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THE ROLE OF SPECIFIC CYTOCHROMES P450 IN THE FORMATION OF 7,12-DIMETHYLBENZ(*a*)ANTHRACENE–PROTEIN ADDUCTS IN RAT LIVER MICROSOMES *IN VITRO*

SARA E. LAMBARD,*† ALAN K. BURNETT,‡ C. ROLAND WOLF§ and JOHN A. CRAFT*

*Department of Biological Sciences, Glasgow Polytechnic, Glasgow G4 0BA; ‡Leukaemia Research Fund Laboratories, Department of Haematology, Glasgow Royal Infirmary, Glasgow G4 0SF; and §Imperial Cancer Research Fund Laboratory of Molecular Pharmacology and Drug Metabolism, Department of Biochemistry, Hugh Robson Building, George Square, Edinburgh EH8 9HD, U.K.

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Abstract—The role of specific cytochrome P450 (P450) isoforms in the formation of adducts of 7,12-dimethylbenz(*a*)anthracene metabolites and membrane proteins has been investigated *in vitro* with microsomal fractions prepared from rats pretreated with various isoenzyme selective inducers. The effects of isoenzyme selective inhibitors were also evaluated. Adduct formation was shown to be mediated by P450 catalysed reactions but was unaltered, relative to untreated animals, in membranes from pyrazole- and clofibrate-treated animals suggesting that CYP2E1 and CYP4A1 are not involved in this process. However, adduct formation was significantly increased in microsomes from Sudan III-, phenobarbital- and dexamethasone-treated rats, suggesting the involvement of the CYP1A, CYP2B and CYP3A subfamilies, respectively. These conclusions were further supported by the finding that adduct formation in these microsomes could be inhibited by the isoenzyme-selective inhibitors α-naphthoflavone, metyrapone and troleandomycin, respectively.

The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(*a*)anthracene (DMBA‖) is a potent procarcinogen, causing mammary [2, 3] and skin tumours [3] and leukaemia [3, 4] in rats. DMBA is metabolized via a sequence of reactions catalysed by cytochromes P450 and epoxide hydrolase, to give a variety of metabolites, amongst them the bay-region diol-epoxide DMBA-3,4-diol-1,2-oxide, which is thought to be the ultimate carcinogen [5, 6]. It has also been suggested that the bay-region diol-epoxide of 7-hydroxymethyl-12-methylbenz(*a*)anthracene may be important in carcinogenesis [7] but DMBA 3,4-diol-1,2-oxide adducts predominate, and hydroxylation of the C7 methyl group reduces carcinogenic activity [8].

DMBA-induced tumourgenicity in rats is influenced by a number of factors including age, sex and strain [2, 3]. Alteration in DMBA metabolism, resulting in differential production of the ultimate carcinogen(s) could contribute to this variation. Cytochrome P450 isoenzymes have a major role in determining the route of DMBA metabolism. These enzymes exhibit regio- and stereo-selectivity during metabolism of this polycyclic aromatic hydrocarbon, producing a variety of metabolites determined by the isoenzyme composition of the membrane preparation used [9, 10]. Many of the cytochrome P450s are present constitutively at low levels while others are not expressed in the uninduced state. Induction of specific P450 isoenzymes with xenobiotics *in vivo*, results in alterations in the pattern of DMBA diols produced *in vitro* [11]. These changes can be linked to altered carcinogenic potential. For example the azo dye, Sudan III [1-(4-phenylazo-phenylazo)-2-naphthol], induces CYP1A1 and is associated with a lower incidence of leukaemia in DMBA-treated Long–Evans rats [12]. DMBA epoxide formation as measured by diol formation is increased overall in microsomes from Sudan-treated rats; however, production of the proximate carcinogen DMBA-3,4-diol is decreased [13].

Metabolites of DMBA can bind covalently to DNA [14] and this may result in the activation of proto-oncogenes [15–17]. Binding to proteins also occurs [18] and the formation of active metabolites can be conveniently measured by quantification of the resultant membrane–protein adducts. Previous work in this laboratory indicated that DMBA–protein adduct formation probably involved secondary metabolites and is increased by the isoenzyme-selective inducers phenobarbital and Sudan III [18]. The involvement in this process of CYP2B was further indicated since the selective inhibitor metyrapone significantly inhibited adduct formation in microsomes from phenobarbital-treated rats [18].

These studies have been extended using other P450 inducers and inhibitors to gain a better insight into the P450 isoenzymes responsible for adduct formation. The isoenzyme-selective inducers utilized in these studies were: Sudan III (S), phenobarbital (PB), pyrazole (PY), clofibrate (CLO) and dexamethasone (DEX). In the rat Sudan III induces the CYP1A1 isoenzyme [11, 13]. Phenobarbital has pleiotropic effects and causes a large increase in CYP2B1 which is not detectable before induction [19]. Smaller increases are also found for the

† To whom correspondence should be addressed.

‖ Abbreviations: P450, cytochrome P450; DMBA, 7,12-dimethylbenz(*a*)anthracene; UT, untreated; S, Sudan III; PB, phenobarbital; PY, pyrazole; CLO, clofibrate; DEX, dexamethasone; mRNA, messenger ribonucleic acid. The abbreviations used for the P450 isoenzymes are those of Nebert *et al.* [1].

constitutively expressed CYP2A1 and 2B2 and various members of the CYP2C and 3A subfamilies [20]. Pyrazole induces CYP2E1 [21] while clofibrate induces two P450s of the CYP4A subfamily which are present constitutively in rat liver and are involved in fatty acid hydroxylation [20]. Dexamethasone induces CYP3A1, an isoenzyme involved in the metabolism of endogenous steroids [22].

A number of P450 isoenzyme-selective inhibitors were used in these studies. Piperonyl butoxide is a general P450 inhibitor [23] while metyrapone is selective for CYP2B [23], α-naphthoflavone for CYP1A [23] and troleandomycin for CYP3A [24].

MATERIALS AND METHODS

Chemicals. DMBA, glucose-6-phosphate (sodium salt), glucose-6-phosphate dehydrogenase (yeast type VII), metyrapone, troleandomycin, NADP-Na^+, NADPH-Na^+, Sudan III, pyrazole, dexamethasone, clofibrate and phenobarbital were purchased from the Sigma Chemical Co. (Poole, U.K.). Piperonyl butoxide and α-naphthoflavone were obtained from the Aldrich Chemical Co. (Gillingham, U.K.). Trichloroacetic acid (TCA), ethanol, diethylether and chloroform were all analytical grade. [G-^{3}H]DMBA (87.4 Ci/mmol) was purchased from Amersham International (Amersham, U.K.). Radiochemical purity of this material has been confirmed previously, by normal phase HPLC, to be at least 97% [25].

Animals and treatments. Female rats of the Long–Evans strain, aged approximately 6 weeks and weighing 200 g when killed, were taken from a random-bred colony maintained on a commercial diet CRMX (BS and F, Scotland Ltd, Portobello, East Lothian, U.K.). Treated animals received xenobiotics at the frequency, duration and dose stated: Sudan III (single intragastric administration of 7.5 mg in 0.5 mL sesame oil, 24 hr before being killed), phenobarbital (0.1% w/v in drinking water for 7 days prior to being killed], pyrazole (single intraperitoneal injection of 37 mg in isotonic saline given 24 hr before being killed), clofibrate [0.5% (w/w) blended with diet as in Ref. 11 and fed *ad lib.* for 10 days prior to being killed], dexamethasone (daily intraperitoneal injections of 37 mg suspended in corn oil given for 2 days prior to being killed).

Microsome preparation. These studies used two separate membrane preparations. Microsomes were prepared from the pooled livers of two animals in Tris–HCl (0.05 M, pH 7.4 at 4°)/KCl (1.15% w/v)/EDTA (1 mM) as described previously [18]. The microsomal pellets were resuspended in potassium phosphate buffer (0.2 M, pH 7/1 mM EDTA) to a protein concentration of 10–20 mg/mL. Protein was estimated by the method of Bradford [26] against BSA standards. P450 content was determined by the method of Omura and Sato [27] and mean values from determinations on both preparations used in these studies, given as nmol/mg protein, were: untreated, 0.59 ± 0.13; Sudan, 0.73 ± 0.06; phenobarbital, 2.36 ± 0.5; pyrazole, 0.71 ± 0.21; clofibrate, 0.61 ± 0.08; dexamethasone, 0.81 ± 0.01.

Adduct formation. Adduct formation was measured essentially as described in Ref. 18 and was linear with respect to protein concentration (up to 2 mg) and time (up to 60 min) and was dependent on the presence of NADPH. [^{3}H]DMBA (10 μCi, 50 μM) was incubated with microsomal samples (1 mg) in the presence of a NADPH recycling system containing NADP (0.4 mM), glucose-6-phosphate (4 mM) and glucose-6-phosphate dehydrogenase (4 units) in Tris–HCl buffer (0.07 M, pH 7.5 at 37°)/potassium chloride (215 mM)/EDTA (0.1 mM)/magnesium chloride (14 mM) in a final volume of 1 mL. Incubations were performed at 37° and continued for 30 min. CYP inhibitors were prepared as ethanolic solutions with the exception of α-naphthoflavone which was prepared in chloroform. The inhibitor solutions were added to the reaction tubes and the solvent evaporated under argon before the addition of the remaining reaction components. In all cases the reaction was stopped by the addition of ice-cold acetone and the aqueous phase was extracted twice with 1 mL ethyl acetate. Microsomal protein was precipitated from the aqueous phase with 10% TCA and collected onto a glass-fibre disc (Type GF/C, Whatman, Maidstone, U.K.), washed twice with 2 mL ethanol/ether (2:1) and dried prior to determination of radioactivity in a liquid scintillation spectrometer. Adduct formation was calculated as the difference between radioactivity incorporated in the presence and absence of NADPH. The limits of detection of this assay were 3 pmol of incorporated DMBA. All samples were evaluated in triplicate and pooled results from two microsome preparations are shown. Differences in adduct formation between untreated, induced and inhibited microsome preparations were analysed by a paired Student's *t*-test.

Western blotting. Western blotting was performed essentially as described by Towbin [28]. After separation on 9% polyacrylamide reducing gels, proteins were transferred electrophoretically to nitrocellulose filters (Schleicher and Schull) and probed with various antisera to rat liver cytochromes P450. Antibodies to the purified enzymes were isolated as described previously [29]. Anti-CYP4A1 was generously provided by Dr G. G. Gibson. The specificities of these antibodies when used in Western blots have been described previously [18, 30]. Anti-CYP2B reacts with CYP2B1 and CYP2B2, anti-CYP2C6 recognises several proteins of the IIC subfamily and anti-CYP3A recognises both CYP3A1 and CYP3A2. The immunoreactive polypeptides were visualized using horseradish peroxidase-labelled second antibody in conjunction with an enhanced chemiluminescence detection system (Amersham) following the supplier's protocol. The intensity of the signal on the X-ray film was directly proportional to the amount of protein loaded on the gel.

RESULTS

DMBA–protein adduct formation

The extent of DMBA–protein adduct formation was determined in microsomes prepared from the livers of untreated female Long–Evans rats or animals treated with Sudan III, phenobarbital, pyrazole, clofibrate or dexamethasone. DMBA–

Table 1. Effects of various inducers of P450 enzymes on the rate of production of DMBA–protein adducts and diols by hepatic microsomes

Animal treatment	Adduct formation (pmol/min/mg)	Adduct formation (% untreated control)	Diol formation† (pmol/min/mg)	Diol formation† (% untreated control)
UT	0.5 ± 0.13	100	31.3	100
S	1.36 ± 0.38*	272	237.6	760
PB	1.55 ± 0.38*	310	90.3	290
PY	0.43 ± 0.15	86	22.4	40
CLO	0.62 ± 0.14	124	60.6	200
DEX	2.83 ± 0.95*	566	22.6	70

UT, untreated; S, Sudan III; PB, phenobarbital; PY, pyrazole; CLO, clofibrate; DEX, dexamethasone.
* $P \leq 0.01$.
† Diol formation represents the mean production of three diols (3,4-diol, 5,6-diol, and 8,9-diol) calculated from results presented in Ref. 11. Individual diol data was obtained from three different membrane preparations, with duplicate determinations for each preparation.

protein adduct formation in microsomes from untreated rats averaged 0.5 ± 0.13 pmol/min/mg protein. Of the five xenobiotic inducers tested, DMBA–protein adduct formation was significantly increased in microsomes prepared from Sudan-, phenobarbital- and dexamethasone-treated rats (see Table 1). Sudan treatment increased adduct formation by 2.7-fold, phenobarbital by 3-fold and dexamethasone by 5.7-fold. Microsomes prepared from pyrazole- and clofibrate-treated rats did not exhibit significantly altered adduct formation. The rate of adduct formation in these membranes might be related to the rate of DMBA–epoxide formation. However, no correlation was found between adduct formation and the formation of primary epoxides as measured by diols (Table 1). This was true when the rates were compared to total diols (DMBA-3,4- + -5,6- + -8,9-diols) (Table 1) or individual diols (data not shown; see Ref. 11).

Inhibition of adduct formation

The role of P450s in adduct formation was further investigated using inhibitors of P450-mediated reactions. A high inhibitor concentration (1 mM) was chosen initially to ensure enzyme saturation. Piperonyl butoxide, a general inhibitor of P450-catalysed reactions [23], reduced DMBA–protein adduct formation in all microsome preparations to less than 22% of the rate observed in the absence of inhibitor (uninhibited control) (see Fig. 1a).

α-Naphthoflavone is selective for CYP1A [23], and this inhibitor reduced DMBA–protein adduct formation to 28% of uninhibited control levels in microsomes from Sudan-treated rats and 41% in microsomes from both pyrazole- and clofibrate-treated rats (see Fig. 1b). There was no significant effect with membranes from untreated, phenobarbital- or dexamethasone-treated animals. Metyrapone (2-methyl-1,2,di-3-pyridyl-1-propanone) is selective for CYP2B [23], the group of isoenzymes which is most sensitive to phenobarbital induction. DMBA–protein adduct formation was most significantly reduced by this inhibitor in microsomes from rats treated with phenobarbital (23% of uninhibited control) (see Fig. 1c). Reduced adduct formation with this agent at 1 mM was also observed in microsomes from dexamethasone-treated rats, Sudan-treated rats and untreated rats (38, 37 and 61% of uninhibited control, respectively). However, when metyrapone was used at 80 μM adduct formation was only inhibited in membranes from the phenobarbital-treated animals (data not shown). Troleandomycin is selective for the CYP3A isoforms [24] and reduced adduct formation in microsomes from dexamethasone- and phenobarbital-treated rats to 16 and 40% of uninhibited control, respectively (see Fig. 1d). Membranes from untreated, Sudan-, pyrazole- and clofibrate-treated rats were unaffected by this inhibitor. The results shown in Fig. 2 were obtained with single concentrations of inhibitors but further work showed inhibition of DMBA–protein adduct formation to be concentration dependent. In these experiments α-naphthoflavone, metyrapone and troleandomycin, were used with microsomes from Sudan-, phenobarbital- and dexamethasone-treated rats. Half maximal effects were determined and the results are shown in Table 2.

Induction of P450s

The membrane preparations used in the adduct measurements were also analysed by Western blotting using antibodies raised against CYP1A1, 1A2, 2B1, 2C6, 3A1 and 4A1 (see Fig. 2). The majority of the antisera produced a single dominant band with each of the membrane preparations used. The sole exception was anti-CYP2B1 which revealed two faint bands in membranes from untreated animals, but only one of these bands was altered by xenobiotic treatment. The signal intensity produced by the antibodies in membranes from xenobiotic-treated animals is considered relative to the signal in membranes from untreated controls.

Antiserum raised against CYP1A1 gave an enhanced signal in microsomes from Sudan-, pyrazole- and clofibrate-treated rats. Chemiluminescence was far less intense in microsomes from untreated and phenobarbital-treated rats, and was absent in microsomes from those animals treated with dexamethasone. Anti-CYP1A2 reacted with

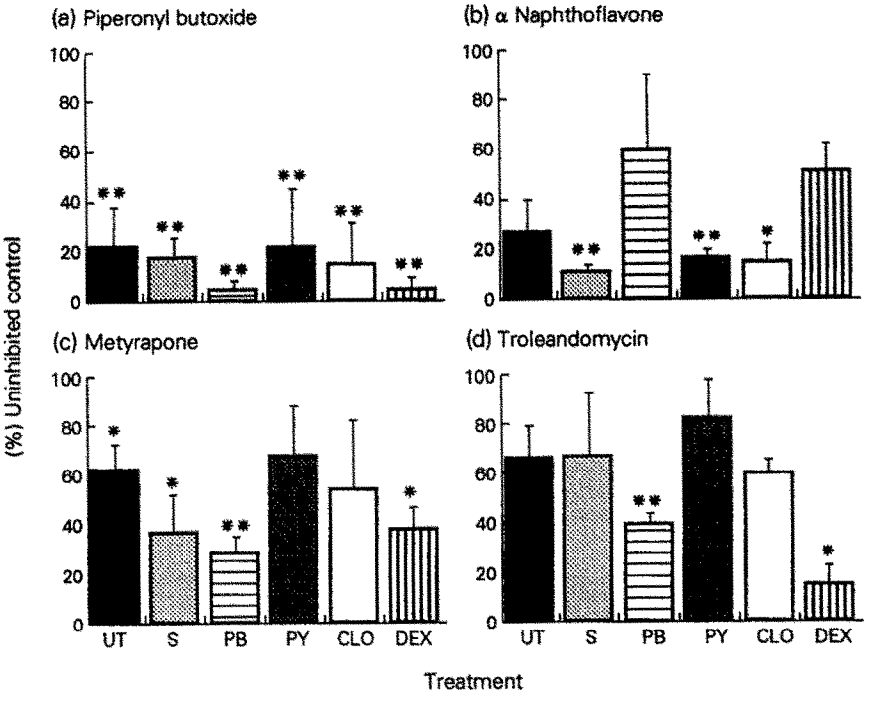

Fig. 1. The formation of DMBA–protein adducts was investigated in the absence and presence of the various inhibitors (final concentration 1 mM) as described in Materials and Methods.

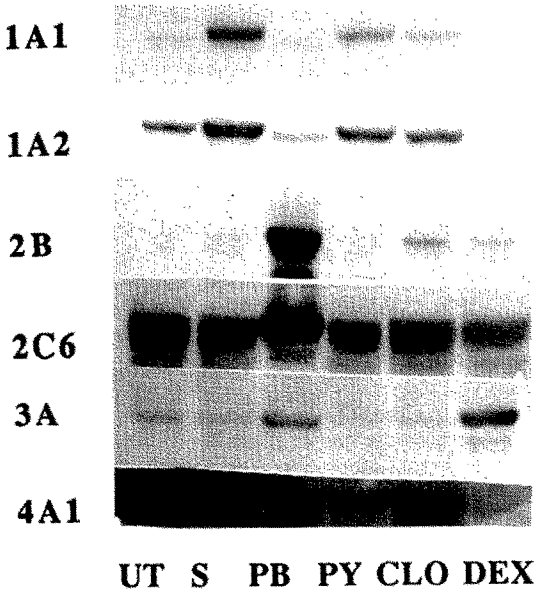

Fig. 2. Western blots of rat liver microsomes. Membranes from untreated and xenobiotic-treated animals were analysed with various antisera as in Materials and Methods. Ten micrograms of membrane protein was loaded in each channel.

protein in all membrane preparations. Chemiluminescence was increased in microsomes from Sudan-, pyrazole- and clofibrate-treated animals and reduced in membranes from phenobarbital- and dexamethasone-treated animals. Protein reacting with anti-CYP2B was detectable at low levels in all microsome preparations. However, chemiluminescence was greatly increased in microsomes from phenobarbital-treated animals and to a much lesser extent in those from clofibrate- and dexamethasone-treated rats. Anti-CYP2C6 reacted with protein in all membrane preparations to give a broad band signal. The intensity of this signal was increased in microsomes from phenobarbital-treated rats but reduced in those from Sudan-, pyrazole- and dexamethasone-treated animals. Chemiluminescence produced by antiserum to CYP3A1 was much increased in microsomes from dexamethasone-treated animals and to a lesser extent in those treated with phenobarbital while the signal was reduced in microsomes from pyrazole-treated rats. Chemiluminescence produced with antibody to CYP4A1 was increased in microsomes from clofibrate-treated animals and decreased in those from dexamethasone-treated rats.

DISCUSSION

In these studies we have examined the formation of covalent DMBA–microsomal protein adducts *in vitro* following induction of different P450 subfamilies *in vivo*. This *in vitro* system is not representative of the *in vivo* situation in which detoxification

Table 2. EC_{50} values for inhibition of DMBA–protein adduct formation by metyrapone, troleandomycin and naphthoflavone in microsomes from phenobarbital- (PB), dexamethasone- (DEX) and Sudan-treated (S) rats

	Inhibitor (μM)		
Inducer	Metyrapone	Troleandomycin	Naphthoflavone
PB	28	200	ND
DEX	500	100	ND
S	1000	ND	4

Reactions were performed as in Materials and Methods.
ND, not determined.

mechanisms, such as glutathione transferase, would reduce the number of active metabolites. However, protein adduct formation in microsomes is indicative of the potential for the production of active metabolites and as such is a useful experimental tool.

Induction of P450 isoenzymes has been monitored by Western blotting. Moreover, the contribution to adduct formation by P450 subfamilies and individual P450 isoenzymes has been evaluated using selective inhibitors of P450 action. DMBA–protein adduct formation is dependent upon metabolism. This is illustrated by the low level of radioactivity bound to protein when NADPH is omitted from the reaction [18] and also by the dramatic reduction in adduct formation in all microsomal preparations in which a general P450 inhibitor, piperonyl butoxide, was included (see Fig. 1a).

DMBA–protein adduct formation was altered significantly by treatment of animals with certain xenobiotics which induce P450s. There was no increase in DMBA–protein adduct formation in membranes from pyrazole- or clofibrate-treated rats, indicating that CYP2E1 and CYP4A1 are not likely to be involved in increased adduct formation. However, Sudan, phenobarbital and dexamethasone increased adduct formation by 2.7-, 3- and 5.7-fold, respectively, when compared to adduct formation in microsomes from untreated rats (Table 1). This implicates members of the CYP1A family (induced by Sudan III), CYP2A, CYP2B and CYP2C families (all induced by phenobarbital), and members of CYP3A (induced by dexamethasone and phenobarbital) in increased adduct formation in these membranes. Thus, members of more than one P450 isoenzyme family are likely to be involved in DMBA–protein adduct formation. Previous work has shown that several of the P450 inducers also alter the profile of DMBA metabolites. Induction with Sudan increases formation of the 5,6- (9-fold) and 8,9-diols (13-fold), whilst reducing the formation of the 3,4-diol (0.5-fold) resulting in a 7.6-fold increase overall in the production of these diols [11]. Induction with phenobarbital increases the formation of the 3,4- (2-fold), 5,6- (4-fold) and 8,9-diols (2-fold) (2.9-fold overall), whilst dexamethasone alters the formation of the 3,4-diol only (0.2-fold) (diols reduced to 0.7-fold of control). Clearly the changes in DMBA metabolism as indicated by production of individual diols do not correlate with the increases observed in DMBA–protein adduct formation with these inducers, whether the diols are considered separately or are summed. This suggests that the reactive species involved in adduct formation, in these microsomes, are not primary metabolites. Epoxide hydrolase activity, measured previously in this laboratory, in both untreated and treated rat microsomes, exceeds diol formation by at least three orders of magnitude (data not shown) indicating efficient metabolism of primary epoxides. Moreover, Bigger *et al.* [31] have shown that at the ratio of DMBA:protein used in these studies, there is rapid further metabolism of primary metabolites to secondary and tertiary compounds. Thus, the majority of DMBA–protein adducts measured here are unlikely to involve primary epoxides.

In microsomes from Sudan-treated rats, induction of both CYP1A1 (see Fig. 2) and CYP1A2 was observed. Moreover, adduct formation in these membranes was reduced by α-naphthoflavone to levels seen in microsomes from untreated rats (Fig. 1b). The P450-selectivity of this inhibitor suggests that in Sudan membranes, CYP1A1 and/or 1A2 probably catalyses increased DMBA–protein adduct formation. Induction of both CYP1A1 and CYP1A2 was also evident in microsomes from pyrazole- and clofibrate-treated rats although no increase in adduct formation was observed. This observation presumably results from the much lower induction of CYP1As by these agents and suppression of other P450s involved in adduct formation. It is noteworthy that adduct formation in these membranes was reduced by α-naphthoflavone.

Phenobarbital induces P450s from several subfamilies. The major induced protein is CYP2B1 [19], but CYP2B2 and members of the CYP2C [11] and CYP3A [22] families are increased to a lesser extent (Fig. 2). Metyrapone, an inhibitor selective for the CYP2B subfamily [23], reduced adduct formation most effectively (to levels 28% of control) in microsomes from phenobarbital-treated rats, emphasizing the role of this subfamily in adduct formation. The specificity of metyrapone action is further indicated by the low value of EC_{50} found with these membranes (see Table 2). Adduct formation was also reduced by metyrapone in microsomes from untreated (61% of control) and Sudan- (37% of control) and dexamethasone-treated rats (37% of control). However, the EC_{50} values for these effects were found to be very high (Table 2) and presumably

reflect non-specific action of the inhibitor. CYP2C is expressed constitutively [32] and is induced following phenobarbital treatment, but to a lesser extent than CYP2B. CYP2C has been implicated in the formation of the proximate carcinogen DMBA-3,4-diol [11, 33], but it is unlikely that these isoforms are involved in DMBA–protein adduct formation. Metyrapone is selective for CYP2B and does not effect DMBA-3,4-diol formation but reduced adduct formation in membranes from phenobarbital-treated animals to levels observed in microsomes from untreated rats (see Fig. 1c).

Dexamethasone treatment increased microsomal DMBA–protein adduct formation by 5.7-fold, indicating a role for CYP3A in this process. It is interesting to note that Shimada *et al.* [34] concluded that CYP3A was responsible for conversion of DMBA-3,4-diol to the ultimate carcinogen in human liver microsomes. CYP3A1 mRNA is undetectable in untreated rats [35] but is induced by glucorcorticoids [36], and Western blotting showed that CYP3A was induced in microsomes from dexamethasone-treated rats (Fig. 2). Troleandomycin, an inhibitor selective for this subfamily [24], forms a stable complex with these enzymes preventing further activity [37]. This inhibitor reduced adduct formation to 15% of uninhibited control levels in microsomes from dexamethasone-treated rats (see Fig. 1) and with a low EC_{50} (Table 2). Interestingly, adduct formation was also significantly reduced by troleandomycin in microsomes from phenobarbital-treated rats (to levels 40% of uninhibited control) and with an EC_{50} similar to that observed for membranes from dexamethasone-treated animals. Western blotting showed that CYP3A was also induced by phenobarbital, although to a lesser extent, as has been found previously [11, 22].

In summary, metabolism of DMBA is essential for DMBA–protein adduct formation in rat hepatic microsomes. Although the extent of protein adduct formation for a carcinogenic hydrocarbon may not correlate with carcinogenic potential as well as the extent of DNA adduct formation for that hydrocarbon, protein adduct formation may provide an important indicator *in vitro* for the formation of reactive metabolites, and *in vivo* for exposure assessment [38]. Also protein adduct formation *in vivo*, may be important indirectly in the process of chemical carcinogenesis by limiting any further metabolism of the activated procarcinogen. Adduct formation is increased in microsomes from rats treated with Sudan III, phenobarbital and dexamethasone, indicating an important role for several P450 families in this process. Investigation of individual P450s and subfamilies participating in adduct formation, using isoenzyme selective inhibitors, confirmed the involvement of the CYP2B subfamily in microsomes from phenobarbital-treated rats, the CYP3A subfamily in microsomes from dexamethasone-treated rats and the CYP1A subfamily in microsomes from Sudan-treated rats.

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