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Research Article

Group VIB Phospholipase A₂ Promotes Proliferation of INS-1 Insulinoma Cells and Attenuates Lipid Peroxidation and Apoptosis Induced by Inflammatory Cytokines and Oxidant Agents

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Group VIB Phospholipase A₂ (iPLA₂γ) is distributed in membranous organelles in which β-oxidation occurs, that is, mitochondria and peroxisomes, and is expressed by insulin-secreting pancreatic islet β-cells and INS-1 insulinoma cells, which can be injured by inflammatory cytokines, for example, IL-1β and IFN-γ, and by oxidants, for example, streptozotocin (STZ) or t-butyl-hydroperoxide (TBHP), via processes pertinent to mechanisms of β-cell loss in types 1 and 2 diabetes mellitus. We find that incubating INS-1 cells with IL-1β and IFN-γ, with STZ, or with TBHP causes increased expression of iPLA₂γ mRNA and protein. We prepared INS-1 knockdown (KD) cell lines with reduced iPLA₂γ expression, and they proliferate more slowly than control INS-1 cells and undergo increased membrane peroxidation in response to cytokines or oxidants. Accumulation of oxidized phospholipid molecular species in STZ-treated INS-1 cells was demonstrated by LC/MS/MS scanning, and the levels in iPLA₂γ-KD cells exceeded those in control cells. iPLA₂γ-KD INS-1 cells also exhibited higher levels of apoptosis than control cells when incubated with STZ or with IL-1β and IFN-γ. These findings suggest that iPLA₂γ promotes β-cell proliferation and that its expression is increased during inflammation or oxidative stress as a mechanism to mitigate membrane injury that may enhance β-cell survival.

1. Introduction

Diabetes mellitus (DM) is the most common human endocrine disease and is reaching pandemic proportions in the US and elsewhere [1]. DM represents a constellation of disorders that are grouped into the major categories types 1 and 2 (T1DM and T2DM). T1DM is caused by autoimmune destruction of insulin-secreting pancreatic islet β-cells [2, 3], and inflammatory cytokines released by invading leukocytes during insulinitis are believed to participate in these processes [4, 5]. Among them are IL-1β, which impairs insulin secretion and inflicts islet injury [6], and IFN-γ, which greatly potentiates the destructive effects of IL-1β [7]. These effects

are mediated in part by induction of nitric oxide (NO) synthase expression and overproduction of NO [8–13], which can induce apoptosis of cells by mechanisms that involve generation of reactive oxygen species that cause oxidative stress [13–15].

T2DM is thought to evolve after a period of initial insulin resistance in which nearly normal glucose tolerance is maintained by compensatory hypersecretion of insulin by β-cells [16, 17]. At some point there is failure to sustain insulin secretion at sufficiently high levels and glucose intolerance and then overt DM ensue [18]. One contributor to the eventual failure of β-cell compensation is a reduction in β-cell mass by 50% or more, and this occurs at least in part

by apoptotic β -cell death [16, 19]. Although many mechanisms probably participate in these processes, production of reactive oxygen species induced by metabolic stress has been proposed to represent a final common pathway of injury that ultimately results in β -cell failure [17, 20–29]. Among supporting observations are that β -cells express low levels of antioxidant defense enzymes compared to other tissues [30–33] and that antioxidant compounds confer protection from glucose toxicity to islets *in vitro* and against development of T2DM in animal models *in vivo* [34, 35].

Beta cells must sustain a high level of metabolic activity, which provides critical signals in the coupling of nutrient sensing to insulin secretion [36–50], in order to meet the unceasing demand for insulin biosynthesis and processing. Prolonged overstimulation of β -cells may eventually contribute to their failure as a consequence of stresses imposed on the endoplasmic reticulum (ER) and mitochondria [51–53]. Protein synthesis, including that of proinsulin, occurs in ER, and nascent proteins must be properly folded, which involves formation of disulfide bonds. This is an oxidative reaction that requires a prooxidant environment to be maintained in ER. Sustained hyperstimulation can result in ER stress and overproduction of reactive oxygen species (ROS) that exceed ER reductive capacity, resulting in ROS leakage from ER and cellular oxidative stress [54–57].

Mitochondrial metabolism is a major source of ROS production via incomplete reduction of molecular oxygen in the respiratory chain to yield superoxide anion ($O_2^{\bullet-}$) [58, 59], and $O_2^{\bullet-}$ production increases with metabolic activity [21, 60]. $O_2^{\bullet-}$ is removed by superoxide dismutase (MnSOD)-catalyzed dismutation to H_2O_2 that can be reduced to H_2O by catalase or by glutathione peroxidase and GSH. If generation exceeds removal, excess H_2O_2 can undergo Fe^{2+} -catalyzed conversion to $\bullet HO$ (Fenton reaction) or to $O_2^{\bullet-}$ and $\bullet HO$ (Haber-Weiss reaction). These ROS can injure mitochondria by mechanisms that include membrane phospholipid peroxidation [14, 61] and activation of stress pathways [27–29]. Mitochondrial phospholipid peroxidation can precipitate cytochrome c release from the inner membrane into the cytosol [14, 62–65], and this can initiate apoptosis [66, 67]. Released cytochrome c interacts with caspase-9 in formation of the apoptosome [68], which leads to activation of the executioner caspases-3, -6, and -7 that dismantle the cell [66, 69].

It has been proposed that phospholipases A_2 (PLA₂) can prevent or abort apoptosis by repairing peroxidized membrane phospholipids [63–65, 70–76]. PLA₂ enzymes catalyze hydrolysis of the *sn*-2 ester bond of glycerophospholipids to yield a free fatty acid and a 2-lysophospholipid, and at least 16 major groups within the PLA₂ superfamily are recognized [77, 78]. Among their proposed functions are the membrane remodeling and the protection or repair of membranes from oxidative damage [63–65, 70–72] in a sequence that involves PLA₂-catalyzed removal of oxidized fatty acid residues [73] to yield a lysophospholipid that can be reacylated with an unoxidized fatty acid to preserve membrane integrity [74].

A PLA₂ is suited for such a role because oxidized fatty acid substituents usually occur at the *sn*-2 position of phospholipids where most polyunsaturated fatty acid (PUFA) substituents, such as linoleate (C18:2) and arachidonate

(C20:4), are esterified [73, 74]. PUFA are especially susceptible to oxidation because they contain *bis*-allylic methylene moieties with a labile H atom that can be abstracted to yield a carbon-centered radical that readily reacts with molecular oxygen to form a fatty acid hydroperoxide [73]. Oxidation reduces hydrophobicity of the *sn*-2 fatty acid substituent and allows it to approach the hydrophilic phospholipid head-group more closely [73]. This increases separation between head groups, which causes the *sn*-2 ester bond to be more accessible to PLA₂. Liberated peroxy fatty acids can then be reduced to alcohols by glutathione peroxidases after release from phospholipids by PLA₂ enzymes [75, 76].

Two members of Group VI PLA₂ family have been suggested to play such a role in repairing oxidized mitochondrial membrane phospholipids [63–65, 70–72]. Group VIA PLA₂ (iPLA₂ β) localizes to mitochondria in insulinoma cells and protects against oxidant-induced apoptosis, and pancreatic islets from iPLA₂ β -null mice exhibit increased susceptibility to oxidant-induced apoptosis [63–65]. Oxidant-induced lipid peroxidation and death of renal proximal tubule cells (RPTC) is potentiated by the Group VI PLA₂ inhibitor bromoenol lactone (BEL) [71], and R-BEL, which selectively inhibits Group VIB PLA₂ (iPLA₂ γ), accelerates oxidant-induced lipid peroxidation and renal cortical mitochondrial injury [72]. Moreover, when small hairpin ribonucleic acid (shRNA) adenovirus is used to reduce RPTC iPLA₂ γ expression, lipid peroxidation and sensitivity to apoptosis induced by the oxidant tert-butyl hydroperoxide (TBHP) increase [70].

The latter observations [70–72] suggest that iPLA₂ γ acts to reduce lipid peroxidation and to protect against oxidant-induced apoptosis in renal proximal tubule cells, and this may reflect iPLA₂ γ -catalyzed removal of oxidized PUFA residues from glycerophospholipids that are formed in mitochondria under conditions of oxidative stress. This could permit the resultant lysophospholipid to be reacylated with an unoxidized PUFA residue, which would restore functions that are impaired as a result of membrane oxidation. In the absence of iPLA₂ γ or when its activity is impaired, this repair mechanism cannot operate fully, and this could result in progressive mitochondrial injury that eventually triggers the mitochondrial pathway of apoptosis [70–72].

Here we have conducted experiments to determine whether iPLA₂ γ might play a similar role in insulin-secreting β -cells because mitochondrial injury induced by oxidative stress appears to be an important mechanism underlying β -cell loss during the development of diabetes mellitus [16–35]. Our studies involved preparation of iPLA₂ γ -knockdown INS-1 insulinoma cell lines in which iPLA₂ γ expression is reduced by stable expression of shRNA and comparing these lines to control INS-1 cell lines for sensitivity to lipid peroxidation and apoptosis induced by the inflammatory cytokines IL-1 β and IFN- γ and the oxidant agents TBHP [70] and streptozotocin (STZ) [79].

2. Materials and Methods

2.1. Materials. Rainbow molecular mass standards, PVDF membranes, and Triton X-100 were obtained from Bio-Rad

(Richmond, CA, USA); SuperSignal West Femto Substrate was from Thermo Fisher; Coomassie reagent and SDS-PAGE supplies were from Invitrogen (Carlsbad, CA, USA); bovine serum albumin (BSA, fatty acid free, fraction V) were from MP Biomedicals (Solon, OH, USA); Streptozotocin (STZ) and tert-butyl hydroperoxide (TBHP) were from Sigma (St. Louis, MO, USA).

2.2. Cell Culture. INS-1 rat insulinoma cells that had been stably transfected and mock-transfected INS-1 cells were generated and cultured in RPMI 1640 medium containing 11 mM glucose, 10% fetal calf serum, 10 mM Hepes buffer, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM β mercaptoethanol, 100 units/mL penicillin, and 100 μ g/mL streptomycin, essentially as previously described [80]. The medium was exchanged every 2 days, and the cell cultures were split once a week. Cells were grown to 80% confluence and harvested after treatment as indicated in the figure legends or the text of the Results section. All incubations were performed at 37°C under an atmosphere of 95% air/5% CO₂.

2.3. Establishing iPLA₂ γ Knockdown INS-1 Insulinoma Cell Lines Using siRNA and a Lentiviral Vector. Two hairpin-forming oligonucleotides directed against iPLA₂ γ mRNA were cloned into FIV H1 Lentivector according to instructions from the manufacturer (SBI System Biosciences, Mountain View, CA, USA) by described procedures [80]. Targeting sequences within the synthetic oligonucleotides are italicized and underlined below. The sequence of the first was 5'-GATCCGCAAGAGTGAGTATTGATAACTTAA-GAGAGTTATCAATACTCACTCTTGCTTTTTT-G-3'. The second oligonucleotide was 5'-GATCCGGGCCATATTAGCATTTCATGCTTCAAGAGAGCATGAATGCTAATATGGCCCTTTTTTG-3'. Constructs that express the shRNAs are designated FIVH1-iPLA₂-1 and FIVH1-iPLA₂-2. Cells were selected with neomycin.

2.4. Immunoblotting Analyses. Cells were harvested and sonicated, and an aliquot (30 μ g) of lysate protein was analyzed by SDS-PAGE (8–12% Tris-Glycine gel, Invitrogen), transferred onto Immobilon-P polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA, USA), and processed for immunoblotting analyses, essentially as previously described [81]. The primary antibody concentration for iPLA₂ γ (Sigma, St. Louis, MO, USA) was 1:500. The secondary antibody concentration was 1:10,000. The concentrations of other antibodies are described in the figure legends. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL).

2.5. Determination of INS-1 Cell Proliferation Rate. INS-1 cell proliferation rates were measured by two approaches, as previously described [80]. One assay is based on fluorescence enhancement when CyQuant GR binds to nucleic acids, which reflects the amount of cell DNA [82]. Cells were seeded onto 96-well plates (3 \times 10³ cells/well). Medium was removed after 1 or 3 days, and cells were frozen (–20°C). DNA was measured with a CyQuant assay kit (Molecular

Probes, Inc., Eugene, OR, USA) with reference to a standard curve. CyQuant GR solution (200 μ L) was added to each well and incubated (5 min, room temperature). Fluorescence was measured on a microplate fluorimeter (excitation, 480 nm; emission, 538 nm). A second assay is based on incorporation of thymidine analog 5-bromo-2'-deoxyuridine (BrdU) into DNA in proliferating cells [83]. Cells were seeded (10⁴ cells/well) and cultured (3 days) before assay with an enzyme-linked immunoassay detection kit III (Roche Applied Science) after BrdU labeling.

2.6. Lipid Peroxidation. Lipid peroxidation was quantitated using a Cayman TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions, as previously described [64, 84]. Lipid peroxides derived from polyunsaturated fatty acids decompose to form a complex series of compounds that include reactive carbonyl species, such as MDA. Measurement of thiobarbituric acid reactive substances (TBARS) by determining absorbance at 530 nm is used to assess the extent of lipid peroxidation [84]. Results are expressed as μ mol/ μ g protein.

2.7. HPLC-ESI-MS/MS Analysis of Oxidized Lipids. Lipids extracted from INS-1 cells were stored in sealed vials (under N₂ at –20°C) to suppress artifactual oxidation, and extracts were then analyzed by LC/MS/MS in a manner similar to that previously described [85] on a Surveyor HPLC (Thermo-Electron, San Jose, CA, USA) using a modified gradient [86] on a C8 column (15 cm \times 2.1 mm, Sigma Chemical Co., St. Louis, MO, USA) interfaced with the ion source of a Thermo-Electron Vantage triple quadrupole mass spectrometer with extended mass range operated in negative ion mode. Tandem MS scans for precursors of m/z 295, m/z 319, and m/z 343 were performed to identify glycerolipid molecular species that contained singly oxygenated forms of the polyunsaturated fatty acids (PUFA) linoleate (C18:2), arachidonate (C20:4), or docosahexaenoate (C22:6), respectively. The major oxylipid species identified was (1-stearoyl, 2-hydroxyeicosatetraenoyl)-sn-glycerophospho-ethanolamine [(C18:0/HETE)-GPE], and it was quantified by MRM of 782.76 \rightarrow 319.3, which is a transition that corresponds to production of the HETE carboxylate anion from the [M-H][–] ion of the parent oxy-phospholipid species.

2.8. Assessment of Apoptosis by Flow Cytometry. INS-1 cell apoptosis was determined using an Annexin-VFLUOS Staining Kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions, essentially as previously described [64, 87]. Briefly, harvested cells were washed with PBS and resuspended in Annexin-VFLUOS labeling solution (100 μ L). After incubation (10–15 min, 15–25°C), cells were transferred to fluorescence-activated cell sorting (FACS) tubes and diluted 1:5 with buffer provided in the kit. Fluorescence in cells was analyzed with a FACScan flow cytometer (BD Biosciences, Sparks, MD, USA) at an excitation wavelength of 488 nm, and data were processed with WinMDI 2.9 software.

2.9. Statistical Analyses. Results are expressed as mean \pm SEM. Data were evaluated by unpaired, two-tailed Student's *t*-test for differences between two conditions or by analysis of variance with appropriate posthoc tests for larger sets, as previously described [80, 81, 87]. Significance levels are described in the figure legends, and a *P* value <0.05 was considered to reflect a significant difference.

3. Results

3.1. INS-1 Cell iPLA₂ γ Expression and the Influence of Inflammatory Cytokines and Oxidative Agents. INS-1 insulinoma cells were found to express iPLA₂ γ mRNA and iPLA₂ γ -immunoreactive protein by quantitative PCR and by Western blotting, respectively (Figure 1), and also to exhibit iPLA₂ activity (not shown). Incubation with the inflammatory cytokines IL-1 β and IFN- γ resulted in increased INS-1 cell expression of iPLA₂ γ mRNA in a concentration-dependent manner (Figure 1(a)), and expression of iPLA₂ γ immunoreactive protein exhibited a similar pattern (Figure 1(b)).

The major iPLA₂ γ -immunoreactive band (Figure 1(b)) migrated with an apparent MW of about 74 kDa on SDS-PAGE and Western Blotting analyses of INS-1 cell lysates, but other bands of variable intensity were observed with apparent MW between 40 and 88 kDa (not shown), as previously reported [88, 89]. Although full-length iPLA₂ γ cDNA encodes an 88 kDa protein, transcriptional and translational regulatory mechanisms result in production of multiple gene products of various sizes, and the major species often migrate with apparent MW of 74–77 kDa [88, 89].

The increased expression of iPLA₂ γ mRNA induced by IL-1 β and IFN- γ was dependent on the incubation time (Figure 1(c)). Incubation of INS-1 cells with the oxidative agents streptozotocin (STZ) [79] (Figure 2(a)) or t-butylhydroperoxide (TBHP) [70] (Figure 2(b)) also resulted in a concentration-dependent increase in expression of iPLA₂ γ mRNA (Figure 2) and protein (not shown).

These findings suggested the possibility that upregulation of iPLA₂ γ expression might represent a compensatory response to injurious agents in order to enhance β -cell survival in the settings of inflammation or oxidative stress. To examine this possibility, effects of suppressing INS-1 cell iPLA₂ γ expression were examined.

3.2. Establishing iPLA₂ γ -Knockdown INS-1 Cell Lines. INS-1 cells were infected with FIV constructs containing inserts that produced either scrambled RNA (control) or shRNA directed against sequences in iPLA₂ γ mRNA. Selection of neomycin-resistant cells resulted in isolation of two clones that had stably incorporated knockdown constructs and expressed less than 20% of the control cell iPLA₂ γ mRNA content when analyzed by real-time PCR (Figure 3(a)) or Northern blots (not shown) and reduced amounts of iPLA₂ γ immunoreactive protein on Western blots (Figure 3(b)). The iPLA₂ γ -knockdown (iPLA₂ γ -KD) cell lines also exhibited a reduction in iPLA₂ activity (not shown) that was comparable in magnitude to the reduction in mRNA levels (Figure 3(a)). The level of iPLA₂ γ expression was a stable property of

control and iPLA₂ γ -KD INS-1 cell lines that persisted on serial passage in culture.

3.3. INS-1 Cell Line Proliferation Rates. Cell proliferation was measured using an indicator that exhibits strong fluorescence enhancement upon association with nucleic acids [82]. Identical numbers of cells of each INS-1 cell line were seeded at time 0, and their growth rates were monitored for 72 hr. INS-1 iPLA₂ γ -KD lines proliferated at rates that were significantly lower than those for control INS-1 cells (Figure 4). Similar results were obtained when proliferation was measured by BrdU incorporation into DNA [83] and when seeding was performed at different initial cell densities (not shown).

3.4. Lipid Peroxidation in INS-1 Cell Lines. Lipid peroxidation was monitored by measuring TBARS [64, 84] in INS-1 cell lines incubated with IL-1 β and IFN- γ or with the oxidant agents STZ or TBHP under conditions similar to those in Figures 1 and 2. Relative to cells incubated only with vehicle, incubation with the cytokine mixture induced a significant increase in lipid peroxidation in both control INS-1 cells (1.60 ± 0.03 -fold) and in iPLA₂ γ -KD cells (2.71 ± 0.47 -fold), and the level achieved in the latter (0.92 ± 0.16 pmol/ μ g protein) significantly exceeded that in the former (0.54 ± 0.01 pmol/ μ g) (Figure 5). A similar pattern was observed upon incubation with STZ, which induced a significant rise in lipid peroxidation in control INS-1 cells (1.44 ± 0.04 -fold) and in iPLA₂ γ -KD cells (2.12 ± 0.09 -fold), and the level achieved in the latter (0.73 ± 0.07 pmol/ μ g) significantly exceeded that in the former (0.47 ± 0.04 pmol/ μ g) (Figure 5). Incubation with TBHP also induced a rise in the lipid peroxide content of both control INS-1 cells (2.20 ± 0.09 -fold) and in iPLA₂ γ -KD cells (2.56 ± 0.15 -fold), and there was a nonsignificant trend for the level achieved in the latter (0.86 ± 0.05 pmol/ μ g) to exceed that in the former (0.74 ± 0.03 pmol/ μ g).

3.5. HPLC-ESI-MS/MS Analysis of Oxidized Lipid Molecular Species That Accumulate in INS-1 Cells Incubated with Streptozotocin. To examine oxidized lipid molecular species in INS-1 cells, LC/ESI/tandem mass spectrometric scanning was used to detect parent ions that liberate an oxidized polyunsaturated fatty acid carboxylate anion (Figure 6(a)) upon collisionally activated dissociation (CAD) [85]. Hydroxyeicosatetraenoate (HETE) (*m/z* 319.3) arising from the oxidized analog of the glycerophosphoethanolamine (GPE) species 18:0/20:4-GPE (oxy-analog *m/z* 782.76) was found to represent the most abundant of the oxidized lipid species in INS-1 cells (Figure 6(b)), which is consistent with the facts that this is also the most abundant oxidized GPE lipid in activated platelets [85] and that 18:0/20:4-GPE is the most abundant GPE lipid in INS-1 cells [90] and rat islets [91]. Figure 6(b) displays an MS/MS scan for parent [M-H]⁻ ion precursors over the range *m/z* 400 to *m/z* 2000 that yield the HETE [M-H]⁻ (*m/z* 319.3) upon collisionally activated dissociation, and *m/z* 782.76 is the predominant parent ion observed. Figure 6(c) is an expansion of that mass spectrum

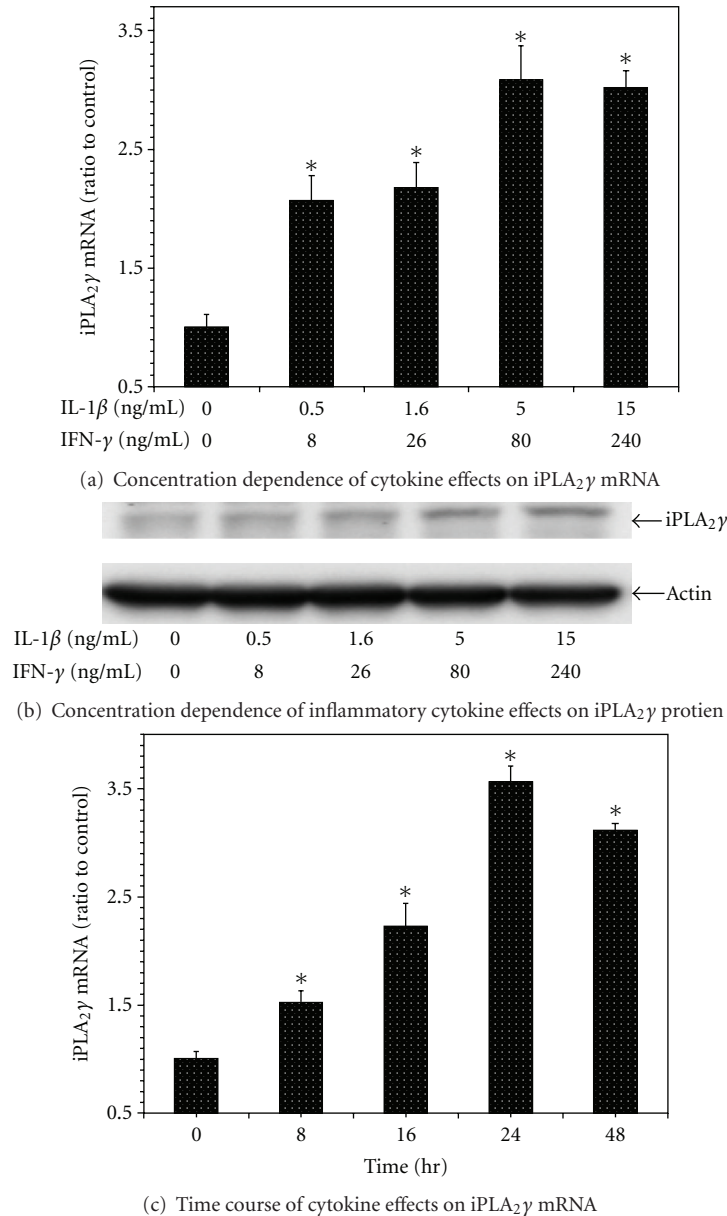


FIGURE 1: Influence of the inflammatory cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) on iPLA₂γ expression by INS-1 cells. Control INS-1 cells were incubated with vehicle alone or with various concentrations of IL-1β and IFN-γ for 16 hr, and iPLA₂γ mRNA levels were then determined by quantitative PCR (panel (a)) and iPLA₂γ protein levels by Western blotting (panel (b)), as described in Experimental Procedures. In panel (c), control INS-1 cells were incubated with IL-1β (5 ng/mL) and IFN-γ (80 ng/mL) for various intervals (0, 8, 16, 24, and 48 hr), at the end of which iPLA₂γ mRNA levels were determined by quantitative PCR. In panels (a) and (c), mean values ± SEM ($n = 4$) are displayed, and an asterisk (*) indicates a significant difference ($P < 0.05$) from the condition in which the concentration (panel (a)) or time (panel (b)) parameter value was zero. The immunoblot in panel (b) is representative of four experiments.

over the range m/z 782.5 to 785.0 that illustrates the [¹³C] isotopomer distribution of the $[M-H]^-$ ion.

The content of the oxylipid species (C18:0/HETE)-GPE in INS-1 cells was quantified by LC/ESI/MS/MS MRM scanning of the transition 782.76 → 319.5 (Figures 6(d)–6(g)), and incubation with STZ was found to induce an increase in the (C18:0/HETE)-GPE content of both control INS-1 cells transfected with vector only (Figures 6(d) and 6(e)) and in iPLA₂γ-knockdown INS-1 cells (Figures 7(f) and 7(g)). Both basal levels of (C18:0/HETE)-GPE and those achieved after

incubation with STZ for the iPLA₂γ-knockdown INS-1 cells exceeded those for control INS-1 cells (Figure 7(h)), and this is consistent with the proposal that iPLA₂β acts to excise oxidized PUFA residues from phospholipids so that the resultant lysophospholipid can be reacylated with an unoxidized fatty acid substituent to restore the structure and function of the parent phospholipid [63, 64].

Oxidized cardiolipin species were not observed directly due to the relatively low abundance of the parent lipid among all cellular lipids and the tendency of linoleate residues,

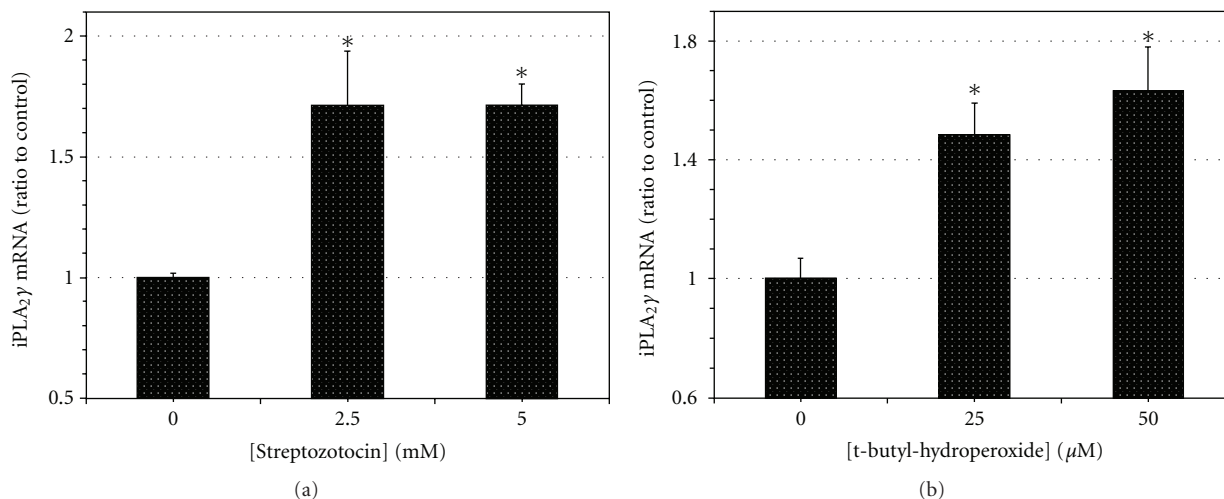


FIGURE 2: Influence of the oxidant agents streptozotocin (STZ) and tert-butylhydroperoxide (TBHP) on *iPLA₂γ* expression by INS-1 cells. Control INS-1 cells were incubated with various concentrations of STZ (panel (a)) or TBHP (panel (b)) for 16 hr, and *iPLA₂γ* mRNA levels were then determined by quantitative PCR, as in Figure 1(a). Mean values \pm SEM ($n = 4$) are displayed, and an asterisk (*) indicates a significant difference ($P < 0.05$) from the condition in which the concentration of the oxidant agent was zero.

which are the principal fatty acid substituents of cardiolipin, upon oxidation to undergo chain scission reactions that yield a variety of truncated *sn*-2 substituents rather than a single-predominant species [92]. Similar behavior has been observed for other polyunsaturated fatty acids [93]. Mitochondria contain substantial amounts of GPE lipids, however, and they undergo the largest fractional modification of measured mitochondrial lipid classes upon induction of apoptosis [94], which suggests that GPE lipid oxidation represents a surrogate marker for mitochondrial phospholipid oxidation.

3.6. Apoptosis of INS-1 Cell Lines. Apoptosis was monitored by determination of Annexin V binding by FACS [64, 87] with INS-1 cell lines incubated with IL-1 β and IFN- γ under conditions similar to those in Figure 1. The inflammatory cytokine mixture induced a robust increase in apoptosis of both control INS-1 cells and *iPLA₂γ*-KD cells, and the percentage of apoptotic cells for the latter ($30.2 \pm 0.5\%$) than for the former ($25.7 \pm 0.9\%$) (Figure 7(a)). Incubation of the INS-1 cell lines with the oxidant agent STZ also induced a concentration-dependent increase in the percentage of apoptotic cells that was significantly higher for *iPLA₂γ*-KD cells (12.1 ± 0.9) than for control INS-1 cells (4.5 ± 0.9) at the highest STZ concentration (7.5 mM) tested (Figure 7(b)).

4. Discussion

Loss of insulin-secreting β -cells occurs in both type I and type II diabetes mellitus (T1DM and T2DM), and apoptosis is thought to be the major mechanism by which β -cell death occurs [16, 19]. Lipid oxidation plays an important role in initiating apoptosis [17, 20–29], and it has been suggested

that generation of reactive oxygen species results in cardiolipin peroxidation in mitochondrial membranes, which destabilizes the lipid bilayer and potentiates membrane permeabilization, cytochrome c release, and apoptosis [61–67]. Understanding the mechanisms that the β -cell uses to protect its mitochondrial membranes from oxidative injury could yield insight into the pathogenesis of β -cell loss and development of means to treat or prevent T1DM and T2DM.

Phospholipases A₂ (PLA₂) hydrolyze glycerophospholipids to yield a free fatty acid and a 2-lysophospholipid [77, 78], and PLA₂ are thought to participate in signaling and membrane-remodeling processes that include repairing of oxidative damage to membranes in order to preserve their functional integrity [70–74]. When lipid peroxidation occurs, the oxidized *sn*-2 fatty acid substituent of phospholipids becomes less hydrophobic and more accessible to phospholipases [73]. The lysophospholipid that results from PLA₂-catalyzed removal of oxidized fatty acid substituents can be reacylated with an unoxidized fatty acid to restore the native structure and function of the parent phospholipid.

Two members of a lipase family [95] that has been designated Group VI PLA₂ [96] or patatin-like phospholipase domain-containing (PNPLA) proteins [97] may play such a role in remodeling mitochondrial cardiolipin, and neither enzyme requires Ca²⁺ for catalytic activity. Group VIA PLA₂ (*iPLA₂β*) was the first member of this family to be recognized [98, 99] and is also designated PNPLA9. Group VIB PLA₂ (*iPLA₂γ*) was recognized thereafter [88, 100] and is also designated PNPLA8 [96, 97]. *iPLA₂γ*, is expressed in mitochondria and peroxisomes [89, 101, 102], which are both membranous organelles that produce reactive oxygen species, and *iPLA₂γ* cooperates with *iPLA₂β* in stimulated phospholipid hydrolysis in some circumstances [103].

Mitochondria also contain *iPLA₂β*, and observations in a *Drosophila* model of the human Barth syndrome have raised

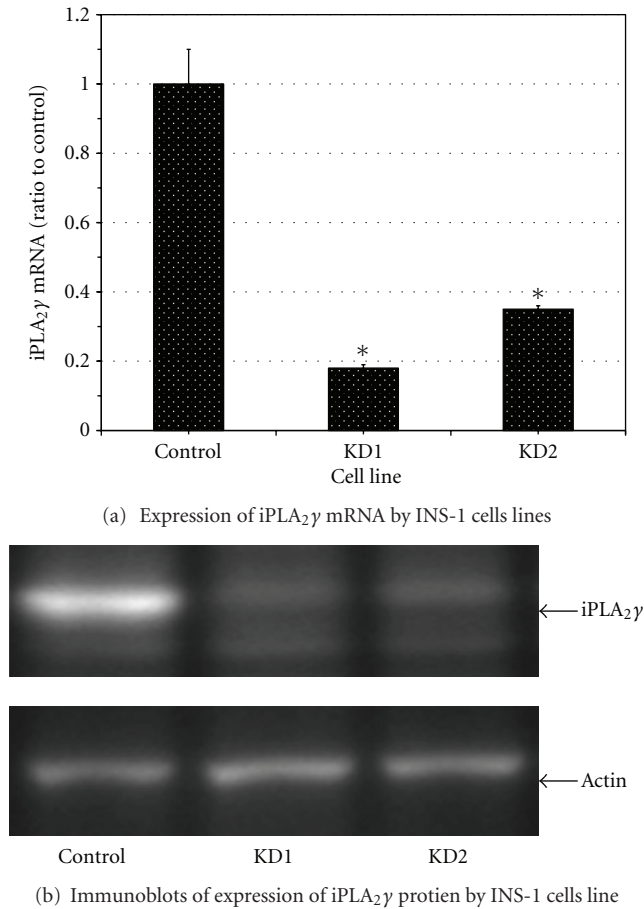


FIGURE 3: Establishing iPLA₂γ-knockdown INS-1 cell lines. INS-1 cells were transfected with FIV constructs containing inserts that produced either scrambled RNA (control) or siRNA directed against sequences in iPLA₂γ mRNA (KD1, KD2). The relative iPLA₂γ expression levels in control INS-1 cells and in the iPLA₂γ-knockdown (KD) cell lines KD1 and KD2 were assessed by quantitative PCR for mRNA (panel (a)) and by Western blotting analysis for iPLA₂γ-immunoreactive protein (panel (b)). In panel (a), mean values ± SEM are displayed ($n = 4$). An asterisk (*) denotes a significant difference ($P < 0.05$) from the value for control INS-1 cells. The immunoblot in panel (b) is representative of four experiments.

interest in the possibility that iPLA₂β participates in cardiolipin remodeling [104]. Barth syndrome results from mutations in the tafazzin gene, which encodes a mitochondrial phospholipid-lysophospholipid transacylase, and the disorder is characterized by severe cardioskeletal myopathy, low-cardiolipin content, and abnormal cardiolipin fatty acyl composition [105]. Tafazzin-deficient *Drosophila* have similar abnormalities in cardiolipin content and mitochondrial function associated with monolysocardiolipin accumulation, and this phenotype is suppressed by inactivation of the iPLA₂β gene, suggesting that iPLA₂β contributes to monolysocardiolipin formation [104].

Several observations indicate that iPLA₂γ is also involved in cardiolipin remodeling. Selective overexpression of

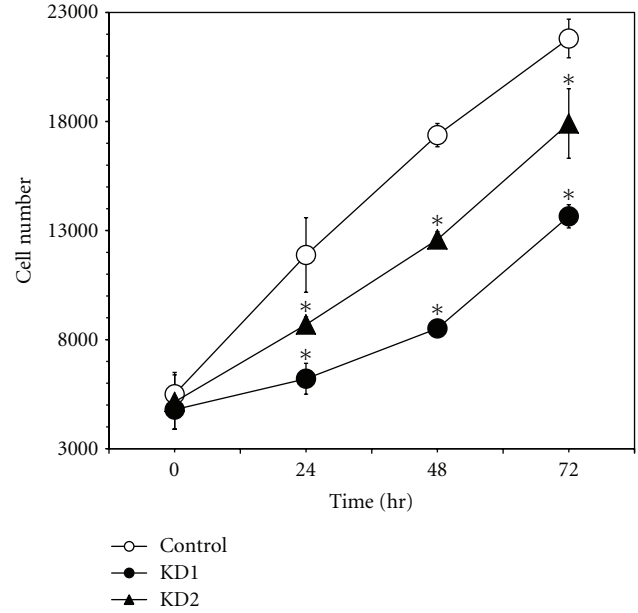


FIGURE 4: Proliferation rates of INS-1 cell lines. Control INS-1 cells (open circles) or the iPLA₂γ-Knockdown cell lines KD1 (closed circles) or KD2 (closed triangles) were seeded onto the wells of microtiter plates (density 3×10^3 cells/well) and incubated for various intervals (24, 48, or 72 hr), at the end of which cell number was estimated based on DNA content by using a fluorescent indicator as described in Experimental Procedures. Mean values ± SEM are displayed ($n = 4$). An asterisk (*) denotes a significant difference ($P < 0.05$) from the value for the control INS-1 cell line at the indicated time point.

iPLA₂γ in mouse myocardium results in altered mitochondrial function associated with cardiac dysfunction [106, 107], and genetic ablation of iPLA₂γ produces a deficient mitochondrial bioenergetic phenotype [108] associated with cognitive dysfunction and hippocampal abnormalities that include mitochondrial degeneration and alterations in cardiolipin content and molecular species distribution [109]. iPLA₂γ-null mice also exhibit exaggerated high-fat diet-induced changes in tissue cardiolipin content and composition and altered patterns of mitochondrial fatty acid oxidation [110, 111]. Cardiolipin remodeling in myocardial mitochondria that occurs during heart failure in rats also appears to involve iPLA₂γ [112].

iPLA₂β and iPLA₂γ cooperate in effecting certain cell fate decisions [113], and both enzymes may participate in determining whether a cell survives or succumbs to oxidative injury via their roles in cardiolipin metabolism. In β-cells, stimuli that induce apoptosis cause iPLA₂β to redistribute from cytosol to mitochondria [63–65, 92, 114–119]. Staurosporine, for example, stimulates INS-1 cell mitochondrial superoxide production, and this results in mitochondrial membrane peroxidation, cytochrome c release, and apoptosis [63–65]. Staurosporine-induced membrane peroxidation and apoptosis in β-cells are attenuated by overexpressing iPLA₂β and amplified by its pharmacologic inhibition or genetic ablation [63–65]. This may reflect a role for iPLA₂β

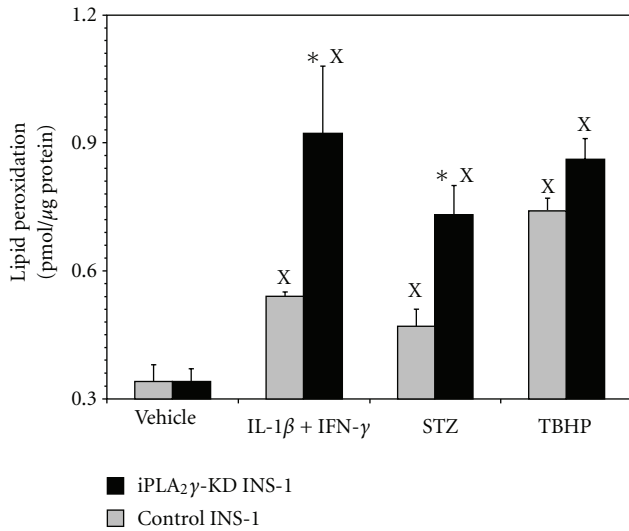


FIGURE 5: Influence of inflammatory cytokines and oxidant agents on lipid peroxidation of INS-1 Cell Lines. Control INS-1 cells (light bars) or iPLA₂γ-Knockdown INS-1 cells (dark bars) were incubated with vehicle only or with the combination of IL-1β (5 ng/mL) and IFN-γ (80 ng/mL) or with STZ (5 mM) or TBHP (50 μM) for 16 hr, and lipid peroxidation was then determined by the TBARS assay, as described in Experimental Procedures. Mean values ± SEM ($n = 4$) are displayed, and an asterisk (*) indicates a significant difference ($P < 0.05$) from control INS-1 cells. An (X) indicates a significant difference ($P < 0.05$) from the vehicle-treated condition.

to excise oxidized cardiolipin fatty acid residues to generate monolysocardiolipin species that can be recycled to restore the native structure [63–65].

Pharmacologic [120] observations suggest that iPLA₂γ may play a similar role in cardiolipin remodeling [112], and this is consistent with the abnormalities in cardiolipin content and composition that result from genetic manipulation of iPLA₂γ expression [108–110]. In renal proximal tubular cells, pharmacologic inhibition or molecular biologic suppression of expression of iPLA₂γ increases susceptibility to oxidant-induced lipid peroxidation, mitochondrial dysfunction, and cell death [70–72]. These observations prompted us to determine whether iPLA₂γ might play a similar role in β-cells, given the importance of β-cell loss via oxidative injury in the development of diabetes mellitus [20–35].

Our findings indicate that iPLA₂γ could participate in maintaining the aggregate mass of β-cells by promoting their proliferation and by protecting them from oxidative membrane injury induced by inflammatory cytokines or by oxidant agents that leads to apoptosis. Our iPLA₂γ-knockdown (KD) INS-1 cell lines exhibited significantly lower growth rates than control INS-1 cells did. The inflammatory cytokines IL-1β and IFN-γ increased INS-1 cell iPLA₂γ expression, and a similar response occurred when INS-1 cells were incubated with the oxidant agents STZ and TBHP. Those findings suggest that iPLA₂γ may be upregulated as a compensatory repair mechanism in response to agents that injure β-cells, and this is consistent with the observations that iPLA₂γ-KD INS-1 cells were also more

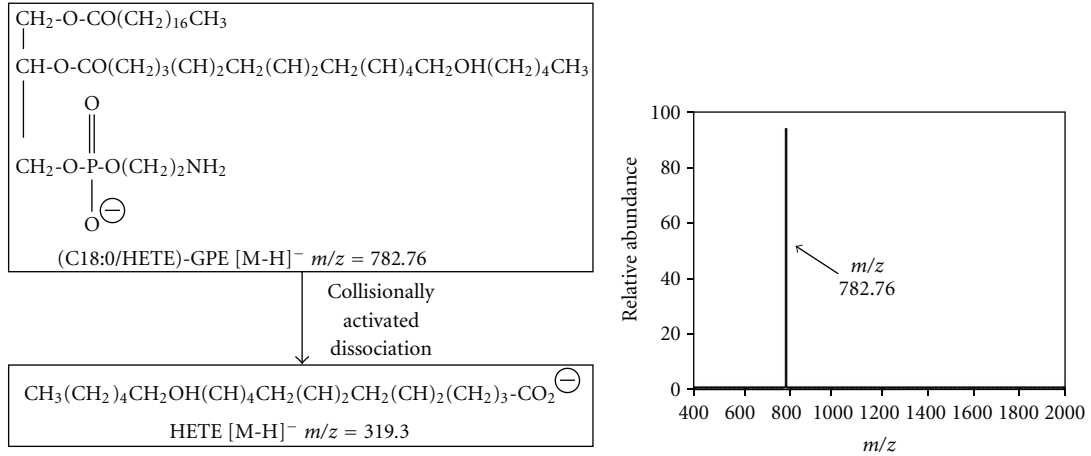
sensitive than control cells to injury from inflammatory cytokines and oxidative agents. These findings in β-cell lines are consistent with the increased sensitivity to oxidant-induced lipid peroxidation and apoptosis of renal proximal tubular cells with reduced iPLA₂γ expression [70] and suggest that iPLA₂γ plays a role in repairing oxidized membranes and mitigating oxidant-induced cellular injury.

The mechanisms by which cytokines and oxidant agents increase the expression of iPLA₂γ have not yet been determined experimentally, but the accumulation of iPLA₂γ mRNA suggests that increased transcription is involved. Current experiments to examine potential mechanisms are focused on three possibilities. One is that the redox sensitive transcription factor NF-κB is activated via ROS-mediated inactivation of its inhibitory subunit I-κB [121–124] and that NF-κB stimulates transcription of the iPLA₂γ gene directly or indirectly. NF-κB activation is known to contribute to β-cell injury induced by cytokines [125] under conditions similar to those employed in the studies described here. Two is that transcriptional activation of the iPLA₂γ gene might occur via p38 MAPK-dependent pathways, since stimuli that induce β-cell ER stress and apoptosis result in p38 MAPK activation [87], and ROS-induced p38 MAPK activation contributes to apoptosis in other cells [126, 127].

Three is that ROS-induced oxidation of cellular phospholipids yields agonistic ligands for the transcription factor PPARγ, as previously reported [128], which then activates transcription of the iPLA₂γ gene. It is of interest in this regard that conditions that result in differentiation of 3T3L1 fibroblasts to adipocytes lead to increased expression of PPARγ and in transcriptional upregulation of iPLA₂γ and iPLA₂β and that siRNA directed against either enzyme blocks differentiation [113].

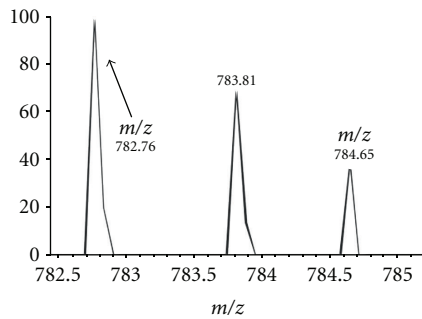
The presence of oxidized phospholipids in INS-1 cells treated with oxidant agents in the studies described here was determined by performing LC/MS/MS scans of lipid extracts for precursors of m/z 295, m/z 319, and m/z 343 in order to identify glycerolipid molecular species that contained singly oxygenated forms of the polyunsaturated fatty acids (PUFA) linoleate (C18:2), arachidonate (C20:4), or docosahexaenoate (C22:6), respectively. The major oxylipid species identified was (stearoyl, hydroxyicosatetraenoyl)-glycerophosphoethanol-amine [(C18:0/HETE)-GPE], and it was quantified by MRM of the transition 782.6 → 319.3, which corresponds to production of the HETE carboxylate anion from the [M-H]⁻ ion of the parent oxy-phospholipid species. Minor species were observed at other m/z values but were not further characterized because of the low signal obtained from the limited amount of lipid contained in the quantities of INS-1 cells with which it was practical to work.

Although C18:0/HETE-PE is the most abundant oxidized phospholipid observed here, it is probably not the only oxidized species formed under these conditions. Oxidized lipids represent only a tiny fraction (substantially below 1%) of their unoxidized precursors, and all but the most abundant species will likely fall below the limit of quantitation, even if present in the mixtures, when the amount of membrane lipid available for analysis is limiting. In addition, other phospholipid oxidation products, for example, those that contain

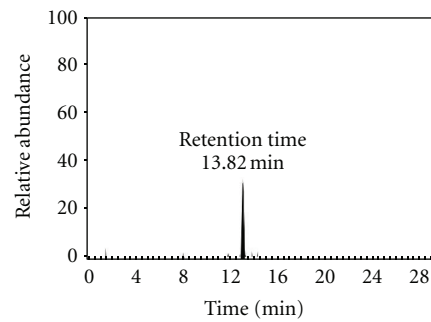


(a) MRM of the transition 782.76 → 319.3 for oxy-pe

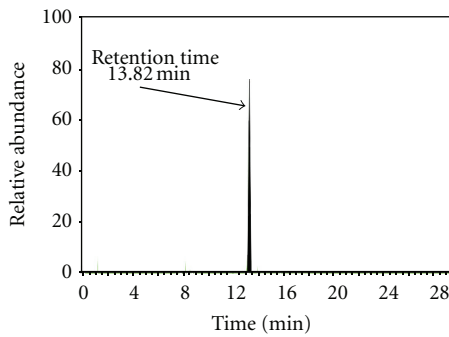
(b) Parents of m/z 319.3 MS/MS scan



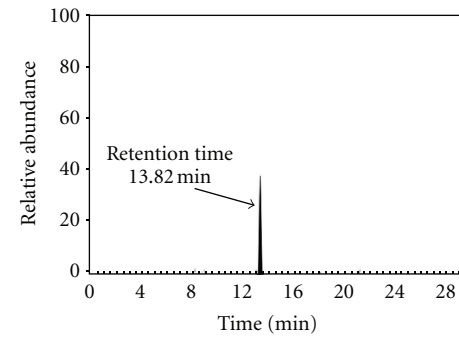
(c) $\{[\text{M-H}]^- \text{ } ^{13}\text{C}\}$ isotopomers parents of m/z 319.3 scan



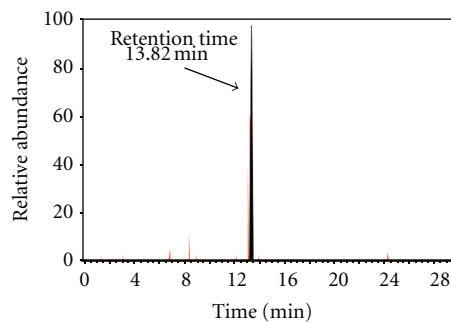
(d) LC/MS/MS MRM m/z 782.76 → m/z 319.3 control INS-1 cells



(e) LC/MS/MS m/z 782.76 → m/z 319.3 control INS-1 cells incubated with STZ

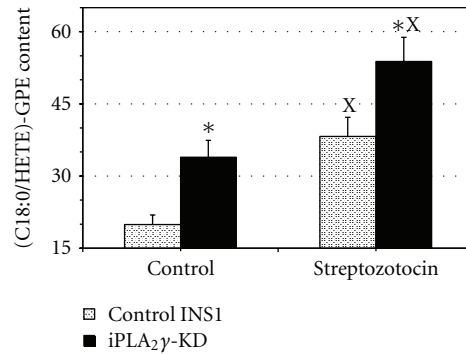


(f) LC/MS/MS MRM m/z 782.76 → m/z 319.3 iPLA₂γ-knockdown INS-1 cells



(g) LC/MS/MS m/z 782.76 → m/z 319.3 INS-1-KD cells treated with STZ

FIGURE 6: Continued.



(h) Streptozotocin effect on INS-1 cell oxidized pe

FIGURE 6: HPLC-ESI-MS/MS analysis of oxidized lipid molecular species that accumulate in INS-1 cells incubated with streptozotocin. Control INS-1 cells (light bars) or iPLA₂γ-knockdown INS-1 cells (dark bars) were incubated with vehicle only or with STZ (5 mM) for 16 hr as in Figure 5, and lipids were then extracted and analyzed by HPLC-LC-MS/MS as described in Experimental Procedures. Panel (a) illustrates the MS/MS transition monitored, which is the production of the hydroxyeicosatetraenoate (HETE) [M-H]⁻ ion (*m/z* 319.3) from the parent (C18:0/C20:4)-GPE [M-H]⁻ ion (*m/z* 782.76) upon collisionally activated dissociation. Prior survey scans monitoring parents of oxidized linoleate (C18:2) and docosahexaenoate (C22:6) species had revealed that HETE (HO-C20:4) was the dominant oxidized fatty acid residue esterified in INS-1 cell phospholipids. Panel (b) is an MS/MS scan over the *m/z* range 400–2000 in which parent ions that generate the HETE anion (*m/z* 319.3) are monitored, and *m/z* 782.76 is the vastly predominant parent, which was found to represent the (C18:0/C20:4)-GPE [M-H]⁻ ion upon analysis of the complete MS/MS spectrum/ Panel (c) is an expansion of that mass spectrum over the *m/z* range 782.5 to 785.0 to illustrate the [¹³C] isotopomer distribution of the [M-H]⁻ ion. In panels (d) and (e) represent HPLC/ESI-MS/MS scans in which the transition *m/z* 782.76 to *m/z* 319.3 is monitored as a function of LC retention time to quantitate the (C18:0/C20:4)-GPE content of control INS-1 cells (panels (d) and (e)) or iPLA₂γ-knockdown (KD) INS-1 cells (panels (f) and (g)) incubated without (panels (d) and (f)) or with STZ (panels (e) and (g)). Panel (h) represents a summary of four such experiments, and mean values are displayed and SEM indicated. An asterisk (*) denotes a significant (*P* < 0.05) difference between control and iPLA₂γ-KD INS-1 cell lines, and an X denotes a significant difference between cells incubated with or without STZ.

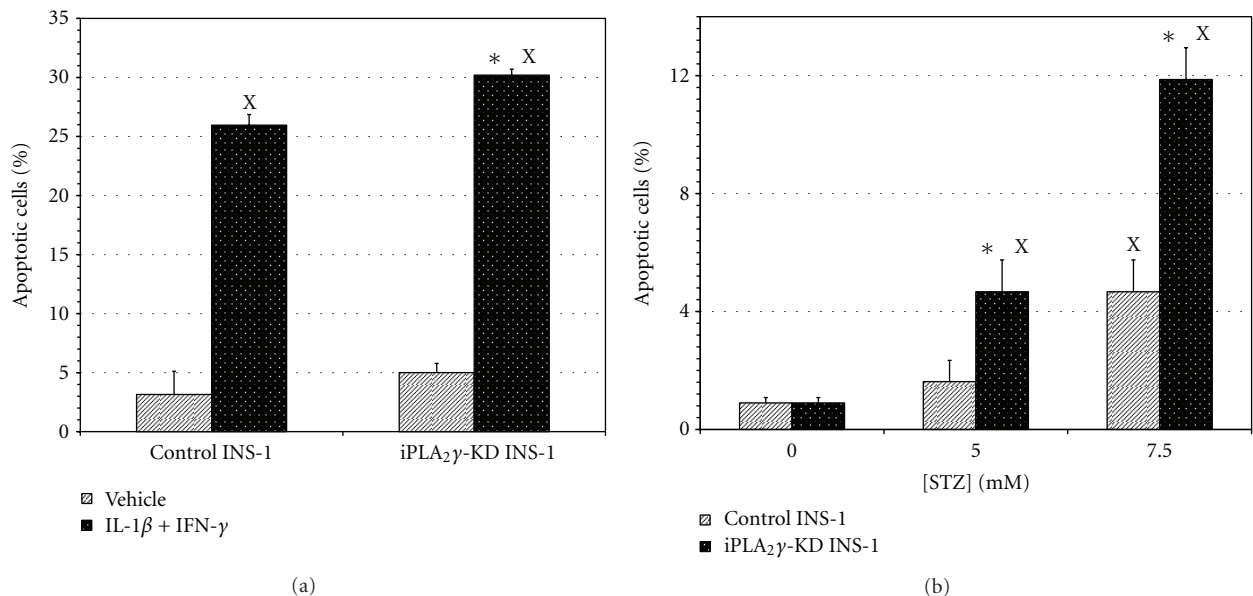


FIGURE 7: Influence of inflammatory cytokines and streptozotocin (STZ) on apoptosis of INS-1 cell lines. In panel (a), control INS-1 cells or iPLA₂γ-knockdown INS-1 cells (iPLA₂γ-KD) were incubated with vehicle only (light bars) or with IL-1β (5 ng/mL) and IFN-γ (80 ng/mL) (dark bars) for 16 hr, and the percentages of apoptotic cells were then determined by FACS as described in Experimental Procedures. In panel (b), control INS-1 cells (light bars) or iPLA₂γ-Knockdown INS-1 cells (dark bars) were incubated with vehicle alone or with varied concentrations of STZ (5 mM or 7.5 mM) for 16 hr, and the percentages of apoptotic cells were then determined as in panel (a). Mean values ± SEM (*n* = 4) are displayed. An asterisk (*) indicates a significant difference (*P* < 0.05) between control cells and iPLA₂γ-KD cells. An (X) indicates a significant difference (*P* < 0.05) from the vehicle-treated condition.

esterified hydroperoxy- or ketofatty acid derivatives [129], would not have been detected by the approach used here, which would also have failed to detect esterified short chain substituents arising from PUFA oxidation [130]. Moreover, neither the regio- nor the stereospecificity of oxygenation was determined in our studies because of the limited amount of oxidized lipid available for characterization, and it is possible that C18:0/HETE-PE consisted of several distinct isomers, as reported for other cells [85].

Nonetheless, we think that C18:0/HETE-PE represents a reasonable marker for phospholipid oxidation in our experiments for several reasons. First, oxidized species of PE are much more abundant in stimulated monocytes and platelets than are oxidized species of other phospholipid head group classes, including PC, PI, PS, or PG [131], and C18:0/HETE-PE is the most abundant oxidized diacyl-phospholipid under those conditions [85, 131, 132]. Second, the precursor C18:0/C20:4-PE is the most abundant PE species in INS-1 cells [90, 91]. Moreover, mitochondria contain substantial amounts of PE lipids, and they undergo the largest fractional modification of measured mitochondrial lipid classes upon induction of apoptosis [94], which suggests that PE lipid oxidation serves as a surrogate marker for mitochondrial phospholipid oxidation.

The LC/MS/MS measurements reported here indicate that INS-1 cell C18:0/HETE-PE content rises upon incubation with an oxidant agent and is higher in cells in which iPLA₂γ expression level has been knocked down compared to control cells. This is compatible with a role for iPLA₂γ in remodeling of oxidized phospholipids that involves excision of oxidized PUFA residues to yield lysophospholipid species that can be reacylated with unoxidized fatty acyl-CoA molecules. This would regenerate the native phospholipid structure and restore its normal function, thereby mitigating the effects of oxidative insults that might otherwise induce apoptosis.

5. Conclusions

Group VIB Phospholipase A₂ (iPLA₂γ) is distributed in mitochondria and expressed by insulin-secreting pancreatic islet β-cells and INS-1 insulinoma cells that are susceptible to oxidative injury by inflammatory cytokines, for example, IL-1β and IFN-γ, and by oxidizing toxins, for example, streptozotocin (STZ) or t-butyl-hydroperoxide (TBHP), via processes relevant to β-cell loss in types 1 and 2 diabetes mellitus. We demonstrate here that INS-1 cells incubated with IL-1β and IFN-γ, with STZ, or with TBHP increase their expression of iPLA₂γ mRNA and protein and that INS-1 knockdown (KD) cell lines with reduced iPLA₂γ expression proliferate more slowly than control INS-1 cells and undergo increased membrane peroxidation when incubated with cytokines or oxidants. Accumulation of the oxidized phospholipid species (1-stearoyl, 2-hydroxyeicosatetraenoyl)-sn-glycerophosphocholine was demonstrated in STZ-treated INS-1 cells by LC/MS/MS scanning, and the levels in iPLA₂γ-KD cells exceeded those in control cells. iPLA₂γ-KD INS-1 cells also exhibited higher levels of apoptosis than control cells when incubated with STZ or with IL-1β and IFN-γ. Together, these observations suggest that iPLA₂γ promotes

β-cell proliferation and that its increased expression during inflammation or oxidative stress may serve to mitigate membrane injury and thereby to enhance β-cell survival under these conditions.

Abbreviations

BEL:	Bromo-enol lactone
CAD:	Collisionally activated dissociation
C18:2:	Linoleate
C20:4:	Arachidonate
C22:6:	Docosahexaenoate
DM:	Diabetes mellitus
ER:	Endoplasmic reticulum
ESI:	Electrospray ionization
FACS:	Fluorescence-activated cell sorting
GSH:	Glutathione
HETE:	Hydroxyeicosatetraenoate
IFN-γ:	Interferon-γ
IL-1β:	Interleukin 1-β
iPLA ₂ β:	Group VIA PLA ₂
iPLA ₂ γ:	Group VIB Phospholipase A ₂
LC:	Liquid chromatography
KD:	Knockdown
MDA:	Malondialdehyde
MRM:	Multiple reaction monitoring
MS:	Mass spectrometry
MS/MS:	Tandem mass spectrometry
PC:	Phosphatidylcholine
PE:	Phosphatidylethanolamine
PG:	Phosphatidylglycerol
PI:	Phosphatidylinositol
PS:	Phosphatidylserine
PUFA:	Polyunsaturated fatty acid
ROS:	Reactive oxygen species
RPTC:	Renal proximal tubule cells
SEM:	Standard error of the mean
shRNA:	Small hairpin ribonucleic acid
SOD:	Superoxide dismutase
STZ:	Streptozotocin
TBARS:	Thiobarbituric acid reactive substances
TBHP:	t-butyl-hydroperoxide.

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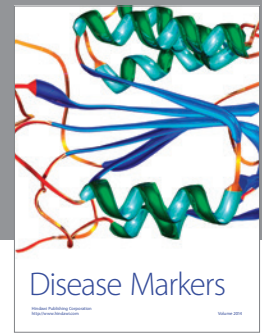
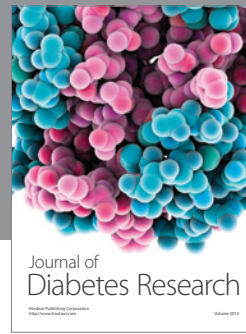
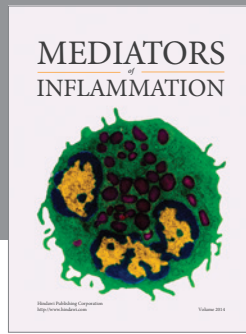
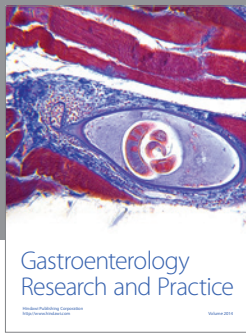
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