Drug Metabolizing Activity in Rats with Chronic Liver Injury Induced by Carbon Tetrachloride: Relationship with the Content of Hydroxyproline in the Liver

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Accepted April 5, 1986

Abstract—The drug metabolizing activity of rat liver during long-term administration of carbon tetrachloride (CCl₄), and its relationship with the content of hydroxyproline (Hyp) in the liver were examined. The contents of cytochrome P-450 (P-450) and cytochrome b₅ (b₅) and the metabolism of aniline, aminopyrine, 7-ethoxycoumarin (7-EC) and benzo(a)pyrene (B(a)P) in the microsomal fraction were examined five days after the final administration of CCl₄. The contents of P-450 and b₅ and the activity to metabolize the four substrates were gradually reduced as the Hyp content in the liver increased. However, aminopyrine N-demethylation and B(a)P hydroxylation, particularly the latter, was more reduced than aniline hydroxylation and 7-EC O-deethylation in the early stage of hepatic fibrosis. Such differences may be due mainly to the different P-450 subtypes affected by CCl₄.

Many hydrophobic compounds, including drugs, chemicals, environmental pollutants and carcinogens, are biotransformed into more hydrophilic metabolites through the mixed-function oxidase system in liver microsomes, in which cytochrome P-450 (P-450) plays an important role as a key enzyme (1, 2). In patients with chronic liver disease, especially with liver cirrhosis, the elimination of several drugs is impaired with the advance in histological and functional impairment of the liver. For example, the elimination half-life of antipyrine is obviously prolonged in chronic active hepatitis and liver cirrhosis (3); the content of P-450 is reduced in proportion to the severity of hepatic parenchymal changes (4).

Carbon tetrachloride (CCl₄) has been widely used to develop an appropriate animal model of liver cirrhosis (5). McLean et al. (6) first showed that phenobarbital-treated rats chronically exposed to CCl₄ rapidly developed liver cirrhosis; subsequently, they demonstrated that in such cirrhotic animals, the content of P-450 and the activity of aminopyrine demethylation were reduced as the severity of cirrhosis increased (7).

We produced chronic liver injury in rats by chronic administration of CCl₄ and examined the metabolism of aniline, aminopyrine, 7-ethoxycoumarin (7-EC) and benzo(a)pyrene (B(a)P), which are widely used substrates for P-450 (8–11), in the microsomal fraction of the liver.

We also measured the content of hydroxyproline (Hyp) in the liver of each CCl₄-treated rat as an indicator of the hepatic fibrosis developed and examined its relationship with the drug metabolizing activity.

Materials and Methods

Animals and treatments: Male Wister rats each weighing 200 g were given a standard pelleted diet and drinking water ad libitum. CCl₄ (2.0 ml/kg body weight) was administered subcutaneously, twice weekly for three to four months, in an equal volume of olive
oil (5). The animals were fasted overnight and killed five days after the final administration of CC14. Blood samples were taken from the jugular vein.

Preparation of microsomes: Rat liver microsomes were prepared as follows (12): Excised livers were thoroughly perfused with cold 0.15 M KCl and homogenized in 4-fold volumes of 0.15 M KCl solution containing 10 mM EDTA using a Potter-type Teflon glass homogenizer. The homogenate was centrifuged at 10,000×g for 15 min in a refrigerated centrifuge (Kubota, KR/20000). The supernatant was then centrifuged at 105,000×g for 60 min in a preparative ultracentrifuge (Hitachi, 70P–72). The pellet of microsomes was suspended in the homogenizing solution in the homogenizer and centrifuged again as described above. The resulting pellet was suspended in 20 mM potassium phosphate buffer, pH 7.4, containing 15% glycerol. These operations were done at 0–4°C.

Assays of the contents of cytochromes P-450 and b5: The content of P-450 was assayed by the method of Omura and Sato (13), and the content of cytochrome b5 (b6) was assayed by the method of Omura and Takesue (14). The microsomal protein was measured by the method of Lowry et al. (15).

Assays of substrate-metabolizing activities: Substrate-metabolizing activities, aniline hydroxylation, aminopyrine N-demethylation, 7-EC O-deethylation and B(a)P hydroxylation were assayed as described by Imai et al. (8), Nash (9), Ullrich and Weber (10) and Nebert and Gelboin (11), respectively.

Assays of the content of hydroxyproline:

Fig. 1. Histological examination of the rat liver (H.E.×100) (A) Grade 0: no increase of connective tissue. (B) Grade I: slight increase of connective tissue. (C) Grade II: definite increase of connective tissue without the formation of pseudolobules. (D) Grade III: marked increase of connective tissue with the formation of pseudolobules.
The content of hydroxyproline (Hyp) in the liver was measured by the method of Blumenkrantz (16).

**Histological study:** The degree of hepatic fibrosis was classified histologically into four grades by microscopic examination as described by Aoto (17) (Fig. 1).

**Assays of serum protein and protein fractions:** Serum total protein was measured by the biuret reaction using an autoanalyzer (Hitachi 736). Serum protein fractions were assayed by cellulose acetate electrophoresis.

**Results**

**Relationship between Hyp content in the liver and hepatic fibrosis:** Figure 2 shows the relationship between the Hyp content in the liver and the grade of hepatic fibrosis. The Hyp content in the liver was increased as hepatic fibrosis advanced; the mean value in Grade III was four times as large as that in Grade 0.

**Correlation between serum protein fractions and Hyp content in the liver:** As shown in Fig. 3, the content of serum albumin decreased with the increase in the Hyp content in the liver with a coefficient value of \(-0.743\) (\(P<0.01\)). In contrast, the content of \(\gamma\)-globulin correlated to the Hyp content in the liver with a coefficient value of 0.821 (\(P<0.01\)).

**Contents of P-450 and \(b_5\) in rats chronically exposed to CCl₄:** The rats used in these experiments were classified into three groups according to the Hyp content in the liver, that is the normal group (Group N) with a Hyp content of less than 1.0 mg/g liver, the group (Group M) with a slightly higher Hyp content (1.0 to 3.0 mg/g liver), and the group (Group S) with a Hyp content of more than 3.0 mg/g liver.

**Substrate-metabolizing activities in rats chronically exposed to CCl₄:** Figure 5 shows the activity to metabolize the four substrates based on g liver. The metabolizing activity was gradually reduced as the Hyp content in the liver increased, as was the content of P-450 (Fig. 4). The level of these metabolizing activities in Group S was significantly and severely decreased to 11.4-17.7% of that of Group N (\(P<0.01\)). The level of \(\alpha\)-naphthylamine N-demethylation and B(a)P hydroxylation in Group M (37.5% and 19.5%, respectively) was lower than that of aniline hydroxylation and 7-EC O-deethylation (50.5% and 51.5%, respectively). The level of B(a)P hydroxylation in Group M was significantly lower than that of the other three metabolizing activities, no significant difference being observed between Group M and Group S. In contrast, significant differences were observed between Group M and Group S (\(P<0.01\)), concerning the other three substrate-metabolizing activities, respectively.

In order to investigate the effects of the relative decrease in the number of liver cells...
Fig. 3. Correlation between the contents of serum albumin (A), γ-globulin (B) and the hydroxyproline content in the liver.

Fig. 4. The contents of cytochrome P-450 (A) and cytochrome b₆ (B) in three groups classified according to the hydroxyproline content in the liver.
following the development of hepatic fibrosis, the contents of P-450 and b5 and the four substrate-metabolizing activities based on microsomal protein (mg) are shown in Table 1, compared with those based on g liver. They were similarly reduced to those based on g liver, but the values based on g liver were 5–10% lower than those based on microsomal protein (mg), in both Group M and Group S.

**Discussion**

The liver is the major site of metabolism of various hydrophobic compounds (1, 2), and drug metabolizing activity decreases in chronic liver disease, especially in liver

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**Fig. 5.** The activities of aniline hydroxylation, aminopyrine N-demethylation, 7-ethoxycoumarin (7-EC) O-deethylation and benzo(a)pyrene (B(a)P) hydroxylation in three groups classified according to the hydroxyproline content in the liver.
Table 1. The contents of cytochromes P-450 and \( b_6 \) and the four substrate-metabolizing activities based on microsomal protein (mg). compared with those based on g liver

|                | P-450 (nmol/mg MS prot.) | \( b_6 \) (nmol/mg MS prot.) | Aniline (nmol/mg MS prot./min) | Aminopyrine (nmol/mg MS prot./min) | 7-EC (pmol/mg MS prot./min) | B(a)P (nmol/mg MS prot./min) |
|----------------|--------------------------|-------------------------------|-------------------------------|-----------------------------------|-----------------------------|-----------------------------|
| **Group N** (n=5) | 1.26±0.14                | 0.59±0.08                     | 1.44±0.30                     | 15.71±1.69                       | 265.01±75.17               | 1.01±0.21                   |
| **Group M** (n=7)  | 0.79±0.23                | 0.33±0.12                     | 0.88±0.33                     | 7.23±3.03                        | 162.29±54.97               | 0.24±0.14                   |
| M/N×100(%)        | 62.7                     | 55.9                          | 61.1                          | 46.0                             | 61.2                        | 23.8*                       |
| **Group S** (n=10) | 0.47±0.16                | 0.29±0.07                     | 0.34±0.27                     | 3.39±1.86                        | 54.19±68.02                | 0.15±0.13                   |
| S/N×100(%)        | 37.3                     | 49.2                          | 23.6                          | 21.6                             | 20.4                        | 14.9                        |

|                | P-450 (nmol/g liver) | \( b_6 \) (nmol/g liver) | Aniline (nmol/g liver/min) | Aminopyrine (nmol/g liver/min) | 7-EC (nmol/g liver/min) | B(a)P (nmol/g liver/min) |
|----------------|----------------------|-------------------------|-----------------------------|--------------------------------|--------------------------|---------------------------|
| **Group N** (n=5) | 28.61±3.86          | 13.28±0.94              | 32.37±4.95                  | 356.05±22.09                   | 5.92±1.20                | 22.72±2.74                |
| **Group M** (n=7)  | 15.22±4.19          | 6.03±1.52               | 16.34±5.01                  | 133.56±42.63                   | 3.05±0.72                | 4.42±1.99                 |
| M/N×100(%)        | 53.2                 | 45.4                    | 50.5                        | 37.5                           | 51.5                     | 19.5**                    |
| **Group S** (n=10) | 7.57±3.31           | 4.55±1.39               | 5.73±5.07                   | 55.58±35.11                    | 0.92±1.22                | 2.60±2.55                 |
| S/N×100(%)        | 26.5                 | 34.3                    | 17.7                        | 15.6                           | 15.5                     | 11.4                      |

(mean±S.D.). *Significantly lower than the data of aniline, 7-EC (P<0.01), and aminopyrine (P<0.05). **Significantly lower than the data of aniline, aminopyrine and 7-EC (P<0.01).
cirrhosis (3, 4). This decrease of the activity mainly depends on the damage of P-450 (4), which is the principal component of the mixed-function oxidase system in liver microsomes.

CCl₄ has been widely used for producing experimental liver cirrhosis (5), and such cirrhotic animals have been used as appropriate models to investigate drug metabolism in human liver cirrhosis (6, 7).

However, a potential problem in the administration of CCl₄ to rats is the acute reduction in the drug metabolizing activity by about 90% within 8 hr, which is normalized in about 8 days (18). Murray and Farrell (19) indicated that the efficiency of binding of substrates to P-450 in rats 48 hr after acute CCl₄ exposure was quite different from that in cirrhotic rats permitted to recover 10 days. They emphasized that the period required for recovery should be carefully examined to minimize the residual acute effects of the drug in studies of experimental cirrhosis.

Villeneuve et al. (20) showed that the elimination of aminopyrine was initially prolonged in CCl₄-induced cirrhotic rats, but fell to a stable plateau by day 7 after the cessation of drug administration; therefore, they carried out subsequent experiments between day 7 and 12. Similarly, Farrell and Zaluzny (21) allowed the rats 10 days to recover.

On the other hand, Marshall and McLean (7) demonstrated that the level of P-450 fell after CCl₄ administration, but was nearly normalized within a few days in the early stages of their cirrhosis-producing schedules, even though it remained depressed in severely cirrhotic rats even after 8 weeks. Histologically, the liver was normalized after the cessation of CCl₄ in the pre-cirrhotic reversible stage, even though the perilobular fibrosis persisted for months in the cirrhotic stage (5).

Although rats with severe liver cirrhosis should be allowed at least 7 days to recover from acute CCl₄ intoxication, this may be too long in the early stage of fibrosis. We chose 5 days as a reasonable period for our experiments.

Hydroxyproline (Hyp) is a specific amino acid for collagen. Aoto (17) showed that in patients with liver diseases, the Hyp content in the liver increased proportionally with the progress of hepatic fibrosis, it increased significantly with the decrease in serum albumin and the increase in γ-globulin. We obtained similar results using rats with chronic liver injury induced by CCl₄; therefore, we considered the Hyp content in the liver as an appropriate indicator of the hepatic fibrosis developed, and we examined its relationship with the drug metabolizing activity.

The contents of P-450 and b₅ and the activity to metabolize the four substrates were gradually reduced with the increase in the Hyp content in the liver, that is the development of hepatic fibrosis. These contents and activities based on g liver were 5–10% lower than those based on microsomal protein (mg). This difference may reflect the relative decrease in the number of liver cells following the development of hepatic fibrosis.

The extent of impairment of the metabolism of the four substrates differed. Aminopyrine N-demethylation and B(a)P hydroxylation, especially the latter, was more reduced in the early stage of hepatic fibrosis. Formerly, Murase et al. (22) showed this kind of non-uniformity in acutely galactosamine-treated rats; in their studies, 7-EC O-deethylation and B(a)P hydroxylation were more reduced.

P-450 is considered to consist of multiple forms, each having loose substrate specificity (23). Ryan et al. (24–26) purified five subtypes of P-450 (P-450ₓ, b, c, d, e) in rat liver microsomes, each of which has a characteristic substrate specificity. P-450ₓ, which is the predominant form induced by 3-methylcholanthrene, has very high catalytic activity for B(a)P and zoxazolamine hydroxylation and 7-EC O-deethylation. Noguchi et al. (27) and Head et al. (28) demonstrated that a specific form(s) of P-450 rapidly disappeared from liver microsomes of phenobarbital-induced rats after the administration of CCl₄.

The differences in the extent of impairment of the metabolism of the four substrates may be due mainly to the different P-450 subtypes affected by CCl₄. The marked
reduction of B(a)P hydroxylation in the early stage of hepatic fibrosis may reflect the predominant impairment of P-450. However, the population of P-450 of untreated rats differs from that of rats treated with various inducers, and a large proportion of B(a)P hydroxylation in untreated rats is not catalyzed by P-450a, P-450b, and P-450c (29). Since the number of P-450 subtypes in untreated rats is still unknown, which subtype(s) was easily affected in these experiments remains unknown.

Sex-related differences exist in drug metabolism (30), e.g., aminopyrine N-demethylation is 4-fold higher in male rats than in female rats, whereas there is no significant sexual difference in aniline hydroxylation. The androgen-dependent drug metabolizing activities such as aminopyrine N-demethylation are more easily impaired under pathological conditions than androgen-independent activities such as aniline hydroxylation. Sasame et al. (31) observed that ethylmorphine N-demethylation (androgen-dependent) was slightly more impaired in male rats than in female rats after the administration of CCl4. In our studies, too, aminopyrine N-demethylation was more reduced than aniline hydroxylation. Kamataki et al. (32) recently reported that the difference in the form(s) of P-450 was responsible for the sexual differences in drug metabolizing activities. The sex-related differences after CCl4 administration may be due to the easier impairment of the metabolism of this form(s).

The P-450 substrates belong to two types, Type I (aminopyrine etc.) and Type II (aniline etc.) (33), each with a different binding site to P-450. Murray and Farrel (19) indicated that the binding capacity of the Type I substrate was more reduced than that of the Type II substrate in CCl4-induced cirrhotic rats. The differences in the extent of impairment of substrate metabolism may be due to the different effects of the cirrhotic process at the two binding sites.

The content of b5 decreased as the Hyp content in the liver increased. Kato et al. (34) reported that the activities of electron transport systems, such as NADPH-cytochrome c reductase, were reduced in rats treated with CCl4. The effects of the reduction of such components of the mixed-function oxidase system on each substrate-metabolizing activity should also be taken into consideration.

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