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Sex-Dependent Induction of Alcohol Dehydrogenase Activity in Rats

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ABSTRACT. The glycolethers 2-methoxyethanol (2-ME), 2-ethoxyethanol (2-EE), and 2-butoxyethanol are widely used organic solvents with teratogenic, spermatotoxic, and hematotoxic effects due to the respective alkoxyacetic acid metabolites formed via alcohol dehydrogenase (ADH). ADH displays sexually dimorphic activities in adult rats, and is probably at least in part under the control of testosterone. The aim of this study was to investigate whether induction of ADH is also sex-dependent. Ethanol, 2-ME, and 2-EE were tested as inducers of hepatic and gastric ADH in female, male, and castrated male rats. The activity of hepatic ADH was higher in female than in male rats, while the activity of gastric ADH was higher in male than in female rats. The activities of ADH increased with increasing chain length of the glycolethers and alcohols. Castration of male rats led to a female pattern of ADH activity, i.e. increased activity of hepatic ADH and decreased activity of gastric ADH. Ethanol had no inducing effect on hepatic ADH in either male or female rats. 2-ME and 2-EE caused an increase in the activity of hepatic ADH in male and castrated male rats only. The present data demonstrate a different expression of ADH isoenzymes in male and female rats, and a sex-dependent induction of ADH isoenzymes. The different possible regulatory mechanisms for the different ADH isoenzymes require further investigation. BIOCHEM PHARMACOL **57**;9:1067–1072, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 2-methoxyethanol; 2-ethoxyethanol; ethanol; sex difference; induction; gastric alcohol dehydrogenase; hepatic alcohol dehydrogenase

ADH§ is a pivotal enzyme in the metabolism of glycolethers such as 2-ME, 2-EE, and 2BE. The glycolethers are widely used organic solvents with teratogenic, spermatotoxic, and hematotoxic effects due to the respective alkoxyacetic acid metabolites formed via ADH [1–16].

Three isoenzymes of ADH have been detected in rat tissues [17]. The liver contains two of these, an anodic isoenzyme, ADH-2, and a cathodic isoenzyme, ADH-3. ADH-3 has a broad substrate specificity towards short-, medium-, and long-chain alcohols, and accounts for most ethanol oxidation *in vivo*. ADH-2 is found in all rat tissues, and catalyzes the metabolism of medium- and long-chain primary alcohols, probably related to a role in metabolism of endogenous alcohols and aldehydes. The highest concentrations of the anodic isoenzyme ADH-1 are found in organs in immediate contact with the exterior, i.e. the cornea, the stomach and the lung, indicating that ADH-1 may play a role as the first metabolic barrier against external alcohols and aldehydes [17].

ADH appears to be one of several enzymes that displays sexual dimorphism, with higher activities in rat and human female livers [18–20]. Gastric ADH activity in humans is also sex-dependent at high ethanol concentrations, but with higher activities in males [21]. We recently showed that 2-ME, 2-EE, and 2-BE are metabolized via ADH-3, and that *in vitro* hepatic glycolether metabolism via ADH was higher in female rats [22], corroborating *in vivo* findings of a higher rate of 2-ME elimination in female rats [23].

A number of xenobiotics induce metabolizing enzymes by stimulation of the synthesis of specific proteins [24, 25]. Sex differences in induction are seen where xenobiotic metabolism itself is sexually differentiated [26]. ADH displays sexually dimorphic activities in adult rats, but induction studies with ethanol [27–29] and 2-ME [30] have only been conducted in male rats. It is therefore not known whether induction of the enzyme is also sex-dependent. We herein report data addressing this question using ethanol, 2-ME, and 2-EE as inducers of hepatic and gastric ADH in female, male, and castrated male rats.

MATERIALS AND METHODS

Chemicals

Dormicum® was obtained from Roche and Hypnorm® from Janssen Pharmaceutica. 2-ME (min 99.5%) and 2-BE (>99%) were obtained from Merck. 2-EE (>99%) was obtained from Fluka AG. Ethanol (96% v/v) was obtained

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§ *Abbreviations:* ADH, alcohol dehydrogease; 2-ME, 2-methoxyethanol; 2-EE, 2-ethoxyethanol; and 2-BE, 2-butoxyethanol.

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from Vinmonopolet. NAD was obtained from the Sigma Chemical Co. Other chemicals used were of reagent grade.

Animals

Sixty-five male (157–319 g) and 52 female (145–210 g) Wistar rats from Charles River Wiga GmbH were maintained on a 12-hr dark/12-hr light cycle with a relative humidity of 50% and room temperature of 21 ± 1°. Rats were fed on Rat and Mouse No. 1 Maintenance Diet (Special Diet Services Limited) and given water *ad lib*. In all instances, animals were allowed free access to food and water throughout the course of the experiments.

This study was carried out according to the laws and regulations controling experiments and procedures in Norway, i.e. the "Animal Protection Act" of December 20th 1974, the "Animal Protection Ordinance concerning Biological Experiments in Animals" of December 22nd 1977, and "The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" of March 18th 1986.

Under Dormicum®/Hypnorm® (midazolam/fentanyl/fluanison) anesthesia (1.80/2.50/3.75 mg/kg), 19 rats were castrated via scrotal incision. After ligation of the spermatic cord, testicle and epididymis were en bloc removed bilaterally. After surgery, all animals were allowed to recuperate for one week.

Induction Studies

The solutions for infusion contained ethanol (5.6 M), 2-EE (4.4 M), and 2-ME (5.3 M) in 0.9% NaCl. All rats were weighed every day prior to the injections. The volumes injected were 0.3–0.8 mL. To keep the number of rats used in the induction study to a minimum, a pilot study was conducted using ethanol, 2-ME, and 2-EE as inducers of hepatic ADH in male and female rats. We observed that ethanol had no inducing effect on hepatic ADH, and 2-EE had an effect comparable to that of 2-ME. Therefore, we decided to use only 2-ME in the remaining study. The results from both studies are presented below. To examine whether the infusion of solutions or handling of the rats affected the experiment, hepatic and gastric ADH activity was determined in 5 female, 5 male, and 5 castrated male untreated rats.

Exposures for Ethanol and 2-EE

Ethanol (22.7 mmol/kg) and 2-EE (7.8 mmol/kg) were administered as daily intraperitonial injections for 7 days. Five male and 7 female rats were exposed to ethanol, and 3 female and 3 male rats were exposed to 2-EE. Five male and 5 female rats were administered 0.5 mL 0.9% NaCl i.p. per day for 7 days, and served as control groups.

Exposures to 2-ME

2-ME (9.2 mmol/kg) was administered as daily i.p. injections in 10 male and 13 female rats for 7 days. Fourteen male and 14 female rats were administered 0.5 mL 0.9% NaCl i.p. per day for 7 days, and served as control groups. Ten castrated male rats were administered 2-ME i.p. for 7 days, and 9 castrated male rats serving as the control group received 0.5 mL 0.9% NaCl i.p. per day for 7 days.

Homogenate Preparations

The animals were killed by decapitation 24 hr after the last i.p. injection, and the livers and stomachs immediately removed and cleaned in 0.9% sodium chloride (4°). The organs were cut into small pieces, transferred to a Teflon-to-glass homogenizer (B. Braun Melsungen), and homogenized at 4°. Livers were homogenized in 10 mL 0.1 M PBS pH 7.4, and stomachs in 4 mL 10mM Tris–HCl pH 7.9.

The homogenate was centrifuged at 20,000 *g* for 20 min and the supernatant fraction was then centrifuged for 1 hr at 100,000 *g*. The 100,000 *g* supernatant (cytosol) was used for ADH assays after dialysis overnight against 0.1 M PBS pH 7.4 (livers) and 10 mM Tris–HCl pH 7.9 (stomachs). All steps were carried out at 4°.

Determination of Alcohol Dehydrogenase Activities

ADH activity was determined at room temperature at 340 nm using an ATI Unicam UV/VIS Spectrometer. ADH activity was assayed in 3-mL cuvettes containing 0.1 M glycine-NaOH buffer pH 10.4, 1.3 mM NAD, and 0.6–1.4 mg protein (20 μL of the liver cytosol and 130 μL of stomach cytosol). The reaction was started by the addition of 2-ME, ethanol, or octanol as the enzyme substrate. In the liver ADH assay, the concentration of 2-ME in the cuvette was 20 mM. In stomach ADH assay, the following concentrations were used: 200 mM 2-ME, 200 mM 2-EE, 200 mM ethanol, and 1 mM octanol. Specific activities were expressed as the rate of NADH formation per min per mg protein. Protein concentrations in cytosol were determined by the Lowry method [31].

Starch Gel Electrophoresis

Electrophoresis of liver homogenates was carried out using horizontal gels containing 13% starch (Sigma) for 24 hr at room temperature according to the method of Smithies [32]. A voltage gradient of 8.8 V/cm was applied, giving a current of about 20 mA. The buffer system used was tris/phosphate pH 7.4, 0.1 M for the electrode compartment and 0.01 M in the gels.

The gel was split along its length in the horizontal plane, and the two gel slices were separated and cut into smaller parts parallel to the length of the gel. The gel slices were stained for liver ADH activity by incubating the gels for approximately 1 hr at room temperature in 30 mL 0.1 M

TABLE 1. Relative body weight gain in female, male, and castrated male rats treated for 7 days with ethanol (22.7 mmol/kg), 2-ME (9.2 mmol/kg), or 2-EE (7.8 mmol/kg)

	Relative body weight gain (%)			
	NaCl	Ethanol	2-ME	2-EE
Female	7.8 ± 3.4*	6.0 ± 1.6	6.0 ± 2.3	9.7 ± 1.5
Male	14.0 ± 2.1	7.2 ± 3.4†	4.7 ± 5.5†	13.4 ± 0.6
Castrated male	9.8 ± 3.3*		5.4 ± 2.1†	

As controls, female, male, and castrated male rats were administered 0.5 mL 0.9% NaCl for 7 days.

*Indicates a significant difference from the male control value.

†Indicates a significant difference from the respective control value.

glycine-NaOH buffer pH 10.4, containing 10 mg NAD, 1 mg *N*-methylphenazonium methosulfate, 10 mg of nitroblue tetrazolium, and 20 mM of the different alcohols or glycolethers. The gel slices were stained for stomach ADH activity by essentially the same procedure, but with different concentrations of the substrates: 200 mM 2-ME, 200 mM ethanol, and 1 mM octanol. The substrate-dependent reduction in NAD was coupled to the reduction in nitroblue tetrazolium to give an insoluble purple formazan at the zones of enzyme activity.

Statistical Analysis

The results are given as means ± SD. Statistical analysis was performed by Two-Sample analyses for paired samples using Statgraphics® (Statistical Graphics Corporation). Values were considered statistically significant at $P < 0.05$.

RESULTS

Weight Gain

Table 1 shows the relative increase in body weight of female, male, and castrated male rats treated for 7 days with NaCl, 2-ME, 2-EE, and ethanol. Ethanol and 2-ME reduced the weight gain for male and castrated male rats, whereas 2-EE had no effect on weight gain.

Hepatic ADH

After electrophoresis in tris/phosphate buffer, the liver homogenate from male control rats and rats treated with 2-ME revealed an enzymatically active cathodal zone on staining with 2-ME and ethanol as substrate, identical to the ADH-3 isoenzyme described by Julià *et al.* [17] (Fig. 1). The electrophoresis revealed both a cathodal and an anodal active zone on staining with octanol as substrate, identical to the ADH-3 and ADH-2 isoenzymes, respectively [17] (Fig. 1). This pattern was identical in liver homogenates from male and female rats (not shown).

An initial experiment was carried out to test the effect of time and handling on hepatic ADH activity. After housing untreated and unhandled rats, hepatic ADH activity did not differ from ADH activity in the corresponding group of rats treated with 0.5 mL 0.9% NaCl i.p. per day for 7 days (Table 2). There was a higher specific activity of hepatic ADH in female as compared to male rats (Table 2). Ethanol had no inducing effect on hepatic ADH in male or female rats. Neither 2-ME nor 2-EE had an effect on the specific activity of hepatic ADH in female rats. In male rats, 2-ME and 2-EE induced an increase in the specific activity of ADH (Table 2).

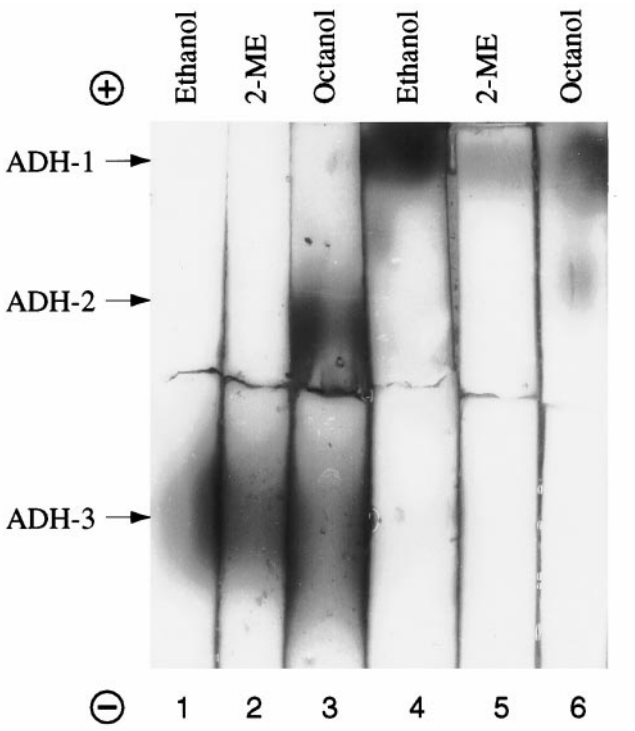

FIG. 1. Starch gel electrophoresis of male rat liver (lanes 1–3) and stomach homogenate (lanes 4–6) at pH 7.4 and 8.8 V/cm over 24 hr. Lanes 1 and 4 were stained with ethanol, lanes 2 and 5 with 2-ME, and lanes 3 and 6 with octanol.

Castration of male rats resulted in an increase in the specific activity of hepatic ADH as shown in Table 2. The activity of hepatic ADH with ethanol as substrate in control male rats was 16.8 ± 2.2 nmol NADH/min/mg prot, and in control castrated rats 23.2 ± 2.1 nmol NADH/min/mg protein. Treatment of male castrated rats with 2-ME for 7 days resulted in an additional increase in the activity, from 23.2 ± 2.1 to 30.9 ± 5.3 nmol NADH/min/mg protein.

In a previous study in our laboratory, we reported K_m and V_{max} values for liver ADH with ethanol and the glycolethers as substrates [22]. Induction of hepatic ADH in male rats resulted in an increase in V_{max}, but not in K_m (data not shown).

Gastric ADH

After electrophoresis in Tris/phosphate buffer, the stomach homogenate revealed an enzymatically active zone on staining with ethanol and 2-ME as substrates (Fig. 1). This

TABLE 2. Specific activity of hepatic ADH in male, castrated male, and female untreated rats, control rats, and rats treated with ethanol (22.7 mmol/kg), 2-ME (9.2 mmol/kg), or 2-EE (7.8 mmol/kg) for 7 days

			nmol/NADH/min/mg prot			
	Substrate	Untreated	NaCl	Ethanol	2-ME	2-EE
Female	Ethanol		29.8 ± 4.3*	38.4 ± 11.5	30.6 ± 5.0	27.7 ± 4.2
	2-ME	21.5 ± 3.5*	20.9 ± 4.6*	23.9 ± 5.9	20.7 ± 4.0	19.7 ± 3.6
Male	Ethanol		16.8 ± 2.2	16.8 ± 4.5	28.5 ± 5.6†	25.2 ± 4.3†
	2-ME	12.0 ± 1.7	10.4 ± 2.0	11.5 ± 4.7	20.5 ± 7.6†	14.8 ± 2.7†
Castrated male	Ethanol		23.2 ± 2.1*		30.9 ± 5.3†	
	2-ME	17.5 ± 4.6*	16.3 ± 1.7*		21.1 ± 3.8†	

As controls, female, male, and castrated male rats were administered 0.5 mL 0.9% NaCl for 7 days. The activity is expressed as nmol NADH/min/mg protein with ethanol (20 mM) and 2-ME (20 mM) as substrates.

*Indicates a significant difference from the respective male value.

†Indicates a significant difference from the respective control value.

pattern was identical in stomach homogenates from male and female rats (not shown). This ADH isoenzyme migrates toward the anode and is identical to the ADH-1 isoenzyme described by Julià *et al.* [17]. On staining with octanol as substrate, the stomach homogenate revealed two enzymatically active anodic zones, identical to the ADH-1 and ADH-2 isoenzymes (Fig. 1).

An initial experiment was carried out to test the effect of time and handling on gastric ADH activity as well. After housing untreated and unhandled rats, gastric ADH activity did not differ from ADH activity in the corresponding group of rats treated with 0.5 mL 0.9% NaCl i.p. per day for 7 days (Table 3). The specific activities of gastric ADH increased with increasing chain length of the glycolethers and alcohols (Table 3). Ethanol and the glycolethers are poor substrates for this isoenzyme. The stomach homogenate from both male and female rats showed the highest specific activity of ADH with octanol. The specific activity of stomach ADH was higher in male than in female rats. The sex difference was only significant with octanol as substrate, as shown in Table 3. Castration of male rats resulted in a decreased activity of ADH, significant only with octanol as substrate. Table 3 shows that treatment with 2-ME had no effect on the specific activity of gastric ADH.

TABLE 3. Specific activity of gastric ADH in male, castrated male, and female untreated rats, control rats, and rats treated with 2-ME (9.2 mmol/kg) for 7 days

			nmol/NADH/min/mg prot	
	Substrate	Untreated	NaCl	2-ME
Female	Ethanol		11.0 ± 5.5	12.7 ± 2.8
	2-ME	0.9 ± 1.0	0.9 ± 0.4	0.5 ± 0.4
	Octanol		19.8 ± 9.2*	21.6 ± 12.2
Male	Ethanol		20.5 ± 7.6	21.0 ± 5.6
	2-ME	1.0 ± 0.5	1.5 ± 0.5	1.7 ± 0.5
	Octanol		47.1 ± 11.1	51.3 ± 10.2
Castrated male	Ethanol		12.9 ± 2.3	21.1 ± 9.4
	2-ME	0.9 ± 0.4	0.9 ± 0.2	1.6 ± 0.9
	Octanol		26.4 ± 6.3*	33.1 ± 20.1

As controls, female, male, and castrated male rats were administered 0.5 mL 0.9% NaCl for 7 days.

The activity is expressed as nmol NADH/min/mg protein with 2-ME (200 mM), ethanol (200 mM), and octanol (1 mM) as substrates.

*Indicates a significant difference from the respective male control value.

DISCUSSION

In this study, we report for the first time a sex-dependent induction of ADH. The specific activity of hepatic ADH was induced following treatment with 2-ME or 2-EE in male rats only. Castration of male rats, known to reduce serum levels of testosterone by approximately 95% after 7 days [33], affected hepatic and gastric ADH activity, and led to a female pattern of ADH activity.

The starch gel electrophoresis of rat liver and stomach homogenates demonstrated the existence of different ADH isoenzymes in liver and stomach. ADH-1 is present in stomach, ADH-3 in liver, and ADH-2 in both liver and stomach. Neither hepatic nor gastric ADH activity was affected by infusion of solutions or handling of the rats.

The glycolethers were poor substrates for the gastric ADH-1 isoenzyme, and as with the hepatic ADH-3 isoenzyme, the substrate specificity increased with increasing chain length of the glycolethers and alcohols. We could not detect significant activity of the gastric ADH isoenzyme with 20 mM of the different glycolethers, and were not able to determine kinetic constants for the glycolethers because we could not reach saturation even at high concentrations (150–200 mM) of substrate.

In contrast to hepatic ADH activity, gastric ADH activity was higher in male rats compared to female rats with octanol as substrate. There was no sex difference with ethanol or glycolethers as substrate. Gastric ADH activity in humans measured at 16 mM ethanol was not affected by age and sex, although both factors did influence ADH activity measured at 580 mM ethanol, with lower gastric ADH activity in females than in males [21]. Different from ethanol and the glycol ethers, octanol is a substrate for both ADH-1 and ADH-2 in the stomach, and the observed sex difference in gastric activity in this study might be due to a sex difference in ADH-2 activity.

Our data indicate a testosterone-dependent regulation of ADH isoenzymes and different regulatory mechanisms for the different ADH isoenzymes. However, there seems to be

no direct effect of androgens in controling liver xenobiotic metabolism [34]. The pattern of growth hormone secretion is influenced by sex steroids, and the result is a different secretion pattern of the hormone in males and females which could be important for observed sex difference in xenobiotic metabolism and body weight gain [35–38]. A female plasma growth hormone pattern has been shown to increase the transcription of the rat class I ADH gene [39]. Our understanding is that rat class I ADH is identical to rat ADH-3 isoenzyme; higher basal amounts of growth hormone can, then, explain higher ADH-3 activity in the liver from female than male rats. In most of these studies, only hepatic ADH in male rats was investigated. However, our data indicate that the different ADH isoenzymes have different regulatory mechanisms.

Castration of male rats and subsequent treatment with ethanol or 2-ME resulted in a significantly slower weight gain compared to controls in this study. Pampori *et al.* [36] have shown that body weight gain in male and female rats is determined by the plasma growth hormone patterns and that a masculine pattern of growth hormone results in a greater weight gain. As feminizing regimens, such as castration of male rats, were associated with a female pattern of growth hormone in plasma [35], this could explain why castrated male rats in our study exhibited a weight gain comparable to female rats. Acute ethanol administration to male rats resulted in a reduction in both serum testosterone and serum growth hormone [40], and could thus explain the reduced weight gain in male rats in our study. There is currently nothing in the literature concerning the effect of glycolethers on serum growth hormone, although it has been reported that treatment of male rats with 2-ME resulted in reduced body weight gain [30].

The mechanisms for the induction of ADH are complex. Hepatic ADH was not induced following treatment with ethanol, but treatment with 2-ME or 2-EE resulted in a significant induction of hepatic ADH in male and castrated male rats, in line with other studies [27–30]. Ethanol did not induce hepatic ADH in male Wistar rats, but did in male SH rats [41]. The increase in hepatic ADH in the male SH rat in the latter study was explained by a decreased plasma testosterone concentration caused by ethanol [42]. However, ethanol reduced plasma and testicular testosterone contents in ethanol-treated male Wistar rats without inducing testicular ADH [29]. Enzyme induction is defined as the enhanced activity of enzymes due to an increase in the rate of synthesis of the enzyme. However, due to lack of pure enzyme, we were not able to establish the mechanism behind increased activity of ADH following treatment with 2-ME and 2-EE. The increased activity may be a combination of induction, stabilization of ADH against degradation, and an indirect effect via hormones.

In conclusion, the present data demonstrate a different expression of two ADH isoenzymes in male and female rats as well as a sex-dependent induction of ADH isoenzymes. The specific activity of hepatic ADH is higher in females than in males, whereas the specific activity of gastric ADH is higher in males than in females. Hepatic ADH is induced in male and castrated male rats following treatment with 2-ME and 2-EE. No induction was observed in female rats. The different possible regulatory mechanisms for the different ADH isoenzymes require further investigation.

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