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DRUG METABOLISM AND DISPOSITION
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DRUG METABOLISM & DISPOSITION

Sexual Dimorphism in the Expression of Cytochrome P450 Enzymes in Rat Heart, Liver, Kidney, Lung, Brain, and Small Intestine

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ABSTRACT

Cytochrome P450 (P450) enzymes are monooxygenases that are expressed hepatically and extrahepatically and play an essential role in xenobiotic metabolism. Substantial scientific evidence indicates sex-specific differences between males and females in disease patterns and drug responses, which could be attributed, even partly, to differences in the expression and/or activity levels of P450 enzymes in different organs. In this study, we compared the mRNA and protein expression of P450 enzymes in different organs of male and female Sprague-Dawley rats by real-time polymerase chain reaction and western blot techniques. We found significant sex- and organ-specific differences in several enzymes. Hepatic *Cyp2c11*, *Cyp2c13*, and *Cyp4a2* showed male-specific expression, whereas *Cyp2c12* showed female-specific expression. *Cyp2e1* and *Cyp4f* enzymes demonstrated higher expression in the female heart and kidneys compared with males; however, they showed no significant sexual dimorphism in the liver. Male rats showed higher hepatic and renal *Cyp1b1* levels. All assessed enzymes were found in the liver, but some were not expressed in other organs. At the protein expression level, CYP1A2, CYP3A, and CYP4A1 demonstrated higher expression levels in the females in several organs, including the liver. Elucidating sex-specific differences in P450 enzyme levels could help better understand differences in disease pathogeneses and drug responses between males and females and thus improve treatment strategies.

SIGNIFICANCE STATEMENT

This study characterized the differences in the mRNA and protein expression levels of different cytochrome P450 (P450) enzymes between male and female rats in the heart, liver, lung, kidney, brain, and small intestine. It demonstrated unique sex-specific differences in the different organs. This study is considered a big step towards elucidating sex-specific differences in P450 enzyme levels, which is largely important for achieving a better understanding of the differences between males and females in the disease's processes and treatment outcomes.

Introduction

Cytochrome P450 (P450) is a superfamilyof membrane-bound hydrophobic heme enzymes that play a pivotal role in health, homeostasis, and metabolism. P450 enzymes are expressed in almost all biologic systems (El-Sherbeni and El-Kadi, 2017). They are so called because their heme pigment absorbs light at a wavelength of 450 nm following reduction and exposure to carbon monoxide (Lynch and Price, 2007). The discovery of P450 enzymes started in the early 1950s and continued until the 1960s (El-Sherbeni and El-Kadi, 2017). P450 enzymes are classified into families, subfamilies, and individual enzymes based on the structural homology of their amino acid sequences (Nebert et al., 1987; Elbekai and El-Kadi, 2006). Microsomal P450s, which are attached to the endoplasmic reticulum membrane, comprise the majority of human P450 enzymes and catalyze a wide array of biologic reactions (El-Sherbeni and El-Kadi, 2017).

P450 enzymes are mainly expressed in the liver, but they are also found in several extrahepatic tissues, including the heart (Elbekai and El-Kadi, 2006), kidney (Fan et al., 2015), lungs (Hukkanen et al., 2002), brain (Dutheil et al., 2008), and other tissues (Zhu and Zhang, 2012; Alonso et al., 2015; Ibrahim et al., 2020). The induction or inhibition of P450 enzymes by xenobiotics or disease states is a major mechanism underlying drug-drug and drug-disease interactions. Moreover, genetic polymorphisms in P450 genes could lead to differences in P450 enzymes that might explain individual and ethnic variations in disease pathogeneses and drug responses (Manikandan and Nagini, 2018). Given their significant biologic effects, P450 enzymes have been the focus of many clinical, experimental, and drug development studies.

P450 enzymes play an essential role in the detoxification and activation of both xenobiotics and endogenous molecules. In humans, there are 18 P450 families with more than 50 individual P450 isoenzymes, nine of which are involved in the metabolism of several drugs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) (Wilkinson, 2005). In addition to their essential role in xenobiotic metabolism, P450 enzymes are also largely involved in the synthesis and metabolism of endogenous molecules such as steroids and prostaglandins. For example, P450s metabolize polyunsaturated fatty acids like arachidonic acid (AA) by the insertion of either an epoxide or hydroxyl group, based on which they can be classified into P450 epoxygenases or hydroxylases, respectively. AA metabolism by P450 enzymes to give multiple epoxy and

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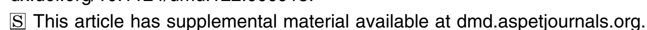
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ABBREVIATIONS: AA, arachidonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; P450, cytochrome P450; PCR, polymerase chain reaction; SD, Sprague-Dawley.

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TABLE 1

Rat cytochrome P450 enzymes and their human orthologs

Rat gene	Human Ortholog
Cyp1a1	*CYP1A1*
Cyp1a2	*CYP1A2*
Cyp1b1	*CYP1B1*
Cyp2a1	—
Cyp2b1	*CYP2B6*
Cyp2b2	—
Cyp2c6	*CYP2C19*
Cyp2c11	*CYP2C9*
Cyp2c12	—
Cyp2c13	—
Cyp2c23	—
Cyp2d2	—
Cyp2d3	*CYP2D6*
Cyp2d4	—
Cyp2e1	*CYP2E1*
Cyp2j3	—
Cyp2j4	*CYP2J2*
Cyp2j10	—
Cyp3a1	*CYP3A5*
Cyp3a2	-
Cyp3a9	*CYP3A7*
Cyp3a18	—
Cyp3a23	*CYP3A5*
Cyp4a1	*CYP4A11*
Cyp4a2	—
Cyp4a3	—
Cyp4a8	—
Cyp4f1	*CYP4F12*
Cyp4f4	*CYP4F8*
Cyp4f5	—
Cyp4f6	*CYP4F3*

hydroxy metabolites has been extensively studied in health and diseases, particularly in the cardiovascular system (Elbekai and El-Kadi, 2006; Shoieb et al., 2019; Gerges and El-Kadi, 2022).

To date, a large body of evidence points out to significant differences between males and females in the pathogenesis and outcomes of different diseases, as well as in drug metabolism and responses (Dahan et al., 2008; Waxman and Holloway, 2009; Ngo et al., 2014; Regitz-Zagrosek and Kararigas, 2017; Holingue et al., 2020; Tramunt et al., 2020; Madla et al., 2021; Gerges and El-Kadi, 2022). Moreover, it was established previously that there are significant sex differences in the expression or activity of several drug-metabolizing enzymes in animals and humans and that this could be responsible for differences in clinical drug effects between men and women (Waxman and Holloway, 2009). For example, multiple studies have shown higher mRNA and protein expression levels of hepatic CYP3A4 in women than in men, which could explain the higher clearance rates of CYP3A4 substrates in women (Tanaka, 1999; Greenblatt and Von Moltke, 2008; Waxman and Holloway, 2009). On the other hand, some CYP1A2, CYP2E1, and CYP2D6 substrates were found to have higher clearance rates in men than in women (Franconi et al., 2007; Schwartz, 2007). Cardiovascular diseases are among the diseases that demonstrate significant sex-specific discrepancies, which could be mediated, even in part, by different expression or activity levels of cardiac P450 enzymes and their metabolites (Gerges and El-Kadi, 2022).

Elucidating sex differences in the expression levels of P450 enzymes in different organs could help explain observed sex differences in diseases and drug effects, decrease the incidence of adverse effects and improve the efficacy of different medications, and approach precision medicine. Thus, the current study is one of a series of studies aiming at investigating sex-specific differences in the expression and activity levels of different P450s, as well as the levels of their metabolites. The aim of the current study was to compare the mRNA and protein expression levels of different P450 enzymes in the heart, liver, lung, kidney, brain, and small intestine between male and female rats.

TABLE 2

Rat primer sequences

Gene	Forward Primer	Reverse Primer
Cyp1a1	CCAAACGAGTTCCGGCCT	TGCCCAAACCAAAGAGAATGA
Cyp1a2	CGCCCAGAGCGGTTTCTTA	TCCCAAGCCGAAGAGCATC
Cyp1b1	GCTTTACTGTGCAAGGGAGACA	GGAAGGAGGATTCAAGTCAGGA
Cyp2a1	CACAGGGCAGCTCTATGACA	CAGACCCAGCAAAGAAGAGG
Cyp2b1	AACCCTTGATGACCGCAGTAAA	TGTGGTACTCCAATAGGGACAAGATC
Cyp2b2	CCATCCCTTGATGATCGTACCA	AATTGGGGCAAGATCTGCAAA
Cyp2c6	CCTGCTGAAGTGTCCAGAGG	CCCATCTAAAAAGTGGCCAG
Cyp2c11	CACCAGCTATCAGTGGATTTGG	GTCTGCCCTTTGCACAGGAA
Cyp2c12	TATAAACTCAATACGTTCTGAG	TTTTACATTAACTTCAGAAACTG
Cyp2c13	CTGGCAATCATGGTGACTGA	GAAACTCCTTGCTGTCATGC
Cyp2c23	GATGCTGTCTTCCGTCATGC	GTAATAGGCTTGATGTCAAG
Cyp2d2	CTACTGCCCATCTATAATCA	CCAAAGCTCTCCTTCAATGT
Cyp2d3	ACCAATGCTGTCATCCATGAGGT	GCTGGACTAGAATTTCTTTCCTT
Cyp2d4	GACCAGTCGGGCTTTGGACCAC	CGAAGGCCTTCTTTCCAGAG
Cyp2e1	AAAGCGTGTGTGTGTTGGAGAA	AGAGACTTCAGGTTAAAATGCTGCA
Cyp2j3	CATTGAGCTCACAAGTGGCTTT	CAATTCCTAGGCTGTGATGTCG
Cyp2j4	GCTCGGACCTTCATTCCACA	GATCGTGGCTACCAGAGAGC
Cyp2j10	TTGAACTTAGCAGAGGGGCTG	TCATACTCAAAGCGCTCCCC
Cyp3a1	GGAAATTCGATGTGGAGTGC	AGGTTTGCCTTTCTCTTGCC
Cyp3a2	GCTCTTGATGCATGGTTAAAGATTTG	ATCACAGACCTTGCCAACTCCTT
Cyp3a9	GGACGATTCTTGCTTACAGG	ATGCTGGTGGGCTTGCCTTC
Cyp3a18	CAACTACGGTGATGGCATGT	CACTCGGTTCTTCTGGTTTG
Cyp3a23	ATGTTCCCTGTCATCGAACAGTATG	TTCACAGGGACAGGTTTGCCT
Cyp4a1	TTGAGCTACTGCCAGATCCCAC	CCCATTTTTGGACTTCAGCACA
Cyp4a2	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATT
Cyp4a3	CTCGCCATAGCCATGCTTATC	CCTTCAGCTCATTCATGGCAATC
Cyp4a8	TGTGGTATCATGAGTGGCTCG	CTTCAGCACGCAGGTCCTTA
Cyp4f1	CCCCCAAGGCTTTTTGATG	GAGCGCAACGGCAGCT
Cyp4f4	CAGGTCTGAAGCAGGTAACTAAGC	CCGTCAGGGTGGCACAGAGT
Cyp4f5	AGGATGCCGTGGCTAACTG	GGCTCCAAGCAGCAGAAGA
Cyp4f6	TCACTTGACCTTGATGAAGAACAAC	AAGAGAGGTGGATATCACGGAAG
B-actin	CCAGATCATGTTTGAGACCTTCAA	GTGGTACGACCAGAGGCATACA
Gapdh	CAAGGTCATCCATGACAACTTTG	GGGCCATCCACAGTCTTCTG

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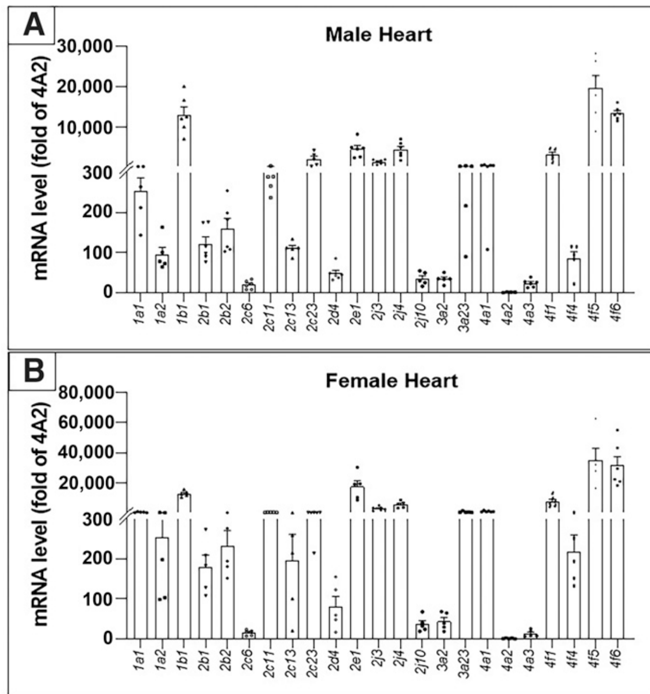

Fig. 1. The mRNA expression of different P450 enzymes in male (A) and female (B) rat heart relative to the least expressed. The mRNA expression of P450 enzymes was determined in the heart of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M, n = 4–6.

Material and Methods

Nomenclature. The nomenclature used throughout the manuscript is following the Guidelines for Formatting Gene and Protein Names, released in 2014. Briefly, enzyme symbols were written in sentence case and italicized when referring to genes or mRNA of mice or rats and were capitalized and nonitalicized when referring to the proteins (https://www.biosciencewriters.com/Guidelines-for-Formatting-Gene-and-Protein-Names.aspx).

Animals. Adult (8 weeks old) male (260–280 g, n = 6) and female (200–220g, n = 6) Sprague-Dawley (SD) rats were purchased from Charles River Canada (Montreal, QC, Canada). All animals were allowed access to food and water ad libitum throughout the experiment period and were maintained on a 12-hour light/dark cycle. Rats were kept in the animal facility for an acclimatization period of 1 week, after which they were euthanized under isoflurane anesthesia. The liver, heart, lung, kidney, brain, and small intestine (20 cm extending from the stomach distally) were isolated and immediately frozen in liquid nitrogen and then stored at −80°C. All procedures involving experimental animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and were approved by Alberta Health Sciences Animal Policy and Welfare Committee.

Chemicals. The TRIzol reagent used for mRNA extraction was Invitrogen brand (Thermo Fisher Scientific, Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Real-time polymerase chain reaction (PCR) primers were formulated by and purchased from Integrated DNA Technologies (Coralville, IA). Trans-Blot Turbo RTA Transfer Kit and 2X Laemmli Sample Buffer were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A2, CYP3A, and CYP4A1 mouse monoclonal primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX); CYP2C23, CYP2E1, and CY4F2 rabbit polyclonal primary antibodies were purchased from Abcam (Cambridge, UK); and CYP2J rabbit polyclonal primary antibody was purchased from MilliporeSigma (St. Louis, MO). Chemiluminescence western blotting detection reagents (enhanced chemiluminescence) were obtained from Cytiva (Marlborough, MA). All other chemicals used were obtained from Sigma Aldrich (St. Louis, MO).

RNA Extraction and cDNA Synthesis. RNA extraction and cDNA synthesis were performed according to the method described by Elshenawy and El-Kadi (2015).

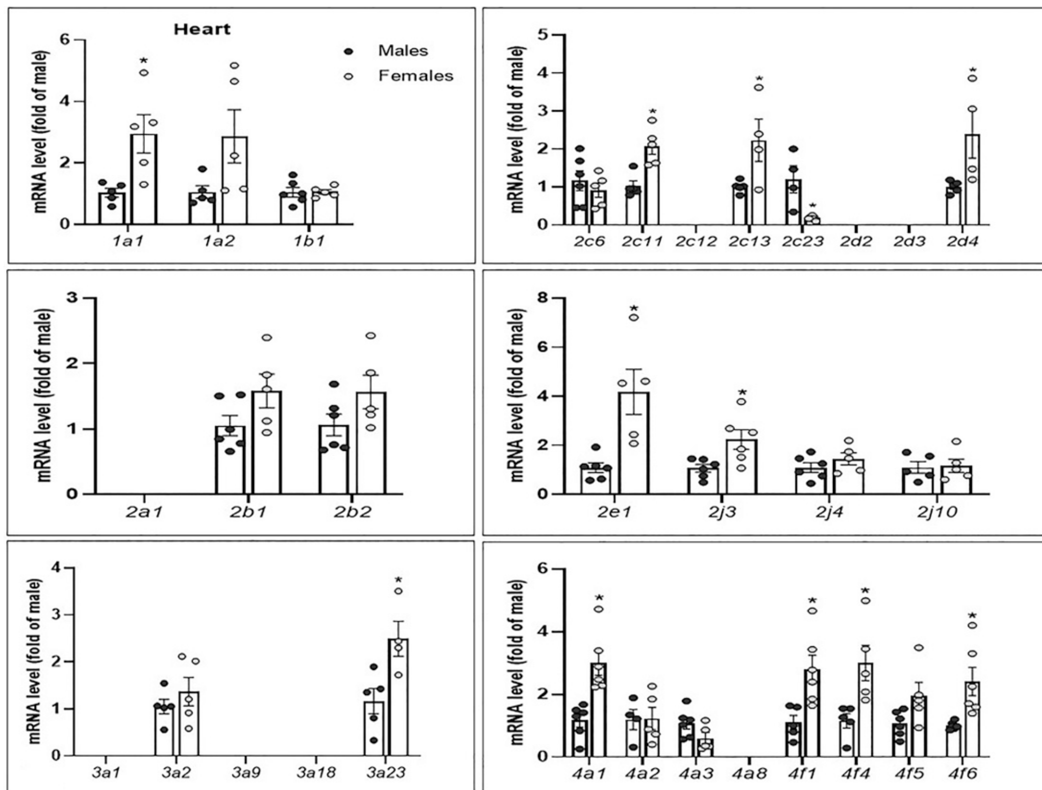

Fig. 2. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat heart. The mRNA expression of P450 enzymes was determined in the heart of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *P < 0.05, significant difference from male rats.

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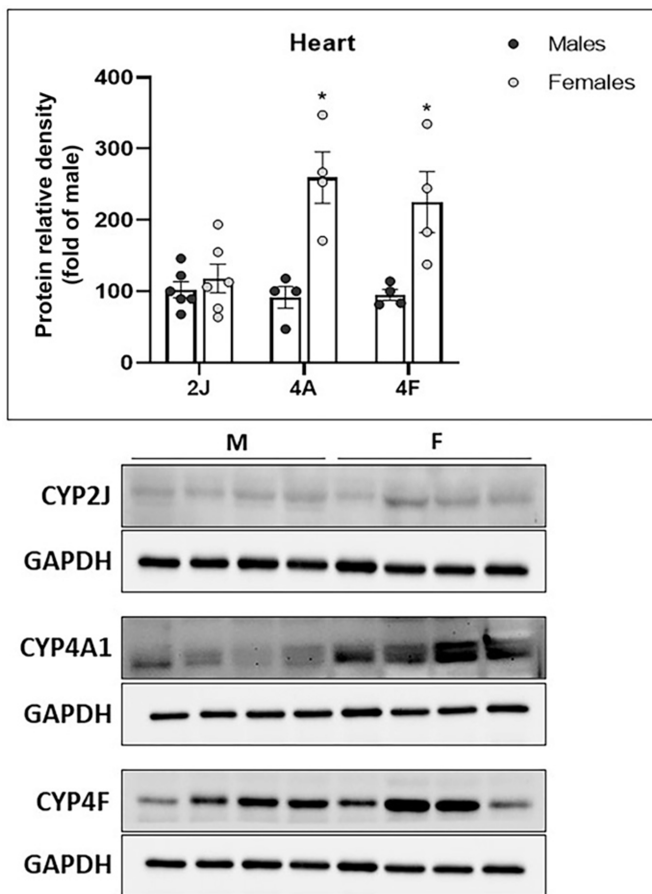

Fig. 3. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat heart. The protein expression of P450 enzymes was determined in the heart of adult male and female Sprague-Dawley rats by western blot and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping protein. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *$P < 0.05$, significant difference from male rats.

Quantification of mRNA Expression by Real-Time PCR. The resulting cDNA was subject to PCR amplification using 384-well optical reaction plates in the QuantStudio 5 (Applied Biosystems). The 20 μL reaction mix contained 0.04 μL of 10 μM forward primers and 0.04 μL of 10 μM reverse primers (20 nM final concentration of each primer), 10 μL SYBR Green Universal Master Mix, 8.92 μL of nuclease-free water, and 1 μL cDNA sample. Thermocycling conditions were as described in previous reports (Shoieb et al., 2022). The rat P450 enzymes and their human orthologs are listed in Table 1. Rat primer sequences used in this study are listed in Table 2. Analysis of the real-time PCR data were performed using the relative gene expression (ΔΔCT) method (Livak and Schmittgen, 2001). In short, the fold change in the level of target genes between female and male rats, corrected for the level of the housekeeping gene, was determined using the following equation: Fold change = $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct(target gene) − Ct(housekeeping gene) and Δ(ΔCt) = ΔCt(females) – mean ΔCt(males). For the comparison of all genes' expression within the same organ, fold change was calculated relative to the least expressed gene.

Preparation of Microsomal Protein. Microsomal fractions were prepared by differential centrifugation of the homogenized organs. Briefly, a weighed mass of each organ was homogenized in cold sucrose solution (0.25 M in distilled water, 0.5 g tissue in 2 mL sucrose solution) containing protease inhibitor cocktail (5 μL/1 mL sucrose solution). The homogenate was centrifuged at 10,000*g* for 20 minutes. The resulting supernatant was centrifuged again at 100,000*g* for 60 minutes to obtain the microsomal pellet. The pellets were dissolved in the homogenization sucrose solution containing protease inhibitor cocktail and stored at −80°C. The Lowry method was used to determine microsomal protein concentrations using bovine serum albumin as a standard (Lowry et al., 1951).

Western Blot Analysis. We determined the protein expression of important P450-metabolizing enzymes (CYP1A2, CYP3A) as well as some main arachidonic acid epoxygenases (CYP2C23, CYP2J) and hydroxylases (CYP2E1, CYP4A1, and CYP4F) using denaturing gel electrophoresis. Briefly, isolated proteins from the different organs of male and female rats (15 μg from the liver; 50 μg from the kidney, lung, and brain; 60 μg from the heart; and 75 μg from the small intestine) were diluted with an equal amount of 2X Laemmli Sample Buffer, boiled for 5 minute, and separated by 10% SDS-PAGE as described by Shoieb et al. (2022). Then, the blots were incubated with the primary antibody: mouse anti-rat CYP1A2 (sc-53241), rabbit anti-rat CYP2C23 (ab53944), rabbit anti-rat CYP2E1 (ab28146), rabbit anti-rat CYP2J (ABS1605), mouse anti-rat CYP3A (sc-271033), mouse anti-rat CYP4A1 (sc-53248), and rabbit anti-human CYP4F2 (ab230709) for 2 hours or overnight at 4°C. Then, blots were incubated with a horseradish peroxidase–conjugated horse anti-mouse or goat anti-rabbit IgG secondary antibody for 45 minutes at room temperature. Bands were visualized using the ChemiDocTM Imaging System (Bio-Rad Laboratories, Hercules, CA) using the enhanced chemiluminescence method.

Statistical Analysis. All results are presented as mean plus or minus S.E.M. Comparisons between male and female groups were carried out using unpaired student t tests. Differences were considered significant at $P < 0.05$. All statistical analyses and graphs plotting were performed using GraphPad Prism software, version 8.4.3. (GraphPad Software, Inc. La Jolla, CA).

Results

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Heart. The mRNA expression levels of different P450 enzymes were determined by real-time PCR. Some P450 enzymes were found to not be expressed in the heart (*Cyp2a1*, *Cyp2c12*, *Cyp2d2*, *Cyp2d3*, *Cyp3a1*, *Cyp3a9*, *Cyp3a18*, and *Cyp4a8*). *Cyp4f5* is the most highly expressed P450 in the hearts of both male and female rats, whereas *Cyp4a2* is the least expressed (Fig. 1). Generally, female hearts showed higher P450 expression levels than male hearts. *Cyp2e1* showed the most marked difference, being nearly fourfold higher in female hearts than male hearts. *Cyp1a1*, *Cyp1a2*, *Cyp4a1*, *Cyp4f1*, and *Cyp4f4* are all approximately threefold higher in female than male hearts. *Cyp4a2*, *Cyp4a3*, and *Cyp4f5* showed no significant difference, whereas all other *Cyp4* family members showed significantly higher expression in female hearts. Only *Cyp2c23* was significantly higher in male hearts than female hearts (5.3-fold) (Supplemental Material). Fig. 2 shows the mRNA expression levels of different P450 enzymes in male hearts compared with female hearts.

Since mRNA expression does not always correlate with protein levels of enzymes, we measured the protein expression of certain P450 enzymes (CYP1A2, CYP2C23, CYP2E1, CYP2J, CYP3A, CYP4A1, and CYP4F) in all organs to investigate sex-specific differences at the protein level. As shown in Fig. 3, CYP2J, CYP4A, and CYP4F enzymes were detected in the heart. In agreement with the mRNA results, CYP4A and CYP4F enzymes showed higher protein levels in female rat hearts (2.8 and 2.4-fold higher than the male level, respectively). However, CYP2J showed no significant difference (Fig. 3).

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Liver. All the investigated 31 P450 enzymes were found to be expressed in the liver. The expression of *Cyp2c12* and *Cyp3a18* was found to be limited to the liver, with no extrahepatic expression. *Cyp2c23* was the highest-expressed P450 in the male liver, whereas *Cyp2e1* was the highest in the female liver. *Cyp4a8* was the lowest in both sexes (Fig. 4). In contrast to the heart, *Cyp2c11* and *Cyp2c13* levels in the liver are male specific, with dramatically higher expression in males, around 1700- and 1300-fold the female hepatic expression levels, respectively. In contrast, *Cyp2c12* in female livers is nearly 200-fold compared with male livers. In the CYP3 family, *Cyp3a9* is nearly

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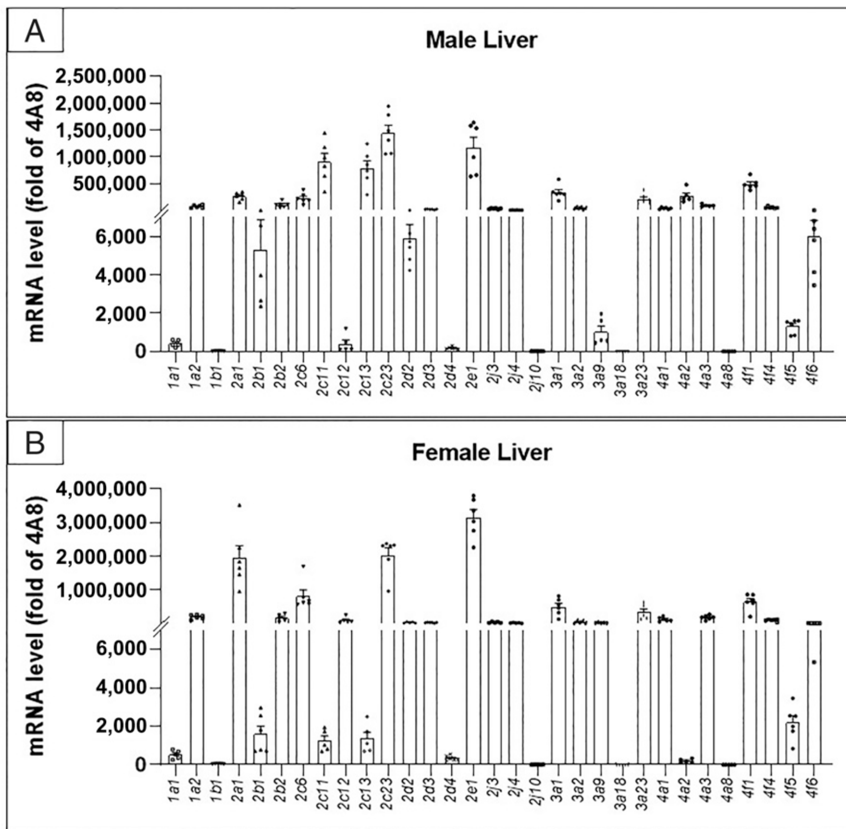

Fig. 4. The mRNA expression of different P450 enzymes in male (A) and female (B) rat liver relative to the least expressed. The mRNA expression of P450 enzymes was determined in the liver of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M, $n = 4–6$.

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10-fold higher in the female liver, whereas *Cyp3a18* is nearly 25-fold higher in the male liver. Most CYP4 family enzymes are significantly higher in male livers, especially *Cyp4a2*, which is also male specific: 3000-fold in the male liver compared with the female liver (Supplemental Material). Fig. 5 shows the mRNA expression levels of different P450 enzymes in male versus female livers.

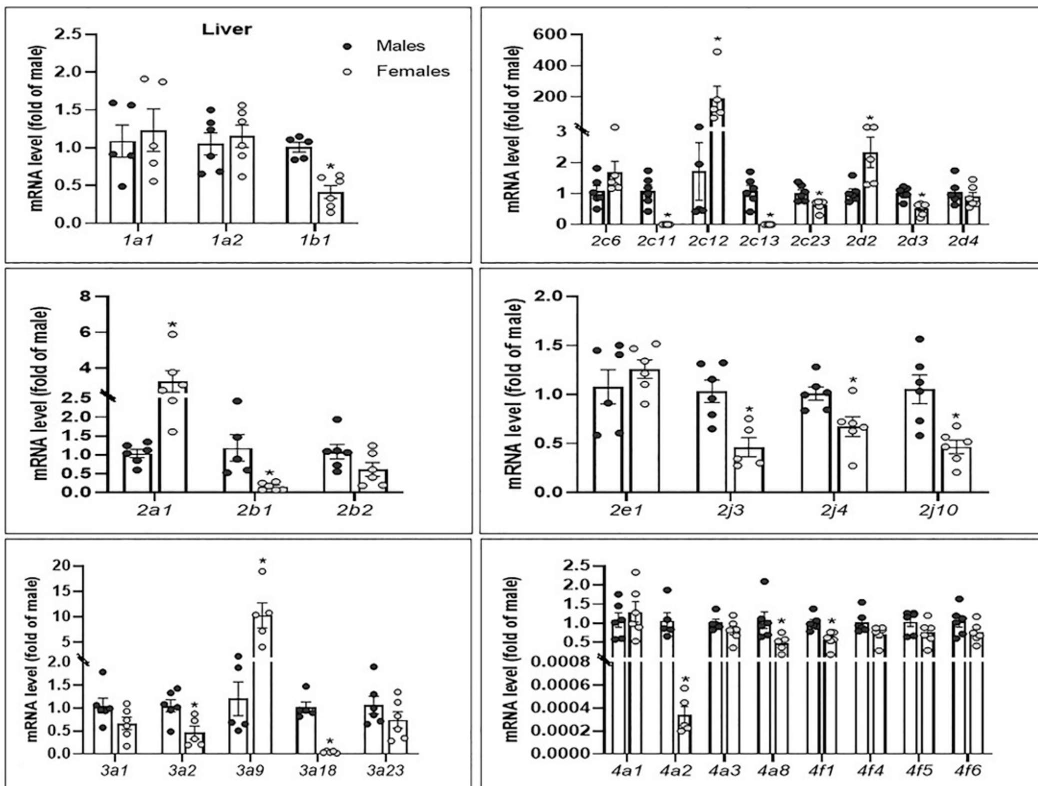

Fig. 5. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat liver. The mRNA expression of P450 enzymes was determined in the liver of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M., $n = 4–6$. Data were analyzed using an unpaired student t test. $*P < 0.05$, significant difference from male rats.

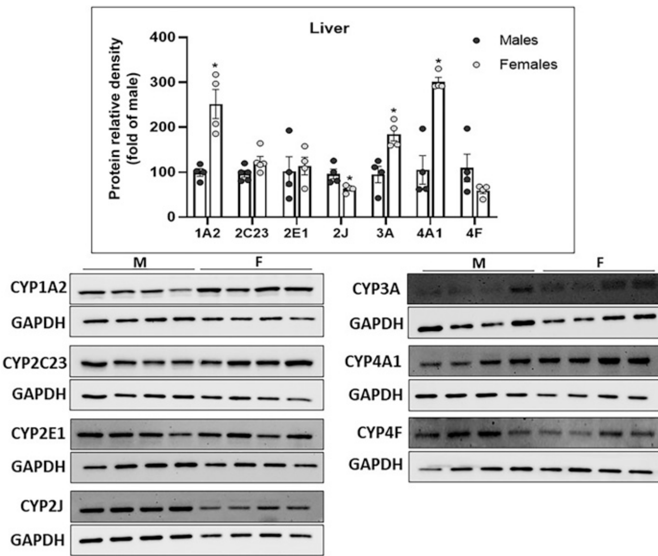

Fig. 6. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat liver. The protein expression of P450 enzymes was determined in the liver of adult male and female Sprague-Dawley rats by western blot and normalized to GAPDH housekeeping protein. Results are presented as mean plus or minus S.E.M., $n = 4–6$. Data were analyzed using an unpaired student t test. $^*P < 0.05$, significant difference from male rats.

As shown in Fig. 6, all the assessed enzymes were found to be expressed in the liver. CYP1A2, CYP3A, and CYP4A1 were significantly higher in female rat livers (2.5-fold, 1.9-fold, and 2.9-fold, respectively) compared with the male livers. In contrast, CYP2J was found to be significantly higher in the male rat liver (1.5-fold female expression level), in agreement with the mRNA result (Fig. 6).

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Kidney. *Cyp2c23* was the most highly expressed, whereas *Cyp3a1* was the least expressed, P450 in both male and female kidneys (Fig. 7). *Cyp2c12*, *Cyp2c13*, and *Cyp3a18* are not expressed in the kidney. Several enzymes demonstrated similar sexual dimorphism in the kidney to that shown in the heart, such as *Cyp1a1*, *Cyp2e1*, *Cyp4f1*, *Cyp4f4*, and *Cyp4f6*, which all showed higher expression in the female organs. *Cyp1a1* showed the greatest difference, with the female expression level 26-fold the male level. However, similar to the liver and in contrast to the heart, *Cyp2c11* expression was significantly higher in male versus female kidneys (13-fold), and in contrast to both heart and liver, *Cyp2c23* expression was significantly higher in female kidneys (approximately twofold). In addition, *Cyp4a8* showed nearly 7.5-fold higher expression in the females' kidney, whereas it was not expressed in the heart (Supplemental Material). Fig. 8 shows the mRNA expression levels of different P450 enzymes in male versus female kidneys.

At the protein expression level, CYP2C23, CYP2E1, CYP3A, CYP4A1, and CYP4F2 were detected in the kidney. Similar to the mRNA expression, CYP2E1 was found to be significantly higher in the female rat kidney (1.7-fold the male expression level). CYP2C23 also appears to be slightly higher in females, but the difference did not achieve statistical significance. Moreover, CYP3A protein levels are significantly higher in the female kidney (2.4-fold), in agreement with CYP3A2 mRNA result (Fig. 9).

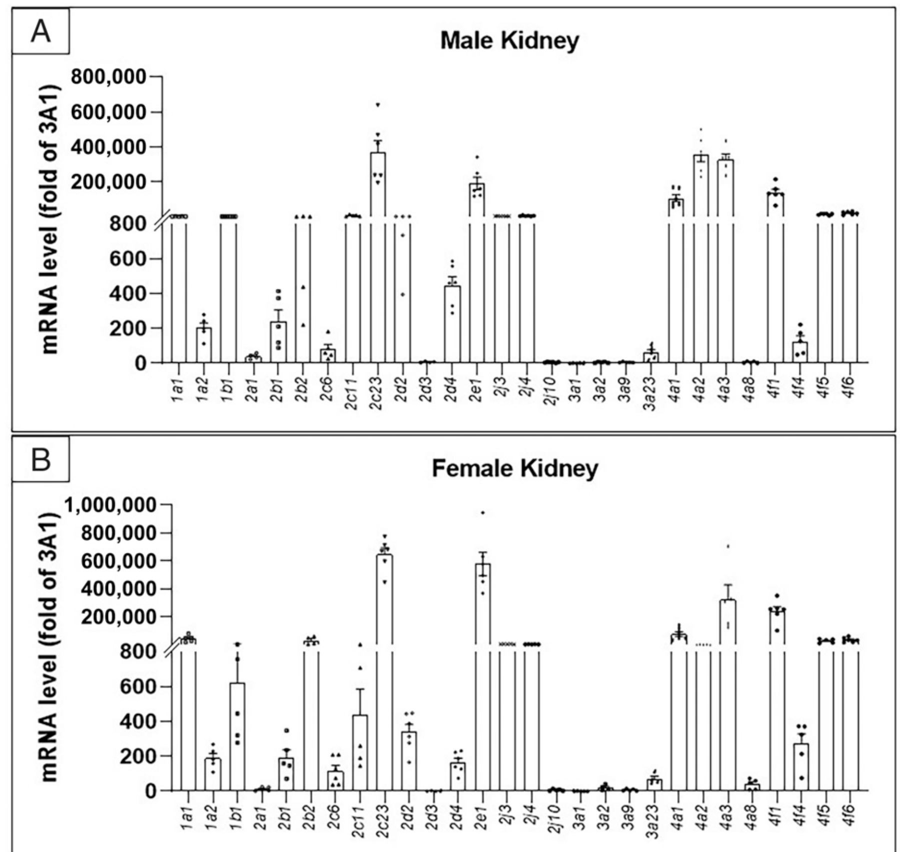

Fig. 7. The mRNA expression of different P450 enzymes in male (A) and female (B) rat kidney relative to the least expressed. The mRNA expression of P450 enzymes was determined in the kidney of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M, $n = 4–6$.

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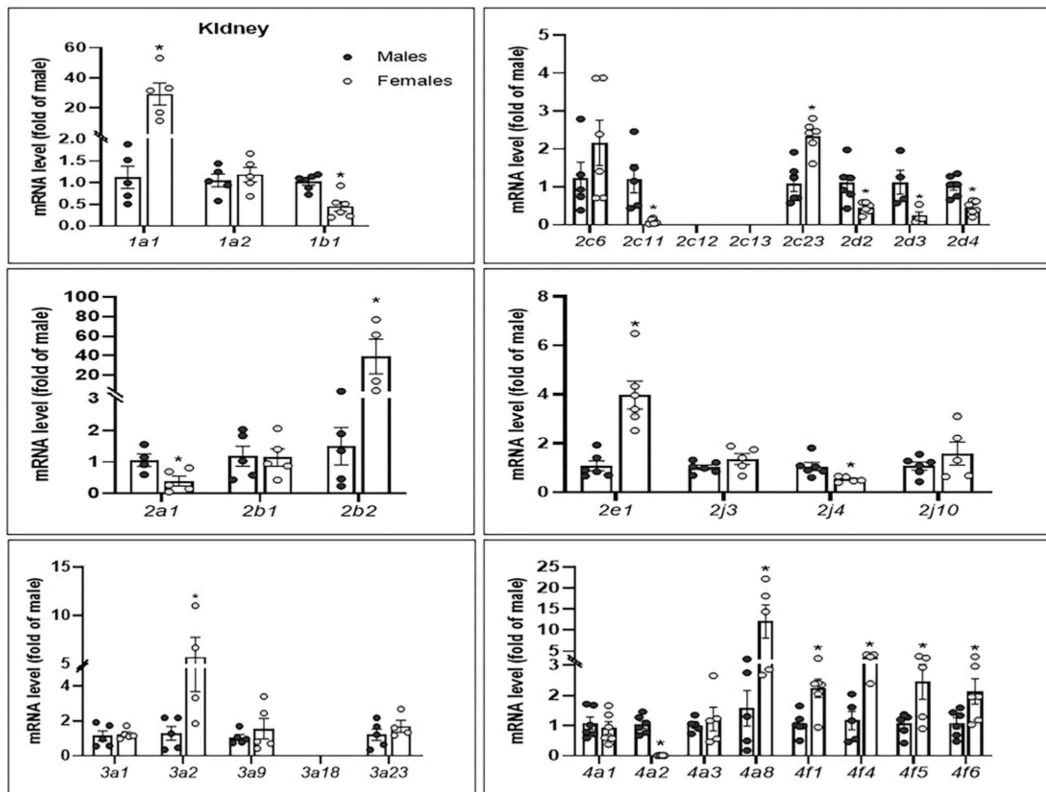

Fig. 8. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat kidney. The mRNA expression of P450 enzymes was determined in the kidney of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *P < 0.05, significant difference from male rats.

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Lung. The most expressed P450 mRNA in the lungs of both male and female rats was *Cyp2b1*, with a marked difference between it and the second enzyme (*Cyp2e1*) (11.5-fold in males and 27-fold in females). On the other hand, *Cyp2j10* and *Cyp3a2* were the least expressed (Fig. 10). In addition to *Cyp2c13* and *Cyp4a2* and except *Cyp3a9*, all the enzymes that were not expressed in the heart were also found to not be expressed in the lung. Except *Cyp1a2* and

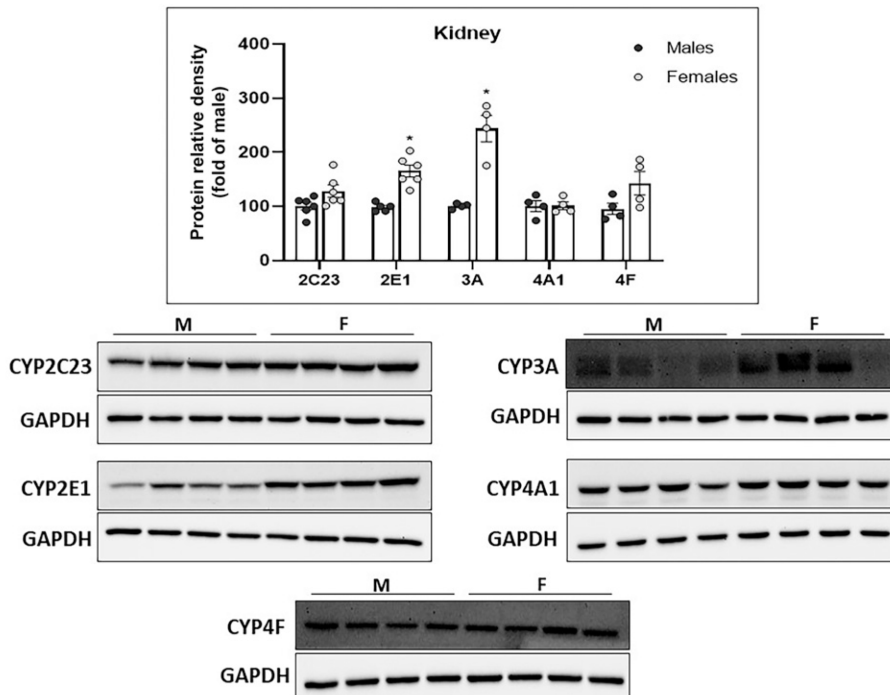

Fig. 9. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat kidney. The protein expression of P450 enzymes was determined in the kidney of adult male and female Sprague-Dawley rats by western blot and normalized to GAPDH housekeeping protein. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *P < 0.05, significant difference from male rats.

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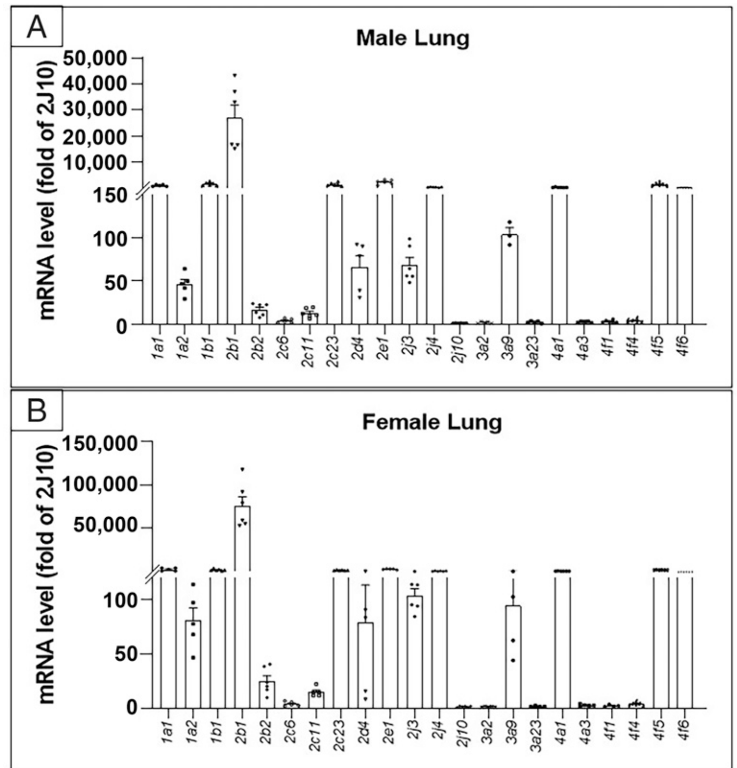

Fig. 10. The mRNA expression of different P450 enzymes in male (A) and female (B) rat lung relative to the least expressed. The mRNA expression of P450 enzymes was determined in the lung of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M, n = 4–6.

Cyp2b1, all other P450 enzymes expressed in the lung did not show statistically significant sex-specific differences between males and females. *Cyp1a2* is around 1.8-fold higher in male lung, whereas *Cyp2b1* is 2.3-fold higher in female lung (Supplemental Material). Fig. 11 demonstrates the male versus female mRNA expression levels of different P450 enzymes in the lungs.

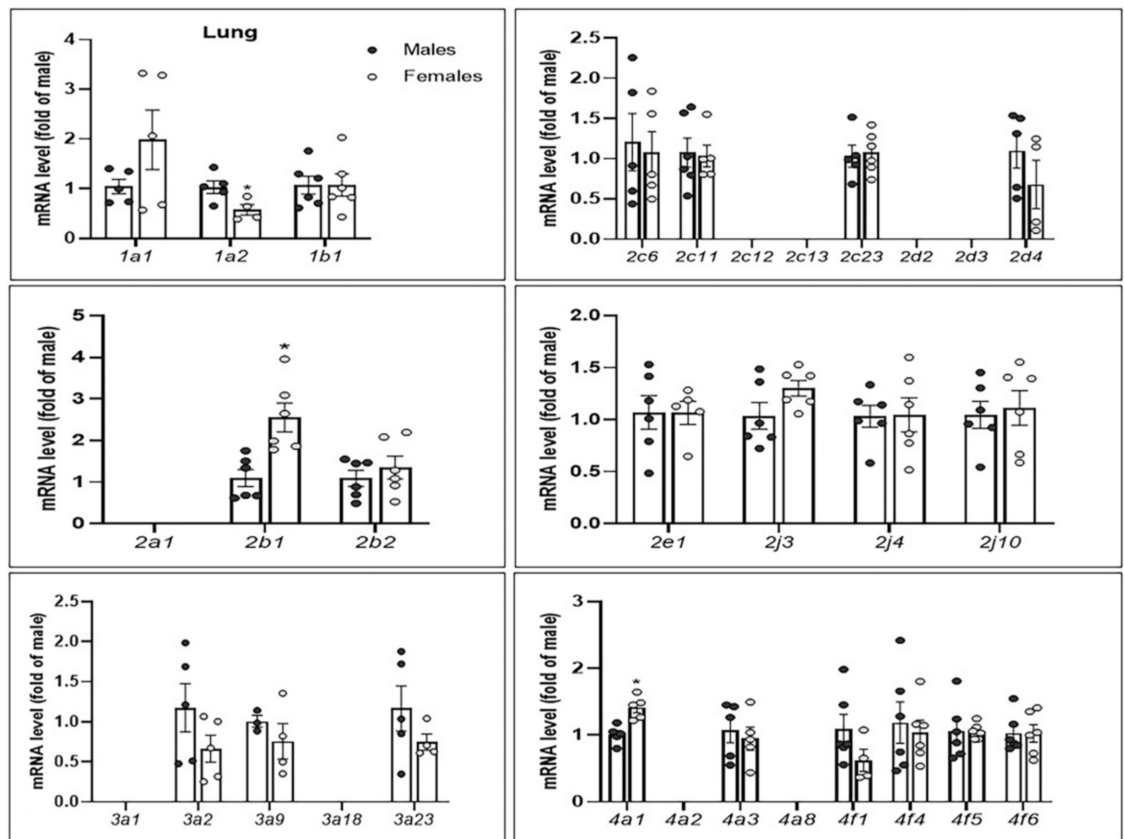

Fig. 11. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat lung. The mRNA expression of P450 enzymes was determined in the lung of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *$P < 0.05$, significant difference from male rats.

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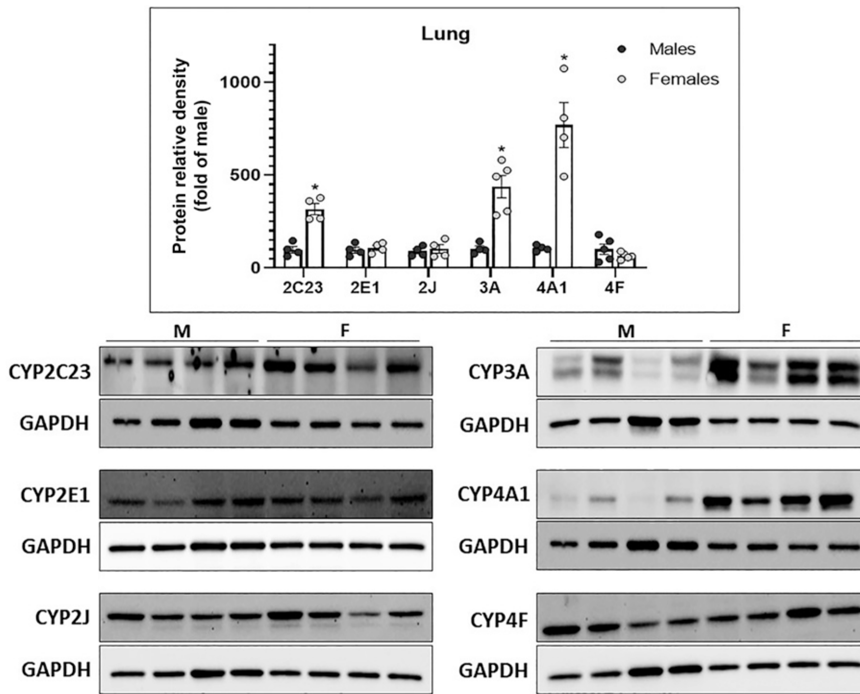

Fig. 12. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat lung. The protein expression of P450 enzymes was determined in the lung of adult male and female Sprague-Dawley rats by western blot and normalized to GAPDH housekeeping protein. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. $^*P < 0.05$, significant difference from male rats.

CYP2C23, CYP3A, and CYP4A1 were all found to be significantly higher in the female lung at the protein expression level (3.2-, 4.2-, and 7.3-fold the male expression level, respectively). On the other hand, CYP2E1, CYP2J, and CYP4F demonstrated no significant difference between males and females, and CYP1A2 was not detected (Fig. 12).

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Brain. *Cyp2j3* was found to be the most expressed enzyme in the brain of male and female rats, whereas *Cyp4a8* and *Cyp2c13* were the least expressed (Fig. 13). *Cyp2c12*, *Cyp2d2*, *Cyp2d3*, *Cyp3a18*, and *Cyp4a2* were found to not be expressed in the brain. Similar to the liver, the brain mRNA expression levels of *Cyp2c11* and *Cyp2c13* were significantly higher in males than in females but with a less marked difference (1.8- and 3.7-fold, respectively). Unlike all other organs, *Cyp1a1* and *Cyp4a3* in the brain were significantly higher in the males (approximately two- and sixfold, respectively). On the other hand, *Cyp4f4* and *Cyp4f5* mRNA levels were significantly higher in the females (1.7- and 1.4-fold, respectively) (Supplemental Material). The mRNA expression levels of different P450 enzymes in male versus female brain are shown in Fig. 14.

At the protein expression level, CYP2J and CYP4F enzymes were detected in the brain, and both showed no significant sex-specific difference (Fig. 15).

Sex-Specific Differences in the mRNA and Protein Expression Levels of P450 Enzymes in the Small Intestine. At the mRNA expression level, *Cyp2b1* and *Cyp2b2* were found to be the most highly expressed P450 enzymes in the small intestine of female and male rats, respectively, whereas *Cyp3a1* was the least expressed in both sexes, similar to the kidney (Fig. 16). *Cyp3a18*, *Cyp2c12*, *Cyp2d2*, *Cyp4a2*, and *Cyp4a3* were found to not be expressed in the small intestine. Only *Cyp2c13* showed significant sex-specific difference in the small intestine, being 1.4-fold higher in the males compared with the females (Supplemental Material). The male-versus-female mRNA expression of P450 enzymes in the small intestine is demonstrated in Fig. 17.

At the protein expression level, CYP1A2 and CYP3A were demonstrated to be significantly higher in the females: 2.4-fold and 3.7-fold, respectively. This result is in agreement with other organs such as the liver, kidney, and lung. CYP4A1 was also detected at the protein

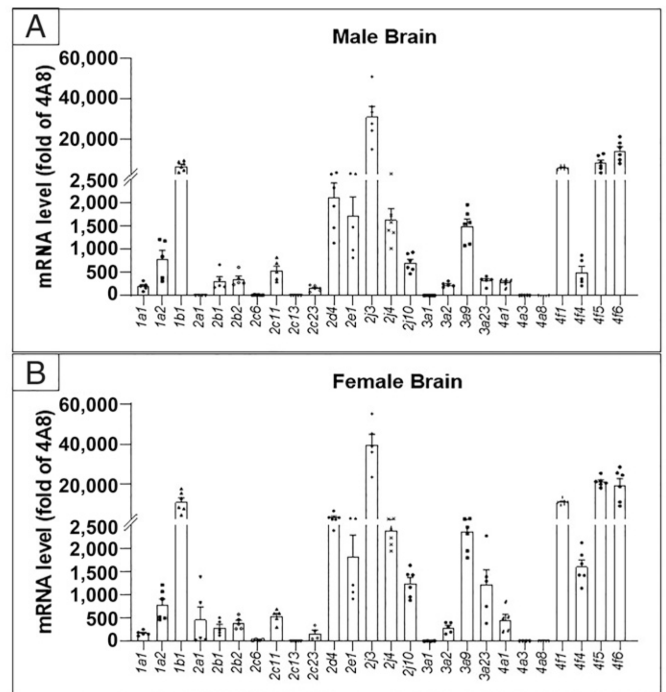

Fig. 13. The mRNA expression of different P450 enzymes in male (A) and female (B) rat brain relative to the least expressed. The mRNA expression of P450 enzymes was determined in the brain of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M, n = 4–6.

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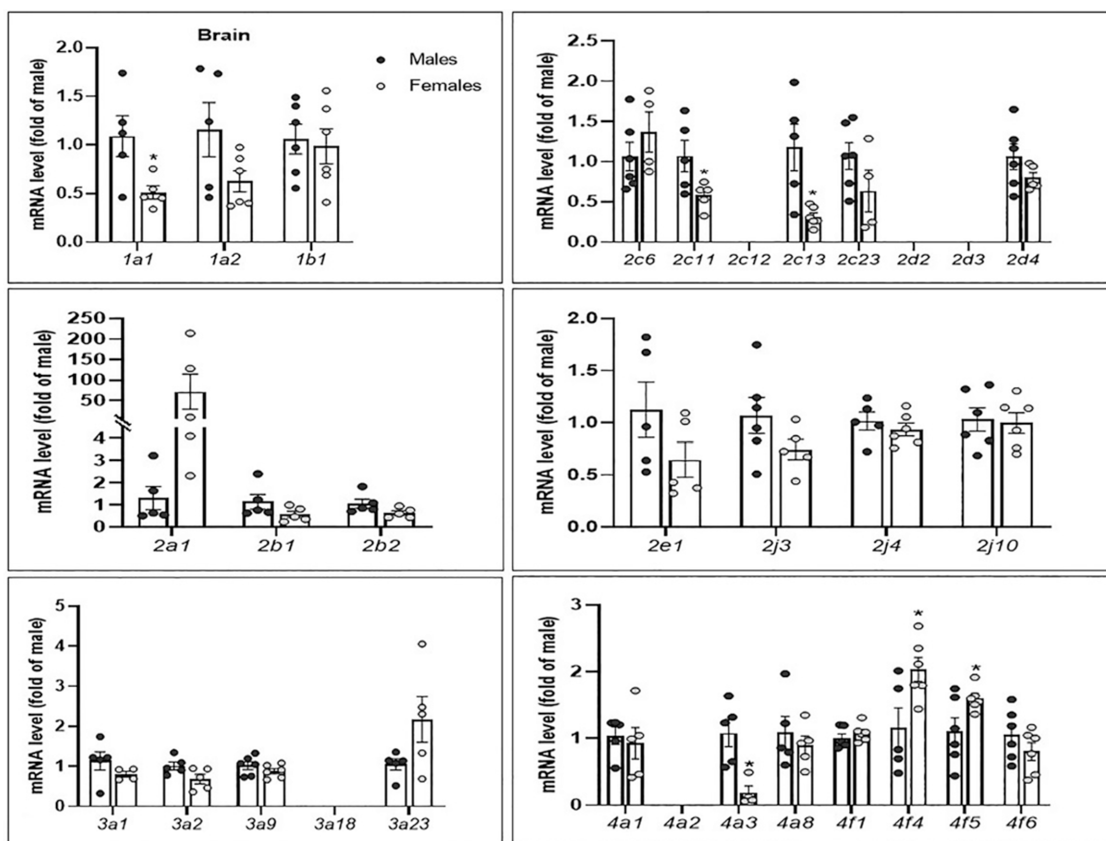

Fig. 14. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat brain. The mRNA expression of P450 enzymes was determined in the brain of adult male and female Sprague-Dawley rats by real-time PCR and normalized to β-actin housekeeping gene. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *P < 0.05, significant difference from male rats.

expression level but showed no significant difference between males and females, similar to CYP4A1 expression in the kidney (Fig. 18).

Discussion

Previously, many studies were conducted on male animals only, and results were generalized to both sexes (Holdcroft, 2007; Lee, 2018). Ignoring sex differences could have undesirable consequences such as increased side effects or decreased efficacy. Thus, sex-specific differences are gaining increasing attention in research, and more studies are starting to include female groups (Wald and Wu, 2010; Lee, 2018). Previous studies have demonstrated sex-specific differences in the expression or activity levels of different P450 enzymes (Waxman and Holloway, 2009; Zhang et al., 2011). However, most of these studies reported just a few enzymes and investigated sexual dimorphism in response to inducers or inhibitors. In this study, we investigated and compared the mRNA and protein expression of different P450s in the heart, liver, lung, kidney, brain, and small intestine of male and female SD rats.

Higher expression levels of CYP1A1 were previously reported in the lungs (Lingappan et al., 2013, 2016) and hearts (Zhang et al., 2015) of female versus male mice. Moreover, CYP1A1 was detectable in the lungs and kidneys of female but not male SD rats and was undetectable in the liver of both sexes (Iba et al., 1999). Our results showed significantly higher cardiac and renal *Cyp1a1* mRNA levels in female rats. Hepatic CYP1A2 activity was reported to be higher in male than in female SD rats (Fonsart et al., 2008). However, a study found higher CYP1A2 levels in female compared with male human liver samples (Zhang et al., 2011), whereas other studies gave conflicting results (Nafziger and Bertino, 1989; Ou-Yang et al., 2000; Zanger and Schwab, 2013). A study in 2016 demonstrated higher *Cyp1a2* brain mRNA levels in female Wistar rats (Nagai et al., 2016), whereas our results showed no significant difference in the brain. In our study, *Cyp1a2* mRNA expression showed significant sex difference only in the lung, being male dominant, whereas CYP1A2 protein was female dominant in the liver and the intestine.

CYP1B1 is constitutively expressed in several tissues, most importantly in the heart (Maayah et al., 2015). A previous study showed that treatment of embryonic rat cardiomyocytes with growth hormone (GH) in a pulsatile pattern, which mimics the male secretory pattern, significantly decreased *Cyp1a1* and increased *Cyp1b1* expression compared with the constant treatment pattern, which mimics the female pattern. Moreover, they found higher *Cyp1b1* mRNA levels in male mice hearts compared with female mice (Zhang et al., 2015). Acute doxorubicin exposure in mice also caused a male-specific increase in cardiac *Cyp1b1* (Grant et al., 2017). In our study, cardiac *Cyp1b1* showed no significant difference, but hepatic and renal *Cyp1b1* levels were significantly higher in male rats. Interestingly, human hepatic CYP1B1 was also previously reported to be significantly higher in men (Yang et al., 2012). Hepatic CYP2A1 was previously found to be female dominant in rats (Martignoni et al., 2006). We found higher hepatic *Cyp2a1* expression in females but higher renal expression in males.

The CYP2C family is known to be highly abundant in the rat liver (Martignoni et al., 2006). CYP2C11 and CYP2C13 were previously reported to be male-specific enzymes in the liver, spleen, and bone marrow, whereas CYP2C12 was reported to be female specific (Thangavel

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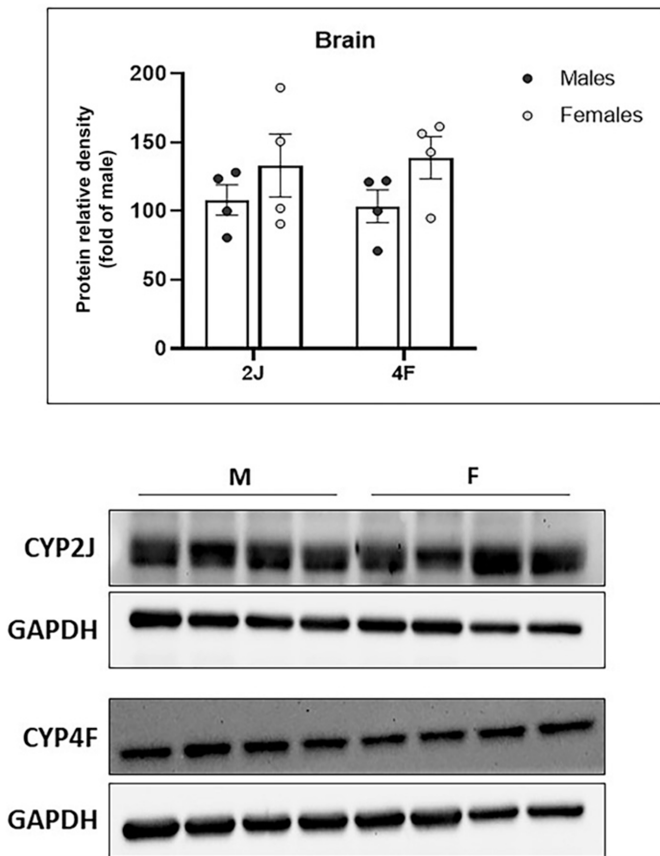

Fig. 15. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat brain. The protein expression of P450 enzymes was determined in the brain of adult male and female Sprague-Dawley rats by western blot and normalized to GAPDH housekeeping protein. Results are presented as mean plus or minus S.E.M., n = 4–6. Data were analyzed using an unpaired student t test. *P < 0.05, significant difference from male rats.

et al., 2007; Huang et al., 2011; Babelova et al., 2015). In addition, previous studies demonstrated significantly lower metabolism of CYP2C substrates in female compared with male Wistar (Ohhira et al., 2006) and SD rats (Fukuno et al., 2018). CYP2C11 sexual dimorphism is attributed to sex differences in the circulating GH profile. Female SD rats could express CYP2C11 after hypophysectomy and infusion with GH in the pulsatile male secretory fashion (Legraverend et al., 1992; Thangavel et al., 2007; Banerjee et al., 2021). We also found significantly higher *Cyp2c11* levels in male versus female kidney and brain; however, interestingly, it was found to be female dominant in the heart. CYP2C23 protein was previously reported to be highly abundant in the liver and kidney of SD rats, whereas it was undetectable in the heart and lungs (El-Sherbeni et al., 2013). We detected CYP2C23 in the lung with a higher expression in female rats.

Our results showed female-dominant expression of *Cyp2e1* in the heart and kidney. In mice, in contrast, renal CYP2E1 expression was found to be higher in males (Freeman et al., 1992; Speerschneider and Dekant, 1995), whereas cardiac CYP2E1 showed no sexual dimorphism (Zhang et al., 2015). However, acute doxorubicin exposure was associated with a female-specific increase in heart CYP2E1 in mice (Grant et al., 2017). Hepatic CYP2J2 levels were found to be significantly higher in female subjects compared with male subjects (Yang et al., 2012). In contrast, our results showed male-dominant hepatic expression of CYP2J enzymes. We found *Cyp2j4* to be significantly higher in male kidneys. A previous study demonstrated significantly higher CYP2J5 levels in male versus female mice kidneys (Ma et al., 2004). As for cardiac expression, we found significantly higher *Cyp2j3* levels in female rat hearts compared with males. In line with that, treatment of rat cardiomyocytes with GH in a male secretory pattern significantly decreased *Cyp2j3* expression compared with the female pattern. Mouse *Cyp2j11* is also higher in female than in male hearts (Zhang et al., 2015). However, cardiac CYP2J protein levels showed no significant difference.

The CYP3A subfamily of enzymes is considered the most important among human drug-metabolizing enzymes. Several studies have shown that women have significantly higher hepatic and intestinal CYP3A enzyme activities compared with men (Tanaka, 1999; Greenblatt and Von Moltke, 2008; Krogstad et al., 2020), as well as higher hepatic CYP3A4 levels (Lamba et al., 2010; Yang et al., 2010, 2012). CYP3A7, the human ortholog of rat Cyp3a9, was previously found to have significantly higher gene expression in female hepatic samples compared with males (Yang et al., 2012). In agreement with human data, our results showed higher hepatic *Cyp3a9* mRNA and CYP3A protein levels in female rats; however, we found *Cyp3a2* mRNA to be higher in male rats. CYP3A2 was previously found to be induced by zolmitriptan in male but not female SD rats (Yu et al., 2008).

A study in 2003 reported *Cyp3a9* mRNA levels to be significantly higher in the livers and lungs of female SD rats compared with male rats and that its expression is affected by ovariectomy and subsequent estrogen administration (Anakk et al., 2003). In agreement, we found significantly higher *Cyp3a9* mRNA and CYP3A protein levels in the livers of female rats. In the lung, kidney, and small intestine, *Cyp3a9* mRNA levels showed no significant difference, whereas CYP3A protein levels were significantly higher in the females. Similar to our findings, previous reports have identified hepatic CYP3A2 and CYP3A18 to be male-dominant isoenzymes and CYP3A9 to be a female-dominant isoenzyme in Wistar (Robertson et al., 1998) and SD rats (Kushida et al., 2021).

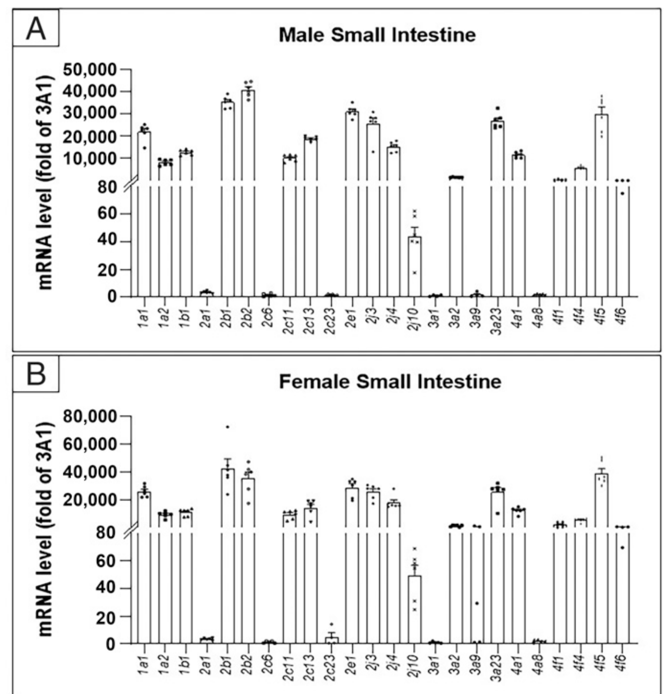

Fig. 16. The mRNA expression of different P450 enzymes in male (A) and female (B) rat small intestine relative to the least expressed. The mRNA expression of P450 enzymes was determined in the small intestine of adult male and female Sprague-Dawley rats by real-time PCR and normalized to *Gapdh* housekeeping gene. Results are presented as mean plus or minus S.E.M, n = 4–6.

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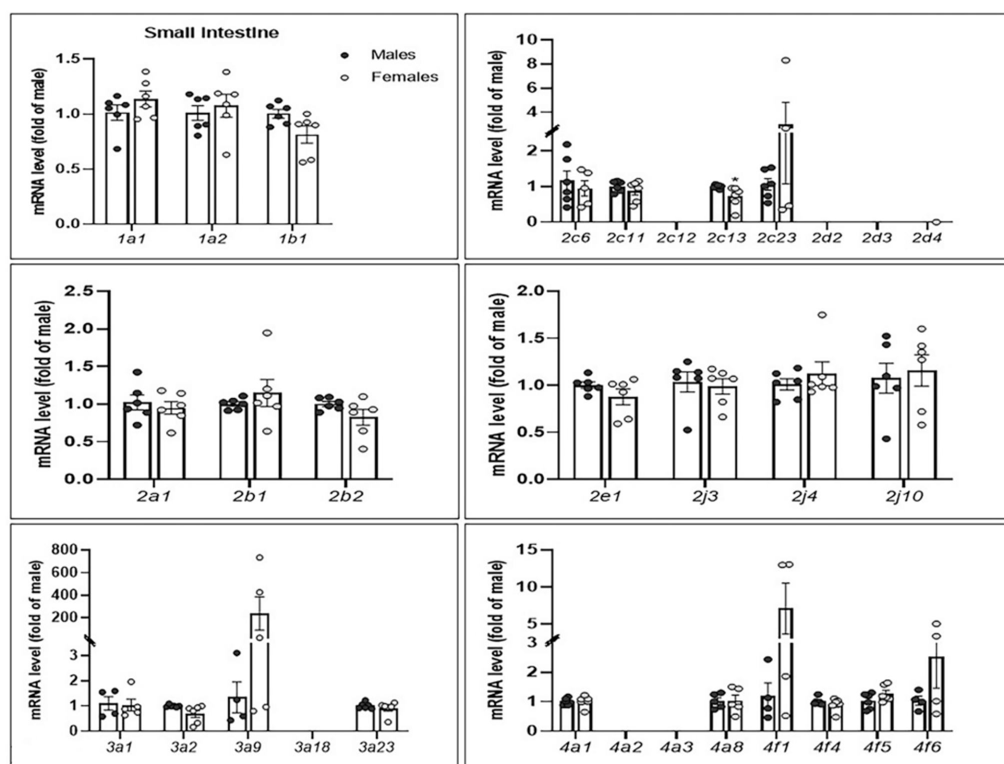

Fig. 17. Sex-specific differences in the mRNA expression levels of P450 enzymes in the rat small intestine. The mRNA expression of P450 enzymes was determined in the small intestine of adult male and female Sprague-Dawley rats by real-time PCR and normalized to *Gapdh* housekeeping gene. Results are presented as mean plus or minus S.E.M., $n = 4–6$. Data were analyzed using an unpaired student t test. $^{*}P < 0.05$, significant difference from male rats.

CYP4A enzymes play an important role in the ω-hydroxylation of AA (El-Sherbeni and El-Kadi, 2017). In line with our results, hepatic *Cyp4a2* was previously reported to be significantly higher in male rats of Fischer 344 and obese ZSF1 strains (Sundseth and Waxman, 1992; Babelova et al., 2015). Moreover, the induction of hepatic *Cyp4a* by endotoxin was found to be male specific in SD and Fischer 344 rats (Mitchell et al., 2001). Although hepatic *Cyp4a1* showed no sex-specific difference at the mRNA level, hepatic CYP4A1 protein levels were found to be significantly higher in female rats.

We found renal *Cyp4a2* levels to be significantly higher in male rats. Similarly, previous studies showed significantly higher renal CYP4A2 levels in male versus female Fischer 344 and SD rats (Sundseth and Waxman, 1992; Bleicher et al., 2001). Interestingly, a previous study demonstrated that treatment of SD rats with dihydrotestosterone lowered the renal *Cyp4a1* levels and enhanced *Cyp4a2/3* levels (Nakagawa et al., 2003). In addition, clofibrate administration significantly enhanced renal *Cyp4a2* expression only in male SD rats (Bleicher et al., 2001). Another study showed an increase of renal CYP4A protein in female SD rats treated with dihydrotestosterone (Zhou et al., 2005).

CYP4F enzymes appear to be female-dominant enzymes. We found significantly higher *Cyp4f* levels in the heart and kidney of females versus males, and *Cyp4f4* and *Cyp4f5* were significantly higher in female brains. At the protein expression level, we found significantly higher CYP4F levels in the heart of female versus male rats. Similarly, a study in 2002 found significantly higher expression levels of CYP4F enzymes in female versus male SD rats in the liver, kidney, lung, and brain and found a significant decrease in hepatic and renal CYP4F expression levels in female rats after ovariectomy, which was significantly restored by estrogen treatment (Kalsotra et al., 2002).

In conclusion, there are significant sex-specific differences in the expression levels of different P450 enzymes. Elucidating sex-specific differences in P450s is crucial for explaining the differences between males and females in diseases processes and treatment outcomes. This study has some limitations. First, species discrepancies in the basal and inducible levels of enzymes could complicate the translation of the results to humans (Hammer et al., 2021). Moreover, P450 expression levels could differ among different rat strains (Nishiyama et al., 2016).

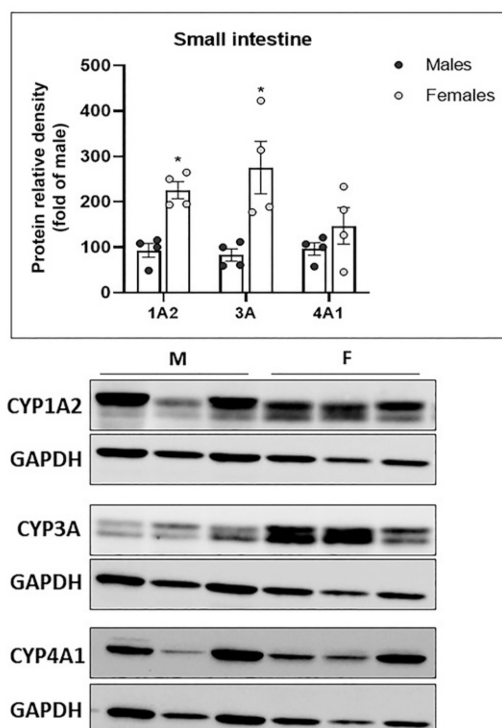

Fig. 18. Sex-specific differences in the protein expression levels of some P450 enzymes in the rat small intestine. The protein expression of P450 enzymes was determined in the small intestine of adult male and female Sprague-Dawley rats by western blot and normalized to GAPDH housekeeping protein. Results are presented as mean plus or minus S.E.M., $n = 4–6$. Data were analyzed using an unpaired student t test. $^{*}P < 0.05$, significant difference from male rats.

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Finally, expression levels of enzymes are not necessarily correlated to their activity levels. However, rats are still considered to be valuable models for preclinical development and have previously been used for the study of sex differences in drug-metabolizing enzymes and the mechanisms underlying these differences (Waxman and Holloway, 2009; Jung et al., 2015; Blais et al., 2017). Moreover, several rat P450 enzymes (e.g., CYP1A1, CYP1A2, CYP2E1, CYP2J3, CYP4F1) show high degrees of structural similarity to their human orthologs (Hammer et al., 2021). Thus, despite the study limitations and some results that are different from human data, we believe that our results could still give valuable insights regarding sex-specific differences in human P450 enzymes. Additional studies investigating the activity of different P450 enzymes and levels of their metabolites in males and females are important for having better insight into sex-specific discrepancies and their potential clinical and therapeutic implications.

Authorship Contributions

Participated in research design: Gerges, El-Kadi.

Conducted experiments: Gerges.

Performed data analysis: Gerges, El-Kadi.

Wrote or contributed to the writing of the manuscript: Gerges, El-Kadi.

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