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Global profiling of double stranded RNA- and IFN-γ-induced genes in rat pancreatic beta cells

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Abstract

Aims/hypothesis. Viral infections and local production of IFN-γ might contribute to beta-cell dysfunction/death in Type 1 Diabetes. Double stranded RNA (dsRNA) accumulates in the cytosol of viral-infected cells, and exposure of purified rat beta cells to dsRNA (tested in the form of polyinosinic-polycytidylic acid, PIC) in combination with IFN-γ results in beta-cell dysfunction and apoptosis. To elucidate the molecular mechanisms involved in PIC + IFN-γ-effects, we determined the global profile of genes modified by these agents in primary rat beta cells.
Methods. FACS-purified rat beta cells were cultured for 6 or 24 h in control condition or with IFN-γ, PIC or a combination of both agents. The gene expression profile was analysed in duplicate by high-density oligonucleotide arrays representing 5000 full-length genes and 3000 EST's. Changes of greater than or equal to 2.5-fold were considered as relevant.
Results. Following a 6- or 24-h treatment with IFN-γ, PIC or IFN-γ and PIC, we observed changes in the expression of 51 to 189 genes. IFN-γ modified the expression of MHC-related genes, and also of genes involved in beta-cell metabolism, protein processing, cytokines and signal transduction. PIC affected preferentially the expression of genes related to cell adhesion, cytokines and dsRNA signal transduction, transcription factors and MHC. PIC and/or IFN-γ up-regulated the expression of several chemokines and cytokines that could contribute to mononuclear cell homing and activation during viral infection, while IFN-γ induced a positive feedback on its own signal transduction. PIC + IFN-γ inhibited insulin and GLUT-2 expression without modifying *pdx*-1 mRNA expression.
Conclusion/interpretation. This study provides the first comprehensive characterization of the molecular responses of primary beta cells to dsRNA + IFN-γ, two agents that are probably present in the beta cell milieu during the course of virally-induced insulitis and Type 1 Diabetes. Based on these findings, we propose an integrated model for the molecular mechanisms involved in dsRNA + IFN-γ induced beta-cell dysfunction and death. [Diabetologia (2003) 46:1641–1657]

Keywords Double stranded RNA, microarray analysis, apoptosis, pancreatic beta cells, interferon-γ, nitric oxide, NF-κB, diabetes mellitus.

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Abbreviations: AS, argininosuccinate synthetase; ADAR, RNA-specific adenosine deaminase; Bax, bcl-2 associated x protein; BB rats, diabetes-resistant BioBreeding rats; bcl-2, B-cell lymphoma/leukemia-2; BH3, bcl-2 homology domain-3; Bid, BH3 interacting domain death agonist; dsRNA, double stranded RNA; CCK, cholecystokinin; eIF2α, eukaryotic initiation factor 2α; EMC-D, encephalomyocarditis virus; ER, endoplasmic reticulum; ESTs, expressed sequence tags; GADD, growth arrest and DNA damage; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GH, growth hormone; GIP, gastric inhibitory peptide; HMGCoA, 3-hydroxy 3-methylglutaryl coenzyme A; HO, heme oxygenase; hsp, heat shock protein; ICE, interleukin converting enzyme/caspase 1; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IRF, interferon regulatory factor; JAK-1/2, Janus tyrosine kinase 1 and 2; MAP, mitogen activated protein; MCP-1, macrophage chemoattractant protein-1; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; MnSOD, manganese superoxide dismutase; MX, myxovirus; NF, nuclear factor; NO, nitric oxide; NOD mouse, non obese diabetic mouse; MGMT, O-6-methylguanine-DNA methyltransferase; OAS, 2′,5′-oligoadenylate synthetase; PC, prohormone convertase; PDX-1, pancreatic duodenal homeobox factor-1; PI, propidium iodide; PIC, polyinosinic-polycytidylic acid; PKR, dsRNA dependent protein kinase; RANTES, regulated upon activation, normal T-cell expressed, and presumably secreted; SERCA-2, sarco(endo)plasmic reticulum Ca^{+2} ATPase type 2; STAT, signal transducers and activators of transcription; T1DM, type 1 diabetes mellitus; TLR, toll-like receptor.

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Type 1 diabetes mellitus is an auto-immune disease associated with a progressive and selective destruction of the insulin-producing pancreatic beta cells [1, 2]. Although a genetic susceptibility seems to be a prerequisite for the development of Type 1 Diabetes [3, 4], it is now clear that environmental factors such as viral infections are also important aetiological determinants [2, 5].

There is extensive epidemiological evidence for the involvement of viral infections in the pathogenesis of Type 1 Diabetes [6]. Up to now, 13 different viruses, most of them belonging to the enterovirus family, have been found to be associated with the onset of Type 1 Diabetes in humans and in various animal models [7]. Different mechanisms have been proposed for the role of viruses in the pathogenesis of Type 1 Diabetes. These include: (i) infection and rapid destruction of beta cells [8]; (ii) triggering of local inflammation, leading to destruction of the beta cells through the production of NO, cytokines and other immune mediators in a mechanism referred to as “innocent bystander killing” [9, 10]; (iii) molecular mimicry, based on a partial sequence homology between a protein of the infected cells (i.e. GADD65) and a viral antigen, leading to autoimmune destruction of the beta cell [11]; (iv) viral infection, coupled with one or more of the factors described above, acting in conjunction to induce beta cell death [12]. For example, mouse infection with a high titre of the D variant of the encephalomyocarditis (EMC-D) virus leads to beta-cell destruction and diabetes mainly as a result of viral replication within beta cells, while mouse infection with a low titre of EMC-D virus leads to diabetes as a chronic process, caused by the destruction of beta cells by soluble mediators such as IL-1β, TNF-α/β and NO produced by macrophages or the beta cells themselves [7].

The molecular mechanisms involved in beta cells damage by viruses, alone or in combination with soluble mediators, remain to be elucidated. During viral infection, accumulation of the viral replicative intermediate double stranded RNA (dsRNA) in the cytosol of the infected cell stimulates antiviral activities, such as dsRNA-dependent protein kinase (PKR) activation, type I interferons and NO production and a general inhibition of protein translation [13, 14]. These antiviral responses can be mimicked by treatment of cells with the synthetic dsRNA polyinosinic-polycytidylic acid (PIC) [15, 16]. PIC triggers the development of hyperglycaemia in diabetes-resistant BioBreeding (BB) rats and accelerates the development of the disease in diabetes-prone BB rats [17, 18]. In vitro, PIC inhibits glucose-stimulated insulin biosynthesis in mouse islets [19] and when used in combination with IFN-γ affects rat islet cell function and viability by a mechanism involving, at least in part, increased inducible nitric oxide synthase (iNOS) expression and NO production [20, 21].

We have previously shown that exposure of fluorescence-activated cell sorting-(FACS)-purified rat beta cells to PIC alone does not induce cell death. However, when these cells are exposed to PIC + IL-1β or PIC + IFN-γ they die mostly by apoptosis. The mechanisms of death induction are either NO-dependent in the case of PIC + IL-1β, or NO-independent, in the case of PIC + IFN-γ [21]. Moreover, PIC regulates the expression of several genes that could participate in the induction of islet inflammation and beta-cell death, such as Fas, iNOS, IL-15 and diverse chemokines [21, 22]. The complete range of genes induced by PIC and cytokines in pancreatic beta cells remains, however, to be clarified.

Evaluation of complex patterns of gene expression is now feasible by the use of microarray analysis [23]. We have previously used high-density oligonucleotide arrays to analyse FACS-purified rat beta cells exposed for 6 or 24 h to IL-1β + IFN-γ [24, 25]. Based on the findings obtained, we proposed that beta-cell fate following cytokines exposure, namely death by apoptosis or survival with or without complete functional recovery, depends on the intricate pattern of dozens of genes up- or down-regulated in parallel and/or sequentially. Moreover, these and subsequent data on INS-1 cells [26] allowed us to start an annotated “Beta Cell Gene Bank”, including information on nearly 3000 genes expressed in beta cells and in INS-1 cells, of which 700 are modified by cytokines. Against this background, we carried out a microarray analysis of FACS-purified rat pancreatic beta cells exposed for 6 or 24 h to PIC, IFN-γ or a combination of both agents. The data obtained provide the first broad picture on how a primary beta cell responds to dsRNA and the inflammatory cytokine IFN-γ, an experimental condition which bears similarity to the in vivo situation during the course of a viral infection. The results obtained allowed us to propose a comprehensive hypothesis for the mechanisms involved in dsRNA and IFN-γ induced beta-cell dysfunction and death.

Materials and methods

Islet cell isolation and culture. Pancreatic islets were isolated from 10–12-week-old male Wistar rats by collagenase digestion, and subsequently dissociated into single cells in a calcium-free medium containing dispase (0.5 mg/ml). Single beta-cells were purified by autofluorescence-activated cell sorting (FACS) [27]. These preparations contain around 90–95% viable beta cells [data not shown, 27]. The purified beta cells were cultured in HAM’s F-10 medium (Invitrogen, Paisley, Scotland) supplemented with 10 mmol/l glucose [28]. For determination of viability, FACS-purified single beta cells (10^4 cells per well) were cultured for 6 days in Falcon 96-well microtitre plates (Becton Dickinson, New Jersey, N.J., USA) pre-coated with poly-L-lysine and containing 200 µl of medium. Culture medium was changed every 3 days and fresh IFN-γ or PIC was added. For RNA extraction for microarray or RT-PCR analysis, single beta cells were re-aggregated for 3 h

in a rotatory shaking incubator [29], cultured for 14–16 h in suspension, and then exposed for 6 or 24 h to IFN-γ (1000 U/ml; 10 U/ng; Invitrogen) or PIC (100 μg/ml; Sigma Chemical, St Louis, Mo., USA). In some experiments, recombinant human IL-1β (50 U/ml, 38 U/ng, a kind gift of Dr. C.W. Reynolds from the National Cancer Institute, Bethesda, Md., USA) was also utilized as a positive control. The concentrations of cytokines and PIC, and the time points for array analysis, were selected based on our previous studies in beta cells [21, 22, 24, 25], and aimed to analyse beta cells at time points which precede non-specific changes in mRNA expression induced by early apoptosis. Culture media were collected after 24 h for nitrite determination (nitrite is a stable product of NO oxidation), which was done spectrophotometrically at 546 nm wavelength after coloured reaction with the Griess reagent [30].

Assessment of beta cell protein synthesis and viability. Total protein biosynthesis was determined at 10 mmol/l glucose using L-[4,5-^{3}H] leucine incorporation and trichloroacetic precipitation [31]. The experiments were carried out in duplicate, using 6×10^4 re-aggregated beta cells per condition. The cells were exposed for 1, 5 and 23 h to IFN-γ + PIC (same concentrations as described above) or left untreated (control) before determination of protein biosynthesis for 2 h in the absence (control) or presence of IFN-γ + PIC. These time points were selected to cover an early time point (3 h), and then two time points placed 1 h after the time points used for microarray analysis (6 and 24 h), the rationale being that protein synthesis usually lags behind mRNA expression. As a positive control for protein biosynthesis inhibition, some control cells were exposed to cycloheximide (10 μmol/l) during the final 2 h of the incubation period.

The percentage of viable, apoptotic and necrotic beta cells was determined after 6 days exposure to IFN-γ and/or PIC [21, 22]. For this purpose, beta cells were incubated for 15 min with propidium iodide (PI, 10 μg/ml) and Hoechst (HO) 342 (10 μg/ml) [32]. This fluorescence assay for single beta cells is quantitative and has been validated by systematic comparisons with electron microscopy observations [32, 33]. The method has been successfully used to evaluate apoptosis/necrosis in rat [21, 22, 32], mouse [34, 35] and human [36] beta cells.

Microarray analysis. For microarray analysis, total RNA was isolated from beta cells (at least 10 μg/sample) and used to prepare biotinylated cRNA. The labelled cRNAs were hybridized in duplicate to the rat U34-A oligonucleotide array (Affymetrix, Santa Clara, Calif., USA) [24, 25]. Due to difficulties in obtaining a sufficient number of primary beta cells in a single isolation, and to decrease putative biases caused by biological variation, the cells were pooled from four independent experiments, using in each experiment $3.5–3.7\times10^5$ cells/group. We have shown before that microarray analysis, carried out in duplicate on pooled beta cell samples, provides a reliable estimation of massive changes in mRNA expression, as confirmed by a greater than 90% confirmation by RT-PCR of genes observed as modified in the array [24, 25, 37, present data]. Analysis of differential expression was carried out using the GeneChip Suite software, version 4.0.1 (Affymetrix, Santa Clara, Calif., USA). Arrays were normalized by global scaling, with the arrays scaled to an average intensity of 150. Genes were considered as modified by PIC and/or IFN-γ in case they fulfilled the following criteria [24, 25]: (i) the mRNA was present in either control or cells exposed to PIC and/or IFN-γ in both duplicates; (ii) the mean average fold change (experimental group vs control) was greater than or equal to 2.5; (iii) the fold change in each individual duplicate was greater than or equal to 2.0. We have used our own curated "Beta Cell Gene Bank" to assign the filtered genes into their respective functional clusters. The expressed sequence tags (ESTs) that had homology to a known sequence were annotated using the Resourcerer 6.0 database [38]. Non-identified ESTs are not shown here.

RT-PCR and real time RT-PCR. RT-PCR was done using poly(A)+ RNA as described [24]. The number of cycles was selected to allow linear amplification of the cDNA under study. The primer sequences used for amplification of rat cDNAs for GAPDH, GADD153 [24], *pdx-1*, GLUT-2 and c-Myc [25] and for sarco(endo)plasmic reticulum Ca^{2+} ATPase type 2 (also called SERCA-2) [39] were as described in the indicated references. For the other genes studied, the primer sequences and their respective PCR fragment lenghts were as follows: dsRNA-dependent protein kinase (PKR), forward (5′-AATCACGCCA-ACATTGTTCA-3′) and reverse (5′-CACCGGGTCTTGTATC-GACT-3′) (107 bp); RNA-specific adenosine deaminase (ADAR), forward (5′-AAGAAACAGGGCAAG-CAAGA-3′) and reverse (5′-TGTTGGTCAGAGCGTTGAAG-3′) (244 bp); CEBP/β, forward (5′-CAAGCTGAGCGACGAG-TACA-3′) and reverse (5′-CAGCTGCTCCACCTTCTTCT-3′) (147 bp) and Bip/GRP78, forward (5′-CTCAAAGAGCGCATTGACAC-3′) and reverse (5′-GCCACTTGGGCTATAGCATT-3′) (446 bp). The identity of the PCR fragments of each gene was confirmed by size after electrophoretic migration on ethidium bromide-stained agarose gels photographed under UV-transillumination using Kodak Digital Science DC120 camera (Kodak, Rochester, N.Y., USA). The data are shown as a representative figure for four similar experiments. Expression of the "housekeeping" gene GAPDH is not affected by exposure to cytokines in both whole islets and FACS-purified beta cells [21, 22, 40].

Real-time RT-PCR was done as described [41] using a Lightcycler instrument in a 20 μl reaction containing 3 mmol/l $MgCl_2$, 0.5 μmol/l forward and reverse primers, 2 μl FastStart SYBR Green mix (Roche), and 2 μl template cDNA. The primer sequences and their respective PCR fragment lenghts were as follows: GADD153, forward (5′-CCAGCAGAGGTCACA-AGCA-3′) and reverse (5′-CGCACTGACCACTCTGTTTC-3′) (126 bp); SERCA-2, forward (5′-TTGTGGCCCGAAACT-ACCT-3′) and reverse (5′-TTCATAATGAGCAGCACAAAG-GG-3′) (121 bp). The method used for quantification is the standard curve approach [42, 43]. To obtain the standard curve, the primer sequences and their respective PCR fragment lenghts were as follows: GADD153, forward (5′-GTCTCTGCC-TTTCGCCTTTG-3′) and reverse (5′-CTACCCTCAGTCCCC-TCCTC-3′) (605 bp); SERCA-2, forward (5′-TCTAGTCAC-CATAGAGATGTG-3′) and reverse (5′-TACTGACTGAGGT-AGCAGGA-3′) (912 bp).

Promoter studies. A plasmid construct containing the human *pdx-1* gene promoter linked to a luciferase reporter gene was kindly provided by Dr D. Melloul, Department of Endocrinology, Hadassah University Hospital, Jerusalem, Israel [44]. Transfected beta cells were exposed to cytokines and/or PIC for 24 h (same concentrations as above). Luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega) as previously described [21, 41]. Test values were corrected for the luciferase activity value of the internal control plasmid, pRL-CMV.

Statistical analysis. Results of microarray analysis are shown as means of two similar determinations. The results for other experiments are presented as means±SEM of at least three independent experiments. Statistical differences between the groups were determined by paired Student's *t*-test or ANOVA, as indicated. A *p* value of less than 0.05 was considered statistically significant.

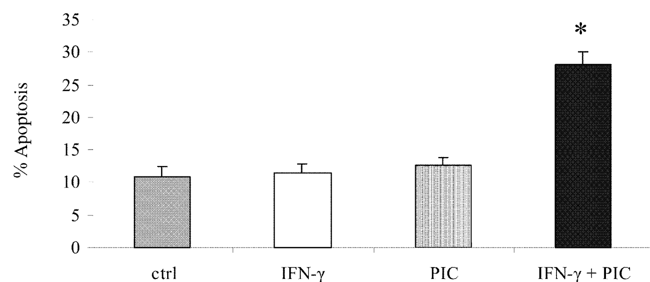

Fig. 1. Percentage of apoptosis observed in FACS purified beta cells exposed for 6 days to PIC (100 μg/ml) and/or IFN-γ (1000 U/ml). Cell viability was determined with the DNA-binding dyes Hoechst 342 and PI. Data are means±SEM of six experiments. * $p<0.001$ vs. control, ANOVA

Table 1. Total number of up- and down-regulated genes in rat pancreatic beta cells after 6 or 24 h

	6 h		24 h	
	Increased	Decreased	Increased	Decreased
IFN-γ	25 (28%)	64 (72%)	46 (88%)	6 (12%)
PIC	81 (94%)	5 (6%)	54 (95%)	3 (5%)
IFN-γ + PIC	130 (80%)	33 (20%)	140 (73%)	51(27%)
TOTAL	236 (70%)	102 (30%)	240 (80%)	60 (20%)

IFN-γ- and/or dsRNA-induced differences in gene expression were considered as present when the mean fold change of the duplicates was ≥2.5, and both individual fold change values were ≥2.0. Data are expressed in absolute numbers, or percentages (between parenthesis)

Results

Viability and nitrite production of beta cells exposed to PIC and/or IFN-γ. Exposure of FACS-purified beta cells to PIC and IFN-γ for 6 days induced a significant ($p<0.001$) increase in the percentage of apoptotic cells when compared against control cells. Neither the treatment with PIC nor with IFN-γ alone led to beta-cell death, indicating a synergistic effect between PIC and IFN-γ to induce beta-cell apoptosis (Fig. 1). We did not detect a significant increase in the percentage of necrotic cells (data not shown).

To identify by microarray analysis early and late IFN-γ and/or PIC induced or decreased genes in pancreatic beta cells, the cells were exposed for 6 or 24 h to the following conditions: control condition; IFN-γ (1000 U/ml); PIC (100 μg/ml); or the combination of both agents. When the beta cells were treated for 6 h with PIC and/or IFN-γ, there was no detectable nitrite production. However, after a 24-h treatment the combination of PIC and IFN-γ significantly increased nitrite production as compared to control, non-treated cells. Thus, the values for PIC + IFN-γ were 5.6±1.1 pmol nitrite·10^{-3}cells·h (means ± SEM; $n=6$; $p<0.05$ vs control) while control values were 0.7±0.4 pmol nitrite·10^{-3} cells·h (mean ± SEM; $n=6$). When tested alone, neither PIC nor IFN-γ affected nitrite production (data not shown). These data on viability and nitrite production are in good agreement with our previous observations [21, 22] and confirm that both IFN-γ and PIC were biologically active.

Identification of IFN-γ and/or PIC -modified genes in beta cells by microarray analysis. Cells from four separate experiments conducted as described above were pooled for RNA extraction, and the resulting biotinylated cRNAs were hybridized in duplicate to the Affymetrix rat U34-A oligonucleotide array containing about 8000 probes (77% known genes and 23% ESTs). Approximately 3759 (3300 to 4217) genes or ESTs were scored as present in each of the six conditions, in fair agreement with our previous observations [24, 25].

Following a 6-h exposure of beta cells to IFN-γ or PIC we observed changes in the expression of respectively 89 and 86 genes, while exposure to PIC + IFN-γ nearly doubled the number of modified genes (163 genes). A 24-h treatment induced a similar pattern, with 52–57 genes modified by IFN-γ or PIC alone, and a nearly four-fold increase in the number of changed genes by the combination of IFN-γ + PIC. Of note, except in the case of a 6-h exposure to IFN-γ, the majority (73–95%) of these modifications in gene expression consisted in up-regulation (Table 1). The PIC and/or IFN-γ responsive genes were clustered in 15 groups according to the putative biological function of their encoded proteins. Table 2 shows the percentages of affected genes observed in each experimental condition, expressed relative to the total number of modified genes. After treatment with IFN-γ alone for 6 h, the most frequent changes in gene expression were observed in beta-cell metabolism, protein processing and cytokine signal transduction. Of note, after 24-h exposure to IFN-γ, the MHC-related genes represented 31% of all modified genes. Exposure to PIC alone for 6 or 24 h affected preferentially the expression of genes related to cell adhesion, cytokine signal transduction, transcription factors and MHC. Beta-cell treatment with both IFN-γ and PIC led to a combined pattern of the modifications described above, but several novel genes were detected as changed when cells were exposed to a combination of both agents.

From the 340 IFN-γ and/or PIC modified genes, 161 genes of special relevance are presented in Table 3. The complete list of modified genes is presented in S1, deposited as "Supporting online material" at Diabetologia home page: http://dx.doi.org/10.1007/s00125-003-1245 .

Among the metabolism-related genes, a decrease in the expression of mRNAs encoding for genes involved in glucose metabolism, such as the beta-cell specific glucose transporter GLUT-2 and lactate dehy-

Table 2. Percentages of differentially expressed genes following a 6 or 24 h exposure to IFN-γ and/or PIC

	Gene clusters	6 h			24 h		
		IFN-γ %	PIC %	IFN-γ + PIC %	IFN-γ %	PIC %	IFN-γ + PIC %
1.	Metabolism	13.5	5.8	12.1	11.8	5	15.3
2.	Protein synthesis, modification and secretion	12.4	8.1	7.9	13.7	5	7.4
3.	Ionic channels, ions transporters and related proteins	7.9	1.2	1.2	2	0	1.6
4.	Hormones, growth factors and related genes	6.7	4.7	3	0	5	8.5
5.	Cytokines, chemokines and related receptors	2.2	7	6.1	3.9	5	2.1
6.	Cytokine processing and signal transduction	14.6	12.8	13.9	9.8	10	11.6
7.	MHC and related genes	9	12.8	11.5	31.4	17	10.1
8.	Cell adhesion and cytoskeleton	9	12.8	9.7	2	15	6.9
9.	Transcription factors and related genes	5.6	11.6	11.5	9.8	15	13.2
10.	RNA synthesis and splicing factors	0	0	0	0	0	1.1
11.	Cell cycle	3.4	2.3	2.4	0	0	1.1
12.	Defense/repair	5.6	5.8	6.7	0	3	4.8
13.	Apoptosis	4.5	1.2	1.2	2	3	2.6
14.	Anti-viral response	0	4.7	2.4	0	5	1.6
15.	Miscellaneous	5.6	9.3	10.3	13.7	12	12.2
	Total number of genes (absolute values)	**89**	**86**	**163**	**52**	**57**	**191**

IFN-γ- and/or dsRNA-induced differences in gene expression were considered as present when the mean fold change of the duplicates was ≥2.5, and both individual fold change values were ≥2.0. Data are expressed as percentage of the total number of modified genes observed in each condition (*bold numbers*), taken as 100%

drogenase (isoforms A and B) was observed (Table 3; item 1.1). Interestingly, expression of glucokinase was not affected by any of the tested conditions. While expression of glycolytic enzymes was down-regulated, several genes involved or related to lipid metabolism, such as ATP-citrate lyase, 3-hydroxy-3 methylglutaryl-coenzyme A (HMGCoA) reductase, LDL receptor, mitochondrial cytochrome P450 and stearyl-CoA desaturase were up-regulated following exposure for 6 or 24 h to both IFN-γ and PIC (Table 3, item 1.4). Of note, exposure to IFN-γ and PIC led to modifications in key genes related to arginine metabolism and nitric oxide formation (Table 3, item 1.2). The modified genes are arginase (decreased), argininosuccinate synthetase, iNOS and the cationic amino acid transporter-1 (all increased) and correlate well with the observed increased production of nitrite after 24-h treatment by both agents (see above).

After 24-h treatment with IFN-γ and PIC, there was down-regulation of mRNAs encoding mitochondrial subunits of respiratory chain genes involved in ATP production, such as NADH dehydrogenase and cytochrome b5 reductase, coupled with an up-regulation of uridine kinase (Table 3, item 1.5). IFN-γ + PIC also down-regulated expression of mRNAs encoding receptors for the incretins cholecystokinin-A and gastric inhibitory peptide and of growth hormone receptor (Table 3, item 4.0). This, associated with the observed decrease in insulin and pro-hormone convertase (PC) 1 mRNA levels, and of genes involved in ATP production, could contribute for the decreased insulin secretion observed after treatment of pancreatic islets or beta cells with IFN-γ + PIC [20, 45].

Several chemokines, cytokines, as well as genes involved in signal transduction, were modified by IFN-γ or PIC, alone and specially in combination (Table 3, items 5.0 and 6.0). When tested alone, IFN-γ affected expression of the chemokines macrophage inhibitory cytokine-1 (MIC-1) and interferon inducible protein (IP)-10 and of the cytokine IL-15 (Table 3, item 5.0). IL-15, IP-10, MIP-3α, macrophage chemoattractant protein (MCP)-1 and fractalkine mRNAs were induced by PIC and/or the combination of IFN-γ + PIC. Induction of RANTES and TNF-β mRNAs were also observed in pancreatic beta cells exposed to PIC and/or PIC + IFN-γ. It has been previously shown that RANTES and IP-10 are secondarily up-regulated by autocrine production of IFN-β in RAW 264.7 cells, a murine macrophage cell line [46]. In the present microarray analysis however, neither PIC nor IFN-γ, nor PIC and IFN-γ together, modified the expression of IFN-α, IFN-β and IL-1β, at least at our selected time points (data not shown).

Interestingly, the concomitant presence of PIC and IFN-γ led to up-regulation of Notch1 receptor and one of his ligands, Delta-1. Presenilin-2, an enzyme required for intramembraneous proteolysis of Notch was also up-regulated (Table 3; item 6.0). PIC and IFN-γ, however, did not modify expression of Jagged, another Notch ligand (data not shown). There was also up-regulation of JAK-2, Pim-3 serine threonine kinase and CL100 protein tyrosine phosphatase which, asso-

Table 3. List of selected genes modified after a 6- or 24-hour exposure to PIC and/or IFN-γ

Cluster/GAN	Gene Name	6h			24h		
		IFN-γ	PIC	IFN-γ+PIC	IFN-γ	PIC	IFN-γ+PIC
1.0 Metabolism							
1.1 Carbohydrates							
X59737	Creatine kinase—ubiquitous *				5.4	11.4	10.9
M86240	Fructose-1,6-bisphosphatase			7.9			
M13979	Glucose transporter type 1 (GLUT 1)						3.1
L28126	Glucose transporter type 2 (GLUT 2) *						–6.8
M54926	Lactate dehydrogenase A						–2.7
L25387	Phosphofructokinase c (PFK-c)			3.9			
1.2 Arginine metabolism and NO formation							
J02720	Arginase						–4.6
X12459	Argininosuccinate synthetase						4.3
AA957917	Cationic amino acid transporter-1						3.1
D44591	iNOS*			10.5			9.1
1.3 Aminoacids (other than arginine)							
AA942685	Cysteine dioxygenase			–3.6			–2.8
M72422	Glutamic acid decarboxylase 65 (GAD 65)	–3.9					–5.2
AI176504	Glutaminase	–6.5		–8.7	–6.3		
AA852004	Glutamine synthetase	–3.4	2.8	2.8			
M84648	L-amino acid decarboxylase						–11.9
1.4 Lipids							
AA799489	Acyl-CoA oxidase	–6.0					
L27075	ATP-cytrate lyase	–5.2	2.7	3.2			
M29249	3-hydroxy-3 methylglutaryl coenzyme A (HMGcoA) reductase			9.9			
X13722	LDL-receptor			4.9			
AI044900	Long-chain acyl-CoA synthetase				5.6		
S81497	Lysosomal acid lipase		4.7	6.5			3.1
M38566	Mitochondrial cytochrome P450 (P450C27)		30.3	42.1			10.2
AF036761	Stearyl-CoA desaturase 2		8.2	9.4			
S70011	Trycarboxilate carrier (mitochondrial)			–2.8			
1.5 ATP production and processing							
D00636	Cytochrome b5 reductase (NADH)*						–11.4
AI169265	NADH dehydrogenase (ubiquinone) 1α subcomplex 4						–2.5
AA859827	Uridine kinase						8.1
1.6 Miscellaneous							
AA799406	Cytochrome P450 monooxygenase	–18.4	–2.6	–2.5			
D87839	GABA transaminase						3.1
U27518	UDP-glucuronosyltransferase					–3.8	–3.9
2.0 Protein synthesis, modification, and secretion							
AA859966	18S rRNA		–3.1				–3.2
AI008852	Elongation factor 1 α	–3.3					
AA892250	Lysyl-tRNA synthetase			3.2	2.7		3.5
M24353	Mannosidase α type II				–4.7		–5.0
AI169327	Metalloproteinase inhibitor*		3.4	5.0	2.7	3.3	5.9
M83676	RAB12	–5.3					
AA893080	Selenocysteine lyase	3.0		4.3	6.9	5.8	12.9
AI007857	SNAP-25 interacting protein hrs-2						2.8
AJ006855	Synaptojanin				7.0	9.0	6.4
U56261	Synaptosomal associated protein (SNAP-25a)	–5.8					–6.4

Table 3. (continued)

Cluster/GAN	Gene Name	6h			24h		
		IFN-γ	PIC	IFN-γ+PIC	IFN-γ	PIC	IFN-γ+PIC
3.0 Ionic channels, ions transporters and related proteins							
U69884	Calcium-activated potassium channel rSK3 (SK)						4.1
M31178	Calbindin	−3.8					
X90642	Multidrug resistance protein (ID:1)						7.0
L35771	Potassium channel		3.4				
AF048828	Voltage dependent anion channel (RVDAC 1)*	−6.4					
4.0 Hormones, growth factors and related genes							
X01032	Cholecystokinin (CCK) precursor		58.4			98.9	
E12746	Cholecystokinin-A receptor*						−7.6
L20913	Endothelial growth factor form 3	−5.8					
L19660	Gastric inhibitory peptide receptor						−2.9
S49003	Growth hormone receptor (short isoform)						−25.6
Z83757	Growth hormone receptor	−5.0					
M25584	Insulin 1						−2.6
Y07534	Mitochondrial vitamin D(3) 25-hydroxylase		19.2	11.5			26.0
M83745	Prohormone convertase-1						−2.9
S40669	Prohormone convertase 2			2.6			
M25804	Rev-ErbA-a *						4.1
M93273	Somatostatin receptor type 2			−7.2			−7.8
X63574	Somatostatin receptor type 3					6.0	6.7
M23643	Thyrotropin releasing hormone*	−3.1					−11.0
M32167	Vascular endothelial growth factor (VEGF)*	−9.7					
AF022952	Vascular endothelial growth factor B						8.3
5.0 Cytokines, chemokines and related receptors							
AA799761	CD-40		10.9	88.3			24.3
D11445	CINC-1		9.3	8.1			
AF030358	Fractalkine			7.6			
U68272	Interferon-γ receptor			5.1			
U69272	Interleukin 15*		2.7	7.9	3.2		7.7
X17053	Macrophage chemoattractant protein-1 (MCP-1)*		7.2	29.3			
AJ011969	Macrophage inhibitory cytokine-1 (MIC-1)	−4.9					16.8
53312	MIP-3α		3.0	2.7			
U17035	Mob-1/ IP-10	26.6	87.0	90.3	17.2	123.0	196.7
U94330	Osteoprotegerin			12.8		2.6	
AI009658	RANTES		155.8	180.4		171.7	284.8
L00981	TNF-β			6.5			
6.0 Cytokine and dsRNA processing and signal transduction							
M64780	Agrin*		3.4	2.8		3.1	3.2
AI170268	β-2 microgobulin	3.0		2.9			3.2
S74351	3CH134/CL100 protein tyrosin phosphatase		4.6	4.9		5.5	
U78889	Delta1			5.0			
AA924925	ER transmembrane protein					6.2	5.6
M80367	Guanylate nucleotide binding protein 2	15.1	18.5	106.1	27.4		63.4
AA891944	INF-γ induced GTPase	37.1	14.3	33.7	17.3	2.9	11.4
U13396	Janus protein tyrosine kinase 2 (JAK-2)*		7.7	14.2			8.3
AA875327	Lim-kinase 1		4.5	5.9			
AF013144	MAP-kinase phosphatase (cpg21)			−2.7			−3.0
D89863	M-Ras*	6.0		4.4			
X57405	Notch-1*			7.0			
D84667	Phosphatidylinositol 4-kinase						8.2
J03806	Phospholipase C				6.4		7.8
AF086624	Pim-3 serine threonine kinase						14.8
X99267	Presenilin-2						9.1
M18330	Protein kinase C delta subspecies		5.3	5.7			5.1
U02553	Protein tyrosine phosphatase		6.2			4.9	
AF077000	Protein tyrosine phosphatase TD14 (PTP-TD14)						21.0

Table 3. (continued)

Cluster/GAN	Gene Name	6h			24h		
		IFN-γ	PIC	IFN-γ+PIC	IFN-γ	PIC	IFN-γ+PIC
AA800318	SERPIG 1	7.2	3.5	9.9	4.6		5.0
S61868	Syndecan-4		5.0	5.2			2.7
7.0 MHC and related genes							
AJ005023	Mature MHC class Ib α chain		6.0	5.6		6.3	7.4
M31038	MHC-I non-RT1 α chain		6.5	10.0	5.2	7.0	10.4
AF029240	MHC-Ib RT1.S3 *	18.1	29.3	54.6	19.5	12.9	25.9
U16025	MHC-Ib RT1*		3.6	6.5	3.4	3.1	7.3
M36151	MHC class II A-β RT1.B-b-β				4.7		8.6
X13044	MHC-II-assoc. invariant chain γ *	4.9	3.9	38.0	39.6	26.0	178.4
X57523	Mtp1*	13.4	4.5	17.6	13.0		19.3
X63854	Mtp2	5.7	3.6	10.0	4.7		8.2
D10729	Proteasome subunit RC1	16.2	14.9	33.5	20.3	6.9	40.4
D10757	Proteasome subunit RING 12 *	15.6	7.4	24.3	16.6	3.0	16.7
D45250	Proteasome activator rPA28-b *			4.3	6.6	3.3	7.8
8.0 Cell adhesion, cytoskeleton and related genes							
AF017437	Antigen CD-47 *		2.7	4.0			2.5
AI070848	β-actin	–3.1					
U72741	36Kd β-galactoside binding lectin *		4.4	4.6		4.0	3.1
Y16898	Connexin36					6.7	9.3
L16532	2,3-cyclic nucleotide 3-phosphodiesterase (CNPII)	4.9	8.4	7.3		3.7	2.9
X67788	Ezrin p81			3.2		2.8	3.3
U82612	Fibronectin*					–3.1	–10.2
D00913	Intercellular adhesion molecule-1 (ICAM-1)		15.4	22.6		4.9	9.7
AA875659	Internexin α	–4.8	–2.9	–5.0			
X81449	Keratin 19*		4.6	3.6		10.6	11.4
Z12152	Neurofilament protein middle (NF-M)			5.2			8.7
U16845	Neurotrimin			–7.1			–5.6
9.0 Transcription factors and related genes							
S77528	C/EBPβ					5.4	21.3
AI045030	C/EBPδ	–3.9					
X17163	c-jun*			8.8			7.6
Y00396	c-Myc*						9.6
S66024	CREM transcriptional repressor*						–4.4
U04835	CREMδ C-G gene						–2.9
J03179	D-binding protein*						8.9
X83094	Heat shock transcription factor 1						8.9
X62875	High mobility group protein I						3.6
Y09507	Hypoxia-inducible factor 1 (HIF1)			4.5			
X63594	I-kB α-chain*		15.6	17.4		6.0	9.8
M34253	Interferon regulatory factor-1 (IRF-1) *	19.1	6.1	15.1	13.1	3.1	19.6
AA799861	Interferon regulatory factor-7 (IRF-7)*	9.7	55.2	78.4	7.7	62.5	93.4
M63282	Leucine zipper protein			3.0			2.6
D32209	Leucine-rich acidic nuclear protein		3.9		6.7	4.6	7.9
S71523	Lim-1						5.7
U72620	LOT 1			–3.1			–2.9
D14448	Max			9.7			
U58279	Mist1			–3.3			
AA900476	MRG 1*						–3.4
L26267	NF-κB-p105		3.4	3.3		2.5	
AF022081	Small nuclear RING finger protein 4			6.0		5.2	6.5
AA892553	STAT-1	56.3	30.5	81.2	33.6	4.4	28.4
X91810	STAT-3			3.2			
AI011498	SWI/SNF related						11.7
AA800613	TIS 11*		4.0	7.1			4.1
M61725	Transcription factor UBF-2						2.7
AF026476	Transcription factor USF-1		4.3	4.9			
AF001417	Zinc finger protein 9		7.2	9.8			9.1

Table 3. (continued)

Cluster/GAN	Gene Name	6h			24h		
		IFN-γ	PIC	IFN-γ+PIC	IFN-γ	PIC	IFN-γ+PIC
10.0 RNA synthesis and splicing factors							
AF044910	Survival motor neuron*						4.8
11.0 Cell cycle							
D16308	Cyclin D2	–5.1					
U24174	p21/WAF1						7.0
U75404	SSeCKs 322	17.2		9.9			
12.0 Defense/repair							
AI070295	GADD45					–2.9	
AF020618	GADD34 (mouse MyD116; rat PEG-3) *						4.2
D42148	Gas-6 growth arrest specific			–7.1			
U73174	Glutathione reductase	–6.0					
AI138143	Glutathione S-transferase			–5.8			
Z27118	Hsp 70 gene 1/2 *	–17.8	3.9	3.8			
AA875620	Hsp 70 gene 3						3.0
AI176456	Metallothionein 2						2.9
Y00497	MnSOD		3.5	4.0			
X52711	MX1		17.7	49.5		3.6	16.8
M76704	O-6-methylguanine-DNA methyltransferase (MGMT)						3.4
J02722	Heme oxygenase						2.7
AA848563	Heat shock protein 70-1	–24.3	4.1	3.5			
AI138143	Glutathione S-transferase			–5.8			
13.0 Apoptosis and ER stress response and related genes							
S76511	Bax						2.9
M31178	Calbindin d28 K	–3.8					
L18889	Calnexin	–2.9					
U77931	Calreticulin	–3.3					
AF025671	Caspase 2				2.9	2.7	4.0
C07012	Cyclophilin c		2.7	3.2	2.7	3.8	4.5
U30186	GADD 153 (Growth arrest DNA damage 153)						9.0
U41853	150 kDa oxygen regulated protein (ORP150)	–4.4		2.6			
X62950	(pBUS30) with repetitive elements						–2.8
M96630	Sec 61 homolog	–14.8					
Z14030	TRAP-complex gamma subunit	–14.6	–3.7	5.0			
14.0 Anti-viral response							
L2928	Double-stranded RNA-dependent protein kinase (PKR)		8.6	9.9			
U18942	Double-stranded RNA-specific adenosine deaminase (ADAR)		5.8	7.2		8.2	9.3
X52713	Mx3		43.9	36.6		4.7	19.7
Z18877	2’,5’-oligoadenylate synthetase (OAS)		11.3	10.7		2.6	2.6
15.0 Miscellaneous							
Y07704	Best-5*	24.4	60.5	60.9	5.2	38.6	99.4
D88250	Complement C1		4.7	15.0	7.1	7.9	22.0
L21711	Galectin-5		23.8	22.8	7.2	17.7	19.5
M62642	Hemopexin		9.1	8.1		13.2	10.9
M81855	P glycoprotein 1			11.7			8.6
AA875037	Serine protease inhibitor 15 (spi15)						6.4

Data are shown as fold-variation corresponding to the gene with the indicated access number. The genes are ordered alphabeticaly in each cluster. The data are means of individual duplicate hybridizations. mRNAs were considered as modified by dsRNA and/or IFN-γ when the mean fold change of the duplicates was ≥2.5, and both individual fold change values were ≥2.0. Decreased expression compared with respective controls (beta cells not exposed to IFN-γ and/or PIC; 6 or 24 h) is indicated by “–” preceding the fold-change value; absence of sign means increased expression. * indicates gene detected by more than one probe. GAN, GeneBank Accession Number

ciated with a decrease in MAP-kinase phosphatase (cpg21) by exposure to PIC alone or in combination with IFN-γ (Table 3; item 6.0), could affect beta-cell signal transduction by acting on key phosphorylation/dephosphorylation steps.

Genes related to antigen presentation were induced to a major extent after both 6 and 24 h (Table 3, item 7.0). Increased expression of MHC-Ib RT1, MHC-II and proteasome subunit RC1 and RING12 were observed with both IFN-γ and PIC alone, but the two agents often showed additive effects. MTP-1 and MTP-2, proteins involved in the "machinery" for MHC class I presentation, were also up-regulated by both agents.

IFN-γ and/or PIC led to up- and down-regulation of numerous transcription factors and associated proteins (Table 3, item 9.0). Of note, among the 41 transcription factors modified, 63% of them were affected only in the presence of both agents, the sole condition leading to beta cell apoptosis. There was up-regulation of c-jun, c-Myc, C/EBPβ, NF-κB, Lim-1 and STAT-1. Up-regulation of these transcription factors was also observed after beta cell exposure to IL-1β and IFN-γ, another treatment leading to apoptosis. The microarray results also showed up-regulation of other transcription factors not described before in beta cells exposed to cytokines, including heat shock transcription factor 1, hypoxia-inducible factor 1, STAT-3, Max and USF-1 (Table 3; item 9.0). Nuclear Ring Finger protein 4 (RNF4) and SWI/SNF, mRNAs encoding for multiprotein complexes remodelling chromatin, which are required for positive and negative control of various cellular pathways, were also up-regulated following beta-cell exposure to PIC and IFN-γ (Table 3; item 9.0).

IFN-γ alone induced an early (6 h) decrease in two mRNAs potentially involved in beta-cell defence/repair, namely glutathione reductase and hsp70 (Table 3; item 12.0), and of two endoplasmic reticulum (ER) chaperones that could contribute to defence against ER stress, i.e. calnexin and calreticulin (Table 3; item 13.0). In contrast, PIC, especially in combination with IFN-γ, up-regulated several defence/repair genes after both 6 and 24 h. Among them, IFN-γ + PIC modified expression of MnSOD, haeme oxygenase, hsp 70, PEG-3/MyD116 (the rat and mouse homologs of human GADD34) and the DNA repair enzyme O-6-methylguanine-DNA methyltransferase (Table 3; item 12.0).

Among genes related to apoptosis, we observed induction of several putative pro-apoptotic genes by PIC alone or combined to IFN-γ. These included Bax, caspase 2, the cyclin-dependent kinase inhibitor p21/WAF1 and GADD153/CHOP, an ER stress-response transcription factor (Table 3, items 11 and 13.0). Of interest, Bip/GRP78 and bcl-2 expression were unaffected by IFN-γ and/or PIC, at least at our selected time points (see below).

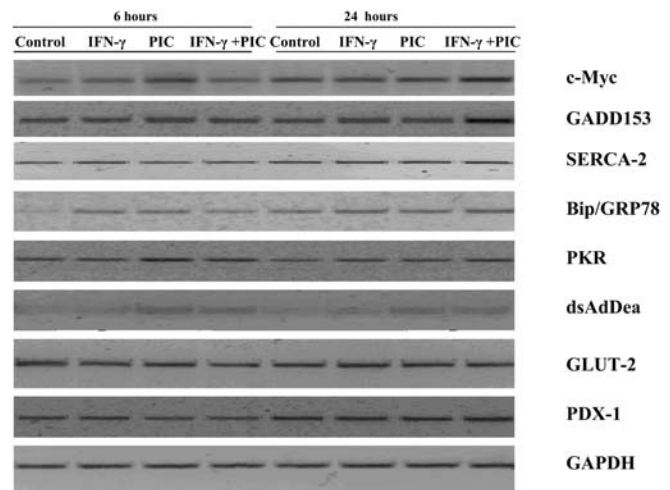

Fig. 2. RT-PCR analysis of PKR, dsRNA-specific adenosine deaminase, c-Myc, GADD153/CHOP, SERCA-2, GLUT-2, PDX-1, Bip/GRP78 and GAPDH mRNA expression by beta cells exposed for 6 to 24 h to: IFN-γ (1000 U/ml), PIC (100 μg/ml) or IFN-γ + PIC. The cDNA samples were amplified in parallel with GAPDH-specific primers, confirming similar loading in all lanes. The figure is representative of four similar experiments

Treatment of beta cells for 6 or 24 h with PIC alone induced expression of genes promoting resistance to viral infection, such as dsRNA-activated protein kinase (PKR, also named eIF-2α kinase), 2′,5′-oligoadenylate synthetase (OAS) and RNA-specific adenosine deaminase (ADAR) (Table 3; item 14.0); addition of IFN-γ neither modified the pattern of expression nor the magnitude of this response. Moreover, IFN-γ alone did not induce any of these genes.

Confirmation by RT-PCR and real-time RT-PCR of genes identified as modified by IFN-γ and/or PIC. Eight genes of special interest were selected for confirmation by RT-PCR. Six of the selected genes were detected as "changed" in at least one of our experimental conditions in the array analysis. Thus, ADAR and PKR, both involved in anti-viral response; the oncogene c-Myc; GADD153, an ER stress-induced transcription factor; and the transcription factor CEBP/β were scored as increased; in contrast, expression of GLUT-2, the beta-cell specific glucose transporter, was detected as decreased by 24 h exposure to the combination of PIC + IFN-γ. The RT-PCR results confirmed the data obtained in the microarray for five of the six genes under consideration (Fig. 2). The altered expression of CEBP/β, however, could not be confirmed (data not shown). The up-regulation of GADD153 mRNA observed after 24-h exposure to IFN-γ and PIC (nine-fold increase, as observed by microarray analysis) was also confirmed by real-time RT-PCR, the ratio of GADD153/GAPDH mRNAs reaching 9.1±1.7-fold increase compared with controls (n=3, p<0.05). Bip/GRP78, a resident ER chaper-

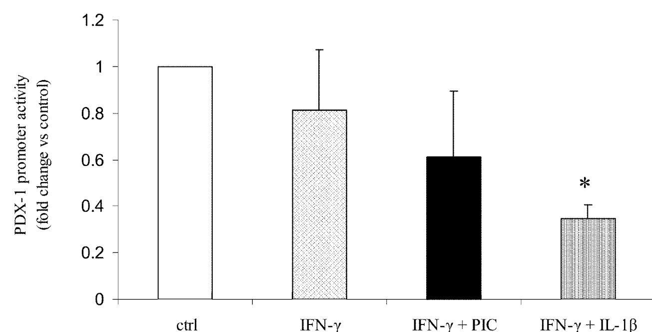

Fig. 3. Effect of IFN-γ, PIC, IFN-γ + PIC and IL-1β + IFN-γ on *pdx-1* promoter activity. After transfection with the *pdx-1* and pRL-CMV plasmid constructs , purified beta cells were treated for 24 h with IFN-γ (1000 U/ml), PIC (100 µg/ml) and/or IL-1β (50 U/ml). Luciferase activities of PDX-1 plasmid were corrected for those obtained with the pRL-CMV plasmid in the same sample. Data are expressed relative to the value obtained in the control condition, taken as 1.0. Results are means±SEM of four to five experiments. * $p<0.0005$ vs. control; Student's paired *t* test

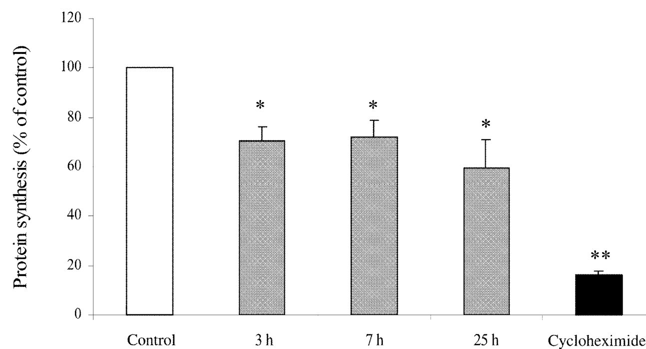

Fig. 4. Effect of IFN-γ and PIC on beta-cell total protein biosynthesis. Beta cells were cultured for 23 h in the absence (control) (*white column*) or presence for 1, 3 and 23 h of IFN-γ (1000 U/ml) + PIC (100 µg/ml) (*grey columns*). A further 2-h incubation was then carried out under the same experimental conditions but in the additional presence of L-[4,5-^{3}H]leucine (370 kBq/ml). As positive control, control cells pre-cultured for 23 h were exposed to cycloheximide (10 µmol/l) during the final 2-h incubation period (*black column*). The results were calculated as percentage of the tritiated leucine incorporated into control cells, taken as 100%. Data are means±SEM of six separate experiments. * $p<0.05$, ** $p<0.001$ vs. control; Student's paired *t* test

one whose expression closely parallels that of GADD153 in other cell types [47, 48], was also examined. There was no clear increase in Bip expression after 24-h exposure to IFN-γ and PIC, the treatment which induced GADD153 expression. In line with these observations, there was also no increase in Bip/GRP78 expression in the microarray analysis of cells exposed for 6 or 24 h to IFN-γ and PIC. Expression of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA-2) was also found unaffected by the different experimental conditions tested in the array, a finding confirmed by RT-PCR (Fig. 2) and real-time RT-PCR.

As mentioned above, we observed a decreased expression of GLUT-2, insulin and pro-hormone convertase-1 (PC-1) in the microarray analysis (Table 3). Against this background, we decided to study by RT-PCR the expression of *pdx-1*, whose probe was not present in the microarray. Surprisingly, our results indicated that *pdx-1* expression was unaffected by exposure to IFN-γ and/or PIC for 6 or 24 h (Fig. 2). These findings were confirmed by studying *pdx-1* promoter activity. After transfection with a luciferase-reporter plasmid containing a −3.7 kb fragment of the human PDX-1 promoter, FACS-purified beta cells were incubated for 24 h in the presence or absence of IFN-γ and/or PIC; none of these conditions affected the promoter activity (Fig. 3). However, exposure of the transfected beta cells to IFN-γ and IL-1β, a treatment previously shown to down-regulate PDX-1 mRNA expression by more than 50%, led to a 60% decrease in the promoter activity.

As mentioned above, some genes related to ER stress were modified by PIC + IFN-γ. Since ER stress could lead to a decrease in protein synthesis initiation, we evaluated total protein biosynthesis in beta cells cultured for 3, 7 or 25 h in the presence or absence (control) of PIC and IFN-γ. As positive controls, the incorporation of tritiated leucine was carried out in the presence of cycloheximide, a well known inhibitor of protein synthesis. Exposure of beta cells to PIC and IFN-γ induced a mild decrease in protein synthesis at each time point (Fig. 4), in the range of 30–40% ($p<0.05$, paired Student's *t* test), while cycloheximide reduced protein biosynthesis by more than 80% ($p<0.0005$, paired Student's *t* test).

Discussion

The present study is the first attempt to comprehensively define the repertoire of dsRNA- and IFN-γ-induced genes in primary pancreatic beta cells. Using microarray analysis, 3759 genes were detected as expressed in beta cells. Of these, 348 (nearly 10% of the total) were found as changed by dsRNA and IFN-γ, alone or in combination, after 6- or 24-h exposure. The large number of genes modified by dsRNA and IFN-γ, encoding for proteins involved in a broad range of beta-cell functions, emphasises the complex nature of beta-cell responses to two putative mediators of early insulitis. From the genes detected as changed, 38% were either induced by IFN-γ or by dsRNA, while 58% showed an additive or a potentiating effect by the combination of both agents; only 4% of the modified genes were similarly increased/decreased by dsRNA and IFN-γ, without an additive effect by both

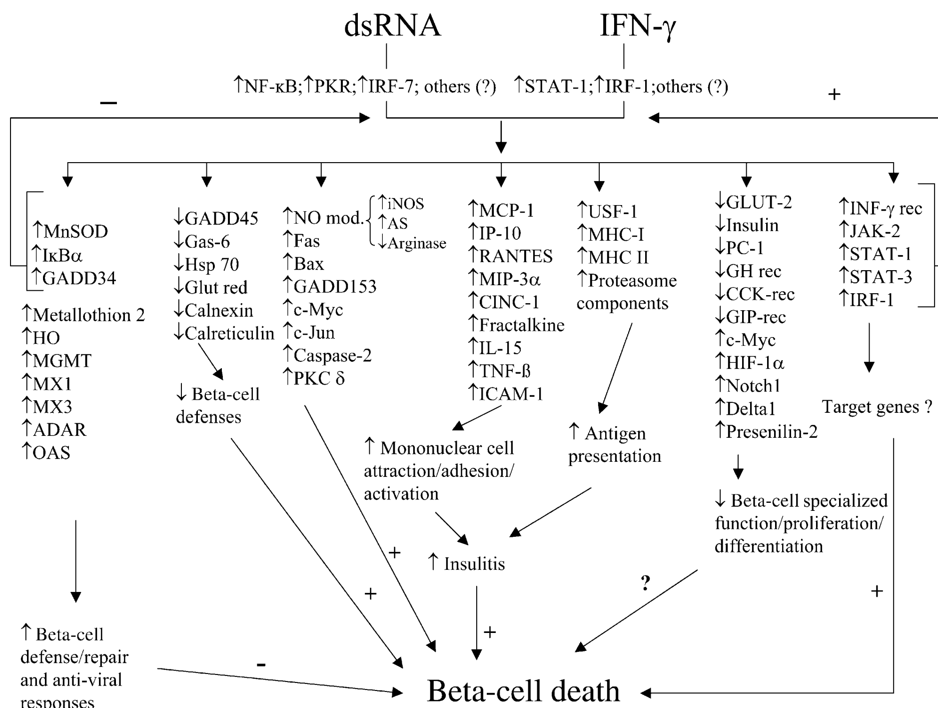

Fig. 5. Proposed model for PIC and/or IFN-γ effects on pancreatic beta cells. Explanations about the figure are provided within the text. *Metallothion 2* metallothionein 2; *HO* haeme oxygenase; *MGMT* O-6-methylguanine-DNA methyltransferase; *ADAR* RNA-specific adenosine deaminase; *OAS* 2′,5′-oligoadenylate synthetase; *glut red* glutathione reductase; *NO mod.* NO module; *AS* argininosuccinate synthetase; *GH rec* growth hormone receptor; *CCK rec* cholecystokinin receptor; *GIP rec* gastric inhibitory peptide receptor; *IFN-γ rec* IFN-γ receptor

agents. This, and the fact that dsRNA mostly increases mRNA expression at 6 h (94% of the genes defined as increased), while IFN-γ has mainly an inhibitory effect at this time point (72% of the genes defined as decreased), suggests that dsRNA and IFN-γ signal via different and complementary pathways. Comparison of the present data with our previous microarray analysis of beta cells exposed to IL-1β, alone or in combination with IFN-γ [24, 25], indicates a nearly 50% difference in the pattern of gene expression, suggesting that dsRNA has also important points of differences in signalling as compared to IL-1β. Of note, and in agreement with our previous observations [22], neither dsRNA nor IFN-γ, nor a combination of both agents, changed beta-cell expression of IL-1β or IL-1α in the microarray analysis. As previously shown [21, 22], and confirmed in the present study, beta cells exposed for 3–6 days to dsRNA + IFN-γ, but not to either agent alone, undergo cell death by apoptosis. These beta cells also present initial adaptive responses that are part of the early host reaction to a viral infection and contribute to amplify immune recognition and immune response against the infective agent [22]. How can we integrate these functional responses with the complex pattern of dsRNA and/or IFN-γ-induced gene expression observed in our microarray analysis? A general model, based on the present data, is proposed in Fig. 5 and discussed below. Due to space limitations, not all genes shown in Fig. 5 and Table 3 are discussed here; additional information on these genes is presented in our previous publications dealing with microarray analysis of pancreatic beta cells [24, 25, 26, 37].

Activation of PKR and of the transcription factor NF-κB are important mediators of dsRNA signal transduction in other cell types [13, 49, 50], and these pathways have been shown to transduce at least part of the effects of dsRNA, including apoptosis, in beta cells [21, 22, 45, 50]. We observed both an induction of the NF-κB precursor p105 and of several well defined beta-cell NF-κB-dependent genes, such as iNOS [51], MCP-1 [41] and MnSOD [52]; there was also a nearly 10-fold increase in PKR mRNA expression. Another gene induced to a major extent (more than 50-fold) by dsRNA, in an effect potentiated by IFN-γ, is the transcription factor interferon regulatory factor (IRF)-7. IRF-7 is induced by dsRNA and/or interferons (mostly IFN-α and IFN-β) in other cell types, an effect mediated by the transcription factors NF-κB and STAT-1 [53, 54]. dsRNA increased STAT-1 expression by more than 50-fold, an effect potentiated by IFN-γ at 6 h (present data). IRF-7 and IRF-3 (not found as modified in the array; data not shown) play critical roles in the innate response to a viral infection [53] and IRF-7 also contributes to IFN-γ-mediated apoptosis [55]. Among the downstream genes regulated by IRF-7, in cooperation with NF-κB, is the CC chemokine RANTES [56]. We observed that PIC increased RANTES expression by more than 100-fold.

The agreement between the effects of dsRNA on the expression of genes upstream (STAT-1) and downstream (RANTES) of IRF-7 suggests that this transcription factor participates in the signal transduction of dsRNA in beta cells. Another pathway of dsRNA signalling is via activation of the toll-like receptor 3 (TLR3) [57], but it is unknown whether this receptor is expressed and functional in beta cells.

Signal transduction by IFN-γ is mediated via binding of the cytokine to the IFN-γ receptor. This is followed by autophosphorylation and activation of the Janus tyrosine kinases 1 and 2 (JAK-1/2), which then phosphorylate and activate STAT-1. Activated STAT-1 migrates to the nucleus, where it regulates the expression of several genes, including the transcription factor IRF-1, contributing to both changes in beta-cell function and the induction of apoptosis [10]. Beta-cell exposure to dsRNA and IFN-γ up-regulates nearly all these key steps for IFN-γ signal transduction (Fig. 5). This, and the lack of detectable effects of dsRNA and/or IFN-γ on the expression of IFN-α and IFN-β (probes for both cytokines were present in the array), at least at the time points studied, indicates that IFN-γ might have a major role in the early responses of beta cells to a viral challenge.

While the observations described above suggest a positive feedback on IFN-γ signal transduction, there seems to be a negative feedback operating for dsRNA signalling. Activation of the transcription factor NF-κB has a pro-apoptotic role in beta cells exposed either to IL-1β + IFN-γ [58, 59] or to dsRNA + IFN-γ [22]. MnSOD and IκBα are two genes that might participate in beta-cell defence against apoptosis by decreasing NF-κB activation [60, 61], and both mRNAs were induced by dsRNA. MnSOD is a NF-κB dependent [52] mitochondrial antioxidant enzyme, and overexpression of MnSOD protects beta cells against immune-mediated damage [62]. NF-κB also regulates IκBα, and increased IκBα concentration both prevents NF-κB migration to the nucleus and removes NF-κB already present in the nucleus [63].

As discussed above, another effect of dsRNA is to up-regulate PKR [64]. Once activated, PKR phosphorylates, among other substrates, the small subunit of the eukaryotic initiation factor 2α (eIF2α), reducing translation initiation and severely decreasing total cellular protein synthesis. This effect hampers viral replication but, if prolonged, could trigger apoptosis [65]. We observed, however, that beta cells exposed for different time points to dsRNA and IFN-γ have only a 30–40% inhibition of total protein biosynthesis. These apparently divergent findings can be explained by the observation that GADD34 (human homolog of mouse Myd116 or rat progression elevated gene-3), a stress-inducible phosphatase, dephosphorylates eIF2α and induces partial translational recovery after 2–8 h of cellular stress [66]. We presently observed that GADD34 mRNA is up-regulated by dsRNA + IFN-γ, suggesting that this phosphatase provides an additional negative feedback on dsRNA effects.

Despite the up-regulation of IκBα, MnSOD, GADD34 and other putative defence/repair genes (Fig. 5), prolonged exposure of beta cells to dsRNA and IFN-γ eventually culminates in apoptosis. This could be at least in part due to inhibition of several important "defence/repair" genes, and induction of genes that directly contribute to beta-cell death (Table 3; Fig. 5). Decreased expression of "defence" genes [24] seems to be mostly an early effect of IFN-γ. Two of these genes, calnexin and calreticulin, are chaperones located in the endoplasmic reticulum (ER) [67], and their early inhibition by IFN-γ could render the beta cells more susceptible to ER stress induced by the subsequent (after 6 h) production of nitric oxide [24, 25, 67, 68]. GADD153, a transcription factor involved in the execution of ER-mediated apoptosis [67], is up-regulated by dsRNA + IFN-γ at 24 h. IL-1β + IFN-γ also induce GADD153 up-regulation in beta cells [24, 25], an effect mediated by NF-κB activation and consequent increase in iNOS expression and nitric oxide formation [25]. Of note, blocking iNOS activity prevents IL-1β + IFN-γ-induced GADD 153 expression [25, 26], but does not prevent apoptosis in human or rodent beta cells [34, 36] or in insulin-producing cells [26]. Similarly, the use of iNOS blockers decreased dsRNA + IFN-γ-induced beta-cell necrosis, but not apoptosis [21, 22]. Thus, it seems that cytokines or dsRNA + IFN-γ lead to ER stress in beta cells via NO production, but ER stress is not the main mechanism leading to beta cell apoptosis.

Probes for several members of the bcl-2 family of pro- and anti-apoptotic genes [69], and for different caspases [70], were present in the array, including bcl-2, bcl-xL, Bak, Bax, Bid, Bad, caspase 1 (ICE), caspase 2, caspase 6 and caspase 7. Of these, only Bax and caspase 2 were found to be modified, both maximally induced by dsRNA and IFN-γ at 24 h. The pro-apoptotic Bax homodimerizes through its BH3 domain, and forms heterodimers with bcl-2 and other proteins. An increased ratio between Bax and bcl-2 contributes to the mitochondrial release of cytochrome c, and other pro-apoptotic proteins, triggering the "execution" phase of apoptosis [71]. dsRNA and IFN-γ increase Bax expression without modifying bcl-2, which could tilt the balance in favour of cell death. Up-regulation of c-Myc was observed under the same experimental conditions and at the same time point as Bax, and it is conceivable that, as described for other cell types [72], the pathways mediated by both proteins synergize to induce cell death. Caspase 2 is an upstream caspase, contributing to apoptosis by activating executioner caspases, such as caspases 3 and 7 [70]. Caspase-2 activity is also required for translocation of Bax to the mitochondria and the consequent release of cytochrome c [73]. Finally, dsRNA-induced Fas expression might render the beta cells more sus-

ceptible to death induced by FasL-expressing mononuclear cells [22].

Beta cells exposed to dsRNA and IFN-γ have a functional inhibition that precedes cell death [20, 21, 22]. This might be due to excessive production of NO and consequent impairment in glucose oxidation, but other potentially contributory elements were observed in our microarray analysis. Thus, there was a decrease in the glucose transporter GLUT-2, of both insulin and PC-1, an enzyme involved in the conversion of proinsulin to insulin, and in the receptors for the incretins GIP and CCK. We have previously observed that IL-1β + IFN-γ decrease the expression of several genes related to differentiated beta-cell functions and preservation of beta-cell mass [24, 25]; and inhibition of these genes was associated to a 50% decrease in the expression of *pdx-1*. PDX-1 has a crucial role in maintaining the differentiated phenotype of beta cells [24, 25, 74]. In contrast, dsRNA + IFN-γ decrease insulin mRNA expression and release, without affecting neither *pdx-1* mRNA expression nor activity of the *pdx-1* promoter (present data). If *pdx-1* is not involved in this process of loss of beta-cell differentiated functions, which other genes could participate in it? c-Myc was detected as up-regulated in the array, and increased expression of this oncogene suppresses insulin gene transcription by inhibiting NeuroD/BETA2 [75]. Another intriguing finding was the up-regulation of Notch1, delta-1 (both induced by dsRNA + IFN-γ at 6 h), and of presenilin-2 (induced by dsRNA and IFN-γ at 24 h). Delta-1 is a ligand of the Notch receptor, while presenilins-1 and 2 are enzymes responsible for the intramembraneous proteolysis and activation of Notch [74, 76, 77, 78]. Differentiation is inhibited in endocrine precursor cells expressing activated Notch receptors, whereas the signalling cells (expressing delta-1) are free to differentiate into endocrine cells [74]. It is thus conceivable that dsRNA and IFN-γ-induced re-expression of genes of the Notch signalling pathway contributes to both the loss of the differentiated beta-cell phenotype, and, together with the observed inhibition of GH receptor, prevents in a paracrine fashion the growth/differentiation of newly generated beta cells.

The first line of cellular defence against a viral infection is provided by the local innate immunity, which is followed by the adaptive immune response. Both processes are, at least to some extent, integrated [79, 80]. We have detected induction of several genes that might impair intracellular viral proliferation, including PKR, MX3, double-stranded RNA-specific adenosine deaminase (ADAR) and 2′,5′-oligoadenylate synthetase (OAS). These mRNAs were up-regulated by dsRNA at both 6 and 24 h, with little or no additive effect by IFN-γ. MXs are large GTPases which interfere with viral replication and spread [81], while ADAR hyperedits dsRNAs by converting adenosine to inosine, targeting the dsRNAs for cleavage and removal from the cytosol [82]. OAS activates RNAseL, which both decreases total protein synthesis and accelerates the degradation of RNA, affecting viral replication but also contributing to cell dysfunction and eventually apoptosis [83]. To mount the "second line" of defence, namely the adaptive immune response, the infected cells must present the viral antigens bound to HLA molecules and attract immune competent cells to the site of infection. As can be seen from Fig. 5, beta cells exposed to dsRNA and/or IFN-γ express several genes related to antigen processing and presentation in the context of MHC class I molecules, and also up-regulate several chemokines, adhesion molecules and cytokines that contribute to homing and activation of macrophages, dendritic cells and T-cells. We and others have described before, by differential display and microarray analysis, induction of several of these chemokines, cytokines and MHC-related molecules by IL-1β and IFN-γ [24, 25, 84], and also confirmed their expression at the mRNA and protein level in both rodent and human islets, and in islets isolated from prediabetic NOD mice [68, 84, 85]. Moreover, by use of RT-PCR and "gene-by-gene' analysis we showed that beta cells exposed to dsRNA and IFN-γ express IP-10, MCP-1, MIP-3α and fractalkine [22], findings confirmed in the present microarray analysis. One chemokine, however, was not described before in beta cells, namely RANTES. The C-C chemokine RANTES (CCL5) attracts monocytes, activated T-cells and immature dendritic cells during inflammation and immune responses, suggesting a role for RANTES in virus-related diseases [86]. Of special interest, RANTES expression in microglia correlates with the initial symptoms of experimental autoimmune encephalomyelitis [87, 88] and the chemokine, together with IP-10, MCP-3 and MCP-5, contributes to the distinct Th1 islet inflammatory infiltrate leading to beta-cell destruction in NOD.scid mice infused with islet-specific TCR transgenic CD4 cells [89].

Most information available on the broad molecular effects of dsRNA have been obtained in tumoural cell lines [90], and little is known about the effects of dsRNA or actual viral infection on gene regulation by non- or poorly-dividing cells, such as beta cells. It is conceivable that some of the mechanisms that allow one cell type to eradicate a viral infection in a non-cytopathic and cytokine-dependent way might cause death in another cell type [79]. This possibility is of special relevance for Type 1 diabetes, where both viruses and their product, dsRNA, together with locally produced cytokines, such as IFN-γ and IL-1β, probably play an important role in the initiation and progression of insulitis. Why and how the cellular attempts to eradicate/neutralise the invading virus go wrong in some individuals, giving rise to progressive inflammation and beta-cell death, remain to be determined. Our study, by showing large scale evaluation of mRNAs modified by dsRNA and IFN-γ in beta

cells, provides valuable information to answer this question, and allowed us to propose a comprehensive hypothesis for the molecular regulation of the different cellular responses involved (Fig. 5). The present "data driven" hypothesis needs now to be tested by both targeted "hypothesis driven" experiments and by new microarray and proteomic analysis of rodent and human islet cells exposed either to dsRNA, in the presence of blockers of key signalling pathways, or infected with viruses with a putative pathogenic role in human Type 1 diabetes.

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