Inhibition of Cytokine-Induced NF-κB Activation by Adenovirus-Mediated Expression of a NF-κB Super-Repressor Prevents β-Cell Apoptosis

Harry Heimberg,[1] Yves Heremans,[1] Christian Jobin,[2] Ruth Leemans,[1] Alessandra K. Cardozo,[1] Martine Darville,[1] and Décio L. Eizirik[1]

Cytokine-induced β-cell death is an important event in the pathogenesis of type 1 diabetes. The transcription factor nuclear factor-κB (NF-κB) is activated by interleukin-1β (IL-1β), and its activity promotes the expression of several β-cell genes, including pro- and anti-apoptotic genes. To elucidate the role of cytokine (IL-1β + γ-interferon [IFN-γ])-induced expression of NF-κB in β-cell apoptosis, rat β-cells were infected with the recombinant adenovirus AdIκB$^{(SA)2}$, which contained a nondegradable mutant form of inhibitory κB (IκB$^{(SA)2}$, with S32A and S36A) that locks NF-κB in a cytosolic protein complex, preventing its nuclear action. Expression of IκB$^{(SA)2}$ inhibited cytokine-stimulated nuclear translocation and DNA-binding of NF-κB. Cytokine-induced gene expression of several NF-κB targets, namely inducible nitric oxide synthase, Fas, and manganese superoxide dismutase, was prevented by AdIκB$^{(SA)2}$, as established by reverse transcriptase–polymerase chain reaction, protein blot, and measurement of nitrite in the medium. Finally, β-cell survival after IL-1β + IFN-γ treatment was significantly improved by IκB$^{(SA)2}$ expression, mostly through inhibition of the apoptotic pathway. Based on these findings, we conclude that NF-κB activation, under in vitro conditions, has primarily a pro-apoptotic function in β-cells.
***Diabetes* 50:2219–2224, 2001**

Prolonged exposure of rodent and human β-cells to the cytokines interleukin-1β (IL-1β) and γ-interferon (IFN-γ) leads to functional suppression and eventual cell death, mostly by apoptosis (rev. in 1–3). The β-cell outcome after immune-mediated injury, i.e., apoptosis or survival with functional recovery, probably depends on the complex pattern of dozens of genes up- or downregulated in parallel and/or sequentially (4,5).

From the [1]Diabetes Research Center, Vrije Universiteit Brussel, Brussels, Belgium; and the [2]Division of Digestive Diseases and Nutrition, University of North Carolina, Chapel Hill, North Carolina.

Address correspondence and reprint requests to H. Heimberg, Diabetes Research Center, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium. E-mail: hheimber@vub.vub.ac.be.

H.H. and Y.H. contributed equally to this work.

Received for publication 6 October 2000 and accepted in revised form 13 July 2001.

ANOVA, analysis of variance; FasL, ligand protein for Fas; GAPDH, glyceraldehyde-phosphate dehydrogenase; GFP, green fluorescent protein; IFN-γ, γ-interferon IκB, inhibitory κB; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; MOI, multiplicity of infection; NO, nitric oxide; PCR, polymerase chain reaction; PI, propidium iodide.

Cytokine-induced modifications in gene expression are mediated by the activation of different transcription factors. The transcription factor nuclear factor-κB (NF-κB) seems to regulate several genes affected by cytokines in β-cells, as suggested by a recent oligonucleotide array analysis of cytokine-treated β-cells performed by our group (5) and by gel shift analysis and transient transfections with promoter-luciferase reporter constructs for inducible nitric oxide synthase (iNOS) (6–8), Fas (8a), and manganese superoxide dismutase (MnSOD) (9). Whereas iNOS and Fas contribute to β-cell death, MnSOD is probably involved in β-cell defense against the toxic oxygen free radicals generated during exposure to cytokines (4). Preponderance of the anti- or pro-apoptotic effects of NF-κB depends on both the cell type and the type of inducer tested (10). It remains to be determined which is the main role for NF-κB in the context of cytokine-induced β-cell apoptosis.

NF-κB is present in an inactive form in the cytoplasm, where it is bound to inhibitory κB (IκB) proteins (rev. in 11). After stimulation by different agents, such as cytokines, bacterial products, and viruses and their proteins (rev. in 12), IκB is phosphorylated at two NH_2-terminal Ser residues (S32 and S36) by an IκB kinase complex, leading to its degradation at the proteasome level. The released NF-κB complex can then enter the nucleus and stimulate or inhibit the transcription of genes containing NF-κB binding sites in their promoter regions (11). Mutation of the amino acids 32 and 36 of IκB prevents its degradation and consequent NF-κB activation (13). Indeed, overexpression of an IκB mutant form, AdIκB$^{(SA)2}$, with serine 32 and 36 substituted by alanine residues, specifically blocked NF-κB activation, without affecting other transcription factors (14). Moreover, infection of intestinal epithelial cells (14) and rat hepatic stellate cells (15) with a recombinant adenovirus expressing AdIκB$^{(SA)2}$ prevented the expression of several cytokine-induced genes whose transcription depends on NF-κB.

To test whether cytokine-induced activation of NF-κB in pancreatic β-cells is mostly pro- or anti-apoptotic, we infected β-cells with AdIκB$^{(SA)2}$ overexpressing the IκB "super-repressor." This approach prevented cytokine-induced nuclear translocation of NF-κB and expression of the NF-κB–dependent genes iNOS, Fas, and MnSOD. Furthermore, it prevented cytokine-induced apoptosis in β-cells. Our data indicate that NF-κB is mostly pro-apoptotic in β-cells.

RESEARCH DESIGN AND METHODS

Isolation and culture of β-cells. Islets were isolated from male Wistar rats. The rats were housed according to the Guidelines of the Belgian Regulations for Animal Care, and the experimental protocol was approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel. Islets were isolated, dissociated, and purified to single β-cells that were ~95% viable, as previously described (16). β-Cells were cultured in Ham's F10 medium supplemented with 10 mmol/l glucose, 50 mmol/l IBMX (3-isobutyl-l-methylxantine), 1% bovine serum albumin (Boehringer Mannheim, Mannheim, Germany), 0.1 mg/ml streptomycin (Continental Pharma, Puteaux, Belgium), 0.075 mg/ml penicillin (Laboratoires Diamant, Brussels, Belgium), and 2 mmol/l L-glutamine (Gibco, Paisley, U.K.) (17). For mRNA and nuclear or total cellular protein extraction, single β-cells were reaggregated for 3 h in a rotatory shaking incubator (18). For viability experiments (see below), β-cells (1×10^4 cells per well) were cultured in Falcon 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ) containing 200 μl medium. For nuclear translocation experiments, 5×10^4 β-cells were plated in 8-well poly-L-lysine–coated chamber slides. During prolonged culture, the culture medium was changed every 3 days.

Cytokine treatment. At 24 h postinfection, cytokines were added. The effects of cytokines after transfection with the different adenoviral constructs were examined after 1 h (nuclear translocation and DNA binding), 24 h (mRNA and protein abundance), 3 days (nitrite concentration), and 6 days (viability) of culture in the presence of recombinant murine IFN-γ (1,000 units/ml, 10 units/ng; Holland Biotechnology, Leiden, the Netherlands) and recombinant human IL-1β (50 units/ml, 38 units/ng; a gift from Dr. C.W. Reynolds from the National Cancer Institute, Bethesda, MD). The concentration of cytokines and the time points of sampling were selected based on our previous studies with rodent pancreatic islets and β-cells (2,8,19,20). Every 3 days, fresh cytokines were added in parallel to medium change.

Transfection with recombinant adenoviruses. After overnight incubation, part of the β-cells were either infected with control virus (AdLuc or AdGFP) or with AdIκB$^{(SA)2}$ expressing the NF-κB super-repressor. The recombinant replicative-deficient adenovirus containing a mutated nondegradable IκB (AdIκB$^{(SA)2}$) was prepared as previously described (14). The recombinant adenoviruses AdGFP and AdLuc were used as controls for transfection with AdIκB$^{(SA)2}$. β-Cells were infected at a multiplicity of infection (MOI) of 7.5 for 2 h at 37°C.

Nuclear extracts and electrophoretic mobility shift assay. DNA binding by nuclear protein from β-cells (4×10^5 β-cells) or rat insulinoma (RIN) cells (4 μg protein) and supershift analysis with anti-p65 (see below) were performed as previously described (9). The sequence of the NF-κB consensus oligonucleotide was 5′-AGCTTCAGAGGGGACTTTCCGAGA (upper strand shown).

Immunofluorescence and immunoblotting. Immunoblotting and immunofluorescence were done as previously described (21). Anti-p65 (sc-372; Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:100 dilution, anti-IκB (sc-371; Santa Cruz Biotechnology) at 1:500, anti-iNOS (BD Transduction Labs, Los Angeles) at 1:3,000, and anti-hemaglutinin (Roche Diagnostics, Mannheim, Germany) at 1:100.

mRNA isolation and reverse transcriptase–polymerase chain reaction. Poly(A)$^+$ RNA was isolated from β-cells (0.5×10^5 cells) using oligo(dT)25-coated polystyrene Dynabeads (Dynal, Oslo). The reverse transcription reaction and subsequent polymerase chain reaction (PCR) were performed as previously described (19,20). The primers used for iNOS, MnSOD, and glyceraldehyde-phosphate dehydrogenase (GAPDH) have been described previously (9,19). The primers for Fas were 5′-GAATGCAAGGGACTGATAGC (forward primer) and 5′-TGGTTCGTGTGCAAGGCTC (reverse primer). The number of cycles was selected to allow linear amplification of the cDNA under study. The ethidium bromide–stained agarose gels were photographed under ultraviolet-transillumination using a Kodak Digital Science DC40 camera (Rochester, NY). The PCR band intensities on the image were quantified by Biomax 1D image analysis software (Kodak) and expressed as pixel intensities (optical density). The target cDNAs present in each sample were corrected for their respective GAPDH values. Expression of the "housekeeping" gene GAPDH is not affected by exposure to cytokines (22).

Determination of nitrite concentration. Culture media were collected after 72 h for colorimetric nitrite determination (23). Nitrite was not determined at subsequent time points, because after 6 days, β-cells exposed to cytokines have a significant decrease in viability (see RESULTS).

Assessment of β-cell viability and function. The percentage of viable, apoptotic, and necrotic β-cells was determined after 6 days of exposure to cytokines, the required time to detect significant increases in β-cell death (24,25). For this purpose, β-cells were incubated for 15 min with propidium iodide (PI; 10 mg/ml) and Hoechst 342 (20 mg/ml) (26). The cells were examined by inverted microscopy with ultraviolet excitation at 340–380 nm.

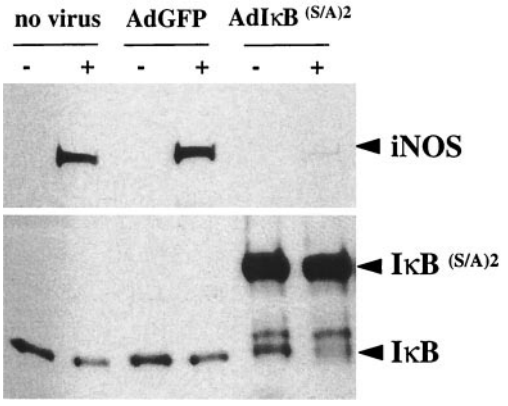


FIG. 1. Expression of IκB$^{(SA)2}$ and its effect on the expression of iNOS in rat β-cells. Protein extracts from rat β-cells uninfected or infected with recombinant adenoviruses expressing GFP (AdGFP) or IκB$^{(SA)2}$ (AdIκB$^{(SA)2}$), followed by cytokine treatment (50 and 1,000 units/ml for IL-1β and IFN-γ, respectively, for 24 h), were immunoblotted as described in RESEARCH DESIGN AND METHODS. Blots were incubated with antisera directed against iNOS (top panel) and IκB (bottom panel). The experiment shown is representative for three independent experiments. The origin of the higher–molecular weight signal detected by anti-IκB in AdIκB$^{(SA)2}$-infected cells is unknown.

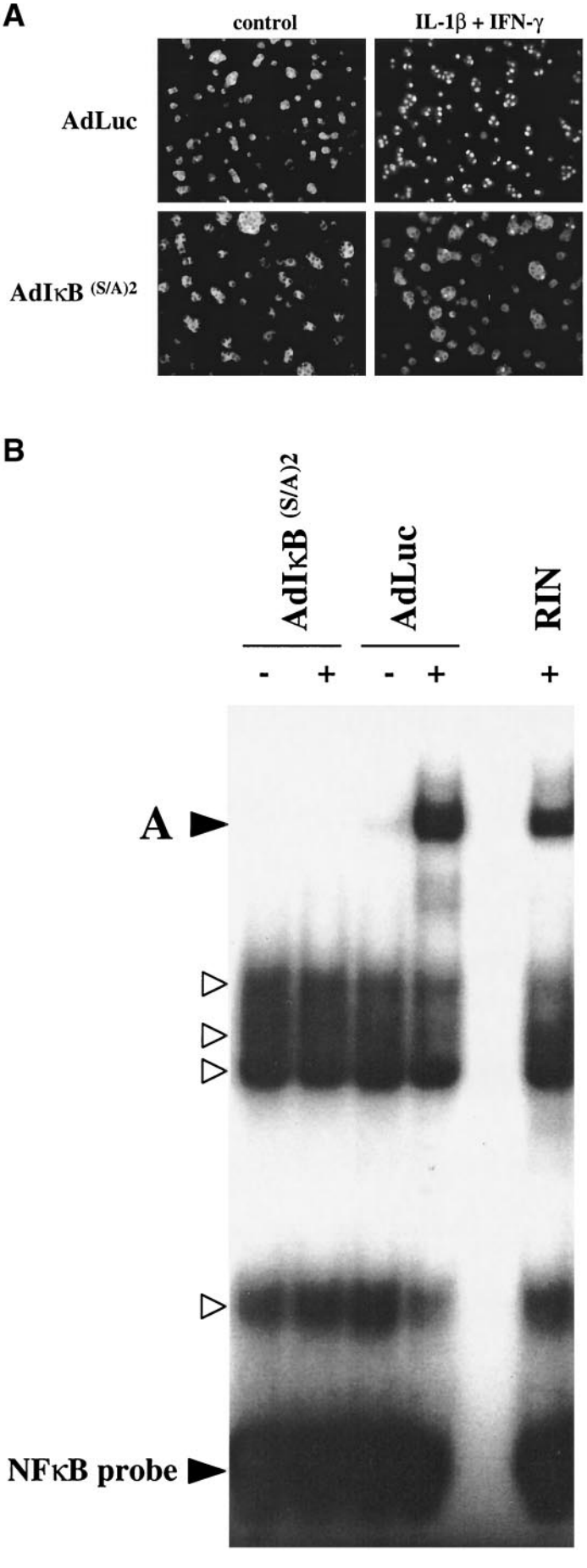
Viable cells were identified by their intact nuclei with blue fluorescence (Hoechst 342), necrotic cells by their intact nuclei with yellow-red fluorescence (Hoechst 342 + PI), and apoptotic cells by their fragmented nuclei, exhibiting either a blue (Hoechst 342; early apoptosis) or yellow-red fluorescence (Hoechst 342 + PI; late apoptosis) (26). This fluorescence assay is quantitative for single β-cells and has been validated by systematic comparisons with electron microscopy observations (26). This method has been used to evaluate apoptosis/necrosis in rat (25,26), mouse (20,27), and human (24) β-cells. The use of purified β-cells in these experiments provides a homogeneous cell population (>95% β-cells), decreasing the problem of apoptosis/necrosis detection in non–β-cells, which is inherent to studies performed in whole islets. Determination of β-cell insulin content and release was performed 48 h after infection, as previously described (18).

Statistical analysis. The results are presented as the means ± SE. Statistical differences between the groups were determined by Student's t test or, when indicated, by analysis of variance (ANOVA) followed by multiple t tests with the Bonferroni correction.

RESULTS

The effect of adenovirus-mediated IκB$^{(SA)2}$ overexpression on β-cell function and cytokine-induced NF-κB activity. In an initial series of experiments, green fluorescent protein (GFP) was used as reporter to determine the number of successfully infected cells. The day after incubation of the β-cells with the adenoviruses, at a MOI of 7.5, we observed that >75% of the β-cells expressed the target protein ($n = 5$). However, GFP was found to influence β-cell survival (see below), forcing us to add an additional control adenovirus expressing luciferase, which did not affect β-cell survival (see below). MOIs >20 caused β-cell death (data not shown).

Putative nonspecific viral effects on basal and glucose-stimulated β-cell functions were determined by incubating the virus-infected β-cells at different glucose concentrations ($n = 3$–4 independent experiments, evaluated in duplicate). At 48 h after infection (MOI 7.5), the insulin content (in nanograms per 10^3 cells) was similar in all conditions under study: noninfected β-cells, 31.7 ± 5.0; AdLuc, 26.3 ± 4.9; and AdIκB$^{(SA)2}$ 26.2 ± 5.9 ($P > 0.05$ vs. control for all comparisons). The functional activity of the β-cells was further evaluated by assessment of insulin release in response to glucose. The fraction of the total cellular insulin secreted during a 2-h incubation in the



presence of 2.5 or 20 mmol/l glucose, respectively, was: 0.8 ± 0.2 and 8.9 ± 2.0% in noninfected β-cells; 0.9 ± 0.3 and 9.2 ± 1.3% in AdLuc-infected β-cells; and 0.7 ± 0.2 and 8.5 ± 1.6% in AdIκB$^{(SA)2}$-infected β-cells ($P > 0.05$ vs. control for all comparisons). Insulin mRNA abundance was also unaffected after infection ($n = 2$; data not shown). These findings suggest that at an MOI of 7.5, neither AdLuc nor AdIκB$^{(SA)2}$ compromise the synthesis and secretion of insulin by β-cells.

The AdIκB$^{(SA)2}$-specific effects were determined 2 days after infection with AdIκB$^{(SA)2}$. The expression level of IκB$^{(SA)2}$ exceeded the endogenous IκB expression by more than fivefold, and the IκB$^{(SA)2}$ mutant protein was well preserved after the addition of a combination of cytokines (IL-1β and IFN-γ), whereas endogenous IκB was degraded (Fig. 1). Cytokine signaling downstream of IκB was severely affected by expressing IκB$^{(SA)2}$. Although the cytokines caused nuclear translocation of the p65 subunit of NF-κB, in noninfected β-cells or β-cells infected with control viruses, the subcellular localization of NF-κB remained cytosolic in AdIκB$^{(SA)2}$-infected cells (Fig. 2*A*). Moreover, gel shift analysis of control β-cells exposed to cytokines showed specific DNA binding of NF-κB, which was absent in AdIκB$^{(SA)2}$-infected β-cells (Fig. 2*B*). Specificity was established by causing supershift of the binding complex when incubated with antiserum directed against the NF-κB subunit p65 as well as by competition between labeled and cold target sequence, in 50-fold molar excess (data not shown). The observations described above indicate that AdIκB$^{(SA)2}$ successfully infects primary rat β-cells and leads to overexpression of IκB$^{(SA)2}$, which is effective in preventing NF-κB activation and nuclear binding, without affecting β-cell–specific functions.

The effect of NF-κB activity on cytokine-induced gene expression and β-cell survival. To investigate the downstream effects of inhibiting NF-κB signaling, we evaluated cytokine-induced gene expression in β-cells. Gene expression of MnSOD, Fas, and iNOS was switched on after the addition of cytokines to uninfected β-cells or to β-cells infected with the control viruses AdGFP or AdLuc (Fig. 3). On the other hand, the abundance of these mRNAs remained low in cytokine-treated β-cells infected with AdIκB$^{(SA)2}$ (Fig. 3). After cytokine exposure, the relative abundance of iNOS, Fas, and MnSOD mRNAs (optical density corrected per GAPDH abundance) was, respectively, 4-, 8-, and 10-fold lower in β-cells infected with AdIκB$^{(SA)2}$ compared with the control conditions ($n = 3$). Expression of the housekeeping gene GAPDH was not affected by exposure to cytokines or by infection with the different adenoviruses used in this study (Fig. 3). Inhi-

FIG. 2. The effect of IκB$^{(SA)2}$ expression on nuclear translocation and DNA binding of NF-κB. *A*: Rat β-cells infected with recombinant adenoviruses expressing luciferase (AdLuc), as a control condition, or IκB$^{(SA)2}$ (AdIκB$^{(SA)2}$) were treated for 1 h with cytokines (50 and 1,000 units/ml for IL-1β and IFN-γ, respectively). Fixed cells were immunostained for subcellular localization of p65, a NF-κB subunit. The experiment shown is representative for four independent experiments. *B*: Nuclear protein extracts from rat β-cells infected with AdLuc or AdIκB$^{(SA)2}$ and treated with cytokines (50 and 1,000 units/ml for IL-1β and IFN-γ, respectively) were prepared as described in RESEARCH DESIGN AND METHODS and incubated with labeled oligoprobe representing the NF-κB consensus sequence. Nuclear protein extracts from cytokine-treated RIN cells were used as a positive control. A, NF-κB–specific DNA-binding protein complex; ▷, nonspecific complexes. The experiment shown represents two independent experiments.

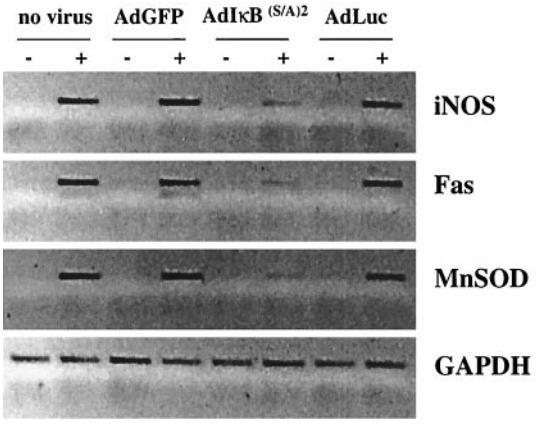


FIG. 3. The effect of IκB$^{(SA)2}$ expression on cytokine-induced transcription of NF-κB target genes. A representative agarose gel of three similar experiments, containing reverse transcriptase–PCR fragments specific for iNOS, Fas, MnSOD, or GAPDH. mRNA was isolated from uninfected β-cells and from β-cells infected with recombinant adenoviruses expressing GFP (AdGFP), IκB$^{(SA)2}$ (AdIκB$^{(SA)2}$), or luciferase (AdLuc), reverse transcribed, and amplified with specific oligonucleotide primers, as described in RESEARCH DESIGN AND METHODS. Incubation with cytokines (50 and 1,000 units/ml for IL-1β and IFN-γ, respectively) was for 24 h.

bition of cytokine-induced iNOS mRNA expression by AdIκB$^{(SA)2}$ was confirmed at the protein (Fig. 1) and reaction product level (see below). Whereas iNOS protein was absent in non–cytokine-treated β-cells, it was abundant after cytokine treatment of uninfected, AdGFP-infected (Fig. 1), or AdLuc-infected cells (data not shown). On the other hand, there was hardly any detectable iNOS protein expression in AdIκB$^{(SA)2}$-infected cytokine-treated β-cells (Fig. 1). The iNOS enzymatic activity was estimated by measurements of medium nitrite (a stable product of nitric oxide [NO] oxidation) accumulation during a 72-h exposure to cytokines (50 and 1,000 units/ml for IL-1β and IFN-γ, respectively). Basal nitrite production, in the absence of cytokines, was low (means ± SE) and similar in all experimental groups under study (65 ± 20 pmol/10^3 cells × 72 h for noninfected control β-cells). In the presence of cytokines, nitrite production was similar in noninfected β-cells (222 ± 26 pmol/10^3 cells × 72 h, $n = 4$; $P < 0.01$ vs. respective control that was not exposed to cytokines, ANOVA) and in β-cells infected with AdLuc or AdGFP (241 ± 23 and 211 ± 24 pmol/10^3 cells × 72 h, respectively) but was reduced by more than twofold in β-cells overexpressing IκB$^{(SA)2}$ (92 ± 29 pmol/10^3 cells × 72 h, $n = 4$; $P < 0.01$ vs. uninfected cells and AdLuc infected cells; $P < 0.05$ vs. AdGFP infected cells, ANOVA).

Finally, we evaluated whether cytokine-induced NF-κB expression is also required for the induction of β-cell death. β-Cell survival was not affected when cells were infected with AdLuc or AdIκB$^{(SA)2}$ (Fig. 4). Expression of GFP, however, significantly decreased β-cell viability, mainly through the induction of apoptosis (Figs. 4*A* and *B*). GFP has recently been shown to modify survival and/or function of other cell types (28), raising doubts about the utility of this protein as a "nonfunctional control" for viral transfections or as a tool to sort viable β-cells. As previously described (25), IL-1β + IFN-γ caused the death of >50% of the exposed rat β-cells (Fig. 4*A*). β-Cell death was caused by apoptosis (Fig. 4*B*) as well as by necrosis (Fig. 4*C*). In regard to overexpression of IκB$^{(SA)2}$-rescued β-cells from the cytokine-induced death pathway, the number of viable β-cells in the presence of cytokines was two- to fourfold higher in AdIκB$^{(SA)2}$-infected β-cells compared with cells from the different control groups (Fig. 4*A*). These beneficial effects of the IκB superrepressor were due to inhibition of both cytokine-induced apoptosis (Fig. 4*B*) and necrosis (Fig. 4*C*).

DISCUSSION

To investigate the role of NF-κB in cytokine-induced β-cell gene expression and apoptosis, we infected purified rat β-cells with recombinant adenoviruses expressing the nondegradable AdIκB$^{(SA)2}$, a super-repressor of NF-κB (13,14). Infection with AdIκB$^{(SA)2}$ induced an at least fivefold higher expression of IκB$^{(SA)2}$ compared with the endogenous IκB, and the protein was not degraded after

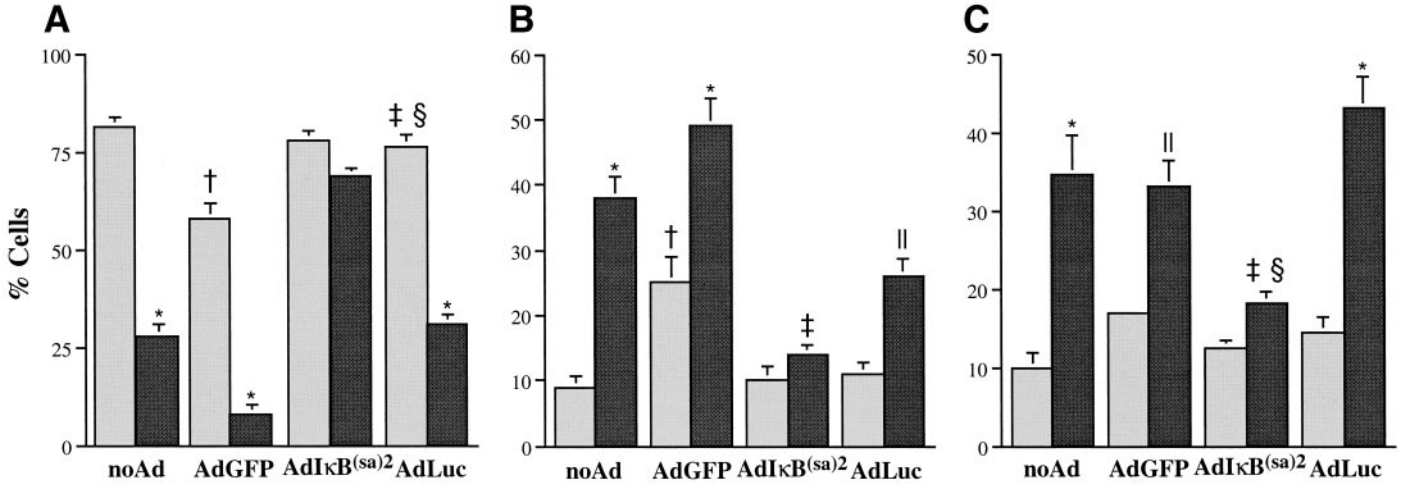


FIG. 4. The effect of IκB$^{(SA)2}$ expression on β-cell survival. Cytokine-induced overall cell death (*A*), apoptosis (*B*), and necrosis (*C*) in uninfected β-cells (NoAd) or in β-cells infected with recombinant adenoviruses expressing either GFP (AdGFP), IκB$^{(SA)2}$ (AdIκB$^{(SA)2}$), or luciferase (AdLuc). The fraction of viable, apoptotic, or necrotic cells was established as described in RESEARCH DESIGN AND METHODS. Data were expressed as percentages and statistically analyzed by ANOVA, followed by multiple *t* tests with the Bonferroni correction ($n = 5$). *$P < 0.001$ vs. respective controls, not exposed to cytokines; †$P < 0.05$ vs. noAd, not exposed to cytokines; ‡$P < 0.001$ vs. noAd exposed to cytokines; §$P < 0.001$ vs. AdLuc exposed to cytokines; ‖$P < 0.05$ vs. respective controls, not exposed to cytokines. Cytokines were 50 and 1,000 units/ml for IL-1β and IFN-γ, respectively, during 6 days. ■, Cytokines; □, no cytokines.

the addition of cytokines. This confirms the resistance of NF-κB super-repressor to cytokine-induced phosphorylation and consequent degradation (13,14).

The adenovirus-mediated expression of the IκB super-repressor prevented cytokine-induced NF-κB translocation to the β-cell nucleus, as judged by cellular immunostaining for p65 and gel shift analysis. This led to inhibition of the expression of iNOS, Fas, and MnSOD mRNAs. AdIκB$^{(SA)2}$ also prevented, to a large extent, cytokine-induced iNOS protein expression and NO release. We have previously suggested, based on studies with transient transfections with promoter-luciferase reporter constructs, that NF-κB activation is crucial for cytokine-induced iNOS (8), MnSOD (9), and Fas (8a) expression. For MnSOD, the reporter construct data were not confirmed by the use of nonspecific NF-κB chemical inhibitors (i.e., pyrrolidine dithiocarbamate [PDTC]). Thus, PDTC prevented IL-1β–induced iNOS, but not MnSOD, expression (7). The present observations, based on a specific NF-κB blocker (13, 14), validate the promoter construct studies and confirm that NF-κB activation and nuclear translocation are necessary steps for cytokine-induced iNOS, MnSOD, and Fas mRNA expression in primary β-cells.

The inhibition of Fas gene expression is probably not of primary importance for the β-cell fate in our model system, because purified β-cells do not express a functional ligand protein for Fas (FasL) (29; D.L. and D.L.E., unpublished data). However, during insulitis in animal models of autoimmune diabetes, FasL-expressing mononuclear cells invade the islets and may induce apoptosis in β-cells expressing Fas (Fas is probably upregulated in these cells by the local production of cytokines) (30–32). It is noteworthy that Fas expression (32,33) and apoptosis (33) have also been reported in human β-cells in the early stages of type 1 diabetes. In this context, the blocking of NF-κB could have a beneficial effect by preventing upregulation of Fas expression in β-cells.

Prolonged exposure of rodent pancreatic β-cells to combinations of cytokines leads to cell death by both apoptosis and necrosis (20,25,27). Whereas cytokine-induced necrosis is probably NO-mediated, apoptosis seems mostly NO-independent in mouse and human β-cells (20,24). We have recently suggested that the "non-NO" signal for β-cell apoptosis is mediated by an intricate pattern of up- and downregulation of the expression of dozens of genes (4,5). As suggested by microarray analysis and promoter studies, several of these genes are regulated by NF-κB (5). We observed that AdIκB$^{(SA)2}$ prevents both the necrotic and apoptotic components of cytokine-induced β-cell death. As discussed above, the inhibition of necrosis is probably related to a AdIκB$^{(SA)2}$-induced decrease in iNOS expression and NO production. On the other hand, the observation that the IκB super-repressor also prevents β-cell apoptosis indicates that at least part of the pro-apoptotic gene expression pattern in these cells is regulated by NF-κB. To further investigate this issue, we intend to perform additional microarray analysis of gene expression in β-cells exposed to cytokines with or without previous infection with AdIκB or AdLuc.

After the submission of this article, another study was published indicating that an IκB repressor protects human islets against IL-1β–induced NO production, inhibition of insulin release (apparently mediated by NO), and Fas-triggered apoptosis (34). These results are apparently similar to the present observations. There are, however, some methodological differences between these two studies. We discriminated among living, apoptotic, and necrotic cells on the basis of their nuclear features and provided an absolute number of apoptotic/necrotic cells, whereas Giannoukakis et al. (34) evaluated cell death by measuring whole-islet caspase 3 activity. In addition, our experiments were performed on purified rat β-cells; thus, we could identify the nature of the cells undergoing apoptosis. Because Giannoukakis et al. (34) analyzed cell death in whole human islet preparations, it is unclear whether the cells apparently undergoing apoptosis in their experiments are indeed β-cells.

The present data indicate that the transcription factor NF-κB has a mostly pro-apoptotic role in β-cells exposed to cytokines. This in vitro data provide a rationale for targeting NF-κB by genetic intervention in attempts to prevent β-cell death in the early stages of insulitis and after islet transplantation. However, it needs to be remarked that the anti- and pro-apoptotic roles of NF-κB in different tissues may vary depending on the type of assault inflicted on the target cells (10). Furthermore, there may be differences between in vitro (as in the present study) and in vivo conditions. Thus, additional in vivo experiments are required to evaluate whether blocking β-cell NF-κB activation will indeed protect these cells against the complex immune response leading to β-cell death in islets transplanted in animal models of type 1 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Foundation International, Fund for Scientific Research Flanders (FWO), and by a Shared Cost Action in Medical and Health Research of the European Community. H.H. was supported by a postdoctoral research fellowship (Fund for Scientific Research, Flanders, Belgium) and a Career Development Award (Juvenile Diabetes Foundation International). The technical assistance from the Diabetes Research Center personnel involved in β-cell purification is gratefully acknowledged.

REFERENCES

1. Mandrup-Poulsen T: The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39:1005–1029, 1996
2. Eizirik DL, Pavlovic D: Is there a role for nitric oxide in beta-cell dysfunction and damage in IDDM? *Diabete Metab Rev* 13:293–307, 1997
3. Rabinovitch A: An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus. *Diabete Metab Rev* 14:129–151, 1998
4. Eizirik DL, Darville MI: β-Cell apoptosis and defense mechanisms: lessons from type 1 diabetes. *Diabetes* 50 (Suppl. 1):S64–S69, 2001
5. Cardozo AK, Kruhøffer M, Leeman R, Ørtonft T, Eizirik DL: Identification of novel cytokine-induced genes in pancreatic β-cells by high-density oligonucleotide arrays. *Diabetes* 50:909–920, 2001
6. Saldeen J, Welsh N: Interleukin-1 β induced activation of NF-κB in insulin producing RINm5F cells is prevented by the protease inhibitor N alpha-p-tosyl-L-lysine chloromethylketone. *Biochem Biophys Res Commun* 203: 149–155, 1994
7. Bedoya FJ, Flodstrom M, Eizirik DL: Pyrrolidine dithiocarbamate prevents IL-1-induced nitric oxide synthase mRNA, but not superoxide dismutase mRNA, in insulin producing cells. *Biochem Biophys Res Commun* 210: 816–822, 1995
8. Darville MI, Eizirik DL: Regulation by cytokines of the inducible nitric

oxide synthase promoter in insulin-producing cells. *Diabetologia* 41:1101–1108, 1998
8a. Darville MI, Eizirik DL: Cytokine induction of Fas gene expression in insulin-producing cells requires the transcription factors NK-κB and C/EBP. *Diabetes* 50:1741–1748, 2001
9. Darville MI, Ho YS, Eizirik DL: NF-κB is required for cytokine-induced manganese superoxide dismutase expression in insulin-producing cells. *Endocrinology* 141:153–162, 2000
10. Barkett M, Gilmore TD: Control of apoptosis by Rel/NF-κB transcription factors. *Oncogene* 18:6910–6924, 1999
11. Karin M: How NF-κB is activated: the role of the IκB kinase (IKK) complex. *Oncogene* 18:6867–6874, 1999
12. Pahl HL: Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* 18:6853–6866, 1999
13. Brown K, Gerstberger S, Carlson L, Franzoso G, Siebenlist U: Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. *Science* 267:1485–14888, 1995
14. Jobin C, Panja A, Hellerbrand C, Iimuro Y, Didonato J, Brenner DA, Sartor, RB: Inhibition of proinflammatory molecule production by adenovirus-mediated expression of a nuclear factor κB super-repressor in human intestinal epithelial cells. *J Immunol* 160:41041–41048, 1998
15. Hellerbrand C, Jobin C, Iimuro Y, Licato L, Sartor RB, Brenner DA: Inhibition of NF-κB in activated rat hepatic stellate cells by proteasome inhibitors and an IκB super-repressor. *Hepatology* 27:1285–1295, 1998
16. Pipeleers DG, in't Veld PA, Van de Winkel M, Maes E, Schuit FC, Gepts W: A new in vitro model for the study of pancreatic A and B cells. *Endocrinology* 117:806–816, 1985
17. Ling Z, Hannaert JC, Pipeleers D: Effect of nutrients, hormones and serum on survival of rat islet beta cells in culture. *Diabetologia* 37:15–21, 1994
18. Ling Z, Chen M-C, Smismans A, Pavlovic D, Schuit F, Eizirik DL, Pipeleers DG: Intercellular differences in interleukin-1β-induced suppression of insulin synthesis and stimulation of noninsulin protein synthesis by rat pancreatic β-cells. *Endocrinology* 139:1540–1545, 1998
19. Chen MC, Schuit F, Eizirik DL: Identification of IL-1β-induced messenger RNAs in rat pancreatic beta cells by differential display of messenger RNA. *Diabetologia* 42:1199–1203, 1999
20. Liu D, Pavlovic D, Chen MC, Flodstrom M, Sandler S, Eizirik DL: Cytokines induce apoptosis in β-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS$^{-/-}$). *Diabetes* 49:1116–1122, 2000
21. Heimberg H, Bouwens L, Heremans Y, Van De Casteele M, Lefebvre V, Pipeleers D: Adult human pancreatic duct and islet cells exhibit similarities in expression and differences in phosphorylation and complex formation of the homeodomain protein Ipf-1. *Diabetes* 49:571–579, 2000
22. Eizirik DL, Björklund A, Cagliero E: Genotoxic agents increase expression of growth arrest and DNA damage–inducible genes gadd 153 and gadd 45 in rat pancreatic islets. *Diabetes* 42:738–745, 1993
23. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126:131–138, 1982
24. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL: Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138:2610–2614, 1997
25. Pavlovic D, Andersen NA, Mandrup-Poulsen T, Eizirik DL: Activation of extracellular signal-regulated kinase (ERK)1/2 contributes to cytokine-induced apoptosis in purified rat pancreatic β-cells. *Eur Cytokine Netw* 11:267–274, 2000
26. Hoorens A, Van de Casteele M, Kloppel G, Pipeleers D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568–1574, 1996
27. Pavlovic D, Chen MC, Gysemans CA, Mathieu C, Eizirik DL: The role of interferon regulatory factor-1 in cytokine-induced mRNA expression and cell death in murine pancreatic β-cells. *Eur Cytokine Netw* 10:403–412, 1999
28. Liu HS, Jan MS, Chou CK, Phen PH, Ke I: Is green fluorescence protein toxic to the living cells? *Biochem Biophys Res Commun* 260:712–717, 1999
29. Ingelsson E, Saldeen J, Welsh N: Islet expression of perforin, Fas/Apo1 and interleukin-1 converting enzyme (ICE) in non-obese diabetic (NOD) mice. *Immunol Lett* 63:125–129, 1998
30. Suarez-Pinzon W, Sorenson O, Bleackey RS, Elliot JF, Rajotte RV, Rabinovitch A: β-Cell destruction in NOD mice correlates with Fas (CD95) expression on β-cells and proinflammatory cytokine expression in islets. *Diabetes* 48:21–28, 1999
31. Amrani A, Verdaguer J, Thiessen S, Bou S, Santamaria P: IL-1a, IL-1β, and IFN-γ mark β cells for Fas-dependent destruction by diabetogenic CD4+ T lymphocytes. *J Clin Invest* 105:459–468, 2000
32. Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, Iwahashi H, Nakajima H, Namba M, Nagata S, Hanafusa T, Matsuzawa Y: Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset type I diabetes mellitus. *Diabetologia* 42:1332–1340, 1999
33. Stassi G, De Mari R, Trucco G, Rudert W, Testi R, Galluzzo A, Giordano C, Trucco M: Nitric oxide primes pancreatic β-cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J Exp Med* 186:1193–1200, 1997
34. Giannoukakis N, Rudert WA, Trucco M, Robbins PD: Protection of human islets from the effects of interleukin-1β by adenoviral gene transfer of an IκB repressor. *J Biol Chem* 275:36509–36513, 2000