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A combination of dietary N-3 fatty acids and a cyclooxygenase-1 inhibitor attenuates nonalcoholic fatty liver disease in mice☆

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Abstract

We sought to determine whether a combination of purified n-3 fatty acids (n-3) and SC-560 (SC), a cyclooxygenase-1-specific inhibitor, is effective in ameliorating nonalcoholic fatty liver disease in obesity. Female wild-type mice were fed a high-fat and high-cholesterol diet (HF) supplemented with n-3 in the presence or absence of SC. Mice treated with SC alone exhibited no change in liver lipids, whereas n-3-fed mice tended to have lower hepatic lipids. Mice given n-3+SC had significantly lower liver lipids compared with HF controls indicating enhanced lipid clearance. Total and sulfated bile acids were significantly higher only in n-3+SC-treated mice compared with chow diet (CD) controls. Regarding mechanisms, the level of pregnane X receptor (PXR), a nuclear receptor regulating drug/bile detoxification, was significantly higher in mice given n-3 or n-3+SC. Studies in precision-cut liver slices and in cultured hepatoma cells showed that n-3+SC enhanced not only the expression/activation of PXR and its target genes but also the expression of farnesoid X receptor (FXR), another regulator of bile synthesis/clearance, indicating that n-3+SC can induce both PXR and FXR. The mRNA level of FGFR4 which inhibits bile formation showed a significant reduction in Huh 7 cells upon n-3 and n-3+SC treatment. PXR overexpression in hepatoma cells confirmed that n-3 or SC each induced the expression of PXR target genes and in combination had an enhanced effect. Our findings suggest that combining SC with n-3 potentiates its lipid-lowering effect, in part, by enhanced PXR and/or altered FXR/FGFR4 signaling.

Keywords: Nonalcoholic fatty liver disease; Fish oil; Omega-3 fatty acids; PXR; Bile

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), for which no approved pharmacotherapy is available, is an independent risk factor for cardiovascular disease (CVD). Fish oil (FO), containing n-3 fatty acids (n-3), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is the most widely used dietary supplement and is known to have protective effects against CVD and neurodegenerative diseases, and in alleviating arthritis. In addition to its well-known plasma triglyceride (TG) lowering effects, FO or n-3 reduced hepatic lipid accumulation in animal models [1–3]. Moreover, we and others demonstrated that FO or n-3 each attenuates liver lipid accumulation by reducing both TGs and cholesterol esters in liver [1,3,4]. Because of its potential to reduce hepatic lipid accumulation, clinical studies were conducted to determine the efficacy of FO in attenuating NAFLD. They found that FO treatment exhibited some protective effects in reducing NAFLD in adult human subjects [5]. A randomized controlled trial reported that DHA supplementation was associated with attenuation of liver steatosis in pediatric patients with obesity [6]. However, another recent study in pediatric patients, with NAFLD, measured by ultrasound, showed no reduction of hepatic lipid accumulation by dietary n-3 fatty acids [7]. Together, these reports suggest that although FO has lipid-lowering properties, the effect is moderate. Therefore, strategies to improve the benefit of n-3 fatty acids in promoting hepatic lipid clearance must be developed.

We reported that combining FO with indomethacin, an isoform nonspecific cyclooxygenase (COX) inhibitor, potently reduced hepatic steatosis and liver inflammation in LDLR$^{-/-}$ mice [2]. This effect was associated with increased expression of hepatic pregnane X receptor (PXR) and its target genes. Regarding COX inhibitors, the general COX

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inhibitors (that inhibit both COX-1 and -2) were associated with gastrointestinal side effects; therefore, COX-2-specific inhibitors were developed. However, prolonged use of the latter drugs is reportedly associated with cardiovascular side effects. It is increasingly recognized that inhibition of COX-1 may be more beneficial in reducing inflammatory events and vascular injury than COX-2 (reviewed in Refs. [8,9]), thereby offering a safer approach than inhibiting both enzymes or COX-2 alone. Therefore, we hypothesized that a combination of purified n-3 and SC-560 (SC), a COX-1-specific inhibitor, will be effective in promoting hepatic lipid catabolism in mice *via* improved PXR signaling.

Using wild-type (WT) mice fed a high fat and high cholesterol (HF) diet in the presence or absence of n-3 and/or SC, we demonstrated that a combination of n-3 and SC exhibit a greater reduction in hepatic lipid accumulation. Here we present these and other results that support the therapeutic effects of these agents.

2. Materials and methods

2.1. Mice and diet

Female WT C57BL/6 mice were purchased from Jackson lab and fed a chow diet (CD, Purina Lab Diet #5001) or an HF diet (Dyets Inc., Bethlehem, PA, US) for 12 weeks. The diet provided 45% fat-derived calories and was supplemented with 1% cholesterol (w/w). Oleic acid was added at 0.56% (w/w) to this diet as a control for n-3. N-3s were added at 0.56%, and EPA and DHA were used at a ratio of 1.5:1 to reflect their ratio in conventional FO supplements. We administered SC (Cayman Chemical, Ann Arbor, MI, USA) in drinking water at 1 mg/L. A stock of SC was made in DMSO, which was then mixed in drinking water. The water containing SC was changed every day. Mice not treated with SC received DMSO as a vehicle control in their drinking water. We had five study groups with five mice per group: (1) CD, (2) HF, (3) HF+SC, (4) HF+n-3 and (5) HF+n-3+SC. Twelve week post-diet and treatment, the mice were euthanized after 5-h fasting. All animal care procedures were carried out with approval from the Institutional Animal Care and Use Committee of VA Nebraska–Western Iowa Health Care System.

2.2. Metabolic variables

Blood glucose was measured using the Accuchek Aviva glucometer. Plasma total cholesterol and TGs were measured using kits from Raichem. Plasma insulin was measured using kits from Mercodia. Resolvin D1 (RvD1) was measured in plasma samples using the immunoassay kit from Cayman Chemical as we reported previously [10].

2.3. Markers of toxicity

Serum markers of liver toxicity such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), bilirubin and albumin were determined using the Vitros Clinical Analyzer.

2.4. Bile acid analysis

Bile was collected from each gall bladder at sacrifice. The total, sulfated and unsulfated bile acids were determined using a highly sensitive, high-throughput liquid chromatography/mass spectrometry as we described previously [11–13]. Briefly, a Waters ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a 4000 Q TRAP quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Applied Biosystems, MDS Sciex, Foster City, CA, USA) was used. Chromatographic separations were performed with an ACQUITY UPLC®BEH C18 column (1.7 μm, 150×2.1 mm) equipped with an inline precolumn filter. The mobile phase consisted of 7.5 mM ammonium bicarbonate, adjusted to pH 9.0 using ammonium hydroxide (mobile phase A), and 30% acetonitrile in methanol (mobile phase B), at a total flow rate of 0.2 ml/min. The gradient profile was held at 52.5% mobile phase B for 12.75 min, increased linearly to 68% in 0.25 min, held at 68% for 8.75 min, increased linearly to 90% in 0.25 min and held at 90% for 1 min, and finally brought back to 52.5% in 0.25 min followed by 4.75 min re-equilibration. Data were acquired in multiple reaction monitoring-negative ESI mode as described previously [11–13].

For preparation of calibration curve, blank bile was obtained by charcoal stripping as described previously [11–13]. Calibration curve was prepared in stripped matrices by spiking 10 μl of appropriate standard solution into 100 μl blank bile at final concentrations ranging from 1 to 1000 ng/ml. For extraction of bile samples, Supelclean LC-18 SPE cartridges (Sigma-Aldrich, St. Louis, MO, USA) were used. Bile samples were diluted 100-fold with deionized water, and 100 μl of diluted bile samples was spiked with 10 μl IS, vortexed, and loaded onto SPE cartridges preconditioned with 2 ml MeOH, followed by 2 ml H_2O. Loaded cartridges were washed with 2 ml H_2O and eluted with 4 ml MeOH. Elute was evaporated under vacuum at room temperature and reconstituted in 100 μl of 50% MeOH, and 10 μl of sample was injected for analysis.

2.5. Western blot analysis

The liver tissue homogenates for Western analysis were prepared as we described earlier [2]. Briefly, liver samples were homogenized in lysis buffer containing 20 mmol/l Tris–HCl (pH 8.0), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA and 0.5% NP-40. Sodium pyrophosphate and sodium orthovanadate were used to inhibit phosphatase activity. Protease inhibitor cocktail (Roche) was added to the lysis buffer. Antibodies against PXR, peroxisome proliferator-activated receptor alpha (PPARα), CYP3A, SULT2A, GSTA2, farnesoid X receptor (FXR) and FGF15 were from Santa Cruz Biotechnology. Antibodies for β-actin and TATA binding protein (TBP) were from Cell Signaling Technology, and CYP7A1 antibody was obtained from Abcam. IRDye 680 and 800 conjugated secondary antibodies were obtained from LI-COR Biosciences.

2.6. Cell culture and fatty acid treatment

Huh 7 hepatoma cells were cultured in HEPES-buffered Dulbecco's modified Eagle's medium with nutrient mixture (DMEM/F12) containing 10% fetal bovine serum (FBS). The confluent cells were pretreated with SC at 5 μM for 2 h followed by co-treatment with n-3. Treatment with n-3 fatty acids was carried out using media containing serum as the source of albumin as we described previously [14]. Briefly, EPA and DHA (Nu-Chek Prep, Elysian, MN, USA) were first dissolved in ethanol and were added to DMEM containing 5% serum at a final concentration of 90 μM before treating the cells. This resulted in a fatty acid to albumin ratio of 3:1 which is within a physiological range [15]. The cells were treated for 24 h with a mixture of EPA and DHA at a combined final concentration of 90 μM as we reported earlier [16]. The fatty acids were used at a ratio of 1.5:1 (EPA 54 μM and DHA 36 μM) to reflect the fatty acid composition of commercially available FO supplements used in clinical settings. The fatty acids were added to DMEM/F12 containing 5% FBS at a final concentration of 90 μM.

2.7. Collection and treatment of precision-cut liver slices

Precision-cut liver slices (PCLSs) were prepared as we described previously [17]. The liver was excised and placed into oxygenated V-7 cold preservation buffer (Vitron, Tucson, AZ, USA). Cylindrical tissue cores (8 mm) were cut with a hand-held coring tool, and loaded into the Vitron Tissue Slicer (Vitron). Liver slices of 250 μm thickness were cut with a 45-mm rotary blade and floated into ice-cold oxygenated V-7 preservation buffer. Slices were equilibrated in serum-free Williams E Medium (Sigma Chemical, St. Louis, MO, USA) containing D-glucose and gentamicin (WEM) under 95% O_2–5% CO_2 (carbogen) at 37 °C for 30 min. The slices were floated onto titanium screen-containing rollers (Vitron). These rollers were inserted into sterile 20-ml glass vials containing 1.7 ml of WEM containing 5% FBS in the presence or absence of n-3, SC or n-3+SC. The liver slices were pretreated with SC at 5 μM for 2 h followed by co-treatment with n-3 (a mixture of EPA and DHA at 1.5:1 ratio) at 90 μM for 24 h. as described in Section 2.6. The vials were capped with lids containing a 1-mm hole for oxygen transfer. Vials were placed into the dynamic organ culture incubator (Vitron) and were incubated at 37 °C with carbogen (1.5 l/min). RNA samples were collected from the liver slices at the end of 24 h.

2.8. Transient transfection

PXR cDNA cloned in pCMV vector was obtained from Origene Technologies (Rockville, MD, USA). Transfection was performed by mixing PXR cDNA with Lipofectamine 2000 (Invitrogen) at a final volume of 100 μl OPTI-MEM without antibiotics. The procedure was carried out according to the manufacturer's instructions. Huh 7 cells were incubated with the transfection mixture for 24 h after which the transfection medium was exchanged with growth medium (DMEM/F12 with 10% FBS). Cells were incubated for 24 h before treatment with n-3 SC, or n-3+SC. Twenty-four hour posttreatment, the RNA samples were collected for reverse transcriptase polymerase chain reaction (PCR) analysis to quantify PXR and its target genes.

2.9. Real-time PCR

Real-time PCR analysis was performed using the Taqman primer-probes from Applied Biosystems. RNA was isolated using the Trizol reagent. Reverse transcription was performed using 200 ng of total RNA in 20 ul reaction volume using iScript reverse transcriptase from BioRad. The cDNA samples were diluted 1:10 and a real-time PCR was carried out using iQ supermix from Bio-Rad and Taqman predesigned primer-probe sets from Life Technologies. The details of Taqman primer-probe sets are listed in Table 1. A ΔΔCT method was used to calculate gene expression and the values were normalized to 18S ribosomal RNA.

2.10. Preparation of nuclear extracts

Nuclear extracts were prepared as described previously [18]. Briefly, the cells were lysed in a lysis buffer containing 10 mmol/l Tris–HCl, 150 mmol/l KCl, 1 mmol/l EDTA and 1 mmol/l dithiothreitol for 5 min. The lysate was centrifuged at 2500 rpm for 5 min. The pellet was incubated with nuclear extract buffer containing 20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.2 mmol/l EDTA, 1.5 mmol/l MgCl and 0.1% glycerol for 5 min on ice. The