Nuclear Factor κB Protects Pancreatic β-Cells From Tumor Necrosis Factor-α–Mediated Apoptosis

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Recent studies incriminating tumor necrosis factor (TNF)-α as the final effector in pancreatic β-cell death in type 1 diabetes underscore the potential role of TNF-α–dependent NF-κB activation as an important modulator of pancreatic β-cell death in autoimmune diabetes. Although nuclear factor (NF)-κB activation has been implicated in the protection of target cells against apoptosis by a variety of death effectors, its role in pancreatic islet cell death is not clear. We studied the role of NF-κB activation in pancreatic islet cell death by using a γ-interferon (IFN-γ)/TNF-α synergism model we had previously reported. TNF-α induced inhibitor of κB (IκB) degradation and p65 translocation from cytoplasm to nuclei in MIN6N8 insulinoma cells. The NF-κB DNA-binding nuclear complex activated by TNF-α contained both the p65 and p50 subunit. IFN-γ pretreatment did not affect TNF-α–induced NF-κB activation. Treatment with a proteasome inhibitor blocked p65 translocation and induced susceptibility to TNF-α in otherwise resistant insulinoma cells or primary pancreatic islet cells. Specific inhibition of NF-κB activation by adenoviral transduction of IκB "superrepressor" also sensitized insulinoma cells and primary islet β-cells to TNF-α–induced apoptosis. These results suggest the protective role of NF-κB activation against cytokine-mediated pancreatic β-cell death, contrary to previous reports implicating NF-κB as a mediator of pancreatic islet cell death. *Diabetes* 52:1169–1175, 2003

Although apoptosis of pancreatic β-cells is a critical step in the development of type 1 diabetes (1,2), it has not yet been clearly elucidated which molecules are the real effectors of pancreatic β-cell death. We have recently published in vitro and in vivo data suggesting that γ-interferon (IFN-γ) and tumor necrosis factor (TNF)-α synergism is responsible for apoptosis of pancreatic β-cells (3). IFN-γ seems to sensitize otherwise resistant pancreatic islet cells to TNF-α–mediated apoptosis, and TNF-α is thought to exert the final apoptosis on pancreatic islet cells. The role of TNF-α as the final death effector molecule is consistent with other studies that use genetic ablation models (4,5). However, other data showing the opposite effect of TNF-α in autoimmune diabetes have been published, reflecting the complexity of the pathogenesis and probably different role of cytokines in the different stages of the disease progression (6,7).

Although TNF-α is one of the most important death effector molecules, most primary or immortalized cells are not susceptible to apoptosis by TNF-α alone because of the concomitant activation of the antiapoptotic process by TNF-α (8–10). Many studies have implicated nuclear factor (NF)-κB as an important player in the protection of target cells against TNF-α–induced apoptosis (11–13). However, other studies have reported increased cell death by NF-κB activation using neuronal cells, pancreatic islet cells/insulinoma cells, or others (14–20), reflecting a complex interplay of cytokines and transcriptional factors that could be different according to the cell types or modes of cell death.

This investigation was carried out to determine the role of NF-κB activation in cytokine-induced pancreatic β-cell death. We found evidence supporting the role of NF-κB in the protection of pancreatic β-cells against TNF-α–induced apoptosis.

RESEARCH DESIGN AND METHODS

Cell line and reagents. MIN6N8 cells, SV40 T-transformed insulinoma cells derived from nonobese diabetic (NOD) mice (provided by Prof. Jun-ichi Miyagaki, Osaka University, Osaka, Japan) (21), were grown in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, 2 mmol/l glutamine, and penicillin-streptomycin (Life Technologies, Gaithersburg, MD). Recombinant rat IFN-γ that is active on murine cells was provided by Dr. van der Meide (TNO Primate Center, the Netherlands). For some experiments, murine IFN-γ (R&D Systems, Minneapolis, MN) was used to confirm the results obtained using rat IFN-γ. Recombinant mouse TNF-α and interleukin (IL)-1β were also purchased from R&D Systems. Caspase inhibitors (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone [z-VAD-fmk]) were purchased from Enzyme Systems Products (Livermore, CA). Carbobenzoxyl-leucinyl-leucinyl-leucinal-H (MG132) and Hoechst 33342 dye were from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise.

Isolation of mouse pancreatic islets. Islets were isolated from overnight-fasted ICR mice by the collagenase digestion technique as previously described (3). In brief, after 2.5 ml collagenase P (0.8 mg/ml) was injected into the bile duct of anesthetized mice, swollen pancreas was gently pulled out and other attached tissues were removed. The pancreas was then digested in collagenase P solution at 37°C for 15 min with gentle shaking. After stopping the digestion with cold Hanks' balanced salt solution, tissue was passed through a 400-μm screen and centrifuged on 25, 23, 21.5, and 11.5% Ficoll gradients. Islets collected from the interface were washed with M199 media, and individual islets were handpicked using micropipettes. The islets were

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Ab, antibody; IFN-γ, γ-interferon; IκB, inhibitor of κB; IL, interleukin; MG132, carbobenzoxyl-leucinyl-leucinyl-leucinal-H; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylte-trazolium bromide; NF, nuclear factor; NMMA, *N*-monomethyl L-arginine; SR, superrepressor; TNF, tumor necrosis factor; z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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treated with trypsin-EDTA for 5 min to yield single islet cells before treatment with TNF-α. All animal experiments in this work were done in accordance with the institutional guidelines of Samsung Medical Center.

Western blot analysis. Cells were lysed in triple-detergent lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 0.02% sodium azide, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholoate, and 1 mmol/l phenylmethylsulfonyl fluoride) as described (22). Protein concentration in cell lysate was determined using a protein assay kit (Bio-Rad, Hercules, CA). An equal amount of protein for each sample was separated by 10 or 12% SDS-PAGE and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham, Piscataway, NJ). After blocking with 5% skim milk, the membranes were sequentially incubated with anti-IκBα antibody (Ab) (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated anti-rabbit IgG (Vector, Burlingame, CA), followed by enhanced chemiluminescence detection (Amersham). In experiments exploring the cleavage of caspases, CM1 Ab (provided by Idun Pharmaceuticals, La Jolla, CA) was used as the primary Ab.

Immunofluorescence staining. MIN6N8 cells or primary islet cells seeded on chamber slides (Lab-Tek; Nalge Nunc International, Naperville, IL) were fixed in 4% paraformaldehyde at room temperature for 30 min and then in cold methanol at −20°C for 10 min. After three washes with PBS, nonspecific binding was blocked with 2% horse serum/0.5% Triton X-100/0.02% NaN_3 in PBS for 20 min. Fixed cells were then sequentially incubated with anti-p65 Ab (Santa Cruz), biotinylated anti-mouse IgG, and streptavidin fluorescein isothiocyanate (Vector). Stained cells were examined on a fluorescent microscope. In some experiments, anti-FLAG (Stratagene, La Jolla, CA) or anti-insulin Ab (Dako, Glostrup, Denmark) was used as the primary Ab.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from MIN6N8 cells treated with cytokines as previously described (23). Synthetic double-stranded oligonucleotides of the consensus NF-κB binding sequence GAT CCC AAC GGC AGG GGA (Promega, Madison, WI) were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear extract was incubated with the labeled probe in the presence of poly(dI-dC) in a binding buffer containing 20 mmol/l HEPES at room temperature for 30 min. For supershift assays, a total of 0.2 μg Ab against the p65 or p50 subunit of NF-κB (Santa Cruz) was included in the reaction. DNA-protein complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel and visualized by autoradiography.

MTT assay. Cells were seeded on 96-well microtiter plates (3×10^4/well for MIN6N8 cells, 2×10^4/well for single islet cells) and treated with cytokines for indicated time periods. In some experiments, cells were pretreated with caspase inhibitors or MG132 for 15–30 min before cytokine treatment. After cytokine treatment, medium was removed and 12.2 mmol/l 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added, followed by incubation in a CO_2 incubator at 37°C for 2 h. After a brief centrifugation, supernatant was carefully removed, and DMSO was added. After insoluble crystals were completely dissolved, absorbance at 540 nm was read using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). In some experiments, a trypan blue exclusion test or Hoechst staining was used instead of MTT assays as a measure of cell death. A trypan blue exclusion test was performed by incubating cells in 0.02% trypan blue solution (Life Technologies) for 5 min before observation on a light microscope. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with 4.1 μmol/l DNA-binding bisbenzimide Hoechst 33342 fluorochrome, followed by an examination on a fluorescence microscope. *N*-monomethyl L-arginine (NMMA) was added to the culture in experiments to study the role of nitric oxide (NO) in insulinoma cell death. NO production was measured using the Griess reaction. In brief, 50 μl of sample aliquots were mixed with 50 μl Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2% phosphoric acid) in 96-well plates and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard for calculation.

Adenoviral transduction of IκBα superrepressor. Adenovirus compromising human IκBα-superrepressor (SR), whose serines 32 and 36 were replaced with alanines (Ad5IκBα-SR) and control adenovirus (Ad5LacZ), was provided by Dr. Choon-Taek Lee (Seoul National University Hospital, Seoul, Korea) (24). Adenovirus was propagated in HEK 293 cells and concentrated by standard CsCl ultracentrifugation. MIN6N8 cells or primary islet cells seeded on 96-well plates were infected with Ad5IκBα-SR at a multiplicity of infection of 50 in a serum-free medium for 1 h. After incubation for 24 h, the cells were treated with TNF-α before MTT assays.

Statistical analysis. The Student's *t* test was used to compare the differences in cell viability and death between the groups. *P* values <0.05 were regarded statistically significant.

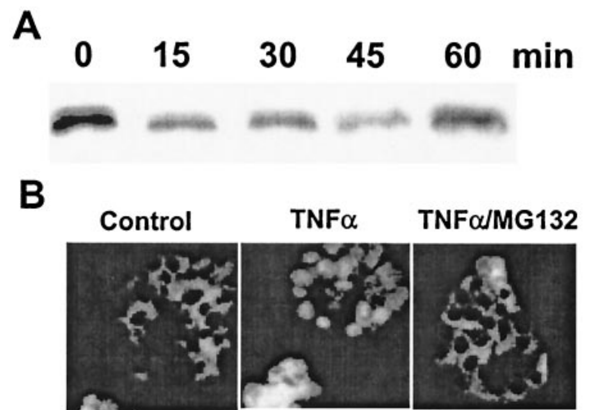

FIG. 1. ***A*: Degradation of IκBα by TNF-α. Western blot analysis showed that treatment of MIN6N8 insulinoma cells with 10 ng/ml TNF-α induced the degradation of IκBα, which reached the nadir at 45 min after TNF-α treatment. IκBα band intensity increased after 60 min. *B*: Nuclear translocation of p65 by TNF-α. Immunocytochemical staining demonstrated that treatment of MIN6N8 cells with 10 ng/ml TNF-α for 30 min induced the translocation of p65 subunit of NF-κB complex from cytosol to nuclei. Pretreatment with 1 μmol/l MG132 that inhibits proteasomal degradation of IκBα inhibited nuclear translocation of p65 by TNF-α.**

RESULTS

NF-κB activation by TNF-α in insulinoma cells. We first studied if TNF-α could activate NF-κB in MIN6N8 insulinoma cells by accelerating the degradation of inhibitor of κB (IκB)α. Western blot analysis using an anti-IκBα Ab demonstrated that IκBα expression began to decrease 15 min after treatment with 10 ng/ml TNF-α and reached the nadir 45 min after TNF-α treatment, suggesting the time-dependent degradation of IκBα in MIN6N8 cells by TNF-α (Fig. 1*A*). We next studied the intracellular movement of NF-κB after degradation of IκBα. Immunocytochemical staining showed that the p65 subunit of the NF-κB complex was in the cytoplasm of MIN6N8 cells before TNF-α treatment; however, it was translocated to the nuclei 30 min after treatment with 10 ng/ml TNF-α (Fig. 1*B*). Electrophoretic mobility shift assay also showed that the NF-κB DNA-binding complex was activated in the nuclei of MIN6N8 cells 45 min after TNF-α treatment (Fig. 2). Supershift assays demonstrated further shift of NF-κB nuclear complex by anti-p65 and anti-p50 Ab, suggesting that NF-κB DNA-binding complex activated in MIN6N8 cells comprises both the p65 Rel A subunit and p50 subunit (Fig. 2). Because we have previously observed that IFN-γ synergizes with TNF-α in the apoptosis of cancer cells by decreasing TNF-α–induced NF-κB activation (23), we next asked if IFN-γ could affect TNF-α–induced NF-κB activation in MIN6N8 insulinoma cells. However, pretreatment with 20 ng/ml IFN-γ for 24 h did not affect NF-κB activation in MIN6N8 cells, indicating that the synergism between IFN-γ and TNF-α in insulinoma cell death is not directly related to the modulation of NF-κB activation (Fig. 2). Despite the absence of an IFN-γ effect on TNF-α–induced NF-κB activation, MTT assays showed that treatment of MIN6N8 cells with 20 ng/ml IFN-γ and 10 ng/ml TNF-α for 72 h induced a significant death of MIN6N8 cells (43.7 ± 6.4% viability compared with control cells without treatment) as reported (3). Treatment with 20 ng/ml IFN-γ alone or 10 ng/ml TNF-α alone for 72 h did

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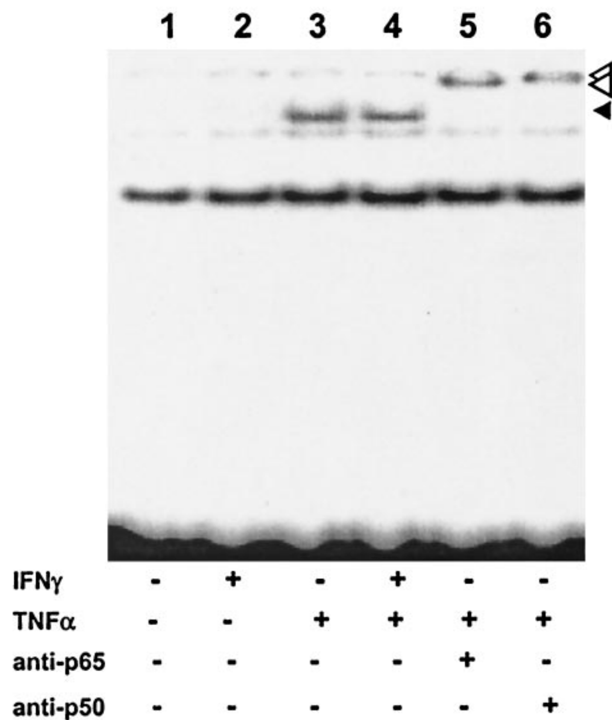

FIG. 2. Activation of NF-κB binding nuclear complex by TNF-α. Electrophoretic mobility shift assay showed that treatment of MIN6N8 cells with 10 ng/ml TNF-α for 45 min activated NF-κB DNA binding nuclear complex (black arrowhead) (*lane 3*). NF-κB complex comprised the p65 and p50 subunit because it was "supershifted" by anti-p65 or -p50 Ab (white arrowheads) (*lanes 5* and *6*). Pretreatment with 20 ng/ml IFN-γ did not affect NF-κB activation by TNF-α (*lane 4*).

not induce a significant death (87.0 ± 8.7 and 94.3 ± 6.0%, respectively).

Insulinoma cell death by TNF-α in combination with a proteasome inhibitor. We next studied whether the modulation of NF-κB activation could affect MIN6N8 cell death. First, the effect of MG132, a proteasome inhibitor that prevents IκB degradation, was examined. Pretreatment with 1 μmol/l MG132 for 15 min almost completely inhibited the nuclear translocation of p65 after TNF-α treatment, indicating the successful inhibition of NF-κB activation by a proteasome inhibitor (Fig. 1*B*). As previously reported, treatment with TNF-α alone did not induce death of MIN6N8 cells after incubation for 24 h. However, MTT assay showed that the same treatment exerted a strong cytotoxic activity on MIN6N8 cells after pretreatment with 1 μmol/l MG132 for 15 min, whereas MG132 itself had only a minor effect on MIN6N8 cell survival (Fig. 3*A*). Viability of MIN6N8 cells treated with TNF-α/MG132 was significantly lower than that without treatment or that treated with either TNF-α or MG132 alone ($P < 0.0001$ for all three comparisons). Trypan blue exclusion assay also demonstrated that >75% of MIN6N8 cells treated with TNF-α/MG132 for 24 h were dead, whereas <10% of the cells treated with either TNF-α or MG132 were dead (Fig. 3*B*). Death of MIN6N8 cells treated with TNF-α/MG132 was significantly higher than that without treatment or that treated with either TNF-α or MG132 alone ($P < 0.0001$ for all three comparisons). Cell death by TNF-α in combination with MG132 was a typical apoptosis characterized by nuclear condensation and internucleosomal DNA fragmentation (data not shown). Furthermore, 50 μmol/l z-VAD-fmk as a pan-caspase inhibitor significantly decreased MIN6N8 cell death by TNF-α/MG132, indicating that TNF-α/MG132-induced insulinoma cell death is a classic caspase-dependent apoptosis (Fig. 3*A*). Western blot analysis using CM1 Ab, which recognizes active caspase-3 but not the proform, also demonstrated that casapase-3 was cleaved in insulinoma cells after treatment with TNF-α/MG132 (Fig. 3*C*).

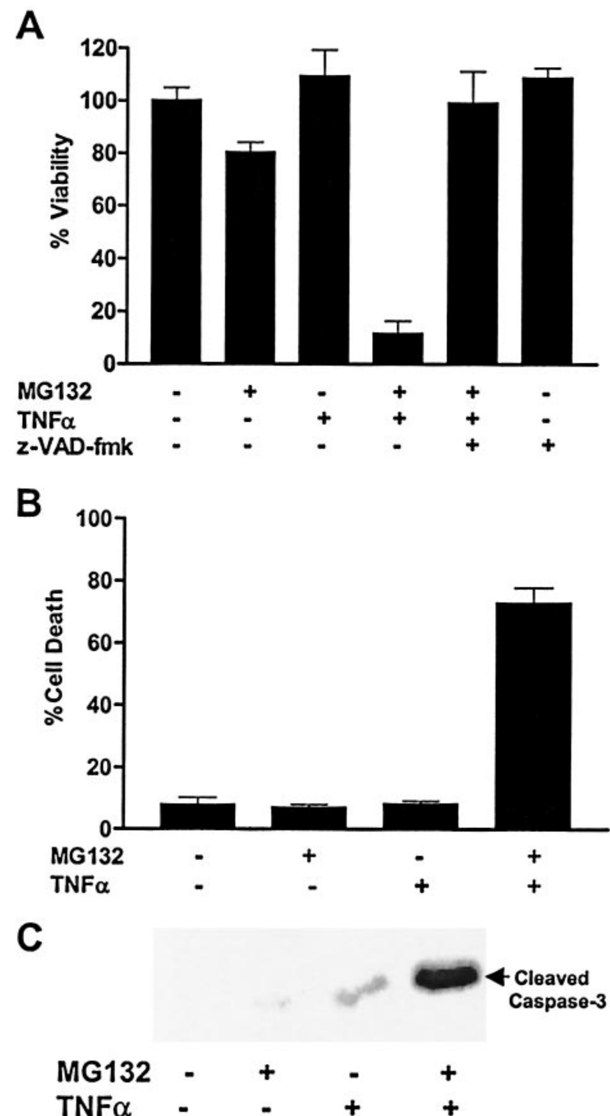

FIG. 3. *A*: MTT assay showing the induction of TNF-α susceptibility by MG132. MIN6N8 cells were resistant to the treatment with 10 ng/ml TNF-α alone. However, pretreatment with 1 μmol/l MG132 rendered them susceptible to TNF-α, which was inhibited by 50 μmol/l z-VAD-fmk, a pan-caspase inhibitor. MG132 alone induced insulinoma cell death to a small degree. Results are representative of four independent experiments performed in triplicate, showing similar tendency. *B*: Trypan blue exclusion test. MIN6N8 cells were dead after treatment with TNF-α/MG132 but not with either reagent alone. Results are representative of two independent experiments performed in triplicate, showing similar tendency. *C*: Cleavage of caspase-3. Western blot using CM1 Ab showed that caspase-3 was cleaved by TNF-α/MG132 but not by either reagent alone.

We also studied if MG132 could modulate the effect of cytokines other than TNF-α on insulinoma cell viability. Treatment with 100 ng/ml IL-1β, which could induce NF-κB activation, 20 ng/ml murine IFN-γ, or their combi-

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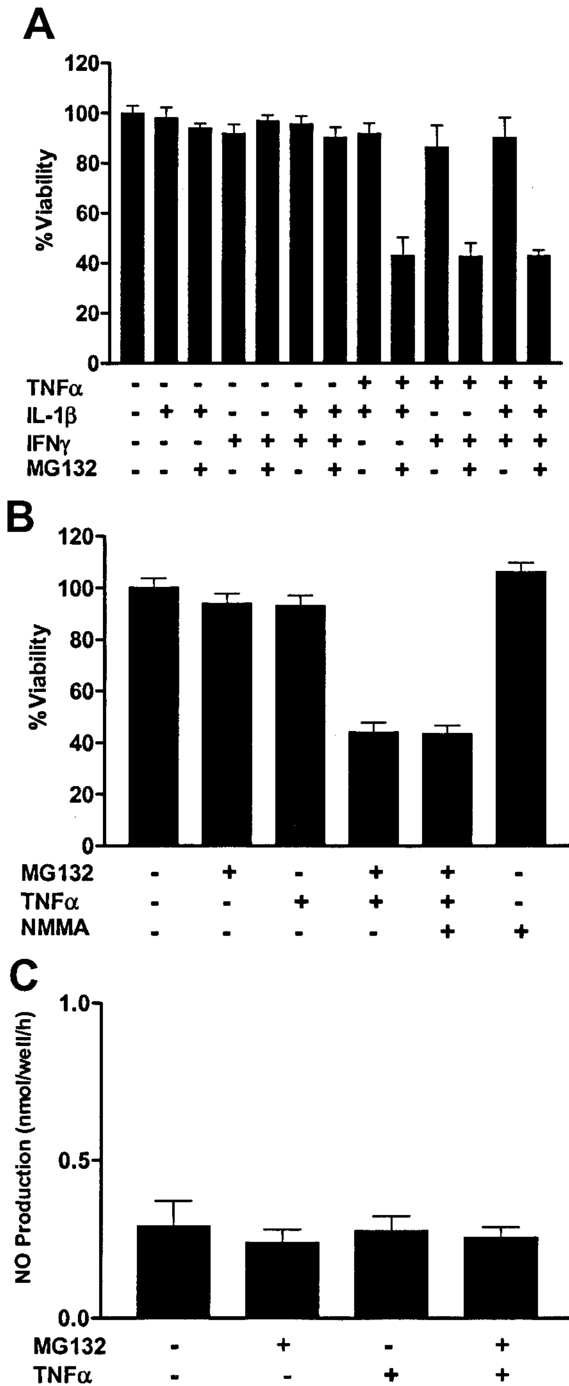

FIG. 4. ***A*: MG132 did not render insulinoma cells sensitive to treatment with various cytokine combinations without TNF-α for 24 h. However, MG132 pretreatment significantly enhanced MIN6N8 cell death by IL-1β/TNF-α, IFN-γ/TNF-α, or IL-1β/IFN-γ/TNF-α treatment for 24 h. Treatment with IFN-γ/TNF-α or IL-1β/IFN-γ/TNF-α for 24 h had a negligible effect on MIN6N8 cell viability in contrast to the same treatment for 72 h. Results are representative of four independent experiments performed in triplicate, showing similar tendency. *B* and *C*: No role of NO in insulinoma cell death by TNF-α/MG132. NMMA did not affect MIN6N8 cell death by TNF-α in combination with MG132 pretreatment (*B*). NO production was not affected by TNF-α/MG132 (*C*). Results are representative of four independent experiments performed in triplicate, showing similar tendency.**

nation after 1 μmol/l MG132 pretreatment, did not affect MIN6N8 cell viability, suggesting that the effect of MG132 is specific for TNF-α–induced cell death (Fig. 4*A*). Viability of MIN6N8 cells treated with IL-1β/MG132, IFN-γ/MG132, or IL-1β/IFN-γ/MG132 was not significantly different from that without treatment ($P > 0.1$ for all three comparisons). However, MG132 pretreatment significantly enhanced MIN6N8 cell death by IL-1β/TNF-α, IFN-γ/TNF-α, or IL-1β/IFN-γ/TNF-α treatment for 24 h ($P < 0.001$ for all three comparisons), suggesting that a TNF-α–specific antiapoptotic effect of NF-κB persists despite the presence of other cytokines (Fig. 4*A*). Although treatment of MIN6N8 cells with IFN-γ/TNF-α or IL-1β/IFN-γ/TNF-α for 72 h induced significant death, the same treatment for 24 h did not induce significant death of insulinoma cells (Fig. 4*A*; data not shown). Next we studied if NO production is responsible for insulinoma cell death by TNF-α/MG132 because inducible NO synthase could be a prominent target of cytokine(s) in islet cell death. NMMA (10 mmol/l), an inhibitor of NO synthase, did not affect insulinoma cell death by TNF-α/MG132, indicating that NO does not play a significant role in the insulinoma cell death in this condition (Fig. 4*B*). Viability of MIN6N8 cells treated with TNF-α/MG132/NMMA was not significantly different from that of cells treated with TNF-α/MG132 ($P > 0.1$). NO production from insulinoma cells treated with TNF-α/MG132 was also not different from the basal level or that after treatment with either TNF-α or MG132 ($P > 0.1$ for all three comparisons) (Fig. 4*C*).

Effect of IκBα-SR on insulinoma cell death. Because MG132 could have effects other than the inhibition of proteasomal degradation, we next studied if specific inhibition of NF-κB activation by adenoviral transduction of IκB-SR that is resistant to proteasomal degradation could sensitize otherwise resistant MIN6N8 cells to TNF-α–induced apoptosis. Western blot analysis demonstrated that the infection of MIN6N8 cells with Ad5IκBα-SR at a multiplicity of infection of 50 induced a strong expression of IκBα, which was not decreased by TNF-α treatment (Fig. 5*A*). Immunocytochemistry using anti-FLAG Ab showed that >90% of insulinoma cells were expressing FLAG tagged to the IκBα-SR construct and infected with Ad5IκBα-SR (data not shown). Treatment with 10 ng/ml TNF-α for 48 h after Ad5IκBα-SR infection induced death of MIN6N8 cells (Fig. 5*B*), strongly indicating that NF-κB activation by TNF-α plays a protective role against insulinoma cell death, and the inhibition of NF-κB activation facilitates insulinoma cell death. Viability of MIN6N8 cells treated with TNF-α in combination with an Ad5IκBα-SR infection was significantly lower than that after an Ad5IκBα-SR infection alone or TNF-α after a control Ad5LacZ infection that did not have a significant effect on MIN6N8 cell viability ($P < 0.005$ and $P < 0.001$, respectively). Pretreatment with z-VAD-fmk significantly decreased death of insulinoma cells by TNF-α in combination with Ad5IκBα-SR infection, indicating that the death of MIN6N8 cells by TNF-α in combination with Ad5IκBα-SR infection is a caspase-dependent apoptosis ($P < 0.005$) (Fig. 5*C*).

Primary islet cell death by TNF-α. Next, primary islet cells were used to examine the role of NF-κB activation in TNF-α–mediated apoptosis because primary islet cells and insulinoma cells could differ in their susceptibility to TNF-α or NF-κB activation. Treatment with 10 ng/ml TNF-α alone for 48 h induced primary islet cell death of only a marginal degree as reported (3) (Fig. 6*A*). However, TNF-α in combination with MG132 pretreatment for 15 min had a

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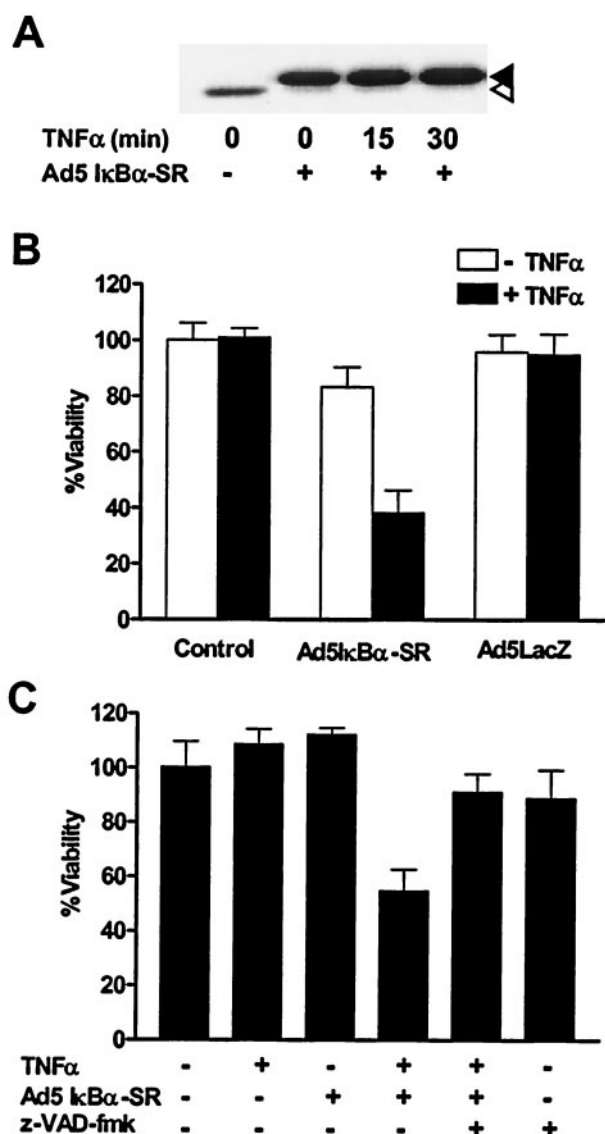

FIG. 5. Induction of TNF-α susceptibility by adenoviral transduction of IκBα-SR. *A*: Western blot analysis showed that IκBα was expressed after infection with adenovirus compromising IκBα-SR, which was not degraded by 10 ng/ml TNF-α treatment in contrast to endogenous IκBα (Fig. 1*A*) (black arrowhead, IκBα-SR; white arrowhead, endogenous IκBα). *B*: MTT assay showed that adenoviral transduction of IκBα-SR induced susceptibility to TNF-α–mediated apoptosis. Ad5κBα-SR infection itself or TNF-α treatment after control Ad5LacZ infection did not significantly affect insulinoma cell viability. Results are representative of three independent experiments performed in triplicate, showing similar tendency. *C*: Pretreatment with z-VAD-fmk significantly inhibited insulinoma cell death by TNFα/Ad5IκBα-SR infection. Results are representative of four independent experiments performed in triplicate, showing similar tendency.

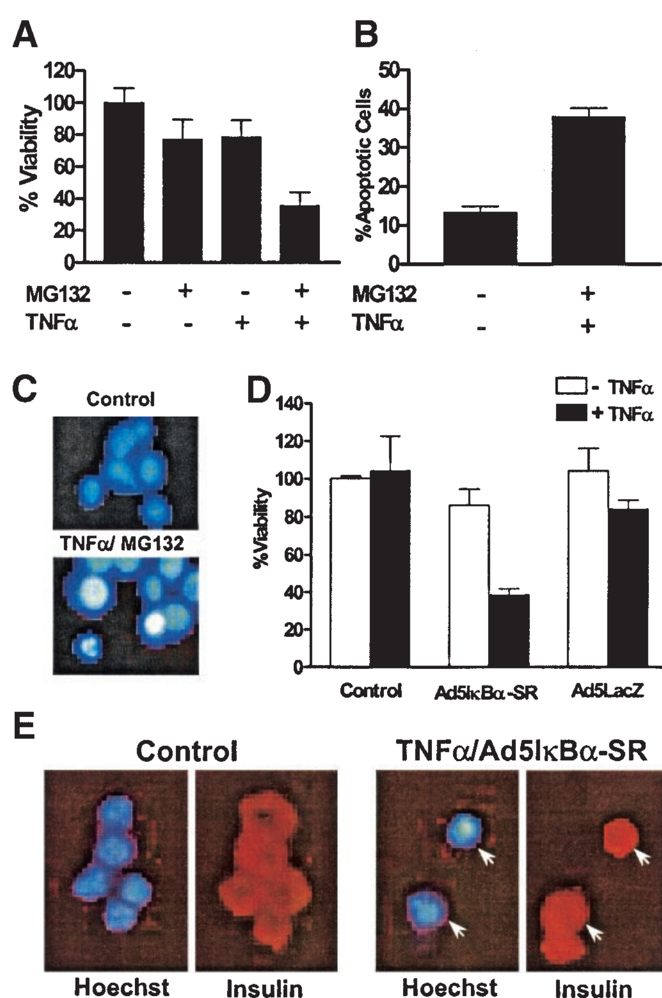

FIG. 6. Primary pancreatic islet cell death by TNF-α in combination with NF-κB inhibitors. *A*: MTT assay showed that pretreatment with 1 μmol/l MG132 for 15 min induced TNF-α susceptibility in primary pancreatic islet cells, whereas 10 ng/ml TNF-α alone had only a marginal effect on primary islet cell viability. Results are representative of three independent experiments performed in triplicate, showing similar tendency. *B* and *C*: Hoechst 33342 staining demonstrated nuclear condensation/fragmentation in primary islet cells treated with TNF-α/MG132. Results are representative of two independent experiments performed in triplicate, showing similar tendency. *D*: TNF-α treatment in combination with Ad5IκBα-SR infection also exerted cytotoxicity on primary islet cells, whereas Ad5IκBα-SR infection alone or TNF-α treatment after control Ad5LacZ infection did not induce significant islet cell death. Results are representative of three independent experiments performed in triplicate, showing similar tendency. *E*: Immunofluorescent staining showed that >90% of apoptotic primary islet cells showing nuclear condensation/fragmentation by Hoechst 33342 staining after treatment with TNF-α/Ad5IκBα-SR were β-cells expressing insulin (arrows).

significant apoptotic activity on primary islet cells as determined by MTT assays, suggesting that the inhibition of NF-κB activation induces TNF-α susceptibility in otherwise resistant primary islet cells similar to MIN6N8 cells (Fig. 6*A*). Viability of islet cells treated with TNF-α/MG132 was significantly lower than that without treatment or that treated with either TNF-α or MG132 alone ($P < 0.01$ for all three comparisons). Hoechst 33342 staining also demonstrated nuclear condensation and/or fragmentation in 37.9% of primary islet cells after treatment with TNF-α/MG132 for 48 h in contrast to 13.4% of control islet cells without treatment, suggesting that primary islet cell death by TNF-α/MG132 is a classic apoptosis (Fig. 6*B* and *C*). Death of islet cells treated with TNF-α/MG132 was significantly higher than that without treatment ($P < 0.001$). TNF-α treatment for 96 h after Ad5IκBα-SR infection also induced a significant death of islet cells, whereas Ad5IκBα-SR infection alone or TNF-α treatment after control Ad5LacZ infection did not induce significant islet cell death (Fig. 6*D*). Viability of islet cells treated with TNF-α in combination with Ad5IκBα-SR infection was significantly lower than that after Ad5IκBα-SR infection alone or that treated with TNF-α in combination with Ad5LacZ infection ($P < 0.0005$ and $P < 0.001$, respectively). More than 90% of primary islet cells showing nuclear

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condensation/fragmentation by Hoechst 33342 staining after TNF-α/Ad5IκBα-SR treatment were stained with anti-insulin Ab, suggesting that most apoptotic cells after TNF-α/Ad5IκBα-SR were islet β-cells (Fig. 6*E*).

DISCUSSION

Our observation that TNF-α treatment activated NF-κB in MIN6N8 cells of NOD mouse origin is similar to the results observed using another SV40T-transformed NOD-derived insulinoma cell model (NIT-1) (25). The activation of NF-κB in TNF-α–treated MIN6N8 cells was due to the degradation of IκBα and subsequent nuclear translocation of the p65 subunit of the NF-κB complex. Our current observation that IFN-γ did not significantly affect TNF-α–induced NF-κB activation in MIN6N8 insulinoma cells despite its ability to synergize MIN6N8 cell death is different from our previous results showing a significant decrease in TNF-α–induced NF-κB activation by concomitant IFN-γ treatment in ME-180 cervical cancer cells (23,26). Other studies have reported of an increase in NF-κB activation by IFN-γ using INS-1 rat insulinoma cells (18). These discrepancies might be due to the difference in cell types, species, or reporter constructs used.

Our observation that MG132 rendered otherwise resistant MIN6N8 cells and primary murine islet cells susceptible to TNF-α–induced apoptosis is consistent with many previous studies using other types of cells (9,23,24). Because MG132 blocked the translocation of p65, probably by inhibiting proteasomal degradation of IκBα, the abrogation of MIN6N8 cell resistance to TNF-α by MG132 strongly suggests that NF-κB activation by TNF-α plays a protective role against TNF-α–induced cell death. MG132 has been shown to inhibit cytokine-induced IκB degradation and NF-κB activation in islet cells (27). The role of NF-κB activation in protecting insulinoma cells against TNF-α was further supported by the ability of IκBα-SR, which inhibits NF-κB activation to induce cell death after TNF-α treatment. A larger degree of MIN6N8 cell death by TNF-α/MG132 compared with that by TNF-α/IκB-SR adenoviral transduction and a small degree of MIN6N8 cell death by MG132 alone could be due to the inhibition of nonspecific proteases other than proteasomal protease by MG132 (28). These results are consistent with those of many other studies showing the protective role of NF-κB activation against apoptosis by death effector molecules (8,12,13); however, they are contradictory to previous studies that use primary islet cells or insulinoma cells (16–18,20). This difference could be due to the difference in cell types, species, or death effectors. Most studies showing the proapoptotic activity of NF-κB used rat insulinoma cells or rat primary islet cells, whereas murine insulinoma cells and murine primary islet cells were used in the current investigation. The difference in the mode of cell death between islet cells from different species has been well documented (3,29–31). NF-κB might also play a different role in apoptosis by different cytokines or death effectors. Most previous studies of the role of NF-κB in pancreatic islet cell death used IL-1 or chemical toxins as death effectors (16,17,19,20,32), whereas the current investigation or other studies supporting the protective role of NF-κB used TNF family members. Different cross-talk between NF-κB activation and intracellular events such as Jun NH_2-terminal kinase activation or cell death in response to different cytokines (TNF-α vs. IL-1) has been reported (33).

If NF-κB activation plays a protective role against pancreatic β-cell death by TNF-α in vivo as suggested by the above in vitro findings, some pathways should be implemented to abrogate or suppress NF-κB activation for the development of type 1 diabetes in vivo. Because IFN-γ renders otherwise resistant MIN6N8 cells or primary murine pancreatic islet cells susceptible to TNF-α (3), IFN-γ could act by modulating NF-κB activation by TNF-α. However, we observed that IFN-γ did not affect TNF-α–induced NF-κB activation in MIN6N8 insulinoma cells, in contrast to other cell types (18,26). While IFN-γ seems to be unable to suppress NF-κB activation by TNF-α in insulinoma cells, IFN-γ may suppress NF-κB–dependent activation of antiapoptotic machinery such as inhibitor of apoptosis protein (IAP) or TNF receptor–associated factor (12) through its inhibitory action on their translation. IFN-γ has been reported to inhibit the translation of a variety of cellular proteins by inducing or activating double-stranded RNA-activated protein kinase (PKR) (34). Our preliminary data suggest that IFN-γ suppresses the expression of IAP proteins in MIN6N8 cells during the progression of the apoptotic process (M.-S.L., J. Inik Chang, H.S.K., M.-S.L., unpublished data).

In addition to its role in modulating β-cell death, NF-κB may have other roles in the pathogenesis of type 1 diabetes, such as modulation of autoimmune responses or insulin secretion from islet cells. Recent studies reported defective or enhanced NF-κB activation in splenocytes or dendritic cells from NOD mice, which might be related to decreased antigen presentation or increased Th1 cytokine production (35,36). Further studies will be necessary to clarify the role of NF-κB in abnormal immunoregulation or antigen presentation in NOD mice.

Although we observed the protective role of NF-κB activation in pancreatic islet cell death by TNF-α in vitro, the real significance of NF-κB activation in the apoptosis of pancreatic β-cells and the development of type 1 diabetes in vivo has yet to be determined. It will be addressed using in vivo systems such as transgenic mice expressing IκBα-SR in their pancreatic islet cells.

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