Role of calcium-independent phospholipase A2β in human pancreatic islet β-cell apoptosis

Xiaoyong Lei,1 Sheng Zhang,2 Alan Bohrer,2 Suzanne E. Barbour,3 and Sasanka Ramanadham1

1Department of Cell, Developmental, and Integrative Biology and Comprehensive Diabetes Center, University of Alabama at Birmingham, Birmingham, Alabama; and 2Department of Medicine, Mass Spectrometry Resource and Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, St. Louis, Missouri; and 3Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, Virginia

Submitted 8 May 2012; accepted in final form 9 October 2012

Lei X, Zhang S, Bohrer A, Barbour SE, Ramanadham S. Role of calcium-independent phospholipase A2β in human pancreatic islet β-cell apoptosis. Am J Physiol Endocrinol Metab 303: E1386–E1395, 2012.—Death of β-cells due to apoptosis is an important contributor to β-cell dysfunction in both type 1 and type 2 diabetes mellitus. Previously, we described participation of the Group VIA Ca2+-independent phospholipase A2 (iPLA2) in apoptosis of insulinoma cells due to ER stress. To examine whether islet β-cells are similarly susceptible to ER stress and undergo iPLA2-mediated apoptosis, we assessed the ER stress response in human pancreatic islets. Here, we report that the iPLA2β protein is expressed predominantly in the β-cells of human islets and that thapsigargin-induced ER stress promotes β-cell apoptosis, as reflected by increases in activated caspase-3 in the β-cells. Furthermore, we demonstrate that ER stress is associated with increases in islet iPLA2β message, protein, and activity. iPLA2β-dependent induction of neutral sphingomyelinase and ceramide accumulation, and subsequent loss of mitochondrial membrane potential. We also observe that basal activated caspase-3 increases with age, raising the possibility that β-cells in older human subjects have a greater susceptibility to undergo apoptotic cell death. These findings reveal for the first time expression of iPLA2β protein in human islet β-cells and that induction of iPLA2β during ER stress contributes to human islet β-cell apoptosis. We hypothesize that modulation of iPLA2β activity might reduce β-cell apoptosis and this would be beneficial in delaying or preventing β-cell dysfunction associated with diabetes.

Address for reprint requests and other correspondence: S. Ramanadham, Dept. of Cell, Developmental, and Integrative Biology, Univ. of Alabama at Birmingham, 1205 Shelby Biomedical Research Bldg., 1825 University Blvd., Birmingham, AL 35294 (e-mail: sramvem@uab.edu).

DIABETES MELLITUS (DM) is the most prevalent human metabolic disease, and it results from loss and/or dysfunction of β-cells in pancreatic islets. Type 2 diabetes mellitus (T2DM) results from a progressive decline of β-cell function and chronic insulin resistance and accounts for 90–95% of diagnosed cases of diabetes (9). Autopsy studies indicate that β-cell mass in obese T2DM patients is smaller than in obese nonobdiabetic subjects and that the loss of β-cell function in nonobese T2DM is associated with decreases in β-cell mass (4). Experimental and clinical studies reveal that the decrease in β-cell mass in T2DM is a consequence of increased β-cell apoptosis (3, 41). Furthermore, cytokine-mediated β-cell apoptosis contributes to the development of autoimmune type 1 diabetes mellitus (T1DM) (36), and this form of diabetes accounts for 5–10% of diagnosed cases of diabetes. β-Cell apoptosis also limits the feasibility of islet transplantation as a potential cure for T1DM (8). These observations raise the need to gain a better understanding of the molecular mechanisms underlying β-cell apoptosis.

Prolonged ER stress induces stress factors and the caspase (casp) signaling cascade, which ultimately leads to activation of casp-3, a protease that is central to the execution of apoptosis (7). The association of ER stress-induced β-cell apoptosis in experimental and clinical diabetes settings (10, 35, 42, 47) raises the likelihood that ER stress is a critical factor in the onset and progression of DM. Thapsigargin, which depletes ER Ca2+ stores by inhibiting sarcoendoplasmic reticulum Ca2+-ATPase (SERCA), is widely used to induce ER stress in vitro. Our work revealed that exposure of rat pancreatic islets to thapsigargin promotes hydrolysis of arachidonic acid (AA) from β-cell membrane phospholipids and that such AA release is suppressed by inactivation of Ca2+-independent phospholipase A2β (iPLA2β) (33).

As a member of the diverse family of phospholipases A2β (PLA2s), the iPLA2β catalyzes hydrolysis of the sn-2 substituent from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid. iPLA2β is implicated in multiple biological processes, and this is most likely facilitated by unique features in its protein sequence (27, 40). These include ankyrin repeats, casp-3 consensus sequence, bipartite nuclear localization sequence, calmodulin-binding domain, and acyl-CoA esterase activity, and the iPLA2β gene contains a sterol regulatory element. iPLA2β is recognized to play a role in phospholipid remodeling and signal transduction in the central nervous, musculoskeletal, cardiovascular, and immune systems.

Our recent findings in rodent insulinoma cells reveal participation of iPLA2β in ER stress-induced apoptosis. To determine whether islet β-cells are susceptible to ER stress and whether the subsequent β-cell apoptosis occurs via an iPLA2β-mediated pathway, we assessed the ER stress response in human pancreatic islets.

MATERIALS AND METHODS

Materials. This study was approved by the Institutional Review Boards of the Washington University School of Medicine, St. Louis, MO, and the University of Alabama at Birmingham, Birmingham, AL, under the designation of Not Human Subjects Research. Human islets were obtained through the Islet Cell Resource Centers for Islet Distribution Program, the Juvenile Diabetes Research Foundation, and the University of Alabama at Birmingham (UAB) Islet Resource Facility. The islets were isolated at various procurement centers from subjects with the following features: males: n = 34, age = 37.2 ± 1.8 yr, BMI = 28.2 ± 0.90, islet viability = 90 ± 1%, and islet purity = 50%.
PVDF membrane (Millipore, Billerica, MA); DIOC(6)3, Slow Fade Carlsbad, CA); FITC-conjugated donkey anti-guinea pig IgG (Jackson, Minneapolis, MN); Accumax (Innovative Cell Technologies, San Diego, Ft. Washington, PA); DNase-free RNase A (Gentra Systems, Minnetonka, CA); paraformaldehyde (Electron Microscopy Sciences, Ann Arbor, MI); Coomassie reagent, SDS-PAGE supplies, and Triton (Avanti Polar Lipids, Alabaster, AL); (PLPC, 55 mCi/mmol), rainbow molecular mass standards, and enzyme for iPLA2 (LCS), 2

Other materials obtained were as follows: (16:0/14[18]C)18:2-GPC (PLPC, 55 mCi/mmol), rainbow molecular mass standards, and enzyme for iPLA2 (LCS), 2

Casp-3 (Cell Signaling Technology, Danvers, MA); mitochondrial membrane potential detection kit (Cell Technology, Mountain View, CA); paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA); DNase-free RNase A (Genta Systems, Minneapolis, MN); Accumax (Innovative Cell Technologies, San Diego, CA); Alexa Fluor 594 goat anti-rabbit and SuperScript II and SYBR Green PCR kits and SYBR Gold nucleic acid gel stain (In Vitrogen, Carlsbad, CA); FITC-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratory, West Grove, PA); RT-PCR reagents (Invitrogen, Carlsbad, CA); 1° antibody for insulin, Immobilon-P PVDF membrane (Millipore, Billerica, MA); DIO6C(6), Slow Fade light antifade kit (Molecular Probes, Eugene, OR); RNasey kit (Qiagen, Valencia, CA); TUNEL kit (Roche Diagnostic, Indianapolis, IN); 1° antibody for iPLA2-β, goat anti-guinea pig IgG-TR for insulin and Alexa Fluor for glucagon, 20 µl of 1:100, 2–3 h), washed with PBS (3 × 10 min), stained with TUNEL reagent (2 h), washed with PBS (3 × 10 min), covered with Vector shield mounting medium containing DAPI, and sealed with a coverslip using nail polish. Fluorescence was recorded using a Nikon Eclipse TE300 microscope, and images were captured at ×20 magnification.

Immunostaining of islet sections. Islets were fixed in 10% formalin containing 5 µl of tissue-marking dye prior to addition of low-melting agarose (150 µl). The mixture was spun down quickly to settle the islets at one surface of the agarose, which was then allowed to solidify. The islet-containing blocks were then processed, and paraffin sections (8–10 µm) were prepared for immunostaining for insulin, iPLA2-β, and activated (cleaved) casp-3 (aC3). The sections were incubated overnight with 1° antibodies (1:25), washed with PBS (4 × 30 min), incubated for 2–3 h, followed by 2° antibodies (goat anti-guinea pig IgG-TR for insulin and Alexa Fluor for glucagon, 20 µl of 1:100, 2–3 h), washed with PBS (3 × 10 min), rinsed with PBS, and counterstained with 1 µg/ml DAPI in PBS for 10 min to identify cellular nuclei. Incidence of apoptosis was assessed under a fluorescence microscope using a FITC filter. Islets with TUNEL-positive nuclei were considered apoptotic. DAPI staining was used to determine the total number of islet cells in a field. A minimum of six fields per slide was used to calculate the percentage of apoptotic islet cells.

Immunoblotting analyses. Human islets were harvested at various times (0–48 h) following induction of ER stress and sonicated to obtain an islet homogenate. An aliquot (containing 30 µg of protein) of homogenate was analyzed by SDS-PAGE (8 or 15%), transferred onto Immobilon-P PVDF membranes, and processed for immunoblotting analyses as described (38). The targeted proteins and the antibody (1:20) concentrations were iPLA2-β, GRP78, pancreatic ER kinase (PERK), and phosphorylated (p)PERK (1:1000/5,000). Tubulin (1,2,000/1:10,000) was used as loading control. Immunoreactive bands were visualized by enhanced chemiluminescence.

In situ detection of DNA cleavage by terminal deoxynucleotidyl-mediated dUTP nick-end labeling staining. Islets were harvested and washed twice with ice-cold phosphate-buffered saline (PBS), immunobilized on slides by cytospin, and fixed with 4% paraformaldehyde [in PBS, pH 7.4, for 1 h at room temperature (rt)]. The islets were then washed with PBS and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate in PBS for 30 min at rt). The permeabilization solution was then removed, terminal deoxynucleotidyl-mediated dUTP nick-end labeling (TUNEL) reaction mixture (50 µl) was added, and the islets were incubated (1 h, 37°C) in a humidified chamber. The islets were washed again with PBS and counterstained with 1 µg/ml DAPI in PBS for 10 min to identify cellular nuclei. Incidence of apoptosis was assessed under a fluorescence microscope using a FITC filter. Islets with TUNEL-positive nuclei were considered apoptotic. DAPI staining was used to determine the total number of islet cells in a field. A minimum of six fields per slide was used to calculate the percentage of apoptotic islet cells.

iPLA2-β enzymatic activity assay in islets. Cytosol was prepared from human islets, as described previously (38). Protein concentration was determined and iPLA2-β catalytic activity (in a 30-µg protein aliquot) was assayed and quantified, as described (38).

Immunostaining of islet sections. Islets were dispersed into cells using Accumax solution (1 ml, 37°C, 1 h), cytosplun on glass slides and dried [rt, overnight (O/N)]. The cells were then fixed (4% paraformaldehyde, 60 min), permeabilized (0.1% Triton X-100 + 0.1% sodium citrate, 30 min), washed with PBS, blocked (5% FBS + 3% BSA + 0.5% Tween-20 in TBS, 1 h), and washed. The cells were then incubated with 1° antibodies (1:25, O/N) for insulin or glucagon, washed with PBS (4 × 30 min), incubated with 2° antibodies (goat anti-guinea pig IgG-TR for insulin and Alexa Fluor for glucagon, 20 µl of 1:100, 2–3 h), washed with PBS (3 × 10 min), stained with TUNEL reagent (2 h), washed with PBS (3 × 10 min), covered with Vecta shield mounting medium containing DAPI, and sealed with a coverslip using nail polish. Fluorescence was recorded using a Nikon Eclipse TE300 microscope, and images were captured at ×20 magnification.

Casp-3 activity. To obtain a quantitative measurement of apoptosis in islet sections, aC3 activity was assayed according to the manufacturer’s instructions (Sigma Chemical). Islets were harvested, resuspended in 1× lysis buffer (100 µl/200 islets, 20 min), sonicated, and centrifuged at 18,000 g for 20 min at 4°C. The supernatants were then collected for the activity assay, which is based on measurement of aC3-catalyzed generation of p-nitroaniline (pNA) from Ac-DEVD-pNA or Ac-DEVD-pNA and cell homogenate or casp-3 positive control was incubated (3 h, 37°C, total volume of 1 ml) with 1× assay buffer and the casp-3 substrate. The samples were then transferred to 1-ml quartz cuvettes, and the absorbance of released pNA was read at 405 nm. The assay was performed using a 96-well plate reader and the casp-3 inhibitor (Ac-DEVD-CHO, 200 µM) was used to verify aC3 activity.

Ceramide and sphingomyelin analyses by electrospray ionization/mass spectrometry. Lipids were extracted from islets under acidic conditions as described (17, 18). Briefly, islets were harvested and gently pelleted, and extraction buffer (chloroform-methanol-2% acetic acid, 2:2:1.8, vol/vol/vol) was added. Internal standards C8-ceramide (m/z 432, 500 ng) and 14:0/14:0-GPC (m/z 684, 8 µg) were added to the islet pellet, and the relative abundances
Expression of iPLA2β in human islet β-cells. Previously, we reported that rodent and human islets express iPLA2β message (2, 26, 28) and that rodent islet β-cells, but not non-β-cells, express iPLA2β catalytic activity (14). To establish the potential role of iPLA2β in islet β-cell apoptosis, we examined whether iPLA2β protein is indeed expressed in human β-cells. Human islet sections were stained for cell nuclei (D), insulin (I), and iPLA2β, and the individual and merged images are presented in Fig. 1A. Prominent iPLA2β green and insulin red fluorescence are seen in the islet, and the merged image reveals an association of iPLA2β with insulin-containing cells but not with other cells. These findings indicate that iPLA2β is expressed predominantly in islet β-cells and strengthen the likelihood that it has a functional role in the β-cell.

Thapsigargin induces ER stress factors in human islets. The freshly obtained human islets, procured from autopsied sub-

of individual ceramide and sphingomyelin species, relative to the respective internal standard, were measured by electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) and quantified relative to lipid phosphorous as described (22–24). Because we observed subject-to-subject variation in basal ceramide and sphingo-

myelin mass in human islets, the values for each subject were normalized to corresponding vehicle control, and the data are presented as percent increase relative to control. Basal ceramide and sphingomyelin values ranged from 4 to 24 nmol/nmol PO₄⁻⁻ and 123–409 pmol/nmol PO₄⁻⁻, respectively.

Assessment of mitochondrial membrane potential. Loss of mito-

chondrial membrane potential (ΔΨ) is an important step in the induction of cellular apoptosis (12). Following vehicle or thapsigargin treatment, islets were dispersed into cells by incubating them in equal volumes of PBS and Accumax reagent (2 h, 37°C). Islet cell ΔΨ was determined using a commercial kit by flow cytometry (BD Biosciences, San Jose, CA) as described (24). Briefly, dispersed cells were washed once with PBS and resuspended in 100 μl of PBS (~1 × 10⁵ cells/ml). An aliquot (5 μl) of Mito Flow fluorescent reagent was added, and the cell suspension was incubated at 37°C for 30 min. The cells were then transferred to appropriate fluorescence-activated cell-sorting tubes and diluted 1:5 with a buffer provided in the kit. Cell fluorescence was analyzed by flow cytometry (BD Biosciences, San Jose, CA) at an excitation wavelength of 488 nm. Although these analyses yielded qualitatively interpretable results, they were hampered by the tendency of the cells to clump together, causing the fluorescence peaks to be broad. To address this issue, a second method to monitor ΔΨ was established to substantiate the findings from flow cytometry analyses. Dispersed cells were incubated with a mitochondria-associated DIOC(6)3 stain (175 nM) for 15 min under an atmosphere of 5% CO₂-95% air (37°C). Hoechst (5 μg/ml) was then added to stain cell nuclei. After 20 min, the cells were cytospun onto glass slides and examined using a Zeiss confocal laser-scanning microscope at excitation/emission wavelengths of 488/405 nm.

Quantitative RT-PCR. To determine message expression, total RNA was isolated from human islets using the RNeasy kit, and cDNA was synthesized using the SuperScript III kit. PCR amplifications were performed using the SYBR Green PCR kit in an ABI 7000 detection system (Applied Biosystems). The primers were designed on the basis of known human sequences for iPLA2β (Gene ID 8398), NSMase (Gene ID 6610), and internal control 18S (Gene Bank M100981). The sense/antisense primer sets are as follows: iPLA2β, gcagacctgcgcaaatccccctct-gagagaacttca; NSMase, ggctgctgcctgctgaa/gcccttgaagtcccgagttt; 18S, gc-gcctagaggtttaattcctcattctggaatctcctt-gagagaacttca. As seen, TUNEL-positive cells did not costain with glucagon-containing cells but did costain with insulin-containing cells, as reflected by visualization of merged yellow fluorescence (Fig. 1C, right). These findings reveal that prolonged thapsigargin-induced ER stress promotes apoptosis and that the predominant cells in the islet that undergo apoptosis due to ER stress are the β-cells.

Thapsigargin-induced ER stress causes apoptosis of human islet β-cells. To determine whether induction of ER stress with thapsigargin promotes apoptosis, whole intact islets treated with vehicle (DMSO) or thapsigargin were assessed for TUNEL positivity. As seen in Fig. 1C, left, TUNEL fluorescence increased significantly (P < 0.05) in islets treated with thapsigargin (8 ± 1%) relative to DMSO-treated controls (0.5 ± 0.01%). Pretreatment of islets with S-BEL to inactivate iPLA2β resulted in significant (P < 0.05) attenuation of TUNEL positivity due to thapsigargin (0.8 ± 0.10%). Although this protocol provided evidence of ER stress-induced apoptosis and its suppression by iPLA2β inactivation, it did not identify which islet cells undergo apoptosis. To address this, human islets were dispersed into single cells following exposure to thapsigargin. The cells were then loaded with DAPI (D) and TUNEL (T) stains to identify cell nuclei and apoptotic cells, respectively, and costained for insulin (I) or glucagon (G) detection. The individual and merged (D + G + T and D + I + T) images are presented in Fig. 1C, right. As seen, TUNEL-positive cells did not costain with glucagon-containing cells but did costain with insulin-containing cells, as reflected by visualization of merged yellow fluorescence (Fig. 1C, right). These findings reveal that prolonged thapsigargin-induced ER stress promotes apoptosis and that the predominant cells in the islet that undergo apoptosis due to ER stress are the β-cells.

Thapsigargin-induced ER stress promotes iPLA2β-mediated casp-3 activation in human islet β-cells. To verify that the β-cells are the most sensitive of islet cell stress to ER stress, paraffin sections were prepared from islets following treatment with thapsigargin. To assess a role for iPLA2β in this process, islets were cultured in the absence and presence of S-BEL. The sections were then stained for aC3, insulin (I), and cell nuclei (D), and the individual and merged images are shown in Fig. 2A. Exposure to thapsigargin (Fig. 2A, top) promoted cleavage of casp-3 to aC3, as reflected by the bright staining above background fluorescence (Fig. 2A, top, image of aC3 alone). Costaining for insulin revealed nearly complete overlapping of aC3 with insulin-containing cells (I + aC3). In contrast, in islets in which iPLA2β was inactivated with S-BEL, there was minimal evidence of aC3 (Fig. 2A, bottom). These findings are supported by enzymatic assays (Fig. 2B), which reveal increased aC3 activity following exposure to thapsigargin and its inhibition in islets pretreated with S-BEL. Complete inhibition of thapsigargin-induced activity in the presence of casp-3 inhibitor Ac-DEVD-CHO verified that the measured activity was manifested by cleaved casp-3 (data not shown). Because of the difficulty in quantifying TUNEL-positive cells in whole intact islets, we reason that the aC3 enzymatic assay is a more reliable quantitative measure of apoptosis. These findings suggest that ER stress induces human islet β-cell apoptosis that is mediated by iPLA2β activation.
Intriguingly, during the course of our studies an age-related effect on endogenous αC3 activity was revealed (Fig. 2C). Linear regression analyses revealed a significant correlation between donor age and islet αC3 expression (17–61 yr, $r = 0.82$ and $P < 0.05$; and 21–61 yr, $r = 0.94$ and $P < 0.005$). This supports the possibility that susceptibility to β-cell apoptosis increases with age. Consistent with the immunofluorescence analyses, a significant increase in mean thapsigargin-induced casp-3 activity was evident relative to vehicle-treated controls (Fig. 2D). Furthermore, paired comparison of αC3 activity in individual preparations at each age revealed a significant increase at both 24 ($P = 0.0021$) and 48 h ($P = 0.0098$) of thapsigargin treatment relative to corresponding endogenous activity. This is evidence for susceptibility of islets, irrespective of the age of donor, to ER stress. However, to circumvent potential influence of high endogenous αC3, subsequent analyses were performed in islets obtained from donors between the ages of 20 and 30 yr.

**Thapsigargin-induced ER stress induces iPLA2β in islets.** We next examined whether exogenous exposure to an ER stressor agent induces iPLA2β in islets. Total RNA was isolated from the islets following exposure to thapsigargin and processed for quantitative (q)RT-PCR analyses of iPLA2β. Such analyses revealed a temporal increase in iPLA2β mRNA (Fig. 3A). As might be expected, S-BEL did not have a significant effect on iPLA2β message (not shown) or protein (Fig. 3A, top). As shown in Fig. 3B, islet cytosol-associated iPLA2β enzymatic activity was significantly inhibited by S-BEL and increased nearly fourfold after 16 h of thapsigargin treatment, consistent with the increase in iPLA2β mRNA (Fig. 3A). The ER stress-induced activity was
also significantly inhibited by S-BEL (Fig. 3B), suggesting that this increase in PLA2 activity is indeed manifested by iPLA2/H9252.

Taken together with the observations in the Akita/H9252-cells (22), these findings indicate that ER stress induces iPLA2/H9252 at the message, protein, and activity levels in human islet/H9252-cells.

Thapsigargin-induced ER stress promotes ceramide accumulation in human islets that is suppressed by S-BEL. To examine whether ER stress is associated with ceramide generation in human pancreatic islets, ceramide molecular species were analyzed by ESI/MS/MS. The fatty amide substituents of the major ceramide species endogenous to human islets are 16:0 (m/z 544), 18:0 (m/z 572), 20:0 (m/z 600), 22:0 (m/z 628), 24:1 (m/z 654), and 24:0 (m/z 656) (Fig. 4A). As illustrated in Fig. 4B, thapsigargin exposure for 24 h resulted in increases in ceramides 16:0, 24:1, and 24:0 in particular, and these were all restored to basal levels in islets in which iPLA2/H9252 was inactivated. These findings suggest that ceramide generation due to ER stress in pancreatic islets occurs via an iPLA2/H9252-dependent pathway.

Thapsigargin-induced ER stress reduces sphingomyelins in human islets by inducing neutral sphingomyelinase in an iPLA2/H9252-dependent manner. Analyses of sphingomyelins (SMs) in islets revealed a decrease (by 35 ± 16%; n = 3) in the pool of 16:0-SM, 24:1-SM, and 24:0-SM following 24-h exposure to thapsigargin relative to vehicle-treated islets. These SM mo-
were pretreated with either vehicle (EtOH) or washed, and then treated with vehicle (DMSO) or T (2 μM). iPLA2 message and protein. The islets were harvested at various times, and total RNA was prepared for quantitative (q)RT-PCR analyses of iPLA2 message. Cytosol-associated iPLA2 activity was measured as means ± SE. *Significantly different from DMSO vehicle group, P < 0.05; n = 7–15. †Significantly different from T group, P < 0.05; n = 4.

Fig. 3. T-induced ER stress promotes iPLA2β expression and activity in human islets. Islets were cultured O/N at 37°C under an atmosphere of 5% CO2-95% air and then treated with either vehicle (EtOH) or S-BEL (10 μM) for 1 h, washed, and then treated with vehicle (DMSO) or T (2 μM). A: iPLA2β message and protein. The islets were harvested at various times, and total RNA was prepared for quantitative (q)RT-PCR analyses of iPLA2β. *Significantly different from corresponding 0-h group, P < 0.05; n = 3–5. A: top: the islets were pretreated with either vehicle (EtOH) or S-BEL (B; 10 μM) for 1 h, washed, and then treated with vehicle (DMSO) or T (2 μM). iPLA2β protein at 16 h was then assessed by immunoblotting analyses. B: iPLA2β enzymatic activity. Cytosol-associated iPLA2β activity was measured ± S-BEL (10 μM) or following exposure of islets to T for 16 h (the activity values are expressed as means ± SE). *Significantly different from DMSO vehicle group, P < 0.05; n = 7–15. †Significantly different from T group, P < 0.05; n = 4.

The onset and progression of diabetes mellitus is associated with losses in β-cells due to apoptosis (3–6, 13, 30, 31, 37, 41, 45, 47, 48). Therefore, it is important to gain a better understanding of the processes that lead to apoptotic β-cell death so that more targeted therapeutic measurements can be used to prevent or delay this process. β-Cell apoptosis due to ER stress is a recognized contributor to the development of diabetes mellitus in both experimental and clinical settings (13, 34, 35, 42, 45, 47). However, very little is known about the underlying cellular events that are triggered by ER stress and lead to β-cell apoptosis.

Our previously published studies indicate that ER stress-induced apoptosis occurs via a iPLA2β-mediated pathway (21, 25). This conclusion was based on comparing findings from empty vector-expressing and iPLA2β-overexpressing INS-1 cells following exposure to thapsigargin. Conclusions drawn from the INS-1 cells (23, 24, 38) were subsequently confirmed in mouse Akita β-cells, which develop ER stress spontaneously and express higher iPLA2β than wild-type β-cells (22) and thus present a system that does not require chemical induction of ER stress or genetic manipulation of iPLA2β expression. Although these observations are intriguing, if we

Thapsigargin-induced ER stress causes loss in human islet-cell ΔΨ. Loss of ΔΨ is a hallmark of cellular apoptosis, and ceramides generated during ER stress promote mitochondrial abnormalities (22, 24). To assess ΔΨ in human islets, following treatment they were dispersed into single cells, and flow cytometry and DIOC staining protocols were used to monitor ΔΨ in the islet cells. Flow cytometry analyses were done in a suspension of cells stained with Mito Flow. This reagent concentrates in the mitochondria of healthy cells, but mitochondria of cells undergoing apoptosis become compromised and accumulate less of the reagent. This is reflected by a decrease in the fluorescence signal and the appearance of a second peak that is shifted to the left of the original. The spectra (Fig. 5A) reflect fluorescence measurement in 10,000 cells, and the percentage of cells losing ΔΨ, analyzed by the application software, is indicated as M1. Quantification (n = 3–5 in each group) of the M1 peak revealed that ER stress promoted a significantly higher incidence of ΔΨ loss relative to vehicle-treated control cells (Fig. 5A, right). However, inactivation of iPLA2β with S-BEL significantly reduced the incidence of cells losing ΔΨ. Although it might be argued that the dispersed islet cell suspension includes non-β-cells, the finding that iPLA2β is localized to β-cells (Fig. 1) strongly suggests that the loss in ΔΨ and its maintenance following inactivation of iPLA2β predominantly reflect events triggered in β-cells. We further find that inhibition of NSMase with GW-4869 decreases the incidence of islet cells losing ΔΨ, whereas inhibition of the rate-limiting enzyme in de novo ceramide synthesis (serine palmitoyltransferase) with LCS was ineffective. These findings support the notion that ceramides generated via SM hydrolysis but not via de novo synthesis participate in ER stress-induced mitochondrial abnormalities in human islet β-cells and that these events are downstream of iPLA2β.

Because the islet cells had a tendency to clump, the fluorescence peaks tended to be broad even in the vehicle-treated group. Therefore, to verify the loss in ΔΨ during ER stress, islet cell mitochondria were labeled with DIOC(6)3 following thapsigargin treatment and visualized by fluorescence microscopy. The ΔΨ-sensitive DIOC(6)3 fluorochrome accumulates in the mitochondria of healthy cells but is not retained in the mitochondria of compromised cells (11). As seen in Fig. 5B, vehicle-treated cells retained DIOC(6)3 fluorescence, whereas the signal was largely lost in thapsigargin-treated cells, confirming ΔΨ loss due to ER stress in islet cells.

**DISCUSSION**

The onset and progression of diabetes mellitus is associated with losses in β-cells due to apoptosis (3–6, 13, 30, 31, 37, 41, 45, 47, 48). Therefore, it is important to gain a better understanding of the processes that lead to apoptotic β-cell death so that more targeted therapeutic measurements can be used to prevent or delay this process. β-Cell apoptosis due to ER stress is a recognized contributor to the development of diabetes mellitus in both experimental and clinical settings (13, 34, 35, 42, 45, 47). However, very little is known about the underlying cellular events that are triggered by ER stress and lead to β-cell apoptosis.

Our previously published studies indicate that ER stress-induced apoptosis occurs via a iPLA2β-mediated pathway (21, 25). This conclusion was based on comparing findings from empty vector-expressing and iPLA2β-overexpressing INS-1 cells following exposure to thapsigargin. Conclusions drawn from the INS-1 cells (23, 24, 38) were subsequently confirmed in mouse Akita β-cells, which develop ER stress spontaneously and express higher iPLA2β than wild-type β-cells (22) and thus present a system that does not require chemical induction of ER stress or genetic manipulation of iPLA2β expression. Although these observations are intriguing, if we
Fig. 4. iPLA₂β mediates ceramide (CM) generation due to T-induced ER stress. Human islets were cultured O/N at 37°C under an atmosphere of 5% CO₂-95% air and then prepared for electrospray ionization/mass spectrometry/mass spectrometry (ESI/MS/MS) analyses, as described in MATERIALS AND METHODS. 

A: control human islet CM spectrum. B: ER stress-induced CM accumulation ± S-BEL. Islets were treated with vehicle (DMSO; control) or T (2 μM) in the absence or presence of S-BEL (10 μM). At 24 h, the islets were processed for ESI/MS/MS analyses and the CMs quantified relative to total phosphate content. The CM species exhibiting the most prominent fold change relative to control are presented as means ± SE. *T group significantly different from other groups, P < 0.05; n = 3–5. 

C: ER stress-induced neutral sphingomyelinase (NSMase) ± S-BEL. Human islets were cultured O/N at 37°C under an atmosphere of 5% CO₂-95% air and then treated with either vehicle (EtOH) or S-BEL (10 μM) for 1 h, washed, and then treated with vehicle (DMSO) or T (2 μM). The islets were harvested at various times, and total RNA was prepared for qRT-PCR analyses of NSMase (n = 3–5). The fold increase values are expressed as means ± SE. *−S-BEL group significantly different from 0-h group, P < 0.05. #S-BEL group significantly different from corresponding −S-BEL group, P < 0.05. 

D: ER stress-induced aC3 activity ± GW-4869 (GW). Human islets were cultured O/N at 37°C under an atmosphere of 5% CO₂-95% air and pretreated with either vehicle (EtOH) or GW (20 μM) for 1 h, washed, and then treated with vehicle (DMSO) or T (2 μM). The islets were harvested at 16 h and assayed for aC3 activity. *T group significantly different from all other groups, P < 0.05.
T-induced ER stress promotes loss of mitochondrial membrane potential (ΔΨ) in human pancreatic islet cells. Human islets were cultured O/N at 37°C under an atmosphere of 5% CO2-95% air and then treated with either vehicle (DMSO, control) or with T (2 μM) for 24 h and dispersed into individual cells, and ΔΨ was monitored by Mito Flow or DIOC(6)3 staining. A: representative flow cytometry analyses. The spectra were obtained from analyses of 10,000 cells, and the % cells with compromised ΔΨ is indicated by M1. The table at right shows compiled data from 3–5 determinations (values are means ± SE). B: DIOC(6)3 staining. Cells were loaded with blue nuclear (Hoechst; left images) and green mitochondrial [DIOC(6)3; middle images] stains and examined by confocal microscopy. The merged images are shown at right (n = 3–5). *Significantly different from the vehicle control group, P < 0.05. #Significantly different from the T-treated group, P < 0.05.

are to understand the contribution of ER stress to β-cell death in the onset and progression of diabetes, it is necessary to translate these observations in human β-cells. Therefore, we undertook studies to assess the ER stress response in human pancreatic islet β-cells.

In the original characterization of pancreatic iPLA2β (14), we found that rodent islet iPLA2β activity was localized predominantly in β-cells, but not in non-β-cells, and subsequently demonstrated that iPLA2β message is expressed in rodent and human islets (1, 26, 28). These findings raised the possibility of a functional role for iPLA2β in islet β-cells. The likelihood of this is enhanced by the present finding of iPLA2β protein expression in human islet β-cells, and not in non-β-cells, as reflected by its predominant colocalization in insulin-containing cells. To our knowledge, this is the first demonstration of iPLA2β protein expression in human pancreatic islet β-cells, strengthening the possibility that iPLA2β activation contributes to biological processes in human β-cells.

Therefore, we considered the possibility that iPLA2β is involved in β-cell apoptosis secondary to ER stress in human β-cells. Herein, we report that induction of ER stress in human pancreatic islets leads to increased iPLA2β expression, induction of NSMase, ceramide generation, mitochondrial compromise, activation of caspase-3 in β-cells, and β-cell apoptosis. Using islets isolated from human donors, we find that thapsigargin induces ER stress, as evidenced by induction of GRP78 (BiP), a member of the Ca2+-binding chaperone proteins, and subsequent autophosphorylation of PERK. These are characteristics of the unfolded protein response to ER stress (20). As expected, ER stress leads to apoptosis of human islet cells. TUNEL staining of whole islets provided evidence of prolonged ER stress causing islet cell apoptosis. Although this signal was quantified, accurate analyses were limited by visualization of the islets in one plane. Nevertheless, the conspicuous absence of TUNEL-positive cells in islets pretreated with S-BEL provides support to the notion that activation of iPLA2β contributes to islet cell apoptosis. To verify that iPLA2β participates in apoptosis of the islet β-cells, immunostaining protocols were performed using dispersed islet cells and islet sections prepared from islets treated with thapsigargin. These analyses revealed colocalization of TUNEL and insulin staining. Furthermore, activation of the executioner of aC3 was evident only in insulin-containing cells in islet sections, and this was suppressed significantly by inhibition of iPLA2β activity. These observations are consistent with previous reports suggesting that β-cells are particularly sensitive to ER stress (46). Interestingly, PERK-null mice have a normal complement of islet cells, but with time the β-cells diminish, whereas α-cells increase, suggesting that the α-cells are less sensitive to defects in the UPR response (16). Also consistent
with this conclusion are earlier descriptions of expression of iPLA2β activity predominantly in β-cell islets (14) and reversal of ER stress-mediated outcomes in the Akita β-cells by knockdown of iPLA2β with siRNA (22).

Several lines of evidence support our hypothesis that iPLA2β ER stress induced apoptosis of human pancreatic islet β-cells: 1) expression of iPLA2β protein in human islets being limited to insulin-containing cells, 2) ER stress inducing iPLA2β message, protein, and activity in human islets prior to the appearance of ER stress-mediated outcomes, 3) nearly complete overlap of TUNEL positivity and insulin staining in thapsigargin-treated islet cells, 4) exclusive colocalization of aC3 and insulin-containing cells, and 5) suppression of ER stress-mediated increases in mitochondrial dysfunction, NSMase expression, ceramide accumulation, aC3, and β-cell apoptosis following iPLA2β inactivation. Together, these observations suggest that the iPLA2β/ceramide axis pathway contributes to apoptosis of human islet β-cells.

Another novel observation in our studies concerns endogenous aC3 in islets. The human islets were isolated at various islet procurement centers from male and female nondiabetic human donors ranging in age between 17 and 61 yr. Intriguingly, although endogenous aC3 is measurable in all islet preparations, there appears to be an age-dependent increase in aC3. The endogenous expression of aC3 activity is most likely related to the trauma associated with the islet isolation and shipment processes. Nevertheless, our observations suggest that the islet β-cells may become increasingly susceptible to apoptosis with age and that this may be a contributing factor to decreased β-cell function in the elderly (15, 29, 44). Irrespective of endogenous aC3 levels, preparations from all ages respond to ER stress with increases in aC3, which also tends to be higher in the older subjects. Theses findings suggest the importance of considering the age of the donor subjects when assessing functional, survival, or other parameters in human islet β-cells.

In summary, our findings provide evidence for iPLA2β protein expression specifically in islet β-cells, susceptibility of human islet β-cells to ER stress, and a pivotal role for iPLA2β in the consequential β-cell apoptosis via promotion of ceramide generation. We recognize that alternate pathways might contribute to iPLA2β-mediated effects on β-cells, since we described previously that other ER stress (C/EBP homologous protein, caspase-12) and intrinsic apoptotic (cytochrome c and Smac) factors are induced in β-cells by thapsigargin and that their expression is amplified with higher iPLA2β expression (23, 24). Furthermore, a recent study suggests that p38 MAPK is activated downstream of iPLA2β in response to thapsigargin (43). These observations suggest that continued study of the role of iPLA2β in β-cell apoptosis is needed for identification of additional loci of iPLA2β regulation or action that could serve as future targets for therapeutic intervention in the context of preventing or delaying β-cell dysfunction accompanying type 1 and type 2 diabetes.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Karen Goodwin and Ying Gai and advice from the Washington University Diabetes Research and thank the Training Center (DRTC)-supported Morphology and β-cell Morphology Cores and the DRTC at UAB for their support. We also thank the National Institute of Diabetes and Digestive and Kidney Diseases-sponsored Integrated Islet Distribution Program, the Washington University/Juvenile Diabetes Research Foundation (Award no. 31-2008-382 to Dr. Thalachatallour Mohanakumar), and the UAB Islet Resource Facility, supported by UAB DRTC grant (NIH P60-DK-079626), awarded to the UAB Comprehensive Diabetes Center for providing (to approved user S. Ramanadham) the human islets.

GRANTS

This work was supported by grants from the National Institutes of Health (RO1-69455, P01-HL-57278, P41-RR-09545, P60-DK-20579, and P30-DK-56341) and the American Diabetes Association (S. Ramanadham).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.L., S.E.B., and S.R. did the conception and design of the research; X.L., S.Z., A.B., and S.R. performed the experiments; X.L., A.B., and S.R. analyzed the data; X.L., S.E.B., and S.R. interpreted the results of the experiments; X.L., S.Z., A.B., and S.R. prepared the figures; X.L. and S.R. drafted the manuscript; X.L., S.E.B., and S.R. edited and revised the manuscript; X.L., S.E.B., and S.R. approved the final version of the manuscript.

REFERENCES

14. Gross RW, Ramanadham S, Kruzska KK, Han X, Turk J. Rat and human pancreatic islet cells contain a calcium ion independent phospholipase A2 activity selective for hydrolysis of arachidonate which is stim-


