Reduced Sensitivity of Inducible Nitric Oxide Synthase–Deficient Mice to Multiple Low-Dose Streptozotocin-Induced Diabetes

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Nitric oxide (NO), synthesized by the inducible isoform of nitric oxide synthase (iNOS), has been proposed as a mediator of immune-induced β-cell destruction in type 1 diabetes. To evaluate the role of iNOS for β-cell dysfunction and death, we investigated the sensitivity of β-cells from mice genetically deficient in this enzyme (iNOS$^{-/-}$, background C57BL/6x129SvEv, H-2^{b}) both to interleukin (IL)-1β–induced β-cell dysfunction in vitro and to multiple low-dose streptozotocin (MLDS)–induced diabetes in vivo. Exposure of islets isolated from C57BL/6 mice to IL-1β for 24 h in vitro resulted in an induction of iNOS mRNA expression, an increase in nitrite formation, and a decrease in insulin release and proinsulin biosynthesis as compared with untreated C57BL/6 islets. IL-1β failed to induce iNOS mRNA expression and increase nitrite formation by islets isolated from iNOS knockout mice (iNOS$^{-/-}$), and no impairment in islet function was observed. The iNOS$^{-/-}$ mice showed a reduced incidence of hyperglycemia after treatment with MLDS as compared with wild-type C57BL/6 (H-2^{b}) and 129 SvEv (H-2^{b}) mice. On day 21 after the first streptozotocin (STZ) injection, 75% of the C57BL/6 mice and 100% of the 129SvEv mice had blood glucose levels >11 mmol/l, whereas the corresponding number for iNOS$^{-/-}$ mice was only 23%. This protection was not due to a delay in the onset of hyperglycemia, since no increase in number of hyperglycemic iNOS$^{-/-}$ mice was observed when the animals were followed up to 42 days. Moreover, islets isolated from iNOS$^{-/-}$ mice were susceptible to the in vitro deleterious effects of STZ. In conclusion, the present study provides evidence that iNOS may contribute to β-cell damage after exposure to IL-1β in vitro and treatment with MLDS in vivo. *Diabetes* 48:706–713, 1999

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BG, blood glucose; BSA, bovine serum albumin; FCS, fetal calf serum; IL, interleukin; iNOS, inducible isoform of nitric oxide synthase; KRBH, Krebs-Ringer bicarbonate buffer solution; MLDS, multiple low-dose streptozotocin; NOS, nitric oxide synthase; PCR, polymerase chain reaction; RT, reverse transcriptase; STZ, streptozotocin; TCA, total protein biosynthesis.

A hallmark of type 1 diabetes is the selective destruction of the insulin-producing pancreatic β-cells (1). Although the etiopathogenesis of the disease remains largely unknown, it is generally hypothesized that β-cells are destroyed via an immune-mediated process. In the NOD mouse and BB rat, a mononuclear cell infiltration of the pancreatic islets precedes β-cell destruction, and it has been suggested that the infiltrating cells, either directly or through the release of inflammatory mediators such as cytokines and free radicals, are responsible for β-cell damage and death (2,3).

The radical nitric oxide (NO) is a molecule that regulates complex, physiological functions, such as memory formation, vascular tone, and immunological host-defense reactions (4,5). Large amounts of NO, however, may cause tissue damage, and in recent years, expression of the inducible nitric oxide synthase (iNOS) has been connected with autoimmune diseases (6,7). iNOS is expressed by islet β-cells and/or macrophages during insulitis in the NOD mouse and BB rat (8–10). Moreover, isolated intact pancreatic islets or purified β-cells express iNOS mRNA when exposed to proinflammatory cytokines in vitro (11). By using pharmacological inhibitors of nitric oxide synthase (NOS), it has been concluded that several of the deleterious actions of cytokines on rodent islet functions in vitro are mediated by NO formation (12). Some in vivo studies with animal models of type 1 diabetes have shown a delay or prevention of the outbreak of disease after treatment with NOS blockers (13–17). On the other hand, recent reports have highlighted the involvement of NO-independent mechanisms for cytokine-induced β-cell damage in vitro (18–22), and several studies failed to reproduce the protection by NOS inhibitors against development of diabetes in rodents (23–26). One possible explanation for these discrepant in vivo results is the lack of specificity of NOS pharmacological inhibitors, which to a varying degree block both iNOS and the constitutive isoforms of the enzyme (cNOS and eNOS) (27), leading to uncontrolled side effects on blood pressure, neurotransmission, body weight, etc., all of them possibly interfering with the diabetogenic process.

To circumvent the limitations inherent in the in vivo use of NOS inhibitors, we presently used a mouse deficient in iNOS (iNOS$^{-/-}$) (28) to reevaluate the role of NO for immune-mediated β-cell damage. More specifically, we tested the iNOS$^{-/-}$ mouse sensitivity to *1*) hyperglycemia induced by the injection of multiple low doses of streptozotocin (MLDS) and *2*) the damaging effect of IL-1β on islet β-cell function in vitro.

RESEARCH DESIGN AND METHODS

Animals. Breeding pairs of mice deficient in iNOS were kindly provided by J.S. Mudgett (Merck Research Laboratories, Rahway, NJ) and J.D. MacMicking and C. Nathan (Cornell University Medical College, New York, NY). The mice (background C57BL/6Jx129SvEv, H-2^b) were generated by gene targeting in embryonic stem cells as previously described (28). Homozygous iNOS mutants were maintained by interbreeding the F2 generation. The mice have subsequently been bred under specific pathogen-free conditions in the Animal Department, Biomedical Centre (Uppsala, Sweden). For wild-type controls, male C57BL/6 (H-2^b, in vitro and in vivo studies) (BK-Universal, Sollentuna, Sweden, and Bomholtgaard, Ry, Denmark) and male 129SvEv (H-2^b, in vivo studies) (Taconic, Germantown, NY) were used. Mice were 8–10 weeks when experiments were initiated. In the text, the iNOS-deficient mice have been designated by the abbreviation iNOS$^{-/-}$ and the wild-type (iNOS$^{+/+}$) mice as C57BL/6 and 129SvEv, respectively.

In vitro studies: isolation and culture of pancreatic islets. Mice were killed by cervical dislocation, and islets were isolated from collagenase-digested pancreatic tissue. Islets were cultured free-floating in medium RPMI-1640 supplemented with 10% fetal calf serum (FCS, vol/vol), streptomycin (0.1 mg/ml), benzylpenicillin (100 U/ml), and 11.1 mmol/l glucose (29). Islet cultures were kept at 37°C and subjected to medium changes every second day. All islet preparations were cultured for 6–7 days before exposure to IL-1β. For the latter purpose, human recombinant IL-1β (25 U/ml; kindly provided by Klaus Bendtzen, Copenhagen, Denmark) was added for 24 h.

Analysis of medium nitrite contents. Medium contents of nitrite was determined using the Griess reagent (30). Briefly, 10 µl of freshly prepared Griess reagent (0.5% naphtyletylenediamine dihydrochloride, 5% sulphanilamide, and 25% H_2PO_4) was added to a 100-µl aliquot of culture medium. After a 2-min incubation at 60°C, the reaction product was determined spectrophotometrically (λ = 546 nm). Medium that had been incubated without cells was used as blank. All determinations were performed in triplicate, and a standard curve with known concentrations of nitrite was used to calculate the amount of nitrite in each sample. The intra- and interassay coefficients of variation were <15% and the lower detection limit was 3 pmol/100 µl medium.

Isolation of RNA and reverse transcriptase-polymerase chain reaction analysis. For gene expression analysis, polyA$^+$RNA was isolated from 70–80 islets using oligo(dT)25-coated polystyrene Dynabeads (DYNAL, Oslo, Norway). Reverse transcription was performed using the GeneAmp RNA PCR kit (Perkin-Elmer, Norwalk, CT). The reaction mixture contained mRNA equivalent to 3 × 10^3 cells, 10 mmol/l Tris-HCl (pH 8.3), 5 mmol/l $MgCl_2$, 50 mmol/l KCl, 1 mmol/l dNTP, 2.5 µmol/l random hexamer primers, 1 U/µl RNase inhibitor, and 2.5 U/µl M-MLV reverse transcriptase (RT), and the transcriptions were performed according to the description of the manufacturer. Polymerase chain reaction (PCR) reactions contained 5 µl cDNA, 2 mmol/l $MgCl_2$, 0.4 mmol/l of each primer, 0.2 µmol/l of each dNTP, and 0.625 U AmpliTaq DNA polymerase (Perkin Elmer). Samples were subjected to a first denaturation step of 5 min at 94°C, whereafter cycles were run with denaturation at 94°C for 45 s; annealing at 58°C for 45 s; extension at 72°C for 90 s. In the last cycle, the extension step was prolonged to 10 min. To exclude for contamination of genomic DNA, PCR amplifications without previous RT amplifications were run in parallel. GAPDH expression was used as an internal control in all experiments (31). The PCR reactions were run for 30 and 33 cycles to detect GAPDH and iNOS mRNA expression, respectively. The following primers were used for detecting GAPDH; F: 5′-TCCCTCAA GATTGTCAGCAA-3′; R: 5′-AGATCCACAACGGATACATT-3′ and iNOS; F: 5′-GACTGCACAGAATGTTCCAG-3′; R: 5′-TGGCCAGATGTTCCTCTATT-3′. PCR reactions were separated by electrophoresis in ethidium bromide–containing agarose gels, and the gels were photographed under ultraviolet transillumination.

Determinations of medium insulin accumulation, glucose-induced insulin release, insulin, and DNA contents. Insulin accumulating in the culture medium was determined by radioimmunoassay (32). To determine acute insulin release, duplicate groups of 10 islets previously exposed to IL-1β or control condition (no IL-1β added) for 24 h were preincubated for 30 min in 100 µl Krebs Ringer bicarbonate buffer solution (KRBH, pH 7.4), supplemented with 2 mg/ml bovine serum albumin (BSA), 10 mmol/l HEPES, and 1.7 mmol/l glucose, followed by a 90-min incubation in KRBH solution containing 1.7 or 16.7 mmol/l glucose, respectively. Insulin released during the 90 min was then measured. To determine islet insulin and DNA contents, the islets were ultrasonically disrupted in redistilled water. An aliquot of the homogenate was mixed with acid ethanol, and insulin was extracted at 4°C before analysis. A second aliquot was used to determine DNA contents by the fluorometric method described by Hinegardner (33).

Total protein and proinsulin biosynthesis. Duplicate groups of 10 islets, previously exposed to IL-1β or control condition for 24 h, were cultured during 90 min in 100 µl KRBH solution containing 1.7 or 16.7 mmol/l glucose, 2 mg/ml BSA, and 50 µCi/ml L-[4,5-^{3}H]leucine. Islets were rinsed once in Hanks' solution supplemented with 10 mmol/l nonradiolabeled L-leucine and ultrasonically disrupted in redistilled H_2O. Total protein biosynthesis rates (TCA) were determined by trichloroacetic acid precipitation, and the levels of proinsulin biosynthesis were estimated by the immunoprecipitation method described by Halban et al. (34).

In vitro toxicity of streptozotocin. Isolated cultured islets from C57BL/6 or iNOS$^{-/-}$ mice, in groups of 50, were preincubated in sterile 1 ml KRBH solution containing 5.6 mmol/l glucose for 30 min at 37°C. Then 1.5 mmol/l streptozotocin (STZ) or 10 µl citrate buffer (vehicle) was added for another 30 min. The incubation was interrupted by the addition of 2 ml KRBH solution, and the islets were transferred to culture dishes containing RPMI-1640 + 10% FCS. After culture for 18 h, the islets were harvested and homogenized in water, and the insulin and DNA content was determined as described above.

In vivo studies. Male mice were treated with intraperitoneal injections of streptozotocin (STZ; 40 mg/kg body wt, dissolved in citrate buffer, pH 4.5) or vehicle (citrate buffer, pH 4.5) during five consecutive days (35). Day 1 was defined as the day of the first injection of STZ. Blood glucose concentrations were measured in blood obtained from the tail vein on days 0, 4, 7, 10, 14, and 21, and in one additional experimental series, also on day 42, using ExacTech blood glucose meter (Baxter Travenol, Deerfield, IL). Hyperglycemia was defined as a nonfasting blood glucose level higher than 11.1 mmol/l. The cumulative incidence of hyperglycemia in the different strains of mice was calculated as the percentage of hyperglycemic mice among treated mice at each time point.

Histology. Pancreases from mice killed on day 21 were dissected free of fat and surrounding tissues, fixed in 10% formalin for 24 h, paraffin-embedded, and sectioned in 5-µm thin sections. Immunostaining was performed with an anti-bovine insulin antibody (1:200; ICN Pharmaceuticals, Costa Mesa, CA) and a peroxidase-anti peroxidase/diamino benzidine detection system (DAKO, Glostrup, Denmark and Kem-En-Tec, Copenhagen, Denmark). Slides were counterstained with Mayer's hematoxylin. In each pancreas, 15 ± 1 islets were evaluated blindly with semiquantitative methods for the following parameters: *1*) lymphocyte infiltration (graded as no infiltration, minor infiltration, minor peri-insulitis, clear peri-insulitis, or insulitis [with intra-islet invasion by mononuclear cells]); and *2*) β-cell fraction (graded as >80, 60–80, 40–60, 20–40, or <20% β-cells). The evaluations are based on islets from ≥6 different areas from each pancreas.

Statistical analysis. Data are presented as means ± SE, and statistical analysis was performed using either χ^2 test, Wilcoxon's signed-rank sum test, or analysis of variance (followed by unpaired comparisons with Student's t test and correction of P values for multiple comparisons by the Bonferroni method (36) as indicated. When experiments were performed in duplicate, the mean of these two values was considered as one independent observation.

RESULTS

Expression of iNOS mRNA and nitrite formation by cytokine-stimulated islets. Exposure of islets isolated from C57BL/6 mice to IL-1β (25 U/ml) for 24 h in vitro resulted in the expression of iNOS mRNA and increased nitrite production, as compared with control C57BL/6 islets (Fig. 1). iNOS mRNA expression was not detected in untreated C57BL/6 islets. IL-1β failed to induce iNOS mRNA expression and increase nitrite formation by islets isolated from iNOS$^{-/-}$ mice (Fig. 1). Control iNOS$^{-/-}$ and C57BL/6 islets produced low amounts of nitrite, indicating that these cells express a constitutive form of NOS, as previously suggested (37).

Functional responses of islets exposed to IL-1β in vitro. Islets isolated from C57BL/6 and iNOS$^{-/-}$ mice were exposed in vitro to IL-1β (25 U/ml) for 24 h. Islet DNA and insulin contents were not significantly altered in either strain by the exposure to IL-1β (Table 1). A minor reduction in medium insulin accumulation was observed in IL-1β–exposed C57BL/6 islets, while islets from iNOS$^{-/-}$ mice responded to the cytokine by increasing medium insulin accumulation.

Neither islets obtained from C57BL/6 mice nor islets of iNOS$^{-/-}$ mice showed a change in total protein biosynthesis (TCA) rates after exposure to IL-1β (Table 2). However, the cytokine induced a 30–35% decrease in proinsulin biosynthesis and proinsulin/TCA at 16.7 mmol/l glucose in C57BL/6 islets (Table 2). On the other hand, there was neither a decrease in proinsulin biosynthesis nor in the ratio between proinsulin and TCA at 16.7 mmol/l glucose in iNOS$^{-/-}$ islets

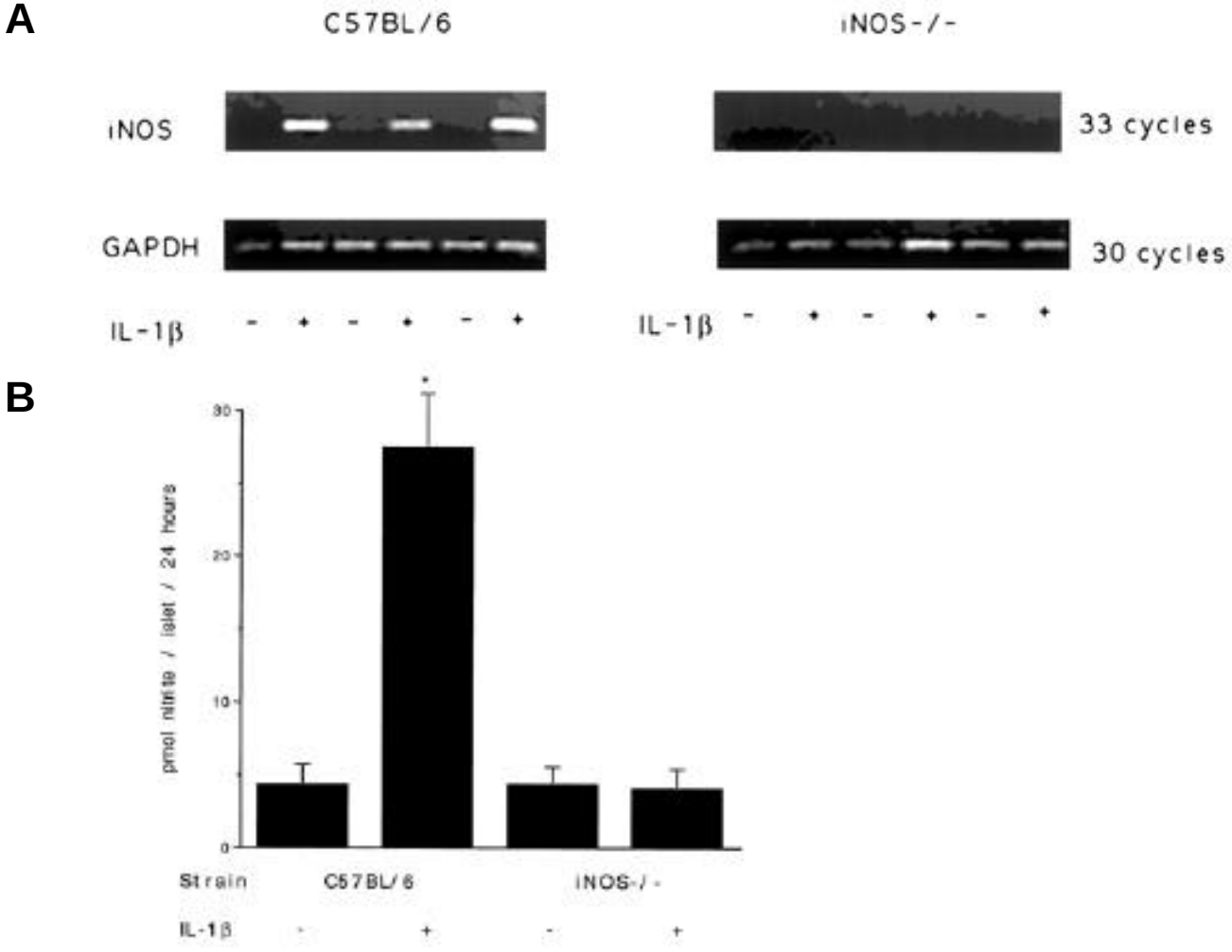

FIG 1. IL-1β effects on islet iNOS mRNA expression and medium nitrite accumulation. Islets from C57BL/6 and iNOS$^{-/-}$ mice were exposed to IL-1β (25 U/ml) during 24 h. At the end of this period, islets were retrieved for RT-PCR analysis of iNOS and GAPDH expression (*A*), and culture medium was collected for determination of nitrite levels (*B*) as described in METHODS. *A*: Expression of iNOS and GAPDH mRNA; the figure shows three separate experiments. *B*: Islet nitrite production; data are presented as means ± SE for seven separate experiments. *P < 0.05 vs. untreated islets from the same strain, Wilcoxon's signed-rank sum test.

treated with IL-1β (Table 2). At 1.7 mmol/l glucose, iNOS$^{-/-}$ islets exposed to IL-1β had an increased ratio between proinsulin rates and TCA rates.

The ability of IL-1β–exposed islets to respond to an acute glucose challenge was determined by measuring insulin released during a 90-min incubation at 16.7 mmol/l glucose (Fig. 2). Basal levels of insulin release were determined in parallel by culturing islets at 1.7 mmol/l glucose. IL-1β–treated islets from C57BL/6 mice showed a significant impairment of insulin release at 16.7 mmol/l glucose (40–50% reduction compared with untreated C57BL/6 islets), whereas insulin release was well preserved in iNOS$^{-/-}$ islets exposed to the cytokine. IL-1β did not affect basal insulin release in both mouse strains.

TABLE 1
Effect of in vitro exposure to IL-1β on insulin release to the medium, islet insulin, and DNA contents in pancreatic islets from C57BL/6 and iNOS$^{-/-}$ mice

	C57BL/6		iNOS $^{-/-}$	
IL-1β	–	+	–	+
DNA content (μg · 10 islets^{-1})	0.24 ± 0.04 (7)	0.24 ± 0.04 (8)	0.24 ± 0.02 (7)	0.22 ± 0.05 (6)
Insulin content (ng · 10 islets^{-1})	321 ± 33 (9)	362 ± 26 (9)	305 ± 39 (8)	208 ± 21 (8)
Medium insulin (ng · 10 islets^{-1} · 24 h^{-1})	193 ± 34 (9)	139 ± 18 (9)	163 ± 27 (8)	354 ± 43* (8)

Data are means ± SE (n). Pancreatic islets isolated from C57BL/6 and iNOS$^{-/-}$ mice were exposed to IL-1β (25 U/ml) for 24 h. Islets and culture medium were retrieved for DNA and insulin determinations as described in METHODS. *P < 0.05 vs. non-IL-1β–treated iNOS$^{-/-}$ islets, Wilcoxon's signed-rank sum test.

TABLE 2
TCA and proinsulin biosynthesis in islets isolated from C57BL/6 and iNOS$^{-/-}$ mice and exposed to IL-β for 24 h

	C57BL/6				iNOS$^{-/-}$			
	1.7 mmol/l		16.7 mmol/l		1.7 mmol/l		16.7 mmol/l	
IL-1β	–	+	–	+	–	+	–	+
Proinsulin (10^3 dpm · 10 islets^{-1} · 90 min^{-1})	3.1 ± 0.1	3.7 ± 0.7	22.6 ± 3.9	15.5 ± 1.5*	4.2 ± 0.4	7.8 ± 1.2	29.6 ± 2.3	35.4 ± 4.9
TCA (10^3 dpm · 10 islets^{-1} · 90 min^{-1})	69.4 ± 11.5	71.6 ± 6.2	157.2 ± 17.9	168.4 ± 17.9	97.0 ± 9.5	114.4 ± 10.3	240.6 ± 23.4	292.7 ± 28.0
Proinsulin/TCA (%)	5.1 ± 0.8	5.4 ± 1.1	15.0 ± 3.0	9.8 ± 1.6*	4.7 ± 1.0	7.0 ± 1.0*	12.9 ± 1.6	13.2 ± 2.7

Data are means ± SE for total of six independent experiments. Islets were exposed to IL-1β (25 U/ml) for 24 h. TCA and proinsulin biosynthesis were determined during a 90-min incubation at either 1.7 or 16.7 mmol/l glucose as described in METHODS. *P < 0.05 vs. non-IL-1β–exposed islets from the same strain cultured at an equimolar concentration of glucose, Wilcoxon's signed-rank sum test.

Studies in mice treated with MLDS. C57BL/6 and 129SvEv mice treated with MLDS gradually developed hyperglycemia (Fig. 3). On day 21, 12 of 16 (75%) of STZ-treated C57BL/6 and 9 of 9 (100%) of MLDS-treated 129SvEv mice had blood glucose (BG) levels >11.1 mmol/l (Fig. 4). Vehicle-treated C57BL/6 and 129SvEv mice showed no increase in BG levels throughout the study (Fig. 3), and none of the animals had a BG level >11.1 mmol/l on day 21 (data not shown).

During the 21-day study period, normal BG levels were maintained by vehicle-treated iNOS$^{-/-}$ mice, and only a minor increase was observed in MLDS-treated iNOS$^{-/-}$ mice (Fig. 3). Among the MLDS-treated iNOS$^{-/-}$ mice, 4 of 17 (23%) developed hyperglycemia (Fig. 4), and all control iNOS$^{-/-}$mice had BG levels <11.1 mmol/l (data not shown). Histological examination of pancreas from iNOS$^{-/-}$ and C57BL/6 mice killed on day 21 (Fig. 5) revealed an increased number of infiltrating cells, and a decrease in islet β-cell fraction in animals treated with MLDS, as compared with respective controls (P < 0.001 for all comparisons, χ^2-test, data not shown). The degree of infiltration and reduction in islet β-cell fraction was, however, lower in pancreas from MLDS-treated iNOS$^{-/-}$ as compared with MLDS-treated C57BL/6 mice (Table 3).

To evaluate whether iNOS$^{-/-}$ mice have a delayed onset of hyperglycemia after MLDS, a new series of experiments was performed where animals were followed up to 42 days after the first injection of STZ. On day 42, 6 of 9 (67%) of the C57BL/6 mice and 8 of 8 (100%) of 129SvEv mice exposed to MLDS were diabetic (BG level >11.1 mmol/l glucose), whereas only 1 of 8 (13%) of MLDS-treated iNOS$^{-/-}$ showed hyperglycemia (data not shown).

Finally, a series of experiments were undertaken to investigate whether there was a different sensitivity to STZ toxicity in vitro between islets isolated from C57BL/6 and iNOS$^{-/-}$ mice. When examined 18 h after exposure for 30 min to 1.5 mmol/l STZ, there was a similar reduction in islet DNA content in C57BL/6 islets (75.2 ± 6.3% of control [n = 7]; P < 0.01) and iNOS$^{-/-}$ islets (80.4 ± 6.4% of control [n = 7]; P < 0.01). The absolute values for DNA contents (μg/10 islets) were for C57BL/6 islets 0.28 ± 0.06 (control) and 0.21 ± 0.05 (STZ) and for iNOS$^{-/-}$ islets 0.32 ± 0.06 (control) and 0.25 ± 0.06 (STZ). The two groups of islets exhibited also a similar decrease in their insulin content after STZ treatment (C57BL/6 islets [54.7 ± 7.0% of control, n = 6; P < 0.01] and iNOS$^{-/-}$ islets [67.8 ± 11.9% of control, n = 6; P < 0.05]). The absolute values for insulin contents (ng/10 islets) were for

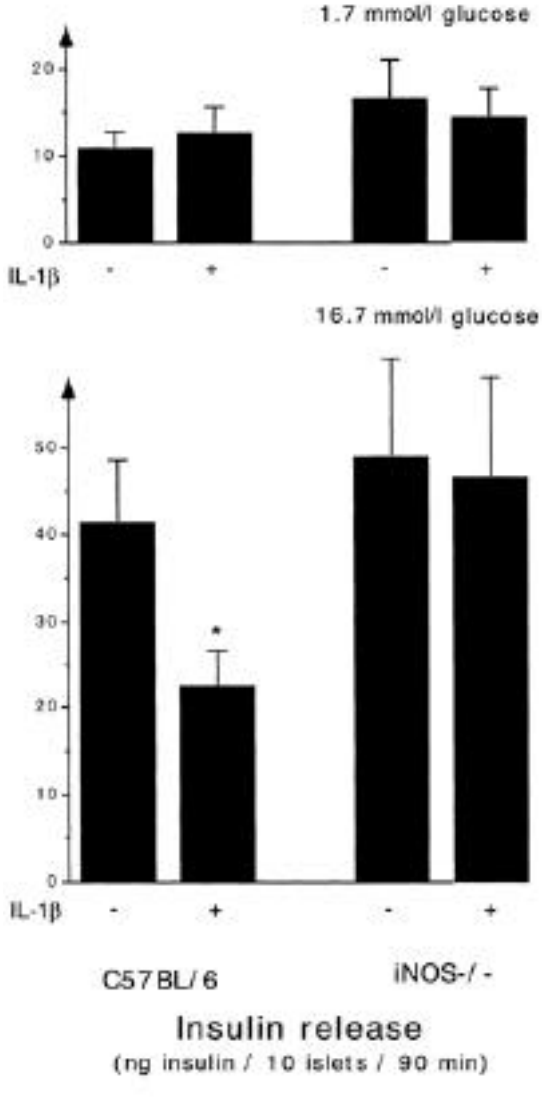

FIG 2. IL-1β effects on glucose-stimulated insulin release. Islets from C57BL/6 and iNOS$^{-/-}$ mice were exposed to IL-1β (25 U/ml) or control condition for 24 h and then incubated at 1.7 mmol/l glucose for 30 min followed by a second period of 90 min at 1.7 or 16.7 mmol/l glucose. Insulin released during the incubation period was determined as described in METHODS. Results are presented as means ± SE for nine (C57BL/6) or eight (iNOS$^{-/-}$) independent experiments. *P < 0.05 vs. untreated islets from the same strain, Wilcoxon's signed-rank sum test.

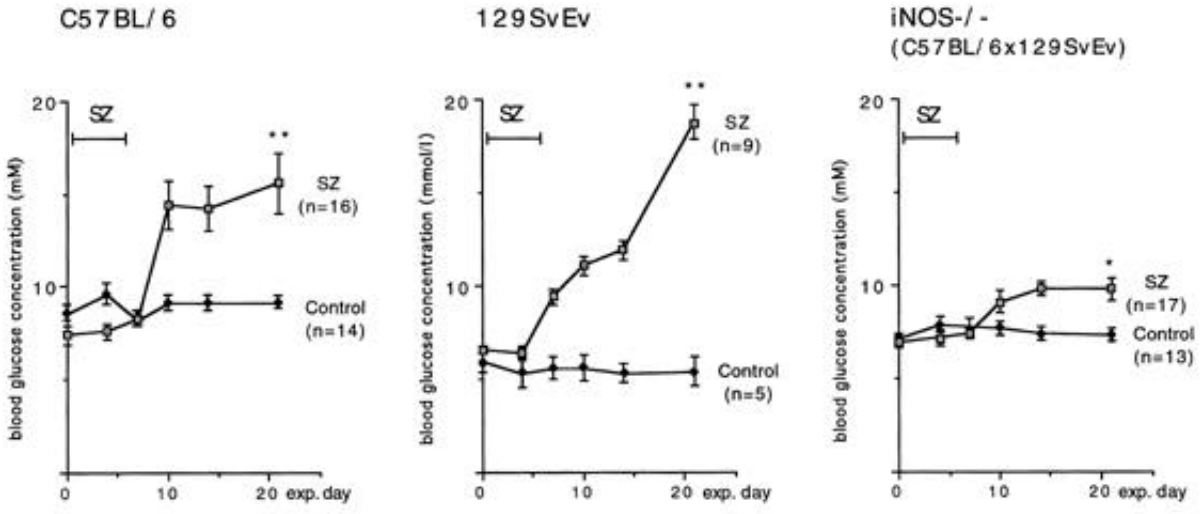

FIG 3. Blood glucose levels in C57BL/6, 129SvEv, and iNOS$^{-/-}$ mice treated with MLDS. STZ (40 mg/kg body wt) or vehicle (citrate buffer) was injected intraperitoneally during five consecutive days. Blood glucose levels were measured on days 0, 4, 7, 14, and 21 as described in METHODS. Data are presented as means ± SE for the number of animals given in the figure.*P < 0.01 and **P < 0.001 vs. vehicle-treated animals from the same strain on day 21, analysis of variance.

C57BL/6 islets 218 ± 31.1 (control) and 110 ± 12.6 (STZ) and for iNOS$^{-/-}$ islets 185 ± 15.2 (control) and 129 ± 27.4 (STZ).

DISCUSSION

The pathogenesis of type 1 diabetes is complex, involving both environmental and genetic factors (1). Data obtained from animal models of the disease, and in restricted cases also from diabetic patients, suggest that β-cell destruction is mediated by the immune system (38). The results presented in this report indicate that the enzyme iNOS plays an important role both in the cascade of events culminating in MLDS-induced diabetes in mice and in the mechanism(s) mediating IL-1β–induced murine β-cell dysfunction in vitro. These observations reinforce the hypothesis that NO is one of the mediators used by the immune system to damage and destroy the β-cells.

The present in vitro results corroborate the findings of numerous studies using NOS inhibitors, showing that NO contributes to the suppressive effects of IL-1β on rodent islet functions in vitro (reviewed in 12). It is noteworthy that while IL-1β inhibits medium insulin accumulation by islets isolated from C57BL/6 mice, it stimulates islet insulin release by islets from iNOS$^{-/-}$ mice (present data). Exposure of rodent islets to IL-1β results in an early stimulatory phase (1–2 h) and a later inhibitory phase (12–24 h) of insulin release (39). A rapid activation of protein kinase C by IL-1β has been proposed to mediate the early stimulation of insulin release in mouse islets, whereas the induction of iNOS and NO synthesis mediates the latter inhibition (2). Thus, it is conceivable that in the absence of iNOS, the initial stimulatory phase will be prolonged, explaining the observed cytokine-induced increase in medium insulin accumulation over 24 h in iNOS$^{-/-}$. In line with this possibility, IL-1β alone does not induce iNOS expression in human islets and leads to a prolonged stimulation of insulin release (40), similar to the present observations with iNOS$^{-/-}$ islets.

Several mechanisms may contribute to the iNOS-dependent suppressive effect of IL-1β on insulin release during culture both at basal glucose levels and during a challenge with a high glucose concentration. Earlier studies have shown that enzymes involved in glucose metabolism and mitochondrial energy production are affected by NO (12). In addition, IL-1β exposure and induction of iNOS results in a suppression of proinsulin biosynthesis (2,39, present data), which may further impair the capacity of the islet to release adequate amounts of insulin. It remains unclear how NO exerts this selective effect on the synthesis of insulin, but one possible

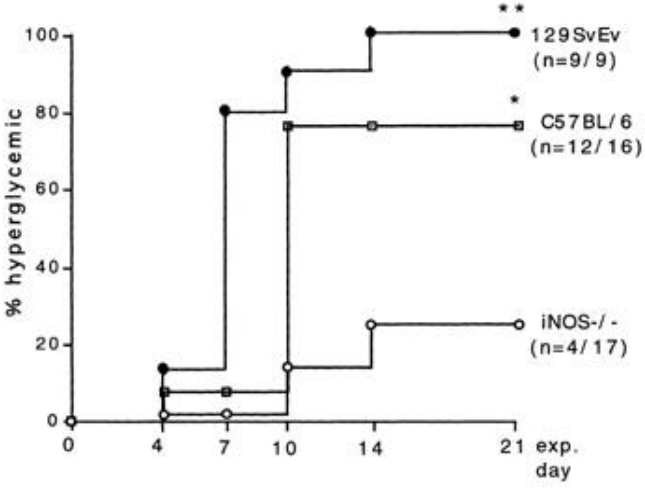

FIG 4. Cumulative incidence of hyperglycemia in C57BL/6, 129SvEv, and iNOS$^{-/-}$ mice treated with MLDS. Animals were treated with STZ (40 mg/kg body wt) for five consecutive days. Blood glucose levels were monitored during a 21-day period after the first injection and animals were considered hyperglycemic when blood glucose levels were >11.1 mmol/l. Data are presented as the percentage of hyperglycemic mice among all treated mice at the indicated time points. The numbers of hyperglycemic mice among the total numbers of treated mice are given in parentheses. *P < 0.01 and **P < 0.001 vs. STZ-treated iNOS$^{-/-}$ mice on day 21, χ^2 test.

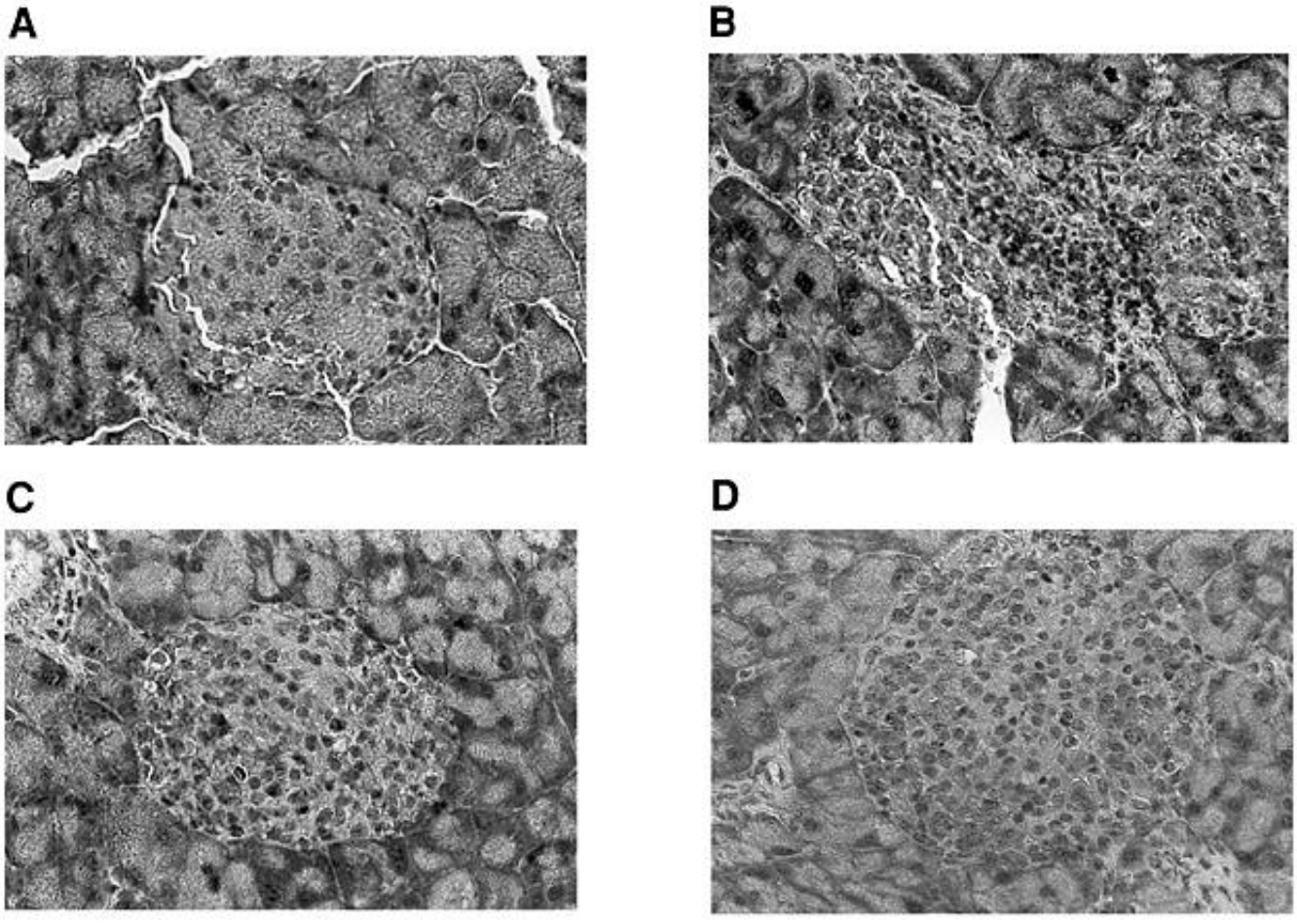

FIG 5. Pancreatic tissue sections from MLDS-treated C57BL/6 and iNOS$^{-/-}$ mice. Animals were killed on day 21 and histology performed as described in METHODS. Light photomicrographs show pancreatic tissue from vehicle-treated (*A*) or MLDS-treated (B) C57BL/6J mice and vehicle-treated (*C*) or MLDS-treated (*D*) iNOS$^{-/-}$ mice. Original magnification ×400.

explanation is that NO interferes with transcription factors involved in preproinsulin mRNA expression.

In the MLDS model, hyperglycemia and diabetes are induced in genetically predisposed mice by multiple injections of subdiabetogenic doses of the nitrosamide methylnitrosourea (MNU)–containing compound STZ (35). STZ has a direct cytotoxic effect on β-cells by inducing alkylation of nuclear and mitochondrial DNA (41), and perhaps also by releasing NO (42–44). It is conceivable that each injection of STZ will result in damage of a fraction of β-cells eventually leading to a local inflammation and infiltration by mononuclear cells, which culminates in widespread β-cell destruction and diabetes (45,46).

In the present study, both C57BL/6 and 129SvEv mice developed hyperglycemia within 10–14 days after treatment with MLDS, whereas there was only a minor increase in BG levels in iNOS$^{-/-}$ mice. Histological examination revealed less infiltration and β-cell destruction in islets from iNOS$^{-/-}$ mice

TABLE 3
Histological evaluation of MLDS-treated mice at day 21

	Infiltration (%)					β-Cell fraction per islet (%)				
Strain	No infiltration	Minor infiltration	Minor peri-insulitis	Clear peri-insulitis	Insulitis	>80	60–80	40–60	20–40	<20
iNOS$^{-/-}$	18.6	53.7	23.7	1.7	2.3	31.1	46.7	13.9	6.7	1.7
C57BL/6	1.7	38.7	47.9	8.4	3.4	5.0	21.8	46.2	17.6	9.2

Data are percentage of islets in each group among the total number of islets evaluated. Pancreases were taken from C57BL/6 and iNOS$^{-/-}$ mice treated with MLDS. Histological examination was performed as described in METHODS. A total number of 177 islets from nine iNOS$^{-/-}$ mice and 119 islets from six C57BL/6 mice were evaluated as described in METHODS. $P < 0.001$ for iNOS$^{-/-}$ strain (infiltration and β-cell fraction); χ^2 test vs. C57BL/6 strain.

as compared with islets from C57BL/6 mice. Genetic differences between iNOS$^{-/-}$ mice and C57BL/6 and 129SvEv mice regarding susceptibility to MLDS is an unlikely explanation for the observed differences, since all three strains share the same H-2^b complex, iNOS$^{-/-}$ and C57BL/6 mice accept skin grafts from each other with no rejection over a 6-day observation period (data not shown), and both C57BL/6 and 129SvEv are highly susceptible to MLDS treatment. Moreover, preliminary data indicate a similar high sensitivity to MLDS treatment in C57BL/6J × 129Sv F2 hybrids (M.F., unpublished data). MLDS-treated iNOS$^{-/-}$ mice show a small increase in BG levels, appearing in parallel to the initial increase in BG levels observed in the two other mouse strains (day 7–10). Islets isolated from iNOS$^{-/-}$ mice were found to show a similar sensitivity as islets isolated from C57BL/6 to the direct toxic effect of an exposure to STZ in vitro. This suggests that β-cells from the iNOS$^{-/-}$ mice are sensitive to the direct toxic effect of STZ but are protected against the subsequent immune-mediated processes resulting in hyperglycemia. Previous studies have shown that pancreatic islets from mice exposed to MLDS are heavily infiltrated, mainly by macrophages, 10–14 days after the first injection with STZ (45,46). Activated macrophages can contribute to β-cell dysfunction and damage by releasing both NO (47) and IL-1β (IL-1β will trigger β-cell iNOS expression (48). Data obtained in the present study show that iNOS expression is required for IL-1–induced β-cell dysfunction in vitro. A recent study also provided evidence that ectopical expression of iNOS by the rat insulin II promoter results in β-cell destruction and development of diabetes (49). Thus, a lack of iNOS expression and NO synthesis by both infiltrating macrophages and cytokine-stimulated β-cells may be a mechanism underlying the protection against MLDS-induced β-cell destruction in iNOS$^{-/-}$ mice. On the other hand, it cannot be excluded that iNOS deficiency affects MLDS-induced diabetes by a more generalized effect on the immune system, and it may be of interest to characterize whether MLDS-treated iNOS$^{-/-}$ mice have a different pattern of mononuclear cell-types infiltrating the islets at the different stages of islet inflammation.

The role of iNOS and NO in human type 1 diabetes remains unknown. While some studies suggest that NOS inhibitors protect human islets against cytokine-induced functional inhibition (50–52), other studies using the same inhibitors have failed to prevent human β-cell dysfunction (53, 54) and death (22) ensuing from cytokine exposure in vitro. On the other hand, there are data to suggest that human islets are susceptible to the DNA-damaging effects of the high concentrations of NO (55) and peroxynitrite (56) liberated by chemical donors. Human β-cells (52) and ductal cells (57), present inside or in near vicinity to human islets, express iNOS on exposure to proinflammatory cytokines. This may lead to high local NO production during insulitis, and it therefore is not excluded that this radical contributes to the injurious events resulting in β-cell depletion and diabetes development in humans. Further studies on histological material from human type 1 diabetic subjects may provide more information about iNOS involvement in the pathogenesis of the disease. In the meantime, the iNOS$^{-/-}$ mice provide a good model for evaluating the function of iNOS and NO in inflammatory processes mediating β-cell destruction and death.

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