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ETHANOL-INDUCED INHIBITION OF TESTOSTERONE BIOSYNTHESIS IN RAT LEYDIG CELLS: ROLE OF L-GLUTAMATE AND PYRUVATE

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Summary—The mechanisms by which ethanol (EtOH) inhibits testicular testosterone biosynthesis were studied with isolated rat Leydig cells *in vitro* comparing the effects of EtOH in six different culture media. The actual sites of inhibition by EtOH, identified by measuring the steroidogenic precursors, varied depending on the medium used. In Krebs–Ringer bicarbonate buffer, EtOH inhibited both the conversion of pregnenolone to progesterone and androstenedione to testosterone. In the pyruvate (Pyr) supplemented Dulbecco's Modified Eagle medium, the decreased progesterone concentrations in the presence of EtOH were reflected to all successive steroids 17-OH-progesterone, androstenedione and testerone. The presence of L-glutamate (Glu) in the medium elevated testosterone production, but EtOH still inhibited the conversion of pregnenolone to progesterone, and also the androstenedione/testosterone ratio was elevated because of the decreased testosterone concentrations. In the presence of both Glu and Pyr in the medium the EtOH-induced decreases in the steroid concentrations were fully recovered in isolated Leydig cells. These results demonstrate that both Pyr and Glu supplementations are essential for the maintenance of maximal rate of testosterone synthesis *in vitro* in the presence of EtOH.

INTRODUCTION

Ethanol (EtOH) intoxication is known to affect the functions of many components of the male endocrine system along the hypothalamic–pituitary–gonadal axis [1]. As a result, decreased circulating testosterone concentrations are seen in rat [2–4] and man [5–8]. The direct gonadal effects have been studied by using *in vitro* models based on testis perfusions or incubations of dispersed Leydig cells [9–16]. The actual mechanisms by which EtOH inhibits testosterone biosynthesis *in vivo* and *in vitro* are in some cases dissimilar [17–19], because the inhibition *in vitro* totally depends upon the metabolism of EtOH whereas the metabolism does not seem to play an essential role in the inhibition of testosterone synthesis by EtOH *in vivo* [4, 17, 18]. For that reason we have performed a set of experiments to study more closely the different factors involved in the EtOH-induced inhibition of testosterone synthesis in isolated and Percoll purified Leydig cells incubated *in vitro*. We have previously shown that there exists a difference in the action of EtOH if we employed Dulbecco's Modified Eagle's Medium (DME) or HAM F12 nutrient mixture (F12) for the Leydig cell incubations. When F12 was used no EtOH-induced inhibition could be observed. In that report we also showed that Glu was very effective in abolishing the EtOH-induced inhibition in DME [20].

In the present work we have studied six different culture media to find out whether this stimulatory effect of Glu is a common feature, and why EtOH inhibits testosterone synthesis in Glu-containing Medium 199 [10–15]. The sites of EtOH-induced inhibition and Glu-induced reversal in different culture media were identified by measuring the relevant steroidogenic precursors in addition to the end product, testosterone. Because both DME and F12, but not medium 199, are supplemented with pyruvate (Pyr), the possible role of Pyr supplementation in the Glu-containing media was also studied.

EXPERIMENTAL

Animals

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

Culture media

Basal Medium (BME, contains no Glu or Pyr), Dulbecco's Modified Eagle medium (DME, contains

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Pyr), HAM-F12 nutrient mixture (F12, contains both Glu and Pyr), Medium 199 (M199, contains Glu) and RPMI 1640 (RPMI, contains Glu) were purchased from Gibco (Grand Island, N.Y.). HEPES buffered media M199, RPMI, and HAM-F10 nutrient mixture (F10) which is similar to F12, were also from Gibco. Krebs–Ringer bicarbonate buffer (KR) and phosphate-buffered saline (PBS), both pH 7.4 and supplemented with D-glucose (5 mmol/l) were prepared prior to use by dilution from stock solutions. The culture media were supplemented with bovine serum albumin (1 mg/ml, Sigma Chemicals, St Louis, Mo.). All other compounds were from Sigma Chemicals.

Preparation and purification of Leydig cells

Rats were killed by decapitation. The interstitial cells were isolated from the decapsulated testes by collagenase digestion and the Leydig cells were purified in Percoll density gradients [21, 22] as described earlier [20]. Isolation and purification procedures were performed in KR and before the incubations the pooled cell suspensions were suspended in PBS.

Cell incubations

The Leydig cells were incubated in different culture media in the presence or absence of EtOH (25 mmol/l) or Glu (0.5 mmol/l) for 3 h at 33°C under O_2–CO_2 (95:5) with continuous shaking. In the second experiment the HEPES buffered media F10, M199 and RPMI were adjusted to pH 6.9, 7.4 or 7.9, and the Leydig cells were incubated in the presence or absence of EtOH (25 mmol/l) or Pyr (0.5 mmol/l) as described above.

The testosterone synthesis was stimulated with human chorionic gonadotropin, hCG (Pregnyl, Organon, Oss, The Netherlands, 10 mIU/ml). The incubations were terminated with PCA (perchloric acid, E. Merck, Darmstadt, F.R.G.) and the supernatants were stored at −20°C until neutralized with K_3PO_4 before determinations.

Analytical procedures

EtOH was determined by head-space gas chromatography with a Perkin Elmer Sigma 2000 [23] and the steroid precursors were measured from the neutralized supernatants after Lipidex-5000™ chromatography as described earlier [15]. In the second part of the experiment the testosterone concentrations were determined using RIA kits supplied by Farmos Diagnostica (Oulunsalo, Finland) without chromatography.

Statistical analysis

The means ± SD are present in table and figures. Analysis of variance and *t*-tests were used to detect the effects of EtOH and Glu on steroid precursor concentrations. Means were compared by *t*-tests after regrouping the samples according to the presence or absence of Pyr and Glu in the medium. The culture media KR and BME, which did not contain Glu or Pyr, were considered as control group. DME contained only Pyr (Pyr group) and the Glu group consisted of M199, RPMI with KR and BME after Glu supplementation. F12 and DME after Glu supplementation were taken as Glu + Pyr group.

Table 1. Effects of ethanol (ETOH, 25 mmol/l) and L-glutamate (GLU, 0.5 mmol/l) on the concentrations of pregnenolone (PREG), progesterone (PROG), 17-OH-progesterone (17PROG), androstenedione (ADIONE), testosterone (TESTO) and dehydroepiandrosterone (DHEA) of the hCG-stimulated Leydig cells incubated in different culture media. Values are mean ± SD of triplicate incubations

Medium	Group	PREG (nmol/l)	PROG (nmol/l)	17PROG (nmol/l)	ADIONE (nmol/l)	TESTO (nmol/l)	DHEA (nmol/l)
KR	Control	2.7 ± 0.5	1.4 ± 0.2	5.4 ± 1.0	41.7 ± 5.0	57.6 ± 7.7	2.2 ± 0.3
	ETOH	4.9 ± 1.0[a]	1.1 ± 0.1[a]	5.6 ± 0.5	53.4 ± 4.8[a]	48.8 ± 0.1[a]	5.1 ± 0.4[a]
	GLU	2.7 ± 0.6	1.6 ± 0.1	4.3 ± 0.6	28.3 ± 1.0[a]	106.3 ± 4.5[a]	3.1 ± 0.1[a]
	GLU + ETOH	3.5 ± 0.9	1.4 ± 0.2	3.7 ± 0.3[b]	27.7 ± 3.5[b]	92.7 ± 8.6[b]	5.2 ± 1.2[c]
BME	Control	3.0 ± 0.8	1.8 ± 0.2	6.7 ± 0.5	54.6 ± 10.8	50.7 ± 4.2	2.6 ± 0.3
	ETOH	3.5 ± 0.3	1.4 ± 0.2[a]	6.5 ± 0.3	58.0 ± 8.9	31.4 ± 2.6[a]	2.9 ± 0.1
	GLU	2.4 ± 1.2	2.0 ± 0.2	6.9 ± 0.5	38.0 ± 4.1[a]	88.6 ± 4.5[a]	2.3 ± 0.3
	GLU + ETOH	3.4 ± 0.3	1.5 ± 0.2[c]	7.2 ± 0.3	46.8 ± 15.1	80.3 ± 1.3[bc]	3.7 ± 0.3[bc]
DME	Control	2.4 ± 0.5	1.8 ± 0.3	6.2 ± 0.7	26.4 ± 2.0	75.8 ± 2.8	2.4 ± 1.0
	ETOH	3.7 ± 1.5	1.6 ± 0.1	4.8 ± 0.4[a]	18.2 ± 0.8[a]	62.7 ± 4.5[a]	2.0 ± 0.3
	GLU	2.3 ± 1.1	1.9 ± 0.2	6.1 ± 0.4	37.3 ± 5.4[a]	85.0 ± 2.8[a]	2.4 ± 0.0
	GLU + ETOH	2.1 ± 1.2	1.8 ± 0.1[b]	5.4 ± 0.4	29.5 ± 2.0[b]	90.8 ± 9.7[b]	2.0 ± 0.3
M199	Control	4.0 ± 1.0	2.2 ± 0.1	8.3 ± 0.9	49.3 ± 2.1	93.1 ± 8.1	2.7 ± 0.4
	ETOH	5.2 ± 0.6	1.5 ± 0.1[a]	7.0 ± 0.2	50.0 ± 2.4	80.7 ± 3.4[a]	5.9 ± 0.7[a]
	GLU	4.2 ± 0.7	2.1 ± 0.0	8.4 ± 1.0	48.2 ± 3.0	84.0 ± 7.1	3.0 ± 0.1
	GLU + ETOH	5.9 ± 0.7[c]	1.6 ± 0.0[c]	7.1 ± 0.8	44.3 ± 7.4	84.0 ± 3.1	5.5 ± 1.5[c]
F12	Control	1.3 ± 0.2	1.2 ± 0.1	4.4 ± 0.4	20.9 ± 5.1	93.3 ± 13.1	0.7 ± 0.1
	ETOH	2.6 ± 0.1[a]	1.4 ± 0.2	5.4 ± 0.7	21.5 ± 1.1	98.6 ± 1.4	0.7 ± 0.2
	GLU	1.9 ± 0.6	1.4 ± 0.3	5.3 ± 0.4	21.6 ± 5.0	89.3 ± 3.6	0.7 ± 0.2
	GLU + ETOH	1.1 ± 0.8[b]	1.4 ± 0.2	4.9 ± 0.9	22.6 ± 2.4	103.8 ± 5.6[c]	0.8 ± 0.6
RPMI	Control	1.8 ± 1.1	1.5 ± 0.3	5.7 ± 0.5	26.5 ± 5.9	93.1 ± 2.8	0.7 ± 0.4
	ETOH	2.4 ± 0.6	1.1 ± 0.2[a]	4.4 ± 0.6[a]	27.9 ± 7.3	80.0 ± 2.2[a]	1.7 ± 0.5[a]
	GLU	1.0 ± 0.2	1.4 ± 0.1	4.8 ± 0.7	27.9 ± 5.5	94.5 ± 9.0	1.1 ± 0.7
	GLU + ETOH	2.4 ± 0.5[c]	1.2 ± 0.1	5.0 ± 0.7	30.1 ± 3.3	86.0 ± 0.3	1.7 ± 1.1

Different from [a]control, [b]ETOH, or [c]GLU group ($P < 0.05$).

RESULTS

The concentrations of the steroids after incubations of the Leydig cells in different culture media are shown in Table 1. EtOH had an overall raising effect on pregnenolone concentrations in all media tested (ANOVA test d.f. = 1, $n = 72$, $F = 7.2$, $P < 0.01$), and Glu reversed this effect in DME and F12, whereas in KR this reversal was partial.

EtOH lowered the progesterone concentrations in each medium studied except in F12. In DME this decrease was not as evident as in other media, and only in this medium the effect of EtOH was reversed by Glu supplementation. In KR the reversal by Glu was only partial.

EtOH had no decreasing effect on the concentrations of 17-OH-progesterone in KR, BME or F12, while in DME and RPMI EtOH lowered 17-OH-progesterone concentrations. Addition of Glu lowered 17-OH-progesterone concentrations in KR and partly abolished EtOH inhibition in DME.

EtOH raised androstenedione concentrations in KR whereas lowered them in DME. In BME, M199, F12, or RPMI no significant EtOH-induced changes could be observed in androstenedione concentrations. When Glu was added to the culture media, the concentrations of adrostenedione decreased in KR and BME. In DME, where the basic androstenedione concentrations were low, Glu actually raised its concentrations. In F12 and RPMI no changes by EtOH or additional Glu were observed.

EtOH lowered testosterone concentrations in all the media except in F12. The basic testosterone concentrations were lowest in KR, BME and DME, but the addition of Glu elevated the testosterone production to the level of the Glu containing media M199, F12, and RPMI. The effects of EtOH were blocked by Glu in DME, but in KR and BME the recovery from EtOH inhibition was not complete. EtOH raised the concentrations of DHEA in KR, M199 and RPMI, and Glu had no effect on this raise. In other media no effects could be observed.

Figure 1 shows the recalculated effects of EtOH on testosterone and its steroid precursors after grouping the samples into Cont, Glu, Pyr and Glu + Pyr groups.

This recalculated data shows that only in the presence of both Glu and Pyr EtOH did not raise the concentrations of pregnenolone or decrease the concentrations of progesterone. The lack of significances in the Pyr group is probably caused by the small number of observations in that particular group, which contained only medium DME. The EtOH-induced decreases in the concentrations of progesterone, 17-OH-progesterone and androstenedione in that medium are analogous to our recent results using this medium [24].

If the culture media (KR and BME) did not contain Glu or Pyr, or if it contained only Pyr (DME) or Glu (M199, RPMI together with KR and BME after Glu supplementation), EtOH inhibited testosterone synthesis. If the culture medium contained both Glu and Pyr (F12 and DME after Glu supplementation) EtOH, if anything, increased testosterone production.

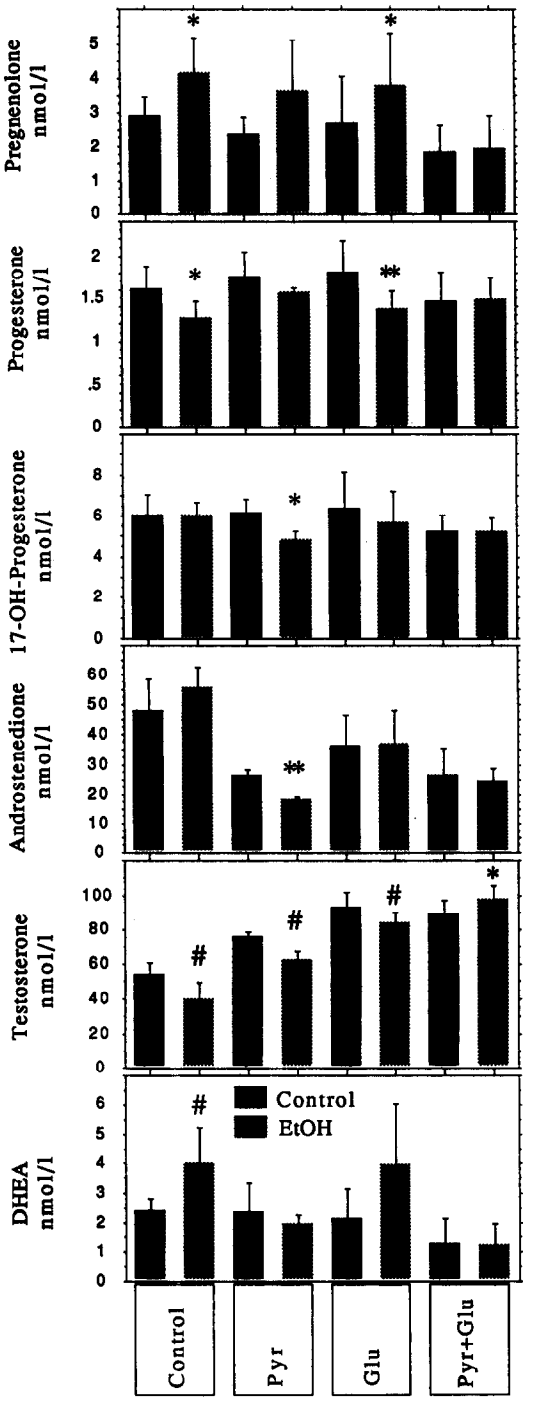

Fig. 1. Effects of ethanol (EtOH, 25 mmol/l) on the steroid concentrations after grouping the samples according to L-glutamate (Glu) and pyruvate (Pyr) content as described in Methods section. See other details from the legend to Table 1. Values are mean ±SD $^{**}P < 0.01$, $P < 0.02$, $^{*}P < 0.05$. Cont ($n = 6$), Glu ($n = 18$), Pyr ($n = 3$), Glu + Pyr ($n = 9$).

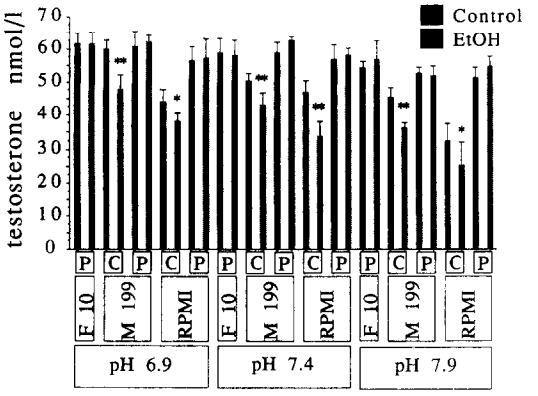

Fig. 2. Effects of ethanol (EtOH, 25 mmol/l) on testosterone concentrations in hCG-stimulated Leydig cells incubated in HEPES buffered F10, M199 or RPMI culture media buffered to three different pH levels, with (P) or without (C) pyruvate (0.5 mmol/l) supplementation. Values are mean ± SD of 4 cell pools. $^{**}P < 0.01$.

The DHEA concentrations show that Pyr prevented EtOH-induced rises indicating that Pyr supplementation abolished the block at the pregnenolone-to-progesterone reaction.

Figure 2 shows the effects of Pyr on the EtOH-induced inhibition of testosterone synthesis in the HEPES-buffered media F10, M199 and RPMI buffered to acidic (pH = 6.9), normal (pH = 7.4) and alkaline (pH = 7.9) conditions. The medium pH had only minor effects on the EtOH-induced inhibition of testosterone synthesis in these Glu containing media, but the inhibition was fully prevented if the media were supplemented with Pyr. In F10 medium containing Pyr and Glu EtOH had no effects on testosterone concentrations.

DISCUSSION

It is well known that EtOH metabolism elevates the cytosolic NADH/NAD^+ ratio in the liver, which can be calculated from the lactate/pyruvate (L/P) ratio (for review, see [25]). Similarily, EtOH-induced raises in the L/P ratio have been observed in the Leydig cells [15] and EtOH probably inhibits several NAD^+-dependent reactions in testis as well [26]. Thus, the EtOH-induced inhibition of the NAD^+-dependent 3β-hydroxysteroid dehydrogenase/oxosteroid isomerase (3βHSD) reaction has been explained as the decreased availability of NAD^+ [9, 13, 27] caused by the NAD^+-dependent metabolism of EtOH by the cytosolic alcohol dehydrogenase. The present results, with EtOH increasing the pregnenolone/progesterone ratios, confirm these previous findings. The EtOH-induced shift in the pregnenolone/progesterone ratio did not appear in Glu + Pyr group, where no reduction in testosterone concentrations by EtOH was observed, either. It seems that there was some kind of interaction between the action of these supplements because neither had this effect when present alone in the incubation medium.

In the Glu-containing media M199 and RPMI, the EtOH-induced decreases in the testosterone concentrations were not affected by additional Glu. However, when these media were supplemented with Pyr in the second experiment, no EtOH-induced inhibition was observed. After Pyr supplementation these media acted like the tested Glu and Pyr containing media F10 or F12 in which no EtOH-induced inhibition was observed in the present or our earlier study [20]. Similar results were also obtained using Iscove's Modified Eagle Medium, which is DME medium supplemented with non-essential amino acids and thus contains both Pyr and Glu (result not shown).

The mechanisms involved in the EtOH-induced inhibition of the steroidogenesis in the Leydig cell are supposed to be connected with the decreased activity of the substrate shuttles transporting the NADPH of mitochondrial origin (for review, see [28, 29]) to smooth endoplasmic reticulum (SER) as citrate, because the shuttles utilize the same metabolites and transport systems that are used for the transport of NADH-reducing equivalents from cytosol to mitochondria [24].

The central role of Pyr in the reversion of the EtOH-induced inhibition in the presence of Glu supports the hypothesis that one of the shuttle systems that might be involved is the citrate-pyruvate shuttle [24]. The citrate transported from mitochondria to SER could be cleaved to oxalacetate and acetyl-CoA by the ATP-citrate lyase. Oxalacetate could then be reduced to malate by NADH-dependent malate dehydrogenase (MDH). If the NAD(H) pool of 3βHSD is the same as that of MDH, then sufficient oxalacetate supply could support 3βHSD reaction converting pregnenolone to progesterone. Malate could be decarboxylated to Pyr by the malic enzyme generating NADPH for steroidogenesis in SER. After this Pyr enters mitochondria and the shuttle is completed when Pyr is converted back to citrate using the citric acid cycle.

The availability of oxalacetate in SER and the activities of the transport shuttles can be limited by the competition with other shuttles, like the malate-aspartate shuttle, and the inhibition of the citric acid cycle reactions during EtOH metabolism [30]. The shuttles can also be inhibited if part of the Pyr entering mitochondria is converted to lactate during EtOH metabolism. The major mechanism of the Glu and Pyr interaction is probably that the continuous oxalacetate supply due to high Pyr concentrations, when combined with increased α-ketoglutarate supply from Glu supplementation, probably increased, or at least maintained the production of citrate and other shuttle metabolites essential for the proper function of these transport shuttles during EtOH metabolism [30,32]. The finding that only Pyr effectively abolished the EtOH-induced raises in the DHEA concentrations further indicated its central role in the reversion of the inhibition at the 3βHSD reaction [33].

The present results demonstrated that the NADPH-dependent reduction of androstenedione to testosterone was stimulated by Glu in media lacking this amino acid, indicating elevated NADPH production in SER. This step has been reported as the second major EtOH-induced inhibition site in steroidogenesis [10, 12, 14, 15], especially if only the steps after progesterone formation are studied [34], and the stimulation of this reaction may explain Glu's ability to overcome the EtOH-induced inhibition. The present results show that the stimulation of the NADPH-dependent conversion of androstenedione to testosterone in KR was too high for the progesterone to 17-OH-progesterone reaction to respond, and so the precursor steroids 17-OH-progesterone and androstenedione were depleted. Decreases in the androstenedione/testosterone ratios were also observed in BME indicating similar but milder stimulatory responses to Glu supplementation. It seems likely that the stimulation by Glu was higher when other amino acids were present in lower concentrations, as the stimulation was highest in KR and in lowest DME, where the amino acid concentrations are about 4 times higher than in BME. It is also possible that other amino acids could supply "non-essential" Glu or some of its stimulatory metabolite like α-ketoglutarate, and the need for added Glu is minor, although not abolished. However, the situation is somewhat complex because of the finding that only in KR the pregnenolone/progesterone ratio was nearly recovered by Glu alone. This indicates that the metabolism of amino acids could also be involved in the inhibition processes and the relative concentration of amino acids can play an important role.

As a conclusion it seems that during maximally hCG-stimulated conditions both Glu and Pyr are obligatory culture medium components and the substrate shuttle metabolites generated from them probably maintain sufficient transport shuttle activities and thus overcome the metabolic effects of EtOH on the NAD(P)H/NAD(P)$^+$ ratios in different cell compartments of the Leydig cells.

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