

# Nitric Oxide Production and Fas Surface Expression Mediate Two Independent Pathways of Cytokine-Induced Murine $\beta$ -Cell Damage

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Activated T-cells and macrophages infiltrate pancreatic islets early in the pathogenesis of type 1 diabetes. Their secretion of different pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$ , interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$  affects  $\beta$ -cell function. Here we report that a combination of these cytokines inhibits insulin release, stimulates inducible nitric oxide synthase (iNOS), and upregulates the surface expression of Fas in NIT-1  $\beta$ -cells and intact mouse islets. Using iNOS-deficient and Fas-deficient islets, respectively, we investigated the relative contribution of NO and Fas upregulation in cytokine-induced  $\beta$ -cell damage. Interestingly, inhibition of insulin release did not occur in the absence of NO production. However, de novo expression of Fas-specific mRNA and Fas cell surface expression were detected and thus appear to be NO-independent. The lack of NO production partially protected islets from cytokine-induced apoptosis but had no effect on cell death induced by cell surface cross-linking of Fas with soluble Fas ligand (FasL). The absence of FasL on  $\beta$ -cells and the degree of apoptosis observed in Fas-deficient islets exclude the possibility of cytokine-induced fratricide. In conclusion, pro-inflammatory cytokines exert a cytotoxic effect on  $\beta$ -cells via an NO-dependent pathway and, in parallel, render  $\beta$ -cells susceptible to Fas:FasL-mediated, NO-independent cell death triggered by activated T-cells. *Diabetes* 49:39–47, 2000

**T**ype 1 diabetes is the result of autoimmune destruction of insulin-producing  $\beta$ -cells within the islets of Langerhans (1–3). The precise cellular and molecular mechanisms effective for  $\beta$ -cell death have yet to be defined. Histologic examination of pancreatic tissue early in the pathogenesis of type 1 diabetes has revealed an infiltration of islets with macrophages and

T-cells. Macrophages are the first inflammatory cells to infiltrate the islets, where they may engulf  $\beta$ -cells completely (4). A pathogenic role for macrophages in type 1 diabetes has been suggested by the use of agents such as silica, which inhibit macrophage function and prevent the development of diabetes in rodent models (5). The importance of T-cells for the pathogenesis of type 1 diabetes has been demonstrated in NOD/SCID mice, where the generation of mature T-cells was blocked (6,7) or, alternatively, where T-cell function was inhibited by the immunosuppressive agent cyclosporin A (8).

Macrophages and T-cells both secrete pro-inflammatory cytokines. The critical role of these molecules in the pathogenesis of type 1 diabetes was first detailed when crude cytokine preparations derived from activated mononuclear cells were demonstrated to mediate functional and structural damage to isolated murine islets of Langerhans (9). Further studies with rat islets revealed that their exposure to the inflammatory cytokine interleukin (IL)-1 $\beta$  was sufficient to inhibit  $\beta$ -cell function (10) and that other cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  potentiated this cytotoxic effect (11).

Despite their identification as soluble effectors, the  $\beta$ -cell-specific molecular changes induced by these cytokines remain incompletely understood. Toxic free radicals have been suggested as important mediators of  $\beta$ -cell destruction (12). IL-1 $\beta$ , alone or in combination with TNF- $\alpha$  and IFN- $\gamma$ , induces in rodent and human islets the transcription of the inducible nitric oxide synthase (iNOS) gene (11,13,14). This enzyme catalyzes the generation of nitric oxide (NO) from L-arginine, and importantly, NO production appears to correlate with the inhibition of insulin secretion (13,15,16). The molecular mechanisms operational in this effect include a loss of optimal oxidative phosphorylation, a decrease in glycolysis, and consequently an impairment of ATP concentrations (17). The cellular source of oxygen radicals, however, was not specifically determined in experiments where isolated pancreatic islets were exposed to a mixture of cytokines (18). NO production has also been correlated with DNA fragmentation and subsequent cell death in macrophages and mesangial cells (19). Despite these observations, inhibition of NO synthesis in isolated pancreatic  $\beta$ -cells (18) and in vivo (20) achieved only a partial protection from cytokine-mediated cytotoxicity.

In addition to NO, other intra- and intercellular mechanisms have been demonstrated to initiate programmed cell death. One important mechanism is the interaction of the cell surface receptor Fas (CD95/APO-1) with its specific ligand, FasL (21). Fas is expressed on a wide variety of cells and,

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ANOVA, analysis of variance; FasL, Fas ligand; G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NAME, N<sup>w</sup>-nitro-L-arginine methylester; MHC, major histocompatibility complex; PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor.

upon cross-linking, generates an apoptotic signal via activation of cysteine proteases called caspases. In contrast, FasL expression is much more limited and is found typically on the surface of activated T-cells and natural killer (NK) cells. Inappropriate Fas expression in the pathogenesis of autoimmune thyroiditis has been proposed by some (22) but not others (23). Although normal human pancreatic  $\beta$ -cells do not express Fas on their surface, exposure of these cells to IL-1 $\beta$  induces Fas expression (24,25). Moreover, the production of NO may link cytokine exposure to Fas expression on human  $\beta$ -cells (26), which provides a rational basis for therapeutic strategies using free radical scavengers (e.g., nicotinamide) to prevent the occurrence of programmed cell death.

To test whether inflammatory cytokines induce changes in  $\beta$ -cell function and viability via mechanisms dependent on inducible NO production, we analyzed insulin secretion, Fas expression, and induction of apoptosis in pancreatic  $\beta$ -cells from wild-type animals and mice deficient for iNOS or Fas. We demonstrate that NO radicals are critical for the decrease in insulin secretion by  $\beta$ -cells, but that their Fas cell surface expression is NO-independent and that pro-inflammatory cytokines promote a certain degree of apoptosis independently of NO and Fas.

## RESEARCH DESIGN AND METHODS

**Mice.** OF1 and C57BL/6-*Ipr/Ipr* mice (5 to 7 weeks old) were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland), and iNOS<sup>-/-</sup> mice (27) (5 to 7 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at the Department of Research. All animal experiments were approved by the animal welfare committee, and mice were kept according to federal guidelines.

**Reagents.** Recombinant human IL-1 $\beta$  (PeproTech, Rocky Hill, NJ), recombinant mouse IFN- $\gamma$  (Genzyme, Cambridge, MA), and recombinant human TNF- $\alpha$  (Biogen, Cambridge, MA) were obtained commercially. N<sup>ω</sup>-nitro-L-arginine methylester (L-NAME) was obtained from Fluka Chemie AG (Buchs, Switzerland). For flow cytometric analysis of surface molecules, biotin-conjugated hamster anti-mouse Fas antibodies (Jo2) (28) and phycoerythrin (PE)-conjugated streptavidin were used (Pharmingen, San Diego, CA). Biotin-conjugated hamster IgG isotype (anti-trinitrophenyl) (Pharmingen) was used as a negative control. Soluble recombinant mouse FasL (29) was a gift from Dr. A. Filipowicz, Department of Research, Kantonsspital Basel, Switzerland.

**Islet isolation and culture.** The method of islet isolation was established previously (30) and adapted for our needs. In short, after laparotomy, the bile duct was clamped at the junction with the duodenum. Cannulation of the bile duct and infusion of 2 mg/ml cold collagenase type IV (Worthington Biochemical, Freehold, NY) was performed by retrograde injection. After swift surgical removal of the pancreas, the entire organ was incubated in a petri dish at 37°C for 40 min. The islets were washed three times and filtered through a 425- $\mu$ m metal grid to remove undigested tissue and debris. Islets were subsequently purified twice by centrifugation (10 min at 800g) on a noncontinuous Ficoll gradient (type 400 DL; Sigma-Aldrich, Buchs, Switzerland). Cells in the interphase between the 23 and 21% and the 21 and 11% gradients, respectively, were harvested. This procedure yielded single islets of high purity, which were subsequently cultured for 1 week in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 11 mmol/l D-glucose supplemented with 10 mmol/l HEPES, L-glutamine, penicillin/streptomycin solution, fungizone, and 10% heat-inactivated fetal calf serum (Amimed, Allschwil, Switzerland). During that time, further purification was achieved by two to three additional rounds of hand-picking to achieve a homogeneous sample of single viable islets. To assess the functional capacity of the islets, glucose challenge experiments (described below) were performed in Krebs-Ringer HEPES buffer (KRHB) containing either 1.67 or 16.7 mmol/l D-glucose. The insulin secretion increased from  $1.55 \pm 0.8$  to  $10.18 \pm 7 \mu\text{U} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$  upon glucose challenge. Of further note, the spontaneous insulin release of wild-type (OF1) islets and iNOS<sup>-/-</sup> islets was not statistically different ( $2.02 \pm 0.54$  and  $1.84 \pm 0.61 \mu\text{U} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$ , respectively;  $P > 0.5$ ).

**Cell line.** NIT-1 cells (31), a murine  $\beta$ -cell line established from a transgenic NOD/It mouse, were obtained from ATCC (Rockville, MD). NIT-1 cells were grown in F-12 Ham's medium with L-GlutaMAX I (Gibco BRL) containing 10 mmol/l D-glucose supplemented with penicillin/streptomycin solution and 10% heat-inactivated fetal calf serum. The medium was changed 24 h before each experiment. Glucose challenges were performed, resulting in a fourfold increase of the insulin

response after high-glucose stimulation ( $0.35 \pm 0.05 \mu\text{U} \cdot 10^5 \text{ cells}^{-1} \cdot \text{h}^{-1}$  for basal insulin release and  $1.42 \pm 0.25 \mu\text{U} \cdot 10^5 \text{ cells}^{-1} \cdot \text{h}^{-1}$  after glucose challenge).

**Insulin determination.** Cells were plated into 96-well plates with different combinations of 15 U/ml IL-1 $\beta$ , 80 U/ml IFN- $\gamma$ , and 10 U/ml TNF- $\alpha$  in F-12 Ham's medium containing 10 mmol/l D-glucose (for NIT-1 cells) and RPMI 1640 medium containing 11 mmol/l D-glucose (for islets). For glucose challenge experiments, cells were first grown in KRHB containing 1.67 mmol/l D-glucose for 1 h and subsequently challenged with exposure to 16.7 mmol/l D-glucose for 2 h. Insulin release was quantified in the supernatant using a radioimmunoassay (INSIK-5; Sodiag SA, Losone, Switzerland). For measurement, each sample was first diluted to a typical insulin concentration between 25 and 50  $\mu\text{U}/\text{ml}$  corresponding to the linear part of the standard curve.

**Nitrite determination.** From the same samples used for insulin determination, NO production was determined by measuring NO<sub>2</sub><sup>-</sup>, a stable NO oxidation product (32). To this end, 90  $\mu\text{l}$  cell-free medium was mixed with 10  $\mu\text{l}$  of 5 $\times$  Griess reagent, consisting of equal volumes of 0.5% N-naphthylethylenediamine and 5% sulfanilamide/25% H<sub>3</sub>PO<sub>4</sub>. Nitrite concentrations were determined in triplicate by spectrophotometry at 540 nm within a concentration range that corresponds to a linear portion of the standard curve.

**Polymerase chain reaction.** RNA was extracted using TRIzol reagent (Gibco BRL) according to the manufacturer's recommendations, and samples were diluted to 1  $\mu\text{g}$  RNA (total amount). Reverse transcription (RT) was performed for 60 min at 42°C using 0.2 U SuperScript II (RNase Reverse Transcriptase, 200 U/ml; Gibco BRL). Equal amounts of sample were used for polymerase chain reaction (PCR) amplification of cDNAs specific for iNOS, Fas, FasL, and glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH). Amplification of specific cDNA was performed with 5 U/ml Taq DNA polymerase (Gibco BRL). For quantification of Fas and G-3-PDH amplicons, [<sup>32</sup>P]-labeled dCTP (1  $\mu\text{Ci}$ ) was added to each reaction. The following primers were used: iNOS, sense 5'-CCCTCCGAAGTTTCTG GCAGCAGC-3' and antisense 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'; Fas, sense 5'-CGGAATCCAAGGTAATAAGCATCTCCGAG-3' and antisense 5'-CGAAGCTTTCAGCGATTTCTGGACTTTGTTTCTGCA-3'; FasL, sense 5'-GGAATGTATGAGCTCGTCCACCTGCAGAAGG-3' and antisense 5'-GCTCTAG AATATTCCTGGTGCATGAT-3'; G-3-PDH, sense 5'-TGAAGGTCGGTGTCAAC GGATTTGGC-3' and antisense 5'-CATGTAGCCATGAGGTCCACCAC-3'. Fas and FasL primers were kindly provided by Dr. S. Hahn (Institute for Medical Microbiology, University of Basel). PCR was performed for iNOS, Fas, and G-3-PDH as follows: 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C for 35 cycles. For FasL, 30 cycles were performed (30 s at 95°C, 1 min at 55°C, and 1 min at 72°C). The PCR product was analyzed by gel electrophoresis (1% Tris acetate EDTA agarose gel) and visualized under ultraviolet light after staining with ethidium bromide. [<sup>32</sup>P]-labeled products were visualized on a PhosphorImager (Molecular Dynamics, Waysata, MN) and analyzed by ImageQuant Software (Molecular Dynamics).

**Flow cytometric analysis of Fas expression.** NIT-1 cells and islets were incubated for 28–30 h with different cytokines, washed twice with Hanks' buffered salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup>, and incubated in prewarmed cell dissociation buffer (Sigma, St. Louis, MO) (8 min at 37°C). A single-cell suspension was obtained by gently pipetting (i.e., assisted mechanical disruption). The physical disruption of  $\beta$ -cells results, independently of cytokine exposure, in a moderate increase of non-viable cells (33) but is critical to obtain single cells. The cells were subsequently washed with phosphate-buffered saline, and  $5 \times 10^5$  cells were collected and stained for Fas expression using mouse monoclonal antibody (Jo2). Immediately before flow cytometry, a 1:500 propidium iodide (PI) solution (1 mg/ml) was added to each sample to discriminate live from necrotic cells (by exclusion of cells positive for PI). Live gated cells were analyzed using a FACScan and Lysis II software (Becton Dickinson, Sparks, MD).

**Detection of apoptosis by cell-cycle analysis.** Apoptotic cell death and subsequent fragmentation of DNA results in decreased PI staining of DNA, referred to as the sub-G<sub>1</sub> peak (34,35). The percentage of apoptotic cells can therefore be measured by determining the cells within the distinct sub-G<sub>1</sub> peak. To this end, cells were cultured in 24-well plates with or without a combination of the three cytokines. After 28–30 h, cells were washed, dissociated (as above), and further incubated for 20 h in a 3% (vol/vol) solution of FasL diluted in incubation medium. Dissociation of whole islets into single cells was performed to achieve adequate binding of soluble FasL. After this period, cells were stained (40 min at 4°C) with a solution containing PI (50  $\mu\text{g}/\text{ml}$ ) and 0.1% Triton X-100. Cells were analyzed by flow cytometry on a linear scale (FACScan and Lysis II software). Necrotic cells were excluded from the analysis according to PI staining intensity below the sub-G<sub>1</sub> peak.

**Statistical analysis.** Data are presented as mean values  $\pm$  SD. In Figs. 1, 4, and 5, data of groups were compared by analysis of variance (ANOVA). The level of significance was  $P = 0.05$ . If ANOVA revealed significant differences, comparison within groups was performed, and the P values were adjusted by Bonferroni's correction for multiple comparisons. In Table 1 and Fig. 3, a paired Student's t test (two-tailed) was performed. Statistical calculations were done with StatView (version 5.0).

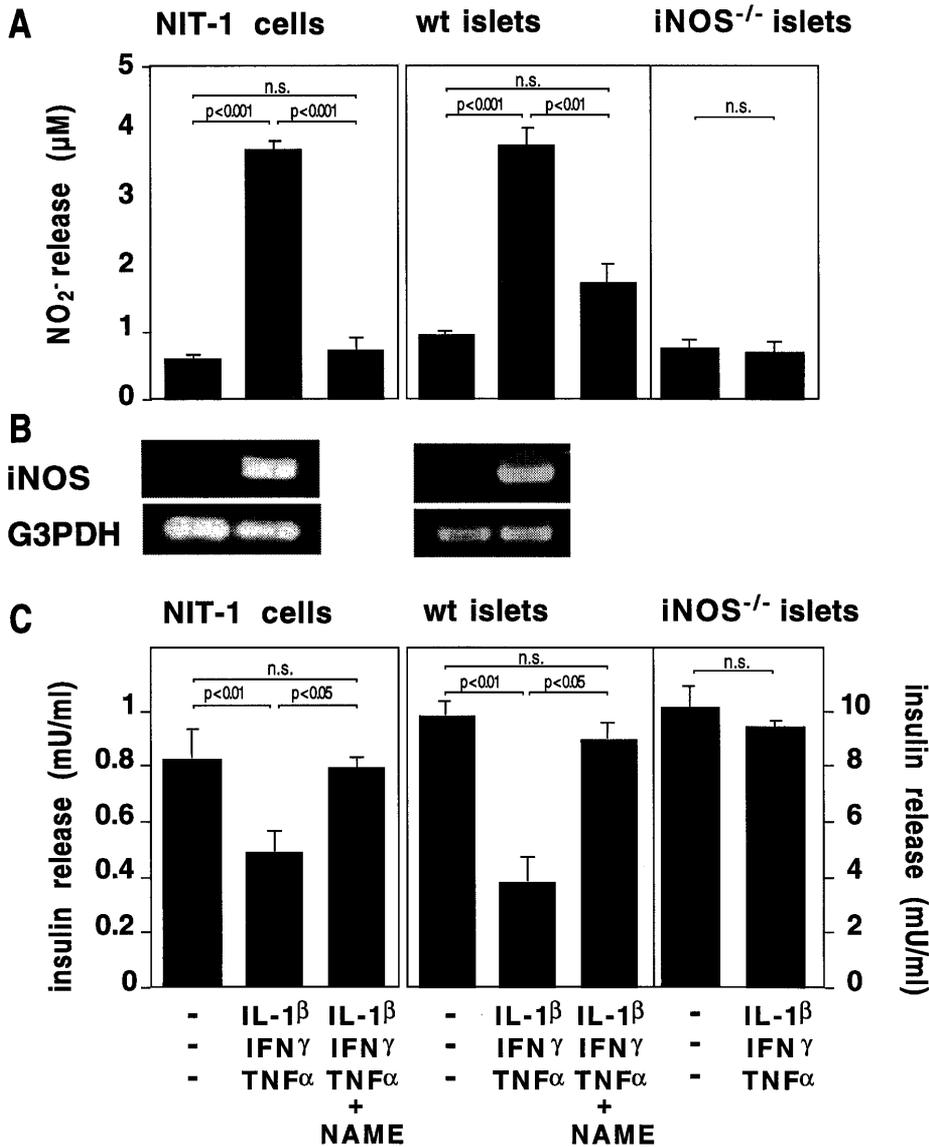


FIG. 1. Blocking of iNOS function restores insulin production. Insulin release, NO production and iNOS expression by NIT-1 cells, wild-type (wt) islets, and iNOS<sup>-/-</sup> islets exposed during 24 h to 15 U/ml IL-1β plus 80 U/ml IFN-γ plus 10 U/ml TNF-α in the presence or absence of 2.5 mmol/l L-NAME are shown. A: Supernatants from NIT-1 cells, wild-type islets, and iNOS<sup>-/-</sup> islets were assayed for NO<sub>2</sub><sup>-</sup> content by the Griess reaction. B: Cells were harvested, RNA was isolated and reverse-transcribed, and cDNA was amplified using specific iNOS and G-3-PDH primers. C: Insulin release was quantified by determining insulin in the supernatants using a radioimmunoassay. The bars delineate insulin accumulation by 2 × 10<sup>5</sup> NIT-1 cells or 20 primary islets, respectively, per milliliter of supernatant in the course of 24 h. Data represent mean values ± SD of at least three independent experiments. P values (ANOVA) were calculated separately for NIT-1 cells and islets.

## RESULTS

NO release and iNOS expression in cytokine-exposed β-cells. The previously described effect of the pro-inflammatory cytokines IL-1β, IFN-γ, and TNF-α on NO production and iNOS expression (10,13) was tested in the pancreatic β-cell line NIT-1 and in isolated primary wild-type (OF1) islets of Langerhans. NIT-1 cells and wild-type islets were cultured for 24 h in the presence or absence of 15 U/ml IL-1β, 80 U/ml IFN-γ, and 10 U/ml TNF-α. Samples exposed to a combination of all three cytokines showed a significant increase in NO generation, as measured by accumulation of NO<sub>2</sub><sup>-</sup> (Fig. 1A). Comparable results were obtained using a combination of IL-1β plus IFN-γ, whereas exposure to all other combinations or single cytokines had no effect on NO release (data not shown). Semiquantitative RT-PCR analysis of individual cultures revealed an upregulation of iNOS-specific message in cytokine-exposed cultures compared with unstimulated cultures (Fig. 1B). These results confirm previous data that a combination of pro-inflammatory cytokines upregulates iNOS mRNA in β-cells and that these stimulated cells release increased amounts of NO (10,11).

Generation of NO causes a decrease in insulin secretion by β-cells. Previous studies suggested that NO may exert a degenerative effect on β-cells (36). Using NIT-1 cells and primary islets, we measured insulin release in the supernatants. NIT-1 cells exposed to IL-1β plus IFN-γ plus TNF-α for 24 h showed an inhibition in insulin release of 25–30% compared with controls (Fig. 1C). This effect was more pronounced in the supernatant of primary cytokine-exposed wild-type islets, where insulin accumulation was decreased by 50% compared with naive islets. To test whether the decrease of insulin release was due to the production of NO, islets genetically deficient for the expression of iNOS were analyzed. As expected, iNOS-deficient islets produced no NO upon stimulation with cytokines (Fig. 1A). Islets from iNOS-deficient mutants, both unstimulated and exposed to IL-1β plus IFN-γ plus TNF-α, showed comparable amounts of insulin accumulation over a period of 24 h (Fig. 1C). Interestingly, insulin secretion in response to a glucose challenge was also unaltered in cytokine-stimulated iNOS-deficient islets compared with iNOS-deficient islets not exposed to the three pro-inflammatory cytokines (6.3 ± 2.5 vs. 7.63 ± 4.6 μU · islet<sup>-1</sup> · h<sup>-1</sup>).

TABLE 1  
Fas mRNA expression in cytokine-stimulated and unstimulated NIT-1 cells, wild-type islets, and iNOS<sup>-/-</sup> islets

Stimulation		Fold increase above background
NIT-1 cells	None	1.0
	IL-1 $\beta$ + IFN- $\gamma$ + TNF- $\alpha$	4.87 $\pm$ 1.9*
	IL-1 $\beta$ + IFN- $\gamma$ + TNF- $\alpha$ + L-NAME	4.35 $\pm$ 1.4*
Wild-type islets	None	1.0
	IL-1 $\beta$ + IFN- $\gamma$ + TNF- $\alpha$	4.73 $\pm$ 1.8*
	IL-1 $\beta$ + IFN- $\gamma$ + TNF- $\alpha$ + L-NAME	7.69 $\pm$ 2.5*
iNOS <sup>-/-</sup> islets	None	1.0
	IL-1 $\beta$ + IFN- $\gamma$ + TNF- $\alpha$	7.88 $\pm$ 4.6†

Data are means  $\pm$  SD for five independent experiments. NIT-1 cells and islets were exposed for 24 h to a combination of 15 U/ml IL-1 $\beta$  plus 80 U/ml IFN- $\gamma$  plus 10 U/ml TNF- $\alpha$  in the presence or absence of 2.5 mmol/l L-NAME. For semiquantification, hot PCR was performed and G-3-PDH was used as an internal control. For comparison between and within each experimental group, the background (i.e., Fas/GAPDH) of unstimulated samples was arbitrarily set to 1. The ratio of Fas/GAPDH for unstimulated cells was compared with the background value of 1. It is of note, however, that Fas expression on unstimulated  $\beta$ -cells could be detected by neither hot PCR nor flow cytometry. The statistical differences between cytokine-exposed (with or without L-NAME) and untreated cells was calculated within each group (\*P < 0.01; †P < 0.05 by Student's t test). Comparison between the groups of cytokine-exposed (with or without L-NAME) cells did not reveal any statistical difference (ANOVA).

This result was specific for the lack of NO, since identical results were observed in NIT-1 cells and wild-type islets treated with the pharmacologic agent L-NAME, which specifically blocks iNOS activity (Fig. 1C). These results demonstrate a direct involvement of intracellular NO for the impairment of insulin production or secretion or both. Importantly, this immediate effect is independent of an induction of cell death, since  $\beta$ -cell viability was not altered within the first 24 h of culture when comparing the two experimental groups (Fig. 4 and data not shown).

Fas expression is independent of NO. The Fas/FasL interaction is an effective mechanism by which activated T-cells induce apoptosis in a large number of different target cells (21,37). To investigate whether exposure to cytokines induces expression of Fas or FasL or both, mRNA from stimulated  $\beta$ -cells was reverse-transcribed and analyzed for the presence of Fas-specific cDNA. To this end, total RNA was extracted from isolated pancreatic islet cells incubated for 24 h with or without the triple combination of cytokines. The amplification was performed in the presence of [<sup>32</sup>P]dCTP to quantify the PCR products by radioactivity. Unstimulated islets did not express Fas mRNA (data not shown). This finding is in accordance with the lack of Fas cell surface expression as analyzed by flow cytometry (see below and Fig. 3).

Fas mRNA expression was induced in wild-type islets exposed to a combination of IL-1 $\beta$  plus IFN- $\gamma$  plus TNF- $\alpha$ , whereas unstimulated cells failed to express Fas mRNA (Table 1 and data not shown). To further determine the role of NO in Fas expression as a consequence of cytokine expo-

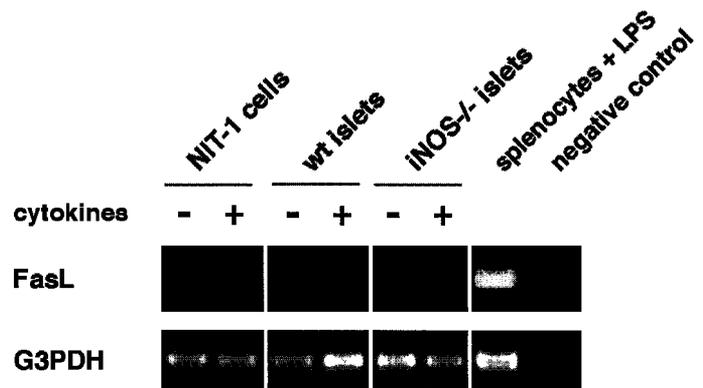


FIG. 2. Lack of FasL mRNA expression in naive  $\beta$ -cells and  $\beta$ -cells exposed to a combination of cytokines. NIT-1 cells, wild-type (wt) islets, and iNOS<sup>-/-</sup> islets were harvested after 24-h culture in the presence or absence of 15 U/ml IL-1 $\beta$  plus 80 U/ml IFN- $\gamma$  plus 10 U/ml TNF- $\alpha$  and reverse-transcribed, and cDNA was amplified using specific FasL and G-3-PDH primers. For a positive control, lipopolysaccharide-stimulated mouse splenocytes (C57BL/6) were used; the negative control reaction represents the same cellular source but in the absence of reverse transcription.

sure, iNOS<sup>-/-</sup> islets were compared with wild-type islets. The induction of Fas-specific mRNA in response to cytokines could also be detected in cells deficient for iNOS. Thus, and in contrast to other species (26), Fas transcription in murine islets is independent of NO production.

Because islets are composed of a number of different cell types, we next sought to determine whether  $\beta$ -cells are competent to upregulate Fas expression when exposed to cytokines. To this end, we analyzed NIT-1 cells exposed to cytokines. The results obtained with this  $\beta$ -cell line were comparable to the results generated with primary pancreatic islet cells. When exposed to IL-1 $\beta$  plus IFN- $\gamma$  plus TNF- $\alpha$ , NIT-1 cells expressed Fas mRNA (Table 1), which was already detectable after 15 h of cytokine exposure and appeared to reach a plateau after 24 h (data not shown). NIT-1 cells cultured with cytokines in the presence or absence of 2.5 mmol/l L-NAME to block iNOS activity resulted in amplicons of comparable intensities when analyzed by gel electrophoresis (Table 1). This result confirms the finding in islets that NO is not essential for Fas expression. Moreover, RT-PCR analysis of wild-type islets, iNOS<sup>-/-</sup> islets, and NIT-1 cells showed that neither cytokine-exposed nor control cells expressed mRNA specific for FasL (Fig. 2), even when tested at different time points in the course of 24 h. This result is in accordance with previous findings (38).

Next, we tested the functional surface expression of Fas on  $\beta$ -cells by flow cytometry. As demonstrated in Fig. 3A and B, staining of unstimulated NIT-1 and primary  $\beta$ -cells, respectively, with either anti-Fas antibodies or isotype control antibodies produced histograms with comparable shapes and identical median fluorescence intensities. This finding establishes that neither cell type expresses detectable Fas surface protein. In contrast, Fas cell surface expression (measured by median fluorescence intensity) was noted in both NIT-1 cells and wild-type islet cells following exposure to pro-inflammatory cytokines. Thus, the accumulation of Fas-specific mRNA (Table 1) correlated directly with an increased surface expression of Fas. Comparable results

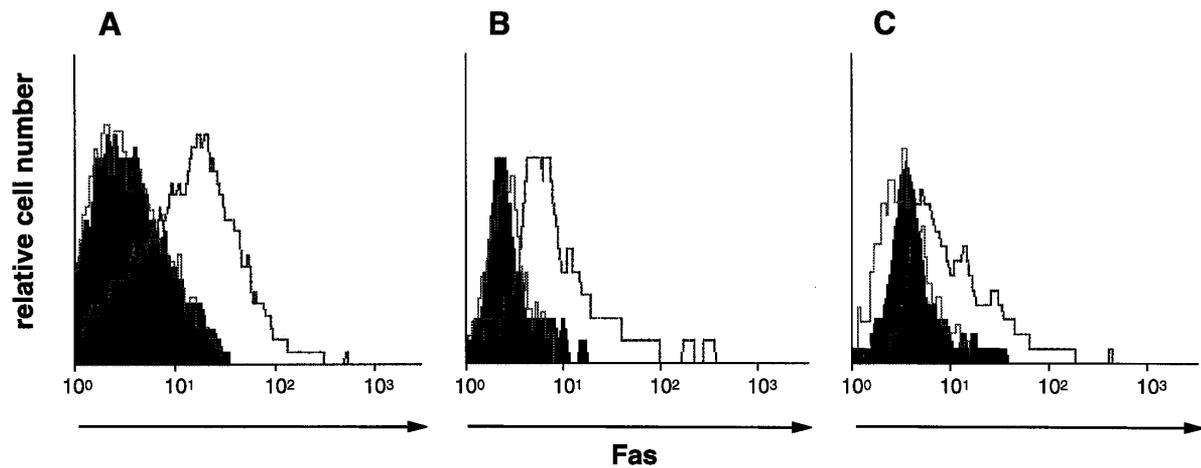


FIG. 3. Cytokines upregulate Fas surface expression on  $\beta$ -cells. Fas surface expression was assayed in NIT-1 cells (A), wild-type islets (B), and  $iNOS^{-/-}$  islets (C) after exposure to 15 U/ml IL-1 $\beta$  plus 80 U/ml IFN- $\gamma$  plus 10 U/ml TNF- $\alpha$ . In each panel, filled histograms represent Fas cell surface expression of unstimulated cells, open histograms with solid black lines depict Fas expression of cytokine-stimulated cells, and histograms with gray lines reveal the staining of cytokine-stimulated cells with the isotype control antibody. After 28–30 h of incubation with cytokines, staining was performed using a biotinylated monoclonal antibody directed against murine Fas, and the cells were assayed by flow cytometry; 10,000 events were counted, and mean fluorescence intensity was determined. Data are representative for at least three independent experiments. The statistical analysis (paired Student's *t* test) reveals a significant difference in mean fluorescence intensities between unstimulated and cytokine-exposed cells within each group (NIT-1 cells,  $P < 0.05$ ; wild-type islets,  $P < 0.05$ ;  $iNOS^{-/-}$  islets,  $P < 0.01$ ).

were obtained with islet cells from  $iNOS$ -deficient mice (Fig. 3C) and with wild-type islet cells cocultured with cytokines plus 2.5 mmol/l L-NAME (data not shown). Induction of apoptosis through cross-linking of the Fas receptor. Cross-linking of the Fas receptor by its natural ligand, FasL, serves as the appropriate stimulus to generate apoptotic signals (21). To test whether the induced Fas molecules are functional, islets were first cultured during 28–30 h with cytokines (incubation 1) to achieve sufficient cell surface Fas expression. Subsequently, islets were gently dissociated into single cells and then exposed to soluble FasL (incubation 2). Cross-linking of Fas resulted in increased apoptotic cell death of cells initially exposed to inflammatory cytokines (Fig. 4). In contrast, islet cells cultured in the absence of cytokines did not display an increase in programmed cell death when exposed to soluble FasL. These results demonstrate the functional expression of Fas on the cell surface of  $\beta$ -cells exposed to a mixture of pro-inflammatory cytokines. Apoptosis of cytokine-exposed  $\beta$ -cells is determined by different pathways. Primary  $\beta$ -cells demonstrated a low degree of apoptosis after initial culture for 1 day regardless of the presence or absence of cytokines (Fig. 4 and data not shown). A moderate degree of spontaneous apoptosis was noted in cells cultured for an additional 20 h independent of cytokine exposure, detailing the effect of cell dissociation (33). However, prolonged incubation with cytokines revealed increased apoptotic cell death in single wild-type islet cells compared with unstimulated cells (40.1 vs. 24.4%; Figs. 4 and 5). Thus, cytokines appear to induce programmed cell death in  $\beta$ -cells independent of any Fas:FasL-mediated signaling. This contention was further corroborated by prolonged exposure of islets from Fas-deficient  $lpr/lpr$  mice to the combination of pro-inflammatory cytokines. Stimulated  $Fas^{-/-}$  islets demonstrated decreased insulin secretion and enhanced NO production at a level comparable to wild-type islets (data not shown). Moreover, the relative increase in apoptotic cell death (in the absence of soluble FasL) was

similar when comparing  $Fas^{-/-}$  islets with wild-type islets exposed to pro-inflammatory cytokines (data not shown).

To determine the role of NO in cytokine-induced apoptosis, islets from  $iNOS$ -deficient mice and wild-type animals were compared. The two genetically different groups of islet cells revealed an identical degree of apoptosis when cultured in the absence of cytokines. In contrast, exposure of islet cells to the mixture of inflammatory cytokines demonstrated that programmed cell death was in part dependent on NO synthesis, since  $iNOS$ -deficient cells showed a lower degree of apoptosis than wild-type  $\beta$ -cells (Fig. 5). Islets from  $iNOS^{-/-}$  mice still displayed some degree of programmed cell death when exposed to pro-inflammatory cytokines, however, suggesting that these molecules exerted a direct apoptotic effect on  $\beta$ -cells.

To assess whether the lack of NO could also reduce the degree of apoptosis triggered by FasL, cytokine-exposed wild-type and  $iNOS^{-/-}$  islets were incubated with soluble FasL (Fig. 5). DNA fragmentation analysis disclosed that the extent of apoptosis was comparable for both experimental groups, suggesting an NO-independent, Fas-triggered mechanism of apoptosis. Importantly, the percentage of necrotic cells, as determined by PI staining of cells below the sub-G<sub>1</sub> peak (mean fluorescence intensity  $< 40$ ), was comparable for all stimulations ( $28 \pm 4.7\%$ ).

Taken together, these results clearly demonstrate that loss of  $\beta$ -cell function in the course of exposure to pro-inflammatory cytokines can be effected in at least three independent ways: cytokine-induced induction of programmed cell death, Fas:FasL-mediated apoptosis, and  $iNOS$ -triggered dysfunction and cell death.

#### DISCUSSION

The pathomechanisms responsible for the initiation and progression of pancreatic  $\beta$ -cell destruction in type 1 diabetes have not been fully characterized, although animal models have greatly enhanced our understanding of the immuno-

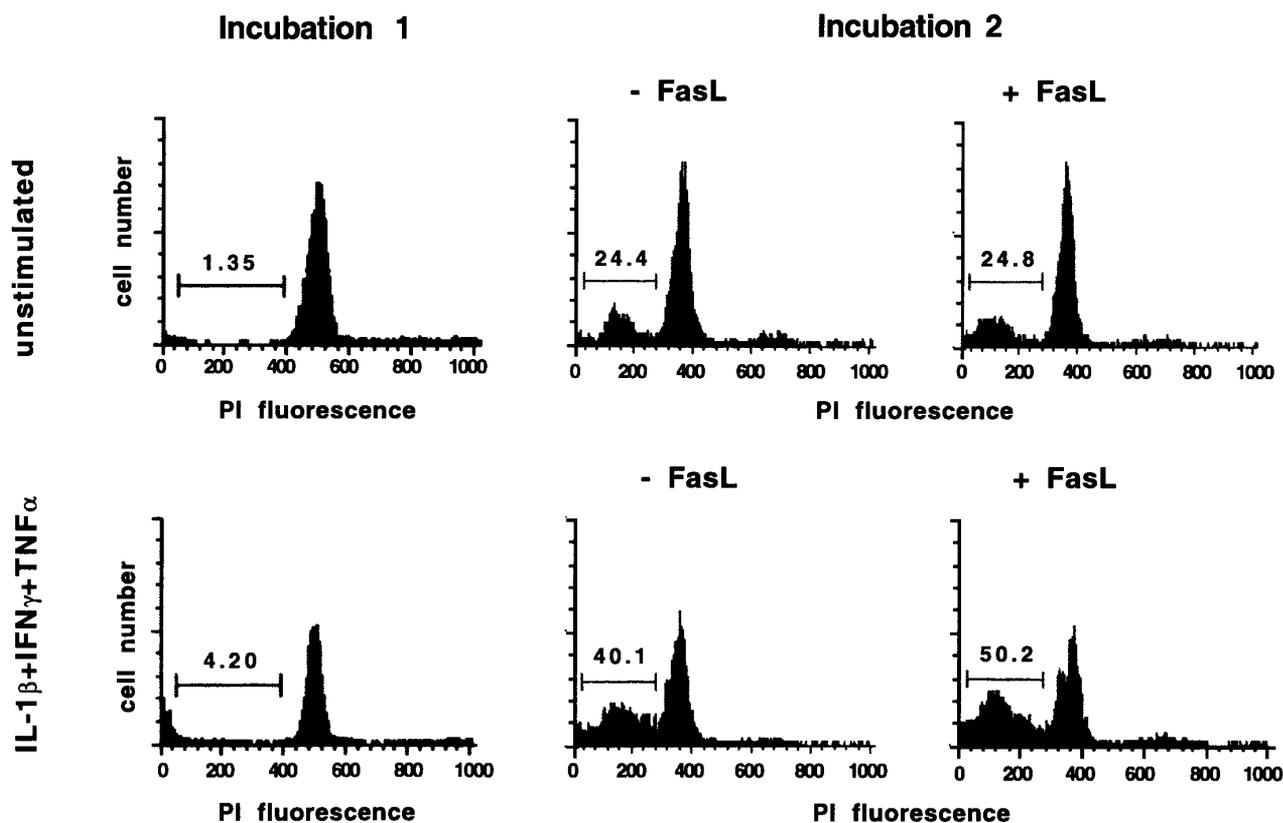


FIG. 4. Induction of apoptosis through Fas/FasL interaction in wild-type islets. After 28–30 h of exposure to 15 U/ml IL-1 $\beta$  plus 80 U/ml IFN- $\gamma$  plus 10 U/ml TNF- $\alpha$ , cells were washed and dissociated as described in RESEARCH DESIGN AND METHODS (incubation 1). Dispersed islet cells were then further exposed to 3% (vol/vol) FasL for an additional 20 h (incubation 2), stained with 1  $\mu$ g/ml PI solution containing 0.1% Triton X-100, and analyzed by flow cytometry. Cells with a staining intensity within the sub-G<sub>1</sub> peak (as indicated by the horizontal bar) were scored as apoptotic cells. Data in the figure represent the mean of five independent experiments: unstimulated islet cells, 24.42  $\pm$  3.3; unstimulated islet cells plus FasL, 24.8  $\pm$  2.1; cytokine-exposed islet cells, 40.18  $\pm$  2.1; and cytokine-exposed islet cells plus FasL, 50.16  $\pm$  2.34. Statistical comparison revealed a significant difference between unstimulated islet cells and cytokine-exposed islet cells cultured in the presence ( $P < 0.05$ ) or absence ( $P > 0.05$ ) of FasL. No statistical difference was detected between unstimulated islet cells cultured in the presence or absence of FasL ( $P > 0.05$ ). A statistically significant increase of apoptosis was detected between cytokine-exposed islet cells grown in the absence of FasL and cytokine-exposed islet cells cultured with FasL ( $P < 0.05$ ).

logic effector cells and their soluble mediators involved. The current understanding is that an unknown (environmental) incident triggers an autoimmune reaction that initiates a slow but progressive course of events, ultimately leading to specific  $\beta$ -cell death. In NOD mice, macrophages or dendritic cells (or both) are the first cell types to infiltrate the islets. Although T-cells are critical for the unfolding of disease, the role of particular T-cell subpopulations has not been unequivocally established. Moreover, it is not at all clear whether the destruction of  $\beta$ -cells occurs via direct contact with T-cells or, alternatively, whether soluble factors secreted by activated T-cells or macrophages are responsible for the loss of  $\beta$ -cells. In the former instance, direct killing is provoked by CD8<sup>+</sup> T-cells recognizing antigens on  $\beta$ -cells. Indeed, CD8<sup>+</sup> T-cells are often the dominant lymphocytes infiltrating the islets. Cytotoxicity mediated by CD8<sup>+</sup> T-cells could then occur via either secretion of perforin or engagement of Fas (39,40). The blocking of CD8<sup>+</sup> or major histocompatibility complex (MHC) class I molecules or both results, consequently, in the reduction of overt diabetes but has no bearing on insulinitis. However, diabetes in NOD mice can also be induced by CD4<sup>+</sup> T-cells and in the absence of MHC class I expression. For this second mechanism, CD4<sup>+</sup> T-cells are activated by  $\beta$ -cell-specific autoantigens present in the context of MHC

class II molecules on the surface of antigen-presenting cells (APC). The nature of the self-antigen(s) specific for  $\beta$ -cells remains to be elucidated, although an increasing number of candidates have been listed as possible targets.  $\beta$ -Cell cytotoxicity is effected indirectly via IFN- $\gamma$  produced by activated T-cells, which in turn also stimulate macrophages to release IL-1 $\beta$ , TNF- $\alpha$ , and oxygen free radicals. Whereas different mechanisms may account for the destruction of  $\beta$ -cells, the direct role of these cells in the pathophysiology of type 1 diabetes has received little attention. Here we demonstrate that the murine  $\beta$ -cell line NIT-1 and primary islets of Langerhans actively participate in the pathologic events leading to their destruction. This hastening of their own demise is achieved by at least three independent pathways: cytokine-mediated apoptosis, production of NO, and cell surface expression of Fas.

Cultures of NIT-1 cells and intact murine islets showed a marked inhibition of glucose-induced insulin release after exposure to a combination of IL-1 $\beta$  plus IFN- $\gamma$  plus TNF- $\alpha$  (Fig. 1C). This effect was not reversed even after removal of the inciting cytokines (data not shown). The molecular mechanism that is responsible and sufficient for this finding was clearly demonstrated to be the production and secretion of NO by  $\beta$ -cells (and in the case of intact islets, possibly

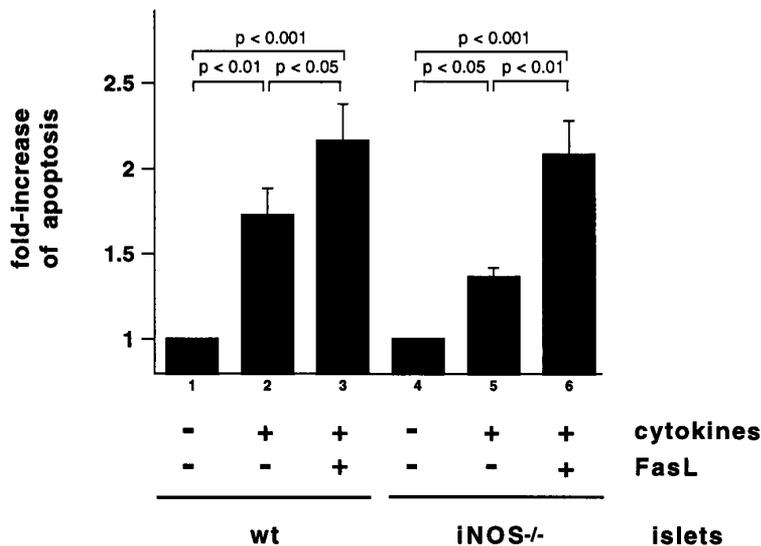


FIG. 5. Role of cytokines, NO, and Fas:FasL in  $\beta$ -cell apoptosis. Wild-type (wt) and  $iNOS^{-/-}$  islets were exposed to a combination of 15 U/ml IL-1 $\beta$  plus 80 U/ml IFN- $\gamma$  plus 10 U/ml TNF- $\alpha$  during 28–30 h. After cell dissociation and a further culture for 20 h in the presence or absence of FasL, single islet cells were collected and stained with a PI/Triton X-100 solution, and DNA fragmentation was analyzed by flow cytometry. The percentage of cells within the sub-G<sub>1</sub> peak of PI staining were scored as apoptotic cells. Background apoptosis (as determined in unstimulated samples) was arbitrarily set to 1. Data represent the mean values  $\pm$  SD of fold-increase above background for five independent determinations. P values were determined by ANOVA. In addition to the data shown, a statistical difference ( $P < 0.05$ ) was also found between cytokine-exposed wild-type islets and treated  $iNOS^{-/-}$  islets (column 2 vs. 5).

other cells as well). Importantly,  $\beta$ -cells from mice deficient for functional iNOS continued to display normal insulin release despite their protracted exposure to cytokines (Fig. 1C). Comparable results were observed by blocking the catalytic activity of iNOS with the arginine analog L-NAME (Fig. 1C). Thus, insulin secretion is inhibited by NO production. Further analysis showed a similar effect using IL-1 $\beta$  plus IFN- $\gamma$ , whereas all other combinations or single cytokines had no effect on NO production and insulin accumulation (data not shown). The lack of a response using only IL-1 $\beta$  may be explained by a species-specific, dose-related resistance of mouse islets to this cytokine (41). Because a deficiency in the physiologic insulin response to glucose was also observed in intact islets, it is implied that the normal environment of  $\beta$ -cells does not suffice to scavenge these pathogenic oxygen radicals.

Recent advances in the understanding of T-cell function disclosed that the Fas/FasL interaction serves as a second cytotoxic mechanism independent of the perforin/granzyme-based lytic pathway. Fas surface expression is not detectable on freshly isolated  $\beta$ -cells (24), and programmed cell death cannot be induced with soluble FasL (Fig. 4). However, exposure to pro-inflammatory cytokines resulted in a functional upregulation of Fas receptors (Fig. 3 and Table 1), which in turn rendered the  $\beta$ -cells susceptible to Fas-induced apoptosis (Fig. 4). According to our experiments (Fig. 2 and data not shown) and the experience of others (38,42,43), murine  $\beta$ -cells express FasL neither spontaneously nor after exposure to pro-inflammatory cytokines, excluding the possibility of suicide/fratricide triggered by Fas:FasL interactions. However, these results are contrasted by a recent report by Suarez-Pinzon et al. (44), which found FasL expression on a minority of R2D6<sup>+</sup> islet cells of NOD mice. Furthermore, a single report (45), but not other publications (26,46), recently described a constitutive expression of FasL on human  $\beta$ -cells. A uniform explanation for this discrepancy in FasL expression is not currently available.

The *in vivo* importance of Fas expression for the pathogenesis of type 1 diabetes has been detailed in several models. For example, NOD mice constitutively expressing FasL on their  $\beta$ -cells displayed heightened  $\beta$ -cell death due to self-destruction following T-cell-induced Fas upregulation

(39). Similarly, the lack of Fas expression in NOD mice prevented the occurrence of spontaneous diabetes in this animal model (47).

NO has been reported to hinder Fas-mediated apoptosis in different cell types via inhibition of caspase activity and sustaining of cellular bcl-2 concentrations but without altering Fas expression (48,49). Although  $\beta$ -cells had not been tested for NO-induced inhibition of Fas signaling until now, overexpression of the anti-apoptotic protein bcl-2 was partially protective for  $\beta$ -cells when exposed to pro-inflammatory cytokines (50). To test whether cytokine-triggered iNOS upregulation also exerted a protective effect in  $\beta$ -cells, we analyzed wild-type and iNOS-deficient  $\beta$ -cells for apoptosis (Fig. 5). In contrast to results in leukocytes (49), iNOS function was not associated with decreased  $\beta$ -cell apoptosis upon Fas triggering. Furthermore, a difference in programmed cell death was noted (in the absence of FasL) between cytokine-exposed  $\beta$ -cells from wild-type and iNOS-deficient mice, implying that NO acts directly as a pro-apoptotic messenger (51) inducing DNA strand breaks in murine  $\beta$ -cells.

Concomitant cross-linking of Fas resulted in maximal apoptosis, suggesting that NO has a priming effect for cytotoxicity (Fig. 5). It is of general note that the extent of apoptotic cell death was intermediate in the experimental system employed; despite appreciable cell surface Fas expression (Fig. 3), signaling via FasL-triggered apoptosis occurred in only a percentage of  $\beta$ -cells (Fig. 4). Although this result is statistically significant, one may have expected a higher efficiency in the induction of programmed cell death. However, limitations inherent to the experimental system account for this finding and have been noted in other experimental systems (52).

Our results reveal that Fas expression is a dominant cytotoxic effector mechanism for murine  $\beta$ -cells and that Fas/FasL-induced apoptosis is not modulated by NO production (Fig. 5). Conversely, NO causes DNA fragmentation in  $\beta$ -cells via a direct (i.e., Fas independent) pathway (Fig. 5). Although mutually independent, the relative contribution of these two cytotoxic mechanisms to the self-inflicted susceptibility for apoptotic  $\beta$ -cell death *in vivo* remains to be determined. Finally, pro-inflammatory cytokines also have a direct, NO- and Fas-independent effect on  $\beta$ -cell viability, as shown in Fig. 5. Thus, three separate pathways are operational

in parallel in murine  $\beta$ -cell death and may have implications for early intervention strategies in the pathogenesis of type 1 diabetes.

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