Hepatic and pulmonary enzyme activities in horses

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Objective—To determine hepatic and pulmonary phase-I and phase-II enzyme activities in horses.

Sample Population—Pulmonary and hepatic tissues from 22 horses that were 4 months to 32 years old.

Procedure—Pulmonary and hepatic tissues from horses were used to prepare cytosolic (glutathione S-transferase and soluble epoxide hydrolase) and microsomal (cytochrome P450 monooxygenases) enzymes. Rates of microsomal metabolism of ethoxyresorufin, pentoxysresorufin, and naphthalene were determined by high-performance liquid chromatography. Activities of glutathione S-transferase and soluble epoxide hydrolase were determined spectrophotometrically. Cytochrome P450 content was determined by carbon monoxide bound-difference spectrum of dithionite-reduced microsomes. Activity was expressed relative to total protein concentration.

Results—Microsomal protein and cytochrome P450 contents were detectable in all horses and did not vary with age. Hepatic ethoxyresorufin metabolism was detected in all horses; by comparison, pulmonary metabolism of ethoxyresorufin and hepatic and pulmonary metabolism of pentoxysresorufin were detected at lower rates. Rate of hepatic naphthalene metabolism remained constant with increasing age, whereas rate of pulmonary naphthalene metabolism was significantly lower in weanlings (ie, horses 4 to 6 months old), compared with adult horses. Hepatic glutathione S-transferase activity (cytosol) increased with age; however, these changes were not significant. Pulmonary glutathione S-transferase activity (cytosol) was significantly lower in weanlings than adult horses. Hepatic and pulmonary soluble epoxide hydrolase did not vary with age of horses.

Conclusions and Clinical Relevance—Activity of cytochrome P450 isoforms that metabolize naphthalene and glutathione S-transferases in lungs are significantly lower in weanlings than adult horses, which suggests reduced ability of young horses to metabolize xenobiotics by this organ. (Am J Vet Res 2000;61:152–157)

Virtually all aspects of an animal’s existence involve exposure to natural and synthetic compounds. Enzymatic metabolism of xenobiotics represents one way an organism may handle these compounds upon exposure to them. In general, action of phase-I enzymes results in addition of functional groups that are acted upon by phase-II enzymes. The resulting hydrophobic compounds are larger and more polar, allowing improved excretion. For most chemicals, increased enzyme activities are associated with a decrease in toxicity, whereas a decrease or deficiency in enzyme activities results in increased toxicity. Organ, tissue, cellular, and subcellular expression of these enzymes, as well as coordinated activities of phase-I and -II enzymes, often determine whether specific compounds will result in cellular injury. Lack of a particular enzyme, a defect in an enzymatic pathway, or an increase in expression of one isoenzyme relative to another isoenzyme may result in species or tissue susceptibility to a particular xenobiotic.

The purpose of our study was to quantify changes in selected cytochrome P450 mediated metabolism of xenobiotics and subsequent conjugative capabilities in liver and lung tissues of horses as they mature. In doing so, we hoped to provide a database of expression and activity of enzymes that metabolize xenobiotics in clinically normal horses that may be compared with those data available in other species. Because similar studies, under identical conditions, have been performed in our laboratory, we compare results of our work in horses with these reported studies and others available in the literature. This information may allow comparison of data obtained from laboratory species to that of horses and should provide information on which to base clinical decisions on dose and treatment intervals for those treatment agents that are cleared primarily by xenobiotic metabolism in early postnatal and geriatric horses.

Materials and Methods

Horses—Tissues from 22 horses that were 4 months to 32 years old were obtained at necropsy. Horses were of both sexes and were Thoroughbreds, Arabians, Quarter Horses, Peruvian Paso Horses, Morgans, and crossbreds. Horses were classified into 4 groups on the basis of their ages at necropsy as follows: 5 horses were 4 to 6 months old (mean ± SD, 0.4 ± 0.09 years old; range 0.333 to 0.50 years), 4 horses were considered yearlings (0.92 ± 0.1 years old;
range 0.83 to 1.0 years), 5 horses were 2 to 4.5 years old (3.1 ± 1.1 years old; range 2.0 to 4.5 years), and 8 horses were > 4.5 years old (18.4 ± 9.6 years old; range 6.0 to 32 years).

On the basis of physical examination findings, all horses included in our study were determined to be free of pulmonary and hepatic disease prior to euthanasia. These horses had not been given pharmacologic agents for ≥ 2 weeks prior to euthanasia. After sedation of horses with IV administration of 1.1 mg of xylazine hydrochloride/kg of body weight, euthanasia was performed by rapid IV infusion of 40 mg of pentobarbital sodium/kg. Four- and 6-month-old weanlings were given 1.1 mg of xylazine/kg IV followed by ketamine (2.2 mg/kg, IV) for short surgical procedures (muscle biopsies) prior to euthanasia with pentobarbital sodium. The thorax and abdomen were opened immediately after loss of corneal reflexes, and the lungs and right liver lobe were removed. Liver and lungs of all horses were normal in appearance. Sections of liver, approximately 10 g, were taken and placed in ice-cold phosphate buffered sodium chloride (0.01 M phosphate, 0.154 M NaCl, pH 7.4; 300 mOsm). The caudodorsal portion of the right caudal lung lobe was homogenized in fresh buffer and centrifuged for 1 hour at 100,000 × g. Supernatant fractions were centrifuged at 100,000 × g pieces were cut and placed on ice.

Preparation of microsomes—Microsomes were prepared by differential centrifugation. Briefly, 20 g of lung and 10 g of liver were homogenized in 150 mM KCl, 50 mM Tris-HCl, 75 mM EDTA, and 30% glycerol buffer solution (pH 7.4), using a vertical tissue homogenizer. All procedures were performed on ice. Supernatant fractions were centrifuged at 100,000 × g for 1 hour. Supernatant was removed and saved for analysis of glutathione S-transferases and soluble epoxide hydrolase activities. The pellet was resuspended in fresh buffer and centrifuged for 1 hour at 100,000 × g. The resulting microsomal pellet was suspended in 75 mM phosphate buffer (pH 7.4) and stored on ice for enzyme incubations. Enzyme incubations were performed in triplicate. Microsomal protein concentration was estimated by the Coomassie blue dye binding procedure, using bovine serum albumin as the standard.

Cytochrome P450 assays—Assay conditions for cytochrome P450 0-dealkylase, naphthalene monooxygenase, glutathione S-transferase, and soluble epoxide hydrolase were as described (Table 1). Cytochrome P450 content was estimated by the carbon monoxide bound-difference spectrum of dithionite-reduced microsomes. Assays of 7-ethoxyresorufin 0-dealkylase and pentoxyresorufin O-dealkylase activities were performed as described. Reactions were incubated at 37°C for 6 minutes in the presence of NADPH (nicotinamide adenine dinucleotide phosphate, the reduced form)-generating solution (β-NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase), 1 µM ethanol or pentoxysorufin, 250 µg microsomal protein, and 100 mM phosphate buffer (pH 7.4) to a total volume of 1 ml. The reaction was terminated by adding 2 ml of ice-cold high-performance liquid chromatography (HPLC) gradient methanol to precipitate proteins. Resorufin was detected by HPLC fluorescence (excitation wavelength 535 nm, emission wavelength 585 nm) after separation with a radial pak cartridge (0.8 × 10 cm). Eluent was 68% KH2PO4/32% methanol, pH 6.8, with a flow rate of 1.3 ml/min. Standard curves were constructed by use of known concentrations of resorufin with 250 µg boiled microsomal protein, and examined. Enzyme assay conditions were chosen on the basis of incubation time and protein concentration in other species studied in our laboratory.

Naphthalene monooxygenase activity was determined by quantitation of naphthalene conjugate formation (Table 1). Naphthalene incubations were prepared on ice in a final volume of 2 ml in 0.1 M phosphate buffer, pH 7.4. Incubations consisted of microsomal protein (2 mg), NADPH-generating system (β-NADP [1 µM], glucose-6-phosphate [30 µM], glucose 6-phosphate dehydrogenase [1 U]), MgCl2 [10 µmol]), reduced glutathione (5 mM), and 10 µM CDNB of mouse hepatic glutathione S-transferase. Naphthalene (0.5 mM final concentration) was added as the substrate. Reaction vessels were tightly capped and transferred to a 37°C shaking water bath for 20 minutes. Four milliliters of ice-cold HPLC grade methanol were added to terminate reactions. Enzyme assay conditions were chosen on the basis of time and protein concentration in other species studied in our laboratory. Measurement of glutathione adducts of naphthalene and naphthalene dihydrodiol was performed as reported. Protein was removed by centrifugation at 9,000 × g for 11 minutes. Methanol/water supernatant was evaporated to dryness and the residue reconstituted for HPLC analysis in 200 µl distilled water. A radial pak cartridge (0.8 × 10 cm) was used for all analyses. Dihydrodiol and 3 naphthalene-glutathione conjugates were eluted between 35 to 70 minutes in a 3.5 to 3.8% acetonitrile/1% glacial acetic acid/water eluent at 1.5 ml/min flow rate. Quantitation was on the basis of integration of peaks detected at 254 nm. Standards were prepared with naphthalene dihydrodiol, which has an extinction coefficient identical to glutathione adducts.

Activity of glutathione S-transferase was estimated using 1-chloro-2,4-dinitrobenzene and measuring the product spectrophotometrically (Table 1). Briefly, the reaction mixture consisted of 2.8 ml of phosphate buffer (0.1 M; pH 6.5), 100 µl of 30 mM 1-chloro-2,4-dinitrobenzene, 100 µl of 30 mM glutathione in pH 6.5 buffer, and 5 to 40 µl cytosol, depending on the tissue type being stud-

Table 1—Descriptions of microsomal and cytosolic enzyme incubations and the conditions under which they were performed

<table>
<thead>
<tr>
<th>Phase I or phase II isoenzyme/enzyme</th>
<th>Substrate/Solvent</th>
<th>Concentration</th>
<th>Incubation time (min)</th>
<th>Quenching method</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1/1A2</td>
<td>EROD/methanol</td>
<td>1.0 µM</td>
<td>6</td>
<td>2 ml methanol</td>
<td>4, 5</td>
</tr>
<tr>
<td>2B1</td>
<td>PROD/methanol</td>
<td>1.0 µM</td>
<td>6</td>
<td>2 ml methanol</td>
<td>4, 5</td>
</tr>
<tr>
<td>1A1/2B1/2F</td>
<td>NAMO/methanol</td>
<td>0.5 mM</td>
<td>20</td>
<td>4 ml methanol</td>
<td>8</td>
</tr>
<tr>
<td>GSTx</td>
<td>CDNB/ethanol</td>
<td>1 mM</td>
<td>3</td>
<td>none</td>
<td>24</td>
</tr>
<tr>
<td>SEH</td>
<td>TSO/ethanol</td>
<td>50 µM</td>
<td>10</td>
<td>none</td>
<td>9, 10</td>
</tr>
</tbody>
</table>

CDNB = 1-Chloro-2,4-dinitrobenzene. EROD = Ethoxyresorufin. PROD = Pentoxyresorufin. NAMO = Naphthalene. GSTx = Glutathione S-transferase. SEH = Soluble epoxide hydrolase. TSO = Trans stilbene oxide.
ied. Change in absorbance was measured continuously at 340 nm for 3 minutes. In reactions where rates of conjugation exceeded 0.05 absorbance U/min, cytosolic protein was diluted to maintain activity within the range where product formation was proportional to protein concentration and time.

Determination of soluble epoxide hydroxal activity in cytosolic supernatants of lung and liver tissue was performed by use of a spectrophotometric assay and a partition assay with the same substrate (Table 1). The 2 methods gave similar rates of conversion. Data are reported for the spectral assay. Briefly, assays were performed in a UV/Vis spectrophotometer at 23 C. Typically 30 to 50 μl of enzyme were added to reference and sample cuvettes containing 940 μl (pH 7.4) sodium/potassium phosphate buffer (0.2 M) and 100 μg of bovine serum albumin/ml. After equilibration at 23 C, 10 μl ethanol were added to the reference cuvette, and 10 μl trans-stilbene oxide (TSO) in ethanol were added to the sample cuvette. Typical final substrate concentrations were 50 μM. Change in absorbance was monitored at 229 nm for TSO where there is maximal difference between epoxide and dihydriodiol concentrations (ε_M = 15,000). In some instances, radiolabeled TSO was used, and the conversion of TSO to trans-stilbene dihydriodiol was monitored by determining the amount of product dihydriodiol in the aqueous phase versus the epoxide substrate in the isocantane phase.

**Statistical Analyses**—Results of triplicate analyses of each enzyme assay were summed, and mean values were determined for each horse. Data obtained for each age group and each measured variable for equine liver and lung tissue were tabulated and the mean (± SD) calculated. For each measured variable in each organ and for each age group were tested for normality by use of the Kolmogorov-Smirnov goodness-of-fit test. Data that were normally distributed were further analyzed by use of a one-way ANOVA, with pairwise comparisons made with the Tukey procedure. For data that were not normally distributed, the nonparametric, Kruskal-Wallis test was used to compare different age groups, with all pairwise comparisons between groups made using the Tukey procedure. Additionally, because large variations were observed at all ages, data for each protein or enzyme activity for all ages were analyzed as a group, and data for liver tissue were compared with that observed in lung tissue by use of a Student t-test. Data reported were the values for all ages analyzed together (Table 2). Significance was assigned when P < 0.05.

**Results**

Substantial interindividual variation in microsomal cytosolic protein content and enzyme activities was observed among all ages of horses. When protein content, cytochrome P450 content, and enzyme activities were compared by age groups, the only significant differences recorded were low pulmonary naphthalene monooxygenase and glutathione S-transferase activities in weanlings, compared with that of adult horses (P = 0.015, P = 0.014 respectively).

Hepatic microsomal protein and cytochrome P450 contents were significantly greater than that observed in pulmonary microsomal preparations (Table 2; P = 0.05). Likewise, liver metabolized ethoxyresorufin at rates that were far greater than that observed in pulmonary microsomal incubations when comparing data from all ages of horses together (Table 2; P = 0.001). Compared with lung tissue, hepatic microsomal metabolism of ethoxyresorufin was approximately 30-fold greater. However, hepatic microsomal metabolism of pentoxyresorufin or naphthalene was not significantly different from that observed in pulmonary microsomal preparations (P = 0.087 and P = 0.98, respectively). With these 2 substrates, there was less than a twofold difference in activities observed in liver tissue, compared with lung tissue.

Although hepatic cytosolic glutathione S-transferase activity was twofold greater than that observed in lung, the difference was not significant, most likely because of substantial variability in individual horses (P = 0.077). Activity of cytosolic epoxide hydrolase was significantly greater in hepatic homogenates than in pulmonary preparations with approximately 17-fold greater activity in hepatic cytosol, compared with lung, when all ages were examined together (P < 0.001).

**Discussion**

Although several diseases of horses may involve metabolism of inhaled or ingested xenobiotics, little information is available regarding drug metabolism in this species. Our search of the literature provided a total of 5 references. The purpose of our study was to define expression of the pulmonary cytochrome P450 monooxygenase system and conjugative capabilities in equine lungs by defining activities of these
enzymes toward selected substrates. We compared the activity of pulmonary cytochrome P450 and phase-II enzyme reactions to that detected in equine livers. We chose to define the O-dealkylase, naphthalene monoxygenase, glutathione S-transferase, and epoxide hydrolase activities to that observed in liver tissue, because these substrates have been used to define the contribution of these enzymes to susceptibility to toxicants in other species.2-22 There are substantial species differences in relative activity and specific locations of enzymes within the respiratory system in which they are expressed.6,12,13,14

Appearance of cytochrome P450 in many species develops postnatally.6,30,31 In some species (rabbits, sheep, goats, and cattle), there is less cytochrome P450 in young animals, compared with adults of that species.31,34 In others, the specific content of cytochrome P450 does not vary as widely in young animals as in adults (dog, monkey, swine, and horses).26,30,33-35 When comparing the cytochrome P450 content of adults, variation among species appears to be greater, with rabbits33 expressing twofold higher amounts of cytochrome P450 than horses,28 sheep34,30-40,41 goats,34 and rats.16,33,41 In all species, hepatic cytochrome P450 content varies from twofold (goats) to as much as 20-fold (sheep) higher than in pulmonary tissue from young animals.13,40 Hepatic cytochrome P450 content of horses in our study was approximately threefold greater than that observed in lung tissue.

Similarly, O-dealkylase activity of liver varies considerably among species, age, and substrate. On the basis of results of our study, it appears that even young horses are capable of metabolizing ethoxyresorufin, and in vitro hepatic metabolism is similar to that observed in goats.34,42 sheep32,39,40,41 and cattle.40,44 but greater than that described for rats,16,33,40 dogs,26,33,35 and swine.30,38 In horses, in vitro metabolism of ethoxyresorufin by liver was approximately 30-fold greater than that observed in lungs. This is in contrast to sheep,34,30,40,41 and goats,34-35 where in vitro pulmonary metabolism of ethoxyresorufin is similar to that of liver. In contrast, in vitro metabolism of pentoxysorufin by liver and lungs of horses is low, compared with that of rats (liver and lung),30,33,42 rabbits (lung),33 and goats (liver).34,42 Pentoxysorufin metabolism by microsomes prepared from horse lungs is low when compared with that observed in other species.31

Naphthalene metabolism has been studied extensively in the mouse lung.13,17 In mice, naphthalene is metabolized stereo- and regioselectively, with a correlation between activity of a specific isofrom of cytochrome P450 and cell injury.13,15,17 The hallmark of this injury is dose- and time-dependent swelling of endoplasmic reticulum, loss of Clara cell granulation, and exfoliation of bronchiolar epithelium.11,17 Some of these characteristic changes in equine Clara cells are associated with chronic respiratory conditions, although metabolism of xenobiotics in these conditions has not been defined.21 A similar cytochrome P450 isoform with the naphthalene metabolizing enzyme in mice is found in goat lungs and causes pulmonary injury after administration of 3-methyl indole.21 Horses metabolized naphthalene in vitro at low rates, whereas relative contribution of pulmonary tissue to overall naphthalene metabolism was substantial in horses, when compared with liver tissue.20

In vitro metabolism of naphthalene by equine pulmonary microsomes resulted primarily in dihydrodiol of naphthalene oxide.15 Horses do not appear to metabolize naphthalene stereoselectively, and our data suggest that detoxification of naphthalene is primarily via epoxide hydrolase. Naphthalene monoxygenase activity is significantly lower in young horses, compared with adults. This is a finding similar to that reported from studies on developing mouse lungs.19 In mice, the pattern of injury associated with naphthalene administration to neonatal mice differs considerably from that of adults.17 These findings may prove relevant to pulmonary injury in young horses.

Glutathione S-transferases are dimeric proteins that have been grouped into 6 classes on the basis of their subunit composition.34,40 These enzymes provide key mechanisms for detoxification by liver, lung, and other tissues. On the basis of findings in our study, development of conjugative abilities of equine hepatic cytosol appears to be greater by 4 months of age than in young sheep,33,39,42 goats,14 cattle,13 swine,30,38 and humans32 but less than in dogs.13,34 and rats.13 Activity of glutathione S-transferase in pulmonary tissue from weanlings was significantly lower than that observed in adult horses. Lack of sufficient conjugative ability in young horses may also indicate susceptibility to reactive intermediates.

Soluble epoxide hydrolases have wide-ranging substrate specificity and are important in mammalian cellular metabolism.21 Soluble epoxide hydrolases have been found in all tissues thus far examined.20 This enzyme facilitates hydration of epoxides on acyclic systems including mono, cis, and trans-1, 2, di-, tri-, and tetra-substituted epoxides.21 For example, these enzymes rapidly hydrate chemically stable squalene oxides and lanosterol epoxides and are thought to be involved in metabolism of fatty acid epoxides, including arachidonate oxides as well as many epoxides that are natural components of plants.21 Activity of these enzymes generally reduces biological activity of moderate electrophiles.21 Results of our study indicate that, similar to humans, activity of soluble epoxide hydrolase in horses declines with age.21

Compared with other species, our data indicate marked differences in in vitro rates and substrate selectivity of hepatic and pulmonary cytochrome P450 monooxygenases and conjugative enzymes in horses. In horses, age-related expression appeared important for some of these reactions. Low or deficient activity of specific enzymes may be indicative of species- or age-related susceptibility to injury when exposed to xenobiotics. Direct metabolism and toxicity relationships are difficult to define, however, because these preparations contain multiple forms of enzymes with overlapping substrate selectivity.22 When extrapolating metabolism or toxicity data from one species or age of an animal to another, these variations should be considered. The importance of these differences awaits determination of the location and cell types that express these enzymes, specific forms present, their substrate selec-
tivity, and relative expression, compared with detoxifi-
cation enzymes. Localization to airway epithelia would
be expected in horses, because their Clara cells contain
large amounts of endoplasmic reticulum, similar to
rabbits. Compared with adult horses, our data sug-
gest that young horses, having less ability to metabo-
lize aromatic hydrocarbons and less conjugative ability,
may be at greater risk when exposed to xenobiotics
requiring metabolism by these systems.

1. deBethizy JD, Hayes JR. Metabolism a determinant of cyto-
3. Bradford MM. A rapid and sensitive method for the quanti-
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