# Cytokines Induce Apoptosis in β-Cells Isolated From Mice Lacking the Inducible Isoform of Nitric Oxide Synthase (iNOS$^{-/-}$)

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**Prolonged exposure of rodent β-cells to combinations of cytokines induces the inducible form of nitric oxide synthase (iNOS) expression and Fas expression, nitric oxide (NO) production, and cell death. It also induces the expression of potential "defense" genes, such as manganese superoxide dismutase (MnSOD) and heat shock protein (hsp) 70. NO is a radical with multifaceted actions. Recent studies have shown that NO, in addition to having cytotoxic actions, may regulate gene transcription. It remains unclear whether NO mediates cytokine-induced gene expression and subsequent β-cell death. Previous studies using NO synthase blockers yielded conflicting results, which may be due to nonspecific effects of these agents. In this study, we examined the effects of cytokines on gene expression, determined by reverse transcriptase–polymerase chain reaction (RT-PCR), and viability, determined by nuclear dyes, of pancreatic islets or fluorescence-activated cell sorter (FACS)-purified β-cells isolated from iNOS knockout mice (iNOS$^{-/-}$, background C57BL/6x129SvEv) or their respective controls (C57BL/6x129SvEv). The combination of cytokines used was interleukin-1β (50 U/ml) plus γ-interferon (1,000 U/ml) plus tumor necrosis factor-α (1,000 U/ml). The lack of cytokine-induced iNOS activity in the iNOS$^{-/-}$ islet cells was confirmed by RT-PCR and nitrite determination. Cytokines induced a >3-fold increase in Fas and MnSOD mRNA expression in wild-type (WT) and iNOS$^{-/-}$ islets. On the other hand, hsp 70 was induced in WT but not in iNOS$^{-/-}$ islets. Prolonged (6–9 days) exposure of WT islets to cytokines leads to an 80–90% decrease in islet cell viability, whereas viability decreased by only 10–30% in iNOS$^{-/-}$ islet cells. To determine the mode of cytokine-induced cell death, FACS-purified β-cells were exposed to the same cytokines. After 9 days, the apoptosis index was similarly increased in WT (39 ± 3%) and iNOS$^{-/-}$ (33 ± 4%) β-cells. On the other hand, cytokines increased necrosis in WT (20 ± 4%) but not in iNOS$^{-/-}$ (7 ± 3%) β-cells. From these data, we concluded that *1*) NO is required for cytokine-induced hsp 70 mRNA expression but not for Fas and MnSOD expression, *2*) cytokines induce both apoptosis and necrosis in mouse β-cells, and *3*) cytokine-induced apoptosis is mostly NO-independent, whereas necrosis requires NO formation. *Diabetes* 49:1116–1122, 2000**

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ANOVA, analysis of variance; FACS, fluorescence-activated cell sorter; HO, Hoechst; hsp, heat shock protein; IFN-γ, γ-interferon; IL, interleukin; iNOS, inducible form of nitric oxide synthase; L-NMMA, $N^G$-monomethyl-L-arginine; MnSOD, manganese superoxide dismutase; NO, nitric oxide; OD, optical density; PI, propidium iodide; RT-PCR, reverse transcriptase–polymerase chain reaction; TNF, tumor necrosis factor; WT, wild-type.

Inflammatory mediators, such as cytokines and free radicals, probably contribute to β-cell demise in the preclinical phase of type 1 diabetes (1,2). Among the free radicals, nitric oxide (NO) has attracted special attention (3). Expression of the inducible form of nitric oxide synthase (iNOS) has been observed in different autoimmune diseases (4,5), and iNOS is expressed by islet cells and/or invading macrophages in the insulitis present in diabetes-prone BB rats and NOD mice (6–8). Moreover, iNOS mRNA expression is detected in both rodent and human pancreatic islets exposed in vitro to cytokines (3). Transgenic expression of iNOS in β-cells induces β-cell destruction and diabetes (9), whereas lack of iNOS expression (iNOS knockout or iNOS$^{-/-}$ mice) prevents diabetes induced by multiple subdiabetogenic doses of streptozotocin and the in vitro inhibitory effects of interleukin (IL)-1β on mouse β-cell function (10). On the other hand, there are conflicting data on whether blocking of iNOS activity by pharmacological agents prevents cytokine-induced β-cell dysfunction and death in both rodent and human islets (3). Furthermore, it remains unclear whether the radical NO contributes to cytokine-induced β-cell necrosis and/or apoptosis (11–13). A possible reason for these conflicting observations is the use of relatively nonspecific pharmacological blockers of iNOS activity. Indeed, pancreatic β-cells also express a constitutive isoform of NOS (nNOS), which may participate in β-cell physiology (14,15). Thus, it is conceivable that pharmacological NO synthase inhibitors will affect both isoforms of the enzyme, confounding the experimental data obtained. Moreover, some of the analogs used to inhibit iNOS activity, such as aminoguanidine (16,17), may directly interfere with β-cell function (18–20), making it difficult to evaluate their potential impact on cytokine-induced β-cell dysfunction.

When pancreatic β-cells are exposed to cytokines, they express several genes and proteins that may either contribute to cell dysfunction and death or participate in cell repair (21,22). Among these genes are the putative protective agents



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heat shock protein (hsp) 70 (23–25), manganese superoxide dismutase (MnSOD) (26,27), and the pro-apoptotic gene Fas (28,29). It remains to be clarified whether induction of these genes is a direct effect of IL-1β or whether iNOS induction and NO production is a required intermediary step for mRNA expression.

To define the exact role for iNOS in cytokine-induced β-cell gene expression and cell death, we used islets and fluorescence-activated cell sorter (FACS)-purified β-cells from mice genetically deficient in iNOS (iNOS$^{-/-}$) (30). These islets, or purified β-cells, were exposed for different periods of time to combinations of cytokines (IL-1β + interferon-γ [IFN-γ] + tumor necrosis factor [TNF]-α), and both mRNA expression and cell viability were evaluated by reverse transcriptase–polymerase chain reaction (RT-PCR) and DNA-binding dyes, respectively. The data obtained indicate that NO production mediates cytokine-induced hsp 70 mRNA expression but that the radical is not required for MnSOD or Fas expression. Finally, iNOS activation participates in cytokine-induced β-cell necrosis but is not a major factor for cytokine-induced apoptosis.

## RESEARCH DESIGN AND METHODS

**Animals.** Breeding pairs of iNOS$^{-/-}$ mice were a gift from Dr. J.S. Mudget (Merck Research Laboratories, Rahway, NJ) and Drs. J.D. MacMicking and C. Nathan (Cornell University Medical College, New York, NY). The animals have been subsequently bred under specific pathogen-free conditions at the Animal Department, Biomedical Centre (Uppsala, Sweden) (10). The iNOS mice (background C57BL/6x129SvEv) were generated by gene targeting in embryonic stem cells as previously described (30). Homozygous iNOS mutants were maintained by interbreeding the F2 generations. The homozygosity of iNOS$^{-/-}$ mice was confirmed in islet cells by the absence of iNOS mRNA expression in response to cytokines. The function, DNA and insulin content, and histology of islets from iNOS$^{-/-}$ mice were described in a recent article by our group (10). For wild-type (WT) controls, male C57BL/6x129SvEv were used (Taconic, Germantown, NY). Mice were 8–12 weeks old when they were killed for islet isolation.

**Islet and β-cell isolation and culture.** Islets were obtained from male iNOS$^{-/-}$ and WT mice. Pancreases were digested with collagenase and filtered through 500-μm pore mesh nylon screens. From the filtered material, islets were hand-picked using a siliconized glass pipette, placed into the culture medium, and cultured for 16 h at 37°C (31). After this step, the islets were dissociated with trypsin (20 μg/ml) and sorted by FACStar flow cytometer (Becton Dickinson, Sunnyvale, CA), as previously described for rat β-cells (32). Evaluation by electron microscopy showed that these preparations contained >95% viable β-cells (data not shown).

Cell culture was performed in Ham's F-10 medium supplemented with 10 mmol/l glucose, 50 μmol/l 3-isobutyl-1-methylxanthine, 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany), 0.1 mg/ml streptomycin (Continental, Puteaux, Belgium), 0.075 mg/ml penicillin (Laboratoires Diamant, Brussels, Belgium), and 2 mmol/l L-glutamine (Gibco, Paisley, Scotland, U.K.) (33). These culture conditions, originally developed for the maintenance of rat β-cells, have been validated for mouse islet or β-cell culture (31). Islets were cultured in suspension culture dishes of 3 cm diameter (Nunc, Naperville, IL) at a concentration of 50–100 islets in 1 ml of medium. FACS-purified single β-cells ($3 \times 10^3$ cells per well) were cultured in Falcon 96-well microtiter plates (Becton Dickinson, NJ) containing 200 μl medium. For long-term culture (6 or 9 days), culture medium was changed every 3 days, and fresh cytokines were added.

Human pancreatic islets from 4 human donors (age 21 ± 6 [mean ± SE], range 8–36) were isolated and cultured at the central unit of the β-Cell Transplant Program as previously described (34). Light microscopic examination of immunohistochemical stained islets indicated the prevalence of insulin- and glucagon-positive cells to be 59 ± 8 and 18 ± 2%, respectively (mean ± SE; $n = 4$).

**Cytokine treatment and nitrite determination.** The effect of cytokines was examined after 6 h and after 6 and 9 days of culture in the presence of recombinant murine IFN-γ (1,000 U/ml, 10 U/ng; Holland Biotechnology, Leiden, the Netherlands), recombinant human IL-1β (50 U/ml, 38 U/ng; a gift from Dr. C.W. Reynolds from the National Cancer Institute, Bethesda, MD), and recombinant murine TNF-α (1,000 U/ml, 220 U/ng; Innogenetics, Gent, Belgium). These concentrations of cytokines were selected based on our previous studies with mouse pancreatic islets and β-cells (31,35). Culture media were collected after 24 or 72 h for nitrite determination (nitrite is a stable product of NO oxidation), which was performed spectrophotometrically at 546-nm wavelength after colored reaction with the Griess reagent (36). For the experiments with human islets, the cytokines used were recombinant human IL-1β (50 U/ml) and recombinant human IFN-γ (1,000 U/ml, $4.7 \times 10^4$ U/μg; Genzyme, Cambridge, MA), with or without 1.0 mmol/l iNOS inhibitor $N^G$-monomethyl-L-arginine (L-NMMA). We have previously shown that this concentration of L-NMMA prevents cytokine-induced nitrite production by human pancreatic islets (37).

**mRNA isolation and RT-PCR.** Poly(A)$^+$ RNA was isolated from islets (0.5 to $1 \times 10^5$ cells) using oligo(dT)25-coated polystyrene Dynabeads (Dynal, Oslo, Norway). The reverse transcription reaction was performed at 42°C for 1 h and contained (per 10 μl) mRNA equivalent to $6 \times 10^3$ cells, 1× reverse transcription buffer, 5 mmol/l $MgCl_2$, 1 mmol/l each dNTP, 2.5 μmol/l random hexamer primers, and 100 U of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT). The subsequent PCR contained the following (in 25 μl reaction solution): 5 μl cDNA, 0.4 μmol/l forward and reverse primers, 200 μmol/l each dNTP, 1× PCR buffer, 2 mmol/l $MgCl_2$, and 0.625 U AmpliTaq Gold DNA polymerase (Perkin Elmer) (31). PCR specificity and efficiency was improved by using hot start PCR with 12 min predenaturation at 95°C and then 30 cycles for GAPDH and iNOS, 28 cycles for MnSOD, 40 cycles for Fas, 33 cycles for hsp 70 (mouse islets); or 33 cycles for iNOS, 33 cycles for Fas and 31 cycles for GAPDH (human islets) at 94°C for 45 s, 58°C for 45 s, and 72°C for 80 s. The number of cycles was selected to allow linear amplification of the cDNA under study. The primer sequences used for determination of mouse cDNAs were as follows: iNOS-F 5′-GACAGCACAGAAT GTTCCAG-3′; iNOS-R 5′-TGGCCAGATGTTCCTCTATT-3′; GAPDH-F 5′-TCA CTCAAGATTGTCAGCAA-3′; GAPDH-R 5′-AGATCCACGACGGACACATT-3′; MnSOD-F5′-GACCTGCCTTACGACTATGG; MnSOD-R 5′-GACCTTGCTCCT TATTGAAGC; Fas-F 5′-CACAGTTAAGAGTTCATAC-3′; Fas-R 5′-TGGTTGCT GTGCATGGCTC-3′; hsp 70-F 5′-GGCAAGGTGGAGATCATCGC-3′; and hsp 70-R 5′-AGAGTCGTTGAAGTAGGCGG-3′.

The primer sequences for determination of human Fas were as follows: Fas-F 5′-CAAGTGACTGACATCAACTCC-3′ and Fas-R 5′-CCTTGGTTTTCCT TTCTGTGC-3′. The primers used for amplification of human iNOS and GAPDH were as previously described (38).

The ethidium bromide-stained agarose gels were photographed under ultraviolet transillumination using a Kodak Digital Science DC40 camera (Kodak, Rochester, NY), and the PCR band intensities on the image were quantified by Biomax 1D Image analysis software (Kodak) and expressed in pixel intensities (optical density [OD]). The target cDNAs present in each sample were corrected for the respective GAPDH value. Expression of the "housekeeping" gene GAPDH is not affected by exposure to cytokines (39).

**Assessment of β-cell viability.** The viability of islet cell preparations was assessed after 6–9 days of exposure to cytokines. The islets were incubated for 15 min with propidium iodide (PI) (10 μg/ml) and Hoechst (HO) 342 (20 μg/ml) (40). PI is a highly polar dye that penetrates only cells with damaged membranes, staining their nuclei in red; HO 342 freely crosses the plasma membrane, entering both cells with damaged and intact membranes and staining the DNA blue (40). The approximate percentage of dead islet cells was estimated by 3 individual observers, 2 of them unaware of the sample identity (31). Evaluation of cell death in whole islets is difficult because of superposition of cells, and these observations should thus be considered as semiquantitative. The percentages of apoptotic and necrotic cells in the single β-cell preparations were assessed after 6 or 9 days of exposure to cytokines—the amount of time required to detect significant increases in β-cell death (12,31). The cells were examined in an inverted fluorescence microscope with ultraviolet excitation at 340–380 nm. Viable cells were identified by their intact nuclei with blue fluorescence (HO 342), necrotic cells by their intact nuclei with yellow-red fluorescence (HO 342 + PI), and apoptotic cells by their fragmented nuclei, exhibiting either a blue (HO 342; early apoptosis) or yellow-red fluorescence (HO 343 + PI; late apoptosis) (40). This fluorescence assay for single β-cells is quantitative and has been validated by systematic comparisons with electron microscopy observations (13,40). In each experimental condition, a minimum of 500 cells was counted. The necrosis and apoptosis indexes were calculated as [(% necrotic or apoptotic cells in experimental condition – % necrotic or apoptotic cells in control)/(100 – % dead cells in control)] × 100 (41). The mean values for necrosis and apoptosis in single control β-cells (not exposed to cytokines) were 27 and 16%, respectively. These values were similar after 6 or 9 days of culture and when comparing WT and iNOS$^{-/-}$ β-cells (data not shown).

**Statistical analysis.** Results are presented as means ± SE. The statistical differences between the groups were determined by paired Student's $t$ test or, when indicated, by analysis of variance (ANOVA) followed by multiple $t$ test with the Bonferroni correction. In all experiments, islets or pure β-cell prepa-

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rations obtained from 1 set of animals (2–3 mice) were considered as 1 individual experiment. Thus, each independent experiment was performed with a separate set of animals on a separate day.

## RESULTS

**Cytokine-induced mRNA expression in islets isolated from WT and iNOS$^{-/-}$ mice.** There was a low basal expression of hsp 70 mRNA in WT islets that was increased by >15-fold after addition of the combination of cytokines (IL-1β + IFN-γ + TNF-α) (Fig. 1). On the other hand, there was no detectable hsp 70 expression in iNOS$^{-/-}$ islets, independent of the presence of cytokines. MnSOD mRNA was expressed under basal conditions in WT and iNOS$^{-/-}$ islets and showed a marked increase in both strains after exposure to cytokines. There was no iNOS expression in the absence of cytokines in either WT or iNOS$^{-/-}$ islets, but WT islets responded with a clear iNOS expression after a 6 h exposure to cytokines. As expected, the iNOS$^{-/-}$ islets failed to express iNOS in response to cytokines.

There was no detectable Fas expression in control WT and iNOS$^{-/-}$ islets, but upon exposure to cytokines, islets from both mouse strains presented a marked and similar increase in Fas expression (Fig. 2). This observation contrasts with previous observations, suggesting that cytokine-induced Fas expression in human pancreatic islets is mediated via NO production (29). To assess whether this discrepancy was due to species differences (i.e., human × mouse islets), we exposed human pancreatic islets obtained from 4 separate donors to IL-1β (50 U/ml) and/or IFN-γ (1,000 U/ml), with or without the iNOS inhibitor L-NMMA (1.0 mmol/l) for 6 or 24 h before cells were collected for mRNA extraction and RT-PCR for Fas, iNOS, and GAPDH (data presented as ODs, corrected per GAPDH; means ± SE of 4 experiments). iNOS was not detectable in islets exposed for 6 or 24 h to control conditions, L-NMMA, IL-1β, or IFN-γ, but it was present in islets exposed to IL-1β + IFN-γ for 6 h (OD 9.1 ± 1.6) or 24 h (OD 7.1 ± 1.4). A similar iNOS expression was observed in human islets exposed to IL-1β plus IFN-γ in the presence of L-NMMA (data not shown). There was no detectable Fas expression in human islets exposed for 6 or 24 h to control conditions, L-NMMA, or IL-1β alone. For the other groups, the OD values were as follows: for 6 h: IFN-γ, 9.2 ± 0.2; IL-1β + IFN-γ, 9.2 ± 0.6; IL-1β + IFN-γ + L-NMMA, 8.1 ± 0.5; for 24 h: IFN-γ, 6.8 ± 0.9; IL-1β + IFN-γ, 8.7 ± 1.1; IL-1β + IFN-γ + L-NMMA, 7.7 ± 1.2. Thus, the main inducer of Fas expression in human islets is IFN-γ, a cytokine that does not induce iNOS expression (see above). A combination of IL-1β plus IFN-γ induced both iNOS and Fas expression, but the iNOS blocker L-NMMA did not prevent Fas mRNA induction. These data indicate that cytokine-induced Fas expression in human islets is not mediated via NO production.

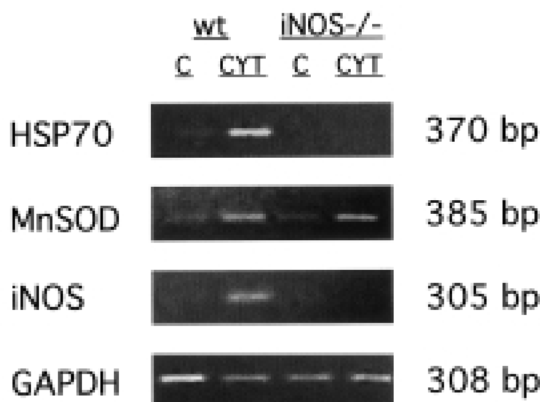


**FIG. 1. RT-PCR analysis of hsp 70, MnSOD, iNOS, and GAPDH mRNA expression by WT or iNOS$^{-/-}$ islets exposed for 6 h to control conditions (C, no cytokines added) or cytokines (CYT, 50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α). The cDNA samples were amplified in parallel with GAPDH-specific primers, confirming loading in all lanes. ODs (corrected for GAPDH) are as follows: for hsp 70: WT: C, 0.13; CYT, 2.16; iNOS$^{-/-}$: C, not detectable (ND); CYT, ND; for MnSOD: WT: C, 0.08; CYT, 0.61; iNOS$^{-/-}$: C, 0.17; CYT, 0.5; for iNOS: WT: C, ND; CYT, 0.72; iNOS$^{-/-}$: C, ND; CYT, ND. The figure is representative of 3–4 similar experiments.**

**FIG. 2. Fas expression by WT or iNOS$^{-/-}$ islets exposed for 6 h to control condition (C, no cytokines added) or cytokines (CYT, 50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α; 2 separate experiments are shown), analyzed by RT-PCR. The cDNA samples were amplified in parallel with GAPDH-specific primers, confirming loading in all lanes. ODs (corrected for GAPDH) are as follows: experiment 1: WT: C, not detectable (ND); CYT, 0.42; iNOS$^{-/-}$: C, ND; CYT, 0.73; experiment 2: WT: C, ND; CYT, 0.21; iNOS$^{-/-}$: C, ND; CYT, 0.25. The figure is representative of 4 similar experiments.**

**Viability and nitrite production of islets or purified β-cells from WT and iNOS$^{-/-}$ mice exposed for 6–9 days to cytokines.** WT islets exposed for 72 h to cytokines (50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α) showed a near 5-fold increase in medium nitrite accumulation, whereas iNOS$^{-/-}$ islets did not increase the nitrite production above basal levels (Fig. 3; cytokine-exposed iNOS$^{-/-}$ islets were not different from iNOS$^{-/-}$ non–cytokine-exposed control islets but have a significance of $P < 0.01$ when compared with WT islets exposed to cytokines [determined by ANOVA]). Similar results were observed in whole WT and iNOS$^{-/-}$ islets exposed for 24 h to cytokines (data not shown) or in FACS-purified

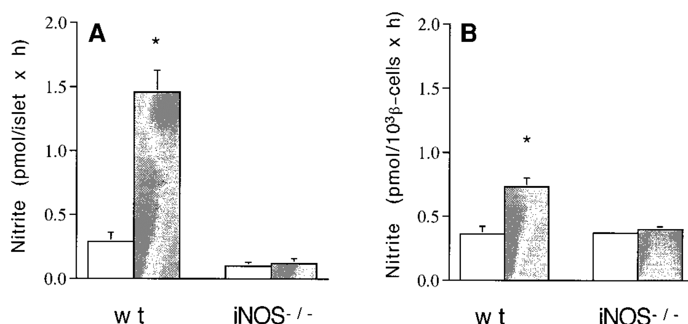


**FIG. 3. Cytokine-induced nitrite production by WT or iNOS$^{-/-}$ islets (*A*) or FACS-purified β-cells (*B*) exposed for 72 h to control condition (□, no cytokines added) or cytokines (■, 50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α). Results are means ± SE of 5–6 experiments. *$P < 0.01$ vs. respective non–cytokine-treated control (ANOVA).**



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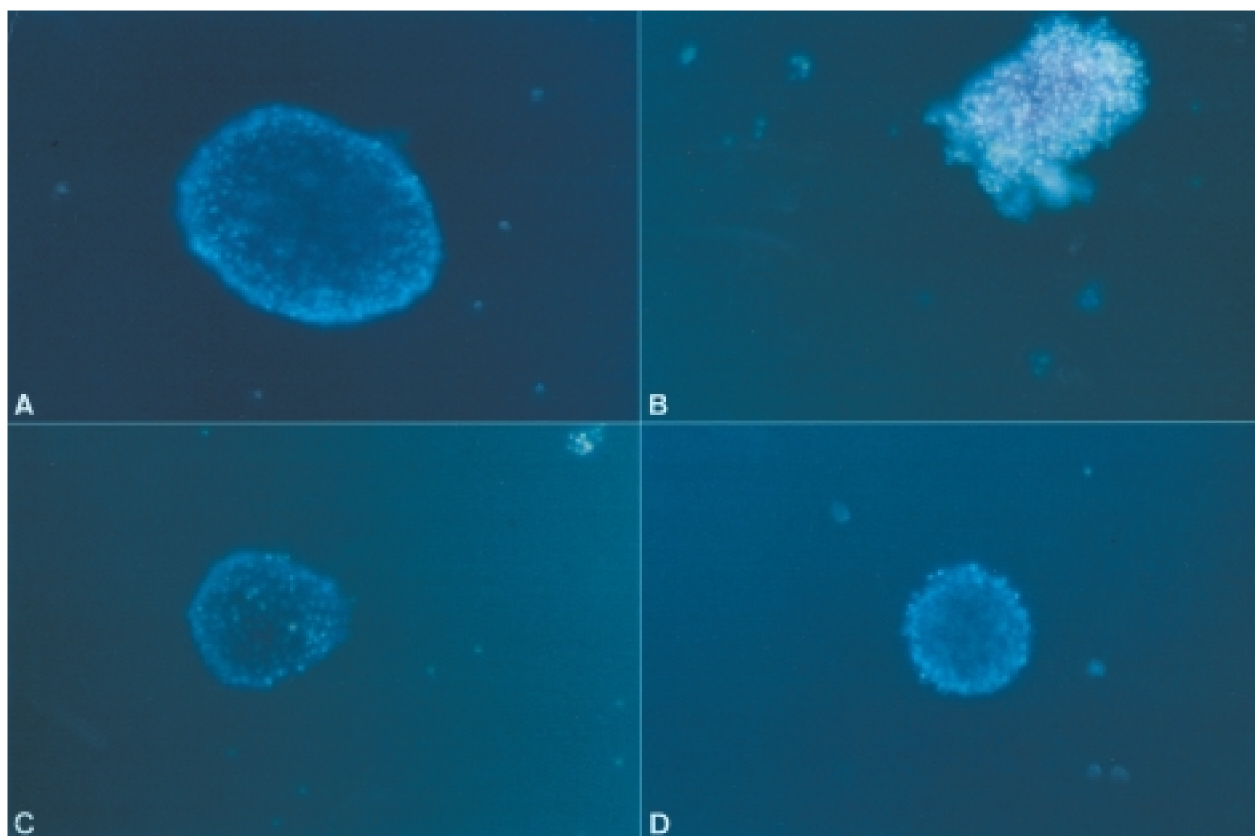


**FIG. 4. Effect of cytokines on islet cell survival in WT (*A* and *B*) and iNOS$^{-/-}$ (*C* and *D*) islets. Islets were isolated and cultured for 6 days under control conditions (no cytokines added; *A* and *C*) or in the presence of cytokines (50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α; *B* and *D*). Cell viability was determined with DNA binding dyes HO 342 and PI, as described in RESEARCH DESIGN AND METHODS. Nuclei of nonviable cells are stained in red. The figure is representative of 5–6 similar experiments. An estimate of the percentage of viable cells is presented in Table 1.**

β-cells from WT or iNOS$^{-/-}$ mice exposed for 72 h to cytokines (Fig. 3). In pure β-cell preparations from WT mice, however, cytokine-induced nitrite production was of a lower magnitude (2-fold) than the increase observed in whole pancreatic islets (5-fold). We have previously observed this difference when comparing nitrite production by β-cells and whole islets isolated from WT C57BL/6 or IRF-1$^{-/-}$ mice exposed to combinations of cytokines (31). The higher nitrite production by whole islets compared with β-cells is probably due to the effects of cytokines on nonendocrine cells (e.g., macrophages and ductal cells) present in these preparations (31,38). Medium nitrite accumulation was not determined at later time points because of the decreased cellular viability observed in some of the experimental groups exposed to cytokines (see below).

Culture of WT islets in the presence of cytokines (50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α) for 6 days induced a major decrease in islet cell viability, whereas the survival of iNOS$^{-/-}$ islet cells was not affected by this treatment (Fig. 4 and Table 1). In parallel experiments (control values as in Table 1), these islets were exposed for 6 days to either IL-1β alone, IL-1β plus TNF-α, or IL-1β plus IFN-γ (same concentrations as above). Whereas IL-1β did not significantly decrease the viability of WT or iNOS$^{-/-}$ islets (data not shown), IL-1β plus TNF-α or IL-1β plus IFN-γ reduced the viability of WT islets to 50 ± 8 and 13 ± 3%, respectively ($P < 0.01$ vs. control WT islets; $n = 5$). On the other hand, these combinations of cytokines failed to decrease the viability of iNOS$^{-/-}$ islets (viability of 86–88%, similar to control values, Table 1). A more prolonged exposure of WT islets to IL-1β plus IFN-γ plus TNF-α reduced cell viability even further (Table 1). At this time point, iNOS$^{-/-}$ islets exposed to the same cytokines showed a significant decrease in the number of viable cells (Table 1) but to a much less degree than the values observed

TABLE 1
Effects of cytokines on islet cell viability in whole mouse pancreatic islets isolated from WT and iNOS$^{-/-}$ mice and exposed for 6 or 9 days to cytokines

| | 6 Days | | 9 Days | |
|---|---|---|---|---|
| | WT | iNOS$^{-/-}$ | WT | iNOS$^{-/-}$ |
| Control | 89 ± 1 | 90 ± 1 | 85 ± 4 | 86 ± 2 |
| Cytokines | 12 ± 4† | 88 ± 2‡ | 7 ± 1† | 72 ± 3*‡ |

Data are means ± SE for 5–6 experiments. Whole mouse islets obtained from WT or iNOS$^{-/-}$ mice were cultured for 9 days either without cytokines (control) or with IL-1β (50 U/ml) + IFN-γ (1,000 U/ml) + TNF-α (1,000 U/ml). Total islet cell death was estimated by fluorescence microscopy, as described in RESEARCH DESIGN AND METHODS. *$P < 0.001$ and †$P < 0.001$ vs. respective controls (same mouse strain); ‡$P < 0.001$ vs. WT islets exposed to the same treatment. Data were analyzed by ANOVA.

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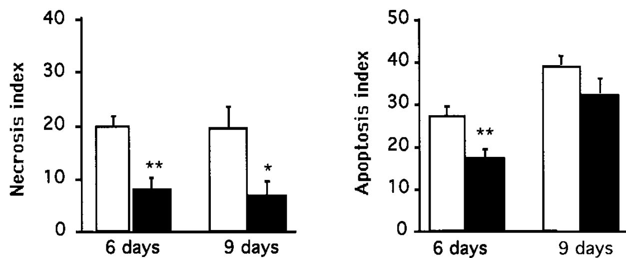


**FIG. 5. Prevalence of necrosis and apoptosis in FACS-purified β-cells from WT (□) or iNOS$^{-/-}$ (■) mice exposed to 50 U/ml IL-1β + 1,000 U/ml IFN-γ + 1,000 U/ml TNF-α. The necrosis and apoptosis indexes were calculated as described in RESEARCH DESIGN AND METHODS. Cell viability was determined with the DNA binding dyes HO 342 and PI. Data are means ± SE of 7–8 experiments. *$P$ < 0.05, **$P$ < 0.01 vs. WT (ANOVA).**

in WT islet cells (72 vs. 7% viable cells in cytokine-treated iNOS$^{-/-}$ and WT islet cells, respectively).

The data described above indicate that absence of iNOS expression decreases cytokine-induced cell death in mouse pancreatic islets. These experiments, however, did not allow a clear determination of the type of cell death induced by cytokines (necrosis or apoptosis). To answer this question, β-cells were purified by FACS sorting from both WT and iNOS$^{-/-}$ mice. These cells were exposed for 6 or 9 days to IL-1β plus IFN-γ plus TNF-α (Fig. 5). Under these conditions, there was a significant increase in both apoptosis and necrosis in WT β-cells ($P$ < 0.05 vs. WT noncytokine treated controls), with apoptosis as the predominant form of cell death. Interestingly, iNOS$^{-/-}$ β-cells exposed to cytokines showed only a minor increase in necrosis (not significantly different from iNOS$^{-/-}$ noncytokine-treated controls; $P$< 0.05 vs. WT β-cells exposed to cytokines), whereas the prevalence of apoptosis was significantly increased compared with that of iNOS$^{-/-}$ controls ($P$< 0.001 at both days 6 and 9), reaching values similar to those observed in WT β-cells after 9 days of exposure to cytokines (Fig. 5). These data indicate that the lack of iNOS expression prevents necrosis, but not apoptosis, in mouse β-cells.

## DISCUSSION

In this study, we used pancreatic islets, or FACS-purified β-cells, from iNOS deficient mice (30) to define the role for this radical in cytokine-induced gene expression and β-cell death. That islet cells from these mice are indeed iNOS deficient was confirmed by the lack of iNOS expression or increased nitrite production in response to cytokines. On the other hand, islet cells obtained from 4 different WT mouse strains expressed iNOS and produced NO in response to combinations of cytokines (10,31,35; present data).

The induction of MnSOD and Fas mRNA expression by cytokines was well preserved in iNOS$^{-/-}$ mice, indicating that NO is not a necessary intermediate for the induction of these genes. On the other hand, IL-1β plus IFN-γ plus TNF-α induced hsp 70 mRNA expression in WT, but not iNOS$^{-/-}$, islets, suggesting that hsp 70 expression is mediated via iNOS activation and NO production. The data on MnSOD and hsp 70 mRNA are in agreement with previous observations showing that chemical iNOS blockers prevent IL-1β–induced hsp 70 (42,43), but not MnSOD (44), expression in pancreatic islets or insulin-producing cells and that NO donors fail to increase the cellular content of MnSOD mRNA in these cells (44). On the other hand, the finding that cytokines induce Fas expression in iNOS$^{-/-}$ islets (45; present data) does not confirm 2 previous studies (from the same group) suggesting that NO is the mediator of Fas induction by IL-1β in human pancreatic islets (29,46). In another series of experiments, we observed that chemical iNOS inhibitors do not prevent IL-1β–induced Fas mRNA expression in FACS-purified rat β-cells (M. Darville, D.L.E., unpublished data). Similar observations were made in islets isolated from NOD mice and exposed to cytokines and/or iNOS inhibitors (47). These differences may be due to the use of islets from different species in these studies, i.e., human (29,46) versus mouse islets (45,47; present data) or rat β-cells (M. Darville, D.L.E., unpublished data). It is worth noting that in the studies by Stassi et al. (29) and Giannoukakis et al. (46), it was reported that IL-1β alone induces NO production, Fas upregulation, and functional suppression of human islets. Several other studies dealing with large numbers of well-characterized human islet preparations reported induction of iNOS expression, NO production, and β-cell functional suppression in human islets when these cells were exposed to combinations of 2 or 3 cytokines (IL-1β + IFN-γ or IL-1β + IFN-γ + TNF-α) but not when they were cultured in the presence of IL-1β alone (18,48–51; present data). Because there is no detailed description of the cellular composition or hormonal concentration of the human islet preparations used in the studies by Stassi et al. (29) and Giannoukakis et al. (46), it cannot be ruled out that their surprising results with IL-1β alone are due to a contribution by nonendocrine cells not separated from the islet endocrine cells during the isolation procedure. Indeed, our current data indicate that IFN-γ or IL-1β plus IFN-γ, but not IL-1β alone, induce Fas mRNA expression in well-characterized human islet preparations ($n$ = 4), but this induction is not prevented by iNOS blockers.

We presently observed that prolonged exposure of whole WT mouse islets to combinations of cytokines induces a severe decrease in islet cell viability, confirming previous observations with islets obtained from different mouse strains (31,35). Islets isolated from iNOS$^{-/-}$ mice were more resistant to cytokine-induced cell death than WT islets and presented only a significant decrease in islet cell viability after a 9-day exposure to IL-1β plus IFN-γ plus TNF-α. This finding indicates that iNOS-mediated NO formation is an important component for cytokine-induced cell death in whole islets, as previously suggested by the observation that iNOS blockers prevent cytokine-induced DNA loss in NMRI mouse islets (35). Evaluation of cell viability in whole islets is difficult because of both cellular superposition and limited access to the central area of the islets. The use of nuclear dyes in whole islets allows detection of major differences in cell viability (31), as presently observed, but does not allow a reliable determination of the type of cell death, i.e., necrosis or apoptosis. To address this issue, we examined the effects of cytokines on FACS-purified β-cells attached to polylysine-coated microtiter cups and stained with the nuclear dyes HO 342 and PI. This method allows a reliable and quantitative evaluation of the percentage of necrotic and apoptotic β-cells (13,40), was validated by systematic comparisons with electron micrographs (13,40), and has been successfully used to evaluate apoptosis/necrosis in rat (40,52), mouse (31; present data), and human (12) β-cells. The use of purified β-cells in these experiments provides a homogeneous and well-defined

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cell population (>95% β-cells), obviating detection of apoptosis or necrosis in non–β-cells—a problem inherent to studies performed in whole isles.

The combination of cytokines induced cell death by both apoptosis and necrosis in WT (C57BL/6x129SvEv) β-cells, with predominance of apoptotic cell death. Similar observations were made in β-cells purified from Wistar rats (13,52) and C57BL/6 mice (31). Interestingly, cytokine-induced necrosis was nearly completely prevented in $iNOS^{-/-}$ mice, whereas the lack of iNOS expression seemed only to postpone apoptosis, which was partially decreased at 6 days, but reached values similar to that observed in WT β-cells after 9 days of continued exposure to cytokines. Thus, it can be concluded from these data that NO contributes to the necrotic, but not the apoptotic, component of cytokine-induced β-cell death in mouse β-cells. In line with these observations, iNOS blockers failed to prevent cytokine-induced apoptosis in both human (12) and rat (13) β-cells.

It is worth noting that whereas IL-1β plus IFN-γ plus TNF-α induces both apoptosis and necrosis in mouse β-cells, with the necrotic component dependent on iNOS expression and NO formation (present data), the same cytokines induced only apoptosis in human β-cells by an NO-independent mechanism (12). The cellular decision to undergo necrosis or apoptosis may be determined by the intracellular ATP concentration. Classic apoptotic triggers will cause apoptosis in the presence of normal stores of cellular ATP, whereas they induce necrosis when ATP is predepleted in the target cell (53). We have previously observed that a 48-h exposure of mouse islets to IL-1β plus IFN-γ plus TNF-α leads to a 70% decrease in glucose oxidation, an effect prevented by iNOS inhibitors (35). On the other hand, a 6-day exposure of human islets to the same combination of cytokines also increases NO production but fails to significantly decrease glucose oxidation (18), probably because of the increased resistance of human islets to this radical (54–56). It is thus conceivable that the well-preserved human islet mitochondrial function will enable these cells to maintain near-normal ATP production in the face of a prolonged pro-apoptotic stimulus (i.e., cytokines), allowing the human islet cells to complete the apoptotic program. On the other hand, mouse islets exposed to the same pro-apoptotic stimulus will suffer an early decrease in mitochondrial function and ATP production. Under these conditions, some cells may preserve enough ATP to complete apoptosis, whereas others will suffer a more severe ATP depletion and thus undergo necrosis. This heterogeneous fate of β-cells is compatible with our recent observation that IL-1β induces different degrees of functional suppression in distinct β-cell subpopulations (57). Cytokine-induced inhibition of glucose oxidation in mouse islets is NO dependent (35), which may explain why $iNOS^{-/-}$ mouse β-cells respond to cytokines similarly to human β-cells, i.e., they undergo cell death mostly by apoptosis.

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