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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Dietary olive oil prevents carbon tetrachloride-induced hepatic fibrosis in mice

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Abstract
Aim The specific purpose of this study was to investigate the effects of dietary olive oil on hepatic fibrosis induced by chronic administration of carbon tetrachloride (CCl_4) in the mouse. In addition, the effects of oleic acid, a major component of olive oil, on activation of hepatic stellate cells (HSCs) were investigated in vitro.
Methods Mice were fed liquid diets containing either corn oil (control, AIN-93) or olive oil (6.25 g/L) throughout experiments. Animals were treated with CCl_4 for 4 weeks intraperitoneally. The mRNA expression of TGF-β1 and collagen 1α2 (col1α2) in the liver was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). The HSCs were isolated from mice, and co-cultured with either oleic acid (100 μM) or linoleic acid (100 μM) for 2 days. The expression of alpha-smooth muscle actin (α-SMA) was assessed by immunohistochemistry. In addition, the production of hydroxyproline was determined.
Results Serum alanine aminotransferase levels and the mRNA expression of TGF-β and collα2 were significantly reduced by treatment of olive oil. Dietary olive oil blunted the expression of α-SMA in the liverand liver injury and hepatic fibrosis were prevented by treatment of olive oil. The number of α-SMA positive cells was significantly lower in HSCs co-cultured with oleic acid than in those co-cultured with linoleic acid. Concentration of hydroxyproline in culture medium was significantly lower in cells co-cultured with oleic acid than in the control.
Conclusions Dietary olive oil prevents CCl_4-induced tissue injury and fibrosis in the liver. Since oleic acid inhibited activation of HSCs, oleic acid may play a key role on this mechanism.

Keywords Fatty acid · Hepatic stellate cells · Transforming growth factor-β1

Abbreviations

CCl_4	Carbon tetrachloride
HSC	Hepatic stellate cell
ALT	Alanine aminotransferase
TGF-β1	Transforming growth factor-β1
α-SMA	α-Smooth muscle actin

Introduction

Nutrition is an important factor in the pathogenesis of acute and chronic liver disease [1]. Indeed, it has been previously reported that fatty acids exhibit protective effects on alcohol- or endotoxin-induced liver injury [2, 3]. Traditional Mediterranean diets chiefly containing olive oil reduced the risk of coronary heart disease [4]. Beneficial effects of oleic acid, a major component of olive oil, have been observed not only in aortic atherosclerosis [5], but also in gastroprotective actions on the stomach [6] and in lipogenesis and cholesterologenesis [7]. The protective effects of olive oil on liver injury caused by oral carbon tetrachloride (CCl_4) intake have been reported in animal models [8]. Thus, fatty acids play an important role in hepatic fibrosis.

During initiation of hepatic fibrosis, hepatic stellate cells (HSCs), which are key producers of the extracellular

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matrix (ECM) under conditions of liver injury, proliferate and acquire the characteristics of contractile cells (myofibroblasts) [9]. Thus, activated HSCs play a key role on hepatic fibrogenesis. In culture conditions, they spontaneously undergo transformation and activation. This transformation is useful in understanding the molecular mechanism underlying the activation of HSC in the injured liver, and the transformed myofibroblast-like cells can be detected by immunohistochemical staining for alpha-smooth muscle actin (α-SMA) [10].

Accordingly, the aim of the present study was to examine the effects of olive oil on hepatic fibrosis in a mouse model induced by chronic intraperitoneal administration of CCl_4. The effects of oleic acid, a major component of olive oil, on fibrogenesis were also investigated in isolated HSCs.

Various studies have reported the effects of fatty acids on hepatic fibrosis induced by CCl_4 [11–17]; in this study, mice were treated with a liquid diet containing fatty acids for wide applications of clinical nutrition.

Materials and methods

Animals and treatments

Male C57BL/6N mice (9 weeks old) were obtained from Charles River (Yokohama, Japan). All animals used for this study were housed in sterilized cages in a facility with a 12-h night/day cycle. The experimental protocol followed the institutional and National Research Council criteria for the care and use of laboratory animal research. Furthermore, all animals were given humane care in compliance with governmental regulations and institutional guidelines. The powder diet without oil (AIN-93) was purchased from Oriental Yeast (Tokyo, Japan). Animals were randomly allocated into the two groups. Mice were fed liquid diets containing either corn oil (the control group) or olive oil (the olive oil group) (Table 1). The liquid diets were prepared every morning. After 1 week of feeding of the liquid diets, intraperitoneal injection of CCl_4 (1 ml/kg) was started to induced hepatic fibrosis twice a week for 4 weeks [16], and mice were sacrificed 48 h after the last injection. At sacrifice, mice were anesthetized with diethyl ether. After exsanguination, livers were removed and weighed. Sections from the right median and left lateral lobe were fixed in 10% formalin and embedded in paraffin. Remaining tissue specimens were snap frozen in liquid nitrogen and stored at −80°C until assayed. All animals had free access to water throughout the study.

Table 1 Content of diets

Components	Control diet (g/L)	Olive oil diet (g/L)
Casein	19.32	19.32
L-cystine	0.25	0.25
Maltodextrin	90.00	90.00
Corn oil	6.25	0
Olive oil	0	6.25
Cellulose	6.90	6.90
Mineral mix, AIN-93G-MX	4.83	4.83
Vitamin mix, AIN-93-VX	1.38	1.38
Choline bitartrate	1.25	1.25
Xanthan gum	1.50	1.50

Quantitative analysis of hepatic fibrosis

The Sirius red reaction was used for analysis of hepatic fibrosis. The area of hepatic fibrosis was calculated as means of the randomly selected six different fields in each liver sample, and indicated as a percentage of the total area of the field using PhotoShop and Image J software [16].

Immunohistochemistry for α-SMA in the liver

The expressions of α-SMA in the liver were detected by immunohistochemical staining as described elsewhere [18]. Briefly, formalin-fixed and paraffin-embedded tissue sections were deparaffinized. After incubation with 0.3% hydrogen peroxide to block endogenous peroxidase and subsequently with normal horse serum to inhibit non-specific reactions, the sections were incubated with monoclonal mouse anti-human smooth muscle actin antibody (Dako A/S, Glostrup, Denmark, 1:100 dilution) for 1 h at room temperature. Following incubation, immunoperoxidase staining was completed using a Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) and diaminobentizine (DAB) as a chromogen.

Determination of serum ALT levels

Serum samples were collected from the inferior vena cava, and assessed for alanine aminotransferase (ALT) by standard enzymatic procedures [19].

Measurement of the mRNA expression of TGF-β and col1α2 by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of TGF-β1 and col1α2 was assessed by semiquantitative RT-PCR as previously reported ($n = 6$ in each group) [20]. Total RNA was isolated using an RNA purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RT of total RNA (2 μg) was performed in a final volume of 100 μl containing 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM/L each

deoxy-unspecified nucleoside 5′-triphsophate, 2.5 μM random hexamers, 0.4 U/ml RNase inhibitor, and 1.25 U/ml multiscribe RT. PCR primers for TGF-β1 [21], col1α2 [22], and β-actin [14] contained the following sequences: TGF-β1 sense (5′-AAAATCAAGTGTGGAGCAAC-3′), and antisense (5′-CAAGAGACTTCCAGCCAGTTGC-3′); col1α2 sense (5′-GGAACAGCGATTACTACTGG-3′), and antisense (5′-TCTCCTAACCAGACATGCTT-3′); β-actin sense (5′-TGGAATCCTGTGGCATCCATGAAAC-3′), and antisense (5′-TAAAACGCAGCTCAGTAACAGTC CG-3′).

The size of amplified PCR products was 224 base pairs (bp) for TGF-β1, 172 bp for col1α2, and 348 bp for β-actin. Aliquots (5 μL) of synthesized cDNA were added to 45 μL PCR mix containing 5 μL 10× PCR buffer, 1 μL each deoxynucleotide (1 mmol/L each), 0.5 μL sense and antisense primers (0.15 mmol/L), and 0.25 μL DNA polymerase (Gene Amp PCR kit, Perkin Elmer Cetus, Norwalk, CT). The reaction mixture was covered with a wax gem (Perkin Elmer Cetus), and amplification was initiated by 1 min denaturation at 94°C for 1 cycle, followed by multiple (28–30) cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using a GeneAmp PCR system 9800 DNA thermal cycler (Perkin Elmer Cetus). After the last cycle of amplification, samples were incubated for 7 min at 72°C. The amplified PCR products were subjected to electrophoresis at 100 V through a 2% agarose gel (Gibco Laboratories Life Technologies) for 30 min. The agarose gels were stained with 0.5 mg/mL ethidium bromide Tris–borateethylene diaminetetraacetic acid buffer (ICN, Costa Mesa, CA) and photographed with Type 55 Polaroid positive/negative film. Densitometric analysis of the captured image was performed using Image J Analysis software. The area under the curve was normalized for β-actin content.

Isolation and culture of the hepatic stellate cell

Hepatic stellate cells were isolated from male C57BL/6N mice (10–12 weeks old) using a modification of the methods of Baba et al. and Kawada et al. [23–26]. Six mice were used for each isolation. Briefly, the inferior vena cava was cannulated with a small length of polypropylene tube, and ligated above the diaphragm, and the portal vein was dissected. The liver was then perfused with Gey's balanced salt solution, pH 7.3 for 120 mL/h 5 min at 37°C followed by 0.07% collagenase solution containing 50 mg of pronase for 10 min. Digested liver tissue was filtered through a 60-μm nylon mesh to eliminate nondigested material. The nonparenchymal fraction was subjected to separation of the HSC by density gradient centrifugation at 1,400×g using 8.2% Nycodenz (Myegaard, Oslo, Norway) for 20 min at 4°C. About 6×10^6 cells were collected. Cells were washed, and cultured on uncoated plastic dishes in DMEM (Invitrogen, Carlsbad, CA).

Immunocytochemical analysis for cultured cells

Isolated HSCs were seeded at a density of 1×10^6 cells/mL [27] in 24-well culture dishes, and cultured in DMEM media containing 10% FCS for 24 h. After washing in phosphate-buffered saline (PBS) to remove non-adherent cells, the cells were divided into three groups; (1) DMEM (control), (2) DMEM containing linoleic acid (the linoleic acid group), and (3) DMEM containing oleic acid (the oleic acid group). For the linoleic acid group, 100 μM of linoleic acid conjugated with bovine serum albumin (BSA) was added to the medium [28]. For the oleic acid group, 100 μM oleic acid conjugated with BSA was added to the medium [28]. Culture medium was changed every 48 h in each group. After incubation for 7 days, the adherent cells were washed with PBS. Subsequently, the cells were fixed with 4% paraformaldehyde fixative overnight at 4°C. After incubation with 0.3% hydrogen peroxide to block endogenous peroxidase, and subsequently with normal horse serum to inhibit non-specific reactions, the cells were incubated with monoclonal mouse anti-human smooth muscle actin antibody (Dako A/S, Glostrup, Denmark, 1:100 dilution) for 1 h at room temperature. Following incubation, immunoperoxidase staining was completed using a Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) and DAB as a chromogen. The number of α-SMA positive cells was counted from five different fields in each dish.

Hydroxyproline concentrations in culture medium of isolated HSC

Isolated HSCs were seeded at a density of 1×10^6 cells/ml in 24-well dishes, and cultured in DMEM for 24 h. After pre-incubation the cells were divided into the three groups, and the HSCs were co-cultured as previously described for 2 days. The supernatant hydroxyproline concentration was determined as described elsewhere [29]. Briefly, 40 μl of the neutralized sample solution was removed to a 96-well ELISA plate and oxidized in each well with a solution containing 5 mL of 7% Chloramine T (Sigma, St Louis, MO, USA) and 20 mL of acetate/citrate buffer. Thereafter, 150 mL of Ehrlich's solution was added. The final mixture was incubated at 60°C for 35 min and then at room temperature for 10 min, and the absorbance was determined at 560 nm.

Statistical analysis

Data are expressed as mean ± SEM. The Student's t test was used for the determination of significance as

appropriate. **A P value less than 0.05 was selected before the study as the level of significance.

Results

Weight gain and food intake in mice fed with liquid diets containing different types of fatty acids

Steady body weight gain was observed throughout experiments in each group (Fig. 1), and there were no significant differences between the control group and the olive oil group (Table 2). Furthermore, there were no significant differences in liver mass per body weight ratio and food intake between the two groups.

Serum ALT levels

Serum ALT levels were about 330 IU/L in the controls. Values were significantly reduced by 55% in mice fed the liquid diet containing olive oil compared with mice fed the control diet (Fig. 2).

Effects of olive oil on fibrosis induced by CCl_4 and the expression of α-SMA in the liver

Steatotic changes were not observed in two groups after treatment of CCl_4 (data not shown). In the control mice, hepatic fibrosis detected by the Sirius red reaction was observed in the periportal area (Fig. 3). The fibrosis was significantly blunted in mice fed the liquid diet containing olive oil. The expression of α-SMA was detected in the control group in the periportal area by immunohistochemical staining, and was concomitant with hepatic fibrosis (Fig. 3). This expression was markedly blunted by dietary olive oil.

Quantitative analysis of fibrosis area by image analysis showed significant reduction of fibrosis by 40% in the olive oil group compared with the controls (Fig. 3e).

Effect of dietary oil on the mRNA expression TGF-β1 and collagen 1α2 in the liver

The mRNA expression of TGF-β1 was detected in the controls (Fig. 4). This expression was significantly blunted by 40% in mice fed on olive oil containing diet compared

Table 2 Weight and daily intake of liquid diets

	Control ($n = 6$)	Olive oil ($n = 6$)	P value
Growth rate of body weight (%)	9.72 ± 4.85	10.4 ± 4.05	NS
Liver/body (weight ratio)	49.4 ± 3.48	58.3 ± 10.3	NS
Daily intake of liquid diet (g)	22.8 ± 6.02	21.8 ± 6.66	NS

Data represent mean ± SD

NS not significant

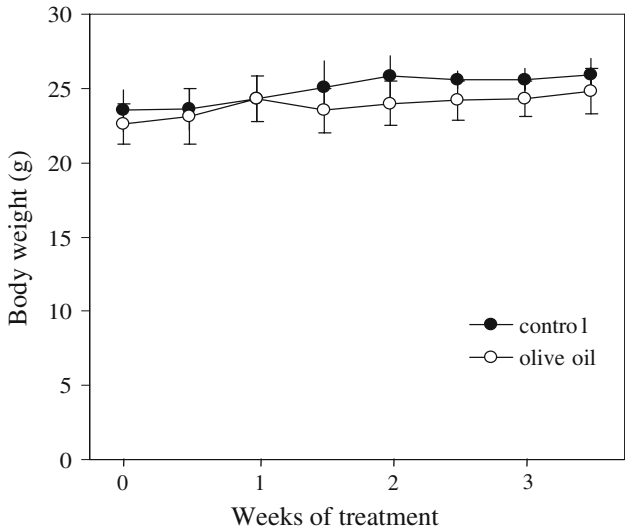

Fig. 1 Body weights. Body weights in mice fed liquid diets containing corn oil (control) or olive oil were measured before injections of CCl_4 and after treatment once a week ($n = 6$ in each group). *Closed circle* mice fed control diets, *open circle* mice fed liquid diets containing olive oil

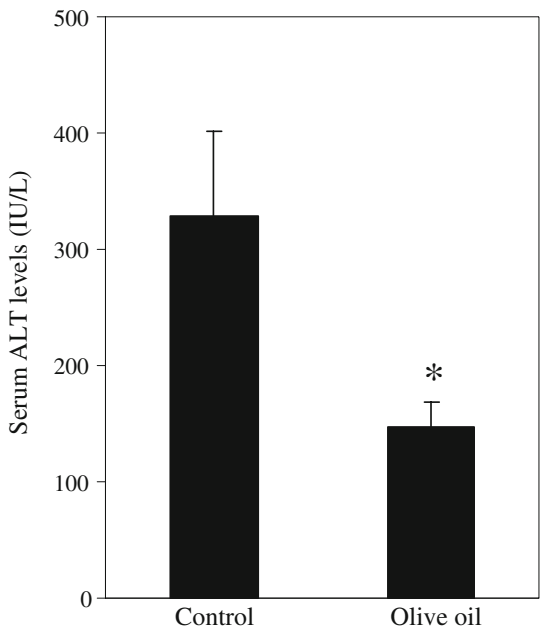

Fig. 2 Effects of fatty acids on serum alanine aminotransferase levels. Mice were treated as described in "Materials and methods". Serum samples were collected 48 h after last treatment of CCl_4. The values are mean ± SE ($n = 6$ in each group). *$P < 0.05$ compared with the control group by the unpaired Student's t test

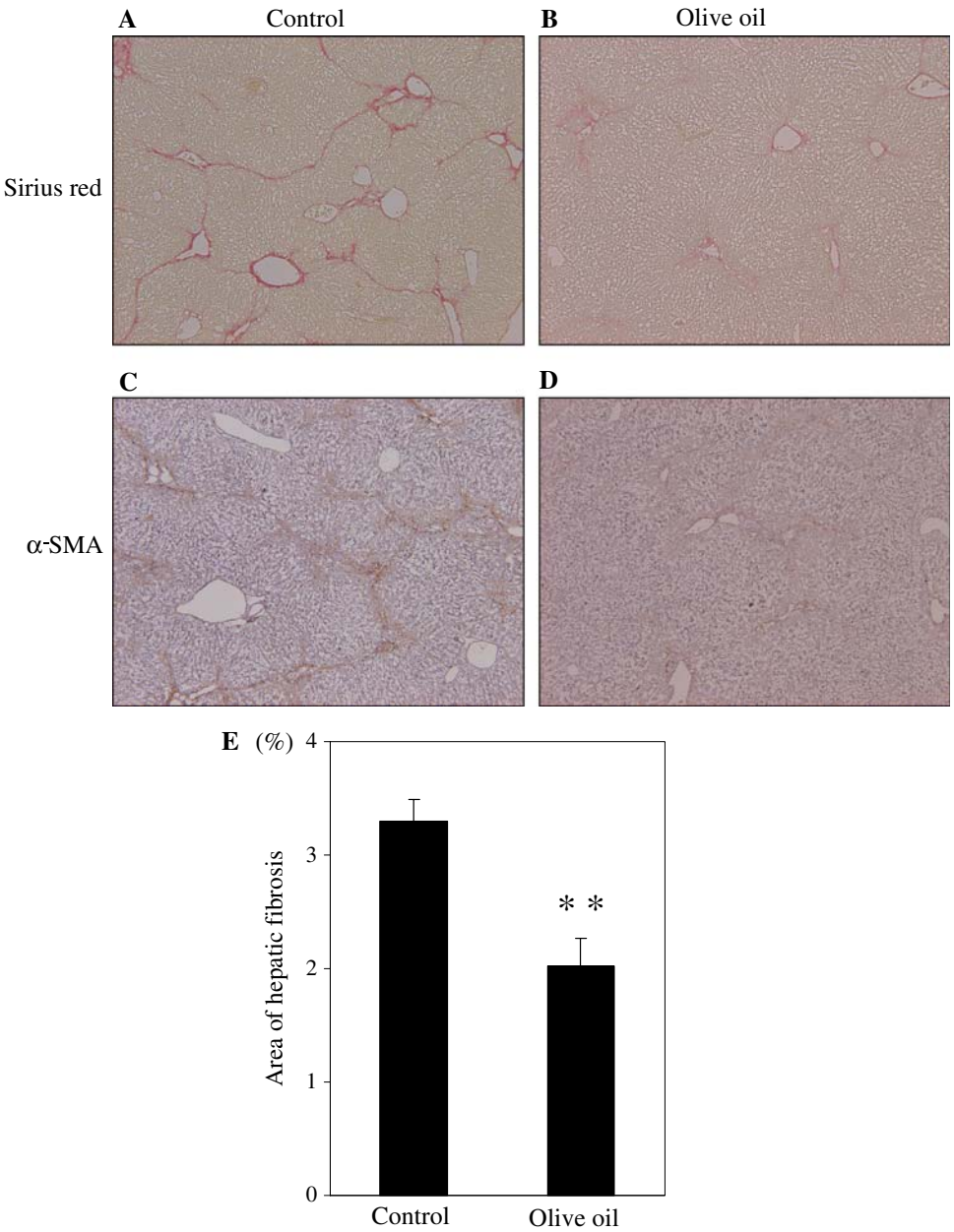

Fig. 3 Effects of fatty acids on hepatic fibrosis. Mice were treated as described in "Materials and methods". **a** The Sirius red reaction in livers from CCl_4-treated mice fed control diets, **b** the Sirius red reaction in livers from CCl_4-treated mice fed liquid diets containing olive oil, **c** immunohistochemistry for α-SMA in livers from CCl_4-treated mice fed control diets, and **d** immunohistochemistry for α-SMA in livers from CCl_4-treated mice fed liquid diets containing olive oil. Representative photomicrographs, ×100. **e** the area of hepatic fibrosis area was calculated as the mean of six different randomly selected fields in liver sections, and indicated as a percentage of the total area of the field using Photo Shop and Image J software. Data represent mean ± SE ($n = 4$ in each group). $^{**}P < 0.01$ compared with the control group by the unpaired Student's t test

with control diet. The mRNA expression of col1α2 was also detected in the control group. This expression was significantly blunted by 40% by olive oil.

Effect of oleic acid on expression of α-SMA in HSC

The expression of α-SMA was detected after 7 days in the HSCs cultured with the control medium (Fig. 5). A similar expression of α-SMA was observed in the HSCs cultured with a medium containing linoleic acid, a major component of corn oil. The expression was reduced in the cells incubated with a medium containing oleic acid, a major component of olive oil. The number of α-SMA positive cells was significantly blunted by about 50% in the oleic acid group compared to the linoleic acid group (Fig. 5d).

Production of hydroxyproline by isolated HSCs

Concentration of hydroxyproline was about 0.21 μg/ml in culture medium in the HSCs cultured with control medium for 2 days. There was no significant difference in hydroxyproline concentration between the control medium group and the linoleic acid group. On the other hand, concentration of hydroxyproline was about 0.10 μg/ml in culture medium in the cells cultured with oleic acid, and the concentration of hydroxyproline was significantly

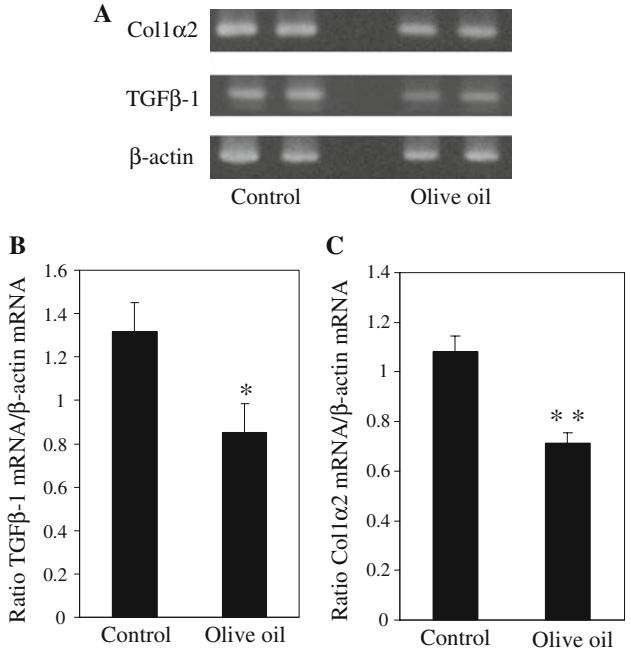

Fig. 4 Effects of fatty acids on the mRNA expression of col1α2 and TGF-β1 in the liver. The mRNA expression of col1α2 and TGF-β1 was determined as described in "Material and methods". **a** Representative bands are shown, **b** densitometric analysis of mRNA expression of TGF-β1, and **c** densitometric analysis of mRNA expression of col1α2 as the ratio of β-actin. Data represent mean ± SE ($n = 4$ in each group). $^*P < 0.05$, $^{**}P < 0.01$ compared with the control group by the unpaired Student's t test

lower in the oleic acid group than the linoleic acid group (Fig. 6).

Discussion

Protective effects of olive oil on the CCl_4-induced hepatic fibrosis

Previous studies have reported that fatty acids prevented liver injury induced by chronic administration of CCl_4 or intragastric alcohol feeding in rats [30, 31]. The protective effects of olive oil against liver injury due to oral intake of CCl_4 have also been reported [8]. In the present study, serum transaminase levels were blunted by dietary olive oil (Fig. 2). Furthermore, the expression of α-SMA in the liver was markedly inhibited by dietary olive oil (Fig. 3) and liver fibrosis induced by intraperitoneal injection of CCl_4 was prevented by olive oil (Fig. 3e). Thus, olive oil had anti-inflammatory and anti-fibrogenic effects.

Alternatively, CCl_4 is converted to a trichloromethyl radical by NADH-dependent cytochrome P-450 in the hepatocyte endoplasmic reticulum membrane, which causes lipid peroxidation at the plasma membrane [32]. Therefore, oxidative stress is one cause of CCl_4-induced liver injury [16]. Indeed, oxidative stress activates HSCs [33–35], which induces the progression of liver fibrosis. FIn the present

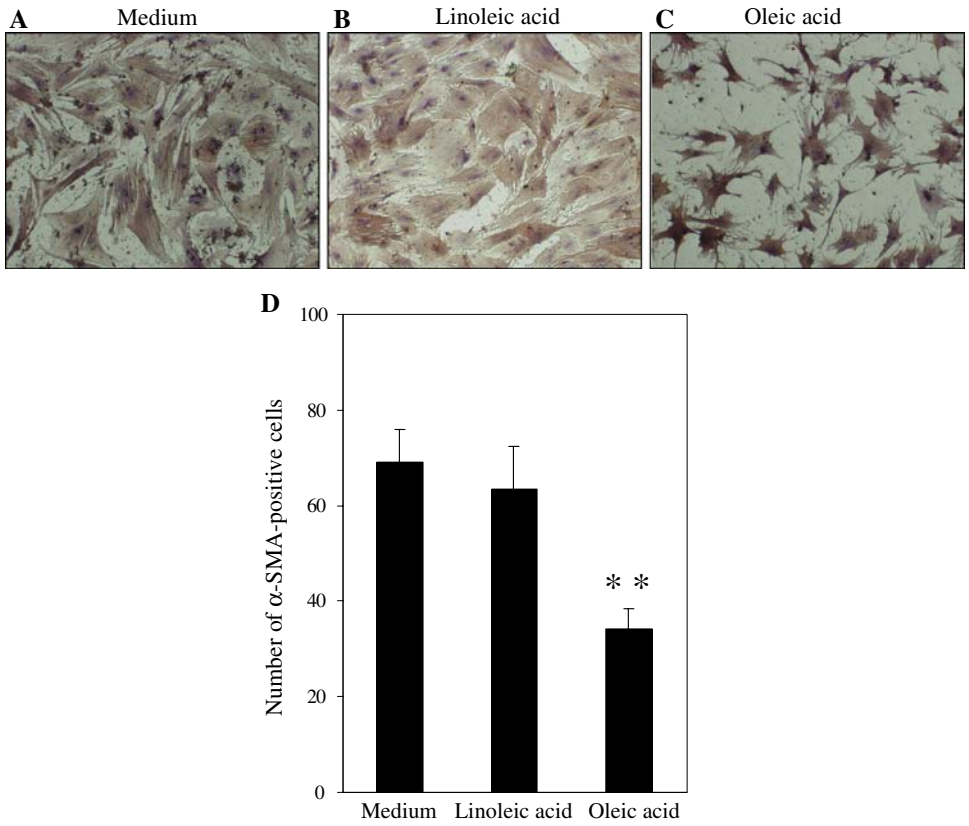

Fig. 5 Effect of fatty acids on activation of hepatic stellate cells. Hepatic stellate cells isolated from C57BL/6N mice were cultured in 24-well uncoated plastic plates for 24 h. After pre-incubation, the cells were divided into three groups: **a** control media, **b** linoleic acid in media, and **c** oleic acid in media. Hepatic stellate cells on day 8 were stained immunohistochemically using anti-α-SMA antibodies. Representative photomicrographs are shown, ×200. **d** The number of α-SMA positive cells was counted from five different fields (×200), and the number of positive cells per field is shown. Data represent means ± SE ($n = 4$). $^{**}P < 0.01$ compared with control media and media containing linoleic acid by the unpaired Student's t test

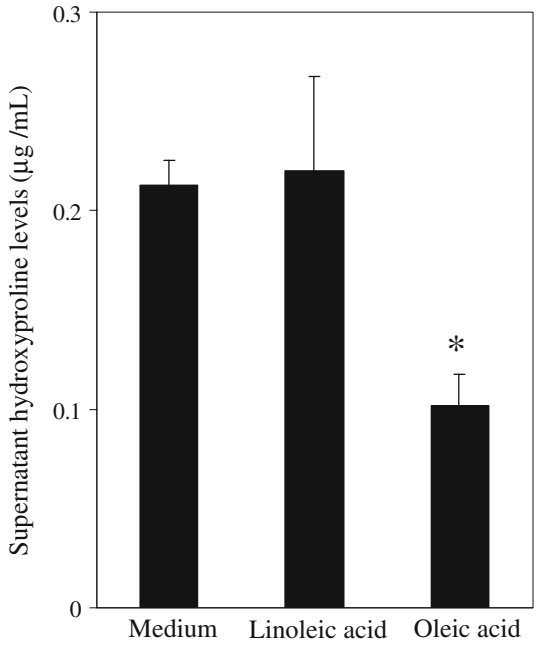

Fig. 6 Effects of fatty acids on production of hydroxyproline concentrations by isolated hepatic stellate cells. After 24 h of incubation, the HSCs were co-cultured with either 100 mM of oleic acid or linoleic acid for 2 days ($n = 4$ in each group). The controls were cultured for another 2 days in medium alone. Data represent mean ± SE. $^*P < 0.05$ compared with control media and media containing linoleic acid by the unpaired Student's t test

study, serum ALT levels were significantly blunted by olive oil (Fig. 2). Therefore, olive oil may suppress intrahepatic oxidative stress on liver fibrosis. Since TNF-α is a key factor in inflammatory cytokine cascade, the mRNA expression of TNF-α was assessed in liver tissues after treatment of CCl_4 in the present study. Although CCl_4 increased the mRNA expression of TNF-α in the liver, there were no significant differences in its expression between the control group and the olive oil group (data not shown). Further investigation is needed on this issue.

Effects of oleic acid on HSC

Mohamed et al. [36] have demonstrated that serum concentration of oleic acid, a major component of olive oil, increased in rats given olive oil. Furthermore, oleic acid is hardly oxidized compared with other unsaturated fatty acids. Moreover, Carluccio et al. [37] have demonstrated that oleic acid contributes to prevention of atherosclerosis by inhibiting activation of the endothelial cells through selective displacement of saturated fatty acids in cell membrane phospholipids. Since dietary olive oil blunted the expression of α-SMA in the liver, and prevented CCl_4-induced hepatic fibrosis in mice (Fig. 3), oleic acid is possibly involved in this mechanism.

Since olive oil inhibited the mRNA expression of TGF-β and collα2 (Fig. 4), olive oil may inhibit activation of cells in the hepatic sinusoid [15]. There are two possible cell types involved in these effects, hepatic macrophage Kupffer cells and HSCs. In a preliminary study, oleic acid had no effect on activation of isolated Kupffer cells by endotoxin in vitro (data not shown). Furthermore, the expression of α-SMA was blunted by olive oil in the liver (Fig. 3). Therefore, the effects of oleic acid on HSCs were investigated in the present study.

As a result, the HSCs cultured with a medium containing oleic acid showed less fibrogenic formation than those cultured with the medium alone or with a medium containing linoleic acid (Fig. 5). Furthermore, the number of α-SMA-positive cells was significantly lower in the cells co-cultured with oleic acid than those co-cultured with linoleic acid (Fig. 5). Moreover, the concentration of hydroxyproline was also significantly lower in the cells co-cultured in a medium with oleic acid than those co-cultured in control medium (Fig. 6). Thus, oleic acid inhibits activation of the HSCs.

Clinical applications

In the present study, hepatic fibrosis was prevented in mice fed a liquid diet containing olive oil. Enteral nutrition is widely used in clinics, and much useful evidence is reported. Although fats and oils are considered as risk factors for liver damage [38], data presented here suggest that olive oil may useful to patients with liver fibrosis.

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