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ETHANOL-INDUCED INHIBITION OF TESTOSTERONE BIOSYNTHESIS IN RAT LEYDIG CELLS: CENTRAL ROLE OF MITOCHONDRIAL NADH REDOX STATE

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Summary—The mechanisms by which ethanol (EtOH) inhibits the human chorionic gonadotropin (hCG)-stimulated testosterone synthesis was studied in isolated rat Leydig cells *in vitro*. EtOH inhibited steroidogenesis, but this inhibition was reversed by L-glutamate (Glu) and an uncoupler of the oxidative phosphorylation, 2,4-dinitrophenol (DNP). The mechanism of EtOH-induced inhibition was studied by measuring steroidogenic precursors and comparing them with the cytosolic and mitochondrial NADH redox states during uncoupling or in the presence of Glu. DNP had a dual effect. Low concentrations abolished the EtOH-induced inhibition of progesterone to testosterone formation suggesting that the inhibitory step was at or before progesterone formation. A large concentration led to an overall decrease in steroidogenesis indicating toxic effects on steroidogenesis. The mitochondrial NADH/NAD^+ ratio, measured as the 3-hydroxybutyrate/acetoacetate ratio, decreased simultaneously when steroidogenesis was stimulated, either during uncoupling or in the presence of Glu, whereas cytosolic NADH/NAD^+ ratio, measured as lactate/pyruvate ratio showed no response. These results demonstrate that the rise in the mitochondrial NADH/NAD^+ ratio rather than in the cytosolic one is connected with the inhibition of testosterone synthesis by EtOH in isolated Leydig cells. The EtOH-induced high mitochondrial NADH/NAD^+ ratio may deplete mitochondrial oxalacetate concentrations. This can decrease the activity of several transport shuttles and interrupt the flow of mitochondrial citrate into the smooth endoplasmic reticulum, which then reflects to decreased rate of steroidogenesis in the presence of ethanol.

INTRODUCTION

It is becoming generally accepted that ethanol (EtOH)-inhibition of testosterone biosynthesis in isolated Leydig cells *in vitro* results from its metabolic consequences, but the actual mechanisms are still unclear. We have previously reported that culture medium composition strongly affects this EtOH-induced inhibition of testosterone biosynthesis, and that L-glutamate (Glu) plays an important role effectively eliminating EtOH-induced inhibition of steroidogenesis when added to culture medium lacking this amino acid [1].

An EtOH-induced lack of NAD^+ [2–6] inhibiting the NAD^+-dependent conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase/oxosteroid isomerase (3βHSD), and decreased NADPH availability [7–9] for the NADPH-dependent conversion of androstenedione to testosterone by 17-ketosteroid reductase (17KSR) have been proposed to be the main inhibitory mechanisms taking place at the smooth endoplasmic reticulum (SER) of the Leydig cell. The increased NADH/NAD^+ ratio, caused by the cytosolic metabolism of EtOH by the alcohol dehydrogenase, can explain the inhibition at the first site, but the mechanism behind the inhibition at the latter step is obscure.

The acute effects of EtOH on NADPH-dependent microsomal drug detoxification reactions in the liver may be caused by interruption of the activities of the transport shuttles carrying NADPH reducing equivalents from mitochondria to cytosol (for review see [10, 11]). These substrate shuttles share many metabolites and transport systems with the malate–aspartate shuttle (MAS) which transports cytosolic NADH reducing equivalents to mitochondria (for review see [12, 13]). Thus, it seemed important to study whether the NADPH transporting shuttles in Leydig cells are controlled by the same mechanisms as MAS in the liver.

One major control site of EtOH metabolism in the liver is the transport of cytosolic NADH reducing equivalents to mitochondria by MAS. It has been shown that the high mitocondrial NADH/NAD^+ ratio, by controlling the mitochondrial oxalectate-to-malate ratio, adjusts the rate of MAS activity to the

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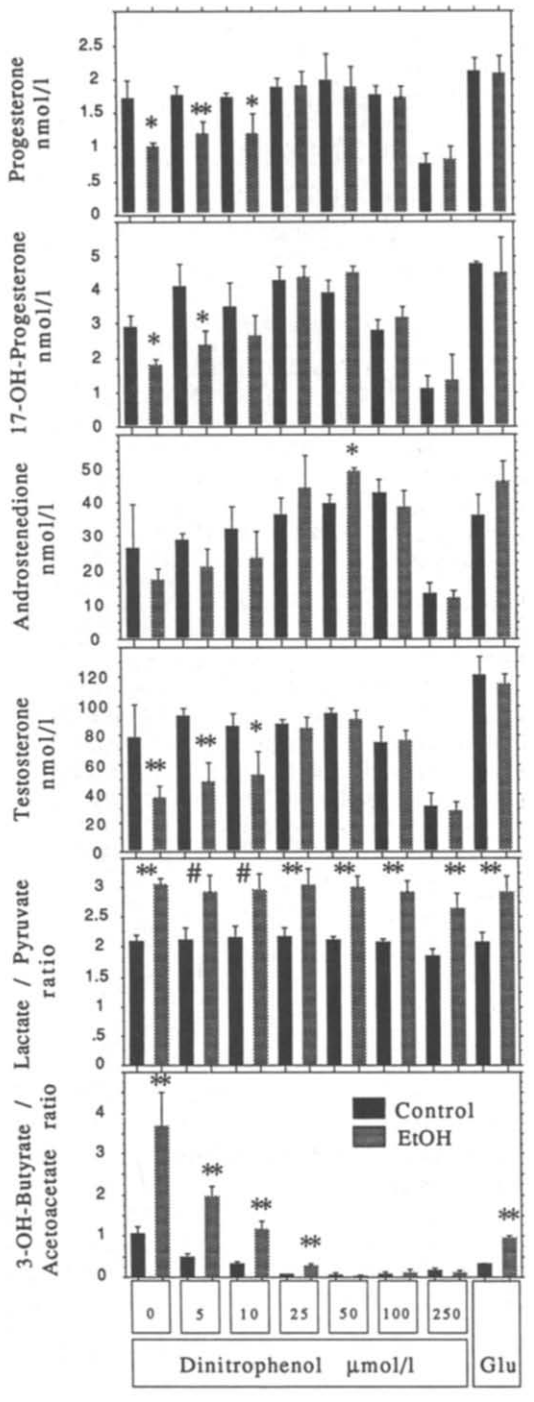

Fig. 1. Effects of 2,4-dinitrophenol or L-glutamate (Glu, 0.5 mmol/l) on the ethanol-induced (EtOH, 25 mmol/l) effects on progesterone, 17-OH-progesterone, androstenedione, and testosterone concentrations, and on the lactate/pyruvate and 3-OH-butyrate/acetoacetate ratios in the hCG-stimulated (10 mIU/ml, 3 h) rat Leydig cells. Values are mean ± SD of triplicate incubations. $^{**}P < 0.01$, $^{*}P < 0.02$, $^{*}P < 0.05$.

rate of respiration (for review see [12,13]), and that mitochondrial NADH reoxidation and EtOH metabolism can be stimulated by uncoupling the oxidative phosphorylation by DNP [14].

In the present work we have studied the roles of EtOH-induced increases in the cytosolic and mitochondrial NADH/NAD^+ ratios in control and Glu-treated conditions and with gradually increasing uncoupling by DNP. The enzymatic steps affected by EtOH were evaluated by measuring the steroidogenic precursors.

EXPERIMENTAL

Culture media and supplements

The incubation medium used in these experiments was Dulbecco's Modified Eagle Medium (DME), supplied by Sigma Chemicals (St Louis, Mo.). It was supplemented with L-lactate (Boehringer, Mannheim, F.R.G., 1 mmol/l), pyruvate-Na (Boehringer, 0.1 mmol/l) and bovine serum albumin (Sigma Chemicals, 1 mg/ml). L-Glutamate (free acid) was from Sigma Chemicals and 2,4-dinitrophenol was from E. Merck (Darmstadt, F.R.G.).

Preparation and incubation of Leydig cells

Preparation and purification of rat Leydig cells were performed as described earlier [1]. Collagenase dispersed and Percoll-purified rat Leydig cells were incubated under O_2–CO_2 (95:5) for 3 h at 33°C. Testosterone production was stimulated with hCG (Pregnyl, Organ, Oss, The Netherlands, 10 mIU/ml), and the EtOH concentration used was 25 mmol/l. Because the redox metabolites are labile, the incubations were stopped by dipping the tubes in liquid nitrogen and transferring them to −70°C. Just prior to metabolite determinations, the tubes were thawed and treated on ice with PCA and K_3PO_4 as described in [1].

Analytical procedures

Head-space gas chromatography with a Perkin–Elmer Sigma 2000 was used for ethanol determinations [15]. Pyruvate [16], lactate [17], acetoacetate [18], and 3-OH-butyrate [19] concentrations were determined fluorometrically with a Transcon 102 FN Analyzer (Elomit, Transcon Instruments Ltd, Helsinki, Finland). The acetoacetate and pyruvate concentrations were determined within 30 min after thawing the samples. Steroids were measured from the neutralized supernatants as described earlier [20] using chromatography on Lipidex-5000™ and antibodies with carefully-defined specificity.

Statistical analysis

Student's *t*-tests were used for comparisons of groups.

RESULTS

The concentrations of progesterone were decreased in the presence of EtOH (Fig. 1). This was in line with our previous findings [5] and indicates that the NAD^+-dependent 3βHSD reaction was inhibited by

EtOH also when DME culture medium containing pyruvate was used. This decrease was reversed by Glu or by uncoupling with 25–100 μmol/l DNP. The pronounced decreases in progesterone concentrations in the presence of 250 μmol/l DNP were associated with decreases in concentrations of all the steroids measured, indicating toxic effects on steroidogenesis.

EtOH lowered the concentrations of 17-OH-progesterone and this inhibition also was reversed by addition of Glu or uncoupling with DNP in concentrations of 25–100 μmol/l (Fig. 1).

As also shown in Fig. 1, there was a tendency for a decreased androstenedione concentration in the presence of EtOH alone, but it was not statistically significant. DNP, in concentrations of 25–100 μmol/l had an effect on androstenedione concentrations comparable to that on progesterone and 17-OH-progesterone concentrations. Addition of Glu also reversed the effect of EtOH.

EtOH lowered the testosterone concentrations (Fig. 1), whereas addition of Glu elevated testosterone production also in control incubations and reversed the inhibition effected by EtOH. The dose-dependent recovery from EtOH-induced inhibition with DNP until 50 μmol/l showed how uncoupling effectively prevented the effects of EtOH on testosterone production.

EtOH raised the cytosolic NADH/NAD$^+$ ratio, measured as lactate-to-pyruvate (L/P) ratio, and addition of Glu or uncoupling with DNP had no effect on this increase (Fig. 1). EtOH also raised the mitochondrial NADH/NAD$^+$ ratio, measured as 3-OH-butyrate-to-acetoacetate (B/A) ratio, as shown in Fig. 1. Supplementation with Glu or uncoupling with the same DNP concentrations that removed the inhibition of steroid production effectively reversed this EtOH-induced increase. These results demonstrate a parallel and dose-dependent recovery from EtOH-induced effects between the mitochondrial NADH-NAD$^+$ ratio and steroid biosynthesis in the presence of the uncoupler DNP. They also show a similar effect to be mediated by addition of Glu in the incubation medium. Table 1 shows the original concentrations of the redox metabolites, and it shows that the concentration of 3-OH-butyrate was most affected by uncoupling with DNP or addition or Glu.

DISCUSSION

The findings that EtOH decreased progesterone concentrations support earlier results which suggested that the raised NADH/NAD$^+$ ratio inhibited the NAD$^+$-dependent formation of this steroid [2–6]. The lowered progesterone concentrations were then probably reflected in the concentrations of progesterone metabolites 17-OH-progesterone, androstenedione and testosterone. Under the conditions used the androstenedione-to-testosterone inhibition site [7–9] was not detectable. When the progesterone concentrations were restored by DNP uncoupling or Glu supplementation, the rate of steroidogenesis increased and some evidence for the androstenedione-to-testosterone inhibition site became evident.

It had previously been assumed that EtOH inhibited steroidogenesis simply by increasing the cytosolic NADH-NAD$^+$ ratio which then directly inhibited progesterone formation. The present findings show that the effect is more complicataed and that the EtOH-induced high NADH/NAD$^+$ ratio in mitochondria, rather than in the cytosol, is connected with the inhibition of steroidogenesis in SER. The present results showing that the upward shift in the cytosolic free NADH/NAD$^+$ ratio (indicated as L/P ratio) was not connected with the fall in progesterone and testosterone concentrations can be explained only if the free NADH/NAD$^+$ ratio controlling 3βHSD reaction was not in rapid equilibrium with that of L/P. Also evidence for different cytosolic NAD(H) pools for lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) has previously been presented for the liver [21, 22].

Table 1. Effects of ethanol (25 mmol/l) on the concentrations of pyruvate, lactate, acetoacetate, and 3-hydroxybutyrate of the hCG-stimulated Leydig cells incubated in DME culture media containing the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (DNP), or L-glutamate (0.5 mmol/l)

DNP (mmol/l)	Group	Pyruvate (μmol/l)	Lactate (μmol/l)	Acetoacetate (μmol/l)	3-OH-butyrate (μmol/l)
0	Control	353 ± 16	742 ± 23	26.1 ± 3.4	26.9 ± 3.0
	Ethanol	274 ± 24	891 ± 32	10.9 ± 1.8	39.1 ± 3.2
0.005	Control	376 ± 20	804 ± 34	26.3 ± 3.0	13.5 ± 2.9
	Ethanol	287 ± 22	842 ± 10	14.6 ± 0.7	28.2 ± 4.5
0.01	Control	364 ± 20	791 ± 34	29.1 ± 1.2	9.3 ± 1.5
	Ethanol	281 ± 20	839 ± 22	19.3 ± 1.4	22.2 ± 1.9
0.025	Control	365 ± 21	799 ± 12	33.6 ± 1.3	3.6 ± 1.9
	Ethanol	276 ± 16	847 ± 35	31.0 ± 0.9	8.2 ± 2.1
0.05	Control	368 ± 17	796 ± 26	35.6 ± 3.4	3.1 ± 3.6
	Ethanol	271 ± 18	820 ± 20	36.5 ± 2.2	2.9 ± 3.2
0.1	Control	363 ± 19	757 ± 24	33.3 ± 4.4	4.7 ± 3.7
	Ethanol	273 ± 18	790 ± 31	33.1 ± 0.8	4.4 ± 3.1
0.25	Control	358 ± 18	672 ± 6	22.0 ± 1.6	5.2 ± 3.4
	Ethanol	267 ± 18	713 ± 13	21.9 ± 1.5	3.6 ± 2.6
L-Glutamate	Control	386 ± 19	804 ± 8	25.8 ± 2.1	8.4 ± 1.4
	Ethanol	290 ± 23	840 ± 8	21.2 ± 1.1	20.9 ± 1.1

Values are mean ± SD of triplicate incubations.

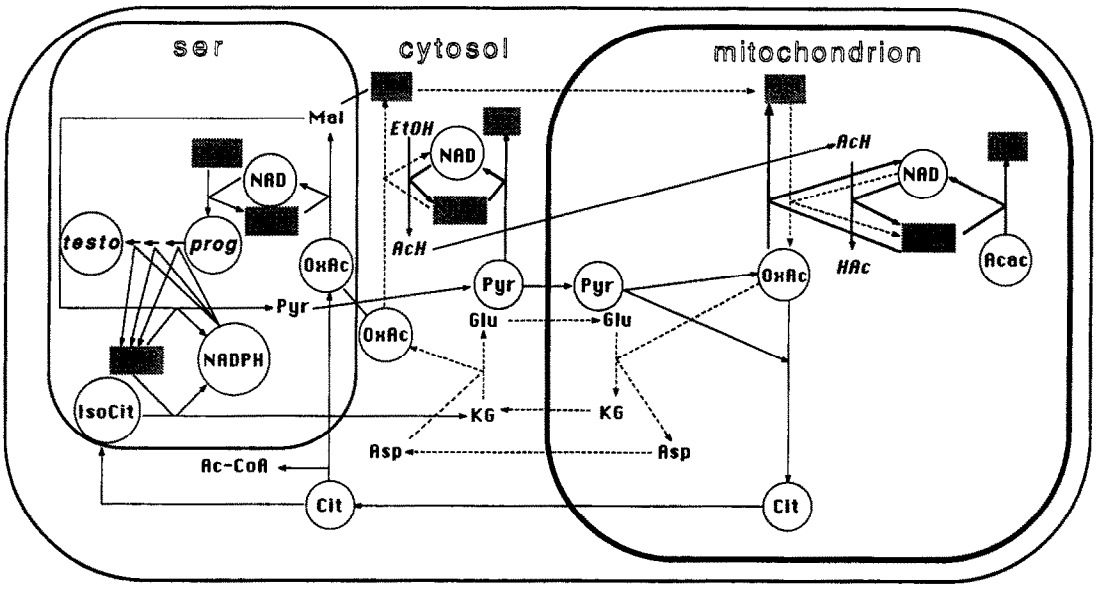

Fig. 2. The substrate shuttles transporting mitochondrial citrate to SER (→) and their connections to steroidogenesis, EtOH metabolism and the malate–aspartate shuttle (----→). Abbreviations: Acetoacetate (Acac), acetyl coenzyme-A (Ac-CoA), aspartate (Asp), citrate (Cit), L-glutamate (Glu), isocitrate (IsoCit), α-ketoglutarate (KG), lactate (Lac), malate (Mal), 3-OH-butyrate (OHB), oxalacetate (OxAc), pyruvate (Pyr) and smooth endoplasmic reticulum (SER). The grey backgrounds indicate EtOH-induced increases in metabolite concentrations and the concentrations of the encircled metabolites are supposed to decrease in the presence of EtOH metabolism.

It is generally accepted that the reducing equivalents produced during ethanol metabolism in the cytosol are primarily transported to mitochondria using MAS as depicted in Fig. 2 (for review see [12, 13]). This process increases the mitochondrial NADH/NAD^+ ratio and subsequently the NADH reoxidation in the respiratory chain. In addition, the mitochondrial NADH/NAD^+ ratio increases as a result of the mitochondrial oxidation of acetaldehyde [23]. The reason why Glu decreased the mitochondrial NADH/NAD^+ ratio could well be an increased NADH reoxidation as a result of increased MAS activity [24].

A suggestion for how the mitochondrial NADH/NAD^+ ratio could control the microsomal NAD^+-dependent 3βHSD reaction is outlined in Fig. 2. It is known that mitochondrial NADH production during EtOH oxidation may deplete mitochondrial oxalacetate, and subsequently citrate, concentrations in the liver (for review see [12, 13, 24]). The lowered citrate could in turn lead to decreased microsomal oxalacetate, malate, and NAD^+ formation in the reactions catalyzed by ATP–citrate lyase and MDH.

In the present results we found similarities to earlier hepatic mechanisms for how EtOH acutely inhibits the NADPH-dependent mixed function oxidations in SER (for review see [12, 13]). It has been proposed that this inhibitory mechanism could be an inhibition of the transport of mitochondrial NADPH as citrate to SER (for review see [12, 13]). The results of these liver studies can be summarized as follows: (1) The metabolism of EtOH is essential for the inhibition of mixed function oxidations. (2) The EtOH concentration needed for maximal inhibition in whole cell preparation is under 10 mmol/l, whereas in microsomal fraction, supplemented with an effective NADPH generating system, the EtOH concentration must be higher to inhibit mixed function oxidations. (3) Addition of Glu (at least partially) abolished the EtOH-induced inhibition of mixed function oxidations. (4) Total uncoupling inhibits, whereas slight uncoupling stimulates mixed function oxidations. These liver findings seem valid also for the EtOH-inhibition in the Leydig cells and so the actual inhibitory mechanisms involved could be related. (For references related to (1), see [2–5], for (2) see [3, 25]. Items (3) and (4) are supported by the present results.)

Experiments studying the mechanism by which EtOH inhibits NADPH-dependent drug metabolism in liver have shown that the pentose phosphate shunt is not the only source of NADPH in SER. Instead, the NADPH of mitochondrial origin, generated via the citric acid cycle and transported to SER using substrate shuttles is essential for the mixed function oxidations in intact hepatocytes [26]. The inhibition of drug metabolism by EtOH is tightly connected to the activity of these shuttles. Because mitochondrial membranes are impermeable to NADPH, there exist substrate shuttles transporting NADPH reducing equivalents from mitochondria to SER (for review see [12, 13]). This NADPH is generated by the malic enzyme system or the isocitrate dehydrogenase system. Malate can also be generated extramitochondrially from citrate using ATP–citrate lyase and

MDH reactions. This provides a way to connect NAD^+-dependent progesterone formation, and subsequent NADPH-dependent reactions in SER, with effective control by the mitochondrial NADH/NAD^+ ratio. Previous studies have shown a close relationship between the mitochondrial NADH/NAD^+ ratio and citrate production mediated by the oxalacetate concentrations [27]. They demonstrate that if the intramitochondrial oxalacetate concentrations decrease, the acetyl units from acetyl-CoA are directed to ketone formation instead of citrate synthesis (for review see [28]). It is probable that Glu (by providing shuttle metabolites [24]), or uncoupling activate the substrate shuttles transporting citrate from mitochondria to SER [for review see [12, 13]). The citrate can be cleaved to oxalacetate and acetyl-CoA or it can be converted to isocitrate for NADPH production. This locally-formed oxalacetate could support the 3βHSD reaction if MDH and 3βHSD shared the same NAD(H) pool in SER, as shown in Fig. 2. These mechanisms could also be involved in the inhibition of the NADPH-dependent reduction of androstenedione to testosterone. In addition, this inhibition site could be related to increased utilization of NADPH by the microsomal ethanol oxidizing system (MEOS) in Leydig cells [29].

The present results show that the steroidogenic pathways cannot be taken as isolated self-controlling systems located in mitochondria and SER. They share many metabolites and transport mechanisms with other active metabolic systems, such as MAS, and therefore all factors affecting any of these other biological systems can also affect steroidogenic pathways. Thus, the EtOH-induced NAD^+-dependent 3βHSD and NADPH-dependent 17KSR inhibitions are both suggested to be connected with the mitochondrial substrate shuttles under mitochondrial NADH-redox regulation, rather than being the result of a direct interaction between cytosol and SER.

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