Exposure to Stearate Activates the IRE1α/XBP-1 Pathway in 3T3-L1 Adipocytes

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INTRODUCTION

For a protein to effectively function, it must form a higher-order structure after synthesis and modification. The endoplasmic reticulum (ER), the site of protein synthesis, contains molecular chaperones and disulfide isomerases, which are involved in the formation of higher-order protein structures. However, when the function of the ER is impaired or excessive protein synthesis occurs, the burden on the ER can result in the accumulation of proteins with inadequate higher-order structures. This condition is known as ER stress. In the late 1980s, Kozutsumi et al. revealed that increased expression of molecular chaperones occurred when proteins with inadequate higher-order structures were over-expressed, which induced ER stress in simian cells. In addition, expression of these malformed proteins inhibited protein synthesis and accelerated protein degradation, thereby reducing ER stress. Furthermore, apoptosis is reportedly initiated in cells exposed to prolonged ER stress. These cellular responses are known as the unfolded protein response (UPR).

The UPR involves three stress sensor proteins: inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-activated-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6α (ATF6α). All of these stress sensors are inactivated when bind to binding immunoglobulin protein (BiP)/glucose-regulated protein 78 (Grp78). However, ER stress causes BiP/Grp78 proteins to dissociate from the stress sensors, resulting in their activation. IRE1α is activated through dimerization (or multimer formation) and phosphorylation. Activated IRE1α leads to X-box binding protein 1 (XBP-1) mRNA splicing and transmission of signals downstream that regulate protein synthesis. Similarly, PERK is activated by phosphorylation and transmits signals downstream. Specifically, expression of the CCAAT/enhancer binding protein (C/EBP)-homologous protein (CHOP) gene is activated by PERK signaling, and both proteins are involved in the induction of apoptosis. CHOP is frequently used as a marker of the UPR. ATF6α is cleaved, and the cleavage product functions as a transcription factor. Thus, the UPR is considered a regulatory reaction that maintains cellular homeostasis or induces apoptosis in cells that cannot maintain homeostasis via ER stress sensors and their downstream signals.

The UPR is induced by changes in various factors surrounding cells such as cytokines, heat, calcium ions, oxygen and glucose. Recently, dietary fats containing saturated fatty acids without a double bond between carbons have been shown to induce the UPR in cells such as pancreatic β cells, muscle cells and liver cells leading to apoptosis of these cells. Apoptosis of such cells impairs insulin sensitivity and the metabolism of glucose and lipids, causing the development of diabetes and metabolic disorders. Thus, the UPR initiated due to saturated fatty acids increases the risk of developing metabolic diseases.

The development of metabolic disorders, coronary heart disease and cancer has been closely related to the func-
tion of adipocytes. Adipocytes store energy sources such as excess sugars and fatty acids in the form of triglycerides and also secrete various physiologically active substances that affect energy metabolism in other organs. One such substance is adiponectin, which improves insulin resistance, promotes glucose metabolism, and exhibits anti-inflammatory effects. These functions are reported to prevent diseases such as diabetes and atherosclerotic disease. On the other hand, the release of inflammatory cytokines and fatty acids from adipocytes that have been enlarged by accumulating excess triglyceride is elevated, resulting in reduced secretion of adiponectin. Based on these reports, the function of adipocytes is clearly related to the onset and progression of metabolic diseases such as diabetes. In adipocytes, the UPR occurs during differentiation and induces expression of enzymes involved in lipid metabolism, but is suppressed in adipocytes deficient in XBP-1 lipid synthesis. In addition, the UPR may be involved in the activation of adiponectin, suggesting that it is an important reaction for adipocyte differentiation as well as maintenance of adipocyte function. However, whether saturated fatty acids induce the UPR in adipocytes remains uncertain. One previous study showed that palmitate (0.5 mM for 6–12 h) can induce CHOP protein expression in fully differentiated 3T3-L1 adipocytes. In contrast, palmitate (1 mM for 48 h) has no effect on the expression levels of CHOP protein and spliced XBP-1 mRNA in the same adipocytes. Thus, the effects of palmitate on the UPR in adipocytes are unclear. Therefore, as the major saturated fatty acids present in the diet are palmitate and stearate, we examined the effects of these saturated fatty acids on the UPR in adipocytes.

**MATERIALS AND METHODS**

**Cell, Primers, and Antibodies** The mouse embryonic fibroblast-derived pre-adipocyte cell line 3T3-L1 was supplied by the Human Science Foundation (JCBR9014, Lot 0125200, Japan). Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L α-glucose (DMEM-high glucose, C1995500; Life Technologies, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS, FB-1380; Biosera, France), 100 units/mL penicillin (P3032; Sigma-Aldrich/Merck KGaA, Darmstadt, Germany), and 0.1 mg/mL streptomycin (190–14342; FUJIFILM Wako, Osaka, Japan) was used to culture the cells. The primers used for PCR were as follows, XBP-1s (spliced): 5'-CTG AGT CCG AAT CAG GTG CAG-3' (antisense) ; 5'-ATG AAC TCC ACT TAC C-3' (sense) and 5'-GGG CAT CAC AGG CCT GTT ATT G-3' (antisense). All primers were supplied by Exigen Co., Ltd. (Tokyo, Japan). Immunoblotting was performed with the following antibodies: IRELα (NB100–2324, NOVUS; Littleton, CO, U.S.A.), Phospho-IRE1α [Ser724] (NB100–2323, NOVUS), PERK (#33192; CST, Danvers, MA, U.S.A.), Phospho-PERK [Thr980] (#3179, CST), ATF6α (73–505, Bio Academia, Osaka, Japan), and β-actin (#4967, CST). Horseradish peroxidase (HRP)-conjugated whole-molecule anti-mouse immunoglobulin G (IgG) (A4416; Sigma-Aldrich/Merck KGaA) and anti-rabbit IgG (#7074, CST) were used as secondary antibodies.

**Cell Culture, Differentiation, and Addition of Fatty Acids to Medium** We cultured 3T3-L1 cells in DMEM-high glucose (containing 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin). For differentiation of the 3T3-L1 pre-adipocytes into mature adipocytes, cells of post-confluent status (designated day 0) were induced to differentiate by adding 0.5 mM 3-isobutyl-1-methylxanthine (I7018, Sigma-Aldrich/Merck KGaA), 1 mM dexamethasone (D4902, Sigma-Aldrich/Merck KGaA), and 5 mg/mL bovine insulin (I5500, Sigma-Aldrich/Merck KGaA) to the culture medium every 2 d until day 4. Then the cells were fed with DMEM-high glucose containing 10% FBS and 5 mg/mL insulin for 2 d. Beginning 6 d after the initiation of differentiation, cells were given DMEM-high glucose containing 10% FBS and 0.5 mg/mL insulin until day 8. To adjust the glucose concentration in the medium to physiological conditions, 5 µL 20 g/dL glucose solution was added to the 5 mL culture on day 7 (the glucose concentration was approximately 130 mg/dL). On day 8, bovine serum albumin (BSA)-conjugated palmitate, stearate, oleate, or BSA alone (vehicle) was added to the medium at 500 µm for 0.5, 1, 3, 6, or 12 h. Fatty acids conjugated to BSA (017–15146; FUJIFILM Wako) were prepared according to the method described in a previous study. Quantitative (q)RT-PCR Cells that had been exposed to fatty acids were washed twice with phosphate-buffered saline (PBS), and then their total RNA was isolated using the RNaiso Plus kit (TaKaRa, Otsu, Japan) according to the manufacturer’s instructions. Reverse transcription was performed with 1 µg RNA using PrimeScript II reverse transcriptase (TaKaRa). RT-PCR amplification of the transcribed cDNA was performed with the SYBR Premix Ex Taq II (TaKaRa). Reaction mixtures were incubated for initial denaturation at 95°C for 30 s followed by 40 cycles consisting of 95°C for 30 s and 60°C for 30 s. The expression levels of CHOP, DNAJB9, Pdia6 and Grp78 mRNA were normalized to 18S rRNA.

**Immunoblotting** Cells were washed twice with PBS, and then cell lysates were prepared in sodium dodecyl sulfate (SDS) sample buffer (133 mM Tris–HCl, pH 6.8, 3% SDS, 26% glycerol). Protein was separated using 10% (w/v) SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Merck Millipore, Billerica, MA, U.S.A.). After blocking with skim milk (Morianaga, Tokyo, Japan) or BSA in Tris-buffered saline (TBS) containing 0.05% Tween for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. The next day, the membranes were washed three times in TBS with 0.05% Tween and incubated with a secondary antibody for 1 h at room
temperature. After washing again in TBS with 0.05% Tween, the membranes were subjected to enhanced chemiluminescence using ImmunoStar LD (FUJIFILM Wako). Detection of immunoreactive bands was performed using the Amer sham Imager 680 (GE Healthcare, WI, U.S.A.). Lysate of cells treated with 5µM Thapsigargin (AdipoGen Life Sciences, Liestal, Switzerland) for 6 h was used as a positive control for the detection of IRE1α and PERK phosphorylation, as well as ATF6α cleavage. Quantification of the bands was conducted through volume densitometry using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, U.S.A.).

RESULTS

Exposure to 100µM Stearate Elevated the XBP-1s/t Ratio in Adipocytes To investigate whether palmitate or stearate induces the UPR in adipocytes, the proportion of spliced XBP-1 mRNA (XBP-1s/t ratio) and the expression level of CHOP mRNA were measured. For evaluation of the nonspecific effects of fatty acid addition to the culture medium, another fatty acid (oleate) was compared to palmitate and stearate. The concentration of each fatty acid was 100–500 µM to mimic the fatty acid concentration in the plasma of healthy subjects, which is several hundred micromolar, and to match the level of palmitate that causes the UPR in various cells. When 100µM stearate was added to the medium of adipocytes for 6 h, the XBP-1s/t ratio was significantly elevated, at 1.4 times that of the vehicle (Fig. 1A). On the other hand, adding palmitate or oleate did not affect XBP-1s/t. Expression levels of CHOP mRNA did not change with adding stearate, oleate, or palmitate (Fig. 1B). These results indicate that stearate induced the UPR in adipocytes.

Stearate Induce the UPR in Adipocytes More Strongly than Palmitate In hepatocytes and pancreatic β cells exposed to palmitate, the XBP-1s/t ratio and expression level of CHOP mRNA are elevated when UPR is induced. However, our results indicate that adipocytes supplemented with 100µM palmitate for 6 h have an unaltered XBP-1s/t ratio and CHOP mRNA expression level, and the UPR was not active. This finding could be due to the concentration of palmitate being too low, or may indicate that the XBP-1s/t ratio and expression level of CHOP mRNA require longer than 6 h to rise. Therefore, the concentration (100, 200, 500 µM) and time (0, 1, 3, 6, 12 h) of the palmitate and stearate additions were changed and analyzed again. The addition of 500 µM palmitate to the medium elevated the XBP-1s/t ratio at 6 h to 2.0-fold higher than the vehicle (Fig. 2A). Moreover, XBP-1s/t did not change at any time in adipocytes supplemented with 100 or 200 µM palmitate, indicating that palmitate has the potential to induce the UPR in adipocytes.

Exposure to 500µM stearate elevated XBP-1s/t after 1 h, and it remained significantly higher than the vehicle after 3, 6, and 12 h (Fig. 2B). XBP-1s/t peaked after 6 h of exposure to stearate, when it exhibited a 3.9-fold increase compared to the vehicle. Moreover, 100 and 200 µM stearate also significantly elevated XBP-1s/t, in contrast with palmitate. With 6 h of exposure, the XBP-1s/t ratio of cells exposed to 500 µM stearate was significantly higher than that of cells exposed to 500 µM palmitate (Fig. 2E). On the other hand, expression levels of CHOP mRNA were slightly elevated at 3 h after adding 500 µM stearate to the medium, but remained almost the same as in the vehicle-only treatment. These results suggest that exposure to stearate induced the UPR in adipocytes more strongly than palmitate.

The IRE1α/XBP-1 Pathway Is Activated in Adipocytes Exposed to Stearate Next, to investigate which UPR pathway was activated in adipocytes exposed to saturated fatty acids, we examined the activation of three stress sensors (IRE1α, PERK, and ATF6α) as well as genes acting downstream of XBP-1 and CHOP proteins in cells exposed to stearate (Fig. 3). Activation of IRE1α and PERK was examined based on the phosphorylation levels of those proteins using antibodies against the phosphorylation site. ATF6α activation was examined using the ratio of unfragmented ATF6α (P) to fragmented ATF6α (N). The phosphorylation level of IRE1α was elevated at 0.5 h after adding stearate to the medium, and was significantly elevated at 1 h relative to the vehicle. Moreover, at 6 h after adding stearate, the phosphorylation level of PERK was significantly elevated. However, this elevation did not coincide with changes in downstream CHOP mRNA. Finally, the fragmentation of ATF6α was comparable to that of the vehicle.

These observations indicate that the IRE1α/XBP-1 pathway might be activated in adipocytes exposed to stearate. As the expression of several genes (DNAJB9, Pdia6, Grp78) is regulated by XBP-1, we examined the effects of stearate on...
the expression levels of those genes using qRT-PCR. DNAJB9 mRNA levels were significantly elevated at 3 h after stearate was added to the culture medium of adipocytes relative to the vehicle. Similarly, Pdia6 mRNA levels were slightly but significantly elevated after 3 h. On the other hand, Grp78 mRNA levels did not increase relative to the vehicle. These results suggest that the IRE1α/XBP-1 pathway is activated in adipocytes exposed to stearate.

**DISCUSSION**

Based on analyses of stress sensors (IRE1α, PERK, ATF6α) and changes in the downstream gene targets of XBP-1 (DNAJB9, Pdia6, Grp78), we revealed the pathway activating the UPR in adipocytes exposed to stearate. Phosphorylation of IRE1α was enhanced 1 h after adding stearate to the culture medium, and the downstream XBP-1s/t ratio increased from 1 h until 6 h. The expression levels of DNAJB9 mRNA and Pdia6 mRNA were elevated after 3 h. In addition, the phosphorylation level of PERK was elevated 6 h after adding stearate, whereas the expression level of CHOP mRNA did not change within 12 h. Fragmentation of ATF6α was similar to the vehicle, consistent with a previous report that activation of the ATF6α pathway does not occur after adding saturated fatty acids.54 These results strongly suggest that stearate activates only the IRE1α/XBP-1 pathway in adipocytes.

The addition of 100–500 µM stearate or 500 µM palmitate to the culture medium increased the XBP-1s/t ratio in adipocytes. However, the expression level of CHOP mRNA remained nearly constant, even in the presence of 500 µM stearate. Previous studies have reported that adding 500 µM palmitate to culture medium of hepatocytes or pancreatic β cells for several hours leads to elevation of XBP-1s/t and the expression level of CHOP mRNA.19,49 In addition, 500 µM palmitate in the medium of undifferentiated 3T3-L1 pre-adipocytes may increase XBP-1s/t and CHOP mRNA expression.

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Fig. 2. The Unfolded Protein Response (UPR) Was Induced in Adipocytes More Strongly by Stearate than by Palmitate

We added 100, 200, or 500 µM palmitate, stearate or BSA (vehicle) to the adipocyte culture medium and examined XBP-1s/t (A, palmitate; B, stearate) and CHOP/18S rRNA (C, palmitate; D, stearate) after 0, 1, 3, 6, and 12 h (mean ± S.D., n = 3–6) *p < 0.05, **p < 0.01, vs. vehicle. (E) XBP-1s/t and CHOP mRNA (CHOP/18S rRNA) expression levels in cells exposed to 500 µM palmitate or stearate at 6 h were extracted from A–D (mean ± S.D., n = 4) * p < 0.05 vs. palmitate (Student’s t-test).
levels. Together, these reports demonstrate elevation of both the XBP-1s/t ratio and the expression level of CHOP mRNA with palmitate addition to the culture medium. Therefore, our observation is a unique phenomenon compared to previous studies. Increased expression of CHOP protein induces apoptosis, and one study showed that cell death was induced in hepatocytes. As adipocytes store fatty acids in the form of triacylglyceride (TAG), lipotoxicity is unlikely and one study focused on the enzyme stearoyl-CoA desaturase 1 (SCD1), which promotes desaturation of fatty acids. When SCD1 was knocked down in HeLa cells, the content and proportion of saturated fatty acids in cell membrane phospholipids increased, and furthermore, the UPR (elevation of XBP-1s/t ratio and expression levels of CHOP mRNA) was induced. Another study showed that an increased proportion of saturated fatty acids in membrane phospholipids enhanced phosphorylation of PERK. In addition, we have found that, while extracellular stearate activates G protein-coupled receptor 120, this activation is not involved in the UPR in adipocytes exposed to stearate (manuscript in preparation). Therefore, the increase in saturated fatty acids among cell membrane phospholipids may be related to the UPR induced by stearate.

XBP-1 positively regulates the expression of diacylglycerol O-acyltransferase, which is involved in TAG synthesis. Because stearate-induced UPR is repressed by oleate (Supplementary Fig. S2), when stearate blood concentrations are higher than those of oleate, as in the case of dietary intake or fasting, the stearate-activated IRE1α/XBP-1 pathway may enhance synthesis of TAG and suppress saturated fatty acid-induced lipotoxicity. The gene expression levels of molecular chaperones such as DNAJB9 and Pdia6 were elevated. The expression of molecular chaperones in adipocytes is involved in the formation of highly active macromolecular adiponectin. As activation of adiponectin promotes glucose metabolism and anti-inflammatory responses, the stearate-induced UPR may help prevent the development of diseases such as diabetes.

Our findings demonstrate that the UPR induced in adipocytes through the addition of stearate to the culture medium is an important reaction that enhances the function of adipocytes and helps maintain homeostasis in vivo. In other words, we believe that this study describes a novel mechanism through
which saturated fatty acids and adipocytes regulate systemic energy metabolism.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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