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Acutely Administered Ethanol Participates in Testosterone Synthesis and Increases Testosterone in Rat Brain

Ahmed A. Alomary, Monique Vallée, Laura E. O'Dell, George F. Koob, Robert H. Purdy, and Robert L. Fitzgerald

Background: The interaction of alcohol and testosterone has long been of interest, mainly due to the effect of alcohol on aggression and sexual behavior. To date, there have been very few, if any, studies examining the effect of acute alcohol administration on testosterone concentrations in the brain. The administration of 1,1-dideuteroethanol ([1,1-$^2H_2$]ethanol) provided the opportunity to trace the deuterium label into newly synthesized deuterotestosterone in brain samples to determine whether ethanol oxidation was directly linked to testosterone synthesis.

Methods: Unoperated and adrenalectomized-gonadectomized (ADX/GDX) rats were given either ethanol or [1,1-$^2H_2$]ethanol in a single intraperitoneal dose of 2 g/kg body weight. We used gas chromatography/mass spectrometry to accurately determine both the amount of steroids present and the degree of deuterium incorporation into specific steroids isolated from brain samples.

Results: Thirty minutes after alcohol administration, the level of total testosterone increased 4-fold in the frontal cortex and 3-fold in the plasma of unoperated male Wistar rats. The relative increase in the abundance of monodeuterated testosterone 30 min after [1,1-$^2H_2$]ethanol administration was significant ($p < 0.05$) in both brain and plasma. ADX/GDX animals treated with alcohol had testosterone concentrations that were 5% of those found in unoperated animals dosed with ethanol.

Conclusions: Acutely administered ethanol increased brain concentrations of testosterone 4-fold in male Wistar rats. ADX/GDX surgery reduced brain concentrations of testosterone in response to alcohol by 95%. The deuterium labeling of testosterone after [1,1-$^2H_2$]ethanol showed that ethanol oxidation is directly linked to testosterone biosynthesis and that the deuterium-labeled testosterone is present in the central nervous system. These results demonstrate that peripherally administered ethanol directly contributes to the concentrations of testosterone in the central nervous system and that the testosterone found in brain samples is primarily synthesized in the periphery. These findings may be important for understanding the behavioral changes associated with acute alcohol consumption.

Key Words: Testosterone, Ethanol, 1,1-Dideuteroethanol, NCI-GC/MS, Adrenalectomized-Gonadectomized Rats.

THE INTERACTION OF alcohol and the male sex hormone testosterone has long been of interest because of the increased alcoholism in men versus women, the association of alcohol intoxication and violence, and the deleterious effect of alcohol abuse on male sexual function (Schuckit, 1998). The purpose of this study was to examine the effects of an acute dose of ethanol on the concentrations of testosterone in brain and plasma of alcohol-naïve male rats and to determine whether ethanol has a direct role in the biosynthesis of testosterone. The use of mass spectrometry allowed us to accurately determine both the amount of steroids present and the degree of deuterium incorporation after acute administration of 1,1-dideuteroethanol into specific steroids isolated from brain samples. On the basis of previous work by Sjövall and Cronholm (1970) that showed the transfer of deuterium from dideuteroethanol to androgens in the periphery (Cronholm et al., 1971), the working hypothesis of this study was that alcohol is involved in the biosynthesis of testosterone in male rats.

Previous studies have shown that alcohol generally decreases circulating testosterone levels in animals and humans (Cicero and Badger, 1977; Cicero and Bell, 1980; Eriksson et al., 1994; Orpana et al., 1990; Renshaw, 1985; Rivier, 1999). Rivier (1999) found that intraperitoneal (ip) administration of a 2 g/kg dose of ethanol decreased the serum level of human chorionic gonadotropin stimulated

From the Department of Neuropharmacology (AAA, MV, LEO, GFK, RHP), The Scripps Research Institute, La Jolla, California; Veterans Affairs Healthcare Center and Veterans Medical Research Foundation (AAA, RHP, RLF), San Diego, California; and the Departments of Psychiatry (RHP) and Pathology (RLF), University of California, San Diego, California.

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Reprint requests: George F. Koob, PhD, Department of Neuropharmacology, CVN-7, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037; Fax: 858-784-7405; E-mail: gkoob@scripps.edu.

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testosterone in male Sprague-Dawley rats. Cicero and Badger (1977) reported that low doses of ethanol (0.75 g/kg) significantly increased serum testosterone, whereas high doses (2.5 g/kg) decreased serum levels of testosterone, in male Sprague-Dawley rats. In humans, intoxicating doses of ethanol that resulted in blood alcohol concentrations of 109 mg/dl decreased plasma testosterone concentrations in men (Mendelson et al., 1977). To our knowledge, there are no similar studies examining the effect of ethanol on the formation or distribution of brain concentrations of testosterone. Measurement of testosterone in the brain is essential for understanding the behavioral consequences of this steroid after acute doses of ethanol.

METHODS

Animals

Male Wistar rats, both unoperated and adrenalectomized-gonadectomized (ADX/GDX), weighing 250 to 350 g, were purchased from Charles River Laboratory (Kingston, NY). The ADX/GDX surgery was performed by Charles River Laboratory; we obtained the animals 2 weeks after surgery. No salt supplementation was required to maintain the animals in a healthy condition during the 1-week quarantine period before experimentation. The ADX/GDX group was designed to provide a model in which the normal sources of peripheral testosterone were removed. The animals were group-housed (three per cage) with food and water available ad libitum in a temperature-controlled vivarium. The animals were acclimated to the handling procedure once before the test day. The lights were on a 12-hr light/dark cycle, with lights on at 6:00 AM. Thirty unoperated rats and 24 ADX/GDX rats were used in this study. We did not have a sham-operated group because the ADX/GDX animals were dosed 3 weeks after surgery. At this time, the effect of the stress induced by the surgery would have dissipated (Ogilvie and Rivier, 1997). All animal procedures were performed in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Treatment

USP alcohol (95%) was diluted in saline (0.9% sodium chloride) and administered ip as a 20% (w/v) solution at a dose of 2 g/kg. 1,1-Dideuteroethanol (98% d_2) also was diluted in saline (0.9% sodium chloride) and administered ip as a 20% (w/v) solution at a dose of 2 g/kg. This moderately high dose of ethanol has been used in several recent studies of neurosteroid concentrations in the brain of male rats (Barbaccia et al., 1999; VanDoren et al., 2000). Unoperated animals were divided into 3 groups: 12 animals were given 2 g/kg of ethanol in saline, 6 animals were given saline, and 12 animals were given 2 g/kg of deuterated ethanol in saline. ADX/GDX animals were divided into 2 groups of 12 animals, each treated with either 2 g/kg of ethanol or 2 g/kg of deuterated ethanol. This was an intoxicating dose of ethanol in most animals, as demonstrated by their ataxia. The animals were killed by decapitation 30 min after alcohol treatment. The 30-min time period was chosen because it was the time of maximally increased plasma cortisol in men acutely administered ethanol and also because it had been used in several other recent brain neurosteroid studies after acute doses of ethanol (Barbaccia et al., 1999; VanDoren et al., 2000). Blood was collected from the trunk in ethylenediaminetetraacetic acid–coated tubes and centrifuged at $1000 \times g$ for 10 min, and the plasma samples were stored at −70°C. The brains were removed and dissected on ice. A coronal section was made on the ventral surface of the brain at the level of the optic chiasm. The frontal cortex then was taken (with the underlying striatal and corpus callosum tissue removed), placed in a preweighed vial, and frozen on dry ice. Samples were kept frozen at −70°C until analysis. The frontal cortex was chosen because numerous studies have demonstrated neurochemical changes in response to acutely administered ethanol (Fadda et al., 1980; Hegarty and Vogel, 1993; Morrow et al., 2001).

Blood Alcohol Level (BAL)

A 200-μl aliquot of serum was stored at −20°C until assayed. Serum was analyzed for BAL by use of an Analox instrument (Lunenburg, MA). We demonstrated that the calibration for ethanol quantification was identical when either ethanol or deuteroethanol was used.

Steroid Analysis

The steroids pregnenolone, allopregnanolone, and testosterone were extracted from both plasma and brain tissue by a simple solid-phase extraction method (Vallée et al., 2000). This method was validated in terms of sensitivity, accuracy, and precision. Run-to-run precision and accuracy were within ±20%. Briefly, the method uses negative chemical ionization gas chromatography/mass spectrometry and involves the formation of pentafluorobenzyloxime/trimethyl silyl ether derivatives of the steroid fraction of brain or plasma extracts to enhance the chromatographic and mass spectrometric analysis. Mass spectra were acquired with a Finnigan TSQ-7000 mass spectrometer (Thermo Finnigan, San Jose, CA). The mass spectrometer was operated in a selective ion-monitoring mode, allowing for picograms of neurosteroids to be quantified from biological extracts (Alomary et al., 2001). The isotope-dilution method was used to achieve accurate quantification. Testosterone-16,16,17-d_3 from MCD Isotopes (Montreal, Canada) was used as the internal standard. The procedure was suitable for measuring concentrations of endogenous neurosteroids in rat plasma and cortex and for measuring the relative amounts of the free neurosteroids and their monodeuterated isomers. In these experiments, testosterone-16,16,17-d_3 was used as an internal standard to quantify both testosterone and pregnenolone, because testosterone-d_3 does not have a significant amount of testosterone-17-d_1, whereas pregnenolone-d_3 contains significant percentages of d_1 isomers. For testosterone, the peak areas of the [536 mass/charge (m/z) ion]/(535 m/z ion) were used to calculate the (A + 1)/A ratio. For pregnenolone, the peak areas of the (567 m/z ion)/(566 m/z ion) were used to calculate the (A + 1)/A ratio.

Testosterone-17-d_1 was prepared by reduction of androstenedione with one equivalent of sodium borodeuteride in deuteroethanol (CH_3CH_2OD) at 0°C for 30 min, followed by treatment with dilute acetic acid, dilution with water, extraction of the product with ethyl acetate, and crystallization of the product.

Statistical Analysis

Results are expressed as mean ± SEM. The magnitude of the effect of alcohol on the steroid level was defined as the change in concentration of the steroid in nanograms per milliliter for plasma and per gram for brain tissue observed after alcohol administration. The significance of results was determined by Student's t test ($p < 0.05$).

RESULTS

Figure 1 shows that in both plasma and brain cortex, concentrations of testosterone increased approximately 4-fold 30 min after an acute dose of ethanol, compared to saline control. Figure 1 also shows that in ADX/GDX animals 30 min after acute alcohol administration, there was a low, but detectable, amount of testosterone in brain cortex (mean ± SEM, 0.05 ± 0.01 ng/g). Figure 1 clearly shows that most of the testosterone found in the brain is derived from the testes and adrenal gland, because ADX/

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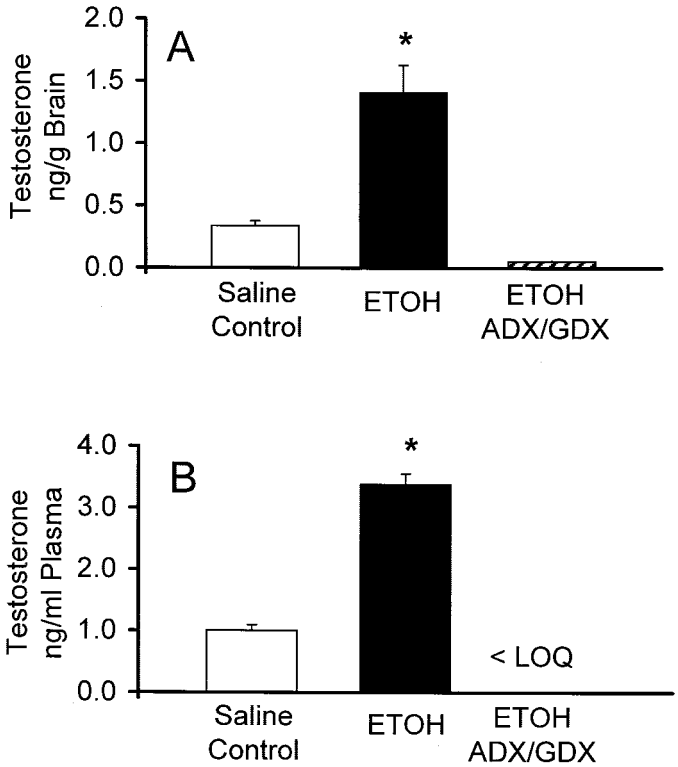

Fig. 1. Total testosterone concentration 30 min after acute alcohol (ETOH) administration (2 g/kg) in controls (n = 6), unoperated animals (n = 12), and ADX/GDX animals (n = 12), as determined by gas chromatography/mass spectrometry from extracts of (A) frontal cortex and (B) plasma. The limit of quantification (LOQ) for testosterone in plasma was 0.05 ng/ml. The asterisks (*) indicate significant difference from saline control.

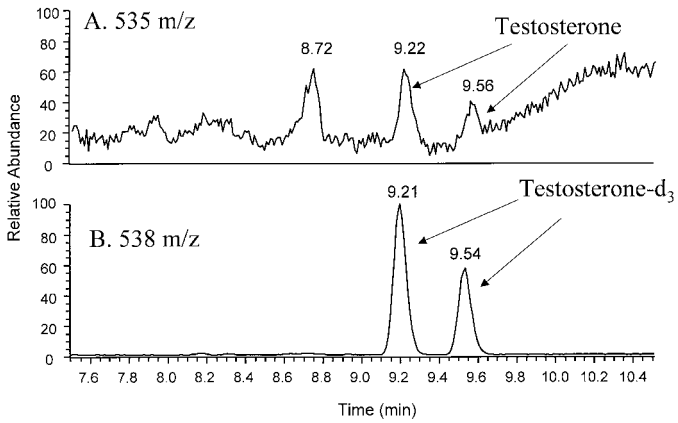

Fig. 2. Chromatogram obtained by selected ion monitoring of pentafluorobenzyloxime/trimethyl silyl ether derivatives of testosterone and testosterone-16,16,17-d_3 ions of m/z 535.2 and 538.2, respectively, obtained with an extract of 400 mg of frontal cortex from an ADX/GDX animal 30 min after acute alcohol administration (2 g/kg). The retention times of the *syn* and *anti* isomers of testosterone were 9.22 and 9.56 min. Dehydroepiandrosterone elutes at 8.72 min.

GDX treatment reduced testosterone concentrations by 95%.

Figure 2 shows a selected ion monitoring trace of the 535 and 538 m/z ion channels of a brain extract from an ADX/GDX animal. The 535 ion channel represents endogenous testosterone, and the 538 ion channel is the exogenously added internal standard. The *syn* and *anti* isomers of the pentafluorobenzyl/trimethyl silyl derivative of the internal standard testosterone-16,16,17-d_3 elute at 9.21 and 9.54 min in the 538 m/z ion channel (Fig. 2B). In the 535 ion channel, the two distinct testosterone peaks were detected in brain samples of ADX/GDX animals (Fig. 2A), indicating the presence of endogenous testosterone in these animals.

Table 1 shows the average BALs 30 min after dosing in each group of the animals used in this study. There was a significantly lower (by 34%) average BAL in ADX/GDX animals, compared to unoperated animals. This suggests that ADX/GDX animals had an increased rate of ethanol metabolism, which is consistent with previous studies demonstrating that castration increases the activity of alcohol dehydrogenase in the liver (Cicero et al., 1980; Mezey et al., 1980).

To demonstrate that our method was suitable for measuring the relative-ratio deuterium/hydrogen isotopes in steroids, the precision of the assay was evaluated by using mixtures of testosterone and testosterone-17-d_1. The precision of measuring specific m/z values with the gas chromatography/mass spectrometry procedure used for plasma and brain samples had a coefficient of variation of 2.8% (0.5 ng of testosterone injected).

Table 2 shows the incorporation of deuterium into steroids isolated from cortex and plasma after ethanol or deuterated ethanol in unoperated rats (i.e., not ADX/GDX). After deuterated ethanol administration to unoperated animals, a significant amount of the deuterium was incorporated into testosterone. The concentration of testosterone in ADX/GDX animals was at the detection limit of our assay; therefore, it was not possible to determine whether excess deuterium was incorporated in these animals.

Table 2 demonstrates that the incorporation of deuterium into testosterone does not result from a generalized increase in deuterium, because pregnenolone shows no deuterium incorporation. There was also a lack of incorporation of deuterium into allopregnanolone after the administration of deuterated ethanol (data not shown). The data shown in Table 2 also indicate that deuterium was incorporated into the testosterone found in plasma.

DISCUSSION

We report a 4-fold increase in total testosterone in brain 30 min after acute ethanol administration (2 g/kg ip). No significant circulating testosterone was found in ADX/GDX animals, and only a small amount of testosterone was identified in brain tissue. After the administration of 1,1-dideuteroethanol, a significant amount of deuterium was incorporated into testosterone. The increase in testosterone observed in Wistar rats could result in part from a decrease in the catabolism of testosterone in the liver (Sarkola et al., 2001), from increased conversion of andro-

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Table 1. Blood Alcohol Levels Measured 30 min After Treatment

Variable	Treatment				
	Saline	Ethanol 2 g/kg	Deuteroethanol 2 g/kg	Ethanol 2 g/kg	Deuteroethanol 2 g/kg
Animal type	Unoperated	Unoperated	Unoperated	ADX/GDX	ADX/GDX
Mean ± SEM (mg/dl)	<LOQ	202 ± 6	177 ± 5	128 ± 6	121 ± 3

LOQ, limit of quantification.
n = 6 animals for saline controls; n = 12 animals for all other groups.

Table 2. Incorporation of Deuterium Into Testosterone and Pregnenolone (Unoperated Animals)

Tissue	Isolated steroid	Mean [(A + 1)/A] ± SEM		p Value
		EtOH	Deuterated EtOH	
Cortex	Testosterone	0.351 ± 0.003	0.395 ± 0.011	<0.001
Plasma	Testosterone	0.361 ± 0.002	0.414 ± 0.017	<0.001
Cortex	Pregnenolone	0.378 ± 0.005	0.377 ± 0.009	0.76
Plasma	Pregnenolone	0.349 ± 0.015	0.345 ± 0.011	0.83

n = 12 animals at each data point.
The p value refers to the difference between ethanol and deuterated ethanol treatments for either the cortex or plasma.

stenedione to testosterone in the liver (Martel et al., 1992), or from increased synthesis in the testes. In this respect, there is approximately 0.1 ng/ml of androstenedione in the peripheral venous blood of adult male Wistar rats, and it is largely of testicular origin (Blacker et al., 1991). Thus, a combination of factors may be involved to explain the increase in testosterone to approximately 3.5 ng/ml in plasma.

In male Sprague-Dawley rats, acutely administered ethanol generally causes a decrease in plasma testosterone; an acute ethanol dose (1.5 to 2 g/kg) decreased testosterone concentrations at 1 to 3 hr after the dose (Cicero and Badger, 1977; Rivier, 1999). These differences from our study could be the result of strain differences. In a preliminary experiment with male Sprague-Dawley rats acutely treated with ethanol, we found a marked decrease in testosterone levels in the frontal cortex. This preliminary result in Sprague-Dawley animals is consistent with other reports that analyzed plasma testosterone (cited previously) and suggests that our results with Wistar rats, which showed an increase of testosterone after acutely administered ethanol, are due to a species difference.

In contrast to most published reports showing a decrease in testosterone in human and rat males after acute ethanol, Sarkola et al. (2001) found that in premenopausal women, ethanol resulted in a marked increase in plasma testosterone. This increase in plasma testosterone was interpreted as a decrease in the oxidation of testosterone to androstenedione in the liver, which was caused by an alcohol-induced increase in the ratio of reduced nicotinamide adenine dinucleotide to oxidized nicotinamide adenine dinucleotide (NAD^+). The increase in testosterone that we observed in Wistar rats could be due in part to a similarly decreased conversion of testosterone to androstenedione by the liver, but the specific incorporation of deuterium into testosterone in our experiments argues that newly synthesized testosterone is also significant in the animals we studied. It therefore seems that there is a polymorphic response to acutely administered ethanol that depends on gender and species.

The mechanism for ethanol's participation in the synthesis of testosterone may involve its well-established effects on the redox state of the liver (Andersson et al., 1986; Cronholm and Sjövall, 1970). 1,1-Dideuteroethanol oxidation by alcohol dehydrogenase leads to an accumulation of reduced cofactors and an increase in the ratio of deuterium-labeled reduced NAD (NAD^2H) to NAD^+ (Fig. 3A). The cofactor NAD^+ is reduced during the oxidation of ethanol through a transfer of deuterium on the C1 carbon of 1,1-dideuteroethanol. The increase in NAD^2H is the driving force for subsequent reduction reactions. The reduced deuterium-labeled pyridine dinucleotides NAD^2H and $NADP^2H$ are interconvertible through malate or citrate pathways in the liver (Cronholm et al., 1976). These authors concluded that 1,1-dideuteroethanol is suitable for studying a variety of compounds that use NAD^2H or $NADP^2H$ as cofactors, including the androgens at C17. $NADP^2H$ then causes an increase in the stereospecific reduction of androstenedione to testosterone (Fig. 3C) by 17β-hydroxysteroid dehydrogenase (17β-HSD) (Labrie et al., 1997).

To determine the role of ethanol in the biosynthesis of testosterone, we measured the A and A + 1 ion intensities after administering 1,1-dideuteroethanol. The A ion is the mass of the ion composed of the isotopes with the lowest mass (e.g., carbon-12, hydrogen-1, nitrogen-14, and so on), for example, the 535 m/z testosterone ion (Fig. 2). The term "A + 1" refers to the natural abundance of heavier isotopes of elements such as carbon (e.g., carbon-13 at 1.1%) and silicon (silicon-29 at 5.1%), for example, the 536 m/z testosterone ion. The (A + 1)/A ratio for testosterone (i.e., the peak area of the 536 m/z ion relative to the peak area of the 535 m/z ion) in the absence of an extra source of deuterium is expected to be approximately 0.37, because we measured testosterone as the pentafluorobenzyl/trimethyl silyl derivative with a molecular formula of $C_{29}H_{38}F_5SiNO_2$. The A + 1 and A peaks for testosterone were measured, and the (A + 1)/A ratio was determined to be 0.351 ± 0.003 after unlabeled ethanol administration. If deuterium was incorporated from deuterated ethanol, then the (A + 1)/A ratio was expected to increase, because the deuterium contributes to the A + 1 peak. Indeed, after a dose of deuterated ethanol, the (A + 1)/A ratio for testosterone increased to 0.395 ± 0.011. By using successive approximations, it was estimated that approximately 5% of

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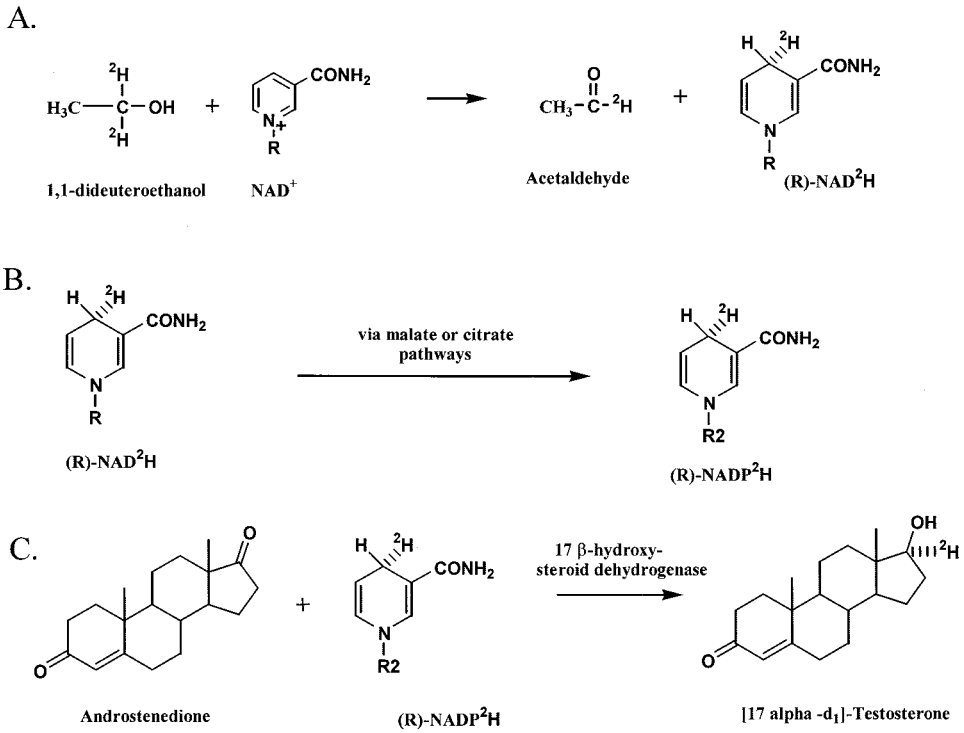

Fig. 3. Metabolic pathway for the formation of 17-monodeuterotestosterone from 1,1-dideuteroethanol.

the testosterone molecules incorporated one deuterium 30 min after treatment with deuterated ethanol. This presumably reflects the relative percentage of $NADP^2H$ in the total pool of reduced pyridine nucleotides present in vivo at this time point.

To demonstrate that the incorporation of deuterium into testosterone was not due to random incorporation in steroids that increased after ethanol, we also measured the (A + 1)/A ratio for pregnenolone. Because pregnenolone is formed from cholesterol by side-chain cleavage and not by reduction of a ketosteroid precursor, we expected no change in the (A + 1)/A ratio for pregnenolone; this was confirmed. The results of the experiment with deuterated ethanol indicate that ethanol oxidation is directly linked to testosterone formation.

17β-HSD modulates the biological activity of androgens by catalyzing reactions between 17-keto- and 17β-hydroxysteroids (Nokelainen et al., 1998). Martel et al. (1992) studied the tissue distribution of 17β-HSD activity in the male rat and found significant enzyme activity in the order of liver > testes > adrenals > brain, and so on. These authors clearly demonstrated that liver tissue homogenates converted androstenedione into testosterone and that the activity of 17β-HSD for this conversion was approximately 30-fold higher in the liver than in testis. Martel et al. concluded that the "widespread distribution of 17β-HSD in rat clearly indicates the importance of this enzyme in peripheral sex steroid formation."

Norsteen-Hoog et al. (1992) investigated the transfer of deuterium from 1,1-dideuteroethanol to steroids in isolated rat testis; no deuterium was found in pregnenolone or testosterone. The transfer of deuterium from 1,1-dideuteroethanol to testosterone requires a change in the redox state of the oxidized nicotinamide adenine dinucleotide phosphate system, as shown in Fig. 3. In addition, to observe an increase in deuterium incorporation, it is necessary that there also be a significant increase in newly synthesized testosterone. In male Sprague-Dawley rats, acutely administered ethanol generally causes a decrease in testosterone (Cicero and Badger, 1977; Rivier, 1999); consequently, no incorporation of deuterium would be expected in this species.

These findings may be relevant to the behavioral changes associated with alcohol consumption. Ethanol was found to increase plasma levels of testosterone and aggression in male adolescent hamsters (Ferris et al., 1998). It also has been found that increased testosterone levels altered behavioral sensitivity to ethanol in mice (De Bold and Miczek, 1985). How these animal studies translate to the human condition remains to be determined, but our results provide new insights of critical importance to understanding alcohol/neuroactive steroid interactions that may have implications for individual differences in the behavioral and endocrine pathology associated with alcohol abuse.

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