Stimulation of Lipogenesis as Well as Fatty Acid Oxidation Protects against Palmitate-Induced INS-1 β-Cell Death

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Saturated fatty acids are generally cytotoxic to β-cells. Accumulation of lipid intermediates and subsequent activation of lipid-mediated signals has been suggested to play a role in fatty acid-induced toxicity. To determine the effects of lipid metabolism in fatty acid-induced toxicity, lipid metabolism was modulated by up- and down-regulation of a lipogenic or fatty acid oxidation pathway, and the effects of various modulators on palmitate (PA)-induced INS-1 β-cell death were then evaluated. Treatment with the liver X receptor agonist T0901317 reduced PA-induced INS-1 cell death, regardless of its enhanced lipogenic activity. Furthermore, transient expression of a lipogenic transcription factor sterol regulatory element binding protein-1c (SREBP-1c) was also protective against PA-induced cytotoxicity. In contrast, knockdown of SREBP-1c or glycerol-3-phosphate acyltransferase 1 significantly augmented PA-induced cell death and reduced T0901317-induced protective effects. Conversely, T0901317 increased carnitine PA transferase-1 (CPT-1) expression and augmented PA oxidation. CPT-1 inhibitor etomoxir or CPT-1 knockdown augmented PA-induced cell death and reduced T0901317-induced protective effects, whereas the peroxisome proliferator-activated receptor (PPAR)-α agonist bezafibrate reduced PA-induced toxicity. In particular, T0901317 reduced the levels of PA-induced endoplasmic reticulum (ER) stress markers, including phospho-eukaryotic initiation factor-2α, phospho-C-Jun N terminal kinase, and CCAAT/enhancer-binding protein homologous protein. In contrast, knockdown of SREBP-1c or glycerol-3-phosphate acyltransferase 1 augmented PA-induced ER stress responses. Results of these experiments suggested that stimulation of lipid metabolism, including lipogenesis and fatty acid oxidation, protected β-cells from PA-induced lipotoxicity and that protection through enhanced lipogenesis was likely due to reduced ER stress. (Endocrinology 152: 816–827, 2011)

Free fatty acid (FFA)-induced toxicity may recapitulate lipotoxicity seen in obese type 2 diabetes and may mediate β-cell loss (1). In vitro studies have clearly demonstrated that long-term exposure to FFAs could induce β-cell death in culture and isolated islets. Death was primarily apoptotic with cytochrome c release, caspase-3 activation, and DNA fragmentation (2). Saturated fatty acids, including palmitic and stearic acids, were particularly cytotoxic to β-cells (3, 4).

The role of lipid metabolism in FFA-induced β-cell cytotoxicity is not fully understood. Long-chain acyl-coenzyme A (LC-CoAs), active form of FFAs, are transported into the mitochondria via carnitine palmitoyl trans-
ferase-1 (CPT-1) and undergo β-oxidation when used as energy fuel. FFAs are generally known to be nontoxic to β-cells if they are properly oxidized (5). However, FFA metabolism can be shifted from an oxidation to esterification pathway, particularly with a sufficient energy level or a simultaneous supply of glucose. Increased glucose flux and enhanced glycolysis promote mitochondrial anaplerotic and cataplerotic pathway (6). Enhanced intracellular flux of citrate and subsequent production of malonyl-coenzyme A through acetyl-coenzyme A carboxylase have been reported to inhibit activity of CPT-1, a rate-limiting enzyme for long-chain fatty acid oxidation (FAO), not for medium or short chain, and therefore augment lipogenesis (7, 8). Conclusively, accumulation of LC-CoA and lipid intermediate molecules including lysophosphatidic acid, phosphatidic acid, and diacylglycerol (DAG) was suggested to play a critical role in FFA-induced toxicity (7). Ceramide synthesized from LC-CoA and serine may serve as an important mediator of FFA-induced β-cell death (2, 4, 9). Activation of protein kinase C (PKC)-δ through DAG was also reported to be necessary for FFA-induced β-cell apoptosis (10, 11).

Accumulation of lipid intermediates has been associated with FFA-induced β-cell toxicity (5, 12); however, triacylglycerol (TG) itself is not likely to be toxic because it is a biologically inert molecule (13). Some studies have suggested that FFA-induced TG accumulation was beneficial to β-cell survival, potentially due to sequestration of toxic lipid intermediates (14, 15). In particular, enhanced TG synthesis through stearoyl-CoA desaturase 1 (SCD1) overexpression reduced FFA-induced cytotoxicity (16). Recently glycerolipid/FFA cycling, enhanced lipid esterification in conjunction with increased lipolysis, was suggested as a mechanism for decrease of FFA-induced toxicity (17).

Endoplasmic reticulum (ER) stress was postulated as a critical mediator of FFA-induced β-cell death (18, 19). ER stress is a response to unfolded proteins in the ER as well as conditions breaking ER homeostasis. Adaptive responses for dissipation of ER stress are initially activated by general translation attenuation, transcriptional up-regulation of molecular chaperones, and activation of ER-associated protein degradation (20). The three initiation sensors for dissemination of ER stress responses include activation of RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor (ATF)-6α, and inositol requirement 1 (IRE1) (21). PERK released from binding immunoglobulin protein (BiP), a molecular chaperone in the ER lumen, dimerizes and triggers its phosphorylation and activation. This leads to eukaryotic initiation factor (eIF)-2α phosphorylation, which attenuates the rate of general translation initiation but activates translation of a specific transcription factor ATF4. IRE1 release from BiP activates endonuclease activity, initiating the splicing of a 26-base intron from the X-box-binding protein 1 mRNA. The spliced X-box-binding protein 1 subsequently elicits transcriptional activation of several molecular chaperones, including BiP. BiP release from the ER membrane allows for ATF6 translocation to the cis-Golgi compartment, in which it is cleaved by site-1 and -2 proteases and acts as a transcription factor to augment ER folding capacity. The apoptotic pathway can be triggered if ER stress responses were prolonged and/or the adaptive response fails. Signals through PERK and ATF6 induce death signals by CCAAT enhancer binding protein homologous protein (CHOP) activation, whereas IRE1α provides death signals through activation of C-Jun N terminal kinase (JNK) and caspase-12 (22).

In the present study, we examined the effects of lipid metabolism on palmitate (PA)-induced INS-1 β-cell death. Lipogenesis was augmented through treatment with the pharmacological liver X receptor (LXR) agonists T0901317 or GW3965 or by transient overexpression of a master lipogenic transcription factor sterol regulatory element binding protein (SREBP)-1c. The effects of diminished lipogenesis on PA-induced cell death were examined through knockdown of SREBP-1c or glycerol-3-phosphate acyltransferase (GPAT) 1. FAO was stimulated by treatment with the peroxisome proliferator-activated receptor-α (PPARα) activator bezafibrate, whereas it was inhibited by CPT-1 knockdown or treatment with the CPT-1 inhibitor etomoxir. The effects of various metabolic modulators on PA-induced death were examined through DNA fragmentation and caspase-3 activation. Because ER stress was known to be a key mediator for FFA-induced β-cell death, we evaluated the effect of PA on induction of ER stress responses and also investigated the question of whether enhanced lipogenesis by T0901317 reduces PA-induced ER stress responses. We also evaluated the question of whether inhibition of lipogenesis or FAO resulted in further augmentation of PA-induced ER stress. Finally, PA-induced loss of ER integrity and the recovery of the integrity loss by enhanced lipogenesis were investigated through the analysis of the fluorescence intensity of brefeldin A-boron dipyrromethene (BODIPY) and the capacity of Ca²⁺ release from the ER.

Materials and Methods

Details on materials, preparation of PA, and treatment of INS-1 cells with the PA, DNA fragmentation assays, viability assay, Nile Red staining, analysis of TG accumulation, RT-PCR, immunoblotting, measurement of PA oxidation, DNA and RNA transfection (Supplemental Fig. 1, pub-
lished on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org/), quantitative analysis of brefeldin A-BODIPY-stained ER, measurement of intracellular Ca\(^{2+}\), and statistics are described in Supplemental Materials and Methods.

**Results**

**Apoptotic death of INS-1 \(\beta\)-cells by PA treatment**

We examined the cytotoxic effects of PA on INS-1 cell \(\beta\)-cells. INS-1 cells were treated with different PA concentrations, and cell death was determined by DNA fragmentation assay at 15 h after PA treatment. Cell death was induced by PA in a concentration-dependent manner (Fig. 1A). Treatment with 0.4 mM PA was sufficient to increase fragmented DNAs by 3.5-fold, and 0.6 mM PA enhanced fragmented DNAs by 4-fold. PA-induced cell death was primarily due to apoptotic death. Treatment with PA increased cleavage forms of caspase-3 and poly(ADP-ribose) polymerase (PARP), demonstrating that PA induced caspase-3 activation (Fig. 1B). PA-induced caspase-3 activation was also PA concentration dependent.

**Protective effect of LXR agonists on PA-induced INS-1 cell death**

PA-induced cytotoxicity may be induced by accumulation of lipid intermediates and subsequent activation of lipid-mediated signals (5, 12). Therefore, we examined the effects of enhanced lipogenic metabolism on PA-induced INS-1 \(\beta\)-cell death. Lipogenesis was stimulated by treatment with the LXR agonists T0901317 or GW3965. Stimulation of lipogenesis was determined by increase of Nile Red-stained lipid droplets and enhancement of accumulated TG in the cytoplasm. Treatment with LXR agonists T0901317 (1 \(\mu\)M) or GW3965 (1 \(\mu\)M) in the presence of 0.4 mM PA for 24 h induced lipid droplets in INS-1 cells, whereas the same dose of T0901317 or GW3965 without PA was not sufficient for lipid droplet induction in 24 h (Fig. 2A). Treatment with PA without an additional LXR agonist was also not sufficient for induction of lipid droplets in 24 h. On the other hand, TG content was increased by treatment with PA for 12 h, and PA-induced TG accumulation was further increased by treatment with LXR agonists T0901317 or GW3965 (Fig. 2B and Supplemental Fig. 2). In accordance with TG accumulation, the mRNA level of lipogenic transcription factor SREBP-1c and fatty acid synthetic enzyme fatty acid synthase (FAS) was significantly increased by treatment with T0901317 in the presence of PA. mRNA levels of the SREBP-1c and FAS genes in PA/T0901317-treated cells were 3- to 8-fold higher, compared with PA-treated cells (Fig. 2, C and D, and Supplemental Fig. 3). Expression levels increased at 6 h and were maintained at steady levels until 15 h after treatment with PA/T0901317. These data demonstrated that T0901317- and GW3965-induced LXR activation stimulated lipogenesis, particularly in the presence of PA. Treatment with LXR agonists prevented PA-induced INS-1 cell death (Fig. 2E). Treatment with 1 \(\mu\)M T0901317 or 1 \(\mu\)M GW3965 significantly reduced PA-induced DNA fragmentation. The preventive effect of T0901317 or GW3965 on PA-induced cell death was due to reductions of apoptotic death. Both T0901317 and GW3965 significantly reduced PA-induced cleavage of caspase-3 and PARP (Fig. 2F and Supplemental Fig. 4). These data strongly suggest that an enhanced lipogenesis could protect against PA-induced INS-1 \(\beta\)-cell death.

**Protective role of enhanced lipogenesis in PA-induced INS-1 cell death**

To clarify the protective effect of enhanced lipogenic metabolism on PA-induced \(\beta\)-cell death, the nuclear form of SREBP-1c, a master lipogenic transcription factor as a
downstream target of LXR (23), was transiently expressed in INS-1 cells and the effects of SREBP-1c overexpression on PA-induced INS-1 cell death were then examined. Transient transfection of SREBP-1c increased the level of SREBP-reactive bands in immunoblotting analysis in a transfection dose-dependent manner (Fig. 3B) and SREBP-1c overexpression increased PA-induced TG accumulation (Supplemental Fig. 5A). Overexpression of nuclear SREBP-1c significantly reduced PA-induced DNA fragmentation, and the protective effect of SREBP-1c on PA-induced DNA fragmentation was transfection dose dependent (Fig. 3A). In addition, overexpression of SREBP-1c significantly reduced PA-induced caspase-3 and PARP cleavage (Fig. 3B). SREBP-1c-induced inhibitory effects on PA-induced caspase-3 activation were also transfection dose dependent. However, SREBP-1c overexpression in the absence of PA did not influence DNA fragmentation (Fig. 3A) and caspase-3 cleavage (Fig. 3B). Conversely, knockdown of SREBP-1c using small interfering RNA (siRNA) further increased PA-induced caspase-3 and PARP cleavage, although SREBP-1c knockdown in the absence of PA did not influence caspase-3 activation (Fig. 3C). In addition, SREBP-1c knockdown significantly prevented T0901317-induced inhibitory effects on PA-induced caspase-3 cleavage (Fig. 3C). Next, the effect of the first lipogenic enzyme, GPAT, catalyzing lysophosphatidic acid synthesis from glycerol-3-phosphate and acyl-coenzyme A, on PA-induced INS-1 cell death was investigated. Mitochondrial GPAT1 was chosen because of its preference for PA as a substrate and its role in directing FAO to lipogenesis and because it is a direct target enzyme of SREBP-1c. Knockdown of GPAT1 augmented PA-induced caspase-3 cleavage. It also prevented T0901317-induced protective effects on PA-induced caspase-3 cleavage (Fig. 3C). These data collectively demonstrated that stimulation of lipogenesis protected against PA-induced β-cell death; however, inhibition of the lipogenic pathway augmented the death. To determine the knockdown effect of GPAT1 or SREBP-1c on TG accumulation, TG content was also analyzed by thin-layer chromatography. As shown in Fig. 3D (Supplemental Fig. 5B), PA increased TG content at 12 h after treatment; however, knockdown of GPAT1 or SREBP-1c significantly reduced PA-induced TG accumulation. Furthermore, the knockdown showed the same reductive effect on PA/T0901317-induced TG accumulation. These data confirmed that knockdown of GPAT1 or SREBP-1c prevented PA- or PA/T0901317-induced lipogenesis.

Enhanced FAO metabolism by LXR agonist in INS-1 cells

The CPT-1 inhibitor etomoxir augmented PA-induced β-cell death and CPT-1 overexpression protected PA-induced cytotoxicity; therefore, enhanced FAO was reported to be protective against FFA-induced toxicity (5, 24). Colin et al. (25) recently reported that LXR activation could stimulate PPARα expression and subsequently increase expression of its target CPT-1 gene. This report suggested that LXR activation increased FAO in PA-treated INS-1 β-cells and that enhanced metabolism of FAO had a role in LXR’s protective effect on PA-induced INS-1 cell death. We initially examined the question of whether PA treatment inhibited FAO. PA oxidation was directly investigated by measurement of C14-labeled carbon dioxide produced from C14-labeled PA. Pretreatment with PA reduced the production rate of CO2 from PA, and
the inhibitory effects of PA treatment on PA oxidation was PA treatment time dependent (Fig. 4A). A 9-h PA treatment reduced the rate of PA oxidation by 17%, whereas a 15-h treatment reduced the oxidation rate by 55%. We then examined the question of whether LXR activation could increase FAO metabolism. PA alone slightly increased the mRNA levels of FAO-related genes including CPT-1 and medium-chain acyldehydrogenase (MCAD) at 3 h after treatment (Fig. 4, B and C, and Supplemental Fig. 6). T0901317 further increased PA-induced CPT-1 and MCAD mRNA levels (Fig. 4, B and C) and significantly increased the oxidation rate of PA (Fig. 4D) in accordance with up-regulation of CPT-1 and MCAD expression. The level of carbon dioxide produced from labeled PA was increased to 142% in T0901317-treated cells. PPAR agonist bezafibrate as a positive control significantly increased the oxidation rate of PA; however, CPT-1 inhibitor etomoxir as a negative control significantly reduced the oxidation rate of PA (Fig. 4D). T0901317 in particular demonstrated a restorative effect on PA-induced reduction of FAO to nearly 100% levels (Fig. 4D). These data demonstrated that LXR activation had a stimulatory effect on FAO metabolism in INS-1 cells.

Role of FAO metabolism in PA-induced INS-1 cell death

To determine the effect of FAO metabolism on PA-induced INS-1 cell death, β-oxidation was stimulated by a PPARα agonist or inhibited by CPT-1 inhibitor or CPT-1 knockdown, and the stimulatory or inhibitory effects of FAO metabolism on PA-induced INS-1 cell death were then investigated. Treatment with bezafibrate as a PPARα agonist significantly reduced PA-induced cleavage of caspase-3 and PARP; however, etomoxir as a CPT-1 inhibitor further increased PA-induced cleavage of caspase-3 and PARP.
caspase-3 and PARP (Fig. 5A), demonstrating that stimulation of FAO metabolism protected INS-1 cells against PA-induced cell death, whereas inhibition of FAO augmented PA-induced death. In addition, etomoxir prevented T0901317-induced protective effects on PA-induced caspase-3 and PARP cleavage (Fig. 5A). Knockdown of CPT-1 through transient transfection of CPT-1 siRNAs showed similar augmentation effects on PA-induced caspase-3 and PARP cleavage and slightly prevented a T0901317-induced protective effect (Fig. 5B). On the other hand, T0901317 reduced etomoxir and CPT-1 knockdown-induced augmentation effects on PA-induced cell death (Fig. 5A). As shown in Fig. 5C, knockdown of CPT-1 significantly inhibited FAO and further augmented PA-induced FAO inhibition. These data suggested that inhibition of FAO metabolism played a role in PA-induced INS-1 cell death and that LXR activation by T0901317 could provide protective effects on PA-induced cell death through stimulation of FAO metabolism.

**Role of lipid metabolism on PA-induced ER stress**

ER stress has been identified as a critical mediator of FFA-induced β-cell death (18, 19). We therefore examined PA-induced ER stress responses in INS-1 cells. The levels of ER stress response markers including phospho-eIF2α, phospho-JNK, CHOP, and cleaved calnexin were increased by PA treatment (Fig. 6A and B). The level of phospho-eIF2α peaked at 6 and 9 h after PA treatment, whereas phospho-JNK increased from 9 h after PA treatment (Fig. 6A and B). CHOP levels were increased from 9 h after PA treatment and maintained continuously (Fig. 6C). Cleaved calnexin was highly induced at 12 and 15 h after PA treatment (Fig. 6D). Chaperone BiP level was mildly increased by PA treatment; however, the increase was not significant (Fig. 6E). On the other hand, treatment with LXR agonist T0901317 significantly reduced levels of PA-induced ER stress markers. In particular, CHOP and cleaved calnexin levels were dramatically reduced by T0901317 treatment (Fig. 6, C and D). These results suggested that PA-induced ER stress responses; however, enhanced lipid metabolism by T0901317 prevented PA-induced ER stress responses. To further clarify the effects of lipid metabolism on PA-induced ER stress responses, knockdown of CPT-1, GPAT1, and SREBP-1c was facilitated by siRNA transfection and the knockdown effects on PA-induced ER stress responses were then examined. CPT-1 knockdown reduced PA-induced phospho-eIF2α and phospho-JNK levels but augmented PA-induced CHOP levels (Fig. 7). On the other hand, knockdown of GPAT1 or SREBP-1c augmented levels of all ER stress markers including phospho-eIF2α, phospho-JNK, and CHOP. Treatment with T0901317 prevented augmented effects by knockdown of GPAT1 or SREBP-1c on PA-induced ER stress responses. These data demonstrated that lipogenic and FAO metabolism modulated PA-induced ER stress differently because inhibition of lipogenic pathways augmented PA-induced ER stress responses (phospho-eIF2α, phospho-JNK, and CHOP), whereas inhibition of FAO resulted in reduction of PA-induced ER stress (phospho-eIF2α and phospho-JNK) (Fig. 7).

**Restoration of PA-induced loss of ER integrity by LXR agonist**

We examined the question of whether PA treatment disrupted ER integrity and whether disruptions in integrity were prevented by enhanced lipid metabolism. ER
integrity was investigated through the change of brefeldin A-BODIPY staining (26) and the capability of Ca\(^{2+}\)/H\(^{11001}\) release from ER (27). PA-treated INS-1 cells were stained with brefeldin A-BODIPY 558/568 conjugate isomer 1, and the stained fluorescence intensity was measured by flow cytometric analysis. Fluorescence intensity of brefeldin A-BODIPY was reduced by PA treatment (Fig. 8A), suggesting an alteration of ER integrity from the reticular ER to a punctuate/aggregate distribution (26). On the other hand, sarcoplasmic reticulum calcium ATPase inhibitor thapsigargin, an ER stress inducer by Ca\(^{2+}\)/H\(^{11001}\) depletion in ER rumen, increased fluorescence intensity (Fig. 8B), suggesting that alteration of ER integrity by thapsigargin was due to ER expansion. T0901317 treatment in PA-treated cells partially restored the reduced intensity of fluorescence to that of PA-untreated cells. Events in the M1 region were reduced from 83.91 to 67.81% and mean fluorescence intensity (MFI) increased from 23.43 to 28.79 by treatment with T0901317 (Fig. 8, A and B). The capability of Ca\(^{2+}\)/H\(^{11001}\) release from intracellular Ca\(^{2+}\)/H\(^{11001}\) stores was examined by measurement of intracellular Ca\(^{2+}\) level released by ionomycin (A23187) under conditions with ethylene glycol tetraacetic acid-mediated chelating extracellular Ca\(^{2+}\). Intracellular Ca\(^{2+}\)/H\(^{11001}\) levels were increased by ionomycin stimulation; however, treatment with PA significantly reduced ionomycin-induced Ca\(^{2+}\) release (Fig. 8C). T0901317 treatment significantly restored the capability of ionomycin-induced Ca\(^{2+}\) release in PA-treated INS-1 cells (Fig. 8C). These data suggested that integrity of ER was lost by PA treatment and that this PA-induced loss of ER integrity might be due to inhibition of lipid metabolism.

**Discussion**

We wanted to examine the effects of lipid metabolism on PA-induced \(\beta\)-cell death. Enhanced lipogenesis through treatment with LXR agonists or transient SREBP-1c transfection resulted in a reduction of PA-induced INS-1 cell death.
inhibition of FAO through CPT-1 knockdown increased PA-induced CHOP levels but reduced PA-induced phospho-eIF2α and phospho-JNK levels. Collectively these data suggested that inhibition of the lipogenic pathway significantly contributes to PA-induced ER stress responses.

Because INS-1 cell were treated with PA in the presence of relatively high glucose concentrations (11 mM glucose in RPMI 1640 medium), the PA-induced toxicity studied in our experiments was thought to be a glucolipotoxicity augmented by high glucose. Previous studies have speculated that the detrimental actions of FFAs stemmed from the influence of glucose on intracellular fatty acid metabolism, promoting synthesis of cellular lipids (5, 12). Activation of lipid-derived signals has been reported to be critical in induction of glucolipotoxicity (7, 28). In particular, ceramide has been reported as a critical mediator of FFA-induced β-cell death (2, 4, 9); however, because a ceramide synthase inhibitor did not block FFA-induced toxicity (14, 15, 29), a role for ceramide in FFA-induced toxicity has been suspected. We reevaluated the role of ceramide in PA-induced INS-1 cell death. We observed that fumonisin B1 and myriocin, inhibitors of sphingosine N-acetyl transferase and serine palmitoyltransferase, respectively, afforded little protection against PA-induced cytotoxicity (Supplemental Fig. 9A). Although PKC-δ activation was required for FFA-induced β-cell apoptosis (10, 11), Welters et al. (30) reported that PKC-δ was not required for PA-induced β-cell toxicity. The role of PKC in PA-induced β-cell death is still unclear. In addition, it is still unknown whether PKC-δ is activated by DAG generated through glucose/FFA-mediated lipid partition. Our preliminary experiments using PKC inhibitors demonstrated weak involvement of PKC in PA-induced cytotoxicity (Supplemental Fig. 9B). Previous studies have suggested that accumulation of lipid intermediates (lysophosphatidic acid, phosphatidic acid, LC-CoA) exerted adverse effects on β-cells through the activation of lipid-mediated signals or protein acylation (7, 31, 32). However, the role of these intermediates in FA-induced toxicity has not been clearly identified. Reactive oxygen species have also been suggested as a critical mediator of lipotoxicity because they accompanied lipid accumulation, and antioxidants prevented LXR agonist-induced lipotoxicity (33). However, because the antioxidants N-acetyl cysteine or reduced glutathia-
higher SCD1 expression (36) was consistent with protective effects of T0901317 seen in our study. Although accumulation of lipid intermediates was initially suggested to play a role in PA-induced β-cell death, GPAT downstream intermediates, including lysophosphatidic acid, phosphatidic acid, and DAG, would not be direct toxic mediators for PA-induced β-cell toxicity because knockdown of GPAT1, an enzyme determining the first step of lipogenesis, could not result in accumulation of GPAT-downstream intermediates. In addition, the result showing that GPAT1 knockdown augmented PA-induced cytotoxicity suggested that inhibition of lipogenic pathways, rather than insufficient desaturation of palmitoyl-coenzyme A, had a more important role in PA-induced cytotoxicity. Knockdown of GPAT1 may increase FAO because of its inhibitory role in the lipogenic pathway. However, FAO does not appear to be enhanced by GPAT1 knockdown because we did not observe any protective effect of GPAT1 knockdown on PA-induced INS-1 cell death.

Stimulation of FAO also provided a protective role in PA-induced cell death. Stimulation of FAO through the LXR agonist T0901317 as well as the PPARα agonist bezafibrate was protective; however, inhibition of FAO through a CPT-1 inhibitor or CPT-1 knockdown was more toxic to PA-treated INS-1 cells. El-Assaad et al. (5) reported that AMPK activator as a stimulator of β-oxidation protected β-cells against glucolipotoxicity, whereas etomoxir as an inhibitor of β-oxidation amplified toxicity. In addition, PPARα agonists were reported to be protective against PA-induced toxicity (37). Recent reports demonstrated that CPT-1 overexpression was protective against PA-induced β-cell death, supporting the protective role of enhanced FAO metabolism in FFA-induced toxicity (24). These studies have hypothesized that accumulation of lipid intermediates resulting from inhibition of FAO could render β-cells susceptible to PA-induced toxicity. In particular, controlled expression of the active form of SREBP-1c (38) or overexpression of ER-GPAT (39) in INS-1 cells induced apoptosis with massive accumulation of lipid droplets. Their hypothesis that promotion of lipid accumulation is instrumental in β-cell glucolipotoxicity is somewhat contradictory to ours. Their glucolipotoxicity mechanism with hyperinduction of lipid

one exhibited limited protection against PA-induced death (Supplemental Fig. 9C), findings from our experiments did not support a role for reactive oxygen species in PA-induced β-cell death.

Several studies have suggested that β-cells were resistant to FFA cytotoxicity under conditions that increased lipogenesis. An inverse relationship between FFA cytotoxicity and TG accumulation has been reported (14), and FFAs converted to TG were reported to be less toxic than FFAs alone (15). In particular, unsaturated FFAs were reported to channel FFA into TG pools, and promotion of lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of saturated FFA through lipid esterification could detoxify saturated FFA-induced toxicity (4, 34). Desaturation of satu
accumulation may be different from the fatty acid-induced glucolipotoxic mechanism.

In particular, LC-CoA was thought to be a candidate for toxicity induction because it could act as a substrate for protein acylation and subsequently activate toxic signals (40). Findings from knockdown studies of CPT-1 as a catabolic downstream enzyme of LC-CoA have strongly suggested that accumulation of LC-CoA could serve as a toxic mediator for PA-induced cell death. However, because CPT-1 knockdown provided different toxic signals from GPAT1 knockdown (Fig. 7), LC-CoA was not supposed to be a common toxic mediator for PA-induced INS-1 cell death. Impaired FAO metabolism itself, rather than LC-CoA, was likely to play a critical role in PA-induced cell death. Our preliminary data demonstrated that the energy level in β-cells was reduced by long-term treatment with PA (Supplemental Fig. 10). A report that incomplete FAO played a role in lipid-induced insulin resistance (41) might support that impaired FAO metabolism itself could cause PA-induced toxicity.

On the other hand, a report that enhanced GL/FFA cycling could prevent FFA-induced toxicity (17) suggested that impaired lipid metabolism including synthesis and lysis might play a critical role in PA-induced toxicity. Results from our additional experiment showing that inhibition of lipolysis through knockdown of hormone-sensitive lipase augmented PA-induced INS-1 cell death and reduced T0901317-induced protective effects (Supplemental Fig. 11) supported the suggestion that impaired whole lipid metabolism, rather than accumulation of specific lipid intermediates, plays a more critical role in PA-induced toxicity. In particular, the LXR agonist T0901317 may provide protective effects against PA-induced toxicity through stimulation of whole lipid metabolism, including synthesis/lysis and FAO because it simultaneously stimulated the synthetic and oxidative metabolism of lipids. A recent report showing that LXR agonists were able to activate GL/FFA cycling through increased expression of hormone-sensitive lipase in human islets supports our assumption (42).

ER stress was suggested as a critical mediator for PA-induced lipotoxicity. Chemical chaperon 4-phenylbutyrate significantly prevented PA-induced viability reduction (Supplemental Fig. 9D). PA-induced ER stress responses are widespread as the activation of the PERK-dependent arm, including phospho-eIF2α, ATF4, and CHOP and IRE1-dependent JNK responses (18). However, PA failed to promote expression of the ER chaperone BiP (19). IRE1-mediated JNK activation and PERK-mediated CHOP expression were thought to play primary roles in PA-induced β-cell death (43). These reports were consistent with our data showing that PA increased the level of phospho-JNK and CHOP. Of particular interest, knock-
down of GPAT1 or SREBP-1c augmented PA-induced ER stress responses with caspase-3 activation, whereas T0901317 reduced PA-induced ER stress responses. This finding suggested that blockage of lipogenic pathways augmented PA-induced toxicity through cytotoxic ER stress induction. The mechanism of ER stress augmentation by inhibition of the lipogenic pathway and the reason that GPAT1, located in the outer membrane of mitochondria, affects ER stress remains to be studied. Our assumption is that the capacity of the lipogenic pathway may determine sensitivity of ER stress responses. Limited supply of glycerophospholipids or acylglycerol, possibly through limited lipogenesis, may prevent expansion of ER rumen in response to ER stress and the limited expansion of ER rumen may reduce the capacity of ER to cope with fatty acid-induced toxicity. Conclusively, insufficient activity of GPAT, even in mitochondrial form, reduces lipogenic capacity, which may reduce ER capacity, and, finally, augment PA-induced ER stress. In contrast, inhibition of FAO reduced PA-induced phospho-eIF2α and phospho-JNK levels, whereas the same inhibition increased PA-induced CHOP. These data suggested that FAO metabolism and TG synthetic metabolism had different effects on PA-induced ER stress and also suggested that inhibition of FAO augmented β-cell death through different toxic signals from inhibition of lipogenesis. Collectively PA-induced toxicity appeared to be caused by CHOP with inhibition of FAO and through phospho-JNK and CHOP with inhibition of lipogenic pathways. A recent report showing that inhibition of the mitochondrial respiratory chain induced CHOP without induction of ER stress responses (44) suggested that PA could augment CHOP expression in β-cells though inhibition of FAO and depletion of energy.

PA appeared to reduce reticular structure and Ca²⁺ release from ER stores along with ER stress induction. Reduction of brefeldin A-BODIPY intensity suggested that the ER morphology changed from the reticular to the punctuate/aggregate form, consistent with our previous data (26). Although a previous report demonstrated that PA did not alter ER Ca²⁺ concentrations (45), a recent report by Gwiazda et al. (27) clearly demonstrated that PA reduced Ca²⁺ levels in ER. Findings from this recent report were consistent with our data. Another report suggested that enhanced ER membrane saturation occurred when cells were exposed to saturated fatty acids, like PA, and that this saturation resulted in impairment of ER integrity and induction of ER stress through depletion of ER Ca²⁺ (46). This may explain why stimulation of general lipid metabolism results in reduction of PA-induced loss of ER integrity and PA-induced ER stress responses because stimulation of lipid metabolism can reduce PA-induced saturation in the ER membrane.

In conclusion, stimulation of lipid metabolism including synthesis and oxidation, protected PA-induced cytotoxicity to β-cells, whereas inhibition of lipid metabolism augmented PA-induced toxicity. These data suggest that blockage of whole lipid metabolism may play a critical role in PA-induced toxicity, rather than accumulation of specific lipid intermediates. These data also suggest that PA-induced toxicity can potentially occur when FFA overload overwhelms the cellular capacity for storage of FFAs as TGs or to use them for energy. Therefore, stimulation of the synthetic or oxidative pathways of lipid metabolism can potentially serve as therapeutic targets for prevention of the loss of β-cell mass seen in type 2 diabetes.

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