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Effects and mechanisms of rifampin on hepatotoxicity of acetaminophen in mice

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ABSTRACT

This study examined the effects and possible mechanisms of rifampin against acetaminophen-induced hepatotoxicity in mice. Rifampin significantly enhanced the biotransformation of acetaminophen, evidenced by the increase in *p*-aminophenol formation in rifampin-treated microsomes and the increase in plasma clearance rate of acetaminophen. Pretreatment with rifampin significantly decreased serum alanine transaminase (ALT) activities, aspartate transaminase (AST) activities and prevented severe liver necrosis following acetaminophen overdose. The contents and activities of microsomal drug-metabolizing enzyme were less affected in rifampin-pretreated mice in comparison to the animals treated with acetaminophen alone. Rifampin was capable of increasing glutathione (GSH) level and GSH reductase activity and reducing GSH depletion and the decrease in GSH reductase activity by acetaminophen in mice. In addition, it was found that the microsomal Ca^{2+}-ATPase activity was not directly related to acetaminophen toxic species generated in the P450 enzyme system *in vitro*. These findings suggest that rifampin has species-specific effects on the liver against acetaminophen-induced hepatotoxicity in mice, which increase the level of GSH by promoting GSH regeneration.

1. Introduction

Acetaminophen, an *N*-acetylated derivative of *p*-aminophenol, is one of the most widely used antipyretic analgesics in the world because of its efficacy and safety when given in therapeutic doses. In contrast to aspirin and nonsteroidal anti-inflammatory agents, it does not cause gastrointestinal bleeding. However, acetaminophen is a metabolism-dependent hepatotoxicant, which could cause hepatotoxic and nephrotoxic to man and animals in overdoses. Cytochrome P450 (CYP) 2E1 and CYP 3A4 (Manyike et al., 2000; Cheng et al., 2009) are involved in the formation of *N*-acetyl-*p*-benzoquinone imine (NAPQI), the toxic intermediate of acetaminophen, which causes the depletion of glutathione (GSH) and subsequent covalent binding (Jollow et al., 1973), oxidative stress and lipid peroxidation (Fairhurst et al., 1982), and inhibition of plasma membrane Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase) activity that disrupts Ca^{2+} homeostasis (Moore et al., 1985). In an effort to reduce the cytotoxicity of acetaminophen, *N*-acetylcysteine has been used as an antidote primarily by elevating intracellular GSH concentrations (Cacciatore et al., 2010). It has been shown that acetaminophen-mediated hepatotoxicity can be reduced by treatment with zinc sulfate, presumably through the replenishment of hepatic GSH levels (Woo et al., 1995), and by treatment with lobenzarit likely by its antioxidant effect and/or its ability to stimulate hepatic GSH reductase (Armesto et al., 1993).

Rifampin, an antituberculosis drug, is usually administered with other antituberculosis agents and/or medication from other classes. Studies have shown that rifampin significantly relieves pruritus in patients with primary biliary cirrhosis (Ghent and Carruthers, 1988). The potential for drug interaction between rifampin and numerous other drugs such as opioids, fluconazole, cyclosporine, glucocorticoids, and oral anticoagulant, often exists due to the induction of microsomal drug-metabolizing enzymes by rifampin (Borcherding et al., 1992). There are evidences that rifampin elevates the levels of P450 and cytochrome b_5 and the activities of cytochrome *c* reductase and substrate-metabolizing enzymes both in liver and small intestine (Huang et al., 1996). Some patients experienced hepatotoxic reactions after using acetaminophen while underwent treatment for active tuberculosis with rifampin, isoniazid, and other agents (Nolan et al., 1994). Human pregnane X receptor (PXR) is involved in regulation of acetaminophen-induced toxicity through CYP3A4 mediated hepatic metabolism of acetaminophen in the presence of PXR ligands, thus, some PXR activators, such as rifampin may induce acetaminophen's toxicity (Cheng et al., 2009). But, species differences existed in human PXR and rodent PXR, different pharmacological or toxic effects may be embodied by using different animal models, even

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strain-specific responses to toxicant exposure have been observed in cultured genetically distinct mouse hepatocytes (Martinez et al., 2010).

In order to clarify more possible mechanisms of acetaminophen hepatotoxicity, and interaction effects with rifampin in mice, a species-specific cytochrome P450 (CYP) inducer, we examined the effects of rifampin on acetaminophen-induced hepatotoxicity using mouse model and P450 enzyme system prepared by mice *in vitro*, and possible mechanism involved would be clarified in detail.

2. Materials and methods

2.1. Chemicals

Rifampin and serum alanine transaminase (ALT) and aspartate transaminase (AST) kits were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Phenobarbital was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Carbon tetrachloride (CCl_4), *p*-aminophenol, 3-(*N*-mopholino) propanesulfonic acid, ethyleneglycol-bis-β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), leupeptin hemisulfate monohydrate, dithiothreitol, phenylmethylsulfonyl fluoride, and 5,5′-dithio-bis-(2-nitrobenzoic acid) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). NADPH, NADH, GSSG, GSH and adenosine-5′-triphosphate Tris salt (5′-ATP-Tris) were obtained from Kohjin Co., Ltd. (Tokyo, Japan). Acetaminophen, cytochrome *c* (Type III, horse heart) and GSH reductase (Type III, bakers yeast) were the products of Sigma–Aldrich Chemical Co. (St, Louis, MO, USA).

2.2. Animals and treatments

Male ICR mice, weighing 24–26 g (Charles River Japan, Yokohama, Japan) were used. The animals were housed in a temperature-controlled room at 22 ± 2 °C and fed a standard diet. Rifampin was dissolved in 0.02 N HCl and administered orally to mice at doses of 50, 100, 200 mg/kg for 4 days. Phenobarbital dissolved in distilled water was used, i.p., at a dose of 80 mg/kg for 4 days, which used in 2.8. Acetaminophen, dissolved in dimethyl sulfoxide, was used, i.p. given by a bolus injection with 700 mg/kg on the 5th day. The last dose of rifampin and acetaminophen was given concurrently in the co-treatment groups. In order to measure the biochemical and metabolic functions of the liver, the animals were anesthetized with ether and the livers were isolated at 1, 3, 8, 16 h following acetaminophen administration. For mortality analysis, the animals were observed up to 7 days after administration of acetaminophen (700 mg/kg, i.p.) with or without daily administration of rifampin (50, 100, or 200 mg/kg, p.o.). Equivalent amount of dimethyl sulfoxide were given to four groups (acetaminophen alone-treated group, rifampin and acetaminophen co-treatment groups) according to mice weight. For monitoring the blood levels of drug and metabolite, the blood samples were collected and diluted to one-third with 100 mM potassium phosphate buffer (PPB), pH 7.4, at 20-min intervals within 100 min after administration of acetaminophen. The experiments were performed according to the guideline of "how to use laboratory animal".

2.3. Preparation of liver microsomes

For the determination of the content and activity of microsomal drug-metabolizing enzyme, the livers were immediately removed, perfused with an ice-cold 0.15 M KCl, and homogenized in 8 volumes of 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 collected and then centrifuged at 105,000*g* for 60 min using Hitachi 70P-I ultracentrifuge, (Hitachi, Japan). The pellet was washed three times using an equal volume of the homogenization solution and then suspended in 20 mM PPB, pH 7.4, containing 15% glycerol, and stored at −80 °C. The resulting supernatant was also collected and kept at −80 °C for measurement of total GSH content and GSH reductase activity.

The preparation of liver microsomes (endoplasmic reticulum vesicles) for the determinations of Ca^{2+}-ATPase and Mg^{2+}-ATPase activities was carried out as described above in 8 volumes of ice-cold 0.25 M sucrose containing 1 μM leupeptin hemisulfate monohydrate, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (Lytton et al., 1991; Rossi et al., 1979). The final pellet was washed, suspended in 0.25 M sucrose containing 1 mM dithiothreitol, and stored at −80 °C. All procedures were performed at 0–4 °C.

2.4. Biochemical liver function tests and microsomal drug-metabolizing enzyme assays

The activities of serum ALT and AST were measured by the UV rate method (Pitot and Pries, 1964) and expressed as international units per liter. The contents of total serum proteins and liver microsomal protein (MS Pr.) were determined by the method developed by Lowry et al. (1951). The P450 content was measured by the method developed by Omura and Sato (1964), and cytochrome b_5 content and NADPH-cytochrome *c* reductase activity were assayed using the reported method (Omura and Takesue, 1970) using Beckman DU-64 UV–vis spectrophotometer (Beckman Instruments, USA).

Substrate-metabolizing enzyme activities, aniline hydroxylation and aminopyrine *N*-demethylation were assayed as described in the literature (Imai et al., 1966; Nash, 1953). 7-Ethoxycoumarin *O*-deethylase activity was measured by the method developed by Ullrich and Weber (1972) with Hitachi 650-60 spectrofluorometer (Hitachi, Japan). Each substrate-metabolizing enzyme activity was assayed using NADPH as the sole electron source.

2.5. Measurement of total GSH content and GSH reductase activity in liver cytosolic fraction

The content of GSH was measured as described previously by James and Harbison (1982) with modification, 1 ml of medium containing 100 mM PPB, pH 7.4, 5 mM EDTA, 0.2 mM NADPH, 1.7 U of GSH reductase, 0.2 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), and 20 μl of sample. The reaction was started at 25 °C by adding 5,5′-dithio-bis-(2-nitrobenzoic acid) and the change in absorbance at 412 nm was monitored for 30 s using Beckman DU-64 UV–vis spectrophotometer (Beckman Instruments, USA). GSH reductase activity was assayed according to the method described by Abraham et al., 1978) with modification, 1 ml of mixture was consisted of 100 mM PPB, pH 7.4, 5 mM EDTA, 0.2 mM NADPH, 1.1 mM GSSG, 0.2 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), and 50 μl of sample. The reaction was initiated at 25 °C by adding NADPH 1 min after the addition of 5,5′-dithio-bis-(2-nitrobenzoic acid) and monitored as described above.

2.6. Measurement of the concentrations of p-aminophenol and acetaminophen in blood

The concentration of acetaminophen metabolite, *p*-aminophenol, was measured using aniline hydroxylation assay (Imai et al., 1966) within 3 h of blood sample collection. Blood samples were diluted and centrifuged at 5000 rpm for 10 min using high-speed MRX-150 microcentrifuge (Tomy Disital Biology Co., Japan). A 150 μl of diluted plasma and 50 μl of 100 mM PPB, pH 7.4, were mixed with 60 μl of 30% trichloroacetic acid and centrifuged at 5000 rpm for 10 min. Following the centrifugation, 200 μl of supernatant was combined with 100 μl of 10% Na_2CO_3, and 200 μl of 2% phenol in 0.2 M NaOH and incubated at 37 °C for 60 min and measured at 630 nm using a spectrophotometer. The concentration of acetaminophen was assayed in 200 μl of medium containing 150 μl of diluted plasma, 10 μl of 100 mM PPB, pH 7.4, and 20 μl of 10 mM NADPH, and 20 μl of liver microsomes (final protein concentration 0.6 mg/ml). The liver microsomes were prepared in 0.15 M KCl with 10 mM EDTA from phenobarbital-treated mice. The reaction was initiated by adding microsomes and terminated by adding 60 μl of 30% trichloroacetic acid after 60 min of incubation at 37 °C. The concentration of acetaminophen was calculated from standard curve plotted from the concentrations of *p*-aminophenol produced from *in vitro* metabolism of known acetaminophen, measured by the same method.

2.7. Measurement of Ca^{2+} and Mg^{2+}-ATPase activities in liver

The activities of Ca^{2+} and Mg^{2+}-ATPase in liver were measured according to spectrophotometric determination of inorganic phosphate liberated as described previously (Inagaki et al., 1985; Shiroya et al., 1989) and the method developed by Lytton et al. (1991) with modification. The assay of Ca^{2+}-ATPase activity was carried out in 0.4 ml of medium containing 25 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0, 100 mM KCl, 0.2 mM $CaCl_2$, 0.4 mM EGTA, 2.5 mM ATP (Tris salt) and 0.24 mg liver microsomes (final concentration, 0.6 mg/ml) prepared in 0.25 M sucrose at 37 °C for 20 min. The assay of Mg^{2+}-ATPase was performed by using 2 mM $MgCl_2$ and 1 mM EDTA instead of $CaCl_2$. The values of Ca^{2+} and Mg^{2+}-ATPase activities of blank run in the presence of 1 mM EDTA without $CaCl_2$ or $MgCl_2$ were subtracted.

The effects of acetaminophen and rifampin on Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were determined as described above in the presence of 10 mM acetaminophen, 0.5 mM NADPH, and 9 μM GSH with or without 3.2×10^{-5} M rifampin using *in vitro* P450 enzyme system prepared in 0.25 M sucrose from untreated animals. ATP (Tris salt) was added to the medium after 20, 40 and 60 min of incubation at 37 °C and incubated for additional 20 min. The changes in GSH content in the enzyme system were also measured as described above.

2.8. Measurement of acetaminophen-metabolizing enzyme activity

The activity of acetaminophen-metabolizing enzyme was assayed in 0.5 ml of mixture containing 100 mM PPB, pH 7.4, 0.3 mg of liver microsomes isolated in 0.15 M KCl/10 mM EDTA from the rifampin- and phenobarbital-treated (1 mM NADPH, and 10 mM acetaminophen) and untreated animals. The reaction was started by the addition of microsomes and stopped by the addition of 0.25 ml of 20% trichloroacetic acid after 60 min of incubation at 37 °C. The mixture was centrifuged at 3500 rpm for 12 min. A 0.5 ml of supernatant was added to 0.25 ml of 10% Na_2CO_3, and 0.5 ml of 2% phenol in 0.25 M NaOH in an assay tube and incubated at 37 °C for 60 min and then the absorbance at 630 nm was measured spectrophotometrically.