Palmitic Acid-induced Apoptosis in Pancreatic β-Cells Is Increased by Liver X Receptor Agonist and Attenuated by Eicosapentaenoate

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Abstract. Saturated fatty acids are implicated in the development of diabetes via the impairment of pancreatic islet β-cell viability and function. Liver X receptors (LXRs) and eicosapentaenoate (EPA) are known regulators of fatty acid metabolism. However, their roles in the pathogenesis of diabetes remain incompletely understood. The aim of this study was to determine the effects of EPA and the LXR agonist T0901317 on saturated fatty acid (palmitic acid)-induced apoptosis in the insulinoma β-cell line INS-1, a model for insulin-secreting β-cells. T0901317 significantly promoted palmitic acid-induced apoptotic cell death in the INS-1 cells. Consistent with these results, caspase-3 activity and BAX and sterol regulatory element binding protein-1c (SREBP-1c) mRNA levels were markedly increased in INS-1 cells co-administered palmitic acid and T0901317. The production of reactive oxygen species was considerably higher in the cells cultured concurrently with T0901317 and palmitic acid than in the cells incubated with either agent alone. EPA treatment attenuated the cellular death promoted by palmitic acid and T0901317 in the INS-1 cells, disclosing a possible mediating mechanism involving the inhibition of SREBP-1c. Finally, T0901317 up-regulated the palmitic acid-induced expression of p27KIP1, transforming growth factor beta 1, and SMAD3 proteins in INS-1 cells. These results demonstrate that palmitic acid-induced apoptosis in β-cells is enhanced by T0901317 via the activation of LXRs and is blocked by EPA via the inhibition of SREBP-1c, suggesting that the regulation of lipogenesis and lipotoxicity affecting pancreatic β-cell viability and insulin production may be a unique strategy for diabetes therapy.

Diabetes mellitus is a complex metabolic syndrome characterized by absolute insulin deficiency or the development of insulin resistance, producing hyperglycemia and altered glucose, fat and protein metabolism (1). In diabetes mellitus, the activities of desaturases decline, consequently decreasing the enzymatic conversion of saturated fatty acids into beneficial polyunsaturated fatty acids and thereby altering the fatty acid composition to include increased amounts of saturated fatty acids (2-4). Nonessential saturated fatty acids, specifically palmitic acid, induce insulin resistance in diabetic patients (5-8).

Several important factors are involved in the regulation of lipid metabolism leading to fatty acid synthesis and production. Sterol regulatory element binding protein-1c (SREBP-1c) is a key lipogenic transcription factor responsible for regulating the genes required for de novo lipogenesis. Recent evidence has shown that SREBP-1c is highly expressed in the islets of diabetic animals, where it has been implicated in the stimulation of fatty acid synthesis (9, 10). Oxygen free radicals produced by fatty acids are believed to play an important role in the destruction of β-cells in diabetes. Fatty acids, including palmitic acid, have been demonstrated to be cytotoxic to β-cells in models of obesity and diabetes, as well as normal β-cells (11-13). Fatty acid-induced apoptosis of pancreatic β-cells is considered to be a factor in the progression from obesity and insulin resistance to type 2 diabetes (14). SREBP-1c serves as a key sensor of lipids and glucose and is involved in the physiological response to lipotoxicity (15, 16). Moreover, ectopic overexpression of mature SREBP-1c has been shown to increase intracellular lipid deposition, thereby hindering glucose-stimulated insulin secretion and stimulating β-cell apoptosis (17, 18). The omega-3 fatty acid eicosapentaenoic acid (EPA) is an inhibitor of SREBP-1c, and evidence suggests that EPA may improve the clinical outcome in type 2 diabetes (19-22).
The liver X receptors (LXRs) belong to the nuclear receptor family of transcription factors. The target genes of the two isoforms of this nuclear hormone receptor, LXRα and LXRβ, play crucial roles in cholesterol and fatty acid metabolism (23-25). LXRs are expressed in a number of metabolically active tissues, including islets (26). A recent study has reported that the mRNA levels of both LXRα and LXRβ are significantly elevated in the pancreatic islets of animal models of type 2 diabetes (27). In addition to their effects on cholesterol and glucose homeostasis, LXRs also directly regulate the expression of lipogenic genes such as SREBP-1c in fat and liver (28, 29). The synthetic nonsteroidal LXR agonist T0901317 has shown efficacy similar to that of natural ligands such as oxy-cholesterols, but it is significantly more potent and binds selectively to LXRs (30).

The effects of LXR activation on β-cell dysfunction are largely unknown. The activation of the LXR pathway has been reported to contribute to insulin resistance (31). In addition, LXRs have been shown to regulate β-cell proliferation by promoting apoptosis in insulin-secreting cells, and LXR activation has been demonstrated to be an important event in β-cell apoptosis (32), suggesting that the activation of LXR transcription factor may elicit a pathological pathway leading to cell apoptosis. Given that LXR activation stimulates lipogenic gene expression in β-cells (33, 34), the pharmacological activation of LXRs may increase lipogenesis, leading to the accumulation of excess lipid in pancreatic β-cells and eventually to β-cell death.

The number of pancreatic β-cells in the body represents the balance between β-cell proliferation and apoptosis. Elevated β-cell apoptosis in type 2 diabetes has recently been reported as a major reason for the decreased number of β-cells in the disease state (35). As the lipid-laden β-cells undergo apoptosis, β-cell number and mass are reduced. In the current study, the effects of palmitic acid and regulators of fatty acid synthesis on cellular proliferation and apoptosis were investigated in the insulinoma β-cell line INS-1, a model for insulin-secreting β-cells.

**Materials and Methods**

**Cell line and cell culture.** The insulinoma cell line INS-1 was obtained from Bochuan Bio Co. (Shenzhen, Guangdong, P.R. China). The INS-1 cells were cultured as previously described (36) in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 11 mmol/L D-glucose and supplemented with 10% bovine serum albumin (BSA), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate and 50 μmol/L β-mercaptoethanol at 37°C in a humidified atmosphere with 5% CO2.

**Treatment of INS-1 cells with T0901317, palmitic acid, and EPA.** T0901317 (Cayman Chemical, Ann Arbor, MI, USA) was dissolved at 50 mg/mL in DMSO and further diluted (1:5) in 0.9% PBS. Palmitic acid (Sigma-Aldrich, St. Louis, MO, USA) and EPA (Sigma-Aldrich) were each dissolved at 100 mmol/L in methanol. For use in the cell treatment, the solutions were diluted in RPMI-1640 supplemented with 0.5% bovine serum albumin (BSA) to final concentrations of 10 μmol/L T0901317, 250 μmol/L palmitic acid, and 50 μmol/L EPA, respectively. INS-1 cells were treated for 48 h before the indicated experiments. Control cells were cultured in RPMI containing BSA, unless otherwise stated.

**MTT assay for cell viability.** An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay kit was purchased from Sigma-Aldrich. The INS-1 cells were seeded onto 96-well plates at 4,000 cells/well with 10% BSA and incubated with T0901317 (10 μmol/L), palmitic acid (250 μmol/L), a combination of T0901317 and palmitic acid or PBS (mock control) for 24 or 48 h. After the treatment incubation, 20 μl of MTT solution (5 mg/mL MTT in PBS, pH 7.4) were added to each well, followed by incubation at 37°C for 4 h. The supernatants were removed from the wells and DMSO (15 μl/well) was added to dissolve the crystals. The optical density at a wavelength of 490 nm was measured using a microplate reader (model 2550; Bio-Rad, Hercules, CA, USA).

**Caspase-3 activity assay.** Caspase-3 activity in cell lysates was measured with the modified method previously described (37, 38), using a caspase-3 DEVD-R110 fluorometric and colorimetric assay kit (Hedebio, Beijing, P.R. China) according to the manufacturer’s protocol. Cleavage of the specific caspase-3 substrate was quantified fluorometrically at excitation and emission wavelengths of 485 and 535 nm, respectively, using a microplate reader. The results are expressed as the quantity (μM) of substrate converted, as deduced from a standard curve generated from known concentrations of the dye R110.

**Real-time quantitative PCR.** The total RNA was extracted from the cells using TRIzol (Sigma-Aldrich) and used for real-time quantitative PCR to quantify the levels of BAX and SREBP-1c mRNA in INS-1 cells, as described previously (39). Real-time quantitative PCR was performed with a Quantitect SYBR Green kit (Invitrogen) and an ABI Prism 7700 real-time PCR instrument and software (Applied Biosystems, Foster City, CA, USA); following the manufacturer’s instructions. The PCR primers for SREBP-1c (GenBank accession no. AF286469) and BAX (GenBank accession no. NM 017059) were designed and synthesized by Invitrogen. All the results were quantified based on GAPDH (GenBank accession no. XM132897) as an internal standard. The optimized PCR program was 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 57°C for 1 min.

**Reactive oxygen species (ROS) assay.** The production of ROS was estimated by fluorescence using the redox-sensitive fluorescent probe CM-H2DCFDA (Invitrogen). The oxidation of H2DCFDA by ROS converts the molecule to 2',7'-dichlorodihydrofluorescein (DCF), which is highly fluorescent. Thus, ROS production by stimulated cells causes an increase in the fluorescence signal over time. ROS assays were performed as previously described (13). Briefly, the INS-1 cells were seeded in 96-well plates (3×104 cells/well) and allowed to attach for 48 h. After 48 h treatment with T0901317 (10 μmol/L), palmitic acid (250 μmol/L), a combination of T0901317 and palmitic acid, or PBS (untreated control), the cells were washed three times with 0.1 M PBS, incubated for 60 min with 5 μg/mL DCFDA and then washed again with PBS. Fresh PBS was added (100 μL/well) and DCF fluorescence was detected using
a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), at excitation and emission wavelengths of 485 and 528 nm, respectively. DCF fluorescence was determined by subtracting background fluorescence measured under identical conditions except without DCF.

Annexin-V assay. The INS-1 cells were incubated with T0901317 (10 μmol/L) plus palmitic acid (250 μmol/L), with and without EPA (50 μmol/L) for 48 h, harvested by trypsinization, and incubated with 10 μl of annexin-V-PE/7-AAD (Biosea, Beijing, P.R. China) at 37˚C for 15 min, as recommended by the manufacturer. The annexin-V-positive cells were measured by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) (40-42).

Western blot analysis. Western blot analysis was performed as previously described (40-42). In brief, the cells were harvested by trypsinization and lysed in Triton lysis buffer. Whole-cell lysates were collected and sonicated three times for 3 s, followed by centrifugation. Sample aliquots containing protein were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked using 5% nonfat dry milk for 1 h and then incubated in TBS buffer at room temperature for 1 h or at 4˚C overnight with one of the following primary antibodies: mouse anti-p27KIP1, anti-TGF-β1, or anti-SMAD3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-ACTIN (Cell Signaling Technology, Inc., Beverly, MA, USA). After washing, the membrane was incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Immune complexes were detected by chemiluminescence (LumiGLO; Cell Signaling Technology).

Statistical data analysis. Values are expressed as means±SD from at least three separate experiments. Differences between groups were assessed with Student’s t-test or one-way ANOVA using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). Differences were considered significant for p<0.05.

Results

Effect of palmitic acid and LXR activation by T0901317 on INS-1 cell viability. As shown by MTT assay (Figure 1), T0901317 (10 μmol/L), and palmitic acid (250 μmol/L) alone reduced INS-1 cell viability. Treatment with both T0901317 and palmitic acid significantly reduced INS-1 cell viability compared with that of the cells treated with PBS (p=0.001), T0901317 alone (p=0.003), and palmitic acid alone (p=0.023).

Caspase-3 analysis. To investigate the mechanism by which T0901317 enhanced palmitic acid-induced apoptosis in the INS-1 cells, caspase-3 activity was evaluated. Co-treatment with palmitic acid and T0901317 increased caspase-3 activity in the INS-1 cells compared with the activity in the cells treated with T0901317 or palmitic acid alone (p=0.041; Figure 2).

BAX and SREBP-1c mRNA expression in INS-1 cells. Real-time quantitative PCR analysis revealed that the expression of BAX mRNA was increased remarkably in the INS-1 cells at 48 h after co-treatment with T0901317 and palmitic acid compared with the levels in the cells treated with PBS, T0901317 alone, and palmitic acid alone (p=0.001, 0.013, and 0.040, respectively) (Figure 3). Similarly, SREBP-1c mRNA levels were significantly higher in the INS-1 cells at
48 h after co-treatment with T0901317 and palmitic acid compared with levels in the cells treated with PBS, T0901317 alone, and palmitic acid alone (p=0.001, 0.005, and 0.001, respectively), based on real-time quantitative PCR (Figure 4).

**Cytotoxic oxidative stress in INS-1 cells.** ROS levels were significantly higher in the INS-1 cells treated with both T0901317 and palmitic acid than in the cells treated with PBS (p=0.001), T0901317 alone (p=0.028), and palmitic acid alone (p=0.032) (Figure 5).

**Effect of EPA on apoptosis induced by palmitic acid and T0901317.** Increased expression of lipogenic enzymes has been linked to diabetes progression, and free radical-mediated apoptosis related to LXR activation and palmitic acid has been shown to be dependent upon the lipogenic activity of these enzymes (32, 43). Treatment with T0901317 plus palmitic acid for 48 h induced apoptotic INS-1 cell death as determined by annexin-V staining, and treatment with EPA (50 μmol/L) significantly attenuated the cell death induced by palmitic acid and T0901317 (Figure 6).

**Discussion**

LXRs have been shown to play a critical role in regulating β-cell growth and proliferation, and recent evidence has demonstrated that LXRs are activated in type 2 diabetes (44). In the present study, the LXR agonist T0901317 significantly enhanced palmitic acid-induced INS-1 cell death and demonstrated a direct association between LXR activation and apoptosis in β-cell cells. Increasing evidence...
indicates the importance of saturated fatty acids or LXR activation in the reduction of β-cell numbers during diabetes pathogenesis (39, 45).

However, the mechanism underlying the induction of apoptosis by LXR activation and palmitic acid is not well understood. In the current study, caspase-3 activity was significantly higher in the cells treated with both T0901317 and palmitic acid than in the cells treated with each agent alone, suggesting that LXR activation enhances palmitic acid-induced INS-1 cell apoptosis via a caspase-3-dependent pathway. Given that β-cell survival during diabetes progression is controlled by the relative expression levels of pro- and anti-apoptotic molecules, we also investigated the involvement of the BAX/BCL-2 family of cellular proteins in our model system. Previous studies have shown that palmitic acid induced cell apoptosis by up-regulating the critical pro-apoptotic molecule Bax and down-regulating the apoptosis inhibitor Bcl-2, leading to the activation of caspases (46, 47). In the present study, the combination of T0901317 and palmitic acid up-regulated BAX expression and significantly increased caspase-3 activity in INS-1 cells, thereby promoting apoptosis. These findings suggest that BAX overexpression as a consequence of LXR activation and palmitic acid induction contributes to apoptosis in INS-1 cells. These results were consistent with the findings of Wente and colleagues (39), who show that the activation of LXRs induces apoptosis and reduces cell proliferation in insulin-secreting cells.

Lipid peroxidation, the oxidative catabolism of palmitic acid, is widely accepted as a general mechanism of cellular injury and death. Lipid peroxidation and oxygen free radical generation are complex and deleterious processes that are closely related to lipotoxicity (47, 48). Oxidative stress occurs in a system when the generation of ROS exceeds the neutralization or elimination of ROS, as ROS can damage DNA, RNA, lipids, and proteins. ROS production is characteristic of the early stages of apoptosis, and oxidative damage is thought to be involved in the development of many diseases such as cancer, diabetes, and cardiovascular diseases. Several reports have implicated ROS in the toxicity of LXR activation in INS-1 cells (27, 39). In the current study, the levels of ROS were significantly higher in the INS-1 cells exposed to T0901317 plus palmitic acid compared with the levels in control cells and cells treated with T0901317 or palmitic acid alone. This demonstrates that LXR activation played a critical role in the toxicity of palmitic acid, supporting the hypothesis that cellular damage is the result of a combined effect of LXR activation and palmitic acid-induced toxicity. ROS production induced by LXR activation may lead to impairment of the antioxidant system, consequently enhancing oxidative damage in palmitic acid-exposed cells.
Furthermore, the combination of LXR activation and palmitic acid treatment in INS-1 cells induced mRNA expression of SREBP-1c, which is the master regulator of fatty acid synthesis. LXRs regulate fatty acid synthesis by influencing the expression of genes involved in cholesterol and fatty acid homeostasis, including genes for SREBP-1c and for the cellular cholesterol efflux regulator ABCA1 (member 1 of human transporter sub-family ABCA). The loss of LXRs contributes to the derepression of the ABCA1 gene, and the SREBP-1c gene remains transcriptionally silent (49). Zitzer and coworkers reported that LXRs induce insulin secretion mainly via SREBP-1c (34). The activity of mature SREBP-1c is regulated by phosphorylation during the cell cycle (50). SREBP-1c has also been shown to play a dominant role in palmitic acid-mediated lipotoxicity in insulin-secreting cells, and EPA, a key inhibitor of SREBP-1c, reverses the lipotoxicity (43). Taken together, these observations suggest that SREBP-1c may serve to link lipid synthesis, cell proliferation, and cell growth and that SREBP-1c may be an essential mediator of apoptosis induced by LXR activation and palmitic acid.

TGF-β acts as an anti-proliferative factor in normal epithelial cells, but plays a role in diabetes and other diseases by inducing apoptosis via the SMAD pathway in numerous cell types. In this pathway, TGF-β dimers bind to the type II receptor, which recruits and phosphorylates the type I receptor. The phosphorylated type I receptor recruits and phosphorylates receptor-regulated SMAD (R-SMAD). One R-SMAD, SMAD3, has been implicated in apoptosis induction. R-SMAD then binds to SMAD4, forming a heterodimeric complex that enters the cell nucleus, where it acts as a transcription factor for various genes such as activating genes for the mitogen-activated protein kinase 8 pathway, which triggers apoptosis. In the current study, both palmitic acid and T0901317 induced increases in TGF-β1 and SMAD3 protein levels in INS-1 cells, indicating that TGF-β1 and SMAD3 play important roles in mediating apoptotic INS-1 cell death induced by saturated fatty acid and LXR activation.

Intriguingly, the present results demonstrate that INS-1 cell death induced by palmitic acid and T0901317 was not completely reversed by EPA inhibition of SREBP-1c, suggesting that other mechanisms in addition to lipotoxicity and apoptosis may be involved in the growth arrest and cell death induced by saturated fatty acid and LXR activation in insulin-secreting cells. As a member of the universal cyclin-dependent kinase (CDK) inhibitor family, p27KIP1 functions mainly to stop or slow the cell division cycle. Specifically, p27KIP1 binds to and prevents the activation of cyclin D-CDK4 and cyclin E-CDK2 complexes, thereby controlling cell cycle progression at G1 phase. The expression of p27KIP1 is regulated by SMAD and by specific growth factors such as TGF-β. Our data revealed that palmitic acid and/or T0901317 increased the p27KIP1 protein levels in the INS-1 cells. Given that elevated levels of p27KIP1 protein typically cause cells to arrest in G1 phase, this finding, together with observations by others (45, 51), indicates that LXR activation altered cell cycle distribution and blocked cell cycle progression.

In summary, this study shows that palmitic acid-induced apoptosis in INS-1 cells is enhanced by T0901317 through the activation of LXRs and is suppressed by EPA through the inhibition of SREBP-1c. Thus, saturated fatty acid accumulation and LXR activation may induce growth arrest and cell death in insulin-secreting cells by lipogenesis and lipotoxicity (oxygen free radicals) via the modulation of SREBP-1c expression, by direct triggering of apoptosis and by the blockade of cell cycle progression or slowing of cell division.
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References


