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Effect of Cellular Retinol-Binding Protein on Retinol Oxidation by Human Class IV Retinol/Alcohol Dehydrogenase and Inhibition by Ethanol[1]

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All-*trans* retinoic acid (atRA) is a powerful morphogen synthesized in a variety of tissues. Oxidation of all-*trans* retinol to all-*trans* retinal determines the overall rate of atRA biosynthesis. This reaction is catalyzed by multiple dehydrogenases *in vitro*. In the cells, most all-*trans* retinol is bound to cellular retinol binding protein (CRBP). Whether retinoic acid is produced from the free or CRBP-bound retinol *in vivo* is not known. The current study investigated whether human medium-chain alcohol/retinol dehydrogenases (ADH) can oxidize the CRBP-bound retinol. The results of this study suggest that retinol bound to CRBP cannot be channeled to the active site of ADH. Thus, the contribution of ADH isozymes to retinoic acid biosynthesis will depend on the amount of free retinol in each cell. Physiological levels of ethanol will substantially inhibit the oxidation of free retinol by human ADHs: class I, $\alpha\alpha$ and $\beta_2\beta_2$; class II, $\pi\pi$; and class IV, $\sigma\sigma$.

All-*trans* retinoic acid (atRA) is a powerful hormone that serves as an activating ligand for retinoic acid receptors (RARs) (1). RAR α, β, and γ regulate the transcription of multiple genes during embryonic development and in adulthood (1). Whereas the effects of atRA on gene transcription have been studied intensively during the past decade, the exact enzymes responsible for atRA biosynthesis in all atRA-responsive tissues have not been identified yet. It has been established that atRA is synthesized from all-*trans* retinol in two oxidative steps: retinol is oxidized to retinal and further to retinoic acid (2). The rate of the first reaction determines the overall rate of retinoic acid production (2). *In vitro,* all-*trans* retinol is oxidized to all-*trans* retinal by multiple enzymes found both in cytosol and in microsomes (3, 4). The cytosolic activity has been linked to the medium-chain alcohol dehydrogenases (ADHs) that have 40 kDa subunits (5). ADHs are dimeric zinc metalloenzymes that are encoded by nine different genes in humans (reviewed in ref. 6). The isozymes have been grouped into six classes (7), based on their catalytic properties and primary structures. All ADH isozymes are NAD^+-dependent cytosolic dehydrogenases that oxidize a variety of primary, secondary and a number of cyclic alcohols (6). One of the suggested physiological substrates for these ADHs is retinol. Several human ADH isozymes (class I, II and IV) oxidize retinol to retinaldehyde *in vitro* (8). Class IV ADH is the most catalytically efficient isozyme for retinol oxidation (8). Based on this finding, it was suggested that class IV may contribute to RA biosynthesis *in vivo*. The expression pattern of class IV ADH appears to be consistent with this hypothesis. For example, Ang *et al.* (9) reported that class IV ADH colocalizes with several spots of retinoic acid biosynthesis during early stages of mouse development, suggesting that ADH enzymes may be responsible for atRA production in these tissues. Retinol is a hydrophobic molecule poorly soluble in aqueous solutions and binds to specific extra and intracellular binding proteins. In the cells, most retinol is bound to cellular retinol binding protein (CRBP) type I and type II (10). Type I is present in most cells, whereas type II appears to be specific for absorptive cells of the small intestine (11). CRBP-I exhibits about 100 times higher affinity for retinol than CRBP-II (12). The concentration of CRBP-bound retinol ($\sim$2-5 μM) is much higher than that of the free

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retinol (<0.1 μM) (13). Several microsomal enzymes, lecithin-retinol acyl transferase (14, 15), retinol dehydrogenases RoDH-1 and 2 (16, 17), and retinal reductase (18) were shown to utilize the CRBP-bound form of retinol. We were interested whether retinol-active ADH isozymes are also capable of oxidizing the CRBP-I-bound form of retinol.

In addition to retinol, ADHs oxidize a variety of substrates. Ethanol is the best known dietary substrate for these enzymes. Ethanol consumption during pregnancy leads to Fetal Alcohol Syndrome (19) that is characterized by fetal malformations similar to those seen in vitamin A deficiency (20). The molecular basis for Fetal Alcohol Syndrome is not clear. It was suggested that ethanol may act as a competitive inhibitor of retinol oxidation (21). The only retinol dehydrogenases that are inhibited by ethanol are cytosolic ADHs. In this study, we investigated whether physiological concentrations of ethanol achieved in tissues after drinking (20-50 mM) are sufficient to inhibit retinol oxidation by human ADH isozymes.

MATERIALS AND METHODS

Expression and Purification of Recombinant Human ADH Isozymes

The human recombinant $\alpha\alpha$, $\beta_2\beta_2$, $\pi\pi$, and $\sigma\sigma$ ADH were expressed in *E. coli* and purified as described in the literature (22-25). The cDNA for γ_2-ADH was obtained from Dr. J. O. Höög (Department of Medical Biochemistry and Biophysics, Berzelius Laboratory, Karolinska Institutet, Stockholm, Sweden). The expression and purification procedure was similar to that for other ADH isozymes (Parsons *et al*., unpublished). From a 20 L culture, 41 units of enzyme with specific activity of 1.3 U/mg were obtained.

Preparation of CRBP-Bound Retinol

The coding region of CRBP (type I) cDNA (26) was amplified from rat liver total RNA by RT-PCR using *pfu* polymerase. The gene specific nucleotide primers carried restriction sites for *Bam*H I and *Eco*R I endonucleases. The PCR product was subcloned into the corresponding sites in pGEX-2T expression vector and sequenced. The recombinant protein was produced at 30°C overnight in TG-1 *E. coli* cells in the presence of 0.2 mM IPTG and 200 μg/ml ampicillin as a fusion with glutathione S-transferase. The cell pellet was resuspended in ice-cold PBS, 2 mM EDTA, 0.1% β-mercaptoethanol, and lysed using a French Press. The fusion protein was purified to homogeneity using affinity chromatography on a glutathione agarose column. The purified fusion protein was cleaved with thrombin in 50 mM Tris, pH 8.0, 0.1% β-mercaptoethanol, 150 mM NaCl, 2.5 mM $CaCl_2$. CRBP was separated from glutathione S-transferase by elution with 0-500 mM NaCl gradient in 10 mM Tris, pH 7.4, 1 mM DTT, from a Q Sepharose column. The yield of CRBP was about 14 mg per liter culture. The final preparation exhibited a single protein band of approximately 16 kDa on SDS-PAGE. The amount of functional protein was determined from the fluorescence titration curve of apo-CRBP with retinol (27). Excitation was at 350 nm; emission was measured at 480 nm. The fluorescence values were corrected for contribution of free retinol.

The purified recombinant CRBP was incubated with excess of free retinol for 1-2 h in the dark. The free retinol was separated from the bound by elution of the S Superose column with 10 mM sodium phosphate, pH 7.4. The final preparation of CRBP-retinol had a ratio A_{350}/A_{280} of 1.76, indicating that CRBP-bound retinol constituted 94%.

Determination of Kinetic Constants

Steady-state kinetics were performed at 37°C in 90 mM potassium phosphate, pH 7.3, 40 mM KCl, 0.02% Tween. All-*trans* retinol (Aldrich) stock solution was prepared in acetone (HPLC quality). The concentration of the acetone stock solution of retinol was determined in ethanol using extinction coefficient of ϵ_{325}=51,000 $M^{-1}cm^{-1}$. Aqueous solution of retinol was prepared by adding the calculated amount of acetone stock solution to the reaction buffer. The extinction coefficient used to determine the concentration of retinol in aqueous solution was ϵ_{328} 39,500 $M^{-1}cm^{-1}$ (8). The effect of increasing concentrations of acetone on the rate of oxidation of 30 μM retinol was determined for all isozymes. Only $\alpha\alpha$ was inhibited 15%. Other ADHs were inhibited less than 2%. The effect of 0.02% Tween-80 on the activities of ADH isozymes was determined in reactions with ethanol used at K_M values. A maximum of 35% inhibition in the presence of Tween-80 was observed for $\gamma_2\gamma_2$. Other ADHs were inhibited less than 10%. All rates were normalized to a standard assay. The standard assay for class I, II and IV ADH was performed with 2.4 mM NAD^+ and 100 mM ethanol in 0.1 M glycine, pH 10.0, 25°C. Initial velocities (micromoles of product formed per micromole of enzyme) were obtained by linear regression of the enzyme rate data. The amount of product formed was less than 5% within the 1.2 min during which the reaction was monitored. The production of NADH was monitored at 340 nm assuming an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. The K_M values for substrates were determined at a fixed NAD^+ concentration of 2.4 mM. Initial rates were evaluated in duplicate. Values of V_{max} were calculated from a standard assay, assuming a specific activity of 2 units/mg for $\alpha\alpha$, 15 units/mg for $\beta_2\beta_2$, 3 units/mg for $\gamma_2\gamma_2$, 1.7 units/mg for $\pi\pi$, and 92 units/mg for $\sigma\sigma$ in the standard assay, and two active sites per molecule (22-25).

Inhibition kinetics of all-*trans* retinol with ethanol were performed by monitoring the production of all-*trans* retinal at 425 nm (molar extinction coefficient 14,600 $M^{-1}cm^{-1}$) (28). Ethanol inhibition of retinol oxidation by $\beta_2\beta_2$, $\pi\pi$ and $\sigma\sigma$ with varied ethanol was evaluated by mixing enzymes with 2.4 mM NAD^+, 4 to 5 ethanol concentrations (0-50 mM), and 4-5 retinol concentrations (30-90 μM) in reaction buffer. Analysis was performed with duplicate data points totaling about 50 observations. Each data set was evaluated for fit to competitive, non-competitive and uncompetitive inhibition equations (29). Kinetics of initial velocities were evaluated by nonlinear regression of inhibition equations using the method of Marquart (30).

The activity of $\sigma\sigma$ ADH with 10 μM CRBP-bound retinol was determined by monitoring the fluorescence of the bound retinol in 0.1 M sodium phosphate buffer, pH 7.4, in the presence of 2.4 mM NAD^+ at 37°C during a 30 min incubation (31). The excitation was at 350 nm and emission was measured at 480 nm every 5 minutes. The enzyme was omitted from the control sample. To minimize the exposure of retinol to light the shutter of the spectrofluorometer for the excitation light was opened only when a measurement was being taken.

RESULTS AND DISCUSSION

Most tissues and cells seem to be able to synthesize atRA (2). However, the exact enzymes responsible for atRA biosynthesis in this wide variety of tissues have not been identified yet. The predominant form of retinol in the cells is the CRBP-bound retinol (13). The apparent dissociation constant for CRBP-retinol was initially determined by fluorometric titration (32). From the K_d value for CRBP-retinol of ~16 nM and 5 μM CRBP-retinol in the liver, free retinol concentration was esti-

mated to be <0.1 μM (13). Later, the K_d value for CRBP-retinol was determined by fluorine nuclear magnetic resonance analysis and appeared to be even lower, 0.1 nM (33). From the K_d value for CRBP of ~0.1 nM, the concentration of CRBP in rat liver cytosol of ~7 μM, and the total retinol concentration of 5 μM, the concentration of free retinol in the liver cells was calculated to be ~2.5 nM (34). Accordingly, studies were conducted to see whether retinol metabolizing enzymes can utilize the CRBP-bound form of retinol (35). Two microsomal dehydrogenases that belong to the superfamily of short-chain dehydrogenases/reductases, RoDH-1 and RoDH-2 from rat, were shown to oxidize CRBP-bound retinol with K_M values of ~0.9 and 2 μM, respectively (16, 17). These K_M values are lower than those of ADH isozymes for the free retinol. However, RoDH-1 and 2 prefer $NADP^+$, which is present mainly in the reduced form (NADPH) in the cells (36). Therefore, it was suggested that, *in vivo*, RoDH-1 and 2 may function in the reductive rather than oxidative direction, reducing retinal to retinol (37). In addition, RoDH-1 and RoDH-2 have limited tissue distribution and are present predominantly in the liver and kidney (only RoDH-2) (17), but not in such atRA-target tissues as lung, brain, skin, etc. Thus, RoDH-1 and RoDH-2 may not be responsible for biosynthesis of atRA in all tissues. On the other hand, ADH isozymes exhibit the right cofactor preference (NAD^+), and are expressed in a wide variety of tissues. Human class I ADH isozymes, - the homo and heterodimers of α, β, and γ subunits, - are present in liver, kidney, skin, gastrointestinal tract and lung to a lesser extent (reviewed in ref. 6). $\alpha\alpha$ ADH appears in fetal liver, $\gamma\gamma$ in fetal intestine and kidney, and $\beta\beta$ in fetal lung. Class II $\pi\pi$ is found in fetal and adult liver. Class IV $\sigma\sigma$ is the extrahepatic form of ADH found in the epithelial cells, especially the lining of stomach and esophagus (38). The message for $\sigma\sigma$ ADH is also detected in the human cornea and skin (Kedishvili *et al.*, unpublished).

It is necessary to know how much of the cellular retinol is actually available to ADHs to determine the actual contribution of ADH isozymes to atRA biosynthesis. The kinetics of retinol oxidation by human ADHs were determined previously with a detergent-solubilized retinol (8). Considering that retinol is present mainly in the bound form in the cells, it is important to determine whether the CRBP-bound form of retinol can be oxidized by ADHs. Ong *et al.* (27) reported that retinol bound to CRBP was slightly oxidized by unspecified liver ADH after 5 min, and retinol bound to CRBP (II) was about 40% oxidized in the same period. Retinol bound to BSA or β-lactoglobulin was completely oxidized in 5 min. These data suggested that CRBP-bound retinol can be oxidized by ADH isozymes, although at a lower rate than the free retinol. Similarly, RoDH-1 exhibited 10 times lower rate for oxidation of CRBP-bound retinol versus free (13). Thus,

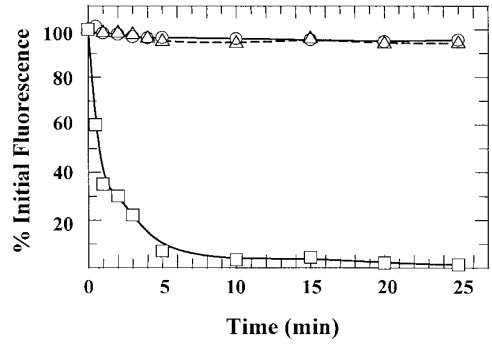

FIG. 1. Oxidation of retinol bound to CRBP-I by class IV ADH. CRBP-retinol (10 μM) was incubated with 2.4 mM NAD^+ in 0.1 M sodium phosphate buffer, pH 7.4, in the presence (○) and absence (△) of 0.26 units of human class IV ADH at 37°C. In the control reaction, equimolar delipidated bovine serum albumin (BSA) was added to 10 μM retinol and incubated in the presence (□) of class IV ADH under the same conditions. No decrease in fluorescence was observed for BSA-bound retinol in the absence of enzyme (not shown).

we asked whether ADH isozymes may recognize the CRBP-bound retinol, which would significantly increase their contribution to RA synthesis. The purpose of the present study was to determine the rate of CRBP-bound retinol oxidation by human ADH isozymes. Since our studies suggested that the epithelial form of ADH, class IV, is the most catalytically efficient enzyme for oxidation of free retinol (8), the focus of this study was primarily on class IV ADH. The role of class IV ADH in retinoic acid production has been intensively studied during the past several years, mainly by Duester's group (9, 38-44). The expression of class IV ADH in mouse embryos was shown to coincide with sites of RA detection, suggesting that this ADH isozyme may play a role in regulating the initial turn-on of atRA synthesis during embryogenesis.

The cDNA for CRBP-I was subcloned into pGEX expression vector and expressed in *E. coli*. The purified CRBP was incubated with all-*trans* retinol and the unbound retinol was removed by size-exclusion chromatography. The binding of retinol to CRBP was monitored by fluorescence, since fluorescence of retinol is greatly enhanced in the CRBP complex. The activity of ADH was assayed by monitoring the decrease in fluorescence upon conversion of the bound retinol into retinal (27). We found that retinol bound to CRBP was completely protected from oxidation by human class IV $\sigma\sigma$ ADH over the 30-min incubation period at 37°C. The drop in fluorescence in the presence of ADH did not exceed 5% and this value was identical to the control cuvette that did not contain the enzyme (Fig. 1). When the production of retinal from free retinol by ADH was monitored spectrophotometrically by the increase in absorbance at 400 nm, the addition of CRBP to the reaction mixture completely abolished this increase (not shown), suggesting that CRBP sequestered retinol

TABLE I

Kinetic Parameters of ADH Isozymes for Oxidation of Free All-*trans* Retinol and Ethanol

ADH isozyme	K_M for retinol, mM	k_{cat}/K_M for retinol, $min^{-1}mM^{-1}$	K_M for ethanol, mM	k_{cat}/K_M for ethanol, $min^{-1}mM^{-1}$
Class I, $\alpha\alpha$	0.11 ± 0.003	150 ± 15	8.1 ± 1.2	4.3 ± 0.5
Class I, $\beta_2\beta_2$	0.052 ± 0.010	1,800 ± 170	1.2 ± 0.13	580 ± 50
Class I, $\gamma_2\gamma_2$	0.043 ± 0.013	420 ± 60	0.59 ± 0.11	200 ± 30
Class II, $\pi\pi$	0.030 ± 0.002	240 ± 10	23 ± 4	0.44 ± 0.05
Class IV, $\sigma\sigma$	0.062 ± 0.011	3,800 ± 300	69 ± 13	7.7 ± 0.9

from oxidation by human ADH. The products of CRBP-bound retinol oxidation were also analyzed by HPLC. CRBP-bound retinol and free retinol were incubated with different amounts of σ-ADH in the dark for 30 and 60 min. The reaction products were extracted with hexane, hexane was evaporated and retinoids were analyzed by HPLC as described previously (45). Retinal peak appeared in the reaction with free retinol and increased proportionally to the amount of enzyme used. No retinal peak was detectable in the reaction with CRBP-bound retinol. Thus, cytosolic ADH can efficiently oxidize free retinol, dispersed in a Tween-containing aqueous solution, but not retinol bound to CRBP. This finding implies that the actual amount of retinol available to ADH in the cells is likely to be at the nanomolar level.

Recently, it was demonstrated that ethanol treatment of mouse embryo leads to a reduction in endogenous RA (21). Similarly, ethanol treatment of quail embryos induced cardiovascular abnormalities that resemble those observed in the vitamin A deficient embryo and can be prevented by the administration of RA (46). These findings support the hypothesis that ethanol is a competitive inhibitor of retinol oxidation via ADHs, causing a deficiency of RA in target tissues that require RA for normal development. The kinetic parameters of mouse ADHs for oxidation of retinol and ethanol are significantly different from those of human ADH isozymes. For example, the K_M value of mouse class IV ADH for ethanol is 232 mM (47) versus 69 mM in humans (current study; Table I). In addition, humans have three isoforms of class I ADH α, β and γ, whereas lower mammals have only one form of class I ADH. Thus, the effect of ethanol on retinol oxidation in humans may be different than in mice.

We measured the ethanol inhibition constants for ADH class I $\alpha\alpha$, $\beta_2\beta_2$, and $\gamma_2\gamma_2$, class II $\pi\pi$ and class IV $\sigma\sigma$ ADH to determine the extent to which human ADH isozymes would be inhibited by ethanol. The inhibition constants were determined from experiments where both retinol and ethanol concentrations were varied. The kinetic data were fitted to competitive, non-competitive, full non-competitive and uncompetitive inhibition equations (29). The data fitted equally well to the competitive and the two K_i term full non-competitive inhibition model. The simple competitive inhibition model was chosen and the apparent K_i values for ethanol inhibition were calculated from a fit of the kinetic data to the equation $(V=V_{max}B/(K_B(1+I/K_i) + B)$, where B and I are retinol and ethanol concentrations, respectively (Table II). The inhibition was greatest for class I isozymes $\beta_2\beta_2$ and $\gamma_2\gamma_2$, indicating that retinol oxidation by these isozymes would be the most sensitive to the presence of ethanol (Table II). The next most sensitive ADH was class IV $\sigma\sigma$, whereas class I $\alpha\alpha$ and class II $\pi\pi$ had much higher ethanol K_i values (Table II).

We then calculated the activity of each ADH isozyme toward oxidation of 2.5 nM free retinol, based on K_d value for CRBP-retinol of 0.1 nM (33). The activity in the presence of 20 mM ethanol (blood alcohol level of 0.1% that equals ~21 mM is considered legally intoxicating) was calculated as $v_i/v_0=(K_B + B)/K_B (1+I/K_i) + B$, where v_i is the initial velocity at 2.5 nM retinol in the presence of ethanol, v_0 is the initial velocity in the absence of ethanol, I is the concentration of ethanol, K_i is the ethanol inhibition constant, B is the concentration of retinol, and K_B is the K_M for retinol. The percent of ethanol inhibition of ADH activity with retinol was determined as $(1-v_i/v_0)\cdot 100$ (29). Based on the K_i values determined in this study, 20 mM ethanol would almost completely inhibit oxidation of 2.5 nM free retinol by $\beta_2\beta_2$, strongly inhibit $\sigma\sigma$ (~60%), but have less effect (~20%) on the activi-

TABLE II

The Apparent Ethanol K_i Values and Calculated % Inhibition of ADH-Catalyzed Oxidation of 2.5 nM Free All-*trans* Retinol by 20 mM Ethanol

ADH isozyme	K_i, mM	% Ethanol inhibition
Class I, $\alpha\alpha$	67 ± 13	23
Class I, $\beta_2\beta_2$	0.7 ± 0.06	97
Class I, $\gamma_2\gamma_2$	—	~100
Class II, $\pi\pi$	84 ± 8	19
Class IV, $\sigma\sigma$	12 ± 1	62

Note. Retinol oxidation by class I $\gamma_2\gamma_2$ was almost completely inhibited. K_i value could not be calculated because retinol production was at the detection limit.

ties of $\alpha\alpha$ and $\pi\pi$ with retinol. In the stomach mucosal cells, ethanol concentration can reach 100 to 580 mM. Importantly, the retinol-oxidizing activity of $\sigma\sigma$ ADH present in the stomach mucosa would be inhibited 89% at 100 mM ethanol.

Han *et al.* (48) have just reported the results of a similar study which determined the ethanol inhibition constants for retinol oxidation by human ADHs purified from liver and stomach. The reaction conditions (2% acetonitrile) and temperature (25°C) used in the above study were different from the conditions employed in this study (0.6% acetone, 37°C), which can partially explain the differences in the absolute values of kinetic constants. Han *et al.* (48) have not evaluated CRBP-bound retinol as substrate, and calculated the percent ethanol inhibition of retinol oxidation based on the free retinol concentration of 10 μM. As our study has shown, the concentration of retinol available to ADH isozymes in the cells is about 4,000 times less than 10 μM. Han *et al.* concluded, as we did, that retinol oxidation can be substantially perturbed by ethanol via ADH pathways.

Thus, our data suggest that CRBP-bound retinol cannot be channeled directly to the active site of ADH. This result is expected, since the X-ray structure of holo-CRBP suggests that the hydroxyl group of retinol is hidden inside the binding pocket and is not readily available for oxidation or esterification (49). Therefore, retinol would have to dissociate from CRBP to become available to retinol-active enzymes, if it is not directly channeled to their active sites. The contribution of class IV ADH to oxidation of retinol appears to be limited by the rate of dissociation of retinol from the CRBP complex to form free retinol. Millimolar concentrations of ethanol would almost completely inhibit oxidation of physiological levels of retinol by ADHs. The expression level of CRBP varies in different cells, and is upregulated by retinoic acid (50), leading to a further decrease in the amount of retinol available to ADHs. One can hypothesize that CRBP serves as a feedback regulator of the amount of free retinol available for oxidation: as the level of retinoic acid increases, the increased amount of CRBP decreases free retinol concentration and prevents its further oxidation by ADH to retinaldehyde and further to retinoic acid.

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