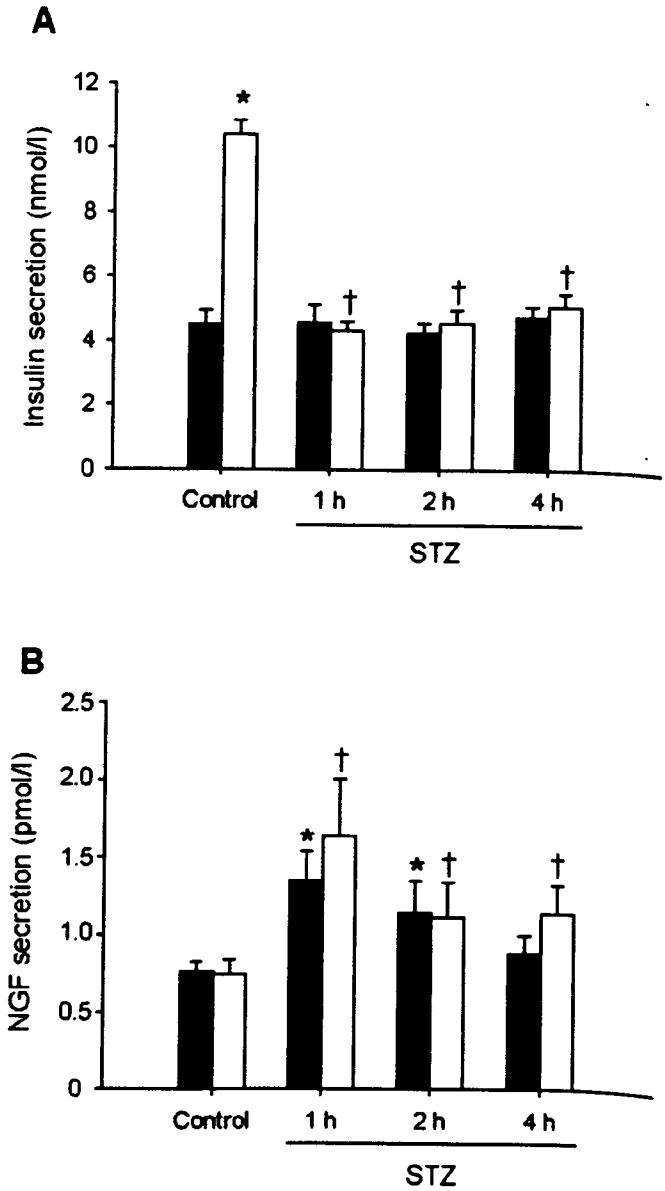

Figure 4. Insulin and NGF secretion of β cells treated *in vitro* with STZ. Pancreatic β cells were incubated with 1 m*M* STZ for 1, 2, or 4 hrs (see Materials and Methods). (A) Concentration of secreted insulin in 5.6 m*M* glucose (black bars) or 15.6 m*M* glucose (white bars). $^*P < 0.001$ with respect to control in 5.6 m*M* glucose; $\dagger P < 0.001$ with respect to control in 15.6 m*M* glucose. (B) Concentration of secreted NGF in 5.6 m*M* glucose (black bars) or 15.6 m*M* glucose (white bars). $^*P < 0.05$ compared with control in 5.6 m*M* glucose; $\dagger P < 0.05$ compared with control in 15.6 m*M* glucose; $\ddagger P < 0.05$ compared with 1-hr STZ in 5.6 m*M* glucose. Data represent mean ± SE of three to four experiments.

Discussion

It is well established that STZ causes destruction of pancreatic β cells, leading to a marked reduction in serum insulin level and hyperglycemia. The early responses of β cells to STZ damage are not entirely understood. We analyzed and compared changes in the physiology of the adult rat pancreas and isolated β cells exposed to a single dose of STZ.

We observed that after 4 hrs of a single, high-dose ip injection of STZ, rats showed markedly elevated blood glucose concentrations and a severe reduction in serum insulin levels; these conditions persisted throughout the course of the study. After 4 weeks, we did not find any sign of recovery from the treatment. Previous studies show that pancreatic β cell damage starts 6 hrs after STZ injection, leading to diabetes mellitus development (11). However, there are differences in the conditions of both studies, for instance, STZ potency can vary depending on the lot and source.

Insulin secretion by isolated β cells obtained from STZ-treated rats was minimal both in basal- and high-glucose concentrations, and *insulin* mRNA level was considerably diminished as compared with controls. In contrast, NGF secretion and *NGF* mRNA increased in the same isolated β cells. This observation is interesting because NGF has been proposed to play a role in the acute-phase response after damage (12). To our knowledge, this is the first study that provides evidence of pancreatic NGF overproduction after STZ damage.

NGF production by pancreatic β cells as a primary response to injury could be analogous to NGF responses in the nervous system. It has been shown that brain levels of

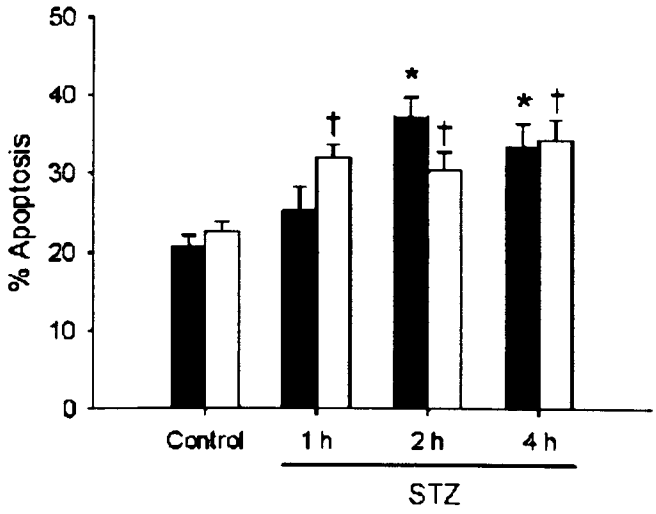

Figure 5. Apoptosis of β cells treated *in vitro* with STZ. Cells were incubated with 1 m*M* STZ for 1, 2, or 4 hrs; after these periods, apoptosis was measured. Percentage of apoptosis of β cells previously incubated in 5.6 m*M* glucose (black bars) or 15.6 m*M* glucose (white bars). $^*P < 0.05$ with respect to control in 5.6 m*M* glucose; $\dagger P < 0.05$ with respect to control in 15.6 m*M*. Data represent mean ± SE of 3–4 experiments.

NGF increase after hypoxic-ischemic injury (13), neurotoxin administration (14, 15, 16), and excitotoxic lesions (17, 18). Moreover, elevated NGF levels have been also reported after seizures (19, 20), and brain trauma (21). In the peripheral nervous system, within 6 hrs after a sciatic nerve transection, a large increase in *NGF* mRNA level is observed in Schwann cells and fibroblasts at nerve segments proximal and distal to the lesion (22).

We have previously demonstrated that rat pancreatic β cells synthesize and secrete NGF (3). Pierucci *et al.* (23) showed that NGF withdrawal induces apoptosis in cultured human β cells and in the βTC6-F7 cell line. We have also demonstrated that the apoptosis of isolated β cells increases when autocrine regulation by insulin and NGF are blocked by specific antibodies against these hormones (5). Thus, it is possible that a primary response of β cells to STZ exposure is to synthesize and secrete NGF to protect them; as it is clear from Figures 2–4.

Both insulin and NGF receptors have tyrosine kinase activities that trigger intracellular phosphorylation signaling cascades that converge in downstream-located effector proteins, such as PI3K/Akt, which are associated with the antiapoptotic systems in different cell types (23–25).

Data presented in this study indicate that increased NGF expression and secretion after injury could be an endogenous protective mechanism to maintain cell survival in damaged tissue, as has been observed in the nervous system. In our model, the increase in NGF was not enough to prevent STZ damage. However, an increase in NGF could be an early sign of β cell damage at the onset of diabetes mellitus.

We thank Héctor Malagón for assistance in animal care and Angélica Zepeda and Alvaro Caso for proofreading and discussion of this manuscript.

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