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Cytokines activate the nuclear factor κB (NF-κB) and induce nitric oxide production in human pancreatic islets

Malin Flodström[a,*], Nils Welsh[a], Décio L. Eizirik[a,b]

[a]*Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden*
[b]*Department of Metabolism and Endocrinology, Vrije Universiteit Brussel, Brussels, Belgium*

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Abstract We studied the ability of cytokines to activate the nuclear transcription factor NF-κB in human pancreatic islets and the putative role of NF-κB for cytokine-induced NO production. Brief exposure (20 min) of human islets of Langerhans to a combination of interleukin-1β + interferon-γ + tumor necrosis factor-α induced a 2.6-fold increase in nuclear NF-κB activity in gel shift analysis. This increase was prevented by the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), which also counteracted NO production by human islets exposed for 14 h to the cytokine combination. High concentrations of interleukin-1β alone (150 and 250 U/ml) increased NF-κB nuclear binding but failed to induce NO formation in human islets. The present data are the first to demonstrate that cytokines activate NF-κB in primary adult human pancreatic islets and suggest that activation of NF-κB may be a necessary but not sufficient signal for cytokine-induced iNOS expression in human islets of Langerhans.

Key words: Nitric oxide; Nuclear factor κB; Inducible nitric oxide synthase; Insulin-producing cell; Pancreatic islet; Insulin-dependent diabetes mellitus

1. Introduction

Cytokines may be mediators of β-cell damage in insulin dependent diabetes mellitus (IDDM) [1]. These inflammatory proteins bind to islet cell receptors and induce a cascade of signals, culminating with the transcription of the inducible form of nitric oxide synthase (iNOS) and NO production [2,3]. The nature of these intracellular signals remains to be clarified. At high concentrations, NO has many detrimental effects to the β-cells, including inhibition of the mitochondrial enzyme aconitase and DNA damage [4,5]. Based on these data, a central role has been proposed for NO in immune-mediated β-cell death in early IDDM [6].

NF-κB is a dimeric transcription factor consisting of homo- or heterodimers of the subunits p50 and/or p65 (for a recent review see [7]). In its inactivated state it stays in the cytosol bound to an inhibitory subunit, IκB. The signal(s) leading to activation of NF-κB remains to be identified, but the fact that the antioxidant pyrrolidine dithiocarbamate (PDTC) blocks NF-κB activation suggests that cytokine-induced production of free oxygen radicals may play a role in NF-κB activation [8]. Upon activation, IκB is released and proteolytically degraded, whereas the p50/p65 dimer is transported to the nucleus. In the nucleus the dimer binds to NF-κB binding sites in promoter regions of specific genes, affecting their transcription. The promoter of the murine macrophage iNOS gene contains several binding motifs for transcription factors, including two NF-κB binding sites, 10 IRE (Interferon-γ responsive element), two ISRE (IFN-α stimulated response elements), two AP-1, etc. An analysis of the putative human iNOS promoter revealed 66% identity to the murine iNOS gene and consensus sequences for three IRE and one NF-κB binding site [9].

It has previously been shown that IL-1β activates NF-κB in the rodent insulinoma cell line RINm5F [10] and that this is a necessary step in the induction of iNOS mRNA [11-13]. It is noteworthy that while IL-1β alone is enough to induce iNOS transcription and NO formation in rat islets, it is necessary to use a combination of IL-1β + IFN-γ + TNF-α to achieve iNOS mRNA expression and NO formation in human islets of Langerhans [14]. Thus, it remains unclear whether NF-κB is also of relevance for NO production by human pancreatic islets. The present study evaluated the role for NF-κB activation in NO formation by human islets of Langerhans.

2. Materials and methods

2.1. Materials

Human recombinant IL-1β (bioactivity 50 U/ng) was kindly donated by Dr K. Bendtzen (Laboratory of Medical Immunology, Rigshospitalet, Copenhagen, Denmark). Recombinant human IFN-γ and murine TNF-α (bioactivity 6.7 and 10 U/ng, respectively) were purchased from AMS Biotechnology (Sweden). The cytokine concentrations used in the present experiments are based upon previous data obtained in rat [15] and human pancreatic islets [14]. Double stranded oligomers for EMSA were produced by Dr. J. Seibt (Dept. of Immunology, Uppsala University, Uppsala, Sweden) and labelled with a Megaprime labelling kit (Amersham International, Aylesbury, UK). Culture medium RPMI-1640 (containing 0 or 11 mM glucose) and fetal calf serum were purchased from Northumbria Biologicals (Irvine, UK). All other chemicals of analytical grade were obtained from E. Merck (Darmstadt, Germany) or Sigma.

2.2. Methods

Human islets of Langerhans were isolated from 14 heart-beating organ donors at the Central Unit of the β-Cell Transplant, Brussels as described elsewhere [16]. The age of the donors was 38 ± 4 years (range 15–54 years). Aliquots of the preparations were examined by electron microscopy (n = 12), indicating 5 ± 1% dead cells and 1% exocrine cells. The prevalence of insulin- and glucagon-positive cells was determined by light microscopical examination of immuno histochemically stained islets, showing 48 ± 5% insulin-positive cells and 9 ± 2% glucagon-positive cells. After isolation islets were cultured in Brussels for 2–18 days (7 ± 1 days) [16], before being sent by air to Uppsala. Rat islets were isolated from adult male Sprague Dawley rats. Both human and rat islets were cultured free-floating in RPMI-

*Corresponding author. Fax: (46) (18) 556401.
E-mail: malin.flodstrom@medcellbiol.uu.se

Abbreviations: iNOS, nitric oxide synthase; NO, nitric oxide; IDDM, insulin-dependent diabetes mellitus; IL-1β, interleukin-1β; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; NF-κB, nuclear transcription factor κB; PDTC, pyrrolidine dithiocarbamate; IκB, inhibitor of NF-κB; IRE, Interferon-γ responsive element; ISRE, IFN-α stimulated response elements; EMSA, electrophoretic mobility shift assay; IRF-1, interferon regulatory factor-1.

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1640 medium containing 10% (v/v) fetal calf serum, benzylpenicillin (100 U/ml), streptomycin (0.1 mg/ml) and 5.6 or 11 mM glucose (human and rat islets, respectively) before the experiments were performed. During this period medium was changed every second day. After 4–6 days in culture, human islets were exposed to IL-1β alone (150 or 250 U/ml) or to the combination of IL-1β (50 U/ml) + TNF-α (1000 U/ml) + IFN-γ (1000 U/ml) whereas rat islets were exposed to IL-1β (50 U/ml). After 14 h of culture medium was retrieved for nitrite determination using the Greiss reagent as previously described [12], and islet DNA contents measured [14]. In some experiments human and rat islets were pre-treated for 1 h with PDTC before exposure to cytokines. Note that PDTC was also present during the 14 h exposure to cytokines. PDTC was previously found to interact with the nitrite assay, reducing the nitrite values by 20% [11]. Thus all experiments where PDTC was added were corrected accordingly.

For the electrophoretic mobility shift assay (EMSA) 1000–1500 human islets were exposed to cytokines for 20 min, washed with cold PBS and nuclear protein fractions extracted (for a detailed description see [10]). Note that the extreme shortage of adult human islets much limits the number of experiments that can be performed with these cells. In some experiments PDTC was added for 1 h before cytokine exposure. Protein concentrations were determined using the Bradford reagent. For the EMSA a double-stranded 26 mer oligonucleotide containing the κB binding site; 5'-AGCTTCAGAGGGGACTTTCC-GAGAGG, was used. A 1000-fold excess of non-labelled oligonucleotide was used as a negative control. The oligonucleotide was labelled with [^{32}P]dCTP using the Megaprime labelling kit and extracted once with an equal volume of phenol/chloroform/isoamyl acohol (25:25:1, v/v). Before incubation with the oligonucleotide, nuclear fractions (5–10 μg) were denatured with formamide (27%). The fractions were then allowed to react with the nucleotide for 30 min at room temperature in a solution containing 10 mM Tris (pH 7.5), 0.2% deoxycholic acid, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2 μg polydeoxyinosinic acid and 0.1 ng DNA (14000 cpm). The samples were then separated on 5% non-denaturing polyacrylamide gels in 0.5×TBE. Band intensities were quantified by densitometric scanning with a Quick Scan Jr densitometer (Helena Laboratories, Beaumont, TX) and values corrected for the amount of loaded protein.

Growing RINm5F cells were trypsinized and subcultured in RPMI 1640 supplemented as described above for rat islet culture [11,12]. When reaching 60–80% confluence, cells were pre-treated with PDTC for 30 min before cytokine exposure. After 6 h culture, medium was taken and nitrite measured as mentioned above.

Data are presented as mean ± S.E.M. Results were compared using Student's unpaired or paired *t*-test. When multiple comparisons were performed, ANOVA with corrections by the Bonferroni method was used. Experimental results obtained from each human islets preparation (i.e. islets obtained from one donor) were considered as one individual observation, even when experiments were performed in duplicates of triplicates.

3. Results and discussion

To evaluate the role for NF-κB in the human islet response to cytokines, in seven separate experiments islets were exposed to IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) (7) for 20 min and NF-κB binding activity was determined by EMSA. Densitometric scanning of the auto radiographs revealed a 2.6-fold increase in nuclear NF-κB binding in islets exposed to the cytokine combination ($n = 7$, $P < 0.05$, see representative gel in Fig. 1; lanes 1,4, and lanes 5–6). In two separate experiments islets were exposed to IL-1β alone (150 and 250 U/ml) or to a combination of cytokines (same as above) for 20 min. A clear increase in NF-κB binding was seen in both experiments using IL-1β, but the NF-κB induction was less marked than that observed with the three cytokines (Fig. 1). As previously described [15,17] human islet exposure to IL-1β alone for 14 h failed to induce nitrite formation (pmol/μg DNA per h; mean of 2 similar experiments; control, 6.6; IL-1β (150 U/ml), 2.2; IL-1β (250 U/ml), 2.9; IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml), 11.6). These findings indicate that the combination of cytokines induces NF-κB in human islets of Langerhans and that, although to a lesser extent, this is also true for IL-1β alone. Considering that the motif for NF-κB binding is present in the human iNOS promoter region [9], it is possible that this transcription factor may play a role in the cytokine-induced iNOS transcription in human islets of Langerhans. To further investigate this hypothesis, blockage of NF-κB activation by PDTC in human islets was tested. Firstly, in two separate experiments human islets were pre-treated with 100 μM PDTC for 1 h and subsequently exposed to IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) for 20 min before extraction of nuclear proteins for EMSA (Fig. 1; lanes 5–8). It was found that PDTC completely inhibited cytokine-induced NK-κB activation. Secondly, in six separate experiments human islets were treated with PDTC (50 or 100 μM) for 1 h [11,12] before exposure to the three cytokines. After 14 h incubation, medium was retrieved for nitrite determination (Fig. 2). Control islets and islets exposed to PDTC alone, produced low and similar levels of nitrite. When exposed to cytokines, medium nitrite levels increased 56-fold ($P < 0.001$ vs. control). Both concentrations of PTDC were able to block most of the cytokine-induced increase in NO production ($P < 0.001$ vs. islets exposed to cytokines alone; $P > 0.05$ vs. controls).

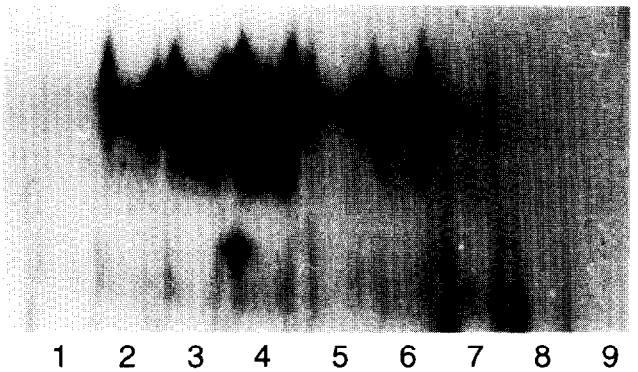

Fig. 1. EMSA showing nuclear NF-κB binding activity in human pancreatic islets exposed to cytokines and/or PDTC. Islets were exposed to IL-1β (150 or 250 U/ml) alone or to the combination of IL-1β (50 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) for 20 min and then retrieved for EMSA as described in section 2. In some experiments islets were pre-treated for 1 h with PDTC (100 μM) before cytokine exposure. Lanes: 1, control; 2, IL-1β (150 U/ml); 3, IL-1β (250 U/ml); 4, IL-1β + IFN-γ + TNF-α; 5, control; 6, IL-1β + IFN-γ + TNF-α; 7, PDTC; 8, PDTC + IL-1β + IFN-γ + TNF-α; 9, negative control using 1000-fold excess of non-labelled oligonucleotide. The autoradiograph is representative for 2 separate experiments.

We have previously shown that PDTC prevents IL-1β-induced iNOS mRNA expression and nitrite formation in RINm5F cells [11,12], a finding recently reproduced by Kwon and co-workers [13]. In order to investigate the role for NF-κB in primary adult rat islets, in 3–4 experiments rat islets pre-treated with 50 or 100 μM PDTC for 1 h were exposed to IL-1β (25 U/ml). After 14 h exposure to IL-1β and/or PDTC, nitrite accumulation to the medium was measured. Low and similar nitrite levels were found in medium from control islets (0.33 ± 0.10 pmol/islet per h) and islets cultured with PDTC alone (0.17 ± 0.06 and 0.20 ± 0.12 pmol/islet per h, for islets treated with 50 and 100 μM PDTC,

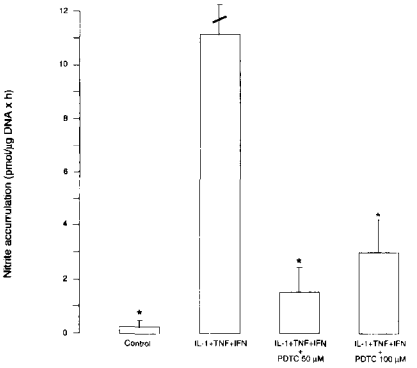

Fig. 2. PDTC effects on cytokine-induced nitrite formation by human islets of Langerhans. Islets were pre-treated with PDTC for 1 h before exposure to cytokines and/or PDTC. After 14 h culture medium nitrite accumulation was determined as described in section 2. Results are presented as mean ± S.E.M. of 6 separate experiments. $^{*}P < 0.001$ vs islets exposed to cytokines, ANOVA.

respectively). Islets exposed to IL-1β produced 5 times more nitrite than control islets (1.65 ± 0.21 pmol/islet per h, $P<0.01$ vs. control islets). Pre-incubation with PDTC significantly decreased this cytokine-induced increase in nitrite levels (0.23 ± 0.03 and 0.33 ± 0.13 pmol/islet per h for islets treated with 50 and 100 μM PDTC, respectively, $P < 0.05$ vs. islets exposed to cytokines). In order to test whether PDTC could also prevent nitrite production by a combination of three cytokines in rodent insulin-producing cells, RIN cells were pre-treated with 50 μM PDTC for 30 min and then exposed to IL-1β (25 U/ml) or IL-1β (25 U/ml) + IFN-γ (1000 U/ml) + TNF-α (1000 U/ml) and/or PDTC. After 6 h, nitrite accumulation into the medium was measured. Control cells and cells incubated with PDTC alone produced low and similar levels of nitrite (control cells, 30 ± 18 pmol/10^6 cells per h; PDTC treated cells, 49 ± 30 pmol/10^6 cells per h). IL-1β alone and the three cytokines induced a 30- and 70-fold increase, respectively, in nitrite levels compared to control cells ($P < 0.01$ for both comparisons, data not shown). Both of these increases were completely blocked in cells pre-treated with PDTC ($P < 0.01$ vs. cells exposed to cytokines alone and $P > 0.05$ vs. control islets).

These data confirm our previous observations that PDTC prevents cytokine-induced iNOS expression and NO formation in RIN cells [6], and implicate the activation of the transcription factor NF-κB as a necessary step in the signal-transduction for NO production in rodent insulin-producing cells. The above-described results also indicate that activation of NF-κB is needed for the induction of iNOS and NO production in adult human pancreatic islets. To our knowledge, this is the first demonstration for such a role of NF-κB in primary adult human cells. It is unclear why human islet cells need three cytokines for the induction of iNOS whereas rodent islet cells require only IL-1β. However, it is conceivable that in human islets additional cytosolic signal(s) besides NF-κB are required for iNOS expression, as compared to rodent islets. Indeed, NF-κB activation may be necessary but not sufficient for cytokine-induced NO production in human islets, as judged by; (a) the ability of PDTC to block NF-κB activation and nitrite formation; (b) the observation that IL-1β alone activates NF-κB but fails to induce NO formation.

NF-κB exists preformed in the cytoplasm until its activation [7]. In this context, the observation that the protein synthesis inhibitor cycloheximide prevents cytokine-induced iNOS mRNA expression in several cell types [9], including insulin-producing cells [12,18], suggests that other factor(s) besides NF-κB is (are) also important for iNOS induction. Recent data suggest that interferon regulatory factor-1 (IRF-1), a transcription factor which requires de novo synthesis after cytokine exposure, may play a role for IL-1β-induced iNOS expression in rat islets [19]. Considering that IFN-γ is necessary for iNOS mRNA expression in human islets [14] and that there are binding sites for IRF-1 both in the murine and in the human iNOS promoter [9], it is conceivable that both species need activation of NF-κB and IRF-1 (and possibly other factor(s)) to induce the iNOS gene. Further characterisation of the molecular regulation of human iNOS may open new alternatives for an early therapeutic intervention to prevent β-cell destruction in IDDM.

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