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Beneficial effect of docosahexaenoic acid on cholestatic liver injury in rats

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Abstract

Bile duct obstruction and subsequent cholestasis are associated with hepatocellular injury, cholangiocyte proliferation, stellate cell activation, Kupffer cell activation, oxidative stress, inflammation and fibrosis. Docosahexaenoic acid (DHA) is an essential polyunsaturated fatty acid that has been shown to possess health beneficial effects, including hepatoprotection. However, the molecular mechanism of DHA-mediated hepatoprotection is not fully understood. In the present study, we report the protective effect of DHA on cholestatic liver injury. Cholestasis was produced by bile duct ligation (BDL) in male Sprague–Dawley rats for 3 weeks. Daily administration of DHA was started 2 weeks before injury and lasted for 5 weeks. In comparison with the control group, the BDL group showed hepatic damage as evidenced by histological changes and elevation in serum biochemicals, ductular reaction, fibrosis, inflammation and oxidative stress. These pathophysiological changes were attenuated by chronic DHA supplementation. DHA alleviated BDL-induced transforming growth factor beta-1 (TGF-β1), intereukin-1beta, connective tissue growth factor and collagen expression. The anti-fibrotic effect of DHA was accompanied by reductions in α-smooth muscle actin-positive matrix-producing cells and Smad 2/3 activity critical to the fibrogenic potential of TGF-β1. DHA also attenuated BDL-induced leukocyte accumulation and nuclear factor-κB (NF-κB) activation. Further studies demonstrated an inhibitory effect of DHA on redox-sensitive intracellular signaling molecule extracellular signal-regulated kinase (ERK). Taken together, the hepatoprotective, anti-inflammatory and anti-fibrotic effects of DHA seem to be multifactorial. The beneficial effects of chronic DHA supplementation are associated with anti-oxidative and anti-inflammatory potential as well as down-regulation of NF-κB and transforming growth factor beta/Smad signaling probably via interference with ERK activation.

Keywords: Cholestasis; DHA; Fibrosis; Inflammation; Oxidative stress

1. Introduction

Cholestasis is characterized by an abnormal accumulation of bile acids due to defects in the process of bile acid transport. Chronic cholestasis is a key histopathological change that occurs in biliary atresia, primary biliary cirrhosis and primary sclerosing cholangitis and contributes to later development of hepatocellular injury, progressive hepatic fibrosis, cirrhosis and death from liver failure [1–3]. The abnormal accumulation of hydrophobic bile acids in the liver and consequent bile duct proliferation is a hallmark of cholestatic change. Histologically, the increase in hepatic bile ductular structures is generally accompanied by hepatocyte death, inflammatory response and periductular fibrosis. Although mechanisms of liver damage in cholestasis are multifactorial, retained bile acids, oxidative stress and inflammation have been implicated as important factors in cholestatic liver injury. On a cellular level, cholangiocyte activation/proliferation, hepatocyte apoptosis, hepatic stellate cell activation/proliferation and Kupffer cell/leukocyte activation/infiltration all play a crucial role in cholestasis-associated pathophysiological changes [4–10]. Therefore, bile acids, oxidative stress, inflammation and these cellular events are considered to be potential targets for therapeutic intervention in treating cholestatic liver disorders.

Fatty acids are important for the histological, anatomical and biochemical integrity of tissues/cells and fatty acid composition influences various physiological and biochemical processes. Epidemiological, clinical and biochemical studies have shown that dietary fatty acids can modify the risks of diversity of human diseases. Usually, polyunsaturated fatty acids (PUFA) are recognized as health-promoting agents and saturated fatty acids are regarded as unhealthy, but nutritionists believe that both types of fatty acids may be beneficial or deleterious depending on disease types and/or status. Specifically, PUFA and saturated fatty acids have a dual role in inflammatory disorders [11–15]. Inflammation plays a relevant role in the onset and development of liver diseases; its nutritional or pharmacological control is the

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focus of studies. At the crossroad between nutrition and pharmacology, dietary fatty acids have the potential to modulate the severity of liver injury. Dietary saturated fatty acids have been shown to protect against alcohol-induced liver injury [16,17]. PUFA deficiency is often observed in patients with advanced liver cirrhosis and a low n-3 PUFA diet increases the development of alcoholic fatty liver and fibrosis [18,19]. Animal studies show that dietary fish oil or PUFA supplementation has beneficial effects against liver damage caused by thioacetamide, monocrotaline or carbon tetrachloride [20–22]. Puder et al. [23] have reported that parenteral fish oil improves outcomes in patients with parenteral nutrition-associated liver injury. Although the consequences of fatty acid supplementation vary, these findings imply that the beneficial effects of fatty acids are most accompanied by the alleviation of oxidative stress and inflammation.

Cholestasis is a clinically important primary event that contributes to hepatic damage. Parenteral nutrition-associated liver disease is characterized by a spectrum of fatty liver disease, inflammation, bile duct proliferation, fibrosis and cirrhosis [24]. The beneficial effect of fish oil against parenteral nutrition-associated liver injury [23] encourages the possibility that fatty acids, particularly n-3 PUFA, may be effective against liver damage secondary to cholestasis. Recently, we found that pre-treatment with saturated fatty acid stearic acid (18:0) but not palmitic acid (16:0) protected against cholestasis-induced liver injury [25]. Chronic fish oil supplementation reduced hepatocellular injury after bile duct obstruction [26]. Currently, the detailed hepatoprotective mechanisms of fatty acids on chronic cholestatic liver injury remain largely unknown. PUFAs have been implicated in the prevention of various human diseases, including inflammation-associated diseases [13,27]. Docosahexaenoic acid (DHA) is an essential n-3 PUFA and has been shown to possess beneficial health effects. Common bile duct ligation (BDL) and scission in animals represents a classical experimental model for the analysis of cholestasis and consequent hepatic damage. BDL produces cholestasis, triggers free radical generation and inflammation, induces progressive portal fibrosis, causes secondary biliary cirrhosis and finally leads to liver failure [25,28]. Our hypothesis is that chronic DHA supplementation reduces hepatocellular damage in a rat model of BDL and to characterize the mechanisms by which this may occur.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were randomly divided into six experimental groups. The control (n=30) and BDL group (n=30) rats underwent sham and BDL operations, respectively. Intraperitoneal administration of saline and DHA (250 and 1000 nmol/kg per day, respectively) was carried out and modified in accordance with the method used in our previous reports [25]. The rats in both groups received saline and DHA (10 animals/each group) for 5 weeks starting from 2 weeks before operation. All animals were sacrificed 3 weeks after surgery under pentobarbital anesthesia. The animal study was approved by the Animal Care and Use Committee of Taichung Veterans General Hospital.

2.2. BDL operation

Rats (200–250 g) were anesthetized with pentobarbital (50 mg/kg), and the common bile duct was exposed and ligated by double ligatures with 3–0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature was made above the entrance of the pancreatic ducts. The common bile duct was then cut between the double ligatures. Thus, animals had total, permanent biliary obstruction [25]. In sham-operated rats, an abdominal incision was made without a ligation.

2.3. Biochemical analysis

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (Alk-P), γ-glutamyl transpeptidase (GGT), total bilirubin, creatinine, triacylglycerol (TG) and total cholesterol were measured by automated standardized procedures (Roche Hitachi 917/747, Mannheim, Germany). The serum levels of interleukin-1 beta (IL-1β) and transforming growth factor beta-1 (TGF-β1) were measured by enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA).

2.4. Bile acids extraction and analysis

The extraction and analysis of total bile acids were carried out and modified as previously reported method [29]. Briefly, liver samples were homogenized in a 1:1 solution of *t*-butanol/water (200 mg of tissue/ml). Samples were centrifuged at 10,000×*g* for 20 min, and the supernatants were collected and dried. Samples were then reconstituted with normal saline and stored at −80°C. Serum samples were mixed with chilled acetone (1:4), shaken vigorously and left on ice for 5 min before centrifugation at 10,000×*g* for 3 min. The supernatants were collected and stored at −80°C. Both extracted samples were analyzed for total bile acids concentration in a reaction catalyzed by 3α-hydroxysteroid dehydrogenase using the total bile acids assay kit by Diazyme Laboratories (Poway, CA, USA).

2.5. Histological and immunohistochemical examination

Excised liver specimens were fixed in 10% formalin and embedded with paraffin. Hematoxylin and eosin (H&E) staining and Sirius Red staining were performed according to standard procedures. For immunohistochemical examination, deparaffinized sections were incubated with α-smooth muscle actin (α-SMA) and CD68 primary antibody and biotinylated secondary antibody, followed by the avidin-biotin-peroxidase complex. The immunoreactive signal was developed by color deposition using diaminobenzidine as substrate.

2.6. Tissue preparation and Western blot

The resected liver tissues were extracted with lysis buffer (1% Triton X-100; 50 mmol/l Tris-HCl, pH 7.6; 150 mmol/l NaCl; and 1% protease inhibitor cocktail). For Western blot, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated for 1 h at room temperature with the indicated antibodies including α-SMA (Calbiochem Biotechnology, San Diego, CA, USA), CD68 (Invitrogen, Carlsbad, CA, USA), GGT, Smad2/3, phospho-Smad2/3, extracellular signal-regulated kinase (ERK), phospho-ERK and GAPDH (glyderaldehyde 3-phosphate dehydrogenase; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were developed using ECL Western blotting reagents and quantified using optical densitometry. The intensity of each signal was corrected by the values obtained from the immunodetection of GAPDH and the relative protein intensity was expressed as folds of the content in the sham control group.

2.7. Collagen measurement

Proteins (50 μg) extracted from liver tissues were subjected to ELISA with antibody against collagen (Sigma-Aldrich, St. Louis, MO, USA) for the determination of collagen [25].

2.8. Gelatinase zymography

Liver homogenates (50 μg) were loaded onto 8% SDS-polyacrylamide gels containing gelatin (0.5 mg/ml). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 1 h and incubated at 37°C for 16 h in 100 mmol/l Tris-HCl, pH 7.4 and 10 mmol/l $CaCl_2$. Thereafter, gels were stained with Coomassie Brilliant R-250 and destained in a solution of 7.5% acetic acid and 5% methanol. The intensity of each signal was corrected by the values obtained from the immunodetection of GAPDH and the relative protein intensity was expressed as folds of the content in the sham control group.

2.9. Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

The isolation of RNA and synthesis of cDNA were carried out as previously reported [30]. DNA fragments of specific genes and internal controls were co-amplified in one tube containing *Taq* DNA polymerase (Promega, Madison, WI, USA) and 0.8 μM of each sense and antisense primers. The PCR reaction was performed with a DNA thermal cycler under the following conditions: one cycle of 94°C for 3 min, 28 cycles of (94°C for 50 s, 58°C for 40 s and 72°C for 45 s) and then 72°C for 5 min. The amplified DNA fragments were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide. DNA band intensity was determined by a computer image analysis system (Alpha Innotech, IS1000). Relative mRNA levels were expressed as the intensity ratio of each gene and internal control (β-actin). Oligonucleotides used in this study were as follows: 5′-CTGGATGCCATCAAGGTCTAC and 5′-CGCTGTTCTTGCAGTGATAGG for procollagen I; 5′-CTTGGAATTGCAGGGCTAAC and 5′-TTGCCACCAGGAGATCCGTCT for procollagen III; 5′-GACAGCTTGTGGCAAGTGAA and 5′-TTCCTCGTGGAAATCTGACC for connective tissue growth factor (CTGF); 5′-TCCTGTGGCATCCACGAAACT and 5′-GGAGCAATGATCTTGATCTTC for β-actin.

2.10. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted from liver tissues. The isolation of nuclear extract and EMSA was conducted as described previously [25]. The oligonucleotides specific for nuclear factor-κB (NF-κB) (5′-AGTTGAGGGGACTTTCCCAGGC) were synthesized and labeled with biotin. Nuclear extract (5 μg) was used for EMSA. The binding reaction mixture included 1 μg of poly (dI-dC), 0.1 μg of poly L-lysine and 100

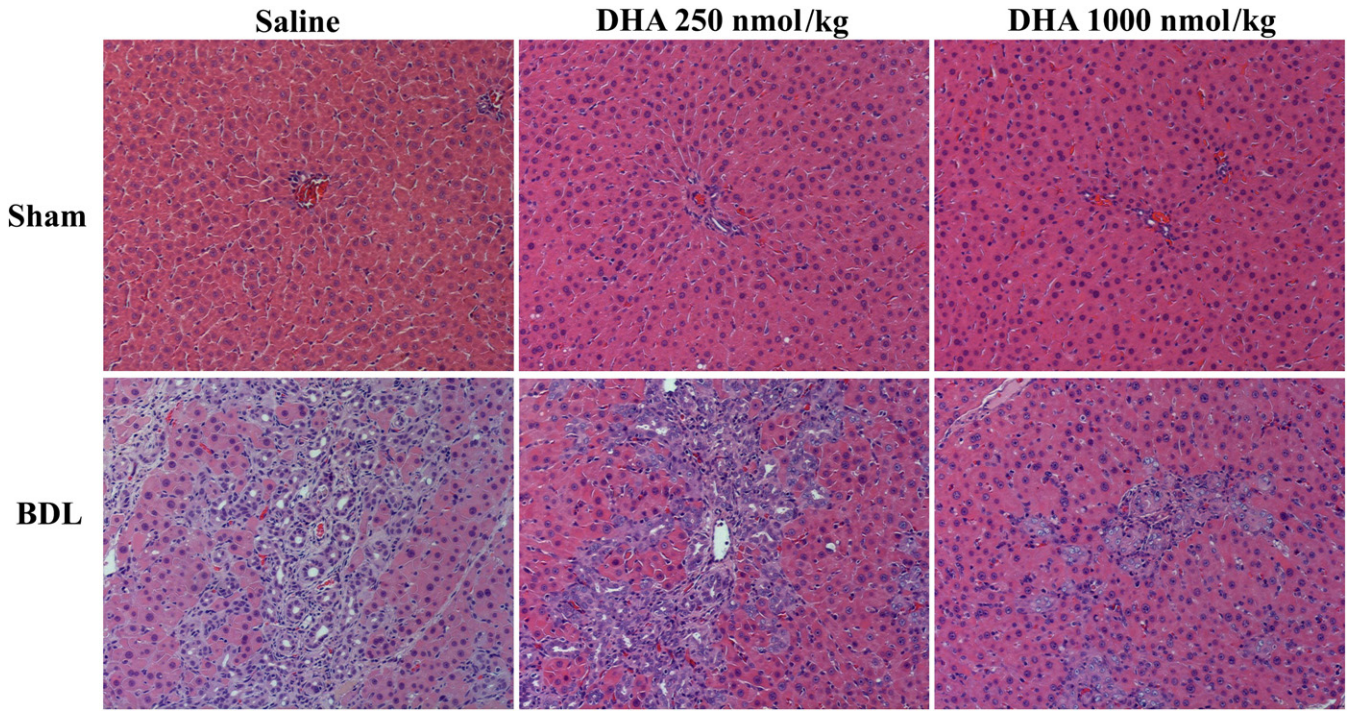

Fig. 1. DHA-attenuated, BDL-induced liver injury. The obtained liver sections were subjected to histological examination. Representative photomicrographs of H&E staining are shown (original magnification ×100).

fmol of biotin-labeled DNA probe in a 20 μl binding buffer (10 mM HEPES, pH 7.6; 50 mM NaCl; 0.5 mM $MgCl_2$; 0.5 mM EDTA; 1 mM dithiothreitol; and 5% glycerol). The DNA/protein complex was analyzed on 6% native polyacrylamide gels.

2.11. Measurement of lipid peroxidation

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix, Buffalo, NY, USA) was used to measure the lipid peroxidation products, malondialdehyde (MDA) equivalents. In brief, liver tissues were homogenized with 0.1 mol/l sodium phosphate buffer (pH 7.4). One hundred microliters of homogenate were mixed with 2.5 ml reaction buffer (provided by the kit) and heated at 95°C for 60 min. After the mixture had cooled, the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The lipid peroxidation products are expressed in terms of MDA equivalents.

2.12. Measurement of antioxidant enzymes

Catalase, manganese-superoxide dismutase (Mn-SOD), copper/zinc-superoxide dismutase (Cu/Zn-SOD) and glutathione peroxidase (GPx) activities were determined using commercially available assay kits (Cayman, Ann Arbor, MI, USA). Briefly, liver tissues were weighed and homogenized with appropriate buffers (provided by the kits). Mitochondria and cytosol were obtained from the liver homogenate by differential centrifugation. The homogenates were then determined following procedures provided by the respective manufacturers. The Catalase Assay Kit utilizes the peroxidative function of catalase for determination of enzyme activity. The Superoxide Dismutase Assay Kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The Glutathione Peroxidase Assay Kit measures GPx activity indirectly by a coupled reaction with glutathione reductase. The specific activities of the various enzymes in the rat liver are expressed in U/μg of the protein with the protein content determined as stated above.

2.13. Measurement of reduced glutathione (GSH)

GSH was determined using a commercially available glutathione assay kit (Cayman, Ann Arbor, MI, USA). Briefly, liver tissues were weighed and homogenized with 0.1 M sodium phosphate buffer (pH 7.4). The homogenates were then centrifuged with 5% trichloroacetic acid to remove the proteins. An aliquot of 50 μl of homogenate was mixed with 150 μl reaction buffer (kit provided). The mixture was vortexed and the absorbance read at 405 nm within 30 min. The content was calculated using a standard solution of GSH.

2.14. Statistical analysis

All data are presented as mean±S.D. For comparison, the statistical significance between means was determined using one-way analysis of variance followed by Dunnett's *t* test. A *P* value of less than .05 was considered significant.

Table 1
Serum parameters

	Group (DHA, nmol/kg)					
	Sham 0	BDL 0	Sham 250	BDL 250	Sham 1000	BDL 1000
AST (U/l)	159±72	685±117**	139±24	583±87** #	186±70	454±205** #
ALT (U/l)	37±9	149±41**	36±3	111±29**	41±23	72±49* #
Alk-P (U/l)	114±33	278±38**	138±37	277±50**	92±12	195±75* ##
GGT (U/l)	1.8±1.2	55.4±15.4**	1.7±1.0	35.8±22.6**	1.5±0.9	31.4±10.1** ##
Total bilirubin (mg/dl)	0.9±0.3	9.0±1.4**	0.7±0.3	10.0±1.3**	1.1±0.4	4.6±3.0** ##
Creatinine (μmol/l)	55.6±5.4	39.8±3.9**	53.1±6.1	41.6±4.1**	56.1±3.9	45.6±3.4** #
TG (mg/dl)	35±15	182±43**	50±21	118±39**	47±23	67±43* ##
Total cholesterol (mg/dl)	54±10	104±16**	54±8	91±16**	55±9	75±10* #
IL-1β (pg/ml)	47±31	1443±419**	62±47	1016±367**	47±22	261±191** ##
TGF-β1 (pg/ml)	419±305	1613±397**	424±191	1189±473**	295±126	818±321** ##

Data are expressed as mean±S.D. *P<.05 and **P<.01 vs. sham control group and #P<.05 and ##P<.01 vs. BDL control group, n=10.

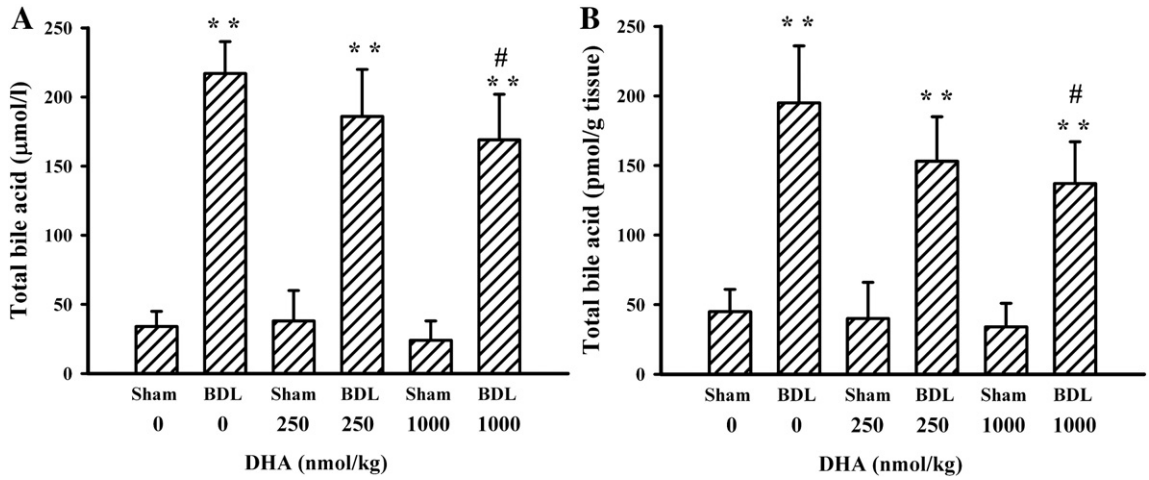

Fig. 2. DHA-attenuated, BDL-induced total bile acid accumulation. The obtained serum (A) and liver (B) samples were subjected to enzymatic reaction for the measurement of total bile acids. Values are expressed as mean±S.D. **P<.01 vs. the sham control group. #P<.05 vs. the BDL control group.

3. Results

3.1. DHA-attenuated, BDL-induced liver injury

Grossly, body mass and average food intake of BDL rats were lower than those of sham control rats. There were no significant differences in body mass and average food intake between saline- and DHA-supplemented animals (data not shown). Liver damage was first analyzed by histological examination (Fig. 1). No morphological abnormalities were observed in saline- and DHA-supplemented sham-operated control rats showing regular morphology of liver parenchyma with intact hepatocytes, sinusoids and portal tracts. Diffuse severe/high bile duct hyperplasia, portal edema and mild portal infiltrates, all features of extrahepatic cholestasis, were present in BDL rats. BDL rats also showed a loss of hepatic structure in periportal areas. Serum levels of AST, ALT, Alk-P, total bilirubin, TG and total cholesterol, common biochemical indexes of hepatocellular injury, were significantly elevated in BDL rats (Table 1). BDL rats showed a decreased serum level of creatinine (Table 1) and increased levels of serum (Fig. 2A) and hepatic (Fig. 2B) total bile acids. These histological (Fig. 1), serum biochemical (Table 1) and total bile acid (Fig. 2) changes were improved by DHA supplementation. The results indicate that DHA supplementation attenuates BDL-induced liver damage.

3.2. DHA-attenuated, BDL-induced ductular proliferation

In most cases, primary damage to the biliary epithelium leads to the development of cholestasis. Long-standing cholestasis causes ordered bile duct proliferation or typical ductular reaction [5]. To examine the effect of DHA on cholestasis-related changes, the structure and level of biliary epithelial cells of newly formed ductular structures were evaluated histologically and biochemically. In BDL rats, an abundance of newly formed bile ducts was observed, as evidenced by the degree of histopathological score of bile duct hyperplasia (Table 2) and the parallel elevations in serum GGT activity (Table 1) and GGT protein (Fig. 3). GGT is known to be selectively expressed by bile duct cells [31]. The incidence of BDL-induced histopathological changes was not affected by DHA supplementation (Table 2). However, the serum GGT activity (Table 1), histopathological score of bile duct hyperplasia (Table 2) and GGT protein expression (Fig. 3) were improved by DHA supplementation, reflecting a potential inhibition of the development of ductular reaction. Thus, supplementation with DHA was accompanied by a reduction in the severity of BDL-induced ductular reaction.

3.3. DHA-attenuated, BDL-induced fibrosis

Biliary fibrosis, such as the type observed in conditions of chronic obstruction of the biliary tree, is characterized by accumulation of extracellular matrix at portal tracts, surrounding newly formed bile ducts emerging during the course of ductular reaction and originating from proliferation of pre-existing bile duct epithelial cells [5]. DHA alleviated BDL-induced ductular reaction. Thus, the next experiment was conducted to determine whether DHA has a role in BDL-induced fibrosis. Fibrosis was assessed by a classical histopathological technique using Sirius Red staining. In BDL rats, significant hepatic collagen deposition/accumulation was present in periportal and portal tracts. The presence of this deposition/accumulation and the bridging fibrosis were reduced in DHA-treated groups (Fig. 4). The extent of liver fibrosis was also measured biochemically as hepatic collagen content. In parallel to the observed improvement in liver histology, DHA reduced BDL-induced elevation

Table 2
Scores of bile duct hyperplasia

Histopathological findings	Group (DHA, nmol/kg)					
	Sham 0	BDL 0	Sham 250	BDL 250	Sham 1000	BDL 1000
Hyperplasia, bile duct, focal, moderate to severe/high[a]	0/10	10/10	0/10	10/10	0/10	10/10
Histopathology score of bile duct hyperplasia[b]	0	4.2±0.8	0	3.8±0.8	0	3.0±0.9[c]

Data are expressed as mean±S.D.
[a] Incidence: affected rats/total examined rats.
[b] Score of bile duct hyperplasia. Severity of lesions was graded according to the methods described by Shackelford et al. [77]. Degree of lesions was graded from one to five depending on severity: 1=minimal (< 1%); 2=slight (1–25%); 3=moderate (26–50%); 4=moderate/severe (51–75%); 5=severe/high (76–100%).
[c] P<.01 vs. BDL control group.

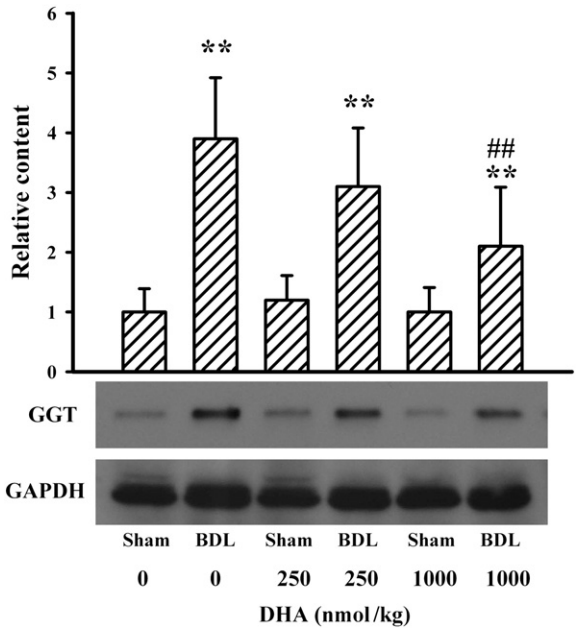

Fig. 3. DHA-attenuated, BDL-induced GGT protein expression. The obtained proteins from liver tissues were subjected to Western blot with antibodies against GGT and GAPDH. Representative blots and the quantitative data are shown. The relative GGT protein content in sham control group is defined as 1. Values are expressed as mean±S.D. $^{**}P<.01$ vs. the sham control group. $^{\#\#}P<.01$ vs. the BDL control group.

in collagen content (Fig. 5A). Deposition of extracellular matrix components is a common phenomenon in liver fibrosis, in which collagen is a major protein associated with basement membranes and fibrosis. Matrix metalloproteinases (MMP) are a class of secreted enzymes with major functions in the degradation and remodeling of all components of the extracellular matrix [32]. Gelatinase zymography study (Fig. 5B) showed an increased activity of hepatic MMP-9 and MMP-2 in BDL rats. However, the increased MMP-9 (Fig. 5C) and MMP-2 (Fig. 5D) activities were not changed after DHA treatment. These results indicate that DHA has an attenuating effect against BDL-induced liver fibrosis independently of modulation of MMP activity.

3.4. DHA-attenuated, BDL-induced myofibroblast activation

Initial injury to bile ducts can lead to the organization of a fibrotic reaction, which involves different matrix-producing cells of mesenchymal origin such as α-SMA-positive portal myofibroblasts and activated hepatic stellate cells [33]. Immunohistochemical results showed that BDL caused an elevation in α-SMA immunopositive signals in portal and periportal areas and the immunoreactive signals were reduced by DHA treatment (Fig. 6). Western blotting revealed an elevated α-SMA protein expression in BDL rats and DHA caused a reduction of α-SMA protein (Fig. 7A). These results indicate that DHA decreases the activation of α-SMA-positive matrix-producing cells in hepatic tissues after BDL injury. Numerous studies indicate that TGF-β1 plays an important role in liver fibrogenesis through acting on matrix-producing cells and enhances extracellular matrix deposition through its effector molecule Smad [34,35]. Among the Smad family, the phosphorylation/activation of Smad2/3 is a critical determinant in transmitting the TGF-β1 signal to regulate cellular activity. Generally, TGF-β1/Smad signaling triggers myofibroblasts to synthesize CTGF, collagen I and collagen III [34–37]. The elevation of serum level of TGF-β1 in BDL rats was reduced by DHA in this study (Table 1). Western blot study revealed that BDL activated Smad2/3 by increasing its phosphorylation and the increased Smad2/3 activity was attenuated by DHA treatment (Fig. 7B). In BDL rats, there were elevated mRNA expressions in procollagen I, procollagen III and CTGF (Fig. 7C). DHA caused a reduction in procollagen I (Fig. 7D), procollagen III (Fig. 7E) and CTGF (Fig. 7F) mRNA expression. These data suggest that the anti-fibrotic effect of DHA is associated with reduction of hepatic TGF-β1.

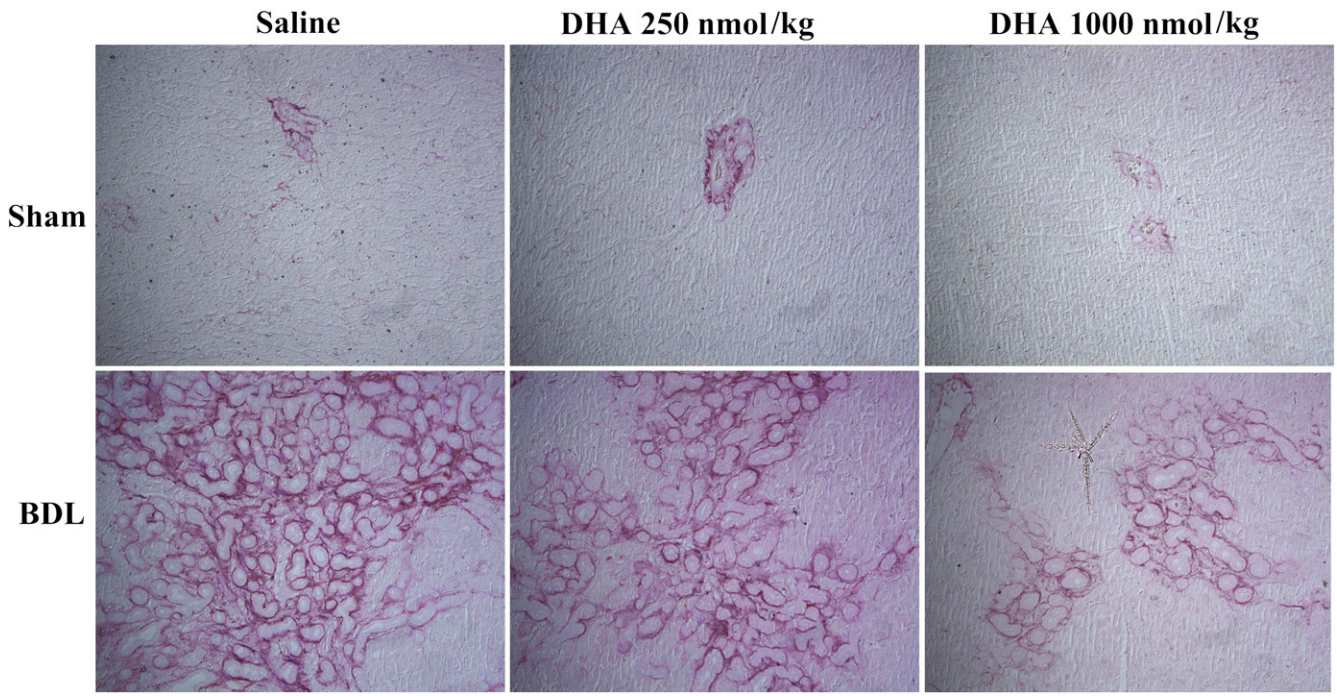

Fig. 4. DHA-attenuated, BDL-induced liver fibrosis. The obtained liver sections were subjected to histological examination. Representative photomicrographs of Sirius Red staining are shown (original magnification ×100).

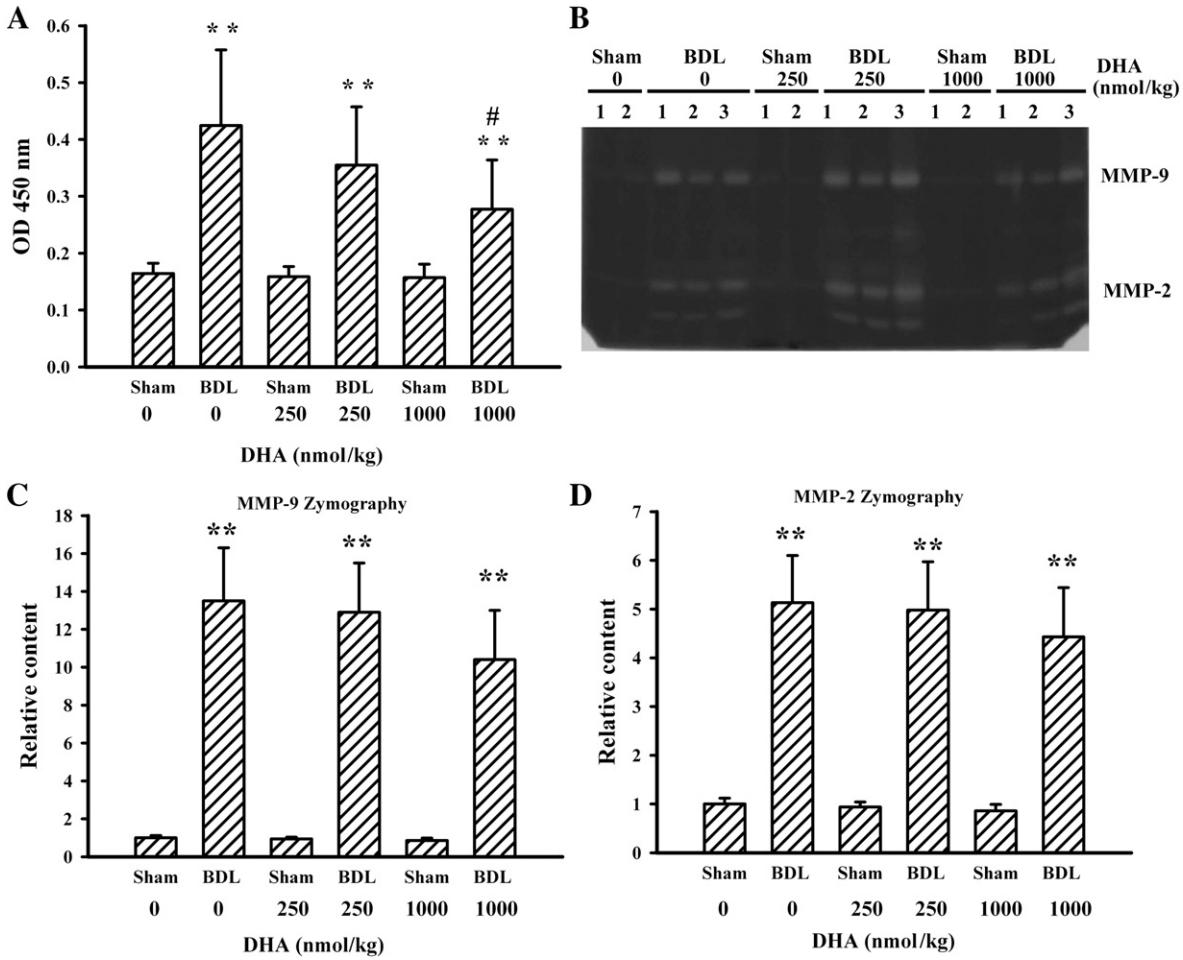

Fig. 5. DHA-attenuated, BDL-induced liver fibrotic changes. Proteins were isolated from liver tissues and subjected to ELISA for the measurement of collagen. The absorbance was measured at 450 nm using a spectrophotometer (A). Proteins were isolated from liver tissues and subjected to zymographic assay for the determination of MMP-2 and MMP-9 activity. Representative zymographs are shown (B). The quantitative data are depicted in C (MMP-9) and D (MMP-2). The relative content in sham control group is defined as 1. Values are expressed as mean±S.D. $^{**}P<.01$ vs. the sham control group and $\#P<.05$ vs. the BDL control group.

3.5. DHA-attenuated, BDL-induced inflammation

Prolonged biliary obstruction is associated with inflammatory cell infiltration, NF-κB activation and cytokine over-production. Inhibition of inflammatory responses prevents cholestatic liver damage [6,10,38,39]. Immunohistochemical staining (Fig. 8) and Western blotting (Fig. 9A) revealed an accumulation of monocytes/macrophages in the liver tissues of BDL rats. The recruitment/accumulation of inflammatory cells was attenuated by DHA. The activation of NF-κB occurs in cholestatic liver damage and contributes to inflammatory responses [6]. EMSA revealed an increase of NF-κB activity in the liver tissues of BDL rats (Fig. 9B). BDL-induced NF-κB DNA binding activity was attenuated by DHA (Fig. 9C). Signaling molecule ERK is an upstream activator of NF-κB and the ERK/NF-κB signaling cascade has a crucial role in the expression of inflammatory cytokines and activation of myofibroblasts [40]. Stimulated ERK activity was detected in BDL rats, as demonstrated by an increase in the level of phosphorylated ERK. As shown in Fig. 9D, the level of phosphorylated ERK was reduced by DHA. These data show that DHA can attenuate BDL-induced inflammation by suppressing inflammatory cell recruitment/accumulation and ERK/NF-κB activation.

3.6. DHA-attenuated, BDL-induced oxidative stress

It has been postulated that oxidative stress modulates inflammatory and fibrotic responses and may be a link between liver injury and fibrosis [41]. To evaluate the effect of DHA on hepatic redox potential, the status of oxidative stress in liver tissues was determined. The level of lipid peroxidation product MDA, an index of oxidative stress, was elevated in BDL rats. The elevation of MDA in BDL rats was reduced by DHA treatment (Fig. 10A), indicating an anti-oxidative capacity of DHA. The changes in the activities of hepatic anti-oxidative enzymes were inversely related to the increase in MDA levels. The hepatic Mn-SOD (Fig. 10B), Cu/Zn-SOD (Fig. 10C), catalase (Fig. 10D) and GPx (Fig. 10E) activities were significantly decreased in BDL rats compared to those in sham-operated control rats. DHA treatment caused a moderate attenuation in BDL-induced reduction in anti-oxidative enzyme activities except for Cu/Zn-SOD. BDL rats showed a significant reduction in liver GSH content compared with sham control rats. The reduction of liver GSH content was attenuated by DHA (Fig. 10F). These data suggest that DHA can attenuate BDL-induced oxidative stress.

4. Discussion

Obstruction of the biliary tree by BDL causes cholestasis. Among the serum biochemicals, total bilirubin level and Alk-P activity are highly reflective of cholestasis and cholangiocyte injury, respectively [26,39,42]. Evidence shows that the hepatoprotection is not always accompanied by the reduction of total bilirubin and Alk-P. Simvastatin reduced ALT and AST and protected against cholestasis-induced

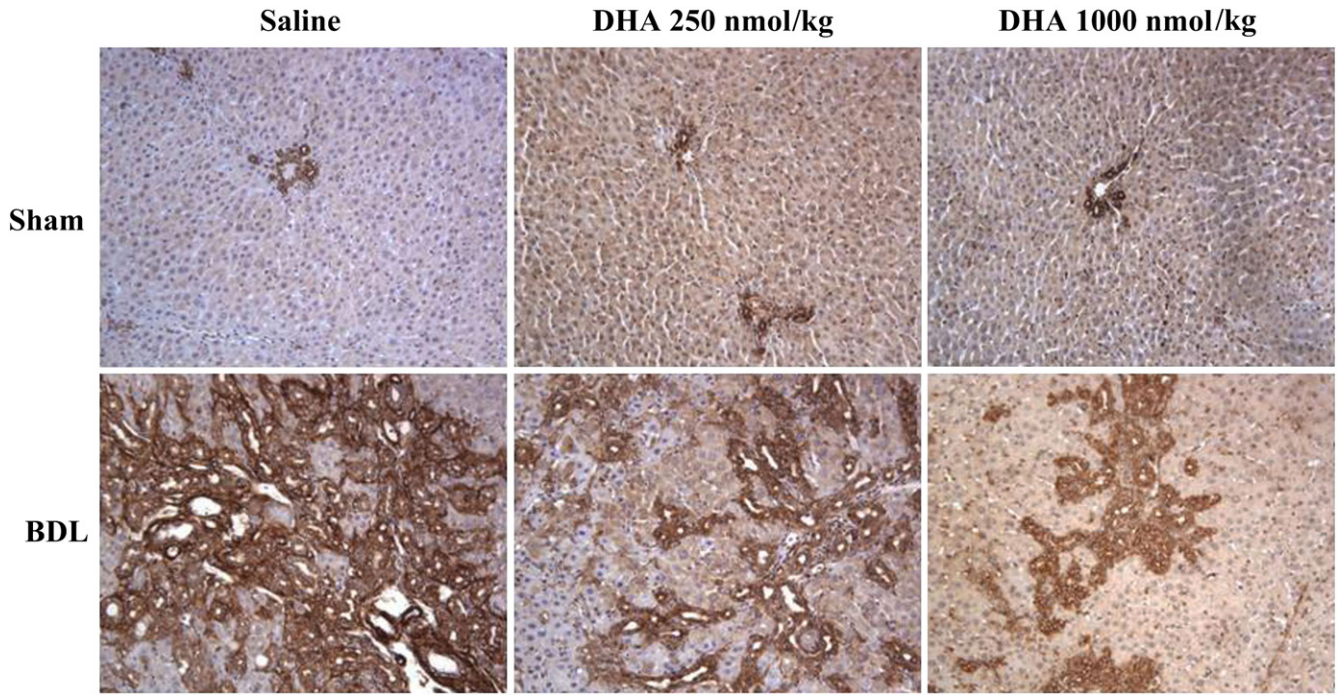

Fig. 6. DHA-attenuated, BDL-induced α-SMA immunoreactive signal. The obtained liver sections were subjected to immunohistochemical examination with antibody against α-SMA. Representative photomicrographs of α-SMA immunoreactivity are shown (original magnification ×100).

liver injury after BDL, but left total bilirubin unchanged [39]. Polyphenolic extracts from *Camellia sinensis*-attenuated BDL-induced ALT but had negligible effects on total bilirubin and Alk-P [42]. Three weeks of n-3 PUFA pre-treatment caused a marked reduction in hepatocyte death and inflammation. There were no significant differences in total bilirubin and Alk-P compared with control 8 days after BDL [26]. The reduction of total bilirubin and Alk-P was not observed in the hepatoprotective features of stearic acid against cholestatic liver injury [25]. These studies suggest that there might be other hepatoprotective mechanisms against cholestatic liver damage acting independently on the reduction of total bilirubin and Alk-P. Using a rat BDL model, we showed chronic DHA supplementation had a beneficial effect against cholestatic liver injury, as evidenced by the alleviation of histopathological changes; the improvement of serum biochemicals such as AST, ALT, Alk-P, total bilirubin, creatinine, TG, total cholesterol and total bile acids; the decreases in ductular reaction, fibrosis and inflammatory cell accumulation; the resolution of oxidative stress as well as the suppression of ERK, TGF-β1 and NF-κB activity. In comparison with our previous stearic acid study [25], our findings suggest a global beneficial effect of DHA against cholestatic hepatic changes.

In investigating potential intervention targets by DHA, we observed the alleviation of bile duct proliferation/ductular reaction, serum GGT activity and GGT protein expression in DHA-treated BDL rats. Since the proliferation of bile ducts is an early event in cholestasis-related changes, the attenuation of hepatic injury and fibrosis in BDL rats by DHA might be associated with alleviation of ductular reaction. In consideration of relevant studies, it is hypothesized that DHA possesses at least two beneficial effects against ductular reaction and hepatic damage. In the former condition, bile acid synthesis, bile flow, bile acid composition and bile acid effects might be potential targets for the beneficial effects of DHA. Hydrophobic bile acids have biologically active potential. The retained hydrophobic bile acids are able to generate free radicals, trigger inflammatory reactions, cause hepatocyte damage and stimulate cholangiocyte proliferation [5,9]. Bile acids are biosynthesized in the liver by cytochrome P450-mediated oxidation of cholesterol [43]. Since we observed a decreased serum cholesterol level and serum/hepatic total bile acid level in DHA-treated BDL rats, the elevated biosynthesis and abnormal accumulation of bile acids after BDL might be improved by DHA. A cell study further pointed out the directly hepatoprotective effect of n-3 PUFA due to bile acid exposure [44]. Thus, our study and other reports suggest that the interference of bile acid synthesis and accumulation and the blockade of bile acid-mediated responses might be actions targeted by DHA.

Biliary fibrosis is characterized by the accumulation of matrix proteins due to the imbalance between fibrogenesis and fibrolysis [5]. MMPs are a family of extracellular zinc- and calcium-dependent proteases that degrade the ECM (extracellular matrix) and other extracellular proteins [32]. In this study, BDL injury increased hepatic MMP-2 and MMP-9 activities. Although histological examination and hepatic collagen measurements showed a beneficial effect against BDL-induced liver fibrosis, DHA had a negligible effect on MMP-2 and MMP-9 activities. That is, hepatic MMP-2 and MMP-9 were unlikely to be the anti-fibrotic targets of DHA in the BDL model. Matrix-producing cells play an important role in the positive regulation of fibrogenesis. Among fibrogenic mesenchymal cells in the liver, the most extensively studied populations include hepatic stellate cells and portal myofibroblasts, in particular, the former. BDL injury caused activation in α-SMA-positive matrix-producing cells and increased α-SMA protein expression. DHA inhibited the development of hepatic fibrosis and suppressed α-SMA-positive cells and α-SMA protein. These findings indicate that DHA exerted a negative regulatory effect on fibrogenesis by suppression of α-SMA-positive matrix-producing cells. Evidence indicates that mitogenic and fibrogenic factors are critical for the activation of matrix-producing cells. Tumor necrosis factor alpha, IL-1β, TGF-β1 and IL-6 are the most extensively studied mitogenic and fibrogenic factors. Among them, TGF-β1 as a key fibrogenic mediator can enhance extracellular matrix deposition by activating stellate cells to turn on fibrogenic gene expression and inhibiting collagenase activity [34,45]. Investigation of intracellular

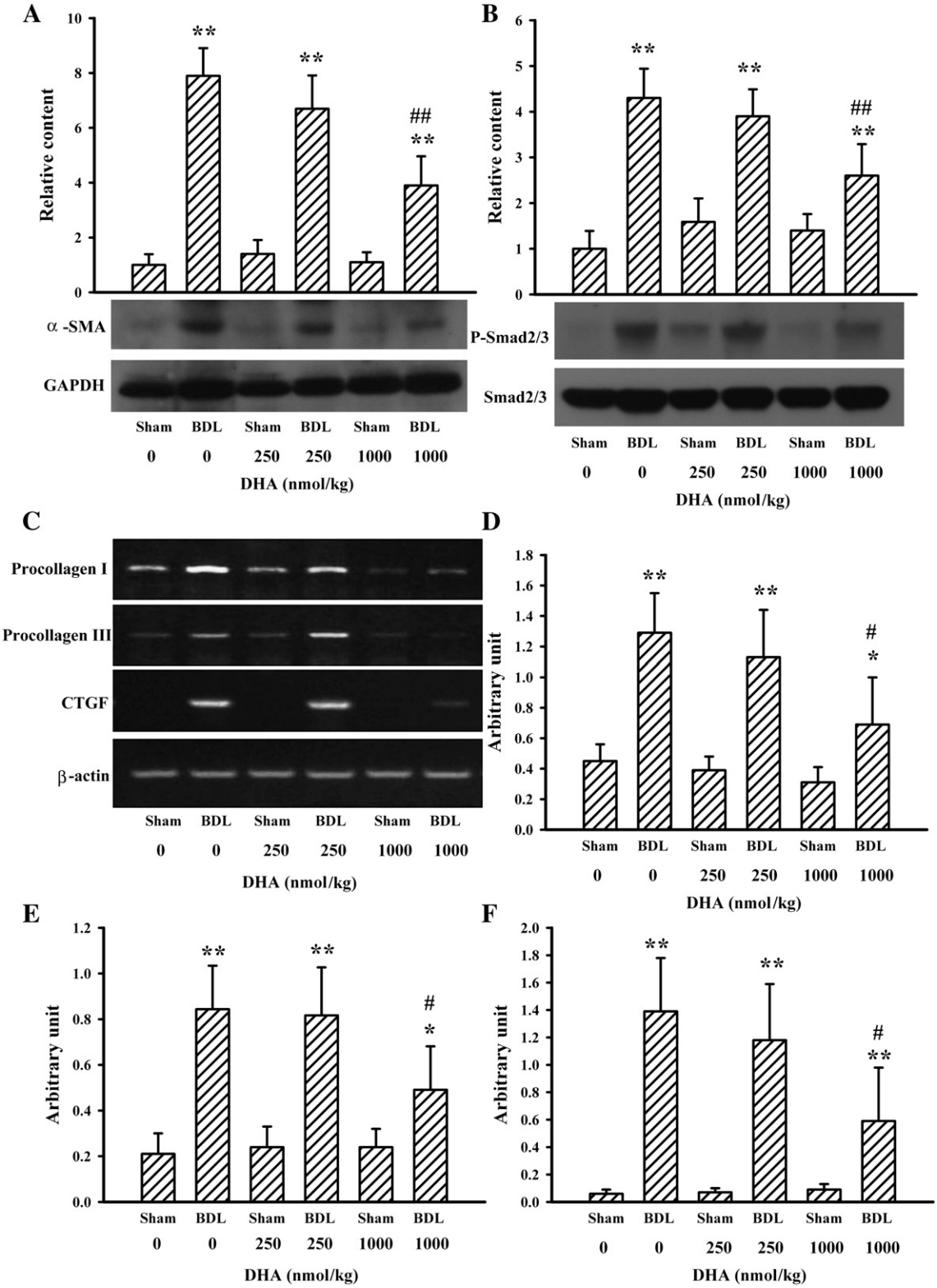

Fig. 7. DHA-attenuated, BDL-induced fibrotic signaling. Proteins were isolated from liver tissues and subjected to Western blot with antibodies against α-SMA/GAPDH (A) and P-Smad2/3/Smad2/3 (B). Representative blots and the quantitative data are shown. The relative protein content in sham control group is defined as 1. Total RNAs were isolated from liver tissues and subjected to RT-PCR. Representative blots (C) and the quantitative data of procollagen I (D), procollagen III (E) and CTGF (F) are shown. Values are expressed as mean ±S.D. *P<.05 and **P<.01 vs. the sham control group and #P<.05 and ##P<.01 vs. the BDL control group.

signal transduction has revealed that Smad2/3 is a major signaling molecule downstream of TGF-β1 cell surface receptors involved in the activation TGF-β1 gene targets [3,34–36]. Thus, they might be key therapeutic targets in the treatment of liver fibrosis. DHA possesses anti-inflammatory effects and is able to suppress pro-inflammatory cytokine expression [46]. This study demonstrated that DHA decreased mitogenic IL-1β and fibrogenic TGF-β1 expression and down-regulated BDL-induced hepatic Smad2/3 phosphorylation.

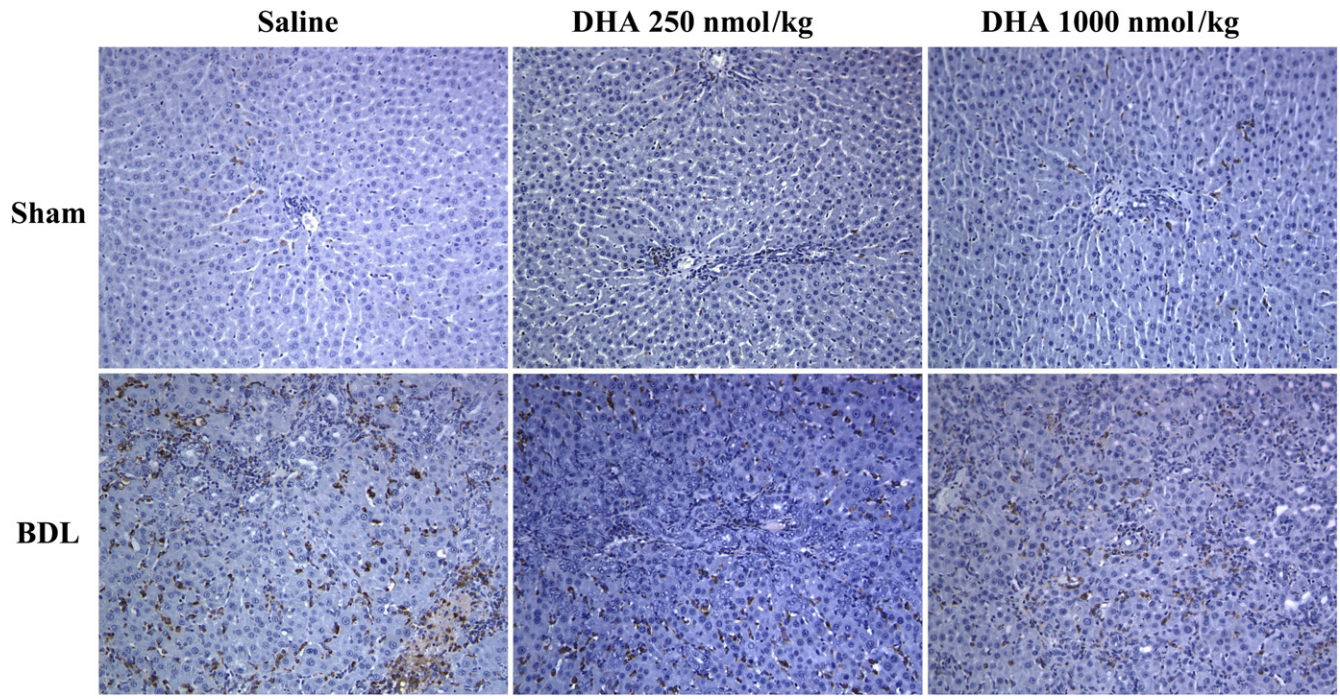

Fig. 8. DHA-attenuated, BDL-induced inflammatory cell infiltration. The obtained liver sections were subjected to immunohistochemical examination with antibody against CD68. Representative photomicrographs of CD68 immunoreactivity are shown (original magnification ×100).

These findings suggest that the inhibitory mechanisms of DHA on hepatic fibrosis are mediated by the suppression of α-SMA-positive matrix-producing cells involving mitogenic and fibrogenic inhibition. Many studies have shown strong evidence regarding the role of CTGF in liver fibrosis as a mediator of TGF-β1. Hepatic stellate cells are the major cellular source of CTGF in the liver during fibrogenesis. The importance of CTGF in stellate cell biology may be a downstream mediator of the pro-fibrotic and mitogenic actions of TGF-β1, which promotes extracellular matrix deposition and fibrogenesis [47]. Blockade of CTGF synthesis resulted in down expression of collagen mRNA in an animal model with experimental liver fibrosis [48]. The role and importance of TGF-β1/Smad2/3 signaling in DHA-mediated anti-fibrotic effects were further supported by the findings that DHA decreased BDL-induced mRNA expressions in CTGF, procollagen I and procollagen III. Taken together, these results indicate that the anti-fibrotic effect of DHA is associated with the blockade of mitogenic and fibrogenic signaling in α-SMA-positive matrix-producing cells.

Persistent cholestasis induces bile duct proliferation and dilation, attracts low-grade inflammatory infiltration, activates matrix-producing cells and leads to periportal and perineoductular fibrosis [4–10]. Bile duct epithelial cells, hepatocytes, Kupffer cells and recruited leukocytes are important sources for inflammatory responses. The consequences of an inflammatory reaction contributes to regenerative processes and/or augmented liver damage. In these processes, NF-κB plays an important role in cholangiocyte survival/damage, hepatocyte survival/damage, stellate cell and inflammatory cell activation and inflammatory cytokine production [6,49]. Inhibition of inflammatory responses prevents cholestatic liver damage and fibrosis [6,10,38,39,49]. Specifically, NF-κB inhibition attenuates hepatic injury after biliary obstruction [49]. Our results showed that DHA supplementation was accompanied by the suppression of inflammatory cell recruitment/accumulation, inflammatory cytokine production and NF-κB activation. That is, DHA attenuated BDL-induced hepatic inflammation. However, the detailed anti-inflammatory mechanisms of DHA in hepatic inflammation are not fully understood. Evidence shows that the immunosuppressive effects of n-3 PUFAs or fish oil are attributable to the elevated production of immunomodulatory cytokines such as IL-10 and/or decreased production of pro-inflammatory cytokines [46,50,51]. In addition to being an immunosuppressive mediator, IL-10 also possesses a hepatoprotective effect [52]. Free radicals are recognized as potent activators for gene expression, including IL-1β and TGF-β1 [8,53]. n-3 PUFAs possess the potential to neutralize oxidative stress [54]. Thus, the anti-inflammatory effect of DHA might be attributable to its NF-κB inhibition, antioxidant and/or immunosuppressive cytokine producing effects. In addition, the mechanisms underlying the anti-inflammatory effects of DHA may also be related to altered gene transcription and translation via direct or indirect actions on intracellular signaling pathways. Peroxisome proliferator-activated receptors (PPARs) has been demonstrated to act as a negative regulator of genes involved in the inflammatory response by antagonizing the transcription factors, such as NF-κB [55]. PPAR agonists ameliorate hepatic inflammation, fibrosis and damage in chronic cholestasis [56,57]. One of the biological activities of n-3 PUFAs such as DHA is being a potent PPAR agonist [58]. Evidence shows that the activation of AMP-activated protein kinase (AMPK) exerts anti-inflammatory and hepatoprotective effects [59,60]. Dietary PUFAs are able to enhance hepatic AMPK activity in rats [61]. It has been reported that n-3 PUFAs are able to alter the integrity of functional membrane lipid microdomains (known as caveolae/lipid rafts) and down-regulate endotoxin-related CD14 expression and toll-like receptor 4-induced signaling pathways [62,63]. Studies show that portal endotoxemia also plays an important role in cholestasis-induced inflammation [64]. Therefore, the activation of PPARs and AMPK, disruption of lipid raft integrity or interference of endotoxin-induced signaling pathways might be alternative mechanisms for DHA-mediated immunosuppression.

Evidence indicates that oxidative stress occurs during cholestasis and likely plays a role in cholestasis-induced liver injury. Cholestasis-derived reactive oxygen species are involved in the regulation of mitogenic and fibrogenic gene expression and cholangiocyte, stellate

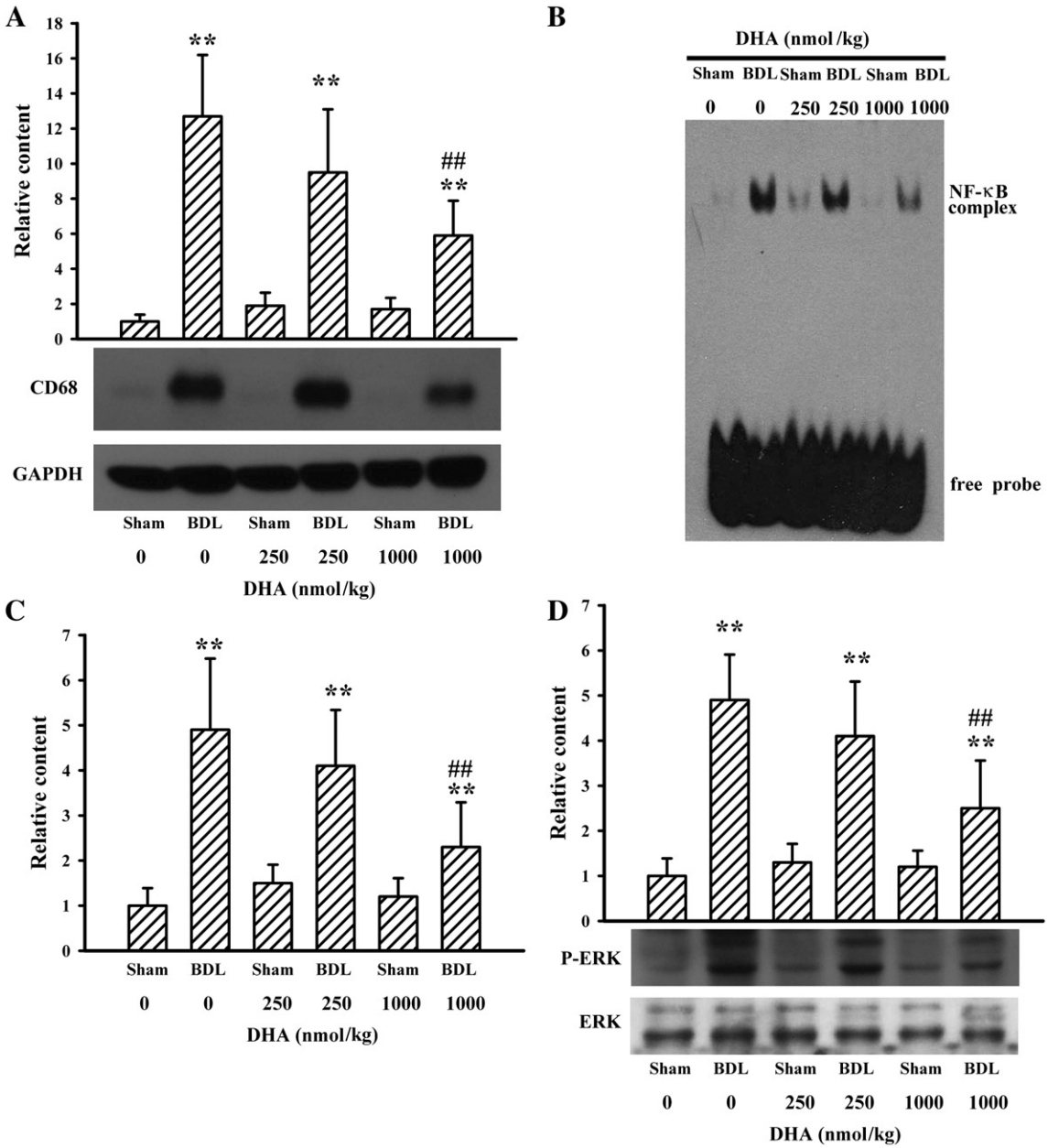

Fig. 9. DHA-attenuated, BDL-induced inflammation. Proteins were isolated from liver tissues and subjected to Western blot with antibodies against CD68 and GAPDH. Representative blots and the quantitative data are shown (A). Nuclear proteins were isolated from liver tissues and subjected to EMSA for the measurement of NF-κB DNA binding activity. Representative blots (B) and the quantitative data (C) are shown. Proteins were isolated from liver tissues and subjected to Western blot with antibodies against P-ERK and ERK. Representative blots and the quantitative data are shown (D). The relative content in sham control group is defined as 1. Values are expressed as mean±S.D. $^{**}P<.01$ vs. the sham control group and $^{\#\#}P<.01$ vs. the BDL control group.

cell and Kupffer cell activation [8,53,65–67]. BDL in rats caused changes in the equilibrium between antioxidant and pro-oxidant activity, favoring the latter, since it increased production of hepatic MDA and reduced free radical scavenging activities. The effects of n-3 PUFAs on oxidative stress are still equivocal and, in part, counterintuitive. There is considerable in vitro and in vivo evidence that points to an anti- rather than pro-oxidant action of DHA, though the precise nature of this antioxidant activity is yet to be fully clarified. It is hypothesized that trapping of free radicals, inhibition of free radical generation, elevation of antioxidant enzyme expression or enhancement of antioxidant enzyme activity may be favorable for the resolution of oxidative stress. Although chronic n-3 PUFAs supplementation has been shown to increase endogenous antioxidant enzyme activity and decrease MDA level [13,54], these effects were not observed in this study. However, we still found that DHA alleviated BDL-induced reductions in antioxidant enzyme activities and GSH content and BDL-induced hepatic oxidative stress. In consideration of these related findings, the mechanisms underlying the anti-inflammatory, anti-fibrotic and hepatoprotective effects of DHA most likely involve anti-oxidative potential.

The activation of hepatic stellate cells and other hepatic originated cells are closely associated with activation of transcription factor NF-κB and TGF-β/Smad [36,49,68]. A potential crosstalk between the NF-κB and TGF-β/Smad is proposed because that NF-κB plays a central role in the regulation of TGF-β1 expression [6]. In addition to Smad recognition site, potential NF-κB binding sites are found in the promoter of the gene encoding CTGF [36,40]. Therefore, NF-κB might regulate mitogenic/fibrogenic gene expression through itself or

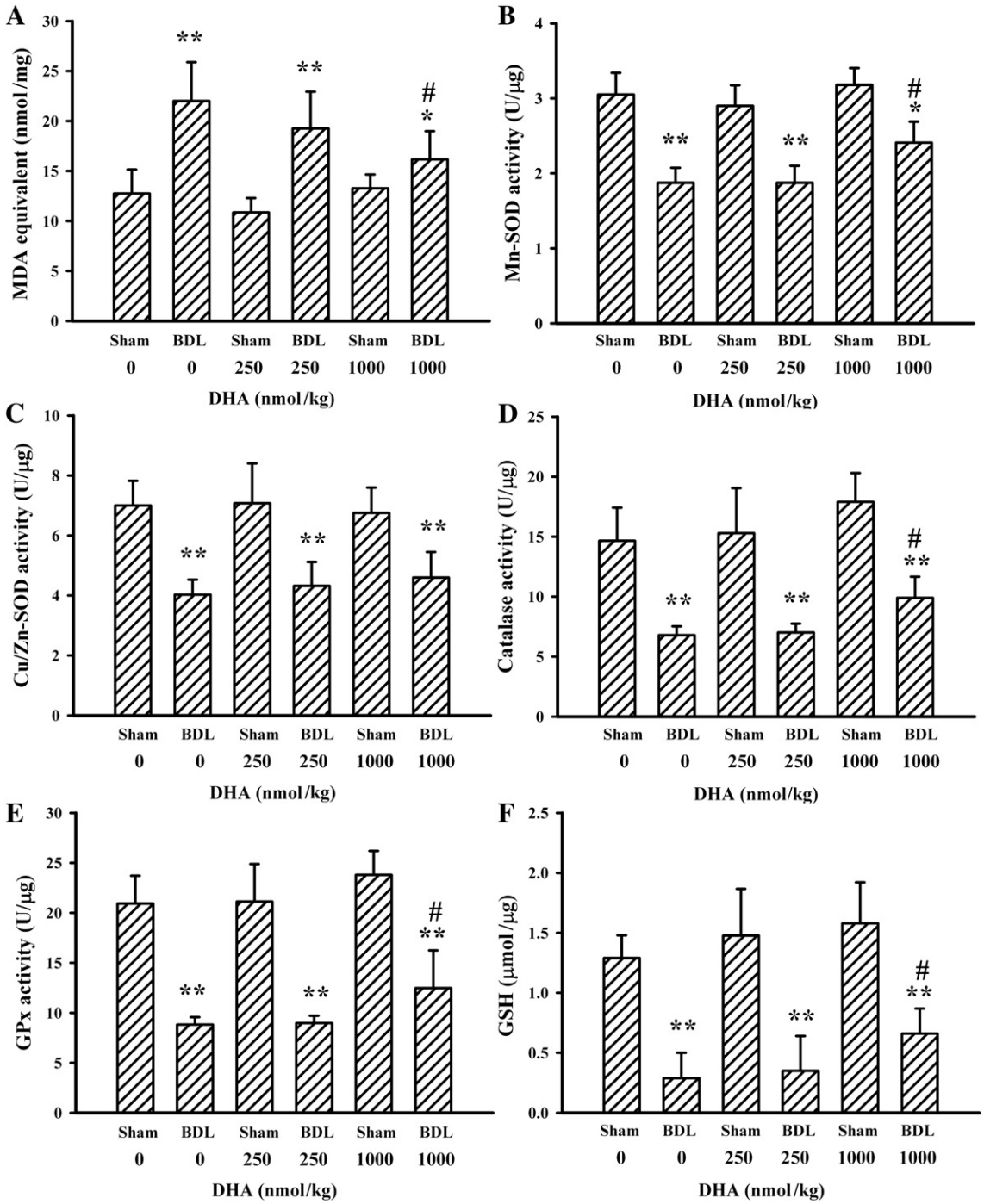

Fig. 10. DHA-attenuated, BDL-induced oxidative stress. Liver tissues were extracted and subjected to TBARS assay for the measurement of lipid peroxidation product MDA. The quantitative data are shown (A). Proteins were isolated from liver tissues and subjected to enzymatic assays for the determination of Mn-SOD (B), Cu/Zn-SOD (C), catalase (D) and glutathione peroxidase (GPx, E) activities. The quantitative data are shown. Liver tissues were extracted and subjected to the measurement of GSH content. The quantitative data are shown (F). Values are expressed as mean±S.D. *P<.05 and **P<.01 vs. the sham control group and #P<.05 vs. the BDL control group.

TGF-β/Smad-mediated mechanism. Evidence shows that CTGF gene expression is mediated through the activation of the ERK signaling pathway. Inhibition of ERK activity results in the suppression of gene expression of CTGF and collagen in hepatic stellate cells [40,69]. The ERK is an upstream regulator of NF-κB [70]. Besides, there is evidence for crosstalk between the ERK and TGF-β signaling pathways [71]. Studies also point out the importance of ERK in cholangiocyte survival/proliferation and stellate cell activation [72,73]. Therefore, the DHA inhibition of ERK activity might interfere with signaling pathways, including NF-κB and TGF-β/Smad signaling in BDL rats. ERK activity is up-regulated by phosphorylation. Our data showed that ERK activity was inhibited by DHA, manifested by decreased phosphorylation levels. Activation of protein phosphorylation cascade is regulated by kinases and phosphatases. It is not yet clear whether inactivation of kinases or activation of phosphatases orchestrates the phosphorylation change of ERK in response to DHA treatment. The integrity and function of lipid rafts and free radicals are potential upstream activators of ERK phosphorylation [74,75]. DHA alleviated oxidative burden in BDL rats. The modification of lipid composition of lipid rafts by DHA caused a change in signal

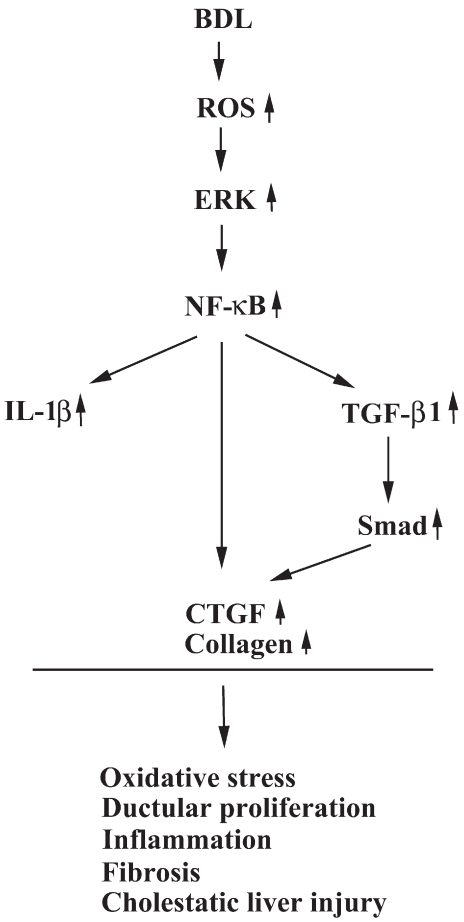

Fig. 11. A possible beneficial mechanism of DHA against BDL-induced liver injury is proposed. This schematic diagram indicates the intracellular signaling molecules employed in mediating oxidative stress, ductular proliferation, inflammation, fibrosis and cholestatic liver injury after BDL in rats. Some additional signaling molecules and cascades have been omitted for the sake of clarity. The label ↑ in each molecule indicates its activity or expression was stimulated by BDL. DHA supplementation attenuated those BDL-induced alterations.

transduction from these specialized plasma membrane microdomains [63]. In addition, DHA was able to up-regulate protein phosphatase activity [76]. These observations suggest that the inhibition of ERK activity by DHA in BDL rats might be multifactorial. The resolution of oxidative stress, the interference of lipid raft-derived signals and the activation of protein phosphatases are possibilities.

The results of the current study support the concept that DHA could serve as a hepatoprotective agent, and chronic DHA supplementation could attenuate cholestasis-related hepatic damage. The experimental findings provide evidence that supplementation with DHA is effective in alleviating BDL-induced cholestatic liver injury, ductular proliferation, oxidative stress, inflammation and fibrosis. Transcription factors represent a group of important effectors that could cause the convergence of multiple extrinsic and intrinsic signals resulting in the regulation of cell proliferation, cell activation and pro-inflammatory cytokine expression. We have shown that inhibition of BDL-induced cholangiocyte, stellate cell and inflammatory cell activation by DHA is attributed to down-regulation of NF-κB and TGF-β/Smad activities probably via interference of ERK activation. The hepatoprotective, anti-inflammatory and anti-fibrotic effects of DHA seem to be multifactorial. It is reasonable to propose that resolution of oxidative stress by DHA represents a potential way to attenuate cholestatic liver injury. A schematic diagram showing the intracellular signaling molecules employed in mediating potential beneficial mechanism of DHA against cholestatic liver injury is depicted in Fig. 11. However, other beneficial mechanisms and action targets by which DHA protects against cholestatic liver injury require further investigation.

Acknowledgment

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