Intraislet Release of Interleukin 1 Inhibits β Cell Function by Inducing β Cell Expression of Inducible Nitric Oxide Synthase

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Summary

Cytokines, released in and around pancreatic islets during insulitis, have been proposed to participate in β-cell destruction associated with autoimmune diabetes. In this study we have evaluated the hypothesis that local release of the cytokine interleukin 1 (IL-1) by nonendocrine cells of the islet induce the expression of inducible nitric oxide synthase (iNOS) by β cells which results in the inhibition of β cell function. Treatment of rat islets with a combination of tumor necrosis factor (TNF) and lipopolysaccharide (LPS), conditions known to activate macrophages, stimulate the expression of iNOS and the formation of nitrite. Although TNF + LPS induce iNOS expression and inhibit insulin secretion by intact islets, this combination does not induce the expression of iNOS by β or α cells purified by fluorescence activated cell sorting (Facs). In contrast, IL-1β induces the expression of iNOS and also inhibits insulin secretion by both intact islets and Facs-purified β cells, whereas TNF+LPS have no inhibitory effects on insulin secretion by purified β cells. Evidence suggests that TNF+LPS inhibit insulin secretion from islets by stimulating the release of IL-1 which subsequently induces the expression of iNOS by β cells. The IL-1 receptor antagonist protein completely prevents TNF+LPS–induced inhibition of insulin secretion and attenuates nitrite formation from islets, and neutralization of IL-1 with antisera specific for IL-1α and IL-1β attenuates TNF+LPS–induced nitrite formation by islets. Immunohistochemical localization of iNOS and insulin confirm that TNF+LPS induce the expression of iNOS by islet β cells, and that a small percentage of noninsulin-containing cells also express iNOS. Local release of IL-1 within islets appears to be required for TNF+LPS–induced inhibition of insulin secretion because TNF+LPS do not stimulate nitrite formation from islets physically separated into individual cells. These findings provide the first evidence that a limited number of nonendocrine cells can release sufficient quantities of IL-1 in islets to induce iNOS expression and inhibit the function of the β cell, which is selectively destroyed during the development of autoimmune diabetes.

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by a local inflammatory reaction in and around islets that is followed by selective destruction of the insulin-secreting β cell. Macrophages and monocytes comprise the early islet insulitis, followed by the presence of T cells (1–3). It has been proposed that the cytokine IL-1, released during islet insulitis, participates in β cell destruction (4–6). Treatment of rat islets with IL-1 results in a time- and concentration-dependent inhibition of insulin secretion that is followed by islet degeneration (7–10). IL-1β inhibits insulin secretion by inducing the expression of inducible nitric oxide synthase (iNOS)[1] and the production of nitric oxide (11–13). Inhibitors of iNOS, N^G-monomethyl-L-arginine (NMMA), aminoguanidine, and nitro-L-arginine methylester, prevent both the inhibitory effects of cytokines on insulin secretion, and cytokine-induced nitric oxide formation by rat, mouse, and human islets (11, 14–17). IL-1β has been shown to induce the accumulation of iNOS mRNA in two different insulinoma cell lines (18, 20).

In vivo evidence suggests that nitric oxide participates in the development of autoimmune diabetes. Lukic et al. (21) have shown that NMMA prevents the development of diabetes in male mice injected for 5 d with streptozotocin. In this model, streptozotocin produces an inflammatory reaction characterized by the presence of lymphocytes and macrophages followed by β cell necrosis. In the nonobese spontaneous diabetic mouse (NOD), the transfer of spleen cells from a diabetic female NOD mouse to an irradiated nondiabetic male NOD mouse induces diabetes 11–13 d after transfer. We have shown that islets isolated from recipient male NOD mice produce nitric oxide in a time-dependent fashion that

[1] *Abbreviations used in this paper:* iNOS, inducible nitric oxide synthase; IRAP, IL-1 receptor antagonist protein; KRB, Kregs-Ringer bicarbonate buffer; NOD, nonobese diabetic; NMMA, N^G-monomethyl-L-arginine.

mimics the time-dependent infiltration of T cells and macrophages (22). Treatment of recipient male NOD mice with two daily injections of aminoguanidine delays the development of diabetes in this model by 7–10 d (22). Also, the presence of iNOS mRNA has recently been demonstrated in and around islets of prediabetic BB rats (23).

Islets of Langerhans contain a heterogeneous population of both endocrine (β, α, δ, and polypeptide secreting cells) and nonendocrine cells (macrophages, endothelial cells, dendritic cells, and fibroblasts). The cellular sources of iNOS in animal models of diabetes are unknown, but in vitro treatment of purified populations of β cells with IL-1β stimulates the accumulation of nitrite and cGMP, whereas this cytokine does not induce nitric oxide formation by purified populations of α cells (24). IL-1β–induced expression of iNOS by islet β cells has recently been confirmed by immunoprecipitation using antiserum specific for mouse macrophage iNOS (20).

β cell destruction associated with autoimmune diabetes is characterized by an early peri-insulitis in which lymphocytes and macrophages are located on the periphery of islets. This peri-insulitis is followed by invasion of these immune cells into islets (25). Under these conditions, cytokine release may have marked effects on islet function. In this study, conditions have been found that stimulate the release of IL-1 in islets. Evidence is presented which shows that cytokines released in islets by nonendocrine cells induce the expression of iNOS and inhibit insulin secretion by islet β cells. These results provide the first experimental evidence demonstrating that local release of cytokines in islets can directly modulate β cell function.

Materials and Methods

Materials and Antiserum. CMRL-1066 tissue culture medium, fetal bovine serum, and glutamine were obtained from GIBCO BRL (Gaithersburg, MD). Collagenase, human recombinant TNF-α, and human recombinant IFN-γ were from Boehringer Mannheim (Indianapolis, IN). NMMA acetate was from Calbiochem-Novabiochem Corp., (La Jolla, CA), and recombinant human IL-1β was purchased from Cistron Biotechnology, Inc. (Pine Brook, NJ). [^{35}S]-trans-labeled methionine (1,117 Ci/mmol) was from ICN (Costa Mesa, CA). Rabbit antiserum specific for mouse macrophage iNOS used for immunohistochemistry and rabbit antiserum specific for the COOH-terminal 27 amino acids of mouse macrophage iNOS used for immunoprecipitation studies were kind gifts from Drs. Michael Marletta (University of Michigan, Ann Arbor, MI) and Thomas Misko (Monsanto Corporate Research, St. Louis, MO), respectively. Hamster anti–mouse IL-1β (B122) and hamster anti–mouse IL-1α (ALF) were gifts from Dr. Robert Schreiber (Washington University School of Medicine). Human and mouse recombinant IL-1 receptor antagonist proteins (IRAP) were gifts from Dr. Charles Hall (Upjohn, Kalamazoo, MI). Indocarbocyanine (CY3)-conjugated donkey anti–rabbit, and FITC-conjugated donkey anti–guinea pig antisera were from Jackson Immuno Research (West Grove, PA). All other chemicals were from commercially available sources.

Islet Isolation and Culture. Islets were isolated from male Sprague-Dawley rats (200–250 g; Sasco, O'Fallon, MO) by collagenase digestion as described previously (26). Islets were cultured overnight in an atmosphere of 95% air and 5% CO_2 in complete CMRL-1066 tissue culture medium (2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) before the start of experiments. Experiments were initiated by the addition of cytokines and endotoxin, 5 U/ml IL-1β, 150 U/ml IFN-γ, 0.7 nM (240 U/ml) TNF, 10 μg/ml LPS, or 0.5 mM NMMA, and the islets were then incubated for the indicated periods of time. In some experiments islets were pretreated with either human or mouse recombinant IRAP (1 μg/ml) or antibodies against IL-1α and IL-1β (1 μg/ml for both antisera) for 1 h before the addition of cytokines.

Purification of β and α Cells by Fluorescence-activated Cell Sorting (Facs). Islets isolated from 12 male Sprague-Dawley rats were cultured overnight (~2,500 islets/2.5 ml) in complete CMRL-1066 media in an atmosphere of 95% air and 5% CO_2. Islets were dispersed into individual cells by treatment with dispase (0.33 mg/ml) in Ca^{2+} and Mg^{2+} free Hank's solution (27) at 31°C for 15 min (28). The dispersed islet cells were incubated for 45–60 min in complete CMRL-1066 media at 37°C and then purified by the method of Pipeleers et al. (29) using an Epics 733 flow cytometer (Coulter Corp., Hialeah, FL). The cells were illuminated at 488 nm and emission was monitored at 515–535 nm. The sorting process yields a 90–95% population of β cells, and an 80–85% population of α cells. The α cell population also contains some δ and β cells (28).

Glucose-stimulated Insulin Secretion by Islets. After treatment with cytokines, islets were washed three times in Krebs-Ringer bicarbonate buffer (KRB; 25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4, containing 3 mM D-glucose, and 0.1% BSA). Islets were counted (20 islets/tube) into 10 × 75 mm siliconized borosilicate tubes and preincubated for 30 min at 37°C with shaking in a Dubnoff water bath in an atmosphere of 95% air and 5% CO_2. The KRB buffer was then replaced with fresh KRB buffer supplemented with either 3 or 20 mM D-glucose and the incubation continued for 30 min. The supernatant was withdrawn and insulin content was determined by RIA.

Glucose-induced Insulin Secretion from Facs-purified β Cells. Purified β cells pretreated for 18 h in complete CMRL-1066, or complete CMRL-1066 containing the indicated cytokines, LPS and NMMA, were washed three times with KRB containing 3 mM D-glucose and 0.1% BSA. β cells were then aliquoted into 96-well microtiter plates (15,000 cells/100 μl of KRB containing 3 mM D-glucose and 0.1% BSA). Insulin secretion was initiated by the addition of an equal volume of KRB containing either 3 mM D-glucose or 37 mM D-glucose plus 10 mM theophylline (final concentration, 20 mM glucose and 5 mM theophylline). The phosphodiesterase inhibitor theophylline was included to elevate cAMP levels which are required for glucose-stimulated insulin secretion from purified β cells (29, 30). Cells were incubated for 3 h in an atmosphere of 95% air, 5% CO_2 at 37°C. After the incubation, cells were pelleted by centrifugation (200 *g* for 10 min), and the supernatant was aspirated and analyzed for insulin by RIA.

Immunoprecipitation of iNOS. iNOS was immunoprecipitated using a modification (31) of methods described previously (32). In brief, islets (100) or purified β or α cells (100,000 cells) were cultured with the indicated cytokines and LPS for 5 h in MEM methionine-deficient media (9 parts MEM without methionine/1 part MEM containing methionine), 300 μCi of [^{35}S]methionine trans-label was added and the islets were incubated for an additional 13 h at 37°C. iNOS was immunoprecipitated using rabbit anti-iNOS (1:750 dilution) antiserum specific for the COOH-terminal 27 amino acids of mouse macrophage iNOS. Immunoprecipitates were analyzed on 8% SDS-polyacrylamide gels (33) and visualized by fluorography.

Nitrite Determination. Nitrite release was determined by mixing 50-μl portions of culture media with 50 μl of the Griess reagent (34), and the absorbance at 540 was measured using a Multiskan (model MCC/340; Titertek, Elfab Oy, Finland) plate reader. In experiments comparing the effects of IL-1β and TNF+LPS on nitrate formation by intact islets and islet cells, islets were dispersed into single cells as described above and cultured overnight. The cells were then washed three times with complete CMRL-1066 and added to 24-well microtiter plates at a concentration of 100,000 cells/400 μl of the same medium. Islets (90/400 μl of complete CMRL-1066) were counted into the same plate and the experiments were initiated by the addition of IRAP followed by the addition of cytokines and LPS as described above. The supernatant was isolated by centrifugation and used for nitrite determinations.

Immunohistochemistry. Immunohistochemistry was performed as described previously for sequential double staining (35). In brief, isolated islets were either untreated or incubated with the indicated concentrations of IL-1β, TNF, LPS, and IRAP for 24 h, isolated, washed three times with 0.1 M PBS, pH 7.4, and then fixed in 1 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. Islets were then cryopreserved overnight in 0.1 M phosphate buffer, pH 7.4, containing 10% sucrose, frozen, and then cut into 10-μm sections. Sections were incubated overnight at 4°C with the primary antisera at the following dilutions: rabbit anti-iNOS prepared against mouse macrophage iNOS, 1:500; guinea pig anti-human insulin, 1:500. Slides were washed and then incubated for 2 h with secondary antiserum, CY3-conjugated donkey anti-rabbit, and FITC-conjugated donkey anti-guinea pig, at dilutions of 1:200. Staining was visualized by fluorescent microscopy where insulin- and iNOS-containing cells stain green and red, respectively.

Statistics. Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences ($p < 0.05$) were evaluated using a Scheffe's F-test posthoc analysis.

Results

Effects of Cytokines and LPS on iNOS Expression by Intact Islets and Facs-purified β and α Cells. The effects of cytokines and endotoxin on iNOS expression by islets and Facs-purified populations of both β and α cells are shown in Fig. 1, *a* and *b*, and Table 1. IL-1β stimulates the expression of iNOS by both intact islets and purified β cells, but does not induce iNOS expression in purified populations of α cells. Individually, TNF, IFN-γ, or LPS stimulate low levels of iNOS expression by islets but do not induce the expression of iNOS by purified β cells (Table 1). In combination, TNF+LPS stimulate iNOS expression to a level that is 50% lower than the level induced by IL-1β in islets. TNF+LPS do not induce the expression of iNOS by purified populations of β or α cells. In combination with TNF, IFN-γ induces iNOS expression to a level that is 47% of that observed with IL-1β in islets. IFN-γ also appears to reduce the level of IL-1β–induced iNOS expression (45% inhibition) by both intact islets and purified β cells. These findings show that IL-1β stimulates the expression of iNOS by the β cell. The combinations of TNF+LPS and IFN-γ +TNF also induce the expression of iNOS by intact islets, but this does not appear to be a direct effect of these cytokines and endotoxin on β

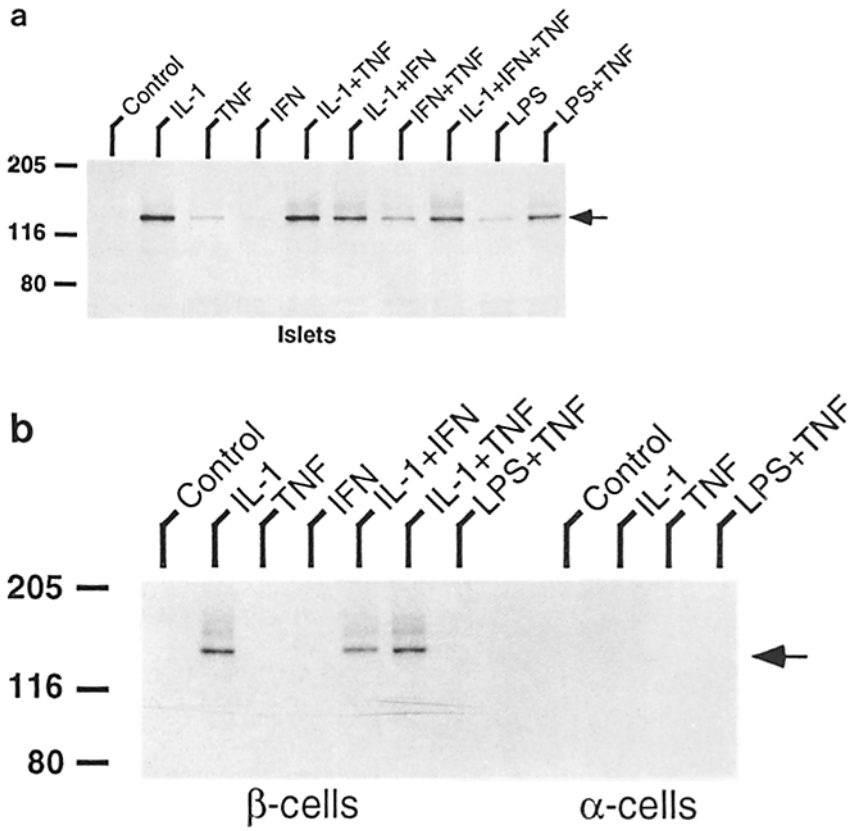


Figure 1. Effects of cytokines and endotoxin on iNOS expression by rat islets and Facs-purified β and α cells. Intact islets (*a*), and Facs-purified β and α cells (*b*) were incubated for 5 h with 5 U/ml IL-1β, 150 U/ml IFN-γ, 0.7 nM TNF, and 10 μg/ml LPS alone or in combination as indicated. [^{35}S]Methionine was added and the islets and cells were incubated for an additional 13 h. iNOS was immunoprecipitated using antiserum specific for the COOH-terminal 27 amino acids of mouse macrophage iNOS. Immunoprecipitates were separated on 8% SDS-polyacrylamide gels and visualized by fluorography. Results are representative of two to three individual experiments.

Table 1. *Effects of Cytokines and LPS on iNOS Expression by Islets and Purified β Cells*

Treatment	Optical density (IL-1–induced iNOS)
	%
Islets	
Control	0
IL-1	100
TNF	16 ± 5
IFN	11 ± 7
IL-1 + TNF	101 ± 17
IL-1 + IFN	59 ± 8
TNF + IFN	47 ± 18
IL-1 + TNF + IFN	53 ± 16
LPS	14 ± 4
LPS + TNF	50 ± 9
Purified β cells	
Control	0
IL-1	100
TNF	0
IFN	3 ± 4
IL-1 + TNF	103 ± 7
LPS	9 ± 7
IL-1 + IFN	45 ± 16
TNF + LPS	0

Densitometry was performed on immunoprecipitation data shown in Fig. 1. The level of iNOS expression after treatment with IL-1 was set at 100%. Results are the average ± SEM from two experiments with two replicates/condition.

or α cells. These combinations of cytokines and endotoxin (TNF+LPS, IFN-γ +TNF) are known to activate macrophages to release cytokines (IL-1 and TNF) and to produce nitric oxide (36–39), suggesting that the resident islet macrophage is a source of iNOS.

Effects of IL-1β and TNF+LPS on Insulin Secretion by Islets. The effects of nitric oxide production on insulin secretion by islets were examined under conditions that induce iNOS expression by the β cell (IL-1β) or conditions that induce the highest level of iNOS expression by nonendocrine islet cells (TNF+LPS, see Table 1). As shown in Fig. 2, treatment of rat islets for 18 h with IL-1β completely inhibits glucose-stimulated insulin secretion, and this effect is attenuated by 0.5 mM NMMA. Under these conditions, IL-1β stimulates a threefold increase in nitrite release from the same islets used for insulin secretion, and this increase is completely prevented by NMMA. TNF+LPS treatment of islets for 18 h inhibits glucose-stimulated insulin secretion by 40%, and this inhibition is completely attenuated by NMMA. TNF+LPS induce a 50% increase in the release of nitrite from the same islets used for insulin secretion, and this is also completely prevented by NMMA. Our previous studies (15) have shown that NMMA completely prevents the inhibitory effects of IL-1β on insulin secretion by rat islets. The lack of complete protection in these studies appears to result from the production of nitric oxide during the insulin secretion portion of these experiments (See Materials and Methods). When NMMA is included in the buffers used for insulin secretion (KRB containing 3 or 20 mM glucose and 0.1% BSA), complete prevention of the inhibitory effects of IL-1β are observed (data not shown).

Effects of IL-1β and TNF+LPS on Insulin Secretion by Facs-purified β Cells. The effects of IL-1β and TNF+LPS on glucose-stimulated insulin secretion and nitrite formation by Facs-purified β cells are shown in Fig. 3, *a* and *b*. Treatment of purified β cells with IL-1β for 18 h results in a 70% inhibition of insulin secretion that is attenuated by NMMA. This inhibition of insulin secretion correlates with a 10-fold increase in nitrite release from the same β cells used for secretion, and the release of nitrite is completely prevented by NMMA. TNF+LPS have no inhibitory effects on insulin secretion, and do not induce the formation of nitrite by purified β cells. As shown in Fig. 1 *b* this combination does not induce iNOS expression by purified β cells. The inhibitory effects of TNF+LPS on glucose-stimulated insulin secretion by islets suggest that either TNF+LPS induce the expression of iNOS

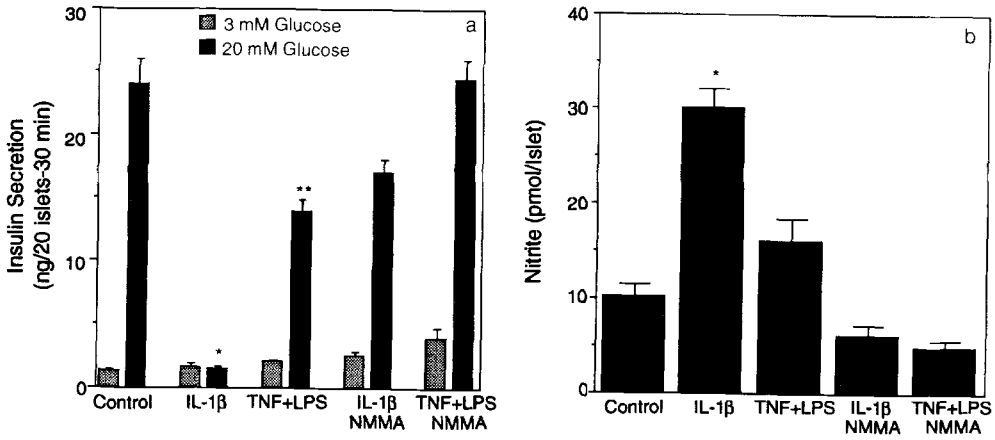


Figure 2. Effects of IL-1β and TNF+LPS on glucose-stimulated insulin secretion by islets. Islets were incubated for 18 h with or without 5 U/ml IL-1β, 0.7 nM TNF, 10 μg/ml LPS, and 0.5 mM NMMA as indicated. Islets were isolated for insulin secretion (*a*) and nitrite formation was determined on the incubation media (*b*). Results are the average ± SEM of four individual experiments containing three replicates per condition. Statistically significant inhibition of insulin secretion and nitrite formation as compared with controls are indicated (*, $p < 0.001$; and **, $p < 0.05$).

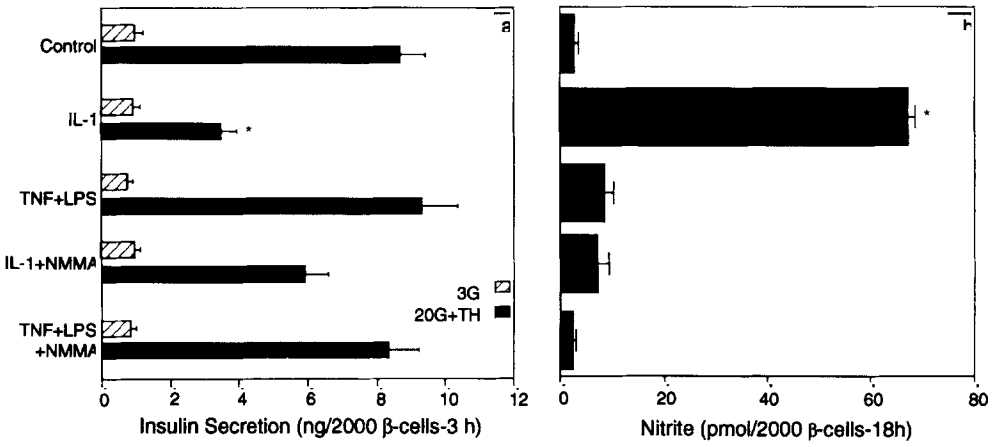


Figure 3. Effects of IL-1β and TNF+LPS on glucose-stimulated insulin secretion by Facs-purified β cells. β cells purified by Facs were incubated for 18 h with 5 U/ml IL-1β, 0.7 nM TNF, 10 μg/ml LPS, or 0.5 mM NMMA as indicated. The cells were then isolated for insulin secretion (*a*) and nitrite formation was determined on the culture supernatant (*b*). Results are the average ± SEM of two individual experiments containing four replicates per condition. Statistically significant inhibition of insulin secretion and nitrite formation as compared with controls are indicated (*, p <0.001).

by nonendocrine islet cells (macrophages or endothelial cells) and the resulting production of nitric oxide inhibits β cell function, or that TNF+LPS stimulate the release of cytokines that induce iNOS expression by β cells.

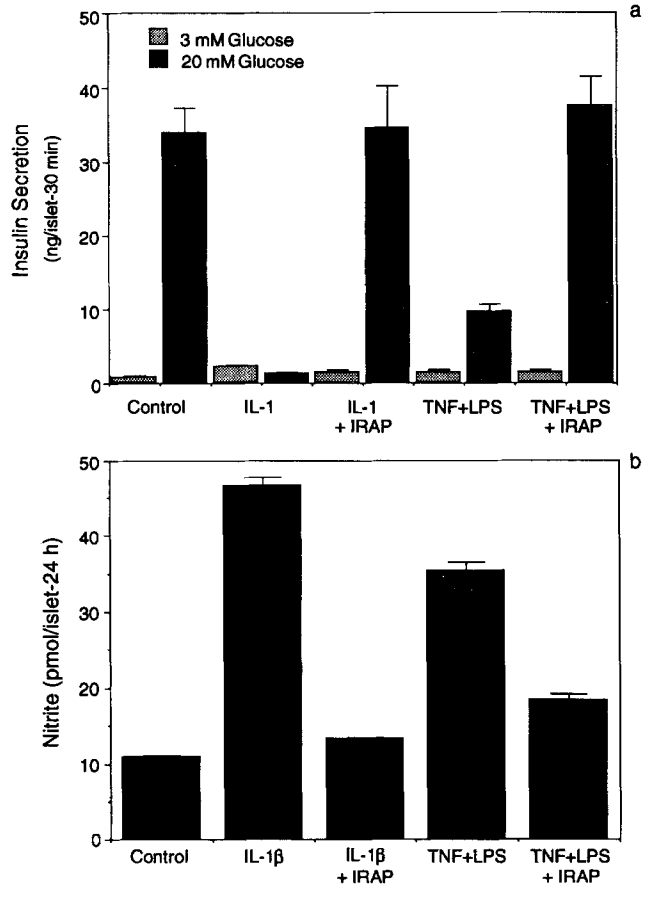


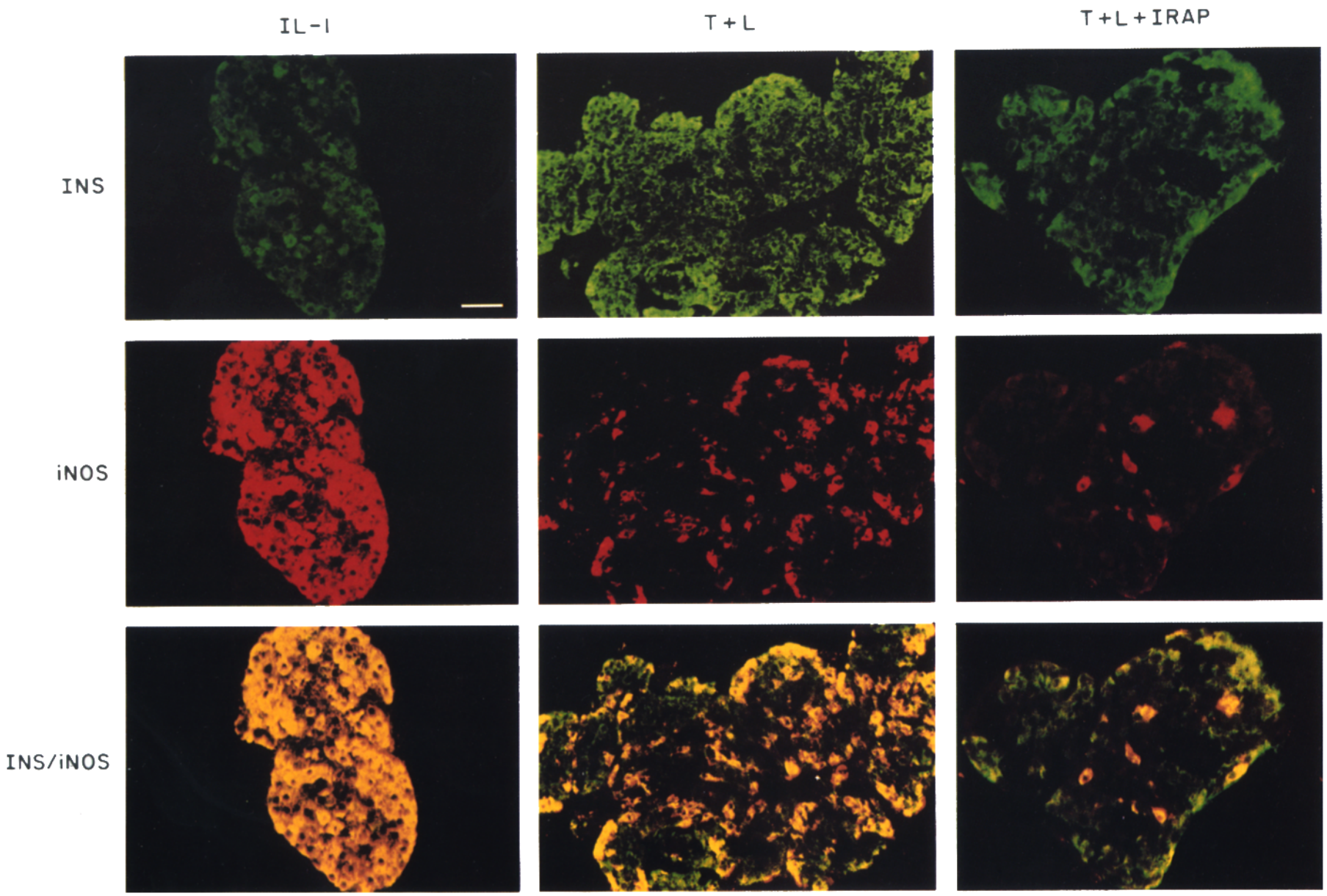
Figure 4. Effects of IRAP on IL-1β and TNF+LPS–induced inhibition of glucose-stimulated insulin secretion by islets. Islets were pretreated for 1 h with 1 μg/ml human recombinant IRAP. The islets were then incubated for an additional 24 h with 5 U/ml IL-1β, or 0.7 nM TNF and 10 μg/ml LPS as indicated. After the 24-h incubation, the culture media were removed for nitrite determination (*b*) and insulin secretion was examined on the islets (*a*). Results are the average ± SEM of an individual experiment containing three replicates per condition, and are representative of three individual experiments.

IRAP Prevents TNF+LPS–induced Inhibition of Insulin Secretion by Intact Islets. The effects of IRAP on TNF+LPS–induced inhibition of insulin secretion are shown in Fig. 4 *a*. After a 24-h incubation, TNF+LPS inhibit glucose-stimulated insulin secretion by 70%, and this inhibitory effect is completely prevented by coincubation of islets with 1 μg/ml IRAP in addition to TNF+LPS. Incubation of islets for 24 h with IL-1β results in complete inhibition of insulin secretion that is also prevented by IRAP. The effects of IRAP on TNF+LPS- and IL-1β–induced nitrite formation are shown in Fig. 4 *b*. IL-1β induces a 4.3-fold increase and TNF+LPS induce a 3.3-fold increase in the release of nitrite from the same islets used for insulin secretion. IL-1β–induced nitrite formation is completely prevented by IRAP, whereas TNF+LPS–induced nitrite formation is significantly attenuated by IRAP. These findings suggest that TNF+LPS stimulate IL-1 release, and that IL-1 then induces the expression of iNOS by the β cell. IRAP does not completely prevent TNF+LPS–induced nitrite formation by islets, suggesting that TNF+LPS induce the formation of nitric oxide by islets independent of IL-1 release. The higher levels of TNF+LPS–induced nitrite production and inhibition of insulin secretion observed in Fig. 4 compared with Fig. 2 is believed to reflect different incubation times of 24 and 18 h, respectively.

Table 2. *Antibodies to IL-1α and IL-1β Inhibit TNF+LPS–induced Nitrite Formation by Rat Islets*

Conditions	Nitrite (pmol/Islet)
Control	11.1 ± 2.4
LPS + TNF	26.8 ± 2.4
LPS + TNF + anti-IL-1	15.9 ± 1.7*

Rat islets (70/200 μl of complete CMRL-1066) were pretreated for 1 h with 1 μg/ml of both anti-IL-1α and anti-IL-1β antiserum. TNF (0.7 nM) and LPS (10 μg/ml) were added and the islets were cultured for an additional 24 h. The medium was then removed for nitrite determinations. Results are the average ± SEM of three individual experiments containing two to three replicates/condition. Statistically significant inhibition of TNF+LPS–induced nitrite formation is as indicated (*p = 0.002).


IL-1
T+L
T+L+IRAP
INS
iNOS
INS/iNOS

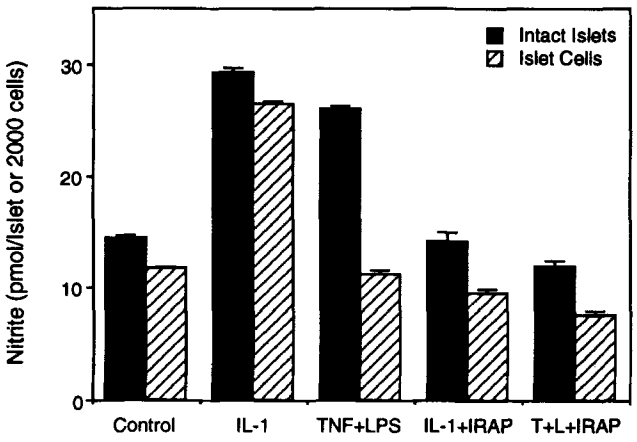


Figure 6. Effects of IRAP on IL-1β– and TNF+LPS–induced nitrite formation by intact islets and dispersed islet cells. Islets or dispersed islet cells were incubated for 24 h with 5 U/ml IL-1β, 0.7 nM TNF, 10 μg/ml LPS, or 1 μg/ml mouse recombinant IRAP. The culture media were then removed and analyzed for the formation of nitrite. Results are the average ± SEM of an individual experiment that is representative of two individual experiments.

To confirm that TNF+LPS stimulate the release of IL-1 from nonendocrine islet cells, the effects of antisera specific for IL-1β and IL-1α on nitrite formation by islets was examined. As shown in Table 2, hamster anti–mouse IL-1α and IL-1β (1 μg/ml for each antiserum) attenuate TNF+LPS–induced nitrite formation by islets (Table 2). Consistent with the effects of IRAP, IL-1 antisera does not completely prevent TNF+LPS–induced nitrite formation, suggesting that TNF+LPS also induce the expression of iNOS in islet cells independent of IL-1.

Immunohistochemical Cellular Localization of Insulin and iNOS. To directly determine the cellular source of iNOS, the effects of IL-1β, TNF+LPS, and TNF+LPS+IRAP on iNOS expression by intact islets were examined by immunohistochemistry. Rabbit antiserum specific for mouse macrophage iNOS and guinea pig antiserum specific for human insulin were used to determine if iNOS expression localizes with insulin under the indicated conditions. In these experiments (Fig. 5), fluorescent secondary antisera used to stain iNOS and insulin were goat anti–rabbit CY3 (red), and donkey anti–guinea pig FITC (green), respectively. IL-1β induces the expression of iNOS in nearly every insulin containing cell (left column), as shown by the intense red staining of iNOS-containing cells which localizes with insulin (green)-containing β cells (yellow on double exposure). A few cells appear to stain for insulin that do not stain for iNOS (approximately four to five per islet) after IL-1β exposure, however we did not observe iNOS staining in the absence of insulin colocalization. TNF+LPS stimulate the expression of iNOS in a lower percentage of islet cells (middle column) as compared with IL-1β, and TNF+LPS–induced iNOS expression localizes predominately with insulin-containing β cells (yellow). IRAP inhibits TNF+LPS–induced expression of iNOS in almost all of the insulin-containing β cells (right column). Six cells stain positive for iNOS (red), and only one of these cells contains insulin (yellow cell located on the right edge of islet, bottom row of right column). The remaining five cells positive for iNOS are believed to be resident macrophages which comprise ~0.5% (10–15/islet) of all islet cells and are known to express iNOS after treatment with TNF+LPS (36, 37, 39, 40, 41). It is also possible that these iNOS-containing cells could be endothelial or dendritic in origin. Untreated control islets stain for insulin but iNOS staining was not observed (data not shown). Also, cross-reactivity of second antibodies with the tissue or with nonspecific antisera of the same species as the primary antiserum was not observed.

TNF+LPS Fail to Induce Nitrite Formation by Dispersed Islet Cells. Results of Figs. 4 and 5 suggest that TNF+LPS stimulate the release and accumulation of sufficient concentrations of IL-1 to induce the expression of iNOS by islet β cells. To further examine the potential role of local concentrations of cytokines, nitrite formation by intact islets was compared with nitrite formation from dispersed islet cells treated with IL-1 or TNF+LPS. Dispersion of islets prevents the accumulation of high local concentrations of IL-1 by physically separating islets into single cells. In this experiment, IL-1β stimulates a twofold increase in nitrite production by both islets and dispersed islet cells, and nitrite formation is completely prevented by coincubation with 1 μg/ml IRAP (Fig. 6). TNF+LPS induce a 1.8-fold increase in nitrite formation by intact islets, and this is also prevented by 1 μg/ml IRAP. Importantly, TNF+LPS do not stimulate the formation of nitrite by dispersed islet cells. These results indicate that in intact islets, TNF+LPS stimulate the release and accumulation of sufficient concentrations of IL-1 to induce the expression of iNOS by β cells. If islets are physically separated into single cells by dispersion, the concentration of IL-1 does not attain sufficient levels to induce iNOS expression.

Discussion

IDDM is characterized by a local inflammatory reaction in the vicinity of islets that are undergoing autoimmune destruction. Cytokines released during this inflammatory reaction have been proposed to participate in the destruction of β cells (5). In this study we have used an in vitro approach

Figure 5. Immunohistochemical localization of iNOS and insulin. Islets were incubated for 24 h with 5 U/ml IL-1β, 0.7 nM TNF (*T*), 10 μg/ml LPS (*L*), or 1 μg/ml human recombinant IRAP as indicated. The islets were then isolated, sectioned, and stained for both insulin (INS; 1:500 dilution of guinea pig anti–human insulin) and iNOS (1:500 dilution of rabbit anti–mouse macrophage iNOS). Insulin and iNOS were visualized with FITC-conjugated donkey anti–guinea pig, and CY3-conjugated donkey anti–rabbit IgG second antibodies, respectively. Results are representative of two individual experiments.

to directly examine the effects of IL-1 release in islets on both iNOS expression and β cell function. Exposure of rat islets to TNF+LPS results in an inhibition of insulin secretion that is mediated by the intraislet release of IL-1. Although TNF+LPS induce the expression of iNOS and the production of nitric oxide by nonendocrine islet cells, TNF+LPS directly, or indirectly by inducing the production of nitric oxide by nonendocrine islet cells, do not appear to inhibit β cell function. TNF+LPS–induced inhibition of insulin secretion is mediated by the intraislet release of IL-1 and subsequent expression of iNOS by islet β cells. These results are contrary to the hypothesis of Kolb, Kolb-Bachofen and co-workers (42–44) who suggest that nitric oxide production by nonendocrine islet cells mediates cytokine-induced inhibition of islet function. Our results indicate that nonendocrine production of nitric oxide may contribute to TNF+LPS–induced inhibition of insulin secretion, but this production of nitric oxide, per se, is not sufficient to inhibit insulin secretion by islets.

These studies also support the concept that local release of cytokines in an islet markedly influence β cell function. TNF+LPS stimulate nitrite formation by islets, but this combination does not induce nitrite formation by dispersed islet cells. Dispersion of islets into single cells prevents IL-1 from attaining sufficient local concentrations required to induce iNOS expression by the β cell. This finding provides the first experimental evidence demonstrating that it is possible to attain intraislet concentrations of IL-1 sufficient to induce iNOS expression and impair β cell function. These results also have implications for the regulation of iNOS expression during conditions of infection, inflammation, and transplantation rejection. The ability of cytokines to regulate the expression of iNOS in local areas of inflammation and infection suggest that nitric oxide may participate as a specific effector molecule regulated at the level of expression by cytokine release at sites of inflammation or injury.

Animal models of IDDM have shown that macrophages and monocytes are two of the first cells to infiltrate islets under autoimmune attack and this is followed by the appearance of T cells (1–3). In an activated state these cell types are known to release IL-1, TNF, and IFN-γ. We have shown that NOD mouse islets produce nitric oxide in a time-dependent manner that precedes the development of diabetes (22). The results of the current study suggest that during this immune attack the release of cytokines in and around islets results in the generation of local concentrations of these cytokines sufficient for the expression of iNOS by β cells. The induction of iNOS in mouse and human islets appears to require IL-1 and an additional signal, either IFN-γ or TNF, whereas IL-1 alone is sufficient to induce iNOS in rat islets (17, 45, and our unpublished observations). Recently we have shown that IRAP and antibodies specific for IL-1α and IL-1β inhibit the production of nitric oxide by mouse islets treated with TNF and IFN-γ (our unpublished observations). mRNA for these cytokines have been demonstrated in NOD mouse islets undergoing autoimmune attack (46). These preliminary findings provide further support of the potential importance of local cytokine release and the induction of iNOS in the pathogenesis of autoimmune diabetes.

Nitric oxide appears to participate in the inhibition of β cell function, β cell destruction, and in the inflammatory response observed during the development of diabetes. IL-1β induces the coexpression of inducible cyclooxygenase (COX-2) and iNOS by islets, and nitric oxide directly activates COX-2 resulting in the production of proinflammatory prostaglandins (31, 47). These findings suggest that local release of cytokines may influence both the inflammatory response stimulated by the induction of COX-2, and cellular destruction resulting from the expression of iNOS and the production of nitric oxide.

The authors thank Connie Marshall and Joan Fink for technical assistance; Denise Dorsey for immunohistochemical staining of islets; and Parveen Chad for Facs purification of β and α cells. We thank Drs. Paul Lacy, Kevin Roth, and Emil Unanue for many helpful discussions during the course of this work.

This study was supported by National Institutes of Health grants DK-06181 and F32 DK-08748, and a Washington University/Monsanto grant.

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Received for publication 23 August 1994 and in revised form 6 October 1994.

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