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Phenacetin and chlorzoxazone biotransformation in aging male Fischer 344 rats

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Abstract

We evaluated the role of specific isoforms in the biotransformation of phenacetin and chlorzoxazone and examined the effect of age on these reactions using liver microsomes from Fischer 344 rats between 3 and 26 months of age. Using rat cDNA-expressed cytochrome P450 (CYP) enzymes, we found that phenacetin biotransformation was primarily mediated by CYP2C6 and CYP1A isoforms, while chlorzoxazone biotransformation was largely mediated by CYP2E1 and CYP1A1. Incubations with liver microsomes prepared from rats of varying ages demonstrated that both phenacetin and chlorzoxazone biotransformation declined with age. Metabolite formation rates in the old rats (25–26 months) were reduced by approximately 60–70% for these reactions. This study suggests that the activity of CYP2E and CYP1A enzymes decline with age in the rat liver. Also, the relative specificity of the index substrates phenacetin (for CYP1A2) and chlorzoxazone (for CYP2E1) in man appears not to be applicable in rats.

Introduction

With the projected 30% increase in the elderly population in the USA (Administration on Aging, Health and Human Services 2001) and the frequent use of pharmacological agents in this population (Vestal 1997; von Moltke & Greenblatt 1999), an understanding of age-related differences in drug metabolism is becoming increasingly important.

Many studies have demonstrated a reduced clearance in cytochrome P450 (CYP) substrates in aging humans in-vivo (Greenblatt et al 1982; Schmucker 1985; Vestal 1997; von Moltke & Greenblatt 1999; Cotreau et al 2004). Age-related reductions in CYP activity have also been observed in rats in-vivo (Boonstra-Nieveld & van Bezooijen 1989; Fujita et al 1990b) and in-vitro (Kamataki et al 1985; Fujita et al 1990a; Warrington et al 2003, 2004).

To date, many aging studies on CYP expression and function in rats have focused on age-related changes in reactions mediated by CYP3A and CYP2C enzymes (Kamataki et al 1985; Lee et al 1994; Warrington et al 2003), the two most abundant subfamilies in the human and rat liver (Imaoka et al 1990; Shimada et al 1994).

A more limited number of studies have examined age-related differences in the activity of CYP1A or CYP2E isoforms, which represent the third and fourth most abundant subfamilies in the human liver (Shimada et al 1994). Studies in man on the effect of age on CYP1A and CYP2E expression in-vitro have been inconsistent. For example, while Hunt et al (1990) found no change in CYP2E expression with age, George et al (1995) found an age-related reduction in CYP2E expression. On the other hand, George et al (1995) found no difference with age in CYP1A expression. A few studies in rats have demonstrated a decline in aniline *p*-hydroxylation, which may be mediated by a combination of CYP1A2 and CYP2E1 isoforms (Rikans 1989; Horbach et al 1992). Wynne et al (1987) studied the effect of age on 7-ethoxyresorufin *O*-deethylation (EROD), which has recently been shown to be largely mediated by CYP1A2 and CYP2C6 isoforms in the absence of induction of CYP1A1 (Kobayashi et al 2002). However, since several of these studies were performed before the commercial availability of rat cDNA-expressed CYPs and immunoinhibitory antibodies,

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the roles of specific enzymes in many of these reactions have not been fully characterized in the rat. Since multiple species differences have been identified in specific CYP-mediated reactions (von Moltke et al 1993; Court et al 1997; Perloff et al 2000; Kobayashi et al 2002; Warrington et al 2003, 2004), it is critical to identify the involvement of specific CYP enzymes in commonly studied reactions in rodent models.

Despite the presence of identifiable species differences in CYP-mediated reactions, the study of age-related differences in CYP1A and CYP2E isoforms in the rat may provide insight into factors that modulate drug metabolism with age in man. First, the genetic composition of CYP2E and CYP1A subfamilies in the rat share greater than 80% similarity with their human homologues (Rebhan et al 1997). Second, in rats, fewer sexual dimorphisms have been identified in these enzymes (Parkinson 1996; Anderson et al 1998). Since age-related differences in CYP3A and CYP2C isoforms has been shown to be dependent upon androgen exposure (Fujita et al 1990a) and CYP3A and CYP2C isoforms demonstrate significant sexual dimorphisms in the rat (Parkinson 1996), an understanding of age-related changes in CYP subfamilies that exhibit fewer sexual dimorphisms may provide further insight into how age can affect CYP-mediated reactions.

In man, phenacetin *O*-deethylation and chlorzoxazone hydroxylation have been frequently used as markers for CYP1A and CYP2E1 activity, respectively (von Moltke et al 1996; Court et al 1997; Venkatakrishnan et al 1998). We evaluated the role of specific CYPs in these reactions in the male Fischer 344 rat model and examined the effect of age on these reactions.

Materials and Methods

Chemicals and reagents

Chlorzoxazone and 6-hydroxychlorzoxazone (6-OH-CLZ) were kindly provided by R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). Phenacetin and acetaminophen (paracetamol) were purchased from Sigma-Aldrich Co. (Indianapolis, IN). cDNA-expressed enzymes were obtained from Gentest Corporation, a division of Becton-Dickinson sciences (Woburn, MA). Other reagents were also purchased from commercial sources.

Animals

Young (3–4 months, n = 10), intermediate (13–14 months, n = 10) and old (25–26 months, n = 12) male Fischer 344 rats were obtained from the National Institutes of Aging (held at Harlan Sprague-Dawley Inc., Indianapolis, IN), as described previously (Warrington et al 2003). The Fischer 344 male rat model has been the most extensively studied rat model for aging studies (Weindruch & Masoro 1991) and allows for direct comparisons to previous studies on age-related differences in hepatic CYP activity and expression. The research protocols utilized in this study were approved by the Tufts-New England Medical Center Animal Research Committee. Four rats from the old group were not included in the study due to the presence of disease: infection (n = 1), superficial gross tumours (n = 2) and hepatomegaly (n = 1). The remaining rats appeared healthy and did not exhibit any gross pathology, including no evidence of tumours or renal pathology. After sacrifice by decapitation, livers were removed and stored at −80°C until microsomal preparation.

Microsomal preparations

Hepatic microsomes were prepared as described previously (von Moltke et al 1993, 1996; Warrington et al 2002). Briefly, liver segments were homogenized in a 0.05 M potassium phosphate buffer solution containing 0.15 M KCl and 0.25 M sucrose. Samples were centrifuged at 11 960 *g* for 22 min at 4°C. Supernatants were transferred to ultracentrifugation tubes and centrifuged for 70 min at 111 884 *g* at 4°C. Pellets were washed with 0.05 M potassium phosphate buffer and resuspended in an 80% 0.1 M potassium phosphate buffer–20% glycerol solution. Samples were stored at −80°C, until use.

In-vitro biotransformation of chlorzoxazone

The biotransformation of chlorzoxazone to 6-OH-CLZ was conducted as described previously (Court et al 1997). Due to the limited supply of microsomal protein, hepatic microsomes (0.8 mg mL^{-1}) from young (n = 10), intermediate (n = 10) and old (n = 8) rats were incubated with a single concentration of chlorzoxazone (300 μM) and an NADPH-regenerating system for 20 min at 37 °C. Acetonitrile (2/5th volume) was added to stop the reactions and phenacetin was used as an internal standard. Microsomal protein was removed by centrifugation at 15 996 *g* for 10 min. Supernatants were transferred to autosampling HPLC vials. Incubation times and microsomal protein concentrations were conducted within a linear range (data not shown). Increasing quantities of 6-OH-CLZ (0–13.5 nmol) were used to construct a calibration curve.

In-vitro biotransformation of phenacetin

The biotransformation of phenacetin was examined using both pooled microsomes at increasing concentrations of phenacetin (0–837 μM) and with individual microsomal samples at a single phenacetin concentration (100 μM). In both assays, phenacetin was incubated with liver microsomes (0.25 mg mL^{-1}), as described previously for man (von Moltke et al 1996; Venkatakrishnan et al 1998). Samples containing phenacetin were subjected to similar conditions as described above for chlorzoxazone hydroxylation. However, for phenacetin, 2-acetamidophenol (2-AAP) was used as an internal standard. Incubation times and microsomal protein concentrations were conducted within a linear range (data not shown). Increasing quantities of acetaminophen (0–62.5 nmol) were used to

construct a calibration curve for determination of the product formation kinetics of paracetamol.

Substrate biotransformation using rat cDNA-expressed CYPs

The role of specific CYPs in the biotransformation of phenacetin (100 μM) and chlorzoxazone (300 μM) was evaluated using rat cDNA-expressed CYPs. Substrates were incubated with one of ten CYPs (CYP1A1, -1A2, -2B1, -2C6, -2C11, -2C12, -2C13, -3A1 or -3A2 at 50 pmol cytochrome P450 mL^{-1} or CYP2E1 at 1 mg mL^{-1}; BD Gentest, Woburn, MA) or a vector control (1 mg mL^{-1}; from Hi5 insect cell line or AHH-1 TK+/− human lymphoblastoid cell line). All rat CYP enzymes, except for CYP2E1, were transfected into Hi5 insect cells and are referred to as Supersomes by their manufacturer. Rat CYP2E1 was transfected into a human B lymphoblastoid cell line. Each CYP from the Hi5 insect cell line was co-transfected with a P450 reductase (rat or human) and human cytochrome b_5 except for CYP1A1 and CYP1A2, which were co-transfected with rat P450 reductase alone. For phenacetin biotransformation, CYP2E1 was added at 0.93 mg mL^{-1}, while the remaining CYPs were added at 46.9 pmol mL^{-1}.

Incubations were conducted in the same manner as described above. Samples containing rat liver microsomes were analysed in parallel. In addition, several negative controls were evaluated, including samples in which the substrate, the cofactor system or microsomes were omitted.

HPLC analysis

Data was collected using an HPLC system (Waters Associates, Milford, MA) with the following components: a 700-series Satellite Wisp autoinjector, a 486 absorbance detector and a 500-series pump and a 30 cm × 3.9 mm reverse-phase C_{18} MicroBondaPak column.

For chromatographic separation of samples containing phenacetin, paracetamol and 2-AAP, incubations were subjected to a mobile phase of 50 mM $KH_2PO_4 - CH_3CN$ (85:15) at a flow rate of 1.5 mL min^{-1}. Constituents were detected at a wavelength of 254 nm. Samples containing chlorzoxazone, 6-OH-CLZ and phenacetin were analysed using a mobile phase of 50 mM $KH_2PO_4 - CH_3CN$ (75:25) at a flow rate of 1.8 mL min^{-1}. Samples were analysed at 295 nm.

Data analysis

Formation of paracetamol was fit to a single-enzyme Michaelis–Menten model using nonlinear regression:

$$V = V_{max}S/(K_m + S) \quad (1)$$

where V is the reaction velocity, S is the substrate concentration, V_{max} is the maximum reaction velocity and K_m is the substrate concentration corresponding to 50% V_{max}. Chlorzoxazone samples were only analysed at a single concentration of chlorzoxazone and thus were not evaluated by Michaelis–Menten kinetics.

Statistical analysis

Age-related differences were compared using a one-way analysis of variance. Student–Newman–Keul's multiple comparison tests were used to evaluate differences between individual age groups. For both statistical tests, an alpha value was set at $P = 0.05$.

Results

The biotransformation of phenacetin was largely mediated by CYP2C6 and CYP1A isoforms, while chlorzoxazone hydroxylation was largely mediated by CYP2E1 and CYP1A1 (Figure 1). While paracetamol formation by CYP2C6 and CYP1A isoforms greatly exceeded that observed in rat liver microsomes, 6-OH-CLZ formation was similar for both cDNA-expressed CYP2E1 and CYP1A1 as observed for rat liver microsomes.

Phenacetin biotransformation was best approximated by a single-enzyme Michaelis–Menten model in all age groups (Figure 2A). However, age-related differences were apparent in the rate of phenacetin biotransformation across all ages (analysis of variance, $P < 0.001$; Figure 2B). Age-related differences were apparent in intrinsic clearance (Cl_{int}, equal to V_{max}/K_m), V_{max} and K_m values; however, differences in K_m values did not reach statistical significance (Table 1; analysis of variance, $P = 0.06$). Similar age-related trends were apparent when values were expressed per gram of liver or for total liver (Table 1).

Age-related differences were detected in the biotransformation of chlorzoxazone (analysis of variance, $P < 0.001$, Figure 3). Age-related differences were found between all age groups (Student–Newman–Keuls, $P < 0.05$). The old livers displayed the greatest degree of variability.

Discussion

The biotransformation of phenacetin by rat cDNA-expressed CYPs suggests that the formation of paracetamol is largely mediated by CYP1A isoforms and, to a lesser degree, by CYP2C6. These findings are consistent with a recent study, which had examined phenacetin to a more limited extent (Kobayashi et al 2002). Immunoinhibitory antibodies had previously suggested that CYP1A2 contributed to the formation of paracetamol (Sesardic et al 1990). However, since hepatic CYP2C6 expression may be 7- to 8-fold greater than CYP1A expression in the rat (Imaoka et al 1990), CYP2C6 may also contribute significantly to the biotransformation of phenacetin in this model. The role of multiple enzymes in phenacetin *O*-deethylation is consistent with previous studies in rat and human liver microsomes in which a low-affinity component was identified (Boobis et al 1981; von Moltke et al 1996; Venkatakrishnan et al 1998, 1999). Venkatakrishnan et al (1999) found that the low-affinity enzyme in man was also a CYP2C isoform.

The metabolite formation rates for chlorzoxazone are similar to those observed previously in the rat (Court et al

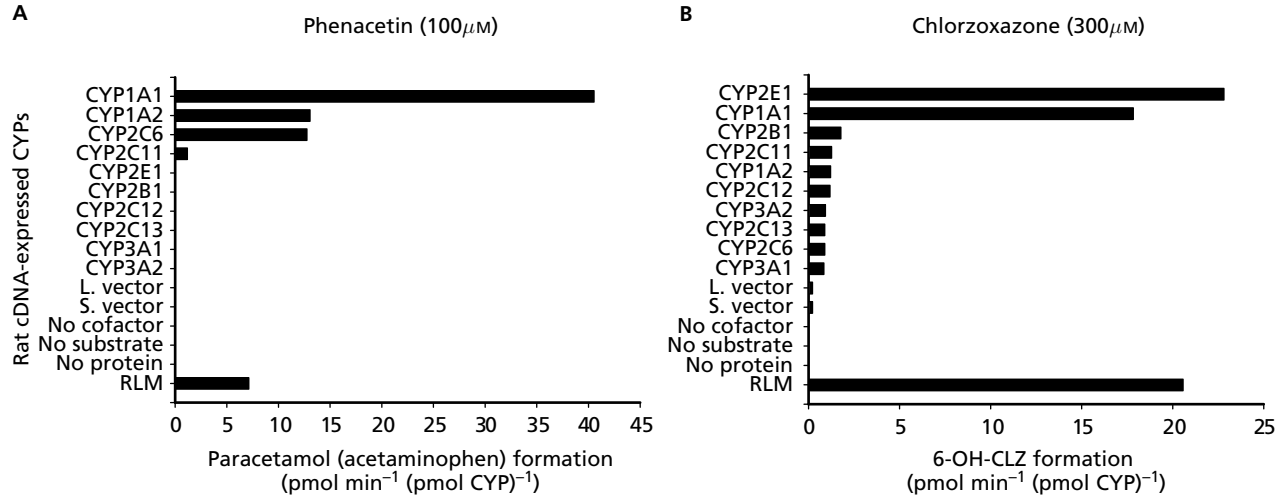

Figure 1 Phenacetin and chlorzoxazone biotransformation using rat cDNA-expressed CYPs. Phenacetin 100 μM (A) or chlorzoxazone 300 μM (B) were incubated with a variety of CYPs (CYP1A1, -1A2, -2B1, -2C6, -2C11, -2C12, -2C13, -2E1, -3A1, or -3A2) for 20 min at 37°C. As negative controls, lymphoblastoid vector (L. vector), supersomes vector (S. vector) and samples containing no cofactor, no substrate or no protein were included. Samples containing rat liver microsomes (RLM) served as positive controls and values are expressed as the mean of duplicate samples.

1997). Previous studies in our laboratory identified a biphasic kinetic profile for chlorzoxazone biotransformation using rat liver microsomes, which suggested the involvement of multiple isoforms (Court et al 1997). Consistent with this and other findings (Kobayashi et al 2002), we found that formation of 6-OH-chlorzoxazone is predominantly mediated by two isoforms in the CYP2E and CYP1A subfamilies. Although this study has identified the primary involvement of CYP2E1 and CYP1A1 isoforms at 300 μM chlorzoxazone, Kobayashi et al (2002) demonstrated the predominant involvement of CYP2E1 and CYP1A2 isoforms at 20 μM chlorzoxazone. Both studies identified a minor involvement of CYP2C and CYP3A family members in the formation of 6-OH-CLZ. Chemical and antibody inhibition may help to determine the relative contribution of CYP2E1 and CYP1A isoforms more fully in the rat. Using human cDNA-expressed CYPs, Gorski et al (1997) demonstrated that CYP2E1, CYP3A4 and CYP2D6 enzymes may participate in the biotransformation of chlorzoxazone in man.

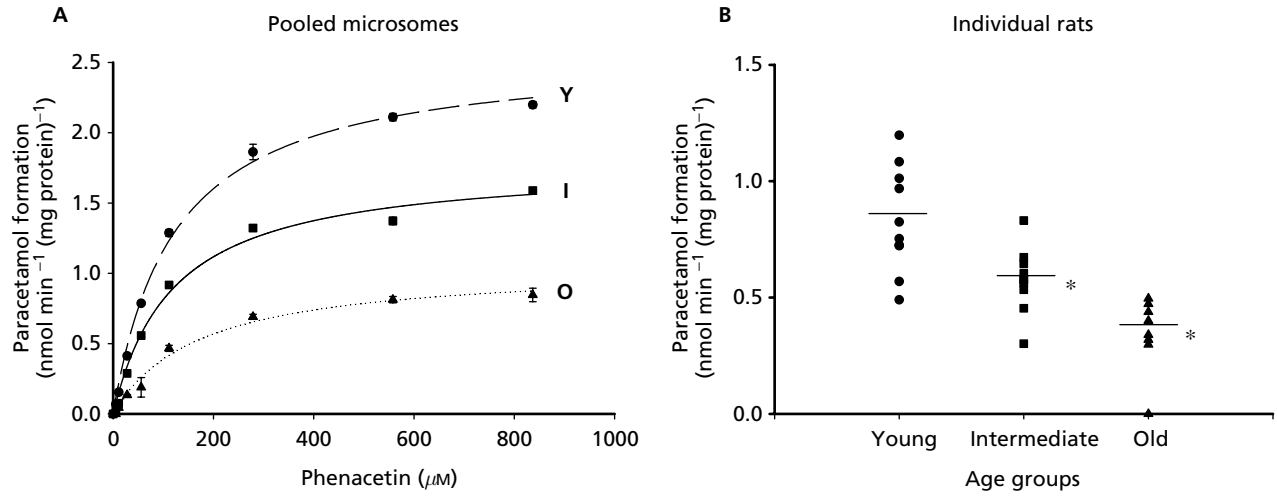

Figure 2 Paracetamol (acetaminophen) formation in rat liver microsomes from young (3–4 months), intermediate (13–14 months) and old rats (25–26 months). A. Increasing concentrations of phenacetin (0–837 μM) were incubated with pooled hepatic microsomes from young (pooled from 10 rats; circles and dashed line), intermediate (pooled from 10 rats; squares and solid line) and old (pooled from 8 rats; triangles and dotted line) rats. Samples were performed in triplicate and values represent the mean ± s.e. B. Phenacetin (100 μM) was incubated with hepatic microsomes from individual rats from each of the age groups (young, circles, n = 10; intermediate, squares, n = 10; old, triangles, n = 8). Lines represent the mean values for each group. *$P < 0.001$ (analysis of variance) and $P < 0.05$ (Student–Newman–Keuls test), difference among all age groups.

Table 1 Kinetic parameters for the biotransformation of phenacetin.

Kinetic parameters	Young (3–4 months, n = 3)	Intermediate (13–14 months, n = 3)	Old (25–26 months, n = 3)	Value of F from analysis of variance
V_{max}[a]	2.6 ± 0.2	1.9 ± 0.1	1.10 ± 0.04	111, $P < 0.001$
K_m	121.8 ± 2.8	129.8 ± 10.9	172.2 ± 18.0	4.9, NS
Cl_{int}[a]	21.2 ± 0.5	14.6 ± 0.4	6.2 ± 0.5	263, $P < 0.001$
Cl_{int} per mg liver[b]	0.4 ± 0.1	0.30 ± 0.01	0.10 ± 0.01	20, $P < 0.05$
Cl_{int} per total liver/1000[c]	4.5 ± 0.4	3.3 ± 0.1	1.6 ± 0.1	13.4, $P < 0.05$

Kinetic parameters were determined for assays that used pooled microsomes. Samples were performed in triplicate. NS, not significant; V_{max} values are expressed as nmol min^{-1} (mg protein)$^{-1}$; K_m values are expressed in μM; Cl_{int}, intrinsic clearance (1000 V_{max}/K_m) is expressed as L min^{-1} (mg protein)$^{-1}$. [a]A difference was detected between all age groups (analysis of variance, $P < 0.001$; Student–Newman–Keuls test, $P < 0.05$). [b]A difference was detected between all age groups (analysis of variance, $P < 0.05$; Student–Newman–Keuls test, $P < 0.05$). [c]A difference was detected between the old group and the other two age groups (analysis of variance, $P < 0.05$, Student–Newman–Keuls test, $P < 0.05$).

Chemical and antibody inhibition assays in their study further confirmed the role of CYP3A enzymes in the formation of 6-OH-CLZ.

For chlorzoxazone biotransformation, the estimation of the relative contribution of CYP1A1 and CYP2E1 is complicated by the fact that these two CYPs are heterologously expressed in different vector systems that contain different enzyme–coenzyme ratios. For example, while CYP1A1 was expressed in a B-lymphoblastoid cell line, CYP2E1 was expressed in a Supersomes vector (from a Hi5 insect cell line). The underlying vector system may alter the activity in this in-vitro system.

We observed a reduction in both phenacetin and chlorzoxazone biotransformation with age. These findings are similar to those observed for other putative CYP1A and CYP2E substrates (Wynne et al 1987; Horbach et al 1992). Imaoka et al (1991) observed a small reduction in CYP2E1 expression by western blot analysis. However, these authors and others (Horbach et al 1992) did not observe a change in the expression of CYP1A isoforms with age. In addition, mRNA expression of CYP1A1 and CYP1A2 in untreated rats did not change with age (Horbach et al 1990).

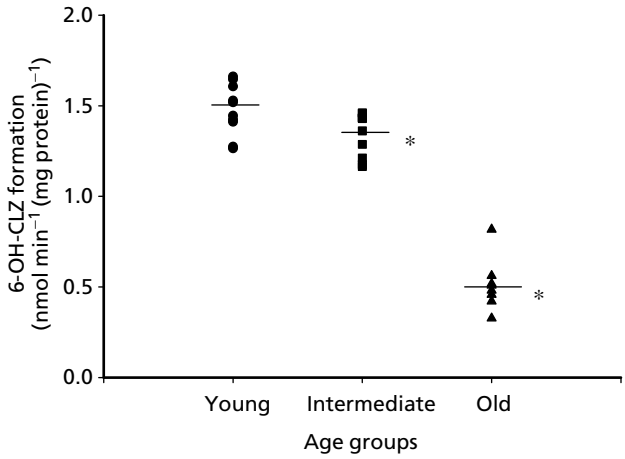

Figure 3 6-OH-CLZ formation in rat liver microsomes from young (3–4 months), intermediate (13–14 months) and old rats (25–26 months). Chlorzoxazone (300 μM) was incubated with hepatic microsomes from young (circles; n = 10), intermediate (squares; n = 10) and old (triangles; n = 8) rats. Lines represent the mean values for each group. *$P < 0.001$ (analysis of variance) and $P < 0.05$ (Student–Newman–Keuls) vs young rat microsomes.

Age-related decrements observed for phenacetin and chlorzoxazone biotransformation are of a similar magnitude to that previously reported for triazolam biotransformation, which is largely mediated by CYP3A and CYP2C enzymes (Warrington et al 2003). However, the effect of age on sildenafil biotransformation, a marker for CYP2C11 activity (Warrington et al 2002, 2003), is more marked than that observed in this study. These findings are consistent with previous studies in which the relative age-related decrements were found to be substrate specific (Rikans & Notley 1982). It is likely that the differential expression of specific CYPs with age contributes to differences among specific substrates (Rikans & Notley 1982).

Animal models are an important tool for the study of drug metabolism and drug toxicology. Yet, surprisingly little is known about the similarity between rodent models and man in substrate specificity and the role of particular CYP enzymes in their biotransformation (Court et al 1997; Bogaards et al 2000). In this study, we have characterized the role of specific CYPs in phenacetin and chlorzoxazone biotransformation in the male rat. As the role of particular CYPs in the biotransformation of specific substrates is elucidated in the rat, it will become increasingly possible to evaluate age-related differences in other CYPs in the rat liver.

Conclusion

In conclusion, our results indicate that age-related differences in phenacetin and chlorzoxazone biotransformation are apparent in the male rat liver. However, these age-related decrements are of lesser magnitude than previously

identified age-related changes in other enzymatic reactions (Warrington et al 2003, 2004). Since phenacetin *O*-deethylation is largely mediated by CYP1A isoforms and chlorzoxazone biotransformation may be predominantly mediated by CYP2E1 and CYP1A1 enzymes, age-related differences in these reactions may reflect age-related changes in CYP2E and CYP1A activity or expression.

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