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ROLE OF ETHANOL METABOLISM IN THE INHIBITION OF TESTOSTERONE BIOSYNTHESIS IN RATS *IN VIVO*: IMPORTANCE OF GONADOTROPIN STIMULATION

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Summary—The mechanisms by which ethanol (EtOH, 1.5 g/kg) inhibits testicular testosterone synthesis were studied in nonstimulated and human chorionic gonadotropin (hCG, 50 IU/kg)-treated male rats. To dissociate the effects caused by ethanol metabolism, the alcohol dehydrogenase inhibitor 4-methylpyrazole (4MP, 10 mg/kg) was given to half of the rats 30 min before EtOH.

The 4MP had little or no effect in the nonstimulated rats on the EtOH-induced decreases in the concentrations of serum testosterone and of the intratesticular steroids of the testosterone biosynthetic pathway measured, but reduced the EtOH-induced elevation in the intratesticular pregnenolone-to-progesterone ratio. In contrast, 4MP pretreatment markedly reversed the EtOH-induced decrease in serum and intratesticular testosterone and increase in intratesticular pregnenolone concentrations in the hCG-stimulated rats. Simultaneously, the EtOH-induced elevations in the intratesticular pregnenolone/progesterone and androstenedione/testosterone ratios were abolished. In the EtOH-treated rats whose EtOH metabolism was blocked by 4MP pretreatment, the intratesticular testosterone concentrations were negatively correlated with the elevated serum corticosterone levels.

It is concluded that: (1) EtOH metabolism is involved in the inhibition of testicular steroidogenesis *in vivo*. This effect is pronounced during gonadotropin-stimulated conditions. Thus, previously reported "discrepancies" between the *in vivo* and *in vitro* results are clarified; (2) corticosterone seems also to be involved in the EtOH-induced inhibition of steroidogenesis. This effect is also pronounced during gonadotropin-stimulated conditions; and (3) without external gonadotropin stimulation other inhibitory mechanisms, such as decreased stimulation by luteinizing hormone, are prevalent.

INTRODUCTION

Ethanol (EtOH) has various effects on the male endocrine system [1]. EtOH affects the male hypothalamic–pituitary–testicular axis (for review see Ref. [2]), and at moderate doses it may decrease circulating serum concentrations of testosterone in men [3–6]. Rats have been used as the animal model for examining this decrease [7–9]. Incubation of dispersed Leydig cells [10–18] or testis perfusion [19, 20] have been used as *in vitro* tools to study the role of the direct gonadal effects of EtOH. However, the actual inhibitory mechanisms *in vitro* seemed to differ from those observed *in vivo* [21, 22]. EtOH metabolism was apparently the direct cause of the inhibition *in vitro*, but with one exception [23] *in vivo* experiments have indicated that it was not involved in the inhibition processes [8, 9]. The great majority of the *in vitro* experiments have studied hCG-stimulated testosterone synthesis, whereas the *in vivo* studies have focused on the basal testosterone production without exogenous gonadotropin stimulation. The exception [23] noted above was an *in vivo* study in which the biosynthesis of testosterone was stimulated with injection of human chorionic gonadotropin (hCG) to the rats.

The aim of the present work, therefore, was to see whether the discrepancy between *in vivo* and *in vitro* results in rats could be explained by differences between stimulated and non-stimulated synthesis. The serum corticosterone levels were measured to resolve the possible role of adrenal stimulation associated with EtOH

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administration, as corticosterone has been shown to inhibit testicular testosterone synthesis [24, 25]. The intratesticular steroid precursor profiles were studied to localize the enzymatic steps inhibited.

METHODS

Animals

Male rats of Alko mixed strain, 2.5–3 months old, were used in the experiment. Animals were caged in temperature and light controlled rooms and given a standard laboratory rat chow (Ewos AB, Södertälje, Sweden) and water *ad libitum*.

Treatments

The rats were divided into eight treatment groups of 12–15 rats (Table 1). Each rat received two injections. The treatments were: ethanol (EtOH, 1.5 g/kg, 12% (w/v) in physiological saline), 4-methylpyrazole (4MP, 10 mg/kg, Labkemi AB, Gothenburg, Sweden), and human chorionic gonadotropin (hCG, 50 IU/kg, Pregnyl™, Organon, Oss, The Netherlands).

The rats were decapitated 2.5 h after the second injection and serum was prepared from the trunk blood for corticosterone, testosterone, and EtOH determinations. The right testis was dissected and immersed into liquid nitrogen: the frozen tissue was then freeze-dried for the assay of intratesticular steroids.

Analytical procedures

EtOH was determined by head-space gas chromatography with a Perkin–Elmer Sigma 2000 [26]. Testosterone and corticosterone were measured from the serum samples using radioimmunoassay kits supplied by Farmos Diagnostica (Oulunsalo, Finland), and ICN Biomedicals (Carson, Calif., U.S.A.), respectively. Serum samples from the hCG-treated rats were diluted (1:10) with saline before testosterone determinations, and all serum samples were diluted (1:200) for corticosterone determinations. Freeze-dried testes were homogenized with a glass–glass homogenizer in distilled water and the steroids were extracted with diethyl ether–ethyl acetate (90:10, v/v). Steroids were then fractionated in Lipidex-5000™ micro columns (Packard-Becker, Chemical Operations, Groningen, The Netherlands), followed by RIA of each steroid from appropriate fractions [27]. The protein concentration was measured according to Bradford [28].

Table 1. Summary of 8 treatment groups

Group	First injection $t = 0$ min	Second injection $t = 30$ min
Control	Saline	Saline
EtOH	Saline	EtOH
4MP	4MP	Saline
4MP + EtOH	4MP	EtOH
hCG	Saline	hCG
hCG + EtOH	Saline	hCG + EtOH
hCG + 4MP	4MP	hCG
hCG + 4MP + EtOH	4MP	hCG + EtOH

Statistical analysis

The means ± SE are presented in the figures. Mann–Whitney U-test and 3-way ANOVA tests were used for comparisons of groups [29].

RESULTS

Inhibition of EtOH metabolism by 4MP pretreatment resulted in the expected higher EtOH levels in 4MP + EtOH-treated rats ($P < 0.001$) (Table 2). These rats also had elevated serum corticosterone concentrations ($P < 0.01$) (Table 2). The hCG treatment had no effects on serum EtOH levels, but elevated serum testosterone levels. EtOH treatment lowered testosterone levels (Table 2). In non-stimulated rats, 4MP did not prevent EtOH-induced decreases in the serum testosterone

Table 2. Effects of ethanol (EtOH, 1.5 g/kg), 4-methylpyrazole (4MP, 10 mg/kg), and human chorionic gonadotropin (hCG, 50 IU/kg) on the serum concentrations of corticosterone and testosterone

	EtOH (mmol/l)		Corticosterone (ng/ml)		Testosterone (ng/ml)	
Group	Mean	SEM	Mean	SEM	Mean	SEM
Control	<1.0		174	36	3.3	0.5
EtOH	43.2**	1.4	219	49	1.0**	0.1
4MP	<1.0		172	41	3.4	0.6
4MP + EtOH	69.5**	3.0	403**	29	1.2**	0.2
hCG	<1.0		132	34	17.5	1.1
hCG + EtOH	41.1**	1.8	143	38	12.7**	1.0
hCG + 4MP	<1.0		152	28	17.1	1.6
hCG + 4MP + EtOH	68.1**	1.9	436**	14	14.5	0.9

Mean ± SEM, $n = 12$–15, **$P < 0.01$ between EtOH-treated and -untreated groups.

Table 3. Effects of ethanol (EtOH, 15 g/kg), 4-methylpyrazole (4MP, 10 mg/kg), and human chorionic gonadotropin (hCG, 50 IU/kg) on the intratesticular steroid concentrations.

	Pregnenolone (pg/mg protein)		Progesterone (pg/mg protein)		17-OH-progesterone (pg/mg protein)		Androstenedione (pg/mg protein)		Testosterone (pg/mg protein)		DHEA (pg/mg protein)	
Group	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	255	40	192	46	249	49	261	59	1453	264	39	5
EtOH	193	25	72**	11	70***	7	100*	19	496***	51	32	4
4MP	244	25	153	17	263	47	190	32	1368	187	39	5
4MP + EtOH	123***	11	58***	6	67***	9	85***	11	462***	48	26	3
hCG	1178	117	1747	164	3190	498	1016	91	7095	434	89	9
hCG + EtOH	1908**	200	1815	365	1751	205	1086	116	4721***	364	98	11
hCG + 4MP	1106	161	1948	388	2085	364	1057	113	6569	651	93	13
hCG + 4MP + EtOH	1120	100	3023*	361	1888	219	1038	131	6480	563	89	9

Mean ± SEM, $n = 12$–15, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between EtOH-treated and -untreated groups. DHEA = dehydroepiandrosterone.

levels, but in the hCG-stimulated rats, 4MP partly reversed the EtOH-induced inhibition.

The intratesticular steroid concentrations are presented in Table 3. In the nonstimulated rats, the intratesticular pregnenolone concentrations did not decrease significantly after EtOH treatment, but if EtOH metabolism was blocked by 4MP pretreatment and the serum EtOH and corticosterone were high, the pregnenolone concentrations decreased significantly ($P < 0.001$). In the hCG-stimulated rats, on the other hand, EtOH elevated pregnenolone concentrations ($P < 0.01$), and this elevation was blocked by 4MP. The intratesticular progesterone concentrations were decreased strongly in the nonstimulated rats ($P < 0.01$); 4MP pretreatment had no effect on this decrease. In the 4MP + hCG-treated rats, EtOH treatment induced an elevation in the intratesticular progesterone levels ($P < 0.05$). As the progesterone levels decreased relatively more than the pregnenolone levels in the nonstimulated rats, and the pregnenolone concentrations elevated in the hCG + EtOH-treated rats, an EtOH-induced elevation in the intratesticular pregnenolone/progesterone ratio was observed in both nonstimulated and gonadotropin-stimulated rats ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 1). In both the gonadotropin-stimulated and nonstimulated rats, this elevation was reduced by 4MP pretreatment. In fact, the pregnenolone/progesterone ratio was even lower in the 4MP + hCG + EtOH-treated rats than in the 4MP + hCG controls because of the elevated progesterone concentrations in the latter group (Fig. 1).

The intratesticular concentrations of 17-OH-progesterone, androstenedione and testosterone decreased, and 4MP pretreatment had no effects on these decreases in the nonstimulated rats. In the gonadotropin-stimulated rats, no significant effects by EtOH or 4MP could be observed on the intratesticular 17-OH-progesterone or androstenedione concentrations, but the intratesticular testosterone concentrations decreased ($P < 0.001$) (Table 3). After 4MP pretreatment no EtOH-induced inhibition could be observed in the intratesticular testosterone concentrations of the gonadotropin-stimulated rats. Thus, the elevation in the androstenedione/testosterone ratio in the hCG + EtOH-treated rats returned to control level with 4MP pretreatment (Fig. 2). The ANOVA test showed a significant 3-way interaction between the hCG, EtOH and 4MP treatments (d.f. = 1/102, $F = 4.15$, $P < 0.05$) supporting that the reversion of the EtOH-induced inhibition by 4MP was dependent upon hCG stimulation.

There existed a negative correlation between the serum corticosterone levels and the intratesticular androstenedione (d.f. = 11, $r = 0.64$, $P < 0.03$) and testosterone (d.f. = 12, $r = 0.66$, $P < 0.02$) concentrations suggesting that the elevated serum corticosterone levels of the

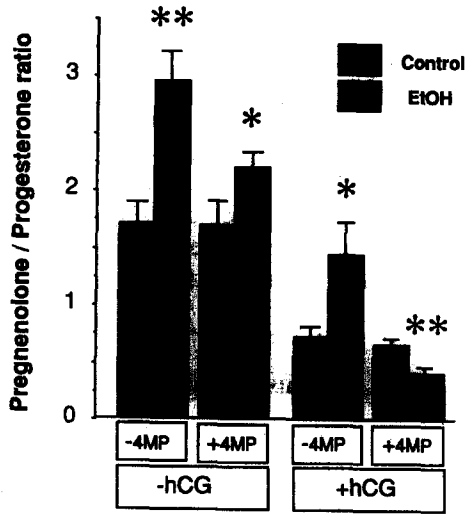

Fig. 1. Effects of ethanol (EtOH, 1.5 g/kg) on the intratesticular pregnenolone/progesterone ratio of the nonstimulated (−hCG) and human chorionic gonadotropin (50 IU/kg)-stimulated (+hCG) rats treated (+4MP) or untreated (−4MP) with 4-methylpyrazole (10 mg/kg). Mean ± SEM, $n = 12$–15, $^*P < 0.05$, $^{**}P < 0.01$.

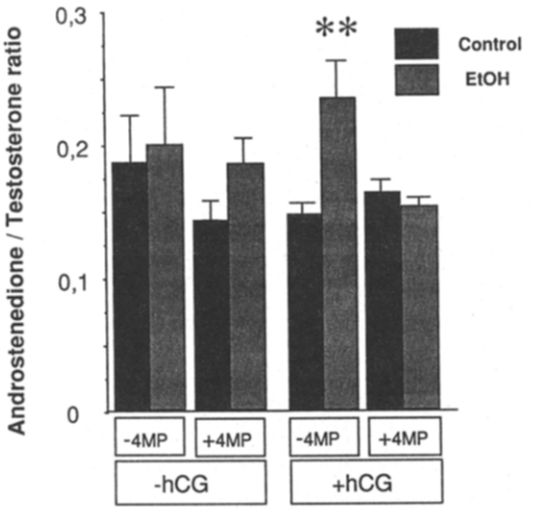

Fig. 2. Effects of ethanol (EtOH, 1.5 g/kg) on the intratesticular androstenedione/testosterone ratio of the nonstimulated (−hCG) and human chorionic gonadotropin (50 IU/kg)-stimulated (+hCG) rats treated (+4MP) or untreated (−4MP) with 4-methylpyrazole (10 mg/kg). Mean ± SEM, $n = 12$–15, $^{*}P < 0.05$, $^{**}P < 0.01$.

4MP + hCG + EtOH-treated rats could be involved in the inhibition processes. Furthermore, the elevated progesterone concentrations in these rats were observed to correlate with the, elevated serum corticosterone levels (d.f. = 12 $r = 0.54$, $P < 0.05$). The probable inhibition site was the steroid 17-hydroxylase/C_{17-20} lyase, because the progesterone/17-hydroxyprogesterone, progesterone/androstenedione and 17-hydroxyprogesterone/androstenedione ratios all showed a positive correlation with serum corticosterone concentrations (d.f. = 12, $r = 0.52$, $P < 0.07$; d.f. = 12, $r = 0.53$, $P < 0.06$; and d.f. = 11, $r = 0.55$, $P < 0.07$, respectively). Also the dehydroepiandrosterone/androstenedione ratio—often compared with the pregnenolone/progesterone ratio, because they both represent the 3β-hydroxysteroid dehydrogenase reaction—showed a clear positive correlation with serum corticosterone levels in the 4MP + hCG + EtOH group (d.f. = 11, $r = 0.76$, $P < 0.01$).

DISCUSSION

The role of EtOH metabolism in the inhibition of testosterone production has been a matter of controversy. In the previous studies using similar doses of EtOH and 4MP as in the present study, the inhibition of EtOH metabolism by 4MP had no effect on the EtOH-induced fall in the basal serum testosterone levels, although the rise in the intratesticular lactate/pyruvate ratio was blocked by 4MP indicating effective inhibition of EtOH metabolism [8]. This inability to abolish the EtOH-induced inhibition of the unstimulated testosterone synthesis was also observed in the present study. The central effects of EtOH, decreasing LHRH secretion from the hypothalamus [30] and thus the LH secretion from the anterior pituitary [7, 31], were probably responsible for the observed inhibition in the nonstimulated rats. This is also in line with the results of the present (Table 3) and previous studies [9, 32] showing that concentrations of all intratesticular steroids in testosterone biosynthetic pathway were decreased following EtOH administration. However, the increased pregnenolone/progesterone ratio observed in the present study and previously [9, 32] suggest that the inhibition of 3β-hydroxysteroid dehydrogenase/oxosteroid isomerase may also contribute to the inhibition of testosterone synthesis. On the other hand, a recent study also showed that stress induced by physical training elevated serum corticosterone levels in rats [34]. Simultaneously, the intratesticular progesterone, androstenedione, and testosterone concentrations decreased. No effects were seen in the LH levels after the exercise. Thus, the elevated corticosterone levels of the nonstimulated 4MP + EtOH-treated rats could inhibit some steps prior to pregnenolone formation without affecting LH secretion.

When the effects on LH secretion were bypassed by gonadotropin injection, and the rate of testosterone production was maximal, the metabolic component of the inhibition became apparent in the present study. This metabolic inhibition mechanism has been extensively studied *in vitro* [10–18]. The sites of the EtOH-induced inhibition in the steroidogenic pathway observed *in vitro* are the reactions from pregnenolone to progesterone by the NAD^+-dependent 3β-hydroxysteroid dehydrogenase/oxosteroid isomerase [10, 14, 17], and from androstenedione to testosterone by the NADPH-dependent 17-ketosteroid reductase [11–13, 15–17]. Our recent studies have indicated that the inhibition probably resulted from the EtOH-induced lack of essential metabolites of the transport shuttles involved in the maintenance of the proper NAD(P)H redox states in the mitochondrial and smooth endoplasmic reticulum compartments of the Leydig cell [34, Orpana *et al.*, submitted for publication].

The present results confirm the findings by Cicero *et al.* [23] that inhibition of alcohol dehydrogenase by pyrazole derivates can reduce the decrease in the serum testosterone concentrations after EtOH treatment, and indicate that this occurs if the rate of steroidogenesis was increased by gonadotropin injections. The present increases in the pregnenolone /progesterone and androstenedione/testosterone ratios support the hypothesis that similar inhibition mechanisms are active in the hCG-stimulated Leydig cells of the intact rats *in vivo*, as observed in isolated Leydig cells incubated and stimulated with hCG *in vitro* [15, 17].

The observed accumulation of progesterone in the testes of the 4MP + hCG + EtOH-treated rats was most likely caused by the inhibition of the conversion of progesterone to androstenedione by the high corticosterone concentration [24, 25]. The corticosterone-induced elevation in the progesterone concentration and the decrease in the androstenedione concentration may in some conditions mask the changes in the opposite direction caused by EtOH metabolism.

In conclusion, the present results show that in gonadotropin-stimulated rats, inhibition of steroidogenesis by EtOH metabolism is also an important inhibition mechanism *in vivo*. The high circulating EtOH levels after EtOH treatment, in rats whose alcohol dehydrogenase is inhibited, results in high circulating corticosterone concentration, which can also disturb the steroidogenic machinery of the Leydig cell. If no external stimulation is used, the central inhibitory mechanisms decreasing internal LH stimulation can mask both the metabolic and corticosterone effects. Thus, the present study indicates that the "discrepancies" between the previous *in vivo* and *in vitro* results are caused by the fact that only one of the three effective *in vivo* mechanisms can be studied *in vitro*.

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