Apoptosis in Insulin-secreting Cells

Evidence for the Role of Intracellular Ca^{2+} Stores and Arachidonic Acid Metabolism

Yun-Ping Zhou, Deeling Teng, Flora Dralyuk, Diane Ostrega, Michael W. Roe, Louis Philipson, and Kenneth S. Polonsky
Department of Medicine, Section of Endocrinology, The University of Chicago, Chicago, Illinois 60637

Abstract

This study investigated the role of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in apoptosis in MIN6 cells, an insulin secreting cell line, and in mouse islets. Thapsigargin, an inhibitor of sarcoendoplasmic reticulum Ca^{2+}-ATPases (SERCA), caused a time- and concentration-dependent decrease in the viability of MIN6 cells and an increase in DNA fragmentation and nuclear chromatin staining changes characteristic of apoptosis. Two structurally distinct SERCA inhibitors, cyclopiazonic acid and 2,5-di-[t-butyl]-1,4-hydroquinone also caused apoptosis, but agents that increased $[Ca^{2+}]_i$ by other mechanisms did not induce apoptosis in MIN6 cells. Carbachol- or ionomycin-releasible intracellular Ca^{2+} stores were completely depleted in cells treated by SERCA inhibitors, but not by other agents that increase $[Ca^{2+}]_i$. The ability of thapsigargin to induce cell death was not affected by blocking Ca^{2+} influx or by clamping $[Ca^{2+}]_i$ with a cytosolic Ca^{2+} buffer suggesting that the process did not depend on changes in $[Ca^{2+}]_i$ per se. However, application of the lipoxygenase inhibitors 5,8,11-eicosatrienoic acid and nordihydroguaiaretic acid partially prevented MIN6 cell apoptosis, while exposure of cells to the product of lipoxygenase, 12-hydroxy-[5,8,10,14]-eicosatetraenoic acid, caused apoptosis. In contrast, inhibition of cyclooxygenase with indomethacin did not abolish thapsigargin-induced apoptosis in MIN6 cells. Our findings indicate that thapsigargin causes apoptosis in MIN6 cells by depleting intracellular Ca^{2+} stores and leading to release of intermediate metabolites of arachidonic acid metabolism. (*J. Clin. Invest.* 1998. 101:1623–1632.) Key words: apoptosis • lipoxygenase • calcium • islets of Langerhans • thapsigargin

Introduction

Recent studies have highlighted the importance of apoptosis or programmed cell death in the physiological and pathophysiological regulation of tissue development (1), neoplastic expansion (2), ischemic destruction of cardiac myocytes (3), and toxic cell injury (4). Apoptosis also plays a key role in the reduction in β-cell mass that occurs postpartum (5) and has been proposed to mediate β-cell destruction in insulin-dependent diabetes mellitus (6) and the reduction in β-cell mass that occurs in non–insulin-dependent diabetes mellitus (NIDDM)[1] (7).

Several agents have been shown to induce apoptosis in β-cells, including superoxide radicals (8, 9), cytokines (9), sulfonylureas (10), staurosporine (11), fructose (12), agents that activate G-proteins (13), actinomycin, and cycloheximide (14). However, the basic biochemical events that trigger and mediate apoptosis in insulin-secreting cells are not known. Several of these agents affect the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and previous studies have implicated Ca^{2+} as a mediator of apoptosis in other cells (15). Therefore, these studies were undertaken to investigate the role of $[Ca^{2+}]_i$ in death of MIN6 cells, a well-differentiated mouse insulinoma cell line (16), and in mouse pancreatic islets.

In pancreatic β-cells, $[Ca^{2+}]_i$ is primarily regulated by the interplay between Ca^{2+} entry via L-type voltage-dependent calcium channels, sequestration of Ca^{2+} into intracellular stores, and removal of Ca^{2+} via plasma membrane Ca^{2+} pumps (17, 18). Inhibition of microsomal Ca^{2+} pumps, termed the sarcoendoplasmic reticulum Ca^{2+}-dependent adenosine trisphosphatases (SERCA), results in the depletion of the intracellular Ca^{2+} stores (19, 20) and activates voltage-independent Ca^{2+} or Na^+ currents, which can contribute to the elevation in $[Ca^{2+}]_i$ (21). Agents such as thapsigargin that inhibit the two isoforms of SERCA (SERCA 2b and SERCA 3) in β-cells (22, 23) cause depletion of the intracellular Ca^{2+} stores and increase in $[Ca^{2+}]_i$ (24, 25).

We investigated the role of $[Ca^{2+}]_i$ in triggering apoptosis in insulin-secreting cells. Cells were exposed to agents that are known to increase $[Ca^{2+}]_i$ and the signaling pathways necessary for initiation of apoptosis were identified using well-characterized Ca^{2+} transport inhibitors as well as intracellular and extracellular Ca^{2+} chelators. We found that inhibition of SERCA activity with thapsigargin caused apoptosis. In addition, our studies indicated that thapsigargin induced β-cell apoptosis in the absence of rises in $[Ca^{2+}]_i$, but was related to cellular lipoxygenase activity. Our findings suggest that sustained depletion of intracellular Ca^{2+} pools may trigger apoptosis in pancreatic β-cells and that products of arachidonate metabolism play a role in mediating this process. Emptying of the intracellular stores of Ca^{2+} thus appears to be one of the

Address correspondence to Dr. Kenneth S. Polonsky, M.D., Department of Medicine, (MC 1027) University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637. Phone: 773-702-6217; FAX: 773-834-0486; E-mail: polonsky@medicine.bsd.uchicago.edu

Received for publication 21 July 1997 and accepted in revised form 4 February 1998.

J. Clin. Invest.

0021-9738/98/04/1623/10 $2.00
Volume 101, Number 8, April 1998, 1623–1632
http://www.jci.org

1. *Abbreviations used in this paper:* AA, arachidonic acid; AVP, arginine vasopressin; BAPTA, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*[1],*N*[1]-tetra acetic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CPA, cyclopiazonic acid; DPC, diphenylamine carboxylate; ER, endoplasmic reticulum; ETI, 5,8,11-eicosatrienoic acid; 12-HETE, 12-hydroxy-(5,8,10,14)-eicosatetraenoic acid; HO342, Hoechst 33342; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NDGA, nordihydroguaiaretic acid; NIDDM, non–insulin-dependent diabetes mellitus; PI, propidium iodide; SERCA, the sarco/endoplasmic Ca^{2+}-ATPase; tBHQ, 2,5-di-(t-butyl)-1,4-hydroquinone.

important mechanisms whereby apoptosis may be triggered in insulin secreting cell lines.

Methods

Cell culture and islet isolation. MIN6 cells (16) were grown in Dulbecco's modified Eagle's medium (DME) containing 25 mM glucose, 1 mM sodium pyruvate, 15% FCS, 100 IU/ml penicillin, and 100 μg/liter streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were used between passages 20 and 30. Islets of Langerhans were isolated from the pancreata of 8–10-wk-old normal C57BL/KsJ mice (The Jackson Laboratory, Bar Harbor, ME) by collagenase digestion and discontinuous Ficoll gradient separation, a modification of the original method of Lacy and Kostianovsky (26). Islets were cultured in RPMI 1640 medium supplemented with 11 mM glucose, 5% FCS, and antibiotics as above.

Cell viability assay. MIN6 cells were grown to ~ 50% confluence in flat-bottom 96-well plates before being exposed to a variety of agents known to affect $[Ca^{2+}]_i$ by different mechanisms. These experiments were carried out in DME complete culture medium for 48 h unless otherwise indicated. Cell viability was determined by a colorimetric assay (27). This assay is based on the ability of viable cells, but not dead cells, to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), the reaction generates a dark blue formazan product. After exposure of the cells to the test agents, 10 μl of 5 g/liter MTT in PBS was added to 100 μl of medium in each well. After a 2–3-h incubation at 37°C, 100 μl of 40 mM HCl/isopropanol was added to stop MTT reduction and to homogenize the cells. After an additional 5–10 min incubation at 37°C, the absorbance at 540 nm was measured using a Titertek Multiskan MC™ Immuno-Reader (Titertek, Helsinki, Finland). For determining islet cell viability the MTT assay was modified as follows: 30 islets were incubated in 100 μl medium with MTT in an Eppendorf tube for 3 h and homogenized by sonification before measuring absorbance.

Nuclear chromatin staining. Hoechst 33342 (HO342; 10 μg/liter) and propidium iodide (PI; 5 μM) were used to detect differences between normal and apoptotic nuclei as described previously (14). Cells grown on glass coverslips were stained with a mixture of the two dyes for 5 min and washed with PBS before examination by fluorescence microscopy. HO342 freely enters cells with intact membranes as well as cells with damaged membranes and stains DNA blue when visualized under the fluorescent microscope (ultraviolet excitation at 340–380 nm). Intact plasma membranes are impermeable to PI, a highly polar dye that only enters cells that have lost integrity of their cell membranes (necrotic or late apoptotic cells) and stains DNA red. Apoptotic cells were identified by the presence of condensed or fragmented nuclei stained either blue or pink depending on the stage in the apoptotic process.

Determination of internucleosomal DNA cleavage (DNA laddering). DNA was extracted by a modification of a previous method (9) and precipitated with ethanol. DNA fragments (~ 10 μg) were separated by electrophoresis in 1.8% agarose gels, and visualized by staining with ethidium bromide (1 mg/liter).

Quantification of DNA fragmentation. Fragmented DNA was separated from chromatin DNA, and quantified as described previously (28). Lysates from 200 mouse islets or ~ 2×10^6 MIN6 cells were centrifuged at 15,000 *g* for 15 min. The supernatant and pellet fractions were extracted with phenol-chloroform. The amount of DNA in the two fractions was determined spectrofluorometrically after incubation with the fluorescent dye Hoechst 33258 (0.2 μg/ml).

Measurements of $[Ca^{2+}]_i$ in MIN6 cells. MIN6 cells were seeded on glass coverslips and grown for 2 d before exposure to various agents that affect $[Ca^{2+}]_i$. Cells were loaded with fura-2 acetoxymethyl ester (5 μM; Molecular Probes, Eugene, OR) for 30 min at 37°C in a Krebs-Ringer bicarbonate medium containing (in mM): 119 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 2 glucose. Coverslips were mounted into a microperifusion chamber on the specimen stage of a microscope equipped for epifluorescence and perifused with Krebs-Ringer bicarbonate medium at a rate of 2.5 ml/min at 37°C as described in detail elsewhere (24). $[Ca^{2+}]_i$ was measured using dual-wavelength fluorescence video microscopy and expressed as the ratio of the fluorescence intensity (detected at 510 nm) of fura-2 stimulated with light at excitation wavelengths of 340 and 380 nm (ratio 340:380) as described previously (24).

Materials. DME (high glucose), RPMI 1640, PBS, and penicillin/streptomycin were purchased from Life Technologies, Inc. (Gaithersburg, MD), FCS was from Hyclone (Logan, UT). Proteinase K and RNAase A were from Boehringer Mannheim Biochemicals (Indianapolis, IN). A23187, arachidonic acid, SK&F 96365, *N*-ethylmaleimide, cyclopiazonic acid, 2,5-di-(t-butyl)-1,4-hydroquinone, ionomycin, and MTT were from Calbiochem (La Jolla, CA), Hoechst 33258, HO342, and Fura-2 acetoxymethyl ester from Molecular Probes, Inc. (Eugene, OR). Thapsigargin, nordihydroguaiaretic acid, PI and others were obtained from Sigma Chemical Co. (St. Louis, MO). Agents with low aqueous solubility were dissolved in DMSO.

Statistics. Results were expressed as mean±SEM. The statistical significance of differences between treatment groups was determined using either paired Student's *t* test or one-way ANOVA.

Results

Relationship between $[Ca^{2+}]_i$ and viability of MIN6 cells (Fig. 1). To test the hypothesis that agents that cause an increase in $[Ca^{2+}]_i$ can decrease β-cell viability, MIN6 cells were exposed to a variety of test reagents that increase $[Ca^{2+}]_i$ by different mechanisms (17, 29). As shown in Fig. 1, few reagents decreased MIN6 cell viability significantly. Exposure of cells for

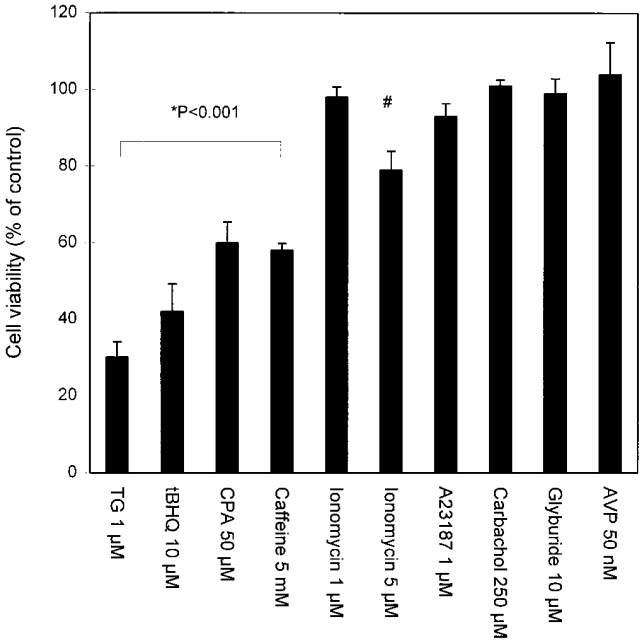

Figure 1. MIN6 cell viability is reduced by agents that affect intracellular Ca^{2+} stores, but not by agents that increase $[Ca^{2+}]_i$. MIN6 cells were exposed 48 h to thapsigargin (*TG*; 1 μM), tBHQ (10 μM), CPA (50 μM), caffeine (5 mM), ionomycin (1 and 5 μM), A23187 (1 μM), carbachol (250 μM), glyburide (10 μM), and AVP (50 nM). The MTT assay was used to measure cell survival. Control cells were exposed to solvent alone. Data represent results of at least four independent experiments and are expressed as percent of control response. Statistical significance ($*P < 0.001$; $\#P < 0.01$) is indicated.

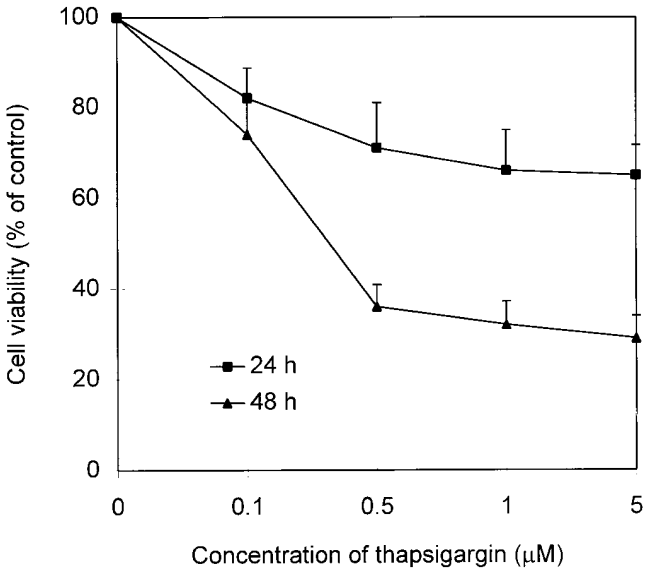

Figure 2. Effects of thapsigargin on MIN6 cell viability. MIN6 cell viability as a function of concentration of thapsigargin exposed for 24 and 48 h is shown. Control responses were measured in cells incubated with dimethylsulfoxide (0.01 ~ 0.5%, vol/vol) for identical periods of time. The results are the mean±SEM of five experiments.

48 h to arginine vasopressin (AVP; 50 nM), glyburide (10 μM), or carbachol (250 μM) did not significantly affect MIN6 cell viability. The cells also were resistant to prolonged incubation with calcium ionophores: 1 μM of either A23187 or ionomycin did not reduce viability significantly. However, incubation of the MIN6 cells with a higher concentration of ionomycin (5 μM) significantly reduced ($P < 0.01$) viability by 21% (Fig. 1).

Drugs that deplete endoplasmic reticulum (ER) Ca^{2+} stores induced death of MIN6 cells. Cyclopiazonic acid (CPA; 50 μM), 2,5-di-(t-butyl)-,4-hydroquinone (tBHQ; 10 μM), and thapsigargin (1 μM) decreased viability by 39, 58, and 70%, respectively (Fig. 1). Exposure of cells to 0.1–5 μM thapsigargin for 24 h caused a concentration-dependent increase in MIN6 cell death that was more profound after a 48-h incubation (Fig. 2). Inhibition of SERCA activity by thapsigargin is irreversible (30) Thus a transient exposure of cells to 1–5 μM thapsigargin for 4 h (followed by removal of thapsigargin from the incubation medium) also triggered MIN6 cell death within 24–48 h (data not shown).

Caffeine (5 mM), a methylxanthine that discharges β-cell intracellular Ca^{2+} stores (31), reduced MIN6 cell viability by 40% (Fig. 1). However, this was not associated with the appearance of DNA laddering characteristic of apoptosis (data not shown). Therefore, the reduction in cell viability induced by caffeine was likely due to necrosis rather than apoptosis.

Evidence that apoptosis mediates the thapsigargin-induced reduction in viability of MIN6 cells. We measured DNA fragmentation and stained the cell nuclei using the fluorophores HO342 and PI to determine if the reduction in viability of MIN6 cells induced by thapsigargin was due to apoptosis. Fig. 3 *A* shows the time course of the effects of 1 μM thapsigargin on DNA fragmentation in MIN6 cells. DNA laddering started to appear after 6 h and became further evident by 24 and 48 h after thapsigargin exposure (Fig. 3 *A*). The amount of fragmented cell DNA (expressed as a percent of total DNA) revealed a similar temporal pattern (Fig. 3 *B*). After 6- and 12-h exposures to 1 μM thapsigargin, DNA fragmentation in-

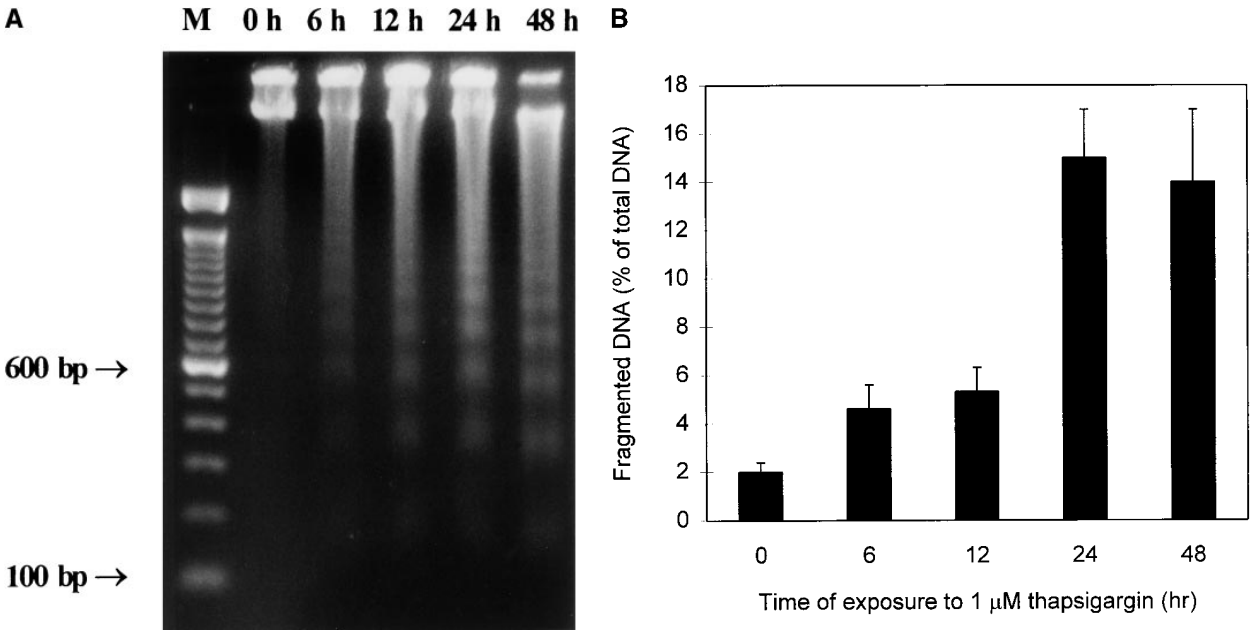

Figure 3. Thapsigargin triggers DNA fragmentation in MIN6 cells. *(A)* DNA fragmentation in MIN6 cells treated with 1 μM thapsigargin. Cells were exposed to the SERCA inhibitor for 6, 12, 24, and 48 h, respectively, as indicated. Typically, a DNA laddering pattern began to appear after 6 h of treatment with thapsigargin, and it became evident after 24 h of treatment. Band formation from a DNA molecular weight marker (*M*) is shown in lane 1, and the migration of 100- and 600-bp fragments are indicated. The amount of DNA fragmentation was determined as described in Methods; the results are summarized in *B*. Data are mean±SEM of three independent experiments. Note the temporal concordance between the onset DNA laddering (*A*) and increase in DNA fragmentation (*B*).

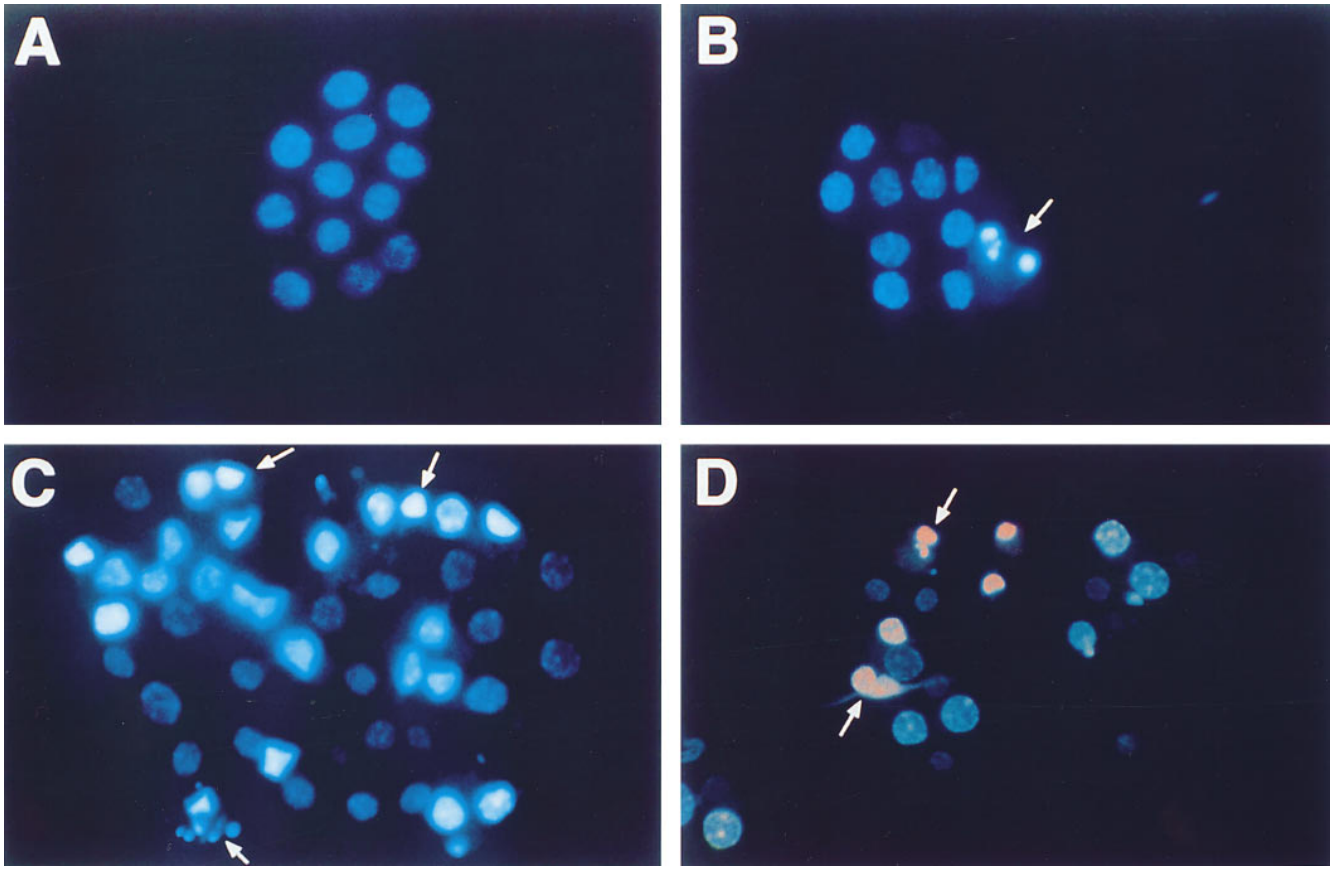

Figure 4. Effects of thapsigargin on MIN6 cell nuclear morphology. Photomicrographs of untreated (*A*) and thapsigargin-treated MIN6 cells (*B–D*) are shown. Cells were stained with HO342 (*blue*) and propidium iodide (*pink*) and visualized using fluorescence microscopy. Control cell nuclei were homogeneously stained with HO342. In contrast, the nuclei of MIN6 cells exposed to 1 μM (*B*) or 5 μM (*C*) thapsigargin for 48 h showed evidence of condensation and chromatin fragmentation (*arrows*). (*D*) Some of the apoptotic cells have lost their membrane integrity (stained by PI) during the late stage of apoptosis.

creased 2.1- and 2.5-fold above controls, respectively. Thapsigargin induced 7.1- and 6.9-fold elevations in the amount of DNA fragmentation after 24 and 48 h, respectively (Fig. 3 *B*). The other two SERCA inhibitors CPA and tBHQ also caused DNA fragmentation similar to that seen with thapsigargin (data not shown). That thapsigargin induced apoptosis was further documented using HO342 and PI staining (Fig. 4). In unexposed control MIN6 cells, the HO342 fluorescence was homogenously distributed throughout the cell nuclei and no chromatin condensation or nuclear fragmentation was evident (Fig. 4 *A*). This staining pattern was found in $> 95\%$ of the control cells studied. In contrast, the nuclei of MIN6 cells exposed to 1 μM (Fig. 4 *B*) or 5 μM (Fig. 4 *C*) thapsigargin for 48 h appeared smaller than control cells and exhibited condensed or fragmented chromatin with punctate or nonhomogeneous regions of fluorescence staining (Fig. 4, *B* and *C*; *arrows*), a staining pattern consistent with apoptosis (14). Fig. 4 *D* shows the appearance of cells during the later stages of apoptosis at which time the nuclei are stained with HO342 and PI. The number of thapsigargin-treated cells containing apoptotic nuclei ranged between 15–40% per field-of-view. It is conceivable that a higher proportion of the cells might exhibit apoptotic nuclei, a finding that would parallel the MTT assay results shown in Fig. 1. However, we noted that some apoptotic cells detached during the staining process, suggesting that the actual number of apoptotic MIN6 cells may be underestimated by the HO342 fluorescence technique. Nevertheless, our findings derived from three separate assays (MTT, DNA fragmentation, and fluorescent staining) clearly indicate that thapsigargin exposure triggers apoptotic cell death in MIN6 cells.

The role of cellular protease and protein biosynthesis. Application of ethylmaleimide (5 μM), a nonspecific proteinase inhibitor (32), as well as cycloheximide (10 μM), an inhibitor of protein biosynthesis (14), significantly blocked thapsigargin activation of apoptosis in MIN6 cells. After incubation in thapsigargin plus ethylmaleimide for 48 h, $45\pm4\%$ of the cells remained viable as judged by the MTT assay compared with $27\pm4\%$ with thapsigargin alone ($n = 5$, $P < 0.05$). Corresponding results after incubation in thapsigargin plus cycloheximide ($51\pm4\%$) versus $30\pm2\%$ in thapsigargin alone ($n = 5$, $P < 0.01$) also showed an increase in cell viability.

Role of $[Ca^{2+}]_i$ in thapsigargin-induced MIN6 cell apoptosis. Next, we determined whether rises in $[Ca^{2+}]_i$ contributed to thapsigargin-induced MIN6 cell apoptosis (Fig. 5, *A* and *B*; Table I). Diazoxide (100 μM), a K_{ATP} channel opener that prevents membrane depolarization in β-cells, failed to attenuate

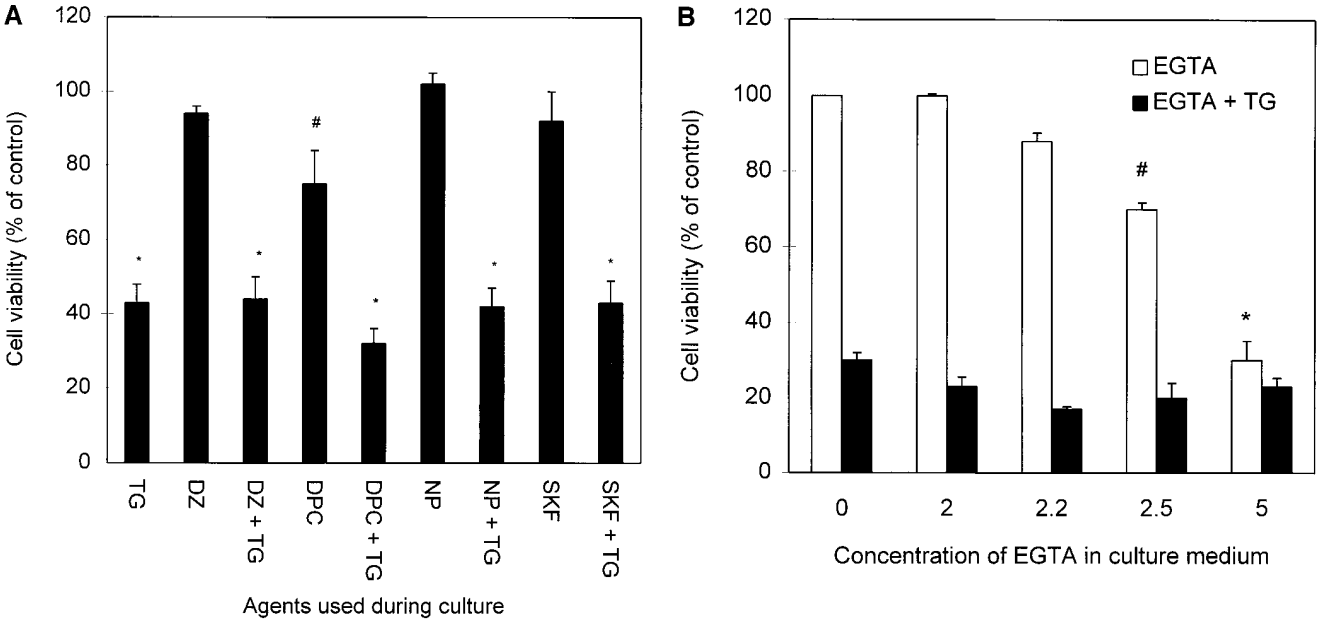

Figure 5. Effect of extracellular Ca^{2+} on thapsigargin-induced MIN6 cell death. (*A*) There was no significant attenuation of thapsigargin-induced cell death by drugs that block influx of extracellular Ca^{2+}. Inhibiting voltage-dependent Ca^{2+} channel activity either indirectly with diazoxide (*DZ*; 100 μM), an ATP-sensitive inward rectifier K^+ channel opener, or directly with nitrendipine (*NP*; 0.5 μM), did not prevent apoptotic cell death induced by a 48-h exposure to thapsigargin (*TG*; 1 μM). Application of the Ca^{2+} store-operated channel blockers DPC (250 μM) or SKF-96365 (*SKF*; 25 μM) were without effect either in suppressing cell death consequent to SERCA inhibition. (*B*) There was no significant reduction in thapsigargin-induced apoptosis by lowering extracellular Ca^{2+} concentration with 2–5 mM EGTA. In fact, the 48-h incubation with 2.5 and 5 mM EGTA alone triggered significant (#$P < 0.05$; * $P < 0.01$) loss of MIN6 cell viability. Data are expressed as mean±SEM of at least four independent experiments.

the decrease in cell viability induced by thapsigargin (Fig. 5 *A*). This suggested that influx of Ca^{2+} through voltage-dependent calcium channels was not important in triggering cell death by thapsigargin, and is supported by the finding that nitrendipine (0.5 μM), an L-type Ca^{2+} channel blocker did not suppress thapsigargin-stimulated apoptosis (Fig. 5 *A*). Equally ineffective were diphenylamine-2-carboxylate (DPC; 250 μM) and the imidazole compound, SKF-96365 (25 μM), inhibitors of Ca^{2+} store-operated channels (33) (Fig. 5 *A*). With the exception of DPC, which caused 25% cell death, neither diazoxide, nitrendipine, nor SKF-96365 decreased MIN6 cell viability (Fig. 5 *A*).

These findings provide compelling evidence that influx of Ca^{2+} consequent to thapsigargin exposure did not contribute to apoptosis. This was studied further using Ca^{2+} chelators, EGTA and 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*^{1},*N*^{1}-tetra acetic acid (BAPTA). Fig. 5 *B* shows that application of EGTA did not protect MIN6 cells from cell death induced by thapsigargin. In fact, the experiments revealed that application of 2.2–5 mM EGTA alone for 48 h triggered cell death (Fig. 5 *B*). Evidence further excluding a role of rises in $[Ca^{2+}]_i$ in death of MIN6 cells was provided by loading cells with BAPTA. Clamping $[Ca^{2+}]_i$ with the cytosolic Ca^{2+} chelator failed to prevent thapsigargin-triggered cell death (Table I). Loading the cells with BAPTA for 1 h appeared to be sufficient to prevent a rise in $[Ca^{2+}]_i$ by thapsigargin. Thus exposure of MIN6 cells to the SERCA blocker (2 μM) 4 and 24 h after BAPTA loading did not cause an increase in $[Ca^{2+}]_i$ (data not shown).

The effect of thapsigargin on intracellular Ca^{2+} stores. We directly measured $[Ca^{2+}]_i$ in MIN6 cells treated with thapsigargin. Resting levels of $[Ca^{2+}]_i$ in cells exposed to thapsigargin (1 μM) for 48 h (A340/A380: 0.58±0.03, $n = 43$) were not different from controls (A340/A380: 0.54±0.03, $n = 89$). However, mobilizable intracellular Ca^{2+} stores were entirely depleted (Fig. 6 *B*). Carbachol, a muscarinic agonist that increases β-cell $[Ca^{2+}]_i$ by receptor-stimulated activation of phospholipase C, production of inositol 1,4,5-trisphosphate and consequent discharge of ER-sequestered Ca^{2+} pools, did not increase $[Ca^{2+}]_i$ in MIN6 cells treated with 1 μM thapsigargin (compare Fig. 6, *A* and *B*). Similar results were observed with MIN6 cells

Table I. Effect of BAPTA Preloading on Thapsigargin-induced Cell Death in MIN6 Cells

	Time of exposure to thapsigargin	
	24 h	48 h
Control (loaded with BAPTA)	100±0 (3)	100±1 (5)
Thapsigargin (loaded with BAPTA)	72±1* (3)	34±5* (5)
Thapsigargin (unloaded)	66±9* (3)	38±7* (5)

Cells were preincubated with 5 μM BAPTA/AM for 1 h before being exposed to 1 μM thapsigargin in DME medium for 24 or 48 h and followed by MTT assays. Data are means±SEM of three to five separate experiments (numbers in parentheses). *$P < 0.01$ or less compared to control.

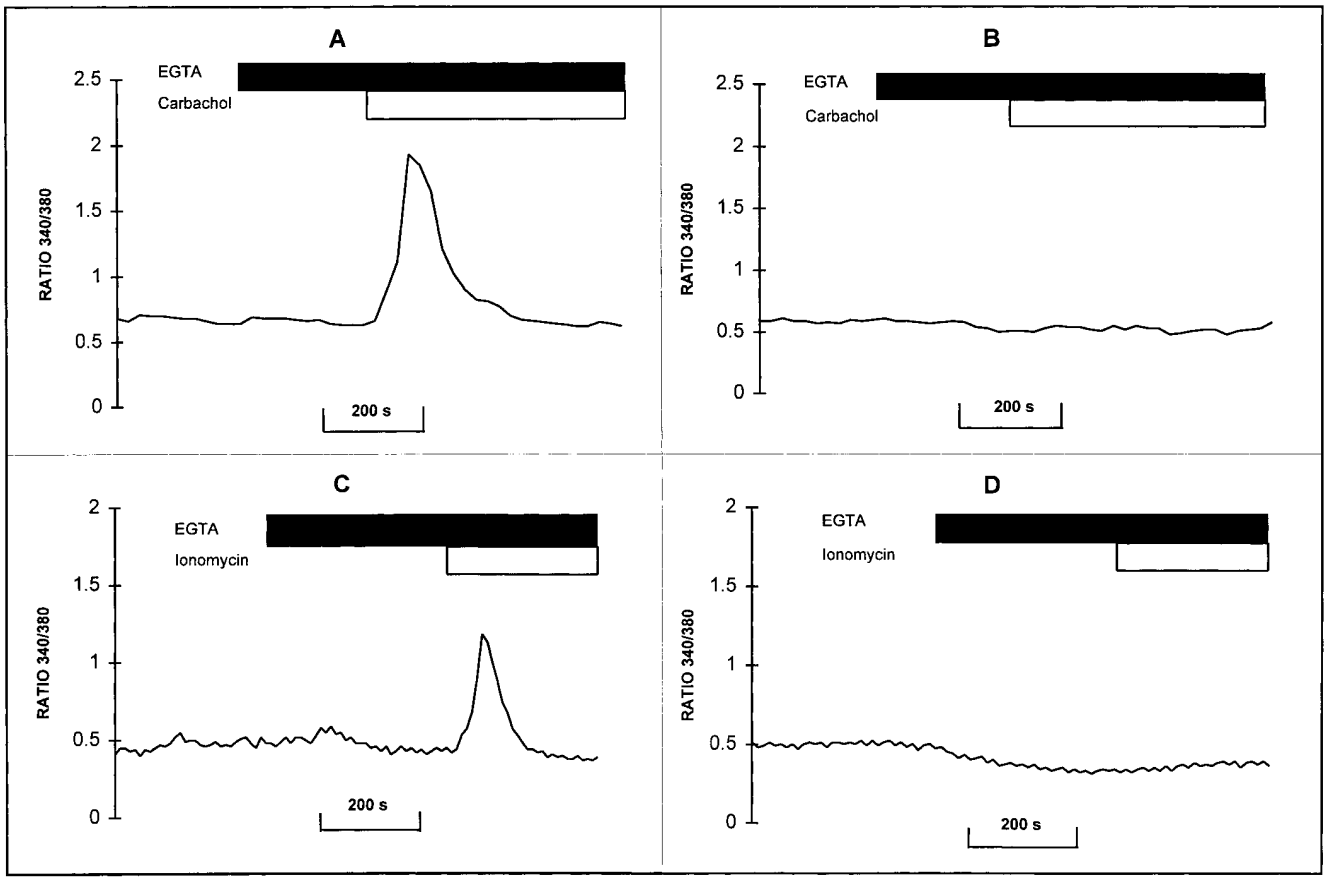

Figure 6. Thapsigargin depletes mobilizable Ca^{2+} stores in MIN6 cells. (*A*) A typical transient $[Ca^{2+}]_i$ rise in a single fura-2–loaded MIN6 cell stimulated with carbachol (*open bar*; 250 μM) in the absence of extracellular Ca^{2+} (perifusion buffer contained 50 μM EGTA and no $CaCl_2$; *filled bar*). (*B*) Depletion of carbachol-sensitive (*open bar*; 250 μM) intracellular Ca^{2+} stores in MIN6 cells after a 48-h treatment with thapsigargin (1 μM). The prolonged exposure (48 h) of MIN6 cells to 1 μM thapsigargin did not increase resting $[Ca^{2+}]_i$. (*C*) Release of intracellular Ca^{2+} by 5 μM ionomycin (*open bar*). (*D*) After a 48-h exposure to 1 μM thapsigargin, ionomycin-induced mobilization of Ca^{2+} stores was completely ablated. All traces are representative recordings from three to five separated experiments (each experiment measured 10–18 cells simultaneously).

treated with 0.5 μM thapsigargin, 50 μM CPA, or 10 μM tBHQ as well, whereas cells treated with AVP (50 nM) and glyburide (10 μM) retained intact responses to carbachol (data not shown).

Additional experiments with ionomycin, a Ca^{2+} ionophore known to be able to release intracellular Ca^{2+} stores at high concentration, confirmed the observation that the induction of apoptosis by thapsigargin is related to depletion of intracellular stores regulated by SERCA. As shown in Fig. 6, *C* and *D*, release of intracellular Ca^{2+} stimulated by 5 μM ionomycin was completely inhibited after a 48-h treatment of MIN6 cells with 1 μM thapsigargin. The absence of stores of intracellular Ca^{2+} that could be released by ionomycin (up to 10 μM) was also documented after a 4- or 24-h exposure to thapsigargin, and after incubation for 24 h with 10 μM tBHQ and 50 μM CPA (data not shown).

Role of arachidonate metabolism in thapsigargin-induced apoptosis. Recent studies in smooth muscle cells (34) and U937 cells (35) demonstrated that the irreversible depletion of ER-sequestered Ca^{2+} pools by thapsigargin, generated arachidonate by activating a Ca^{2+}-independent phospholipase A_2. Exposure of MIN6 cells to arachidonate alone (AA; 100 μM) did not reduce cell viability (Fig. 7). Nonetheless, 5,8,11-eicosatrienoic acid (ETI; 5 μM) and nordihydroguaiaretic acid (NDGA; 20 μM), inhibitors of lipoxygenase activity (36), significantly blocked thapsigargin-induced cell death (Fig. 7). These results suggested that a product of the lipoxygenase pathway generated consequent to thapsigargin-induced ER Ca^{2+} store depletion contributed to cell death. Consistent with this hypothesis was the finding that 12-hydroxy-(5,8,10,14)-eicosatetraenoic acid (12-HETE; 5–10 μM), the major product of the lipoxygenase pathway in β-cells (37), caused a significant reduction in MIN6 viability (Fig. 7). The role of activation of lipoxygenase activity in triggering cell death appeared to be specific since inhibition of the cyclooxygenase pathway with indomethacin (IND, 10 μM) did not significantly impair the thapsigargin-induced decrease in MIN6 cell viability (Fig. 7). Indomethacin alone was without effect. However, exposure of MIN6 cells to AA (100 μM) plus indomethacin (10 μM) for 48 h reduced MIN6 cell viability by 22% (Fig. 7). This result further suggested the role of lipoxygenase pathway in β-cell death.

Effects of thapsigargin in mouse pancreatic islets. Exposure of islets to 10 μM thapsigargin caused 35% of islet cells to die

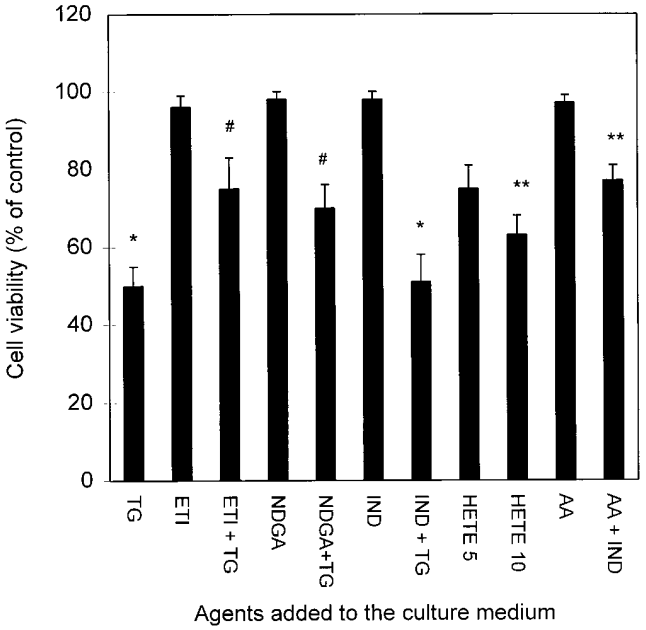

Figure 7. Effects of arachidonic acid metabolism on MIN6 cell death. Cells were exposed to lipoxygenase inhibitors ETI (5 μM) and NDGA (20 μM), a cyclooxygenase blocker, IND (10 μM) in the presence or absence of 1 μM thapsigargin (*TG*) for 4 h. After this period, thapsigargin was removed and the cells incubated for 48 h in fresh medium containing only the arachidonate inhibitors. In parallel experiments, HETE (5 and 10 μM), AA (100 μM), and AA (100 μM) plus IND (10 μM) were added to MIN6 cells. Data are expressed as mean±SEM of three experiments. Statistical significance of the effects of ETI and NDGA on thapsigargin-induced cell death are depicted (#$P < 0.01$ compared with thapsigargin alone). The level of statistical significance between the viability in untreated control cells and cells treated with thapsigargin, IND + thapsigargin, or HETE, are indicated (*$P < 0.001$ and **$P < 0.01$).

(Fig. 8 *A*) an effect associated with a threefold increase in the amount of DNA fragmentation (Fig. 8 *B*) and formation of DNA laddering (data not shown). Exposure of mouse islets to 0.5–10 μM thapsigargin caused a progressive, but partial depletion of carbachol-releasible intracellular Ca^{2+} stores determined in the absence of extracellular Ca^{2+} (data not shown).

Discussion

Although increases in $[Ca^{2+}]_i$ have been implicated in apoptotic cell death in a wide variety of cell types, including pancreatic β-cells (38), the mechanisms that trigger programmed cell death in insulin-secreting cells have not been defined clearly. We investigated the effect of chronic Ca^{2+} overloading on MIN6 cell and mouse islet cell death and found that chemical agents capable of producing sustained rises in $[Ca^{2+}]_i$ did not cause a significant decrease in cell viability. However, thapsigargin, CPA, and tBHQ, structurally distinct inhibitors of SERCA, were potent inducers of apoptotic cell death in β-cells. Furthermore, the increase in apoptosis induced by the SERCA blockers was unrelated to changes in $[Ca^{2+}]_i$, but was triggered by a signal that involved activation of the lipoxygenase pathway of arachidonate metabolism consequent to the depletion of ER Ca^{2+} stores.

Inhibition of SERCA activity after the application of 0.1–1 μM thapsigargin causes multiphasic increases in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular Ca^{2+} stores and influx of Ca^{2+} either through voltage-independent Ca^{2+} channels or Ca^{2+} store-operated channels that open in response to depletion of the ER Ca^{2+} stores (20). Previous studies have demonstrated that exposure to thapsigargin induce apoptosis in several cell types, and elevation of $[Ca^{2+}]_i$ is widely considered as an important signal for apoptosis (39). This hypothesis is based on earlier experiments showing blockade of thapsigargin-induced apoptosis by agents that prevent elevation of $[Ca^{2+}]_i$ either by inhibiting Ca^{2+} influx through the plasma membrane (40) or by chelating cytosolic Ca^{2+} with BAPTA, a Ca^{2+} buffer (41).

Our data in MIN6 cells, however, suggest that the thapsigargin-induced increase in β-cell death is not due to an increase in $[Ca^{2+}]_i$. Incubation of MIN6 cells with nitrendipine, a blocker of L-type Ca^{2+} channels, SKF-96365 or DPC, blockers of Ca^{2+} store-operated channels, removal of extracellular Ca^{2+} with EGTA, or chelation of cytosolic Ca^{2+} with BAPTA, failed to inhibit thapsigargin-induced apoptosis. These findings are in accord with studies in human hepatoma cells (42), cerebellar granule neurons (43), and S49 mouse lymphoma cells (39), in which the rises in $[Ca^{2+}]_i$ produced by thapsigargin were dissociated from induction of apoptotic cell death. In fact, the MIN6 cells were resistant to cell death consequent to Ca^{2+} overload; exposure to agents that cause prolonged increases in β-cell $[Ca^{2+}]_i$ did not cause cell death.

Consistent with the well-characterized inhibitory actions of thapsigargin on SERCA (19, 30), our findings suggest that thapsigargin-induced apoptosis is triggered by depletion of ER Ca^{2+} stores. SERCA, a critical regulatory component of Ca^{2+} signaling and Ca^{2+} homeostasis, uses the energy derived from hydrolysis of ATP to transport cytosolic Ca^{2+} against a concentration gradient into the lumen of the ER. In a wide variety of cell types, inhibiting SERCA activity with thapsigargin, or with structurally unrelated SERCA antagonists, CPA or tBHQ, rapidly depletes the mobilizable Ca^{2+} pool in the ER (44). In this study, we assessed the amount of depletion of the ER Ca^{2+} stores induced by thapsigargin by monitoring $[Ca^{2+}]_i$ responses evoked by carbachol and ionomycin in the absence of extracellular Ca^{2+}. Two possible mechanisms might explain the inhibition of carbachol-induced intracellular Ca^{2+} mobilization by thapsigargin: (*a*) inhibition of IP_3-dependent signaling consequent to muscarinic receptor stimulation, or (*b*) depletion of Ca^{2+} stores mobilizable by carbachol. Since the discharge of intracellular Ca^{2+} pools by ionomycin does not depend on IP_3, our results with ionomycin suggest that the inhibitory effect of thapsigargin on carbachol-stimulated Ca^{2+} release (Fig. 6, *A* and *B*) was most likely due to the depletion of mobilizable Ca^{2+} stores, rather than defects in IP_3 formation or binding of IP_3 to its receptor. These conclusions are consistent with other studies that reveal no effect of thapsigargin on IP_3 formation in insulin secreting cells (45).

Thapsigargin-induced MIN6 cell death was progressive and irreversible; incubation with the SERCA antagonist for 4 h was sufficient to produce a significant and progressively increasing percentage of cells undergoing apoptosis over a subsequent 48-h period. That depletion of intracellular Ca^{2+} stores was sufficient to trigger apoptosis in the β-cells was supported further by the observation that incubation of MIN6 cells with 5 mM EGTA for 48 h caused cell death (Fig. 5 *B*).

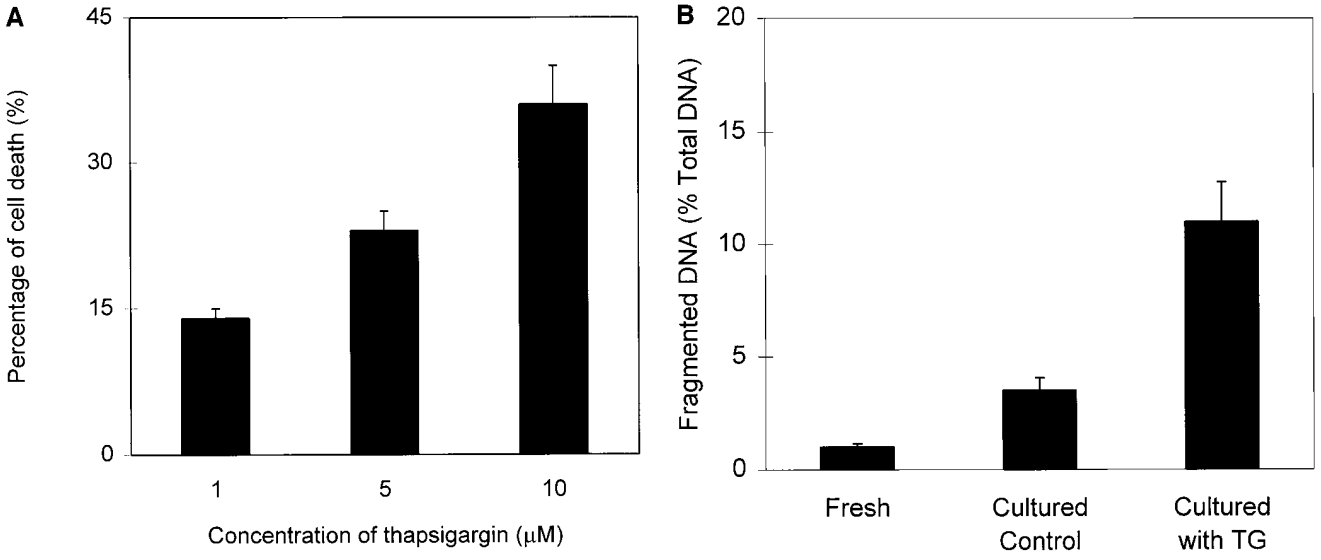

Figure 8. Effect of thapsigargin on viability of mouse islets of Langerhans. (*A*) The effect of a 48-h incubation with different concentrations of thapsigargin on islet cell viability. Results are expressed as mean±SEM of five independent experiments. (*B*) A 48-h exposure to thapsigargin (*TG*; 10 μM) significantly increases the amount of fragmented DNA (*$P < 0.001$ when compared with untreated islets maintained in culture) in isolated from islets. Data are mean±SEM of three experiments.

Previously, we have shown that high extracellular concentration of EGTA depletes β-cell Ca^{2+} pools and activates Ca^{2+} store–operated cation currents (25). Taken together, our findings suggest that a sustained lowering of the ER Ca^{2+} concentration is a signal that initiates apoptosis in β-cells. This premise is in accord with the results of a recent study in which apoptosis in WEHI7.2 lymphoma cells exposed to thapsigargin is not prevented by reducing extracellular Ca^{2+} concentration (which inhibits capacitative entry), but was inhibited by overexpression of Bcl-2 (46). He et al. found that Bcl-2, which localizes to the ER membrane, attenuated thapsigargin-induced cell death by preventing ER Ca^{2+} pool depletion, and suggested that the regulation of Ca^{2+} homeostasis within the ER plays an important role in apoptosis.

The precise identity of the cell death signal distal to thapsigargin-induced β-cell Ca^{2+} store depletion is not known. It is likely that prolonged reduction of the ER lumenal Ca^{2+} concentration is an important proximal event in apoptotic signal transduction. In addition to activating Ca^{2+} store–operated cation currents (23), depletion of ER Ca^{2+} pools by thapsigargin inhibits protein biosynthesis (47), activates phospholipase A_2, and mobilizes arachidonic acid in smooth muscle cells and U937 promonocytes (34, 35). In this study, since cycloheximide significantly reduced cell death induced by thapsigargin, it is unlikely that inhibition of protein biosynthesis stemming from depletion of intracellular Ca^{2+} accounted for apoptosis in MIN6 cells. On the other hand, exogenous application of arachidonic acid while blocking cyclooxygenase, or exposure to 12-HETE, the major metabolite of the lipoxygenase pathway in β-cells did cause significant cell death. A role of the lipoxygenase activation in mediating β-cell apoptosis was further substantiated using ETI and NDGA, inhibitors of lipoxygenase: both were effective blockers of cell death induced by thapsigargin. In contrast, the likelihood that cyclooxygenase activity did not contribute to β-cell death consequent to ER Ca^{2+} depletion was supported by the finding that indomethacin did not suppress thapsigargin-induced apoptosis. Inhibition of cyclooxygenase actually facilitates the conversion of AA to HETE, thereby rendering MIN6 cell sensitive to AA toxicity. These conclusions are in accord with results of previous studies examining mechanisms underlying islet cell death induced by nitric oxide: NDGA fully protected the islet cells from nitric oxide–mediated lysis, whereas, inhibition of cyclooxygenase with indomethacin or acetylsalicylic acid was without effect (48). Furthermore, NDGA has been shown to prevent apoptotic cell death associated with neurotoxicity (49) and CD95 ligand–induced apoptosis of human malignant glioma cells (50). Nevertheless, despite the recent evidence documenting the expression and enzyme activity of Ca^{2+}-independent phospholipase A_2 activity in β-cells (51), the mechanisms controlling this process remain to be determined.

In summary, we have shown that inhibition of SERCA activity with thapsigargin causes apoptotic cell death in MIN6 insulinoma cells and in mouse islet cells. The induction of β-cell apoptosis by thapsigargin did not require increased $[Ca^{2+}]_i$, but likely was initiated by irreversible depletion of ER Ca^{2+} stores and consequent activation of the lipoxygenase pathway of arachidonate metabolism. Our results thus illustrate the critical importance of intracellular Ca^{2+} pools in maintaining β-cell viability. Although previous studies have demonstrated reduced expression of SERCA in islets isolated from the *db*/*db* mouse (22), GK (23), and Zucker diabetic fatty rat (52) models of NIDDM, the role of organelle-sequestered Ca^{2+} pools and β-cell apoptosis in the pathogenesis of insulin-dependent diabetes mellitus and NIDDM is not yet understood. Our findings do suggest, however, that preservation of β-cell mass might be accomplished by targeting regulatory components of ER Ca^{2+} stores or lipoxygenase activity.

Acknowledgments

The authors thank Dr. R.J. Miller and J. Jordan for their help with the Hoechst 33342 staining experiments.

This work was supported by National Institutes of Health grants DK-31842, DK-20595, DK44840, the Blum-Kovler and Mazza Foundations and the Jack and Dollie Galter Center of Excellence of the Juvenile Diabetes Foundation International.

References

1. Wyllie, A.H., J.F.R. Kerr, and A.R. Currie. 1980. Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68:251–305.

2. Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science.* 267:1456–1462.

3. Gottlieb, R.A., K.O. Burleson, R.A. Kloner, B.M. Babior, and R.L. Engler. 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest.* 94:1621–1628.

4. Wertz, I.E., and M.R. Hanley. 1996. Diverse molecular provocation of programmed cell death. *TIBS (Trends Biochem. Sci.).* 21: 359–364.

5. Scaglia, L., F.E. Smith, and S. Bonner-Weir. 1996. Apoptosis contributes to the involution of β-cell mass in the post partum rat pancreas. *Endocrinology*. 136:5461–5468.

6. Kurrer, M.O., S.V. Pakala, H.L. Hanson, and J.D. Katz. 1997. β cell apoptosis in T cell–mediated autoimmune diabetes. *Proc. Natl. Acad. Sci. USA*. 94: 213–218.

7. Lorenzo, A., B. Razzabonl, G.C. Weir, and B.A. Yankner. 1994. Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature.* 369:756– 760.

8. Dypbukt, J.M., M. Ankarcrona, M. Burkitt, A. Sjoholm, K. Strom, S. Orrentius, and P. Nicotera. 1994. Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. *J. Biol. Chem*. 269: 30553–30560.

9. Kaneto, H., J. Fujii, H.G. Seo, K. Suzuki, T.-A., Matsuoka, M. Nakamura, H. Tatsumi, Y. Yamasaki, T. Kamada, and N. Taniguchi. 1995. Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes.* 44:733–738.

10. Kagimot, S., Y. Yamad, A. Kubota, H. Ishida, and Y. Seino. 1996. Apoptotic cell death of β-cells triggered by glibenclamide. *Diabetologia.* 39: A107.

11. Sanchez-Margalet, V., M. Luca, F. Solano, and R. Goberna. 1993. Sensitivity of insulin-secreting RINm5F cells to undergoing apoptosis by the protein kinase C inhibitor staurosporine. *Exp. Cell Res.* 209:160–163.

12. Kaneto, H., J. Fuji, T. Myint, N. Miyazawa, K.N. Islam, Y. Kawasaki, K. Suzuki, M. Nakamura, H. Tatsumi, Y. Yamasaki, and N. Taniguchi. 1996. Reducing sugars trigger oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress through the glycation reaction. *Biochem. J.* 320: 855–863.

13. Loweth, A.C., G.T. Williams, J.H.B. Scarpello, and N.G. Morgan. 1996. Heterotrimeric G–proteins are implicated in the regulation of apoptosis in pancreatic beta-cells. *Exp. Cell Res.* 229:69–76.

14. Hoorens, A., M. van de Casteele, G. Kloppel, and D. Pipeleers. 1996. Glucose promotes survival of rat pancreatic β-cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J. Clin. Invest.* 98: 1568–1574.

15. McConkey, D.J., and S. Orrenius. 1995. The role of calcium in the regulation of apoptosis. *J. Leukocyte Biol.* 59:775–783.

16. Miyazaki, J.-I., K. Araki, E. Yamato, H. Ikegami, T. Asano, Y. Shibasaki, Y. Oka, and K.-J. Yamamura. 1990. Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology.* 127:126–132.

17. Prentki, M., and F.M. Matschinsky. 1987. Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol. Rev*. 67:1185–1248.

18. Ashcroft, F.M., P. Prokes, P.A. Smith, C. Ammala, K. Bokvist, and P. Rorsman. 1994. Stimulus-secretion coupling in pancreatic β-cells. *J. Cell. Biochem*. 55: S54–S65.

19. Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA*. 87:2466–2470.

20. Putney, J.W., Jr., and G.S.J. Bird. 1993. The inositol phosphate-calcium signalling system in non-excitable cells. *Endocr. Rev.* 14:610–631.

21. Berridge, M.J. 1993. Capacitative calcium entry. *Biochem. J.* 312:1–11.

22. Roe, M.W., L.H. Philipson, C.J. Frangakis, A. Kuznetsov, R.J. Mertz, M.E. Lancaster, B. Spencer, J.F. Worley III, and I.D. Dukes. 1994. Defective glucose-dependent endoplasmic reticulum Ca^{2+} sequestration in diabetic mouse islets of Langerhans. *J. Biol. Chem.* 269:18279–18282.

23. Varadi, A., E. Molnar, C.-G. Ostenson, and S.J.H. Ashcroft. 1996. Isoforms of endoplasmic reticulum Ca $^{2+}$-ATPase are differentially expressed in normal and diabetic islets of Langerhans. *Biochem. J.* 319:521–527.

24. Roe, M.W., R.J. Mertz, M.E. Lancaster, J.F. Worley III, and I.D. Dukes 1994. Thapsigargin inhibits the glucose-induced decrease of intracellular Ca^{2+} in mouse islets of Langerhans. *Am. J. Physiol.* 266: E852–E862.

25. Worley, J.F., III, M.S. McIntyre, B. Spencer, and I.D. Dukes. 1994. Depletion of intracellular Ca^{2+} stores activates a maitatoxin-sensitive nonselective cationic current in beta-cells. *J. Biol. Chem*. 269:32055–32058.

26. Lacy, P.E., and M. Kostianovsky. 1967. Method for isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 16:35–39.

27. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *J. Immunol. Methods.* 65:55–63.

28. Villalba, M., J. Bockaert, and L. Journot. 1997. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. *J. Neurosci*. 17:83–90.

29. Clapham, D.E. 1995. Calcium signaling. *Cell.* 80:259–268.

30. Inesi, G., and Y. Sagara. 1992. Thapsigargin, a high affinity and global inhibitor of intracellular Ca^{2+} transport ATPases. *Arch. Biochem. Biophys*. 298: 313–317.

31. Roe, M.W., M.F. Lancaster, R.J. Mertz, J.F. Worley III, and I.D. Dukes. 1993. Voltage-dependent intracellular calcium release from mouse islets stimulated by glucose. *J. Biol. Chem*. 268:9953–9956.

32. McGowan, A.J., M.C. Ruiz-Ruiz, A.M. Gorman, A. Lopez-Rivas, and T.G. Gotter. 1996. Reactive oxygen intermediate(s) (ROI): common mediator(s) of poly(ADP-ribose) poylmerase (PARP) cleavage and apoptosis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 392:299–303.

33. Silva, A.M., L.M. Rosario, and R.M. Santos. 1996. Background Ca^{2+} influx mediated by a dihydropyridine- and voltage-insensitive channel in pancreatic β-cells, modulation by Ni^{2+}, diphenylamine-2-caroxylate, and glucose metabolism. *J. Biol. Chem*. 269:17095–17103.

34. Wolf, M.J., J. Wang, J. Turk, and R.W. Gross. 1997. Depletion of intracellular calcium stores activates smooth muscle cell calcium-independent phospholipase A_2, a novel mechanism underlying arachidonic acid mobilization. *J. Biol. Chem*. 272:1522–1526.

35. Rzigalinsky, B.A., P.F. Blackmore, and M.D. Rosenthal. 1996. Arachidonate mobilization is coupled to depletion of intracellular calcium stores and influx of extracellular calcium in differentiated U937 cells. *Biochim. Biophys. Acta.* 1299:342–352.

36. Tang, D.G., Y.Q. Chen, and K.V. Honn. 1996. Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. *Proc. Natl. Acad. Sci. USA*. 93:5241–5246.

37. Ma, Z., S. Ramanadham, J.A. Corbett, A. Bohrer, R.W. Gross, M.L. McDaniel, and J. Turk. 1996. Interleukin-1 enhances pancreatic islet arachidonic acid 12-lipoxygenase product generation by increasing substrate availability through a nitric oxide–dependent mechanism. *J. Biol. Chem.* 271:1029–1042.

38. Juntti–Berggren, L., O. Larsson, P. Rorsman, C. Ammala, K. Bokvist, K. Wahlander, P. Nicotera, J. Dypbukt, S. Orrenius, A. Hallberg, and P.-O. Berggren. 1994. Increased activity of L-type Ca^{2+} channels exposed to serum from patients with type I diabetes. *Science.* 261:86–90.

39. Bian, X., F.M. Hughes, Jr., Y. Huang, J.A. Cidlowski, and J.W. Putney, Jr. 1997. Roles of cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores in induction and suppression of apoptosis in S49 cells. *Am. J. Physiol.* 272:C1241–C1249.

40. Jiang, S., S.C. Chow, P. Nicotera, and S. Orrenius. 1994. Intracellular Ca^{2+} signals activates apoptosis in thymocytes: studies using the Ca^{2+}-ATPase inhibitor thapsigargin. *Exp. Cell Res*. 212:84–92.

41. Furuya, Y., P. Lundmo, A.D. Short, D.L. Gill, and J.T. Issaacs. 1994. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. *Cancer Res*. 54:6167–6175.

42. Kaneko, Y., and A. Tsukamoto. 1994. Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells. *Cancer Lett.* 97:147–155.

43. Levick, V., H. Coffey, and S.R. D'Mello. 1995. Opposing effects of thapsigargin on the survival of developing cerebellar granule neurons in culture. *Brain Res*. 676:325–335.

44. Mason, M.J., C. Garcia-Rodriguez, and S. Grienstein. 1991. Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane: comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4,-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* 266: 20856–20862.

45. Gilon, P., M. Nenquin, and J.-C. Henquin. 1995. Muscarinic stimulation exerts both stimulatory and inhibitory effects on the concentration of cytoplasmic Ca^{2+} in the electrically excitable pancreatic β-cell. *Biochem. J.* 311:259–267.

46. He, H., M. Lam, T.S. McCormick, and C.W. Distelhorst. 1997. Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2. *J. Cell Biol.* 138:1219–1228.

47. Guest, P.C., E.M. Bailyes, and J.C. Hutton. 1997. Endoplasmic reticulum Ca^{2+} is important for the proteolytic processing and intracellular transport of proinsulin in the pancreatic beta-cell. *Biochem. J.* 323:445–450.

48. Burkart, V, and H. Kolb. 1993. Protection of islet cells from inflammatory cell death in vitro. *Clin. Exp. Immunol.* 93:273–278.

49. Rothman, S.M., K.A. Yamada, and N. Lancaster. 1993. Nordihydroguaiaretic acid attenuates NMDA neurotoxicity-action beyond the receptor. *Neuropharmacology.* 32:1279–1288.

50. Wagenknecht, B., E. Gulbins, F. Lang, J. Dichgans, and M. Weller. 1997. Lipoxygenase inhibitors block CD95 ligand-mediated apoptosis of human malignant glioma cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 409:17–23.

51. Ma, Z., S. Ramanadham, K. Kempe, X.S. Ci, J. Ladenson, and J. Turk. 1997. Pancreatic islets express a Ca^{2+}-independent phospholipase A_2 enzyme that contains a repeated structural motif homologous to the integral membrane protein binding domain of ankyrin. *J. Biol. Chem.* 272:11118–11127.

52. Tokuyama, Y., J. Sturis, A.M. Depaoli, J. Takeda, M. Stoffel, J. Tang, X. Sun, K.S. Polonsky, and G.I. Bell. 1995. Evolution of beta-cell dysfunction in the male Zucker diabetic fatty rat. *Diabetes.* 44: 1447–1457.