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Multiple Low-Dose and Single High-Dose Treatments With Streptozotocin Do Not Generate Nitric Oxide

Gianpaolo Papaccio,[1]* **Francesco Aurelio Pisanti,**[2,3] **Michael V.G. Latronico,**[4] **Eduardo Ammendola,**[1] **and Michela Galdieri**[1]

[1]Institute of Histology and Embryology, School of Medicine, Second University of Naples, 5–80138 Naples, Italy
[2]Department of Cell Biology, University of Calabria, 87100 Cosenza, Italy
[3]Department of Clinical Pathology, ASL NA/4, 80044 Ottaviano, Naples, Italy
[4]I.N.M. Neuromed, Pozzilli, 80077 Venafro (IS), Italy

Abstract Streptozotocin (STZ) is a widely used diabetogenic agent that damages pancreatic islet β cells by activating immune mechanisms, when given in multiple low doses, and by alkylating DNA, when given at a single high dose. Actually, STZ contains a nitroso moiety. Incubation of rat islets with this compound has been found to generate nitrite; moreover, photoinduced NO production from STZ has been demonstrated. These reports have suggested that direct NO generation may be a mechanism for STZ toxicity in diabetogenesis. Several other studies have denied such a mechanism of action. This study has shown that (1) the multiple low-dose (MLDS) treatment does not stimulate NO production at the islet level; in fact, nitrite + nitrate levels and aconitase activity (also in the presence of an NO-synthase inhibitor, namely NAME) remain unmodified; RT-PCR analysis demonstrates that this treatment does not stimulate iNOS activity; (2) the high-dose (HDS) treatment does not stimulate NO production; in fact nitrite + nitrate levels remain unmodified and iNOS mRNA levels are not altered, although aconitase activity is significantly decreased. Moreover, we have confirmed that the MLDS treatment is able to decrease SOD activity by day 11 and that STZ, given in a single high dose, transiently increases superoxide dismutase (SOD) values (24 h from the administration), then dramatically lowers SOD levels. On the basis of our results, we conclude that STZ, "in vivo" is unable to generate NO, both as a MLDS or HDS treatment, thus excluding that NO exerts a role in streptozotocin-dependent diabetes mellitus. J. Cell. Biochem. 77:82–91, 2000.

Key words: nitric oxide; mitochondrial aconitase; pancreatic islets; streptozotocin

Streptozotocin (STZ) is an antibiotic produced by *Streptomyces achromogenes*. It damages islet β cells selectively by two different mechanisms. First, when given in multiple low doses (MLDS treatment) to some strains of mice, it induces an inflammation of the islets by recruitment of mononucleates from extra-islet areas with subsequent destruction of islet β cells within a few days [Like and Rossini, 1976; Kolb-Bachofen et al., 1988; Papaccio et al., 1991a, 1993a]; macrophages have been mainly implicated in this mechanism [Papaccio et al., 1991b, 1993b]; their nitric oxide (NO) production seems to be involved as well [Andrade et al., 1993]. Second, when STZ is given in a single high-dose (HDS treatment), it rapidly destroys islet β cells by a direct cytotoxic action, most probably due to alkylation, damaging DNA [Yamamoto et al., 1981]. A controversial hypothesis has been also postulated regarding the action of this compound when given in multiple low doses because, in addition to the immune cell-mediated mechanism, a synergistic direct cytotoxic action seem also to occur [Papaccio et al., 1992].

Many effects of STZ on pancreatic islets are similar to those exerted in vitro by interleukin-1β (IL-1β) and some studies indicate that IL-1β-induced islet injury [Eizirik et al., 1992; Corbett et al., 1992] as well as STZ-induced islet cytotoxicity [Kwon et al., 1994] are mediated by NO production.

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*Correspondence to: Gianpaolo Papaccio, Institute of Histology and Embryology, School of Medicine, Second University of Naples, 5 via L. Armanni, 80138 Napoli, Italy. E-mail: gpapacc@tin.it

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A protective role of NO-inhibiting drugs (e.g., L-arginine analogues) on MLDS-induced diabetes has been reported by some investigators [Kolb et al. 1991; Lukic et al., 1991], but controversial results have also been documented on this topic by others [Holstad et al., 1993; Eizirik et al., 1994; Welsh et al., 1994; Papaccio et al., 1995].

NO, generated from mammalian cells, has been considered a regulator of vasodilation [Palmer et al., 1987; Ignarro et al., 1987] and neurotransmission [Shibuki et al, 1991; O'Dell et al., 1991]. It is also toxic for islet β cells and may be produced by macrophages [Krönke et al., 1991], endothelial cells [Steiner et al., 1997], ductal cells [Pavlovic et al., 1999], or β cells themselves [Corbett et al., 1991, 1992]. Moreover, mitochondrial iron-containing enzymes (e.g., aconitase) are inactivated by NO; this accounts for the reduced cellular respiration and death of cells by macrophages [Welsh and Sandler, 1992]. It has also been demonstrated that STZ liberates NO directly within cultured islets [Turk et al., 1993]. It has been suggested that this compound would be a useful NO generator, indicating that direct NO generation may be a mechanism of STZ toxicity in diabetogenesis [Kwon et al., 1994]. Moreover, recent studies yielded evidence that isolated islets from inducible nitric oxide synthase (iNOS)-deficient mice were susceptible to the in vitro deleterious effects of STZ and that iNOS may contribute to islet β-cell damage after MLDS treatment in vivo [Flodström et al., 1999]. Other studies have shown just the reverse; that is, that NO is not involved in initiating insulin secretion from the islets of Langerhans [Jones et al., 1992; Welsh et al., 1994; Rabinovitch et al., 1994].

The aim of this study is to clarify if the direct NO generation is the major mechanism of toxicity in both multiple low and single high-dose models of STZ-induced diabetes. Our data demonstrate that NO is not involved in the MLDS or HDS treatments.

MATERIALS AND METHODS

Animals and General Design

Male C57Bl6/J mice, bred in our facility, fed ad libitum, age 8 weeks, weighing 25–30 g, were used for the experiment. Diabetes was induced using 40 mg/kg body wt streptozotocin (STZ) (Sigma Chemical Co., Milan, Italy), freshly prepared daily and given for 5 consecutive days, using the Like and Rossini schedule [1976] (MLSD treatment) or using a single high dose of 75 mg/kg body wt (HDS treatment) of the same compound, dissolved immediately before administration in a 0.025 mol/L sodium citrate-buffered solution at pH 4.0.

Animals were subdivided into the following groups: group 1 (n = 20) MLDS-treated; group 2 (n = 20) HDS treated. In addition to receiving the MLDS and HDS treatment, respectively, group 3 (n = 20) and 4 (n = 20) animals were pretreated with an inhibitor of NO-synthase (L-arginine analogue), namely N-nitro-L-arginine methylester (NAME) (Sigma) in 0.5 ml of phosphate-buffered saline (PBS). This pretreatment was given intraperitoneally at 15 mg/mouse, in three daily doses, just before STZ treatment (in order to avoid interactions with STZ), for 10 consecutive days (NAME-MLDS, group 3; NAME-HDS, group 4). Untreated controls (group 5, n = 8 for MLDS; n = 8 for HDS) were administered only the vehicle.

Blood glucose levels were tested on day 0, before STZ treatment, then every 2 days and on the day of killing, using the hexokinase method (Boehringer-Mannheim, Germany). Animals were considered hyperglycemic when their blood glucose levels were >8 mmol/L but <12 mmol/L in two successive determinations. Mice were considered diabetics when their blood glucose levels exceeded 12 mmol/L.

Animals were sacrificed according to the following schedule: MLDS-treated and NAME-MLDS-treated mice on days 6, 11, 15, and 21 (n = 5/day + 2 controls/day); HDS-treated and NAME-HDS-treated mice after 12, 24, and 48 h; and after 5 days (n = 5/time point + 2 controls/time point).

When scheduled, animals were put under ether anesthesia and killed by decapitation, and the pancreas was removed. Pancreatic tail fragments belonging to groups 1 (MLDS-treated) and 3 (MLDS + NAME-treated) and to MLDS controls were used for histological examination. The remaining part of the glands of these groups and the pancreas from the other groups were used for islet isolation. Islets were isolated by collagenase type IV digestion (Sigma) and density-gradient Ficoll centrifugation. After isolation, islets were precultured for 3–7 days in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (FCS) at 37°C in humidified atmospheric air. Then, at least 150 islets/300 µl medium from each pancreas fragment were incubated for 24 h in RPMI 1640 supplemented with 0.2% bovine serum albumin

(BSA); the culture was continued for 6 days, with the medium changed after 3 days. All the islets were pooled for each time point and then used for assays, as specified below.

Histology

Pancreas tail fragments from MLDS controls; group 1 and 3 mice were dissected free of fat and fixed in 10% formalin for 24 h, paraffin-embedded, and sectioned in serial sections 5 µm thick. Slides were stained with hematoxylin & eosin (H&E); in each pancreas, at least 20 islets were evaluated blindly for lymphocyte infiltration grading score as follows: 0 = no infiltration; 1 = minor (focal) infiltration; 2 = minor peri-islet infiltration); 3 = intra-islet infiltration; and 4 = islet atrophy with cytoarchitectural derangement.

Nitrite and Nitrate Determination

Isolated islets (at least 150/condition) were incubated for 30 min at 37°C under 95% air/5% CO_2 in KRB medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3 mM D-glucose, 0.1% BSA) to a final concentration of 1^{-10} mM. Medium nitrite content was measured after conversion of nitrate to nitrite with *Aspergillus* nitrate reductase (Sigma). Nitrite was then measured spectrophotometrically (540 nm by a Gilford spectrophotometer) after mixing medium (0.1 ml) with Griess reagent (0.1 ml of a solution of 1 part of 1.32% sulfanilamide in 60% acetic acid and 1 part of 0.1% naphthyl ethylene diamine HCl in water) and incubation for 10 min at room temperature. The concentration of nitrite plus nitrate was measured using a Cu^{2+}-Cd column to reduce nitrate to nitrite (automated procedure) [Feelisch and Noack, 1987]. All determinations were made in triplicate.

Isolation of RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

RNA was extracted from pancreatic islets, using the guanidinium thiocyanate method [Chirgwin et al., 1979], modified as follows. Islets were homogenized in 4-mol/L guanidinium thiocyanate solution containing 17 mmol/L sodium N-lauroylsarcosine, 25 mmol/L sodium citrate, 0.1 mol/L 2-ME, and 0.1% Antifoam A, 30% aqueous emulsion (Sigma), then precipitated with ethanol, pelleted, and re-extracted with 8 mol/L guanidine hydrochloride:0.5 mol/L EDTA (19:1). After pelleting and drying, samples were extracted twice with phenol/chloroform (1:1) and precipitated with ethanol. cDNA synthesis was carried out on total RNA from each animal with Superscript reverse transcriptase (RT) kit (Life Technologies, Gibco-BRL, Milan, Italy), using oligo$(dt)_{12–18}$ and Moloney murine leukemia virus RT (20 U) in a 25-µl reaction at 37°C for 1.5 h. The RT reaction containing cDNA was diluted 1:30, 1:90, and 1:270 in sterile H_2O. Polymerase chain reaction (PCR) amplification was carried out on the cDNA from each animal, using 3 µl of each dilution of cDNA in a 20-µl reaction with 80 ng of each primer, 0.25 mmol/L of each dNTP, 2.5 µCi of [α-^{32}P] dCTP (3,000 Ci/mmol; DuPont-NEN, Milan, Italy), 1 U of AmpliTaq (Perkin-Elmer-Cetus, Monza, Italy), and 3 mmol/L Mg^{2+}. The sequences of the specific oligonucleotide primer pairs, 5′ and 3′, are as follows: for iNOS, AGC TTC TGG CAC TGA GTA AAG ATA A and TTC TCT GCT CTC AGC TCC AAG; and for cyclophilin, GAC AGC AGA AAA CTT TCG TGC, and TCC AGC CAC TCA GTC TTG G-3′. Samples were amplified through 40 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s in a Gene Amp PCR System 9600 (Perkin-Elmer-Cetus). The PCR reaction was electrophoresed on 1.5% agarose gels and transferred to nylon membranes, and ^{32}P incorporation in cytokine and cyclophilin DNA bands was determined by phosphoimager analysis. The values obtained of PCR product were normalized as a percentage of ^{32}P incorporated in cyclophilin PCR product amplified from the same cDNA preparation. In these experiments, the template used for the PCR amplification was cDNA from NOD splenocytes activated with concanavalin A (Con A) for 3 days to express the different cytokine messages. Also, this cDNA was used as a positive control in all PCR runs. Under the conditions used, the PCR product signal was proportional to the amount of RNA/cDNA subjected to PCR amplification. All PCR products compared were produced in the same PCR run.

Mitochondrial Aconitase Activity

Islets (≥3,000/condition) were dispersed into individual cells with dispase (0.25 mg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's balanced saline solution (HBSS) (15 min at 31°C) [Corbett et al., 1992], filtered (60-µm nylon screen), and placed in medium CMRL-1066 (Gibco) supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin.

They were then incubated (18 h at 37°C), isolated by centrifugation (800*g*, 4°C), resuspended in buffer (5 ml of 250 mM sucrose, 20 mM Hepes, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EGTA, pH 7.4), permeabilized with digitonin (0.007%, 30 min on ice), agar isolated by centrifugation, lysed by treatment with Triton X-100, 30 mM NaCl, 30 mM Tris HCl, pH 7.4, and centrifuged (5,000*g* 4°C, 15 min). Supernatant aconitase activity was measured spectrophotometrically (340 nm, 21°C in 20 mM citrate, 0.5 mM NADP, 0,5 mM $MnCl_2$, 50 mM Tris-Cl, pH 7.4, 1 U isocitrate dehydrogenase, total volume 1 ml).

Superoxide Dismutase Activity

Isolated islets were prepared according to Grankvist et al. [1981]. When insulitis occurs, the infiltrating mononuclear cells form a translucent capsule around the islet, which can be easily distinguished and microdissected away. Isolated islets or spleen were homogenized (Ultra Turrax mechanical blender) in 100 vol of 10 mM potassium phosphate buffer (pH 7.4) supplemented with 30 mM KCl. The homogenates were then sonicated (Sonifier Branson B12, 1 min at 4°C) and left for 30 min at 4°C to solubilize both CuZn-SOD and Mn-superoxide dismutase (SOD) from the tissue. After centrifugation at 20,000*g* for 30 min at 4°C, the supernatant was removed and stored at −70°C. The Harboe method [1979] for the determination of hemoglobin content of tissue homogenates was used. Because of the absence of hemoglobin, there was no need for correction of enzyme activity. The concentration of proteins was determined by Lowry's method [1951]. Measurement of SOD activity (done in triplicate with an intravariation coefficient of 2.7%) employed in this study was based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol as described by Marklund and Marklund [1974]. Results are given as units per mg of protein. The detection limit was found to be 2 U/ml. SOD levels were detected at the same time points as for glycemia.

Statistical Analysis

Student's *t*-test and analysis of variance (ANOVA) were used for statistical analysis. The level of significance was set at $P < 0.05$.

RESULTS

1. *Glycemic values are increased after both HDS and MLDS treatment. NAME administration fails to counteract diabetes progression*: Data are shown in Table I. Control animals are euglycemic throughout the experiment. MLDS treatment increases glycemic levels from day 15 after the first STZ injection. By day 18, animals are considered overtly diabetic with values exceeding 11 mmol/L. At day 21, glycemic values are further increased. Beneficial effects of the L-arginine derivative compound are not seen with NAME-MLDS treatment: their blood glucose levels are similar to those found in MLDS-treated animals. HDS treatment dramatically enhances glycemia 24 h after the injection of STZ. NAME-HDS-treated animals show slightly lower blood glucose levels with respect to those found in HDS-treated animals, but NAME does not significantly counteract the effects of STZ on glycemia.
2. *Insulitis progression is not counteracted by NAME in MLDS-treated animals*: Data are shown in Table II. By day 11, MLDS-treated

TABLE I. Nonfasting Blood Glucose Levels†

	Treatment		
Day	Controls	MLDS	MLDS-NAME
0	5.39 ± 0.35	5.43 ± 0.28	5.37 ± 0.30
6	5.46 ± 0.34	6.22 ± 0.24	5.93 ± 0.27
11	5.44 ± 0.28	7.12 ± 0.28*	7.44 ± 0.33*
15	5.36 ± 0.30	7.69 ± 0.41*	7.76 ± 0.32*
18	5.44 ± 0.26	12.1 ± 0.81**	12.2 ± 0.82**
21	5.50 ± 0.30	18.0 ± 1.54**	17.8 ± 1.2**
	Controls	HDS	HDS-NAME
0	5.19 ± 0.55	5.37 ± 0.34	5.4 ± 0.30
1	5.26 ± 0.64	20.82 ± 1.81**	15.46 ± 2.46**
5	5.34 ± 0.38	24.9 ± 1.52**	20.21 ± 2.96**
15	5.20 ± 0.30	30.22 ± 1.09**	24.04 ± 3.93**

†Values (mmol/L) are expressed as means ± SD. Blood glucose was tested in all animal groups as indicated under Methods.
*$P < 0.01$ vs day 0, 6, and vs controls.
**$P < 0.0001$ vs day 0 and vs controls.

TABLE II. Mean of Insulitis Grading Scores*

Day	Controls	MLDS	MLDS + NAME
5	0	0.5 ± 0.5	0.5 ± 0.5
11	0	1.3 ± 0.3	0.9 ± 0.3
15	0	1.9 ± 0.3	2.1 ± 0.3
21	0	3.0 ± 0.5	3.0 ± 0.8

*Insulitis was observed in pancreas tail fragments belonging to MLDS controls (n = 10), group 1 (MLDS-treated, n = 20), and group 3 (HDS-treated, n = 20) animals.

animals show a focal peri-insulitis and by day 15 a peri-insulitis (Fig.1). By day 18, infiltrating cells are found scattered within the islet parenchyma, which is clearly and massively infiltrated by day 21 (Fig. 2). NAME does not show effects upon the progression of insulitis in the MLDS treatment.

3. *RT-PCR analysis shows that iNOS are not stimulated by both MLDS and HDS treatment*: Expression of iNOS mRNA is very low in MLDS treated islets (Fig. 3). Moreover, iNOS mRNA expression is not detected in HDS-treated mice, as well as in untreated control C57BL6/J animals (Fig. 3). Data are homogeneous and clearly demonstrate that STZ completely fails to induce iNOS mRNA expression from isolated islets of mice.
4. *Nitrite + nitrate levels are not enhanced after MLDS or HDS treatment*: Levels of nitrite + nitrate are not increased after

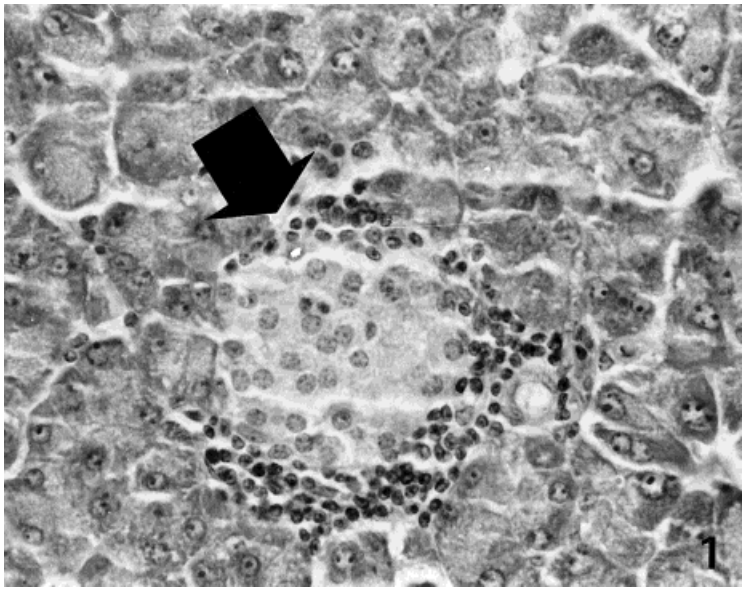

Fig. 1. Light micrograph showing a peri-insulitis (arrow) in an MLDS-treated animal at day 15. ×380.

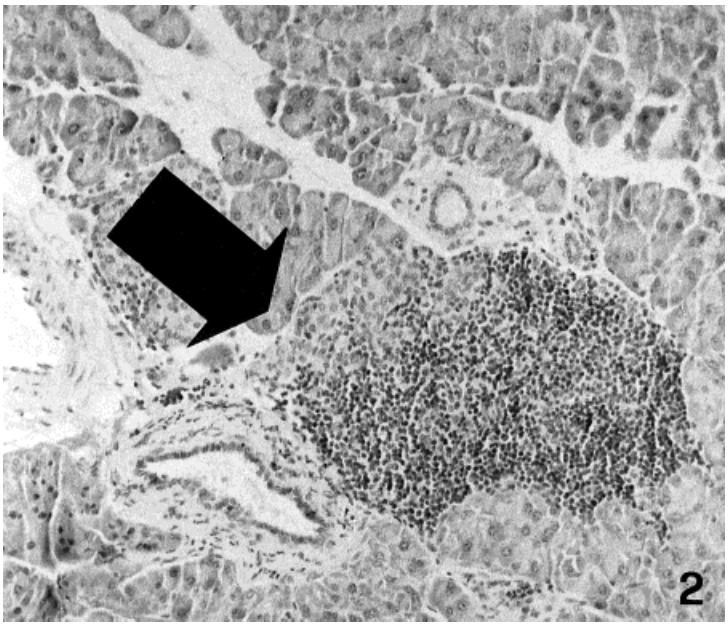

Fig. 2. Light micrograph showing an intraislet insulitis and parenchymal derangement (arrow) in an MLDS-treated animal at day 21. ×320.

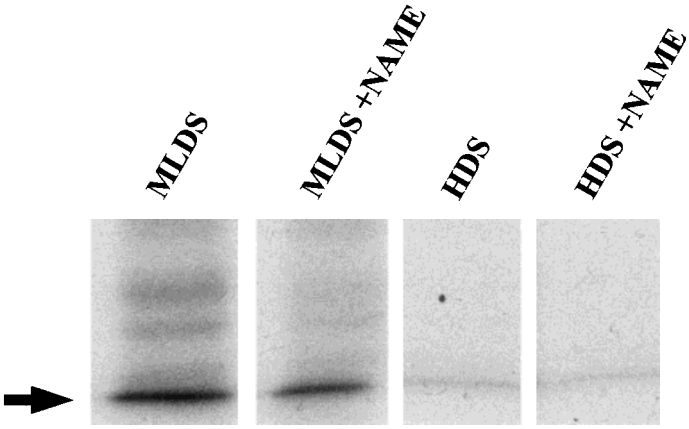

Fig. 3. Fig. showing iNOS mRNA detected by PCR analysis in isolated islets of MLDS-, MLDS + NAME-, HDS-, and HDS + NAME-treated mice. iNOS mRNA levels (PCR product) are expressed as a percentage of the cyclophyllin PCR product amplified from the same cDNA preparation (see under Materials and Methods).

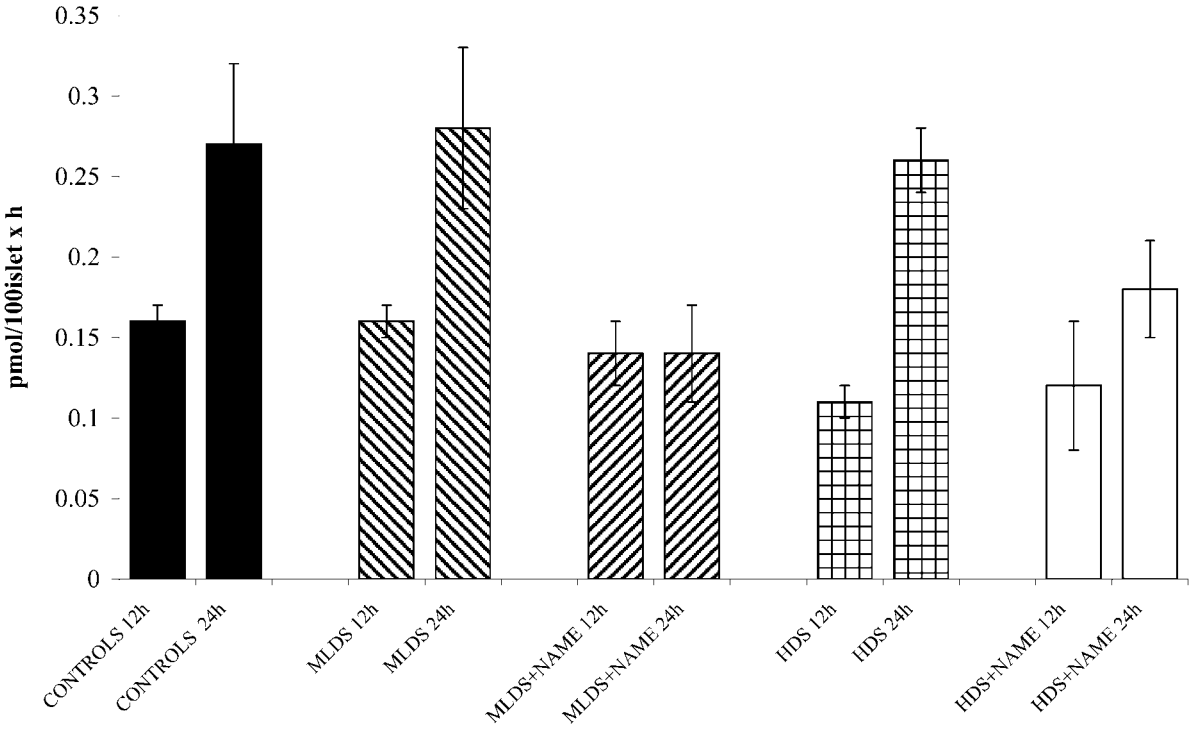

Fig. 4. Nitrite + nitrate production. Data are presented as means ±SD (pmol/100 islet × h). Both HDS and MLDS treatment do not change nitrite + nitrate values. The addition of NAME exerts only slight effects.

HDS administration, as compared with controls. NAME supplementation further lowers these values (Fig. 4). MLDS administration is unable to affect nitrite + nitrate levels, whose values are found similar to those seen in controls. NAME-MLDS treatment decreases nitrite + nitrate levels, when compared to those found in controls (Fig. 4), which express a constitutive form of NO, as known.

5. *Mitochondrial aconitase activity is significantly decreased after HDS treatment. NAME is able to counteract this increase*: Mitochondrial aconitase activity is significantly decreased in HDS-administered animal islets (Fig. 5). In addition to the HDS treatment, NAME treatment is able to counteract this decrease significantly. MLDS treatment does not significantly change the aconitase activity with respect to control values, while

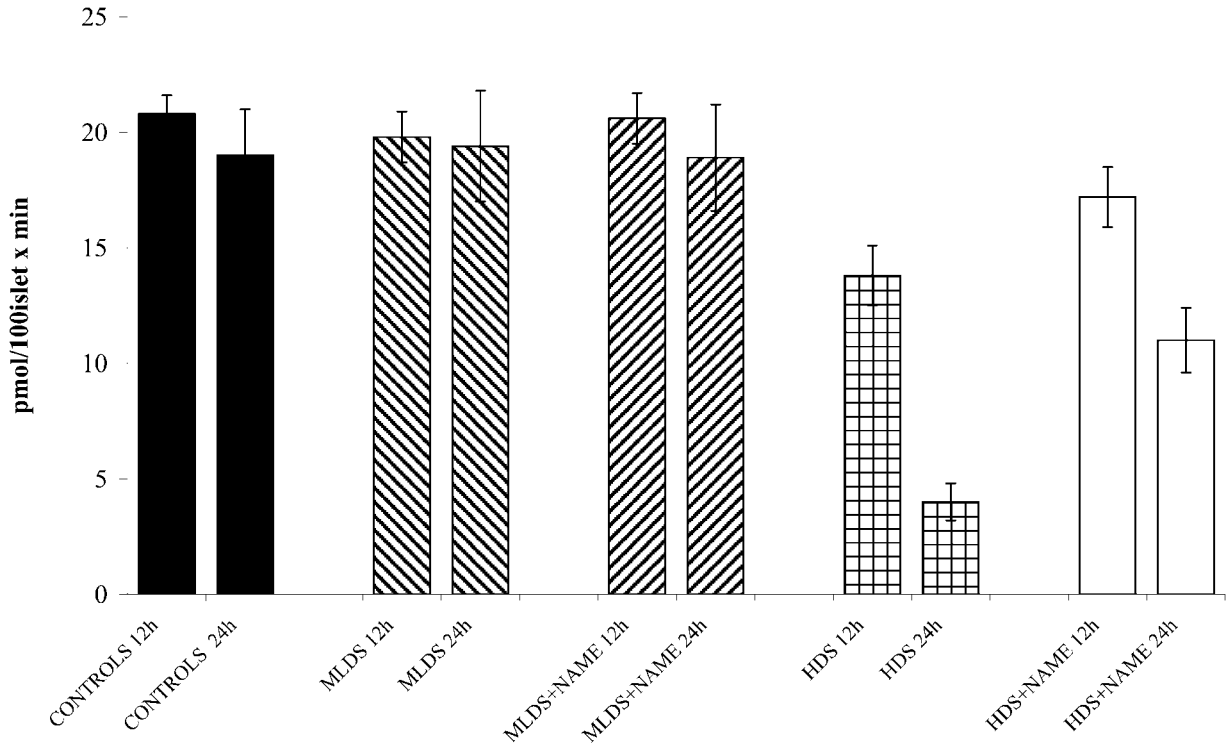

Fig. 5. Aconitase activity. Data are presented as means ±SD (pmol/100 islet × h).

NAME, added to MLDS-administered animals also does not produce significant changes in its activity (Fig. 5).

6. *Superoxide activity is significantly lowered by both MLDS and HDS treatment*: Levels of SOD are considerably decreased in MLDS-treated animals by day 11 (Fig. 6). This decrease can be observed up to day 18, when SOD levels are extremely low. NAME treatment, in addition to the MLDS administration, does not show appreciable effects on the said decrease of SOD levels. HDS-administered animals show a transient, but significant increase of SOD levels within 24 h after treatment (Fig. 7). In fact, after this increase, a dramatic fall of SOD levels is observable. Also in this case, NAME is not capable of counteracting the STZ effects on the SOD values (Fig. 7).

DISCUSSION

Involvement of NO in STZ-induced diabetes is puzzling and is interpreted differently by different investigators. Several studies have demonstrated its direct involvement; many others show just the contrary. A possible involvement in particular experimental conditions appears to be possible albeit, not crucial, in the events leading to islet β-cell destruction, but the direct involvement both in vivo and in vitro under normal experimental conditions has not been fully demonstrated.

This study has demonstrated that (1) MLDS treatment does not stimulate NO production at the islet level or, in other words, MLDS treatment does not appear to act against islet β-cells by NO production; this is clearly shown by the findings that nitrite + nitrate levels and aconitase activity (also in the presence of NAME) remain unmodified and by RT-PCR analysis, which demonstrate that this treatment does not stimulate, in normal experimental conditions, iNOS activity; and (2) HDS treatment does not stimulate NO production; after a single high dose of STZ nitrite + nitrate levels remain unmodified and iNOS mRNA levels are not altered, although aconitase activity is significantly decreased.

Moreover, we have confirmed that STZ, when given in multiple low doses (MLDS treatment), decreases SOD activity by day 11. We have also shown that, when given in a single high dose, STZ first, transiently increases SOD values (24 h from the administration), then dramatically lowers SOD levels.

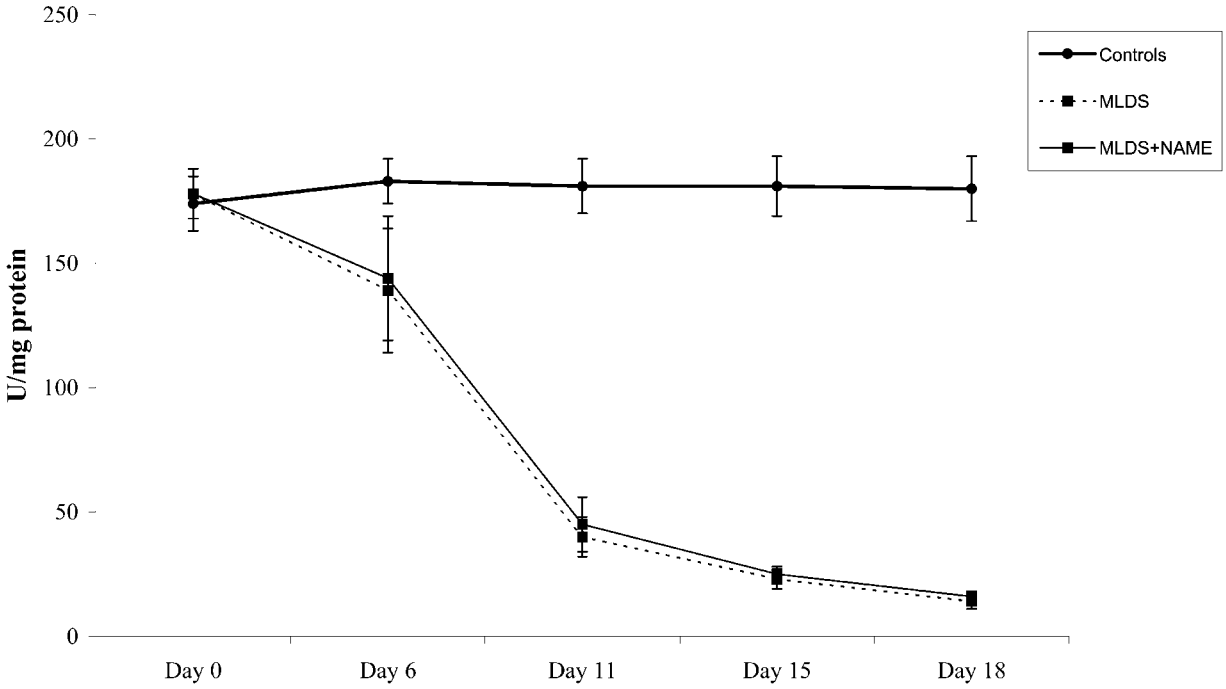

Fig. 6. Fig. showing superoxide dismutase (SOD) values in MLDS-treated animals. Data are presented as U/mg proteins and are means ±SD. MLDS mice showed decreased SOD levels by week 11.

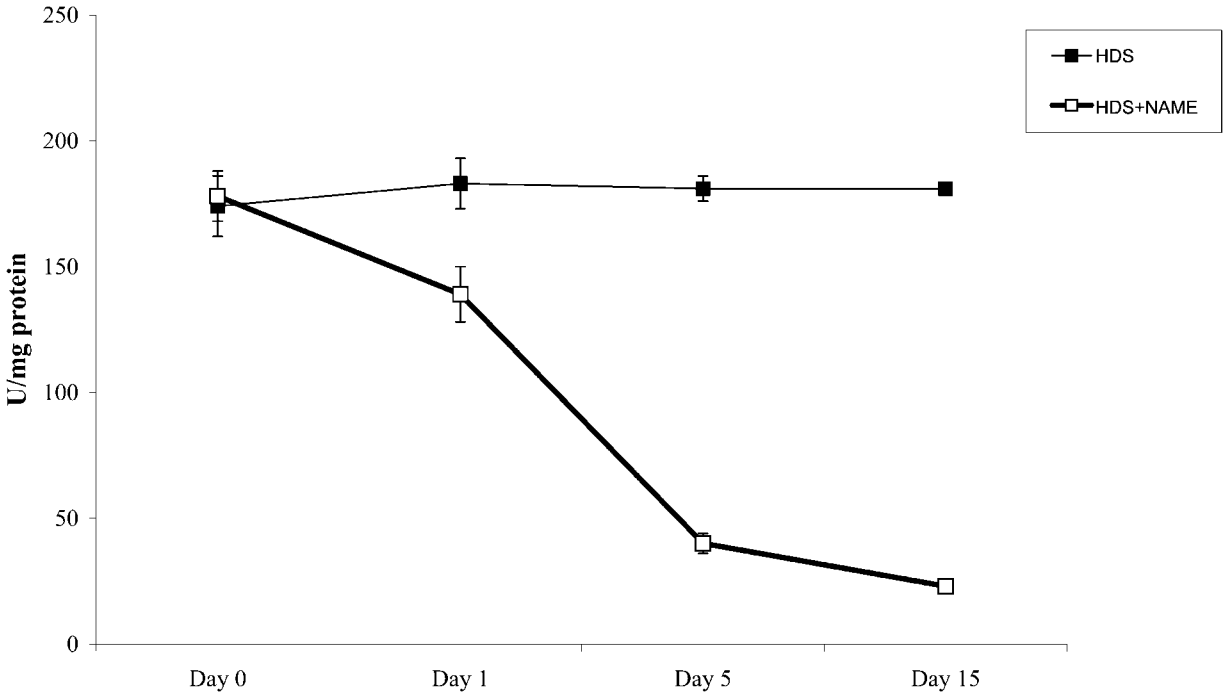

Fig. 7. Superoxide dismutase (SOD) values in HDS-treated animals. Data are presented as U/mg proteins and are means ±SD. HDS-treated mice showed a transient increase of SOD levels (24 h after treatment), after which values fall dramatically.

On the basis of our results, NO is not involved in the mechanism of action of MLDS-induced diabetes. Our findings seem to disagree with recent results of Flodström et al. [1999], which have shown that NO is one of the mediators used by the immune system to destroy islet β cells. As previously reported, opinions regarding this argument are totally in contrast. We think that the main reason and explanation of the disagreement may regard the use of the animals and the experimental conditions. Actually, Flodström et al. [1999], in their experiments used inducible nitric oxide synthase (iNOS)-deficient mice, which were found highly susceptible to the in vitro deleterious effects of STZ, thus leading to the consideration that iNOS may contribute to islet β-cell damage after MLDS treatment in vivo. Here, in normal experimental conditions we show that NO not only is not involved in the MLDS mechanism of action but also that iNOS mRNA are not stimulated. MLDS treatment was previously reported to be mediated by macrophages [Kolb-Bachofen et al, 1988; Papaccio et al., 1991a; Papaccio and Esposito, 1992] and several reports have shown that these cells may act as cytotoxic cells via NO. Our present data appear to be in contrast with these findings, but we believe that macrophages could effectively be involved in MLDS-induced diabetes and their role, also as cytotoxic elements, may be confirmed, taking into consideration that they may act by producing other cytotoxic agents and by critically reducing the levels of SOD. Moreover, we previously showed that, in MLDS diabetes, direct cytotoxic action of STZ against islet β can be observed [Papaccio et al., 1991b, 1992], and that the "immunity" is also "not obligatory" [Papaccio et al., 1992], but only one mechanism involved in its rather complex activity. Indeed, further mediators of cytotoxicity may be the numerous structures involved in islet derangement, such as endothelia and ducts. With regard to the finding of NO, several other investi gators have expressed their critiques and doubts and have clearly written that NO should not be considered an answer to insulin-dependent diabetes mellitus [Welsh and Sandler, 1994].

Therefore, the recent findings of Flodström et al. [1999], as well as the present study, may be regarded as contributions to the understanding of the exact mechanism of action of STZ, which remains largely unknown.

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