Elucidation of Catalytic Specificities of Human Cytochrome P450 and Glutathione S-Transferase Enzymes and Relevance to Molecular Epidemiology

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A number of different approaches have been used to determine the catalytic selectivity of individual human enzymes toward procarcinogens. Studies with cytochrome P450 (P450) enzymes and glutathione S-transferases are summarized here, and recent work with pyrrolizidine alkaloids, aflatoxins, 4,4′-methylenebis(2-chloroaniline), and ethyl carbamate is discussed. In some cases a single enzyme can catalyze both activation and detoxication reactions of a chemical. The purification and characterization of human lung P450IA1 and the development of a noninvasive assay for human P4502E1 are also described.

Introduction

Most of the potentially genotoxic natural and synthetic chemicals to which humans are exposed are not biologically active in themselves but require transformation to reactive electrophiles (1). Metabolism of xenobiotic chemicals is complex, and various enzymes can activate or detoxify compounds, depending on the particular situation. Even different enzymes of a family [e.g., the cytochrome P450 (P450) group] can exert different influences by catalyzing different reactions. Levels of the various enzymes known to be involved in the metabolism of xenobiotics (2) often vary dramatically among individual people, owing to both genetic and environmental influences (3,4). There is considerable precedent from experimental animal models to hypothesize that these differences in enzyme levels may contribute to the overall risk of an individual to a particular chemical. Thus, discernment of the catalytic specificities of families of enzymes is of interest for risk assessment. If a dominant enzyme can be implicated in a particular reaction, then it may be further considered as a potential risk factor. In people with low levels of a particular enzyme, other enzymes may participate but their enzymatic contributions will not be so important if the overall activity is low (5).

Several in vitro approaches have been used in our laboratories to identify which procarcinogens are oxidized by individual human P450 enzymes and conjugated by human glutathione (GSH) S-transferases. The rationale and some recent studies are presented here, with consideration of the further development of noninvasive assays which can be used in molecular epidemiology biomarker studies with humans at risk (Table 1).

Rationale

At least five major approaches can be used in the assigning of catalytic activities to particular P450 enzymes (5): a) Selective inhibitors may be used with microsomes. The degree to which the activity is inhibited reflects the contribution of a particular P450, if the inhibitor is truly specific. This approach may also be of use with iso-
lated cells and in vivo. b) In a similar way inhibitory antibodies can be used with microsomes. Of course, this approach usually requires purification of an antigen to raise the antibody; hence this strategy has been employed in a limited number of cases. c) The catalytic activity can be measured in preparations (microsomes) derived from different individuals and compared to a known reaction catalyzed by a particular P450. The degree of associative attribution (the parameter \( r^2 \)) should reflect the fraction of the variation that can be ascribed to a relationship between the two activities, usually interpreted as being catalyzed by the same or similarly regulated enzymes. d) Purified enzymes can be compared directly for catalytic activity; however, some P450s do not appear to retain their full activities expressed in vivo after purification (6) and, of course, comparison of the activities of a limited set of enzymes has implications only among the set. e) A cDNA can be expressed in an artificial vector system, and catalytic activities can be assessed. Some eukaryotic cell lines can be used, with transient or stable expression.

The latter two approaches are very useful for distinguishing the activities of closely related enzymes, while the first three procedures provide better estimates of the extent to which a particular enzyme contributes to the overall activity. Given the relative advantages and disadvantages of each technique, the most reliable conclusions are derived with a battery of techniques as opposed to a single one. Such knowledge is of course hypothetical in its relevance and must be considered in light of findings in vivo. A current list of assignments of major human P450s involved in the activation of protocarcinogens and procarcinogens is presented in Table 2 (5).

Of course, many of these same general approaches can be applied to studies with other enzymes, with some modification; however, in no other case (other than the P450s) so extensive a menu of technical methods been applied in vitro, and often the noninvasive assays are more limited.

**Pyrrolizidine Alkaloids**

Senecionine has been studied as a prototype of the pyrrolizidine alkaloids, which can be toxic and carcinogenic in laboratory animals. Studies with rats have indi-

### Table 1. Potential noninvasive assays for human P450s.

| P450 | Assay end point                                                                 |
|------|--------------------------------------------------------------------------------|
| 1A2  | phenacetin O-deethylation                                                      |
| 2A6  | caffeine 3-demethylation                                                       |
| 2C10 (also 2C8, 2C9) | tolbutamide hydroxylation                                                      |
| 2D6  | debrisoquin 4-hydroxylation                                                    |
|       | nortriptyline 5-hydroxylation                                                 |
|       | dextromethorphan                                                               |
| 2E1  | chlorzoxazone 6-hydroxylation                                                 |
| 3A4  | purified antibodies                                                            |
|      | N-demethylation                                                                |
|      | benzphetamine                                                                  |
|      | N-demethylation                                                                |
|      | nifedipine hydroxylation                                                       |
|      | erythromycin N-demethylation                                                   |
|      | dapsone N-hydroxylation                                                        |
|      | lidocaine N-deethylation                                                       |
|      | cortisol 6-p-hydroxylation                                                     |

### Table 2. Protocarcinogens and procarcinogens activated by human P450s (5).a

| P450 | Protocarcinogens and procarcinogens | human P450s (5).a                      |
|------|-------------------------------------|---------------------------------------|
| 1A1  | benz[a]pyrene and other polycyclic hydrocarbons                              | 1A1                                   |
|      | 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine                             | 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine |
| 1A2  | 2-Acetylaminofluorene                                                          | 2-Acetylaminofluorene                 |
| 1A2  | 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ)                                   | 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) |
|      | 2-Amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQ)                             | 2-Amino-3,8-dimethylimidazo[4,5-f]quinoline (MeIQ) |
|      | 2-Amino-3,4,8-trimethylimidazo[4,5-f]quinoline (DiMeIQ)                       | 2-Amino-3,4,8-trimethylimidazo[4,5-f]quinoline (DiMeIQ) |
|      | 2-Amino-6-methylpyridine [2,2'-d]imidazole (Glu P-1)                          | 2-Amino-6-methylpyridine [2,2'-d]imidazole (Glu P-1) |
|      | 3-Amino-1-methyl-5H-pyridine [4,3-b] indole (Trp P-2)                         | 3-Amino-1-methyl-5H-pyridine [4,3-b] indole (Trp P-2) |
|      | 2-Aminodipyridine [1,2-a'3',2'-d]imidazole (Glu P-2)                         | 2-Aminodipyridine [1,2-a'3',2'-d]imidazole (Glu P-2) |
|      | 4-Nitroanisole (phenacetin)                                                   | 4-Nitroanisole (phenacetin)            |
|      | 4,4'-Methylenebis(2-chloroaniline) (MOCA)                                     | 4,4'-Methylenebis(2-chloroaniline) (MOCA) |
|      | N-Nitroso-N-ethylbenzylamine                                                   | N-Nitroso-N-ethylbenzylamine           |
|      | N-Nitrosomethylbutylamine                                                      | N-Nitrosomethylbutylamine              |
|      | benzene                                                                       | benzene                               |
|      | carbon tetrachloride                                                          | carbon tetrachloride                   |
|      | chloroform                                                                     | chloroform                            |
|      | methylene chloride                                                            | methylene chloride                    |
|      | trichloroethylene                                                             | trichloroethylene                     |
|      | ethylene dichloride                                                            | ethylene dichloride                   |
|      | ethylene dibromide                                                            | ethylene dibromide                    |
|      | 1,2-Dichloropropane                                                           | 1,2-Dichloropropane                   |
|      | styrene                                                                       | styrene                               |
|      | vinyl chloride                                                                | vinyl chloride                        |
|      | vinyl bromide                                                                 | vinyl bromide                         |
|      | acrylonitrile                                                                 | acrylonitrile                         |
|      | ethyl carbamate                                                               | ethyl carbamate                       |
|      | vinyl carbamate                                                               | vinyl carbamate                       |
|      | acetaminophen                                                                 | acetaminophen                         |
|      | 3A4                                                                           | 3A4                                   |
|      | aflatoxin B1                                                                  | aflatoxin B1                          |
|      | aflatoxin G1                                                                  | aflatoxin G1                          |
|      | sterigmatocystin                                                              | sterigmatocystin                      |
|      | senecionine                                                                   | senecionine                           |
|      | 7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene                                        | 7,8-Dihydroxy-7,8-dihydrobenzo[a]pyrene |
|      | 9,10-Dihydroxy-9,10-dihydrobenzo[a]fluoranthen                                  | 9,10-Dihydroxy-9,10-dihydrobenzo[a]fluoranthen |
|      | 3,4-Dihydroxy-3,4-dihydro-7,12-dihydrobenzo[a]lanthraene                     | 3,4-Dihydroxy-3,4-dihydro-7,12-dihydrobenzo[a]lanthraene |
|      | 6-Aminochrysene                                                               | 6-Aminochrysene                       |
|      | tris-(2,3-Dibromopropyl)phosphate                                             | tris-(2,3-Dibromopropyl)phosphate     |
|      | 1-Nitropyrene                                                                 | 1-Nitropyrene                         |
|      | 4,4'-Methylenebis(2-chloroaniline) (MOCA)                                     | 4,4'-Methylenebis(2-chloroaniline) (MOCA) |
|      | 1β-Estradiol                                                                  | 1β-Estradiol                          |

aThe purpose of this table is to summarize evidence for a major role of a single (human liver) P450 in the activation of each chemical. Of course, other P450s may contribute to some extent, particularly in individuals in whom the major enzyme is deficient. For further discussion, see Guengerich and Shimada (5).

bP4502A6 appears to make some contribution but does not appear to be the dominant enzyme in either case.
fore be raised as to whether higher levels of P450 3A4 are detrimental or protective.

**Aflatoxin B$_1$ Oxidation**

Aflatoxin B$_1$ (AFB$_1$) has several fates of relevance to its genotoxicity (Fig. 1). Our previous work led to the conclusion that, although several forms of human P450 can oxidize AFB$_1$ to its 8,9-oxide, P4503A4 plays a very major role in this reaction, due to both the intrinsic rate and the high levels of P4503A4 that can exist in human liver (12,13). In human fetal liver, the closely related P4503A7 appears to play a dominant role (14). The results of others have demonstrated that cDNA-expressed P4503A4 that has high activity (per nanomole P450) and that antibodies raised against P4503A enzymes are most inhibitory to activation in human liver microsomes (7,15). Any role for P4501A2 in bioactivation would appear to be minimal because of the stimulation seen with 7,8-benzoflavone in vitro (12,13) and the demonstrated detoxifying influence of rodent P4501A2 in vivo (13,16).

Studies in our laboratories have shown that when the activation of AFB$_1$ in human liver microsomes is examined by trapping the GSH conjugate (in the presence of mouse liver cytosol), the results are consistent with those in which *umu* gene response or DNA N$^7$-guanyl adducts are measured (12,13). For instance, GSH conjugate formation is well correlated with nifedipine oxidation in different microsomal preparations (Fig. 2). AFB$_1$ 3a-hydroxylation (to form AFQ$_1$) was also well correlated with nifedipine oxidation ($r^2 = 0.97$) [see also Forrester et al. (7)] and P4503A4 levels. Such ratios of GSH conjugate and AFQ$_1$ formation (Fig. 2) were seen at AFB$_1$ concentrations as low as 2μM; it appears that the $K_m$ for AFB$_1$ epoxidation is approximately 7 μM but that for AFB$_1$ 3-hydroxylation is much higher. Even at the low AFB$_1$ concentration, the results of immunoassay, chemical inhibition, and correlation experiments are consistent with the view that both AFB$_1$ 8,9-epoxidation and 3-hydroxylation are catalyzed primarily by P4503A4. In microsomes, however, 7,8-benzoflavone stimulated epoxidation but inhibited 3-hydro-
xylolation. This phenomenon was also demonstrated with purified yeast recombinant P4503A4 (Table 3) and is interpreted to reflect an allosteric effect on the enzyme which has at least two potential transition states. At low AFB₁ concentrations, below the $K_m$, the ratio of AFB₁: epoxide formation should approach a constant value. Whether the presence of more P4503A4 is detrimental or beneficial may depend upon the in vitro assay, and the in vivo consequences are not yet clear; however, a correlation between urinary excretion of $N^2$-guanyl AFB₁ adducts and the P4503A4 marker 6β-hydroxy cortisol (Table 1) has been seen in studies in the Gambia.

**GSH Conjugation of AFB₁ 8,9-Oxide**

In rats, biliary excretion of the GSH conjugate is a major fate of AFB₁ (17). Mice are thought to be protected from AFB₁ by their high rates of enzymatic conjugation of AFB₁ 8,9-oxide, and rats can be rendered resistant by treatment with compounds that induce GSH S-transferases (19). In work done in our laboratories, it appears that human liver cytosol has considerably less GSH S-transferase activity towards synthetic AFB₁ 8,9-oxide than rat liver cytosol. The consequences may be of significance in the evaluation of strategies for chemoprotection.

When a group of purified rat liver GSH S-transferases was examined, a $\mu$-class enzyme (4-4) (19) and an $\alpha$ class enzyme (1-1) were the most active. It is of considerable interest that what appear to be two different stereoisomeric forms of the GSH:AFB₁ 8,9-oxide conjugate are formed by different enzymes in both rat and human liver, and the $\alpha$ and $\mu$ enzymes form different products. In rats, the conjugation activity is induced by dithiothreitol, which induces $\alpha$ class enzymes AFB₁. Of the human enzyme classes examined, the activities varied in the order of $\mu > \alpha > 7$. The relevance of this specificity in vivo remains to be determined.

**4,4'-Methylenebis(2-chloroaniline)**

4,4'-Methylenebis(2-chloroaniline) (MOCA) can be carcinogenic in rodents and dogs and is of concern because of its use in the curing of polyurethane. Although many of the carcinogenic arylamines are oxidized to hydroxylamines, mainly by P450 1A2 (Table 2), MOCA has been known to be an exception (21). Recently, we purified P4502A6 from human liver—the enzyme activates AFB₁ but does not appear to play an important role because of its low level (<1% of total P450 (22)). Purified P4502A6 catalyzed MOCA

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**Table 3. Oxidation of aflatoxin B₁ (AFB₁) by human P4503A4.**

|        | AFQ₁ | AFB₁, 8,9-oxide: GSH conjugate |
|--------|------|-------------------------------|
| Yeast P450 | 286 ± 9  | 70 ± 1          |
| Yeast P4503A4 plus | 141 ± 13 | 260 ± 24 |
| 20 M 7,8-benzoflavone |       |                 |

*Yeast recombinant P4503A4 (0.2 μM) was incubated with rabbit liver NADPH-P450 reductase, L-α-dilauroyl-sn-glyceryl-3-phosphocholine, 0.1 M potassium phosphate buffer (pH 7.7), 50 μM AFB₁, mouse liver cytosol, and an NADPH-generating system for 30 min (liver P4503A4) or 60 min (yeast P4503A4). The amounts of AFQ₁ and glutathione S-transferase aflatoxin 8,9-oxide conjugate were determined by high-performance liquid chromatography.
6-hydroxylation of the muscle relaxant drug chlorozoxazone, at least as judged by studies in vitro (27). In collaborative studies with G. R. Wilkinson at Vanderbilt University, the usefulness of this reaction as a measure of P4502E1 in vivo is being considered. In the studies done to date, the conversion to 6-hydroxychlorozoxazone in vivo is inhibited by the P4502E1-selective agent disulfiram (24) and increased in individuals after withdrawal from consumption of large amounts of ethanol.

**Lung P4501A1**

Recently, what appears to be P4501A1 has been purified to electrophoretic homogeneity from human lung microsomes. The total amount of spectrally detectable P450 in human lung microsomes is very low, in the order of 10 pmole (P450)/mg protein. The properties of the enzyme are consistent with a role in the metabolism of several carcinogens. In samples with relatively high rates of oxidation of 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, the role of P4501A1 is a major one, but in samples with lower activity its role appears to be less dominant. P4501A2 seems to be absent in human lung microsomes, as judged by several criteria. Another human lung microsomal P450 was partially purified, but its relevance in carcinogen metabolism is unknown.

**Conclusions**

A number of approaches can be used to characterize the selectivity of individual human enzymes in vitro, and our current views regarding catalytic selectivity are summarized here. In vitro approaches and observations on the metabolism of drugs have led to the development of non-invasive assays which can be used to phenotype individuals for enzymes hypothesized to contribute to the metabolism of procarcinogens. In some cases, a single enzyme can both activate and detoxify a single chemical, and work in vivo will be required to evaluate the significance of variation in enzyme levels.

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