Identification of IL-1β-induced messenger RNAs in rat pancreatic beta cells by differential display of messenger RNA

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Abstract

Aims/hypothesis. Interleukin-1β is a putative mediator of pancreatic beta-cell dysfunction and damage in Type I (insulin-dependent) diabetes mellitus. To better understand the molecular mechanisms involved in IL-1β effects, we carried out a differential display of mRNA by RT-PCR to identify novel cytokine-regulated genes.
Methods. Fluorescence activated cell sorting-purified rat pancreatic beta-cells were exposed for 6 or 24 h to IL-1β. Differentially expressed cDNA bands were cloned and then identified by comparing their sequences with data from the GenBank. Differential gene expression was confirmed by RT-PCR using specific primers.
Results. Interleukin-1β increased the expression of adenine nucleotide translocator-1, phospholipase D-1 and cytokine-induced neutrophil chemoattractant-1 and decreased expression of the protein tyrosine phosphatase-like protein IA-2. Interleukin-1β-in-

duced differential expression of these genes in beta cells was confirmed by RT-PCR. In additional studies, IL-1β was shown to induce chemokines other than cytokine-induced neutrophil chemoattractant-1, including cytokine-induced neutrophil chemoattractant-3 and monocyte chemotactic protein-1.
Conclusion/interpretation. Our observations indicate that IL-1β modifies the expression of several genes in pancreatic beta cells. These genes may affect both function, viability and beta-cell recognition by the immune system. Functional characterization of the mRNAs which have been identified could facilitate a better understanding of the mechanisms leading to beta-cell destruction in Type I diabetes.
[Diabetologia (1999) 42: 1199–1203]

Keywords Beta cells, chemokine, phospholipase-D, DDRT-PCR, interleukin-1, monocyte chemoattractant protein-1, adenine nucleotide translocator, CINC-1, CINC-3.

Type I (insulin-dependent) diabetes mellitus is an autoimmune disease, where T-cells and macrophages invade the islets of Langerhans and lead to specific destruction of pancreatic beta cells [1, 2]. Cytokines such as interleukin-1β (IL-1β) and interferon-γ are released by these infiltrating mononuclear cells and can contribute to beta-cell death [2, 3]. Recent observations indicate that genes and proteins activated by the beta cells are crucial to determine whether these cells will survive or undergo apoptosis when faced with immune-mediated cell damage [4–6].

We and others have previously adopted the "candidate gene" approach to characterize beta-cell responses to immune-mediated assaults and observed that cytokines induce the expression of different genes in rodent and human pancreatic islets, encoding both proteins involved in beta-cell damage and

defence or repair or both [4, 7]. Unfortunately, the "candidate gene" approach allows only screening for known and individual genes. To overcome this limitation, in this study we used differential display of messenger RNA with the polymerase chain reaction (DDRT-PCR) [8, 9] to identify novel cytokine-induced genes of potential relevance for beta-cell damage, defence or repair, and apoptosis. For this purpose, DDRT-PCR was done on FACS-purified adult beta cells exposed for 6 or 24 h to IL-1β or control conditions (no cytokine added). These times were selected to detect both early and late responses to IL-1β [4] and the use of FACS-purified beta cells [10], instead of whole islets, increased the chance of identifying cytokine-induced, beta-cell genes.

We identified seven mRNAs whose expression is modified by IL-1β in rat beta cells. These genes could be involved in beta-cell damage and repair and in the process of mononuclear cell homing to the islets during insulitis.

Materials and Methods

Pancreatic islets were isolated from 10-week-old male Wistar rats by collagenase digestion and islet beta cells were purified by autofluorescence-activated cell sorting (FACStar, Beckton-Dickinson, Sunnyvale, Calif., USA) [10], and pre-cultured overnight at 37 °C in Ham's F-10 medium (Gibco Brl-Life Technologies, Scotland) supplemented with 5 mg/ml charcoal-treated BSA (fraction V, RIA grade, Sigma, St. Louis, Mo., USA), 0.1 mg/ml streptomycin, 12.5 U/ml penicillin, 0.3 mg/ml L-glutamine and 10 mmol/l glucose. This was followed by culture at 37 °C in Ham's F-10 medium (Gibco) supplemented with 10 mmol/l glucose, 5 mg/ml BSA, 0.1 mg/ml streptomycin, 12.5 U/ml penicillin, 0.3 mg/ml L-glutamine and 50 µmol/l 3-isobutyl-l-methylxanthine (IBMX; Jansen Chimica, Beerse, Belgium) [11]. For DDRT-PCR 10^6 beta cells were treated with 30 U/ml recombinant human IL-1β (kindly provided by Dr. C.W. Reynolds, National Cancer Institute, USA) for 6 or 24 h before mRNA isolation. Poly(A)$^+$ RNA was isolated from cell aggregates using oligo(dT)$_{25}$-coated polystyrene Dynabeads (Dynal, Oslo, Norway). The DDRT-PCR procedure was adapted from the original protocol [8], using the primer combinations described previously [9]. The protocol used for DDRT-PCR was described in detail in a previous publication, where we reported the induction of serine protease inhibitor-3 by IL-1β [12]. Differentially expressed cDNA bands detected by DDRT-PCR were reamplified and made visible on a 1.5% agarose gel to verify size and then cloned into the pCR 2.1 vector using the TA cloning system (Invitrogen, San Diego, Calif., USA). Clones of interest were further analysed by automated DNA sequencing (ABI Prism 310 Genetic Analyzer) using M13 primer and the Dye Terminator Cycle Sequencing kit (Perkin Elmer, Vaterstetten, Germany). The cDNA sequences were compared with those contained in the GenBank and EMBL database using GCG sequencing analysis software (Genetics Computer Group, Madison, Wis.,USA). For RT-PCR, rat beta cells (10^5 cells) were exposed for either 6 or 24 h to IL-1β (30 U/ml). The insulinoma cell line RINm5F (provided by Å. Lernmark, then at the Hagerdon Institute, Copenhagen, Denmark) was cultured at 5×10^4 cells/well in medium RPMI-1640 supplemented with 10% (vol/vol) fetal calf serum (FCS), 0.1 mg/ml streptomycin and 12.5 U/ml penicillin.

Reverse transcription-polymerase chain reaction. Gene specific primers and RT-PCR were used to confirm the differential expression of the genes detected during DDRT-PCR. Samples of cDNA (equivalent to 1500 cells) were amplified by PCR using AmpliTaq Gold DNA polymerase (Perkin Elmer). Polymerase chain reactions included 12 min predenaturation (hot start PCR) at 95 °C and then cycles of: 94 °C for 45s, 58 °C for 45s and 72 °C for 80s. Polymerase chain reaction cycles were 25 for islet antigen (*IA*)-2; 27 for adenine nucleotide translocator (*ANT*)-1; 28 for *GAPDH* and *IA-2β*; 29 for inducible nitric oxide synthase (*iNOS*), 30 for cytokine-induced neutrophil chemoattractant (*CINC*)-1, (*CINC*)-3 and monocyte chemoattractant proteinn (*MCP*)-1; 31 for phospholipase D (*PLD*)-1. The cycles were selected to allow amplification within the linear range. The gene specific primer sequences and their respective PCR fragment lengths are *ANT-1*: 5'-GCCAG-CAAACAGATCAGTGC-3', 5'-TGGGCAATCATCCAGC-TCAC-3', (533 bp); *CINC-1*: 5'-ACAGTGGCAGGGATTC-ACTT-3', 5'-CTAGCACAGTGGTTGACACT-3', (468 bp); *CINC-3*: 5'-CAAGAACATCCAGAGCTTGA-3', 5'-TCA-GACAGCGAGGCACATCA-3', (274 bp); *GAPDH*: 5'-TCCCTCAAGATTGTCAGCAA-3', 5'-AGATCCACAAC-GGATACATT-3', (308 bp); *IA-2*: 5'-ATGGAGTAGAGCT-GCGTCAG-3', 5'-CCCACCACACTGATGTTGAT-3', (502 bp); *IA-2β*: 5'-GGTACAGGTTTCACGTGGCA-3', 5'-GCTGAGCTCATCTGGCTGGA-3', (411 bp); *iNOS*: 5'-GACTGCACAGAATGTTCCAG-3', 5'-TGGCCAGATG-TTCCTCTATT-3', (308 bp); *MCP-1*: 5'-ACCTGCTGC-TACTCATTCAC-3', 5'-CTACAGAAGTGCTTGAGGTG-3', (325 bp); *PLD-1*: 5'-TCCATCCGTAGTGTGCAGAC-3', 5'-GCAGCAGATCGGAGCAACTG-3', (371 bp). The identity of PCR fragments of each gene were confirmed by DNA sequencing (data not shown). The ethidium bromide-stained agarose gels were photographed under UV-transillumination using a Kodak Digital Science DC40 camera (Kodak, Rochester, N.Y., USA). Abundance of the PCR products of interest was assessed by Biomax 1D Image analysis software (Kodak) and expressed in pixel intensities [optical density (OD)], normalized for the abundance of the GAPDH signal amplified from the same cDNA sample. We have previously shown that IL-1β does not modify expression of the "housekeeping" gene GAPDH [13].

Statistical analysis. Values are expressed as means ± SEM and comparisons were done by Student's paired t test. A p value of less than 0.05 was considered to be significant.

Results

With the use of DDRT-PCR we identified several fragments whose expression was changed by IL-1. Of them five had a high homology ($> 95\%$) to published rat gene sequences, namely *ANT*-1, *IA-2*, *PLD-1*, and *CINC-1* (Table 1). The fifth gene, encoding for the putative cell defence protein serine protease inhibitor-3 (*SPI-3*), was described in a separate publication [12].

Using gene specific primers and RT-PCR we confirmed that the genes described in Table 1 are differentially regulated by IL-1 in rat beta cells (Fig. 1 and

Table 1. cDNA clones isolated by DDRT-PCR

clone	gene	GenBank	matched region	homology
DD134	*ANT-1*	X61667	4710–4820	97%
DD165	*IA-2*	D45414	1540–1900	97%
DD175	*PLD-1*	AB000779	2300–2700	98%
DD185	*CINC-1*	D11444	230–700	98%

DDRT-PCR was done on beta cells exposed for 6 or 24 h to IL-1β or control condition. cDNA bands detected by DDRT-PCR were cloned into the pCR2.1 vector and analysed by automated DNA sequencing. The cDNA sequences obtained were compared with those available in the GenBank. A fifth clone isolated by DDRT-PCR, DD10, represents the gene encoding for serine protease inhibitor-3 [12]
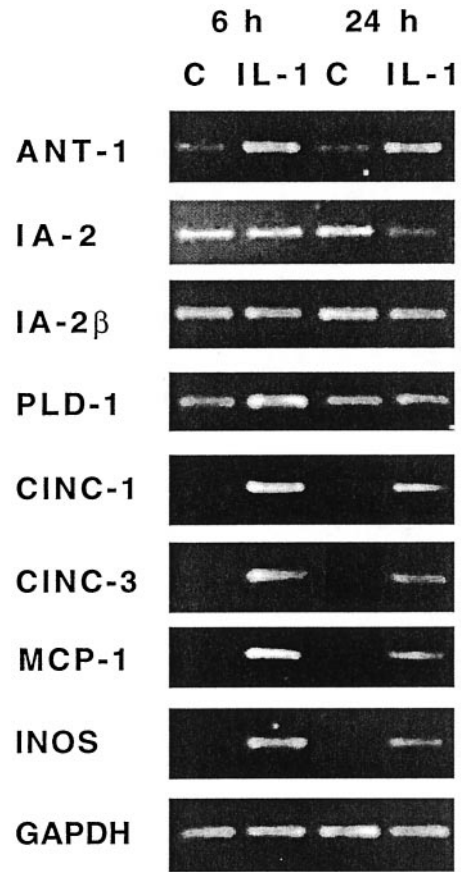
Table 2. Effects of 6-h and 24-h exposure to IL-1β on beta cell mRNA expression

	6-h		24-h	
	Control	IL-1β	Control	IL-1β
ANT-1	0.39 ± 0.10	0.80 ± 0.20[a]	0.55 ± 0.13	0.84 ± 0.15[b]
IA-2	0.37 ± 0.07	0.38 ± 0.10	0.79 ± 0.17	0.43 ± 0.12[b]
IA-2β	0.95 ± 0.06	0.82 ± 0.06	1.17 ± 0.21	0.75 ± 0.16[a]
PLD-1	0.35 ± 0.08	0.81 ± 0.08[b]	0.63 ± 0.08	0.62 ± 0.14
CINC-1	BD	1.24 ± 0.14	BD	0.62 ± 0.05
CINC-3	BD	0.73 ± 0.08	BD	0.31 ± 0.05
MCP-1	BD	0.94 ± 0.13	BD	0.45 ± 0.13
iNOS	BD	0.98 ± 0.13	BD	0.55 ± 0.17

Beta cells were exposed for 6 or 24 h to IL-1β (30 U/ml) and the cells then retrieved for poly $(A)^+$ mRNA extraction and RT-PCR. PCR band intensities were expressed as OD and corrected for GAPDH expression. The results obtained are means ± SEM for 3–6 experiments. [a] $p < 0.05$, [b] $p < 0.01$ vs corresponding control groups. BD, below detection limit. No statistical analysis was done when control values were below detection limit

Fig. 1. Interleukin-1β-induced gene expression in rat beta cells. Rat beta cells (5×10^4 cells/well) were exposed to IL-1β (30 U/ml) or control condition (C; no cytokine added) for 6 or 24 h. After these times the cells were harvested, mRNA extracted and RT-PCR done with the equivalent of 3×10^3 cells. The pictures shown are representative for 3–6 similar experiments. Quantification of these results as OD are shown in Table 2

Table 2). The expected PCR fragment lengths were detected and DNA sequencing of the PCR products confirmed the identity of the genes (data not shown). The activity of IL-1 was confirmed by its induction of iNOS gene expression in beta cells (Fig. 1), a well characterized effect of the cytokine in insulin-producing cells [4]. As previously described [14], IL-1β induced a high iNOS expression after 6 h, with subsequent decline after a 24-h exposure to the cytokine (Table 2).

Interleukin-1β increased the expression of the nuclear DNA-encoded ANT-1 mRNA by twofold after 6 h, and this increased expression was maintained after a 24-h exposure to the cytokine (Fig. 1, Table 2). The mRNAS encoding for the tyrosine phosphatase-like proteins IA-2 and IA-2β were expressed in beta cells under basal conditions, and this expression was not modified by a 6 h exposure to IL-1β (Fig. 1; Table 2). A more prolonged treatment with the cytokine (24 h), lead, however, to a statistically significant decrease in IA-2 and IA-2β expression (Table 2).

Phospholipase D-1 mRNA expression increased by twofold after a 6-h exposure to IL-1β (Table 2). After a 24-h culture in the presence of IL-1β PLD-1 mRNA expression returned to basal levels (Table 2).

Expression of the mRNA encoding for the chemokine CINC-1 was undetectable in control beta cells but it was induced by IL-1 after 6 h, with a slight decline after 24 h (Fig. 1 and Table 2). To analyse whether IL-1β induces chemokines other than CINC-1, we examined CINC-3 and MCP-1 expression. There was a similar pattern as observed for CINC-1, namely the cytokine induced a clear expression of the mRNAs encoding for CINC-3 and MCP-1 after both 6 and 24 h of exposure. To exclude that IL-1β-induced chemokine mRNA expression was due to the small contamination of non-beta cells (< 10%) present in our beta-cell preparations, these experiments were reproduced using the clonal rat insulin-producing cell line RINm5F. W observed, smilarly to the data on beta cells, undetectable basal CINC-1,

CINC-3 and MCP-1 mRNA expression, followed by a clear IL-1β-induced mRNA expression after 6 h (data not shown).

Discussion

Since its original development [8], DDRT-PCR has been widely used to detect changes of gene expression in eukaryotic cells exposed to different stimuli. We have used this technique to detect IL-1β-induced genes in rat pancreatic beta cells. Based on the DDRT-PCR, and on additional experiments to complement the differential display, we detected seven IL-1β-induced genes in rat pancreatic beta cells. These genes include the adenine translocator gene *ANT-1*; chemokines *CINC-1*, *CINC-3* and *MCP-1*; and three genes potentially involved in beta-cell signal transduction, namely *PLD-1*, *IA-2* and *IA-2β*. It is noteworthy that we did not detect by the DDRT-PCR some well-known, cytokine-induced genes, such as *iNOS* and Mn superoxide dismutase [4]. This suggests that the present method, although useful to identify some cytokine-affected genes, does not guarantee the identification of all mRNAs whose expression is modified by IL-1β.

The three isoforms of ANT (1–3) are nuclear DNA-encoded mitochondrial proteins which control the ATP supply of the cell by regulating the exchange of ADP and ATP across the mitochondrial internal membrane [15]. Adenine nucleotide translocator-1 is expressed in heart and skeletal muscle [16] and in beta cells ([17], present data). We observed that ANT-1 expression in beta cells is enhanced by IL-1β both after 6 and 24 h of exposure. It has been previously shown that ANT-1 mRNA expression decreases after inhibition of beta-cell function by streptozotocin or culture at low glucose concentrations [17]. Exposure of FACS-purified beta cells for 20 h to IL-1β, under experimental conditions similar to the present study, leads to severe inhibition of beta-cell function [18], which is accompanied by an increased ANT-1 mRNA expression (present data). This indicates that ANT-1 expression and beta-cell function can be dissociated.

Chemokines are small molecules secreted by a variety of cell types, which play an important part in the recruitment and activation of immune cells [19]. Monocyte chemoattractant protein-1 is a CC chemokine, attracting mostly monocytes and T lymphocytes [20]. Cytokine-induced neutrophil chemoattractant proteins are the rat counterpart of human growth-regulated gene products [21] and belong to the CXC group of chemokines. The expression of the chemokines CINC and MCP-1 can be induced by pro-inflammatory cytokines [19, 20] and these cytokines are probably secreted in the vicinity of the beta cells during insulitis [2, 3]. We observed that IL-1β induces the expression of three chemokines in beta cells, MCP-1, CINC-1 and CINC-3. Monocyte chemoattractant protein-1 – a chemoattractant for mononuclear cells – is probably the most relevant in the context of insulitis. It has been previously shown that transgenic expression of MCP-1 in pancreatic islets induces a monocyte-rich insulitis without diabetes [22]. This, taken together with the present data, raises the possibility that beta-cell exposure to IL-1β during early insulitis leads to MCP-1 production, further potentiating immunocyte infiltration and beta-cell damage.

Phospholipase-1 catalyses the hydrolysis of phosphatidylcholine to form phophatidic acid and choline, which play an important part in signal transduction, cell growth and the intracellular protein trafficking and secretion [23]. Phophatidic acid generated by PLD can be further converted to diacylglycerol which has been described as being increased by in beta cells IL-1β [24]. Interleukin-1β-induced diacylglcycerol production leads to protein kinase C activation and stimulation of insulin release [24]. Thus, it is conceivable that the early and transitory induction of PLD-1 by IL-1β observed contributes to the previously described early and transitory stimulatory effects of IL-1β on insulin release [24]. In line with this possibility, exogenous phosphatidic acid or PLD stimulates insulin release by rat pancreatic islets [25].

Islet antigen-2 and IA-2β are protein tyrosine phosphatase-like proteins and are known as major islet cell autoantigens in Type I diabetes [26]. The functional role for this protein in beta cells is still to be clarified. Circulating autoantibodies against IA2 or another beta-cell autoantigen, the enzyme glutamate decarboxylase, are detected in the sera of up to 80% of patients at the clinical onset of the disease and in sera of pre-diabetic people [27]. We observed that prolonged (24-h) exposure of beta cells to IL-1β inhibits IA-2 and IA-2β mRNA expression. Note that IL-1β also inhibits the expression of the beta-cell autoantigen glutamate decarboxylase [28].

Our study identified seven mRNAs whose expression is modified by IL-1β in rat beta cells. Clarification of the functional role of these genes for in vitro beta-cell damage and repair, and for the in vivo progression of insulitis in animal models of diabetes mellitus, will hopefully broaden our understanding of the pathogenesis of Type I diabetes.

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