Pancreatic nitric oxide and oxygen free radicals in the early stages of streptozotocin-induced diabetes mellitus in the rat

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Abstract

The objective of the present study was to explore the regulatory mechanisms of free radicals during streptozotocin (STZ)-induced pancreatic damage, which may involve nitric oxide (NO) production as a modulator of cellular oxidative stress. Removal of oxygen species by incubating pancreatic tissues in the presence of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (1 U/ml) produced a decrease in nitrite levels (42%) and NO synthase (NOS) activity (50%) in diabetic but not in control samples. When NO production was blocked by N\textsubscript{G}-monomethyl-L-arginine (L-NMMA) (600 µM), SOD activity increased (15.21 ± 1.23 vs 24.40 ± 2.01 U/mg dry weight). The increase was abolished when the NO donor, spermine nonoate, was added to the incubating medium (13.2 – 1.32). Lipid peroxidation was lower in diabetic tissues when PEG-SOD was added (0.40 – 0.02 vs 0.20 – 0.03 nmol/mg protein), and when L-NMMA blocked NOS activity in the incubating medium (0.28 ± 0.05); spermine nonoate (100 µM) abolished the decrease in lipoperoxide level (0.70 ± 0.02). We conclude that removal of oxygen species produces a decrease in pancreatic NO and NOS levels in STZ-treated rats. Moreover, inhibition of NOS activity produces an increase in SOD activity and a decrease in lipoperoxidation in diabetic pancreatic tissues. Oxidative stress and NO pathway are related and seem to modulate each other in acute STZ-induced diabetic pancreas in the rat.

Introduction

Streptozotocin (STZ) is an agent widely employed to induce experimental diabetes due to its ability to selectively target and destroy insulin-producing pancreatic islet β-cells (1). Its diabetogenic action has been ascribed to the enhancement of intracellular methylation reactions (2) and to production of nitric oxide (NO) (3,4) and free radicals (5). NO has been demonstrated to be the effector molecule responsible for pancreatic islet destruction (6). We have recently shown that the NO synthase (NOS) inhibitors N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) and N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) can...
prevent metabolic disorders in tissues and plasma of STZ-induced diabetic rats (7,8).

With respect to oxidative stress, an increased free radical generation was reported in diabetic plasma and tissues. Vitamin C levels are lower (9) and altered levels of the antioxidant defense systems are present in diabetic human plasma compared to control (10). Elevated plasma lipid leads to changes in lipid oxidation (11). Lipid peroxides are higher (12), an early sign of oxygen activation (13). Superoxide dismutase (SOD) catalyzes the removal of the superoxide radical, and the addition of exogenous SOD in vitro also protects cellular membranes from chemical damage attributable to superoxide production (14). The use of a covalent conjugated form of SOD, such as polyethylene glycol-SOD (PEG-SOD), increases the half-life of the enzyme in the circulation and the efficacy of its protective effect (15,16).

The fact that β-cells contain much more SOD than α-cells (17) suggests that this enzyme may play an important role in β-cell homeostasis.

As stated, NO is significantly involved in pancreatic destruction, and seems to be closely related to the events leading to oxidative stress. The interaction between NO and O$_2^-$ may be biologically significant. NO is able to combine with reactive oxygen species (ROS) (18) and to modulate oxidative damage (19). This study aims at exploring the regulatory mechanisms of free radicals during STZ-induced pancreatic damage, which may involve NO production as a modulator of cellular oxidative stress.

**Material and Methods**

**Chemicals**

Citrate buffer, STZ, nitroblue tetrazolium, xanthine, xanthine-oxidase, polyethylene glycol-superoxide dismutase, sodium thiobarbiturate, malondialdehyde (1,1,3,3 tetraethoxypropane), L-NMMA, L-NAME, NADPH, L-valine and HEPES were obtained from Sigma Chemical Co., St. Louis, MO, USA. Glucostix reagent strips were obtained from Bayer Diagnostics, Buenos Aires, Argentina.

The nitrate/nitrite assay kit and spermine nonoate (SN) were purchased from Cayman Chemical Co., Ann Arbor, MI, USA. Dowex AG50W-X8 columns (Na$^+$ form) were purchased from BioRad Laboratories, Richmond, CA, USA. [$^{14}$C]-L-Arginine was from New England Nuclear, Boston, MA, USA.

**Animal preparations**

Female albino Wistar rats bred in the laboratory, weighing 200-230 g were used. The animals were kept in a temperature- (20-23°C) and illumination- (12-h light/12-h dark) controlled room, and were made diabetic by a single injection of STZ (55 mg/kg body weight, ip) dissolved in 0.1 M citrate buffer, pH 4.5 (day 1). Blood glucose from the tail vein was assayed 5 days later (day 5) using glucostix reagent strips and a refractance meter. Control animals showed glucose levels between 80 and 110 mg/dl, and STZ-injected animals between 300 and 500 mg/dl. Animals were killed by cervical dislocation on day 5 after STZ injection, and their pancreas was removed and placed on Petri dishes containing Krebs-Ringer bicarbonate solution for subsequent assays. The ionic composition of this medium was described previously (20). The guidelines for the care and use of animals approved by the local institution were followed and they conformed to the standards for use of laboratory animals established by the Institute of Laboratory Animals Resources, U.S. National Academy of Sciences.

**Nitrate/nitrite assay**

Control tissues and tissues previously incubated in a metabolic shaking bath under an atmosphere of 95% O$_2$ and 5% CO$_2$ for 60
min, with the addition of L-NMMA (600 µM) or PEG-SOD (1 U/ml), were homogenized and deproteinized. Nitrates in the supernatant were reduced to nitrites using nitrate reductase, and total nitrites were measured by the Griess method (21), employing an assay kit for nitrate/nitrite determination. Absorbance was measured at 540 nm in a microplate using NaNO₃ and NaNO₂ as standard. Results are reported as nmol/mg protein.

**Assay of NOS activity**

NOS enzyme activity was quantified in pancreatic tissues from control and diabetic rats previously incubated in a metabolic shaking bath under an atmosphere of 95% O₂ and 5% CO₂ for 60 min with the addition of the NOS blocker L-NMMA (600 µM) or the ROS scavenger PEG-SOD (1 U/ml), by the [¹⁴C]-arginine to [¹⁴C]-citrulline conversion assay as described by Bredt et al. (22), modified by Salter et al. (23). Briefly, tissues were homogenized in 1.5 ml 20 mM HEPES buffer, pH 7.4. The homogenates of each sample were fractionated in 3 tubes, with 0.45 mM Ca²⁺ (control tube), and 1 mM EGTA + 2 mM L-NAME (nonspecific tube) added, respectively. Each fraction was incubated at 37°C with 0.1 µCi of [¹⁴C]-arginine and 0.5 mM NADPH. L-Valine (50 mM) was added to the reaction buffer to minimize any interference from arginase. After 15 min of incubation, samples were centrifuged for a period of 10 min at 10,000 g and the supernatant was applied to a 1-ml Dowex AG50W-X8 column (Na⁺ form) balanced with 20 mM HEPES, pH 7.4, and [¹⁴C]-citrulline was eluted with 3 ml of water. Radioactivity was measured by liquid scintillation counting. Total NOS activity was calculated as the difference between the [¹⁴C]-citrulline produced by samples with Ca²⁺ and by samples containing both EGTA + L-NAME (nonspecific). Ca²⁺-independent enzyme activity was calculated from the difference between samples containing EGTA and samples containing both EGTA + L-NAME (nonspecific). Ca²⁺-dependent activity was calculated by subtracting Ca²⁺-independent activity from total activity. As formation of L-citrulline from L-arginine is stoichiometric with respect to the formation of NO, we could assume that an equal amount of NO was formed. Enzymatic activity is expressed as pmol NO min⁻¹ 100 mg of wet tissue⁻¹. Intra- and interassay variations were less than 10%.

**Superoxide dismutase activity**

To perform SOD determination, tissue samples were previously incubated in a metabolic shaking bath under an atmosphere of 95% O₂ and 5% CO₂ for 60 min with the addition of the NO donor SN (100 µM) or the NOS blocker L-NMMA (600 µM). Tissues were homogenized in 100 mM Tris (hydroxymethyl) aminomethane (Tris-HCl) buffer. SOD activity was assayed according to the method of Yamanaka et al. (24) modified by Sun et al. (25). The method consists of determining the ability of the enzyme to inhibit the superoxide anion-mediated reduction of nitroblue tetrazolium (25 µM) to formazan. The latter was determined spectrophotometrically at 560 nm. The superoxide anion necessary for this reaction is generated by xanthine (100 µM) and xanthine oxidase (200 U/l). Enzymatic activity is expressed as units (U) per mg of dry tissue.

**Lipid peroxidation**

Lipid peroxidation, which gives origin to malondialdehyde, was carried out by the thiobarbituric acid reaction (11) as follows: tissue samples were incubated in a metabolic shaking bath under an atmosphere of 95% O₂ and 5% CO₂ for 60 min with the addition of the NO donor SN (100 µM), the ROS scavenger PEG-SOD (1 U/ml) or the NOS blocker L-NMMA (600 µM). Tissues
were homogenized in 100 mM Tris (hydroxymethyl) aminomethane (Tris-HCl) buffer, the chemical reaction was performed and peroxidation was quantified spectrophotometrically at 530 nm (26). Lipoperoxides are expressed as nmol per mg of protein.

**Statistical analysis**

Results are reported as mean ± SEM, and were analyzed by ANOVA and the Tukey-Kramer multiple comparisons test. Differences between means were considered significant if $P<0.05$.

**Results**

**Influence of SOD on pancreatic nitrate/nitrite levels**

Nitrate/nitrite levels were evaluated after incubation of pancreatic tissues in the presence of the NOS blocker L-NMMA (600 µM) and the ROS scavenger PEG-SOD (1 U/ml). Figure 1 shows that the blockade of NO synthesis is reflected by a decrease in tissue nitrate/nitrite levels in control (P<0.05) and diabetic rats (P<0.05). Nitrate/nitrite values were higher in diabetic than in control pancreas (P<0.05). When PEG-SOD was added to the incubating bath, no effects could be detected in non-diabetic tissues, but the ROS scavenger decreased the nitrate/nitrite increase due to a diabetic state (P<0.05).

**Pancreatic NOS activity: influence of SOD**

NOS activity from pancreatic tissues was evaluated after incubation of pancreatic tissues in the presence of L-NMMA (600 µM) and PEG-SOD (1 U/ml). Figure 1 shows that the blockade of NO synthesis is reflected by a decrease in tissue nitrate/nitrite levels in control (P<0.05) and diabetic rats (P<0.05). Nitrate/nitrite values were higher in diabetic than in control pancreas (P<0.05). When PEG-SOD was added to the incubating bath, no effects could be detected in non-diabetic tissues, but the ROS scavenger decreased the nitrate/nitrite increase due to a diabetic state (P<0.05).
µM on (data not shown). The influence of
the inactive stereoisomer D-NMMA was also
tested, but NOS activity was unaffected,
showing a specific inhibitory action of L-
NMMA (data not shown). The presence of
600 µM L-NMMA significantly reduced the
enzymatic activity in control and diabetic
tissues (P<0.05). The addition of PEG-SOD
improved NOS activity in control animals
(P<0.05). It seems that the increase occurred
due to a Ca\(^{2+}\)-dependent isoform. Decreased
Ca\(^{2+}\)-independent NOS levels were observed
when PEG-SOD was added to the incubat-
ing medium in pancreas from diabetic rats
(P<0.05), showing a different modulatory
profile compared to that of control animals.

**SOD activity in pancreatic tissues from
control and STZ-diabetic rats: influence of
L-NMMA and SN**

Figure 3 shows that SOD activity was
lower in diabetic than in control rats (P<0.05).
We can observe that the NOS blocker L-
NMMA (600 µM) or L-NMMA plus the NO
donor SN (100 µM) could not modify pan-
creatic SOD activity in control animals.

When L-NMMA was added to the incuba-
ting bath of diabetic tissues, an increase in SOD
level was detected (P<0.01). The addition of
L-NMMA and SN abolished the increase of
SOD activity due to the presence of L-NMMA
in the incubating medium (P<0.05). Similar
experiments were carried out with the addition of
L-NAME (1 mM), showing an increase in SOD levels, and were also carried out with L-
NAME plus SN (600 µM), showing a
decrease of enzyme activity (data not shown).
When NO production was blocked, the anti-
oxidative defense seemed to improve in pan-
creatic tissue from diabetic rats.

**Lipoperoxide levels in pancreatic tissues
from control and STZ-diabetic rats: influence
of NOS blockade and NO donors**

Lipoperoxidation was higher in diabetic
than in control tissues (P<0.05), as shown in
Figure 4.

Lipoperoxide levels were evaluated in
the presence of the NOS blocker L-NMMA
(600 µM), L-NMMA plus the NO donor SN
(100 µM) or the ROS scavenger PEG-SOD
(1 U/ml). L-NMMA, SN or PEG-SOD could
not modify pancreatic lipoperoxidation lev-
els of control animals. The addition of PEG-
SOD in the incubating medium of diabetic
tissues caused a decrease of lipoperoxides
(P<0.05). In diabetic pancreas, NOS inhibi-
tion decreased lipoperoxidation (P<0.05),
and NO generation abolished L-NMMA ef-
teffects and increased pancreatic lipoperoxide
levels (P<0.05). Similar experiments were
carried out with the addition of the NOS
blocker L-NAME, which decreased lipoper-
oxidation levels, and L-NAME plus SN (100
µM), which increased lipid peroxidation (data
not shown). The presence of NO increased
lipid peroxidation in pancreatic tissues from
STZ-treated animals.
Discussion

STZ is widely used in studies of experimental diabetes because it selectively destroys the pancreatic β-cell. A considerable body of evidence indicates that the generation of superoxide radicals may mediate the cytotoxic effect. NO overproduction has also been pointed out as a main instrument of destruction in STZ-damaged pancreatic islets. Our hypothesis is that both mechanisms are interconnected, since NO acts as a modulator of pancreatic oxidative damage in STZ diabetes mellitus.

We performed our experiments 5 days after STZ treatment, considering that this is an adequate interval to address the time point of cell necrosis when STZ is used. In this sense, Doi (27) has determined that 48 h after injection, rats were completely diabetic and microscopic examinations showed pyknosis, degranulation and marked degeneration of β-cells. Buchanan and Mawhinney (28) observed similar degeneration and necrosis, indicating that only a small number of α-cells were affected. Metabolic alterations were usually found in animals which received STZ injected 3 to 5 days earlier (29,30). Our own studies indicate that pancreatic endothelin and prostanoid levels are altered at day 5 after STZ treatment (31). Over longer periods of time, the extent of damage is higher. Gruber et al. (32) have pointed out that islets of rats that were sacrificed 10 days after STZ injection were smaller and much less frequent than those of control animals, and endocrine functions were highly affected, but some islet cells retained features of β-cells.

Pancreatic nitrate/nitrite levels from diabetic animals were higher than in control rats. NO has been proposed as a mechanism mediating the diabetogenic effects of STZ (3), and our previous studies have indicated that there is an overproduction of NO when animals are injected with STZ and diabetes mellitus develops (7,8).

The metabolic process leading to pancreatic NO generation in STZ-diabetic rats is not fully explained in the literature, and may involve the metabolism or decay of STZ (33).

The decrease in pancreatic nitrite production in diabetic animals when the tissues were incubated with PEG-SOD is unclear. We suggest that SOD might be blocking NO oxidation to nitrites/nitrates and peroxynitrates (34), scavenging STZ-generated peroxides. Our observations agree with reports about the protective role of SOD against diabetogenic drugs in vitro (35) and in vivo (5).

Our data confirm that the excess of NO production in STZ pancreatic damage involves an increased activity of NOS, whose level is higher in diabetic than in control tissues. The increase in NOS activity in diabetic tissues is the result of the induction of the Ca\(^{2+}\)-independent isoform. This agrees with McDaniel et al. (36), who stated that β-cells, selectively destroyed in diabetes, seem to express the inducible isoform of NOS and to overproduce NO, which exerts deleterious effects on their function.

Previous studies from our laboratory (8) have shown that NOS inhibition ameliorates diabetic signals in STZ-diabetic rats. The presence of the ROS scavenger PEG-SOD increases Ca\(^{2+}\)-dependent NOS activity in control tissues, while it seems to decrease Ca\(^{2+}\)-independent NOS in diabetic pancreas, showing a different modulatory profile for the two enzymatic isoforms, and resulting in an inhibition of total NOS activity in STZ-induced diabetic pancreas.

A compensatory increase of superoxide dismutase activity has been reported in several systems when an increase of superoxide anions is produced (37). In our experimental model the imbalance between oxygenated species and cellular defense mechanisms seems to be critical in influencing tissue injury. The diminished levels of SOD from STZ-induced diabetic pancreas may contri-
bute to β-cell injury. On the other hand, when diabetic tissues are assayed, inhibition of NO synthesis and the presence of NO donors increase and decrease SOD activity, respectively. In this sense, the antioxidant capacity of the diabetic pancreas seems to be related to NO levels in the tissue.

NO exerts a deleterious effect on β-cells through the inactivation of enzymes that are specifically protective against oxidative stress damage. ROS has toxic effects on membrane phospholipids, resulting in the formation of malondialdehyde. Membrane peroxidation alters membrane fluidity and permeability, and leads to a loss of membrane integrity (38). We found here that NO level and ROS scavenging also modulate lipid peroxides in pancreatic tissues from STZ-diabetic animals, increasing lipid peroxides and lowering lipoperoxidation levels, respectively.

In the present study we found that oxidative stress and NO pathway are related and seem to modulate each other, leading to β-cell destruction after STZ administration.

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References

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