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

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A B S T R A C T

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.

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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 activate 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...
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 XBPI mRNA, a bZIP-family transcription factor. XBPI 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 Obiogene (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 No-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% CO2, 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 plated at a density of 1.5 × 104 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 No-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, USA). Homogenates were centrifuged to remove cell debris and supernatants were subjected to 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 bands 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 activity studies 2 × 104 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 (AppIera, 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
were immediately read in the CyFlow ML cytometer (Partec, Münster, Germany). A total of 20,000 events were acquired. Data were analyzed by FlowJo software (Tree Star, Ashland, OR). The data are expressed as % of positive cells without exposure to cytokines.

2.8. RNA isolation and cDNA preparation

Total RNA was obtained using Nucleo-Spin® RNA II kit (Macherey-Nagel, Düren, Germany). The quality of the total RNA was verified by agarose gel electrophoresis. Thereafter, 2 μg of RNA was reverse transcribed into complementary DNA using random-hexamer primers and a reverse transcriptase.

2.9. Real-time RT-PCR

The QuantiTect SYBR Green™ technology (QIAGEN) was employed. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). A total volume of 25 μl was used for the PCR reactions. Samples were first denatured at 94 °C for 2 min followed by up to 30 PCR cycles. Each PCR cycle comprised a melting at 94 °C for 30 s, an annealing at 58 °C for 30 s, and an extension at 72 °C for 30 s. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined. The purity of the amplified PCR products was verified by melting curves. The primers used in this study are shown in Table 3.

2.10. Data analysis

Analyses of the Real-time RT-PCR data and of the standard curve for the genes were performed using the Opticon Monitor v. 1.07 (MJ Research, Inc., Waltham, MA). Densitometry for the immunoblottings was done using specific software (Gel-Pro Analyzer v. 4.0, Media Cybernetics, Silver Spring, MD). All data are expressed as means ± SEM. Statistical analyses for each group were performed using the Prism analysis program (Graphpad, San Diego, CA).

3. Results

3.1. Cytotoxicity after exposure to different cytokines in the absence or presence of an iNOS blocker in insulin-secreting INS1E cells

3.1.1. Cell viability

The proinflammatory cytokines IL-1β, TNFα and IFNγ led to INS1E cell death, however, to a different extent (Table 1). The low concentration of IL-1β 60 U/ml decreased INS1E cell viability marginally by 13%, while the high concentration of 600 U/ml caused approximately 50% cell death (Table 1). Low concentrations of TNFα (185 U/ml) or IFNγ (14 U/ml) did not significantly affect INS1E cell viability (data not shown). High concentrations of TNFα (1850 U/ml) or IFNγ (140 U/ml) decreased the cell viability by around 20% (Table 1). TNFα (185 U/ml) as well as IFNγ (14 U/ml) at low concentration significantly potentiated IL-1β-induced INS1E cell death (Table 1). Treatment of INS1E cells with a combination of TNFα (185 U/ml) and IFNγ (14 U/ml) diminished cell viability by around 45% as compared to untreated cells (Table 1). The mixture of all three proinflammatory cytokines was the most toxic combination leading to a 70% cell loss (Table 1).

The incubation with an iNOS blocker, L-NOARG (5 mM), did not have any toxic effects on INS1E cell viability (Table 1). Interestingly, the blockade of iNOS prevented cell death related to IL-1β toxicity. The improved cell viability was also observed after exposure of INS1E cells to a combination of IL-1β and TNFα, or of IL-1β and IFNγ in the presence of the iNOS blocker (Table 1). The addition of the iNOS blocker did not significantly counteract the toxic effect of TNFα and IFNγ on INS1E cell viability (Table 1). Notably, however, insulin-secreting INS1E cells were not fully rescued from the toxicity caused by the mixture of all three cytokines, leaving behind still approximately a 50% cell death rate.

3.1.2. Caspase-3 activation

Incubation of insulin-secreting INS1E cells with IL-1β (60 U/ml) as well as 600 U/ml alone (1850 U/ml) failed to induce caspase-3 activation (Fig. 1A). The activation of caspase-3 was basically an IL-1β-dependent phenomenon, with a slight aggravating effect of TNFα (185 U/ml) (Fig. 1A). Importantly IFNγ alone (140 U/ml) was not able to induce caspase-3 activation (Fig. 1A). Moreover, incubation with TNFα (185 U/ml) and IFNγ (14 U/ml) caused less caspase-3 activation than TNFα alone (1850 U/ml) (Fig. 1A). The combination of all three proinflammatory cytokines (60 U/ml IL-1β, 185 U/ml TNFα and 14 U/ml IFNγ) was the most potent inducer of caspase-3 activation (Fig. 1A). The use of an iNOS blocker significantly prevented caspase-3 activation caused by IL-1β and by the mixture of all three cytokines (Fig. 1A).

3.2. NfκB-iNOS pathway activation after exposure to different cytokines in the absence or presence of an iNOS blocker in insulin-secreting INS1E cells

3.2.1. NfκB activation

The incubation of insulin-secreting INS1E cells with proinflammatory cytokines led to the activation of the transcription factor NfκB (Table 2). The low concentrations of any of the cytokines alone did not cause a significant rise in NfκB activation. TNFα (1850 U/ml) slightly activated NfκB, while even a high concentration of IFNγ (140 U/ml) failed to induce NfκB activation (Table 2). In contrast the higher concentration of IL-1β (600 U/ml) induced

<table>
<thead>
<tr>
<th>INS1E</th>
<th>Nitrite (pmol/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100 ± 0(10) 0 ± 0(5)</td>
</tr>
<tr>
<td>IL-1 [60]</td>
<td>87 ± 3(8) 1.1 ± 0.6(4)</td>
</tr>
<tr>
<td>IL-1 [600]</td>
<td>47 ± 3(7) 5.2 ± 1.6(9)</td>
</tr>
<tr>
<td>IL-1 [60] + TNF [185]</td>
<td>45 ± 3(5) 5.0 ± 0.4(6)</td>
</tr>
<tr>
<td>IL-1 [60] + IFN [14]</td>
<td>46 ± 5(5) 5.3 ± 1.6(9)</td>
</tr>
<tr>
<td>IL-1 [60] + TNF [185] + IFN [14]</td>
<td>28 ± 2(8) 7.3 ± 1.0(7)</td>
</tr>
<tr>
<td>TNF [1850]</td>
<td>83 ± 3(8) 0 ± 0(6)</td>
</tr>
<tr>
<td>IFN [140]</td>
<td>80 ± 4(7) 0 ± 0(6)</td>
</tr>
<tr>
<td>TNF [185] + IFN [14]</td>
<td>55 ± 6(4) 1.8 ± 0.4(6)</td>
</tr>
<tr>
<td>iNOS blocker</td>
<td>100 ± 0(6) 0 ± 0(7)</td>
</tr>
<tr>
<td>IL-1 [60]</td>
<td>91 ± 2(6) 0 ± 0(6)</td>
</tr>
<tr>
<td>IL-1 [600] + entry align = char*</td>
<td>1.4 ± 0.3(6)</td>
</tr>
<tr>
<td>char =&gt; 85 + 2(5)</td>
<td></td>
</tr>
<tr>
<td>IL-1 [60] + TNF [185]</td>
<td>77 ± 6(5) 1.3 ± 0.3(7)</td>
</tr>
<tr>
<td>IL-1 [60] + IFN [14]</td>
<td>61 ± 5(5) 2.2 ± 0.4(6)</td>
</tr>
<tr>
<td>IL-1 [60] + TNF [185] + IFN [14]</td>
<td>47 ± 3(4) 3.5 ± 0.3(6)</td>
</tr>
<tr>
<td>TNF [1850]</td>
<td>82 ± 3(5) 0 ± 0(6)</td>
</tr>
<tr>
<td>IFN [140]</td>
<td>78 ± 2(4) 0 ± 0(7)</td>
</tr>
<tr>
<td>TNF [185] + IFN [14]</td>
<td>68 ± 1(4) 0.2 ± 0(7)</td>
</tr>
</tbody>
</table>

INS1E cells were exposed for 24 h to the indicated cytokines and cytokine combinations (concentrations given in U/ml in brackets) in the absence or presence of the iNOS blocker. Numbers in brackets represent the concentration of the cytokines in U/ml. Cell viability was measured using the MTT assay. Nitrite formation was measured by Griess assay. Data are means ± SEM with the number of independent experiments in parentheses, each measured in at least triplicates.

P < 0.05 vs. no cytokines in the absence or presence of the iNOS blocker.

P < 0.05 vs. treated in the same way in the absence of the iNOS blocker, ANOVA followed by Bonferroni test.
NFκB activation significantly to the level of 2.7-fold higher or 2-fold higher than in the case of untreated cells, after 6 and 24 h, respectively (Table 2). TNFα (185 U/ml) as well as IFNγ (14 U/ml) potentiated IL-1β (60 U/ml) action and led to a significant 3-fold induction after 6 h and 2-fold induction of NFκB activation after 24 h (Table 2). The activation of NFκB was more pronounced in the case of a 6 h incubation (Table 2) and it was also counteracted by the addition of an iNOS blocker (Table 2). Interestingly, the iNOS blocker was not able to prevent cytokine-induced NFκB activation in the case of a prolonged incubation period (Table 2).

3.2.2. iNOS protein expression

The iNOS protein expression was significantly induced already after a 6 h incubation with cytokines and it was further increased after a 24 h incubation (Fig. 2). The iNOS protein expression was fully dependent on the IL-1β presence (Fig. 2) and it was not induced to any significant extent by addition of even high concentrations of TNFα (1850 U/ml) or IFNγ (140 U/ml) (Fig. 2). The iNOS blocker significantly diminished iNOS protein expression after 6 h exposure to cytokines, but had no effect in the case of a prolonged incubation (Fig. 2).

3.2.3. Nitrite accumulation

In order to estimate the iNOS enzyme activity state nitrite production was measured in the cell culture medium. In line with the iNOS protein expression studies, an IL-1β-dependent nitrite production was observed (Table 1). The experiments with an iNOS blocker showed a significant inhibitory effect of L-NOARG, even though the suppression of NO production was not complete (Table 1). Upon 6 h exposure to cytokines lower concentrations of nitrite were observed, which were completely blocked by the iNOS blocker (data not shown).

3.3. ER stress activation after exposure to different cytokines in the absence or presence of an iNOS blocker in insulin-secreting INS1E cells

The involvement of different proinflammatory cytokines in the ER stress response was analyzed by measurements of the ER-stress-related caspase-12 activation as well as by the estimation of ER stress marker gene expression (real-time PCR primers sequences are provided in Table 3).

Caspase-12 was significantly activated in the presence of IL-1β (60 U/ml and 600 U/ml) and its activation was an IL-1β-dependent event (Fig. 1B). IL-1β at the higher concentration (600 U/ml) did...
not cause a higher induction of caspase-12 that did IL-1β at the low concentration (60 U/ml) (Fig. 1B). TNFα (1850 U/ml) and IFNγ (140 U/ml) both failed to induce caspase-12 activation in the absence of IL-1β (Fig. 1B). The iNOS blocker significantly decreased cytokine-induced caspase-12 activation (Fig. 1B). A 24 h incubation of insulin-secreting INS1E cells with cytokines caused a significant induction of Chop gene expression (Fig. 3A). This induction was IL-1β dependent (Fig. 3A). Although neither TNFα (1850 U/ml) nor IFNγ (140 U/ml) alone did induce Chop transcription, each of them potentiated IL-1β-induced Chop gene expression (60 U/ml IL-1 β + 185 U/ml TNFα, or 60 U/ml IL-1 β + 14 U/ml IFNγ) (Fig. 3A). The iNOS blocker completely prevented cytokine-mediated Chop gene expression (Fig. 3A).

The expression of Bip was significantly diminished in the presence of cytokines (Fig. 3B). In contrast to the Chop expression, the Bip transcription was significantly affected by IL-1β (600 U/ml), TNFα (1850 U/ml) and also by IFNγ (140 U/ml) (Fig. 3B). The combination of low concentrations TNFα (185 U/ml) and IFNγ (14 U/ml) significantly reduced Bip transcription (Fig. 3B). Also the mixture of 60 U/ml IL-1β with TNFα (185 U/ml) or with IFNγ (14 U/ml) significantly diminished Bip gene expression (Fig. 3B). The cytokine-mediated decrease of Bip gene expression was not counteracted by an iNOS blocker (Fig. 3B).

4. Discussion

In this study we aimed to elucidate the potential differential effects and the individual interactions of the proinflammatory cytokines IL-1β, TNFα and IFNγ in insulin-producing INS1E cells in beta cell death and the involvement of NO in their intracellular effects with respect to ER stress and apoptosis induction.

Each of these cytokines possesses distinct mechanisms of action, and activates several different signalling cascades. IL-1β and TNFα additionally to other specific signalling pathways both induce the activation of the master transcription factor in beta cells, namely NFκB [1,3], while IFNγ affects gene transcription via activation of JAK/STAT pathway [1]. Thus, each of these cytokines can induce or repress gene transcription via different mechanisms and the combination of either cytokines can cause an aggravation or suppression of single cytokine effects. The focus of this study was to estimate which of these cytokines has the greatest toxic potential in insulin-producing cells and which combinations potentiate cytokine toxicity.

The mixture of all three cytokines was the most toxic combination causing nearly 70% cell death in the MTT assay. None of cytokines alone, at the concentration used in the cytokine mixture, caused any significant toxic effect in insulin-producing INS1E cells, as also observed in insulin-producing RINm5F cells [3]. However, a significant induction of cell death was achieved by incubation of INS1E cells with IL-1β, TNFα or IFNγ at 10-fold higher concentrations, with the most toxic effects of nearly 50% cell death caused by IL-1β. An incubation of insulin-producing INS1E cells with the combination of TNFα and IFNγ however, caused a 45% cell death rate, though this cytokine combination induced neither a significant NFκB activation nor a stimulation of iNOS expression and activity. Thus, the toxicity mechanism must be independent from the NFκB-iNOS pathway, and could possibly originate from induction of oxidative stress or the extrinsic caspase pathway.

The incubation with an iNOS blocker provided a strong, significant protection against IL-1β-induced toxicity. The inhibition of iNOS activity provided only a partial protection against the toxicity caused by the mixture of all three cytokines, leaving behind still an almost 50% cell death rate. This could be caused either by a partial abolishment of the iNOS activity only and NO production, which we confirmed in the accumulated nitrite measurements, or, additionally also by the cumulative action of other cell damage and death inducing pathways. Importantly the residual nitrate measured in the presence of an iNOS blocker is apparently not produced by nNOS, because we detected only a very weak expression of this NOS isoform, thus confirming an earlier report [26], which was further reduced in the presence of cytokines (Gurgul-Convey, unpublished observation).

The activation of the transcription factor NFκB was strongly induced by the high concentration of IL-1β. TNFα at a high concentration caused a mild induction of NFκB, but did not stimulate iNOS expression. It is possible therefore that either a strong NFκB activation is required for cytokine-induced iNOS expression in INS1E cells or that TNFα induces other signalling pathways which in turn inhibit iNOS expression. IFNγ failed to induce NfκB. TNFα and IFNγ in combination with IL-1β induced NfκB activation to almost the same extent as in the case of the mixture of all three

Fig. 2. iNOS protein expression after 6 and 24 h exposure to different cytokines in the absence or presence of an iNOS blocker in insulin-secreting INS1E cells. INS1E cells were seeded 24 h before the incubation with different cytokines (concentrations given in U/ml in brackets) for 6 or 24 h in the absence or presence of the iNOS blocker L-NAME (5 mM). Thereafter samples were collected and Western blot analyses performed. (A) 6 h incubation, (B) 24 h incubation; (upper panel) shown is a representative blot of 6 independent experiments, (lower panel) quantitative densitometric analyses of all experiments. The iNOS expression level was corrected to β-actin and presented as a fold increase. *P < 0.05 vs. untreated, ANOVA followed by Bonferroni.
cytokines. The use of an iNOS blocker diminished cytokine-induced NFκB activation during a short time incubation of 6 h, but failed to prevent NFκB activation by cytokines after prolonged incubation. The effects of L-NOARG on cytokine-induced NFκB correlated with the concentrations of nitrite measured after exposure of INS1E cells to cytokines in the presence of L-NOARG. The iNOS protein expression was stimulated in an IL-1β-dependent manner, and was diminished by an iNOS blocker, but only in the case of a short time exposure to cytokines in parallel with a decreased NFκB activation. However, no effect of L-NOARG on cytokine-induced iNOS protein expression was observed in the case of a long-time incubation. This suggests the existence of a regulatory feedback mechanism controlling the NFκB-iNOS pathway by its end product NO. This conclusion is supported by the observation that the NO donor SNAP has been shown to activate NFκB [27].

To further elucidate the mechanisms underlying the toxic effects of proinflammatory cytokines we performed an analysis of the activation of the effector caspase-3 in insulin-producing INS1E cells. The results showed a significant induction of caspase-3 activation by IL-1β and its combination with either TNFα or IFNγ, or all three cytokines. TNFα alone induced caspase-3 activation, while IFNγ failed to do so. Thus, although IFNγ decreased cell viability measured in the MTT assay, it failed to induce caspase 3 activation, indicating that in its deleterious effect a necrotic component is of crucial importance. The IL-1β- as well as cytokine mixture-induced caspase-3 activation was almost completely blocked by the use of an iNOS blocker, supporting the idea that in IL-1β-mediated toxicity to INS1E cells nitric oxide plays a crucial role.

Because it is known from earlier studies that NO can play a role in cytokine-induced ER stress in insulin-producing cells, we analyzed the effects of proinflammatory cytokines in the absence and presence of an iNOS blocker on the ER stress-associated caspase-12 activation as well as on the expression of ER stress markers.

Caspase-12 associates with activated Ire1, resulting in proteolytic processing of caspase-12 [17]. Mice lacking caspase-12 genes display a partial resistance to pharmacological ER stress inducers [28]. Caspase-12 activation was an IL-1β-dependent event while both TNFα and IFNγ when used alone or in combination failed to induce it. The induction of caspase-12 was prevented by an iNOS blocker. This clearly indicates that caspase-12 activation in insulin-producing INS1E cells depends on the presence of IL-1β-derived NO.

Caspase-12 activation has been shown to be strongly associated with ER stress [17,18]. Therefore we measured the expression of two important players in the ER stress response, namely the stress-induced transcription factor Chop and the ER chaperone Bip. A high concentration of IL-1β strongly induced Chop expression, thus confirming earlier reports [29]. Interestingly, although low concentrations of IL-1β, TNFα or IFNγ failed to induce Chop expression, the combinations of IL-1β with either TNFα or IFNγ stimulated the Chop expression. It seems therefore that the induction of Chop expression requires either a cooperative action of more than one cytokine or a high level of IL-1β. The Chop expression was completely abolished by the iNOS blocker, in line with earlier studies [20,30]. It has been shown before that a chemical NO donor, SNAP, depletes ER calcium in insulin-producing MIN6 cells and that triggers Chop expression and apoptosis [20]. Also, IL-1β and IFNγ, via NO synthesis, decrease the expression of SERCA in primary beta cells and insulin-producing INS1E, depleting ER calcium stores [31,32]. The present study shows clearly that in insulin-producing INS1E cells Chop expression is related and dependent on IL-1β-induced NO formation.

**Table 3**

Sequences of the primers used for the real-time RT-PCR gene expression quantification.

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′-Primer sequence-3′</th>
</tr>
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<tbody>
<tr>
<td>Chop</td>
<td>Fw – forward (sense) primer; Rev – reverse (antisense) primer. β-actin was used as a housekeeping gene.</td>
</tr>
<tr>
<td>Bip</td>
<td>Rev AGGGCTCCACTTCCATAGA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Fw GAA CAC GGC ATT GTA ACC AAC TGG</td>
</tr>
</tbody>
</table>

Fw – forward (sense) primer; Rev – reverse (antisense) primer. β-actin was used as a housekeeping gene.
In contrast to a strong induction of Chop expression, the transcription of another ER stress marker, namely Bip, was significantly reduced after incubation with all cytokines and their combinations. While a decreased Bip expression after IFNγ treatment has been already shown [33], no information about the role of TNFα has been available so far. Interestingly, there was no protective effect of an iNOS blocker on the cytokine-reduced Bip expression. These observations indicate also that in TNFα and IFNγ toxicity the reduction of the availability of Bip in the ER lumen may play an important role making insulin-producing cells with their enormous production of insulin and their great secretory capacity particularly sensitive to cytokine toxicity.

It has been suggested that the aggravating effects of IFNγ on IL-1β-induced stress and beta cell damage are due to a decrease of beta cell defenses, like downregulation of the ER chaperone Bip [31]; however, from these studies it was not clear whether the observed acceleration of the IL-1β action was due to the IFNγ effect itself or the additional stimulation of iNOS-derived NO production in beta cells. In our study we could show that in insulin-producing INS1E cells IFNγ in the chosen concentration failed to accelerate IL-1β-stimulated iNOS expression and nitrite formation, but significantly decreased Bip. Thus, the IFNγ-induced reduction of Bip expression did not depend on the presence of NO. No rescue of Bip expression after co-incubation with IFNγ and an iNOS blocker provides another independent proof for a NO-independent effect of IFNγ on Bip in insulin-producing INS1E cells.

The study shows that the ER stress component of cytokine-induced pancreatic beta cell damage and death only partially depends on IL-1β-induced NO formation. It becomes apparent that TNFα and IFNγ, which do not induce iNOS expression and NO production, but nevertheless exhibit together significant toxicity to insulin-producing cells, have accelerating effects on ER stress, namely causing an additive induction of Chop and suppression of Bip. These findings widen our knowledge about the mechanisms underlying cytokine-induced toxicity in insulin-producing cells and can help to develop rational therapeutic strategies for prevention of cytokine toxicity in cytokine-mediated autoimmune diabetes.

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References