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Prevention of alcoholic fatty liver and mitochondrial dysfunction in the rat by long-chain polyunsaturated fatty acids

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Abstract

Background/Aims—We reported that reduced dietary intake of polyunsaturated fatty acids (PUFA) such as arachidonic (AA,20:4n6, omega-6) and docosahexaenoic (DHA,22:6n3, omega-3) acids led to alcohol-induced fatty liver and fibrosis. This study was aimed at studying the mechanisms by which a DHA/AA-supplemented diet prevents alcohol-induced fatty liver.

Methods—Male Long-Evans rats were fed an ethanol or control liquid-diet with or without DHA/AA for 9 weeks. Plasma transaminase levels, liver histology, oxidative/nitrosative stress markers, and activities of oxidatively-modified mitochondrial proteins were evaluated.

Results—Chronic alcohol administration increased the degree of fatty liver but fatty liver decreased significantly in rats fed the alcohol-DHA/AA-supplemented diet. Alcohol exposure increased oxidative/nitrosative stress with elevated levels of ethanol-inducible CYP2E1, nitric oxide synthase, nitrite and mitochondrial hydrogen peroxide. However, these increments were normalized in rats fed the alcohol-DHA/AA-supplemented diet. The number of oxidatively-modified mitochondrial proteins was markedly increased following alcohol exposure but significantly reduced in rats fed the alcohol-DHA/AA-supplemented diet. The suppressed activities of mitochondrial aldehyde dehydrogenase, ATP synthase, and 3-ketoacyl-CoA thiolase in ethanol-exposed rats were also recovered in animals fed the ethanol-DHA/AA-supplemented diet.

Conclusions—Addition of DHA/AA prevents alcohol-induced fatty liver and mitochondrial dysfunction in an animal model by protecting various mitochondrial enzymes most likely through reducing oxidative/nitrosative stress.

Keywords

Alcoholic fatty liver; polyunsaturated fatty acids; Long-Evans rat; Oxidative/nitrosative stress; Protein oxidation; β -oxidation of fatty acids; Mitochondrial dysfunction

1. Introduction

A diet deficient in essential fatty acids (EFA) causes a cascade of anomalies in mammals, such as scaly skin conditions, impaired vision, poorer cognitive function [1], and cytoplasmic

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vacuolation, *i.e.* fatty liver, due to an inability to form the lipoproteins responsible for transporting lipids out of the liver [2]. Alcohol consumption was shown to compromise EFA status, either by affecting the biosynthesis, absorption, or catabolism of fatty acids [3-8], or via a reduced dietary intake of proper nutrients [9]. Furthermore, alcohol-mediated increased oxidative/nitrosative stress can inhibit the enzymes, involved in the β -oxidation of fatty acids, and other mitochondrial proteins including the electron transport chain, resulting in fat accumulation and mitochondrial dysfunction [10-13]. During periods of heavy drinking, alcoholics often lack proper diets or consume diets which are compromised in various nutrients, such as EFAs and vitamins. For example, a 24% decrease in total polyunsaturated fatty acids (PUFA) in patients with alcohol-related liver damage, has been reported [14]. Monkeys on a diet low in n-3 EFAs but with an adequate level of linoleic acid (n-6) developed liver fibrosis with only a mean alcohol intake of 26% energy [7], whereas it had previously been observed that even much higher levels of ethanol consumption (36% energy derived from ethanol) failed to produce severe liver pathology except for hepatosteatosis [15].

The relative proportions of fatty acids of various tissues are influenced by both the total caloric intake and the fatty acid composition of the diet [16]. For instance, continuous intragastric feeding with high unsaturated fat diets was shown to cause liver fibrosis in rats [17], although saturated fats protected against this alcohol-mediated fibrosis [18,19], possibly through increased membrane resistance to oxidative stress with elevated fatty acid metabolism and/or production of adiponectin [19,20]. A high fat diet (35 energy%) containing high levels of long-chain PUFAs supplied as fish oil, in conjunction with ethanol exposure, led to an increased incidence of fatty liver and moderate inflammation in rats given total enteral infusion [21]. This diet caused an elevation of ethanol-inducible cytochrome P450 2E1 (CYP2E1) content and activity for increased production of reactive oxygen species (ROS) in the gastrointestinal tract [21]. Elevated ROS in turn can cause markedly increased oxidative modification of proteins, nucleic acids, and lipids. For instance, oxidation of many cytosolic and mitochondrial proteins leads to their inactivation and increased susceptibility toward tissue damage [11,13, 22] especially in the presence of another pathological factor in a synergistic or additive manner [23,24]. Oxidation of lipids also leads to the formation of toxic lipid peroxides 4-hydroxynonenal and malondialdehyde (MDA) [7-9]. Acute massive doses of ethanol alone cause intensified lipid peroxidation within liver microsomes and mitochondria [25]. High fat diets containing a very high content of PUFAs may thus be disastrous to the liver when combined with alcohol [21]. However, these results do not reflect physiological conditions since the concentration of fatty acids used in that study was more than 100-fold above the mean Western intake of long chain n-3 PUFAs. The aims of this current study are to investigate the effect of physiologically relevant concentrations of long chain n-3 and n-6 PUFAs on alcohol-induced fatty liver and to study the underlying mechanisms.

2. Materials and Methods

2.1. Diet and experimental design

The present investigation comprises a 2×2 full factorial design with the two independent variables: dietary long-chain PUFA and ethanol. Two nutritionally adequate liquid diet compositions were prepared according to Olsson et al [26] as shown in Table 1. The basal diet (BASE), had low but adequate levels of the EFAs (18:2n6 and 18:3n3) and vitamin E. The PUFA diet was identical to the BASE-diet except for the addition of low levels of arachidonic (AA:20:4n6) and docosahexaenoic (DHA:22:6n3) acids (AA:DHA=1:1 with 0.56 gm each/Liter liquid-diet). The long-chain PUFAs (20:4n6 and 22:6n3) in the PUFA-diet were supplied by ARASCO® and DHASCO® (Martek Biosciences Corporation; Columbia, MD, USA). The compositions of liquid diets were based in part on both the AIN-93M [27] and the Lieber-DeCarli liquid diets (Bio-Serv) [28].

2.2. Animals treatment, tissue collection, and histopathology

Male Long-Evans rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). The 56 rats were weight matched (mean; 294 ± 36 g) and randomly divided into 4 groups. All animal procedures were approved by the NIAAA Animal Care and Use Committee. Individually housed animals of the two control groups received as much BASE-control and PUFA-control diets, per energy basis, as their respective ethanol groups (BASE-EtOH and PUFA-EtOH) had consumed during the previous 24 hour period where ethanol concentration was gradually increased to 36 en% over a period of one week. After isocaloric pair-feeding for 9 weeks, all rats were fasted overnight and euthanized by decapitation. The killing sequence was randomized in order to eliminate any time dependent variation due to length of fasting. Collected liver and plasma samples were coded and the codes were broken only after completion of all analyses. Immediately after decapitation, whole trunk blood was collected into plastic tubes containing EDTA and spun at 2,700 rpm for 15 min. The plasma isolated from each rat was used to measure alanine transaminase (ALT) and aspartate transaminase (AST) activities. A frontal piece of liver from each rat was fixed in 10% formalin and stained with hematoxylin and eosin (H & E) [20,21]. The degree of fatty liver in all samples was read blind by Dr. Miriam Anver at NCI-SAIC Inc (Frederick, MD, USA). Scoring of hepatocellular vacuolation and fat content were performed semi-quantitatively with the following parameters: 0 = no fat; 1+ <25%, 2+ = 25–50%, 3+ = 51–75%, 4+ = 76–95%, and 5+ = close to 100%, as previously described [21]. The expanded scoring system was used to possibly detect subtle differences between the treatment groups.

2.3. Determination of blood alcohol concentration and fatty acids

Blood alcohol concentrations (BAC) were determined 16 hours after feeding (90 min after the end of the dark cycle) on tail vein blood using an enzymatic alcohol test kit (Sigma Diagnostics). Total lipid extracts were obtained according to a method [29]. Internal standard (23:0 free fatty acid) was added to the lipid extracts along with 50 μ g/sample of butylated hydroxy toluene to minimize the risk of degradative oxidation. All extraction steps were conducted under a protective blanket of nitrogen to minimize oxidative degradation of the PUFA. The organic phase, containing the lipids, was subjected to acidic methylation by BF_3 according to Morrison and Smith [30] with modifications [31].

2.4. Determination of nitrite, hydrogen peroxide, triglyceride, cholesterol and malondialdehyde concentrations

Total nitrite concentration and the levels of triglycerides or cholesterol in the liver homogenates were determined and normalized for the protein concentration, as described [11]. Hepatic levels of MDA were measured by using a Lipid Peroxidation Assay kit (Calbiochem, San Diego, CA, USA). Hydrogen peroxide produced from isolated mitochondria was determined by using the Amplex[®] Red Hydrogen Peroxide assay kit (Molecular Probes, Eugene, OR) by following the manufacturer's instructions [32].

2.5. Identification of oxidatively-modified mitochondrial proteins

Mitochondrial fractions were prepared by differential centrifugation from pooled rat livers ($n \geq 8$ per group) of differently treated groups, as described previously [11,22]. Labeling of oxidatively-modified proteins with biotin-conjugated *N*-maleimide (biotin-NM) was performed using a published method [11,22]. Purified biotin-NM labeled oxidized proteins bound to the streptavidin-agarose beads were washed twice with phosphate buffered saline with 1% CHAPS to remove non-specific protein interactions before they were resolved by 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), silver-stained, and compared.

2.6. Data evaluation

All study data represent results from at least 3 different measurements, unless otherwise stated. Each point represents the average $\pm$ SD. Statistical significance was evaluated using the Student's t-test at the 95% confidence level. Other materials and methods not described here were as previously described [7,11,22,26].

3. Results

3.1. BAC, liver histology, fatty acids and fat accumulation in alcohol-exposed rats

During our treatment protocol, the rats of BASE-EtOH and pair-fed group (BASE-control) received a calculated average of 74.2 and 74.5 kcal/day, respectively, verifying the accuracy of the isocaloric pair-feeding protocol. BAC of alcohol-fed rats used in our experiments averaged 101 mg/dl and was thus above 0.08%, which is the level commonly used as a measure of legal intoxication in humans [33]. Our results showed that PUFA did not significantly change the BAC in different groups, suggesting that PUFA diets did not affect the rate of alcohol metabolism by alcohol and aldehyde dehydrogenases. No obvious signs of EFA deficiency, such as scaly skin, were observed in the animals during the diet feeding period or at termination. Significantly higher levels of 22:5n6 were observed in BASE-EtOH group (mean=61 μ g/g) compared to the PUFA-EtOH animals (mean=23 μ g/g). Other fatty acids revealed statistically significant differences between the respective groups, both after ethanol treatment, and between the BASE and PUFA groups (Table 2).

Fig. 1A shows the best liver histological slides of rats fed a) BASE-control, b) BASE-EtOH, c) PUFA-control, and d) PUFA-EtOH diets, respectively. Histological data indicated no severe liver damage with little differences in the ALT and AST values between the groups (Table 3). The lack of hepatic necrosis or inflammation could be due to the relatively low BAC during our pair-feeding protocol. However, macrovesicular and microvesicular steatosis was easily seen in BASE-EtOH group while much less fat accumulation observed in other groups. Mean histopathology scores were evaluated in a masked fashion and were significantly lower ($p<0.01$) in PUFA-EtOH group compared with the corresponding BASE-EtOH group (Fig. 1B). Addition of PUFAs to BASE diets significantly decreased fat accumulation regardless of ethanol exposure. To further confirm the PUFA-mediated prevention of alcoholic fatty liver, hepatic contents of triglyceride and cholesterol were measured for each group. Although the amounts of hepatic triglyceride were similar between PUFA-treated groups, the triglyceride level in the BASE-EtOH group was increased by 60% compared to that of BASE-control group (Fig. 2A). Similarly, the level of hepatic cholesterol was also elevated by 60% in BASE-EtOH group over that of BASE-control group (Fig. 2B). Both triglyceride and cholesterol contents elevated in BASE-EtOH group, were significantly reduced in PUFA-EtOH group.

Crabb and colleagues reported that ethanol can cause fatty liver by increasing the activity of acetyl-CoA carboxylase (ACC), a key rate-limiting enzyme in fatty acid biosynthesis [34]. To determine whether ACC is modulated in our samples and study the mechanism of fat accumulation in Base-EtOH group, the degree of phosphorylation (inactivation) of ACC was evaluated by immunoblot analysis with the specific anti-phospho-ACC antibody (from Cell Signaling Technology). The densitometric level of phospho-ACC was significantly decreased in BASE-ethanol group (Fig. 2C and D), while the levels of β -actin, as a loading control, and were similar in all groups. The phospho-ACC level was significantly elevated in PUFA-EtOH group. These data suggest that alcohol increased the rate of fat biosynthesis in the BASE-diet while the PUFA supplement efficiently blocked the increased fat synthesis despite ethanol exposure.

3.2. Increased levels of reactive oxygen/nitrogen species (ROS/RNS), CYP2E1, and nitric oxide synthase (NOS) in alcohol-exposed rats

We determined the levels of surrogate marker proteins for increased oxidative and nitrosative stress [11,15]. The hepatic levels of microsomal CYP2E1 (Fig. 3A, top) were elevated in rats fed BASE-EtOH diet compared to those in the control (BASE-Control). Mitochondrial inducible nitric oxide synthase (*i*NOS) levels (middle) were virtually undetected in BASE-diet control rats but significantly elevated in BASE-EtOH group. The levels of mitochondrial prohibitin, used as a loading control, were similar in all samples examined (bottom). The activities of CYP2E1 (Fig. 3B) and NOS (Fig. 3C) were increased approximately 55% and 220%, respectively, in BASE-EtOH group compared to those in BASE-control group. However, the elevated CYP2E1 and NOS activities in BASE-EtOH group were significantly reduced in PUFA group despite ethanol treatment. Mitochondrial hydrogen peroxide levels (Fig. 3D) were elevated more than 4.1-fold in BASE-EtOH group compared to BASE-Control group ($2.18 \pm 0.20 \mu\text{M/hr/mg protein}$). The increased hydrogen peroxide levels returned to control levels in PUFA-EtOH group. Similarly, nitrite levels were significantly elevated from approximately $0.78 \pm 0.03 \mu\text{M}$ in BASE-control group to $2.62 \pm 0.09 \mu\text{M}$ in BASE-EtOH group but the elevated nitrite level was decreased to 0.72 ± 0.10 and $0.95 \pm 0.08 \mu\text{M}$ in PUFA-control and PUFA-EtOH groups, respectively (Fig. 3E). The altered levels of hydrogen peroxide and nitrite are consistent with the changes in the amounts and activities of CYP2E1 and mitochondrial NOS in the different dietary groups.

3.3. Increased levels of oxidatively-modified proteins in alcohol-exposed rats

Because of increased oxidative/nitrosative stress in BASE-EtOH group, we then sought to identify the oxidatively-modified mitochondrial proteins in this group compared to those in other groups fed the various diets by a method newly developed in our laboratory [11,22]. Our 2D-PAGE results revealed that only a few oxidized mitochondrial proteins were detected in the livers of BASE-control group (Fig. 4A). Consistent with the elevated levels of ROS/RNS, the number and intensity of oxidatively-modified mitochondrial proteins were markedly increased in the livers of BASE-EtOH group (Fig. 4B). The overall pattern of oxidatively-modified proteins in ethanol-fed (the BASE-EtOH diet) Long-Evans rats are qualitatively very similar to those of Wistar rats that received chronic ethanol treatment [11]. However, the number and intensity of oxidized proteins were significantly decreased in PUFA-control and PUFA-EtOH groups, respectively (Fig. 4C and 4D).

3.4. Mitochondrial dysfunction following oxidative inactivation of mitochondrial enzymes

Since we consistently observed protein spots with multiple pI values with a similar molecular mass (47-kDa), we hypothesized that these multiple proteins spots were hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase [11], components of the tri-functional enzyme involved in the mitochondrial β -oxidation of fatty acids. To directly determine oxidative modification of 3-ketoacyl-CoA thiolase in BASE-EtOH group, the biotin-NM labeled (oxidized) mitochondrial proteins in different diet groups were purified by using streptavidin-agarose beads and subjected to immunoblot analysis with the specific antibody to this protein (kindly provided by Dr. Nancy E. Braverman). Fig. 4E shows that 3-ketoacyl-CoA thiolase was detected only in BASE-EtOH group but not in the other groups. Similarly, α -ATP synthase (57-kDa) was present only in BASE-EtOH group but not in the other groups (Fig. 4F). These results strongly suggest that the PUFA supplement efficiently prevented oxidative modifications of 3-ketoacyl-CoA thiolase and ATP synthase despite the ethanol treatment. Although we have not fully identified every oxidized mitochondrial protein in this study, it is likely that other mitochondrial proteins were oxidatively-modified in Base-EtOH group, as recently reported [11].

To study oxidative inactivation of 3-ketoacyl-CoA thiolase and ATP synthase, we measured their activities in different dietary groups. The 3-ketoacyl-CoA thiolase activities were 4.97 ± 0.50 , 2.74 ± 0.33 , 4.32 ± 0.43 , and 4.40 ± 0.43 units in BASE-control, BASE-EtOH, PUFA-control, and PUFA-EtOH groups, respectively (Fig. 5A). These results suggest that ethanol treatment decreased the activity of 3-ketoacyl-CoA thiolase by 45% in BASE group. However, the PUFA-diet protected against inactivation of 3-ketoacyl-CoA thiolase activity, possibly through an efficient blockade of oxidative modification of the enzyme. To further establish the role of oxidative inactivation of 3-ketoacyl-CoA thiolase, we incubated the mitochondria of BASE-EtOH group with 15 mM dithiothreitol (DTT) or sodium ascorbate (Asc) for 30 min at room temperature, before measuring the activity of 3-ketoacyl-CoA thiolase. Our results showed that the suppressed activity of 3-ketoacyl-CoA thiolase in BASE-EtOH group was significantly recovered following treatment with DTT or Asc, which effectively removes S-nitrosylated and/or S-glutathionylated Cys residues (Fig. 5B) [11].

The activity of mitochondrial ATP synthase was also decreased significantly by 68% in the BASE-EtOH compared to the BASE-control group. However, the ATP synthase activity in PUFA-EtOH group was comparable to that in the PUFA-control group (Fig. 6A). To further study the mechanism of reversible inhibition of ATP synthase with or without PUFA, mitochondrial proteins from different groups were immunoprecipitated with the specific anti- β -ATP synthase antibody followed by immunoblot analysis. The levels of β -ATP synthase were similar in all four diet groups (Fig. 6B, left panel). However, immunoblot analysis with anti-3-nitro-tyrosine (3-NT) antibody revealed that the 3-NT band was only detected in BASE-EtOH group. Its level in the other groups was low or undetectable under our conditions. These results strongly suggest that the active site Tyr residues of ATP synthase were likely nitrated in BASE-EtOH group and that PUFA prevented nitration of Tyr residues of this enzyme, most likely through prevention of increased levels of peroxynitrite.

The earlier results from this laboratory showed that ethanol exposure coupled with a low n-3 PUFA diet markedly increased the levels of lipid peroxides [7]. Since aldehyde dehydrogenase (ALDH) is involved in the metabolism of the lipid aldehydes [35], we tested whether mitochondrial ALDH2 was inactivated with corresponding elevation of a lipid peroxide MDA in the rats fed BASE-EtOH diet [11]. The hepatic levels of MDA in BASE-EtOH group was significantly elevated (by 75%) compared to that of BASE-control group (Fig. 7A). The level of MDA in PUFA-EtOH group was significantly lower than that in the BASE-EtOH group. The hepatic ALDH2 activity in Long-Evans rats fed BASE-control diet was 0.98 ± 0.08 units. The ALDH2 activity in BASE-EtOH group was significantly inhibited by approximately 60% compared to that in BASE-control group. However, the reduced ALDH2 activity was restored in PUFA-EtOH group. Oxidative inactivation of these mitochondrial enzymes and other enzymes not tested here could contribute to mitochondrial dysfunction in BASE-EtOH group and the PUFA diet prevents oxidative inactivation of these proteins.

4. Discussion

Ethanol-mediated liver damage progresses through the course of alcoholic fatty liver, alcoholic hepatitis, fibrosis and cirrhosis [9,15]. The severity and prognosis of ethanol-mediated liver diseases generally depend on the amount, frequency, and duration of alcohol intake, the presence of inflammation, diets, nutritional status and genetic make-up of the host. Because of the accumulation of the reducing equivalents in the cytosol following the metabolism of ethanol and acetaldehyde, the rates of fatty acid biosynthesis and subsequent esterification into triglycerols are markedly increased [15]. Recent biochemical data clearly showed that ethanol activates ACC, a major rate-limiting enzyme involved in fatty acid biosynthesis in the liver, through inhibiting the activity of AMP kinase, a master switch of intermediary metabolism [34]. In addition to increased rates of fat synthesis, alcoholic fatty liver could also result from

decreased fat transport to mitochondria and subsequent degradation. Accumulation of malonyl-CoA, following activation of ACC [34], can potentially inhibit carnitine palmitoyltransferase, resulting in reduced rates of fat transport to mitochondria. Furthermore, decreased fat degradation could be caused by oxidative inactivation of the enzymes involved in the degradation of fatty acids [10,11]. Therefore, this study was aimed at investigating the mechanism of the protective effect of a DHA-containing PUFA diet against alcoholic fatty liver [7,10,11,36–38] by specifically studying the key enzymes in fat oxidation and biosynthesis pathways as well as ROS/RNS production.

It is well-established that ethanol exposure inhibits the mitochondrial electron transport chain, producing more ROS production [12,13]. Ethanol can also induce and/or activate iNOS, which play a key role in worsening the alcohol-mediated hepatotoxicity [39–41]. In addition, chronic alcohol exposure induces (stabilizes) CYP2E1 protein mainly through interfering with ubiquitin-mediated proteasomal degradation of CYP2E1 protein [42]. All these data strongly indicate that ethanol directly or indirectly promotes the production of ROS/RNS, which further leads to the generation of the more toxic peroxynitrite (ONOO^-), which can nitrate and *S*-nitrosylate various proteins, leading to cellular damage [43]. Our results clearly show that DHA/AA diets restored the suppressed activities of 3-ketoacyl-CoA thiolase, ATP synthase, and ALDH2, most likely through preventing *S*-nitrosylation of Cys and/or nitration of Tyr residues. Furthermore, based on our result that PUFAs blocked the CYP2E1 protein stabilization (Fig. 3), we expect that PUFA may stimulate ubiquitin-dependent degradation of CYP2E1 and that PUFAs may restore the ubiquitin-dependent proteasomal activity by preventing oxidative inactivation of proteasome subunit proteins, as recently discussed [22]. Although this hypothesis needs further experimental verification, the reduction of ethanol-induced CYP2E1 by the PUFA diet represents the first report to our knowledge.

The molecular mechanism by which small amounts of DHA/AA exert their protective effects against liver steatosis is poorly understood. A PUFA diet may decrease the rates of fat biosynthesis while it increases the fat transport and degradation, all of which are significantly affected by alcohol administration. In addition, these PUFAs may restore the expression of the peroxisome proliferator activated receptor α , whose expression is dys-regulated by ethanol [44]. Alternatively, PUFAs may facilitate the uptake of fatty acids by directly activating the carnitine-palmitoyl transferase 1 for efficient fat oxidation [38]. Our current results (Fig. 2C and 3–5) demonstrated that PUFAs directly inhibit the fat biosynthesis pathway by blocking ethanol-mediated ACC activation and that PUFAs protect against oxidative inactivation of 3-ketoacyl-CoA thiolase and possibly other enzymes, though not measured in this study, involved in fat oxidation. Because we did not observe fulminant hepatitis accompanied by elevated transaminase activities in the current study, we do not expect that the DHA effect is due to suppressed production of pro-inflammatory cytokines such as $\text{TNF}\alpha$, as reported [45]. Based on these results, our study provides a new mechanism of regulating fatty liver by dietary manipulation through inhibition of CYP2E1 and iNOS, sources of ROS/RNS. The beneficial effects of small amounts of DHA/AA (PUFAs) in this study were contrary to the adverse effects of the extremely large amounts of PUFA from fish oil where the severity of alcohol-induced liver damage was worsened [21]. However, our current results are consistent with protective roles of physiologically relevant quantities of PUFA against alcoholic liver fibrosis or cirrhosis in non-human primates [7,46]. Therefore, our current data and the data from other investigators re-emphasize the importance of the total content and the type of fatty acids in developing or preventing alcohol-induced mitochondrial dysfunction and liver steatosis.

In conclusion, our current data strongly indicate beneficial effects of physiologically relevant amounts of the PUFAs (DHA and AA) on the incidence of alcoholic fatty liver in an animal model, most likely through prevention against increased ROS/RNS following alcohol exposure.

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Abbreviations

PUFA	polyunsaturated fatty acids
AA	arachidonic acid
DHA	docosahexaenoic acid
EFA	essential fatty acids
CYP2E1	ethanol-inducible cytochrome P450 2E1
iNOS	inducible nitric oxide synthase
MDA	malondialdehyde
ALT	alanine transaminase
AST	aspartate transaminase
H & E	hematoxylin-eosin
BAC	blood alcohol concentration
Biotin-NM	biotin- <i>N</i> -maleimide
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
ACC	acetyl-CoA carboxylase
ROS/RNS	reactive oxygen/nitrogen species
α-ATP synthase	ATP synthase α -subunit

β-ATP synthase

ATP synthase β-subunit

DTT

dithiothreitol

Asc

sodium ascorbate

3-NT

3-nitro-tyrosine

ALDH

aldehyde dehydrogenase

References

1. Das UN. Essential fatty acids: biochemistry, physiology and pathology. *Biotechnol J* 2006;1:420–439. [PubMed: 16892270]
2. Schaefer EJ, Lichtenstein AH, Lamon-Fava S, McNamara JR, Schaefer MM, Rasmussen H, et al. Body weight and low-density lipoprotein cholesterol changes after consumption of a low-fat ad libitum diet. *JAMA* 1995;274:1450–1455. [PubMed: 7474191]
3. Salem N Jr, Reyzer M, Karanian J. Losses of arachidonic acid in rat liver after alcohol inhalation. *Lipids* 1996;31:S153–S156. [PubMed: 8729111]
4. Salem, N., Jr; Olsson, NU. Abnormalities in essential fatty acid status in alcoholism. In: Yehuda, S.; Mostofsky, DI., editors. *Handbook of Essential Fatty Acid Biology: Biochemistry, Physiology and Behavioral Neurobiology*. Totowa: Humana Press; 1997. p. 67-87.
5. Joly J-G, Hetu C. Effects of chronic ethanol administration in the rat: relative dependency on dietary lipids - I. Induction of hepatic drug-metabolizing enzymes in vitro. *Biochem Pharmacol* 1975;24:1475–1480. [PubMed: 811224]
6. Reitz RC. The effects of ethanol ingestion on lipid metabolism. *Prog Lipid Res* 1979;18:87–115. [PubMed: 399682]
7. Pawlosky RJ, Flynn BM, Salem N Jr. The effects of low dietary levels of polyunsaturates on alcohol-induced liver disease in the Rhesus monkey. *Hepatology* 1997;26:1386–1392. [PubMed: 9397975]
8. Pawlosky RJ, Salem N Jr. Ethanol exposure causes a decrease in docosahexaenoic acid and an increase in docosapentaenoic acid in feline brains and retinas. *Am J Clin Nutr* 1995;61:1284–1289. [PubMed: 7762532]
9. Lands, W.; Pawlosky, RJ.; Salem, N, Jr. Alcoholism, antioxidant status, and essential fatty acids. In: Papas, AM., editor. *Antioxidant Status, Diet, Nutrition, and Health*. Boca Raton, FL: CRC Press; 1999. p. 299-314.
10. Cederbaum AI, Lieber CS, Beattie DS, Rubin E. Effect of chronic ethanol ingestion on fatty acid oxidation by hepatic mitochondria. *J Biol Chem* 1975;250:5122–5129. [PubMed: 1171098]
11. Moon KH, Hood BL, Kim BJ, Hardwick JP, Conrads TP, Veenstra TD, et al. Inactivation of oxidized and S-nitrosylated mitochondrial proteins in alcoholic fatty liver of rats. *Hepatology* 2006;44:1218–1230. [PubMed: 17058263]
12. Cunningham CC, Coleman WB, Spach PI. The effects of chronic ethanol consumption on hepatic mitochondrial energy metabolism. *Alcohol* 1990;25:127–136.
13. Venkatraman A, Landar A, Davis AJ, Chamlee L, Sanderson T, Kim H, et al. Modification of the mitochondrial proteome in response to the stress of ethanol-dependent hepatotoxicity. *J Biol Chem* 2004;279:22092–22101. [PubMed: 15033988]
14. De la Maza MP, Hirsch S, Nieto S, Peterman M, Bonout D. Fatty acid composition of liver total lipids in alcoholic patients with and without liver damage. *Alcohol Clin Exp Res* 1996;20:1418–1422. [PubMed: 8947319]
15. Lieber CS, DeCarli LM. Hepatotoxicity of ethanol. *J Hepatol* 1991;12:394–401. [PubMed: 1845298]

16. Cha MC, Jones PJH. Tissue fatty acid deposition is influenced by an interaction of dietary oil source and energy intake level in rats. *J Nutr Biochem* 1996;7:650–658.
17. Tsukamoto H, Tower SJ, Ciofalo LM, French SW. Ethanol-induced liver fibrosis in rats fed high fat diet. *Hepatology* 1986;6:814–822. [PubMed: 3758935]
18. Nanji AA, Mendenhall CL, French SW. Beef fat prevents alcoholic liver disease in the rat. *Alcohol Clin Exp Res* 1989;13:15–19. [PubMed: 2646971]
19. Ronis MJ, Korourian S, Zipperman M, Hakkak R, Badger TM. Dietary saturated fat reduces alcoholic hepatotoxicity in rats by altering fatty acid metabolism and membrane composition. *J Nutr* 2004;134:904–912. [PubMed: 15051845]
20. You M, Considine RV, Leone TC, Kelly DP, Crabb DW. Role of adiponectin in the protective action of dietary saturated fat against alcoholic fatty liver in mice. *Hepatology* 2005;42:568–577. [PubMed: 16108051]
21. Nanji AA, Zhao S, Sadrzadeh SMH, Waxman DJ. Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish-oil-ethanol fed rats. *Alcohol Clin Exp Res* 1994;18:1280–1285. [PubMed: 7847620]
22. Kim BJ, Hood BL, Aragon RA, Hardwick JP, Conrads TP, Veenstra TD, et al. Increased oxidation and degradation of cytosolic proteins in alcohol-exposed mouse liver and hepatoma cells. *Proteomics* 2006;6:1250–1260. [PubMed: 16408314]
23. McClain CJ, Kromhout JP, Peterson FJ, Holtzman JL. Potentiation of acetaminophen hepatotoxicity by alcohol. *J Amer Med Assoc* 1980;244:251–253.
24. Dean RA, Harper ET, Dumaual N, Stoeckel DA, Bosron WF. Effects of ethanol on cocaine metabolism: formation of cocaethylene and norcocaethylene. *Toxicol Appl Pharmacol* 1992;117:1–8. [PubMed: 1440602]
25. Zloch Z. Temporal changes of the lipid peroxidation in rats after acute intoxication by ethanol. *Z Naturforsch* 1994;49:359–362.
26. Olsson NU, Shoaf SE, Salem N Jr. The effect of dietary polyunsaturated fatty acids and alcohol on neurotransmitter levels in rat brain. *Nutr Neurosci* 1998;1:133–140.
27. Reeves PG, Nielsen FH, Fahet GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on reformulation of the AIN-76A rodent diet. *J Nutr* 1993;123:1939–1951. [PubMed: 8229312]
28. Lieber CS, DeCarli LM. Liquid diet technique of ethanol administration: 1989 update. *Alcohol Alcohol* 1989;24:197–211. [PubMed: 2667528]
29. Bligh EG, Dyer JW. A rapid method of total lipid extraction and purification. *Can J Biochem Phys* 1959;37:911–917.
30. Morrison WR, Smith LM. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. *J Lipid Res* 1961;5:600–608. [PubMed: 14221106]
31. Salem N Jr, Reyzer M, Karanian J. Losses of arachidonic acid in rat liver after alcohol inhalation. *Lipids* 1996;31:S153–S156. [PubMed: 8729111]
32. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria. Central role of complex III. *J Biol Chem* 2003;278:36027–36031. [PubMed: 12840017]
33. Ruwe WD, Bause L, Flemons WW, Veale WL, Pittman QJ. Alcohol dependence in the rat. An effective means of induction and assessment. *J Pharmacol Met* 1986;15:225–234.
34. You M, Matsumoto M, Pacold CM, Cho WK, Crabb DW. The role of AMP-activated protein kinase in the action of ethanol in the liver. *Gastroenterology* 2004;127:1798–1808. [PubMed: 15578517]
35. Hartley DP, Ruth JA, Petersen DR. The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. *Arch Biochem Biophys* 1995;316:197–205. [PubMed: 7840616]
36. Alwayn IP, Gura K, Nose V, Zausche B, Javid P, Garza J, et al. Omega-3 fatty acid supplementation prevents hepatic steatosis in a murine model of nonalcoholic fatty liver disease. *Pediatr Res* 2005;57:445–452. [PubMed: 15659701]
37. Geelen MJ, Schoots WJ, Bijleveld C, Beynen AC. Dietary medium-chain fatty acids raise and (n-3) polyunsaturated fatty acids lower hepatic triglycerol synthesis in rats. *J Nutr* 1995;125:2449–2456. [PubMed: 7562078]

38. Surette ME, Whelan J, Broughton KS, Kinsella JE. Evidence for mechanisms of the hypotriglyceridemic effect of n-3 polyunsaturated fatty acids. *Biochim Biophys Acta* 1992;1126:199–205. [PubMed: 1627623]
39. Baraona E, Zeballos GA, Shoichet L, Mak KM, Lieber CS. Ethanol consumption increases nitric oxide production in rats, and its peroxynitrite-mediated toxicity is attenuated by polyenylphosphatidylcholine. *Alcohol Clin Exp Res* 2002;26:883–889. [PubMed: 12068258]
40. McKim SE, Gabele E, Isayama F, Lambert JC, Tucker LM, Wheeler MD, et al. Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. *Gastroenterology* 2003;125:1834–1844. [PubMed: 14724835]
41. Venkatraman A, Shiva S, Wigley A, Ulasova E, Chhieng D, Bailey SM, et al. The role of iNOS in alcohol-dependent hepatotoxicity and mitochondrial dysfunction in mice. *Hepatology* 2004;40:565–573. [PubMed: 15349894]
42. Roberts BJ, Song BJ, Soh Y, Park SS, Shoaf SE. Ethanol induces CYP2E1 by protein stabilization. Role of ubiquitin conjugation in the rapid degradation of CYP2E1. *J Biol Chem* 1995;270:29632–29635. [PubMed: 8530344]
43. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990;87:1620–1624. [PubMed: 2154753]
44. Fischer M, You M, Matsumoto M, Crabb DW. Peroxisome proliferator-activated receptor α (PPAR α) agonist treatment reverses PPAR α dysfunction and abnormalities in hepatic lipid metabolism in ethanol-fed mice. *J Biol Chem* 2003;278:27997–28004. [PubMed: 12791698]
45. Schmocker C, Weylandt KH, Kahlke L, Wang J, Lobeck H, Tiegs G, et al. Omega-3 fatty acids alleviate chemically induced acute hepatitis by suppression of cytokines. *Hepatology* 2007;45:864–869. [PubMed: 17393517]
46. Lieber CS, Leo MA, Aleynik SI, Aleynik MK, DeCarli LM. Polyenylphosphatidylcholine decreases alcohol-induced oxidative stress in the baboon. *Alcohol Clin Exp Res* 1997;21:375–379. [PubMed: 9113278]

Fig. 1.

Comparison of liver histology scores of four different dietary groups. (A) photomicrographs (200 ×) after H & E staining of rat livers fed; a) BASE-Control diet, b) BASE-EtOH diet showing a high degree of hepatocellular cytoplasmic vacuolation (macrovesicular and microvesicular steatosis), c) PUFA-Control diet, and d) PUFA-EtOH diet with minimal changes. (B) Histopathology scores in four different groups were evaluated microscopically in a double-blind manner after H & E staining of liver specimens. Each bar represents the average score ± SD (n = 11–12/group). * and **, significantly different (* $p < 0.03$ and ** $p < 0.01$) from the BASE-EtOH group.

Fig. 2.

Biochemical analysis of fat accumulation. Hepatic levels of total triglyceride (A) and cholesterol (B) in the four dietary groups, as indicated ($n \geq 8$ per group) are presented. (C) Equal amounts of the hepatic cytosolic proteins (10 μ g/well) from the four dietary groups were separated on 12 % SDS-PAGE, transferred to PVDF-immunobilon membranes, and subjected to immunoblot analysis (IB) by using a specific antibody to phospho-ACC (top) or β -actin (bottom). (D) The density of each band was determined by using Image J (v.1.38) software. The relative density was calculated by the ratio of the band in BASE-EtOH group which shows the smallest value. *significantly different from the BASE-Control group at $p < 0.0005$; **significantly different from the BASE-EtOH group at $p < 0.0005$; *** significantly different

from the BASE-Control group at $p < 0.05$; ****significantly different from the BASE-EtOH group at $p < 0.05$; *****significantly different from the PUFA-Control group at $p < 0.05$.

Fig. 3.

Levels of mitochondrial CYP2E1, iNOS, nitrite concentration, NOS activity in rat livers from the four dietary groups. (A) Equal amounts of hepatic mitochondrial proteins (20 μg/well) from different groups were separated on 12 % SDS-PAGE, transferred to PVDF-immobilon membranes, and subjected to immunoblot analysis (IB) by using anti-CYP2E1, iNOS, and prohibitin antibody. (B) The activities of microsomal CYP2E1 (*, significantly different ($p < 0.025$) from the BASE-Control group; **, significantly different ($p < 0.01$) from the BASE-EtOH group.) and (C) mitochondrial NOS in different groups (*, significantly different ($p < 0.005$) from the BASE-Control group; **, significantly different ($p < 0.005$) from the BASE-EtOH group.) were determined (0.5 mg protein/assay) and compared. Mitochondrial proteins (0.5 mg protein/assay) were used to determine (D) the hydrogen peroxide production (*, significantly different ($p < 0.001$) from the BASE-Control group; ** and ***, significantly different ($p < 0.0005$ and $p < 0.005$) from the BASE-EtOH group.) using Amplex red dye in the presence of pyruvate (5.0 mM) and malate (2.0 mM) or (E) nitrite concentrations (* $p < 0.0005$) from the BASE-Control group; **, significantly different ($p < 0.00005$) from

the BASE-EtOH group). Each point represents the average $\pm$ S.D. of three determinations. This figure represents a typical result from two separate experiments.

Fig. 4. Comparison of oxidized mitochondrial proteins by 2D-PAGE in the four dietary groups. Oxidized mitochondrial proteins (10 mg/sample) from BASE-Control (A), BASE-EtOH (B), PUFA-Control (C), and PUFA-EtOH (D) were labeled with biotin-NM in the presence of DTT and purified with streptavidin-agarose. Purified biotin-NM labeled proteins (0.25 mg/sample) were resolved by 2D-PAGE, and silver stained. Squares are used as an internal standard for comparison of the different gels (A – D). These data represent similar results from two independent experiments. Another batch of purified biotin-NM labeled proteins were separated by 1D- PAGE and subjected to immunoblot analysis (IB) by using the specific antibody to 3-ketoacyl-CoA thiolase (E) or α -ATP synthase (F).

Fig. 5. Reversible inactivation of 3-ketoacyl-CoA thiolase in alcohol-exposed groups in the absence and presence of PUFA. (A) Mitochondrial 3-ketoacyl-CoA thiolase activities in four groups treated with different diets were determined and are presented. One unit of thiolase was defined as the amount of thiolase that catalyzes the cleavage of 1 nmol acetoacetyl-CoA to acetyl-CoA/min/mg protein at room temperature. (B) Mitochondrial proteins (1 mg/sample) from the BASE-EtOH group were incubated without and with 15 mM DTT or Asc for 30 min before 3-ketoacyl-CoA thiolase activity was determined. *, significantly different ($p < 0.005$) from the BASE-Control group; **, significantly different ($p < 0.01$) from the BASE-EtOH group.

Fig. 6.

Reversible inactivation of ATP synthase in the alcohol-exposed groups with and without PUFA. (A) Mitochondrial ATP synthase activities in the four dietary groups were determined by using an ATP bioluminescence assay kit (Roche, Mannheim, German) following the manufacturer's protocol. One unit of ATP synthesis activity represents 1 nM ATP produced/min/mg protein at room temperature. (B) Mitochondrial proteins (1 mg/sample) from the 4 different groups were immunoprecipitated (IP) with anti- β -ATP synthase antibody, as described [11]. Immunoprecipitated proteins from each group were then subjected to 12 % SDS-PAGE, transferred to Immobilon membrane, and immunoblot analysis (IB) with anti- β -ATP synthase (left) or anti-3-NT antibody (right). *, significantly different ($*p < 0.001$) from the BASE-Control group; ** and ***, significantly different ($**p < 0.0005$ and $***p < 0.01$) from the BASE-EtOH group.

Fig. 7.

Levels of malondialdehyde (MDA) and ALDH2 activity in ethanol-exposed groups with and without PUFA. (A) Levels of MDA (*, significantly different ($p < 0.005$) from the BASE-Control group; ** and ***, significantly different ($p < 0.05$ and $p < 0.01$) from the BASE-EtOH group.) and (B) mitochondrial ALDH2 activity (*, significantly different ($p < 0.001$) from the BASE-Control group; **, significantly different ($p < 0.005$) from the BASE-EtOH group.) in different groups were determined.

Table 1
Diet Composition

	Base-Control (en%)	Base-EtOH (en%)	PUFA-Control (en%)	PUFA-EtOH (en%)
Caloric Ingredients				
Protein	22.4	22.4	22.4	22.4
Carbohydrates	66.2	30.2	66.2	30.2
Fat (total)	11.4	11.4	11.4	11.4
<i>Essential fatty acids;</i>				
18:2n6	0.3	0.3	0.3	0.3
18:3n3	0.3	0.3	0.3	0.3
20:4n6	-	-	0.5	0.5
22:6n3	-	-	0.5	0.5
Ethanol	-	36.0	-	36.0
Total	100.0	100.0	100.0	100.0
<i>Non-caloric ingredients (g/L)</i>				
Stabilizing agents (propylene glycol alginate, xanthan gum, Tween 80)				3
Mineral mix (AIN-93M-MX)				8.8
Custom vitamin mix				2.5
Choline bitartrate				0.53
Cellulose fiber				10

* All values expressed in mg/L diet unless otherwise noted: Nicotinic acid, 7.5; Ca pantothenate, 4; Pyridoxine, 1.8; Thiamine, 1.5; Riboflavin, 1.5; Folic acid, 0.5; Biotin, 0.05; Choline, 250; Inositol, 25; para-aminobenzoic acid, 13; Vitamin B-12 (cobalamin), 5.6 (µg/L); Vitamin K, 125 (µg/L); Menadione, 83 (µg/L); Vitamin E, 2.3 (IU/L); Vitamin A, 903 (IU/L); Vitamin D, 400 (IU/L).

Table 2**Fatty Acid Composition of Rat Livers**

Fatty Acid	BASE-Control (mg/g wet tissue) (n=14, ±SEM)	BASE-EtOH (mg/g wet tissue) (n=14, ±SEM)*	PUFA-Control (mg/g wet tissue) (n=13, ±SEM)	PUFA-EtOH (mg/g wet tissue) (n=14, ±SEM)*
Total saturate	14.79 ± 1.15	17.41 ± 0.88	15.63 ± 1.12	14.39 ± 0.92
Total monoene	11.21 ± 1.10	13.53 ± 1.23	8.63 ± 0.85	7.72 ± 0.70
18:2n6	1.7 ± 0.12 ^e	2.9 ± 0.19 ^{def}	1.1 ± 0.10 ^f	1.7 ± 0.13 ^{cd}
20:4n6	4.2 ± 0.33 ^{cf}	4.6 ± 0.27 ^{ad}	8.1 ± 0.44 ^{df}	8.5 ± 0.41 ^{ac}
22:5n6**	0.05 ± 0.015	0.06 ± 0.016 ^{ab}	0.03 ± 0.003 ^a	0.02 ± 0.003 ^b
22:6n3	3.3 ± 0.22 ^{bc}	3.8 ± 0.19 ^a	4.1 ± 0.25 ^{ab}	3.8 ± 0.23

*a,b,c,d,e,f Pairwise significant differences are denoted by the same annotation letter: a, b, c, d, e, or f. For example, 18:2n6 values are significantly different ($p < 0.05$) between the BASE-Control and BASE-EtOH groups, both denoted with the letter “e”.

** A marker of n-3 fatty acid deficiency.

Table 3

The Plasma Levels of Transaminases

	BASE-Control (n=14)	BASE-EtOH (n=14)	PUFA-Control (n=13)	PUFA-EtOH (n=14)
ALT [*]	65 ± 3.8	65 ± 5.8	57 ± 4.6	68 ± 2.3
AST [*]	400 ± 28	470 ± 40	400 ± 26	460 ± 33

* Measured in plasma (mmol/L ± SEM).