malignant potential of HCC with subsequent unfavorable prognosis after treatment [12–16]. However, there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities, such as hepatectomy and percutaneous ablative therapy.

The aim of this collaborative retrospective and prospective study was to evaluate the clinical usefulness of measuring AFP-L3 for prognostic predictor in patients with HCC after curative treatment.

Patients and Methods

Study Design

A total of 336 HCC patients underwent curative treatment at four participating hospitals (Niigata University Hospital, Ehime University Hospital, Shinsyu University Hospital, and Gunma University Hospital) from January 1998 to March 2005 and were investigated retrospectively. Of these patients, 232 underwent percutaneous ablative therapy and 104 underwent hepatectomy. Percutaneous ablative therapy comprised PEI in 90 patients, MCT in four patients, and RFA in 138 patients. Long-term survival data on these patients were confirmed as of the end of March 2005.

To evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we prospectively investigated 189 patients diagnosed with early stage HCC initially at four hospitals from April 2005 to October 2007. We considered patients who had multiple (up to three) tumors measuring 3 cm or less in diameter as having early stage HCC. Forty-eight of 189 patients were excluded in this study, as they were received transcatheter treatment. As a result, 141 HCC patients, 99 who underwent percutaneous ablative therapy and 42 who underwent hepatectomy, were enrolled in the prospective study. Percutaneous ablative therapy comprised PEI in ten patients, MCT in two patients, and RFA in 87 patients. In these 141 patients, HCC recurrence was assessed by imaging modalities every 3 or 4 months after treatment and recurrence free survival was evaluated as of the end of December 2007. Informed consent was obtained from each patient, and the study protocol conformed with the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in the a priori approval by our institution's human research committee.

Diagnosis of HCC and Laboratory Examination

In our study, the diagnosis was based essentially on imaging findings together with increments of tumor marker levels. We employed methods such as computed tomography (CT), magnetic resonance imaging, and CT during hepatic arteriography, considering hyperattenuation in the arterial phase with washout in the late phase to be a typical feature of HCC. In nine cases that showed atypical features on imaging, ultrasound-guided biopsies were performed.

Hepatic functional reserve was ranked by the criteria of the Child-Pugh scoring system. Serum alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were determined at each hospital by using commercially available kits. AFP-L3 percentage was measured at each hospital by liquid-binding assay (Wako Pure Chemical Industries Ltd, Osaka, Japan) [17]. AFP, AFP-L3, and DCP were measured in the same serum before treatment. Cut-off values for positivity for AFP, AFP-L3, and DCP were set at 20 ng/ml, 15%, and 40 mAU/ml, respectively, based on previous studies [18–20].

Treatment

Therapeutic modalities for individual patients were chosen according to hepatic functional reserve, tumor multiplicity, and tumor size. Percutaneous local ablative therapies were performed under a US-guided procedure, and its efficacy was evaluated with dynamic CT within a few days after treatment. Complete ablation of HCC was defined as non-enhancement of the lesion with surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete. During the study, a Cool-tip RF System attached to a 200-W power generator (Radionics, Burlington, Massachusetts, USA) was the main device used for RFA treatment and Microtaze OT-110M (Alfresa-Pharma Co., Inc., Osaka, Japan) was used for MCT.

Statistical Analysis

Differences in the proportions of the independent binary variables were determined by Fisher's exact test. Continuous variables were compared by Student's *t*-test. Univariate survival and recurrence-free survival were determined by the Kaplan–Meier method. Log-rank test was used to test for equality of long-term survival and recurrence-free survival between the groups. Multivariate analyses of prognostic factors in the clinical features were performed by using Cox's stepwise proportional hazard model. The factors included for multivariate analyses were patient age, gender (female/male), HBsAg (negative/positive), Anti-HCV (negative/positive), Child-Pugh class (A/B, C), AFP (ng/ml) ($<20/\geq 20$), DCP (mAU/ml) ($<40/\geq 40$), AFP-L3 (%) ($<15/\geq 15$), tumor size (cm) ($<3/\geq 3$ or $\leq 2/>2$), and number of tumors (single/multiple). Statistical analyses were performed with SPSS 15.0 software (SPSS Japan Inc. Tokyo, Japan). A *P*-value of less than 0.05 was considered as statistically significant.

Results

Retrospective Study

Clinical Features of Patients Classified by Therapeutic Modality

A total of 336 HCC patients who underwent hepatectomy and percutaneous ablative therapy were investigated retrospectively. Patients who underwent percutaneous ablative therapy were characterized by older age ($P < 0.05$), positivity for antibody to hepatitis C virus (anti-HCV) ($P < 0.05$), and advanced Child-Pugh classification ($P < 0.05$). In contrast, patients who underwent hepatectomy were characterized by positivity for hepatitis B surface antigen (HBsAg) ($P < 0.05$), AFP-L3 ($P < 0.05$), and DCP ($P < 0.05$) elevation, as well as large tumor size ($P < 0.05$). No significant differences were observed between the two groups in terms of gender, AFP level, or number of tumors (Table 1A).

Univariate and Multivariate Analyses of the Factors Predicting Long-Term Patient Survival

The median observation time after treatment was 38.3 months (range, 1.0–146.2 months). Of the 232 patients who underwent percutaneous ablative therapy, 172 were alive and 60 had died from HCC, hepatic failure, and/or complications of cirrhosis. Of the 104 HCC patients who underwent hepatectomy, 68 were alive and 36 had died. The median survival time was 69.0 months in patients who had undergone percutaneous ablative therapy and 114.9 months in those who had undergone hepatectomy.

In the univariate analysis, anti-HCV status ($P = 0.034$), AFP status ($P = 0.007$), AFP-L3 status ($P = 0.001$), tumor size ($P = 0.001$), and number of tumors ($P = 0.045$) were significant prognostic factors of long-term survival in patients who underwent percutaneous ablative therapy. AFP status ($P = 0.011$), tumor size ($P = 0.006$), and number of tumors ($P < 0.001$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Multivariate analysis by Cox's stepwise proportional hazard model revealed that tumor size ($P = 0.018$) and AFP-L3 status ($P = 0.013$) were significant independent prognostic factors for long-term survival in patients who underwent percutaneous ablative therapy. Tumor size ($P = 0.013$) and number of tumors ($P = 0.004$) were significant independent prognostic factors in patients who underwent hepatectomy (Table 3). We showed the long-term survival curves of two groups (with or without AFP-L3 elevation) in patients who underwent percutaneous ablative therapy and in those who underwent hepatectomy (Fig. 1). No significant difference in survival was observed

Table 1 Clinical features of patients with HCC classified by therapeutic modality in the retrospective and prospective studies

Variables	Percutaneous ablation ($n = 232$)	Hepatectomy ($n = 104$)
(A) Retrospective study		
Age (median, range)	68 (39–89)	65 (35–81)*
Gender		
Male	145 (62.5%)	66 (63.5%)
Female	87 (37.5%)	38 (36.5%)
HBsAg		
Negative	209 (90.1%)	73 (70.2%)
Positive	23 (9.9%)	31 (29.8%)*
Anti-HCV		
Negative	28 (12.1%)	45 (43.3%)
Positive	204 (87.9%)	59 (56.7%)*
Child-Pugh class		
A	177 (76.3%)	95 (91.3%)
B and C	55 (23.7%)	9 (8.7%)*
AFP (ng/ml)		
<20	65 (28.0%)	22 (21.2%)
≥20	167 (72.0%)	82 (78.8%)
DCP (mAU/ml)		
<40	149 (67.4%)	48 (51.1%)
≥40	72 (32.6%)	46 (48.9%)*
AFP-L3 (%)		
<15	181 (78.0%)	61 (58.7%)
≥15	51 (22.0%)	43 (41.3%)*
Tumor size (cm)		
<3	185 (79.7%)	33 (31.7%)
≥3	47 (20.3%)	71 (68.3%)*
Tumor number		
Single	148 (63.8%)	75 (72.1%)
Multiple	84 (36.2%)	29 (27.9%)
Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
(B) Prospective study		
Age (median, range)	69 (36–85)	65 (40–80)
Gender		
Male	66 (66.7%)	24 (57.1%)
Female	33 (33.3%)	18 (42.9%)
HBsAg		
Negative	85 (85.9%)	29 (69.0%)
Positive	14 (14.1%)	13 (31.0%)*
Anti-HCV		
Negative	27 (27.3%)	15 (35.7%)
Positive	72 (72.7%)	27 (64.3%)
Child-Pugh class		
A	79 (79.8%)	39 (92.9%)
B and C	20 (20.2%)	3 (7.1%)

Table 1 continued

Variables	Percutaneous ablation ($n = 99$)	Hepatectomy ($n = 42$)
AFP (ng/ml)		
<20	64 (64.6%)	22 (52.40%)
≥20	35 (35.4%)	20 (47.6%)
DCP (mAU/ml)		
<40	63 (63.6%)	27 (64.3%)
≥40	35 (35.4%)	15 (35.7%)
AFP-L3 (%)		
<15	85 (85.9%)	33 (78.6%)
≥15	14 (14.1%)	9 (21.4%)
Tumor size (cm)		
≤2	63 (63.6%)	27 (64.3%)
>2	36 (36.4%)	15 (35.7%)
Tumor number		
Single	78 (78.8%)	34 (81.0%)
Multiple	21 (21.2%)	8 (19.0%)

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. Percentages are shown in parentheses

* $P < 0.05$ between groups by Fisher's exact test and Student's *t*-test

between the two AFP-L3 groups in patients who underwent hepatectomy ($P = 0.308$). In contrast, patients in the ablative therapy group whose AFP-L3 levels were below 15% lived significantly longer than those whose values were more than 15% ($P = 0.001$).

Prospective Study

Clinical Features of Patients with Early Stage HCC Classified by Therapeutic Modality

A total of 141 patients with early stage HCC were evaluated prospectively. Patients who underwent hepatectomy were characterized by positive for hepatitis B surface antigen (HBsAg) ($P < 0.05$). No significant differences were observed in age, gender, anti-HCV positivity, AFP status, AFP-L3 status, DCP status tumor size, and number of tumors between the two groups. Patients who underwent percutaneous ablative therapies tended to have an advanced Child-Pugh classification ($P = 0.055$) (Table 1B).

Univariate and Multivariate Analysis of the Factors Predicting Recurrence-Free Survival in Patients with Early Stage HCC

The median follow-up time after treatment was 12.0 months (range, 1.0–30.5 months). Among the 99 patients who underwent percutaneous ablation, recurrences were observed in 36 (36.4%). Among the 42 patients who underwent hepatectomy, recurrences were observed in six (14.3%).

In the univariate analysis, we found no significant difference in recurrence-free survival rates by pretreatment variables in patients who underwent percutaneous ablation, although AFP-L3 elevation ($P = 0.054$) tended to decrease recurrence-free survival. In contrast, tumor size ($P = 0.038$) and number of tumors ($P = 0.034$) were significant prognostic factors in patients who underwent hepatectomy (Table 2).

Although this prospective study was conducted over a short period of time, multivariate analysis of prognostic factors among the clinical features was performed and Cox's stepwise proportional hazard model revealed that HBsAg status ($P = 0.033$), DCP status ($P = 0.011$), and AFP-L3 status ($P = 0.006$) were significant independent prognostic factors of recurrence-free survival in patients who underwent percutaneous ablative therapies. On the other hand, we found no significant independent prognostic factors in patients who underwent hepatectomy (Table 3).

We showed recurrence-free survival rates between two groups—with or without AFP-L3 elevation—among

Table 2 Univariate analysis of the factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Variables	Long-term survival		Recurrence-free survival	
	Percutaneous ablation *P*-value	Hepatectomy *P*-value	Percutaneous ablation *P*-value	Hepatectomy *P*-value
Gender (female/male)	0.907	0.525	0.225	0.194
HBsAg (negative/positive)	0.139	0.801	0.151	0.314
Anti-HCV (negative/positive)	0.034	0.963	0.194	0.171
Child-Pugh class (A/B,C)	0.083	0.235	0.293	0.487
AFP (ng/ml) (<20/≥20)	0.007	0.011	0.117	0.994
DCP (mAU/ml) (<40/≥40)	0.328	0.153	0.075	0.059
AFP-L3 (%) (<15/≥15)	0.001	0.308	0.054	0.530
Tumor size (cm) (<3/≥3)	0.001	0.006	0.063	0.038
Tumor number (single/multiple)	0.045	<0.001	0.667	0.034

HBsAg hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin. *P*-value was calculated using Log-rank test

Table 3 Multivariate analysis of factors predicting long-term survival in the retrospective study and recurrence-free survival in the prospective study for patients who underwent percutaneous ablation and in those who underwent hepatectomy

Long-term survival			Recurrence-free survival		
Variables	Hazard ratio (95% CI)	*P*-value	Variables	Hazard ratio (95% CI)	*P*-value
Percutaneous ablation			Percutaneous ablation		
AFP-L3 (%)			HBsAg		
<15	1		Negative	1	
≥15	2.098 (1.169–3.765)	0.013	Positive	2.823 (1.090–7.310)	0.033
Tumor size (cm)			DCP		
<3	1		<40 (mAU/ml)	1	
≥3	1.998 (1.123–3.553)	0.018	≥40 (mAU/ml)	2.767 (1.267–6.046)	0.011
			AFP-L3		
			<15 (%)	1	
			≥15 (%)	3.463 (1.437–8.347)	0.006
Hepatectomy			Hepatectomy		
Tumor size (cm)			Tumor number		
<3	1		Single	1	
≥3	6.162 (1.457–26.064)	0.013	Multiple	4.654 (0.936–23.149)	0.060
Tumor number					
Single	1				
Multiple	3.170 (1.442–6.921)	0.004			

Hazard ratio and *P*-value were calculated using Cox's stepwise proportional hazard model

CI confidence interval, *AFP* alpha-fetoprotein, *HBsAg* hepatitis B surface antigen, *DCP* des-gamma-carboxy prothrombin

patients with early stage HCC who underwent percutaneous ablation and patients who underwent hepatectomy (Fig. 1). No significant difference was observed between groups with or without AFP-L3 elevation ($P = 0.53$) in patients who underwent hepatectomy. In contrast, a close-to-significant ($P = 0.054$) difference was observed between the groups of patients with and without AFP-L3 elevation who underwent percutaneous ablative therapy.

In summary, the results of the retrospective and prospective studies demonstrated that AFP-L3 status was a statistically significant prognostic factor of long-term survival and recurrence-free survival in patients who underwent percutaneous ablative therapy, but did not affect prognosis in patients who underwent hepatectomy.

Discussion

AFP-L3, a fucosylated species of AFP, is the product of alpha 1-6 fucosyltransferase (FUT8) in the presence of GDP-fucose. Our previous result revealed that FUT8 levels in HCC tissue were higher than those in the surrounding non-cancerous tissues and that FUT8 levels of HCC tissue increased in accordance with tumor dedifferentiation [21]. Several reports have shown the relationship between AFP-L3 status and histologic grade in HCC. Miyaaki et al. [16] showed that the frequency of poorly differentiated HCC was significantly higher in AFP-L3-positive patients than in AFP-L3-negative patients. Oka et al. [14] reported that AFP-L3-positive HCC was characterized by portal vein invasion and poorer differentiation, and that tumors in AFP-L3-positive HCC were advanced, even if they were small and the patient had a low serum AFP concentration. These results indicate the relationship between increased AFP-L3 level and increased degree of malignant behavior of HCC tissue.

Recurrence after treatment is an important factor affecting prognosis. Vascular invasion is an established adverse prognostic indicator of recurrence of HCC [22, 23]. Yamashita et al. [24] suggested that portal vein invasion is associated with AFP-L3 positivity, and that there is a strong possibility of intrahepatic invasion when there is positive conversion of this marker. Hayashi et al. [13] reported the relationship between AFP-L3 status and pattern of recurrence in patients with HCC. In their report, intrahepatic metastasis was significantly more common in AFP-L3-positive patients than in negative patients, although the recurrence rate of multicentric tumors did not differ significantly between the two groups with or without AFP-L3 elevation. From this point of view, hepatectomy—especially anatomical resection, which can remove venous tumor thrombi together with the primary lesion—is more suitable than local ablative therapies for the treatment of AFP-L3-positive patients.

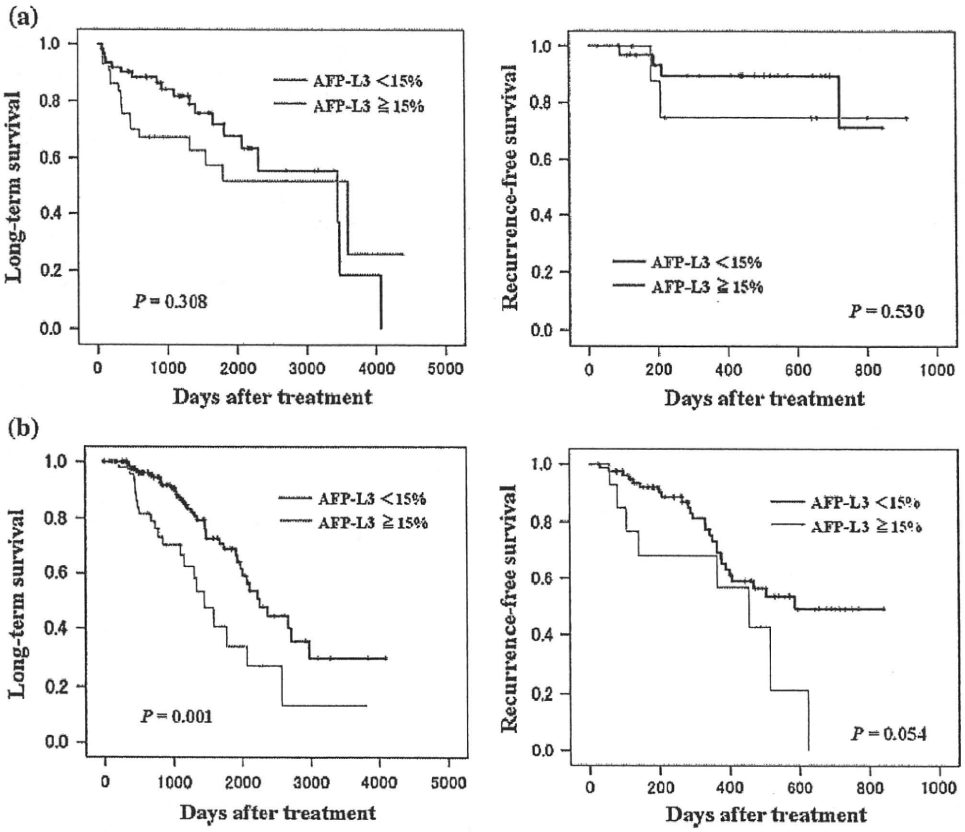

Fig. 1 Comparison of long-term survival rates and recurrence-free survival rates between patients with and without AFP-L3 elevation who underwent hepatectomy (**a**) and who underwent percutaneous ablation (**b**)

In our study, the pathological diagnosis was made by individual pathologists at each hospital. At Niigata University Hospital, 58 HCC patients underwent hepatectomy, of whom 23 had an elevated serum AFP-L3 level (≥15%) and the remaining 35 were negative for AFP-L3 (<15%). Among the 23 patients with AFP-L3 elevation, only two (8.7%) were diagnosed as having well-differentiated HCC on the basis of the resected specimens, 14 (60.9%) had moderately differentiated HCC, and seven (30.4%) had poorly differentiated HCC. In contrast, among the 35 patients who were negative for AFP-L3, 7 (20.0%) were diagnosed as having well-differentiated HCC, 24 (68.6%) had moderately differentiated HCC, and only four (11.4%) had poorly differentiated HCC. Although no statistically significant differences were observed by Fisher's exact test, the group showing AFP-L3 elevation tended to have a poorer histopathological grading ($P = 0.141$). Only eight out of 331 patients who underwent percutaneous ablative therapy were diagnosed as having HCC on the basis of histological findings in four hospitals. Therefore, we were unable to investigate whether poorly differentiated tumors were more frequent in the groups who underwent percutaneous ablative therapy and hepatectomy. Portal vein invasion was investigated similarly in 58 patients, and was found to be present in six of 23 AFP-L3-positive patients and six of 35 AFP-L3-negative patients. No significant difference was observed between AFP-L3 and portal vein invasion in this limited investigation.

We demonstrated here in a multicenter retrospective study that AFP-L3 status was a significant prognostic factor affecting the long-term survival of patients who underwent percutaneous ablative therapy. In addition, to evaluate the prognostic influence of AFP-L3 in two subgroups comparable for tumor extension, we performed a multicenter prospective study to identify the prognostic factors for recurrence-free survival in patients with early stage HCC. Although this evaluation was conducted over a short period of time, we confirmed that AFP-L3 status was a significant prognostic predictor of recurrence-free survival in patients who underwent percutaneous ablative therapy, but it did not affect the prognosis of patients who underwent hepatectomy.

A number of studies have shown that AFP-L3 status is an independent prognostic factor in patients with HCC [12, 13, 15]. We previously reported that AFP-L3-positive (>15%) patients had a lower survival rate than negative (<15%) patients in subgroups with a low serum AFP concentration. Moreover, the statistically significant differences were more distinct in the subgroups with lower AFP concentrations [20]. However, the patients in these studies had received various treatments such as hepatectomy, RFA, and transcatheter arterial embolization, and

there have been few reports of the relationship between AFP-L3 status and prognosis in subgroups of HCC patients receiving different therapeutic modalities. Tateishi et al. [15] demonstrated that pre-treatment AFP-L3 positivity (>15%) was a significant predictor of HCC recurrence in patients who underwent curative ablation, and that AFP-L3 positivity after ablation was the strongest predictor of HCC recurrence by multivariate analysis. Although their study was performed in only one center and did not evaluate long-term survival, their results are compatible with ours.

Treatment of HCC patients with cirrhosis faces a dilemma in that minimization of damage to noncancerous liver tissue improves long-term survival, but incomplete treatment of subsequent HCC recurrences results in a poor prognosis. Accordingly, if a useful indicator of choice of therapeutic modality were to be available before the initial therapy, there would be several advantages in not only the treatment, but also the follow-up, of patients with HCC.

In conclusion, present results revealed that AFP-L3 had different impacts on prognosis in patients with HCC who underwent percutaneous ablative therapy and hepatectomy. Although this study was not a randomized control trial, AFP-L3 might be a promising scale to improve the prognostic estimate and appraisal of therapeutic outcome in patients with HCC.

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Eicosapentaenoic acid improves hepatic steatosis independent of PPARα activation through inhibition of SREBP-1 maturation in mice

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ABSTRACT

Eicosapentaenoic acid (EPA) in fish oil is known to improve hepatic steatosis. However, it remains unclear whether such action of EPA is actually caused by peroxisome proliferator-activated receptor α (PPARα) activation. To explore the contribution of PPARα to the effects of EPA itself, male wild-type and *Ppara*-null mice were fed a saturated fat diet for 16 weeks, and highly (>98%)-purified EPA was administered in the last 12 weeks. Furthermore, the changes caused by EPA treatment were compared to those elicited by fenofibrate (FF), a typical PPARα activator. A saturated fat diet caused macrovesicular steatosis in both genotypes. However, EPA ameliorated steatosis only in wild-type mice without PPARα activation, which was evidently different from numerous previous observations. Instead, EPA inhibited maturation of sterol-responsive element-binding protein (SREBP)-1 in the presence of PPARα through down-regulation of SREBP cleavage-activating protein and site-1 protease. Additionally, EPA suppressed fatty acid uptake and promoted hydrolysis of intrahepatic triglycerides in a PPARα-independent manner. These effects were distinct from those of fenofibrate. Although fenofibrate induced NAPDH oxidase and acyl-coenzyme A oxidase and significantly increased hepatic lipid peroxides, EPA caused PPARα-dependent induction of superoxide dismutases, probably contributing to a decrease in the lipid peroxides. These results firstly demonstrate detailed mechanisms of steatosis-ameliorating effects of EPA without PPARα activation and ensuing augmentation of hepatic oxidative stress.

1. Introduction

Recent lifestyle alterations, such as increased consumption of saturated fats and decreased physical activity, have raised the prevalence of obesity, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD) [1,2]. Nonalcoholic steatohepatitis (NASH) is the progressive type of NAFLD and may develop into cirrhosis, liver cancer, and ultimately death [1–4]. Since NAFLD is also associated with a high susceptibility to atherosclerosis and ischemic heart disease [3,5], the increased prevalence of NAFLD is becoming a pressing issue worldwide. Thus, establishment of strategies to treat and prevent NAFLD and related metabolic disturbances is required.

Eicosapentaenoic acid (EPA) is one of the major components of n-3 polyunsaturated fatty acids (PUFA) preferentially contained in fish oil. From the first report of high EPA levels in the diet and blood of the Greenland Inuit [6], who rarely exhibit atherosclerotic diseases, numerous epidemiological and clinical studies have been

Abbreviations: ACC, acetyl-CoA carboxylase; ALT, alanine aminotransferase; apo, apolipoprotein; AOX, acyl-CoA oxidase; AST, aspartate aminotransferase; CoA, coenzyme A; CPT-I, carnitine palmitoyl-CoA transferase-I; EPA, eicosapentaenoic acid; FA, fatty acid; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; FF, fenofibrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; GPx, glutathione peroxidase; 4-HNE, 4-hydroxynonenal; HTGL, hepatic triglyceride lipase; Insig, insulin-induced gene product; LACS, long-chain acyl-CoA synthase; L-FABP, liver fatty acid-binding protein; LXR, liver X receptor; MCAD, medium-chain acyl-CoA dehydrogenase; MDA, malondialdehyde; mRNA, messenger RNA; MTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NEFA, non-esterified fatty acid; NL, neutral lipase; Nrf2, nuclear factor-E2-related factor 2; PGC, PPARγ coactivator; PMP, peroxisomal membrane protein; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SCAP, SREBP cleavage-activating protein; S1P, site-1 protease; SD, standard deviation; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; TG, triglyceride; TNF, tumor necrosis factor.

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Table 1
Changes in anthropometric and biochemical parameters from a 16-week saturated fat diet.

Genotype	*Ppara* (+/+)		*Ppara* (−/−)	
Diet	Con ($n=6$)	Sat ($n=6$)	Con ($n=6$)	Sat ($n=6$)
Body weight (g)	23.9 ± 1.9	28.3 ± 1.5*	26.5 ± 2.7	41.0 ± 5.2**,##
Liver/body weight (%)	3.8 ± 0.2	4.6 ± 0.4*	4.4 ± 0.2	5.2 ± 0.6*
Epididymal fat/body weight (%)	2.5 ± 0.3	3.7 ± 0.6*	3.0 ± 1.5	6.1 ± 0.5**,##
Serum TG (mg/dL)	61 ± 1	123 ± 41*	124 ± 50	233 ± 49**,##
Serum NEFA (mEq/L)	0.75 ± 0.30	1.33 ± 0.3*	1.19 ± 0.25	1.54 ± 0.15#
Serum glucose (mg/dL)	92 ± 23	89 ± 24	98 ± 14	103 ± 22
Serum insulin (ng/mL)	0.51 ± 0.09	1.21 ± 0.58	0.48 ± 0.06	2.24 ± 0.46**
Serum AST (U/L)	129 ± 66	243 ± 62	149 ± 92	203 ± 46
Serum ALT (U/L)	13 ± 6	43 ± 16*	18 ± 10	99 ± 21**,#
Liver TG (mg/g)	10 ± 1	30 ± 3**	17 ± 3	52 ± 7**,##

Results are expressed as mean ± SD. Con, control standard diet; Sat, saturated fat diet; TG, triglyceride; NEFA, non-esterified fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
* $P<0.05$ compared with mice of the same genotype fed a control diet.
** $P<0.01$ compared with mice of the same genotype fed a control diet.
# $P<0.05$ compared with *Ppara* (+/+) mice fed the same diet.
$P<0.01$ compared with *Ppara* (+/+) mice fed the same diet.

undertaken to show the efficacy of n-3 PUFA and EPA on reducing serum triglyceride (TG) concentrations and preventing cardiovascular events [7–9]. Some data on the steatosis-ameliorating effect of n-3 PUFA have also been obtained [10,11], creating the intriguing possibility that EPA might be beneficial for the treatment of NAFLD.

It has been considered that n-3 PUFA exhibited TG-reducing effects through regulation of peroxisome proliferator-activated receptor α (PPARα) and sterol regulatory element-binding protein (SREBP)-1, which control hepatic fatty acid (FA) catabolism and synthesis, respectively [12]. PPARα is a nuclear receptor expressed primarily in the liver and is involved in not only FA/TG metabolism,

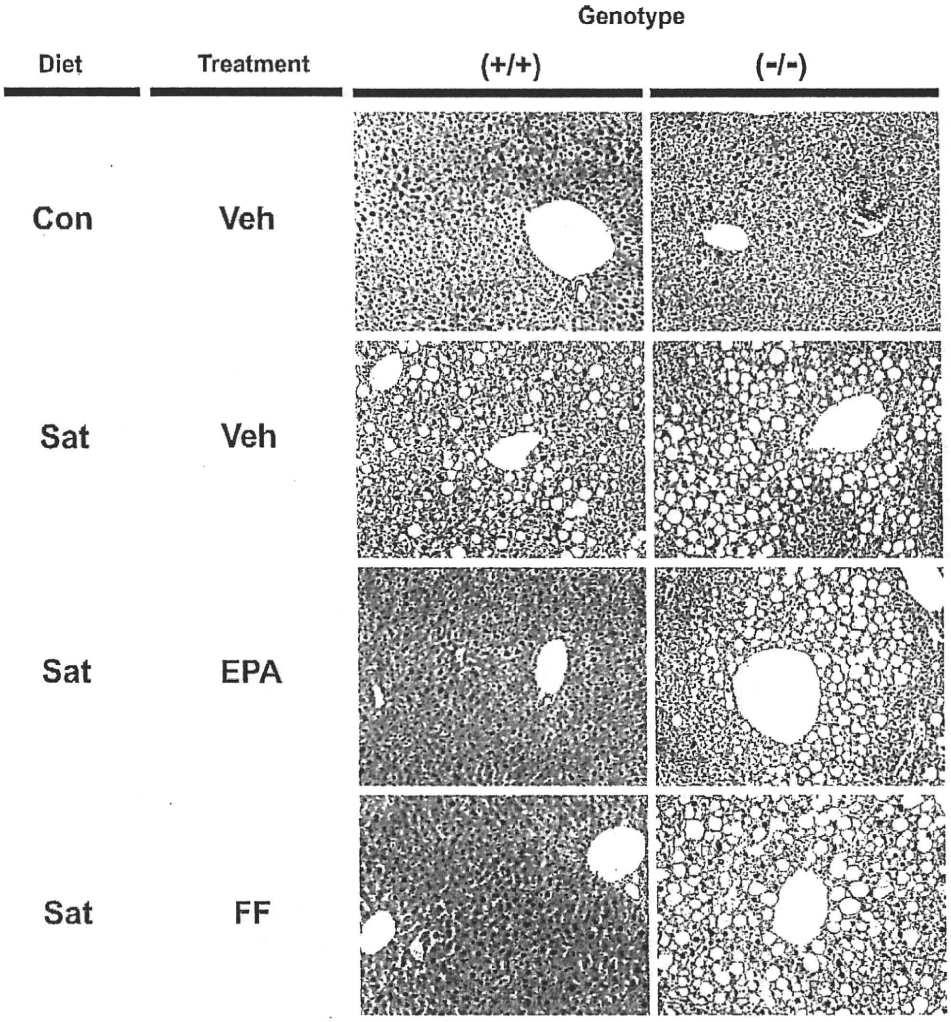

Fig. 1. Histological findings in the livers of wild-type and *Ppara*$^{-/-}$ mice. Male 8-week-old wild-type (+/+) and *Ppara* (−/−) mice were fed a control standard (Con) or saturated fat diet (Sat) for 16 weeks. After 4 weeks on the saturated fat diet, treatment with highly-purified EPA or FF was initiated and continued for 12 weeks. Liver sections were stained by hematoxylin and eosin method. Original magnification, 200×. Veh, vehicle.

but also cell proliferation and inflammatory response [13]. SREBP is a transcription factor belonging to the basic helix–loop–helix leucine zipper family. Depending on various intracellular signals, SREBP is cleaved by several proteases and the N-terminus region is transferred into the nucleus as a mature protein where it regulates transcription of several target genes. SREBP-1 plays an important role in the development of NAFLD, and insulin and synthetic agonists of liver X receptor α (LXRα) enhance its transcription [14].

Several studies have demonstrated an activating effect of EPA on PPARα [15–20]. However, because most of these studies were *in vitro* experiments [15–17] and used crude fish oil [18–20], it has not been determined whether EPA alone can activate PPARα *in vivo* as well. Additionally, since fish oil is reported to lower serum TG levels even in PPARα-null (*Ppara*$^{-/-}$) mice [21], it remains unclear whether such action occurs via PPARα activation. In order to clarify the contribution of PPARα to the effects of EPA *in vivo*, highly (>98%)-purified EPA was administered to wild-type and *Ppara*$^{-/-}$ mice fed a saturated fat diet, and the expression of genes and proteins involved in hepatic FA/TG metabolism was investigated. Furthermore, the changes caused by EPA treatment were compared to those elicited by fenofibrate (FF), a typical PPARα activator.

2. Materials and methods

2.1. Mice and treatment

Ppara$^{-/-}$ mice on a 129/Sv genetic background (129S4/SvJae) were generated as described previously [22]. Male 8-week-old wild-type and *Ppara*$^{-/-}$ mice weighing 24 ± 5 g (n = 30 in each genotype) were maintained in pathogen-free conditions at constant humidity and temperature with a light/dark cycle of 12 h. At the beginning of this study, mice in each genotype were randomly divided into 5 groups (n = 6/group) and pair-fed a diet. As a control, one group was treated with a standard diet for 16 weeks composed of 20.0% (per weight basis) casein, 53% corn starch, 10% sucrose, 7% olive oil, 5% cellulose, 3.5% mineral mix, 1% vitamin mix, and 0.25% choline. The other 4 groups were fed a saturated fat diet (Oriental Yeast Co. Ltd., Tokyo, Japan) in which all fat contained in the standard diet was completely hydrogenated to eliminate the effects of naturally contained PUFA. We chose 16 weeks as a saturated fat diet feeding period, since our preliminary experiments demonstrated that this protocol could induce obvious hepatic steatosis not only in *Ppara*$^{-/-}$ mice but also in wild-type mice. Body weight and food intake were recorded every day. After 4 weeks on the saturated fat diet, administration of the test agents was initiated in the 4 groups. One group was given highly (>98%)-purified EPA ethyl ester (ethyl all-cis-5, 8, 11, 14, 17-icosapentaenoate) (Mochida Pharmaceutical Co., Ltd, Tokyo, Japan) (1000 mg/kg of body weight/day) for 1 week, one group was given highly (>98%)-purified EPA ethyl ester at the same dose for 12 weeks, and another group was administered FF (Wako Pure Chemicals Industries, Osaka, Japan) (25 mg/kg of body weight/day) for 12 weeks. EPA and FF were dissolved in Arabic gum, mixed, and administered once a day at 10 a.m. by gastric gavage. The last test group was given the same amount of Arabic gum as a vehicle for 12 weeks. After the administration periods, the mice were anesthesized and sacrificed in a fasting state for collection of livers and blood. All experiments were conducted in accordance with the animal study protocols outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and approved by the Animal Studies Committee at Shinshu University School of Medicine.

2.2. Immunoblot analysis

Preparation of hepatocyte nuclear fractions was carried out as described previously [23,24]. Protein concentration was measured

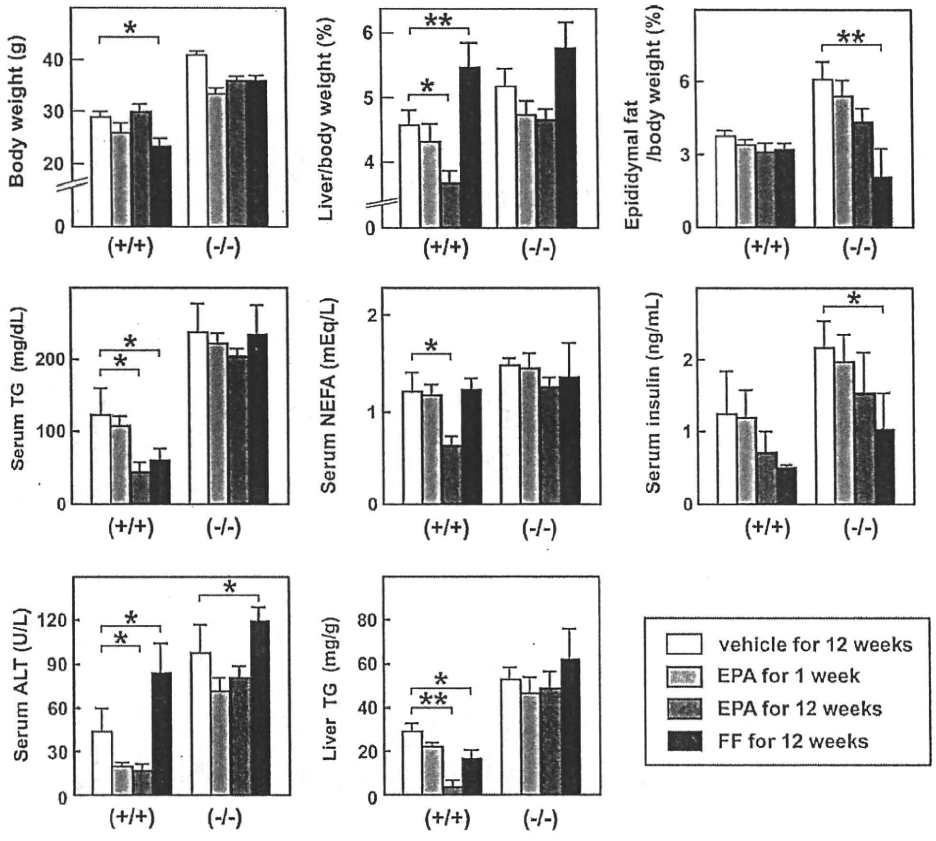

Fig. 2. Effects of EPA and FF on anthropometric and biochemical parameters. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

colorimetrically with a BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). Whole liver lysates (20–80 μg of protein) or nuclear fractions (80 μg of protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [23–28]. A sample from one mouse in each group was loaded into each electrophoresis assay and all samples were examined (n = 6/group). After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with primary antibody followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The antibodies against FA-metabolizing enzymes were described previously [28], and those against other proteins were purchased commercially. Suppliers and dilutions of primary antibodies are summarized in Supplementary Table 1. Actin or histone H1 was used as the loading control. Band intensities were measured densitometrically, normalized to those of actin or histone H1, and subsequently expressed as fold-changes of those of control wild-type mice fed a saturated fat diet. For confirmation of data reproducibility, immunoblot analysis using the same samples was done twice. Overall, the data on 12 band intensities were obtained from each mouse group for each target molecule and subjected to statistical analysis.

2.3. Analysis of mRNA

Total liver RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan), and cDNA was generated by SuperScript II reverse transcriptase (Gibco BRL, Paisley, Scotland). Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed as described elsewhere [29,30] with the primer pairs listed in Supplementary Table 2. Measured mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and subjected to statistical analysis.

2.4. Measurement of hepatic lipids and lipid peroxides

Total hepatic lipids were extracted according to the method developed by Folch et al. [31]. Lipid extracts were dissolved in distilled water, and the concentrations of TG and lipid peroxides

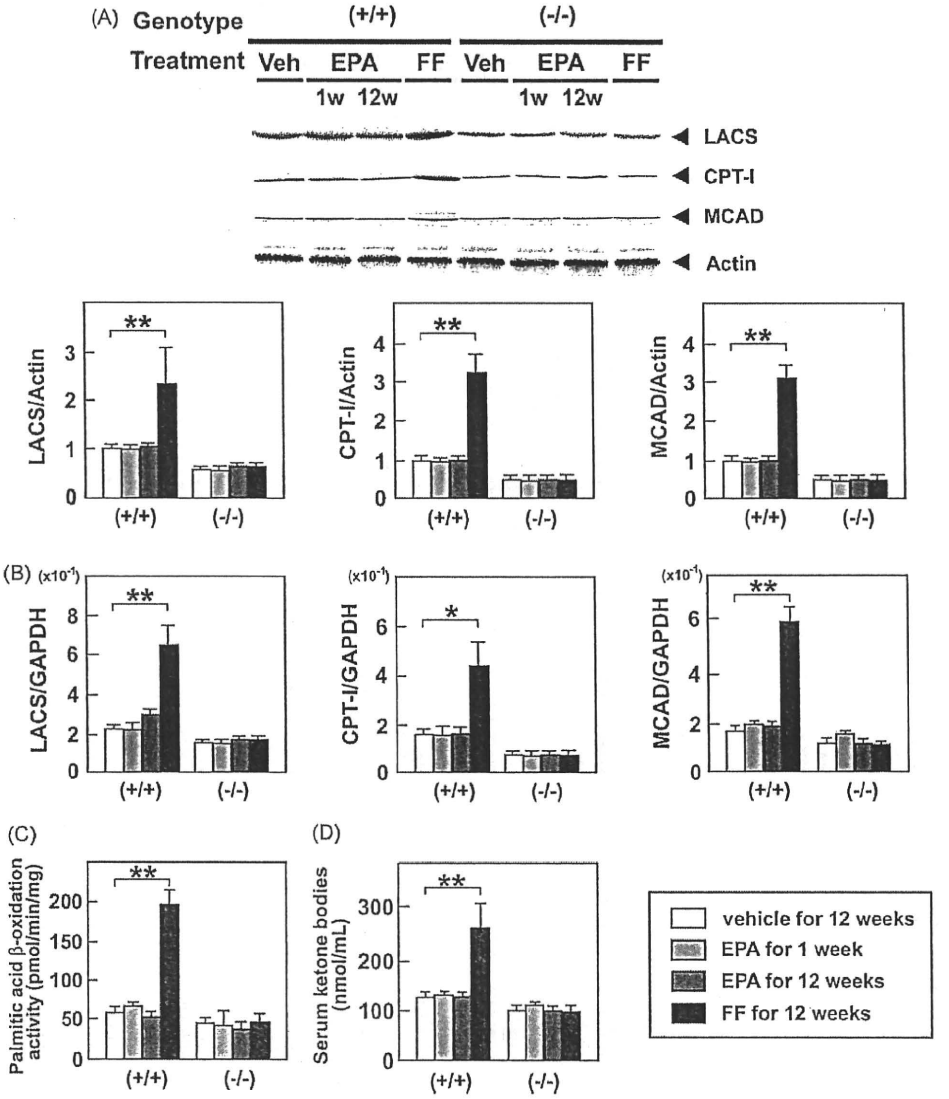

Fig. 3. Immunoblot analysis of LACS, CPT-I, and MCAD. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and whole liver lysates (20 μg of protein) obtained from mice were loaded into each well. Band intensities were measured densitometrically, normalized to those of actin, and subsequently normalized to those in control wild-type mice [(+/+)Veh]. Results are expressed as mean ± SD (n = 6/group). $^{**}P < 0.01$. (B) Hepatic mRNA levels of LACS, CPT-I, and MCAD. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and mRNA levels were determined by quantitative RT-PCR. Measured mRNA levels were normalized to those of GAPDH. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in mitochondrial β-oxidation activity in the liver. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and β-oxidation activity was measured using palmitic acid as a substrate. Results are expressed as mean ± SD (n = 6/group). $^{**}P < 0.01$. (D) Serum concentrations of ketone bodies. Figure presentation is identical to that in Fig. 2.

[the sum of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)] were colorimetrically measured using a TG C-test (Wako Pure Chemicals Industries) and LPO-586 kit (OXIS International, Portland, OR, USA), respectively [32].

2.5. Assessment of FA β-oxidation and uptake abilities

FA β-oxidation activity was measured according to a method described previously with palmitic acid as a substrate, and expressed as pmol/min/mg of liver tissue [28]. FA uptake ability was assessed as described elsewhere and expressed as a fold-change of that in control wild-type mice fed a saturated fat diet [33].

2.6. Assay of hepatic neutral lipase (NL) activity

Fresh liver samples (approximately 100 mg) were homogenized in 20 mM phosphate buffer (pH 7.5) containing 250 mM sucrose and 1 mM EDTA, sonicated, and centrifuged at 20,000 × g for 10 min at 25 °C. NL activity of the supernatant was colorimetrically measured using a MONOTEST (Boehringer Mannheim, Tokyo,

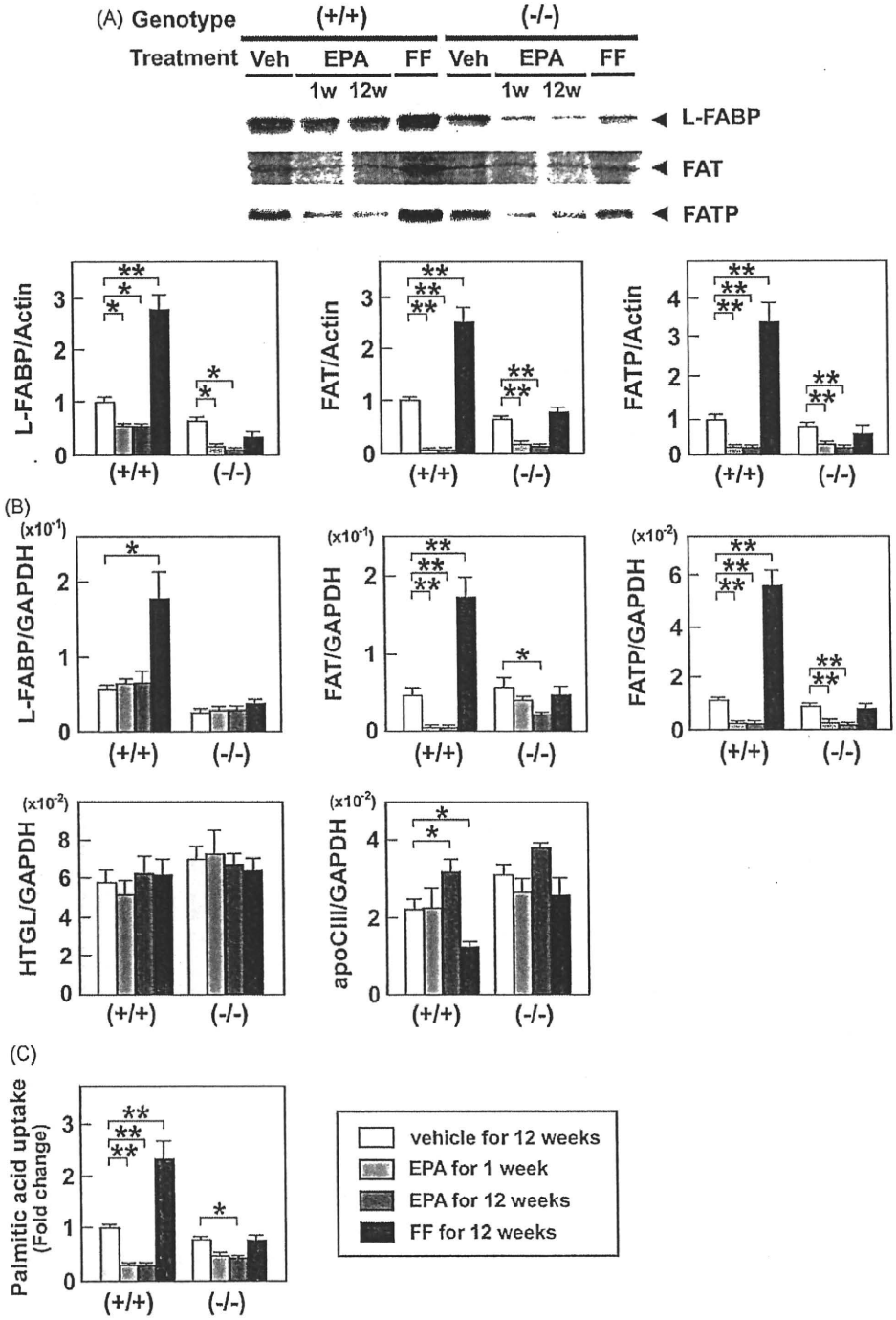

Fig. 4. Effects of EPA and FF on the hepatic FA uptake system. (A) Immunoblot analysis of L-FABP, FAT, and FATP. The same samples used in Fig. 3A were loaded. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of molecules associated with FA uptake. The same samples in Fig. 3B were adopted. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in palmitic acid uptake ability into the liver. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and FA uptake activity was assessed as described in Methods. Results are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

Japan) after adjusting the pH of the substrate-containing buffer to 7.2 and expressed as mU/mg of supernatant protein. Triolein was used as a substrate.

2.7. Histological examination

Small blocks of liver tissue from each mouse were fixed in 10% formalin in phosphate-buffered saline and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin or Azan-Mallory method. The procedure for cytochemical staining for peroxisomal catalase was described elsewhere [24].

2.8. Other assays

Serum levels of TG, non-esterified FA (NEFA), glucose, aspartate and alanine aminotransferases (AST and ALT), and ketone bodies were determined with commercial kits purchased from Wako Pure Chemicals Industries. Serum insulin concentrations were mea-

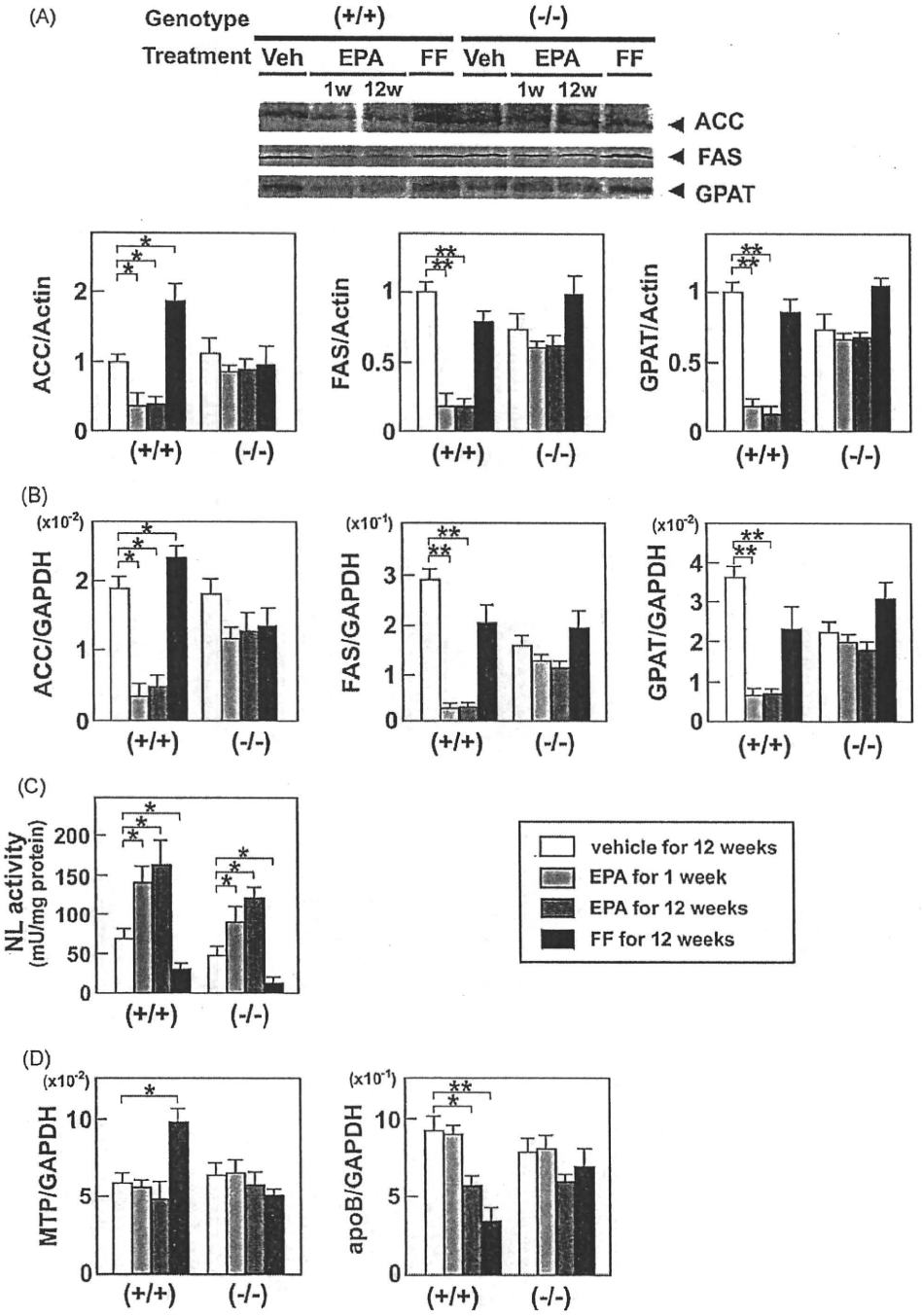

Fig. 5. Effects of EPA and FF on hepatic *de novo* lipogenesis and the TG hydrolysis and secretion pathway. (A) Immunoblot analysis of ACC, FAS, and GPAT. Whole liver lysates (40–80 μg of protein) obtained from wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were loaded into each well. Band intensities were measured densitometrically, normalized to those of actin, and subsequently normalized to those in control wild-type mice [(+/+) Veh]. Results are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of lipogenic enzymes. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Changes in hepatic NL activity. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, highly-purified EPA for 1 week or 12 weeks, or FF for 12 weeks, and hepatic NL activity was determined as described in Methods. Results are expressed as mean ± SD ($n = 6$/group). $^{*}P < 0.05$. (D) Hepatic mRNA levels of molecules associated with TG secretion. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$.

sured using a mouse insulin ELISA KIT (AKRIN-011T, Shibayagi, Gunma, Japan).

2.9. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the two-tailed Student's *t*-test. A probability value of less than 0.05 was considered statistically significant. All calculations were performed with SPSS version 11.0 software for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. A saturated fat diet induces hepatic steatosis in mice

The phenotypic changes caused by a 16-week saturated fat diet are shown in Table 1. At the endpoint, body weight, ratios of liver-to-body weight and epididymal fat-to-body weight, serum TG and ALT concentrations, and hepatic TG contents were all significantly increased by the saturated fat diet. These changes were observed in both genotypes, but were more prominent in *Ppara*$^{-/-}$ mice. Histological analysis revealed that the saturated fat diet induced mild-to-moderate macrovesicular steatosis mainly in zone 3 in both genotypes, but ballooned hepatocytes, lobular inflammation, or Mallory hyaline were not found (Fig. 1).

3.2. EPA ameliorates hepatic steatosis in a PPARα-dependent manner

FF treatment for 12 weeks decreased body weight and serum/liver TG levels in wild-type mice only, but significantly increased serum ALT levels in both genotypes (Fig. 2). When EPA was administered for 1 week, there were no remarkable changes in either genotype. However, EPA treatment for 12 weeks significantly decreased liver-to-body weight ratio, serum levels of TG, NEFA, and ALT, and hepatic TG content in wild-type mice without decreases in body weight or epididymal fat weight (Fig. 2). Histologically, EPA markedly attenuated macrovesicular

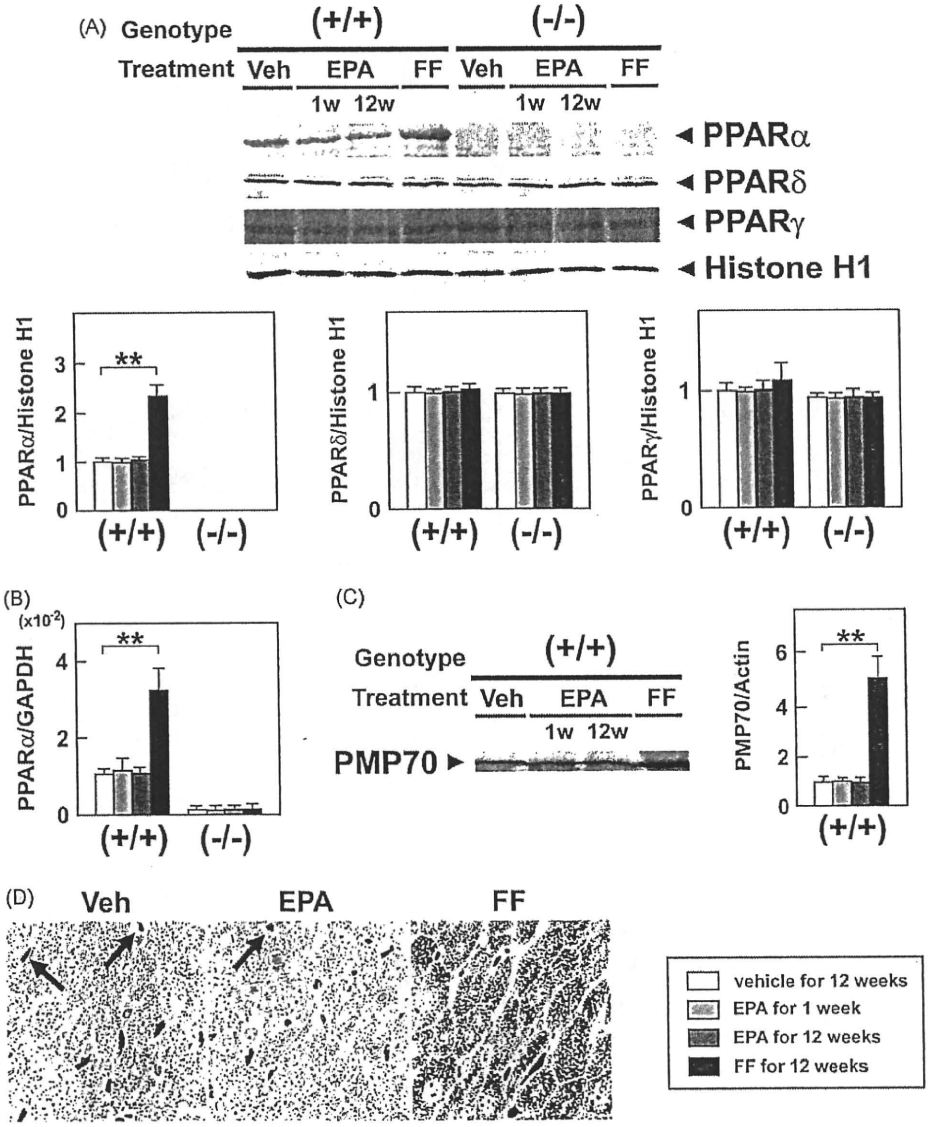

Fig. 6. Effects of EPA and FF on hepatic PPAR. (A) Immunoblot analysis of PPAR. Hepatocyte nuclear fractions (80 μg of protein) obtained from wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were loaded into each well. Band intensities were measured densitometrically, normalized to those of histone H1, and subsequently normalized to those in control wild-type mice [(+/+) Veh]. Results are expressed as mean ± SD (n = 6/group). $^{**}P < 0.01$. (B) Hepatic mRNA levels of PPARα. The same samples in Fig. 3B were used. $^{**}P < 0.01$. (C) Immunoblot analysis of PMP70, a typical PPARα target. The same samples used in Fig. 3A were loaded. $^{**}P < 0.01$. (D) Cytochemical staining for hepatic peroxisomes. Liver tissues were obtained from saturated fat diet-fed wild-type mice treated with a vehicle (Veh), highly-purified EPA, or FF for 12 weeks and subjected to cytochemical staining for peroxisomal catalase. Peroxisomes are seen as darkly-stained particles. Arrows indicate erythrocytes. Original magnification, 400×.

steatosis in wild-type mice only (Fig. 1). These results confirmed that EPA ameliorated saturated fat diet-induced steatosis and hypertriglyceridemia preferentially in wild-type mice.

3.3. EPA does not enhance mitochondrial β-oxidation

To investigate the precise mechanism of the PPARα-dependent steatosis-attenuating effect of EPA, we first analyzed hepatic β-oxidation pathway. FF markedly increased the expression of representative mitochondrial β-oxidation enzymes [long-chain acyl-CoA synthase (LACS), carnitine palmitoyl-CoA transferase-1 (CPT-I), and medium-chain acyl-CoA dehydrogenase (MCAD)] at the protein and mRNA levels (Fig. 3A and B), enhanced mitochondrial β-oxidation activity (Fig. 3C), and elevated serum ketone body concentrations (Fig. 3D) in wild-type mice only. However, these changes were not observed in the EPA-treated mice (Fig. 3), suggesting that the effect of EPA on hepatic steatosis was not derived from enhancement of mitochondrial β-oxidation.

3.4. EPA suppresses FA uptake in a PPARα-independent manner

FF markedly increased the levels of liver fatty acid-binding protein (L-FABP), fatty acid translocase (FAT), and fatty acid transport protein (FATP) and lowered apolipoprotein CIII (apoCIII) in wild-type mice only (Fig. 4A and B). In contrast, EPA significantly increased the levels of mRNA encoding apoCIII (Fig. 4B), and strongly decreased the expression of L-FABP, FAT, and FATP in a PPARα-independent manner (Fig. 4A). A reduction in the latter two proteins was also confirmed by quantitative RT-PCR analysis (Fig. 4B). Furthermore, EPA significantly suppressed palmitic acid uptake into hepatocytes (Fig. 4C). Both agents did not affect the mRNA levels of hepatic TG lipase (HTGL) (Fig. 4B).

3.5. EPA suppresses de novo lipogenesis in a PPARα-dependent manner

FF increased the expression of acetyl-CoA carboxylase (ACC) in wild-type mice, but had no impact on the expression of fatty acid

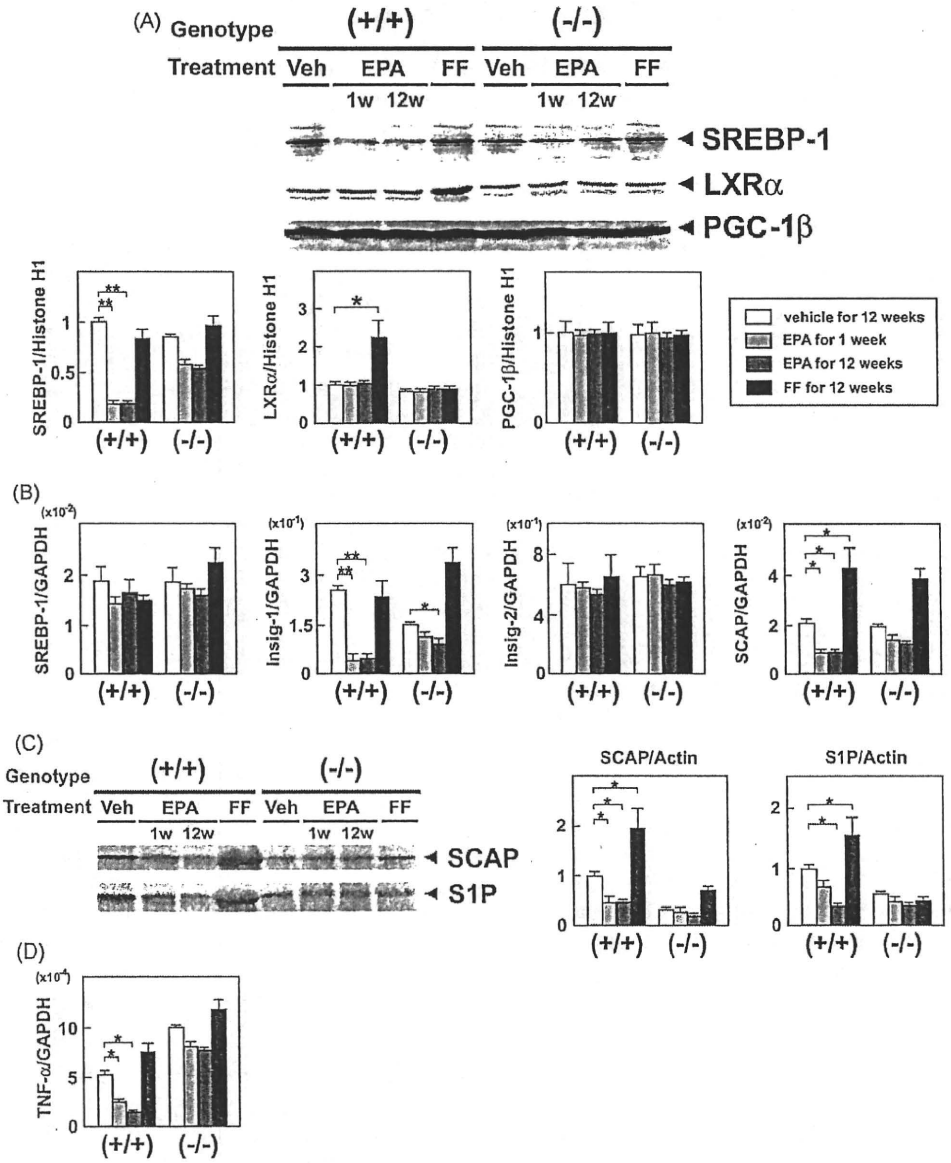

Fig. 7. Effects of EPA and FF on hepatic SREBP-1. (A) Immunoblot analysis of SREBP-1 and its co-regulators, LXRα and PGC-1β. The same samples used in Fig. 6A were loaded. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of SREBP-1 and its activating proteins. The same samples in Fig. 3B were used. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) Immunoblot analysis of SCAP and S1P. The same samples in Fig. 3A were loaded. $^{*}P < 0.05$. (D) Hepatic mRNA levels of TNF-α. The same samples in Fig. 3B were used. $^{*}P < 0.05$.

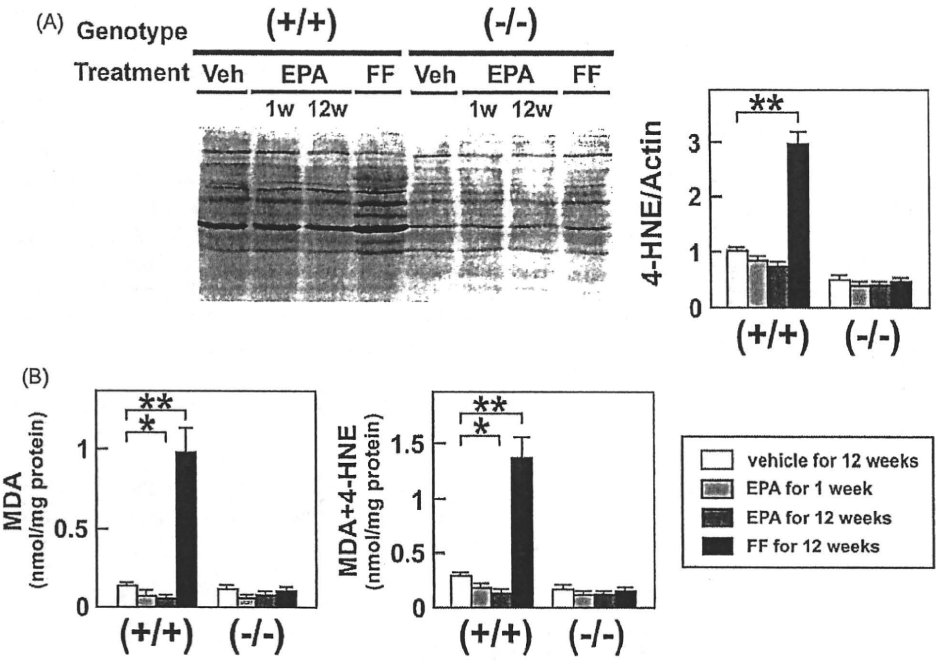

Fig. 8. Analysis of hepatic lipid peroxide content. (A) Immunoblot analysis of 4-HNE. The same samples in Fig. 3A were loaded. $^{**}P < 0.01$. (B) Hepatic levels of MDA and MDA + 4-HNE. Wild-type (+/+) and *Ppara* (−/−) mice fed a saturated fat diet were treated with a vehicle for 12 weeks, EPA for 1 week or 12 weeks, or FF for 12 weeks. Total lipids were extracted from mouse liver tissues and the amounts of MDA and MDA + 4-HNE were determined. Results are expressed as mean ± SD (n = 6/group). $^{*}P < 0.05$; $^{**}P < 0.01$.

synthase (FAS) or glycerol-3-phosphate acyltransferase (GPAT) (Fig. 5A and B). On the other hand, EPA markedly decreased the expression of these three enzymes in wild-type mice (Fig. 5A and B). These results showed that EPA inhibited *de novo* lipogenesis in a PPARα-dependent fashion.

3.6. Effects of EPA on the hepatic TG degradation and secretion pathway

In contrast to FF, EPA significantly enhanced NL activity in both genotypes (Fig. 5C), indicating that it strongly facilitated hydrolysis of intrahepatic TG. EPA did not affect the expression of microsomal TG transfer protein (MTP) or apoB (Fig. 5D).

3.7. EPA does not activate PPARα

Although EPA lowered hepatic TG levels in a PPARα-dependent manner, it did not increase the nuclear or mRNA levels of PPARα (Fig. 6A and B), induce expression of representative PPARα target genes [LACS, CPT-I, MCAD (Fig. 3), L-FABP, FAT, FATP (Fig. 4), or peroxisomal membrane protein 70 (PMP70) (Fig. 6C)], or cause hepatic peroxisome proliferation (Fig. 6D), all of which were distinct from the actions of FF. These results provide compelling evidence that the steatosis-ameliorating effect of EPA is not through PPARα activation. In addition, EPA did not influence the nuclear levels of the other PPARs: PPARδ and PPARγ (Fig. 6A).

3.8. EPA suppresses SREBP-1 maturation in a PPARα-dependent manner

EPA significantly decreased the mature SREBP-1 levels only in hepatocyte nuclei of wild-type mice, but did not affect the levels of LXRα and PPARγ coactivator-1β (PGC-1β), known to be involved in SREBP-1 regulation [34] (Fig. 7A). Decreases in mature SREBP-1 levels correlated with those in SREBP-1 target genes, including ACC, FAS, and GPAT (Fig. 5A and B). However, EPA did not lower SREBP-1 mRNA levels (Fig. 7B), suggesting that EPA modulated the expression of SREBP-1 at the post-transcriptional level. When factors associated with SREBP-1 maturation were examined, the expression of SREBP cleavage-activating protein (SCAP) and site-1 protease (S1P) were significantly decreased by EPA in a PPARα-dependent manner (Fig. 7B and C). The levels of mRNA encoding insulin-induced gene product (Insig)-2, another SREBP-1-activating molecule expressed exclusively in the liver, remained unchanged by EPA treatment (Fig. 7B). Collectively, these results demonstrated that EPA inhibited maturation of SREBP-1 in the presence of PPARα through down-regulation of SCAP and S1P.

A recent study has shown that the expression of SCAP was induced by pro-inflammatory cytokines [35]. The mRNA levels of tumor necrosis factor-α (TNF-α) were decreased in EPA-treated wild-type mice only (Fig. 7D).

3.9. EPA reduces hepatic oxidative stress

Persistent PPARα activation may increase generation of reactive oxygen species (ROS) [33,36]. As expected, FF markedly increased hepatic lipid peroxide content (Fig. 8) and the expression of ROS-generating enzymes, such as NADPH oxidase ($gp91^{phox}$ and $p47^{phox}$) and acyl-CoA oxidase (AOX), in wild-type mice only (Fig. 9). On the other hand, EPA reduced hepatic lipid peroxides in wild-type mice (Fig. 8), likely due to increased expression of manganese- and copper, zinc-superoxide dismutases (Mn- and Cu, Zn-SOD) (Fig. 9). EPA did not affect the expression of glutathione peroxidase (GPx) (Fig. 9). Thus, EPA can ameliorate fatty liver without activation of PPARα and ensuing augmentation of hepatic oxidative stress.

4. Discussion

The present study demonstrated detailed mechanisms of steatosis-attenuating effects of highly-purified EPA in mice, which were unexpectedly unrelated to PPARα activation. They can be summarized as follows: (1) suppression of SREBP-1 processing, (2) suppression of FA uptake from the blood into hepatocytes, and (3)

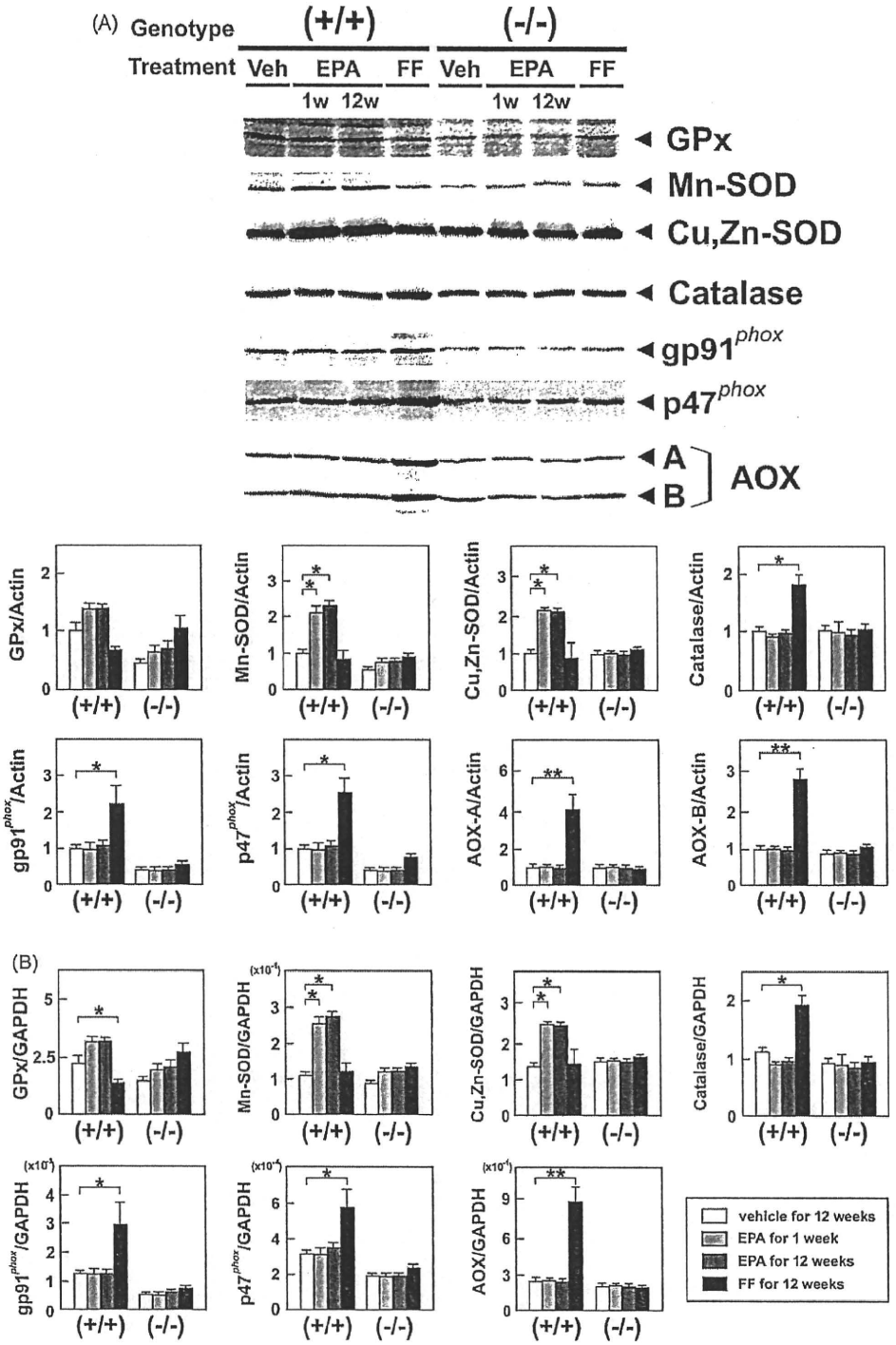

Fig. 9. Effects of EPA and FF on hepatic ROS-scavenging and ROS-generating enzymes. (A) Immunoblot analysis of ROS-scavenging and ROS-generating enzymes. Whole liver lysates (40–80 μg of protein) were loaded into each well. The same samples used in Fig. 3A were loaded. A and B bands of AOX, full-length and truncated AOX, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Hepatic mRNA levels of ROS-scavenging and ROS-generating enzymes. The same samples in Fig. 3B were adopted. $^{*}P < 0.05$; $^{**}P < 0.01$.

enhancement of intrahepatic TG hydrolysis. Interestingly, down-regulation of SCAP and S1P and the resultant suppression of SREBP-1-mediated pathway by EPA occurred in a PPARα-dependent manner. These actions clearly differed from those of FF, a typical PPARα activator.

We uncovered that highly-purified EPA cannot singularly activate PPARα in mice. This fact is quite different from the results of the previous *in vivo* studies using fish oil [10,18–20]. This discrepancy probably stems from PPARα-activating properties of constituents of fish oil other than EPA. Indeed, tuna oil, in which docosahexaenoic acid is dominant, has been shown to be a much stronger PPARα activator than EPA in mice [37]. Thus, it is conceivable that pure EPA may possess pharmacological actions distinct from crude fish oil.

Furthermore, Ishii et al. [38] have reported that EPA-supplemented diet increased the expression of PPARα in *Pten*-deficient mice, which is inconsistent with the present results. EPA contained in the diet is easily oxidized at room temperature and several types of oxidized EPA derivatives are generated. Sethi et al. [39] have demonstrated that oxidized EPA markedly activated PPARα and its

PPARα-activating properties were approximately 2.5-fold stronger than native non-oxidized EPA and as potent as fenofibric acid in bovine aortic endothelial cells. Based on these observations, we suppose that such a discrepancy in PPARα expression may result from the difference in the amount of oxidized EPA.

In the preliminary experiments, we administered highly-purified EPA at a daily dose of 200, 400, 600, and 1000 mg/kg of body weight to wild-type mice fed a saturated fat diet for 12 weeks, and found that EPA treatment at the latter three doses was safe and effective to reduce hepatic TG contents. Down-regulation of SREBP-1 and the lack of PPARα activation were also detected by EPA administration at each dose. To confirm the absence of PPARα activation by EPA, we adopted the highest dose (1000 mg/kg of body weight/day) in this study.

A novel and unexpected finding in the present study was that EPA-induced decreases in the mature SREBP-1 protein in hepatocyte nuclei occurred by down-regulating the expression of SCAP and S1P, but not by lowering the SREBP-1 mRNA levels. This phenomenon was not observed in *Ppara*$^{-/-}$ mice, suggesting that down-regulation of SCAP and S1P by EPA are related to the presence of PPARα. The detailed molecular mechanism regarding the contribution of PPARα to SREBP-1 processing/activation system still remains unclear. However, it has been reported that TNF-α increased the expression of SCAP in the livers of casein-injected apoE knockout mice [35]. Furthermore, binding motifs for Sp1 have been detected in the promoter region of S1P gene [40]. Indeed, TNF-α- and lipid peroxide-reducing effects were observed only in EPA-treated wild-type mice (Figs. 7D and 8). Therefore, we can speculate that PPARα-dependent alleviation of inflammatory stress by EPA is associated with decreases in the expression of SCAP and S1P.

EPA markedly suppressed the expression of L-FABP, FAT, and FATP and inhibited FA uptake into hepatocytes by a PPARα-independent mechanism. EPA-induced suppression of L-FABP appeared at the post-transcriptional level. L-FABP is prone to S-thiolation, N-acetylation, phosphorylation, and conformational changes, all of which are related to protein stability [41]; EPA might influence such modifications. Since the enhancement of FA uptake has also been reported in the livers of patients with NAFLD [42], its correction caused by EPA may also lead to the improvement of hepatic steatosis.

It is noteworthy that EPA significantly increased the expression of Mn- and Cu, Zn-SODs in a PPARα-dependent fashion. These increases occur via nuclear stabilization of nuclear factor-E2-related factor 2 (Nrf2) [43]. It has been documented that EPA stabilized Nrf2 by reacting directly with Keap1, a negative regulator of Nrf2, and disrupting its function [44]. Thus, EPA might affect the stabilization process of Nrf2 via PPARα. Further studies are needed to address its precise mechanism.

It is well recognized that a saturated fat-rich diet is associated with the development of NAFLD in humans, and that lower levels of hepatic n-3 PUFA predispose livers to steatosis by favoring *de novo* lipogenesis [12,45]. In this study, mice fed a saturated fat diet exhibited elevation of serum ALT levels, hypertriglyceridemia, and macrovesicular steatosis, which resembled the clinical features of patients with NAFLD. Furthermore, EPA treatment in these mice decreased hepatic TG levels without affecting body weight or serum insulin concentrations; these findings were also observed in NASH patients treated with highly-purified EPA [46]. Thus, the molecular mechanism of EPA action found in this study may at least in part be translated to humans.

It is known that lipotoxicity, oxidative stress, and mitochondrial dysfunction play important roles in the pathogenesis of NAFLD/NASH [2,36]. Although FF decreased hepatic TG/FA contents, it enhanced mitochondrial β-oxidation activity and induced the expression of NADPH oxidase and AOX, which may result in production of ROS inside and outside the mitochondria. On the other hand, EPA not only down-regulated two major pathways to increase intrahepatic FA contents, but also elevated the levels of mitochondrial Mn-SOD and extra-mitochondrial Cu, Zn-SOD, which may lead to alleviation of lipotoxicity and oxidative stress. Therefore, we believe that highly-purified EPA may be useful for the treatment of NAFLD/NASH [46].

In conclusion, this study clarified that the main action of EPA in hepatic steatosis improvement was not based on the activation of PPARα. In contrast to FF, EPA significantly inhibited *de novo* lipogenesis and hepatic FA uptake with a reduction in hepatic oxidative stress. These data raise the possibility that EPA may be a promising candidate for various types of liver diseases associated with hepatic fat accumulation and oxidative stress, including NASH, alcoholic liver disease [32,47], and chronic hepatitis C [33]. Further studies are needed to confirm the efficacy of highly-purified EPA against these diseases.

Conflicts of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.07.031.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Nonalcoholic fatty liver disease in Japanese junior high school students: its prevalence and relationship to lifestyle habits

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Abstract
Background Despite the increase in nonalcoholic fatty liver disease (NAFLD) in Japanese adults, its prevalence in adolescents remains unclear. This prompted us to evaluate the incidence and clinical characteristics of NAFLD among junior high school students.
Methods A population-based cross-sectional study was conducted among students in a single junior high school in Nagano prefecture. Serum alanine aminotransferase (ALT) and γ-glutamyltransferase (γGT) measurements and abdominal ultrasonography were performed in 249 and 288 students in 2004 and 2007, respectively. In the latter survey, student lifestyle habits were also assessed, using questionnaires.
Results The prevalence of NAFLD was 4.4% and 4.5% in 2004 and 2007, respectively, which was lower than that of obesity (10.0% and 5.9%). Body mass index and ALT and γGT levels increased significantly with hepatic steatosis severity. Multivariate logistic regression analysis demonstrated that the presence of obesity and an ALT level of 30 U/L or more were independent predictors of NAFLD (odds ratio 16.9, $P < 0.001$ and odds ratio 16.6, $P = 0.001$, respectively). The ratios of students commuting to and from school by car and not doing sports outside of school were higher in NAFLD students compared with non-NAFLD ones. Such tendencies were observed independently of the presence of obesity. Additionally, one obese student with severe steatosis and liver dysfunction was diagnosed as having nonalcoholic steatohepatitis (NASH).
Conclusions Approximately 4% of junior high school students had NAFLD that was primarily associated with obesity and reduced daily physical activity. Serum ALT measurement during school check-ups is recommended for the early detection of young adolescent NAFLD/NASH.

Keywords Obesity · ALT · Physical activity · Skipping breakfast · Nonalcoholic steatohepatitis

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Introduction

Due to increasing sedentary lifestyles and the rising prevalence of obesity, nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease. NAFLD encompasses a spectrum of histological findings that range from macrovesicular steatosis alone (simple

steatosis) to macrovesicular steatosis with hepatocyte ballooning and/or lobular inflammation (steatohepatitis). Non-alcoholic steatohepatitis (NASH) is the severe and progressive form of NAFLD and may develop into cirrhosis, hepatocellular carcinoma, and ultimately death [1–3]. Based on current health screening data, the prevalence of NAFLD in Japanese adults is estimated to be around 10% [1].

Of great recent concern is the fact that NAFLD/NASH exists even in children and adolescents and that pediatric NASH can also progress to cirrhosis. Tominaga et al. [4] reported that 2.6% of Japanese children aged 4–12 years had NAFLD. Furthermore, Kinugasa et al. [5] described seven Japanese obese children aged between 9 and 15 years having hepatic steatosis with various degrees of lobular inflammation and/or portal fibrosis and one child having cirrhosis. Therefore, the early detection of NAFLD/NASH and appropriate disease management are now important in the pediatric population as well.

According to data from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the prevalence of obesity among Japanese junior high school students was double in 2003 compared to that in 1977 (from 5% to 10%). Given the strong association of NAFLD with obesity, NAFLD is suspected to affect a substantial, but as yet unidentified, proportion of junior high school students. As such, this population-based cross-sectional study was planned to evaluate the prevalence of NAFLD in young adolescents, along with any lifestyle habits associated with its development.

Materials and methods

Participants

The study population consisted of all students attending a public junior high school in a village located in southern Nagano prefecture. Real-time liver function tests and abdominal ultrasonography (US) were performed in 2004 and 2007 after explaining the significance and protocol of the study to all students and their parents and obtaining written informed consent. This study was approved by the ethics committee of Shinshu University School of Medicine and Showa Inan General Hospital and adheres to the principles of the Declaration of Helsinki. In this study, NAFLD was defined by the existence of hepatic steatosis based on abdominal US, regardless of liver function tests.

Data collection

Anthropometric, biochemical, and ultrasonographic examinations were carried out on the same school-day mornings in June 2004 and July 2007 after an overnight fast. Body height and weight were measured by a school nurse, with subjects barefoot and in light clothing. Body mass index (BMI) and the age-gender-adjusted Japanese standardized weight index for height (JSI) were used as anthropometric parameters. The JSI was calculated as [(body weight − standard body weight)/standard body weight] × 100 (%), where the standard body weight for each subject's age, sex, and height was determined from data on 700 000 Japanese children aged 5–17 years in 1990. The JSI is considered to be more suitable for the evaluation of the physiques of children and adolescents than the BMI in Japan. According to the JSI, subject weight status was classified as lean (≤−20% of JSI), moderately lean (−19.9% to −10.1%), normal (−10% to +10%), overweight (+10.1% to +19.9%), or obese (≥+20%).

Venous blood samples were drawn just after an anthropometric examination. Serum levels of alanine aminotransferase (ALT), γ-glutamyltransferase (γGT), triglycerides (TG), and high-density-lipoprotein cholesterol (HDL-C) were determined using standard automated analyzers. Normal ranges for serum ALT and γGT levels were set as 0–30 U/L each, in accordance with previous studies in pediatric populations [6–8].

Real-time abdominal US was performed by two experienced ultrasonographers (K.H. and C.I.) using a LOGIQ book equipped with a 4.0 MHz convex-type transducer (GE Yokogawa Medical Systems, Tokyo, Japan). Representative US images of each student were kept in the LOGIQ book and evaluated afterwards in a blinded manner by three independent hepatologists (G.T., N.T., and M.K.). The degrees of hepatorenal contrast, profound attenuation of the diaphragm, and blurring of the vascular wall were each scored as 0 (absent), 1 (present), or 2 (marked) [4, 9–11] The sum of these scores from each diagnostician ranged from 0 to 6, and a mean total score of 1 or more was judged as the presence of hepatic steatosis. Total scores of 1–2 and 3 or more were classified as the presence of mild steatosis and moderate-to-severe steatosis, respectively.

Assessment of lifestyle habits

In the 2007 survey, lifestyle habits were assessed in addition to abdominal US and liver function tests. A 15-item questionnaire focusing on personal dietary and exercise habits was prepared and distributed to all students. All questionnaires were completed and submitted by the students themselves, and were then analyzed for any relationships with NAFLD.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS, Chicago, IL, USA). Qualitative