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Role of Calcium in Pancreatic Islet Cell Death by IFN-γ/TNF-α[1]

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We studied the intracellular events associated with pancreatic β cell apoptosis by IFN-γ/TNF-α synergism. IFN-γ/TNF-α treatment of MIN6N8 insulinoma cells increased the amplitude of high voltage-activated Ca^{2+} currents, while treatment with IFN-γ or TNF-α alone did not. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was also increased by IFN-γ/TNF-α treatment. Blockade of L-type Ca^{2+} channel by nifedipine abrogated death of insulinoma cells by IFN-γ/TNF-α. Diazoxide that attenuates voltage-activated Ca^{2+} currents inhibited MIN6N8 cell death by IFN-γ/TNF-α, while glibenclamide that accentuates voltage-activated Ca^{2+} currents augmented insulinoma cell death. A protein kinase C inhibitor attenuated MIN6N8 cell death and the increase in $[Ca^{2+}]_c$ by IFN-γ/TNF-α. Following the increase in $[Ca^{2+}]_c$, calpain was activated, and calpain inhibitors decreased insulinoma cell death by IFN-γ/TNF-α. As a downstream of calpain, calcineurin was activated and the inhibition of calcineurin activation by FK506 diminished insulinoma cell death by IFN-γ/TNF-α. BAD phosphorylation was decreased by IFN-γ/TNF-α because of the increased calcineurin activity, which was reversed by FK506. IFN-γ/TNF-α induced cytochrome c translocation from mitochondria to cytoplasm and activation of caspase-9. Effector caspases such as caspase-3 or -7 were also activated by IFN-γ/TNF-α treatment. These results indicate that IFN-γ/TNF-α synergism induces pancreatic β cell apoptosis by Ca^{2+} channel activation followed by downstream intracellular events such as mitochondrial events and caspase activation and also suggest the therapeutic potential of Ca^{2+} modulation in type 1 diabetes.   *The Journal of Immunology,* 2004, 172: 7008–7014.

Apoptosis of pancreatic β cells is now becoming one of the important fields in diabetes research due to recent realization of the mechanism and significance of apoptosis in a variety of physiological or pathological conditions. In autoimmune diabetes, apoptosis of pancreatic β cells is the most critical and final step in the development of autoimmune diabetes (1, 2). It may also be important in the pathogenesis of type 2 diabetes, while direct evidence for such a theory has not yet been obtained (3–5).

Although the importance of pancreatic β cell apoptosis in autoimmune diabetes has been accepted, it is still not clearly elucidated which molecule(s) is the real effector(s) in autoimmune diabetes. The role for Fas ligand as the death effector in autoimmune diabetes has been questioned (6–8). Others and we have published in vitro and in vivo data suggesting that IFN-γ/TNF-α synergism is responsible for pancreatic β cell death (9, 10). The role of TNF-α as the final death effector molecule is consistent with other articles using genetic ablation models (6, 11), while that of IFN-γ in the development of autoimmune diabetes is not unanimously supported by other studies using the gene targeting technique (12–

14). Intracellular events associated with the apoptosis of pancreatic β cells are also largely unknown. Although the elucidation of intracellular events associated with apoptosis is regarded as one of the most important breakthroughs in the cell biology field, such events in the death of pancreatic β cells have been addressed only in a few reports.

Pancreatic β cells are electrically excitable, displaying action potentials and subsequent rise of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$).[3] Regulation of glucose-induced insulin secretion critically depends on glucose metabolism and the electrical activity controlled by various plasma membrane ion channels, among which the ATP-sensitive K^+ (K_{ATP}) channel and the voltage-activated Ca^{2+} channel (VACC) play key roles. Beside their physiological roles in the regulation of insulin secretion, ion channels of β cells could be targets of various cytokines or other effector molecules. For instance, it has been demonstrated that serum from type 1 diabetes patients activated L-type Ca^{2+} channels of insulinoma cells while the molecular identity was not characterized (15). In contrast, treatment of β cells with the IL-1β/IFN-γ combination has been reported to induce low-voltage activated Ca^{2+} current (LVA-I_{Ca}), leading to their death (16). In fact, Ca^{2+} has been implicated in a variety of cell death modes such as glutamate-induced excitotoxicity of neuronal cells (17) or reperfusion necrosis of cardiac cells (18). Ca^{2+} is also a prototypic agent inducing mitochondrial permeability transition (MPT) (19), which has been considered as the "point of no return" in several cell death models (20). However, the role of Ca^{2+} in classical apoptosis is not clearly established, and it remains to be clarified whether MPT precedes

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[3] Abbreviations used in this paper: $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; K_{ATP}, ATP-sensitive K^+ channel; VACC, voltage-activated Ca^{2+} channel; LVA, low-voltage activated; I_{Ca}, Ca^{2+} current; MPT, mitochondrial permeability transition; NOD, nonobese diabetic; ALLN, N-acetyl-Leu-Leu-Nle-CHO; I/V, current/voltage; GC, glibenclamide; HM, heavy membrane; HVA, high-voltage activated; RMP, resting membrane potential; PKC, protein kinase C; CsA, cyclosporin A.

and induces cytochrome c translocation or ensues after critical mitochondrial events (21).

This study was conducted to investigate the apoptotic cascade associated with apoptosis of pancreatic β cells by IFN-γ/TNF-α synergism with a particular emphasis on the role of Ca^{2+}.

Materials and Methods

Cell line and reagents

MIN6N8 cells, SV40 T-transformed insulinoma cells derived from nonobese diabetic (NOD) mice (kindly provided by Prof. Jun-ichi Miyazaki, Osaka University, Osaka, Japan), were grown in DMEM-15% FBS. Recombinant murine IFN-γ and TNF-α were purchased from R&D Systems (Minneapolis, MN). Calpeptin, N-acetyl-Leu-Leu-Nle-CHO (ALLN), and FK506 were obtained from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Whole-cell patch clamp study

Cells were transferred into a bath on an inverted microscope (IX-70; Olympus, Tokyo, Japan). The bath was superfused with Tyrode's solution containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, and 5 mM glucose (pH 7.4), and voltage clamp experiments were performed at room temperature. Patch pipettes were connected to the head stage of a patch-clamp amplifier (Axopatch-1D; Axon Instruments, Foster City, CA). Liquid junction potentials were corrected with an offset circuit before each experiment. Voltage and current data were low-pass filtered (5 kHz) and analyzed using Origin version 6.1 software (Microcal Software, Northampton, MA). The membrane potential was monitored under the zero-current clamp mode of whole-cell configuration using KCl pipette solution (135 mM KCl, 5 mM NaCl, 0.5 mM $MgCl_2$, 3 mM MgATP, and 10 mM HEPES (pH 7.3) titrated with KOH). Ca^{2+} currents were recorded using CsCl pipette solution containing 140 mM CsCl, 0.5 mM $MgCl_2$, 3 mM MgATP, and 10 mM HEPES (pH 7.3). After making a whole-cell configuration, Ca^{2+} currents were elicited by step-depolarizing pulses. The membrane voltage was held at -90 mV and various levels of step-like depolarizing pulses were applied. In another series of experiments, K_{ATP} current in each group of cells was measured. ATP-free KCl pipette solution was used for the whole-cell patch clamp, and cells were superfused with glucose-free KCl bath solution, where the K^+ equilibrium potential was located at 0 mV. The membrane conductance was monitored as brief current/voltage (I/V) curves obtained by ramp-like pulses. The superfusion with a glucose-free KCl condition alone slowly increased the membrane conductance. When the membrane conductance reached a steady state, 50–100 μM diazoxide was added, which further increased the membrane conductance. Then, 0.2 μM glibenclamide (GC) was applied, and the size of K_{ATP} current in each cell was measured as a GC-sensitive current at -80 mV (see Fig. 2C).

Measurement of $[Ca^{2+}]_c$

Cells were loaded with the acetoxymethyl ester form of 2 μM fura-2 (Molecular Probes, Eugene, OR) in Tyrode's solution at room temperature for 20 min and then rinsed twice with fresh solution. The recording of $[Ca^{2+}]_c$ was performed with a microfluorometric system comprising an inverted fluorescence microscope (Olympus IX-70) with a dry-type fluorescence objective lens ($\times$40; aperture, 0.85), a type R1527 photomultiplier tube (Hamamatsu, Japan), and a Deltascan illuminator (Photon Technology International, Lawrence Ville, NJ). $[Ca^{2+}]_c$ was calculated from the fluorescence emission ratio R at 340 nm/380 nm excitation according to a reported formula (22).

Calpain and calcineurin activity

Calpain activity was measured using t-BOC-Leu-Met-CMAC fluorochrome (Molecular Probes) as a substrate. After incubating cells in 0.15 M NaCl-5 mM HEPES-2 mM EDTA (pH 7.35) containing the substrate, fluorescence was measured at 530 nm using a fluorescence microplate reader (Bio-Tek, Winooski, VT). Serine/threonine phosphatase activity was determined as a measure of calcineurin activity using a commercial kit (Promega, Madison, WI). Briefly, cell lysate was added to the reaction premix in each well of 96-well plates. After the reaction was stopped with premade molybdate dye/additive mix, absorbance at 600 nm was measured with a microplate reader.

Cell fractionation

Cells were fractionated according to a previously published protocol (23). Briefly, cells were lysed in a buffer containing 10 mM HEPES (pH 8.0), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA,1 mM PMSF, and 100 μg/ml leupeptin. After scraping of the cells and douncing, unbroken cells and nuclear fraction were discarded after centrifugation at 600 $\times$ g for 10 min. After further centrifugation at 15,000 $\times$ g for 10 min, pellet was suspended in heavy membrane (HM) buffer containing 10 mM HEPES, 5 mM $MgCl_2$, 42 mM KCl, and 1% Triton X-100 (HM fraction). Supernatant was divided into the light membrane fraction and cytosolic fraction after ultracentrifugation at 100,000 $\times$ g for 60 min. Purity of the HM fraction and cytosolic fraction was confirmed by immunoblot using anti-Cox4 and -IκB Ab, respectively.

Western blot analysis

Cells were lysed in a buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM PMSF, and 1% Nonidet P-40. Protein concentration in the cell lysate was measured using the Bradford assay. After adding a loading buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 8% glycerol, and 0.4% 2-ME, an equal amount of protein for each sample was separated by 10% SDS-PAGE and transferred to ECL membranes (Amersham Pharmacia, Uppsala, Sweden). After blocking with 5% skim milk, membranes were incubated with Ab to caspase-9 (BD PharMingen, La Jolla, CA), caspase-7 (kindly provided by Dr. P. Vandenabeele, University of Ghent, Ghent, Belgium), activated caspase-3 (Idun Pharmaceutical, La Jolla, CA) (24), BAD, or phospho-BAD (Ser[112]; Cell Signaling, Beverly, MA). Blots were probed with HRP-conjugated secondary Ab (Amersham Pharmacia). Bound Ab was visualized using ECL reagent (Pierce, Rockford, IL).

Measurement of mitochondrial potential

Mitochondrial potential was measured using JC-1 (Molecular Probes). After incubating 5×10^6 cells in 0.5 ml of PBS containing 10 μg/ml JC-1 at 37°C for 10 min, emission at 510 and 590 nm was measured using a fluorescence microplate reader. The ratio of A_{590}:A_{510} was calculated as an indicator of mitochondrial potential.

Isolation of mouse pancreatic islets

Islets were isolated from overnight-fasted ICR mice by the collagenase digestion technique as previously described (10). Briefly, pancreas was gently pulled out after 2.5 ml of collagenase P (0.8 mg/ml) was injected into the bile duct. The pancreas was then digested in collagenase P solution at 37°C for 15 min with gentle shaking. After stopping the digestion with cold HBSS, tissue was then passed through a 400-μm screen and centrifuged on 25, 23, 21.5, and 11.5% Ficoll gradients, respectively. Islets collected from the interface were washed with M199 medium, and individual islets were hand-picked using micropipettes. The islets were treated with trypsin-EDTA for 5 min to yield single islet cells before treatment with test reagents. All animal experiments in this work were done in accordance with the institutional guidelines of Samsung Medical Center Animal Facility, an Association for Assessment and Accreditation for Laboratory Animal Care International-accredited facility.

Statistical analysis

All values were expressed as means $\pm$ SEM. Student's t test was used to compare the differences between the groups. Values of $p < 0.05$ were regarded as statistically significant.

Results

Increased Ca^{2+} flux and intracellular Ca^{2+} contents by IFN-γ/TNF-α combination

Because recent articles reported the expression of the LVA-Ca^{2+} channel in islet β cells from NOD mice (25) and activation of the LVA-Ca^{2+} channel in pancreatic islet cell death by cytokines (16), we first studied whether LVA-Ca^{2+} channel was activated in our model of pancreatic islet cell death by 100 U/ml IFN-γ in combination with 10 ng/ml TNF-α (IFN-γ/TNF-α) using MIN6N8 insulinoma cells. Under the whole-cell patch clamp condition, an inward Ca^{2+} current appeared at above -40 mV and reached its peak at 0 mV. The peak inward current to voltage relation showed an inverted bell-shaped I/V curve, suggesting the dominance of high-voltage activated (HVA)-Ca^{2+} channels (Fig. 1A). Current
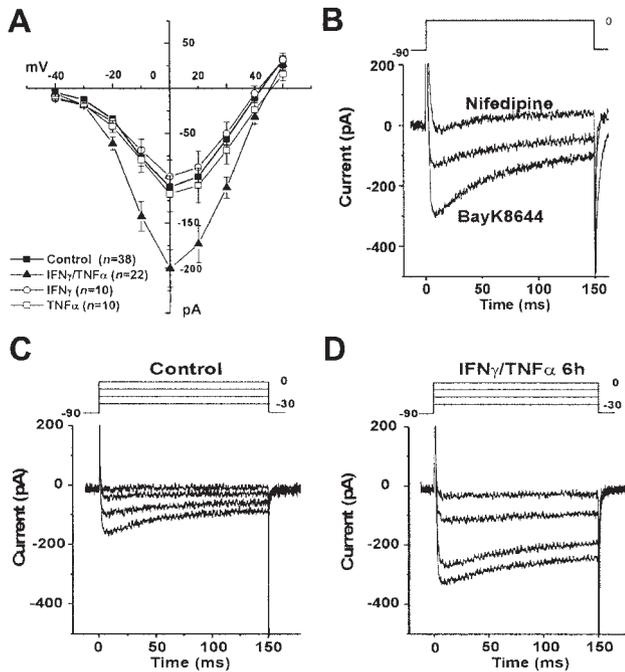
FIGURE 1. Effects of cytokine treatment on voltage-activated Ca^{2+} currents in MIN6N8 cells. *A*, I/V curves of voltage-activated Ca^{2+} currents showing the predominance of HVA-Ca^{2+} channel. Treatment with IFN-γ/TNF-α but not with either cytokine alone increased Ca^{2+} currents. *B*, A step depolarization from -30 to 0 mV induced an inward current showing time-dependent inactivation. BayK8644 facilitated and nifedipine blocked the Ca^{2+} current, respectively. *C* and *D*, Ca^{2+} current tracing in control (*C*) and IFN-γ/TNF-α-treated (*D*) cells showed an increased inward Ca^{2+} current through the HVA-Ca^{2+} channel.

FIGURE 2. The effects of IFN-γ/TNF-α on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), RMP, and K_{ATP} currents. *A*, $[Ca^{2+}]_c$ measured at different time points showed that IFN-γ/TNF-α treatment for 4–10 h increased $[Ca^{2+}]_c$ ($n = 15$, respectively). *B*, Both initial and steady-state RMP 5 min after making a whole-cell configuration was not significantly different between control and IFN-γ/TNF-α-treated cells ($n = 23$, respectively). *C*, Dialysis of cytosol with ATP-free pipette solution containing KCl was accompanied by an increase of membrane conductance. The application of 100 μM diazoxide further increased the membrane conductance and the addition of 0.2 μM GC suppressed the membrane conductance to the initial level. The I/V curve displayed a weak inward rectification. *D*, The size of the K_{ATP} current measured as the GC-sensitive current at -80 mV under glucose-free conditions (0 ATP, 180 s) or full activation by diazoxide was not changed by IFN-γ/TNF-α treatment for 6 h.

tracing showed that the inward current was markedly augmented by 1 μM BayK8644 and blocked by 10 μM nifedipine, the L-type Ca^{2+} channel agonist and antagonist, respectively (Fig. 1*B*), again indicating that the L-type Ca^{2+} channels showing high threshold voltage are predominantly expressed in MIN6N8 cells. Recording of membrane current response to step-depolarizing pulse and I/V curve analysis showed that treatment of MIN6N8 cells with IFN-γ/TNF-α for 6 h increased the amplitude of inward Ca^{2+} currents through HVA-Ca^{2+} channels (HVA-I_{Ca}) by 43% compared with control cells (Fig. 1, *A*, *C*, and *D*). The increase in Ca^{2+} current was due to the synergistic action of IFN-γ and TNF-α because treatment with MIN6N8 cells with either IFN-γ or TNF-α alone did not increase Ca^{2+} current (Fig. 1*A*). I/V curves from 22 cells showed that there is no clear evidence demonstrating the activation of LVA-Ca^{2+} channel current (Fig. 1*A*).

Next, we compared $[Ca^{2+}]_c$ of control and IFN-γ/TNF-α-treated cells using the fura-2 method. Treatment of MIN6N8 cells with IFN-γ/TNF-α for 4 h induced a significant increase in $[Ca^{2+}]_c$. The increase in $[Ca^{2+}]_c$ reached the peak 6 h after IFN-γ/TNF-α treatment and was sustained until 10 h ($p < 0.05$ for all comparisons; Fig. 2*A*). The measurement of $[Ca^{2+}]_c$ at later time points was not attempted because death of MIN6N8 cells might affect intracellular $[Ca^{2+}]_c$ and cause artifactual effects on $[Ca^{2+}]_c$. Treatment with MIN6N8 cells with either IFN-γ or TNF-α alone did not affect $[Ca^{2+}]_c$, again indicating the synergistic action of IFN-γ and TNF-α in modulating Ca^{2+} influx (data not shown). Because the increased $[Ca^{2+}]_c$ could be due to an increased membrane depolarization in addition to increased Ca^{2+} current through HVA-I_{Ca}, we next measured the membrane potential of MIN6N8 cells. The mean of initial resting membrane potential (RMP) within 30 s after making a whole-cell configuration was -45 ± 1.9
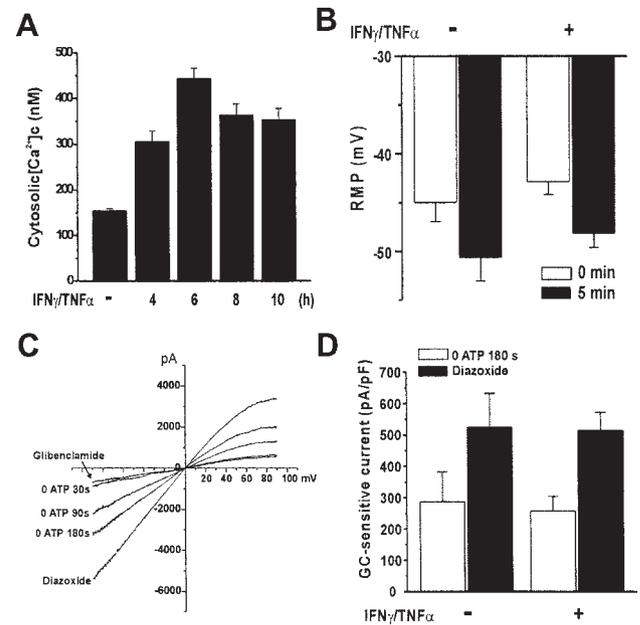
mV ($n = 23$) and -43 ± 1.3 mV ($n = 24$) for control and IFN-γ/TNF-α-treated cells, respectively. There was no significant difference between them ($p > 0.1$), suggesting that the increased $[Ca^{2+}]_c$ after treatment of MIN6N6 cells with IFN-γ/TNF-α is due not to an increase in membrane depolarization but to an increased Ca^{2+} influx (Fig. 2*B*). The steady-state RMP 5 min after making a whole-cell configuration was also not significantly different between control (-50.6 ± 2.47 mV, $n = 23$) and IFN-γ/TNF-α-treated cells (-48 ± 1.45 mV, $n = 24$) ($p > 0.1$; Fig. 2*B*). We also compared the size of K_{ATP} current before and after treatment with IFN-γ/TNF-α because the activity of the K_{ATP} channel plays a crucial role in the determination of RMP of β islet cells and in the release of insulin (26). K_{ATP} current in MIN6N8 cells measured as GC-sensitive current under the glucose-free condition or as full activation by diazoxide was not significantly changed by IFN-γ/TNF-α treatment ($p > 0.1$ for both comparisons; Fig. 2, *C* and *D*). As a whole, the positive modulation of HVA-Ca^{2+} channel current with no significant changes in RMP and K_{ATP} current suggests that the increase in $[Ca^{2+}]_c$ after IFN-γ/TNF-α treatment is due to an increased Ca^{2+} influx through the HVA-Ca^{2+} channel during action potentials.

Death of insulinoma cells due to increased intracellular Ca^{2+}

Because the above change in $[Ca^{2+}]_c$ and HVA-I_{Ca} by IFN-γ/TNF-α raised the possibility that the observed increase in $[Ca^{2+}]_c$ might cause death of MIN6N8 cells, we next studied whether blockade of Ca^{2+} influx in MIN6N8 cells could affect their death

after IFN-γ/TNF-α treatment. Death of MIN6N8 cells by IFN-γ/TNF-α was a classical apoptosis associated with DNA fragmentation/nuclear condensation by Hoechst staining and sub-G_1 peak in DNA ploidy assay as previously reported (10) (data not shown). Ten micromolar nifedipine, a classical blocker of the L-type HVA-Ca^{2+} channel, significantly inhibited death of MIN6N8 cells by IFN-γ/TNF-α (Fig. 3A), suggesting that the increase in Ca^{2+} influx after IFN-γ/TNF-α treatment induced or triggered their death ($p < 0.01$). One hundred micromolar $NiCl_2$, a nonspecific blocker of Ca^{2+} channels, also inhibited MIN6N8 cell death by IFN-γ/TNF-α ($p < 0.01$; Fig. 3A). Similar effects were observed when VACC was indirectly modified. The application of 50 μM diazoxide, a K_{ATP} channel opener that hyperpolarizes the membrane and prevents the activation of VACC (Fig. 2C), significantly inhibited the death of MIN6N8 cells after treatment with IFN-γ/TNF-α (Fig. 3B), further suggesting the role of increased Ca^{2+} influx in MIN6N8 cell death ($p < 0.01$). In contrast, GC that blocks K_{ATP} channel activity and augments the activation of VACC (Fig. 2C) significantly accentuated the death of MIN6N8 cells by IFN-γ/TNF-α in a dose-dependent manner ($p < 0.05$; Fig. 3B).

To study the mechanism of the increase in $[Ca^{2+}]_c$ by IFN-γ/TNF-α synergism, we investigated the effect of protein kinase C (PKC) inhibitors because the PKC activation by growth factors or cytokines is reportedly related to an increased Ca^{2+} influx (27, 28). One micromolar chelerythrine, a PKC inhibitor, substantially attenuated the increase in $[Ca^{2+}]_c$ by IFN-γ/TNF-α treatment for 6 h ($p < 0.01$), suggesting that the activation of PKC is probably involved in the activation of the Ca^{2+} channel and the increase in Ca^{2+} influx (Fig. 4A). Consistent with these results, 1 μM chelerythrine partly inhibited death of MIN6N8 cells by IFN-γ/TNF-α treatment for 48 h ($p < 0.05$; Fig. 4B).

Calcineurin-mediated dephosphorylation of BAD in insulinoma cell death by IFN-γ/TNF-α

Because these results indicated the role of Ca^{2+} influx as a triggering event in MIN6N8 cell apoptosis by IFN-γ/TNF-α, we next asked whether calpain, an abundant Ca^{2+}-activatable protease, was activated in MIN6N8 cells after treatment with IFN-γ/TNF-α. Calpain substrate assay showed a significantly increased fluorescence 3–24 h after IFN-γ/TNF-α treatment ($p < 0.05$ for all comparisons), indicating calpain activation by increased intracellular Ca^{2+} (Fig. 5A). Thus, we next studied whether calpain inhibitors could block MIN6N8 cell death by IFN-γ/TNF-α. Both 1 μM calpeptin and 1 μM ALLN, specific and cell-permeable inhibitors of calpain I and II, inhibited the increase in calpain activity by
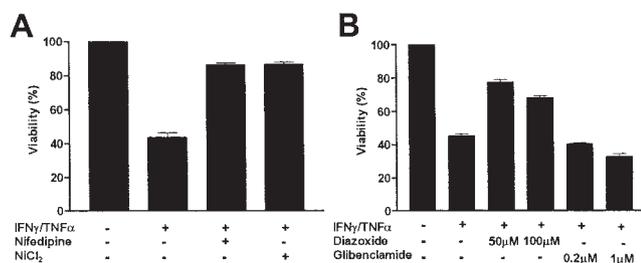
FIGURE 4. Inhibition of MIN6N8 cell death by a PKC inhibitor. *A,* Chelerythrine inhibited the increase in $[Ca^{2+}]_c$ by IFN-γ/TNF-α. *B,* Chelerythrine also attenuated the death of MIN6N8 cells by IFN-γ/TNF-α. Chelerythrine itself did not affect $[Ca^{2+}]_c$ or the viability of MIN6N8 cells.

IFN-γ/TNF-α treatment for 24 h ($p < 0.05$ for both comparisons; Fig. 5A) and also significantly attenuated MIN6N8 cell death by IFN-γ/TNF-α treatment for 48 h ($p < 0.01$ for both comparisons), suggesting that activated calpain plays a role in MIN6N8 cell death by IFN-γ/TNF-α (Fig. 5B). Because previous articles reported that calpain activates calcineurin, leading to Ca^{2+}-induced apoptosis (29, 30), we next conducted a serine/threonine phosphatase assay as a measure of calcineurin activity after treatment of MIN6N8 cells with IFN-γ/TNF-α. Calcineurin activity began to rise 3 h after IFN-γ/TNF-α treatment, which became significant 6 h after cytokine treatment and reached the peak at 12 h ($p < 0.05$ at 6, 12, and 24 h; Fig. 5C). Furthermore, inhibition of calcineurin activity with 1 μM FK506 or 1 μM cyclosporin A (CsA) significantly inhibited death of MIN6N8 cells after IFN-γ/TNF-α treatment for 48 h ($p < 0.01$ for both comparisons), again supporting the role of increased calcineurin activity as an important step in MIN6N8 cell death by IFN-γ/TNF-α (Fig. 5D). We next studied the phosphorylation status of BAD using Ab specific for phospho-BAD because previous articles described calcineurin-dependent dephosphorylation of BAD as a mechanism of Ca^{2+}-induced apoptosis (30, 31). As hypothesized, a decreased phosphorylation of Ser^{112} of BAD was observed between 3 and 24 h after treatment of MIN6N8 cells with IFN-γ/TNF-α. In addition, the decrease in BAD phosphorylation was reversed by FK506, suggesting that calcineurin activation after treatment of MIN6N8 cells with IFN-γ/TNF-α was responsible for BAD dephosphorylation (Fig. 5E). In contrast, the expression of total BAD was not changed by IFN-γ/TNF-α (Fig. 5F).

Early cytochrome c translocation and delayed loss of mitochondrial potential by IFN-γ/TNF-α

Because dephosphorylated BAD could translocate to mitochondria and lead to mitochondrial events critically involved in the apoptotic pathway (32, 33), we next studied the possible translocation of cytochrome c and change in mitochondrial potential. As expected from the dephosphorylation of BAD, cytochrome c was translocated from the HM fraction to the cytosolic fraction 3 h after IFN-γ/TNF-α treatment (Fig. 6A). Cytochrome c translocation was obvious at 6 h and became maximal 48 h after cytokine treatment when almost all cytochrome c of the HM fraction was translocated to cytosol. Cytosolic cytochrome c was not observed 72 h after IFN-γ/TNF-α treatment probably because cytochrome c passed through damaged plasma membrane during the secondary necrosis as observed in other studies (34). In contrast to the early cytochrome c translocation, notable dissipation of mitochondrial potential was observed only 48 h after IFN-γ/TNF-α treatment when overt cell damage was apparent (Fig. 6B).

Cleavage of caspases by IFN-γ/TNF-α

We next studied the postmitochondrial events occurring in the course of MIN6N8 cell death by IFN-γ/TNF-α. We studied

FIGURE 3. Inhibition of MIN6N8 cell death by Ca^{2+} channel blockers. *A,* Nifedipine, an L-type HVA-Ca^{2+} channel blocker and $NiCl_2$, a nonspecific Ca^{2+} channel blocker significantly inhibited death of MIN6N8 cells by IFN-γ/TNF-α. *B,* Diazoxide, a K_{ATP} opener that prevents VACC activation (Fig. 2C), significantly inhibited the death of MIN6N8 cells after treatment with IFN-γ/TNF-α, while GC, a K_{ATP} channel blocker augmenting VACC activation (Fig. 2C), increased MIN6N8 cell death by IFN-γ/TNF-α.
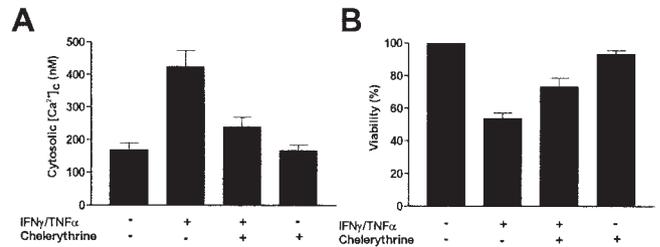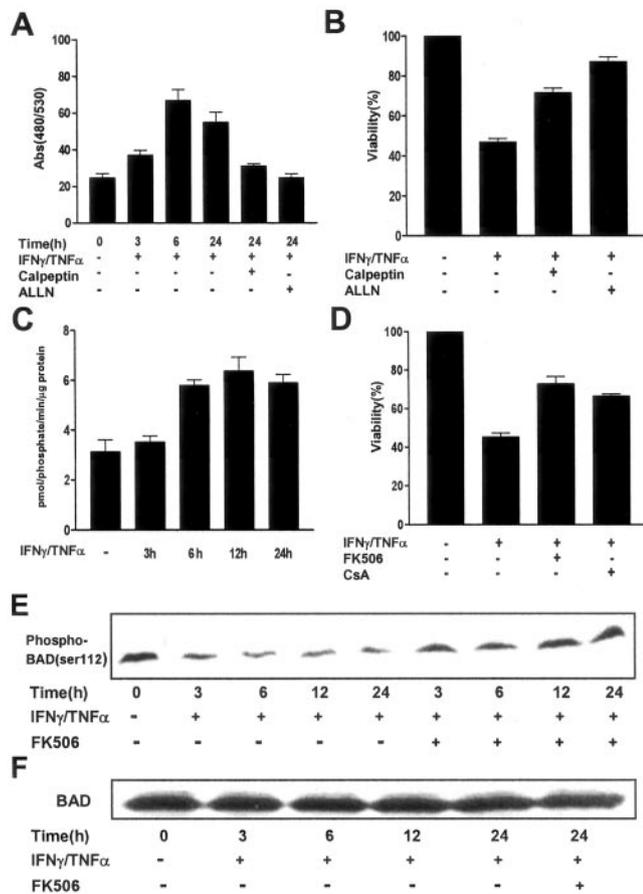
FIGURE 5. Activation of calpain/calcineurin and BAD dephosphorylation. *A,* The calpain substrate assay showed that calpain activity was induced by IFN-γ/TNF-α, which was inhibited by calpeptin or ALLN, specific calpain inhibitors. *B,* MIN6N8 cell death by IFN-γ/TNF-α was inhibited by calpeptin or ALLN. *C,* Calcineurin activity was also induced by IFN-γ/TNF-α. *D,* FK506 or CsA, inhibitors of calcineurin, attenuated MIN6N8 cell death by IFN-γ/TNF-α. *E,* IFN-γ/TNF-α decreased phosphorylation of BAD, which was reversed by FK506. *F,* Total BAD expression was not changed by IFN-γ/TNF-α.

whether caspase-9 was cleaved after cytochrome *c* translocation. Immunoblot analysis showed that caspase-9 was cleaved 36 h after treatment of MIN6N8 cells with IFN-γ/TNF-α (Fig. 7*A*), suggesting that caspase-9 was cleaved by apoptosome following cytochrome *c* translocation from the mitochondria to the cytoplasm. We then asked whether effector caspases were cleaved as the final executors of insulinoma cell apoptosis. Immunoblot analysis using CM1 Ab recognizing cleaved caspase-3 but not its proform demonstrated that caspase-3 was cleaved yielding a 15-kDa fragment by IFN-γ/TNF-α treatment of MIN6N8 cells for 36 h (Fig. 7*B*). IFN-γ or TNF-α alone did not induce cleavage of caspase-3. Caspase-7, another effector caspase, was also cleaved 36 h after IFN-γ/TNF-α treatment (Fig. 7*C*).

Changes of [Ca^{2+}]$_c$ in primary islet cells

To confirm that the change in Ca^{2+} concentration by cytokine treatment is an important step in the death of primary islet cells as well as insulinoma cells, we treated islet cells from ICR mice with IFN-γ/TNF-α and measured [Ca^{2+}]$_c$. [Ca^{2+}]$_c$ in primary islet cells after IFN-γ/TNF-α treatment for 4 h was significantly increased compared with that of untreated islet cells (Fig. 8*A*). Single cytokine was ineffective. Ten micromolar nifedipine and 1 μM chelerythrine substantially inhibited the death of primary islet cells by
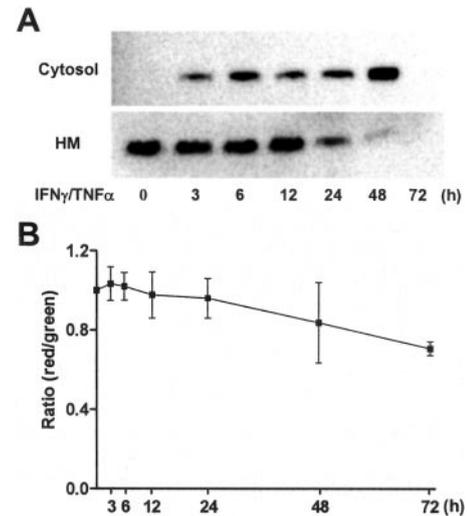

FIGURE 6. Mitochondrial events in insulinoma cell death by IFN-γ/TNF-α. *A,* Cytochrome *c* translocation from the mitochondrial fraction to the cytosol occurred 3 h after cytokine treatment and was most prominent at 48 h. Cytochrome *c* was not detected in the cytoplasm 72 h after cytokine treatment probably because cytochrome *c* passed through the damaged plasma membrane. *B,* Changes in mitochondrial potential was not observed until 48 h after IFN-γ/TNF-α treatment in contrast to the obvious changes in the cytochrome *c* distribution in the earlier times.

IFN-γ/TNF-α, suggesting that IFN-γ/TNF-α treatment induces death of islet cells by essentially the same mechanism involving Ca^{2+} (Fig. 8*B*).

Discussion

Although TNF-α induces apoptosis by activating apical caspases in most cell death models, several previous articles have shown that TNF-α could induce apoptosis by increasing [Ca^{2+}]$_c$ after mobilization of Ca^{2+} from an extracellular or intracellular source (35–37). Consistent with those reports, we also observed an increased Ca^{2+} current and increased [Ca^{2+}]$_c$ in the current investigation studying the mechanism of IFN-γ/TNF-α-induced apoptosis of pancreatic β cells (10). At first, we expected a possible increase in Ca^{2+} current through the LVA-Ca^{2+} channel because previous articles reported that the LVA-Ca^{2+} channel was abnormally expressed in β cells of NOD mice and the Ca^{2+} current through the LVA-Ca^{2+} channel mediates pancreatic β cell death by the IL-1β/IFN-γ combination (16, 25). In contrast to those reports, we could not find evidence supporting the role of LVA-Ca^{2+} channels in MIN6N8 cell death by IFN-γ/TNF-α. A single peak of the I/V curve with the threshold voltage of -40 mV and an inward current markedly augmented by BayK8644 and blocked by nifedipine favored the presence of HVA-Ca^{2+} channels rather than LVA-Ca^{2+} channels. This discrepancy might be due to the difference in the types of cells or cytokines used. Although the RMP was not different between control insulinoma cells and IFN-γ/TNF-α-treated ones, an increased Ca^{2+} influx through the HVA-Ca^{2+} channel during action potentials could lead to an increased [Ca^{2+}]$_c$ observed in this study. The increase in Ca^{2+} influx and [Ca^{2+}]$_c$ occurring 4 h after IFN-γ/TNF-α treatment seems to be responsible for the following apoptotic cascade because an L-type Ca^{2+} channel blocker inhibited the death of insulinoma cells by IFN-γ/TNF-α. The inhibition and exaggeration of IFN-γ/TNF-α-induced MIN6N8 cell death by diazoxide and GC, respectively, further support the role of Ca^{2+} influx in MIN6N8 cell apoptosis by IFN-γ/TNF-α, and are also consistent with a previous report showing
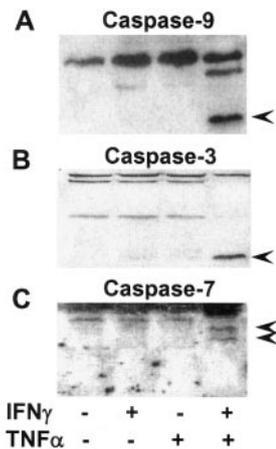
FIGURE 7. Cleavage of caspase-9, -3, and -7. IFN-γ/TNF-α treatment for 36 h induced cleavage of caspase-9 (*A*), caspase-3 (*B*), and caspase-7 (*C*) as downstream events of cytochrome *c* translocation (arrowheads, cleaved caspases).

FIGURE 8. Role of $[Ca^{2+}]_c$ in primary islet cells. *A*, $[Ca^{2+}]_c$ was significantly increased after treatment of primary islet cells with IFN-γ/TNF-α but not after treatment with a single cytokine. *B*, Nifedipine and chelerythrine decreased primary islet cell death by IFN-γ/TNF-α, similar to MIN6N8 cells.

the inhibition of tolbutamide-induced β cell death by a calcium channel blocker or diazoxide (38).

Activation of calpain was also observed in MIN6N8 cells after IFN-γ/TNF-α treatment. Furthermore, calpeptin or ALLN significantly decreased MIN6N8 cell death after IFN-γ/TNF-α treatment, consistent with previous reports showing a critical role of calpain in Ca^{2+}-mediated apoptosis (39). Calcineurin, a serine/threonine phosphatase activated by Ca^{2+}, was also activated in MIN6N8 cells after IFN-γ/TNF-α treatment. Its abrogation by FK506 or CsA also inhibited insulinoma cell death by IFN-γ/TNF-α, suggesting an important role of calpain and calcineurin activation following Ca^{2+} influx in this model. Although CsA could affect MPT, the inhibition of insulinoma cell death by another inhibitor of calcineurin without such an effect on MPT, FK506, strongly suggests that calcineurin activation plays a role in MIN6N8 cell death by IFN-γ/TNF-α. Although the relationship between calpain and calcineurin activation remains largely unknown, a recent article suggested a possible role of cain as a missing link between those two processes in Ca^{2+}-triggered cell death (29). Calpain-induced degradation of cain that binds to and inhibits calcineurin (40, 41) could lead to calcineurin activation, while such a possibility was not tested in this study. The diabetogenic effect of FK506 or CsA reported in patients with islet transplantation is observed only after prolonged administration (42) and appears to be due to their effect on insulin secretion (43) resulting from the blockade of FK506-binding protein-cADP ribose interaction or K_{ATP} (44, 45). Such an observation is unrelated to islet cell death and not incompatible with our finding of the beneficial effect of FK506 or CsA on islet cell viability after a short-term treatment.

Our data showing dephosphorylation of BAD at Ser^{112} probably by calcineurin activation is consistent with previous articles showing a critical role of BAD dephosphorylation as a downstream event following calcineurin activation Ca^{2+}-mediated cell death models (30, 31). As a downstream event of BAD dephosphorylation, we observed translocation of cytochrome *c*. In contrast to the early cytochrome *c* translocation, dissipation of mitochondrial potential was not apparent until 48 h after IFN-γ/TNF-α treatment, suggesting that the loss of mitochondrial potential is not a cause of cytochrome *c* translocation observed in this study. The causal relationship among cytochrome *c* translocation, loss of mitochondrial potential, and MPT is yet to be solved (21).
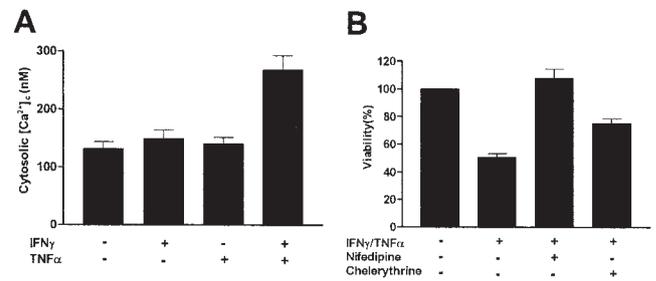
Subsequent to mitochondrial events, we observed activation of caspase-9, which was evident 36–48 h after IFN-γ/TNF-α treatment of MIN6N8 cells. As the final effector molecules in the apoptotic cascade, caspase-3 and caspase-7 were also cleaved. Because we have reported that IFN-γ/TNF-α synergism is one of the in vivo effector mechanisms of pancreatic β cell death in autoimmune diabetes (10), the apoptotic process observed in this study may reflect that occurring in vivo, while other effector molecules such as IL-1β or NO would play some role in the destruction of islet β cells. However, MIN6N8 insulinoma cell death by IFN-γ/TNF-α does not entail NO or Fas as previously reported, and Fas expression or NO production was not induced by IFN-γ/TNF-α (Refs, 10 and 46; I. Chang and M.-S. Lee, unpublished results).

The mechanism of Ca^{2+} channel activation is obscure. Protein kinases have been reported to modulate Ca^{2+} current or $[Ca^{2+}]_c$ as downstream mediators of cytokines or hormones (27, 47, 48). Consistent with those reports, we observed a significant inhibition of IFN-γ/TNF-α-induced increase in $[Ca^{2+}]_c$ and insulinoma cell death by a PKC inhibitor. Death of primary islet cells by IFN-γ/TNF-α was also inhibited by the PKC inhibitor. Ca^{2+} has diverse roles in pancreatic β cells beside its involvement in islet cell death and well-recognized role in insulin secretion. Ca^{2+} influx through the HVA-Ca^{2+} channel is involved in the growth (49) or survival of islet cells (50). Thus, islet cells seem to maintain an intricate balance of Ca^{2+} influx and $[Ca^{2+}]_c$ to achieve optimal growth and survival while avoiding a dangerous level of $[Ca^{2+}]_c$.

Taken together, we report a mechanism of IFN-γ/TNF-α-induced apoptosis of insulinoma and pancreatic islet cells involving Ca^{2+} channel activation, increased Ca^{2+} influx, calpain activation, calcineurin activation, BAD dephosphorylation, cytochrome *c* translocation, and the activation of effector caspases. Such a pathway involving Ca^{2+} influx does not appear to play a role in Fas ligand-mediated apoptosis of target cells like Jurkat cells, indicating the uniqueness of this Ca^{2+} pathway in islet cell death (I. Chang and M.-S. Lee, unpublished results). Although the mechanism of Ca^{2+} channel activation by cytokine combination is not addressed further in this study, our model provides a new insight into the apoptotic cascade in pancreatic islet cells leading to autoimmune diabetes and suggests the therapeutic potential of Ca^{2+} modulation in the prevention of type 1 diabetes.

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