# Docosahexaenoic acid (DHA) blunts liver injury by conversion to protective lipid mediators: protectin D1 and 17*S*-hydroxy-DHA

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ABSTRACT **Docosahexaenoic acid (DHA) is a ω-3 essential fatty acid that reduces the incidence and severity of a number of diseases. Recently, a novel series of DHA-derived lipid mediators with potent protective actions has been identified. In this study we demonstrate that dietary amplification of these DHA-derived products protects the liver from necroinflammatory injury. *In vitro*, supplementation of hepatocytes with DHA significantly reduced hydrogen peroxide-induced DNA damage, evaluated by the "comet assay," and oxidative stress, determined by measurement of malondialdehyde levels. *In vivo*, dietary supplementation of mice with DHA ameliorated carbon tetrachloride-induced necroinflammatory damage. In addition, hepatic cyclooxygenase-2 expression and $PGE_2$ levels were significantly reduced in mice fed DHA-enriched diets. In these animals, increased hepatic formation of DHA-derived lipid mediators (*i.e.*, 17*S*-hydroxy-DHA (17*S*-HDHA) and protectin D1) was detected by HPLC-gas chromatography/mass spectrometry analysis. Consistent with these findings, synthetic 17-HDHA abrogated genotoxic and oxidative damage in hepatocytes and decreased TNF-α release and 5-lipoxygenase expression in macrophages. In a transactivation assay, 17-HDHA acted in a concentration-dependent manner as a PPARγ agonist. Taken together, these findings identify a potential role for DHA-derived products, specifically 17*S*-HDHA and protectin D1, in mediating the protective effects of dietary DHA in necroinflammatory liver injury.—González-Périz, A., Planagumà, A., Gronert, K., Miquel, R., López-Parra, M., Titos, E., Horrillo, R., Ferré, N., Deulofeu, R., Arroyo, V., Rodés, J., Clària, J. Docosahexaenoic acid (DHA) blunts liver injury by conversion to protective lipid mediators: protectin D1 and 17*S*-hydroxy-DHA. *FASEB J.* 20, E1844–E1855 (2006)**

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Virtually every type of liver disease has a significant inflammatory component as its underlying cause (1, 2). Although beneficial as a limited wound-healing process, if the inflammatory response remains uncontrolled or is not properly resolved, it results, in time, in tissue damage, scar accumulation, and fibrosis and eventually in the life-threatening condition of liver cirrhosis (1, 2). Therefore, the development of novel strategies aimed to modulate the factors that govern hepatic inflammation or promote its timely resolution represents a primary focus for disrupting the sequence of events leading to liver injury.

Long chain ω-3 essential fatty acids, found in high proportions primarily in fish oils, display potent anti-inflammatory properties (3). In fact, dietary interventions rich in eicosapentaenoic (EPA, C20:5n-3) and/or docosahexaenoic acid (DHA, C22:6n-3) have been shown to keep inflammation under control without side effects, and therefore are used as preventive measures against a number of illnesses such as rheumatoid arthritis, cystic fibrosis, ulcerative colitis, asthma, atherosclerosis, cancer, and cardiovascular disease (3). However, the molecular mechanisms underlying the beneficial actions of ω-3 essential fatty acids remain to be clearly defined. Recently, a new series of lipid mediators generated from ω-3 essential fatty acids has been identified by lipidomic analyses in exudates that were collected during the resolution phase of acute inflammatory response in mice (4–7). Specifically, during spontaneous resolution, cell-cell interactions and transcellular biosynthesis lead to the production of novel bioactive lipid mediators from DHA and EPA, which have been termed resolvins (resolution phase interaction products) and protectin D1 (10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid or neuroprotectin D1) (4–7). These ω-3-derived lipid mediators display potent anti-inflammatory actions *in vivo* in experimental models of colitis, peritonitis, brain

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ischemia-reperfusion, and corneal injury (8–13). In particular, protectin D1 (PD1), a bioactive DHA product generated from the metabolic intermediate 17*S*-hydro(peroxy)-DHA (17*S*-H(p)DHA), potently regulates critical events associated with inflammation and its resolution, including inhibition of PMN infiltration and T cell migration and reduction of TNF-α and IFNγ secretion, chemokine formation, and interleukin 1 (IL-1)-induced NFκB activation (10–13).

Considering the beneficial effects associated with DHA in several clinical disorders, in the current investigation we addressed the question of whether this ω-3 essential fatty acid could protect liver cells from oxidative damage and whether dietary supplements enriched in DHA could exert hepatoprotective actions in mice submitted to an experimental model of liver injury. In this study we also explored potential mechanisms of action by monitoring the generation of bioactive DHA-derived lipid mediators in the liver and testing their biological effects on key events involved in the pathogenesis of necroinflammatory liver injury.

## MATERIALS AND METHODS

### Materials

Male 129S2/SvPasCrl mice were purchased from Charles River (Saint Aubin les Elseuf, France). Isoflurane was from Abbott Laboratories (Abbott Park, IL, USA). Carbon tetrachloride ($CCl_4$), olive oil, choline bitartrate, EDTA, NaCl, Triton-X-100, hydrogen peroxide, propidium iodide, and Dulbecco's modified Eagle medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Tris, NaOH, and HEPES were from Merck (Darmstadt, Germany). Murine Raw 264.7 macrophages and CC-1 hepatocytes were purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK). Synthetic DHA, 17-HDHA, 17*R*-HDHA, 14-HDHA, 7-HDHA, 4-HDHA, 15*S*-hydroxyeicosatetraenoic acid (HETE), 12*S*-hydroxyeicosatetraenoic acid, 5*S*-hydroxyeicosatetraenoic acid, and $LTB_4$ and enzyme immunoassay (EIA) kits for leukotriene (LT) $B_4$ and prostaglandin (PG) $E_2$ were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The TNF-α EIA kit was purchased from Assay Designs (Ann Arbor, MI, USA). Sep–Pak $C_{18}$ cartridges were from Waters (Milford, MA, USA). Trizol, penicillin-streptomycin, trypsin-EDTA, low melting agarose, and nonessential amino acids were from Invitrogen (Carlsbad, CA, USA). Normal melting agarose was from Serva (Heidelberg, Germany). L-Glutamine was from Biological Industries (Kibbutz Beit Haemek, Israel). EMEM, phenol red-free EMEM, and DPBS were purchased from BioWhittaker (Cambrex Co., East Rutherford, NJ, USA). FBS was from Biowest (Nuaillé, France). 40% Acrylamide/bis, polyvinylidene difluoride (PVDF) membranes, and Bio-Safe Coomassie were from Bio-Rad. The enhanced chemiluminescence (ECL) system was from Amersham (Buckinghamshire, UK). The first-strand cDNA synthesis kit was from Promega (Madison, WI, USA). The cyclooxygenase-2 (COX-2) and β-actin assays-on-demand were from Applied Biosystems (Foster City, CA, USA). Purified ω-3 essential fatty acids were kindly provided by Dr. D. Raederstorff (Roche, Basel, Switzerland) and mouse diets were prepared by U.A.R. (Villemoisson-sur-Orge, France). PPARγ and luciferase constructs were kindly provided by Dr. Ronald Evans (Salk Institute, La Jolla, CA, USA). Polyclonal rabbit antisera specific for 5-lipoxygenase (5-LO) (LO-32) was generously provided by Dr. Jilly Evans (Merck Research Laboratories, Rahway, NJ, USA).

### Assessment of hepatocyte DNA damage by single-cell gel electrophoresis assay (comet assay)

DNA damage was assessed in murine CC-1 hepatocytes growing in 75 $cm^2$ flasks in EMEM with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 1% nonessential amino acids, 50 U/ml penicillin, and 50 μg/ml streptomycin following a method adapted from Singh *et al.* (14) and Klaude *et al.* (15). Briefly, CC-1 cells were transferred to 60 mm culture dishes and exposed to hydrogen peroxide (200 μM) for a total of 1 h in a humidified 5% $CO_2$ atmosphere at 37°C in the presence of serum-free EMEM medium alone or supplemented with DHA (10 μM). For experiments examining the biological activity of DHA-derived lipid mediators, CC-1 cells were grown as described above, then treated with 17-HDHA (1 μM) for 1 h. Subsequently, cells were trypsinized and suspended in DPBS ($50\times10^3$ cells/0.2 ml), centrifuged at 800 *g* for 5 min at 4°C, and pellets were resuspended in 200 μl of 0.5% low melting agarose at 37°C. One hundred microliters of this mixture were spread on an agarose-coated glass slide, covered with a glass coverslip, and left at 4°C for 10 min. Agarose-coated slides were prepared by dipping regular-sized glass slides into 1.5% normal melting agarose, followed by air-drying. Thereafter, coverslips were removed and slides exposed to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100; pH 10) at 4°C for 1 h. After lysis, slides were exposed to electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH 13) for 20 min, then subjected to electrophoresis for 15 min (300 mA, 25 V) at 4°C. Finally slides were neutralized with 400 mM Tris buffer pH 7.5, rinsed with distilled water, stained with propidium iodide (20 μg/ml), covered with a coverslip, and visualized in a fluorescence microscope (Nikon Eclipse E600, Japan) equipped with a 510–560 nm excitation filter. DNA damage was evaluated following a visual classification of relative intensity of fluorescence in the tail (tail length) and scored into five categories (0–4), with 0 representing undamaged cells and 4 the most severely damaged. Fifty comets per slide were analyzed and DNA damage was expressed as arbitrary units ranging from 0 (all undamaged) to 200 (all maximally damaged).

### Measurement of oxidative stress levels

Oxidative stress levels in CC-1 hepatocytes were determined by measuring malondialdehyde (MDA) concentrations in cell lysates by HPLC, as described elsewhere (16). Briefly, CC-1 cells were seeded in 12-well plates at a density of $4 \times 10^5$ cells/ml in complete EMEM medium for 16 h and exposed to hydrogen peroxide (200 μM) for 1 h in a humidified 5% $CO_2$ atmosphere at 37°C in the presence of fresh serum-free EMEM medium (without phenol red) alone or supplemented with DHA (1 and 10 μM). For experiments assessing the biological effects of DHA-derived lipid mediators, cells were grown as described above, then treated with 17-HDHA (1 μM) for 1 h. At the end of the incubation period, 2 vol of cold methanol were added and cell lysates were collected and evaporated under a stream of nitrogen. Pellets were resuspended in DPBS, homogenized in 0.3% trichloroacetic acid and centrifuged at 8000 *g* for 3 min at 4°C. Supernatants were subsequently exposed to phosphoric and thiobarbituric acids and heated at 80°C for 30 min prior to analysis by HPLC.

### Animal studies

Male 129S2/SvPasCrl mice were housed in plastic cages on wood chip bedding and provided free access to water and

standard mouse chow for an acclimation period of 1 wk. Subsequently, mice were assigned to three groups (8 mice each) that were fed a control diet (control group), an experimental diet enriched with DHA (DHA group), or an experimental diet enriched with DHA and EPA (DHA+EPA group). The experimental fatty acids were incorporated into a base fabricated diet composed of starch (522.1 g/kg), sucrose (100 g/kg), cellulose (50 g/kg), casein (190 g/kg), L-cystine (1.8 g/kg), lipids (84.1 g/kg), salt mixture (40 g/kg, ICN 960401), vitamin mixture (10 g/kg, ICN 960402), and choline bitartrate (2 g/kg) (**Table 1**). The lipid part (84.1 g/kg) was composed of 40 g/kg cocoa butter and either 44.1 g/kg sunflower seed oil (control group) or 30.4 g/kg sunflower seed oil + 13.7 g/kg ω-3 polyunsaturated fatty acids supplied as either purified DHA 70% (DHA group) or a mixture containing 80% DHA and EPA (DHA+EPA group). These diets have been used before as dietary ω-3 essential fatty acid supplements in different animal models of disease (17, 18). The diets were stored at –20°C and provided fresh daily. After 1 wk of feeding the experimental diets, mice received an i.p. injection of $CCl_4$ (1 ml/kg b.w. in olive oil as a carrier) twice a week for a total of 5 wk. Body weight and food intake were monitored throughout the experiment. After the intervention period, animals were sacrificed under isoflurane anesthesia, and the liver was excised, rinsed in DPBS, and fixed in 10% formol for histological analysis. Portions of liver tissue were also snap-frozen in liquid nitrogen for analysis of gene expression and measurement of eicosanoids and DHA-derived lipid mediators. All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Hospital Clinic and the European Community laws governing the use of experimental animals.

**Histological analysis**

Liver samples fixed in 10% formol were embedded in paraffin, cut at 5 μm sections, and stained with hematoxylin/eosin and Masson's trichrome and analyzed by a registered pathologist (R.M.) unaware of the treatments. The degree of hepatocellular damage was quantified by assessing necroinflammatory injury scored as Grade 0, (absent), Grade 1 (spotty necrosis; one or few necrotic hepatocytes), Grade 2 (confluent necrosis), and Grade 3 (bridging necrosis); and hepatocyte ballooning scored as 1 (low), 2 (mild), and 3 (severe). Liver fibrosis was scored as Stage 0 (absent, normal lobular architecture), Stage 1 (pericentral fibrosis, increased thickness of the central vein), Stage 2 (central anastomoses, some fibrous septa connecting central veins), Stage 3 (precirrhotic stage, fibrous septa with marked distorsion of the liver lobules), and Stage 4 (cirrhosis, nodule regeneration surrounded by broad connective tissue septa). Steatosis was scored by the percentage (%) of liver cells containing fat as 1 (<33%), 2 (<66%), and 3 (>66%).

TABLE 1. *Composition of the experimental diets*[a]

| Components (g/kg) | Control | DHA | DHA+EPA |
|---|---|---|---|
| Starch | 522.1 | 522.1 | 522.1 |
| Sucrose | 100 | 100 | 100 |
| Cellulose | 50 | 50 | 50 |
| Casein | 190 | 190 | 190 |
| L-Cystine | 1.8 | 1.8 | 1.8 |
| Salt mixture | 40 | 40 | 40 |
| Vitamin mixture | 10 | 10 | 10 |
| Choline bitartrate | 2 | 2 | 2 |
| Cocoa butter | 40 | 40 | 40 |
| Sunflower seed oil | 44.1 | 30.4 | 30.4 |
| DHA | 0 | 13.7 | 0 |
| DHA+EPA | 0 | 0 | 13.7 |

[a]All diets contained the same amount of lipids (84.1 g/kg). DHA: diet enriched in purified docosahexaenoic acid (C22:6n-3); DHA+EPA: diet enriched in a mixture containing docosahexaenoic acid and eicosapentaenoic acid (C20:5n-3).

**Analysis of mRNA expression by real-time RT-polymerase chain reaction (RT-PCR)**

Total RNA was obtained by the Trizol reagent method. RNA concentration was assessed in a UV spectrophotometer and its integrity was tested on a 6000 LabChip in a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). First-strand cDNA synthesis was performed by incubating 1 μg of total RNA with 4 μl of RT buffer, 2 μl of 10 mM dNTPs, 0.5 μl RNasin, 1 μl of Oligo $dT_{15}$ (500 μg/ml), and 1.5 μl of avian myeloblastosis virus reverse transcriptase (13.5 U) (20 μl final vol) at 42°C for 45 min in a MJ Research PTC-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Real-time quantitative polymerase chain reaction (PCR) was performed with an ABI Prism 7900 Sequence Detection System (Applied Biosytems, Foster City, CA, USA) using the fluorescent TaqMan methodology. Ready-to-use primer and probe sets predeveloped by Applied Biosystems (TaqMan Gene Expression Assays) were used to quantify COX-2 (ID: Mm00478374_m1) gene expression using β-actin (ID: Mm00607939_s1) as an endogenous control. Briefly, PCR reactions were performed in duplicate using the Universal TaqMan 2 × PCR master mix in a volume of 25 μl containing 1 μl cDNA. The thermal profile included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. PCR results were analyzed with the Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantitation of gene expression was performed using the standard curve method. Every set of reactions included four serial 2-fold dilutions of the same liver cDNA sample, which was used to generate standard curves for both the target gene (COX-2) and the endogenous reference (β-actin). The amount of COX-2 was then calculated from the standard curve and divided by the amount of β-actin to obtain a normalized value.

**Reverse phase (RP)-HPLC and gas chromatography (GC)/ mass spectrometry (MS) analysis**

Frozen liver samples were placed in methanol/water (5 ml, 65/35, v/v, 4°C). $PGB_2$ (100 ng) was added to samples as an internal standard for recovery and livers were gently homogenized at 4°C and extracted by C18-ODS solid phase as previously detailed (11, 19). In brief, homogenized liver suspensions were placed at –20°C for at least 60 min, centrifuged, and supernatants were collected. Supernatants were diluted with 10 volumes of HPLC grade water (4°C) and acidified to pH 4.0 with HCl (1 N). Acidified samples were immediately loaded onto primed C18-ODS cartridges (AccuBond II, 500 mg, Agilent Technologies). Cartridges were washed with 10 ml of HPLC grade water followed by hexane and compounds eluted in methyl formate, then by a final elution in methanol. Methyl formate fractions were taken to dryness under a gentle stream of nitrogen, resuspended in methanol (100 μl), and stored at –80°C.

DHA-derived products were analyzed using RP-HPLC and capillary GC/MS analysis as described previously (5, 11). Briefly, analysis was performed with an Agilent 1100 series HPLC system (Agilent Technologies) equipped with a diode array detector (DAD), a binary pump, membrane degasser, heated column compartment, and a microflow cell. Samples

were injected in mobile phase and eluted on a Luna C18–2 minibore column (2×150 mm, 5 μm, Phenomenex, Torrance, CA, USA) or a Beckman Coulter Ultrasphere column (4.6×250 mm, 5 μm, Phenomenex) using a mobile phase system that consisted of methanol/water/acetate (65/35/0.02, v/v/vol) that was run as a linear gradient to reach 100% methanol/acetate (99.98/0.02, v/v). Collected UV data were recalled at 234 and 270 nm and analyzed by LC 3D ChemStation software (Agilent Technologies). Calibration curves were established for each compound and peak areas were integrated for quantitation.

GC/MS analysis was performed as in ref. 11 with a 6890N GC System with a HP5MS cross-linked ME siloxane column (30 m×0.25 mm×0.25 μm), autosampler, and a 5973N Mass Selective Detector (Agilent Technologies). The helium flow rate was 1.5 ml/min and the initial temperature was 150°C, followed by 230°C (2 min) and 280°C (10 min). For selected samples, HPLC fractions that corresponded to established retention times of authentic standards were collected and derivatized to generate pentafluorobenzyl (PFB) esters and trimethylsilyl (TMS) ether derivatives, and 10–1000 pg was injected into 2 μl iso-octane for GC/MS analysis. Reference material for 10,17*S*-dihydroxy-DHA (PD1) and 17*S*-HDHA was prepared as described previously (4, 5, 10, 11). Physical criteria for identification such as HPLC retention times, specific UV chromophores, as well as GC/MS and LC/MS major and signature ions have already been established (4, 5, 10). Endogenous arachidonic acid LO products such as $LTB_4$, 5,12-diHETE isomers, 12*S*-HETE, 15*S*-HETE, 5*S*-HETE, as well as standards for the docosanoids 7*S*,17*S*-dihydroxy-DHA (resolvin D5), 14*S*-HDHA, 7*S*-HDHA, and 4*S*-HDHA, which exhibit similar UV characteristics, had distinct retention times in our HPLC system and did not coelute with 17*S*-HDHA or PD1.

### Analysis of eicosanoids by EIA

Liver tissue samples (0.2 g) were homogenized in cold $DPBS^{2+}$ with an Ultra-Turrax T 25 Basic homogenizer (IKA-Werke, Staufen, Germany). Homogenates were centrifuged at 400 g for 10 min at 4°C. Supernatants were collected and brought to a final volume of 10 ml with distilled water, transferred into syringes, acidified to pH 3.5 and loaded onto $C_{18}$-silica reverse-phase cartridges. The eluted methyl formate fraction was rapidly evaporated under a stream of nitrogen, resuspended in 1 ml of methanol and kept at –20°C until analysis of $PGE_2$ by EIA.

### Macrophage TNF-α release

The murine macrophage cell line Raw 264.7 was grown in 150 $cm^2$ flasks in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were transferred to 75 $cm^2$ flasks at a density of $6 \times 10^6$ cells/flask and grown in serum-free DMEM for 16 h before the addition of vehicle (0.5% ethanol) or 17-HDHA (1 μM) for 2 h in a humidified 5% $CO_2$ atmosphere at 37°C. At the end of the incubation period, supernatants were collected in tubes containing butylated hydroxytoluene (BHT) (0.01% final concentration) and TNF-α levels determined by a specific EIA kit.

### Peroxisome proliferator-activated receptor (PPAR) γ transactivation assay

A transactivation assay based on a fusion protein containing the yeast GAL4 DNA binding domain linked to the ligand binding domain of PPARγ (PPARγ-GAL4 plasmid) and a luciferase reporter construct containing four copies of a GAL4 upstream activating sequence ($UAS_G$) and a thymidine kinase (tk) promoter (MH100-tk-luc plasmid) was used to assess the ability of compounds to activate PPARγ independent of endogenous receptors (20). To this end, COS-7 cells ($3\times10^4$ cells/well) were plated in 12-well plates at 70% confluence and cotransfected with 1 ml of DMEM containing 0.3 μg of the luciferase reporter construct MH100-tk-luc, 0.1 μg of PPARγ-GAL4, and 0.002 μg of β-galactosidase expression vector pCMV (an internal control plasmid containing a cytomegalovirus (CMV) promoter) by using the Effectene transfection reagent at a ratio 1:10 to DNA, according to the manufacturer's instructions. Thirty hours after transfection, the medium was removed and cells were washed twice with $DPBS^{2+}$ before addition of vehicle (0.5% ethanol), 7-HDHA (0.1, 1, 5, and 10 μM), 14-HDHA (0.1, 1, 5, and 10 μM), or 17-HDHA (0.1, 1, 5, and 10 μM) for another 18 h in serum-free DMEM. To determine the stereoselectivity of the alcohol group in position 17, 17*R*-HDHA (0.1, 1, 5, and 10 μM) was also tested in the PPARγ reporter assay. At the end of the incubation, cells were harvested in luciferase lysis buffer and light units from firefly luciferase and β-galactosidase activities were measured in a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany). Luciferase values were expressed as relative light units and normalized to the level of β-galactosidase activity. Changes in PPARγ activity were expressed as "fold induction" relative to the vehicle control values.

### Analysis of 5-LO protein expression by Western blot

Raw 264.7 cells were incubated for 2–24 h with vehicle (0.5% ethanol), DHA (1 and 10 μM), and 17-HDHA (1 μM) in a humidified 5% $CO_2$ atmosphere at 37°C. At the end of the incubation period, cells were washed in DPBS, scraped from the culture flask, and centrifuged at 1500 *g* for 5 min. Total protein was extracted in lysis buffer containing 1 M Tris/HCl pH 7.4, 1% triton X-100, 10% glycerol, 137 mM NaCl, 0.5 mM EDTA, and protease inhibitors. Thereafter, the cell lysate was centrifuged at 1700 *g* for 5 min at 4°C and 5-LO protein expression in lysate supernatants was analyzed by Western blot as described previously (21). Briefly, aliquots from each sample containing 30 μg of total protein, determined by the Bradford protein assay, were resuspended in SDS-containing Laemmli sample buffer, heated for 5 min at 95°C, and separated through 12.5% SDS-PAGE. Proteins were electroblotted overnight at 4°C onto PVDF membranes and the efficiency of the transfer was visualized by Ponceau staining. Membranes were then soaked for 1 h at room temperature in Tris-buffered saline (20 mM Tris/HCl pH 7.5 and 0.5 M NaCl) containing 0.05% (v/v) Tween 20 (0.05% T-TBS) and 5% (w/v) nonfat dry milk. Blots were washed twice for 5 min each with 0.05% T-TBS and subsequently treated for 2 h at room temperature with an anti-5-LO polyclonal antibody (pAb) (dilution 1:1000) in 0.05% T-TBS containing 1% dry milk. After washing the blots twice for 5 min each with 0.05% T-TBS, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-linked donkey anti-rabbit antibody (Ab) (dilution 1:2000) in 0.05% T-TBS and subsequently developed using an enhanced chemiluminescence (ECL) detection system.

Statistical analysis of the results was performed using the ANOVA and unpaired Student's *t* test. Results are expressed as mean ± SE and differences were considered significant at a $P$ value $< 0.05$.

## RESULTS

To test *in vitro* the potential protective effects of ω-3 essential fatty acids on liver cells, hepatocytes were grown in medium supplemented with DHA; DNA damage, an early event in the initiation and progression of liver injury, was assessed by means of a single-cell gel electrophoresis assay, the so-called comet assay. As shown in **Fig. 1A**, the length of comet tails, and therefore the extent of DNA strand breaks induced by hydrogen peroxide, was significantly lower in hepatocytes growing in medium supplemented with DHA than that in hepatocytes growing in medium alone. In addition, oxidative stress levels, as determined by measurement of MDA levels by HPLC in cell lysates, was also significantly decreased in hepatocytes growing in a medium rich in DHA (Fig. 1*B*).

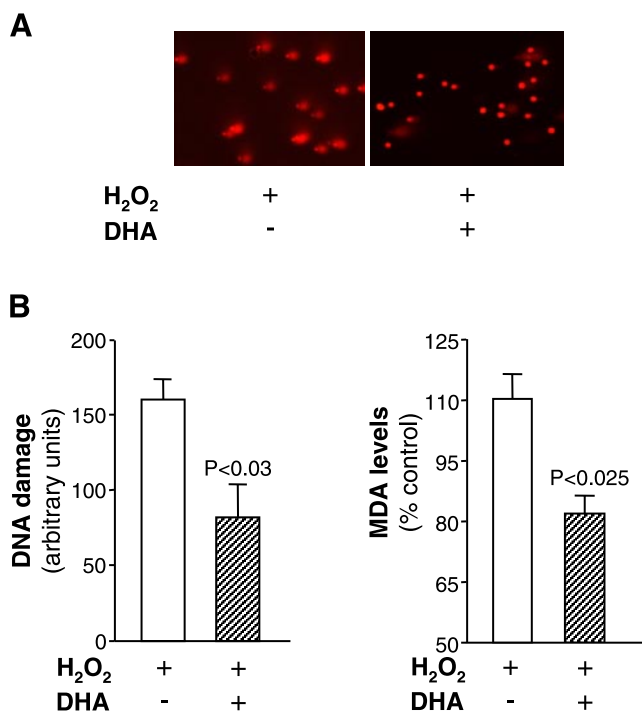


**Figure 1.** Effects of DHA supplementation on liver cells in culture. *A)* Effect on DNA damage. Murine CC-1 hepatocytes were exposed to hydrogen peroxide (200 μM) for a total of 1 h in a humidified 5% $CO_2$ atmosphere at 37°C in the presence of serum-free EMEM medium alone or supplemented with DHA (10 μM). DNA damage was assessed by the single-cell gel electrophoresis assay (comet assay). Comet images (original magnification ×200) are representative of 3 different experiments. The relative intensity of fluorescence in the tail (tail length) was visually scored in 50 comets per slide and assigned as arbitrary units ranging from 0 (all undamaged) to 200 (all maximally damaged) (see Material and Methods for more details). Data are expressed as mean ± SE and *P* value is referred with respect to vehicle. *B)* Effect on oxidative stress. CC-1 cells were exposed to hydrogen peroxide (200 μM) for 1 h in a humidified 5% $CO_2$ atmosphere at 37°C in the presence of fresh serum-free EMEM medium (without phenol red) alone or supplemented with DHA (10 μM). Oxidative stress levels were determined by measuring MDA concentrations in cell lysates as described in Material and Methods. Results are the mean ± SE of 4 different experiments and *P* values are given with respect to untreated cells.

To extent these protective actions of DHA observed in hepatocytes at the *in vivo* level, we administered diets supplemented with DHA to mice submitted to the experimental model of $CCl_4$-induced liver injury and compared the hepatic necroinflammatory damage with that of mice receiving a control diet. As expected, histological examination of livers from mice treated with $CCl_4$ for 5 wk revealed massive and severe hepatocyte necrosis, inflammation, and ballooning at the centrilobular zone with bridging of necrosis that severely disrupted the sinusoidal and lobular architecture of the liver (**Fig. 2A**, upper panels). A significant improvement in hepatic pathology occurred in mice fed the experimental diets enriched with either DHA (Fig. 2*A*, middle panels) or DHA+EPA (Fig. 2*A*, lower panels). In fact, the scores for necroinflammatory liver injury were significantly lower in mice fed with ω-3-enriched experimental diets than in those receiving a control diet (Fig. 2*B*). In addition, the diet enriched with DHA+EPA reduced ballooning degeneration in hepatocytes (Fig. 2*B*). Mice consumed between 2.11 and 2.36 g of food per day, and there were no significant differences in food intake, body weight gain, and relative liver weight among the animals receiving DHA-enriched diets and controls (data not shown). Feeding ω-3-enriched diets did not affect the degree of hepatic steatosis (score from 1 to 3: control: 1.0±0.0; DHA: 1.1±0.1; DHA+EPA: 1.0±0.0). Fibrosis was not observed in any group of the study (data not shown).

We next assessed the profile of bioactive lipid mediators generated in livers from mice fed DHA-enriched diets. We first monitored changes in the proinflammatory COX-2 pathway by quantitative real-time RT-PCR analysis and the formation of the ω-6-derived eicosanoid, $PGE_2$, by EIA. As shown in **Fig. 3A**, administration of diets enriched with ω-3 essential fatty acids led to significant decreases in COX-2 mRNA expression in the liver. Accordingly, the hepatic levels of $PGE_2$ were significantly reduced in both DHA and DHA+EPA groups (Fig. 3*B*). We also assessed by RP-HPLC and GC/MS analysis the formation of potent anti-inflammatory and proresolution DHA-derived lipid mediators in livers from mice fed a DHA-enriched diet. Specifically, PD1 as well as 17*S*-HDHA, a marker of activation of the PD1 pathway and potential metabolic precursor for the trihydroxy 17*S*-resolvins (RevD1-D4), were identified as unique products in liver samples obtained from the DHA group and were not detected in liver samples from the control diet group (**Fig. 4A, C**). PD1 was identified by coelution with the biosynthetic standard and by its characteristic UV spectrum, which is consistent with the presence of a conjugated triene double bond structure (Fig. 4*A*, inset). 17*S*-HDHA was identified as a peak that matched the retention time (Fig. 4*C*) and the characteristic conjugated diene chromophore of the synthetic 17*S*-HDHA standard. 14*S*-Hydroxy-DHA (14*S*-HDHA) was also detected in livers obtained from animals receiving the DHA diet and, like 17*S*-

HDHA, was not detected in mice from the control diet group (Fig. 4*C*). To further confirm the identity of the DHA-derived products in liver samples, materials beneath the PD1 and 17*S*-HDHA peaks were individually collected and their methyl ester trimethylsilyl derivatives were prepared and taken for GC/MS analysis. As shown in Fig. 4*B*, PD1 was identified by its MS spectrum, which displayed ions at *m/z* 503, 413, and 323, which are diagnostic for a dihydroxy DHA structure, whereas material eluting beneath 17*S*-HDHA (Fig. 4*D*)

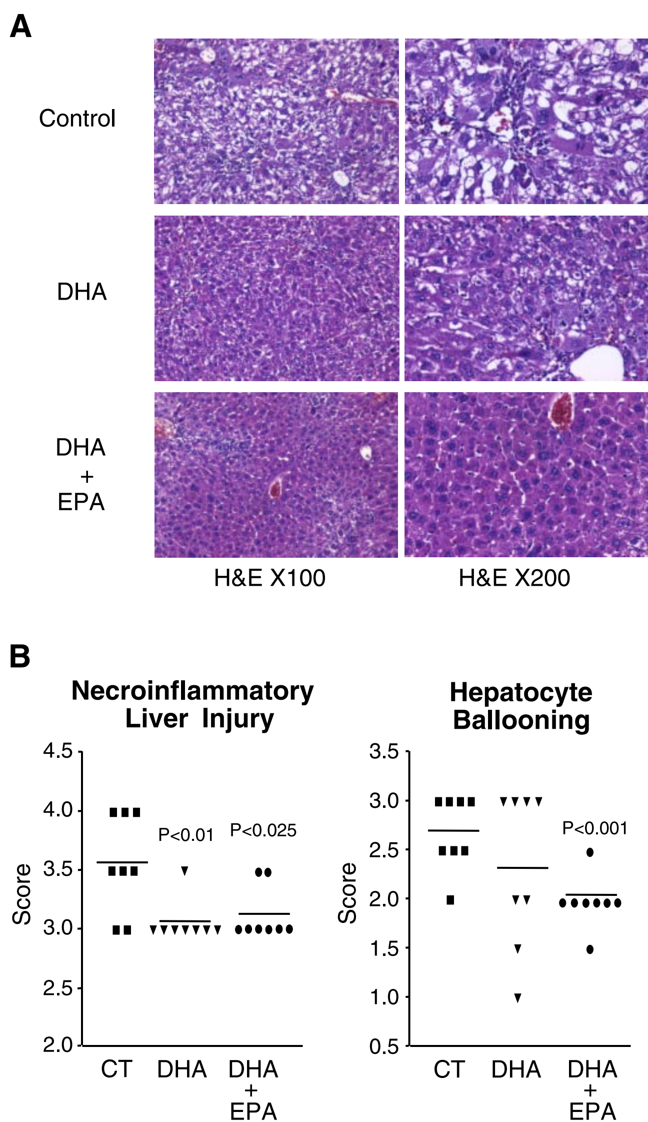


**Figure 2.** DHA-enriched diets prevent $CCl_4$-induced hepatocellular necrosis. *A)* Representative photomicrographs of liver sections stained with hematoxylin/eosin from 5 wk $CCl_4$-treated mice receiving a control diet (upper panels), a diet supplemented with DHA (middle panels), and a diet supplemented with a mixture of DHA and EPA (lower panels). These data are representative of 24 mice. *B)* The hepatocellular damage observed in hematoxylin/eosin-stained liver sections was analyzed by a registered pathologist unaware of the treatments and scored as described in Material and Methods. Results are expressed as mean ± SE and *P* values are calculated with respect to the control (CT) group.

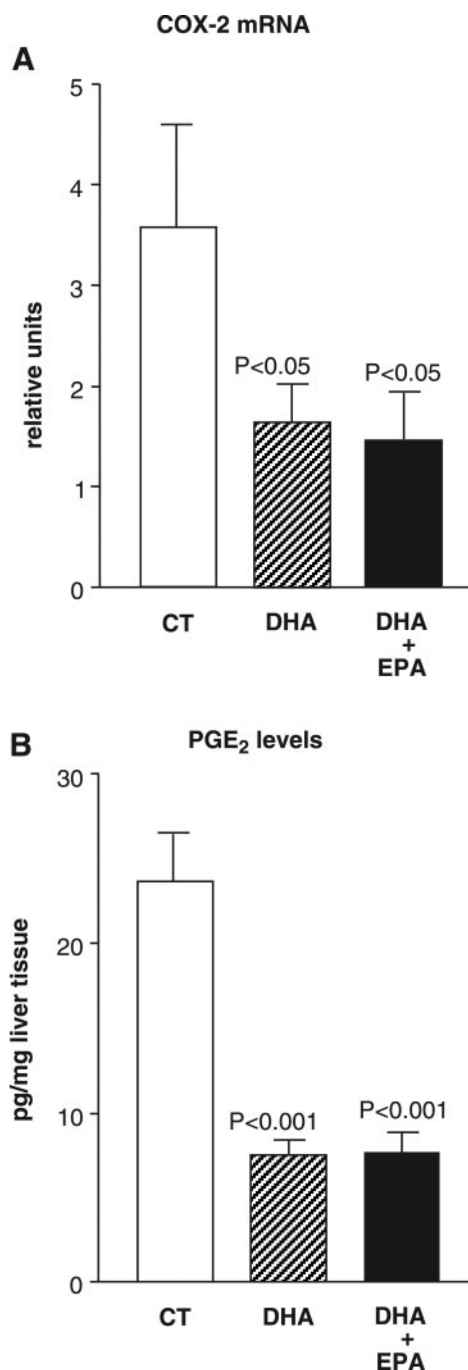


**Figure 3.** Effects of DHA-enriched diets on COX-2 mRNA expression and $PGE_2$ levels in livers from $CCl_4$-treated mice. *A)* Liver tissue was obtained after 5 wk of $CCl_4$ treatment from mice receiving either a control diet (CT) ($n$=8), a diet enriched with DHA (DHA) ($n$=8), or a diet enriched with a mixture of DHA and EPA (DHA+EPA) ($n$=8). After RNA extraction, COX-2 mRNA expression was determined by real-time RT-PCR. Results are expressed as the mean ± SE and *P* values are given *vs.* the CT group. *B)* Liver tissue was obtained after 5 wk of $CCl_4$ treatment from the three groups of mice (CT, DHA, and DHA+EPA) and samples were homogenized, extracted in $C_{18}$-silica reverse-phase cartridges, and $PGE_2$ levels were determined by a highly specific EIA. Results are expressed as the mean ± SE and *P* values are given *vs.* the CT group.

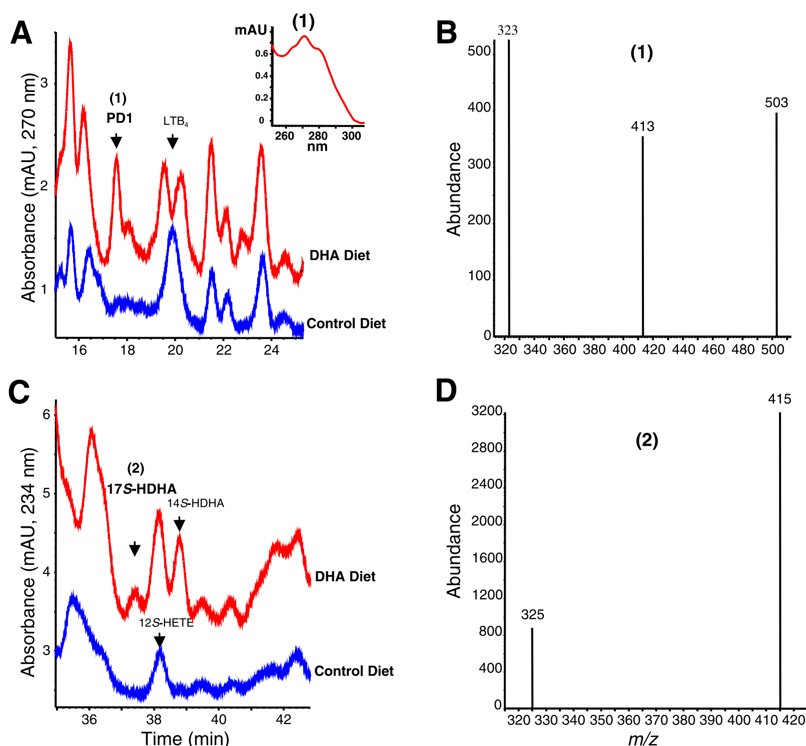


**Figure 4.** Endogenous formation of PD1 and 17*S*-HDHA in the liver of mice receiving a DHA-enriched diet. Liver tissue was obtained after 5 wk of $CCl_4$ treatment from mice receiving either a control diet or a diet enriched with DHA. Samples were extracted and analyzed by DAD/RP-HPLC analysis at 270 nm (*A*) and 234 nm (*C*) for diene-carrying and triene-carrying compounds, respectively. Retention times of authentic standards are indicated by arrows. The UV chromophore of the peak (1) coeluting with 10,17*S*-dihydroxy-docosatriene (PD1) is shown in inset (*A*). GC/MS chromatograms for peaks coeluting with PD1 (1) and 17*S*-HDHA (2) are shown in panels *B*, *D*, respectively. Results are representative of $n = 4$.

displayed ions at *m/z* 415 and 325, which are diagnostic for a monohydroxy DHA structure.

To explore whether DHA-derived lipid signals could mediate the hepatoprotective effects associated with DHA-enriched diets, we set out a series of experiments in hepatocytes as well as in macrophages, the cell type that plays a major role in initiating the cascade of events leading to liver inflammation and injury (22). As shown in **Fig. 5**, 17-HDHA, a lipid signal identified in the liver of mice fed with a DHA-enriched diet and a marker of activation of the PD1 pathway, displayed potent biological activity in these cells. This lipid mediator, in addition to reducing the genotoxic damage (Fig. 5*A*) and improving the oxidative stress status (Fig. 5*B*) in hepatocytes, significantly inhibited TNF-α release by murine macrophages (Fig. 5*C*).

Given that PPARγ is a central inhibitor of the inflammatory and fibrogenic responses in the liver (23), we next assessed whether 17-HDHA and other DHA-derived monohydroxy acids were able to bind and activate this nuclear receptor. To avoid any interference from the endogenous receptor, we tested the compounds in a cell-based PPARγ reporter assay. In this assay, 17-HDHA acted in a concentration-dependent manner as an agonist of PPARγ (**Fig. 6*A***). This effect was stereoselective since 17*R*-HDHA did not activate this nuclear receptor (Fig. 6*A*, inset). Whereas 14-HDHA appeared not to be active in this reporter assay (Fig. 6*B*), 7-HDHA was a potent agonist and activated PPARγ to a similar extent that 17-HDHA (Fig. 6*C*).

To further characterize the mechanisms underlying the hepatoprotective effects of ω-3 essential fatty acids, we assessed the effects of these compounds in the formation of leukotrienes (LTs), which are key effectors of hepatocellular injury (21, 24, 25). As shown in **Fig. 7*A***, hepatic levels of $LTB_4$ were lower in mice fed a diet rich in DHA that in those receiving a control diet. This finding was consistent with a reduction in 5-LO protein expression in murine macrophages growing for > 2 h in a medium supplemented with DHA as well as in macrophages exposed to 17-HDHA (Fig. 7*B*). On the other hand, no changes in COX-2 mRNA expression were observed in murine macrophages incubated with either DHA or 17-HDHA (relative expression to vehicle; DHA 10 μM: 1.2±0.3 and 17-HDHA 1μM: 1.3±0.3).

## DISCUSSION

The major findings of our study were that the ω-3 essential fatty acid, DHA, prevented DNA damage and oxidative stress in liver cells, and significantly reduced necroinflammatory liver injury in mice submitted to an experimental model of liver injury. These hepatoprotective effects were associated with a decrease in the hepatic formation of ω-6-derived eicosanoids (*i.e.*, $PGE_2$) and a concomitant increase in the generation of protective DHA-derived lipid mediators (*i.e.*, PD1 and 17*S*-HDHA). The beneficial role of these recently discovered DHA-derived lipid signals was further supported by experiments *in vitro* demonstrating that synthetic 17-HDHA exerts potent protective actions in hepatocytes and reduces TNF-α release and 5-LO activity in macrophages.

Our findings provide a molecular mechanism for the protective effects of dietary ω-3 fatty acid supplementation, namely via the formation of anti-inflammatory DHA-derived lipid mediators. Our results, which demonstrate abrogation of necroinflammatory liver injury

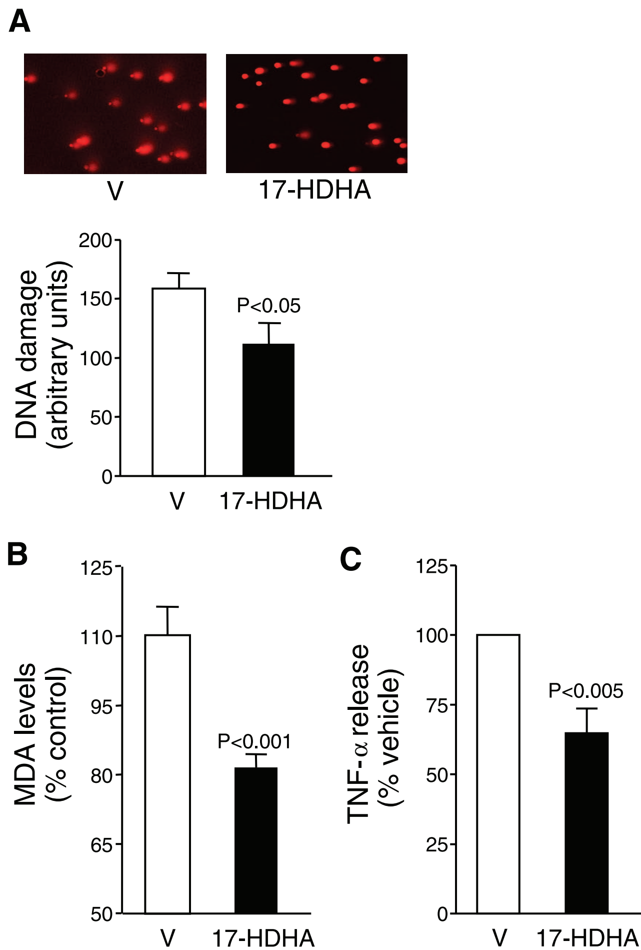


**Figure 5.** Biological effects of 17-HDHA in hepatocytes and macrophages. Murine CC-1 hepatocytes were incubated with vehicle (0.5% ethanol) or 17-HDHA (1 μM) in the presence of hydrogen peroxide for 1 h at 37°C, and genotoxic damage (*A*) and oxidative stress (*B*) were determined by the comet assay and by measuring MDA concentrations in cell lysates, respectively. Comet images (original magnification ×200) are representative of 4 different experiments, and comet tail length was scored as described in Fig. 1 legend and Material and Methods section. Results are the mean ± SE and *P* values are given with respect to untreated cells. *C)* Murine raw 264.7 macrophages were incubated with vehicle or 17-HDHA (1 μM) for 2 h at 37°C and TNF-α levels in cell supernatants determined by EIA. Results are the mean ± SE of 4 different experiments. *P* values are given with respect to untreated cells.

with ω-3 dietary supplementation, are consistent with earlier observations suggesting that the amount and type of dietary essential fatty acids may modulate the progression of liver disease. Along these lines, previous studies in Rhesus monkeys provided evidence that feeding with an experimental diet low in ω-3 fatty acids predisposes these animals to develop alcoholic fatty liver and fibrosis (26). Moreover, in Long-Evans Cinnamon rats, an experimental model of Wilson disease in which copper accumulates in the liver because of a mutation in the copper-transporting ATPase gene, ω-3 fatty acids were previously shown to suppress the development of acute hepatitis and to prolong survival (27). However, our findings differ from those reported by Nanji *et al.* in rats submitted to alcohol-induced liver injury, in which a diet composed entirely of fish oil containing both DHA and EPA combined with ethanol was shown to exacerbate fatty liver, inflammation, and fibrosis (28, 29). Whether differences in the species or the experimental model of liver injury account for this discrepancy is a subject that deserves further investigation. On the other hand, we did not find changes in the extent of hepatic steatosis between mice receiving the ω-3-enriched diets and those fed a control diet. This subject also deserves further investigation using a proper animal model of steatohepatitis, since ω-3 fatty acids are negative regulators of hepatic lipogenesis and their supplementation has been shown to protect the liver against hepatic lipid deposition in obese mice (30, 31).

The mechanisms underlying the hepatoprotective effects of long chain ω-3 essential fatty acids are not completely understood. A rather widely accepted mechanism of action for these compounds is that they

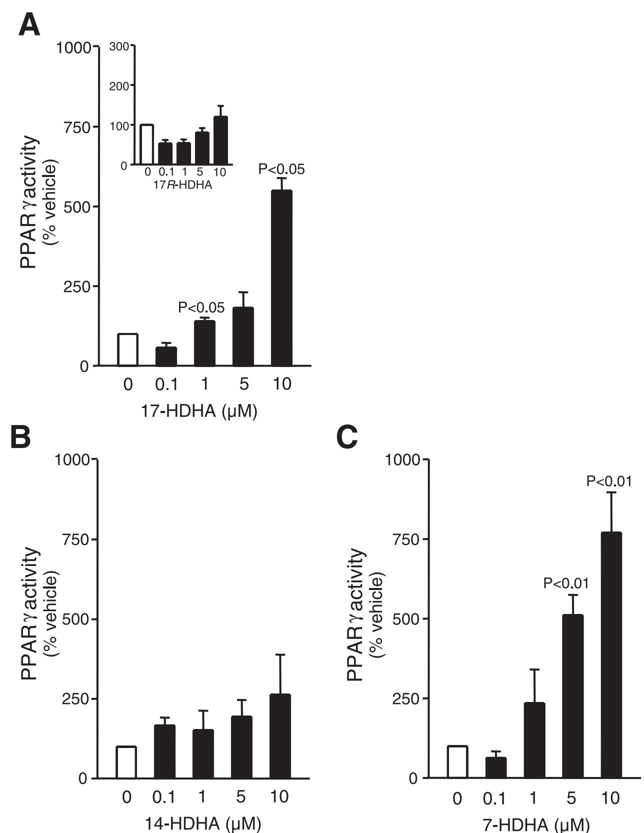


**Figure 6.** Effects of DHA-derived products on PPARγ activity. The activation of PPARγ by selected DHA-derived products was assessed in a cell-based luciferase reporter transactivation assay after incubation with increasing concentrations (0.1, 1, 5, and 10 μM) of 17-HDHA (*A*), 14-HDHA (*B*), and 7-HDHA (*C*) for 18 h. Luciferase activity was normalized to the level of β-gal activity and results expressed as fold induction relative to untreated cells. The stereoselective effects of the alcohol group in the position 17 (17*R*-HDHA, μM) are shown in the inset. Results are the mean ± SE of 3 different experiments with duplicate determinations. *P* values are given *vs.* untreated cells.

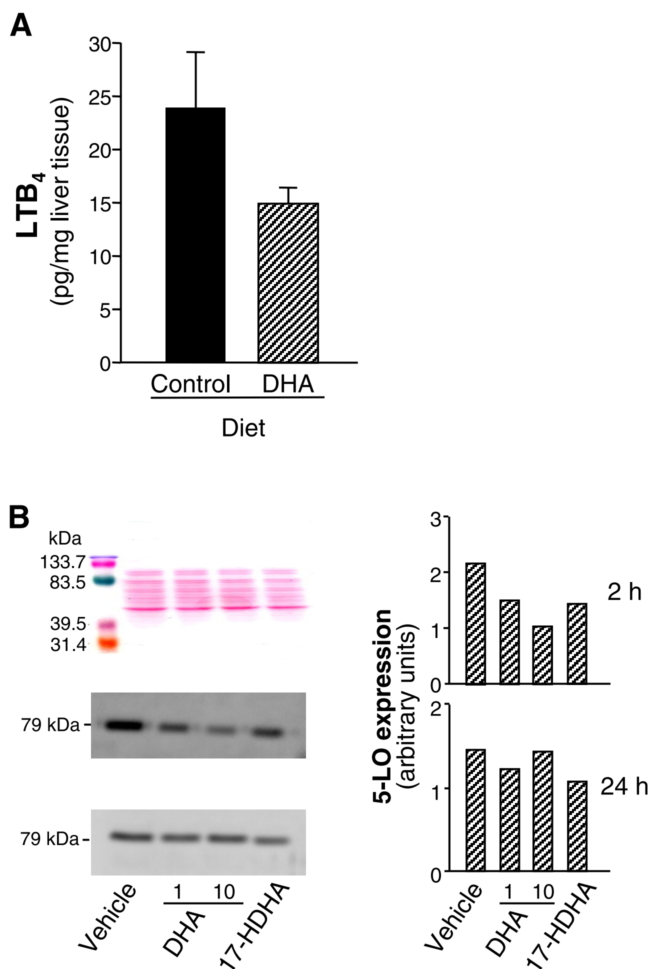


**Figure 7.** Dietary modulation of the 5-LO pathway and inhibition of macrophage 5-LO protein expression by 17-HDHA. *A)* Liver tissue was obtained after 5 wk of $CCl_4$ treatment from mice receiving either a control diet or a diet enriched with DHA. Samples were extracted and $LTB_4$ levels determined by RP-HPLC. *B)* Murine macrophages were incubated in the presence or absence of medium supplemented with two concentrations of DHA (1 and 10 μM) or treated with synthetic 17-HDHA (1 μM). Equal quantities of total protein (30 μg) were separated on a 12.5% SDS-PAGE gel, electrotransferred onto PVDF membranes, and analyzed by Western blot. Upper blot is representative of a Ponceau-stained membrane used as a loading control protein. Middle and lower blots are representative of protein bands detected by a specific anti-5-LO Ab after exposing cells to the compounds for 2 and 24 h, respectively. The densitometric analysis of 5-LO positive bands from these blots is shown on the right.

prevent the conversion of arachidonic acid into proinflammatory eicosanoids through the COX and 5-LO pathways either by competing with the substrate or by inhibiting the activity of eicosanoid-generating enzymes (32, 33). In fact, in our study a significant reduction in COX-2 mRNA expression associated with decreased hepatic levels of $PGE_2$ was observed in mice fed the experimental diets enriched with ω-3 fatty acids. A similar inhibitory effect on the expression of this key inflammatory gene was previously reported in men with prostate cancer fed a fish oil-supplemented diet, in bovine chondrocytes and rat hepatoma cells exposed to α-linoleic acid, and in human colon cancer cells incubated with either DHA or fish oil (33–37). Given that COX-2 is markedly increased in rats with $CCl_4$-induced liver injury and selective COX-2 inhibition prevents liver fibrosis in these animals (23), we speculate that down-regulation of hepatic COX-2 levels contributes to the protective actions of ω-3 fatty acids in this organ. Note that by real-time PCR we did not observe a decrease in COX-2 mRNA expression in murine macrophages incubated with DHA and DHA-derived products, suggesting that the reduction of COX-2 expression in livers from mice receiving DHA is likely to reflect a decrease in the number of COX-2-expressing inflammatory cells rather than a decrease in COX-2 expression in liver cells.

The most important observation of our study was that the decrease in COX-2 expression and $PGE_2$ levels in livers from animals receiving a DHA-enriched diet was associated with an increase in the formation of DHA-derived anti-inflammatory mediators, namely PD1 and 17*S*-HDHA. PD1, 10*R*,17*S*-dihydroxy-docosatriene, is of particular interest as this DHA-derived mediator was originally identified in neural tissues and termed neuroprotectin (reviewed in ref. 7). However, the protective actions of neuroprotectin D1 as well as its endogenous formation are not restricted to the nervous system. Hence, it was recently renamed protectin D1 (PD1) and the complete stereochemistry has been assigned (12, 13). PD1 is a potent regulator of inflammation, and its formation may play a role in the anti-inflammatory and protective properties attributed to DHA. In fact, PD1 has been shown to decrease COX-2 mRNA expression, block NFκB activation in the mouse hippocampus as well as in neural cells in culture (10), and inhibit IL-1β-induced COX-2 expression in human retinal pigment epithelial cells (38). In addition, both PD1 and 17*S*-HDHA potently inhibit TNF-α-induced IL-1β gene expression in human microglial cells (4).

Macrophages, which in response to a noxious agent release an array of soluble factors such as cytokines, reactive oxygen species (ROS), and eicosanoids, are recognized as the predominant effector cells involved in the inflammatory cascade leading to liver damage (22). 17-HDHA, a DHA-derived lipid mediator that is a marker of activation of the PD1 pathway, is also a potential metabolic precursor to the trihydroxy 17*S*-resolvins (RevD1-D4) by a mechanism analogous to the metabolism of 15-HETE by 5-LO, which generates lipoxin $A_4$. In our study, 17-HDHA consistently inhibited TNF-α release in murine macrophages. A similar effect was recently reported by Ariel *et al.* in lymphocytes exposed to increasing concentrations of PD1 (12). Similar inhibitory effects on TNF-α release have also been demonstrated in human monocytes exposed to DHA and EPA and in rodent macrophages growing in the presence of fish oil (39, 40). *In vivo*, supplementation of the diet of human volunteers with fish oil containing both DHA and EPA or feeding fish oil to

rodents is also associated with a decrease in the production of TNF-α (41, 42). Since an increased production of TNF-α by liver macrophages is clearly implicated in the pathogenesis of alcoholic liver disease and liver fibrosis (43), down-regulation of this key proinflammatory cytokine in macrophages treated with 17-HDHA may in part account for the protective effects of dietary DHA in necroinflammatory liver injury. We also noted a decrease in 5-LO protein expression in macrophages treated with 17-HDHA, a finding consistent with the reduction of $LTB_4$ formation seen in livers from mice supplemented with a DHA-enriched diet. Given that the presence of an active 5-LO pathway in the liver is restricted to Kupffer cells and inhibition of the 5-LO pathway in these resident macrophages has been shown to attenuate necroinflammatory liver injury and fibrosis (21, 24, 25), these data provide an additional beneficial action of DHA and DHA-derived lipid mediators in the liver.

Another interesting finding of the current study was that 17-HDHA acted in a concentration-dependent manner as a PPARγ ligand. This effect appears to show positional selectivity of the alcohol group in the DHA monohydroxy acid since 7-HDHA, but not 14-HDHA or 17*R*-HDHA, also exerted a potent activation of PPARγ. Nevertheless, the biological significance of this 7-HDHA-induced PPARγ activation is uncertain in the context of the liver because contrary to 17*S*-HDHA and 14*S*-HDHA, 7*S*-HDHA was not detected in the liver of mice fed a DHA-enriched diet. In any event, since PPARγ is widely recognized as an anti-inflammatory and antifibrogenic system in the liver (23), its activation by DHA-derived products is likely to contribute to the protective actions exerted by DHA-enriched diets in this organ.

The results of the current study strongly support the concept that patients with liver disease might benefit from a dietary supplementation with ω-3 essential fatty acids. A status characterized by essential fatty acid deficiency is a common finding in alcoholic and nonalcoholic patients either with compensated or decompensated cirrhosis as well as in other liver disorders such as acute hepatitis and cholestasis (44). This deficiency appears to be generalized since levels of fatty acids are not only decreased in plasma and blood cells (erythrocytes and platelets), but also in adipocytes and gastric mucosa (45–48). Moreover, in cirrhotic patients, an inverse relationship between plasma levels of DHA and the severity of liver disease has been established (49). The high prevalence of essential fatty acid deficiency in liver diseases is not surprising considering that the enzymatic machinery involved in long chain polyunsaturated fatty acid biosynthesis largely predominates in the liver (50).

In conclusion, using a well-established animal model of liver injury, we demonstrate that dietary ω-3 fatty acids are protective by reducing the incidence and severity of liver damage. More important, we demonstrate for the first time that dietary supplementation amplifies formation of DHA-derived anti-inflammatory lipid signals, namely PD1 and 17*S*-HDHA, in the liver. These novel lipid signals attenuated DNA damage and oxidative stress in hepatocytes as well as key markers of inflammation in macrophages, and thus provide a mechanism of action for the protection afforded by DHA dietary supplementation against necroinflammatory liver injury (**Fig. 8**). Hence, these findings may provide the rational basis for dietary supplementation with ω-3 fatty acids in patients with liver disease.

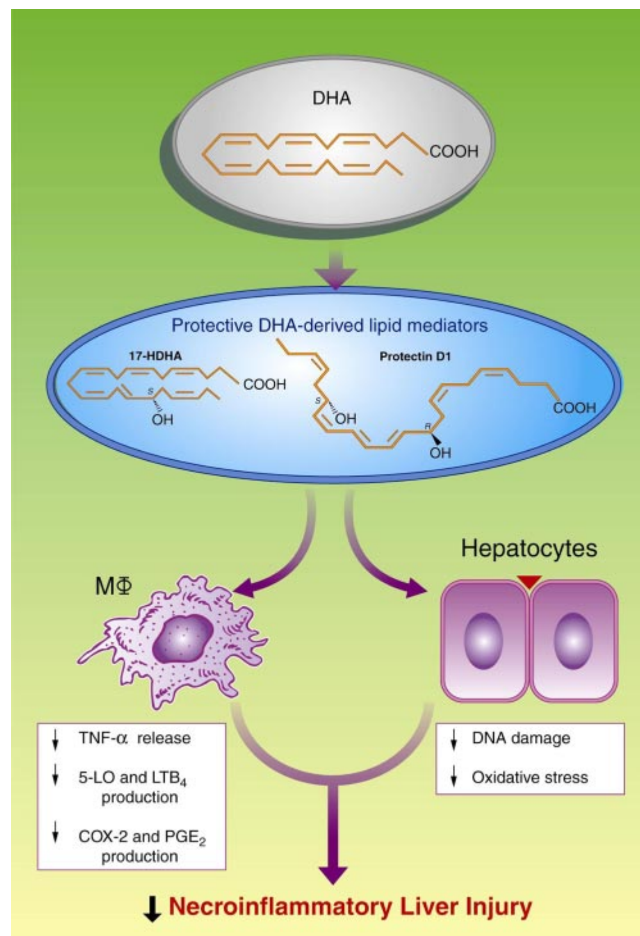


**Figure 8.** Schematic diagram illustrating the formation and protective actions of DHA-derived lipid mediators in the liver. Protectin D1 and 17*S*-HDHA are novel bioactive lipid mediators derived from DHA with potent protective actions. These lipid signals may ameliorate necroinflammatory liver injury by reducing DNA damage and oxidative stress in hepatocytes and by down-regulating TNF-α release and 5-LO and COX-2 activities in macrophages (MΦ).

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# Docosahexaenoic acid (DHA) blunts liver injury by conversion to protective lipid mediators: protectin D1 and 17*S*-hydroxy-DHA

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## SPECIFIC AIM

A new family of bioactive lipid mediators generated from docosahexaenoic acid (DHA) that carry potent anti-inflammatory properties has recently been identified. The aim of the current study was to assess the formation of these DHA-derived products in the liver and to explore whether these lipid signals protect liver cells from necroinflammatory injury.

## PRINCIPAL FINDINGS

### 1. Supplementation of hepatocyte cultures with DHA significantly reduces genotoxic and oxidative damage

To test *in vitro* the potential protective effects of ω-3 essential fatty acids on liver cells, hepatocytes were grown in medium supplemented with DHA; DNA damage was assessed by a single-cell gel electrophoresis assay, the so-called comet assay. Results indicate that the length of comet tails, and therefore the extent of DNA strand breaks induced by hydrogen peroxide, was significantly lower in hepatocytes growing in medium supplemented with DHA than in hepatocytes growing in medium alone. Oxidative stress levels [*i.e.*, malondialdehyde (MDA) levels measured by HPLC in cell lysates] were significantly decreased in hepatocytes growing in a DHA-rich medium.

### 2. Dietary supplementation of mice with DHA blunts carbon tetrachloride ($CCl_4$) -induced necroinflammatory damage

To extend the protective actions of DHA observed in hepatocytes at the *in vivo* level, we administered diets supplemented with DHA to mice submitted to the experimental model of $CCl_4$-induced liver injury and compared the hepatic necroinflammatory damage with that of mice receiving a control diet. Histological examination of livers from mice treated with $CCl_4$ for 5 wk revealed massive and severe hepatocyte necrosis, inflammation, and ballooning at the centrilobular zone with bridging of necrosis that severely disrupted the sinusoidal and lobular architecture of the liver (**Fig. 1*A***, upper panels). A significant improvement in hepatic pathology occurred in mice fed experimental diets enriched with DHA (Fig. 1*A*, middle and lower panels). The scores for necroinflammatory liver injury and hepatocyte ballooning were significantly lower in mice fed ω-3-enriched experimental diets than in those receiving a control diet (Fig. 1*B*).

### 3. Detection of the novel bioactive lipid mediators 17*S*-hydroxy-DHA (17*S*-HDHA) and protectin D1 in the liver of mice fed a DHA-enriched diet

We next assessed the profile of bioactive lipid mediators generated in livers from mice fed DHA-enriched diets. We first monitored changes in the proinflammatory cyclooxygenase (COX)-2 pathway and found that administration of diets enriched with ω-3 essential fatty acids led to significant decreases in hepatic COX-2 mRNA expression and prostaglandin (PG) $E_2$ levels. When we analyzed the profile of lipid mediators in the liver of mice fed a DHA-enriched diet by HPLC gas chromatography/mass spectrometry, we identified the

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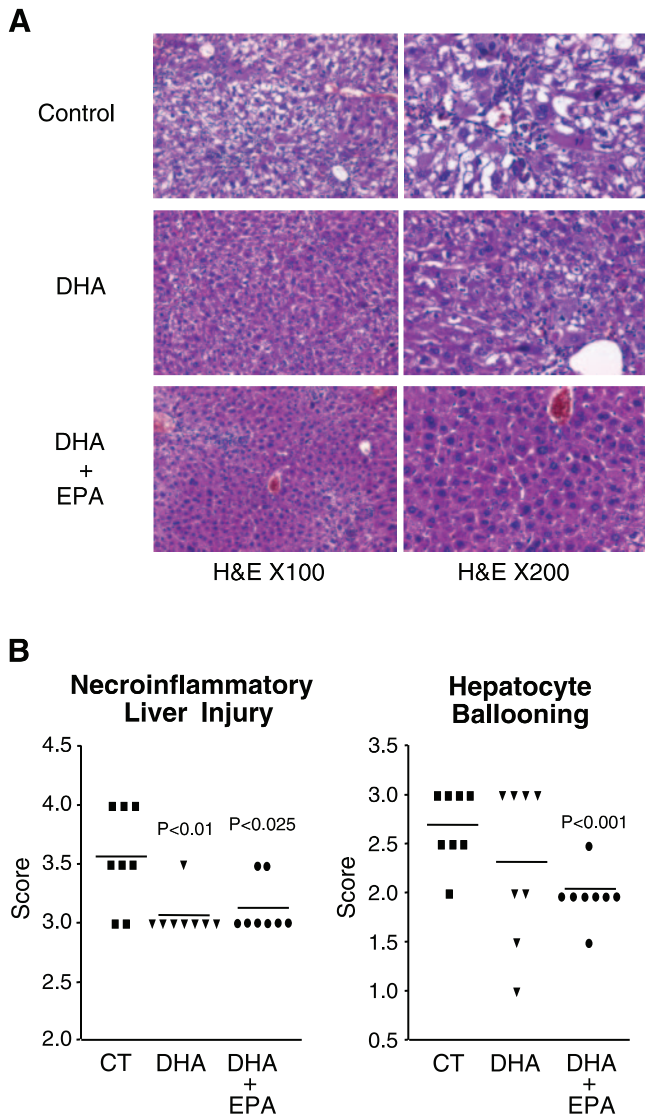


**Figure 1.** DHA-enriched diets prevent $CCl_4$-induced hepatocellular necrosis. *A)* Representative photomicrographs of liver sections stained with hematoxylin-eosin from 5 wk $CCl_4$-treated mice receiving a control diet (upper panels), a diet supplemented with DHA (middle panels), and a diet supplemented with a mixture of DHA and EPA (lower panels). These data are representative of 24 mice. *B)* The hepatocellular damage observed in hematoxylin-eosin stained liver sections was scored by a registered pathologist unaware of the treatments. Results are expressed as mean ± SE and *P* values are calculated with respect to the control CT group.

potent anti-inflammatory and proresolution DHA-derived lipid signals, protectin D1 and 17*S*-HDHA. These unique products were identified by coelution with the biosynthetic standards and by their characteristic UV spectra consistent with the presence of a conjugated triene double bond structure for protectin D1 and a conjugated diene chromophore for 17*S*-HDHA. The identity of these DHA-derived products was further confirmed by mass spectrometry analysis, which displayed ions at *m/z* 503, 413, and 323 (diagnostic for a dihydroxy DHA structure) and at *m/z* 415 and 325.

### 4. Lipid signals identified in the liver of mice supplemented with DHA display potent biological activity on hepatocytes and macrophages

To explore whether DHA-derived lipid signals could mediate the hepatoprotective effects associated with DHA-enriched diets, we set out a series of experiments in hepatocytes and macrophages, the cell type that plays a major role in initiating the cascade of events leading to liver inflammation and injury. As shown in **Fig. 2**, 17-HDHA, a lipid signal identified in the liver of mice fed a DHA-enriched diet and a marker of activation of the protectin D1 pathway, displayed potent biological activity in these cells. This lipid mediator abrogated genotoxic damage (Fig. 2*A*), improved oxidative stress status (Fig. 2*B*) in hepatocytes, and signif-

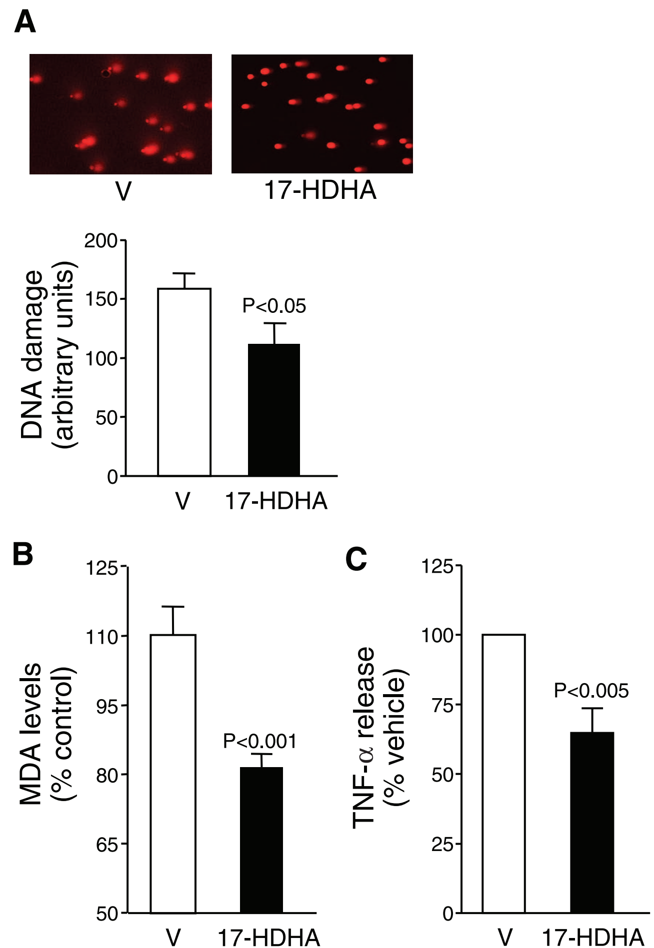


**Figure 2.** Biological effects of 17-HDHA in hepatocytes and macrophages. Murine CC-1 hepatocytes were incubated with vehicle (0.5% ethanol) or 17-HDHA (1 μM) in the presence of hydrogen peroxide for 1 h at 37°C, and genotoxic damage (*A*) and oxidative stress (*B*) were determined by comet assay and measuring MDA concentrations in cell lysates, respectively. Results are the mean ± SE of 4 experiments; *P* values are given with respect to untreated cells. *C)* Murine raw 264.7 macrophages were incubated with vehicle or 17-HDHA (1 μM) for 2 h at 37°C and TNF-α levels in cell supernatants were determined by enzyme immunoassay. Results are mean ± SE of 4 different experiments. *P* values are given with respect to untreated cells.

icantly inhibited TNF-α release by murine macrophages (Fig. 2*C*). In addition, 17-HDHA decreased macrophage 5-LO expression. In a cell-based PPARγ and luciferase reporter transactivation assay, synthetic 17-HDHA acted in a concentration-dependent manner as an agonist of PPARγ, a central inhibitor of inflammatory and fibrogenic responses in the liver.

## CONCLUSIONS AND SIGNIFICANCE

Virtually every type of liver disease has an inflammatory component as its underlying cause. Although beneficial as a limited wound-healing process, if the inflammatory response is not resolved it results in tissue damage, scar accumulation, fibrosis, and eventually in the life-threatening condition of liver cirrhosis. Therefore, developing novel strategies to modulate the factors that govern hepatic inflammation represents a primary focus for disrupting the sequence of events leading to liver injury.

Long chain ω-3 essential fatty acids display potent anti-inflammatory properties. Dietary interventions rich in DHA and/or eicosapentaenoic acid (EPA) have been shown to keep inflammation under control without side effects and are used as preventive measures against illnesses such as rheumatoid arthritis, cystic fibrosis, ulcerative colitis, asthma, atherosclerosis, cancer, and cardiovascular disease. Although the molecular mechanisms underlying the beneficial actions of ω-3 essential fatty acids remain to be clearly defined, a new series of bioactive lipid mediators generated from these fatty acids has recently been identified. During the resolution phase of acute inflammatory response, cell-cell interactions and transcellular biosynthesis lead to production of novel bioactive lipid mediators from DHA and EPA, termed resolvins (resolution phase interaction products) and protectin D1. Protectin D1, a bioactive lipid mediator derived from DHA, potently regulates critical events associated with inflammation and its resolution, including inhibition of PMN infiltration and T cell migration, and reduction of cytokine and chemokine formation and IL-1-induced NFκB activation.

Results of this study demonstrate that dietary interventions rich in DHA modulate hepatic inflammation and may help prevent necroinflammatory liver injury. The decrease in COX-2 expression and $PGE_2$ levels noted in livers from animals receiving a DHA-enriched diet was associated with an increase in the formation of DHA-derived anti-inflammatory mediators, protectin D1 and 17*S*-HDHA. The potential protective actions of these DHA-derived lipid signals in the liver was further supported by experiments *in vitro* demonstrating that synthetic 17-HDHA attenuated DNA damage and oxidative stress in hepatocytes, and key markers of inflammation in macrophages (**Fig. 3**). 17-HDHA inhibited TNF-α release in murine macrophages. Since an increased production of TNF-α by liver macrophages is

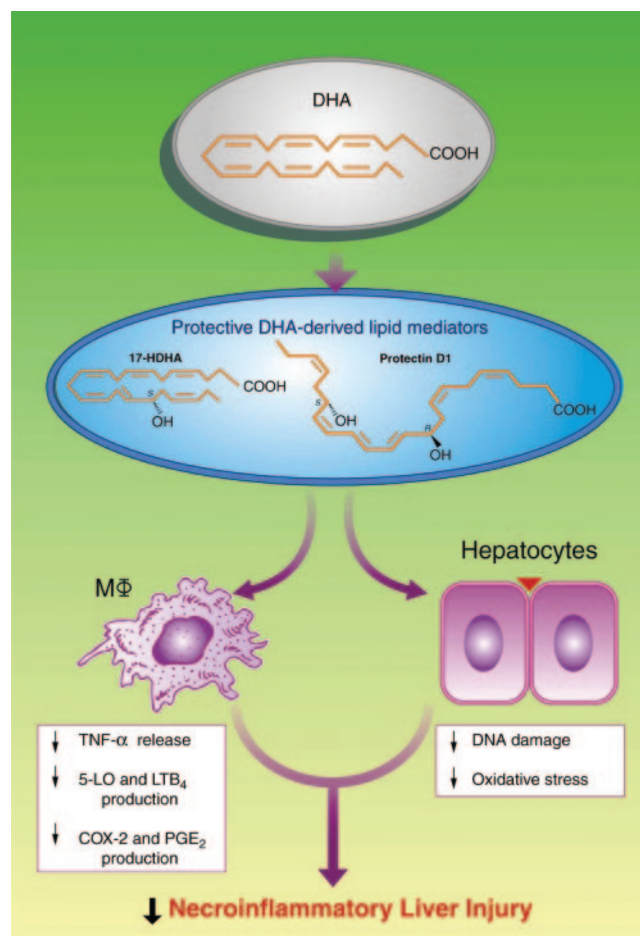


**Figure 3.** Schematic diagram illustrating the formation and protective actions of DHA-derived lipid mediators in the liver. Protectin D1 and 17*S*-HDHA are novel bioactive lipid mediators derived from DHA with potent protective actions. These lipid signals may ameliorate necroinflammatory liver injury by reducing DNA damage and oxidative stress in hepatocytes and by down-regulating TNF-α release and 5-LO and COX-2 activities in macrophages (MΦ).

implicated in the pathogenesis of alcoholic liver disease and liver fibrosis, down-regulation of this key proinflammatory cytokine in macrophages treated with 17-HDHA may in part account for the protective effects of dietary DHA in necroinflammatory liver injury. We noted a decrease in 5-LO protein expression in macrophages treated with 17-HDHA. Earlier studies established that inhibition of 5-LO attenuates necroinflammatory liver injury and fibrosis.

Using a well-established animal model of liver injury, we demonstrate that dietary ω-3 fatty acids are protective by reducing the incidence and severity of liver damage. We demonstrate for the first time that dietary supplementation amplifies formation of DHA-derived anti-inflammatory lipid signals, namely protectin D1 and 17*S*-HDHA in the liver. These findings strongly support the concept that patients with liver disease might benefit from a dietary supplementation with ω-3 essential fatty acids. FJ