Double-Stranded RNA–Dependent Protein Kinase Is Not Required for Double-Stranded RNA–Induced Nitric Oxide Synthase Expression or Nuclear Factor-κB Activation by Islets

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Environmental factors, such as viral infection, have been implicated in the destruction of β-cells during the development of autoimmune diabetes. Double-stranded RNA (dsRNA), produced during viral replication, is an active component of a viral infection that stimulates antiviral responses in infected cells. Previous studies have shown that treatment of rat islets with dsRNA in combination with γ-interferon (IFN-γ) results in a nitric oxide–dependent inhibition of glucose-stimulated insulin secretion. This study examines the role of nuclear factor-κB (NF-κB) and the dsRNA-dependent protein kinase (PKR) in dsRNA + IFN-γ–induced nitric oxide synthase (iNOS) expression and nitric oxide production by rat, mouse, and human islets. Treatment of rat and human islets with dsRNA in the form of polyinosinic-polycytidylic acid (poly IC) and IFN-γ resulted in iNOS expression and nitric oxide production. Inhibitors of NF-κB activation—the proteasome inhibitor MG-132 and the antioxidant pyrrolidinedithiocarbamate (PDTC)—prevented poly IC + IFN-γ–induced iNOS expression and nitric oxide production. Incubation of rat islets for 3 h or human islets for 2 h with poly IC alone or poly IC + IFN-γ resulted in NF-κB nuclear translocation and degradation of the NF-κB inhibitor protein, IκB, events that are prevented by MG-132. PKR has been shown to participate in dsRNA-induced NF-κB activation in a number of cell types, including mouse embryonic fibroblasts. However, poly IC stimulated NF-κB nuclear translocation and IκB degradation to similar levels in islets isolated from mice devoid of PKR ($PKR^{-/-}$) and wild-type mice ($PKR^{+/+}$). Furthermore, the genetic absence of PKR did not affect dsRNA + IFN-γ–induced iNOS expression, nitric oxide production, or the inhibitory actions of these agents on glucose-stimulated insulin secretion. These results suggest that *1*) NF-κB activation is required for dsRNA + IFN-γ–induced iNOS expression, *2*) PKR is not required for either dsRNA-induced NF-κB activation or dsRNA + IFN-γ–induced iNOS expression by islets, and *3*) PKR is not required for dsRNA + IFN-γ–induced inhibition of glucose-stimulated insulin secretion by islets. *Diabetes* 50:283–290, 2001

Autoimmune diabetes is characterized by a local inflammatory reaction in and around the pancreatic islets, followed by selective destruction of insulin-producing β-cells (1). Viral infection has been implicated as one environmental factor that may trigger the initial autoimmune reaction that targets and destroys β-cells in genetically susceptible individuals (2–5). Viruses have been isolated from the pancreata of acutely diabetic deceased patients, and viral-specific IgM responses have been identified in newly diagnosed diabetic patients (2,5). Autoimmune diabetes can also be induced in genetically susceptible strains of rats and mice by infection with Kilham rat virus (6) and encephalomyocarditis virus (7), respectively. In these animal models, viral-induced diabetes is associated with increased cytokine expression and nitric oxide production (6,7). Although this evidence supports a role for viral infection in the development of diabetes, the mechanisms by which viruses initiate β-cell damage have been difficult to determine because multiple viruses from both RNA and DNA viral families have been implicated in this disease. One common feature of a viral infection is the formation of double-stranded RNA (dsRNA), which accumulates during replication. dsRNA is an active component of a viral infection that stimulates host antiviral responses (8). The synthetic dsRNA molecule polyinosinic-polycytidylic acid (poly IC) also activates the antiviral response (8), and has been shown to stimulate the development of diabetes in diabetes-resistant BB rats and accelerate disease development in diabetes-prone BB rats (9,10).

One cellular target activated in response to dsRNA is the transcriptional regulator nuclear factor-κB (NF-κB) (11). dsRNA has been shown to stimulate NF-κB nuclear translocation in endothelial cells (12) and murine macrophages (13). NF-κB is a heterodimer comprised of p50 and p65 (RelA) subunits, and is found sequestered in the cytoplasm of unstimulated cells as an inactive complex with the NF-κB inhibitory protein (IκB). Upon stimulation, IκB is phosphorylated and

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AG, aminoguanidine; dsRNA, double-stranded RNA; HRP, horseradish peroxidase; IFN, interferon; IκB, NF-κB inhibitor protein; IKK, IκB kinase; IL-1, interleukin-1; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; KRBB, Krebs-Ringer bicarbonate buffer; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NOS-2, nitric oxide synthase-2; PDTC, pyrrolidinedithiocarbamate; PKR, dsRNA-dependent protein kinase; poly IC, polyinosinic-polycytidylic acid; RT-PCR, reverse transcriptase–polymerase chain reaction.

degraded in a ubiquitin-dependent manner. Free of IκB, the NF-κB heterodimer translocates to the nucleus and activates mRNA transcription (14). NF-κB is activated by several cytokines, including interleukin-1 (IL-1) and tumor necrosis factor-α (15), and appears to play a primary role in the regulation of inducible nitric oxide synthase (iNOS) gene expression (16–18). NF-κB activation is required for IL-1β and IL-1β + IFN-γ–induced iNOS expression by rat and human islets, respectively (19,20).

The dsRNA-dependent protein kinase (PKR) is an important regulator of the antiviral response. NF-κB activation in response to dsRNA appears to be dependent on functional PKR. dsRNA-induced NF-κB activation is attenuated in mouse embryonic fibroblasts isolated from PKR-deficient mice (21,22). PKR has been shown to phosphorylate IκB in vitro (11), although recent evidence suggests that PKR may mediate dsRNA-induced NF-κB nuclear localization by activating the IκB kinase (IKK) (23,24). PKR is a 65–68 kDa serine/threonine kinase whose expression is induced by interferons (IFNs) (25). Binding to dsRNA, an event that leads to dimerization and autophosphorylation, activates PKR (26). Once activated, PKR participates in the antiviral response by inhibiting translation through phosphorylation of eukaryotic initiation factor-2α (25). However, PKR is also thought to participate in other cellular and antiviral responses, such as transcription factor activation, cell cycle control, and apoptosis (21,27,28).

In this study, we examined the roles of NF-κB and PKR in dsRNA + IFN-γ–induced iNOS expression and nitric oxide production by rat, mouse, and human islets. We show that dsRNA (in the form of poly IC) stimulates the activation of NF-κB in rat and human islets. Pyrrolidinedithiocarbamate (PDTC) and MG-132, inhibitors of NF-κB, prevent dsRNA and dsRNA + IFN-γ–induced NF-κB nuclear localization and IκB degradation as well as dsRNA + IFN-γ–induced iNOS expression and nitric oxide production by islets. Furthermore, the genetic absence of PKR does not adversely affect dsRNA-induced NF-κB activation or dsRNA + IFN-γ–induced iNOS expression by mouse islets. These findings suggest that *1*) NF-κB activation is required for dsRNA + IFN-γ–induced iNOS expression by islets, and *2*) dsRNA-induced NF-κB activation and dsRNA + IFN-γ–induced iNOS expression by islets occur by PKR-independent mechanisms.

RESEARCH DESIGN AND METHODS

Materials and animals. CMRL-1066 tissue culture medium, L-glutamine, penicillin, streptomycin, and rat recombinant IFN-γ were obtained from Gibco BRL-Life Technologies (Grand Island, NY). Fetal calf serum was obtained from Hyclone (Logan, UT); human recombinant IL-1β from Cistron Biotechnology (Pine Brook, NJ); human IFN-γ from Boehringer Mannheim (Indianapolis, IN); and mouse IFN-γ from R & D Systems (Minneapolis, MN). Poly IC, PDTC, MG-132, and collagenase type XI were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP and enhanced chemiluminescence reagents were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), and rabbit antiserum specific for the C-terminal 27 amino acids of mouse macrophage iNOS was a generous gift from Dr. Thomas Misko (G.D. Searle, St. Louis, MO). NF-κB consensus oligonucleotide, rabbit anti-human IκBα, and rabbit anti-human nitric oxide synthase-2 (NOS-2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Male SD rats (250–300 g) and male C57BL/6J mice (7–8 wks) were purchased from Harlan (Indianapolis, IN). PKR$^{-/-}$ mice in a C57BL/6 × 129 background (22) were a generous gift from Dr. Randal J. Kaufman (University of Michigan Medical Center, Ann Arbor, MI). C57BL/6 × 129 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Human islets were obtained from the Juvenile Diabetes Federation International Islet Isolation Centers at Washington University School of Medicine (St. Louis, MO), the Diabetes Research Institute at the University of Miami (Miami, FL), and the Diabetes Institute for Immunology and Transplantation (University of Minnesota). All other reagents were from commercially available sources.

Islet isolation. Islets were isolated from male SD rats, male or female PKR$^{-/-}$ mice, male C57BL/6J or male C57BL/6 × 129 (PKR$^{+/+}$) mice by collagenase digestion as previously described (29). After isolation, islets were cultured overnight in complete CMRL-1066 (containing 2 mmol/l L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin) under an atmosphere of 95% air and 5% CO_2 at 37°C. Human islets were incubated for 48 h at 37°C in complete CMRL-1066 before the initiation of experiments. Before each experiment, islets were cleaned by hand picking, washed three times in complete CMRL-1066, counted, and cultured for an additional 2 h at 37°C. No differences in islet function or cytokine signaling were observed between male and female PKR$^{-/-}$ mice. Data presented in Figs. 5 and 6 were obtained using islets isolated from C57BL/6J mice. To control for potential strain differences, the effects of dsRNA + IFN-γ on iNOS expression, nitric oxide production, and NF-κB activation were compared among islets isolated from C57BL/6 × 129 and C57BL/6J mice; no differences were observed (data not shown).

Nitrite determination. Nitrite formation was determined by mixing 50 μl of culture medium with 50 μl of Griess reagent (30). Absorbance was measured at 540 nm, and nitrite concentrations were calculated from a sodium nitrite standard curve.

Western blot analysis. Western blot analysis of iNOS and IκB was performed as previously described (31), using the following antibody dilutions: rabbit anti-mouse iNOS (1:2,000 dilution), rabbit anti-human IκBα (1:1,500 dilution), rabbit anti-human NOS-2 (1:1,000 dilution), and HRP-conjugated donkey anti-rabbit IgG (1:7,000 dilution). Antigen was detected by enhanced chemiluminescence according to manufacturer's specifications.

Insulin secretion. Glucose-stimulated insulin secretion was performed as previously described (29). In brief, islets isolated from PKR$^{-/-}$ and PKR$^{+/+}$ mice (180/ml of complete CMRL-1066) were cultured for 40 h with the indicated concentrations of poly IC, IFN-γ, IL-1, and aminoguanidine (AG). The islets were washed three times in Krebs-Ringer bicarbonate buffer (KRBB; 25 mmol/l HEPES, 115 mmol/l NaCl, 24 mmol/l $NaHCO_3$, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, and 0.1% bovine serum albumin, pH 7.4) containing 3 mmol/l D-glucose, and then preincubated for 30 min in 200 μl KRBB containing 3 mmol/l glucose. The preincubation buffer was removed and the islets were incubated for 30 min in 200 μl of KRBB containing either 3 or 20 mmol/l glucose. The incubation buffer was removed and insulin content was determined by radioimmunoassay (32).

Nuclear extraction and gel shift analysis. After treatment with poly IC and cytokines, islets were dispersed into single cells by trypsin treatment (33) and nuclear proteins were extracted as previously described (13). The probe consisted of a double-stranded oligonucleotide containing the consensus binding sequence for NF-κB (5′-AGTTGAGGGGAC TTTCCCAGGC-3′) end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega; Madison, WI). Binding reactions consisted of 10 μg of nuclear protein extract, 0.5 ng of DNA probe, and 1 μg/ml of poly (dI-dC) in a buffer containing 10 mmol/l HEPES (pH 7.8), 50 mmol/l NaCl, 1 mmol/l EDTA, 10% glycerol, and 1 mmol/l dithiothreitol. Reactions were incubated at 30°C for 30 min, and the NF-κB/oligonucleotide complex was separated on 4.5% Tris-glycine nondenaturing polyacrylamide gels in a 2× Tris-glycine (50 mmol/l Tris-HCl [pH 8.3], 0.38 mol/l glycine, and 2 mmol/l EDTA) buffer system (34). Gels were dried on Whatman paper and subjected to autoradiography.

Polymerase chain reaction. Total RNA was isolated from islets using the RNeasy RNA isolation kit (QIAGEN, Santa Clarita, CA) and was used to prepare first-strand cDNA by reverse transcription using the SuperScript Preamplification System (Life Technologies, Grand Island, NY) as described (35). iNOS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PKR primers were designed according to these published sequences: *1*) iNOS forward primer 5′-CCAACCGGAGAAGGGGACGAACT-3′, reverse primer 5′-GGAGGG TGGTGCGGCTGGAC-3′ (PCR product size = 297 bp); *2*) GAPDH forward primer 5′-GCTGGGGCTCACCTGAAGGG-3′, reverse primer 5′-GGATGACCT TGCCACGCC-3′ (PCR product size = 343 bp); and *3*) murine PKR forward primer 5′-GCCAGATGCACGGAGTAGCC-3′, reverse primer 5′-GAAAACTTG GCCAAATCCACC-3′ (PCR product size = 722 bp). A standard 25-μl PCR reaction of 30 cycles was performed (35), and PCR products were separated on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide and visualized by ultraviolet light exposure.

Statistical analysis. Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences between groups ($P < 0.05$) were determined by Scheffe's F test post-hoc analysis.

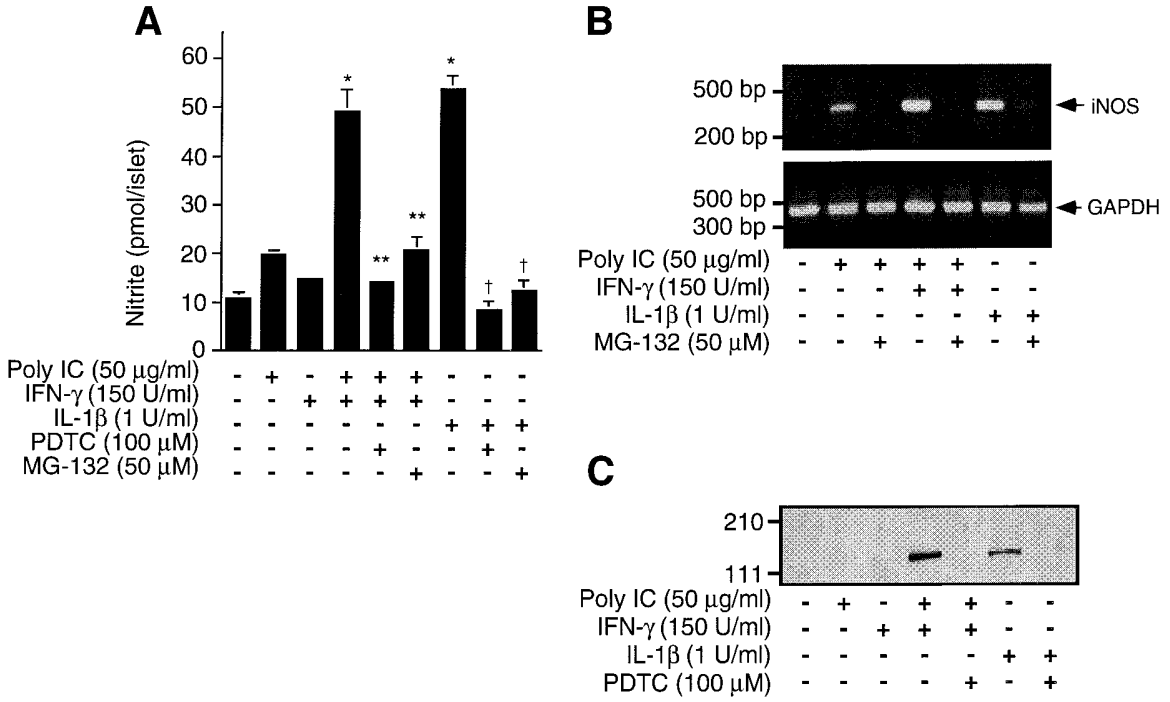

FIG. 1. MG-132 and PDTC inhibit poly IC + IFN-γ–induced nitrite formation and iNOS expression by rat islets. Rat islets (120/400 µl of complete CMRL-1066) were treated with the indicated concentrations of poly IC, IFN-γ, or IL-1β for 40 h at 37°C; 100 µmol/l PDTC was added to islets 2 h before the addition of cytokines. After treatment, medium was removed for nitrite determination (*A*). iNOS expression by islets was examined by Western blot analysis (*C*), as described in RESEARCH DESIGN AND METHODS. *B*: Rat islets (100/400 µl of complete CMRL-1066) were treated with the indicated concentrations of poly IC, IFN-γ, or IL-1β for 18 h at 37°C; 50 µmol/l MG-132 was added to islets 90 min before the addition of cytokines. cDNA was generated from total RNA isolated from islets, and iNOS and GAPDH mRNA expression was examined by RT-PCR, as described in RESEARCH DESIGN AND METHODS. Results for nitrite are averages ± SE of three independent experiments, and iNOS mRNA accumulation and protein expression are representative of three independent experiments. *$P < 0.05$ vs. untreated control; **$P < 0.05$ vs. poly IC + IFN-γ treatment; †$P < 0.05$ vs. IL-1β treatment.

RESULTS

Effects of PDTC and MG-132 on poly IC + IFN-γ–induced nitrite formation and iNOS expression by rat islets. Recently we showed that dsRNA + IFN-γ stimulates iNOS expression and nitrite production by rat islets, and that nitric oxide mediates the inhibitory actions of these agents on glucose-stimulated insulin secretion (36). The role of NF-κB in dsRNA + IFN-γ–stimulated iNOS expression and nitric oxide production was evaluated using PDTC and MG-132, which are inhibitors of NF-κB activation (37,38). As shown in Fig. 1*A*, the combination of 50 µg/ml poly IC and 150 U/ml IFN-γ stimulated an approximately fivefold increase in nitrite formation by rat islets. Alone, neither poly IC nor IFN-γ stimulated nitrite production by rat islets. Pretreatment of rat islets for 2 h with 100 µmol/l PDTC or for 90 min with 50 µmol/l MG-132 inhibited dsRNA + IFN-γ–induced nitrite formation by ~90%. At these concentrations, PDTC (19) and MG-132 (39; data not shown) have been shown to maximally inhibit IL-1–induced NF-κB activation by rat islets without adversely affecting islet total protein synthesis or oxidative metabolism (19,39). As a control, the inhibitory effects of PDTC and MG-132 on IL-1–induced nitrite production are also shown. In addition, neither PDTC nor MG-132 alone stimulated nitrite production by rat islets, and DMSO, the vehicle control for both inhibitors, neither stimulated nor inhibited nitrite production in response to dsRNA + IFN-γ (data not shown).

We previously reported that poly IC + IFN-γ stimulates maximal iNOS mRNA accumulation in rat islets after an 18-h incubation (36). As shown in Fig. 1*B*, pretreatment of rat islets for 90 min with MG-132 (50 µmol/l) prevented poly IC– and poly IC + IFN-γ–induced iNOS mRNA accumulation, as determined by reverse transcriptase–polymerase chain reaction (RT-PCR). Similar to iNOS mRNA accumulation, MG-132 (50 µmol/l; data not shown) and PDTC (100 µmol/l) also prevented poly IC + IFN-γ–induced iNOS expression at the protein level, as determined by Western blot analysis (Fig. 1*C*). These findings indicate that NF-κB activation may be required for dsRNA + IFN-γ–induced iNOS expression by rat islets. It is also clear from these experiments that poly IC alone stimulates iNOS mRNA accumulation, but does not induce iNOS protein expression or nitrite formation by rat islets. Previously, we were unable to detect poly IC-induced iNOS mRNA accumulation in rat islets by Northern blot analysis (36). The detection of iNOS mRNA accumulation in response to dsRNA in these studies appears to reflect the increased sensitivity of PCR over Northern blot analysis in the evaluation of mRNA expression.

Effects of poly IC on NF-κB activation in rat islets. To determine whether dsRNA stimulates NF-κB activation in rat islets, NF-κB nuclear localization was examined by gel shift analysis and IκB degradation was determined by Western blot analysis. Treatment of rat islets for 3 h with 50 µg/ml poly IC alone or in combination with 150 U/ml IFN-γ resulted in the maximal nuclear translocation of NF-κB, as demonstrated by the reduced mobility of the radio-labeled DNA probe containing the NF-κB consensus sequence (Fig. 2*A*;

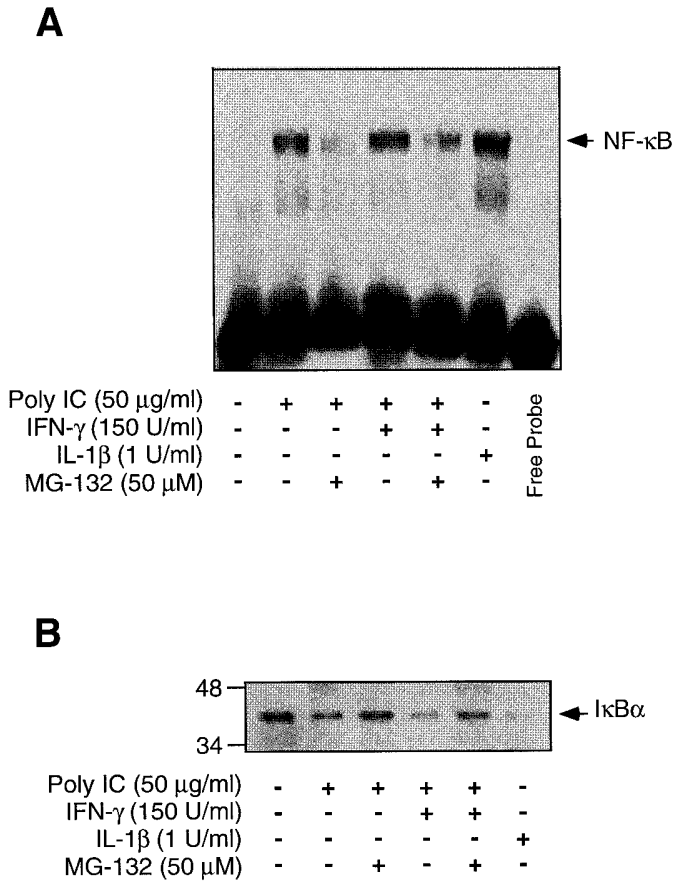

FIG. 2. Poly IC induces NF-κB nuclear translocation and IκBα degradation in rat islets. *A*: Rat islets (400/ml of complete CMRL-1066) were treated with indicated concentrations of poly IC or IFN-γ for 3 h, or IL-1β for 30 min at 37°C; 50 μmol/l MG-132 was added 90 min before the addition of cytokines. Islets were then dispersed by trypsin treatment, nuclear proteins were extracted, and NF-κB activation was determined by gel shift analysis as described in RESEARCH DESIGN AND METHODS. *B*: Rat islets (120/400 μl of complete CMRL-1066) were treated with the indicated concentrations of poly IC and IFN-γ for 3 h, or IL-1β for 30 min at 37°C; 50 μmol/l MG-132 was added 90 min before the addition of cytokines. Islets were isolated and IκBα degradation was evaluated by Western blot analysis. Results are representative of three independent experiments.

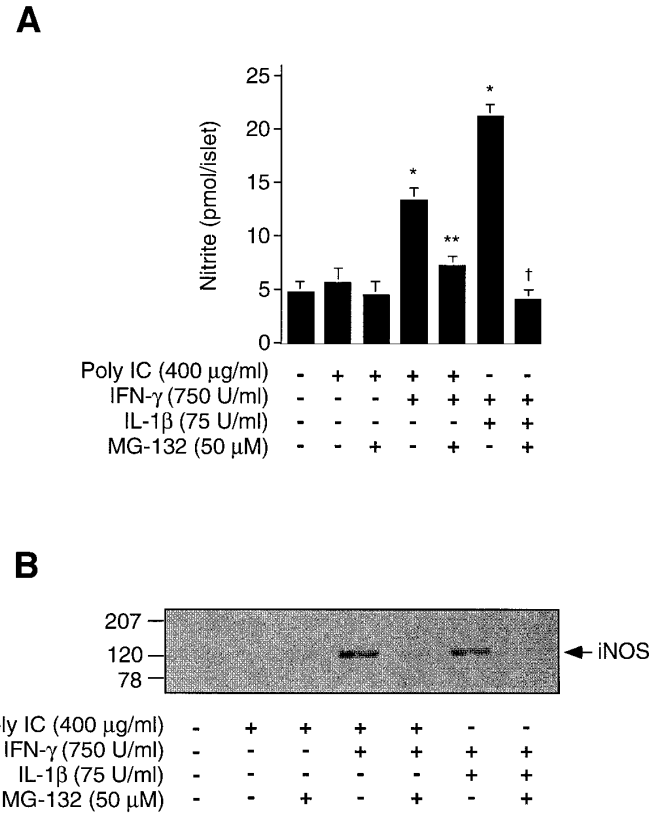

FIG. 3. MG-132 inhibits poly IC + IFN-γ–induced nitrite formation and iNOS expression by human islets. Human islets (220/400 μl of complete CMRL-1066) were incubated with the indicated concentrations of poly IC, IFN-γ, or IL-1β for 48 h at 37°C; 50 μmol/l MG-132 was added to islets 90 min before the addition of cytokines. After treatment, medium was removed for nitrite determination (*A*). iNOS expression by islets was examined by Western blot analysis (*B*). Results for nitrite determination are averages ± SE of four independent experiments; results for iNOS protein expression are representative of two independent experiments. **P* < 0.05 vs. untreated control; ***P* < 0.05 vs. poly IC + IFN-γ treatment; †*P* < 0.05 vs. IL-1β + IFN-γ treatment.

data not shown for time-dependence of NF-κB nuclear translocation). As controls, inclusion of antiserum specific for the p65 subunit of NF-κB in the binding reaction further reduced the NF-κB/DNA complex migration (supershift), and the addition of excess unlabeled NF-κB oligonucleotide prevented the NF-κB/DNA complex formation (data not shown). Consistent with NF-κB nuclear localization, treatment of rat islets for 3 h with 50 μg/ml poly IC and poly IC + 150 U/ml IFN-γ resulted in the degradation of IκBα. Pretreatment of islets for 90 min with MG-132 (50 μmol/l) attenuated poly IC- and poly IC + IFN-γ–induced NF-κB nuclear localization and IκBα degradation (Fig. 2). Alone, neither MG-132 nor IFN-γ stimulated NF-κB nuclear translocation or IκBα degradation (data not shown). As a positive control, the stimulatory effects of IL-1 on NF-κB nuclear translocation and IκBα degradation in rat islets are also shown (Fig. 2).

Effects of MG-132 on poly IC + IFN-γ–induced nitrite formation and iNOS expression by human islets. Treatment of human islets with dsRNA + IFN-γ resulted in the concentration-dependent expression of iNOS and production of nitric oxide that was maximal at 400 μg/ml poly IC and 750 U/ml IFN-γ (M.R.H. and J.A.C., unpublished observation). To determine if NF-κB activation is required for dsRNA + IFN-γ–induced nitric oxide formation, human islets were pretreated for 90 min with 50 μmol/l MG-132, followed by incubation with poly IC + IFN-γ for 48 h. Alone, neither poly IC nor IFN-γ stimulated nitrite production by human islets (Fig. 3*A*) (40); however, in combination, poly IC + IFN-γ stimulated an approximately threefold increase in nitrite formation. Similar to rat islets, MG-132 (50 μmol/l) prevented nitrite formation by human islets in response to poly IC + IFN-γ. As a positive control, the inhibitory actions of MG-132 on IL-1 + IFN-γ–induced nitrite formation by human islets are also shown (Fig. 3*A*) (20,40).

Consistent with its inhibitory actions on nitrite formation, MG-132 prevented poly IC + IFN-γ–induced iNOS expression by human islets (Fig. 3*B*). Treatment of human islets for 48 h with poly IC + IFN-γ resulted in the expression of iNOS to levels similar in magnitude to that induced by IL-1 + IFN-γ. MG-132 prevented both poly IC + IFN-γ– and IL-1 +

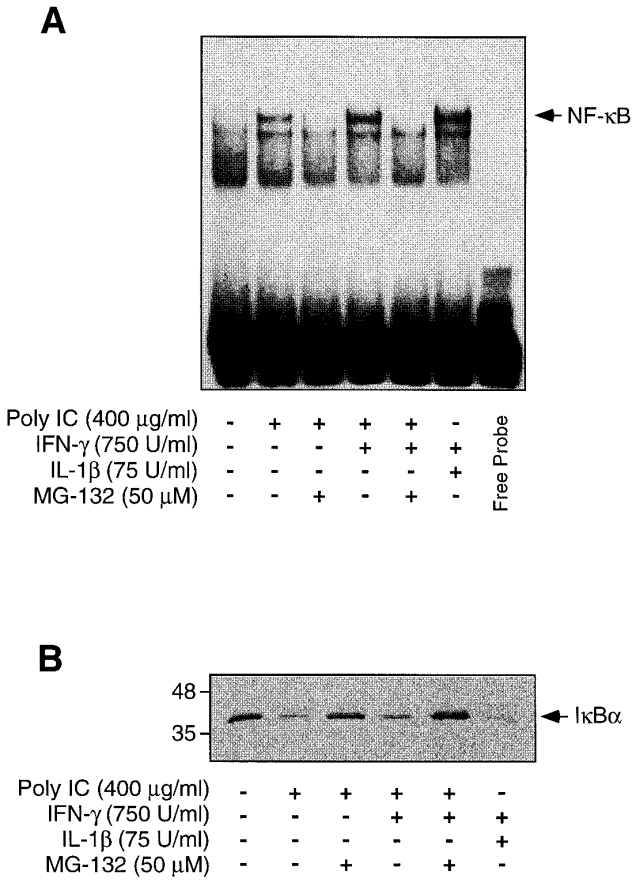

FIG. 4. Poly IC induces NF-κB nuclear translocation and IκBα degradation in human islets. *A*: Human islets (500/ml of complete CMRL-1066) were treated with the indicated concentrations of poly IC or IFN-γ for 2 h, or IL-1β for 1 h at 37°C; 50 μmol/l MG-132 was added 90 min before the addition of cytokines. Islets were then dispersed by trypsin treatment, nuclear proteins were extracted, and NF-κB activation was determined by gel shift analysis as described in RESEARCH DESIGN AND METHODS. *B*: Human islets (150/400 μl of complete CMRL-1066) were treated with the indicated concentrations of poly IC and IFN-γ for 2 h, or IL-1β for 1 h at 37°C; 50 μmol/l MG-132 was added 90 min before the addition of cytokines. Islets were isolated and IκBα degradation was evaluated by Western blot analysis. Results are representative of three independent experiments.

IFN-γ–induced iNOS expression by human islets. Alone, neither poly IC (Fig. 3*B*) nor IFN-γ (39; data not shown) stimulated iNOS protein expression by human islets.

Poly IC stimulates NF-κB activation in human islets. To determine whether poly IC alone or in combination with IFN-γ activates NF-κB in human islets, NF-κB nuclear translocation was examined by gel shift analysis. Treatment of human islets for 2 h with 400 μg/ml poly IC or poly IC + 750 U/ml IFN-γ stimulated maximal NF-κB nuclear translocation (Fig. 4*A*; data not shown for time-dependence of NF-κB nuclear translocation). MG-132 attenuated NF-κB nuclear translocation induced by poly IC and poly IC + IFN-γ. Alone, neither IFN-γ (20) nor MG-132 (data not shown) stimulated NF-κB nuclear localization in human islets. Consistent with the effect of dsRNA on NF-κB nuclear localization, poly IC- and poly IC + IFN-γ–induced IκBα degradation was maximal after a 2-h incubation (Fig. 4*B*; data not shown for time-dependence of IκBα degradation); the addition of MG-132 (50 μmol/l) attenuated IκBα degradation under these conditions.

The genetic absence of PKR does not affect islet expression of iNOS or production of nitrite in response to poly IC + IFN-γ. PKR is believed to regulate dsRNA-induced NF-κB activation by either directly phosphorylating IκB or by activating the upstream IκB kinase, IKK (11,22). As shown in Figs. 1–4, NF-κB activation appears to be required for dsRNA-induced iNOS expression by islets. Therefore, the effects of dsRNA + IFN-γ on iNOS expression and nitric oxide production were examined in islets isolated from both $PKR^{+/+}$ and $PKR^{-/-}$ mice. Islets isolated from either $PKR^{-/-}$ or $PKR^{+/+}$ mice were treated with poly IC + IFN-γ, which resulted in the production of similar levels of nitrite after a 40-h incubation (Fig. 5*A*). Poly IC + IFN-γ also stimulated iNOS expression to similar levels in islets isolated from both $PKR^{-/-}$ and $PKR^{+/+}$ mice (Fig. 5*B*). Similar to human islets, a concentration of 400 μg/ml poly IC + 150 U/ml IFN-γ was required to stimulate maximal iNOS expression and nitrite production by mouse islets. Also, similar to rat and human islets, neither poly IC nor IFN-γ alone stimulated nitrite production by islets isolated from $PKR^{-/-}$ and $PKR^{+/+}$ mice. The level of nitrite produced by mouse islets in response to poly IC + IFN-γ was similar to the levels induced in response to 15 U/ml IL-1 + 150 U/ml IFN-γ. RT-PCR was used to confirm the lack of PKR mRNA accumulation in islets isolated from $PKR^{-/-}$ mice (Fig. 5*C*).

Poly IC stimulates NF-κB nuclear translocation and IκB degradation in islets isolated from $PKR^{-/-}$ and $PKR^{+/+}$ mice. The effects of dsRNA on IκB degradation and NF-κB nuclear translocation in islets isolated from $PKR^{-/-}$ and $PKR^{+/+}$ mice were examined by Western blot and gel shift analyses, respectively. As shown in Fig. 6*A* and *B*, a 1-h incubation with poly IC or poly IC + IFN-γ stimulated IκB degradation and NF-κB nuclear localization in islets isolated from $PKR^{-/-}$ mice. The effects of poly IC and poly IC + IFN-γ on IκB degradation and NF-κB nuclear localization were similar to those observed in islets isolated from $PKR^{+/+}$ mice. As a positive control, we also showed that a 30-min incubation with IL-1 + IFN-γ stimulated IκB degradation and NF-κB nuclear translocation in islets isolated from both $PKR^{-/-}$ and $PKR^{+/+}$ mice.

Poly IC + IFN-γ inhibits glucose-stimulated insulin secretion by islets isolated from $PKR^{-/-}$ mice. We have previously shown that dsRNA + IFN-γ inhibits glucose-stimulated insulin secretion by rat islets in a nitric oxide–dependent manner (36). To determine if PKR is required for the inhibitory actions of poly IC + IFN-γ on insulin secretion, the effects of these agents on glucose-stimulated insulin secretion by islets isolated from $PKR^{-/-}$ mice were examined. Treatment of islets isolated from $PKR^{-/-}$ mice for 40 h with 400 μg/ml poly IC + 150 U/ml IFN-γ resulted in ~75% inhibition of glucose-stimulated insulin secretion (Fig. 7). AG, the iNOS selective inhibitor, prevented poly IC + IFN-γ–induced inhibition of insulin secretion by islets isolated from $PKR^{-/-}$ mice, indicating that nitric oxide mediates the inhibitory actions of these agents on islet function. Alone, neither poly IC nor IFN-γ inhibited glucose-stimulated insulin secretion by $PKR^{-/-}$ mouse islets (Fig. 7). The inhibitory actions of poly IC + IFN-γ were similar in magnitude to the inhibitory effects of 15 U/ml IL-1 + 150 U/ml IFN-γ on glucose-stimulated insulin secretion by islets isolated from $PKR^{-/-}$ mice. Similar results were obtained in islets isolated from $PKR^{+/+}$ mice (data not shown). These results suggest that PKR is not required for the

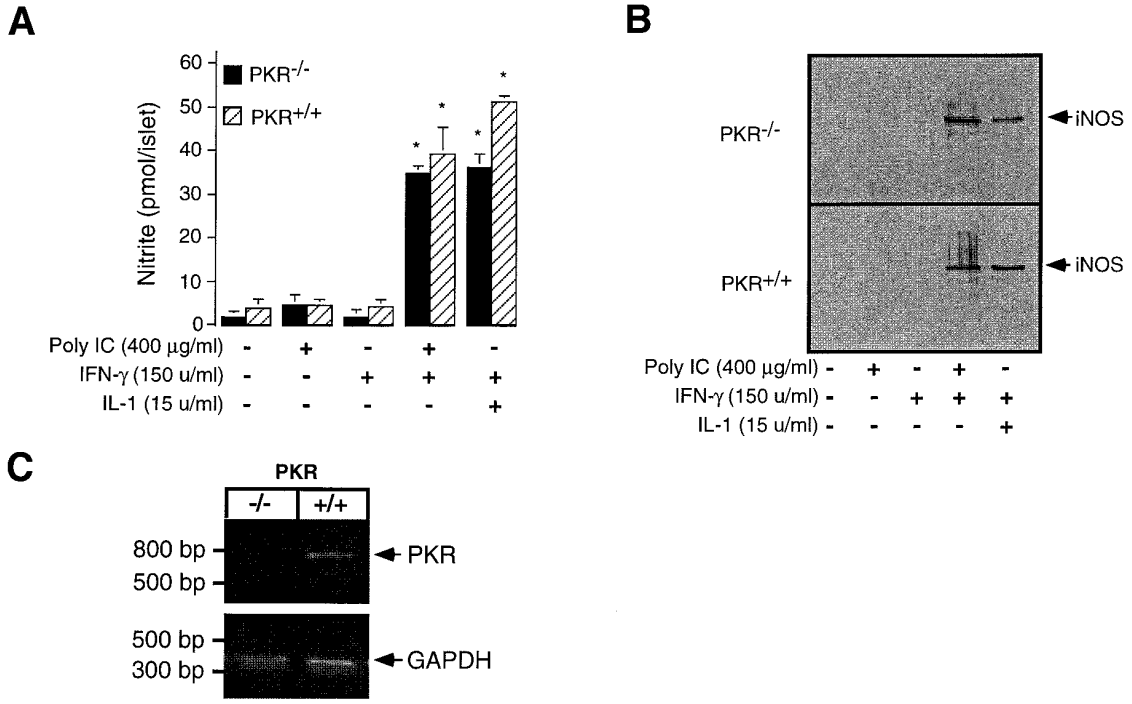

FIG. 5. Poly IC + IFN-γ induces nitrite formation and iNOS expression by islets isolated from PKR$^{-/-}$ mouse islets. PKR$^{-/-}$ or C57BL/6J (PKR$^{+/+}$) mouse islets (120/400 μl of complete CMRL-1066) were treated with the indicated concentrations of poly IC, IFN-γ, or IL-1β for 40 h at 37°C. After treatment, medium was removed for nitrite determination (*A*). iNOS expression was examined by Western blot analysis (*B*), as described in RESEARCH DESIGN AND METHODS. *C*: Total RNA was isolated from PKR$^{-/-}$ or C57BL/6J (PKR$^{+/+}$) mouse islets (120/400 μl of complete CMRL-1066), and PKR and GAPDH mRNA expression was examined by RT-PCR, as described in RESEARCH DESIGN AND METHODS. Results for nitrite are averages ± SE of three independent experiments; both iNOS protein expression and PKR mRNA accumulation and protein expression are representative of three independent experiments. *P < 0.05 vs. untreated control.

inhibitory actions of dsRNA + IFN-γ on glucose-stimulated insulin secretion by mouse islets.

DISCUSSION

Viral infection has been implicated as one environmental factor that may trigger β-cell destruction during the development of autoimmune diabetes. However, the mechanism by which a viral infection induces β-cell damage has been difficult to examine because multiple viruses from both DNA and RNA genome families have been associated with the development of diabetes (2–5). dsRNA is an active component of a viral infection that stimulates antiviral responses in infected cells (8). Recently we showed that treatment of rat islets and primary β-cells with dsRNA + IFN-γ results in a potent inhibition of glucose-stimulated insulin secretion and islet degeneration, events that require β-cell production of nitric oxide (36). In this study, the role of NF-κB in dsRNA + INF-γ–induced iNOS expression and nitric oxide production by islets was examined. We showed that dsRNA (in the form of poly IC), in the presence or absence of IFN-γ, stimulated NF-κB nuclear translocation and IκB degradation, and that inhibition of NF-κB activation prevented dsRNA + IFN-γ–induced iNOS expression and nitric oxide production by rat and human islets. These findings indicate that NF-κB activation is required for iNOS expression by islets in response to dsRNA, and provide further evidence supporting a key role for NF-κB activation in the regulation of iNOS expression by rat and human islets (19,20).

One mechanism by which dsRNA may activate NF-κB is by PKR-dependent phosphorylation of IκB, an event that results in IκB degradation by the proteasome complex (11). In addition, PKR has been shown to activate IKK in mouse embryonic fibroblasts, and this activation results in IκB degradation and NF-κB nuclear localization (23,24). In this study, the role of PKR in both dsRNA + IFN-γ–induced iNOS expression and NF-κB activation was examined using islets isolated from PKR-deficient mice. In contrast to previous studies (21,22), the genetic absence of PKR did not prevent dsRNA or dsRNA + IFN-γ-induced NF-κB nuclear translocation or IκB degradation in islets isolated from PKR$^{-/-}$ mice. In addition, dsRNA + IFN-γ stimulated iNOS expression and nitric oxide production to similar levels in islets isolated from PKR$^{-/-}$ and PKR$^{+/+}$ mice. Consistent with iNOS expression and nitric oxide production, treatment of islets isolated from PKR$^{-/-}$ mice with poly IC + IFN-γ resulted in a nitric oxide–dependent inhibition of glucose-stimulated insulin secretion. These findings indicate that PKR is not required for dsRNA-induced NF-κB activation or dsRNA + IFN-γ–induced iNOS expression by isolated mouse islets. The mechanisms responsible for the differences in dsRNA responsiveness of mouse embryonic fibroblasts as compared to islets are unknown. However, in support of our findings in islets, we recently showed that dsRNA + IFN-γ–induced iNOS expression and NF-κB activation in mouse peritoneal macrophages occur by PKR-independent mechanisms (41).

Although our findings indicate that PKR is not required for dsRNA-induced NF-κB activation, the signaling pathways activated by dsRNA in islets have yet to be identified. The mitogen-activated protein kinases (MAPKs) p38 and c-Jun NH_2-terminal kinase (JNK), along with their upstream MAPK

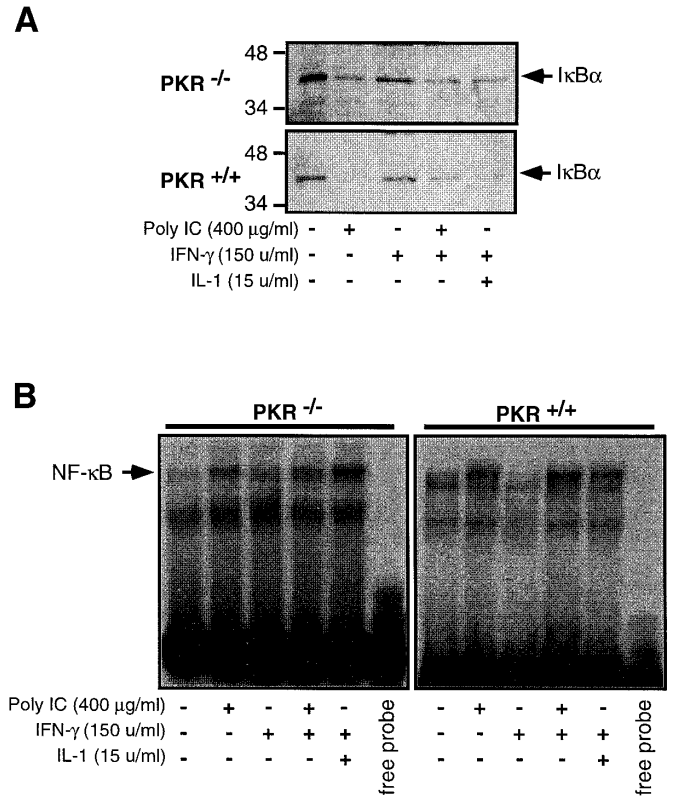

FIG. 6. Poly IC induces NF-κB nuclear translocation and IκBα degradation in islets isolated from PKR$^{-/-}$ mouse islets. *A*: PKR$^{-/-}$ or C57BL/6J (PKR$^{+/+}$) mouse islets (120/400 μl of complete CMRL-1066) were treated with indicated concentrations of poly IC and IFN-γ for 1 h, or IL-1β for 30 min at 37°C. Islets were isolated and IκBα degradation was evaluated by Western blot analysis. *B*: PKR$^{-/-}$ or C57BL/6J (PKR$^{+/+}$) mouse islets (500/ml of complete CMRL-1066) were treated with indicated concentrations of poly IC or IFN-γ for 1 h, or IL-1β for 30 min at 37°C. Islets were then dispersed by trypsin treatment, nuclear proteins were extracted, and NF-κB nuclear translocation was determined by gel shift analysis, as described in RESEARCH DESIGN AND METHODS. Results are representative of three independent experiments.

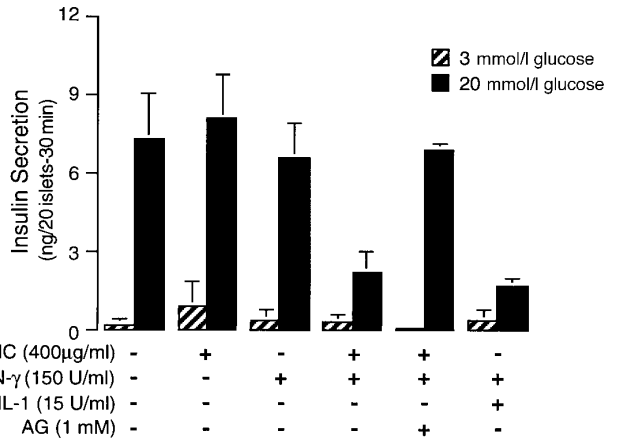

FIG. 7. Poly IC + IFN-γ inhibit glucose-stimulated insulin secretion by islets isolated from PKR$^{-/-}$ mice. Islets isolated from PKR$^{-/-}$ mice were treated for 40 h with the indicated concentrations of poly IC, IFN-γ, IL-1, and AG. The islets were isolated and glucose-stimulated insulin secretion was examined. Results are averages ± SE of three independent experiments.

kinases (MKK3, MKK4, and MKK6), are activated by dsRNA and viral infection in mouse embryonic or immortalized 3T3 fibroblasts, HeLa, and Rat-1 cell lines (23,42). Importantly, dsRNA-induced p38 and JNK activation appears to be PKR-independent; however, these stress kinases do not appear to directly activate NF-κB in response to dsRNA (23,42). It is also possible that dsRNA may interact with other cellular proteins, distinct from PKR, that contain dsRNA-binding motifs (43), and that this interaction may result in the activation of NF-κB. A number of novel candidate dsRNA-binding proteins have been identified; however, a protein that contains dsRNA-dependent kinase activity has yet to be described (44–47). Although we have not identified the kinase(s) that regulate dsRNA-induced NF-κB activation in islets, our findings provide novel evidence that dsRNA + IFN-γ–induced iNOS expression and nitric oxide production require NF-κB activation and occur by mechanisms that are independent of PKR.

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