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Modulation of prolactin binding sites in vitro by membrane fluidizers. Effects on male prostatic and female hepatic membranes in alcohol-fed rats

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The objectives of this study were (i) to determine if in vivo administration of ethanol to rats produced changes in apparent lipid fluidity and prolactin binding capacity of male prostatic and female hepatic membranes and (ii) to compare the effects of membrane fluidizers (aliphatic alcohols) in vitro on prolactin binding of prostatic and hepatic membranes in control and alcohol-fed animals. In vitro ethanol has been shown by us previously to increase prolactin receptor levels presumably by unmasking cryptic prolactin receptors. The degree of fluidization was monitored by a fluorescence polarization method using 1,6-diphenylhexatriene. Adult male and female rats were given either water or 4% ethanol as the sole source of drinking fluid for a period of 6 weeks. No significant changes in plasma prolactin were observed between control and ethanol-treated groups of either sex. However, the microviscosity parameter, inversely related to lipid fluidity, was increased approx. 34% and 40%, respectively, in male prostatic and female rat hepatic membranes after ethanol feeding. Furthermore, ^{125}I-prolactin binding capacity was decreased approx. 30% and 26%, respectively, in prostatic and hepatic membranes of alcohol fed animals. In vitro treatment with aliphatic alcohols had no effect on either microviscosity or prolactin binding in hepatic or prostatic membranes from ethanol-fed rats, but both fluidized and increased prolactin binding in the same membrane preparations from control rats. Our observations are consistent with the direct relationship between membrane fluidity and prolactin receptor levels. The changes in prostatic and hepatic membranes after alcohol feeding, namely decreased prolactin receptor levels, decreased fluidity and increased resistance to the fluidizing effects of in vitro aliphatic alcohols may reflect a fundamental membrane defect.

Introduction

Ethanol is known to produce non-specific membrane fluidizing effects that are similar to those of the anesthetic agents [1]. Earlier studies by other investigators have suggested that mammalian cells may adapt to chronic alcohol in their environment by changing the make up of the membrane lipid bilayer and in so doing, overcome the fluidizing effect of the drug [1]. Furthermore, these changes in membrane lipid are thought to alter the physical properties of the membrane.

In recent studies on murine hepatic and rat prostatic membranes the factors known to modulate prolactin binding in vitro and in vivo were also reported to alter membrane lipid microviscosity [2–5]. These and other studies have suggested that changes in membrane lipid microviscosity may alter prolactin and other peptide hormone receptor levels, possibly by modifying the physical nature of the surrounding lipid bilayer [6–8]. In a recent

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study we also reported that aliphatic alcohols, known to fluidize certain membranes, increased in vitro the apparent fluidity and prolactin binding capacity of prostatic membranes in a dose-dependent and age-dependent manner [9,10]. Presumably, this technique increases the availability of cryptic prolactin receptors [9,10].

The studies reported herein were undertaken to (1) document changes in the lipid microviscosity and prolactin binding capacity of rat prostatic and female rat hepatic membranes following prolonged alcohol-ingestion, and (2) compare the effect of membrane fluidizers (aliphatic alcohols) on membrane lipid microviscosity and prolactin binding capacity in control and alcohol-fed rats.

Materials and Methods

Animals. Sprague-Dawley male and female rats (80–100 days old), obtained from Charles River Breeding Laboratories, were maintained in our colony in a 12 h light-dark cycle with water (controls) or 4% ethanol (alcohol-fed) and Purina rat chow available ad libitum. The period of treatment was six weeks.

Reagents. Ovine prolactin (oPRL, NIH-P-S-13, 35 IU/mg), obtained from the National Pituitary Agency, was dissolved in 0.01 M NH_4OH in an ice bath and then diluted in 25 mM Tris/0.9% NaCl at pH 7.4.

Tissue preparation. After decapitation, the ventral prostate glands from male and liver from female rats were excised, frozen in liquid nitrogen, and pulverized. The tissues were homogenized for 1 min using a Brinkmann Polytron set at 6 in approx. 10 vol. of 0.3 M sucrose buffered to pH 7.6 with 25 mM Tris-HCl. The supernatant from a 10 min preliminary $125 \times g$ centrifugation was recentrifuged at $15\,000 \times g$ for 20 min to obtain a membrane pellet. This membrane pellet ($15\,000 \times g$) of ventral prostate exhibited higher prolactin binding (per mg protein) than the whole homogenate, or $125 \times g$ or $100\,000 \times g$ pellets [5,11]. On the basis of marker enzyme analysis, this membrane fraction of ventral prostate gland was found to be rich in both Golgi and plasma membranes with some mitochondrial contamination (Dave, J.R. and Witorsch, R.J., unpublished data). In female liver, the supernatant fluid obtained from $15\,000 \times g$ centrifugation was further centrifuged at $100\,000 \times g$ for 1 h to obtain the hepatic microsomal fraction, which has been reported to exhibit higher prolactin binding than the other membrane fractions.

Prolactin binding assay. Ovine prolactin was iodinated with ^{125}I (Amersham) by a modification of the lactoperoxidase method of Thorell and Johansson [5,12]. The specific activity of iodinated prolactin was 97 μCi/μg. The membrane pellet was resuspended in 10 mM $MgCl_2$/25 mM Tris at pH 7.6 to provide approx. 3 mg protein/ml [13]. When indicated membranes were exposed to aliphatic alcohols in two ways, either by coincubation with ligand or preincubation prior to the exposure of ligand. 100 μl of membrane suspension was incubated overnight at room temperature with varying concentrations of ethanol, 1-propanol or 1-butanol and 75 000 to 85 000 cpm of ^{125}I-labeled ovine prolactin with and without unlabeled ovine prolactin in a final volume of 0.5 ml of buffer (10 mM $MgCl_2$/0.1% bovine serum albumin/25 mM Tris-HCl, pH 7.6). In other experiments 100 μl of membrane suspension was exposed to the varying concentrations of alcohols for 15 min at room temperature in 0.5 ml of buffer (25 mM Tris-HCl, pH 7.6) and washed free of alcohols (by spinning the tubes at 2500 rpm for 10 min and washing the pellets twice with 1.0 ml buffer) prior to the addition of ligand. We previously reported that both methods of fluidizing membranes produced essentially identical results on prostatic prolactin receptors [9,10]. The binding assay was terminated by adding 1.0 ml of chilled buffer. The tubes were centrifuged at 2500 rpm for 20 min. The pellets were washed with an additional 1 ml of buffer and counted in a Nuclear-Chicago gamma counter. Each sample was assayed in triplicate. The diminution of radioactivity by coincubation with 1 μg unlabeled ovine prolactin represented specificially bound hormone. Under these assay conditions we routinely obtained 25–35% total and 6–12% non-specific binding of added iodinated prolactin to the prostatic and hepatic membranes. Scatchard analysis was performed by incubating the iodinated ovine prolactin with varying amounts of unlabeled hormone (0 to 1000 ng) [14].

Radioimmunoassay of prolactin. All prolactin

samples were assayed in a single radioimmunoassay (RIA). The prolactin RIA was conducted with the kit provided by the NIAMDD Rat Pituitary Hormone Distribution Program. The reference preparation was NIAMDD Rat Prl RP-1. The intra-assay coefficient of variation was 2.5% at 1.0 ng/tube.

Membrane microviscosity. The fluorescence polarization technique, with 1,6-diphenylhexatriene was used for derivation of the steady-state polarization constant, P, and microviscosity parameter [15,16]. Membrane suspensions were incubated at 24°C with an equal volume of dispersion of $2 \cdot 10^{-6}$ M 1,6-diphenylhexatriene in phosphate-buffered saline at pH 7.1 for 45 min. Alcohols were added for a further 15 min of incubation. The unincorporated 1,6-diphenylhexatriene was removed by two washes with phosphate-buffered saline. This treatment also removed the alcohols whose presence otherwise might contribute some probe partitioning effects on the steady-state polarization measurements. Steady-state polarization measurements were made at 25°C on a SLM subnanosecond fluorometer (4800 series). The ratio output ($I_{\parallel}/I_{\perp}$) and $I_{\perp}$ from the photomultiplier tubes were entered directly into a 9815 Hewlett-Packard calculator for programming. $I_{\perp}$ and $I_{\parallel}$ are the fluorescence intensities polarized, respectively, perpendicular and parallel to the polarization of the excitation beam [16]. Appropriate measures were taken to avoid light scattering and signal-to-noise effects on the polarization measurements. The values of steady-state polarization were used to determine the microviscosity parameter defined as $((r_o/r)-1)^{-1}$, where r is the value of the anisotropy calculated from the measured polarization constant, P, according to the equation $r = 2P/(3-P)$ and r_o is the limiting value of the anisotropy of 1,6-diphenylhexatriene [16] which in the present study was taken as 0.362. Data presented in the text are expressed both as P values and as 'microviscosity parameter' $((r_o/r)-1)^{-1}$, for comparison with other systems.

Results

No significant effects on circulating levels of prolactin were detected in male or female rats after 6 weeks ingestion of 4% alcohol. In male rats, control levels of prolactin were 40.5 ± 7.8 ng/ml (range 20.0–93.7 ng/ml, $n = 8$) and alcohol-fed levels were 38.2 ± 11.0 ng/ml (range 14.6–112.1 ng/ml, $n = 8$). In female rats, control levels were 84.0 ± 20.0 ng/ml (range 18.4–241.9 ng/ml, $n = 11$) and alcohol-fed levels were 56.5 ± 15.5 ng/ml (range 21.3–136.3, $n = 7$).

Table I shows that alcohol ingestion increased membrane microviscosity (polarization constant and microviscosity parameter) in prostatic and hepatic membranes obtained from alcohol-fed animals. Alcohol feeding increased the microviscosity parameter of prostatic and hepatic membranes 34% and 40%, respectively. Table II shows that alcohol ingestion decreased prolactin binding capacity of prostatic and hepatic membranes in the rat approximately 38% and 26%, respectively.

Table II also presents the Scatchard analysis of ^{125}I-labeled ovine prolactin binding data for prostatic and hepatic membrane preparations obtained from control and alcohol-fed animals. The total number of prostatic prolactin binding sites decreased 34% in alcohol-fed male rats, whereas in hepatic membranes, the total number of prolactin binding sites decreased 31% in alcohol-fed female rats. No apparent effect of alcohol-feeding on the

TABLE I

DEMONSTRATION THAT ALCOHOL INGESTION INCREASED THE APPARENT MICROVISCOSITY OF PROSTATIC AND FEMALE HEPATIC MEMBRANES IN THE RAT

Steady-state polarization, proportional to the apparent microviscosity, was measured at 25°C using 1,6-diphenylhexatriene. The microviscosity parameter was calculated according to Ref. 16, as described in the text. Each value represents the mean of 7–15 animals assayed individually in triplicate ± S.D. Values in parentheses are percent of control values. Values marked with asterisk are significantly different from control values at $p < 0.005$ (*) or $p < 0.001$ (**).

Sex	Group	Polarization constant, P	Microviscosity parameter $((r_o/r)-1)^{-1}$)
M	control	0.162 ± 0.004	0.461 ± 0.007
	alcohol-fed	0.194 ± 0.009* (120)	0.618 ± 0.017** (134)
F	control	0.225 ± 0.006	0.811 ± 0.011
	alcohol-fed	0.263 ± 0.008* (117)	1.132 ± 0.015** (140)

TABLE II

DEMONSTRATION THAT ALCOHOL INGESTION DECREASED THE APPARENT NUMBER OF PROLACTIN BINDING SITES IN MALE PROSTATIC AND FEMALE HEPATIC MEMBRANES IN THE RAT

Male and female rats were given either water (control) or 4% ethanol (alcohol-fed) as the sole source of drinking fluid for a period of 6 weeks. Scatchard analysis of ^{125}I-labeled ovine prolactin binding was carried out as described in the text according to Ref. 14. Values for specific binding are means ± S.D. of ten determinations each assayed individually in triplicate. Values of Scatchard analysis are means ± S.D. of four Scatchard plots each assayed individually in duplicate. Values in parentheses are percent of control values. Values marked with asterisk are significantly different from control values at $p < 0.001$ (*), $p < 0.025$ (**) or $p < 0.05$ (***). $^{+}$ values not significantly different.

Sex	Group	^{125}I-prolactin specific binding (cpm/mg protein)	Number of prolactin binding sites (fmol prolactin per mg protein)	Affinity constant, K_a (nM^{-1})
M	control	32853 ± 3133	58 ± 6	7.6 ± 1.5
	alcohol-fed	20369 ± 3053* (62)	38 ± 4** (66)	7.4 ± 0.9^{+} (97)
F	control	85212 ± 4653	70 ± 9	9.0 ± 1.0
	alcohol-fed	62998 ± 5053* (74)	48 ± 6*** (69)	9.3 ± 0.8^{+} (103)

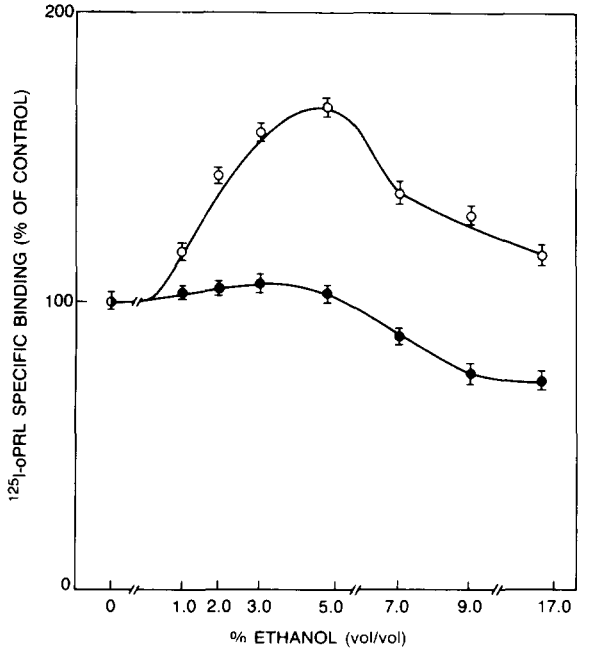

Fig. 1. A representative experiment showing that membrane fluidizers in vitro produced different effects on specific prolactin binding of prostatic membranes obtained from control (open circles) or alcohol-fed (filled circles) male rats. Alcohol-fed rats received 4% ethanol in drinking water for a period of 6 weeks. For in vitro study, ethanol was coincubated overnight with membranes and ^{125}I-labeled ovine prolactin (^{125}I-oPRL) as described in the text. Each point represents mean ± S.D. of triplicate determinations.

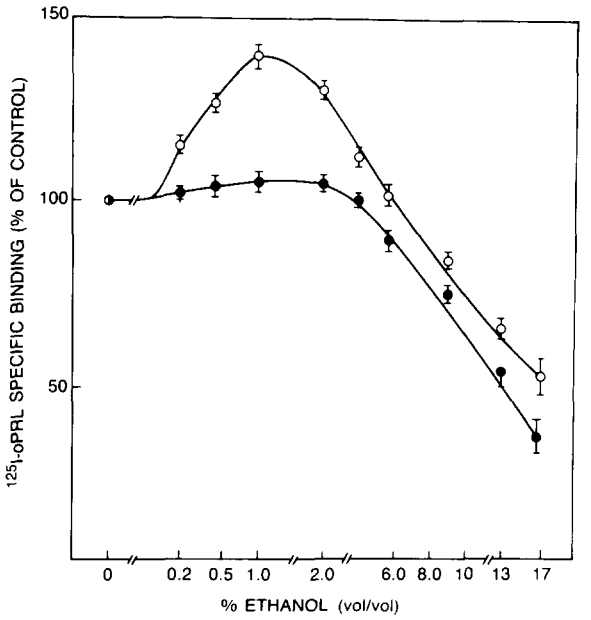

Fig. 2. A representative experiment showing that membrane fluidizers in vitro produced differential effects on specific prolactin binding of hepatic membranes obtained from control (open circles) or alcohol-fed (filled circles) female rats. Alcohol-fed rats received 4% ethanol in drinking water for a period of 6 weeks. For the in vitro study, ethanol was coincubated overnight with membranes and ^{125}I-labeled ovine prolactin (^{125}I-oPRL) as described in the text. Each point represents mean ± S.D. of triplicate determinations.

affinity constant, K_a, of prolactin binding was demonstrable (Table II).

Fig. 1 shows that overnight exposure of prostatic membranes to ethanol in vitro along with ^{125}I-labeled ovine prolactin produced different effects on prolactin binding in control and alcohol-fed rats. In alcohol-fed rats, in vitro ethanol at concentrations ranging from 1.0% to 4.8% had no significant effects on specific prolactin binding. Higher concentrations of ethanol progressively decreased specific prolactin binding in this group. However, in control rats, ethanol produced a dose-dependent increase and decline in prolactin binding of prostatic membranes. In several different experiments a maximal increase in prolactin binding (of approx. 65% in control rats) was observed at 4.8% ethanol.

1-Propanol at concentrations ranging from 1% to 16.7% and 1-butanol at concentrations ranging from 1% to 9% produced a progressive decline in prolactin binding to prostatic membranes obtained from alcohol-fed animals and a dose-dependent increase and decline in the same in control rats (data not shown). A maximal increase in prolactin binding of approximately 52% and 44% was observed in control rats at 3.8% 1-propanol and 1.0% 1-butanol, respectively (data not shown). Consistent with our previous report [9], a 15 min exposure of prostatic membranes to the above optimal concentrations of aliphatic alcohol (4.8% ethanol, 3.8% 1-propanol, or 1.0% 1-butanol), prior to the addition of ^{125}I-labeled ovine prolactin, produced essentially the same changes in prolactin binding as reported above for the experiment in which ethanol and prolactin are incubated together (data not shown).

Fig. 2 shows that overnight incubation of female rat hepatic membranes with ^{125}I-labeled ovine prolactin and varying concentrations of ethanol produced changes in specific prolactin binding which were similar to that observed in prostatic membranes. Ethanol at concentrations ranging

TABLE III

EFFECTS OF IN VITRO EXPOSURE OF MALE PROSTATIC AND FEMALE HEPATIC MEMBRANES OBTAINED FROM CONTROL OR ALCOHOL-FED RATS TO ALIPHATIC ALCOHOLS ON STEADY-STATE POLARIZATION AND MICROVISCOSITY PARAMETER

The membrane preparations were exposed to various concentrations of aliphatic alcohols, as described in the text, for 15 min at 25°C. Steady-state polarization, proportional to the apparent microviscosity, was measured at 25°C using 1,6-diphenylhexatriene. Microviscosity parameter was calculated according to Ref. 16, as described in the text. Each value represents the mean of five animals each assayed individually in triplicate ± S.D. Values in parentheses are percent of control values.

Sex	Group	Treatment (in vitro)	Polarization constant, P	Microviscosity parameter $((r_o/r)-1)^{-1}$
M	control	none	0.160 ± 0.005	0.452 ± 0.009
		ethanol 4.8%	0.137 ± 0.002 (86)	0.359 ± 0.004 (79)
		propanol 3.8%	0.148 ± 0.003 (93)	0.401 ± 0.006 (89)
		butanol 1.0%	0.150 ± 0.002 (94)	0.410 ± 0.004 (91)
	alcohol-fed	none	0.198 ± 0.008	0.640 ± 0.015
		ethanol 4.8%	0.192 ± 0.004 (97)	0.607 ± 0.007 (95)
		propanol 3.8%	0.199 ± 0.003 (101)	0.646 ± 0.006 (101)
		butanol 1.0%	0.197 ± 0.003 (99)	0.635 ± 0.006 (99)
F	control	none	0.221 ± 0.005	0.784 ± 0.009
		ethanol 1.0%	0.199 ± 0.004 (90)	0.646 ± 0.007 (82)
		propanol 0.75%	0.205 ± 0.003 (93)	0.681 ± 0.006 (87)
		butanol 0.2%	0.210 ± 0.004 (95)	0.712 ± 0.007 (91)
	alcohol-fed	none	0.260 ± 0.009	1.102 ± 0.017
		ethanol 1.0%	0.255 ± 0.005 (98)	1.054 ± 0.009 (96)
		propanol 0.75%	0.259 ± 0.004 (100)	1.092 ± 0.007 (99)
		butanol 0.2%	0.262 ± 0.005 (101)	1.122 ± 0.009 (102)

from 0.2% to 4.0% had no significant effects on specific prolactin binding to hepatic membranes obtained from alcohol-fed female rats, whereas, higher ethanol concentrations progressively decreased specific prolactin binding in this group. However, in control rats, ethanol produced a dose-dependent increase and decline in prolactin binding of hepatic membranes. In several different experiments a maximal prolactin binding increase (approx. 45% in control female rats) was observed at 1.0% ethanol.

1-Propanol at concentrations ranging from 0.2% to 10% and 1-butanol at concentrations ranging from 0.05% to 5% produced a progressive decline in prolactin binding to hepatic membranes obtained from alcohol-fed female rats and a dose-dependent increase and decline in the same in control rats (data not shown). A maximal increase in prolactin binding of approximately 35% and 30% was observed in control female rats at 0.75% 1-propanol and 0.2% 1-butanol, respectively. As seen for prostatic membranes, a 15 min exposure of hepatic membranes to the above optimal concentrations of aliphatic alcohol (1.0% ethanol, 0.75% 1-propanol and 0.2% 1-butanol) prior to the addition of ^{125}I-labeled ovine prolactin mimicked the effects seen above in the coincubation experiment.

Table III shows the effect of in vitro treatment of alcohols on apparent microviscosity expressed both as the measured values of steady-state polarization and calculated values of microviscosity parameter $((r_o/r) - 1)^{-1}$ in control and alcohol-fed rats. Exposure of prostatic and hepatic membranes of control rats to optimal aliphatic alcohol concentrations decreased membrane microviscosity. However, the same treatment had little or no effect on microviscosity in prostatic and hepatic membranes from alcohol-fed rats.

Discussion

This study shows that chronic ethanol ingestion increased lipid microviscosity and decreased prolactin receptor number, without affecting affinity constants, of male prostatic and female hepatic membranes. Furthermore, membranes from alcohol fed rats resisted the fluidizing effects and concomitant enhancement of prolactin binding activity of in vitro treatment with aliphatic alcohol. Estimates of lipid microviscosity on crude membrane preparations employed in this and earlier studies [2–6] represent averaged measures of changes throughout the membranes. However, these changes in specific or localized domains within the membranes may be more extensive than the averaged estimates detected with crude membrane fractions.

Our demonstration of increased microviscosity of prostatic and female rat hepatic membranes in alcohol-fed animals is in agreement with the findings of Johnson et al. [20] who reported that reconstituted membranes prepared from lipid extracts of synaptosomal membranes from alcohol tolerant mice were less fluid and in vitro ethanol was less able to fluidize these membranes than those prepared from controls. On the other hand, Chin et al. [18] showed that dietary ethanol for 8 days had no significant effect on the fluidity of either erythrocyte or synaptosomal membranes in male mice measured by the electron paramagnetic resonance technique. The difference between the findings of Chin et al. and our observation may be related either to the duration of alcohol-exposure, to differences in the membranes used, or to the use of different techniques for the measurement of membrane fluidity. However, Chin et al. did find that membranes from ethanol fed rats had increased cholesterol/phospholipid molar ratios which is generally associated with increased viscosity [16,18]. They also showed that membranes from ethanol-fed rats resisted the fluidizing effects of in vitro ethanol [19].

The non-specific fluidizing effects of aliphatic alcohols in vitro on a number of membrane systems have been well documented [8,21,22]. The effects of alcohols in vitro on prolactin binding suggests a direct relationship between prolactin receptor number and membrane lipid fluidity. These and earlier observations in rat prostatic and in other prolactin target tissues support the hypothesis that an alteration in the fluidity of the membrane lipid bilayer affects the accessibility of the receptor for its hormone [2–6,9,10]. Membrane proteins, including receptor proteins, are now considered to float within the matrix of the lipid bilayer. Changes in the receptor protein functionality are presumably due to changes in the mobil-

ity or orientation of these proteins within the bilayer. Increased lipid fluidity would tend to increase the mobility of receptor protein and, thus, increase the detectability of cryptic prolactin receptors. Such effects of increased membrane fluidity leading to exposure of a larger proportion of the cryptic prolactin and other peptide hormone receptors have been reported by other investigators [23,24].

Prolactin is known to modulate its own receptor level in target tissue and this modulation also appears to involve membrane fluidity [3]. Therefore, the possibility that alcohol-ingestion might produce a decrease in prostatic and hepatic prolactin receptors via a prolactin-mediated self-regulatory mechanism should be considered. However, the present observation that chronic alcohol ingestion produced no effect on serum prolactin levels, which is consistent with a previous report [17], suggests that changes in prostatic and hepatic prolactin receptor levels occurred independent of serum prolactin.

Ethanol treatment of membranes in vitro, by the process of fluidization, has been used in the past to unmask cryptic prolactin receptors [10]. We showed previously that although rat prostatic prolactin receptors declined progressively from weaning to senscence, along with a progressive increase in membrane microviscosity, the total number of prostatic prolactin receptors estimated after ethanol exposure was similar at all ages. Therefore, the fact that ethanol in vitro failed to enhance prolactin receptor levels in membranes from alcohol-fed rats would suggest that the diet reduced the total number of prolactin receptors in these tissues. This may or may not be the case since alcohol treatment in vitro failed to fluidize these membranes. The lack of fluidizing response suggest that ethanol feeding produced a fundamental change in the membrane lipid and/or protein composition.

The differences in the effects of the various aliphatic alcohols in vitro and the responses of the two tissues also deserve comment. It appears that the longer the carbon chain length of the alcohol, the lower the concentration required to produce a maximal increase in prolactin binding, whereas the magnitude of the peak response is inversely related to length of the alcohol. These differences in the magnitude of the peak response and in the concentration of alcohols required to produce a maximal increase in prolactin binding in the present study are in agreement with earlier [8,10] studies and may be related to the differences in stability of prolactin receptors to various alcohols. The differences in the critical concentrations seen with prostate and liver may reflect fundamental differences in the chemical composition of the tissues.

In conclusion, the present study documents changes in prostatic and hepatic membrane lipid microviscosity and prolactin binding in rat after ethanol feeding. The fact that in vitro alcohol failed to fluidize membranes from ethanol fed rats suggests some fundamental and as yet undefined change(s) in the composition of prostatic and hepatic membranes occurred after ethanol ingestion.

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