Tauroursodeoxycholic acid inhibits TNF-α-induced lipolysis in 3T3-L1 adipocytes via the IRE-JNK-perilipin-A signaling pathway

WENYAN XIA1,2, YU ZHOU1, LIJING WANG2,3, LINXI WANG1, XIAOYING LIU3, YICHUAN LIN1, QING ZHOU1, JIANQING HUANG1 and LIBIN LIU1,3

Departments of 1Endocrinology and 2Geriatrics, Fujian Medical University Union Hospital; 3Fujian Institute of Endocrinology, Fuzhou, Fujian 350001, P.R. China

Received December 15, 2015; Accepted December 9, 2016

DOI: 10.3892/mmr.2017.6209

Abstract. The present study investigated the effects of tauroursodeoxycholic acid (TUDCA) on the lipolytic action of tumor necrosis factor (TNF)-α in 3T3-L1 adipocytes. Following treatment with TNF-α, cell viability was determined by MTT assay to select the optimum concentration and duration of TNF-α treatment in 3T3-L1 adipocytes. Intracellular lipid droplet dispersion and glycerin content in culture media were determined to evaluate the effect of TUDCA on TNF-α-induced lipolysis in 3T3-L1 adipocytes. Western blotting was performed to detect protein expression levels of perilipin-A and protein markers of endoplasmic reticulum stress: Immunoglobulin-binding protein (BiP), inositol-requiring enzyme (IRE), c-Jun N-terminal kinase (JNK), phosphorylated (p)-IRE and p-JNK. Following treatment with 50 ng/ml TNF-α for 24 h, glycerin content increased significantly and lipid droplets were dispersed. Glycerin content was reduced significantly and dispersal of lipid droplets reduced following pretreatment of 3T3-L1 adipocytes with 1 mmol/l TUDCA. TNF-α additionally activated the expression of BiP, p-IRE and p-JNK in a time-dependent manner; following pretreatment of 3T3-L1 adipocytes with 1 mmol/l TUDCA, the expression levels of these three proteins decreased. Therefore, TUDCA may inhibit TNF-α-induced lipolysis in 3T3-L1 adipocytes and reduce production of free fatty acids. Its underlying molecular mechanisms are potentially associated with the inhibition of activation of the IRE-JNK signaling pathway, which influences perilipin-A expression levels.

Introduction

Lipolysis refers to the breakdown of lipids, and involves the hydrolysis of triglycerides into glycerol and free fatty acids (FFAs). To maintain glucose homeostasis, the secretion of glucagon, epinephrine and norepinephrine increases, which stimulates lipase secretion to promote lipolysis. In addition, increases in levels of tumor necrosis factor (TNF)-α produced by adipose cells promote lipolysis in metabolic diseases including obesity. High rates of lipolysis in adipose tissues leads to the excess production of FFAs, which may be important components of insulin resistance and metabolic syndrome.

The majority of research on lipolysis has focused on lipases. A previous study demonstrated that the protein perilipin-A contributes to lipolysis by adjusting lipase activity. The primary underlying mechanisms of the action of perilipin-A are not known; however, a negative association between perilipin-A content and lipase activity in human adipose cells has been identified (1). TNF-α may inhibit perilipin-A expression levels via the c-Jun N-terminal kinase (JNK) signaling pathway, which promotes lipolysis and induces insulin resistance (2). Ozcan et al (3) revealed that endoplasmic reticulum (ER) stress (ERS) serves an important role in metabolic disease. ERS often results in the unfolded protein response (UPR); therefore, markers of UPR may be used to estimate the extent of ERS. Such marker proteins include three types of ER transmembrane protein: Inositol-requiring enzyme (IRE) 1, protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6). In addition, sensitivity to ERS is associated with immunoglobulin-binding protein (BiP). JNK in the IRE signaling pathway is considered a key factor that regulates insulin; therefore, activation of the JNK signaling pathway is involved in the pathogenesis of obesity, type-2 diabetes mellitus (T2DM) and insulin resistance in the periphery (4). The present study hypothesized that TNF-α may inhibit perilipin-A expression levels via activation of ERS in 3T3-L1 adipocytes. A previous study demonstrated that molecular chaperones including tauroursodeoxycholic acid (TUDCA) may affect ER function, which alleviates the increased ERS observed in obesity, and reverses insulin resistance and T2DM (5).

TUDCA is a hydrophilic bile acid that is normally produced endogenously in the liver, and is used as a bile acid replacement therapy for the treatment of cholestasis and hepatocellular necrosis. Furthermore, TUDCA may inhibit PERK and eukaryotic translation initiation factor 2A (eIF2a)
phosphorylation to reduce ER stress by facilitating correct protein folding.

The aim of the present study was to investigate the effects of TUDCA on the lipolytic action of TNF-α in 3T3-L1 adipocytes, and the underlying mechanisms of action.

Materials and methods

Reagents. The 3T3-L1 preadipocyte cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and trypsin were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum was purchased from PAA Laboratories; GE Healthcare Life Sciences (Chalfont, UK). Oil Red O, dexamethasone and 1-methyl-3-isobuthylxanthine were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Insulin was obtained from Novo Nordisk (Bagsværd, Denmark, Germany). TNF-α was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). TUDCA was obtained from Sigma-Aldrich; Merck Millipore. A glycerol-3-phosphate oxidase-peroxidase assay kit was obtained from Chaoyan Biotechnology Co., Ltd. (Shanghai, China). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Antibodies. The primary antibodies used were rabbit anti-mouse β-actin monoclonal antibody (catalog no. BA2305; BOSTER, Wuhan, Hubei, China). Bovine anti-mouse Perilipin-A antibody (catalog no. PB1061; Bioss, Beijing, China). Rabbit anti-mouse phosphorylated (p)-IRE polyclonal antibody (catalog no. ab48187; Abcam, Cambridge, UK). Rabbit anti-mouse IRE polyclonal antibody (catalog no. ab37037; Abcam). Rabbit anti-mouse BIP monoclonal antibody (catalog no. 3183; Cell Signaling Technology Inc., Danvers, MA, USA). Rabbit anti-mouse JNK polyclonal antibody (catalog no. 9252; Cell Signaling Technology Inc.). Rabbit anti-mouse p-JNK monoclonal antibodies (catalog no. 9251; Cell Signaling Technology Inc.). The secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit IgG (catalog no. ZDR-5401; ZSGB-Bio, Beijing, China). Horseradish peroxidase-conjugated anti-bovine IgG (catalog no. ZDR-5105; ZSGB-Bio).

Cultivation and differentiation of 3T3-L1 preadipocytes. Cultivation and differentiation of 3T3-L1 preadipocytes was performed as previously described by Wang et al (6).

Determination of cell viability by MTT assay. Cells were counted and seeded into 96-well culture plates at ~100 μl/well (~5x10^4 cells). The method previously described by Wang et al (6) was used to induce production of 3T3-L1 preadipocytes following fusion. TNF-α (0.1, 1, 10, 50 or 100 ng/ml) and control (untreated) was added to 3T3-L1 adipocytes for 24 h. Following this, 10 μl MTT solution (5 mg/ml) was added to each well and the plates incubated at 37°C for 4 h. Following incubation, the remaining MTT solution was removed and dimethyl sulfoxide was added to each well to dissolve the formazan crystals. Plates were agitated for 10 min on a shaker to ensure adequate solubility. Absorbance readings for each well on plates were performed at a wavelength of 490 nm. Cell viability was subsequently calculated. All experiments were carried out in triplicate. From the procedures described above, 50 ng/ml was determined to be the optimal concentration of TNF-α; therefore, 3T3-L1 adipocytes were subsequently treated with TNF-α at this concentration for 0, 2, 4 and 6 h.

Experimental groups. The present study used six well plates and seeded 3T3-L1 cells at 80% density.

For western blot analysis following TNF-α treatment, 3T3-L1 adipocytes were divided into four groups: A control group, treated with serum-free DMEM media only; TNF-α, treated with TNF-α at 50 ng/ml for 4 h; TUDCA + TNF-α, cultured in serum-free medium for 12 h after which 1 mmol/l TUDCA was added, incubated for 3 h, and 50 ng/ml TNF-α was added for a further 4 h; and TUDCA, cultured in serum-free medium for 12 h and 1 mmol/l TUDCA was added for 3 h followed by culture for 4 h.

Lipolysis measurement. Lipolysis was evaluated in 3T3-L1 adipocytes from different groups by measuring the amount of glycerin released into the medium using the glycerol-3-phosphate oxidase-peroxidase assay, following a 4 h incubation period. The enzyme label plate contained five wells/group. The microplate spectrophotometer and 96-well enzyme label plate were used to measure the absorbance. In order to create a standard curve, five wells in every group were set in a 96-well enzyme label plate. Samples (50 μl) 1x10^4/ml were incubated with 150 μl operating fluid from the aforementioned glycerol assay kit for 15 min at 37°C. The absorbance was measured at a wavelength of 550 nm using an automatic microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). A standard curve was used to calculate the glycerin concentration.

Staining with Oil Red O. 3T3-L1 adipocytes were washed three times with PBS and fixed with paraformaldehyde. Each well in the 6-well culture plates was stained with 1.5 ml diluted Oil Red O. A total of 0.05 g Oil red powder was dissolved in 10 ml isopropanol as storage liquid. Following this, 6 ml storage solution was added for a further 4 h; and Oil Red O was added for 3 h followed by culture for 4 h.

Western blotting. Cells were lysed with radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) for 20 min on ice. Cell lysates were centrifuged at 4°C, 12,000 x g for 20 min and supernatants were saved for analysis. Protein samples (2 mg/ml) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following separation, proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skimmed milk powder at room temperature, incubated overnight at 4°C with different primary antibodies: Perilipin A (1:1,000), IRE (1:500), p-IRE (1:500), Bip (1:1,000), JNK (1:1,000), p-JNK.
(1:1,000), β-actin (1:1,000) and membranes washed three times with TBS containing Tween-20 (TBST). The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary anti-bovine IgG antibody (Perilipin, 1:2,000) and anti-rabbit IgG antibody (IRE, 1:500; p-IRE, 1:500; Bip, 1:1,000; JNK, 1:1,000; p-JNK, 1:1,000; β-actin, 1:1,000) for 1 h at room temperature. Membranes were washed three times with TBST, visualized by Enhanced Chemiluminescence (EMD Millipore, Billerica, MA, USA) and quantified using Bandscan analysis software version 5.0 (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Statistical analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Significance was assessed by one-way analysis of variance and the least-significant difference test, as the post hoc test for multiple group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

TNF-α influences the survival of 3T3-L1 adipocytes. Following treatment with 0.1, 1, 10 or 50 ng/ml TNF-α for 24 h, cell survival decreased as TNF-α concentration increased (0.9871±0.069, P=0.6662; 0.9753±0.062, P=0.3740; 0.9669±0.032, P=0.0523 and 0.9568±0.108, P=0.3722 respectively) in 3T3-L1 adipocytes, compared with the control group (the value of control group was 1.0000±0.043). Cell survival was significantly decreased following treatment with 100 ng/ml TNF-α for 24 h (0.8598±0.053; P<0.01) compared with the control group (Fig. 1). Thus, the concentration of 50 ng/ml was selected for further experiments.

TUDCA inhibits TNF-α-induced lipolysis in 3T3-L1 adipocytes. Lipolysis was evaluated by measuring the quantity of glycerin released into the medium. Glycerin content in the TNF-α group (3.890±0.240) was significantly increased compared with the control group (2.303±0.131; P<0.0001). Glycerin content in the TUDCA+TNF-α group (2.417±0.174) was significantly reduced compared with the TNF-α group (P<0.0001). No significant differences were observed in glycerin content between the control and TUDCA+TNF-α groups (P=0.2755; Fig. 2).

Forms of intracellular lipid droplets in 3T3-L1 adipocytes. Lipid droplets were dispersed in the TNF-α group (Fig. 3A) following treatment with TNF-α for 24 h, compared with the control group. However, this degree of dispersion was not observed in the TUDCA (Fig. 3B) or TUDCA+TNF-α (Fig. 3C) groups, compared with the control group (Fig. 3D).

TUDCA inhibits TNF-α-induced expression of perilipin-A in 3T3-L1 adipocytes. Western blotting revealed that perilipin-A protein expression levels in 3T3-L1 adipocytes were significantly reduced in the TNF-α group compared with the control group (P=0.0031); however, they were not altered in the TUDCA+TNF-α group compared with the control group (P=0.0792; P=0.0055 compared with the TNF-α group; Fig. 4).

Figure 1. TNF-α influences the survival of 3T3-L1 adipocytes. Quantification of cell survival following treatment with 0 (control), 0.1, 1, 10, 50 or 100 ng/ml TNF-α for 24 h. Data are presented as the mean ± standard deviation. *P<0.01 vs. control. TNF-α, transforming growth factor-α.

Figure 2. TUDCA inhibits TNF-α-induced lipolysis in 3T3-L1 adipocytes. Quantification of the amount (mmol/l) of glycerin released into the medium. Data are presented as the mean ± standard deviation. *P<0.01 vs. control and TUDCA+TNF-α groups. TNF-α, transforming growth factor-α; TUDCA, tauroursodeoxycholic acid.

Figure 3. Formation of intracellular lipid droplets in 3T3-L1 adipocytes. Representative images of liquid droplet dispersion in the (A) TNF-α (B) TUDCA (C) TUDCA+TNF-α and (D) control groups. TNF-α, transforming growth factor-α; TUDCA, tauroursodeoxycholic acid.

Figure 4. TNF-α induces ERS in a time-dependent manner in 3T3-L1 adipocytes. Following treatment with 50 ng/ml TNF-α for 0,
2, 4 and 6 h, western blotting revealed that the expression of marker proteins of ERS (BiP, p-IRE and p-JNK) was induced by TNF-α, and that their expression levels peaked at 4 h (BiP at 4 h, P=0.0026 P=0.0099, P=0.0073 compared with 0, 2 and 6 h groups; p-IRE at 4 h, P=0.0003, P=0.0003, P=0.0009 compared with 0, 2 and 6 h groups; p-JNK at 4 h, P<0.0001, P=0.0001, P=0.0045 compared with 0, 2 and 6 h groups) and began to decline at 6 h (BiP at 6 h, P=0.0073 compared with 4 h groups; p-IRE at 6 h, P=0.0009 compared with 4 h groups; p-JNK at 6 h, P=0.0045 compared with 4 h groups; Fig. 5). Therefore, TNF-α treatment for 4 h was selected to assess the effect of TUDCA on the expression levels of these proteins.

**TUDCA inhibits TNF-α-induced ERS in 3T3-L1 adipocytes.** Western blotting revealed that the greatest expression levels of marker proteins of ERS (BiP, p-IRE and p-JNK) were present in the TNF-α group (P=0.0003, P=0.0027, P=0.0017 compared with the control group). The expression levels of these three proteins were reduced in the TUDCA+TNF-α group compared with the TNF-α group (P=0.0003, P=0.0005, P=0.0017; P=0.0011; P>0.05 compared with the control group; Fig. 6).

**Discussion**

Obesity and T2DM are primary global health concerns, associated with disorders of lipid metabolism and accompanied by increased levels of FFAs. Prolonged exposure to FFAs causes reduced glucose-stimulated insulin secretion, and apoptosis of beta cells in the pancreas (7). An increase in lipolysis causes levels of FFAs to increase.

The present study demonstrated that increased levels of TNF-α promote lipolysis in terms of intracellular lipid droplets and glycerin content. TNF-α may increase lipolysis via the nuclear factor-κB (NF-κB)-JNK and p44/42 signaling pathways (8). Perilipin-A has been the primary target of various mechanistic studies involving lipolysis (9); perilipin-A expression has previously been demonstrated to be blocked by inhibitors of NF-κB-JNK and p44/42 signaling pathways (10). Perilipin-A surrounds lipid droplets in adipocytes and restricts the access of lipases to the triglyceride core within the droplet, thus inhibiting lipolysis. However, perilipin-A additionally regulates lipase phosphorylation, thereby enhancing lipolytic reactions. A previous study revealed that perilipin-A-null mice exhibit reduced adipose mass, increased basal lipolysis in adipose tissues, and increased expression and activity of hormone-sensitive and adipose triglyceride lipases (11). Increased lipolytic activity may accelerate the efflux of FFAs from adipose tissues to the bloodstream, and therefore insulin resistance in perilipin-A null mice (11).

Obesity and T2DM are additionally closely associated with ERS. ERS is the accumulation of un- or misfolded proteins resulting from various causes in ER lumina. Signal transduction of ERS is mediated by IRE1, PERK and ATF6, which combine with BiP to remain inactive under non-pathologic conditions. In the presence of ERS, these proteins may transduce ERS signals from the membrane to the nucleus by oligomerization and phosphorylation. IRE (a type-1 transmembrane protein that contains silk threonine kinase and ribonuclease domains) increases JNK phosphorylation upon activation.

Low-level inflammation is present in obesity and T2DM; inflammatory factors are produced primarily in adipose tissue. TNF-α may induce ERS in L929 murine fibrosarcoma cells after 4 h to increase levels of the ERS marker proteins BiP, ATF6 and PERK (12). To ascertain if ERS is involved in TNF-α-stimulated lipolysis in 3T3-L1 adipocytes, the present study assessed the survival of 3T3-L1 adipocytes treated with different concentrations of TNF-α for 24 h. The concentration of 50 ng/ml TNF-α was selected for use in the present study. Following treatment, expression levels of ERS marker proteins were measured at various time points. The present study revealed significant differences after 2-h treatment, compared with the control group, and the expression levels of the ERS marker proteins BiP, IRE and JNK increased in a time-dependent manner, peaking at 4 h and declining at 6 h. Thus, treatment with 50 ng/ml TNF-α for 4 h was selected for TUDCA intervention experiments.

Ozcan et al (5) were the first to propose that TUDCA may alter ER function, alleviating the increased ERS observed in obesity, and reversing insulin resistance and T2DM. Jiao et al (13) demonstrated that TUDCA may inhibit FFA-induced ERS and inflammation to improve insulin sensitivity. TUDCA has previously been demonstrated to reverse obesity-associated metabolism alterations in C57BL/6 mice, including glucose intolerance, systemic and hepatic insulin resistance, hyperinsulinemia, and increases in blood pressure (14). Based on this information, the present study selected to use TUDCA to further investigate the effects of ERS on FFA-induced lipolysis.

Based on previous literature, and a previous experiment (data not shown), the present study selected to treat cells with 1 mmol/l TUDCA for 3 h in vitro to ensure cell survival (15). The present study demonstrated that glycerin content in the TUDCA+TNF-α group was reduced significantly, and that
Figure 5. TNF-α influences expression of the endoplasmic reticulum stress marker proteins BiP, p-IRE and p-JNK. Representative western blot images and quantification of p-IRE, IRE, p-JNK, JNK and BiP protein expression levels in 3T3-L1 adipocytes, following treatment with 50 ng/ml TNF-α for 0, 2, 4 or 6 h. β-actin served as an internal control. Data are presented as the mean ± standard deviation.* P<0.01 vs. 0 h treated group; ^P<0.01 vs. 2 h treated group; ∆P<0.01 vs. 6 h treated group. p, phosphorylated; JNK, c-Jun N-terminal kinase; BiP, immunoglobulin-binding protein; IRE, inositol-requiring enzyme; TNF-α, transforming growth factor-α.

Figure 6. TUDCA inhibits TNF-α-induced endoplasmic reticulum stress in 3T3-L1 adipocytes. Representative western blot images and quantification of p-IRE, IRE, p-JNK, JNK and BiP protein expression levels in 3T3-L1 adipocytes treated with TNF-α, TNF-α + TUDCA, or TUDCA. Data are presented as the mean ± standard deviation. *P<0.01 vs. control and TUDCA + TNF-α groups. TUDCA, tauroursodeoxycholic acid; p, phosphorylated; JNK, c-Jun N-terminal kinase; BiP, immunoglobulin-binding protein; IRE, inositol-requiring enzyme; TNF-α, transforming growth factor-α.
dispersal of lipid droplets in the TUDCA+TNF-α group was reduced, compared with the TNF-α group. Therefore, TUDCA may inhibit the downregulation of perilipin-A expression. Additionally, the present study revealed that expression levels of the ERS marker proteins BiP, p-IRE and p-JNK in 3T3-L1 adipocytes reduced significantly following pretreatment with TUDCA. Thus, TUDCA may alleviate TNF-α-induced ERS.

In conclusion, TUDCA may inhibit TNF-α-induced lipolysis in 3T3-L1 adipocytes. The underlying mechanism of action is potentially associated with the inhibition of the IRE-JNK signaling pathway, which affects perilipin-A expression levels. The underlying mechanisms of action of TNF-α-induced lipolysis merit further studies to identify potential targets for therapy against obesity, dyslipidemia, insulin resistance and DM. TUDCA may reduce insulin resistance and improve insulin sensitivity. Furthermore, it may alleviate insulin ER stress and protect microvessels. Therefore, TUDCA may act as a potential therapeutic agent for the treatment of obesity and T2DM in the future.

Acknowledgements

The present study was supported by the National and Fujian Province’s Key Clinical Specialty Discipline Construction Programs (grant no. 2015-SLN-11).

References