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Hormonal regulation of renal multidrug resistance-associated proteins 3 and 4 (Mrp3 and Mrp4) in mice☆

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Abbreviations:
Mrp, multidrug resistance-associated protein
Cyp, cytochrome P450
ABCC, ATP-binding cassette transporter, subfamily C
DHT, 5α-dihydroxytestosterone
HPX, hypophysectomized
GNX, gonadectomized mice
E2, 17β-estradiol
GH, growth hormone
GHRH, growth hormone-releasing hormone
MP, male-pattern
FP, female-pattern

ABSTRACT

Multidrug resistance-associated proteins 3 and 4 (Mrp3 and Mrp4) are expressed at much higher levels in female than male kidney. Sex steroids and sex-specific growth hormone (GH) secretion patterns often mediate gender-predominant gene expression. Thus, three models were used to investigate potential endocrine regulation of Mrp3 and Mrp4: (1) gonadectomized (GNX) mice with 17β-estradiol (E2) or 5α-dihydroxytestosterone (DHT) replacement; (2) hypophysectomized (HPX) mice receiving E2, DHT, or simulated male-pattern (MP) or female-pattern (FP) GH secretion; (3) *lit/lit* mice, which have a spontaneous mutation in the growth-hormone releasing-hormone (GHRH) receptor, with simulated MP- or FP-GH secretion. GNX and HPX decreased Mrp3 mRNA levels compared with intact females. In both respective models E2 administration increased Mrp3 expression in GNX and HPX mice. DHT markedly repressed Mrp3 from GNX + placebo levels, however, this was not observed in the HPX model. In *lit/lit* mice, Mrp3 expression was lower than in wild-type controls, and MP-GH and FP-GH simulation slightly increased Mrp3 expression. Whereas GNX increased Mrp4 in males to female levels, HPX actually increased Mrp4 expression in both genders +375% and +66%, respectively. In both models DHT markedly repressed Mrp4. Furthermore, Mrp4 was higher in *lit/lit* than wild-type male mice, and simulation of MP-GH secretion suppressed female-predominant Mrp4 expression. In conclusion, these data indicate that E2 contributes to higher Mrp3 mRNA expression in females, yet a role for androgens in Mrp3 repression cannot be discounted. In contrast, Mrp4 mRNA is higher in females due to repression by both DHT and MP-GH secretion in males.

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1. Introduction

Multidrug resistance-associated proteins (Mrp) 3 and Mrp4 are efflux transporters that transport a broad range of conjugated and unconjugated endo- and xenobiotics. Both Mrp3 and Mrp4 are capable of transporting chemotherapeutic drugs and antivirals, as well as several endogeneous ligands, including conjugated bile acids, glucuronidated estrogens, and leukotrienes [1–4]. Because Mrp3 and Mrp4 can transport bile acids, induction of Mrp3 and Mrp4 during cholestasis may serve as a protective response to decrease potentially toxic levels of bile acids in liver and kidney [3,5,6].

The renal functions of Mrp transporters are not well characterized. At the mRNA level, Mrp3 is moderately expressed in mouse kidney, as compared with liver and intestine [7]. Mrp3 is localized to the basolateral membrane in liver and kidney [8]. Contrary to Mrp3, Mrp4 expression is very low in liver, with high expression in kidney. Mrp4 is also localized to the basolateral membrane in hepatocytes, but in kidney, Mrp4 is apically expressed, and is localized primarily in proximal tubules [9]. Previous studies from this laboratory have demonstrated that renal mouse Mrp3 and Mrp4 mRNA expression is markedly female-predominant, with onset of gender-divergent expression occurring at approximately 30 days of age for both transporters [7].

Many genes have gender-specific expression patterns in liver. In rat liver, classic examples of gender-dimorphic expression are Cyp2c11 and 2c12, which are male- and female-specific, respectively [10,11]. Likewise, in mouse liver, Cyp2d9 and 2a4 are male- and female-specific, respectively, and are regulated primarily by gender-specific GH patterns [12,13]. Examples of renal P450s that are regulated by sex hormones or GH patterns are much fewer, but male predominant expression of mouse Cyp2J5 has recently been shown to occur via up-regulation by androgens [14].

In addition to biotransformation genes, there are several examples of gender-dimorphic transporter expression. Male-predominant expression of organic anion transporter 3 (Oat3) is upregulated by androgens, and suppressed by female-pattern GH secretion [15]. Likewise, male-predominant renal expression of organic anion transporting polypeptide 1a1 (Oatp1a1) in rats and mice is due to stimulation by androgens [16,17]. Furthermore, multidrug resistance protein 1b (Mdr1b), an efflux transporter similar to Mrps, has female-predominant expression in mice [18]. In rats, Mrp4 is male-predominant, but the mechanism is not known [19]. It is important to note that exposure times and amount of protein loaded were optimized for quantification between genders, and does not reflect absolute quantification between Mrp3 and Mrp4; under homeostatic conditions, Mrp4 is much higher than Mrp3 in kidney, and vice versa in liver (data not shown).

Gender-divergent transporter expression can manifest in differential disposition of endogenous substrates, toxicants, and therapeutics. For instance, the lack of luminal expression of Oatp1a1 on the brush-border membrane in female kidney may be responsible for the 250-fold higher rate of urinary excretion of exogenously administered estradiol-17β-D-glucuronide in female compared to male rats [20]. Additionally, marked female-predominant Oat2 mRNA expression in rat kidney correlates with a 70-fold higher urinary excretion rate of perfluorooctanoic acid in females compared to males [21].

The mechanisms of gender divergent gene expression may occur by direct action of androgens or estrogens, by gender-specific GH-secretion patterns, or by a combination of these systems. Androgens and estrogens may alter gene expression by directly stimulating gene transcription through nuclear hormone receptors [22–24]. Furthermore, differential male and female patterns of GH are also known to mediate sex-dependent gene expression. In rats, males secrete GH in high-amplitude pulses with a regular frequency every three to four hrs. Between pulses, serum GH levels are non-detectable [25]. In contrast, female rats secrete GH in low-amplitude pulses with greater frequency and higher trough levels than males, resulting in a continuously detectable serum GH concentrations [26,27]. These secretion patterns are responsible for masculinization and feminization of livers, respectively. Growth-hormone-secretion patterns in male mice are similar to those in male rats [28]. In female mice, GH is secreted at regular intervals with a non-detectable baseline between pulses; however, the pulses are more frequent (1–1.5 h) than those in male mice [28]. The male-GH-secretion pattern is responsible for induction of male-predominant Cyp2d9 and repression of female-predominant Cyp2a4 in mouse liver [29,30].

Hypophysectomy (HPX) and the *little* (*lit*/*lit*) mouse are models for examining the influence of gender-specific GH-secretion patterns on gene expression. Total hypophysectomy involves ablation of the pituitary gland, resulting in loss of several hormones such as (1) luteinizing hormone, (2) follicle-stimulating hormone, and (3) adrenocorticotropic hormone, which are critical mediators of sex steroid production, as well as growth hormone, which can also masculinize or feminize expression of some genes. Although the HPX model is complicated by the complete removal of all pituitary-derived hormones, each hormone can be administered individually to assess the contribution of each hormone to gender-specific expression. The *lit*/*lit* mouse has a spontaneous mutation in the growth-hormone releasing-hormone (GHRH) receptor, resulting in a mutated, non-functional GHRH receptor, thus preventing GH secretion [31–34]. The *lit*/*lit* mice are known to respond to GH therapy in a manner comparable to the HPX model with the benefit of other endocrine factors not being disrupted [30,35].

Gender-divergent expression of Mrp3 and Mrp4 exists in mouse kidney, although the mechanism of this expression pattern is unknown. Therefore, the purpose of this study is to determine whether the gender differences in renal expression of Mrp3 and Mrp4 are due to sex hormones, GH-secretion patterns, or a combination of these two factors.

2. Materials and methods

2.1. Materials and reagents

Rat growth hormone (GH; Lot #AFP611) was purchased through the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrance, CA). Pellets for subcutaneous (sc) release of the hormones used in this study [GH (1 mg; 21-day release), 5α-dihydroxytestosterone (DHT; 5 mg; 21-day release), and

17β-estradiol (E2; 0.5 mg; 21-day release) were formulated by Innovative Research of America (Sarasota, FL)] to deliver approximately physiological levels of these hormones [36–38]. Antibodies specific for mouse Mrp3 (M_3II2) and Mrp4 (M_4I10) were provided by Dr. George Scheffer. The M_4I10 (Mrp4) antibody design was described previously [39].

2.2. Mice

All mice were maintained on automatically timed 12-h dark/light cycles in an American Animal Associations Laboratory Animal Care-accredited facility and allowed water and rodent chow ad libitum (Teklad, Harlan, Indianapolis, IN).

2.3. Gonadectomy and sex hormone replacement

C57BL/6 mice were castrated or ovariectomized at 37 days of age by Charles River Laboratories. At 54 days of age, DHT (5 mg), E2 (0.5 mg), or placebo 21-day release pellets (Innovative Research of America, Sarasota, FL) were sc implanted interscapularly in intact and gonadectomized (GNX) male and female mice under isoflurane anesthesia. The mice were separated into four treatment groups, with six mice/gender/treatment: (1) intact mice + placebo, (2) GNX + placebo, (3) GNX + DHT, and (4) GNX + E2. Kidneys were removed at 64 days of age from GNX and intact control mice. No statistical difference in mRNA expression between GNX untreated and GNX + placebo mice, or untreated intact and intact mice + placebo was observed (data not shown), thus only placebo-treated data was shown.

2.4. Hypophysectomy with growth-hormone or sex-hormone replacement

Hypophysectomy (HPX) surgeries were performed by Charles River when the mice were 38 days of age. Unsuccessful HPX surgeries were excluded from the experimental design by close monitoring of weight gain before pellet insertion and by physical observation of remaining pituitary on inspection after treatment. The mice were separated into six treatment groups (n = 4–6/gender/treatment), with male and female mice in each group: (1) intact mice, (2) HPX mice, (3) male-pattern GH was given to HPX mice by a twice-daily sc injection of 25 μg of rat GH (50 μg GH/day), (4) female-pattern GH was mimicked in HPX mice by continuous infusion by implant of a 21-day-time-release pellet (1 mg GH/pellet), (5) DHT (5 mg) pellet insertion, and (6) E2 (0.5 mg) pellet insertion. Kidneys were removed at 64 days of age from all HPX and age-matched intact control mice. No statistical difference in mRNA expression between HPX untreated mice and HPX + placebo was observed (data not shown), thus only placebo data was shown.

2.5. Lit/lit Mice with growth-hormone replacement

Breeding pairs of C57BL/6J-Ghrhr^{lit} mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred as described. For experimental purposes, *lit/lit* and *lit/+* littermates of 8–16 weeks of age were used (n = 6). The mice were separated into four treatment groups (n = 4–6/gender/treatment), with male and female mice in each group: (1) Intact mice + placebo, (2) *lit/lit* + placebo, (3) *lit/lit* + male-pattern GH given twice-daily sc injection of 25 μg of rat GH (50 μg GH/day), and (3) *lit/lit* + female-pattern given by continuous sc infusion by implant of a 21-day-time-release pellet (1 mg GH/pellet). No statistical difference in mRNA expression between *lit/lit* untreated and *lit/lit* mice + -placebo was observed (data not shown).

2.6. RNA isolation

Total RNA was isolated using RNAzol Bee® reagent (Tel Test, Friendswood, TX) according to the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. The integrity of each RNA sample was evaluated by formaldehyde–agarose gel electrophoresis before analysis. RNA was stored at −80 °C until use.

2.7. Branched DNA signal amplification assay

Mouse Mrp3 and Mrp4 transcripts were measured using the branched signal amplification assay as previously described (QuantiGene®, High Volume bDNA Signal Amplification Kit, Genospectra, Fremont, CA) [7].

2.8. Preparation of crude kidney homogenates and membranes

To analyze Mrp protein expression, crude membrane fractions were isolated from kidney based on a previously described method [40]. Kidneys from mice were removed, placed in four volumes of ice-cold homogenization buffer (10 mM Tris, 250 mM sucrose, pH 7.4, containing 50 mM phenylmethylsulfonyl fluoride), and homogenized by 20 up-down strokes with a dounce homogenizer. The homogenate was centrifuged at 100,000 × g at 4 °C for 1 h. The resulting pellet was resuspended in 0.3 mM sucrose, 20 mM Hepes, pH 7.4, containing 50 mM phenylmethylsulfonyl fluoride. Protein concentrations of kidney homogenates and crude membrane fractions were determined with the bicinchoninic acid (BCA) Protein Assay Reagent Kit (Pierce, Rockford, IL) as described by the manufacturer.

2.9. Western blotting

Kidney membrane preparations containing 150 μg (Mrp3) and 75 μg (Mrp4) of total protein were loaded per well and separated on 10% SDS-polyacrylamide Criterion gels (Biorad, Hercules, CA). Proteins were transferred overnight (30 V) at 4 °C to polyvinylidene difluoride membranes. Blots were blocked with 1% non-fat dry milk (NFDM) for 1 h in Tris-buffered saline with 0.5% Tween 20 (TBS-T), incubated for 2 h at room temperature in 1% NFDM-TBS-T containing a 1:2000 dilution of the primary antibodies (Mrp3, M3II2; Mrp4 M4I10) and washed three times in TBS-T. Blots were subsequently incubated in secondary antibody (1:10,000) in 1% NFDM-TBS-T. Blots were then incubated with horseradish peroxidase-conjugated IgG (Amersham Biosciences, Piscataway, NJ). Each blot was incubated in the enhanced chemiluminescence (ECL™) substrate reagent for horseradish peroxidase (Amersham Biosciences, Piscataway, NJ), and immunopositive

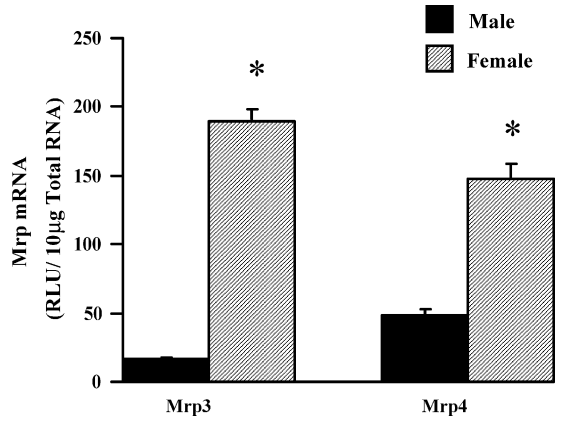

Fig. 1 – Mrp3 and Mrp4 mRNA expression in male and female kidneys from mice, expressed in relative light units (RLU). Error bars represent ±S.E.M. Asterisks (*) represent a statistically significant difference between gender ($p \geq 0.05$) as determined by a two-tailed *t*-test.

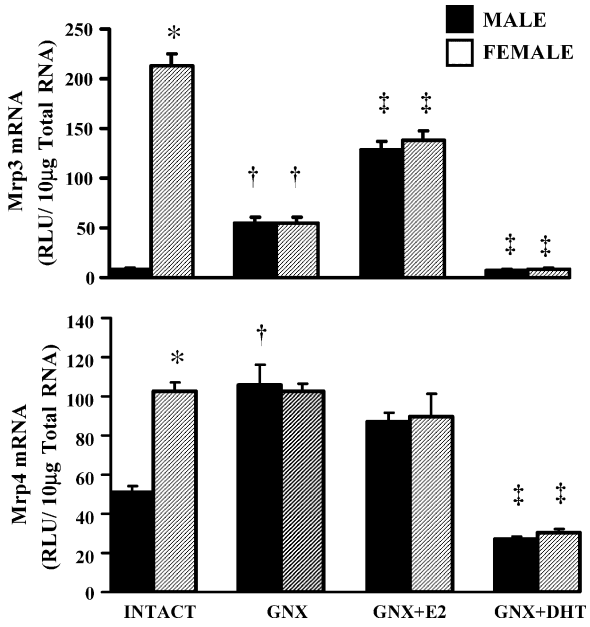

Fig. 3 – Effects of gonadectomy (GNX) and sex steroid replacement in GNX mice on Mrp3 and Mrp4 mRNA expression in male and female kidneys from mice. Data are expressed as relative light units ±S.E.M. Asterisks (*) represent a statistically significant difference between intact male and female mice. Daggers (†) represent statistical differences between GNX mice and the respective intact mice of the same gender. Double daggers (‡) represent a statistical difference between GNX + placebo and GNX + E2 and GNX + DHT groups. The level of statistical significance was $p \geq 0.05$.

interactions were visualized by exposure of the blot to X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan). Densitometric analysis of Western blots was completed using Scion Image® software (Scion, Frederick, Maryland).

2.10. *Analysis of 5′ flanking regions for transcriptional response elements*

The 5′ flanking regions of Mrp3 and Mrp4 were identified from the Ensembl database (http://www.ensembl.org/). Promoter analysis of 10 kb upstream of the hypothetical transcriptional start site were analyzed using Alibaba2 (Niels Grabe, http://www.gene-regulation.com/pub/programs/alibaba2/) as well as response element analysis with DS Gene® software (Accelrys, San Diego, CA).

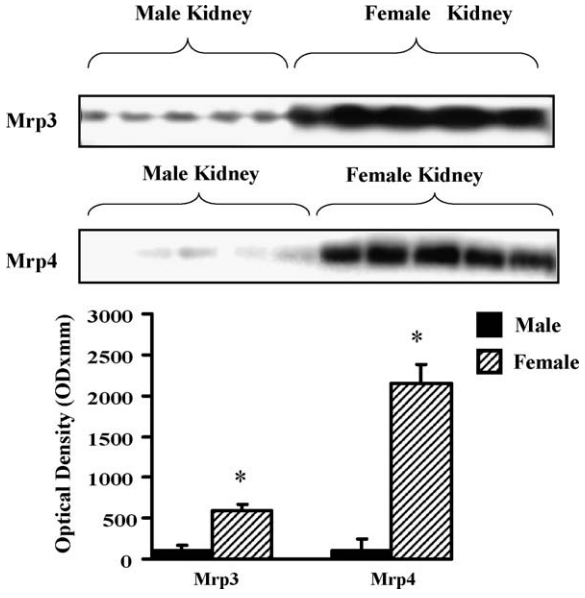

Fig. 2 – Mrp3 and Mrp4 protein expression in kidneys of male and female mice. Quantification of the Western Blot is in arbitrary units of optical density, with error bars representing ±S.E.M. Asterisks (*) represent a statistically significant difference between gender ($p \geq 0.05$) as determined by a two-tailed *t*-test.

2.11. *Statistics*

For differences between genders in Figs. 1 and 2, a two-tailed *t*-test with significance set at $p \geq 0.05$ was utilized. For Figs. 3–5, data were analyzed by one-way analysis of variance, followed by Tukey's post hoc test. Bars represent standard error of the mean.

3. Results

3.1. *Gender differences in Mrp3 and Mrp4 expression*

As previously reported, at the mRNA level, Mrp3 is about 25-fold higher in female than male kidneys, and Mrp4 mRNA is about twice as high in female than male kidneys (Fig. 1) [7]. However, using membrane protein extracts, approximately a 6- and 21-fold higher protein expression were observed in females than in males, respectively, for Mrp3 and Mrp4 (Fig. 2).

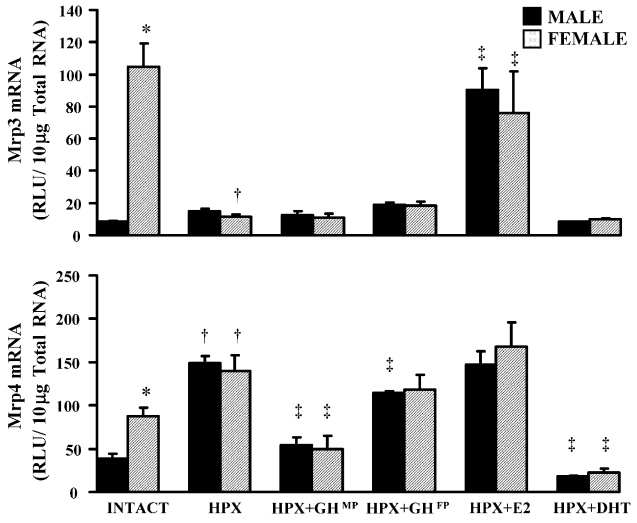

Fig. 4 – Effects of hypophysectomy (HPX) and HPX mice administered sex hormone or gender-specific growth-hormone secretion patterns on Mrp3 and Mrp4 mRNA expression in male and female kidneys from mice. Error bars represent ±S.E.M. Asterisks (*) represent a statistically significant difference between intact male and female mice. Daggers (†) represent statistical differences between HPX mice and the respective intact mice of the same gender. Double daggers (‡) represent a statistical difference between HPX + placebo and the HPX + MP-GH, HPX + FP-GH, HPX + E2 and HPX + DHT groups. Statistical differences were determined by one-way ANOVA, followed by a Tukey's post hoc test.

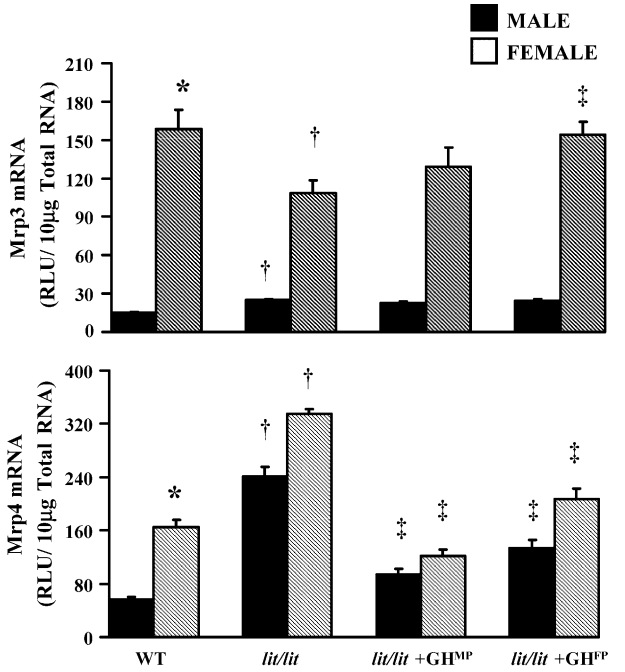

Fig. 5 – Effects of the *lit*/*lit* mutation and gender-specific growth-hormone secretory patterns on Mrp3 and Mrp4 mRNA expression in *lit*/*lit* mice. Mrp3 and Mrp4 mRNA expression in kidney of male and female control, *lit*/*lit*, *lit*/*lit* + MP-GH, and *lit*/*lit* + FP-GH. Data are expressed as relative light units ±S.E.M. Asterisks (*) represent a statistically significant difference between intact male and female mice. Daggers (†) represent statistical differences between lit/lit mice and the respective intact mice of the same gender. Double daggers represent a statistical difference between *lit*/*lit* + placebo and the *lit*/*lit* + MP-GH and *lit*/*lit* + FP-GH groups. The level of statistical significance was $p \geq 0.05$.

3.2. *Gonadectomy*

Gonadectomy increased Mrp3 mRNA expression compared with intact male levels, and decreased Mrp3 mRNA compared with intact female levels (Fig. 3). Administration of E2 to GNX mice elevated Mrp3 mRNA levels to about 60% of intact female expression, whereas GNX mice given DHT had Mrp3 mRNA levels similar to intact male mice. GNX elevated Mrp4 mRNA levels in male mice to intact female levels. Gonadectomized mice with E2 pellets had Mrp4 mRNA levels similar to intact female and control GNX mice (Fig. 3). However, DHT administration markedly decreased the expression of Mrp4 mRNA in GNX mice to levels observed in intact male mice.

3.3. *Hypophysectomy*

HPX female mice had markedly lower expression of Mrp3 when compared to intact female mice. Furthermore, FP-GH and MP-GH administration did not alter expression of Mrp3 in HPX males or females, and similarly DHT had little effect in increasing Mrp3 expression. However, E2 administration almost completely restored Mrp3 mRNA levels to those observed in intact female mice.

Hypophysectomy increased renal expression of Mrp4 mRNA to levels higher than those observed in intact female mice (Fig. 4). Simulation of the male-GH-secretory pattern in HPX mice decreased Mrp4 mRNA levels compared to HPX controls, whereas Mrp4 mRNA levels were unchanged following simulation of the female-GH-secretory pattern compared to HPX controls. Following administration of DHT to male and female HPX mice, Mrp4 mRNA expression was decreased.

3.4. *Little mice (lit/lit)*

In *lit*/*lit* mice, Mrp3 mRNA levels were similar to those observed in control mice, with a female-predominant pattern of expression (Fig. 5). In *lit*/*lit* females, Mrp3 mRNA levels were about 30% lower, whereas *lit*/*lit* male expression was approximately 40% higher than the respective intact controls. MP-GH administration was ineffective in altering expression of Mrp3 in male or female *lit*/*lit* mice. FP-GH secretion slightly increased Mrp3 expression in *lit*/*lit* mice, elevating *lit*/*lit* female Mrp3 mRNA expression in kidney to control female kidney levels.

Mrp4 expression was higher in both male and female *lit*/*lit* mice compared to control male and female mice, respectively (Fig. 5). Although Mrp4 expression is higher in *lit*/*lit* than control male and female mice, a gender difference was still apparent in *lit*/*lit* mice. Male-pattern-GH treatment decreased

Table 1 – Putative response elements in the 5′ flanking regions of mouse Mrp3 and Mrp4

Mrp promoter sequence	Transcription factor	Cluster	Binding site (bp)	Transcription factor sequence	Percent homology
Mrp3	ER	768–2068	2056–2068	tgaccttgagct	>80
	ER	768–2068	768–779	agggtcaaggt	>80
Mrp4	HNF3b	1684–1718	1700–1712	ttttatttattt	87.9
	HNF3b	1684–1718	1684–1696	atttatttattc	87.3
	HNF3b	1684–1718	1696–1708	atttatttattt	89.6
	HNF3b	1684–1718	1692–1704	atttatttattt	89.6
	Stat5b	6274–6403	6274–6283	ttcccagaa	96.2
	Stat5b	6274–6403	6394–6403	tttccagtt	76.7
	AR-like	None	7191–7177	gcttctgttgttct	>80

expression of Mrp4 in male and female *lit/lit* mice to control male levels, and no gender difference was observed. FP-GH also decreased Mrp4 expression, but a gender difference was clearly observed. This suggests that GH alone is suppressive of Mrp4, but MP-GH is more suppressive, and able to absolve the gender-specific Mrp4 expression pattern.

3.5. 5′ *flanking region analysis*

Several putative response elements for known *cis*-acting transcription factors exist for Mrp3 and Mrp4 (Table 1). Two estrogen response elements were discovered at approximately −0.8 and −2.1 kb upstream of the translational start site of the Mrp3 gene. For Mrp4, a group of elements responsive to growth-hormone secretory patterns and androgens were analyzed. A large cluster of potential HNF3b response elements were located at −1.7 kb. A more distal pair of Stat5b elements, and an androgen response element were located at approximately −6.3 and −7.2 kb upstream, respectively.

4. Discussion

This study aimed to determine the contributions of sex hormones and GH-secretory patterns on the expression of renal Mrp3 and Mrp4. Three different model systems were utilized (GNX, HPX, and *lit/lit* mice), and the results integrated to obtain a thorough understanding of the contribution of both sex hormones and GH patterns in the expression of Mrp3 and Mrp4 (Table 2). Interestingly, gender-specific Mrp3 expression exists in kidney but not in liver or intestine, whereas female predominant expression of Mrp4 is observed in liver and kidney [7]. Thus, tissue-specific expression of certain transcription factors may ultimately be responsible for the gender-specific expression of Mrp3 and Mrp4 observed in mice.

Table 2 – Summary of the effects of the hormonal treatments on Mrp3 and Mrp4 expression

	Male	Female	E2	DHT	FP-GH	MP-GH
Mrp3						
GNX	↑↑	↓↓	↑↑↑	↓↓	NA	NA
HPX	–	↓↓↓	↑↑↑	–	–	–
lit/lit	–	↓	NA	NA	↓	–
Mrp4						
GNX	↑↑	–	–	↓↓↓	NA	NA
HPX	↑↑↑	↑	–	↓↓↓	↓	↓↓↓
lit/lit	↑↑↑	↑	NA	NA	↓	↓↓↓

In mouse kidney, female-predominant expression of Mrp3 occurs at least in part via the stimulatory effects of E2. However, some evidence in this study also suggests that DHT and GH-secretory patterns may be inhibitory in nature. Data in the present study indicate that in GNX and HPX mice, renal Mrp3 is clearly increased after E2 administration (Figs. 3 and 4). For Mrp3, there seems to be some discrepancy between the results from the GNX model, and the HPX model in regard to the contribution of androgens. Castration increased expression of Mrp3, whereas ovariectomy decreased expression, suggesting that E2 increases and androgen decreases Mrp3 expression (Fig. 3). In the HPX model, HPX mice and intact male controls had almost identical Mrp3 expression, and DHT did not alter expression (Fig. 4). Therefore, the data from the GNX model suggest that in intact mice, androgens may be able to decrease Mrp3 expression, whereas the HPX model suggests no role for androgens. If the actions of androgen require the contribution of other hormones or factors supplied by the pituitary, however, the contributions of androgens in the intact animal could be overlooked by a negative result in the HPX mice. Also, male-pattern GH was ineffective in altering Mrp3 levels in either HPX or *lit/lit* mice, repression of Mrp3 by FP-GH secretion was observed. Thus, what can be concluded is that female-predominant expression of Mrp3 is due at least in part to E2, with some evidence that androgens and the FP-GH secretory pattern may be able to inhibit Mrp3 expression.

In contrast, female-predominant expression of Mrp4 occurs by repression of Mrp4 in male mice by DHT and MP-GH. Mrp4 is markedly repressed by androgens, as observed when GNX and HPX were administered DHT (Figs. 3 and 4). In addition, Mrp4 was clearly down-regulated after administration of MP-GH to both HPX and *lit/lit* mice, suggesting that Mrp4 is under control of both androgens and GH. Whereas E2 treatment did not increase Mrp4 expression in either GNX or HPX mice, FP-GH secretory patterns repressed expression of Mrp4 in *lit/lit* mice (Fig. 5). Perhaps the influence of GH on Insulin-like Growth Factor-1 (IGF-1) expression may also lead to subsequent changes in expression of Mrp4 via alternate signaling pathways. Taken together, the data suggests that androgen and MP-GH secretory patterns repress Mrp4 expression in male mice, leading to female-predominant expression of Mrp4.

Regulation of drug-metabolizing enzymes is thought to occur through the influences of a multitude of transcription factors and receptors. Male-pattern GH secretion causes Stat5b activation, and Stat5b and HNF4α are thought to work together to influence male-specific transcription patterns in liver [41]. Stat5b is activated by high-amplitude peaks of male-pattern GH release, and such peaks are absent in female mice [42]. Stat5b activation has well-known effects in liver, and leads to male-specific expression of genes such as Cyp2C11. In contrast, HNF3b has stimulatory effects on expression of female-specific genes, such as Cyp2C12 [43,44]. When the liver is masculinized, Stat5b has been proposed to interfere with the transcriptional activation of Cyp2C12 by HNF3b, in combination with HNF6 [44].

Whereas information on the transcriptional regulation of Mrp3 and Mrp4 is poorly understood, potential regulation may be gleaned by examination of the 5′ flanking regions of Mrp3 and **Mrp**4. Because the data clearly suggest potential regulation of Mrp3 via E2, promoter analysis to search for conserved estrogen receptor response elements was undertaken. Two potential estrogen receptor response elements exist that may play a role in Mrp3 regulation. Thus, an attractive concept is that the observed female-predominant expression of Mrp3 occurs directly via the estrogen receptor in females. The kidney is responsive to GH patterns [45,46], and potential regulation of Mrp4 by Stat5b and HNF3b is suggested. Highly homologous response elements for Stat5b and HNF3b in the 5′ flanking region of the Mrp4 gene can also be found in two distinct clusters (Table 1). Thus, MP-GH secretion may activate Stat5b in males, leading to deactivation of HNF3b, and repressing Mrp4 expression. In females, Stat5b may not be activated, and HNF3b could increase expression of Mrp4 in a similar manner as that observed for Cy2C12 [43]. Furthermore, studies have suggested that androgens can aid in masculinization of GH patterns, and may explain why Mrp4 can be dually repressed by both androgen and male-pattern GH secretion [33,47]. Future in vitro analysis of these binding sites could help understand the molecular basis of these in vivo observations.

Sex steroids have been shown to affect renal expression of some drug transporters. The direct involvement of the estrogen receptor in multidrug-resistance phenotypes has been observed previously, including known regulation of Mdr1 in mouse by the ERα receptor, which directly leads to alterations in disposition of chemotherapeutic drugs [48,49]. Furthermore, treatment with the ER antagonist tamoxifen is effective in negating the multidrug-resistance phenotype in colorectal carcinomas [49]. Similarly, tamoxifen might also inhibit female-predominant Mrp3 expression in kidney by functioning as an antagonist of the estrogen receptor.

Distinct female-predominant expression of Mrp3 and Mrp4 exist in the kidney at both the mRNA and protein level in mice. Although alterations in murine Mrp3 and Mrp4 by hormones are not necessarily indicative of alterations in the corresponding human Mrps, the frequent use of rodents in pharmacokinetic studies makes these data relevant when extrapolating between species. This is apparent even between mouse and rat; whereas rat Mrp4 is male-predominant [19], mouse Mrp4 is female-predominant.

The mechanisms behind gender-specific expression of Mrp3 and Mrp4 seem to be quite different. Evidence from this study suggests E2 contributes to female-predominant Mrp3 expression. However, because Mrp3 is markedly repressed in the GNX model but not in the HPX model, a possible role for androgens in Mrp3 repression cannot be discounted. Furthermore, female predominant Mrp4 expression may be due to suppression of Mrp4 in males by androgens and MP-GH secretion. Such hormonal alterations of Mrp transporters may indeed manifest into differences in renal clearance of Mrp substrates and lead to differences in pharmacokinetics and/or in the toxicity of xeno- and endobiotics.

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