Double-Stranded RNA Induces Pancreatic β-Cell Apoptosis by Activation of the Toll-Like Receptor 3 and Interferon Regulatory Factor 3 Pathways

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OBJECTIVE—Viral infections contribute to the pathogenesis of type 1 diabetes. Viruses, or viral products such as double-stranded RNA (dsRNA), affect pancreatic β-cell survival and trigger autoimmunity by unknown mechanisms. We presently investigated the mediators and downstream effectors of dsRNA-induced β-cell death.

RESEARCH DESIGN AND METHODS—Primary rat β-cells and islet cells from wild-type, toll-like receptor (TLR) 3, type I interferon receptor (IFNAR1), or interferon regulatory factor (IRF)-3 knockout mice were exposed to external dsRNA (external polyinosinic-polycytidylic acid [PICex]) or were transfected with dsRNA ([PICin]).

RESULTS—TLR3 signaling mediated PICex-induced nuclear factor-κB (NF-κB) and IRF-3 activation and β-cell apoptosis. PICin activated NF-κB and IRF-3 in a TLR3-independent manner, induced eukaryotic initiation factor 2α phosphorylation, and triggered a massive production of interferon (IFN)-β. This contributed to β-cell death, as islet cells from IFNAR1$^{-/-}$ or IRF-3$^{-/-}$ mice were protected against PICin-induced apoptosis.

CONCLUSIONS—PICex and PICin trigger β-cell apoptosis via the TLR3 pathway or IRF-3 signaling, respectively. Execution of PICin-mediated apoptosis depends on autocrine effects of type I IFNs. ***Diabetes*** **57:1236–1245, 2008**

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Received for publication 22 July 2007 and accepted in revised form 18 January 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 25 January 2008. DOI: 10.2337/db07-0844.

Additional information can be found in an online appendix at http://dx.doi.org/10.2337/db07-0844.

ATF-4, activating transcription factor-4; CHOP, CCAAT/enhancer-binding protein homologous protein; dsRNA, double-stranded RNA; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFNAR1, type I interferon receptor; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; LF, lipofectamine; NF-κB, nuclear factor-κB; PIC, polyinosinic-polycytidylic acid; PKR, dsRNA-dependent protein kinase; TLR, toll-like receptor.

Type 1 diabetes is an immune-mediated disease resulting from the loss of insulin-producing pancreatic β-cells. Besides genetic susceptibility, environmental factors play a role in the development of type 1 diabetes. Several studies (1,2) have implicated viral infections in the pathogenesis of the disease. Insulin-producing β-cells are highly susceptible to enteroviral infections, and studies (3,4) on postmortem pancreatic specimens of type 1 diabetic patients demonstrated enterovirus RNA–positive cells in islet cells but not in the exocrine pancreas. During viral infection, the local production of cytokines by both invading immune cells and the pancreatic β-cells themselves interact to initiate and/or accelerate the transition from innate immune response to the chronic autoimmune assault (5). The molecular mechanisms involved in these steps remain to be clarified.

Double-stranded RNA (dsRNA) is generated during the life cycle of most viruses, and it accumulates in and around infected cells during viral infections (6,7). The antiviral activities induced by dsRNA are mimicked by polyinosinic-polycytidylic acid (PIC), a synthetic dsRNA. In vivo, PIC triggers hyperglycemia in diabetes-resistant BB rats and accelerates the development of diabetes in diabetes-prone BB rats (8,9); in vitro, PIC inhibits glucose-stimulated insulin biosynthesis (10) and, when used in combination with interferon (IFN)-γ, impairs islet cell function and viability (11).

Toll-like receptors (TLRs) act as sensors of microbial components and mediate innate and adaptive immunity, modulating production of proinflammatory cytokines and chemokines (12). TLR3 is involved in the cellular recognition of viral dsRNA, and both cytosolic dsRNA and dsRNA released from dying/damaged cells serve as TLR3 ligands. Expression of TLRs in rodent and human pancreatic islets was recently described (13–15). dsRNA or viral infection increase expression of mRNAs encoding for TLR3 and for genes downstream of its signaling pathway in rat and human β-cells (13,16). dsRNA-induced activation of TLR3 leads to activation of nuclear factor-κB (NF-κB) and of IFN regulatory factor (IRF) 3 in other cell types (17,18). Activated IRF-3 and NF-κB translocate to the nucleus and induce type I IFN expression. Secreted IFN-α and -β stimulate the expression of IFN-inducible genes, such as proinflammatory cytokines and chemokines (19). Type I IFNs have been implicated as potential mediators of viral/dsRNA-induced type 1 diabetes (20–22).

In the present study, we characterized the early mediators and downstream effectors of dsRNA-induced apoptosis in primary pancreatic β-cells. Both fluorescence-activated cell sorting (FACS)-purified rat β-cells and islet

cells isolated from wild-type and TLR3, IRF-3, or type I IFN receptor (IFNAR1) knockout mice were studied. Cells were exposed to either dsRNA added in the culture medium (external dsRNA; PICex) in the absence or presence of IFN-γ or were subjected to dsRNA transfection using lipofectamine (LF) (internal dsRNA; PICin). The data obtained indicate major differences in the signaling of internal and external dsRNA and point to novel mechanisms by which this crucial byproduct of viral infection triggers β-cell apoptosis and contributes for initiation/amplification of inflammation and insulitis.

RESEARCH DESIGN AND METHODS

Animals. Adult male Wistar rats (Charles River Laboratories, Brussels, Belgium), wild-type C57BL/6 mice (Harlan CBP, Zeist, the Netherlands), TLR3$^{-/-}$ mice (23), IRF-3$^{-/-}$ mice (Riken BioResource Center, Ibaraki, Japan, with the approval of T. Taniguchi, University of Tokyo), and IFNAR1$^{-/-}$ mice (received from C. Libert, courtesy of Dr. Demengeot, Instituto Gulbenkian de Ciencia, Oeiras, Portugal) were housed and used according to the guidelines of the Belgian regulations for animal care. The number of islets obtained per pancreas was comparable among wild-type, TLR3$^{-/-}$, IFNAR1$^{-/-}$, and IRF3$^{-/-}$ mice (online appendix Table S1 [available at http://dx.doi.org/10.2337/db07-0844]). Glycemia in the fed state was somewhat higher in TLR3$^{-/-}$ and IRF-3$^{-/-}$ mice when compared with wild-type mice (online appendix Table S1), but TLR3$^{-/-}$ mice showed a normal glucose tolerance test (online appendix Fig. S1). Islet histology was similar in wild-type and TLR3$^{-/-}$ mice (not shown).

Islet cell isolation and culture. Pancreatic islets were isolated by collagenase digestion, and β-cells were purified by autofluorescence-activated cell sorting (FACStar; Becton Dickinson, Sunnyvale, CA) as previously described (13,24). Purified β-cells (>90% pure) were cultured in HAM's F-10 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10 mmol/l glucose, 2 mmol/l L-glutamine, 0.5% BSA, 50 μmol/l 3-isobutyl-1-methylxanthine, 50 units/ml penicillin, and 50 μg/ml streptomycin. During the first 36 h of culture, 5% heat-inactivated fetal bovine serum was added.

Assessment of IFN-β production and cell viability. Rat β-cells or mouse dispersed islet cells (10^4 cells per well) were cultured in Falcon 96-well microtiter plates precoated with poly-L-lysine. Cells were maintained in control condition or were exposed to external dsRNA (PICex; 100 μg/ml; Sigma), IFN-γ (500 units/ml; R&D Systems, Oxon, U.K.), and/or interleukin (IL)-1β (50 units/ml; NCI, National Institutes of Health, Bethesda, MD). The medium was replaced every 2 days. Foreign dsRNA was introduced into the cells (PICin) by treating them for 4 h with a mix of LF and PIC (1 μg/ml for rat β-cells and 10 μg/ml for mouse dispersed islet cells); control cells were treated with LF alone or left untreated. Selection of intra- and extracellular PIC concentrations was based on dose-response studies (data not shown). The PIC-transfected cells were then maintained in culture (see above) for up to 5 days. In some experiments, an antibody raised against rat IFN-β (10 μg/ml; PBL Biomedical Laboratories, Piscataway, NJ) was added to the culture medium.

After 2 and 5 days' culture, cell supernatants were pooled and murine IFN-β was measured by enzyme-linked immunosorbent assay (PBL Biomedical Laboratories). The percentage of viable, apoptotic, or necrotic cells was assessed by incubation for 15 min with propidium iodide (10 μg/ml) and Hoechst 342 (10 μg/ml) (25,26). Counting of viable, apoptotic, and necrotic cells was done by two observers, with one of them unaware of the tested conditions. At least 500 cells were counted per experimental condition, and the agreement between the observers was always >90%. Similar amounts of apoptosis were obtained via determination of caspase 3 activation using the NunView 488 Caspase-3 Kit for Live Cells (data not shown; Biotium, Hayward, CA).

RT-PCR and real-time RT-PCR. Semiquantitative RT-PCR was done using poly(A)+ RNA as described (27). The primer sequences for amplification of rat cDNAs for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (13), TLR3 (13), and dsRNA-dependent protein kinase (PKR) (28) were described in the indicated references. Expression of the housekeeping gene GAPDH is not affected by exposure to dsRNA (26) and was used as a control. Real-time RT-PCR for IFN-α, IFN-β, CCAAT/enhancer-binding protein homologous protein (CHOP), activating transcription factor-4 (ATF-4), Bip, X-box binding protein-1 spliced, and GAPDH was done as described (13,29).

Immunocytochemistry. FACS-purified rat β-cells or dispersed mouse islet cells were fixed with 4% formaldehyde and permeabilized with a mix of 70% acetone and 30% methanol. The cells were blocked with PBS containing 0.8% BSA and 10% normal goat serum and then incubated with a rabbit antibody raised against the p65 subunit of NF-κB (C-20; Santa Cruz) alone or in combination with a mouse antibody recognizing insulin (clone K36AC10; Sigma) at 1/500 or 1/1,000 dilution in PBS, respectively. After washing, cells were incubated with goat anti-rabbit Alexa 488 (used at 1/500 dilution; Molecular Probes) alone or in combination with donkey anti-mouse rhodamine (used at 1/200 dilution; Jackson Laboratories). The cells were visualized at a magnification of ×40 with a Zeiss Axiovert 200 microscope (Germany). The number of negative and positive cells for NF-κB nuclear localization was evaluated by two observers (one of them unaware of the tested conditions) who counted at least 300 cells for each experimental condition.

Western blot analysis and protein biosynthesis. Total cell extracts were prepared by lysing equal numbers of cells (30,31). The following primary antibodies were used: rabbit polyclonal antibodies to phospho (p)-IRF-3 (Ser396, no. 4961; Cell Signaling), p–eukaryotic initiation factor (eIF) 2α (Ser51, no. 9721; Cell Signaling), total eIF2α (FL-315; Santa Cruz), or β-actin (no. 4967; Cell Signaling) at 1/1,000 dilution and horseradish peroxidase–conjugated donkey anti-rabbit antibody (Amersham Life Science, Amersham, U.K.) at 1/5,000 dilution. The membranes were incubated with detection system of enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce). The protein-specific signals were quantified using Biomax1D image analysis software (Kodak). Total protein biosynthesis was determined in the presence of 10.0 mmol/l glucose using L-[4,5-^{3}H] leucine incorporation and trichloroacetic precipitation, as previously described (32). As a positive control for protein biosynthesis inhibition, cells were exposed to cycloheximide (10 μmol/l).

Statistical analysis. Data are presented as means ± SE. Statistical differences between groups were determined either by paired Student's t test or by one-way ANOVA, followed by Student's t test with the Bonferroni correction, as indicated. A P value of <0.05 was considered statistically significant.

RESULTS

dsRNA induces apoptosis in pancreatic β-cells. Exposure of purified rat β-cells to PICex (100 μg/ml) for 5 days induced a less than twofold increase in apoptosis (Fig. 1*A*); this effect was magnified fourfold by IFN-γ (Fig. 1*A*). There was a sevenfold increase in apoptosis when foreign dsRNA was introduced into β-cells by lipofection (PICin) (Fig. 1*A*). IFN-γ treatment alone did not affect cell viability (Fig. 1*A*). Addition of 1 mmol/l monomethyl-L-arginine, an inhibitor of the inducible form of nitric oxide (NO) synthase, did not prevent PICin-induced β-cell death (Fig. 1*A*). Similar results were observed in β-cells exposed to PICex+IFN-γ (26), suggesting that the proapoptotic effects of both PICex and PICin are NO independent. No significant modifications in β-cell necrosis were observed in any of the experimental conditions tested (data not shown).

To evaluate whether the detrimental effects of PICex and PICin on β-cell viability are mediated via the TLR3 pathway, dispersed islet cells isolated from wild-type or TLR3 knockout (TLR3$^{-/-}$) mice were utilized. As observed in rat β-cells (Fig. 1*A*), exposure of wild-type mouse islet cells to PICex+IFN-γ induced a clear increase in apoptosis (Fig. 1*B*). Disruption of the TLR3 pathway prevented the deleterious effects of PICex+IFN-γ (Fig. 1*B*). PICin-induced apoptosis was partially prevented by TLR3 disruption, but the percentage of cell death remained higher than in control cells (Fig. 1*B*). Suppression of the TLR3 pathway did not protect against IL-1β+IFN-γ–induced cell death, which was used as a positive control. These results demonstrate that TLR3 mediates both PICex- and PICin-induced apoptosis; in the latter case, however, other pathway(s) contribute to β-cell death.

TLR3 is required to activate NF-κB in response to dsRNA. Activation of the transcription factor NF-κB is a key mediator of β-cell apoptosis induced by cytokines or PICex (33,34). We therefore analyzed whether dsRNA activates NF-κB in purified rat β-cells. Both PICex- and

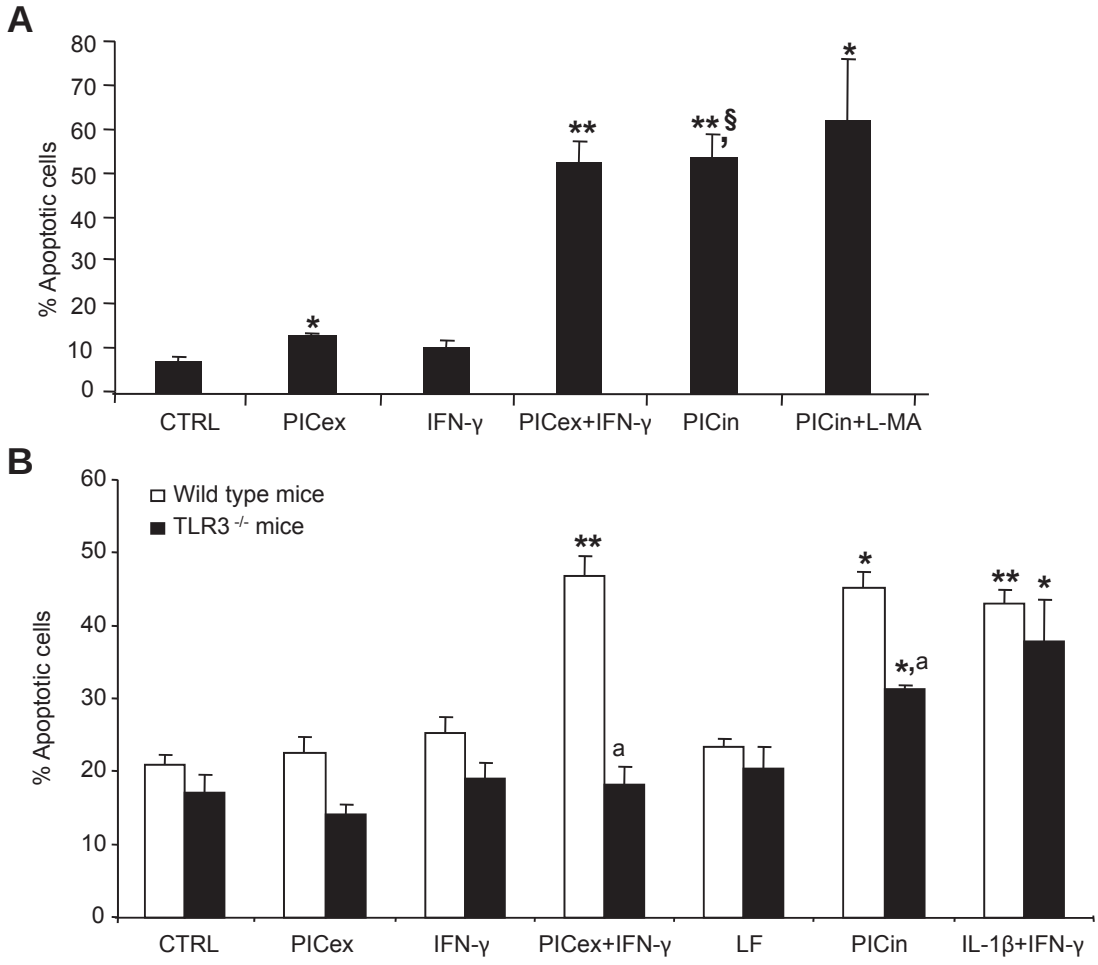

FIG. 1. Suppression of the TLR3 signaling pathway protects β-cell against dsRNA-induced apoptosis. *A*: Percentage of apoptosis observed in FACS-purified rat β-cells cultured for 5 days in the absence (CTRL) or presence of PICex (100 μg/ml), PICex+IFN-γ (500 units/ml), PICin (1 μg/ml), or monomethyl-L-arginine (L-MA) (1 mmol/l). Cell viability was determined with the DNA-binding dyes Hoechst 342 and propidium iodide. The results are means ± SE of five individual experiments. *$P < 0.05$; **$P < 0.001$ vs. control; §$P < 0.001$ vs. PICex, ANOVA followed by Student's *t* test with the Bonferroni correction. *B*: Percentage of apoptosis observed in wild-type or TLR3$^{-/-}$ dispersed islet cells cultured for 5 days in the absence (CTRL) or presence of PICex (100 μg/ml), PICex+IFN-γ (1,000 units/ml), LF alone (LF), or PICin (10 μg/ml). The results are means ± SE of four to five individual experiments. *$P < 0.05$; **$P < 0.005$ vs. respective controls; $P < 0.005$ vs. wild-type mice; ANOVA followed by Student's *t* test with the Bonferroni correction.

PICin-induced nuclear translocation of NF-κB in β-cells (54 ± 12 and 80 ± 6% positive cells, respectively; $P < 0.05$ and $P < 0.005$ vs. control, respectively; $n = 4$), while IL-1β, used as positive control, activated NF-κB in 86 ± 7% of the cells ($P < 0.005$ vs. control; $n = 3$). To examine whether dsRNA-induced activation of NF-κB depends on TLR3 signaling, dispersed islet cells from wild-type or TLR3$^{-/-}$ mice were evaluated (Fig. 2). NF-κB activation was induced by both PICex and PICin in wild-type cells, with a more potent effect of PICin (Fig. 2*B*).

In TLR3$^{-/-}$ cells, PICex failed to activate NF-κB (Fig. 2*A* and *B*), while PICin induced NF-κB nuclear translocation in 63 ± 6% of the cells (Fig. 2*B*). NF-κB activation by PICin, however, was somewhat lower in TLR3$^{-/-}$ than in wild-type cells (Fig. 2*B*). IL-1β–induced NF-κB activation, used as positive control, was similar in TLR3$^{-/-}$ and wild-type cells (data not shown). Of note, inhibition of NF-κB activation by the use of an adenoviral construct expressing a nondegradable form of IκBα (33) did not prevent PICin-induced apoptosis (online appendix Fig. S2), suggesting that NF-κB activation is not a key mediator of PICin-triggered β-cell death. These results demonstrate that in β-cells PICex activates NF-κB exclusively via TLR3 signaling, while PICin-induced NF-κB activation depends both on TLR3 and on other pathways.

TLR3 mediates external, but not internal, dsRNA-induced IRF-3 activation in β-cells. IRF-3 is an ubiquitous transcriptional regulator constitutively expressed in the cytosol. Stimulation of TLR3 induces IRF-3 phosphorylation, nuclear translocation, and production of type I IFNs (35). As shown in Fig. 3*A*, treatment of rat β-cells with PICex or PICin induced IRF-3 phosphorylation. Following correction for β-actin expression, PICin induced a higher activation of IRF-3 than PICex in both wild-type mouse and rat β-cells (Fig. 3*A* and *B*). To investigate the role of TLR3 in dsRNA-mediated IRF-3 activation, islet cells from wild-type and TLR3$^{-/-}$ mice were treated with external or internal PIC. As shown in Fig. 3*B*, exposure to PICex for 4 h induced IRF-3 phosphorylation in wild-type cells but not in TLR3$^{-/-}$ cells. On the other hand, IRF-3 activation was observed after PICin treatment in both wild-type and TLR3$^{-/-}$ islet cells (Fig. 3*B*). These results indicate that IRF-3 activation by PICex depends on TLR3 stimulation, while PICin induces IRF-3 phosphorylation via TLR3-independent mechanism(s).

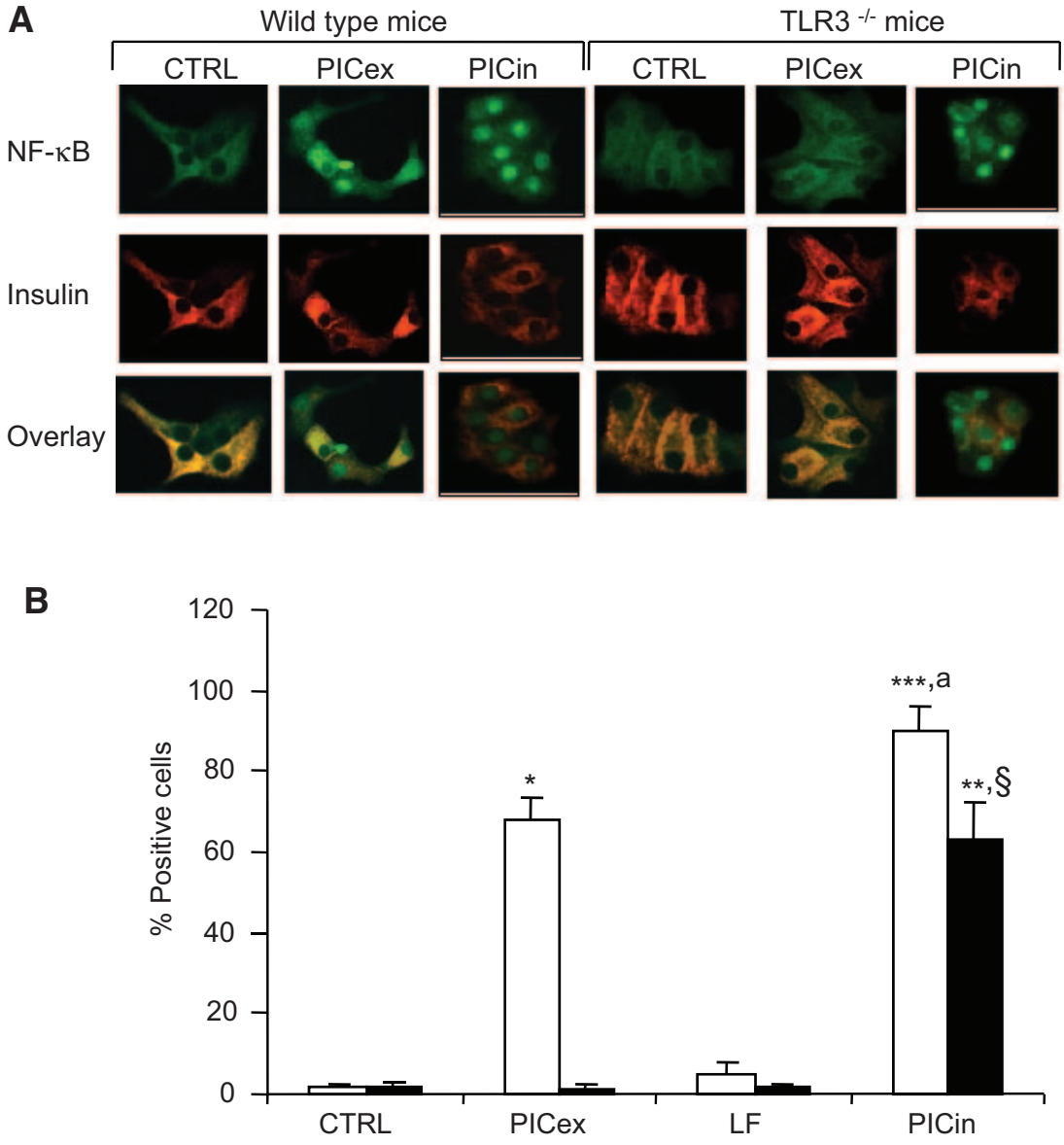

FIG. 2. External, but not internal, dsRNA-induced NF-κB activation is TLR3 dependent in β-cells. *A*: **Wild-type and TLR3$^{-/-}$ dispersed mouse islet cells were cultured for 4 h in the absence (CTRL or LF) or presence of PICex (100 μg/ml) or PICin (1 μg/ml). Activation of NF-κB and expression of insulin were analyzed by double immunocytochemistry. Cells were fixed and fluorescently double stained with anti–NF-κB p65 and anti-insulin antibodies, and the preparations were then analyzed by fluorescence microscopy. NF-κB fluorescence is shown in green (fluorescein isothiocyanate, ×400) and insulin fluorescence in red (rhodamine, ×400) (Fig. 2*A*).** *B*: **The percentage of cells positive for NF-κB nuclear localization is shown in Fig. 2*B*. The results represent the means ± SE of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$ vs. control; [a]$P < 0.01$ vs. external PIC; §$P < 0.001$ vs. wild-type mice; ANOVA followed by Student's *t* test with the Bonferroni correction. □, wild-type mice; ■, TLR3$^{-/-}$.**

Suppression of IRF-3 signaling prevents internal dsRNA-induced β-cell apoptosis. To evaluate the role of IRF-3 signaling in dsRNA-mediated apoptosis, islet cells were isolated from wild-type and IRF-3$^{-/-}$ mice. As shown in Fig. 4, exposure to PICex+IFN-γ induced a comparable degree of apoptosis in wild-type and IRF-3$^{-/-}$ islet cells. On the other hand, PICin markedly increased apoptosis in wild-type cells but failed to trigger cell death in IRF-3$^{-/-}$ cells (Fig. 4). To further examine whether the IRF-3 pathway plays a role in the deleterious effects of PIC on β-cell function, glucose-stimulated insulin secretion was performed in islet cells isolated from wild-type and IRF-3$^{-/-}$ mice. The insulin content of islet cells was not affected by IFN-γ and PICex+IFN-γ treatment in both cell types (online appendix Table S2). Exposure to PICex or PICin decreased insulin content in wild-type ($P < 0.05$ and $P < 0.005$ vs. control, respectively; $n = 12$) but not in IRF-3$^{-/-}$ ($P > 0.05$; $n = 9$) (online appendix Table S2) cells.

Basal insulin release was similar between wild-type and IRF3$^{-/-}$ cells and was not affected by the different experimental conditions (Fig. 5*A* and *B*). Exposure to glucose plus forskolin increased insulin secretion by threefold in control wild-type and IRF-3$^{-/-}$ islet cells (Fig. 5*A* and *B*). Stimulated insulin release was decreased after exposure to PICex+IFN-γ in both cell types (Fig. 5*A* and *B*), as previously reported for wild-type islets (10,11,36). In islet cells isolated from wild-type mice, PICin treatment caused a trend ($P = 0.07$) for a decrease in glucose plus forskolin–induced insulin secretion when compared with control cells (Fig. 5*A*). By contrast, insulin release was not affected by PICin in IRF-3$^{-/-}$ islet cells ($P = 0.181$) (Fig. 5*B*).

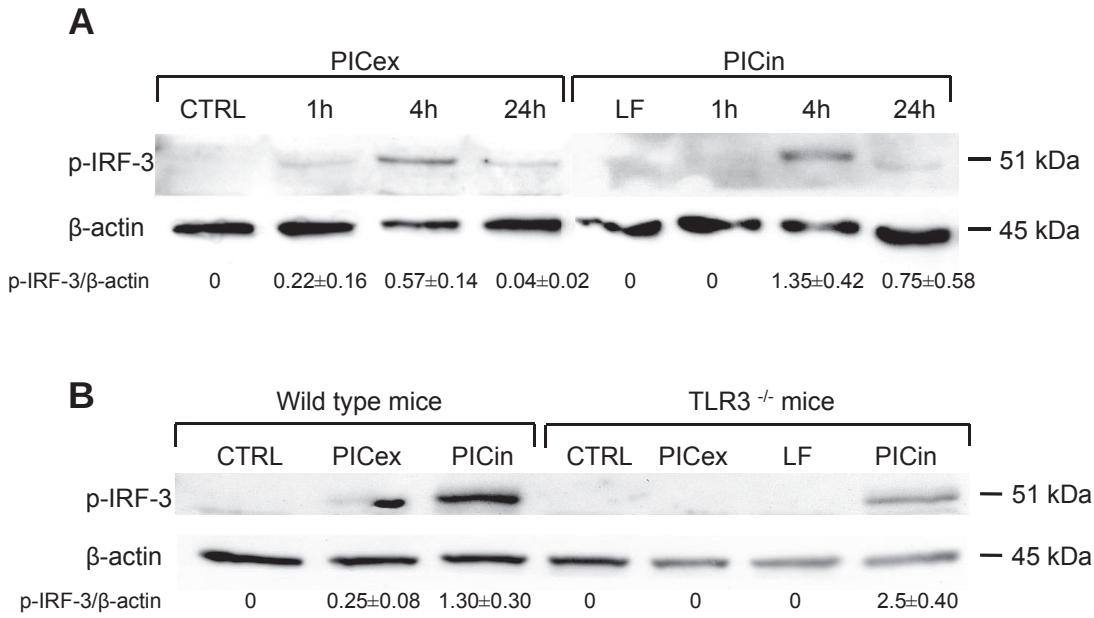

FIG. 3. TLR3 signaling pathway mediates external, but not internal, dsRNA-induced IRF-3 activation in pancreatic β-cells. FACS-purified rat β-cells (*A*) or wild-type and TLR3$^{-/-}$ dispersed mouse islet cells (*B*) were cultured in the absence (CTRL or LF) or presence of PICex (100 μg/ml) or PICin (1–10 μg/ml) for the indicated time points. Whole-cell lysates were isolated and resolved on 10% SDS-PAGE followed by immunoblotting with *p*–IRF-3 antibody. The membranes were stripped and probed with β-actin antibody, used as a loading control. Expression of β-actin and total IRF-3 were similar (data not shown), but since total IRF-3 formed dimers it was therefore not used for quantification. The figure is representative of three to six similar experiments.

Islet cells from IRF-3$^{-/-}$ mice are thus more resistant to the deleterious effects of PICin on viability and function, while they are affected in a similar way as wild-type islet cells when exposed to PICex+IFN-γ (Figs. 4 and 5) (online appendix Table S2). Taken together, these data suggest a key role for IRF-3 signaling in internal dsRNA-induced β-cell dysfunction and death, while IRF-3 plays a minor role in β-cell apoptosis triggered by PICex+IFN-γ.

Autocrine production of IFN-β contributes to dsRNA-induced apoptosis. Exposure of β-cells to PICex increased expression of IFN-α and IFN-β 2–10 times above control values, while PICin induced a 50- to 2,000-fold upregulation of type I IFN mRNAs (Fig. 6*A* and *B*). Genes downstream of dsRNA signaling, including PKR, TLR3, and RANTES, were upregulated to the same extent by PICex and PICin (data not shown). In line with these data, IFN-β production by PICin-treated mouse islet cells was 58.1 ± 8.1 pg · 10^3 cells^{-1} · 5 days^{-1} (Fig. 6*C*), while it was below detection in wild-type islet cells exposed to PICex or in control condition. Of interest, IFN-β secretion was markedly reduced in TLR3$^{-/-}$ islet cells treated with PICin and it was virtually absent in IRF-3$^{-/-}$ mice (Fig. 6*C*).

To further evaluate the role of such a massive autocrine production of type I IFNs in PICin-induced apoptosis, islet cells from wild-type or IFNAR1$^{-/-}$ mice were treated with PICin for 5 days. IFNAR1$^{-/-}$ cells were protected against PICin-induced apoptosis (Fig. 6*D*). Of note, IFNAR1$^{-/-}$ cells secreted five times less IFN-β than wild-type cells (Fig. 6*C*). In accordance with these observations, an antibody raised against rat IFN-β also induced a 70% protection against PICin-induced apoptosis (online appendix Fig. S3). These results show that PICin markedly upregulates IFN-α/β mRNA expression and triggers secre-

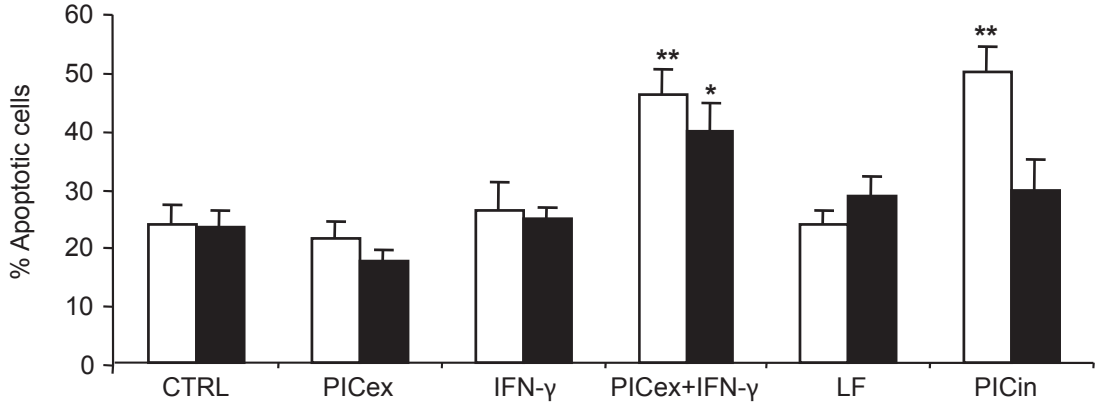

FIG. 4. Suppression of IRF-3 signaling prevents internal dsRNA-induced β-cell apoptosis. Percentage of apoptosis observed in wild-type and IRF-3$^{-/-}$ dispersed mouse islet cells cultured for 5 days in the absence (CTRL) or presence of PICex (100 μg/ml), PICex+IFN-γ (1,000 units/ml), or PICin (10 μg/ml). Cell viability was determined with the DNA-binding dyes Hoechst 342 and propidium iodide. The results are means ± SE of four to seven individual experiments. **P* < 0.05; ***P* < 0.01 vs. control; ANOVA followed by Student's *t* test with the Bonferroni correction. □, wild-type mice; ■, IRF-3$^{-/-}$ mice.

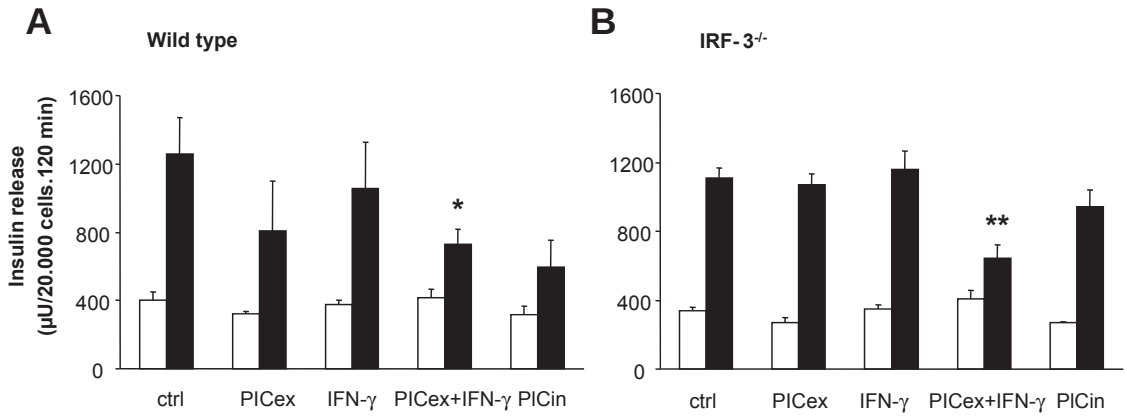

FIG. 5. Glucose-stimulated insulin secretion in wild-type and IRF-3$^{-/-}$ islet cells. Insulin release measured in dispersed islet cells isolated from wild-type (*A*) or IRF-3$^{-/-}$ (*B*) mice treated for 2 days with PICex (100 µg/ml) and/or IFN-γ (1,000 units/ml) or with PICin (10 µg/ml) and then exposed for 2 h to 1.7 mmol/l glucose (□) or 16.7 mmol/l glucose plus forskolin (10 µmol/l) (■). Results are means ± SE of three to four experiments. *P < 0.05 vs. control (ctrl); **P < 0.005 vs. ctrl.

tion of IFN-β by β-cells. IFN-β production is regulated by IRF-3 signaling and, to a lesser extent, through TLR3 stimulation. These data suggest that execution of PICin-induced β-cell apoptosis depends on autocrine effects of type I IFNs.

Internal dsRNA induces eIF2α phosphorylation, inhibits protein biosynthesis, and upregulates endoplasmic reticulum stress marker genes in β-cells. Exposure of β-cells to PICex did not induce eIF2α phosphorylation, while internalization of dsRNA resulted in a

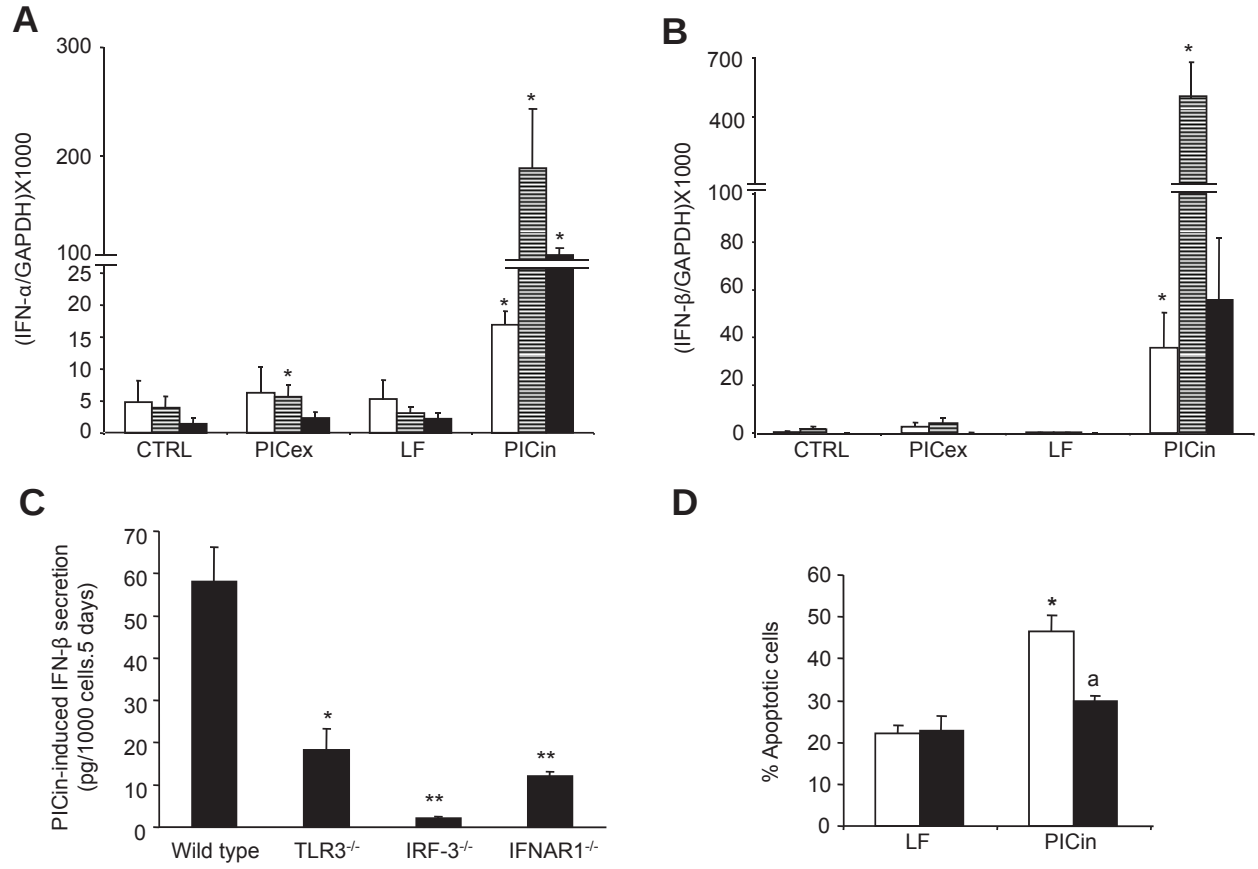

FIG. 6. Autocrine production of type I IFNs mediates internal dsRNA-induced β-cell death. Rat β-cells were cultured in absence (CTRL or LF) or presence of PICex (100 µg/ml) or PICin (1 µg/ml) for 2–24 h. Expression of IFN-α (*A*) and IFN-β (*B*) mRNAs were analyzed by real-time RT-PCR, and the data were normalized for the housekeeping gene GAPDH. The results represent means ± SE of three to four independent experiments. *P < 0.05 vs. control; Student's paired t test. *A* and *B*: □, 2 h; ▤, 6 h; ■, 24 h. *C*: IFN-β secretion was determined by ELISA in PICin-stimulated islet cells from wild-type, IRF-3, TLR3, or IFNAR1 knockout mice. The results are means ± SE of 5–10 individual determinations. *D*: Percentage of apoptosis observed in dispersed islet cells of wild-type or IFNAR1$^{-/-}$ mice transfected (PICin) or not (LF) with PIC and cultured for 5 days. Cell viability was determined with the DNA-binding dyes Hoechst 342 and propidium iodide. The results are means ± SE of four experiments. *P < 0.005 vs. LF; $^a P$ < 0.001 vs. wild type; ANOVA followed by Student's t test with the Bonferroni correction. □, wild-type mice; ■, IFNAR1$^{-/-}$.

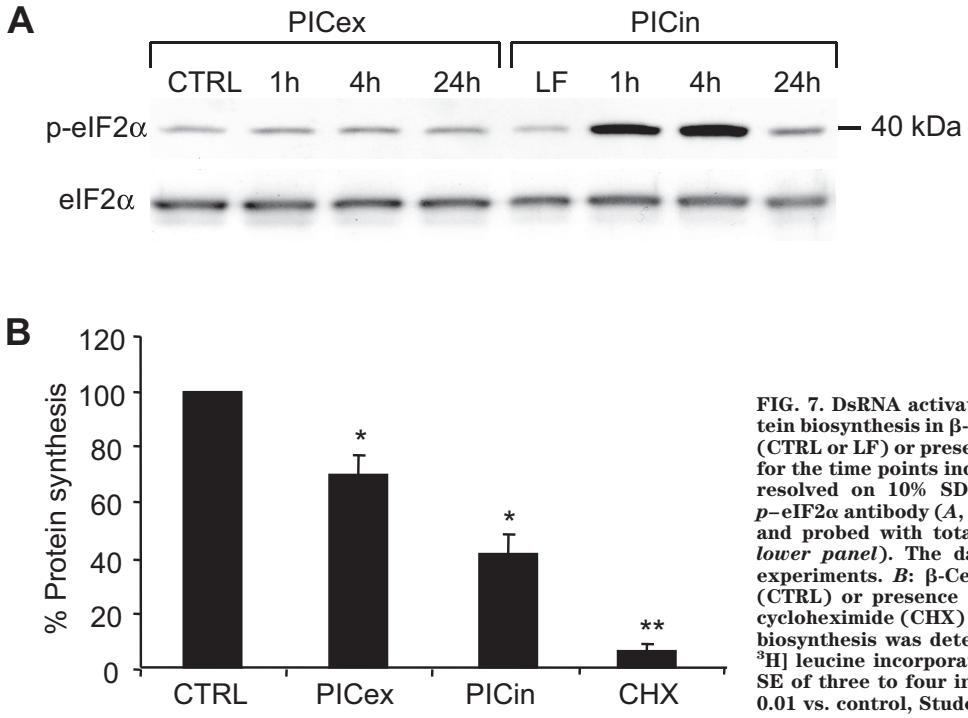

FIG. 7. DsRNA activates eIF2α phosphorylation and inhibits protein biosynthesis in β-cells. *A*: β-Cells were cultured in the absence (CTRL or LF) or presence of PICex (100 μg/ml) or PICin (1 μg/ml) for the time points indicated. Whole-cell lysates were isolated and resolved on 10% SDS-PAGE followed by immunoblotting with *p*–eIF2α antibody (*A*, *upper panel*). The membranes were stripped and probed with total eIF2α antibody as a loading control (*A*, *lower panel*). The data are representative of three individual experiments. *B*: β-Cells were cultured for 24 h in the absence (CTRL) or presence of PICex (100 μg/ml), PICin (1 μg/ml), or cycloheximide (CHX) (10 μmol/l). The percentage of total protein biosynthesis was determined at 10.0 mmol/l glucose using L-[4,5-^{3}H] leucine incorporation. The results are expressed as means ± SE of three to four individual experiments. *$P < 0.05$ and **$P < 0.01$ vs. control, Student's paired *t* test.

rapid phosphorylation of eIF2α, which persisted through 4 h before returning to basal level (Fig. 7*A*). In line with these data, PICin reduced protein biosynthesis to 50% of the control value (Fig. 7*B*), while PICex induced a less marked decrease in protein biosynthesis (Fig. 7*B*). Cycloheximide suppressed translation by 90% (Fig. 7*B*).

To evaluate whether eIF2α phosphorylation was part of a broader endoplasmic reticulum (ER) stress response, we examined the expression of four ER stress–related genes. As shown in Fig. 8, PICin markedly induced expression of the transcription factors ATF-4 (Fig. 8*A*) and CHOP (Fig. 8*B*) and of the ER chaperone Bip (Fig. 8*D*). Activation of the ER stress response was confirmed by increased expression of the spliced form of X-box binding protein-1

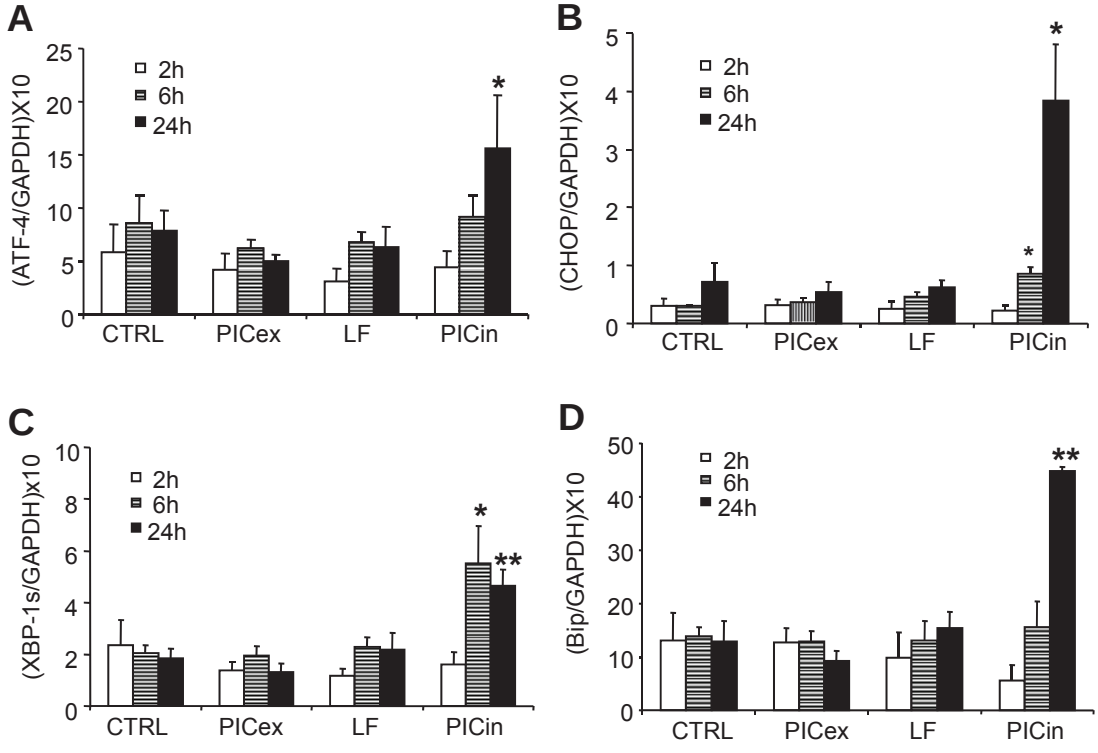

FIG. 8. Internal dsRNA upregulates expression of ER stress marker genes in β-cells. Rat β-cells were cultured in the absence (CTRL or LF) or presence of PICex (100 μg/ml) or PICin (1 μg/ml) for 2–24 h. Expression of activating transcription factor-4 (ATF-4), CCAAT/enhancer-binding protein homologous protein (CHOP), BiP, and X-box binding protein-1 (XBP-1) spliced mRNAs was analyzed by real-time RT-PCR, and the data were normalized for the housekeeping gene GAPDH. The results represent means ± SE of four to six independent experiments. *$P < 0.05$; **$P < 0.02$ vs. control, Student's paired *t* test.

(Fig. 8*C*). Exposure to PICex did not affect expression of these genes (Fig. 8*A–D*).

DISCUSSION

Epidemiological evidence, postmortem studies, and in vitro experimental data support the hypothesis that enterovirus infections play a role in the development of type 1 diabetes. The molecular mechanisms involved in viral/dsRNA-mediated β-cell apoptosis and in triggering the early cellular events that will lead to insulitis and autoimmunity, however, remain to be clarified.

TLR3 recognizes viral and synthetic dsRNA and plays an important role in the cellular responses to several viruses (37,38). The existence of viral evasion mechanisms targeting the TLR3 pathway confirms that TLR3 signaling is a key component of the host antiviral response (39). We have shown that TLR3 is expressed and functional in pancreatic β-cells (13 and present data).

To dissect the signaling pathways downstream of dsRNA in pancreatic β-cells, we used dsRNA, tested in the form of PIC, either added to the extracellular medium or introduced in the cytosolic compartment by lipofection. PICex, when tested alone, induces a minor increase in β-cell apoptosis; in the presence of IFN-γ, however, it induces apoptosis in >50% of the cells. Similar results were previously observed in the presence of type I IFNs plus dsRNA (13). Disruption of the TLR3 pathway completely abrogates PICex+IFN-γ–induced cell death (present data). Stimulation of TLR3 by PICex activates NF-κB and IRF-3. Inhibition of NF-κB activation prevents both cytokine- and PICex+IFN-γ–induced β-cell apoptosis, suggesting that NF-κB has proapoptotic effects (34,40,41). The PICex-induced activation of NF-κB via TLR3 could therefore contribute to the presently observed proapoptotic effects of TLR3 stimulation.

Cytosolic accumulation of dsRNA induces a more intense activation of NF-κB and IRF-3 and a higher prevalence of apoptosis compared with PICex, suggesting that NF-κB and IRF-3 may play a role in this process. Inhibition of NF-κB activation, however, did not protect against PICin-induced cell death. In other cell types, TLR3-mediated IRF-3 and NF-κB activation transactivates IFN-β and -α. Once secreted, IFN-α/β act in an autocrine/paracrine manner, leading to the upregulation of a secondary set of genes involved in antiviral responses and in triggering apoptosis (42). Part of the observed discrepancy between the effects of external versus internal dsRNA could be due to the marked PICin-induced upregulation of IFN-β mRNA expression and secretion by the β-cells. In line with this possibility, suppression of the IRF-3 signaling pathway abolished secretion of IFN-β and prevented PICin-induced islet cell dysfunction and death, whereas it had only a minor effect on the deleterious effects of PICex+IFN-γ. In addition, blocking type I IFNs signaling by use of IFNAR1$^{-/-}$ mice or by an antibody against IFN-β prevents the detrimental effects of PICin, confirming that IFN-α/β signaling contributes to β-cell death in an autocrine/paracrine manner. Type I IFNs may have a dual biological role: elicit an antiviral state in uninfected cells while selectively inducing apoptosis in virally infected cells, thus limiting viral replication and spreading of the infection. Depending on the type of viral infection and on the cellular context, IFN-α/β effects may favor tissue damage or eradication of the viral infection (43). Type I IFNs have been implicated in the development of type 1 diabetes (21,44); high levels of these cytokines were detected in the pancreas or islets of type 1 diabetic patients (20,45), and IFN-α was proposed as a mediator of viral-induced experimental type 1 diabetes (21,22,46).

Viral infection inhibits protein synthesis through phosphorylation of eIF2α by PKR. PKR activation has been involved in islet cell defense during viral infection (47), and we presently observed that PICin triggers a marked translational repression associated with eIF2α phosphorylation.

eIF2α phosphorylation may also occur in response to other cellular stresses, resulting from activation of kinases such PKR-like ER kinase, which responds to ER stress. Pancreatic β-cells are highly sensitive to ER stress, and a tight control of ER homeostasis is crucial to preserve β-cell function and survival (48), and prolonged eIF2α phosphorylation was described as proapoptotic in β-cells (31). It is conceivable that a massive and prolonged PICin-triggered production of type I IFN–induced proteins, such as cytokines, chemokines, and HLA-1 components (16,28), overloads the ER capacity triggering an excessive ER stress response and favoring β-cell apoptosis. In line with this hypothesis, PICin, but not PICex, upregulates ER stress marker genes in β-cells (present data). Additional experiments are required to validate the role of ER stress in PICin-triggered apoptosis.

In summary, we demonstrate that external and internal dsRNA trigger β-cell apoptosis by essentially different mechanisms (online appendix Fig. S4). Both external and internal dsRNA induce activation of NF-κB and IRF-3 in pancreatic β-cells, but activation of these transcription factors is entirely dependent of TLR3 signaling for external, but not for internal, dsRNA. In line with these observations, the TLR3 pathway is the key mediator of β-cell apoptosis triggered by PICex, while IRF-3 signaling plays a major role in PICin-induced islet cell dysfunction and death. Internal dsRNA induces a massive production of type I IFNs, which contributes to PICin-induced apoptosis.

The present work represents the first detailed mechanistic study on innate immunity signaling pathways activated by dsRNA in pancreatic β-cells and clarifies part of the complex molecular responses of β-cells faced with viral components in the intra- or extracellular space. We propose that activation of immune pathways and production of type I IFNs by β-cells play a relevant role in the development of insulitis and eventually type 1 diabetes. As recently described by Baccala et al. (49), type I IFNs induction by nucleic acids or apoptotic materials via TLR-dependent and -independent pathways might be a central pathogenic event in autoimmunity.

ACKNOWLEDGMENTS

This study was supported by grants from the European Foundation for the Study of Diabetes/Lilly European Diabetes Research Programme, the "Fonds David et Alice Van Buuren," the "Fonds de la Recherche Scientifique Médicale" (convention no. 3.4514.03/avenant 3.4554.05), the "Actions de Recherche Concertées" (convention no. 04/09-311), the Belgium Program on Interuniversity Poles of Attraction initiated by the Belgium State (interuniversitaire attractiepolen P6/40) and The European Union Project SAVEBETA (contract no. 036903 in the FP6 of the European Commission).

We thank Prof. T. Taniguchi (University of Tokyo) for kindly providing the IRF-3 knockout mice. We are also

grateful to N. El Amrite, J. Schoonheydt, M. Urbain, and G. Vandenbroeck for expert technical assistance.

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