Dig Dis Sci
DOI 10.1007/s10620-010-1400-5

ORIGINAL ARTICLE

Eicosapentaenoic Acid Attenuates Progression of Hepatic Fibrosis with Inhibition of Reactive Oxygen Species Production in Rats Fed Methionine- and Choline-Deficient Diet

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Received: 8 April 2010 / Accepted: 12 August 2010

Abstract
Background and Aims Nonalcoholic steatohepatitis (NASH) is associated with fat accumulation in the liver, and develops to cirrhosis with the progression of hepatic fibrosis. Eicosapentaenoic acid (EPA) is used to treat hyperlipidemia, and suppresses hepatic fat accumulation. As the effect of EPA on NASH remains unclear, we assessed the therapeutic effect of EPA and its mechanisms in an animal model of NASH.
Methods Wistar rats were fed a methionine- and choline-deficient (MCD) diet for 20 weeks, and given EPA ethyl ester (EPA-E, 1,000 mg/kg/day) or vehicle by gavage from week 12, at which hepatic fibrosis has already established. The liver was histologically analyzed for fibrosis and α-smooth muscle actin (αSMA) expression, and hepatic levels of transforming growth factor-β1 (TGF-β1), fibrogenic gene expression, reactive oxygen species (ROS), and triglyceride (TG) content were determined. Serum oxidative markers were also measured.
Results EPA-E treatment significantly suppressed the MCD-induced increase in fibrotic area of liver sections, with repressed macronodule formation. EPA-E also suppressed increases in hepatic fibrogenic factors, αSMA expression, TGF-β1 level, and messenger RNA (mRNA) levels of procollagens and connective tissue growth factor. EPA-E reduced MCD-induced increases in hepatic ROS level, serum oxidative markers, 8-isoprostane and ferritin, and hepatic TG content. Attenuation of hepatic fibrosis by EPA-E was significantly correlated with hepatic ROS level, but not TG content.
Conclusions EPA-E attenuates progression of hepatic fibrosis in developed steatohepatitis, and this effect is likely mediated by inhibition of ROS production. These actions may elicit the therapeutic effect of EPA-E against NASH.

Keywords Eicosapentaenoic acid · Fibrosis · Reactive oxygen species · Oxidative stress · Nonalcoholic steatohepatitis · Methionine- and choline-deficient diet

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Introduction

Nonalcoholic steatohepatitis (NASH) is characterized by excess fat accumulation in the liver and is associated with hepatic inflammation and fibrosis [1]. NASH is considered to be the progressive form of nonalcoholic fatty liver disease (NAFLD), developing from simple steatosis to cirrhosis with the progression of fibrosis, and occasionally to hepatocellular carcinoma [1].

NASH is closely associated with metabolic syndromes, such as obesity, insulin resistance, and hyperlipidemia [1],

Published online: 17 September 2010

and these disorders cause increased free fatty acids in plasma and their overload in tissues, resulting in excessive synthesis and accumulation of TG in the liver [1, 2]. The accumulated TG in hepatocytes is metabolized via mitochondrial and peroxisomal oxidation, which increases reactive oxygen species (ROS) production [3]. ROS are considered to be responsible for the further progression from steatosis to steatohepatitis. ROS and ROS-triggered peroxidated products cause cell necrosis and apoptosis, and increased production of proinflammatory cytokines in Kupffer cells, such as tumor necrosis factor (TNF)-α, which leads to activation of hepatic stellate cells (HSCs) [1, 4, 5]. TNF-α also induces ROS by interference with the normal electron flow in mitochondria [6]. Moreover, proliferation and activation of HSCs can be directly induced by ROS and peroxidated lipid products in vitro [7–9], which is associated with collagen synthesis [7, 8]. In humans, increases in oxidation products, including 4-hydroxy-2′-nonenal (HNE), 8-hydroxydeoxyguanosine (8-OHdG), and nitrotyrosine (NYT), have been detected in the livers of NASH patients with hepatic fibrosis [10, 11]. Moreover, serum content of lipid peroxidation products and oxidative stress markers, such as malondialdehyde, 8-isoprostane, and ferritin, is also increased in NASH patients [12–14]. This evidence supports an important role for ROS in the pathogenesis of NASH and the progression of hepatic fibrosis.

Eicosapentaenoic acid (EPA) is the only component of omega-3 long-chain polyunsaturated fatty acids (*n*-3 LCPUFAs) that is used clinically as a single-agent treatment for hypertriglyceridemia [15]. *n*-3 LCPUFAs, including EPA, have been reported to provide improvement to NAFLD patients [16, 17]. EPA suppresses hepatic TG accumulation, via inhibition of sterol regulatory element binding protein (SREBP)-1c-mediated lipogenesis [18] and an increase in *β*-oxidation, regulated by the peroxisome-proliferator activated receptor α (PPARα)-mediated pathway [19, 20]. Moreover, we showed that EPA suppressed hepatic ROS level with inhibition of hepatic fat accumulation in mice [21]. EPA also reduced ROS production in vitro, likely via a superoxide scavenging pathway [22]. The mechanistic details underlying the suppressive effect of EPA on ROS production are being intensively investigated. Activation of PPARα-responsive genes by EPA is accompanied by increased expression and/or activity of defensive molecules against ROS, such as superoxide dismutase (SOD), uncoupling protein-2 (UCP-2), and glutathione *S*-transferase (GST) [23–25]. Several recent reports have demonstrated that EPA downregulates activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [26], which generates large amounts of superoxide anions and contributes to the development of hepatic fibrosis [27, 28]. EPA also reduces hepatic peroxidated lipid content in mice by enhanced activities of catalase and glutathione-requiring detoxification enzymes [29]. These findings suggest that EPA has an inhibitory effect on ROS production.

We have previously reported that EPA prevents the development of hepatic fibrosis in a NASH model using methionine- and choline-deficient (MCD) diet [30]. In that study, EPA ethyl ester (EPA-E) was administered from onset of steatohepatitis induction, thus the therapeutic effect of EPA against hepatic fibrosis has not been clarified. Moreover, EPA also inhibited MCD-induced fat deposition in the liver, hence it is not clear whether EPA can prevent hepatic fibrosis independently from hepatic TG accumulation. In the current work, we investigated the effect of EPA on hepatic fibrosis in MCD diet-induced steatohepatitis, in which hepatic TG was sustained at an elevated level. We also evaluated the effect of EPA on ROS induction in the liver, which may play a crucial role in progression of hepatic fibrosis.

Materials and Methods

Drugs and Diets

EPA-E (98% pure; Mochida Pharmaceutical, Tokyo, Japan) was suspended in 5% arabic gum at 100 mg/mL (Wako Pure Chemical Industries, Osaka, Japan) using a homogenizer (POLYTRON PT3100; KINEMATICA, Bohemia, NY) and administered orally. The MCD diet and the methionine- and choline-sufficient (MCS) diet were obtained from Dyets Inc. (#518810 and #518811, respectively; Bethlehem, PA). The composition of each diet is detailed in our previous report [30].

Animals and Experimental Protocol

Male Wistar rats (6 weeks of age) were purchased from Charles River Japan (Yokohama, Japan). They were housed individually in a room under controlled temperature (20–26°C), humidity (35–75%), and lighting (12-h light/dark cycle) conditions, and were provided with a standard pellet diet containing no fish products (F1; Funabashi Farm, Funabashi, Japan). All animals were fed ad libitum, with free access to water, and received humane care throughout the study. All study protocols complied with the institutional guidelines.

Two different studies were conducted. In the first study, animals were randomly divided into two groups of ten rats after 1 week of acclimation, and fed the MCD diet, or MCS diet as control, for 12 weeks. In another study, animals acclimated for 1 week were randomly divided into two groups. One group of 20 rats was fed the MCS diet as

control, and the other group was fed the MCD diet. At 12 weeks, the MCD-fed group was again randomly divided into two groups of 20 rats, and one group was administered EPA-E (1,000 mg/kg) daily by gavage with MCD feeding until 20 weeks. The dose of EPA-E (1,000 mg/kg/day) was considered to be appropriate for investigation of the pharmacological effects of EPA-E and its underlying mechanisms in rats, because plasma EPA level following administration of EPA-E to rats at 1,000 mg/kg/day for 4 weeks has been reported to be 62 μg/mL [31], which is almost comparable to that in humans given a clinical dose of EPA-E (1,800 mg/day) for 3 months (143 μg/mL) [32]. Groups not given EPA-E were also administered 5% arabic gum solution as vehicle from week 12 to 20 with feeding of the respective diet.

At the end of both experiments, blood was collected from the inferior vena cava under isoflurane anesthesia and the liver was removed. A portion of the liver tissue was fixed in 10% buffered formalin for histological analysis, a second portion was frozen in liquid nitrogen for measurement of TG and transforming growth factor β1 (TGF-β1) content, and a third portion was immersed in RNAlater (Ambion, Austin, TX) and frozen for isolation of total RNA. Frozen samples were stored at −80°C until use. A portion of fresh liver tissue was additionally collected for measurement of hepatic ROS level.

Measurement of Serum Biochemical Markers

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, albumin, total protein, choline esterase, TG, and hyaluronic acid were measured using commercial kits (Wako Pure Chemical Industries and Fujirebio, Tokyo, for hyaluronic acid) and an automatic analyzer (7170; Hitachi, Tokyo). Serum levels of collagen IV (Exocell, Philadelphia, PA), 8-isoprostane (Cayman Chemical, Ann Arbor, MI), and ferritin (Mitsubishi Chemical Medience, Tokyo) were measured using commercially available enzyme immunoassay (EIA) kits.

Liver Histology and Measurement of Fibrotic Area

Formalin-fixed left lobe of the liver was embedded in paraffin. Sections were stained with hematoxylin and eosin (HE), or with Masson-trichrome (MT) staining to determine the degree of hepatic fibrosis. For immunohistochemical analysis, liver sections were processed with a monoclonal mouse anti-human α-smooth muscle actin (αSMA) antibody (dilution 1:400; Dako Japan, Tokyo) and a detection reagent (Histofine Simple Stain Rat MAX PO; Nichirei Biosciences, Tokyo). The immunoreactive products were visualized using a 3,3′-diaminobenzidine tetrahydrochloride reagent followed by counterstaining with hematoxylin. MT- and αSMA-positive areas were photographed digitally, measured using an image analysis system (Win ROOF ver. 5.5; Mitani Corporation, Fukui, Japan), and expressed as percentages of total sectional area. Five randomly selected areas per specimen were analyzed. We represented fibrotic area of 12- and 20-week study as fold increase from respective time-matched MCS-fed rats, thereby minimizing the difference between studies.

Measurement of Hepatic Levels of TG and TGF-β1

To assess hepatic TG content, liver tissue was homogenized and lipid was extracted according to Folch et al. [33]. TG levels were determined using a commercial kit (Triglyceride E-test Wako; Wako Pure Chemical Industries). Hepatic TGF-β1 levels were measured by EIA as previously described [30].

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Hepatic messenger RNA (mRNA) expression of type I procollagen α1 [collagen α1(I)], collagen α2(I), and connective tissue growth factor (CTGF) were evaluated using quantitative real-time RT-PCR as previously reported [30]. The relative amount of target mRNA in each sample was determined by applying the threshold cycle to the standard curve. The result for each sample was normalized to the respective glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value measured as an internal control.

Measurement of Hepatic ROS Level

The hepatic ROS level was determined as previously described [34]. Briefly, 500 μL liver homogenate, freshly prepared in phosphate-buffered saline using a Teflon homogenizer, was mixed with 4.4 mL 100 mmol/L potassium phosphate buffer (pH 7.4) and incubated with 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma–Aldrich, St Louis, MO) at final concentration of 5 μmol/L, for 15 min at 37°C. After centrifuging at 10,000 rpm for 10 min at 4°C, the pellet was suspended in 5 mL potassium phosphate buffer (pH 7.4) on ice and incubated for 60 min at 37°C. Fluorescence was measured using a microplate fluorometer (Powerscan HT; Themo Scientific, Waltham, MA) at wavelengths of 485 nm for excitation and 528 nm for emission. The hepatic ROS level was determined as the amount of 2′,7′-dichlorofluorescein (DCF) quantified from a standard DCF curve. The protein concentration in the homogenate was measured with the BCA protein assay kit (Thermo Scientific, Rockford, IL).

Statistical Analysis

Values are expressed as mean ± standard error of mean (SEM). All results were tested using the *F*-test for homogeneity of variance between two groups, specifically: MCS-fed versus MCD-fed groups, and MCD-fed versus MCD-fed EPA-E-treated groups. If the variance was homogeneous ($F > 0.05$), Student's *t*-test was performed; if the variance was heterogeneous ($F < 0.05$), the Aspin–Welch *t*-test was performed. Correlation was determined by simple linear regression analysis. In all analyses, SAS statistical program was used (ver. 9.1.3; SAS Institute Japan, Tokyo). *P* values less than 0.05 were considered statistically significant.

Results

Pathophysiological Characteristics in Rats Fed the MCD Diet for 12 and 20 Weeks

Rats receiving the MCD diet for 12 weeks had decreased body and liver weights (Table 1). Hepatic TG content in MCD-fed rats was markedly increased from MCS-fed rats, while serum TG level was decreased, which is likely due to reduced TG secretion from the liver in MCD-fed animals [35]. Serum levels of markers for liver and bile duct injury, ALT, AST, ALP, and total bilirubin, and those for fibrosis, hyaluronic acid, and collagen IV, were increased, and markers for liver function, albumin, and total protein were decreased by the MCD diet as compared with MCS-fed rats (Table 1). Fibrotic area, which was measured as MT-positive areas in liver sections, was increased 11.4-fold in MCD-fed rats compared with MCS-fed rats (measured values 7.0 ± 0.6% and 0.6 ± 0.1%, respectively) (Fig. 1g). These findings indicate that steatohepatitis with hepatic fibrosis has already developed in rats fed the MCD diet for 12 weeks.

In rats fed the MCD diet for 20 weeks, body and liver weights were decreased similarly as in 12-week MCD-fed rats (Table 1). Liver nodule formation, which was absent in 12-week MCD-fed rats, was observed in 89.5% (17/19 rats) of MCD-fed rats (Table 1). Hepatic TG content was significantly increased by MCD feeding for 20 weeks, but was not higher than that in 12-week MCD-fed rats (Table 1). As in 12-week MCD-fed rats, serum markers for liver and bile duct injury and fibrosis were increased, and those for liver function were decreased (Table 1). Histological analysis clarified that rats fed the MCD diet for 20 weeks showed steatosis (Fig. 1b) and severe hepatic fibrosis observed as portal–portal and central–portal bridging fibrosis (Fig. 1e), while MCS-fed rats remained normal

Table 1 Effects of the MCD diet and EPA-E treatment on pathophysiological characteristics in rats

	12 weeks		20 weeks		
	MCS	MCD	MCS	MCD	MCD + EPA-E
Body weight (g)	534.6 ± 9.6	174.9 ± 3.7$^{\#\#}$	603.9 ± 14.3	178.7 ± 2.3$^{\#\#}$	174.3 ± 2.2
Liver weight (g)	11.36 ± 0.16	7.11 ± 0.21$^{\#\#}$	12.14 ± 0.39	5.94 ± 0.16$^{\#\#}$	6.09 ± 0.22
Hepatic TG content (mg/g liver)	19.3 ± 1.6	110.2 ± 7.3$^{\#\#}$	34.7 ± 5.6	90.7 ± 3.5$^{\#\#}$	73.0 ± 2.1**
Liver nodule formation (no. of rats observed/total)	0/10	0/10	0/20	17/19	11/20
Serum markers					
ALT (IU/L)	23 ± 2	80 ± 5$^{\#\#}$	24 ± 1	95 ± 5$^{\#\#}$	82 ± 3*
AST (IU/L)	78 ± 3	272 ± 15$^{\#\#}$	78 ± 3	380 ± 26$^{\#\#}$	336 ± 25
ALP (IU/L)	137 ± 5	421 ± 29$^{\#\#}$	139 ± 7	718 ± 69$^{\#\#}$	648 ± 71
Total bilirubin (mg/dL)	0.13 ± 0.01	2.15 ± 0.16$^{\#\#}$	0.10 ± 0.00	1.98 ± 0.10$^{\#\#}$	1.49 ± 0.08**
Albumin (g/dL)	3.5 ± 0.0	2.9 ± 0.1$^{\#\#}$	3.7 ± 0.0	2.5 ± 0.1$^{\#\#}$	2.8 ± 0.1**
Total protein (g/dL)	5.5 ± 0.1	4.4 ± 0.1$^{\#\#}$	6.1 ± 0.0	3.9 ± 0.1$^{\#\#}$	4.4 ± 0.1**
Choline esterase (IU/L)	108 ± 8	89 ± 12	148 ± 11	33 ± 2$^{\#\#}$	59 ± 8**
TG (mg/dL)	94 ± 10	14 ± 1$^{\#\#}$	77 ± 6	55 ± 10$^{\#\#}$	37 ± 10
Hyaluronic acid (ng/mL)	35 ± 2	41 ± 1$^{\#}$	43 ± 3	127 ± 21$^{\#\#}$	63 ± 6**
Collagen IV (μg/mL)	1.24 ± 0.05	1.87 ± 0.09$^{\#\#}$	0.93 ± 0.07	4.76 ± 0.33$^{\#\#}$	3.37 ± 0.31**

Rats were fed the MCS or MCD diet for 12 or 20 weeks

EPA-E (1,000 mg/kg/day) was administered by gavage from 12 to 20 weeks after onset of MCD feeding in 20-week study

Values are expressed as mean ± SEM ($n = 10$ or 19–20, in 12- or 20-week study, respectively)

$^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$ versus time-matched MCS-fed rats

* $P < 0.05$, ** $P < 0.01$ versus 20-week MCD-fed rats

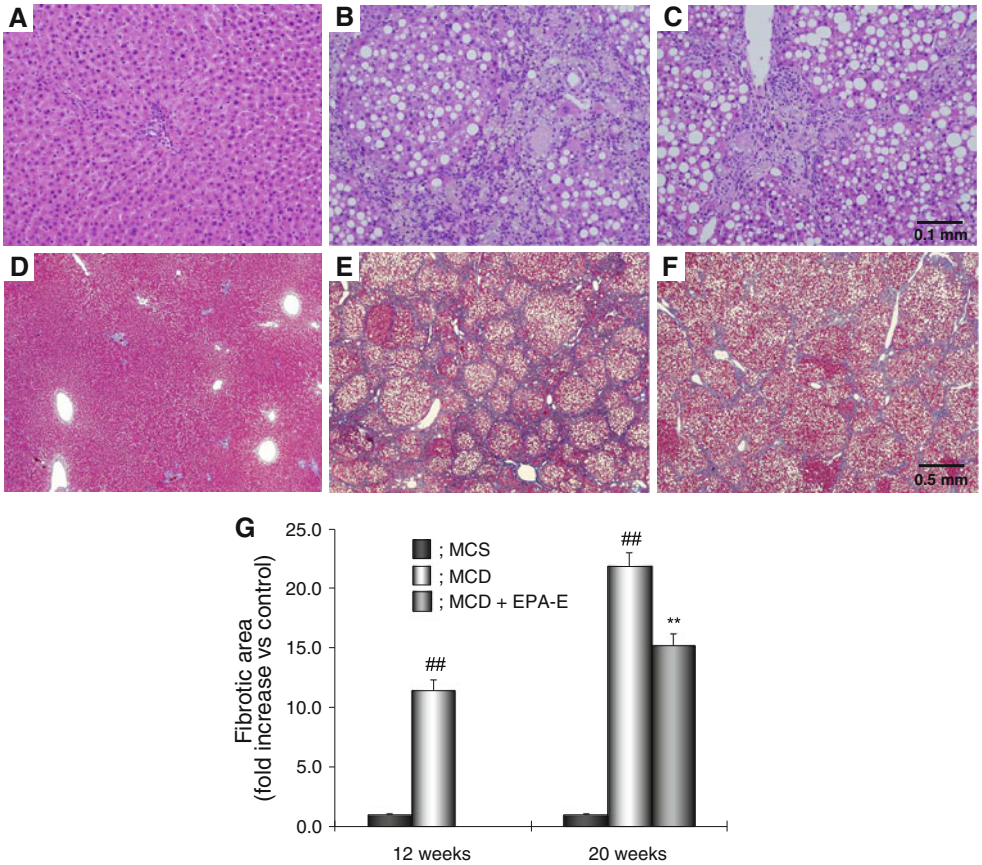

Fig. 1 Effects of the MCD diet and EPA-E treatment on hepatic steatosis and fibrosis. Liver sections stained with HE (**a**, **b**, **c**) and MT (**d**, **e**, **f**) from a rat fed the MCS diet (**a**, **d**) or the MCD diet for 20 weeks with administration of vehicle (**b**, **e**) or EPA-E (1,000 mg/kg/day) from week 12 to 20 (**c**, **f**) by gavage (original magnification: ×40 in HE, and ×8 in MT). The percentage of MT-positive area (*blue*) in the total area of liver sections was quantified using an image analysis system, and represented as fold increase from each time-matched MCS-fed rat (**g**). Values are expressed as mean ± SEM ($n = 10$ or 19–20, in 12- or 20-week study, respectively). $^{\#\#} P < 0.01$ versus time-matched MCS-fed rats. $^{**} P < 0.01$ versus 20-week MCD-fed rats

(Fig. 1a, d). Assessment of fibrotic areas in liver sections also revealed a 21.9-fold increase in MCD-fed rats compared with MCS-fed rats (measured values 8.1 ± 0.4% and 0.4 ± 0.0%, respectively), this increase being higher than that in 12-week MCD-fed rats (Fig. 1g). These data indicate that steatohepatitis induced by 12-week MCD feeding in rats is progressed to cirrhosis by additional MCD feeding to 20 weeks, which was associated with further progression of hepatic fibrosis, while fat accumulation in liver was not increased.

Effects of EPA-E on Developed Steatohepatitis and Hepatic Fibrosis

Eight-week administration of EPA-E, from 12 to 20 weeks after the start of MCD feeding, lowered the ratio of rats with liver nodule formation (55%, 11/20 rats) compared with MCD-fed rats, despite body and liver weights remaining unchanged (Table 1). EPA-E also decreased hepatic TG content in MCD-fed rats without change in serum TG level. Levels of other serum markers exacerbated by the MCD diet were ameliorated by EPA-E treatment, except AST and ALP (Table 1). Histologically, EPA-E treatment from 12 weeks after the start of MCD feeding reduced the development of liver fibrosis in 20-week MCD-fed rats (Fig. 1f), although a pronounced change in steatosis was not observed (Fig. 1c). This was also evidenced by significant decrease of fibrotic area in MCD-fed EPA-E-treated rats (measured value 5.6 ± 0.4%) compared with MCD-fed rats without EPA-E treatment (Fig. 1g). These findings suggest that EPA-E treatment, from 12 weeks after onset of MCD feeding, attenuated further progression of hepatic fibrosis in MCD-induced steatohepatitis.

Effects of EPA-E on Fibrogenic Factors

Immunohistochemical analysis of αSMA, an HSC activation marker, showed that αSMA-positive area in liver sections was significantly increased in rats fed the MCD

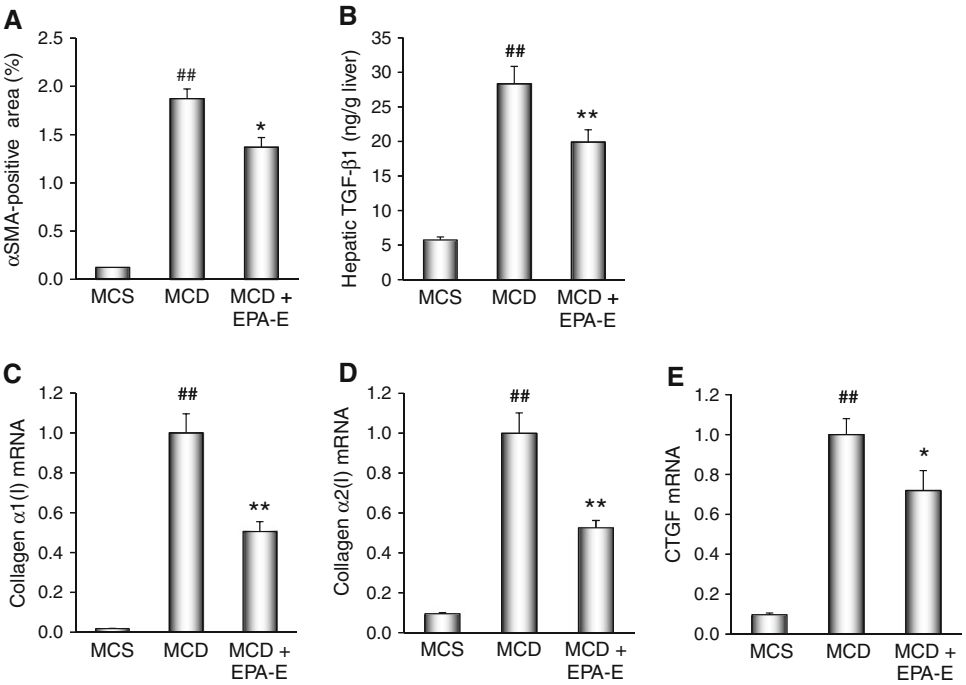

Fig. 2 Effects of the MCD diet and EPA-E treatment on hepatic fibrogenic factors in rats fed the MCS or MCD diet for 20 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage from week 12 to 20. Liver sections were performed with immunostaining of αSMA (**a**). Hepatic TGF-β1 content (**b**) was determined as described in the "Materials and Methods" section. Hepatic mRNA levels of collagen α1(I) (**c**), α2(I) (**d**), and CTGF (**e**) were examined by quantitative real-time RT-PCR and normalized to GAPDH levels. The ratio of mRNA levels to the mean value of MCD-fed rats is shown. Values are expressed as mean ± SEM ($n = 19$–20). ## $P < 0.01$ versus MCS-fed rats; * $P < 0.05$, ** $P < 0.01$ versus MCD-fed rats

diet for 20 weeks compared with MCS-fed rats. EPA-E treatment suppressed MCD-induced increase in αSMA-positive areas in MCD-fed rats (Fig. 2a). Hepatic content of TGF-β1, a key profibrogenic cytokine that regulates HSC activation and synthesis of extracellular matrix (ECM) [5], was also increased in rats fed the MCD diet compared with MCS-fed rats, and EPA-E treatment significantly reduced its level (Fig. 2b). Further analysis of mRNA levels in the liver revealed that expression of genes for fibrogenesis, collagen α1(I), α2(I), and CTGF, were increased in MCD-fed rats, and reduced by EPA-E treatment (Fig. 2c–e). Our findings confirm that attenuation of hepatic fibrosis by EPA-E coincided with suppressed HSC activation and reduced levels of hepatic TGF-β1 and TGF-β-regulated fibrogenic gene expression.

Effects of EPA-E on Oxidative Stress

We determined hepatic ROS level as the amount of fluorescent DCF formed from nonfluorescent DCFH-DA by ROS contained in liver homogenate [34]. Rats fed the MCD diet for 20 weeks showed an increase in DCF level compared with MCS-fed rats. EPA-E treatment reduced the increase in DCF level of MCD-fed rats (Fig. 3a). Serum level of the oxidative stress marker 8-isoprostane was also increased by the MCD diet (Fig. 3b). For another marker, ferritin, the MCD-fed rats showed a 2.3-fold higher value than that in MCS-fed rats; however, the difference between those groups was not significant ($P = 0.0710$) (Fig. 3c). This was due to an extra high value (65.8 μg/mL) in 1 of the 20 MCS-fed rats, due to an undetermined cause. Therefore, we consider that the MCD diet increased serum ferritin level. EPA-E treatment also significantly repressed these increases in both serum markers (Fig. 3b, c). These data confirm that EPA-E reduces hepatic ROS production in MCD diet-induced steatohepatitis.

The Relationship Between Hepatic Levels of Fibrosis, ROS, and TG

In rats fed the MCD diet with or without EPA-E treatment, fibrotic area was significantly correlated with hepatic ROS level ($R^2 = 0.2058$; $P = 0.0037$) (Fig. 4). In contrast, fibrotic area and hepatic ROS level were not significantly correlated with hepatic TG level in EPA-E-treated and untreated rats fed the MCD diet ($R^2 = 0.0183$; $P = 0.4110$ and $R^2 = 0.0727$; $P = 0.0970$, respectively).

Discussion

In rats, MCD diet induces severe steatohepatitis that is histologically similar to human NASH [36]. In this model, pathogenic events during MCD feeding involve early lipid accumulation and increased lipid peroxidation in the liver, followed by inflammation and hepatic fibrosis [37, 38]. In the present study, we demonstrated that rats fed the MCD diet for 12 weeks showed increased hepatic TG content, serum markers for hepatic injury, and liver fibrosis, indicating the development of fibrotic steatohepatitis.

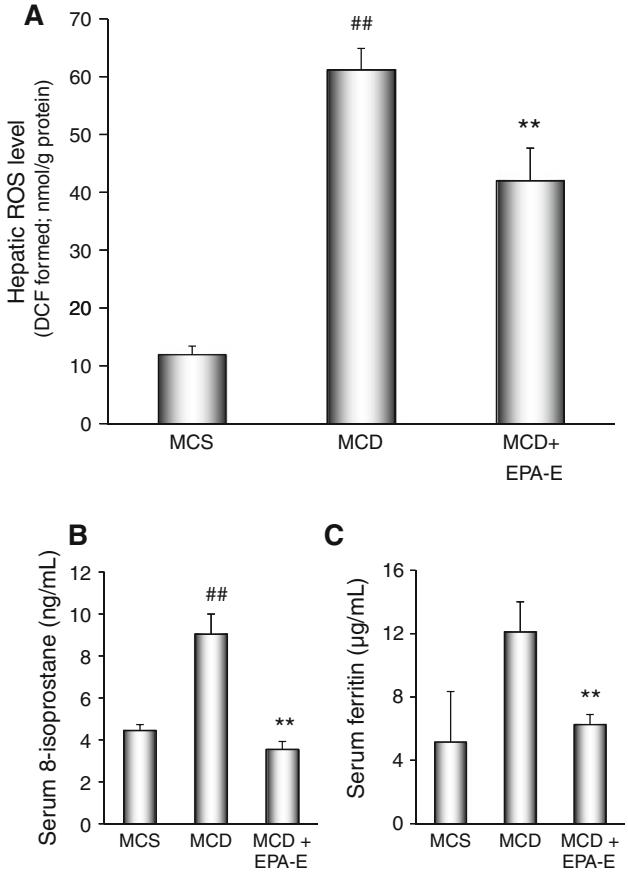

Fig. 3 Hepatic ROS level (**a**), measured as the amount of DCF formed, and serum levels of 8-isoprostane (**b**) and ferritin (**c**) in rats fed the MCS or MCD diet for 20 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage from week 12 to 20. The amount of DCF formed from DCFH-DA was determined using liver homogenate as described in the "Materials and Methods" section. Values are expressed as mean ± SEM ($n = 19$–20). $^{\#\#}P < 0.01$ versus MCS-fed rats; ** $P < 0.01$ versus MCD-fed rats

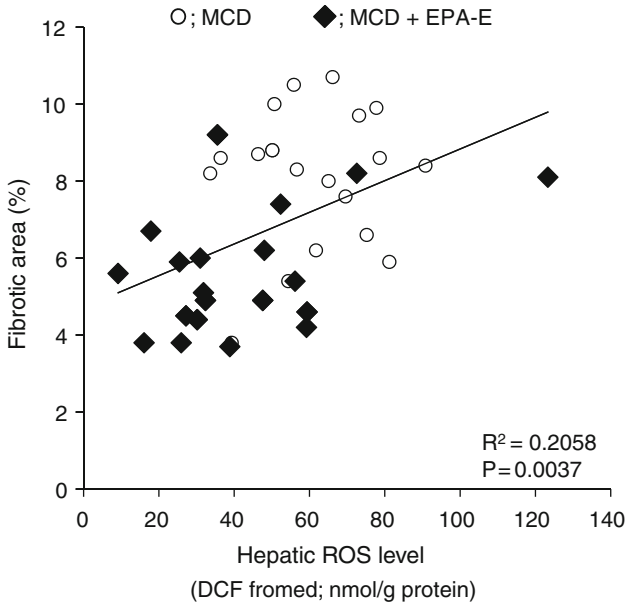

Fig. 4 The relationship between hepatic ROS level (measured as the amount of DCF formed) and MT-positive fibrotic areas in liver sections. Rats were fed the MCD diet for 20 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage from week 12 to 20 ($n = 19$–20 in each group). The correlation between fibrotic area and hepatic ROS level was evaluated by simple linear regression analysis

Additionally, we previously showed that rats fed the MCD diet for 8 weeks had already developed severe hepatic steatosis with increased hepatic fat content; however, this was not accompanied by fibrosis [30]. Based on these findings, to assess the therapeutic effect of EPA-E on developed fibrosis in steatohepatitis, we initiated EPA-E treatment at 12 weeks after the start of MCD feeding. The results indicated that EPA-E reduced the increase in hepatic fibrosis induced by 20-week MCD feeding. In comparison with rats fed the MCD diet for 12 weeks, 20-week MCD-fed rats showed further progression in hepatic fibrosis. Meanwhile, the degree of hepatic fibrosis in EPA-E-treated rats was almost comparable to that in 12-week MCD-fed rats. These findings potently suggest that EPA-E attenuates progression of hepatic fibrosis even in already developed fibrotic steatohepatitis.

In the present study, hepatic TG content was increased by MCD feeding for 12 weeks, and was sustained without increase up to week 20. EPA-E treatment caused a 20% decrease in hepatic TG content at week 20, suggesting a reversal effect on fat accumulation. Hepatic fat accumulation is an underlying disorder in the progression of steatohepatitis; thus, it is implied that attenuation of hepatic fibrosis by EPA-E may be caused by improvement in hepatic TG content. However, EPA-E attenuation of fibrotic area, indicating the degree of hepatic fibrosis, did not correlate with the hepatic TG content in the progressing stage of MCD-induced hepatic fibrosis. These findings suggest that the antifibrotic effect of EPA-E against further fibrosis progression in steatohepatitis may involve mechanisms independent of hepatic fat accumulation. Indeed, dietary fish oil has been reported to have an antifibrotic role in a bleomycin-induced pulmonary fibrosis model in rats [39], which is independent of tissue TG content but requires ROS production [40].

We also showed that attenuation of hepatic fibrosis by EPA-E is significantly correlated with inhibition of ROS production. Meanwhile, hepatic levels of ROS and TG were not significantly correlated. It has been reported that administration of EPA-E increases hepatic EPA levels in rats [41, 42] and mice [18, 43]. These results suggest that ROS inhibition by EPA in liver may contribute to the attenuation of hepatic fibrosis as a mechanism independent from hepatic TG accumulation. ROS are generated from various cells, including hepatocytes, Kupffer cells, and HSCs [1, 5], and induce proliferation and activation of

HSCs with collagen synthesis directly [7–9], or indirectly via production of cytokines, such as TNF-α and TGF-β1, in Kupffer cells and HSCs [4, 5]. Thus, ROS are considered to be responsible for the progression of hepatic fibrosis. Meanwhile, TGF-β also plays a key role in the induction of hepatic fibrosis, because it upregulates expression of various genes involved in ECM synthesis [44]. In this study, EPA-E significantly suppressed HSC activation and decreased hepatic TGF-β1 level and mRNA levels of fibrogenic genes regulated by TGF-β1, collagen, and CTGF, with ROS reduction. Recent studies indicate the close relationship between ROS and TGF-β in hepatic fibrosis. ROS upregulate TGF-β secretion in rat HSCs, and activate latent TGF-β by oxidation and/or cleavage of latency-associated protein, in a complex with TGF-β when secreted from cells [45–47]. Inversely, TGF-β also induces ROS production in mitochondria and microsomes and suppresses antioxidative enzyme expression in rat hepatocytes [48–50]. Moreover, induction of TGF-β-responsive genes is mediated by ROS via mitogen-activated protein kinase pathways, which is also important in the TGF-β-signaling pathway, in addition to the Smad pathway [51]. Taken together, inhibition of ROS production by EPA-E, independent of fat accumulation, is suggested to play a crucial role in the attenuation of hepatic fibrosis, involving the inhibition of TGF-β-signaling pathway.

Inhibition of ROS production by EPA-E may include several mechanisms. EPA has been reported to increase β-oxidation via activation of PPARα [19]. Among PPARα-responsive genes, EPA increases expression and/or activity of defensive molecules against ROS, such as SOD, UCP-2, and GST [23–25]. Therefore, excess ROS generation may be quenched. Indeed, EPA has been reported to suppress ROS production in vivo and in vitro, at least in part by induction of a superoxide scavenging pathway [22, 29]. Moreover, several reports demonstrated that EPA downregulates activity of NADPH oxidase [26], which generates large amounts of superoxide anions and contributes to the development of hepatic fibrosis [27, 28]. In addition, *n*-3 PUFA downregulates expression of the $p47^{phox}$ subunit of the NADPH oxidase complex [52], which is required for ROS production [40]. The various antioxidative properties of EPA, independent of fat accumulation, may contribute to the inhibition of ROS induction.

Increased oxidative stress has been detected in the liver of NASH patients as overproduction of HNE, 8-OHdG, and NYT [10, 11]. Thus, according to our considerations, inhibition of ROS production by EPA-E possibly causes the amelioration of hepatic fibrosis in NASH. To date, EPA-E has been reported to improve NAFLD score in NASH patients with decreased levels of serum oxidative markers in a pilot study [53]. This implies that reduction of oxidative stress by EPA-E in NASH patients may be involved in the amelioration of NASH conditions, while additional studies are needed to confirm the effects of EPA-E on fibrosis and oxidative stress in the liver of NASH patients.

In conclusion, we demonstrated that EPA-E attenuates progression of hepatic fibrosis that has already developed in steatohepatitis. Moreover, the antifibrotic effect of EPA-E may be mediated by direct inhibition of ROS production, which involves a TGF-β-stimulated signaling pathway and/or fatty acid oxidation. These mechanisms, independent of the effect against TG accumulation, may contribute to the therapeutic efficacy of EPA in NASH patients, although further investigation will be needed to clarify the relationship between the antifibrotic properties of EPA and ROS inhibition.

Acknowledgments We thank Ms. Reiko Ono and Mr. Shin-ichi Akimoto for their skillful assistance.

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