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Inhibition of Human Prenatal Biosynthesis of all-*trans*-Retinoic Acid by Ethanol, Ethanol Metabolites, and Products of Lipid Peroxidation Reactions

A Possible Role for CYP2E1

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ABSTRACT. Biotransformation of all-*trans*-retinol (t-ROH) and all-*trans*-retinal (t-RAL) to all-*trans*-retinoic acid (t-RA) in human prenatal hepatic tissues (53–84 gestational days) was investigated with HPLC using human adult hepatic tissues as positive controls. Catalysis of the biotransformation of t-ROH by prenatal human cytosolic fractions resulted in accumulation of t-RAL with minimal t-RA. Oxidations of t-ROH catalyzed by prenatal cytosol were supported by both NAD^+ and $NADP^+$, although NAD^+ was a much better cofactor. In contrast, catalysis of the oxidation of t-RAL to t-RA appeared to be solely NAD^+ dependent. Substrate K_m values for conversions of t-ROH to t-RAL and of t-RAL to t-RA were 82.4 and 65.8 μM, respectively. At concentrations of 10 and 90 mM, ethanol inhibited the conversion of t-ROH to t-RAL by 25 and 43%, respectively, but did not inhibit the conversion of t-RAL to t-RA significantly. In contrast, acetaldehyde reduced the conversion of t-RAL to t-RA by 25 and 87% at 0.1 and 10 mM respective concentrations. Several alcohols and aldehydes known to be generated from lipid peroxides also exhibited significant inhibition of t-RA biosynthesis in human prenatal hepatic tissues. Among the compounds tested, 4-hydroxy-2-nonenal (4-HNE) was highly effective in inhibiting the conversion of t-RAL to t-RA. A 20% inhibition was observed at a concentration of only 0.001 mM, and nearly complete inhibition was produced at 0.1 mM. Human fetal and embryonic hepatic tissues each exhibited significant *CYP2E1* expression as assessed with chlorzoxazone 6-hydroxylation, a highly sensitive western blotting technique, and reverse transcriptase-polymerase chain reaction (PCR) (RT-PCR), suggesting that lipid peroxidation can be initiated via CYP2E1-catalyzed ethanol oxidation in human embryonic hepatic tissues. In summary, these studies suggest that ethanol may affect the biosynthesis of t-RA in human prenatal hepatic tissues directly and indirectly. Ethanol and its major oxidative metabolite, acetaldehyde, both inhibit the generation of t-RA. Concurrently, the CYP2E1-catalyzed oxidation of ethanol can initiate lipid peroxidation via generation of a variety of free radicals. The lipid peroxides thereby generated could then be further converted via CYP2E1-catalyzed reactions to alcohols and aldehydes, including 4-HNE, that act as potent inhibitors of t-RA synthesis. BIOCHEM PHARMACOL **57**;7:811–821, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ethanol; retinoids; lipid peroxidation; CYP2E1; human embryos; fetuses; hepatic biotransformation

t-RA†, the biologically active metabolite of t-ROH and t-RAL, plays a highly important role in embryonic development mediated by nuclear receptor proteins known as retinoic acid receptors (RARs) and retinoid "X" receptors (RXRs) [1–4]. In addition, it is accepted that t-RA has highly potent teratogenic potential and is capable of markedly disrupting the normal development of human embryos and fetuses, resulting in profound developmental abnormalities in the offspring. Numerous previous studies have well demonstrated that both excesses and deficiencies of retinoids are capable of eliciting severe birth defects, making it apparent that a stringent regulation of levels of retinoid receptor ligands in embryonic tissues is essential for normal developmental processes. In spite of these facts, studies of the biosynthesis of t-RA in human prenatal tissues appear not to have been reported [5]. The objective of these investigations, therefore, was to initiate a filling of this highly important gap in our fundamental knowledge of developmental processes as they specifically relate to humans. Because of the similarities in the enzymic processes relating to t-RA synthesis from its major precursors (t-ROH and t-RAL) and ethanol biotransformation, and because

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† *Abbreviations:* t-RA, all-*trans*-retinoic acid; t-ROH, all-*trans*-retinol; t-RAL, all-*trans*-retinal; 4-HNE, 4-hydroxy-2-nonenal; CYP2E1, cytochrome P4502E1; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; HAHC, human adult hepatic cytosolic fraction; HPHC, human prenatal hepatic cytosolic fraction; CHZ, chlorzoxazone; 6-OH CHZ, 6-hydroxy chlorzoxazone; and RT-PCR, reverse transcriptase-polymerase chain reaction.

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ethanol is deemed to be the most important of all known human chemical teratogens [6–9], studies were also directed toward an ascertainment of the potential interactive effects of ethanol, ethanol biotransformation, and ethanol metabolites with t-RA synthesis in human embryonic and fetal tissues.

In adult mammalian hepatic tissues, biosynthesis of t-RA is catalyzed primarily by ADH and ALDH [10, 11], but other enzymes including P450 heme-thiolate proteins [12], a cytosolic retinal oxidase also designated as a retinoic acid synthase [13], xanthine dehydrogenase [14], and aldehyde oxidase [14, 15] also have been reported to catalyze the biosynthesis of t-RA from precursor retinoids. It may be expected that some or all of these enzymes/enzyme systems would function to catalyze t-RA biosynthesis in human prenatal tissues, although there is as yet little documentation for such an expectation. Possibly even more importantly, it is not yet known which of such enzymes/enzyme systems would act as the predominant catalysts in human prenatal tissues. However, since the oxidation of ethanol and the biosynthesis of t-RA via retinol oxidation are catalyzed by similar enzymes, it would seem reasonable that ethanol might inhibit t-RA biosynthesis. Indeed, it has been documented that catalysis of the conversion of t-ROH to t-RAL by purified class IV alcohol dehydrogenase is inhibited by ethanol [16], and inhibition of the biosynthesis of t-RA by ethanol in adult human hepatic tissues [17] as well as other adult tissues [17, 18] *in vitro* also has been documented. A number of investigators [10, 19–22], in fact, have proposed that ethanol may produce embryotoxic effects including dysmorphogenesis via inhibition of t-RA biosynthesis, resulting in an effective deficiency of hormonal t-RA in prenatal tissues and thereby eliciting some of the characteristic defects of the fetal alcohol syndrome. A rigorous testing of this hypothesis appears warranted for at least three important reasons: first, both ethanol and retinoids are well-established and highly important human teratogens, and the interactive effects of these agents should be better understood; second, a number of recent studies have documented the enzymic participation of t-RA biosynthesis in rodent prenatal tissues [23–26]; finally, inhibition of the oxidation of t-ROH to t-RA by ethanol in adult rodent hepatic tissues [16–18] suggests the possibility of a similar inhibition in prenatal human tissues.

An additional impetus for such investigations is the fact that recent studies have shown that cytochrome P4502E1, a P450 isoform that actively catalyzes the oxidation of ethanol, is expressed in human prenatal cephalic tissues even during embryogenesis [27]. The human prenatal brain is a primary target for the teratogenic effects of ethanol as well as retinoids. Carpenter *et al.* [28] earlier demonstrated the expression of *CYP2E1* at relatively high levels in human prenatal hepatic tissues during later (fetal) stages of gestation. In addition to catalysis of the conversion of ethanol to acetaldehyde (a highly embryotoxic metabolite), P4502E1 is known to generate reactive oxygen species and organic free radicals (such as the hydroxyethyl radical) that are capable of initiating lipid peroxidation reactions [29]. Lipid peroxidation results in the formation of lipid hydroperoxides, and these products will undergo P4502E1-catalyzed conversion to highly toxic aldehydes such as malondialdehyde and 4-HNE among several others [30]. Recent studies from this laboratory [31] have shown that 4-HNE and certain other products of lipid peroxidation are highly potent inhibitors of t-RA biosynthesis in prenatal rat tissues.

Collectively, the studies cited above suggest that biosynthesis of t-RA in human prenatal tissues may be inhibited by ethanol by at least two possible mechanisms. As indicated by the investigations of Duester and coworkers [10, 19, 22] and others [16–18, 20, 21, 24], ethanol can act as a competitive inhibitor of retinol dehydrogenase(s) and thereby directly inhibit the dehydrogenation of t-ROH to t-RAL, normally regarded as the rate-limiting step in t-RA biosynthesis in adult tissues. Concurrently, ethanol biotransformation can result in the generation of a large number of reactive species, some of which may also be capable of secondarily inhibiting prenatal t-RA biosynthesis via a variety of mechanisms. A primary objective of this study, therefore, was to investigate the potential roles of ethanol, reactive intermediates generated as the result of the oxidation of ethanol, and other reactive chemicals associated with ethanol oxidation on the biosynthesis of t-RA in human prenatal tissues. For these investigations, we chose to study human prenatal hepatic tissues (because they have been better characterized to this point) at days 53–84 of gestation. At this institution, these human tissues may be obtained in excellent condition during these stages of gestation, which represent both embryonic and fetal stages of development. For these initial investigations, we focused primarily upon the potential for interactive effects involving the dehydrogenase-catalyzed oxidations of ethanol and t-ROH. In view of the established importance of CYP2E1 in generating reactive ethanol metabolites that could also inhibit t-RA biosynthesis, we also investigated the capacity of the human prenatal tissues under study to effect CYP2E1-catalyzed oxidation reactions.

MATERIALS AND METHODS

Materials

Retinoids, ethanol, acetaldehyde, and other alcohols and aldehydes were purchased either from the Sigma Chemical Co. or from the Aldrich Chemical Co. Prestained SDS-PAGE standards were obtained from the Bio-Rad Laboratories. Nitrocellulose membrane (Nitrobind, 0.45 μm pore size) was from Micron Separations, Inc. Rabbit anti-human cytochrome P4502E1 antibody was purchased from Research Diagnostics, Inc. Horseradish peroxidase-conjugated goat anti-rabbit IgG and SuperSignal ULTRA chemiluminescent substrate were obtained from the Pierce Chemical Co. All other chemicals and reagents utilized were of the highest purity commercially available.

Preparation of Cytosolic Fractions

Adult human hepatic tissues were obtained from the Solid Organ Transplant Program, Life Center Northwest, via courtesy of Dr. Kenneth Thummel, Department of Pharmaceutics, University of Washington. Fetal and embryonic hepatic tissues were obtained from the Birth Defects Research Laboratory of the University of Washington (Department of Pediatrics). Handling of all human tissues was in accordance with the guidelines of the Human Subjects Review Committee at the same institution. Tissues were snap frozen in liquid nitrogen immediately after removal; fetal and embryonic tissues obtained after dilatation and curettage were delivered to the laboratory within 3–4 hr after removal and were processed immediately for analyses or were stored under liquid nitrogen for not more than 4–5 days prior to processing for analyses. Gestational ages ranged between 53 and 84 days as estimated from measurements of foot lengths. Tissues were transferred to a Duall Tissue Grinder containing 3 mL of 0.1 M sodium phosphate buffer (pH 7.5)/g of human tissue (wet weight) and were homogenized by hand on ice. Homogenates were centrifuged at 700 *g* for 10 min to remove large particulates. The resultant supernatant was further centrifuged at 105,000 *g* at 4° for 1.0 hr, and the subsequent supernatant fraction was defined as liver cytosol. The liver cytosol was stored at −80° for not more than 2 weeks until utilized as enzyme source in enzymic incubations. The 105,000 *g* × 1 hr particulate fractions were also used as enzyme sources in studies of the monooxygenation of CHZ, described below.

Oxidative Conversions of t-ROH and t-RAL

HAHC and HPHC were mixed well with 0.1 M sodium phosphate buffer (pH 7.5) containing 4 mM NAD^+ (or other cofactors where indicated), 40 mM NaCl, and 0.02% Tween-80 in a 5-mL glass tube. t-ROH or t-RAL in acetone (1 mg/mL) was added to the test tube at various concentrations, and the final volume was brought to 1.0 mL with buffer. Additions of retinoids were conducted in a darkened room under yellow lights to prevent photoisomerization and autoxidation. The reactants were then incubated in total darkness for various times at 37° in a continuously shaking water bath. At the end of the incubation, an equal volume of ice-cold isopropanol was added to the incubation mixture, which was then vortexed for 1 min and centrifuged for 30 min at 16,000 *g* at 4°. The supernatant fraction was decanted and stored at −20° for HPLC analyses.

Identification and Quantitation of Retinoid Metabolites by HPLC

The solvent delivery system for HPLC consisted of two model 100 A dual piston Beckman pumps linked together for activation of a binary gradient. The system was interfaced with a Shimadzu SPD-10A UV-VIS detector (set at 354 nm) and a Shimadzu C-R5A Chromatopac data processor. The HPLC system was equipped with a Beckman mixing chamber and manual injector. Identification and quantitation of retinoid metabolites (t-RAL, t-RA) were conducted with a 5 μm Zorbax octadecylsilane (ODS) column (0.46 × 25 cm) (MacMod Analytical, Inc.) following the procedures described by Kim *et al.* [32] with slight modifications. The analytical eluents consisted of solvent A (acetonitrile:H_2O:acetic acid, 49.75:49.75:0.5, by vol.) and solvent B (acetonitrile:H_2O:acetic acid, 90:10:0.04, by vol.), both containing 10 mM ammonium acetate. The HPLC conditions were as follows: 80% solvent A plus 20% solvent B with a flow rate of 1.2 mL/min for 25 min. A mixture of authentic retinoid standards was analyzed with the same procedure. One hundred microliters of a mixture of standard retinoids or supernatant fraction from the incubation mixture was loaded onto the HPLC column, and the retention times of the retinoid standards were used to identify peaks eluting from the HPLC column. The detection limit of the HPLC system for concentrations of measurable retinoids was 1 ng/100 μL. Concentrations of retinoids below 1 ng/100 μL were designated as not detectable.

Enzymic Conversion of CHZ to 6-OH CHZ

Catalytic activities of CYP2E1 in human adult, fetal, and embryonic tissues were estimated by measurements of rates of conversion of CHZ to 6-OH CHZ with high speed particulate fractions (105,000 *g* × 1.0 hr sedimentation following an initial 700 *g* × 10 min centrifugation) utilized as enzyme source. The procedures used were those described by Lucas *et al.* [33] with slight modifications. Briefly, 400 μM CHZ was incubated with human prenatal or adult hepatic particulate fractions in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 1.0 mM NADPH in a total volume of 1 mL. Reactions were initiated, following a 3-min preincubation at 37°, by addition of NADPH and were carried out for 30 min in a shaking water bath at 37°. The reactions were terminated by adding 50 μL of 43% H_3PO_4. The incubation mixtures were extracted with 2 mL of chloroform:2-propanol (85:15, v/v). After centrifugation at 3000 *g* for 10 min, the organic phases were dried by filtration on Na_2SO_4 and then evaporated to dryness under a stream of nitrogen at 37°. Dry residues were dissolved in 200 μL of an HPLC mobile phase consisting of 0.5% (v/v) glacial acetic acid in H_2O:acetonitrile (75:25, v/v) and subjected to HPLC analyses.

Analyses of CHZ Monooxygenation with HPLC

CHZ and its major metabolite, 6-OH CHZ, were analyzed with HPLC on a 5-μm Nucleosil C18 column (250 × 4.6 mm). The column was interfaced with a Shimadzu LC-600 double-plunger reciprocating pump, a Shimadzu SPD-10A UV-VIS detector, and a Shimadzu C-R5A Chromatopac data processor. Two hundred microliters of sample was injected onto the column, and the flow rate of the mobile

phase was 1.6 mL/min. Quantitation of 6-OH CHZ was performed utilizing a quantitative calibration program installed in the C-R5A with detection at 287 nm.

Immunoblot Analyses

Samples of microsomal protein and prestained standards were separated by SDS-PAGE [34] using the Mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories). The running and stacking gels were composed of 10 and 5% polyacrylamide, respectively, and both gels contained 0.1% SDS. The protein gel was electroblotted for 2 hr onto a nitrocellulose membrane using the Bio-Rad Trans-Blot electrophoretic transfer system. The membrane was blocked for 1 hr in 2% nonfat milk and rinsed briefly; TBST buffer, containing 0.1 M Tris, 0.9% NaCl, and 0.1% Tween 20, was used for all washes and dilutions. The membrane was incubated overnight with rabbit anti-human CYP2E1 antibody (1:10,000 dilution), and then washed three times. After incubating for 1 hr with peroxidase-conjugated secondary antibody (1:250,000 dilution), the membrane was washed six times and immersed in Super-Signal ULTRA chemiluminescent substrate (Pierce Chemical Co.) for 15 min. The immunoblot was exposed to Hyperfilm ECL (Amersham, Inc.) for several minutes, and the film was developed for visualization of signal.

Protein Determinations

Protein concentrations of the subcellular fractions were determined by the method of Lowry *et al.* [35], using BSA as the reference standard.

NADH Generation

Generation of the reaction product (NADH) as a measure of NAD^+-dependent, dehydrogenase-catalyzed ethanol or acetaldehyde oxidation was assessed spectrophotometrically by measurements of increases in UV absorbance in assay solutions at 340 nm. Ethanol (30 mM) or acetaldehyde (10 mM) was incubated with cytosolic fractions in 0.1 M sodium phosphate buffer (pH 7.5) containing 4 mM NAD^+ at room temperature or 37° for various time periods.

Statistical Analyses

Student's *t*-test was used to evaluate statistical comparisons. Differences were considered to be statistically significant at $P < 0.05$. Data are presented as means ± standard deviations from at least three separate experiments run in duplicate or triplicate.

RESULTS

The biotransformations of t-ROH and t-RAL to t-RA were analyzed using HPHC from 54 to 81 days of gestation and HAHC as enzyme sources. The results are presented in panels A–C of Fig. 1. Figure 1A shows that HPHC preparations from gestational ages ranging from 54 to 81 days exhibited similar specific activities under the incubation conditions utilized, with a slight tendency to increase as a function of gestational age. With 18 μM t-ROH as substrate and HPHC as enzyme source, the major metabolite generated was t-RAL, which appeared in metabolite profiles at 2–3 times the level of t-RA. This contrasted with the use of HAHC preparations as enzyme source; in those experiments (not shown) the major metabolite generated from t-ROH was t-RA, with only minimal quantities of t-RAL appearing in the metabolite profiles. Using the same HPHC preparations as enzyme source but with 18 μM t-RAL as substrate (Fig. 1B), rates of generation of t-RA were only marginally higher than those observed with t-ROH as substrate (Fig. 1A). The accumulation of t-RAL indicated that, with HPHC preparations as enzyme source and t-ROH as substrate, the conversion of generated t-RAL to t-RA proceeded at slower rates than the preceding conversion of t-ROH to t-RAL. In the case of HPHC-catalysis of the conversion of 18 μM t-RAL to t-RA, however (Fig. 1B), rates of t-RAL conversion to t-RA appeared to increase as a function of gestational age. Under conditions for which substrate concentrations were equal (18 μM), HPHC-catalyzed conversions of t-RAL to t-RA were slightly higher than HPHC-catalyzed conversions of t-ROH to t-RAL (Fig. 1C), and retinoid metabolites generated from HAHC-catalyzed reactions were 8- to 16-fold more abundant than those generated from HPHC-catalyzed reactions.

Representative rates of the HPHC-catalyzed, NAD^+-dependent conversion of ethanol to acetaldehyde and of the further conversion of acetaldehyde to acetate are illustrated in Fig. 2. Noteworthy in this case are the extremely low rates of ethanol oxidation observed with HPHC as enzyme source when compared with the same reactions using HAHC as enzyme source (Fig. 2B). In HPHC-catalyzed reactions (Fig. 2A), generation of NADH from NAD^+ tended to be more rapid with acetaldehyde (10 mM) as substrate than with ethanol (30 mM) as substrate. With HAHC as enzyme source (Fig. 2B), the reverse was observed, i.e. generation of NADH was more rapid with ethanol as substrate. The apparently very low rates of ethanol oxidation observed with HPHC as enzyme source were generally concordant with several earlier studies in which prenatal hepatic tissues from experimental animals were used as enzyme sources.

With t-ROH as substrate and HPHC as enzyme source, generation of t-RAL was most rapid when NAD^+ was included as a cofactor in the reaction mixture. $NADP^+$ also supported the reaction, but was only approximately one-sixth as effective as NAD^+. Neither NADH nor NADPH supported the reaction detectably. With t-RAL as substrate and HPHC as enzyme source, generation of t-RA was supported by NAD^+ but not detectably by $NADP^+$, NADH, or NADPH. The results indicated that the reactions were catalyzed primarily by NAD^+-dependent dehy-

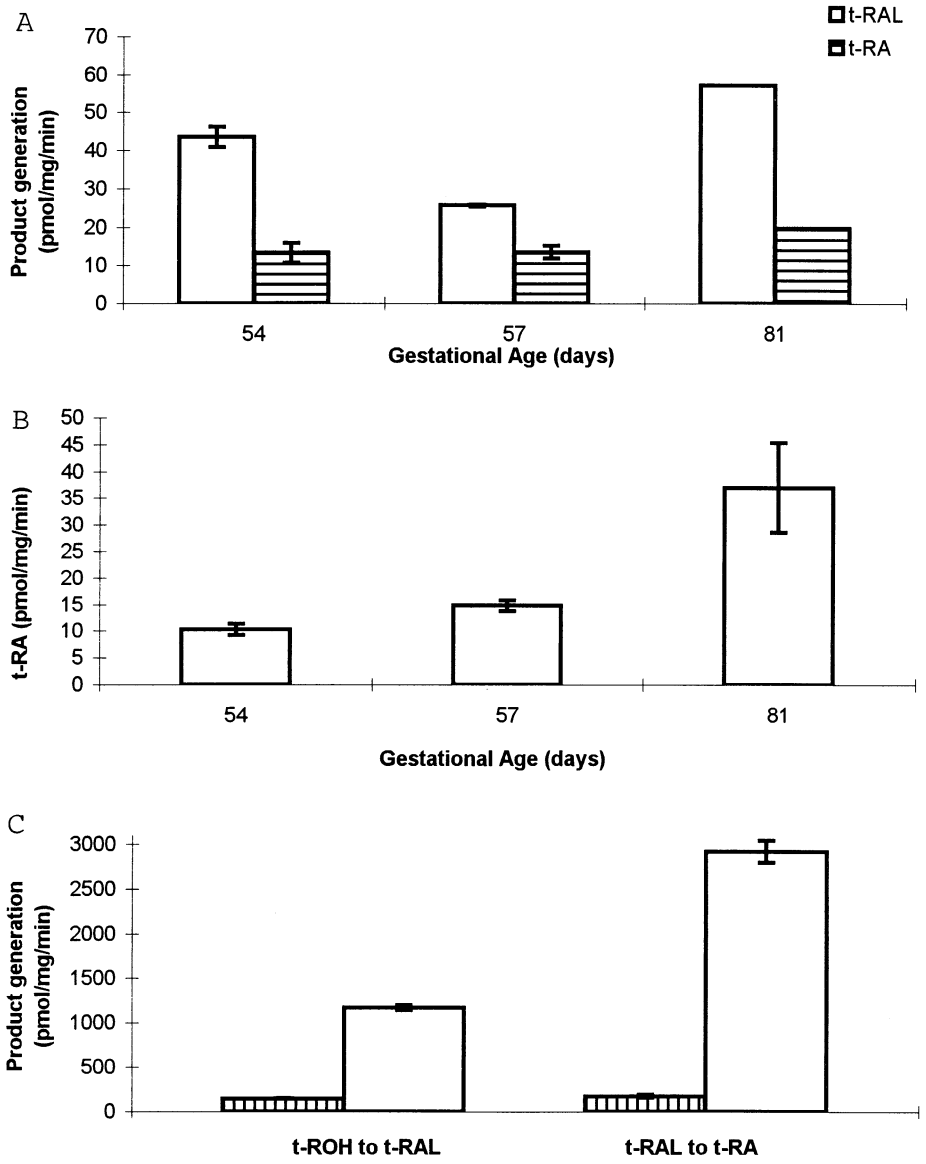

FIG. 1. Biotransformation of t-ROH and t-RAL with human hepatic cytosolic fractions as enzyme sources. (A) HPHC-catalyzed conversion of t-ROH to t-RAL and t-RA at different stages of gestation; (B) HPHC-catalyzed conversion of t-RAL to t-RA at different stages of gestation; and (C) comparisons of the HPHC- and HAHC-catalyzed conversions of t-ROH to t-RAL and of t-RAL to t-RA. Forty μg of HPHC protein (hatched bars) or 70 μg of HAHC protein (open bars) was incubated for 30 min with 36 μM t-ROH or 18 μM t-RAL at 37° in the presence of 4 mM NAD^+ in total darkness. HPHC protein was from prenatal hepatic tissues at day 54 of gestation. Reaction products were analyzed with HPLC as described in Materials and Methods. Values are means ± SD; N = 3 or 4.

drogenases present in the human prenatal hepatic tissues. Kinetics of the HPHC-catalyzed, NAD^+-dependent reactions are illustrated in panels A and B of Fig. 3. With t-ROH as substrate and t-RAL as the measured metabolite (Fig. 3A), the K_m was approximately 82 μM. With t-RAL as substrate and t-RA as the measured metabolite (Fig. 3B), the K_m was approximately 66 μM. Corresponding V_{max} values were calculated as 97 and 49 pmol/mg/min, respectively. K_m and V_{max} values were determined twice on the same tissue preparation and were within 10%.

The inhibitory effects of ethanol, ethanol metabolites, and other chemicals associated with ethanol biotransformation were investigated next. Ethanol *per se* proved to be an effective inhibitor of the HPHC-catalyzed conversion of t-ROH to t-RAL, exhibiting a 25% inhibition at 10 mM and a 43% inhibition at 90 mM. Ethanol did not inhibit the HPHC-catalyzed conversion of t-RAL to t-RA significantly under the reaction conditions utilized, even at concentrations as high as 90 mM. Acetaldehyde, a primary oxidative metabolite of ethanol, failed to inhibit the HPHC-catalyzed conversion of t-ROH to t-RAL significantly at concentrations as high as 100 mM (data not shown) but did elicit effective inhibition of the HPHC-catalyzed conversion of t-RAL to t-RA at relatively low concentrations (Fig. 4). A 25% inhibition was observed at a concentration of only 0.1 mM, and 87% inhibition was observed at 10 mM. A number of other alcohols also were examined for their inhibitory effects, and the results are given in Fig. 5. Short

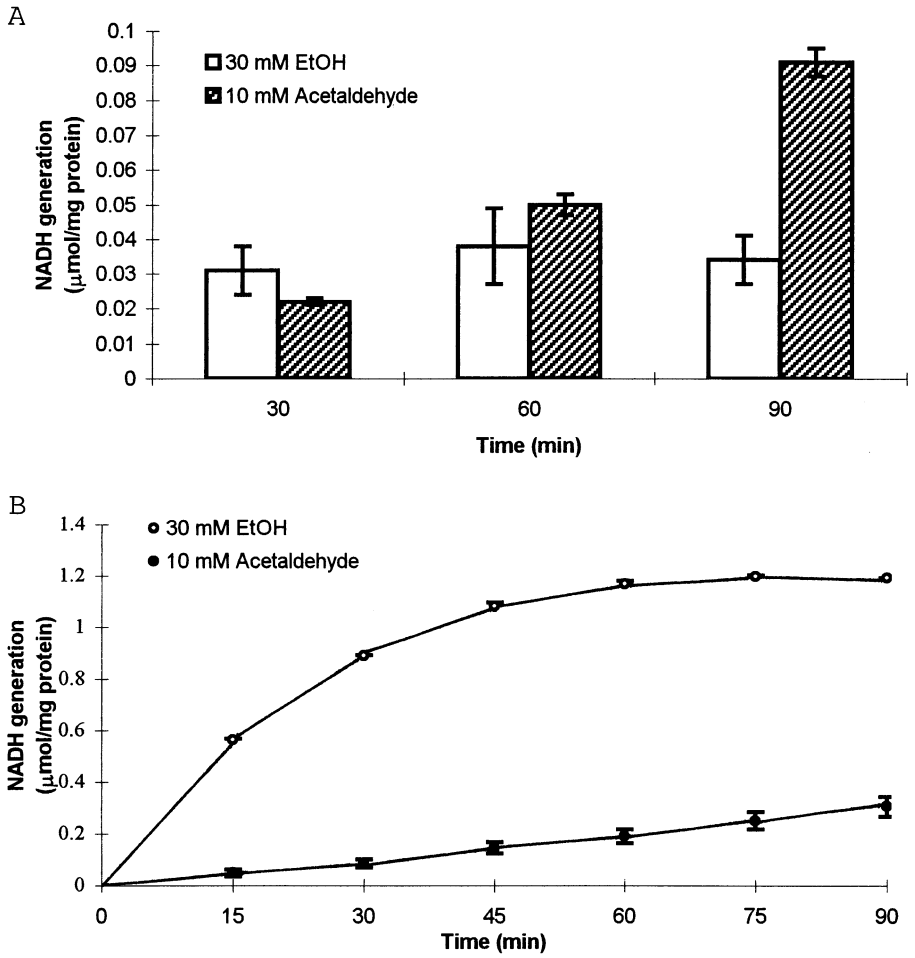

FIG. 2. Comparisons of NAD^+-dependent oxidations of ethanol and acetaldehyde catalyzed by human liver cytosolic fractions. Sixty micrograms of HPHC (A) or HAHC (B) protein was incubated with 4 mM NAD^+ and 30 mM ethanol at 37° or 10 mM acetaldehyde at room temperature. Incubations run without additions of ethanol or acetaldehyde served as controls, and the values obtained were subtracted from those of the test samples. Values are means ± SD of 3 or 4 experiments. Spectrophotometric analysis of the reaction product (NADH) is described in Materials and Methods.

chain alcohols such as methanol and isopropanol, like ethanol, were relatively weak inhibitors of the HPHC-catalyzed conversion of t-ROH to t-RAL (Fig. 5A) at concentrations of 10 mM. Longer chain alcohols such as 2-hexanol and 2-nonanol (products of the biotransformation of lipid hydroperoxides), however, inhibited that reaction by approximately 51 and 65%, respectively, at concentrations of 10 mM. The same longer chain alcohols also produced a much lesser but statistically significant inhibition of the HPHC-catalyzed conversion of t-RAL to t-RA (not shown), and the inhibition of t-ROH to t-RA (Fig. 5B) followed the same pattern, i.e. an increasing inhibition with increasing chain length and molecular weight of the alcohol inhibitor under study. The ADH inhibitor, 4-methylpyrazole (10 mM), inhibited the HPHC-catalyzed conversion of t-ROH to t-RAL by approximately 58% and the HPHC-catalyzed conversion of t-ROH to t-RA by approximately 27% (Fig. 5, A and B).

Several additional aldehydes also were studied as potential inhibitors of the HPHC-catalyzed conversion of t-RAL to t-RA, and the results are given in Table 1. Formaldehyde, the aldehyde of lowest molecular weight and shortest chain length, was also the least effective inhibitor among those tested in that series, producing only a 34% inhibition at concentrations of 0.1 mM. Acetaldehyde, likewise of short chain length and the major product of ethanol oxidation, was approximately equally effective as described above (Fig. 4). At the same concentrations, however, hexanal and t-2-nonenal, aldehydes of relatively long chain length and major products of the further biotransformation of lipid hydroperoxides, produced nearly complete inhibition (Table 1). Citral and disulfiram produced almost complete inhibition at concentrations of only 0.01 mM (Table 1). 4-HNE, a principal aldehydic product of the further biotransformation of lipid hydroperoxides and of currently high interest in toxicology because of its high toxicity and relatively rapid rates of generation during lipid peroxidation, was also a particularly effective inhibitor of the HPHC-catalyzed conversion of t-RAL to t-RA. This aldehyde compound was studied separately at four different concentrations (Fig. 6). Significant inhibitory effects were observed at micromolar concentrations.

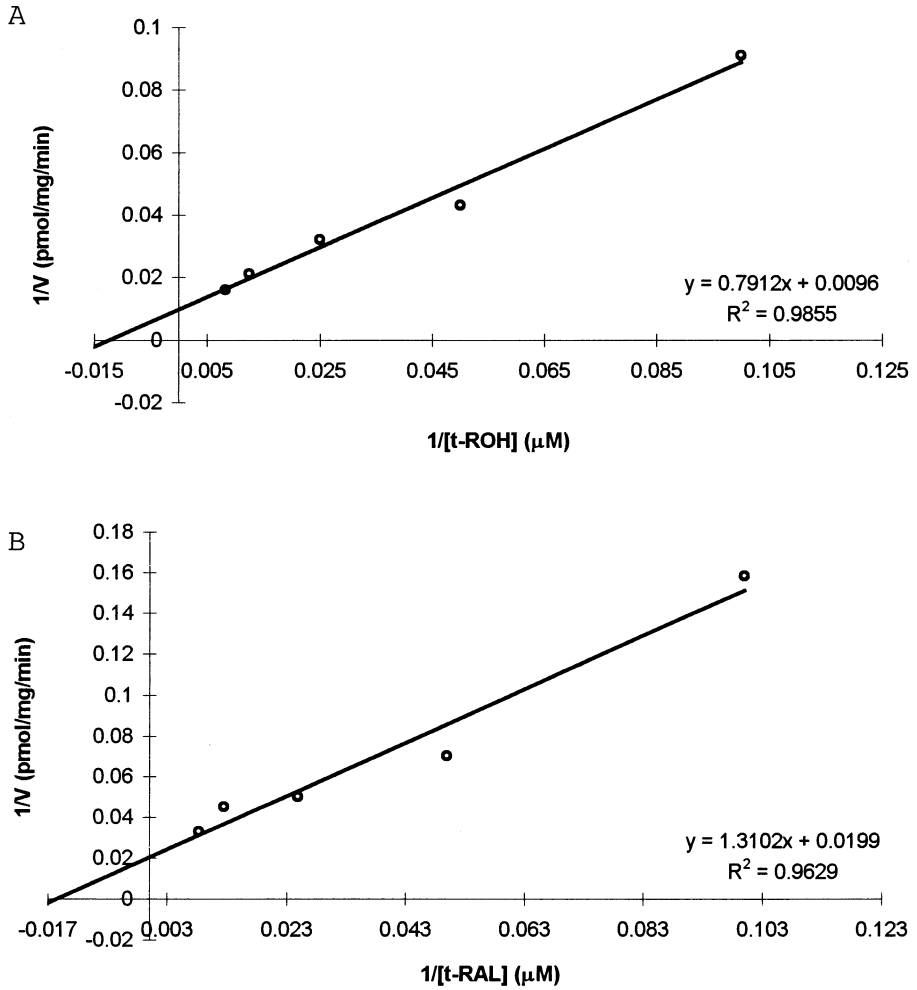

FIG. 3. Lineweaver-Burk plots for retinoid biotransformation reactions. K_m and V_{max} values were determined for catalysis of the conversion of t-ROH to t-RAL (A) and of t-RAL to t-RA (B) with 70 μg of HPHC protein as enzyme source. Incubations were conducted at 37° for 20 min in total darkness with 10–120 μM substrate concentrations and 4 mM NAD^+ as cofactor. Reaction products were analyzed with HPLC as described in Materials and Methods. Values are means of triplicate determinations.

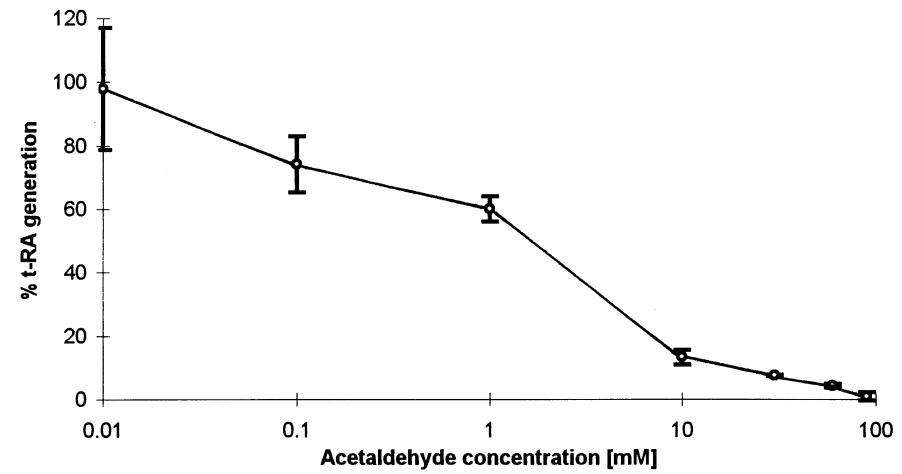

FIG. 4. Effects of various acetaldehyde concentrations on the HPHC-catalyzed conversion of t-RAL to t-RA. HPHC protein (60 μg) was incubated with 18 μM t-RAL, 4 mM NAD^+, and acetaldehyde for 30 min at 37° in total darkness. Acetaldehyde concentrations ranged from 0.01 to 90 mM. Values are the means ± SD of triplicate determinations. The value for the control was 127 ± 59 pmol/mg/min. Reaction products were analyzed with HPLC as described in Materials and Methods.

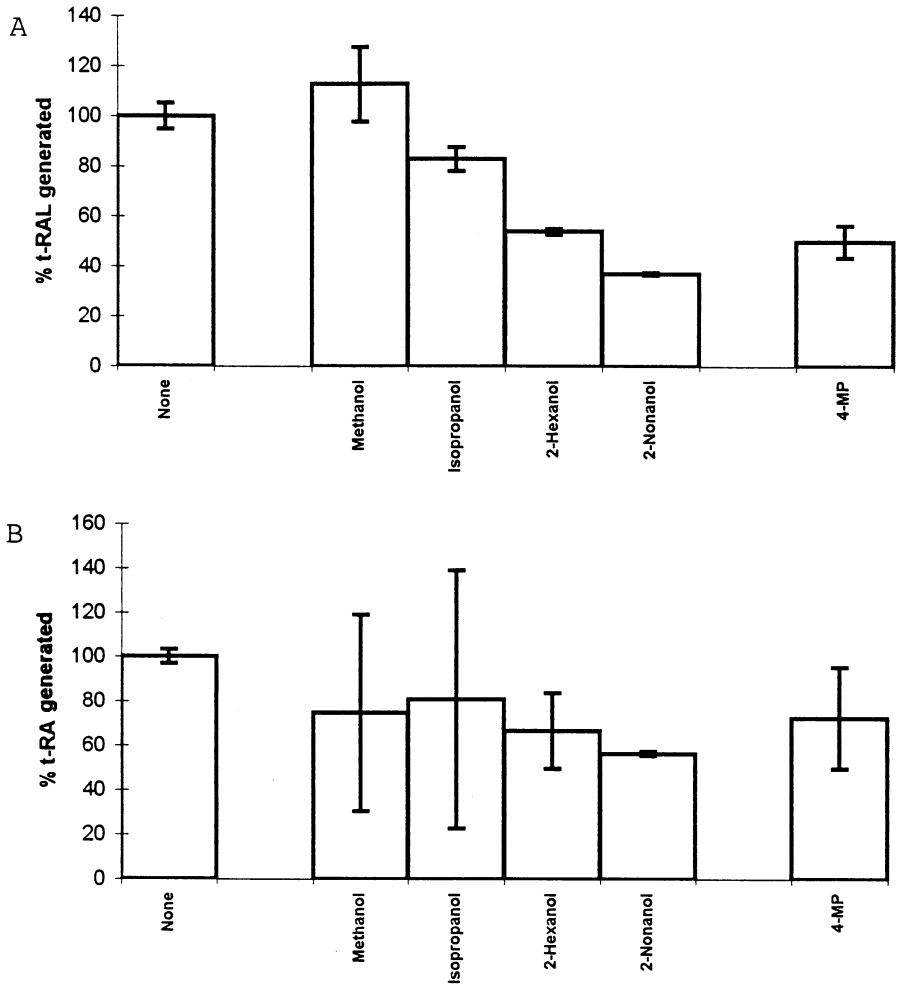

FIG. 5. Effects of various alcohols on the HPHC-catalyzed oxidation of t-ROH. Inhibitions of the HPHC-catalyzed conversion of t-ROH to t-RAL (A) and of t-ROH to t-RA (B) by various alcohols and 4-methylpyrazole (4-MP) were determined. HPHC protein (60 μg) was incubated with 36 μM t-ROH, 4 mM NAD^+, and 10 mM inhibitor at 37° for 30 min in total darkness. Control values ranged from 83 to 123 pmol/mg/min for generation of t-RAL and from 37 to 49 pmol/mg/min for generation of t-RA. Values are reported as mean percentages of inhibition ± SD for 3–4 determinations. Reaction products were analyzed with HPLC as described in Materials and Methods.

TABLE 1. Inhibition of HPHC-catalyzed* conversion of t-RAL to t-RA by various aldehydes and disulfiram

	% Inhibition†		
Inhibitor	**1 μM**	**10 μM**	**100 μM**
Formaldehyde	0†	0	34
Hexanal	0	50	> 98
t-2-Nonenal	12	91	> 98
Citral	22	95	> 98
Disulfiram‡	21	> 98	> 98

*HPHC (50 μg protein) was incubated at 37° for 15 and 30 min in the presence of inhibitor plus 4 mM NAD^+ and 18 μM t-RAL in total darkness. Products were analyzed by HPLC as described in Materials and Methods.

†Values represent percentages of inhibition observed at the concentrations (1, 10 and 100 μM) of inhibitors tested; > 98 indicates that activities measured were indistinguishable from background, indicating essentially total inhibition. Values are the means of two separate experiments. Absolute value for the control was 75.7 ± 7.8 pmol/mg protein/min.

‡Disulfiram, a classical aldehyde dehydrogenase inhibitor, was included in these experiments for comparative purposes.

Because of the observed effective inhibition of t-RA synthesis by products of lipid peroxidation, the capacity of reactive intermediates generated via ethanol oxidation reactions to initiate lipid peroxidation in adult tissues, and the implications of human prenatal *CYP2E1* expression in the hepatic tissues under study in terms of ethanol/P4502E1-elicited increases in initiation of lipid peroxidation, we were specifically interested in the expression of P4502E1 in the human prenatal hepatic tissues investigated. Thus, we evaluated the expression of this P450 isoform in human prenatal hepatic tissues at the same gestational ages (days 54–84) with RT-PCR, western immunoblotting, and analyses of CHZ 6-hydroxylation. RT-PCR analyses yielded somewhat weak but positive signals (data detailed previously [27]) with human embryonic livers at days 54 and 59 of gestation and with a human fetal liver at day 78 of gestation. At that point, we were unable to obtain detectable signals with northern blotting during this period of gestation. For these studies, however,

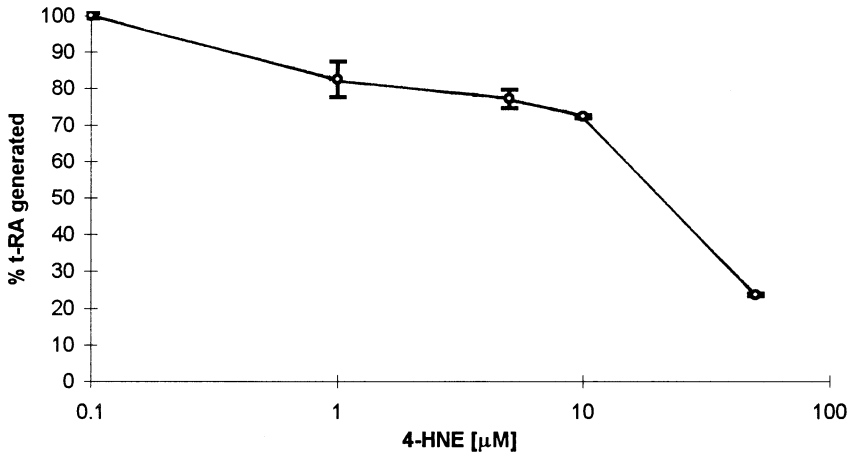

FIG. 6. Effects of various 4-HNE concentrations on the HPHC-catalyzed conversion of t-RAL to t-RA. Incubations were conducted for 30 min at 37° in total darkness with 70 μg HPHC protein, 18 μM t-RAL, 4 mM NAD^+, and 4-HNE. Concentrations of 4-HNE ranged from 1 to 50 μM. Values are means ± SD of triplicate determinations. The value for the control was 47.6 ± 0.7 pmol/mg protein/min. Reaction products were analyzed with HPLC as described in Materials and Methods.

a highly sensitive western blotting technique (see Materials and Methods) yielded relatively weak but definitively positive signals when human embryonic livers from days 54 and 57 and a human fetal liver from day 84 were analyzed (Fig. 7).

Data pertaining to catalysis of the 6-hydroxylation of CHZ to 6-OH CHZ by human prenatal hepatic particulate fractions are presented in Table 2. Monooxygenase activities were detectable although low in comparison with activities measured in adult human hepatic tissues and adult rat hepatic tissues. Significant inhibition of the prenatally catalyzed hydroxylation reaction by carbon monoxide as well as by a selective P4502E1 inhibitor, diethyldithiocarbamate, was also demonstrated (Table 2). The inhibition observed with prenatal human hepatic high speed particulate fractions as enzyme source was somewhat less than the inhibition observed with the same concentrations of inhibitors and adult human hepatic particulate fractions as enzyme source.

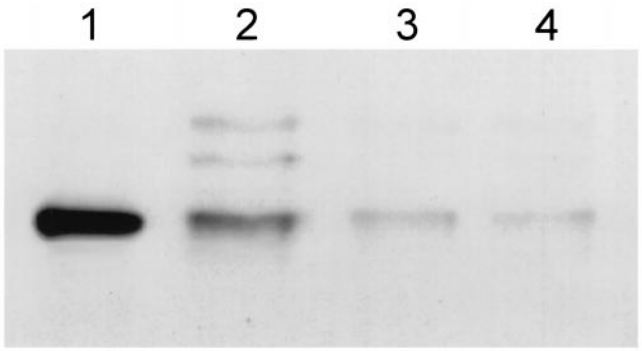

FIG. 7. Immunoblot analysis of human hepatic particulate fractions using anti-CYP2E1 antibody. Particulate samples prepared from adult and prenatal hepatic tissues were subjected to SDS-PAGE and immunoblotting (western blotting) as described in Materials and Methods. Aliquots of tissues are shown from adult (lane 1; 1 μg), gestational day 84 (lane 2; 20 μg), gestational day 57 (lane 3; 20 μg), and gestational day 54 (lane 4; 30 μg). A predominant band of immunoreactive protein is apparent in each lane corresponding to P4502E1 (53 kDa). Higher molecular weight bands of unknown identity are also visible in lanes containing prenatal samples. This experiment was performed twice with similar results.

DISCUSSION

The presented data provide insights into the potential interactive toxic effects of ethanol and retinoids on human embryos from several perspectives. They demonstrate that previous investigations of prenatal rodent tissues with respect to the biotransformation of ethanol and t-ROH [23–26] appear not to apply to prenatal human tissues, at least insofar as human prenatal *hepatic* tissues are concerned. It will be of great future interest to determine the

TABLE 2. Catalysis of CHZ hydroxylation to 6-OH CHZ* by particulate fractions† of human prenatal hepatic tissues, human adult hepatic tissues, and rat adult hepatic tissues: Inhibition by diethyldithiocarbamate (DETC) and carbon monoxide (CO)

Hepatic particulate fraction	Inhibitor (concn)	Specific activities (nmol/mg protein/30 min)
Human prenatal	None	0.48 ± 0.21
Human prenatal	DETC (0.1 mM)	0.28 ± 0.13
Human prenatal	CO (80:20, $CO:O_2$)‡	0.26 ± 0.19
Human adult	None	12.3 ± 1.7
Human adult	DETC (0.1 mM)	1.20 ± 0.34
Human adult	CO (80:20, $CO:O_2$)‡	1.47 ± 0.56
Rat adult	None	26.2 ± 7.6

*Specific activities reported represent the means ± SD of three experiments. Prenatal human hepatic tissues were pooled from three embryonic livers ranging from days 53 to 59 of gestational age.

†Particulate fractions were prepared by an initial centrifugation of the whole homogenate at 700 *g* for 10 min and a subsequent centrifugation of the supernatant fraction at 105,000 *g* for 1 hr. The sediment of the second centrifugation was resuspended and utilized as enzyme source.

‡Controls for these experiments were run under an atmosphere of $N_2:O_2$ (80:20).

extent to which such a statement can be made pertaining specifically to prenatal cephalic tissues because the prenatal brain represents such a highly important toxicologic target of both ethanol and retinoids. Investigations [36] of the actual interactive dysmorphogenic effects of ethanol and t-ROH on developing whole rodent embryos cultured *in vitro* showed a lack of antagonistic effects such as would be predicted by the hypothesis propounded by Duester and coworkers [10, 19, 22] and others [20, 21]. That hypothesis would predict antagonistic embryotoxic effects of ethanol and t-ROH (mutually decreased effects) due to competitive inhibition by ethanol and/or t-ROH of enzymes catalyzing oxidation via dehydrogenation of either the retinoid or the alcohol to reactive metabolites—acetaldehyde and t-RA, respectively. Instead, *greater* than additive and apparently synergistic effects were observed. Investigations of the biotransformation of retinol and ethanol in the same prenatal rodent tissues [23–25] suggested probable reasons for the observed lack of antagonistic effects in the whole embryos. For example, they showed a total lack of inhibition of oxidation of retinol by ethanol, even at extremely high concentrations (10 M) and at the very highest concentrations, even an increase in conversion of t-ROH to t-RA. Thus, competition of retinol and ethanol for common oxidative enzymes was not evident in that rodent model. In addition, the studies detected only extremely low rates of ethanol oxidation in embryonic rodent tissues, compatible with the lack of ethanol inhibition of t-ROH oxidation.

The lack of competitive inhibition was consistent with a lack of antagonistic effects of ethanol and t-ROH, but the observed *greater*-than-additive and apparently synergistic effects still remained to be explained. It seemed probable that the observed synergistic effects produced by the combination of ethanol and retinol on cultured whole rodent embryos might be attributable to biotransformation-independent factors such as enhanced effects at the receptor level via a number of possible mechanisms or greater-than-additive effects on other crucial macromolecules involved in developmental processes. Ethanol-treated embryos did, in fact, exhibit significantly higher levels of t-RA and t-RAL than were measured in the corresponding non-treated controls [36].

The present studies, however, show several potentially important differences between the above-described rodent model and the human prenatal hepatic model reported here. First, in the rodent model, ethanol failed to inhibit t-ROH oxidation significantly, even at extremely high (1 M) concentrations [24]. By contrast, in these investigations, ethanol exhibited significant inhibition of t-ROH oxidation at concentrations as low as 10 mM and nearly 50% inhibition at 90 mM. Blood ethanol concentrations ranging between 10 and 90 mM are commonly observed in human alcoholics. Total inhibition was not achieved at the highest ethanol concentration studied (90 mM), which might be expected in view of kinetic studies demonstrating very low affinity of ethanol versus t-ROH for various alcohol dehydrogenase enzymes [10, 19, 22]. Alternatively, weak inhibition by ethanol of t-RA synthesis from t-ROH in prenatal human hepatic tissue preparations could also suggest separate enzyme systems for each alcohol.

Second, the characteristics of t-ROH oxidation in human hepatic tissue preparations differed from those observed in rodent embryo preparations. For example, in the prenatal rodent preparations, it appeared to be quite conclusive that the first step in the conversion of t-ROH to t-RA (oxidation to t-RAL) was the slow and rate-limiting step [23]. In the presently reported investigations with prenatal human hepatic tissues, the converse appeared to be the case; oxidation of t-ROH resulted in significant accumulation of t-RAL with very limited quantities of t-RA appearing in HPLC profiles. Even with comparisons at high substrate concentrations of t-ROH and t-RAL, rates of conversion of t-RAL to t-RA were only slightly higher than rates of conversion of t-ROH to t-RA.

A third consideration is that prenatal rodent hepatic tissues reportedly do not express P4502E1 in measurable quantities [30], whereas the human prenatal liver expresses measurable quantities at very early stages of gestation, even as early as day 53 [27, 28]. Although expression levels in human embryonic hepatic tissues were very low, they could nevertheless be contributory to the control of t-RA synthesis because of the several known mechanisms whereby P4502E1 and ethanol can interact to initiate lipid peroxidation [30] and, pertinently, because of the profound inhibitory effects on t-RA synthesis of the products of lipid peroxidation as observed in the present studies as well as in previous studies with rodents [31]. It should be emphasized that although these results provide evidence for a *potential* role for CYP2E1 to contribute to the generation of lipid peroxidation products capable of inhibiting t-RA biosynthesis, a conclusive and definitive role has not yet been demonstrated. While the likelihood of a contributory role seems very high, the magnitude of such a role remains to be defined in future investigations, and we are now pursuing these questions. It is clear that 4-HNE and other aldehydic products of lipid peroxidation can be generated via several other pathways and that such a role for CYP2E1 in human prenatal brain tissues could be relatively minor.

In addition, differences in the capacity of other chemicals to inhibit t-RA synthesis in prenatal rodent tissues versus prenatal human tissues were noted. Both 4-HNE and hexanal appeared to be more effective inhibitors of t-RA synthesis in prenatal human hepatic cytosol than in rat conceptal cytosol [31], although rigorous direct comparisons will be needed to fully confirm this observation. Thus, while the hypothesis of Duester and coworkers [10, 19, 22] and others [20, 21]—suggesting that adverse effects of ethanol on developing embryos/fetuses may be due to direct competitive inhibition by ethanol of t-RA biosynthesis catalyzed by dehydrogenases—appears not to be viable in terms of the developing rat, further work will be required to adequately test the viability of the hypothesis in humans. In any event, it seems evident that extrapolations from the

rodent to the human in terms of the interactive embryotoxic effects of retinoids and ethanol cannot be justified at this time. Of particular future interest will be investigations of human prenatal cephalic tissues, important targets for the embryotoxic effects of both ethanol and retinoids. Discovery of a consistent and potentially significant level of expression of P4502E1 in human prenatal cephalic tissues [27, 37] adds considerable importance to this aspect.

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