Regulation of aflatoxin B₁-metabolizing aldehyde reductase and glutathione S-transferase by chemoprotectors

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Ingestion of aflatoxin B₁ (AFB₁) represents a major risk factor in the aetiology of human hepatocellular carcinoma. In the rat, the harmful effects of AFB₁ can be prevented by the administration of certain drugs which induce hepatic detoxification enzymes. We have previously shown that treatment of rats with the chemoprotector ethoxyquin (EQ) results in a marked increase in expression of the Alpha-class glutathione S-transferase (GST) Yc₂ subunit which has high activity towards AFB₁-8,9-epoxide [Hayes, Judah, McLellan, Kerr, Peacock and Neal (1991) *Biochem. J.* 279, 385–398]. To allow an assessment of whether the increased expression of GST Yc₂ represents a general adaptive resistance mechanism to chemical stress, that is invoked by both chemoprotectors and carcinogens, we have examined the effects of EQ, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), phenobarbital (PB), AFB₁, 3-methylcholanthrene (3-MC) and clofibrate on the AFB₁-glutathione-conjugating activity and the GST subunit levels in rat liver. In addition, the effect of these drugs on the hepatic levels of an aldehyde reductase (AFB₁-AR) that metabolizes the cytotoxic dialdehydic form of AFB₁ has been studied as this enzyme also appears to be important in chemoprotection. Administration of the anti-oxidants EQ, BHA or BHT, as well as PB, led to a marked increase in levels of the GST Yc₂ subunit in rat liver, and this increase coincided with a substantial rise in the GST activity

towards AFB₁-8,9-epoxide; neither AFB₁, 3-MC nor clofibrate caused induction of Yc₂ or any of the GST subunits examined. Among the xenobiotics studied, EQ was found to be the most effective inducing agent for the Yc₂ subunit as well as Yc₁, Yb₁ and Yf. However, PB was equally as effective as EQ in increasing levels of the Ya-type subunits, although it was not found to be as potent an inducer of the other GST subunits, including Yc₂. In addition to induction of GST, EQ caused a substantial increase in the hepatic content of AFB₁-AR. Both BHA and BHT were also able to induce this enzyme but, by contrast, PB was found to be a poor inducer of AFB₁-AR. AFB₁, 3-MC and clofibrate were unable to serve as inducers of this reductase. The presence of Alpha-class GST, including the Yc₂ subunit, was examined in various rat tissues. Constitutive expression of Yc₂ was found in the epididymis at levels comparable with that observed in the liver from EQ-treated rats. Epididymal expression of Yc₂ was accompanied by a high GSH-conjugating activity towards AFB₁-8,9-epoxide which was not found in any other organ from normal rats. The highest levels of AFB₁-AR were found in the kidney, followed by moderate levels in the liver and testis. Other extrahepatic organs such as heart, lung, spleen, adrenal gland, epididymis, vas deferens, seminal vesicles and ovary contain much lower levels of AFB₁-AR.

INTRODUCTION

Aspergillus flavus, a mould widely encountered in tropical and subtropical areas as a contaminant of poorly stored grain crops, produces aflatoxin B₁ (AFB₁) as a secondary metabolite. AFB₁ is a potent hepatotoxin and carcinogen, and, along with Hepatitis B virus, is suspected to represent a major factor contributing to the high incidence of liver cancer in man in the developing world (for a review, see Groopman et al., 1988).

Hepatic metabolism of ingested AFB₁ is extensive. Much of its toxicity can be attributed to the metabolite, AFB₁-8,9-epoxide, the formation of which is catalysed by the microsomal monooxygenase system (Neal, 1987). The AFB₁-epoxide is a highly reactive electrophile and reacts readily with cellular macromolecules. There are, however, endogenous defence mechanisms which can protect cells from the toxicity of AFB₁-epoxide, including conjugation reactions, active efflux from the cell and repair of AFB₁-DNA adducts (Hayes et al., 1991a). In many mammalian cells, detoxification of AFB₁-epoxide by phase-II drug metabolism is of principal importance. Activated AFB₁ can

be conjugated with glutathione, a reaction catalysed by the glutathione S-transferases (GSTs), and this step is thought to represent a major detoxification mechanism. Alternatively, the AFB₁-epoxide can hydrolyse spontaneously to AFB₁-dihydrodiol. This is not a true detoxification process as the dihydrodiol product can rearrange at neutral pH values to form a dialdehydic phenolate ion. This AFB₁-dialdehyde can undergo Schiff-base formation with primary amine groups in proteins and is therefore likely to be cytotoxic. Recently, a novel AFB₁-aldehyde reductase (AFB₁-AR) purified from ethoxyquin (EQ)-treated rat liver has been shown to metabolize the dialdehyde form of AFB₁-dihydrodiol to an AFB₁-dialcohol and its relative importance in AFB₁ detoxification may be considerable (Hayes et al., 1993; Judah et al., 1993a).

The toxicity of AFB₁ is selective towards certain species. In contrast with the mouse and hamster, the rat, guinea pig and man are susceptible to the hepatotoxic effects of AFB₁ (O'Brien et al., 1983; Hayes et al., 1991a). The toxicity of the mycotoxin is based on a balance between the rate of primary activation of AFB₁ and the rate of detoxification of primary metabolites or

Abbreviations used: AFB₁, aflatoxin B₁; GST, glutathione S-transferase; EQ, ethoxyquin; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; PB, phenobarbital; 3-MC, 3-methylcholanthrene; AFB₁-AR, AFB₁-aldehyde reductase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one.

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repair of cellular damage, determined by the relative activity of enzymes responsible for these reactions; the differential toxicity of AFB₁ between species is thought to be due mainly to different levels of activity of xenobiotic-metabolizing enzymes. In this regard, the livers of mice which are resistant to the hepatotoxic effects of AFB₁ contain high concentrations of a Yc-type GST subunit (McLellan et al., 1991) that has considerable GSH-conjugating activity towards AFB₁-epoxide (Quinn et al., 1990; Ramsdell and Eaton, 1990; Hayes et al., 1991a, 1992; Beutler and Eaton, 1992; Beutler et al., 1992). By contrast, the Fischer rat, an inbred strain that is five times more susceptible to AFB₁-induced liver cancer than the Wistar rat (Hayes et al., 1991a), possesses 20-fold less hepatic AFB₁-GSH-conjugating activity than the mouse. Fischer rats can, however, be protected against AFB₁ by treatment with the antioxidant EQ. We have shown that following EQ-treatment the livers of Fischer rats express a GST subunit that is immunochemically related to the mouse Yc subunit (Hayes et al., 1991b). Moreover, this inducible polypeptide (Yc₂, subunit 10) has high activity towards AFB₁-epoxide (Hayes et al., 1991b; Raney et al., 1992). Thus, the Yc₂ subunit is thought to confer protection against AFB₁, and its induction by EQ is likely to be one of the key mechanisms for the protective action of this anti-carcinogen.

Chemoprotectors are a group of structurally diverse compounds that have the ability to protect animals from the toxic effects of carcinogenic xenobiotics (Wattenberg, 1985). Many of these anti-carcinogens are thought to act by increasing levels of hepatic drug-metabolizing enzymes, thus enhancing detoxification and elimination of chemical carcinogens (Prochaska and Talalay, 1988; Talalay, 1989). EQ, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are all antioxidants that have been described as having anti-carcinogenic properties (Ito et al., 1985). Furthermore, all of these compounds have been shown to protect rats from AFB₁-mediated hepatocarcinogenesis. In addition to antioxidants, both phenobarbital (PB) and 3-methylcholanthrene (3-MC) have been shown to be capable of protecting rodents from carcinogens (Miller et al., 1958; Lotlikar et al., 1989). Also, low-level feeding of AFB₁ to rats before injection of a higher dose has been shown to reduce the incidence of covalent binding of AFB₁ to DNA (Neal et al., 1981). It is, however, unknown whether these compounds with chemoprotective properties act in a similar manner to EQ by inducing enzymes able to detoxify AFB₁ metabolites. The control of enzyme activities responsible for the detoxication of AFB₁ is of particular interest, because of its relevance to human liver disease. In the present study we have examined the effects of several chemoprotectors on hepatic levels of enzymes capable of detoxifying AFB₁-epoxide: specifically, GST Yc₂ and AFB₁-AR. In addition, we have investigated the tissue distribution of GST Yc₂ and AFB₁-AR in Fischer rats to establish whether their constitutive expression is co-ordinated.

MATERIALS AND METHODS

Chemicals

Androst-5-ene-3,17-dione was a gift from Dr. P. K. Stockman (Royal Infirmary, Edinburgh, U.K.). All other chemicals were of analytical grade and readily available commercially.

Animals

Adult male Fischer 344 rats (300–450 g), bred and maintained at the Imperial Cancer Research Fund Animal Unit, Clare Hall,

London, were used to examine the effects of xenobiotics on levels of hepatic drug-metabolizing enzymes. Each group of rats (four animals per group) were fed the same basic powdered diet (standard laboratory diet) to which the following compounds were added: (1) no additives, (2) peanut oil (2%, v/w), (3) EQ (0.5%, w/w) dissolved in peanut oil at a final concentration of 2% (v/w) in the diet, (4) BHA (0.75%, w/w), or (5) BHT (0.5%, w/w). An additional group of rats were given (6) phenobarbital (PB) (0.1%, w/v) in their drinking water with no additives to the food. Dietary additives were administered for 14 days before the animals were killed. Alternatively, the rats were injected intraperitoneally with the following: (7) corn oil only (0.5 ml/day for 4 days), (8) AFB₁ (0.2 mg/kg) in corn oil (0.5 ml/day for 4 days), (9) clofibrate acid (200 mg/kg) in corn oil (0.5 ml/day for 4 days), or (10) 3-MC (100 mg/kg) in corn oil (0.5 ml/day for 3 days). Fischer 344 rats (150–200 g; Bantin and Kingman, Hull, U.K.) were used to examine the tissue distribution of AFB₁-epoxide-metabolizing enzymes. Adult males (four animals) were used for the excision of all tissues except for ovarian samples where four adult females were used. In all instances, the rats were allowed free access to food and water until killed.

Subcellular fractionation

The organs used for subcellular fractionation were stored at –80 °C until required. Cytosols and microsomes were prepared by a similar method to that described by McLellan et al. (1991).

Analytical methods

Enzyme assays for GST activity towards the model substrates and glutathione peroxidase activity were carried out at 37 °C as described previously (McLellan and Hayes, 1989; McLellan et al., 1991). GST activity towards AFB₁-epoxide was measured as described by Hayes et al. (1991b). The AFB₁-AR activity was measured by the method of Judah et al. (1993). Protein determination was by the method of Bradford (1976) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts., U.K.).

Antisera

Polyclonal antisera raised against rat hepatic GST [Alpha-class (Ya, Ya₂, Yc₁Yc₁ and YkYk), Mu-class (Yb₁Yb₁), Pi-class (YfYf) and microsomal GST] were used in immunoblotting experiments as described in previous publications (Hayes and Mantle, 1986a,b; McLellan and Hayes, 1989). Antibodies that cross-reacted specifically with the rat Yc₂ subunit, without cross-reactivity with Yc₁, were prepared from polyclonal antisera raised against the mouse Yc subunit [a constitutive hepatic subunit with high amino-acid-sequence similarity to rat Yc₂ and previously designated Ya₃ (McLellan et al., 1991; Hayes et al., 1992)] as described elsewhere (Judah et al., 1994). Briefly, serum (5 ml) from a female New Zealand White rabbit immunized with mouse YcYc was diluted to 10 ml with 20 mM Tris/HCl, pH 7.5, containing 0.85 M NaCl and incubated overnight at 4 °C with rat Yc₁Yc₁-Sepharose (1.5 ml) in the presence of Tween 20 (0.05%, v/v). The Yc₁Yc₁-Sepharose was prepared by coupling purified rat transferase AA (GST Yc₁Yc₁) to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's guidelines. The antiserum was separated from the Yc₁Yc₁-Sepharose by filtration on a sintered-glass funnel and retained. It was then incubated overnight at 4 °C with mouse YcYc-Sepharose (prepared with purified mouse YcYc and CNBr-activated Sepharose as above). The serum was removed

Table 1 Effect of xenobiotics on hepatic GST activity in Fischer 344 rats

Specific activity values are expressed as the mean of triplicate values $\pm$ S.D. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; AD, androst-5-ene-3,17-dione; tPBO, *trans*-4-phenylbut-3-en-2-one; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; CH, cumene hydroperoxide; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; PB, phenobarbital; AFB₁, aflatoxin B₁; 3-MC, 3-methylcholanthrene; i.p., intraperitoneally.

Treatment	Specific activity (nmol/min per mg of protein)							
	AFB ₁ -8,9-epoxide	CDNB	AD	tPBO	DCNB	EA	CH	H ₂ O ₂
No treatment	0.030 $\pm$ 0.006	1233 $\pm$ 11	6.05 $\pm$ 0.85	17.99 $\pm$ 0.46	31.7 $\pm$ 1.6	23.1 $\pm$ 1.0	1115 $\pm$ 11	980 $\pm$ 4
Peanut oil	0.038 $\pm$ 0.005	1567 $\pm$ 11	7.09 $\pm$ 0.33	18.41 $\pm$ 0.69	32.2 $\pm$ 3.1	21.3 $\pm$ 0.9	1001 $\pm$ 2	852 $\pm$ 11
EQ	0.194 $\pm$ 0.007	4134 $\pm$ 299	15.31 $\pm$ 0.59	25.13 $\pm$ 0.50	123.9 $\pm$ 8.1	34.2 $\pm$ 2.6	897 $\pm$ 14	587 $\pm$ 6
BHA	0.129 $\pm$ 0.009	2733 $\pm$ 72	10.90 $\pm$ 0.59	20.93 $\pm$ 0.67	73.6 $\pm$ 6.4	35.1 $\pm$ 2.9	1045 $\pm$ 50	827 $\pm$ 22
BHT	0.103 $\pm$ 0.001	3016 $\pm$ 55	15.66 $\pm$ 0.73	23.94 $\pm$ 0.35	98.1 $\pm$ 3.6	30.9 $\pm$ 1.3	929 $\pm$ 9	695 $\pm$ 5
PB	0.117 $\pm$ 0.004	3248 $\pm$ 62	20.15 $\pm$ 1.01	25.92 $\pm$ 0.67	71.6 $\pm$ 0.4	39.1 $\pm$ 1.4	968 $\pm$ 18	716 $\pm$ 27
Corn oil (i.p.)	0.024 $\pm$ 0.001	1181 $\pm$ 89	7.81 $\pm$ 0.42	18.48 $\pm$ 0.53	27.0 $\pm$ 2.0	25.2 $\pm$ 3.3	1003 $\pm$ 5	876 $\pm$ 9
AFB ₁ (i.p.)	0.029 $\pm$ 0.001	1349 $\pm$ 21	6.75 $\pm$ 0.28	17.81 $\pm$ 0.64	30.4 $\pm$ 3.0	25.0 $\pm$ 1.8	1060 $\pm$ 11	906 $\pm$ 9
3-MC (i.p.)	0.028 $\pm$ 0.001	1441 $\pm$ 88	8.67 $\pm$ 0.67	13.49 $\pm$ 0.36	26.3 $\pm$ 1.2	25.1 $\pm$ 1.1	863 $\pm$ 5	734 $\pm$ 1
Clofibrate (i.p.)	0.028 $\pm$ 0.001	856 $\pm$ 4	5.66 $\pm$ 0.13	13.33 $\pm$ 0.47	19.2 $\pm$ 2.0	22.9 $\pm$ 1.6	1020 $\pm$ 7	896 $\pm$ 9

by filtration on a scintered-glass funnel and discarded. The mouse YcYc-Sepharese was retained and washed with approx. 50 ml of 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl and 0.05 % (v/v) Tween 20, followed by approx. 50 ml of 20 mM sodium phosphate buffer, pH 7.2, containing 0.25 M NaCl. The gel was eluted with 0.1 M glycine buffer, pH 2.5, and the eluate immediately titrated to approx. pH 7.5 with 1 M Tris solution. The eluate was dialysed against two changes of 2 litres of 20 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl. Finally, the semi-purified IgG was incubated overnight with rat Ya₁Yc₁-Sepharese (prepared with GST Ya₁Yc₁ purified from rat liver and CNBr-activated Sepharose as described above) in the presence of Tween 20 (0.05 %, v/v). The mixture was filtered on a sintered-glass funnel and the eluate retained as the affinity-purified antibody specific for the rat Yc₂ subunit.

Electrophoresis and Immunoblotting

The methods described previously by Hayes and Mantle (1986a,b) were used. The immunoblots were quantified by visual estimation.

RESULTS

Effect of chemoprotective xenobiotics on hepatic GST activity in rats

Several xenobiotic compounds, of which many have been reported to increase GST activity towards model substrates in rodents, were tested for their ability to modulate GST activity towards AFB₁-8,9-epoxide (Table 1). Of these, the antioxidants BHA, BHT and EQ, as well as PB, were found to increase activity towards AFB₁-epoxide between 3- and 5-fold, with EQ being the most effective inducing agent. Neither AFB₁, 3-MC nor clofibrate had any effect on AFB₁-epoxide-conjugating activity. When the 'general' substrate, 1-chloro-2,4-dinitrobenzene (CDNB) was employed in enzyme assays, EQ, BHA, BHT and PB were all found to increase GST activity by approx. 2.5-fold. The compounds AFB₁ and 3-MC had little effect on GST activity towards CDNB. By contrast, clofibrate-treatment was found to cause a decrease in the specific activity towards CDNB. Of the other GST substrates tested [androst-5-ene-3,17-dione, *trans*-4-phenylbut-3-en-2-one (tPBO), 1,2-dichloro-4-nitroben-

zene (DCNB) and ethacrynic acid], EQ, BHA, BHT and PB were all found to be effective inducers of activity. Neither AFB₁ nor 3-MC proved to be good inducers of GST activity for any of the substrates tested. Clofibrate did not increase GST activity for any of the substrates, and for all the activities examined, except that of glutathione peroxidase, cytosol from clofibrate-treated animals had a lower specific activity than control cytosol.

None of the compounds tested was effective in inducing glutathione peroxidase activity towards either cumene hydroperoxide or H₂O₂. Furthermore, as reported previously by Hayes et al. (1991b), EQ was found to cause a reduction in glutathione peroxidase activity that was most pronounced when H₂O₂ was employed as a substrate. BHT, BHA and PB were found to cause modest reductions in this activity. This was also true of 3-MC. However, neither AFB₁ nor clofibrate caused any obvious changes in glutathione peroxidase activity.

Induction of AFB₁-epoxide-metabolizing enzymes by compounds that protect against AFB₁ hepatotoxicity

In order to determine which GST isoenzymes were responsible for the increases in activity seen in the cytosol obtained from xenobiotic-treated rats, levels of individual subunits were examined by Western-blotting analysis (Figure 1a). Of particular interest is the hepatic content of the Yc₂ subunit, which has been shown to have a high specific activity towards AFB₁-epoxide and is known to increase following administration of EQ (Hayes et al., 1991b). However, the regulation of the Yc₂ subunit by BHA, BHT, PB, 3-MC, clofibrate or AFB₁ has not been studied previously. To examine levels of this subunit specifically we purified an antibody that would only react with the rat Yc₂ subunit from antisera raised to mouse YcYc (see the Materials and methods section). This strategy circumvented the problem of cross-reactivity with the other rat Alpha-class subunits, Yc₁ and Ya.

Of all the hepatic GST subunits examined, the most pronounced antioxidant-mediated induction was that of the Yc₂ subunit, which was induced by between 10- and 15-fold by EQ treatment. BHA, BHT and PB were also effective inducing agents for the Yc₂ subunit, although to a slightly lesser extent than EQ. Neither AFB₁, 3-MC nor clofibrate caused elevated levels of the Yc₂ subunit. This result is compatible with the rises in GST activity towards AFB₁-epoxide seen after treatment with

Figure 1 Effect of chemoprotectors on drug-metabolizing enzymes in rat liver

Levels of the drug-metabolizing enzymes, GST (a), cytochrome *P*-450 1A2 (b) and AFB₁-AR (c), in Fischer 344 rats treated with xenobiotics were examined by Western blotting. Rats (four per group) were fed either powdered diets containing additives or were injected intraperitoneally with various compounds as described in the text. Liver protein (40 µg for both cytosolic (a, A–F, and c) and microsomal (a, (G), and b) fractions) was loaded on to polyacrylamide gels, and the separate lanes containing samples from rats treated in the following way: lane 2, control powdered diet; lane 3, dietary BHA; lane 4, dietary BHT; lane 5, PB in drinking water; lane 6, dietary peanut oil; lane 7, dietary EQ; lane 8, corn oil by injection; lane 9, AFB₁ by injection; lane 10, clofibrate by injection; lane 11, 3-MC by injection. Lane 1 contained the antigen against which the relevant antibody was raised, except in (a; G) and (b), where prestained molecular-mass markers were used. The resolved proteins were transferred to nitrocellulose and probed with antisera raised against the following GST (a): (A) mouse Yc (specific for rat Yc₂, see the Materials and methods section), (B) rat Yc₁, (C) rat Ya, (D) rat Yk, (E) rat Yb₁, (F) rat Yf and (G) rat microsomal GST. (b) Western blot of microsomes probed with antiserum raised to CYP450 1A2. (c) Western blot of cytosol probed with antiserum raised against AFB₁-AR.

these compounds. The Yc₁ subunit was considerably less responsive than Yc₂ to the inducing effects of EQ, with only a maximal increase of 2-fold found in cytosol from EQ-treated animals. None of the other compounds examined had a pro-

nounced effect on levels of the Yc₁ subunit. EQ was found to induce the Ya-type subunits in rat liver by approx. 3-fold, as did PB. Both BHA and BHT caused a 2-fold induction of Ya. Neither AFB₁, 3-MC nor clofibrate had any effect on the concentration of Ya. A further Alpha-class GST subunit, Yk, was examined for potential inducibility by chemoprotectors, but the levels of this subunit did not appear to be increased by any of the compounds examined.

Levels of both Mu-(Yb₁) and Pi-(Yf) class GST subunits in the liver cytosols from treated animals were examined. The concentration of the Pi-class Yf subunit was found to be increased by both EQ and BHA (2–3-fold), but not by BHT or PB. As with all the other GST subunits examined, Yf was not induced after treatment with AFB₁, 3-MC or clofibrate. The Mu-class Yb₁ subunit concentration was elevated by approx. 4-fold and 3-fold following treatment with EQ and PB respectively. Both BHA and BHT caused a more modest increase (1.5–2-fold) in the level of the Yb₁ subunit, and concentrations of this subunit did not appear to be affected by AFB₁, 3-MC or clofibrate. The presence of the microsomal GST was also examined, but none of the compounds tested caused any detectable changes in level of this enzyme.

Contrary with the findings presented here, earlier studies have reported that 3-MC causes an increase in GST activity in rat liver, as well as transcriptional activation of GST genes (Igarashi et al., 1987; Ding and Pickett, 1985). Thus, it was critical to establish whether the lack of GST induction found in the present study was accompanied by induction of members of the cytochrome *P*-450 1A family; CYP450 1A genes are transcriptionally activated by 3-MC via the Ah receptor and xenobiotic response elements (XRE), and failure to induce CYP450 1A proteins would indicate that this mechanism of induction was not active under the experimental conditions used. Figure 1(b) shows the Western blot obtained when the rat liver microsomal fractions were probed with antisera raised against CYP450 1A2 protein. It is clear that members of the CYP450 1A family have been induced by greater than 30-fold in response to 3-MC. It is interesting to note that EQ can also induce 1A cytochrome *P*-450(s).

In addition to GSTs, a recently described aldehyde reductase (AFB₁-AR) is thought to play a pivotal role in EQ-mediated chemoprotection against activated metabolites of AFB₁ (Hayes et al., 1993; Ellis et al., 1993). It was important to determine whether other chemoprotectors besides EQ would cause induction of AFB₁-AR. The cytosols from the xenobiotic-treated rats were examined by Western blotting (Figure 1c) using antisera raised against purified AFB₁-AR. Although EQ was found to be the most effective inducing agent for this enzyme, causing an approx. 10–15-fold induction, BHA, BHT and PB also caused induction. BHA caused a 5–8-fold elevation in levels of AFB₁-AR and BHT a 3–4-fold increase. PB was the poorest of the dietary inducing agents, causing only a 2-fold increase in the reductase. Neither AFB₁, 3-MC or clofibrate was an effective inducing agent for AFB₁-AR.

Tissue distribution of AFB₁-detoxifying enzymes

In untreated adult Fischer 344 rats, the Yc₂ subunit is normally undetectable in the liver. It was of interest to determine whether the enzyme is expressed constitutively at high levels in rat organs other than the liver; this could perhaps be indicative of a potential endogenous function for Yc₂-containing enzymes.

Levels of GST activity towards AFB₁-epoxide in several extrahepatic organs from untreated Fischer 344 rats were determined (Table 2). Of all the organs examined (liver, kidney,

Table 2 Levels of GST activity in extrahepatic organs of Fischer 344 rats

Specific activity values are expressed as the mean of triplicate measurements $\pm$ S.D. Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; AD, androst-5-ene-3,17-dione; tPBO, *trans*-4-phenylbut-3-en-2-one; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; CH, cumene hydroperoxide; nd, not determined.

Treatment	Specific activity (nmol/min per mg of protein)							
	AFB ₁ -8,9-epoxide	CDNB	AD	tPBO	DCNB	EA	CH	H ₂ O ₂
Liver	0.013 $\pm$ 0.001	1194 $\pm$ 23	7.66 $\pm$ 0.17	13.13 $\pm$ 0.33	39.9 $\pm$ 1.0	19.2 $\pm$ 0.8	746 $\pm$ 3	712 $\pm$ 7
Kidney	0.008 $\pm$ 0.001	102 $\pm$ 2	1.01 $\pm$ 0.05	0.29 $\pm$ 0.02	< 0.1	8.6 $\pm$ 0.4	495 $\pm$ 10	476 $\pm$ 25
Heart	0.004 $\pm$ 0.001	122 $\pm$ 4	0.41 $\pm$ 0.09	0.67 $\pm$ 0.02	0.4 $\pm$ 0.1	8.0 $\pm$ 0.3	583 $\pm$ 6	619 $\pm$ 15
Lung	0.005 $\pm$ 0.001	47 $\pm$ 2	0.27 $\pm$ 0.03	0.30 $\pm$ 0.02	< 0.1	4.2 $\pm$ 0.1	309 $\pm$ 4	338 $\pm$ 14
Spleen	0.005 $\pm$ 0.001	95 $\pm$ 5	0.32 $\pm$ 0.01	0.18 $\pm$ 0.02	< 0.1	4.3 $\pm$ 0.4	460 $\pm$ 4	503 $\pm$ 10
Adrenal	0.003 $\pm$ 0.001	519 $\pm$ 12	< 0.10	0.05 $\pm$ 0.01	1.0 $\pm$ 0.4	30.0 $\pm$ 2.5	nd	nd
Testes	0.009 $\pm$ 0.001	2756 $\pm$ 113	0.62 $\pm$ 0.09	12.26 $\pm$ 0.67	52.6 $\pm$ 0.9	27.2 $\pm$ 0.8	222 $\pm$ 5	138 $\pm$ 5
Epididymis	0.199 $\pm$ 0.004	2382 $\pm$ 129	< 0.1	9.95 $\pm$ 0.56	96.2 $\pm$ 1.1	41.6 $\pm$ 2.3	434 $\pm$ 2	223 $\pm$ 9
Vas deferens	0.010 $\pm$ 0.001	644 $\pm$ 11	< 0.1	1.87 $\pm$ 0.13	11.6 $\pm$ 0.6	16.6 $\pm$ 1.4	nd	nd
Seminal vesicles	0.007 $\pm$ 0.001	182 $\pm$ 6	< 0.1	3.61 $\pm$ 0.39	< 0.1	3.4 $\pm$ 0.7	152 $\pm$ 6	116 $\pm$ 6
Ovary	0.005 $\pm$ 0.001	691 $\pm$ 33	< 0.1	5.85 $\pm$ 0.23	11.1 $\pm$ 0.6	23.9 $\pm$ 1.0	327 $\pm$ 6	227 $\pm$ 12

heart, lung, spleen, adrenal gland, testis, epididymis, vas deferens, seminal vesicles and ovary), only the epididymis was found to exhibit high constitutive specific activity towards AFB₁-epoxide. The level of activity in this organ was shown to be 15 times higher than that found in untreated liver and 20–60 times higher than that in any of the other organs studied. The epididymis was also found to have a high specific activity towards CDNB, DCNB and tPBO, indicating high levels of Mu-class subunits. Ethacrynic acid also served as a good substrate for epididymal GST, but the specific activity towards androst-5-ene-3,17-dione, a substrate for which Ya-type subunits have a high specific activity, was low. In the epididymis, glutathione peroxidase activity towards cumene hydroperoxide was approx. twice as high as that towards H₂O₂. This indicates that a substantial portion of the activity is being contributed by a selenium-independent glutathione peroxidase, such as a GST, that cannot utilize H₂O₂ as substrate. Of the other organs examined, only the liver and kidney contained appreciable levels of activity towards androst-5-ene-3,17-dione, with the liver containing around seven times as much activity as the kidney. The liver also contained the highest level of glutathione peroxidase activity. As has been reported previously, the testis has a high specific activity towards CDNB, DCNB and tPBO, probably attributable to the presence of the Yn₁ subunit and Yb₂ (Hayes, 1984; Hayes and Mantle, 1986a). By contrast, the ovary contained a much lower level of activity towards those substrates.

In an attempt to establish which GST is responsible for the high specific activity towards AFB₁-epoxide in the epididymis, the extrahepatic organ cytosols were examined by Western blotting, using antisera raised against the Alpha-class GSTs: members of the Alpha class of GSTs are thought most likely to be responsible for GST activity towards AFB₁-epoxide (Hayes et al., 1991a). Figure 2 clearly demonstrates that the epididymis is the only organ that expresses the Yc₂ subunit constitutively. The Yc₁ subunit was found to be expressed in all organs examined, with only the heart containing very low levels. It is notable that the kidney appeared to contain two bands with similar mobility that strongly cross-reacted with the antibody raised to Yc₁. Neither of these polypeptides cross-reacted with the affinity-purified antibody specific for Yc₂-type subunits. When antisera raised to Ya subunits were used to probe the extrahepatic organs, the liver was found to express the highest levels of this subunit, with the kidney containing lesser but substantial quantities. The

Figure 2 Tissue distribution of Alpha-class GST

The concentration of Alpha-class GST was examined in extrahepatic organs by Western blotting. Cytosolic protein (30 μ g) from the separate organs was resolved on SDS/polyacrylamide gels before being electrophoretically transferred to nitrocellulose paper. The gel was loaded as follows: lane 1, the antigen against which the relevant antibody was raised; lane 2, ovary; lane 3, testis; lane 4, epididymis; lane 5, vas deferens; lane 6, seminal vesicles; lane 7, adrenals; lane 8, liver; lane 9, heart; lane 10, lung; lane 11, spleen; and lane 12, kidney. The resolved proteins were probed for cross-reactivity with antisera raised to the following GST: (a) mouse Yc (specific for rat Yc₂, see the Materials and methods section for details), (b) rat Yc₁ and (c) rat Ya.

ovary was also found to contain Ya-type subunits and the testis contained more modest, but detectable, levels of Ya. None of the other organs examined contained detectable levels of Ya subunits.

It was of interest to examine the tissue distribution of AFB₁-AR, again for the reason that high-level constitutive expression of the enzyme in organs where xenobiotic metabolism was not a primary function could indicate an additional role within the cell for AFB₁-AR. The highest levels of AFB₁-AR were found in the kidney, which contained approx. five times that of the liver. Testis contained levels of AFB₁-AR that are comparable with the liver. It was found that the ovary and the seminal vesicles contain trace amounts of a band with a similar mobility to the major protein seen in testis, liver and kidney, and that the adrenal,

heart, lung and spleen contain low, but detectable, levels of AFB₁-AR (results not shown).

DISCUSSION

It has been known for more than 35 years that chemically mediated carcinogenesis can be inhibited by administering compounds such as polycyclic aromatic hydrocarbons at low doses to rats (Miller et al., 1958). The potential of xenobiotic compounds to protect animals from chemical carcinogenesis has since been further explored and developed, and a plethora of chemoprotectors (or anti-carcinogens) identified [see Wattenberg (1985) for a review]. The means by which chemoprotectors reduce the incidence of chemical carcinogenesis is diverse and multifactorial, but it is likely that the principal mechanism of action of many of these chemicals is to alter levels of xenobiotic-metabolizing enzymes, thus increasing the capacity for carcinogen detoxification and excretion.

AFB₁ is a naturally occurring carcinogen and, as with many synthetic chemical carcinogens, its carcinogenic potency can be modulated in rats by the administration of chemoprotective agents [for a review, see Hayes et al. (1991a)]. It has been demonstrated previously in our laboratories that the chemoprotector, EQ, causes elevations in the level of a GST subunit, Yc₂, that has high specific activity towards AFB₁-8,9-epoxide (Hayes et al., 1991b). An aldehyde reductase (AFB₁-AR) capable of detoxifying a dialdehydic product of AFB₁ metabolism has also been identified as an EQ-inducible protein (Hayes et al., 1993). The hepatic induction of both of these two cytosolic proteins is likely to make a major contribution to the anti-carcinogenic mechanism of EQ, although it should also be noted that EQ modestly enhances the ability of liver microsomes to detoxify AFB₁ to aflatoxins Q₁ and M₁ (Mandel et al., 1987). It was previously unknown if other compounds with chemoprotective properties acted in a similar way to EQ by inducing AFB₁-detoxifying enzymes, in particular, the GST subunit Yc₂ and AFB₁-AR.

Control of AFB₁-detoxifying enzymes by antioxidants and PB

Of all the compounds studied, EQ was found to be the most effective inducer of GST activity towards AFB₁-8,9-epoxide, although not necessarily towards all of the other substrates examined. The rise in specific activity towards AFB₁-epoxide coincided with a substantial increase in the level of the Yc₂ subunit, which was induced by between 10- and 15-fold by EQ. It should be noted, however, that the 10–15-fold increase in concentration of the Yc₂ subunit was accompanied by only a 5-fold increase in specific activity towards AFB₁-epoxide. This result suggests that constitutively expressed GSTs are responsible for the basal level of AFB₁-epoxide-conjugating activity. Indeed, we have shown previously that Yc₁-containing GSTs possess a very modest specific activity towards AFB₁-epoxide (Hayes et al., 1991b), and as the Yc₁ subunit is abundant in untreated rat liver it is likely that it contributes to the basal specific activity towards AFB₁-epoxide.

The Yc₂ subunit appears to be induced by a variety of drugs. In the present study we have shown that the Yc₂ subunit is induced in rat liver after treatment with each of the phenolic antioxidants, BHA, BHT and EQ, as well as by PB. Recently, Meyer et al. (1993) have demonstrated that Yc₂ expression is markedly increased after administration of 1,2-dithiole-3-thiones, compounds which also possess antioxidant properties. It is apparent therefore, that as a group, the synthetic antioxidants are of major importance in regulating genes that protect against AFB₁. This class of compound (e.g. 1,2-dithiole-3-thiones) can

also occur naturally in cruciferous vegetables [for a review see Wattenberg (1992)], leading to the possibility that natural antioxidants in the diet can significantly alter susceptibility to AFB₁ carcinogenesis through modulation of hepatic levels of GST Yc₂.

The three antioxidants tested here, and PB, all increase the hepatic levels of Ya-type subunits, with EQ and PB having the greatest effect. Both of these inducing agents caused an approx. 3-fold induction of Ya subunits, whereas BHA and BHT caused a 2-fold induction. Although GST Ya subunits have little catalytic activity towards AFB₁-8,9-epoxide (Hayes et al., 1991b), the increase in this subunit type may help protect against AFB₁ through the ability of Ya-containing GST to sequester the mycotoxin, thereby preventing it from interacting with DNA. The Ya-containing GSTs have been shown to be capable of covalently binding carcinogens such as 3-MC (Ketterer, 1972), and it should be noted that following exposure of chicken embryo primary hepatocytes to AFB₁, Iwaki et al. (1990) found that the mycotoxin specifically associated with a polypeptide of M_r 25000. Although not identified as a GST subunit, this polypeptide, which binds AFB₁ covalently, is closely similar in size to the Ya subunits.

Induction of the Yc₁-type subunits was much less marked than either Ya or Yc₂, with EQ causing the greatest effect (1.5–2-fold). The Yk subunit did not appear to be induced by any of the compounds examined. Hayes et al. (1991b) reported an approx. 2-fold increase in Yk after treatment of rats with EQ. This discrepancy could be simply due to differences in methodology for detection of the Yk subunit, but it is possible that the difference in the age of rats (10–15 weeks cf. 8–12 months) had an effect as it has been shown by McCusker et al. (1989) that Yk is developmentally regulated. Potentially, different basal levels of the Yk subunit in young and mature rats could influence the extent of induction by EQ, in that there may be an upper threshold level of expression that precludes induction by xenobiotics. In mouse liver, basal levels of Yk are comparatively low (L. I. McLellan and J. D. Hayes, unpublished work) but the Yk subunit has been shown to be induced approx. 5-fold by BHA (McLellan et al., 1992).

The Yb₁ subunit was induced by EQ approx. 4-fold, and to a lesser extent by PB (3-fold), BHA and BHT (1.5–2-fold). Compared with Ya and Yf, considerably less is known about the control of activity of the Yb subunit and expression of the gene. It has been demonstrated previously that Yb₁ can be induced by BHA in mouse and rat (Pearson et al., 1983; Sato et al., 1984; Hayes et al., 1991c), and that, in rat, PB can cause transcriptional activation of the gene encoding Yb₁ (Pickett et al., 1987). The mouse Yb₁ gene has been studied in relation to its induction by BHA (Reinhart and Pearson, 1993). It is believed that BHA can be metabolized *in vivo* to the reactive quinone, *t*-butylhydroquinone (Brannen, 1975) which can act either indirectly or directly as a transcriptional activator for the gene encoding the rat Ya₂ subunit via an antioxidant response element (Rushmore and Pickett, 1990; Rushmore et al., 1991). However, Reinhart and Pearson (1993) have examined the 5' regulatory sequence of the mouse Yb₁ gene and have not found any exact matches for an antioxidant response element or AP1-binding sites [the mouse electrophile response element, thought to control BHA-mediated transcriptional activation of a mouse Ya-encoding gene (Friling et al., 1990), comprises tandem AP1-binding sites]. Two introns in the Yb₁ gene were shown to contain AP1 sites and AP1-like sites, as well as xenobiotic response elements and a reverse antioxidant response element. Transfected constructs lacking these two introns were still found to be induced by BHA and β -naphthoflavone, suggesting that

those elements were unlikely to be important in transcriptional activation of this gene.

The Pi-class GST, Yf, showed an interesting response to treatment by antioxidants, in that only BHA and EQ were inducing agents. Neither BHT nor PB had any effect on induction of this subunit. The reason behind this differential induction is not clear; in most cases where BHA has caused induction of a GST subunit, PB and BHT have also been inducing agents, suggesting that there may be some common mechanism. The lack of induction of Yf by PB or BHT clearly demonstrates that this is not the case, at least for Yf.

When levels of AFB₁-AR were measured in rat liver cytosol it was found that EQ was the most effective inducing agent, causing between 10- and 15-fold induction. BHA and BHT were also effective in inducing AFB₁-AR, producing a 6–8-fold and 3–4-fold induction respectively. PB caused only a 2-fold induction of AFB₁-AR. AFB₁-AR was identified only recently as an EQ-inducible protein capable of reducing a potentially toxic dialdehydic product of AFB₁ metabolism (Hayes et al., 1993; Judah et al., 1993) and little is known about the control of its activity. It has been demonstrated that induction of the AFB₁-AR protein by EQ is accompanied by an increase in the level of mRNA (Ellis et al., 1993), although the mechanism by which this happens is still unclear.

The effect of 3-MC on AFB₁-metabolizing enzymes

The Yc₂ subunit was not found to be induced by 3-MC, a compound that has been described as a chemoprotective agent (Miller et al., 1958). However, 3-MC has been reported previously to increase hepatic GST activity in rats, which is in contrast with the results reported in the present study (Ding and Pickett, 1985; Igarashi et al., 1987). Igarashi et al. (1987) demonstrated that 3-MC caused a rise in GST activity and of the concentration of Ya subunits in rat liver. Ding and Pickett (1985) also showed transcriptional activation of Alpha- and Mu-class GST genes in rat liver after 3-MC treatment. The work described herein shows little, if any, increase in GST activity towards any of the substrates tested after 3-MC treatment, and levels of the GST subunits, as determined by Western-blot analysis, appeared to undergo little change. It does not seem likely that these conflicting data can simply be explained by differences in drug regimens between laboratories, as the dose of 3-MC used in this study was two and five times greater respectively than those described by Ding and Pickett (1985) and Igarashi et al. (1987). The only major difference in experimental design is that of age, size and strain of rats. The rats used in the laboratories of both Ding and Pickett (1985) and Igarashi et al. (1987) were young adult male Sprague–Dawley rats, being 150–200 g and 3–7 weeks old in the respective laboratories. By contrast, we used Fischer 344 rats of approx. 400 g (8–12 months old) for our induction study. Like the Yk subunit, Ya is developmentally regulated and basal expression increases with age (McCusker et al., 1989). It is feasible that an age-related loss of the mechanism for induction of GSTs by polycyclic aromatic hydrocarbons occurs that is perhaps related to basal hepatic concentrations. Alternatively, GST gene expression could be regulated differently in Fischer 344 rats and Sprague–Dawley rats. However, this explanation is improbable as the Ah receptor/xenobiotic response element induction mechanism appears to be functional in the induction of the cytochromes P-450 1A family in mature Fischer rats (Figure 1b). As one of the genes encoding a rat Ya subunit contains a xenobiotic response element (Rushmore et al., 1990), some measure of induction of Ya would be expected, particularly when Ya was shown to be inducible by antioxidants and PB. It is unclear why

the CYP450 1A2 should be induced by 3-MC and not the Ya subunit.

The role of oxidative stress in induction of Yc₂

At present, the nature of the inducing signal for increased GST Yc₂ expression is unknown. However, Rushmore et al. (1991) have shown that the antioxidant response element on the rat Ya subunit is responsive to H₂O₂ and reactive-oxygen species in transfected human hepatoma cells. It was thus of interest to determine whether drugs that can generate oxidative stress *in vivo* could induce the Yc₂ and Ya subunits. Clofibrate is a hepatocarcinogen that has been shown to increase the number of peroxisomes in hepatic parenchymal cells (Rao and Reddy, 1987). The associated elevation in H₂O₂-generating peroxisomal β -oxidation may cause increased oxidative stress in the liver, a phenomenon that could potentially activate transcription of genes containing an antioxidant response element. We found that clofibrate caused no induction of Yc₂ or the Ya subunit. None of the GSTs examined was induced by clofibrate, nor was AFB₁-AR. Foliot et al. (1986) and Furukawa et al. (1985) also found that GST activity was not increased by peroxisome proliferators.

Tissue-specific regulation of enzymes capable of detoxifying AFB₁ metabolites

Among the extrahepatic organs studied (kidney, heart, lung, spleen, adrenal gland, testis, epididymis, vas deferens, seminal vesicles and ovary), the only one found to contain detectable quantities of the Yc₂ subunit was the epididymis. The concentration of Yc₂ in the epididymis is probably comparable with that seen in EQ-treated rat liver. Furthermore, the level of AFB₁-8,9-epoxide-conjugating activity compared favourably with that of EQ-treated rat liver, with specific activities of 0.194 nmol/min per mg of protein and 0.199 nmol/min per mg of protein found in EQ-treated rat liver and epididymis respectively. The presence of Yc₂ in the epididymis is intriguing and does imply a role for Yc₂ that is separate from AFB₁-epoxide metabolism. Although it is feasible that its function in the epididymis is solely to conjugate GSH with xenobiotics that can enter the seminal fluid, the Yc₂ subunit has relatively high glutathione peroxidase activity (Hayes et al., 1991b) and it is possible that the role of Yc₂ in the epididymis is that of a glutathione peroxidase. The epididymis is the site of sperm maturation and storage, and as spermatozoa are very sensitive to lipid peroxidation (with loss of motility and fertility being the consequence), it is important that this organ contains enzymes that can combat oxidative stress. In some mammals, glutathione peroxidase(s) is thought to be a major factor in preventing the loss of sperm fertility associated with oxidative damage (Mann and Lutwak-Mann, 1981). A novel glutathione peroxidase has recently been isolated from rat epididymis (Perry et al., 1992) and like the Yc₂ subunit it appears to be specific to this organ. The epididymal glutathione peroxidase was found to be regulated by androgens, although its possible regulation by androgens in organs other than the epididymis was not determined. It is possible that Yc₂ is regulated in a similar manner; Robaire and Hales (1982) have shown that epididymal GST activity towards CDNB and tPBO is influenced by androgen status.

Apart from the kidney, AFB₁-AR was not expressed at particularly high levels in any of the extrahepatic organs examined. The testis was the only other organ examined that contained appreciable quantities of AFB₁-AR, containing levels comparable with that of liver from untreated rats. Nevertheless, the fact that the testis was the only extrahepatic organ apart from kidney

to contain more than a trace of AFB₁-AR signifies that the reductase may play a role in testis that is not directly involved in drug metabolism. However, until more is known about the substrate specificity of AFB₁-AR, it is difficult to predict potential functions in testis.

Concluding comments

Both of the phase-II detoxification enzymes that appear to be most influential in protecting against the activated metabolites of AFB₁ (i.e. GST Yc₂ and AFB₁-AR) are present in low concentrations in normal adult male rat liver. In the present study we have shown that the Yc₂ subunit seems, in many instances, to be regulated in a co-ordinate manner to AFB₁-AR. Both enzymes are induced by feeding rats with the antioxidants BHA, BHT and EQ. Moreover, both enzymes are present in high concentrations in hepatic tumours derived from AFB₁-fed rats (Hayes et al., 1993). There are, however, also some notable differences in the regulation of Yc₂ and AFB₁-AR. Hayes et al. (1993) demonstrated that developmental regulation of the two proteins is distinct, in that fetal rat liver contains high concentrations of Yc₂ but very little AFB₁-AR. The present study demonstrates that tissue-specific regulation is a further example where regulation of Yc₂ and AFB₁-AR differs. Unlike AFB₁-AR, high levels of Yc₂ are present in the epididymis. Substantial quantities of AFB₁-AR were also found to be present in the kidney, whereas Yc₂ was undetectable. It has yet to be determined whether the mechanisms governing drug induction of these AFB₁-detoxifying enzymes are similar for both Yc₂ and AFB₁-AR. This is currently being examined in our laboratories.

We are grateful to the MRC for financial support (project grant G 9027099 SA) and to the Scottish Office Home and Health Department for funding for the Cobas Fara centrifugal analyser (grant no. K/CSO/2/20/3/7). We thank Dr. E. M. Ellis for critical comments and Professor C. R. Wolf for providing the antiserum raised against CYP450 1A2.

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