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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 17β-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- to 2.0-fold in mice lacking Mrp1. Additionally, the ratio of 17β-hydroxysteroid dehydrogenase to 3β-hydroxysteroid dehydrogenase, and steroidogenic factor 1 to 3β-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.

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 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

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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 CO_2 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 0.1 M potassium phosphate buffer, pH 7.4, and testicular steroid hormones were extracted twice by the addition of 2 mL of diethyl ether. The two extracts were combined, evaporated under nitrogen and resuspended in 1 mL 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, 10 μL of a 1:20 dilution of the testes extracts or 10 μL of serum was used. For progesterone concentrations, 25 μL of the testes extract and serum were used. For estradiol concentrations, 25 μL serum and 100 μL of the testicular extracts was used. For androstenedione, 5 μL of both serum and testes extract were used. For DHEA, 50 μL of serum and 100 μL of the testicular extracts were used, while for DHEAS, 1 μL of serum or 100 μL 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 (2 μg) was incubated with 50 ng random hexamers, RNAsin, 10 mM dNTP mix, and 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase at 37 °C for 1 h. Quantitative PCR was performed in Bio-Rad's I-Cycler (Hercules, CA) using 0.2 mM dNTPs, 0.25× Sybr green, 1 U 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), lutenizing hormone receptor (LHR), Cyp17, Cyp11a, steriodogenic 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 s, an annealing/extension step at 61 °C (51 °C for GAPDH) for 1 min for a total of 40 cycles. The cycle threshold values obtained from the real-time PCR were converted into starting number of molecules per 100 ng 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 (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, containing 2 μg/mL each aprotinin, leupeptin, and pepstatin) with a Dounce homogenizer. Microsomes were prepared by centrifuging the homogenate at 10,000*g* for 10 min. The supernatant was removed and centrifuged at 100,000*g* for 60 min. 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 (1976) using bovine serum albumin as the standard. Estrogen sulfotransferase (EST) activity was determined by preincubating 100 μg of either liver or testes cytosolic protein in 50 mM Tris–HCl, 7 mM $MgCl_2$ buffer, pH 7.4, containing 100 nM [^{3}H] estradiol (100 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) at 37 °C for 3 min. The reactions were started by the addition of 20 μM PAPS