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Molecular impact of omega 3 fatty acids on lipopolysaccharide-mediated liver damage

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ABSTRACT

Background: Growing evidence suggests that omega 3 fatty acid containing lipid emulsions have a beneficial effect on parenteral nutrition associated liver disease (PNALD). However, the cellular and molecular mechanisms responsible for this effect are unclear. In this study, we investigated whether Omegaven™ fish oil emulsion could inhibit lipopolysaccharidase (LPS) mediated liver damage.

Methods: We examined the effects of Omegaven™ and LPS alone and synergistically on hepatic paraoxonase 1 (PON1), a potent antioxidant protein, ERK1/2 activity, and TLR4 regulation.

Results: LPS did not alter PON1 release from HepG2 cells but did significantly decrease PON1 protein synthesis (44%, $P < 0.05$). Omegaven™ alone had no direct effect on PON1 release. However, it did significantly reverse LPS-mediated decrease in PON1 protein levels (control: 100%; LPS alone: 56 +/4%; LPS + Omegaven™: 87 +/6%, $P < 0.05$). Furthermore, molecular analysis indicated that Omegaven™ blocked LPS-mediated increase in ERK1/2 activity (35% increase), an important LPS signal transduction pathway. TLR4, the receptor for LPS, was down-regulated in the presence of Omegaven™.

Conclusion: Omegaven™ may be beneficial in patients with PNAC because of its ability to reverse LPS-mediated inhibition of antioxidant promoting PON1 expression, and this activity may be in part mediated by the ERK1/2 pathway.

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Short bowel syndrome (SBS) is a disease with high morbidity and mortality and linked to the development of end-stage parenteral nutrition–associated liver disease (PNALD) [1]. The clinical spectrum includes steatosis, cholestasis, cholelithiasis, hepatic fibrosis, and ultimately progression to biliary cirrhosis, portal hypertension, and end-stage liver failure. Total parenteral nutrition (TPN) has profoundly improved the prognosis and quality of life for these patients, who are otherwise unable to absorb adequate enteral nutrients [2]; however, TPN is also associated with serious complications including septicemia, metabolic imbalance, and hepatobiliary dysfunction.

The pathophysiology of non-alcoholic fatty liver disease (NAFLD), including PNALD, is multifactorial and not completely understood. Increased levels of fatty acids and triglycerides are associated with the production of free radicals and oxidative stress, the result of an imbalance in pro-oxidant and anti-oxidant processes [3]. It is believed that free radical production causes lipid peroxidation and activation of pro-inflammatory and fibrogenic cytokines, promoting the establishment of nonalcoholic steatohepatitis (NASH) [3]. TPN can improve progressive malnutrition in patients who cannot be fed enterally. However, lack of enteral nutrition can lead to infectious complications and multiple organ failure. During the past several decades, the mechanisms for the increased susceptibility to infections in patients fed parenterally have been extensively studied. Lipopolysaccharide (LPS) may play an important role in this process. LPS is a well-known component of the gram-negative outer bacterial membrane that triggers the production of pro-inflammatory mediators such as, cyclooxygenase-2 (COX-2), cytokines interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and reactive oxygen species (ROS) [4,5]. These pro-inflammatory intermediates are associated with the pathogenesis of chronic liver diseases characterized by the concomitant presence of oxidative stress and a severe inflammatory response. The available literature states that the LPS signaling receptor is Toll-like Receptor 4 (TLR4) [6]. LPS signaling is lost when TLR4 is defective, suggesting that TLR4 plays an important role in LPS activity.

Human PON1 is an antioxidant enzyme with lactonase and esterase activity, synthesized mainly by the liver. It hydrolyses lipid peroxides and is bound to high-density lipoproteins (HDL) as it circulates in plasma [7]. PON1 is protective against liver impairment, attenuating the production of pro-inflammatory factors [8,9]. Recent evidence indicates that PON1 over-expression provides strong protection against the development of experimental liver disease. Conversely, low PON1 levels are associated with an enhanced sensitivity to the development of liver damage [10,11]. Recent evidence from animal models, as well as data from humans, has suggested that the replacement of the typical soy-based, predominantly omega-6 fatty acid (v6FA) TPN lipid emulsion Intralipid®, with a fish oil-based lipid emulsion containing omega-3

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fatty acids (v3FA) Omegaven™, has the potential to reverse liver disease in children with SBS [12–14]. Omegaven™ has been reported to attenuate liver damage mediated by TPN, but the molecular mechanisms remain unclear [15]. Whether PON1 specifically plays an important role in Omegaven's™ ability to prevent TPN-mediated liver damage is unknown. In this study, we examined whether Omegaven™ could alter LPS-mediated liver damage by assessing PON1 synthesis and release from HepG2 cells. PON1 enzymatic activity in response to Omegaven™ was also examined. Our data show that Omegaven™ prevents LPS-mediated PON1 protein decrease and provides insight into a potential mechanism by which Omegaven™ may prevent LPS-mediated liver damage.

1. Methods

1.1. Cell culture

Human hepatic HepG2 cancer cells were cultured and maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS in 100 mm plastic plates. Cells were treated with media supplemented with 20% Omegaven™ [Fresenius SE & Co. KgaA, Bad Homburg, Germany] in medium for one hour and LPS, 10 ng/mL, was added for another 16 h. Cells were harvested and lysed, and PON1 expression was measured by western blot. The cell lysate supernatant was used to measure PON1 enzymatic activity. LPS-mediated endogenous levels of phosphorylated ERK1/2 were measured using Fluorescence Energy Transfer (FRET) technology [Cisbio, Bedford, MA].

1.2. PON1 activity assay

Determination of PON1 arylesterase activity was done by the standard phenyl acetate (PA) method. Arylesterase activity of PON1 was measured in a buffer solution using PA as the substrate at a final concentration of 2 mM, pH 8, at room temperature (RT). Formation of phenol was measured after 30 min of incubation at 270 nm using a nanodrop ND-1000 [ThermoFisher, Waltham, MA]. Enzymatic activity was calculated from the molar extinction coefficient of phenol ($\varepsilon 270 = 1310\ M - 1\ cm - 1$). All measurements were carried out in triplicates; error bars represent the standard deviation of three independent experiments.

1.3. Western blotting

HepG2 cell lysates were prepared by homogenization by RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl flouride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecylsulfate) containing 5 μg/ml of aprotinin, and 5 μg/ml of leupeptin. Cell debris was removed by centrifugation and protein concentration determined by using the Bio-Rad protein assay. Lysate aliquots were stored at −20 °C for future use. Cell lysates were boiled in sample buffer at 100 °C for 5 min. 10 μg of total protein was used for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for 1–2 h at 100 V. The gel was placed in 1× transfer buffer for 15 min and protein transferred to a PVDF membrane [Thermo Scientific, Rockford IL] overnight at 4 °C. The membrane was blocked in 5% milk for 1 h at RT, or overnight at 4 °C. Incubation with the appropriate primary antibody dilutions anti-PON1 [abcam®, Cambridge, MA] or TLR-4 [abcam®, Cambridge] in blocking buffer was done overnight at 4 °C. The membrane was washed three times for 5 min in Tris-Buffered Saline-Tween 20 (TBST) and then incubated with the recommended dilutions of horseradish peroxidase (HRP) - conjugated secondary antibody, goat anti-mouse [Thermo Scientific, Rockford, IL] or goat anti-rabbit [abcam®] in blocking buffer at room temperature for 1 h, followed by three washes, 5 min each. Signals were developed using enhanced chemiluminesence (ECL) kit [Millipore, Billerica, MA] as per the manufacturer's instructions. Images were acquired using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

1.4. Phospho-ERK signaling pathway

To determine whether LPS alters the ERK signaling pathway, HepG2 cells were treated with LPS or LPS + 20% Omegaven™ in DMEM. LPS-mediated endogenous levels of phosphorylated ERK1/2 were measured using FRET technology. HepG2 cells were incubated with 0.5 ml Earle's Balanced Salt Solution (EBSS), containing LPS (5 ng/ml and 10 ng/ml), for 20 min at 37 °C. ERK1/2 levels were measured according to the instructions accompanying the assay kit. Each experiment was performed a minimum of three times with duplicate wells.

1.5. Real time PCR

RNA extraction was performed in an ABI Prism™ 6100 nucleic acid prep-station [Applied Biosystems, Waltham, MA]. Reverse transcription to cDNA, from 1 μg of total RNA, was done using random hexamer primers [Invitrogen, Carlsbad, CA]. Real-time PCR analysis was performed using the Thermo Fisher Scientific Luminaris Higreen Fluorescein qPCR kit. Primers for *PON1* is forward primer: 5′-GAT TGG CAC AGT GTT TC −3′; reverse primer: 5'CCT CAG TTT CTA TGG CA-3′) and GAPDH (forward primer: 5′-TCA CCA CCA TGG AGA AGG C-3′; reverse primer: 5′-GCT AAG CAG TTG GTG GTG CA-3′). Quantitative real-time PCR (qRT-PCR) was conducted by also using the ThermoFisher Scientific Luminaris Higreen Fluorescein and qPCR master mix reagent [ThermoFisher Scientific, Waltham, MA] with an iQ5 RealTime Thermal Cycler [Bio-Rad, Hercules, CA]. Briefly, HepG2 cells (3×10 [6] cells) were seeded in 6 well plates for RNA isolation by TRIzol [Life Technologies, CA] according to the manufacturer's instructions. The concentration of RNA was quantified by the Nanophotometer [Implen, Munich, Germany]. 1 μg of RNA was then reverse transcribed to cDNA using SuperScript III Reverse Transcriptase [Life Technologies, Carlsbad, CA] with 0.5 μg of oligo (dT) 18 primer. The qRT-PCR reaction contained 1 μg of cDNA, primers, and Luminaris Higreen Fluorescein PCR Premix. The thermal cycle program was set as follows: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. *PON1* gene expression was normalized to the threshold cycle (Ct) values of the housekeeping gene actin. The relative index (2-ΔΔCt) was calculated by comparing the average expression level for control samples with the index defined as 1.00.

1.6. Statistics

Each experiment was performed three separate times as duplicates. Data are expressed as mean ± SEM. Significant differences were determined by Student's t test, with $p < 0.05$ considered to be statistically significant.

2. Results

2.1. Omegaven™ prevents LPS-mediated liver PON1 protein decrease

To examine Omegaven's™ effect on LPS-mediated liver damage, the hepatic cancer line HepG2 was used. We assessed PON1 function, in response to LPS treatment by measuring PON1's enzymatic activity and protein expression. HepG2 cells were treated with LPS (10 ng/ml) for 2 h, after which supernatant paraoxonase activity was measured. Our results indicate that LPS did not alter PON1 release from HepG2 cells (Fig. 1A). Next, cells were treated with LPS (10 ng/ml) for 16 h, harvested, lysed, and PON1 protein expression assessed by western blotting, where we found LPS to significantly decrease PON1 protein synthesis in a dose dependent manner averaging to 44% of control ($P < 0.05$) (Fig. 1B). To determine the effect of Omegaven™ on PON1 release from HepG2 cells, they were treated with DMEM containing 20% Omegaven™ for 2 h and PON1 levels in the media determined. We found that Omegaven™ alone had no

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