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Multiple retinoid dehydrogenases in testes cytosol from alcohol dehydrogenase negative or positive deermice

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Abstract—Retinoic acid syntheses from retinol by cytosol from testes of alcohol dehydrogenase negative or positive deermice were similar in specific activity and in their insensitivity to 1 M ethanol or 100 mM 4-methylpyrazole. Anion-exchange followed by size-exclusion chromatography revealed multiple and similarly migrating peaks in each cytosol that had both retinol and retinal dehydrogenase activities. Thus, the effects of ethanol on testes cannot be caused by direct inhibition of cytosolic retinoic acid synthesis because retinoid dehydrogenases distinct from mouse class A_2 alcohol dehydrogenases, which corresponds to human class I, occurred in testes and they were not inhibited by ethanol. These data also demonstrate the occurrence of multiple cytosolic retinoic acid synthesis activities and indicate that the two reactions of cytosolic retinoic acid synthesis, retinol and retinal dehydrogenation, may be catalyzed by enzymes that occur as complexes.

Mammalian testes are retinoic acid dependent [1, 2] and are damaged functionally and morphologically in alcoholism [3, 4]. This poses the fundamental questions of whether retinol and alcohol dehydrogenases in testes are cospecific, and if not, whether ethanol impairs retinol conversion into retinoic acid by direct competition. To examine these possibilities, retinoic acid synthesis was measured in testes cytosol of *Peromyscus maniculatus*, an inbreed strain of deermouse that has an ADH^- mutant* [5]. This report will show that testes cytosols from the ADH^- mutant and the wild-type ADH^+ deermouse not only synthesized retinoic acid with similar specific activities and were insensitive to ethanol, but also had similar and multiple retinol and retinal dehydrogenases that comigrated through anion-exchange and size-exclusion columns as though they were complexed.

Materials and Methods

Preparation of testes cytosol. Breeding pairs of deermice (*P. maniculatus*), from Dr. Michael R. Felder (USC, Columbia, SC), were fed mouse chow supplemented with 10 g/cage/week of: oatmeal (450 g); wheat germ (56 g); brewers' yeast (28 g); and cod liver oil (14 g). Adult males were starved overnight before cervical dislocation. Cytosol was prepared as described [6].

Retinoid dehydrogenase assays. Retinol and retinal were purified by normal-phase HPLC. Unless noted otherwise, assays were done in duplicate for 20 min at 37° with 50–100 μg of protein and 10 μM substrate in 0.5 mL of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM KCl, 2 mM dithiothreitol (DTT) and 2 mM NAD, at pH 7.5 for retinol and pH 8.0 for retinal. Substrates were added in 2.5 μL of dimethyl sulfoxide. Inhibitors were added either neat (ethanol, pentanol) or in buffer (4-methylpyrazole, 1,10-phenanthroline) 2 min prior to substrate. Hexane extracts of the assays were analyzed for retinoids by HPLC as described [6, 7].

Fast-protein liquid chromatography (FPLC). Ten to twenty milligrams of cytosolic protein was exchanged with 10 mM Tris–HCl and 2 mM DTT, pH 8.0, and reduced to 1 mL with an Amicon ultrafilter. Samples were eluted from a Mono-Q column (0.5 × 7.5 cm, Pharmacia LKB) at 1 mL/min with 10 mM Tris-HCl, 2 mM DTT, pH 8.0 (buffer A), for 10 min, followed by buffer A at pH 7.5 with 200 mM NaCl for 20 min. The column was washed for 10 min with 1 M NaCl in buffer A. Size-exclusion chromatography was done with a TSK G3000SW column (0.8 × 7.5 cm) eluted at 0.5 mL/min with 20 mM HEPES, 150 mM KCl and 2 mM DTT (pH 7.5).

*Abbreviations: ADH^+, alcohol dehydrogenase positive; ADH^-, alcohol dehydrogenase negative; DTT, dithiothreitol; FPLC, fast-protein liquid chromatography; and HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Results and Discussion

Testes cytosol from both strains of deermice converted retinol into retinoic acid with activity linear to 100 μg of protein and for 20 min, and maximum in the pH range of 7.5 to 8.0. With these conditions, the specific activity of retinoic acid synthesis from retinol was similar with cytosol from either strain (Table 1). Ethanol and pentanol concentrations 5 and 3 orders of magnitude, respectively, greater than the retinol concentration did not depress retinoic acid production significantly in either cytosol. Pentanol concentrations 4 orders of magnitude greater than the retinol concentration did inhibit 60% in both preparations. 4-Methylpyrazole, a potent inhibitor of alcohol dehydrogenases with a K_i in the micromolar range [8], did not diminish significantly the conversion of retinol into retinoic acid in either cytosol. This total insensitivity to ethanol and inhibitors of alcohol dehydrogenase and partial inhibition by high concentrations of pentanol are distinct from the behavior of the known alcohol dehydrogenases. 1,10-Phenanthroline, a metal chelator, inhibited retinoic acid production by 40% at 5 mM in both cytosols, indicating that these dehydrogenases require a divalent metal as cofactor.

To further examine the possibility of differences in retinoic acid syntheses, testes cytosols were fractionated

Table 1. Effects of inhibitors on retinoic acid synthesis by deermouse testes cytosol

Addition	Retinoic acid* (pmol)	
	ADH^+	ADH^-
None	72 ± 5	80 ± 7
1000 mM Ethanol	64 ± 6	73 ± 3
25 mM 1-Pentanol	60 ± 3	65 ± 6
100 mM 1-Pentanol	27 ± 1†	32 ± 5†
100 mM 4-Methylpyrazole	65 ± 3	55 ± 8
0.5 mM 1,10-Phenanthroline	52 ± 3	51 ± 1
5.0 mM 1,10-Phenanthroline	44 ± 4†	46 ± 6†

* Values are means ± SD (N = 3). No retinoic acid was observed when retinol was incubated in the absence of protein.

† Significantly different from control, $P < 0.005$.

over an anion-exchange FPLC column. Fractions were assayed for retinoic acid synthesis from retinol and retinal, and for retinal synthesis from retinol. Two major peaks of retinoic acid synthesis activity from both substrates were observed in each of the samples. The first major peak in each sample (Peak 1) eluted with the low salt buffer; the second major peak (Peak 2) eluted with 6 mL of the high salt buffer (Fig. 1). A minor peak of activity eluted with 4 mL of the high salt buffer. In each case, the retinoic acid synthetic activities that recognized retinol as substrate co-eluted with the activities that recognized retinal as substrate. No separate peak of activity was observed that converted retinol into retinal, nor was net retinal synthesis noted.

The major activity peaks recovered from the two anion-exchange analyses were analyzed individually on a size-exclusion FPLC column. Each Peak 1 produced a narrow peak of activity, with either retinol or retinal as substrate, that eluted in the molecular weight range of 66 to 80 kDa (Fig. 2, A and B). Each Peak 2 produced a broad peak in the molecular weight range of 60 to 120 kDa (Fig. 2, C and D), suggesting heterogeneity. The activities that converted retinol into retinoic acid migrated with those that converted retinal into retinoic acid. No separate activity was observed that converted retinol into retinal and no net retinal production was noted.

There has been long-standing interest in an interaction between retinol and ethanol metabolism in the testes, because retinoids are essential for normal testicular morphology and function [9, 10], and chronic alcoholics suffer testicular atrophy and loss of testes function. Two hypotheses have emerged. One contends that a biochemical mechanism for the aspermatogenesis of chronic alcoholics could be interference by ethanol with retinal synthesis, based on apparent competitive inhibition by ethanol of retinal formation in rat testes cytosol [11, 12]. A second perspective stems from work with ADH^- deermice, which have morphologically and functionally normal testes and therefore must be synthesizing retinoic acid. A conclusion that direct interaction of ethanol with a cytosolic dehydrogenase could not be responsible for the aspermatogenesis of alcoholics was based on undetectable cytosolic retinal synthesis in the testes of the ADH^- deermice, suggesting that cytosolic retinol dehydrogenases must not be significant to retinoic acid formation [13]. Both of these studies used non-specific and insensitive spectrophotometer assays to measure "retinal" and neither measured retinoic acid.

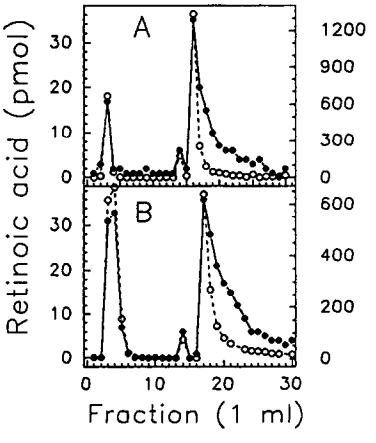

Fig. 1. Anion-exchange FPLC of deermice testes cytosol. Panel A: 18 mg of protein from ADH^+ cytosol was loaded; panel B, 12 mg of protein from ADH^- was loaded. Retinoic acid synthesis was measured from retinol (filled circles, solid line, left axes) or from retinal (open circles, dashed line, right axes). Data are pmol retinoic acid/fraction/mg of protein applied.

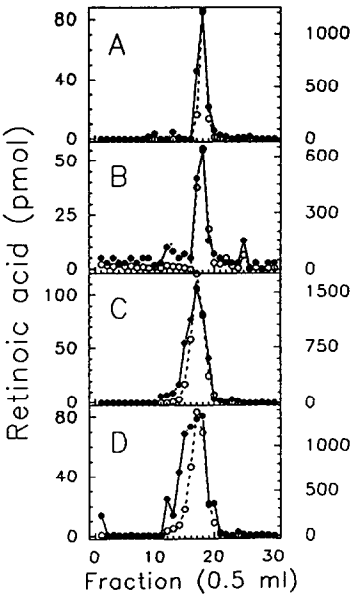

Fig. 2. Size-exclusion FPLC of Peaks 1 and 2 from anion-exchange FPLC. Retinoic acid synthesis was assayed from retinol (filled circles, solid lines, left axes) and retinal (open circles, dashed lines, right axes). The column was calibrated with (kDa, elution volume): alcohol dehydrogenase (150, 6 mL); bovine serum albumin (66, 9 mL); carbonic anhydrase (29, 11.5 mL); and cytochrome *c* (12.4, 13.5 mL). Panel A, Peak 1 from ADH^+; panel B, Peak 1 from ADH^-. Less than 200 μg of protein was recovered from the anion-exchange column for each Peak 1, precluding accurate determinations of the amounts of protein loaded onto the size-exclusion column. Therefore, data are expressed as pmol of retinoic acid/fraction. Panel C, 0.85 mg of protein from ADH^+ Peak 2 was applied; panel D, 3.6 mg of protein from ADH^- Peak 2 was applied. Data for panels C and D are expressed as pmol of retinoic acid/fraction/mg of protein loaded.

This work relied on FPLC fractination of cytosol and sensitive and specific HPLC assays for retinoic acid to provide the first evidence of multiple cytosolic retinoid dehydrogenases in ADH^- deermice testes identical with those in ADH^+ testes. Thus, contrary to two previous models, impairment of testes function by ethanol cannot be attributed to direct inhibition of retinol activation because *multiple cytosolic retinoid dehydrogenases are expressed in testes cytosol that are not inhibited by ethanol concentrations which could be lethal in vivo*. The unexpected observation of retinol and retinal dehydrogenases co-eluting through anion-exchange and size-exclusion FPLC, and the lack of retinal accumulation with retinol as substrate, suggest that these activities may exist as complexes.

A distinct *microsomal* retinol dehydrogenase, expressed in testes and other tissues, also has been reported recently, which is not inhibited by ethanol or 4-methylpyrazole [14]. As was pointed out in that report, both the cytosolic and microsomal retinol dehydrogenases may contribute physiologically to retinoic acid synthesis—contributions by both are not mutually exclusive. Perhaps the relative contribution of each enzyme will depend on the cell type, stage of development/differentiation, or other factors, as has been observed with the multiple distinct retinoic acid receptors and retinoid binding proteins. Future investigations will address this issue.

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