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INHIBITION OF TESTOSTERONE SYNTHESIS BY ETHANOL AND ACETALDEHYDE*

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Abstract—Ethanol administration decreases plasma testosterone levels acutely in man and animals. The specific mechanism responsible for this effect is, at present, unknown. We have examined the hypothesis that ethanol or acetaldehyde directly inhibits the enzymes required for testosterone synthesis from pregnenolone. Microsomes prepared from testes of normal adult rats were isolated and assayed, in the presence and absence of ethanol or acetaldehyde, for activity of 3β-hydroxy-Δ^5-steroid dehydrogenase (E.C.1.1.1.145)/3-oxosteroid Δ^5-Δ^4 isomerase (EC5.3.3.1) complex, testosterone 17β-dehydrogenase (EC1.1.1.64), and 17α-hydroxyprogesterone aldolase (EC4.1.2.30), three of the four enzymes required for the biosynthesis of testosterone from pregnenolone. Ethanol at levels commonly seen in the blood of chronic alcohol-ingesting men was shown to inhibit the activity of 17α-hydroxyprogesterone aldolase in a concentration-dependent manner. In addition, acetaldehyde was shown to act as an inhibitor of 17α-hydroxyprogesterone aldolase in the presence of androstenedione.

Hypogonadism is seen frequently in alcoholic men [1–4]. It has been shown to develop in such men as a result of alcohol-induced gonadal and hypothalamic-pituitary injury [5–7]. The specific pathogenic mechanism of such injury, however, remains undetermined. Ethanol in concentrations of 50–300 mg/dl acutely inhibits the production of testosterone in isolated perfused rat testes [8]. Moreover, acetaldehyde has been shown to be about 200 times as potent as ethanol on a molar basis in inhibiting testosterone synthesis in the system [8]. Other recent studies utilizing decapsulated rat testes [9, 10] or isolated Leydig cells [11,12] also have implicated acetaldehyde as the substance principally responsible for inhibiting testosterone synthesis within the testes as a result of alcohol exposure.

Several possible mechanisms could potentially explain the inhibiting effect of ethanol or acetaldehyde upon testicular androgen synthesis. First, alcohol or one of its metabolic products might inhibit testosterone synthesis directly by inhibiting one or more of the required enzymes. Second, the oxidation of ethanol and/or its metabolites within testis might increase the NADH/NAD ratio in testicular cells and thus inhibit 3β-hydroxysteroid dehydrogenase/steroid isomerase. Finally, ethanol or one of its metabolites might interfere with hormone receptors or with the action of cyclic AMP within the testis.

In the present study, we have examined the first of these hypotheses. Specifically, we have assessed the effects of ethanol and acetaldehyde upon three of the four enzyme complexes involved in the synthesis of testosterone from pregnenolone: 3β-hydroxy-Δ^5 steroid dehydrogenase (EC1.1.1.145)/3-oxosteroid-Δ^{4-5}-isomerase, (EC5.3.3.1), 17α-hydroxyprogesterone aldelase (EC4.1.2.30) and testosterone 17β-dehydrogenase (EC1.1.1.64).

MATERIALS AND METHODS

Animals. One hundred and thirty-six adult male white Wistar rats matched for body size and age (all 60 days of age) were obtained from Charles River Breeding Laboratories, Wilmington, MA. Each had been fed a standard rat chow (Wayne Rat Blox F, obtained from Best Foods, Oakdale, PA) and water *ad lib*. for 2 weeks prior to being killed. Animals were caged individually. Prior to killing the rats, blood was obtained via aortic puncture, for assay of plasma testosterone and luteinizing hormone levels.

Materials. Unlabeled progresterone and 17α-hydroxyprogesterone (Sigma Chemical Co., St. Louis, MO), dehydroepiandrosterone (Serdary Research Laboratories, London, Ontario), and androstenedione (Steraloids, Wilton, NH) were recrystallized prior to use. [1,2-^{3}H]Dehydroepiandrosterone (48.5 Ci/mmole) and [1,2-^{3}H]androstenedione (45 Ci/mmole), obtained from the New England Nuclear Corp., Boston, MA, were purified prior to use by silica gel thin-layer chromatography, with methylene chloride–butyl acetate (9:1, v/v) as the developing solvent.

Tissue preparation. Immediately after exsanguination, the testes were removed and weighed. The

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decapsulated testes were homogenized in three parts 0.25 M sucrose containg 0.01 M HEPES,* pH 7.4. The 12,000 *g* supernatant fraction was subjected to centrifugation at 100,000 *g* for 60 min. The pellet was then resuspended in 0.15 M KCl containing 0.01 M HEPES and again subjected to centrifugation at 100,000 *g* for an additional 60 min. The final pellet was resuspended in a small volume of KCl–HEPES to yield a concentrated microsomal preparation (10–15 mg protein/ml).

Assay procedures. The assay for testosterone 17β-dehydrogenase (EC1.1.1.64) was based on the method of Menard and Purvis [13] with slight modification. The reaction mixture contained 0.15 M KCl, 50 mM HEPES (pH 7.4) at 37°, 500 μmoles NADPH, 50 mM glucose-6-phosphate (titrated to pH 7.4 before use), 5 units of glucose-6-phosphate dehydrogenase (from *Leuconostoc Mesenteroides*, "Type XXII" from the Sigma Chemical Co.), 5 mM $MgCl_2$, 0.5 μCi [1,2-^{3}H]androstenedione, and 1 μmole of unlabeled androstenedione in a final volume of 0.3 ml. The reaction mixture and the microsomal protein were preincubated separately at 37°. The reaction was started by the addition of 0.1 ml of microsomal protein to the reaction mixture. Samples (0.1 ml) were withdrawn at 2, 4 and 8 min to verify linearity of the reaction velocity. The samples so obtained were introduced rapidly into tubes containing 0.1 ml of ice-cold methanol. An aliquot of the reaction mixture was then applied to Eastman Chromogram silica gel thin-layer plates along with 100 μg each of unlabeled product and appropriate substrate standards. Chromatograms were developed in a solvent system of methylene chloride–butyl acetate (9:1, v/v). Spots were located with the use of an iodine vapor. After evaporation of the iodine, the product and substrate areas were cut out and washed from the chromatographic material with chloroform–methanol (1:1). Equal aliquots were assayed for total radioactivity and fraction of assayed radioactivity contained in the specific product by repetitive recrystallization (×3) to constant specific activity after the addition of 10 mg of unlabeled carrier steroid. The percentage conversion of substrate to product was calculated and the reaction rates were determined.

The assays for the 17α-hydroxyprogesterone aldolase (EC4.1.2.30) and the 3β-hydroxy-Δ^5-steroid dehydrogenase (EC1.1.1.145)/3-oxosteroid-Δ^5-$\Delta^{4\alpha}$-isomerase (EC5.3.3.1) complex were similar to the above described procedure. For the adolase assay, 1 μCi of 17α-hydroxy-[1,2-^{3}H]progesterone and 1 μmole of unlabeled progesterone were added to each incubation. In the assay for the 3β-hydroxysteroid dehydrogenase/steroid isomerase, the NADPH-generating system was omitted and replaced with 500 μmoles NAD^+. In addition, [1,2-^{3}H]dehydroepiandrosterone (0.5 μCi) and 1 μmole unlabeled dehydroepiandrosterone were added to each incubation to obtain the desired substrate concentration. The same solvent system (methylene chloride–butyl acetate) was used for the 17α-hydroxyprogesterone aldolase assay, and the 17α-hydroxyprogesterone and androstenedione areas of the t.l.c. plates were recovered, counted and recrystallized to constant specific activity after addition of unlabeled carrier. A solvent system of chloroform–methanol (98.5:1.5, v/v) was used for both the testosterone 17β-dehydrogenase and the 3β-hydroxysteroid dehydrogenase/steroid isomerase assays. For the 3β-hydroxysteroid dehydrogenase/steroid isomerase assay, the dehydroepiandrosterone and androstenedione areas were recovered counted and recrystallized, and for the testosterone 17β-dehydrogenase assay, the androstenedione and testosterone spots were recovered, counted and recrystallized to constant specific activity.

For each set of assays a reaction was quenched with methanol before the addition of microsomes. An aliquot of this quenched reaction was chromatographed with the appropriate solvent systems to serve as a zero time point.

Hormone assays. Serum LH was measured by double antibody RIA with highly purified LH obtained from the National Pituitary Agency. Results are expressed in terms of the reference preparation supplied by the NPA (NIAMDD-Rat LH - RP-1). All samples were tested in duplicate. Hormone was iodinated with ^{125}I using the chloramine T method [14]. ^{125}Iodinated hormones were purified by polyacrylamide gel electrophoresis using 9% acrylamide with 2% crosslinking [15]. Other details of the assay have been described previously [16].

Testosterone was measured by specific RIA [17]. All samples were tested in duplicate in a single assay. The intra-assay variation for laboratory standards was less than 8 per cent. The detection limit using 200 μl of rat serum for each assay is 1.0 pg.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [18] with bovine serum albumin as the standard.

Statistical analysis. Analysis of variance with appropriate paired comparison test procedures (Tukey's honestly significant difference) was used for statistical analysis of the data. A P value < 0.05 was considered probably significant, whereas a P value < 0.01 was considered significant.

RESULTS

Plasma hormone levels. The plasma testosterone concentration in the animals studied was 4.6 ± 0.2 ng/ml (mean ± S.E.M.). Plasma luteinizing hormone in the animals studied was 92.0 ± 1.3 mIU/ml.

Inhibition of microsomal enzymes of testosterone synthesis. Ethanol at concentrations up to 600 mg/dl did not inhibit testosterone 17β-dehydrogenase or the complex of 3β-hydroxysteroid dehydrogenase and steroid Δ^{4-5}-isomerase. Up to 4800 mg/dl of ethanol failed to inhibit the testosterone 17β-dehydrogenase (Figures 1A, 1B and 2A). In contrast, 17α-hydroxyprogesterone aldolase was inhibited by ethanol ($P < 0.05$) at 150 mg/dl ($P < 0.05$) and at 300 and 600 mg/dl ($P < 0.01$), compared to the control without ethanol, in a concentration-dependent manner (Fig. 3A).

Acetaldehyde did not consistently cause

* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphoric acid.

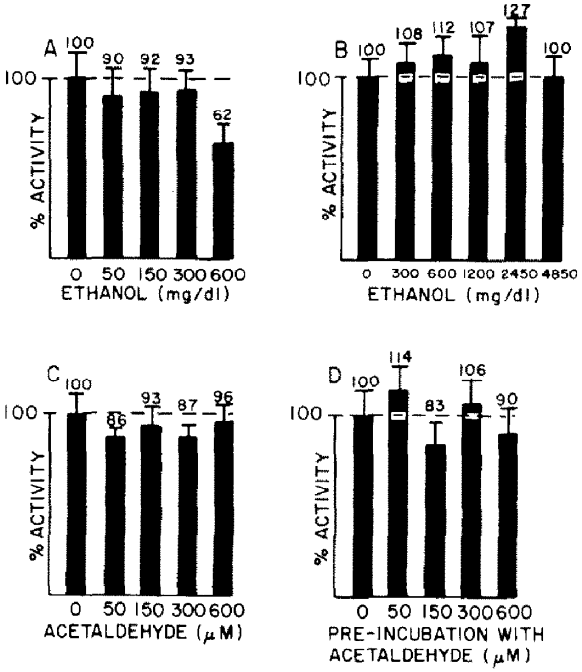

Fig. 1. Testosterone 17β-dehydrogenase activity. Results are expressed as percentages of the control rates ± S.E.M. Each reaction mixture contained 0.5 μM androstenedione; mixtures A, C and D contained 400 μg of microsomal protein; mixture B contained 1300 μg of microsomal protein. The control rates without inhibitors were: A and C, 8.30 nmoles/min; B, 13.5 nmoles/min; D, 7.73 nmoles/min.

concentration-dependent inhibition of any of the three enzymes studied (Figs. 1C, 2B and 3B). In the samples of microsomes that were pre-incubated with acetaldehyde for 1 hr before assay no inhibition could be demonstrated (Figs. 1D, 2C and 3C). The reaction mixtures for the pre-incubated microsomes contained at least as much acetaldehyde as the corresponding samples that were not pre-incubated. Therefore, the fact that none of the reactions carried out in the presence of acetaldehyde in Fig. 3B was inhibited, despite quite unphysiologic concentrations of acetaldehyde, suggests that no inhibition of 17α-hydroxyprogesterone aldolase occurred in the presence of acetaldehyde.

The interaction of androstenedione and acetaldehyde is seen in Fig. 4. Figure 4A demonstrates possible slight stimulation by 1 μM androstenedione (NS), questionable inhibition by 600 μM acetaldehyde (NS), and definite inhibition ($P < 0.01$) in the presence of both of these additions. Figure 4B shows data from a similar experiment, using 16 μM androstenedione and/or 600 μM acetaldehyde. Once again the combination of acetaldehyde and androstenedione inhibited the reaction ($P < 0.01$) to a rate that was 72 percent of the control value.

DISCUSSION

We have examined the effects of acetaldehyde and ethanol on three of the four enzymes involved in testosterone biosynthesis from pregnenolone using isolated microsomes obtained from the testes of normal male rats. Reactions with the testosterone

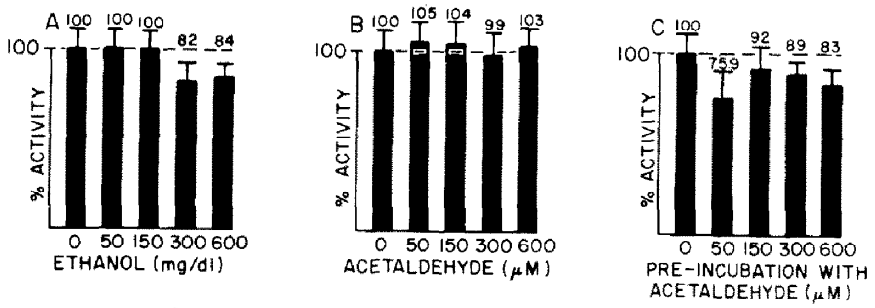

Fig. 2. 3β-Hydroxy-Δ^5-steroid dehydrogenase/3-oxosteroid-Δ^{5-4} isomerase activity. Results are expressed as percentages of the control rates ± S.E.M. Each reaction contained 12 μg of microsomal protein and 0.25 μM dehydroepiandrosterone. The control rate of A and B was 5.55 nmoles/min. The control rate for C was 5.90 nmoles/min.

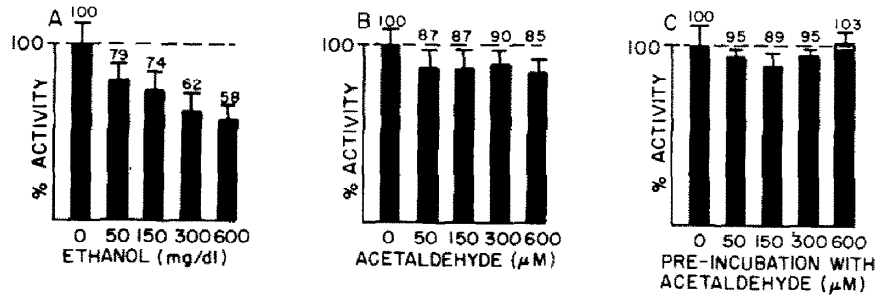

Fig. 3. 17α-Hydroxyprogesterone aldolase activity. Results are expressed as percentages of the control rates ± S.E.M. Each reaction contained 260 μg of microsomal protein and 0.25 μM 17α-hydroxyprogesterone. Control rate for A and B was 7.25 nmoles/min. Control rate for C was 6.45 nmoles/min.

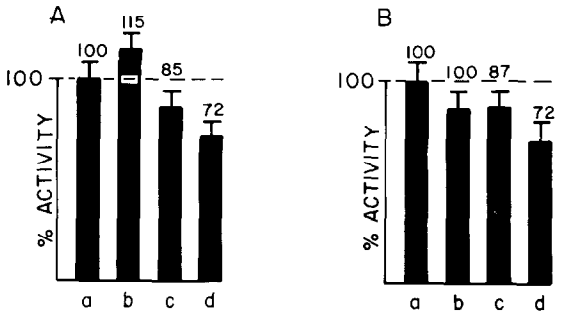

Fig. 4. 17α-Hydroxyprogesterone aldolase: interaction of androstenedione and acetaldehyde. Results are expressed as percentages of the control rates ± S.E.M. Panel A shows the effect of 1 μM androstenedione and/or 600 μM acetaldehyde. Each reaction mixture contained 260 μg of microsomal protein and 0.25 μM 17α-hydroxyprogesterone. The control rate for A was 7125 nmoles/min. Reaction a was the control, b contained androstenedione, c contained acetaldehyde, and d contained both androstenedione and acetaldehyde. Panel B shows the effect of 16 μM androstenedione and/or 600 μM acetaldehyde. Each reaction contained 350 μg of microsomal protein and 0.25 μM 17α-hydroxyprogesterone. The control rate for B was 4.46 nmoles/min. Reaction a was the control, b contained androstenedione, c contained acetaldehyde, and d contained both androstenedione and acetaldehyde.

17β-dehydrogenase and 17α-hydroxyprogesterone aldolase were carried out at an NADPH concentration of 500 μM, a figure about ten times the K_m for NADPH for these enzymes. Under these conditions, an inhibitor that acts competitively with respect to NADPH would have to be present in a concentration nine times its K_i to produce 50 percent inhibition. Intracellular concentrations of NADPH, however, are probably at least as large as the ones we have chosen. The enzymes we examined have K_m values for their steroid substrate of several micromolar.

Other investigators have suggested recently that redox changes that occur in Leydig cells, either as a result of testicular [12] or hepatic [10] metabolism of alcohol, might be responsible for the reduced testicular secretion of testosterone associated with alcohol exposure. In our studies, excess exogenous cofactor (NADPH for testosterone 17β-dehydrogenase and 17α-hydroxyprogesterone aldolase; NAD for the 3β-hydroxysteroid dehydrogenase/steroid isomerase complex) was purposely added to each reaction vessel, such that cofactor availability would not be limiting. Rather, enzymic activity itself would be the limiting factor, should we observe any changes. Using such an experimental design, we found that ethanol, and to a lesser degree acetaldehyde, combined with androstenedione, reduced 17α-hydroxyprogesterone aldolase activity. In contrast to our studies utilizing isolated testicular microsomes, Cicero and Bell [11] have examined the effect of acetaldehyde on isolated Leydig cells. On the basis of a decreased conversion of labeled pregnenolone and pregesterone to testosterone and an increased conversion to androstenedione, these investigators have concluded that levels of acetaldehyde as low as 50 μM can inhibit testosterone 17β-dehydrogenase. These investigators, however, did not examine the enzyme directly. In our studies utilizing isolated microsomes, neither ethanol nor acetaldehyde inhibited this enzyme, even at very large (quite unphysiologic) inhibitor concentrations. Likewise, neither ethanol nor acetaldehyde had an effect on 3β-hydroxysteroid dehydrogenase/steroid Δ^{4-5}-isomerase. This later finding is consistent with the recent report of Gordon *et al.* [10] in which 3β-hydroxy-steroid dehydrogenase activity was found to be normal when the assay was carried out in the presence of saturating amounts of exogenous cofactor.

Moreover, in contrast to the findings of Cicero and Bell [11], our results suggest that ethanol may inhibit 17α-hydroxyprogesterone aldolase. A comparison of our results with those obtained using the isolated perfused rat testes [8] suggests that ethanol itself could be responsible for the observed inhibition of testosterone synthesis. In the isolated perfused testes, 300 mg/dl of ethanol reduced the testosterone production to 60 percent of that observed in the testes perfused with ethanol-free medium. In the present studies, at the same ethanol concentration, we observed a 17α-hydroxyprogesterone aldolase activity that was 62 percent of control. Thus, the inhibition of steroid aldolase by ethanol could explain the decreased testosterone synthesis seen in the isolated perfused testes, if the aldolase were the rate-determining step in the pathway.

We also noted some reproducible, but probably unphysiologic, effects of acetaldehyde on the activity of 17α-hydroxyprogesterone aldolase. Early in these experiments we wondered whether acetaldehyde might serve as a substrate for oxidation by this steroid aldolase. The aldolase with O_2 and NADPH acts to cleave 17α-hydroxy-20-ketosteroids to produce 17-ketosteroids and acetic acid. Consideration of a likely enzyme mechanism led us to the idea that acetaldehyde might also serve as a substrate in place of the 20-ketosteroid side chain. This might be especially true if the steroid-binding site of the enzyme were occupied by androstenedione. Thus, we looked for a synergism between acetaldehyde and androstenedione in the inhibition of the aldolase activity. Our results show a slight but insignificant inhibitory effect of acetaldehyde, an effect that could be enhanced arithmetically with the addition of androstenedione. We did not see a significant synergism, however, between androstenedione and acetaldehyde in inhibiting 17α-hydrozyprogesterone aldolase.

A major difficulty in assessing the significance of acetaldehyde as an inhibitor of 17α-hydroxyprogesterone aldolase is that we do not know the acetaldehyde concentration within the testes during ethanol consumption. In the human, the blood level averages about 50 μM for a wide range of blood ethanol levels [19], but acetaldehyde is produced and possibly consumed in the testis. If the aldolase were inhibited by acetaldehyde in the intact testis, then this inhibition could explain the observations of Cicero and Bell [11]. Some kinetic studies in cell free extracts of rat testis suggest that testosterone may be perferentially produced via the Δ^5-pathway by way of dehydroepiandrosterone in this species.

In this pathway, the aldolase may be rate-limiting [20]. Thus, in alcohol-treated rodent testes, androstenedione may be formed perferentially via the Δ^4-pathway, as suggested by Cicero and Bell [11].

We have not yet examined steroid 17α-hydroxylase to determine whether it is inhibited by ethanol or acetaldehyde. It also remains for us to investigate the hypothesis that an increase in the NADH/NAD ratio occurs *in vivo* in the testes, and that this inhibits testosterone synthesis by decreasing the activity of 3-oxosteroid Δ^5-Δ^4 isomerase, as recently suggested by Gordon *et al.* [10]. Nevertheless, our data would suggest that the acute inhibitory effect of ethanol and possibly of acetaldehyde upon testosterone synthesis by the rat testes is due, at least in part, to inhibition of 17α-hydroxyprogesterone aldolase, enzyme required for testosterone synthesis from pregnenolone.

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