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ORIGINAL ARTICLE

Highly Purified Eicosapentaenoic Acid Ethyl Ester Prevents Development of Steatosis and Hepatic Fibrosis in Rats

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Abstract
Background and Aims Pathogenesis of nonalcoholic steatohepatitis (NASH) is considered to be involved in fat accumulation, oxidative stress, inflammation, and fibrosis in liver, but no drug therapy has been established as yet. Eicosapentaenoic acid (EPA) is an agent used clinically to treat hypertriglyceridemia, and has been reported to suppress reactive oxygen species and inflammation. Here, we aimed to assess the effect of EPA on progression of hepatic fibrosis in an animal model of NASH.
Methods Wistar rats were fed a methionine- and choline-deficient (MCD) diet and given EPA ethyl ester (EPA-E) (1,000 mg/kg/day) or vehicle by gavage for 8 or 20 weeks.
Results The MCD diet caused development of hepatic fibrosis and nodule formation at 20 weeks. EPA-E treatment significantly suppressed MCD-induced increase in fibrosis and hepatic hydroxyproline, and inhibited nodule formation. EPA-E treatment also decreased hepatic transforming growth factor (TGF)-β1, and messenger RNA (mRNA) levels of connective tissue growth factor. EPA-E suppressed MCD-induced elevation of serum levels of ferritin, 8-isoprostane, soluble tumor necrosis factor receptor 1 (sTNFR1), and sTNFR2 at 20 weeks, and hepatic triglyceride accumulation at 8 weeks.
Conclusions EPA-E prevents progression of hepatic fibrosis in an MCD-induced NASH model with reduction of oxidative stress, inflammation, and initial hepatic steatosis. Thus, EPA-E treatment may be a potential therapy to treat NASH.

Keywords Eicosapentaenoic acid · Nonalcoholic steatohepatitis · Methionine- and choline-deficient diet · Steatosis · Fibrosis · Transforming growth factor β1

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Introduction

Nonalcoholic steatohepatitis (NASH) is characterized by accumulation of excess fat in the liver and is associated with hepatic inflammation and fibrosis [1]. NASH is considered as the progressive form of nonalcoholic fatty liver disease (NAFLD), and may progress to cirrhosis and occasionally to hepatocellular carcinoma [1]. Although aberrant accumulation of triglyceride (TG) in the liver is a clear underlying condition of NASH, little is known about the pathogenesis and mechanisms of progression from simple fatty liver to steatohepatitis, and then to cirrhosis.

Nonalcoholic steatohepatitis is frequently associated with metabolic syndromes, such as obesity, insulin resistance (IR), and hyperlipidemia [1]. IR is believed to be a central mechanism in progression of NASH, because the resulting hyperinsulinemia increases glucose levels in the blood and free fatty acids in adipose tissue, which then cause excessive accumulation of TG in the liver [1]. Hyperlipidemia, especially hypertriglyceridemia, which

results from plasma lipid overflow and tissue overload, also increases hepatic synthesis of TG [2]. Accumulated TG in hepatocytes is metabolized via mitochondrial or peroxisomal oxidation, which increases production of reactive oxygen species (ROS) [1]. ROS cause lipid peroxidation followed by activation of an inflammatory response and hepatic stellate cells (HSCs), leading to fibrosis [1, 3].

Recently, several clinical trials using insulin-sensitizing medications have been shown to improve hepatic steatosis and insulin sensitivity in NASH patients [4, 5]. Antioxidants are also used to improve oxidative stress [6]. However, successful therapies have not been reported for NASH in large-scale randomized controlled trials. Antihyperlipidemic therapies have been examined for treatment of NASH. Bezafibrate, a peroxisome-proliferator activated receptor α (PPARα) agonist with lipid-lowering properties, showed preventive effects on steatohepatitis and fibrosis in mice fed a methionine- and choline-deficient (MCD) diet [7]. Clinical efficacy, however, has not yet been reported.

Omega-3 long-chain polyunsaturated fatty acids (*n*-3 LCPUFAs), for which effects on hyperlipidemia and IR have been extensively reviewed [8], have been shown to provide improvement to NAFLD patients [9]. Eicosapentaenoic acid (EPA) is the only component of *n*-3 LCPUFA that is used clinically as a single-agent treatment for hypertriglyceridemia, and its TG-lowering effect in serum has been clinically proven [10]. Moreover, the efficacy of EPA for treatment of major coronary events was evidenced in a large-scale prospective randomized clinical trial [11]. EPA has been reported to decrease de novo lipogenesis via downregulation of sterol regulatory element binding protein 1c (SREBP-1c) [12], and induces fatty acid catabolism through the activation of the PPARs-mediated pathway in the liver [13]. Moreover, EPA decreases oxidative stress in the liver [14, 15], and suppresses production of proinflammatory cytokines in mice likely through the inhibition of nuclear factor (NF)-κB activity [16, 17]. While these suppressive effects of EPA on hepatic TG accumulation, oxidative stress, and inflammation suggest the preventive effect of EPA on hepatic fibrosis in NASH, this effect has not been fully clarified. Here, to assess the effects of EPA on progression of hepatic fibrosis and learn more about its mechanistic details, we investigated the effects of EPA on hepatic fibrosis in rats fed an MCD diet, which induces steatohepatitis histologically similar to NASH patients [18].

Materials and Methods

Drugs and Diets

EPA ethyl ester (EPA-E) (98% pure; Mochida Pharmaceutical, Tokyo, Japan) was suspended in 5% arabic gum at 100 mg/mL (Wako Pure Chemical Industries, Osaka, Japan) with 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 shown in Table 1.

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 (22–24°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 study periods of 8 and 20 weeks were examined. After 1 week of acclimation, animals were randomly divided into three groups of 10 rats in the 8-week study or three groups of 20 rats in the 20-week study. In both studies, two groups were fed the MCD diet, and one group was also administered EPA-E (1,000 mg/kg) daily by gavage. Another group was fed the MCS diet as a control. 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 [19], which is less than that in humans given a clinical dose of EPA-E (1,800 mg/day) for 3 months (143 μg/mL) [20]. Therefore, 1,000 mg/kg/day

Table 1 Composition of MCS and MCD diets

Nutrient	MCS	MCD
L-Amino acids	142.0	142.0
L-Methionine	1.7	0.0
Choline chloride	8.0	0.0
Cornstarch	100.0	100.0
Dextrin	100.0	100.0
Sucrose	398.7	408.6
Cellulose	50.0	50.0
Corn oil	50.0	50.0
Sodium bicarbonate	4.3	4.3
Primex (hydrogenated vegetable oil)	100.0	100.0
Salt mix	35.0[a]	35.0[b]
Vitamin mix	10.0	10.0
Ferric citrate	0.3	0.1

Note: Values are expressed as g/kg of each diet

[a] No Fe added

[b] Fe (0.21 g/kg) added

was considered to be an appropriate dose for investigation of the pharmacological effects of EPA-E in rats. Groups not given EPA-E were administered 5% arabic gum solution as vehicle. At the end of the experiment, blood was collected from inferior vena cava under isoflurane anesthesia and the liver was removed. A portion of liver tissue was fixed in 10% buffered formalin for histology, another portion was frozen in liquid nitrogen for measurement of hepatic content of TG, hydroxyproline, and transforming growth factor β1 (TGF-β1), and a third portion was immersed in RNAlater (Ambion, Austin, TX) and frozen for isolation of messenger RNA (mRNA). Frozen samples were stored at −80°C until use.

Measurement of Serum Biochemical Markers

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, albumin, total protein, choline esterase, and hyaluronic acid were measured using commercial kits (Wako and Fujirebio, Tokyo, for hyaluronic acid) and an automated blood analyzer (BiOLis 24i, Tokyo Boeki Medical System, Tokyo). Serum levels of collagen IV (Exocell, Philadelphia, PA), 8-isoprostane (Cayman Chemical, Ann Arbor, MI), and ferritin (Mitsubishi Chemical Safety Institute, Tokyo) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Serum levels of soluble tumor necrosis factor α receptor 1 (sTNFR1), and sTNFR2 were also measured with ELISA (R&D Systems Inc., Minneapolis, MN).

Liver Histology and Quantitation of Fibrosis

Formalin-fixed liver tissue was embedded in paraffin. Sections of the left lobe were stained with hematoxylin and eosin (HE) in the 8-week study.

To determine degree of hepatic fibrosis, Masson-trichrome (MT) staining was performed with the 20-week livers. MT-positive areas were photographed digitally, quantified with an image analysis system (Win ROOF version 5.5, Mitani Corporation, Fukui, Japan), and expressed as percentage of total area. Five randomly selected areas per specimen were analyzed.

Measurement of Hepatic Levels of TG, Hydroxyproline, and TGF-β1

To assess hepatic TG content, liver tissue was homogenized and lipid was extracted according to Folch et al. [21]. TG levels were determined using a commercial kit (Triglyceride E-test Wako, Wako). Hepatic hydroxyproline was quantitated as previously described [22]. Briefly, 0.5 mL liver homogenate was hydrolyzed in 6 mol/L hydrochloric acid (HCl) at 110°C for more than 13 h. After centrifugation, supernatant was neutralized with sodium hydroxide and mixed with chloramine T solution (Sigma–Aldrich, St Louis, MO). Following a 20-min incubation, perchloric acid (Wako) was added. Reaction was developed by addition of *p*-dimethylaminobenzaldehyde (Wako) and absorbance at 557 nm was measured. Hydroxyproline levels were determined from a standard curve and expressed as μg/g liver. Hepatic TGF-β1 levels were

Table 2 Primer pairs used for quantitative real-time RT-PCR

Gene	Forward primer	Reverse primer	Amplicon size (bp)	GenBank accession no.
Collagen α1(I)	TCCCCCAAAGACACAGGAAA	TGGGACGATTCAGCATTGC	67	NM_053304
Collagen α2(I)	CCCAGCCAAGAATGCATACA	TGGCTGCCACCATTGATG	78	NM_053356
CTGF	AGACGTTTGTGCCTATTGTTCTTG	TGTCCTATCGATGGTGTTTGGA	87	NM_022266
GAPDH	GCTACACTGAGGACCAGGTTGTCT	CCCAGCATCAAAGGTGGAA	71	NM_017008

Table 3 Effects of the MCD diet and treatment with EPA-E in rats (8-week study)

	MCS	MCD	MCD + EPA-E
Body weight (g)	471 ± 11.6	176 ± 2.0$^{\#\#}$	175 ± 2.5
Liver weight (g)	11.0 ± 0.4	8.1 ± 0.3$^{\#\#}$	8.1 ± 0.2
Hepatic TG content (mg/g liver)	19.8 ± 1.8	277.6 ± 11.1$^{\#\#\#}$	208.1 ± 14.7**

Rats were fed the MCS or the MCD diet for 8 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage

Values are expressed as mean ± SEM for ten rats in each group

$^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$, compared with the MCS diet

** $P < 0.01$, compared with the MCD diet

measured as previously described [23]. Briefly, liver tissue was homogenized in tissue protein extraction reagent (T-PER, Pierce, Rockford, IL) containing protease inhibitors (Halt Protease Inhibitor Cocktail Kit, Pierce) and centrifuged. TGF-β1 in the supernatant was measured using an ELISA kit (R&D Systems). TGF-β1 was assessed as the active form by addition of HCl, and expressed as ng/g liver.

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

Total RNA from liver tissue stored in RNAlater was isolated with TRIzol LS reagent (Invitrogen, Carlsbad, CA) and an RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA (5 μg) from each sample was reverse-transcribed to complementary DNA (cDNA) using the SuperScript III first-strand synthesis system (Invitrogen), according to the manufacturer's instructions. Hepatic 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 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Quantitative real-time RT-PCR and analysis were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with the SYBR Green PCR master mix (Applied Biosystems). 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 GAPDH value. Primer sequences are shown in Table 2.

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 the two groups, that is MCS-fed and MCD-fed group, and MCD-fed and MCD-fed plus EPA-E-treated group, respectively. If the variance was homogenous ($F > 0.05$), Student's *t*-test was performed; if the variance was heterogeneous ($F < 0.05$), the Aspin–Welch *t*-test was performed. In all analyses, SAS statistical programs were used (version 9.1.3, SAS Institute Japan, Tokyo). *P* values less than 0.05 were considered statistically significant.

Results

Effect of EPA-E in the 8-Week Study

Wistar rats fed the MCD diet for 8 weeks exhibited decreased body weight and liver weight compared with rats fed the MCS diet (Table 3). The livers of MCD-fed rats developed severe steatosis with large vacuoles in hepatocytes (Fig. 1b). Severe hepatic fibrosis was not observed at 8 weeks. Administration of EPA-E (1,000 mg/kg/day) by gavage in MCD-fed rats suppressed development of

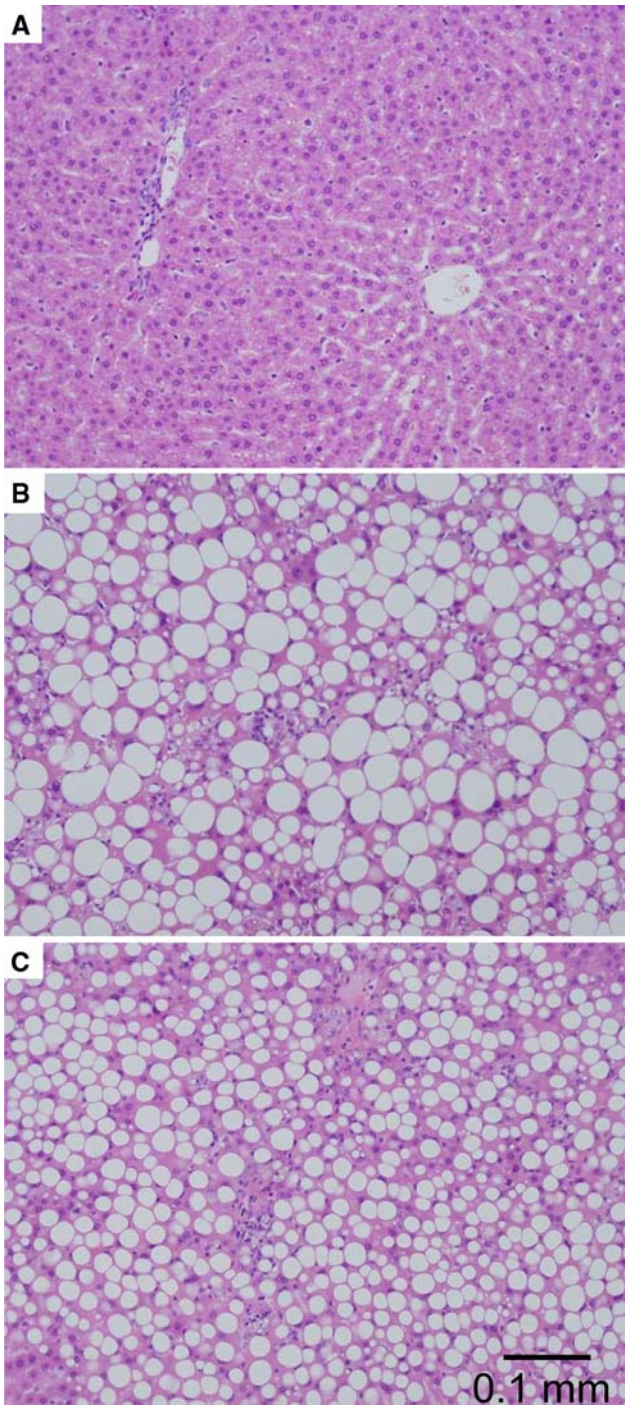

Fig. 1 Effects of the MCD diet and treatment with EPA-E on hepatic steatosis. Liver sections stained with HE from a rat fed with the MCS diet (**a**) or the MCD diet for 8 weeks with administration of vehicle (**b**) or EPA-E (1,000 mg/kg/day) (**c**) by gavage (original magnification: ×40)

vacuoles (Fig. 1c), and significantly decreased hepatic TG content by 25% (Table 3). These findings indicate that EPA-E suppresses hepatic steatosis induced by the MCD diet.

Effect of EPA-E in the 20-Week Study

Effect of EPA-E on Physiological Characteristics and Hepatic Disorder

Rats receiving the MCD diet for 20 weeks had decreased body weight and liver weight, as in the 8-week study, and EPA-E treatment suppressed the reduction in liver weight without further altering the body weight reduction seen with the MCD diet (Table 4). Macroscopically, the MCD diet caused nodule formation on the surface of the liver in 9 out of 20 rats. In contrast, no nodule formation was seen in MCD-fed rats also given EPA-E treatment. Serum levels of markers for hepatocyte injury (ALT and AST), bile duct injury (ALP), and total bilirubin were increased by the MCD diet. Reduced levels of indicators of liver function, including albumin, total protein, and choline esterase, were also observed. Additionally, compared with the MCS diet, the MCD diet caused an increase in the fibrosis markers hyaluronic acid and collagen IV. EPA-E treatment significantly improved these alterations in serum biochemical markers, except for ALT (Table 4). These data suggest that the MCD diet induces a hepatic disorder that may progress to cirrhosis, which is likely accompanied by fibrosis. EPA-E appears to prevent disease progression.

Effect of EPA-E on Hepatic Fibrosis and Level of Hepatic Hydroxyproline

We examined MT-stained liver sections from MCD-fed rats and observed severe fibrosis with perivenular and periportal fibrosis in MCD-fed rats (Fig. 2b), but not in MCS-fed rats (Fig. 2a). In contrast, EPA-E visibly reduced development of liver fibrosis (Fig. 2c). Quantitative image analysis showed that the extent of liver fibrosis in MCD-fed rats treated with EPA-E was reduced by about 60% compared with MCD-fed rats not treated with EPA-E (Fig. 2d). Quantification of hepatic hydroxyproline (Fig. 2e) supported the observation that the MCD diet increased, and EPA-E treatment reduced, hepatic fibrosis. These findings confirm that EPA-E prevents MCD-induced hepatic fibrosis.

Effect of EPA-E on Hepatic Expression of Fibrogenic Genes

To elucidate the molecular pathways by which EPA-E suppresses hepatic fibrosis, we examined mRNA expression of fibrogenic genes. MCD feeding increased hepatic mRNA levels of factors involved in fibrillar extracellular matrix (ECM) synthesis [collagen α1(I), α2(I), and CTGF] [24] compared with MCS-fed rats (Fig. 3). Treatment with EPA-E significantly reduced gene expression of collagen α1(1), α2(1), and CTGF, suggesting inhibition of fibrogenesis (Fig. 3).

Table 4 Effects of the MCD diet and treatment with EPA-E on physiological characteristics and serum markers in rats (20-week study)

	MCS	MCD	MCD + EPA-E
Body weight (g)	615 ± 12.0	169 ± 3.7$^{\#\#}$	176 ± 3.4
Liver weight (g)	11.8 ± 0.3	6.6 ± 0.2$^{\#\#}$	7.9 ± 0.3**
Nodule formation on the liver (no. of rats observed/total)	0/20	9/20	0/20
Serum markers			
ALT (IU/L)	23 ± 1	72 ± 4$^{\#\#}$	76 ± 3
AST (IU/L)	69 ± 2	321 ± 29$^{\#\#}$	239 ± 11*
ALP (IU/L)	131 ± 6	530 ± 35$^{\#\#}$	406 ± 24**
Total bilirubin (mg/dL)	0.08 ± 0.00	1.48 ± 0.10$^{\#\#}$	0.80 ± 0.06**
Albumin (g/dL)	3.5 ± 0.0	2.7 ± 0.1$^{\#\#}$	3.4 ± 0.1**
Total protein (g/dL)	6.0 ± 0.0	4.5 ± 0.1$^{\#\#}$	5.3 ± 0.1**
Choline esterase (IU/L)	151 ± 9	53 ± 6$^{\#\#}$	105 ± 9**
Hyaluronic acid (ng/mL)	20 ± 1	44 ± 6$^{\#\#}$	31 ± 2*
Collagen IV (μg/mL)	2.92 ± 0.13	4.33 ± 0.26$^{\#\#}$	3.15 ± 0.17**

Rats were fed the MCS or the MCD diet for 20 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage

Values are expressed as mean ± SEM for 20 rats in each group

$^{\#\#}$ $P < 0.01$, compared with the MCS diet

* $P < 0.05$, ** $P < 0.01$, compared with the MCD diet

Effect of EPA-E on Hepatic TGF-β1 Level

We next analyzed the effect of EPA-E on hepatic content of TGF-β1, which is a key profibrogenic cytokine that regulates expression of collagen and CTGF in liver [24]. Whereas MCD-fed rats exhibited increased hepatic TGF-β1 content compared with MCS-fed rats, EPA-E treatment significantly reduced MCD-induced increase in hepatic TGF-β1 levels (Fig. 4). These findings suggest that EPA-E reduces hepatic TGF-β1 level, leading to suppression of hepatic fibrosis.

Effect of EPA-E on Markers of Oxidative Stress and Inflammation

Further examination of serum biochemical markers revealed that the MCD diet induced significant elevation of serum levels of the oxidative stress markers, 8-iso-prostane [25] and ferritin, which indicates hepatic iron accumulation and enhanced oxidative stress in the liver [26]. EPA-E treatment significantly decreased these serum parameters that were increased by the MCD diet (Fig. 5a, b).

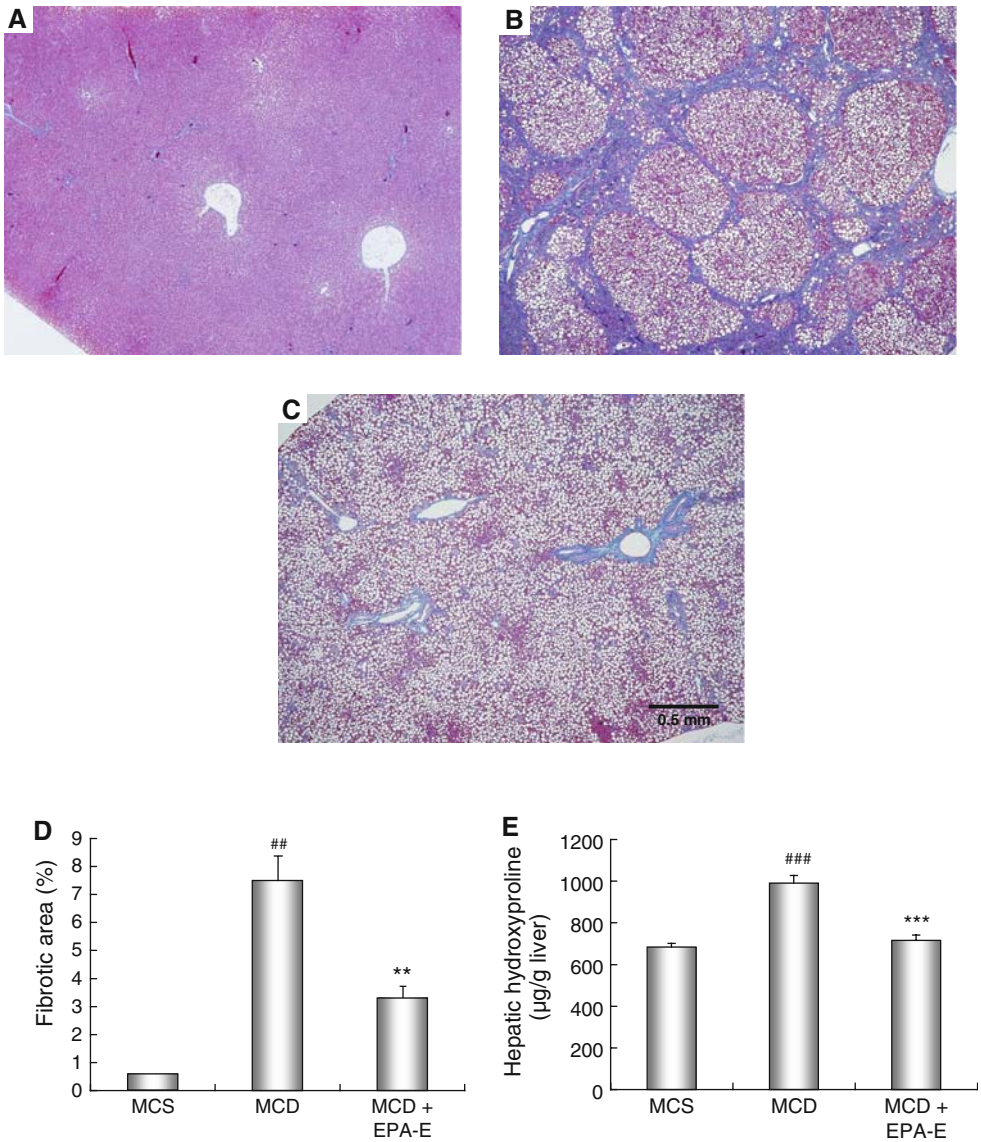

Fig. 2 Effects of the MCD diet and treatment with EPA-E on hepatic fibrosis. Liver sections stained with MT from a rat fed the MCS diet (**a**) or the MCD diet for 20 weeks with administration of vehicle (**b**) or EPA-E (1,000 mg/kg/day) (**c**) by gavage (original magnification: ×8). The MT-positive area (*blue*) was quantified with an image analysis system, and expressed as percentage of total area (**d**). Hepatic hydroxyproline level was determined as described in the "Materials and Methods" section (**e**). Values are expressed as mean ± SEM for 20 rats in each group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus MCS-fed rats; ** $P < 0.01$, *** $P < 0.001$ versus MCD-fed rats

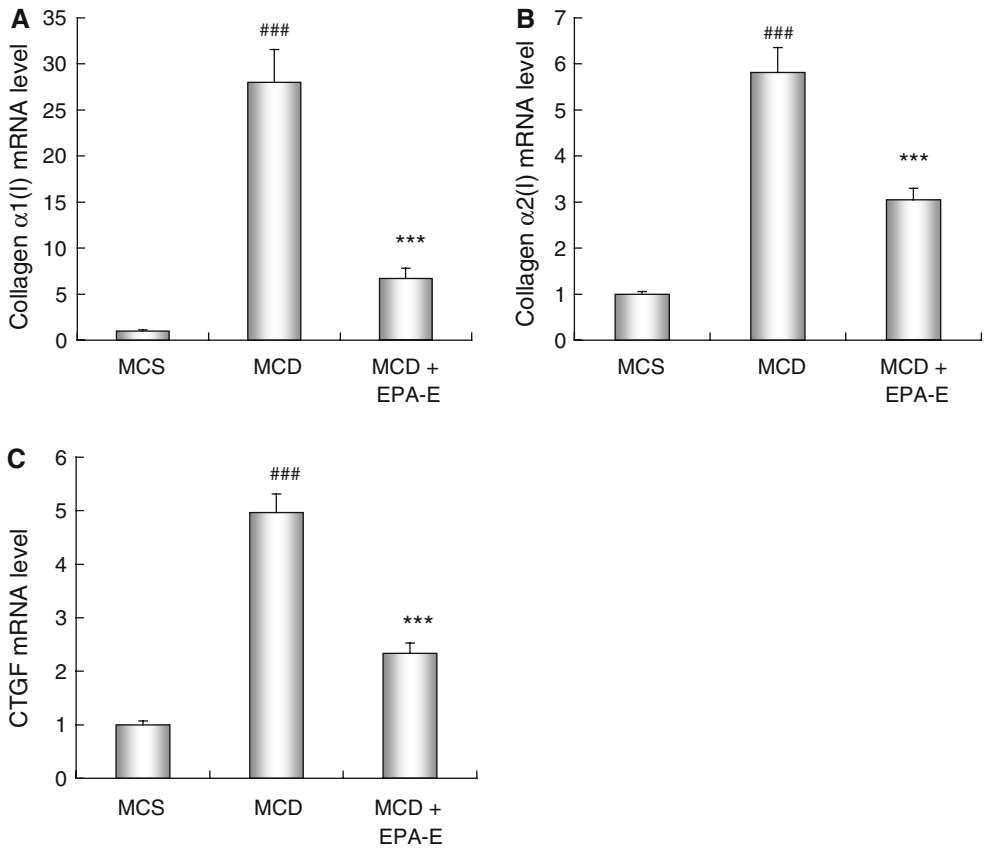

Fig. 3 Effects of the MCD diet and treatment with EPA-E on hepatic mRNA levels for fibrillar ECM synthesis [collagen α1(I) (**a**), α2(I) (**b**), and CTGF (**c**)]. Rats were fed the MCS or the MCD diet for 20 weeks and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage. mRNA levels were examined by quantitative real-time RT-PCR and normalized to GAPDH levels. The ratio of mRNA levels to the mean value of MCS-fed rats is shown. Values are expressed as mean ± SEM for 20 rats in each group. ### $P < 0.001$ versus MCS-fed rats; *** $P < 0.001$ versus MCD-fed rats

Enhanced oxidative stress may lead to inflammation during progression of NASH [1]. TNF-α plays an important role in induction of oxidative stress and inflammation [1]. We determined serum sTNFR1 and sTNFR2 levels, which are proposed as good indicators for TNF-α production because they remain elevated longer than serum level of TNF-α [27, 28], while serum TNF-α level was not detected (<5 pg, data not shown). Serum sTNFR1 and sTNFR2 levels, however, were increased by the MCD diet, and EPA-E treatment significantly decreased levels of both sTNFRs (Fig. 5c, d). These findings indicate that EPA-E suppresses the MCD-induced increases in oxidative stress and inflammation that may cause induction of hepatic fibrosis.

Discussion

We used a rat MCD dietary model to examine the effect of EPA on hepatic fibrosis. Rats fed MCD display fibrotic steatohepatitis that was histologically similar to that in NASH patients [18]. Furthermore, in this model, hepatic TG accumulation causes increased oxidative stress and inflammation, resulting in development of hepatic fibrosis [29, 30]. Finally, portal-portal and central-portal bridging fibrosis is established that partly develops to macronodular cirrhosis [29]. We have demonstrated in this paper that highly purified EPA-E completely represses MCD-induced nodule formation and suppresses bridging fibrosis. This effect of EPA-E was accompanied by decreased area of fibrosis in the liver as judged by histological analysis, decreased hepatic hydroxyproline content, and decreased gene expression of collagen and CTGF. These results indicate that the preventive effect of EPA on progression of liver cirrhosis is due to inhibition of hepatic fibrosis.

In MCD-fed rats, the condition that underlies the development of fibrosing steatohepatitis is considered to be hepatic fat accumulation. In our study, EPA significantly suppressed the increase in the level of TG within the liver that was induced by MCD feeding. The mechanisms by which EPA inhibits hepatic fat accumulation are likely to involve a reduction in de novo lipogenesis mediated by

downregulation of SREBP-1c [12], and an increase in fatty acid oxidation through PPARα activation [13]. In addition, we have recently reported that reduction in hepatic fat mediated by EPA prevents progression of steatotic liver injury and reduces oxidative stress and inflammation [15]. Thus, the inhibitory effect of EPA on fat accumulation may contribute to its ability to prevent progression of hepatic fibrosis, at least in part, via a reduction in oxidative stress and inflammation. However, EPA only decreased hepatic TG content by 25% compared with control. This makes it difficult to contend that the main mechanism by which EPA inhibits fibrosis is mediated by suppression of hepatic TG accumulation. Moreover, it has been reported that EPA has an antifibrotic role in a bleomycin-induced pulmonary fibrosis model, which was not dependent on tissue TG content [31]. Therefore, we must assume that EPA can suppress fibrosis by several pharmacological mechanisms that are independent of EPA-induced reduction of hepatic TG.

Hepatic fibrosis is caused by accumulation of excess ECM that is mainly synthesized from HSCs. The activity of HSCs is increased by several factors, including ROS, peroxidated lipids, and proinflammatory cytokines such as TNF-α and TGF-β1 [32]. We therefore considered that the following three mechanisms might be involved in the inhibitory effects of EPA on hepatic fibrosis in MCD-fed rats. The first mechanism that we examined was oxidative stress. It is known that, in steatohepatitis, excess fat accumulation in hepatocytes causes ROS induction via enhanced fatty acid oxidation and mitochondrial dysfunction [1]. ROS triggers lipid peroxidation of plasma or mitochondrial membranes, and also induces expression of proinflammatory cytokines [1]. In this study, MCD feeding caused severe steatosis that was accompanied by increased levels of markers for oxidative stress. This result is consistent with a previous report that MCD feeding increases the level of hepatic peroxidated lipids as steatosis increases [30]. These findings suggest that, in our study, MCD feeding induced an increase in hepatic ROS. EPA has been reported to suppress ROS production in vivo and in vitro, at least in part by induction of a superoxide scavenging pathway [14, 33]. Therefore, EPA may inhibit hepatic fibrosis by directly reducing the level of ROS.

The second mechanism that we investigated to explain the effect of EPA on hepatic fibrosis in MCD-fed rats was via an effect on TNF-α production. TNF-α provokes liver inflammation and hepatocyte injury, and is considered to play an important role in progression of steatohepatitis [34]. TNF-α also directly induces ROS by interference with the normal electron flow in the mitochondria [35]. In our study, EPA suppressed the MCD-induced increase in sTNFR levels, which are considered to be good indicators of TNF-α production [27, 28]. Consistent with previous reports, MCD feeding did not induce increased body weight and obesity [30]. Hepatic TNF-α expression has been reported to be increased in MCD-fed rats [7]. These findings suggest that sTNFR levels in this study reflect TNF-α production in the liver, but not in adipose tissue, which is considered to be a major source of TNF-α in NASH patients [34]. It has also been reported that EPA inhibits the secretion of TNF-α from macrophages and lymphocytes by suppressing activation of NF-κB [16]. Based on these data, we propose that EPA will directly suppress hepatic TNF-α production, leading to inhibition of inflammation and further ROS generation.

The third mechanism that we considered was an effect of EPA on TGF-β1 production. TGF-β1 is considered a key molecule for induction of hepatic fibrosis [24]. An elevated level of TGF-β1 has been detected in fibrotic human and rat liver [29, 36], and blockade of TGF-β1 synthesis and signaling prevents hepatic fibrosis in various animal models [37, 38]. In our study, prevention of the MCD-induced increase in hepatic fibrosis by EPA-E was accompanied by suppression of TGF-β1. Therefore, inhibition of the TGF-β1-stimulated signaling cascade is considered to be a key mechanism by which EPA suppresses fibrosis. Although it has been reported that EPA suppresses expression of TGF-β1 in vivo and in vitro [39, 40], the pharmacological mechanism by which EPA suppresses TGF-β1 is not fully understood. However, several reports suggest that EPA inhibits expression of TGF-β1 via direct activation of a PPARγ-mediated pathway and/or direct

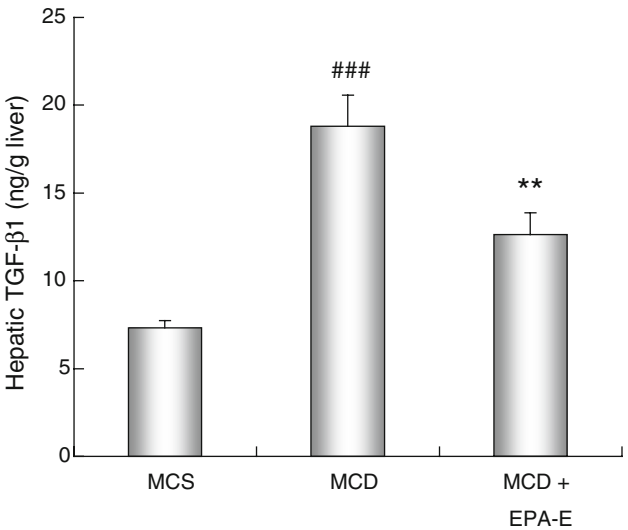

Fig. 4 Effect of the MCD diet and treatment with EPA-E on hepatic TGF-β1 content. Rats were fed the MCS or the MCD diet for 20 weeks, and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage. Hepatic TGF-β1 level was determined as described in the "Materials and Methods" section. Values are expressed as mean ± SEM for 20 rats in each group. ### $P < 0.001$ versus MCS-fed rats; ** $P < 0.01$ versus MCD-fed rats

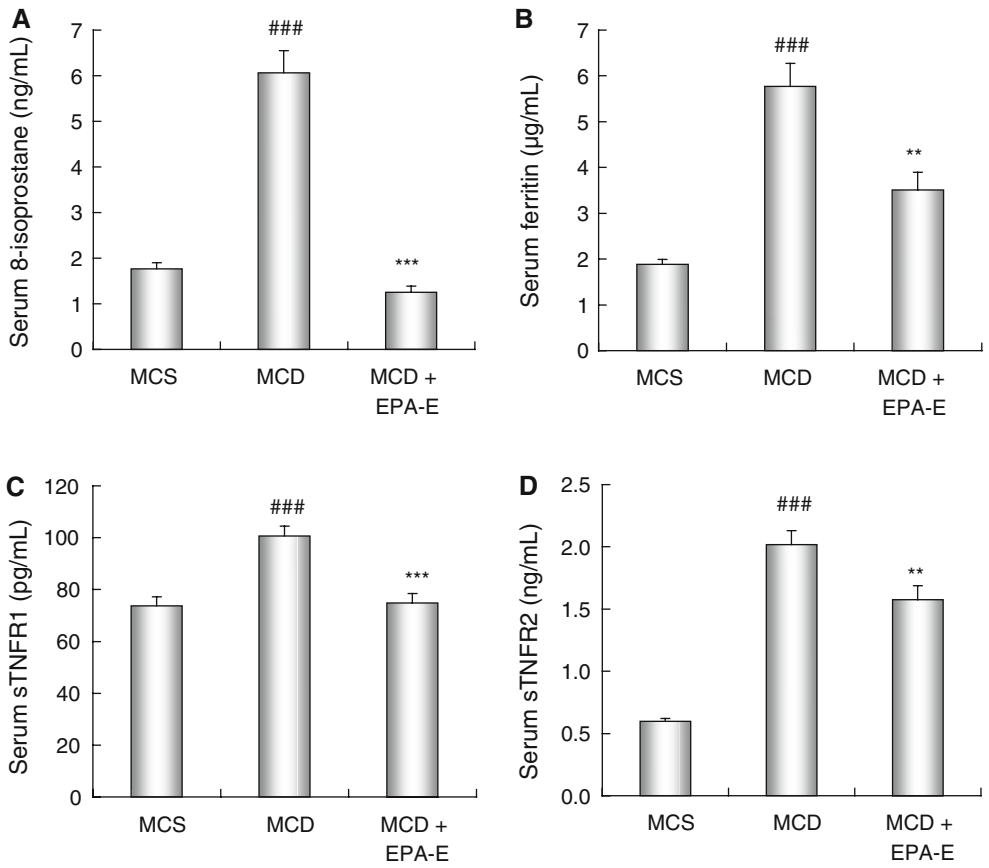

Fig. 5 Effects of the MCD diet and treatment with EPA-E on serum levels of 8-isoprostane (**a**), ferritin (**b**), sTNFR1 (**c**), and sTNFR2 (**d**). Rats were fed the MCS or the MCD diet for 20 weeks, and administered EPA-E (1,000 mg/kg/day) or vehicle by gavage. Values are expressed as mean ± SEM for 20 rats in each group. ### $P < 0.001$ versus MCS-fed rats; ** $P < 0.01$, *** $P < 0.001$ versus MCD-fed rats

inhibition of a protein kinase C (PKC) pathway. EPA has been reported to have the potential to bind and interact with PPARγ, and to influence a PPARγ-signaling pathway [41–43]. It has been reported that a PPARγ agonist suppresses MCD-induced hepatic fibrosis concomitant with impairment of the MCD-induced increase in TGF-β1 expression in vivo [44], and also inhibits TGF-β1 gene expression in vitro [45]. Moreover, EPA has been reported to suppress TGF-β expression via inhibition of a PKC pathway in vascular smooth muscle cells [39, 46, 47]. Therefore, these findings imply that expression of TGF-β1 could be inhibited by EPA via activation of PPARγ and inhibition of a PKC pathway.

Based on the above considerations, it is suggested that EPA inhibits hepatic fibrosis by direct suppression of several factors involved in progression of fibrotic steatohepatitis. Indeed, EPA-E has been reported to improve the NAFLD activity score of NASH patients in a pilot clinical trial [48]. Therefore, EPA-E treatment may attenuate hepatic fibrosis due to inhibitory effects on various pathogenic factors in NASH. Further studies are needed to clarify the mechanisms involved in suppression of hepatic fibrosis by EPA.

In the present study, we have shown that EPA-E prevents progression of hepatic fibrosis by reducing fibrillar ECM synthesis in MCD-fed rats. EPA may suppress progression of hepatic fibrosis by inhibiting the TGF-β1-stimulated signaling pathway in association with reduced hepatic steatosis, oxidative stress, and inflammation. Therefore, EPA-E treatment may be a potential therapy for treating NASH. Further investigation will be needed to clarify the precise mechanisms by which EPA prevents progression of this pathology in a NASH model.

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