METABOLISM OF DRUGS BY SUBFRACTIONS OF HEPATIC MICROSONES FROM PROLONGED ETHANOL-TREATED RATS

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In a previous paper, it was reported that in rats treated chronically with ethanol, the side-chain oxidation of hexobarbital, N-demethylation of aminopyrine and p-hydroxylation of aniline in vitro from either 9,000 g supernatant fraction of liver homogenates or washed microsomes was identical with that of control rats when ethanol was withdrawn and substituted for tap water 24 hr prior to sacrifice. In contrast, the activity of aniline hydroxylase of the rats which continued to ingest ethanol ad libitum up to the time of sacrifice was approx. 1.5-fold increased, compared with that of controls, in spite of no change being detected in hexobarbital oxidase and aminopyrine demethylase activities (1, 2).

It is known that pretreatment of rats with phenobarbital markedly increases the activities of several hepatic drug-metabolizing enzymes in smooth-surfaced microsomes relative to rough-surfaced ones. The ratio of enzyme activity in smooth microsomes to that in rough microsomes increases in the liver from the phenobarbital-treated animal as compared with that from the control animal (3, 4). Fouts and Gram (5) reported that ratios of smooth-versus rough-surfaced microsomal aminopyrine N-demethylase and hexobarbital oxidase of rabbits decreased after 3-methylcholanthrene treatment.

The present experiments were conducted to determine the intramicrosomal distribution of drug-metabolizing enzyme activity in the rats treated chronically with ethanol. Parallel experiments were done to determine the intramicrososomal distributions of cytochrome P-450, protein and phospholipid phosphorous contents of the liver from the rats treated chronically with ethanol.

METHODS

Male Wistar rats weighing 80 g initially were used. These were exposed to a daily schedule of 12-hr light and 12-hr darkness. All had free access to adequate laboratory chow (CLEA, CE-2), and ethanol treatments were the same as in the previous experiments (1, 2). In the present experiments, the rats were sacrificed 5 to 6 months after the beginning of ethanol treatments and the hepatic aniline hydroxylase and aminopyrine demethylase activities, microsomal cytochrome P-450 and phospholipid phosphorous contents were determined. According to the drinking fluid, the animals were divided into two groups, one
drinking tap water (control group), and the other only ethanol (ethanol group). The rats of ethanol group were given 10 v/v% ethanol for the final week prior to sacrifice. For the enzyme assay, the latter was further divided into two subgroups. In one, ethanol was withdrawn and substituted for tap water 24 hr prior to sacrifice (EtOH-1), whereas in the other, the animals continued to drink ethanol ad libitum up to the time of sacrifice (EtOH-2). All the animals were deprived of food 12 hr prior to sacrifice to reduce glycogen contents in the liver, and sacrificed by decapitation between 8 : 30 and 9 : 00 a.m. to minimize the circadian variations of drug-metabolizing enzyme activities (6).

The procedure of microsomal subfractionation was essentially that of Bergstrand and Dallner (7). The following procedures were carried out in a room temperature of 4°C. The animals were sacrificed and the livers perfused in situ with ice-cold isotonic KCl solution. The livers were totally removed, washed in ice-cold isotonic KCl solution until clear of blood, minced, weighed and homogenized in 3 vols of 0.25 M sucrose solution by the use of a Potter homogenizer fitted with a loose Teflon pestle. The homogenate was centrifuged at 10,000 g for 20 min. Six ml of the 10,000 g supernatant, which contained 15 mM CsCl, was layered over 3 ml of 1.3 M sucrose containing 15 mM CsCl, and the gradients were centrifuged at 102,000 g for 140 min in a Hitachi 65P ultracentrifuge. Rough microsomes formed a pellet at the bottom of the centrifuge tube. A band at the interphase was transferred from two centrifuge tubes to another tube, diluted to 8.5 ml with 0.25 M sucrose solution, and sedimented by centrifugation at 105,000 g for 90 min. Smooth microsomes were obtained as a pellet. A pellet was suspended with 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4.

Whole microsomes were prepared by centrifuging a portion of the 10,000 g supernatant containing 15 mM CsCl at 102,000 g for 104 min. Those microsomes were resuspended in 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4 and centrifuged at 105,000 g for 90 min. A pellet was finally suspended in 0.15 M KCl-0.02 M Tris-HCl buffer, pH 7.4 and used to assay enzyme activity.

The soluble fraction of the liver homogenates for the assay of the enzyme activity was prepared as follows. The livers were homogenized in 2 vols of 0.15 M KCl solution by the use of a Teflon-glass homogenizer. The 10,000 g supernatant was centrifuged at 105,000 g for 70 min. The soluble fraction obtained was carefully aspirated to avoid microsomal contamination.

Incubation mixtures for the assay of the activity of aniline hydroxylase and aminopyrine demethylase were the same as those described previously (2) (8). Aniline and aminopyrine concentrations were 4 mM and 5 mM respectively. Microsomal protein concentrations from 1.3 to 2.1 mg per ml of incubation mixtures were used. The reaction mixtures were incubated in 30 ml Erlenmeyer flasks in an atmosphere of air with a Dubnoff metabolic shaker at 37°C for 10 min. The p-hydroxylation of aniline was estimated from p-aminophenol formation, with the amount of p-aminophenol being determined as described previously (2). The N-demethylation of aminopyrine was measured by formaldehyde production, the amount of formaldehyde formed was determined as previously described (8). Protein contents in the
soluble fraction and microsomes were estimated by the method of Lowery et al. (9). The ethanol concentration in 10,000 g supernatant of liver homogenates was determined by gas chromatography as described previously (2). The phospholipid phosphorous in subfractions of microsomes was estimated as described by Chen et al. (10). The cytochrome P-450 contents of smooth- and rough-surfaced microsomes were determined by the method of Holtzman et al. (11) except that 0.1 M phosphate buffer, pH 7.5 was used instead of 0.02 M Tris buffer.

Electron microscopic studies were performed on submicrosomal pellets to determine the degree of contamination of the smooth microsomes with the rough microsomes.

RESULTS

The electron microscopic appearance of submicrosomal pellets showed little contamination of the smooth-surfaced microsomes mingled with the rough-surfaced microsomes.

1. Cytochrome P-450 contents

The cytochrome P-450 content of whole microsomes in EtOH-2 group was approx. 1.3-fold increased as compared with that in control group. The cytochrome P-450 content of smooth microsomes in EtOH-2 group was significantly increased as compared with that in control or EtOH-1 group but ethanol treatment had no effect on the enzyme contents of rough-surfaced microsomes in all groups. The smooth/rough ratio of cytochrome P-450 in EtOH-2 group was 1.72 (average of five experiments) and that in control group was 1.50 (average of five experiments). There was no difference observed in the cytochrome P-450 content of each subfraction between control and EtOH-1 groups (Table 1).

| Groups | Whole microsomes | Rough microsomes | Smooth microsomes | Smooth/Rough ratio |
|--------|-----------------|-----------------|-----------------|-------------------|
| Control | 0.077±0.006 | 0.054±0.013 | 0.077±0.010 | 1.50 |
| EtOH-1  | 0.082±0.012 | 0.065±0.009 | 0.087±0.007 | 1.39 |
| EtOH-2  | 0.102±0.010*† | 0.062±0.008 | 0.105±0.007*‡ | 1.72*‡ |

P-450: △ E_{450-600} per mg of microsomal protein per ml suspension
* p<0.01 in control vs EtOH-2
‡ p<0.05 in EtOH-1 vs EtOH-2
† p<0.01 in EtOH-1 vs EtOH-2

2. Activities of drug-metabolizing enzymes

The enzyme activities were expressed per 100 mg protein of microsomal subfractions. With aminopyrine demethylase activity in whole microsomes no significant difference was observed between control, EtOH-1 and EtOH-2 groups. This finding confirmed previous laboratory work (1). The smooth/rough ratio of the activity of aminopyrine demethylase in the liver from control rats was 1.39 (average of five experiments). No significant difference in the smooth/rough ratio of the enzyme activity was observed among three groups (Table 2).
TABLE 2. Aminopyrine demethylase activities of rough- and smooth-microsomes from liver of the rat treated chronically with ethanol.

| Groups  | Whole microsomes | Rough microsomes | Smooth microsomes | Smooth/Rough ratio |
|---------|------------------|------------------|-------------------|--------------------|
| Control | 366.0±49.0       | 362.7±44.0       | 498.8±48.1        | 1.39               |
| EtOH-1  | 407.2±45.5       | 426.8±67.0       | 562.2±62.6        | 1.34               |
| EtOH-2  | 337.0±64.9       | 348.0±69.0       | 466.4±84.3        | 1.36               |

Enzyme activity: µg formaldehyde formed/100 mg protein/10 min

TABLE 3. Aniline hydroxylase activities of rough- and smooth-microsomes from liver of the rat treated chronically with ethanol.

| Groups  | Whole microsomes | Rough microsomes | Smooth microsomes | Smooth/Rough ratio |
|---------|------------------|------------------|-------------------|--------------------|
| Control | 1.24±0.18        | 1.03±0.03        | 1.47±0.15         | 1.43               |
| EtOH-1  | 1.34±0.19        | 1.21±0.27        | 1.66±0.23         | 1.41               |
| EtOH-2  | 2.16±0.26*†      | 1.73±0.26*†      | 2.34±0.44*†       | 1.37               |

Enzyme activity: µmoles p-aminophenol formed/100 mg protein/10 min
* p<0.01 in control vs EtOH-2
† p<0.05 in EtOH-1 vs EtOH-2

The activity of aniline hydroxylase of whole microsomes in EtOH-2 group was significantly higher than that of the control or EtOH-1 group. This was also in agreement with previously reported results (1). The activities of aniline hydroxylase in both rough and smooth microsomes were significantly increased in the liver from EtOH-2 group as compared with those in control and EtOH-1 groups. The smooth/rough ratio of aniline hydroxylase activity in EtOH-2 group was 1.37 (average of five experiments) this value being approx. the same as that of the control group. From the facts described above, we may conclude that hepatic microsomal aniline hydroxylase activity in EtOH-2 was increased as compared with that of control or EtOH-1 group but the intramicrosomal distribution of the enzyme activity was identical with that in control or EtOH-1 group. There was no observable difference in aniline hydroxylase activity of each subfraction among control and EtOH-1 groups (Table 3).

When the enzyme activities were expressed per unit of cytochrome P-450, the aniline hydroxylase activity of whole microsomes in EtOH-2 was also higher than that in control or EtOH-1 group. The aminopyrine demethylase activity of whole microsomes in EtOH-2 group was the same as that in the control or EtOH-1 group. The activity of aniline hydroxylase in both smooth and rough microsomes was increased in the liver from EtOH-2 group as compared with that in control or EtOH-1 group, but the increase in the activity of the enzyme of rough microsomes was considerable as compared with that of smooth microsomes. There was no difference in the activity of the enzyme of smooth and rough microsomes among control and EtOH-1 groups.

3. Protein and phospholipid phosphorous contents

No differences were observed in protein and phospholipid phosphorous contents among the three groups (Tables 4 and 5).
TABLE 4. Protein contents of rough- and smooth-microsomes from liver of the rat treated chronically with ethanol.

| Groups   | Whole microsomes | Rough microsomes | Smooth microsomes | Smooth/Rough ratio |
|----------|------------------|------------------|-------------------|--------------------|
| Control  | 14.85 ± 1.85     | 8.46 ± 1.28      | 4.86 ± 1.24       | 0.58               |
| EtOH-1   | 14.73 ± 1.97     | 8.30 ± 1.60      | 4.07 ± 1.09       | 0.49               |
| EtOH-2   | 14.52 ± 2.04     | 8.42 ± 1.37      | 4.82 ± 1.16       | 0.59               |

Protein content was expressed as mg/g liver

TABLE 5. Phospholipid phosphorous contents of rough- and smooth-microsomes from liver of the rat treated chronically with ethanol.

| Groups   | Whole microsomes | Rough microsomes | Smooth microsomes | Smooth/Rough ratio |
|----------|------------------|------------------|-------------------|--------------------|
| Control  | 244.3 ± 42.4     | 157.5 ± 25.4     | 62.3 ± 15.0       | 0.40               |
| EtOH-1   | 244.3 ± 34.8     | 155.5 ± 21.6     | 59.2 ± 15.4       | 0.38               |
| EtOH-2   | 255.0 ± 47.1     | 157.5 ± 22.2     | 72.5 ± 19.5       | 0.43               |

Phospholipid phosphorous content was expressed as μg P/g liver

DISCUSSION

Hepatic NADPH-dependent drug-metabolizing enzymes are localized in the microsomal fraction of the liver cell (12), however, hepatic microsomes are morphologically heterogeneous. Since the definition of the microsomal fraction is operational rather than functional, the composition of the pellet after ultracentrifugation is dependent on both the tissue and the technique (13). As far as the liver is concerned, the rough microsomal subfraction derives from the rough-surfaced endoplasmic reticulum and the smooth vesicles from the smooth-surfaced endoplasmic reticulum (14).

In the present experiments the procedure of Bergstrand and Dallner (7) was employed to subfractionate microsomes. The electron microscopic studies showed little contamination of the smooth-surfaced microsomes mingled with the rough-surfaced microsomes. The smooth/rough ratios of aminopyrine demethylase and aniline hydroxylase activities in the liver from normal rats were 1.39 and 1.43 respectively. The smooth/rough ratio of cytochrome P-450 of control group was 1.50. The smooth/rough ratio of phospholipid phosphorous of control group was 0.40.

On the other hand, it is known that pretreatment of rats with phenobarbital markedly increases the activities of several hepatic drug-metabolizing enzymes in smooth-surfaced microsomes relative to rough-surfaced microsomes. The ratios of activity of aminopyrine N-demethylase and cytochrome P-450 content in smooth-surfaced to that in rough-surfaced microsomes were higher in the liver from the phenobarbital-treated rats than from the control rats (3). In contrast, Fouts and Gram (5) reported that the ratios of smooth- versus rough-surfaced microsomal aminopyrine N-demethylase and hexobarbital oxidase decreased after 3-methylcholanthrene treatment. In the present experiments the ratio of aniline hydroxylase activity in smooth-surfaced to that in rough-surfaced microsomes in EtOH-2 group was almost identical with that in control rats. The ratio of aminopyrine demethylase activity
in smooth-surfaced to that in rough-surfaced microsomes was also identical in all the three groups. The cytochrome P-450 contents of whole microsomes were about 1.3-fold increased in the liver from EtOH-2 group as compared with those from control and EtOH-1 groups.

The ratio of content of cytochrome P-450 in smooth-surfaced to that in rough-surfaced microsomes was 1.72 in EtOH-2 group, this value being significantly higher than that in control or EtOH-1 groups. Therefore, in the EtOH-2 group, both the P-450 content and the aniline hydroxylase activity were increased though the intramicrosomal distributions differed. It is surprising that the cytochrome P-450 content and aniline hydroxylase activity in EtOH-1 group returned to the levels of control group after only one day of withdrawal of ethanol. More recently Mezey (15) has reported that the microsomal ethanol-oxidizing system, cytochrome P-450 and aniline hydroxylase were enhanced by ethanol feeding. The activities of the microsomal enzymes did not fall to control values until 7 days after withdrawal of ethanol. These results are not in agreement with our present data. Presumably this can be attributed to different experimental conditions. It was also observed that the increased ethanol-oxidizing activity of hepatic microsomes by ethanol feeding returned to control level after only one day withdrawal of ethanol (16). Furthermore electron microscopic studies show the qualitative difference between EtOH-1 and EtOH-2 groups.

SUMMARY

Studies have been performed prolonged ethanol-treated rats to determine the intramicrosomal distribution of drug-metabolizing enzyme activities.

In rats where ethanol was withdrawn and substituted for tap water 24 hr prior to sacrifice (EtOH-1 group), aniline hydroxylase activity was not increased in whole microsomes, smooth and rough microsomes as compared with that in control group. Only in those rats continuing to drink ethanol ad libitum up to the time of sacrifice (EtOH-2 group), was aniline hydroxylase activity increased significantly in whole microsomes, rough and smooth microsomal fractions. The smooth/rough ratio of aniline hydroxylase activity in EtOH-2 group was the same as that in EtOH-1 group or control group.

In contrast to aniline hydroxylase activity, a greater concentration of the cytochrome P-450 in the smooth microsomal fraction was observed in EtOH-2 group.

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