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

Kleber L A Souza[1,2,*], **Ewa Gurgul-Convey**[1,*], **Matthias Elsner**[1] and **Sigurd Lenzen**[1]

[1]Hannover Medical School, Institute of Clinical Biochemistry, 30625 Hannover, Germany

[2]Department of Cell Biology and Genetics, University of Maringá, 87020-900 Maringá, Brazil

(Correspondence should be addressed to E Gurgul-Convey; Email: Gurgul-Convey.Ewa@mh_hannover.de)

(Present address of K L A Souza is the Department of Physiology and Biophysics, University of Campinas, Brazil)

*(K L A Souza and E Gurgul-Convey equally contributed to this work)

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, NFκB activation using a SEAP reporter gene assay, protein expression by western blotting and caspase-3 activity using the DEVD cleavage method. IL-1β, tumour necrosis factor α (TNFα) and a combination of all three pro-inflammatory cytokines increased while IFNγ alone did not affect NFκB activity and iNOS gene and protein expression. Interestingly, the anti-inflammatory cytokines IL-4, IL-13 and IL-10 decreased IL-1β-stimulated NFκB activation and iNOS expression. IL-1β, TNFα 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-1β-stimulated MnSOD expression. Caspase-3 activity was increased by IL-1β and the pro-inflammatory cytokine combination, and to a lesser extent by TNFα. In contrast, IFNγ 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.

Journal of Endocrinology (2008) **197,** 139–150

Introduction

The pro-inflammatory cytokines, such as IL-1β, tumour necrosis factor α (TNFα) and IFNγ, 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 (Zaccone *et al.* 1999, Kaminski *et al.* 2007) and the prevention of destructive insulitis (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 (NFκB; Eizirik *et al.* 1996*b*), 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.* 1996*a*,*b*, Eizirik & Mandrup-Poulsen 2001, Bast *et al.* 2002). β-Cells produce a large amount of NO when exposed to IL-1β or a combination of pro-inflammatory cytokines (Cetkovic-Cvrlje & Eizirik 1994, Eizirik & Leijerstam 1994, Eizirik *et al.* 1996*b*, 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

0022–0795/08/0197–139 © 2008 Society for Endocrinology *Printed in Great Britain*
DOI: 10.1677/JOE-07-0638
Online version via http://www.endocrinology-journals.org

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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, Ledeboer *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 & Grencis 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-1β effects, and reduced NO production has been considered an important element for this beneficial effect (Perretti *et al.* 1995, Ledeboer *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 TNFα activates caspases 3, 8 and 9 in different cell types (Alikhani *et al.* 2004) and that exposure of insulin-producing cells to IFNγ causes activation of caspase-1 (Karlsen *et al.* 2000). IL-1β 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-CHO_2 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% CO_2. For RNA extraction and protein analysis, cells were platted 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-1β (4·4 ng/ml); 600 U/ml IL-1β (44 ng/ml); 185 U/ml TNFα (8·7 ng/ml); 1850 U/ml TNFα (87 ng/ml); 14 U/ml IFNγ (10·3 ng/ml); 140 U/ml IFNγ (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

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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 (ΔFU) 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 ΔFU/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

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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: TTCAGATTGTTCACGTAGGTCGCG

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

Table 2 Effects of pro- or anti-inflammatory cytokines in insulin-producing RINm5F cells on NFκB 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

	NFκB induction	**iNOS expression**	**MnSOD expression**
RINm5F			
Untreated	1·0±0·1 (20)	1·0±0·1 (20)	1·0±0·1 (12)
IL-1 (60)	1·8±0·2 (13)*	800±109 (6)*	6·2±1·2 (4)*
IL-1 (600)	2·5±0·2 (12)[*,†]	1982±223 (5)[*,†]	10±2 (5)*
TNF (185)	1·4±0·1 (18)	22±4 (7)	2·6±0·2 (4)
TNF (1850)	1·5±0·0 (18)	37±15 (7)	3·9±0·8 (4)*
IFN (14)	1·2±0·1 (10)	2·2±0·3 (4)	0·7±0·1 (7)
IFN (140)	1·2±0·1 (20)	3·3±0·8 (5)	1·4±0·2 (4)
IL-1+TNF	2·5±0·2 (20)[*,†,‡]	844±75 (4)[*,‡]	8·3±1·7 (4)[*,‡]
IL-1+IFN	2·2±0·2 (20)[*,§]	833±209 (4)[*,§]	6·5±1·8 (4)[*,§]
TNF+IFN	1·3±0·1 (20)	5·2±1·1 (4)	2·6±0·2 (5)
Mix	2·4±0·1 (40)[*,†,‡,§]	1024±122 (6)[*,‡,§]	9·3±2·0 (5)[*,‡,§]
IL-4 (500)	1·3±0·1 (20)	0·9±0·2 (4)	1·2±0·2 (4)
IL-1+IL-4	1·2±0·1 (20)[†]	61±7 (4)[†]	9·9±2·6 (3)[*,‖]
IL-13 (500)	1·1±0·1 (20)	1·4±0·4 (4)	1·7±0·4 (2)
IL-1+IL-13	1·4±0·1 (20)[†]	65±20 (4)[†]	9·2±2·2 (5)[*,¶]
IL-10 (500)	1·3±0·1 (20)	1·0±0·3 (3)	1·0±0·1 (4)
IL-1+IL-10	1·4±0·1(20)[†]	133±40 (3)[†]	11±2 (3)[*,a]
Camptothecin (0·5)	0·8±0·1 (4)	1·0±0·2 (3)	0·9±0·2 (3)

Numbers in brackets represent the concentration of the compound in U/ml or μM (for camptothecin). *$P<0·05$ versus control; [†]$P<0·05$ versus IL-1β 60 U/ml; [‡]$P<0·05$ versus TNFα; [§]$P<0·05$ versus IFNγ; [‖]$P<0·05$ versus IL-4; [¶]$P<0·05$ 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-1β, 185 U/ml TNFα, 14 U/ml IFNγ, the same concentrations of cytokines were used in case of co-incubations with two cytokines.

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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

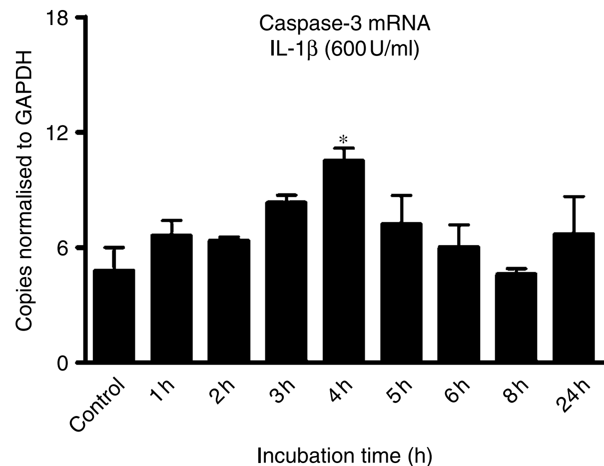

Figure 1 Time-dependent effect of IL-1β on the caspase-3 gene expression in insulin-producing cells. RINm5F cells were incubated with 600 U/ml IL-1β. The mRNA was obtained using the guanidine–thiocyanate method. After reverse transcription, gene expression was measured by quantitative real-time PCR at 0 (no cytokines added, control), 1, 2, 3, 4, 5, 6, 8 and 24 h after incubation. $n=4$. $*P<0{\cdot}05$, 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)

$*P<0{\cdot}05$ versus control, Dunnett's test; $^{\dagger}P<0{\cdot}05$ 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.

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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-1β (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 TNFα 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 IFNγ (14 and 140 U/ml; Table 3). TNFα showed a synergistic effect with IL-1β (Table 3). The combination of IL-1β with IFNγ did not have an additive effect when compared with IL-1β alone (Table 3). The combination of TNFα and IFNγ did not induce iNOS protein expression (Table 3). A combination of all three pro-inflammatory cytokines (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) 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-1β-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-1β (60 U/ml) insulin-producing RINm5F cells exhibited a significant increase of MnSOD protein expression when compared with control cells. Higher concentrations of IL-1β (600 U/ml) further potentiated this effect. Both, low and high concentrations of TNFα significantly enhanced MnSOD protein expression (Table 3). IFNγ (14 and 140 U/ml) did not affect MnSOD protein expression and did not induce any synergistic effect in combination with IL-1β or TNFα (Table 3). The combination of IL-1β 60 U/ml and TNFα 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-1β (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-1β with anti-inflammatory cytokines did not significantly affect IL-1β-stimulated MnSOD protein expression when compared with IL-1β alone. Camptothecin did not affect the protein expression of iNOS or MnSOD.

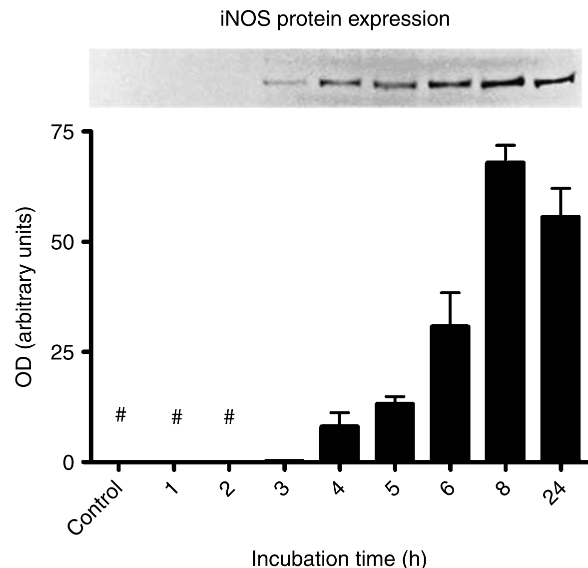

Figure 3 Time-dependent effect of IL-1β on the iNOS protein expression in insulin-producing cells. RINm5F cells were seeded 24 h before the incubation with 600 U/ml IL-1β. The cells were lysed and used for western blot analyses at 0 (no cytokines added, lane C, control), 1, 2, 3, 4, 5, 6, 8 and 24 h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

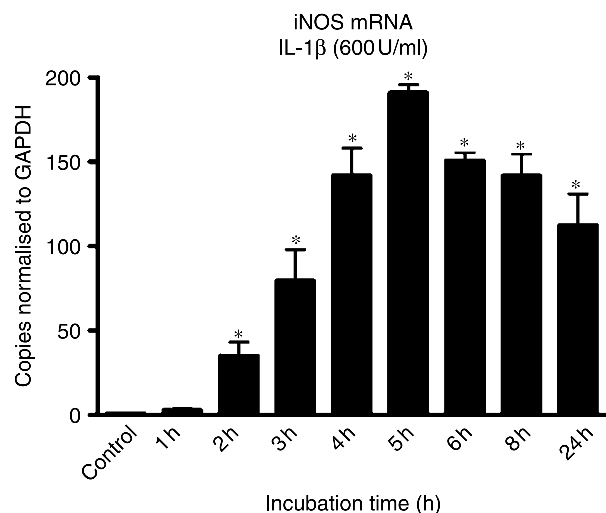

Figure 2 Time-dependent effect of IL-1β on the iNOS gene expression in insulin-producing cells. RINm5F cells were seeded 24 h before the incubation with 600 U/ml IL-1β. The mRNA was obtained using the guanidine–thiocyanate method. After reverse transcription, gene expression was measured by quantitative real-time PCR at 0 (no cytokines added, control), 1, 2, 3, 4, 5, 6, 8 and 24 h after incubation. The means of absolute values from four to eight independent experiments are shown. $*P<0{\cdot}05$, ANOVA followed by Bonferroni.

Effect of cytokines on cell viability

Incubation of control RINm5F cells with the pro-inflammatory cytokines IL-1β, TNFα and IFNγ 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-1β and the combination of TNFα and IFNγ 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-1β, TNFα and IFNγ (60, 185, and 14 U/ml respectively) as evident from a more than 30% decrease in cell viability (Table 4). Even high concentrations of TNFα or

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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)*,†,a
IL (600)+iNOS blocker	82±2 (4)*	74±3 (5)*a	404±43 (4)*,‡,a
Mix+iNOS blocker	86±2 (4)*,¶	76±4 (6)*,¶,a	362±29 (6)*,¶,a

*$P<0{\cdot}05$ versus control; †$P<0{\cdot}05$ versus IL-1β 60 U/ml; ‡$P<0{\cdot}05$ versus IL-1β 600 U/ml; §$P<0{\cdot}05$ versus TNFα 185 U/ml; ‖$P<0{\cdot}05$ versus IFNγ 14 U/ml; ¶$P<0{\cdot}05$ versus Mix; [a]$P<0{\cdot}05$ 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).

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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-CHO_2 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

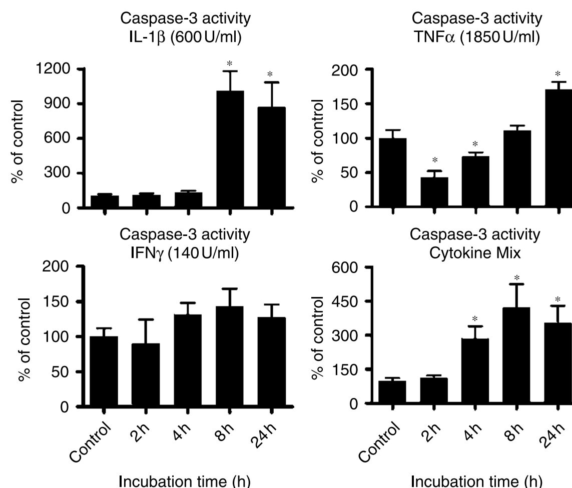

Figure 4 Time-response curve for caspase-3 activity after exposure of insulin-producing cells to cytokines. Insulin-producing RINm5F cells were exposed to cytokines in the indicated concentration for different periods of incubation. After lysis of the cells and extraction of the total intracellular protein, 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.

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

600 U/ml) plus the iNOS blocker L-NOARG significantly decreased caspase-3 activity when compared with IL-1β 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*. 1996*b*, Mandrup-Poulsen 1996, 2003*a*,*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, 1996*b*, 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-1β was the most potent inducer of NFκB and iNOS and MnSOD gene expression, effects that could be potentiated by other pro-inflammatory cytokines. Our results showed that TNFα 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. IFNγ (14 U/ml) by itself did not affect IL-1β- or TNFα-induced iNOS or MnSOD gene and protein expression. However, a potentiation of the effects of IL-1β was observed in the presence of TNFα. A potentiation by IFNγ was observed only in the combination with IL-1β or TNFα. 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 NFκB and the gene and protein expression of iNOS but not of MnSOD. In the same way, the effects of IL-1β 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 NFκB-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-1β 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 NFκB activation by anti-inflammatory cytokines, which may account for the decreased iNOS expression, range from *de novo* synthesis of IκBα (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 NFκB binding to the promoter regions of the genes coding NFκB subunits (Bennett *et al*. 1997, Abu-Amer 2001) or prevention of NFκB translocation to the nuclear compartment (Ohmori & Hamilton 2000, Molina-Holgado *et al*. 2002, Al-Ashy *et al*. 2006).

Previous studies have shown that TNFα activates caspases 3, 8 and 9 in different cell types (Alikhani *et al*. 2004) and that exposure of insulin-producing cells to IFNγ causes activation of caspase-1 (Karlsen *et al*. 2000). IL-1β 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),

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

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-1β 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-1β-induced iNOS protein expression is reached at the same time point (8 h) as maximal IL-1β-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-1β. 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 NFκB activation. Our studies indicate that NFκB 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-1β 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.

Acknowledgements

This work was supported by a grant from the Ministry of Science and Culture of Lower Saxony (K S) and by the European Union (STREP SAVEBETA in the Framework programme 6 of the European Community). The skilful technical assistance of B Lueken and S Lippold is gratefully acknowledged. The authors are grateful to Dr K Dobashi (Kitakyushu, Japan) for the antibodies against rat MnSOD. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Abu-Amer Y 2001 IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-κB. *Journal of Clinical Investigation* **107** 1375–1385.

Al-Ashy R, Chakroun I, El-Sabban ME & Homaidan FR 2006 The role of NF-κB in mediating the anti-inflammatory effects of IL-10 in intestinal epithelial cells. *Cytokine* **36** 1–8.

Alikhani M, Alikhani Z, Raptis M & Graves DT 2004 TNF-α *in vivo* stimulates apoptosis in fibroblasts through caspase-8 activation and modulates the expression of pro-apoptotic genes. *Journal of Cellular Physiology* **201** 341–348.

Azevedo-Martins AK, Lortz S, Lenzen S, Curi R, Eizirik DL & Tiedge M 2003 Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-κB activation in insulin-producing cells. *Diabetes* **52** 93–101.

Bast A, Wolf G, Oberbaumer I & Walther R 2002 Oxidative and nitrosative stress induces peroxiredoxins in pancreatic beta cells. *Diabetologia* **45** 867–876.

Bennett BL, Cruz R, Lacson RG & Manning AM 1997 Interleukin-4 suppression of tumor necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-κB. *Journal of Biological Chemistry* **272** 10212–10219.

Berkman N, Robichaud A, Robbins RA, Roesems G, Haddad EB, Barnes PJ & Chung KF 1996 Inhibition of inducible nitric oxide synthase expression by interleukin-4 and interleukin-13 in human lung epithelial cells. *Immunology* **89** 363–367.

Callard RE, Matthews DJ & Hibbert L 1996 IL-4 and IL-13 receptors: are they one and the same? *Immunology Today* **17** 108–110.

Cetkovic-Cvrlje M & Eizirik DL 1994 TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* **6** 399–406.

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

Chen HQ, Tannous M, Veluthakal R, Amin R & Kowluru A 2003 Novel roles for palmitoylation of Ras in IL-1 beta-induced nitric oxide release and caspase 3 activation in insulin-secreting beta cells. *Biochemical Pharmacology* **66** 1681–1694.

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162** 156–159.

Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S & Eizirik DL 2005 Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* **54** S97–S107.

Darville MI & Eizirik DL 1998 Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* **41** 1101–1108.

Delaney CA, Tyrberg B, Bouwens L, Vaghef H, Hellman B & Eizirik DL 1996 Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. *FEBS Letters* **394** 300–306.

Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG & Eizirik DL 1997 Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138** 2610–2614.

Eizirik DL 1988 Interleukin-1 induced impairment in pancreatic islet oxidative metabolism of glucose is potentiated by tumor necrosis factor. *Acta Endocrinologica* **119** 321–325.

Eizirik DL 1991 Interleukin-1 beta induces an early decrease in insulin release, (pro)insulin biosynthesis and insulin mRNA in mouse pancreatic islets by a mechanism dependent on gene transcription and protein synthesis. *Autoimmunity* **10** 107–113.

Eizirik DL & Leijerstam F 1994 The inducible form of nitric oxide synthase (iNOS) in insulin-producing cells. *Diabetes and Metabolism* **20** 116–122.

Eizirik DL & Pavlovic D 1997 Is there a role for nitric oxide in beta-cell dysfunction and damage in IDDM? *Diabetes/Metabolism Reviews* **13** 293–307.

Eizirik DL & Darville MI 2001 Beta-cell apoptosis and defense mechanisms: lessons from type 1 diabetes. *Diabetes* **50** S64–S69.

Eizirik DL & Mandrup-Poulsen T 2001 A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44** 2115–2133.

Eizirik DL, Bendtzen K & Sandler S 1991 Short exposure of rat pancreatic islets to interleukin-1 beta induces a sustained but reversible impairment in beta-cell function: influence of protease activation, gene transcription, and protein synthesis. *Endocrinology* **128** 1611–1616.

Eizirik DL, Cagliero E, Bjorklund A & Welsh N 1992 Interleukin-1 beta induces the expression of an isoform of nitric oxide synthase in insulin-producing cells, which is similar to that observed in activated macrophages. *FEBS Letters* **308** 249–252.

Eizirik DL, Sandler S, Welsh N, Cetkovic-Cvrlje M, Nieman A, Geller DA, Pipeleers DG, Bendtzen K & Hellerstrom C 1994 Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *Journal of Clinical Investigation* **93** 1968–1974.

Eizirik DL, Delaney CA, Green MH, Cunningham JM, Thorpe JR, Pipeleers DG, Hellerstrom C & Green IC 1996*a* Nitric oxide donors decrease the function and survival of human pancreatic islets. *Molecular and Cellular Endocrinology* **118** 71–83.

Eizirik DL, Flodstrom M, Karlsen AE & Welsh N 1996*b* The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* **39** 875–890.

Flodstrom M, Welsh N & Eizirik DL 1996 Cytokines activate the nuclear factor kappa B (NF-κB) and induce nitric oxide production in human pancreatic islets. *FEBS Letters* **385** 4–6.

Gurgul E, Lortz S, Tiedge M, Jorns A & Lenzen S 2004 Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. *Diabetes* **53** 2271–2280.

Joshi SG & Sahni SK 2003 Immunofluorescent detection of activation of initiator caspases-8 and -9 during pharmacologically induced apoptosis of cultured HeLa and endothelial cells. *Histochemistry and Cell Biology* **119** 463–468.

Joshi SG, Francis CW, Silverman DJ & Sahni SK 2003 Nuclear factor kappa B protects against host cell apoptosis during Rickettsia rickettsii infection by inhibiting activation of apical and effector caspases and maintaining mitochondrial integrity. *Infection and Immunity* **71** 4127–4136.

Kaminski A, Kaminski ER & Morgan NG 2007 Pre-incubation with interleukin-4 mediates a direct protective effect against the loss of pancreatic beta-cell viability induced by proinflammatory cytokines. *Clinical Experimental Immunology* **148** 583–588.

Kaneko M, Takahashi T, Niinuma Y & Nomura Y 2004 Manganese superoxide dismutase is induced by endoplasmic reticulum stress through IRE1-mediated nuclear factor (NF)-kappaB and AP-1 activation. *Biological and Pharmaceutical Bulletin* **27** 1202–1206.

Karlsen AE, Pavlovic D, Nielsen K, Jensen J, Andersen HU, Pociot F, Mandrup-Poulsen T, Eizirik DL & Nerup J 2000 Interferon-gamma induces interleukin-1 converting enzyme expression in pancreatic islets by an interferon regulatory factor-1-dependent mechanism. *Journal of Clinical Endocrinology and Metabolism* **85** 830–836.

Kotowicz K, Callard RE, Friedrich K, Matthews DJ & Klein N 1996 Biological activity of IL-4 and IL-13 on human endothelial cells: functional evidence that both cytokines act through the same receptor. *International Immunology* **8** 1915–1925.

Kraich M, Klein M, Patino E, Harrer H, Nickel J, Sebald W & Mueller TD 2006 A modular interface of IL-4 allows for scalable affinity without affecting specificity for the IL-4 receptor. *BMC Biology* **4** 13.

Kuhn K, Shikhman AR & Lotz M 2003 Role of nitric oxide, reactive oxygen species, and p38 MAP kinase in the regulation of human chondrocyte apoptosis. *Journal of Cellular Physiology* **197** 379–387.

Kurrer MO, Pakala SV, Hanson HL & Katz JD 1997 Beta cell apoptosis in T cell-mediated autoimmune diabetes. *PNAS* **94** 213–218.

Laffranchi R & Spinas GA 1996 Interleukin 10 inhibits insulin release from and nitric oxide production in rat pancreatic islets. *European Journal of Endocrinology* **135** 374–378.

Ledeboer A, Breve JJ, Poole S, Tilders FJ & Van Dam AM 2000 Interleukin-10, interleukin-4, and transforming growth factor-beta differentially regulate lipopolysaccharide-induced production of pro-inflammatory cytokines and nitric oxide in co-cultures of rat astroglial and microglial cells. *Glia* **30** 134–142.

Lenzen S, Drinkgern J & Tiedge M 1996 Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radical Biology and Medicine* **20** 463–466.

Lieb K, Engels S & Fiebich BL 2003 Inhibition of LPS-induced iNOS and NO synthesis in primary rat microglial cells. *Neurochemistry International* **42** 131–137.

Liu D, Pavlovic D, Chen MC, Flodstrom M, Sandler S & Eizirik DL 2000 Cytokines induce apoptosis in beta-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS−/−). *Diabetes* **49** 1116–1122.

Lortz S & Tiedge M 2003 Sequential inactivation of reactive oxygen species by combined overexpression of SOD isoforms and catalase in insulin-producing cells. *Free Radical Biology and Medicine* **34** 683–688.

Lortz S, Tiedge M, Nachtwey T, Karlsen AE, Nerup J & Lenzen S 2000 Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* **49** 1123–1130.

Lortz S, Gurgul-Convey E, Lenzen S & Tiedge M 2005 Importance of mitochondrial superoxide dismutase expression in insulin-producing cells for the toxicity of reactive oxygen species and proinflammatory cytokines. *Diabetologia* **48** 1541–1548.

Lubberts E, Joosten LA, van de Loo FA, van den Gersselaar LA & van den Berg WB 2000 Reduction of interleukin-17-induced inhibition of chondrocyte proteoglycan synthesis in intact murine articular cartilage by interleukin-4. *Arthritis and Rheumatism* **43** 1300–1306.

Mandrup-Poulsen T 1996 The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* **39** 1005–1029.

Mandrup-Poulsen T 2003*a* Apoptotic signal transduction pathways in diabetes. *Biochemical Pharmacology* **66** 1433–1440.

Mandrup-Poulsen T 2003*b* Beta cell death and protection. *Annals of the New York Academy of Sciences* **1005** 32–42.

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access

Mandrup-Poulsen T, Bendtzen K, Dinarello CA & Nerup J 1987 Human - tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic beta-cell cytotoxicity. *Journal of Immunology* **139** 4077–4082.

Mandrup-Poulsen T, Helqvist S, Wogensen LD, Molvig J, Pociot F, Johannesen J & Nerup J 1990 Cytokine and free radicals as effector molecules in the destruction of pancreatic beta cells. *Current Topics in Microbiology and Immunology* **164** 169–193.

Molina-Holgado E, Arevalo-Martin A, Castrillo A, Bosca L, Vela JM & Guaza C 2002 Interleukin-4 and interleukin-10 modulate nuclear factor kappaB activity and nitric oxide synthase-2 expression in Theiler's virus-infected brain astrocytes. *Journal of Neurochemistry* **81** 1242–1252.

Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65** 55–63.

Nelson G, Wilde GJ, Spiller DG, Kennedy SM, Ray DW, Sullivan E, Unitt JF & White MR 2003 NF-κB signalling is inhibited by glucocorticoid receptor and STAT6 via distinct mechanisms. *Journal of Cell Science* **116** 2495–2503.

Nerup J, Mandrup-Poulsen T, Helqvist S, Andersen HU, Pociot F, Reimers JI, Cuartero BG, Karlsen AE, Bjerre U & Lorenzen T 1994 On the pathogenesis of IDDM. *Diabetologia* **37** S82–S89.

Nishisaka F, Sohen S, Fukuoka H, Okamoto Y, Matukawa M, Fukuda K & Hamanishi C 2001 Interleukin-4 reversed the interleukin-1-inhibited proteoglycan synthesis through the inhibition of NO release: a possible involvement of intracellular calcium ion. *Pathophysiology* **7** 289–293.

Ohmori Y & Hamilton TA 2000 Interleukin-4/STAT6 represses STAT1 and NF-kappa B-dependent transcription through distinct mechanisms. *Journal of Biological Chemistry* **275** 38095–38103.

Paintlia AS, Paintlia MK, Singh I & Singh AK 2006 IL-4-induced peroxisome proliferator-activated receptor gamma activation inhibits NF-kappaB trans activation in central nervous system (CNS) glial cells and protects oligodendrocyte progenitors under neuroinflammatory disease conditions: implication for CNS-demyelinating diseases. *Journal of Immunology* **176** 4385–4398.

Pennline KJ, Roque-Gaffney E & Monahan M 1994 Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clinical Immunology and Immunopathology* **71** 169–175.

Pennock JL & Grencis RK 2006 The mast cell and gut nematodes: damage and defence. *Chemical Immunology and Allergy* **90** 128–140.

Perretti M, Szabo C & Thiemermann C 1995 Effect of interleukin-4 and interleukin-10 on leucocyte migration and nitric oxide production in the mouse. *British Journal of Pharmacology* **116** 2251–2257.

Plunkett JA, Yu CG, Easton JM, Bethea JR & Yezierski RP 2001 Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat. *Experimental Neurology* **168** 144–154.

Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J & Simon FR 2004 Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. *Biochemical Journal* **378** 919–928.

Rabinovitch A & Suarez-Pinzon WL 2003 Role of cytokines in the pathogenesis of autoimmune diabetes mellitus. *Reviews in Endocrine and Metabolic Disorders* **4** 291–299.

Racke MK, Bonomo A, Scott DE, Cannella B, Levine A, Raine CS, Shevach EM & Rocken M 1994 Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *Journal of Experimental Medicine* **180** 1961–1966.

Rapoport MJ, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS & Delovitch TL 1993 Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *Journal of Experimental Medicine* **178** 87–99.

Raz I, Eldor R & Naparstek Y 2005 Immune modulation for prevention of type 1 diabetes mellitus. *Trends in Biotechnology* **23** 128–134.

Ronn SG, Billestrup N & Mandrup-Poulsen T 2007 Diabetes and suppressors of cytokine signaling proteins. *Diabetes* **56** 541–548.

Saldeen J 2000 Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. *Endocrinology* **141** 2003–2010.

Sandler S & Sternesjo J 1995 Interleukin 4 impairs rat pancreatic islet function *in vitro* by an action different to that of interleukin 1. *Cytokine* **7** 296–300.

Sandler S, Eizirik DL, Svensson C, Strandell E, Welsh M & Welsh N 1992 Are cytokines mediators in the development of insulin-dependent diabetes mellitus? *Lakartidningen* **89** 1777–1779.

Serafin A, Rosello-Catafau J, Prats N, Gelpi E, Rodes J & Peralta C 2004 Ischemic preconditioning affects interleukin release in fatty livers of rats undergoing ischemia/reperfusion. *Hepatology* **39** 688–698.

Storling J, Binzer J, Andersson AK, Zullig RA, Tonnesen M, Lehmann R, Spinas GA, Sandler S, Billestrup N & Mandrup-Poulsen T 2005 Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. *Diabetologia* **48** 2039–2050.

Suarez-Pinzon WL, Mabley JG, Strynadka K, Power RF, Szabo C & Rabinovitch A 2001 An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. *Journal of Autoimmunity* **16** 449–455.

Tiedge M, Lortz S, Drinkgern J & Lenzen S 1997 Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* **46** 1733–1742.

Tiedge M, Lortz S, Munday R & Lenzen S 1999 Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. *Diabetologia* **42** 849–855.

Todaro M, Di Gaudio F, Lavitrano M, Stassi G & Papaccio G 2003 Islet beta-cell apoptosis triggered *in vivo* by interleukin-1β is not related to the inducible nitric oxide synthase pathway: evidence for mitochondrial function impairment and lipoperoxidation. *Endocrinology* **144** 4264–4271.

Veluthakal R, Amin R & Kowluru A 2004 Interleukin-1 beta induces posttranslational carboxymethylation and alterations in subnuclear distribution of lamin B in insulin-secreting RINm5F cells. *American Journal of Physiology. Cell Physiology* **287** C1152–C1162.

Verri WA Jr, Cunha TM, Parada CA, Poole S, Cunha FQ & Ferreira SH 2006 Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? *Pharmacology and Therapeutics* **112** 116–138.

Walsh GM 2006 Targeting airway inflammation: novel therapies for the treatment of asthma. *Current Medicinal Chemistry* **13** 3105–3111.

Wright K, Kolios G, Westwick J & Ward SG 1999 Cytokine-induced apoptosis in epithelial HT-29 cells is independent of nitric oxide formation. Evidence for an interleukin-13-driven phosphatidylinositol 3-kinase-dependent survival mechanism. *Journal of Biological Chemistry* **274** 17193–17201.

Zaccone P, Phillips J, Conget I, Gomis R, Haskins K, Minty A, Bendtzen K, Cooke A & Nicoletti F 1999 Interleukin-13 prevents autoimmune diabetes in NOD mice. *Diabetes* **48** 1522–1528.

Zaitseva II, Sharoyko V, Storling J, Efendic S, Guerin C, Mandrup-Poulsen T, Nicotera P, Berggren PO & Zaitsev SV 2006 RX871024 reduces NO production but does not protect against pancreatic beta-cell death induced by proinflammatory cytokines. *Biochemical and Biophysical Research Communications* **347** 1121–1128.

Zumsteg U, Frigerio S & Hollander GA 2000 Nitric oxide production and Fas surface expression mediate two independent pathways of cytokine-induced murine beta-cell damage. *Diabetes* **49** 39–47.

Received in final form 20 December 2007
Accepted 4 February 2008
Made available online as an Accepted Preprint
5 February 2008

Downloaded from Bioscientifica.com at 08/24/2022 07:18:18PM
via free access