

Double-stranded RNA induces pancreatic beta cell apoptosis by activation of the TLR3 and IRF-3 pathways

Zeynep Dogusan¹, Mónica García¹, Daisy Flamez¹, Lena Alexopoulou², Michel Goldman³, Conny Gysemans⁴, Chantal Mathieu⁴, Claude Libert⁵, Decio L. Eizirik¹, Joanne Rasschaert¹.

¹ Laboratory of Experimental Medicine, Université Libre de Bruxelles, Brussels, Belgium.

² Centre d'Immunologie de Marseille-Luminy, CNRS-INSERM-
Université de la Méditerranée, Marseille, France.

³ Institute of Medical Immunology, Université Libre de Bruxelles, Charleroi, Belgium.

⁴ Laboratory of Experimental Medicine and Endocrinology (LEGENDO), UZ Gasthuisberg
O&N, Katholieke Universiteit Leuven, Leuven, Belgium.

⁵ Department for Molecular Biomedical Research, VIB - Ghent University, Ghent, Belgium.

Running Title: DsRNA-induced apoptosis of pancreatic beta cells.

Corresponding Author:

Dr Joanne Rasschaert, Laboratory of Experimental Medicine, Université Libre de Bruxelles,
Route de Lennik, 808, CP 618, B-1070 Brussels, Belgium.
jrasscha@ulb.ac.be

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

Additional information can be found in an online appendix at
<http://diabetes.diabetesjournals.org>.

ABSTRACT

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

Research Design and Methods: Primary rat beta cells and islet cells from wild type, Toll-like receptor 3 (TLR3), type I interferon receptor (IFNAR1) or interferon regulatory factor 3 (IRF-3) knockout mice were exposed to external dsRNA (polyinosinic polycytidylic acid; PICex) or were transfected with dsRNA (PICin).

Results: TLR3 signaling mediated PICex-induced NF- κ B and IRF-3 activation and beta cell apoptosis. PICin activated NF- κ B and IRF-3 in a TLR3-independent manner, induced eIF2 α phosphorylation and triggered a massive production of IFN- β . This contributed to beta cell death as islet cells from IFNAR1^{-/-} or IRF-3^{-/-} mice were protected against PICin-induced apoptosis.

Conclusions: External and internal dsRNA trigger beta cell apoptosis via respectively the TLR3 pathway or IRF-3 signaling. Execution of PICin-mediated apoptosis depends on autocrine effects of type I IFNs.

Type 1 diabetes is an immune-mediated disease resulting from the loss of insulin-producing pancreatic beta cells. Besides the genetic susceptibility, environmental factors play a role in the development of type 1 diabetes. Several studies have implicated viral infections in the pathogenesis of the disease (1;2). Insulin-producing beta cells are highly susceptible to enteroviral infections and studies on post-mortem pancreatic specimens of type 1 diabetes patients demonstrated enterovirus RNA positive cells in islets cells but not in the exocrine pancreas (3;4). During viral infection, the local production of cytokines by both invading immune cells and the pancreatic beta 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 IFN- γ , impairs islet cell function and viability (11).

Toll-like receptors (TLR) act as sensors of microbial components and mediate innate and adaptive immunity, modulating production of pro-inflammatory 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 beta cells (13;16). dsRNA-induced activation of TLR3 leads to activation of NF- κ B and of IFN regulatory factor (IRF) 3 in other cell types (17;18). Activated IRF-3 and NF- κ B translocate to the nucleus and induce type I interferons (IFNs) expression. Secreted IFN- α and β stimulate the expression of IFN-inducible genes, such as pro-inflammatory 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 beta cells. Both FACS-purified rat beta cells and islet cells isolated from wild type and TLR3, IRF-3 or type I interferon 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 (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 by-product of viral infection triggers beta cell apoptosis and contributes for initiation/amplification of inflammation and insulinitis.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (Charles River Laboratories, Brussels, Belgium), wild type (WT) C57BL/6 mice (Harlan CBP, Zeist, The Netherlands), TLR3^{-/-} (23), IRF-3^{-/-} (obtained from the Riken BioResource Center (Ibaraki, Japan) with the approval of T. Taniguchi, University of Tokyo, Japan) and IFNAR1^{-/-} mice (received from C. Libert by 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 between WT, TLR3^{-/-}, IFNAR1^{-/-} and IRF3^{-/-} mice (Table S1). Glycemia in the fed state was somewhat higher in TLR3^{-/-} and IRF3^{-/-} mice when compared to WT (Table S1), but TLR3^{-/-} mice showed normal glucose tolerance test (Fig. S1). Islet histology was similar in WT and TLR3^{-/-} mice (not shown).

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

Assessment of IFN- β production and cell viability. Rat beta cells or mouse dispersed islet cells (10⁴ cells per well) were cultured in Falcon 96-well microtiter plates pre-coated with poly-L-lysine. Cells were maintained in control condition or were either exposed to external dsRNA (tested in the form of polyinosinic-polycytidilic acid; PICex; 100 μ g/ml; Sigma), IFN- γ (500 U/ml; R&D Systems, Oxon, UK) and/or IL-1 β (50 U/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 hours with a mix of lipofectamine and PIC (1 μ g/ml for rat beta cells and 10 μ g/ml for mouse dispersed islet cells); control cells were treated with lipofectamine alone (LF) or left untreated. Selection of intra- and extra-cellular 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 μ g/ml; PBL Biomedical

Laboratories, New Jersey, USA) was added to the culture medium.

After 2 and 5 days culture, cell supernatants were pooled and murine IFN- β was measured by ELISA (PBL Biomedical Laboratories; New Jersey, USA). The percentage of viable, apoptotic or necrotic cells was assessed by incubation for 15 min with propidium iodide (PI, 10 μ g/ml) and Hoechst (HO) 342 (10 μ g/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 NunViewTM 488 Caspase-3 Kit for Live Cells (Biotium, Inc – Hayward-CA, EUA; data not shown).

RT-PCR and real time RT-PCR. Semi-quantitative RT-PCR was done using poly(A)⁺ RNA as described (27). The primer sequences for amplification of rat cDNAs for GAPDH (13), TLR3 (13) and 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 control.

Real-time RT-PCR for IFN- α , IFN- β , CHOP, ATF-4, Bip, xbp-1 spliced (xbp-1s) and GAPDH was done as described (13;29).

Immunocytochemistry. FACS-purified rat beta 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- κ B (C-20, Santa Cruz) alone or in combination with a mouse antibody recognizing insulin (clone K36AC10, Sigma) at 1/500 or 1/1000 dilution in PBS, respectively. After washing, cells were incubated with goat anti-rabbit Alexa 488 (Molecular Probes; used at 1/500 dilution) alone or in combination with donkey anti-mouse rhodamine (Jackson Laboratories;

used at 1/200 dilution). The cells were visualized at a magnification of X 40 with a Zeiss Axiovert 200 microscope (Germany). The number of negative and positive cells for NF- κ B 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; Cell Signaling; #4961), p-eIF2 α (Ser51; Cell Signaling; #9721), total eIF2 α (FL-315; Santa Cruz) or β -actin (Cell Signaling; #4967) at 1/1000 dilution and horseradish peroxidase conjugated donkey anti-rabbit antibody (Amersham Life Science, Amersham, UK) at 1/5000 dilution. The membranes were incubated with detection system of enhanced chemiluminescence (ECL; 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 mM 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 μ M).

Statistical analysis. Data are presented as means \pm SEM. 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 less than 0.05 was considered as statistically significant.

RESULTS

dsRNA induces apoptosis in pancreatic beta cells. Exposure of purified rat beta cells to PICex (100 μ g/ml) for 5 days induced a < 2-fold increase in apoptosis (Fig. 1A); this

effect was magnified 4-fold by IFN- γ (Fig. 1A). There was a 7-fold increase in apoptosis when foreign dsRNA was introduced into beta cells by lipofection (PICin) (Fig.1A). IFN- γ treatment alone did not affect cell viability (Fig. 1A). Addition of 1 mM monomethyl-L-arginine (L-MA), an inhibitor of the inducible form of nitric oxide synthase, did not prevent PICin-induced beta cell death (Fig.1A). Similar results were observed in beta cells exposed to PICex+IFN- γ (26), suggesting that the pro-apoptotic effects of both PICex and PICin are NO-independent. No significant modifications in beta 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 beta cell viability are mediated via the TLR3 pathway, dispersed islet cells isolated from wild type (WT) or TLR3 knock-out (TLR3 $^{-/-}$) mice were utilized. As observed in rat beta cells (Fig.1A), exposure of WT mouse islet cells to PICex+IFN- γ induced a clear increase in apoptosis (Fig. 1B). Disruption of the TLR3 pathway prevented the deleterious effects of PICex+IFN- γ (Fig. 1B). PICin-induced apoptosis was partially prevented by TLR3 disruption, but the percentage of cell death remained higher than in control cells (Fig. 1B). Suppression of the TLR3 pathway did not protect against IL-1 β +IFN- γ -induced cell death, 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 beta cell death.

TLR3 is required to activate NF- κ B in response to dsRNA. Activation of the transcription factor NF- κ B is a key mediator of beta cell apoptosis induced by cytokines or PICex (33;34). We therefore analysed whether dsRNA activates NF- κ B in purified rat beta cells. Both PICex and PICin induced nuclear translocation of NF- κ B in beta cells, (54 \pm 12 and 80 \pm 6 % positive cells, respectively; *p* < 0.05 and *p* < 0.005 vs control; *n* = 4), while IL-1 β , used as positive

control, activated NF- κ B in 86 ± 7 % of the cells ($p < 0.005$ vs ctrl; $n = 3$).

To examine whether dsRNA-induced activation of NF- κ B depends on TLR3 signaling, dispersed islet cells from WT or TLR3^{-/-} mice were evaluated (Fig. 2). NF- κ B activation was induced by both PICex and PICin in WT cells, with a more potent effect of PICin (Fig. 2B).

In TLR3^{-/-} cells, PICex failed to activate NF- κ B (Fig. 2A and B) while PICin induced NF- κ B nuclear translocation in 63 ± 6 % of the cells (Fig. 2B). NF- κ B activation by PICin, however, was somewhat lower in TLR3^{-/-} than in WT cells (Fig. 2B). IL-1 β -induced NF- κ B activation, used as positive control, was similar in TLR3^{-/-} and WT cells (data not shown). Of note, inhibition of NF- κ B activation by the use of an adenoviral construct expressing a non degradable form of I κ B α (33) did not prevent PICin-induced apoptosis (Fig. S2), suggesting that NF- κ B activation is not a key mediator of PICin-triggered beta cell death.

These results demonstrate that in beta cells PICex activates NF- κ B exclusively via TLR3 signaling while PICin-induced NF- κ B activation depends both on TLR3 and on other pathways.

TLR3 mediates external -but not internal-dsrRNA-induced IRF-3 activation in beta 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 interferons (35).

As shown in Fig. 3A, treatment of rat beta 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 WT mouse and rat beta cells (Fig. 3 A and B).

To investigate the role of TLR3 in dsRNA-mediated IRF-3 activation, islet cells from WT and TLR3^{-/-} mice were treated with external or internal PIC. As shown in figure 3B, exposure to PICex for 4 hours induced IRF-3 phosphorylation in WT cells but not in TLR3^{-/-} cells. On the other hand, IRF-3 activation was observed after PICin

treatment in both WT and TLR3^{-/-} islet cells (Fig. 3B).

These results indicate that IRF-3 activation by PICex depends on TLR3 stimulation, while PICin induces IRF-3 phosphorylation via TLR3 independent mechanism(s).

Suppression of IRF-3 signaling prevents internal dsRNA- induced beta cell apoptosis.

To evaluate the role of IRF-3 signaling in dsRNA-mediated apoptosis, islet cells were isolated from WT and IRF-3^{-/-} mice. As shown in Fig. 4, exposure to PICex+IFN- γ induced a comparable degree of apoptosis in WT and IRF-3^{-/-} islet cells. On the other hand, PICin markedly increased apoptosis in WT 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 beta cell function, glucose-stimulated insulin secretion was performed in islet cells isolated from WT 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 (Table S2). Exposure to PICex and PICin decreased insulin content in WT ($p < 0.05$ and $p < 0.005$ vs ctrl, respectively; $n=12$) but not in IRF-3^{-/-} cells ($p > 0.05$; $n=9$; Table S2).

Basal insulin release was similar between WT and IRF3^{-/-} cells and was not affected by the different experimental conditions (Fig. 5, A and B). Exposure to glucose+forskolin increased insulin secretion by 3-fold in control WT 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 WT islets (10;11;36). In islet cells isolated from WT mice, PICin treatment caused a trend ($p = 0.07$) for a decrease in glucose+forskolin-induced insulin secretion when compared to control cells (Fig. 5 A). By contrast, insulin release was not affected by PICin in IRF-3^{-/-} islet cells (Fig. 5 B; $p = 0.181$). 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 WT islet cells when exposed to PICex+IFN- γ (Figs. 4 and 5; Table S2).

Taken together, these data suggest a key role for IRF-3 signaling in internal dsRNA-induced beta cell dysfunction and death, while IRF-3 plays a minor role in beta cell apoptosis triggered by PICex+IFN- γ .

Autocrine production of IFN- β contributes for dsRNA-induced apoptosis. Exposure of beta cells to PICex increased expression of IFN- α and IFN- β 2-10 times above control values, while PICin induced a 50-2000 fold up-regulation of type I IFN mRNAs (Fig. 6A and 6B). Genes downstream of dsRNA signaling, including PKR, TLR3 and RANTES were up-regulated 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.5 days (Fig. 6C), while it was below detection in WT 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. 6C).

To further evaluate the role of such a massive autocrine production of type I IFNs in PICin-induced apoptosis, islet cells from WT or type I IFN receptor knockout mice (IFNAR1^{-/-}) were treated with PICin for 5 days. IFNAR1^{-/-} cells were protected against PICin-induced apoptosis (Fig. 6D). Of note, IFNAR1^{-/-} cells secreted 5 times less IFN- β than WT cells (Fig. 6C). In accordance with these observations, an antibody raised against rat IFN- β also induced a 70% protection against PICin-induced apoptosis (Fig. S3).

These results show that PICin markedly up-regulates IFN- α/β mRNA expression and triggers secretion of IFN- β by the beta 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 beta cell apoptosis depends on autocrine effects of type I IFNs.

Internal dsRNA induces eIF2 α phosphorylation, inhibits protein

biosynthesis and up-regulates ER-stress marker genes in beta cells. Exposure of beta cells to PICex did not induce eIF2 α phosphorylation, while internalization of dsRNA resulted in a rapid phosphorylation of eIF2 α which persisted through 4 hours before returning to basal level (Fig. 7A). In line with these data, PICin reduced protein biosynthesis to 50% of the control value (Fig. 7B) while PICex induced a less marked decrease in protein biosynthesis (Fig. 7B). Cycloheximide (CHX) suppressed translation by 90 % (Fig. 7B).

To evaluate whether eIF2 α 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. 8A) and CHOP (Fig. 8B) and of the ER chaperone BiP (Fig. 8D). Activation of the ER stress response was confirmed by increased expression of the spliced form of X-box binding protein-1 (Xbp-1) (Fig. 8C). Exposure to PICex did not affect expression of these genes (Fig. 8 A-D).

DISCUSSION

Epidemiological evidence, post-mortem 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 beta cell apoptosis and in triggering the early cellular events that will lead to insulinitis and autoimmunity remain, however, 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 beta cells (13); present data).

To dissect the signaling pathways downstream of dsRNA in pancreatic beta 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 beta cell apoptosis; in the presence of IFN- γ , however, it induces apoptosis in more than 50% of the cells. Similar results were previously observed in the presence of type I IFNs + dsRNA (13). Disruption of the TLR3 pathway completely abrogates PICex+IFN- γ -induced cell death (present data). Stimulation of TLR3 by PICex activates NF- κ B and IRF-3. Inhibition of NF- κ B activation prevents both cytokine- and PICex+IFN- γ -induced beta cell apoptosis, suggesting that NF- κ B has pro-apoptotic effects (34;40;41). The PICex-induced activation of NF- κ B via TLR3 could therefore contribute for the presently observed pro-apoptotic effects of TLR3 stimulation.

Cytosolic accumulation of dsRNA induces a more intense activation of NF- κ B and IRF-3, and a higher prevalence of apoptosis as compared to PICex, suggesting that NF- κ B and IRF-3 may play a role in this process. Inhibition of NF- κ B activation, however, did not protect against PICin-induced cell death. In other cell types TLR3-mediated IRF-3 and NF- κ B activation transactivates IFN- β and IFN- α . Once secreted, IFN- α/β act in an autocrine/paracrine manner, leading up-regulation 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 *vs* internal dsRNA could be due to the marked PICin-induced up-regulation of IFN- β mRNA expression and secretion by the beta 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 effect of PICin, confirming that IFN- α/β signaling contributes to beta 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 favour 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 diabetes 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 eukaryotic initiation factor 2-subunit (eIF2 α) 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 eIF2 α phosphorylation.

eIF2 α phosphorylation may also occur in response to other cellular stresses, resulting from activation of kinases such as PERK, a PKR-like ER kinase which responds to endoplasmic reticulum (ER) stress. Pancreatic beta cells are highly sensitive to ER stress and a tight control of ER homeostasis is crucial to preserve beta cell function and survival (48) and prolonged eIF2 α phosphorylation was described as pro-apoptotic in beta cells (31). It is conceivable that a massive and prolonged PICin-triggered production of type I IFNs-induced proteins, such as cytokines, chemokines and HLA-1 components (16;28), overloads the ER capacity triggering an excessive ER stress response and favouring beta cell apoptosis. In line with this hypothesis, PICin, but not PICex, upregulates ER stress marker genes in beta 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 beta cell

apoptosis by essentially different mechanisms (Fig. S4). Both external and internal dsRNA induce activation of NF- κ B and IRF-3 in pancreatic beta 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 beta 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 beta cells, and clarifies part of the complex molecular responses of beta cells faced with viral components in the intra- or extra- cellular space. We propose that activation of immune pathways and production of type I interferons by the beta cells play a relevant role in the development of insulinitis and eventually type 1 diabetes.

As recently described by Baccala et al. (49), type I interferons induction by nucleic acids or apoptotic materials via TLR-dependent and -independent pathways might be a central pathogenic event in autoimmunity.

ACKNOWLEDGEMENTS

This study was supported by grants from the EFSD/Lilly European Diabetes Research Programme, the "Fonds David et Alice Van Buuren", the "Fonds de la Recherche Scientifique Médicale" (convention n° 3.4514.03/avenant 3.4554.05), the "Actions de Recherche Concertées (ARC – convention 04/09-311). The Belgium Program on Interuniversity Poles of Attraction initiated by the Belgium State (IUAP P6/40) and The European Union Project SAVEBETA (contract n° 036903 in the FP6 of the EC). We thank Prof. T. Taniguchi (University of Tokyo, Japan) for kindly providing the IRF-3 knock-out mice. We are grateful to N. El Amrite, J. Schoonheydt, M. Urbain, and G. Vandebroek for expert technical assistance.

REFERENCES

1. Jun, HS, Yoon, JW: A new look at viruses in type 1 diabetes. *Diabetes Metab Res Rev* 19:8-31, 2003
2. Knip, M, Veijola, R, Virtanen, SM, Hyoty, H, Vaarala, O, Akerblom, HK: Environmental triggers and determinants of type 1 diabetes. *Diabetes* 54 Suppl 2:S125-136, 2005
3. Roivainen, M: Enteroviruses: New findings on the role of enteroviruses in type 1 diabetes. *Int J Biochem Cell Biol* 38:721-725, 2006
4. Sarmiento, L, Cabrera-Rode, E, Lekuleni, L, Cuba, I, Molina, G, Fonseca, M, Heng-Hung, L, Borroto, AD, Gonzalez, P, Mas-Lago, P, Diaz-Horta, O: Occurrence of enterovirus RNA in serum of children with newly diagnosed type 1 diabetes and islet cell autoantibody-positive subjects in a population with a low incidence of type 1 diabetes. *Autoimmunity* 40:540-545, 2007
5. Jun, HS, Yoon, JW: The role of viruses in type I diabetes: two distinct cellular and molecular pathogenic mechanisms of virus-induced diabetes in animals. *Diabetologia* 44:271-285, 2001
6. Jacobs, BL, Langland, JO: When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology* 219:339-349, 1996
7. Majde, JA, Guha-Thakurta, N, Chen, Z, Bredow, S, Krueger, JM: Spontaneous release of stable viral double-stranded RNA into the extracellular medium by influenza virus-infected MDCK epithelial cells: implications for the viral acute phase response. *Arch Virol* 143:2371-2380, 1998
8. Sobel, DO, Newsome, J, Ewel, CH, Bellanti, JA, Abbassi, V, Creswell, K, Blair, O: Poly I:C induces development of diabetes mellitus in BB rat. *Diabetes* 41:515-520, 1992
9. Ewel, CH, Sobel, DO, Zeligs, BJ, Bellanti, JA: Poly I:C accelerates development of diabetes mellitus in diabetes-prone BB rat. *Diabetes* 41:1016-1021, 1992
10. Heitmeier, MR, Scarim, AL, Corbett, JA: Double-stranded RNA inhibits beta-cell function and induces islet damage by stimulating beta-cell production of nitric oxide. *J Biol Chem* 274:12531-12536, 1999
11. Scarim, AL, Arnush, M, Blair, LA, Concepcion, J, Heitmeier, MR, Scheuner, D, Kaufman, RJ, Ryerse, J, Buller, RM, Corbett, JA: Mechanisms of beta-cell death in response to double-stranded (ds) RNA and interferon-gamma: dsRNA-dependent protein kinase apoptosis and nitric oxide-dependent necrosis. *Am J Pathol* 159:273-283, 2001
12. Beutler, B: Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* 430:257-263, 2004
13. Rasschaert, J, Ladriere, L, Urbain, M, Dogusan, Z, Katabua, B, Sato, S, Akira, S, Gysemans, C, Mathieu, C, Eizirik, DL: Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells. *J Biol Chem* 280:33984-33991, 2005
14. Vives-Pi, M, Somoza, N, Fernandez-Alvarez, J, Vargas, F, Caro, P, Alba, A, Gomis, R, Labeta, MO, Pujol-Borrell, R: Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to endotoxin (LPS) in islet beta cells. *Clin Exp Immunol* 133:208-218, 2003
15. Wen, L, Peng, J, Li, Z, Wong, FS: The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets. *J Immunol* 172:3173-3180, 2004
16. Ylipaasto, P, Kutlu, B, Rasilainen, S, Rasschaert, J, Salmela, K, Teerijoki, H, Korsgren, O, Lahesmaa, R, Hovi, T, Eizirik, DL, Otonkoski, T, Roivainen, M: Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. *Diabetologia* 48:1510-1522, 2005
17. Oshiumi, H, Matsumoto, M, Funami, K, Akazawa, T, Seya, T: TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 4:161-167, 2003
18. Yamamoto, M, Sato, S, Mori, K, Hoshino, K, Takeuchi, O, Takeda, K, Akira, S: Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 169:6668-6672, 2002
19. Taniguchi, T, Takaoka, A: The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr Opin Immunol* 14:111-116, 2002

20. Huang, X, Yuang, J, Goddard, A, Foulis, A, James, RF, Lernmark, A, Pujol-Borrell, R, Rabinovitch, A, Somoza, N, Stewart, TA: Interferon expression in the pancreases of patients with type I diabetes. *Diabetes* 44:658-664, 1995
21. Devendra, D, Jasinski, J, Melanitou, E, Nakayama, M, Li, M, Hensley, B, Paronen, J, Moriyama, H, Miao, D, Eisenbarth, GS, Liu, E: Interferon-alpha as a mediator of polyinosinic:polycytidylic acid-induced type 1 diabetes. *Diabetes* 54:2549-2556, 2005
22. Lang, KS, Recher, M, Junt, T, Navarini, AA, Harris, NL, Freigang, S, Odermatt, B, Conrad, C, Ittner, LM, Bauer, S, Luther, SA, Uematsu, S, Akira, S, Hengartner, H, Zinkernagel, RM: Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat Med* 11:138-145, 2005
23. Alexopoulou, L, Holt, AC, Medzhitov, R, Flavell, RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732-738, 2001
24. Pipeleers, DG, in't Veld, PA, Van de Winkel, M, Maes, E, Schuit, FC, Gepts, W: A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806-816, 1985
25. Hoorens, A, Van de Casteele, M, Kloppel, G, Pipeleers, D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568-1574, 1996
26. Liu, D, Darville, M, Eizirik, DL: Double-stranded ribonucleic acid (RNA) induces beta-cell Fas messenger RNA expression and increases cytokine-induced beta-cell apoptosis. *Endocrinology* 142:2593-2599, 2001
27. Cardozo, AK, Kruhoffer, M, Leeman, R, Orntoft, T, Eizirik, DL: Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. *Diabetes* 50:909-920, 2001
28. Rasschaert, J, Liu, D, Kutlu, B, Cardozo, AK, Kruhoffer, M, ORntoft, TF, Eizirik, DL: Global profiling of double stranded RNA- and IFN-gamma-induced genes in rat pancreatic beta cells. *Diabetologia* 46:1641-1657, 2003
29. Pirot, P, Eizirik, DL, Cardozo, AK: Interferon-gamma potentiates endoplasmic reticulum stress-induced death by reducing pancreatic beta cell defence mechanisms. *Diabetologia* 49:1229-1236, 2006
30. Dogusan, Z, Hooghe, R, Verdood, P, Hooghe-Peters, EL: Cytokine-like effects of prolactin in human mononuclear and polymorphonuclear leukocytes. *J Neuroimmunol* 120:58-66, 2001
31. Cnop, M, Ladriere, L, Hekerman, P, Ortis, F, Cardozo, AK, Dogusan, Z, Flamez, D, Boyce, M, Yuan, J, Eizirik, DL: Selective inhibition of EIF2alpha dephosphorylation potentiates fatty acid-induced er stress and causes pancreatic beta -cell dysfunction and apoptosis. *J Biol Chem* 282:3989-3997, 2007
32. Eizirik, DL: Interleukin-1 beta induces an early decrease in insulin release, (pro)insulin biosynthesis and insulin mRNA in mouse pancreatic islets by a mechanism dependent on gene transcription and protein synthesis. *Autoimmunity* 10:107-113, 1991
33. Heimberg, H, Heremans, Y, Jobin, C, Leemans, R, Cardozo, AK, Darville, M, Eizirik, DL: Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. *Diabetes* 50:2219-2224, 2001
34. Liu, D, Cardozo, AK, Darville, MI, Eizirik, DL: Double-stranded RNA cooperates with interferon-gamma and IL-1 beta to induce both chemokine expression and nuclear factor-kappa B-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulinitis and beta-cell death in type 1 diabetes mellitus. *Endocrinology* 143 :1225-1234, 2002
35. Servant, MJ, Grandvaux, N, Hiscott, J: Multiple signaling pathways leading to the activation of interferon regulatory factor 3. *Biochem Pharmacol* 64:985-992, 2002
36. Rhodes, CJ, Taylor, KW: Effect of interferon and double-stranded RNA on B-cell function in mouse islets of Langerhans. *Biochem J* 228:87-94, 1985
37. Guillot, L, Le Goffic, R, Bloch, S, Escriou, N, Akira, S, Chignard, M, Si-Tahar, M: Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 280:5571-5580, 2005
38. Wang, T, Town, T, Alexopoulou, L, Anderson, JF, Fikrig, E, Flavell, RA: Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 10:1366-1373, 2004

39. Li, K, Foy, E, Ferreon, JC, Nakamura, M, Ferreon, AC, Ikeda, M, Ray, SC, Gale, M Jr, Lemon, SM: Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A* 102:2992-2997, 2005
40. Robbins, MA, Maksimova, L, Pockock, E, Chantler, JK: Nuclear factor-kappaB translocation mediates double-stranded ribonucleic acid-induced NIT-1 beta-cell apoptosis and up-regulates caspase-12 and tumor necrosis factor receptor-associated ligand (TRAIL). *Endocrinology* 144:4616-4625, 2003
41. Gysemans, CA, Ladriere, L, Callewaert, H, Rasschaert, J, Flamez, D, Levy, DE, Matthys, P, Eizirik, DL, Mathieu, C: Disruption of the gamma-interferon signaling pathway at the level of signal transducer and activator of transcription-1 prevents immune destruction of beta-cells. *Diabetes* 54:2396-2403, 2005
42. Doyle, SE, O'Connell, R, Vaidya, SA, Chow, EK, Yee, K, Cheng, G: Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. *J Immunol* 170:3565-3571, 2003
43. Heylbroeck, C, Balachandran, S, Servant, MJ, DeLuca, C, Barber, GN, Lin, R, Hiscott, J: The IRF-3 transcription factor mediates Sendai virus-induced apoptosis. *J Virol* 74:3781-3792, 2000
44. Pelegrin, M, Devedjian, JC, Costa, C, Visa, J, Solanes, G, Pujol, A, Asins, G, Valera, A, Bosch, F: Evidence from transgenic mice that interferon-beta may be involved in the onset of diabetes mellitus. *J Biol Chem* 273:12332-12340, 1998
45. Foulis, AK, Farquharson, MA, Meager, A: Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus. *Lancet* 2:1423-1427, 1987
46. Devendra, D, Eisenbarth, GS: Interferon alpha--a potential link in the pathogenesis of viral-induced type 1 diabetes and autoimmunity. *Clin Immunol* 111:225-233, 2004
47. Flodstrom-Tullberg, M, Hultcrantz, M, Stotland, A, Maday, A, Tsai, D, Fine, C, Williams, B, Silverman, R, Sarvetnick, N: RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during coxsackievirus infection. *J Immunol* 174:1171-1177, 2005
48. Scheuner, D, Mierde, DV, Song, B, Flamez, D, Creemers, JW, Tsukamoto, K, Ribick, M, Schuit, FC, Kaufman, RJ: Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. *Nat Med* 11:757-764, 2005
49. Baccala, R, Hoebe, K, Kono, DH, Beutler, B, Theofilopoulos, AN: TLR-dependent and TLR-independent pathways of type I interferon induction in systemic autoimmunity. *Nat Med* 13:543-551, 2007

FIGURE LEGENDS

Figure 1. Suppression of the TLR3 signaling pathway protects beta cell against dsRNA-induced apoptosis. A. Percentage of apoptosis observed in FACS-purified rat beta cells cultured for 5 days in the absence (CTRL) or presence of PICex (100 µg/ml), PICex + IFN-γ (500 U/ml), PICin (1 µg/ml) or monomethyl-L-arginine (LMA; 1 mM). Cell viability was determined with the DNA-binding dyes HO 342 and PI. The results are means ± SEM of 5 individual experiments. *p<0.05; **p<0.001 vs. control; § p < 0.001 vs PICin, ANOVA followed by Student's *t*-test with the Bonferroni correction.

B. Percentage of apoptosis observed in WT or TLR3^{-/-} dispersed islet cells cultured for 5 days in the absence (CTRL) or presence of PICex (100 µg/ml), PICex+ IFN-γ (1000 U/ml), lipofectamine alone (LF) or PICin (10 µg/ml). The results are means ± SEM of 4-5 individual experiments; *p<0.05; **p<0.005 vs. respective controls; a p<0.005 vs. WT mice; ANOVA followed by Student's *t*-test with the Bonferroni correction.

Figure 2. External, but not internal, dsRNA-induced NF-κB activation is TLR3-dependent in beta cells. WT and TLR3^{-/-} dispersed mouse islet cells were cultured for 4 hours in the absence (CTRL or lipofectamine, LF) or presence of PICex (100 µg/ml) or PICin (1 µg/ml). Activation of NF-κB and expression of insulin were analysed by double immunocytochemistry. Cells were fixed, 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 (FITC, X400) and insulin fluorescence in red (rhodamine, X400) (Fig. 3A). The percentage of cells positive for NK-κB nuclear localization is shown in Fig. 3B. The results represent means ± SEM of 3 independent experiments; *p<0.05, **p<0.01, ***p<0.005 vs. control; ^a p<0.01 vs. external PIC; [§] p<0.001 vs WT mice; ANOVA followed by Student's *t*-test with the Bonferroni correction.

Figure 3. TLR3 signaling pathway mediates external -but not internal- dsRNA-induced IRF-3 activation in pancreatic beta cells. FACS-purified rat beta cells (Fig. 4A) or WT and TLR3^{-/-} dispersed mouse islet cells (Fig. 4B) were cultured in absence (CTRL or LF), or presence of PIC external (100 µg/ml) or PIC internal (1 – 10 µg/ml) for the indicated time points. Whole cell lysates were isolated and resolved on 10 % SDS-PAGE gel followed by immunoblotting with p-IRF-3 antibody (upper panel). The membranes were stripped and probed with β-actin antibody, used as a loading control (lower panel). 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 3 to 6 similar experiments.

Figure 4. Suppression of IRF-3 signaling prevents internal dsRNA-induced beta cell apoptosis. Percentage of apoptosis observed in WT 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 (1000 U/ml) or PICin (10 µg/ml). Cell viability was determined with the DNA-binding dyes HO 342 and PI. The results are means ± SEM of 4 -7 individual experiments; *p<0.05, **p<0.01 vs. control; ANOVA followed by Student's *t*-test with the Bonferroni correction.

Figure 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^{-/-} mice (B) treated for 2 days with PICex (100 µg/ml) and/or IFN-γ (1000 U/ml) or with PICin (10µg/ml) and then exposed for 2 hours to glucose 1.7 mM (white bars) or 16.7 mM +

