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Male-specific induction of CYP3A2 in rats by zolmitriptan

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Abstract

We report here a novel observation that zolmitriptan induced CYP3A2 in male but not female rats. As part of our research programme to evaluate sex differences in the response to zolmitriptan, we studied the effects of zolmitriptan on CYP3A activity, protein and gene expression in male and female rats. Zolmitriptan was found to induce CYP3A activity, measured as testosterone and diazepam metabolism in-vitro, as well as midazolam pharmacokinetics in-vivo, in male but not female rats. The sex difference in response to zolmitriptan was further evaluated by analysis of CYP3A1/2 mRNA levels using real-time PCR, and CYP3A1/2 protein levels using immunoblotting. Zolmitriptan preferentially induced CYP3A2 in male but not female rats. No obvious effects on CYP3A1 were observed at any dose in either sex. Thus, we concluded that the observed sex-dependent induction of CYP3A by zolmitriptan was largely due to induction of CYP3A2 in male rats.

Introduction

The cytochromes P450 (CYP) are a superfamily of haem-containing enzymes that play a major role in the oxidative and reductive metabolism of drugs, xenobiotics and endogenous compounds. Induction of CYP enzymes can have important clinical consequences such as bioactivation and reduced systemic exposure (Wrighton & Stevens 1992; Gonzalez & Gelboin 1994; Parkinson 1996). In addition, induction of CYPs is sometimes sex dependent, which makes the possibility of clinical drug–drug interactions even more complex (Agrawal & Shapiro 1996; Sharma et al 1998). Sex differences in hepatic drug metabolism occur in numerous species, including fish, reptiles, birds and humans (Chen et al 2006; Orlando & Guillette 2007). Research concerning sexual dimorphisms in drug metabolism is expected to have clinical implications, particularly for individualized drug therapy. Prediction of the therapeutic and toxicological effects of drugs and xenobiotics can be improved when the mechanisms of CYP induction are understood.

Zolmitriptan is a novel and highly selective 5-$HT_{1B/1D}$ receptor agonist used as an acute oral treatment for migraine. The potential for drug interactions has been reported when zolmitriptan is co-administered with other drugs such as propranolol (Peck et al 1997), diazepam (Yang et al 1998; Mei et al 1999) and moclobemide (Rolan 1997), which could increase the plasma concentrations of zolmitriptan. No studies, however, have reported whether zolmitriptan affects the expression of any of the CYPs. If this is shown to be true, potential interactions may occur and may be clinically relevant. Understanding the potential for drug–drug interactions and induction or inhibition of CYPs is therefore very important.

In preliminary studies, the activity of CYP3A, but not CYP1A2, CYP3A1/2, CYP2B, CYP2C or CYP2E1, was induced by zolmitriptan. Furthermore, the effect of zolmitriptan on CYP3A showed sex differences. Thus, the purpose of this study is to further assess the effects of zolmitriptan on CYP3A1/2 mRNA and protein expressions, and activity in male and female rats.

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Materials and Methods

Animals and treatments

Male and female Sprague–Dawley rats weighing 190 ± 10 g were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The animal experimental study was approved by the Animal Ethics Committee of this institution.

Rats were maintained under standardized conditions of light and temperature, with free access to standard rat chow and water. After acclimation, rats were randomized into treatment groups of five rats of each sex for the pharmacokinetic study of midazolam, and into treatment groups of three or four rats of the same sex for the in-vitro studies of enzyme activity, protein and mRNA levels. In each case, each group included three batches. The animals for the pharmacokinetic study of midazolam were treated with zolmitriptan (2.5 mg kg^{-1}) or vehicle (0.1% sodium carboxymethylcellulose (CMC-Na) in saline) or dexamethasone (100 mg kg^{-1}) dissolved in saline for 3 consecutive days. Zolmitriptan was provided by the Chemistry Department of Zhejiang University and had a purity of 99.8%.

Rats for the analysis of enzyme activity and protein and mRNA levels were treated with zolmitriptan (0.5, 2.5 or 12.5 mg kg^{-1}), vehicle or dexamethasone (100 mg kg^{-1}) dissolved in saline for 3 consecutive days. Dexamethasone groups were used as positive control groups.

Pharmacokinetics of midazolam

Rats were given a single i.v. injection of midazolam (Sanmen Kangning Chem Co., Zhejiang, China), 10 mg kg^{-1}, into the tail vein 24 h after the last administration of zolmitriptan. Blood was collected from the reverse side of the tail vein at 5, 10, 20, 30, 60, 90 and 120 min (Watanabe et al 1998). The concentrations of midazolam in plasma were measured by HPLC as described by Watanabe et al (1998). Pharmacokinetic parameters for midazolam were obtained from the plasma concentration–time profile and were calculated using software 3p87 (Chinese Pharmacology Association, Beijing, China).

Preparation of liver tissue and microsomes

Rats were decapitated 24 h after zolmitriptan administration and the liver was excised and perfused with ice-cold physiological saline to remove the blood. Each liver was quickly minced and a portion of the liver was saved for mRNA isolation; the rest was used for preparation of microsomes. Rat liver microsomes for Western blot and assay of enzyme activities were prepared by the method of Gibson & Skett (1994). All samples were stored at −80°C. Protein concentration was determined using the Lowry method (Lowry et al 1951).

Measurement of CYP3A activity

Testosterone and diazepam (Sigma Aldrich, St Louis, MO, USA) were used as the probe substrates to determine CYP3A activity in rat liver microsomes. An NADPH generation system (Yu et al 2003) and the probe substrates (75 μM testosterone, 70 μM diazepam) were added to rat liver microsomes in a total volume of 0.5 mL. The solution of NADP/NADPH (5 μL) was added to each reaction mixture after 5 min' pre-incubation. After incubation at 37°C for 10 min, 0.8 mL acetonitrile was added to terminate the reaction. The mixture was then centrifuged and 20 μL supernatant was analysed using isocratic HPLC with UV detection. For testosterone, the mobile phase was 50/50 (v/v) acetonitrile/water delivered at a flow rate of 1 mL min^{-1}, with detection at 240 nm. For diazepam, the mobile phase was acetonitrile/water (70/30, v/v) delivered at a flow rate of 1 mL min^{-1}, with detection at 254 nm. The CYP3A enzyme activity was assessed as percentage of the probe substrates remaining.

Quantitative real-time PCR

Total RNA of liver tissue was isolated by a single-step method using Trizol reagent (Invitrogen, Inc., Carlsbad, CA, USA). The concentration and purity of isolated RNA were measured in triplicate by UV spectrophotometry. The isolated RNA had an OD 260/280 ratio no less than 1.8. Reverse transcription was carried out using M-MuLV reverse transcriptase (Fermentas Life Sciences, Glen Burnie, MD, USA) following the manufacturer's protocol. Samples were stored at −80°C until analysis.

Levels of CYP3A1/2 mRNA levels were determined using real-time quantitative PCR using the SYBR green dye, performed using the Mastercycler Ep Realplex detection system (Eppendorff, Hamburg, Germany). The reaction mixture for CYP3A1/2 was prepared as follows: Real Master Mix/SYBR solution (1 ×), primers (0.15 μM), and the RT product from 80 ng of RNA (reagents from Qiangen Biotech, Beijing, China). After incubation at 95°C for 15 min, the PCR reaction was performed for 40 cycles: denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 68°C for 30 s. Triplicate samples from each of three animals were used for each treatment condition. Within each experiment the fold change between treatment and control was determined by transforming logarithmic data to linear data using the equation: fold change = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = (average C_T P450 in treated samples − average C_T β-actin in treated samples) − (average C_T P450 in control samples − average C_T β-actin in control samples). The value represents the fold change in mRNA level in the treatment group compared with the control group.

Specific primer pairs were as follows: CYP3A1, 5′-GATGTTGAAATCAATGGTGTGT-3′ and 5′-TTCAGAGGTATCTGTGTTTCC-3′; CYP3A2, 5′-AGTAGTGACGATTCCAACATAT-3′ and 5′-TCAGAGGTATCTGTGTTTCCT-3′; β-actin, 5′-ACTGGCATTGTGATGGACTC-3′, and 5′-CAGCACTGTGTTGGCATAGA-3′ (Rekka et al 2002).

Determination of CYP3A by Western Blot

SDS-PAGE was performed as described previously (Laemmli 1970) using a 10% polyacrylamide separating

gel. Liver microsomes (20 μg per lane) were separated by SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. All membranes were blocked with blotto (5% dry milk in phopshate-buffered saline, pH 7.4, with 0.1% Tween 20) at room temperature for 1 h. The blots were probed with rabbit polyclonal anti-CYP3A1 (1:4000, Abcam, Cambridge, MA, USA) overnight at 4°C and sheep polyclonal anti-CYP3A2 (1:1000, Abcam) for 1 h at room temperature. The membranes were then incubated with horseradish peroxide-conjugated goat anti-rabbit (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-sheep (1:2000, Abcam) immunoglobulin G for recognizing CYP3A1 and CYP3A2 primary antibodies, respectively. The incubation time was 1 h at room temperature. After exposure to enhanced chemiluminescence reagents (LumiGlO, Gaithersburg, MD, USA), the proteins were detected by fluorescence recorded on radiographic film.

Statistical analysis

Data are given as mean ± s.d. Individual differences were evaluated using Dunn's test. Non-parametric data was examined by the Kruskal–Wallis test. All calculations were performed using the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Sex-dependent induction of CYP3A enzyme activity in zolmitriptan-treated rat liver microsomes

Since 6β-OH testosterone is not commercially available, CYP3A activity was assessed by measuring the percentage of testosterone or diazepam remaining (Figure 1). Here, the low dose of zolmitriptan (0.5 mg kg^{-1}) was equivalent to the therapeutic dose for humans (5 mg per 60 kg body weight). In male rat liver microsomes, the metabolism of testosterone and diazepam in any zolmitriptan or dexamethasone groups was faster than that of the control group. The greatest induction was observed at the moderate dose of zolmitriptan (2.5 mg kg^{-1}). By contrast, CYP3A enzyme activities in treated female rat liver microsomes were not significantly different from that of the control group.

Sex-dependent induction of midazolam pharmacokinetics by zolmitriptan

The results of enzyme activities agree with midazolam pharmacokinetics. Midazolam is a sensitive CYP3A probe substrate in-vivo, recommended by the US Food and Drug Administration (FDA). The mean pharmacokinetic parameters

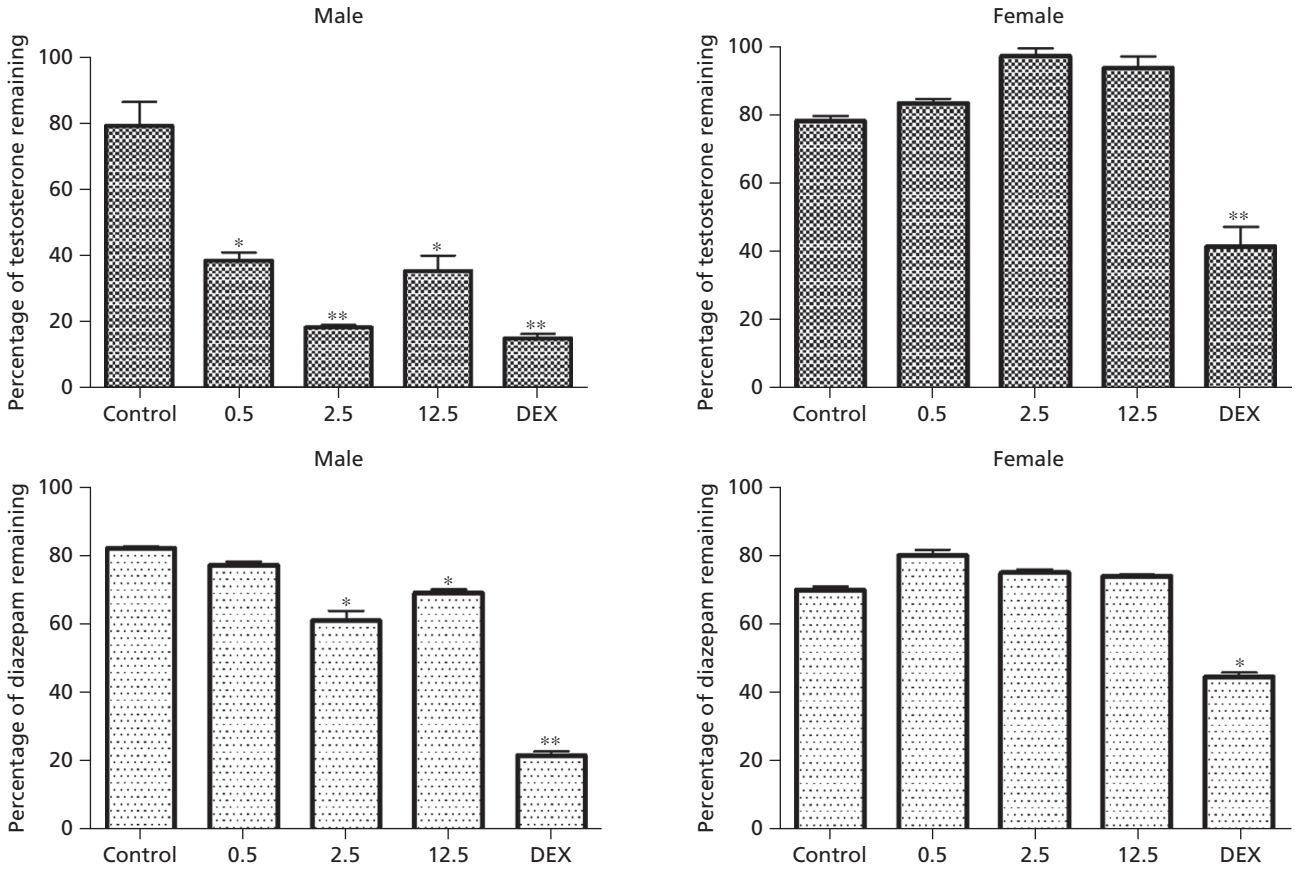

Figure 1 The metabolism of testosterone (top) and diazepam (bottom) in liver microsomes from rats pretreated for 3 days with zolmitriptan (0.5, 2.5 and 12.5 mg kg^{-1}) or dexamethasone (DEX; 100 mg kg^{-1}) and control group. Dexamethasone groups were used as positive control groups. Metabolism is shown as proportion of parent compound remaining. Data are mean ± s.d. n ≥ 3. *P < 0.05; **P < 0.01 vs control.

Table 1 Pharmacokinetic parameters of midazolam in zolmitriptan pretreated rats

	Male			Female		
	Control	Zolmitriptan group	Dexamethasone group	Control	Zolmitriptan group	Dexamethasone group
Half-life	21.5 ± 1.9	16.6 ± 1.2*	13.0 ± 1.8**	21.6 ± 2.9	22.4 ± 2.0	14.0 ± 2.6*
AUC (μmol min L^{-1})	3014.7 ± 142.3	2103.0 ± 163.8**	2070.0 ± 171.3**	3417.5 ± 247.6	3953.3 ± 425.7	1672.5 ± 143.5**
Clearance (mL min^{-1} kg^{-1})	8.8 ± 0.5	13.2 ± 0.9**	13.3 ± 1.8**	7.4 ± 0.9	6.0 ± 0.2	16.6 ± 2.0**

Data are mean ± s.d (n = 5). *$P < 0.05$; **$P < 0.01$ vs control.

estimated for midazolam are summarized in Table 1. The half-life ($t_{1/2}$) and area under the plasma–concentration time curve (AUC) of midazolam were significantly reduced by zolmitriptan pretreatment to 77.2% and 69.8%, respectively, of the control group. The clearance of midazolam was significantly increased by the zolmitriptan pretreatment. Although the $t_{1/2}$ and the AUC of midazolam were significantly reduced in dexamethasone-induced female rats, no significant changes were observed in the zolmitriptan pretreatment groups (Table 1). The in-vitro and in-vivo data indicate that the induction effect of zolmitriptan on rat liver CYP3A was male specific. CYP3A can be induced by zolmitriptan in male rats, but zolmitriptan displayed no obvious effect on CYP3A in female rats.

Induction of CYP3A2 by zolmitriptan at the protein level in male rats

CYP3A1 and CYP3A2 are two main CYP3A isoforms which catalyse the metabolism of numerous xenobiotics. To further confirm the apparent sex-dependent induction, the expression of CYP3A1 and CYP3A2 were determined immunochemically (Figure 2). No effects on CYP3A1 expression were observed at any dose in either sex. Western blots showed that the level of CYP3A2 was increased in zolmitriptan-treated males, whereas CYP3A2 was undetectable in all female rats. The maximum increase (5-fold) in males was observed at 2.5 mg kg^{-1}. These results are in agreement with previous reports in which CYP3A2 was found to be a constitutive male-specific hepatic isoform but was undetectable in female rats (Schenkman 1992; Agrawal & Shapiro 2003). In addition, CYP3A activities and the CYP3A2 protein level showed a good correlation in male rats (Figure 3; $r = 0.8927$, $P < 0.01$). These findings suggest that the observed sex-dependent inducibility of CYP3A by zolmitriptan in rats (evident from enzyme activity in-vitro, and midazolam pharmacokinetics in-vivo) is largely due to induction of CYP3A2 protein in male rats.

Induction of CYP3A2 by zolmitriptan at the mRNA level in male rats

Gene activation at the mRNA level, representing the possibility of functional cellular events, can be used to gauge CYP3A induction. We therefore examined the effects of zolmitriptan on CYP3A1 and CYP3A2 mRNA expression in rats, using real-time PCR. Overall, CYP3A2 mRNAs were induced in males, with its maximum effect (3.3-fold) at the intermediate dose of zolmitriptan. In agreement with the measurement of protein levels, CYP3A2 gene could not be detected in either treated or untreated female rats (Table 2). In contrast, CYP3A1 mRNA levels were not markedly affected by zolmitriptan in either sex. Minor increases of CYP3A1 mRNA of approximately 0.38-fold and 0.44-fold were observed in males and females, respectively. Although this was in slight discordance with protein levels, the trend appeared similar. CYP3A2 was thus more sensitive to zolmitriptan pretreatment than CYP3A1 in terms of mRNA level.

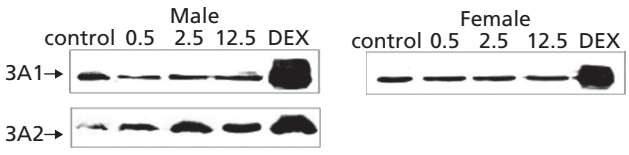

Figure 2 Representative blots for CYP3A1 and CYP3A2 protein expression in male and female rat liver. Rats were treated with vehicle, zolmitriptan (0.5, 2.5 or 12.5 mg kg^{-1}) or dexamethasone (100 mg kg^{-1}) for 3 consecutive days. Dexamethasone groups were used as positive controls. CYP3A proteins were monitored by Western blotting in at least three animals per group.

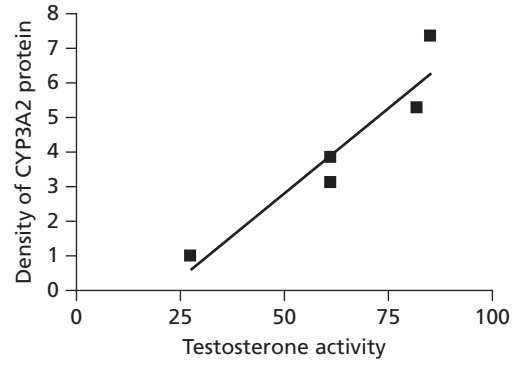

Figure 3 Correlation between testosterone activity (proportion of testosterone metabolized) and liver CYP3A2 protein density (fold change between treatment and control groups) in male rats pretreated with zolmitriptan (0.5, 2.5 and 12.5 mg kg^{-1}), dexamethasone (100 mg kg^{-1}) or vehicle for 3 consecutive days ($r = 0.8927$; $P < 0.01$). Dexamethasone groups were used as positive controls.

Table 2 Effects of zolmitriptan on the expression of CYP3A in male and female rat livers

Dose (mg kg^{-1})	Normalized gene expression (control = 1)			
	Male		Female	
	CYP3A1	CYP3A2	CYP3A1	CYP3A2
Zolmitriptan				
12.5	1.40 ± 0.84	1.60 ± 0.24	1.36 ± 0.31	ND
2.5	1.78 ± 0.33*	3.30 ± 0.28*	1.55 ± 0.23	ND
0.5	0.97 ± 0.12	2.05 ± 0.49*	1.40 ± 0.27	ND
Dexamethasone (10)	8.13 ± 3.7*	3.8 ± 0.49**	20.7 ± 7.14**	NC

Gene expression is given in relative levels, mean ± s.d.; n ≥ 3. *$P < 0.05$; **$P < 0.01$ vs control. ND, not detected; NC, could not be calculated. CYP3A2 gene in rats treated with dexamethasone is detectable in light of C_T and the melting curve. However, CYP3A2 mRNA was not detected in female control rats. Thus, the normalized gene expression cannot be calculated.

Discussion

In the present study, the effects of zolmitriptan on the induction of CYP3A isoforms in male and female rats was evaluated in terms of enzyme activities, protein and gene expression.

The effect of zolmitriptan on CYP3A was evaluated from the breakdown of testosterone and diazepam in-vitro, and midazolam pharmacokinetics in-vivo. Testosterone and diazepam are substrates for two distinct groups of CYP3A enzymes, and it is recommended that multiple CYP3A probes are used for the in-vitro assessment of CYP3A-mediated drug interactions (Kenworthy et al 1999). Although only testosterone 6β-hydroxylation (Krauser & Guengerich 2005) and diazepam N-demethylation (Kenworthy et al 1999) correlate with CYP3A activities, the results with similar trends concluded from the two substrate assays indicated that CYP3A was the major isoform induced by zolmitriptan in male rats but not in female rats. According to FDA guidance for drug interaction studies, when an in-vitro evaluation cannot rule out the possibility that an investigational drug is an inducer of CYP3A, an in-vivo evaluation can be conducted using a sensitive substrate such as midazolam (FDA CDER/CBER 2006). Therefore, midazolam pharmacokinetics was investigated to further confirm the sex-specific induction of CYP3A by zolmitriptan.

Testosterone and diazepam assays and midazolam pharmacokinetics suggested that rat CYP3A may show sex-differences in response to zolmitriptan. Our results showed that male rats were significantly more sensitive than female rats in terms of the induction potential of zolmitriptan, as measured by typical in-vitro and in-vivo CYP3A substrates. Zolmitriptan can induce CYP3A in male rats, but had no obvious such effect on CYP3A in female rats.

CYP3A1 and CYP3A2 are two main CYP3A isoforms. To investigate whether the sex difference is due to the differential effects of zolmitriptan on these two CYP3A isoforms, we also quantified CYP3A1 and CYP3A2 protein levels in male and female rats. Though CYP3A1 and CYP3A2 share an 89% sequence similarity (Debri et al 1995), the antibodies we used are specific enough to recognize their own antigens. Even if cross-reaction does occur, it is negligible, as only CYP3A1 bands, but not CYP3A2 bands, were detected in tissue lysates from female rats in Western blot experiments. Furthermore, male rat liver expresses far more CYP3A2 protein than CYP3A1. Thus, it seems reasonable to conclude that the two antibodies can distinguish CYP3A1 and CYP3A2. Immunoblotting showed that zolmitriptan increased CYP3A2 protein levels in males but not females, whereas no obvious effects were observed on CYP3A1 protein level in male or female rats. Pearson's product-moment correlation coefficient (r) was used to assess the relationship between CYP3A activity and the CYP3A2 protein level. The testosterone activities were significantly correlated with the CYP3A2 protein level ($r = 0.8927$, $P < 0.01$). Thus, we conclude that the observed sex-difference in inducibility of CYP3A in rats by zolmitriptan (in terms of enzyme activity and midazolam pharmacokinetics) is largely due to male-specific induction of CYP3A2 protein.

We also examined the effects of zolmitriptan on CYP3A1 and CYP3A2 mRNA expression in rats. Real-time PCR showed that zolmitriptan can induce both CYP3A2 and CYP3A1 mRNAs. However, CYP3A2 was more sensitive than CYP3A1 to zolmitriptan pretreatment. Although the effect on CYP3A1/2 mRNA levels was in slight discordance with the effect on protein levels, the trend appeared similar. The reason for this discrepancy may be that CYP3A1 and CYP3A2 possess slightly different post-transcriptional controls. Meanwhile, the gene products at the protein and mRNA levels could possibly be disconnected, as demonstrated; it may therefore be better to conclude the induction of CYP3A1/2 on the basis of the elevated protein level rather than the mRNA expression (Zhang et al 2006).

Taking the results of enzyme activity, protein and mRNA level assays together, the effect of zolmitriptan on CYP3A1/2 was found to be sex-dependent with two different patterns. First, zolmitriptan preferentially induced CYP3A2 in male rats but had less effect on CYP3A1. This may be due to the fact that two isoforms have two different regulation pathways, although CYP3A1 and CYP3A2 share a high degree of structural homology (Sierra-Santoyo et al 2000). Secondly, zolmitriptan, at a therapeutic dose, induced CYP3A2 in males not females, suggesting its action is sex selective. The rationale for such a selective induction is unclear, but is not without precedent. For example, marginal effects of 1,4-dithiothreitol on CYP2C11, a male-specific

and growth-hormone-dependent isozyme, were also observed in males but not in females (Li et al 1995).

Animal studies are used to help determine the metabolism of chemicals in an attempt to extrapolate the risk of human exposure to these agents. In our studies, the lowest dose of zolmitriptan was selected on the basis of the therapeutic dose. Zolmitriptan is metabolized by the same isoenzyme, CYP1A2, in both human and rat liver microsomes, forming N-demethylzolmitriptan as the major metabolite (Wild et al 1999; Yu et al 2003); however, we cannot extrapolate the risk of human exposure to zolmitriptan from the present limited findings obtained in rats because of differences in CYP isoform compositions between humans and rats. Further studies with primary human hepatocytes are planned to investigate the inducible effect on human CYP3A by zolmitriptan.

The pregnane X receptor (PXR) is known to be a key component mediating most xenobiotic-related induction of CYP3A (Moore et al 2000; Ma et al 2007). However, noone has reported that PXR-mediated induction of CYP3A is sex specific. Rat liver, which has been the focus of the majority of investigations, is known to contain at least a dozen sex-dependent isoforms of P450. CYP2A2, 2C11 and 3A2 are specific to male rat hepatocytes; female rat hepatocytes express CYP2C12 and 3–4-fold greater levels of CYP2C7 (Shapiro et al 1995; Pampori & Shapiro 1999). Growth hormone is known as the major mechanistic determinant of sexually dimorphic gene expression, such as of CYP3A2, in the rat liver model (Colby et al 1973; Denef 1974; Gustafsson & Stenberg 1974; Ahluwalia et al 2004). The facts stated above suggest that CYP3A2 may have an unconventional regulation pathway that can be sexually dimorphic. Further research is needed on the molecular events involved in the male-specific induction of rat CYP3A2 by zolmitriptan.

Conclusion

Our report here is the first to demonstrate sex differences in the induction of CYP3A activity by zolmitriptan. This sex-specific induction by zolmitriptan is largely due to male-specific induction of CYP3A2 rather than CYP3A1. However, it remains to be established whether this sex-specific modulation has significance in exposed human populations.

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