Activities of Rat Liver Glutathione-requiring Enzymes and Catalase in Comparison to the Action of Phénobarbital

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ABSTRACT

The biochemical effects in the livers of male rats of prolonged administration of the experimental hepatocarcinogen nafenopin, a hypolipidemic agent and peroxisome proliferator, were compared to those of another experimental liver carcinogen, pheno-barbital, which acts as a neoplasm promoter. Feeding of nafenopin, 0.03 mmol/kg basal diet for up to 24 weeks increased the numbers of hepatic peroxisomes, increased catalase activity, markedly decreased cytosolic glutathione transferase activities toward two substrates, decreased cytosolic glutathione peroxidase activities toward H2O2 and two organic peroxides, and suppressed the age-related increase in γ-glutamyl transpeptidase activity. In contrast the livers of rats fed an equimolar concentration of pheno-barbital displayed increases in cytosolic glutathione transferase activities and enhancement of catalase activity but no changes in glutathione peroxidase activities. There was also an enhancement of catalase activity without apparent increase in peroxisome number. Enzyme kinetic analyses revealed that the cytosolic glutathione transferase activities toward two halogenonitrobenzene substrates were inhibited in the rats fed nafenopin and displayed elevated Km and decreased Vmax. Kinetic studies of glutathione transferase activities in which nafenopin was mixed with normal rat liver cytosols in the assay system revealed competitive type inhibition toward 1-chloro-2,4-dinitrobenzene and a noncompetitive type of inhibition toward 3,4-dichloronitrobenzene. Likewise activities of glutathione peroxidases toward H2O2 and cumene hydroperoxide were suppressed by in vitro addition. Thus the effects of nafenopin and pheno-barbital on liver biochemistry were very different. The inhibition of hepatic biotransformation and scavenger systems by nafenopin is suggested to be relevant to its hepatocarcinogenicity.

INTRODUCTION

Several hypolipidemic agents such as clofibrate and nafenopin which induce peroxisome proliferation (1, 2) have caused mainly liver cancer and to a lesser extent neoplasms at other sites in rodents (3, 4). Experimental carcinogens of this type have not been suspected to result in an excessive generation of H2O2 in hepatocytes (17). H2O2 can escape from peroxisomes (18) and the conversion of H2O2 to reactive species and the reaction of the latter with DNA has been suggested to be the basis for the carcinogenicity of peroxisome proliferators (3, 4).

Outside of peroxisomes GSH® peroxidase (EC 1.11.1.9), is a scavenger of H2O2, as well as of a variety of organic hydroperoxides (19). This enzyme therefore would play a key role in the decomposition of any H2O2 that might escape from peroxisomes (18). Cirilo et al. (20) reported that clofibrate and another peroxisome proliferator, fenofibrate, lowered the activity of GSH peroxidase toward an organic hydroperoxide and reduced the activity of superoxide dismutase (EC 1.15.1.1) suggesting an inhibiting effect of the drugs on these extraperoxisomal scavenger enzymes which deal with oxygen radicals. Awasthi et al. (21), however, found only minimal inhibition of GSH peroxidase by ciprofibrate. This aspect requires further study because it is possible that alterations of H2O2 and oxygen radical levels by hypolipidemic peroxisome proliferators could originate not only from peroxisome proliferation but also from effects on scavenger systems outside of peroxisomes.

Another enzyme system which deals with reactive species is GSH transferases (EC 2.5.1.18). GSH transferases are a group of multifunctional enzymes (22) involved in detoxification of a broad spectrum of both endogenous substances and xenobiotics...

The abbreviations used are: GSH, reduced glutathione; GGT, γ-glutamyl transpeptidase; ENPP, 1,2-epoxy-3-(p-nitrophenoxy)propane; GSSG, oxidized glutathione; CDNB, 1-chloro-1,2-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene.
including carcinogens (23–28). Liver GSH transferase activities have been reported to be decreased by clofibrate (29) and ciprofibrate (21). This effect could be of importance to the carcinogenic properties of peroxisome proliferators because GSH transferases possess selenium-independent GSH peroxidase activity which reduces a variety of organic hydroperoxides (30, 31). Also GSH transferases have been postulated to inhibit nonenzymatically the initiation of lipid peroxidation (27, 28).

In a previous study we found that nafenopin inhibited the activity of GGT (EC 2.3.2.2) (32), which like GSH peroxidase and transferases is a GSH-requiring enzyme. In light of all these observations, we undertook the present study to determine the effect of nafenopin on the three GSH-requiring enzyme systems. In this study we compared the effect of nafenopin on these enzymes with that of phenobarbital, which also produces rodent liver neoplasms (33, 34) but acts as a neoplasm promoter (35, 36) in contrast to nafenopin (32, 37). We report here that feeding of nafenopin under conditions which produce a marked hepatic peroxisome proliferation and elevation of catalase activity decreased both cytosolic GSH peroxidase and transferase activities. Also the age-related increase in GGT activity was inhibited. In contrast dietary phenobarbital enhanced the activities of GSH transferases and induced GGT activity in perportal hepatocytes, whereas the GSH peroxidase activities were not altered. Additionally, phenobarbital elevated catalase activity without producing apparent peroxisome proliferation. The relevance of these observations to the liver carcinogenicity of these agents is discussed.

MATERIALS AND METHODS

Animals and Treatments

Male F344 rats weighing 250–280 g were obtained from Charles River Breeding Laboratories, Kingston, NY. They were maintained in a conventional specific-pathogen-free facility accredited by the American Association for the Accreditation of Laboratory Animals’ Care and their care conformed to the Care and Use of Laboratory Animals (NIH-78-23).

The rats were divided into 3 groups of 10 animals each. The control group was fed NIH-07 diet (Bio-Serv, Frenchtown, NJ). The experimental groups were fed NIH-07 diet which contained 0.093% nafenopin (kindly donated by CIBA Pharmaceutical Co., Summit, NJ) or 0.07% phenobarbital (Malinckrodt Chemical Works, St. Louis, MO), which represent approximately 0.03 mmol of each compound per kg diet. Rats were given the diets and water ad libitum until the day of killing.

At 6 and 24 weeks, 4 and 6 rats in each group, respectively, were used for biochemical and histochemical examinations of the livers. Animals were killed over a period of 3 h from 10:00 a.m. to 1:00 p.m. Under anesthesia with ethyl ether animals were exsanguinated by cutting the abdominal inferior vena cava, after which the livers were washed free of blood by perfusion with saline through the portal vein.

Biochemical Assays of the Liver Enzyme Activities

Liver Preparations. Representative liver homogenates were prepared by using portions of every lobe of each rat in each experimental group. The preparation of homogenates and cytosol fractions of liver was conducted at 0–4°C. Liver was homogenized in 10 vol of 0.25 M sucrose containing 5 mM MgCl₂ and 20 mM Tris·HCl, pH 7.6, with 10 strokes of a Potter-Elvehjem homogenizer. The homogenates were then centrifuged at 10,000 × g for 10 min followed by further centrifugation of the supernatant at 105,000 × g for 60 min in a Beckman L-5-75 ultracentrifuge. The final supernatant was used as the cytosol fraction.

For the measurements of catalase and GGT activities homogenates in 10 vol of distilled water and of 0.2 M sodium phosphate buffer, pH 8.0, respectively, were prepared as mentioned above. All assays were carried out at least in duplicate and the data were corrected for nonenzymatic reactions.

Chemicals. GSH, ENPP, GSGS reductase (yeast, type III), cumene hydroperoxide, tert-butyl hydroperoxide, NADPH, L-γ-glutamyl-p-nitroanilide, and glycylglycine were purchased from Sigma Chemical Co., St. Louis, MO; CDNB and DCNB were from Eastman Kodak (Rochester, NY).

Catalase. The activities of GSH transferases were measured at 25°C using a Beckman Model 25 spectrophotometer with CDNB, DCNB, and ENPP as substrates according to the procedure of Habig et al. (38). The reaction mixtures were identical with those reported by Habig and Jakoby (39). One unit of the enzyme activity was defined as 1.0 nmol of GSH conjugate production/min at 25°C.

Cytosolic GSH Peroxidase. GSH peroxidase activities were measured spectrophotometrically by a modified method of Jensen and Clausen (40). The standard assay mixture (1.0 ml) consisted of 48 mM sodium phosphate buffer, pH 7.4, containing 4.8 mM EDTA-Na₂, 3.75 mM NaH₂O₂, 5 mM GSH, 1.0 IU GSGS reductase, 0.28 mM NADPH, and 0.25 mM H₂O₂ (the substrate specific for selenium-dependent GSH peroxidase) or 1.2 mM cumene hydroperoxide, or 0.07 mM tert-butyl hydroperoxide. The mixture containing the cytosol fraction and the chemicals other than H₂O₂ or the hydroperoxides were equilibrated by an incubation for 10 min at room temperature. The reaction was then started with addition of one of the three peroxides. One unit of enzyme activity was defined as the amount of GSH peroxide leading to the oxidation of 1 nmol NADPH by GSSG reductase/min at 25°C.

Catalase. For the assay of catalasic activity an aliquot of homogenate was mixed with an equal volume of 1.0% Triton X-100 and diluted with distilled water. Spectrophotometric measurement of the activity was performed by the method of Beers and Sizer (41). One unit of the enzyme activity was defined as the amount catalyzing decomposition of 1.0 μM H₂O₂/min at 25°C.

GGT Activity. In homogenates was measured according to the method of Novogrodsky et al. (42). The reaction mixture was identical with that reported previously (43). A unit of the enzyme activity was defined as the amount catalyzing the release of 1.0 nmol p-nitroaniline/min at 25°C.

Protein Determination. Protein contents were measured by the Folin method using crystalline bovine serum albumin as the standard.

Enzyme Kinetics. Michaelis-Menten kinetics of liver cytosolic GSH transferases in nafenopin-fed and untreated rats were compared at 24 weeks of feeding using CDNB and DCNB as substrates. GSH concentrations in the assay systems were fixed at 1.0 and 5.0 mM for the assays of the GSH transferase activities toward CDNB and DCNB at 0.05 to 1.0 mM, respectively. The Km and Vmax were calculated by a computer program using least-squares estimation of a nonlinear regression curve (44). Apparent kinetics is illustrated with Lineweaver-Burk plots.

To examine direct actions of nafenopin on the activities of GSH transferase and peroxidase liver cytosol fractions were obtained from 3 normal male F344 rats (body weight, 261 ± 18 g (SD)); liver weight, 9.8 ± 0.9 g). Nafenopin was added to the GSH transferase and GSH peroxidase assays as a 30% ethanol solution in the assay buffers such that the final concentration of ethanol was 3% and the pH of the buffers was identical to that without nafenopin. Addition of nafenopin at concentrations higher than 3.0 mM in the assay substrates led to its precipitation.

As 1.0 nmol of GSH conjugate production/min at 25°C.

The in vitro effect of nafenopin on Michaelis-Menten kinetics of GSH transferase in normal rat liver cytosol fraction was also examined. The activity of GSH transferase toward 0.05 to 1.0 mM CDNB or DCNB was measured in the presence of nafenopin in the assay system. The GSH
concentration for each assay was fixed as for the in vivo kinetics studies. Histochemical Examinations. Liver slices taken from each lobe in a manner described previously (45) were fixed with ice-cold 95% ethanol followed by embedding in soft paraffin for the GGT reaction or fixed with 10% phosphate-buffered formalin for catalase reaction. Liver tissue blocks fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, were also prepared for electron microscopy. To demonstrate the peroxidatic activity of catalase in peroxisomes formalin-fixed sections were washed with distilled water and incubated with the alkaline 3,3'-diaminobenzidine medium for 2 h (46). Deparaffinized sections were reacted for GGT using L-γ-glutamyl-4-methoxy-β-naphthylamide (VEGA Biochemicals, Tucson, AZ) as a substrate (47).

RESULTS

Male rats were fed equimolar concentrations of nafenopin or phenobarbital for both 6 and 24 weeks.

Effects on Body and Liver Weights. In rats fed nafenopin for 6 or 24 weeks body weight gain was inhibited and liver weight was markedly increased (Table 1). Phenobarbital increased liver weights at 24 weeks but to a lesser degree than did nafenopin. The relative liver weight expressed as g liver/100 g body weight was increased in the rats fed nafenopin or phenobarbital for 24 weeks. The food intakes measured for 3 weeks from 12 to 14 weeks were not significantly different between untreated, nafenopin- and phenobarbital-fed rats, i.e., 17.1 ± 1.7, 17.1 ± 1.3, and 17.2 ± 1.8 g/day, respectively.

Microscopically enlargement of centrilobular hepatocytes was observed in the rats fed phenobarbital, whereas nafenopin- and phenobarbital-fed rats, i.e., 17.1 ± 1.7, 17.1 ± 1.3, and 17.2 ± 1.8 g/day, respectively.

Table 1

<table>
<thead>
<tr>
<th>Duration</th>
<th>Exposure</th>
<th>Body wt</th>
<th>Liver wt</th>
<th>g liver wt/100 g body wt</th>
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<tr>
<td>6 weeks</td>
<td>Control</td>
<td>361 ± 12</td>
<td>14.5 ± 2.2</td>
<td>4.0 ± 0.6</td>
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<tr>
<td></td>
<td>Nafenopin</td>
<td>319 ± 14</td>
<td>20.3 ± 1.5</td>
<td>6.4 ± 0.3</td>
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<td></td>
<td>Phenobarbital</td>
<td>379 ± 10</td>
<td>16.9 ± 1.7</td>
<td>4.5 ± 0.5</td>
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<tr>
<td>24 weeks</td>
<td>Control</td>
<td>422 ± 9</td>
<td>12.7 ± 0.26</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Nafenopin</td>
<td>331 ± 15</td>
<td>24.0 ± 1.4</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Phenobarbital</td>
<td>434 ± 21</td>
<td>18.3 ± 1.5</td>
<td>4.2 ± 0.2</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Duration</th>
<th>Catalase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 weeks</td>
<td>332 ± 11†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>555 ± 33‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>475 ± 53‡</td>
</tr>
<tr>
<td></td>
<td>24 weeks</td>
<td>299 ± 65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>464 ± 81†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>508 ± 75†</td>
</tr>
</tbody>
</table>

Effects on Liver Catalase Activity. Both nafenopin and phenobarbital increased catalase activity to a similar level after 6 weeks of feeding (Table 2). The activity did not increase further by 24 weeks of exposure. Histochemical reaction for catalase revealed reaction-positive granules which represent peroxisomes in the cytoplasms of hepatocytes in control rats (Fig. 1A). No increase in peroxisomes was evident in the hepatocytes of rats fed phenobarbital for 6 or 24 weeks (Fig. 1B) in comparison to the density in corresponding untreated hepatocytes. In the rats fed nafenopin for 6 or 24 weeks the cytoplasms of hepatocytes were filled with reaction-positive granules (Fig. 1C), indicating peroxisome proliferation. This was confirmed by electron microscopy (Fig. 1D).

Effects on Rat Liver Cytosolic GSH Transferase Activities. At 6 weeks of feeding of nafenopin the activities of GSH transferases with CDNB and DCNB as substrates were decreased to around 41 and 25%, respectively, of the activity of control liver (Table 3). The activity toward ENPP was only slightly reduced. At 24 weeks the activities toward CDNB and DCNB in nafenopin-fed rats were reduced to the same extent as at 6 weeks, while activity toward ENPP was comparable to that of untreated rats.

The feeding of phenobarbital for 6 weeks enhanced the activities of GSH transferases toward all three substrates (Table 3). The activities toward CDNB and ENP increased further in the rats fed phenobarbital for 24 weeks.

Kinetics of Rat Liver Cytosolic GSH Transferases. In the GSH transferase assay when CDNB and DCNB were used as substrates at different concentrations and the GSH concentration was held constant the apparent kinetics of liver cytosolic GSH transferases in untreated and nafenopin-fed rats at 24 weeks of feeding appeared to fit Michaelis-Menten kinetics as shown in Chart 1, A and B.

For GSH transferase activity toward CDNB, the Km for the enzyme in nafenopin-fed rats was increased from 0.09 ± 0.01 mM in controls to 0.13 ± 0.02 (P < 0.025). The Vmax was decreased from 590 ± 40 units/mg cytosolic protein to 220 ± 20 (P < 0.001). The Km of GSH transferase activity toward DCNB were 0.45 ± 0.05 in untreated rats and 0.90 ± 0.12 mM in nafenopin-fed rats (P < 0.005). The Vmax of GSH transferase activity toward DCNB in untreated and nafenopin-fed rats were 39 ± 2 and 24 ± 3 units/mg cytosolic protein (P < 0.001), respectively. Thus the dietary treatment with nafenopin inhibited GSH transferase activities toward CDNB and DCNB in a similar manner.

In Vitro Effects on the Activities of Liver Cytosolic GSH Transferases and Their Kinetics. Nafenopin was added to normal rat liver cytosol fractions and the reaction was started immediately with addition of CDNB and GSH. At 1.0 and 3.0 mM in the assay system nafenopin inhibited the GSH transferase activity relative to that of corresponding cytosol fractions without nafenopin (Chart 2A). Maximum inhibition at 3.0 mM was about 60%. The GSH transferase activity toward DCNB was also immediately inhibited by addition of nafenopin to the reaction mixture over a range of 0.03 to 3.0 mM (Chart 2B). In this case maximum inhibition at 3.0 mM was about 90%.

Alterations in kinetics of GSH transferases in the normal liver cytosol fractions by addition of nafenopin to the assay systems are illustrated in Chart 3, A and B. In the case of GSH transferase activity with CDNB as a substrate nafenopin appeared to inhibit the activity in a competitive type manner (Chart 3A) which
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Table 3

Effects of dietary nafenopin and phenobarbital on liver cytosolic glutathione transferase activities

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Duration</th>
<th>1-Chloro-1,2-dinitrobenzene</th>
<th>3,4-Dichloronitrobenzene</th>
<th>1,2-Epoxy-3-p-nitrophenoxypropane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 weeks</td>
<td>468 ± 70^a</td>
<td>31.5 ± 5.4</td>
<td>26.5 ± 1.3</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>6 weeks</td>
<td>191 ± 44^b</td>
<td>7.8 ± 1.3^b</td>
<td>22.4 ± 1.4^b</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>6 weeks</td>
<td>800 ± 164^b</td>
<td>45.7 ± 8.0^b</td>
<td>37.5 ± 4.6^b</td>
</tr>
<tr>
<td>Control</td>
<td>24 weeks</td>
<td>478 ± 102</td>
<td>23.8 ± 2.5</td>
<td>29.5 ± 2.4</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>24 weeks</td>
<td>197 ± 36^b</td>
<td>7.9 ± 1.1^b</td>
<td>28.6 ± 4.0</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>24 weeks</td>
<td>1109 ± 181^b</td>
<td>49.8 ± 7.3^b</td>
<td>71.1 ± 16.4^b</td>
</tr>
</tbody>
</table>

^a Mean ± SD of 4 rats/group (6 weeks) or 6 rats/group (24 weeks).

" P < 0.001.
* P < 0.005.

Chart 1. Lineweaver-Burk plots of kinetics of liver cytosolic GSH transferases in nafenopin-fed (A) and untreated control (B) rats at 24 weeks of feeding. The GSH transferase activities when CDNB (A) and DCNB (B) were used as variable substrates and GSH concentration was held constant were measured as described in "Materials and Methods." The cytosolic protein contents were fixed at 20 μg/ml and 200 μg/ml in the assay media for measuring the activities toward CDNB and DCNB, respectively. Points, mean value of duplicate determinations for 6 rats.

Chart 2. In vitro effect of nafenopin on normal rat liver cytosolic GSH transferase activities toward CDNB and DCNB. Nafenopin was mixed with normal rat liver cytosols (20 μg/ml cytosolic protein/ml assay medium) at 0.01 to 3.0 mw (abscissa) in the assay system and the GSH transferase activity was measured immediately against CDNB (A) or DCNB (B). Activity relative to the corresponding cytosolic activities without nafenopin is represented as percentages (ordinate). Points, mean of duplicate determinations for 3 rats; bars, SD, and small deviations are not illustrated; *, P < 0.05.

differed from mode of inhibition on the activity toward this substrate in nafenopin-fed rats (Chart 1A). When DCNB was used as the substrate nafenopin inhibited the activity in a non-competitive manner (Chart 3B) which also was different from the type of inhibition found in the nafenopin-fed rats (Chart 1B) as well as that of the in vitro inhibition by nafenopin of the activity toward CDNB (Chart 3A).

Effects on Rat Liver Cytosolic GSH Peroxidase Activities. In rats fed nafenopin for 6 weeks the activities of GSH peroxidase with H₂O₂ and cumene hydroperoxide as substrates were decreased to around half the levels of untreated rats (Table 4). The levels of these activities did not change further with feeding for 24 weeks. GSH peroxidase activity toward tert-butyl hydroperoxide was measured at 24 weeks of feeding and was decreased in the rats fed nafenopin to about 50% of control activity.

Feeding of phenobarbital to rats for 6 weeks slightly lowered GSH peroxidase activity toward cumene hydroperoxide (Table 4). In the rats fed phenobarbital for 24 weeks GSH peroxidase activity was comparable to that of untreated rats.

In Vitro Effects on Activity of GSH Peroxidase. When nafenopin was mixed with normal rat liver cytosol fractions at 0.01 to 3.0 mw in the standard GSH peroxidase assay system only the highest concentration slightly lowered activity toward H₂O₂ relative to that of the control fractions (Chart 4A). In the case of the activity toward cumene hydroperoxide the activity relative to that of the unexposed fractions was also lowered only when 3.0 mw nafenopin were mixed in the assay system (Chart 4B).

Effects on GGT Activity. In untreated rats liver GGT activity was increased 10-fold by 24 weeks of study as compared with the activity at 6 weeks (Table 5). Feeding of phenobarbital enhanced liver GGT activity at both 6 and 24 weeks. In contrast administration of nafenopin lowered the GGT activity at 24 weeks.

In untreated rats at 6 weeks of study only biliary epithelial cells were histochemically positive for GGT activity. In the rats fed phenobarbital for 6 weeks biliary epithelial cells and a small number of hepatocytes around some portal areas displayed histochemical GGT activity. At 24 weeks of study in untreated rats one to three cell layers of hepatocytes surrounding the portal triads displayed GGT activity (Fig. 2A). By 24 weeks of feeding of phenobarbital large periportal areas of GGT-positive

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Chart 3. Lineweaver-Burk plots showing in vitro effects of nafenopin on kinetics of normal rat liver cytosolic GSH transferases. The GSH transferase activities when CDNB or DCNB was used as variable substrates and GSH concentration was held constant were measured as described in "Materials and Methods." Activity toward CDNB (A) was measured in the presence of nafenopin at 0 (•), 1.0 (O), and 2.0 (a) mw in the assay system with the use of 20 µg cytosolic protein/ml assay medium, while the activity toward DCNB (B) was measured in the presence of nafenopin at 0 (•), 0.1 (O), and 0.2 (a) mw in the assay system with the use of 200 µg cytosolic protein/ml assay medium. Points, mean of duplicate determinations for 3 rats.

Table 4
Effects of dietary nafenopin and phenobarbital on liver cytosolic glutathione peroxidase activity

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Duration</th>
<th>GSH peroxidase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6 weeks</td>
<td>H2O2 712 ± 177*</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>6 weeks</td>
<td>Cumene hydroperoxide 573 ± 621f</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>6 weeks</td>
<td>tert-Butyl hydroperoxide 622 ± 134f</td>
</tr>
<tr>
<td>Control</td>
<td>24 weeks</td>
<td>H2O2 734 ± 80</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>24 weeks</td>
<td>Cumene hydroperoxide 845 ± 83</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>24 weeks</td>
<td>tert-Butyl hydroperoxide 311 ± 15</td>
</tr>
</tbody>
</table>

*a Mean ± SD of 4 rats/group (6 weeks) or 6 rats/group (24 weeks).
bp < 0.01.
cP < 0.001.

Chart 4. In vitro effect of nafenopin on normal rat liver cytosolic GSH peroxidase activities. Nafenopin was mixed with normal rat liver cytosol (20 µg cytosolic protein/ml assay medium) at 0.01 to 3.0 mw (abscissa) in the assay system and the GSH peroxidase activity toward H2O2 (A) or cumene hydroperoxide (B) was measured immediately after the 10-min incubation with GSSG reductase and NADPH. Activity relative to the corresponding cytosolic activities without nafenopin is represented as percentages (ordinate). Points, mean of duplicate determinations for 3 rats; bars, SD, and small deviations are not illustrated; *, P < 0.05.

Table 5
Effects of dietary nafenopin and phenobarbital on liver γ-glutamyltranspeptidase activity

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Duration</th>
<th>GGT activity (units/mg protein)</th>
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<tbody>
<tr>
<td>Control</td>
<td>6 weeks</td>
<td>0.3 ± 0.2*</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>6 weeks</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>6 weeks</td>
<td>1.0 ± 0.4*</td>
</tr>
<tr>
<td>Control</td>
<td>24 weeks</td>
<td>2.6 ± 1.2*</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>24 weeks</td>
<td>0.8 ± 0.3*</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>24 weeks</td>
<td>8.6 ± 3.8*</td>
</tr>
</tbody>
</table>

*a Mean ± SD of 4 rats/group (6 weeks) and 6 rats/group (24 weeks).
bP < 0.025.
cP < 0.01, as compared with rats fed the same diet for 6 weeks.
dP < 0.01.
eP < 0.005. P < 0.005, as compared with rats fed the same diet for 6 weeks.

hepatocytes were present (Fig. 2B). In contrast in the rats fed nafenopin for 24 weeks GGT activity was found only in biliary epithelial cells (Fig. 2C).

DISCUSSION

The present study documented marked differences between the effects of the experimental hepatocarcinogens nafenopin and phenobarbital on liver GSH-requiring enzymes. Nafenopin produced a marked suppression of GSH transferase, GSH peroxidase, and GGT activities in the presence of peroxisome proliferation and enhancement of catalase activity. In contrast phenobarbital enhanced GSH transferase and GGT activities, did not affect GSH peroxidase, and increased catalase activity in the absence of peroxisome proliferation.

Prolonged exposure to nafenopin produced a decrease in GSH transferase activity, as has been reported for clofibrate (29) and ciprofibrate (21). The decrease in activity toward DCNB was greater than that toward CDNB and the activity toward ENPP was not altered. The experiments to examine the in vitro effects of nafenopin on untreated rat liver cytosolic GSH transferase activities also revealed a greater inhibition of the activity toward DCNB than toward CDNB. Nafenopin inhibited in vitro the activity toward CDNB in a competitive manner and that toward DCNB in a noncompetitive manner. The latter finding was similar to that of Awasthi et al. (21) who also provided evidence that inhibition
was due to binding of nafenopin to the enzyme subunits. The in vitro effects of nafenopin, however, may not fully account for the decreases in the GSH transferase activities found in cytosols of nafenopin-fed rats. The activity toward CDNB or DCNB was inhibited in nafenopin-fed rats in a mode different from that of in vitro inhibition by nafenopin. It could be that nafenopin metabolites or generation of H2O2 in vivo contribute to enzyme inhibition. Fucci et al. (48) have reported that inactivation of enzymes by mixed-function oxidation systems can occur through the generation of H2O2 and its conversion to an activated oxygen species which reacts with the enzymes.

Feeding of nafenopin decreased GSH peroxidase activity toward H2O2 and two organic hydroperoxides to around 50% of the levels of corresponding controls. In vitro inhibitions by nafenopin of the activities toward H2O2 and cumene hydroperoxide were also demonstrated. Although the in vitro inhibitions were not marked under the conditions used in the present experiments, in current studies we have found that inhibition is more profound if the concentration of H2O2 is decreased (49). H2O2 is a substrate specific for selenium-dependent GSH peroxidase (30, 50) and 33% of total GSH peroxidase activity toward cumene hydroperoxide in rat liver cytosol depends upon selenium-independent GSH peroxidase (51). Therefore feeding of nafenopin appears to affect both selenium-dependent and selenium-independent GSH peroxidases. The selenium-independent GSH peroxidase has been shown to be an activity of GSH transferase (27, 28, 30, 31) but the inhibition of the selenium-dependent activity demonstrates that inhibition is not attributable solely to effects on GSH transferases.

Cinollo et al. (20) have reported that the activity of GSH peroxidase toward tert-butyl hydroperoxide in rat liver was suppressed by administration of clofibrate or fenofibrate for 30 days and that addition of these hypolipidemic drugs to the assay system was not effective in inhibiting the activity. On the other hand Awasthi et al. (21) found only a minimal inhibition of GSH peroxidase activity (substrate not specified) by ciprofibrate in hand. Awasthi et al. (21) found only a minimal inhibition of GSH peroxidase activity (substrate not specified) by ciprofibrate in hand. The present observation of inhibition of GSH-peroxidase may be important in the overall effects of nafenopin. Jones et al. (18) have reported that selenium-dependent GSH peroxidase, not catalase, plays the major role in decomposing H2O2 released from peroxisomes into the cytoplasm. Increased peroxisomal oxidation of lipids by peroxisome proliferators has been suggested to generate excess H2O2 which could be released into the cytoplasm (4, 17). Therefore the decrease in the GSH peroxidase activity toward H2O2 by nafenopin may be a major factor leading to elevated cytosolic levels of H2O2. Moreover suppression of the GSH peroxidase activity toward organic hydroperoxides by nafenopin could permit increased formation of lipid hydroperoxides. Thus if hepatocarcinogenesis by nafenopin is a consequence of the disturbance of H2O2 metabolism, as proposed by Reddy et al. (3) and Reddy and Lalwani (4) or is related to increased lipid peroxides, it could depend heavily upon the lowering of scavenger enzyme activities.

In a recent study we found suppression by nafenopin of GGT activity in liver altered foci and neoplasms induced in rats by the carcinogen N-2-fluorenylacetamide (32). This inhibition of GGT activity was suggested to contribute to the reported absence of GGT in liver neoplastic lesions and hepatocellular carcinomas in rats exposed to hypolipidemic agents (52–55). In the present study nafenopin suppressed the age-related appearance of GGT activity in periportal hepatocytes (43, 56, 57), in contrast to an enhancement by phenobarbital. The appearance of GGT activity in adult rat hepatocytes in primary cultures has also been delayed by addition of nafenopin (58). GGT is an enzyme involved in the mercapturic acid pathway (26, 38) as well as the γ-glutamyl cycle (59). Therefore the suppression of hepatocellular GGT activity by nafenopin may be an important part of its overall effects on GSH-requiring enzymes.

The present study showed that phenobarbital, an experimental hepatocarcinogen (33, 34) and liver neoplasm promoter (35, 36), exerted biochemical effects quite different from those of nafenopin on conjugation and scavenger enzymes requiring GSH. Phenobarbital at the dose used in this study strongly promotes liver neoplasm development in rats exposed previously to DNA-reactive hepatocarcinogens, whereas studies on the modifying effects of peroxisome proliferators on rat hepatocarcinogenesis have not reached a consistent conclusion (32, 37, 60–62). In consideration of the differences in the biochemical effects between phenobarbital and nafenopin documented in this study, it seems probable that the action of nafenopin in liver carcinogenesis is quite different from that of phenobarbital.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Yoichi Mochizuki for the electron microscopic examinations and for valuable suggestions and to Drs. Edward Butler and Tomiko Shimada for helpful advice. The authors also thank Laura Kanabaic and Arleen Weinstein for typing the manuscript.

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CANCER RESEARCH VOL. 45 OCTOBER 1985


EFFECTS ON GSH-REQUIRING ENZYMES BY NAFENOPIN

Fig. 1. Hepatic peroxisomes detected by catalase histochemistry and electron microscopy. A, control rat liver at 24 weeks. Reaction-positive granules which represent peroxisomes are present in the cytoplasms of hepatocytes. × 330. B, phenobarbital-treated rat liver at 24 weeks. No apparent increase in peroxisome number is noted as compared with that of corresponding untreated hepatocytes shown in A. × 330. C, nafenopin-treated rat liver at 24 weeks. Hepatocytes are filled with confluent granules indicating peroxisome proliferation. × 330. D, electron micrograph of a hepatocyte from a rat fed nafenopin for 24 weeks. Many large peroxisomes are present (arrows). Several peroxisomes contain crystalloid nucleoids or show flocculent matrices. × 20,000.
EFFECTS ON GSH-REQUIRING ENZYMES BY NAFENOPIN

Fig. 2. Effects of nafenopin and phenobarbital on liver GGT activity detected by histochemical reaction. A, control rat liver at 24 weeks. A few hepatocytes surrounding the portal areas and biliary duct epithelial cells have GGT activity. B, phenobarbital-treated rat liver at 24 weeks. Many periportal hepatocytes display GGT activity. C, nafenopin-treated rat liver at 24 weeks. GGT activity is absent in hepatocytes but is still present in biliary duct epithelial cells. All x 22.
Effects of the Hepatocarcinogen Nafenopin, a Peroxisome Proliferator, on the Activities of Rat Liver Glutathione-requiring Enzymes and Catalase in Comparison to the Action of Phenobarbital

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