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A mechanism of inhibition of aflatoxin B_1 hepatocarcinogenesis by β-naphthoflavone pretreatment of rats

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Abbreviations: AFB_1, aflatoxin B_1; AFB_1-epoxide, aflatoxin B_1-8,9-oxide; AFQ_1, aflatoxin Q_1; AFM_1, aflatoxin M_1; AFP_1, aflatoxin P_1; AFB_1-SG, aflatoxin B_1-glutathione conjugate; BNF, β-naphthoflavone; GST, glutathione S-transferase; PB, phenobarbital

Abatract

Pretreatment with β-naphthoflavone (BNF) inhibits aflatoxin B_1 (AFB_1) hepatocarcinogenesis in the rat by stimulation of aflatoxin M_1 (AFM_1) hydroxylation. We have now examined the effect of BNF pretreatment of rats on AFB_1-hydroxylations, AFB_1-DNA binding and AFB_1-glutathione (AFB_1-SG) conjugation in liver *in vitro, in vivo* and in isolated hepatocytes. Male Fischer rats were injected i.p. with either corn oil or BNF (25 mg/kg body weight) 24 h before for *in vitro* and in isolated hepatocyte studies. Cytochrome P-450 contents in BNF-treated rat liver microsomes and hepatocytes were 170% and 200% of controls, respectively. At two AFB_1 concentrations (2 μM and 100 μM), AFB_1-epoxidation measured as AFB_1-DNA binding and AFM_1 hydroxylation were 60% and 300% of controls, respectively. At low AFB_1 level (33 nM), AFB_1-binding and AFM_1 formation in BNF-treated hepatocytes were 40% and 225% of controls, respectively. Formation of AFB_1-SG, aflatoxin Q_1 and aflatoxin P_1 were not affected significantly. Hepatic nuclear AFB_1-DNA binding 2 h after i.p. injection of 0.4 mg [^{3}H]AFB_1/kg body wt. in BNF-treated rats was 60% of controls. The overall data indicate that lower AFB_1-DNA binding in addition to higher inactivation via AFM_1 hydroxylation are responsible for the inhibition of AFB_1 hepatocarcinogenesis by BNF pretreatment of rats. It is suggested that this inhibition of AFB_1-DNA binding may be a direct result of lower levels of constitutive cytochromes P-450 responsible for AFB_1-epoxidation.

Keywords: aflatoxin B_1-DNA binding; AFB_1-epoxide; aflatoxin M_1 hydroxylation; β-naphthoflavone

Introduction

Epidemiological data have indicated that contamination of food with aflatoxin B_1 (AFB_1) may be an important etiological factor in human liver cancer in several parts of Africa and Asia (Van Rensburg *et al.*, 1985; Autrup *et al.*, 1987; Ross *et al.*, 1992). Rat has been shown to be a very sensitive laboratory animal species to AFB_1 hepatocarcinogenesis (Newberne and Butler, 1969). It is well established that AFB_1 undergoes cytochrome P-450 dependent metabolic activation to form an ultimate reactive metabolite, AFB_1-8,9-oxide (AFB_1-epoxide) which interacts covalently with cellular macromolecules including DNA. It is believed that AFB_1-DNA interaction is responsible for the initiation of AFB_1 hepatocarcinogenesis (Miller and Miller, 1977; Essigmann *et al.*, 1977). In addition to AFB_1-epoxidation, AFB_1 is oxidized via cytochrome P-450 dependent enzymes to several hydroxylated metabolites viz. aflatoxin Q_1 (AFQ_1), aflatoxin M_1 (AFM_1) and aflatoxin P_1 (AFP_1) which are non carcinogenic (Wong and Hsieh, 1976; Gurtoo *et al.*, 1978).

Carcinogenicity of AFB_1 is inhibited by pretreatment of rats with either phenobarbital (PB), several antioxidants or β-naphthoflavone (BNF). This inhibition is shown to be due to induction of either cytochromes P-450 phase I enzymes, or glutathione S-transferases (GSTs) phase II enzymes or both (Gurtoo and Dahms, 1979; Degen and Neumann, 1981; Gurtoo *et al.*, 1985; Mandel *et al.*, 1987; Jhee *et al.*, 1989; Lotlikar, 1989; Lotlikar *et al.*, 1989; Kensler, 1994; Stresser *et al.*, 1994a, 1994b). Earlier studies indicated that BNF inhibition of AFB_1 hepatocarcinogenesis in the rat is primarily due to the induction of cytochrome P-450 which specifically enhances AFB_1 inactivation via AFM_1 hydroxylation (Gurtoo *et al.*, 1985). This report confirms earlier observations of Gurtoo *et al.* (1985) indicating enhanced AFM_1 hydroxylation and also provides additional data both *in vivo* and *in vitro* which indicate that BNF pretreatment of rats inhibits hepatic AFB_1-DNA binding. This inhibition of AFB_1-DNA binding may be a direct result of lower levels of constitutive cytochromes P-450 responsible for AFB_1-epoxidation.

Materials and Methods

Chemicals

Non-radioactive AFB_1, AFM, AFQ_1, AFP_1, GSH, calf thymus DNA, bovine pancreatic RNase, bovine serum albumin, BNF, collagenase (type IV), β-glucuronidase (type H_1), and sulfatase (type H-1) were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive [^{3}H]AFB_1 (30 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Scintillation fluid 'Scintiverse II' was obtained from National Diagnostic Co. (Manville, NJ). Radioactive [^{3}H]AFB_1 was diluted with non-radioactive AFB_1 in dimethyl sulfoxide to obtain the required specific activity. C_{18}-μ Bondapak column (0.39 x 30 cm) and C_{18}-Sep-pak cartridges were obtained from Waters Associates, (Milford, MA). NADPH was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). HPLC grade $CHCl_3$, methanol and ethyl acetate were obtained from Fisher Scientific Co. (Fairlawn, NJ).

Animals and treatment

Male Fischer rats (175-200g) obtained from Charles River Breeding Laboratories (Wilmington, MA) were maintained on a Purina rodent laboratory chow and given tap water for drinking for a week before use. Some animals were injected i.p. with BNF (2.5 mg/100 g body weight dissolved in corn oil) 24 h before sacrifice. Control animals were injected i.p. with corn oil alone. For *in vitro* studies, liver microsomes and cytosols were prepared as described previously (Lotlikar *et al.*, 1989).

Microsome-mediated hydroxylation of AFB_1 and binding to exogenous DNA

The incubation medium contained 100 mM phosphate buffer, pH 7.4, 2 mM NADPH, 1 μCi [^{3}H]AFB_1 containing either 2 or 100 nmol AFB_1 dissolved in 0.02 ml dimethyl sulfoxide, 100 mM sucrose, liver microsomes containing 1 mg protein and 0.2 mg native calf thymus DNA in a total volume of 1.0 ml. After incubation of duplicate samples in air for 30 min at 37°C, 1 ml solution containing 0.8 mg calf thymus DNA was added as a carrier to each sample. Out of 2 ml a 1-ml portion was drawn for isolation of DNA and another portion was used for extraction of hydroxylated metabolites of AFB_1. Isolation of DNA and quantitation of DNA by UV spectrophotometry were as described previously (Daoud and Irving, 1977; Lotlikar *et al.*, 1989). Two aliquots of isolated DNA were taken for radioactivity measurements by using 5 ml of Scintiverse II. Samples were counted in a LKB 1219 Rackbeta liquid scintillation spectrometer with an efficiency of about 45% for ^{3}H counting. Results of [^{3}H]AFB_1 binding to DNA are corrected for DNA recovery and are expressed as pmol [^{3}H]AFB_1 bound/mg DNA/mg protein/30 min.

Extraction and separation of hydroxy metabolites on HPLC C_{18}-μ Bondapak column were as described previously (Lotlikar *et al.*, 1989). Results are expressed as pmol of AFP_1, AFM_1 and AFQ_1 formed/mg protein/30 min.

AFB_1-DNA binding, AFB_1-GSH (AFB_1-SG) conjugation and hydroxylation of AFB_1 during incubation of isolated hepatocytes

Hepatocytes were isolated by the collagenase method of Moldeus *et al.* (1978) as described previously (Lotlikar *et al.*, 1989). Duplicate aliquots of hepatocytes (2 x 10^7 cells/5ml) were incubated in the presence of modified Hank's balanced salt solution containing 2 mM $CaCl_2$, 25 mM Hepes buffer (pH 7.4), 0.5% serum albumin and two levels of AFB_1 (33 nM and 2 μM) containing 1 μCi of [^{3}H]AFB_1 per ml in 95% O_2-5% CO_2 at 37°C for 1 h. AFB_1 was dissolved in dimethyl sulfoxide and final concentration of dimethyl sulfoxide was 2.0% (v/v). After incubation, cells were homogenized and nuclei were sedimented at 600 *g* for 10 min. Nuclear supernatants were used for extraction, separation and quantitation of free and conjugated hydroxy metabolites and AFB_1-SG conjugate, as described by our published procedures (Lotlikar *et al.*, 1989).

Isolation of DNA from the sedimented nuclei was as described previously (Lotlikar *et al.*, 1989). Aliquots of isolated DNA were taken for colorimetric determination of DNA (Burton, 1956) and for radioactivity measurements. Results of [^{3}H]AFB_1 bound to DNA are corrected for DNA recovery, which was 50-60% and are expressed in terms of pmol of AFB_1 bound per mg DNA per h.

AFB_1 binding to hepatic nuclear DNA *in vivo*

For these studies rats, 24 hr after i.p. injection with either corn oil or BNF were injected i.p. with [^{3}H]AFB_1 (40 μg AFB_1 containing 10 μCi [^{3}H]AFB_1 in 0.2 ml dimethyl sulfoxide/100 g body weight) 2 h before sacrifice. Isolation of DNA from the hepatic nuclear pellet and quantitation of AFB_1-DNA binding were as described previously (Lotlikar *et al.*, 1989).

Miscellaneous assays

Cytochrome P-450 content in liver microsomes and isolated hepatocytes was determined by the method of Omura and Sato (1984). Protein in microsomes and cytosol was assayed by the Lowry method with bovine serum albumin as the standard (Lowry *et al.*, 1951). The significance of the data was statistically evaluated by using student's *t*-test; *P* values less than 0.05 were considered to be highly significant.

Table 1. Effect of BNF pretreatment of rats on hepatic microsomal cytochrome P-450 content and cytochromes P-450 mediated hydroxylations of AFB_1 and AFB_1 binding to exogenous DNA. Animals were injected i.p. with either corn oil or 2.5 mg BNF/100 g body weightt. 24 h before sacrifice. Isolation of hepatic microsomes, the incubation medium for microsome-mediated AFB_1 binding to calf thymus DNA and formation of hydroxy metabolites of AFB_1 and other experimental details were as described in 'Materials and Methods'. Results are given as means ± SD of three analyses on individual livers from 3 animals.

BNF Pretreatment	Cytochrome P-450 content (nmol/mg protein)	AFB_1 concentration (μM)	Metabolites of AFB_1 (pmol/mg protein/30 min)			
			AFB_1-DNA	AFQ_1	AFM_1	AFP_1
-	0.30 ± 0.03	2	58 ± 4	32 ± 6	120 ± 10	55 ± 15
		100	1,350 ± 55	1,320 ± 300	3,000 ± 200	270 ± 20
+	0.52 ± 0.04[a]	2	37 ± 5[a]	47 ± 9	340 ± 50[a]	50 ± 10
		100	820 ± 80[a]	1,430 ± 120	9,050 ± 1,100[a]	290 ± 100

[a] Data highly significant with *P* values < 0.01 when compared with respective data of animals without BNF pretreatment.

Table 2. AFB_1-DNA binding, AFB_1-SG conjugation and hydroxy metabolite formation during AFB_1 metabolism with isolated hepatocytes from control and BNF treated rats. Isolation and incubation of hepatocytes with either 33 nM or 2 μM [^{3}H]AFB_1 for 1 h were as described in 'Materials and Methods'. Metabolites were calculated for 10^8 cells corresponding to 1 mg DNA/h. Results of [33 nM] AFB_1 incubations are expressed as mean ± SD of three separate analyses on individual livers from three animals whereas data of 2 μM AFB_1 incubations are averages of duplicate samples from one liver with variations of < 10%.

BNF Pretreatment	AFB_1	AFB_1-DNA	AFB_1-SG	AFQ_1	AFM_1	AFP_1
		pmol/10^8 cells/h				
-	33 nM	8.6 ± 2.1	14.0 ± 4.5	14.5 ± 5.0	46.5 ± 3.5	24.0 ± 8.0
+		3.3 ± 0.4[a]	18.7 ± 2.7	18.8 ± 2.8	104 ± 16[b]	16.0 ± 6.0
-	2 μM	404	810	1,350	4,875	2,730
+		271	1,400	1,900	7,950	1,925

[a] Data highly signficant with *P* values < 0.05 when compared with data of animals without BNF pretreatment.
[b] Data highly significant with *P* value < 0.01 when compared with data of animals without BNF pretreatment.

Results

Data on hepatic microsomal cytochrome P-450 content and cytochromes P-450 mediated hydroxylations of AFB_1 and AFB_1 binding to exogenous calf thymus DNA from control and BNF pretreated rats are presented in Table 1. Total cytochrome P-450 content in microsomes from BNF treated rats was increased by about 70% of control levels. At 2 μM AFB_1 concentration, in addition to AFM_1, the formation of AFQ_1 appeared to be substantial. At both AFB_1 concentrations, only AFM_1 formation was increased 2-3 fold by microsomes from BNF pretreated rats. In contrast, AFB_1-epoxidation as measured by AFB_1-DNA binding was decreased by 40% at both AFB_1 levels with microsomes from BNF-treated rats.

Cytochrome P-450 content was twice as much in isolated hepatocytes from BNF-treated compared to untreated controls (46.2 ± 2.0 nmol/10^8 cells vs. 23.3 ± 4.0 nmol/10^8 cells). Metabolism of AFB_1 was also examined in freshly isolated hepatocytes (Table 2). Data on hydroxylated metabolites represent both free and conjugated metabolites. Our preliminary studies indicated that conjugated metabolites comprised only 25% of total hydroxylated metabolites. Since conjugated metabolites were released as free hydroxylated metabolites by a combined treatment of β-glucuronidase and sulfatase, it is not possible to determine which conjugate was more predominant. In hepatocyte studies also, AFM_1 was the predominant hydroxylated metabolite of AFB_1 formed by control hepatocytes. Its formation was elevated by 120-160% above control levels by BNF-treatment of rats. In these studies, epoxidation of AFB_1 was examined by both AFB_1-DNA binding and AFB_1-SG conjugation. At low AFB_1 concentration (33 nM), BNF pretreatment of rats

Table 3. Effect of BNF pretreatment of rats on hepatic nuclear AFB_1-DNA binding *in vivo*. Experimental details were as described in Materials and Methods' section. Results are given as means ± SD of three analyses on individual livers from animals.

BNF Pretreatment 2.5 mg/100 g body weight	AFB_1-DNA binding pmol/DNA
-	107 ± 13
+	62 ± 7[a]

[a] Data statistically significant from untreated controls with P values < 0.02.

lowered AFB_1-DNA binding by 60%. Concomitant with inhibition of AFB_1-DNA binding, there appears to be an increase of 30% in AFB_1-SG conjugation. However, this increase in AFB_1-SG conjugation was not statistically significant. In one experiment, when AFB_1 metabolism was examined at higher AFB_1 concentration (2 μM), BNF pretreatment lowered AFB_1-DNA binding by 30% with an appreciable increase (70%) in AFB_1-SG conjugation.

Data on hepatic nuclear AFB_1-DNA binding in vivo are presented in Table 3. A single i.p. dose of BNF (2.5 mg/100 g body weight) 24 h before AFB_1 administration decreased AFB_1-DNA binding by 40%.

Discussion

Gurtoo's laboratory demonstrated that feeding BNF before and during AFB_1 treatment inhibited to a large extent AFB_1 hepatocarcinogenesis in the rat (Gurtoo *et al.*, 1985). The present study has shown that a single i.p. dose of BNF (25 mg/kg body weight) pretreatment of rats 24 h before AFB_1 administration inhibits genotoxic effect of AFB_1as determined by hepatic AFB_1-DNA binding *in vivo* (Table 3). These data are in agreement with our earlier preliminary data (Lotlikar, 1989) and those reported recently by others (Stresser *et al.*, 1994a).

Data on cytochrome P-450 content and AFB_1 hydroxylations with isolated microsomes and hepatocytes presented in this report are also compatible with microsomal studies from other investigators indicating that AFB_1 inactivation via enhanced AFM_1 hydroxylation as a major mechanism of BNF inhibition of AFB_1 hepatocarcinogenesis in the rat (Gurtoo *et al.*, 1985; Stresser *et al.*, 1994a). A similar mechanism has been proposed for BNF inhibition of AFB_1 hepatocarcinogenesis in rainbow trout (Goeger *et al.*, 1988). However, BNF pretreatment of hamsters, a species less susceptible to AFB_1 hepatocarcinogenesis, has a contrasting effect. In this species, hepatic AFB_1-DNA binding is increased *in vivo* and with isolated microsomes without significantly affecting AFM_1, AFQ_1 and AFP_1 hydroxylations even though cytochrome P-450 levels are increased (Santhanam and Lotlikar, 1989).

Induced GSTs after PB or various antioxidant treatment of rats have been shown to play a significant role in inhibiting hepatic AFB_1-DNA binding and AFB_1 hepatocarcinogenesis by inactivation of the reactive AFB_1-epoxide via AFB_1-SG conjugation (Degen and Neumann, 1981; Mandel *et al.*, 1987; Lotlikar, 1989; Jhee *et al.*, 1989; Lotlikar *et al.*, 1989; Kensler, 1994; Stresser *et al.*, 1994a). In the present study, there was no significant difference in AFB_1-SG conjugate levels when hepatocytes were incubated at low levels of AFB_1 (33 nM). However, our present data indicate that this pathway of inactivation may be operative at higher concentrations of AFB_1. Recent subcellular studies by Stresser *et al.* have suggested that enhanced detoxication of AFB_1-epoxide via AFB_1-SG conjugation due to enhanced levels of hepatic Yc^2 subunit of GST may be partly responsible for BNF protection of AFB_1 hepatocarcinogenesis in the rat (Stresser *et al.*, 1994a).

Previous investigations employing induction of *umu* gene in *Salmonella typhimurium* TA 1535/pSK1002 by activated carcinogens have indicated that constitutive forms of cytochrome P-450 have more important roles for the activation of AFB_1 than 3-methylcholanthrene, BNF and PB-inducible P-450 isozymes in rat liver (Shimada *et al.*, 1987, 1989; Imaoka *et al.*, 1992). Their studies have indicated that P-450 2C11, a major hepatic P-450 in male rat, is responsible for the highest activity in AFB_1 activation (Shimada *et al.*, 1987; Guengerich, 1988; Imaoka *et al.*, 1992). In the present study, both with isolated microsomes and hepatocytes, inspite of higher P-450 contents, AFB_1-DNA binding was lower with BNF-treated rats. Even with 50-fold increase in AFB_1 concentration (2 μM to 100 μM), AFB_1-epoxidation measured with AFB_1-DNA binding was significantly lower with microsomes from BNF-treated rats. Shimada *et al.* (1989) also obtained lower activation of AFB_1 with liver microsomes from BNF treated rats compared to control microsomes when they examined *umu* gene response in a bacterial system. In contrast, previous data by Gurtoo *et al.* (1985) and recent data from another laboratory indicated no significant differences in AFB_1 epoxidation between microsomes from control and BNF treated rats (Stresser *et al.*, 1994b). However, their results of Western blot band densities of various P-450 forms from hepatic microsomes indicated that P-450 2C11 level was decreased by 50% in animals fed the diet containing BNF (Stresser *et al.*, 1994b). Other laboratories have also reported such a decrease in hepatic P-450 2C11 in rats pretreated with BNF

(Guengerich *et al.*, 1982; Shimada *et al.*, 1987; Imaoka *et al.*, 1992). On the basis of our present data and those of others (Guengerich *et al.*, 1982; Shimada *et al.*, 1987; Guengerich, 1988; Shimada *et al.*, 1989; Imaoka *et al.*, 1992; Stresser *et al.*, 1994a), we suggest an additional mechanism of inhibition of AFB_1 hepatocarcinogenesis by BNF pretreatment of rats. Inhibition of AFB_1-DNA binding may be a direct result of lower AFB_1-epoxidation due to lower levels of P-450 2C11 and other constitutive P-450s in livers of BNF treated rats. Additional studies are warranted to examine the contributions of these various mechanisms in inhibition of hepatic AFB_1-DNA binding and hence AFB_1 hepatocarcinogenesis by BNF pretreatment of rats.

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