RESEARCH COMMUNICATION

A novel aldehyde reductase with activity towards a metabolite of aflatoxin B₁ is expressed in rat liver during carcinogenesis and following the administration of an anti-oxidant

David J. JUDAH,* John D. HAYES,† Ji-Chun YANG,‡ Lu-Yun LIAN,‡ Gordon C. K. ROBERTS,‡ Peter B. FARMER,* John H. LAMB* and Gordon E. NEAL*

*MRC Toxicology Unit, Carshalton, Surrey SM5 4EF, U.K., †Biomedical Research Centre, University of Dundee, Dundee DD1 9SY, U.K. and ‡Medical Sciences Building, University of Leicester, Leicester LE1 9HN, U.K.

In contrast with fractions from control animals, an aldehyde reductase, which catalyses the reduction of aflatoxin B₁-dihydrodiol, in the dialdehyde form at physiological pH values, to aflatoxin B₁-dialcohol, is expressed in cytosolic fractions prepared from rat livers bearing pre-neoplastic lesions, or following treatment with the anti-oxidant ethoxyquin. This expression parallels the development of resistance to the toxin. Unlike the aflatoxin B₁-dihydrodiol, the dialcohol does not

undergo binding to protein. This enzyme activity could play a mechanistic role in hepatocarcinogenesis and chemoprotection in the rat. Correlated n.m.r. and m.s. spectra are provided in Supplementary Publication SUP 50171 (3 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1993) 289, 9.

INTRODUCTION

The development of resistance to the cytotoxicity of carcinogens is a frequent feature of carcinogenesis including aflatoxin B₁ (AFB₁)-induced hepatocarcinogenesis in the rat (Judah et al., 1977). It has been hypothesized that it represents an important step in multistage carcinogenesis, in that it permits DNA replication and cell proliferation in the presence of a carcinogen which normally inhibits these processes (Farber et al., 1975). Possible biochemical mechanisms include loss of xenobiotic activating capacity (Farber et al., 1976) and increased detoxification (Neal et al., 1981a). A glutathione S-transferase (GST) isoenzyme Alpha-class subunit Yc₂ is expressed in AFB₁-induced pre-neoplastic rat liver as heterodimers with subunits Ya₁ or Yc₁. These Yc₂-containing GSTs have high AFB₁-conjugating activity, and probably play a major role in resistance in pre-neoplastic and neoplastic rat liver (Hayes et al., 1991a). The Yc₂ subunit is expressed in the livers of rats after administration of the anti-oxidant ethoxyquin, which also induces resistance to AFB₁ (Hayes et al., 1991a), and it also shares extensive sequence similarity with the constitutively expressed Yc GST-isoenzyme subunit (Hayes et al., 1991b, 1992; Buetler and Eaton, 1992) in the AFB₁-resistant mouse.

During previous studies we had noted in incubations using cytosolic fractions from pre-neoplastic and ethoxyquin-treated livers, in addition to increased AFB₁-GSH formation, the capacity to form an apparently previously unreported metabolite. The formation of this metabolite was not evident in incubations using cytosolic fractions from control rats. We now describe the isolation and characterization of this metabolite as AFB₁-dialcohol. It is formed from AFB₁-dihydrodiol (AFB₁-dhd) in the dialdehyde configuration, by a novel AFB₁-dhd aldehyde reductase (AFB₁-AR). AFB₁-dhd is capable of binding to protein via Schiff-base formation and it has been suggested that this reaction is involved in cytotoxicity (Neal et al., 1981b). Therefore

the newly identified AFB₁-AR might have significant detoxifying potential, a role which would be consistent with its expression in pre-neoplastic liver or following the administration of the anti-oxidant, ethoxyquin.

MATERIALS AND METHODS

Animals

Male Fischer F344 rats bred on site at the MRC Toxicology Unit, were fed a basic diet of powdered MRC 41 B with arachis oil (20 ml/kg of diet), or the basic diet plus ethoxyquin (5 ml/kg of diet) or the basic diet plus AFB₁ (2 mg/kg of diet) with arachis oil (20 ml/kg of diet) as the vehicle. Experimental feedings were commenced at 150 g body wt. in the case of the ethoxyquin administration and continued for 5 days. Control and experimental groups of three rats were then killed and the livers rapidly frozen in liquid N₂. Other groups of rats were fed the AFB₁-containing diet for approx. 6 months from weanling stage and were killed at approx. 1 year of age. Livers were removed and rapidly frozen. Small portions of the livers were removed before freezing the tissue, fixed in cold acetone and the presence of altered cell foci and nodules in the rats fed the AFB₁ diet was confirmed by histological examination and histochemistry using γ -glutamyltransferase as a marker (Manson and Neal, 1987). Hepatic microsomal suspensions were prepared from quail liver in 150 mM KCl as described previously (Moss and Neal, 1985).

Chemicals

Ethoxyquin was obtained from Sigma Chemical Co., Poole, Dorset, U.K. AFB₁ was obtained from Makor Chemicals, Jerusalem, Israel, and [³H]AFB₁ (specific radioactivity 22 Ci/mmol) was from Moravsek Biochemicals, Brea, CA, U.S.A. Whatman DEAE-cellulose and CM-cellulose ion-exchange materials were purchased from Chromatography Services,

Wirral, Merseyside, U.K., and hydroxyapatite was from Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K. The Protein-PAK Glass SP-8HR f.p.l.c. column was obtained from Waters Chromatography Division, Millipore (UK), Watford, Herts., U.K. The h.p.l.c.-grade solvents were obtained from Rathburn Chemicals, Walkerburn, Peeblesshire, U.K. and high-purity water from a Milli-Q cartridge system (Millipore).

Enzyme fractionation

(a) GSH-affinity gel

Cytosolic fractions (supernatants from 100 000 *g* centrifugation for 1 h), prepared from frozen livers in 20 mM Tris/HCl (pH 7.8) containing 100 mM NaCl were passed through a column of a glutathione–Sephacrose 6B affinity matrix essentially as described previously (Hayes, 1986). The adsorbed GST activity, after washing the column with the Tris/HCl homogenizing solution, was eluted with a solution of 50 mM GSH in 200 mM Tris/HCl (pH 9.0) and subsequently dialysed against two changes, each of 2 litres, of 20 mM Tris/HCl (pH 7.8) (Hayes, 1986).

(b) Ion-exchange hydroxyapatite chromatography and f.p.l.c.

Liver post-mitochondrial supernatants (10 000 *g*, 30 min) prepared in 20 mM Tris/HCl (pH 7.5) were fractionated sequentially on DEAE-cellulose, CM-cellulose, hydroxyapatite and Protein-PAK Glass SP-8HR columns using essentially the conditions used previously to fractionate GST (Hayes et al., 1991b). Fractions were monitored for the capacity to catalyse the formation of the novel metabolite of AFB₁ throughout the successive column separations.

Electrophoresis

SDS/PAGE was performed by the method of Laemmli (1970). The resolving gels employed 12% (w/v) polyacrylamide which contained 0.32% *NN*-methylenebisacrylamide as described previously (Hayes and Mantle, 1986).

Aldehyde reductase assay

Aldehyde reductase activity was determined at 30 °C by a modification of the method of Ryle and Tipton (1981) using 73 μ M 4-nitrobenzaldehyde as a model substrate and 0.075 mM NADPH in 100 mM sodium phosphate buffer (pH 7.0), monitoring the reaction by $\Delta A_{340\text{ nm}}$.

Protein assays

These were carried out by the dye-binding method of Bradford (1976).

Enzyme incubation and h.p.l.c. procedures

(a) Analytical

Incubations in which activation of AFB₁ to form AFB₁-8,9-epoxide was catalysed by quail microsomes, thus providing the substrate for secondary metabolism by cytosols or fractions of cytosols, were essentially as described previously for assay of AFB₁-GST activity (Hayes et al., 1991a), GSH being omitted from the incubation mixtures where indicated. H.p.l.c. sample preparations and separations of metabolites following incubations were also essentially as described previously (Hayes et al., 1991b). H.p.l.c. eluants were monitored using u.v. absorption (365 nm) and fluorescence (365 nm band-pass excitation/405 nm

long-pass emission). ³H binding to protein was assayed by dissolving the methanol-insoluble pellets (obtained by centrifuging the incubation media after addition of methanol) in NaOH, followed by scintillation-counting in acidified Monofluor (National Diagnostics, Aylesbury, Bucks., U.K.).

In some incubations AFB₁-8,9-epoxide, generated *in situ* by quail microsomes, was replaced by AFB₁-dhd, which was generated in incubation mixtures containing AFB₁ (1.11 μ mol), quail liver microsomes (equivalent to 3.2 g of liver), potassium phosphate buffer (2 mmol, pH 7.4), MgCl₂ (127 μ mol), NADP⁺ (1.1 μ mol), glucose 6-phosphate (157 μ mol), glucose-6-phosphate dehydrogenase (8 units) and distilled water to a total volume of 24.4 ml, and incubated under O₂ at 37 °C for 2 h. This incubation protocol ensured a virtually complete metabolism of the AFB₁ and so avoided its carry-over into the second incubation. The post-incubation reaction mixture was clarified by centrifugation (100 000 *g*, 60 min) and 2 ml aliquots of the supernatant were loaded on to an activated C₁₈ Sep-Pak cartridge (Millipore, Watford, Herts., U.K.), washed (2 $\times$ 2 ml of water) without application of positive pressure to avoid elution of weakly retained AFB₁-dhd, which was then eluted with 2 ml of methanol/water (1:1, v/v) followed by 2 ml of methanol. The combined eluates were dried in a Savant Vacuum Concentrator (IEC Ltd., Dunstable, Beds., U.K.). The residue was dissolved in potassium phosphate buffer (0.8 M, pH 7.4), analysed by h.p.l.c. to confirm > 95% purity of AFB₁-dhd, and stored at -20 °C. Samples were routinely used within one day of preparation because significant degradation occurred on storage.

(b) Preparative

Samples of the novel metabolite were prepared for analysis by n.m.r. spectroscopy and m.s. using the incubation system described in the Materials and methods section, incorporating purified AFB₁-AR [i.e. obtained from the hydroxyapatite pool; see Enzyme fractionation procedure (b)]. Quail microsomes equivalent to 375 mg of liver in a total volume of 7.32 ml were incubated with appropriate cofactors for 20 min. The reactions were terminated by the addition of 4 vol. of ice-cold methanol followed by centrifugation. The extracts were evaporated to dryness in a Savant Vacuum Concentrator and redissolved in 1.5 ml of 5% (v/v) methanol/0.05% formic acid (Solvent A). Aliquots (3 $\times$ 100 μ l) of the solution were used for on-column concentration using the h.p.l.c. system described previously (Hayes et al., 1991b) and a flow rate of 1 ml/min of Solvent A. The on-column concentrated metabolite was subsequently eluted by initiating a linear gradient from 100% of Solvent A to 50% (v/v) methanol (Solvent B) (7 min). The novel metabolite eluted with a retention time (*t_R*) of 6 min, the appropriate fractions monitored by u.v. absorption were collected. This procedure was repeated five times, and the combined metabolite-containing fractions were evaporated to dryness in a Savant Vacuum Concentrator and used subsequently in the n.m.r. and m.s. studies.

N.m.r. analyses

Spectra were obtained, using a Bruker AM 500 spectrometer operating at a proton frequency of 500 MHz at 298 K and at pH 7.4. Two-dimensional dual-quantum-field correlated spectroscopy (DQF-COSY) spectra were acquired using a data matrix of 2 k $\times$ 1 k. Data was processed with zero-filling in the *F*₂ dimension. Sine-bell squared window functions were used. DQF-COSY data have been deposited as Supplementary Publication SUP 50171 at the British Library Document Supply Centre, Boston Spa, West Yorkshire, U.K.

M.s. analyses

Mass spectral analyses were carried out using a VG-70-SEQ instrument. Spectra were obtained after fast-atom-bombardment (FAB) ionization, using glycerol containing 0.1 M *p*-toluenesulphonic acid as the matrix for positive-ion spectra and aminoglycerol as the matrix for negative ionization. Positive-ion tandem m.s. was carried out at m/z 351 (collision energy 10 eV, no added collision gas, i.e. collision in air at 3×10^{-6} Pa).

RESULTS AND DISCUSSION

Detection of the presence of a novel AFB₁ metabolite in incubations *in vitro*

Metabolism *in vitro* using hepatic cytosolic fractions (Figure 1) isolated from control-fed (Figure 1a) or ethoxyquin-induced (Figure 1d) rats incubated with AFB₁ and cofactors, followed by h.p.l.c. analysis, revealed not only the previously reported increase in the formation of the AFB₁-GSH conjugate in the ethoxyquin-treated cytosol (Figure 1d, peak c), but also the presence of an unknown metabolite (Figure 1d, peak a) not observed in incubations using control cytosols. Although most of the results given in this paper relate to experiments using fractions isolated from ethoxyquin-treated liver, similar studies using cytosols from pre-neoplastic liver have revealed the capacity to form an identical metabolite. Subsequent fractionation of cytosols by GSH-Sephadex 6B affinity chromatography [enzyme fractionation (a) in the Materials and methods section] into retained and non-retained subfractions demonstrated that production of the novel metabolite was associated entirely with the non-retained, GST-depleted, fraction (Figure 1e). The Tris-AFB₁-dhd (peak b, Figure 1) is produced by the Schiff-base reaction between AFB₁-dhd in the dialdehyde form and Tris buffer homogenizing solution, and also in the running buffer used in GSH-Sephadex affinity chromatography as detailed in Scheme 1. AFB₁-dhd was formed by either spontaneous or enzymic hydrolysis of the AFB₁-8,9-epoxide formed by the quail

microsomes. Comparison of the levels of Tris-AFB₁-dhd in Figures 1(b) and 1(e) demonstrates a reduced level of production

Figure 1 Formation of novel AFB₁ metabolite (AFB₁-dialcohol) by liver cytosol from ethoxyquin-treated rats

Cytosols prepared and fractionated on the Sephadex-GSH column and incubations carried out using previously described methods (Judah et al., 1977; Hayes et al., 1991a). Control cytosol: (a) complete; (b) Sephadex-GSH non-retained; (c) Sephadex-GSH retained. Ethoxyquin-treated cytosol: (d) complete; (e) Sephadex-GSH non-retained; (f) Sephadex-GSH retained. H.p.l.c. peaks (a) AFB₁-dialcohol; (b) Tris-AFB₁-dhd; (c) AFB₁-GSH; (d) unmetabolized AFB₁.

Scheme 1 Proposed pathway for metabolic formation of AFB₁-dialcohol

Assignment of AFB₁-dialcohol protons is indicated.

Figure 2 AFB₁-dialcohol u.v. absorption spectra

Spectra were obtained from diode-array-detector monitoring (model 1000 S, A.B.I., Warrington, U.K.) of h.p.l.c. eluate, the mobile phase was adjusted to the required pH. The retention time of AFB₁-dialcohol is reduced considerably at neutral pH. Key to spectra: —, pH 5.0; ---, pH 7.1.

Figure 3 Purification of AFB₁-AR

SDS/PAGE of cytosol from livers of control and ethoxyquin-treated rats is shown (lanes A and B respectively). The aldehyde reductase was purified from ethoxyquin-treated cytosols by chromatography on DEAE-cellulose, CM-cellulose and hydroxyapatite (lane C) and subsequently on Protein-PAK Glass SP-8HR columns (lane D). Protein molecular-mass markers are shown in lane E.

of this compound in the incubations using cytosols from ethoxyquin-treated rats which produced the unknown metabolite. This result indicated a competition between Tris and the enzyme catalysing the formation of the novel metabolite for available AFB₁-dhd *in vitro*. The novel metabolite exhibited a high fluorescence/u.v. absorption ratio which was similar to that of AFB₁-dhd (Beebe and Takahashi, 1980) and inconsistent with the metabolite being a GSH conjugate or a breakdown product of an AFB₁-GSH conjugate (Moss et al., 1985). The acid/neutral u.v. absorption spectra, obtained by linear diode-array-detector monitoring of h.p.l.c. eluates, at two pH values (5.0 and 7.1, Figure 2) demonstrated a 400 nm peak at neutral pH. This is consistent with an AFB₁ metabolite, hydroxylated in the 8 and/or 9 carbon atom, forming a phenolate ion at neutral or alkaline pH. All of these preliminary results therefore suggested that the unknown metabolite was structurally closely related to AFB₁-dhd.

Characterization of the novel metabolite

(a) Enzyme fractionation and metabolite sample preparation

The enzyme catalysing the formation of the metabolite was purified from ethoxyquin-treated liver cytosol [procedure (b) in the Materials and methods section]. PAGE of the purified fractions, monitored by their ability to catalyse formation of the novel AFB₁ metabolite, are given in Figure 3. The purification procedures involved and protein sequence data will be dealt with in detail elsewhere (J. D. Hayes, D. J. Judah and G. E. Neal, unpublished work). The protein which catalyses the formation of the metabolite has an M_r value of 36600 (assessed by Lasermat) and can be seen after PAGE of complete liver cytosols prepared from ethoxyquin-treated rats (Figure 3). The metabolite was extracted from incubation mixtures incorporating enzyme purified to the hydroxyapatite stage, purified by h.p.l.c. as described in the Materials and methods section, and used in n.m.r. and m.s. analyses.

(b) N.m.r. and m.s. analyses

Analyses were carried out using examples of the unknown metabolite at physiological pH. The ¹H-n.m.r. spectra of the unknown metabolite in aqueous solution, particularly ¹H-¹H DQF-COSY spectra, provided clear evidence for the presence of a -CH₂-CH-CH-CH₂ fragment in place of the dialdehyde moiety of AFB₁-dhd, with the chemical shifts suggesting that the carbons have electronegative substituents. N.m.r. data (proton notation shown in Scheme 1; chemical shifts relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate): Ha, Ha' 3.39, 3.45 p.p.m. (Jaa' 12 Hz, Jab < 3 Hz, Ja'b 12 Hz); Hb 4.12 p.p.m. (Jbc 12 Hz); Hc 3.76 p.p.m.; Hd, Hd' 3.92, 4.04 p.p.m. (Jdd' 15 Hz, Jcd 6 Hz, Jcd' 12 Hz). These results are consistent with the proposed structure for the novel metabolite (Scheme 1). They are presented in SUP 50171. The positive FAB spectrum of the metabolite showed (MH)⁺ 351 and the negative-ion spectrum (M-H)⁻ 349. Positive-ion tandem m.s. showed major product ions at m/z 333 (MH-H₂O)⁺, m/z 315 (MH-2H₂O)⁺, m/z 303 (MH-CH₄O₂)⁺, m/z 259 (MH-C₃H₈O₃)⁺. These results are also consistent with the structure for the AFB₁-dialcohol given in Scheme 1. The m.s. data have been deposited at the British Library Document Supply Centre as Supplementary Publication SUP 50171.

Evidence that the enzyme is an aldehyde reductase utilizing AFB₁-dhd as substrate

Scheme 1 proposes that the enzyme is an aldehyde reductase

Figure 4 Fractionation of enzyme activity catalysing *p*-nitrobenzaldehyde reductase (■) and AFB₁-AR (○)

Enzyme activity in fractions eluted from a Protein-PAK Glass SP-8HR column (see the Materials and methods section). PAGE of protein present in fractions 44–48 is shown in lane D of Figure 3.

Table 1 Effect of AFB₁-AR activity on binding of AFB₁ to microsomal protein

Results are given as the means of triplicate incubations $\pm$ S.D. The [³H]AFB₁ (Moravsek Biochemicals, Brea, CA, U.S.A.) was added to all incubations (specific radioactivity 354 Bq/nmol), together with quail hepatic microsomes (equivalent to 15.6 mg of liver) and NADPH-generating system. Hydroxyapatite-purified AFB₁-AR (lane C, Figure 3) (19.6 μ g of protein) was added where indicated. Incubations (30 min) were terminated by addition of 1 ml of ice-cold methanol. Samples were clarified by centrifugation (1500 *g*, 45 min), and the pellets were washed twice with 1 ml of methanol. Radioactivity was determined in combined soluble plus washing fractions and in insoluble fractions (the latter after air-drying and dissolving in 0.1 M KOH) by liquid scintillation counting using acidified Monofluor scintillation solution (National Diagnostics, Aylesbury, Bucks., U.K.). Methanol-soluble fractions were evaporated to dryness in a Savant Vacuum Concentrator, redissolved in methanol and subjected to h.p.l.c. analysis.

Incubation constituents	AFB ₁ equivalents (nmol)	
	Methanol-insoluble	Methanol-soluble
+ AR	2.35 $\pm$ 0.06	8.61 $\pm$ 0.08
– AR	5.77 $\pm$ 0.13	4.12 $\pm$ 0.20

utilizing AFB₁-dhd in the dialdehyde configuration at physiological pH. The aldehyde reductase activity assayed with *p*-nitrobenzaldehyde present in the fractions eluted from the Protein-PAK Glass SP-8HR column in the final purification step, co-incided with the activity catalysing the formation of the novel metabolite (Figure 4). This provides strong evidence that the enzyme is an aldehyde reductase, again consistent with the product being AFB₁-dialcohol.

That the substrate for the enzyme was AFB₁-dhd and not AFB₁-8,9-epoxide was demonstrated in incubations incorporating AFB₁-dhd instead of the *in situ* AFB₁-8,9-epoxide-generating quail microsome system. AFB₁-dialcohol was produced in incubations incorporating AFB₁-dhd, NADPH and AFB₁-AR purified up to the hydroxyapatite stage. The reaction

Figure 5 H.p.l.c. detection of [³H]AFB₁-dialcohol

H.p.l.c. separation of methanol-soluble extract following incubation of [³H]AFB₁ with a microsomal-activating system and AFB₁-AR as detailed in Table 1.

was dependent on NADPH, since AFB₁-dialcohol was not produced when this cofactor was omitted.

Effect of aldehyde reductase activity on AFB₁-dhd protein binding *in vitro*

AFB₁-dhd undergoes Schiff-base binding to protein via the pathway indicated in Scheme 1. Alternative pathways to Schiff-base formation have been suggested for the adduction of AFB₁ to protein, but AFB₁-dhd was still involved as the reactive AFB₁ metabolite (Sabbioni and Wild, 1991). It is probable that protein binding via formation of AFB₁-dhd is associated with the *in vivo* cytotoxicity of AFB₁. The relative production *in vitro* of AFB₁-dhd by microsomes from various animal species parallels the susceptibilities *in vivo* to acute AFB₁ toxicity (Neal et al., 1981b). In incubations *in vitro* incorporating [³H]AFB₁ it was found that the production of [³H]AFB₁-dialcohol *in vitro* (Figure 5) paralleled a significant reduction in ³H binding to microsomal protein (Table 1). The residual ³H protein binding, not competed for by the aldehyde reductase, probably represents a fraction of metabolized AFB₁ which reacts with protein immediately ad-

jacent to the site of activation and thus is not accessible for trapping by exogenous factors in the medium (G. E. Neal and D. J. Judah, unpublished work). Addition of [^3H]AFB $_1$ -dialcohol to microsomal preparations did not result in any methanol-insoluble binding of radioactivity.

Conclusions and possible relevance of AFB $_1$ -AR activity

The expression in rat liver of the AFB $_1$ -AR activity, at a time of reduced sensitivity to the cytotoxicity of AFB $_1$, is consistent with a role for the enzyme in the resistance phenomenon associated with pre-neoplasia and the response to anti-oxidants such as ethoxyquin. This enzymic mechanism might also have relevance in terms of the development of resistance to other cytotoxic agents the mechanisms of action of which involve metabolism to a reactive aldehyde. It is clearly necessary to examine other systems, in particular human, to determine if this enzyme activity is expressed, and if so in what circumstances, before its potential significance in the carcinogenic process can be evaluated. It would be of great practical importance to establish whether a similar detoxifying system is expressed in the livers of humans consuming diets contaminated with aflatoxins, since such exposure is frequently monitored by assaying serum albumin-aflatoxin adducts (Wild et al., 1990).

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