Sexual dimorphic expression of ADH in rat liver: importance of the hypothalamic-pituitary-liver axis

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Simon, Francis R., John Fortune, Mieko Iwahashi, and Eileen Sutherland. Sexual dimorphic expression of ADH in rat liver: importance of the hypothalamic-pituitary-liver axis. Am J Physiol Gastrointest Liver Physiol 283: G646–G655, 2002.—Hepatic alcohol dehydrogenase (ADH) activity is higher in female than in male rats. Although sex steroids, thyroid, and growth hormone (GH) have been shown to regulate hepatic ADH, the mechanism(s) for sexual dimorphic expression is unclear. We tested the possibility that the GH secretory pattern determined differential expression of ADH. Gonadectomized and hypophysectomized male and female rats were examined. Hepatic ADH activity was 2.1-fold greater in females. Because protein and mRNA content were 1.7- and 2.4-fold greater, results indicated that activity differences were due to pretranslational mechanisms. Estradiol increased ADH selectively in males, and testosterone selectively decreased activity and mRNA levels in females. Effect of sex steroids on ADH was lost after hypophysectomy; infusion of GH in males increased ADH to basal female levels, supporting a role of the pituitary-liver axis. However, GH and L-thyroxine (T4) replacements alone in hypophysectomized rats did not restore dimorphic differences for either ADH activity or mRNA levels. On the other hand, T4 in combination with intermittent administration of GH reduced ADH mRNA to basal male values, whereas T4 plus GH infusion replicated female levels. These results indicate that the intermittent male pattern of GH secretion combined with T4 is the principal determinant of low ADH activity in male liver.

ADH is principally expressed in liver and is hormonally, nutritionally, and developmentally controlled (2, 36, 42). Although the mechanism(s) is unclear, ADH is dimorphically expressed, with higher specific activity values in female compared with male rats (4, 51). Some authors have shown that estrogen increased and testosterone decreased hepatic ADH activities, whereas others have found no effect (40, 41, 52). Growth hormone (GH) has also been shown to be important in regulation of hepatic ADH-specific activity and transcriptional regulation of mRNA in vivo and in vitro, respectively (39, 49). Sexual dimorphic expression of hepatic genes in rats is most often due to sexual difference in the GH secretory pattern, which is regulated, in part, by sex steroid hormones (20, 54). Therefore, it is unclear whether exogenous sex steroid hormones alter expression of ADH directly or indirectly through changes in GH secretion. Additionally, thyroidectomy increased hepatic ADH in both sexes, which may be a direct effect or possibly secondary to modulation of serum GH levels (14). In contrast, adrenalectomy had no effect in male rats (39).

Previous studies, for the most part, have not directly addressed the possible role of the GH secretory pattern in mediating the effect of sex steroid hormones on expression of hepatic ADH. Therefore, the present studies were undertaken in gonadectomized and hypophysectomized (Hx) male and female rats to examine the molecular mechanisms involved in the sexual dimorphic expression of hepatic ADH.

The results indicate that the pattern of GH secretion in concert with thyroid hormone is principally responsible for regulation of hepatic ADH mRNA and protein levels. A novel observation was that the dimorphic expression of ADH is due to the downregulation of constitutive ADH expression.

MATERIALS AND METHODS

Animals. Adult male and female Sprague-Dawley rats (weighing 185–200 g) were purchased from Harlan (Indianapolis, IN). The supplier performed all surgeries. Animals were allowed to stabilize for a minimum of 6 days within our
facility before treatment. If Hx rats gained weight, they were eliminated from the study. Food and water were allowed ad libitum until 18 h before death, when food was removed. Animals were killed by 9 AM. All animals were exposed to 12:12-h of light-dark. All agents except for bovine GH (bGH) were purchased from Sigma and were the highest purity available. bGH was kindly supplied by Protiva (Monsanto) and the USDA-Animal Research Science research hormone programs. bGH was administered continuously at 60 µg·100 g⁻¹·day⁻¹ for 7 days or intermittently at twice-daily 30 µg/100 g sc for 7 days. The solvent for bGH was 0.05 M Na₂HPO₄, pH 8.8, 1.6% glycerol, and 0.02% NaN₃. For all continuous infusions, Osmonic minipumps (model 2001; Alzet) were placed subcutaneously for the length of time indicated in the figures. 1-ß-Thyroxine, sodium salt (T₄) was dissolved in normal saline and administered once daily at 50 µg·kg⁻¹·day⁻¹ ip for 7 days. 17β-Estradiol (β-E) and testosterone enanthate (TE) were dissolved in corn oil, and injections were administered at 100 and 2 µg·kg⁻¹·day⁻¹ sc, respectively, for 14 days as indicated in the figures and tables.

Enzyme activities. Under ether anesthesia, livers were rapidly removed, and homogenates were prepared as follows: 0.25 g of minced liver was added to 3.0 ml of isolation buffer [15 mM Tris·HCl, 300 mM mannitol, 5 mM EGTA, protease inhibitor tablets (Boehringer Mannheim)]. This sample was Dounce homogenized and ultracentrifuged at 150,000 g for 40 min to prepare cytosolic fractions and was stored at −80°C. Mitochondrial fractions were isolated in mannitol buffers as previously described (55). All procedures were conducted at 4°C. ADH-specific activities were determined spectrophotometrically using 25 mM ethanol as substrate at pH 10.0 and 37°C according to the modified method of Algar et al. (1). ALDH was also measured spectrophotometrically in cytosolic and mitochondrial fractions using the method of Little and Peterson (31) using 50 µM and 5 mM propionaldehyde as substrate, respectively, and 1.2 mM NAD as cofactor at pH 7.4 and 32°C. Protein was measured according to Lowry et al. (32). Specific activities of both enzymes are expressed as nmol·mg protein⁻¹·min⁻¹.

Immunoblots of ADH. Content of ADH protein was measured in liver cytosol fractions using PBS, pH 7.5, containing protease and phosphatase inhibitors. Protein content of ADH was determined by immunoblotting using a monospecific anti-class I ADH antibody (provided by William Bosron, University of Indiana Medical School). SDS-PAGE and immunoblotting were carried out using minigels for enhanced chemiluminescence (ECL). After electrophoresis, cytosolic proteins were transferred to Hybond ECL membranes (Amersham) by the procedure of Towbin et al. (59) at 167 V for 1 h using a high transfer apparatus by Ideal. Gels were blocked for 1 h using 5% Tween/TBS and processed for ECL detection (Amersham) using 1% milk in TBS for antibody dilution. Blots were visualized by streptavidin horseradish peroxidase detection system (Amersham). Washes were with 0.5% Tween/TBS for 5 min (3×). ECL blots were placed in plastic film and exposed to Amersham Hyperfilm for ECL for 30–60 s. Autoradiograms were quantitated by densitometry using a Bio-Rad laser imaging densitometry.

Preparation and analysis of RNA. Total RNA was extracted from whole liver using RNeasy Mini Kit (Qiagen). The RNA was fractionated in 1.2% agarose-formaldehyde gels in borate buffer at 140 V for 4 h. RNA was transferred to Hybond N⁺ (Amersham) with high-efficiency transfer solution (Tel-Test) by capillary action and fixed by ultraviolet crosslinking. cDNA probes were labeled with [³²P]dCTP (Amersham) using Decaprime II (Ambion) random-promim labeled system. Unincorporated label was removed with Probequant G-50 microcolumns (Pharmacia). Membranes were hybridized using a high-efficiency hybridization system (TelTest) for 16 h at 62°C and washed twice in 2x SSC/0.1% SDS followed by two washes in 0.1x SSC/0.1% SDS, all at 55°C for 20 min. Membranes were exposed to Hyperfilm MP with intensifying screen at −70°C for 3 h. Autoradiograms were quantitated with an imaging densitometer. The following probes were used: ADH (provided by Vincent Yang, Johns Hopkins Medical School), ALDH2 (provided by Henry Weiner, Purdue University), CYP2C11 and CYP2C12 (provided by Agneta Mode, Karolinska Institute), and 5α-reductase (provided by David Russell, University of Texas southwestern, Dallas). The relative density of mRNA was normalized to 18S rRNA (Ambion) and expressed as a percentage of the male control.

Data analysis. Data were expressed as ± SE and analyzed statistically by using two-way ANOVA, followed by post hoc analysis with Tukey's test. Other comparisons among groups were made using the Student's t-test. A P value of <0.05 was determined to be statistically significant.

RESULTS

Analysis of sexual differences in ADH and ALDH. ADH-specific activities were measured in liver homogenates and cytosol fractions from male and female rats. Although values are higher in cytosol, similar sexual differences in ADH-specific activities were obtained in liver homogenate as well as cytosol (Fig. 1A). ADH-specific activities in homogenate and cytosol fractions were 2.1- and 1.8-fold (P < 0.001) higher in females compared with males, respectively. To confirm that differential expression of ADH was due to protein content, liver cytosol fractions were isolated and, after separation by SDS-PAGE, were immunoblotted with a monospecific antibody against rat ADH. ADH peptides were recognized as single 40-kDa bands as shown in Fig. 1B. Female rats showed a significantly (P < 0.01) increased ADH protein content compared with males (68%). Because the density of ADH protein and enzyme activity were similar, the dimorphic expression of ADH was due to pretranslational mechanisms.

Steady-state mRNA levels for ADH were analyzed by Northern blots and quantitated by densitometry in male and female livers (Fig. 1, C and D). ADH mRNA was increased 2.4-fold (P < 0.01) in females compared with males, whereas ribosomal 18S mRNA levels were similar. Together, these results indicated that the sexual dimorphic expression of ADH-specific activity was due to pretranslational mechanisms.

To determine whether increased ADH-specific activities were part of a generalized increase in the ethanol-metabolizing system in female rats, specific activities of mitochondrial and cytosolic ALDH were determined in liver fractions. Specific activity (6.8 ± 1.0 vs. 7.2 ± 1.0 U/mg protein) and mRNA content of mitochondrial, low-Kₘ ALDH mRNA content was similar (data not shown). In addition, cytosolic high-Kₘ ALDH-specific activity was not significantly different between sexes (9.8 ± 0.7 vs. 9.4 ± 0.4 U/mg protein). These results confirmed and extended a previous report (4), indicat-
Fig. 1. Sex differences in ADH-specific activity, protein content, and mRNA. Normal male (closed bars) and female (open bars) Sprague-Dawley rats were killed, and liver homogenates and cytosol fractions were prepared as described in MATERIALS AND METHODS. A: ADH-specific activity was determined spectrophotometrically at pH 10.0 using 25 mM ethanol as substrate in total liver homogenates and in cytosol fractions from male and female rats. Results are expressed as U mg protein−1 min−1. B: ADH protein content. Cytosol fractions were prepared and proteins were separated on SDS-PAGE. ADH protein content was identified using monoclonal antibody and quantitated by densitometry. C: Northern blots. Total liver RNA was isolated, and ADH mRNA was identified by 32P-labeled ADH cDNA. 18S was used as a loading control. D: analysis of ADH mRNA. ADH mRNA content from male and female livers was quantitated by densitometry. Females were compared to males that were set at 100%. Number in parentheses is number of individual experiments. Results are expressed as means ± SE.

Table 1. Effect of hormonal states on change in body weight

<table>
<thead>
<tr>
<th>Groups</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>6.6 ± 0.2(13)</td>
<td>3.6 ± 0.3(16)</td>
</tr>
<tr>
<td>Intact + β-E</td>
<td>0.2 ± 0.1(3)*</td>
<td>0.1 ± 0.9(3)*</td>
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<tr>
<td>Castration</td>
<td>6.4 ± 0.4(4)</td>
<td>4.3 ± 0.5(4)</td>
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<tr>
<td>+TE</td>
<td>6.9 ± 0.4(4)</td>
<td>3.8 ± 0.3(4)</td>
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<tr>
<td>Hypophysectomy</td>
<td>−0.5 ± 0.2(14)</td>
<td>0.2 ± 0.4(15)</td>
</tr>
<tr>
<td>+β-E</td>
<td>−4.7 ± 0.7(4)†</td>
<td>−1.0 ± 0.3(5)</td>
</tr>
<tr>
<td>+TE</td>
<td>−0.2 ± 0.3(4)</td>
<td>−0.2 ± 0.3(4)</td>
</tr>
<tr>
<td>+GHp</td>
<td>4.5 ± 0.6(4)†</td>
<td>4.7 ± 0.4(5)†</td>
</tr>
<tr>
<td>+GHp</td>
<td>3.9 ± 0.3(7)†</td>
<td>2.5 ± 0.4(5)†</td>
</tr>
<tr>
<td>+T4</td>
<td>0 ± 1.0(3)−</td>
<td>−2.4 ± 0.9(4)†</td>
</tr>
<tr>
<td>+GHp + T4</td>
<td>4.8 ± 0.5(3)†</td>
<td>1.0 ± 0.4(5)</td>
</tr>
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</table>

Data are means ± SE in grams per day for each group; (n), number of animals in each group. Animals were weighed each day in the morning. Mean weight gain or loss was calculated from the slope of the daily weight gain or loss. Rats were compared with gender-matched controls; *P < 0.01 vs. non-treated control; †P < 0.01 compared with hypophysectomized (Hx) alone animals; ‡P < 0.01 for control male and female rats. ND, not determined; β-E, 17β-estradiol; TE, testosterone enanthate; GHp, growth hormone injections; GHp, growth hormone pump.

Changes in weight gain in male and female rats with hormonal status. Because it is known that fasting and food restriction posttranslationally decrease hepatic ADH activity (3, 27, 33), we measured changes in weight gain with different hormonal manipulations. Patterns of weight gain/loss are shown for male and female rats in Table 1. As other studies (15) have shown, female rats gain significantly less weight than their male littersmates. Thus the effect of β-E, castration, Hx, and TE were compared with gender-matched controls. β-E significantly (P < 0.05) decreased weight gain in both male and female rats, whereas castration and TE had no effect. On the other hand, Hx stopped weight gain in both sexes, as previously shown (47). Administration of GH either by injection or infusion increased weight gain, and as previously shown, the male pattern of GH administration is more effective that the female infusion pattern (6, 47). In contrast, β-E in males and T4 in females decreased weight gain in Hx animals. Weight loss associated with estradiol administration was probably related to its known effects on food aversion (18).

Effect of castration and sex steroid hormones on ADH-specific activity and mRNA levels. Although some studies have shown that estrogens increased and testosterone decreased hepatic ADH-specific activity, other studies (41, 42, 50, 52, 58) failed to confirm these results. These studies used short time courses of hormone treatments, and most did not examine whether sex steroids altered ADH mRNA levels. Therefore, we examined the effect of testosterone and estrogen given intermittently for 14 days to intact and castrated (Cx) male and female rats to determine whether changes in ADH-specific activity were related to pretranslational processes. The results are shown in Table 2 and Fig. 2, A and B. As shown in Table 2, castration in male rats significantly (P < 0.05) increased ADH activity, whereas TE administration restored values to control levels. β-E administration to intact males also significantly (P < 0.01) increased ADH-specific activity; whereas in female rats, ovariectomy (Ovx) and testosterone significantly reduced ADH-specific activity. In contrast to its effects in males, β-E had no effect in female rats. Furthermore, testosterone given to intact females, but not males, reduced activity (data not shown). These results indicated that testosterone and estrogen selectively altered ADH-specific activity, possibly accounting for the difference in reported results.
Sex steroid hormones are known to regulate hepatic proteins by multiple mechanisms, involving both transcriptional and posttranscriptional steps (22). Because previous studies had not reported the effect of sex steroid hormones on hepatic ADH mRNA levels, we evaluated whether androgens and/or estrogens regulated ADH at the pretranslational level. Northern blot analysis of total rat liver RNA was hybridized with 32P-labeled ADH cDNA and quantitated for changes relative to control males. Results from castration and sex steroid hormone administration are shown in Fig. 2A. Similar to changes with ADH-specific activity, mRNA levels in males were increased by Cx and β-E, whereas testosterone replacement in Cx males reduced values to control levels. On the other hand, neither Ovx nor β-E significantly changed ADH mRNA, but TE decreased ADH mRNA levels in Ovx females. These results support the hypothesis that sex steroid hormones regulated ADH activity primarily at the pretranslational level.

Previous studies have demonstrated the important role that GH plays in regulating hepatic gene expression (20, 61) and that, for the most part, sex steroid hormones indirectly mediate changes in hepatic gene expression through alterations in the GH secretory pattern (20, 24). For example, the intermittent male-like secretory pattern increases the expression of the microsomal gene CYP2C11 (male dominant) and the constant infusion of GH increased CYP2C12 (female dominant) (13, 29, 44). To examine the possibility that sex steroid hormones might regulate hepatic ADH by altering the GH secretory pattern, we measured changes in CYP2C11 and CYP2C12 mRNA levels (44). Figure 2B compares changes in CYP2C11 and CYP2C12 mRNA in male and female rats undergoing different hormonal modifications. The male-dominant gene CYP2C11 mRNA is decreased toward female levels by both Cx (50 ± 14) and β-E (16 ± 21) in males, whereas in females Ovx (27 ± 22) and testosterone (19 ± 26) modestly increased mRNA. In contrast, the female-dominant gene CYP2C12 mRNA level was increased by Cx (127 ± 4) and markedly increased with β-E in males (790 ± 5) but reduced with Ovx (620 ± 30) and testosterone (341 ± 21) in females.

Effect of GH secretory patterns on ADH-specific activities and mRNA levels. Two experiments were designed to test the possibility that GH secretion modifies ADH. First, if estrogen and testosterone effects on ADH were mediated through pituitary hormones, we anticipated that Hx would prevent changes in ADH induced by sex steroid hormone administration. The results of Hx and its effect on estrogen and testosterone alteration of ADH activity and mRNA are shown in Table 3 and Fig. 3A. In male rats, ADH-specific activity was increased approximately twofold by Hx to values greater than those measured in intact females (Table 3), whereas values in females were also significantly increased by 46% (P < 0.01). However, the

<table>
<thead>
<tr>
<th>Groups</th>
<th>Specific Activity</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Intact male</td>
<td>44.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Cx</td>
<td>55.5 ± 0.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cx + TE</td>
<td>47.6 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>Intact male + β-E</td>
<td>68.6 ± 1.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Intact female</td>
<td>79.4 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Ovx</td>
<td>66.0 ± 2.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Ovx + TE</td>
<td>56.7 ± 0.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Intact female + β-E</td>
<td>82.2 ± 0.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are units per gram protein expressed as means ± SE determined by ANOVA; 4 individual assays were performed per group. ADH activity was determined in 100,000 g cytosol with a spectrophotometric assay as described in MATERIALS AND METHODS. Enzyme activities and mRNA levels and differences in band density were determined by scanning. Results of Hx and its effect on estrogen and testosterone alteration of ADH activity and mRNA are shown in Table 3 and Fig. 3A. In male rats, ADH-specific activity was increased approximately twofold by Hx to values greater than those measured in intact females (Table 3), whereas values in females were also significantly increased by 46% (P < 0.01). However, the administr-
Table 3. Effect of Hx and sex steroid hormones on ADH-specific activity in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male n</th>
<th>Female n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>44.2 ± 3.9</td>
<td>4</td>
</tr>
<tr>
<td>Hypophysectomy</td>
<td>96.9 ± 9</td>
<td>4*</td>
</tr>
<tr>
<td>+β-E</td>
<td>67.3 ± 3.9</td>
<td>4†</td>
</tr>
<tr>
<td>+TE</td>
<td>97.2 ± 1.1</td>
<td>4</td>
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</tbody>
</table>

Data are expressed as means ± SE in units per milligram protein as determined by ANOVA; (n), number of individual determinations. ADH activity was determined in 100,000 g cytosol spectrophotometrically. After Hx male and female rats were treated, for 2 wk with either β-E or TE. *P < 0.05 compared with sham; †P < 0.05 compared with Hx.

tion of β-estradiol paradoxically decreased ADH-specific activity in both sexes. On the other hand, ADH activity was unchanged with TE administration.

Similar changes were measured in steady-state hepatic ADH mRNA levels (Fig. 3A). After Hx in male and female rats, ADH mRNA was increased 2.6- and 1.5-fold, respectively. β-E significantly decreased ADH mRNA levels selectively in males, whereas TE administration to Hx rats did not alter ADH values in either sex. These results indicate that sex steroid hormones did not directly mediate the dimorphic hepatic ADH activity and mRNA levels and suggest that the pattern of GH secretion regulates ADH.

In the second series of experiments, we examined whether changing the GH secretory pattern in male rats might alter ADH. We reasoned that if the GH secretory pattern was involved in regulation of hepatic ADH, then steady infusion of GH at physiological levels to male rats should increase ADH activity to levels measured in female rats. Results are shown in Fig. 4, A–D. Figure 4A demonstrates that ADH-specific activity was significantly increased by the steady infusion of physiological concentrations of GH to male rats, whereas Fig. 4B shows that protein content was similarly changed. In addition, as shown in Fig. 4C, ADH mRNA levels were also significantly increased in males by GH infusion. Figure 4D shows Northern blots of ADH, 2C11, 2C12, 5α-reductase, and 18S. Both CYP2C12 and 5α-reductase mRNA were increased in the same direction as ADH by GH infusion, whereas CYP2C11 was decreased. 18S was unchanged by treatment. Together, these changes in ADH-specific activity, protein content, and mRNA levels were consistent with alterations in the GH secretory pattern induced by sex steroid hormones.

Pretranslational regulation of hepatic ADH by pituitary hormones in Hx rats. Previous studies have shown complex hormonal regulation of ADH by pituitary hormones, but the mechanisms and interactions of these hormones in determination of the sexually dimorphic expression of ADH are unclear (9, 10, 42). Therefore, we examined whether the mode of GH administration was of primary importance in the regulation of the dimorphic expression of hepatic ADH activity. Table 4 demonstrates the effect of Hx and different GH replacement patterns in male and female rats on hepatic ADH-specific activities. Hx significantly (P < 0.05) increased ADH-specific activity in male and female rats 2.5- and 1.4-fold, respectively. Intermittent administration of GH after Hx in male and female animals only modestly (<0.05) decreased ADH activity, whereas infusion of GH did not significantly change values. Thus loss of pituitary hormones selectively increased ADH activity especially in males, whereas GH administration alone did not restore ADH levels in either sex to basal male values.

Changes in steady-state mRNA levels with Hx and GH replacements are shown in Figure 5, A and B. Hx increased ADH mRNA levels in both sexes, although more strikingly in males such that the new values were similar in both sexes. Intermittent GH administration in males decreased ADH mRNA (<0.05) but not in females, whereas infusion of GH selectively increased ADH mRNA in males. Furthermore, pharmacological doses of GH previously reported (39) or more frequent administration of GH (every 8 h instead of every 12 h) had no significant effect on ADH mRNA levels (data not shown).

Effect of Hx and GH administration on insulin-like growth factor-I (IGF-I), CYP2C11, and CYP2C12 is shown in Fig. 5B. Circulating levels of IGF-I and especially liver mRNA expression are known to be GH dependent (34). Multiple-size classes of IGF-I mRNA are present in rat liver due to variation in the 3′ untranslated region (23). Results demonstrated that Hx markedly reduced IGF-I levels as expected, and with either method of GH administration, values were restored to normal levels. Similarly, changes in CYP2C11 and CYP2C12 confirmed that our patterns of GH replacements produced the expected sex-specific responses (28).

Because infusion of GH to intact male rats increased ADH (however, we were unable to restore ADH to
normal basal values with either GH injections or infusion in Hx rats), we proposed that thyroid hormone, glucocorticoids, or both were required in addition to GH to produce the dimorphic expression of ADH. Administration of corticosterone-21-acetate either alone or with GH to Hx male rats did not alter either ADH-specific activity or mRNA levels (data not shown). On the other hand, thyroid hormone, which was previously implicated in the downregulation of ADH-specific activity and mRNA levels in intact rats (14, 38), was demonstrated to be an important component of ADH sexual dimorphic expression. Because previous studies had not considered the indirect effect of thyroid hormone on GH secretion (7), we examined the effect of T4 both alone and in combination with GH on hepatic ADH-specific activity and mRNA in Hx male and female rats. Table 5 shows changes in ADH-specific activity, whereas Fig. 6, A and B, illustrates mRNA levels after Hx and treatment with T4 alone or in combination with GH.

Increased ADH-specific activity after Hx was modestly ($P < 0.05$) reduced after either T4 or GH injections alone, but neither recapitulated the sexual dimorphic expression of ADH. On the other hand, T4 combined with GH injection reduced enzyme activity to basal male values in both sexes, whereas in contrast, T4 with GH infusions had no effect. Similar changes were measured in ADH mRNA levels (Fig. 6, A and B). Elevated ADH mRNA levels in Hx males ($P < 0.05$) and females ($P < 0.01$) were reduced by T4 administration alone, indicating that T4 in addition to its effects on GH levels also directly modified ADH expression. Because these changes did not reduce ADH to basal levels or simulate the dimorphic expression of ADH, we combined T4 with GH. In both sexes, GH injections plus T4 reduced ADH to male levels, whereas when T4 was combined with GH infusions, ADH remained at female levels. Together, the results strongly indicate that the combination of T4 specifically with intermittent (male-like pattern) GH administration is required to pretranslationally downregulate ADH and recapitulate the dimorphic expression of ADH.

DISCUSSION

Hormonal regulation of ADH has been extensively examined by a number of laboratories using in vivo animal models, hepatocytes, and cultured liver cell lines (8, 36). However, previous studies did not establish whether sex steroid hormones or GH were primarily responsible for the sexual dimorphic expression of ADH. Furthermore, the molecular mechanism(s) involved in increased ADH-specific activity in female rat liver has not been examined. In the present study we used Hx and gonadectomized as well as intact male and female rats given hormones to establish that the primary mechanism for differential sexual expression of ADH is mediated by pretranslational processes through the intermittent secretion of GH in combination with T4.
A number of hepatic proteins are under complex regulatory controls by hormones that determine their sex-specific expression (19, 28). Sexual dimorphic gene expression in liver can be controlled by three distinct but overlapping mechanisms: 1) androgen imprinting during a critical period in development, 2) circulating gonadal hormones in the adult, and 3) pattern of GH secretion. The secretory pattern of GH is perhaps the most common mechanism involved in the sex-specific expression of hepatic genes, especially those involved in drug metabolism. Pituitary GH secretion is pulsatile, with the frequency of pulsations being sex-dependent and under the influence of gonadal hormones (24). In adult female rats, a high pulse frequency results in the continuous presence of GH in the circulation at levels in excess of 10–20 ng/ml of plasma. By contrast, in adult male rats GH is present in plasma intermittently, with regular peaks detected every 3–4 h followed by trough periods of no detectable GH (15). These sexually differentiated plasma GH profiles in turn regulate expression of a number of liver-specific hepatic genes (12). Female-specific CYP2C12 is positively regulated by the continuous plasma GH pattern, whereas expression of male-specific CYP2C11 is stimulated by the male pattern of intermittent GH pulsations (56). However, expression of some male-specific genes is primarily derived from the suppressive influences of the continuous pattern of plasma GH rather than the positive influence of the male intermittent pattern (5, 13, 35, 45). GH pretranslationally and directly regulates these hepatic sex-dependent cytochrome P-450 enzymes (21, 29).

Changes in body weight were measured to determine adequacy of hormonal ablation and replacement protocols. In addition, it is known that starvation and caloric restriction decreases ADH levels primarily by post-translational mechanisms (3, 33). As other studies (24) have shown, untreated males gain weight faster than females. Importantly β-E but not testosterone treatment markedly reduced weight gain in both intact male and female animals (60). Hypophysectomy, as expected, prevented weight gain that was restored with GH replacement, indicating adequacy of pituitary ablation and hormone replacement. In contrast, sex steroid hormones and T4 administration to Hx rats did not restore weight gain; and importantly β-E especially in Hx males and T4 in Hx females significantly reduced weights. These later changes may contribute, in part, to reduced ADH activity and mRNA levels.

Studies have shown that hepatic ADH-specific activity is higher in female rats compared with males (4, 9, 51, 52), but the mechanism(s) is unclear. Androgens downregulated ADH-specific activity in some studies (37, 40, 43), but not others (9, 10), whereas estrogens increased hepatic ADH-specific activity in male rats but not females (41, 57, 58). Furthermore, it is unclear whether sex steroid hormones alter ADH activity directly in the liver or rather through modulation of the GH secretory pattern (9). Previous studies, in addition, did not examine the molecular mechanisms involved in the sexual dimorphic expression of hepatic ADH. The purpose of the present study was, therefore, to fill in these gaps and to determine the physiological and molecular mechanisms involved in the sexual dimorphic expression of ADH.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male</th>
<th>n</th>
<th>Female</th>
<th>n</th>
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<td>Sham</td>
<td>45.7 ± 2.34</td>
<td>18</td>
<td>80.0 ± 3.22</td>
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<tr>
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<td>14*</td>
<td>91.6 ± 5.41</td>
<td>15*</td>
</tr>
<tr>
<td>+GHinj</td>
<td>91.9 ± 7.30</td>
<td>13†</td>
<td>69.0 ± 0.70</td>
<td>3†</td>
</tr>
<tr>
<td>+GHp</td>
<td>115.0 ± 0.80</td>
<td>8</td>
<td>98.0 ± 4.00</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are units per gram protein expressed as means ± SE determined by ANOVA; n, number of individual determinations. ADH activity was measured spectrophotometrically in 100,000 g cytosol. Following Hx, rats were treated for 7 days with bovine growth hormone administered either by divided twice-daily injections (GHinj) or as a continuous infusion (GHp). *P < 0.05 compared with sham control; †P < 0.05 compared with Hx in males and females, respectively.

Fig. 5. Effect of hypophysectomy and GH replacement on ADH mRNA levels in male and female rats.

Changes in body weight were measured to determine adequacy of hormonal ablation and replacement protocols. In addition, it is known that starvation and caloric restriction decreases ADH levels primarily by post-translational mechanisms (3, 33). As other studies (24) have shown, untreated males gain weight faster than females. Importantly β-E but not testosterone treatment markedly reduced weight gain in both intact male and female animals (60). Hypophysectomy, as expected, prevented weight gain that was restored with GH replacement, indicating adequacy of pituitary ablation and hormone replacement. In contrast, sex steroid hormones and T4 administration to Hx rats did not restore weight gain; and importantly β-E especially in Hx males and T4 in Hx females significantly reduced weights. These later changes may contribute, in part, to reduced ADH activity and mRNA levels.

Studies have shown that hepatic ADH-specific activity is higher in female rats compared with males (4, 9, 51, 52), but the mechanism(s) is unclear. Androgens downregulated ADH-specific activity in some studies (37, 40, 43), but not others (9, 10), whereas estrogens increased hepatic ADH-specific activity in male rats but not females (41, 57, 58). Furthermore, it is unclear whether sex steroid hormones alter ADH activity directly in the liver or rather through modulation of the GH secretory pattern (9). Previous studies, in addition, did not examine the molecular mechanisms involved in the sexual dimorphic expression of hepatic ADH. The purpose of the present study was, therefore, to fill in these gaps and to determine the physiological and molecular mechanisms involved in the sexual dimorphic expression of ADH.
SELECTIVE GENDER EFFECTS OF ESTROGENS AND ANDROGENS ON ADH SPECIFIC ACTIVITY AND mRNA LEVELS IN THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

Table 5. Effect of thyroid and GH replacements on ADH-specific activities in Hx male and female rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham/Intact</td>
<td>44.2 ± 1.6</td>
<td>89.9 ± 5.7</td>
</tr>
<tr>
<td>Hx</td>
<td>81.7 ± 2.2</td>
<td>109.9 ± 6.5</td>
</tr>
<tr>
<td>+T4</td>
<td>64.4 ± 5.9</td>
<td>67.8 ± 2.4</td>
</tr>
<tr>
<td>+GHinj</td>
<td>73.3 ± 1.7</td>
<td>90.7 ± 1.2</td>
</tr>
<tr>
<td>+GHinj + T4</td>
<td>38.9 ± 2.5</td>
<td>41.6 ± 1.2</td>
</tr>
<tr>
<td>+GHp</td>
<td>90.2 ± 3.0</td>
<td>112.7 ± 3.9</td>
</tr>
<tr>
<td>+GHp + T4</td>
<td>81.4 ± 1.6</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are units per milligram protein expressed as means ± SE as determined by ANOVA; n, number of animals examined. ADH activity was determined in liver cytosol fractions spectrophotometrically. Male and female rats were hypophysectomized and after stabilization were either untreated, administered 50 μg-kg⁻¹·day⁻¹ T4 or 120 μg-100 g⁻¹·day⁻¹ GH, either by twice-daily injections or infusions, or given a combination of GH + T4 for 7 days. *P < 0.05 compared with sham; †P < 0.05 compared with Hx.

Consistent with previous reports, hepatic ADH-specific activity was approximately twofold greater in both liver homogenates and cytosols from female rats compared with males (4, 52). In addition, we demonstrated that ADH protein content and steady-state mRNA levels were 1.8- and 2.4-fold greater in females, respectively. Thus, indicating that the sexual dimorphic expression of hepatic ADH is pretranslationally regulated. To examine whether sex steroid hormones were responsible for the dimorphic expression of hepatic ADH, male and female rats were either castrated or given hormones. Estrogens increased ADH-specific activity and mRNA levels in males, whereas androgens decreased ADH levels in females, consistent with sex-specific changes in ADH. However, neither administration of estrogens in males nor testosterone in females completely restored the dimorphic expression. This partial response may be due to hormonal imprinting established in the neonatal period (25, 26).

Selective gender effects of estrogens and androgens on ADH suggested that the differential effect might be mediated secondary to alterations in the GH secretory pattern rather than a direct effect on the liver. This proposal was supported by the following observations: 1) β-E administration to Hx rats did not increase ADH activity or mRNA in either sex; 2) testosterone administration had no effect on ADH in Hx rats of either sex; 3) changes in the male-dominant gene CYP2C11 and the male-dominant CYP2C12 with sex steroid hormones and Cx are consistent with previous reports of changes with GH secretory patterns (44); and 4) infusion of GH into male rats increased ADH-specific activity, protein content, and mRNA to levels measured in females. Together, the results support an important role for the differential pattern of GH secretion in the regulation of ADH. Furthermore, because changes in ADH mRNA and protein were coordinately expressed, the results are consistent with pretranslational mechanisms rather than changes in protein turnover (9, 43).

Sexual dimorphic expression of ADH could be due to stimulation by the continuous secretion of GH (female pattern), inhibition by the intermittent malelike pattern, or an interaction of GH with other pituitary hormones. Hx, especially in the male rat, increased ADH activity and mRNA. These results suggested that intermittent GH secretion decreased ADH levels in males, similar to the regulation of s-adenosylmethionine synthetase (46). However, in the Hx rat, intermittent administration of GH only modestly decreased ADH, whereas GH infusions had no significant effect on ADH.

Regulation of CYP genes by pituitary hormones is frequently complex, involving interplay between different hormones. This seemed particularly likely in the regulation of ADH, because several studies have not only implicated GH but glucocorticoids and especially thyroid hormone in regulation of ADH levels (8, 42). Because both glucocorticoids and thyroid hormone can regulate GH levels (62), we examined the effect of these hormone replacements in the Hx male and female rat. Glucocorticoid administration (data not shown) did not significantly alter ADH levels in Hx rats. There-

Fig. 6. Effect of thyroxine and GH administration of hepatic ADH mRNA levels. Male and female rats were Hx and after recovery were either sham treated or administered thyroxine (T4) (50 μg-kg⁻¹·day⁻¹), bGH (120 ng-100 g⁻¹·day⁻¹), either as injections or infusions, or a combination of bGH plus T4 for 7 days. Four separate animals were analyzed in each group. A: liver ADH mRNA levels in males. B: liver ADH mRNA levels in female rats. Total liver RNA was extracted and ADH levels were determined as previously described. 18S rRNA was determined to compare loading in each experimental group. Results are expressed as means ± SE relative to sham male controls. Number of animals was 3–7 as indicated in Table 5.
fore, we then examined T4. Thyroid hormone administration to Hx rats reduced ADH activity and mRNA levels in both sexes but not to basal levels. However, the combination of T4 with intermittent GH administration reduced ADH activity and mRNA to or below basal ADH levels measured in males. This effect was specific for the pattern of GH secretion, because the administration of constant GH infusions with T4 did not significantly alter ADH levels. Furthermore, because the administration of cortisone acetate, in addition to GH and T4, did not contribute to dimorphic expression of ADH (data not shown), T4 and intermittent GH administration are not only specific but also sufficient to lead to sex differences in ADH.

The molecular mechanism(s) accounting for thyroid repression of ADH either alone or with GH is not clear. A putative thyroid response element has not been demonstrated in the rat promoter (14), but Harding and Duester (22a) have demonstrated that thyroid may compete at retinoic acid receptor elements in the human promoter. However, in the intact animal, thyroid hormone was also shown to regulate circulating levels of GH that might, in turn, regulate ADH. Previous reports have demonstrated that the female-dominant gene, 5α-reductase, required thyroid hormone in addition to GH to increase mRNA to normal levels in the Hx rat model (53). Although, the molecular mechanism has not been examined, intermittent GH pulses increase nuclear levels of signal transducer and activator of transcription (Stat5b), resulting in signaling through Stat binding sites. The rat ADH promoter has a putative Stat response element at −210 bp upstream, which has been shown to bind Stat5b (48). However, the mechanism permitting T4 to interact with Stat5b and negatively regulate ADH gene expression is unknown. One possibility is that thyroid hormone may inhibit translocation of Stat5 as shown by Farve-Young et al. (17).

In summary, our experiments demonstrate that neither GH nor thyroid hormone alone can account for the dimorphic expression of hepatic ADH; however, T4 together with the intermittent secretion of GH down-regulates ADH, leading to its sexually dimorphic expression. Thus hormonal regulation of ADH provides an excellent model to understand the complex hormonal regulation of a major liver gene that is rate limiting in ethanol oxidation.

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REFERENCES


