Peroxisome proliferator-activated receptor α attenuates high cholesterol diet-induced toxicity and pro-thrombotic effects in mice

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Abstract

Peroxisome proliferator-activated receptor α (PPARα) is involved not only in the regulation of fatty acid metabolism, but also cholesterol metabolism. A high cholesterol (HC) diet increases the risk of developing cardiovascular diseases (CVD), however, it is unclear whether the toxic effects of cholesterol involves changes in thrombotic factor expression, and whether PPARα is necessary for such effects. To investigate this possibility, we fed a HC diet to wild type (WT) and *Ppara*-null mice and compared contents of cholesterol and triglyceride, liver histological change, serum/plasma levels of coagulation factors, hepatic expression of the coagulation factors, liver/serum sulfatide levels, hepatic sulfatide metabolism, hepatic expression of lipid transporters, and hepatic oxidative stress and its relating enzymes, between WT and *Ppara*-null mice. In the *Ppara*-null mice, the HC diet caused triglyceride accumulation and exacerbated inflammation and oxidative stress in the liver, increased levels of coagulation factors, including tissue factor, plasminogen activator inhibitor-1 and carboxypeptidase B2 in blood and liver, and decreased levels of anti-thrombotic sulfatides in serum and liver. These changes were much less marked in WT mice. These findings imply that cholesterol overload exerts its toxic effects at least in part by enhancing thrombosis, secondary to abnormal hepatic lipid metabolism, inflammation, and oxidative stress. Moreover, we reveal for the first time that PPARα can attenuate these toxic effects by transcriptional regulation of coagulation factors and sulfatides, in addition to its known effects of maintaining fatty acid metabolism and suppressing

inflammation and oxidative stress. Therapies aimed at activating PPARα might prevent HC diet-induced CVD through modulating levels of various pro- and anti-thrombotic factors.

Key words

High cholesterol diet, Peroxisome proliferator-activated receptor α (PPARα), Coagulation factors, Cardiovascular diseases, Sulfatides

Introduction

Peroxisome proliferator-activated receptor α (PPARα) is an intra-nuclear receptor that is widely distributed among organs that are involved in fatty acid (FA) metabolism, such as the liver, heart, kidney, and skeletal muscle (Aoyama et al. 1998; Kersten et al. 2000; Kamijo et al. 2002). PPARα is a master transcription factor for genes involved in triglyceride (TG) and FA metabolism, and is important in the maintenance of energy homeostasis (Aoyama et al. 1998; Kersten et al. 2000). Because FAs are ligands of PPARα, a FA-enriched high fat diet induces hepatic PPARα activation, resulting in greater expression of FA metabolic enzymes and FA catabolism (Aoyama et al. 1998; Kersten et al. 2000). It has also been reported that PPARα activation suppresses oxidative stress and pro-inflammatory responses caused by a high fat diet through up-regulation of reactive oxygen species (ROS)-eliminating enzymes and inhibitor κBα (IκBα) expression (Tanaka et al. 2011; Zúñiga et al. 2011; Valenzuela et al. 2012; Hu et al. 2017).

In addition to these roles, hepatic PPARα is known to play a role in cholesterol metabolism. PPARα influences the metabolic pathway generating cholic acids from cholesterol via transcriptional regulation of 12α-hydroxylase toward 7α-hydroxy cholesterol (Hunt et al. 2000), and that of intracellular cholesterol and cholic acid transporters (Chakravarthy et al. 2005; Badman et al. 2007). Therefore, it is possible that PPARα may ameliorate the toxic effects of a high cholesterol (HC) diet. However, almost all the previous studies that have investigated the relationship between PPARα and

lipids have used a FA-enriched high fat diet (Ye et al. 2001; Patsouris et al. 2006), and these studies have been therefore not capable of demonstrating a direct relationship between PPARα function and cholesterol toxicity. A study using a HC diet, containing FA and TG that are similar levels to a normal diet, is required to clarify this relationship.

Excessive cholesterol intake and the resulting hypercholesterolemia increase the risk of cardiovascular diseases (CVD) through increases in vascular plaque formation and thrombosis following plaque rupture (Abela et al. 1995). However, there may still be additional mechanisms underlying the development of CVD that have not been identified. Various pro-thrombotic molecules are thought to play an important role in the development of CVD (Toschi et al. 1997; Kohler and Grant 2000; Steffel et al. 2006). Many clinical and experimental studies have reported that high levels of representative coagulation factors, including tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1), indicate a high risk of CVD development (Hamsten et al. 1987; Toschi et al. 1997; Kohler and Grant 2000; Steffel et al. 2006), and that feeding a HC diet increases the levels of these coagulation factors and enhances their pro-thrombotic effects (Kato et al. 1996; Ichino et al. 1997). However, the molecular mechanisms underlying the enhanced pro-thrombotic effects of cholesterol overload, as well as the influence of other thrombotic factors, remain to be identified.

Carboxypeptidase B2 (CPB2) is a liver enzyme that suppresses fibrinolysis (Mosnier and Bouma 2006). Sulfatides are major mammalian serum sphingoglycolipids which are synthesized and

secreted mainly from the liver as a component of lipoproteins (Kimura et al. 2012). Our previous experimental and clinical studies have demonstrated that serum sulfatides exert anti-coagulative and anti-platelet functions, and that lower levels of serum sulfatides might be associated with the development of CVD (Kamijo et al. 2012; Kimura et al. 2012; Yuzhe et al. 2015). It has been reported that expression of these important molecules (TF, PAI-1, CPB2, and sulfatides) is regulated by PPARα and oxidative stress (Marx et al. 2001; Kimura et al. 2012; Masuda et al. 2012; Kanbe et al. 2014; Gonsalves et al. 2015; Wojewodzka-Zelezniakowicz et al. 2017). We hypothesized that a HC diet exerts its toxic effects through changes in lipid metabolism, oxidative stress, and production of these thrombosis-related factors, and that PPARα might strongly influence such effects, thereby being involved in the development of CVD. To test this hypothesis, we have fed a HC diet containing normal amounts of FA and TG to wild-type (WT) and *Ppara*-null mice and then compared the effects on serum and hepatic contents of TG and cholesterol, the expression of TF, PAI-1, CPB2, sulfatides, and intracellular transporter of sulfatides, and oxidative stress between these genotypes.

Materials and methods

Mice and experimental design

Male WT and *Ppara*-null mice on a SV/129 genetic background (age 14–18 weeks; body mass 24–30 g) were used, as previously described (Lee et al. 1995). These mice were fed with a cholesterol-free diet (control group) or a 1.5% (w/w) cholesterol diet, both of which contained normal amounts of FA and TG, for 8 weeks (n=6 per group). The detailed composition of each diet is given in **Supplementary Table 1**. The experimental conditions for the current study were determined through preliminary experiments, which are described in the **Supplementary methods**. The mice were maintained in a specific pathogen-free facility and housed in a light- and temperature-controlled environment (12-hour light/dark cycle; 25°C), and were provided with tap water *ad libitum*. All animal experiments and procedures were conducted in accordance with the guidelines approved by the Shinshu University, the National Institutes of Health, and the Association for Assessment and Accreditation of Laboratory Animal Care.

Histopathologic analysis

Mouse livers were fixed in 10% formaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm. The deparaffinized sections were then stained with hematoxylin and eosin (HE).

Analysis of mRNA expression

Total liver RNA was extracted using the RNeasy Mini Kit (QIAGEN, Tokyo, Japan). Total RNA extracted from whole livers was reverse-transcribed using oligo (dT) primers and SuperScript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). Subsequently, the cDNAs were quantified by real-time polymerase chain reactions (PCR) using a SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 system (Takara Bio, Otsu, Japan). The sequences of specific primers are shown in **Supplementary Table 2**. The mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an internal control, and the relative expression of each target mRNA was calculated using the comparative threshold cycle (Ct) method for PCR amplification (Livak et al. 2001).

Immunoblot analysis

Whole liver lysates were prepared as previously described (Aoyama et al. 1989). The nuclear and cytoplasmic fractions of whole liver extracts were prepared for each mouse using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific, Rockford, IL, USA), and the protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Nuclear and cytoplasmic proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose

membranes. The membranes were incubated with the respective primary antibodies, followed by incubation with an alkaline phosphatase-conjugated secondary antibody (Tanaka et al. 2008). Immunoblotting was performed using antibodies targeting tissue factor (TF), carboxypeptidase B2 (CPB2), plasminogen activator inhibitor 1 (PAI-1), cerebroside sulfotransferase (CST), arylsulfatase A (ARSA), ceramide galactosyltransferase (CGT), galactosylceramidase (GALC), fatty acid binding protein 1 (FABP1), glycolipid transfer protein (GLTP), sterol carrier protein 2 (SCP2), microsomal triglyceride transfer protein (MTTP), and 4-hydroxy-2-nonenenal (4-HNE). β-actin was used as the internal control for protein loading. Primary antibodies targeting TF, CPB, PAI-1, FABP, and β-Actin were purchased from Abcam (Cambridge, MA, USA), those targeting CGT, GALC, SCP2, and MTTP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and those targeting GLTP, CST, ARSA, and 4-HNE were purchased from Proteintech (Chicago, IL, USA), Abnova corporation (Jhouzih St., Taiwan), Everest Biotech (Upper Heyford, Oxfordshire, UK, USA), and Alexis Biochemicals (Farmingdale, NY, USA), respectively. Specific protein band intensity was quantified by densitometry using NIH Image J software (Bethesda, MD, USA)

Lipid analysis

Lipids were extracted from liver using the hexane/isopropanol method, as previously described (Hara and Radin 1978). Serum and liver concentrations of total cholesterol (TC) and liver TG were

measured using enzymatic assay kits (Wako Pure Chemical Industries. Ltd, Osaka, Japan).

Measurement of serum levels of alanine aminotransferase (ALT) and TF, and plasma levels of CPB2 and PAI-1

Serum levels of ALT and TF were measured using enzymatic assay kits (ALT, Wako Pure Chemical Industries, Ltd., Osaka, Japan and TF, Assay Pro, Anaheim, CA, USA, respectively). Plasma levels of CPB2 and PAI-1 were also measured using enzymatic assay kits (CPB2, SAB, Baltimore, MD, USA and PAI-1, CUSABIO, Baltimore, MD, USA, respectively).

Analysis of oxidative stress markers

Tissue content of malondialdehyde (MDA), a marker of oxidation, was measured using a lipid peroxidation colorimetric assay kit (Oxis International, Beverly Hills, CA, USA). The abundance of 4-HNE-modified proteins, another marker of oxidation, was measured by immunoblot analysis.

Extraction and measurement of hepatic and serum sulfatides

Sulfatides were extracted from serum and liver tissue. Samples were microsonicated in six volumes of cold water, and lipids were extracted using the hexane/isopropanol method, as previously described (Hara and Radin 1978). Lipid extracts were then treated with methanolic sodium

hydroxide to convert sulfatides to their corresponding lysosulfatides. After purification, lysosulfatide preparations were assayed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Seven species of lysosulfatides were detected, and the total amount of sulfatides in each sample was calculated as the sum of each of these species. Detailed methods of extraction and measurement of sulfatides have been previously described (Li et al. 2007).

Other experiments

The DNA-binding activities of PPARα, δ, and γ were measured in liver nuclear protein fractions using PPARα, δ, and γ Transcription Factor Assay kits (Cayman Chemical, Ann Arbor, MI, USA). These assay kits are enzyme-linked immunosorbent assays that use specific DNA binding sequence-immobilized microplates and specific antibodies for each target transcription factor. This assay system is non-radioactive and sensitive, and has been recently used as an alternative method to the radioactive electrophoretic mobility shift assay system (Harada et al. 2016). The results of each DNA-binding assay are shown as a fold difference from the WT control group.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). Significant interactions between two

factors were identified using two-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software (version 22.0J; SPSS, Inc., Chicago, IL, USA).

Results

PPARα attenuates HC diet-induced TG accumulation and inflammation in liver.

We investigated the effects of feeding a HC diet for 8 weeks on hepatic and serum TC levels. These were identical between WT and *Ppara*-null mice fed the control diet, but were significantly and similarly higher in mice fed with the HC diet (**Fig. 1A and B**). Obvious liver swelling was not detected, but white coloration of the liver was recorded in the HC diet-fed *Ppara*-null mice, indicating the possibility of fatty liver. To evaluate this further, we measured hepatic TG content and histologically assessed hepatic lipid content by HE staining. Hepatic TG was significantly higher in *Ppara*-null mice than in WT mice in the control diet-fed groups, but was significantly higher in the HC diet-fed mice of both genotypes, in particular in *Ppara*-null mice (**Fig. 1C**). Histological analysis demonstrated no hepatic steatosis in the WT groups, whereas mild hepatic steatosis was observed in the *Ppara*-null group fed with the control diet, and this was compounded by the presence of inflammatory cell infiltration in the HC diet-fed *Ppara*-null group, suggestive of the development of steatohepatitis (**Fig. 1D**). To investigate the hepatocyte damage further, we measured serum levels of ALT and AST. Serum ALT levels were identical between WT and *Ppara*-null mice on the control diet, but the level was significantly higher in the HC diet-fed mice of the *Ppara*-null group (**Fig. 1E**). No significant differences in AST levels were detected among the groups (data not shown). These findings suggest that the HC diet can induce TG accumulation and inflammation in the liver, and that

PPARα suppresses these effects. They are consistent with the previous reports which have demonstrated that PPARα maintains TG and FA metabolism, and has anti-inflammatory effects (Aoyama et al. 1998; Kersten et al. 2000; Kamijo et al. 2007; Hashimoto et al. 2012).

PPARα partially suppresses the HC diet-induced increase of pro-coagulatory factors.

To elucidate the effects of the diet and the genotype on representative coagulation factors, we measured the blood content and liver mRNA and protein expression levels of TF, CPB2, and PAI-1. The blood and liver levels of these three molecules did not differ between the groups fed with the control diet. Feeding with the HC diet did not affect or slightly increased the blood content and liver expression of TF and CPB2 in WT mice, whereas the diet dramatically increased the levels of these two molecules in *Ppara*-null mice (**Figs. 1F-1G and Figs. 2E-2F**). In contrast, the blood level and liver expression of PAI-1 were significantly and similarly increased by the HC diet in both WT and *Ppara*-null mice (**Fig. 1H and Fig. 2G**). The changes in hepatic mRNA levels of these factors were similar to those in hepatic protein levels (**Figs. 2A-2C and Figs. 2E-2G**), suggesting that the effects of feeding with the HC diet influence in gene transcription level. These results indicate that the feeding with the HC diet can dramatically increase the expression of two coagulation factors, TF and CPB2, in the absence of PPARα expression. It is known that HC diet can increase blood and hepatic PAI-1 levels (Ichino et al. 1997), but our study is the first to demonstrate that these changes in PAI-1

are not significantly influenced by the absence of PPARα. Taken together, these findings suggest that the HC diet can increase blood and hepatic levels of at least three species of coagulation factors, and that PPARα can ameliorate these effects partially.

PPARα attenuates the HC diet-induced decrease of anti-thrombotic factors, sulfatides.

Next, we investigated the effect of the HC diet-feeding on serum and liver levels of the anti-coagulation and anti-platelet molecules, sulfatides, which are also regulated by PPARα, in WT and *Ppara*-null mice (Marx et al. 2001; Kimura et al. 2012; Masuda et al. 2012; Kanbe et al. 2014; Gonsalves et al. 2015; Wojewodzka-Zelezniakowicz et al. 2017). Liver and serum levels of sulfatides were similar between WT and *Ppara*-null mice fed with the control diet, and these levels became markedly lower in mice of either genotype fed with the HC diet. However, the decrease of sulfatide level was significantly greater in *Ppara*-null mice than that in WT mice (**Figs. 3A-3B**. Throughout the experiments, the composition of the sulfatides in the liver did not differ among the groups (**Fig. 3C**).

Because the HC diet significantly affected hepatic and serum sulfatide levels, we then measured the mRNA and protein expression of sulfatide metabolizing enzymes in the liver. The mRNA and protein expression levels of the key sulfatide synthase, CST, which is known to be regulated by PPARα (Kimura et al. 2012), were similar between WT and *Ppara*-null mice fed with

the control diet. However, these levels were significantly lower in both WT and *Ppara*-null mice fed with the HC diet, and the decrease was significantly larger in *Ppara*-null mice than that in WT mice (**Figs. 4A, 4E, 4F**). The mRNA and protein expression levels of the other three sulfatide metabolizing enzymes, ARSA, CGT, and GALC, did not change among the groups (**Figs. 4B-4D, 4E, 4G-4I**). We also measured the mRNA and protein expression levels of intracellular lipid transport molecules. The mRNA and protein expression levels of FABP1 and GLTP, which respectively transport free FA and glycolipids such as sulfatides (Zou et al. 2011), were not different between WT and *Ppara*-null mice fed with the control diet, but the both levels were significantly lower in mice of both genotypes fed with the HC diet (**Figs. 5A-5B, 5E, 5F-5G**). The mRNA and protein levels of the other lipid transport proteins, SCP2 and MTTP, which respectively transport cholesterol and TG, did not differ among the groups (**Figs. 5C-5D, 5E, 5H-5I**). These new findings suggest that the HC diet can reduce the serum level of sulfatides by reducing the synthesis and transport in liver, and that PPARα is deeply involved in the regulation of these HC diet-induced effects.

PPARα suppresses the HC diet-induced oxidative stress.

It has been reported that HC diet increases oxidative stress (Nazıroğlu et al. 2014); the levels of thrombotic factors such as TF, PAI-1, and sulfatides investigated in the current study are strongly influenced by oxidative stress (Marx et al. 2001; Kimura et al. 2012; Masuda et al. 2012; Kanbe et al.

2014; Gonsalves et al. 2015; Wojewodzka-Zelezniakowicz et al. 2017); and PPARα exerts anti-oxidative effects (Kamijo et al. 2007). Therefore, we investigated the effects of feeding the HC diet on hepatic oxidative stress and the related factors in WT and *Ppara*-null mice. To evaluate overall hepatic oxidative stress level, we measured the liver content of the lipid peroxidation markers, 4-HNE-modified protein and MDA. Both contents of these markers were higher in *Ppara*-null mice than in WT mice fed with the control diet (**Figs. 6A-6B**). Both contents became significantly higher in both genotypes fed with the HC diet, although the increases were larger in *Ppara*-null mice than those in WT mice (**Figs. 6A-6B**). Next, we examined the effects of the HC diet on mRNA expression levels of molecules responsible for generating or eliminating reactive oxygen species (ROS). The hepatic mRNA expression of the ROS-generating molecules, *Ncf1* and *Cybb*, which respectively encode neutrophil cytosolic factor 1 (NCF1) and NADPH oxidase 2 (Nox2), did not differ among the groups (**Figs. 6C-6D**). PPARα is known to regulate the ROS-eliminating molecules such as catalase and Cu/Zn-superoxide dismutase (SOD) (Kamijo et al. 2007). The liver mRNA expression of *Cat* and *Sod1*, which encode catalase and Cu/Zn-SOD, respectively, was similar between WT and *Ppara*-null mice fed with the control diet, but the expression levels were significantly lower in the HC diet-fed mice of both genotypes (**Figs. 6E-6F**). The decrease of *Sod1* expression was much larger in *Ppara*-null mice than that in WT mice (**Fig. 6F**). The expression of hepatic mRNAs encoding other ROS-eliminating molecules, *Sod2* and *Gpx1*, which encode Mn-SOD and glutathione

peroxidase 1, respectively, did not differ among the groups (**Figs. 6G-6H**). These new findings suggest that the HC diet can increase hepatic oxidative stress, and that PPARα represents anti-oxidative effects through maintaining expression of Cu/Zn SOD.

The changes in mRNA expression and DNA-binding ability of PPARs by the HC diet

Last, we evaluated the mRNA expression and DNA-binding ability of PPARα, δ, and γ. The mRNA expression and DNA-binding ability of PPARα in WT mice unchanged by the HC diet-feeding, those of PPARδ and γ were not affected by both the HC diet and the mouse genotype (**Figs. 7A-7B**).

Discussion

In the current study, we fed the HC diet to WT and *Ppara*-null mice to investigate the influence of PPARα deficiency on the changes induced by dietary cholesterol overload. Compared with WT mice, the HC diet-fed *Ppara*-null mice exhibited a number of important differences: accumulation of TG and the induction of inflammation and oxidative stress in the liver, marked increases in liver expression and blood levels of the coagulation factors such as TF, PAI-1, and CPB2, and significant decreases in serum and liver levels of the anti-thrombotic sulfatides. These novel results suggest that cholesterol overload can induce toxic effects, including steatohepatitis, enhanced oxidative stress, and increased pro-thrombotic activity, and that PPARα deficiency can promote these effects. The HC diet used in this study contains normal levels of FA and TG, therefore, the findings are likely to reflect specific effects of cholesterol overload.

In addition to known beneficial effects of PPARα such as maintenance of lipid homeostasis (Aoyama et al. 1998; Kersten et al. 2000; Kamijo et al. 2002), anti-inflammatory effect (Kamijo et al. 2002), and anti-oxidant effect (Kamijo et al. 2002), the present study is the first to show that PPARα might attenuate cholesterol-induced thrombotic effects by partial suppression of the increases in circulating coagulation factors and by antagonizing the decrease in levels of the anti-thrombotic sulfatides. Furthermore, the current study has also revealed that PPARδ and γ seem not to be involved in these protective effects, suggesting that PPARα is the predominant isoform responsible

for protection against the deleterious effects of cholesterol overload in the liver.

The mechanism underlying the development of steatohepatitis and the higher levels of coagulation factors in Ppara-null mice

PPARα maintains TG and FA homeostasis, and exerts anti-inflammatory and anti-oxidative effects, by transcriptionally regulating FA metabolizing enzymes, IκBα, catalase, and Cu/Zn-SOD (Aoyama et al. 1998; Kamijo et al. 2002, 2007; Hashimoto et al. 2012; Harada et al. 2016). It has been reported that severe fatty liver is easily induced in *Ppara*-null mice by insults such as starvation and excess ethanol consumption through abnormal FA metabolism and/or oxidative stress (Lee et al. 1995; Nakajima et al. 2004; Okiyama et al. 2009; Kanbe et al. 2014). It is speculated that the HC diet suppresses *de novo* hepatic cholesterol synthesis and increases a key molecule for FA/TG synthesis, acetyl-CoA, resulting in the intrahepatic accumulation of TG. Moreover, the HC diet induces oxidative stress. Thus, the steatohepatitis induced in the HC diet-fed *Ppara*-null mice might be the result of their abnormal FA/TG metabolic ability and the decreases of anti-oxidative and anti-inflammatory effects, as well as other cases of steatohepatitis (Matsuzawa et al. 2007; Komatsu et al. 2015; Tanaka et al. 2017, Hu et al. 2017).

The inflammatory process can activate inflammatory cells in the liver, resulting in the production of large amounts of coagulation factors including TF, PAI-1, and CPB2, in the damaged

cells. Furthermore, the expression of these coagulation factors are reported to be partially regulated by PPARα (Marx et al. 2001; Kimura et al. 2012; Masuda et al. 2012; Kanbe et al. 2014; Gonsalves et al. 2015). Therefore, the levels of these coagulation factors would increase in the liver of the HC diet-fed *Ppara*-null mice. In addition to these intrahepatic changes, feeding with the HC diet might lead to higher levels of circulating coagulation factors because of damage to vascular endothelial cells through the increased inflammation. In addition, PPARα is expressed in vascular endothelial cells of the systemic circulation (Lefebvre et al. 2006) and can suppress the release of coagulation factors from these cells, which might contribute to the enhanced levels of circulating coagulation factors in the *Ppara*-null mice.

Lower liver and serum levels of sulfatides in the HC diet-fed Ppara-null mice

The current study is the first to report that the HC diet greatly reduces serum levels of sulfatides by suppressing expression of the rate-limiting enzyme for sulfatide synthesis, CST, and that this effect is enhanced under PPARα deficiency. It has been reported that oxidative stress decreases CST expression, and that PPARα regulates the CST expression at transcriptional level (Kamijo et al. 2012; Kimura et al. 2012; Yuzhe et al. 2015). In *Ppara*-null mice, the increased HC diet induced-oxidative stress and the lower ability to maintain CST expression lead to markedly lower CST expression and consequent reduction in serum sulfatide levels. Moreover, this is the first study

to show that the HC diet decreases the expression of an intracellular transporter of sulfatides, GLTP, which would further contribute to reduce serum sulfatides levels.

PPARα-specific function against cholesterol toxicity

In this study, the induction of TG accumulation, oxidative stress, and inflammation in the liver, as well as that of blood levels of coagulation factors, in mice fed with the HC diet, were more marked in *Ppara*-null mice than in WT individuals. Since the mRNA expression and functional activation of PPARα were not change in the HC diet-fed WT mice, and those of PPARδ and γ were also unchanged in the both mouse strains, the presence of a certain level of PPARα seems to be important to suppress the toxicity by the HC diet described above.

Prior studies have reported that constitutive expression of PPARα is maintained by the presence of endogenous ligands such as FAs, which is important for the maintenance of basal FA metabolism in liver and heart (Aoyama et al. 1998, Watanabe et al. 2000). In the case of kidney dysfunction, PPARα expression becomes lower, which is insufficient to maintain its basic functions (Kamijo et al. 2007). As shown in this study, cholesterol toxicity is significantly enhanced in the absence of PPARα. Thus, maintenance of a certain level of PPARα and its function is important to protect against cholesterol toxicity.

Clinical applications of the present findings

High blood levels of TF and PAI-1 have been reported to indicate a high risk of CVD (Toschi et al. 1997; Steffel et al. 2006; Kohler and Grant 2000; Hamsten et al. 1987). The current study has shown for the first time that constitutive level of PPARα expression attenuates the pro-thrombotic changes induced by cholesterol overload. These data suggest that maintaining PPARα function by using a specific agonist would ameliorate and prevent CVD. Indeed, the results of several large scale randomized controlled trials using PPARα agonists, fibrates, have indicated the possibility that they could reduce the risk of CVD (Davis et al. 2011; Ansquer et al. 2005). In addition to its lipid lowering effects, the beneficial preventive effect of PPARα agonism might be derived from the other effects described in the current study. Telmisartan and Irbesartan, which are angiotensin II receptor blockers (ARBs), promote hepatic and renal PPARα expression (Harada et al. 2016; Clemenz et al. 2008), and many clinical studies using these ARBs have demonstrated protective effects against CVD (Yusuf et al. 2008; Brenner et al. 2001). This type of ARB may not only lower blood pressure or suppress the renin-angiotensin-aldosterone system, but may also attenuate cholesterol toxicity by modulating PPARα function. The findings in this study suggest a novel molecular mechanism to explain the existing clinical evidence of CVD risk suppression by these agents. Further investigation of the anti-thrombotic effects of PPARα agonism is required to confirm this.

Limitations of the study

We only examined TF, PAI-1, CPB2 and sulfatides in the many types of pro- and anti-thrombotic factors and found the PPARα-specific regulation in these factors. Because we did not evaluate other factors or thrombotic activity directly, additional investigations will be necessary in the future. However, we believe that our present findings, which demonstrate that cholesterol overload causes pro-thrombotic effects and that PPARα is important in preventing such effects, should provoke further studies of PPARα agonism as a novel therapeutic/preventive strategy for CVD.

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Figure captions

Fig 1 Effects of a high cholesterol (HC) diet in wild type (WT) and *Ppara*-null mice

(A) Effects on total cholesterol (TC) content in the liver and (B) in serum induced by HC diet in WT (+/+) and *Ppara*-null (−/−) mice. (C) Effect on triglyceride (TG) content in liver. (D) Photomicrographs of liver sections stained with hematoxylin and eosin. Arrow indicates inflammatory cell infiltration, suggesting steatohepatitis. Scale bar = 100 μm. (E) Effect on serum level of ALT. (F) Effect on serum tissue factor (TF) concentration, (G) effects on plasma carboxypeptidase B2 (CPB2), and (H) plasminogen activator inhibitor-1 (PAI-1). Black bars indicate control groups and white bars indicate HC diet-fed groups. Values are mean ± SD (n=6 for each group). Data were compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 2 Effects of diet on hepatic mRNA and protein expression of representative coagulation factors

(A-C) Effect on liver mRNA levels of *Tf*, *Cpb2* and *Pai-1* of HC diet in WT (+/+) and *Ppara*-null (−/−) mice. (D-G) Effect on liver protein levels of tissue factor (TF), carboxypeptidase B2 (CPB2), and plasminogen activator inhibitor-1 (PAI-1). Black bars indicate control groups (Con) and white bars indicate HC diet-fed groups (Cho). Values are mean ± SD (n=6 for each group). Data were

compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 3 Effects of diet on sulfatide content of liver and serum

(A) Effect on sulfatide levels in liver, and (B) serum of HC diet in WT (+/+) and *Ppara*-null (−/−) mice. Black bars indicate control groups (Con) and white bars indicate HC diet-fed groups (Cho). (C) Composition of sulfatides in the liver of each group. Values are mean ± SD (n=6 for each group). Data were compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 4 Effects of diet on hepatic mRNA and protein expression of sulfatide metabolizing enzymes

(A-D) Effect on hepatic mRNA expression of *Cst, Arsa, Cgt* and *Galc* of HC diet in WT (+/+) and *Ppara*-null (−/−) mice. (E-I) Effect on protein expression of CST, ARSA, CGT, and GALC. Black bars indicate control groups and white bars indicate HC diet-fed groups. Values are mean ± SD (n=6 for each group). Data were compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 5 Effects of diet on hepatic mRNA and protein expression of sulfatide transport enzymes

(A-D) Effect on mRNA expression of *Fabp1, Gltp, Scp2,* and *Mttp* of HC diet in WT (+/+) and *Ppara*-null (−/−) mice. (E-I) Effect on protein expression of FABP1, GLTP, SCP2, and MTTP. Black bars indicate control groups (Con) and white bars indicate HC diet-fed groups (Cho). Values are mean ± SD (n=6 for each group). Data were compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 6 Effects of diet on oxidative markers and mRNA expression of oxidative stress-related enzymes

(A) Effect on liver protein level of 4-hydroxy-nonenal (HNE)-modified protein of HC diet in WT (+/+) and *Ppara*-null (−/−) mice. (B) Effect on MDA content in the liver. (C-H) Effect on mRNA expression of oxidative stress-related enzymes, *Ncf1, Cybb, Cat, Sod1, Sod2,* and *Gpx1*. Black bars indicate control groups (Con) and white bars indicate HC diet-fed groups (Cho). Values are mean ± SD (n=6 for each group). Data were compared between two groups using two-way ANOVA. Significant differences are indicated by *p <0.05 and **p<0.01.

Fig 7 Effects of diet on mRNA expression and DNA-binding activity of PPARs

(A) Effect on mRNA expression of *Ppara* (*A*), *Ppard* (*D*) and *Pparg (G*), of HC diet in WT (+/+)

and *Ppara*-null (−/−) mice. (B) Effect on the DNA-binding activity of PPARα, PPARδ, and PPARγ. Values are mean ± SD (n=6 for each group). Black, striped and white bars indicate PPARα, PPARδ, and PPARγ, respectively. Con and Cho indicates control and HC diet-fed groups, respectively.

Fig. 1

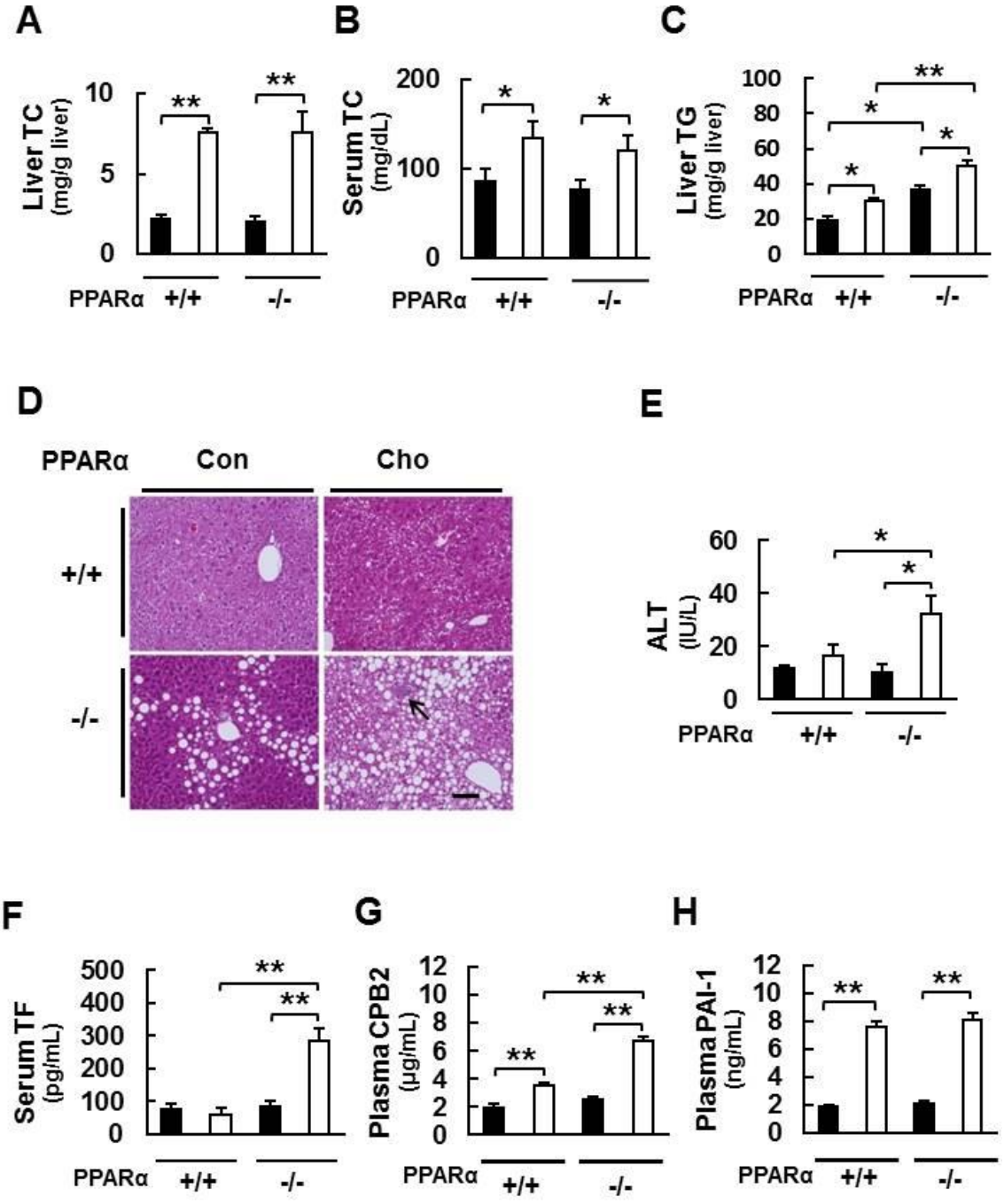

A
Liver TC (mg/g liver)
10
5
0
**
**
PPARα +/+ -/-
B
Serum TC (mg/dL)
200
100
0
*
*
PPARα +/+ -/-
C
Liver TG (mg/g liver)
100
80
60
40
20
0
*
*
*
**
PPARα +/+ -/-
D
PPARα
Con
Cho
+/+
-/-
E
ALT (IU/L)
60
40
20
0
*
*
PPARα +/+ -/-
F
Serum TF (pg/mL)
500
400
300
200
100
0
**
**
PPARα +/+ -/-
G
Plasma CPB2 (μg/mL)
12
10
8
6
4
2
0
**
**
**
PPARα +/+ -/-
H
Plasma PAI-1 (ng/mL)
12
10
8
6
4
2
0
**
**
PPARα +/+ -/-

Fig. 2

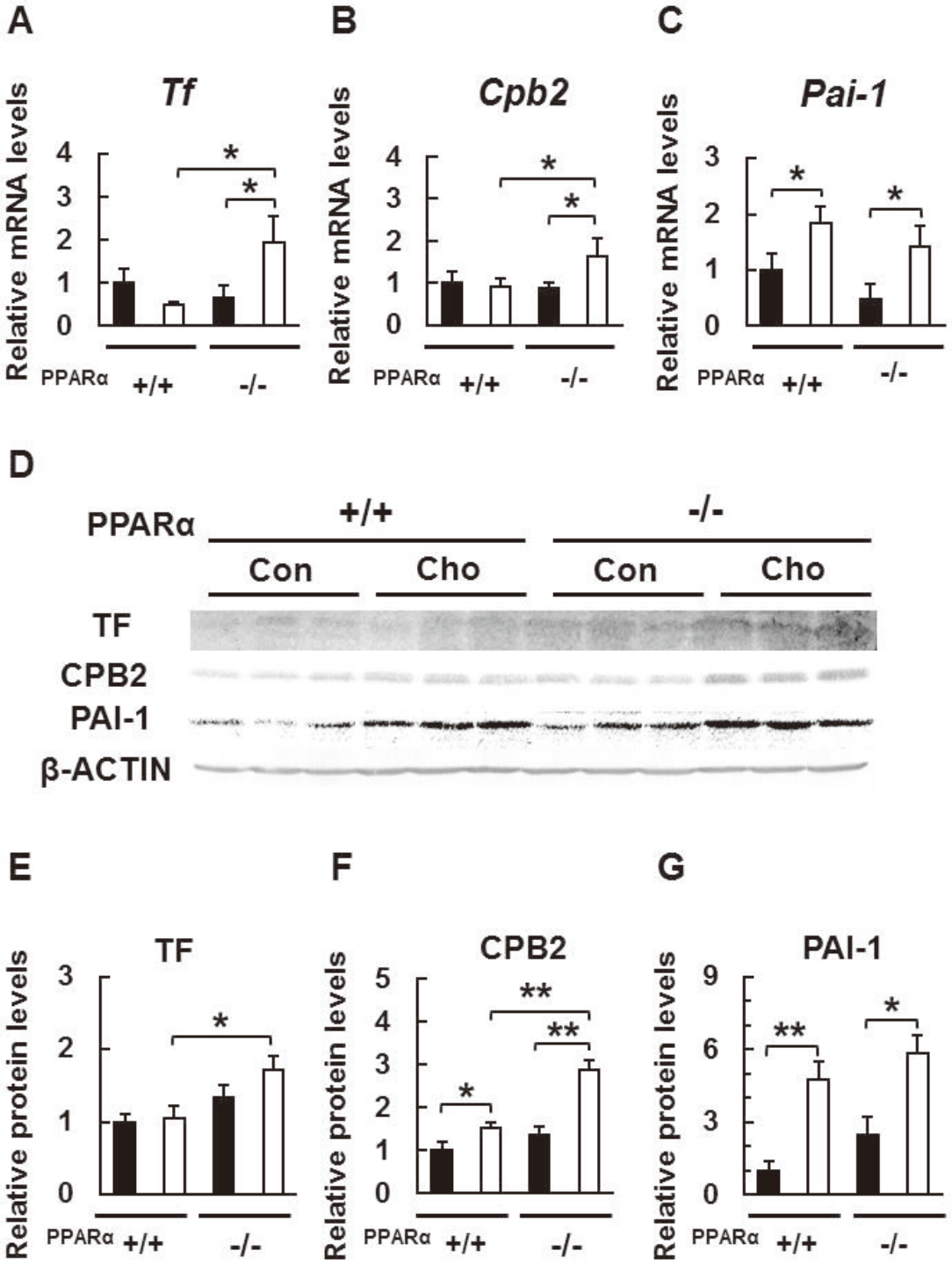

A
Tf
Relative mRNA levels
PPARα +/+ -/-
B
Cpb2
Relative mRNA levels
PPARα +/+ -/-
C
Pai-1
Relative mRNA levels
PPARα +/+ -/-
D
PPARα
+/+
-/-
Con
Cho
Con
Cho
TF
CPB2
PAI-1
β-ACTIN
E
TF
Relative protein levels
PPARα +/+ -/-
F
CPB2
Relative protein levels
PPARα +/+ -/-
G
PAI-1
Relative protein levels
PPARα +/+ -/-

Fig. 3

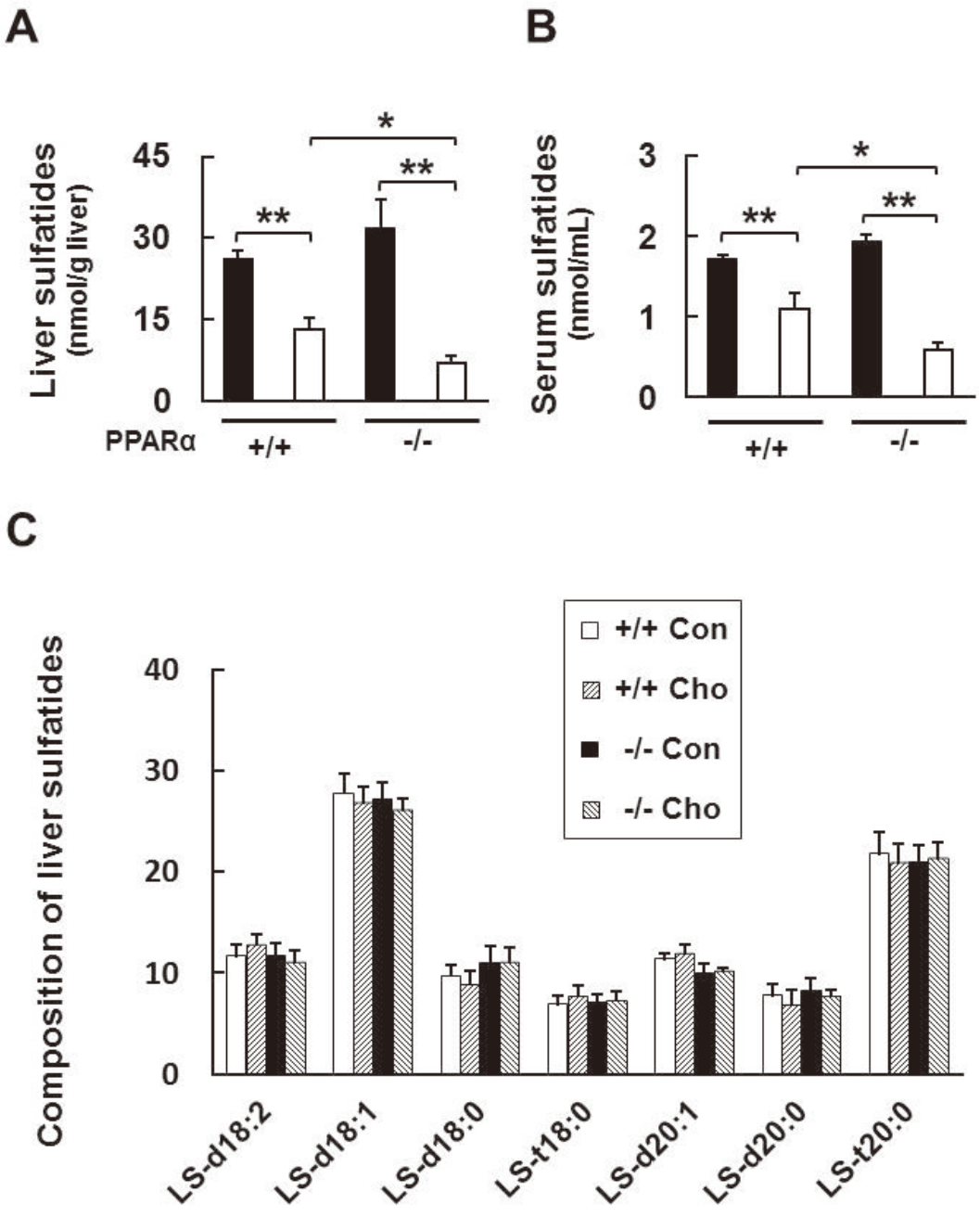
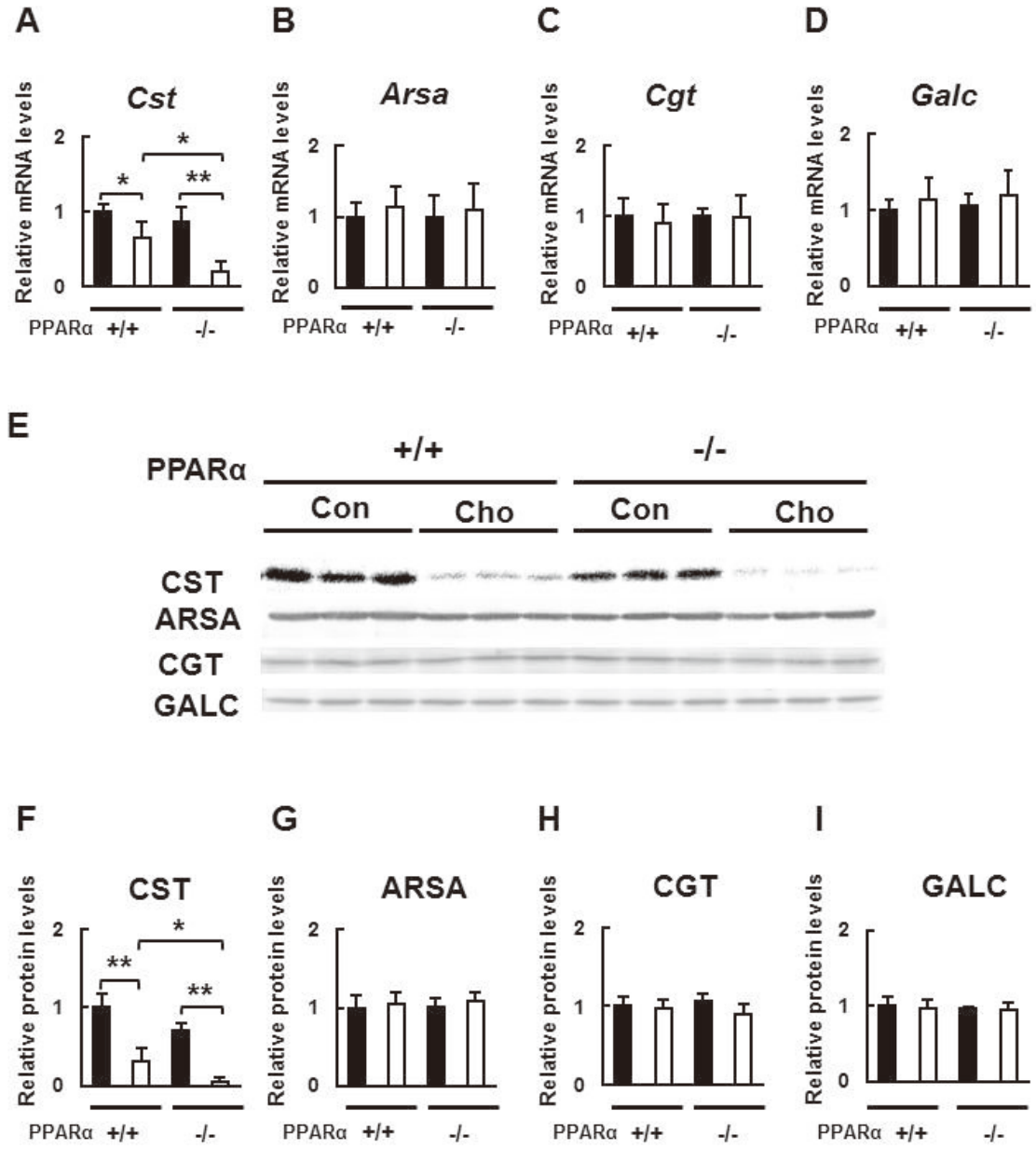

A
B
Liver sulfatides
(nmol/g liver)
45
30
15
0
*
**
**
PPARα
+/+
-/-
Serum sulfatides
(nmol/mL)
3
2
1
0
*
**
**
+/+
-/-
C
Composition of liver sulfatides
40
30
20
10
0
+/+ Con
+/+ Cho
-/- Con
-/- Cho
LS-d18:2
LS-d18:1
LS-d18:0
LS-t18:0
LS-d20:1
LS-d20:0
LS-t20:0

Fig. 4


A
Cst
Relative mRNA levels
2
1
0
*
*
**
PPARα +/+ -/-
B
Arsa
Relative mRNA levels
2
1
0
PPARα +/+ -/-
C
Cgt
Relative mRNA levels
2
1
0
PPARα +/+ -/-
D
Galc
Relative mRNA levels
2
1
0
PPARα +/+ -/-
E
PPARα
+/+
-/-
Con
Cho
Con
Cho
CST
ARSA
CGT
GALC
F
CST
Relative protein levels
2
1
0
**
*
**
PPARα +/+ -/-
G
ARSA
Relative protein levels
2
1
0
PPARα +/+ -/-
H
CGT
Relative protein levels
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1
0
PPARα +/+ -/-
I
GALC
Relative protein levels
2
1
0
PPARα +/+ -/-

Fig. 5

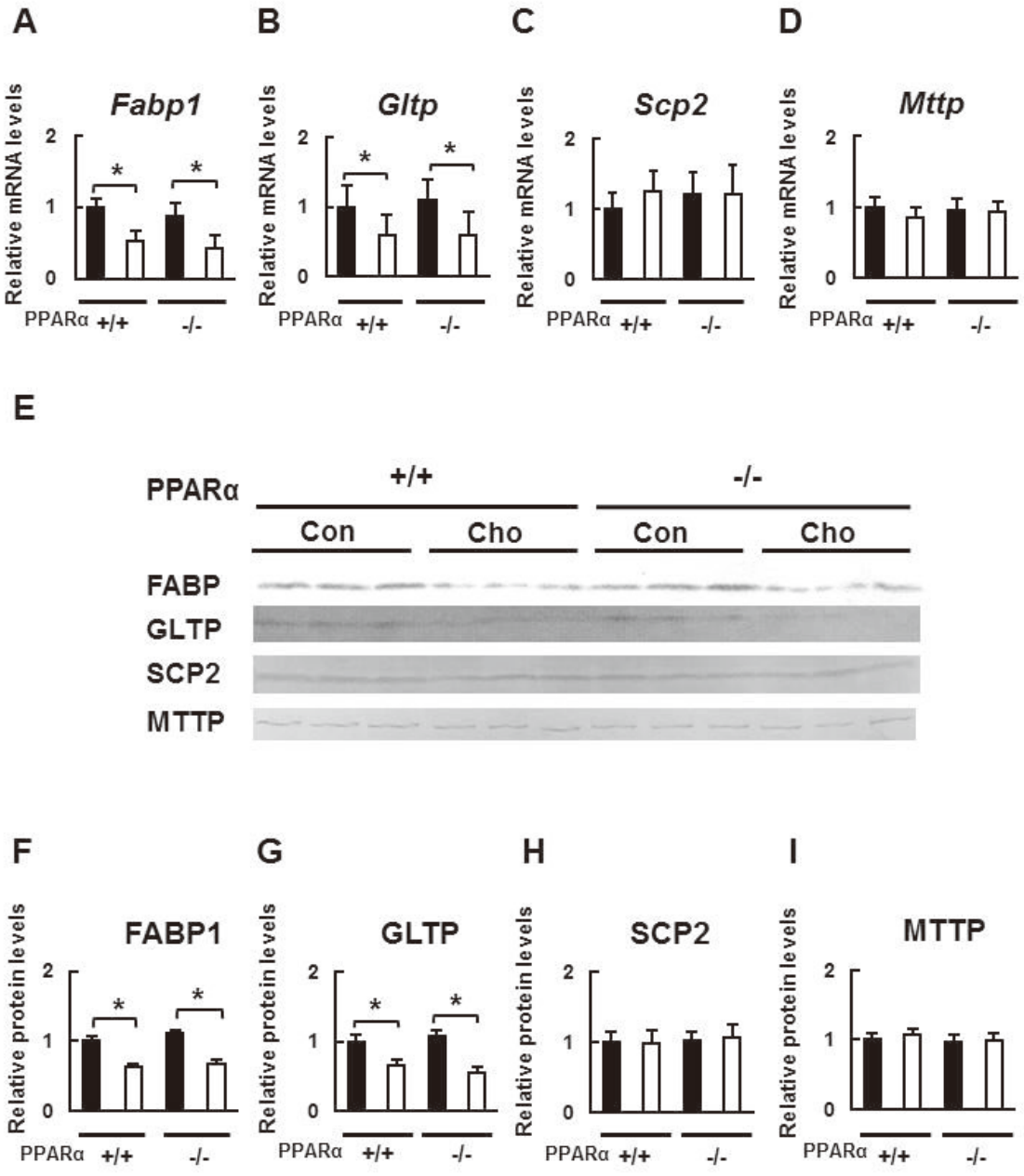

A
B
C
D
Fabp1
Gltp
Scp2
Mttp
Relative mRNA levels
PPARα +/+ -/-
E
PPARα
+/+
-/-
Con
Cho
Con
Cho
FABP
GLTP
SCP2
MTTP
F
G
H
I
FABP1
GLTP
SCP2
MTTP
Relative protein levels

Fig. 6

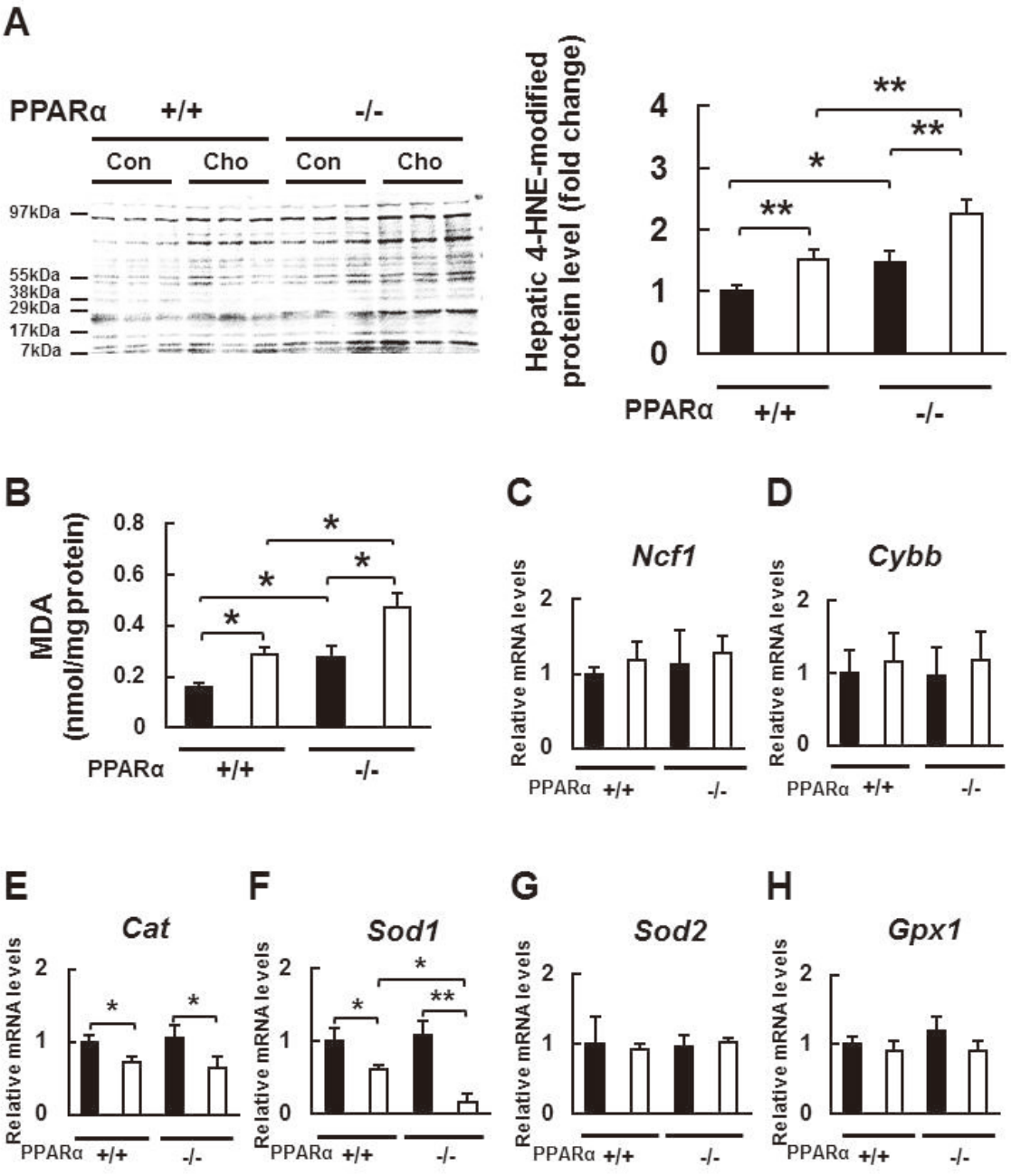
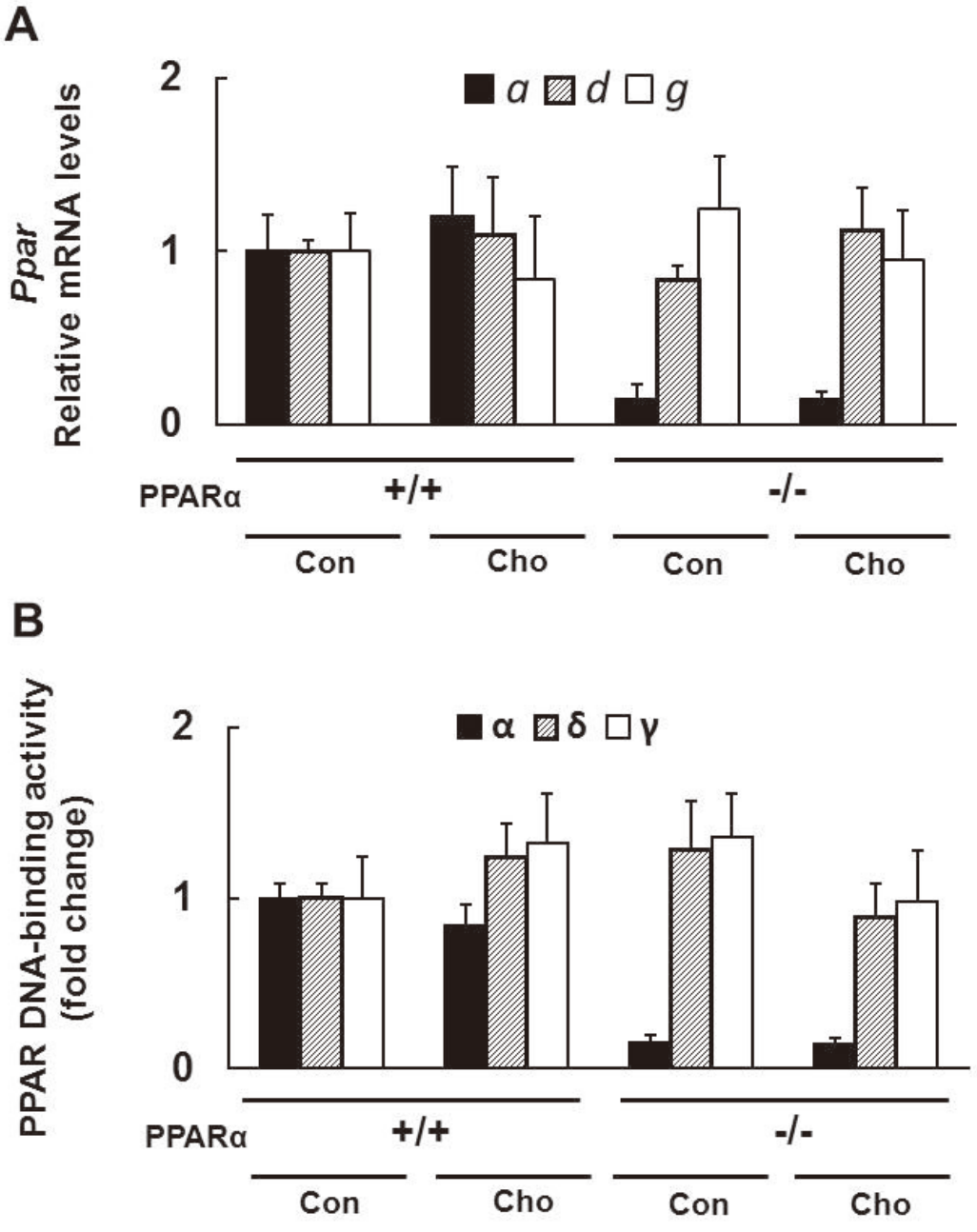

A
PPARα +/+ -/-
Con Cho Con Cho
97kDa
55kDa
38kDa
29kDa
17kDa
7kDa
Hepatic 4-HNE-modified protein level (fold change)
PPARα +/+ -/-
B
MDA (nmol/mg protein)
PPARα +/+ -/-
C
Ncf1
Relative mRNA levels
PPARα +/+ -/-
D
Cybb
Relative mRNA levels
PPARα +/+ -/-
E
Cat
Relative mRNA levels
PPARα +/+ -/-
F
Sod1
Relative mRNA levels
PPARα +/+ -/-
G
Sod2
Relative mRNA levels
PPARα +/+ -/-
H
Gpx1
Relative mRNA levels
PPARα +/+ -/-

Fig. 7


A
Ppar
Relative mRNA levels
2
1
0
a d g
PPARα
+/+
-/-
Con
Cho
Con
Cho
B
PPAR DNA-binding activity
(fold change)
2
1
0
α δ γ
PPARα
+/+
-/-
Con
Cho
Con
Cho