Age-Related Differences in CYP3A Expression and Activity in the Rat Liver, Intestine, and Kidney

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ABSTRACT

We evaluated the effect of age on CYP3A expression and function in the liver, intestine, and kidney from young (3–4 months), intermediate (13–14 months), and old (25–26 months) male Fischer-344 rats. The biotransformation of triazolam to its primary hydroxylated products, 4-OH-TRZ (triazolam) and α-OH-TRZ, was used as a marker of CYP3A activity in rat liver and intestine. Immunoactive CYP3A expression was evaluated by Western blot analysis in the rat intestine, liver, and kidney. Since testosterone and NADPH reductase expression may modulate CYP3A activity, we also examined free plasma testosterone concentrations and NADPH reductase expression in these rats. The effect of age on CYP3A expression was tissue-specific. Although both CYP3A activity and expression were reduced by approximately 50 to 70% in the old livers compared with the young animals, intestinal CYP3A activity and expression did not change significantly with age. The expression of one CYP3A isozyme was increased by 1.5-fold in the old kidneys. NADPH reductase expression was reduced by 23 to 36% with age in all tissues; this reached statistical significance only in the liver. Plasma testosterone levels declined by 74% in the old animals. This study suggests that the effect of age on CYP3A expression and function is tissue-specific. In addition, changes in testosterone levels and NADPH reductase expression may contribute to age-related differences in hepatic CYP3A activity.

Many studies have noted an increased prevalence of adverse drug reactions in the elderly population (Greenblatt et al., 1982; Le Couteur and McLean, 1998). As the proportion of those over the age of 65 years increases in the U.S. population (Administration on Aging, Health and Human Services, 2001, www.aoa.gov/aoa/STATS/profile/2001/1.html), an understanding of the frequency and etiology of adverse reactions in older individuals is becoming increasingly important. Multiple studies have suggested that pharmacokinetic changes with age may contribute to the increase in these adverse effects in the elderly. In particular, many studies have demonstrated a reduced clearance of CYP3A substrates in the elderly (Greenblatt et al., 1983a,b, 1984, 1991; Holazo et al., 1988; Miglioli et al., 1990; Cotreau et al., 2004). However, in some studies, significant age-related changes in clearance have not been observed (Hunt et al., 1992b; Gorski et al., 2003).

Age-related changes in the pharmacokinetics of CYP3A substrates are likely to be multifactorial. Possible contributing causes include reduced expression of hepatic or enteric CYP3A protein, reduction in liver mass, and possibly reduction in hepatic blood flow (Kinirons and Crome, 1997; Schmucker, 1998; Cotreau et al., 2004). CYP3A function in vivo also may be influenced by factors such as disease states, concurrent medications, diet, smoking, and genetics. Several investigators have evaluated the effect of age on hepatic CYP3A expression and function in vitro (Schmucker et al., 1990; Hunt et al., 1992a; Shimada et al., 1994; George et al., 1995; Transon et al., 1996; Patki et al., 2004). Outcomes of these studies have been inconsistent, and a variety of limitations exist for this type of analysis, such as tissue sources and harvesting protocols (Warrington et al., 2000). Rodent models have more consistently displayed a reduction in the clearance of CYP3A substrates with age in vivo (Barnhill et al., 1990) and in vitro (Warrington et al., 2000). However, many studies of age in rodent models were conducted before the availability of specific immunoinhibitory antibodies and index substrates for rodent P450s. These early studies were thus limited in their ability to determine the involvement of individual P450 enzymes in specific reactions. Recent studies have suggested that triazolam biotransformation may be

ABBREVIATIONS: P450, cytochrome P450; TRZ, triazolam; OH, hydroxy; PMSF, phenylmethylsulfonyl chloride; HPLC, high-performance liquid chromatography; V<sub>max</sub>, maximal reaction velocity; K<sub>m</sub>, substrate concentration at 50% V<sub>max</sub>; CL<sub>int</sub>, intrinsic clearance; TBS, Tris-buffered saline; ANOVA, analysis of variance.
largely mediated by CYP3A isoforms in rodents (Perloff et al., 2000). Thus, this reaction may be useful for evaluating age-related changes in CYP3A activity.

The in vitro studies discussed above have been limited to hepatic CYP3A. Although CYP3A is most predominantly expressed in the liver, the importance of enteric CYP3A activity is increasingly recognized (Thummel and Wilkinson, 1998; Tsunoda et al., 1999). Differences in enteric CYP3A activity are likely to contribute, in part, to the interindividual variability observed in the clearance of the same CYP3A substrates after oral dosage. The effect of age on enteric CYP3A expression and activity is not well established. To our knowledge, only one study has examined the effect of age on intestinal CYP3A expression and found no change with age (Lown et al., 1997). However, age differences were not the primary focus of this study, and few older subjects were included.

Using Western blot analysis to evaluate CYP3A expression and triazolam biotransformation as a marker of CYP3A activity, we examined age-related changes in CYP3A expression and activity in the liver, intestine, and kidney of male Fischer-344 rats. Since testosterone (Mäenpää et al., 1998; Schrag and Wienkers, 2001; Patki et al., 2003) and the accessory protein NADPH reductase (Venkatakrishnan et al., 2000, 2001) have been shown to modulate CYP3A activity, we also examined the effect of age on plasma testosterone concentrations and the expression of NADPH reductase in the liver, intestine, and kidney.

Materials and Methods

Rats. The National Institute on Aging supplied 32 male Fischer-344 rats from three age groups (held at Harlan, Indianapolis, IN: young, 2–3 months, n = 10; intermediate, 12–13 months, n = 10; old, 24–25 months, n = 12) (Warrington et al., 2003). Four old rats were removed from the study due to the development of infection (n = 1), superficial gross tumors (n = 2), or hepatomegaly (n = 1). Rats were housed in the animal facilities at Tufts University School of Medicine for an additional 1 to 2 months in a 12 h light/dark cycle. Food and water were provided ad libitum. Animals were sacrificed by decapitation. Livers and kidneys were stored on dry ice for ~30 min and then stored at ~80°C until microsomal preparation. Intestinal microsomes were prepared directly after sacrifice.

Reagents. Triazolam was purchased from Sigma-Aldrich Laboratories (St. Louis, MO), and its metabolites were kindly provided by Pharmacia and Upjohn Co. (Kalamazoo, MI). Antibodies, rat cDNA-expressed enzymes, and other reagents were purchased from commercial sources.

Microsomal Preparations

Intestinal Microsomes. Intestinal microsomes were prepared as previously described (Cotreau et al., 2000, 2001; Kotegawa et al., 2002). Briefly, enterocytes were isolated from the proximal portion (35 cm) of the rat small intestine using four solutions, designated as A, B, C, and D. First, the intestinal segment was washed with solution A [1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH2PO4, 5.6 mM Na2HPO4, and 40 μM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. Cells were then eluted from the intestinal wall using solution B (phosphate-buffered saline without calcium and magnesium, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 40 μM PMSF, pH 7.4) and were washed twice in solution C (5 mM histidine, 0.25 M sucrose, 0.5 mM Na2EDTA, and 40 μM PMSF, pH 7.4). Samples were homogenized using a loose-fitting Dounce homogenizer. Samples were then centrifuged for 10 min at 15,000g, and the supernatants were removed and placed on ice. The pellet was resuspended in solution C and centrifuged at 15,000 g for an additional 10 min. The second supernatant was added to the first and 2.5 ml CaCl2 was added. After a 20-min incubation on ice, samples were centrifuged at 25,000 g for 10 min, and pellets were resuspended in 0.5 ml of 80% 0.1 M potassium phosphate buffer with solution D (20% glycerol). Microsomes were stored at ~80°C until use.

Hepatic and Renal Microsomes. Hepatic and renal microsomes were prepared by differential centrifugation, as previously described (Lake, 1987; Warrington et al., 2000). Briefly, approximately one lobe from the liver and one kidney from each rat were homogenized in a 0.5 M potassium phosphate buffer containing 0.15 M KCl and 0.25 M sucrose. Samples were centrifuged at 10,000 rpm for 22 min at 4°C. Supernatants were then centrifuged for an additional 70 min at 33,000 rpm at 4°C. The pellets were then homogenized in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at ~80°C until used. For all microsomal preparations, protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) with albumin as the protein standard.

In Vitro Biotransformation of Triazolam

Triazolam hydroxylation was examined in intestinal and hepatic microsomes (von Moltke et al., 1996; Perloff et al., 2000). Increasing concentrations of triazolam (0–750 μM) were incubated with microsomes (0.25 and 0.1 mg/ml for liver and intestine microsomes, respectively) in the presence of a NADPH-regenerating cofactor system (6.3 mM MgCl2, 0.5 U of isocitric dehydrogenase, 3.8 mM isocitric acid, and 0.5 mM NADP+ in 0.05 M potassium phosphate, pH 7.4). Incubations were terminated with acetonitrile (2/5 vol) and an internal standard (1 μg of phenacetin) was added. Samples were centrifuged and transferred to autosampling vials for high-performance liquid chromatography (HPLC) analysis. A standard curve of varying known quantities of the α-OH and 4-OH metabolites was constructed for quantification of metabolite formation. Time and protein linearity studies were performed using hepatic microsomes, and incubations were conducted in a linear range. Negative controls without cofactor, protein, or substrate were incubated in parallel.

Chemical and Antibody Inhibition of Triazolam Hydroxylation

Ketoconazole and immunoinhibitory antibodies were used to evaluate the role of CYP3A in the biotransformation of triazolam in the aging male rat. The human CYP3A inhibitor, ketoconazole (2.5 μM), was incubated with TRZ (250 μM) in the presence of either hepatic (0.25 mg/ml for TRZ) or intestinal (0.1 mg/ml for TRZ) microsomes. Reactions were performed in duplicate and as described above. The samples were then analyzed by HPLC.

For immunoinhibition studies, microsomes (10 μg) were preincubated with TRZ at 37°C for 30 min in the presence of either an anti-rat P450 antibody (100 μg; CYP1A1/2, CYP2B1/2, CYP2C11, CYP2E1, and CYP3A1/2; BD Gentest, Woburn, MA), an appropriate serum control (rabbit or goat, 100 μg), or 50 mM potassium phosphate buffer. An NADPH-regenerating system was added and samples were incubated as described above. As negative controls, serum controls and samples containing 50 mM potassium phosphate buffer were included. TRZ was incubated with rat hepatic microsomes as a positive control. Reactions were subjected to HPLC analysis.

HPLC Analysis

Samples were analyzed by HPLC, as previously described (von Moltke et al., 1996; Perloff et al., 2000). Triazolam, phenacetin, α-, and 4-hydroxylated products were separated using a stainless steel 15 cm × 3.9 mm reverse phase C18 NovaPak column (Waters Associates, Milford, MA) with a mobile phase of 70% 10 mM potassium phosphate buffer, 20% acetonitrile, and 10% methanol at a flow rate of 1.4 ml/min. The column effluent was analyzed at a wavelength of 220 nm.
Data Analysis of TRZ Hydroxylation

For both reactions, chromatographic peak height ratios were generated. These ratios were converted into reaction velocities (nanomoles per minute per milligram of protein) using a standard curve of known, increasing concentrations of metabolites subjected to the same incubation conditions. Metabolite formation rates for hepatic and intestinal incubations were fit to appropriate kinetic models by nonlinear regression. A single-enzyme Michaelis-Menten kinetic model was used for the formation of both metabolites in the intestine, 4-OH-TRZ formation in rat liver for all age groups, and for α-OH-TRZ formation in the old rat liver:

\[ V = \frac{V_{\text{max}} S}{K_m + S} \]  
\[ V = \frac{V_{\text{max}} S}{K_m + S} + zS \]

in which \( V \) represents the reaction velocity; \( S \) represents the substrate concentration, \( V_{\text{max}} \) denotes the maximal reaction velocity, and \( K_m \) represents the substrate concentration at which 50% \( V_{\text{max}} \) is achieved.

Formation of α-OH-TRZ in livers of the young and intermediate rats was fit to a single-enzyme Michaelis-Menten model with a second low-affinity component:

\[ V = \frac{V_{\text{max}} S}{K_m + S} + zS \]

where \( z \) represents the slope of the second low-affinity enzymatic component, approximated by a linear function, and the other variables are as previously defined.

The intrinsic clearance (CLint) for each pathway was defined as:

\[ \text{CL}_{\text{int}} = \frac{V_{\text{max}}}{K_m} \]

Net intrinsic clearance represents the sum of the intrinsic clearance values for the two pathways.

### Table 1

Age-related changes in TRZ biotransformation in male rat hepatic and intestinal microsomes

Values are expressed as mean ± S.E. \( V_{\text{max}} \) is expressed as nanomoles per minute per milligram of protein; \( K_m \) is expressed in micromolar concentration, and CLint is intrinsic clearance ([Vmax/Km] · 1000). No significant age-related differences were found for intestinal samples.

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Young (n = 10)</th>
<th>Intermediate (n = 10)</th>
<th>Old (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatic microsomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-OH-TRZ</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>( V_{\text{max}} )</td>
<td>0.29 ± 0.010</td>
<td>0.22 ± 0.014</td>
<td>0.12 ± 0.018</td>
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<tr>
<td>( K_m )</td>
<td>27.36 ± 2.35</td>
<td>20.97 ± 2.06</td>
<td>10.06 ± 1.72</td>
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<tr>
<td>CLint</td>
<td>11.44 ± 0.97</td>
<td>10.86 ± 0.45</td>
<td>13.68 ± 2.10</td>
</tr>
<tr>
<td>Percentage of net CLint</td>
<td>19%</td>
<td>22%</td>
<td>46%</td>
</tr>
<tr>
<td><strong>4-OH-TRZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>0.97 ± 0.043</td>
<td>0.79 ± 0.062</td>
<td>0.25 ± 0.095</td>
</tr>
<tr>
<td>( K_m )</td>
<td>21.08 ± 1.37</td>
<td>20.51 ± 1.29</td>
<td>16.27 ± 2.35</td>
</tr>
<tr>
<td>CLint</td>
<td>47.26 ± 2.64</td>
<td>39.51 ± 1.96</td>
<td>15.99 ± 4.79</td>
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<tr>
<td>Percentage of net CLint</td>
<td>81%</td>
<td>78%</td>
<td>54%</td>
</tr>
<tr>
<td>Net CLint</td>
<td>58.70 ± 2.37</td>
<td>49.37 ± 2.11</td>
<td>29.67 ± 5.05</td>
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<tr>
<td><strong>Intestinal microsomes</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>α-OH-TRZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>0.28 ± 0.031</td>
<td>0.25 ± 0.032</td>
<td>0.26 ± 0.059</td>
</tr>
<tr>
<td>( K_m )</td>
<td>147 ± 30</td>
<td>142 ± 14</td>
<td>134 ± 20</td>
</tr>
<tr>
<td>CLint</td>
<td>2.27 ± 0.27</td>
<td>1.97 ± 0.37</td>
<td>2.21 ± 0.50</td>
</tr>
<tr>
<td>Percentage of net CLint</td>
<td>36%</td>
<td>35%</td>
<td>35%</td>
</tr>
<tr>
<td><strong>4-OH-TRZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>0.69 ± 0.057</td>
<td>0.49 ± 0.061</td>
<td>0.49 ± 0.101</td>
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<tr>
<td>( K_m )</td>
<td>173 ± 36</td>
<td>154 ± 14</td>
<td>160 ± 37</td>
</tr>
<tr>
<td>CLint</td>
<td>4.08 ± 0.56</td>
<td>3.62 ± 0.73</td>
<td>3.94 ± 1.00</td>
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<tr>
<td>Percentage of net CLint</td>
<td>64%</td>
<td>65%</td>
<td>64%</td>
</tr>
<tr>
<td>Net CLint</td>
<td>6.35 ± 0.82</td>
<td>5.59 ± 1.10</td>
<td>6.15 ± 1.49</td>
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</table>

Western Blot Analysis of CYP3A Expression in Rat

Hepatic (0.94–8.0 μg), intestinal (1.3–12.3 μg), and renal (50–98 μg) microsomal protein was evaluated for CYP3A expression, as described previously for hepatic and intestinal microsomes (Cotreau et al., 2000, 2001). Briefly, samples were loaded onto a 7.5% polyacrylamide gel alongside tissue-specific standards and separated by exposure to 120 V for 1.5 h using a running buffer (0.19 M glycine, 250 mM Tris-base, and 0.1% SDS). Protein was transferred onto a nitrocellulose membrane at 100 V for 1 h at 4 °C using a transfer buffer (0.19 M glycine, 250 mM Tris-base, and 20% methanol). After a 1-min exposure to gel fixation buffer (50% methanol, 10% glacial acetic acid), membranes were washed with 1× TBS-Tween 20 (0.15 M NaCl, 40 mM Tris-HCl, 40 mM Tris-base, and 0.06% Tween 20). After incubation with 3% Blotto (dry nonfat milk in 1× TBS-Tween 20) for 1 h at 25 °C, membranes were probed with an anti-rat CYP3A1/2 antibody (1:3000; Xenotech, Kansas City, KS) for 16 to 18 h at 4 °C and 1 h at 25 °C. Membranes were washed with 1× TBS-Tween 20, incubated with 3% Blotto for 30 min at 25 °C, and reprobed with a donkey anti-rabbit secondary antibody coupled to horseradish peroxidase (Pierce Chemical Co.) for 1 h at 25 °C. The membranes were then exposed to a chemiluminescent substrate for detection and analyzed using a Kodak imager and Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

### NADPH Expression Analysis in Rat Microsomal Preparations

NADPH reductase expression was also evaluated in these tissues. Membranes that were probed with an anti-rat CYP3A antibody were stripped and reprobed with an anti-rat NADPH reductase (1:3000; BD Gentest) and a donkey anti-goat secondary antibody that was linked to horseradish peroxidase (Pierce Chemical Co.) for 1 h at 25 °C. The membranes were then exposed to a chemiluminescent substrate for detection and analyzed using a Kodak imager and Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).
glycine, 0.1% SDS, and 1% Tween 20, pH 2.2) for 1 h and washed thoroughly with double-distilled H₂O and 1× TBS-Tween 20 before reprobing. If the net intensity of the bands probed with the NADPH reductase antibody were outside the range of the standard curve, samples were reloaded at lower protein concentrations and subjected to the same conditions described above for CYP3A expression analysis.

Data Analysis for Immunoblotting

Using Kodak 1D software, the relative quantities of CYP3A or NADPH reductase content were assessed by the band's net intensity, the pixels × area – background at the perimeter. These values were compared with those determined for the tissue-specific microsomal protein standards, which represent serially diluted microsomal protein and loaded alongside of the samples. Standard curves in which the net intensity of the standard versus the amount of microsomal protein were generated. Data points were fit to either $y = mx$ or $y = mx^a$ in which $x$ represents the microsomal protein amount loaded, $y$ reflects the net intensity of each band, and $A$ is an exponent.

Plasma Concentrations of Testosterone

Trunk blood was collected into heparinized tubes and stored at −20°C. Samples (∼1 ml; $n = 10$ for young and intermediate, $n = 7$ for old) were evaluated for free testosterone concentrations by immunoassay (Olubodun et al., 2003).

Results

In Vitro Hydroxylation of Triazolam in the Rat Liver. Both 4-OH-TRZ and α-OH-TRZ were formed by rat liver microsomes. Metabolite formation rates declined with age for both metabolites (Table 1; Fig. 1, A and B). Age-related reductions in $V_{\text{max}}$ values were greater for the production of 4-OH-TRZ; $V_{\text{max}}$ values were reduced by 74% in old compared with young animals, whereas formation of α-OH-TRZ was reduced by 59% in the old animals.

$K_m$ values for 4-OH-TRZ formation did not differ significantly with age, whereas $K_m$ values were reduced with age for the α-OH-TRZ pathway. Although $CL_{\text{rat}}$ values declined with age for 4-OH-TRZ formation, $CL_{\text{rat}}$ for α-OH-TRZ did not change with age.

The relative contribution of each pathway to the net $CL_{\text{rat}}$ changed with age. Whereas 4-OH-TRZ formation was the predominant pathway in the young livers, both metabolites had similar relative $CL_{\text{rat}}$ values in the old livers. Normalization of the net $CL_{\text{rat}}$ per milligram of liver or the total liver weight had little impact on age-related trends.

In Vitro Hydroxylation of Triazolam in the Rat Intestine. Both hydroxylated products of TRZ biotransforma-
tion were produced by rat intestinal microsomes. Metabolite formation for both α-OH and 4-OH-TRZ was best fit to a single-enzyme Michaelis-Menten model.

The net Cl_in of TRZ in rat intestinal microsomes was 10% of that observed using rat liver microsomes. This reduced Cl_in was largely attributable to an increase in K_m values for both metabolites (Table 1). The 4-OH-TRZ metabolite pathway accounted for approximately two-thirds of net Cl_in regardless of age. Age-related changes in TRZ hydroxylation by intestinal microsomes were small, and not statistically significant (Fig. 1, Table 1).

Chemical and Antibody Inhibition of Triazolam Hydroxylation. Among five immunoinhibitory antibodies (polyclonal anti-rat CYP1A1/2, 2B1/2, 2C11, 2E1, and CYP3A1/2), only the anti-rat CYP3A1/2 antibody inhibited TRZ biotransformation in the liver and intestine (Fig. 2).

In liver microsomes, the anti-rat CYP3A1/2 antibody inhibited both 4-OH- and α-OH-TRZ formation in the old animals less than the other age groups (Student-Newman-Keuls, p < 0.05). Similar findings were observed at a higher antibody concentration (1:50, data not shown). In intestinal microsomes, the CYP3A1/2 antibody inhibited TRZ hydroxylation in all age groups to a similar degree for both metabolites, although some statistical differences were found (Fig. 2). For both hepatic and intestinal samples, the antibody did not fully inhibit either α-OH- or 4-OH-TRZ formation.

Ketoconazole inhibited metabolite formation in both the liver and intestine (Fig. 3). Age-related differences in chemical inhibition were observed in the liver (Kruskal-Wallis ANOVA, p < 0.05), but not in the intestine. Ketoconazole (2.5 μM) inhibited metabolite formation to a lesser degree in the old livers (Dunn's test, p < 0.05).

Western Blot Analysis of CYP3A Expression in Liver, Intestine, and Kidneys. The polyclonal anti-rat CYP3A1/2 antibody detected two bands in the rat liver, intestinal, and kidney samples (Figs. 4–6). Based on product information, these bands are likely to represent two CYP3A isoforms, CYP3A1 (the upper band) and CYP3A2 (the lower band). The relative amounts of these isoforms differed among the three tissues. Whereas CYP3A1 and -3A2 isoforms were expressed

![Fig. 2. Age-related differences in antibody inhibition of TRZ hydroxylation. A screen of five immunoinhibitory antibodies (anti-rat CYP3A1/2, 2E1, 1A1/2, 2B1/2, 2C11) and a 50 mM potassium phosphate buffer control (Buffer) were used to inhibit α-OH-TRZ (A and C) and 4-OH-TRZ (B and D) formation in hepatic (A and B) and intestinal (C and D) microsomes. Microsomes were pooled by age (black bars, young; white bars, intermediate; gray bars, old). Samples were performed in triplicate and expressed as mean ± S.E. * Student's t test detected a difference of the young samples from the buffer controls; †, difference among the age groups for inhibited samples was detected (ANOVA, p < 0.05), differences from the young are noted (Student-Newman-Keuls test, p < 0.05); ‡, reflects a difference among the age groups for inhibited samples (ANOVA, p < 0.001) and demarcates a difference from the young (Student-Newman-Keuls test, p < 0.05).]
in similar quantities in the liver, CYP3A2 was the predominant isoform in the kidney and the intestine. Also, although there was a strong correlation between these isoforms in the liver \( (r^2 = 0.68) \), there is only a weak correlation with the other two tissues \( (r^2 = 0.35 \text{ for the intestine and } r^2 = 0.23 \text{ for the kidney}) \).

After normalizing renal, intestinal, and hepatic CYP3A expression to a liver microsomal standard, we found that the liver expressed the highest levels of CYP3A. Intestinal CYP3A expression was approximately 25% of hepatic expression, whereas renal CYP3A was less than 1% of the liver.

The effect of age on CYP3A expression was tissue-dependent. In the rat livers, an age-related decline was observed in CYP3A1 (ANOVA, \( p < 0.005 \)) and CYP3A2 (ANOVA, \( p < 0.005 \)).

Fig. 3. Age-related differences in inhibition of TRZ (250 \( \mu \)M) biotransformation by ketoconazole. Production of \( \alpha \)-OH-TRZ (A and C) and 4-OH-TRZ (B and D) in hepatic (A and B) or intestinal (C and D) microsomes from young (Y; black bars, \( n = 10 \)), intermediate (I; white bars, \( n = 10 \)) and old (O; gray bars, \( n = 8 \)) rats was inhibited by ketoconazole (2.5 \( \mu \)M). Samples with inhibitor were compared with controls without inhibitor and values are expressed as mean \( \pm \) S.E. *, difference of the old livers from the young and intermediate groups (Kruskal-Wallis ANOVA, \( p < 0.05 \); Dunn’s test, \( p < 0.05 \)).

Fig. 4. Age-related differences in hepatic CYP3A1/2 expression (panel A). CYP3A1 and CYP3A2 were determined by Western blot analysis (panel B). Mean values of young (black bars, \( n = 10 \)), intermediate (white bars, \( n = 10 \)), and old (gray bars, \( n = 8 \)) samples are represented \( \pm \) S.E. The y-axis represents the net intensity (pixels \( \times \) area – background intensity at the perimeter of each band) relative to a standard curve. Young (Y), intermediate (I), and old (O) samples loaded were standardized to hepatic CYP3A activity, as measured by 4-OH-triazolam production. A Student-Newman-Keuls test and Dunn’s test indicated that the old livers differ statistically from the young and intermediate age groups (*, \( p < 0.05 \)).
A) Intestinal CYP3A expression

B) Representative western blot

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No statistical differences in net CYP3A expression were found among the three age groups in the intestine (Fig. 5) or the kidney (Fig. 6). A Student-Newman-Keuls test detected a statistical difference between the intermediate and old age groups in renal CYP3A1 expression ($p < 0.05$). However, no difference was found with age in renal CYP3A2 expression.

Comparison of TRZ Hydroxylation and CYP3A Expression in Liver and Intestine. Hepatic CYP3A expression and TRZ biotransformation demonstrated a similar decline with age. For example, the net intrinsic clearance of TRZ was reduced, on average, by 49% in the old livers in comparison to the young, whereas the net CYP3A expression
declined by 67% in the old livers. However, a correlation analysis between hepatic net intrinsic clearance of TRZ biotransformation and net CYP3A expression demonstrated only a weak correlation \( (r^2 = 0.35) \). A comparison of intestinal CYP3A activity and expression demonstrated no correlation \( (r^2 = 0.10) \) and no corresponding decline with age.

**NADPH Reductase Expression in the Liver, Intestines, and Kidney.** In all three tissues, the anti-rat NADPH reductase antibody identified one band at \(-80 \text{ kDa}\), which is consistent with the molecular weight of NADPH reductase. In all three tissues, this antibody also detected a second unidentified band of lower intensity \((-100 \text{ kDa})\).

An age-related decline in NADPH reductase expression was detected in all three tissues (Fig. 7). However, these age-related trends did not reach statistical significance in the intestine (ANOVA, \( p = 0.091 \)) or kidney (ANOVA, \( p = 0.06 \)). Correlational analyses of hepatic, intestinal, and renal NADPH reductase expression demonstrated no significant correlation between NADPH reductase expression across tissues.

**Plasma Testosterone in Aging Rats.** Age-related differences were observed in free testosterone concentrations (Fig. 8; ANOVA, \( p < 0.05 \)). These differences reached statistical significance between the young and old groups (Student-Newman-Keuls, \( p < 0.05 \)) with or without inclusion of an outlying data point. The relative decrement in testosterone concentrations between young and old animals was similar to the relative decrement in \( V_{\max} \) for 4-OH-TRZ formation between young and old animals. However free testosterone concentrations were not correlated with hepatic CYP3A activity \( (r^2 = 0.20) \) or with NADPH reductase activity \( (r^2 = 0.04) \).

**Discussion**

Using TRZ hydroxylation as a marker of CYP3A function, we demonstrated an age-related decline in hepatic CYP3A activity in the male rat. The decrement in hepatic TRZ hydroxylation activity with age was evident primarily in the pathway leading to 4-OH-TRZ formation with a much smaller age effect on \( \alpha\)-OH-TRZ formation. Although a low-affinity enzyme component (eq. 2) was identified for hepatic \( \alpha\)-OH-TRZ formation in young and intermediate rats, \( \alpha\)-OH-TRZ formation in old animals was consistent with a single-enzyme Michaelis-Menten model. This finding, along with the age-related differences in susceptibility to chemical and antibody inhibition, suggests that the relative contribution of specific P450 isoforms may change with age. In any case, the value of \( z \) for the low-affinity component was much smaller than the \( V_{\max}/K_m \) value for the high-affinity component, indicating that the high-affinity process for \( \alpha\)-OH-TRZ formation accounted for essentially all of total intrinsic clearance via this pathway in the young and intermediate-aged animals.

The age-related difference in CYP3A activity paralleled changes in protein expression. For instance, a comparable decrement of \(-25\% \) to \(-40\% \) was observed in CYP3A expression and \( V_{\max} \) values for both TRZ metabolites in the old livers. Similar decrements in CYP3A expression and function have been observed previously in mice (Warrington et al., 2000) and humans (Greenblatt et al., 1980, 1983a,b, 1991; Miglioli et al., 1990; Cotreau et al., 2004). Although species differences have been observed between the male rat and humans, these similarities suggest that the study of age-related changes in the male rat may provide a framework for evalu-
Fig. 8. Free plasma testosterone concentrations in the three groups. Free testosterone levels (picogram per milliliter) were analyzed in young (circles, \( n = 10 \)), intermediate (squares, \( n = 10 \)), and old (triangles, \( n = 7 \)). Data points correspond to individual rats, whereas lines represent the mean values for the group.

At the regulatory mechanisms of age-related differences in human CYP3A expression and function. Although age-related decrements in hepatic CYP3A expression and activity were observed, no differences in enteric CYP3A expression, function, or net renal CYP3A expression were observed. To our knowledge, the effect of age on intestinal or renal CYP3A expression has not been previously examined in the aging rat. In one clinical study, intestinal CYP3A expression did not change with age in humans (Lown et al., 1997), although age was not the primary focus of this study. The inconsistent effect of age upon CYP3A expression and function suggest an independent regulation of CYP3A expression and function with age among different tissues.

Coadministration of TRZ with an immunoinhibitory anti-CYP3A antibody and the chemical inhibitor, ketoconazole, did not result in complete inhibition of TRZ hydroxylation. This suggests that TRZ hydroxylation is not a fully specific index reaction for CYP3A function in rats. Further study is warranted to elucidate the possible contribution of other P450 isoforms to triazolam biotransformation.

Age-related declines in NAPDH reductase expression were evident in all three tissues. These findings were consistent with a previous study of NAPDH reductase ontogeny in which Simmons and Kasper (1989) found age-related changes in mRNA levels in the liver, intestine, and kidney. Reduced hepatic NAPDH expression with age may contribute to the age-related decrements in CYP3A function; however, these changes would not contribute to lower levels of immunoreactive CYP3A protein.

We also noted a reduction in free testosterone levels in our aging rats. The reduction in free testosterone levels was similar to the degree of decline in the intrinsic clearance values of TRZ (−25% of the degree of the young). Alterations in testosterone levels may modulate CYP3A function, as suggested by previous studies (Fujita et al., 1985, 1990; Kamataki et al., 1985; Mäenpää et al., 1998; Schrag and Winkens, 2001; Patki et al., 2003). However, correlations between testosterone concentrations and CYP3A activity were weak, suggesting that testosterone is not the only modulator of P450 expression in relation to age. Also, since CYP3A activity and expression changes in the aging male rat are likely to be tissue-dependent, a change in systemic hormone levels alone is unlikely to be the sole cause of changes in P450 activity. This suggests that either local hormone levels or differences in the regulation of CYP3A at these various sites may contribute to CYP3A modulation. Evaluation of age-related differences in CYP3A expression, \( V_{\text{max}} \) values for triazolam hydroxylation, and NAPDH reductase expression in females may provide more insight into the role of hormonal regulation.

Further study of the regulation of CYP3A expression is central to the understanding of age-related differences in CYP3A activity, and examination into tissue-specific effects may help to elucidate factors that regulate CYP3A expression and function with age.

References


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