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Title page

Protectin DX ameliorates hepatic steatosis by suppression of endoplasmic reticulum stress *via* AMPK-induced ORP150 expression

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DHA, Docosahexaenoic acid; ORP150, Oxygen-regulated protein 150; SREBP1, Sterol regulatory element-binding protein 1

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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 docosahexaenoic acid (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 have found 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 (HFD)-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

Non-alcoholic 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 (PERK), and the activating transcription factor 6 (ATF6) (Ma and Hendershot, 2004). These pathways suppress protein synthesis and increase the expression of ER chaperones leading to degradation of mis-folded or unfolded proteins (Schroder and Kaufman, 2005). ER stress also causes hepatic lipid accumulation *via* sterol regulatory element-binding protein (SREBP) 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 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 (ORP) 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 ω-3 polyunsaturated fatty acids (PUFA) have anti-inflammatory and insulin sensitizing effects in animal models with 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 ω-3 fatty acid with anti-inflammatory and anti-diabetic properties (Chen et al., 2009). PDX is formed from DHA through 15-lipoxygenase-mediated double lipoxygenation (O'Flaherty et al., 2012) (Suppl. 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.

While some studies have reported physiological 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 pathological inflammation along with the metabolic disorders (Hisada et al., 2017; Serhan, 2017). Therefore, we investigated the pharmacological effects of PDX on lipid metabolism and 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.

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Material and methods

Cell cultures and reagents. The human hepatoma cells HepG2 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco`s modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/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, USA) was dissolved in ethanol (2 mM). Sodium palmitate (Sigma, St Louis, MO, USA) was conjugated to 2% BSA (fatty acid free grade; Sigma) 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 h 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 (5 animal each) of 8-week-old male C57BL/6J (B6) mice were given a normal diet (ND; Brogaarden, Gentofte, Denmark) and a HFD (D12492; Research Diets, New Brunswick, NJ, USA) containing 60% calories from fat, 20% from carbohydrates and 20% from proteins for 8 weeks respectively.

PDX was dissolved in ethanol (10 μg/ml). The HFD plus PDX group (n=5) were administered intraperitoneally (35 μg/kg/day) (Li et al., 2017) for 8 weeks. The same volume (3.3 μl/g) of

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ethanol was injected to a control group. After a study period, all experimental mice were sacrificed under anaesthesia after fasting overnight (12 h).

Western blot analysis. HepG2 cells were harvested and proteins were extracted with lysis buffer (PRO-PREP; Intron Biotechnology, Seoul, Republic of Korea) for 60 min at 4°C. Protein samples (35 μg) subjected to 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a nitrocellulose membrane (Amersham Bioscience, Westborough, MA, USA), and probed with primary antibody followed by secondary antibody conjugated with horse radish peroxidase (Santa Cruz Biotechnology). The samples were detected with enhanced chemoluminescence (ECL) kits. Anti-IRE-1 (Cat. 3294; 1:2500), anti-phospho eIF2α (Cat. 3597; 1:1000), anti-eIF2α (Cat. 5324; 1:1000), anti-CHOP (Cat. 2895; 1:1000), anti-phospho AMPK (Cat. 2535; 1:1000), anti-AMPK (Cat. 5832; 1:2500), anti-ORP150 (Cat. 13452; 1:2500), and anti-P62 (Cat. 39749; 1:2500) were purchased from Cell Signaling (Beverly, MA, USA). Anti-LC3 (NBP2-46888; 1:1000) was supplied by Novus Biologicals (Littleton, CO, USA). 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-beta actin (SC-47778; 1:5000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho IRE-1 (ab48187; 1:1000) was purchased from Abcam (Cambridge, MA, USA).

Immunoprecipitation. The total protein from HepG2 cells were extracted with immunoprecipitation buffer (IP buffer: 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% IGEPAL

CA630) and diluted into a concentration of 1 mg/mL. Polyclonal antibodies against FOXO1 (Santa Cruz Biotechnology) was 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 h at 4 °C. Samples were centrifuged at 12,000 rpm for 30s; the beads were washed three times in IP buffer. The isolated beads were re-suspended in 1 × SDS-PAGE loading buffer, heated to 95 °C for 5 min, 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 Biological, Beijing, China) were 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.

Histological analysis. HepG2 cells and mouse liver sections were stained using the Oil Red-O method to measure the accumulated cellular neutral lipids, including triglycerides (TG). After fixation with 10% formalin for 40 min, hepatocytes were stained with the Oil Red-O solution (Sigma) for 1 h 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 min. At last, 100

μL isopropanol-extracted samples were analyzed by a spectrophotometer at 510 nm. Steatosis was assessed by a semi-quantitative scoring system by an experienced pathologist.

TG measurement. Total lipids were extracted using a 2:1 chloroform:methanol (2:1, *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, USA).

Serum insulin quantitation. Serum insulin was assayed using the Mouse Insulin Kit (RayBiotech, Norcross, GA, USA), 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, USA). Results are presented as the fold of the highest values (means ± SEM). All of the *in vitro* experiments were performed at least three times. One-way 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. 1A and B; Suppl. Fig. 2A 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 eIF2α phosphorylation and CHOP expression in dose- and time-dependent manners as well (Fig. 1C; Suppl. 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 with previous reports (Jung et al., 2017b; Jung et al., 2017a), 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 markers expression in HepG2 cells (Fig. 2C and D).

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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 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 next investigated whether PDX-induced AMPK could contribute to PDX-mediated upregulation of ORP150 expression. We also examined whether ORP150 is involved in 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. 3E-G). 5 mM 4-phenylbutyrate (4PBA) (Uppala et al., 2017), a chemical chaperone which inhibits ER stress suppressed palmitate-induced nuclear SREBP1 expression and TG accumulation (Fig. 4A 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. 4C and D), the finding which is in line to that reported by Kammoun et al. (Kammoun et al., 2009). Transfection using 4 μg ORP150 more strongly induced ORP150 expression than 2 μM

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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 thence prevents hepatic steatosis in HFD-fed mice. Based on the above mentioned results, we subsequently evaluated the effect of PDX on lipid accumulation in mice. To this end, we performed histological 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. 5A and B). Similar to the 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 mouse model. PDX administration increased the levels of serum adiponectin in HFD-fed mice (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 (Figs. 6A and B). Moreover, PDX administration markedly reduced the weight of liver and epididymal adipose tissue in HFD-fed mice (Figs. 6C and D).

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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. Up to the author`s knowledge, the present investigation shows for the first time that PDX can attenuate the lipid-induced hepatic ER stress and steatosis through AMPK-mediated induction of ORP150 expression both in *in vitro* and *in vivo* studies. First, we have 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 the 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 the AMPK phosphorylation and the ORP150 expression, thereby alleviating HFD-induced ER stress and TG accumulation in the liver. Furthermore, PDX treatment resulted in the up-regulation 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 (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

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inhibition of COX-1 and COX-2, in addition to decreasing ROS production (Liu et al., 2014). PDX has also been shown to improve the insulin resistance in obese diabetic *db*/*db* mice through stimulating the release of IL-6 from skeletal muscle, thereby inhibiting hepatic gluconeogenesis (White et al., 2014). Up to the end, there are currently no studies that deal 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.

Based on 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). ω-3 PUFAs, including EPA and DHA, have been shown to activate AMPK and consequently improve the 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 steeply stimulates AMPK phosphorylation. It 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 of protein folding and degradation, consequently ameliorates 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 up-regulated 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/TNF-related Protein 9 attenuates the hepatic steatosis and apoptosis *via* AMPK-autophagy-dependent suppression of ER stress (Jung et al., 2015). Furthermore, autophagy is regulated by AMPK and mTOR through direct phosphorylation of Ulk1 (Kim et al., 2011). Therefore, we examined whether PDX activates autophagy. Unexpectedly, PDX did not have any effects on autophagy markers including LC3 conversion and P62 degradation in the effective PDX concentration range in *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* vs. 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

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which is reported by Otabe et al. (Otabe et al., 2007). However, the mechanism by which PDX causes weight loss remains to be elucidated in this study. 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 the 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: Tae Woo Jung, Eun Jung Kyung, Hyoung-Chun Kim, Yong Kyu Shin, Sung Hoon Lee, Eon Sub Park, Ahmet Hacımüftüoğlu, A.M. Abd El-Aty, and Ji Hoon Jeong

Conducted experiments: Tae Woo Jung, Eun Jung Kyung, Hyoung-Chun Kim, and Yong Kyu Shin, Sung Hoon Lee

Performed data analysis: Tae Woo Jung, Sung Hoon Lee

Wrote or contributed to the writing of the manuscript: Tae Woo Jung, Abd El-Aty, and Ji Hoon Jeong

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Footnotes

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Figure legends

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 h or 2 μM PDX for 0-48 h. 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 h or 2 μM PDX for 0-48 h. Means ± SEM were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$ and $^{**}P<0.01$ when compared to levels in control. $^{!!!}P<0.001$, $^{!!}P<0.01$, and $^{!}P<0.05$ when compared to 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 h. 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 hr. 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 h. Means ± SEM were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$ and $^{**}P<0.01$ when compared to levels in control or scramble. $^{!!!}P<0.001$, $^{!!}P<0.01$, and $^{!}P<0.05$ when compared to palmitate treatment. $^{###}P<0.001$, $^{##}P<0.01$, and $^{#}P<0.05$ when compared to palmitate plus PDX treatment.

Fig. 3. AMPK-mediated induction of ORP150 expression involves in the effects of PDX on palmitate-induced ER stress and TG accumulation in HepG2 cells. (A) Western blot analysis of various chaperones expression in HepG2 cells treated with PDX (0-2 μM) for 24 hr. (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 h. (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 h. (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 h. (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 h. 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 h. TG accumulation was quantitated by isopropyl alcohol extraction. Means ± SEM were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$ and $^{**}P<0.01$ when compared to levels in control or scramble. $^{!!!}P<0.001$, $^{!!}P<0.01$, and $^{!!}P<0.05$ when compared to palmitate or PDX treatment. $^{###}P<0.001$, $^{##}P<0.01$, and $^{#}P<0.05$ when compared to palmitate plus PDX treatment.

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 4PBA for 24 h. (B) Oil-red O staining in HepG2

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cells treated with palmitate and 4PBA for 24 h. (C) Western blot analysis of processed SREBP1 expression in ORP150 overexpressing (0-4 μg) HepG2 cells treated with 200 μM palmitate for 24 h. (D) Oil-red O staining in ORP150 overexpressing (0-4 μg) HepG2 cells treated with palmitate for 24 h. 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 h. (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 hr. Means ± SEM were calculated from three independent experiments. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$ when compared to levels in control or scramble. $^{!!!}P<0.001$, $^{!!}P<0.01$, and $^{!}P<0.05$ when compared to palmitate treatment.

Fig. 5. Systemic PDX administration ameliorates hepatic steatosis and both AMPK phosphorylation and ORP150 expression. (A) H&E and Oil-red O staining on liver sections of experimental animals – 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 ± SEM were calculated data obtained from five separated animals. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$, $^{**}P<0.01$, and $^{*}P<0.05$ when compared to the ND treatment. $^{!!!}P<0.001$, $^{!!}P<0.01$,

and $^{!}P<0.05$ when compared to the HFD.

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 ± SEM were calculated data obtained from five separated animals. One-way ANOVA with Tukey post-hoc was performed. $^{***}P<0.001$ and $^{**}P<0.01$ when compared to the ND treatment. $^{!!}P<0.01$ and $^{!}P<0.05$ when compared to the HFD.

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Figure 1

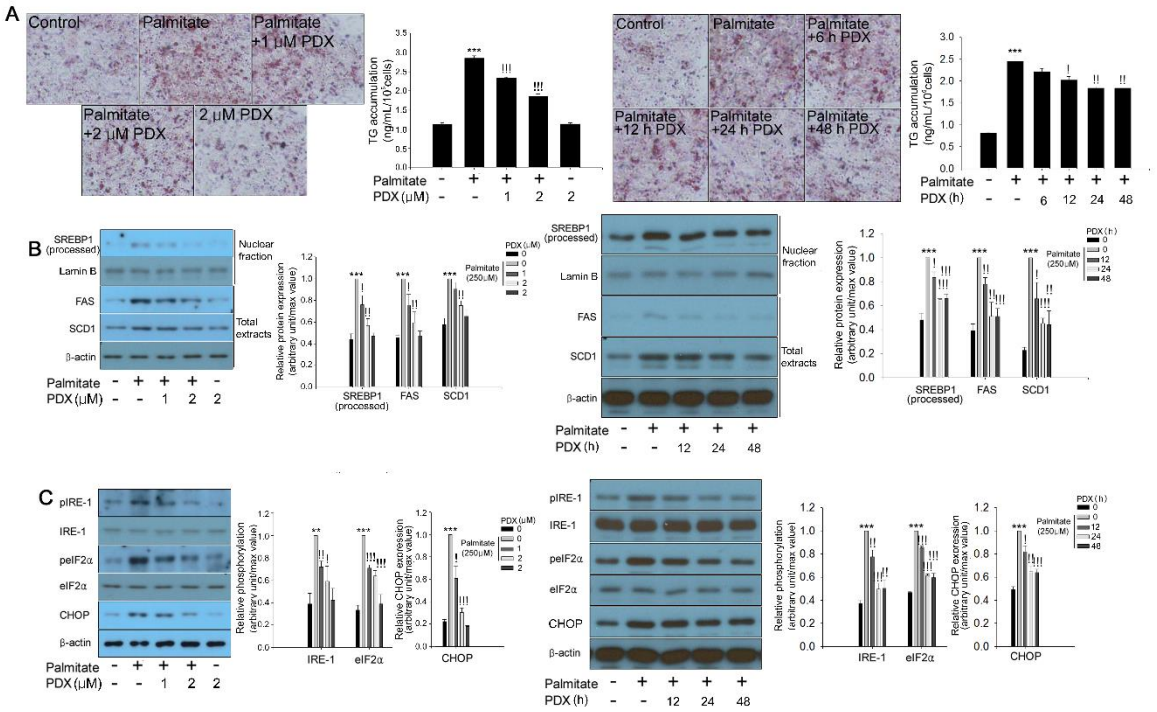

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Figure 2

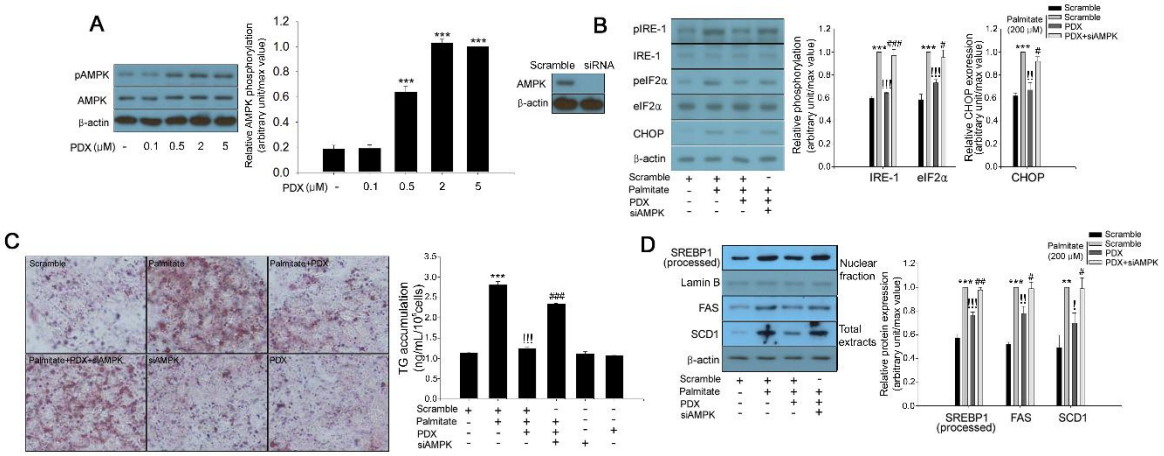

Figure 3

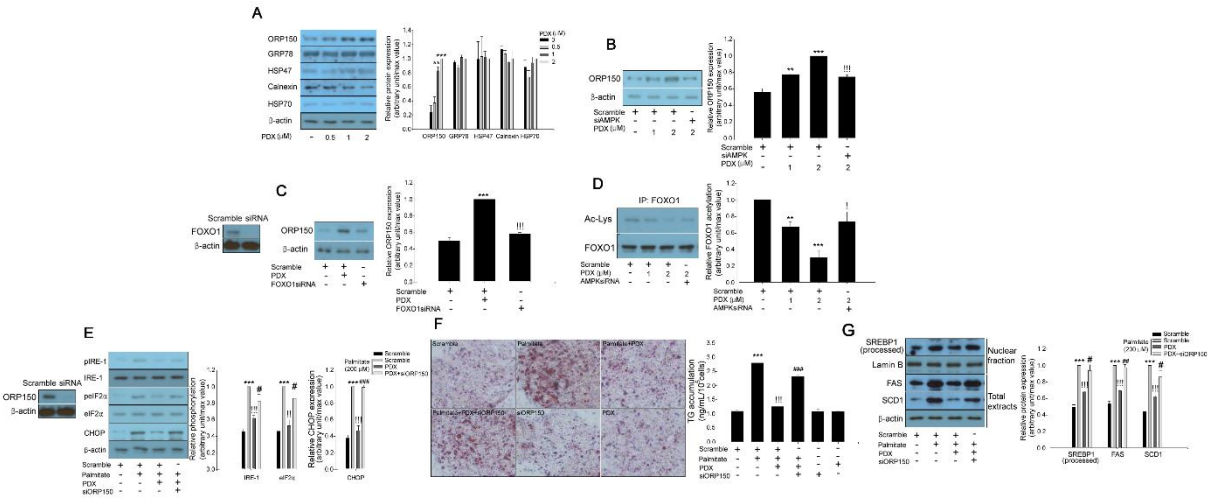

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Figure 4

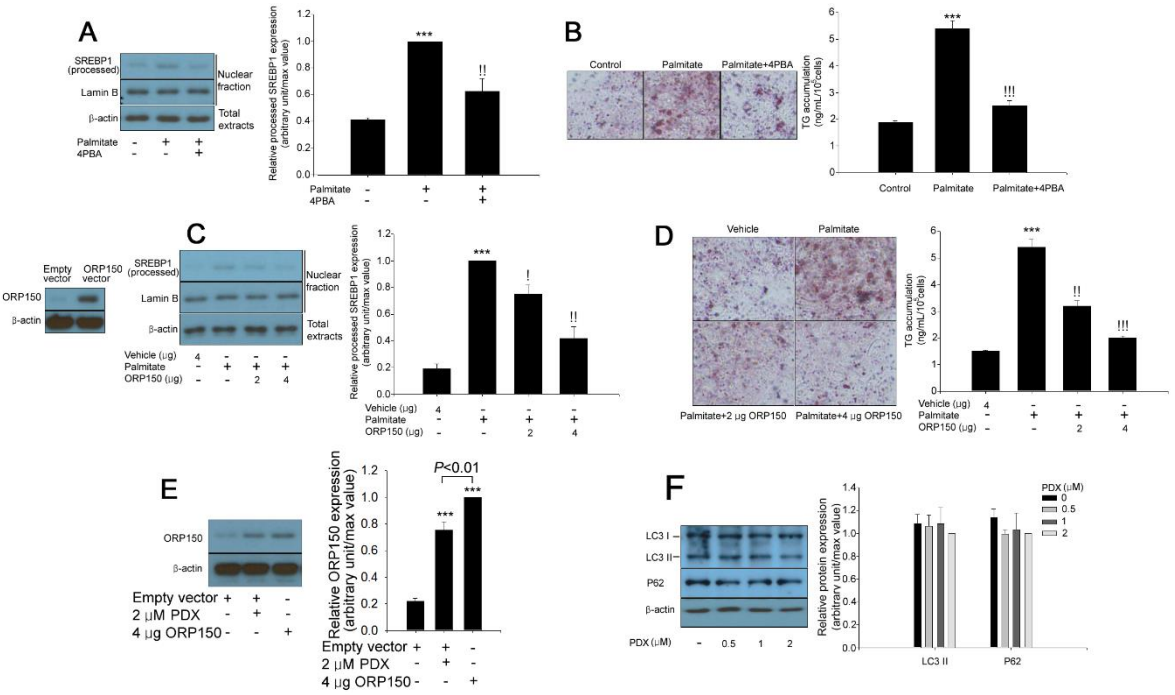

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Figure 5

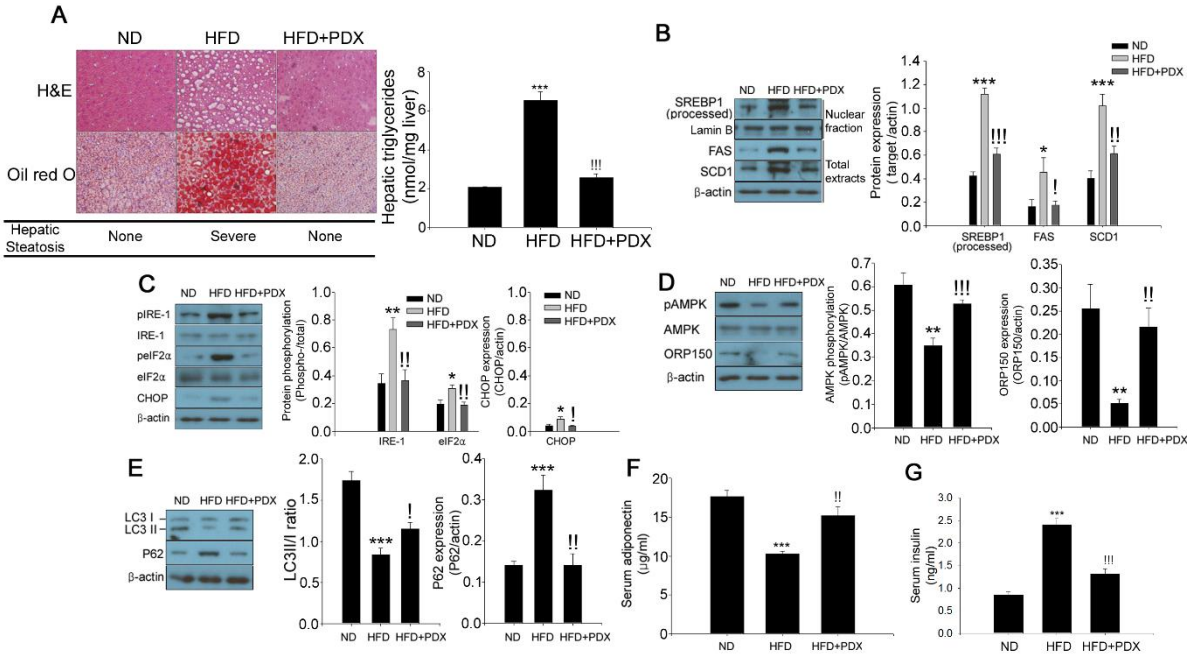

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Figure 6