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Anti-fibrotic Effect of Saturated Fatty Acids via Endoplasmic Reticulum Stress Response in Rat Pancreatic Stellate Cells

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Abstract

Objectives—We investigated the effect of saturated fatty acids on the CP pathogenesis by elucidating the endoplasmic reticulum (ER) stress response in pancreatic stellate cells (PSCs), which are major effector cells in pancreatic fibrosis.

Methods—Wistar Bonn/Kobori (KOB) rats were fed either control diet or high-fat diet (HFD) for 4 weeks. Meanwhile, cultured rat PSCs were stimulated with thapsigargin (Tg), an ER stress inducer, or palmitic acid (PA). Pancreatic fibrosis, expressions of fibrosis-related and ER stress-related proteins and mRNA, cell viability and apoptosis were examined.

Results—HFD reduced fibrosis and α -smooth muscle actin expression (i.e., activated PSCs) but upregulated ER stress-related mRNA expression in the pancreas of young HFD-fed KOB rats. Induction of ER stress response in PSCs with Tg or PA induced apoptosis, activated the PERK pathway, inhibited cell viability, and downregulated fibrosis-related protein and mRNA expression. PERK inhibitor negated PA-induced ER stress response.

Conclusions—Saturated fatty acids can inhibit but may not promote the fibrogenesis of CP, at least in the early stage, via an ER stress response (i.e., the PERK pathway) in PSCs. Moreover, induction of an apoptotic ER stress response in PSCs might be a novel therapeutic strategy for pancreatic fibrosis.

Keywords

Chronic pancreatitis; Pancreatic stellate cells; Endoplasmic reticulum stress; PERK pathway; Saturated fatty acids

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Introduction

Chronic pancreatitis (CP) is characterized by progressive and irreversible structural fibrotic change and acinar cell (AC) destruction, resulting in the loss of pancreatic exocrine and endocrine function.^{1,2} Periacinar stellate-shaped cells, designated pancreatic stellate cells (PSCs), were identified and characterized in 1998.^{3,4} In the normal pancreas, PSCs are in a quiescent state, storing vitamin A-containing lipid droplets in the cytoplasm. However, when PSCs are activated in response to pancreatic inflammation or injury, they transformed to a myofibroblast-like phenotype characterized by increased expression of alpha-smooth muscle actin (α -SMA). Therefore, activated PSCs are considered to play a central role in the pathogenesis of CP by secreting various cytokines/chemokines such as transforming growth factor (TGF) β 1⁵ and producing extracellular matrix components (ECM) such as type I collagen and fibronectin.^{4,6}

Although PSCs are attracting attention as a new therapeutic target for CP, alleviating the cause is currently the only way to prevent disease progression because of the lack of treatments that directly inhibit CP pathogenesis. Alcohol consumption is the major cause of CP,^{1,2} and exposure to ethanol and its oxidative metabolite acetaldehyde are reported to promote CP pathogenesis via the activation of PSC function.^{7,8} Thus, alcohol abstinence is critical. In contrast, the relationship between hyperlipidemia and CP is still “rare and controversial”.¹ Free fatty acids (FFA) are reported to injure AC via lipid peroxidation of the cellular membrane⁹ and by inducing cholecystokinin release from the duodenal endocrine cells, which overstimulates pancreatic exocrine function.¹⁰ Therefore, a high-fat diet (HFD) promotes symptoms in CP patients presenting with abdominal pain.¹¹ In addition, an HFD induces pancreatic injury and fibrosis in the pancreas of normal rats via oxidative stress, activation of nuclear factor- κ B (NF- κ B), and microcirculatory disturbance.¹²⁻¹⁴ However, the direct effects of an HFD on the pathogenesis of CP and the cellular function of PSCs have not been evaluated.

Hyperlipidemia is well known to contribute to the progression of several diseases such as diabetes,^{15,16} nonalcoholic steatohepatitis,¹⁷⁻¹⁹ and atherosclerosis.^{20,21} Recently accumulated evidence shows that the saturated fatty acid (SFA)-induced endoplasmic reticulum (ER) stress response plays a crucial role in these processes. ER stress, accumulation of misfolded proteins in the ER lumen, is induced in variant cells, resulting in cellular dysfunction. The ER stress response, which protects cellular function from ER stress, is activated by 3 pathways: double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme-1 α (IRE1 α), and activating transcription factor (ATF) 6.^{22,23} Accordingly, upregulation of ER chaperones [glucose-regulated protein (Grp) 78, Grp94] promotes protein folding, and activation of the PERK/eukaryotic initiation factor 2 α (eIF2 α) pathway attenuates protein synthesis.²³⁻²⁵ Although augmentation of ER capacity by these mechanisms improves cell survival, the ER stress response induces apoptosis when ER function is severely injured.^{22,23,26} PERK activation also plays a key role in this process via ATF4-mediated C/EBP homologous protein (CHOP) upregulation.^{22, 23, 25-27}

Although the ER stress response is an essential mechanism for maintaining cellular homeostasis, especially in secretory cells, its effect on PSCs function remains unclear. Regarding CP, Sah et al²⁸ recently reported that ER stress is chronically activated in ACs and that the induction of ER stress in pancreatic acini results in NF- κ B activation and cell death, leading to acinar atrophy and chronic inflammation in CP. However, it is unclear whether the ER stress response in PSCs is involved in the progression of CP. Furthermore, the effect of SFA on CP pathogenesis via the ER stress response in PSCs is unknown.

Therefore, the present study investigated the relationship between SFA and CP pathogenesis by elucidating (1) the effect of the ER stress response on the activation and cellular function of PSCs, (2) whether SFA induces the ER stress response in PSCs, and (3) whether SFA promotes CP pathogenesis via PSCs function.

Materials and Methods

Materials

Palmitic acid (PA), thapsigargin (Tg), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St Louis, MO, USA). GSK2606414 was purchased from Calbiochem (La Jolla, CA, USA). Goat anti-rat type I collagen antibody was purchased from Southern Biotech (Birmingham, AL, USA). Rabbit anti-rat fibronectin antibody was purchased from Chemicon International (Temecula, CA, USA). Rabbit anti-rat Grp78 antibody and mouse anti-rat α -smooth muscle actin (α -SMA) antibody were purchased from Abcam PLC (Cambridge, UK). Anti-rabbit Alexa 488-conjugated IgG antibody, anti-mouse Alexa 555-conjugated IgG antibody, and Hoechst 33342 were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents were purchased from Cell Signaling Technology (Beverly, MA, USA) unless mentioned otherwise.

Animals and diet

All animal experiments were performed in accordance with the Guidelines of the Committee on Animal Care of Kyushu University. Male Wistar rats and Wistar/Bonn-Kobori (KOB) rats (Japan SLC Inc., Hamamatsu, Japan), which is widely accepted as a rodent model of CP [29], were used. Because pancreatic fibrosis occurs in KOB rats at 10–12 weeks of age,²⁹ we used 3- and 7-week-old KOB rats (considered the early stage of CP) to investigate the effect of a HFD on CP fibrogenesis. Wistar and KOB rats (3 and 7 weeks old) were fed either control diet (CD) (MF; KBT Oriental, Saga, Japan) or HFD (Test Diet 58Y1; Purina, St Louis, MO, USA) *ad libitum* for 4 weeks (Figure 1). Total calories were matched between CD and HFD, and the percentages of energy derived from fat in the CD and HFD were 4.8% and 61.6%, respectively. Rats were maintained in a 12-hour light/dark cycle and had clean water *ad libitum*. Body weight was monitored weekly. After 4 weeks of feeding, 7- and 11-week-old rats were euthanized, and blood samples from the inferior vena cava and pancreatic tissues were extracted.

Isolation and culture of pancreatic stellate cells (PSCs)

PSCs were isolated from the pancreas of male Wistar rats weighing 200–250 g by density-gradient centrifugation.³⁰ Cell purity was assessed by a typical star-like configuration and by

detecting vitamin A autofluorescence and always exceeded 95%. Isolated stellate cells were resuspended in complete Dulbecco's modified Eagle medium mixed with Ham's F-12 nutrient mixture (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 50 units/mL penicillin, and 50 mg/mL streptomycin (Invitrogen). Upon reaching confluence, the cells were trypsinized with 0.05% trypsin/0.01% EDTA (Invitrogen) and passaged at a ratio of 1:3. All experiments were performed using third-passage PSCs, except for those using freshly isolated PSCs (first passage; 2 days after isolation). Unless specified otherwise, PSCs were incubated in serum-free medium for 24 hours before the addition of experimental reagents. PA was dissolved in medium, making an emulsion with BSA at a molar ratio of 10:1. Fresh PA/BSA complex was prepared before each experiment. We examined the effects of BSA as a control to clarify whether the effects of PA were due to nonspecific protein binding to PSCs. For some experiments, GSK2606414, a specific inhibitor of PERK phosphorylation,³¹ was added 1 hour before PA or BSA treatment.

Serum biochemical analysis

Blood samples were centrifuged at $2000 \times g$ for 5 minutes, and serum levels of FFA, triglycerides, total cholesterol, amylase, and lipase were analyzed using a fully automatic biochemistry analyzer (Hitachi 7600 Auto Analyzer; Hitachi Ltd, Tokyo, Japan) according to the manufacturer's instructions.

Histopathological analysis

Pancreatic tissue from Wistar and KOB rats was fixed by immersion in 4% paraformaldehyde overnight at 4°C. The specimens were embedded in regular paraffin wax and cut into 4- μ m-thick sections for histopathological analysis. Specimens were stained with *hematoxylin & eosin* for routine histologic observations, and Masson-trichrome to detect fibrosis. Ten microscopic fields ($\times 100$) were randomly chosen for each specimen, and the percentage of fibrotic area in the total pancreatic area was calculated using ImageJ (NIH, Bethesda, MD, USA).

Immunofluorescence staining

Immunofluorescence staining for pancreatic tissues was performed as described previously.³⁰ Briefly, paraffin-embedded pancreas tissue sections (4 μ m) were deparaffinized and rehydrated in a graded series of ethanol. After blocking with 2% normal BSA, sections were incubated with mouse anti-rat glial fibrillary acidic protein (GFAP, to stain quiescent PSCs) or α -SMA antibody (1:100) and rabbit anti-rat Grp78 (1:100) overnight at 4°C. After washing, slides were incubated with anti-rabbit Alexa 488-conjugated IgG antibody and anti-mouse Alexa 555-conjugated IgG antibody for 1 hour.

Immunofluorescence staining for PSCs was performed as described previously.³⁰ Briefly, PSCs were serum starved, incubated for 24 hour at 37°C, and fixed in 4% paraformaldehyde. Double-immunofluorescence staining for α -SMA/Grp78 was performed in a similar manner as that described above. Nuclear counterstaining was performed using Hoechst 33342 (Invitrogen). The fluorescence of the slides was analyzed under a confocal laser scanning microscope (Nikon A1/C1, Tokyo, Japan). Sections without primary antibody or with

polyclonal rabbit IgG (Abcam) were included in each staining experiment as negative controls (data not shown).

Cell viability assay

Cell viability was assessed by MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) as described previously [30]. Briefly, serum-starved PSCs were seeded onto 96-well flat-bottomed tissue culture plates at 1×10^4 cells/well. After 24 hours incubation with or without test agents, MTS solution was added to the cells, and the incubation continued at 37°C for 1 hour. Cell proliferation was subsequently quantified by the differences in absorbance at 570 and 690 nm (EnSpire, Perkin Elmer, Waltham, MA, USA).

Cell apoptosis assay

Cell apoptosis was assessed using the Cell Death Detection ELISA Plus Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions; this assay quantifies apoptosis by measuring mono- and oligonucleosomes released in the lysates of apoptotic cells. Briefly, PSCs were seeded onto 96-well flat-bottomed tissue culture plates at 1×10^4 cells/well. After 24 hours incubation with or without test agents, the supernatants were carefully removed, and PSCs were lysed for 30 minutes. Cell lysate solution and immunoreagent containing a mixture of anti-histone-biotin and anti-DNA-POD were transferred onto streptavidin-coated microtiter plates and incubated for 2 hours at room temperature in a shaking incubator at 300 rpm. The plate was washed with incubation buffer to remove unbound antibodies, and ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]) substrate solution was added to each well. Absorbance was measured photometrically at 405 nm, with a reference wavelength at 490 nm (EnSpire, Perkin Elmer).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the pancreatic tail and PSCs using the RNeasy mini kit (Qiagen, Valencia, CA, USA) as described previously.³⁰ For qRT-PCR, 100 ng total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan), and RT-PCR was performed using a LightCycler Real-Time PCR system (Roche, Switzerland) according to the manufacturer's instructions. The reaction mixture (20 µL) contained SYBR Premix Ex Taq II (TLi RNaseH Plus; Takara Bio, Inc.), 4 mM MgCl₂, 0.5 mM upstream and downstream PCR primers [see Supplemental Table in Supplemental Digital Content (SDC) 1], and 2 µL first-strand cDNA template. To control for variations in the reactions, all PCR data were normalized against GAPDH expression.

Western blotting

Western blot analysis was performed as described previously.³⁰ Briefly, cells were lysed with RIPA buffer for 10 minutes on ice. Whole-cell lysates (~100 µg) were fractionated by SDS-PAGE (Bio-Rad, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane (Bio-Rad), which was incubated overnight at 4°C with primary antibodies (1:1000–5000). After incubation with HRP-conjugated secondary antibody (1:10,000) for 1

hour at room temperature, the proteins were visualized by using an ECL kit (Perkin Elmer) and ImageQuant LAS 4000mini (GE Healthcare, Buckinghamshire, UK). Phosphorylated and total levels of PERK and eIF2 α as well as Grp78, Grp94, ATF4, CHOP, α -SMA, TGF β 1, type I collagen, fibronectin, and β -tubulin were determined in a similar manner. Band intensity was evaluated by densitometric analysis using ImageJ (NIH).

Statistical analysis

The results are presented as the mean $\pm$ SEM of 3–4 separate cell preparations unless specified otherwise. Experiments were performed at least 3 times, and similar results were obtained. Representative luminograms and autoradiograms are shown. Student's *t*-test was for comparisons between 2 groups. The level of significance was set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) for Windows.

Results

HFD increases serum FFA levels in Wistar and KOB rats

After 4 weeks, the mean body weight of the HFD-fed 11-week-old KOB (KOB11/HFD) group was significantly greater than that of the CD-fed 11-week-old KOB (KOB11/CD) group; no significant differences were observed among other groups (Table 1). HFD significantly increased serum levels of FFA in all groups and triglycerides in the 11-week-old Wistar (Wistar11) groups, while total cholesterol was significantly decreased in all groups except for 11-week-old KOB (KOB11) groups. There were no remarkable differences in serum pancreatic enzyme levels between the HFD-fed and CD-fed groups.

HFD induces ER stress response and inhibits fibrosis in KOB rats

Histopathological analysis of pancreatic tissues revealed the fibrotic area was significantly smaller in the HFD-fed 7-week-old KOB (KOB7/HFD) group than the CD-fed 7-week-old KOB (KOB7/CD) group, while no differences were observed among the KOB11 groups (see Supplemental Figure A, C in SDC 2). HFD also decreased α -SMA–positive cells in the stromal area in 7-week-old KOB (KOB7) rats (see Supplemental Figure D in SDC 2). In contrast, no histological changes such as inflammatory cell infiltration and fibrosis were observed in HFD-fed Wistar rats (see Supplemental Figure B in SDC 2).

The pancreatic expressions of fibrosis-related mRNA; TGF β 1, type I collagen, and fibronectin, and ER stress-related mRNA; Grp78, Grp94, ATF4, and CHOP were significantly higher in the KOB7/CD and KOB11/CD groups than the CD-fed 7-week-old Wistar (Wistar7/CD) and CD-fed Wistar11 (Wistar11/CD) groups, respectively (see Supplemental Figure E, F in SDC 2). These results are consistent with those of Sah et al, who report that ER stress is chronically activated in CP.²⁸ Although there was no significant difference between CD- and HFD-fed 7-week-old Wistar (Wistar7), Wistar11, or KOB11 groups, HFD significantly reduced fibrosis-related mRNA expression, except for type I collagen; meanwhile, ER stress-related mRNA expression, except for CHOP, was significantly higher in KOB7 rats (see Supplemental Figure E, F in SDC 2). Therefore, we hypothesize that serum FFA elevated by HFD induces an ER stress response in PSCs that

suppressed cellular function, especially ECM synthesis and activation, thus inhibiting fibrogenesis in CP.

Chronic activation of the ER stress response in PSCs

Before elucidating the effect of FFA on the PSCs function, we investigated whether the ER stress response is involved in PSCs activation. Immunofluorescence staining of KOB7/CD and Wistar7/CD rat pancreatic specimens showed Grp78 expression in both quiescent and activated PSCs (Fig. 2A, B), whereas no difference was observed between the CD- and HFD-fed KOB7 or KOB11 groups (data not shown). Fluorescence immunocytochemistry also showed Grp78 expression in both quiescent (first passage) and culture-activated (third passage) PSCs (Fig. 2C). Although the expressions of α -SMA as well as fibrosis-related protein and mRNA were higher in activated PSCs than quiescent PSCs, PSC activation itself did not promote the ER stress response (Fig. 2D, E).

ER stress response induction impairs PSCs function

We subsequently examined the effect of the ER stress response on PSC function using Tg. Tg induced apoptosis and impaired cell viability in activated PSCs in a dose-dependent manner (see Supplemental Figure A, B in SDC 3). Moreover, PERK and eIF2 α phosphorylation was induced in a time-dependent manner (see Supplemental Figure C in SDC 3). Tg also upregulate the expressions of ER chaperones and the downstream signaling of the PERK/eIF2 α pathway (i.e., ATF4 and CHOP), but downregulated the expressions of α -SMA and fibrosis-related protein and mRNA (see Supplemental Figure D, E in SDC 3). Similar results were observed in quiescent (first passage) PSCs (see Supplemental Figure A, B, C in SDC 4). Thus, induction of the ER stress response impairs cellular function in both quiescent and activated PSCs.

Palmitic acid induces the ER stress response in PSC

We subsequently examined the effect of PA on PSC function and determined if PA induces the ER stress response. PA stimulation induced apoptosis and impaired cell viability in activated PSCs in a dose-dependent manner (see Supplemental Figure A, B in SDC 5). Western blotting and qRT-PCR showed PA upregulated ER chaperones and activated the PERK/eIF2 α pathway and its downstream, but downregulated α -SMA and fibrosis-related proteins and mRNA (see Supplemental Figure C, D, E in SDC 5). Similar results were also observed in quiescent (first passage) PSCs (see Supplemental Figure A, B, C in SDC 6).

The PA-induced ER stress response in PSCs is mediated by the PERK/eIF2 α pathway

We used GSK2606414, PERK inhibitor, to determine whether the above-mentioned effects of PA on PSC function are mediated by the ER stress response.³¹ GSK2606414 (10 μ M) inhibited the PA-induced phosphorylation of PERK and eIF2 α (Fig. 3A). Although GSK2606414 itself induced apoptosis and impaired cell viability in the absence of PA, inhibition of the PERK/eIF2 α pathway negated the PA-induced apoptosis and cell viability impairment in activated PSCs (Fig. 3B, C). GSK2606414 also negated the PA-induced upregulation of ER stress-related proteins and downregulation of α -SMA and fibrosis-related proteins and mRNA (Fig. 3D, E). Although western blotting showed no significant

difference, inhibition of the PERK/eIF2 α pathway itself downregulated ATF4, CHOP, α -SMA, and TGF β 1 mRNA expressions, whereas those of ER chaperones and ECM were upregulated in the absence of PA. Thus, the results indicate the PERK/eIF2 α pathway plays a crucial role in the cellular function of PSCs by modulating adaptive and apoptotic signals of ER stress response regardless of the induction of ER stress (Fig. 4).

Discussion

CP is a progressive inflammatory disease characterized by irreversible fibrotic destruction, resulting in the deterioration of exocrine and endocrine functions.^{1, 2} PSCs are activated from their quiescent phenotype in response to pancreatic injury and inflammation during pancreatic fibrosis.^{3, 4} Activated PSCs play a pivotal role in CP pathogenesis by secreting various inflammatory cytokines/chemokines and ECM.⁴⁻⁶ Therefore, PSCs are considered a promising anti-fibrotic therapeutic target in CP.

CP has a multifactorial etiology.^{1, 2} Alcohol consumption is widely recognized as a major cause of CP as well as acute pancreatitis (AP).^{1, 2, 32} Although hyperlipidemia is well known to cause AP,³² the relationship between hyperlipidemia and CP is still “rare and controversial”.¹ Wistar rats chronically exposed to an HFD exhibit pancreatic injury and fibrosis via oxidative stress, NF- κ B activation, and microcirculatory disturbance.¹²⁻¹⁴ Furthermore, HFD increases the number of α -SMA-positive cells in pancreatic parenchyma.¹²⁻¹⁴ Similar results have also been reported using mutant Kras^{G12D} knock-in mice³³ and Zucker diabetic fatty rats.³⁴ Although these previous results suggest HFD induces fibrosis in the normal pancreas, its direct effect on the pathogenesis of CP has not been studied. To address this issue, we examined the morphological changes of CP caused by HFD using KOB rats, a spontaneous developing CP model rat.²⁹ The results show that HFD inhibited fibrosis and decreased α -SMA-positive cells (i.e., activated PSCs) in the stromal area of the pancreas in young KOB rats. These results appear inconsistent with previous reports.^{12-14, 33, 34} However, considering that pancreatic fibrosis was observed after the acute inflammatory change of acinar and islet cells,¹⁴ the PSC activation observed in previous studies may not be directly due to HFD, but the subsequent repair mechanism after acinar/islet cell injury reported by Siech et al.³⁵

To explain the inhibitory effect of HFD on fibrogenesis in CP via PSC function, the present study focused on the SFA-induced ER stress response, which plays a central role in the underlying mechanism encompassing hyperlipidemia and the progression of various diseases.^{22, 26} The ER provides several important functions including assembly and folding of secretory proteins as well as intracellular calcium storage; therefore, the ER is highly developed in pancreatic cells.³⁶ Accumulation of misfolded proteins in the ER lumen, termed “ER stress,” induces cellular dysfunction; accordingly, the ER stress response aims to maintain disturbed ER homeostasis under ER stress conditions.^{22, 23} The ER stress response initially adapts the ER’s capacity by increasing protein-folding capacity via upregulation of ER chaperones such as Grp78 and Grp94 as well as attenuating protein synthesis.²³⁻²⁵ However, if the response fails to resolve excessive ER stress, cells undergo apoptosis mediated by several signals.^{22, 23, 26} Recent accumulating evidence shows that the SFA-induced ER stress response in pancreatic β cells,^{15, 16} hepatocytes,¹⁷⁻¹⁹ macrophages,

and vascular endothelial cells^{20, 21} contributes to hyperlipidemia-mediated disease progression in diabetes, nonalcoholic steatohepatitis, and atherosclerosis. Like previous studies, the present study shows that HFD feeding induced the ER stress response in the pancreas of young KOB rats. Moreover, SFA induced the ER stress response in PSCs that resulted in apoptosis, inhibition of activation, and attenuation of ECM synthesis. These anti-fibrotic effects were limited to young KOB rats *in vivo*, mainly because of the irreversibility of the histological changes in CP. However, the present findings suggest SFA actually inhibit fibrogenesis in CP via PSC function.

We further examined the underlying mechanisms of the SFA-induced ER stress response in PSCs by investigating the PERK pathway, 1 of the 3 ER stress transducers. PERK activation and subsequent phosphorylation of Ser51 on eIF2 α transiently inhibits general mRNA translation, thereby reducing ER overload.^{22, 23, 25} PERK-deficient and eIF2 α mutant mice are reported to develop pancreatic endocrine/exocrine insufficiency via ER failure,³⁷ and cells with PERK deletion exhibit higher ER stress in response to abnormally elevated protein synthesis.²⁷ As PERK is essential for secretory cells, it is most highly expressed in the pancreas.³⁷ On the other hand, phosphorylated eIF2 α induces selected transcriptional upregulation including that of ATF4 and its target CHOP, a transcription factor that activates downstream pro-apoptotic signals.^{22, 23, 25-27} Several studies using CHOP-deficient mice show CHOP deletion protects against cell apoptosis.³⁸ Thus, the PERK pathway contributes significantly to maintaining ER homeostasis by modulating these paradoxical adaptive and apoptotic responses. By using the PERK-specific inhibitor GSK2606414,³¹ we demonstrated that activation of PERK pathway plays an essential role in the SFA-induced ER stress response in PSCs not only by attenuating ECM synthesis, but also by inducing apoptosis and inhibiting PSC activation. These results are consistent with previous reports indicating that PERK/ATF4/CHOP signals are involved in the SFA-induced ER stress response.¹⁵⁻²¹ This further indicates the PERK pathway is a key signaling pathway underlying the hyperlipidemia-mediated disease progression in CP and other diseases.

The ER stress response was recently reported to be activated during pancreatic injury and inflammation in CP²⁸ and AP.³⁹ Sah et al²⁸ reported that ER stress is chronically activated in an animal model of CP induced by recurrent episodes of AP induced by cerulein hyperstimulation. These results are consistent with our finding that ER stress-related mRNA expression was upregulated in KOB rats regardless of age. However, the ER stress response examined by Sah et al was involved in AC injury and not the cellular function of PSCs. The present study shows that the ER stress response, especially the PERK pathway, is chronically activated in both quiescent and activated PSCs, which is essential for the survival of PSCs under ER stress-free conditions. However, in the presence of ER stress inducers such as PA and Tg, the response switches from adaptive to apoptotic. Therefore, the present findings suggest that the induction of an apoptotic ER stress response in PSCs might be a potent therapeutic target for pancreatic fibrosis. Several studies have examined the effect of the ER stress response on the cellular function of hepatic stellate cells (HSCs), the major effector cells in liver fibrosis, which have similar morphology and cellular function to PSCs. Huang et al⁴⁰ reported that induction of ER stress by Tg leads HSCs to apoptosis via intracellular calcium concentration-mediated activation of the Calpain/Caspase and JNK/p38 MAPK pathways. Other ER stress inducers including tunicamycin,⁴¹ cannabidiol⁴² and 2-

APB,⁴³ which are transient receptor potential melastatin-like 7 channel blockers, also exert similar effects. The ER stress-mediated apoptosis of HSCs is observed during the resolution of liver fibrosis in rats that have undergone bile duct ligation and subsequent bile duct diversion.⁴¹ Deletion of heat shock protein 47 in HSCs is reported to inhibit experimentally induced liver fibrosis;⁴⁴ accordingly, Kawasaki et al⁴⁵ report that the ER stress-mediated apoptosis of HSCs may underlie this process. In contrast, recent report from Koo et al showed the strong correlation between ER stress in HSCs and liver fibrosis as a result of PERK-mediated degradation of heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and subsequent SMAD2 overexpression.⁴⁶ Although further studies are required to elucidate the effect of ER stress response on HSCs function, it is attracting attention as new therapeutic strategy for liver fibrosis. Interestingly, Ishiwatari et al⁴⁷ report similar results using a rat model of CP induced by dibutyltin dichloride and cerulein; they report that siRNA treatment against heat shock protein 47 in PSCs induces apoptosis and attenuates pancreatic fibrosis. However, they did not examine ER stress response. Nevertheless, considering the similarities between HSCs and PSCs, it can be inferred that ER stress-induced apoptosis in PSCs was involved in their results. The present and previous findings collectively suggest induction of the “apoptotic” ER stress response in PSCs and HSCs is a potent anti-fibrotic therapeutic strategy for pancreatic fibrosis as well as liver fibrosis.

The present study has several limitations that should be taken into consideration when interpreting the results. First, the 4-week HFD treatment is shorter than that in other studies (>18 weeks);^{12-14, 34} therefore, the long-term effect of HFD on CP pathogenesis remains unclear. As mentioned above, HFD and FFA induce AC and β cell injury via various mechanisms,^{9, 12-16} which might exacerbate several complications of CP, such as abdominal pain¹¹ and diabetes.^{15, 16} Moreover, AC injury could contribute to the fibrogenesis of CP according to the necrosis–fibrosis hypothesis, i.e., the development of fibrosis due to recurrent attacks of hyperlipidemia-induced AP.⁴⁸ Consistent with this hypothesis, Gu et al⁴⁹ recently reported that TGF β 1 can be produced by ACs early during AP onset, which initiates a fibrotic response. Thus, the indirect effect of AC injury on the pathogenesis of CP should be taken into consideration, especially during chronic exposure to HFD. Second, we did not examine other ER stress response pathways including those of IRE1 α and ATF6, which are also reported to be important for maintaining ER homeostasis.^{22, 23} In addition to PERK/ATF4-mediated CHOP upregulation, other apoptotic signals were also activated during the ER stress response, including the PERK-independent upregulation of CHOP,²⁶ IRE1 α -mediated activation of apoptosis signal-regulating kinase 1 (ASK1)/c-Jun amino-terminal kinase (JNK),^{50, 51} activation of procaspase 12,⁵² and ER stress-mediated regulation of the NF- κ B signaling pathway.^{53, 54} Indeed, in the present study, PERK inhibition did not totally abolish the PA-induced ER stress response, particularly apoptosis. Therefore, further studies are required to clarify the ER stress response in PSCs, especially those mediated by IRE1 α and ATF6.

In conclusion, an HFD inhibits the fibrogenesis of CP in young KOB rats. Moreover, the underlying mechanism involves the SFA-induced ER stress response, especially activation of the PERK/ATF4/CHOP pathway. These findings suggest the induction of the apoptotic ER stress response in PSCs may be a novel anti-fibrotic therapeutic strategy for CP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Study protocol

Wistar and Wistar Bonn/Kobori (KOB) rats (3 and 7 weeks old) were fed either control diet (CD) or high-fat diet (HFD) for 4 weeks ($n = 6$ mice per group). Wistar7/CD, CD-fed 7-weeks-old Wistar rats; Wistar7/HFD, HFD-fed 7-weeks-old Wistar rats; KOB7/CD, CD-fed 7-weeks-old KOB rats; KOB7/HFD, HFD-fed 7-weeks-old KOB rats; Wistar11/CD, CD-fed 11-weeks-old Wistar rats; Wistar11/HFD, HFD-fed 11-weeks-old Wistar rats; KOB11/CD, CD-fed 11-weeks-old KOB rats; KOB11/HFD, HFD-fed 11-weeks-old KOB rats.

Figure 2. ER stress is chronically activated in pancreatic stellate cells (PSCs)
Expressions of Grp78 (in green) and α -SMA (in red) in the pancreas of 7-week-old KOB rats fed control diet (CD) (A) and 7-week-old Wistar fed CD (B) were examined by immunofluorescence staining. Representative sections are shown. *Scale bars* = 20 μ m. (C) Expressions of Grp78 (in green) and α -SMA (in red) in quiescent (1 day after isolation; first

passage) and culture-activated (third passage) PSCs were examined by immunofluorescence staining. *Scale bars* = 50 μ m. **(D)** Levels of α -SMA, and fibrosis-related and ER stress-related proteins in first- and third-passage PSCs were assessed by Western blotting. β -tubulin was used as a loading control. **(E)** Expressions of α -SMA, and fibrosis-related and ER stress-related mRNA in first- and third-passage PSCs were assessed by qRT-PCR (mean $\pm$ SEM; $n = 3$). ** $P < 0.01$ vs. first-passage PSCs.

A

B

C

D

Figure 3. Inhibition of PERK negates PA-induced ER stress response in activated PSCs

Activated PSCs were left untreated (UT) or treated with BSA (20 μM) or PA (200 μM) in the presence or absence of the specific PERK inhibitor GSK2606414 (GSK; 1–10 μM) for 4 hours (A) or 24 hours (B, C, D, E). After incubation, total cell lysates and total were prepared. (A) Phosphorylated and total levels of PERK and eIF2α in PSCs were assessed by Western blotting. β-tubulin was used as a loading control. (B) Apoptosis of PSCs was assessed using an ELISA kit (mean ± SEM; $n = 3$). (C) PSC viability was assessed by MTS assay (mean ± SEM; $n = 6$). (D) Levels of α-SMA, and fibrosis-related and ER stress-related proteins in PSCs were assessed by Western blotting. β-tubulin was used as a loading control. (E) Expression of α-SMA, fibrosis-related, and ER stress-related mRNA in PSCs was assessed by qRT-PCR (mean ± SEM; $n = 3$). * $P < 0.05$, ** $P < 0.01$.

Figure 4. Experimental scheme

ER stress responses are chronically activated in both quiescent and activated PSCs. Under ER stress-free conditions, the PERK pathway plays adaptive roles such as attenuating protein synthesis, which is essential for cell survival. However, in response to excessive ER stress induced by Tg or PA, activation of the PERK pathway upregulates ATF-mediated CHOP, leading to PSC apoptosis.

Table 1**Body Weight and Blood Parameters of Wistar and KOB Rats**

	7 weeks old		11 weeks old		
	CD	HFD	CD	HFD	
Body weight (g)					
Baseline	Wistar	39.7 ± 2.0	38.0 ± 1.8	147.5 ± 2.6	147.3 ± 2.8
	KOB	46.3 ± 1.5	46.5 ± 1.3	176.3 ± 4.1	177.0 ± 4.4
Week 4	Wistar	189.3 ± 4.1	186.7 ± 4.1	275.3 ± 4.9	269.0 ± 4.2
	KOB	210.0 ± 4.6	218.7 ± 3.9	305.3 ± 5.6	317.7 ± 2.7 [*]
Serum biochemical parameters					
Free fatty acids (μEq/L)	Wistar	253.7 ± 19.6	860.8 ± 114.6 [†]	482.2 ± 71.6	1741.4 ± 235.3 [†]
	KOB	189.0 ± 8.1	1072.5 ± 211.4 [†]	416.3 ± 28.6	1017.2 ± 97.2 [†]
Triglycerides (mg/dL)	Wistar	151.3 ± 7.3	142.7 ± 16.3	182.2 ± 12.7	300.4 ± 13.0 [†]
	KOB	128.5 ± 8.2	189.8 ± 31.0	168.2 ± 4.4	175.8 ± 15.0
Total cholesterol (mg/dL)	Wistar	92.3 ± 2.6	76.0 ± 1.0 [†]	85.3 ± 3.0	68.8 ± 2.0 [†]
	KOB	86.0 ± 1.2	80.8 ± 1.2 [*]	71.8 ± 0.8	80.8 ± 2.6 [*]
Amylase (IU/L)	Wistar	2261.8 ± 31.2	2136.7 ± 25.4 [*]	2238.5 ± 34.0	2242.4 ± 66.2
	KOB	2071.5 ± 82.5	2201.3 ± 43.3	1932.2 ± 62.4	1853.3 ± 41.0
Lipase (IU/L)	Wistar	6.8 ± 0.3	7.3 ± 0.2	6.3 ± 0.2	6.8 ± 0.2
	KOB	8.8 ± 1.2	7.0 ± 0.0	9.0 ± 2.4	10.5 ± 1.9

CD: control diet, HFD: high-fat diet, KOB: Wistar Bonn/Kobori.

^{*} $P < 0.05$,[†] $P < 0.01$ vs. CD.