Interaction between pro-inflammatory and anti-inflammatory cytokines in insulin-producing cells

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Abstract

Pro-inflammatory cytokines cause β -cell dysfunction and death. The aim of this study was to investigate the interactions between different pro- and anti-inflammatory cytokines and their effects on apoptotic β -cell death pathways. Insulin-producing RINm5F cells were exposed to different combinations of cytokines. Gene expression analyses of manganese superoxide dismutase (MnSOD) and inducible nitric oxide synthase (iNOS) were performed by real-time RT-PCR. Cell viability was measured by the MTT assay, NF κ B activation using a SEAP reporter gene assay, protein expression by western blotting and caspase-3 activity using the DEVD cleavage method. IL-1 β , tumour necrosis factor α (TNF α) and a combination of all three pro-inflammatory cytokines increased while IFN γ alone did not affect NF κ B activity and iNOS gene and protein expression. Interestingly, the anti-inflammatory cytokines IL-4, IL-13 and IL-10 decreased IL-1 β -stimulated NF κ B activation and iNOS

expression. IL-1 β , TNF α and the pro-inflammatory cytokine combination also increased MnSOD gene and protein expression. But IL-4, IL-13 and IL-10 did not affect MnSOD expression and did not modulate IL-1 β -stimulated MnSOD expression. Caspase-3 activity was increased by IL-1 β and the pro-inflammatory cytokine combination, and to a lesser extent by TNF α . In contrast, IFN γ had no effect on caspase-3 activity. IL-4, IL-13 and IL-10 decreased caspase-3 activity and increased viability of insulin-producing cells treated with pro-inflammatory cytokines. The anti-inflammatory cytokines counteracted the cytotoxic effects of pro-inflammatory cytokines in insulin-producing cells. This was achieved through the reduction of nitrosative stress. Thus, a balance between the anti-inflammatory and the pro-inflammatory cytokines is of crucial importance for the prevention of pancreatic β -cell destruction.

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Introduction

The pro-inflammatory cytokines, such as IL-1 β , tumour necrosis factor α (TNF α) and IFN γ , are putative mediators of the progressive loss of pancreatic β -cells in type 1 diabetes mellitus. These cytokines are released by macrophages and T cells in infiltrated islets of Langerhans and cause impaired function and ultimately cell death by apoptosis or necrosis (Eizirik & Mandrup-Poulsen 2001). On the other hand, the release of anti-inflammatory cytokines, in particular IL-4, IL-13 and IL-10, is related to the protection of pancreatic β -cells (Zacccone *et al.* 1999, Kaminski *et al.* 2007) and the prevention of destructive insulinitis (Rabinovitch & Suarez-Pinzon 2003). Cytokines acting alone or in combination induce various transcription factors and signal transduction pathways within β -cells. One of the most important signalling events is the activation of the transcription factor, nuclear factor kappa B (NF κ B; Eizirik *et al.* 1996b), which plays the role of a master switch in β -cells, activating

transcription of a number of genes involved in cytokine-mediated toxicity. Of great importance for cytokine toxicity in β -cells are in particular the generation of nitric oxide (NO) via induction of the inducible nitric oxide synthase (iNOS) and production of reactive oxygen species. Cytokine-induced nitrosative and oxidative stresses trigger eventually β -cell death (Eizirik *et al.* 1996a,b, Eizirik & Mandrup-Poulsen 2001, Bast *et al.* 2002). β -Cells produce a large amount of NO when exposed to IL-1 β or a combination of pro-inflammatory cytokines (Cetkovic-Cvrlje & Eizirik 1994, Eizirik & Leijerstam 1994, Eizirik *et al.* 1996b, Flodstrom *et al.* 1996, Darville & Eizirik 1998). However, it is not clear whether NO is crucial for apoptosis in β -cells, since conflicting results have been published (Wright *et al.* 1999, Saldeen 2000, Zumsteg *et al.* 2000, Kuhn *et al.* 2003). The pro-inflammatory cytokines induce also the expression of manganese superoxide dismutase (MnSOD) in β -cells, the enzyme that resides in the mitochondria and generates there toxic hydrogen peroxide, which cannot be removed in face of lack of appropriate

detoxifying enzymes such as catalase and glutathione peroxidase (Gpx) in insulin-producing cells (Lenzen *et al.* 1996, Tiedge *et al.* 1997). The activation of MnSOD seems to play an important role in cytokine-mediated oxidative stress in β -cells (Lortz *et al.* 2005).

Although the action of anti-inflammatory cytokines has been studied in different cell types during recent years (Perretti *et al.* 1995, Berkman *et al.* 1996, Laffranchi & Spinas 1996, Ledebuer *et al.* 2000, Lubberts *et al.* 2000, Nishisaka *et al.* 2001, Plunkett *et al.* 2001, Lieb *et al.* 2003, Serafin *et al.* 2004, Raz *et al.* 2005, Pennock & Grecis 2006, Verri *et al.* 2006, Walsh 2006), little is known about the effects of these cytokines in pancreatic β -cells. It is known from the studies in different cell models that IL-4 is able to counteract many of the IL-1 β effects, and reduced NO production has been considered an important element for this beneficial effect (Perretti *et al.* 1995, Ledebuer *et al.* 2000, Lubberts *et al.* 2000, Nishisaka *et al.* 2001). The biological effects of IL-13 may be achieved through binding to the IL-4 receptor- α (Callard *et al.* 1996, Kotowicz *et al.* 1996, Kraich *et al.* 2006), and therefore it is generally assumed that these two cytokines overlap in the biological effects. The effects of IL-10 on the nitric oxide pathway are unclear and the opposite findings have been reported (Berkman *et al.* 1996, Laffranchi & Spinas 1996, Plunkett *et al.* 2001, Lieb *et al.* 2003, Serafin *et al.* 2004). Some studies on anti-inflammatory cytokine action in insulin-producing cells have been published recently (Kaminski *et al.* 2007), but opposite effects with respect to β -cell survival have been reported and the underlying molecular mechanisms of the action of anti-inflammatory cytokines still remain unknown.

The pro-inflammatory cytokines may directly activate caspases, the main effectors of programmed cell death. Nuclear chromatin condensation and DNA fragmentation (classical characteristics of programmed cell death), sequential activation of caspases and segmentation in apoptotic bodies have already been described for insulin-producing cells exposed to cytokines (Delaney *et al.* 1997, Kurrer *et al.* 1997). Cell death by apoptosis is ultimately accomplished by effector caspases, i.e. caspases 3, 6 and 7 (Joshi & Sahni 2003, Joshi *et al.* 2003). Previous studies have shown that TNF α activates caspases 3, 8 and 9 in different cell types (Alikhani *et al.* 2004) and that exposure of insulin-producing cells to IFN γ causes activation of caspase-1 (Karlsen *et al.* 2000). IL-1 β is able to activate caspase-3 in β -cells and this effect is possibly linked to NO production (Chen *et al.* 2003, Veluthakal *et al.* 2004). However, also in this case, the differential effects of cytokines are unknown, and no data regarding the combined action of pro- and anti-inflammatory cytokines on caspase-3 activation in insulin-producing cells are available.

Since cytokines may vary in their mechanisms of action, we decided to perform a detailed analysis of the effects of single pro- and anti-inflammatory cytokines as well as of different combinations, in order to analyse potential crosstalk. We studied the different pathways and mechanisms of pro-inflammatory cytokine-mediated β -cell death as well as their interactions with anti-inflammatory cytokines with

particular emphasis upon the early changes in the death signalling pathways.

Materials and Methods

Chemicals

Cytokines and the dNTP mixture were obtained from PromoCell (Heidelberg, Germany). The jetPEI transfection reagent was purchased from Qbiogene (Heidelberg, Germany) and Biotherm Taq polymerase from GeneCraft (Münster, Germany). The SuperScript II RT reverse transcriptase and all tissue culture equipment were purchased from Invitrogen. All primers were from MWG-Biotech (Ebersberg, Germany). The Ac-DEVD-AMC caspase-3 substrate and Ac-DEVD-CHO₂ caspase-3 inhibitor were from Biosource International (Carnarillo, CA, USA). SNAP (S-nitroso-*n*-acetylpenicillamine) was from Alexis Biochemicals (San Diego, CA, USA). Hybond N nylon membranes, ECL detection system and autoradiography films were from Amersham Biosciences and Immobilon-P polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA, USA). All other reagents were from Sigma Chemicals (München, Germany).

Cell culture and cytokine incubation

Insulin-producing RINm5F cells were cultured as described (Lortz *et al.* 2000) in RPMI1640 medium, supplemented with 10 mM glucose, 10% (v/v) fetal calf serum (FCS), penicillin and streptomycin in a humidified atmosphere at 37 °C and 5% CO₂. For RNA extraction and protein analysis, cells were plated at a density of 2×10^6 per 90 mm plastic dish and grown to confluence within 2 days. Thereafter, cells were exposed to the desired concentration of pro- and/or anti-inflammatory cytokines, the nitric oxide-donor SNAP, the iNOS blocker *N* ω -nitro-L-arginine (L-NOARG) and camptothecin. The concentrations of cytokines were 60 U/ml IL-1 β (4.4 ng/ml); 600 U/ml IL-1 β (44 ng/ml); 185 U/ml TNF α (8.7 ng/ml); 1850 U/ml TNF α (87 ng/ml); 14 U/ml IFN γ (10.3 ng/ml); 140 U/ml IFN γ (103 ng/ml); 500 U/ml IL-4 (50 ng/ml); 500 U/ml IL-13 (50 ng/ml) and 500 U/ml IL-10 (50 ng/ml). Control RINm5F cells were grown in the absence of test compounds. The lower concentrations of pro-inflammatory cytokines are commonly used in β -cell research (Mandrup-Poulsen *et al.* 1987, 1990, Nerup *et al.* 1994, Lortz *et al.* 2000). The anti-inflammatory cytokine concentrations were chosen on the basis of preliminary concentration dependencies as the best suited for counteracting the effects of pro-inflammatory cytokines (data not shown).

MTT cell viability assay

In all sets of experiments, the viability of the cells was determined after a 24 h or 48 h incubation period using a

microplate-based MTT assay (Mosmann 1983). The viability was expressed as % of the MTT absorbance at 562/650 nm in the absence of test compounds.

iNOS and MnSOD western blot analyses

RINm5F cells were incubated for 24 h with cytokines, washed with ice-cold PBS and homogenised in ice-cold buffer (50 mM Tris-HCl, 1% Triton T100, 5% glycerol, pH 7.5) using short bursts (Braun-Sonic 125 Homogenisator, Quigley-Rochester, Inc., Rochester, NY, USA) and then heated up to 94 °C for 3 min. Cell homogenates were centrifuged at 9000 *g* and 4 °C for 10 min and the supernatants were used for western blot analyses. Protein content was determined by the BCA assay (Pierce, Rockford, IL, USA). For the assays, 20 µg (for MnSOD) or 40 µg (for iNOS) total protein were resolved in SDS polyacrylamide gel electrophoresis and then electroblotted onto membranes. The Ponceau staining was used as a loading control. Immunodetection was performed by specific primary antibodies against MnSOD (rabbit polyclonal, a kind gift from Dr. K Dobashi, Japan) or iNOS (NOS2 rabbit polyclonal IgG, Santa Cruz Biotechnology, Heidelberg, Germany) followed by exposition to secondary peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (Dianova, Hamburg, Germany). The hybrids were visualised 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).

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 h. The pSEAP-NF-κB construct was used as described in detail earlier (Azevedo-Martins *et al.* 2003). Secreted alkaline phosphatase expression was measured using the Phospha-Light System kit (Applera, Darmstadt, Germany).

Caspase-3 activity assay

The fluorimetric method of cleavage of the DEVD was employed. Caspase-3 cleaves the DEVD (a tetrapeptide composed of Asp-Glu-Val-Asp) in the C side of the aspartate residuum. In case of the cytokine incubations, control and treated incubated RINm5F cells were washed with ice-cold PBS and harvested using a cell scraper and incubated in hypotonic lysis buffer containing Chaps and TritonX for 10 min in microfuge tubes on ice. In contrast to pro-inflammatory cytokines, the chemical compounds used in this study caused significant cell death even after short-time exposures. Therefore, in order to measure caspase-3 activity in all cells, also the dying cells, after indicated time period the cells

were directly harvested using a cell scraper, centrifuged at 800 *g* at 4 °C for 5 min and then the cell pellet was washed with ice-cold PBS and lysed as written for cytokines. Thereafter, the tubes were centrifuged at 12 500 *g* at 4 °C for 10 min and the supernatants were stored at –80 °C for measurement. For the assay, 20 µl lysate, 10 µl substrate and 70 µl assay buffer were placed in 96-well plates in duplicate, and the fluorescence at 360/460 nm excitation/emission was measured every 5 min for 2 h at 37 °C. Total protein content was determined by the Bradford assay. Calculation of the specific activity of caspase-3 was done adjusting the delta fluorescence value at 60 min–0 min (Δ FU) to the protein content and dividing the obtained value by the slope of the standard curve. All caspase-3 activity curves were linear in the time window chosen. The amount of fluorescence produced was calculated against a standard curve of AMC over the time. The threshold for the enzyme activity was set at 1000 Δ FU/h. The results are presented as % of untreated cells.

RNA isolation and cDNA preparation

Total RNA was obtained using the guanidine thiocyanate method (Chomczynski & Sacchi 1987). The quality of the total RNA was verified in agarose gel electrophoresis. RNA was quantified spectrophotometrically at 260/280 nm. Thereafter, 2 µg RNA was reverse transcribed into complementary DNA using an oligo-dT18TNV primer and a reverse transcriptase.

Real-time RT-PCR

The QuantiTect SYBR Green technology (QIAGEN), which uses a fluorescent dye that binds only double-stranded DNA, was employed. The reactions were performed by the DNA Engine Opticon Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). A total volume of 25 µl was used for the PCRs. Samples were first denatured at 94 °C for 2 min followed by up to 30 PCR cycles. Each PCR cycle comprised melting at 94 °C for 30 s, annealing at 61–63 °C for 30 s and extension at 72 °C for 30 s. Each PCR amplification was performed in triplicate. The optimal parameters for the PCRs were empirically defined. The purity of the amplified PCR products was verified by melting curves. The standard curves for each gene were generated using the specific amplified fragment cloned into a plasmid vector (pCR II-TOPO, Invitrogen), and the amounts determined using a fluorimetric assay (PicoGreen DNA Quantitation Kit, Invitrogen). The primers used in this study are shown in Table 1.

Data analysis

Analyses of the real-time RT-PCR data and of the standard curve for the genes were performed by the Opticon Monitor v. 1.07 (MJ Research Inc., Waltham, MA, USA). Densitometry for the immunoblottings was done using specific

Table 1 Sequences of the primers used for the real-time RT-PCR gene expression quantification. All amplicons were in the size ranging from 95 to 170 bp. GAPDH was used as housekeeping gene

Gene	GenBank access	5'-Primer sequence-3'
GAPDH	NM_017008	Fw: TCACCACCATGGAGAAGGC Rv: GCTAAGCAGTTGGTGGTGCA
Caspase-3	NM_012922	Fw: TTGGAACGAACGGACCTGTGG Rv: TGTCTCAATACCGCAGTCCAGCTC
iNOS	D44591	Fw: TCGTACTTGGGATGCTCCATGG Rv: TCCTGCAGGCTCACGGTCAA
MnSOD	NM_017051	Fw: CCTCCCTGACCTGCCTTACGACTA Rv: TTCAGATTGTTACGCTAGTGCGC

Fw, forward (sense) primer; Rv, reverse (antisense) primer.

software (Gel-Pro Analyzer v. 4.0, Media Cybernetics, Silver Spring, MD, USA). All data are expressed as means $\pm$ S.E.M. Statistical analyses for each group were performed by the Prism analysis program (Graphpad, San Diego, CA, USA).

Results

Effects of cytokines on NF κ B enhancer element activation

To study the effects of cytokines on early events in the cytokine-induced cell death signalling cascade, the activation

of the transcription factor NF κ B was analyzed. Incubation of insulin-producing RINm5F cells for 6 h with IL-1 β resulted in a significant, concentration-dependent activation of the NF κ B enhancer element (Table 2). TNF α and to a lesser extent IFN γ enhanced IL-1 β -stimulated NF κ B induction. A combination of all three pro-inflammatory cytokines (60 U/ml IL-1 β , 185 U/ml TNF α , 14 U/ml IFN γ) strongly stimulated NF κ B activation. TNF α (185 U/ml) alone increased NF κ B activity slightly, while IFN γ (140 U/ml) alone did not significantly activate NF κ B (Table 2). The anti-inflammatory cytokines IL-4, IL-13 and IL-10 did not induce NF κ B activation, but rather decreased IL-1 β -stimulated

Table 2 Effects of pro- or anti-inflammatory cytokines in insulin-producing RINm5F cells on NF κ B activation and the inducible nitric oxide synthase (iNOS) and manganese superoxide dismutase (MnSOD) gene expression. Insulin-producing RINm5F cells were exposed for 6 h to the indicated compounds. Results are presented as fold increase comparing against control values obtained from cells incubated without the compounds. Normalisation of the real-time PCR curves was done against GAPDH. Data are mean values for the indicated number (n) of independent experiments, in each measured in at least three repetitions

	NF κ B induction	iNOS expression	MnSOD expression
RINm5F			
Untreated	1.0 $\pm$ 0.1 (20)	1.0 $\pm$ 0.1 (20)	1.0 $\pm$ 0.1 (12)
IL-1 (60)	1.8 $\pm$ 0.2 (13)*	800 $\pm$ 109 (6)*	6.2 $\pm$ 1.2 (4)*
IL-1 (600)	2.5 $\pm$ 0.2 (12)*, [†]	1982 $\pm$ 223 (5)*, [†]	10 $\pm$ 2 (5)*
TNF (185)	1.4 $\pm$ 0.1 (18)	22 $\pm$ 4 (7)	2.6 $\pm$ 0.2 (4)
TNF (1850)	1.5 $\pm$ 0.0 (18)	37 $\pm$ 15 (7)	3.9 $\pm$ 0.8 (4)*
IFN (14)	1.2 $\pm$ 0.1 (10)	2.2 $\pm$ 0.3 (4)	0.7 $\pm$ 0.1 (7)
IFN (140)	1.2 $\pm$ 0.1 (20)	3.3 $\pm$ 0.8 (5)	1.4 $\pm$ 0.2 (4)
IL-1 + TNF	2.5 $\pm$ 0.2 (20)*, [†] , [‡]	844 $\pm$ 75 (4)*, [‡]	8.3 $\pm$ 1.7 (4)*, [‡]
IL-1 + IFN	2.2 $\pm$ 0.2 (20)*, [§]	833 $\pm$ 209 (4)*, [§]	6.5 $\pm$ 1.8 (4)*, [§]
TNF + IFN	1.3 $\pm$ 0.1 (20)	5.2 $\pm$ 1.1 (4)	2.6 $\pm$ 0.2 (5)
Mix	2.4 $\pm$ 0.1 (40)*, [†] , [‡] , [§]	1024 $\pm$ 122 (6)*, [†] , [‡] , [§]	9.3 $\pm$ 2.0 (5)*, [†] , [‡] , [§]
IL-4 (500)	1.3 $\pm$ 0.1 (20)	0.9 $\pm$ 0.2 (4)	1.2 $\pm$ 0.2 (4)
IL-1 + IL-4	1.2 $\pm$ 0.1 (20) [†]	61 $\pm$ 7 (4) [†]	9.9 $\pm$ 2.6 (3)*,
IL-13 (500)	1.1 $\pm$ 0.1 (20)	1.4 $\pm$ 0.4 (4)	1.7 $\pm$ 0.4 (2)
IL-1 + IL-13	1.4 $\pm$ 0.1 (20) [†]	65 $\pm$ 20 (4) [†]	9.2 $\pm$ 2.2 (5)*,
IL-10 (500)	1.3 $\pm$ 0.1 (20)	1.0 $\pm$ 0.3 (3)	1.0 $\pm$ 0.1 (4)
IL-1 + IL-10	1.4 $\pm$ 0.1 (20) [†]	133 $\pm$ 40 (3) [†]	11 $\pm$ 2 (3)*, ^a
Camptothecin (0.5)	0.8 $\pm$ 0.1 (4)	1.0 $\pm$ 0.2 (3)	0.9 $\pm$ 0.2 (3)

Numbers in brackets represent the concentration of the compound in U/ml or μ M (for camptothecin). * P < 0.05 versus control; [†] P < 0.05 versus IL-1 β 60 U/ml; [‡] P < 0.05 versus TNF α ; [§] P < 0.05 versus IFN γ ; ^{||} P < 0.05 versus IL-4; ^a P < 0.05 versus IL-13, ^a P < 0.05 versus IL-10, ANOVA followed by Bonferroni test. The expression level for the housekeeping gene GAPDH did not change significantly. Mix, 60 U/ml IL-1 β , 185 U/ml TNF α , 14 U/ml IFN γ , the same concentrations of cytokines were used in case of co-incubations with two cytokines.

NFκB activation (Table 2). The pro-apoptotic caspase-3 activator camptothecin failed to activate the NFκB enhancer element (Table 2).

Effect of cytokines on expression of genes mediating cell death and survival

The induction of the iNOS gene in insulin-producing RINm5F cells was basically an IL-1β-mediated event (Table 2). IL-1β-stimulated iNOS gene expression achieved a maximum 5 h after addition of the cytokine (Fig. 2). The effect of IL-1β on iNOS gene expression was concentration dependent (Table 2). The pro-inflammatory cytokines TNFα and IFNγ neither alone nor in combination significantly induced iNOS gene expression (Table 2). However, a combination of all three pro-inflammatory cytokines induced a more potent stimulatory effect on iNOS gene expression than 60 U/ml IL-1β alone (Table 2). Interestingly, TNFα in combination with IL-1β also showed an additive effect (Table 2).

IL-4 (500 U/ml), IL-13 (500 U/ml) and IL-10 (500 U/ml) did not affect iNOS gene expression. However, all anti-inflammatory cytokines significantly decreased iNOS gene expression induced by IL-1β, an effect that was particularly evident with IL-4 and IL-13. The pro-apoptotic caspase-3 activator camptothecin did not affect iNOS expression.

MnSOD gene expression was also affected by cytokine treatment (Table 2). After exposure to IL-1β (60 U/ml) insulin-producing RINm5F cells exhibited a significant increase in MnSOD gene expression when compared with control cells (Table 2). Higher concentrations of IL-1β (600 U/ml) further increased this effect. While a low concentration of TNFα (185 U/ml) did not significantly affect MnSOD gene expression, a higher concentration of this cytokine (1850 U/ml) caused a significant increase of MnSOD gene expression. IFNγ (14 and 140 U/ml) did not affect the expression of MnSOD and did not induce any synergistic effect in combination with IL-1β or TNFα. The combination of three pro-inflammatory cytokines increased MnSOD expression to a level similar to that found after exposure to IL-1β plus TNFα. IL-4 (500 U/ml), IL-13 (500 U/ml) and IL-10 (500 U/ml) did not affect MnSOD gene expression. Moreover, combinations of IL-4, IL-13 or IL-10 plus IL-1β did not show a significant additive effect. Again, camptothecin did not modulate MnSOD expression.

The caspase-3 gene expression levels were not affected by any of the different cytokine treatments after a 6 h incubation time (data not shown). A time-dependent effect to the exposure to a high concentration of IL-1β (600 U/ml) revealed a small significant increase in caspase-3 gene expression only after a 4 h incubation time (Fig. 1).

Effects of cytokines on iNOS and MnSOD protein expression

There was no iNOS protein detectable in untreated insulin-producing RINm5F cells (Table 3 and Fig. 3). Induction of iNOS protein was basically an IL-1β-mediated effect, as shown in Table 3 and Fig. 3. A time course analysis of iNOS

Figure 1 Time-dependent effect of IL-1β on the caspase-3 gene expression in insulin-producing cells. RINm5F cells were incubated with 600 U/ml IL-1β. The mRNA was obtained using the guanidine-thiocyanate method. After reverse transcription, gene expression was measured by quantitative real-time PCR at 0 (no cytokines added, control), 1, 2, 3, 4, 5, 6, 8 and 24 h after incubation. $n=4$. * $P<0.05$, ANOVA followed by Bonferroni.

Table 3 Effects of pro- or anti-inflammatory cytokines in insulin-producing RINm5F cells on the inducible nitric oxide synthase (iNOS) and manganese superoxide dismutase (MnSOD) protein expression, insulin-producing RINm5F cells were exposed for 24 h to the indicated compounds. Numbers in brackets represent the concentration of the compound in U/ml. Results are presented as % or fold increase comparing against control values obtained from cells incubated without cytokines. Data are mean values for the indicated number (n) of independent experiments

	iNOS expression (% of IL-1 60)	MnSOD expression (fold induction)
RINm5F		
Untreated	n.d ± 0 (8)	1.0 ± 0.1 (5)
IL-1 (60)	100 ± 2 (6)	2.4 ± 0.2 (10)*
IL-1 (600)	274 ± 42 (8) [†]	2.7 ± 0.6 (5)*
TNF (185)	n.d ± 0 (7)	1.6 ± 0.2 (6)*
TNF (1850)	n.d ± 0 (7)	1.8 ± 0.2 (4)*
IFN (14)	n.d ± 0 (4)	1.2 ± 0.3 (6)
IFN (140)	n.d ± 0 (5)	1.5 ± 0.4 (4)
IL-1 + TNF	145 ± 14 (3) [†]	3.0 ± 0.4 (5)*
IL-1 + IFN	97 ± 18 (4)	1.7 ± 0.1 (4)*
TNF + IFN	n.d ± 0 (4)	1.7 ± 0.1 (6)*
Mix	254 ± 75 (6) [†]	3.0 ± 0.4 (4)*
IL-4 (500)	n.d ± 0.2 (4)	1.1 ± 0.1 (3)
IL-1 + IL-4	60 ± 9 (6) [†]	2.2 ± 0.4 (7)
IL-13 (500)	n.d ± 0 (4)	0.9 ± 0.2 (4)
IL-1 + IL-13	67 ± 18 (4) [†]	2.1 ± 0.7 (7)
IL-10 (500)	n.d ± 0.3 (4)	1.1 ± 0.1 (4)
IL-1 + IL-10	65 ± 18 (4) [†]	2.3 ± 0.6 (7)

* $P<0.05$ versus control, Dunnett's test; [†] $P<0.05$ versus IL-1β 60 t -test. Mix, 60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ, the same concentrations of cytokines were used in case of co-incubations with two cytokines.

protein expression showed a delayed increase of protein expression in comparison with gene expression. The expression of iNOS protein became evident after a 3- to 4 h exposure and achieved a maximal expression level after an 8 h exposure to IL-1 β (600 U/ml), which remained stable up to 24 h (Fig. 3). Thus, maximal iNOS protein expression was delayed by around 3 h when compared with maximal iNOS gene expression (Figs 2 and 3). iNOS protein was not detected after 24 h incubation of the cells with TNF α at both low and high concentrations (185 and 1850 U/ml) and also after exposure of the cells to both low and high concentrations of IFN γ (14 and 140 U/ml; Table 3). TNF α showed a synergistic effect with IL-1 β (Table 3). The combination of IL-1 β with IFN γ did not have an additive effect when compared with IL-1 β alone (Table 3). The combination of TNF α and IFN γ did not induce iNOS protein expression (Table 3). A combination of all three pro-inflammatory cytokines (60 U/ml IL-1 β , 185 U/ml TNF α , 14 U/ml IFN γ) strongly induced iNOS protein expression. IL-4 (500 U/ml), IL-13 (500 U/ml) and IL-10 (500 U/ml) did not affect iNOS protein expression. However, all three anti-inflammatory cytokines significantly decreased IL-1 β -induced iNOS protein expression, an effect that was particularly evident with IL-4 (Table 3).

MnSOD protein expression was affected by 24 h cytokine treatment (Table 3). After exposure to IL-1 β (60 U/ml) insulin-producing RINm5F cells exhibited a significant increase of MnSOD protein expression when compared with control cells. Higher concentrations of IL-1 β (600 U/ml) further potentiated

Figure 2 Time-dependent effect of IL-1 β on the iNOS gene expression in insulin-producing cells. RINm5F cells were seeded 24 h before the incubation with 600 U/ml IL-1 β . The mRNA was obtained using the guanidine-thiocyanate method. After reverse transcription, gene expression was measured by quantitative real-time PCR at 0 (no cytokines added, control), 1, 2, 3, 4, 5, 6, 8 and 24 h after incubation. The means of absolute values from four to eight independent experiments are shown. * $P < 0.05$, ANOVA followed by Bonferroni.

Figure 3 Time-dependent effect of IL-1 β on the iNOS protein expression in insulin-producing cells. RINm5F cells were seeded 24 h before the incubation with 600 U/ml IL-1 β . The cells were lysed and used for western blot analyses at 0 (no cytokines added, lane C, control), 1, 2, 3, 4, 5, 6, 8 and 24 h after incubation. #not detectable. A representative blot of four independent experiments is shown.

this effect. Both, low and high concentrations of TNF α significantly enhanced MnSOD protein expression (Table 3). IFN γ (14 and 140 U/ml) did not affect MnSOD protein expression and did not induce any synergistic effect in combination with IL-1 β or TNF α (Table 3). The combination of IL-1 β 60 U/ml and TNF α 185 U/ml or of three pro-inflammatory cytokines increased MnSOD expression to a level similar to that found after exposure to 600 U/ml IL-1 β (Table 3). IL-4 (500 U/ml), IL-13 (500 U/ml) and IL-10 (500 U/ml) did not affect MnSOD protein expression. A combination of IL-1 β with anti-inflammatory cytokines did not significantly affect IL-1 β -stimulated MnSOD protein expression when compared with IL-1 β alone. Camptothecin did not affect the protein expression of iNOS or MnSOD.

Effect of cytokines on cell viability

Incubation of control RINm5F cells with the pro-inflammatory cytokines IL-1 β , TNF α and IFN γ for 24 and 48 h caused a loss of cell viability in the MTT assay, which was more pronounced in the case of the 48 h incubation (Table 4).

Only high concentrations of 600 U/ml IL-1 β and the combination of TNF α and IFN γ significantly induced cell death after 24 h (Table 4). The most toxic effects were observed in case of a mixture of all three pro-inflammatory cytokines IL-1 β , TNF α and IFN γ (60, 185, and 14 U/ml respectively) as evident from a more than 30% decrease in cell viability (Table 4). Even high concentrations of TNF α or

Table 4 Cell viability and caspase-3 activity of RINm5F cells exposed to cytokines. For cell viability, insulin-producing RINm5F cells were exposed to cytokines for 24 and 48 h in the indicated concentration. The measurements were done using the MTT assay. For caspase-3 activity, the RINm5F cells were exposed to the compounds in the indicated concentration for 8 h, washed with ice-cold PBS and lysed as described in the Methods. After lysis of the cells and extraction of the total intracellular protein, the activity of caspase-3 was measured using the DEVD cleavage method. Data are mean values for the indicated number (*n*) of independent experiments, in each measured in at least three repetitions

	Cell viability (24 h)	Cell viability (48 h)	Caspase-3 activity (8 h)
RINm5F			
Untreated	100 ± 2 (3)	100 ± 8 (8)	100 ± 5 (8)
IL-1 (60)	91 ± 2 (3)	83 ± 3 (6)*	563 ± 44 (4)*
IL-1 (600)	84 ± 2 (3)*	67 ± 2 (4)*	1118 ± 124 (4)*
TNF (185)	92 ± 2 (3)	92 ± 7 (4)	111 ± 7 (4)
TNF (1850)	90 ± 2 (3)	78 ± 2 (6)*,§	115 ± 7 (6)
IFN (14)	95 ± 2 (3)	86 ± 8 (4)*	105 ± 12 (3)
IFN (140)	97 ± 3 (3)	88 ± 4 (6)*	143 ± 25 (4)
IL-1 + TNF	93 ± 5 (4) [¶]	78 ± 5 (4)*,†,¶	495 ± 88 (4)*
IL-1 + IFN	93 ± 3 (4) [¶]	75 ± 9 (4)*,†,¶	269 ± 32 (4)*
TNF + IFN	80 ± 2 (4)*	64 ± 6 (4)*,†,§, ,¶	295 ± 27 (4)*
Mix	68 ± 2 (3)*	55 ± 2 (4)*,†,§,	422 ± 25 (4)*
IL-4 (500)	104 ± 2 (3)	96 ± 4 (4)	110 ± 7 (4)
IL-1 + IL-4	102 ± 3 (3)	90 ± 5 (4)	146 ± 3 (4) [†]
Mix + IL-4	80 ± 3 (3)*	64 ± 2 (4)*,¶	121 ± 12 (5) [¶]
IL-13 (500)	104 ± 2 (3)	105 ± 2 (4)	117 ± 14 (5)
IL-1 + IL-13	94 ± 2 (3)	88 ± 3 (4)	239 ± 51 (4)*
Mix + IL-13	85 ± 3 (4)*,¶	68 ± 2 (4)*,¶	189 ± 19 (6) [¶]
IL-10 (500)	112 ± 3 (3)	88 ± 3 (5)	142 ± 10 (8)
IL-1 + IL-10	94 ± 3 (3)	91 ± 9 (4)	155 ± 14 (6) [†]
Mix + IL-10	84 ± 3 (4)*,¶	65 ± 2 (4)*,¶	165 ± 17 (5) [¶]
ANTI mix	101 ± 1 (4)	106 ± 2 (4)	83 ± 17 (3)
IL-1 60 + ANTI mix	97 ± 2 (4)	99 ± 3 (4) [†]	102 ± 12 (3) [†]
IL-1 600 + ANTI mix	86 ± 2 (4)	81 ± 3 (4)*,‡	74 ± 16 (3) [‡]
Mix + ANTI mix	76 ± 2 (4)*,¶	50 ± 1 (4)*	90 ± 11 (7) [¶]
Control iNOS blocker	97 ± 3 (4)	93 ± 4 (6)	105 ± 11 (4)
IL-1 (60) + iNOS blocker	95 ± 2 (4)	86 ± 2 (6)	340 ± 40 (7)*,†,‡,‡,‡
IL (600) + iNOS blocker	82 ± 2 (4)*	74 ± 3 (5)*,‡	404 ± 43 (4)*,‡,‡
Mix + iNOS blocker	86 ± 2 (4)*,¶	76 ± 4 (6)*,¶,‡	362 ± 29 (6)*,¶,‡

**P* < 0.05 versus control; †*P* < 0.05 versus IL-1β 60 U/ml; ‡*P* < 0.05 versus IL-1β 600 U/ml; §*P* < 0.05 versus TNFα 185 U/ml; ||*P* < 0.05 versus IFNγ 14 U/ml; ¶*P* < 0.05 versus Mix; ‡*P* < 0.05 versus control iNOS blocker. ANOVA followed by Bonferroni. Mix, 60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ.

IFNγ failed to decrease cell viability after 24 h (Table 4). The anti-inflammatory cytokines IL-4 (500 U/ml), IL-13 (500 U/ml) and IL-10 (500 U/ml) did not affect β-cell viability after 24 h incubation either when was used each alone or in the combination (ANTI-mix; Table 4). A combination of anti-inflammatory cytokines with IL-1β (60 U/ml) did not significantly affect cell viability when compared with IL-1β alone (Table 4). Interestingly, however, anti-inflammatory cytokines partially prevented the loss of cell viability in insulin-producing cells caused by a cytokine combination consisting of IL-1β, TNFα and IFNγ (60, 185 and 14 U/ml respectively; Table 4).

After 48 h, IL-1β (600 U/ml) reduced the cell viability by around 30% (Table 4). IL-1β, TNFα and IFNγ added in low concentrations (60, 185 and 14 U/ml respectively) to the incubation medium each decreased the viability by around 5–15% (Table 4). TNFα and IFNγ each slightly potentiated IL-1β-induced β-cell loss leading to a loss of 20–25% (Table 4). Treatment of insulin-producing cells with a combination of TNFα and IFNγ reduced the cell viability

by around 35% (Table 4). As expected the most toxic combination of cytokines was the mixture of IL-1β, TNFα and IFNγ (60, 185 and 14 U/ml, respectively) as evident from a roughly 50% reduction of the cell viability when compared with untreated cells (Table 4).

The anti-inflammatory cytokines IL-4, IL-13 and IL-10 did not affect β-cell viability either after 48 h (Table 4). A combination of anti-inflammatory cytokines with IL-1β (60 U/ml) again, like in case of a 24 h incubation, did not significantly affect cell viability when compared with IL-1β alone (Table 4). Each of the anti-inflammatory cytokines alone was able to partially prevent the loss of cell viability in insulin-producing cells caused by a cytokine combination consisting of IL-1β, TNFα and IFNγ (60, 185 and 14 U/ml respectively; Table 4). The mixture of all three anti-inflammatory cytokines did not affect cell viability after 48 h incubation (Table 4). ANTI-mix significantly protected against IL-1β-induced β-cell loss, failed, however, to counteract the toxicity of the pro-inflammatory cytokine mixture (Table 4).

Table 5 Cell viability and caspase-3 activity of RINm5F cells exposed to cytotoxic chemical compounds. For cell viability, insulin-producing RINm5F cells were exposed for 24 and 48 h to chemical compounds in the indicated concentration. The measurements were done using the MTT assay. For caspase-3 activity, the RINm5F cells were exposed to the compounds in the indicated concentration for 8 h and lysed as described in the Methods. After lysis of the cells and extraction of the total intracellular protein, the activity of caspase-3 was measured using the DEVD cleavage method. Data are mean values for the indicated number (*n*) of independent experiments, in each measured in at least 3 repetitions

	Cell viability (24 h)	Cell viability (48 h)	Caspase-3 activity (8 h)
RINm5F			
Untreated	100 ± 2 (4)	100 ± 8 (4)	100 ± 5 (8)
Camptothecin (0.5 µM)	54 ± 7 (4)*	12 ± 1 (4)*	1150 ± 487 (4)*
SNAP (0.1 mM)	79 ± 4 (4)*	79 ± 3 (3)*	443 ± 75 (6)*
SNAP (1 mM)	50 ± 4 (4)*	3 ± 0 (3)*	844 ± 115 (5)*

**P* < 0.05 versus control. ANOVA followed by Bonferroni. SNAP, S-nitroso-n-acetylpenicillamine.

Exposure of insulin-producing RINm5F cells to cytotoxic chemical compounds resulted in impaired cell viability as shown in Table 5. This was especially evident after treatment with a high concentration of the nitric oxide-donor SNAP (1 mM) and with the pro-apoptotic caspase-3 activator camptothecin (0.5 µM). Incubation of insulin-producing cells with the iNOS blocker L-NOARG (5 mmol/l) significantly decreased toxicity induced by the combination of all three pro-inflammatory cytokines (Table 4).

Effect of cytokines on caspase-3 enzyme activity

The time dependent effect on caspase-3 enzyme activity in response to pro-inflammatory cytokines and camptothecin is shown in Fig. 4. High concentrations of IL-1β strongly increased caspase-3 activity starting at 8 h incubation (Fig. 4).

Caspase-3 activity in response to high concentrations of TNFα (1850 U/ml) showed after a transient decrease in activity at 2 and 4 h, a significant increase of activity at 24 h (Fig. 4). However, the effect was weaker than that of IL-1β 600 U/ml. IFNγ (14 and 140 U/ml) did not affect caspase-3 activity (Fig. 4 and Table 4). A combination of the three pro-inflammatory cytokines (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) increased caspase-3 activity already at 4 h and reached a maximum at 8 h (Fig. 4). However, the increase was less than that of a high concentration of IL-1β (600 U/ml). Camptothecin, used as a positive control, increased caspase-3 activity at 8 h with a further increase at 24 h (data not shown). Prior incubation for 10 min with the caspase-3 inhibitor Ac-DEVD-CHO₂ kept the activity at levels near or below the control values, confirming the specificity of the measurement (data not shown).

Additionally, a comprehensive panel of incubations for 8 h with different combinations of cytokines is shown in Table 4. Incubation of insulin-producing RINm5F cells with a low concentration of IL-1β (60 U/ml) also resulted in increased caspase-3 activity (Table 4). Co-incubation of cells with IL-1β (60 U/ml) and either IL-4 (500 U/ml), IL-13 (500 U/ml) or IL-10 (500 U/ml) decreased the activation of caspase-3 (Table 4). This antagonistic effect of the

anti-inflammatory cytokines IL-4, IL-13 and IL-10 was more evident when the cells were co-incubated with the pro-inflammatory cytokine mixture (Table 4). While the treatment with the three pro-inflammatory cytokines resulted in a more than 400% increase in caspase-3 activity, co-incubation with IL-13 or IL-10 decreased the activity to half this level and co-incubation with IL-4 reduced the activity to levels near the control group (Table 4). Co-incubations of IL-1β alone or of pro-inflammatory cytokine mixture with the ANTI-mix completely prevented caspase-3 activation after 8 h (Table 4).

Use of the iNOS blocker L-NOARG (5 mmol/l) in the absence of pro-inflammatory cytokines did not affect caspase-3 activity. Co-incubation of the cells with IL-1β (60 U/ml or

Figure 4 Time-response curve for caspase-3 activity after exposure of insulin-producing cells to cytokines. Insulin-producing RINm5F cells were exposed to cytokines in the indicated concentration for different periods of incubation. After lysis of the cells and extraction of the total intracellular protein, the activity of caspase-3 was measured using the DEVD cleavage method. *n* = 4–8. **P* < 0.05, ANOVA followed by Bonferroni. Mix, 60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ.

600 U/ml) plus the iNOS blocker L-NOARG significantly decreased caspase-3 activity when compared with IL-1 β alone. Co-incubation with iNOS blocker failed, however, to prevent caspase-3 activation caused by the mixture of the three pro-inflammatory cytokines (Table 5). Camptothecin and a NO-donor SNAP significantly and very pronouncedly induced caspase-3 activity in the present study (Table 5). It is important to mention that the high cell death rate caused by these very toxic chemical compounds even after 8 h incubation forced us to change the cell sample preparation procedure for caspase-3 in order to avoid washing away of dying and dead cells, which decrease the values for caspase-3 significantly.

Discussion

The pro-inflammatory cytokines induce cell death through complex signalling pathways (Eizirik *et al.* 1996b, Mandrup-Poulsen 1996, 2003a,b, Eizirik & Mandrup-Poulsen 2001, Cnop *et al.* 2005). Specific cell death pathways are activated depending on the individual pro-inflammatory cytokine as well as the composition of the cytokine combinations to which the cells are exposed. It is generally accepted that pro-inflammatory cytokines are major effectors of programmed cell death during the onset of type 1 diabetes mellitus (Eizirik 1988, 1991, Eizirik *et al.* 1991, 1994, Sandler *et al.* 1992, Mandrup-Poulsen 1996, Eizirik & Mandrup-Poulsen 2001, Cnop *et al.* 2005, Ronn *et al.* 2007). The mechanisms of action are only partially understood, but it is assumed that increased nitrosative and oxidative stresses (Lortz *et al.* 2000, Suarez-Pinzon *et al.* 2001, Bast *et al.* 2002, Lortz & Tiedge 2003, Gurgul *et al.* 2004, Storling *et al.* 2005) play a crucial role. Therefore, induction of nitrosative pathways, such as generation of NO by iNOS (Eizirik *et al.* 1992, 1996b, Eizirik & Leijerstam 1994, Eizirik & Pavlovic 1997, Tiedge *et al.* 1999), and oxidative stress by upregulation of MnSOD (Azevedo-Martins *et al.* 2003, Qadri *et al.* 2004, Lortz *et al.* 2005) might play a key role in the mechanisms of cytokine-mediated β -cell death. Upregulation of expression of these two genes leads always to increased protein expression and enzyme activity in insulin-producing cells (Tiedge *et al.* 1997). On the other hand, the anti-inflammatory cytokines IL-4, IL-13 and IL-10 may be involved in the protection of β -cells (Rapoport *et al.* 1993, Pennline *et al.* 1994, Racke *et al.* 1994, Kaminski *et al.* 2007) and other cell types (Paintlia *et al.* 2006).

IL-1 β was the most potent inducer of NF κ B and iNOS and MnSOD gene expression, effects that could be potentiated by other pro-inflammatory cytokines. Our results showed that TNF α also activated these genes, although to a much lesser extent, and therefore a significant effect was visible on the protein level only for MnSOD but not for iNOS. IFN γ (14 U/ml) by itself did not affect IL-1 β - or TNF α -induced iNOS or MnSOD gene and protein expression. However, a potentiation of the effects of IL-1 β was observed in the presence of TNF α . A potentiation by IFN γ was observed

only in the combination with IL-1 β or TNF α . An induction of gene and protein expression by these pro-inflammatory cytokines correlated always with a loss of cell viability and an activation of caspase-3 as a sign of apoptotic cell death.

All anti-inflammatory cytokines tested in the present study (IL-4, IL-13 and IL-10) antagonised the effects of pro-inflammatory cytokines on the activation of NF κ B and the gene and protein expression of iNOS but not of MnSOD. In the same way, the effects of IL-1 β and in particular of the pro-inflammatory cytokine mixture on caspase-3 activity and cytotoxicity were reduced. This supports the contention that the anti-inflammatory cytokines act in a protective way through antagonising the activation of the NF κ B-mediated pathway of cell death, thereby suppressing nitrosative stress. Moreover, our results confirm earlier observations in different cell types which have shown that anti-inflammatory cytokines decrease the pro-inflammatory cytokine stimulated iNOS gene and protein expression and reduce NO release (Sandler & Sternesjo 1995, Berkman *et al.* 1996, Plunkett *et al.* 2001, Paintlia *et al.* 2006). In case of a shorter 24 h incubation, the combination of three anti-inflammatory cytokines were also able to reduce pro-inflammatory cytokine toxicity. However, after 48 h, this reduction of cytotoxic effects was seen only in the case of IL-1 β but not in the case of the pro-inflammatory cytokine mixture. This can be due to a very intense attack of pro-inflammatory cytokines which is hard to counteract especially in the face of a very low level of anti-inflammatory cytokine receptor expression in insulin-producing RINm5F cells (E Gurgul-Convey, unpublished observation).

The mechanisms underlying the reduction of NF κ B activation by anti-inflammatory cytokines, which may account for the decreased iNOS expression, range from *de novo* synthesis of I κ B α (Molina-Holgado *et al.* 2002), interactions between STAT6 and upstream factors in signalling cascades activated by pro-inflammatory cytokines (Nelson *et al.* 2003) and transcription blockade by STAT6-mediated inhibition of NF κ B binding to the promoter regions of the genes coding NF κ B subunits (Bennett *et al.* 1997, Abu-Amer 2001) or prevention of NF κ B translocation to the nuclear compartment (Ohmori & Hamilton 2000, Molina-Holgado *et al.* 2002, Al-Ashy *et al.* 2006).

Previous studies have shown that TNF α activates caspases 3, 8 and 9 in different cell types (Alikhani *et al.* 2004) and that exposure of insulin-producing cells to IFN γ causes activation of caspase-1 (Karlsen *et al.* 2000). IL-1 β is able to activate caspase-3 in β -cells and this effect is possibly linked to NO production (Chen *et al.* 2003, Veluthakal *et al.* 2004).

Studies with NO donors have previously shown that NO is toxic to insulin-producing cells (Tiedge *et al.* 1999, Storling *et al.* 2005), an observation that was corroborated through the present study. However, the relevance of NO in triggering cytokine-induced apoptotic cell death in insulin-producing cells is still not entirely clear. While some studies have shown an absolute importance of the NO pathway in cytokine-mediated β -cell apoptosis (Delaney *et al.* 1996, Eizirik & Darville 2001, Storling *et al.* 2005),

other experiments provided opposite results (Liu *et al.* 2000, Todaro *et al.* 2003, Zaitseva *et al.* 2006). From an *in vitro* point of view, our results indicate that the incubation of insulin-producing cells with pro-inflammatory cytokines and the NO-donor SNAP increased caspase-3 activity while the iNOS blocker L-NOARG reduced pro-inflammatory cytokine-induced caspase-3 activity. The pro-inflammatory cytokine-stimulated increase in caspase-3 activity was also substantially blocked by co-incubation with IL-4, IL-13 or IL-10, which reduced iNOS expression. Early activation of apoptotic pathways by pro-inflammatory cytokines seems thus to be NO dependent. Our results also indicate a clear time-dependent induction of caspase-3 by IL-1 β or the pro-inflammatory cytokine combination at a very early stage. Notably, *de novo* synthesis of caspase-3 does not seem to be essential or required for its activity. Interestingly, maximal IL-1 β -induced iNOS protein expression is reached at the same time point (8 h) as maximal IL-1 β -induced caspase-3 activity, which is an additional indication for a link between NO and apoptotic pathways.

Insulin-producing cells are not well equipped with hydrogen peroxide detoxifying enzymes, especially in the mitochondrial compartment (Gurgul *et al.* 2004), but express significant levels of the superoxide dismutating enzymes CuZnSOD and MnSOD (Lenzen *et al.* 1996, Tiedge *et al.* 1997). MnSOD resides in the mitochondrial compartment and is responsible for dismutation of superoxide radicals into hydrogen peroxide. As shown in earlier studies, pro-inflammatory cytokines significantly stimulate the expression of MnSOD and this event appears to play a crucial role in triggering cytokine toxicity in insulin-producing cells (Lortz *et al.* 2005). Nonetheless, it appears from our present studies that induction of MnSOD in the early phase of cytokine toxicity does not account for cytokine-mediated cell damage. Oxidative stress caused by induction of MnSOD requires a prolonged time to cause damage to cellular components and to cause cytotoxic effects mediated by pro-inflammatory cytokines in insulin-producing cells (Lortz *et al.* 2005).

Interestingly, co-incubations with anti-inflammatory cytokines, although beneficial for β -cell survival, did not prevent MnSOD induction stimulated by the pro-inflammatory cytokine IL-1 β . This is an additional indication that oxidative stress does not play a major role in the early phase of cytokine-mediated toxicity. Of importance, the effects of the combined action of pro- and anti-inflammatory cytokines upon MnSOD expression could not be explained by NF κ B activation. Our studies indicate that NF κ B although important for induction of the MnSOD expression in insulin-producing cells is not the only relevant transcription factor involved in the transcription of this gene. It is possible therefore that other transcription factors, such as AP1 or Sp1, are involved in the modulation of MnSOD expression in insulin-producing cells, as has been shown in other cell types (Kaneko *et al.* 2004, Qadri *et al.* 2004).

In conclusion, our results indicate that in insulin-producing cells exposed to cytokines, nitrosative stress plays

an important role in activating apoptotic pathways in the early phase of cytokine toxicity. This might be true especially when the pro-inflammatory cytokine concentration is high. The use of IL-4, IL-13 and IL-10 to block pro-inflammatory cytokine-induced activation of caspase-3 may have important consequences for pancreatic β -cell protection and survival, in particular when the pro-inflammatory cytokine concentration is low or when only IL-1 β is present. Thus, protection might be especially effective, when low concentrations of pro-inflammatory cytokines are released in the vicinity of the β -cells such as in the early phase of type 1 diabetes development or reach the β -cells via the circulation from distant sites of inflammation.

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