

Interleukin-1 Plus γ -Interferon–Induced Pancreatic β -Cell Dysfunction Is Mediated by β -Cell Nitric Oxide Production

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Cytokines have been implicated in pancreatic β -cell destruction leading to type 1 diabetes. *In vitro*, a combination of γ -interferon (IFN- γ) and interleukin-1 (IL-1) stimulate inducible nitric oxide synthase (iNOS) expression in islets, and the resulting increased production of nitric oxide (NO) causes islet cell destruction. Islets contain macrophages, ductal cells, and endothelial cells that, when activated, may mediate islet cell damage by producing either NO themselves or cytokines that then stimulate NO production by β -cells. The aim of this study was to determine whether β -cell damage mediated by cytokine-induced NO production is dependent on β -cell production of NO, or whether NO produced by other cells in the islet is capable of destroying β -cells. To address this aim, we used transgenic mice expressing a dominant-negative IFN- γ receptor in β -cells (RIP- $\Delta\gamma$ R). RIP- $\Delta\gamma$ R islets are resistant to IL-1 + IFN- γ -induced inhibition of insulin secretion and DNA damage, indicating that β -cell IFN- γ responsiveness is required for IL-1 + IFN- γ -mediated β -cell damage. Although islets isolated from RIP- $\Delta\gamma$ R mice are resistant to functional damage, these islets produce NO in response to IL-1 + IFN- γ , but at a lower concentration than that produced by wild-type islets. β -Cells appear to be the primary cellular source of IL-1 + IFN- γ -induced iNOS expression in wild-type islets. In contrast, IL-1 + IFN- γ fail to stimulate iNOS expression by insulin-expressing cells in islets isolated from RIP- $\Delta\gamma$ R mice. IL-1 + IFN- γ -induced expression of iNOS was detected in non- β -cells in both wild-type and RIP- $\Delta\gamma$ R islets. These findings support the hypothesis that NO must be produced by β -cells to induce damage. *Diabetes* 51:311–316, 2002

Type 1 (insulin-dependent) diabetes is the result of T-cell-mediated destruction of pancreatic β -cells. There are three major mechanisms by which this β -cell destruction may occur: 1) perforin and granzymes released from the granules of cytotoxic T-cells; 2) cell death receptors, such as CD95 and TNFR1; and 3) inflammatory cytokines, including interleukin-1 (IL-1), γ -interferon (IFN- γ), and tumor necrosis factor (TNF).

Inflammatory cytokines are likely to be important mediators of β -cell destruction in some animal models of diabetes, and possibly in humans. In the lymphocytic choriomeningitis virus (LCMV)-induced model, β -cells express the glycoprotein (GP) from LCMV, and diabetes is induced by infection with LCMV (1,2). Diabetes did not occur in LCMV-infected IFN- γ -deficient GP mice, suggesting that IFN- γ is important in the disease progression (3). The role of IFN- γ in LCMV-induced diabetes was further studied using transgenic mice, with only the β -cells unresponsive to IFN- γ . These mice were also protected from diabetes, suggesting that IFN- γ is acting on the β -cell to promote disease and may play a role in β -cell destruction (4). Other animal models of diabetes in which cytokines are believed to be important include cyclophosphamide-induced diabetes in the nonobese diabetic (NOD) mouse and BDC2.5 T-cell receptor (TCR) transgenic models (5,6) as well as β -cell destruction caused by infection of mice with the D variant of encephalomyocarditis virus (7).

Cytokines, such as IL-1, TNF, and IFN- γ , are known to induce the production of nitric oxide (NO) by islets, and there is evidence to support a role for NO in diabetes development. The incidence of diabetes induced by injections of multiple low-dose streptozotocin is reduced in mice deficient in inducible NO synthase (iNOS) (8), and iNOS expression has been detected in islets of NOD mice after cyclophosphamide treatment (9). Also, transgenic expression of iNOS under control of the rat insulin promoter results in NO-dependent diabetes (10). NO production may not be the only mechanism by which cytokines damage β -cells. IFN- γ and IL-1 stimulate CD95 death receptor expression by β -cells *in vitro*, and CD95 activation has been implicated in β -cell death caused by T-cells in the TCR transgenic NOD4.1 model (11) and in the NOD mouse (12). These studies support a role for inflammatory cytokines, such as IL-1 and IFN- γ , as well as NO in the development of diabetes in animal models. These animal

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GP, glycoprotein; IFN- γ , γ -interferon; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; KRB, Krebs-Ringer bicarbonate buffer; LCMV, lymphocytic choriomeningitis virus; NMMA, NG-monomethyl-L-arginine; NO, nitric oxide; TCR, T-cell receptor; TNF, tumor necrosis factor.

models potentially reflect aspects of diabetes in humans, which is likely to be a heterogeneous disease involving a variety of effector mechanisms.

Cytokines and NO may contribute to β-cell damage by two mechanisms. Cytokines such as IL-1 and IFN-γ may directly stimulate iNOS expression and NO production by β-cells, resulting in NO-mediated β-cell damage. Alternatively, cytokines may stimulate iNOS expression by non-endocrine cells in islets (e.g., macrophages and endothelial and ductal cells), and in a paracrine fashion, NO may mediate β-cell damage. In support of the first hypothesis, cytokines such as IL-1 and IFN-γ have been shown to stimulate iNOS expression by β-cells in rat and human islets, and the resulting increased production of NO is believed to participate in cytokine-induced inhibition of glucose-stimulated insulin secretion and islet degeneration. In addition, activation of intraislet macrophages results in β-cell damage by a mechanism that is dependent on the intraislet release of IL-1 (13–15). In support of the second hypothesis, a number of studies have shown that macrophage and/or endothelial cell NO production can mediate β-cell damage independent of local cytokine release (16–20).

It has been difficult to directly determine whether β-cell or nonendocrine cell production of NO mediates cytokine-induced β-cell damage because all cell types in cytokine receptor-deficient mice are unresponsive, and the direct effects of cytokines on individual islet cell types cannot be determined. Transgenic NOD mice with β-cell-specific expression of a dominant-negative IFN-γ receptor (RIP-ΔγR) were generated to address the effects of IFN-γ on β-cell destruction (21). β-Cells of these mice are not responsive to IFN-γ in vitro and in vivo because IFN-γ fails to upregulate class I major histocompatibility complex or induce signal transducer and activator of transcription-1 nuclear localization. RIP-ΔγR mice provide a novel experimental system to examine cell-specific cytokine signaling in islets because IFN-γ-unresponsiveness is confined to β-cells, whereas IFN-γ signaling is normal in all other cell types, both within the complex local environment of the islet and in the immune system. Our previous studies with these transgenic mice showed that IFN-γ effects on β-cells are not required for diabetes in the NOD mouse (21). However, in LCMV-induced diabetes, direct effects of IFN-γ on the β-cell are required for β-cell destruction (4). This raised the question of the mechanism by which IFN-γ resistance protects β-cells, given that IFN-γ on its own is not toxic to β-cells.

In the current study, RIP-ΔγR mice were used to understand the extent to which cytokine-mediated β-cell damage depends on the production of NO by non-β-cells. Our data indicate that NO production by β-cells themselves is required for IL-1 + IFN-γ-mediated inhibition of islet function and islet damage, and that NO produced by nonendocrine islet cells does not mediate β-cell damage.

RESEARCH DESIGN AND METHODS

Mice and cytokines. NOD/Lt and RIP-ΔγR mice were housed in the animal facility at the Walter and Eliza Hall Institute of Medical Research. The prevalence of diabetes of female NOD/Lt mice at our institution is 75–80% by 300 days of age. RIP-ΔγR mice on an NOD genetic background have been previously described (21). Mice used as islet donors were ≤50 days of age and always age- and sex-matched with control donors. Young mice were used to minimize the amount of islet inflammation; however, subsequent experiments

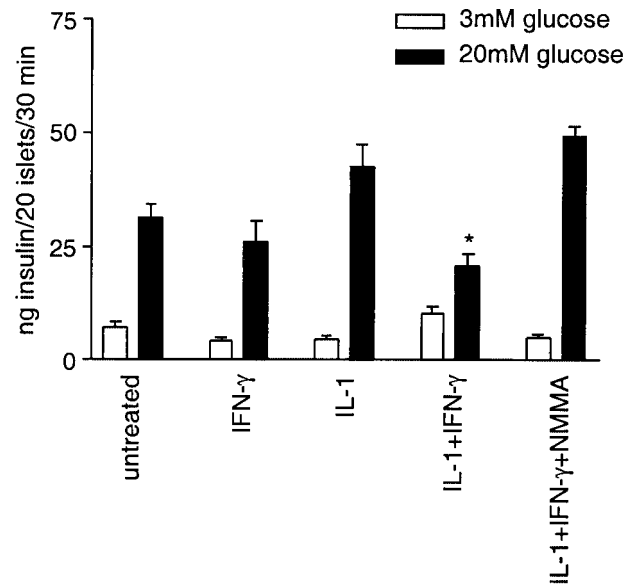


FIG. 1. IL-1 and IFN-γ inhibit glucose-stimulated insulin secretion in NOD islets. Glucose-stimulated insulin secretion assays were performed on cytokine-treated NOD islets as indicated. Insulin was measured by radioimmunoassay. Insulin secreted at 3 and 20 mmol/l glucose is shown, with means ± SE from three independent experiments. **P* < 0.01, untreated vs. IL-1 + IFN-γ (calculated on fold increase from 3–20 mmol/l glucose).

with islets from older NOD mice (90–100 days of age) yielded similar results (not shown).

Recombinant murine IFN-γ was obtained from Genentech (San Francisco, CA), and recombinant hIL-1β was from Genzyme (Cambridge, MA). NG-monomethyl-L-arginine (NMMA) was purchased from Sigma.

Islet isolation. Islets of Langerhans were isolated from mice by pancreas digestion with collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) followed by a BSA density gradient (First Link, U.K.), as previously described (21,22). Islets were hand-picked and cultured at 37°C in 5% CO₂ in CMRL-1066 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS and antibiotics. Islets were precultured overnight before washing, counting, and adding cytokines for experiments.

Glucose-stimulated insulin secretion assays. Insulin secretion assays were performed as previously described (17). Briefly, 200 islets were cultured in complete CMRL in the presence of cytokines for 24 h and then washed three times in Krebs-Ringer bicarbonate buffer (KRB; 25 mmol/l HEPES, 115 mmol/l NaCl, 24 mmol/l NaHCO₃, 5 mmol/l KCl, 1 mmol/l MgCl₂, 2.5 mmol/l CaCl₂, pH 7.4) with 3 mmol/l glucose and 0.1% BSA. Groups of 20 islets were counted into glass tubes in 200 μl of KRB containing 3 mmol/l glucose and incubated for 30 min under 5% CO₂/95% air with shaking at 37°C, followed by a second incubation in 200 μl KRB containing either 3 or 20 mmol/l glucose. Buffer was sampled for insulin after 30 min using a rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO). In each experiment, up to four groups of 20 islets were independently assayed for each condition. Statistical differences between groups was calculated using the fold difference between 3 and 20 mmol/l glucose for each sample.

Nitrite determination. Nitrite was detected in the cultures by mixing 50 μl supernatant with 50 μl Griess reagent (23). Absorbances were read at 540 nm, and nitrite concentrations were calculated using a sodium nitrite standard curve.

Apoptosis assay. Quantitation of apoptosis was determined according to the method of Nicoletti et al. (24). Islets were cultured for 4 days with cytokines, followed by analysis for DNA fragmentation on a flow cytometer (Becton Dickinson, Mountain View, CA) as previously described (25). This assay measures fragmented nuclei, and therefore greater than one fragment can be derived from one apoptotic cell. Data from 3–4 individual experiments were pooled.

Western blotting. Islets were incubated for 24 h with cytokines, transferred to microcentrifuge tubes, washed three times in PBS, and then resuspended in 25 μl SDS sample buffer (0.25 mol/l Tris-HCl, 20% 2-mercaptoethanol, 4% SDS, pH6.6) plus 15 μl H₂O. Samples were then boiled for 5 min, followed by the addition of 4 μl loading dye (0.05% bromophenol blue and 80% glycerol). Samples were separated by SDS-PAGE and transferred to nitrocellulose using

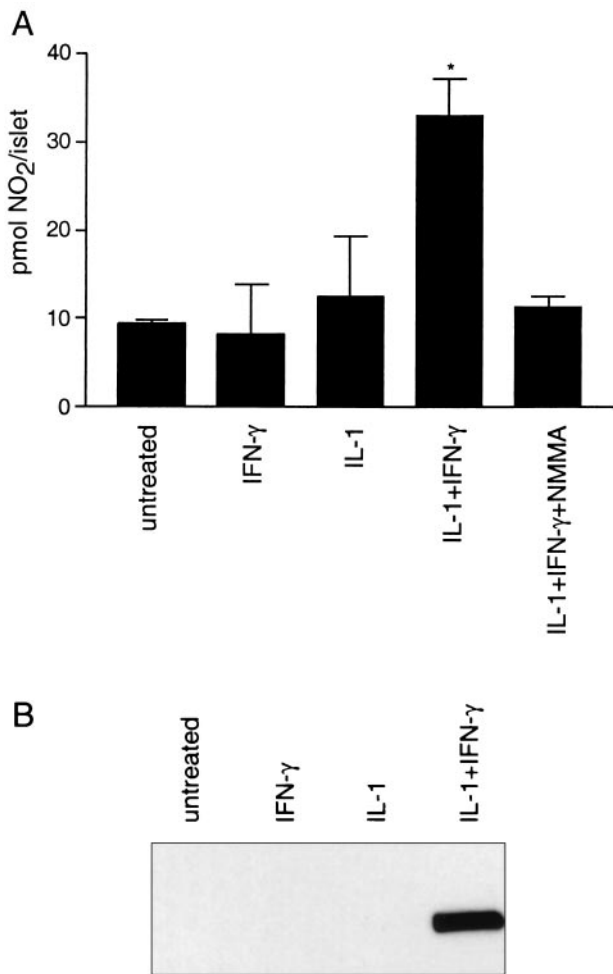


FIG. 2. NO production induced by cytokines in NOD islets. A total of 200 islets per group were incubated for 24 h with the cytokines indicated, followed by analysis of nitrite production and iNOS expression. **A:** Supernatant was sampled for the presence of nitrite. Results are the means \pm SE of 3–6 experiments. * $P < 0.01$, untreated vs. IL-1 + IFN- γ . **B:** Western blot of iNOS expression in islet extracts.

standard procedures. Western blotting was performed with anti-NOS2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-conjugated anti-rabbit Ig and detection with enhanced chemiluminescence (Amersham International, Amersham, U.K.).

Immunostaining. Islets were cultured with cytokines in CMRL-1066 medium containing 10% FCS and antibiotics. For cytopins, islets were dispersed into single cells with 0.2% trypsin (Calbiochem, La Jolla, CA) and 10 mmol/l EDTA in Hank's balanced salt solution. Cell suspensions (4×10^4 cells per slide) were centrifuged onto glass slides. Slides were fixed for 1 h in 4% paraformaldehyde, air-dried, and stored at -20°C until staining. Two-color immunofluorescence was performed, using standard procedures. Blocking was with 5% BSA in PBS, and antisera were incubated for 30 min, with 5-min washes in PBS. Antisera used were anti-NOS2 antibody (Santa Cruz Biotechnology) with anti-rabbit Ig conjugated with Texas red (Molecular Probes, Eugene, OR) and anti-insulin antibody (Dako, Carpinteria, CA) with anti-guinea pig Ig conjugated to fluorescein isothiocyanate (Becton Dickinson, Franklin Lakes, NJ).

Statistics. Analysis of data were performed using the program GraphPad Prism (GraphPad Software, San Diego, CA). Data are represented as the means \pm SE from at least three independent experiments. Data were analyzed using one-way ANOVA with Bonferroni's posttest for comparison of multiple columns.

RESULTS

Effects of IL-1 + IFN- γ on glucose-stimulated insulin secretion by mouse islets. Treatment of mouse islets for 24 h with either IL-1 (100 units/ml) or IFN- γ alone (100

units/ml) failed to inhibit glucose-stimulated insulin secretion (Fig. 1); however, in combination these cytokines inhibited insulin secretion by mouse islets. NMMA (2 mmol/l) completely prevented IL-1 + IFN- γ -induced inhibition of insulin secretion, indicating that the inhibitory actions of IL-1 and IFN- γ were mediated by NO.

The inhibitory actions of IL-1 + IFN- γ on insulin secretion are consistent with the effects of this cytokine combination on NO production by mouse islets. As shown in Fig. 2A and B, neither IL-1 nor IFN- γ alone stimulated nitrite formation or iNOS expression; however, in combination IL-1 and IFN- γ induced a threefold increase in nitrite formation and high levels of iNOS expression. NMMA, which prevented IL-1 + IFN- γ -induced inhibition of insulin secretion (Fig. 1), also prevented the formation of nitrite stimulated by the combination of these two cytokines.

RIP- $\Delta\gamma\text{R}$ islets are not susceptible to NO-mediated inhibition of function. Using islets isolated from RIP- $\Delta\gamma\text{R}$ mice, it is possible to dissect the contributions of β -cell NO production compared with non- β -cell NO production on glucose-stimulated insulin secretion in response to IL-1 + IFN- γ , because IFN- γ signaling in β -cells from these transgenic mice is ablated. In contrast to results presented in Fig. 1 for wild-type islets, islets isolated from RIP- $\Delta\gamma\text{R}$ mice were resistant to IL-1 + IFN- γ -induced inhibition of glucose-induced insulin secretion (Fig. 3). This finding indicates that IFN- γ is acting directly on the β -cell to induce damage caused by IL-1 and IFN- γ . Although IL-1 + IFN- γ failed to inhibit glucose-stimulated insulin secretion by islets isolated from RIP- $\Delta\gamma\text{R}$ mice, these islets were still capable of producing NO in response to this cytokine combination. As shown in Fig. 4A and B, a 24-h treatment with IL-1 + IFN- γ resulted in the expression of iNOS and a twofold increase in nitrite production. Although RIP- $\Delta\gamma\text{R}$ islets produced NO in response to IL-1 and IFN- γ , the levels of nitrite produced

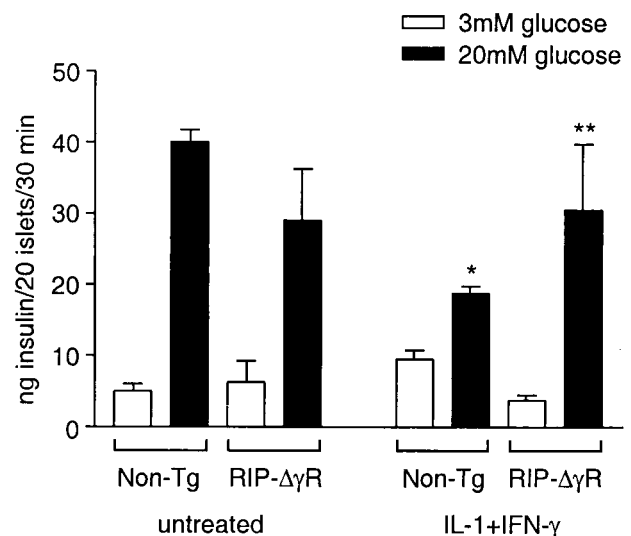


FIG. 3. Cytokines do not cause damage to RIP- $\Delta\gamma\text{R}$ islets in vitro. Glucose-stimulated insulin secretion assays were performed on islets isolated from RIP- $\Delta\gamma\text{R}$ or nontransgenic control mice after a 24-h incubation with cytokines. Data represent the means \pm SE for three independent experiments. * $P < 0.05$, untreated vs. IL-1 + IFN- γ ; ** $P < 0.05$, RIP- $\Delta\gamma\text{R}$ vs. nontransgenic (calculated on fold increase from 3 to 20 mmol/l glucose). Non-Tg, nontransgenic.

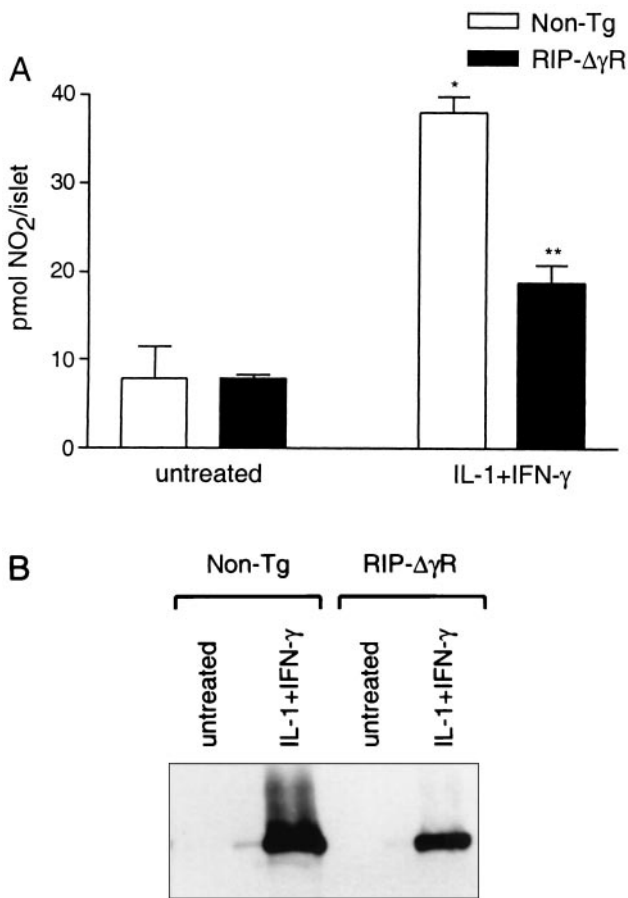


FIG. 4. RIP-ΔγR islets produce nitrite and iNOS. Islets were isolated from RIP-ΔγR and nontransgenic mice and counted into 200 islets per group. **A:** Supernatants were assayed for nitrite production. The means ± SE from three experiments is shown. **P* < 0.001, RIP-ΔγR vs. nontransgenic; ***P* < 0.01, RIP-ΔγR untreated vs. IL-1 + IFN-γ. **B:** Islets were analyzed by Western blotting with anti-iNOS antibody. Non-TG, nontransgenic.

and iNOS expressed are significantly lower than in wild-type islets.

RIP-ΔγR islets are protected from IL-1 + IFN-γ-induced cell death. The protection of RIP-ΔγR islets from IL-1 + IFN-γ-induced damage was not restricted to glucose-stimulated insulin secretion, because these islets were also protected from cytokine-mediated DNA damage. After 4 days' incubation with IL-1 + IFN-γ, islets were analyzed by flow cytometry for DNA fragmentation (Fig. 5). There was a four- to fivefold increase in DNA fragmentation in the nontransgenic islets after treatment with IL-1 + IFN-γ, whereas no such increase was observed in islets isolated from RIP-ΔγR mice. Cell death of nontransgenic islets appears to be mediated by NO, because IL-1 + IFN-γ-induced DNA fragmentation was prevented by NMMA (not shown and [26]).

iNOS is expressed by non-β-cells in RIP-ΔγR islets. The fact that NO was produced by RIP-ΔγR islets in response to IL-1 + IFN-γ, yet these cytokines failed to inhibit insulin secretion or induce DNA fragmentation, prompted us to examine the cell types expressing iNOS in the cytokine-treated islets. Two-color immunofluorescence staining of cytokine-treated islet cell cytopins revealed that insulin-expressing β-cells are the primary cellular source of iNOS in nontransgenic mouse islets (Fig.

6). iNOS expression was also observed in a small percentage of islet cells that did not contain insulin. This second population of iNOS-expressing cells is most likely endothelial and/or ductal because these cell types are known to express iNOS in response to IL-1 + IFN-γ (20,27). In contrast, we did not detect iNOS expression by insulin-containing cells in islets isolated from RIP-ΔγR mice (Fig. 6). These findings are consistent with the idea that the β-cells in these islets are unable to upregulate iNOS expression because of unresponsiveness to IFN-γ.

DISCUSSION

The mechanisms by which cytokines impair β-cell function have primarily been determined using isolated rat and human islets. In rat islets, IL-1 induces a potent inhibition of insulin secretion and islet degeneration (28,29). The iNOS inhibitors aminoguanidine and NMMA prevent IL-1-induced islet damage, suggesting that NO mediates the inhibitory actions of IL-1 on rat islet function (13,30–32). NO also appears to participate, in part, in cytokine-induced human islet damage. Treatment of human islets with IL-1, TNF, and IFN-γ stimulates iNOS expression, nitrite production, inhibition of insulin secretion, and islet cell apoptosis. All of these effects are attenuated, but not completely prevented, by inhibition of iNOS (13,15,18). Our current data shows that IL-1 and IFN-γ are both required to stimulate iNOS expression and induce cellular damage in NOD mouse islets. We also show that NO mediates cytokine-induced inhibition of insulin secretion and islet DNA damage. Consistent with our findings, the combination of IL-1, TNF, and IFN-γ has recently been shown to reduce the viability of wild-type but not iNOS-deficient mouse islets, suggesting the cytokine-induced damage is caused by NO production (33).

Because IL-1 alone does not stimulate iNOS expression in mouse islets (Fig. 2) and RIP-ΔγR β-cells do not respond to IFN-γ, the current data suggest that NO is produced by IFN-γ-responsive non-β-cells within the islets of RIP-ΔγR mice. Our experiments differ from adding IL-1 alone to

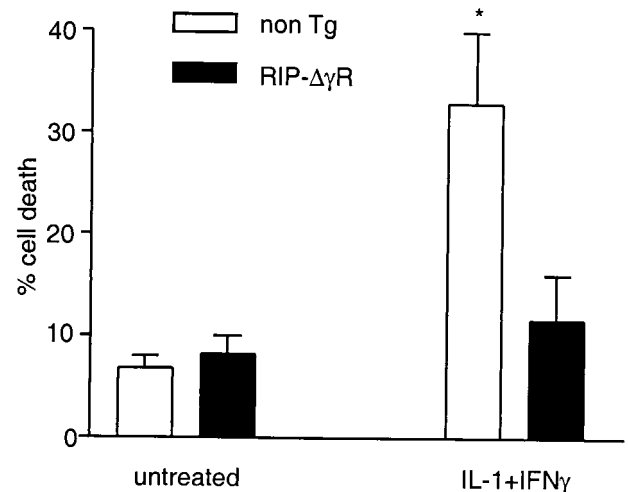


FIG. 5. Apoptosis of islet cells induced by IL-1 + IFN-γ. RIP-ΔγR or nontransgenic islets were isolated from ≤50-day-old mice and cultured in cytokines as indicated for 4 days. Percentage DNA fragmentation was determined by flow cytometry. The means ± SE of data from 3–4 independent experiments is shown. **P* < 0.05 untreated vs. IL-1 + IFN-γ. Non-TG, nontransgenic.

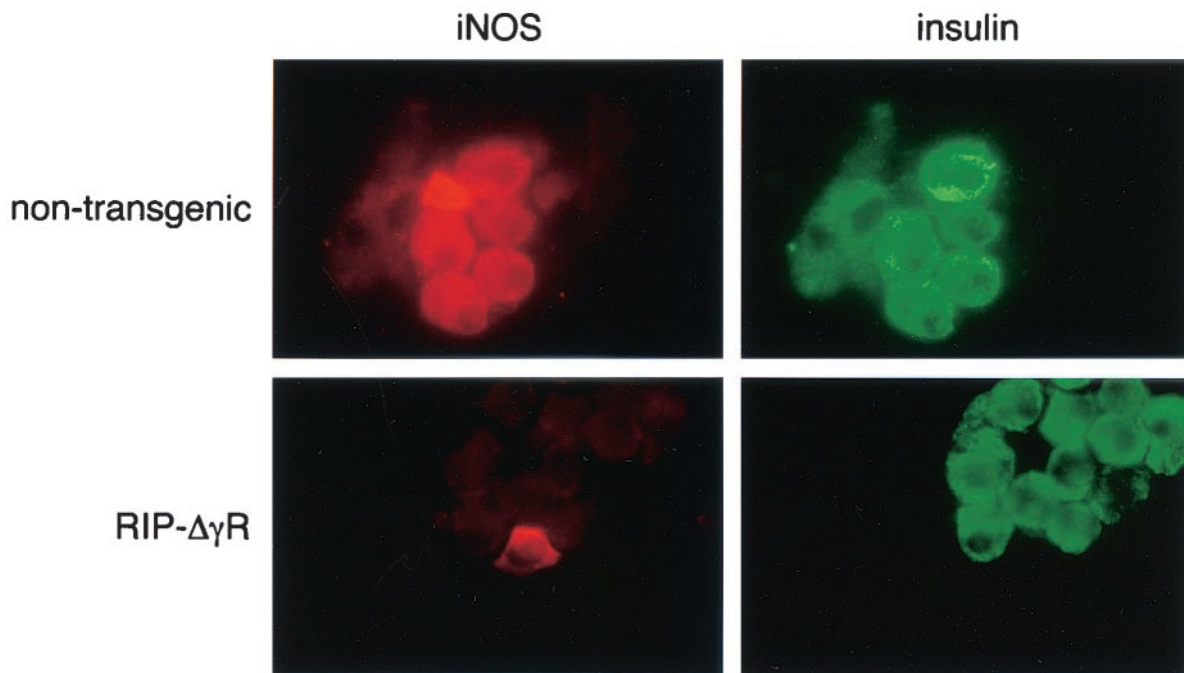


FIG. 6. iNOS is expressed by non- β -cells in RIP- $\Delta\gamma$ R islets. After overnight incubation with IL-1 + IFN- γ , islets from RIP- $\Delta\gamma$ R and nontransgenic mice were dispersed into single-cell suspensions and transferred to microscope slides by cytopsin. Cells were fixed and double-stained with anti-NOS2 + anti-rabbit Ig conjugated with Texas red, followed by anti-insulin + anti-guinea pig Ig conjugated to fluorescein isothiocyanate. Staining was visualized by fluorescence microscopy. The same field was photographed with red and green filters. Magnification was 400 \times . The photograph is a representative of staining performed on five independently isolated groups of islets.

islets (Fig. 2) or using islets isolated from IFN- γ receptor-deficient mice, because all cells except β -cells are able to respond to IFN- γ and have the capacity to produce NO in RIP- $\Delta\gamma$ R islets. Our findings are consistent with nonendocrine cell production of NO (20,27,34); however, NO produced by islet cell types other than β -cells does not mediate β -cell dysfunction *in vitro*.

A critical question concerning cytokine-mediated β -cell damage is the islet cellular sources of NO. A number of islet cell types are capable of producing NO after cytokine stimulation. Colocalization of iNOS and insulin by immunofluorescence indicate that β -cells are the major source of iNOS in rat and human islets treated with IL-1 or IL-1 + IFN- γ , respectively (17,18), and our current data reveal that this is also true in NOD mouse islets. Endocrine α - and β -cells (25 and 70%, respectively) are the primary cell types found in islets. Islets also contain low percentages of macrophages and endothelial and ductal cells. Previous studies have shown that in an activated state, endothelial cells and macrophages can kill β -cells in an NO-dependent manner when cocultured at an effector-to-target ratio of 1:1 (20,34). Although nonendocrine islet cells are capable of producing NO *in vitro*, *in vivo* it is unlikely that NO produced by nonendocrine islet cells mediates β -cell damage because the high effector-to-target ratio required to kill islet cells in coculture experiments probably does not exist in whole islets. This is in agreement with a recent study in which NO-dependent islet cell death only occurred in cultures of aggregated islet cells or whole islets, in which the local NO concentrations were sufficient to induce cell death (35).

Activated macrophages invading an infected tissue or tumor can kill cells in an NO-dependent way (36). It therefore remains possible that *in vivo* β -cells may be

damaged by large numbers of activated macrophages infiltrating the islet. We believe this is unlikely in the NOD mouse because iNOS-deficient islet grafts are not protected from destruction mediated by CD4+ BDC2.5 T-cells (37), and RIP- $\Delta\gamma$ R and IFN- γ receptor-deficient NOD mice develop diabetes, even though their β -cells are protected from NO (5,21,38,39). However, NO may still be an important mediator of β -cell destruction in other animal models and may be important in the progression to diabetes in humans. Strategies to prevent NO-mediated β -cell death are therefore important to consider in the goal of protecting β -cells from autoimmune destruction.

The issue of which effector mechanisms are important in diabetes will be resolved by attempting to block candidate molecules in individual cell types (40). The use of RIP- $\Delta\gamma$ R mice has enabled us to block IFN- γ responsiveness only in β -cells, and in doing so we have protected islets from destruction mediated by IFN- γ -regulated factors, such as iNOS. In addition, other IFN- γ -induced pro- and antiapoptotic molecules and chemoattractant molecules expressed by β -cells (41) may also participate in the pathogenesis of or protection from diabetes. Decreased expression of these molecules may contribute to protection of RIP- $\Delta\gamma$ R mice from diabetes in the LCMV-GP model.

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