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Evidence for cytochrome P-450$_{NF}$, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver

(mutagenicity/drug metabolism/chemical carcinogens)

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ABSTRACT ***In vitro*** **studies with human liver indicate that the major catalyst involved in the bioactivation of the hepatocarcinogen aflatoxin B$_1$ (AFB$_1$) to its genotoxic 2,3-epoxide derivative is cytochrome P-450$_{NF}$ (P-450$_{NF}$), a previously characterized protein that also catalyzes the oxidation of nifedipine and other dihydropyridines, quinidine, macrolide antibiotics, various steroids, and other compounds. Evidence was obtained using activation of AFB$_1$ as monitored by *umuC* gene expression response in *Salmonella typhimurium* TA1535/pSK1002 and enzyme reconstitution, immunochemical inhibition, correlation of response with levels of P-450$_{NF}$ and nifedipine oxidase activity in different liver samples, stimulation of activity by 7,8-benzoflavone, and inhibition of activity by troleandomycin. Similar results were obtained when levels of 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B$_1$ formed in DNA were measured. P-450$_{NF}$ or a closely related protein also appears to be the major catalyst involved in the activation of aflatoxin G$_1$ and sterigmatocystin, the latter compound being more genotoxic than AFB$_1$ in these systems. Several drugs and conditions are known to influence the levels and activity of P-450$_{NF}$ in human liver, and the activity of the enzyme can be estimated by noninvasive assays. These findings provide a test system for the hypothesis that a specific human disease state (liver cancer) is linked to the level of oxidative metabolism in populations in which aflatoxin ingestion is high.**

Aflatoxin B$_1$ (AFB$_1$) is one of the most potent hepatocarcinogens known, and environmental contamination by various aflatoxins is a serious problem in many parts of the world (for review, see ref. 1). The prototype AFB$_1$ is not particularly biologically active in its native form, and oxidation is necessary for interaction with DNA and biological damage (1–6).

Recently, a number of attempts have been made to link the incidence of various tumors in humans to levels of activity of specific drug-metabolizing enzymes (7–9). With regard to the specific forms of cytochrome P-450 involved in AFB$_1$ activation, there is ambiguity in assignment of the involved rat liver P-450s in the literature (see refs. 9 and 10 and references therein) and these studies do not point clearly to human orthologs. If the form of human P-450 involved in aflatoxin bioactivation could be identified and appropriate *in vivo* assays could be devised in populations in which aflatoxin exposure is high (11), then the general hypothesis concerning cancer risk might be tested. In this report, we provide evidence that P-450$_{NF}$, the previously characterized nifedipine oxidase (12), is the main catalyst of bioactivation of two major aflatoxins, AFB$_1$ and AFG$_1$, and the related naturally occurring carcinogen sterigmatocystin (STG). Levels of the enzyme vary widely among humans and can be readily monitored (13, 14).

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MATERIALS AND METHODS

Human liver samples were obtained from organ donors through the Nashville Regional Organ Procurement Agency and microsomes were prepared. Human P-450$_{NF}$ (12),† P-450$_{MP-1}$ (22), P-450j (23), P-450$_{PA}$, P-450$_{DB}$ (24), cytochrome b_5 (22), and rabbit liver NADPH–P-450 reductase (22) were prepared as described elsewhere; all preparations were electrophoretically homogeneous. Antibodies were raised in rabbits (25), including anti-rat P-450$_{ISF-G}$ and anti-rat P-450$_{UT-H}$ (26), and immunoglobulin G fractions were used here.

AFB$_1$, AFG$_1$, STG, and 7,8-benzoflavone were purchased from Aldrich. 2,3-Dihydro-2-(N^7-guanyl)-3-hydroxyalflatoxin B$_1$ (AFB$_1$-N^7-Gua) was prepared by K. D. Raney and T. M. Harris (Vanderbilt University) and troleandomycin was a gift of D. J. Waxman (Harvard University).

The activation of *umu* gene expression by metabolites of aflatoxins in liver microsomal and reconstituted P-450 systems was determined by using the tester strain *Salmonella typhimurium* TA1535/pSK1002 as described elsewhere (27, 28). Briefly, the assay includes a microsomal or P-450-based oxidation system that activates a particular procarcinogen in the presence of a *umuC'lacZ* fusion gene. The chimeric fusion gene is harbored in the plasmid pSK1002, which is carried in *S. typhimurium* TA 1535; the bacterium is permeable and devoid of repair enzymes (4) and acts only as a carrier. The *umu* operon normally plays a role in the SOS response in *Escherichia coli*; after alkylation by ultimate carcinogens in this system, the *lacZ* gene produces increased levels of β-galactosidase, which is detected spectrophotometrically by using the substrate *o*-nitrophenyl β-D-galactopyranoside. The details and advantages of this system over other genotoxicity assays are described elsewhere (27–29). In general, the activation system used here included human liver microsomes containing 10–30 pmol of P-450; 50 mM potassium phosphate buffer (pH 7.4); AFB$_1$, AFG$_1$, or STG; bacteria; and an NADPH-generating system in a total vol of 1.0 ml; incubations proceeded at 37°C for 120 min. Activity is expressed in β-galactosidase units (30) per min per mg of protein or nmol of P-450. A high degree of correlation between AFB$_1$ activation as measured by the *umu* system and *S. typhimurium* reverse mutation (4) has been demonstrated (31). The substrate concentrations used were 10 μM for AFB$_1$ and STG and 20 μM for AFG$_1$; these concentrations were

Abbreviations: P-450, liver microsomal cytochrome P-450; AFB$_1$, aflatoxin B$_1$; AFG$_1$, aflatoxin G$_1$; STG, sterigmatocystin; AFB$_1$-N^7-Gua, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B$_1$.

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†The protein P-450$_{NF}$ has been characterized (12). The sequences of the major cDNA (15) and two minor cDNAs (16) are known. An organizational scheme puts the genes in the P450IIIA3 family (17). Some related preparations from other laboratories include P-450HLFa (18) and P-450p (19). For recent reviews of human P-450s, see refs. 20 and 21.

found to be saturating as judged by *umu* response at P-450 concentrations of 10–50 μM (10). The formation of DNA adducts by liver microsomes and HPLC assay of released AFB_1-N^7-Gua were based on procedures described by Essigman *et al.* (5), using 1 μM P-450 and 20 μM AFB_1 in incubations and a period of 60 min (37°C), during which the formation of DNA adducts was found to be linear with respect to time using rat liver microsomes. Nifedipine oxidation and levels of $P\text{-}450_{NF}$ were estimated by procedures described elsewhere (12).

RESULTS

Effects of Polyclonal Antibodies on Metabolic Activation of AFB_1, AFG_1, and STG in Human Liver Microsomes. Several human liver microsomal preparations showed considerable activity in catalyzing metabolic activation of AFB_1, AFG_1, and STG to genotoxic metabolites, which caused induction of *umu* gene expression in *S. typhimurium* TA1535/pSK1002. With five liver samples, the order of genotoxicity was found to be STG > AFB_1 > AFG_1. Since sample HL 110 contained the greatest activity for all three carcinogens, the effects of specific antibodies to P-450s were first examined with this preparation. Fig. 1 shows that polyclonal antibodies raised against $P\text{-}450_{NF}$ inhibited the microsomal activation of all three aflatoxins. The other antibodies tested had little or no effect on these reactions. In other experiments (not shown), anti-$P\text{-}450_{NF}$ (added at 20 mg per nmol of microsomal P-450) reduced the formation of AFB_1-N^7-Gua to 8% of the control level while the addition of the same amount of preimmune antibody decreased the level of adduct by <10%.

Correlation of Amounts of $P\text{-}450_{NF}$ and Nifedipine Oxidation with Aflatoxin Activation of Microsomes. Fig. 2 shows that there were good correlations between levels of immunochemically determined $P\text{-}450_{NF}$ and rates of metabolic activation of AFB_1, AFG_1, and STG to reactive metabolites in these microsomes. Correlation coefficients (r) in these reactions were all ≥0.8. In addition, similar correlation with rates of nifedipine oxidation was also observed in every case. Included with the liver microsomal preparations assayed were samples that were among the highest in levels of P-450j, $P\text{-}450_{MP}$, $P\text{-}450_{PA}$, and $P\text{-}450_{DB}$ measured in this laboratory, as judged by both marker activities (21) and immunochemical analysis. None of these samples was unusually proficient in the activation of aflatoxins or STG.

Activation of AFB_1, AFG_1, and STG by Purified $P\text{-}450_{NF}$. Table 1 (Exp. 1) shows activation of the aflatoxins by a reconstituted monooxygenase system containing purified human $P\text{-}450_{NF}$. $P\text{-}450_{NF}$ converted AFB_1, AFG_1, and STG to genotoxic products. Omission of P-450, NADPH–P-450 reductase, or the NADPH-generating system from the reaction mixture abolished the AFB_1 activation, but cytochrome b_5 was not an essential component.

On a molar P-450 basis, the reconstituted $P\text{-}450_{NF}$ system gave a higher turnover number for AFB_1 than did the

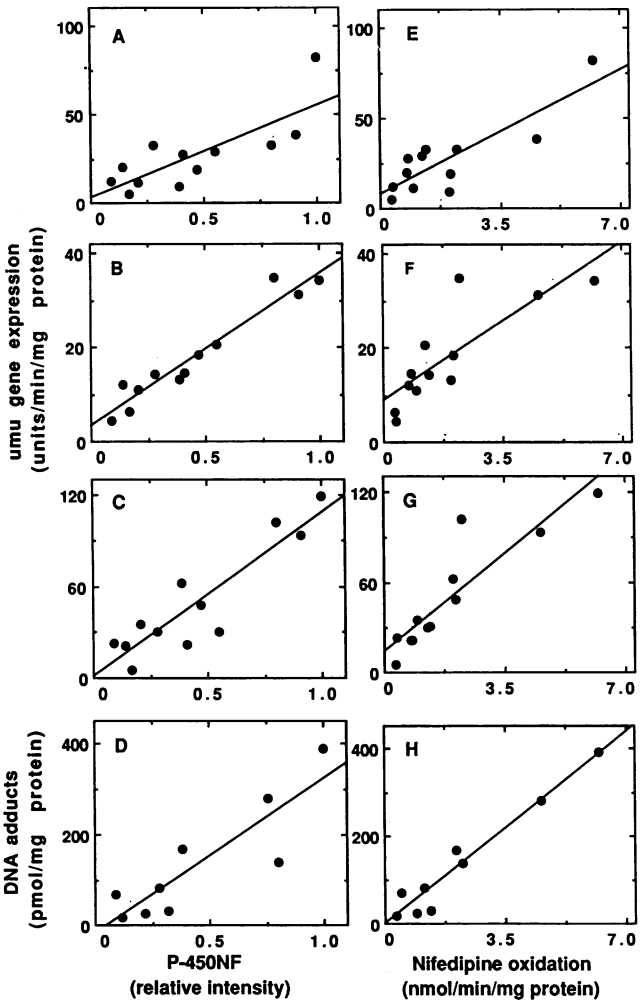

FIG. 2. Correlation between genotoxic activation of AFB_1 (*A* and *E*), AFG_1 (*B* and *F*), and STG (*C* and *G*) or formation of AFB_1-N^7-Gua DNA adducts (*D* and *H*) versus $P\text{-}450_{NF}$ content (*A–D*) or nifedipine oxidase activity (*E–H*) in different human liver microsomes. The respective correlation coefficients (r) obtained in these relations were as follows: *A*, 0.80; *B*, 0.97; *C*, 0.90; *D*, 0.88; *E*, 0.85; *F*, 0.83; *G*, 0.90; *H*, 0.97.

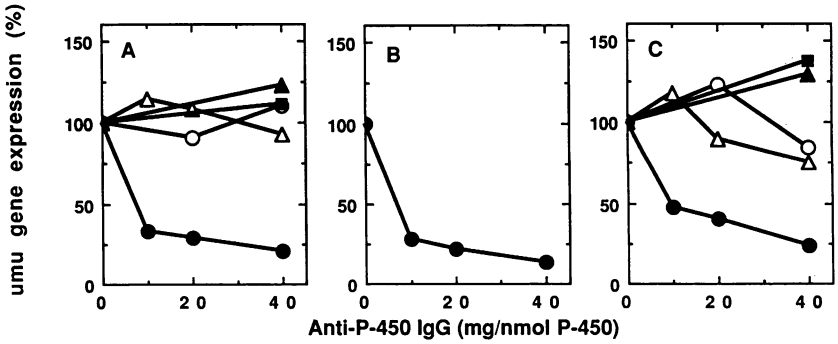

FIG. 1. Inhibition of activation of AFB_1 (*A*), AFG_1 (*B*), and STG (*C*) to genotoxic products in human liver microsomes (sample HL 110) by anti-$P\text{-}450_{NF}$. The uninhibited catalytic activities (in units per min per nmol of P-450) were 240 for AFB_1, 71 for AFG_1, and 1044 for STG. The metabolic activation was measured in the presence of anti-$P\text{-}450_{NF}$ (●) (12); anti-$P\text{-}450_{MP}$ (○) (21); anti-P-450j (△) (22); anti-$P\text{-}450_{UT\text{-}H}$ (▲), an inhibitor of $P\text{-}450_{DB}$ (23, 25); or anti-$P\text{-}450_{ISF\text{-}G}$ (■), an inhibitor of $P\text{-}450_{PA}$ (23). In all cases, preimmune IgG was without effect on the reaction (results not shown). The amounts of P-450j, $P\text{-}450_{MP\text{-}1}$, and $P\text{-}450_{NF}$ present were estimated immunochemically at 0.03, 0.14, and 0.45 nmol per mg of protein, respectively; total spectrally determined cytochrome P-450 was 1.57 nmol per mg of protein in this sample.

Table 1. Metabolic activation of AFB_1, AFG_1, and STG in human liver microsomes and reconstituted P-450 systems

Exp. 1	*umu* gene expression, units per min per nmol of P-450			Exp. 2	AFB_1 activation, units per min per nmol of P-450	
	AFB_1	AFG_1	STG		− 7,8-benzoflavone	+ 7,8-benzoflavone (6 μM)
Liver microsomes (n = 5)	168 ± 88	44 ± 16	393 ± 434	$P\text{-}450_{NF}$	394 ± 29	1535 ± 202
Reconstituted system				$P\text{-}450_{MP\text{-}1}$	167 ± 43	192 ± 38
Complete	418 ± 68	36 ± 7	249 ± 113	P-450j	125 ± 32	182 ± 26
− $P\text{-}450_{NF}$	<10	ND	ND	$P\text{-}450_{DB}$	56 ± 10	87 ± 16
− NADPH–P-450 reductase	<10	ND	ND			
− NADPH	<10	ND	ND			
− cytochrome b_5	381 ± 10	ND	ND			

Results are expressed as means of triplicate determinations ± SD unless indicated otherwise. The reconstituted systems contained 10 pmol each of P-450 and NADPH–P-450 reductase, 20 pmol of cytochrome b_5, and 7.5 nmol of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine. ND, not determined.

microsomal samples. When the AFG_1 and STG were used as substrates, the $P\text{-}450_{NF}$ activity was somewhat less.

Effect of 7,8-Benzoflavone and Troleandomycin on Metabolic Activation of AFB_1. Several flavonoids, including 7,8-benzoflavone, have been reported to enhance microsomal benzo[*a*]pyrene and AFB_1 oxidation (32). Fig. 3 shows the effect of 7,8-benzoflavone on the metabolic activation of AFB_1 by liver microsomes and by purified $P\text{-}450_{NF}$. A 2-fold stimulation was observed in the microsomal system (maximum effect at 125 μM 7,8-benzoflavone), and 7,8-benzoflavone stimulated microsomal nifedipine oxidase activity by ≈50% (data not shown). Activity toward AFB_1 was enhanced 4-fold when 6 μM 7,8-benzoflavone was added to the reconstituted $P\text{-}450_{NF}$ system (Fig. 3*B*).

Table 1 (Exp. 2) shows the metabolic activation of AFB_1 in reconstituted monooxygenase systems containing four forms of human P-450. In the absence of 7,8-benzoflavone, $P\text{-}450_{NF}$ had the highest activity, followed by $P\text{-}450_{MP\text{-}1}$, P-450j, and $P\text{-}450_{DB}$ (cf. Fig. 1). The effect of this flavonoid on the metabolic activation of AFB_1 by $P\text{-}450_{NF}$ was much greater than in the case of any other P-450.

The antibiotic troleandomycin is known to rather specifically inhibit $P\text{-}450_{NF}$ and related enzymes after oxidation to a nitroso derivative, which complexes the P-450 heme (33–35). As shown in Table 2, preincubation of human liver microsomes with troleandomycin decreased both rates of nifedipine oxidation and AFB_1-N^7-Gua formation.

DISCUSSION

The assignment of AFB_1 bioactivation to human $P\text{-}450_{NF}$ is based on the following pieces of evidence. Purified $P\text{-}450_{NF}$ had activity (measured using *umuC* gene activation), on a molar P-450 basis, greater than human liver microsomes. Antibodies highly specific for $P\text{-}450_{NF}$ inhibited activation when added to microsomes, which contain all P-450s capable of activating AFB_1. 7,8-Benzoflavone enhanced AFB_1 bioactivation and nifedipine oxidation, and oxidation of troleandomycin inhibited both activities. Finally, AFB_1-dependent *umuC* gene expression was well correlated with levels of $P\text{-}450_{NF}$ protein and nifedipine oxidation in various human liver samples—in other experiments (not presented), no correlation was observed with levels of $P\text{-}450_{DB}$ $P\text{-}450_{PA}$, $P\text{-}450_{MP}$, or P-450j. Levels of AFB_1-N^7-Gua formed in DNA were also correlated with $P\text{-}450_{NF}$ and anti-$P\text{-}450_{NF}$ lowered rates of AFB_1-N^7-Gua formation. Epidemiological evidence has been interpreted to indicate a role of $P\text{-}450_{DB}$, the debrisoquine hydroxylase, in AFB_1-linked liver cancer (36), but these and previous results (26) argue strongly against the hypothesis.

The results explain some observations previously reported in the literature concerning the effects of 7,8-benzoflavone and other flavones. The mutagenicity of AFB_1 is enhanced by 7,8-benzoflavone (and other flavones) in some human liver microsomal samples but not others (32). Rabbit P-450 3c, an ortholog of $P\text{-}450_{NF}$, is stimulated by 7,8-benzoflavone (37) and recently Schwab *et al.* (38) reported that, in addition to P-450 3c, human liver microsomal estradiol 2-hydroxylase activity is enhanced by 7,8-benzoflavone, with the effect being due to a decrease of the K_m. Estradiol 2-hydroxylation is mediated by $P\text{-}450_{NF}$ (12), and one might expect other $P\text{-}450_{NF}$-linked activities to show this response to flavones. $P\text{-}450_{NF}$ is encoded by a multigene family (15, 16) and the caveat should be presented that proteins closely related to $P\text{-}450_{NF}$ might also exist (see ref. 18) and also activate aflatoxins and be stimulated by flavones. Finally, the obser-

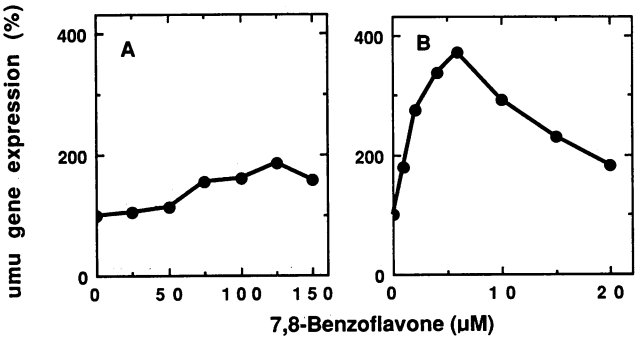

FIG. 3. Effects of 7,8-benzoflavone on genotoxic activation of AFB_1 in liver microsomes (*A*) and $P\text{-}450_{NF}$-containing reconstituted monooxygenase system (*B*). Control activities (units per min per nmol of P-450) without the flavone were 246 for liver microsomes (sample HL 110) and 418 for the $P\text{-}450_{NF}$ system.

Table 2. Inhibition of catalytic activities by troleandomycin

Components present in preincubation	% catalytic activity	
	Nifedipine oxidation	AFB_1-N^7-Gua formation
Control	(100)	(100)
NADPH	90 ± 3	
Troleandomycin	99 ± 8	
Troleandomycin and NADPH	37 ± 6	55 ± 13

Human liver microsomes (sample HL 110) were incubated with the indicated components [1–4 μM P-450, 20 μM troleandomycin, 50 mM potassium phosphate (pH 7.4), and an NADPH-generating system containing final concentrations of 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1.0 unit of yeast glucose-6-phosphate dehydrogenase per ml] at 37°C for 30 min. The original incubate was diluted 1:20 with buffer containing more of the NADPH-generating system, 50 mM Tris·HCl (pH 7.7), and either nifedipine (200 μM) or AFB_1 (40 μM) plus calf thymus DNA (1 mg/ml). The 100% catalytic activity values were 6.3 (±0.5) nmol of nifedipine oxidized per min per nmol of P-450 and 333 (±85) pmol of AFB_1-N^7-Gua formed per hr per nmol of P-450.

vation that P-450$_{NF}$ and its rat and rabbit orthologs often show lower catalytic activity than expected after purification should be mentioned (see refs. 12, 19, 21, 34). The response of P-450$_{NF}$ to flavones has, in these experiments, been considerably greater in the purified state than in microsomes and the possibility could be considered that an endogenous activator is being removed during purification.

P-450$_{NF}$ levels vary widely among humans, apparently in a broad unimodal distribution (14). Several lines of *in vivo* and *in vitro* evidence indicate that the enzyme can be induced by barbiturates, certain steroids, and macrolide antibiotics (19). A noninvasive assay involves measurement of urinary 6β-hydroxycortisol levels‡ (39); alternatively, the pharmacokinetics of nifedipine metabolism may be examined *in vivo* (13, 14). P-450$_{NF}$ oxidizes a rather wide variety of substrates (21, 40), and elevated levels of the enzyme appear to be involved in undesirable interactions with 17α-ethynylestradiol (35). It is possible that elevated levels of P-450$_{NF}$ may predispose individuals to AFB$_1$-induced liver tumors. However, the point should be considered that phenobaribital administration in rats decreases AFB$_1$ adduct levels (*in vivo*) and tumor formation (6, 41, 42), apparently by increasing glutathione conjugation—such a complicating factor might be considered here.

Finally, it is of interest that P-450$_{NF}$ also appears to be the main enzyme involved in the activation of all of the compounds examined, the two aflatoxins and STG. STG consistently yielded a greater *umu* gene response than AFB$_1$ in human liver microsomal samples (Fig. 2, Table 1). In rats, STG has been reported to be an order of magnitude less carcinogenic than AFB$_1$ (43). However, in *S. typhimurium* reversion assays utilizing rat liver activation systems, AFB$_1$ is more mutagenic than STG in strain TA 98 but STG is more potent in TA 100 (4, 44). These results are surprising in consideration of the similar structure and adducts formed by AFB$_1$ and STG (45). Elucidation of the basis of the genetic response to STG may provide insight into mechanisms of mutagenesis by aflatoxins and similar compounds.

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