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Inhibition of testosterone biosynthesis by ethanol: relation to the pregnenolone-to-testosterone pathway

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The concentrations of metabolites in the pregnenolone ⟶ testosterone pathway were determined in freeze-stopped testes in control rats and during ethanol intoxication (2 h after injection of 1.5 g ethanol/kg body wt). Ethanol lowered the mean testicular concentrations of testosterone (by 63–74%), androstenedione (49–81%), 17-hydroxyprogesterone (60–76%), progesterone (29–67%) and pregnenolone (12–25%). 4-Methylpyrazole had no effect on the ethanol-induced changes. The present results reveal no inhibition at the 17-hydroxyprogesterone ⟶ androstenedione ⟶ testosterone steps, but do not exclude inhibition before the step yielding pregnenolone and at the pregnenolone ⟶ progesterone ⟶ 17-hydroxyprogesterone steps.

Rat *Testis* *Steroidogenesis* *Testosterone synthesis* *Ethanol* *4-Methylpyrazole*

1. INTRODUCTION

The biosynthesis of testosterone in rat testis involves the following pathway: cholesterol ⟶ pregnenolone ⟶ progesterone ⟶ 17α-hydroxyprogesterone ⟶ androstenedione ⟶ testosterone [1]. Any of these steps may, in principle, be the target for the ethanol-mediated inhibition of testosterone production, a well-documented effect in rats [2–11]. Inhibition of pregnenolone formation is supported by observations [2,12,13] after ethanol treatment of lowered testosterone levels associated with decreased levels of luteinizing hormone (LH), the primary gonadotropin regulating testicular conversion of cholesterol to pregnenolone [14]. The generality may, however, be questioned, because no changes were found in mice [15] and increased LH levels were found in humans [16,17] under conditions in which ethanol decreased the plasma testosterone levels. The oxidative, NAD^+-dependent, transformation of pregnenolone to progesterone, requiring 3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) [1], could also be the target for the ethanol-mediated inhibition. This possibility is indirectly supported by observations that ethanol competitively inhibited NAD^+-dependent retinol oxidation in testis homogenates [18] and excess NAD^+ reversed ethanol-induced inhibition of testosterone synthesis in isolated lysed Leydig cells [5], and by the fact that NAD^+-dependent ethanol oxidation has been detected in rat testis [11,19,20]. Moreover, chronic ethanol treatment seems to reduce the NAD^+ availability of 3β-hydroxysteroid dehydrogenase [21]. The inhibitory role of the NAD^+ deficiency is, however, questioned in a recent investigation reporting no changes in testicular NAD^+ after ethanol treatment in vivo or in vitro [22]. That the inhibition would take place after progesterone formation has also been suggested [10]. Based on the inhibition of 17α-hydroxyprogesterone aldolase (EC 4.1.2.30), cleavage of 17α-hydroxyprogesterone to androstenedione has been proposed as the target for the ethanol effect [23]. Inhibition, under in vitro conditions, has also been observed at the last step of testosterone biosynthesis, the NADPH-

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dependent reduction of androstenedione to testosterone [8]. According to the proposed mechanism, ethanol-derived acetaldehyde directly inhibits 17β-hydroxysteroid dehydrogenase (EC 1.1.1.64). However, it was recently demonstrated that on stimulation by human chorionic gonadotropin (hCG) in vivo the inhibition probably occurs before the pregnenolone ⟶ progesterone step, in contrast to the situation in vitro with the inhibited androstenedione ⟶ testosterone step [9].

The inhibitory effects of ethanol on hCG-stimulated testosterone biosynthesis have been found in both in vitro and in vivo studies to be blocked by alcohol dehydrogenase (EC 1.1.1.1) inhibitors [4,24,25]. This suggests that ethanol per se is not responsible for the inhibition and that it is instead produced by some factors arising from ethanol metabolism, such as competition for NAD^+ at the pregnenolone ⟶ progesterone step or acetaldehyde-mediated inhibition of the androstenedione ⟶ testosterone step. Both of these possibilities were, however, contradicted by recent findings under nonstimulated in vivo conditions [11]. In fact, an ethanol-induced decrease in the 3-hydroxybutyrate/acetoacetate ratio, indicating a decreased free NADH/free NAD^+ ratio, seemed to be associated with inhibited testosterone production.

Here, the localization of the inhibitory step(s) was studied in a nonstimulated in vivo situation by determination of the concentrations of testosterone and its steroid precursors in testis with direct freeze-stop techniques.

2. MATERIALS AND METHODS

2.1. *Rats*

Male Long-Evans rats, 2.5–3.5 months old, bred in our laboratory (originating from rats purchased from Simonsen, Gilroy, CA), were used in the experiments. The animals were given a standard laboratory diet (Astra-Ewos AB, Södertälje, Sweden) and water ad libitum.

2.2. *General procedures*

All experiments were performed between 08.00 and 11.00 h. In the first series of experiments animals were divided into 4 groups: a control group that received no injections, an ethanol group injected intraperitoneally with 1.5 g ethanol/kg body wt as a 10% (w/v) solution in 0.9% NaCl, a 4-methylpyrazole + ethanol group injected intraperitoneally with 10 mg 4-methylpyrazole/kg body wt (Labkemi AB, Gothenburg, Sweden) as a 0.1% (w/v) solution in 0.9% NaCl 30 min before the 1.5 g ethanol/kg injection, and a 4-methylpyrazole group which received 10 mg 4-methylpyrazole/kg but no ethanol. In the second series of experiments animals were divided into two groups: the control group (no treatment) and ethanol group (1.5 g/kg). Tail-blood samples were taken 2 h after ethanol administration. Immediately after tail-blood sampling rats were cervically dislocated and one of the testes was (within 30 s) excised and freeze-stopped by rapid immersion in liquid N_2. An additional blood sample was taken by decapitation.

Tail blood was hemolysed with ice-cold water. The hemolysates were used directly for ethanol analyses (mean concentrations between 22 and 33 mM as previously reported) [11]. Blood taken after decapitation was centrifuged at 800 × *g* for 15 min at 4°C. Plasma was removed and stored at −17°C for 4–5 weeks before testosterone analysis. The frozen testes were stored for 6–7 weeks at −70°C before testosterone and precursor analysis.

2.3. *Analytical procedures*

Head-space gas chromatography with a Perkin-Elmer F40 instrument was applied for the determination of ethanol [26].

Plasma testosterone was measured as in [27], with a radioimmunoassay kit supplied by Nordiclab (Oulu).

Testicular steroid concentrations were measured as in [28]. Briefly, testis pieces (about 100 mg) were homogenized in water on ice and steroids were extracted with diethyl ether/ethyl acetate. The organic phases were chromatographed on 1-ml Lipidex-5000 columns, and steroids were quantitated from the appropriate fractions by a specific radioimmunoassay system [29]. The recovery was monitored by inclusion of ^3H-labeled steroid in each sample, and samples with recovery of less than 85% were re-analysed. Hence no correction for recovery was needed. The inter-assay coefficients of variation for steroid assays were monitored by inclusion of a known serum sample in each series of 15 testis samples, and were below 13%.

2.4. *Statistical analyses*

The results are given as means ± SD. Group mean comparisons were made with Student's *t*-distribution [30]. Pearson *r* coefficients [31] were used for correlation analyses.

3. RESULTS

3.1. *Effects of ethanol on the pregnenolone ⟶ testosterone pathway*

In the first series of experiments the ethanol group showed markedly decreased testicular testosterone ($p < 0.01$), 17-hydroxyprogesterone ($p < 0.05$) and progesterone ($p < 0.05$) concentrations relative to controls (table 1).

4-Methylpyrazole treatment did not prevent the ethanol-induced decreases; in fact, the statistical results were even more significant and androstenedione was now significantly lowered as well ($p < 0.0005$). No significant effects were observed regarding the pregnenolone concentrations. 4-Methylpyrazole treatment alone had no effects, and the 4-methylpyrazole results were combined with those of the corresponding control and ethanol for some of the subsequent analyses. To examine further the possibility of ethanol inhibiting any of the steps from pregnenolone to testosterone, the product/substrate ratios of this pathway were calculated. The control ratios from combined control and 4-methylpyrazole groups were as follows: progesterone/pregnenolone = 2.04 ± 0.92; 17-hydroxyprogesterone/progesterone = 2.54 ± 0.96; androstenedione/17-hydroxyprogesterone = 0.40 ± 0.15; testosterone/androstenedione = 6.82 ± 2.63. The ethanol ratios from combined ethanol and 4-methylpyrazole + ethanol groups and their probabilities relative to the controls were as follows: progesterone/pregnenolone = 1.20 ± 0.66 ($p < 0.01$); 17-hydroxyprogesterone/progesterone = 1.81 ± 0.92 ($p < 0.05$); androstenedione/17-hydroxyprogesterone = 0.48 ± 0.22 (not significant, n.s.); testosterone/androstenedione = 6.88 ± 4.11 (n.s.).

With a view to the fact that the pregnenolone ⟶ testosterone metabolites have not previously been determined in a nonstimulated situation during ethanol oxidation, and to the relatively large variability in the first results, another series of experiments was conducted on another generation of Long-Evans rats. The 4-methylpyrazole groups were omitted because of the obvious lack of effect on the ethanol-induced inhibition of steroidogenesis. On the other hand, dehydroepiandrosterone and plasma testosterone were now also determined. In accordance with the earlier experiments (table 1), ethanol reduced the levels of 17-hydroxyprogesterone, androstenedione and testosterone (table 2). A reduction was also seen in the dehydroepiandrosterone and plasma testosterone concentrations. The decrease observed previously in progesterone concentrations was not significant in our experiments, perhaps due to the

Table 1

Effects of ethanol and 4-methylpyrazole on the pregnenolone ⟶ testosterone pathway

Metabolite	Metabolite concentration (pmol/g wet wt)			
	Control ($n = 9$)	Control + 4-methylpyrazole ($n = 8$)	Ethanol ($n = 10$)	Ethanol + 4-methylpyrazole ($n = 10$)
Pregnenolone	52 ± 38	41 ± 21	46 ± 30	35 ± 22
Progesterone	87 ± 52	126 ± 49	43 ± 27[a]	42 ± 25[c]
17-Hydroxyprogesterone	246 ± 213	283 ± 143	75 ± 50[a]	67 ± 47[c]
Androstenedione	89 ± 66	125 ± 58	45 ± 31	24 ± 11[c]
Testosterone	590 ± 350	693 ± 207	217 ± 149[b]	181 ± 109[c]

Values are means ± SD from freeze-stopped testes. Samples were taken after cervical dislocation 2 h after ethanol (1.5 g/kg body wt) and 2.5 h after 4-methylpyrazole (10 mg/kg body wt) injections. Compared with corresponding control group, [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.0005$

relatively low levels and high variability, but a decrease in the pregnenolone concentration was now found.

The following product/substrate ratios were obtained in the second series of experiments for the control group: progesterone/pregnenolone = 0.58 ± 0.36; 17-hydroxyprogesterone/progesterone = 3.63 ± 1.81; androstenedione/17-hydroxyprogesterone = 3.67 ± 3.09; testosterone/androstenedione = 2.66 ± 0.95. The corresponding ratios (and probabilities relative to the control group) obtained for the ethanol group were: progesterone/pregnenolone = 0.60 ± 0.28 (n.s.); 17-hydroxyprogesterone/progesterone = 1.97 ± 0.80 ($p < 0.001$); androstenedione/17-hydroxyprogesterone = 2.46 ± 2.17 (n.s.); testosterone/androstenedione = 3.09 ± 1.37 (n.s.). No differences were observed in the testis/plasma testosterone ratio: controls = 9.0 ± 7.3; ethanol rats = 8.1 ± 2.5.

Table 3 lists individual correlations within the pregnenolone ⟶ testosterone pathway from both series of experiments. The purpose of the correlation analysis was to obtain further information about the site(s) of ethanol inhibition of steroidogenesis. For example, if ethanol acts primarily at a particular single step, one could expect that the presumed positive correlation between substrate and product in the control group might be disturbed by ethanol and could even be made negative at that step. However, as demonstrated in table 3, this was not the case. Regardless of ethanol treatment, the correlation between substrate and product concentration seemed high in the single steps. In addition, the low correlation in both the control and ethanol groups between dehydroepiandrosterone and other steroids (except androstenedione) could be noted.

Table 2

Effects of ethanol on the pregnenolone ⟶ testosterone pathway

Metabolite	Metabolite concentration (pmol/ml or g wet wt)	
	Control (n = 29)	Ethanol (n = 30)
Plasma		
Testosterone	13.4 ± 8.1 (n = 16)	7.1 ± 5.0[a] (n = 17)
Testis		
Pregnenolone	40 ± 24	30 ± 14[a]
Progesterone	24 ± 24	17 ± 10
17-Hydroxy-progesterone	81 ± 83	32 ± 19[b]
Dehydroepi-androsterone	5.3 ± 3.7	3.6 ± 2.7[a]
Androstenedione	181 ± 118	63 ± 41[c]
Testosterone	432 ± 261	159 ± 72[c]

Values are means ± SD from freeze-stopped testes. Samples were taken after cervical dislocation 2 h after ethanol (1.5 g/kg body wt) injections. Compared with the control group, [a] $p < 0.06$, [b] $p < 0.005$, [c] $p < 0.001$

4. DISCUSSION

4.1. *Biosynthesis of testosterone from pregnenolone: effects of ethanol*

It has been suggested that the major route for testicular steroidogenesis involves the 4-ene pathway, i.e., pregnenolone ⟶ progesterone ⟶ 17-hydroxyprogesterone ⟶ androstenedione ⟶ testosterone, in rats, and the 5-ene route, i.e., pregnenolone ⟶ 17-hydroxypregnenolone ⟶ dehydroepiandrosterone ⟶ androstenedione (or androstenediol) ⟶ testosterone, in humans [1]. The normal testicular concentrations of the steroids in these pathways (table 1,2) have not been determined in vivo in rats. The present results, showing very low dehydroepiandrosterone concentrations and the absence of correlations between this steroid and pregnenolone, or testosterone, are in line with the earlier view regarding the 4-ene pathway. The positive correlation between dehydroepiandrosterone and androstenedione concentration, and the general pattern of the correlations with dehydroepiandrosterone – positive correlations with other steroids within the 5-ene pathway and negative correlations with steroids in the 4-ene pathway that are not in the 5-ene one – (table 3) suggest, however, that the 5-ene pathway occurs in rats and may play a minor role in steroidogenesis. The fact that pregnenolone, progesterone, 17-hydroxyprogesterone and androstenedione all correlated positively with testosterone suggests that steroidogenesis involves regulatory, rate-limiting sites before these steroids.

Table 3

Interrelations in the pregnenolone ⟶ testosterone pathway

	Pregnen-olone	Proges-terone	17-Hydroxy-progesterone	Dehydro-epiandros-terone	Andros-tene-dione	Testos-terone
Progesterone						
Control 1	331					
Control 2	703[c]					
Ethanol 1	658[b]					
Ethanol 2	390[a]					
17-Hydroxyprogesterone						
Control 1	497[a]	817[c]				
Control 2	814[c]	899[c]				
Ethanol 1	705[b]	807[c]				
Ethanol 2	502[b]	784[c]				
Dehydroepiandrosterone						
Control 2	061	−270	−160			
Ethanol 2	136	−365[a]	−213			
Androstenedione						
Control 1	253	777[c]	903[c]			
Control 2	559[b]	263	447[a]	400[a]		
Ethanol 1	178	411	670[b]			
Ethanol 2	462[a]	076	441[a]	452[a]		
Testosterone						
Control 1	327	708[b]	720[b]	719[b]		
Control 2	385[a]	293	490[a]	804[c]	333	
Ethanol 1	754[c]	579[a]	886[c]	564[a]		
Ethanol 2	408[a]	215	613[c]	831[c]	188	
Testosterone (plasma)						
Control 2	439	621[a]	840[c]	744[b]	072	580[a]
Ethanol 2	125	−121	374	563[a]	155	688[b]

The matrix of individual correlations is shown. Values are product-moment correlation coefficients ($\times 10^3$). Control 1 (i.e., control and 4-methylpyrazole groups combined after normalization) and ethanol 1 (i.e., ethanol and 4-methylpyrazole + ethanol groups combined after normalization) refer to the experiments listed in table 1. Control 2 and ethanol 2 refer to the experiments listed in table 2. ^{a} $p < 0.05$, ^{b} $p < 0.005$, ^{c} $p < 0.0005$

The large standard deviations and the differences between the controls in table 1,2 are to be expected, because the animals of the Long-Evans strains are genetically heterogenous and two different generations were used. We believe it strengthens the significance of our finding that a clear ethanol effect on testosterone, androstenedione and 17-hydroxyprogesterone can be seen in spite of the large basic biological variation.

Our results contradict some earlier suggestions based on in vitro studies. Thus, the view that the inhibitory action of ethanol may be mediated by acetaldehyde inhibiting the androstenedione ⟶ testosterone step [8] seems unlikely, based on the present experiments in which ethanol lowered both these levels similarly in vivo (table 1,2). This confirms similar conclusions reached in a recent investigation using hCG-stimulated in vivo condi-

tions [9]. On the same basis, it seems that the step from 17-hydroxyprogesterone to androstenedione [23] could also be eliminated as a primary site for the ethanol-induced inhibition of steroidogenesis. An inhibitory influence on the progesterone ⟶ 17-hydroxyprogesterone step may be possible, as suggested by the product/substrate ratios of the values listed in table 1,2. In addition, the data listed in table 1 support inhibition at the pregnenolone ⟶ progesterone step, while this effect was not prominent in the second series of experiments (table 2). Inhibition before pregnenolone cannot be excluded, as is clearly demonstrated in table 2. Thus in conclusion, the present steroid concentration results supports inhibitory actions before pregnenolone, and at the pregnenolone ⟶ progesterone and progesterone ⟶ 17-hydroxyprogesterone steps, but not at the 17-hydroxyprogesterone ⟶ androstenedione and androstenedione ⟶ testosterone steps.

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