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The transcriptional and DNA binding activity of peroxisome proliferator-activated receptor α is inhibited by ethanol metabolism: a novel mechanism for the development of ethanol-induced fatty liver

Andrea Galli*, Jane Pinaire, Monika Fischer, Ryan Dorris, and David W. Crabb**

Departments of Medicine and of Biochemistry and Molecular Biology, Indiana University School of Medicine and *Department of Clinical Pathophysiology, University of Florence, Florence, Italy

** To whom correspondence should be addressed:

Emerson Hall 312

545 Barnhill Drive

Indianapolis, IN 46202

317-274-3122

317-274-1437 (fax)

dcrabb@iupui.edu

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Summary

Fatty acids are ligands for the peroxisome proliferator activated receptor α (PPARα). Fatty acid levels are increased in liver during the metabolism of ethanol and might be expected to activate PPARα. However, ethanol inhibited PPARα activation of a reporter gene in H4IIEC3 hepatoma cells expressing alcohol-metabolizing enzymes, but not in CV-1 cells which lack these enzymes. Ethanol also reduced the ability of the PPARα ligand WY14643 to activate reporter constructs in the hepatoma cells or cultured rat hepatocytes. This effect of ethanol was abolished by the alcohol dehydrogenase inhibitor 4-methylpyrazole and augmented by the aldehyde dehydrogenase inhibitor cyanamide, indicating that acetaldehyde was responsible for the action of ethanol. PPARα /retinoid X receptor (RXR) extracted from hepatoma cells exposed to ethanol or acetaldehyde bound poorly to an oligonucleotide containing peroxisome proliferator response elements. This effect was also blocked by 4-methylpyrazole and augmented by cyanamide. Furthermore, *in vitro* translated PPARα exposed to acetaldehyde failed to bind DNA. Thus, ethanol metabolism blocks transcriptional activation by PPARα, in part due to impairment of its ability to bind DNA. This effect of ethanol may promote the development of alcoholic fatty liver and other hepatic consequences of alcohol abuse.

Key words: fatty acids, ethanol, transcription, acetaldehyde, DNA binding proteins

Introduction

The liver coordinates synthesis of fatty acids, esterification of triacylglycerols, and their packaging into very low density lipoproteins (VLDL) for export during fed conditions, while in fasting it controls the rates of β-oxidation and ketogenesis. By balancing these processes, the liver handles large amounts of fat without accumulating triacylglycerol. Many homeostatic responses of the hepatocyte to FFA are modulated by peroxisome proliferator-activated receptor α (PPARα). FFA are endogenous ligands for PPARα (1-4) and numerous genes involved in fat metabolism contain peroxisome proliferator response elements (PPREs) in their promoters. It has been suggested that this constitutes a feed back control system: elevated FFA activate PPARα, inducing a battery of enzymes (peroxisomal β-oxidation, mitochondrial β-oxidation, and microsomal fatty acid hydroxylation (which initiates ω-oxidation)) involved in FFA oxidation (5,6), which serve to reduce the level of FFA. However, in certain forms of liver disease, this fine balance is disrupted, and elevated levels of hepatocellular free fatty acids (FFA) and triacylglycerol occur.

The most common liver disease in which fatty acid metabolism is deranged is alcoholic liver disease. Alcohol metabolism alters the intramitochondrial redox potential via generation of NADH by alcohol dehydrogenase (ADH). This impairs β-oxidation and tricarboxylic acid cycle activity (7), resulting in elevated FFA, increased formation of triacylglycerol, and increased rates of VLDL synthesis and secretion (8,9). Paradoxically, the fatty liver persists despite attenuation of the altered redox state after chronic ethanol administration (10). Fatty liver is not necessarily

benign: the development of liver injury in a rat model is clearly dependent upon the amount and type of fat in the diet (11,12) and a disproportionate elevation of liver FFA after ethanol administration may contribute to the susceptibility of women to alcoholic liver disease (13). Genetically obese mice and rats with hepatic steatosis are unusually sensitive to the effects of endotoxin (14). Furthermore, a number of other compounds, including valproic acid, amiodarone, and perhexilene, are postulated to cause liver injury by way of inhibition of β-oxidation (15,16). Thus, several lines of evidence suggest that liver injury may occur when fatty acid oxidation or esterification and export are inadequate.

One would predict that ethanol consumption would induce the PPARα battery of proteins by elevating intracellular fatty acid levels. Although alcohol consumption resulted in peroxisomal proliferation in humans (17) and alcohol feeding of rats induced cytochrome P450 4A1 (lauryl ω-hydroxylase, (13)) and liver fatty acid binding protein (18), other typical responses to peroxisome proliferators were impaired by ethanol. The excretion of dicarboxylic fatty acids was increased in alcohol-fed animals (13), due to increased lauryl hydroxylase activity but failure of induction of acyl-CoA oxidase (19). Medium chain acyl-CoA dehydrogenase activity, the gene for which has a PPRE in its proximal promoter (20), was reported to be decreased by ethanol feeding (21). Thus, chronic ethanol feeding apparently does not activate a full PPARα response. One group has reported that PPARα mRNA was decreased in the livers of rats chronically fed alcohol via gastric lavage, and that several PPAR-inducible enzymes were not increased in these animals (22). It is noteworthy that fatty liver and steatohepatitis, hallmarks of alcoholic liver injury, are also observed in both PPARα (23) and acyl-CoA oxidase (24)

knockout mice. We therefore examined the effect of ethanol on the function of PPARα in transfected cells.

Experimental Procedures

Materials

Most chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Trypsin and tissue culture media were purchased from GIBCO BRL (Gaithersburg, MD). Fetal bovine serum charcoal-stripped of lipids was purchased from Hyclone Laboratories (Logan, UT). All radioisotopes were purchased from DUPONT NEN Research Products Inc. (Boston, MA). $PPRE_3$-tk-luciferase (containing three copies of the peroxisome proliferator response element (PPRE) from the acyl-CoA oxidase gene ligated to a herpes simplex thymidine kinase promoter upstream of the luciferase gene (25)), and the expression plasmids for murine PPARα, δ, and γ were the kind gifts of Dr. Ronald Evans, Salk Institute (26). pALDH3'-BLCAT was used as an HNF-4 responsive reporter and was previously described (27). Expression plasmids for apolipoprotein regulatory protein 1 (ARP-1) and chicken ovalbumin upstream promoter transcription factor (COUP-TF) were from Dr. H. Nakshatri (Indiana University) and that for hepatocyte nuclear factor 4 (HNF-4) was from Dr. Frances Sladek (University of California, Irvine).

Transfection of tissue-culture cells

All cells were grown in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 63 μg/ml penicillin G. The day before transfection, the cells were plated at 10^6 cells/100 mm dish. For studies on PPAR α and γ, the cells were transfected with 10 μg of reporter plasmid ($PPRE_3$-tk-luciferase), 20 μg of receptor expression plasmid, and 5 μg of pSV_2CAT (as an internal control for transfection efficiency) by calcium phosphate

precipitation (28). For studies on HNF4, the reporter contained four copies of an HNF-4 response element from the aldehyde dehydrogenase 2 promoter cloned in pBL2CAT (28) and the internal control was SV40-luciferase. For studies on ARP-1 and COUP-TF, the reporter was SV40-luciferase (which is activated by these two orphan receptors (29)) and because of problems with effects of these receptors on other promoters, the activity was expressed per μg cell extract protein. Four hours later the cells were exposed to PBS containing 15% glycerol for 3 min. The cells were rinsed twice with PBS and fresh MEM with 10% charcoal-stripped fetal bovine serum was added. Twenty-four to forty-eight hours after transfection, cells were washed twice with PBS and lysed in 150 μl of a buffer containing 25 mM Tris, pH 7.8, 2 mM EDTA, 20 mM DTT, 10% glycerol, and 1% Triton X-100. Fifty μl of cell extract was incubated with luciferase assay reagent based on the original protocol of deWet (30). CAT activity was measured as described previously (31). The conversion of chloramphenicol to its acetylated products was quantified on an AMBIS β-scanner.

Primary hepatocyte suspensions were isolated from male Sprague-Dawley rats (Harlan Laboratory Animals for Research, Indianapolis, IN) as previously described (32,33). Briefly, rats were anesthetized with pentobarbital (50-100 mg/kg body weight), their portal veins were cannulated with a 16-gauge catheter, and the livers were perfused with Ca^{++}, Mg^{++}-free Hanks' A solution, followed by Hanks' B solution containing Ca^{++}, Mg^{++}, and 0.05% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Livers were then excised, minced, and passed through nylon mesh filters and the resultant hepatocytes were suspended in culture medium. Viability of the hepatocytes exceeded 90% by trypan blue exclusion. These cells were cultured in DMEM with 2.4 g/L of sodium bicarbonate, 10 mM glucose, 1 μM each of

dexamethasone and thyroxine, 1 nM insulin, and 10% FBS. They were transfected 4 hours after plating by calcium phosphate precipitation according to the method of Ginot (34). Twenty four hours later the cells were treated 100 μM WY14,643 for an additional 24 hr before harvesting the cells for assay of reporter enzymes as described above.

Isolation of nuclear protein extracts

Nuclear proteins were isolated from cultured cells based on a micropreparation method (35). The nuclear extract was suspended in 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, and aliquots were frozen in liquid nitrogen and stored at -70^{o}C.

In vitro synthesis of receptor proteins

PPARs and RXRα were synthesized using a rabbit reticulocyte lysate system (Promega *in vitro* transcription/translation kit). The production of protein of the expected molecular weight was monitored by labeling with ^{35}S-methionine and autoradiography. Aliquots of the translation reaction were used without further purification.

DNA binding assays

Electrophoretic mobility shift assays (EMSA) were performed by radiolabeling double-stranded oligonucleotides corresponding to the peroxisome proliferator response element of the acyl-CoA oxidase gene. Nuclear extracts (3-5 μg) or reticulocyte lysates (1-2 μl) were incubated

with 1-2 μg of non-specific competitor DNA (poly (dIC)) in binding buffer containing 10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, and 7% (v/v) glycerol on ice for 15 min. Where indicated, specific competitor oligonucleotides were added before the addition of labeled probe and incubated for 15 min on ice. For supershift assays, antibodies were added and the mixture was incubated an additional 1-2 hours. Labeled probe (20,000 cpm) was added last and the reaction incubated an additional 15 min on ice. Reaction mixtures were electrophoresed on a non-denaturing 4% acrylamide gel and subjected to autoradiography. Anti-PPARα antibody was from Santa Cruz Biotechnology and anti-RXR was donated by Drs. C. Rochette-Egly and P. Chambon.

Western blotting

Nuclear extracts (20 μg protein) were fractionated in an 8% SDS-PAGE gel and electroblotted to nitrocellulose filters. PPARα was visualized using antiserum from Santa Cruz Biotechnology. Detection of the protein bands was performed using the Amersham ECL kit.

Results

Effects of ethanol on transcriptional activation by PPARα in cells with and without the enzymatic capacity for ethanol oxidation.

The activity of a PPARα-responsive reporter gene ($PPRE_3$-tk-luciferase, containing 3 copies of the PPRE from the acyl-CoA oxidase gene) was used as an index of PPARα function in CV-1 and H4IIEC3 hepatoma cells with or without co-transfected PPARα (Table I). These cells contain low amounts of PPARα protein on Western blots (Figure 2B, lane 2) but both cell lines contained immunoreactive retinoid X receptor (RXR, (36)), the required dimerization partner for PPARα (25). An important difference between CV-1 and H4IIEC3 cells was the presence of enzymes capable of oxidizing ethanol in the latter cells (37,38). Responses of the reporter were relatively small (no more than two-fold induction) in the absence of co-transfected PPARα. Clofibrate markedly induced the reporter activity in CV-1 cells transfected with PPARα. In the hepatoma cells, the reporter activity was much less dependent on the presence of clofibrate (36). Ethanol at a physiologically relevant concentration of 20 mM inhibited clofibrate-independent and -stimulated activity of $PPRE_3$-tk-luciferase by PPARα by over 50% in the hepatoma cells (Table I). Ethanol had no effect on basal or clofibrate-stimulated PPARα action in the CV-1 cells, either in the presence or absence of transfected PPARα.

The effect of ethanol on the ability of the more potent and specific PPARα agonist WY14,643 was also tested. In duplicate experiments with H4IIEC3 cells transfected with the PPARα expression plasmid and reporter, WY14, 643 at 100 μM increased reporter activity by 591 ± 49 %. Ethanol (20 mM) reduced the basal activity of the reporter to 45 ± 4%, and

decreased the WY14,643-stimulated activity to 216 ± 134% of the control level (means ± standard error). We also tested the effect of WY14,643 on primary hepatocyte cultures that were transfected with the PPAR reporter plasmid to see if ethanol also inhibited the activity of the endogenous rat PPARα. WY14,643 stimulated reporter activity by 433 ± 107. Ethanol (20 mM) reduced basal activity to 57 ± 3 % and WY14,643-stimulated activity to 128 ± 32% of control levels (means ± standard errors for four replicate experiments). Thus, ethanol reduced the activity of the reporter by about 50% in both the basal and WY-14,643-stimulated cells, similar to the magnitude of the effect on clofibrate-stimulated activity. Further, the effect was also seen in primary cultures of hepatocytes, indicating that the rat PPARα is also sensitive to ethanol.

To determine if this effect of ethanol was restricted to PPARα, additional transfection assays were performed using PPARγ, HNF4, ARP-1, or COUP-TF. These receptors are structurally related to PPARα and each recognizes DR-1 promoter elements. Compared with hepatoma cells transfected with PPARγ alone (n= 5, means ± standard errors), clofibrate increased activity of $PPRE_3$-tk-luciferase by 189 ± 20 %, while ethanol reduced activity to 60 ± 3 %, and reduced the clofibrate-stimulated activity to 154 ± 21%. These small differences were statistically significant. Transfection of the H4IIEC3 cells with an HNF-4 expression plasmid stimulated its reporter plasmid expression (pALDH3'-BLCAT containing 4 copies of an HNF-4 response element from the aldehyde dehydrogenase promoter) by 956 ± 159% in the absence and 1034 ± 109% in the presence of ethanol (n= 3, not significant). ARP-1 stimulated its reporter plasmid expression (SV40-luciferase (29)) by 8222 ±1776% in the absence and 12203 ± 6875% in the presence ethanol (n = 4, not significant). COUP-TF stimulated its reporter plasmid

expression (SV40-luciferase) by 10,408 ± 1092% in the absence and 9312 ± 3432% in the presence of ethanol (not significant, n = 4). The large errors observed in the transfections with ARP-1 and COUP-TF were related to the use of cellular protein for normalizing the data, rather than an internal control plasmid (29). Thus, the effect of ethanol was relatively specific for PPARα, although the small effect on the γ isoform was studied further with *in vitro* translated receptor (below).

Effects of inhibitors of ethanol metabolism and acetaldehyde on PPARα function

The ADH inhibitor 4-methylpyrazole and the aldehyde dehydrogenase (ALDH) inhibitor cyanamide were then used to determine if the effect of ethanol on PPARα was dependent on its metabolism (Table II). Neither compound affected $PPRE_3$-tk-luciferase activity in the hepatoma cells in control experiments. However, 4-methylpyrazole completely prevented the effect of ethanol on PPARα function, while cyanamide augmented the effect. This suggested that acetaldehyde generated from ethanol was responsible for the inhibition of PPARα action, and indeed, low levels of exogenous acetaldehyde (50-150 μM) inhibited $PPRE_3$-tk-luciferase reporter activity, both in H4IIEC3 cells and CV-1 cells (Table III). There was no visible evidence of toxicity to the cells of these doses of acetaldehyde. Because many biological effects of acetaldehyde have been attributed to modification of proteins (39,40), we examined the effect of pyridoxal phosphate on ethanol inhibition of PPARα induction of the reporter. Pyridoxal phosphate has been reported to protect proteins from formation of Schiff bases with acetaldehyde by reversibly blocking lysyl residues (41), even in whole cell models (42), and thus is useful in

understanding mechanisms of acetaldehyde effects. Treatment of the cells with 10 mM pyridoxal phosphate completely prevented the effect of ethanol (control activity = 970 ± 10, ethanol-treated cells = 454 ± 43, ethanol plus pyridoxal phosphate = 830 ± 49; activity represents % increase over the untransfected control cells as described for Table I; means ± standard error for 3 replications), indicating that acetaldehyde-protein adduct formation may explain the inhibitory effect of ethanol metabolism.

Effect of ethanol and acetaldehyde on DNA binding ability of PPARα

To understand how ethanol metabolism impaired PPARα function, it was of interest to study the effect of ethanol on the ability of nuclear factors to bind the PPRE in EMSA. In preliminary experiments, ethanol was found to have no effect on the level of endogenous RXRα in the hepatoma cells. Nuclear extracts from untransfected H4IIEC3 cells contained proteins that retarded the mobility of the PPRE oligonucleotide (Figure 1). The major and minor bands appeared to be specific, in that they were competed with unlabeled oligonucleotide (lanes 3-5). These bands were not PPARα, since the hepatoma cells contain very low levels of PPARα (Figure 2B, lane 2) and the bands could not be shifted with antibody to PPARα (not shown). The bands likely represent other nuclear factors present in H4IIEC3 cells that can bind to DR-1 elements, such as HNF-4. When the cells were exposed to ethanol, alone or in the presence of inhibitors of its metabolism, there was no change in the intensity of the bands.

We then analyzed nuclear extracts from H4IIEC3 cells that were transfected with the

PPARα expression plasmid (Figure 2A and B). The intensity of the major band was markedly increased in the transfected cells, which contained large amounts of PPARα seen by western blotting (Figure 2A and B, lane 3), and there was a prominent shift induced with anti-PPARα. Binding was again competed with unlabeled competitor oligonucleotides (lanes 4-6). The major band could also be shifted with antibody to RXR (not shown). The more slowly migrating band was also more intense in the transfected cells. The identity of this band is uncertain; however, it might represent nuclear receptors bound to other factors such as NRBF-1 (43) or PBP165 (44). These factors interact with a number of nuclear receptors including PPARα and HNF4.

The effect of 24 hours of treatment with ethanol on PPRE binding activity in extracts of hepatoma cells transfected with the PPARα expression plasmid was then evaluated (Figures 2 and 3). The Western blots for PPARα protein demonstrated that similar amounts of the receptor were present in all of the nuclear extracts, and that ethanol metabolism did not impair synthesis or nuclear localization of the receptors (Figure 2B). Compared with control transfected cells (lane 7), ethanol treatment reduced the ability of PPARα to bind the oligonucleotide (lane 8); binding was further reduced if cyanamide had been present during the ethanol treatment (lane 9). The band was not completely eliminated by ethanol, as expected from the existence of PPRE-binding proteins in the untransfected cells (Figure 1). DNA-binding activity of the PPARα from cells treated with ethanol plus 4-methylpyrazole was normal (lane 10); 4-methylpyrazole or cyanamide treatment in the absence of ethanol had no effect on the ability of the PPARα to bind DNA (lanes 11 and 12).

Exposure of the cells to progressively increasing concentrations of acetaldehyde (in the

range of those that can be achieved in the liver during ethanol metabolism (45)) reduced the ability of extracted PPARα to bind DNA (Figure 3A). This treatment of the cells did not reduce the amount of immunoreactive PPARα present in the nuclear extracts (Figure 3B). Acetaldehyde also reduced the intensity of the more slowly moving band. Inclusion of pyridoxal phosphate in the culture medium prevented the inhibitory effects of ethanol and acetaldehyde on DNA binding activity (not shown).

To further document that the nuclear factor binding that was reduced by ethanol and acetaldehyde was PPARα, antibody against this factor was used to super-shift the binding complex (Figure 4). This autoradiogram was exposed for a shorter time than Figures 1 and 2 to allow better resolution of the shifted bands, and the binding activity in the untransfected cells is therefore less prominent. The major band of binding activity was shifted with anti-PPARα antibody (lane 4). Addition of ethanol in the presence of cyanamide markedly reduced the intensity of the non-shifted and the shifted bands (land 7 and 8). Similarly, acetaldehyde at 50 or 150 μM reduced the intensity of both the major and the antibody-shifted band (lanes 9-12). Thus, the ethanol, by way of acetaldehyde, dramatically reduces the ability of PPARα to bind to DNA.

Effect of acetaldehyde on DNA binding ability of in vitro translated PPARs

Because there is evidence that acetaldehyde can alter protein function via covalent modification, the effect of acetaldehyde on *in vitro* synthesized PPARα and RXRα was examined (Figure 5A). The receptors were incubated on ice (lanes 1-5), at 37^{o} (lanes 6-11), or at

37° in the presence of 1 mM acetaldehyde (lanes 12-17; the receptor incubated with acetaldehyde is indicated by the dot), and then either tested individually or after mixing PPARα with RXRα for DNA binding ability. In the lanes indicated by (RXR/PPAR), the two receptors were mixed to form heterodimers before the incubation. Under the binding conditions used here, only the mixture of RXR and PPAR bound DNA. The one hour incubation at 37° reduced the intensity of binding of the receptor complex, unless they had been mixed to form heterodimers before the incubation (lane 11). Incubation of RXR with acetaldehyde before mixing reduced the intensity of the binding complex somewhat (lane 15). However, treatment of PPAR with acetaldehyde abolished the band, as did treatment of the pre-formed heterodimer (lane 17).

Because there was a modest reduction in the ability of PPARγ to activate the reporter in transfection studies, we also studied the effect of acetaldehyde on the ability of the PPARγ and δ isoforms to bind DNA (Figure 5B). This experiment was carried out as described for PPARα (Figure 5A). The preformed heterodimers (lanes 10 and 11) appeared to somewhat more stable than the receptor subunits incubated alone (lanes 8 and 9), as was seen with PPARα. Treatment of the RXR with acetaldehyde did not dramatically reduce its ability to form a DNA-binding complex with either PPARγ or δ (lanes 14 and 15). As with PPARα, both PPARγ and δ were sensitive to pre-incubation with acetaldehyde (lanes 16 and 17). The preformed heterodimers appeared to be somewhat more resistant to the effect of acetaldehyde than the corresponding PPARα/RXR complex (lanes 19 and 20).

Discussion

These studies reveal potentially important interactions between ethanol metabolism and the function of PPARα. The presence of ethanol reduced the ability of transfected PPARα to activate a reporter construct in the H4IIEC3 hepatoma cell line. This effect was mediated by acetaldehyde, since inhibition of ethanol oxidation by 4-methylpyrazole blocked the effect completely, while the aldehyde dehydrogenase inhibitor cyanamide enhanced the effect of ethanol. Furthermore, low concentrations of acetaldehyde added directly to the medium inhibited PPARα activity in both the hepatoma and CV-1 cells. Although ethanol modestly reduced the ability of PPARγ to activate the reporter plasmid, this effect of ethanol was not observed with several other members of the nuclear receptor family (HNF4, ARP-1 or COUP-TF), further suggesting that it is not a non-specific, toxic effect of ethanol.

The effect of ethanol and acetaldehyde on expression of the PPARα reporter gene was correlated with the ability of PPARα/RXR extracted from the cells to bind to its response element. The H4IIEC3 cells contain factors that can bind the PPRE oligonucleotide used for the EMSA. These factors have not been identified, but could include HNF4, COUP-TF, ARP-1, or RXR, all of which are known to bind DR-1 sequences. The major band observed is likely to correspond to the dimeric form of these transcription factors, as each of these factors is of similar molecular weight and the mobility was close to that of *in vitro* translated PPARα/RXR. A minor band was also observed whose identity is unknown, but could represent DR-1 binding factors complexed with other nuclear proteins. The intensity of these bands was not affected by treatment of the cells with ethanol, again arguing against the effects of ethanol being non-

specific. Nuclear extracts from H4IIEC3 cells transfected with a PPARα expression plasmid had more prominent shifted bands at both positions, and treatment with ethanol, ethanol plus cyanamide, or acetaldehyde reduced the intensity of both bands. That the reduced intensity of the major band represented decreased ability of PPARα/RXR to bind the PPRE was further demonstrated by the use of antibody to PPARα. The intensity of the shifted bands was decreased by ethanol, ethanol plus cyanamide, and acetaldehyde. The reduction in intensity of the more slowly moving complex cannot be fully interpreted at present.

An attractive explanation for the observed effects of ethanol was the formation of acetaldehyde adducts with PPARα. Acetaldehyde is known to react with lysyl side chains in a number of proteins (40, 46-48), and has been implicated in the dysfunction of liver microtubules in alcohol-fed animals (49). This hypothesis is consistent with the ability of pyridoxal phosphate pre-treatment of the cells to prevent the effects of ethanol and acetaldehyde on both PPARα activation of the reporter gene and on the ability of the receptor to bind DNA. It was possible to show that exposure of *in vitro* synthesized PPARα to acetaldehyde for only one hour interfered with DNA binding by PPARα/RXR. Although the concentration of acetaldehyde used in the *in vitro* experiments was higher than can be achieved *in vivo*, exposure to this concentration had much less effect on the ability of RXRα to form DNA-binding heterodimers with PPARα. This suggests that PPARα is unusually sensitive to acetaldehyde. The DNA binding domain of PPARα contains a number of conserved lysyl residues that are predicted to be directly involved in DNA-protein interactions (50). The ethylation of these residues by acetaldehyde might be

expected to dramatically alter the electrostatic interactions of these side-chains with DNA. Other possible explanations for impaired DNA binding could be the inability of acetaldehyde-treated PPARα to dimerize with RXR and impairment of the activation functions of the receptor. However, additional studies are needed to determine if the intracellular and *in vitro* effects of acetaldehyde on PPARα function involve similar mechanisms. It will also be important to examine the ability of other biologically occurring aldehydes (e.g., aldehydic products of lipid peroxidation or glucose) to affect PPARα function. We also observed sensitivity of PPARγ and δ to acetaldehyde *in vitro*, although the kinetics of inactivation were not formally studied to allow quantitative comparisons of the PPAR isoforms.

Earlier work has shown that exposure to ethanol increases the level of fatty acids in hepatocytes (8,9). The results of the present work show that ethanol also can impair the function of PPARα. The failure of induction of PPARα-controlled genes such as those for peroxisomal β-oxidation and medium chain acyl-CoA dehydrogenase could thus contribute to the development of alcoholic fatty liver. This effect of ethanol may be responsible for the persistence of fatty liver despite a return of the redox state toward normal during chronic ethanol administration (10). Inhibition of PPARα function may also contribute to more serious alcoholic hepatic injury. Indeed, this suggests that pharmacologic or nutritional maneuvers that activate the PPARα system may ameliorate the hepatotoxicity of ethanol. However, ethanol feeding does not uniformly inhibit expression of genes known to contain PPREs (13,18). This presumably results from the presence of multiple factors controlling most promoters. In addition,

the consensus PPRE is a direct repeat with one spacer nucleotide (DR-1 element) that can also be bound by such factors as retinoic acid receptors, HNF-4, COUP-TF, and ARP-1 (51). We speculate that ethanol inhibition of DNA binding by PPARα might permit retinoic acid receptors or HNF-4 to bind and activate certain genes, explaining, for instance, the apparent induction of apoAI and apoAII by ethanol (52). Conversely, binding of COUP-TF and ARP-1 might actively repress certain genes. Such interactions among the steroid receptor family of transcription factors could increase the spectrum of biological actions of ethanol.

The actions of ethanol on PPARγ also deserve additional study. We have shown that PPARγ may play an important role in the control of proliferation of hepatic stellate cells (53). The expression of PPARγ decreases as the cells proliferate after being plated on plastic substrate, and activated PPARγ antagonizes the actions of platelet-derived growth factor, a major contributor to proliferation of stellate cells. We hypothesize that the high intra-hepatic levels of acetaldehyde occurring during prolonged alcohol consumption inhibits PPARγ and renders the stellate cells more susceptible to activation. This could contribute to the pathogenesis of alcoholic cirrhosis as well as the increased risk of hepatic fibrosis in patients with hepatitis C who drink heavily. Further, PPARγ is extremely important for the differentiation of preadipocytes to adipocytes, in the control of sensitivity to the actions of insulin, and in the pathogenesis of atherosclerosis (54, 55). Although the multiple roles of this PPAR isoform is still incompletely understood, inhibition of PPARγ function by heavy ethanol consumption might contribute to insulin resistance, syndrome X, and accelerated cardiovascular disease. This possibility deserves further study.

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Table I. Effect of ethanol on PPARα-induced reporter activity in CV-1 and H4IIEC3 cells.

	Cell Line			
	CV1		H4IIEC3	
	- PPARα	+ PPARα	- PPARα	+PPARα
Control	100	382 ± 62	100	825 ± 112
Clofibrate	198 ± 41	2523 ± 425	153 ± 35	1209 ± 363
Ethanol	107 ± 134	628 ± 403	57 ± 12	399 ± 165*
Clofibrate + ethanol	194 ± 107	2361 ± 617	100 ± 34	582 ± 174**

The cells were transfected using the calcium phosphate procedure with the reporter, $PPRE_3$-tk-luciferase (26), the chloramphenicol acetyltransferase (CAT) expression vector pSV_2CAT as an internal control of transfection efficiency, and, where indicated by columns labeled +PPARα, the PPARα expression plasmid (26). The cells were exposed to 20 mM ethanol and/or 1 mM clofibrate (dissolved in DMSO as vehicle) from 24 to 48 hours after transfection. Control cells were treated with vehicle alone. The concentration of ethanol in the medium was maintained by culturing the cells in an incubator containing a reservoir of 20 mM ethanol in water. Forty-eight hours after transfection, the cells were harvested for luciferase and CAT assays. In each experiment, duplicate plates were transfected and the reporter activities averaged. The results are shown as percentage of luciferase reporter activity in control cells (transfected with the reporter but not the PPARα expression plasmid) after correction for CAT

activity of the internal control vector (means ± standard deviation for 3 or 4 replications of each condition). * denotes a statistically significant difference compared with no ethanol; ** denotes a statistically significant difference compared with clofibrate alone ($p<0.05$ by paired t-test).

Table II. Effect of inhibitors of alcohol and aldehyde dehydrogenase on the effect of ethanol on PPARα-induced reporter activity in H4IIEC3 cells

Condition	Reporter Activity (% of Control)	
	- Ethanol	+ Ethanol
Control	1040 ± 104	693 ± 67*
4-methylpyrazole (0.1 mM)	1034 ± 100	993 ± 110
Cyanamide (0.1 mM)	1043 ± 113	361 ± 54*
4-methylpyrazole + cyanamide	-	1071 ± 77

The cells were transfected as described in Table I with the PPARα expression plasmid, internal control plasmid, and the $PPRE_3$-tk-luciferase reporter; the inhibitors were added 24 hours later and the cells were exposed to ethanol (20 mM) where indicated beginning at the same time. The cells were harvested for assay of the reporter enzymes at 48 hours; data are reported as in Table I. The data are normalized to control cells analyzed in each replication which were not transfected with the PPARα expression plasmid. * denotes significant differences from the activity of the reporter in the control cells ($p<0.05$ by paired t-test).

Table III. Effect of acetaldehyde on PPARα-induced reporter activity in H4IIEC3 and CV-1 cells

	Cell Line	
Acetaldehyde (μM)	H4IIEC3	CV-1
0	954 ± 202	333 ± 58
50	655 ± 83*	297 ± 37
85	481 ± 81*	193 ± 42*
150	329 ± 114*	159 ± 18*

The cells were transfected with the PPARα expression plasmid, internal control, and the $PPRE_3$-tk-luciferase reporter as in Table I; acetaldehyde was added to the medium 24 hours later and the culture dishes were sealed to reduce evaporative losses. In the hepatoma cells, 4-methylpyrazole and cyanamide were present at 0.1 mM to slow the metabolism of the added acetaldehyde. The cells were harvested for assay of the reporter enzymes at 48 hours. Data are reported as in Table I. * denotes statistically significant differences from control ($p<0.05$ by paired t-test).

Figure legends

Figure 1. Effect of exposure of H4IIEC3 hepatoma cells to ethanol on binding of endogenous nuclear factors to bind DNA. EMSAs were performed using nuclear extracts from hepatoma cells exposed to ethanol and inhibitors of ethanol or acetaldehyde metabolism. The probe was a double stranded oligonucleotide containing a copy of the acyl-CoA oxidase PPAR response element (26). Nuclear extract was incubated with 20,000 cpm of the probe then analyzed by electrophoresis through a 4% PAGE gel and autoradiography. Lane 1 represents the probe alone; all other lanes contained the nuclear extract. Cold competitor indicates addition of unlabeled oligonucleotide at the noted molar excess. In lanes 6 through 13, the cells had been pre-treated with the indicated compounds for 24 hours before the cells were harvested for nuclear extraction. 4-Methylpyrazole and cyanamide were added at 0.1 mM and ethanol was added at 20 mM.

Figure 2. Effect of exposure of H4IIEC3 hepatoma cells to ethanol on the ability of PPARα to bind DNA. Panel A. EMSAs were performed using nuclear extracts from hepatoma cells transfected with a PPARα expression plasmid as described in Figure 1. Lane 1 represents the labeled oligonucleotide in the absence of added nuclear proteins, and lane 2 represents extract from cells that had not been transfected. The cold competitor lanes indicate the molar excess of unlabeled oligonucleotide added to the binding reaction. Where noted, ethanol had been present in the medium at 20 mM, cyanamide at 0.1 mM, and 4-methylpyrazole at 0.1 mM for 24 hours before harvesting the cells. Panel B. Western blots of the nuclear extracts used in panel A were performed using anti-PPARα to confirm the presence of similar amounts of PPAR in each lane.

Figure 3. Effect of acetaldehyde on the ability of PPARα to bind DNA. Panel A. Nuclear extracts were prepared as in Figure 1 from hepatoma cells transfected with PPARα that had been incubated with the noted concentrations of acetaldehyde for 24 hours prior to harvest. The culture medium also contained 0.1 mM 4-methylpyrazole and 0.1 mM cyanamide to retard the reductive or oxidative metabolism of added acetaldehyde. Panel B. Western blots of the nuclear extracts for PPARα

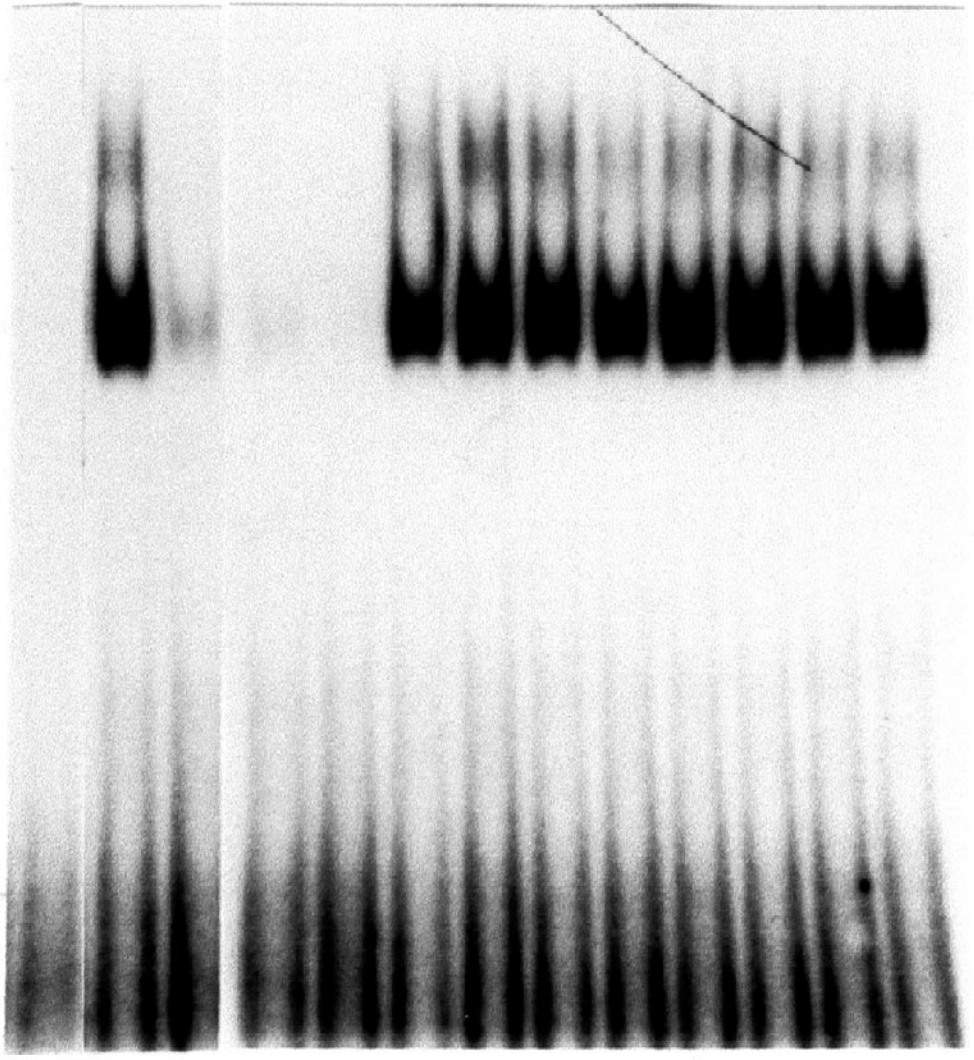
Figure 4. Effect of ethanol and acetaldehyde on PPARα binding to DNA.

H4IIEC3 cells were treated with ethanol (20 mM), ethanol plus cyanamide (0.1 mM), or acetaldehyde (50 or 150 μM in the presence of cyanamide plus 4-methylpyrazole) for 24 hours before preparation of nuclear extracts and EMSAs were performed as described for Figures 1-3. In the lanes 4,6, 8, 10, and 12, antibody to PPARα was added to the binding reaction prior to addition of radiolabeled DNA and incubated for 1-2 hours prior to electrophoresis. With this shorter autoradiographic exposure, the major band is resolved to 2 bands, both of which are shifted by anti-PPAR antibody.

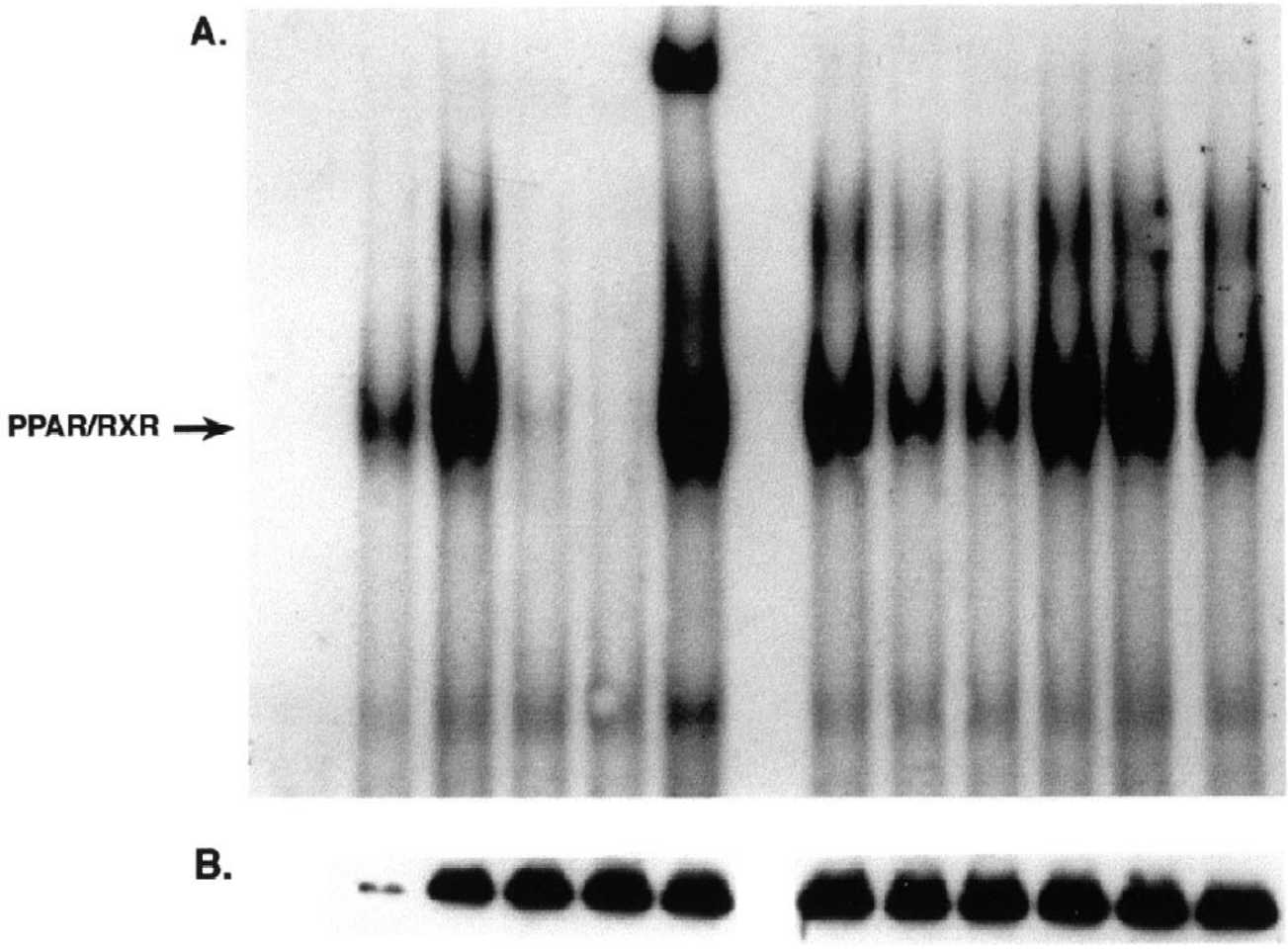
Figure 5. Effect of pre-incubation of *in vitro* synthesized PPAR and RXRα proteins with acetaldehyde on the ability of PPAR/RXR heterodimers to bind DNA. Panel A. Expression plasmids for the receptors were used to prepare proteins using the Promega TNT *in vitro* transcription/translation kit. The proteins were either incubated on ice, at 37^{o}, or treated with 1

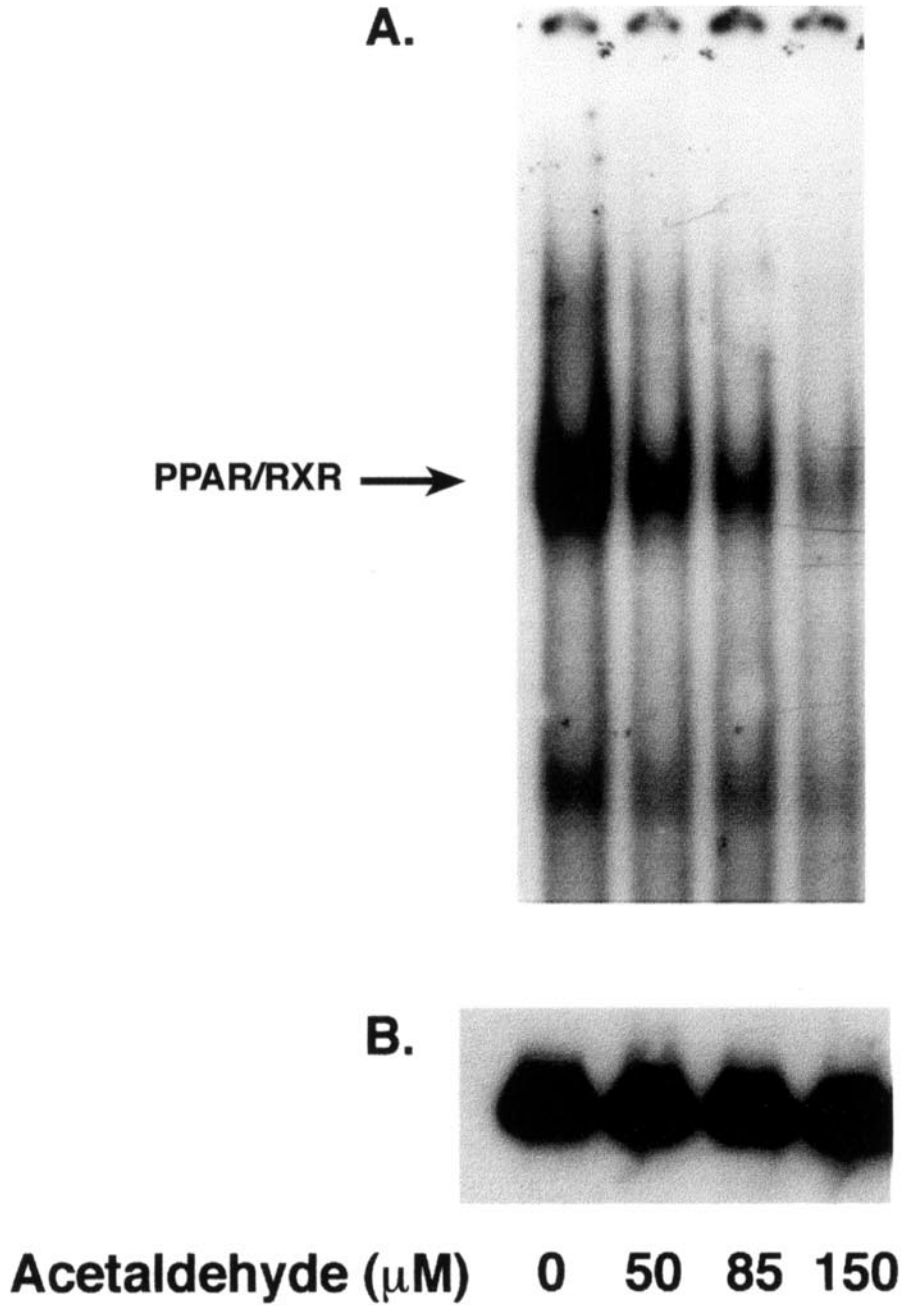
mM acetaldehyde at 37^{o} for 1 hour. The RXR or PPAR proteins were incubated individually, or after mixing to form heterodimers (indicated by (RXR/PPAR)). The receptors were then mixed to form dimers and analyzed by EMSA as described for Figure 1. Lane 1 represents the labeled probe in the absence of added receptors. Lane 2-4 show that neither the *in vitro* translation mixture programmed with a luciferase plasmid ((-)IVT), or RXR or PPAR alone bound the DNA probe, while the mixture of RXR and PPARα bound. The 37^{o} control lanes show that incubation at this temperature reduced the ability of the individually incubated receptors to form a DNA binding complex, but that the pre-formed heterodimer withstood the incubation well. The receptor or mixture that was treated with acetaldehyde is indicated by the solid dots. When RXR was treated with acetaldehyde before mixing, there was still formation of a faint binding complex. When the PPAR receptor, or the pre-formed complex was treated with acetaldehyde, DNA binding was abolished.

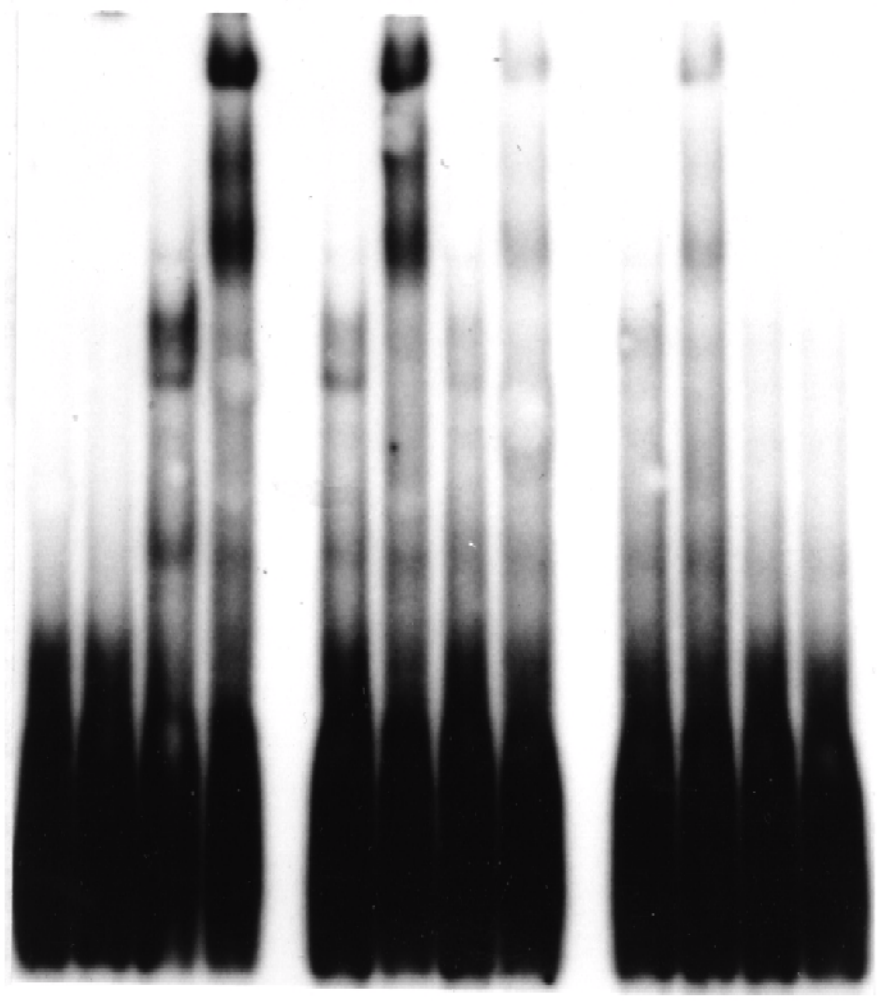
Panel B. This experiment was performed as described for panel A, but with PPARγ and δ. (RXR/γ) indicates the preformed RXR/PPARγ heterodimer, and (RXR/δ) indicates the preformed RXR/PPARδ heterodimer. Treatment of the individual PPARs or the preformed dimmers with acetaldehyde substantially reduced DNA binding.



	1	2	3	4	5	6	7	8	9	10	11	12	13
Nuclear extract	-	+	+	+	+	+	+	+	+	+	+	+	+
Cold Competitor	-	-	50	100	200	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	+	+	+	+
Cyanamide	-	-	-	-	-	-	+	-	+	-	+	-	+
4-methylpyrazole	-	-	-	-	-	-	-	+	+	-	-	+	+

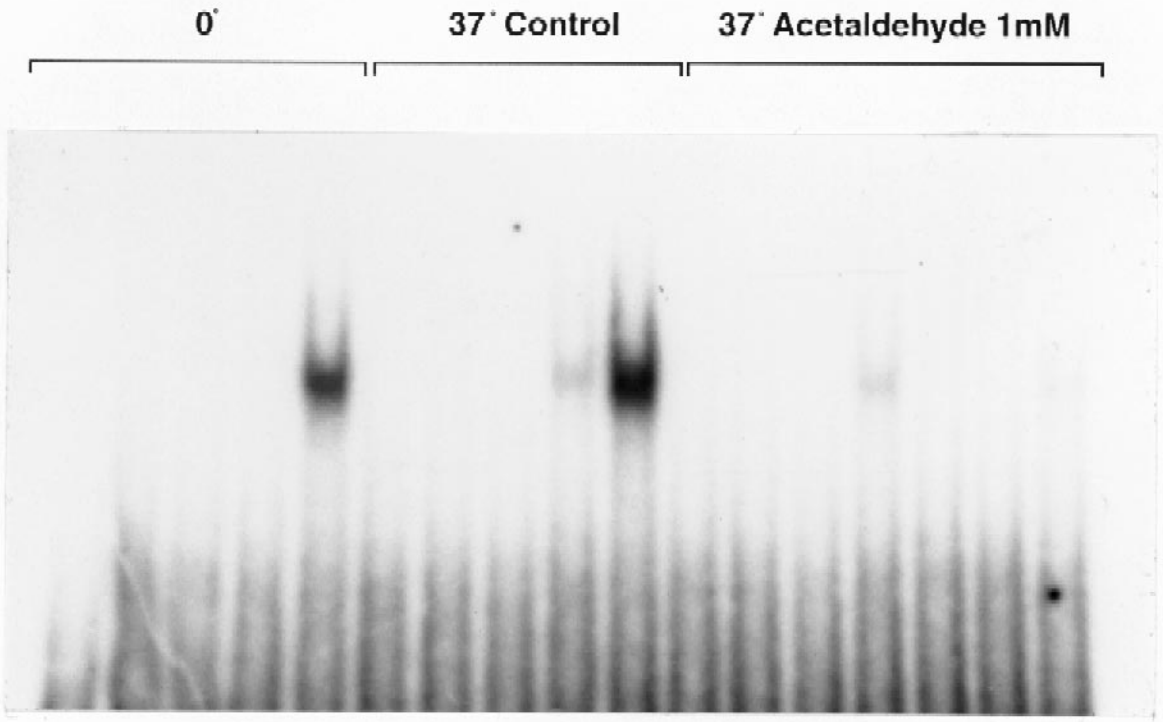

A.
PPAR/RXR
B.
1 2 3 4 5 6 7 8 9 10 11 12
Nuclear extract - + + + + + + + + + + +
Cold competitor - - - 50 100 - - - - - - -
Anti - PPARα - - - - - + - - - - - -
Ethanol - - - - - - - + + + - -
Cyanamide - - - - - - - - + + + -
4-methyl-pyrazole - - - - - - - - - + - +


A.
PPAR/RXR
B.
Acetaldehyde (μM) 0 50 85 150



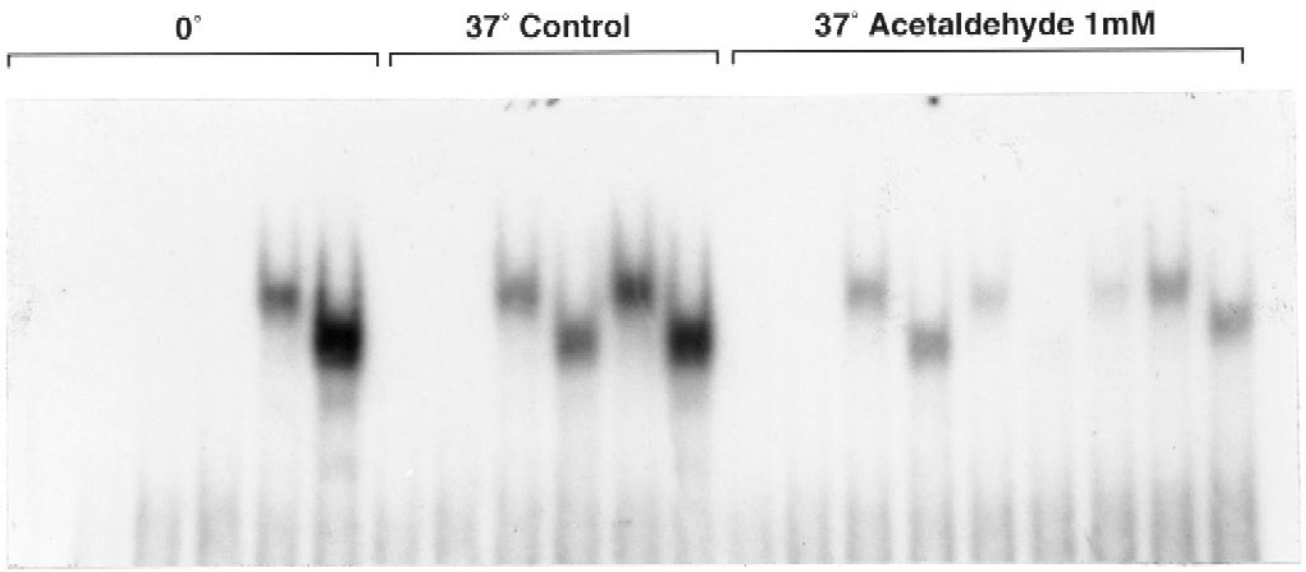
Non - transfected	–	+	–	–	–	–	–	–	–	–	–	–		
PPAR α - transfected	–	–	+	+	+	+	+	+	+	+	+	+		
Ethanol	–	–	–	–	+	+	+	+	–	–	–	–		
Cyanamide	–	–	–	–	–	–	+	+	–	–	–	–		
Acetaldehyde	–	–	–	–	–	–	–	–	50	50	150	150		
PPAR α antibody	–	–	–	+	–	+	–	+	–	+	–	+		

Effect of Acetaldehyde Exposure on DNA Binding Activity of in vitro Translated RXRα and PPARα



(-) IVT	-	+	-	-	-	+	-	-	-	-	●	-	-	-	-	-	-
RXRα	-	-	+	-	+	-	+	-	+	-	-	●	-	●	+	●	-
PPARα	-	-	-	+	+	-	-	+	+	-	-	-	●	+	●	●	-
(RXR/PPAR)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	●

Effect of Acetaldehyde Exposure on DNA Binding Activity of in vitro Translated RXRα, PPARγ and PPARδ



(-) IVT	-	+	-	-	-	+	-	-	-	-	-	●	-	-	-	-	-	-	-	-
RXRα	-	-	+	+	+	-	+	+	+	-	-	-	●	●	●	+	+	●	-	-
PPARγ	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	●	-	●	-	-
PPARδ	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	-	●	-	-	-
(RXR/γ)	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	●	-
(RXR/δ)	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	●

The transcriptional and DNA binding activity of peroxisome proliferator-activated receptor α is inhibited by ethanol metabolism: a novel mechanism for the development of ethanol-induced fatty liver

Andrea Galli, Jane Pinaire, Monika Fischer, Ryan Dorris and David W. Crabb

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