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ROLE OF THE PITUITARY AND NEONATAL ANDROGENIC IMPRINTING IN THE HORMONAL REGULATION OF LIVER ALCOHOL DEHYDROGENASE ACTIVITY

DAVID W. CRABB,* WILLIAM F. BOSRON and TING-KAI LI

Departments of Medicine and Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46223, U.S.A.

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Abstract—Liver alcohol dehydrogenase activity is increased by thyroidectomy, orchidectomy, or hypophysectomy. We investigated the mechanisms of these hormonal effects by examining the effects of testosterone, dexamethasone and thyroid hormone on liver alcohol dehydrogenase activity in hypophysectomized rats and in cultured hepatocytes, and the effect of administration of androgens to neonatal female rats. Testosterone did not lower alcohol dehydrogenase activity in hypophysectomized rats, whereas dexamethasone and thyroxine produced moderate decreases in activity. Triiodothyronine reduced alcohol dehydrogenase activity of cultured hepatocytes from male and hypothyroid female rats in a dose-dependent fashion, confirming that thyroid hormone had pituitary-independent effects on the enzyme activity. Dexamethasone was required for the expression of alcohol dehydrogenase activity in cultured cells, and it increased the enzyme activity when present at supraphysiologic concentrations. Treatment of neonatal female rats with testosterone reduced the activity of the enzyme in adulthood. The difference in alcohol dehydrogenase activity in adult male and female rats appears to be determined in part by neonatal imprinting by androgens and in part by an effect of testosterone that is either mediated by or dependent upon the pituitary. Thyroid hormone reduces alcohol dehydrogenase activity by a direct effect on the liver.

The hormonal control of liver alcohol dehydrogenase (EC 1.1.1.1) has attracted attention because the activity of this enzyme is a rate-determining factor for alcohol elimination in rats [1]. The effects of hormones have largely been deduced from endocrine gland ablation and hormone administration experiments. Hypophysectomy, orchidectomy, and thyroidectomy increase the activity of the enzyme, whereas administration of testosterone to females or castrated males, or administration of thyroid hormone to hypothyroid animals lowers the activity towards normal [2–6]. Adrenalectomy has no effect on enzyme activity [2]. The mechanisms by which these hormones affect alcohol dehydrogenase activity are unknown. Although rat liver contains receptors for gonadal steroids [7, 8], several characteristics of the male liver are determined by the hypothalamic-pituitary axis. Exposure of the neonatal brain to androgens appears to control the expression of certain male-specific enzymes in adulthood. This effect has been termed androgenic imprinting [9–11]. Other effects of androgen administration or castration on the liver have been shown to be indirect actions, which require the presence of an intact pituitary, and are probably mediated by changes in the secretion of growth hormone [12–15]. In earlier studies, we were unable to demonstrate direct effects of androgens or estrogens on the activity of alcohol dehydrogenase of cultured rat hepatocytes [16]. We therefore examined the effects of androgens, thyroid hormone, and dexamethasone in hypophysectomized rats and of triiodothyronine and dexamethasone in cultured hepatocytes to determine whether these hormones act directly on the liver or require the presence of the pituitary. We also tested the hypothesis that androgenic imprinting in the neonatal period influences alcohol dehydrogenase activity in adulthood.

* Address all correspondence to: David W. Crabb, M.D., Emerson Hall, Room 421, 545 Barnhill Drive, Indianapolis, IN 46223.

METHODS

Animals. Sprague–Dawley rats were obtained from Harlan Sprague–Dawley Industries (Indianapolis, IN). Parapharyngeal hypophysectomy was performed on 4-week-old male rats by the supplier, and hormone treatment was begun 1 week later. The adequacy of the hypophysectomy was judged from the failure of the animals to gain weight and from the low levels of plasma hormones (below). The animals were fed a standard rat food (Rodent Laboratory Chow, Ralston Purina, or Wayne Lab Blox F-6, Allied Mills, Inc.) and were housed in a temperature- and humidity-controlled vivarium with a 7:00 a.m.–7:00 p.m. light cycle.

Hormone treatments. One-day-old female rats were given a single subcutaneous injection of testosterone propionate (1.25 mg) in corn oil, a dose which induces tonic gonadotropin secretion and causes sterility [17]. Hypothyroidism was induced by adding propylthiouracil (0.015%) to the drinking water for 4 weeks before using the animals for liver cell isolation [18]. Adult male rats had serum corticosterone, thyroxine, and testosterone levels of 34 ± 1 μg/dl, 4.3 ± 1.0 μg/dl, and 298 ± 133 ng/dl;

hypophysectomy reduced the levels to 2.4 ± 2.0 μg/dl, 0.6 ± 0.1 μg/dl, and 15 ± 8 ng/dl, respectively, at 23 days after surgery. Hormone treatments were begun 1 week after hypophysectomy and were continued for 16 days. L-Thyroxine and testosterone propionate (Sigma Chemical Co., St. Louis, MO) were suspended in corn oil and administered as daily subcutaneous injections. Dexamethasone phosphate was administered in saline as a daily intraperitoneal injection. The dosages were: dexamethasone, 10 μg/100 g body wt/day; L-thyroxine, 1 μg100 g/day; and testosterone, 100 or 500 μg/100 g/day. These regimens restored the serum thyroxine level to 5.1 ± 1.3 μg/dl, and the testosterone level to 369 ± 155 ng/dl for the lower dose and to 1714 ± 144 ng/dl for the higher dose. Blood for hormone assays was obtained on the day enzyme activity was determined and was analyzed for thyroxine and testosterone using commercial kits (T_4 RIA, Micromedic Systems, Inc., and Testosterone Test Kit, Wien Laboratories, Inc.). Corticosterone was measured by a competitive binding assay [19].

Determination of liver alcohol dehydrogenase activity. The animals were killed, and the livers were immediately excised and weighed. An 0.5-g section was homogenized in 4 vol. of 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], pH 8.4, containing 0.5 mM dithiothreitol. The homogenate was centrifuged at 100,000 *g* for 30 min. The supernatant fraction was filtered through an 0.2 μm nylon filter to reduce turbidity and was assayed for alcohol dehydrogenase activity as previously described [1]. The assay buffer contained 0.5 M Tris–HCl, pH 7.2, 2.8 mM NAD^+, and 10 mM ethanol. One unit (U) of activity catalyzes the oxidation of 1 μmole of ethanol/min. Supernatant protein concentration was determined by the biuret reaction [20]. The liver DNA content was measured with a fluorescent dye-binding assay [21].

Cell culture techniques. Liver cells were isolated by collagenase perfusion and were plated at a density of 9×10^6 cells/100 mm culture dish. The plates were precoated with type III collagen (Sigma). The medium was a modification of Leibovitz-15 medium (Grand Island Biological Co., Grand Island, NY), which we have described elsewhere [16]. The cells were incubated under air and the medium was changed daily. After 4 days in culture, the cells were washed in medium and scraped in phosphate-buffered saline containing 0.5 mM dithiothreitol, sonically disrupted, and assayed for alcohol dehydrogenase activity as described above. The enzyme activity was determined in duplicate plates in each experiment, and the data shown are the results of at least three separate hepatocyte preparations. In one set of experiments, the activity of α-glycerol-phosphate dehydrogenase was also assayed according to Fried *et al.* [22]. The α-glycerol-phosphate dehydrogenase activity was expressed as nmoles of tetrazolium dye reduced/min/μg DNA. The DNA concentration of the extracts was determined by a fluorescent dye binding assay [23].

Statistical analysis. Differences between groups of hormone-treated animals were analyzed for significance by one-way analysis of variance using the SSPS-x statistical program package. Differences in alcohol dehydrogenase activity in triiodothyronine-treated cultured liver cells were analyzed by a paired *t*-test.

RESULTS

Effects of testosterone, thyroxine, and dexamethasone on alcohol dehydrogenase activity in hypophysectomized rats. The effects of hypophysectomy and of hormone administration to hypophysectomized rats on liver weight and the total liver content of protein and DNA are shown in Table 1. Hypophysectomy prevented the maturational increase in body weight, liver weight, and in the

Table 1. Effects of hypophysectomy and hormone administration on liver weight, and DNA and protein content

Treatment group (N)	Liver weight (g)	Liver DNA (mg)	Liver protein (g)
Control (5)	7.9 ± 0.4*	22.0 ± 1.8*	1.34 ± 0.11*
Hypophysectomized rats (9)	4.2 ± 0.3	10.8 ± 2.0	0.72 ± 0.05
+Testosterone (5)	5.2 ± 0.4	15.8 ± 2.3*	0.79 ± 0.12
+5x Testosterone (5)	4.3 ± 0.3	10.7 ± 1.9	0.78 ± 0.13
+Dexamethasone (6)	3.5 ± 0.2*	11.1 ± 1.0	0.56 ± 0.02*
+Thyroxine (5)	4.4 ± 0.3	13.9 ± 1.3*	0.80 ± 0.06
+Dexamethasone + testosterone (5)	3.2 ± 0.6*	11.0 ± 0.9	0.52 ± 0.07*
+Thyroxine + testosterone (12)	4.5 ± 0.5	13.3 ± 1.9*	0.75 ± 0.12
+Thyroxine, dexamethasone, testosterone (6)	3.8 ± 0.2	11.4 ± 1.1	0.59 ± 0.07*

Male Sprague–Dawley rats underwent hypophysectomy at 4 weeks of age. Daily hormone injections were begun 1 week later as described in Methods. "5x Testosterone" denotes administration of five times the physiological dose of testosterone. After 16 days of treatment, the animals were killed for determination of liver alcohol dehydrogenase activity and collection of blood for hormone assays. Control animals were 7-week-old males. The number of animals in each group is shown in parentheses. DNA and protein content denote total liver DNA and protein respectively. The data are expressed as mean ± S.D.

* Denotes a significant difference compared with hypophysectomized rats by one-way analysis of variance ($P < 0.05$).

Table 2. Effects of hypophysectomy and hormone replacement on liver alcohol dehydrogenase activity

Treatment group (N)	Alcohol dehydrogenase activity U/g liver	U/g protein	U/mg DNA
Control (5)	3.5 ± 0.3*	21 ± 2*	1.3 ± 0.1*
Hypophysectomized rats (9)	5.8 ± 0.7	33 ± 3	2.3 ± 0.4
+Testosterone (5)	5.9 ± 0.7	39 ± 7*	2.0 ± 0.3
+5x Testosterone (5)	6.9 ± 0.4*	38 ± 2*	2.8 ± 0.5*
+Dexamethasone (6)	5.8 ± 1.0	35 ± 5	1.8 ± 0.2*
+Thyroxine (5)	5.4 ± 0.4	29 ± 3	1.7 ± 0.2*
+Dexamethasone + testosterone (5)	5.2 ± 0.7	32 ± 5	1.6 ± 0.3*
+Thyroxine + testosterone (12)	5.4 ± 0.4	33 ± 6	1.8 ± 0.2*
+Thyroxine, dexamethasone, testosterone (6)	5.0 ± 0.5*	32 ± 2	1.7 ± 0.2*

Animals were treated as described in the legend to Table 1. Enzyme activity is expressed as mean ± S.D.

* Denotes a significant difference when compared with hypophysectomized rats by one-way analysis of variance ($P < 0.05$).

liver content of protein and DNA. The amount of DNA/g liver was also significantly lower in hypophysectomized rats than in the control animals. Physiological doses of testosterone or thyroxine given to hypophysectomized rats increased liver DNA, while dexamethasone treatment reduced liver weight and liver protein content. There was a significant increase in the amount of DNA per g liver and a reduction in the amount of protein per mg DNA in the animals treated with thyroxine or dexamethasone, alone or in combination with testosterone. Because of these effects of the hormone treatments, we have expressed alcohol dehydrogenase activity in several ways (Table 2). Hypophysectomized rats had higher alcohol dehydrogenase activity than age-matched control animals, as has been reported [2]. Testosterone, which decreases the activity of alcohol dehydrogenase in orchidectomized rats of the same age and strain [4], increased the enzyme activity/g cytosolic protein when given in physiological amounts, and increased the activity expressed in any fashion when given in supraphysiological doses. Dexamethasone and thyroxine decreased alcohol dehydrogenase activity per mg liver DNA. There was no further decrease in enzyme activity when animals treated with dexamethasone, thyroxine, or dexamethasone and thyroxine were given physiological doses of testosterone.

Effect of neonatal exposure of female rats to androgens on alcohol dehydrogenase activity in adulthood. Treatment of female rats with a single subcutaneous dose of 1.25 mg of testosterone propionate at 1 day of age had several general effects on the liver (Table 3). The livers of these animals were larger, and contained larger amounts of DNA and protein when compared with control females of similar age and body weight. The size and content of DNA and protein of these livers were, in fact, very similar to those of age-matched males. The activity of alcohol dehydrogenase was reduced in the androgen-treated animals to a level intermediate between that of control females and males. Failure of vaginal opening in the androgen-treated females [17] was confirmed.

Effects of insulin, dexamethasone, and triiodothyronine on alcohol dehydrogenase activity in cultured hepatocytes. We have recently established a cell culture system in which rat liver cells from male rats maintain alcohol dehydrogenase activity at levels

Table 3. Effect of neonatal treatment with testosterone on liver alcohol dehydrogenase activity

	Control Female	Androgen-treated Female	Control Male
Body weight (g)	217 ± 6	196 ± 22	246 ± 37
Liver weight (g)	7.1 ± 0.3	8.4 ± 1.1*	8.6 ± 1.2*
Liver DNA (mg)	18 ± 3	25 ± 5*	26 ± 3*
Liver protein (g)	1.23 ± 0.04	1.40 ± 0.20	1.47 ± 0.14*
Alcohol dehydrogenase activity			
U/g liver	6.4 ± 0.9	4.2 ± 0.4*	3.6 ± 0.7*
U/g protein	37 ± 2	26 ± 2*	21 ± 4*
U/mg DNA	2.6 ± 0.5	1.5 ± 0.2*	1.2 ± 0.2*

Female rats were given 1.25 mg testosterone propionate at 1 day of age. The animals were killed at 8 weeks of age. Control females and males were 9 weeks old. The data are expressed as mean ± S.D.

* Indicates a significant difference compared with the control females by one-way analysis of variance ($P < 0.05$).

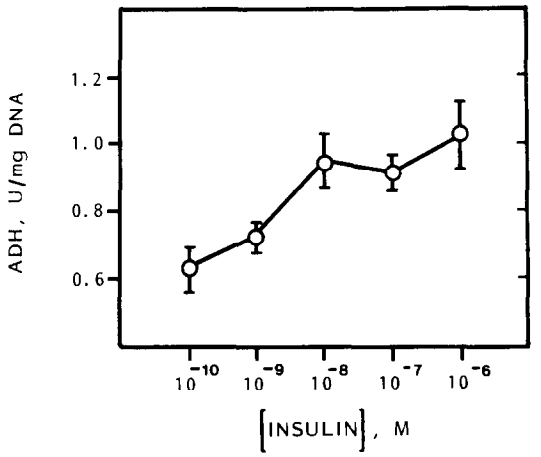

Fig. 1. Effect of insulin concentration on the activity of alcohol dehydrogenase in cultured rat hepatocytes. Liver cells from male rats were cultured in the presence of 10^{-10} M dexamethasone and various concentrations of insulin. After 4 days, the activity of alcohol dehydrogenase (ADH) was determined and is expressed as the mean ± S.E.M. of four experiments.

near that observed *in vivo* [16]. The effects of insulin and dexamethasone on the activity of the enzyme in cultured cells are shown in Figs. 1 and 2. Insulin was required at concentrations of 10^{-8} to 10^{-6} M for full expression of alcohol dehydrogenase activity, and was used at 10^{-6} M in all subsequent experiments. Approximately physiological concentrations of dexamethasone (10^{-9} M) permitted expression of alcohol dehydrogenase activity at *in vivo* levels, but higher concentrations (10^{-8} to 10^{-6} M) increased the activity of the enzyme in cultured cells by 25–30%. The effect of triiodothyronine on alcohol dehydrogenase activity in cultured cells is shown in Table 4. Increasing concentrations of triiodothyronine resulted in a dose-dependent reduction in alcohol

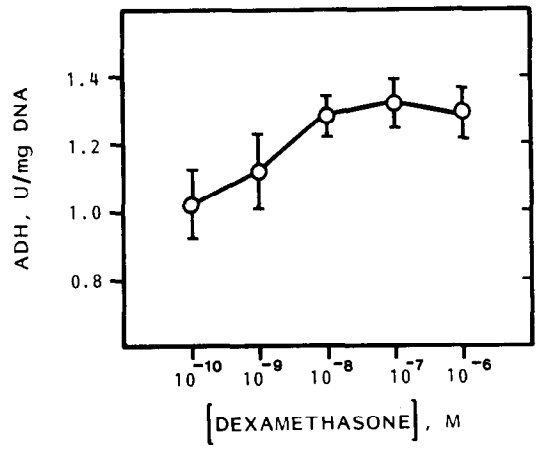

Fig. 2. Effect of dexamethasone concentration on the activity of alcohol dehydrogenase in cultured rat hepatocytes. Liver cells were cultured as described in Fig. 1 in the presence of 10^{-6} M insulin and various concentrations of dexamethasone. Alcohol dehydrogenase (ADH) activity is expressed as the mean ± S.E.M. for four experiments.

dehydrogenase activity. Since this effect was more pronounced in cells prepared from male rats than in those from females, the response of cells from hypothyroid female rats was also studied. The cells from hypothyroid females demonstrated high alcohol dehydrogenase activity, as reported by Mezey and Potter [6], and the activity fell in response to the addition of triiodothyronine to the culture medium. To ensure that this was not the result of a toxic effect of the concentrations of triiodothyronine employed in the study, the activity of α-glycerol-phosphate dehydrogenase, an enzyme induced by thyroid hormone, was assayed. The activity of this enzyme in the cultured cells from hypothyroid animals was increased from 3.0 ± 2.4 to 7.9 ± 3.9 nmoles/min/μg DNA ($P < 0.05$) by 10^{-7} M triiodothyronine, an increase similar to that reported by Ismail-Beigi *et al.* [24].

DISCUSSION

Studies on the hormonal regulation of liver alcohol dehydrogenase activity present certain problems not encountered with several other enzymes which are studied as model systems. Alcohol dehydrogenase is a cytosolic enzyme with a relatively long half-life of about 4 days [25]. In certain nutritional states, such as fasting or food-restriction, alcohol dehydrogenase turns over at the same rate as bulk cytoplasmic proteins. Because of this relationship, and because liver weight can change rapidly owing to glycogen mobilization, a previous study showed that changes in enzyme activity in response to nutritional shifts were obscured when the activity was expressed as U/g liver or U/g cytosolic protein. However, the reduction in activity was clearly evident if expressed as U/mg DNA, because the number of liver cells and their content of DNA did not change appreciably during these relatively short-term experiments [1]. Because hypophysectomy and hormone administration alter not only liver and body weight but also liver protein and DNA content (Table 1), we expressed the activity of alcohol dehydrogenase in the hormone-treated animals in three ways (Tables 2 and 3). We believe, however, that activity per mg DNA best reflects changes in alcohol dehydrogenase activity at the cellular level.

Although testosterone has been shown by several groups of investigators to reduce the activity of alcohol dehydrogenase in female rats or orchidectomized males, it did not reduce the enzyme activity in hypophysectomized animals when given in physiological doses, and, in fact, increased the activity when given in high doses (Table 2). A similar increase in alcohol dehydrogenase activity in intact male rats given large doses of testosterone has been reported [4]. Because the responsiveness of the liver to androgens could require the presence of other, pituitary-dependent hormones, testosterone was administered to the hypophysectomized animals in conjunction with thyroxine, dexamethasone, or the combination of the two. We were unable to detect an inhibitory effect of testosterone under any of the conditions examined. This is consistent with our previous observation that the alcohol dehydrogenase activity of cultured hepatocytes is not sensitive to the presence

Table 4. Effect of triiodothyronine on the alcohol dehydrogenase activity of cultured hepatocytes

	Alcohol dehydrogenase activity (U/mg DNA)		
	Male	Female	Hypothyroid female
Fresh cells	1.08 ± 0.06	1.52 ± 0.23	2.63 ± 0.30
Control incubation	1.08 ± 0.08	1.06 ± 0.12	2.11 ± 0.17
Triiodothyronine (M):			
10^{-10}	0.98 ± 0.22	1.12 ± 0.19	
10^{-9}	0.83 ± 0.08	1.06 ± 0.20	1.90 ± 0.14*
10^{-8}	0.79 ± 0.11	1.09 ± 0.16	1.96 ± 0.11*
10^{-7}	0.69 ± 0.10*	0.98 ± 0.13	1.89 ± 0.15*
10^{-6}	0.77 ± 0.07	0.78 ± 0.11	1.76 ± 0.14*

Liver cells were prepared from adult male, female, or hypothyroid female rats and were cultured for 4 days in modified Leibovitz-15 medium containing 5% fetal bovine serum, insulin, dexamethasone, and various concentrations of triiodothyronine. Control incubations were also carried out for 4 days, but no triiodothyronine was added to the medium. Enzyme activity is reported as mean ± S.E.M.

* Significantly different from control incubations by paired *t*-test ($P < 0.05$).

of androgens in the medium [16]. The results indicate that the effect of testosterone on liver alcohol dehydrogenase in females and castrated males is either mediated by another hormone, presumedly of pituitary origin, or that the liver requires the presence of a pituitary-dependent hormone, other than thyroxine or corticosterone, to be responsive to androgens.

Another way by which androgens are thought to control the activity of liver enzymes is by "imprinting" the hypothalamus and pituitary in the neonatal period. Unlike estrogens, androgens are not tightly bound to α-fetoprotein in the neonatal circulation and can therefore gain access to the central nervous system [26]. Treatment of neonatal females with testosterone has been shown to induce the male pattern of secretion of some pituitary hormones. The best studied example is that of the gonadotropins, which are secreted tonically in the androgen-treated female animals, rather than cyclically. As a result, the animals are sterile [17], and do not undergo normal vaginal opening. In addition, neonatal androgen treatment modifies the pattern of the hepatic steroid-metabolizing enzymes [10]. The studies described here show that androgen treatment of newborn female rats accelerated liver growth as well as reduced alcohol dehydrogenase activity in adulthood. Thus, the activity of alcohol dehydrogenase was imprinted by androgens in the neonatal period. The mediator of this effect is unknown.

Thyroidectomy has been shown to increase alcohol dehydrogenase activity in male and female rats, and administration of thyroid hormone to hypothyroid or normal animals lowers the enzyme activity [5, 6]. Hypophysectomized rats are, of course, hypothyroid, and when they were treated with thyroxine, total liver DNA increased, and the ratio of protein to DNA decreased. This may reflect stimulation of DNA synthesis and cell division. The activity of alcohol dehydrogenase was decreased when expressed in U/mg DNA. The reduction in the ratio of protein to DNA suggests that the hepatocytes in thyroxine-treated rats were smaller and had less cytosolic protein, including alcohol dehydrogenase, and that the lower alcohol dehydrogenase activity/mg DNA was again due to a reduction in the enzyme activity per cell. We also investigated the hepatic effect of thyroid hormone directly by examining its effect on the alcohol dehydrogenase activity of cultured liver cells. As has been reported for intact animals [5, 6], triiodothyronine decreased the activity of alcohol dehydrogenase in cultured hepatocytes from male and hypothyroid female rats (Table 4). This effect was dose dependent and was achieved at concentrations which are near physiological. Hence, thyroid hormone has a direct effect on the liver which results in a reduction of alcohol dehydrogenase activity.

Adrenalectomy or corticosteroid administration does not affect liver alcohol dehydrogenase activity in rats [2]. When we tested the effect of dexamethasone in cultured cells, we found that the activity of alcohol dehydrogenase was maintained at *in vivo* levels with low concentrations of dexamethasone (Fig. 2). Higher concentrations increased the activity by 25–30%. When hypophysectomized rats were treated with dexamethasone, liver weight and protein were reduced, and no change in alcohol dehydrogenase activity expressed as U/g liver or U/g protein was observed. There was a moderate reduction in enzyme activity in U/mg DNA. Since corticosteroids are not required for the expression of alcohol dehydrogenase in either adrenalectomized or hypophysectomized rats, and treatment of cultured hepatocytes with high concentrations of dexamethasone does not reduce the enzyme activity, the effect of dexamethasone in hypophysectomized rats is probably neither physiological nor the result of a direct effect on the liver.

Since hypophysectomy increases the activity of alcohol dehydrogenase, expression of the alcohol dehydrogenase gene apparently does not require any of the pituitary-controlled hormones (thyroxine, corticosterone, growth hormone, prolactin, or gonadal steroids). Our experiments support the hypothesis that thyroid hormone has a direct suppressive effect on liver alcohol dehydrogenase activity. Androgens probably act indirectly on the liver by a mechanism involving the pituitary. A previously unknown aspect

of the sexual difference in alcohol dehydrogenase activity in adult rats is the finding that exposure of female animals to androgens in neonatal life permanently changes the expression of the enzyme in adulthood. We are currently investigating the possibility that the effect of gonadal steroids is mediated by the pattern of growth hormone secretion.

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