XENOBIOTIC METABOLIZING ENZYME ACTIVITIES IN RAT, MOUSE, MONKEY, AND HUMAN TESTES

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ABSTRACT:

The capacity of the testis to metabolize xenobiotics has been proposed to play a role in the susceptibility of different species to testicular toxicity. Since species differences in testicular xenobiotic metabolizing enzyme activities are not well documented, the primary objective of the present study was to compare enzyme activities in subcellular fractions prepared from rat, mouse, monkey, and human testes. In microsomal fractions, enzyme activities measured were pentoxyresorufin O-dealkylase (PROD), ethoxyresorufin O-dealkylase (EROD), and epoxide hydrolase (mEH). In cytosolic preparations, epoxide hydrolase (cEH) and glutathione S-transferase (cGST) activities were measured. PROD activity was not detectable in any of the species studied, while it was readily detected in liver microsomes used as a positive control. Although EROD activity was low, it was measurable in testicular microsomes from rat and mouse, but not monkey or human. No marked species differences in cEH activity were found. In contrast, mEH activity was low in the monkey, intermediate in the rat, and highest in the human and mouse. cGST activity was significantly lower in the two primate species compared with the rat and the mouse. The levels of activity of the xenobiotic metabolizing enzymes studied were generally more than an order of magnitude lower in the testis as compared to the liver. However, in rat and mouse, the levels of mEH and cGST activities in testis were relatively similar to hepatic levels. Overall, these data indicate that species differences in capacity to metabolize xenobiotics may play a role in differential sensitivity to testicular toxicants.

Numerous studies have documented the importance of metabolic activation in chemically-induced organ damage. In the testis, xenobiotic metabolism is of particular interest since reactive metabolites may be formed in the immediate vicinity of the germ cells. Other investigators have documented target organ biotransformation of the testicular toxicants 7,12-dimethylbenz(a)anthracene, benzo(a)pyrene, 1,3-dinitrobenzene, ethylene dibromide, and DBCP(1–5).

Many chemicals undergo metabolic activation via the cytochrome P-450 mixed function oxygenase system. The two major classes of isozymes in this enzyme system have frequently been classified as cytochromes P-450 (phenobarbital-inducible) and cytochromes P-448 (3-methylcholanthrene-inducible). These two isozyme classes were measured by Lake et al. (6) in rat testis 10,000x supernatant using biphenyl as the substrate. They detected cytochrome P-450-type activity as indicated by formation of 4-hydroxy biphenyl, but no cytochrome P-448-type activity measured by the formation of 2-hydroxy biphenyl. In contrast, several other authors have reported detection of cytochrome P-448-type activity in rat testicular microsomes by measuring either aryl hydrocarbon hydroxylase (2,7) or ethoxycoumarin O-dealkylase (8).

Metabolism can also result in detoxification of testicular toxicants, or their toxic metabolites, providing protection from toxic insult. For example, epoxides, which are often mutagenic, carcinogenic, or cytotoxic, can be detoxified by epoxide hydrolases or glutathione S-transferases. There have been several reports of mEH, cEH, and cGST activities in rat or mouse testis (2,8–12). Therefore, in the testis, differential capacities for metabolic activation and detoxification may account for species differences in sensitivity to testicular toxicants. For example, rats, humans, and monkeys are sensitive to DBCP-induced testicular toxicity, whereas mice are insensitive, and a metabolic basis has been proposed to account for this difference (4,5,13–15).

In the present study, testicular xenobiotic metabolism was compared using tissue from humans, rhesus monkeys, Fisher 344 rats, and CD-1 mice. Metabolism via the cytochromes P-450 and P-448 isozymes was measured by the activities of PROD and EROD, respectively. Epoxide detoxification potential was assessed by measuring microsomal and cytosolic activities of epoxide hydrolase (mEH and cEH), and using an epoxide substrate for cGST. In addition, hepatic enzyme activities were measured as a positive control and to allow comparison to testicular activities.

Materials and Methods

Chemicals. TSO, cis-stilbene, diethylmaleate, isooctane (99%), and hexanol (98%) were purchased from Aldrich Chemical Co. (Milwaukee,
Tissue Samples. Human and Monkey. Human tissues were obtained from three adult male organ transplant donors (age 19-37 years) who were maintained on life support systems after sustaining lethal traumatic injuries. Within 30 min after circulatory arrest, both testes were removed from each donor and placed in saline on ice. The testes were frozen within 1-2 h after collection and stored at -80°C. Tissues from three adult male rhesus monkeys (age 6-10 years) were obtained at necropsy. One testis from each monkey and liver from two of the monkeys were immediately frozen and stored in liquid nitrogen. Both human and monkey tissues were frozen prior to preparation of subcellular fractions because transportation to our laboratory from the facility where tissues were obtained was required to ensure long time periods. This practice has been used by numerous other laboratories (17-19), and the stability of xenobiotic metabolizing enzyme activities in whole rat livers frozen for up to 9.5 months at -80°C has been demonstrated by Danner-Rabovsky and Groseclose (20). In addition, composite data from our laboratories (unpublished) have indicated that freezing whole tissue is preferable to freezing subcellular fractions. Moreover, we were concerned about the stability of the enzymes over the uncertain length of time that would be required to obtain all of the human and monkey tissues for simultaneous analysis.

Rodent. Sexually mature male Fisher 344 rats and CD-1 mice were purchased from Charles River Laboratories (Kingston, NY or Wilmington, MA), except rats used for mEH, cEH, and cGST measurements, which were purchased from Bantin & Kingman (Fremont, CA). The animals were allowed to acclimate, for at least 6 days after shipment, in an animal facility approved by the American Association for the Accreditation of Laboratory Animal Care with 12 hr light/dark cycles. Food and water were provided ad libitum. Rats (10-12 weeks of age) and mice (9-11 weeks of age) were euthanized using either CO2 and cervical dislocation, or cervical dislocation alone. Testes and livers were immediately removed, though pooling of organs was not necessary for rats, mouse organs were pooled as described below. For PROD and EROD activities, testes and livers were used from three rats and three pools of mouse tissue (16 mice per pool). Testicular pools included testes from all 16 mice and liver pools included livers from 4 of the 16 mice. Testes and livers were used from all three rats. For measurement of mEH, cEH, and cGST activities, testes and livers were used from four to five rats and three to five mouse pools (five to six mice per pool).

Preparation of Subcellular Fractions. Microsomal and cytosolic fractions were prepared using standard homogenization and centrifugation techniques (21). Human tissues, which had been frozen in saline, were thawed on ice. Monkey tissues were thawed in 150 mM KCl on ice. Human, monkey, and rat testes were decapsulated. Rodent livers were flushed with ice-cold 150 mM KCl to remove blood. Organs were placed in ice-cold 20 mM Tris/1.15% KCl, pH 7.4, and weighed. Additional buffer was added to achieve 2× w/v prior to homogenization with a Polytron. The homogenate was centrifuged at 12,000g for 25 min at 4°C, then the supernatant was ultracentrifuged at 105,000g for 75 min at 4°C. Cytosol (105,000g supernatant) was collected after the fatty layer was drawn off. The microsomal pellet was resuspended in 0.39 M sucrose/77 mM sodium pyrophosphate, pH 7.5 (liver, 10 ml; testes, 5 ml), by 5 sec of homogenization with a Polytron, then ultracentrifuged again as described above to wash the microsomes. Microsomal pellets were resuspended at ½ w/v, except rat and mouse microsomes for epoxide study were at 1× w/v, using either 150 mM KCl (rats and mice) or sodium phosphate buffer, 0.1 M, pH 7.8 (monkeys and humans), as specified above. Microsomes and cytosol were stored at -80°C until use.

Protein Determination. Protein concentrations were determined using a modification of the method of Bradford (22) as per Moody et al. (23).

Enzyme Activities. PROD and EROD Assays. Dehalogenation of PtR was monitored using the method of Lubet et al. (24). The procedure was verified for protein linearity and all measurements were taken from linear portions of the rate curves. The substrate solution was made by weighing out PtR using a microbalance, then dissolving it in HPLC grade methanol. The reactions were carried out at room temperature (23-24°C) in 0.05 M Tris buffer, pH 7.5, with 0.025 M MgCl2, and 10 μM PtR. Concentrations of microsomal protein used were 0.5 mg/ml for rat and mouse liver and 2.0 mg/ml for monkey liver and all testicular samples. Reactions were initiated by the addition of 125 μM NADPH, and the formation of resorufin was monitored spectrophotometrically (excitation = 530 nm, emission = 577 nm) until a concentration of 10-20 nM resorufin was formed. Resorufin (10 μM in DMSO) was diluted in Tris buffer for the standard curve (1.25 nM to 80 nM). Dehalogenylation of EriR was measured using the method of Pohl and Fouts (25). Microsomes were incubated at 37°C in 0.1 M HEPES buffer, pH 7.8, 5 mM glucose-6-phosphate, 1.5 units glucose-6-phosphate dehydrogenase, 5 mM MgSO4, BSA (1.6 mg/ml), and 1.5 μM EriR (in HPLC grade methanol, based on ε100 = 16 mM−1 cm−1) in a total reaction volume of 1.25 ml. Amounts of microsomal protein and times of incubation were varied to utilize linear ranges of time and protein dependency: human, testis (2.0 mg, 60 min); monkey, testis (2.0 mg, 60 min), liver (0.025 mg, 4 min); rat, testis (0.5 mg, 40 min), liver (0.25 mg, 5 min); mouse, testis (1.0 mg, 80 min), liver (0.125 mg, 2 min). The reaction was initiated with 6 nmol NADPH after preincubating for 5 min. Boiled microsomes were used for blanks rather than omitting NADPH, since NADPH quenches the fluorescence (blanks with no NADPH have an increased fluorescence, equivalent of 1 nM resorufin, as compared with boiled microsomes). The reaction was terminated using 2.5 ml methanol and precipitated protein was removed by centrifugation at 830g for 15 min. The supernatant was measured spectrophotometrically for resorufin formed. Resorufin (10 μM in DMSO) was diluted with HEPES/methanol (1:2) for the standard curve, which ranged from 1.25 nM to 80 nM.

mEH, cEH, and cGST Assays. Activities of mEH, cEH, and cGST were assayed in triplicate using partition assays with radiolabeled CSO or TSO as substrates (16). The cis isomer (CSO) was used to measure EH activity in microsomes since it is a better substrate for mEH, whereas the trans isomer (TSO), which is the better substrate for cEH, was used to measure EH activity in the cytosolic fraction. All assays were run under conditions of maximal velocity and reported values were corrected for nonenzymatic reaction. Protein concentrations used for each assay are shown in table 1. mEH activity was measured by the formation of the diol from CSO in 100 mM Tris-HCl at pH 9.0. All samples were incubated for 20 min at 37°C, then the unreacted epoxide was partitioned into isooctane and an aliquot of the aqueous layer was removed for quantitation by LSC. cEH activity was measured by the formation of the diol from TSO in 76 mM sodium phosphate buffer at pH 7.4. The cEH assay was modified by adding diethylmaleate (9) to the cytosolic preparations from all testes and rat livers, since they were found to have contaminating amounts of GSH. Samples were incubated at 37°C for 20 min, after which the unreacted epoxide was partitioned into isooctane.

## Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>mEH Assay</th>
<th>cEH Assay</th>
<th>cGST Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>0.05</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Monkey</td>
<td>0.4</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Rat</td>
<td>0.06</td>
<td>1.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.03</td>
<td>0.8</td>
<td>0.04</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>0.03</td>
<td>0.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Rat</td>
<td>0.04</td>
<td>1.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.02</td>
<td>0.03</td>
<td>0.015</td>
</tr>
</tbody>
</table>
and LSC was used to quantify an aliquot of the aqueous layer. cGST activity was assayed by the formation of GSH conjugate from CSO in 76 mM phosphate buffer containing 5 mM GSH. Samples were incubated at 37°C for either 5 min (mouse) or 10 min (human, monkey, and rat), then the epoxide and diol were partitioned into hexanol while the conjugate remained in the aqueous layer. An aliquot of the aqueous layer was then removed for quantitation by LSC.

Statistics. Data were log transformed to achieve equal variances. The analysis of variance was then employed followed by post-hoc comparison of means by Scheffe’s F test with p < 0.05 accepted as significant.

Results

PROD and EROD Activities. Enzyme activities in testicular microsomes were very low in all species for both substrates (table 2). PROD activity could not be detected in testicular preparations from any of the species, despite using protein concentrations of up to 2.0 mg/ml. In contrast, liver levels of PROD activity were readily detected in microsomes from rat and mouse. Although activity was detected in monkey liver, it was not quantifiable; that is, an increase in fluorescence was evident but it was not quantifiable since it was far below the standard curve (less than 1.25 pmol/min/mg protein). Human liver was not available for comparison. EROD activity could be measured in testicular microsomes from rodents, with the rat showing 3 times the activity found in the mouse. However, both monkey and human had levels that were not quantifiable (less than 0.08 pmol/min/mg protein). In contrast, the activity of this enzyme in liver from all species was high, with monkey levels approximately twice that found in mouse, and the mouse approximately twice the level in rat.

mEH, cEH, and cGST Activities. The potential for testicular detoxification of epoxides by mEH, cEH, and cGST is illustrated in fig. 1. Human and mouse had similar levels of testicular mEH activity. Monkey testis had significantly less mEH activity than all other species, whereas the rat was in an intermediate range that was statistically different from the other species. Testicular cEH activity was comparable in all species, with mouse having significantly less cEH activity than human or rat but not monkey.

TABLE 2

PROD and EROD activity in testicular and liver microsomes from different species

<table>
<thead>
<tr>
<th>Activity</th>
<th>Testis</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROD</td>
<td>pmol/min/mg microsomal protein</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt; 94.8 ± 9.7</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>ND 61.5 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>ND &lt;1.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>EROD</td>
<td>pmol/min/mg microsomal protein</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>0.523 ± 0.325 239 ± 20</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>1.520 ± 0.070 110 ± 4</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>&lt;0.08&lt;sup&gt;b&lt;/sup&gt; 476 ± 20</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>&lt;0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not detectable, no change in fluorescence.

<sup>b</sup> An increase in fluorescence was observed but was not quantifiable since it fell far below the standard curve. The limits of quantitation for the PROD and EROD assays were 1.25 and 0.08 pmol/min/mg protein, respectively.

FIG. 1. Species differences in testicular mEH, cEH, and cGST activity.

Each value represents the mean and SD of N = 5 individual animals or pools of tissue, except mouse testes in cEH and cGST assays, where N = 3 pools. No difference exists (p < 0.05) between species that have the same alphabetical superscript for a given enzymatic activity.

<sup>a</sup> Significantly different (p < 0.05) from <sup>b</sup> or <sup>c</sup>.<sup>b</sup> Significantly different (p < 0.05) from <sup>b</sup> or <sup>c</sup>.<sup>c</sup> Significantly different (p < 0.05) from <sup>b</sup> or <sup>c</sup>.<sup>d</sup> Not significantly different (p < 0.05) from either <sup>a</sup> or <sup>b</sup>.
The most marked species difference was seen in testicular cGST activity. Very low levels of testicular cGST were measured in human and monkey (less than 2 nmol/min/mg protein) in contrast with high levels in rat and mouse (101.6 and 58.6 nmol/min/mg protein, respectively).

For comparison, levels of hepatic enzyme activities are depicted in fig. 2. Comparisons to humans was not possible since human liver was unavailable. Hepatic mEH activity was highest in monkeys and significantly less in rats and mice. In contrast, hepatic cEH activity was highest in the mouse, followed by monkey, then rat, with all species showing statistically significant differences. Hepatic cGST activity was similar in rats and mice and significantly greater than in monkeys.

Discussion

In the present study, xenobiotic metabolizing enzyme activities measured were those associated with the two major classes of isozymes of the cytochrome P-450 mixed function monoxygenase system, cytochromes P-450 (phenobarbital-inducible) and cytochromes P-448 (3-methylcholanthrene-inducible), as well as those of EH and GST. Previous attempts to measure enzymatic activities of the cytochromes P-450 and/or P-448 isozymes in testis revealed low to no detectable amounts of activity using biphenyl (6), benzo(a)pyrene (2, 6–8), or ethoxycoumarin (8) as substrates. In the present study, we employed PrR and EtR as substrates, since the PROD and EROD assays are extremely sensitive and highly specific for cytochromes P-450 and P-448 isozymes, respectively (24, 26, 27).

No one species studied was a good model for humans in terms of the testicular xenobiotic metabolism of the substrates investigated (table 2, fig. 1). However, the monkey was the most similar to the human, with the exception of mEH, which was markedly lower in the nonhuman primate. Although liver generally possessed much higher levels of activity than testis, the patterns of xenobiotic metabolism in testis as compared to liver differed among species (table 2, figs. 1 and 2).

The most marked species difference was observed for testicular cGST activity between rodents and primates. In addition, when cGST activity is expressed per testis, rather than per mg protein, rats have significantly greater activity than do humans (1292 vs. 520 nmol/min/testis, respectively), despite the rat testis weighing 20-fold less than the human. The significance of these findings lies in the fact that metabolism of toxicants via GSH conjugation could play an important role in governing species susceptibility to testicular toxicants.

The data presented here on species differences in xenobiotic metabolism may be useful in explaining species differences in sensitivity to testicular toxicants. For example, DBCP is a potent testicular toxicant in humans, monkeys, rats, rabbits, and guinea pigs, but produced little or no testicular damage in mice and hamsters (5, 13–15). A metabolic basis has been suggested as the possible mechanism of species sensitivity (4, 5). Although the ultimate form of the testicular toxicant is unknown, there are data that indicate that DBCP may be metabolized in vivo by isozymes of cytochrome P-450 to a reactive epoxide that may undergo epoxide hydrolysis and conjugation to GSH (28, 29).

Although the data presented here on species differences in activities of xenobiotic metabolizing enzymes in the testis do not correlate with species sensitivity to DBCP-induced testicular toxicity, the data do support the contention that differences in metabolic capacity could play a role in species sensitivity to DBCP-induced testicular toxicity. Greater potential for hepatic activation, as measured by PROD activity, and lower potential for hepatic detoxification, as measured by cytosolic hydrolysis of the epoxide CSO, were seen in the sensitive species. Therefore, it is possible that the greater capacity for hepatic activation relative to detoxification may be involved in species sensitivity to DBCP-induced testicular toxicity.

**FIG. 2. Species differences in hepatic mEH, cEH, and cGST activity.**

Each value represents the mean and SD of $N = 5$ individual animals or pools of tissue, except rat liver in cEH and cGST assays, where $N = 4$ individual animals. $a,b,c,d$ As for fig. 1 different alphabetical superscripts indicate significant ($p < 0.05$) differences between species for a given enzymatic activity.
Mixed function monooxygenases are frequently involved in the metabolic activation of xenobiotics. Although reactions involving epoxide hydrolysis and GSH conjugation can in some instances activate xenobiotics, these reactions generally produce less reactive metabolites. To date, several monooxygenase enzyme activities have been measured in the testis, including biphenyl 4-hydroxylase (6), aryl hydrocarbon hydroxylase (2, 6–8), ethoxyquin O-dealkylase (8), and p-chloroethylammonium N-demethylase (7). The testicular levels of enzymatic activity are very low (pmol/min/mg protein) both in the monooxygenases previously investigated and those examined in the present study (PROD and EROD). It is intriguing that testicular levels of activity of EH and GST, enzymes that usually serve to detoxify xenobiotics, are much higher, generally at least three orders of magnitude higher, as substantiated by several authors (2, 8–10, 12). These quantitative differences in xenobiotic metabolizing enzymes may be a protective mechanism against toxic insult to the testis.

It should be noted that in the present study, enzyme activities were measured in subcellular fractions obtained from whole testis. The testis is composed of many cell types. The interstitium contains Leydig cells, their major function being steroidogenesis. Spermatogenesis takes place in the seminiferous tubules, which contain the germ cells at various stages of spermatogenesis, as well as Sertoli cells. Differential distribution of xenobiotic metabolizing enzyme activities have been shown to exist in the different testicular cell types. For example, rat spermatogenic cells possess twice the activity of mEH and GST, but only half the microsomal aryl hydrocarbon hydroxylase activity and cytochrome P-450 content of interstitial cells (30), and 7,12-dimethylbenz(a)anthracene metabolism occurs primarily in Leydig cells (1). Likewise, in humans, an isozyme of cytochrome P-450 has been shown to exist in Leydig cells, but not seminiferous tubules, by immunocytochemical examination of whole testis sections (31). Therefore, localized areas of high and/or low enzyme activity in a specific cell population could have a major influence on the potential toxic insult to the testis.

Although no one species studied here was a good model for humans in terms of testicular xenobiotic metabolism, we are gaining further insight as to the capabilities of testis from various species to metabolize xenobiotics. These data may be useful in elucidating mechanisms of action of species-specific testicular toxins. Additional biochemical and immunocytochemical studies are being conducted in rat and mouse to investigate the effect of inducing agents on the low levels of activity associated with the cytochromes P-450 and P-448 isozymes and to visualize the cellular localization of cytochrome P-450 isozymes within the testis.

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References


