Apoptosis

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Cytokine-induced β-cell apoptosis is NO-dependent, mitochondria-mediated and inhibited by BCL-X$_L$

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ABSTRACT

Pro-inflammatory cytokines are implicated as the main mediators of β-cell death during type 1 diabetes but the exact mechanisms remain unknown. This study examined the effects of interleukin-1β (IL-1β), interferon-γ (IFNγ) and tumour necrosis factor α (TNFα) on a rat insulinoma cell line (RIN-r) in order to identify the core mechanism of cytokine-induced β-cell death. Treatment of cells with a combination of IL-1β and IFNγ (IL-1β/IFNγ)-induced apoptotic cell death. TNFα neither induced β-cell death nor did it potentiate the effects of IL-1β, IFNγ or IL-1β/IFNγ. The cytotoxic effect of IL-1β/IFNγ was associated with the expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide. Adenoviral-mediated expression of iNOS (AdiNOS) alone was sufficient to induce caspase activity and apoptosis. The broad range caspase inhibitor, Boc-D-fmk, blocked IL-1β/IFNγ-induced caspase activity, but not nitric oxide production nor cell death. However, pre-treatment with L-NIO, a NOS inhibitor, prevented nitric oxide production, caspase activity and reduced apoptosis. IL-1β/IFNγ-induced apoptosis was accompanied by loss of mitochondrial membrane potential, release of cytochrome *c* and cleavage of pro-caspase-9, -7 and -3. Transduction of cells with Ad-Bcl-X$_L$ blocked both iNOS and cytokine-mediated mitochondrial changes and subsequent apoptosis, downstream of nitric oxide. We conclude that cytokine-induced nitric oxide production is both essential and sufficient for caspase activation and β-cell death, and have identified Bcl-X$_L$ as a potential target to combat β-cell apoptosis.

Keywords: apoptosis ● Bcl-X$_L$ ● caspases ● cytokines ● iNOS ● mitochondria ● nitric oxide ● Type 1 diabetes

Introduction

Type 1 diabetes occurs as a result of the destruction of insulin-secreting pancreatic β-cells by autoreactive immune cells [1]. Interleukin-1β (IL-1β), tumour necrosis factor α (TNFα) and interferon-γ (IFNγ) secreted by T cells and macrophages have been identified as the major mediators of β-cell damage during type 1 diabetes [2–4].

There is growing evidence suggesting that cytokines cause β-cell loss by inducing apoptosis [5, 6]. Apoptosis is a tightly regulated process that culminates in activation of the caspase family of proteases leading to dismantling of the dying cell [7–9]. Caspase activation occurs by two major routes; either *via* activation of death receptors, or through mitochondrial pathways [10, 11]. The latter involves the

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loss of the mitochondrial inner membrane potential ($\Delta\Psi$m), and release of cytochrome c which is often considered as the 'point of no return' in the pathway [12–14]. This step is tightly regulated by the Bcl-2 family of proteins. The balance between the pro-apoptotic (*e.g.* Bax, Bax, Bid, Bim) and anti-apoptotic (*e.g.* Bcl-2, Bcl-X_L) members of this family determines whether cytochrome c can be released and thus controls the fate of the cell [13–16].

Although the importance of cytokines in β-cell destruction is clear, the mechanism by which cytokines kill β-cells is far less understood. Studies of cytokine-treated β-cells have identified induction of iNOS and excess production of nitric oxide as a major contributor to β-cell injury [17, 18]. Furthermore, endogenous production of nitric oxide is required for β-cell injury [19]. Also, nitric oxide can mediate its toxicity through inducing apoptosis [6, 20]. In view of this, we set out to examine the mechanism of β-cell death following both cytokine treatment and forced expression of iNOS in order to gain a clearer understanding of the apoptotic pathways activated and to identify the decisive steps in this pathway.

We demonstrate that IL-1β and IFNγ act in synergy to cause β-cell death while TNFα has no detectable toxic effect on the cells. Both IL-1β and IFNγ induced nitric oxide production, which was solely responsible for mitochondrial damage, caspase activation and apoptosis in rat insulinoma (RIN) cells. The ability of Bcl-X_L to inhibit nitric oxide and cytokine-induced apoptosis proves that the mitochondrial pathway is a critical step in cytokine-induced β-cell death. It further indicates that anti-apoptotic strategies aimed at protecting β-cells from pro-inflammatory cytokines should target events at or above the mitochondria.

Materials and methods

Cell Culture and treatments

RIN-r cells (a gift from Prof. J. Nerup, Steno Diabetes Centre, Gentofte, Denmark) were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 5 mg/ml streptomycin, 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES, pH 7.4) and 1 mM sodium pyruvate in a humidified atmosphere at 37°C and 5% CO_2. All cell culture reagents were from Sigma. For all experiments, unless otherwise stated, cells were seeded at 2×10^5 cells/ml and

allowed to recover for 24 hrs prior to treatment. Cells were treated with recombinant human IL-1β, recombinant human TNFα and recombinant rat IFNγ (all from PromoCell) for the times and concentrations indicated. For inhibition studies cells were pre-treated with the indicated concentrations of L-N^5-(1-Iminoethyl)-ornithine 2HCl (L-NIO) (Alexis Biochemicals) or Boc-Asp(OMe)-fmk (Boc-D-fmk) (Enzyme Systems Products) for 1 hr. All other reagents were from Sigma-Aldrich unless otherwise stated.

MTT assay

Cell viability was measured by adding 200 μg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) to control and treated cells, and incubated for 3 hrs at 37°C. The reaction was stopped and the purple formazan precipitate formed was dissolved using dimethyl sulfoxide (DMSO) and the colour intensity was measured at 550 nm using a Wallac multi-label counter. The control value corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control.

Detection of apoptotic cells

Externalization of phosphatidylserine (PS) on the plasma membrane of apoptotic cells was detected using annexin V-FITC (IQ Corporation). Briefly, cells were collected by trypsinization and allowed to recover for 10 min in growth medium. The cells were then washed in ice-cold calcium buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and incubated with annexin V-FITC for 15 min. in the dark. Cells were then stained with propidium iodide (PI) (1.25 μg/ml) prior to acquisition on a FACSCalibur flow cytometer (Becton Dickinson). PI stains cells that have lost their membrane integrity and thus identifies late apoptotic/ necrotic cells.

Hoechst staining

Cells were fixed in 3.7% formaldehyde for 5 min. at room temperature (RT) and the membranes were permeabilized with methanol for an additional 5 min. at RT. Cells were then rinsed with phosphate buffered saline (PBS) and incubated with Hoechst 33342 (100 μg/ml) for 15 min. at RT. Images were collected on an Olympus BX51 fluorescent microscope at a final magnification of 100×.

Griess assay

The concentration of produced nitrite (reflecting the release of nitric oxide due to iNOS activity) was determined by the Griess reaction in cell-free supernatants from the cultures.

Briefly, 50 μl aliquots of culture medium were taken at appropriate time-points, added to 96-well plates and incubated with 50 μl of Griess reagent for 10–15 min. in the dark at RT. The purple azo-compound produced was then quantified photometrically at 550 nm using a Wallac multi-label counter. Concentration of nitrite (μM) in the samples was calculated using 0–100 μM $NaNO_2$ as a standard.

Western blotting

After treatments cells were lysed in 100 μl buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5mM ethyl-enediamine-tetraacetic acid (EDTA), 0.1 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA), 1% Igepal-630, 0.5 mM dithiothreitol (DTT), 100 μM phenylmethyl-sulphonyl fluoride (PMSF), 2 μg/ml pepstatin A, 25 μM N-Acetyl-Leu-Leu-Nle-CHO (ALLN), 2.5 μg/ml aprotinin and 10 μM leupeptin) for 15 min. on ice. Protein concentration was measured using the Bradford method. Protein samples were denatured in Laemmli's sample buffer and boiled for 5 min. Proteins were separated by 10% SDS–PAGE and transferred onto nitrocellulose membrane. Membranes were blocked for 1 hr in PBS containing 0.05% Tween 20 and 5% (w/v) non-fat dried milk. The membranes were then incubated for 1 hr at RT with antibodies to actin (1:500; Sigma) or overnight at 4°C with antibodies to caspases-3, -7 and -9 (1:1,000; Cell Signaling Technologies), iNOS (1:1,000; BD Biosciences) or Bcl-XL (1:1000; Santa Cruz). This was followed by 2 hrs incubation at RT with appropriate secondary antibodies (1:10,000 for all antibodies except 1:2,000 for detection of caspases-3, -7 and -9). Protein bands were visualized using Supersignal Ultra Chemiluminescent Substrate (Pierce) on X-ray film (Agfa).

Detection of caspase activity

Cells were re-suspended in PBS (25 μl) and were transferred to a microtitre plate and snap-frozen over liquid nitrogen. To initiate the reaction, 50 (M of the caspase substrate DEVD-AMC (carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) (Peptide Institute Inc., Osaka Japan) in assay buffer (100 mM Hepes buffer, 10% sucrose, 0.1% 3[(3cholamidopropyl)-dimethylammonio]-1 propanesulphonate (CHAPS), 5 mM DTT and 0.0001% Igepal 630, pH 7.25) was added to cell lysates. Substrate cleavage leading to the release of free AMC was monitored at 37°C at 60 sec. intervals for 25 cycles using a Wallac multi-label counter (excitation 355 nm, emission 460 nm). Enzyme activity was expressed as nmol AMC released/min/mg protein.

Adenoviral transduction

The Bcl-x_L construct was generated as described before [21]. Briefly, the rat cDNA for Bcl-X_L was amplified with sequence-specific primers by PCR and cloned into the pAC-CMVpLpA plasmid (forw: GCGCCCGAATTCAT-GTCTCAGAGCAA and rev: GCCGCTCTAGATCACTTCC-GACTGAA). The recombinant plasmid was co-transfected into the 911 cell line [22] with the adenoviral plasmid pJM17 using Lipofectamine (Gibco/BRL). Resulting adenovirus clones were analysed by PCR with specific primers. The propagation and purification of recombinant adenovirus clones was performed in the 911 cells as described previously [22].

A recombinant adenoviral vector encoding the human iNOS gene driven by the cytomegalovirus promoter was a kind gift from Imre Kovesdi, Genvec and was generated as previously described [23]. AdEGFP is a replication-deficient, E1- and E3-deficient adenoviral vector that includes a humanized green fluorescence protein (GFP) gene under transcriptional control of the cytomegalovirus promoter. AdNull is a null vector control with E1-deletion but no therapeutic insert. Each of these three adenoviruses was propagated, isolated and titred using the CsCl method as described previously [24]. RIN cells were transduced with adenovirus diluted in RPMI at a multiplicity of infection (MOI) of 25 (AdiNOS) and 50 (AdBcl-XLand AdGFP) for all experiments unless otherwise stated. For all experiments a transduction volume of 500 μl was used. Cells were incubated with the virus for 1 hr then medium was removed and fresh medium was added.

Measurement of Δψm

Mitochondrial inner membrane potential was determined by using the fluorescent probe tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) as previously described [25]. Briefly, cells (1×10^6/ml) were trypsinized and incubated with TMRE at RT for 30 min. in the dark and analysed by flow cytometry using a FACSCalibur (Becton Dickinson).

Immunofluorescence microscopy

Cells were trypsinized, fixed in 3.7% formaldehyde for 10 min. and cytospins were prepared. Cells were then permeabilized with 100% methanol for 5 min. followed by incubation in blocking buffer (5% goat serum and 1% bovine serum albumin [BSA] in PBS) for 30 min., all steps carried out at RT. Incubations with anti-cytochrome *c* 1:250 (Pharmingen clone 7H8.2C12) were for 45 min. in a humidified chamber. Excess antibody binding was removed by three changes of PBS wash and followed by incubation with

fluorescein-5-isothiocynate (FITC) conjugated goat anti-mouse secondary antibody (1:100) for 30 min. in the dark. Following three changes of PBS wash coverslips were mounted onto microscope slides using 80% glycerol containing 100 μg/ml Hoechst 33342. Images were collected on an Olympus BX51 fluorescent microscope at a final magnification of 1000×.

Statistical analysis

The data is expressed as means ± S.D. of at least three separate experiments. Differences between the treatment groups were assessed using one-way ANOVA with pair wise comparisons performed using Tukey's *post hoc* test with a significance level of $P < 0.05$. For the experiments with inhibitors and transfected cells two-way ANOVA was used for the assessment of differences between treatment groups and transfection groups. Following a determination of a statistical significance a pair wise comparison was performed using Tukey's post hoc test with a significance level of $P < 0.05$. All statistical tests were carried out using SPSS version 14.0 software.

Results

IL-1β and IFNγ, but not TNFα, induce loss of cell viability

The effect of IL-1β, TNFα and IFNγ was examined by treating RIN cells with increasing concentrations (2.2, 6.6, 20, 60, 180, 540 U/ml) of each cytokine individually, or with all possible combinations of them. The change in viability was measured by MTT assay after treatment for 48 hrs. This concentration range incorporated concentrations at which toxicity has previously been observed for all three cytokines [26]. Treatment with IL-1β reduced viability to 71.0 ± 4.6% compared to untreated cells at the highest concentration used (540 U/ml, Fig. 1A). IFNγ was slightly more toxic to the cells as incubation with the highest concentration (540 U/ml) decreased cell viability to 54.9 ± 7.1%. TNFα had no effect on cell viability over the concentration range and time course examined. Reports have shown that inflammatory cytokines can potentiate each others action. In particular, the cytotoxic effect of IL-1β has been shown to be potentiated by either TNFγ or IFNα. To investigate the synergistic effect of these cytokines with IL-1β in our

model, RIN cells were treated with a sub-lethal dose of IL-1β (60 U/ml) and increasing concentrations (20–540 U/ml) of TNFα or IFNγ (Fig. 1B). TNFα caused no observable decrease in cell viability compared to the effect of IL-1β alone, even when high concentrations were used (Fig. 1B) In contrast, treatment with a combination of IL-1β (60 U/ml) and IFNγ resulted in a pronounced drop in cell viability (26.6 ± 5.1%), even at the lowest concentration of IFNγ used (20 U/ml, Fig. 1B). Synergism between TNFα and IFNγ was also examined by treating RIN cells with a sub-lethal dose of TNFα (60 U/ml) and increasing concentrations of IFNγ (20–540 U/ml). Addition of IFNγ in combination with TNFα did not show any potentiation, the decrease in viability was not significantly different from that induced by IFNγ alone.

IL-1β/IFNγ-induced loss of cell viability is due to apoptotic cell death

Although recent studies suggest a role for apoptosis in β-cell death induced by cytokines, there is still considerable controversy over this issue [6]. We examined key features of apoptosis, including PS externalisation, caspase activation and nuclear fragmentation to determine whether the loss of viability induced by cytokines was due to apoptotic cell death. As an early marker of apoptosis, PS externalization was detected by annexin V/PI staining (Fig. 2A). A combination of IL-1β and IFNγ (IL-1β/IFNγ), but neither cytokine alone induced PS externalisation after 24 hrs, confirming that the mode of β-cell loss is apoptosis and the two cytokines act synergistically. Caspase activation was monitored using an enzyme assay with the caspase-specific tetrapeptide substrate, DEVD-AMC as well as by detecting pro-caspase-3 and -7 processing with western blotting. Increased caspase enzyme activity was observed following treatment with IL-1β/IFNγ and was maximal at 24 hrs (Fig. 2B). Incubation with either IL-1β or IFNγ alone did not induce caspase-3-like activity up to 24 hrs (Fig. 2B), though, as expected, they did induce DEVDase activity after longer incubation times (48 hrs, data not shown). Analysis of caspase activation by western blotting confirmed the result of the caspase assays; cleavage products corresponding to the active forms of both pro-caspase-3 and -7 were detectable after 24 hrs and 48 hrs treatment with IL-1β/IFNγ (Fig. 2C). To generate a positive control for caspase cleavage, thapsigargin, a known inducer of
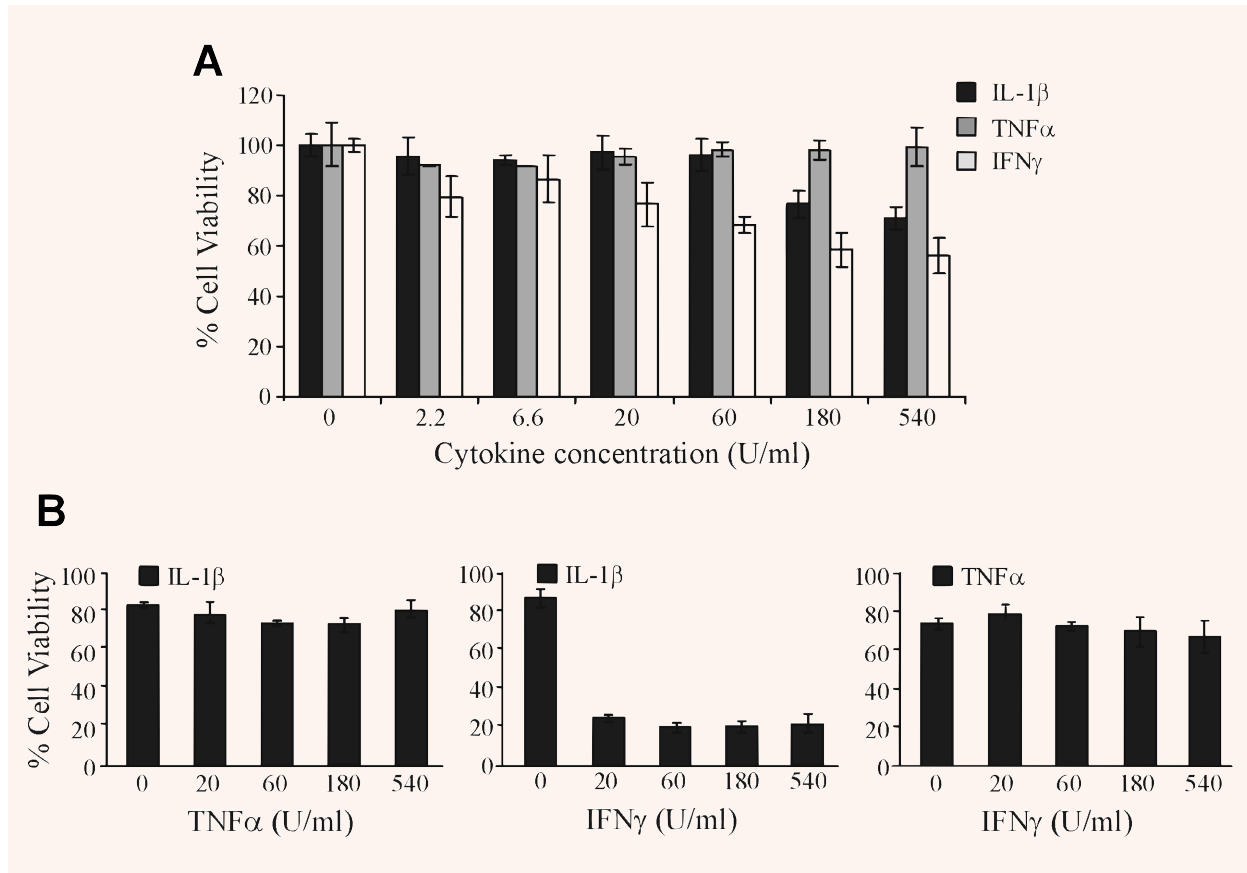
Fig. 1 Assessment of the contributions of IL-1β, TNFα and IFNγ to loss of rat insulinoma (RIN) cell viability RIN cells were treated with the indicated doses of IL-1β, TNFα, IFNγ alone or in combination. Cell viability was measured using MTT assay and expressed as a percentage of the mean absorbance of untreated cells. (**A**) RIN cells were treated for 48 hrs treatment with increasing concentrations (2.2–540 U/ml) of each cytokine alone and cell viability was measured. (**B**) RIN cells were treated for 48 hrs with 60 U/ml of each of the indicated cytokines in combination with increasing concentrations (20–540 U/ml) of another as indicated and cell viability was assessed. Data represents the average of at least three independent experiments ± S.D.

apoptosis was used. Finally, as a morphological hallmark of apoptosis, nuclear fragmentation was studied using Hoechst fluorescent DNA dye. RIN cells treated with IL-1β/IFNγ for 36 hrs displayed condensed and fragmented nuclei typical of apoptosis (Fig. 2D).

IL-1β/IFNγ-mediated iNOS-dependent nitric oxide production is sufficient for β-cell apoptosis

Previous studies have examined individual markers of cell death upon cytokine treatment, but have not identified the core apoptotic pathway activated. Because production of nitric oxide by iNOS has been implicated

as a major mediator of cytokine-induced β-cell injury, we examined whether intracellular nitric oxide production by iNOS was sufficient to cause the β-cell apoptosis detected following IL-1β/IFNγ treatment. First, expression of iNOS following 24 hrs cytokine treatment was examined by Western blotting (Fig. 3A). IL-1β, but neither IFNγ nor TNFα induced a modest but detectable induction of iNOS, which was absent in untreated cells (Fig. 3A). However, when IL-1β and IFNγ were used in combination, IFNγ greatly enhanced IL-1β-mediated iNOS induction (Fig. 3A). Addition of TNFα had no further effect on iNOS expression (Fig. 3A). Accumulation of nitrite, the end product of nitric oxide degradation, in the culture medium was also quantified using the Griess method, which closely
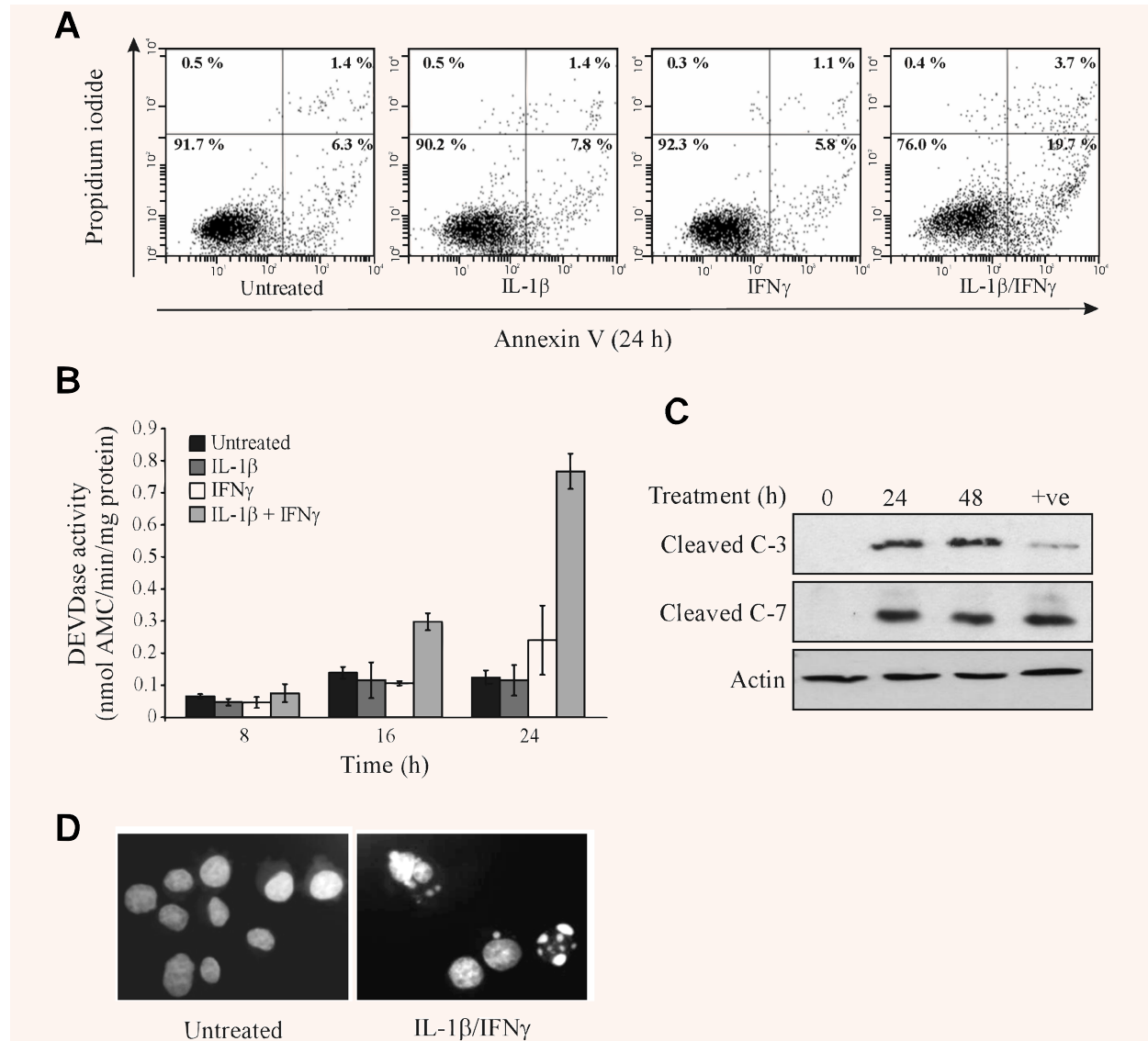
Fig. 2 IL-1β/IFNγ-induced loss of viability is due to apoptotic cell death RIN cells were treated with IL-1β (60 U/ml) and IFNγ (60 U/ml) alone or in combination (IL-1β/IFNγ) for the indicated times. (**A**) Annexin V/PI staining of control and cytokine treated cells was measured by flow cytometry. Dot plots are from one representative experiment and percentages are the average of three independent experiments ± S.D. (**B**) Caspase-3-like activity was detected by monitoring DEVD-AMC cleavage and values were expressed as nmol AMC released per minute by 1 mg of protein. Data represents the mean of at least three independent experiments ± S.D. (**C**) Caspase-3 and -7 processing in response to cytokine treatment. Whole cell lysates from control and cytokine-treated cells were subjected to western blot analysis of cleaved caspase-3, and -7. Thapsigargin-treated cells (1 μM) were used as a positive control (+ve). As a control for protein loading the blot was probed with anti-actin antibody. The image is representative of two independent experiments. (**D**) Cytospins were prepared of IL-1β/ IFNγ-treated cells and then stained with Hoescht 33342. The images shown are representative of at least three independent experiments.

followed the pattern of iNOS induction (Fig. 3B). These data show that the synergistic effect of IL-1β and IFNγ on induction of iNOS and subsequent nitric oxide production correlates with apoptosis (Fig. 1B).

In order to determine if effects other than nitric oxide production are required to mediate cytokine-induced apoptosis, RIN cells were transduced with an iNOS expressing adenovirus (AdiNOS) and
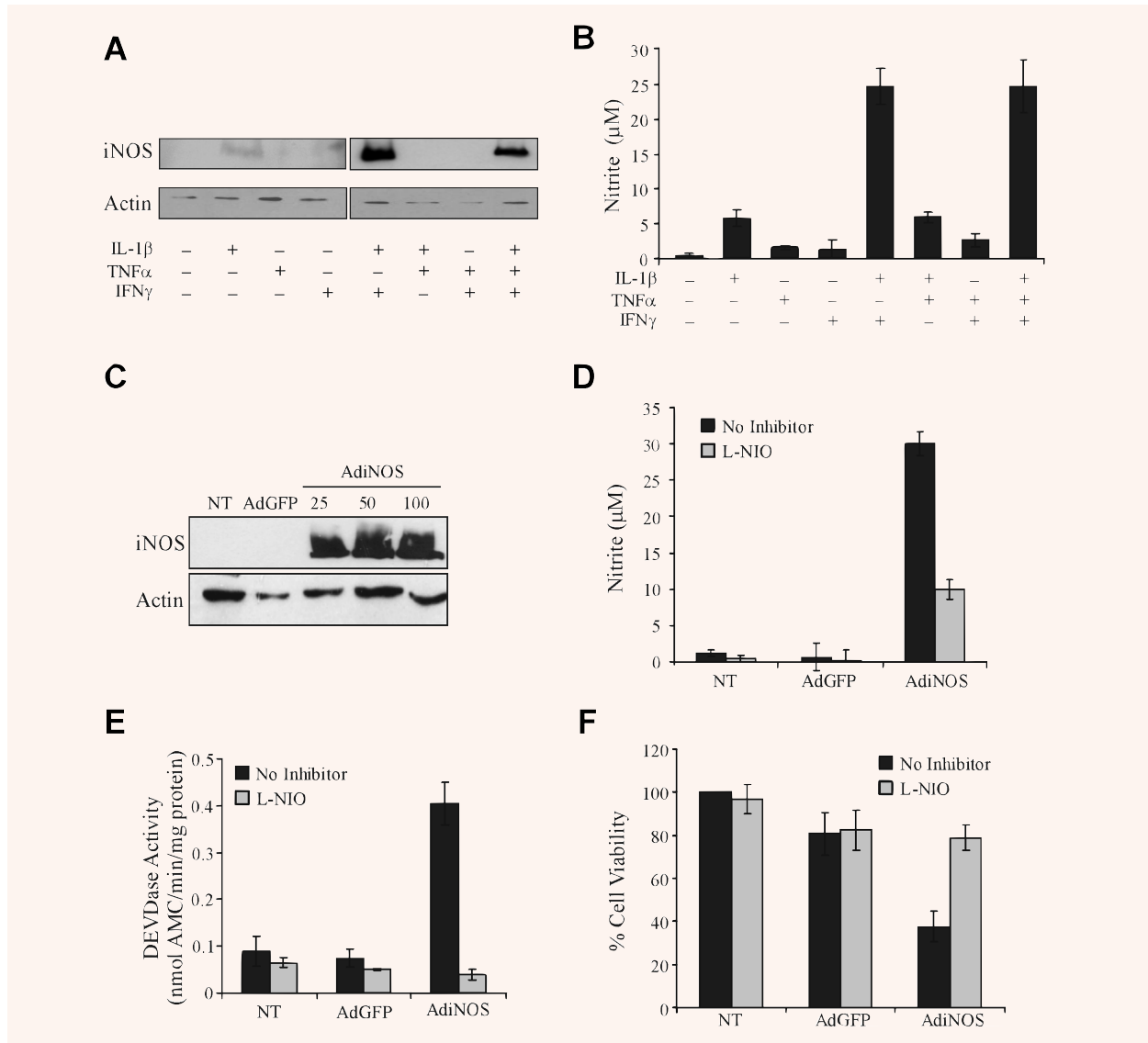
Fig. 3 IL-1β/ IFNγ induce iNOS expression and nitric oxide production which is sufficient to cause apoptosis RIN cells were incubated with IL-1β (60 U/ml), IFNγ (60 U/ml) and TNF((60 U/ml) alone or in combination as indicated for 24 hrs or transduced with an AdiNOS or AdEGFP. (**A**) iNOS induction by cytokines. Whole cell lysates of control and cytokine-treated cells were subjected to western blot analysis for iNOS expression. (**B**) Griess assay was used to measure nitric oxide production and expressed as μM nitrite produced. Data represents the mean of at least three independent experiments ± S.D. (**C**) Cells were transduced with AdiNOS at the indicated MOIs for 24 hrs. Whole cell lysates from non-transduced (NT), AdGFP and AdiNOS cells were subjected to western blot analysis for iNOS. As a control for protein loading the blot was probed with anti-actin antibody. (**D**) Adenoviral gene transfer of iNOS results in nitric oxide production. Griess assay was used to measure nitric oxide production and expressed as (M nitrite produced. Data represents the mean of at least three independent experiments ± S.D. (**E**) Adenoviral gene transfer of iNOS induced caspase activity. Caspase-3-like activity was monitored at 24 hrs fluorimetrically through use of the synthetic substrate DEVD-AMC. Results are expressed as nmol AMC released per min by 1 mg total cellular protein. Data represents the mean of at least three independent experiments ± S.D. (**F**) Adenoviral gene transfer of iNOS results in loss of cell viability. Cell viability of control, GFP and iNOS transduced samples was assessed by MTT assay at 24 hrs. Viability was expressed as a percentage of the mean absorbance measured in untreated cultures. Data represents the mean of at least three independent experiments ± S.D. *$P<0.05$ between the indicated treatment groups.

induction of apoptosis was monitored. Multiplicity of infections (MOIs) was optimized to express a level of iNOS comparable to that observed following IL-1β/IFNγ treatment. This was achieved at an MOI of 25 (Fig. 3C) and not only resulted in similar level of iNOS expression, but also a comparable level of nitric oxide as that produced by IL-1β/IFNγ (Fig. 3D). DEVDase activity and cell viability was monitored 24 hrs following AdiNOS transduction. Overexpression of iNOS induced caspase activation, which was similar to that induced by IL-1β/IFNγ treatment (Fig. 3E). A reduction of cell viability as measured by MTT assay corresponded with this increase in caspase activity indicating that prolonged nitric oxide production alone is sufficient to mediate β-cell death (Fig. 3F). Adenoviral overexpression of GFP (AdGFP) was used as a control to confirm that the effects were not due to viral transduction. Furthermore, all the observed effects were reversible by pre-treatment with L-NIO, an inhibitor of NOS, indicating that the detected β-cell apoptosis required iNOS activity (nitric oxide production by iNOS) thus it was not an artificial event due to protein overexpression (Fig. 3E and 3F).

Nitric oxide is essential for cytokine-induced caspase activation

IL-1β/IFNγ-mediated toxicity seemed to involve both nitric oxide production and caspase activity so inhibitors of both these were employed to determine whether they were essential events during RIN cell apoptosis. RIN cells were treated with IL-1β, IFNγ or IL-1β/IFNγ in the presence or absence of Boc-D.fmk, a broad spectrum caspase inhibitor or L-NIO and caspase activity and cell death were examined in the samples. Pre-treatment of cells with Boc-D.fmk was able to block caspase activity induced by IL-1β/IFNγ (Fig. 4A). However, inhibition of caspases had no effect on nitric oxide production or PS externalization induced by cytokine treatment (Fig. 4B and C). Therefore, although cytokines activate the apoptotic pathway, inhibition of caspases is not sufficient to block β-cell death suggesting activation of alternative cell death pathways in the absence of caspase activity.

Since nitric oxide production by iNOS was sufficient to induce β-cell death and it occurred upstream or independent of caspase activity, we examined whether nitric oxide production is a requisite step for caspase activation and RIN cell death induced by IL-1β/IFNγ. We found that pre-treatment with L-NIO was able to completely block cytokine-induced nitric oxide production (Fig. 4A). More importantly, it could prevent IL-1β/IFNγ-induced caspase activation as measured by DEVDase activity as well as by detecting pro-caspase-3 processing with western blotting (Fig. 4B and 4C). Pre-treatment with L-NIO also reduced the PS externalisation indicating decreased apoptosis. Taken together, these data suggest that IL-1β/IFNγ-mediated nitric oxide production is upstream of, and necessary for activation of caspases and execution of apoptosis.

IL-1β/IFNγ activate the intrinsic apoptotic pathway

As many pro-apoptotic signals, including oxidizing agents, converge on the mitochondria, we examined if IL-1β/IFNγ induces a mitochondrion-dependent, intrinsic apoptotic pathway. RIN cells were treated with IL-1β/IFNγ and three key features of the intrinsic death pathway, namely loss of $\Delta\Psi$m, release of cytochrome c and activation of pro-caspase-9 were studied in response to both cytokines and iNOS. Loss of $\Delta\Psi$m was measured using TMRE, a fluorescent dye that accumulates only in polarized mitochondria. Treatment with IL-1β/IFNγ for 24 hrs caused loss of $\Delta\Psi$m in approximately 25% of cells (Fig. 5A). This value corresponds to the number of annexin V positive cells after the same period of treatment (23.4 ± 5.6%, Fig. 2A). Pre-treatment with L-NIO reduced this loss of $\Delta\Psi$m to approximately 15% of cells. Similarly, transduction of cells with AdiNOS also caused a loss of $\Delta\Psi$m in approximately 40% of cells (Fig. 5A). Re-distribution of cytochrome c was examined by immunocytochemical staining which revealed a high cytochrome c signal with a perinuclear, punctate staining in untreated cells. In contrast, there was marked reduction in the signal in apoptotic cells with intact morphology and cells with fragmented nuclei always exhibited a very weak, dispersed signal if detectable at all (Fig. 5B). Previous studies using the same cytochrome c antibody (7H8.2C12) have shown that it specifically detects mitochondrial localised cytochrome c and changes in cytochrome c distribution during the apoptotic
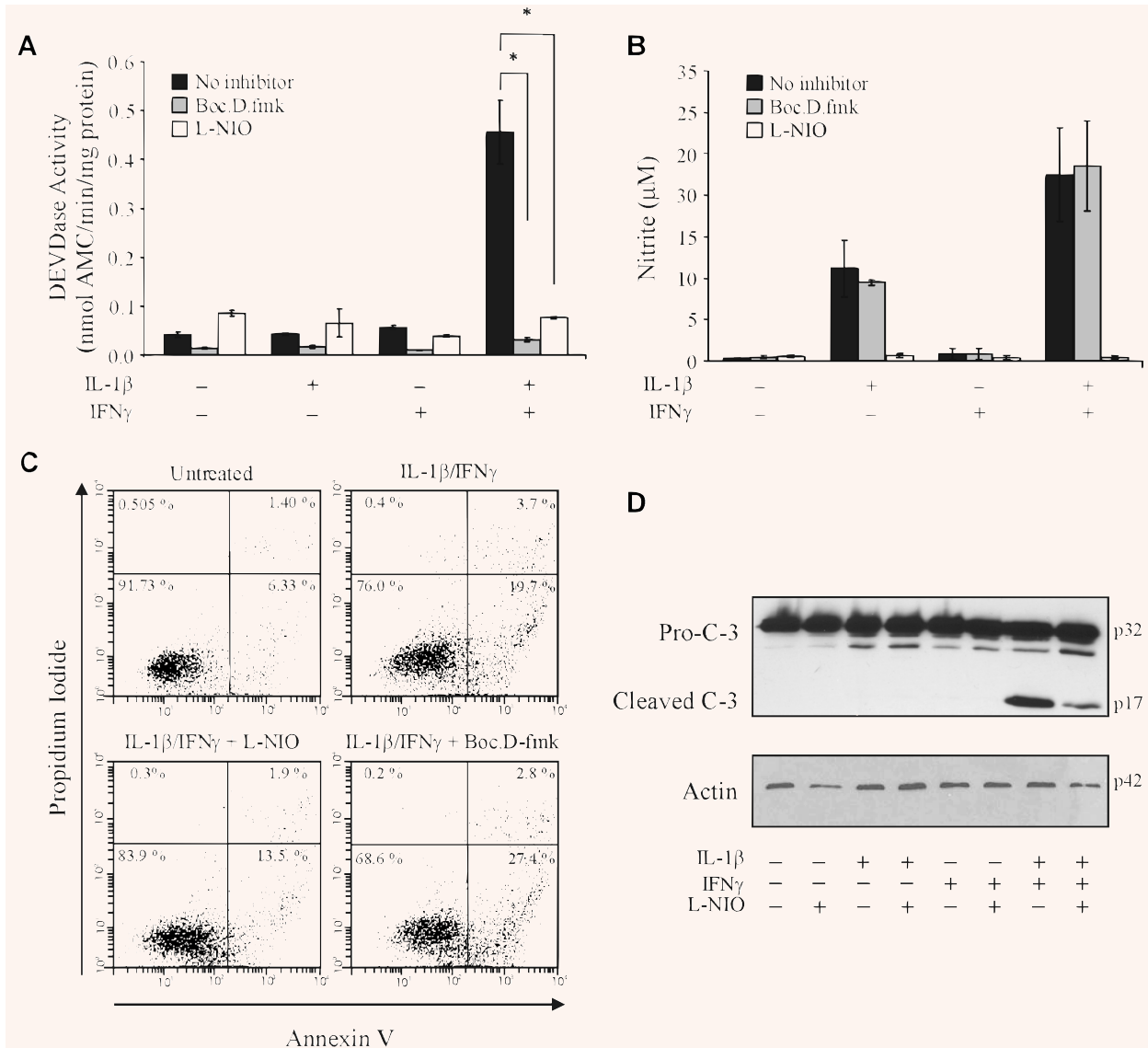
Fig. 4 Assessment of the effect of the broad range caspase inhibitor Boc-D.fmk and the iNOS inhibitor L-NIO on RIN cell death RIN cells were treated with IL-1β (60 U/ml), IFNγ (60 U/ml) and IL-1β/IFNγ with or without prior treatment with Boc-D.fmk (20 μm) or μM L-NIO (500). (**A**) Caspase-3-like activity was monitored 24 hrs after treatment. Caspase-activity is expressed as nmol AMC released per min by 1 mg of total cellular protein. Data represents the mean of at least 3 independent experiments ± SD. *$P < 0.05$ between the indicated treatment groups. (**B**) Supernatants from control and treated samples were assayed at 24 hrs for nitrite accumulation using the Griess method. Data represents the mean of at least three independent experiments ± S.D. (**C**) Annexin V/PI staining was measured by flow cytometry. Dot plots are from one representative experiment and percentages are the average of three separate experiments ± S.D. (**D**) Pre-treatment with L-NIO blocks IL-1β/IFNγ–mediated cleavage of caspase-3 and caspase-9. Whole cell lysates from control and treated samples were subjected to western blot analysis for to detect caspase-3 and -9 cleavage. The image is representative of two independent experiments.

process are reflected by reduced antibody binding and reduction of the cellular cytochrome *c* signal [27]. Staurosporine (STS) treatment, a known inducer of cytochrome *c* release, was used as a positive control. Cleavage of pro-caspase-9, a consequence of cytochrome *c* release was also
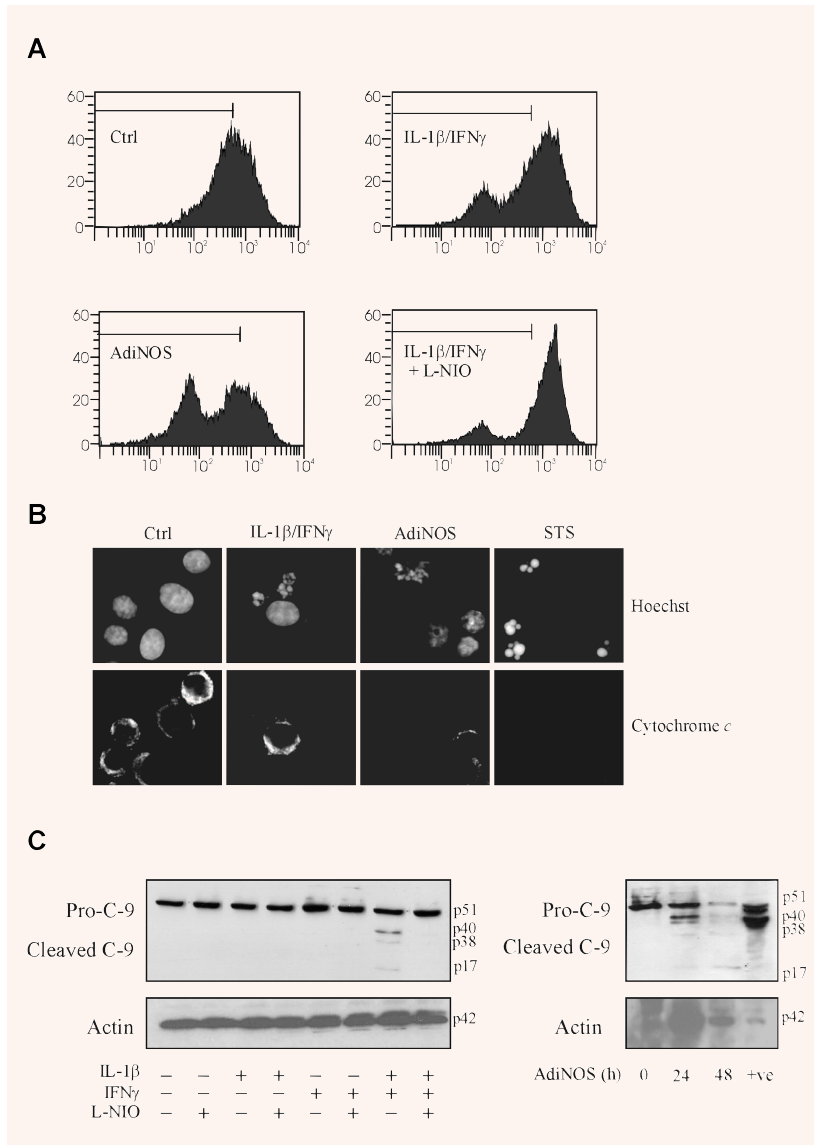
Fig. 5 IL-1β/IFNγ and iNOS cause a drop in ΔΨm, cytochrome c redistribution and caspase-9 cleavage. RIN cells were treated with (IL-1β/IFNγ) or transduced with AdiNOS for the indicated times. (**A**) Mitochondrial membrane potential was measured using TMRE after 24 hrs cytokine treatment with and without L-NIO pre-treatment, and following 24 hrs AdiNOS transduction. Histograms are representative from three independent experiments. (**B**) Redistribution of cytochrome c was examined following 24 hrs cytokine treatment and AdiNOS transduction by immunocytochemical staining using anti-cytochrome c. The top panels represent RIN cells stained with Hoechst following the indicated treatments. The bottom panels are RIN cells which have been stained with a cytochrome c specific antibody. (**C**) Whole cell lysates from control, cytokine-treated (24 hrs) and AdiNOS (24 and 48 hrs) transduced cells were subjected to western blot analysis of cleaved caspase-9. In this case +ve indicates lysates from rat liver cytosols activated with cytochrome c and dATP.

observed following both IL-1β/IFNγ treatment and forced expression of iNOS (Fig. 5C). Following 48 hrs AdiNOS treatment the caspase-9 signal disappeared altogether. This was possibly due to the disintegration of cells and loss of protein at this late time point. Collectively, these results prove the activation of the intrinsic apoptotic pathway in β-cells exposed to cytokines and the necessity of nitric oxide in activating mitochondrial signalling pathways. Thus elimination of nitric oxide, or protection of its target, the mitochondrion, may rescue β-cells from cytokine mediated apoptosis.

Bcl-X$_L$ protects RIN cells from cytokine and iNOS-induced apoptosis

Bcl-X$_L$ can prevent mitochondrial-mediated apoptosis by maintaining outer mitochondrial membrane integrity and thus cytochrome c release [13]. Bcl-X$_L$-expressing adenovirus (AdBcl-X$_L$) was therefore employed to determine whether expression of this anti-apoptotic protein could prevent cytokine and iNOS-mediated β-cell death. Substantial Bcl-X$_L$

expression was observed when cells were transduced with an MOI of 50 and thus, this MOI was chosen for further experiments (Fig. 6A). RIN cells were transduced with AdBcl-X$_L$ and 24 hrs later treated with IL-1β/IFNγ or transduced with AdiNOS. Both the cytokine mix and iNOS expression caused mitochondrial membrane depolarization after 24 hrs. Expression of Bcl-X$_L$ substantially decreased the loss of ΔΨm both after cytokine treatment and AdiNOS transduction (Fig. 6B). In order to determine whether protection of the mitochondria was sufficient to prevent apoptosis, both annexin V binding and caspase-3 activity were measured. We found that AdBcl-X$_L$ was able to prevent PS externalization and to completely block caspase-3 activation (Fig. 6C and D) in both IL-1β/IFNγ-treated and AdiNOS transduced RIN cells. It was also observed that AdBcl-x$_L$ had no effect on nitric oxide production, induced by either cytokines or AdiNOS, indicating that this event occurs upstream of mitochondrial damage (Fig. 6E). These data demonstrate that cytokines directly or indirectly induce apoptosis through mitochondria-mediated caspase activation and induction of iNOS is responsible for mediating the mitochondrial damage.

Discussion

This study examined the cytotoxic effect of IL-1β, IFNγ and TNFα on β-cells and identified the decisive steps of the apoptotic pathway that leads to caspase activation and β-cell death. The data presented here establishes that nitric oxide is solely responsible for IL-1β/IFNγ-mediated β-cell apoptosis by activating the mitochondrial apoptotic pathway. Moreover, the study demonstrates for the first time that blocking the mitochondrial death pathway by Bcl-X$_L$ prevents both cytokine- and nitric oxide-mediated β-cell death.

As different combinations and concentrations of cytokines have been implicated in causing β-cell toxicity, we examined a wide range of doses and combinations of IL-1β, TNFα and IFNγ in order to determine the exact contribution of these cytokines to β-cell death *in vitro*. Our results on IL-1β/IFNγ synergy being central to β-cell death are consistent with previous studies carried out in RIN cells and isolated rat primary β-cells [6]. IL-1α/IFNγ synergy is also

believed to be responsible for cytokine-mediated toxicity in human β-cells [3]. Although TNFα has also been shown to synergize with IFNγ in murine insulinoma cells, we did not observe this effect in the rat RIN cells [28]. This difference might be due to the species difference, that is mouse or rat, or concentrations and treatment times used. The cytotoxic effect of IL-1β/IFNγ correlated with a synergistic induction of iNOS indicating a possible role for nitric oxide in the synergistic action of the two cytokines in β-cell destruction. The molecular mechanism behind this synergy is not known but IL-1β and IFNγ can activate multiple transcription factors which may co-operate to regulate iNOS induction [29]. Previous studies in different cell systems have shown that nuclear factor NF-κB sites are required for iNOS induction [30–33]. Other IFNγ activated transcription factors, such as interferon regulatory factor (IRF)-1, CCAAT box/enhancer binding protein (C/EBP) and signal transducer and activator of transcription (STAT) 1 also are involved in iNOS induction [33–35]. However, in RIN cells IFNγ alone had neither an effect on iNOS expression nor an effect on nitric oxide production. This may indicate that IFNγ potentiates IL-1β-mediated iNOS induction by enhancing NF-κB activation. In support of this we have previously reported that inhibiting NF-κB activity can also prevent IL-1β/IFNγ-mediated nitric oxide production in Rin cells [26].

To investigate the mechanisms responsible for cytokine-induced β-cell death, biochemical and morphological hallmarks of apoptosis were evaluated and confirmed that apoptosis was the primary mode of death induced by IL-1β/IFNγ. Other studies found varying proportion of necrosis and apoptosis after similar treatments, presumably due to different animal/cell models, or time courses and detection techniques used [36]. We also detected a small proportion of cells with morphological features of necrosis. Unfortunately, in *in vitro* conditions the unphagocytosed disintegrating apoptotic cells also display necrosis-like features (secondary necrosis). Thus, based on our model system we cannot exclude the possibility, that cytokines induce necrosis in a small proportion (less than 10% of the dying population) of β-cells.

Induction of iNOS and the resulting sustained production of nitric oxide in response to pro-inflammatory cytokines are well established. The role of iNOS in the development of type I diabetes is however more disputable. Depending on the model system, *i.e. in vivo* model with the immune cells present, *versus in*
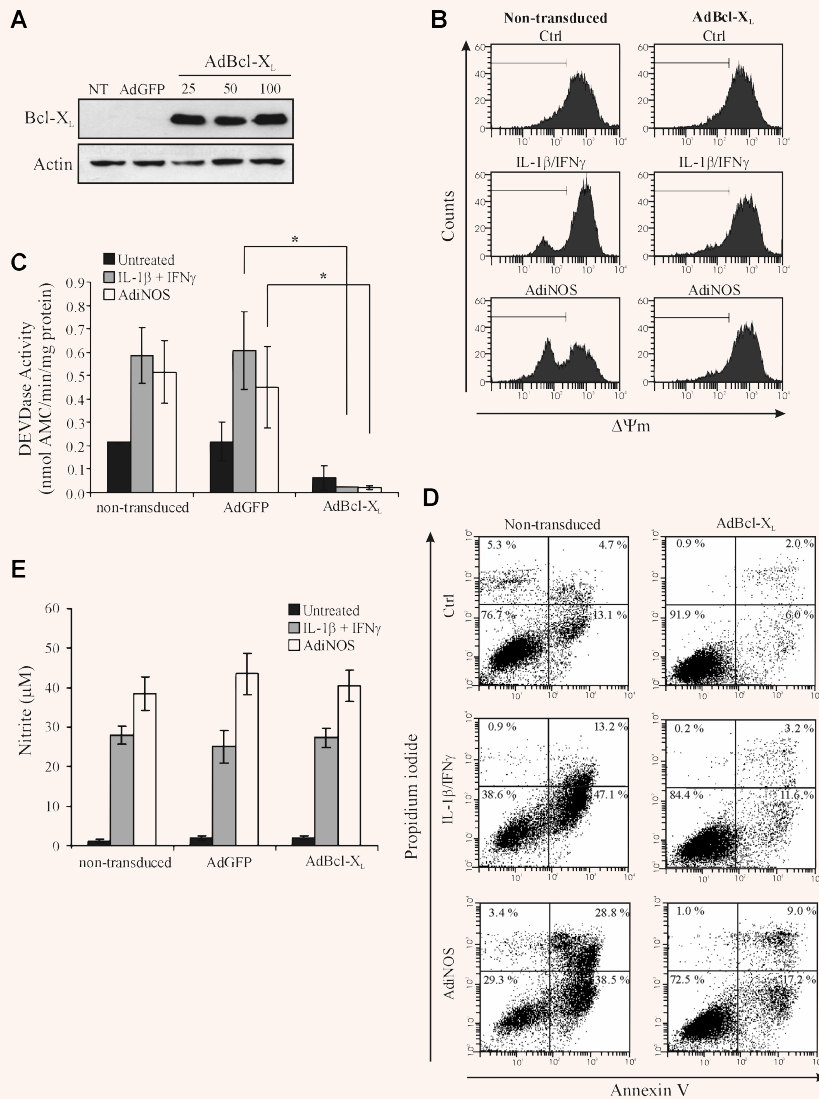
Fig. 6 Bcl-X$_L$ overexpresion protects RIN cells from iNOS-induced apoptosis RIN cells were transduced with AdBcl-X$_L$ or AdGFP (MOI of 50), and 24 hrs later cells were transduced with either AdiNOS (MOI of 25) or treated with IL-1β/IFNγ for 24 hrs (**A**) Cells were transduced with AdBcl-X$_L$ at the indicated MOIs for 24 hrs. Whole cell lysates from non-transduced (NT), AdGFP and AdBcl-X$_L$ cells were subjected to western blot analysis for Bcl-X$_L$. As a control for protein loading the blot was probed with anti-actin antibody. (**B**) Bcl-X$_L$ overexpression prevents iNOS and cytokines mediated loss of membrane potential. Mitochondrial membrane depolarization was determined by staining the fluorescent probe TMRE and measured by flow cytometry. Histograms are of one representative experiment from three repeats. (**C**) Bcl-XL expression prevents iNOS and cytokine-induced caspase activity. Caspase-3-like activity was monitored at 24 hrs fluorimetrically through use of the synthetic substrate DEVD-AMC. Results are expressed as nmol AMC released per min by 1 mg total cellular protein. Data is representative of at least three independent experiments ± S.D. *$P < 0.05$ between the indicated treatment groups. (**D**) Bcl-X$_L$ overexpression protects RIN cells from iNOS and cytokine mediated apoptosis. Annexin V/PI staining was measured by flow cytometry. Dot plots are from one representative experiment and percentages are the average of three separate experiments ± S.D. (**E**) Bcl-X$_L$ expression does not block cytokine and AdiNOS induced nitric oxide production. Supernatants from control and treated samples were assayed at 24 hrs for nitrite accumulation using the Griess method. Data represents the mean of at least three independent experiments ± S.D.

vitro systems of isolated islet cells or insulinoma cell lines, there are different outcomes. In order to resolve some of the controversies, we aimed to clarify the role of nitric oxide produced inside the β-cells in response to pro-inflammatory cytokines. We found that expression of iNOS was sufficient to induce caspase activation and RIN cell apoptosis comparable to that induced by IL-1β/IFNγ. Moreover, inhibition of iNOS could completely prevent IL-1β/IFNγ-induced caspase activation, which indicates that nitric oxide production is not only a sufficient, but an indispensable step in cytokine-induced caspase activation and thus β-cell apoptosis. The ability of the NOS inhibitor to reduce cell loss induced by IL-1β/IFNγ confirms that nitric oxide is the key apoptotic trigger. A recent paper using the same cell model documented a cytoprotective effect against IL-1β after targeted knockdown of iNOS expression directly using a lentiviral based shRNA delivery system [37]. Our findings are supported by a microarray analysis carried out which identified a wide array of genes regulated by IL-1β/IFNγ in INS-1E β-cells, another rat β-cell line [29]. A number of the genes identified, namely Bak, Bid, Bcl-X$_L$ and GADD153 are directly linked to apoptosis [38]. Although the study did not examine the actual function of these genes in IL-1β/IFNγ-induced β-cell death, it is notable that with the exception of Bak, all these genes were regulated in an nitric oxide-dependent manner [29].

There is much debate about how nitric oxide exerts its cytotoxic effects in β-cells. Earlier studies suggested that nitric oxide production leads to a necrotic death and nitric oxide-independent pathways are responsible for apoptosis induction [6]. This view was challenged by recent studies [18, 20]. Messner *et al.* found that cytokines induced β-cell apoptosis through nitric oxide-mediated DNA damage and activation of p53 [39]. Endoplasmic reticulum (ER) also plays a major role in the initiation of apoptosis [38]. A recent report has shown that sustained nitric oxide production triggered the ER stress response and deficiency of the ER stress-induced pro-apoptotic transcription factor, GADD153/CHOP reduces nitric oxide-induced β-cell death [40]. We have also observed an early induction of C/EBP homology protein (CHOP) in an iNOS-dependent manner following cytokine treatment (Holohan, Szegezdi and Samali, unpublished data). These data support the notion that ER stress and mitochondrial pathway may be linked. We are
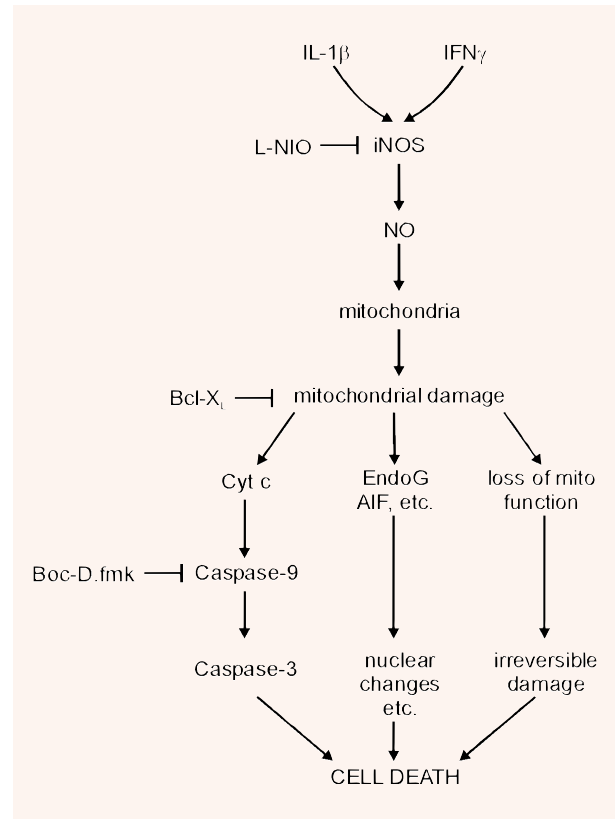

Fig. 7 Mechanisms of cytokine-induced β-cell death IL-1β and IFNγ act in synergy to induce expression of iNOS. Induced expression of iNOS causes excess production of nitric oxide which results in irreversible damage to the mitochondria. This damage results in the release of pro-apoptotic factors (including cytochrome *c*) and loss of mitochondrial function. In the cytosol cytochrome *c* activates a caspase cascade. Under caspase-inhibiting conditions (presence of Boc-D.fmk) cell death still occurs indicating the presence of caspase-independent cell death pathways. However, prevention of mitochondrial damage, through forced expression of Bcl-X$_L$ or inhibition of iNOS, blocks cytokine-mediated cell death.

currently investigating whether blockade of the ER stress pathway may prevent cytokine-mediated apoptosis.

In addition to p53 activation and ER stress, nitric oxide and its peroxynitrite derivatives can directly cause mitochondrial damage through inhibition of components of the mitochondrial electron transport chain [41]. Inhibition of the mitochondrial respiration leads to the dissipation of ΔΨm, mitochondrial swelling and cytochrome *c* release, as it has been

shown with isolated mitochondria exposed to nitric oxide [42]. We found that both IL-1β/IFNγ treatment and overexpression of iNOS activated the mitochondrial apoptosis pathway. Overexpression of Bcl-X$_L$ was able to inhibit mitochondrial membrane depolarization, prevent caspase activation and most importantly stop apoptosis, induced by both IL-1β/IFNγ or forced expression of iNOS. Bcl-X$_L$ exerted these protective effects without affecting nitric oxide production. This indicates that nitric oxide production either directly, or indirectly, targets the mitochondria *via* activating the p53 or the ER stress pathways, which is required for IL-1β/IFNγ-induced β-cell death. It should be noted that although Bcl-X$_L$ is primarily located on the mitochondria [43] it can also localize to the ER and may exert some of its cytoprotective effect at the level of the ER.

Although IL-1β/IFNγ-induced apoptosis was associated with marked caspase activation, inhibition of caspases by Boc-D.fmk failed to reduce cell death. The inability of caspase inhibition to rescue the cells is in line with many other reports which demonstrate that apoptosis induced by stress stimuli that target the mitochondria cannot be blocked by inhibition of caspases [44]. This may suggest that an irreversibly damaged cell is committed to die and if the caspases are inhibited, that is apoptotic pathway is blocked, the cell uses alternate death mechanisms (Fig. 7). Therefore, the point of no return is at the level of the mitochondrion. Once the outer mitochondrial membrane is permeabilized cytochrome *c* and other pro-apoptotic factors, namely endonuclease G (EndoG), apoptosis inducing factor (AIF) and the serine protease Omi, are also released. AIF and EndoG directly cause chromatin condensation and fragmentation, while Omi can cleave many substrates leading to cell death in a caspase independent manner [45–49]. Furthermore, disruption of mitochondria under caspase inhibiting conditions may force cells down a necrotic cell death pathway [44]. Therefore, targeted protection of the mitochondria, but not downstream apoptotic events may prevent cytokine-induced β-cell death.

Acknowledgements

References

1. **Bach JF.** Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev.* 1994; 15: 516–42.
2. **Cetkovic-Cvrlje M, Eizirik DL.** TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly *via* generation of nitric oxide. *Cytokine.* 1994; 6: 399–406.
3. **Rabinovitch A, Suarez-Pinzon WL.** Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol.* 1998; 55: 1139–49.
4. **Sandberg JO, Andersson A, Eizirik DL, Sandler S.** Interleukin-1 receptor antagonist prevents low dose streptozotocin induced diabetes in mice. *Biochem Biophys Res Commun.* 1994; 202: 543–8.
5. **Iwahashi H, Hanafusa T, Eguchi Y, Nakajima H, Miyagawa J, Itoh N, Tomita K, Namba M, Kuwajima M, Noguchi T, Tsujimoto Y, Matsuzawa Y.** Cytokine-induced apoptotic cell death in a mouse pancreatic beta-cell line: inhibition by Bcl-2. 5 *Diabetologia.* 1996; 39: 530–6.
6. **Eizirik DL, Mandrup-Poulsen T.** A choice of death–the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia.* 2001; 44: 2115–33.
7. Cryns V, Yuan J. Proteases to die for. *Genes Dev.* 1998; 12: 1551–70.
8. **Stroh C, Schulze-Osthoff K.** Death by a thousand cuts: an ever increasing list of caspase substrates. *Cell Death Differ.* 1998; 5: 997–1000.
9. **Samali A, Zhivotovsky B, Jones D, Nagata S, Orrenius S.** Apoptosis: cell death defined by caspase activation. *Cell Death Differ.* 1999; 6: 495–6.
10. **Samali A, Gorman AM, Cotter TG.** Apoptosis – the story so far. *Experientia.* 1996; 52: 933–41.
11. **Reed JC.** Mechanisms of apoptosis. *Am J Pathol.* 2000; 157: 1415–30.
12. **Green DR, Reed JC.** Mitochondria and apoptosis. *Science.* 1998; 281: 1309–12.
13. **Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD.** The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* 1997; 275: 1132–6.
14. **Kluck RM, Esposti MD, Perkins G, Renken C, Kuwana T, Bossy-Wetzel E, Goldberg M, Allen T, Barber MJ, Green DR, Newmeyer DD.** The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J Cell Biol.* 1999; 147: 809–22.
15. **Finucane DM, Bossy-Wetzel E, Waterhouse NJ, Cotter TG, Green DR.** Bax-induced caspase activation and apoptosis *via* cytochrome *c* release from mitochondria is inhibitable by Bcl-xL. *J Biol Chem.* 1999; 274: 2225–33.

16. **Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ.** Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol.* 1997; 139: 1281–92.

17. **Suarez-Pinzon WL, Strynadka K, Schulz R, Rabinovitch A.** Mechanisms of cytokine-induced destruction of rat insulinoma cells: the role of nitric oxide. *Endocrinology.* 1994; 134: 1006–10.

18. **Welsh N, Eizirik DL, Sandler S.** Nitric oxide and pancreatic beta-cell destruction in insulin dependent diabetes mellitus: don't take NO for an answer. *Autoimmunity.* 1994; 18: 285–90.

19. **Thomas HE, Darwiche R, Corbett JA, Kay TW.** Interleukin-1 plus gamma-interferon-induced pancreatic beta-cell dysfunction is mediated by beta-cell nitric oxide production. *Diabetes.* 2002; 51: 311–6.

20. **Ankarcrona M, Dypbukt JM, Brune B, Nicotera P.** Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp Cell Res.* 1994; 213: 172–7.

21. **Reeve JL, Szegezdi E, Logue SE, Chonghaile TN, O'Brien T, Ritter T, Samali A.** Distinct mechanisms of cardiomyocyte apoptosis induced by doxorubicin and hypoxia converge on mitochondria and are inhibited by Bcl-xL. *J Cell Mol Med.* 2007; 11: 509–20.

22. **Fallaux FJ, Kranenburg O, Cramer SJ, Houweling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ.** Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther.* 1996; 7: 215–22.

23. **Kibbe MR, Li J, Nie S, Watkins SC, Lizonova A, Kovesdi I, Simmons RL, Billiar TR, Tzeng E.** Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. *J Vasc Surg.* 2000; 31: 1214–28.

24. **Duffy AM, O'Doherty AM, O'Brien T, Strappe PM.** Purification of adenovirus and adeno-associated virus: comparison of novel membrane-based technology to conventional techniques. *Gene Ther.* 2005; 12 Suppl 1: S62–72.

25. **Samali A, Cai J, Zhivotovsky B, Jones DP, Orrenius S.** Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of jurkat cells. *EMBO J.* 1999; 18: 2040–8.

26. **McCabe C, Samali A, O'Brien T.** beta cell cytoprotective strategies: establishing the relative roles for iNOS and ROS. *Biochem Biophys Res Commun.* 2006; 342: 1240–8.

27. **Stahnke K, Mohr A, Liu J, Meyer LH, Karawajew L, Debatin KM.** Identification of deficient mitochondrial signaling in apoptosis resistant leukemia cells by flow cytometric analysis of intracellular cytochrome c, caspase-3 and apoptosis. *Apoptosis.* 2004; 9: 457–65.

28. **Chang I, Cho N, Kim S, Kim JY, Kim E, Woo JE, Nam JH, Kim SJ, Lee MS.** Role of calcium in pancreatic islet cell death by IFN-gamma/TNF-alpha. *J Immunol.* 2004; 172: 7008–14.

29. **Kutlu B, Cardozo AK, Darville MI, Kruhoffer M, Magnusson N, Orntoft T, Eizirik DL.** Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. *Diabetes.* 2003; 52: 2701–19.

30. **Spink J, Cohen J, Evans TJ.** The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor-kappa B. *J Biol Chem.* 1995; 270: 29541–7.

31. **Taylor BS, Alarcon LH, Billiar TR.** Inducible nitric oxide synthase in the liver: regulation and function. *Biochemistry.* 1998; 63: 766–81.

32. **Taylor BS, de Vera ME, Ganster RW, Wang Q, Shapiro RA, Morris SM Jr, Billiar TR, Geller DA.** Multiple NF-kappaB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J Biol Chem.* 1998; 273: 15148–56.

33. **Xie QW, Kashiwabara Y, Nathan C.** Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem.* 1994; 269: 4705–8.

34. **Gao J, Morrison DC, Parmely TJ, Russell SW, Murphy WJ.** An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. *J Biol Chem.* 1997; 272: 1226–30.

35. **Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura T, Green SJ, et al.** Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science.* 1994; 263: 1612–5.

36. **Collier JJ, Fueger PT, Hohmeier HE, Newgard CB.** Pro- and antiapoptotic proteins regulate apoptosis but do not protect against cytokine-mediated cytotoxicity in rat islets and beta-cell lines. *Diabetes.* 2006; 55: 1398–406.

37. **McCabe C, O'Brien T.** Beta cell cytoprotection using lentiviral vector-based iNOS-specific shRNA delivery. *Biochem Biophys Res Commun.* 2007; 357: 75–80.

38. **Szegezdi E, Logue SE, Gorman AM, Samali A.** Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep.* 2006; 7: 880–5.

39. **Messmer UK, Ankarcrona M, Nicotera P, Brune B.** p53 expression in nitric oxide-induced apoptosis. *FEBS Lett.* 1994; 355: 23–6.

40. **Cardozo AK, Ortis F, Storling J, Feng YM, Rasschaert J, Tonnesen M, Van Eylen F, Mandrup-Poulsen T,**

Herchuelz A, Eizirik DL. Cytokines downregulate the sarcoendoplasmic reticulum pump Ca^{2+} ATPase 2b and deplete endoplasmic reticulum Ca^{2+}, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes.* 2005; 54: 452–61.

41. **Brown GC.** Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome *c* oxidase. *Biochim Biophys Acta.* 2001; 1504: 46–57.

42. **Ghafourifar P, Schenk U, Klein SD, Richter C.** Mitochondrial nitric-oxide synthase stimulation causes cytochrome *c* release from isolated mitochondria. Evidence for intramitochondrial peroxynitrite formation. *J Biol Chem.* 1999; 274: 31185–8.

43. **Kaufmann T, Schlipf S, Sanz J, Neubert K, Stein R, Borner C.** Characterization of the signal that directs Bcl-x(L), but not Bcl-2, to the mitochondrial outer membrane. *J Cell Biol.* 2003; 160: 53–64.

44. **Kroemer G, Martin SJ.** Caspase-independent cell death. *Nat Med.* 2005; 11: 725–30.

45. **Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM.** Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature.* 2001; 410: 549–54.

46. **Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P.** Toxic proteins released from mitochondria in cell death. *Oncogene.* 2004; 23: 2861–74.

47. **Cilenti L, Kyriazis GA, Soundarapandian MM, Stratico V, Yerkes A, Park KM, Sheridan AM, Alnemri ES, Bonventre JV, Zervos AS.** Omi/HtrA2 protease mediates cisplatin-induced cell death in renal cells. *Am J Physiol Renal Physiol.* 2005; 288: F371–9.

48. **Cilenti L, Soundarapandian MM, Kyriazis GA, Stratico V, Singh S, Gupta S, Bonventre JV, Alnemri ES, Zervos AS.** Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death. *J Biol Chem.* 2004; 279: 50295–301.

49. **Trencia A, Fiory F, Maitan MA, Vito P, Barbagallo AP, Perfetti A, Miele C, Ungaro P, Oriente F, Cilenti L, Zervos AS, Formisano P, Beguinot F.** Omi/HtrA2 promotes cell death by binding and degrading the anti-apoptotic protein ped/pea-15. *J Biol Chem.* 2004; 279: 46566–72.