Palmitic Acid-Enriched Diet Induces Hepatic Steatosis and Injury in Adult Zebrafish

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Abstract

Palmitic acid (PA) is the most abundant saturated fatty acid in fast foods and is known to induce inflammation and cellular injury in various tissues. In this study, we investigated whether a PA-enriched diet can induce hepatic steatosis and injury in adult zebrafish. The adult zebrafish exhibited increased body weight, hyperlipidemia, hyperglycemia, and steatosis and a hepatic injury phenotype after being fed with a PA-enriched diet for 6 weeks. The quantitative polymerase chain reaction analysis demonstrated that genes associated with hepatic injury were all significantly increased in the liver. Furthermore, livers from the PA-fed group showed an increased messenger RNA (mRNA) expression associated with oxidative stress and endoplasmic reticulum (ER) stress responses. We also found significant upregulation of genes involved in lipid metabolism and triacylglyceride accumulation. Ultrastructural analysis revealed mitochondrial cristae injury and a dilated ER phenotype in the PA-fed hepatocytes, which can be causes of hepatic injury. PA-enriched diet induced hepatic steatosis and injury in adult zebrafish that recapitulated typical metabolic changes and pathophysiological changes as well as increased oxidative stress and ER stress observed in patients with nonalcoholic fatty liver disease.

Keywords: adult zebrafish, ER stress, hepatic injury, liver steatosis, oxidative stress, palmitic acid

Introduction

PALMITIC ACID (PA) is the most abundant saturated fatty acid in fast foods (ranging from 50% to 90% of total fat),¹ because it is the major component of palm oil (45% of total fatty acid).² PA treatment has been known to induce inflammation and cellular injury in various tissues.^{3,4} A recent clinical report showed that even a single dose of palm oil could induce insulin resistance, changes in lipid storage, and gene expression in the liver.⁵ Thus, a chronic PA-enriched diet may be one of the major causes of nonalcoholic fatty liver disease including simple steatosis and hepatic injury. However, few studies have examined the effect of PA in the development of liver disease. Understanding the molecular mechanism of liver pathogenesis induced by dietary PA intake will bring new insights that could lead to the prevention and cure of high-fat-induced hepatic injury. The zebrafish is an excellent model system for studying liver diseases because of the organ system homology to humans including cell types and gene expressions.^{6,7} Zebrafish larvae have been used to study alcoholic and nonalcoholic fatty liver disease^{6,8–10}; however, results from larvae may not represent chronic liver disease in adults. Adult zebrafish have not been used as a model for fatty liver disease, but recent articles reported fatty

liver development after chronic alcohol exposure or trans-fat diet in adult zebrafish.^{11,12} In this article, we used adult zebrafish to investigate the effect of a PA-enriched diet on the liver. The quantity of PA in the food was determined based on the average PA content of typical fast foods.¹ After adult zebrafish were fed with a PA-enriched diet for 6 weeks, we analyzed serum glucose, triglycerides (TG), and alanine aminotransferase (ALT) levels, in addition to pathological defects in the liver. The changes of messenger RNA (mRNA) expression caused by a PA diet were accessed by real-time quantitative polymerase chain reaction (RT-qPCR) analysis of genes associated with inflammation, cell death, and fibrosis as well as lipid metabolism in the liver. Moreover, to determine two major stress signaling involved in high-fat diet-induced liver injury, we evaluated multiple genes associated with oxidative stress and endoplasmic reticulum (ER) stress.

Experimental Section

Zebrafish

Wild-type zebrafish (AB/TU strain) were purchased from the Zebrafish International Resource Center and maintained according to standard protocols. Maintenance

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and experimental procedures for zebrafish were approved by the Medical University of South Carolina (Charleston, SC) Institutional Animal Care and Use Committee (IACUC), protocol number AR3324. The fish were maintained in a system cage (Multi-linking water treatment unit system; Tecniplast) at 28°C during feeding under a 14:10 h light:dark cycle. Twelve-month-old male adult zebrafish were under anesthesia with 0.016% (w/v) tricaine and allocated to two groups ($n = 30$ in each group) with similar body weights. Fish were placed in 3 L tank.

Diets and feeding regime

We used tetramin flake (47% crude protein; 11.0% crude fat; 3.0% crude fiber; Tetra-Fish, Inc., Blacksburg, VA) as the control diet. A PA-enriched diet for adult fish was made by soaking tetramin flake in a diethyl ether solution containing PA to achieve a content of 8% (w/w) PA in the food (Supplementary Table S1). Ether was completely evaporated in the fume hood for at least 3 days. We calculated the amount of PA in the diet to achieve 33% of calories from the fat, whereas control group fish received 23% calories from the fat in the tetramin flake diet (Supplementary Fig. S1A). Each group ($n = 15 \times 2$) consumed the designated diet (21.6 mg of control and 20 mg of PA-enriched diet/day/fish) for 6 weeks. To match total calories per meal between the control and PA-enriched diet, we fed 8% higher amount of food to the control group.

Blood collection and biochemical analysis

Blood collection was performed by inserting a glass capillary needle into the zebrafish's dorsal aorta as previously reported.¹³ Zebrafish blood was obtained by using a heparinized needle for blood collection along the body axis and posterior to the anus in the region of the dorsal aorta. Blood was collected from adult zebrafish after a 20-h fasting period and was diluted 1:10 in phosphate-buffered saline. After centrifugation, the plasma supernatant was collected. Serum glucose level was measured with Bayer Contour NEXT Diabetes EZ meter (Bayer AG) using Contour NEXT Blood Glucose Test Strips. TG, total cholesterol (TC), and ALT levels in diluted plasma were measured with kits according to manufacturer's protocol (Cat. No. TR22421 for TG; Thermo Scientific, Cat. No. 439-17501 for TC; Wako, Cat. No. A7526 for ALT assay; Pointe Scientific).

Liver histology

Liver were fixed in 4% paraformaldehyde from overnight to 2 days at 4°C. Fixed liver were embedded in 1.2% agarose/5% sucrose and saturated in 30% sucrose at 4°C for 1–2 days. Blocks were frozen on the surface of 2-methyl butane chilled using liquid nitrogen. Ten micrometer sections were collected on microscope slides using a Leica cryostat (Leica CM 1950). Sections were kept in –80°C before use.

Quantitative RT-PCR

RNA was isolated from adult zebrafish liver using a Trizol method and complementary DNA (cDNA) was reverse transcribed using a SuperScript III First-Strand kit (Cat. No. 18080-051; Invitrogen). The total RNA was extracted from three livers in the control group and in the PA-fed group, respectively, and then the same amount of RNA was pooled for

cDNA synthesis. Oligo-dT primed cDNA were prepared from total RNA isolated with Trizol® Reagent (Cat. No. 15596-026; Invitrogen) using superscript III First-Strand kit (Cat. No. 18080-051; Invitrogen). Real-time quantitative polymerase chain reaction (qPCR) was performed with thermal cycler (CFX96 real-time system; Bio-Rad) with 45 cycle using 50 ng cDNA, 200 pmol/μL gene-specific primers¹⁴ (Supplementary Table S2), and SsoAdvanced™ Universal SYBR® Green Supermix (Cat. No. 172-5274; Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was used as reference, and relative quantification was calculated using double-delta Ct method. qRT-PCR was run in at least triplicate for each gene.

TG analysis

After 6 weeks of diet experiment, five sets of control diet-fed and PA diet-fed fish were subjected to an overnight fasting and obtained liver. Liver homogenized in tissue homogenization buffer (250 mM sucrose, 25 mM KCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4) with 1× protease and phosphatase inhibitor (Halt™ protease and phosphatase inhibitor cocktail, Cat. No. 78441; Thermo Fisher Scientific) by sonication method. We collected the lysate and determined protein concentration using the Pierce™ BCA protein assay (Cat. No. 23227; Thermo Fisher Scientific); we kept the lysate in –80°C before TG analysis. Liver TG was measured by using Triglycerides Reagent (Cat. No. TR22421; Thermo Scientific). Fatty acid composition analysis in the diet was performed at Eurofins Nutritional analysis center (Eurofins Scientific, Inc., Des Moines, IA).

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) was measured by using OxiSelect. *In Vitro* ROS/reactive nitrogen species (RNS) Kit from Cell Biolabs (San Diego, CA), as per the manufacturer's protocol. Lysates were collected (1 mg/mL) and immediately subjected to the ROS/RNS measurement. The fluorescent intensity of fluorophore dichlorofluorescein (DCF), which was formed by peroxide oxidation of the nonfluorescent precursor dichlorodihydrofluorescein (DCFH), was detected at 480 Ex/530 Em BMG LABTECH CLARIOstar. In contrast, DCFH with lysis buffer was used as a blank control.

Measurement of GSH and GSSG levels

Quantitative determinations of GSH and GSSG levels in liver lysates were performed using the enzymatic-recycling method.¹⁵ Proteins in the extracts from control diet-fed and PA-enriched diet-fed adult livers were precipitated by sulfosalicylic acid and the supernatant was then divided into two tubes. For reduced GSH, the supernatant was incubated with the thiol fluorescent probe IV, and fluorescent intensities were measured at 400 Ex/465 Em. For total GSH (GSH+GSSG), the supernatant was neutralized by triethanolamine and incubated with the reduction system (containing NADPH and glutathione reductase) at 37°C for 20 min. GSSG was calculated based on the results from reduced GSH and total GSH; the ratio of $GSH/GSSG = \frac{[GSH]}{([TOTAL\ GSH] - [GSH])/2}$.

Transmission electron microscopy

Zebrafish liver were immersed overnight in 2.5% glutaraldehyde in phosphate buffer, pH 7.4. Ultrathin sections

(70 nm) were positioned on copper grids and stained with uranyl acetate and lead citrate for 10 min each. The sections were viewed with a JEOL 1010 electron microscope at 80 kV in various magnifications. The images were captured with a Hamamatsu camera. Whole processes were performed in the electron microscopy core facility at the MUSC.

Statistical analysis

Statistical significance was determined by two-tailed Student's *t*-test with a value of $p < 0.05$ considered significant using GraphPad Prism 8 software (GraphPad Software, Inc.). Data were expressed as mean $\pm$ standard deviation.

Results

Effect of dietary PA on metabolic parameters in adult zebrafish

We used tetraamin flake as the control diet that containing 11% crude fat and we added 7% (w/w) PA to the tetraamin flake to make the PA-enriched diet, which was equivalent to at least 41% of all fat being PA. Thus, the adult fish received 22% and 33% of calories from fat under the control diet and the PA diet condition, respectively, which is highly relevant to a normal and moderate high-fat diet condition in humans. After 6 weeks of the calorie-matched control and PA-enriched diet, the PA-fed group showed 30% more body weight gain than the control group that received plain tetraamin diet (Fig. 1A). Blood glucose, plasma TG, and TC levels were significantly elevated after PA feeding, that is, 1.6-, 1.5- and 1.3-fold higher than control group (Fig. 1B–D), respectively. Thus, the chronic PA-enriched diet induced obesity, hypertriglyceridemia, and hyperglycemia phenotype in adult zebrafish.

PA-enriched diet induces hepatic injury in adult zebrafish

Hepatocellular ballooning is characterized by swollen hepatocytes with/without acidophilic body (pink staining on

hematoxylin and eosin [H&E]) or Mallory hyaline and reflects hepatocellular injury. As given in Figure 2, histological analysis of liver sections stained with H&E showed hepatocyte ballooning with accumulation of acidophilic body phenotype in PA-fed groups (Fig. 2A, B). In addition, serum ALT levels indicated a significant increase of liver injury after being fed with the PA-enriched diet (Fig. 2C). The relative mRNA expression of genes associated with inflammation (tumor necrosis factor alpha [*tnfa*] and interleukin 1 beta [*il1b*]), cell death (DNA fragmentation factor subunit alpha [*dffa*]), and fibrosis (collagen, type I, alpha 1a [*colla1a*]) were significantly elevated in the PA-fed group compared with the control group (Fig. 2D). Thus, PA-enriched diet in adult zebrafish mimicked the hepatocellular injury phenotype observed in steatohepatitis.

PA-enriched diet induces hepatic steatosis and increases gene expression associated with lipid metabolism

To investigate whether the PA-enriched diet can induce steatosis in the liver of zebrafish, we performed the Oil red O staining in the liver sections. The result showed increased lipid accumulation in the liver of the PA-fed group (Fig. 3A). Moreover, quantification of TG in the livers of the control group and the PA-fed group showed a threefold higher TG level in the PA-fed group than control group (Fig. 3B). To determine the mechanism underlying the steatosis phenotype in the PA-fed group, we investigated whether the PA consumption affected expression of genes associated with hepatic lipid metabolism. We examined relative mRNA expression of genes including lipogenic transcription factors, lipogenesis, and lipid transport using the quantitative RT-PCR (Fig. 3C). We found significant increases of sterol regulatory element-binding protein 2 (*srebp2*), which is essential for cholesterol biogenesis, and *cebpa*, which encodes a protein that is a key regulator of adipogenesis and the accumulation of lipid in liver, whereas expression of sterol regulatory element-binding protein 1 (*srebp1*) was not

FIG. 1. Assessment of body weight, plasma TG, and blood glucose levels in zebrafish fed control or PA (7% PA) diet. (A) Changes in body weight in male adult zebrafish in control or PA diet groups. Values are given as mean $\pm$ SD. Control or PA group: $n = 15$. (B) Changes in blood glucose levels ($n = 10$, each groups), (C) changes in plasma TG levels ($n = 5$, each groups), and (D) changes in plasma TC. Error bars indicate SD of the mean. * $p < 0.05$; ** $p < 0.01$. PA, palmitic acid; SD, standard deviation; TC, total cholesterol; TG, triglyceride.

FIG. 2. Ballooning of hepatocytes with steatosis and elevation of liver injury after ingestion of a PA-enriched diet. Representative images of H&E staining of liver in control (A) and PA (B) diet for 6 weeks. Yellow rectangles on left side corners showed magnified views of selected areas. Scale bar = 25 μ m. (C) Plasma ALT levels in control and PA diet-fed zebrafish ($n=5$). (D) Relative mRNA expression of *tnfa*, *il1b*, *dffa*, and *coll1a1a* in control and PA diet-fed fish livers ($n=5$). Error bars indicate SD of the mean. * $p<0.05$; ** $p<0.01$. ALT, alanine aminotransferase; *coll1a1a*, collagen, type I, alpha 1a; *dffa*, DNA fragmentation factor subunit alpha; H&E, hematoxylin and eosin; *il1b*, interleukin 1 beta; mRNA, messenger RNA; *tnfa*, tumor necrosis factor alpha. Color images are available online.

changed. The mRNA levels of key lipogenic enzymes involved in fatty acid synthesis were also significantly increased, including acetyl-CoA carboxylase 1 (*acc1*), a gene that synthesizes malonyl-CoA from acetyl-CoA, fatty acid synthase (*fasn*), which catalyzes the synthesis of PA from acetyl-CoA and malonyl-CoA, and diacylglycerol O-acyltransferase 2 (*dgat2*), a gene essential for the synthesis of TG. The mRNA levels of genes involved in the lipid

uptake were also increased. We found that the expressions of CD36 molecule (*cd36*), low-density lipoprotein receptor (*ldlr*), and lipoprotein lipase (*lpl*) were significantly upregulated in the livers of PA-fed zebrafish compared with the livers of the control group, which suggests that the PA-enriched diet increased lipid transport into the liver through increase of free fatty acid uptake and low-density lipoprotein receptor mediated lipid transport into the liver. Thus, the

FIG. 3. Lipid metabolism-associated gene expression and liver TG levels in zebrafish fed the control or the PA diet. (A) Oil red O staining of livers from control diet and PA diet. Images are representative of 10 samples per each. (B) The level of TG level measured from zebrafish liver lysates of control and PA-fed groups ($n=5$, each groups). (C) Relative mRNA expression of genes associated with lipid metabolism include *srebp1*, *srebp2*, *C/EBPα*, *acc1*, *fasn*, *dgat2*, *ppar-γ*, *cd36*, *ldlr*, *lpin1*, and *lpl*. * $p<0.05$; ** $p<0.01$. *acc1*, acetyl-CoA carboxylase 1; *cd36*, CD36 molecule; *C/EBPα*, CCAAT enhancer binding protein alpha; *fasn*, fatty acid synthase; *ldlr*, low-density lipoprotein receptor; *lpin1*, lipin 1; *lpl*, lipoprotein lipase; *ppar-γ*, peroxisome proliferator-activated receptor gamma; *srebp1*, sterol regulatory element-binding protein 1; *srebp2*, sterol regulatory element-binding protein 2. Color images are available online.

PA-enriched diet in adult zebrafish mimicked key pathophysiological features of hepatic injury (Fig. 2) and steatosis (Fig. 3).

PA-enriched diet increases mitochondrial biogenesis and activity β -oxidation, which may lead to increased oxidative stress in the liver of adult zebrafish

Impact of a high-fat diet on enhancement of mitochondrial biogenesis has been studied in the muscle and liver.^{16–18} We analyzed mRNA expression of genes in mitochondrial biogenesis and function in the liver of PA-fed zebrafish compared with control diet-fed zebrafish. We found a significant increase of dynamin-related protein 1 (*drp1*), a gene required for mitochondrial fission; optic atrophy type 1 (*opa1*), which plays a role in mitochondrial fusion and cristae stabilization; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*pgc1a*), a master regulator of mitochondrial biogenesis; DNA polymerase gamma, catalytic subunit (*polg*), mitochondrial DNA polymerase; and NADH-ubiquinone oxidoreductase chain 1 (*nd1*), a subunit of NADH dehydrogenase encoded from the mitochondrial DNA; in addition, expression of mitofusin 1 (*mfn1*), a gene important for mitochondrial fusion (Fig. 4A) showed a moderate increase in PA-fed zebrafish liver. Of importance, we found PA consumption increased transcription of electron transfer flavoprotein subunit alpha (*etfa*), a gene encoding a subunit of electron transfer flavoprotein that catalyzes the initial step of

the mitochondrial fatty acid β -oxidation and carnitine-palmitoyltransferase I (*cpt1*), the key regulatory enzyme for transferring fatty acid acyl-CoA into the mitochondria. These results suggest that PA consumption enhanced mitochondrial homeostasis and fatty acid β -oxidation activity in the liver of adult zebrafish. Enhanced mitochondrial β -oxidation activity may cause an increase of cellular oxidative stress. To test our hypothesis, we analyzed the expression of genes associated with superoxide generation within the cell. We found transcriptional elevation of NADPH oxidases such as NADPH oxidase 1 (*nox1*), *nox2*, and *nox5* after PA-diet ingestion (Fig. 4B). Because these enzymes produce superoxide, we speculated that the PA-enriched diet in zebrafish might elevate the oxidative stress in the liver through the transcriptional activation of *nox* genes. An increase of superoxide can induce superoxide dismutase 2 (*sod2*) mRNA expression and lead to an accumulation of hydrogen peroxide. The expression of nuclear factor erythroid 2-related factor 2 (*nrf2*), the master regulator of antioxidative response, was highly elevated after PA diet. Because increased oxidative stress triggers transcriptional activation of genes associated with oxidative stress response, we analyzed genes in two main systems governing cellular redox reactions: the glutathione system and the thioredoxin system (Fig. 4B). We found that mRNAs of genes associated with both glutathione (glutathione reductase [*gsr*], glutathione S transferase pi 1 and 2 [*gstp1/2*], and glutathione peroxidase 1a [*gpx1a/4a*]) and thioredoxin (*prdx4*, thioredoxin-like 1 [*txnl1*], *txnl4*) systems

FIG. 4. Mitochondrial homeostasis- and oxidative stress-associated gene expression and GSH, GSSG levels in control and PA-fed groups. (A) Relative mRNA expression of genes associated with mitochondrial homeostasis include *drp1*, *mfn1*, *opa1*, *pgc1a*, *polg*, *nd1*, *etfa*, and *cpt1*. (B) Relative mRNA expression of oxidative stress-related genes includes *nox1*, *nox2*, *nox5*, *nrf2*, *sod2*, *gsr*, *gstp1/2*, *gpx1a*, *gpx4a*, *prdx1*, *prdx4*, *txnl1*, and *txnl4*. Quantification of (C) ROS, (D) GSH, (E) GSSG, and (F) GSH/GSSG ratio in control- and PA-fed groups. * $p < 0.05$; ** $p < 0.01$. *cpt1*, carnitine-palmitoyltransferase I; *drp1*, dynamin-related protein 1; *etfa*, electron transfer flavoprotein subunit alpha; *gpx1a*, glutathione peroxidase 1a; *gsr*, glutathione reductase; *gstp1/2*, glutathione S transferase pi 1 and 2; *mfn1*, mitofusin 1; *nd1*, NADH-ubiquinone oxidoreductase chain 1; *nox*, NADPH oxidase; *nrf2*, nuclear factor erythroid 2-related factor 2; *opa1*, optic atrophy type 1; *pgc1a*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *polg*, DNA polymerase gamma, catalytic subunit; *prdx1*, peroxiredoxin 1; ROS, reactive oxygen species; *sod2*, superoxide dismutase 2; *txnl1*, thioredoxin-like 1. Color images are available online.

were highly elevated after ingestion of the PA diet. The quantification of ROS level in the liver showed significant increase of ROS in the PA-fed zebrafish liver (Fig. 4C). Glutathione (GSH) is a key antioxidant that protects cellular components from oxidative stress and injury. A reduction in the ratio of GSH-to-GSSG indicates that cells have increased susceptibility to oxidative stress.^{19,20} The increase in mRNA expression of components of the GSH pathway raised the possibility of imbalance in GSH and oxidized GSH glutathione disulfide (GSSG) ratio. Therefore, we measured reduced GSH and oxidized form of GSH (GSSG). Although GSH level in the liver did not change after ingestion of the PA diet, we found a significant elevation of the GSSG level, which resulted in a reduction of the GSH/GSSG ratio in the PA-enriched diet group (Fig. 4D–F). In aggregate, our results suggest that oxidative stress may play a critical role in PA diet-induced liver injury in adult zebrafish.

PA-enriched diet induces cristae damage in the mitochondria and dilation of ER in hepatocytes

In addition to an increase of oxidative stress, the dramatic elevation of the *opal* expression level given in Figure 4A suggested that the PA diet could induce mitochondrial injury, because *opal* could be induced to protect mitochondrial cristae structure from the oxidative stress. To test this possibility, we performed ultrastructural analysis in the liver of control diet-fed and PA diet-fed adult zebrafish (Fig. 5A). We found that there was accumulation of mitochondria with damaged cristae structures in hepatocytes of PA-fed zebrafish. Thus, steatosis and liver injury may result from mitochondrial injury by PA diet. Of importance, the PA-fed group exhibited extensive dilation of the ER membrane in hepatocytes of PA diet-fed zebrafish (Fig. 5A, right panel).

To investigate ER stress induced by PA-enriched diets, we analyzed the expression of genes associated with the ER stress response in the control and PA-fed groups. The expression of those genes significantly increased, including activating transcription factor 4 (*atf4*), and its target gene growth arrest and DNA damage-inducible 45 alpha (*gadd45a*), activating transcription factor 6 (*atf6*), and targets including X-box binding protein 1 (*xbp1*), DNA damage-inducible transcript 3 (*ddit3*), ER degradation-enhancing alpha-mannosidase-like 1 (*edem1*), binding immunoglobulin protein (*bip*), and DnaJ homolog subfamily C member 3 (*dnajc3*) mRNA expression. In addition, Bcl2 interacting mediator of cell death (*bim*), BH3-interacting domain death agonist a (*bida*), and Bcl2 associated x b (*baxb*) mRNA expression also significantly increased, which is the BCL-2 family of proteins associated with ER stress-induced apoptosis. Although the expression of inositol-requiring enzyme 1 (*ire1*) and its downstream component, nuclear factor kappa-light-chain-enhancer of activated B cells (*nfkb*) did not change after ingestion of the PA-enriched diet (Fig. 5B), *xbp1* splicing by *ire1* was enhanced by PA diet. These results indicate that both mitochondrial injury and ER injury may contribute to the PA diet-induced hepatic injury phenotype we observed in adult zebrafish.

Discussion

Genetic and feeding models of steatohepatitis have been well-established using rodents. However, the majority of

FIG. 5. Transmission electron microscopy imaging of hepatocytes and mRNA expression of genes associated with ER stress. **(A)** 8,000 $\times$ magnification in the liver of control and PA diet-fed zebrafish for 6 weeks. The magnified areas at the bottom (yellow rectangles) depict normal mitochondria in control diet-fed and abnormal mitochondria with damaged cristae in the PA-fed zebrafish liver. Images shown are representative of at least three other zebrafish livers. Scale bar = 800 nm. N (nucleus), m (mitochondria), Lip (lipid droplet) and asterisks mark for injured cristae. **(B)** Relative mRNA expression of ER stress/unfolded protein response-related genes; *atf4*, activating transcription factor 4; *atf6*, activating transcription factor 6; *baxb*, Bcl2 associated x b; *bim*, Bcl2 interacting mediator of cell death; *bida*, BH3-interacting domain death agonist a; *bip*, binding immunoglobulin protein; *ddit3*, DNA damage-inducible transcript 3; *dnajc3*, DnaJ homolog subfamily C member 3; *edem1*, ER degradation-enhancing alpha-mannosidase-like 1; ER, endoplasmic reticulum; *gadd45a*, growth arrest and DNA damage-inducible 45 alpha; *grp94*, glucose-regulated protein 94; *ire1*, inositol-requiring enzyme 1; *nfkb*, nuclear factor kappa-light-chain-enhancer of activated B cells; *xbp1*, X-box binding protein 1; *xbp1-s*, spliced form of *xbp1*. Color images are available online.

models have shown a discrepancy between pathological defect and metabolic context of steatosis or steatohepatitis (reviewed in Schattenberg and Galle²¹ and Lau *et al.*²²), that is, some models replicate the histopathology of steatohepatitis but not the physiological properties such as insulin resistance, dyslipidemia, or obesity, and others vice versa. Although high-fat diet-induced fatty liver disease models recapitulated both metabolic contexts and pathological liver phenotypes including steatosis and hepatic injury,^{23–25} those

models produce variable results depending on the rodent species, strain, fat content, composition of fat, and the duration of treatment (reviewed in Takahashi *et al.*²⁶). In addition, the average content of high fat in an experimental high-fat diet results in 60%–75% of energy uptake from the fat, which may represent an extreme high-fat diet condition in humans. Therefore, development of a new animal model that recapitulates the disease spectrum of steatohepatitis is essential for further study. Zebrafish models of steatosis are well established at larvae stage,^{8,10} however lack of serum analysis and absence of hepatocyte ballooning phenotype, a marker for severe hepatic injury, and difference of physiology compared with adult limited use of larvae model to study chronic liver disease in adults. Adult zebrafish have not been used widely to study chronic liver disease. Although recent studies of overfeeding experiments in adult zebrafish described liver steatosis,^{27,28} the condition of overfeeding in zebrafish was an extreme diet condition (5 mg/fish vs. 60 mg/fish, i.e., 12-fold more feeding for overfeeding group). Thus, previous larvae and overfeeding models may not be ideal as representative model of chronic liver disease. In this study, we used adult zebrafish as a new model to evaluate an impact of PA on the liver. We chose a PA-enriched diet, because PA is the most abundant fatty acid in fast food meals. In addition, PA treatment in human liver cell lines has induced the steatosis phenotype²⁹ and even a single dose of palm oil (44% of fatty acid is PA) could induce insulin resistance, changes in lipid storage, and gene expression in the liver.⁵ To test whether a PA-enriched diet can induce similar results in humans using adult zebrafish, we added 7% (w/w) PA to the control diet, which contained 11% crude fat (14% of total fatty acid being PA), to make a PA-enriched diet (17% total fat) that led to 48% of fatty acid being PA, and a 33% calorie uptake from fat. We used a feeding condition that approximated guidelines for dietary fat intake in adult humans, that is, 22% of energy produced from fat as the control diet, and 33% of energy from fat as the high-fat diet (Supplementary Table S1 and Supplementary Fig. S1A). Furthermore, to determine whether the liver defects resulted from the total fat amount or PA content in the diet, we tested a commercially available fish food that includes 17% crude fat (Aquamax fry powder; Purina, LLC, Largo, FL). We found that both the PA-enriched diet and the Aquamax diet produced a significant increase of body weight compared with the control diet; however, a larger increase in ALT was seen in PA-enriched diet than the Aquamax-fed group (Supplementary Fig. S1B, C). Thus, our results indicate that dietary PA content in diet is essential to our diet-induced steatosis and hepatic injury phenotype. In addition to steatosis and hepatic injury phenotype after 6 weeks of PA-enriched diet, we found significant increase in *colla1a* mRNA expression in PA-fed zebrafish. However, we were not able to detect a typical fibrosis staining (data not shown), possibly because of differences of liver structure between mammals and zebrafish; hepatocytes are not clearly organized and absence of portal triads in zebrafish³⁰ or actual fibrosis may require longer period of PA-enriched diet. In addition, PA-induced hepatic injury (hepatocyte ballooning and increased in mRNA of genes involved in inflammation) seemed obvious in our model. However, we were not able to achieve clear evidence of immune cell infiltration in H&E staining. We performed an antibody staining specific to macrophage and neutrophils, but

the antibody staining did not work (anti-Lcp1 antibody; Genetex, Inc., data not shown). Further investigation may be required by using transgenic lines that label macrophage or neutrophils in the future. While we were preparing the present article, other group reported on the hepatotoxicity of PA in 5 days post-fertilization and 1-month-old zebrafish³¹ focused on ER stress in the liver. Thus, our study confirmed PA diet-mediated increase of ER stress is critical in liver pathogenesis in adult zebrafish. Collectively, we found PA-enriched diet induced hepatic steatosis and injury in adult zebrafish and adult zebrafish can be used as a model of a high-fat diet-induced chronic liver disease model. Our model recapitulated pathological (steatosis and hepatocyte ballooning) and metabolic (hyperglycemia and hyperlipidemia) changes similar to those seen in patients with steatohepatitis. Gene expression analysis showed that two major stress signaling pathways associated with liver injury (oxidative stress and ER stress) were highly elevated in the PA-fed zebrafish liver.

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Authors' Contributions

S.-H.K. formulated ideas, designed the project, performed experiments, prepared figures and wrote the article. K.-H.P. maintained zebrafish colonies, performed experiments, and prepared figures. Z.-W.Y. and J.Z. performed experiments for Figure 4C–F.

Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Supplementary Material

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2

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