Potential Role of Resident Islet Macrophage Activation in the Initiation of Autoimmune Diabetes

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Potential Role of Resident Islet Macrophage Activation in the Initiation of Autoimmune Diabetes

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The purpose of this study was to evaluate the effects of resident islet macrophage activation on β cell function. Treatment of freshly isolated rat islets with TNF-α and LPS results in a potent inhibition of glucose-stimulated insulin secretion. The inhibitory actions of TNF + LPS are mediated by the intraislet production and release of IL-1 followed by IL-1-induced inducible nitric oxide synthase (iNOS) expression by β cells. The IL-1R antagonist protein completely prevents TNF + LPS-induced nitrite production, iNOS expression and the inhibitory effects on glucose-stimulated insulin secretion by rat islets. Resident macrophages appear to be the source of IL-1, as a 7-day culture of rat islets at 24°C (conditions known to deplete islets of lymphoid cells) prevents TNF + LPS-induced iNOS expression, nitrite production, and the inhibitory effects on insulin secretion. In addition, macrophage depletion also inhibits TNF + LPS-induced IL-1α and IL-1β mRNA expression in rat islets. Immunocytochemical colocalization of IL-1β with the macrophage-specific marker ED1 was used to provide direct support for resident macrophages as the islet cellular source of IL-1. IL-1β appears to mediate the inhibitory actions of TNF + LPS on β cell function as TNF + LPS-induced expression of IL-1β is fourfold higher than IL-1α, and Ab neutralization of IL-1β prevents TNF + LPS-induced nitrite production by rat islets. These findings support a mechanism by which the activation of resident islet macrophages and the intraislet release of IL-1 may mediate the initial dysfunction and destruction of β cells during the development of autoimmune diabetes. The Journal of Immunology, 1998, 160: 2684–2691.

It has been suggested that resident islet macrophages may play a primary role in mediating the initial destruction of β cells during the development of autoimmune diabetes (1). Wright et al. have shown that a diet deficient in essential fatty acids prevents the development of diabetes induced by multiple injections of streptozotocin in CD-1 mice by a mechanism that appears to be associated with the depletion of resident tissue macrophages (2). Lefkowith et al. (3) extended these original studies to show that a fatty acid-deficient diet attenuates the natural occurrence of autoimmune diabetes in the Bio Breeding rat. Using a similar approach, Oschilewski et al. have shown that in vivo depletion of macrophages by silica treatment attenuates the development of diabetes in the Bio Breeding rat (4). These two independent lines of evidence support a primary role for macrophages in the development of autoimmune diabetes.

IL-1 is one of the predominant cytokines released by macrophages. Mandrup-Poulsen et al. (5, 6) first showed that treatment of rat islets with IL-1 results in a potent inhibition of insulin secretion followed by islet destruction. We and others have shown that the cellular mechanism by which IL-1 mediates an inhibitory effect on islet function and induces islet destruction involves the expression of iNOS3 and the increased production of nitric oxide (7–9). Treatment of rat islets with IL-1 results in a time-dependent inhibition of insulin secretion that is associated with a similar time-dependent production of nitric oxide (8, 10). Inhibitors of iNOS, N(G)-monomethyl-L-arginine (NMMA), amino-guanidine (AG), and nitro-L-arginine methylster prevent the inhibitory effects of IL-1 on insulin secretion and the destructive effects of IL-1 on islet viability (7, 8, 11–14). The inhibitory effects of IL-1-induced nitric oxide production on islet function and viability are mediated, in part, by the targeting and disruption of mitochondrial electron transport (complexes I and II) and islet aconitase activity (8, 15). IL-1-induced inhibition of mitochondrial glucose oxidation to CO2 and aconitase activity are prevented by NMMA and AG (8, 10, 15, 16). The β cell, selectively destroyed during the development of autoimmune diabetes, appears to be the sole islet cellular source of iNOS following treatment with IL-1. We have shown by immunohistochemical analysis that iNOS expression colocalizes exclusively with insulin in rat islets treated with IL-1 (17).

In 1991, Lacy and Finke (18) showed that depletion of islet lymphoid cells prevents IFN-γ-mediated islet destruction. We have extended these original observations to show that treatment of rat islets with TNF + LPS (classical conditions used to activate macrophages) results in the impairment of β cell function that is mediated by the intraislet release of IL-1 and the subsequent expression of iNOS by β cells (17). In the current study, the effects of TNF + LPS on the time dependence and isoform of IL-1 expressed in islets and the effects of resident islet lymphoid cell depletion on TNF + LPS-induced inhibition of β cell function have been evaluated. Depletion of resident lymphoid cells prevents...
TNF + LPS-induced iNOS expression, nitric oxide production, and the inhibitory effects of this cytokine and endotoxin on glucose-stimulated insulin secretion by rat islets. In addition, we have identified IL-1β as the primary isoform of IL-1 that is expressed in islets under conditions associated with resident islet macrophage activation. These findings provide a mechanism by which the activation of resident islet macrophages may initiate β cell damage during the development of autoimmune diabetes.

Materials and Methods

Materials

CMRL-1066 tissue culture medium, penicillin, streptomycin, 1-glutamine, and PCR oligonucleotide primers were from Life Technologies (Gaithersburg, MD). PBS was obtained from HyClone (Logan, UT). Collagenase and PCR oligonucleotide primers were from Life Technologies (Gaithersburg, MD). FBS was obtained from HyClone (Logan, UT). Collagenase and PCR oligonucleotide primers were from Life Technologies (Gaithersburg, MD). All other reagents were from commercially available sources.

Islet isolation and culture

Islets were isolated from male Sprague-Dawley rats (250 to 300 g; Harlan, Indianapolis, IN) by collagenase digestion as described previously (19). Islets were cultured overnight at 37°C in an atmosphere of 95% air and 5% CO2. Islets were incubated at 37°C for the indicated time periods as described in the figure legends. In some experiments, islets were pretreated with murine rIFN-γ (5 µg/ml) for 30 min before adding cytokines.

Islet lymphoid cell depletion

Resident lymphoid cells were depleted from islets as described previously (18). In brief, isolated islets were cultured for 7 days in complete CMRL-1066 at 37°C in an atmosphere of 95% air and 5% CO2. Islets were removed from the 24°C culture, washed three times with fresh complete CMRL-1066, and then cultured for 3 days at 37°C in complete CMRL-1066. Experiments were conducted as described above for 37°C islets.

Glucose-stimulated insulin secretion

Islets were cultured for 40 h in either complete CMRL-1066, or complete CMRL-1066 containing IL-1β, TNF, LPS, IRAP, and AG as indicated. Islets were then washed three times with Krebs-Ringer bicarbonate buffer (KRB) (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, and 0.1% BSA, pH 7.4) containing 3 mM d-glucose. Islets were aliquoted into siliconized 10-mm × 75-mm borosilicate culture tubes (20 islets/200 µl of KRB containing 3 mM d-glucose), and preincubated for 30 min at 37°C with shaking. Insulin secretion was initiated by removing the preincubation KRB (containing 3 mM d-glucose) followed by the addition of 200 µl of KRB containing either 3 mM d-glucose or 20 µM d-glucose. Islets were incubated for 30 min in an atmosphere of 95% air and 5% CO2 at 37°C. After the incubation, the supernatant was removed and analyzed for insulin by RIA (20).

Nitrite determination

Nitrite production was determined as described previously (21) by mixing 50 µl of culture supernatant with 50 µl of Griess reagent and the absorbance at 540 nm was determined. Nitrite concentrations were calculated from a sodium nitrite standard curve.

Western blot analysis

Rat islets (150/400 µl of complete CMRL-1066) were treated with IL-1, TNF, LPS, IRAP, and AG as indicated. The islets were isolated by centrifugation (6000 × g, 3 min), and washed three times with 0.1 M PBS. Islets were lysed by adding 25 µl of SDS sample mix (0.25 M Tris-HCl, 20% β-mercaptoethanol, and 4% SDS), and 15 µl of distilled H2O, followed by boiling for 4 min, and the addition of 4 µl of loading dye (0.05% bromophenol blue in 80% glycerol). Proteins were separated by SDS gel electrophoresis (22), and transferred to Nitrocellulose membranes (Micron Separations, Inc., Westborough, MA) under semidy transfer conditions. The blots were blocked overnight in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20, pH 7.5) containing 5% nonfat dry milk. Blots were washed once with TBST and then incubated for 1.5 h at room temperature with rabbit anti-mouse iNOS (diluted 1:2000) in TBST containing 1% nonfat dry milk. After incubating in the primary antiserum, blots were washed four times with TBST (5 min each), and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit secondary Ab (1:7000 dilution). The blots were then washed three times in TBST and once in 0.1 M PBS at room temperature. Rat iNOS was detected by ECL according to product specifications.

Immunoprecipitation of IL-1α and IL-1β

Rat islets (700) were cultured with or without 10 ng/ml TNF + 10 µg/ml LPS for 2.5 h in 1 ml of methionine-deficient medium (9 parts MEM minus methionine:1 part MEM containing methionine). [35S]Methionine (500 µCi) was added and the islets were cultured for an additional 6 h. Islets were isolated by centrifugation and then 600 ml of lysis buffer (10 mM Tris, 140 mM NaCl, 1% Nonidet P-40, 1% BSA, 100 µg/ml aprotinin, 100 µg/ml leupeptin, 1 mM iodoacetamide, and 1 mM PMSF) was added and the islets were incubated for 1 h at 4°C. Cellular debris was removed by centrifugation (30 min, 10,000 × g, 4°C), and IL-1α and IL-1β were immunoprecipitated by methods previously described (23). Immunoprecipitations were performed sequentially with IL-1α precipitated first followed by IL-1β. The immunoprecipitates were separated by SDS gel electrophoresis (15% polyacrylamide), and IL-1α and IL-1β (precursor and mature forms) were visualized by fluorography.

Immunocytochemistry

Islets (150/400 µl of complete CMRL-1066) cultured for 4 h with or without 10 ng/ml TNF + 10 µg/ml LPS, were isolated and dispersed into individual cells as described previously (17). Islet cells were washed three times with 0.1 M PBS (pH 7.4) and diluted to a concentration of 40,000 cells/100 µl. The cells were transferred to Superfrost/Plus microscope slides by cytospin. The slides were fixed in 4% paraformaldehyde for 1 h at 4°C and then blocked for 1 h with 5% BSA (in 0.1 M PBS). The slides were incubated overnight at room temperature with a 1:25 dilution (in 0.1 M PBS containing 1% BSA) of goat anti-rat IL-1β antisera (R&D Systems; Minneapolis, MN), followed by a 1-h incubation with a 1:100 dilution of mouse anti-rabbit macrophage antigen (ED1; Serotec, Raleigh, NC). The slides were washed three times with 150 µl of PBS and then incubated for 1 h with a 1:200 dilution of CY3-conjugated donkey anti-rabbit secondary Ab and CY3-conjugated donkey anti-mouse secondary Ab, for IL-1β and ED1, respectively. Immunofluorescence microscopy was used for the detection of IL-1β and ED1.

Polymerase chain reaction

Total RNA was isolated from islets using the Qiagen RNeasy RNA isolation kit. Total RNA (2.5 µg) from each sample was then used to prepare first-strand cDNA using the Superscript Preamplification System from Life Technologies. A standard 25-µl PCR reaction containing 2.5 µl from the reverse transcriptase reaction, 200 µM of each dATP, dCTP, dGTP, dUTP, 50 pmol of each forward and reverse primers, 2 µl of Taq DNA polymerase from Promega (Madison, WI), 1.5 mM MgCl2, and 2.5 µl of the supplied 10× reaction buffer was performed. IL-1α, IL-1β, iNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were designed according to the published sequences: 1) IL-1α forward primer 5′-CCCAAC CCAGATCGACCTCA-3′, reverse primer 5′-TCTCCCTCGGATG TAGGCA-3′ (PCR product size = 204 bp); 2) IL-1β forward primer 5′- CCTTGGCTTTGGCCCTCAA-3′, reverse primer 5′-GCTTGCTGAT TACGATGTTGG-3′ (PCR product size = 204 bp); 3) iNOS forward primer 5′-CCACACCGGAAGAGGGGCAACT-3′, reverse primer 5′-GGAATGTTGCGTGCCTGACAC-3′ (PCR product size = 297 bp); 4) GAPDH forward primer 5′-GCTTGGGGTCTACACAGGAG-3′, reverse primer 5′-GGATGACCTTGGCCACAGCC-3′ (PCR product size = 343 bp).

Each PCR mixture was overlaid with 1 drop of mineral oil, and incubated in a Hybaid Omnimic thermal cycler using the following cycling profile: an initial denaturation step at 94°C for 5 min, 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 75 s. The samples were finally incubated at 30°C for 2 min. To each reaction, 5 µl of 6× loading dye (Promega) was added, and

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then 12 μl from each PCR reaction was run alongside 5 μl of 100-bp ladder (Promega) on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. PCR products were visualized with UV light and photographed.

**Semiquantitative PCR**

The cDNAs samples described above were used in semiquantitative PCR as previously described (24, 25). In brief, each cDNA template was diluted 1:25 and 1:50 for both IL-1α and IL-1β reactions, and 1:125 and 1:250 for GAPDH reactions. Amplification of each cDNA species for each sample was therefore represented by two different concentrations of initial cDNA template. This would later be used to verify that each reaction was amplified within the linear range, and that PCR product yields were proportional to initial concentrations of cDNA template. From each cDNA dilution, 5 μl was used in a standard 25 μl PCR reaction as described above. In addition, 2.5 μCi of [α-35S]-labeled dCTP was added. PCR was conducted according to the standard profile described above with the exception of reducing the cycle number to 22. A total of 12 μl from each PCR reaction was then run on a 1.5% agarose gel; the gels were dried and subjected to phosphorimage analysis using a Molecular Dynamics PhosphorImager and Molecular Dynamics ImageQuant Software Version 3.3 (Molecular Dynamics, Sunnyvale, CA). The values obtained for each IL-1 PCR reaction were normalized as a percentage of the values obtained for each GAPDH control. Under the conditions used, the PCR product signal was proportional to the amount of cDNA subjected to PCR amplification.

**Statistical analysis and figure preparation**

Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences between groups were determined by Scheffe’s F-test posthoc analysis. For figure preparation, ethidium bromide-stained agarose gels and Western blot autoradiograms were scanned into National Institutes of Health Image version 1.59 using a COHU high performance CCD camera (Brookfield, WI). The images were then imported into Canvas 3.5 (Deneba Software, Miami, FL) for the preparation of figures.

**Results**

**A 7-day culture at 24°C prevents TNF + LPS-induced inhibition of insulin secretion and nitrite formation by rat islets**

To determine whether the source of IL-1 released in islets in response to TNF + LPS is resident islet lymphoid cells, the effects of TNF + LPS on nitric oxide production and insulin secretion by rat islets either freshly isolated or cultured for 7 days at 24°C have been evaluated. Treatment of freshly isolated rat islets with IL-1 results in a potent inhibition of insulin secretion and a 2.5-fold increase in nitrite production (Fig. 1A). The inhibitory effects of IL-1β on insulin secretion and stimulatory effects on nitrite production are prevented by the IL-1R antagonist protein (IRAP). We have previously shown that β cells are the islet cellular source of iNOS in response to IL-1 (17). Treatment of freshly isolated rat islets with TNF + LPS also results in a potent inhibition of insulin secretion and a twofold increase in the production of nitrite. IRAP completely prevents the inhibitory effects of TNF + LPS on glucose-stimulated insulin secretion and significantly attenuates nitrite formation by isolated islets. The selective inhibitor of iNOS, AG (12), also prevents TNF + LPS-induced inhibition of insulin secretion and nitrite formation by rat islets, providing evidence that nitric oxide mediates the inhibitory effects of TNF + LPS on insulin secretion. These findings are consistent with our previous studies showing that TNF + LPS-induced inhibition of glucose-stimulated insulin secretion is mediated by the release of IL-1β within islets, followed by IL-1-induced iNOS expression and nitric oxide production by β cells (17).

To provide evidence supporting resident lymphoid cells as the islet source of IL-1, the effects of a 7-day culture at 24°C on nitrite production and insulin secretion were examined. Lacy and Finke have shown that a 7-day culture at 24°C in complete CMRL-1066 tissue culture medium reduces the number of resident islet lymphoid cells by over 98% (18). As shown in Figure 1B, TNF + LPS fails to induce the production of nitrite or to inhibit glucose-stimulated insulin secretion by rat islets cultured for 7 days at 24°C. These findings indicate that lymphoid cell depletion prevents TNF + LPS-induced inhibition of insulin secretion. Although this culture condition prevents TNF + LPS-induced inhibition of insulin secretion, the inhibitory effects of IL-1 on glucose-stimulated insulin secretion and the stimulatory effects of IL-1 on nitrite formation were not modified. IL-1 stimulates a greater than 2-fold increase in nitrite production by both freshly isolated islets, and islets cultured for 7 days at 24°C (~75 pmol nitrite/islet for both culture conditions). Also, 20 mM glucose induced a 6.6-fold increase in the release of insulin (3 mM glucose, 1.86 ng/20 islets, 30 min; 20 mM glucose, 12.3 ng/20 islet, 30 min) from freshly isolated rat islets, and a 4.2-fold increase by islets cultured for 7 days at 24°C (3 mM glucose, 1.96 ng/20 islets, 30 min; 20 mM glucose, 8.0 ng/20 islet, 30 min). These findings indicate that a 7-day culture at 24°C does not impair islet function, and does not reduce β cell production of nitric oxide in response to IL-1.

**A 7-day culture at 24°C prevents TNF + LPS-induced iNOS expression by rat islets**

The effects of TNF + LPS and IL-1 on iNOS expression by freshly isolated rat islets, and islets cultured for 7 days at 24°C are shown in Figure 2. Treatment of freshly isolated islets with TNF + LPS results in the expression of iNOS at the mRNA (Fig. 2A) and protein level (Fig. 2B) as determined by RT-PCR and Western blot.
analysis, respectively. IRAP completely prevents TNF + LPS-induced iNOS protein expression by freshly isolated rat islets (Fig. 4). This finding is consistent with the ability of IRAP to prevent TNF + LPS-induced inhibition of insulin secretion and nitrite production by freshly isolated islets as shown in Figure 1A. Conversely, TNF + LPS fails to stimulate the expression of iNOS at either the mRNA or protein level by rat islets depleted of resident tissue lymphoid cells (Fig. 2, A and B). The lack of iNOS expression is consistent with the inability of TNF + LPS to stimulate nitrite formation or to inhibit insulin secretion by rat islets cultured for 7 days at 24°C (Fig. 1B). These findings indicate that lymphoid cell depletion prevents TNF + LPS-induced iNOS expression by rat islets.

Incubation of rat islets for 7 days at 24°C does not inhibit IL-1-induced iNOS expression by rat islets. As shown in Figure 2, IL-1 induces the expression of iNOS mRNA and protein by either freshly isolated islets or islets cultured for 7 days at 24°C. These results are consistent with the inhibitory effects of IL-1 on insulin secretion and IL-1-induced nitrite production by rat islets under both conditions, and indicate that culture conditions used to deplete resident macrophages do not modify the ability of IL-1 to induce the expression of iNOS by β cells.

**Colocalization of IL-1β and macrophage surface markers**

Immunocytochemistry was used to identify the islet cellular source of IL-1. For these experiments, islets were cultured for 4 h with TNF + LPS, dispersed into individual cells, fixed on slides, and then stained for IL-1β and the macrophage surface marker ED1. As shown in Figure 3, TNF + LPS stimulates the expression of IL-1β in a limited number of islet cells (green fluorescence) as evidenced by the punctate green fluorescence in the two indicated cells (arrows in Fig. 3a). IL-1β expression appears to colocalize

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**FIGURE 2.** Effects of intraislet lymphoid cell depletion on TNF + LPS-induced iNOS mRNA and protein expression by rat islets. A, iNOS mRNA expression by rat islets (100 islets/ml of complete CMRL-1066), either freshly isolated (37°C) or cultured for 7 days at 24°C to deplete resident islet lymphoid cells, was determined by RT-PCR following a 4-h incubation with 10 ng/ml TNF + 10 μg/ml LPS or 5 U/ml IL-1β as stated in Materials and Methods. B, iNOS protein expression was examined by Western blot analysis of rat islets (100 islets/400 μl of complete CMRL-1066) incubated for 40 h with 10 ng/ml TNF + 10 μg/ml LPS or 5 U/ml IL-1β as stated in Materials and Methods. Results are representative of two and three individual experiments for iNOS mRNA and protein expression, respectively.

**FIGURE 3.** Colocalization of IL-1β and the macrophage surface marker ED1. Rat islets were treated for 4 h with 10 ng/ml TNF and 10 μg/ml LPS. The islets were isolated, dispersed into individual cells, and plated onto slides by cytopsin. IL-1β (a) was identified using goat anti-rat IL-1β and FITC-conjugated donkey anti-goat secondary antisera (green fluorescence), and macrophages (b) were identified using mouse anti-rat macrophage (ED1) and CY3-conjugated donkey anti-mouse secondary antisera (red fluorescence). Colocalization of IL-1β and ED1-positive macrophages is shown by the intense orange fluorescence following double exposure (c). Results are representative of two individual experiments.
100 islets/ml of complete CMRL-1066) were cultured with TNF (10 ng/ml). 

Materials and Methods

Untreated islets were cultured for 4 h with TNF. Rat islets, freshly isolated (37°C) or cultured for 7 days at 24°C, were treated with TNF (10 ng/ml) for 1, 2, 4, and 8 h as indicated. Total RNA was isolated and IL-1α and IL-1β mRNA expression was determined by RT-PCR, as stated in Materials and Methods. Relative levels of IL-1α and IL-1β mRNA expression, normalized to GAPDH, were determined by semiquantitative RT-PCR performed on total RNA isolated from rat islets treated with TNF (10 ng/ml) plus LPS (10 μg/ml) for 4 h as described in Materials and Methods. IL-1α and IL-1β mRNA expression in control-untreated islets was below the detection limits of the PCR analysis. Rat islets, freshly isolated (37°C) or cultured for 7 days at 24°C, were treated with TNF (10 ng/ml) LPS. Total RNA was isolated and IL-1α and GAPDH mRNA expression were determined by RT-PCR. To determine whether the expression of IL-1 at the mRNA level correlates with the synthesis of this cytokine, the effects of TNF + LPS on IL-1α and IL-1β protein expression were examined by immunoprecipitation. For these experiments, islets were pretreated for 2.5 h with TNF + LPS, [35S]methionine was added, and the islets were cultured for an additional 6 h. The precursor and mature forms of both IL-1α and IL-1β were then immunoprecipitated using hamster anti-mouse IL-1α and IL-1β antisera as described previously (23, 27). Similar to the accumulation of mRNA, IL-1β mRNA expression appears to require the presence of resident islet macrophages. As shown in Figure 4C, a 4-h incubation with TNF + LPS fails to stimulate IL-1β mRNA expression in islets cultured for 7 days at 24°C. Similar results were obtained for IL-1α (data not shown). The lack of IL-1α and IL-1β mRNA expression by islets cultured for 7 days at 24°C is consistent with the inability of TNF + LPS to stimulate iNOS expression and nitrite production, or to inhibit insulin secretion under similar culture conditions.

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Materials and Methods

Materials and Methods

Identification of the isoform of IL-1 that is expressed in islets and that mediates TNF + LPS-induced iNOS expression. A, Rat islets (100 islets/ml of complete CMRL-1066) were cultured with TNF (10 ng/ml) + LPS (10 μg/ml) for 1, 2, 4, and 8 h as indicated. Total RNA was isolated and IL-1α, IL-1β, and GAPDH mRNA expression was determined by RT-PCR as stated in Materials and Methods. Relative levels of IL-1α and IL-1β mRNA expression, normalized to GAPDH, were determined by semiquantitative RT-PCR performed on total RNA isolated from rat islets treated with TNF (10 ng/ml) + LPS (10 μg/ml) for 4 h as described in Materials and Methods. IL-1α and IL-1β mRNA expression in control-untreated islets was below the detection limits of the PCR analysis. B, Rat islets, freshly isolated (37°C) or cultured for 7 days at 24°C, were treated for 4 h with TNF + LPS. Total RNA was isolated and IL-1β and GAPDH mRNA expression was determined by RT-PCR. D, Rat islets (700/ml of complete CMRL-1066) were cultured for 2.5 h with TNF + LPS in methionine-deficient MEM; 500 μCi of [35S]methionine were added and the islets were cultured for an additional 6 h. The islets were then isolated and IL-1α and IL-1β were immunoprecipitated as stated in Materials and Methods. Immunoprecipitates were separated by SDS gel electrophoresis (15% acrylamide) and IL-1 expression was detected by fluorography, with the precursor form of IL-1β as indicated. E, Isolated rat islets (120/400 μl of complete CMRL-1066) were incubated for 40 h with 10 ng/ml TNF, 10 μg/ml LPS, 10 μg/ml hamster anti-mouse IL-1α, or 10 μg/ml hamster anti-mouse IL-1β antiserum as indicated. Following culture, the supernatant was removed and nitrite production was determined as stated in Materials and Methods. Results are representative of two to three independent experiments (A, C, and D), and are the mean ± SEM of three independent experiments for B and E. Statistical significance: p < 0.05 vs control (*) and p < 0.05 vs TNF + LPS (Δ) are as indicated.

Time course of expression and isoform of IL-1 induced by TNF + LPS in rat islets

Two isoforms of IL-1 have been identified, IL-1α and IL-1β (26). RT-PCR was used to identify the isoform of IL-1, and the time course of IL-1 expression by rat islets treated with TNF + LPS. As shown in Figure 4A, TNF + LPS stimulates the expression of both IL-1α and IL-1β mRNA in rat islets. IL-1α expression is absent in untreated islets, while TNF + LPS-induced IL-1α mRNA expression is detectable following a 1-, 2-, 4-, and 8-h incubation. IL-1α mRNA expression appears to be significantly reduced following a 4-h incubation with TNF + LPS. TNF + LPS stimulates the expression of significantly higher levels of IL-1β mRNA than IL-1α mRNA levels following 1-, 2-, 4-, and 8-h exposures. The levels of IL-1α and IL-1β mRNA accumulation in response to TNF + LPS have been quantitated by semiquantitative RT-PCR and are shown in Figure 4B. TNF + LPS-induced IL-1β mRNA accumulates to levels that are over fourfold higher than the levels of IL-1α. IL-1α and IL-1β mRNA expression appears to require the presence of resident islet macrophages. As shown in Figure 4C, a 4-h incubation with TNF + LPS fails to stimulate IL-1β mRNA expression in islets cultured for 7 days at 24°C. Similar results were obtained for IL-1α (data not shown). The lack of IL-1α and IL-1β mRNA expression by islets cultured for 7 days at 24°C is consistent with the inability of TNF + LPS to stimulate iNOS expression and nitrite production, or to inhibit insulin secretion under similar culture conditions.

To determine whether the expression of IL-1 at the mRNA level correlates with the synthesis of this cytokine, the effects of TNF + LPS on IL-1α and IL-1β protein expression were examined by immunoprecipitation. For these experiments, islets were pretreated for 2.5 h with TNF + LPS, [35S]methionine was added, and the islets were cultured for an additional 6 h. The precursor and mature forms of both IL-1α and IL-1β were then immunoprecipitated using hamster anti-mouse IL-1α and IL-1β antisera as described previously (23, 27). Similar to the accumulation of mRNA, IL-1β mRNA expression appears to require the presence of resident islet macrophages. As shown in Figure 4C, a 4-h incubation with TNF + LPS fails to stimulate IL-1β mRNA expression in islets cultured for 7 days at 24°C. Similar results were obtained for IL-1α (data not shown). The lack of IL-1α and IL-1β mRNA expression by islets cultured for 7 days at 24°C is consistent with the inability of TNF + LPS to stimulate iNOS expression and nitrite production, or to inhibit insulin secretion under similar culture conditions.
There appears to be the major isoform of IL-1 synthesized in islets in response to TNF + LPS (Fig. 4D). We were unable to detect the expression of IL-1α (precursor or mature form), or the mature form of IL-1β. To confirm that the intraislet production of IL-1β stimulates iNOS expression, the effects of IL-1α and IL-1β neutralizing antisera on TNF + LPS-induced nitrite production by rat islets were examined. Neutralization of IL-1β results in nearly complete inhibition of TNF + LPS-induced nitrite production by rat islets (Fig. 4E). Incubation of rat islets for 40 h with hamster anti-IL-1α antisera also reduced TNF + LPS-induced nitrite production; however, the level of neutralization was much less than the effects of hamster anti-IL-1β-specific antisera. In addition, irrelevant hamster antiserum did not modulate TNF + LPS-induced nitrite production by rat islets (data not shown). These findings suggest that IL-1β is the predominant isoform of IL-1 that is expressed in islets and that mediates the actions of TNF + LPS on iNOS expression and nitrite production.

Nitric oxide is not required for intraislet IL-1 release by resident macrophages

It has recently been shown that nitric oxide production is required for the release of biologically active IL-1 from peritoneal exudate cells (PEC) and RAW 264.7 macrophages (23). Nitric oxide appears to participate in IL-1 release, in part, by stimulating the accumulation of cGMP. In Figure 5, A and B, we have evaluated the role of nitric oxide in the release of IL-1 by resident islet lymphoid cells. Treatment of rat islets with TNF + LPS stimulates a threefold increase in nitrite production and high levels of iNOS protein expression. IRAP completely prevents iNOS expression, and significantly attenuates, but does not completely prevent, nitrite formation by rat islets. These findings are consistent with results shown in Figures 1 and 2, and demonstrate that TNF + LPS stimulates iNOS expression and nitrite production by rat islets by a mechanism that involves the intraislet release of IL-1 and the subsequent expression and production of nitric oxide by β cells. The iNOS inhibitor AG completely prevents TNF + LPS-induced nitrite production by rat islets; however, AG does not inhibit iNOS expression under these conditions. In addition, AG does not inhibit TNF + LPS-induced IL-1α or IL-1β mRNA expression, nor does it prevent the inhibitory actions of IRAP on TNF + LPS-induced iNOS protein expression (data not shown). These findings indicate that nitric oxide production is not required for the release of biologically active IL-1 by resident islet macrophages.

Discussion

In this study the effects of intraislet macrophage activation on β cell function have been evaluated. We show that a combination of TNF + LPS, classical conditions used to activate macrophages, induces the expression of both IL-1α and IL-1β mRNA, and that IL-1β mRNA accumulates to levels that are approximately fourfold higher than the accumulation of IL-1α mRNA by rat islets. To investigate the cellular source of IL-1 in islets treated with TNF + LPS, we have used culture conditions previously shown to deplete islets of resident lymphoid cells. Incubation of rat islets for 7 days at 24°C results in the loss of over 98% of islet class II-positive lymphoid cells (18). Islets contain approximately 0.5% resident macrophages (10 to 15/islet), and this cell type is believed to be the cell type lost under these culturing conditions (18). Treatment of freshly isolated rat islets with TNF + LPS results in the expression of iNOS, production of nitric oxide, and a potent inhibition of glucose-stimulated insulin secretion. IRAP, which competes with IL-1 for receptor binding, prevents TNF + LPS-induced iNOS expression, markedly attenuates nitrite production, and prevents the inhibitory effects on glucose-stimulated insulin secretion. Depletion of islet lymphoid cells by culturing islets for 7 days at 24°C also completely prevents TNF + LPS-induced iNOS expression, nitrite formation, and the inhibitory effects on glucose-stimulated insulin secretion. In addition, TNF + LPS fail to stimulate the expression of IL-1α and IL-1β in islets cultured for 7 days at 24°C.

We believe that the resident macrophage is the islet cellular source of IL-1 in response to TNF + LPS; however, islets contain a number of nonendocrine cells that may also be potential sources of this cytokine. These nonendocrine cells include endothelial cells (which comprise ~10% of islet cells, ~150/islet) (28), and a limited number of fibroblasts and dendritic cells. Fibroblasts and endothelial cells do not appear to contribute to the intraislet production of IL-1 in response to TNF + LPS. Fibroblasts are normally found associated with the islet capsule, a structure that is lost during isolation, and islet microcapillaries (endothelial cells) appear to form rounded structures that are extruded from isolated islets following a 48-h culture (Dr. Susan Bonner-Weir; Joslin Diabetes Center, Harvard Medical School, Boston, MA; personal communication). In this regard, TNF + LPS stimulates iNOS and IL-1 expression and nitrite production to nearly identical levels by islets either freshly isolated, or islets cultured for 48 h before stimulation with TNF + LPS (data not shown). Immunocytochemical colocalization of IL-1β and the macrophage surface marker, ED1, was used to provide direct support for resident macrophages as the islet cellular source of IL-1. We show that TNF + LPS stimulates
IL-1β expression in approximately 50% of islet ED1-positive macrophages, and that IL-1 is not detected in other islet endocrine or nonendocrine cells. These results strongly support resident macrophages as the islet cellular source of IL-1 in response to TNF + LPS.

It is somewhat surprising that IL-1β is the primary isoform of IL-1 that mediates the inhibitory actions of TNF + LPS on islet function. IL-1β comprises the majority of secreted IL-1, whereas IL-1α is commonly believed to be a membrane-associated isoform that mediates its actions in a localized fashion. It was originally believed that the isoform of IL-1 expressed in islets under conditions associated with islet macrophage activation would be IL-1α, because target β cells are in close proximity to resident macrophages in the microenvironment of islets. In addition, an increase in IL-1α mRNA expression has been detected in leukocytes purified from islets isolated from nonobese diabetic (NOD) mice (25). Although the identification of IL-1α mRNA expression by leukocytes purified from islets isolated from recently diagnosed diabetic NOD mice does not appear to be consistent with our identification of IL-1β as the predominant isoform of IL-1 expressed by resident macrophages, it is important to emphasize that the aforementioned study primarily has identified cytokine mRNA expression by invading inflammatory leukocytes, and not cytokine mRNA expression by resident islet macrophages. Cytokine expression by resident islet macrophages would be expected to occur very early in the development of diabetes, if this cell type participates in the initial stages of β cell damage. Further support for IL-1β as a potential early mediator of β cell damage includes recent findings by Caileau et al. (29), which show that neutralizing Abs specific for IL-1β prevent cyclophosphamide-induced diabetes in the NOD mouse.

It has been difficult to determine the cellular mechanisms associated with macrophage release of IL-1 because both IL-1α and IL-1β are synthesized as precursor proteins that do not contain signal sequences, and neither isoform appears to be glycosylated (26). These findings support the view that both isoforms of IL-1 are not secreted through the Golgi apparatus as is the case for most secreted proteins. Two potential mechanisms have been proposed for macrophage IL-1 release. Macrophage death, either by apoptosis or necrosis, is known to result in the release of IL-1 (27, 30). Also, Hill et al. (23) have shown that nitric oxide production is required for IL-1 release by a macrophage cell line, RAW 264.7, and by primary mouse peritoneal macrophages (PEC). In these studies, iNOS inhibitors (AG and NMMA) were shown to completely prevent LPS and LPS + IFN-γ-induced release of biologically active IL-1 by PEC and RAW 264.7 cells, respectively (23). Nitric oxide appears to participate in IL-1 release by activating guanylate cyclase and stimulating cGMP accumulation (23). If nitric oxide production is required for cytokine release by resident macrophages, then AG, the selective iNOS inhibitor, should prevent TNF + LPS-induced iNOS expression and nitrite production by rat islets. However, AG does not inhibit TNF + LPS-induced iNOS expression, but completely prevents nitrite production by rat islets. These findings provide evidence to suggest that nitric oxide is not required for cytokine release by resident islet macrophages. It is possible that AG does not completely inhibit TNF + LPS-induced nitric oxide production, and that low levels of nitric oxide may be sufficient to stimulate cGMP accumulation and macrophage release of IL-1 under these conditions. Although this interpretation is possible, it is not likely. We have previously shown that AG completely prevents IL-1-induced cGMP accumulation by rat islets (12), and LPS-induced cGMP accumulation by RAW 264.7 cells (31) under conditions similar to those used in our study. Interestingly, we have recently shown that resident islet macrophage death results in the release of sufficient levels of IL-1 to stimulate iNOS expression by β cells in the presence of IFN-γ (32). Taken together, these findings suggest that cellular damage or death is one mechanism associated with the release of IL-1 by resident islet macrophages.

It is also interesting that the primary isoform of IL-1 released by peritoneal macrophages and RAW 264.7 cells in the study by Hill et al. (23) was IL-1α, while TNF + LPS stimulates the expression and release of IL-1β by resident islet macrophages. This difference suggests that: 1) the primary isoform of IL-1 released by peritoneal macrophages differs from the isoform released by resident tissue macrophages (IL-1α vs IL-1β, respectively); and 2) that IL-1α release may be nitric oxide dependent while IL-1β is released by mechanisms that are independent of the production of nitric oxide.

It has been proposed that resident islet macrophage activation may be one triggering or initiation event that leads to the development of autoimmune diabetes (1). In this hypothesis, activation of islet lymphoid cells (macrophages) mediates the initial destruction of β cells and this stimulates the subsequent induction of an immune-mediated process for the eventual destruction of remaining β cells. Lacy and Finke (18) first demonstrated that depletion of islet lymphoid cells prevents islet destruction stimulated by high concentrations of IFN-γ (1000 U/ml). Although we have not directly examined the effects of high concentrations of IFN-γ on intranest IL-1 release, our studies provide mechanistic information on how the activation of resident macrophages can directly modulate β cell function. Activation of resident macrophages results in the expression and release of IL-1, and IL-1 accumulates in islets to levels that are sufficient to stimulate the expression of iNOS and the production of nitric oxide by β cells. Under these conditions, nitric oxide produced by β cells impairs islet oxidative metabolism resulting in the inhibition of glucose-stimulated insulin secretion and β cell damage. It has been proposed that the initiation of autoimmune diabetes is associated with a chemical or viral agent that targets and stimulates the initial destruction of β cells (33–35). Our studies support a role for the resident islet macrophage as the target of a chemical or viral agent. Chemical or viral activation of resident islet macrophages would result in the release of IL-1 in the microenvironment of the islet, followed by IL-1-induced β cell dysfunction and destruction in a nitric oxide-dependent manner. β Cell death would then result in the release and presentation of neoantigens and β cell-specific Ags by macrophages (or dendritic cells) leading to classical T cell-dependent destruction of β cells and the development of autoimmune diabetes.

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