Age-related differences in CYP3A expression and activity in the rat liver, intestine and kidney

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Tissue-specific differences in CYP3A with age

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Nonstandard abbreviations: CYP, cytochrome P450; TRZ, triazolam; OH, hydroxy; PMSF, phenylmethylsulfonyl chloride; EDTA, ethylenediamine tetraacetic acid; V_{max}, maximal reaction velocity; $K_{m,}$ substrate concentration at 50% V_{max}; SDS, sodium dioctyl sulfate; Cl_{int}, intrinsic clearance

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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 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 levels 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. While both CYP3A activity and expression were reduced by approximately 50-70% in the old livers compared to the young animals, intestinal CYP3A activity and expression did not change significantly with age. The expression of one CYP3A isoform was increased by 1.5-fold in the old kidneys. NADPH reductase expression was reduced by 23-36% 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 (Le Couteur and McLean, 1998; Greenblatt et al., 1982). As the proportion of those over 65 years increases in the U.S. population (Administration on Aging, 2001), 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 (Miglioli et al., 1990; Holazo et al., 1988; Greenblatt et al., 1991, 1983a, b, 1984; Cotreau et al., 2004). However in some studies, significant age-related changes in clearance are not observed (Hunt et al, 1992a; 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 (Schmucker, 1998; Kinirons and Crome, 1997; 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* (Transon et al., 1996; George et al., 1995; Shimada et al., 1994; Hunt et al., 1992b; Schmucker et al., 1990; 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 prior to the availability of specific immunoinhibitory antibodies and index substrates for rodent CYPs. These early studies were thus limited in their ability to determine the involvement of individual CYP enzymes in specific reactions. Recent studies have suggested that triazolam biotransformation may be 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. While 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 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 (Schrag and Wienkers, 2001; Mäenpää et al., 1998; 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 Institutes of Aging supplied thirty-two male Fischer 344 rats from three age groups (held at Harlan Sprague-Dawley, Inc., 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 1-2 months in a 12hr:12hr 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 Laboratories, while 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 (Kotegawa et al., 2002; Cotreau et al., 2000, 2001). Briefly, enterocytes were isolated from the proximal portion (35cm) of the rat small intestine using four solutions, designated as A, B, C and D. First, the intestinal segment was washed with Solution A (1.5mM KCl, 96mM NaCl, 27mM sodium citrate, 8mM KH_2PO_4, 5.6mM Na_2HPO_4 and 40µg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.4). Cells were then eluted from the intestinal wall using Solution B (phosphate buffered saline without calcium and

magnesium: 1.5mM ethylenediamine tetracetic acid (EDTA), 0.5mM dithiothreitol and 40μg/ml PMSF, pH 7.4) and were washed twice in Solution C (5mM histidine, 0.25M sucrose, 0.5mM Na_2EDTA and 40μg/ml 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 15,000g for an additional 10 min. The second supernatant was added to the first and 2.5ml $CaCl_2$ was added. After a 20min incubation on ice, samples were centrifuged at 25,000g for 10 min and pellets were resuspended in 0.5ml 80% 0.1M 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 (Warrington et al., 2000; Lake, 1987). Briefly, approximately one lobe from the liver and one kidney from each rat were homogenized in a 0.05M potassium phosphate buffer containing 0.15M KCl and 0.25M sucrose. Samples were centrifuged at 10,000 rpm for 22 min at 4˚C. Supernatants were then centrifuged for an additional 70min at 33,000rpm at 4°C. The pellets were then homogenized in 0.1M potassium phosphate buffer containing 20% glycerol and stored at -80°C until use.

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 (0-750μM) were incubated with microsomes (0.25 and 0.1mg/ml for liver and intestine microsomes, respectively) in the presence of a NADPH-regenerating cofactor system (6.3mM $MgCl_2$, 0.5 U isocitric dehydrogenase, 3.8mM isocitric acid, 0.5mM $NADP^+$ in 0.05M potassium phosphate, pH 7.4). Incubations were terminated with acetonitrile (2/5 vol) and an internal standard (phenacetin; 1μg) was added. Samples were centrifuged and transferred to autosampling vials for high performance liquid chromotography (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 (2.5μM), was incubated with TRZ (250μM) in the presence of either hepatic (0.25mg/ml for TRZ) or intestinal (0.1mg/ml for TRZ) microsomes. Reactions were performed in duplicate and as described above. The samples were then analyzed by HPLC.

For immunoinhibition studies, microsomes (10μg) were preincubated with TRZ at 37°C for 30 min in the presence of either an antirat CYP antibody (100μg; CYP1A1/2, CYP2B1/2, CYP2C11, CYP2E1 and CYP3A1/2; Gentest, Woburn, MA), an appropriate serum control (rabbit or goat; 100μg) or 50mM potassium phosphate buffer. An NADPH-regenerating system was added and samples were incubated as described above. As negative controls, serum controls and samples containing 50mM 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 (Perloff et al., 2000; von Moltke et al., 1996). Triazolam, phenacetin, α- and 4-hydroxylated products were separated using a stainless steel 15cm x 3.9mm reverse phase C_{18} NovaPak column (Waters Associates, Milford, MA) with a mobile phase of 70% 10mM potassium phosphate buffer, 20% acetonitrile, 10% methanol at a flow rate of 1.4ml/min. The column effluent was analyzed at a wavelength of 220nm.

Data analysis of TRZ hydroxylation

For both reactions, chromatographic peak height ratios were generated. These ratios were converted into reaction velocities (nmols/min/mg 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

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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:

$$V = \frac{V_{max} S}{K_m + S} \quad \text{(Equation 1)}$$

in which V represents the reaction velocity; S represents the substrate concentration, V_{max} denotes the maximal reaction velocity and K_m represents the substrate concentration at which 50% of V_{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:

$$V = \frac{V_{max} S}{K_m + S} + zS \quad \text{(Equation 2)}$$

in which *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 (Cl_{int}) for each pathway was defined as:

$$Cl_{int} = 1000 \bullet \frac{V_{max}}{K_m} \quad \text{(Equation 3)}$$

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

Western blot analysis of CYP3A expression in rat

Hepatic (0.94-8.0µg), intestinal (1.3-12.3µg) and renal (50-98µg) 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 120V for 1.5 hr using a running buffer (0.19M glycine, 250mM Tris-base, 0.1% SDS). Protein was transferred onto a nitrocellulose membrane at 100V for 1 hour at 4ºC using a transfer buffer (0.19M glycine, 250mM Tris-base, 20% methanol). After a 1 min exposure to gel fixation buffer (50% methanol, 10% glacial acetic acid), membranes were washed with 1xTBS-Tween (0.15M NaCl, 40mM Tris-HCl, 40mM Tris-base, 0.06% Tween). After incubation with 3% blotto (dry nonfat milk in 1xTBS-Tween) for 1hr at 25˚C, membranes were probed with an antirat CYP3A1/2 antibody (1:3000; Xenotech, Kansas City, KS) for 16-18hr at 4˚C and 1hr at 25˚C. Membranes were washed with 1xTBS-Tween, incubated with 3% blotto for 30min at 25˚C and reprobed with a donkey antirabbit secondary antibody coupled to horseradish peroxidase (Pierce, Rockford, IL) for 1 hr at 25˚C. The membranes were then exposed to a chemilluminescent substrate for detection and analyzed using a Kodak imager and Kodak 1D image software.

NADPH expression analysis in rat microsomal preparations

NADPH reductase expression was also evaluated in these tissues. Membranes that were probed with an antirat CYP3A antibody were stripped and reprobed with an antirat NADPH reductase (1:3000; Gentest Corp., Woburn, MA) and a donkey antigoat secondary antibody that was linked to horseradish peroxidase (1:10,000; Santa Cruz

Biotechnology; Santa Cruz, CA). The same immunoblotting conditions described for CYP3A expression were used to evaluate NADPH reductase expression.

For stripping, blots were subjected to stripping buffer (0.2M glycine, 0.1% sodium dodecyl sulfate (SDS) and 1% Tween-20, pH 2.2) for one hour and washed thoroughly with ddH_2O and 1xTBS-Tween 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 to 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 vs. 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 -20ºC. Samples (~1ml; 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; Figure 1.A&B). Age-related reductions in V_{max} values were greater for the production of 4-OH-TRZ: V_{max} values were reduced by 74% in old compared to young animals, while 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. While Cl_{int} values declined with age for 4-OH-TRZ formation, Cl_{int} for α-OH-TRZ did not change with age.

The relative contribution of each pathway to the net Cl_{int} changed with age. While 4-OH-TRZ formation was the predominant pathway in the young livers, both metabolites had similar relative CL_{int} values in the old livers. Normalization of the net Cl_{int} per mg 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 biotransformation 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_{int} of TRZ in rat intestinal microsomes was 10% of that observed using rat liver microsomes. This reduced Cl_{int} 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_{int} regardless of age.

Age-related changes in TRZ hydroxylation by intestinal microsomes were small, and not statistically significant (Figure 1, Table 1).

Chemical and antibody inhibition of triazolam hydroxylation

Among five immunoinhibitory antibodies (polyclonal antirat CYP1A1/2, 2B1/2, 2C11, 2E1 and CYP3A1/2), only the antirat CYP3A1/2 antibody inhibited TRZ biotransformation in the liver and intestine.

In liver microsomes, the antirat CYP3A1/2 inhibited both 4-OH- and α-OH-TRZ formation in the old animals less than the other age groups (SNK, $p<0.05$). 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 (Figure 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 (Figure 3). Age-related differences in chemical inhibition were observed in the liver (Kruskal-Wallis ANOVA, $p<0.05$), but not in the intestine. Ketoconazole (2.5μM) inhibited metabolite formation to a lesser degree in the old livers (Dunn's, $p<0.05$).

Western blot analysis of CYP3A expression in liver, intestine and kidneys

The polyclonal antirat CYP3A1/2 antibody detected two bands in the rat liver, intestinal and kidney samples (Figures 4-6). Based on product information, these bands are likely to represents two CYP3A isoforms: CYP3A1 (the upper band) and CYP3A2

(the lower band). The relative amounts of these isoforms differed among the three tissues. While CYP3A1 and -3A2 isoforms were expressed in similar quantities in the liver, CYP3A2 was the predominant isoform in the kidney and the intestine. Also, while 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 for the intestine and r^2=0.23 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, while renal CYP3A was less than 1% in the liver.

The effect of age on CYP3A expression was tissue-dependent. In the rat livers, an age-related decline was observed in both CYP3A1 (ANOVA, $p<0.005$) and CYP3A2 (ANOVA, $p<0.001$) expression (Figure 4). Normalization of expression data for microsomal protein per gram of liver or total liver weight did not affect the results substantially (data not shown).

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

Comparison of TRZ hydroxylation and CYP3A expression in liver and intestine

Both 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, while 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 antirat NADPH reductase antibody identified one band at ~80kDa, 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 (~100kDa).

An age-related decline in NADPH reductase expression was detected in all three tissues (Figure 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 (Figure 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 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 α–OH-TRZ formation. Although a low-affinity enzyme component (Equation 2) was identified for hepatic α-OH-TRZ formation in young and intermediate rats, α-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 CYP 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 α–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 ~25-40% 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 (Miglioli et al., 1990; Greenblatt et al., 1980, 1991, 1983a, 1983b, Cotreau et al., 2004). Although species differences have been identified 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 evaluating the mechanisms of age-related differences in human CYP3A expression and function.

While age-related decrements in hepatic CYP3A expression and activity were observed, no differences in enteric CYP3A expression or 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.

Coincubation of TRZ with an immunoinhibitory antiCYP3A 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 CYP isoforms to triazolam biotransformation.

Age-related declines in NADPH reductase expression were evident in all three tissues. These findings were consistent with a previous study of NADPH reductase ontogeny in which Simmons and Kasper (1989) found that age-related changes in mRNA levels in the liver, intestine and kidney. Reduced hepatic NADPH 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 young). Alterations in testosterone levels may modulate CYP3A function, as suggested by previous studies (Schrag and Wienkers,

2001; Mäenpää et al., 1998; Fujita et al., 1985, 1990a; Kamataki et al., 1985; Patki et al, 2003). However, correlations between testosterone concentrations and CYP3A activity were weak, suggesting that testosterone is not the only modulator of CYP 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 CYP 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_{max} values for triazolam hydroxylation, and NADPH 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 differences may help to elucidate factors that regulate CYP3A expression and function with age.

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Tables

Table 1: Age-related changes in TRZ biotransformation in male rat hepatic and intestinal microsomes

Pharmacokinetic parameters	**Young (n=10)**	**Intermediate (n=10)**	**Old (n=8)**
Hepatic microsomes			
α-OH-TRZ			
V_{max} †	0.29 ± 0.010	0.22 ± 0.014	0.12 ± 0.018
K_m *	27.36 ± 2.35	20.97 ± 2.06	10.06 ± 1.72
Cl_{int}	11.44 ± 0.97	10.86 ± 0.45	13.68 ± 2.10
% of net Cl_{int}	19%	22%	46%
4-OH-TRZ			
V_{max} **	0.97 ± 0.043	0.79 ± 0.062	0.25 ± 0.095
K_m	21.08 ± 1.37	20.51 ± 1.29	16.27 ± 2.35
Cl_{int} †	47.26 ± 2.64	38.51 ± 1.96	15.99 ± 4.79
% of net Cl_{int}	81%	78%	54%
Net Cl_{int}	58.70 ± 3.27	49.37 ± 2.11	29.67 ± 5.05
Intestinal microsomes			
α-OH-TRZ			
V_{max}	0.28 ± 0.031	0.25 ± 0.032	0.26 ± 0.059
K_m	147±30	142±14	134±20
Cl_{int}	2.27 ± 0.27	1.97 ± 0.37	2.21 ± 0.50
% of net Cl_{int}	36%	35%	35%
4-OH-TRZ			
V_{max}	0.60 ± 0.057	0.49 ± 0.061	0.49 ± 0.101
K_m	173±36	154±14	160±37
Cl_{int}	4.08 ± 0.56	3.62 ± 0.73	3.94 ± 1.00
% of net Cl_{int}	64%	65%	64%
Net Cl_{int}	6.35 ± 0.82	5.59 ± 1.10	6.15± 1.49

Values are expressed as mean±S.E. V_{max} is expressed as nmols/min/mg protein; K_m is expressed in μM and Cl_{int} is intrinsic clearance ((V_{max}/K_m)*1000).

* denotes that Student-Newman-Keuls or Dunn's test detected a difference among all age groups ($p<0.05$)

** denotes that a Dunn's test detected a difference of the old from the young ($p<0.05$)

† denotes that a Student-Newman-Keuls or Dunn's test detected a difference of the young from the intermediate and old groups ($p<0.05$)

‡ denotes that a Student-Newman-Keuls or Dunn's test detected a difference of the old from the young and intermediate groups ($p<0.05$)

No significant age-related differences were found for intestinal samples.

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Figure legends

Figure 1: The effect of age on TRZ hydroxylation using liver (A and B) and intestinal (C and D) microsomes: Increasing concentrations of TRZ (0-750μM) were incubated with hepatic or intestinal microsomes from young (circles; solid lines), intermediate (squares; long, dashed lines) and old (triangles; short dashed lines). Production of α-OH-TRZ (A and C), 4-OH-TRZ (B and D) was expressed as the mean±S.E. Incubations from individual rats were performed in duplicate (n=10 for young and intermediate and n=8 for old). The lines represent the best fit Michaelis-Menten model for the mean values.

Figure 2: Age-related differences in antibody inhibition of TRZ hydroxylation: A screen of five immunoinhibitory antibodies (antirat CYP3A1/2, 2E1, 1A1/2, 2B1/2, 2C11) and a 50mM 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 (young, black; intermediate, white; old, grey). Samples were performed in triplicate and expressed as mean±S.E. * denotes that a Student's t-test detected a difference of the young samples from the buffer controls **. † a difference among the age groups for inhibited samples was detected (ANOVA, $p<0.04$) 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, $p<0.05$).

Figure 3: Age-related differences in inhibition of TRZ (250μM) biotransformation by ketoconazole: Production of α-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; hatched bars; n=8) rats was inhibited by ketoconazole (2.5μM). Samples with inhibitor were compared to controls without inhibitor and values are expressed as mean±S.E. * denotes a difference of the old livers from the young and intermediate groups (Kruskal-Wallis ANOVA, $p<0.05$; Dunn's, $p<0.05$).

Figure 4: Age-related differences in hepatic CYP3A1/2 expression: CYP3A1 (A) and CYP3A2 (B) were determined by western blot analysis. Mean values of young (black bars; n=10), intermediate (white bars; n=10) and old (hatched bars; n=8) samples are represented ± S.E. The y-axis represents the net intensity (pixels*area – background intensity at the perimeter of each band) relative to a standard curve (C). The standards (D) were serial dilutions of a young liver (young #8; 0.3-2.9µg). In D) amounts of 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 (A) and Dunn's test (B) indicated that the old livers differ statistically from the young and intermediate age groups (*, $p<0.05$).

Figure 5: Age-related differences in intestinal CYP3A1/2 expression: Expression of CYP3A1 (A) and CYP3A2 (B) were determined by western blot analysis. Mean values of young (black bars; n=10), intermediate (white bars; n=10) and old (hatched bars; n=8) samples are represented ± S.E. The y-axis represents the net intensity (pixels*area – background intensity at the perimeter of each band) relative to a standard curve (C). The standards (D) were serial dilutions of a young intestine (Young #8; 0.9-7.8µg). In D) amounts of young (Y), intermediate (I) and old (O) samples loaded were standardized to intestinal CYP3A activity, as measured by 4-OH-triazolam production. No statistical differences were found among the three age groups for either isoform.

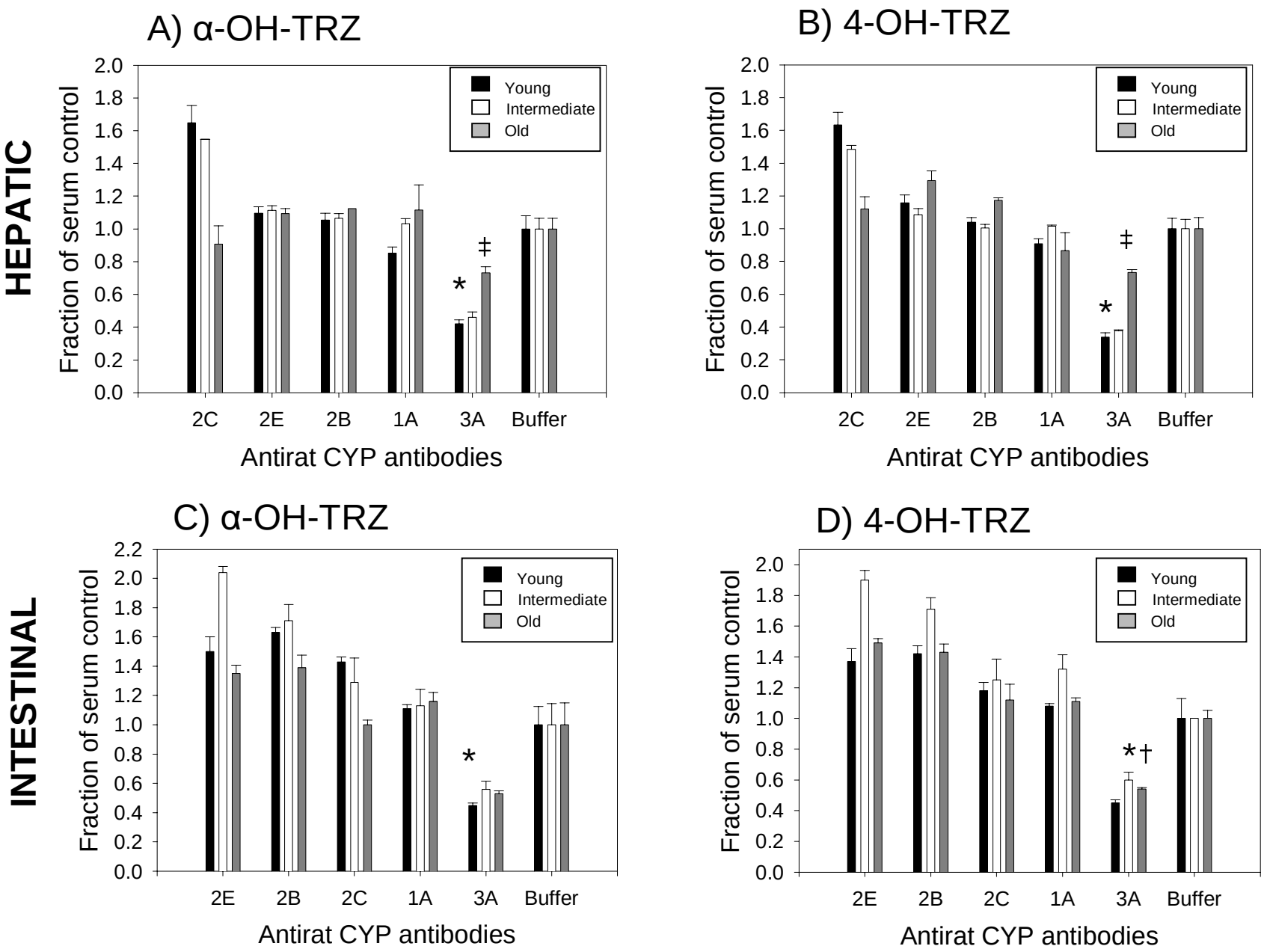
Figure 6: Age-related differences in CYP3A1/2 expression in kidney microsomes: CYP3A1 (A) and CYP3A2 (B) expression were determined by western blot analysis. Mean values of young (black bars; n=9), intermediate (white bars; n=9) and old (hatched bars; n=8) samples are represented ± S.E. The y-axis represents the net intensity (pixels*area – background intensity at the perimeter of each band) relative to a standard curve (C). The standards (D) were serial dilutions of an old kidney (old #3; 10-90µg). In D) varying amounts of young (Y), intermediate (I) and old (O) samples were loaded (ranging between 50-100µg). A Student-Newman-Keuls test detected a difference between the intermediate and old age groups in CYP3A1 expression (*).

Figure 7: Relative decrements in NADPH reductase in the liver, intestine and kidney: Mean values for young (Y), intermediate (I) and old (O) groups are expressed as a fraction of the young for the liver (triangles; dotted lines), intestine (squares; dashed lines) and kidney (circles; solid lines). * denotes a difference of the old from the young group (SNK, $p<0.05$). Values are expressed as relative to the mean young value ± S.E. For the liver and intestine, 10 young and intermediate groups were used. For the kidney, 9 rats were used. 8 old rats were used for all three tissues.

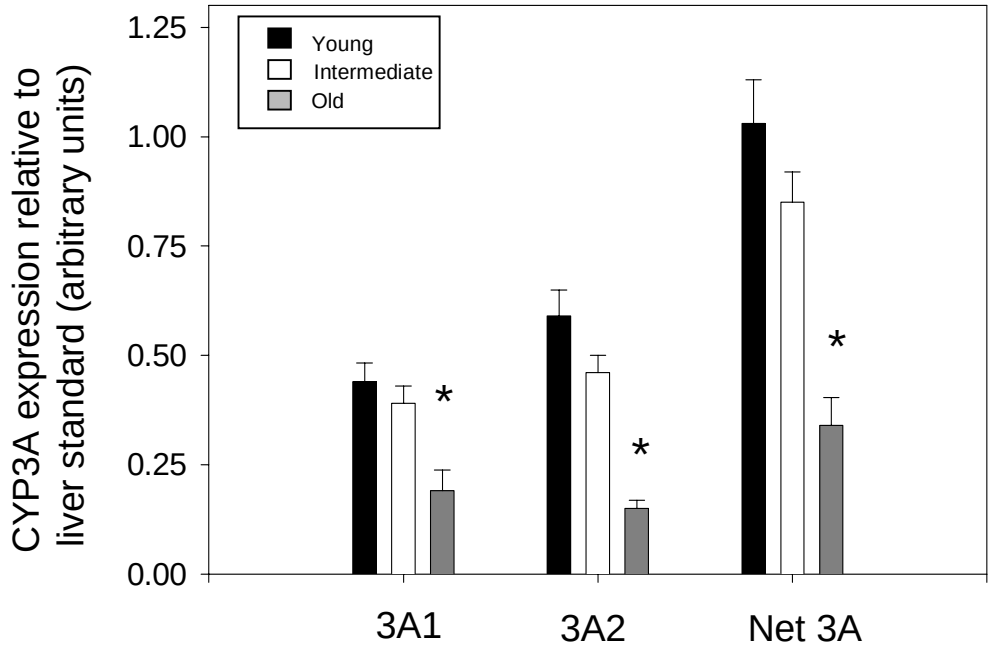
Figure 8: Free plasma testosterone concentrations in aging rats: Free testosterone levels (pg/ml) were analyzed in young (circles; n=10), intermediate (squares; n=10) and old (triangles; n=7). Data points correspond to individual rats, while lines represent the mean values for the group.

HEPATIC

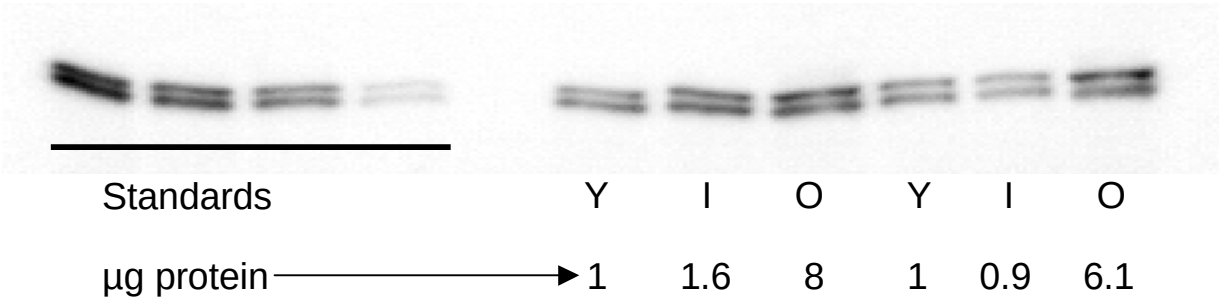
INTESTINAL



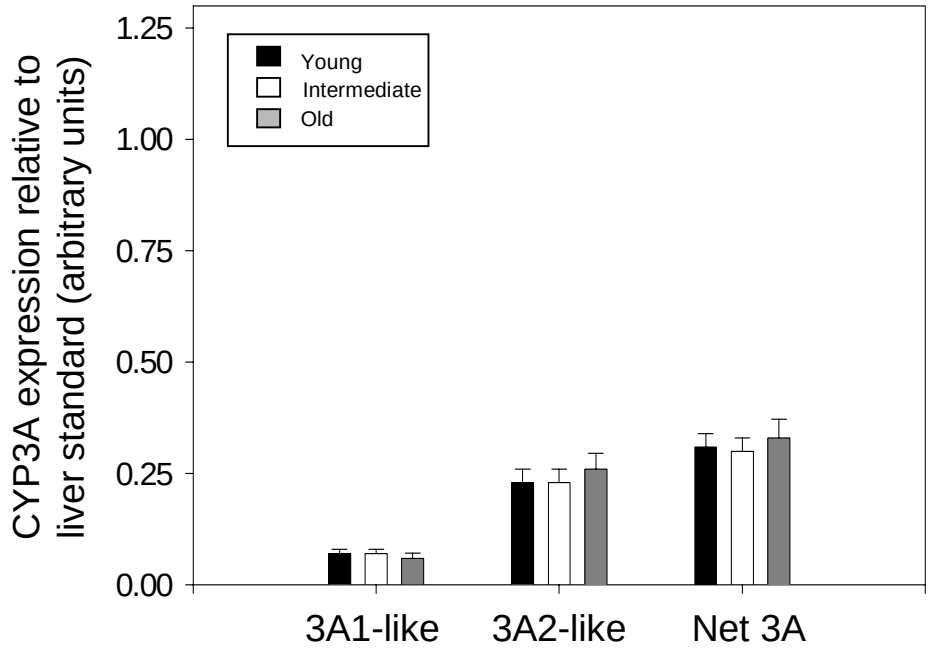
A) Hepatic CYP3A expression



B) Representative western blot

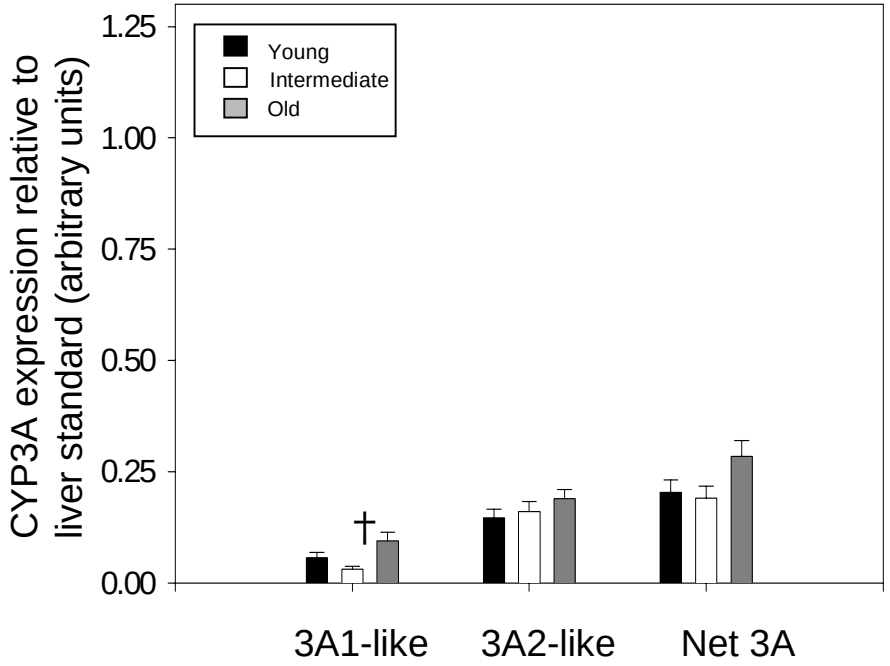


A) Intestinal CYP3A expression

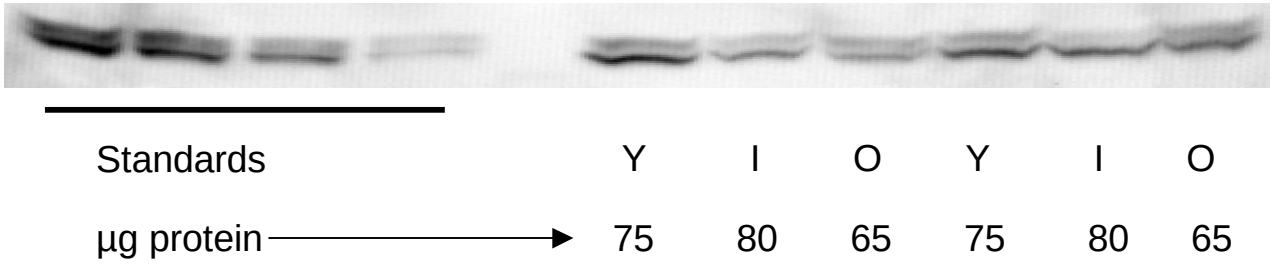


B) Representative western blot

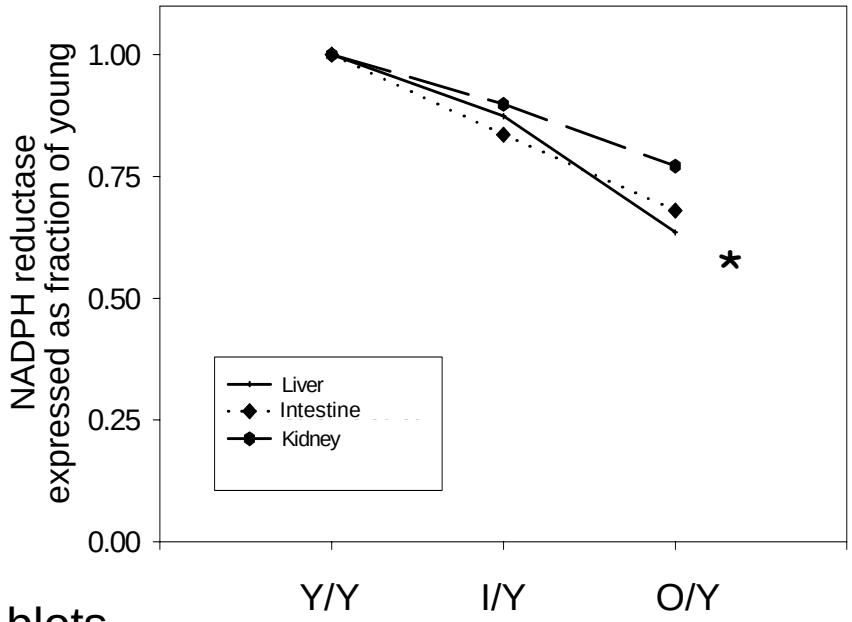
A) Renal CYP3A expression (x100)



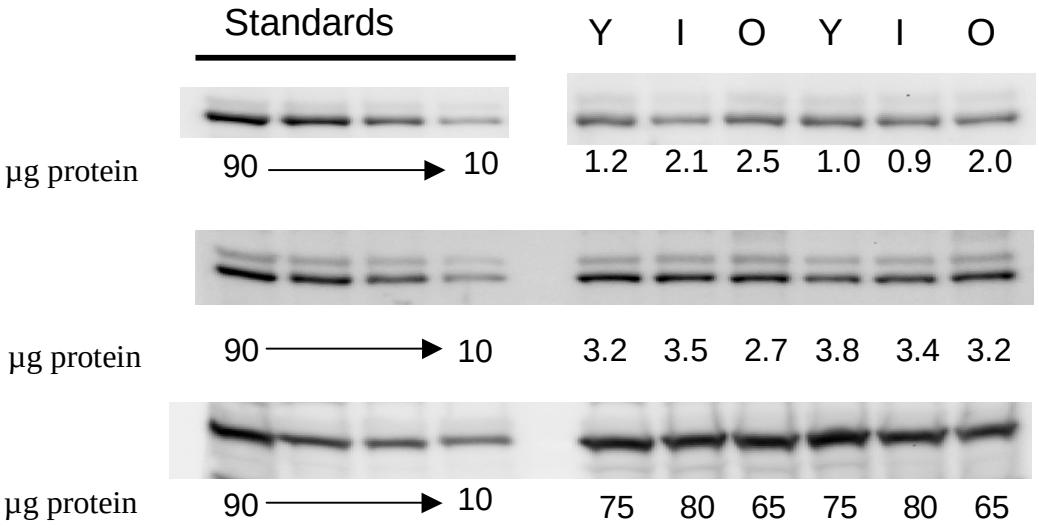
B) Representative western blot



A) NADPH reductase expression



B) Representative blots



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