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INDUCTION OF RAT LIVER MICROSOMAL AND NUCLEAR CYTOCHROME P-450 BY DIETARY 2-ACETYLAMINOFLUORENE AND BUTYLATED HYDROXYTOLUENE

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Abstract—The influence of dietary 2-acetylaminofluorene (AAF) on the cytochrome P-450 content of rat liver microsomal and nuclear fractions was immunochemically probed with monoclonal and polyclonal antibodies to cytochromes P-450c and P-450d. Cytochrome P-450d but not P-450c was immunodetected in microsomes, nuclear envelopes, and nuclei from untreated rats. The levels of both cytochromes P-450c and P-450d were elevated after a diet of either 0.1% AAF for 1 week or 0.05% AAF for 3 weeks. However, the level of cytochrome P-450c relative to P-450d was lower after the more prolonged AAF feeding. Supplementation of AAF-containing diets with 0.3% butylated hydroxytoluene (BHT), which affords protection against AAF hepatocarcinogenesis in high-fat fed rats, protected and/or induced total (spectral) nuclear envelope cytochrome P-450 content. Immunochemical studies of liver fractions showed that BHT enhanced the AAF-dependent induction of cytochrome P-450c, but not of P-450d. This was a concerted effect of AAF + BHT since dietary BHT by itself did not affect the levels of cytochrome P-450c or P-450d as compared to control rats. Since 1- to 3-week dietary AAF had little effect on total (spectral analyses) microsomal cytochrome P-450 but markedly reduced total P-450 in nuclear envelopes, the coordinated induction of specific cytochrome P-450s in the different fractions suggests selective induction and depression of different forms of cytochrome P-450 and provides additional evidence for independent regulation of the drug-metabolizing system in nuclear envelope and microsomes. In addition, these results suggest that regulation of cytochrome P-450 may play a crucial role in the nutritional modulation of AAF hepatocarcinogenesis.

Previous enzymological and immunological studies have demonstrated the identity between some of the drug-metabolizing enzymes of the nuclear envelope and the endoplasmic reticulum [1]. On the other hand, the advent of reliable methods for the isolation of nuclei and of nuclear envelopes free of microsomal contamination has clearly established the presence of nuclear drug-metabolizing activity distinct from that of the endoplasmic reticulum [2]. Furthermore, several investigations have also shown that some of these enzymes exhibit patterns of induction in liver nuclear membranes different from those in microsomes, suggesting independent control of their response to xenobiotics [3–5].

We have shown recently that short-term (1–3 week) feeding of AAF-containing diets to male rats results in marked losses of the total cytochrome P-450 (P-450§) content (CO-reduced vs reduced difference spectrum) in the hepatic nuclear envelopes, whereas the microsomal P-450 remains virtually unchanged [5]. Dietary BHT, which provides protection against hepatocarcinogenesis in rats fed AAF in high fat diets [6], has also been shown to preserve and/or induce nuclear envelope cytochrome P-450 [7].

To investigate the effect of the dietary administration of AAF and BHT on some individual forms of cytochrome P-450 in liver nuclei and microsomes, immunochemical analyses were performed by an immunoblot technique using monoclonal and polyclonal antibodies.

MATERIALS AND METHODS

For short-term AAF feeding experiments, male Sprague–Dawley rats (90–100 g body weight) were fed the semipurified diet of Medes *et al.* [8] which contains 9% of partially hydrogenated vegetable fat. After a 48-hr equilibration on basal diet, the experimental animals were fed basal diet supplemented with 0.05% (w/w) AAF for 3 weeks, or 0.1% AAF for 1 week. For the study of the effect of dietary BHT on AAF-fed animals, weanling male Sprague–Dawley rats (50–60 g body weight) were fed a diet with a high content of saturated fat [25.6% (w/w), of a mixture of 9 parts of beef tallow and 1 part of corn oil). After a 2-week equilibration on basal diet with or without BHT supplementation (0.3%, w/w), the experimental animals were fed basal diet with AAF (0.05%) or AAF plus BHT, for 9 weeks. Under

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§ Abbreviations: AAF, 2-acetylaminofluorene; P-450, cytochrome P-450; MAb, monoclonal antibody; MC, 3-methylcholanthrene; NE, nuclear envelope; NUC, nuclei; and BHT, butylated hydroxytoluene.

these conditions, BHT preserves nuclear envelope cytochrome P-450 in the AAF-fed rats [9].

All rats were purchased from SASCO (Omaha, NE), and the diets were prepared by the Life Sciences Division of ICN Nutritional Biochemicals (Cleveland, OH). Additions of AAF and of BHT were carried out as previously described [5, 7]. All handlings of AAF and AAF-containing diets were carried out following strict institutional and federal safety regulations [10]. The rats were fed *ad lib.* with free access to drinking water, and killed by decapitation at 9:00 to 10:00 a.m. to minimize diurnal variation. Livers were quickly excised, chilled in ice-cold saline and, after removal of blood clots and extraneous tissue, blotted, weighed and stored at −80° until cell fractionation and enzyme assays were performed. Highly purified nuclei, nuclear envelopes and microsomes were isolated as previously described [5, 11, 12]. Electron microscopic examination of the isolated nuclei and nuclear membranes utilized for these studies showed clean preparations free of adhering cytoplasmic membranes. Total cytochrome P-450 content was determined by the CO-reduced versus reduced difference spectrum [13] as previously described [5, 7]. Protein was measured by the method of Lowry *et al.* [14] using crystalline bovine serum albumin as the standard.

Immunochemical analysis of cytochrome P-450 was performed by the immunoblot technique. Samples of nuclei, nuclear envelopes and microsomes were subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis [15], followed by electrophoretic transfer to nitrocellulose paper and immunoblot analysis [16]. Microsomes isolated from the livers of rats treated with i.p. injections of 3-methylcholanthrene (MC) in corn oil [17] were utilized as markers of cytochromes P-450c and P-450d.* For primary antibodies, mouse ascites fluids containing monoclonal antibodies (MAbs) 1-7-1 and 1-36-1 to P-450c [18] were diluted to 0.25 mg/ml, and rabbit antiserum to P-450c, purified according to Ryan *et al.* [19], was diluted 1:1000. The paper was incubated with MAbs or antiserum for 16 hr and 1 hr, respectively, followed by 0.5 μg/ml of either alkaline phosphatase-coupled rabbit anti-mouse or goat anti-rabbit antibody (KPL, Gaithersburg). The blot was then developed and the areas corresponding to immunostained protein were quantitated by densitometry with a Beckman DU-8 spectrophotometer with scanning accessory.

RESULTS

Two MAbs to P-450c, 1-36-1 and 1-7-1, were used to detect the P-450 by the immunoblot technique. MAb 1-36-1 recognizes only P-450c whereas MAb 1-7-1 recognizes P-450c as well as the structurally similar and cross-reactive P-450d form [18, 20]. This is shown in immunoblots of MC microsomes with MAb 1-36-1 (Fig. 1, lane 13) which revealed a single band corresponding to P-450c, whereas immunodetection with MAb 1-7-1 (Fig. 2, lane 13) revealed two bands corresponding to P-450c and P-450d as the higher and lower molecular weight bands respectively. These results with MAbs 1-36-1 and 1-7-1 demonstrate their utility for immunodetecting P-450c exclusively or in combination with P-450d.

The immunoblot with MAb 1-36-1 (Fig. 1) revealed immunospecific protein corresponding to P-450c in liver microsomes, nuclear envelope, and nuclear fractions from rats fed 0.1% AAF for 1 week or 0.05% AAF for 3 weeks. The fractions from control rats did not reveal immunodetectable material, up to the 0.5 mg practical limit of protein loading on the gel. The immunoblot with MAb 1-7-1 (Fig. 2) revealed immunospecific protein corresponding to P-450d in all fractions from AAF-fed rats, but these bands were barely visible in the control fractions. MAb 1-7-1 did not immunodetect the P-450c seen with MAb 1-36-1 (Fig. 1), presumably owing to the lower sensitivity of MAb 1-7-1 for this P-450.† In all fractions, administration of the diet containing 0.1% AAF for 1 week yielded higher levels of both immunospecific P-450c and P-450d than administration of the diet with 0.05% AAF for 3 weeks.

The combined results with MAbs 1-36-1 and 1-7-1 demonstrate that both P-450c and P-450d are present in the liver fractions of AAF-treated rats. However, owing to the limited sensititivity with these MAbs, maximal amounts of nuclear fractions (0.5 mg) were required for immunodetection. At this high protein load, numerous bands migrating faster than P-450 and appearing near the dye front were immunostained with both MAbs. A more sensitive polyclonal antiserum to P-450c that also recognizes the structurally similar P-450d form was thus subsequently used to better visualize and quantitate the P-450s.

Immunodetection with anti-P-450c antiserum revealed the P-450c and P-450d bands in nuclear fractions using much less (12.5-fold) protein than was required with the MAbs, and less nonspecific staining was observed (Fig. 3). The results with MC microsomes and the fractions from AAF-fed rats were consistent with those obtained with MAb 1-7-1 and 1-36-1. Furthermore, because of the higher sensitivity of the antiserum, P-450d was observed clearly in all fractions from control rats and, therefore, the degree of induction by AAF could be measured by densitometry. P-450c remained undetectable in all controls. An additional band of unknown identity that migrated faster than P-450d was also visible in fractions from control livers but not in those from AAF-fed rats. Its presence did not interfere with densitometric evaluation of immunostained P-450c and P-450d.

Figure 3 shows clear differences in the patterns of induction of liver microsomal P-450s observed following treatment with AAF and with MC. The relative staining intensities of the P-450c and P-450d bands are presented in Table 1. MC treatment resulted in a microsomal P-450c band that was twice as intense as the P-450d band (Fig. 3, lanes 1 and 14). On the other hand, the bands were of nearly equal intensity in microsomes from rats fed 0.1% AAF for 1 week (lane 3), whereas the 0.05% AAF feeding for 3 weeks yielded a P-450c band that was

* P-450c and P-450d correspond to P-450IA1 and P-450IA2 respectively.

† Friedman FK, unpublished observations.

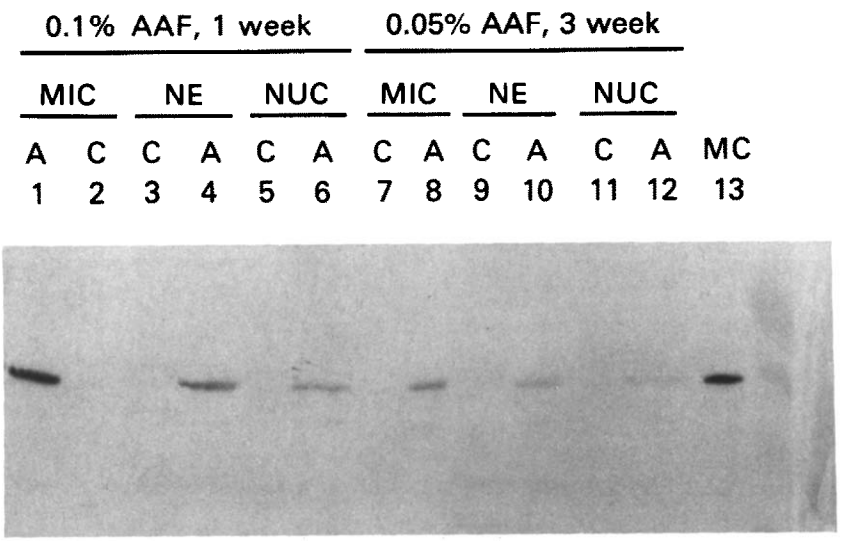

Fig. 1. Immunoblot of subcellular liver fractions from rats fed basal diet [8] with or without AAF supplementation. The bands were detected using MAb 1-36-1 to cytochrome P-450c. Lanes 1, 4, and 6 contained microsomes, nuclear envelopes and nuclei from rats fed 0.1% AAF for 1 week. Lanes 2, 3 and 5 contained microsomes, nuclear envelopes and nuclei from 1-week control rats. Lanes 8, 10, and 12 contained microsomes, nuclear envelopes and nuclei from rats fed 0.05% AAF for 3 weeks. Lanes 7, 9, and 11 contained microsomes, nuclear envelopes and nuclei from 3-week control rats. The microsomal samples contained 25μg protein and the nuclear samples 500 μg protein. A and C denote samples from AAF-treated and from control rats; MIC, NE and NUC denote microsomal, nuclear envelope, and nuclear fractions, respectively. Lane 13 contained 0.2 μg of rat MC microsomes.

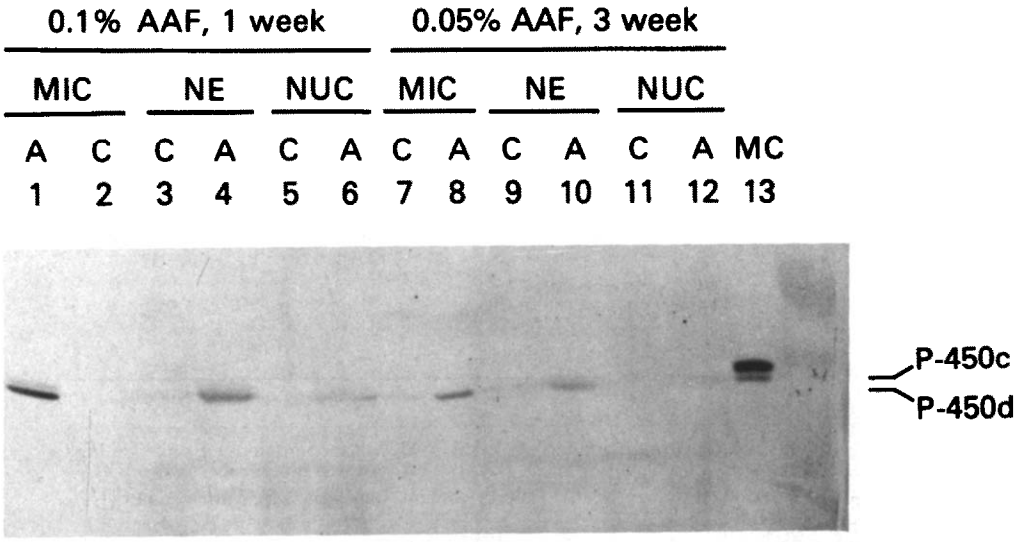

Fig. 2. Immunoblot of subcellular liver fractions from control and AAF-treated rats, using MAb 1-7-1 to cytochrome P-450c and P-450d. Samples, loading sequence, and protein loads were the same as in Fig. 1.

less intense than the P-450d band (lane 9). Measurement of the relative levels of microsomal P-450c and P-450d induced by these agents (Table 1) demonstrated that MC treatment yielded a greater ratio of P-450c to P-450d (ratio = 2.25) than AAF feeding (ratios = 0.89 and 0.45 for rats fed diets containing 0.1% and 0.05% AAF, respectively). In addition, the contents of cytochromes P-450c and P-450d in MC microsomes were 25- and 10-fold greater, respectively, than the levels in microsomes from rats fed 0.1% AAF. The corresponding differences between the microsomes of MC- and 0.05% AAF-treated rats were even larger.

AAF feeding also induced both P-450c and P-450d in the nuclear envelope and nuclear fractions (Fig. 3 and Table 1). Their specific contents of P-450c and

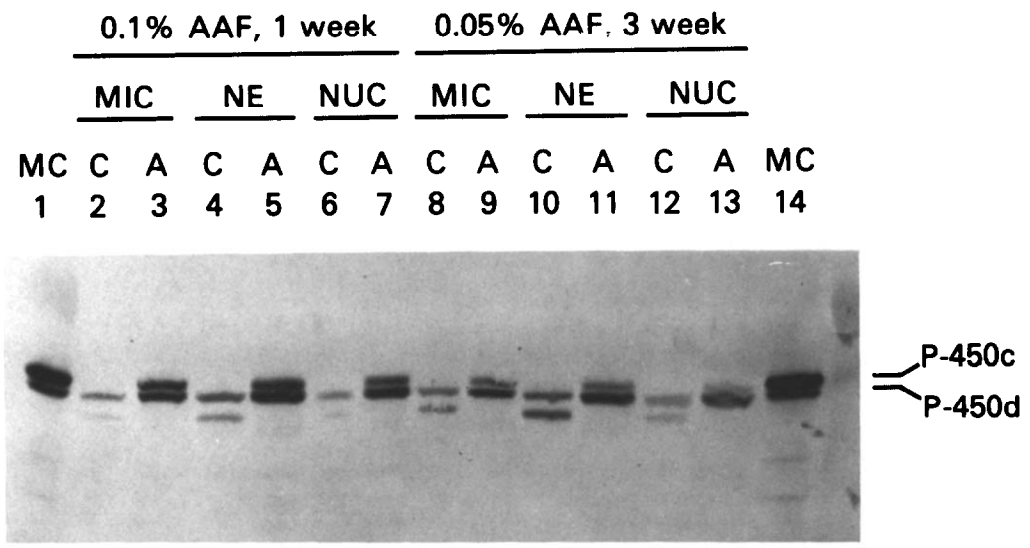

Fig. 3. Immunoblot of subcellular liver fractions from control and AAF-treated rats, using anti-P-450c antiserum. Lanes 1 and 14 contained 0.2 μg of rat MC microsomes. Lanes 2, 4, and 6 contained microsomes, nuclear envelopes and nuclei from control rats fed basal diet [8] for 1 week. Lanes 3, 5, and 7 contained microsomes, nuclear envelopes and nuclei from rats fed basal diet supplemented with 0.1% AAF for 1 week. Lanes 8, 10, and 12 contained microsomes, nuclear envelopes and nuclei from 3-week control rats. Lanes 9, 11, and 13 contained microsomes, nuclear envelopes and nuclei from rats fed 0.05% AAF for 3 weeks. Protein loads were 2 μg for AAF-microsomal samples and 40 μg for nuclear envelopes and nuclei samples.

Table 1. Effect of AAF feeding on relative cytochrome P-450c and P-450d content of rat hepatic microsomal and nuclear fractions

	Immunostain intensity per μg protein*					
	0.1% AAF, 1 week			0.05% AAF, 3 weeks		
Specimen†	P450c	P450d	c/d Ratio	P450c	P450d	c/d Ratio
C-MIC	ND‡	75	—	ND	53	—
A-MIC	357	401	0.89	88	195	0.45
C-NE	ND	10	—	ND	6	—
A-NE	19	32	0.61	7	23	0.30
C-NUC	ND	3	—	ND	2	—
A-NUC	12	26	0.44	3	11	0.28
MC-MIC	8995	4005	2.25			

* Immunostained areas corresponding to P-450c and P-450d in the immunoblot shown in Fig. 3 were determined densitometrically.

† C, control rat; A, AAF-fed rat; MC, 3-methylcholanthrene i.p.; MIC, microsomes; NE, nuclear envelopes; NUC, nuclei.

‡ Not detectable (<1).

P-450d ranged from 3 to 12% of the amounts immunodetected in microsomes and, in addition, these fractions had a lower P-450c to P-450d ratio than microsomes. The AAF content of the diet and the length of the dietary treatment also influenced the P-450 levels since the P-450c:P-450d ratios for the 3-week treatment with 0.05% AAF were lower than those for the 1-week treatment with the diet containing 0.1% AAF.

In separate experiments we studied the effect of the antioxidant BHT on AAF-induction of P-450. It has been shown that the anticarcinogenic effect of BHT in rats fed AAF in high fat diets [6] correlates with protection against AAF-induced loss of total nuclear envelope P-450 [7]. In recent studies with rats fed 0.05% AAF in a diet with a high content (25.6%, w/w) of saturated fat (beef tallow + corn oil, 9:1) the protective effect of dietary BHT (0.3%) on total nuclear envelope P-450 was observed up to week 9 of the feeding protocol [9]. Immunoblots of microsomes, nuclear envelopes and nuclear fractions from rats fed this diet with 0.05% AAF and/or

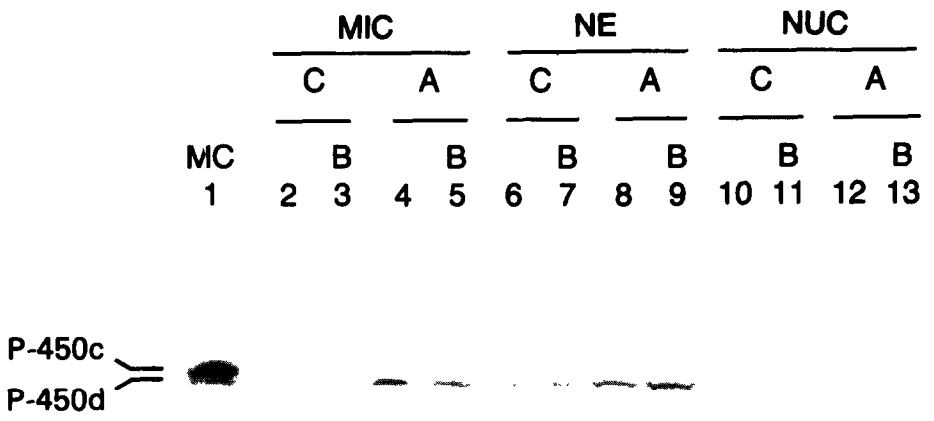

Fig. 4. Immunoblot of subcellular liver fractions from rats fed high saturated-fat diet [9] with or without AAF (0.05%) and/or BHT (0.3%) supplementation for 9 weeks. The bands were detected using anti-P-450c antiserum. Specimens from rats fed AAF-free diets appear under C; A denotes diet supplemented with AAF; B denotes BHT supplementation. Lane 1 contained 0.2 μg of rat MC microsomes. Lanes 2 and 3 contained microsomes from control rats and from rats fed BHT-supplemented diet. Lanes 4 and 5 contained microsomes from AAF-fed rats and from rats fed both AAF and BHT. The sequence in lanes 2–5 is repeated in lanes 6–9 for nuclear envelopes, and in lanes 10–13 for nuclei. Protein loads were 2 μg for microsomes and 40 μg for nuclear envelopes and nuclei.

0.3% BHT for 9 weeks were compared to fractions from control rats fed the unsupplemented high saturated fat diet. BHT did not affect the P-450d levels in any of the fractions from either control or AAF-fed rats (Fig. 4). However, BHT accentuated AAF induction of P-450c, as the staining intensities for nuclear envelope, nuclear, and microsomal fractions from rats fed both AAF and BHT were 2-, 4- and 7-fold higher, respectively, than those from rats fed AAF alone. Unlike AAF, BHT alone did not induce P-450c; in rats fed BHT in an AAF-free diet no P-450c staining was visible. Thus, the inductive effect of BHT on P-450c levels was only observed in concert with AAF feeding.

DISCUSSION

AAF is a hepatocarcinogen that has proven useful for studying the mechanism of chemical carcinogenesis. This arylamine has been shown to modulate cytochrome P-450 content as well as drug-metabolizing activities in rat liver microsomes [5, 7, 21–25].

In previous studies conducted in our laboratory, we showed that a relatively short-term dietary administration of AAF (1–3 weeks) to male rats results in a marked decrease of the total cytochrome P-450 in the hepatic nuclear envelopes whereas the microsomal P-450 remains virtually undiminished [5]. BHT, which is known to protect against AAF hepatocarcinogenesis, protects against the AAF-induced loss of nuclear envelope cytochrome P-450 [7]. Therefore, we decided to examine the effects of AAF and BHT treatment both separately and concurrently on the expression of nuclear and microsomal P-450s.

In this study we conducted immunochemical analyses using monoclonal and polyclonal antibodies to P-450c, the major P-450 induced by the carcinogenic polycyclic aromatic hydrocarbon 3-methylcholanthrene, in order to evaluate the effect of AAF and/or BHT feeding on rat liver microsomal, nuclear, and nuclear envelope P-450.

Immunoblot analysis following SDS-gel electrophoresis revealed that AAF induced P-450c and the related P-450d form in all these subcellular fractions. However, some differences were observed in the induction of these two forms of P-450. Cytochrome P-450c was undetectable in any of the control fractions but appeared after AAF feeding, while P-450d, which was present in all control fractions, increased 3- to 9-fold following AAF feeding.

Another interesting observation is that the levels of both P-450c and P-450d after 1 week on a diet containing 0.1% AAF were higher than after a 3-week treatment with a diet containing 0.05% AAF. The lower P-450 levels after the prolonged treatment may result from the lower levels of AAF used (0.05% vs 0.1% used in the 1-week protocol), or from destruction of P-450 upon extensive exposure to AAF [5].

The presence of MC-inducible cytochrome P-450 in rat liver nucleus, both as a fibrillar network and in nucleoli, was established by immunohistochemical and immunodiffusion studies [26, 27], using monospecific rabbit antibody to rat microsomal cytochrome P-450c. More recent immunochemical studies using MAbs to MC-induced microsomal cytochrome P-450 demonstrated that MAb-specific P-450 was present in both endoplasmic reticulum and nuclear envelope [28, 29]. The identity of the MAb-specific P-450 could not be determined, however,

because the MAbs did not distinguish between the P-450c and the P-450d forms. The immunoblot procedure used in the current report satisfactorily resolved these P-450s electrophoretically.

The coordinated induction of P-450c and P-450d by AAF in nuclei and microsomes contrasts with the behaviour of the total P-450 content which decreases markedly in the nuclear envelope but remains virtually unchanged in microsomes following 1–3 weeks of dietary AAF administration [5]. In comparing measurements of total spectral P-450, one must be aware that only spectrally active P-450 is determined, and these data yield no information on P-450 protein, in which the heme prosthetic group may have been destroyed or removed. The immunoblot data specifically detect protein irrespective of the state of heme and catalytic efficiency. These measurements thus provide insight into regulation of P-450 apoprotein levels but are less useful for drawing conclusions regarding catalytic activity levels.

The phenolic antioxidant BHT is a common food additive which has been reported to protect against AAF-induced liver carcinogenesis [6, 30]. *In vitro* studies have shown that BHT markedly inhibits AAF mutagenesis [31], and *in vivo* studies suggest that the BHT stimulates the pathways responsible for the hepatic detoxification and urinary excretion of AAF [30, 32]. This increased excretion of AAF causes a decrease in the amounts of AAF available for metabolic activation and, therefore, a decrease in the amounts of AAF covalently bound to hepatic nuclear DNA [32]. Dietary BHT administration is known to cause an increase in the cytochrome P-450 content as well as an induction of mixed-function oxidase activities in rat liver microsomes [33, 34].

Our immunoblot analyses of microsomes, nuclei and nuclear envelopes showed that, although BHT alone had no effect on the levels of P-450c or P-450d, it enhanced the AAF-induced expression of P-450c in all fractions. This effect is specific since the level of P-450d, which is also induced by AAF, was not affected by BHT.

In summary, our data demonstrate that AAF coordinatively induced both P-450c and P-450d in rat liver microsomal and nuclear envelope membranes. The AAF-dependent induction of these P-450s in nuclear envelope contrasts with the general loss of total P-450. Dietary BHT, which protects against the AAF-dependent loss of total P-450, had no effect on P-450d and had an inductive effect on P-450c but only when administered concurrently with AAF. The selective induction and loss of different forms of P-450 strengthen the concept of independent regulation of the P-450 system in nuclei and microsomes and provide new insights into the role of cytochrome P-450s in the nutritional modulation of AAF hepatocarcinogenesis.

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