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Attenuation of Parenteral NutritionAssociated Liver Disease by Omega-3 Long-Chain Polyunsaturated Fatty Acids

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Comments

One of the most devastating complications affecting infants and children with intestinal failure is parenteral nutrition-associated liver disease (PNALD). PNALD is a progressive disease that can lead to liver failure and death. In many cases, children require liver and small bowel transplantation, prolonged hospitalization and home care. Intravenous infusion of a fish oil emulsion has been shown to be efficacious for the treatment of PNALD. The therapeutic mechanism is unknown, but is thought to occur via anti-inflammatory pathways, as of yet undefined. Fish oil contains omega-3 polyunsaturated long-chain fatty acids (ω3PUFA) that have anti-apoptotic and anti-inflammatory effects. This body of work is focused on my central hypothesis that PNALD is attenuated by ω3PUFA via anti-apoptotic and anti-inflammatory mechanisms. I have used both cultured hepatocytes and macrophages and a preterm neonatal pig model to show that ω3PUFA attenuate both apoptosis and inflammation associated with PNALD. Although there are still many unanswered questions concerning the mechanism of ω3PUFA in the treatment of PNALD, this work supports the efficacy of this novel therapy for treating PNALD.

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By
Emma Monique Tillman
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ABSTRACT

One of the most devastating complications affecting infants and children with intestinal failure is parenteral nutrition-associated liver disease (PNALD). PNALD is a progressive disease that can lead to liver failure and death. In many cases, children require liver and small bowel transplantation, prolonged hospitalization and home care. Intravenous infusion of a fish oil emulsion has been shown to be efficacious for the treatment of PNALD. The therapeutic mechanism is unknown, but is thought to occur via anti-inflammatory pathways, as of yet undefined. Fish oil contains omega-3 polyunsaturated long-chain fatty acids (ω3PUFA) that have anti-apoptotic and anti-inflammatory effects. This body of work is focused on my central hypothesis that PNALD is attenuated by ω3PUFA via anti-apoptotic and anti-inflammatory mechanisms. I have used both cultured hepatocytes and macrophages and a preterm neonatal pig model to show that ω3PUFA attenuate both apoptosis and inflammation associated with PNALD. Although there are still many unanswered questions concerning the mechanism of ω3PUFA in the treatment of PNALD, this work supports the efficacy of this novel therapy for treating PNALD.

PREFACE

In December 2007 during my clinical pharmacy practice residency, I became very interested in parenteral nutrition-associated liver disease (PNALD). In the first six months of my residency training, I observed the deaths of two infants from PNALD. I had read case studies reporting the use of a fish oil-based intravenous fat emulsion for the treatment of PNALD. This product is not FDA-approved; therefore, we were unable to obtain it for use at our institution. I approached my faculty preceptors about using enteral fish oil for the treatment of PNALD, and the treatment was initiated in six patients with PNALD. Complete reversal of disease occurred in four patients, and improvement was observed in the remaining two patients. This experience was the basis for the work presented in chapter 2.

In July 2008, I completed my residency and began a fellowship training program specializing in pediatric nutrition and metabolic support. I realized that, at best, a clinical study would yield safety and efficacy data within the next few years. However, we would probably still know very little about the mechanism by which fish oil attenuates PNALD. My curiosity led me to develop a research program to determine the mechanism.

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ω3PUFA	Omega-3 polyunsaturated fatty acid
ω6PUFA	Omega-6 polyunsaturated fatty acid
ω9PUFA	Omega-9 polyunsaturated fatty acid
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CDCA	Chenodeoxycholic acid
CFLAR	CASP8- and FADD-like apoptosis regulator
CFTR	Cystic fibrosis transmembrane conductance regulator
CVC	Central venous catheter
DB	Direct bilirubin
DHA	Docosahexaenoic acid
DR	Death receptor
DSS	Dextran sulfate sodium
EB/AO	Ethidium bromide/acridine orange
EFAD	Essential fatty acid deficiency
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
EN	Enteral nutrition
EPA	Eicosapentaenoic acid
Fas-L	Fas ligand
FDA	Food and drug administration
FFA	Free fatty acids

G	Gastrostomy
GGT	Gamma-glutamyl transpeptidase
GPR	G-coupled protein receptor
H & E	Hematoxylin and eosin
HepG2	Human liver hepatocellular cells
IFALD	Intestinal failure-associated liver disease
IL-1β	Interleukin one beta
IL-6	Interleukin six
IND	Investigational new drug
INR	International normalized ratio
IVFE	Intravenous fat emulsion
LPS	Lipopolysaccharide
LXR	Liver X receptor
NAFLD	Nonalcoholic fatty liver disease
NEC	Necrotizing enterocolitis
NFκB	Nuclear factor kappa-B
NG	Nasogastric
PMA	Phorbol 12-myristate 13-acetate
PN	Parenteral nutrition
PNAC	Parenteral nutrition-associated cholestasis
PNALD	Parenteral nutrition-associated liver disease
PPARα	Peroxisome proliferator-activated receptor alpha
PPRE	Peroxisome proliferator response element

RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SBS	Short bowel syndrome
siRNA	Small interfering RNA
SMOF	Soybean oil, medium-chain triglycerides, olive oil and fish oil
STEP	Serial transverse enteroplasty
TB	Total bilirubin
TBA	Total bile acids
THP-1	Human acute monocytic leukemia cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-associated apoptosis-inducing ligand
TRAIL-R2	Tumor necrosis factor-associated apoptosis-inducing ligand receptor 2
T/T	Triene:tetraene ratios

CHAPTER 1. PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE*

Introduction

Definition and etiology

Parenteral nutrition (PN)-associated liver disease (PNALD) is defined as a decrease in bile flow, independent of mechanical obstruction, in patients receiving prolonged PN and with no other underlying cause of liver disease (1-6). PNALD is also referred to as PN-associated cholestasis (PNAC), PN-associated liver injury (PNALI), or more recently intestinal failure-associated liver disease (IFALD). Because PNALD typically improves with the advancement of enteral nutrition (EN) and the discontinuation of PN (7), and IFALD encompasses patients that necessitate specialized nutrition support such as EN and/or PN, I have chosen to use PNALD throughout the manuscript.

Clinical diagnosis of PNALD includes biochemical markers for liver disease such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB), and direct bilirubin (DB), and ruling out all other potential causes of liver disease. Although liver biopsy is needed to definitively diagnose PNALD, because of the invasive nature of biopsy, many centers will not perform diagnostic liver biopsies and consider DB > 2 or 3 mg/dL in the absence of any other liver etiology reflective of PNALD. PNALD is reported to occur in 50–66% of children receiving long-term PN, with a higher incidence in premature neonates (1-3, 8, 9). Although the incidence of PNALD is much higher in children, PNALD also occurs in adult patients, but may present as cholestasis, hepatosteatosis, or cholelithiasis.

PNALD is one of the most devastating complications affecting infants receiving PN. The etiology of PNALD is not well understood and is likely multi-factorial (1-3, 8, 10). Risk factors include immature liver function, inflammation, oxidative stress, infection (specifically sepsis), nutrient deficiencies, and contaminants in parenteral products, and macronutrient provisions including intravenous fat emulsion (IVFE) and proteins (11-14). PNALD can progress from cholestasis to cirrhosis, liver failure, and death if not treated by advancement to full EN with discontinuation of PN. In cases where full enteral feeding is not possible, the patient may require liver and small bowel transplantation, which is also associated with significant morbidity and mortality.

*Adapted with permission. Tillman EM. Review and clinical update on parenteral nutrition-associated liver disease. Nutr Clin Pract 2012.28(1):30-9.

Pathophysiology of PNALD

The lack of EN can lead to a decrease in the release of counter-regulatory hormones secreted from the gut, which can decrease bile flow. In premature infants, organ systems are not developed to the extent they are in a term infant and/or adult, which can result in a build-up of toxic intermediates from parenteral products, which in turn can have harmful effects on the hepatobiliary system (15). Methionine, as an example, is known to be hepatotoxic in immature rabbit pups, and the very high concentrations of methionine in the plasma of neonates and infants receiving standard adult amino acid formulations may result in liver injury in the neonate (16). Aluminum contamination is unavoidable in parenteral products. Neonates receiving PN have been estimated to receive aluminum ranging from 10-60 μg/kg/day (17). Exposure to aluminum has been shown to decrease bile flow by decreasing bile acid transport proteins (17). This may be even more profound in neonates with immature bile acid transport systems (15). Alemmari *et al*. developed a pig model to study aluminum effects on hepatic function. They showed that bile acid concentrations and hepatic aluminum concentration correlated with duration of aluminum exposure (18). The same authors evaluated incidence and severity of PNALD in this neonatal pig model given standard PN solution vs. a low aluminum PN solution. The investigators observed more severe histological changes in the standard PN group compared to the low aluminum group, but they did not find a significant difference in serum direct bilirubin or bile acids (17).

Intravenous fat emulsions have been of particular interest in PNALD, as ω6 polyunsaturated fatty acid (PUFA) soybean-based lipid emulsions are thought to be pro-inflammatory, as well as contain potentially toxic phytosterols (19). Composition and dose of soybean-based IVFE have been associated as risk factors for cholestasis, as well as recent packaging changes of IVFE associated with fat globules > 5 μm (13, 20). In animal studies, unstable IVFE administration resulted in increased oxidative stress and liver injury (21).

The development of PNALD is associated with increased morbidity and mortality, and, if not reversed, PNALD can progress to liver fibrosis, hepatic failure and death. PNALD improves with the initiation of enteral feeding. While most PNALD reverses with advancement of EN and discontinuation of PN, this is not an option in many patients with anatomic or functional short bowel syndrome (SBS).

Treatments for PNALD

Many treatments for PNALD have been reported to have limited success. They include PN cycling, drug therapy with ursodeoxycholic acid, cholecystokinin, oral antibiotics and nutrient restriction, including limiting soybean-based IVFE and providing conservative protein and dextrose calories to prevent overfeeding (22-26). Advancement to full EN and discontinuation of PN is the best treatment for PNALD. In a retrospective study by Javid *et al.* 12 patients were evaluated and determined to have PNALD; only two of these patients had improvement in serum bilirubin prior to the achievement of full

EN, whereas the mean time of normalization of bilirubin in the remainder of the patients was four months after discontinuation of PN (7). Although achievement of full EN is associated with resolution of PNALD, this is not possible for many patients with SBS. Surgical approaches, such as the serial transverse enteroplasty (STEP) bowel lengthening procedure, have been used to decrease need for PN (5). Intestine/intestine + liver transplantation have also been lifesaving for patients with progressive PNALD (4, 27). However, these are invasive surgical procedures that have a high risk of complications, including bleeding, infection and rejection (in transplant) (4, 27). Most of the recent progress in treating PNALD has been related to IVFE. This includes the use of parenteral fish oil-based IVFE, Omegaven®, (Fresenius Kabi AG, Bad Homburg v.d.h., Germany) (28, 29), dose reduction of soybean-based IVFE, enteral administration of fish oil, and the use of contemporary IVFE products that provide a blend of ω3-, ω6-, and ω9PUFA (30).

Cyclic parenteral nutrition. Cyclic PN has been anecdotally thought to decrease the incidence and severity of PNALD, but there is very limited evidence to support this observation. Jensen *et al.* performed a retrospective review to evaluate if prophylactic cycling decreased the incidence and the time of onset of PNALD in patients with gastroschisis. Thirty-six infants were prophylactically cycled and were compared to 71 control patients that received continuous PN. The authors reported that infants receiving continuous PN were 2.86 times more likely to develop PNALD as compared to controls, but after adjusting for confounding factors, this failed to meet statistical significance (26). Salvador *et al.* conducted a prospective randomized study to evaluate the effect of cycling PN in very low birth weight infants on the incidence of PNALD. In this study, the control group (n = 36) received continuous PN (amino acid and dextrose solution over 24 hours and lipids over 18 hours in accordance with hospital protocol), and the treatment group (n = 34) received cyclic PN (amino acid solution over 20 hours, lipids over 18 hours, and dextrose over 24 hours). Infants requiring abdominal surgery were excluded from this study because they required transfer to a surgical referral center. The incidence of PNALD was similar in both groups (32% in the treatment group and 31% in the control group). The authors concluded that early prophylactic cyclic PN is not a significant predictor for the development of PNALD (31). In this study, the only macronutrient that was provided over different lengths of time in this study was protein. All patients received continuous dextrose and all patients received cycled IVFE. Additionally, patients requiring abdominal surgery typically have a longer duration of PN dependence and are at higher risk for the development of PNALD. Exclusion of these patients may be a flaw of the study.

Parenteral ω3 polyunsaturated fatty acids. The first reports of using fish oil-based IVFE in children came from the Boston Children's Hospital. They reported the case of a 17-year-old male who developed essential fatty acid deficiency (EFAD) due to a soy allergy preventing administration of soybean-based IVFE. The patient was treated with parenteral fish oil comprised primarily of ω3PUFA and limited ω6PUFA; EFAD was reversed. Clinicians also observed decreases in AST, ALT, TB, and DB (28). In 2006, the Boston group reported the complete reversal of PNALD in two infants with

intestinal failure treated with fish oil-based IVFE at 1 g/kg/day. Cholestasis resolved in both infants within the first 60 days of treatment (29).

The same group published safety and efficacy data for 18 patients treated with fish oil-based IVFE at 1 g/kg/day infused over 12 h per day from September 2004 – August 2006 compared to 21 historical control patients who received standard soy-based lipid emulsions 1 – 4 g/kg/day infused over 24 h per day between 1986 - 1996. They reported the time to reversal of cholestasis of 9.4 weeks in the fish oil-based IVFE group compared to 44 weeks in the control group ($p = 0.002$; (32). There were two deaths and no liver transplants reported in the fish oil-based IVFE group compared to seven deaths and two liver transplants in the control group. Death occurred at 6.7 and 10.7 weeks respectively in the two patients in the fish oil group, whereas the time to death in the historic control group was reported as a median of 41 weeks. Although the initial findings are interesting, this study is limited by the small sample size and a control group that is from an observation period 10 – 20 years prior to the treatment group. Standards of care and infant survival have changed in the last 20 years. Also, the dose of IVFE was significantly decreased in the treatment group. These results could be interpreted as suggesting that reduction of IVFE resulted in the improved outcomes. Puder *et al.* published results of an open-label study of 42 infants treated with fish oil-based IVFE between August 2006 and November 2007 compared to a historical cohort of 49 patients with SBS and PNALD treated with soybean-based IVFE from 1999 – 2006 (33). This report is an extension of the previously published work from this group with a slightly more contemporary historical cohort. Death occurred in three of 42 (7%) patients in the fish oil group and 12 of 49 (24%) in the control group. One patient receiving fish oil underwent liver transplant compared to six patients in the control group.(33) The median time to discontinuation of PN was four weeks in the fish oil group as compared to 20 weeks in the control group. PNALD resolved while patients were still receiving PN in 19 patients in the fish oil group and only two patients in the control group (33). Patients in the fish oil group had lower international normalized ratio (INR) values and increased platelet counts compared to the control group, suggesting that fish oil has a limited effect on coagulation, but this conclusion is confounded by the higher incidence of advanced liver disease in the control group.

Similar success has also been observed by Italian clinicians in a case of an infant with severe PNALD (34). The patient was started at 0.2 g/kg/day of fish oil-based IVFE and advanced by 0.2 g/kg/day to achieve a dose of 1.5 g/kg/day, which is higher than has been previously reported (34). Chinese clinicians reported the reversal of PNALD in three of four infants with intestinal failure treated with fish oil-based IVFE (35). In these patients, standard soybean oil-based IVFE was discontinued and fish oil-based IVFE was initiated at 1 g/kg/day (35). Calhoun and Sullivan report successful resolution of PNALD in a 17-month-old infant with SBS after treatment with fish oil-based IVFE (36). Chung *et al.* reported the use of fish oil-based IVFE to treat four patients with SBS and PNALD (37).

A study from Toronto recently reported the reversal of PNALD in nine of 12 infants with SBS that were treated with a combination of a soybean oil-based IVFE and

fish oil-based IVFE. Four patients had complete reversal of PNALD while receiving a combination of soybean-based IVFE and fish oil-based IVFE, whereas the other five patients did not have reversal of disease until after soybean-based IVFE was discontinued (38).

Early reports of fish oil-based IVFE used outcomes based on clinical presentation and biochemical markers of cholestasis (AST, ALT, TB and DB) and not based on liver biopsy results. Investigators from Boston have now reported 83 biopsy results taken in 66 children. Of the 83 biopsies, 74 demonstrated fibrosis; eight of these also demonstrated cirrhosis. Forty-one of the 74 fibrotic biopsies (55%) were obtained in patients without biochemical evidence of cholestasis (39). Seventy percent of these patients were treated with fish-oil-based IVFE during his/her clinical course (39). Soden *et al.* reports similar findings with two infants who had improvement in biochemical markers of cholestasis, but persistent portal fibrosis despite treatment with fish oil-based IVFE (40). This report has been questioned because of the timing of the biopsy and the treatment with fish oil-based IVFE. In contrast, an abstract from Dimmitt *et al.* reported multiple biopsy evaluation that showed fibrosis had resolved or was stable after switching to a fish oil-based IVFE (41). Hepatocellular injury associated with PNALD has been reported to occur within the first few weeks of PN, and the reversal of fibrosis associated with any type of liver injury may never occur, but, if it does, reversal often takes a long time (42, 43).

While much of the use of fish oil-based IVFE has been in pediatric patients, Burns and Gill reported similar success in treating a 50-year-old female patient with PNALD with fish oil-based IVFE (44). Although, the past five years has resulted in many publications evaluating fish-oil based IVFE, a recent systematic review of five randomized controlled trials and three high-quality prospective cohort studies concluded that there is still a lack of high-quality data to support the use of fish-oil based IVFE in children and future trials are needed to evaluate the safety and long-term outcomes (45).

Modification of soybean-based IVFE and enteral fish oil. While fish oil-based IVFE appears to be a promising therapy for PNALD, its use in the United States is hindered by lack of FDA approval, thereby necessitating the use of an investigational new drug (IND) application. Because of the difficulty in obtaining the parenteral fish oil product, some institutions have restricted or eliminated soybean-based IVFE and used enteral fish oil (250 mg/kg/d; J.R. Carlson Laboratories, Arlington Heights, IL) to treat infants with PNALD. Rollins *et al.* retrospectively evaluated 26 patients with SBS receiving soybean-based IVFE 2 – 3 g/kg/day: 23 patients developed PNALD. PNALD resolved in 10 patients with the advancement of EN, one patient developed PNALD and remained on 2 g/kg/day of soybean-based IVFE, three patients required liver transplant, three patients died, and six patients had complete resolution of PNALD with the removal of soybean-based IVFE. Four of these six patients also received enteral fish oil (46).

Our group has reported six infants with PNALD treated with enteral fish oil (dose ranging from 0.15-1 g/kg/d using three different commercially available products) (47). PNALD completely reversed in four of the six infants supplemented with enteral fish oil

within a mean of five weeks after initiation of therapy. The two infants that did not have complete resolution of disease had some improvement in bilirubin and liver enzymes while on enteral fish oil. One of these non-responding infants had an extremely short gut, estimated as 8 cm of small bowel remaining, and no ileocecal valve. This would suggest that absorption is limited in the extremely short gut patient, but does not rule out an intraluminal effect of fish oil. Similar results were observed by Sharma *et al.* when four infants had resolution of PNALD after being treated with enteral fish oil (48).

Cober *et al*. conducted a prospective study evaluating dose reduction of soybean-based IVFE to improve PNALD in infants. Soybean-based IVFE were reduced to 1 g/kg/day two days per week in the treatment group and these patients were matched with historical controls that received daily soybean-based IVFE at a dose of 3 g/kg/day. Patients receiving the restricted IVFE dosing had a significant decrease in direct bilirubin as compared to historical controls. Eight of the 31 infants in the restricted IVFE group were reported as having mild essential fatty acid deficiency, but this was reversed when an additional day of IVFE was added (49). Nehra *et al*., retrospectively evaluated 61 patients that received either 1 g/kg/day soybean-based IVFE or 2-3 g/kg/day soybean based IVFE. They found that restricting soybean-based IVFE to 1 g/kg/day did not prevent PNALD in neonates (50).

Other contemporary lipids. European clinicians and researchers have utilized other IVFE products in addition to fish oil-based IVFE. An IVFE comprised of soybean oil, medium-chain triglycerides, olive oil and fish oil (SMOFlipid 20%, Fresenius Kabi AG, Bad Homburg v.d.h., Germany) has also been used for the treatment of PNALD. In a randomized, double-blind trial of 22 patients treated with SMOFlipid and 22 patients treated with olive oil and soybean oil IVFE for five days postoperatively there was no significant difference in baseline AST and ALT between the two groups; however, significantly lower AST and ALT values were observed at day two and day five in the SMOFlipid group as compared to the olive oil and soybean oil group (51). Muhammed *et al.* retrospectively compared serum bilirubin of 17 neonates with PNALD. Eight were treated with SMOFlipid and nine were continued on standard soybean-based IVFE. After six months of treatment, 63% of patients in the SMOFlipid group had resolution of PNALD compared to 22% in the soybean-based IVFE group (52).

Adverse side effects associated with novel IVFE

As with any new therapy, there may be potential adverse side effects. In the initial Boston cohort study, only one of 18 patients and two of 42 patients in the second cohort exhibited biochemical evidence of essential fatty acid deficiency (EFAD) based on triene:tetraene ratios (T/T); however, total fatty acid profiles were not reported in either study (32, 33). With decreased lipid dosing, it is possible to have T/T within the reference range, but have concentrations of individual fatty acids below the age-related reference range. A prospective evaluation of 10 patients receiving fish oil-based IVFE as a sole fat source at a dose of 1 g/kg/day resulted in T/T within the reference range in all 10 patients, but 50% of patients had linoleic acid concentrations below the reference range and 90%

had at least a 17% reduction in linoleic acid concentration after six weeks of treatment with fish oil-based IVFE (53). In the lipid restriction study by Cober *et al*., 25% of patients developed mild EFAD, which necessitated increasing the weekly dose of IVFE (49). All cases of EFAD corrected with the increase in lipid dose. Animal studies have not clarified the adequacy of linoleic acid intake with fish oil. A study of pair-fed mice provided with chow containing either soybean-based or fish oil-based fat showed fish oil both prevents EFAD and enhances growth in mice. EFAD was determined based on T/T. All T/T were within the reference range, but linoleic acid concentrations were not reported (54).

One infant with SBS and PNALD treated with fish oil-based IVFE developed burr cell anemia that resolved upon discontinuation of fish oil-based IVFE (55). The authors speculated that incorporation of ω3PUFA within red blood cell membranes made erythrocytes susceptible to trapping and destruction within the spleen. The product labeling for the fish oil-based IVFE suggests that the product could increase bleeding risk (56). In contrast, Bays and Harris both conclude that ω3PUFA does not increase bleeding risk (57, 58).

Animal Studies of ω3PUFA for the Treatment of Liver Disease

Several animal models have been utilized to study the effects of ω3PUFA supplementation on liver injury. Alwayn *et al.* report the use of both enteral and parenteral ω3PUFA in a mouse model of NAFLD (59). Animals were randomized to receive standard chow or an enteral fat-free, high carbohydrate diet identical to parenteral nutrition solutions given to pediatric patients, with and without ω3PUFA supplementation. Mice fed standard chow had the lowest hepatic fat content and served as controls. Hepatic fat content was the highest in mice fed the liquid high carbohydrate diet, and was significantly decreased by both enteral and parenteral ω3PUFA supplementation (59).

In a surgical model of liver injury, mice underwent a common bile duct ligation and were then randomized to receive a control soy diet rich in ω6PUFA or a Menhaden diet rich in ω3PUFA (60). In this study, there were trends that a Menhaden diet was hepato-protective against injury, but this was not statistically significant (60).

A study in rabbits receiving a balanced PN regimen with 3 g/kg/day IVFE evaluated the effects of a soybean oil emulsion alone, an olive oil emulsion alone, and a combination soybean (2.8 g/kg/day) and fish oil (0.2 g/kg/day) emulsion. Animals that received the fish oil were found to have more extensive hepatic fibrosis than those that received the soybean or olive oil regimens (61). The authors speculated that this finding might be due to the omega-3 to omega-6 ratio of 1:6 in the emulsion studied, whereas optimal omega-3 to omega-6 ratios in animal models have ranged from 1:2 to 1:4 (62, 63).

Potential Mechanism of Action of ω3PUFA

Omega-3 PUFA supplementation has been used for the treatment of many inflammatory diseases, including cardiovascular disease, arthritis, asthma, sepsis, autoimmune disease and malignancy (57). ω3PUFA are thought to exhibit anti-inflammatory effects by causing a shift from ω6PUFA-derived pro-inflammatory eicosanoids to the anti-inflammatory variety derived from ω3PUFA (64). This pathway is regulated by the Δ-5-desaturase enzyme system, which preferentially metabolizes ω3PUFA. A number of studies have suggested that positive changes in membrane phospholipid content, as well as in clinical outcomes, can be achieved with relatively low intakes of fish oil (65-68). This suggests that ω3PUFA compete very effectively as substrates for the Δ-5-desaturase enzyme system despite the presence of large amounts of ω6PUFA (**Figure 1-1**).

Tumor necrosis factor (TNF)-α is one of the major pro-inflammatory cytokines involved in liver disease (69). In a murine macrophage model, the reduction of TNF-α gene transcription, via inactivation of the nuclear factor kappa-B (NF-κB) signal transduction cascade secondary to decreased IκB phosphorylation at serine 32, has been proposed as a mechanism for the anti-inflammatory effects of ω3PUFA (70). TNF-α and other pro-inflammatory cytokines transcribed via NF-κB, as well as stimulation of death receptors on the hepato-cellular surface, have been linked to liver injury, causing both apoptosis and necrosis in many types of liver diseases (cholestasis, NAFLD, alcoholic cirrhosis and hepatitis) (71). While the exact mechanism of action of ω3PUFA in PNALD is unclear, this anti-inflammatory pathway would seem to make sense based on potential etiologies of the disease. Li *et al*. reported that anti-tumor necrosis factor-α ameliorated the progression of PNALD and improved the expression of hepatic ABC transporter genes in a mouse model of PNALD (72).

In addition to the anti-inflammatory effects of ω3PUFA, many investigators are studying the effects of phytosterols found in soybean-based IVFE on liver injury. Phytosterols act as antagonists to the nuclear receptors that are critically involved in hepato-protection from cholestasis (73), therefore the elimination or dose limitation of soybean-based IVFE may play a key role in the mechanism of liver injury observed in PNALD. Kurvinen *et al.* measured serum cholesterol, non-cholesterol sterols and liver biochemistries in 28 neonates and 11 children. Neonates with PNALD had significantly higher ratios of plant sterols and cholesterol compared to neonates without PNALD. Additionally, duration of PN was associated with concentrations of cholestanol, stigmasterol, avenasterol, AST, and ALT (74). These data suggest that plant sterols found in soybean-based IVFE may be responsible for liver injury observed in PNALD.

The past five years have resulted in many changes in how clinicians are treating PNALD. There has also been increased excitement for research in this very important area. While there are still many unanswered questions, the current data suggest IVFE play a key role in PNALD. Restricting soybean-based IVFE, or providing fish oil either parenterally or enterally may be a potential treatment for patients with PNALD. It is clear from the treatment failures reported from many centers across the world that fish oil is

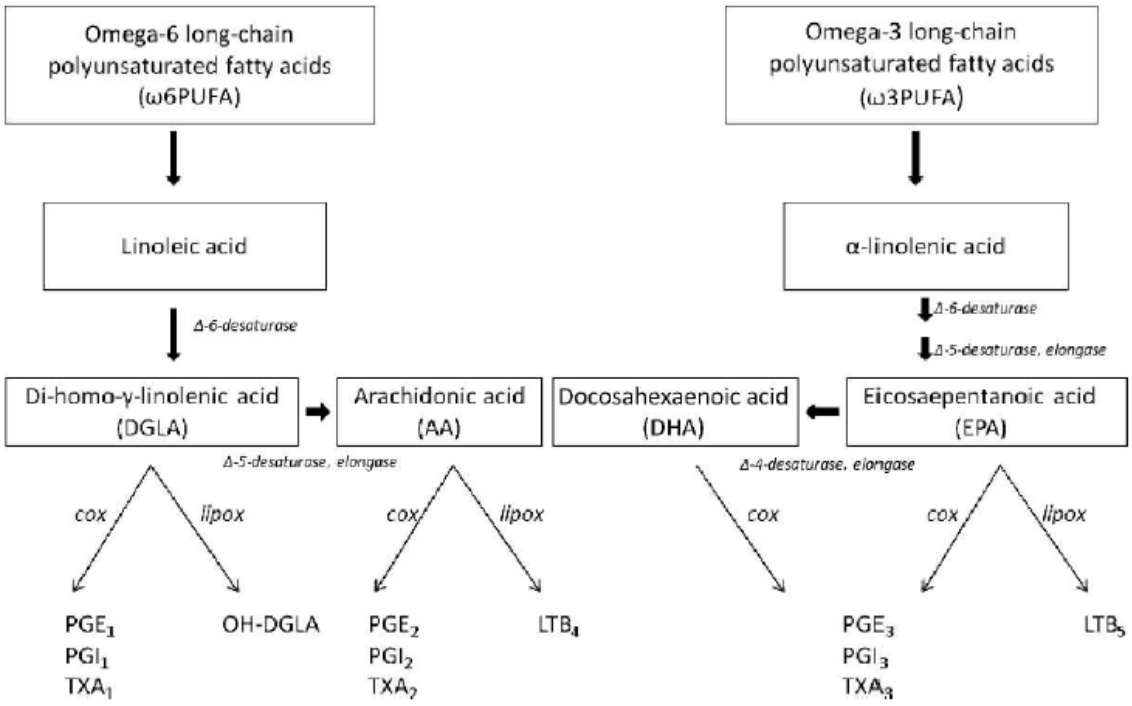

Figure 1-1: Omega-3 and omega-6 long-chain polyunsaturated fatty acid metabolism.

not a universal cure for all patients. Recent biopsy reports from patients treated with fish oil-based IVFE that showed hepatic fibrosis with no biochemical evidence of cholestasis is of concern (39, 40). Contemporary IVFE solutions, such as the SMOFlipid product that offers a blend of ω3PUFA, ω6PUFA and ω9PUFA, may be an advantage in the treatment of PNALD. A well-balanced lipid product may have advantages over a single-source product. Several centers leading research efforts against intestinal failure and PNALD have established inter-professional teams to attack this multifactorial disease from all angles (19, 75, 76). This approach to the care of this disease will likely result in the most favorable patient outcomes. More research in the form of randomized controlled trials evaluating dosing of ω3PUFA, as well as other potential drug therapies, are needed to advance our knowledge and treatment of PNALD.

CHAPTER 2. IMPROVEMENT OR REVERSAL OF PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE IN SIX INFANTS WITH SHORT BOWEL SYNDROME TREATED WITH ENTERAL FISH OIL*

Introduction

Parenteral nutrition (PN)-associated liver disease (PNALD) is defined as a decrease in bile flow that occurs independent of mechanical obstruction in patients receiving prolonged parenteral nutrition (1-3). It is diagnosed by clinical presentation, and elevation in direct bilirubin, liver enzymes, and serum bile acids (1-3). PNALD occurs in approximately 25 – 66% of patients maintained on long-term PN, and incidence increases in infants with low birth weight, low gestational age and short bowel syndrome (SBS) (1-3, 8). The etiology of PNALD is not well understood and likely multifactorial. If not reversed, PNALD can progress from cholestasis to liver fibrosis, hepatic failure and death (1). PNALD improves with the initiation of enteral nutrition (EN) and discontinuation of PN, but many patients with SBS are dependent on PN.

Fish oil-based intravenous fat emulsion (IVFE), Omegaven® (Fresenius Kabi AG, Bad Homburg v.d.h., Germany) appears to be a promising therapy in the treatment of PNALD.(28, 29, 32-38) However, its lack of food and drug administration (FDA) approval requires use under an investigational new drug (IND) application. Due to the difficulty in obtaining the parenteral fish oil, restriction of soybean-based IVFE and enteral fish oil have been utilized. Rollins *et al.* reported complete resolution of PNALD with the removal of soybean-based IVFE in six patients, four of whom also received enteral fish oil (46). In contrast, we report the use of enteral fish oil to treat PNALD with no change in soybean-based IVFE. Fish oil products are shown in **Table 2-1**.

Case Reports

Six infants with PNALD (diagnosis based on elevated total bilirubin, PN dependence and ruling out other potential causes of liver disease) receiving a combination of PN and EN were supplemented with enteral fish oil and no change in parenteral lipids. All patients received the institutional standard of care treatment for PNALD including the initiation and advancement of EN as tolerated, cyclic PN to provide a PN-free period each day, and ursodiol. Enteral fish oil doses were targeted at 1 g/kg/day based on previously published reports using fish-oil based IVFE, but dosing and product varied among patients due to the discretion of the clinician and doses were rounded to the nearest capsule, so dose ranged from 1 – 6 g/kg/day. Patients were required to have enteral access determined by his/her physician, but no minimum feeding

*Adapted with permission. Tillman EM, Crill CM, Black DD, Hak EB, Lazar LF, Christensen ML, *et al.* Enteral fish oil for treatment of parenteral nutrition-associated liver disease in six infants with short-bowel syndrome. Pharmacotherapy 2011.31(5):503-9.

Table 2-1: Fish oil product comparison.

Product constituents	Lovaza®	Nature Made®	Nature's Bounty®	Omegaven®
Linoleic acid	----	----	10	10 - 70
α-Linolenic acid	----	----	300	< 20
EPA	465	160	180	125 - 282
DHA	375	320	120	144 - 309
Arachidonic acid	----	----	----	10 – 40
Glycerol	----	----	----	250
Egg phospholipid	----	----	----	120
Tocopherol	4	----	1	1.5 - 2.9

---- Signifies that the information is not available from the manufacturer. Amounts given as mg/g of each product. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

was necessary to qualify for initiation of enteral fish oil. Fish oil was administered by withdrawing the oil from the gelatin capsule and administering via mouth, nasogastric (NG), or gastrostomy (G) tube. Fish oil dosing and response to therapy are shown in **Table 2-2.**

Patient 1

A male born at 33 weeks gestation (birth weight 1053 grams) developed necrotizing enterocolitis (NEC) requiring a small bowel resection leaving approximately 45 cm of small bowel and an intact ileocecal valve. At the time of arrival to our institution, the patient had developed PNALD. Postoperatively he was continued on PN and EN was slowly advanced. At nine months of age he was discharged home receiving 65% PN cycled over 18 hours with 35% of calories provided enterally.

By 11 months of age, he was receiving 40% of calories from EN and 60% from PN. Total bilirubin was 7.9 mg/dL, and he was prescribed 1 gram of fish oil four times per day (approximately 1 g/kg/day). Two weeks after starting enteral fish oil, the bilirubin had decreased to 1.4 mg/dL. At the clinic visit four weeks after starting fish oil, his previously notable jaundice had resolved and the total bilirubin had decreased to 0.7 mg/dL. This dramatic improvement in bilirubin and clinical appearance occurred with only a modest 2 mL/hr increase in EN and no change to the PN regimen. Over the next four months, enteral feeding tolerance dramatically improved and by 17 months the patient was off PN and receiving full EN. At a clinic visit three months after initiation of enteral fish oil, it was discovered that his parents were giving him 1 gram of fish oil per day (0.15 g/kg) and not the 4 grams per day originally prescribed. At this time, PNALD had completely reversed and therefore, the dose was not increased.

Table 2-2: Nutrition, treatment and outcome data.

Patient outcomes	1	2	3	4	5	6
Total nutrition PN + EN (kcal/kg/day)	100	120	115	105	115	130
% EN at initiation of fish oil[*]	37%	56%	94%	100%	25%	20%
Dose of fish oil (g/kg/day)	0.15	0.87	1	0.8	0.6	0.6
Dose of fish oil (g/day)	1	3	6	3	3	2
Fish oil product[‡]	1	2	2	2	3	3
Dose of IVFE (g/kg/day)[§]	0.9	0.6	1	1	0.8	0.8
Omega-3 to omega-6 ratio	1:20	1:5	1:5	1:8	1:5	1:5
Total bilirubin at initiation of fish oil (mg/dL)	7.9	7.2	6.2	9.5	8.6	9.6
AST/ALT at initiation of fish oil (units/L)	218/119	132/95	206/169	398/232	233/113	173/110
Weeks of fish oil prior to reversal	2	4	6	N/A	6	N/A
Total bilirubin 6 weeks after fish oil (mg/dL)	0.5	1.5	0.7	5.3	2	77
AST/ALT 6 weeks after initiation of fish oil (units/L)	35/65	120/65	89/65	199/91	109/117	144/88

[*]All patients were receiving continuous feedings with an infant hydrolysate formula. Although some patients were at or near goal EN initially, they had a long history of EN intolerance and poor weight gain that resulted in varying amounts of EN given during the time of enteral fish oil supplementation. [‡]Fish oil products: 1) Nature Made; 2) Nature's Bounty; 3) Lovaza. [§]A soybean oil-based IVFE was used in all patients. PN, parenteral nutrition; EN, enteral nutrition; IVFE, intravenous fat emulsions; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Patient 2

A female born at 31 weeks gestation (birth weight 692 grams) developed a small bowel obstruction that required a small bowel resection and jejunostomy, leaving approximately 35 cm of small bowel with an intact ileocecal valve. Postoperatively she continued on PN and developed PNALD. She underwent reanastamosis at three months of age, and by nine months of age she was still receiving a combination of PN and EN was slowly being advanced. Bilirubin remained elevated and enteral fish oil was initiated at 0.9 g/kg/day, feeding tolerance improved, and PNALD reversed after four weeks. Six weeks after initiation of enteral fish oil, the patient was tolerating full EN and PN was discontinued.

Patient 3

A male born at 30 weeks gestation (birth weight 1425 grams) developed NEC that required a small bowel resection, jejunostomy and mucous fistula, leaving approximately 20 cm of small bowel with an intact ileocecal valve. Postoperatively he was maintained on PN with supplemental EN and developed PNALD. He underwent reanastamosis approximately three months after his small bowel resection and, by six months of age, he was still receiving a combination of PN and EN was slowly being advanced. At six months of age enteral fish oil was initiated at 1 g/kg/day. Bilirubin decreased, enteral feeding tolerance improved and six weeks after initiation of enteral fish oil, PNALD had reversed. Of note, PNALD resolved despite interruptions in EN due to feeding intolerance. He was advanced to full EN and PN was discontinued ten weeks after initiation of enteral fish oil.

Patient 4

A male born at 28 weeks gestation (birth weight 1001 grams) developed NEC requiring ileal resection and jejunostomy placement, leaving approximately 60 cm of small bowel and an intact ileocecal valve. Postoperatively he was maintained on PN and developed PNALD. He underwent reanastamosis and gastrostomy tube placement at three months of age. He continued on PN while EN was slowly being advanced. At six months of age, the patient had progressive PNALD with a bilirubin of 9.5 mg/dL and international normalized ratio (INR) of 1.3. Enteral fish oil was initiated at 1 g/kg/day, and within four weeks the bilirubin decreased to 4.6 mg/dL. A gastrointestinal tract bleed developed and he was made NPO, requiring discontinuation of EN and enteral fish oil, and treated with parenteral vitamin K. Bilirubin, transaminases, and INR subsequently increased and platelet count decreased over the next several weeks. Blood cultures were positive for *E. coli* and Gram-positive cocci, and clinical status declined, requiring transfer to the intensive care unit where his bleeding was managed with vitamin K, platelets, and fresh frozen plasma. Overwhelming sepsis and hepatic failure ultimately led to the patient's death.

Patient 5

A female born at 27 weeks gestation (birth weight 626 grams) developed NEC at one week of age requiring a small bowel resection and jejunostomy placement, leaving approximately 32 cm of small bowel with an intact ileocecal valve and three ostomies. Postoperatively she was maintained on PN with slow advancement of EN and developed PNALD. She underwent reanastamosis approximately three months after her small bowel resection and by eight months of age, she was still receiving a combination of PN and EN was slowly being advanced. At eight months of age enteral fish oil was initiated at 0.6 g/kg/day. Bilirubin decreased, enteral feeding tolerance improved and six weeks after initiation of enteral fish oil, PNALD had reversed. She was advanced to full EN and PN was discontinued nine weeks after initiation of enteral fish oil.

Patient 6

A female born at 26 weeks gestation (birth weight 600 grams) developed NEC with multiple perforations requiring extensive resections including the loss of the ileocecal valve. A jejunostomy was performed, leaving approximately eight centimeters of small bowel that was reanastamosed three months later. Postoperatively she was maintained on PN and trophic EN and developed PNALD. At six months of age, she was receiving PN with minimal EN (2 mL/hr continuous feedings) and bilirubin remained increased at 9.6 mg/dL. Enteral fish oil was started (0.6 g/kg/day) and bilirubin decreased to 7 mg/dL; however, she continued to experience feeding intolerance. After six weeks of enteral fish oil, and bilirubin was 7 mg/dL. She developed bloody stools, and EN and fish oil were stopped for two weeks. Her INR had not changed from values prior to starting enteral fish oil. During the two weeks that she was NPO, her bilirubin increased to 9.4 mg/dL. When EN and fish oil were restarted bilirubin decreased. Six months after initiation of enteral fish oil, the patient was still on PN with supplemental EN and bilirubin was approximately 7 mg/dL. She was discharged on a combination of PN, EN, and enteral fish oil. This patient had multiple readmissions to the hospital for central venous line infections and there have been several occasions where enteral fish oil was temporarily discontinued. Each time enteral fish oil was stopped, bilirubin increased, and then it decreased upon re-initiation of enteral fish oil. The patient is now three years old and is currently on the small bowel and liver transplant list.

Discussion

Notable improvement in PNALD and EN advancement was associated with initiation of enteral fish oil in four of the six patients. EN is thought to be the most important factor in the prevention and treatment of PNALD. Horslen *et al.* reported improved enteral tolerance in infants with short bowel syndrome and end stage liver disease after liver transplantation (77). This suggests that the resolution of liver disease, whether it occurs via transplantation or medical management, may have a positive impact on enteral tolerance. In contrast, Javid *et al.* retrospectively evaluated 12 infants with

PNALD and observed reversal of PNALD in only two patients on PN while enteral feeds were advancing. PNALD did not reverse in the other 10 patients until approximately four months after PN was discontinued and patients were receiving full EN (78). In our patients, it is not clear if the addition of enteral fish oil is entirely responsible for the dramatic improvement in PNALD and EN tolerance, or if the reversal of PNALD may have been attributed to increased EN.

Improvement of liver function was first described in case reports after administration of fish oil-based IVFE (28, 29). These initial reports were followed by safety and efficacy data for 18 patients treated with fish oil based-IVFE compared to 21 historical control patients who received standard soybean oil-based IVFE. The time to reversal of cholestasis was 9.4 weeks in the fish oil group compared to 44 weeks in the control group (32). Although these initial findings are interesting, this study is limited by the small sample size and a control group that is from an observation period 10 – 20 years prior to the treatment group. Also, the dose of lipids was significantly decreased in the treatment group. In addition, these investigators published results of an open-label study of 42 infants treated with fish oil-based IVFE compared to a more contemporary historical cohort of 49 patients (33). The median time to discontinuation of PN was four weeks in the fish oil group compared to 20 weeks in the control group. PNALD resolved while patients were still receiving PN in 19 patients in the fish oil group but only two patients in the control group (33).

A study from Toronto recently reported the reversal of PNALD in nine of 12 infants with SBS treated with a combination of a soybean oil-based IVFE and fish oil-based IVFE (1 g/kg/day soybean oil-base IVFE and 1 g/kg/day fish oil-based IVFE). Four patients had reversal of PNALD while receiving a combination of soybean-based IVFE and fish oil-based IVFE, whereas the other five patients did not have reversal of disease until after soybean-based IVFE was discontinued (38).

Italian clinicians reported success with fish oil-based IVFE in a case report of an infant with severe PNALD (34). The patient was started at 0.2 g/kg/day of fish oil-based IVFE and advanced by 0.2 g/kg/day to achieve a dose of 1.5 g/kg/day, which is higher than has been previously reported (34). Chinese clinicians report the reversal of PNALD in three of four infants with intestinal failure treated with fish oil-based IVFE (35). In these patients, standard soybean oil-base IVFE was discontinued and fish oil-based IVFE was initiated at 1 g/kg/day (35). Calhoun and Sullivan report resolution of PNALD in a 17-month-old infant with SBS after treatment with fish oil-based IVFE (36). Chung *et al.* reported successful treatment of PNALD with fish oil-based IVFE in four patients (37).

Rollins *et al.* retrospectively evaluated 26 patients with SBS and PNALD receiving soybean-based IVFE 2 – 3 g/kg/day. Ten patients had complete resolution of PNALD with advancement of EN, one patient with PNALD remained on 2 g/kg/day of soybean-based IVFE, three patients required liver transplant, three patients died, and six patients had complete resolution of PNALD with the removal of soybean-based IVFE. Four of these six patient also received enteral fish oil (46). This suggests that removal of soybean-based IVFE alone may be beneficial in the treatment of PNALD.

The optimal dosing of enteral fish oil has not been elucidated. Reports of fish oil-based IVFE have utilized doses ranging from 1 – 1.5 g/kg/day as the exclusive source of fats (29, 32-38). In this case series, the dramatic improvement of PNALD observed in patient 1 with a dose of 0.15 g/kg/day suggests that doses much lower than what has been previously thought may provide benefit.

We report bleeding in the two patients that did not have reversal of PNALD. Although bleeding is a possible adverse effect of fish oil, because omega-3 long-chain fatty acids competitively inhibit cyclooxygenase, thereby decreasing synthesis of thromboxane A_2 from arachidonic acid leading to platelet aggregation, bleeding has not been significant in previous reports (28, 29, 32-38). Additionally, bleeding is often seen with the progression of liver disease; it is unclear if the bleeding these patients experienced was due to the enteral fish oil supplementation or the progression of liver disease. When the bleeding events occurred in our patients, the INR values were not greater than reported prior to starting enteral fish oil, suggesting that bleeding was not related to the enteral fish oil. Furthermore, evaluations of safety of fish oil in the adult cardiology population by Bays and Harris have both concluded that fish oil does not increase bleeding risk (57, 58).

These cases have several variables that could have influenced time to reversal of PNALD independently of the administration of enteral fish oil. The literature reports a 27% fatality rate and 11% transplant rate in historical controls receiving soybean-based IVFE (32, 33). In our six patients, only one patient died and no patients have yet required transplant. This case series report was retrospective, therefore laboratory evaluations were not conducted under a specific protocol and serial direct bilirubin concentrations were not available for all patients. Patients were outside of the period of neonatal jaundice; therefore, the total bilirubin should be highly reflective of the direct bilirubin.

Because these patients all had SBS and questionable enteral absorption, fatty acid profiles are needed to confirm absorption. These observations are not part of routine care and therefore were not done. It is also possible that systemic absorption is not necessary to see some beneficial effect, but that the local effects of fish oil on the gastrointestinal mucosa and liver may play a role in the attenuation of inflammation, thereby attenuating hepatocellular damage associated with PNALD. Alwayn *et al.* report the use of both enteral and parenteral fish oil administration in a mouse model of nonalcoholic fatty liver disease (59). Animals were randomized to receive standard chow or an enteral fat-free, high carbohydrate diet identical to PN, with and without fish oil. Mice fed standard chow had the lowest hepatic fat content and served as controls. Hepatic fat content was the highest in mice fed the liquid high carbohydrate diet, and was significantly decreased by both enteral and parenteral fish, but the lowest hepatic fat content was observed in mice receiving enteral fish oil (59).

PNALD remains a potentially devastating disease with limited treatment options. Enteral fish oil is not a treatment option for all patients with PNALD, but for those patients with some enteral access and adequate intestinal surface area for absorption,

enteral fish oil supplementation may be a safe and effective adjunctive treatment. These initial cases have prompted us to design a multicenter, randomized, controlled prospective study to evaluate the use of enteral fish oil for the treatment of PNALD.

CHAPTER 3. EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID SYNERGISTICALLY ATTENUATE BILE ACID-INDUCED HEPATOCELLULAR APOPTOSIS*

Introduction

Parenteral nutrition (PN)-associated liver disease (PNALD) is a common complication in infants receiving long-term PN. PNALD can progress from cholestasis to liver fibrosis, hepatic failure and death (1, 8). The etiology of PNALD is not well understood; it is likely multi-factorial and possibly attributed to immature bile secretion, inflammation, oxidative stress, infection, nutrient deficiencies, and toxic components in parenteral products. including lipids or amino acids (1, 24).

Lipophilic bile acids, which are often increased in PNALD, are known to cause cellular apoptosis. Many lipophilic bile acids have been shown to induce apoptosis in both cellular and animal models (79-85). Apoptosis occurs by activation of death receptors (DR) located on the cell surface. There are six known death receptors, but the two that have been shown to be important in apoptosis in the liver are Fas and tumor necrosis factor-associated apoptosis-inducing ligand receptor 2 (TRAIL-R2) (86). Apoptosis occurs via different pathways depending on cell type. In type 1 cellular response, effector caspases (caspase-3, -6, -7) are activated by the cleavage of upstream caspases (79). In the type 2 cellular response, which occurs in hepatocytes, caspase activation requires mitochondrial pathway amplification (79). Bile acid-induced apoptosis is thought to occur via the Fas and TRAIL-R2 death receptors (86).

Hydrophilic bile acids, primarily ursodeoxycholic acid, have been shown to protect hepatocytes from apoptosis by activating survival pathways (87) and have been used for the treatment of PNALD (88). Recent clinical research has shown reversal of PNALD with fish oil-based intravenous fat emulsions rich in omega-3 long-chain polyunsaturated fatty acid (ω3PUFA) (28, 29, 32, 89). Although, ω3PUFA supplementation has shown much promise in the treatment of PNALD, the mechanism of action is not fully understood. The aims of this study were to determine (1) if ω3PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) attenuate hepatocyte apoptosis associated with exposure to chenodeoxycholic acid (CDCA), a potent lipophilic bile acid; (2) if this attenuation is due to the down regulation of the Fas and TRAIL-R2 receptors, thereby reducing cellular apoptosis; and (3) if EPA and DHA equally attenuate apoptosis associated with CDCA toxicity.

*Adapted with permission. Tillman EM, Helms RA, Black DD. Eicosapentaenoic acid and docosahexaenoic acid synergistically attenuate bile acid-induced hepatocellular apoptosis. JPEN J Parenter Enteral Nutr 2012. 36(1):36-42.

Materials and Methods

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in EMEM supplemented with 10% FBS, 50 U/ml penicillin, and 37.5 U/ml streptomycin (growth medium). Cells were incubated at 37° C with 5% CO_2 in a humidified incubator. Passages 25 – 45 were used for these experiments.

Experimental design

HepG2 cells were plated and grown to 95% confluence for all experiments, except for fluorescent cell staining, where cells where grown to 50% confluence. Cells were treated with CDCA 50 – 200 mM (Sigma-Aldrich, St. Louis, MO), ± EPA 1 - 10 μM (Nu-check Prep, Elysian, MN), and ± DHA 10 μM (Nu-check Prep, Elysian, MN). HepG2 cells were treated for 4, 8, 12, 18 and 24 hours for cell viability and caspase assays. Cells were treated for 0.5, 1, 1.5, 2 and 4 hour for mRNA analysis by quantitative real time RT-PCR.

Cell viability

Cell viability was evaluated after treating cells, as described above, followed by trypsinization in order to disperse cells into a 0.2% trypan blue (Sigma-Aldrich) cell suspension mixture. After staining, 10 μL of cell suspension was placed on a hemocytometer with a glass cover slip and evaluated using an inverted microscope (25x magnification). All cells were counted and viability was accessed.

Nuclear staining

Cells were grown on glass inserts and, once 50% confluence was achieved, cells were treated with CDCA ± EPA for 2 hours. Immediately following treatment, cells were washed with phosphate buffered saline (PBS), fixed with methanol (-10° C) for 15 minutes, then washed with PBS. Fixed cells were then stained for 5 minutes with ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) (EB/AO) dye mixture (Sigma-Aldrich) in PBS. Following staining, cells underwent 5 washes prior to being mounted on glass slides using Vectashield® mounting medium and analyzed using an Olympus America Inc., IX50-FLA inverted reflected light fluorescence microscope (Lake Success, NY).

Caspase assay

Apoptosis was evaluated using the Apo-ONE® Homogeneous Caspase-3/7 Assay purchased from Promega Corporation (Madison, WI) and performed according to the manufacturer's instructions. HepG2 cells were treated for 4, 8, 12, 18 or 24 hours followed by the addition of caspase-3/7 reagent and incubation for 4 hours in the dark on a rocking shaker at low speed. Results were read at fluorescein 485 nm/535 nm with a Victor 2, Perkin-Elmer Wallace 1420 multilabel counter (Shelton, CT).

Quantitative RT-PCR

Total RNA was isolated from confluent cultures according to the manufacturer's instructions with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized from 2 µg of total RNA in a 21 µL reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) in accordance with the manufacturer's instructions. Quantitative PCR was performed in triplicate using the Model 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Specific primers were designed for the genes of interest (Fas and TRAIL-R2). See **Table 3-1** for primer sequences. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the 7500 Sequence Detection System. The change in fluorescence of SYBR Green in every cycle was monitored by the system software, and the threshold cycle (C_T) above the background for each reaction was calculated. The C_T value of 18S rRNA was subtracted from that of the gene of interest to obtain a ΔC_T value. The ΔC_T value of an arbitrary calibrator (e.g., sample treated with ethanol as a vehicle control) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The mRNA expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$.

Table 3-1: **Cell viability demonstrated by trypan blue staining.**

Treatment conditions	Total number of cells per surface area	Viable cells per surface area	% Viable cells
Control	161	160	99
EPA 10 µM	160	159	99
CDCA 200 µM	99	77	78
CDCA 200 µM + EPA 10 µM	105	105	100

Statistical analysis

All data represent at least three separate and independent experiments. Data are provided as mean ± SEM. A one-way ANOVA was used to compare differences between groups and a *post-hoc* Tukey's HSD (honestly significant difference) test was used to correct for multiple comparisons. A single-tailed *P* value of 0.05 was used to reject the null hypothesis.

Results

Cell viability

After a 2-hour treatment with CDCA, 78% of cells were viable, whereas 100% of cells were viable after treatment with CDCA and EPA (**Table 3-1**). Although cell viability was 100% in cells treated with CDCA and EPA, the total cell count was only 75% of cells exposed to medium alone. Cells treated with EPA alone resulted in similar viability with control and vehicle control, whereas cells treated with CDCA alone had a decrease in both viability and total number of dead cells. When cells were treated with CDCA+EPA the cell number was similar, but there were no dead cells.

Nuclear staining

Cells treated with EPA alone appear similar to control cells, with all green viable cells (**Figure 3-1**). When cells were treated with CDCA alone, not only is there free chromatin, which is stained orange, but cellular integrity is lost. Cells treated with CDCA+EPA exhibited some early stages of apoptosis, as shown by the orange staining, but cellular integrity was maintained.

Caspase 3/7 activity

Caspase activity was evaluated at 4, 8, 12, 18 and 24 hours (**Figure 3-2**). Activity peaked between 12 and 18 hours, therefore, a 12-hour time point was chosen for data reporting. A dose-dependent apoptotic effect of CDCA was observed across all time points, and a significant attenuation effect was observed with 1 and 10 μM EPA with treatment with 200 μM CDCA (**Figure 3-3**). Peak caspase-3/7 activity was observed with CDCA 200 μM. Treatment with EPA alone and DHA alone resulted in 22% and 9% caspase-3/7 attenuation, respectively (**Figure 3-4**). Caspase-3/7 activity was attenuated by 52 % when treated with a combination of EPA and DHA ($p = 0.0034$; **Figure 3-4**).

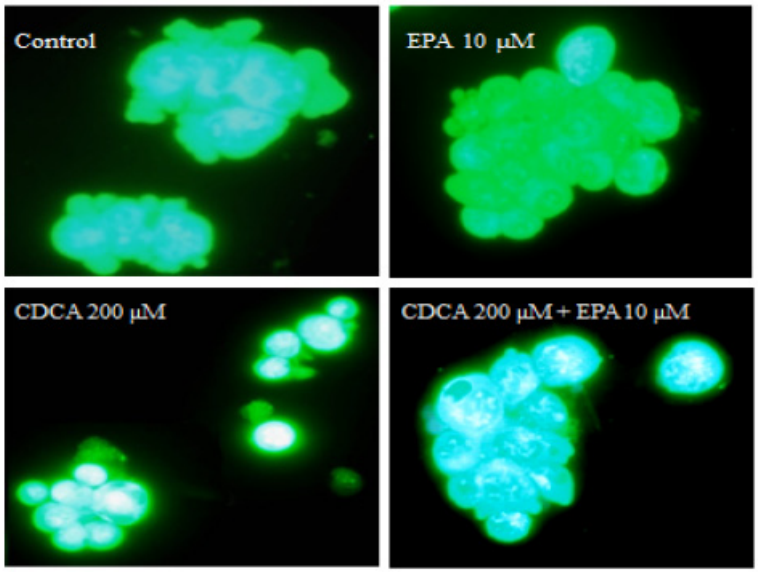

Figure 3-1: EB/AO staining and fluorescent microscopy.
Cells stained were stained with ethidium bromide and acridine orange and viewed with fluorescent microscopy (1400x magnification).

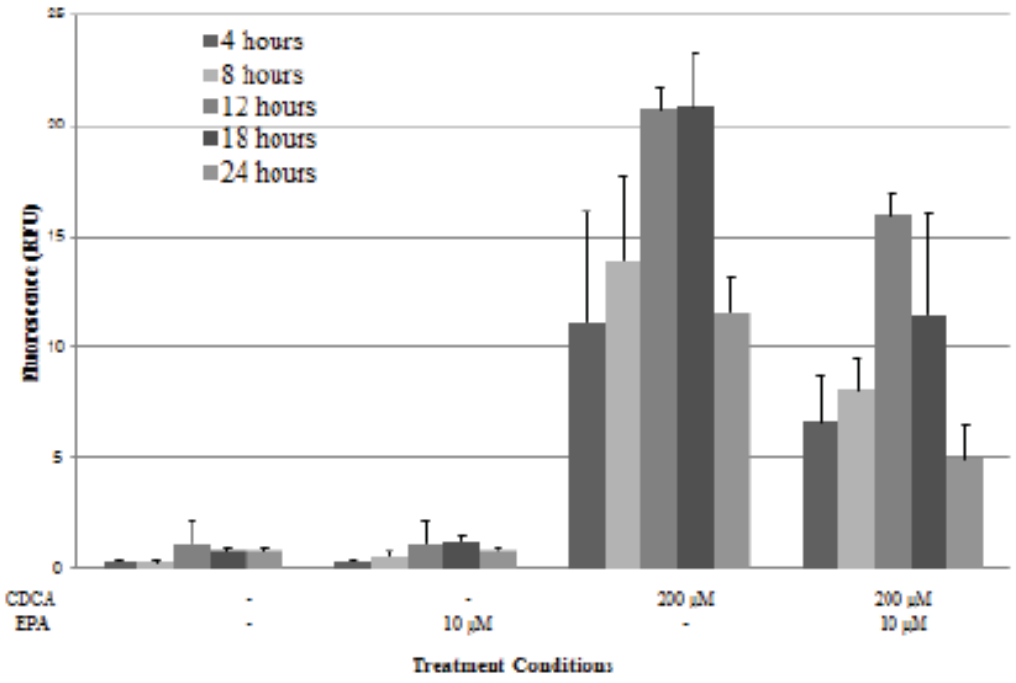

Figure 3-2: Time course of caspase-3/7 activity.
Data are represented as relative fluorescence units above control. Each bar represents mean ± SEM for three independent experiments. Statistical significance was determined using analysis of variance (ANOVA) with Tukey's HSD.

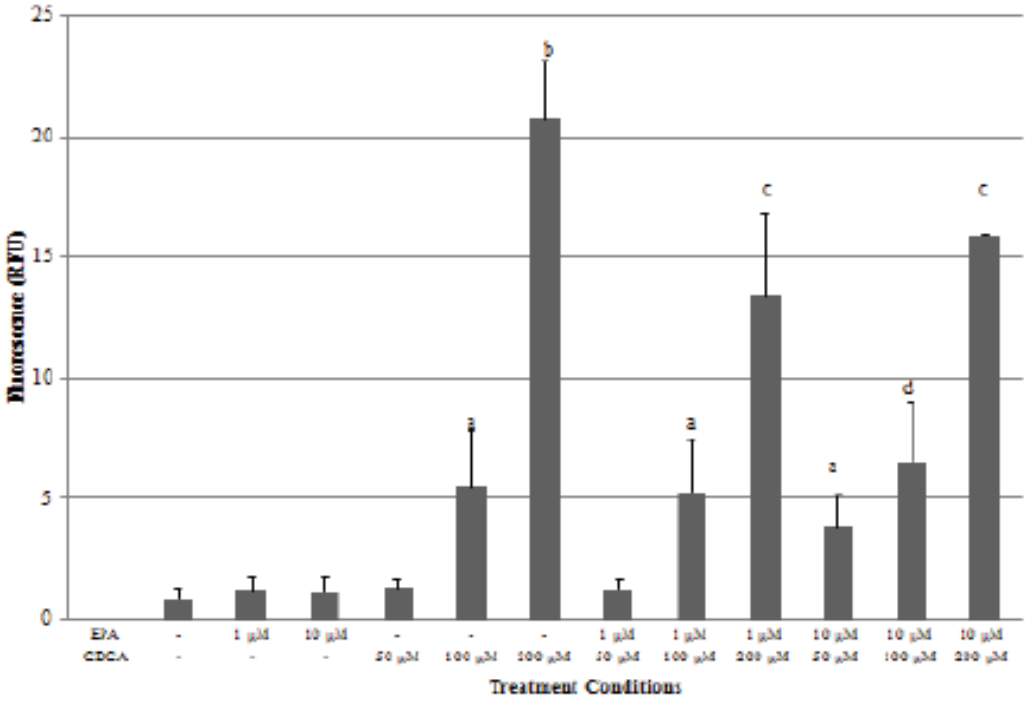

Figure 3-3: Caspase-3/7 activity – Dose response at 12 hours.
Data are represented by relative fluorescence units. Each bar represents mean ± SEM for three independent experiments. Statistical significance was determined using analysis of variance (ANOVA) with Tukey's HSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other.

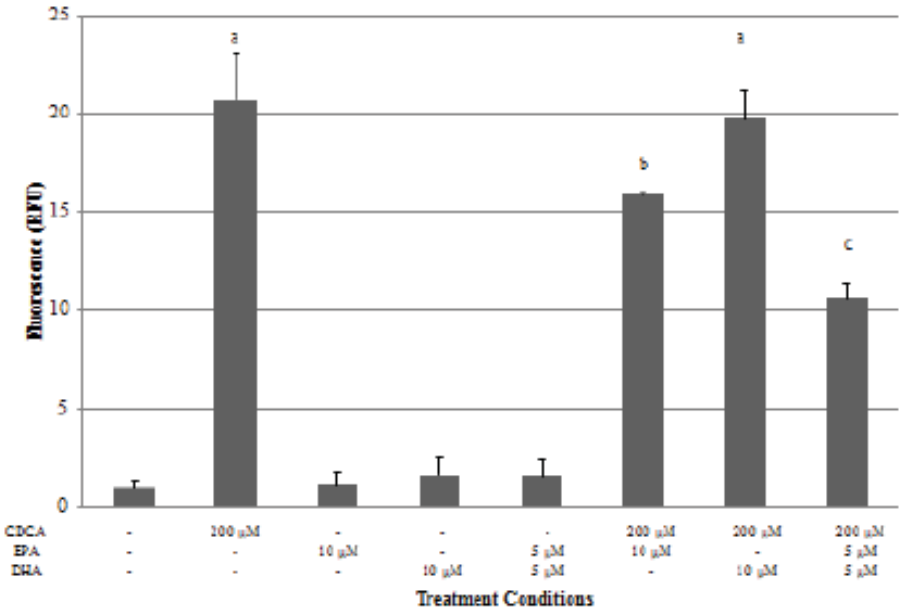

Figure 3-4: Caspase-3/7 activity – EPA and DHA synergy.
Data are represented based on relative fluorescence units above control. Each bar represents mean ± SEM for three independent experiments. Statistical significance was determined using an ANOVA with Tukey's HSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Fas and TRAIL-R2 mRNA levels

Peak Fas and TRAIL-R2 mRNA expression was observed at 0.5 hours (**Figures 3-5** and **3-6**). There was a 4.7-fold increase in Fas mRNA levels when cells were incubated with 200 μM CDCA alone, as compared to no increase in Fas mRNA levels when incubated with CDCA 200 μM with the addition of EPA 1 μM, EPA 10 μM, or 5 μM EPA + 5 μM DHA ($p < 0.01$; **Figure 3-5**). There was a 2-fold increase in TRAIL-R2 mRNA levels when cells were incubated with CDCA 100 μM and 200 μM alone, as compared to no increase in TRAIL-R2 mRNA levels when incubated with both CDCA and EPA 1 μM, EPA 10 μM, or 5 μM EPA + 5 μM DHA ($p < 0.01$; **Figure 3-6**). No significant difference between treatment with EPA alone, DHA alone, or combination of EPA/DHA was observed in Fas or TRAIL-R2 mRNA expression. Interestingly, EPA and DHA separately appeared to induce a low level of both Fas and TRAIL-R2 mRNA when no CDCA was present. However, this effect was not observed with the combination of the two fatty acids with or without CDCA (**Figures 3-5** and **3-6**).

Fas and TRAIL-R2 mRNA levels were measured by quantitative RT-PCR. Values are based on fold change relative to the vehicle control. Statistical significance was determined using an ANOVA with Tukey's HSD. Each bar represents mean $\pm$ SEM for data from three culture wells. Statistical significance was determined using an ANOVA with Tukey's HSD. Means of data from various treatment conditions with different letters above the bars are significantly different from each other at $p < 0.05$ by ANOVA. Those sharing the same letter or no letter are not significantly different from each other.

Discussion

The results from this study show for the first time that the ω3PUFA EPA and DHA significantly attenuate CDCA-induced apoptosis in cultured hepatocytes. The results demonstrate: 1) CDCA dose-dependent induction of apoptosis documented by caspase 3/7 activity and Fas and TRAIL-R2 mRNA expression; 2) attenuation of CDCA-induced apoptosis via caspase 3/7 by EPA; 3) synergistic attenuation of CDCA-induced apoptosis by the combination of EPA and DHA as documented by caspase-3/7 activity, but not Fas or TRAIL-R2 mRNA expression.

HepG2 cells treated with CDCA for 0.5 hour exhibited increased expression of both Fas and TRAIL-R2 mRNA in a dose-dependent manner. This up-regulation of expression of Fas and TRAIL-R2 mRNA with CDCA treatment was attenuated when cells were treated with EPA, DHA, or a combination of both EPA and DHA. Lipophilic bile acids such as deoxycholic acid (DCA), CDCA, and the glycine-conjugated form of deoxycholic acid (GDCA) have been previously shown to up-regulate expression of Fas and TRAIL-R2 in liver cells (79, 86, 90). Merino *et al.* (91) suggested that TRAIL-R3 and TRAIL-R4 inhibit apoptosis by acting as decoy receptors that bind TRAIL, but do not trigger activation of initiator caspases to lead to apoptosis.(91) These receptors are

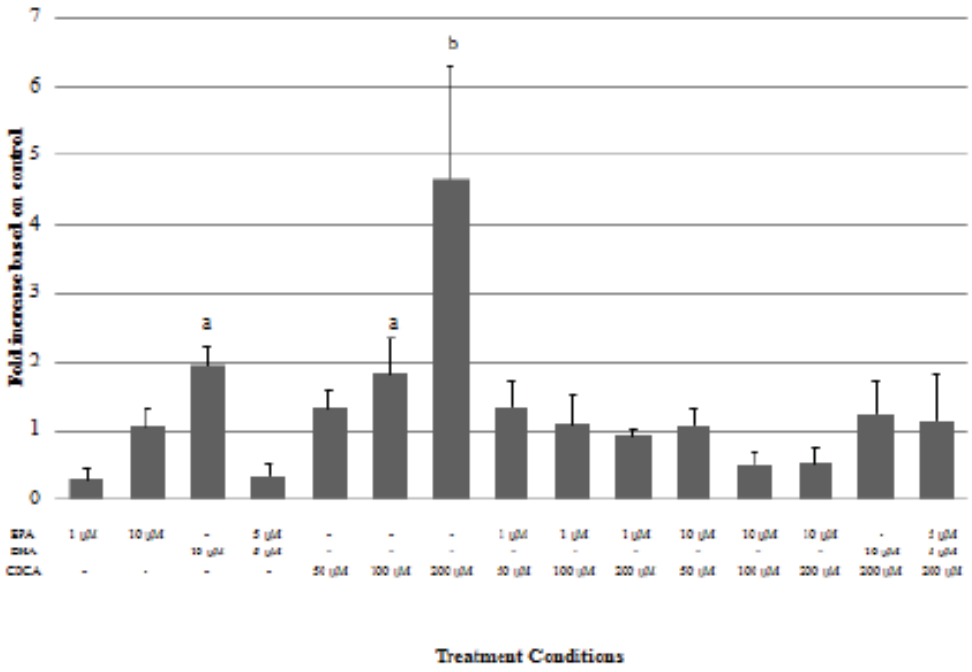

Figure 3-5: Fas mRNA levels.

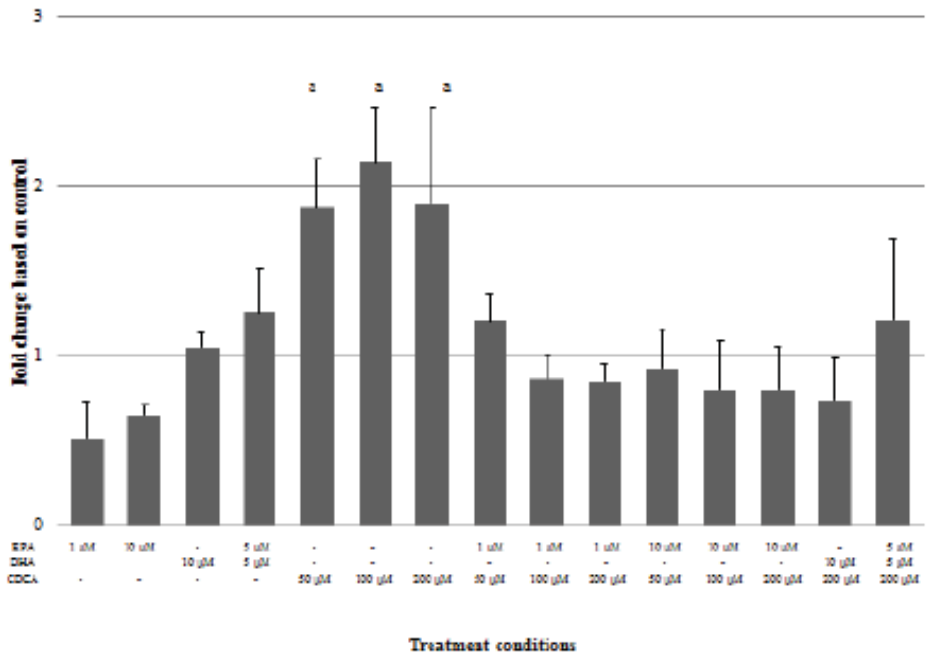

Figure 3-6: TRAIL-R2 mRNA levels.

found in the cell membrane within lipid rafts. The fluidity of ω3PUFA and the rigidity of cholesterol can result in incompatibility and can greatly alter the composition of lipid rafts (92, 93). Treatment with ω3PUFA may attenuate Fas- and TRAIL-R2-induced apoptosis by incorporation of ω3PUFA into membranes, causing fluidity or other alterations in lipid rafts and resulting in changes in death receptor function. However, such a mechanism involving direct modulation of protein function would not account for the pre-translational regulation of Fas and TRAIL-R2 mRNA expression that we observed.

It is established that composition and levels of free fatty acids (FFA) can significantly alter cell survival pathways. Free fatty acids have been shown to inhibit serum-starved apoptosis in a murine cell model via GPR120 (94). In these experiments both saturated and unsaturated FFA enhanced cell survival, but linolenic acid, palmitoleic acid, and DHA had the most potent effects on cell survival (95). GPR120 has recently been shown to be a receptor or sensor for ω3PUFA, mediating regulation of inflammation (96). GPR 120 inhibits both Toll-like receptors (TLR) 2/3/4 and the TNF-α cascade, suggesting that IKKβ and JNK may be iatrical transcription factors affected by GPR120 binding (96). NFκB, IKKβ, and JNK have all been shown to play a role in the up-regulation of death receptors in the presence of noxious stimuli (79, 97). The up-regulation of Fas and TRAIL-R2 mRNA by CDCA and the attenuation of this increase by ω3PUFA that we observed may also be independent of the aforementioned transcription factors and may result from regulation of mRNA stability. Nakata *et al.* (98) have previously demonstrated up-regulation of TRAIL-R2 mRNA independent of transcription factors when treated human cancer cells were treated with 15-deoxy-delta(12,14)-prostaglandin J(2), and apoptosis was attenuated in the presence of a double-stranded small interfering RNA (siRNA) targeting the DR5 gene. Small interfering RNA duplexes, as well as micro-RNAs, have been studied for their roles in interference with expression of Fas, TRAIL-R2, and other pro-apoptotic and anti-apoptotic genes (99-101).

We observed a synergistic attenuation of CDCA-induced apoptosis via caspase-3/7 with a combination of EPA and DHA, yet Fas and TRAIL-R2 mRNA expression was not significantly different from that observed with EPA alone, DHA alone, or a 1:1 molar ratio of EPA to DHA. This discrepancy may suggest that DHA and EPA are involved in multiple pathways of apoptosis attenuation. This is not surprising because EPA and DHA are structurally different and have been shown to have varying effects on cellular functions. For example, the additional double bond in DHA compared to EPA creates an increased number of bends in the acyl chain that cause shortening. Length and saturation of hydrocarbon chains can greatly affect mitochondrial membrane permeability (102). As discussed previously, hepatocyte apoptosis is mediated via the mitochondria, and changes in mitochondrial membrane permeability could influence hepatocellular apoptosis. The EPA and DHA synergy in attenuation of CDCA-induced apoptosis that we observed may represent the end result of ω3PUFA functioning via multiple mechanisms, including alteration of cell membrane fluidity, receptor binding, transcriptional regulation, and non-transcriptional regulation of RNA levels, resulting in the anti-apoptotic effects. To our knowledge, there are no published studies illustrating a synergistic attenuation of apoptosis by the combination of EPA and DHA.

Although we have shown differences in both Fas and TRAIL-R2 expression in HepG2 cells treated with CDCA in the presence and absence of EPA, there may also be other mechanisms that are responsible for some of the protective effects of ω3PUFA in the face of bile acid-induced cell injury. Additional studies are underway in our laboratory to clearly define the precise mechanism(s) of action of ω3PUFA in reversing bile acid-induced cell injury. Collectively, our findings clearly demonstrate the attenuation of CDCA-induced hepatocellular apoptosis by ω3PUFA. With CDCA-induced apoptosis serving as a model for cholestatic liver disease, these results support the therapeutic use of fish oil rich in ω3PUFA for the treatment of PNALD.

CHAPTER 4. ATTENUATION OF BILE ACID-INDUCED HEPATOCELLULAR APOPTOSIS BY OMEGA-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS VIA DEATH RECEPTORS

Introduction

Parenteral nutrition (PN)-associated liver disease (PNALD) occurs in patients receiving long-term PN and may progress from cholestasis to liver cirrhosis, hepatic failure and death. Human studies have shown omega-3 long chain polyunsaturated fatty acids (ω3PUFA) to be beneficial in attenuating PNALD (103). Although ω3PUFAs seem to be effective in attenuating disease, the mechanism is unknown.

Lipophilic bile acids, which are often increased in cholestasis associated with PNALD, are known to cause cellular injury and may trigger pro-inflammatory cytokine release by both hepatocytes and Kupffer cells. These cytokines are potent inhibitors of hepatobiliary transporter gene expression, which may be responsible for impaired bile secretion, resulting in conjugated hyperbilirubinemia and cholestasis, leading to accumulation of hepatotoxic bile acids (104, 105). Lipophilic bile acids, such as chenodeoxycholic acid (CDCA), have been shown to induce apoptosis in both cellular and animal models (79-85). Apoptosis occurs by activation of death receptors (DR) located on the cell surface. There are six known death receptors, but the two that have been shown to be most important in apoptosis in the liver are Fas and tumor necrosis factor-associated apoptosis-inducing ligand receptor 2 (TRAIL-R2) (86). Apoptosis occurs via different pathways depending on cell type. In type 1 cellular response, effector caspases (caspase-3,6,7) are activated by the cleavage of upstream caspases (79). In type 2 cellular response, which occurs in hepatocytes, caspase activation requires mitochondrial pathway amplification (79). Bile acid-induced apoptosis is thought to occur predominantly via the Fas and TRAIL-R2 death receptors (86), although bile acids may also act directly on the mitochondria and increase permeability, thereby increasing the release of cytochrome *c* (106). In our *in vitro* model of PNALD, we previously reported that apoptosis was associated with increased mRNA expression of death receptors Fas and TRAIL-R2 (107). The aims of this study were to: 1) determine whether hepatocellular apoptosis induced by the hydrophobic bile acid CDCA occurs solely via the death receptors Fas and TRAIL-R2 in an *in vitro* model; 2) determine specific apoptotic pathways involved in the attenuation of PNALD by ω3PUFA in an *in vitro* model; and 3) determine if Fas and TRAIL-R2 are involved in the attenuation of PNALD by ω3PUFA in an *in vivo* model.

Materials and Methods

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 50 U/ml penicillin, and 37.5 U/ml streptomycin (growth medium). Cells were incubated at 37° C with 5% CO_2 in a humidified incubator. Passages 10 – 32 were used for these experiments.

Experimental design for cell culture studies

HepG2 cells were plated and grown to 95% confluence for all experiments. Cells were treated with CDCA 200 mM (Sigma-Aldrich, St. Louis, MO), ± EPA 5 μM (Nu-check Prep, Elysian, MN), and ± DHA 5 μM (Nu-check Prep). Controls included cells incubated with vehicle alone (EtOH). HepG2 cells were treated for 12 hours for caspase assays. Cells were treated for 0.5 hour for mRNA analysis by quantitative real time RT-PCR. Time points were determined by previous studies (107).

Apoptosis array and quantitative RT-PCR

Total RNA was isolated from confluent cultures according to the manufacturer's instructions with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized from 2 μg of total RNA in a 21 μL reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Quantitative PCR was performed in triplicate using the Model 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems). Eighty-four key genes involved in apoptosis were evaluated with The Human Apoptosis RT² Profiler PCR Array profiles (SABiosciences, Qiagen, Valencia, CA) using quantitative real-time RT-PCR.

Antibody treatment

Cells were treated with 200 μM CDCA, CDCA + Fas antibody 300 ng/mL (Abcam, Cambridge, MA), CDCA + TRAIL-R2 antibody 300 ng/mL (Abcam), and CDCA + Fas antibody + TRAIL-R2 antibody. Controls included cells incubated with vehicle alone (EtOH). Apoptosis was evaluated using the Apo-ONE® Homogeneous Caspase-3/7 Assay.

Caspase assay

Cultured HepG2 cells were treated with 200 μM CDCA, CDCA + Fas antibody, CDCA + TRAIL-R2 antibody, and CDCA + Fas antibody + TRAIL-R2 antibody. Apoptosis was evaluated using the Apo-ONE® Homogeneous Caspase-3/7 Assay purchased from Promega Corporation (Madison, WI) and performed according to the manufacturer's instructions. HepG2 cells were treated for 12 hours followed by the addition of caspase-3/7 reagent and incubation for 4 hours in the dark on a rocking shaker at low speed. Results were read at fluorescein 485nm/535nm with a Victor 2, Perkin-Elmer Wallace 1420 multilabel counter (Shelton, CT).

Murine model

C57BL/6 wild-type adult male mice (8-10 weeks old; The Jackson Laboratory, Bar Harbor, ME) were exposed *ad libitum* to 2.5% dextran sulfate sodium (DSS) in drinking water for 4 days. Mice then received regular drinking water for 24 hours before placement of a central venous catheter (CVC; Silastic tubing, 0.012 inches internal diameter; Dow Corning) into the right jugular vein. The proximal end of the CVC was tunneled subcutaneously, exited between the shoulder blades, and connected to an infusion pump (Harvard Apparatus, Holliston, MA). Mice were placed in a rubber harness (Instech Laboratories, Plymouth Meeting, PA) and recovered from surgery for 24 hours with intravenous normal saline (NS) infusion at a rate of 0.23 mL/hour and given *ad libitum* access to chow and water. After 24 hours, mice were continuously infused for 7 or 28 days with PN (PN7d/DSS and PN28d/DSS, respectively) at a rate of 0.29 mL/hour, providing a caloric intake of 8.4 kcal/24 hours, as previously described (108). All PN-infused mice had access to water *ad libitum* but not to chow. All mice were individually housed in metabolic cages. Blood was collected from the retro-orbital plexus. Serum was analyzed by the University of Colorado Hospital Clinical Chemistry Laboratory for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin levels. Total bile acids (TBA) were analyzed using a total bile acid detection kit (Diazyme Laboratories, Poway, CA). All animals were treated humanely, and all animal procedures were approved by the Institutional Animal Care and Use Committee.

Tissue RNA isolation and quantitative gene expression analysis

RNA was extracted using TRIzol (Invitrogen), DNAse-treated (Ambion, Austin, TX) and reverse-transcribed with iScript (BioRad, Hercules, CA). Gene expression was analyzed by qRT-PCR on an Applied Biosystems 7300 cycler using commercially available TaqMan gene expression assays (Applied Biosystems). Data are expressed as normalized gene expression relative to chow mice or NS/DSS-treated mice using the Δ/Δ Ct method. RNA was extracted, prepared, and analyzed from whole liver tissue as previously described (108).

Statistical analysis

All data represent at least three separate and independent experiments. Data are provided as mean ± SEM. A one-way ANOVA was used to compare differences between groups and a *post-hoc* Tukey's HSD test was used to correct for multiple comparisons. A single-tailed p value of 0.05 was used to reject the null hypothesis.

Results

Apoptosis focused array

There was a 286-fold increase in Fas ligand (FasL) mRNA levels when cells were incubated with CDCA alone, as compared to 9.8-fold increase in FasL mRNA levels when incubated with CDCA with the addition of ω3PUFA ($p < 0.01$). There was a 1.4-fold increase in Fas with CDCA alone, and a 2.0-fold decrease when treated with CDCA+ ω3PUFA. There was a 38-fold increase in FADD mRNA levels when cells were incubated with CDCA alone, as compared to a 3.8-fold increase in FADD mRNA levels when incubated with both CDCA+ ω3PUFA ($p < 0.01$). There was a 142-fold increase in caspase 8 (CASP8) mRNA levels when cells were incubated with CDCA alone, as compared to a 44.8-fold increase in CASP8 mRNA levels when incubated with CDCA+ ω3PUFA ($p < 0.01$). Interesting, CASP8- and FADD-like apoptosis regulator (CFLAR) was increased 1.5-fold when cells were incubated with CDCA alone, as compared to a 2.4-fold increase in CFLAR mRNA levels when incubated with CDCA+ ω3PUFA ($p < 0.01$; **Figures 4-1** and **4-2**).

Apoptosis independent of Fas and TRAIL-R2

Treatment of HepG2 cells with 200 μM CDCA resulted in peak caspase activity at 12 hours (**Figure 4-3**). Incubation with an antibody to Fas resulted in attenuation of caspase-3/7 by 44%. When cells were incubated with an antibody to TRAIL-R2, caspase-3/7 activity was attenuated by 9%. The addition of antibodies to both Fas and TRAIL-R2 resulted in 44% attenuation of caspase-3/7.

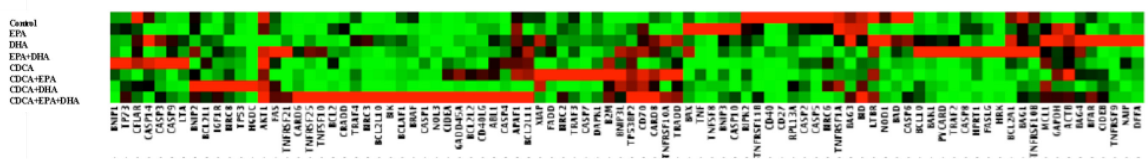

Figure 4-1: Heat map of apoptosis focused array.

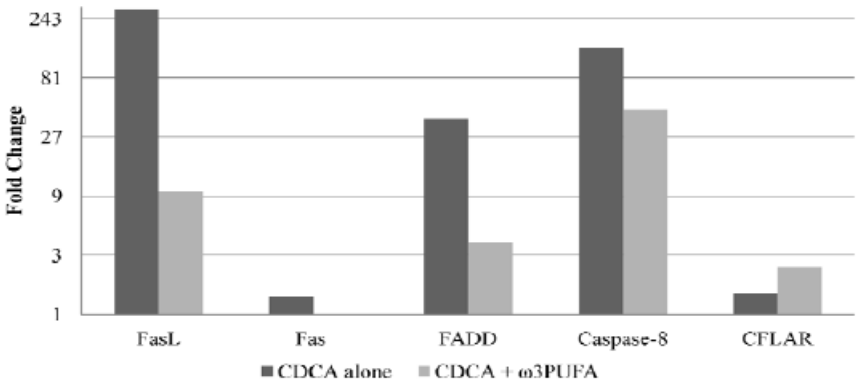

Figure 4-2: Summary of apoptosis focused array.

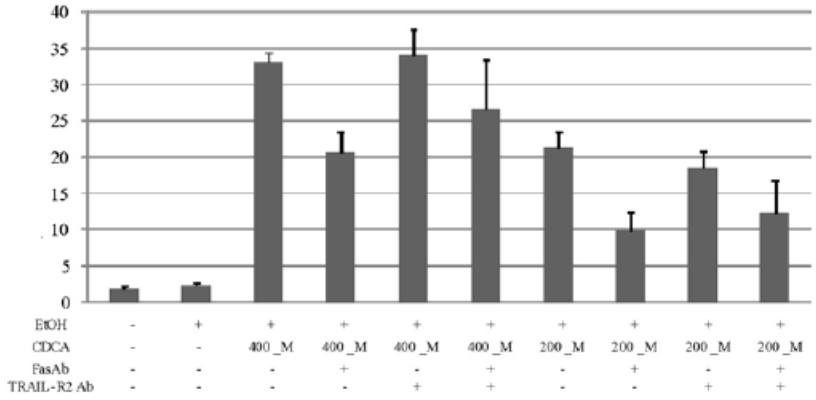

Figure 4-3: Caspase-3/7 activity.

Liver injury in mice exposed to PN+DSS

Mice were pretreated with DSS and randomized into two groups that were infused with either PN (PN7d/DSS mice; n = 30) or NS (NS/DSS mice: n = 20) for 7 days. PN7d/DSS treatment resulted in significantly elevated AST (≈4-fold; $p = 0.0006$), ALT (≈4 fold; $p = 0.0008$), and total bilirubin (≈2-fold; $p = 0.0137$) levels compared with NS/DSS, DSS (n = 9), DSS+8d chow (n = 6), and untreated chow mice (n = 20; **Figure 4-4**). We next tested whether infusion with PN by itself would promote liver injury. PN infusion for 7 days by itself did not cause significant liver injury as measured by AST, ALT, and bilirubin levels in PN mice (n = 10) compared with NS (n = 12) or untreated chow mice (n = 20; $p > 0.05$). Infusion of PN in DSS-pretreated mice was performed for 28 days (PN28d/DSS mice; n = 5). AST and bilirubin levels remained elevated and were comparable to those in PN7d/DSS mice (n = 30), while ALT levels were even further increased after 28 days of PN (**Figure 4-4**).

Death receptor expression in PN-fed mice

Expression of Fas was increased 1.5-fold in mice pretreated with DSS followed by 7 days of PN. In mice that received DSS pretreatment followed by 28 days of PN, Fas was increased 2-fold (**Figure 4-5**). TRAIL mRNA expression was increased 1.25-fold over control in mice fed standard chow, decreased 0.75-fold with DSS and 7 days of PN, and decreased 0.5-fold with DSS and 28 days of PN (**Figure 4-6**). Interestingly, there was no change in Fas-L or TRAIL-R2 (data not shown).

Discussion

The results from this study show that apoptosis associated with PNALD occurs via the death receptor Fas, but also independent of Fas. The results demonstrate that: 1) the attenuation of bile acid-induced apoptosis by ω3PUFA in an *in vitro* model occurs independent of death receptors; 2) hepatocellular apoptosis induced by the hydrophobic bile acid CDCA occurs mainly via the death receptors Fas and to a lesser extent TRAIL-

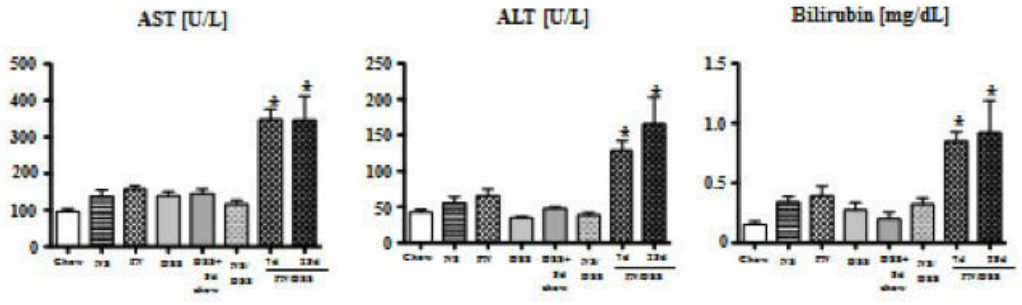

Figure 4-4: Liver function tests in mice exposed to PN+DSS.

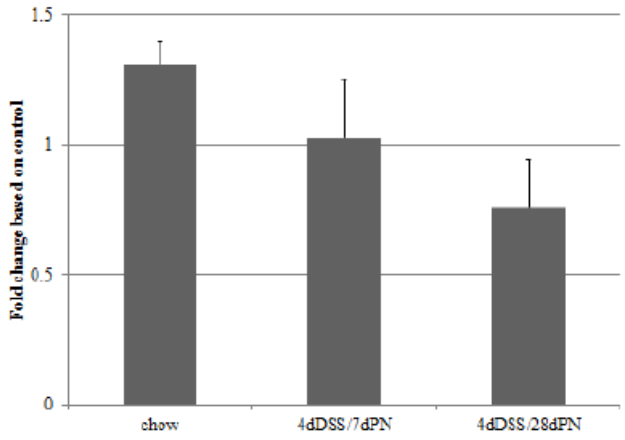

Figure 4-5: Fas expression in mice treated with PN+DSS.

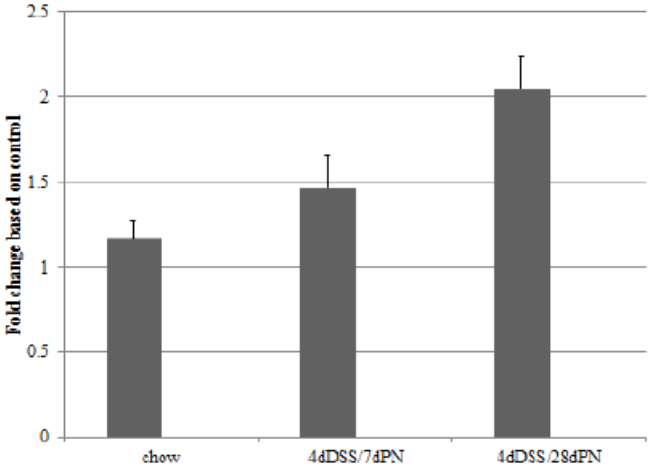

Figure 4-6: TRAIL expression in mice treated with PN+DSS.

R2 in an *in vitro* model; and 3) the death receptor Fas and TRAIL are involved in PNALD in an *in vivo* model.

Treatment with bile acid alone increased gene expression of FasL, Fas, FADD, and CASP8. Treatment with CDCA and the addition of ω3PUFA resulted in less fold-increase in each of these apoptotic mediators. This suggests that attenuation of bile acid-induced apoptosis occurs via a decrease in Fas ligand, thereby reducing binding to Fas and the activation of FADD and CASP8 necessary for mitochondrial amplification to trigger the release of cytochrome *c* and the apoptosome formation. These results suggest that the attenuation of bile acid induced apoptosis occurs via Fas-dependent pathways (**Figure 4-7**).

Bile acid-induced apoptosis has been shown to occur that is both dependent on and independently of hepatic death receptors (79). However, the effect of fatty acids on bile acid-induced apoptosis is not well defined. In a model of NAFLD, ω9PUFA (oleic acid), was shown to increase glycochenodeoxycholic-acid induced apoptosis and increase transcription of pro-inflammatory cytokines (109). Free fatty acids have also been shown to enhance apoptosis via TRAIL, but not via Fas (110). Conversely, free fatty acids have been shown to bind to GPR40 and GPR120 and inhibit serum deprivation-induced apoptosis in a mouse enteroendocrine cell line (94). Additionally, free fatty acids have been shown to increase cholecystokinin production via GPR120 (111), which could also play a role in the attenuation of liver injury.

Here we have shown that bile acid-induced hepatocellular apoptosis occurs predominately via Fas and to a lesser extent via TRAIL-R2. These data confirm our earlier mRNA findings (107). Although there was a 44% attenuation of caspase-3/7 activity when both Fas and TRAIL-R2 were blocked with corresponding antibodies, these data suggest that bile acid-induced apoptosis also occurs via mechanisms that are independent of the death receptors Fas and TRAIL-R2. Yang *et al.* has suggested that bile acid induced apoptosis occurs partially via activation of the novel G protein-coupled cell-surface bile acid receptor TGR5, which activates JNK and leads to formation of a complex between JNK and caspase-8 (81).

We have previously shown that both Fas and TRAIL-R2 are increased in an *in vitro* model of PNALD (107); this had not yet been shown in an *in vivo* model. Here we have shown that the expression of both Fas and TNFS10 were altered in an *in vivo* model of PNALD. Fas expression increased with duration of PN treatment. This also corresponds to increased AST, ALT, and total bile acids, indicating that Fas-dependent apoptosis is directly related to the severity of PNALD. Expression of the ligand TRAIL decreased with exposure to PN, which could be explained by increased binding of TRAIL to the TRAIL-R2 death receptor. Although this is the first report of death receptor-dependent apoptosis associated with PNALD, this is not the first report of apoptosis associated with PNALD. Hong *et al.* suggested that PNALD in an infant rabbit model is associated with mitochondria-initiated apoptosis. After 10 days of PN, rabbits had increased hepatic superoxide dismutase activity and a significant increase in cytochrome

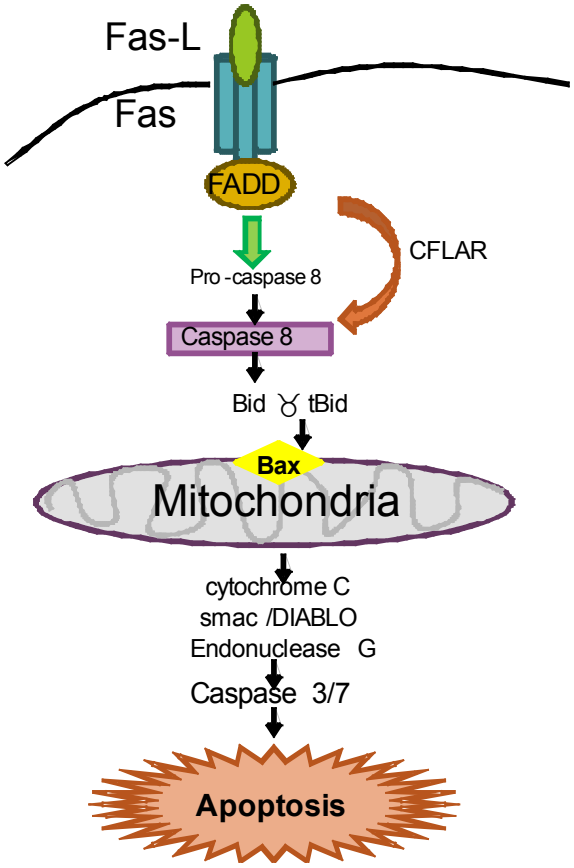

Figure 4-7: Proposed mechanism of Fas-dependent apoptosis.

c release from the mitochondria, and increased caspase-3 activity, resulting in apoptosis (112).

Although these data suggest that bile acid-induced apoptosis primarily occurs via Fas-dependent pathways in HepG2 cells treated with CDCA in the presence and absence of EPA, there may also be other mechanisms that are responsible for some of the protective effects of ω3PUFA in the face of bile acid-induced cell injury. The animal data reported here confirm the *in vitro* findings that death receptors play an important role in apoptosis associated with PNALD. Additional studies are underway in our laboratory to clearly define the precise mechanism(s) of action of ω3PUFA in reversing bile acid-induced cell injury. Collectively, our findings clearly demonstrate the attenuation of CDCA-induced hepatocellular apoptosis by ω3PUFA. With CDCA-induced apoptosis serving as a model for cholestatic liver disease and an *in vivo* mouse model, these results support the therapeutic use of fish oil rich in ω3PUFA for the treatment of PNALD.

CHAPTER 5. BILE ACID-INDUCED APOPTOSIS AND INFLAMMATION ARE ATTENUATED BY OMEGA-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN A MACROPHAGE MODEL

Background

Parenteral nutrition (PN)-associated liver disease (PNALD) is one of the most alarming complications of long-term PN. PNALD is a progressive disease that can lead to liver failure and death. In many cases, children require liver and small bowel transplantation, prolonged hospitalization and home care. PN is associated with a loss of epithelial barrier function, which can lead to intestinal dysfunction and the transit of luminal toxins [lipopolysaccharide (LPS) and tumor necrosis factor (TNF)] into the host (113). When these toxins migrate from the leaky gut into the portal circulation they activate Kupffer cells that act as macrophages in the liver and secrete pro-inflammatory cytokines. This is thought to play a role in the development of PNALD. Investigators have shown that activation of Kupffer cells is necessary to evoke parenchymal liver injury and that attenuation of Kupffer cell-mediated response in turn attenuates liver injury.

Studies have shown improvements in PNALD with omega-3 long-chain polyunsaturated fatty acids (ω3PUFA) containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Anti-inflammatory and anti-apoptotic effects have been attributed to ω3PUFA. The purpose of this study was to examine 1) anti-inflammatory and anti-apoptotic effects of ω3PUFA on macrophage response to bile acid-induced and LPS cellular injury, and 2) the effects of macrophage activation on hepatocytes.

Materials and Methods

Cell culture

Human acute monocytic leukemia cells **(**THP-1) were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 medium supplemented with 10% FBS, 50 U/ml penicillin and 37.5 U/ml streptomycin. THP-1 cells were used as a sergeant for the Kupffer cells. THP-1 cells were plated and differentiated with phorbol 12-myristate 13-acetate (PMA) to a final concentration of 5 ng/mL for 48 hr prior to being treated with conditions described below.

HepG2 cells were obtained from the American Type Culture Collection and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 50 U/ml penicillin and 37.5 U/ml streptomycin. All cells were incubated at 37° C with 5% CO_2 in a humidified incubator. Passages 10 – 32 were used for these experiments.

Cell treatment conditions

THP-1 cells were treated with 200 μmol/L of chenodeoxycholic acid (CDCA) ± ω3PUFA (5 μM EPA and 5 μM DHA). Salmanilla LPS 20 μmol/L, ethanol (EtOH), ω3PUFA alone, and staurosporine 10 μmol/L served as controls. After treatment, cellular supernatants and cells were collected for ELISA assays and/or caspase-3/7 reagents.

THP-1 cells were treated with LPS 20 ng/mL for 30 minutes. After treatment, the supernatant was withdrawn, added to confluent HepG2 cells and allowed to incubate for one hour. RNA was isolated for RT-PCR and caspase-3/7 was evaluated.

Caspase activity

Apoptosis was evaluated using the Apo-ONE® Homogeneous Caspase-3/7 Assay purchased from Promega Corporation (Madison, WI) and performed according to the manufacturer's instructions. HepG2 cells were treated for 12 hours followed by the addition of caspase-3/7 reagent and incubation for 4 hours in the dark on a rocking shaker at low speed. Results were read at fluorescein 485nm/535nm with a Victor 2, Perkin-Elmer Wallace 1420 multilabel counter (Shelton, CT).

Cytokine evaluation

Cytokines (IL-1β, IL-6, TNF-α) were measured in both supernatant and nuclear protein using enzyme-linked immunosorbent assays (ELISAs).

Quantitative RT-PCR

Total RNA was isolated from confluent cultures according to the manufacturer's instructions with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized from 2 μg of total RNA in a 21 μL reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Quantitative PCR was performed in triplicate using the Model 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Independent PCRs were performed using the same cDNA for both the gene of interest and the 18S rRNA, using the SYBR Green PCR Master Mix (Applied Biosystems).

Statistical analysis

All data represent at least three separate and independent experiments. Data are provided as mean ± SEM. A one-way ANOVA was used to compare differences between groups and a *post-hoc* Tukey's HSD test was used to correct for multiple comparisons. A single-tailed p value of 0.05 was used to reject the null hypothesis.

Results

Caspase-3/7 evaluation in THP-1 cells

We first performed a time course study to determine the optimal treatment time for THP-1 cells. Cells were treated for 15 min, 30 min, 45 min and 1 hour. We determined that peak caspase-3/7 activity resulted within 15 minutes of treatment (**Figure 5-1**). Treatment of THP-1 cells with CDCA and ω3PUFA resulted in a 26% decrease of apoptosis in comparison to CDCA alone ($p < 0.01$; **Figure 5-2**).

Cytokine production

Both TNF-α and IL-1β production decreased by 16% ($p = 0.038$) and 19% ($p = 0.03$), respectively (**Figures 5-3** and **5-4**). There was no change in IL-6 concentration with either treatment group **(Figure 5-5**).

When HepG2 cells were treated with medium containing cytokines (IL-1β, IL6 and TNF-α) excreted from THP-1 cells exposed to lipophilic bile acid, there was a 2-fold increase in NF-κB. Treatment with endotoxin alone or bile acid and endotoxin did not result in a significant increase in NF-κB expression over control (**Figure 5-6**).

Discussion

The results from this study show that apoptosis and inflammation associated with PNALD is not limited to hepatocyte injury and may also involve the activation of Kupffer cells in the liver. The results demonstrate: 1) ω3PUFAs attenuate bile acid-induced apoptosis in a macrophage model; 2) ω3PUFA attenuated inflammation via IL-1β and TNF-α in a macrophage model; and 3) macrophage stimulation with lipophilic bile acid and endotoxin increased NFκ-B expression in hepatocytes.

THP-1 cells treated with CDCA for 0.5 hour exhibited increased expression of secretion of IL-1β and TNF-α. This inflammatory response observed with CDCA treatment was attenuated when cells were treated with combination of both EPA and DHA. ω3PUFAs are known for having important anti-inflammatory effects and have been used for the treatment of many inflammatory diseases, including cardiovascular disease, arthritis, asthma, sepsis, autoimmune disease and malignancy (57).

The anti-inflammatory role of ω3PUFA is well known. The ability of ω3PUFA to attenuated liver injury has been studied, but these specific mechanisms are not fully

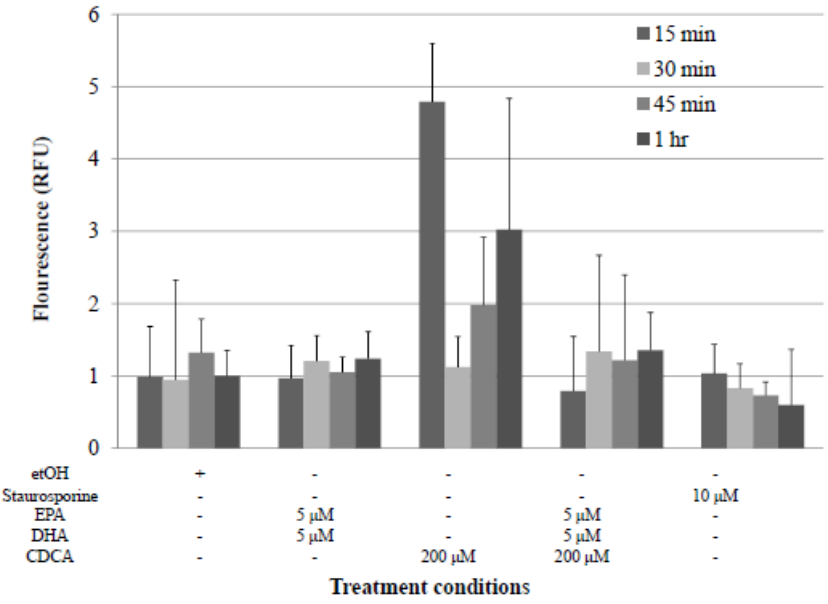

Figure 5-1: Time course of caspase-3/7 activity in THP-1 cells.

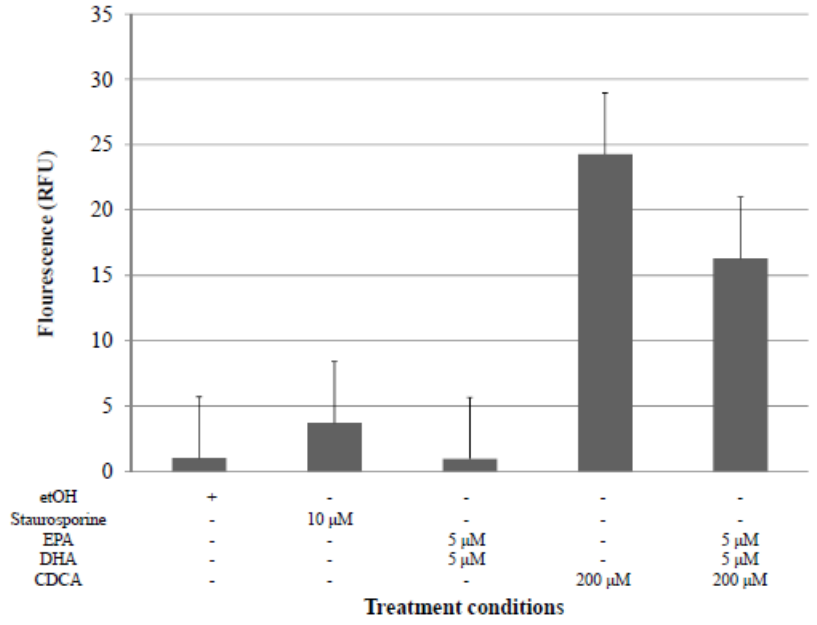

Figure 5-2: Caspase-3/7 activity in THP-1 cells treated for 15 minutes.

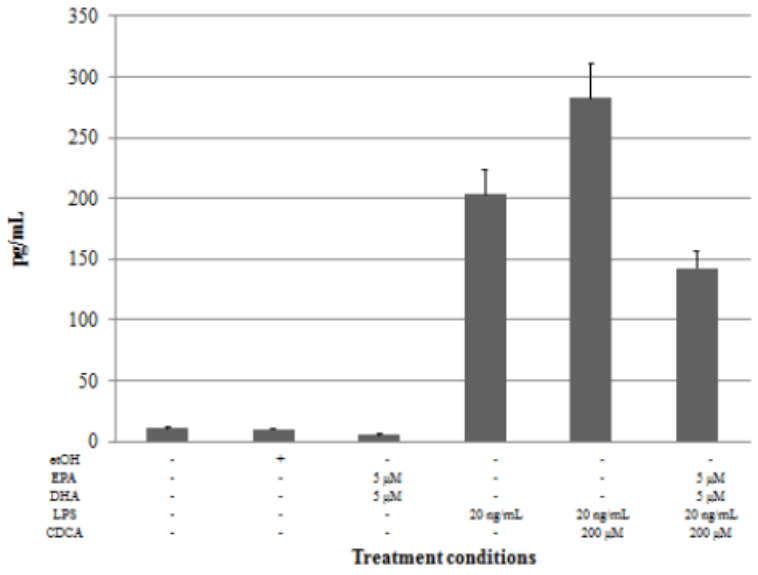

Figure 5-3: TNF-α production in THP-1 cells.

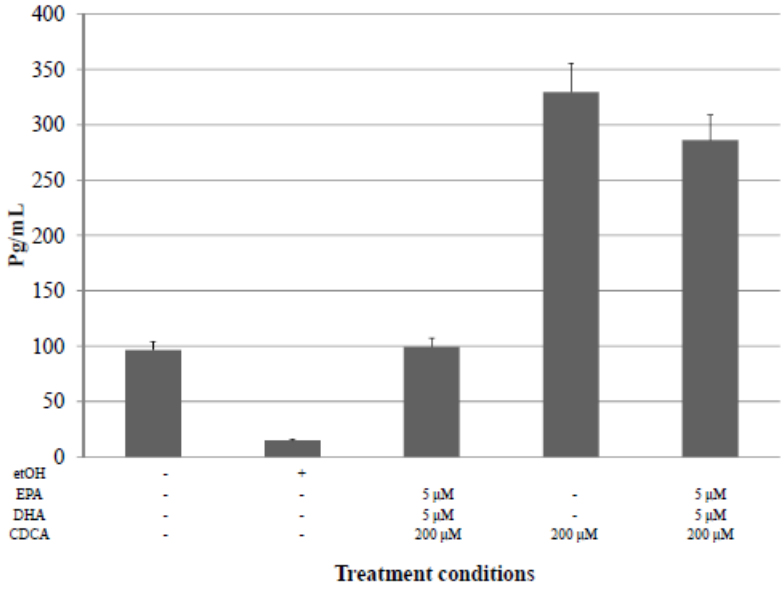

Figure 5-4: IL-1β production in THP-1 cells.

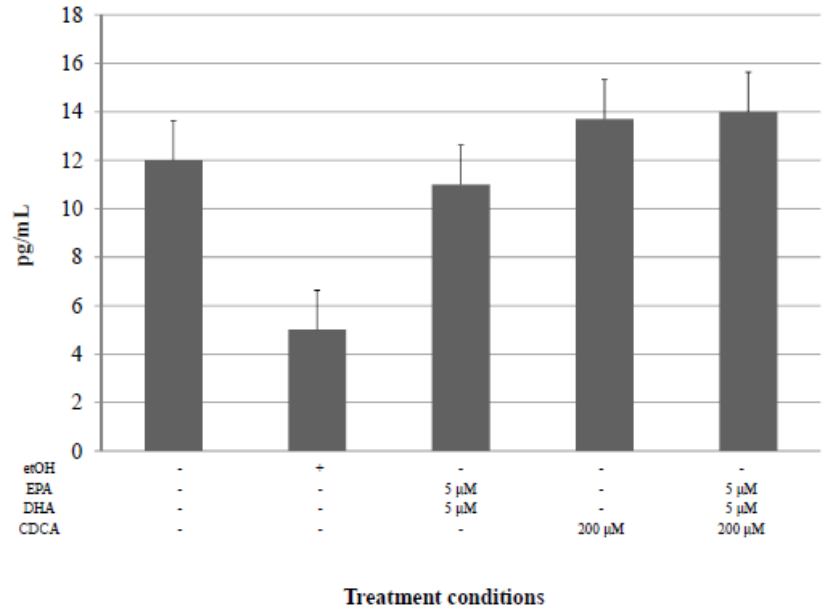

Figure 5-5: IL-6 production in THP-1 cells.

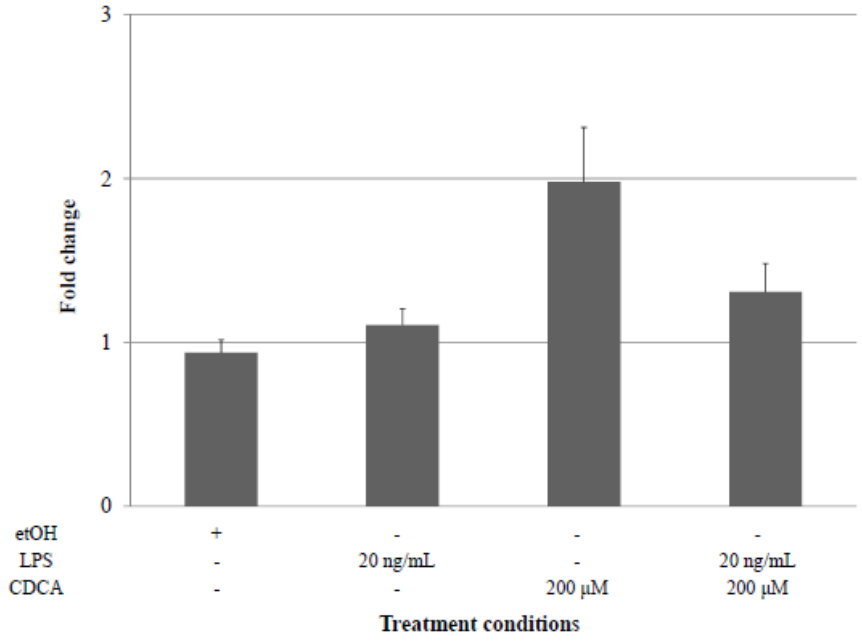

Figure 5-6: NF-κB expression in HepG2 cells stimulated with conditioned medium from THP-1 cells.

elucidated (69, 108, 114). Specifically, there is little known about the role of ω3PUFA in the attenuation of Kupffer cell activation. Kupffer cell activation in the progression of liver injury has been studied in many different types of liver diseases (115, 116). Kupffer cells can be activated by endotoxin, reactive oxygen species, bile acids, or other cellular toxins (117-120). Regardless of the stimulus the activation of the Kupffer cell leads to a cellular inflammatory response which may activate apoptosis (121). Kitagawa *et al.* has shown that branched-chain amino acids have a role in the attenuation of Kupffer cell activation in a rate model of ischemia-reperfusion-induced liver injury (122).

Apoptosis in THP-1 cells was induced by CDCA alone and attenuated by co-incubation of CDCA and ω3PUFA. The attenuation of cellular injury in this model of Kupffer cells provides the basis on which to determine the interaction of Kupffer cells and hepatocytes in order to understand the role of ω3PUFA in the treatment of PNALD.

When THP-1 cells were stimulated with CDCA and then the medium containing cytokines (IL-1β, IL6, and TNF-α) was added to HepG2 cells, we observed a 2-fold increase in NF-κB. This model of treating THP-1 cells with various stimuli and applying conditioned medium to HepG2 cells will allow us to evaluate the mechanisms of hepatotoxicity related to macrophage activation.

Additional studies are underway in our laboratory to clearly define the precise mechanism(s) of action of ω3PUFA in reversing bile acid and endotoxin-induced cell injury, as well as the interplay between parenchymal and non-parenchymal cells. Collectively, our findings clearly demonstrate the attenuation of CDCA- and LPS-induced inflammation and apoptosis by ω3PUFA. With this serving as a model for cholestatic liver disease, these results support the therapeutic use of fish oil rich in ω3PUFA for the treatment of PNALD.

CHAPTER 6. PPAR-A IN THE ATTENUATION OF PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE BY OMEGA-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN AN *IN VITRO* CELLULAR MODEL

Background

Peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear receptor transcription factor that regulates lipid metabolism. When bound to a ligand, PPARα forms a heterodimer with retinoid X receptor (RXR) that binds to the peroxisome proliferator response element (PPRE) in the promoter region of target genes and modulates expression. Activation of PPARα inhibits NFκB activation, thereby repressing inflammation (123, 124). Hepatic nuclear receptor RXR has also been shown to be important in hepatic responses to inflammation (125). Alternatively, NFκB activation increases expression of anti-apoptotic genes, such as the caspase-8 inhibitor c-FLIP, which could lead to resistance to Fas ligand and/or TNF-dependent apoptosis (126). Previous studies have shown ω3PUFA to be a PPARα agonist, thereby inhibiting NFκB activation (124). In studies in PPARα null mouse hepatocytes treated with nafenopin (a peroxisome proliferator), apoptosis was not suppressed, although treatment with TNFα was able to suppress apoptosis in PPARα null hepatocytes (127). These studies demonstrated that PPARα mediates hepatocyte apoptosis, yet high concentrations of TNFα can modulate this response (127). This result suggests that PPARα inhibition of NFκB may play an important role in the overall cellular innate immune response and cell survival. The pathways affected by PPARα and NFκB are complex and, in some cases, seemingly contradictory. The suppression of pro-inflammatory cytokine transcription by PPARα via NFκB would support our hypothesis, but the NFκB-mediated suppression of anti-apoptotic pathways would suggest that ω3PUFA activation of PPARα could result in increased apoptosis. These contradictory effects of NFκB on apoptosis have perplexed investigators studying interferon-mediated activation of NFκB, which results in anti-viral and pro-apoptotic activity. There is also evidence that micro-RNAs may have an important role in this complex signaling pathway (128). Finally, PPARα is known to inhibit apoptosis via NFκB-independent pathways, such as up-regulation of the 14-3-3 proteins (129).

Omega-3 PUFA have been shown to be anti-inflammatory and immunomodulatory and to attenuate cellular injury (59, 65, 69, 130-132). Interestingly, ω3PUFA have been shown to paradoxically both inhibit apoptosis and sensitize cells to apoptosis (109, 110, 133, 134). It is established that the composition and levels of free fatty acids (FFA) can significantly alter cell survival pathways. Free fatty acids have been shown to inhibit serum-starved apoptosis in a murine cell model via the G-coupled protein receptor, GPR120 (94). In these experiments both saturated and unsaturated FFA enhanced cell survival, but linolenic acid, palmitoleic acid, and DHA had the most potent effects on cell survival (95). GPR120 has recently been shown to be a receptor or sensor for ω3PUFA, mediating regulation of inflammation, and inhibits both Toll-like receptor

(TLR) 2/3/4 and TNF-α cascades (96). NFκB and JNK have both been shown to play a role in the up-regulation of death receptors in the presence of noxious stimuli (79, 97).

The objective of this study is to determine if ω3PUFA attenuate PNALD-induced apoptosis and inflammation via PPARα. The rationale for this is that these studies will elucidate a possible pathway by which ω3PUFA attenuate hepatocellular injury by focusing on the transcription factor PPARα, for which ω3PUFA are known ligands.

Materials and Methods

Cell culture

Cell culture experiments were performed using HepG2 cells obtained from the American Type Culture Collection (Rockville, MD). Cell culture experiments used standard medium, incubation conditions, and passages according to the supplier's instructions.

Cell treatment conditions

HepG2 cells were treated with CDCA 200 μM (Sigma-Aldrich, St. Louis, MO) with and without the addition of EPA and DHA (Sigma-Aldrich).

PPARα activation

Activation of PPARα was evaluated by isolation of nuclear protein after 4-hr treatment and measured by assessing transcription factor activity in a non-radioactive, sensitive ELISA method for detecting PPARα DNA binding activity in nuclear extracts.

PPARα expression

HepG2 cells treated with chenodeoxycholic acid 200 μM (CDCA) ± ω3PUFA (EPA 5 μM + DHA 5 μM) for 0.5 hours and expression of PPARα, liver X receptor (LXR), and retinoid X receptor (RXR) were evaluated by RT-PCR using an ABI 7500 Sequence Detector and SYBR Green PCR Master Mix according to the manufacturer's instructions. Levels of mRNA were normalized to 18s ribosomal RNA quantified simultaneously to the selected mediators in a multiplex RT-PCR reaction. All samples were analyzed in triplicate

Gene-specific knockdown using RNAi technique

HepG2 cells were transfected with non-target siRNA or human PPARα-specific siRNA using DharmaFECT transfection reagents (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. To determine transfection efficiency, Western blotting for PPARα was performed with both control and transfected cells.

Results

PPARα activity

Treatment of HepG2 cells with ω3PUFA alone resulted in PPARα activity equal to that observed with recombinant PPARα, and DNA binding of PPARα was decreased by 70% in the presence of CDCA ($p < 0.001$). Treatment with CDCA and ω3PUFA resulted in less reduction of PPARα activity compared to controls. Although not significantly different from that of CDCA alone, there was a trend to restoration of PPARα activity with the addition of ω3PUFA (**Figure 6-1**).

PPARα, LXR, and RXR expression

Treatment with CDCA alone resulted in 67% reduction in PPARα expression as compared to cells treated with a vehicle control. Treatment with CDCA and the addition of ω3PUFA resulted in PPARα restored to the level of control (**Figure 6-2**). There was no statistical difference in expression of RXR (**Figure 6-3**). Treatment with CDCA alone resulted in a 2-fold increase in LXR expression that was attenuated by the addition of ω3PUFA (**Figure 6-4**).

PPARα siRNA knockdown

We were not successful in efficiently and consistently knocking down PPARα with siRNA. Studies were performed at time points from 24-96 hours; 48 hours had the most efficient knockdown, but this was not consistent and reliable enough to be able to perform experiments with confidence of achieving reliable results (**Figure 6-5**).

Discussion

Activity and expression of PPARα were reduced by treatment with lipophilic bile acid. When hepatocytes were exposed to ω3PUFA, PPARα expression and activity were increased. When cells were treated with CDCA with the addition of ω3PUFA, PPARα

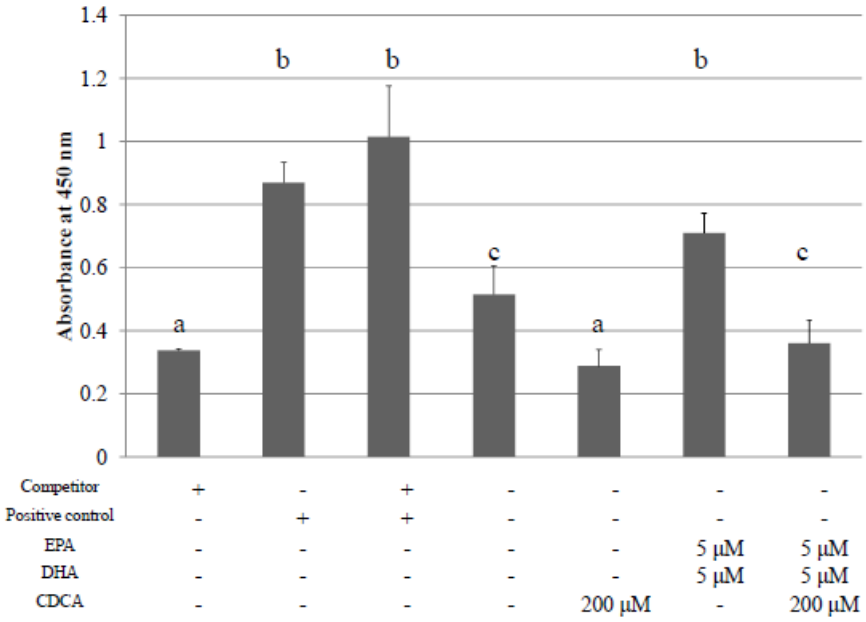

Figure 6-1: PPARα activity.

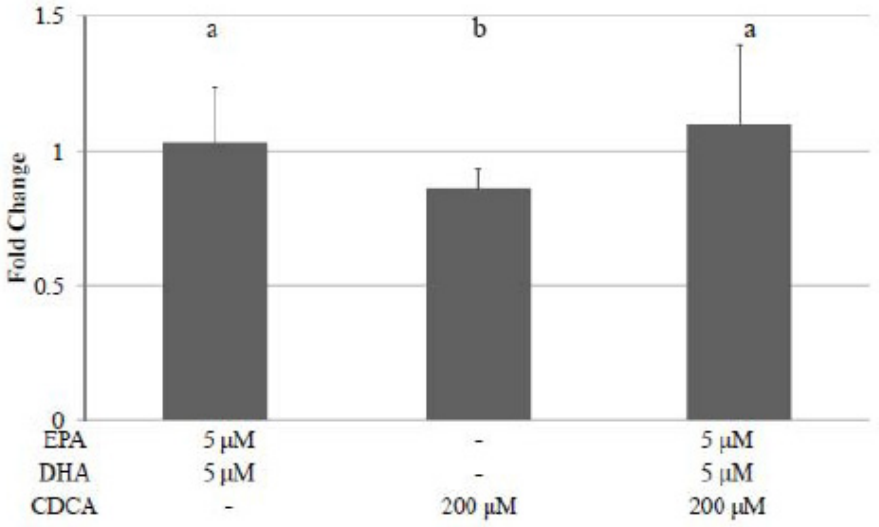

Figure 6-2: PPARα mRNA expression.

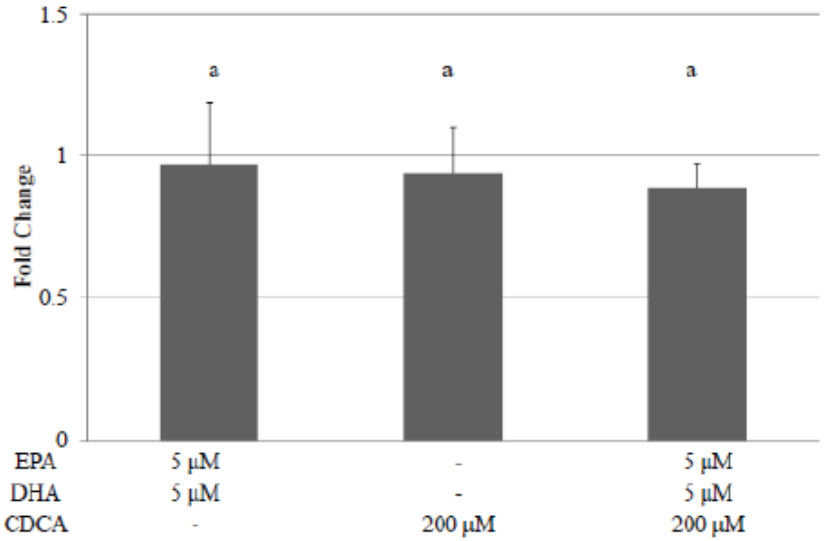

Figure 6-3: RXR mRNA expression.

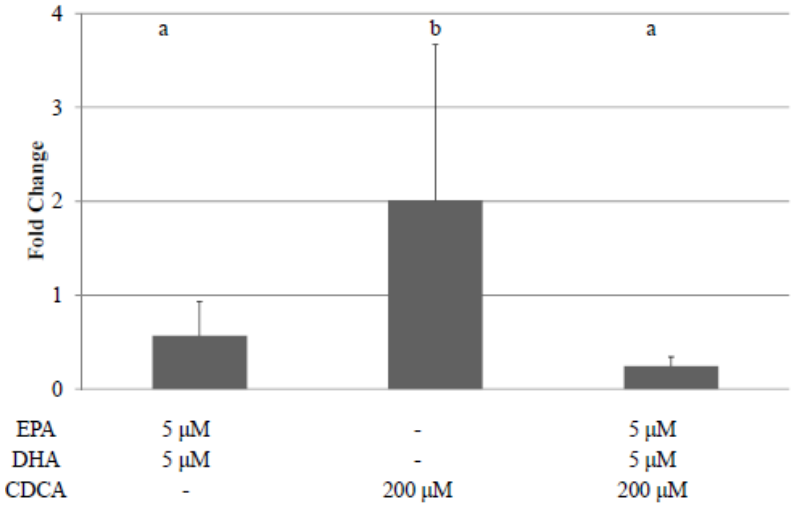

Figure 6-4: LXR mRNA expression.

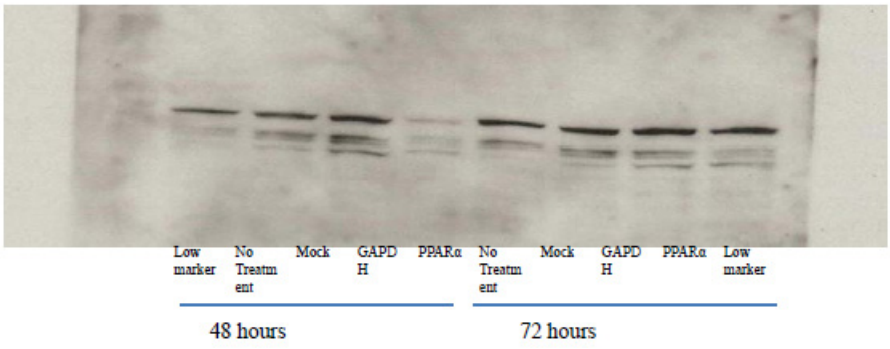

Figure 6-5: PPARα siRNA knockdown.

expression and activity were similar to control cells. In studies of CFTR-knockout mice and isolated macrophages from CFTR-knockout mice pretreatement with DHA resulted in increased PPARα expression. This increase in PPARα expression was blocked by the addition of a PPARα antagonist (135, 136).

We observed a 67% reduction in PPARα expression when cells were treated with CDCA as compared to cells treated with a vehicle control. The addition of ω3PUFA resulted in PPARα restored to the level of control. We did not observe a significant difference in expression of RXR, which was expected because of the abundance of RXR where it is not typically the rate limiting factor. However, treatment with CDCA alone resulted in a 2-fold increase in LXR expression that was attenuated by the addition of ω3PUFA. Typically, PPARα will form a heterodimer with RXR in order to be active and effect gene expression associated with inflammation and lipid regulation (137-140). Although RXR is usually associated with binding PPARα, recent studies have shown a high degree of cross-reactivity in binding among LXR, RXR, and FXR (141, 142).

We completed several time studies with various concentrations of siRNA. We did successfully knock down PPARα, but these results were not consistent and we determined that proceeding forward with treatment studies exposing cells to CDCA and ω3PUFA might result in false results due to insufficient knockdown. Although we were not successful in achieving sufficient and consistent knockdown of PPARα, we plan to use a tetracycline inducible system in order to turn on/off PPARα. We believe this system will be more efficient and predictable.

The decrease in PPARα with exposure to CDCA and subsequent increase of PPARα with ω3PUFA aligns with results in other studies of the anti-inflammatory and immunomodulatory effects of ω3PUFA (59, 65, 69, 130-132). These results suggest that PPARα activation may be a promising mechanism by which ω3PUFA attenuate the bile acid-induced hepatocellular injury that occurs in cholestasis, such as that seen in PNALD.

CHAPTER 7. COLITIS ENHANCES LIVER INJURY AND INFLAMMATION IN A PRETERM PORCINE MODEL OF PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE

Background

One of the most devastating diseases affecting premature neonates is necrotizing enterocolitis (NEC). NEC often requires surgical resection of the necrotic bowel, leaving the infant with short bowel syndrome (SBS). Infants and children with SBS have poor nutrient absorption and often require parenteral nutrition (PN). Although PN is lifesaving for many neonates and infants, it is not benign. Patients receiving long-term PN are at high risk for PN-associated liver disease (PNALD), which is one of the most devastating complications affecting infants receiving PN. The etiology of PNALD is not well-understood and is likely multi-factorial (5-9). Risk factors include immature liver function, inflammation, oxidative stress, infection, nutrient deficiencies, and contaminants in parenteral products, as well the components of the PN, including lipid emulsions and amino acids (10-14). Lipid emulsions have been of particular interest in PNALD, as ω6PUFA soybean-based lipid emulsions are thought to be pro-inflammatory, as well as contain potentially toxic phytosterols (15). PNALD is reported to occur in 50–66% of children receiving long-term PN, with a higher incidence in premature neonates (6-9, 16). If not reversed, PNALD can progress from cholestasis to liver fibrosis, hepatic failure, and death. Because of the complexity of this disease and need for prolonged hospitalization and home care, these patients are especially taxing to the healthcare system. Recent studies in human infants have shown improvement and even reversal of PNALD with intravenous omega-3 polyunsaturated fatty acid (ω3PUFA) supplementation (17-21). Although ω3PUFA supplementation has shown much promise in the treatment of PNALD, its mechanism of action is not well understood.

Several animal models have been utilized to study PNALD and the effects of ω3PUFA supplementation on liver injury. A term neonatal pig model of PNALD has been used to study disease progression as well as treatment (143-145). In a mouse model of non-alcoholic fatty liver disease (NAFLD), hepatic fat content was highest in mice fed a liquid high-carbohydrate diet, and was significantly decreased by both enteral and parenteral ω3PUFA supplementation (59). In a surgical model of liver injury, mice underwent common bile duct ligation and were then randomized to receive a control diet rich in ω6PUFA or a diet rich in ω3PUFA. The diet rich in ω3PUFA resulted in a trend to be protective against injury, but this was not statistically significant (60). Interestingly, rabbits on PN that received fish oil were found to have more extensive hepatic fibrosis than those that received soybean or olive oil regimens (61), although the authors speculated that this adverse finding might be due to the ω-3 to ω-6 ratio of 1:6 in the emulsion studied, whereas optimal ω-3 to ω-6 ratios in animal models have ranged from 1:2 to 1:4 (62, 63). Fish oil-based intravenous lipid emulsions have been shown effective in decreasing impairment of bile flow associated with PN administration in a term neonatal pig model, although the hepatic pathology was not characterized and the mechanism of action was not evaluated (145).

While a term neonatal pig model of PNALD has been used to study disease progression, as well as treatment (143-145), there are currently no published studies using a preterm pig model for the study of PNALD. A preterm pig model has been to study NEC (146-151), but has not been used for PNALD research. The objective of this study was to use a novel preterm neonatal pig model to evaluate enteral ω3PUFA as a treatment for PNALD and to evaluate liver injury after exposure to PN and colitis in a preterm porcine model.

Materials and Methods

Porcine model

Preterm pigs were obtained via caesarian section at 92% gestation (day 106 of 114-day gestation) from timed pregnant sows as described previously (147). After being stabilized, a central venous catheter (CVC) was inserted via the internal jugular vein, and PN solution administered as previously described (151). All animals received PN for the first 24 hours that provided (g/kg-d) glucose (22.3), amino acids (11.6) and lipids (6) Enteral nutrition was administered intermittently via an orogastric tube inserted at the time of CVC placement. Neonatal pigs were confined to metabolic incubators. All animals were euthanized and necropsied at the end of the study.

Evaluation of ω3PUFA for the treatment of PNALD

A brief pilot study with one litter of preterm (gestation 105/114 days) pigs was performed. Six pigs were sustained on PN and six pigs were enterally fed for one week. An additional six pigs were given PN with enteral ω3PUFA 1 g/kg/day (Lovaza®, Reliant Pharmaceuticals Inc., Liberty Corner, NJ) (152). This dose of ω3PUFA is based on preliminary data in human infants (153).

Induction of colitis

All animals received PN for the first 24 hours and were then randomized to enteral nutrition (EN), EN + colitis, PN, or PN + colitis. Colitis was induced with dextran sodium sulfate (DSS) given enterally every 12 hours for three doses starting 24 hours after delivery. Necropsy was performed on day 12.

Sample collection

Blood was collect at baseline and three days per week for the duration of the study. Direct bilirubin (DB), total bile acids (TBA), gamma-glutamyl transpeptidase

(GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were measured (performed by a contract reference laboratory). At the time of necropsy, serum, as well as snap-frozen and formalin-fixed liver, were collected for analysis.

Histology

At the time of necropsy, liver tissue was immediately placed in formalin. Slides were prepared with standard hematoxylin and eosin (H&E) staining. Liver histology was scored by a pathologist blinded to the experimental groups.

Gene expression

Liver RNA was extracted from frozen tissue to evaluate 84 key genes involved in inflammation using the Pig Inflammatory Cytokines and Receptors RT Profiler PCR Array system (SABiosciences, Frederick, MD) using quantitative RT-PCR.

Results

Evaluation of ω3PUFA for the treatment of PNALD

In this one-week pilot study, there was a significant difference in direct bilirubin (DB) between pigs receiving PN and EN at days 3 and 7 ($p = 0.000$ and 0.0369, respectively; **Figure 7-1**).

Induction of colitis

Eighteen pigs were initiated. Nine pigs treated with DSS expired prior to the scheduled necropsy, and nine completed the 12-day protocol. Evaluation of weight, serum transaminases, direct bilirubin (DB), or total bile acids (TBA) was significantly higher in 12-day-old pigs compared to baseline. There was no difference in baseline AST, ALT, DB, or TBA among the four groups. At necropsy, mean DB and TBA were higher in the PN and PN+DSS groups compared to control, but this did not reach statistical significance (**Figure 7-2**).

Histology

Animals treated with PN developed mild steatosis, whereas animals treated with PN+DSS had moderate steatosis. In addition, PN+DSS pigs had significant ballooning

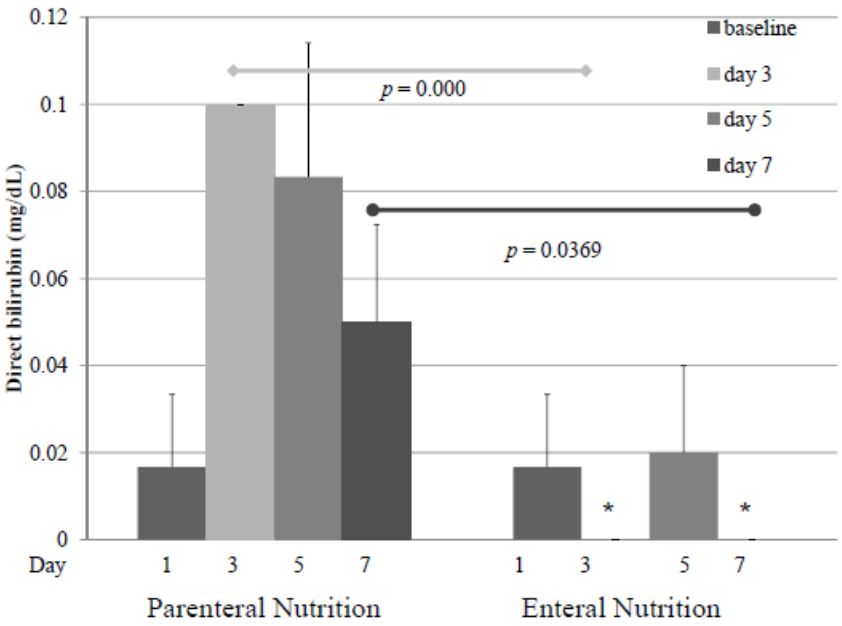

Figure 7-1: Direct bilirubin measurements in 7-day pilot study.

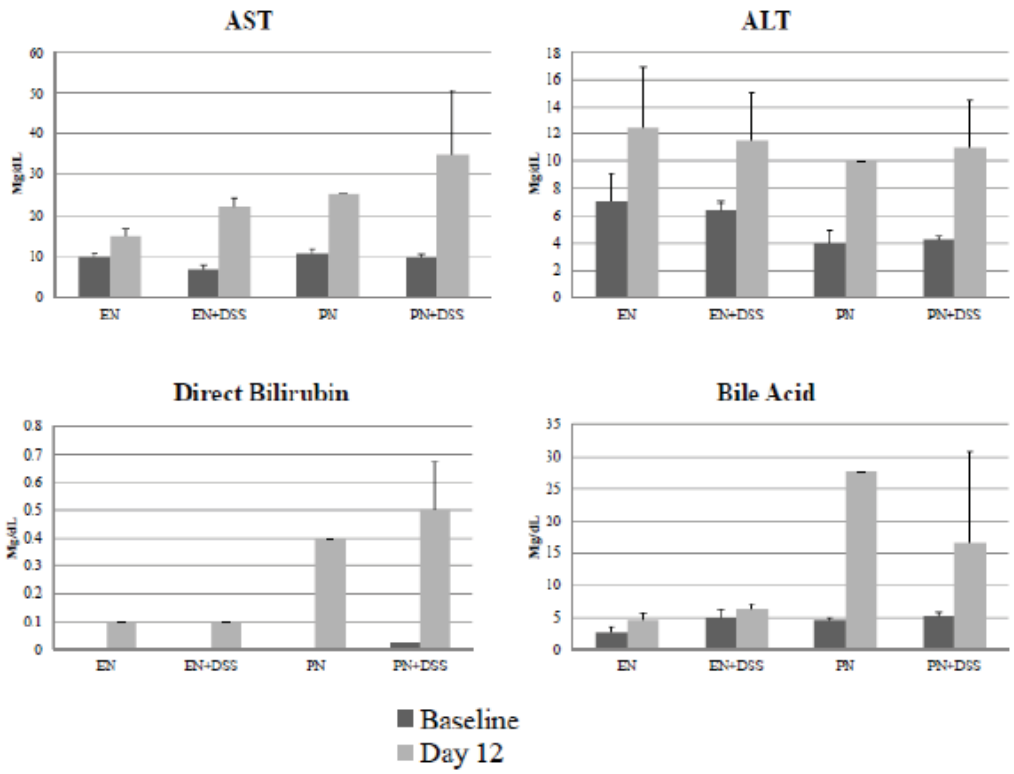

Figure 7-2: Liver functions at baseline and necropsy in 12 day-old pigs.
AST, aspartate aminotransferase; ALT, alanine aminotransferase.

degeneration and presence of Mallory bodies, but this was not observed in the other groups (**Figure 7-3**).

Inflammatory gene expression

Analysis of liver tissue from animals treated with PN+DSS compared to control (EN) identified 26 genes that were up- or down-regulated using a boundary of 4 when the two groups were plotted using $2^{-\Delta CT}$ (**Figure 7-4**). While these results are interesting, their validity is being confirmed using RT-PCR.

Discussion

The results from this study show that liver injury occurs in preterm pigs treated with PN. The results demonstrate: 1) treatment with enteral ω3PUFA results in significantly lower direct bilirubin in pigs treated with PN for seven days; 2) induction of colitis and PN results in significant liver injury observed by histology, but not clinically significant using biochemical markers after 12 days of PN; and 3) treatment with PN and induction of colitis results in up-regulation of pro-inflammatory gene expression in liver tissue from pigs treated for 12 days.

After seven days of PN treatment we only observed a modest increase in direct bilirubin. Although these concentrations were within the normal limits for direct bilirubin, values were significantly higher in pigs that did not receive ω3PUFA supplementation in addition to PN. These modest increases in direct bilirubin are similar to the elevations in biochemical markers of liver disease reported by Kumar *et al.* in pigs treated with PN for 14 days (154).

Because we only observed mild elevations in biochemical markers of liver injury in preterm pigs treated with PN for 7 day, for our next study we lengthened the treatment time to 12 days and induced colitis using DSS. El Kasmi *et al.* induced colitis in mice using DSS treatment combined with PN therapy (108). Animals treated with the combination of PN and DSS had lobular inflammation, hepatocyte apoptosis, peliosis, and Kupffer cell hypertrophy and hyperplasia. Colitis alone or PN alone did not result in liver injury or Kupffer cell activation (108).

Based on these studies in mice, we induced colitis in a similar manner in the preterm pigs. Unfortunately, preterm pigs developed only mild hepatic biochemical changes after twelve days of PN. All biochemical markers were elevated in each of the four groups compared to baseline and animals treated with PN + DSS colitis had higher AST and direct bilirubin levels compared to control, but this was not statistically significant because of the high variability among animals. Bile acids were increased in animals treated with PN, but again this finding was not significant. Given the lack of statistical differences in biochemical markers of liver injury it would be very difficult to use this model for studies involving interventions to treat PNALD.

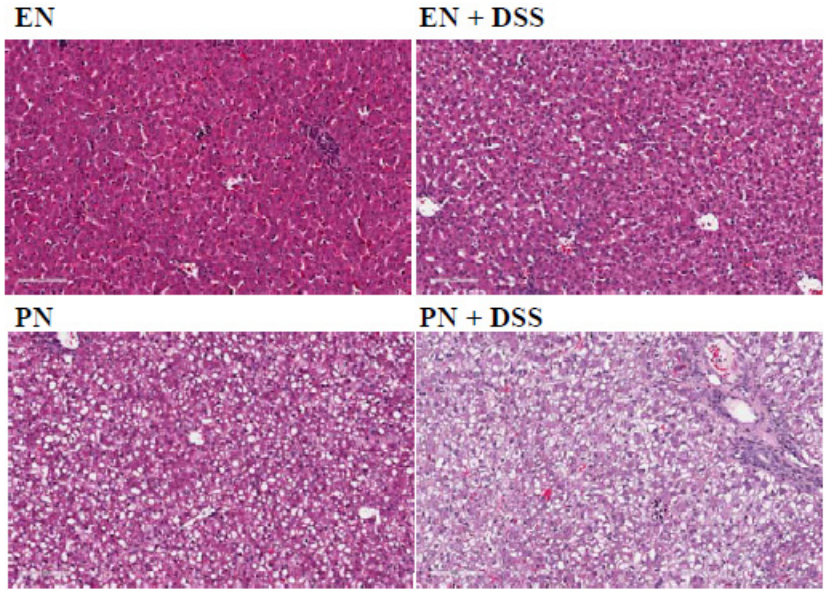

Figure 7-3: Liver histology in 12 day-old pigs.
DSS, dextran sodium sulfate; EN, enteral nutrition; PN, parenteral nutrition.

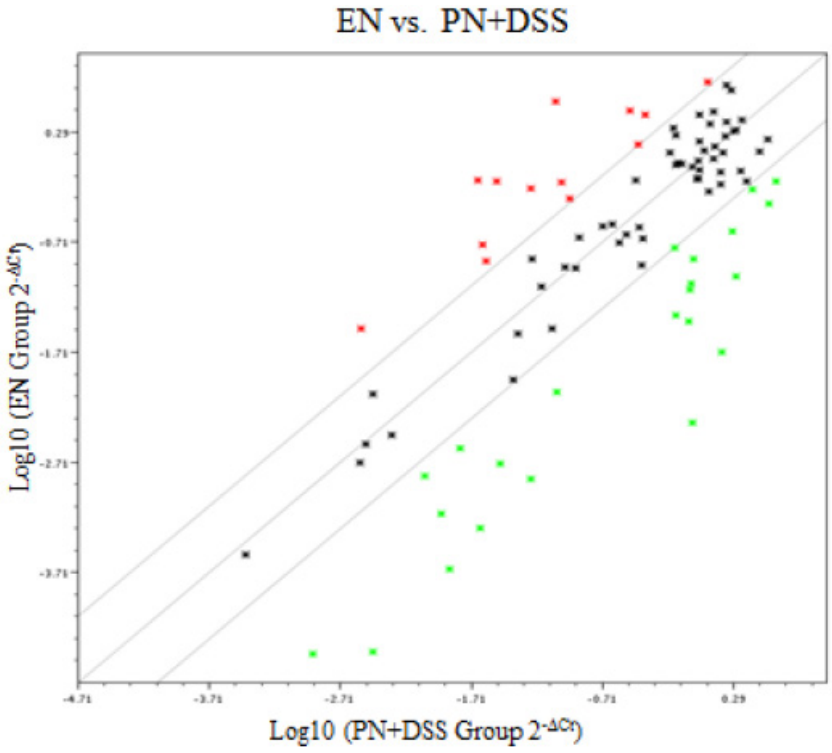

Figure 7-4: Inflammatory gene changes in preterm pigs.
Red stars above the lines signify genes that were up-regulated and green stars below the line signify genes that were down-regulated.

Despite the lack of biochemical evidence of liver injury, histological changes were present. The degree of steatosis increased when a colitis insult was combined with PN treatment. The histological evidence is encouraging that we were successful in inducing PNALD in the preterm pig using both PN and colitis, but without clear biochemical evidence the utility of this model is limited.

Other porcine models of PNALD using term pigs have not induced colitis (155), yet they have been successful in seeing only modest biochemical changes and histological evidence of liver disease. This has allowed expansion of the treatment time to several weeks. This group is using term pigs rather than preterm pigs, which, because of the increased size and stability securing intravenous access and maintaining overall stability, may be slightly easier, yet with term pigs one loses the risk factor of prematurity.

Liver mRNA analysis revealed up and down-regulation of 26 key inflammatory genes in pigs treated with PN+DSS compared to EN. While these findings are interesting, further evaluation of these genes is needed in order to elucidate specific inflammatory pathways affected by ω3PUFA in this model of PNALD. This supports others findings that an initial intestinal insult that increases gut permeability leads to translocation of toxins and inflammatory mediators from the gut into the hepatic circulation, which leads to activation of Kupffer cells (108).

These studies have shown that only modest biochemical changes in liver functions occur in preterm pigs treated with PN and DSS for a 12-day period. Histological changes including increased hepatic fat accumulation are present in pigs treated with PN and DSS. The addition of DSS-induced colitis increases the severity of PNALD and liver inflammation. These data suggest that gut inflammation has a significant impact on liver health in the preterm pig exposed to PN.

CHAPTER 8. DISCUSSION AND CONCLUSION

Summary of Results

My entire research program thus far was inspired by a clinical observation. This dissertation tells the story of my research pursuits over the past five years. I first focused on bile acid-induced apoptosis. I have shown that the lipophilic bile acid CDCA can induce hepatocellular apoptosis and that this is attenuated by ω3PUFA. Interestingly, we found that the combination of EPA and DHA was synergistic in the attenuation of CDCA-induced apoptosis measured by evaluation of caspase-3/7. I was the first to show the synergy between EPA and DHA and I have experiments currently underway to evaluate specific ratios of EPA to DHA in hopes of determining the most optimal ratio.

I also showed that apoptosis primarily occurs via death receptors on the cell surface; in particular, the Fas receptor is associated with bile acid-induced apoptosis, and when Fas is blocked with an antibody, apoptosis is attenuated. Collaboration with colleagues using a mouse model of PNALD allowed us to evaluate Fas, Fas-L, TRAIL-R2, and TRAIL expression *in vivo*. We observed an increase in Fas expression in mice treated with PN compared to mice fed standard chow, and a decrease in TRAIL in mice treated with PN compared to mice fed standard chow. These trends, whether up (as in the receptor Fas) or down (as in the ligand TRAIL), were more exaggerated with duration of time spent on PN. This *in vivo* observation in the mouse model of PNALD strengthened our *in vitro* findings that bile acid-induced apoptosis occurs via death receptor-dependent mechanisms. While apoptosis has been studied in models of injury (156), it has not been extensively evaluated with respect to the attenuation of PNALD by ω3PUFA. We are the first to show alteration in death receptors and ligands in an *in vivo* model of PNALD.

Apoptosis and inflammation associated with PNALD is not limited to hepatocyte injury and may also involve the activation of Kupffer cells in the liver. I have shown that ω3PUFA attenuate bile acid-induced apoptosis in a macrophage model, ω3PUFA attenuated inflammation via IL-1β and TNF-α in a macrophage model, and macrophage stimulation with lipophilic bile acid and endotoxin increased NFκ-B expression in hepatocytes. While these experiments are very elementary, they provide the basis for more exploration in the role of macrophage activation in PNALD. Whether activation of Kupffer cells occurs via LPS migration from the gut (118), or via toxic components from the PN solution such as phytosterols (19, 73), most investigators studying liver injury agree that Kupffer cell activation is key in the development of fibrosis and end stage liver disease (156).

In addition to exploring mechanisms of bile acid-induced apoptosis we also evaluated mechanisms by which ω3PUFA attenuate hepatocellular injury. For these experiments we evaluated the effects of both EPA and DHA. We showed that EPA and DHA were synergistic in attenuating apoptosis when evaluating caspase-3/7 activity, but no synergy was observed with Fas and TRAIL-R2 expression. These findings led me to explore other possible mechanisms by which ω3PUFA could be attenuating

hepatocellular injury. Omega-3 PUFA are known ligands of PPARα, therefore we evaluated both PPARα activity and expression in hepatocytes treated with CDCA with and without EPA and DHA. Treatment with CDCA resulted in a decrease in both PPARα activity and expression, and this was restored to the level of control when hepatocytes were treated with ω3PUFA. We also evaluated the nuclear receptors RXR and LXR, which are essential for the formation of active PPARα heterodimer. RXR expression was unchanged with treatment with CDCA and ω3PUFA, but LXR expression was increased 2-fold with treatment with CDCA and attenuated to the level of control with the addition of ω3PUFA. We attempted to use RNA interference to decrease expression of PPARα so that we could further elucidate mechanisms by which ω3PUFA were altering this PPARα and expression. We were unsuccessful at achieving a consistent and efficient knock-down. We tried transfecting cells with several different primer designs for PPARα and tried a double transfection with two different sets of primers, but did not achieve sufficiently consistent knock-down. Rather than spend more time and resources to try to make this technique work, we will try a tetracycline inducible system to turn off PPARα. The role of PPARα is important in both inflammation and apoptosis. The other nuclear receptors, RXR, LXR, FXR and PXR, have all been identified as important factors in cholestasis and bile acid transport (135, 136, 157).

Besides using an isolated cell culture model of hepatocytes and macrophages involved in PNALD, we also used a preterm neonatal pig model of PNALD. The results from this study show that liver injury occurs in preterm pigs treated with PN. We showed that treatment with enteral ω3PUFA results in significantly lower direct bilirubin levels in pigs treated with PN for seven days, yet this difference was modest. In hopes of increasing the severity of PNALD, we lengthened the treatment time and induced colitis. Induction of colitis and PN resulted in significant liver injury as observed by histology, but it was not clinically significant, as judged by biochemical markers after 12 days of PN. Treatment with PN and induction of colitis results in up-regulation of pro-inflammatory gene expression in liver tissue from pigs treated for 12 days. Although we were not impressed with the degree of change in biochemical markers of liver injury in pigs treated with PN, our findings were similar to serum markers in term pigs treated with PN (155). The results of the inflammatory array will be a valuable tool to use to confirm important inflammatory genes involved in colitis that may be early indicators of PNALD.

Future Directions

Thus far I have shown that ω3PUFA attenuate apoptosis in hepatocytes and have proposed several mechanisms by which this may be occurring. As mentioned in the introduction, PNALD is a multi-factorial disease and a clear etiology is unknown. However, we do know that PN is associated with a loss of intestinal epithelial barrier function, which can lead to intestinal dysfunction and the transit of luminal toxins (such as LPS and TNF) into the host. This increased intestinal permeability is thought to play a role in the development of PNALD (113). We also know that PN alone may not result in PNALD, as animal models require induction of an experimental colitis in addition to PN in order to observe substantial liver injury. Although the liver is the site of disease and we

have shown that ω3PUFA attenuate bile acid-induced apoptosis in hepatocytes, the liver may not be the root of the problem.

I hypothesize that ω3PUFA are attenuating inflammation and apoptosis in the gut, leading to a decrease in intestinal permeability and less migration of inflammatory mediators from the gut to the liver. Our initial observations in human infants treated with enteral fish oil resulted in improvement in liver injury within two weeks of initiation of therapy. This was much sooner than what was observed in human infants treated with IV fish oil where the mean time to resolution of PNALD was four weeks. We hypothesized that ω3PUFA administered enterally may have an added benefit over the IV preparation as it may have a localized anti-inflammatory effect on the gut. While this hypothesis has not been proven in this dissertation, my next step in my pursuit of understanding how ω3PUFA attenuate PNALD will be to focus on the effects of ω3PUFA on the gut and the impact on liver injury.

My next aim will be to identify attenuation of intestinal injury and PNALD *in vivo* by ω3PUFA. For this aim I will use a mouse model of PNALD. In contrast to the pig model, the mouse model has been shown by other investigators to produce reliable biochemical markers of liver injury so that many treatments may be evaluated prior to necropsy. Corollaries of my central hypothesis are that [i] both enteral and parenteral administration of ω3PUFA will be associated with attenuation of intestinal insult and PNALD when compared to animals receiving PN without ω3PUFA, and [ii] animals receiving enteral ω3PUFA will have enhanced attenuation of both intestinal and liver injury compared to animals receiving parenteral ω3PUFA. While I will be interested in the overall progression or presence of PNALD in animals, I would like to specifically evaluate intestinal permeability in regards to ω3PUFA. To test this, I will evaluate both protein and RNA expression of the tight junction proteins occludin and claudin-1, which are essential in maintaining barrier function in the gut. I will also orally administer a radio-labeled protein to determine changes in barrier function related to PN. At the time of necropsy, radioactivity will be measure in serum, gut tissue, and liver tissue and compared to control animals. I will also examine intestinal apoptosis. At first thought one would expect that significant apoptosis would automatically result in loss of barrier function and disruption of tight junctions, but because of the crypt-villus organization of the gut, substantial apoptosis can occur in the crypt without significant disruption of tight junctions.

In my second aim, I will evaluate ω3PUFA effect in endotoxin stimulated intestinal epithelial cells. I anticipate that ω3PUFA will attenuate intestinal injury via GPR120 dependent pathways. Omega-3 PUFA are a ligands for G protein-coupled receptor 120 (GPR120). The therapeutic mechanism of ω3PUFA attenuation of in PNALD is unknown, but is thought to occur via anti-inflammatory pathways, as of yet undefined. Although GPR120 has been shown to be an important pathway for ω3PUFA positive impact on obesity and diabetes, its role in PNALD has not been explored.

For these experiments I will use Caco2 cells and treat with LPS with and without the addition of ω3PUFA. I will first evaluate changes in barrier function using

unidirectional flux of inulin. I will also use immunoflurescence microscopy tight junction and apoptotic proteins. I will then specifically look at the role of GPR120 in regards to the protective effect of ω3PUFA on preserving intestinal barrier function. I will first measure RNA expression of GPR120 and then I will use siRNA techniques to knockdown GPR120 and evaluate tight junctions as described above. Once this hypothesis has been validated, I would like to further confirm this as a mechanism of ω3PUFA attenuating PNALD by using GPR120 knockout mice (96) and treat with PN with and without ω3PUFA.

With respect to expected outcomes, the work proposed in Aim 1 will determine if there is a local intraluminal effect of enterally administered ω3PUFA in an animal model of PNALD. The work proposed in Aim 2 will focus on mechanisms in which ω3PUFA modify inflammation, apoptosis, and ultimately intestinal epithelial barrier function. The results are expected to have an important positive impact by filling a key gap in the understanding of the mechanism of ω3PUFA in the attenuation of intestinal insult. My research will provide explanation into the interplay between intestinal and liver disease, which is imperative to the development of novel therapeutic interventions.

Conclusions

The results presented here have made some progress in determining how ω3PUFA attenuate PNALD, but there are still potentially a lifetime of questions that are still left unanswered. I believe the skills that I have learned from course work and research experience as a part of the requirements for my Doctor of Philosophy degree have prepared me to refine a research question, make a valid hypothesis, and design experiments to test the hypothesis. I hope that my future aims will have an important positive impact by filling a key gap in the understanding of the mechanism of ω3PUFA in the attenuation of both intestinal and liver insult.

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VITA

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