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EXPRESSION OF ALCOHOL DEHYDROGENASE IN PRIMARY MONOLAYER CULTURES OF RAT HEPATOCYTES

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With the use of an extensively modified Leibovitz-15 medium, the alcohol dehydrogenase activity of hepatocytes prepared from male rats was successfully maintained in primary culture at the level observed in freshly isolated hepatocytes. Enzyme activity was higher in freshly isolated cells from female rats than from male rats, but it fell to the level characteristic of the male animals after four days in culture. The levels of activity of the cells in culture from both sexes were unaffected by treatment with estrogens or androgens. The results suggest that the sex-determined differences in alcohol dehydrogenase activity in rats do not arise from direct effects of gonadal steroids on the liver.

Alcohol dehydrogenase (E.C.1.1.1.1) catalyzes the first reaction in the major pathway of ethanol oxidation. The content of this enzyme in liver determines in large measure the rate of ethanol elimination in rats (1) and it appears to be under hormonal regulation. Female rats exhibit a higher activity of liver alcohol dehydrogenase than males, and castration or estrogen treatment of males raises the activity of the enzyme. Testosterone administration to castrated males or to females lowers the activity (2,3). However, we have recently observed that androgens are ineffective in altering alcohol dehydrogenase activity in hypophysectomized animals. This finding has suggested that either the effects of sex steroids are mediated by the pituitary or a pituitary factor is required for the liver to respond to the steroids. We have now tested this hypothesis by studying the effects of androgens and estrogens on alcohol dehydrogenase activity in primary

Abbreviations: L-15, Leibovitz-15 culture medium; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid.

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monolayer cultures of adult rat liver cells. We report here the first culture system in which hepatocytes express alcohol dehydrogenase activity at a level comparable to that seen *in vivo*, and the failure of gonadal steroids to affect the enzyme activity *in vitro*.

Methods

Sprague-Dawley rats weighing 150-200 g were purchased from Harlan Sprague-Dawley, Indianapolis, IN. Suspensions of hepatocytes were obtained by perfusion of the liver with calcium-free Hank's buffer containing 0.3 mg type I collagenase/ml according to the method of Lin and Snodgrass (4). Cell viability was checked by trypan blue exclusion, and routinely exceeded 80%. The cells were plated at a density of 9×10^6 cells per 100 mm culture dish (Corning Glass Works, Corning, NY) which had been precoated with 0.5 mg calf skin collagen (type III) per dish.

The culture medium was a modification of Leibovitz-15 (L-15) medium. The additions are listed in Table I. Penicillin (63 mg/l), streptomycin (100 mg/l), and bacitracin (500 mg/l) were added and the medium was buffered (pH 7.4) with Hepes (6.7 g/l). These modifications were based on the formulation of Ham's MCDB 301 medium (5) and the suggestions of Barnes and Sato (6) and Pitot (7). The medium was supplemented with 10% fetal bovine serum, 10^{-6} M insulin and 10^{-10} M dexamethasone. The approximate concentrations of sex steroids in the final medium, due to their presence in the serum, were: testosterone, 10^{-10} M, and estradiol, 4×10^{-12} M. These concentrations are 100 and 40 times less than those of testosterone and estradiol encountered *in vivo* in the circulation of male and female rats, respectively. Testosterone, 5-α-dihydrotestosterone, 17-β-estradiol, and diethylstilbestrol were dissolved in acetone and added directly to the medium (0.025 ml acetone/10 ml medium). Powdered L-15 medium and fetal bovine serum were obtained from Grand Island Biologicals, Grand Island, NY. All other reagents and hormones were obtained from Sigma Chemical Co., St. Louis, Mo.

The hepatocytes were cultured under air for 4 days in 10 ml of medium, which was changed daily. The cells were harvested by scraping in ice-cold phosphate-buffered saline containing 0.5 mM dithiothreitol. The nonionic detergent NP-40 was added at 0.5%, and the cells were subjected to sonic disruption. An aliquot was reserved for DNA determination. The remainder was centrifuged in a Brinkman tabletop centrifuge at 14,000 x g for 2 min. Alcohol dehydrogenase activity was determined in the supernatant by spectrophotometric assay (1). The assay buffer contained Tris-Cl, 0.5 M, NAD^+, 2.8 mM, and ethanol, 10 mM. The assay was carried out at 37°. Duplicate dishes were assayed for each experimental condition. DNA was measured by a fluorescent dye-binding assay according to Caesarone (8).

Results and Discussion

Cultured eukaryotic cells provide a useful experimental system for the study of intermediary metabolism, hormone action, and gene expression. Hepatocytes in primary culture which are able to metabolize ethanol would be valuable for investigations into the control of the rate of ethanol oxidation, the development of steatosis and liver cell injury, and the effects of ethanol on metabolism. The previously reported system of Lad et

al. (9), which utilized Dulbecco's modified Eagle's medium supplemented with inosine, ornithine, insulin and hydrocortisone, failed to maintain the activity of alcohol dehydrogenase in primary cultures of hepatocytes. The enzyme activity fell to less than 10% of that measured in fresh liver cells in three days, then rose again after 14 days. As with the culture system of Lad et al. (9), we found in preliminary experiments that alcohol dehydrogenase activity is rapidly lost when hepatocytes are cultured in L-15 medium: activity fell from 1.05 to less than 0.1 U/mg DNA within four days, when hepatocytes were cultured in L-15 medium, L-15 containing 10% fetal bovine serum, or L-15 containing insulin (10^{-6} M) and dexamethasone (10^{-6} M). The activity of the enzyme was 0.25 U/mg DNA, when the cells were cultured in L-15 containing 10% serum, insulin, and dexamethasone. However, empiric supplementation of the L-15 medium with the compounds listed in Table I permitted the culture of hepatocytes which maintained alcohol dehydrogenase activity at the level present in freshly prepared hepatocytes from male rats (Table II). Freshly prepared hepatocytes from female rats had higher alcohol dehydrogenase activity than those from male rats, in agreement with many

TABLE I

Modifications of Leibovitz-15 Medium for Hepatocyte Culture

Organic Compounds (M)		Metals (M)		Vitamins (M)	
glucose	1×10^{-2}	$ZnSO_4\ 7H_2O$	3×10^{-6}	ascorbate	1.7×10^{-4}
proline	1.3×10^{-3}	$FeSO_4\ 7H_2O$	3×10^{-6}	tocopherol	2×10^{-5}
putrescine	1×10^{-3}	$(NH_4)_6Mo_7O_{24}\ 4H_2O$	7×10^{-8}	vitamin B_{12}	1×10^{-6}
hypoxanthine	3×10^{-5}	Na_2SeO_3	1×10^{-8}	thiamin-HCl	3×10^{-6}
thymidine	3×10^{-6}	$CuSO_4\ 5H_2O$	1×10^{-8}	riboflavin	3×10^{-6}
linoleic acid	1.6×10^{-5}	$MnSO_4\ 5H_2O$	1×10^{-9}	lipoic acid	2×10^{-6}
4-aminolevulinic acid	2×10^{-6}	NH_4VO_3	1×10^{-9}	biotin	3×10^{-8}
inositol	1×10^{-4}			menadione	1×10^{-7}
choline-Cl	1×10^{-4}			retinol	3×10^{-7}

The linoleic acid was dissolved in 1 ml of a solution of bovine albumin (5 mg/ml) and then added to the medium. Retinol and tocopherol were dissolved in acetone and added after filtration sterilization of the medium. Insulin was added at 10^{-6} M, dexamethasone at 10^{-10} M, and fetal bovine serum at 10%.

TABLE II

Effects of Sex Steroids on Alcohol Dehydrogenase Activity in Primary Hepatocyte Monolayer Cultures*

Male Rats		Female Rats	
	Alcohol Dehydrogenase Activity (U/mg DNA)		Alcohol Dehydrogenase Activity (U/mg DNA)
fresh cells	1.05 ± 0.07	fresh cells	1.94 ± 0.20**
control	1.08 ± 0.10	control	1.06 ± 0.12
estradiol, 10^{-10}M	1.08 ± 0.10	testosterone, 10^{-9}M	1.02 ± 0.17
10^{-9}M	1.02 ± 0.09	10^{-8}M	1.08 ± 0.16
10^{-8}M	1.10 ± 0.10	10^{-7}M	1.22 ± 0.19
10^{-7}M	1.07 ± 0.12	10^{-6}M	1.13 ± 0.24
10^{-6}M	1.09 ± 0.10	10^{-5}M	1.13 ± 0.20
DES, 10^{-10}M	1.01 ± 0.08	DHT, 10^{-9}M	0.98 ± 0.21
10^{-9}M	1.06 ± 0.07	10^{-8}M	1.09 ± 0.12
10^{-8}M	1.05 ± 0.13	10^{-7}M	0.99 ± 0.20
10^{-7}M	1.04 ± 0.10	10^{-6}M	0.93 ± 0.14
10^{-6}M	1.12 ± 0.10	10^{-5}M	0.91 ± 0.14

*Hepatocytes were prepared from male or female Sprague-Dawley rats. Alcohol dehydrogenase activity was determined in the freshly isolated cells and after four days in culture with estrogen or androgen at the noted concentrations or without the steroid hormones (control). DES and DHT denote diethylstilbestrol and 5 α-dihydrotestosterone, respectively. Enzyme activity is reported as mean ± SEM for at least four different hepatocyte preparations.

**Significantly different from fresh male hepatocytes and control incubations ($P<0.001$).

previous reports of alcohol dehydrogenase activity in liver homogenate-supernatants from female and male rats (2,3). After four days in culture, cells prepared from female rats exhibited the same alcohol dehydrogenase activity as that of cells from the male rats.

We then tested the effects of sex steroids on the activity of alcohol dehydrogenase in cells from both male and female rats. In contrast to the increase in alcohol dehydrogenase activity seen in male rats treated with estrogens (2), there was no effect of the estrogens estradiol or diethylstilbestrol, in concentrations up to 10^{-6} M, on the enzyme activity of cells prepared from male rats (Table II). Diethylstilbestrol was chosen because it is not metabolized, yet it is bound to and causes translocation of the estrogen receptor (10); thus the lack of effect of estrogens is probably

not due to rapid metabolic inactivation by the liver cells. It is notable that the activity of alcohol dehydrogenase in cells from male animals after four days in culture was the same as that in freshly isolated cells, and did not rise in the absence of added testosterone, i.e., after "castration" of the cells. As discussed in Methods, the concentration of testosterone in the medium is about 100 times less than that in the plasma of adult male rats.

Liver cells from female animals were also cultured in the presence of testosterone or dihydrotestosterone (Table II). In the absence of added androgens, the activity of alcohol dehydrogenase fell to the level observed in fresh cells from male rats. No further reduction in enzyme activity was observed in cells treated with androgens. It is known from in vivo studies that the high level of alcohol dehydrogenase in female rats is not dependent upon the presence of estrogens, because spaying female rats has no effect on the enzyme activity (2). In light of the absence of demonstrable effects of sex steroids on the activity of alcohol dehydrogenase and the fall of enzyme activity during culture of female cells, it seems likely that the higher enzyme activity in female rats than in male rats is due to a serum factor other than the sex steroids. Other data which we have obtained, in addition to work on the sexual dimorphism of other rat liver enzymes (11), have suggested that this factor may be of pituitary origin, specifically, growth hormone.

It may be argued that exposure of the cells to hormones for only four days is inadequate to allow the establishment of a new steady-state level of alcohol dehydrogenase. The time course of a change in enzyme concentration after a hormonal or nutritional perturbation is determined by the degradation rate constant of the enzyme (12), which for alcohol dehydrogenase in adult male rats is 0.16 d^{-1} (13). However, the rate of bulk protein turnover in perfused liver or cultured liver cells is considerably higher than that in vivo (14-16), even in the presence of insulin and high concentrations of amino acids, which suppress protein degradation. It seems likely, therefore, that the rate of alcohol dehydrogenase degradation would be higher in the

cultured cells and a significant effect of gonadal steroids, if present, should have been observed after four days.

In summary, hepatocytes cultured in the modified L-15 medium here described and containing insulin, dexamethasone, and 10% fetal serum retain alcohol dehydrogenase at the level seen in freshly isolated hepatocytes from male rats. Alcohol dehydrogenase activity in cultured cells from male animals is not affected by the presence of even high concentrations of estrogens, and the activity in cells from female animals is not affected by exposure to high concentrations of androgens. These results support the hypothesis that the sexually determined difference in alcohol dehydrogenase activity in rats is not the result of direct effects of sex steroids on the liver, but rather to other, possibly pituitary, hormones which maintain the enzyme activity at a higher level in females than in males.

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