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# Decrease in the Metabolic Activating Capacities of Arylamines in Livers Bearing Hyperplastic Nodules: Association with the Selective Changes in Hepatic P-450 Isozymes

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**The mechanism of the alteration in carcinogenic arylamine-activating capacities in livers bearing pre-neoplastic (or hyperplastic) nodules induced by the Solt-Farber protocol was investigated in relation to the changes in hepatic cytochrome P-450 isozymes. In the *Salmonella* mutagenesis test, the numbers of revertants induced with 2-amino-3-methylimidazo[4,5-*f*]quinoline and 2-aminofluorene were significantly lower in the presence of microsomes of nodule-bearing livers than of control livers. A similar tendency was also observed with another heterocyclic arylamine, 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole. In Western blots using specific antibodies against 5 different forms of cytochrome P-450, hepatic contents of P-450-male (a main constitutive form) and P-450b (a main phenobarbital-inducible form) were decreased in the livers with hyperplastic nodules to 63% and 35% of the corresponding controls, while no significant decrease was observed in the contents of P-448-H (a main 3-methylcholanthrene-inducible form), P-450$_{6\beta\text{-}1}$ (testosterone 6$\beta$-hydroxylase) and P-450e (a phenobarbital-inducible form). In accordance with the reduction in P-450-male, capacities for microsomal 16$\alpha$- and 2$\alpha$-hydroxylations, but not 6$\beta$-hydroxylation, of testosterone were decreased in the livers with hyperplastic nodules. Although P-448-H has higher capacities for the activation of arylamines than does P-450-male, the hepatic content of P-450-male is more than ten-fold higher than that of P-448-H in both normal and nodule-bearing livers. These results indicate that the selective decrease in hepatic content of P-450-male is likely to be a main cause of the decrease in arylamine metabolic activating capacities in livers with hyperplastic nodules.**

Key words: Arylamine — Metabolic activation — Preneoplastic liver lesion — Cytochrome P-450

Preneoplastic hepatic nodules or hyperplastic nodules (HNs[2]) are considered to be an established site of origin for liver cancer in rats.[6] These preneoplastic lesions show a characteristic phenotype, in which phase I drug-metabolizing enzymes decrease and phase II conjugating enzymes increase.[7–16] Among phase I drug-metabolizing enzymes, phenobarbital (PB)- and 3-methylcholanthrene (3-MC)-inducible cytochrome P-450 (P-450) isozymes were found to decrease in preneoplastic liver nodules by using an immunohistochemical method.[15, 16] Several investigators have also reported that the binding of a carcinogenic arylamine, 2-acetylaminofluorene (2-AAF), to DNA, RNA and proteins decreased in the hepatic nodules.[17] Lack of acetylaminofluorene-DNA adduct formation was also observed in the enzyme-altered foci of rat liver.[18] These results are in agreement with the characteristic alterations of microsomal drug-metabolizing enzymes and suggest that changes occur in the population of hepatic cytochromes P-450 in neoplastic tissues. Nevertheless, decreases in the contents of individual P-450 forms in livers with HNs have not been assessed in detail.

Carcinogenic arylamines including 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-aminofluorene (2-AF) undergo metabolic activation before exerting their carcinogenic effects. Glu-P-1 is activated through N-hydroxylation by a 3-MC-inducible form, P-448-H.[19–21] In addition, P-450-male, a constitutive male-specific form of cytochrome P-450, was also shown to have appreciable activity in the mutagenic activation of IQ.[22] In carcinogenicity experiments, arylamines are fed for considerable periods. Although continual activation by hepatic enzymes might be necessary for arylamine carcinogenesis, the level of the activating enzyme during the course of the hepatocarcinogenesis is still not clearly understood at the isozyme level. In the present report, we have investigated the relationship between changes in the activating capacities for these carcino-

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[2] Abbreviations used: HNs, hyperplastic nodules; PB, phenobarbital; 3-MC, 3-methylcholanthrene; P-450, microsomal cytochrome P-450; 2-AAF, 2-acetylaminofluorene; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; 2-AF, 2-aminofluorene; P-450-male, a constitutive male-specific form of cytochrome P-450[1]; P-450b and P-450e, phenobarbital-inducible forms of cytochrome P-450s[2]; P-450$_{6\beta\text{-}1}$, a major microsomal testosterone 6$\beta$-hydroxylase[3,4]; P-448-H, a high-spin form of cytochrome P-450 which probably corresponds to P-450d[5]; IgG, immunoglobulin G; DEN, diethylnitrosamine.

genic arylamines and in the contents of 5 different P-450 isozymes in livers bearing HNs. The results obtained suggest that in HN-bearing liver, metabolic activation of 2-AF and IQ significantly decreases due mainly to the reduced content of P-450-male in the liver.

## MATERIALS AND METHODS

**Chemicals** Goat anti-rabbit and rabbit anti-goat immunoglobulin G (IgG) were purchased from Cappel Lab. (Cochranville, PA, USA) and horseradish peroxidase-rabbit (or goat) anti-peroxidase complex from Miles Yeda (Rohovot, Israel). Alkaline phosphatase-rabbit IgG was purchased from Binding Site Ltd. (Birmingham, UK). All other chemicals used were of the highest grade available.

**Induction of preneoplastic liver nodules** Male F344 rats were purchased from Charles River Japan, Tokyo. Preneoplastic liver lesions were induced according to the resistant phenotype model presented by Solt and Farber.[23,24] Male Fischer 344 rats, weighing 200 to 220 g, were given diethylnitrosamine (DEN) dissolved in saline, 200 mg/kg intraperitoneally. Two weeks later, 2-acetylaminofluorene was administered, 20 mg/kg intragastrically[25] for 3 consecutive days. On the following day, the rats were subjected to standard two-thirds partial hepatectomy. Control rats were given vehicle only instead of DEN and 2-AAF and they were not subjected to partial hepatectomy. All the animals were killed at 8 weeks after partial hepatectomy. Grayish white nodules appeared on the livers and the livers were homogenized with 1.15% potassium chloride. Then, hepatic microsomes were prepared by sequential centrifugations at 9000*g* and 105,000*g*.

**The activation of carcinogenic arylamines** The incubation mixture (200 $\mu$l) for the activation of the carcinogenic amines consisted of 100 $\mu$g of microsomal protein, an NADPH generating system (1.6 m*M* NADP, 16 m*M* glucose-6-phosphate, 6 m*M* $MgCl_2$, and 0.2 IU of glucose-6-phosphate dehydrogenase), 50 m*M* KCl buffer (pH 7.4) and 0.2 m*M* Glu-P-1 or IQ in a total volume of 200 $\mu$l. After preincubation at 37°C for 10 min, the mixture was filtered and the mutagenicity was assessed as previously described.[26] In the case of 2-AF, a 2 $\mu M$ concentration of the chemical was incubated for 10 min at 37°C and the resulting mixture was added without filtration to *Salmonella typhimurium* TA98 culture.

**Immunochemical quantification of P-450 isozymes** P-450 isozymes were quantified immunochemically by methods using horseradish peroxidase[27] and alkaline phosphatase.[28] Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli[29] using 7.5% gels for analysis of P-450s. Microsomal proteins were electrophoretically transferred to nitrocellulose paper.[30] For the measurement of P-450 isozymes except P-448-H, specific rabbit antibodies were applied. Goat anti-P-448-H antibody was used for the measurement of P-448-H. After addition of goat anti-rabbit IgG or rabbit anti-goat IgG (in the case of P-448-H), the nitrocellulose paper was subsequently treated with peroxidase-antiperoxidase complex for the estimation of P-450s other than P-450-male. Alkaline phosphatase-rabbit IgG complex was used for the measurement of P-450-male. P-450s except P-450-male were visualized with 3,3′-diaminobenzidine and hydrogen peroxide. P-450-male was visualized with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium. Densitometry was carried out with the nitrocellulose sheets by using a Helena Quick Scan, model R&D, in the transmission mode.

**Assay of the microsomal testosterone hydroxylation and epoxide hydrolase activities** The activity of testosterone hydroxylases was quantified according to the method described previously.[4] Microsomal epoxide hydrolase activity was determined using 3-(*p*-nitrophenoxy)-1,2-propene oxide as the substrate according to the method described by Giuliano *et al.*[31]

## RESULTS

**Activation of three carcinogenic arylamines by microsomes of F344 rat liver with preneoplastic nodules** The results on mutagenic activation of three carcinogenic arylamines, IQ, 2-AF and Glu-P-1, by microsomes from control of HN-bearing livers are summarized in Table I. These three arylamines were activated to their mutagenic metabolites in the presence of microsomes of control or HN-bearing livers. Numbers of revertants induced by IQ

Table I. Mutagenic Activation of Three Carcinogenic Arylamines by Microsomes of F344 Male Rats with Preneoplastic Liver Nodules

| Arylamines | Mutagenicity ($10^{-2}\times$Rev/mg protein) | |
|---|---|---|
| | Control | Preneoplastic |
| IQ | 90±17 (100) | 40±15 (44)[a] |
| 2-AF | 0.59±0.07(100) | 0.39±0.10(66)[a] |
| Glu-P-1 | 61±17 (100) | 38±5 (62) |

The data presented are the number of $His^+$ revertants of *Salmonella typhimurium* TA98 (mean±SD of five different animals for control and three different animals for preneoplastic group). Numbers in parentheses indicate the relative percentage with respect to their controls. Rev, revertants. Other experimental details are described in "Materials and Methods."
*a*) Significantly different from the respective control ($P<0.05$).

Table II. Microsomal Contents of Total Cytochrome P-450, P-450 Isozymes and Epoxide Hydrolase Activity in Control and HN-bearing Liver of F344 Rats

| Biochemical parameters | Control | Preneoplastic |
|---|---|---|
| Epoxide hydrolase[a] | 3.3±0.6 (100) | 12.0±0.3(360)[c] |
| Total cytochrome | | |
| P-450[b,e] | 870±140(100) | 530±60 (61)[c] |
| P-450-male[b] | 300±50 (100) | 190±20 (63)[c] |
| P-450b[b] | 5.0±1.4 (100) | 1.8±0.8 (35)[d] |
| P-450e[b] | 8.5±1.2 (100) | 6.5±1.0 (76) |
| $P\text{-}450_{6\beta\text{-}1}$[b] | 49±8 (100) | 43±8 (88) |
| P-448-H[b] | 18±3 (100) | 14±1 (78) |
| Cytochrome $b_5$[b] | 500±60 (100) | 430±10 (86) |

Experimental details are described in "Materials and Methods." See Table I for other details.
*a*) nmol/mg protein/min.
*b*) pmol/mg protein
*c*) Significantly different from the respective control ($P<0.01$).
*d*) Significantly different from the respective control ($P<0.05$).
*e*) As determined from the CO-difference spectra by the method of Omura and Sato.[42]

Table III. Microsomal Testosterone Hydroxylation in F344 Male Rat Livers with Preneoplastic Nodules

| Metabolic activities | Control (nmol/mg protein/min) | Preneoplastic |
|---|---|---|
| 16α-Hydroxylation | 1.98±0.12(100)[a] | 1.33±0.27 (67)[b] |
| 2α-Hydroxylation | 1.15±0.04(100) | 0.69±0.15 (60)[c] |
| 6β-Hydroxylation | 1.57±0.15(100) | 1.68±0.19(107) |

Experimental details are described in "Materials and Methods." See Table I for other details.
*a*) Numbers in parentheses indicate the relative percentage with respect to the control.
*b*) Significantly different from the respective control ($P<0.01$).
*c*) Significantly different from the respective control ($P<0.05$).

and 2-AF were, however, lower in the presence of microsomes from HN-bearing livers, amounting to 44% and 66% of the controls, respectively. Although the number of revertants induced by Glu-P-1 also tended to decrease in livers with HNs, the decrease was not significant due to the large individual differences in control rats.

**Microsomal contents of P-450 isozymes and epoxide hydrolase activity in HN-bearing liver** Microsomal epoxide hydrolase (epoxide hydrase) is considered to be a reliable marker for preneoplastic lesions.[13, 32-34] As can be seen in Table II, the activity of epoxide hydrolase increased to 360% of the control in the livers with preneoplastic lesions. In contrast, hepatic content of total P-450 in HN-bearing livers was decreased to 61% of the control, in accordance with the results reported previously.[7-16] In Western blots, the mean content of a major constitutive form, P-450-male, was reduced from 300 pmol/mg protein to 190 pmol/mg protein in the HN-bearing livers. A major phenobarbital-inducible P-450b is expressed at low levels in untreated rats.[2] The constitutive level of P-450b was also significantly decreased to 35%, whereas another phenobarbital-inducible form, P-450e, and a testosterone 6β-hydroxylase, $P\text{-}450_{6\beta\text{-}1}$, did not significantly decrease in the HN-bearing livers. P-448-H was somewhat decreased from the control, but the difference was not significant. These results suggest that the change in the levels of P-450 isozymes during the process of hepatocarcinogenesis is not uniform, but differs depending on the isozymes measured.

**Testosterone hydroxylation by microsomes in F344 rat liver with preneoplastic lesions** Although P-450 has a broad substrate specificity for the metabolism of drugs and other chemicals, P-450 isozymes show fairly rigid substrate specificity in the metabolism of testosterone. $P\text{-}450_{6\beta\text{-}1}$[4] and P-450-male[35] were reported to catalyze testosterone 6β- and 2α(16α)-hydroxylation, respectively. As described in Table III, testosterone 2α- and 16α-hydroxylations were decreased in the microsomes of HN-bearing livers to 60% and 67% of their respective controls, which are consistent with the reduced level of P-450-male in the microsomes of livers with preneoplastic nodules. On the other hand, no significant change in 6β-hydroxylation, which is mainly catalyzed by $P\text{-}450_{6\beta\text{-}1}$, was observed. These results further support the changes in hepatic content of P-450-male in HN-bearing livers.

## DISCUSSION

Feo and his colleagues reported that liver preneoplastic nodules (2–5 mm in diameter) developed at ten weeks after initiation[36] and the percentage of liver parenchyma occupied by γ-glutamyltranspeptidase-positive foci reached its maximum (22%) at 9 weeks after initiation[37] in the Solt-Farber protocol. In our experimental system too, about 25% of the liver surface was occupied by grayish white nodules (ca. 5 mm in diameter) at 10 weeks after initiation. The putative preneoplastic lesions are considered to represent an adaptive response to xenobiotics, with an increased detoxification metabolism, and the preneoplastic cells may have a proliferative advantage over normal ones.[18] Loss of the ability to form 2-AF-DNA adducts in hepatic enzyme-altered foci is a

common event in different hepatocarcinogenesis models.[18] These results suggest the alteration of metabolic activating capacity in liver during hepatocarcinogenesis. In fact, we have demonstrated a significant decrease in the capacities for metabolic activation of IQ and 2-AF. Mutagenic activation of Glu-P-1 also showed a tendency to decrease, although the value was not significantly different from the control (Table I). IQ is known to be activated by both P-448-H and P-450-male.[22] The specific activity of IQ activation by purified P-448-H is 7.4 times that by P-450-male.[22] On the contrary, the content of P-448-H is one-seventeenth of P-450-male in the liver of normal rats. In HN-bearing liver, the mean level of P-448-H was decreased to 78%, but was not significantly different from the control. In contrast, P-450-male showed a significant decrease on Western blots. Therefore, these results indicate that decreased metabolic activation of IQ by HN-bearing liver is mainly attributable to the reduction of P-450-male level.

Mutagenic activation of 2-AF by untreated rat liver microsomes is partly inhibited by the addition of anti-P-450-male IgG, but not of anti-P-448-H IgG (data not shown). These results suggest that the decrease in the activation of 2-AF in HN-bearing liver is at least in part due to the reduced content of P-450-male. On the other hand, the extent of mutagenic activation of Glu-P-1 in HN-bearing liver was 62% of the control, but it was not significantly different from the control because of the large individual differences in the control group. In the present study, the level of P-448-H was not significantly altered in liver with HNs. In our previous papers, Glu-P-1 is shown to be activated effectively by P-448-H and the addition of anti-P-448-H IgG completely inhibits the mutagenic activation of Glu-P-1 by untreated rat liver microsomes.[19, 20, 21, 38] These results accord with the idea[19, 20] that activation of Glu-P-1 is more selectively mediated by P-448-H than the activation of the other two arylamines.

Hepatocarcinogenic effects of arylamines including 2-AAF[39] and azo dyes[40] are higher in male than female rats. The observed sex difference in liver carcinogenesis has been considered to be due mainly to the difference in hepatic sulfation of N-hydroxyarylamines. However, the sex difference may also possibly arise from the difference in cytochrome P-450-mediated initial N-hydroxylation. Although P-448-H content is higher in female than male rat liver,[41] another activating enzyme, P-450-male, exists sex-specifically in male rats.[1] These results might be indicative of a role of P-450-male in the arylamine activations *in vivo*.

In conclusion, our present study indicates that microsomal activating capacities for carcinogenic arylamines are decreased in HN-bearing liver of F344 rats. The decreases were mainly accompanied by the selective reduction of P-450-male in the HN-bearing liver. These results indicate that male specific P-450-male plays an important role in the metabolic activation of arylamines.

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