DO MEN AND WOMEN DIFFER IN PROXIMAL SMALL INTESTINAL
CYP3A OR P-GLYCOPROTEIN EXPRESSION?

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Abbreviations: P-gp, P-glycoprotein; FDA, Food and Drug Administration; PVDF, polyvinylidene difluoride; PBS, phosphate buffered saline; IOD, integrated optical density
ABSTRACT

The higher systemic clearance of some CYP3A4 (whether also P-gp) drug substrates in women vs. men is attributed in part to a higher hepatic CYP3A4 content in women. This, combined with the general paucity of reported sex differences in the apparent oral clearance of CYP3A4 substrates suggested a sex-dependent expression of CYP3A4 in the intestine but in a pattern opposite to the liver. Accordingly, duodenal biopsies obtained from healthy men (n=46) and women (n=45) were analyzed, by Western blot, for relative CYP3A4, as well as for CYP3A5 and P-gp, expression levels. Among all subjects, CYP3A4 and P-gp varied 8- and 10-fold, respectively. CYP3A5, which was readily detected in 27% of these predominantly Caucasian individuals, varied 7-fold. For all 3 proteins, a sex difference was not detected (p ≥ 0.55). The lack of a difference remained for CYP3A4 and P-gp when the analysis was restricted to Caucasians (n=74) or to individuals with undetectable CYP3A5. Comparing the 21 pre-menopausal women (all were aged <45 years) with the 43 men aged <45 years, again no sex differences were detected in CYP3A4 and P-gp. Comparing the pre- with post-menopausal women, mean CYP3A4 content was 20% lower in the post-menopausal individuals (p=0.01). The lack of a sex-dependent difference in proximal intestinal CYP3A4 could account in part for the lack of reported sex differences in the oral, relative to systemic, clearance of some CYP3A4 substrates. Ramifications of lower CYP3A4 content in post- vs. pre-menopausal women require further investigation.
INTRODUCTION

In 1993, to ensure adequate representation of women in all phases of clinical drug trials, the FDA revised earlier (1977) guidelines to allow the inclusion of women of childbearing potential in phase I and early phase II studies (Merkatz et al., 1993). In addition, because inclusion alone of both women and men would be insufficient to assess potential sex differences in drug response, the earlier guidelines were further revised to request an assessment, among others, of potential pharmacokinetic differences between the sexes (Merkatz et al., 1993). Consequently, sex-related effects on pharmacokinetics have gained considerable attention over the past decade. Such differences in the pharmacokinetics of a number of drugs have been described in several recent reviews (Cummins et al., 2002; Meibohm et al., 2002; Schwartz, 2003; Gandhi et al., 2004). Although some differences can be explained by physiologic disparities between men and women (e.g., body weight, percent body fat, glomerular filtration rate, gastric motility), biochemical disparities may also exist (Meibohm et al., 2002).

Metabolism by the liver represents the most common biochemical means of drug elimination. A superfamily of enzymes prominently involved in drug metabolism is the cytochromes P450 (CYPs). Of the numerous isoforms, CYP3A4 is one of the most important, as it constitutes a major portion (~30% on average) of total hepatic CYP content and is involved in the metabolism of >50% of drugs on the market (Thummel and Wilkinson, 1998). Hepatic metabolism also frequently represents the major component of total systemic clearance. Many drugs that are substrates for CYP3A4 have shown, with intravenous dosing, a higher clearance in women compared to men, even after correcting for body weight (e.g., cyclosporine, erythromycin, methylprednisolone, tacrolimus, tirilazad, verapamil) (Cummins et al., 2002). This suggested that hepatic CYP3A4 activity might be higher in women compared to men.

Another biochemical component that can contribute to hepatic drug clearance is the
widely studied efflux transporter P-glycoprotein (P-gp), the product of the multidrug resistance gene 1 (*MDR1* or *ABCB1*) (Ayrton and Morgan, 2001). Due to its canalicular location, P-gp functions to enhance the biliary excretion of its substrates, many of which are also substrates for CYP3A4 (e.g., those aforementioned). A sex difference in hepatic P-gp could therefore represent an additional determinant of sex-dependent differences in the hepatic clearance of dual CYP3A4/P-gp substrates. Moreover, based on results with a small number of healthy liver specimens, some investigators have proposed that P-gp expression in men might be greater than that in women (i.e., a pattern opposite to that for CYP3A4) (Schuetz et al., 1995; Cummins et al., 2002).

To investigate sex differences in hepatic CYP3A4 and P-gp expression, Wolbold et al. (2003) analyzed 94 normal liver specimens obtained from a well-characterized group of patients undergoing hepatic surgery. The median CYP3A4 immunoreactive protein content in specimens obtained from women (n=46) was significantly greater, by 2.4-fold, than that in specimens obtained from men. This difference was not explained by preferential induction in women by concomitant medications because when the subset of specimens from individuals not taking medications (before surgery) was analyzed (23 men, 16 women), not only did the difference remain, it was more pronounced (3.1-fold) (Wolbold et al., 2003). In contrast to CYP3A4, a sex difference in P-gp expression was not detected. Collectively, these results indicated that a sex difference in hepatic CYP3A4, but not P-gp, underlies sex-related differences in the systemic clearance of some CYP3A4 substrates.

In addition to the liver, CYP3A4 and P-gp are expressed in relatively high levels in epithelial cells lining the small intestinal mucosa (enterocytes). While the existence of sex differences in CYP3A4 and P-gp has been carefully investigated in the liver, the question remains whether such differences exist in the upper small intestine, the major site of drug absorption. It cannot be assumed that the findings reported for the liver will be the same in the intestine, since both CYP3A4 and P-gp appear to be regulated by different mechanisms in the
two organs, as evidenced by a lack of an intra-subject correlation between hepatic and intestinal CYP3A4 (Lown et al., 1994; Paine et al., 1997; von Richter et al., 2004) and P-gp (von Richter et al., 2004). In addition, sex differences in the apparent oral clearance of CYP3A4 substrates, whether also P-gp substrates, have not been consistently reported (unlike with systemic clearance) (Cummins et al., 2002). A sex difference in CYP3A4 expression may exist in the intestine, but in a pattern opposite to that in the liver (i.e., men > women). Indeed, Cummins et al. (2002) implied that such reverse patterns of sex-specific expression in liver and intestine would “cancel each other”, thereby resulting in no net difference in oral clearance. To test this hypothesis, we analyzed 93 duodenal biopsy specimens, obtained from approximately equal numbers of healthy men and women, for CYP3A4 relative protein content. Relative CYP3A5 protein and P-gp contents were also determined. To our knowledge, this is the first report to address whether sex differences exist in CYP3A4, CYP3A5, and P-gp content in the proximal small intestine.
MATERIALS AND METHODS

Materials and Chemicals

Sodium dodecyl sulfate, acrylamide/bis (37.5:1), ammonium persulfate, and TEMED were purchased from Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) membrane (Hybond-P) and enhanced chemiluminescence reagents were purchased from Amersham Biosciences, Inc. (Piscataway, NJ). cDNA-expressed CYP3A4 and CYP3A5 and the anti-CYP3A4 and -CYP3A5 antibodies (WB-3A4 and -3A5, respectively) were purchased from BD Gentest (Woburn, MA). WB-3A4 and -3A5 were confirmed to not cross-react with CYP3A5 and CYP3A4, respectively, using the cDNA-expressed enzymes as reference standards (not shown). Homogenate prepared from the human intestinal cell line Caco-2, known to constitutively express relatively high levels of P-gp and villin, was from a previous study (Paine et al., 2002). A murine monoclonal antibody raised against human villin was purchased from Chemicon International (Temecula, CA). A rabbit polyclonal antibody raised against a peptide of human P-gp was a kind gift from Dr. Erin G. Schuetz (St. Jude’s Children’s Research Hospital, Memphis, TN). The secondary antibodies, rabbit anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG, were purchased from Zymed Laboratories (South San Francisco, CA). All other reagents were of electrophoresis grade.

Human Duodenal Biopsy Specimens

Duodenal pinch biopsies were previously obtained, by upper intestinal endoscopy, from 93 healthy volunteers (45 women), each of whom participated in 1 of 5 clinical studies from 1993 to 1997 (Table 1). Studies A, D, I, and M investigated whether various CYP3A4 or P-gp substrates/modulating agents [A: fexofenadine (KS Lown and PB Watkins, unpublished data); D: grapefruit juice (Lown et al., 1997), chargrilled meat (Fontana et al., 1999), cruciferous vegetables (Lown et al., 1995); I: rifampin, phenobarbital, dexamethasone, clotrimazole (Wille et al., 1997); M, efavirenz (Mouly et al., 2002)] influenced, among other measures, intestinal
CYP3A4 and/or P-gp content. Study E investigated whether intestinal and/or hepatic CYP3A4 predicted estrogen levels in post-menopausal women about to undergo hormone replacement therapy (DK Turgeon and PB Watkins, unpublished data). Only 4 of the subjects (2 women) were smokers. The men ranged in age from 19 to 49 years (mean, 28 years), and the women from 18 to 64 years (mean, 41 years).

Because the biopsy specimens were collected between 8 and 12 years ago, there was concern that the proteins of interest had degraded with time. Such effects were believed to be minimal, as all of the specimens had been stored at -80°C since their time of collection (except for an overnight transfer – on dry ice – from the University of Michigan to the University of North Carolina), and only material from unopened vials was used. To substantiate this notion, relatively “new” biopsy specimens obtained from 8 healthy men and 12 healthy women (SJ Mouly and PB Watkins, unpublished data) – and from unopened vials – were recently analyzed using the same methodology as for the “old” specimens. The median (range) of the relative P-gp, CYP3A4, and CYP3A5 contents for the new specimens were comparable to the old specimens, indicating minimal or no degradation after longer term storage.

Importantly, only those biopsies obtained under baseline conditions (i.e., before any treatment was initiated) were analyzed for the proteins of interest (P-gp, villin, CYP3A4, CYP3A5). That is, none of the subjects were taking medications or supplements known to induce or inhibit CYP3A or P-gp, and none of the post-menopausal women were on hormone replacement therapy at the time such biopsies were collected.

For the endoscopic procedure, briefly, after a 4-hour fast, the subject was sedated with intravenous midazolam (Roche Pharmaceuticals, Nutley, NJ) and meperidine (Sanofi Winthrop, New York), and a fiberoptic endoscope was passed into the second portion of the duodenum, where 4-5 mucosal biopsies were obtained. The biopsies were immediately placed into ~1 mL of an ice-cold homogenizing solution (0.05 M Tris hydrochloride, 20% glycerol, 2 mM ethylenediaminetetraacetic acid, and 0.1 mM phenylmethylsulfonylfluoride) and homogenized in
a glass tissue grinder. The homogenate was divided into aliquots, snap frozen in liquid nitrogen, and stored at -80°C until analyzed by Western blot. Total protein concentrations were re-measured by the method of Lowry (1951) and were similar to those measured at the time the biopsies were originally obtained.

**Western Blot Analysis**

**Preparation of Reference Standards and Control Specimens.** In a pinch biopsy taken from the intestinal mucosa, the specific content of the protein of interest could vary with the depth of the biopsy (i.e., the deeper the biopsy, the lower the proportion of enterocytes, the lower the specific content) (Lown et al., 1994). Therefore, our goal was to obtain relative expression levels, rather than specific contents, of the proteins of interest. As has been previously validated (Lown et al., 1994), the constitutive, cytoskeletal protein villin was used as a control protein to correct for biopsy depth. In addition, because it was not technically feasible to analyze all 93 specimens on one day, and in triplicate, ‘reference standards’ and ‘control specimens’ were used to allow for day-to-day and blot-to-blot comparisons, respectively.

To minimize inter- and intra-day variations, all reference standards and controls were prepared at one time and stored at -80°C until use. For the reference standards, the 20 biopsy specimens from the recent clinical study described above (SJ Mouly and PB Watkins, unpublished data) were pooled and supplemented with Caco-2 cell homogenate (to maximize P-gp and villin concentrations) and cDNA-expressed CYP3A4 and CYP3A5 (to maximize CYP3A4 and CYP3A5 concentrations), then divided into aliquots (~200 µL each). Total protein concentrations were determined by the method of Lowry (1951), and each aliquot was diluted with sample buffer (2% sodium dodecyl sulfate, 5% sucrose, 5% 2-mercaptoethanol, 50 mM Tris•HCl; pH, 6.8) to yield a final concentration of 1 mg/mL (or 60 µg/60 µL). Each aliquot was then serially diluted to yield a 16-fold range in concentrations (60, 30, 15, 7.5, and 3.75 µg per 60 µL); preliminary studies indicated these concentrations would span the ranges in protein concentrations of P-gp, villin, CYP3A4, and CYP3A5 contained in the study specimens. For the
control specimens, a separate set of aliquots (0.5 mg/mL, or 30 µg/60 µL) was prepared in the same manner as the 60 µg/60 µL reference standard, but the aliquots were not further diluted. To monitor the status of protein migration during electrophoresis, the red dye Pyronin-Y (4 µL of 0.125%) was added to all reference standards and control specimens.

**Protocol.** On a given day, 3 sets of reference standards and 3 aliquots of the control specimen were retrieved from -80°C and allowed to thaw on ice. Thereafter, a maximum of 12 specimens from a single clinical study were diluted in sample buffer (to 30 µg/60 µL) to total volumes sufficient for three gels (~200 µL each); to avoid a potential sex bias with studies E (100% women) and M (95% men), 3-4 specimens from one study were swapped with 3-4 specimens from the other study. Caco-2 homogenate and cDNA-expressed human CYP3A5 were diluted similarly to yield 30 µg/60 µL and 300 fmol/60 µL, respectively. The Caco-2 homogenate was used as a positive control for the detection of P-gp and villin; cDNA-expressed CYP3A5 was used as a positive control for the detection of CYP3A5. As with the reference standards and control specimens, Pyronin-Y was added to the study specimens, Caco-2 homogenate, and cDNA-expressed CYP3A5.

Four 15-well gels were prepared, each consisting of a 0.1% SDS-7% polyacrylamide separating gel and a 0.1% SDS-3% polyacrylamide stacking gel. The study specimens, control specimen, Caco-2 cell homogenate, and cDNA-expressed CYP3A5 were loaded (60 µL each) onto 3 of the gels. The 3 sets of reference standards (5 standards/set) were loaded onto the fourth gel. The proteins were electrophoretically separated over ~3.5 hours (i.e., when the dye front reached the bottom of the gel) using a Hoefer SE 600 vertical unit (Amersham). The proteins were then transferred to the PVDF membranes overnight at 4°C (~30 V) using a Hoefer TE 62 Transphor unit (Amersham). The following morning, the immunoblot containing the reference standards was cut into 3 ‘reference strips’. Each reference strip was further processed (see under *Immunoblot Development*), simultaneously, with 1 of the 3 immunoblots
that contained the study and control specimens, Caco-2 homogenate, and cDNA-expressed CYP3A5.

To substantiate the average relative P-gp and CYP3A4 protein contents obtained with the 93 individual specimens, all specimens obtained from the men were pooled, and all specimens from the women were pooled. The total protein concentration of each pooled homogenate was determined by the method of Lowry (1951), then diluted to 30 µg/60 µL and Pyronin-Y added in the same manner as described for the individual specimens. Each homogenate was loaded, in triplicate, onto a single 15-well gel. Because only 1 gel was needed to determine relative P-gp and CYP3A4 contents, reference standards and a control specimen were not used. The gels were transferred to PVDF membranes in the same manner as described for the individual specimens.

To substantiate the average relative CYP3A5 protein contents obtained with the 25 specimens containing readily detectable CYP3A5, each specimen was diluted to a concentration of 30 µg/60 µL, and Pyronin-Y added, in the same manner as described for the individual specimens. Because only 2 gels were needed to determine relative CYP3A5 contents, 1 of the specimens was loaded onto both gels to serve as a between-blot comparator, and no reference standards were used. The gels were transferred to PVDF membranes as described for the individual specimens.

**Immunoblot Development.** For the 93 individual specimens, each of the 3 immunoblots containing the study specimens was placed, along with a reference strip, into 3 separate pans. The blots in each pan were developed simultaneously for the proteins of interest. First, the blots were blocked with 5% nonfat dry milk in PBS containing 0.3% Tween 20 (PBS-T). After 1 hour, the blots were rinsed in PBS-T, incubated with the anti-P-gp antibody (1:10,000 in PBS-T) for 2 hours, rinsed in PBS-T, then incubated with the goat anti-rabbit secondary antibody (1:1000). After 2 hours, the blots were rinsed, then visualized for P-gp by enhanced chemiluminescence using the Chemi-Doc imaging system (Bio-Rad). The blots were
blocked again for 1 hour, rinsed, incubated with the anti-CYP3A5 antibody (1:3000) for 2 hours, rinsed, incubated with the goat anti-rabbit secondary antibody (1:3000) for 2 hours, rinsed, then visualized for CYP3A5. The blots were stripped of the primary antibodies (see under Stripping of Immunoblots), blocked again for 1 hour, rinsed, incubated with the anti-villin antibody (1:3000) for one hour, rinsed, then incubated with the rabbit anti-mouse secondary antibody (1:3000). After 1 hour, the blots were rinsed, incubated with the anti-CYP3A4 antibody (1:500) for 1 hour, rinsed, incubated with the goat anti-rabbit secondary antibody (1:500) for 1 hour, rinsed, then visualized simultaneously for villin and CYP3A4 by enhanced chemiluminescence. Preliminary studies indicated that this order of development with the various primary antibodies allowed the use of the same immunoblot for the visualization of the 4 proteins of interest with the least background.

The 1 immunoblot containing the 2 pooled homogenates was developed for P-gp, then for villin and CYP3A4 as described above for the individual specimens/reference standards. The 2 immunoblots containing the specimens with readily detectable CYP3A5 were developed for CYP3A5 then villin as described above for the individual specimens/reference strips, but the blots were not stripped.

Stripping of Immunoblots. After sitting at room temperature for 1 hour, the reference strips and/or immunoblots containing the individual or pooled specimens were submerged in 400 mL stripping buffer (2% sodium dodecyl sulfate, 100 mM 2-mercaptoethanol, 62.5 mM Tris•HCl; pH, 6.7) in a glass pan, which was placed in a shaking water bath at 70°C. After 30 minutes, the blots were rinsed twice with large volumes (~300 mL) of PBS-T then developed for CYP3A4 and villin as described under Immunoblot development. Stripped blots incubated with the appropriate secondary antibodies showed that the anti-P-gp and -CYP3A5 primary antibodies were effectively removed (not shown). Preliminary studies indicated that the stripping procedure diminished the detection of CYP3A4 (but not villin) protein, although consistently, by 40-50% (hence relative CYP3A4 content would remain unchanged).
**Determination of Relative Protein Contents.** Integrated optical densities (IODs) of the protein bands of interest were obtained using the Bio-Rad software program Quantity One (v4.2). For the determination of relative P-gp, CYP3A4, and CYP3A5 expression levels in the 93 individual biopsy specimens, calibration curves were generated by plotting the IODs of the reference standards against the corresponding amounts of protein loaded (i.e., 3.75-60 µg). The calibration curves were used to convert IODs obtained for the study (and control) specimens to protein equivalents (or PE, in µg). For each of the study and control specimens, the triplicate PE values obtained for P-gp, CYP3A4, CYP3A5, and villin were averaged. Relative P-gp, CYP3A4, or CYP3A5 content was calculated as the ratio of the average P-gp, CYP3A4, or CYP3A5 PE to the average villin PE (designated as the PE ratio). Finally, the P-gp, CYP3A4, or CYP3A5 PE ratio was normalized to that obtained for the control specimen, which was set to a value of 1.00.

For the 1 immunoblot containing the triplicate sets of the 2 pooled homogenates (1 consisting of specimens from the men and 1 from specimens from the women), because reference standards and a control specimen were not used (nor needed), relative P-gp or CYP3A4 content was calculated as the ratio of the IOD of P-gp or CYP3A4 to that of villin. For the 2 immunoblots containing the CYP3A5-expressing specimens, because reference standards were not used (nor needed), relative CYP3A5 content was calculated as the ratio of the IOD of CYP3A5 to that of villin. The CYP3A5/villin IOD ratio was then normalized to that of the control specimen, which was set to a value of 1.00.

**Statistical Analysis**

The distributions of the data sets were assessed by the Kolmogorov-Smirnov test. The comparison of men and women with respect to mean duodenal P-gp (log10 scale) and CYP3A4 relative content used 95% confidence intervals and two-sided \( t \)-tests (each with significance level \( \alpha = 0.05 \)). The robustness of these results was evaluated via complementary tabulations of medians, nonparametric 95% confidence intervals for the medians, and Wilcoxon-Mann-
Whitney U-tests. Sex differences in interindividual variance were investigated using 95% confidence intervals for standard deviations and F-tests for variance ratios. Similar methods were applied for CYP3A5 relative content (in specimens with readily detectable CYP3A5). A chi-square test was used to compare the proportion of men to the proportion of women with detectable CYP3A5. The average of the triplicate values obtained for relative CYP3A4 and P-gp contents in the pooled homogenates prepared from the men and from the women were compared using the unpaired \( t \)-test (\( \alpha = 0.05 \)). Relationships among log \( 10 \) P-gp (or P-gp), CYP3A4, and CYP3A5 relative expression values were evaluated from both Pearson correlation coefficients (\( r_p \)) and Spearman rank correlation coefficients (\( r_s \)) (\( \alpha = 0.05 \)). The uneven age distributions in both men and women (Table 1) precluded evaluation of the effect of age on relative P-gp, CYP3A4, and CYP3A5 content.
RESULTS

Consistency of the Methodology. For each protein analyzed (P-gp, CYP3A4, and CYP3A5, and villin), the band intensities were qualitatively similar among each set of triplicate immunoblots (Figure 1). With the study specimens, the coefficients of variation of the triplicate PE values for P-gp, CYP3A4, CYP3A5, and villin were virtually identical, with an average ± SD of 26 ± 15%, 27 ± 14%, 26 ± 16%, and 24 ± 16%, respectively. These systematic variations demonstrated the consistency of our methodology and thus the robustness of the results. With the control specimens (prepared such that 30 µg were loaded/well), the average of the triplicate PE values, which were determined relative to the reference standards (3.8-60 µg), ranged from 15-70 µg for P-gp, from 30-60 µg for CYP3A4, from 30-50 µg for CYP3A5, and from 30-50 µg for villin. The average PE ratios (i.e., the ratio of the PE value of P-gp, CYP3A4, or CYP3A5 to the PE value of villin) for the control specimens, which in theory should have yielded values of unity, ranged from 0.4-1.4 for P-gp, from 0.8-1.6 for CYP3A4, and from 0.7-1.3 µg for CYP3A5. These variations appeared minimal, however, as the same statistical outcomes ensued if "non-normalized" PE ratios (i.e., not normalized to the PE ratio of the control specimen) were used, further demonstrating the robustness of the results.

Variation in Relative Duodenal CYP3A4 Protein and P-gp Expression. For specimens from 2 of the Caucasian men, all protein bands were too faint to produce reliable IODs. Similarly, for a specimen from 1 of the East Indian men, the CYP3A4 protein band was too faint to produce a reliable IOD. As such, meaningful relative CYP3A4 and P-gp expression values (i.e., PE ratios) were obtained for 90 and 91, respectively, out of the original 93 specimens.

Among all specimens, relative duodenal CYP3A4 and P-gp expression values varied 8- and 10-fold, respectively. The relative CYP3A4 values followed a normal distribution. The relative P-gp values did not follow a normal distribution and therefore required log
transformation prior to applying the parametric statistical tests. For both CYP3A4 (PE ratio scale) and P-gp (log₁₀ PE ratio scale), the distribution of values for the men was similar to that for the women in terms of mean, median, minimum, maximum, interquartile range, and standard deviation (Figure 2). A comparison of the standard deviation for the men to that for the women showed no sex difference for CYP3A4 (p = 0.23) or for log₁₀ P-gp (p = 0.26). Likewise, a sex difference was not detected between mean CYP3A4 or log₁₀ P-gp (Table 2). Consistent with this result, a sex difference was not detected in the median CYP3A4 or P-gp values (Table 2).

The lack of sex differences in mean and median relative CYP3A4 and (log₁₀) P-gp expression values determined with the 90 (or 91) individual subjects was confirmed when the pooled preparation containing specimens obtained from men was compared with that from women (Figure 3). A difference was not detected between the average (± SD) of the 3 CYP3A4/villin IOD ratios determined for the men (0.98 ± 0.05) and that for the women (1.01 ± 0.04) (p = 0.34). Similarly, a difference was not detected between the average (± SD) of the 3 P-gp/villin IOD ratios determined for the men (1.00 ± 0.07) and for the women (1.14 ± 0.08) (p = 0.08).

Secondary investigations included comparison of men and women within the sufficiently large subgroups. Restricting analysis to the subjects self-identified as Caucasian or to the CYP3A5 non-expressors had negligible effects on the comparisons of interest (Table 2). Restricting comparison to subjects younger than 45 years of age, thereby omitting 3 men and the 24 post-menopausal women, had minimal effect except for a small increase in mean and median relative CYP3A4 content for the women (Table 2). Sex differences in means, medians, and standard deviations were not detected in any of these subgroup analyses (Table 2).

Whereas the uneven age distributions in both men and women precluded evaluation of the effect of age on duodenal CYP3A4 and P-gp expression, the relatively even proportions of
pre- and post-menopausal women allowed comparison of these two subgroups. The mean relative CYP3A4 content in pre-menopausal women was 1.05 (Table 2) compared to a mean of 0.82 in the post-menopausal women (p = 0.01). Similarly, the median CYP3A4 contents for the pre- and post-menopausal women were 1.01 (Table 2) and 0.80, respectively (p = 0.02). No such differences were detected in mean log_{10} P-gp content (-0.16 vs. –0.21, p = 0.39) or in median P-gp content (0.65 vs. 0.63, p = 0.36).

A correlation was not evident between relative CYP3A4 and log_{10} P-gp expression values in both men (r_{p} = 0.26, p = 0.08) and women (r_{p} = 0.09, p = 0.55). Similar results ensued with the nonparametric correlation analysis (r_{s} = 0.25, p = 0.09 and r_{s} = 0.19, p = 0.20, for men and women, respectively).

**Frequency and Variation in Relative Duodenal CYP3A5 Protein Expression.** The frequency of readily detectable immunoreactive CYP3A5 protein was 27% in this predominately Caucasian group of individuals (Table 3). A sex difference in this frequency (30% for men vs. 24% for women) was not detected (p = 0.48). Among these 25 individuals, relative CYP3A5 expression values (PE ratios) varied 7-fold. Variation in the 14 men was the same as that in the 11 women (6-fold). As with relative CYP3A4 and P-gp expression values, a sex difference was not detected in both mean ± SD (1.09 ± 0.57 and 1.10 ± 0.54 for men and women, respectively) (p = 0.96) and median (1.01 and 0.90 for men and women, respectively) (p = 0.99) relative CYP3A5 expression values. The lack of a sex difference in CYP3A5 expression was confirmed with results from the 2 immunoblots containing only the CYP3A5-expressing specimens (Figure 4). The mean (± SD) relative CYP3A5 expression value (CYP3A5/villin IOD ratio) was 3.0 ± 1.0 for the men and 3.5 ± 2.4 for the women (p = 0.58). Likewise, median relative CYP3A5 expression values were similar between men and women (2.9 vs. 2.6) (p = 0.79).
As expected, the relative CYP3A5 expression values obtained from the 93 individual specimens (i.e., PE ratios) correlated with the values obtained from the 2 immunoblots containing the CYP3A5-expressing specimens (CYP3A5/villin IOD ratios), whether using parametric ($r_p = 0.56$, $p = 0.004$) or nonparametric ($r_s = 0.63$, $p = 0.002$) methods. A correlation was not detected between relative CYP3A5 and CYP3A4 expression values in the 25 CYP3A5-expressing specimens, using either set of CYP3A5 expression values, and whether using parametric ($r_p \leq 0.34$, $p \geq 0.10$) or nonparametric ($r_s \leq 0.27$, $p \geq 0.19$) methods. Likewise, a correlation was not detected between relative CYP3A5 and $\log_{10}$ P-gp (or P-gp) expression values using either set of CYP3A5 values ($r_p \leq 0.01$, $p \geq 0.66$ and $r_s \leq -0.05$, $p \geq 0.68$).
DISCUSSION

The frequent reports of a higher systemic clearance of some CYP3A4 drug substrates in women compared to men are consistent with the recent demonstration that women express higher hepatic CYP3A4 specific content compared to men (Wolbold et al., 2003). In contrast to systemic clearance, sex differences in the apparent oral clearance of CYP3A4 substrates have generally not been reported (Cummins et al., 2002). This raised the possibility that a sex-dependent expression of CYP3A4 exists in the small intestine but in a pattern opposite to that in the liver. Our analysis of duodenal biopsies obtained from 91 healthy volunteers not receiving known inducers or inhibitors of CYP3A4 does not support this hypothesis, however, as exemplified by the very small estimate of the mean difference (0.002) between men and women. Assuming that the current specimens are representative of healthy adult Americans, the 95% confidence interval for this difference, [-0.14, 0.14], implies that the probability that duodenal CYP3A4 content in men and women differ by more than ~15% is <5%. Although catalytic activities were not measured in the current study (due to insufficient material), it is reasonable to assume that a large sex difference also does not exist in duodenal CYP3A4 activity, as evidenced by a strong correlation between duodenal protein content and activity ($r \geq 0.84$, $p < 0.001$) (Lown et al., 1994; Paine et al., 1997). Opposing effects of sex differences in hepatic and intestinal CYP3A4 activity are therefore unlikely mechanisms underlying the general paucity of reported sex differences in the oral clearance of CYP3A4 substrates.

It should be noted that diets were not strictly controlled in many of these subjects. Ingestion of juice prepared from some citrus fruits (e.g., grapefruit, Seville orange) has been shown to markedly reduce enterocyte CYP3A4 content (Schmiedlin-Ren et al., 1997; Edwards et al., 1999). It is therefore possible that interindividual differences in dietary habits resulted in changes in enterocyte CYP3A4 content that partially masked a true sex-related difference.
However, all subjects were fasted overnight prior to the endoscopic biopsy procedure, and the dietary habits of these individuals should otherwise represent a typical American population.

In rats, while sex differences in intestinal CYP expression are not evident (at the mRNA level) (Lindell et al., 2003), differences in hepatic CYP expression are well established (Agrawal and Shapiro, 2001; Ahluwalia et al., 2004). The differences in hepatic expression appear not to be the direct effect of sex hormones on the liver, but to sex differences in the time-dependent pattern of circulating growth hormone (GH) concentration (Agrawal and Shapiro, 2001; Ahluwalia et al., 2004). A sex difference in circulating GH has also been reported in humans, and a previous study involving patients without anterior pituitary function demonstrated that the pattern of circulating GH could influence hepatic CYP3A4 activity (Jaffe et al., 2002). The sex difference in CYP3A4 expression in human liver may therefore reflect in part GH secretion patterns. Although enterocytes contain the GH receptor, it was recently reported that post receptor binding events are not the same in hepatocytes and enterocytes (Dahly et al., 2004).

Regardless of the mechanisms underlying the sex difference in CYP3A4 expression in the human liver, our observation of the lack of a difference in the duodenal biopsy specimens indicates such mechanisms are not operative at the level of the proximal small intestinal mucosa.

The clinical relevance of the 20% lower mean duodenal CYP3A4 content in post- vs. pre-menopausal women is unclear. None of the women in the post-menopausal group was on hormone replacement therapy, and none of the women in the pre-menopausal group was taking birth control pills (or any other medications known to influence CYP3A4 expression) at the time the baseline biopsies were obtained. To our knowledge, menopausal status has not been reported to influence the apparent oral clearance of CYP3A4 substrates; at least one study designed to address this issue did not detect such an effect (Gorski et al., 2000). This may reflect the relatively small difference observed between pre- and post-menopausal women.
in the current study combined with the reported lack of a difference in hepatic CYP3A4 expression between the two groups (Wolbold et al., 2003).

The polymorphic CYP3A5, which shares many of the same substrates as CYP3A4, was readily detected in 23% of the specimens obtained from the individuals self-identified as Caucasian. This is consistent with earlier findings involving liver microsomal preparations obtained from American Caucasian individuals (23%) (Lin et al., 2002). These higher frequencies compared to that reported by Wolbold et al. (8.5%), whose liver specimens were obtained from European Caucasians, likely reflected the greater genetic diversity in the American groups. Interindividual variation in expression levels, however, was in good agreement among all 3 groups (7 to 13-fold) (Lin et al., 2002; Wolbold et al., 2003). Finally, consistent with an earlier report regarding hepatic CYP3A5 (Wrighton et al., 1990), a sex difference was not detected in CYP3A5 content in the current group of duodenal specimens.

In accord with the study by Wolbold et al. (2003), where median hepatic P-gp levels did not significantly differ between men and women, a sex difference also was not evident for this transporter in the current duodenal specimens. Together, these results do not support the hypothesis (Cummins et al., 2002) that sex differences in the oral clearance of dual CYP3A4/P-gp substrates can be attributed to higher P-gp expression in men compared to women.

In summary, this work represents the largest number of specimens examined to date to determine whether sex influences expression levels of CYP3A4 or P-gp in the upper small intestine. The lack of evidence of a sex difference in intestinal P-gp expression is consistent with the reported lack of a difference in hepatic P-gp expression. The absence of a substantial sex difference in CYP3A4 expression in the intestine, unlike in the liver, further supports the contention that the enzyme is regulated by different mechanisms in the 2 organs. The lack of a sex-related difference in intestinal CYP3A4 could account in part for the paucity of reported sex differences in the oral, relative to systemic, clearance of certain CYP3A4 substrates. The
clinical relevance of the lower mean duodenal CYP3A4 content in post-menopausal compared to pre-menopausal women requires further investigation.
REFERENCES


25


FOOTNOTES

This work was supported by the Office of Women’s Health, Food and Drug Administration, and the National Institutes of Health (RR00046).

1The terms *sex* and *gender* are used to describe differences between men and women; *sex* is the biological difference between men and women that is hormonal and reproductive in nature, whereas *gender* describes variability between men and women in society, culture, or history (Kim and Nafziger, 2000).
LEGENDS FOR FIGURES

Figure 1. Representative triplicate Western blots showing the inter-blot consistency and the interindividuation variation in (A) P-gp, (B) villin, (C) CYP3A5, and (D) CYP3A4 expression among the duodenal pinch biopsies obtained from 12 of the subjects from cohort study D (left panels) and corresponding reference standards (right panels). Lanes 1-12 represent the subject specimens, lane 13 represents the control specimen, lane 14 represents Caco-2 cell homogenate (used as a positive control for the detection of P-gp and villin), and lane 15 represents cDNA-expressed CYP3A5 (used as a positive control for the detection of CYP3A5); 60-3.8 represent the reference standards (µg).

Figure 2. Comparison of men and women with respect to the distributions of CYP3A4 content and log_{10} P-gp content. Each schematic box-and-whisker plot shows the mean (filled circles), median (horizontal bar), interquartile range (box), relatively extreme values (open circles), and the minimum and maximum of non-extreme values (vertical whiskers).

Figure 3. Western blot of pooled biopsy specimens obtained from all of the men (M) and from all of the women (W).

Figure 4. Western blot showing the interindividual variation in CYP3A5 expression among the duodenal biopsy specimens containing readily detectable CYP3A5 (n = 25) and corresponding villin. Specimen no. 12 was used as a between-blot comparator. 3A5 denotes cDNA-expressed CYP3A5 (used as a positive control for the detection of CYP3A5).
Table 1. Characteristics of the 48 men and 45 women from whom duodenal pinch biopsies were obtained.

<table>
<thead>
<tr>
<th>Ethnicity(^a)</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>39</td>
<td>37</td>
</tr>
<tr>
<td>AA</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>EI</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age (years)</th>
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<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-24</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>25-34</td>
<td>18</td>
<td>3</td>
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<tr>
<td>35-44</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>45-64</td>
<td>3</td>
<td>24(^b)</td>
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</table>

<table>
<thead>
<tr>
<th>Study Cohort</th>
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<th>Women</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>M</td>
<td>18</td>
<td>1</td>
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<table>
<thead>
<tr>
<th>CYP3A5</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td>-</td>
<td>34</td>
<td>34</td>
</tr>
</tbody>
</table>

\(^a\)Ethnicity was self-identified: C, Caucasian; AA, African American; A, Asian; EI, East Indian; H, Hispanic.
\(^b\)All post-menopausal women were ≥ 45 years of age.
Table 2. Relative duodenal CYP3A4 and P-gp expression values (arbitrary units) in men and women with corresponding age ranges.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>All Subjects</th>
<th>Caucasians</th>
<th>CYP3A5 Non-expressors</th>
<th>&lt; 45 years of age</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
<td>Women</td>
</tr>
<tr>
<td>Mean Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>28</td>
<td>41</td>
<td>28</td>
<td>44</td>
</tr>
<tr>
<td>Range</td>
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<td>18-64</td>
<td>19-48</td>
<td>18-64</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>n = 45</td>
<td>n = 45</td>
<td>n = 37</td>
<td>n = 37</td>
</tr>
<tr>
<td>Mean</td>
<td>0.93</td>
<td>0.93</td>
<td>0.89</td>
<td>0.89</td>
</tr>
<tr>
<td>95% CI for diff. of means</td>
<td>[-0.14, 0.14]</td>
<td>[-0.16, 0.16]</td>
<td>[-0.16, 0.19]</td>
<td>[-0.33, 0.06]</td>
</tr>
<tr>
<td>p-value(^b)</td>
<td>0.98</td>
<td>0.97</td>
<td>0.84</td>
<td>0.14</td>
</tr>
<tr>
<td>Median</td>
<td>0.95</td>
<td>0.89</td>
<td>0.94</td>
<td>0.89</td>
</tr>
<tr>
<td>95% CI for diff. of medians</td>
<td>[-0.30, 0.26]</td>
<td>[-0.33, 0.24]</td>
<td>[-0.42, 0.33]</td>
<td>[-0.22, 0.55]</td>
</tr>
<tr>
<td>p-value(^c)</td>
<td>0.89</td>
<td>0.88</td>
<td>0.79</td>
<td>0.18</td>
</tr>
<tr>
<td>P-gp</td>
<td>n = 46</td>
<td>n = 45</td>
<td>n = 37</td>
<td>n = 37</td>
</tr>
<tr>
<td>Mean</td>
<td>0.66</td>
<td>0.73</td>
<td>0.64</td>
<td>0.77</td>
</tr>
<tr>
<td>Mean (log(_{10}) scale)</td>
<td>-0.22</td>
<td>-0.18</td>
<td>-0.21</td>
<td>-0.18</td>
</tr>
<tr>
<td>95% CI for diff. of means (log(_{10}) scale)</td>
<td>[-0.10, 0.5]</td>
<td>[-0.12, 0.04]</td>
<td>[-0.10, 0.08]</td>
<td>[-0.15, 0.03]</td>
</tr>
<tr>
<td>p-value(^b)</td>
<td>0.55</td>
<td>0.34</td>
<td>0.23</td>
<td>0.80</td>
</tr>
<tr>
<td>Median</td>
<td>0.69</td>
<td>0.65</td>
<td>0.68</td>
<td>0.68</td>
</tr>
<tr>
<td>95% CI for diff. of medians</td>
<td>[-0.19, 0.18]</td>
<td>[-0.18, 0.18]</td>
<td>[-0.26, 0.16]</td>
<td>[-0.20, 0.32]</td>
</tr>
<tr>
<td>p-value(^c)</td>
<td>0.91</td>
<td>0.58</td>
<td>0.40</td>
<td>0.50</td>
</tr>
</tbody>
</table>

\(^a\)All pre-menopausal women were younger than 45 years of age.
\(^b\)Comparison of men to women (log\(_{10}\) scale) by the student’s t-test.
\(^c\)Comparison of men to women by the Wilcoxon-Mann-Whitney U test.
Table 3. Frequency of readily detectable CYP3A5 protein in duodenal pinch biopsies obtained from healthy volunteers.

<table>
<thead>
<tr>
<th>SUBJECT ID</th>
<th>TOTAL COUNT</th>
<th>CYP3A5+</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>91</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Women</td>
<td>45</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>Men</td>
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<td>14</td>
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</tr>
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<td>23</td>
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</tr>
<tr>
<td>H</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^{a}\) C, Caucasian; AA, African American; A, Asian; EI, East Indian; H, Hispanic.
Figure 2

Log10 P-gp Content (Relative PE Ratio)

CYP3A4 Content (Relative PE Ratio)

Women    Men

Women    Men