ALCOHOLIC LIVER INJURY IN THE RAT IS ASSOCIATED WITH REDUCED
EXPRESSION OF PEROXISOME PROLIFERATOR-ALPHA (PPARα) REGULATED
GENES AND IS AMELIORATED BY PPARα ACTIVATION

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List of Abbreviations

ALD, alcoholic liver disease; L-FABP, liver fatty acid binding protein; PPAR, peroxisome proliferator-activated receptor; FACO, fatty acylcoa oxidase; FE, fish-oil-ethanol; FD, fish oil-dextrose; NFκB, nuclear factor kappa B; TNF, tumor necrosis factor; CYP2E1, cytochrome P4502E1; CYP4A, cytochrome P4504A; FE-C, fish oil-ethanol-clofibrate; COX, cyclooxygenase.

Keywords: Transcription factors, cytokines, Peroxisome-proliferator activated receptors; Liver disease; Fatty acids

Number of text pages 31

Number of tables 2

Number of figures 6

Number of references 73

Number of words in abstract 283

Number of words in introduction 688

Number of words in discussion 1,454
ABSTRACT

Alcoholic liver disease (ALD) is associated with a state of hepatic fatty acid overload. We examined the effect of ethanol and different types of dietary fat on the expression of mRNA for liver fatty acid binding protein (L-FABP), peroxisome proliferator-activated receptor-alpha (PPARα) and peroxisomal fatty acyl coA oxidase (FACO). Four groups of rats (n=5) were fed intragastrically, a liquid diet with or without ethanol (E) (10-16 g/kg/day) for 4 weeks. Pair-fed controls received isocaloric amounts of dextrose (D). The source of fat was either corn oil (C) or fish oil (F). Ethanol-fed rats developed fatty liver, necrosis and inflammation; the changes were more severe in the FE rats. PPARα mRNA levels were not different between groups although there was a trend towards increased levels in ethanol-fed rats. We calculated L-FABP/PPARα and FACO/PPARα ratios as a measure of FACO and L-FABP upregulation relative to PPARα expression. Both FACO/PPARα and L-FABP/PPARα ratios were significantly decreased in FE rats. However, only L-FABP/PPARα was decreased in CE rats. Also, the level of L-FABP/mRNA correlated inversely with the degree of fatty liver in ethanol-fed rats. Since expression of PPARα response genes was impaired in ethanol-fed rats, we determined whether activation of PPARα would normalize the PPARα-response and prevent the pathological changes in ethanol-fed rats. Treatment with clofibrate, a PPARα-activating ligand, led to a marked decrease in fatty liver and complete abrogation of necroinflammatory changes in FE rats. Also, NFκB-activation and upregulation of TNF-α and cyclooxygenase-2, was also abolished in clofibrate-treated rats. We conclude that adaptive gene regulation of FACO and L-FABP by PPARα is impaired in ethanol-fed rats and that treatment with clofibrate, a PPARα ligand, prevents alcohol-induced pathological liver injury, possibly by reversing the above changes.
Numerous experimental studies have shown that ethanol impairs mitochondrial oxidation of fatty acids and reduces the activity of some enzymes involved in the mitochondrial β-oxidation pathway (Lieber and Schmid, 1961; Reitz, 1979; Fromenty, et al., 1995; Eaton et al., 1996). The importance of fatty acid utilization pathways in the maintenance of cellular lipid homeostasis in alcohol-fed rats is underscored by the dramatic pathological consequences in the liver that characterize alcoholic liver disease. Inhibition of mitochondrial fatty acid metabolism, particularly in the face of enhanced fatty acid mobilization, results in enhanced oxidation of fatty acids by extramitochondrial pathways in the endoplasmic reticulum and peroxisomes (Guzman and Geelan, 1993; Lieber, 1994). The oxidation of fatty acids in the endoplasmic reticulum involves the initial formation of ω-hydroxy or (ω-1)-hydroxy fatty acids catalyzed by microsomal cytochromes P450, mainly those of the 4A (CYP4A) sub-family (Sharma et al., 1989; Mortenson, 1990) and cytochrome P450 2E1 (CYP 2E1) (Amet et al., 1994; Fukuda et al., 1994). Dicarboxylic fatty acids, derived from ω-hydroxy fatty acids, are increased in states of impaired mitochondrial fatty acid β-oxidation (Mortensen, 1990; Ma et al., 1993). Also, microsomal ω-hydroxylation via CYP 2E1 and CYP 4A1 is increased in alcohol-fed rats (Amet et al., 1994; French et al., 1997; Amet et al., 1998). Importantly, increased generation of dicarboxylic acids because of enhanced lauryl ω-hydroxylase activity and failure of ethanol to induce acyl-CoA oxidase leads to augmented production of the potentially toxic dicarboxylic acids in both rats and man. (Wan et al, 1995, Ma et al, 1999)

Considerable evidence points to the fact that fatty acids (Forman, et al, 1997; Kliewer et al., 1997) as well as their ω-oxidation products (Gibson, 1992; Bass, et al., 1996) activate peroxisome proliferator-activated receptors (PPARs). PPARs are members of the nuclear receptor superfamily that includes that steroid, thyroid hormone and retinoid receptors (Kersten et al., 2000; Vamecq 1999). PPARs are ligand-activated transactivating factors that enhance the transcription of a variety
PPAR – alpha and alcoholic liver injury

of target genes involved in key steps of lipid metabolism (Vanden-Heuvel, 1999). RXRα is the required heterodimeric partner of PPARα and thus RXR and PPAR are actively involved in regulating genes in fatty acid metabolism which include enzymes of the extramitochondrial fatty acid oxidation pathways such as peroxisomal fatty acyl CoA oxidase (FACO), cytochrome P450 4A1 (CYP 4A1) and liver fatty acid binding protein (L-FABP) (Kaikaus et al., 1993; Vanden-Heuvel, 1999, Wan et al, 2000). The PPAR isoform PPARα predominates in the liver (Braissant, et al., 1996). The induction of hepatic extramitochondrial pathways of fatty acid oxidation via PPARα serves to provide the liver cell with alternative means for the catabolism of fatty acids under conditions of marked increased fatty acid flux and fatty acid “overload”. L-FABP is clearly integral to this response and may act to reduce the toxicity of long-chain unesterified fatty acids by binding them in the cytosolic compartment and facilitating their intracellular diffusion and utilization (Bass, 1992; Bass et al., 1993 a.c; Glatz et al., 1996;; Weisiger, 1996). Thus, PPARα appears to act as cellular transducer that senses the presence of fatty acid “overload” states and directs the appropriate adaptive hepatocellular gene response.

In this study, we initially investigated the expression of PPARα, L-FABP and FACO in alcohol-fed rats. For this purpose, we used the intragastric feeding rat model of alcoholic liver disease in which feeding dietary polyunsaturated fatty acids with ethanol results in the development of fatty liver, necrosis and inflammation (Nanji et al., 1989a,b; Nanji, et al., 1994a). In particular feeding fish oil as the source of polyunsaturated fatty acids causes more severe liver injury than when corn oil is fed with ethanol (Nanji et al., 1994b). We observed that expression of PPARα -response genes was impaired in animals exhibiting pathological liver damage. We therefore reasoned that strong activation of PPARα would normalize the PPARα-mediated gene response and prevent the pathological changes associated with liver injury in alcohol-fed rats. We use clofibrate, a potent
PPAR – alpha and alcoholic liver injury

PPARα ligand/activator, to test this hypothesis. Our findings indicate that pharmacological PPARα activation can prevent not only the pathological manifestations of alcohol-induced perturbations in fatty acid metabolism but also alcohol-induced biochemical and histological inflammatory changes in liver.
MATERIALS AND METHODS

Animal Model

The experimental animals were male Wistar rats weighing between 225 and 250g. In the first set of experiments, four groups of rats (five rats per group) were fed by continuous infusion of liquid diet through permanently implanted gastric tubes as described previously (French et al., 1986; Tsukamoto et al., 1990). The diets contained corn oil plus dextrose (CD) or ethanol (CE) or fish oil plus dextrose (FD) or ethanol (FE). Dietary fatty acids contributed 35% of total calories. The diet also contained protein, minerals and vitamins as described previously (French et al., 1986; Tsukamoto et al., 1990). The amount of ethanol administered was initially 10 g/kg/day and increased up to 16 g/kg/day as tolerance developed. Blood alcohol levels were maintained between 150 and 350 mg/dL. All animals were killed four weeks after the start of feeding. The care of animals and euthanasia procedures were in accordance with the National Institutes of Health criteria for use of laboratory animals.

In the second part of the study, 4 groups of rats (6 rats/group) were studied to evaluate the effect of clofibrate on the pathological and biochemical changes. Rats in the first group were fed fish oil and ethanol FE for 4 weeks, the second group was administered FE plus clofibrate (FE-C) and the third group fish oil dextrose plus clofibrate (FD-C). The control group was fed fish oil and dextrose. Clofibrate (given as clofibric acid, dissolved in 2% Tween 80) was given daily via intragastric tube at a dose of 100 mg/kg body weight. After killing, a portion of the liver was obtained for histopathological analysis and the remainder was placed in liquid nitrogen and stored at -80°C until analysis.
Histopathology

A small sample of the liver was obtained and formalin-fixed when the rats were killed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the percentage of liver cells containing fat) was scored 1+ with <25% of the cells containing fat; 2+, with 26%-50% of the cells containing fat; 3+, with 51%-75% of the cells containing fat; and 4+, with >75% of the cells containing fat. Necrosis was quantified as the number of necrotic foci per square millimeter, and inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample liver. The pathologist evaluating the sections was unaware of the treatment groups when assessing the histology.

RNA Preparation and Northern Blots

[α-32P]-dCTP (3000 Ci/mol) was obtained from Amersham (Arlington Heights, Il). The cRNA probe for L-FABP were prepared from plasmid pTZ 18R-L-FABP as previously described (Kaikus et al., 1993). For transcription of the cRNA for ribosomal protein S14, the cDNA for human ribosomal protein S14 was excised from plasmid pCS14-19 (Rhoads et al., 1986) with PstI and ligated in sense orientation into the unique PstI site in vector pGEM-4Z (Promega). For the synthesis of cRNA probes for rat FACO and PPARα, the following plasmids were constructed. A 501-based pair fragment of the rat peroxisomal fatty acyl CoA oxidase cDNA encompassing bases 268-769 from the transcription start site was synthesized by reverse transcription PCR as previously described (Kaikus et al., 1993) and directly cloned into pCR™II (Invitrogen) using the protocol suggested by the manufacturer. A 568-base pair rat PPARα cDNA encoding the ligand-binding domain of the receptor (Gottlicher et al., 1992) was synthesized from total rat liver RNA by reverse transcription PCR for 30 cycles in a 9600 Thermcycler (Perkin-Elmer Cetus Instruments), using
primers GCCAGTGCATGTCCGTGGACA (upstream, nucleotides 993-1016) and TCTGTAGATCTCTTGCAACAGTGG (downstream, nucleotides 1561-1538). The cDNA was purified by gel electrophoresis, blunted with Klenow fragment, end-adapted with EcoRI linkers and ligated into the EcoRI site of plasmid pGEM-11Zf(+) (Promega) as previously described (Rhoads et al., 1986). Following amplification of plasmid constructs in *Escherichia coli* JM101, orientation was determined by restriction mapping and the antisense [α-32P]-dCTP-labeled cRNA probes were transcribed from the linearized plasmids using either T7 or SP6 DNA polymerase, depending on cDNA orientation, using a MAXIscript™ in vitro transcription kit (Ambion, Austin, Texas) as described (Enoch et al., 1986; Wang et al., 1989).

Total RNA was extracted from 0.05-0.1 g pieces of liver following homogenization in 5 volumes of Trizol reagent (Gibco BRL) according to the method of Chiomczynski and Sacchi (1997). Total cellular RNA (10-20 µg) was fractionated on a 0.8% agarose-2.2 M formaldehyde gel, and blotted on nylon membranes (*Hybond™*-N, Amersham) by capillary transfer. Hybridization to cRNA probes was performed as previously described (Kaikaus et al., 1993; Enoch et al., 1986). Hybridizations with the RNA blots were carried out overnight at 50ºC except in the case of the L-FABP probe for which hybridizations were conducted at 65ºC, and the final wash was also at 65ºC. Membranes were washed once for 15 min at room temperature in 2x SSC, 0.1% SDS and then for 15 min at room temperature in 1x SSC, 0.1% SDS. Normalization for input RNA was done by rehybridizing the blots with the ribosomal protein S14 cRNA probe. Prior to reprobing, membranes were stripped by washing with H₂O at 100ºC. Autoradiography was performed by exposing hybridized blots to Kodak X-OMAT AR for 24h. Signal densities for each mRNA were quantified by densitometric analysis of the autoradiograms. Complete removal of probes was confirmed autoradiographically.
Determination of Thiobarbituric Acid-Reactive Substances and Conjugated Dienes

Levels of liver thiobarbituric acid-reactive substances (TBARS) were measured according to the method of Ohkawa et al (Ohkawa et al., 1979). Conjugated dienes in the total lipid extracted from liver homogenates were identified by their optical density of between 220 and 300 nm as previously described (Nanji et al., 1995).

Determination of NF-κB Binding Activity and IκB-α Protein Levels in Liver

Electrophoretic mobility shift assays (EMSA) were used to determine the binding activity of NF-κB and were performed essentially as described in prior studies (Liu et al., 1995; Lin et al., 1997; Nanji et al., 1999). Equal amounts of nuclear protein were incubated with a 5’ – [32P] labeled oligonucleotide containing the NF-κB consensus site. The incubation mixtures were separated in a 7% nondenaturing polyacrylamide gel and bands were detected by autoradiography. The specificity of binding was determined by prior addition of 100-fold excess of unlabeled competitor consensus oligonucleotide and supershift analysis (Nanji et al, 1999).

Western blot analysis for IκB-α was conducted using 50 µg of cytosolic protein. Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and proteins were then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Sigma). Membranes were incubated with the primary antibody against IκB-α (Santa Cruz Biotechnology) at a dilution of 1:500 in 1% nonfat milk Tween-phosphate buffered saline. Membranes were then incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G) at a dilution of 1:10,000.
Determination of mRNA Levels for TNF-α, COX-2, COX-1, and β-actin

To examine the expression of COX-1, COX-2, TNF-α and β-actin in liver tissue, total RNA was isolated according to the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1997). The integrity of RNA was assessed by agarose gel electrophoresis and ethidium bromide staining. Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described (Nanji et al., 1997a,b). The sequences of primer pairs, 5’ and 3’, and predicted sizes of the amplified PCR fragments of COX-1, COX-2, TNF-α and β-actin have been reported (Nanji et al., 1994a,b; Nanji et al., 1997a,b; Nanji et al., 1997; Kono et al., 2000). PCR products and molecular size markers were subjected to electrophoresis on 1% agarose gels and visualized by means of ethidium bromide staining. Each experiment included a negative control (sample RNA that had not been subjected to RT). This sample did not yield a PCR product confirming the absence of extraneous genomic DNA or PCR products contaminating the samples. Varying the number of PCR cycles did not change the relative differences between the samples indicating that the PCR conditions were not within the plateau phase of amplification. All amplification reactions of one experiment were performed in parallel in the same heating block to ensure compatible conditions.

Statistical Analysis

Data are presented as mean ± SD. Statistical comparison between different groups was done using analysis of variance (ANOVA) using the Student-Newman-Keuls or Bonferroni Multiple comparison post-hoc tests as applicable. For comparisons of pathological scores, the Mann-Whitney test was used. A p value of <0.05 was considered significant.
RESULTS

Liver histopathology

In the first experiment where rats were fed different dietary fats with ethanol or dextrose, there was no difference in the amount of weight gain as seen in the different groups (data not shown). There was also no difference in the blood alcohol levels between the ethanol-treated groups. The details of severity of liver injury in the ethanol-treated groups is shown in Table 1. Rats fed fish oil and ethanol (FE) showed the most severe liver injury (figure 1). None of the animals in the control dextrose-fed groups showed any evidence of pathological injury.

Alterations in the expression of PPARα and PPARα-regulated genes

Changes in the mRNA levels for PPARα, CYP4A1, FACO and L-FABP, corrected for ribosomal S14 mRNA levels, are shown in Figure 2. Neither PPARα nor FACO showed any significant changes between groups, although there were trends toward increased PPARα in the alcohol-fed animals and toward increased FACO in the CE rats. CY4A1 mRNA levels were significantly higher in the CE, FD, and FE groups compared to the CD group. L-FABP mRNA was significantly reduced in FE rats compared with the FD group (p<0.05).

Expression of L-FABP in relationship to the severity of fatty liver

L-FABP, by promoting cellular transport and utilization of fatty acids, may affect the amount of fat accumulation in the liver in response to ethanol feeding. To assess a role for L-FABP in the pathogenesis of fatty liver, the level of L-FABP mRNA was correlated with the severity of fatty liver in ethanol-fed rats. The lowest level of L-FABP expression was seen in FE rats, the group that developed the most severe degree of fatty liver (Figure 3). For all the ethanol-fed animals, a significant inverse correlation was seen between the degree of fatty liver and L-FABP mRNA
(r=0.86, p<0.01) (Figure 3). Dextrose-fed animals were not included in the correlation analysis since none of the animals in these groups developed fatty liver.

**Relationship between PPARα, FACO, and L-FABP expression**

Since the expression of FACO and L-FABP is regulated by PPARα, we calculated FABP/PPARα and FACO/PPARα ratios in the different experimental groups to determine the relative magnitude of FACO and L-FABP mRNA responses to changes in PPARα mRNA abundance. (Figure 4). The lowest FACO/PPARα ratio was seen in the FE group which was significantly decreased compared to the other (p<0.05) groups. The L-FABP/PPARα ratios were decreased in both ethanol-fed groups, with a significant reduction in both the CE and FE groups compared to dextrose-fed controls (Figure 4). Thus in the corn oil-ethanol group, only the L-FABP/PPARα ratio was decreased whereas in the fish oil-ethanol groups, both the L-FABP/PPARα and FACO/PPARα ratios were decreased.

**Clofibrate treatment significantly reduced the severity of fatty liver and lipid peroxidation in ethanol-fed rats**

As shown in the first part of the study, feeding the fish oil-ethanol diet caused severe fatty liver, necrosis and inflammation (Table 2). Treatment with clofibrate led to a marked decrease in the severity of fatty liver and inflammation (Figure 5). Additionally, clofibrate treatment decreased levels of conjugated dienes and TBARS in ethanol-fed rats (Table 2). There were no pathological changes seen in the fish oil-dextrose and fish oil-dextrose-clofibrate treated rats. We have also previously shown that Tween-80 does not induce liver pathology in rats and has no ameliorating effect on liver pathology in fish oil-ethanol-fed rats (unpublished observations).
Clofibrate treatment abrogates necroinflammatory changes, NF-κB activation and upregulation of TNF-α and COX-2

Rats fed fish oil and ethanol developed necrosis and inflammation which was prevented in rats treated with clofibrate (Table 2). To evaluate activation of NF-κB, electrophoretic mobility shift assay of nuclear extracts from whole liver were carried out. NF-κB activation was increased in fish oil-ethanol-fed rats; activation was absent in the clofibrate and dextrose-fed groups (Figure 6). To confirm the specificity of the protein/DNA complex, a 100-fold excess of unlabeled NF-κB or STAT oligonucleotide was added to the EMSA binding reaction (Nanji et al, 1999). Addition of NF-κB oligonucleotide abrogated complex formation, addition of STAT3 oligonucleotide had no effect (data not shown).

Effect of clofibrate on COX-2 and TNF-α

We have previously proposed that COX-2 and TNF-α contribute to alcohol-induced pathological changes in the liver (Nanji et al., 1994a; Nanji et al., 1997a). Thus, we also measured the effect of clofibrate on levels of mRNAs for TNF-α, COX-2, COX-1 and β actin. Since the level of the mRNAs are too low to be detected by Northern blot or ribonuclease protection assay (Nanji et al., 1994a), we used RT-PCR for these measurements. Rats fed fish oil and ethanol showed increased levels of COX-2 and TNF-α mRNAs, treatment with clofibrate downregulated their expression (Figure 6B). The level of COX-1 mRNA, the constitutive isoform of COX was similar in all groups.
DISCUSSION

Alcohol is one of many known causes of impaired mitochondrial fatty acid oxidation (Fromenty and Pessayre, 1995). It has been suggested that impaired mitochondrial oxidation of long-chain fatty acid results in “fatty acid overload,” whereby fatty acids that accumulate in the cell are diverted into esterification (triglyceride synthesis) and extramitochondrial fatty acid oxidation pathways (Kaikaus et al., 1993; Ockner et al., 1993). The latter consist of ω-oxidation in the endoplasmic reticulum and β-oxidation in the peroxisomes. The fatty acid overload hypothesis indicates a role for long-chain fatty acids in producing the hepatotoxic sequelae associated with conditions such as alcoholism which cause impaired fatty oxidation.

Effect of alcohol on PPARα regulated genes

Omega (ω) or omega (ω-1) hydroxylation of fatty acids is catalyzed by CYP 4A1 and CYP 2E1 (Amet et al., 1993, 1994). Both CYP 2E1 and CYP 4A1 are increased in rats fed ethanol and polyunsaturated fatty acids and are expressed in a predominantly perivenular distribution (Tsutsumi et al., 1989; Bell et al., 1992) which is also the site at which the most severe pathological injury occurs in alcoholic liver disease (Lieber, 1994). Alcohol induced perturbations in cytochrome P450-dependent metabolism of fatty acids, is therefore of considerable interest because metabolites of fatty acids generated via P450-dependent oxidative pathways, such as dicarboxylic acids and other long-chain fatty acid metabolites, are potential regulators of gene expression (Duplus et al., 2000).

A key mechanism by which fatty acids and fatty acid metabolites affect gene expression is via the activation of PPARs, which are members of the nuclear receptor superfamily of transactivating factors that mediate the pleiotropic hepatic response to peroxisome proliferator xenobiotics (Kersten et al., 2000; Vamecg 1999; Vanden-Heuvel, 1999). Several lines of evidence suggest that PPARα,
the major isoform in liver, induces enzymes of the peroxisomal \( \beta \)-oxidation pathway and L-FABP. (Duplus et al., 2000). Thus PPAR\( \alpha \) acts as a cellular “lipostat” which tranduces alterations in levels of cellular fatty acids to regulation of genes involved in the utilization of fatty acids. In alcohol-fed rats, therefore, PPAR\( \alpha \)-mediated upregulation of FACO and L-FABP would appear to be an appropriate and important adaptive response in the prevention of cell toxicity resulting from the accumulation of free fatty acids. Scrutiny of the FACO/PPAR\( \alpha \) and L-FABP/PPAR\( \alpha \) mRNA ratios in the ethanol-fed rats provides evidence for an impaired PPAR\( \alpha \)-mediated upregulation of FACO and L-FABP (Figure 4). In particular, rats that had the most severe liver injury (fish oil and ethanol) had decreased rations of both FACO/PPAR\( \alpha \) and L-FABP/PPAR\( \alpha \). In contrast, rats fed corn oil and ethanol had less severe liver injury and had a decreased FACO/PPAR\( \alpha \) ratio only. Furthermore, an inverse correlation between L-FABP and degree of fatty liver was observed (Figure 3). L-FABP is believed to facilitate the intracellular transport and utilization of long chain fatty acids and protects cells from the deleterious effects of free fatty acids (Bass, 1996; Fan et al., 1998). Furthermore, L-FABP may serve to increase the threshold for fatty acid and fatty acyl metabolite activation of PPAR\( \alpha \) and thus prevent chronic induction of the peroxisomal \( \beta \)-oxidation pathway and generation of toxic oxygen species (Bass, 1996).

In support of the interpretation of the results of this study, reduced expression of genes controlled by PPAR\( \alpha \) is seen in PPAR\( \alpha \)-null mice (Lee et al., 1995) which also show markedly increased liver fat accumulation with fasting (Hashimoto et al., 2000) and aging (Costet et al., 1998). In addition, mice with homozygous disruption of the gene expressing FACO develop steatohepatitis at an early age (Fan et al., 1998). Our data point to a similar scenario in alcohol-fed rats where a failure of upregulation (or even downregulation) of PPAR\( \alpha \)-responsive genes under conditions of alcohol-related increased fatty acid flux in the liver may contribute to the observed pathological changes in
the liver. The recent finding that ethanol impairs induction of PPARα controlled genes in hepatoma cells (Galli et al., 2001) is consistent with our in-vivo observations. Also consistent with our observations is the study by Fischer et al (2003) who evaluated the effect of ethanol feeding on PPARα mediated responses in C57BL/6J mice. Ethanol decreased PPARα/retinoid X receptor α binding binding in electromobility shift assays in liver nuclear extracts. MNRA levels for PPARα regulated genes such as long chain and medium chain acyl CoA dehydrogenases were reduced or failed to be induced in the rate of fatty oxidation. The mice showed development of fatty liver only, which is in contrast to the presence of fatty liver necrosis inflammation and fibrosis seen in the rat intragastric feeding model used in the current study.

**Effect of clofibrate on fatty liver alcohol-fed rats**

Based on the observation in the present study that decreased levels of PPARα-responsive genes contribute or are at least permissive to alcoholic liver injury, it was of interest to determine whether administration of clofibrate, a potent activator of PPARα, would ameliorate the adverse effects of alcohol on the liver. Clofibrate, in addition to being a PPARα ligand, also stimulates the transport of fatty acids in hepatocytes (Milliano et al., 2001) and increases levels of L-FABP (Kaikaus et al., 1993). Clofibrate administration to ethanol-fed rats led to a marked decrease in the degree of fatty liver (Table 2 and Figure 5). Although we did not directly evaluate PPARα-regulated gene expression in the clofibrate-treated rats, it is reasonable to assume in light of our previous and other studies (Bass et al., 1989; Kaikaus et al., 1993; Milliano and Luxon, 2001; Luxon and Milliano, 2000) that clofibrate treatment restored the parameters such as L-FABP and FACO that are downregulated by ethanol.
Effect of clofibrate on inflammatory changes in liver

It has been shown that PPARα may also be involved in modulating the inflammatory response. Mice lacking PPARα display a prolonged response to inflammation indicating that PPARα has an anti-inflammatory action (Kersten et al., 2000). Oxidant stress in alcoholic liver injury results in activation of NF-κB and upregulation of pro-inflammatory mediators such as TNF-α and COX-2 (Nanji et al., 1999). The studies presented herein demonstrated that administration of a specific PPARα activator reduced the ethanol-induced inflammatory changes, elevation in NF-κB activity and expression of TNF-α and COX-2. Activation of PPARα has previously been shown to decrease activation of NF-κB and inflammatory cytokine production as a result of oxidative stress (Poynter and Daynes 1998). Fibrates inhibit activation of NF-κB by inducing IκBα expression (Delerive et al., 2000). The results in the present study show that a reduction in NF-κB activity in clofibrate-treated-rats was accompanied by stabilization of IκBα; whether the absence of changes in the amount IκBα in the livers of clofibrate-treated rats is due to failure of degradation or increased synthesis cannot be deduced from these experiments. Downregulation of NF-κB activation was accompanied by a decrease in the NF-κB regulated genes, TNF-α and COX-2.

Relevance to human alcoholic liver disease

The relevance of our findings to human alcoholic liver disease needs to be considered. Human and rodent responses to inhibition of mitochondrial fatty acid oxidation are different (Vamecg et al., 1999; Galli and Crabb, 2000). For example, in rodents the initial insult is counteracted by strong peroxisomal and moderate mitochondrial induction of β-oxidation pathways (Vamecg and Lautruffe, 1999). In humans, strong induction of mitochondrial β-oxidation occurs but peroxisomal β-oxidation is not induced (Vamecg and Lautruffe, 1999). A marked difference between rodents and humans in the response of hepatocyte genes to PPARα activation is well-recognized (Chevalier
and Roberts, 2000). Reasons for the lack of responsiveness of human liver to potent PPARα activators include an order of magnitude lower level of PPARα expression in liver (Palmer et al., 1998), and, at least in the case of FACO, an inactive PPRE motif within the gene promoter (Lambe et al., 1999). There is also evidence for an increased frequency of a functional PPARα mutation in humans with non-alcoholic steatohepatitis (Merrimen et al., 2001). Thus, available evidence, including the results of the present study, supports a protective role for the PPARα-mediated gene response in liver under conditions of fatty acid overload. This adaptive transcriptional pathway may be less robust in humans than in rodents, while varying degrees of impairment of this mechanism may be a determinant of individual susceptibility to fatty liver disease. The role of impaired peroxisomal β-oxidation and disturbances in PPARα inducible genes in steatosis and steatohepatitis has been reviewed recently (Reddy, 2001).

In summary, we have shown that peroxisomal β-oxidation and L-FABP responses to PPARα are impaired in ethanol-fed rats and that the severity of fatty liver correlates inversely with the level of L-FABP. Treatment with clofibrate, a potent PPARα activating ligand, prevented ethanol-induced oxidative stress, fat accumulation and inflammatory changes in the liver. If the results shown here for rats hold true for humans, the pharmacological manipulation of PPARα might allow for the treatment of ALD.
REFERENCES


Fan CY, Pan J, Ususa N, Yeldandi AV, Rao MS, Reddy JK. (1998) Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-


Gottlicher M, Widmark E, Li Q, Gustafsson JA. Fatty acids activate a chimera of the clofibrate acid-activated receptor and the glucocorticoid receptor. Proc Natl Acad Sci USA 89:4653-4657, 1992.


TABLE 1

Pathological Changes in the Ethanol-Fed and Control Rats

<table>
<thead>
<tr>
<th>GROUP (n=5)</th>
<th>FATTY LIVER (0 – 4)</th>
<th>NECROSIS (foci/mm²)</th>
<th>INFLAMMATION (cells/mm²)</th>
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</thead>
<tbody>
<tr>
<td>CORN OIL + DEXTROSE</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>CORN OIL + ETHANOL</td>
<td>2.4 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.9 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>FISH OIL + DEXTROSE</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FISH OIL + ETHANOL</td>
<td>3.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>23.2 ± 8.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> p<0.01 vs. dextrose-fed groups

<sup>b</sup> p<0.01 vs. corn-oil ethanol
TABLE 2

Pathological Changes and Lipid Peroxidation Conjugated Dienes, A232, and TBARS in the Different Experimental Groups

<table>
<thead>
<tr>
<th>GROUP (n=5)</th>
<th>FATTY LIVER (0 – 4)</th>
<th>NECROSIS (foci/mm²)</th>
<th>INFLAMMATION (cells/mm²)</th>
<th>A232 (MMOL/MG PROTEIN)</th>
<th>TBARS (MMOL/MG PROTEIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH OIL – ETHANOL (FE)</td>
<td>4.0 ± 0.0ª</td>
<td>1.5 ± 0.3ª</td>
<td>28.3 ± 5.9ª</td>
<td>0.83 ± 0.17ª</td>
<td>1.54 ± 0.41ª</td>
</tr>
<tr>
<td>FE + CLOFIBRATE</td>
<td>1.3 ± 0.4</td>
<td>0.2 ± 0.1</td>
<td>3.2 ± 1.6</td>
<td>0.46 ± 0.11</td>
<td>0.79 ± 0.21</td>
</tr>
<tr>
<td>FISH OIL – DEXTROSE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.36 ± 0.09</td>
<td>0.62 ± 0.11</td>
</tr>
<tr>
<td>FD + CLOFIBRATE</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.33 ± 0.07</td>
<td>0.68 ± 0.13</td>
</tr>
</tbody>
</table>

ª p<0.01 vs. other groups
FOOTNOTE

Financial Support

This study was supported in part by a grant from the National Institute of Alcoholism and Alcohol Abuse (NIAAA) (AA12893). Dr. Kalle Jokelainen was supported by grants from the Academy of Finland, Finnish Cultural Foundation and Yrjö Jahnsson Foundation. We thank Diana Peters, Timothy Cloutier and Lili Miao for technical assistance.
LEGENDS TO FIGURES

Figure 1
Liver section from a rat fed fish oil and ethanol for four weeks showing the presence of fatty liver, necrosis and inflammation (Hematoxylin and eosin, magnification x155).

Figure 2
Levels of mRNA for PPARα and its target genes (FACO, CYP4A1 and L-FABP) in the different experimental groups. Total cellular RNA was isolated from the liver and was used to perform the Northern blot analysis. The signals on the Northern blot autoradiograms were quantified by laser densitometry. Values were normalized to the signals obtained with the 14s ribosomal cRNA probe. The bars represent the mean ±SD of five animals from each group. The asterisk denotes a statistically significant difference (p<0.05) compared with the values obtained in the other treatment groups for L-FABP mRNA, CYP4A1, and FACO

Figure 3
A correlation between the degree of fatty liver (graded 0-4 depending on the percentage of liver cells containing fat) and L-FABP mRNA in the 10 rats fed ethanol with either corn oil or fish oil. A significant inverse correlation (r= -0.86, p<0.01) was obtained between the degree of fatty liver and L-FABP mRNA.

Figure 4
L-FABP/PPAR and FACO/PPAR ratios in the different experimental groups. In both corn oil- (CE) and fish oil-ethanol (FE) fed groups, the L-FABP/PPAR ratio was decreased compared to the respective dextrose-fed controls (p<0.01). Only the FE group showed a significant decrease in the FACO/PPAR ratio (p<0.05). *p<0.01, †p<0.05.
Figure 5

A) Liver section from a rat fed fish oil-ethanol with clofibrate showing a reduction in the degree of fatty liver, necrosis and inflammation in comparison to the liver obtained from a rat fed fish oil and ethanol (Figure 1). Hematoxylin and eosin, X155.

B) Liver section from a rat fed fish oil-dextrose and clofibrate showing absence of any pathological changes. Rats fed fish oil and dextrose also showed absence of pathology (Figure not shown). Hematoxylin and eosin X155.

Figure 6

A) Representative electrophoretic mobility shift assay for NF-κB and Western blot analysis for IKBa. Increased NF-κB binding activity was seen in rats fed fish oil and ethanol (FE). Binding activity was absent or markedly reduced in fish oil-ethanol rats treated with clofibrate (FE-C), dish-oil dextrose fed rats (FD) and FD rats treated with clofibrate (FD-C). IKBa in cytosol was reduced in the fish oil-ethanol group compared with other groups.

B) Reverse-transcription PCR analysis of TNF-α, Cox-2, Cox-1 and β actin mRNA in liver samples obtained from the different experimental groups. One microgram of RNA was subjected to reverse transcription and the amplification reactions run in ethidium-bromide stained agarose gel. Cox-1 mRNA and β actin were detected in all groups. TNF-α, Cox-2, mRNA were detected only in the FE group but were absent in the other treatment groups.
FIGURE 1
Figure 2
Figure 3

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Figure 4
Figure 5
Figure 6