International Immunopharmacology 53 (2017) 56–62

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Docosahexaenoic acid attenuates carbon tetrachloride-induced hepatic fibrosis in rats

Jianlin He[a,b], Kaikai Bai[b], Bihong Hong[b], Feng Zhang[a], Shizhong Zheng[a,*]

[a] Department of Pharmacology, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing 210029, PR China
[b] Third Institute of Oceanography, State Oceanic Administration, Xiamen 361005, PR China

ARTICLE INFO

Keywords:
Hepatic fibrosis
Docosahexaenoic acid
PPARγ
Carbon tetrachloride
TGFβ
PDGF

ABSTRACT

Fish oil containing docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) has been reported to exert beneficial health effects, including hepatoprotection. However, the effect of DHA alone has not been well studied, and the mechanism is not fully understood. In the present study, we reported the protective effect of DHA on carbon tetrachloride (CCl_4) induced hepatic fibrosis. Compared with the control group, the CCl_4 group showed hepatic damage as evidenced by histological changes and elevation in serum transaminase activity, fibrosis, inflammation and oxidative stress levels. These pathophysiological changes were attenuated by chronic DHA supplementation. The anti-fibrotic effect of DHA was accompanied by reductions in gene and protein expression of α-smooth muscle actin (α-SMA), fibronectin, and collagen in the liver tissue. DHA also attenuated CCl_4-induced elevation of lipid peroxidation (LPO) and decrease of glutathione (GSH)/oxidized GSH (GSSG) ratio. The upregulated inflammatory cytokines tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6 by CCl_4 were also ameliorated by DHA. Peroxisome proliferator-activated receptor (PPAR)-γ upregulation and type I and II receptors for transforming growth factor (TGF)-β (Tβ-RI and Tβ-RII) and platelet-derived growth factor (PDGF)-β receptor (PDGF-βR) downregulation on both mRNA and protein levels were observed by DHA treatment compared to CCl_4 group. Moreover, *in vitro* study showed that DHA inhibited HSC activation, being associated with elevating PPARγ level and reducing the phosphorylation levels of Smad2/3 and ERKs, which are downstream intermediates of TGFβ and PDGF receptors, respectively. Taken together, the hepatoprotective, anti-inflammatory and anti-fibrotic effects of DHA appeared to be multifactorial. Further, one of the mechanisms of the anti-fibrotic effect of chronic DHA supplementation is probably through PPARγ signaling to interrupt TGFβ/Smad and PDGF/ERK pathways in HSCs.

1. Introduction

Hepatic fibrosis resulting from a wide range of liver injury, leading to the imbalance of extracellular matrix (ECM) formation and degradation, is a pathological process that presents as the critical prestage of liver cirrhosis, which will eventually progress to hepatocellular carcinoma [1]. Left untreated, it can ultimately cause organ failure and death. It has been well considered that hepatic fibrosis is a reversible state, which is supported by experimental and clinical evidence [2]. Quiescent hepatic stellate cells (HSCs) are non-parenchymal cells which reside in the space of Disse, containing bunches of vitamin A-rich lipid droplets. Its activation is the key event of hepatic fibrosis. The activation and differentiation into myofibroblasts and subsequent proliferation of HSCs drive the fibrogenic process. Activated HSCs account for nearly 80% of total fibrillar collagen I in the fibrotic liver [3]. Regression of liver fibrosis can be stimulated with drugs that target the activities of HSCs [4]. PPARγ belongs to the superfamily of nuclear receptors controlling the transcription of a subset of genes and it is predominantly present in liver and adipose tissue [5]. The key roles of PPARγ and its regulatory mechanisms in the pathogenesis of hepatic fibrosis have been gradually revealed. Both *in vivo* and *in vitro* studies showed that the level and activity of PPARγ decreased significantly with the activation of HSCs [6,7]. In contrast, the restoration of PPARγ reverses the activated HSCs to the quiescent phenotype and suppresses AP-1 activity via a physical interaction between PPARγ and JunD [8]. A great deal of animal studies has demonstrated that stimulation of PPARγ regulatory system through gene therapy approaches and PPARγ ligands has therapeutic promise for hepatic fibrosis induced by a variety of etiologies [9]. ω-3 polyunsaturated fatty acids, a collection of polyunsaturated fatty acids, including EPA and DHA like fish oil, has been reported to exert a hepatic protection effect in several liver disorders, including cirrhosis, acute liver failure, and fatty liver disease [10–12].

* Corresponding author at: 138 Xianlin Avenue, Nanjing, 210023, Jiangsu, PR China.
E-mail address: nytws@163.com (S. Zheng).

http://dx.doi.org/10.1016/j.intimp.2017.09.013
Received 18 June 2017; Received in revised form 15 September 2017; Accepted 15 September 2017

DHA is a long-chain polyunsaturated fatty acid considered essential for maturation of the developing brain, retina, and other organs in newborn infants [13,14]. However, as a natural ligand of PPARγ [15], the role of pure DHA in liver injury and inflammation has not been well investigated. In this study, CCl_4 treated rats were used to evaluate the protective effect of DHA free acid on hepatic fibrosis.

2. Materials and methods

2.1. Reagents and antibodies

DHA was purchased from Nu-chek Prep (Elysian, MN, US). Primary antibodies against Glyceraldehyde phosphate dehydrogenase (GAPDH), PDGF-βR, PPARγ, Tβ-RI, Tβ-RII, α-SMA and Fibronectin were purchased from Abcam (Cambridge, MA, US). Primary antibodies against Col1a1, p-Smad2/3, Smad2/3, p-ERK, ERK, p-JNK, JNK, p-p38, and p38 were purchased from Cell Signaling Technology (Danvers, MA, US).

2.2. Animals

Male Sprague-Dawley rats were purchased from Animal Centre of Fujian Medical School (Fujian, China). The rats were housed and maintained in a temperature and humidity-controlled environment with 12 h light/dark cycle, and fed with a standard rat diet. The experimental protocol was approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China), and all animals received humane care according to the National Institutes of Health (USA) guidelines.

Forty male Sprague-Dawley rats (180–220 g) were randomly divided into four groups (10 rats/group). Group 1 was the normal control in which rats were intraperitoneally (i.p.) injected with saline. Group 2 was the CCl_4 group in which rats were i.p. injected with CCl_4. Group 3 was a treatment group in which rats were injected with CCl_4 and DHA at 250 nmol/kg. Group 4 was another treatment group in which rats were injected with CCl_4 and DHA at 1000 nmol/kg. Rats in groups 2, 3 and 4 were i.p. injected with a mixture of CCl_4 (0.1 ml/100 g body weight) and olive oil [1:1 (v/v)] for totally 8 weeks, 3 times a week for the first 4 weeks, and 2 times a week for the second 4 weeks. DHA was dissolved in ethanol as stock solution and suspended in sterile saline (adjust the concentration to 250 nmol/kg or 1000 nmol/kg body weight before use) and i.p. injected once daily. Blood was collected when sacrificed. A small portion of the liver was removed for histopathological study by being fixed with 10% formalin and subsequently embedded with paraffin. The remaining liver was cut into pieces and rapidly frozen with liquid nitrogen for hepatic RNA isolation, cDNA synthesis, real time PCR and protein extraction.

2.3. RNA isolation and real-time PCR

Total RNA was extracted from rat liver samples using Trizol reagent (Biouniquer Technology Co., Ltd., Nanjing, China) according to the manufacturer's protocol. Amplification kit was purchased from Bio-Rad Laboratories (Berkeley, CA, USA). GAPDH was used as the invariant control. Sequences of the primers were shown in Table 1.

2.4. Cell culture and DHA treatment

Rat HSC cell line T6 was purchased from The Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Sijiqing Biological Engineering Materials, Hangzhou, China) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. The cells were subjected to no > 20 cell passages. Stock solution of DHA in ethanol were stored at − 20 °C and diluted in complete growth medium before experiments (final concentration of ethanol, 0.05% v/v). Cells were treated with ethanol control or DHA at different concentrations for 24 h in complete growth medium.

Table 1
The RT-qPCR gene-specific primers.

Genes	Forward primers	Reverse primers
α-SMA	ACC ATC GGG AAT GAA CGC TT	CTG TCA GCA ATG CCT GGG TA
Tβ-RI	CAG TCA CCG AGA CCA CAG AC	ATG ACA GTG CGG TTA TGG CA
Tβ-RII	TTC ACC TAC CAC GGC TTC AC	ATG ACC AGC AAC AGG TCA GG
PDGF-βR	GCA AGA AGC AGC CAT GAA CC	TCC TCA GAG TCC ATC GGG AG
Fibronectin	CCC CAC CTC AGG ACT TTT CC	CCG TTG TCA AAA CAG CCA GG
Col1a1	GGC AAC CTC AAG AAG TCC C	GTG CAG CCA TCC ACA AGC
PPARγ	GAC CAC TCC CAT TCC TTT	GCT CTA CTT TGA TCG CAC T
GAPDH	AGG TGAT CGA TAT ATC CAA GCC GTT	TGC GCT TGT GTA GCT ACT GTG T

2.5. Western blot analysis

Liver samples were homogenized in radio-immunoprecipitation assay (RIPA) buffer containing protease inhibitors. The whole cell lysate of HSC T6 was prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. The protein levels were determined using a bicinchoninic acid (BCA) assay kit (Pierce, USA). Proteins (50 μg/well) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to a poly (vinylidene fluoride) (PVDF) membrane (Millipore, Burlington, MA, USA). Equivalent loading was confirmed using an antibody against GAPDH. The levels of target protein bands were densitometrically determined using Quantity One 4.4.1 (Bio-Rad Laboratories, Hercules, CA, USA). The variation in the density of bands was expressed as fold changes compared to the control in the blot after normalized to GAPDH.

2.6. Enzyme-linked immunosorbent assays

Levels of hepatic IFN-γ, TNF-α and IL-6 in rats were determined with a corresponding ELISA kit purchased from Abcam (Cambridge, MA) according to the protocols provided by the manufacturer. Serum levels of Hyaluronic acid (HA), Laminin (LN), Hydroxyproline (HYP), Procollagen type III (PCIII) were determined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). HYP levels in liver were determined using a Hydroxyproline Assay Kit (Mlbio, Shanghai, China) following the manufacturer's instructions. Following the protocols provided by the manufacturer, levels of hepatic GSH, GSSG in liver were determined using Glutathione (GSH/GSSG/Total) Assay Kit (Biovision, Milpitas, CA) and LPO levels in liver were determined using LPO Assay Kit (Cayman Chemical, Ann Arbor, MI).

2.7. Liver histopathology

Liver tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Liver slices of 5 μm thickness were prepared and stained with hematoxylin-eosin using standard methods. For Sirius red collagen staining, thin sections were deparaffinized and stained with picro-Sirius red for 1 h at room temperature. After being washed, sections on the slides were dehydrated in 100% ethanol and in xylene, and then they were mounted in permount. Images were taken in a blinded manner at random fields by a pathologist, and representative views of liver sections are shown.

2.8. Statistical analysis

Results were from at least triplicate experiments. Data are presented