Microsomal Mixed-Function Oxidase and Activities of Some Related Enzymes in Hyperplastic Nodules Induced by Long-Term Griseofulvin Administration in Mouse Liver

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ABSTRACT

Hepatic hyperplastic nodules induced in mice by long-term griseofulvin administration were examined for selected microsomal activities and responses to enzyme inducers. Despite a decrease in microsomal cytochrome P-450 in hyperplastic nodules, aminopyrine N-demethylase was at control levels. Benzopyrene hydroxylase activity was slightly lower in microsomes derived from hyperplastic nodules than in those of control liver. Reduced nicotinamide adenine dinucleotide-cytochrome b5 reductase was at control level, but reduced nicotinamide adenine dinucleotide- and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductases and the NADPH-ferricyanide reductase were increased. NADPH-supported lipid peroxidation was lower in microsomes from hyperplastic nodules than in those from control liver, whereas microsomal stearoyl coenzyme A desaturase activity was almost doubled in the nodules. NADPH-cytochrome c reductases isolated and semipurified from hyperplastic nodules and from control liver microsomes showed almost identical affinity for NADPH. Microsomal enzymes of hyperplastic nodules responded readily to phenobarbital induction, but sodium dodecyl sulfate-polyacrylamide gel electrophoresis disclosed differences in the polypeptide patterns in the molecular weight range from 47,000 to 54,000 between microsomes derived from hyperplastic nodules and control livers.

INTRODUCTION

HN induced in the liver by a variety of carcinogens are considered to represent premalignant lesions (Refs. 12 to 14 and 29; see also Ref. 2 for discussion of the nomenclature). The cells of these lesions are relatively resistant in vivo as well as in vitro to the cytotoxic action of carcinogens and other hepatotoxins (13, 21). This resistance may be caused by one or a combination of the following factors: (a) reduced levels of components involved in the activation of chemical carcinogens or hepatotoxins, e.g., the cytochrome P-450-dependent mixed-function oxidase system; (b) reduced uptake of the toxic molecules or their precursors; and (c) increased amounts of intracellular molecules that detoxify activated hepatotoxins (21).

In order to further explore the distinguishing features of hepatic HN, we have selected, as an experimental model, griseofulvin-fed mice. The mouse hepatic HN induced by long-term griseofulvin administration were examined for selected microsomal enzyme activities and for responses to enzyme inducers. Liver surrounding HN (PT) was tested in parallel studies to provide a basis for comparison.

MATERIALS AND METHODS

Induction of HN in Mouse Liver by Long-Term Griseofulvin Treatment. Male Swiss albino mice (strain Him:OF 1, specific-pathogen-free; Institute of Laboratory Animal Research, University of Vienna School of Medicine, Himberg, Austria; 30 g body weight) were used. The animals were assigned to 2 groups: one receiving a powdered standard diet (Altromin, Lippe, Germany) containing 2.5% griseofulvin (Glaxo Laboratories, Ltd., Greenford, United Kingdom) for at least 6 months and the other receiving griseofulvin-free standard diet for the same time period, serving as control. After this feeding period, grayish white HN had appeared in the liver of almost every mouse treated with griseofulvin; the control group was consistently tumor free. After tumor development, griseofulvin feeding was substituted by standard diet for a 2-month period in order to eliminate the direct drug effect.

Preparation of Microsomes. HN were removed from the liver, taking care not to mix them with nonneoplastic liver tissue. The tissue was chilled on ice and homogenized in 0.15 m KCl at 0°C, and microsomes were prepared by conventional differential centrifugation techniques, as described previously (10). PT and livers from control mice were treated identically. It should be stated, however, in this context that PT still contained small hyperplastic areas and HN, as revealed by gross inspection and light microscopy, which were too small to be removed. PT, therefore, must be regarded as a mixture of neoplastic and nonneoplastic tissue. Microsomal protein was determined by the method of Lowry et al. (25).

Enzyme Determinations. Cytochromes P-450 and b5 were measured as described previously (10). Spectra were recorded with a Pye-Unicam SP 1800 double-beam spectrophotometer. Microsomal aminopyrine N-demethylation in vitro was determined according to the method of Schenkman et al. (38), and benzopyrene hydroxylation was determined according to the method of Dehnen et al. (8), as modified by Robie et al. (34). Lipid peroxidation was measured by determination of malondialdehyde according to the methods of Ottolenghi (32) and Jansson and Schenkman (19). Stearoyl-CoA desaturase was determined by the method of Oshino et al. (31) by measuring oleyl-CoA formation from stearoyl-CoA. Cytochrome c reductase...
and ferricyanide reductase assays were performed and evaluated as described by Jansson and Schenkman (19). NADPH-cytochrome c reductase was isolated from HN and control liver microsomes and semipurified according to the method of Omura et al. (30). Cytochrome c reductase-containing fractions from 3 preparations were pooled, and the apparent Km for various amounts of NADPH (final concentration, 0.5 to 20 μM), potassium phosphate buffer (0.05 M, pH 7.7), and EDTA (0.1 mM). The reaction was started at 25°C with various amounts of NADPH (final concentration, 0.5 to 20 μM).

Induction of Microsomal Enzymes. In order to test the inducibility of the mixed-function oxidase system in HN by different inducers, phenobarbital (sodium salt; Merck, Darmstadt, Germany; 1% in drinking water), 3-methylcholanthrene (Schwarz/Mann, New York, N. Y.; 20 mg/kg body weight in olive oil, i.p.), and griseofulvin (2.5%, incorporated into the diet) were administered for 4 days.

Separation of Microsomal Proteins by SDS-Polyacrylamide Gel Electrophoresis. Microsomal samples in Tris (50 mM)-KCl (0.15 M) buffer, pH 7.5, were adjusted to 2.5 nmol P-450 per ml (for isochromic application) with sodium phosphate buffer (10 mM), pH 7.2, containing 10% β-mercaptoethanol, 5% SDS, and 10% glycerol, and were heated in a boiling water bath for 2 min. Five μl were applied to a 10% polyacrylamide gel (1.5 mm thick), containing 0.1% SDS, and electrophoresis was carried out essentially according to the method of Laemmli (20), using the Bio-Rad (Bio-Rad, Richmond, Calif.) electrophoresis chamber and a Desaga (Desaga, Heidelberg, Germany) power supply. Electrophoresis buffer was Tris (0.02 M)-glycine (0.19 M)-0.1% SDS, pH 8.6. After electrophoresis, gels were stained with Coomassie blue (0.25% in 45% methanol-0.2% acetic acid) for 15 min and then destained in 5% methanol-7.5% acetic acid. Molecular weights were determined by coelectrophoresis of standard proteins (M.W.: phosphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000).

RESULTS

Microsomal Mixed-Function Oxidase and Related Enzymes in HN, PT, and Normal Control Liver. Cytochrome P-450 content of HN microsomes was depressed to approximately 58 to 72% of control microsomes (see Tables 1, 4, and 5) and to about 80% of PT microsomes (Tables 1 and 4). Cytochrome b₅ was not significantly different in HN and control microsomes and was slightly but significantly elevated in microsomes prepared from PT (Tables 1 and 4). Measurement of microsomal NADH-cytochrome b₅ reductase by its ability to reduce ferricyanide revealed no significant difference between control and HN microsomes, but its level in PT microsomes was only 60% of that in HN. NADH-cytochrome c reductase was considerably increased (about 400% of control liver) in HN microsomes despite an unchanged b₅ level. The activity of NADPH-cytochrome P-450 reductase was elevated in HN microsomes by about 50% irrespective of whether it was determined by its ability to reduce cytochrome c or ferricyanide (Table 1). It is interesting to note in this context that the level of this enzyme in PT was somewhat (but not significantly) lower than that in control liver microsomes, despite the presence of small HN and hyperplastic foci, which should actually have elevated the enzyme level above control due to their higher enzyme activities. This may indicate that long-term griseofulvin treatment induced profound (and probably irreversible) microsomal alterations in nonneoplastic liver, which is also suggested by the elevated specific microsomal cytochrome b₅ content in PT microsomes as compared to HN and control (Table 1). Despite decreased microsomal cytochrome P-450 content, the capability of HN microsomes to demethylate aminopyrine remained unaltered (see values of apparent Vₘₜₐₜ in Table 2). The apparent Km value of aminopyrine N-demethylation was significantly lower in HN microsomes than in PT and control microsomes (Table 2). Benzopyrene hydroxylation by HN and PT microsomes was almost identical by slightly (yet significantly, p < 0.05) lower than in control microsomes (Chart 1). NADPH-supported lipid peroxidation by HN and PT microsomes was lower than that catalyzed by control microsomes (Chart 2). Microsomal fatty acid desaturase activity was almost doubled in HN and PT microsomes (Table 3). In order to test the affinity of HN microsomal NADPH-cytochrome c reductase for its electron donor NADPH, the Km for NADPH was determined using cytochrome c as electron acceptor and was found to be 2.25 μM, which is in the same order of magnitude as the NADPH Km (2.86 μM) obtained with control microsomes.

Effects of Inducers on Cytochrome P-450, Cytochrome b₅, and Mixed-Function Oxidase Activities in HN Microsomes as Compared to PT and Control Liver. Specific microsomal cytochrome P-450 content is a reliable indicator of microsomal enzyme induction exerted by phenobarbital administration. Treatment of mice with phenobarbital caused elevation of the specific microsomal P-450 content of HN microsomes to about the same relative extent as that of PT and control microsomes (Table 4). Aminopyrine N-demethylation activities were also significantly increased in HN and PT derived from phenobarbital-treated mice (Table 2); the apparent Km of aminopyrine N-demethylation remained unaltered in HN, whereas it decreased.

Table 1

Microsomal cytochromes P-450 and b₅ and flavine enzymes in HN, PT, and control liver

Values for treated mice are from 4 to 7 experiments, each done with the microsomes of HN and PT tissue derived from 3 to 5 mice. Four experiments were performed with control mice, each with the microsomes from 9 pooled mouse livers.

<table>
<thead>
<tr>
<th>Cytochrome P-450 (nmol/mg microsomal protein)</th>
<th>Cytochrome b₅ (nmol/mg microsomal protein)</th>
<th>Cytochrome c reductase (nmol/sec/mg microsomal protein)</th>
<th>Ferricyanide reductase (nmol/sec/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>NADPH</td>
<td>NADH</td>
<td>NADPH</td>
</tr>
<tr>
<td>HN  0.54 ± 0.06¹,b</td>
<td>0.43 ± 0.04</td>
<td>33.90 ± 2.46¹,b</td>
<td>2.18 ± 0.14¹,b</td>
</tr>
<tr>
<td>PT  0.68 ± 0.06¹,b</td>
<td>0.51 ± 0.01</td>
<td>18.50 ± 2.60¹,b</td>
<td>1.47 ± 0.17¹,b</td>
</tr>
<tr>
<td>Control</td>
<td>0.92 ± 0.05</td>
<td>8.87 ± 1.87</td>
<td>1.83 ± 0.24</td>
</tr>
</tbody>
</table>

¹ Mean ± S.D.
² Statistically significant difference (p < 0.01) from control.

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in PT microsomes; apparent $K_m$ values were not determined with liver microsomes derived from phenobarbital-treated control mice since it is known that phenobarbital treatment does not influence $K_m$ values of aminopyrine $N$-demethylation in control livers (Ref. 6; Table 2). The effect of phenobarbital on cytochrome $b_5$ was less pronounced but equal in HN and PT microsomes (Table 4). The mouse strain used in these studies did not respond to induction by 3-methylcholanthrene, and neither a change in cytochrome P-450 content nor a blue shift of the Soret maximum of the reduced hemoprotein-CO complex occurred in HN, PT, and control liver microsomes (Table 4). Benzopyrene hydroxylation was not induced by 3-methylcholanthrene (data not shown). Griseofulvin, administered for 4 to 12 days, decreased microsomal cytochrome P-450 but elevated cytochrome $b_5$ levels in mouse liver (11). These previous results were confirmed in the present study (Table 5, compare control animals). This peculiar griseofulvin effect, however, did not occur in HN because, after a 4-day-long griseofulvin administration, the specific HN microsomal cytochrome P-450 concentration was elevated whereas cytochrome $b_5$ remained unchanged (Table 5).

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Apparent $K_m$* (nmol aminopyrine)</th>
<th>Apparent $V_{max}$* (nmol formaldehyde/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>0.48 ± 0.15</td>
<td>5.33 ± 1.58</td>
</tr>
<tr>
<td>PT</td>
<td>0.88 ± 0.18</td>
<td>6.23 ± 0.72</td>
</tr>
<tr>
<td>HN(PB)</td>
<td>0.36 ± 0.05</td>
<td>16.07 ± 1.03</td>
</tr>
<tr>
<td>PT(PB)</td>
<td>0.42 ± 0.08</td>
<td>11.97 ± 1.62</td>
</tr>
<tr>
<td>Control</td>
<td>0.76 ± 0.04</td>
<td>5.62 ± 0.62</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Stearoyl-CoA desaturase (mmol oleic acid formed/min/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>1.71 ± 0.30</td>
</tr>
<tr>
<td>PT</td>
<td>2.34 ± 0.87</td>
</tr>
<tr>
<td>Control</td>
<td>0.95 ± 0.31</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

Table 4

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome P-450* (nmol/mg microsomal protein)</th>
<th>Cytochrome $b_5$* (nmol/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>0.54 ± 0.08</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>PT</td>
<td>0.68 ± 0.06</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>HN(PB)</td>
<td>1.21 ± 0.08</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td>PT(PB)</td>
<td>1.76 ± 0.08</td>
<td>0.73 ± 0.06</td>
</tr>
<tr>
<td>HN(MC)</td>
<td>0.56 ± 0.06</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td>PT(MC)</td>
<td>0.71 ± 0.06</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>Control(PB)</td>
<td>1.92 ± 0.09</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>Control(MC)</td>
<td>0.95 ± 0.06</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>Control</td>
<td>0.92 ± 0.05</td>
<td>0.40 ± 0.01</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

PB, phenobarbital; MC, 3-methylcholanthrene.

HN-control, p < 0.01; PT-control, p < 0.01; PT-PB, p < 0.01; control-control(MC), not significant.

HN(PB)-control, p < 0.01; PT(PB), p < 0.01; PT-MC, not significant; control-control(PB), p < 0.01; control-control(MC), not significant.

HN(PB)-control, p < 0.01; PT(PB), p < 0.01; PT-MC, not significant; control-control(PB), p < 0.01; control-control(MC), not significant.
tochrome P-450) of isochromically applied liver and HN microsomes (Fig. 1) revealed a diminution of a band with a molecular weight of approximately 48,000 and an increase of an approximately M.W. 50,000 species in phenobarbital-treated liver microsomes (Fig. 1, Slots 2 and 3). Changes of this type also occurred in HN microsomes but seemed to be less pronounced (Fig. 1, Slots 4 and 5). Furthermore, phenobarbital administration resulted in a conspicuous increase of a M.W. 54,000 band (Fig. 1, Slots 4 and 5). Changes of this type also in liver microsomes, and a band with identical molecular weight value also showed up in induced HN microsomes (Fig. 1, slots 3 and 4) but in contrast to liver microsomes, where a band of identical molecular weight was already present prior to induction and was only accentuated by phenobarbital treatment, a M.W. 54,000 species was not detectable in noninduced HN microsomes. A band corresponding to a M.W. of 47,000 seemed to be specific for HN microsomes (Fig. 1, Slots 4 and 5).

### DISCUSSION

Numerous studies, mostly performed in rats, have shown that the activities of several microsomal cytochrome P-450-dependent enzymes are diminished in primary as well as transplanted hepatomas and in HN (1, 5, 7, 13–17, 21, 24, 27, 29, 35–37, 40–42). Liver tumors of different degrees of differentiation, however, vary considerably in the extent of this impairment. In HN, enzyme activities are less affected than in hepatomas (5, 15, 16, 29), and tumors with less pronounced deviation from normal tissue are enzymatically more active than those with higher degree of aberration (17, 35, 41, 42) and more readily inducible (24, 27, 41, 42). The results presented in this paper show that, in HN induced in mice by long-term griseofulvin treatment, some enzyme activities almost match those of control and perinodular liver despite loss of specific microsomal cytochrome P-450 (see Tables 1 and 2). Unchanged enzyme activities, despite decreased P-450 levels, may at least in some circumstances be due to a compensation of cytochrome P-450 loss by increased NADPH-cytochrome c reductase activity, which is considered to be rate limiting in several cytochrome P-450-dependent mixed-function oxidase reactions (Ref. 11; see also Ref. 4 for review). Indeed, enhanced activity of NADPH-cytochrome c reductase is another distinctive feature of microsomes derived from griseofulvin-induced HN because this enzyme is reduced or unchanged in activity in 2-acetylaminofluorene- and $\alpha$-ethionine-induced nodules in rats (5, 16, 27). An additional explanation, however, for the apparent dissociation between cytochrome P-450 levels and enzyme activities could be the existence of different P-450 species with different catalytic properties (18) in griseofulvin-induced HN, as suggested by altered $K_m$ values and by the results of gel electrophoresis.

Decreased affinity of tumor NADPH-cytochrome c reductase for the electron donor NADPH has been found in the "minimal-deviation" Morris hepatoma 5123tc (H) by Saine and Strobel (37). In this tumor, the $K_m$ of the reductase for NADPH was one order of magnitude higher than that in liver microsomes. However, diminished affinity of the reductase for NADPH cannot be expected to be a general feature of tumor microsomes. This has been shown by the same group (36) with microsomes of the "multiple-deviation" Novikoff hepatoma that did not differ in this respect from normal liver microsomes and now by us in this paper.

Cytochrome P-450 and P-450-dependent enzyme reactions were inducible in HN by phenobarbital, and the extent of induction was similar in HN, PT, and control liver microsomes in agreement with the observations of Feo et al. (15) and Okita et al. (29) in rat liver nodules produced by administration of different carcinogens. In their lack of response to 3-methylcholanthrene, HN matched normal liver in our animal strain. By increasing microsomal cytochrome $b_5$ along with loss of cytochrome P-450, griseofulvin presented itself as a special type of inducer in previous studies (11). HN, however, differed from

<table>
<thead>
<tr>
<th>Cytochrome P-450</th>
<th>Cytochrome b$_5$</th>
<th>Value (nmol/mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN</td>
<td>0.62 ± 0.01</td>
<td>0.50 ± 0.08</td>
</tr>
<tr>
<td>HN(GF)</td>
<td>0.79 ± 0.05</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td>Control</td>
<td>0.86 ± 0.01</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Control(GF)</td>
<td>0.55 ± 0.06</td>
<td>0.49 ± 0.01</td>
</tr>
</tbody>
</table>

**Table 5**

*Effect of GF on cytochromes P-450 and b$_5$ in microsomes of HN and control liver*

Values for treated mice are from 3 experiments, each done with the microsomes of HN derived from 3 to 5 mice. Four experiments were performed with control mice, each with the microsomes from 9 pooled mouse livers.

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**Fig. 1.** SDS-polyacrylamide gel electrophoresis of HN and control microsomes adjusted to identical cytochrome P-450 values. Slot 1, standards; Slot 2, control microsomes; Slot 3, phenobarbital-induced control microsomes; Slot 4, phenobarbital-induced HN microsomes; Slot 5, HN microsomes. Right, molecular weights.
nonneoplastic liver in this respect because microsomal cytochrome P-450 increased whereas cytochrome b5 remained almost constant upon griseofulvin administration. Moreover, griseofulvin is not porphorygenic in HN in contrast to nonneoplastic liver. In HN, therefore, in contrast to nonneoplastic liver, griseofulvin acts like other lipid-soluble inducers, indicating that not only a specific chemical constitution (9) and the mode of application (23, 43) but also characteristics of the target tissue play a role in determining the porphorygenic potential of griseofulvin.

SDS-polyacrylamide gel electrophoresis of liver and HN microsomes disclosed differences in the polypeptide pattern in the M.W. 47,000 to 54,000 region. As far as nonneoplastic liver is concerned, the effects of phenobarbital treatment closely resemble the results presented by Mull et al. (28) and Bell and Hodgson (3). In agreement with these authors, we observed a diminution of a M.W. 48,000 and an increase of a M.W. 54,000 species in liver microsomes following phenobarbital treatment. We have, in addition, seen an induction of a M.W. 50,000 band (less pronounced in HN microsomes) but not of the M.W. 56,000 band, the latter described by Mull et al. (28) and Bell and Hodgson (3). In HN microsomes, the M.W. 54,000 species was not visible in the uninduced state but appeared upon phenobarbital treatment. On the basis of molecular weights as well as the association with heme, these proteins were considered by Bell and Hodgson (3) as being cytochrome P-450 related. If this holds true, then our electrophoretic results may indeed reflect differences in cytochrome composition and inducibility between liver and HN microsomes. However, in the absence of a positive identification of the bands as cytochrome P-450 related, these interpretations must remain speculative. The band clearly discernible in the M.W. 47,000 region in HN microsomes but not seen in liver microsomes also remains to be identified. Epoxide hydratase may be a candidate (22).

 Besides the cytochrome P-450-dependent mixed-function oxidase system, liver microsomes contain at least 2 other major electron transfer pathways involved in lipid metabolism, which share the same pyridine nucleotide-linked electron input enzymes: the fatty acid desaturase and the lipid peroxidase (see Ref. 19 for further information). It was an additional goal of our investigations to determine components and activities of these pathways in griseofulvin-induced HN in order to further define their metabolic background. Lipid peroxidase activity was slightly depressed in griseofulvin-HN microsomes, which in principle, agrees with the results of Gravela et al. (16) obtained with ethionine-induced rat liver tumors. Although cytochrome b5, a component of the desaturase system (See Ref. 39 for further information), was almost unchanged, desaturase activity in HN microsomes was almost doubled, which agrees with the notion that the cyanide-sensitive factor rather than cytochrome b5 limits this reaction (31). In 2 different types of "minimal-deviation" transplanted hepatomas (5123C and 7800), increased (7800) and decreased (5123C) activities of stearoyl-CoA desaturase have been found but, in contrast to nonneoplastic liver, the tumor-associated enzymes were unaffected by dietary manipulations (33). Whether this altered desaturase activity as determined in vitro has any relevance to the situation in vivo, particularly to membrane composition and fluidity, remains to be clarified.

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