Hormonal regulation of hepatic multidrug resistance-associated protein 2 (Abcc2) primarily involves the pattern of growth hormone secretion

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Hormonal regulation of hepatic multidrug resistance-associated protein 2 (Abcc2) primarily involves the pattern of growth hormone secretion. Am J Physiol Gastrointest Liver Physiol 290: G595–G608, 2006; doi:10.1152/ajpgi.00240.2005.—Biliary excretion is the rate-limiting step in transfer of bilirubin, other organic anions, and xenobiotics across the liver. Multidrug resistance-associated protein 2 (Mrp2, Abcc2) is the major transporter for conjugated endo- and xenobiotic-conjugated compounds into bile. Hormones regulate bilirubin and xenobiotic secretion into bile, which have dimorphic differences. Therefore, we examined the possible role of sex steroids and growth hormone in the regulation of Mrp2. In ~8-wk-old rats, mRNA, transcriptional activity, and hepatic content of Mrp2 were selectively increased fourfold (P < 0.001) in females compared with males. In males, estrogens increased and testosterone decreased Mrp2 mRNA and protein, whereas no significant effect was measured in females, suggesting either a direct effect on the liver or an alteration in growth hormone secretory pattern. After hypophysectomy, Mrp2 mRNA was markedly reduced and the effects of estrogens and testosterone on Mrp2 were prevented, supporting the role of pituitary hormones in controlling Mrp2 expression. Mrp2 increased following growth hormone infusion in males. Mrp2 mRNA was decreased in growth hormone-deficient “Little” mice. Growth hormone infusions in hypophysectomized rats partially restored Mrp2 levels, whereas thyrroxine addition returned Mrp2 mRNA and protein to basal levels. Morphology as well as biochemical measurements demonstrated that Mrp2 was localized to the bile canaliculus in equal density in both genders, whereas hormone replacements increased Mrp2 in hypophysectomized animals. In cultured hepatocytes, thyrroxine did not have an effect, but growth hormone alone and combined with thyrroxine increased Mrp2 mRNA levels. In conclusion, Mrp2 levels are regulated by the combination of thyrroxine and different growth hormone secretory patterns.

estrogen; thyroid; glucocorticoids; bilirubin
bin and BSP excretion is biliary excretion (2, 80), these studies suggest that hormones may regulate Mrp2/Mrp2. Therefore, we examined the effect of pituitary and sex steroid hormones on the expression of Mrp2 protein and mRNA in rats. Mrp2 protein and mRNA levels were greater in females than males due to increased transcription. The sexual dimorphic expression was not due to the direct effect of sex steroid hormones but was controlled from a combination of growth hormone and thyroid hormone. In contrast, glucocorticoids had a direct effect on Mrp2 expression but did not affect growth hormone/thyroid effects.

**METHODS**

**Experimental animals and hormone therapy.** Adult male and female Sprague-Dawley rats, weighing between 170 and 225 g, were purchased from Harlan (Indianapolis, IN). Rats were obtained following hypophysectomy (Hx), thyroidectomy (Tx), adrenalectomy (Adx), ovariec-tomy, or castration along with age-matched controls and were maintained under standardized conditions of temperature (22–24°C), humidity, and light and darkness. Animals were provided water and rat chow ad libitum until 18 h before death, when food was removed. Surgery was performed by the supplier on rats weighing 185–200 g, followed by stabilization for a period of at least 6 days within our animal facilities before any treatments. Hx rats that gained weight were eliminated from further study (50). Animals were killed by 9:00 AM. As indicated under each figure, Hx, Tx, and Adx rats were given replacement doses of cortisone 21-acetate (50 μg·kg⁻¹·day⁻¹) and l-thyroxine (Tx; 50 μg·kg⁻¹·day⁻¹) for 7 days as daily subcutaneous injections. Recombinant bovine growth hormone was a generous gift from Protiva (Monsanto). The daily dose of growth hormone was 60 μg·100 g body wt⁻¹·day⁻¹. Bovine growth hormone was given either continuously (femalelike pattern) by means of an Alzet model 2/H11003 osmotic minipump (Alza, Palo Alto, CA) at 60 μg·100 g body wt⁻¹·day⁻¹ × 7 days or intermittently (malelike pattern) twice daily at 30 μg·100 g as subcutaneous injections for 7 days. The osmotic minipumps were implanted subcutaneously on the backs of rats under light anesthesia. The solvent for bovine growth hormone was 0.05 M Na₂PO₄ (pH 8.8), 1.6% glycerol, and 0.02% NaN₂.

**Isolation of bile canaliculus membrane fractions.** Purification of the fractions was as previously described (59). Briefly, 25 mM MgCl₂ was added to homogenized liver tissue in 15 ml buffer [in mM: 300 mannitol, 5 EGTA, 18 Tris (hydroxymethyl)aminomethane hydrochloride, and 0.1 PMSF at pH 7.5] to give a final concentration of 15 mM MgCl₂ and centrifuged at 2,400 g for 15 min. The supernatant from the MgCl₂ step was centrifuged at 45,000 g for 30 min to obtain the bile canalicular membrane fraction. Purification of the fractions was monitored by immunoblotting for Mrp2 and Bsep.

**Nuclear run-on assay.** Transcriptional activity was determined using the run-on assay. RNA transcripts that have already been initiated are fully elongated when using nuclei isolated as described by Gorski et al. (20) from fresh livers of male and female rats. Livers from male and female rats were homogenized in ice-cold homogenization buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 0.15 mM DTT, and 0.5 mM PMSF) using a Dounce homogenizer. Nuclei (1 × 10⁸) were separated by sucrose gradient centrifugation, resuspended in 100 μl glycerol storage buffer (50 mM Tris, pH 8.0, 40% glycerol, 5 mM MgCl₂, and 0.1 SDS-10 μg RNase A/ml) and 0.1× SSC-0.1% SDS, and exposed to film with screens at −70°C. Density of the slot blots was determined by densitometry and expressed relative to β-actin.

**Immunoblotting.** Total liver fractions were prepared for immunoblotting by Na₂CO₃ extractions of liver homogenates (3), and bile canalicular membrane fractions were isolated (59) before SDS-PAGE and immunoblotting as previously reported (64). After electrophoresis, proteins were transferred to Hybond ECL membranes (Amersham) by the procedure of Towbin et al. (74). Geometrical areas for 4 h using 5% Tween-TBS and processed for ECL detection (Amersham) using 1% milk in TBS for antibody diluent. Blots were visualized by Strept-Avidin horseradish peroxidase detection system (Amersham). After being washed with 0.5% Tween-TBS for 5 min × 3, ECL blots were exposed to Amersham Hyperfilm for ECL for 30–60 s. Autoradiograms were quantitated by imaging densitometry. The following antibodies were used: polyclonal antibodies to Ntcp (64), Bsep, and Mrp2 (35). Monoclonal antibodies to Mdr2 (Signet Laboratories, Dedham, ME) and Mrp2 (MII3, 6; Alexis Biochemical-San Diego, CA) were also used. Protein loading was corrected by using amnioblack staining of gels for total protein content.

**Immunofluorescence morphology.** Slices of liver (4 mm thick) were made, immersed in optimal cutting temperature compound (Sakura Fine Tech, Torrence, CA), rapidly frozen in a dry-ice acetate bath, and stored at −70°C. Cryostat sections (4 μm) were taken at −20°C, mounted on charged microscope slides by briefly thawing, and immediately fixed by being plunged into methanol at −30°C. After being fixed for at least 30 min, the sections were stored in PBS at 4°C until being processed the next day for immunofluorescence.

Labeling for immunofluorescence was carried out by the series of incubations at room temperature as follows: 1) 15 min in PBS containing 10% BSA and 0.25% glycine; 2) 60 min in mouse anti-Mrp2 (Alexis) diluted 1:20 in PBS containing 10% BSA; 3) washed twice, 12 min each, with PBS; 4) 30 min in goat anti-mouse IgG conjugated with Alexa 595 (Molecular Probes, Eugene OR); and finally washed as in step 3. The sections were mounted in Mowiol and observed with a Nikon Diaphot microscope equipped with the appropriate fluorescence filter sets. The images were captured with a Coolspot charge-coupled device camera while using identical camera settings. Five regions of each tissue section were photographed and evaluated in a blinded fashion by assigning a numerical value of 0–4 for fluorescence brightness. The final figure was assembled using Photoshop without altering the image histograms until the final figure was assembled.

**Isolated rat hepatocytes.** Rat hepatocytes were isolated by the in situ collagenase perfusion method of Berry and Friend (6) as previ-
ously described. Isolated rat hepatocytes (>95% viable by trypan blue exclusion) were plated at a density of 3.2 × 10⁶ cells on Matrigel-coated plates (100 μg/100 μl) in defined Waymouth’s media containing insulin (10⁻⁷ M). After 3 h, the media were changed to fresh Waymouth’s media plus insulin (10⁻⁹). The added hormones and their concentrations were IGF-I (3 × 10⁻⁸ M), T₃ (10⁻⁸ M), dexamethasone (10⁻⁷ M), bovine growth hormone (100 ng/ml), or a combination of growth hormone plus T₃. Cultured hepatocytes were harvested 24 and 48 h after the addition of hormones, and RNA was extracted as previously described (72).

**Data analysis.** Data are expressed as means ± SE and were analyzed statistically by using one-way ANOVA, followed by post hoc analysis with Tukey’s test using the Prism program. 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**

Levels of bile canicular ABC transporters in male and female rats. In humans as well as rats, uptake, conjugation, and excretion of bilirubin and BSP are greater in females than in males (4, 48, 51, 84). Therefore, we initially examined the possibility that gender differences in organic anion transport capacity may be related to expression of bile canicular transporters. Eight-week-old male and female Sprague-Dawley rats were killed, and total liver homogenates were analyzed for protein and mRNA content. Three bile canicular ABC transporters were determined including Bsep, the primary bile salt transporter Mdr2, which is involved in phospholipid excretion, and Mrp2, which is involved in bilirubin transport (Fig. 1). Carbonate-extracted liver homogenate proteins were separated on SDS-PAGE gels and blotted with monospecific antibodies. Figure 1A shows the protein levels of Bsep, Mdr2, and Mrp2. Neither Bsep nor Mdr2 content was differentially expressed in males compared with females. In contrast, hepatic protein levels for Mrp2 increased 4.5-fold (P < 0.001) in the female liver compared with the male liver. Thus hepatic protein content of Mrp2 was selectively expressed in a sexually dimorphic pattern.

To determine whether increased Mrp2 was pretranslationally regulated, steady-state mRNA levels for these ABC transporters were examined. Northern blot analysis was performed using 3²P-labeled cDNA probes. Steady-state mRNA levels for Bsep, Mdr2, and Mrp2 are shown in Fig. 1B. Bsep and Mdr2 mRNA levels were not significantly different. Liver Mrp2 mRNA levels were 3.5-fold (P < 0.01) greater in females compared with males, indicating that the differences in protein levels are selective and pretranslationally regulated.

Because mRNA and protein for Mrp2 were similarly increased, we next determined whether increased steady-state mRNA levels were transcriptionally regulated. Liver nuclei were isolated, and transcript initiation was determined by nuclear run-on analysis. The results are shown in Fig. 1C. The Mrp2 transcription rate was fourfold (P < 0.01) greater in nuclei from the female liver compared with the male rat liver indicating that the gender differences in Mrp2 expression are due to transcriptional mechanisms.

Sex hormone regulation of gender differences in Mrp2 expression. Bilirubin conjugation and maximal biliary excretion are influenced by sex steroid hormones (48). To determine the possible role of sex steroid hormones in regulating Mrp2, we examined the effect of estrogens, testosterone, and gonadectomy in male and female rats on Mrp2 mRNA and protein. Mrp2 mRNA levels in male and female rats are shown in Fig. 2, A and B. In males, βE increased Mrp2 mRNA 2.4-fold (P < 0.001). Similarly, male castration, with loss of testosterone, increased (P < 0.01) Mrp2 mRNA, which was restored to control levels with testosterone replacement. In contrast, in female rats (Fig. 2B), βE had no effect on the already high level of Mrp2 (3.4-fold greater than in males), indicating that the effect of estrogen administration to males is selective. Both ovariectomy and testosterone reduced Mrp2 mRNA levels, but the results did not reach statistical significance.
Hepatic Mrp2 protein levels after sex steroid hormone administration are shown in Fig. 2, C and D. In males, βE administration increased (P < 0.001) hepatic Mrp2. Although Mrp2 protein content did not change after castration, testosterone addition to castrated male rats significantly (P < 0.01) reduced Mrp2 levels. As previously shown, in females, Mrp2 protein levels were greater than in males. Similar to the results with mRNA, βE did not significantly change Mrp2, but ovariec-tomy and ovarioectomy plus testosterone decreased (P < 0.01) Mrp2 protein content. Thus sex hormone modification changed expression of Mrp2, suggesting an important role for estrogen and testosterone in determining constitutive sex differences in Mrp2.

The sex-specific effects of estrogen and testosterone suggest these hormonal effects may be mediated directly on the liver or, alternatively, indirectly possibly by changing the growth hormone secretory pattern. To determine whether sex steroids directly regulated Mrp2, we hypophysectomized male and female animals before administration of estrogens or testosterone. Figure 3, A and B, shows that in both genders, hypophysectomy markedly (P < 0.01) reduced Mrp2 mRNA, indicating the importance of pituitary hormones in regulation of hepatic Mrp2. Furthermore, administration of βE or testosterone to male or female Hx animals did not significantly change Mrp2 levels. Taken together, these results indicate the importance of pituitary hormones in regulating Mrp2 and that estro-
GROWTH HORMONE SECRETION DETERMINES HEPTIC MRP2 EXPRESSION

G599

gens and testosterone probably exert an indirect effect on Mrp2, possibly by changing the growth hormone secretory pattern (31).

Growth hormone regulation of Mrp2. Sexual dimorphic expression of hepatic genes is more commonly due to differences in the pituitary growth hormone secretory pattern (23, 42). If the pattern of growth hormone secretion is important, one would predict that changing the male secretion pattern to a “femalelike” steady pattern would increase Mrp2 expression. Therefore, osmotic minipumps were placed into male rats, and a “femalelike” steady pattern would increase Mrp2 expression. If the pattern of growth hormone secretion is important, one would predict that changing the male secretion pattern to another “female-dominant” hepatic gene. Thus changing the pattern of growth hormone secretion in intact male rats infused for 7 days. Figure 4 shows the effect of growth hormone infusion in male animals for 7 days. Growth hormone significantly (P < 0.01) increased total hepatic Mrp2 protein content compared with sham-treated male controls. Mrp2 mRNA content was also increased following growth hormone infusion in male animals (Fig. 4B). These values were not significantly different from the results in sham control female rats, indicating that the pattern of growth hormone secretion is an important determinant of Mrp2 levels and that these changes are due to transcriptional regulation. In contrast, no changes in the bile canalicular ABC transporter Bsep with growth hormone infusion were observed, indicating that the increase in Mrp2 is selective. A similar response to growth hormone infusion was measured for 5α-reductase, another “female-dominant” hepatic gene. Thus changing the pattern of growth hormone secretion in intact male rats increased both Mrp2 protein and mRNA levels. Similar changes were previously reported for 5α-reductase (57), suggesting that the growth hormone secretory pattern is principally responsible for pretranslational regulation of the sexual dimorphic levels of Mrp2.

Changes in Mrp2 expression in Little mice. To examine the selective contribution of growth hormone independent of other pituitary hormone deficiencies, we next determined hepatic Mrp2 mRNA levels in the mutant mouse model, Little mouse, which has marked deficiencies in growth-hormone secretion (15, 19). Figure 5, A and B, shows Mrp2 mRNA levels from wild-type (C57 strain +/+), heterozygotic Little mice (lit+/−), and homozygotic Little (lit−/−) mice. Neither wild-type C57+/+ (the original strain) nor heterozygous (+/−) mice demonstrated significant gender differences in Mrp2 mRNA levels, indicating that dimorphic expression of Mrp2 may be specific to rats. However, Mrp2 mRNA levels were significantly reduced in male (P < 0.01) and female (P < 0.001) homozygotic (−/−) mice, which were selectively deficient in growth hormone. These results indicate that, although dimorphic differences in Mrp2 expression may be unique to rats, growth hormone is the major hormone regulating expression of Mrp2 in mice as well as rats.

To examine directly the role of the growth hormone secretory pattern in the regulation of Mrp2, male and female rats were hypophysectomized and, after stabilization, were administered growth hormone either in the intermittent “male pattern” or by constant infusion to mimic the “female pattern” for 7 days as previously described (66). Female (Fig. 6, A and B, shows that hypophysectomy significantly (P < 0.001) reduced Mrp2 mRNA in male and female rats. Replacement of growth hormone by an intermittent injection in both male and female Hx animals failed to increase Mrp2 mRNA levels compared with hypophysectomy alone. However, infusion of growth hormone (female pattern) increased (P < 0.01) Mrp2 mRNA compared with hypophysectomy, especially in females. Although Mrp2 values obtained with growth hormone infusion in males were not significantly different from basal values, the effect in females was still significantly (P < 0.001) different from basal values. Statistical significance was determined by one-way ANOVA. Numbers in parentheses indicate numbers of individual experiments.
female levels. This observation suggested other factors may be required to attain female Mrp2 levels.

Figure 6, C and D, shows the changes in protein levels following hypophysectomy and growth hormone replacement. Hypophysectomy markedly reduced Mrp2 protein levels, but neither growth hormone injections nor infusions restored Mrp2 protein levels to the basal state, further indicating that other hormones are required in conjunction with growth hormone. Because hypophysectomy leads not only to reduced growth hormone but also to reduced levels of thyroid and adrenal steroids, we next explored the possibility that these hormones are involved in sexual dimorphic expression of Mrp2.

Combination of hormone replacements on Mrp2 expression. Growth hormone frequently works together with other hormones to regulate hepatic gene expression (73). Therefore, we examined whether corticosterone and T4 in addition to different patterns of growth hormone administration might recapitulate the gender-dependent differences in Mrp2 expression.

Fig. 5. Effect of GH deficiency in C57 and mutant mice on hepatic Mrp2 mRNA levels. A: males (filled bars). B: females (open bars). Mice liver RNA was extracted from C57, heterozygous “Little” (lit; +/-), and homozygous Little (-/-) mice. Mrp2 mRNA levels were quantitated by densitometry and corrected for loading differences, and levels were compared with male C57 levels set at 100%. Statistical significance was determined by one-way ANOVA. Four animals in each group were analyzed.

Fig. 6. Effect of GH deficiency in male and female rats. Rats were hypophysectomized at ~200 g and allowed to recover from surgery for 1 wk, followed by GH administration for 1 wk. Males are shown in A, and female values are shown in B. One set of animals of each sex was administered GH (60 μg/day sc) by injection every 12 h (GHinj), whereas the other group had GH (120 μg/day) continuously infused by osmominipump (GHp). Liver RNA was extracted, separated, and probed with 32P-labeled Mrp2 cDNA. Northern blots were quantitated by scanning densitometry, and male values were normalized to 100%. Numbers in parentheses indicate numbers of animals in each group. Significance between groups was compared by one-way ANOVA. Statistical differences are shown between experimental groups and control male or female values (values above bars) except where indicated by lines. Mrp2 protein content was measured by Western blots, and the results are shown in C for males and D for females. Results are from 2 separate experiments, and the mean %difference (%Δ) compared with intact male is shown.
After hypophysectomy, male and female rats were administered either the combination of corticosterone and thyroid hormone alone or with different gender-specific patterns of growth hormone administration. Mrp2 mRNA and protein levels were determined after 7 days of treatment. Figure 7, A and B, shows the changes in hepatic Mrp2 mRNA in response to the combination of thyroid hormone and corticosterone alone or with growth hormone in Hx rats. Hypophysectomy reduced Mrp2 content in both genders as previously shown. Addition of thyroid and corticosterone hormones together significantly increased Mrp2 mRNA levels compared with hypophysectomy but only to the male value in both genders. The addition of growth hormone injections (male pattern) in addition to corticosterone and thyroid hormone did not further significantly increase Mrp2 values in either sex. In contrast, growth hormone infusions (female pattern) with thyroid and corticosterone hormones in both sexes significantly (P < 0.001) increased Mrp2 mRNA compared with Hx rats to values not significantly different from those measured in normal basal females.

Figure 7, C and D, shows changes in hepatic Mrp2 protein levels. The pattern of changes in Mrp2 protein was similar to mRNA, strongly suggesting that the molecular alterations were associated with functional changes. Importantly, addition of growth hormone in different secretory patterns resulted in gender differences in total hepatic Mrp2 protein expression. Thus these hormone replacement studies recapitulated the in vivo observations.

Effect of glucocorticoids or thyroid hormone in combination with growth hormone on Mrp2 expression. The previous studies demonstrated that corticosterone and/or thyroid hormone were required along with growth hormone not only to achieve female values of Mrp2 but also to establish the sexual dimorphic expression of Mrp2. However, it is not clear whether both thyroid and corticosterone hormones were required with growth hormone or rather just one of the hormones was necessary. Therefore, in Hx rats, we next examined whether corticosterone or thyroid hormone administered alone with different growth hormone administration patterns was essential for the differential Mrp2 response. Figure 8, A and B, shows the effect of individual hormones in association with growth hormone on the increase in Mrp2 mRNA levels. In male Hx rats, corticosterone plus growth hormone only modestly increased Mrp2 mRNA compared with hypophysectomy. On the other hand, thyroid hormone administration with growth hormone recapitulated the gender differences in Mrp2 expression. In particular, thyroid hormone plus growth hormone infusions increased (P < 0.001) Mrp2 mRNA, and this increase was greater (P < 0.05) than growth hormone given by injection. A similar pattern of response to hormone replacement was measured in female rats except that a small but significant (P < 0.05) increase in Mrp2 mRNA was measured with growth hormone infusions plus corticosterone. Thus thyroid hormone with different growth hormone secretion patterns is required for gender differences in Mrp2 mRNA.

Figure 8, C and D, shows the effect of hormone replacement on Mrp2 liver protein expression in male and female rats. The combination of corticosterone with different patterns of growth hormone infusions significantly increased total hepatic Mrp2 protein content in both genders. Similarly, thyroid hormone with growth hormone given in the male pattern demonstrated modest (P < 0.05) increases of Mrp2 protein. In contrast, female levels of Mrp2 were attained with the combination of growth hormone infusions and thyroid hormone. These results
indicate that increases in Mrp2 result from pretranslational mechanisms for thyroid hormone plus growth hormone; both are necessary and sufficient to restore the sexual dimorphic expression of Mrp2.

**Immunofluorescence analysis of changes in Mrp2 protein with hormonal modifications.** To determine the cellular location of Mrp2, we used immunofluorescence to examine changes in Mrp2. Figure 9 shows localization of Mrp2 in male and female rat livers and the effect of hypophysectomy and hormone replacement. Figure 9, A and B, demonstrates immunofluoresence intensity of Mrp2 protein for males (C) and females (D). Numbers in parentheses for the Northern blots indicate numbers of animals analyzed in each group. The results of 4 individual samples in each group were analyzed for Western blots. Significance between groups was determined by one-way ANOVA. Statistical differences are shown between experimental groups and control male or female values except where specifically indicated by bars.

**Immunoblotting of liver bile canalicular membrane fractions.** To confirm the apparent changes in bile canalicular protein content, we prepared bile canalicular membrane fractions from male and female rat liver. Figure 10, A and B, demonstrates that Mrp2 content was not significantly different between male and female membrane fractions. We also measured the relative enrichment of Bsep, a canalicular transporter that is not regulated by hormones, and Mrp2 to determine whether the lack of changes in Mrp2 content was due to differences in membrane fraction recovery. Relative enrichment of Bsep was not significantly different (38 ± 5 vs. 32 ± 6), whereas Mrp2 enrichment in males was 27 ± 2 compared with 13 ± 3 in females (P < 0.001). These results confirm the morphological observations and furthermore suggest that the lower recovery of Mrp2 in females may reflect its location in an unidentified pool.

Figure 10 also demonstrates that the immunofluorescence differences in Mrp2 with hypophysectomy and hormone replacement in both male and female rats was quantitatively similar. Hypophysectomy significantly reduced bile canalicular Mrp2 protein in male and female livers (P < 0.001). Similar to morphological changes, growth hormone injections with thyroid hormone modestly increased Mrp2 (P < 0.01) compared with hypophysectomy to values similar to male control levels. On the other hand, growth hormone infusions in both males and females restored female levels of Mrp2 protein in bile canalicular fractions to values greater than male basal values (P < 0.001). Thus biochemical changes confirm the morphological studies.

**Effect of corticosterone and thyroid hormone on Mrp2 mRNA and protein content.** Next, we explored the selective importance of adrenal steroids and thyroid hormone in regula-
tion of Mrp2 mRNA and protein content of Mrp2. Pharmacological doses of synthetic glucocorticoids induce Mrp2 (16, 71), but their importance in physiological regulation is less clear. Furthermore, the effect of thyroid hormone in the regulation of Mrp2 has not been examined. Figure 11 shows the effect of adrenalectomy or thyroidectomy plus specific hormone replacement on Mrp2 mRNA and protein levels in male rats. Adrenalectomy significantly reduced Mrp2 to 25% ($P < 0.05$) of control values (Fig. 11A). This decrease is due to loss of glucocorticoids because corticosterone replacement doses restored values to control. Protein changes demonstrated the same trend; however, the differences were not statistically significant (Fig. 11C).

Figure 11, B and D, shows the effect of thyroidectomy and specific replacement on Mrp2 mRNA and protein levels. Mrp2 mRNA was not significantly changed, indicating that the effect of thyroid hormone may be to enable growth hormone to modify Mrp2 expression. In contrast, Mrp2 protein levels (Fig. 11D) after thyroidectomy and thyroid hormone replacement demonstrated significant changes in Mrp2 protein content. These studies suggest that, compared with growth hormone, both glucocorticoids and thyroid hormone have a small but possibly functionally important effect on Mrp2 levels.

Hormonal regulation of Mrp2 mRNA in cultured isolated liver cells. The animal studies suggest that growth hormone with thyroid hormone is important in the regulation of Mrp2. In addition, glucocorticoids may contribute to expression of Mrp2, but independently of growth hormone secretion. Therefore, we examined whether these effects were mediated directly at the hepatocyte level or perhaps indirectly by growth hormone induction of IGF-I, which by autocrine secretion stimulates Mrp2 mRNA induction. To examine these possibilities, we cultured rat hepatocytes on Matrigel. Figure 12A demonstrates that IGF-I addition to hepatocyte cultures did not significantly change Mrp2 mRNA levels over 48 h. In contrast, glucocorticoids (dexamethasone) significantly increased Mrp2 mRNA twofold ($P < 0.05$), indicating that this culture system responds to hormonal induction. Figure 12B shows the effect of thyroid hormone, growth hormone, and the combination of T3 and growth hormone on Mrp2 mRNA levels over 48 h. Thyroid hormone alone did not significantly increase Mrp2 mRNA, but growth hormone alone and especially in combination with thyroid significantly increased Mrp2 mRNA levels, consistent with the in vivo results. These results strongly suggest that growth hormone and thyroid hormone directly induce Mrp2 mRNA levels.

Fig. 9. Detection of Mrp2 by immunofluorescence. The images show immunodetection of Mrp2 at the canalicular level for males (left) and females (right); for controls (A and B); hypophysectomy (HYPOX; C and D); HYPOX + T4 and GH (E and F); and HYPOX + T4 and GHp (G and H). Images are representative of 3 independent experiments per group. No apparent differences in the immunodensity of Mrp2 are noted between control male and female liver sections. Hypophysectomy markedly decreased Mrp2 detection. Hormonal replacement restored Mrp2 content in the bile canalicular domain. Intracellular staining of Mrp2 was not detected in either male or female liver sections. Bar = 10 μm.
DISCUSSION

Sex differences in hepatic transport of a variety of endo- and xenobiotics have been described in rats (43, 67). Hepatic excretion of bilirubin glucuronide and the glutathione conjugate of BSP are significantly greater in female compared with male rats (48), (69). Furthermore, studies in humans and rats have revealed that both growth hormone and thyroid hormone are involved in the regulation of maximum excretion of bilirubin and BSP (18, 36, 44, 55). Therefore, the present studies were undertaken to determine whether Mrp2 is sexually dimorphically expressed and whether hormones, including growth hormone and thyroid hormone, regulate its expression. In rats, our results demonstrate that Mrp2 is increased in females compared with males, and this differential expression is transcriptionally regulated by the sexually dimorphic growth hormone secretory pattern in combination with thyroid hormone. However, Mrp2 content at the bile canalicular membrane was similar in both genders, and mRNA levels in mice were not dimorphically expressed.

Bilirubin clearance involves a putative bilirubin uptake transporter (SLC21A6), intracellular binding protein GSTA1, the phase II conjugating enzyme, UDP-gluronosyltransferase (UGT1A1), and Mrp2. In humans, serum bilirubin levels are significantly lower in females and bilirubin clearance is greater (4, 51). In addition, studies in rats have shown that GSTA1 and UGT1A1 are greater in females compared with males (49, 70), whereas only one study has addressed possible gender differences in the expression of Mrp2 (32). Therefore, we examined the hypothesis that Mrp2 expression was similarly increased in female rats compared with males. Hepatic levels of Mrp2 protein and mRNA were measured in normal male and female rats, and the values were compared with those of other bile
canalicular ABC transporters for bile acids (Bsep) and phospholipids (Mdr2). Although expression of Bsep and Mdr2 were similar in males and females, Mrp2, the transporter responsible for excretion of conjugated bilirubin, was significantly increased in females. This difference was transcriptionally regulated as measured by nuclear run-on experiments. A previous report (14) indicated that female protein and mRNA values were elevated compared with males between 25 and 40 days, similar to the age of the animals in our study. Although the exact mechanism for the increase in Mrp2 and the sexual dimorphic difference was not investigated by Johnson et al. (32), it is interesting to note that Eden (14) has shown that beginning with 25- to 30-day-old rats, there is a sex difference in the secretion of growth hormone. Thus these latter studies strengthen the argument that Mrp2 is sexually dimorphic and extend the mechanism to the level of transcription possibly involving the dimorphic secretion of growth hormone.

Sexually dimorphically regulated genes are well characterized in the rat liver (22, 78). Particularly well characterized are cytochrome P-450s, which are also regulated by growth hormone secretion (83). Other characterized genes showing dimorphic expression are involved in phase II reactions including UDP glucuronyltransferase, sulfotransferase, and glutathione transferase (79). Thus endogenous compounds such as bilirubin, steroids, and xenobiotics may be conjugated for higher-capacity excretion into bile. A number of these phase II conjugating enzymes has been investigated and shown to be regulated by sex steroid hormones and growth hormone (21). Therefore, we examined the effect of estrogens, testosterone, and the sexual dimorphic pattern of Mrp2 mRNA and protein levels similar to those measured in female rats. Third, in mice selectively deficient in growth hormone, so-called Little mice, homozygous deficient male and female Little mice had markedly decreased Mrp2 mRNA levels. Finally, growth hormone infusions but not injections significantly increased Mrp2 mRNA in Hx rats. These studies strongly support a direct role for growth hormone in the regulation of Mrp2. However, although growth hormone alone increased Mrp2 in Hx rats, we did not reproduce dimorphic expression, suggesting that other hormones indirectly regulated by the pituitary (glucocorticoids and/or thyroid hormone) may also be involved. We observed that thyroid hormone in combination with different patterns of growth hormone administration produced both in vivo levels and the sexual dimorphic pattern of Mrp2 mRNA and protein levels. Similar results were not observed with corticosterone, suggesting that thyroid hormone was important in reproducing the sexual dimorphic levels of Mrp2. Similar results have previously been described for 5α-reductase (57).

In contrast to rats, Mrp2 mRNA levels in mice did not display gender differences. It is not unusual to observe differences in dimorphic gene expression between mice and rats especially related to drug metabolism and transporters (7, 72). Because the sexual dimorphic expression of Mrp2 may be age
related and we measured Mrp2 at only a single time point, it is also possible that we missed the dimorphic differences (32).

Mrp2 is primarily localized to the apical membrane in hepatocytes (62). Therefore, we attempted to identify the location of the increased Mrp2 proteins in female rats by imaging techniques and cell membrane fractionation. Surprisingly, we did not detect any significant difference between male and female rats in the bile canalicular density of Mrp2 using both monoclonal and polyclonal antibodies. Because morphological techniques are not quantitative, we also prepared bile canalicular membrane fractions and quantitated Mrp2 protein content. Mrp2 protein levels in bile canalicular membrane fractions were also similar in male and female fractions (Fig. 10).

Intracellular location of Mrp2 has been described after cholestasis, cell swelling, inflammation with lipopolysaccharide, phalloidin, estrogens, and increased reactive oxygen species (37, 38, 45, 60, 75, 76). Similar to previous reports (46), we were unable to identify an intracellular compartment of Mrp2 in normal female livers using confocal microscopy (data not shown). Possibilities to account for these results are diffuse location of the “pool” and altered conformation of intracellular Mrp2 protein such that it was not recognized with our antibodies. In contrast to the lack of gender differences between intact male and female livers, hypophysectomy dramatically reduced expression of Mrp2 protein density. In particular, growth hormone infusion plus thyroid hormone produced the sexual dimorphic differences in Mrp2 at the bile canalicular domain. These results are consistent with our biochemical observations and suggest that the hormonal changes may be related to functional alterations in transport of conjugated endo- and xenobiotics as shown by others (18, 44, 55, 63).

Pharmacological doses of both glucocorticoids and thyroid hormone increased bile flow and bilirubin secretion in rats (21, 43, 44, 47). Also several studies demonstrate that dexamethasone induces Mrp2 mRNA and protein in rats and isolated hepatocytes (14, 16, 19, 41), but neither the effect of physiological levels of glucocorticoids nor T4 has been examined in vitro or in vivo. To examine physiological changes in hormone levels, male rats were adrenalectomized or thyroidectomized. Levels of Mrp2 mRNA and protein content following Adx and corticosterone replacement decreased and increased, respectively. These observations correlated with reported alterations in bile flow. Because the rat Mrp2 promoter has numerous putative glucocorticoid response elements, the results suggest that these changes may be regulated at the transcriptional level (33). Thyroid hormone also changes bile flow and bilirubin secretion, but the mechanism is not well established (39). Thyroidectomy and T4 replacement markedly altered expression of Mrp2 protein and, to a lesser extent, mRNA levels. Thus, although the Mrp2 promoter has thyroid response elements (33), these changes may be due to posttranslational modifications. In addition, these studies further support the primary role of growth hormone in the regulation of Mrp2.

Growth hormone has many effects on the liver and other tissues (27). In particular, the growth hormone-IGF-I axis is the major determinant of growth and regulates expression of a number of genes (61, 81). Therefore, we examined the effect of IGF-I as well as glucocorticoids, growth hormone, and thyroid hormone on isolated rat hepatocyte cultures. IGF-I and thyroid hormone alone had no effect on Mrp2 mRNA levels, strongly suggesting that the effect of growth hormone was directly on the hepatocyte. Pharmacological doses of dexamethasone increased Mrp2 mRNA, as others have shown (11). In addition, growth hormone alone and especially with thyroid hormone significantly increased Mrp2 mRNA compared with basal levels. These in vitro studies support the results obtained in animals and suggest that growth hormone directly stimulates gene expression both alone and in conjunction with thyroid hormone. The mechanism is not clear, but STAT binding sites have been identified in the Mrp2 promoter (33).

These studies in rodents may also have important implications for understanding human biliary secretion. It is well known that growth hormone regulates biliary secretion of conjugated BSP, a substrate for Mrp2 (55, 63). In particular, growth hormone excess and deficiency are associated with changes in BSP maximal transport. In addition, the sexual dimorphic pattern of growth hormone secretion in humans is related to different levels of drug-metabolizing enzymes (28, 29, 82). Thus there may be coordinate regulation between levels of phase I and II drug-metabolizing enzymes and their excretion into bile.

In conclusion, Mrp2 in rats is expressed in a sexually dimorphic pattern that is regulated primarily by the growth-hormone secretory pattern in conjunction with thyroid hormone. These studies are consistent with previous studies on the hormonal control of serum bilirubin levels and clearance in rats and humans. The results suggest coordinate regulation of multiple steps in bilirubin and xenobiotic metabolism and transport primarily by growth hormone.

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REFERENCES


