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DEPRESSION OF ALCOHOL DEHYDROGENASE ACTIVITY IN RAT HEPATOCYTE CULTURE BY DIHYDROTESTOSTERONE

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Abstract—Hepatocytes harvested from castrated rats retained a higher alcohol dehydrogenase (EC 1.1.1.1) activity than hepatocytes harvested from normal rats during 7 days of culture. Dihydrotestosterone (1 μM) decreased the enzyme activity, after 2 and 5 days of culture, in hepatocytes from castrated and control animals respectively. Dihydrotestosterone decreased the enzyme activity to similar values in both groups of hepatocytes by the end of 7 days of culture. Testosterone (1 μM) had no effect on the enzyme activity in normal hepatocytes and only a transitory effect in decreasing the enzyme activity in hepatocytes from castrated animals. The increases in alcohol dehydrogenase activity after castration and their suppression by dihydrotestosterone were associated with parallel changes in the rate of ethanol elimination. Additions of substrates of the malate-aspartate shuttle or dinitrophenol did not modify ethanol elimination. These observations indicate that dihydrotestosterone has a direct suppressant effect on hepatocyte alcohol dehydrogenase and that the enzyme activity is a major determinant of the rate of ethanol elimination.

The activity of hepatic alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) in the rat is affected by a variety of hormones. Castration increases the activity of alcohol dehydrogenase, and this enhanced activity is suppressed by the chronic administration of pharmacological doses of testosterone [1–3]. Chronic administration of testosterone to intact rats resulted in a small paradoxical increase in the enzyme activity in one study [2], but in no change in other studies [1, 3]. The different responses of alcohol dehydrogenase to testosterone in normal rats suggest that the effects are mediated by other hormonal changes not present in the castrated rats. Effects of androgens on various hepatic drug-metabolizing enzymes were found to be indirect and mediated by other androgen-sensitive organs, such as the pituitary [4], whose secretions are altered by castration [5]. Alcohol dehydrogenase activity has also been found to be increased following stress [6], hypophysectomy [7], and thyroidectomy [8]. The effect of chronic administration of dihydrotestosterone, which is derived from the peripheral conversion of testosterone, and is a more potent androgen than testosterone [9], has not been determined.

The complex hormone interactions that occur *in vivo* limit the further exploration of hormonal regulation of alcohol dehydrogenase activity in the whole animal. The purpose of the study was to determine the effects of testosterone and dihydrotestosterone in concentrations which approach physiological levels and in the absence of other potential interacting hormones on alcohol dehydrogenase activity in hepatocyte cultures.

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MATERIALS AND METHODS

Materials. NAD^+ (grade I) was obtained from Boehringer-Mannheim, Indianpolis, IN. Collagenase (Type I), ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N*^{1},*N*^{1}-tetraacetic acid (EGTA), tricine, trypan blue, 4-methylpyrazole, 2,4-dinitrophenol, semicarbazide, asparagine, testosterone, dihydrotestosterone, bovine albumin, and calf thymus DNA were purchased from the Sigma Chemical Co., St. Louis, MO. Insulin and penicillin were purchased from E. R. Squibb & Sons, Inc., Princeton, NJ. Streptomycin was obtained from Eli Lilly, Indianapolis, IN. Gentamicin was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ. Plastic culture dishes were obtained from Allied Fisher Scientific, Plainview, PA. Vitrogen 100 purified bovine dermal collagen (Type I) was obtained from the Collagen Corp., Palo Alto, CA. Amino acids for the culture media were purchased from the Grand Island Biological Co., Grand Island, NY and the Sigma Chemical Co., St. Louis, MO.

Animals. Male Sprague–Dawley rats obtained from the Charles River Breeding Laboratories, Wilmington, MA, were kept at a constant temperature of 25° in separate cages with light/dark cycles alternating every 12 hr starting at 7:00 a.m. They were provided water and Purina Chow *ad lib.* In some of the animals, castration was performed under ether anesthesia 10 days before they were used for the preparation of isolated hepatocytes.

Hepatocyte isolation and Cell culture. Hepatocytes were isolated and cultured according to the methods of Bissell and Guzelian [10]. Rats weighing 150–200 g were anesthetized with ether. The livers were perfused *in situ* through the portal vein initially with Hanks' balanced salt solution containing 0.5 mM EGTA and 2.5 mM tricine, followed by the complete

culture medium [11] containing 0.03% collagenase. The soft livers were removed and the hepatocytes separated from non-parenchymal cells by centrifugation at 60 *g* for 2 min, a process that was repeated three times, each time after washing and resuspending the cells in cool collagenase-free medium. Cell number and percent viability were assessed by counting an aliquot in the presence of 0.1% trypan blue. Only hepatocyte suspensions with a viability greater than 85% were cultured. The cells were cultured in 60 mm × 15 mm petri culture dishes previously coated with 50 μg of collagen. A total of 3.5×10^6 cells in 3.0 ml of culture medium was placed on each dish. The culture medium was serum-free and consisted of modified Waymouth 752 medium [11]. It did not contain arginine, but it contained ornithine (0.15 mM), insulin (250 units), penicillin (1×10^5 units), streptomycin (0.13 mM), and gentamicin (0.18 mM). The medium was prepared in our laboratory directly from the individual components and saturated with 95% O_2 and 5% CO_2. The cultures were incubated at 37° in a humidified atmosphere of 95% O_2 and 5% CO_2.

Alcohol dehydrogenase activity. The effect of each hormone was tested by adding the hormone to the culture medium used for one set of cultures, while an equal number of control cultures did not contain the hormone. Both the hormone-containing medium and the control medium were changed every 24 hr. Hepatocytes were harvested for determination of enzyme activity from a few plates each day for 7 days. This was done by replacement of the medium with 2 ml of 0.5 M Tris–HCl buffer containing 0.25% Triton X-100, followed by scraping with a rubber policeman. The hepatocyte suspensions were homogenized and then sonicated in pulses for a total of 20 sec. Alcohol dehydrogenase was determined in the homogenate at 37° by the method of Crow *et al.* [12]. The volume of the reaction mixture was 1.0 ml and consisted of 0.5 M Tris–HCl buffer, pH 7.2, 18 mM ethanol, 2.8 mM NAD^+, and 0.01 to 0.10 ml of the liver cell homogenate. A blank reaction without ethanol was run in each case. The alcohol dehydrogenase activities were then calculated from the molar extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$ for NADH. One unit of enzyme activity is defined as the formation of 1 μmole of NADH per min under these conditions. Alcohol dehydrogenase activity was expressed per mg of protein, per mg of DNA, and per cell count. Protein was determined by the method of Lowry *et al.* [13] with bovine serum albumin used as a standard. DNA was assayed by the method of Richards [14]. Cell counts were calculated from the activity of lactate dehydrogenase in the hepatocyte homogenates as described by Jauregui *et al.* [15], using a standard plot of enzyme activity versus cell counts obtained in the fresh hepatocyte preparation prior to plating. The viability of the cells was assessed by measurement of the appearance of lactate dehydrogenase in the medium in the 24-hr periods. Media lactate dehydrogenase was expressed as a percentage of total (cellular + medium) lactate dehydrogenase [15]. Lactate dehydrogenase activity was determined by the method of Plagemann *et al.* [16]. The functional capability of the hepatocytes was monitored from measurements of urea synthesis, by determinations of the appearance of urea [17] in the medium over periods of 24 hr. Urea concentration was determined by the method of Gutmann and Bergmeyer [18]. The morphological appearance of the monolayers was monitored by inverted phase-contrast microscopy and was characteristic of adult hepatocytes.

Ethanol elimination. The rate of ethanol elimination by hepatocytes in culture was determined in separate experiments at times when hormonally-induced changes in alcohol dehydrogenase activity had been obtained. The determination was done by incubation of the hepatocyte culture in fresh culture medium, which did not contain the hormones that produced the enzyme changes, and in the presence of 5 mM pyruvate with 8 mM ethanol for 90 min at 37° as described by Crow *et al.* [12]. Samples of the incubation media were obtained at 0, 20, 40, 60, and 90 min for the determination of ethanol by gas liquid chromatography [19]. A set of culture plates, containing medium but no cells, was included as a control to monitor for evaporation losses during the 90-min incubation. Ethanol elimination was calculated from the rate of decrease in ethanol concentration by the method of least squares. The hepatocytes were harvested at the end of the incubation for determinations of alcohol and lactate dehydrogenases, protein, and DNA using the methodology already described. Acetaldehyde concentration was determined in the medium at the end of the 90-min incubation by measurement of the absorbance at 224 nm following semicarbazide trapping as described by Cederbaum and Rubin [20].

Statistical analysis. Four to fifteen plates were analyzed for each experimental condition each day. The data are expressed as means ± S.E. Statistical significance was determined by Student's *t*-test.

RESULTS

Alcohol dehydrogenase activity in isolated hepatocytes from normal rats prior to plating was 21.27 ± 1.60 mIU/mg protein. During the first 24 hr of culture, alcohol dehydrogenase activity decreased to a mean of 43% of initial activity. The enzyme activity remained relatively stable during the first 6 days of culture and then decreased (Fig. 1). Exposure of the hepatocytes to dihydrotestosterone resulted in a much lower activity of alcohol dehydrogenase on days 5–7 of culture. By contrast, testosterone had no effect on alcohol dehydrogenase activity (data not shown). The activity of lactate dehydrogenase was not altered by either hormone. The results were the same when the enzyme activities were expressed per cell count or DNA concentration. The concentrations of DNA per mg of protein were not changed significantly by dihydrotestosterone or testosterone. Cell viability was also not affected by either hormone. The release of lactate dehydrogenase into the medium was a mean of 3.2 ± 0.5% per 24 hr between days 1 and 7 of culture without the addition of the hormones compared with 3.0 ± 0.4% and 2.7 ± 0.6% per 24 hr in the presence of dihydrotestosterone and testosterone respectively. Alcohol dehydrogenase activity was not detected in the culture media. Urea synthesis was a mean of 51.7 ± 5.0

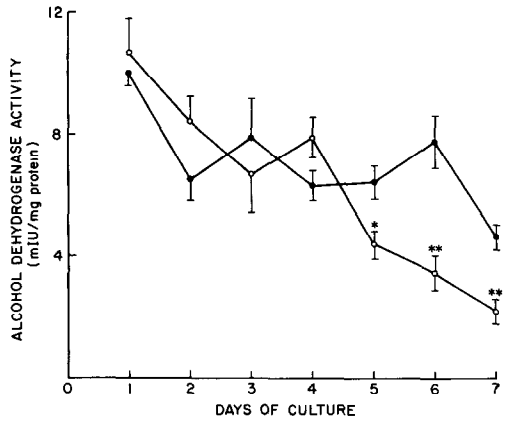

Fig. 1. Effect of dihydrotestosterone on alcohol dehydrogenase activity in hepatocytes cultured from a normal rat. The hepatocytes were exposed to media either containing 1 μM dihydrotestosterone (○) or lacking the hormone (●). The media were changed every 24 hr. The enzyme activities are indicated as means of five cultured plates and the vertical lines indicate ± S.E. of the mean. Statistical significance versus control is indicated: (*) $P < 0.05$, (**) $P < 0.01$.

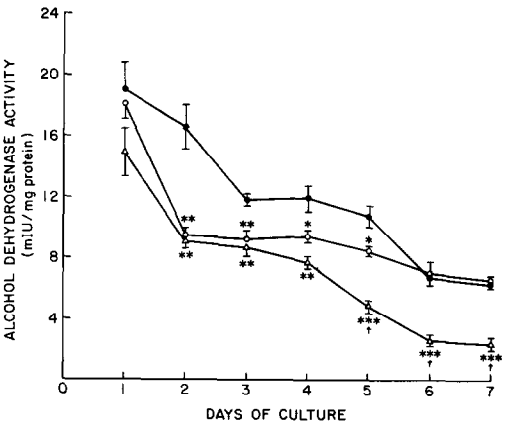

Fig. 2. Effects of dihydrotestosterone and testosterone on alcohol dehydrogenase activity in hepatocytes cultured from a castrated rat. The hepatocytes were exposed to media containing either 1 μM dihydrotestosterone (△) or 1 μM testosterone (○), or lacking the androgens (●). The enzyme activities are indicated as means of four to five culture plates, and the vertical lines indicate means ± S.E. of the mean. Statistical significance versus control is indicated: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$. Statistical significance versus testosterone is indicated: (†) $P < 0.01$.

nmoles/24 hr/mg protein throughout the 7 days of the control culture. Testosterone and dihydrotestosterone did not alter the rate of urea synthesis.

Alcohol dehydrogenase activity in isolated hepatocytes from five castrated animals prior to plating was 31.86 ± 2.99 mIU/mg protein. This value was significantly higher than that obtained in hepatocytes from normal rats ($P < 0.05$). During the first 24 hr of culture in the experiment shown in Fig. 2, alcohol dehydrogenase activity decreased to 54, 52 and 42% of initial activity in the presence of medium alone, testosterone and dihydrotestosterone respectively. Testosterone decreased alcohol dehydrogenase activity between 2 and 5 days of culture but not thereafter. Dihydrotestosterone decreased alcohol dehydrogenase activity markedly throughout 7 days of culture. By day 7 of culture, dihydrotestosterone had resulted in a 74% inhibition of the enzyme activity as compared to the control value on the same day. The activity of lactate dehydrogenase decreased gradually from a value of 2.81 ± 0.26 IU/mg protein on day 1 of culture to 1.86 ± 0.14 IU/mg protein on day 7 of culture with no hormone additions. Neither testosterone nor dihydrotestosterone had a significant effect on lactate dehydrogenase activity. The results were the same when the enzyme activities were expressed per cell count or DNA concentration. The concentrations of DNA per mg of protein were not changed significantly by dihydrotestosterone or testosterone. Cell viability was not affected in the hepatocytes of castrated animals by either hormone. The release of lactate dehydrogenase between days 1 and 7 of culture was 4.7 ± 0.6, 4.8 ± 0.7, and 4.9 ± 0.5% per 24 hr in the control, testosterone, and dihydrotestosterone cultures respectively. Alcohol dehydrogenase activity was not detected in the culture media. Urea synthesis was not affected by the hormones. The rates of urea synthesis were 71.6 ± 1.8, 65.0 ± 1.2, and 60.0 ± 5.0 nmoles/24 hr/mg protein throughout the 7 days in the control, testosterone, and dihydrotestosterone cultures respectively.

The rate of ethanol elimination by hepatocytes

Table 1. Effects of testosterone and dihydrotestosterone on ethanol elimination in hepatocyte cultures prepared from normal and castrated rats

Treatment	Ethanol elimination (nmoles/min/mg cell protein)	
	Normal	Castrated
Control	9.33 ± 0.50	11.91 ± 0.56
Testosterone (1.0 μM)	10.32 ± 0.46	9.77 ± 0.35*
Dihydrotestosterone (1.0 μM)	7.03 ± 0.59†‡	7.16 ± 0.57†§

All values are expressed as means ± S.E. of 14–15 culture plates.
* $P < 0.002$ as compared to control.
† $P < 0.001$ as compared to control.
‡ $P < 0.001$ as compared to testosterone.
§ $P < 0.01$ as compared to testosterone.

Table 2. Effects of 4-methylpyrazole and of other compounds on alcohol dehydrogenase activity and ethanol elimination in cultured hepatocytes from a castrated rat

Type of culture and compound added	Alcohol dehydrogenase (nmoles/min/mg/cell protein)	Ethanol elimination
Control culture	9.70 ± 0.800	9.22 ± 0.27
4-Methylpyrazole, 4 mM	1.29 ± 0.55*	2.19 ± 0.56*
Dinitrophenol, 0.1 mM	8.31 ± 0.39	12.47 ± 2.10
+ Malate, 10 mM	9.56 ± 0.63	7.05 ± 0.62
+ Asparagine, 1 mM	10.01 ± 1.93	8.04 ± 0.55
Dihydrotestosterone culture	5.07 ± 0.27*	6.30 ± 1.13†
+ Dinitrophenol, 0.1 mM	4.96 ± 0.52	7.22 ± 0.38
+ Malate, 10 mM	6.94 ± 0.94	6.58 ± 0.80
+ Asparagine, 1 mM	5.92 ± 1.94	5.70 ± 1.23

The hepatocytes had been in culture with or without the addition of 1.0 μM dihydrotestosterone for 5 days. Fresh medium (devoid of dihydrotestosterone) containing 8 mM ethanol, 5 mM pyruvate, and the compound whose effect was being tested, was added to the cultures. Ethanol elimination was determined over 90 min following which the hepatocytes were harvested for the determination of alcohol dehydrogenase activity. All values are expressed as means ± S.E. of four cultures plates.

* $P < 0.001$ as compared to control culture without additions.

† $P < 0.01$ as compared to control culture without additions.

from a normal rat after 5 days of culture was decreased by dihydrotestosterone but not affected by testosterone (Table 1). In hepatocytes obtained from a castrated animal, both testosterone and dihydrotestosterone decreased the rate of ethanol elimination. The effect of dihydrotestosterone in decreasing the rate of ethanol elimination was significantly greater than that of testosterone ($P < 0.01$). Acetaldehyde concentration in the media at the end of the 90-min incubation of the cells with ethanol, ranged from 20.7 to 40.3 μM in the cultures of normal hepatocytes and from 12.2 to 81.0 μM in cultures of hepatocytes from castrated rats.

4-Methylpyrazole resulted in 87% inhibition of alcohol dehydrogenase activity and in 76% inhibition in the rate of elimination in hepatocytes from a castrated rat after 5 days of culture (Table 2). Dinitrophenol, which uncouples oxidative phosphorylation or substrates of the malate-aspartate shuttle, specifically malate, which was not present in the original medium, and asparagine, which readily enters the cell and is converted to aspartate [21], did not alter ethanol elimination in control and dihydrotestosterone-treated cultures.

DISCUSSION

This study demonstrates that alcohol dehydrogenase activity is easily detectable and remains relatively uniform in primary non-proliferating rat liver cell cultures. This is in contrast to one prior study showing a rapid decrease in alcohol dehydrogenase activity to almost undetectable levels during the first 3 days of culture of proliferating rat hepatocytes grown in the presence of fetal bovine sera [22]. Only after 8–14 days of culture, as the cells approached a stationary phase, did alcohol dehydrogenase activity increase to levels compatible to those obtained initially. The rapid changes in alcohol dehydrogenase activity in this latter study may be due to the finding that rapidly proliferating cells frequently lose their metabolic functions and often become mesenchymal rather than parenchymal [23].

The greater alcohol dehydrogenase activity in hepatocytes harvested from castrated than from normal rats agrees with previous measurements of the enzyme activity in the supernatant fraction of liver homogenates [1–3]. In addition, this study shows that an enhanced enzyme activity persists in the hepatocytes harvested from the castrated as compared to the normal animals throughout the 7 days of culture. The increased liver alcohol dehydrogenase activity in castrated animals was demonstrated previously to be due to increased enzyme protein rather than activation of the enzyme [24]. The greater effectiveness of dihydrotestosterone than of testosterone in suppressing alcohol dehydrogenase activity is compatible with the known relative potency of these two androgens *in vivo* [9]. Addition of higher unphysiologic concentrations of testosterone may have an effect in depressing the enzyme, but this was not tested. The effect of testosterone on the enzyme probably depends on its metabolism to dihydrotestosterone which is catalyzed by a microsomal 5α reductase [25]. The known decrease in the activity of microsomal enzymes during primary hepatocyte culture [26] could explain the loss of the effect of testosterone in depressing alcohol dehydrogenase activity in hepatocytes from castrated rats during the last 2 days of culture. The superior effect of dihydrotestosterone in lowering alcohol dehydrogenase activity in hepatocytes harvested from castrated rats may be due to a larger number of androgen receptors in hepatocytes with prior androgen deficiency. Of interest is that, under the influence of dihydrotestosterone, the enzyme activity fell to similar values in both normal and castrated hepatocytes after 7 days of culture.

Dihydrotestosterone could suppress alcohol dehydrogenase activity by direct enzyme inhibition or by an effect on enzyme turnover. Dihydrotestosterone at high unphysiological levels is both a substrate of alcohol dehydrogenase in the reductive

direction and an inhibitor of ethanol oxidation by the enzyme [27]. However, this is an unlikely mechanism for the decrease in alcohol dehydrogenase demonstrated in this study since the k_i of dihydrotestosterone for the inhibition of ethanol oxidation of 11.5 μM is ten times higher than the 1 μM concentration of dihydrotestosterone which was added to the culture medium. The most likely mechanism for the effect of dihydrotestosterone in suppressing alcohol dehydrogenase activity is an effect on increasing enzyme degradation. In a previous study, we found that a decrease in the rate of degradation was the principal mechanism for the increase in liver alcohol dehydrogenase following castration [24]. Androgens in *in vivo* experiments augment the release of lysosomal enzymes [28, 29]. Parallel changes in the rate of proteolysis and in lysosome size and fragility suggest that lysosomal enzymes participate in the degradation of endogenous protein [30].

The increased activity of alcohol dehydrogenase in hepatocytes harvested from castrated rats was associated with increased rates of ethanol elimination in agreement with previous *in vivo* studies [1–3]. In addition, decreases in alcohol dehydrogenase activity in the presence of testosterone or dihydrotestosterone were associated with decreases in rates of ethanol elimination. Similar parallel decreases in enzyme activity and ethanol elimination occur after fasting *in vivo* [31]. Factors which determine the rate of ethanol oxidation are the activity of alcohol dehydrogenase and the rate of reoxidation of NADH. The parallel changes in enzyme activity after castration and exposure of the hepatocytes to androgens suggest that, under these circumstances, alcohol dehydrogenase activity is the major determinant of the rate of the ethanol oxidation. This is further supported by the decrease of ethanol elimination in association with inhibition of alcohol dehydrogenase activity with 4-methylpyrazole. The lack of significant change in ethanol elimination upon the addition of substrates of the malate-aspartate shuttle or in the presence of an uncoupler of oxidative phosphorylation (dinitrophenol) suggests that mitochondrial transfer or oxidation of NADH is not rate limiting in ethanol oxidation by cultured hepatocytes.

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