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Mice lacking Mrp1 have reduced testicular steroid hormone levels and alterations in steroid biosynthetic enzymes

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Abstract

The multidrug resistance-associated protein 1 (MRP1/ABCC1) is a member of the ABC active transporter family that can transport several steroid hormone conjugates, including 17 β -estradiol glucuronide, dehydroepiandrosterone sulfate (DHEAS), and estrone 3-sulfate. The present study investigated the role that MRP1 plays in maintaining proper hormone levels in the serum and testes. Serum and testicular steroid hormone levels were examined in both wild-type mice and Mrp1 null mice. Serum testosterone levels were reduced 5-fold in mice lacking Mrp1, while testicular androstenedione, testosterone, estradiol, and dehydroepiandrosterone (DHEA) were significantly reduced by 1.7- to 4.5-fold in Mrp1 knockout mice. Investigating the mechanisms responsible for the reduction in steroid hormones in *Mrp1*^{-/-} mice revealed no differences in the expression or activity of enzymes that inactivate steroids, the sulfotransferases or glucuronosyltransferases. However, steroid biosynthetic enzyme levels in the testes were altered. Cyp17 protein levels were increased by 1.6-fold, while Cyp17 activity using progesterone as a substrate was also increased by 1.4-2.0-fold in mice lacking Mrp1. Additionally, the ratio of 17 β -hydroxysteroid dehydrogenase to 3 β -hydroxysteroid dehydrogenase, and steroidogenic factor 1 to 3 β hydroxysteroid dehydrogenase were significantly increased in the testes of *Mrp1*^{-/-} mice. These results indicate that *Mrp1*^{-/-} mice have lowered steroid hormones levels, and suggests that upregulation of steroid biosynthetic enzymes may be an attempt to maintain proper steroid hormone homeostasis.

Keywords

Multidrug resistance-associated protein 1; ATP-binding cassette; testosterone; androstenedione; testes; Cyp17; 17 β -hydroxysteroid dehydrogenase

1. Introduction

Members of the multidrug resistance-associated protein (MRP or ABCC) subfamily of transporters are responsible for the elimination of numerous endogenous ligands, drugs, and toxicants (Bakos and Homolya, 2007; Deeley et al., 2006). One member of the family, the multidrug resistance-associated protein 1 (MRP1 or ABCC1), extrudes phase II metabolites

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of steroid hormones such as 17β -estradiol glucuronide, dehydroepiandrosterone sulfate (DHEAS), and estrone 3-sulfate (Leslie et al., 2005; Loe et al., 1996; Qian et al., 2001; Zelcer et al., 2003), along with other endogenous and exogenous substrates. MRP1 and other members of the ABCC family help maintain the blood-brain barrier, blood-cerebrospinal fluid barrier, and the blood-testes barrier, by removing xenobiotics and endogenous compounds from the nervous system or testes and transporting them back into the bloodstream (Dallas et al., 2003; Nies et al., 2004; Wijnholds et al., 1998). In human tissues, MRP1 is expressed in the testes, adrenals, prostate, skin, esophagus, small intestine, large intestine, lung, heart, amnion epithelium, and the pancreas (Aye et al., 2007; Flens et al., 1996; Zelcer et al., 2003). In mice, Mrp1 is expressed in testes in both Sertoli and Leydig cells, as well as in the colon, heart, small intestine, kidney, and lungs (Peng et al., 1999; Stride et al., 1996). Organs that synthesize and respond to steroid hormones, such as the adrenals, testes and ovaries appear to have the highest levels of expression (Maher et al., 2005).

In addition to cellular and organ localization, evidence from knockout mice also supports the idea that one function of MRP1 is to protect cells from damage. For example, etoposide treatment damages the mucosal layer of the tongue in mice lacking Mrp1 (Wijnholds et al., 1998), while vincristine treatment of Mrp1 knockout mice results in toxicity to the bone marrow and reduces survival by 4-fold (Johnson et al., 2001; van Tellingen et al., 2003). In addition, the presence of Mrp1 reduces tissue accumulation of the antibiotic grepafloxacin (Li et al., 2005; Sasabe et al., 2004). Triple knockout mice that lack Mdr1a, Mdr1b, and Mrp1, exposed to cigarette smoke for 6 months, had reduced numbers of inflammatory cells and IL-8 levels in the lungs of mice compared to control mice, which suggests an impaired inflammatory response (van der Deen et al., 2007). In the testes, Mrp1 is thought to play a role in maintaining the blood-testes barrier, preventing accumulation of toxicants as well as preventing the build-up of estrogen-like compounds (Tribull et al., 2003; Wijnholds et al., 1998).

Although it has long been known that MRP1 can transport a variety of steroid hormones using *in vitro* models, its role in modulation of steroid hormone homeostasis in an animal model has not been previously described. In the testes, hormones are derived from pregnenolone after conversion through two different potential pathways. The Δ^5 steroidogenic pathway involves the conversion of pregnenolone to DHEA and androstenediol, prior to the formation of testosterone. This is the dominant pathway in humans (Fluck et al., 2003). The Δ^4 pathway begins with the conversion of pregnenolone into progesterone, with androstenedione ultimately being converted into testosterone. This is the predominant pathway in rodents (Fevold et al., 1989; Mathieu et al., 2002), although 3β -hydroxysteroid dehydrogenase 2 can convert hormones from the Δ^5 pathway into substrates involved in the Δ^4 pathway.

Testosterone and estradiol are both synthesized in the testes. Although both are needed for proper testicular functioning and for spermatogenesis, estradiol concentrations must be maintained at low levels to prevent testicular feminization and protect developing spermatozoa. To maintain proper estrogen levels, sulfotransferase 1E1 (sult1e1 or EST) catalyzes the formation of estrone 3-sulfate, which inactivates the hormone (reviewed in (Song and Melner, 2000; Strott, 1996). Because the resulting product is hydrophilic, investigators have hypothesized that estrone 3-sulfate is transported out via Mrp1 (Qian et al., 2001). Therefore, this study examined the differences in steroid hormones levels and steroid hormone metabolizing enzymes in Mrp1 knockout mice to determine whether Mrp1 played a role in regulating circulating and testicular steroid hormone levels.

2. Materials and methods

2.1 FVB and FVB/*Mrp1*^{-/-} mice

Three sets of male FVB (control or wild-type mice) and *Mrp1* knockout mice (FVB/*Mrp1*^{-/-}) were obtained from Taconic Farms (Germantown, NY) at 4-5 weeks of age. They were individually housed until 10 weeks of age at 25±2°C and 50% humidity and fed TestDiet 5001 rodent chow (Richmond, IN) before euthanization via a CO₂ overdose. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at El Paso. In the first set of mice, testes, livers, and blood were removed from six animals from each strain. One testis was snap-frozen in liquid nitrogen to prepare cytosol and microsomes, while the other was placed in Tri-Reagent (Sigma Chemical, St. Louis, MO) to obtain RNA. All samples were stored at -80°C. Blood was collected, serum prepared by centrifugation, and stored at -20°C for steroid hormone analyses. In the second and third set of experiments, six additional male FVB and *Mrp1*^{-/-} mice were obtained, housed, and euthanized as above. The testes were removed and weighed. Blood was collected, serum prepared by centrifugation, and stored at -20°C for steroid hormone analyses. One testis was snap-frozen in liquid nitrogen to prepare cytosol and microsomes. The other testis was homogenized in 0.1M potassium phosphate buffer, pH 7.4, and testicular steroid hormones were extracted twice by the addition of 2mL of diethyl ether. The two extracts were combined, evaporated under nitrogen and resuspended in 1mL phosphate buffer (Jeyaraj et al., 2005). The aqueous portions of the testes extracts were used to examine dehydroepiandrosterone sulfate (DHEAS). Extracts were stored at -20°C.

2.2 Steroid hormone levels in serum and testes

Testosterone, progesterone, estradiol, and DHEAS concentrations were determined by EIA kits (Calbiotech, Spring Valley, CA for DHEAS; Cayman Chemical, Ann Arbor, MI for testosterone, progesterone, and estradiol), while androstenedione and DHEA concentrations were determined by RIA (Diagnostic Systems Laboratories, Webster, TX). To determine testosterone concentrations, 10µL of a 1:20 dilution of the testes extracts or 10µL of serum was used. For progesterone concentrations, 25µL of the testes extract and serum were used. For estradiol concentrations, 25µL serum and 100µL of the testicular extracts was used. For androstenedione, 5µL of both serum and testes extract were used. For DHEA, 50µL of serum and 100µL of the testicular extracts were used, while for DHEAS, 1µL of serum or 100µL of the aqueous portion of the testes sample was used. All samples were run in duplicate or triplicate. Results are expressed as amount of hormone per mL serum or amount of hormone per gram of tissue.

2.3 Changes in RNA abundance by QPCR

Total RNA from the testes and liver of each mouse was isolated using TRI-Reagent (Sigma, St. Louis, MO) and then treated with RNase-free DNase. To prepare cDNA, total RNA (2µg) was incubated with 50ng random hexamers, RNAsin, 10mM dNTP mix, and 200U Moloney murine leukemia virus (MMLV) reverse transcriptase at 37°C for 1 hour. Quantitative PCR was performed in Bio-Rad's i-Cycler (Hercules, CA) using 0.2mM dNTPs, 0.25X Sybr green, 1U Taq polymerase (SABiosciences, Frederick, MD), along individual sets of primers for uridineglucuronosyltransferase 2b (*Ugt2b*), sulfotransferase 1e1 (*Sult1e1*), 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1), 17β-HSD3, androgen receptor (AR), luteinizing hormone receptor (LHR), *Cyp17*, *Cyp11A*, steroidogenic acute regulatory protein (*StAR*), *Cyp19*, estrogen receptor α (*Esr1*), estrogen receptor β (*Esr2*), and steroidogenic factor-1 (*SF-1*) (Table 1). 18S rRNA was used as the housekeeper for *Ugt2b* and *Sult1e1* to facilitate comparisons between the liver and testes, while *GAPDH* were used as housekeeper for all other genes. All PCR products had a denaturing step of 95°C for 15 seconds, an annealing /extension step at 61°C (51°C for *GAPDH*) for 1 minute for a total of 40 cycles. The cycle threshold values

obtained from the real-time PCR were converted into starting number of molecules per 100ng cDNA using known concentrations of the specific gene product, which was normalized to the housekeeping gene. The standards were prepared by RT-PCR and sequenced to confirm their identity.

2.4 Estrogen sulfotransferase activity

One testis and a portion of the liver from each mouse was individually homogenized in buffer (250mM sucrose, 1mM EDTA, 10mM Tris, pH 7.4, containing 2 μ g/mL each aprotinin, leupeptin, and pepstatin) with a Dounce homogenizer. Microsomes were prepared by centrifuging the homogenate at 10,000xg for 10 minutes. The supernatant was removed and centrifuged at 100,000xg for 60 minutes. The cytosol was removed and stored at -80°C. The microsomal pellet was resuspended and recentrifuged. The final pellet containing the microsomes was resuspended in homogenization buffer containing 10% glycerol and stored at -80°C. Protein concentrations were determined according to Bradford (Bradford, 1976) using bovine serum albumin as the standard. Estrogen sulfotransferase (EST) activity was determined by preincubating 100 μ g of either liver or testes cytosolic protein in 50 mM Tris-HCl, 7mM MgCl₂ buffer, pH 7.4, containing 100nM [³H] estradiol (100Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) at 37°C for 3 minutes. The reactions were started by the addition of 20 μ M PAPS and incubated for 30 minutes at 37°C. The reactions were terminated by the addition of 250 mM Tris-HCl (pH 8.7) to alkalize the solution and chloroform to separate the estradiol from its sulfated product (Miki et al., 2002). EST formation was determined by removing 100 μ l of the aqueous phase and counting on a liquid scintillation counter.

2.5 Immunoblotting

Testicular microsomal proteins (2-20 μ g) were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose (Harlow and Lane, 1988). The nitrocellulose membrane was blocked in 5% nonfat milk and then incubated with the primary antibody in TBST (Ugt2b: 1:400 dilution; Cyp17: 1:200 dilution; both from Santa Cruz Biotechnology, Santa Cruz, CA). After rinsing, the blot was incubated with the appropriate secondary antibody (1:200-1:5000 dilution; Santa Cruz) in TBST. The blot was developed and the intensity of the bands quantified using the ImmunStar AP kit (BioRad) or the Luminol HRP kit (Santa Cruz) on a ChemiDoc XRS molecular imager (BioRad). Blots were stripped in 100mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl, pH 6.7 at 50°C. After rinsing in TBST, blots were reprobed using an actin (1:500 dilution; Sigma) or GAPDH antibody (1:1000 dilution; Imgenex, San Diego, CA) as a loading control, and were quantified as above.

2.6 Progesterone metabolism by CYP17

CYP17 hydroxylase assays were performed to assess hormone production using progesterone as a precursor. Testicular microsomal protein (50 μ g) was preincubated in 100mM potassium phosphate buffer (pH 7.4) containing 1 μ M [³H]progesterone (120Ci/mmol, American Radiolabeled Chemicals) in a shaking water bath at 37°C for 3 minutes. 1mM NADPH was added to initiate the reactions and the tubes were incubated for 20 minutes. The reaction was terminated and progesterone metabolites were extracted twice by the addition of 2mL ethyl acetate. The extracts were evaporated under nitrogen, dissolved in 35 μ L ethyl acetate, and spotted onto TLC plates. The TLC plates were resolved using a 3:1 mixture of chloroform and ethyl acetate. The identities of metabolites were determined by co-migration of authentic standards (Steraloids, Newport, RI). The TLC plates were exposed to autoradiography film, and individual metabolites were cut from the plates and quantitated by scintillation counting to determine the specific activity for each metabolite.

2.7 Statistical Analysis

Samples from each group were averaged and significant differences were determined by Student's t-test or Mann-Whitney ($p \leq 0.05$) using Graphpad Prism Software (San Diego, CA). Linear regression analyses were also performed using Graphpad Prism.

3. Results

3.1 Reductions in steroid hormone concentrations in Mrp1 knockout mice

Because MRP1 is known to transport several steroid hormones *in vitro*, their concentrations in the serum of wild-type and Mrp1 knockout mice were examined. Serum testosterone concentrations were reduced by 5-fold in the FVB/Mrp1^{-/-} mice, although there were no differences in serum progesterone, androstenedione, DHEA, DHEAS, or estradiol levels (Figure 1). We hypothesized that since testosterone concentrations were reduced in the serum, its levels in the testes would be increased. Surprisingly, testosterone levels were actually reduced by 2.4-fold in the testes of mice lacking Mrp1, indicating low testosterone production (Figure 2). Progesterone levels were similar between both strains of mice. However, the other progesterone metabolites examined, androstenedione and estradiol, were significantly reduced by 4.5-fold and 1.7-fold, respectively, in Mrp1 knockout mice. Likewise, testicular DHEA concentrations were lowered by 2.1-fold (Figure 2).

Next, we determined whether testicular hormone concentrations correlated with one another using linear regression. Progesterone levels tended not to correlate with the other hormones, but the levels of DHEA, androstenedione, testosterone, and estradiol did highly correlate with one another (Table 2). Indeed, when comparing the ratio of two hormones in the testes of an individual mouse, there is always a higher level of progesterone in the Mrp1 knockout mice versus the control mice, relative to the other downstream hormones, including androstenedione and testosterone, as well as DHEA (Table 3). In contrast, there is a trend towards a reduction in testicular androstenedione levels relative to all other downstream hormones. The changes in steroid hormone concentrations in the Mrp1^{-/-} mice, coupled with a higher relative ratio of progesterone versus androstenedione suggests that either (1) there is increased conjugation, inactivation, and/or elimination of androstenedione, testosterone, and estradiol in mice lacking Mrp1, or (2) an enzyme in the steroid hormone biosynthetic pathway is altered in mice lacking Mrp1, likely immediately preceding the formation of androstenedione.

3.2 Alterations in steroid conjugation

To test these hypotheses, the expression and activity of sulfotransferase 1e1 (SULT1E1) and uridine diphosphoglucuronoyltransferase 2b (UGT2B) were examined in the testes to determine whether increased conjugation was responsible for the reductions in testicular steroid hormone concentrations. There were no differences in SULT1E1 RNA expression or enzyme activity, or in Ugt2b RNA and protein levels in the testes of wild-type and Mrp1^{-/-} mice (Figure 3). There are also no changes in SULT1E1 activity and in UGT2b RNA expression in the liver of the two strains of mice (data not shown). Thus, an increase in androgen inactivation due to increased conjugation does not appear to cause the reductions in testicular androstenedione, testosterone, or estradiol levels.

3.3. Changes in steroid biosynthetic enzymes

To test the second hypothesis, the levels of steroidogenic enzyme mRNA expression in the testes along with the transcription factor SF-1, as well as the androgen, estrogen, and lutenizing hormone receptors, were examined by QPCR to determine whether there were differences between control and Mrp1 knockout mice. There were no changes in the expression of StAR, Cyp11a, Cyp19, LHR, ER α , or ER β mRNA levels (data not shown). AR levels were increased

in the mice lacking Mrp1, but this was not quite statistically significant ($p=0.06$; data not shown). We also examined changes in other enzymes in the steroid biosynthetic pathway, including 3β -HSD1, which catalyzes the conversion of pregnenolone into progesterone, 17β -HSD3, which converts androstenedione into testosterone, and SF-1, which is a transcription factor involved in regulation of the enzymes in this pathway. Levels of 3β -HSD1 in the testes were unchanged between wild-type and *Mrp1*^{-/-} mice (Table 4). 17β -HSD3 expression appeared to be upregulated in the mice lacking Mrp1, but this was not quite statistically significant ($p=0.068$; Table 4). However, the ratio of 17β -HSD3/ 3β -HSD1 expression and SF-1/ 3β -HSD1 in each individual mouse was significantly increased (Figure 4). This indicates that mice lacking Mrp1 have upregulated steroidogenic gene expression to compensate for the lowered production of hormones.

Ultimately, it is the conversion of progesterone to androstenedione along the Δ^4 pathway that appears to be most impacted, because progesterone levels in the testes were similar between FVB and *Mrp1*^{-/-} mice while androstenedione levels were reduced in *Mrp1*^{-/-} mice. Therefore, we examined Cyp17 protein and activity. Cyp17 catalyzes the formation of 17-OH progesterone from progesterone, and then the formation of androstenedione from 17-OH progesterone. Transcript levels of Cyp17 were increased by 1.6-fold but this was not quite statistically different (data not shown). There was a significant 1.6-fold increase in Cyp17 protein in the testes of Mrp1 knockout mice (Figure 4A and B). The increase in protein expression was corroborated by with an increase in Cyp17 activity, with the production of 17-OH progesterone increased by 1.4-fold and the production of androstenedione increased by 2-fold (Figure 4C). Taken together, these data indicate that mice lacking the Mrp1 transporter have upregulated steroid biosynthetic enzymes in an attempt to mitigate the reduction in serum and testicular hormones.

4. Discussion

Maintaining steroid hormone homeostasis is essential for ensuring sexual differentiation, development, and reproduction. Estrogens are required for proper development and maintenance of the male reproductive tract, as well as for male fertility (Akingbemi, 2005; Hess, 2003), but too high of estrogen levels have shown to be detrimental (Akingbemi, 2005; Delbès et al., 2006; Toppari, 2008). For example, administration of exogenous estradiol to adult male rats resulted in significant decreases in serum and testicular testosterone levels. Depending on the estradiol concentration administered, serum testosterone levels were reduced between 4.4- to 18-fold while testicular testosterone levels were reduced 7.8- to 11.6-fold (D'Souza et al., 2005). Although the true endogenous function of MRP1 is not known, it has been hypothesized that MRP1 acts to maintain steroid hormone homeostasis because it can actively transport the estrogen metabolites 17β -estradiol glucuronide and estrone 3-sulfate, as well as DHEAS (Chen et al., 2005; Loe et al., 1996; Zelcer et al., 2003). Thus, because Mrp1 expression is quite high in the testes (Flens et al., 1996; Maher et al., 2005; Peng et al., 1999; Stride et al., 1996), one role that Mrp1 likely plays is to help protect developing spermatozoa from excessively high steroid hormone levels.

The present study indicates that mice lacking the Mrp1 transporter have reduced concentrations of androstenedione, testosterone, estradiol, and DHEA in their testes, which to our knowledge, is the first time tissue reductions in steroid hormone levels has been shown in mice lacking one of the MRP family members. Furthermore, linear regression demonstrated that all of the testicular hormone concentrations correlated with one another except for progesterone. For example, mice lacking Mrp1 have 2.8- and 3.3-fold more testicular progesterone than testosterone and androstenedione, respectively. There is a similar trend with a higher ratio of progesterone to estradiol in the knockout mice. In contrast, testicular androstenedione levels were lower relative to all other downstream hormones. Although these were not statistically

significant, the hormone ratios in the Mrp1 knockout mice were consistently half the values of the control mice (0.46-0.55 fold reduction), with all *p*-values being 0.075-0.099. These data suggest that the enzyme that converts progesterone into androstenedione, Cyp17, might be altered in these mice.

Indeed, Cyp17 protein levels and activity were significantly increased between 1.4- to 2-fold in the testes of Mrp1 knockout mice. Transcript levels of Cyp17 were also increased by 1.6-fold but this was not quite statistically different (data not shown). It appears that Cyp17 is therefore upregulated in *Mrp1*^{-/-} mouse testes to compensate for the low levels of androstenedione and testosterone. Although there are several transcription factors that regulate Cyp17 expression, the predominant one appears to be SF-1 (Nr5a1) (Busygina et al., 2005; Mellon et al., 1998; Ozbay et al., 2006; Patel et al., 2009; Shi et al., 2009; Zhang et al., 2001). SF-1 mRNA levels were increased by 1.7-fold in the testes of mice lacking Mrp1, although this was not statistically different. Earlier studies had demonstrated that Cyp17 is regulated by LH secretion via the cAMP signaling pathway (Anakwe and Payne, 1987; Sewer and Waterman, 2002). cAMP can increase Cyp17 mRNA levels, and this effect can be reversed with testosterone exposure (Payne and Sha, 1991). These studies suggest that reduced testosterone levels would cause an increase in Cyp17 expression, consistent with our findings.

The compensatory upregulation in Cyp17 is corroborated by a 1-6-fold increase in the transcript levels of 17 β -Hsd3, which is the enzyme that converts androstenedione into testosterone. mRNA levels of the third enzyme in the pathway, 3 β -Hsd1, which converts pregnenolone into progesterone, was not altered between the two strains. However, the ratio of 17 β -Hsd3 to 3 β -Hsd1 in the testes was significantly increased in mice lacking Mrp1, again suggesting an upregulation of steroidogenic enzymes after the formation of progesterone. A recent study determined that testicular 17 β -Hsd activity in mice was 0.0153 μ mol NAD/minute/mg protein and 3 β -Hsd activity was 0.0246 μ mol NADH/minute/mg protein, for a ratio of 0.62 (Harini et al., 2009). This ratio of 0.62 in their study is in line with our findings in FVB mice, in which the ratio of 17 β -Hsd to 3 β -Hsd transcript levels was 0.7. In *Mrp1*^{-/-} mice, this ratio is much higher, again indicating an upregulation of 17 β -Hsd3. Like Cyp17, 17 β -Hsd3 also requires LH and androgen stimulation to maintain proper expression (Baker et al., 1997; Tsai-Morris et al., 1999). However, cAMP stimulation is not needed for the expression of 3 β -HSD (reviewed in (Payne and Youngblood, 1995)). Indeed, the promoter region of 3 β -HSD does not have typical steroid regulatory elements, and so it is assumed that its regulation is mediated by indirect interactions with other transcription factors, such as Stat proteins and NF- κ B (Reichardt et al., 1998; Simard et al., 2005). Because of the indirect interactions, many investigators consider 3 β -HSD to be a constitutively expressed enzyme. This is in agreement with the present study, in which 3 β -HSD1 levels were unchanged between the two strains of mice.

Additional potential mechanisms responsible for the reductions in testicular steroid hormones might be due to changes in cholesterol uptake. However, we did not see changes in StAR gene expression or in serum cholesterol levels (data not shown), and other studies have not reported pathological differences in the testes between untreated wild-type and Mrp1 knockout mice that would indicate altered cholesterol uptake or storage (Tribull et al., 2003; Wijnholds et al., 1998). Mice lacking estrogen sulfotransferase have increased lipid deposition in Leydig cells, but the accumulation of cholesterol esters is found only in older animals (>18 months). Two- to three-month old *Sult1e*^{-/-} mice did not display this phenotype (Tong et al., 2004). The mice in our study were 10 weeks old, and this may have impeded our ability to detect additional differences such as lipid deposition between the two strains. Another mechanism causing changes in steroid hormones might be due to alterations in LH levels. Serum LH was assayed, but due to the limited amount of serum, we were never able to obtain values above the detection limits of the assay. However, LH receptor gene expression was unchanged between the two strains of mice (data not shown). Additionally, reduced steroid hormone concentrations do not

always translate into increased LH levels. For example, administration of exogenous estradiol to adult male rats resulted in significant decreases in serum and testicular testosterone levels. At the lowest concentration of estradiol, both serum FSH and LH were reduced, but returned to control levels with higher estradiol concentrations (D'Souza et al., 2005). Indeed, constant stimulation of gonadal cells by LH may alter negative feedback loops either in the HPG axis or short loops within the gonadal tissue itself (Taniguchi et al., 2007).

It has been long known that steroid hormone concentrations in the gonads are regulated, in part, through negative feedback loops. However, after chronic stimulation or exposure to a stressor, an organism may over- or under-compensate for that given perturbation, which has been part of the difficulty in modeling dose-responses to stressors in the reproductive system (Andersen et al., 2005). The results of the present study indicate that mice have the ability to compensate for the loss of Mrp1 in their testes by upregulating Cyp17 and altering the ratio of 17 β Hsd to 3 β Hsd. The compensatory responses in the testes seen in our study are corroborated by recent studies in a model fish species. Fathead minnows were exposed to the Cyp19/ aromatase inhibitor fadrozole, which reduced estradiol levels in the serum and ovaries, but increased Cyp19, Cyp11a, StAR, and FSH receptor mRNA levels (Villeneuve et al., 2009). Exposure to prochloraz, which inhibits both Cyp19 and Cyp17, along with acting as an androgen receptor antagonist, reduced testosterone and estradiol levels in the serum of male fish, but increased testicular expression of AR, Cyp17, and Cyp11a mRNA (Ankley et al., 2009). In both of these studies, the investigators noticed an overcompensation once the stressor was removed, indicating that the increase in transcript levels serve to offset the reductions in steroid hormone levels. This is similar to the findings of the current study, in which androstenedione and testosterone concentrations were low, yet transcript, protein, and activity of Cyp17 were increased.

In conclusion, mice lacking Mrp1 have reduced concentrations of testicular steroids, including androstenedione, testosterone, estradiol, and DHEA, but have increased steroid biosynthetic enzymes. These results suggest that upregulation of steroid biosynthetic enzymes may be an attempt to maintain proper steroid hormone homeostasis due to the loss of the Mrp1 transporter.

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Figure 1. Alterations in steroid hormone levels in the serum

All serum samples were run in duplicate or triplicate. Values are the average $\pm$ standard deviation of 4-6 mice per group. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Figure 2. Alterations in steroid hormone concentrations in the testes

All samples testicular samples were run in duplicate or triplicate. Values are the average $\pm$ standard deviation for 5-6 mice per group. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Figure 3. Sulfotransferase and glucuronosyltransferase expression and activity do not differ in the testes of FVB and FVB/*Mrp1*^{-/-} mice

Sult1e1 and *Ugt2b* RNA expression was determined by QPCR (A). The data is expressed in number of molecules/100ng cDNA $\pm$ standard deviation. All samples were normalized to 18S rRNA, with each sample run in triplicate ($n=5=6$). SULT1E1 activity was measured by EST metabolite formation and is expressed as pmol/mg protein (B). All samples were run in triplicate ($n=6$) and the data is expressed as the average of two separate assays. UGT2B protein levels were determined by immunoblotting (C) and quantified by densitometry, using GAPDH as a loading control. Data is expressed as GAPDH-corrected raw intensity values ($n=4$) and are the average of two separate blots (D).

Figure 4. The ratio of 17 β -HSD3 to 3 β -HSD1 and SF-1 to 3 β -HSD1 mRNA expression is increased in *Mrp1*^{-/-} mice

The ratio of 17 β -HSD3 to 3 β -HSD1 mRNA expression (A) and SF-1 to 3 β -HSD1 (B) for each individual mouse was calculated. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Figure 5. Increased testicular Cyp17 protein expression and activity in *Mrp1*^{-/-} mice
 Protein levels of Cyp17 were determined by immunoblotting (A) and quantified by densitometry (B), using actin as a loading control. Data is expressed as actin-corrected raw density values (n=4) and are representative of three separate blots. Cyp17 activity was determined by scintillation counting (C), and the data is presented as the average $\pm$ standard deviation (n=6) of 2 different assays. Statistical differences (*) were determined using Student's t-test ($p \leq 0.05$).

Table 1

Primer sets for QPCR

Name	Accession #	Forward	Reverse
Ugt2b	NP_690024	5'-agttgagacaatgggccaag-3'	5'-gttgggtgaggaaactccaa-3'
Sult1e1	BC034891	5'-tgatgccagaggaaatgatg-3'	5'-tgggaagtgggttctccagt-3'
3 β HSD1	BC052659	5'-tagcaagtacagaggcacaagcca-3'	5'-tgtgagtgggttagtgactggcaa-3'
17 β HSD3	U66827	5'-taacaagatgaccaagaccgccga-3'	5'-gatttcagagcaaggcagccaca-3'
AR	X53779	5'-tcaaggagggttacccaaggat-3'	5'-acagagccagcggaaagttagt-3'
LHR	M81310	5'-cgcatgttcacgaagcatttca-3'	5'-tccttctgtaaagttcagcccggt-3'
Esr1	NM_007956	5'-gaagcccgaatgaaatgggtgct-3'	5'-tcaaggacaaggcagggtctattct-3'
Esr2	U81451	5'-agctggctgggctggtatttatct-3'	5'-tgcccacttctctcacacactt-3'
SF-1	NM_139051	5'-agtctgacttgaaggattgcct-3'	5'-aggtcatttgatgaccacaccgt-3'
Cyp17	NM_007809	5'-accgtcttcaatgaccggactca-3'	5'-ttatcgtgatgcagtgccagaga-3'
Cyp11a	NM_019779	5'-caggccaacattaccgagat-3'	5'-cgcatctctctgtacctt-3'
StAR	NM_011485	5'-gatgtgggcaaggtgttct-3'	5'-gcggtcacacaagttctcat-3'
Cyp19	NM_007810	5'-ccaggtgaagacactgcaaa-3'	5'-atttcacaagggtgcctgtc-3'
18S rRNA	NR_003278	5'-ttgacggaaggcaccaccag-3'	5'-cgattccgtgggtggtgtgc-3'
GAPDH	BC096042	5'-gccttcggtgttctacc-3'	5'-gcctgttcaccaccttc-3'

Table 2
Correlations between testicular concentrations of steroid hormones

Hormone concentrations were determined by EIA or RIA in the testes of FVB and *Mtp1*^{-/-} mice. Correlations were determined by linear regression.

	<u>Progesterone</u>	<u>Androstenedione</u>	<u>DHEA</u>	<u>Testosterone</u>	<u>Estradiol</u>
Progesterone	-----	N.S.	N.S.	$r=0.66$ $p=0.027$	N.S.
Androstenedione		-----	$r=0.87$ $p=0.001$	$r=0.83$ $p=0.030$	$r=0.71$ $p=0.021$
DHEA			-----	$r=0.87$ $p=0.001$	$r=0.83$ $p=0.003$
Testosterone				-----	$r=0.79$ $p=0.004$
Estradiol					-----

N.S. is not significant

Table 3**Ratios of testicular hormones**

The ratios of testicular hormones in each individual mouse was averaged and compared between FVB and *Mrp1* $-/-$ mice. Statistical differences were determined by Student's t-test ($p < 0.05$).

	<u>FVB ratio</u>	<u><i>Mrp1</i>$-/-$ ratio</u>	<u>Fold difference</u>
Progesterone/androstenedione	0.03 $\pm$ 0.03	0.10 $\pm$ 0.05	3.3 [*]
Progesterone /testosterone	1.5 $\pm$ 0.5	4.2 $\pm$ 2.0	2.8 [*]
Progesterone /estradiol	10.0 $\pm$ 2.2	24.4 $\pm$ 16.4	2.4
Progesterone /DHEA	0.8 $\pm$ 0.3	1.7 $\pm$ 0.8	2.3 [*]
Androstenedione/testosterone	79.3 $\pm$ 35.1	44.2 $\pm$ 15.7	0.6
Androstenedione/estradiol	591.5 $\pm$ 356.9	282.3 $\pm$ 97.8	0.5
Androstenedione/DHEA	39.1 $\pm$ 23.1	18.0 $\pm$ 5.8	0.5
Testosterone/estradiol	7.2 $\pm$ 2.3	5.9 $\pm$ 3.7	0.8
Testosterone/DHEA	0.5 $\pm$ 0.2	0.4 $\pm$ 0.2	0.8
Estradiol/DHEA	0.07 $\pm$ 0.02	0.07 $\pm$ 0.03	0.9

* statistically significant using Student's t-test ($p < 0.05$)

Table 4
mRNA concentrations of steroidogenic enzymes

The number of mRNA molecules for each enzyme was determined by QPCR and normalized to GAPDH expression. Fold difference was determined by divide the transcript levels in the *Mrp1*^{-/-} mice by the transcript levels in the FVB mice.

	<u>FVB mRNA</u>	<u><i>Mrp1</i>^{-/-} mRNA</u>	<u>Fold difference</u>
3β-hydroxysteroid dehydrogenase 1	121.8 $\pm$ 50.4	122.0 $\pm$ 47.8	1.0
17β-hydroxysteroid dehydrogenase 3	80.0 $\pm$ 23.6	130.8 $\pm$ 56.2	1.6
SF-1	48.4 $\pm$ 21.0	83.2 $\pm$ 44.3	1.7

*statistically significant using Student's t-test ($p \leq 0.05$)

N=6-7 mice; values are the average of two experiments