Protective effect of testosterone on early apoptotic damage induced by streptozotocin in rat pancreas

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Abstract

Beta-cell apoptosis is responsible for the development of insulin-dependent diabetes mellitus in the streptozotocin (STZ) rat model. It has been demonstrated that steroid hormones possess antioxidant and protective antiapoptotic effects in many tissues. The aim of the present study was to investigate the early apoptotic damage induced by STZ in rat pancreas, and the effect of testosterone in preventing apoptosis of pancreatic β cells. Intact and castrated adult male Wistar rats were subjected to a unique injection of STZ 60 mg/kg (body weight) in citrate buffer, and the kinetics of apoptosis in β cells was assessed. Insulin and glucose were measured by RIA and a glucometer respectively, and in pancreatic tissue by immunohistochemistry. At 6 h after STZ injection, a marked increase in apoptotic β cells was detected; however, glucose and insulin serum

levels were not significantly different from the controls. The castrated animals presented higher percentages of apoptotic β cells ($65.75 \pm 5.42\%$) than intact males ($20.6 \pm 4.38\%$) and castrated, testosterone-substituted males ($30.66 \pm 1.38\%$). The decrease in apoptotic β cells induced by testosterone was reversed by the antiandrogen flutamide ($67.69 \pm 3.45\%$). The overall results indicate that early apoptotic damage produced by STZ in castrated animals was reversed by testosterone, suggesting that this hormone exerts a natural protective effect in rat pancreas. This effect could help to explain some sexual differences in diabetes mellitus incidence in man, reinforcing the idea that new approaches in steroid hormone therapies should be considered for treatment of this disease.

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Introduction

Streptozotocin (STZ) is an alkylating agent that produces β -cell death by the mechanism of DNA damage that induces poly-ADP-ribose synthetase activation, followed by lethal nicotinamide adenine dinucleotide (NAD) depletion, a mechanism well documented in rodent islets (Yang & Wright 2002). During STZ metabolism, various toxic intermediates are produced, including methyl cations, methyl radicals, reactive oxygen species (ROS) and nitric oxide (NO) (Peschke *et al.* 2000, González *et al.* 2002). Beta cells are very susceptible to oxidative changes since they possess a low antioxidative capacity (Hotta *et al.* 1998, Kajimoto & Kaneto 2004).

It is accepted that oxidative stress and apoptosis cause β -cell death in STZ-diabetic mice and nonobese diabetic (NOD) mice. NOD mice spontaneously develop autoimmune diabetes with remarkable similarity to human insulin-dependent diabetes mellitus (IDDM) (O'Brien *et al.* 1997). Injection of STZ induces progressive hyperglycemia, accompanied by lymphocytic infiltration of pancreatic

islets. In some basic aspects, this model mimics recent-onset IDDM in human patients (O'Brien *et al.* 1996).

There is evidence of sexual dimorphism in the incidence and prevalence of diabetes mellitus in animal models. In NOD mice, a greater incidence of diabetes in females (70%) than in males (15%) is found; this sexual dimorphism is related to the effects of sex steroid hormones (Baxter *et al.* 1989, Rosmalen *et al.* 2001).

The antioxidant properties of steroid hormones have been shown in different cells and tissues (Ahlbom *et al.* 2001, Aragno *et al.* 2002, Deroo *et al.* 2004, Miyake *et al.* 2004). For example, testosterone protects cerebellar granule cells from oxidative stress-induced cell death by a receptor-mediated mechanism (Ahlbom *et al.* 2001). *In vivo*, dehydroepiandrosterone (DHEA) exerts a multi-targeted antioxidant activity in rats, protecting various organs against lipid peroxidation (Boccuzzi *et al.* 1997), and also prevents oxidative damage by hyperglycemia in rats (Aragno *et al.* 1997, Brignardello *et al.* 1998). Estradiol regulates several mechanisms that protect the mouse uterus against oxidative stress (Deroo *et al.* 2004).

Several studies have shown that treatment with antioxidants reduces diabetic complications (Wohaieb & Godin 1987, Siman & Eriksson 1997, Aragno *et al.* 2002). These compounds attenuate the development of peripheral nerve dysfunction (Obrosova *et al.* 2002, Yorek *et al.* 2002), protect from diabetes-dependent brain damage (Aragno *et al.* 2002), normalize the endothelial function in diabetes (Ido *et al.* 1997, Haidara *et al.* 2004) and exert beneficial effects on platelet hyperaggregability (Ruf *et al.* 1992). Moreover, antioxidants decrease the occurrence of malformation in the offspring of diabetic rats (Viana *et al.* 1996, Siman & Eriksson 1997).

The present study aimed to assess whether testosterone, the most abundant natural androgen in male rats, exerts a protective effect against the early apoptotic damage produced by STZ in pancreatic β cells *in vivo*.

Materials and Methods

Animals

All animal maintenance and handling was in accordance with the guidelines of Mexican law on animal protection (NOM-062-ZOO-1999). Six groups of six male Wistar rats (200–250 g) each were formed: 1. intact; 2. intact plus STZ; 3. intact plus flutamide plus STZ; 4. castrated plus STZ; 5. castrated plus testosterone enanthate plus STZ; 6. castrated plus flutamide plus testosterone enanthate plus STZ. The animals were housed under controlled conditions of temperature and light: dark period (12:12 h). Glucose determinations were made with a drop of blood obtained by cutting the tail tip. The glucometer employed for determinations was Prestige Smart System (Home Diagnostics, Ft Lauderdale, FL, USA).

Treatments

Groups of rats were gonadectomized under ketamine-xilazine anesthesia, a single, cutaneous incision was made through the scrotal sac and the peritoneal cavity was entered to expose the testes. The testicular bundles were ligated with 4–0 silk suture and the testes removed. The cutaneous incision was closed with 5–0 silk suture. At 72 h after gonadectomy, the animals received testosterone enanthate replacement therapy in a 200 mg/kg (body weight) single dose or vehicle (corn oil); 24 h after replacement, the animals were treated intraperitoneally with 60 mg/kg (body weight) STZ in citrate buffer 100 mM, pH 4.5, in a single dose, or citrate buffer as control. To confirm that the protective effect was mediated by the androgen receptor, the antiandrogen flutamide was tested. For this purpose, a group of castrated rats were substituted with testosterone enanthate similarly to the scheme described before, and treated with 100 mg/kg (body weight) of flutamide for 2 days, and then STZ was administered.

Histology

After 6 h of STZ treatment, the rats were killed and the pancreas was immersion-fixed in ice-cold 4% (w/v) paraformaldehyde in PBS (100 mM), overnight at 4 °C. Tissues were dehydrated through a series of increasing ethanol concentrations (25%, 50%, 70%, 80%, 96% and absolute) and finally cleared with xylene. Tissues were then embedded in Paraplast plus (Oxford Labware, St Louis, MO, USA). Tissue sections (5 μ m) were cut and mounted on poly-L-lysine-coated slides. Sections were cleared of paraplast with xylene, rehydrated and processed for the techniques detailed below.

Double immunohistochemistry staining

Paraffin sections were dewaxed, rehydrated, permeabilized and subsequently incubated overnight with guinea pig anti-porcine insulin antibody (1:4000) (Incstar, Stillwater, MN, USA), as recommended by the supplier's technical bulletins. Afterward, a second fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG antibody for insulin detection (1:100) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After this procedure, sections were incubated for 4 h with mouse anti-rat glucagon (1:6000) (Sigma) and a second CY5-conjugated goat anti-mouse IgG (1:100) (Jackson ImmunoResearch Laboratories).

Sections were observed by confocal microscopy with a BioRad MRC-1024 system, equipped with a Kr/Ar laser attached to an inverted Nikon Diaphot TMD 300 microscope, with an oil-immersion, 40 objective (Nikon Corporation, Tokyo, Japan). Iris aperture, gain and laser power remained fixed in each session, FITC was excited with a 494 nm wavelength, and emitted light was bandpassed with a 520 nm filter, while CY5 was excited with a 650 nm wavelength, and emitted light was bandpassed with a 670 nm filter.

The following controls were performed to achieve reliable double-immunostaining (data not shown):

1. Negative control of antibodies: experimental protocols were carried out without the addition of primary antisera or without the secondary antibody.

2. Controls of antisera specificity were performed with anti-insulin and anti-glucagon antibodies, preadsorbed overnight with their respective antigens and processed by the same protocol described above.

No fluorescent signal could be detected in either control. Confocal images were viewed and processed by Confocal Assistant 4.02 (Todd Clark, University of Minnesota, MN, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

An *in situ* cell death detection kit (Roche) was used to visualize the apoptotic pancreatic cells. After being

dewaxed through xylene and absolute ethanol, the slides were rehydrated through decreasing concentrations of ethanol and rinsed in PBS buffer saline. Rehydrated sections were incubated at room temperature for 30 min with proteinase K (20 µg/ml in 10 mM Tris-HCl, pH 8). After rinsing with PBS, sections were permeabilized with 0.1% Triton X-100 in PBS for 2 min at 4 °C. Positive control sections were treated for 10 min at 37 °C with DNase. Slides were rinsed with PBS and incubated for 1 h at 37 °C in the TUNEL reaction mixture (terminal deoxynucleotidyl transferase enzyme (TdT) with nucleotide-fluorescein-conjugated mixture in reaction buffer). In negative controls, TdT enzyme was omitted from the reaction mixture. The slides were rinsed in 0.01 M PBS buffer, pH 7.4, and mounted in fluorescent mounting medium (Dako, Carpinteria, CA, USA).

Apoptotic nuclei analysis

The slides were analyzed in an epi-fluorescence microscope, and the image of each preparation was acquired in an Axioscope 401 Zeiss system. The fluorescent nuclei were counted in at least 100 islets per animal and expressed as percentage of apoptotic nuclei with respect to the total nuclei in the pancreatic islets.

Hormonal determinations

The levels of testosterone, estradiol and insulin were assessed by specific RIA (Diagnostic Product Corporation, Los Angeles, CA, USA). The sensitivity of the assays was 5.43 pg/ml to estradiol, 2.47 pg/ml to testosterone and 0.05 ng/ml to insulin. The intra- and interassay coefficients of variation were 5.3% and 8%, 4.9% and 8.2%, and 3.6% and 6% respectively.

Statistical analysis

All values reported are expressed as mean $\pm$ S.E.M. Differences between means were analyzed for significance by one-way ANOVA, followed by Tukey multiple comparison as post hoc test. The statistically significant value was $P < 0.05$.

Results

Time course of pancreatic damage by STZ

To establish the period of time in which the STZ damage was evident, we performed a kinetic analysis for different times: 1, 2, 3, 4, 6, 12 and 24 h in castrated male rats. During the first hour considered (1, 2, 3 and 4 h), the apoptotic index was almost undetectable; however, a small number of islets presented scarce apoptotic nuclei (data not shown). At 6 h, a marked increase in apoptotic β cells was

detected ($65.75 \pm 5.42\%$), although glucose levels were in normal range of 4.6–5.2 mmol/l. In contrast, at 12 and 24 h, the animals were hyperglycemic, 10.6 ± 0.2 and 26.4 ± 0.4 mmol/l respectively, and the apoptotic index was over 80% in both groups. Insulin concentration along time course of STZ administration (1, 2, 3, 4 and 6 h) was normal (1.1–1.7 ng/ml), while at 12 and 24 h it was under 0.5 ng/ml.

Protective effect of testosterone in apoptotic-STZ produced damage

The apoptosis of β cells in pancreas was studied by the TUNEL method and observed by fluorescence microscopy. Fluorescent nuclei in pancreatic islets were counted and reported as percentage in relation to total nuclei (see Materials and Methods). As shown in Figs 1 and 2, STZ treatment induced a higher percentage of β -cell apoptosis in the castrated animals ($65.75 \pm 5.42\%$) than in intact males ($20.6 \pm 4.38\%$) and castrated, testosterone-substituted males ($30.66 \pm 1.38\%$). The decrease in apoptotic β cells induced by testosterone was reversed by the antiandrogen flutamide, which reached similar values to those in castrated male animals ($67.69 \pm 3.45\%$). The apoptotic index observed in intact animals treated with flutamide was $64.35 \pm 6.35\%$ (data not included in the figures). In addition, the apoptotic index was tested in intact animals in all experiments and was lower than 0.5% in all cases (data not shown).

Immunodetection of insulin and glucagon in pancreatic tissue

Insulin and glucagon were assessed by double immunohistochemistry staining in slides of paraffin-embedded pancreatic tissue (Fig. 3). As expected, the insulin immunoreactive β cells in the pancreas of all animals were localized in the core of pancreatic islets. Apoptosis was also detected in this area, which indicates that it is exhibited preferentially by β cells. In contrast, glucagon-positive α cells were observed in the periphery of islets.

Hormonal and glucose analysis

Glucose levels in all animals are presented in Table 1. Interestingly, in all animals studied, the glucose levels were in the normal range.

To assess the hormonal status in intact, castrated and substituted animals, the levels of insulin, testosterone and estradiol were analyzed; the results are summarized in Table 2. As expected, testosterone values in castrated animals were almost undetectable; in contrast, substituted males presented increased testosterone values. Estradiol values were normal in all the groups. Insulin serum levels presented similar values in all experimental groups (Table 1).

Figure 1 Apoptotic β cells detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (see Materials and Methods) in pancreas slides from STZ-treated rats. Apoptotic nuclei in islets of Langerhans from intact male plus STZ (A), castrated male plus STZ (B), castrated plus testosterone plus STZ (C) and castrated plus testosterone plus flutamide plus STZ (D). Positive nuclei from cells were identified by fluorescent labeling. Bar=20 μ m in all panels.

Discussion

In this study, we demonstrated the protective effect of testosterone in the early apoptotic damage induced by STZ to pancreatic β cells. At 6 h after STZ administration to intact and castrated Wistar rats, pancreatic β -cell apoptosis without hyperglycemia was observed. We demonstrated that the presence of male gonads partially prevents β -cell apoptosis. Moreover, this result suggests that testosterone is involved in β -cell protection, since the administration of testosterone enanthate to castrated animals significantly reduced the apoptotic index, compared with castrated animals, and this effect was completely

reversed by the androgen receptor antagonist flutamide, suggesting an androgen receptor-mediated mechanism.

STZ is widely used to produce experimental diabetes in rodents. This drug causes β -cell death by apoptosis and consequently diabetes (O'Brien *et al.* 1996). Our data demonstrate that STZ produced early apoptotic damage in pancreatic islets, producing higher apoptotic damage in castrated animals. Although a high apoptotic index was observed, it was not enough to produce hyperglycemia, because glucose levels in all the animals treated with STZ were in the normal range. These results agree with previous observations in similar experimental conditions of STZ administration at similar doses (Park *et al.* 1999).

Figure 2 Apoptotic index in pancreatic islets in intact and treated animals. CAS: castrated rats treated with STZ; INT: intact rats treated with STZ; CAS+T: rats castrated and substituted with testosterone enanthate plus STZ; CAS+T+F: castrated rats treated with flutamide and substituted with testosterone enanthate plus STZ. Positive nuclei were counted in 100 islets per group and expressed as percentage of apoptotic nuclei with respect to the total nuclei in pancreatic islets. * $P < 0.05$ between groups.

The authors demonstrated that β -cell alteration began 6 h after STZ injection. At this stage, many β cells showed condensation of their nuclear chromatin, while the animals maintained their normal blood-glucose level (80–150 mg/dl) for at least 12 h after STZ injection, followed by a steep glucose increase (>300 mg/dl) at 12–24 h after STZ injection.

The protection exhibited by testosterone against STZ-induced apoptotic damage was more than 50%, considering that apoptosis induced by STZ in castrated male was about 65.75% and in castrated substituted male was 30.66%. The apoptotic index observed in intact males was slightly lower than that observed in castrated/

Table 1 Glucose and insulin concentrations in the experimental groups

Animals	Glucose (mmol/L)	Insulin (ng/ml)
Intact	5.1 ± 0.7	1.2 ± 0.1
Intact+STZ	4.9 ± 0.7	1.1 ± 0.7
Intact+F+STZ	5.1 ± 0.6	0.8 ± 0.2 ^a
Castrated+STZ	4.9 ± 0.6	1.7 ± 0.7 ^b
Castrated+T+STZ	5.2 ± 0.9	1.6 ± 0.5
Castrated+F+T+STZ	4.6 ± 0.6	1.5 ± 0.4

Insulin and glucose levels in control and treated rats. STZ=streptozotocin; T=testosterone; F=flutamide. Data are shown as mean $\pm$ s.e. $P < 0.01$ compared between ^a and ^b. Three independent experiments were performed with $n=3-5$ per group.

Table 2 Testosterone and estradiol concentrations in experimental animals

Animals	Testosterone (ng/ml)	Estradiol (pg/ml)
Intact	1.6 ± 0.20	13.64 ± 1.64 ^a
Intact+STZ	1.7 ± 0.25	12.89 ± 1.5 ^a
Intact+F+STZ	1.53 ± 0.50	15.25 ± 3.0
Castrated+STZ	0.2 ± 0.05 ^a	15.77 ± 1.70
Castrated+T+STZ	6.3 ± 1.03 ^b	17.67 ± 2.0
Castrated+F+T+STZ	0.2 ± 0.015 ^a	12.01 ± 1.21 ^a

Testosterone and estradiol serum levels, in intact and castrated male rats. STZ=streptozotocin; T=testosterone; F=flutamide. Data are shown as mean $\pm$ s.e. ^{a,b} $P < 0.01$ compared with all animal groups.

testosterone treated animals, but not significantly different (Fig. 2). This could be due to the fact that in intact animals, in addition to testosterone, dihydrotestosterone (DHT) and other gonadal factors can also protect against STZ-induced apoptosis (Fox 1992). This protection was similar to that observed in other steroid-protection models. In rat cerebellar granule neurons, testosterone decreased nitric oxide-induced apoptosis by 45% and hydrogen peroxide-induced apoptosis by 30% (Ahlbom *et al.* 1999, 2001). In human primary neurons; testosterone decreased serum deprivation-mediated apoptosis by 20% (Hammond *et al.* 2001). To our knowledge, the present study is the first to use testosterone to protect pancreatic β cells against STZ-induced apoptosis.

Previous reports have indicated that the pancreatic islets can respond to androgens (Rosmalen *et al.* 2001). The administration of exogenous androgens is likely to prevent the effects of castration on glucose homeostasis, since it was shown in a multiple-dose STZ diabetes model that testosterone administered to control females or orchidectomized males results in a glucose response similar to that observed in control males (Rossini *et al.* 1978). It has also been reported that testosterone may protect against diabetes in the NOD model of type I diabetes, that female mice are more prone to diabetes development than males (Fitzpatrick *et al.* 1991) and that androgen treatment of NOD females reverses differences between the sexes (Fox 1992). In NOD mice, mega-islet formation is indicative of initial damage and the number of mega-islets is reduced by testosterone, as compared with controls (Rosmalen *et al.* 2001).

It is well recognized that steroids act through nuclear receptors that are ligand-regulated transcription factors and participate in many cellular process such as proliferation, differentiation and cell death (Altucci & Gronemeyer 2001). Other mechanisms described for steroids in protection of cells against damage are related to their antioxidant properties via a membrane mechanism of action (Sotiriadou *et al.* 2003, Alexaki *et al.* 2004). Our data

Figure 3 Double immunohistochemistry staining for insulin (core of islets) and glucagons (periphery of islets) in pancreas slides from STZ-treated male rats: intact plus STZ (A), castrated plus STZ (B), castrated plus testosterone plus STZ (C), castrated plus testosterone plus flutamide plus STZ (D). Bar=50 μ m in all panels.

demonstrate that testosterone acts directly to protect β cells against apoptotic damage produced by STZ. This effect could be mediated by the androgen receptor, since the androgen receptor antagonist flutamide completely abolished this effect. Activation of androgen receptors in this process is supported by previous observations demonstrating androgen receptor expression and regulation in rat pancreas (Díaz-Sánchez *et al.* 1995), and by the fact that testosterone increases insulin gene expression in rat islets *in vitro* (Morimoto *et al.* 2001a). Moreover, changes in insulin expression correlate well with changes in steroid hormone serum content during the estrous cycle (Morimoto *et al.* 2001b).

The cellular mechanism by which testosterone protects β cells against early apoptotic damage in STZ-treated rats has not been fully established. However, there exist some possibilities. The main effectors in STZ damage are free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO) (Peschke *et al.* 2000, González *et al.* 2002). Androgens modulate enzymes that help cells to escape this type of damage (Ahlbom *et al.* 1999, 2001, Aragno *et al.* 1999). In rat ventral prostate, testosterone regulates a set of oxidative stress-related genes, including thioredoxin, peroxiredoxin 5, superoxide dismutase 2, glutathione peroxidase 1, selenoprotein 15 kDa, microsomal glutathione-S-transferase, glutathione reductase and epoxide hydrolase

(Pang *et al.* 2002, Tam *et al.* 2003), and inhibits cell death by controlling caspase-3 and -6 mRNA levels as well as procaspase and active caspase-3 and -6 proteins levels (Omezzine *et al.* 2003). Further investigation is required to determine whether those processes are involved in the protection of pancreatic β cells by testosterone, and investigation of this possible mechanism is in progress in our laboratory.

The overall results suggest that steroids hormones exert some natural protective effects in rat pancreas and could help to explain some sex differences in diabetes mellitus in man, reinforcing the idea that new approaches in steroid hormone therapies should be considered for treatment of this disease.

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