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Protectin DX Ameliorates Hepatic Steatosis by Suppression of Endoplasmic Reticulum Stress via AMPK-Induced ORP150 Expression[S]

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ABSTRACT

Docosahexaenoic acid (DHA) and its bioactive compounds may have suppressive effects on inflammation, endoplasmic reticulum (ER) stress, and insulin resistance. Protectin DX (PDX), a double lipoxygenase product from DHA has shown a suppressive effect on inflammation and insulin resistance. However, the effects of PDX on ER stress and hepatic steatosis have not been elucidated yet. Herein we report that PDX could stimulate the AMP-activated protein kinase (AMPK) phosphorylation, thereby upregulating oxygen-regulated protein 150 (ORP150) expression in a dose-dependent manner. Treatment of HepG2 cells with PDX attenuated the palmitate-induced triglyceride accumulation through regulation of the sterol regulatory element-binding protein 1 (SREBP1)–mediated pathway. To deal with the pharmacological significance in the protective effects of PDX on hepatic steatosis, we performed in vivo experiments. In a mouse model, the PDX administration would alleviate the high-fat diet–induced hepatic steatosis and trigger the hepatic AMPK phosphorylation and ORP150 expression. PDX improved palmitate-induced and HFD-induced impairment of hepatic lipid metabolism and steatosis through suppression of ER stress via an AMPK-ORP150–dependent pathway.

Introduction

Nonalcoholic fatty liver disease (NAFLD) has been emerged as the most common cause of chronic liver disease in developed countries (Argo and Caldwell, 2009). Moreover, patients with NAFLD might have a high risk of developing obesity-mediated diseases, such as cardiovascular disease and type 2 diabetes mellitus (T2DM) (Tonjes et al., 2010). A previous study showed that hepatic endoplasmic reticulum (ER) stress seems to play a crucial role in metabolic dysregulation, which results in NAFLD (Puri et al., 2008). Endoplasmic reticulum stress activates the unfolded protein response (UPR) to attenuate this stress and restore the ER homeostasis. The UPR is regulated by three ER transmembrane proteins: type I transmembrane inositol-requiring enzyme 1 (IRE-1), PKR-like ER kinase, and the activating transcription factor 6 (Ma and Hendershot, 2004). These pathways suppress protein synthesis and increase the expression of ER chaperones leading to degradation of misfolded or unfolded proteins (Schroder and Kaufman, 2005). ER stress also causes hepatic lipid accumulation via sterol regulatory element-binding protein 1c (Zhang et al., 2012), which is expressed exclusively in the liver. SREBP1c regulates the fatty acid synthase (FAS) (Sato, 2010) and stearoyl CoA desaturase (SCD1). These enzymes stimulate lipogenesis (Esfandiari et al., 2010), making upregulation of SREBP1c, a putative causative factor for NAFLD (Knebel et al., 2012).

Adenosine monophosphate-activated protein kinase (AMPK), a master regulator of cellular energy status, plays a central role in the regulation of the cellular energy homeostasis (Hardie, 2007). Activated AMPK has various protective properties, including suppression of inflammation, oxidative stress, and ER stress. Interestingly, several studies have

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ABBREVIATIONS: AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide; [(2*R*, 3*S*,4*R*,5*R*)-5-(4-carbamoyl-5-aminoimidazol-1-yl)-3,4-dihydroxyoxolan-2-yl]methyl dihydrogen phosphate; AMPK, adenosine monophosphate-activated protein kinase; ANOVA, analysis of variance; DHA, docosahexaenoic acid; ER, endoplasmic reticulum; HFD, high-fat diet; IRE-1, inositol-requiring enzyme 1; NAFLD, nonalcoholic fatty liver disease; ORP150, oxygen-regulated protein 150; PDX, protectin DX; SCD1, stearoyl CoA desaturase; SREBP1, sterol regulatory element-binding protein 1; T2DM, type 2 diabetes mellitus; TG, triglycerides; UPR, unfolded protein response.

shown that AMPK activation attenuates atherosclerosis (Dong et al., 2010) and NAFLD (Jung et al., 2015) through suppression of ER stress. Furthermore, activation of AMPK by AICAR prevents hepatic apoptosis via amelioration of ER stress. This effect is mediated by induction of oxygen regulated protein 150 (ORP150), an ER-associated chaperone that plays a protective role in ER stress, indicating that AMPK is a positive regulator of ORP150 (Wang et al., 2011).

A previous investigation has demonstrated that ομεγα-3 polyunsaturated fatty acids have anti-inflammatory and insulin-sensitizing effects in animal models of T2DM (Gonzalez-Periz et al., 2006). Protectin DX (PDX), an isomer of protectin/neuroprotectin D1 (Serhan et al., 2006), is derived from docosahexaenoic acid (DHA), an ομεγα-3 fatty acid with anti-inflammatory and antidiabetic properties (Chen et al., 2009). PDX is formed from DHA through 15-lipoxygenase-mediated double lipoxygenation (O'Flaherty et al., 2012) (Supplemental Fig. 1). PDX has been reported to suppress influenza virus replication through RNA export machinery (Morita et al., 2013). Recently, PDX was shown to attenuate insulin resistance in mice through suppression of hepatic gluconeogenesis via IL-6 secretion from skeletal muscle (White et al., 2014). However, the effects of PDX on ER stress and HFD-induced hepatic steatosis remain to be elucidated.

Although some studies have reported physiologic roles of PDX along with other specialized pro-resolving mediators (SPMs) in resolving inflammatory responses (Bannenberg and Serhan, 2010; Chiang and Serhan, 2017; Vik et al., 2017), others have questioned the production of physiologically relevant levels of the lipids (Skarke et al., 2015). Nevertheless, other studies have shown that pharmacological application of SPMs may be clinically useful as tools for preventing and resolving a wide range of pathologic inflammation along with the metabolic disorders (Hisada et al., 2017; Serhan, 2017). Therefore, we investigated the pharmacological effects of PDX on lipid metabolism and triglycerides (TG) accumulation under hyperlipidemic conditions. We verified the mechanisms of PDX-mediated protection from palmitate-induced ER stress and hepatic steatosis by examining AMPK-ORP150–mediated signal transduction in HepG2 cells. Furthermore, we investigated the effects of PDX on hepatic AMPK phosphorylation, ORP150 expression, ER stress, and hepatic steatosis in animal models.

Materials and Methods

Cell Cultures and Reagents. The human hepatoma cells HepG2 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells (passages: <10) were cultured in a humidified atmosphere of 5% CO_2 at 37°C and mycoplasma was not detected in HepG2 cells. PDX (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol (2 mM). Sodium palmitate (MilliporeSigma, St. Louis, MO) was conjugated to 2% bovine serum albumin (BSA; fatty acid-free grade; MilliporeSigma) dissolved in DMEM. The final concentration of ethanol did not affect cell viability. In all experiments, cells were treated with palmitate-BSA and PDX for 24 hours. and 2% BSA-ethanol was used as a control.

Animals, Feeding, and Treatment. This study was approved by the institutional animal review board (Institutional Animal Care and Use Committee of Korea University, Seoul, Republic of Korea). Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication, 8th edition, 2011). A control group ($n = 5$) and two experimental groups (five animals each) of 8-week-old male C57BL/6J (B6) mice were given a normal diet (ND; Brogaarden, Gentofte, Denmark) and an HFD (D12492; Research Diets, New Brunswick, NJ) containing 60% calories from fat, 20% from carbohydrates, and 20% from proteins for 8 weeks respectively.

PDX was dissolved in ethanol (10 μg/ml) and administered intraperitoneally to the HFD-plus-PDX group ($n = 5$) (35 μg/kg per day; Li et al., 2017) for 8 weeks. The same volume (3.3 μl/g) of ethanol was injected to a control group. After a study period, all experimental mice were sacrificed under anesthesia after fasting overnight (12 hours.).

Western Blot Analysis. HepG2 cells were harvested and proteins were extracted with lysis buffer (PRO-PREP; iNtRON Biotechnology, Seoul, Republic of Korea) for 60 minutes at 4°C. Protein samples (35 μg) were subjected to 12% SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Bioscience, Westborough, MA), and probed with primary antibody followed by secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX). The samples were detected with enhanced chemiluminescence (ECL) kits. Anti-IRE-1 (cat. no. 3294; 1:2500), anti-phospho eIF2α (cat. no. 3597; 1:1000), anti-eIF2α (cat. no. 5324; 1:1000), anti-CHOP (cat. no. 2895; 1:1000), anti-phospho AMPK (cat. no. 2535; 1:1000), anti-AMPK (cat. no. 5832; 1:2500), anti-ORP150 (cat. no. 13452; 1:2500), and anti-P62 (cat. no. 39749; 1:2500) were purchased from Cell Signaling (Beverly, MA). Anti-LC3 (NBP2-46888; 1:1000) was supplied by Novus Biologicals (Littleton, CO). Anti-SREBP1 (SC-13551; 1:2500), anti-FAS (SC-8009; 1:2500), anti-SCD1 (SC-515844; 1:2500), anti-GPR78 (SC-376768; 1:2500), anti-HSP47 (SC-5293; 1:2500), anti-calnexin (SC-11397; 1:2500), anti-HSP70 (SC-221731; 1: 2000), anti-lamin B (SC-6216; 1: 2000), and anti-β actin (SC-47778; 1:5000) were obtained from Santa Cruz Biotechnology. Anti-phospho IRE-1 (ab48187; 1:1000) was purchased from Abcam (Cambridge, MA).

Immunoprecipitation. The total protein from HepG2 cells was extracted with immunoprecipitation buffer (IP buffer: 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% IGEPAL CA630) and diluted to a concentration of 1 mg/ml. Polyclonal antibodies against FOXO1 (Santa Cruz Biotechnology) were added to the mixture at a dilution rate of 1:150, and the samples were incubated overnight at 4°C. After incubation, 50 μl of protein A/G-Sepharose bead suspension (Santa Cruz Biotechnology) was added to each sample and gently mixed for 1 hour at 4°C. Samples were centrifuged at 12,000 rpm for 30 seconds; the beads were washed three times in IP buffer. The isolated beads were resuspended in 1 × SDS-PAGE loading buffer, heated to 95°C for 5 minutes, vortexed, and flash-centrifuged. The supernatants were loaded onto a 12% SDS-polyacrylamide gel for electrophoretic separation and Western blot analysis.

Transient Transfection for Gene Silencing or Overexpression. At 70% confluence, 20 nmol/l small-interfering (si)RNA oligonucleotides for AMPKα1/2 (SC-45312) and ORP150 (SC-96695) were purchased from Santa Cruz Biotechnology and transfected to suppress gene expression. Scramble siRNA was used as a control. Two or 4 μg pCMV3-ORP150 (HG11342-ACG; Sino Biologic, Beijing, China) was transiently transfected to overexpress ORP150 expression. pCMV3 empty vector was used as a control. Transfection was performed with Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's directions.

Histologic Analysis. HepG2 cells and mouse liver sections were stained using the Oil Red-O method to measure the accumulated cellular neutral lipids, including triglycerides. After fixation with 10% formalin for 40 minutes, hepatocytes were stained with the Oil Red-O solution (MilliporeSigma) for 1 hour at 37°C. Oil Red-O stained TG content was quantified by adding isopropanol to each sample. The mixtures were gently agitated at 25°C for 8 minutes. Finally, 100 μl isopropanol-extracted samples were analyzed by a spectrophotometer at 510 nm. Steatosis was assessed by a semiquantitative scoring system by an experienced pathologist.

TG Measurement. Total lipids were extracted using a 2:1 chloroform/methanol (v/v) mixture. The organic layer was dried and immediately dissolved in 60% methanol. The extracted TG were measured using a colorimetric TG assay kit according to manufacturer's directions (BioVision, Milpitas, CA).

Serum Insulin Quantitation. Serum insulin was assayed using the Mouse Insulin Kit (RayBiotech, Norcross, GA), in accordance with the manufacturer's directions.

Statistical Analysis. All statistical analyses were conducted using SPSS/PC statistical program (version 12.0 for Windows; SPSS, Chicago, IL). Results are presented as the fold of the highest values (means ± S.E.M.). All of the in vitro experiments were performed at least three times. One-way analysis of variance (ANOVA) with Tukey post-hoc was used for statistical analysis.

Results

PDX Suppresses Palmitate-Induced TG Accumulation Caused by ER Stress in HepG2 Cells. We found that palmitate-induced TG accumulation and expression of lipogenesis-associated genes, including SREBP1, FAS, and SCD1, were suppressed by PDX in dose- and time-dependent manners in HepG2 cells (Fig. 1, A and B; Supplemental Fig. 2, A and B). Since palmitate augments ER stress, thereby resulting in lipid accumulation via SREBP1-mediated pathways (Li et al., 2014), we also evaluated the effect of PDX on palmitate-induced ER stress. Consistent with a previous report (Jung et al., 2017b), treatment of HepG2 cells with PDX significantly attenuated palmitate-induced IRE-1 and eukaryotic initiation factor 2α (eIF2α) phosphorylation and CHOP expression in dose- and time-dependent manners as well (Fig. 1C; Supplemental Fig. 2B).

PDX Suppresses Palmitate-Induced ER Stress and TG Accumulation through AMPK Activation. Since AMPK has been reported to attenuate ER stress and TG accumulation in hepatocytes (Li et al., 2014), we next evaluated the effect of PDX on AMPK phosphorylation. It has to be noted that PDX was able to induce AMPK phosphorylation in a dose-dependent manner in HepG2 cells. Similar to previous reports (Jung et al., 2017a,b), there is no response in 0- and 0.1-μM PDX-treated HepG2, cells and the optimal concentration of PDX to stimulate AMPK phosphorylation is 2 μM (Fig. 2A). siRNA-mediated silencing of AMPK abrogated the inhibitory effects of PDX on palmitate-induced IRE-1 and eIF2α phosphorylation and CHOP expression (Jung et al., 2017b) (Fig. 2B). Moreover, we found that suppression of AMPK by siRNA ameliorated palmitate-induced TG accumulation and lipogenic-marker expression in HepG2 cells (Fig. 2, C and D).

AMPK-Mediated Induction of ORP150 Expression Contributes to the Suppressive Effect of PDX on ER Stress and TG Accumulation in HepG2 Cells. To elucidate AMPK-mediated protective mechanism of PDX on ER

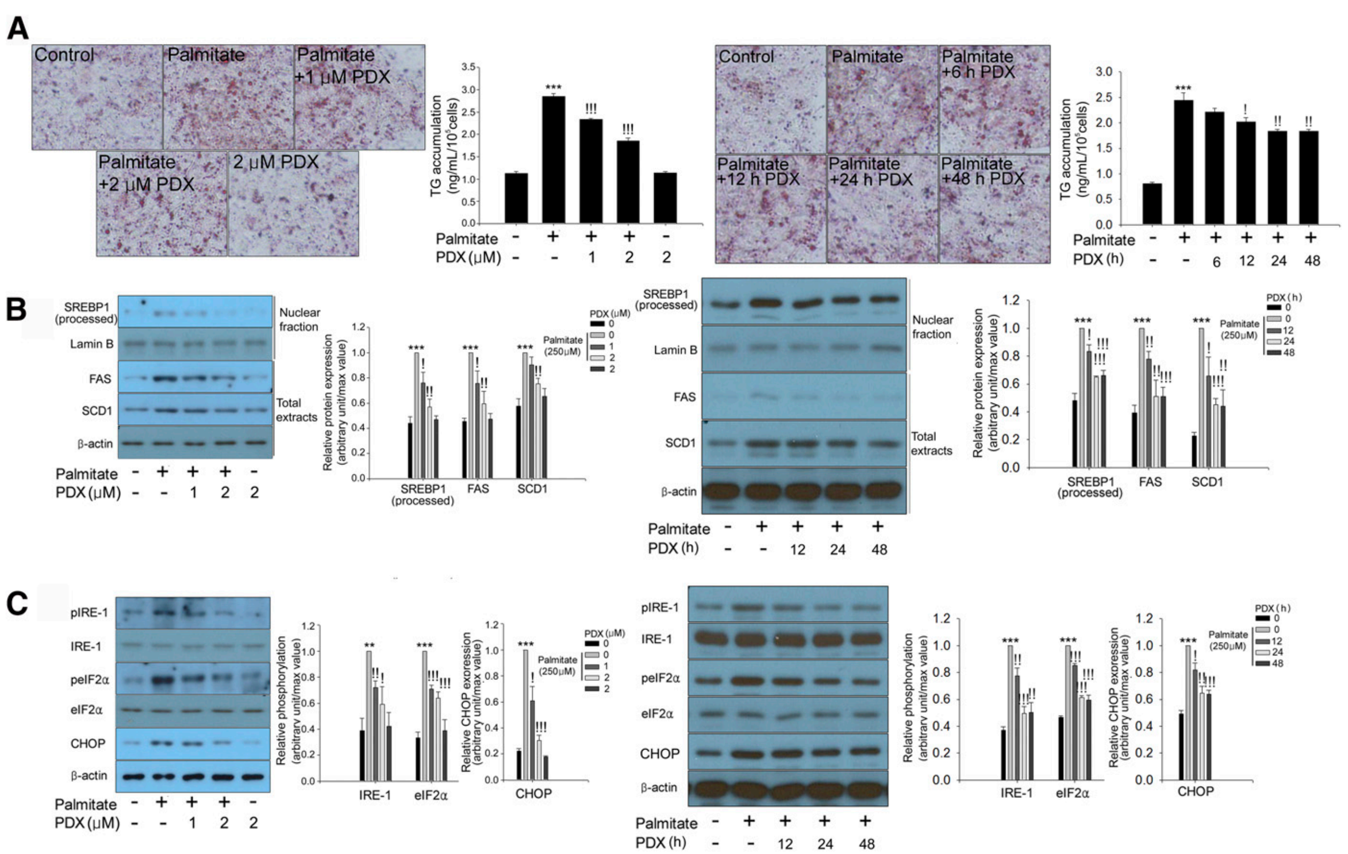

Fig. 1. PDX attenuates palmitate-induced TG accumulation and lipogenic genes and ER stress in HepG2 cells. (A) Oil Red-O staining in HepG2 cells in the presence of 200 μM palmitate and PDX (0–2 μM) for 24 hours, or 2 μM PDX for 0–48 hours. TG accumulation was quantitated by modified TG assay kit. Western blot analysis of SREBP1 (processed), FAS, and SCD1 expression (B) and ER stress markers (IRE-1, eIF2α, and CHOP) phosphorylation and expression (C) in HepG2 cells in the presence of 200 μM palmitate and PDX (0–2 μM) for 24 hours or 2 μM PDX for 0–48 hours. Means ± S.E.M. were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. ***$P<0.001$; **$P<0.01$ compared with levels in control. !!!$P<0.001$; !!$P<0.01$; !$P<0.05$ compared with palmitate treatment.

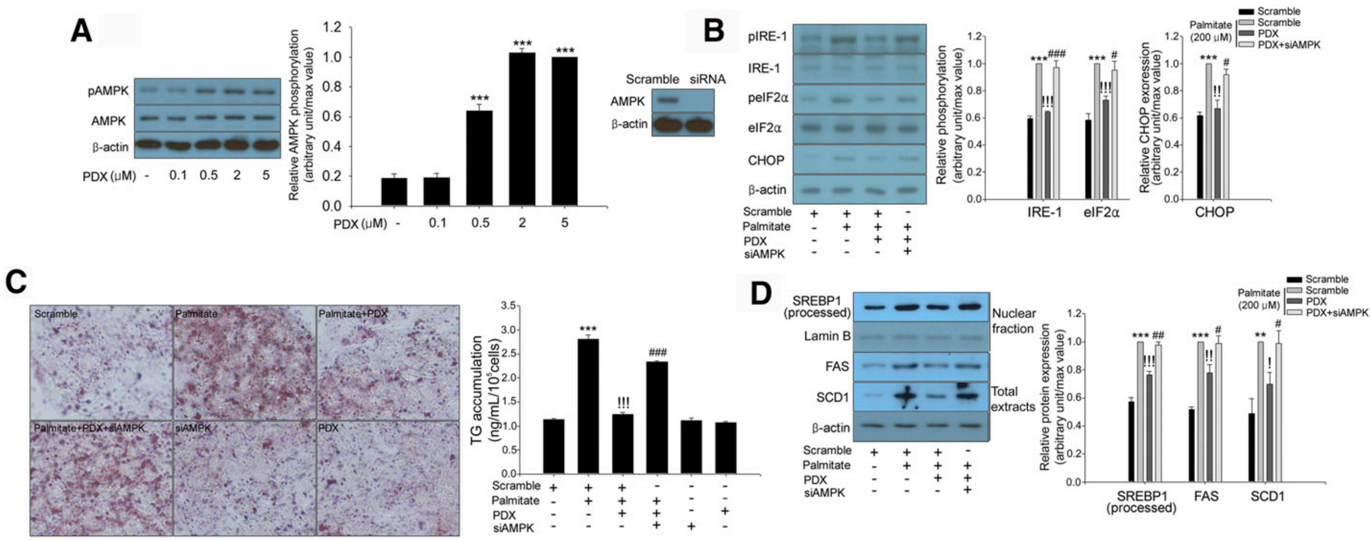

Fig. 2. AMPK contributes to the effects of PDX on palmitate-induced TG accumulation and ER stress. (A) Western blot analysis of AMPK phosphorylation in HepG2 cells treated with PDX (0–5 μM) for 24 hours. Western blot analysis of IRE-1 and eIF2α phosphorylation and CHOP expression (B) and Oil Red-O staining (C) in transfected HepG2 cells with scramble siRNA or AMPK siRNA in the presence of 200 μM palmitate and PDX (2 μM) for 24 hours TG accumulation was quantitated by modified TG assay kit. (D) Western blot analysis of SREBP1 (processed), FAS, and SCD1 expression in transfected HepG2 cells with scramble siRNA or AMPK siRNA in the presence of 200 μM palmitate and PDX (2 μM) for 24 hours. Means ± S.E.M. were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P < 0.001$; $^{**}P < 0.01$ compared with levels in control or scramble. $^{!!!}P < 0.001$; $^{!!}P < 0.01$; $^{!}P < 0.05$ compared with palmitate treatment. $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ compared with palmitate-plus-PDX treatment.

stress, we further evaluated the effects of PDX on the expression of various chaperones and autophagy in HepG2 cells. We found that PDX treatment did not affect the expression of most chaperones tested. However, ORP150 expression was upregulated by PDX treatment (Fig. 3A). Thereafter, we investigated whether PDX-induced AMPK could contribute to PDX-mediated upregulation of ORP150 expression. We also examined whether ORP150 is involved in

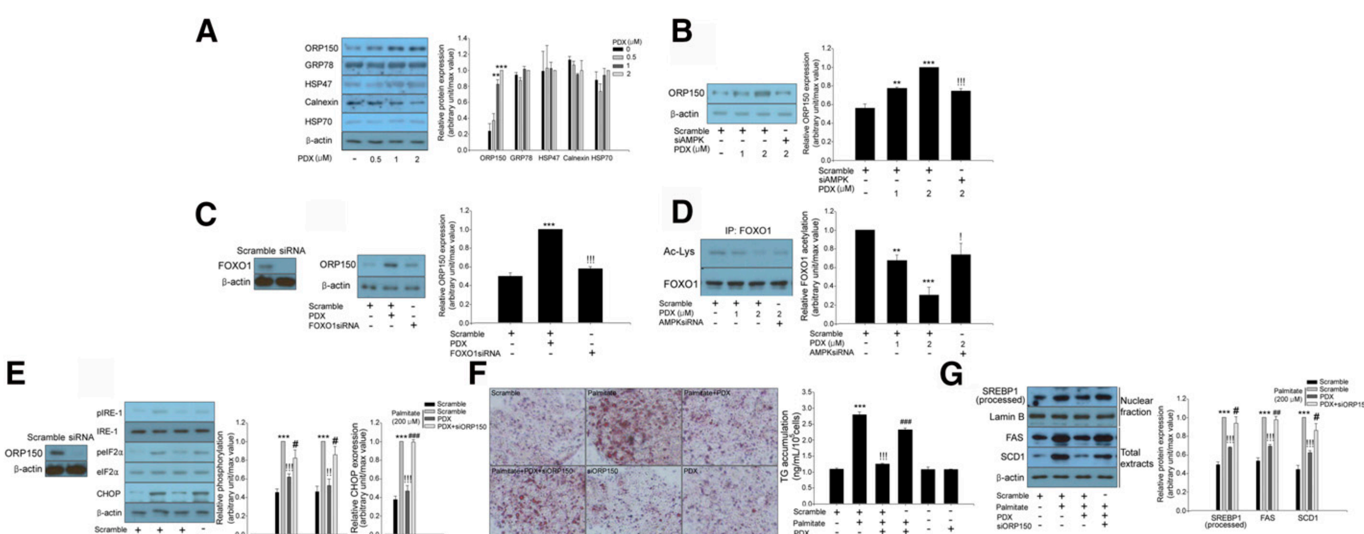

Fig. 3. AMPK-mediated induction of ORP150 expression involved in the effects of PDX on palmitate-induced ER stress and TG accumulation in HepG2 cells. (A) Western blot analysis of the expression of various chaperones in HepG2 cells treated with PDX (0–2 μM) for 24 hours. (B) Western blot analysis of ORP150 expression in transfected HepG2 cells with scramble siRNA or ORP150 siRNA (siORP150) in the presence of palmitate (200 μM) and PDX (2 μM) for 24 hours. (C) Western blot analysis of ORP150 in HepG2 cells in transfected HepG2 cells with scramble siRNA or siFOXO1 in the presence of 2 μM PDX for 24 hours. (D) Immunoprecipitation with FOXO1. Western blot analysis of acetylated lysine (Ac-Lys) in HepG2 cells in transfected HepG2 cells with scramble siRNA or siAMPK in the presence of 0–2 μM PDX for 24 hours. (E) Western blot analysis of IRE-1 and eIF2α phosphorylation and CHOP expression in transfected HepG2 cells with scramble siRNA or ORP150 siRNA (siORP150) in the presence of palmitate (200 μM) and PDX (2 μM) for 24 hours. Oil Red-O staining (F) and Western blot analysis of SREBP1 (processed), FAS, and SCD1 expression (G) in transfected HepG2 cells with scramble siRNA or siORP150 in the presence of 200 μM palmitate and PDX (2 μM) for 24 hours. TG accumulation was quantitated by isopropyl alcohol extraction. Means ± S.E.M. were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P < 0.001$; $^{**}P < 0.01$ compared with levels in control or scramble. $^{!!!}P < 0.001$; $^{!!}P < 0.01$; $^{!!}P < 0.05$ compared with palmitate or PDX treatment. $^{\#\#\#}P < 0.001$; $^{\#\#}P < 0.01$; $^{\#}P < 0.05$ compared with palmitate-plus-PDX treatment.

the suppressive effects of PDX on ER stress and TG accumulation. As shown in Fig. 3B, siRNA-mediated silencing of AMPK markedly reduced the effect of PDX on ORP150 expression (Fig. 3B). Forkhead box protein O1 (FOXO1) has been reported to play a vital role in the AMPK-mediated regulation of ORP150 expression (Wang et al., 2011). Therefore, we examined the effects of FOXO1 on PDX-mediated induction of ORP150 expression. siRNA-mediated suppression of FOXO1 expression abrogated PDX-induced ORP150 expression (Fig. 3C). PDX caused FOXO1 deacetylation in a dose-dependent manner. However, AMPK siRNA reversed the changes (Fig. 3D). As well, siRNA-mediated silencing of ORP150 significantly reduced the effects of PDX on palmitate-induced TG accumulation through suppression of ER stress in HepG2 cells (Fig. 3, E–G). Five mM 4-phenylbutyrate (4PBA) (Uppala et al., 2017), a chemical chaperone that inhibits ER stress suppressed palmitate-induced nuclear SREBP1 expression and TG accumulation (Fig. 4, A and B). Furthermore, overexpression of ORP150 in HepG2 cells markedly suppressed palmitate-induced nuclear SREBP1 expression and TG accumulation in a dose-dependent manner (Fig. 4, C and D), a finding in line with that reported by Kammoun et al. (2009). Transfection using 4 μg ORP150 more strongly induced ORP150 expression than 2 μM PDX (Fig. 4E). Unexpectedly, PDX treatment did not influence the autophagy-associated markers, such as LC3 conversion and P62 degradation (Fig. 4F).

PDX Administration Augments Phosphorylation of AMPK and ORP150 Expression, and Then Prevents Hepatic Steatosis in HFD-Fed Mice. On the basis of the above mentioned results, we subsequently evaluated the effect of PDX on lipid accumulation in mice. To this end, we performed histologic analysis using H&E and Oil Red-O staining. In the animal model, HFD treatment increased hepatic TG accumulation and lipogenesis-associated gene expression in the liver. However, PDX administration significantly reversed these changes (Fig. 5, A and B). As with TG accumulation in the liver, hepatic ER stress was also attenuated by PDX administration (Fig. 5C). Furthermore, HFD-induced suppression of AMPK phosphorylation and ORP150 expression in the liver was markedly restored by PDX treatment (Fig. 5D). At variance to the in vitro results, PDX administration increased the levels of autophagy markers in the liver of HFD-fed mice (Fig. 5E). Since adiponectin stimulates autophagy in myocytes (Liu et al., 2015) and hepatocytes (Nepal and Park, 2013), we further evaluated the levels of serum adiponectin in the mouse model. PDX administration increased the levels of serum adiponectin in HFD-fed mice

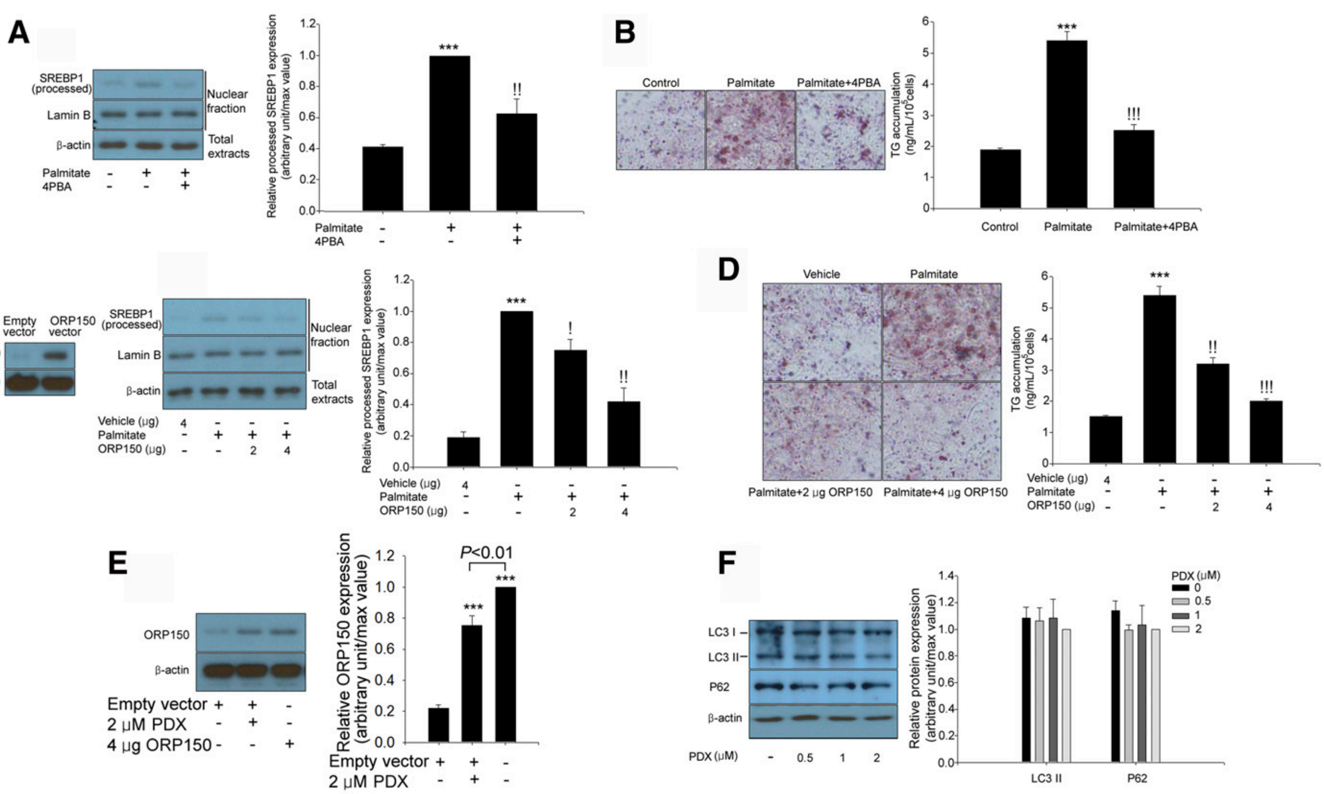

Fig. 4. Palmitate-induced ER stress causes lipid accumulation, and PDX does not affect autophagic markers. (A) Western blot analysis of processed SREBP1 expression in HepG2 cells treated with 200 μM palmitate and 5 mM 4-phenylbutyrate (4PBA) for 24 hours. (B) Oil Red-O staining in HepG2 cells treated with palmitate and 4PBA for 24 hours. (C) Western blot analysis of processed SREBP1 expression in ORP150-overexpressing (0–4 μg) HepG2 cells treated with 200 μM palmitate for 24 hours. (D) Oil Red-O staining in ORP150 overexpressing (0–4 μg) HepG2 cells treated with palmitate for 24 hours. TG accumulation was quantitated by isopropyl alcohol extraction. (E) Western blot analysis of ORP150 expression in 2 μM PDX treated or 4 μg-ORP150-vector–transfected HepG2 cells for 24 hours. (F) Western blot analysis of LC3 and P62 expression in transfected HepG2 cells with scramble siRNA or siORP150 in the presence of 200 μM palmitate and PDX (2 μM) for 24 hours. Means ± S.E.M. were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. ***$P < 0.001$ compared with levels in control or scramble. !!!$P < 0.001$; !!$P < 0.01$; !$P < 0.05$ compared with palmitate treatment.

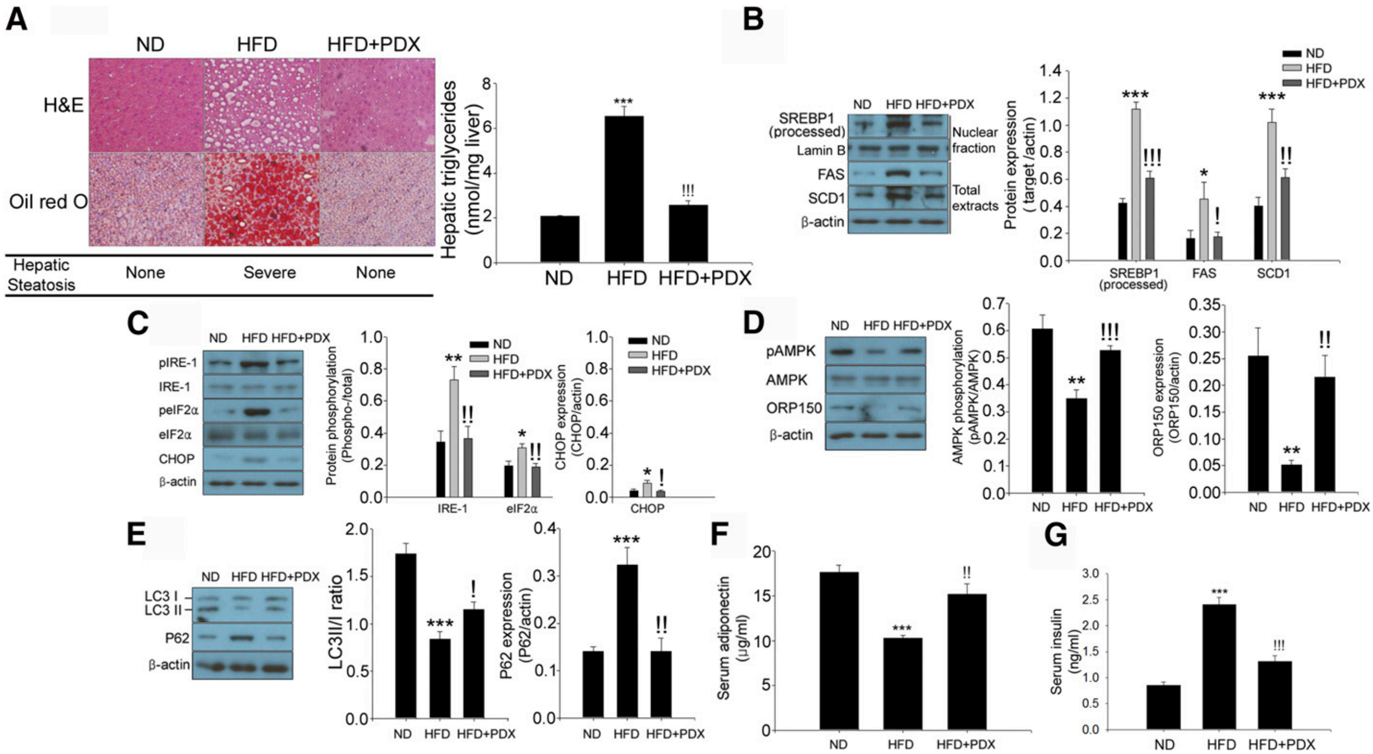

Fig. 5. Systemic PDX administration ameliorates hepatic steatosis and both AMPK phosphorylation and ORP150 expression. (A) Hematoxylin and eosin and Oil Red-O staining on liver sections of experimental animals fed a normal diet (ND), high-fat diet (HFD), and HFD-plus-PDX (HFD+PDX). TG accumulation was quantitated by TG assay kit. Hepatic steatosis scoring was performed by an experienced pathologist. Western blot analysis of SREBP1 (processed), FAS, and SCD1 expression (B), IRE-1 and eIF2α phosphorylation and CHOP expression (C), and AMPK phosphorylation and ORP150 expression (D) in liver of experimental mice. (E) Western blot analysis of LC3 and P62 expression in liver of experimental mice. Serum analysis of adiponectin (F) and insulin (G) of experimental mice. Means ± S.E.M. were calculated from data obtained from five separate animals. One-way ANOVA with Tukey post-hoc was performed. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ compared with the ND treatment. $^{!!!}P < 0.001$; $^{!!}P < 0.01$; $^{!}P < 0.05$ compared with the HFD.

(Fig. 5F). However, HFD-induced serum insulin levels were decreased by PDX treatment (Fig. 5G). PDX treatment did not influence calorie intake, although it significantly decreases the body weight gain by HFD (Fig. 6, A and B). Moreover, PDX administration markedly reduced the weight of liver and epididymal adipose tissue in HFD-fed mice (Fig. 6, C and D).

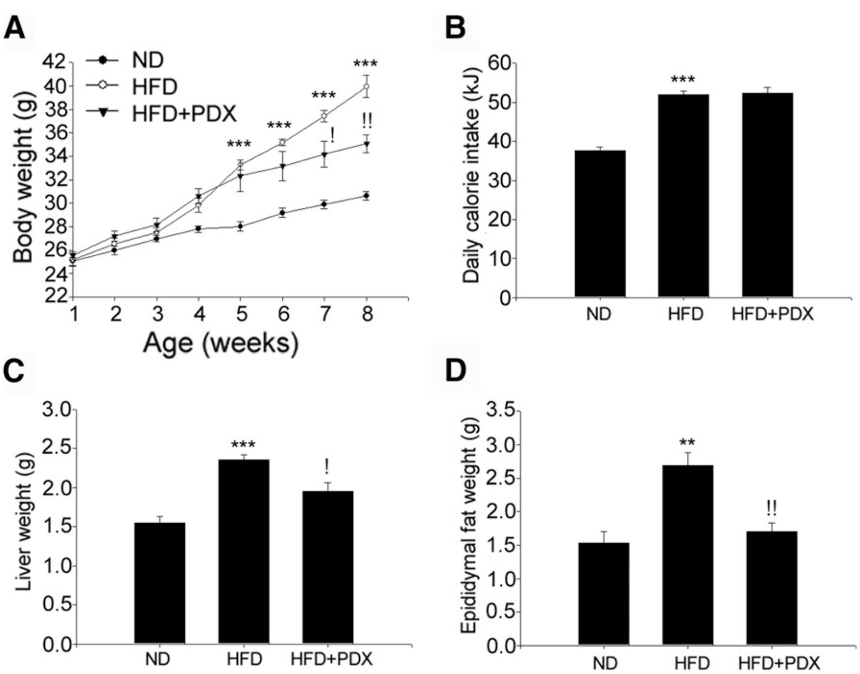

Fig. 6. PDX administration reduces body weight and liver weight in mice. (A) Body weight measurement, (B) daily energy intake, (C) liver weight, and (D) epididymal fat weight in mice (five animals per treatment group). Closed circles, ND; opened circles, HFD; closed triangles, HFD+PDX. Means ± S.E.M. were calculated from data obtained from five separate animals. One-way ANOVA with Tukey post-hoc was performed. $^{***}P < 0.001$; $^{**}P < 0.01$ compared with the ND treatment. $^{!!}P < 0.01$; $^{!}P < 0.05$ compared with the HFD.

Discussion

Elevated ER stress in the liver could contribute considerably to the alteration of lipid metabolism, thereby leading to hepatic steatosis (Flamment et al., 2010) and apoptosis (Malhi et al., 2010). Therefore, regulation of hepatic ER stress is viewed as a promising therapeutic strategy for the treatment of hepatic diseases, including NAFLD. To our knowledge the present investigation has shown for the first time that PDX can attenuate lipid-induced hepatic ER stress and steatosis through AMPK-mediated induction of ORP150 expression in both in vitro and in vivo studies. First, we demonstrated that PDX-induced AMPK activation would provide significant protection against palmitate-induced ER stress and TG accumulation in HepG2 cells. Second, PDX markedly induced ORP150 expression through AMPK-mediated pathway. Third, PDX did not alter classic autophagy markers, such as LC3 conversion and P62 degradation, in HepG2 cells. Finally, in mice, the PDX administration increased AMPK phosphorylation and ORP150 expression, thereby alleviating HFD-induced ER stress and TG accumulation in the liver. Furthermore, PDX treatment resulted in the upregulation of autophagy markers and increased serum adiponectin in the same mouse model.

Chronic low-grade inflammation has been linked to the development of obesity-mediated disorders. The resolution of the inflammatory response is controlled by various mechanisms (Rius et al., 2012). One of them involves protectins, pro-resolving mediators [specializing pro-resolving mediators (SPM)], with anti-inflammatory properties derived from DHA. For instance, SPM exerts protective effects against necroinflammatory hepatic injury by suppressing hepatic COX-2 expression and reducing the oxidative burden (Gonzalez-Periz et al., 2006). PDX also exerts protective effects against neutrophil invasion in chronic inflammatory diseases via the inhibition of COX-1 and COX-2, in addition to decreasing reactive oxygen species production (Liu et al., 2014). PDX has also been shown to improve the insulin resistance in obese diabetic *db/db* mice by stimulating the release of IL-6 from skeletal muscle, thereby inhibiting hepatic gluconeogenesis (White et al., 2014). Up to submission for publication, no other studies have dealt with the cellular effects and the underlying mechanisms of PDX on lipid-induced hepatic ER stress and steatosis.

Previous reports have suggested that elevated ER stress in the liver may contribute to the development of NAFLD through the disruption of ER homeostasis, which leads to hepatic apoptosis and lipid accumulation during altered lipid metabolism (Werstuck et al., 2001). Moreover, abnormally increased ER stress has been observed in the adipose tissue and livers of patients with NAFLD (Boden et al., 2008). Activation of the UPR by ER stress has been documented to be associated with hepatic lipid metabolism, obesity, insulin resistance, and T2DM (Hotamisligil, 2010). Thus, exploring the mechanisms that regulate hepatic ER stress could aid the development of novel therapeutic strategies for the treatment of NAFLD. In support of this idea, chronic disorders such as diabetes (Wu and Kaufman, 2006), atherosclerosis (Haas et al., 2016), and NAFLD (Kammoun et al., 2009) are all attenuated by the suppression of ER stress. In this study, we noticed that PDX markedly suppressed hyperlipidemia-induced ER stress markers (e.g., IRE-1, eIF2α, and CHOP) and TG accumulation in both in vitro and in vivo models.

On the basis of these results, we next investigated PDX-associated mechanisms through which palmitate-induced ER stress is alleviated. AMPK activation plays an important role in the attenuation of NAFLD (Jung et al., 2015). Ομεγα-3 polyunsaturated fatty acids, including EPA and DHA, have been shown to activate AMPK and consequently improve lipid metabolism in the liver and skeletal muscle (Deng et al., 2015). Previously, it was reported that PDX could activate AMPK in skeletal muscle (White et al., 2014). Herein, we demonstrated that PDX significantly stimulates AMPK phosphorylation. Moreover, we found that siRNA-mediated suppression of AMPK markedly reduced the effect of PDX on palmitate-induced ER stress and TG accumulation in HepG2 cells. These results suggest that AMPK is required for PDX suppression of lipid-mediated hepatic steatosis. However, it remains unclear whether PDX activates AMPK directly or indirectly in hepatocytes. Therefore, further studies are needed to identify the specific receptors for PDX. Interestingly, we observed that PDX acutely stimulates AMPK phosphorylation, which is probably a feature of PDX action in hepatocytes. Further studies are required to address this unusual phenomenon.

Overexpression of chaperones increases the ER capacity for protein folding and degradation, consequently ameliorating the ER stress (Ni and Lee, 2007). ER-mediated chaperone GRP78 ameliorates ER stress-induced apoptosis and lipid accumulation in HepG2 cells (Werstuck et al., 2001). Furthermore, overexpression of HSP70 prevented ER stress-induced cell death in PC12 cells (Gupta et al., 2010). ORP150 is an ER-resident stress protein that functions as a chaperone and is upregulated by extrinsic stresses such as hypoxia and glucose deprivation (Bando et al., 2000). AMPK activation by AICAR induces ORP150 mRNA and protein expression in hepatocytes (Wang et al., 2011). In this study, we found that PDX stimulates ORP150 expression via AMPK-mediated FOXO1 deacetylation in hepatocytes. However, FOXO1 binding sequences on ORP150 promoter necessitated further research work. Induction of ORP150 is crucial for the alleviation of ER stress, deregulation of calcium homeostasis, and apoptosis (Sanson et al., 2009); these findings suggest that up-regulation of ORP150 expression is potentially an effective therapeutic strategy for treating ER stress-related diseases. Therefore, we investigated whether ORP150 is involved in the protective effects of PDX against lipid-induced ER stress. siRNA-mediated silencing ORP150 expression abrogated the effect of PDX on palmitate-induced ER stress markers and TG accumulation in HepG2 cells.

Autophagy has been suggested as a defense mechanism against ER stress (Yin et al., 2012). For example, autophagy activation by rapamycin has been shown to improve the ER stress-mediated damage in pancreatic β-cells (Bartolome et al., 2012). We previously reported that C1q/tumor necrosis factor-related protein 9 attenuates hepatic steatosis and apoptosis via AMPK-autophagy-dependent suppression of ER stress (Jung et al., 2015). Furthermore, autophagy is regulated by AMPK and mammalian target of rapamycin (mTOR) through direct phosphorylation of Ulk1 (Kim et al., 2011). Therefore, we examined whether PDX activates autophagy. Unexpectedly, PDX had no effect on autophagy markers, including LC3 conversion and P62 degradation, in the effective PDX concentration range in the in vitro model. In contrast to the in vitro results, PDX administration augmented autophagy markers in the liver of HFD-fed mice.

Thus, we measured the levels of serum adiponectin, which has been reported to stimulate autophagy through an AMPK-mediated pathway (Liu et al., 2015), to elucidate the difference between the effects of PDX in the in vitro versus the in vivo models. We found that PDX treatment significantly increased the serum adiponectin levels in mice, although it does not increase adiponectin production in differentiated 3T3-L1 cells (unpublished data). These results suggest that PDX may activate hepatic autophagy in mice by inducing adiponectin in an indirect pathway. Furthermore, we also found that PDX administration reduced liver weight and body weight, although it does not affect the calorie intake in mice. These results could imply the possibility of hepatic steatosis attenuation by PDX- or PDX-induced adiponectin-mediated weight loss; a similar finding which is reported by Otabe et al. (2007). However, the mechanism by which PDX causes weight loss remains to be elucidated. Therefore, further studies in adiponectin-null animal models are needed to elucidate the precise mechanism of PDX-mediated induction of autophagy in the liver and body weight loss in mice. In sum, our results indicate that PDX directly attenuates lipid-induced ER stress and hepatic steatosis through a signal transduction pathway distinct from the autophagy-mediated pathway or weight loss.

In the current study, we demonstrated that PDX prevents lipid-induced ER stress through AMPK-mediated induction of ORP150 expression, thereby ameliorating hepatic steatosis and lipid metabolism. Our results suggest that PDX-mediated regulation of lipid-induced ER stress through the AMPK-ORP150 pathway is a novel potential therapeutic strategy for treating NAFLD.

Authorship Contributions

Participated in research design: Jung, Kyung, Kim, Shin, Lee, Park, Hacımüftüoğlu, Abd El-Aty, Jeong.

Conducted experiments: Jung, Kyung, Kim, Shin, Lee.

Performed data analysis: Jung, Lee.

Wrote or contributed to the writing of the manuscript: Jung, Abd El-Aty, Jeong.

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