Species Susceptibility to Aflatoxin B\(_1\) Carcinogenesis: Comparative Kinetics of Microsomal Biotransformation

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ABSTRACT

The biotransformation of the potential human carcinogen aflatoxin B\(_1\) (AFB\(_1\)) was studied using hepatic microsomes from the rat, mouse, monkey, and human. Initial rates of AFB\(_1\) oxidation to aflatoxins Q\(_2\), M\(_1\), and P\(_1\), as well as the reactive intermediate AFB\(_1\)-8,9-epoxide, were determined using a high performance liquid chromatography assay. The rates of generation of these AFB\(_1\) metabolites were investigated at low substrate concentrations (more representative of environmental exposures) and also at high (“saturating”) concentrations commonly utilized in studies in vitro. Striking differences in ratios of the metabolites were observed. At an AFB\(_1\) concentration of 124 \(\mu\)M, mouse and monkey microsomes had the highest rates of AFB\(_1\)-8,9-epoxide formation. Primate liver microsomes formed aflatoxin Q\(_2\) in large amounts but failed to produce detectable aflatoxin P\(_1\). Determination of the rates of formation over initial AFB\(_1\), concentrations ranging from 15 to 475 \(\mu\)M revealed that the proportion converted to AFB\(_1\)-8,9-epoxide increased at lower substrate concentrations in the case of the rat and human microsomes but not with mouse or monkey microsomes. The differences in patterns of metabolite formation with varying concentrations have implications for interspecies comparisons of carcinogenic potency of AFB\(_1\).

INTRODUCTION

Aflatoxin B\(_1\) is a potent hepatocarcinogen in several animal species (1), and there is epidemiological evidence that it is involved in the induction of human liver cancer (2). Activation of AFB\(_1\) by microsomal cytochrome(s) P-450 is required for its carcinogenic effect, with formation of AFB\(_1\)-8,9-epoxide, leading to covalent binding to biological macromolecules (3, 4). The AFB\(_1\)-8,9-epoxide may also be conjugated enzymatically with GSH, constituting an important detoxification pathway (5). Microsomal oxidation of AFB\(_1\) can also result in formation of the metabolites AFQ\(_1\), AFM\(_1\), and AFB\(_1\), in each case producing a compound with substantially lower carcinogenic potency than the parent AFB\(_1\) (6, 7).

Biotransformation of AFB\(_1\) appears to be a major determinant of the potency of its effects. The amount of hepatic glutathione S-transferase activity toward AFB\(_1\)-8,9-epoxide is inversely related to the susceptibility of rodent species to AFB\(_1\)-induced hepatocarcinogenesis (5, 8). Primates appear to have low levels of glutathione S-transferase activity (9) but form detectable AFB\(_1\)-8,9-epoxide at higher rates than the parent compound. The AFB\(_1\)-8,9-epoxide metabolite may play a crucial role in AFB\(_1\) detoxification in primates. The amount of an AFB\(_1\) dose that ultimately binds to DNA will be determined by both the proportion converted to the epoxide and the fraction of the epoxide that is detoxified by conjugation with GSH.

In the case of a substrate that may be biotransformed by multiple enzymes (or isoenzymes), extrapolation of results obtained at saturating substrate concentrations to lower levels will be valid only if the apparent \(K_m\) values for each isoenzyme are equal. This is a critical point because tissue concentrations of a chemical due to typical environmental exposures are much lower than concentrations routinely used for in vitro experiments. Meaningful comparisons also demand that initial biotransformation rates be measured because multiple reaction pathways may be linear for differing periods of time.

We have determined the initial rates of biotransformation of AFB\(_1\) by hepatic microsomes from rats, mice, monkeys, and humans. The initial rates of biotransformation of AFB\(_1\) to the major oxidative metabolites were determined using a specific and sensitive HPLC assay (8), as well as the AFB\(_1\) concentration dependence of these rates for microsomes from each species. These studies provide useful results for comparisons of biotransformation differences between species and emphasize the important of kinetic considerations.

MATERIALS AND METHODS

Chemicals. Aflatoxins B\(_1\), Q\(_2\), M\(_1\), and P\(_1\); BHA, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, GSH, and Tris base were obtained from Sigma Chemical Co., St. Louis, MO. HPLC-grade solvents were from J. T. Baker, Inc., Phillipsburg, NJ. All other solvents and salts were analytical reagent grade from commercial sources.

Animals and Tissue Sources. Male Sprague-Dawley rats and male Swiss-Webster mice were obtained from Tyler Laboratories, Bellevue, WA. Livers from 2 male and 1 female adult *Macaca nemestrina* monkeys were obtained from the Regional Primate Research Center at the University of Washington. Livers were pooled from 3 rats and 18 mice for microsome preparation (12). Microsomes prepared from individual monkeys were used. Human liver samples were obtained from 9 organ donors through the University of Washington hospitals (7 male, 2 female, ages 14–46 years), frozen immediately in liquid N\(_2\), and stored at –80\(^\circ\)C until microsomes were prepared as previously described (12). Microsomes were also prepared from the livers of 5 additional organ donors obtained through the University of Arizona Medical Center (13) (2 male, 2 female, 1 not identified, ages 18–49 years) and were then stored at –80\(^\circ\)C until they were used. The drug history of the donors was as follows: no known drug treatment, 6; labetalol, 1; heavy alcohol use, 1; propranolol and heavy alcohol use, 1; no drug history was available for five of the donors. Results obtained from each of the two sources of pooled human microsomes were combined because no differences in AFB\(_1\) biotransformation specific activities (per mg microsomal protein) were observed.

Assays. Microsomal biotransformation of AFB\(_1\) was determined essentially as previously described (8). Incubations of AFB\(_1\) with microsomes (in duplicate) were carried out in the presence of hepatic cytosol from BHA-treated mice and GSH to trap AFB\(_1\)-8,9-epoxide as the GSH conjugate. The incubation mixtures included 2–3 mg BHA-treated mouse liver cytosolic protein/ml, 5 mM GSH, 5 mM glucose 6-phosphate, 0.5 unit/ml glucose 6-phosphate dehydrogenase, and 2 mM NADPH in a buffer containing 190 mM sucrose, 60 mM potassium phosphate, 80 mM Tris, 15 mM NaCl, 5 mM KCl, and 4 mM MgCl\(_2\) (pH 7.6 at room temperature). The AFB\(_1\), and microsomal protein concentrations used are given in the legends to the figures. The GSH conjugate, AFQ\(_1\), AFM\(_1\), and AFB\(_1\), were quantified by reversed-phase...
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HPLC using aflatoxin G₁ as an internal standard. Dilutions of AFB₁ in dimethyl sulfoxide were prepared for use in the substrate concentration dependence experiments so that equal volumes of the solvent (4% v/v) were always added to the incubation mixtures. The concentration of stock solutions of AFB₁ was determined by UV absorbance spectrophotometry, and substrate concentrations were calculated from these values (14). Protein concentrations were measured using the bicinchoninic acid assay (15).

RESULTS

Time Course of AFB₁ Oxidation by Microsomes. Fig. 1 shows the time dependence of formation of AFB₁ metabolites by hepatic microsomes from the rat, mouse, monkey, and human at a substrate concentration of 124 µM. For a given species, the duration of a linear rate of formation varied between metabolites. In the case of mouse microsomes, the production of AFQ₁ reached a plateau after just 15 min, whereas AFB₁-8,9-epoxide formation was linear for up to 30 min. Similarly, with human liver microsomes, the appearance of AFB₁-8,9-epoxide and AFM₁ was generally linear for 30 min, but AFQ₁ formation was appreciably nonlinear beyond 10 min. Deviation from linearity by 10 min was noted for all three metabolites formed by hepatic microsomes from monkey. With rat liver microsomes, the rates of formation of AFB₁-8,9-epoxide and AFQ₁ were parallel for the first 15 min of incubation, whereas at the later time points AFQ₁ formation approached a plateau but that of the epoxide did not.

The data shown in Fig. 1 demonstrate that AFB₁ metabolite formation rates determined from prolonged incubations can substantially underestimate initial rates. For all four metabolites, the rates determined from 30-min data averaged 70, 67, 27, and 72% of those calculated from 5-min data for rat, mouse, monkey, and human microsomes, respectively. In some cases, there were considerable differences between metabolites in the disparity between rates calculated with 5- and 30-min data. With human liver microsomes, data from 30-min incubations yielded rate values that were 99, 54, and 62% of those from 5-min incubations for AFB₁-8,9-epoxide, AFQ₁, and AFM₁, respectively.

These results illustrate the importance of using initial rate data in comparisons between metabolites and species. In most cases, rates of metabolite formation were approximately linear for the first 10 min of incubation time. In subsequent experiments, initial rates were calculated on the basis of the amount of metabolites formed in the first 5 or 10 min.

The drop in the amount of AFQ₁ found in incubations of AFB₁ with monkey microsomes observed after 20 min was accompanied by an increase in size of at least two as-yet-unidentified HPLC peaks (data not shown), suggesting the possibility of secondary oxidation of AFQ₁. Mouse microsomal incubations produced via secondary oxidation a metabolite previously identified as 4,9a-dihydroxy AFB₁ (16), denoted in Fig. 1 as AFM-P.

Comparison of Initial Rates of Microsomal Oxidation of AFB₁. Initial rates of formation of AFB₁-8,9-epoxide, AFQ₁, AFM₁, and AFP₁ are shown in Fig. 2. Liver microsomes from mouse, monkey, and human formed AFB₁-8,9-epoxide at initial rates 189, 150, and 40% of that of the rat, respectively. Initial rates of AFQ₁ formation by human and monkey liver microsomes were 7-8-fold higher than their respective rates of epoxide formation, whereas the rate of AFQ₁ formation in mouse microsomes was only 25% that of epoxide formation. The rate of AFM₁ formation was lower than that of epoxide for all four species (32, 60, 10, and 29% for the rat, mouse, monkey, and human liver microsomes, respectively).

Fig. 1. Time course of AFB₁ oxidation by hepatic microsomes from rat, mouse, monkey, and human. The assays were conducted as described in "Materials and Methods" with an initial AFB₁ concentration of 124 µM and containing 1.1, 0.77, 1.4, and 1.5 mg/ml of pooled microsomal protein for the rat (n = 3), mouse (n = 18), monkey (n = 3), and human (n = 14) microsomes, respectively. Points, average of values obtained from duplicate incubations of pooled microsomes. AFM-P, 4,9a-dihydroxy AFB₁.
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Fig. 2. Initial rates of AFB<sub>1</sub> oxidation by hepatic microsomes from rat, mouse, monkey, and human. The assays were conducted as described in the legend to Fig. 1 at 5- or 10-min incubations. Rates of AFB<sub>1</sub>-8,9-epoxide formation as a percentage of that observed with rat liver microsomes are also shown. The rates of formation of AFQ<sub>1</sub>, AFM<sub>1</sub>, and AFP<sub>1</sub>, were calculated as a percentage of the rate of epoxidation observed for the respective species; these values are shown above each column.

human, respectively). Formation of AFP<sub>1</sub>, occurred at a significant rate only with mouse microsomes. Oxidative demethylation of AFB<sub>1</sub> to form AFP<sub>1</sub>, did not occur or was below our limits of detection in both human and monkey microsomes. The sum of rates for the formation of these four microsomal metabolites was 2.0, 6.4, and 1.5 times that of the rat for mouse, monkey, and human, respectively.

The activation ratio, defined as the ratio of the rate of AFB<sub>1</sub>-8,9-epoxide formation to the sum of the rates of the oxidative metabolites observed (5), was calculated as a measure of the distribution of oxidative biotransformation between activation and deactivation pathways. The activation ratios were 0.454, 0.42, 0.11, and 0.12 for the rat, mouse, monkey, and human, respectively. These results, obtained at an initial AFB<sub>1</sub> concentration of 124 μM, are consistent with the hypothesis that primates are relatively resistant to the carcinogenic effects of AFB<sub>1</sub>, because of the large fraction of dose accounted for by AFQ<sub>1</sub> (11).

Microsomal Oxidation of AFB<sub>1</sub> with Varying Substrate Concentrations. The biotransformation of AFB<sub>1</sub> was measured in vitro with hepatic microsomes from rat, mouse, monkey, and human over the range of 15.6-478 μM. The metabolites were quantified after both 5- and 10-min incubations in order to verify the linearity of product appearance with time. The initial rates of AFB<sub>1</sub>-8,9-epoxide, AFQ<sub>1</sub>, AFM<sub>1</sub>, and AFP<sub>1</sub>, formation at different initial AFB<sub>1</sub> concentrations are shown in Fig. 3. It is apparent that AFB<sub>1</sub>, at 124 μM (used in previous work in this laboratory) was not a saturating concentration for the cytochrome(s) P-450 involved in the formation of AFQ<sub>1</sub> or, in the case of the rodent microsomes, AFB<sub>1</sub>-8,9-epoxide. While clearly not maximal, the rates of metabolite formation at 124 μM AFB<sub>1</sub> are generally representative of rates that would be observed at truly saturating substrate conditions.

Fig. 3. Concentration dependence of AFB<sub>1</sub> oxidation by hepatic microsomes from rat, mouse, monkey, and human. The incubation mixtures contained 1 mg/ml of microsomal protein, except for the mouse (0.77 mg/ml). AFB<sub>1</sub> concentrations ranged from 15.6 to 478 μM. Points, average of duplicate incubations.
The data in Fig. 3 suggest that enzyme systems with different AFB, affinities and maximal velocities are involved in formation of the various metabolites in each species. Reciprocal transformations of the data using Lineweaver-Burk, Eadie-Hofstee, or Hanes-Woolf plots revealed nonlinear kinetics in almost all cases (data not shown). These results indicate that multiple isoenzymes are involved in oxidation of AFB, to a given metabolite. The only exceptions were AFM, and AFP, formation by mouse microsomes, whereby each was produced by isozyme(s) with an apparent $K_m$ value of approximately 30 $\mu$M.

As an alternative assessment of the relative contribution of each pathway to the biotransformation of AFB, at different substrate concentrations, the initial rate for each metabolite, expressed as a fraction of the sum of total AFB, oxidation rates, is shown in Fig. 4. For monkey microsomes, the rate of formation of each metabolite was an essentially constant proportion of the total over the range of AFB, concentrations tested. The rates of AFM, and AFP, formation accounted for an increasing proportion of the rate sum as AFB, concentration decreased with mouse microsomes, whereas AFQ, and AFB,-8,9-epoxide rates represented a decreasing portion of the total. With rat microsomes, the rate of AFB,-8,9-epoxide formation accounted for an increasing proportion of the rate total as AFB, concentration decreased, whereas the rates for each of the other metabolites decreased. The rates of AFB,-8,9-epoxide and AFM, formation constituted an increasing proportion of the total at lower AFB, concentrations with human microsomes, whereas the AFQ, formation rate was a lower fraction.

The rate of AFB,-8,9-epoxide formation as a fraction of the total, as shown in Fig. 4, is equivalent to the activation ratio. Thus, the activation ratio increases as AFB, concentration decreases with rat and human microsomes, whereas the changes are much less pronounced for mouse and monkey microsomes.

Oxidation of AFB, by Human Microsomes at Low Substrate Concentrations. The observations for human microsomes were extended to lower AFB, concentrations in order to determine whether the trends seen in the range tested initially would be continued. The data shown in Fig. 4 for human microsomes were obtained at AFB, concentrations from 478 $\mu$M to 1.9 $\mu$M. It is apparent that the activation ratio increases with decreasing AFB, concentration over this range for human liver microsomes. While AFQ, was the predominant metabolite formed at AFB, concentrations above 16 $\mu$M, AFB,-8,9-epoxide accounts for over one-half of the total at concentrations below 8 $\mu$M.

These results show that the patterns of AFB, oxidation at doses encountered in dietary exposures or experimental carcinogenesis studies are quite different from those observed at the higher substrate concentrations typically used in biotransformation studies. In addition, the ways that these patterns change with varying AFB, concentration differ between species.

**DISCUSSION**

The biotransformation of AFB, is a complex multi-pathway process. In these studies, we have focused attention on the critical first steps of AFB, biotransformation, microsomal oxidations, that may help illuminate the mechanisms underlying species differences in the carcinogenic potency of AFB, as well as providing a basis for extrapolation of AFB, biotransformation patterns to low doses.

To be biochemically meaningful, these comparisons require determination of initial rates of reactions. The results in Fig. 1 show the limited duration of linear production of oxidative
metabolites of AFB₁. The importance of measuring initial rates is underscored by the observation that the linearity of AFB₁ oxidation with time depends on the metabolite measured. This approach is in contrast with many previous studies on microsomal AFB₁ biotransformation, in which incubation times of 15–40 min have been used (5, 11, 17–19). In addition, the method utilized in the present study provides accurate quantification of the production of AFB₁-8,9-epoxide (8), unlike the exogenous DNA binding assay, which provides unknown recovery of the epoxide.

The initial rate data obtained illustrate striking differences in the capacity to oxidize AFB₁ between microsomes from different species. When measured at a relatively high AFB₁ concentration, AFB₁-8,9-epoxide was the major metabolite produced by rodent microsomes. In contrast, AFQ₁ is the predominant species. When measured at a relatively high AFB₁ concentration, AFB₁-8,9-epoxide was the major metabolite produced in vitro. Inactivation of production of AFB₁-8,9-epoxide (8), unlike the exogenous DNA binding assay, which provides unknown recovery of the epoxide.

In contrast, humans appear to have a high capacity for GSH conjugation with AFB₁-8,9-epoxide (11); therefore, the potential role of AFQ₁ formation as a detoxification pathway is of greater importance.

Quantification of multiple microsomal biotransformation pathways at a single substrate concentration defines behavior of the system as fractions of the maximal velocities of the reactions. If the affinities for the substrate (AFB₁) of the enzymes involved are equal, then there will be no variation in relative rates with substrate concentration. However, if substrate affinities vary between enzymes which catalyze different pathways, the pattern of biotransformation will change with substrate concentration. The picture will be additionally complicated, if multiple enzymes with different substrate affinities are involved in the formation of a single metabolite. In vitro studies typically use AFB₁ concentrations much higher than those that would be expected from dietary exposure (5, 17). Binding of AFB₁ to rat liver DNA was linear with dose (i.p.) over the range of 10–1000 ng/kg (21), providing evidence that activation of AFB₁ can occur at very low levels. Liver AFB₁ concentrations of 0.1–10 nm were measured in that study, comparable with those that may occur in humans with dietary exposure.

The only AFB₁ oxidation pathways in mouse microsomes that appeared to be catalyzed by single cytochrome P-450 isozymes were those responsible for formation of AFM₁ and AFP₁. AFM₁ has been shown to be produced by mouse cytochrome P₄₅₀ (22). In all of the other cases, multiple liver cytochrome P-450 isozymes appear to be involved in the oxidation of AFB₁. With such overlapping isozyme specificity for production of a given metabolite, meaningful Michaelis-Menten kinetic parameters could not be obtained, so we sought an alternative approach to describe the behavior at different AFB₁ concentrations. Expression of each metabolic rate as a fraction of the rate sum, as shown in Fig. 4, clearly demonstrates dose dependent changes in liver microsomal biotransformation of AFB₁. AFQ₁, by rat, mouse, and human microsomes by an isozyme(s) with relatively low affinity for AFB₁. Rat cytochrome P-450p has been implicated in the formation of AFQ₁ from AFB₁ by liver microsomes (23). There are isoenzymes present in rat and human liver microsomes that show high affinity for AFB₁ and convert it to the 8,9-epoxide. Recent work using purified liver cytochromes P-450 has shown that both species have multiple isoenzymes with activity for AFB₁ activation (24, 25). The formation of AFM₁ by human liver microsomes is catalyzed by a high affinity enzyme(s) (Fig. 4). The fraction of AFB₁ activated by human liver microsomes, represented by the activation ratio, increases with increasing AFB₁ concentration due to the high affinity of a cytochrome P-450 that forms AFB₁-8,9-epoxide combined with the low AFB₁ affinity of that which forms AFQ₁. Since human liver cytosolic glutathione S-transferase appears to have little capacity for conjugation of AFB₁-8,9-epoxide (11), our results suggest that based on the activation ratio at low AFB₁ concentrations, human liver tissue may be relatively efficient at generating AFB₁-DNA adducts when exposed to dietary levels of AFB₁.

We are currently investigating species differences in liver cytosolic glutathione S-transferase activity toward AFB₁-8,9-epoxide with the aim of quantifying the inactivation of the epoxide formed by microsomal oxidation. This appears to be another important determinant of susceptibility to AFB₁ toxicity (5, 20).

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