Effects of Rifampin on the Glutathione Depletion and Cytochrome c Reduction by Acetaminophen Reactive Metabolites in an In Vitro P450 Enzyme System

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ABSTRACT—The present study examined whether rifampin attenuated glutathione (GSH) depletion by acetaminophen reactive metabolites generated in the in vitro P450 enzyme system prepared from mouse liver and the possible mechanism involved in this effect. The results showed that GSH concentration was decreased concentration-dependently by acetaminophen in the in vitro P450 enzyme system. Rifampin significantly attenuated acetaminophen-mediated GSH depletion in a concentration-dependent manner. The concentration-response curve for GSH depletion of acetaminophen was shifted to the right in a parallel fashion in the presence of rifampin at the concentration of \(3.2 \times 10^{-5}\) M, which appeared to result from the competitive binding of rifampin to acetaminophen metabolites. Cytochrome c was markedly reduced by acetaminophen metabolites in this enzyme system, and GSH concentration-dependently increased the cytochrome c reduction by acetaminophen metabolites. These findings suggested that cytochrome c was reduced by the GSH conjugate of acetaminophen metabolites rather than by acetaminophen-derived superoxide anion \((O_2^-)\) and other unbound free radicals. Rifampin was shown to possess an effect similar to that of GSH. It is concluded that the decrease in GSH depletion by rifampin is most likely attributable to the binding of rifampin to the acetaminophen toxic species, and the increase in cytochrome c reduction by rifampin is attributable to the conjugate formed between rifampin and acetaminophen metabolites.

Keywords: Acetaminophen, Rifampin, GSH depletion, Cytochrome c reduction, P450

Accumulating evidence has demonstrated that acetaminophen must be biotransformed by P450 to reactive intermediates in order to initiate liver injury. The P450 isoforms responsible for the production of electrophilic metabolites from acetaminophen are P450 1A1 and 1A2 (1, 2), P450 3A (3, 4) and P450 2E1 (2, 5) that is also involved in the bioactivation of carbon tetrachloride \((\text{CCl}_4)\) (6, 7). Several metabolites of acetaminophen, including N-acetyl-p-benzoquinone imine \((\text{NAPQI})\) and p-amino-phenol, have been identified in both humans and animals, and they may contribute to the toxic effects of acetaminophen (8–10). NAPQI is thought to be a major toxic species responsible for glutathione (GSH) depletion, covalent binding to protein molecules and cytotoxicity (11–14). Although an initial conversion of acetaminophen to reactive metabolites including superoxide anion \((O_2^-)\) is well documented, the subsequent processes leading to cell necrosis are less well understood.

GSH is the most abundant nonprotein thiol in most mammalian cells and present in high concentration as GSH that is maintained at a level of more than 98% of the intracellular GSH by GSH reductase, with minor fractions being desulfide and other cellular thiols (15). The role of GSH in the cellular response to toxic exposures including disruption of Ca\(^{2+}\) homeostasis and loss of cellular thiols has been described. GSH acts both as a nucleophilic scavenger of numerous compounds such as acetaminophen, and their reactive metabolites, and as a substrate in the GSH peroxidase-mediated destruction of hydroperoxides such as hydrogen peroxide (16). A well-known mechanism of acetaminophen-induced liver injury is depletion of hepatic GSH by the toxic metabolites of acetaminophen. In another study, we found that rifampin, as a potent inducer of microsomal drug-metabolizing enzyme, significantly protected against acetaminophen hepatotoxic-
ity, which seems to be associated primarily with decreased GSH depletion and increased GSH regeneration from oxidized glutathione (GSSG) by induction of GSH reductase activity (R. Huang et al., unpublished data). In the present study, an in vitro P450 enzyme system that catalyzed the biotransformation of acetaminophen to the reactive metabolites was developed to investigate the mechanism by which rifampin attenuated the GSH depletion.

As previously reported, cytochrome c can be reduced by $\text{O}_2^-$ generated by the xanthine oxidase-xanthine and horseradish peroxidase-acetaminophen model systems (17–19). Hence, we compared the effects of rifampin, GSH and superoxide dismutase (SOD) on cytochrome c reduction by acetaminophen reactive metabolites generated in the in vitro P450 enzyme system to further understand the mechanism of cytochrome c reduction and their scavenging efficacies for acetaminophen-derived free radicals.

MATERIALS AND METHODS

Chemicals

Rifampin was obtained from Wako Pure Chemical Industries, Ltd., Osaka. Phenobarbital was purchased from Tokyo Kasei Kogyo Co., Tokyo. NADPH, GSH and GSSG were obtained from Koken Co., Ltd., Tokyo. Acetaminophen, cytochrome c (Type III from horse heart, for measurement of microsomal NADPH-cytochrome c reductase activity), cytochrome c (Type VI from horse heart, for measurement of cytochrome c reduction by acetaminophen reactive metabolites), SOD and GSH reductase (Type III from baker's yeast) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatments

The experiments were performed under conditions consistent with accepted guidelines for the humane treatment of experimental animals. Male ICR mice, weighing 24–26 g (Charles River Japan, Yokohama), were used, and the animals were housed in a temperature-controlled room at 22 ± 2°C and fed a standard diet. Rifampin dissolved in 0.02 N HCl was administered orally to mice at a dose of 200 mg/kg for 4 days, and phenobarbital dissolved in distilled water was given i.p. at a dose of 80 mg/kg for 4 days. The animals were anesthetized with ether and sacrificed by exsanguination 16 h after the last doses of drugs.

Preparation of liver microsomes

Liver was removed immediately, perfused with ice-cold 0.15 M KCl and homogenized in 8 vol 0.15 M KCl, pH 7.4, containing 10 mM EDTA using a Potter-type Teflon glass homogenizer. The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was then centrifuged at 105,000 × g for 60 min in a refrigerated ultracentrifuge (70P-I; Hitachi, Tokyo). The final microsomal pellet was washed three times using an equal volume of the homogenization solution, suspended in 20 mM potassium phosphate buffer (PPB), pH 7.4, containing 15% glycerol and stored at −80°C. All procedures were performed at 0–4°C.

Biochemical liver function tests and microsomal drug-metabolizing enzyme assays

The content of liver microsomal protein was determined by the method of Lowry et al. (20). P450 content was measured by the method of Omura and Sato (21), and NADPH-cytochrome c reductase activity was assayed as described by Omura and Takesue (22) using a spectrophotometer (DU-64; Beckman, Palo Alto, CA, USA).

Effects of rifampin and GSH on acetaminophen-metabolizing enzyme activity in vitro

Acetaminophen-metabolizing enzyme activity was measured by the aniline hydroxylation assay (23), with some modifications, in 0.5 ml of a mixture containing 100 mM PPB (pH 7.4); 0.3 mg of liver microsomes prepared from untreated, rifampin-treated and phenobarbital-treated mice; 1 mM NADPH; and 10 mM acetaminophen, with or without 3.2 × 10⁻³ M rifampin and GSH. The reaction was started by the addition of microcosms and stopped by the addition of 0.25 ml of 20% trichloroacetic acid after 60 min of incubation at 37°C, and the mixture was centrifuged at 3,500 rpm for 12 min. A 0.5-ml aliquot of supernatant, 0.25 ml of 10% Na₂CO₃, and 0.5 ml of 0.25 M NaOH containing 2% phenol were mixed, incubated at 37°C for 60 min and measured at 630 nm spectrophotometrically. The enzyme activity was expressed as $\mu$mol acetaminophen metabolite, p-aminophenol, per milligram of microsomal protein per minute.

Effects of rifampin and SOD on the GSH depletion by acetaminophen reactive metabolites generated in an in vitro P450 enzyme system

The incubation was carried out at 37°C with vigorous shaking in 1 ml of medium containing 100 mM PPB (pH 7.4); 0.6 mg of liver microsomes prepared from untreated, rifampin-treated and phenobarbital-treated mice; 0.5 mM NADPH; 9 $\mu$M GSH; and 10⁻¹ M or various concentrations of acetaminophen from 10⁻⁶ to 10⁻² M, with or without rifampin and SOD. The reaction mixture, after 30-min incubation or various time of incubations from 0 to 30 min at 37°C, was transferred to a 25°C water bath shaker for 3 min, and the GSH content was assayed according to the method described previously by James and Harbison (24), with modifications, by adding 20 $\mu$l of 10 mM NADPH, 20 $\mu$l of GSH reductase (85 U/ml) and 20 $\mu$l of 10 mM of 5',5'-dithiobis-(2-nitrobenzoic acid). The reac-
tion was initiated at 25°C by adding 5',5'-dithiobis (2-nitrobenzoic acid) and the change in absorbance at 412 nm was monitored for 30 s using the DU-64 scanning spectrophotometer. GSH concentration was calculated from the value of standard GSH measured by the same method. GSH content in microsomes was determined as described above, and GSH was added to reaction medium of the in vitro P450 enzyme system described above to bring the final GSH concentration to 9 μM.

**Effects of rifampin, GSH and SOD on the cytochrome c reduction by acetaminophen reactive metabolites generated in an in vitro P450 enzyme system**

For the experiments on the effects of rifampin, GSH and SOD on the cytochrome c reduction by the reactive metabolites of acetaminophen in the in vitro P450 enzyme system, a) the incubation was carried out at 37°C for 30 min in 1 ml of medium containing 100 mM PPB (pH 7.4), 0.6 mg of liver microsomes prepared from phenobarbital-treated mice, 0.5 mM NADPH, and 10 mM of various concentrations of acetaminophen from 1 to 10 mM, with or without the additions of rifampin, GSH and SOD. The acetaminophen metabolism was stopped by inactivating P450 enzymes and NADPH-cytochrome c reductase at 60°C with vigorous shaking for 2 min, and the sample was then transferred to a 25°C water bath shaker for 5 min. b) The incubation was performed as described above. Rifampin, GSH and SOD were added to reaction mixture after 30 min of incubation at 37°C followed by the inactivation of microsomal drug-metabolizing enzyme at 60°C, and the medium was incubated for additional 30 min. c) The medium as described above was incubated at 37°C for 80 min, and then the sample was treated with or without the incubation of 60°C for 2 min to determine the effect of this high temperature on the cytochrome c reduction by acetaminophen reactive metabolites. The reaction of cytochrome c reduction was started at 25°C by adding 100 μl of 0.2 mM cytochrome c (Type VI) and measured at 550 nm for 15 or 30 s using the DU-64 scanning spectrophotometer. Meantime, NADPH consumption in acetaminophen metabolism was measured at 340 nm spectrophotometrically under the same incubation conditions.

For the cytochrome c reduction by rifampin, GSH and acetaminophen alone, the reaction mixture consisted of 100 mM PPB, 0.02 mM cytochrome c (Type VI) with various concentrations of rifampin, GSH and acetaminophen in a final volume of 1 ml. The reaction was started at 25°C by adding cytochrome c and measured at 550 nm spectrophotometrically.

**Statistical analyses**

The values were expressed as means ± S.D. or means of triplicate experiments. Data were analyzed by Wilcoxon's test for unpaired variables. Differences were considered statistically significant when P<0.05.

**RESULTS**

**Decrease by rifampin in the GSH depletion by acetaminophen reactive metabolites**

The concentration of GSH was decreased markedly and incubation time-dependently by acetaminophen reactive metabolites generated in the in vitro P450 enzyme system prepared from rifampin-treated animals; the GSH concentration was decreased by 40%, 57% and 65% at 10, 20 and 30 min after exposure to acetaminophen, respectively. Rifampin markedly attenuated the GSH depletion in this enzyme system, the concentration of GSH was reduced only by 14%, 25% and 29% at 10, 20 and 30 min after incubation, respectively, in the presence of 3.2 × 10⁻⁵ M rifampin (Fig. 1A). The GSH content was depleted concentration-dependently by acetaminophen metabolites in the P450 enzyme system described above. The decrease in GSH concentration was reduced by rifampin; the concentration-response curve for the GSH depletion of acetaminophen was shifted to the right in a parallel fashion in the presence of 3.2 × 10⁻⁵ M rifampin (Fig. 1B). The GSH content was depleted more rapidly in the P450 enzyme systems prepared from rifampin- and phenobarbital-treated animals than in the system prepared from untreated mice. The decrease by acetaminophen reactive metabolites of GSH content in these P450 enzyme systems was attenuated by rifampin in a concentration-dependent manner; the GSH concentration was significantly higher in the presence of rifampin at the concentration range of 3.2 × 10⁻⁶ to 10⁻⁴ M (Fig. 1C).

SOD was able to attenuate the GSH depletion by acetaminophen reactive metabolites at the concentration of 100 U/ml, but not at the concentration of 30 U/ml in the phenobarbital-induced enzyme system. When the effects of rifampin and SOD were compared, the efficacy of the former at a concentration of 3.2 × 10⁻⁵ M was much more powerful than that of the latter at a concentration of 100 U/ml. The GSH concentration was increased by 14% and 110% in the presence of SOD and rifampin, respectively, when compared with the control value, which was taken as 100% in the presence of acetaminophen alone (Fig. 2).

**Increase by rifampin in the cytochrome c reduction by acetaminophen reactive metabolites**

Cytochrome c was reduced concentration-dependently by acetaminophen reactive metabolites generated in the in vitro P450 enzyme system prepared from phenobarbital-treated animals (Fig. 3A) and in the system prepared from untreated and rifampin-treated mice (data not shown). Both rifampin and GSH markedly increased the cyto-
chrome c reduction in the presence of various concentrations of acetaminophen (Fig. 3A) and concentration-dependently increased this reduction rate at the concentration ranges of $10^{-5}$ to $10^{-4}$ M and $6 \times 10^{-6}$ to $3.2 \times 10^{-5}$ M, respectively (Fig. 3B). On the other hand, rifampin alone was capable of reducing cytochrome c in a concentration-dependent manner at the high concentration range of $10^{-5}$ to $3.2 \times 10^{-3}$ M. However, neither GSH nor acetaminophen alone could reduce cytochrome c (Fig. 4).

SOD did not obviously influence the cytochrome c reduction by acetaminophen metabolites. During acetaminophen metabolism, the additions of rifampin and GSH simultaneously to the reaction mixture significantly increased the cytochrome c reduction by 215% of the control value which was expressed as 100% in the presence of 10 mM acetaminophen without the additions of rifampin, GSH and SOD in vitro. The increased value of cytochrome c reduction by cotreatment with rifampin and GSH was

Fig. 1. Effect of rifampin (RFP) on the decrease in glutathione (GSH) concentration by the reactive metabolites of acetaminophen (APAP) in the in vitro P450 enzyme system. A: Incubation was carried out at 37°C for various times from 0 to 30 min in 1 ml of medium containing 100 mM PEP (pH 7.4), 0.6 mg of liver microsomes prepared from rifampin-treated mice, 0.5 mM NADPH, 9 μM GSH and 1 mM acetaminophen, without or with $3.2 \times 10^{-3}$ M rifampin. Each point represents the mean of triplicate assays. APAP + RFP (■), APAP (●). B: Incubation was performed as above at 37°C for 30 min in the presence of various acetaminophen concentrations from $10^{-6}$ to $10^{-2}$ M. Each point represents the mean of triplicate assays. APAP + RFP (■), APAP (●). C: Incubation was performed as above at 37°C for 30 min in the absence or in the presence of various rifampin concentrations from $10^{-4}$ to $10^{-1}$ M. Untreated microsomes (■), RFP-treated (●), phenobarbital-induced (▲). Values represent the means ± S.D., n = 4 animals. *P<0.05, significantly different from the corresponding control values with acetaminophen alone; ^P<0.05, significantly different from control value with acetaminophen alone in untreated microsomes.
Fig. 2. Comparative effects of superoxide dismutase (SOD) and rifampin (RFP) on the decrease in GSH concentration by the reactive metabolites of acetaminophen (APAP) in the in vitro P450 enzyme system. Incubation was carried out at 37°C for 30 min in 1 ml of medium containing 100 mM PPH (pH 7.4), 0.6 mg of liver microsomes prepared from phenobarbital-treated mice, 0.5 mM NADPH, 9 μM GSH and 1 mM acetaminophen with or without SOD and rifampin. Values represent the means ± S.D. of quadruplicate experiments. *P<0.05, significantly different from control value in the presence of acetaminophen alone.

Fig. 3. Effects of rifampin (RFP) and glutathione (GSH) on the cytochrome c reduction by the reactive metabolites of acetaminophen (APAP) in the in vitro P450 enzyme system. A: Incubation was carried out at 37°C for 30 min in 1 ml of medium containing 100 mM PPH (pH 7.4), 0.6 mg of liver microsomes prepared from phenobarbital-treated mice, 0.5 mM NADPH and various acetaminophen concentrations from 0.5 to 10 mM, with 3.2 × 10⁻⁶ M rifampin and GSH or without the additions of rifampin and GSH (with 6 × 10⁻⁶ M GSH contained in microsomes). B: Incubation was performed as above in the presence of various concentrations of rifampin and GSH. Each point represents the mean of triplicate experiments. APAP+GSH (■), APAP+RFP (●), APAP (▲).
Rifampin and Acetaminophen GSH Depletion

![Graph showing the reduction of cytochrome c by rifampin (RFP), GSH, and acetaminophen (APAP). The reaction mixture consisted of 100 mM PPB (pH 7.4), 0.02 mM cytochrome c and various concentrations of rifampin, GSH, and acetaminophen in a final volume of 1 mL. The reaction was started by the addition of cytochrome c at 25°C. Each point represents the mean of triplicate assays. RFP (●), GSH (■), PAPA (▲).]

**Effects of rifampin and GSH on the activity of microsomal acetaminophen-metabolizing enzyme in vitro**

As shown in Table 1, acetaminophen-metabolizing enzyme activity was increased by 15% and 14% in rifampin- and phenobarbital-induced microsomes, respectively. In vitro, this enzyme activity in untreated, rifampin-induced and phenobarbital-induced microsomes was not affected in the presence of 3.2 × 10^{-5} M rifampin and GSH.

**Inactivation of P450 and NADPH-cytochrome c reductase by heat treatment and NADPH oxidation during acetaminophen metabolism**

Microsomal P450 content and NADPH-cytochrome c reductase activity in phenobarbital-treated mice were 1.47 ± 0.08 nmol/mg microsomal protein and 136.8 ± 9.1 nmol/mg microsomal protein/min (n = 4), respectively, and they were completely inactivated after 2 min of incubation at 60°C. NADPH, as a necessary cofactor of cytochrome c reductase activity, has fully disappeared after 80 min of incubation at 37°C in the presence of acetaminophen in the in vitro P450 enzyme system. Therefore, the cytochrome c reduction by acetaminophen metabolites was not influenced by microsomal NADPH-cytochrome c reductase under the conditions of these experiments.

**DISCUSSION**

To elucidate the molecular mechanism of protection by rifampin against acetaminophen hepatotoxicity, we have established an in vitro P450 enzyme system model, in which the reactive metabolites of acetaminophen were effectively generated and the marked acetaminophen concentration- and incubation time-dependent depletion of GSH content was observed after challenge of acetaminophen. A proposed mechanism suggests that P450 initiates a one-electron oxidation of acetaminophen to produce phenoxyl free radicals, in turn, generating O_2^{-}, hydrogen peroxide and reactive electrophilic metabolites, NAPQI, that are causally related to cell injury mediated by acetaminophen (19, 25 – 27). Much of the damage done by O_2^{-} and hydrogen peroxide in vivo is thought to be due to their conversion into more reactive species, including hydroxyl radical (OH·) (28, 29). The free radical scavengers, GSH and promethazine, afford protection against acetaminophen-induced liver damage (30 – 33).

It was reported that SOD prevented the in vivo metabolism-dependent cytotoxicity of acetaminophen by scavenging O_2^{-} and hydrogen peroxide derived from acetaminophen (34). The present experiment found that SOD was also able to reduce the GSH depletion caused by acetaminophen in the in vitro P450 enzyme system, but its effect was much less marked compared with that of rifampin, suggesting that superoxides such as O_2^{-} and hydrogen peroxide, generated from acetaminophen, could not play a major role in the GSH depletion. On the other hand, NAPQI formed as the P450 oxidation product of acetaminophen (35) likely was a major reactive metabolite responsible for the GSH depletion, which arylated and oxidized the GSH thiol.

Rifampin was capable of significantly and concentration-dependently attenuating the GSH depletion by the reactive metabolites of acetaminophen generated in the in vitro P450 enzyme system. Because rifampin did not inhibit the biotransformation of acetaminophen, as shown by the observation that p-aminophenol, a acetaminophen metabolite, production was not suppressed in the presence of rifampin in the in vitro P450 enzyme system, the effect of rifampin on the GSH depletion was suggested to be a direct antagonistic action of toxic metabolites rather than an inhibition of its formation, presumably a competitive binding of rifampin to the reactive metabolites of acetaminophen such as NAPQI, leading to the decreased GSH depletion as a result of competitive conjugation. Our previous study on the protective effect of rifampin against CCl_4 hepatotoxicity showed a possible mechanism that rifampin directly inhibited the lipid peroxidation mediated by CCl_4-derived free radicals without influencing CCl_4 bioactivation by P450 2E1 (36). Both findings indicated that rifampin is act...
Fig. 5. Comparative effects of SOD, rifampin (RFP) and GSH on the cytochrome c reduction by the reactive metabolites of acetylsalicylic acid (APAP) in the in vitro P450 enzyme system. A: The incubation was carried out at 37°C for 30 min in 1 ml of medium containing 100 mM PPB (pH 7.4), 0.6 mg of liver microsomes prepared from phenobarbital-treated mice, 0.5 mM NADPH and 10 mM acetylsalicylic acid with SOD (30 U/ml and 100 U/ml), rifampin (3.2 x 10⁻⁵ M) or glutathione (3.2 x 10⁻⁵ M). The acetylsalicylic acid metabolism was stopped by the inactivation of P450 enzymes and cytochrome c reductase at 60°C for 2 min. B: The incubation was performed as above without the addition of rifampin and GSH during acetylsalicylic acid metabolism, and both the drugs were added to the medium and incubated at 37°C for an additional 30 min after the inactivation of microsomal enzymes. C: The medium described above was incubated at 37°C for 80 min followed by the treatment without (I) or with (II) 2 min of incubation at 60°C. Values represent the means ± S.D. of quadruplicate experiments. *P<0.05, significantly different from corresponding control values in the presence of acetylsalicylic acid alone (with 6 x 10⁻⁶ M GSH contained in microsomes); †P<0.01, significantly different from control value in the presence of acetylsalicylic acid alone without heat treatment of 60°C.

Table 1. Effects of rifampin and GSH on the activity of acetylsalicylic acid-metabolizing enzyme in the in vitro P450 enzyme system

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity of acetylsalicylic acid-metabolizing enzyme (μmol/mg microsomal protein per minute)</th>
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<tbody>
<tr>
<td></td>
<td>Without treatment</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.921 ± 0.155</td>
</tr>
<tr>
<td>Rifampin</td>
<td>1.059 ± 0.106</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1.047 ± 0.031</td>
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</table>

Microsomes were prepared from untreated, rifampin-treated and phenobarbital-treated animals. The concentrations of rifampin and GSH were 3.2 x 10⁻⁵ M. Values represent the means ± S.D., n = 4 - 8 animals. *Not significantly different from the corresponding control values without the additions of rifampin and GSH in vitro.
ing as a scavenger of the toxic metabolites generated from these compounds in the microsomal monoxygenase system.

Rosen et al. (19) demonstrated that cytochrome c was reduced by acetaminophen free radicals generated in the horseradish peroxidase-acetaminophen model system. The reduction reaction was inhibited completely through removing O$_2^-\cdot$ that was converted by SOD-catalyzed dismutation into hydrogen peroxide. The present experiment demonstrated that cytochrome c could be reduced in an acetaminophen concentration-dependent manner by the reactive metabolites of acetaminophen generated in the in vitro P450 enzyme system. However, SOD was not shown to markedly affect the cytochrome c reduction in the in vitro P450 enzyme system, which suggested that acetaminophen-generated O$_2^-\cdot$ did not play an important role in the reduction reaction, but the other bioactive metabolites of acetaminophen such as NAPQI, likely played a key role in this process.

At the same time, the present experiment found the rate of cytochrome c reduction by acetaminophen was not decreased but increased significantly and GSH concentration-dependently by adding GSH to the incubation medium. It is well known that GSH can rapidly and effectively bind to and scavenge the reactive metabolites of acetaminophen; therefore, it is tempting to speculate that the GSH conjugates of acetaminophen metabolites was increased and the unbound reactive metabolites such as phenoxyl free radical and NAPQI were decreased with the elevation of GSH concentrations in the reaction mixture. This suggested that cytochrome c was reduced primarily by GSH conjugates of acetaminophen metabolites, and the cytochrome c reduction without addition of GSH in vitro also resulted from the GSH conjugates formed in the medium, because liver microsomes contained some GSH. However, we still do not know whether cytochrome c was reduced directly by the GSH conjugate or by the product derived from the GSH conjugate.

It was interesting that rifampin showed an effect similar to that of GSH, and the rate of cytochrome c reduction was also increased with the increase in rifampin concentrations. Although the molecule of rifampin, a complex macrolide antibiotics derived from rifamycin B (37), is structurally different from that of GSH, a tripeptide, it was possible that the active site of rifampin is similar to that of GSH, which could bind to the reactive metabolites of acetaminophen, and subsequently, rifampin conjugates of acetaminophen metabolites or its derived products reduced cytochrome c. As the velocity of cytochrome c reduction by rifampin plus GSH was an approximate total value of the respective velocities of rifampin and GSH, it would be expected that the rifampin conjugate and the GSH conjugate possess a similar efficacy for the cytochrome c-binding site. When GSH is added to the medium after inactivation of microsomal enzymes, the efficacy of GSH on cytochrome c reduction was significantly lower compared with that observed when it is added during the biotransformation of acetaminophen; this was probably due to the decreased GSH conjugate of acetaminophen metabolites. In contrast, the same capacity of rifampin on cytochrome c reduction was obtained in both experiments mentioned above, suggesting that the amount of rifampin conjugate of acetaminophen metabolites was the same in both mediums.

As compared between the two experimental results obtained in this study, the rate of cytochrome c reduction by acetaminophen metabolites after incubation followed by inactivation of NADPH-cytochrome c reductase with heat treatment was higher than that after the same incubation without heat treatment, indicating that to a certain extent, the increase in cytochrome c reduction rate was due to the rapid increased metabolism of acetaminophen before inactivation of microsomal drug-metabolizing enzyme and the enhanced interaction between acetaminophen metabolites and GSH or rifampin at the high temperature. The mechanism by which rifampin and GSH reduce cytochrome c is not known at present, and more investigations will be required to identify and characterize rifampin and GSH conjugates and to further confirm the molecular mechanism that cytochrome c reduction is increased by rifampin and GSH in the in vitro P450 enzyme system in which acetaminophen reactive metabolites are effectively generated.

In conclusion, the present study showed that rifampin significantly attenuated the GSH depletion by acetaminophen reactive metabolites generated in the in vitro P450 enzyme system, which is thought to result from the competitive binding of rifampin to acetaminophen metabolites and to be involved partly or primarily in hepatoprotection by rifampin (R. Huang et al., unpublished data). It was also found that the cytochrome c reduction was increased by both rifampin and GSH in this enzyme system, presumably by their conjugates of acetaminophen reactive metabolites or the products derived from these conjugates.

REFERENCES


