Prolonged STAT1 Activation Is Associated with Interferon-γ Priming for Interleukin-1-induced Inducible Nitric-oxide Synthase Expression by Islets of Langerhans*

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In this study, the ability of interferon-γ (IFN-γ) to prime rat and nonobese diabetic (NOD) mouse islets for interleukin-1 (IL-1)-stimulated expression of inducible nitric-oxide synthase (iNOS) has been examined. IL-1-induced iNOS expression by rat islets is concentration-dependent with maximal expression occurring in response to 1.0 unit/ml. Individually, neither 0.1 unit/ml IL-1 nor 150 units/ml IFN-γ stimulates iNOS expression or nitrite production by rat islets. However, a 30–60-min pulse of rat islets with IFN-γ, followed by washing to remove the cytokine and continued culture with 0.1 unit/ml IL-1 for 40 h, results in iNOS expression and nitrite production to levels similar in magnitude to the individual effects of 1.0 unit/ml IL-1. A 1-h pulse with IFN-γ primes for IL-1-induced islet degeneration that is mediated by the expression of iNOS and increased production of nitric oxide. IFN-γ also primes for IL-1-induced iNOS expression and nitrite formation by NOD mouse islets. The priming actions of IFN-γ appear to be selective for β-cells, as IFN-γ primes for IL-1-induced nitrite formation by primary β-cells and RINm5F insulinoma cells, but not primary α-cells. The priming actions of IFN-γ for IL-1-induced iNOS expression do not require de novo protein synthesis as preincubation of RINm5F cells with cycloheximide does not inhibit iNOS mRNA accumulation under priming conditions. The priming actions of IFN-γ on IL-1-induced iNOS expression persists for extended periods of up to 7 days and are associated with persistent signal transducers and activators of transcription (STAT)-1 activation. A 30-min pulse of rat islets with IFN-γ stimulates STAT1 phosphorylation, and STAT1 remains phosphorylated for up to 7 days following IFN-γ removal. In addition, STAT1 remains nuclear for up to 7 days after IFN-γ removal. These results indicate that IFN-γ primes for IL-1-induced islet degeneration via a nitric oxide-dependent mechanism. These findings also provide evidence that the priming actions of IFN-γ for IL-1-induced iNOS expression are associated with the prolonged phosphorylation and activation of STAT1.

Insulin-dependent diabetes mellitus is an autoimmune disea characterized by an inflammatory reaction in and around pancreatic islets followed by selective destruction of insulin-secreting β-cells. Cytokines such as IL-1 and IFN-γ, and the free radical nitric oxide, have been implicated as effector molecules that participate in the initial destruction of β-cells leading to the development of disease. Southern et al. first demonstrated that treatment of rat islets with IL-1 results in the inhibition of insulin secretion that is attenuated by co-incubation with the iNOS inhibitor, L-arginine (1). We and others have shown that IL-1 stimulates the time- and concentration-dependent expression of iNOS and formation of nitric oxide by rat islets (2–5). In addition, human and NOD mouse islets express iNOS and produce nitric oxide in response to IL-1 + IFN-γ (6–9). Cytokine-induced nitric oxide formation by rat, human, and NOD mouse islets results in an inhibition of insulin secretion and islet degeneration, events that are attenuated by iNOS inhibitors, L-arginine, and aminoguanidine (AG) (1–2, 10–11). The inhibitory and destructive effects of cytokines on islet function and viability are mediated, in part, by the ability of nitric oxide to target and inhibit the activity of mitochondrial enzymes, including aconitase and the electron transport chain complexes I and II (2, 4, 12). IL-1 has also been shown to reduce islet cellular levels of ATP and to inhibit glucose oxidation in a nitric oxide-dependent manner (2–3).

The T-cell cytokine IFN-γ appears to play an important role in the development of insulin-dependent diabetes mellitus. Transgenic mice expressing IFN-γ under control of the insulin promoter develop insulitis and diabetes (13). Also, IFN-γ mRNA expression correlates with the development of insulitis and diabetes in the NOD mouse, and antisera specific for IFN-γ attenuates the development of diabetes in these mice (8, 14–15). We have shown that IFN-γ reduces the concentration of IL-1 required to stimulate iNOS expression and nitric oxide production by rat islets, primary β-cells, and insulinoma RINm5F cells by 10-fold from 1.0 to 0.1 unit/ml IL-1 (5). Individually, IFN-γ and 0.1 unit/ml IL-1 do not induce iNOS expression or nitric oxide formation; however, in combination, IFN-γ and 0.1 unit/ml IL-1 potently inhibit insulin secretion and induce islet degeneration in a nitric oxide-dependent manner (5). In 1999, Baquerizo et al. (16) demonstrated that a short pulse of islet-cell monolayers with IFN-γ sensitizes or primes islet cells for IL-1-induced cytolsys as determined by a modified 51Cr release assay. The priming effect was observed only when the islet cells were pulsed with IFN-γ, not IL-1, and

1 The abbreviations used are: IL-1β, interleukin-1β; IFN-γ, interferon-γ; AG, aminoguanidine; iNOS, inducible nitric-oxide synthase; STAT, signal transducers and activators of transcription; NOD, nonobese diabetic; JAK, Janus kinase; GAS, gamma-activated sequence; N-OMargino-L-arginine (1).
for persisted up to 3–6 days following the removal of IFN-γ. The mechanism by which IFN-γ primes for IL-1-induced cytotoxicity, however, is not known.

In this study, we have examined the mechanism by which IFN-γ primes for IL-1-induced islet degeneration. IFN-γ activates a family of transcription factors known as the signal transducers and activators of transcription (STATs) (reviewed in Ref. 17). In response to IFN-γ, STAT1 is recruited to the IFN-γ receptor where it is tyrosine phosphorylated by Janus kinases, JAK1 and JAK2. Thus activated, STAT1 homodimers translocate to the nucleus and activate a new gene transcription by binding to consensus gamma-activated sequences (GAS) sites. The 5′-untranslated region of the mouse iNOS gene contains three GAS sites (18). In this study, we show that a 30-min pulse of islets with IFN-γ followed by removal of IFN-γ by washing and further incubation with submaximal concentrations of IL-1 (0.1 unit/ml) results in islet degeneration that is mediated by iNOS expression and increased nitric oxide production. The priming actions of IFN-γ persist for extended periods of up to 7 days and appear to be associated with increased phosphorylation and activation of transcription factor STAT1. These findings suggest that prolonged activation of STAT1 may be one mechanism by which IFN-γ primes islets for IL-1-induced iNOS expression and islet degeneration.

**EXPERIMENTAL PROCEDURES**

**Materials and Animals**—RINm5F cells were obtained from Washington University Tissue Culture Support Center (St. Louis, MO). RPMI 1640 medium containing 1×t-glutamine, CMRL-1066 tissue culture medium, l-glutamine, penicillin, streptomycin, and rat recombinant IFN-γ were from Life Technologies, Inc. (Grand Island, NY). Mouse IFN-γ was obtained from Genzyme Corp. (Cambridge, MA). Fetal calf serum was obtained from Hyclone Labs. (Logan, UT). Male Sprague Dawley rats (250–300 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN), and NOD mice were obtained from Taconic Farms. AG and collagenase type XI were from Sigma. [α-32P]dCTP and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. Horse-radish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit anti-serum specific for the C-terminal 27 of STAT1 and iNOS were gifts of Dr. Jon Omenn (University of Michigan, Ann Arbor, MI) and Dr. Charles Rodi (Monsanto ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit anti-human insulin was used as an internal control for RNA loading. Hybridization and autoradiography were performed as described previously (5). Cyclophillin was used as an internal control for RNA loading. Hybridization and autoradiography were performed as described previously (27).

**Northern Blot Analysis**—RINm5F cells were pulsed with 30 units/ml IFN-γ, washed with complete CMRL-1066, and cultured as indicated in 24-well microtiter plates in the absence of cytokine. The islets were isolated and dispersed into single cells by trypsin treatment as described above. The cells were isolated (400×g, 2 min), washed three times with 0.1 M phosphate-buffered saline (pH 7.4), and transferred to SuperFrostPlus microscope slides by cytopsin. The slides were fixed in 4% paraformaldehyde for 30 min at room temperature, and immunohistochemistry was performed as described (26). STAT1 αβ primary antibody (1:200) was obtained from Santa Cruz Biotechnology, Inc., guinea pig anti-human insulin was from Linco Research, Inc. (St. Louis, MO), CY3-conjugated donkey anti-guinea pig and fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary (1:200) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Immuno-fluorescence microscopy was used for the detection of STAT1 and insulin.

**Gel Shift Analysis**—Rat islets (500 islets/ml of complete CMRL-1066) were pulsed with 150 units/ml IFN-γ for 30 min, washed three times in complete CMRL-1066, and cultured for 0 or 24 h in complete CMRL-1066. The islets were then dispersed into single cells by trypsin treatment as described above. Nuclear proteins were isolated and gel shift analysis was performed as described (28) using an end-labeled STAT1 oligonucleotide probe containing the consensus sequence for STAT1 binding (5′-CATGGTATGATCTCTGAACTG-3′) Santa Cruz Biotechnology. The STAT1 probe was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega).

**Densitometry and Image Analysis**—Autoradiograms were scanned into NIH Image, Version 1.59, using a COHU high performance CCD camera (Brookfield, WI). Densities were determined using NIH Image, Version 1.59, software. Phosphoimaging analysis of mRNA accumulation was performed using a Molecular Dynamics PhosphorImager and Molecular Dynamics ImageQuant Software, Version 3.3.

**Statistical Analysis**—Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences between treatment groups were evaluated using a Scheffe's F-test post hoc analysis.

**RESULTS**

**IFN-γ Priming of Islets Involves Prolonged STAT1 Activation**

**Islet Isolation and Culture**—Islets were isolated from male Sprague Dawley rats or NOD mice by collagenase digestion as described previously (19). Following isolation, islets were cultured overnight in complete CMRL-1066 medium containing 2 mM l-glutamine, 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 95% air and 5% CO2 at 37 °C. Prior to each experiment, islets were washed three times in complete CMRL-1066, counted, and then cultured for an additional 3 h at 37 °C. Experiments were initiated by incubating islets for 30–60 min (pulse) with IFN-γ or IL-1, washing three times with complete CMRL-1066, and then culturing the islets for 0–7 days in complete CMRL-1066. The prime for IL-1 or IFN-γ was then added, and the islets were cultured for an additional 24–48 h.

**Islet Dispersion**—Isolated rat islets were pulsed for 1 h with 150 units/ml rat IFN-γ, washed three times with complete CMRL-1066, and then dispersed into individual cells by treatment with trypsin (1.0 mg/ml in Ca2⁺− and Mg2⁺-free Hank’s solution at 37 °C for 3 min as described previously (20). The dispersed islet cells were then counted and plated (200,000 cells/200 μl of complete CMRL-1066) into 16-well tissue culture plates and cultured for 24 h in the presence of or without IFN-γ, IL-1, and interleukin-1 receptor antagonist protein (IRAP) as indicated.

**Purification of β- and a-Cells by Fluorescence-activated Cell Sorting (FACS)**—Islets, isolated from 12 rats, were cultured overnight (~1, 200 islets/ml) in complete CMRL-1066 media under an atmosphere of 95% air and 5% CO2 at 37 °C. Islets were then dispersed into individual cells by trypsin treatment as described above. Dispersed islet cells were incubated for 1 h at 37 °C in complete CMRL-1066 prior to cell sorting. Islet cells were purified as described previously (12, 21–22) using a FACSTAR + flow cytometer (Becton Dickinson). The cells were illuminated at 488 nm, and emission was monitored at 515–535 nm. The sorting process yielded a 95% pure population of β-cells and an 80–85% pure population of α-cells.

**Islet Viability**—Islets (25/500 μl of complete CMRL-1066) were pulsed for 1 h with the indicated cytokines, washed three times with complete CMRL, and cultured for 96 h in 24-well microtiter plates with the indicated concentrations of IL-1, IFN-γ, and AG. Islet degeneration was determined in a double-blind manner by phase-contrast microscopy. Islet degeneration is characterized by the loss of islet integrity, disintegration, and partial dispersion of islets as described previously (5, 23–24).

**Western Blot Analysis**—After treatment, rat islets were prepared for Western analysis as described previously (5). Proteins were separated by SDS gel electrophoresis (25) and transferred to Nitrocellulose membranes (Amersham Pharmacia Biotech) under semi-dry transfer conditions. Incubation of blots with primary antisera (iNOS, 1:2000; STAT1 α- and β-32P]dCTP and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. Horseradish peroxidase-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit anti-serum specific for the C-terminal 27 of STAT1 and iNOS were gifts of Dr. Jon Omenn (University of Michigan, Ann Arbor, MI) and Dr. Charles Rodi (Monsanto ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit anti-human insulin was used as an internal control for RNA loading. Hybridization and autoradiography were performed as described previously (5). Cyclophillin was used as an internal control for RNA loading. Hybridization and autoradiography were performed as described previously (27).

**Gel Shift Analysis**—Rat islets (500 islets/ml of complete CMRL-1066) were pulsed with 150 units/ml IFN-γ for 30 min, washed three times in complete CMRL-1066, and cultured for 4 or 24 h in complete CMRL-1066. The islets were then dispersed into single cells by trypsin treatment as described above. Nuclear proteins were isolated and gel shift analysis was performed as described (28) using an end-labeled STAT1 oligonucleotide probe containing the consensus sequence for STAT1 binding (5′-CATGGTATGATCTCTGAACTG-3′) Santa Cruz Biotechnology. The STAT1 probe was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega).
removed by washing, and the islets were incubated for an additional 40 h with or without 0.1 or 1.0 unit/ml IL-1. Under IFN-γ pulse conditions, both 0.1 and 1.0 unit/ml IL-1 stimulate ~3-fold increase in nitrite production that is similar in magnitude to the levels produced in response to a 40-h continuous incubation with 1.0 unit/ml IL-1. Importantly, neither 0.1 unit/ml IL-1 nor 150 units/ml IFN-γ primes for IL-1-induced nitrite formation by rat islets (5), nor does a 1-h pulse of rat islets with 1.0 unit/ml IL-1 followed by a 6- or 12-h incubation with 150 units/ml IFN-γ (data not shown). Although the levels of iNOS mRNA which accumulate in response to 1.0 unit/ml IL-1 following a 6 h exposure are ~5-fold higher than the levels which accumulate under priming conditions, the levels of iNOS protein that accumulate under both conditions are similar. As shown in Fig. 1c, a 1-h pulse of rat islets with 150 units/ml IFN-γ followed by a 40-h incubation with 0.1 unit/ml IL-1 results in the expression of iNOS to levels that are similar in magnitude to the levels observed in response to a 40-h continuous incubation with 1.0 unit/ml IL-1. Importantly, neither 0.1 unit/ml IL-1 nor 150 units/ml IFN-γ primes for IL-1-induced iNOS expression, nor does a 1-h pulse with 1.0 unit/ml IL-1 followed by a 40-h incubation with IFN-γ (Fig. 1c). These findings indicate that IFN-γ primes for IL-1-induced nitrite formation and iNOS mRNA and protein expression by rat islets.

**IFN-γ Priming for IL-1-induced iNOS Expression and Nitrite Formation by NOD Mouse Islets**—The priming actions of IFN-γ on iNOS expression by NOD mouse islets were examined because: 1) IFN-γ appears to play a primary role in the development of autoimmune diabetes in NOD mice (5, 8, 13–14); and 2) NOD mouse islets require a combination of IL-1 (at 15 units/ml) and IFN-γ to stimulate iNOS expression (6, 8–9). In these experiments, isolated NOD mouse islets were pulsed for 1 h with 150 units/ml mouse IFN-γ, washed, and then incubated for an additional 40 h with 15 units/ml IL-1. As shown in Fig. 2, a pulse of NOD mouse islets with mouse IFN-γ followed by incubation with IL-1 results in a ~6-fold increase in nitrite formation and high levels of iNOS expression (inset). Under these priming conditions, the levels of iNOS expressed and nitrite produced are similar in magnitude to the effects of a 40-h continuous incubation of NOD mouse islets with IFN-γ + IL-1 (Fig. 2, compare lanes C and F, respectively). Importantly, IL-1 does not prime NOD mouse islets for IFN-γ-induced iNOS expression or nitrite formation (Fig. 2, lane E); and NOD mouse islets treated for 40 continuous h with IL-1 or IFN-γ alone do...
not produce nitrite (Fig. 2). These results indicate that IFN-γ primes for IL-1-induced iNOS expression and nitric oxide formation by NOD mouse islets.

**IFN-γ Primes for IL-1-induced Islet Degeneration in a Nitric Oxide-dependent Manner**—Incubation of rat islets for 96 h with 1.0 unit/ml IL-1, or 150 units/ml IFN-γ + 0.1 unit/ml IL-1, results in islet degeneration, an effect that is attenuated by the iNOS inhibitor, AG (5). Alone, 0.1 unit/ml IL-1 and 150 units/ml IFN-γ do not induce islet degeneration following a 96-h incubation; however, a 1-h pulse of rat islets with 150 units/ml IFN-γ followed by washing to remove the cytokine and further incubation for 96 h with 0.1 unit/ml IL-1 results in the degeneration of ∼70% of the islets (Fig. 3). AG prevents islet degeneration under these priming conditions, indicating that the destructive effects of this treatment are mediated by nitric oxide. A 1-h pulse with 150 units/ml IFN-γ, followed by incubation for 96 h in the absence of cytokine, or a 1-h pulse with 1.0 unit/ml IL-1, followed by incubation for 96 h in the presence of 150 units/ml IFN-γ, does not induce islet degeneration. These findings indicate that IFN-γ primes islets for IL-1-induced islet degeneration and that the destructive effects are mediated by the production of nitric oxide.

**IFN-γ Primes for IL-1-induced iNOS Expression by FACS-purified β-cells**—Because β-cells are the islet cellular source of iNOS in response to IL-1 (30), and the cell type selectively destroyed during the development of insulin-dependent diabetes mellitus, the effects of IFN-γ priming on iNOS expression by this cell type were examined. Treatment of β-cells purified by FACS with 1.0 unit/ml IL-1 or 150 units/ml IFN-γ + 0.1 unit/ml IL-1 stimulates high levels of iNOS expression following a 40-h incubation (Fig. 4, lower panel). Alone, neither 0.1 unit/ml IL-1 nor 150 units/ml IFN-γ stimulates iNOS expression by primary β-cells. However, β-cells pulsed for 1 h with 150 units/ml IFN-γ, followed by washing and a 40-h incubation with 0.1 unit/ml IL-1, express iNOS to levels similar in magnitude to the effects of a 40-h continuous incubation with 1.0 unit/ml IL-1 or 150 units/ml IFN-γ + 0.1 unit/ml IL-1. A 1-h pulse of primary β-cells with 150 units/ml IFN-γ followed by washing and a 40-h incubation with 1.0 unit/ml IL-1 results in iNOS expression that slightly exceeds the levels induced in response to 1.0 unit/ml IL-1 or 150 units/ml IFN-γ + 0.1 unit/ml IL-1; however, the levels of nitrite that accumulate under these conditions are similar (data not shown). In addition, similar results have been obtained in the insulinoma cell line, RINm5F. A 5-min pulse of RINm5F cells with 150 units/ml IFN-γ followed by washing and a further 24-h incubation with 0.1 unit/ml IL-1 results in iNOS expression and nitric oxide formation to levels similar in magnitude to those observed in response to a 24-h incubation with 1.0 unit/ml IL-1 alone (33.6 pmol/2000 cells under priming conditions, 40.1 pmol/2000 cells for IL-1 treated versus 1.3 pmol/2000 cells for control untreated RINm5F cells). The priming actions of IFN-γ appear to be specific for β-cells, as this cytokine fails to prime α-cells for IL-1-induced iNOS expression (Fig. 4, upper panel). These findings suggest that the priming actions of IFN-γ on iNOS expression by rat islets appear to be due to a direct interaction with β-cells and not α-cells.

**Persistence of IFN-γ Priming for IL-1-induced iNOS Expression**—To determine how long the priming actions of IFN-γ on iNOS expression and nitrite formation persist, rat islets were pulsed with IFN-γ, the cytokine was removed by washing, and the islets were incubated in the absence of cytokine for 8 h 7 days prior to IL-1 stimulation. As shown in Fig. 5a, a 1-h pulse of rat islets with 150 units/ml IFN-γ, followed by an 8–24-h incubation in the absence of cytokine and then a further 24-h incubation with 0.1 or 1.0 unit/ml IL-1, results in an ∼8–10-fold increase in nitrite production. The levels of nitrite produced under these conditions are similar in magnitude to the levels of nitrite produced in response to a 24-h continuous incubation with 150 units/ml IFN-γ + 0.1 unit/ml IL-1 or 1.0 unit/ml IL-1 alone. A 1-h pulse of rat islets with 150 units/ml IFN-γ, followed by a 48-h incubation in the absence of cytokine and a further 24-h incubation with 0.1 or 1.0 unit/ml IL-1 results in nitrite production to levels that are slightly higher than the levels of nitrite produced in response to a 24-h continuous incubation with 1.0 unit/ml IL-1 or 0.1 unit/ml IL-1 + 150 units/ml IFN-γ (11- and ∼14-fold above control versus ∼8-fold above control, respectively).

The priming effects of IFN-γ for IL-1-induced iNOS expression persists for up to 7 days. A 1-h pulse of rat islets with 150
units/ml IFN-γ, followed by a 7-day culture in the absence of cytokine and further incubation with 0.1 unit/ml IL-1 for 24 h, results in iNOS expression to levels that are only slightly lower than the levels expressed in response to a 24-h continuous incubation with 150 units/ml IFN-γ + 0.1 unit/ml IL-1 (Fig. 5b). Cytokine-induced iNOS expression by rat islets is not inhibited by the extended 7-day culture, as a 24-h incubation with 1.0 unit/ml IL-1 or IL-1 (0.1 or 1.0 unit/ml) + 150 units/ml IFN-γ stimulates high levels of iNOS expression following this extended culture period (Fig. 5b). Importantly, rat islets do not express iNOS following: 1) a 7-day culture in the absence of cytokine; 2) a 24-h incubation with 0.1 unit/ml IL-1 (following a 7-day culture, data not shown); or 3) a 1-h pulse with 1.0 unit/ml IL-1, 7-day culture in the absence of cytokine and a 24-h incubation with 150 units/ml IFN-γ (Fig. 5b). These results indicate that the priming actions of IFN-γ on IL-1-induced nitrite formation and iNOS expression persist for extended periods of up to 7 days.

De Novo Protein Synthesis Is Not Required for the Priming Actions of IFN-γ on iNOS Expression—Because the priming actions of IFN-γ on iNOS expression by rat islets persist for extended periods, the requirement for de novo protein synthesis on iNOS mRNA accumulation was examined. RINm5F cells were pulsed for 1 h with 150 units/ml IFN-γ in the presence or absence of 10 μM CHX, washed, and then incubated for an additional 6 h in the presence or absence of CHX and 0.1 unit/ml IL-1. At 10 μM, CHX inhibits islet total protein synthesis by −98% (31). As shown in Fig. 6, CHX does not inhibit the priming actions of IFN-γ on IL-1-induced iNOS mRNA accumulation as compared with similar treatment in the absence of CHX (lanes 2 and 5, respectively). Also, a 1-h pulse of RINm5F cells with 150 units/ml IFN-γ, followed by a 6-h incubation with 1.0 unit/ml IL-1, stimulates high levels of iNOS mRNA accumulation that are not diminished by co-incubation with CHX (lanes 6 and 3, respectively). These results are consistent with previous studies showing that CHX does not alter the levels of iNOS mRNA that accumulate in response to 1.0 unit/ml IL-1 (Ref. 37; Fig. 6, lanes 4 and 7) and the potentiating actions of IFN-γ on IL-1-induced iNOS expression (5). These findings indicate that de novo protein synthesis is not required for the priming actions of IFN-γ on iNOS mRNA accumulation by RINm5F cells.

Physical Dispersion of Islets by Trypsin Treatment Does Not Prevent the Priming Actions of IFN-γ on IL-1-induced Nitrite Formation by Rat Islets—Each islet contains 5–10 resident macrophages. Damage to this resident macrophage population during the physical dispersion of islets into single cells results in the endogenous release of IL-1 to levels sufficient to induce iNOS expression and nitrite production in the presence of exogenously added IFN-γ (5). To determine whether IFN-γ primes for nitrite formation stimulated by the endogenous release of IL-1, rat islets were treated with 150 units/ml IFN-γ for 1 h, dispersed into single cells by trypsin treatment, and then incubated for 24 h in the presence or absence of 0.1 μg/ml IRAP. IRAP competes with IL-1 for receptor binding and thereby prevents IL-1-induced signaling events (32). As shown in Fig. 7, islet cells primed for 1 h with 150 units/ml IFN-γ prior to dispersion produce nitrite to levels similar in magnitude to the levels observed in response to a 24-h continuous incubation with 1.0 unit/ml IL-1 or 150 units/ml IFN-γ. IRAP inhibits nitrite production by dispersed islet cells incubated with 1.0 unit/ml IL-1 (5) and 150 units/ml IFN-γ and by islet cells primed for 1 h with 150 units/ml IFN-γ prior to dispersion (Fig. 7). These results suggest that IFN-γ primes islet cells for nitrite formation induced by IL-1 released endogenously from resident macrophages. Dispersion of rat islets by trypsin treatment results in the destruction of 2–3% of islet cells (trypsin blue exclusion, data not shown) and may be a harsh enough enzymatic treatment to result in the dissociation of any IFN-γ that remains bound to the receptor after repeated washing. These results, therefore, also suggest that the priming actions of IFN-γ on nitric oxide production may be associated with intra-cellular signaling events as islet dispersion does not inhibit the priming actions of IFN-γ on IL-1-induced nitrite formation.

A 30-min Pulse with IFN-γ Stimulates Prolonged STAT1 Activation—The results from Fig. 5 indicate that IFN-γ primes for IL-1-induced iNOS expression for extended periods of up to 7 days. To examine whether the extended priming actions of IFN-γ are associated with prolonged activation of IFN-γ sig-

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**FIG. 5.** Persistence of IFN-γ priming for IL-1-induced nitrite formation and iNOS expression. Rat islets (150 islets/400 μl of complete CMRL-1066) were incubated for 1 h in the presence or absence of 150 units/ml IFN-γ as indicated. The islets were washed, incubated for 0–7 days in the absence of cytokine, followed by incubation for 24 h with IL-1 and/or IFN-γ as indicated. Following the final incubation, nitrite production (a) and iNOS expression (b) were determined by the Griess assay and Western blot analysis, respectively. Results for nitrite are the average ± S.E. of five independent experiments, and iNOS expression is from an individual experiment that is representative of three independent experiments. Statistical significance: p < 0.05 versus control (*) and p < 0.01 versus 24 h IL-1 and 24 h IFN-γ + 0.1 unit/ml IL-1 (**) as indicated.

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**FIG. 6.** Effects of cycloheximide on IFN-γ priming for IL-1-induced iNOS expression by RINm5F cells. RINm5F cells (5 × 10⁶ cells/3 ml of complete CMRL-1066) were pulsed for 1 h with IFN-γ in the presence or absence of 10 μM CHX as indicated. The cells were washed and then incubated for 6 h with the indicated concentrations of IL-1 and/or CHX. iNOS and cyclophilin (internal control) mRNA accumulation was determined by Northern blot analysis as described under “Experimental Procedures” and is representative of three independent experiments.

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**FIG. 7.** Persistence of IFN-γ priming for IL-1-induced nitrite formation. Rat islets were treated with 150 units/ml IFN-γ for 1 h, dispersed into single cells by trypsin treatment, and then incubated for 24 h in the presence or absence of 0.1 μg/ml IRAP. IRAP competes with IL-1 for receptor binding and thereby prevents IL-1-induced signaling events (32). As shown in Fig. 7, islet cells primed for 1 h with 150 units/ml IFN-γ prior to dispersion produce nitrite to levels similar in magnitude to the levels observed in response to a 24-h continuous incubation with 1.0 unit/ml IL-1 or 150 units/ml IFN-γ. IRAP inhibits nitrite production by dispersed islet cells incubated with 1.0 unit/ml IL-1 (5) and 150 units/ml IFN-γ and by islet cells primed for 1 h with 150 units/ml IFN-γ prior to dispersion (Fig. 7). These results suggest that IFN-γ primes islet cells for nitrite formation induced by IL-1 released endogenously from resident macrophages. Dispersion of rat islets by trypsin treatment results in the destruction of 2–3% of islet cells (trypsin blue exclusion, data not shown) and may be a harsh enough enzymatic treatment to result in the dissociation of any IFN-γ that remains bound to the receptor after repeated washing. These results, therefore, also suggest that the priming actions of IFN-γ on nitric oxide production may be associated with intra-cellular signaling events as islet dispersion does not inhibit the priming actions of IFN-γ on IL-1-induced nitrite formation.
naling components, the effects of IFN-γ on STAT1 phosphorylation and nuclear localization were examined. As shown in Fig. 8a, STAT1 remains phosphorylated following a 30-min pulse of rat islets with 150 units/ml IFN-γ, washing, a 7-day incubation in the absence of cytokine, and a 24-h incubation with 0.1 or 1.0 unit/ml IL-1 (Fig. 8a, upper panel). Under these conditions, an ~4–5-fold increase in the expression of STAT1 α/β is also observed (Fig. 8a, lower panel). Following a 7-day culture in the absence of cytokine, a 24-h incubation of rat islets with 150 units/ml IFN-γ + IL-1 (0.1 or 1.0 unit/ml) results in STAT1 phosphorylation to levels that are ~2-fold higher than the levels induced under IFN-γ priming conditions; however, the levels of STAT1 expression induced in response to these conditions are similar (~4–5-fold above control, Fig. 8a, lower panel). In addition, a 30-min pulse of rat islets with 150 units/ml IFN-γ, followed by incubation for 7 days in the absence of cytokine stimulates STAT1 phosphorylation and an ~4–5-fold increase in STAT1 α/β expression (Fig. 8b). Also, a 7-day incubation in the absence of cytokine followed by a 24-h incubation of rat islets with 0.1 or 1.0 unit/ml IL-1 does not result in the phosphorylation or increased expression of STAT1 α/β (Fig. 8, a and b). These results suggest that a short exposure of rat islets to IFN-γ results in the increased expression of STAT1 α/β and increased STAT1 phosphorylation, effects that are sustained for up to 1 week. These results also indicate that IL-1 does not stimulate STAT1 expression or phosphorylation.

Following IFN-γ treatment, phosphorylated STAT1 homodimers translocate to the nucleus and bind to consensus GAS sequences to activate gene transcription. To determine whether STAT1 remains nuclear localized for extended periods following IFN-γ priming, immunohistochemical analysis of STAT1 cellular localization was performed. In these experiments, rat islets were incubated for 30 min with IFN-γ, washed to remove the cytokine, and then incubated for 0, 48, or 7 days in the absence of further treatment. The islets were dispersed into individual cells, fixed on slides and then stained for STAT1; however, sustained nuclear localization was not observed 48 h or 7 days after the removal of IFN-γ (data not shown). These results indicate that a short pulse of dispersed islet cells with IFN-γ results in the nuclear localization of STAT1 for extended periods of up to 7 days in insulin-containing cells.

To confirm that STAT1 remains activated and able to bind DNA, gel shift analysis was performed. As shown in Fig. 8c, a 30-min incubation of rat islets with 150 units/ml IFN-γ results in the nuclear translocation and activation of STAT1 as evi-
Effects of IFN-γ Priming on STAT1 Nuclear Localization

![Control](image1)

![IFN-γ 30 min](image2)

![IFN-γ 30 min, Wash 48 h Culture](image3)

![IFN-γ 30 min, Wash 7 Day Culture](image4)

**Fig. 9. Immunohistochemical co-localization of STAT1 and insulin.** Rat islets (50/400 µl of complete CMRL) were pulsed for 30 min with or without 150 units/ml IFN-γ followed by washing and incubation for 0 h, 48 h, or 7 days in the absence of cytokine. Following treatment, the islets were dispersed by trypsin treatment and centrifuged onto slides. STAT1 cellular localization was determined using rabbit antihuman STAT1 α/β and fluorescent isothiocyanate-conjugated donkey anti-rabbit secondary. β-Cells were detected using guinea pig antihuman insulin and CY3-conjugated donkey anti-guinea pig secondary. Results are representative of three independent experiments.

...denced by reduced mobility of the probe containing the consens... (16) have shown that IFN-γ primes islet cell monolayers for IL-1-induced cytotoxicity. One purpose of this study was to determine whether the priming effects of IFN-γ for IL-1-induced islet cell cytotoxicity are mediated by iNOS expression and increased nitric oxide formation. We show that a short pulse with IFN-γ primes rat islets for iNOS expression and nitrite formation induced by concentrations of IL-1 that alone do not stimulate iNOS expression. In addition, we show that IFN-γ also primes rat islets for IL-1-induced islet degeneration, an effect that is prevented by the iNOS inhibitor, AG. β-cells appear to be one islet cellular source of iNOS as IFN-γ primes RINm5F insulinoma cells and primary β-cells, but not α-cells, for IL-1-induced iNOS expression. Taken together, these results suggest that the destructive priming effects of IFN-γ for IL-1-induced islet degeneration are mediated by β-cell expression of iNOS and increased production of nitric oxide.

Analysis of the time-dependent effects of IFN-γ priming for IL-1-induced nitrite formation and iNOS expression indicates that the priming actions of IFN-γ persist for extended periods (up to 7 days for iNOS expression). The priming actions of IFN-γ do not appear to be associated with de novo protein synthesis as the protein synthesis inhibitor, CHX, does not inhibit iNOS mRNA accumulation by RINm5F cells under priming conditions. Dispersion of islets by trypsin treatment damages the resident islet macrophage population, resulting in the release of endogenous IL-1. In the presence of exogenous IFN-γ, dispersed islet-cells produce nitrite. Dispersion of islets by trypsin treatment would presumably be a sufficiently harsh enzymatic treatment to dissociate any IFN-γ that may remain bound to the membrane after repeated washing; however, islet dispersion does not inhibit the priming actions of IFN-γ on nitrite formation by dispersed islet cells. These results indicate that the persistent priming actions of IFN-γ for IL-1-induced iNOS expression and nitrite formation occur independently of de novo protein synthesis and may be associated with intracellular signaling events.

One mechanism by which IFN-γ may prime rat islets for IL-1-induced iNOS expression is via sustained activation of JAK/STAT signaling proteins. Rat islets have been shown to express JAK2 (35), and the insulinoma cell line, INS-1 expresses both JAK1 and JAK2 (26). Tyrosine phosphorylation of STAT1 by the receptor-associated JAKs (JAK1 and JAK2) in response to IFN-γ results in STAT1 homodimerization and nuclear translocation. Activated nuclear STAT1 homodimers stimulate new gene transcription by binding to GAS sites (17). Subsequent inactivation of nuclear STAT1 appears to involve dephosphorylation by an as yet unidentified nuclear protein tyrosine phosphatase (36). Evidence presented in Fig. 8 indicates that a short pulse of rat islets with IFN-γ results in prolonged phosphorylation and activation of STAT1. Although the mouse iNOS 5'-untranslated region contains three GAS sites, IFN-γ alone is not sufficient to induce iNOS expression or nitric oxide formation by islets; a second signal (such as IL-1-induced NF-κB activation) is required (5, 37). Because STAT1 nuclear translocation in most cell types appears to be maximal following a 30-min exposure to IFN-γ, and is ablated following an additional ~4-h incubation (17, 36), it is unclear why STAT1 remains phosphorylated and active for such extended time periods in rat islets. A pulse of IFN-γ also appears to up-regulate the expression of STAT1; however, because STAT1 is not autophosphorylated, and the priming actions of IFN-γ on iNOS mRNA accumulation appear to be independent of de novo protein synthesis, increased expression of STAT1 alone does not account for the prolonged activation of this transcriptional
regulator. One possible explanation for this finding may be that islets, more specifically β-cells, lack the protein tyrosine phosphatase required for dephosphorylation and inactivation of STAT1. Alternatively, constitutive phosphorylation of the JAKs would result in sustained activation of newly synthesized STAT1, thus resulting in continued phosphorylation and prolonged activation of STAT1. The latter explanation seems plausible since a 30-min pulse of rat islets with IFN-γ results in an ~4–5-fold increase in STAT1 expression and an ~2-fold increase in phosphorylated STAT1 (Fig. 8, a and b). Constitutive nuclear localization of JAK2 has been observed in INS-1 cells (26), rat islets (35), and primary rat β-cells; however, the role of nuclear JAK2 in the activation or deactivation of STAT1 remains to be elucidated. These potential signaling mechanisms are currently under investigation in our laboratory.

Similar to human islets, NOD mouse islets require a combination of IFN-γ and IL-1 to induce iNOS expression and nitrite production (29). In this study, we show that IFN-γ primes NOD mouse islets for IL-1-induced iNOS expression and nitrite formation. These findings suggest that continuous exposure of islets to both IFN-γ and IL-1 during the natural progression of autoimmune diabetes may not be required for iNOS expression and nitrite formation by NOD mouse islets. Release of IFN-γ by perisinusitis T-cells may prime islets for subsequent IL-1-induced iNOS expression and nitric oxide production by β-cells leading to an inhibition of β-cell function and eventual islet cell death. One potential cellular source of IL-1 in islets may be the resident islet macrophage. We have shown that IFN-γ is able to prime dispersed islet cells for nitric oxide formation induced by endogenous IL-1, released by islet macrophages damaged during the dispersion process (5). Environmental factors, such as a bacterial or viral infection, which activate and/or damage resident islet macrophages, may also stimulate intra-islet IL-1 release. We have recently shown that, in the presence of IFN-γ, double-stranded RNA, an active component of a viral infection which stimulates antiviral responses in infected cells, stimulates IL-1 release from resident macrophages (28). In addition, treatment of rat and human islets with TNF + LPS (TNF + LPS + IFN-γ for human islets) stimulates the intra-islet release of IL-1 by resident macrophages which then accumulate to levels sufficient to induce iNOS expression and nitrite formation by β-cells, resulting in a potent inhibition of insulin secretion (30, 38–39). In summary, these findings provide novel evidence that IFN-γ primes rat and NOD mouse islets and primary β-cells for IL-1-induced iNOS expression and nitric oxide production by a mechanism that is associated with the prolonged phosphorylation and activation of the IFN-γ-activated JAK/STAT signaling cascade.

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REFERENCES

1. Southern, C., Schulster, D., and Green, I. C. (1990) FEBS Lett. 276, 42–44
2. Welsh, N., Eizirik, D. L., Bendtzen, K., and Sandler, S. (1991) Endocrinology 129, 3167–3173
3. Corbett, J. A., Wang, J. L., Hughes, J. H., Wolf, B. A., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992) Biochem. J. 287, 229–235
4. Corbett, J. A., and McDaniel, M. L. (1994) Biochem. J. 299, 719–724
5. Heitmeier, M. R., Scarrin, A. L., and Corbett, J. A. (1997) J. Biol. Chem. 272, 13697–13704
6. Sorenson, R. L., and Stout, L. E. (1995) Endocrinology 131, 812–817
7. Corbett, J. A., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992) J. Clin. Invest. 90, 2384–2391
8. Corbett, J. A., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992) J. Clin. Invest. 90, 2384–2391
9. Sarvetnick, N., Liggitt, D., Pits, S. L., Hansen, S. E., and Stewart, T. A. (1988) J. Clin. Invest. 81, 523–531
10. Mandrup-Poulsen, R., Bendtzen, K., Nielsen, H., Bendixen, G., and Nerup, J. (1995) Allergy 40, 424–429
11. Eizirik, D. L., Fjodstrom, M., Karlsen, A., and Welsh, N. (1996) Diabetesologia 39, 875–880
12. Corbett, J. A., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992) J. Clin. Invest. 90, 2384–2391
13. Sarvetnick, N., Liggitt, D., Pits, S. L., Hansen, S. E., and Stewart, T. A. (1988) Cell 52, 773–782
14. Campbell, I. L., Kay, T. W. H., Oxhrow, L., and Harrison, L. (1991) J. Clin. Invest. 87, 739–742
15. Debray-Sachs, M., Carnaud, C., Boitard, C., Cohen, H., Gresser, I., Bedossa, P., and Bach, J.-F. (1991) J. Autoimmun. 4, 237–248
16. Baquerizo, H., and Rabinovitch, A. (1990) J. Autoimmun. 3, Suppl. 1, 123–130
17. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
18. Xie, Q. W., Whisnant, R., and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784
19. McDaniel ML., Colca, J. R., Ketagal, N., and Lacy, P. E. (1983) Methods Enzymol. 98, 182–200
20. Ono, J., Takaki, R., and Fukuma, M. (1977) Endocrinol. Jpn. 24, 265–270
21. Corbett, J. A., Wang, J. L., Sweetland, M. A., Lancaster, J. R., Jr., and McDaniel, M. L. (1992) Am. J. Physiol. 267, C48–C54
22. Pipelers, D. G., Int Veld, P. A., Van De Winkel, M., Maes, E., Schult, F. C., and Gepts, W. (1985) Endocrinology 117, 806–816
23. Corbett, J. A., Kwon, G., Hill, J. R., and McDaniel, M. L. (1995) in The Diabetes Annual 9 (Marshall, S. M., Home, P. D., and Rizza, R. A., eds) pp. 265–294, Elsevier Science Publishers B.V., Amsterdam
24. Lacy, P. E., and Finke, E. H. (1991) Am. J. Pathol. 136, 1183–1190
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Stout, L. R., Svensson, A. M., and Sorenson, R. L. (1997) Endocrinology 138, 1592–1603
27. Purdy, R. R., Rogers, H. W., Gordon, J. R., Martin, C. A., Jayaraman, S., Wilson, S. D., Dvorak, A. M., Galli, S. J., and Dorf, M. E. (1989) J. Exp. Med. 170, 245–257
28. Heitmeier, M. R., Scarrin, A. L., and Corbett, J. A. (1998) J. Biol. Chem. 273, 15301–15307
29. Green, L. C., Wagner, D. A., Glogowski, J., Skipper P. L., Wishnock, J. S., and Tannenbaum, S. R. (1982) Anal. Biochem. 126, 131–138
30. Corbett, J. A., and McDaniel, M. L. (1995) J. Exp. Med. 181, 559–568
31. Hughes, J. H., Colca, J. R., Easom, R. A., Turk, J., and McDaniel, M. L. (1990) J. Clin. Invest. 86, 856–863
32. Arend, W. P. (1991) J. Clin. Invest. 88, 1445–1451
33. Wang, B., Andre, I., Gonzalez, A., Katz, J. D., Aguet, M., Benoist, C., and Mathis, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13844–13849
34. Hultgren, B., Huang, X., Dybdal, N., and Stewart, T. A. (1996) Diabetes 45, 812–817
35. Sorenson, R. L., and Stout, L. E. (1995) Endocrinology 136, 4092–4098
36. Haspel, R. L., Salditt-Georgieff, M., and Darnell, D. E., Jr. (1996) EMBO J. 15, 6262–6268
37. Kwon, G., Corbett, J. A., Rodi, C. P., Sullivan, P., and McDaniel, M. L. (1995) Endocrinology 136, 4780–4785
38. Ar纳斯, M. A., Scarrin, A. L., Heitmeier, M. R., Kelly, C. B., and Corbett, J. A. (1998) J. Immun. 160, 2684–2691
39. Ar纳斯, M. A., Heitmeier, M. R., Scarrin, A. L., and Corbett, J. A. (1998) J. Clin. Invest. 102, 514–526

2 J. A. Corbett and M. R. Heitmeier, unpublished observation.