Protective Effect of Rifampicin against Acute Liver Injury Induced by Carbon Tetrachloride in Mice

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ABSTRACT—Rifampicin conferred significant protection against carbon tetrachloride (CCl_4)-induced liver injury. Serum alanine transaminase (ALT) and aspartate transaminase (AST) activities were not markedly altered and only hepatocellular fatty degeneration was found in mice pretreated with rifampicin (200 mg/kg), whereas severe centrilobular necrosis was observed and serum ALT and AST activities were as high as 281 and 271 I.U./l, respectively, in the control group following administration of CCl_4 (400 $\mu\text{l/kg}$). The contents and activities of microsomal drug-metabolizing enzymes in rifampicin-pretreated animals were also much higher than those of the controls. CCl_4 -mediated malondialdehyde (MDA) formation was increased in rifampicin-treated liver microsomes, demonstrating that rifampicin was capable of increasing the NADPH-dependent metabolism of CCl_4 catalyzed by P-450 2E1 to produce free radicals. However, MDA formation was obviously depressed by rifampicin at varying concentrations from 2 to 32×10^{-6} M in an *in vitro* cytochrome P-450 (P-450) enzyme system. On the other hand, NADPH oxidation in the metabolism of CCl_4 and aniline hydroxylation were not suppressed in the presence of rifampicin in these systems, suggesting that rifampicin did not influence the biotransformation of CCl_4 by P-450 2E1 *in vitro*. Therefore, the protective effect of rifampicin against CCl_4 hepatotoxicity appeared to result from the direct inhibition of lipid peroxidation generated by CCl_4 -derived free radicals.

Keywords: Rifampicin, Carbon tetrachloride, Cytochrome P-450, Liver injury, Malondialdehyde

Rifampicin, an antituberculosis drug, is usually administered for a long period with other antituberculosis drugs or medication from other classes. It was recently reported that rifampicin significantly relieved pruritus in adult patients with primary biliary cirrhosis and children with cholestatic liver disease (1, 2). The potential for drug interaction often exists because rifampicin is a potent inducer of liver microsomal drug-metabolizing enzymes, as demonstrated by proliferation of smooth endoplasmic reticulum and an increase in cytochrome P-450 (P-450) in the liver (3). Clinically important drug interactions have been documented between rifampicin and numerous other drugs such as oral anticoagulants, oral contraceptives, several antiarrhythmics, cyclosporine and digitalis (4). Rifampicin accelerates the biotransformation of various other compounds and decreases their plasma concentration, efficacy and half-life by induction of microsomal enzymes. In contrast, Adachi et al. (5) reported that liver bilirubin-conjugating enzymes were induced by rifampicin, but mixed-function oxidases, including P-450, cytochrome b_5 , aniline hydroxylase and aminopyrine *N*-de-

methylase, were not induced in rats.

As reported previously, phenobarbital enhanced biotransformation of carbon tetrachloride (CCl_4) to bioactive metabolites that cause liver injury by induction of hepatic mixed-function oxidase. It is not understood whether rifampicin, as an inducer of microsomal enzymes, can potentiate CCl_4 hepatotoxicity. In this study, we investigated the effects and mechanism of rifampicin on liver damage induced by CCl_4 and compared the differences between the effects of rifampicin and phenobarbital on CCl_4 hepatotoxicity and hepatic microsomal enzymes in mice.

MATERIALS AND METHODS

Chemicals and reagents

Rifampicin and serum alanine transaminase (ALT) and aspartate transaminase (AST) kits were purchased from Wako Pure Chemical Industries Ltd., Osaka. Phenobarbital was obtained from Tokyo Kasei Kogyo Co., Tokyo. CCl_4 was purchased from Nacalai Tesque, Inc., Kyoto.

NADPH and NADH were obtained from Kohjin Co., Ltd., Tokyo. Cytochrome c was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatments

Male Crj:CD-1 (ICR) mice, 24–26 g (Charles River Japan, Yokohama), were used. Animals were housed in a temperature-controlled room and fed a standard diet. Rifampicin dissolved in 0.02 N HCl was administered orally to mice at doses of 50, 100 and 200 mg/kg for four days. Phenobarbital dissolved in distilled water was injected intraperitoneally at a dose of 80 mg/kg on four consecutive days. CCl₄ dissolved in olive oil with an injection volume of 5 ml/kg was given subcutaneously at a single dose of 50, 100, 200 or 400 μ l/kg in different groups. The last dose of inducer and CCl₄ were given concurrently in the cotreatment groups. The animals were sacrificed 20 hr after treatment with CCl₄ or the last dose of rifampicin and phenobarbital.

Preparation of liver microsomes

The livers were removed immediately, perfused with cold 0.15 M KCl and homogenized in 4 volumes of 0.15 M KCl solution containing 10 mM EDTA using a Potter-type Teflon glass homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 15 min in a refrigerated centrifuge (KR/20000; Kubota, Tokyo). The supernatant was then centrifuged at $105,000 \times g$ for 60 min in a preparative ultracentrifuge (70P-I; Hitachi, Tokyo). The pellet of microsomes was suspended in the homogenization solution in 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 procedures were performed at 0°C–4°C.

Biochemical liver function tests and microsomal enzyme assays

The activities of serum ALT and AST were measured by the UV Rate method and expressed as international units per liter. The contents of serum total protein and liver microsomal protein were determined by the method of Lowry et al. (6). The content of P-450 was measured by the method of Omura and Sato (7). The content of cytochrome b₅ and the activity of NADPH-cytochrome c reductase were assayed as described by Omura and Takesue (8) using a spectrophotometer (DU-64; Beckman, Fullerton, CA, USA).

Substrate-metabolizing enzyme activities, aniline hydroxylation, aminopyrine *N*-demethylation, 7-ethoxycoumarin (7-EC) and 7-methoxycoumarin (7-MC) *O*-dealkylation, and benzo(a)pyrene (B(a)P) hydroxylation were measured as described by Imai et al. (9), Nash (10),

Ullrich and Weber (11) and Nebert and Gelboin (12), respectively. Each substrate-metabolizing enzyme activity was assayed using NADPH as the sole electron source.

Measurement of malondialdehyde (MDA) formation

In vitro metabolism of CCl₄ was determined by measuring CCl₄-dependent lipid peroxidation in the mono-oxygenase system prepared from mouse livers according to the thiobarbituric acid assay (13). Incubation was carried out with liver microsomes (1 mg/ml) in 100 mM potassium phosphate buffer (pH 7.4), containing 0.4 mM NADPH and 10 mM CCl₄ (dissolved in methanol), in a final volume of 1 ml. Methanol was used in the reference tube. Inhibition of MDA production by rifampicin was assayed as described above. Rifampicin was added to the reaction mixtures at varying concentrations of 0.5, 2, 8 and 32×10^{-6} M. The comparative inhibitory effect of rifampicin and glutathione (GSH) on MDA formation was determined by the same method. The final concentrations of rifampicin and GSH in vitro were varied from 2.2×10^{-7} to 10^{-4} M and 4.6×10^{-6} to 10^{-3} M, respectively. The reaction was started by the addition of NADPH and CCl₄ concurrently with vigorous shaking after preincubation at 37°C for 3 min, and it was stopped by 30% trichloroacetic acid after incubation for 6 min. Thiobarbituric acid reactive substance MDA was expressed as nmol/mg microsomal protein (MS Pr.)/min.

Determination of NADPH oxidation

NADPH oxidation during metabolism of CCl₄ was assayed in an in vitro P-450 enzyme system. The incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.4), liver microsomes (1 mg/ml), 5 mM CCl₄ dissolved in methanol and 0.1 mM NADPH. The reference mixture received vehicle alone. The effect of rifampicin on the oxidative metabolism of NADPH was assayed as described above in the presence of rifampicin at a concentration of 8×10^{-6} M. The reaction was started by adding CCl₄ and NADPH concurrently after preincubation at 37°C for 3 min. NADPH disappearance was monitored fluorometrically for 3 min at 450 nm with an excitation wavelength of 365 nm (13) using a fluorescence spectrophotometer (Hitachi 650-60).

Effect of rifampicin on aniline hydroxylase activity in vitro

Aniline hydroxylase activity was assayed according to the method of Imai et al. (9). Incubation was carried out with liver microsomes (1 mg/ml) in 100 mM Tris-acetate buffer (pH 8.0), containing 3 mM MgCl₂, 2 mM aniline and 1 mM NADPH, in a final volume of 0.5 ml. The effect of rifampicin on aniline hydroxylase activity was assayed as described above in the presence of rifampicin

at a concentration of 8×10^{-6} M. The reaction was started by addition of NADPH with vigorous shaking after preincubation at 37°C for 3 min, and it was stopped by 20% trichloroacetic acid after incubation for 15 min.

Histopathology

Livers were fixed in neutral 10% formalin. Tissues were routinely processed and embedded in paraffin. Sections were stained with hematoxylin and eosin and then examined histopathologically by light microscopy.

Statistical analyses

All values are expressed as means $\pm$ S.D. Data were analyzed by Wilcoxon's test for unpaired variables. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Effects of rifampicin on biochemical liver functions

The effects of rifampicin on serum biochemical

parameters in mice treated with and without CCl₄ are shown in Fig. 1, Table 1 and Table 2. Serum ALT and AST activities were not significantly altered in the rifampicin (RFP)- and phenobarbital-treated groups. In mice with liver injury induced by CCl₄, serum ALT and AST were markedly elevated by increasing the dose of CCl₄ to 281 and 272 I.U./l (CCl₄, 400 μ l/kg), respectively, whereas these activities rose only to 70 and 47 I.U./l (RFP, 200 mg/kg) and 150 and 159 I.U./l (RFP, 100 mg/kg), respectively, at the same dose of CCl₄ in the rifampicin-pretreated group. The serum levels of ALT and AST in the other rifampicin-pretreated groups (200 mg/kg) were also much lower than those in the corresponding CCl₄-treated groups at the same dose of CCl₄. The activities of serum ALT and AST in the groups that received combination treatment with phenobarbital and CCl₄ were not significantly different from those of the controls at low doses of CCl₄ (50 and 100 μ l/kg), but they were much higher than those in the controls on treatment with a high dose: serum ALT and AST were as high as 532 and 460 I.U./l, respectively, at the dose of 400 μ l/kg

Fig. 1. Effects of rifampicin on serum ALT and AST activities and content of P450 in mice treated with and without CCl₄. Rifampicin (RFP) and phenobarbital (PB) were given at doses of 200 and 80 mg/kg, respectively, for 4 days. Values represent means $\pm$ S.D., $n = 5$ animals. * $P < 0.05$, ** $P < 0.01$: RFP- and PB-treated groups are compared with the untreated group; RFP + CCl₄- and PB + CCl₄-treated groups are compared with the corresponding same dose of CCl₄ alone-treated group. RFP- and RFP + CCl₄-treated groups (○), PB and PB + CCl₄ (●), untreated and CCl₄ alone (△).

Table 1. Effects of rifampicin on the contents of serum total protein, microsomal protein and cytochrome b_5 and the activity of NADPH-cytochrome c reductase in mice treated with and without CCl_4

Group		Serum total protein (g/l)	Microsomal protein (mg/g liver)	Cytochrome b_5 (nmol/mg MS Pr.)	NADPH-Cyt. c reductase (nmol/mg MS Pr./min)
Untreated	(n=5)	60.92 $\pm$ 5.50	19.66 $\pm$ 1.04	0.328 $\pm$ 0.030	82.90 $\pm$ 12.89
CCl_4 :50	(n=5)	62.39 $\pm$ 3.63	21.76 $\pm$ 1.71	0.261 $\pm$ 0.018	64.06 $\pm$ 13.61
CCl_4 :100	(n=5)	60.14 $\pm$ 4.38	16.90 $\pm$ 1.52	0.245 $\pm$ 0.035	65.92 $\pm$ 14.09
CCl_4 :200	(n=5)	59.90 $\pm$ 2.24	16.74 $\pm$ 1.37	0.197 $\pm$ 0.043	58.92 $\pm$ 5.56
CCl_4 :400	(n=5)	62.47 $\pm$ 6.02	17.02 $\pm$ 1.52	0.191 $\pm$ 0.008	54.04 $\pm$ 3.84
PB	(n=5)	61.01 $\pm$ 4.70	26.54 $\pm$ 3.37**	0.433 $\pm$ 0.032**	136.78 $\pm$ 9.06**
PB + CCl_4 :50	(n=5)	63.76 $\pm$ 5.86	22.26 $\pm$ 2.80*	0.382 $\pm$ 0.043**	122.66 $\pm$ 17.96**
PB + CCl_4 :100	(n=5)	60.22 $\pm$ 3.72	20.77 $\pm$ 1.24*	0.376 $\pm$ 0.064*	112.71 $\pm$ 29.00*
PB + CCl_4 :200	(n=5)	60.37 $\pm$ 6.31	17.98 $\pm$ 2.10	0.307 $\pm$ 0.049*	86.82 $\pm$ 18.03*
PB + CCl_4 :400	(n=5)	62.70 $\pm$ 4.36	16.93 $\pm$ 1.24	0.210 $\pm$ 0.031	70.82 $\pm$ 8.52**
RFP	(n=5)	66.43 $\pm$ 6.25	22.69 $\pm$ 3.12	0.457 $\pm$ 0.069*	157.06 $\pm$ 5.63**
RFP + CCl_4 :50	(n=5)	63.71 $\pm$ 6.59	25.51 $\pm$ 1.91**	0.397 $\pm$ 0.056**	139.39 $\pm$ 13.02**
RFP + CCl_4 :100	(n=4)	63.83 $\pm$ 4.93	23.78 $\pm$ 2.62**	0.426 $\pm$ 0.055**	145.74 $\pm$ 30.58**
RFP + CCl_4 :200	(n=4)	62.35 $\pm$ 4.78	21.82 $\pm$ 4.34*	0.423 $\pm$ 0.066**	139.52 $\pm$ 23.33**
RFP + CCl_4 :400	(n=5)	63.86 $\pm$ 6.65	22.04 $\pm$ 1.42*	0.410 $\pm$ 0.039**	148.44 $\pm$ 13.83**

The numbers following the colons are doses of CCl_4 ($\mu\text{l/kg}$). Rifampicin (RFP) and phenobarbital (PB) were administered at doses of 200 and 80 mg/kg, respectively, for 4 days. Values represent means $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$: PB- and RFP-treated groups are compared with the untreated group, and PB + CCl_4 - and RFP + CCl_4 -treated groups are compared with the corresponding same dose of CCl_4 alone-treated groups.

Table 2. Effect of varying doses of rifampicin on serum ALT and AST activities and the content of P-450

Group		ALT (I.U./l)	AST (I.U./l)	P-450 (nmol/mg MS Pr.)
Untreated	(n=5)	40.25 $\pm$ 4.69**	21.65 $\pm$ 4.17**	0.853 $\pm$ 0.126**
CCl_4	(n=5)	280.63 $\pm$ 58.17	271.36 $\pm$ 78.00	0.293 $\pm$ 0.033
CCl_4 + RFP:50	(n=5)	292.42 $\pm$ 79.64	287.88 $\pm$ 90.44	0.525 $\pm$ 0.153**
CCl_4 + RFP:100	(n=5)	150.14 $\pm$ 58.18*	159.35 $\pm$ 71.39*	0.979 $\pm$ 0.128**
CCl_4 + RFP:200	(n=5)	70.23 $\pm$ 23.75**	46.57 $\pm$ 24.49**	1.421 $\pm$ 0.289**

Rifampicin (RFP) was administered for 4 days, and the numbers following the colons are doses of RFP (mg/kg). CCl_4 was given at a single dose of 400 $\mu\text{l/kg}$. Values represent means $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$: compared with the CCl_4 alone-treated group.

CCl_4 . The content of serum total protein was increased by 9% in the rifampicin-treated group (200 mg/kg), but this was not significantly different from that in untreated mice. There was no obvious change in the content of serum total protein in the groups cotreated with rifampicin or phenobarbital and CCl_4 , which was similar to that in animals that received the hepatotoxin alone.

Effect of rifampicin on the contents of P-450 and cytochrome b_5 and the activity of NADPH-cytochrome c reductase

As shown in Fig. 1, rifampicin and phenobarbital increased the content of P-450 by 110% and 72%, respec-

tively. After administration of CCl_4 , the content of P-450 was decreased obviously at varying doses from 50 to 400 $\mu\text{l/kg}$ in the CCl_4 -treated groups. In mice pretreated with phenobarbital, although P-450 was markedly induced prior to use of CCl_4 , it was also dose-dependently reduced after CCl_4 , and was much lower than in the control at a dose of 400 $\mu\text{l/kg}$. The amount of P-450 was slightly affected in the groups pretreated with rifampicin (200 mg/kg), the decrease in which was much less than in other groups following treatment with CCl_4 . The contents of P-450 in the groups pretreated with rifampicin (200 mg/kg) and phenobarbital were 485% and 72% of the control level (CCl_4 , 400 $\mu\text{l/kg}$), respectively. As shown in

Table 2, the P-450 content was reduced to a certain extent in mice pretreated with rifampicin at doses of 50 and 100 mg/kg after dosing with CCl₄, but it remained much higher than in the group treated with CCl₄ alone.

The contents of microsomal protein and cytochrome b₅ and the activity of NADPH-cytochrome c reductase are shown in Table 1. Rifampicin and phenobarbital were capable of inducing increases in the contents of microsomal protein and cytochrome b₅ and the activity of NADPH-cytochrome c reductase, which were 135%,

132% and 165% of the control values in phenobarbital-treated mice, respectively, and 115%, 139% and 189% of the controls in rifampicin-treated mice, respectively. After administration of CCl₄, the contents of microsomal protein and cytochrome b₅ and the activity of NADPH-cytochrome c reductase were markedly reduced in the mice treated with CCl₄ alone and cotreated with phenobarbital and CCl₄, especially at high CCl₄ doses. In contrast, these values were not obviously reduced in the groups cotreated with rifampicin and CCl₄. The contents

Fig. 2. Effects of rifampicin on the activities of drug-metabolizing enzymes in mice treated with and without CCl₄. Rifampicin (RFP) and phenobarbital (PB) were given at doses of 200 and 80 mg/kg, respectively, for 4 days. Each column represents a mean $\pm$ S.D. Columns A represent untreated ($\square$), PB ($\square$)- and RFP ($\square$)-treated groups; Columns B represent CCl₄ (50 μ l/kg, $\square$), PB + CCl₄ (50 μ l/kg, $\square$) and RFP + CCl₄ (50 μ l/kg, $\square$); and Columns C represent CCl₄ (400 μ l/kg, $\square$), PB + CCl₄ (400 μ l/kg, $\square$) and RFP + CCl₄ (400 μ l/kg, $\square$).

of microsomal protein and cytochrome b_5 and the activity of NADPH-cytochrome c reductase were 129%, 215% and 275% of the control levels, respectively, in animals that received combination treatment with rifampicin and CCl_4 , and 99%, 110% and 131% of the controls in those that received combination treatment with phenobarbital and CCl_4 at a dose of 400 $\mu\text{l/kg}$ CCl_4 , respectively.

Effects of rifampicin on drug-metabolizing enzyme activities

Rifampicin is an inducer of the activities of aniline hydroxylase, aminopyrine *N*-demethylase, 7-EC and 7-MC *O*-dealkylases and B(a)P hydroxylase (Fig. 2). As compared with phenobarbital treatment, the effects of rifampicin on the activities of drug-metabolizing enzymes were less marked with the exception of aminopyrine *N*-demethylase, the activity of which was higher in rifampicin-treated mice than that in those treated with phenobarbital, and B(a)P hydroxylase which was the same in both groups. The activities of drug-metabolizing enzymes were markedly reduced in the groups treated with CCl_4 alone and cotreated with phenobarbital and CCl_4 , but the degree of decline was greater in the former at the dose of 50 $\mu\text{l/kg}$ of CCl_4 . After treatment with CCl_4 , although the activities of these enzymes in the animals cotreated with rifampicin and CCl_4 were also decreased to a certain extent, they remained much higher than control levels. At a dose of 400 $\mu\text{l/kg}$ of CCl_4 , the activities of aniline hydroxylase, aminopyrine *N*-demethylase, 7-EC and 7-MC *O*-dealkylases and B(a)P hydroxylase were 390%, 604%, 1260%, 1910% and 394% of controls, respectively, in the mice cotreated with rifampicin and CCl_4 . In contrast, these values were only 72%, 67%, 106%, 31% and 54% of the controls, respectively, in the group with cotreatment with phenobarbital and CCl_4 .

Table 3. Stimulation of lipid peroxidation by CCl_4 in the in vitro P-450 enzyme systems prepared from untreated and inducer-treated mice

Group		MDA (nmol/mg MS Pr./min)	% control
Untreated	(n=5)	1.036 $\pm$ 0.248	100.0
Phenobarbital	(n=5)	1.921 $\pm$ 0.423**	185.4
Rifampicin	(n=5)	1.502 $\pm$ 0.063**	144.9
CCl_4	(n=5)	0.101 $\pm$ 0.030**	9.7

Microsomes were prepared from untreated, phenobarbital (80 mg/kg for 4 days)-, rifampicin (200 mg/kg for 4 days)- and CCl_4 (400 $\mu\text{l/kg}$)-treated mice. Values represent means $\pm$ S.D. ** $P < 0.01$; compared with the untreated group.

CCl_4 -dependent MDA formation and inhibition of its formation by rifampicin in an in vitro monooxygenase system

As shown in Table 3, the stimulation of lipid peroxidation by CCl_4 was noticeably enhanced in the in vitro P-450 enzyme systems prepared from rifampicin- and phenobarbital-induced mice, especially from the latter, whereas it was almost suppressed in the system from CCl_4 -intoxicated animals (CCl_4 , 400 $\mu\text{l/kg}$). MDA production was 185%, 145% and 10% of control level in the phenobarbital-, rifampicin- and CCl_4 (400 $\mu\text{l/kg}$)-treated P-450 enzyme systems, respectively.

Rifampicin is a powerful inhibitor of CCl_4 -dependent MDA formation. NADPH-linked lipid peroxidation was significantly depressed in the presence of rifampicin at varying concentrations of 2, 8 and 32 $\times 10^{-6}$ M in the in vitro P-450 enzyme systems prepared from untreated and inducer-treated mice. A similar inhibitory effect, which was markedly increased with elevation of concentration, was obtained in the three enzyme systems described above, with rifampicin treatment. Inhibitory rates of rifampicin at a concentration of 32 $\times 10^{-6}$ M on MDA production were 79%, 83% and 79% in untreated,

Fig. 3. Inhibition by rifampicin of MDA formation stimulated by CCl_4 in the in vitro P-450 enzyme systems. Microsomes were prepared from untreated ($\triangle$, n=5), rifampicin ($\circ$, n=5)- and phenobarbital ($\bullet$, n=4)-treated groups. Rifampicin and phenobarbital were given at doses of 200 and 80 mg/kg, respectively, for 4 days. CCl_4 was given at a single dose of 400 $\mu\text{l/kg}$. Values represent means $\pm$ S.D. * $P < 0.05$, ** $P < 0.01$; compared with the respective value of each group in the absence of rifampicin in vitro.

Fig. 4. Comparison of inhibition by rifampicin and GSH of MDA formation mediated by CCl₄ in the in vitro P-450 enzyme system. Microsomes were prepared from mice treated with phenobarbital at dose of 80 mg/kg for 4 days. The final concentrations of rifampicin (●) and GSH (○) in vitro were varied from 2.2×10^{-7} to 10^{-4} M and from 4.6×10^{-6} to 10^{-3} M, respectively. Each point is the mean value of duplicate assays.

rifampicin- and phenobarbital-induced enzyme systems, respectively, as shown in Fig. 3. GSH showed a similar inhibitory effect on CCl₄-generated lipid peroxidation as rifampicin did. The 50% inhibitory concentration (IC₅₀) of rifampicin and GSH on CCl₄-mediated MDA formation in phenobarbital-induced microsomes of mice was 6×10^{-6} and 10^{-4} M, respectively, the former being much lower than the latter (Fig. 4).

Effect of rifampicin on NADPH oxidation in the metabolism of CCl₄ and aniline hydroxylase activity in the in vitro monooxygenase system

The results are shown in Table 4, NADPH oxidation in the metabolism of CCl₄ and aniline hydroxylation were markedly increased in the in vitro P-450 enzyme systems prepared from rifampicin- and phenobarbital-induced mice, while they were obviously diminished in the system prepared from the CCl₄-treated group. In the presence of rifampicin at a concentration of 8×10^{-6} M, which markedly suppressed CCl₄-dependent MDA formation in these systems, NADPH oxidation and aniline hydroxylase activity were not significantly different from the control values.

Histopathological findings

Histopathological examination revealed that cytoplasmic vacuoles were present in hepatocytes, indicating fatty degeneration in mice treated with rifampicin alone (200 mg/kg) and in those cotreated with rifampicin (200 mg/kg) and CCl₄ (400 μ l/kg), but there was no significant difference between the two groups. Severe centrilobular necrosis of the liver was observed in mice treated with CCl₄ alone and in those cotreated with phenobarbital and CCl₄, and the necrosis was more severe in the latter at a dose of 400 μ l/kg. Mice treated with phenobarbital alone had normal liver histology.

DISCUSSION

Rifampicin is a potent inducer of hepatic microsomal enzymes, and it has been shown to increase cytochrome (CYP, gene) 3A6 gene expression in rabbits (14) and elevate the CYP1A1 mRNA level and rate of transcription in cycloheximide-treated rabbit hepatocytes (15). The present study showed that rifampicin elevated the contents of P-450 and cytochrome b₅, and the activities of NADPH-cytochrome c reductase and drug-metabolizing

Table 4. Effect of rifampicin on NADPH oxidation in the metabolism of CCl₄ and aniline hydroxylase activity in the in vitro P-450 enzyme systems prepared from untreated and inducer-treated mice

Group	NADPH oxidation (nmol/mg MS Pr./min)		Aniline hydroxylase activity (nmol/mg MS Pr./min)	
	Absence of RFP	Presence of RFP	Absence of RFP	Presence of RFP
Untreated	1.544 $\pm$ 0.521	1.587 $\pm$ 0.561 ^{ns}	1.527 $\pm$ 0.115	1.491 $\pm$ 0.107 ^{ns}
Phenobarbital	1.924 $\pm$ 0.571	2.028 $\pm$ 0.600 ^{ns}	2.056 $\pm$ 0.195	1.955 $\pm$ 0.221 ^{ns}
Rifampicin	2.389 $\pm$ 0.908	2.559 $\pm$ 0.776 ^{ns}	1.928 $\pm$ 0.242	1.837 $\pm$ 0.258 ^{ns}
CCl ₄	0.389 $\pm$ 0.176	0.326 $\pm$ 0.147 ^{ns}	0.331 $\pm$ 0.056	0.321 $\pm$ 0.050 ^{ns}

Microsomes were prepared from untreated, phenobarbital (80 mg/kg for 4 days)-, rifampicin (RFP) (200 mg/kg for 4 days)- and CCl₄ (400 μ l/kg)-treated mice. The concentration of RFP was 8×10^{-6} M. Values represent means $\pm$ S.D., n = 4–5 animals. ^{ns} not significantly different from the corresponding control values without RFP in vitro.

enzymes to some extent in mice. These results are different from those of a previous study that showed that rifampicin did not induce the mixed-function oxidase in rats (5). Whether this discrepancy is due to the species difference in experimental animals used in the two studies is not clear at present.

It is known that the inducers of microsomal drug-metabolizing enzymes can potentiate the hepatotoxicity of xenobiotics such as acetaminophen and CCl_4 , which are biotransformed to active metabolites by the liver mixed-function oxidase system, as it has been reported that patients on treatment with acetaminophen experienced hepatotoxic reactions with rifampicin, isoniazid and other agents for active tuberculosis (16) and that phenobarbital increased CCl_4 hepatotoxicity (17). However, a significant protective effect of rifampicin against CCl_4 -induced liver injury was found in this investigation. In mice treated with CCl_4 alone (50–400 $\mu\text{l/kg}$), serum ALT and AST activities were increased and reached 281 and 272 I.U./l, respectively, at 400 $\mu\text{l/kg}$ of CCl_4 , while cotreatment with phenobarbital raised them further to 532 and 460 I.U./l, respectively. In both groups of animals, severe centrilobular necrosis was histologically found; Marked reduction in the contents of cytochromes P-450 and b_5 , and the activities of NADPH-cytochrome c reductase and drug-metabolizing enzymes with increasing doses of CCl_4 were observed. In contrast, in mice cotreated with rifampicin (200 mg/kg) and CCl_4 , the activities of serum ALT and AST remained almost normal, and only hepatocellular fatty degeneration was observed; The decreases in the contents and the activities of liver microsomal enzymes induced by CCl_4 were much less than those in CCl_4 - or phenobarbital plus CCl_4 -treated groups. These results demonstrate a significant protection from CCl_4 hepatotoxicity by pretreatment with rifampicin.

It is known that hepatotoxicity of CCl_4 is caused by several of its active metabolites generated by P-450. The primary step in CCl_4 metabolism is reductive and consists of transfer of an electron to CCl_4 by the P-450 system yielding $\text{CCl}_3^\cdot$ (18). There is also evidence for a two-electron reduction yielding dichlorocarbene, but this step seems to be quantitatively minor (19). These reductive steps are carried out by P-450. $\text{CCl}_3^\cdot$ is a reactive species and yields a variety of secondary metabolites. Under certain conditions, $\text{CCl}_3^\cdot$ reacts largely with polyunsaturated fatty acids by $\text{H}^\cdot$ abstraction, forming CHCl_3 and lipid free radicals or by covalent binding at site of a double bond, forming an adduct that is also a free radical (20). In the presence of oxygen, oxygen reacts with free radicals, and lipid peroxidation occurs with destruction of lipids causing liver cell damage. When oxygen reacts with $\text{CCl}_3^\cdot$, it forms $\text{CCl}_3\text{OO}^\cdot$, which may promote the des-

truction of P-450 responsible for catalyzing CCl_4 metabolism and generation of $\text{CCl}_3^\cdot$. This limits CCl_4 metabolism in the liver (21).

CCl_4 hepatotoxicity is relieved by inhibition of hepatic microsomal enzymes. Lauriault et al. (22) reported that the thiol drug diethyldithiocarbamate and its two metabolites, disulfiram and carbon disulfide, could be used as inhibitors of P-450 to protect hepatocytes from damage induced by CCl_4 . In contrast, CCl_4 hepatotoxicity is increased by induction of P-450. It was reported that CCl_4 -evoked liver injury was enhanced in phenobarbital-induced animals (23). Noguchi et al. (24) further showed that the phenobarbital-inducible form of P-450 was capable of generating the trichloromethyl free radical in the reconstituted enzyme system. The present study also showed similar results in that phenobarbital considerably increased the amount of P-450 and the activities of drug-metabolizing enzymes, and it markedly increased liver degeneration and necrosis induced by a large dose of CCl_4 . However, liver injury was not significantly different from that in the controls following treatment with low doses of CCl_4 , and thus it seemed that the rate of biotransformation for a low plasma concentration of CCl_4 in normal mice was similar to that in phenobarbital-induced animals.

The isoform of P-450 that is responsible for the reductive activation of CCl_4 has not been identified conclusively. Recently, anti-(P-450 2E1)-Ig G experiments performed with microsomes showed that addition of anti-(P-450 2E1)-Ig G to the in vitro P-450 enzyme system was capable of inhibiting CCl_4 -mediated microsomal lipid peroxidation by 70%, suggesting that P-450 2E1 is involved in metabolic activation of CCl_4 (25–27). Hepatocytes isolated from rats treated with pyrazole, which is an inducer of P-450, were found to be much more susceptible to CCl_4 -induced cytotoxicity than those isolated from normal rats. Microsomal enzymes prepared from pyrazole-treated rats also underwent a CCl_4 -catalyzed lipid peroxidation with NADPH much more rapidly than microsomal enzymes from controls. Secondly, microsomal enzymes prepared from rats treated with pyrazole also showed enhanced aniline hydroxylation, whereas the activities of ethoxyresorufin *O*-dealkylase, which is somewhat specific for P-450 1A1/1A2; pentoxyresorufin *O*-dealkylase, which is specific for P-450 2B1/2B2; and aminopyrine *N*-demethylase were not markedly altered. In addition, diethyldithiocarbamate, which is an inhibitor of microsomal enzymes, not only reduced lipid peroxidation and cytotoxicity of CCl_4 , but also depressed aniline hydroxylase (22). These findings indicated that aniline hydroxylase is specific, at least to a certain extent, for P-450 2E1.

As compared to the effects of phenobarbital on liver

drug-metabolizing enzymes, rifampicin was less effective on aniline hydroxylase activity, which increased by 26% and 35% in rifampicin- and phenobarbital-treated animals, respectively, but was more effective on aminopyrine *N*-demethylase activity, which showed increases of 163% and 87% in rifampicin- and phenobarbital-induced mice, respectively. Furthermore, CCl₄-dependent MDA formation was moderately elevated in the *in vitro* P-450 enzyme system from rifampicin-induced mice and was significantly enhanced in this system from phenobarbital-induced animals. These experiments indicated that both rifampicin and phenobarbital increased the activity of P-450 2E1 which catalyzed bioactivation of CCl₄ as well as other isoforms of P-450. However, these results failed to explain why rifampicin did not increase but rather reduced liver injury caused by CCl₄. To elucidate the protective mechanism of rifampicin against CCl₄-induced liver damage, the inhibitory effect of rifampicin on lipid peroxidation caused by CCl₄ was investigated in the *in vitro* mixed-function oxidase systems from untreated and inducer-treated mice. The lipid peroxidation generated by CCl₄ was noticeably suppressed in the presence of rifampicin at varying concentrations from 2 to 32×10^{-6} M in the three enzyme systems described above. Meanwhile, another investigation also demonstrated that NADPH oxidation during CCl₄ metabolism was markedly increased in the microsomal enzymes prepared from rifampicin- and phenobarbital-induced mice. However, NADPH oxidation was not inhibited by rifampicin at a concentration of 8×10^{-6} M in these enzyme systems. In addition, rifampicin was also incapable of inhibiting the activity of aniline hydroxylase in the *in vitro* microsomal enzyme systems. These suggested that *in vitro* rifampicin was unable to inhibit the bioactive metabolism of CCl₄ catalyzed by P-450 2E1 and to reduce free radical production.

Therefore, from the results of this study, the protective effect of rifampicin against liver injury induced by CCl₄ was suggested to be due to direct inhibition of lipid peroxidation in liver cells as a result of free radicals generated by metabolism of CCl₄. Although rifampicin accelerated the bioactivation of CCl₄ by induction of P-450 2E1, its direct inhibition of free radical-mediated lipid peroxidation appeared to play the most important role in its effect on the CCl₄ hepatotoxicity, i.e., antagonism of the effects of active metabolites of CCl₄. There have been no similar reports published previously concerning the effects of inducers on CCl₄ hepatotoxicity.

The antioxidant role of vitamin E has been well-reviewed. It has a similar protective effect against CCl₄ hepatotoxicity (28), which is thought to exert its action through scavenging free radicals (29). GSH was a potent antioxidant and could markedly inhibit lipid peroxidation

generated by CCl₄-derived free radicals. As compared with the ability of GSH, the lipid peroxidation inhibitory effect of rifampicin was much more powerful than that of GSH. The IC₅₀ of rifampicin on CCl₄-mediated MDA formation in phenobarbital-induced microsomes of mice was 17 times lower than that of GSH. At present, it is not understood whether the lipid antioxidant activity of rifampicin is acting by direct scavenging of CCl₄-derived peroxy radicals or another mechanism. Our other study also revealed that rifampicin was able to reduce significantly liver injury and animal mortality caused by acetaminophen in mice (R. Huang et al., unpublished data), and studies on its protective mechanism are currently in progress in our laboratory.

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