Cytokine 55 (2011) 195–201

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Differential effects of proinflammatory cytokines on cell death and ER stress in insulin-secreting INS1E cells and the involvement of nitric oxide

Stella Kacheva, Sigurd Lenzen, Ewa Gurgul-Convey *

Institute of Clinical Biochemistry, Hannover Medical School, Carl-Neuberg-Str.1, 30625 Hannover, Germany

ARTICLE INFO

Article history:
Received 27 October 2010
Received in revised form 18 March 2011
Accepted 5 April 2011
Available online 29 April 2011

Keywords:
Cytokines
Diabetes
ER stress
NFκB
Nitric oxide

ABSTRACT

Proinflammatory cytokines produced by immune cells destroy pancreatic beta cells in type 1 diabetes. The aim of this study was to investigate the cytokine network and its effects in insulin-secreting cells. INS1E cells were exposed to different combinations of proinflammatory cytokines. Cytokine toxicity was estimated by MTT assay and caspase activation measurements. The NFκB-iNOS pathway was analyzed by a SEAP reporter gene assay, Western-blotting and nitrite measurements. Gene expression analyses of ER stress markers, *Chop* and *Bip*, were performed by real-time RT-PCR. Cytokines tested in this study, namely IL-1β, TNFα and IFNγ, had deleterious effects on beta cell viability. The most potent toxicity exhibited IL-1β and its combinations with other cytokines. The toxic effects of IL-1β towards cell viability, caspase activation and iNOS activity were dependent on nitric oxide and abolished by an iNOS blocker. IL-1β was the strongest inducer of the NFκB activation. An iNOS blocker inhibited IL-1β-mediated NFκB activation in the first, initial phase of cytokine action, but did not affect significantly NFκB activation after prolonged incubation. Interestingly iNOS protein expression was induced predominantly by IL-1β and decreased in the presence of an iNOS blocker in the case of a short time exposure. The changes in the expression of ER stress markers were also almost exclusively dependent on the IL-1β presence and counteracted by iNOS blockade. Thus cytokine-induced beta cell death is primarily IL-1β mediated with a NO-independent enhancement by TNFα and IFNγ. The deleterious effects on cell viability and function are crucially dependent on IL-1β-induced nitric oxide formation.

1. Introduction

The proinflammatory cytokines IL-1β, TNFα and IFNγ are released by macrophages and T cells from infiltrated islets of Langerhans of the pancreas and cause impaired function and ultimately cell death by apoptosis during type 1 diabetes development [1]. Proinflammatory cytokines induce nitrosative and oxidative stresses, disturb mitochondrial function and eventually lead to the induction of the caspase cascade in beta cells [1–3].

The mechanisms of action of IL-1β, TNFα and IFNγ are different; IL-1β and, to a lesser extent, TNFα can active the NFκB signalling cascade. The transcription factor NFκB plays a crucial role as a master switch in pancreatic beta cells, activating transcription of a number of genes involved in cytokine-mediated toxicity [1,2,4]. One of those genes is the inducible NO synthase, triggering the expression of the iNOS protein and leading to the production of NO [5], causing nitrosative stress. Other genes induced by cytokines are involved in oxidative stress, like manganese superoxide dismutase (MnSOD) [3,6–8], or in endoplasmic reticulum (ER) stress, like Chop [9]. IFNγ acts via the signal transducer and activator of transcription-1 (STAT-1) signalling pathway, accelerating for instance IL-1β-stimulated iNOS expression in insulin-producing cells [5,10,11].

Proinflammatory cytokines can directly activate caspases, the main effectors of programmed cell death. It has been shown in earlier reports that IL-1β is able to activate the effector caspase 3 in beta cells and this effect is possibly linked to NO production [3,12,13]. Previous studies have shown that besides activation of caspase-3 TNFα can also induce caspase 8, which triggers an extrinsic apoptotic signal, and caspase 9 activation, which is induced via mitochondrial stress [14]. The exposure of insulin-secreting cells to IFNγ causes activation of caspase 1 [15]. The activation of ER associated caspase-12 in insulin-secreting cells by various cytokines has not been studied in depth. However, it has been shown that prevention of caspase-12 activation by use of the shRNA technology improves

Abbreviations: BCA, bicinchoninic acid; BiP, binding immunoglobulin protein; Chop, C/EBP-homologous protein; IFNγ, interferon γ; IL-1β, interleukin-1 β; iNOS, inducible nitric synthase; IRE, inositol requiring ER-tonucleus signal kinase; NFκB, nuclear factor κB; NO, nitric oxide; L-NOARG, Nω-nitro-L-arginine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; STAT, signal transducer and activator of transcription; TRAF2, tumor necrosis factor α receptor-associated factor 2; TNFα, tumor necrosis factor α; XBP1, X-box binding protein-1.

* Corresponding author. Tel.: +495115326780; fax: +495115323584.
E-mail address: Gurgul-Convey.Ewa@mh-hannover.de (E. Gurgul-Convey).

1043-4666/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.cyto.2011.04.002

cytokine-treated beta cell survival only mildly [16]. Although there are some reports showing a strong caspase 3 activation by a NO donor [3], the role and the influence of NO in the cytokine-induced caspase activation still needs to be clarified.

Pancreatic beta cells have a well-developed ER [17]. ER plays an important role in beta cell survival and its dysfunction contributes to beta cell death caused by cytokines [17–19]. The response of ER to cytokines consists of activation/suppression of many different pathways. One of those is the induction of the ER chaperone Grp78 (Bip) release from the ER membranes, which is accelerated by the accumulation of misfolded proteins in the ER lumen [17]. The translocation of Bip activates other cascades, like the Ire1 pathway. The Ire1 protein possesses both a kinase and an endoribonuclease domain. The kinase domain is responsible for TRAF2 phosphorylation, which in turn leads to NFκB activation and induction of JNK and p38 MAPK kinases [17]. The endonuclease domain splices XBP1 mRNA, a bZIP-family transcription factor. XBP1 can regulate gene transcription of several genes involved in the unfolded protein response [18]. Another important player involved in the ER stress is the transcription factor CHOP [17,18]. CHOP (GADD153) is a member of the C/EBP family of bZIP transcription factors and its expression is induced by a variety of stimuli leading to ER stress. In beta cells Chop expression induced by IL-1β can be partially prevented when NO formation is blocked [20]. It has been shown that CHOP protein can be phosphorylated by the activated p38 MAPK kinase which increases its transcriptional and apoptotic activity [21]. CHOP can suppress gene transcription of the antiapoptotic protein bcl-2 [22]. Both IL-1β and IFNγ have been shown to induce ER stress in beta cells, through, respectively, NO-mediated depletion of ER calcium and inhibition of ER chaperones, thus decreasing beta cell defence and amplifying proapoptotic pathways [18]. Although the ER stress induction by IL-1β and IFNγ is well established [18], the influence of other cytokines on ER function in beta cells as well as the involvement of NO still require research efforts in this context.

The interactions between the three main proinflammatory cytokines mediating beta cell death and the involvement of NO in their intracellular effects, especially with regard to the induction of ER stress and apoptosis are still not fully understood. It was thus the aim of the present study to elucidate the crosstalk between the different proinflammatory cytokines in the context of ER stress and apoptosis.

2. Materials and methods

2.1. Chemicals

Cytokines and the dNTP mixture were obtained from PromoCell (Heidelberg, Germany). jetPEI™ transfection reagent was purchased from Qbiogene (Heidelberg, Germany), Biotherm™ *Taq* polymerase from GeneCraft (Münster, Germany). The SuperScript II RT™ reverse transcriptase and all tissue culture equipment as well as all primers were from Invitrogen (Karlsruhe, Germany). The inducible NO synthase (iNOS) blocker Nω-nitro-^{l}-arginine (L-NOARG) was from Sigma Aldrich (San Diego, CA, USA). Hybond N nylon membranes, the ECL detection system, and autoradiography films were from Amersham Biosciences (Freiburg, Germany) and Immobilon-P PVDF membranes from Millipore (Bedford, MA, USA). All other reagents were from Sigma Chemicals (München, Germany).

2.2. Cell culture and cytokine incubation

Insulin-secreting INS1E cells were cultured as described [23] in RPMI1640 medium, supplemented with 10 mM glucose, 10% (v/v) foetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2, with addition of 10 mM HEPES, 1 mM pyruvate, 1 mM L-glutamine and 50 μM 2–mercaptoethanol. For cell viability estimation 40,000 cells were seeded onto 96-well plates. For RNA extraction and protein analysis cells were platted at a density of 1.5×10^6 per 60-mm plastic dish and grown to confluence within 2 days. Thereafter cells were exposed to the desired concentration of proinflammatory cytokines and the iNOS blocker Nω-nitro-L-arginine (L-NOARG). Concentrations of cytokines were: 60 U/ml of IL-1β (4.4 ng/ml); 600 U/ml of IL-1β (44 ng/ml), 185 U/ml of TNFα (8.7 ng/ml); 1850 U/ml of TNFα (87 ng/ml); 14 U/ml of IFNγ (10.3 ng/ml); 140 U/ml of IFNγ (103 ng/ml). The iNOS blocker was used at a concentration of 5 mM. Control INS1E cells were grown in the absence of cytokines.

2.3. MTT cell viability assay

In all sets of experiments, the viability of the cells was determined after 24 h incubation with cytokines in the absence or presence of an iNOS blocker using a microplate based MTT assay [24]. Viability was expressed as % of the MTT absorbance at 562/650 nm in the absence of test compounds.

2.4. iNOS Western blot analysis

INS1E cells were incubated for 6 or 24 h with cytokines, washed with ice-cold PBS and homogenized using short bursts (Braun-Sonic 125 Homogenisator, Quigley-Rochester, Inc., Rochester, NY). Protein content was determined by the BCA assay (Pierce, Rockford, IL). 40 μg of total protein was resolved in SDS polyacrylamide gel electrophoresis and then electroblotted onto membranes. Immunodetection was performed using specific primary antibodies against iNOS (NOS2 rabbit polyclonal IgG) or beta-actin (goat polyclonal IgG) (both from Santa Cruz Biotechnology, Heidelberg, Germany) followed by exposition to secondary peroxidase-conjugated AffiniPure donkey anti-rabbit IgG or anti-goat IgG (H + L) (Dianova, Hamburg, Germany). The hybrids were visualized through chemiluminescence using the ECL detection system after short exposure (2–3 min) to autoradiography films. The intensity of the bands was quantified through densitometry with the Gel-Pro Analyzer 4.0 program (Media Cybernetics, Silver Spring, MD, USA).

2.5. Nitrite measurements

Nitrite accumulation after 6 or 24 h of cytokine exposure in the presence or absence of L-NOARG was determined spectrophotometrically at 562 nm by the Griess reaction as described previously [25].

2.6. Reporter gene assay

For the NFκB enhancer element activity studies 2×10^4 cells/well were seeded in 96-well plates 24 h before transient transfection was performed (jetPEI™ transfection reagent) and 48 h before addition of tested compounds for 6 or 24 h. The pSEAP-NF-κB construct was used as described earlier in detail [6]. Secreted alkaline phosphatase expression was measured using Phospha-Light™ System kit (Applera, Darmstadt, Germany).

2.7. Caspase-3 and -12 activity assay

Activation of caspase-3 and -12 was quantified after 24 h exposure to cytokines using a red Caspase-3 or a green Caspase-12 Staining Kits (PromoCell, Heidelberg, Germany) according to the instruction manual. After staining and washing, cell suspensions