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Alteration in Metabolism and Toxicity of Acetaminophen Upon Repeated Administration in Rats

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Abstract. Our previous studies showed that administration of a subtoxic dose of acetaminophen (APAP) to female rats increased generation of carbon monoxide from dichloromethane, a metabolic reaction catalyzed mainly by cytochrome P450 (CYP) 2E1. In this study we examined the changes in metabolism and toxicity of APAP upon repeated administration. An intraperitoneal dose of APAP (500 mg/kg) alone did not increase aspartate aminotransferase, alanine aminotransferase, or sorbitol dehydrogenase activity in serum, but was significantly hepatotoxic when the rats had been pretreated with an identical dose of APAP 18 h earlier. The concentrations and disappearance of APAP and its metabolites in plasma were monitored for 8 h after the treatment. APAP pretreatment reduced the elevation of APAP-sulfate, but increased APAP-cysteine concentrations in plasma. APAP or APAP-glucuronide concentrations were not altered. Administration of a single dose of APAP 18 h before sacrifice increased microsomal CYP activities measured with *p*-nitrophenol, *p*-nitroanisole, and aminopyrine as probes. Expression of CYP2E1, CYP3A, and CYP1A proteins in the liver was also elevated significantly. The results suggest that administration of APAP at a subtoxic dose may result in an induction of hepatic CYP enzymes, thereby altering metabolism and toxicological consequences of various chemical substances that are substrates for the same enzyme system.

Keywords: acetaminophen, metabolic activation, cytochrome P450 (CYP), reactive metabolite, hepatotoxicity

Introduction

The metabolism and toxicity of acetaminophen (APAP), a widely used analgesic-antipyretic, have been the subjects of numerous studies. APAP is a safe drug at therapeutic levels, but upon large or chronic doses, severe liver and kidney injury may result in experimental animals and humans. Development of liver injury by APAP is attributed to formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinonimine (NAPQI) (1), through oxidative metabolism mediated by cytochrome P450 (CYP) enzymes. The reactive metabolite generated is normally detoxified via conjugation with glutathione (GSH). When the generation of NAPQI exceeds the availability of GSH for the conjugation reaction, covalent binding of this reactive metabolite to cellular macromolecules may result, an event that correlates with hepatic necrosis.

Since the induction of APAP-hepatotoxicity is associated with the formation of a reactive metabolite beyond the detoxifying capacity of the liver, most studies have focused on the toxic consequences resulting from an excessively large dose of APAP. On the other hand, its potential adverse effects at a lower or therapeutic dose have hardly been explored. Recently we observed that APAP, at a dose incapable of inducing hepatic injury, enhanced the metabolic conversion of dichloromethane to carbon monoxide (2), a reaction mediated mostly by CYP2E1 (3, 4). This implies that a low dose of APAP may paradoxically increase the biotransformation and toxicity of a xenobiotic that is metabolically converted to a toxic metabolite by the

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same CYP enzymes. Considering the wide use of APAP in humans, a better understanding of its biological activities at a lower dose seems to be relevant. In this study we investigated the effects of pretreating rats with a subtoxic dose of APAP on the metabolic fate and toxicity of a repeating dose of this drug.

Materials and Methods

Animals and treatments

Female Sprague-Dawley rats, 8 – 10-week-old, were purchased from Dae-Han Laboratory Animal (Seoul, Korea). Rats were acclimated in temperature (22 ± 2°C)– and humidity (55 ± 5%)–controlled rooms with a 12-h light-dark cycle for 1 week before use. The use of rats was in compliance with the guidelines established by the Animal Care Committee of this institute. Regular laboratory rat diet and tap water were allowed ad libitum. Rats, weighing 200 – 220 g, were treated intraperitoneally with APAP (500 mg/kg) dissolved in 10% Tween 80. The volume of injection was 15 ml/kg body weight. At 18 h after the initial treatment, the rats were challenged with a following dose of APAP (500 or 1000 mg/kg, i.p.). Control animals were treated with an identical volume of the vehicle.

For sequential sampling of blood in the experiments determining concentrations of APAP and the metabolites in plasma, a jugular vein was catheterized with silicon tubing (Dow Corning, Midland, MI, USA) under ether anesthesia. The cannula was exteriorized to the dorsal side of neck and secured with a mounting post, which allowed attachment of an extensor for blood sampling. Following surgery, the rats were housed individually in plastic metabolic cages (Myungjin, Seoul, Korea) and allowed to recover for 48 h. Every 10 to 15 h until the onset of experiment, the cannula was flushed with a heparin solution (20 units/ml physiological saline) to prevent blood clotting.

Assessment of hepatotoxicity

Hepatotoxicity of APAP was estimated by elevation of serum enzyme activities. At 24 h after APAP treatment, blood was sampled by cardiac puncture from a rat under ether anesthesia. Serum sorbitol dehydrogenase (SDH) activity was determined spectrophotometrically (5). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using the method of Reitman and Frankel (6).

Measurement of APAP metabolites in plasma

Blood samples were obtained from the catheter inserted into the jugular vein at predetermined time points after treatment with an intraperitoneal dose of APAP. Plasma concentrations of APAP and its metabolites were measured using an HPLC method described elsewhere (7). Briefly, plasma was mixed with an aliquot of acetonitrile containing theophylline as an internal standard. After extraction and centrifugation, the resulting supernatant was evaporated to dryness under nitrogen. The residue was diluted with distilled water as necessary before being injected into the HPLC apparatus. APAP and its major metabolites, APAP-GSH, APAP-cysteine, APAP-mercapturate, APAP-glucuronide, and APAP-sulfate, were separated in a reverse phase C_{18} μBondapack column (30 cm × 3.9 mm; Waters Associates, Milford, MA, USA). The APAP metabolites were kind gifts from Dr. José E. Manautou (University of Connecticut, Storrs, CT, USA). APAP and the metabolites, eluted with 1.8% aqueous acetic acid – methanol – H_2O (66:9:100) at a flow rate of 1.5 ml/min, were monitored at 254 nm.

Measurement of microsomal CYP enzyme activities and contents

Rats were euthanized 18 h after treatment with a single dose of APAP. Whole liver was homogenized in an ice-cold buffer consisting of 0.154 M KCl / 50 mM Tris-HCl, pH 7.4, with 1 mM EDTA. The homogenate was centrifuged at 10,000 × *g* for 20 min, and the supernatant was further centrifuged at 104,000 × *g* for 60 min. The microsomal pellet suspended in the homogenizing buffer was recentrifuged at 104,000 × *g* for 60 min. The total CYP was quantified from the CO difference spectrum (8). *p*-Nitrophenol hydroxylase activity was determined by measuring the formation of *p*-nitrocatechol (9). The production of formaldehyde was quantified for measurement of aminopyrine *N*-demethylase activity (10). *p*-Nitroanisole *O*-demethylase was determined using the method of Shigematsu et al. (11).

The microsomal proteins, separated by SDS-polyacrylamide gel electrophoresis, were transferred to nitrocellulose membranes by electroblotting. The membranes were blocked in 5% nonfat dry milk in PBS-T (0.05% Tween 20 in PBS). The blots were incubated overnight with antibodies diluted in 5% bovine serum albumin in PBS-T at 4°C followed by incubation with secondary antibodies conjugated to horseradish peroxidase (diluted in 5% milk powder in PBS-T) for 3 h at room temperature. Rabbit polyclonal antibodies against human CYP2E1 and 3A4 and mouse monoclonal anti-rat 1A1/2 antibodies (Detroit R&D, Detroit, MI, USA) were used as probes. Proteins were detected by enhanced chemiluminescence.

Data analysis

All results, expressed as the mean ± S.E.M., were analyzed by a two-tailed Student's *t*-test. The acceptable level of significance was established at $P<0.05$, except when otherwise indicated.

Results

APAP-induced liver toxicity

Serum AST, ALT, and SDH activities were measured to estimate the APAP-induced hepatotoxicity. A single intraperitoneal dose of APAP (500 mg/kg) to the rats did not result in elevation of the serum enzyme activities when determined 24 h after the treatment (Table 1). In preliminary experiments, the serum enzyme activities were not altered as long as 48 h after treatment with a single 500 mg/kg dose of this drug. Therefore, APAP at this dose was considered to be subtoxic to the female rats under the conditions employed. Serum enzyme activities were increased slightly by APAP at a dose of 1000 mg/kg. However, in the rats pretreated with a subtoxic dose of APAP 18 h earlier (Treatment I), a following dose of APAP (500 or 1000 mg/kg, i.p.) (Treatment II) elevated the AST, ALT, and SDH activities in serum significantly.

APAP and metabolites in plasma

Plasma concentrations of APAP and its metabolites were monitored for 8 h following administration of this drug at a dose of 500 mg/kg. The concentrations of APAP did not differ between the rats pretreated with an identical dose of APAP and the rats pretreated with the vehicle (Fig. 1A). The half-life of APAP in plasma was not changed by APAP pretreatment (control rats, 89.4 ± 7.8 min; APAP-pretreated rats, 100.8 ± 2.1 min). The concentrations of APAP-glucuronide conjugate were not altered (Fig. 1B), but APAP-sulfate concentrations were decreased significantly (Fig. 1C). In contrast, APAP-cysteine concentrations in plasma were greater in the APAP-pretreated rats (Fig. 1D). APAP-mercapturate and APAP-GSH conjugates were below the detectable limits. The area under the concentration–time curve (AUC) of APAP and the major metabolites is summarized in Table 2. The AUC of APAP-sulfate was decreased to less than a half by APAP pretreatment. On the other hand, the AUC of APAP-cysteine was significantly greater in the rats pretreated with APAP.

Xenobiotic-metabolizing enzyme system

Hepatic CYP enzymes were determined 18 h after treatment of rats with a single dose of APAP. The total CYP contents were not changed (control rats, 0.86 ± 0.03 nmol/mg protein; APAP-treated rats, 0.84 ± 0.04 nmol/mg protein), but the enzyme activities measured with *p*-nitrophenol, *p*-nitroanisole, and aminopyrine as probes were all increased significantly (Fig. 2B). Western blot analysis showed results comparable to the changes in the CYP enzyme activities. Administration of APAP induced expression of CYP2E1, CYP1A, and CYP3A proteins significantly (Fig. 2A). Meanwhile, hepatic GSH contents were not altered by a single APAP dose administered 18 h before (control rats, 5.2 ± 0.5 μmol/g liver; APAP-treated rats, 5.4 ± 0.4 μmol/g liver), suggesting that depletion of GSH might not play a role in the potentiation of the APAP-induced liver toxicity.

Discussion

In this study, treatment of the female rats with a single 500 mg/kg dose of APAP did not result in elevation of the serum AST, ALT, or SDH activities. Therefore, a 500 mg/kg dose of APAP was considered to be nontoxic to the female rats under the conditions employed in this study. However, administration of APAP at this dose elevated the serum enzyme activities significantly in the rats that had been pretreated with an identical dose of this drug 18 h earlier. A dose of APAP at 1000 mg/kg

Table 1. Elevation of serum enzyme activities in rats treated with APAP

APAP (mg/kg)		AST	ALT	SDH
Treatment I	Treatment II	(units/ml)		
0	0	85.9 ± 5.5	40.7 ± 6.3	8.0 ± 4.0
0	500	85.2 ± 8.6	47.2 ± 1.9	11.4 ± 3.2
500	500	224.5 ± 3.3**	65.9 ± 1.4**	27.8 ± 4.0**
0	1000	125.2 ± 16.5$^{\#}$	71.7 ± 7.3$^{\#\#}$	22.1 ± 9.2
500	1000	487.1 ± 31.1**	355.9 ± 40.6**	112.3 ± 28.6*

Treatment I was made 18 h before Treatment II. Rats were sacrificed for the assay 24 h after Treatment II. Each value represents the mean ± S.E.M. for six rats. $^{\#}P<0.05$, $^{\#\#}P<0.01$: significantly different from normal control rats. *$P<0.05$, **$P<0.01$: significantly different from the rats treated with a single dose of APAP as Treatment II.

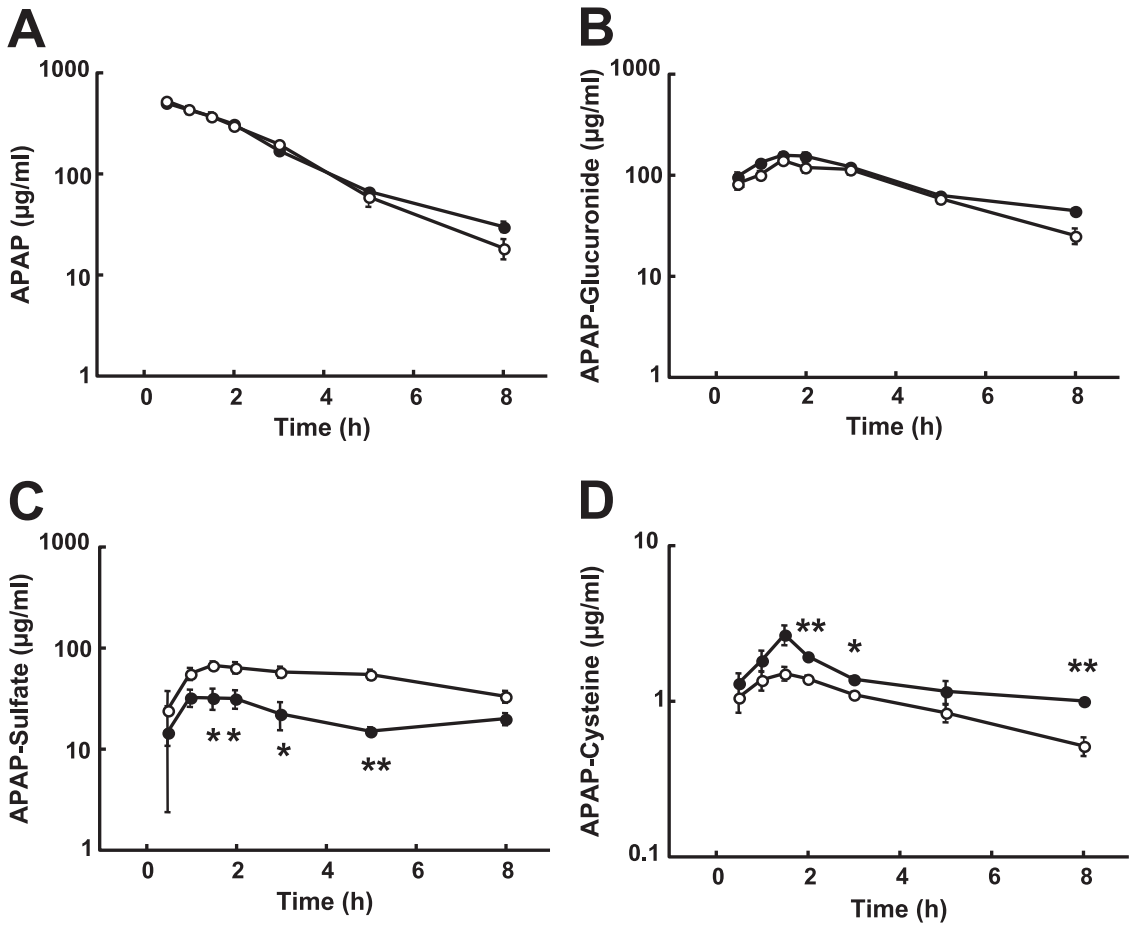

Fig. 1. Concentrations of APAP (A), APAP-glucuronide (B), APAP-sulfate (C), and APAP-cysteine (D) in plasma. Rats were pretreated intraperitoneally with APAP (500 mg/kg) (filled circles) or the vehicle (open circles) 18 h before a challenging dose of APAP (500 mg/kg). Each value represents the mean ± S.E.M. for three or four rats. **P*<0.05, ***P*<0.01: significantly different from the rats pretreated with the vehicle.

Table 2. AUC of APAP and its metabolites in plasma

Pretreatment	APAP	APAP-Glucuronide	APAP-Sulfate	APAP-Cysteine
	(μg·h/ml)			
Vehicle	1365.0 ± 81.8	577.2 ± 40.2	391.2 ± 21.7	7.53 ± 0.70
APAP	1370.9 ± 54.3	677.7 ± 13.5	164.9 ± 34.0**	10.56 ± 0.95*

Each value represents the mean ± S.E.M. for three or four rats. **P*<0.05, ***P*<0.01: significantly different from the rats pretreated with the vehicle.

was slightly hepatotoxic as determined by elevation of the serum enzyme activities. In the rats pretreated with a 500 mg/kg dose of APAP, administration of this drug at 1000 mg/kg resulted in a marked potentiation of the elevation of serum enzyme activities. Pretreatment with APAP changed the concentrations and disappearance of APAP metabolites in plasma resulting from a following dose of APAP. The plasma concentrations of APAP or APAP-glucuronide were not altered, but APAP-sulfate was reduced whereas APAP-cysteine was increased significantly. Hepatic microsomal CYP enzymes involved in the metabolic conversion of APAP into a reactive metabolite were induced by a dose of APAP administered 18 h earlier. These results indicate that administration of APAP, at a dose incapable of elevating the serum enzyme activities per se, may enhance the metabolic activation and resulting toxicity of a following dose of this drug.

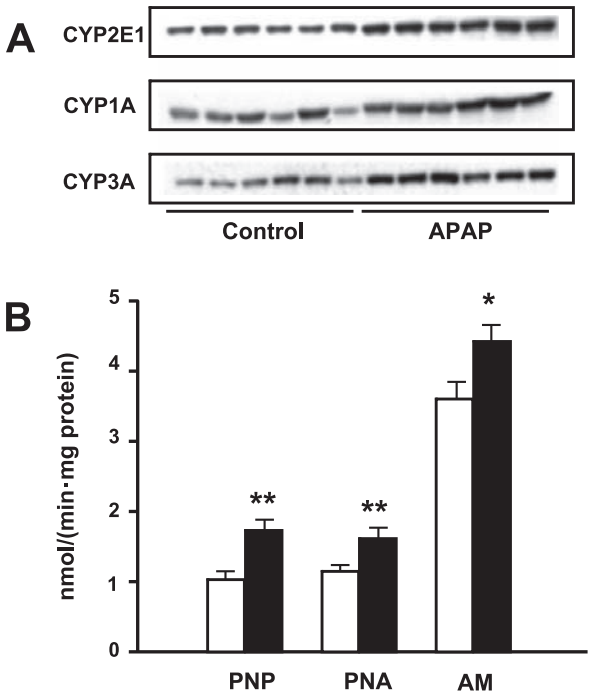

Fig. 2. Hepatic CYP activities and expression of CYP enzymes. Rats were sacrificed 18 h after treatment with an intraperitoneal dose of APAP (500 mg/kg) or the vehicle. A) Western blotting analysis using CYP2E1, CYP3A, and CYP1A antibodies. B) CYP activities in the rats treated with the vehicle (open bars) and the rats treated with APAP (filled bars). Each value represents the mean ± S.E.M. for six rats. PNP, *p*-nitrophenol hydroxylase; PNA, *p*-nitroanisole *O*-demethylase; AM, aminopyrine *N*-demethylase. **P*<0.05, ***P*<0.01: significantly different from the control rats.

APAP, at a low or therapeutic dose, is detoxified primarily through sulfation and glucuronidation (12). Following an overdose, however, APAP is increasingly metabolized via an oxidative pathway into a reactive metabolite, NAPQI. Multiple forms of CYP including CYP2E1, 1A2, and 3A4 are implicated in the oxidative metabolism of APAP (13 – 15), although it is generally accepted that CYP2E1 has the principal role in generation of the reactive metabolite both in humans and rodents.

The reactive metabolite is normally detoxified by conjugation with GSH. APAP-GSH is further converted to the cysteine conjugate before it is acetylated to form APAP-mercapturate. However, when formation of the reactive metabolite exceeds the GSH-conjugation capacity of the liver, covalent binding of NAPQI to cellular macromolecules may result, which initiates the events ultimately leading to cytotoxicity. Therefore, the hepatic metabolizing enzyme activities responsible for generation of the reactive metabolite have a critical role in the development of APAP toxicity.

In this study, a single dose of APAP provoked significant alterations in hepatic xenobiotic-metabolizing enzyme activities in the rats. *p*-Nitrophenol is suggested to be a selective substrate for CYP2E1 (9). Both CYP2E1 and 1A2 are involved in demethylation of *p*-nitroanisole (16), whereas multiple forms of CYP, including 2B1 and 3As, are associated with *N*-demethylation of aminopyrine (17, 18). Therefore, the present results indicate that administration of APAP, at a dose presumed to be nontoxic, may increase all the major CYP activities involved in the metabolic activation of this drug. Western blotting analysis also revealed that a dose of APAP administered 18 h before sacrifice enhanced expression of CYP2E1, 1A, and 3A proteins in the liver.

The present results suggest that pretreatment of rats with APAP may enhance the metabolism of this drug via an oxidative pathway. Concentrations of APAP-mercapturate and APAP-GSH in plasma were below the detectable limits, but APAP-cysteine was elevated significantly. The reason for failure to quantify the APAP-GSH and APAP-mercapturate conjugates in plasma is not clear. However, APAP-GSH conjugates are mainly excreted via bile and biotransformed in intestine to APAP-cysteine before it is reabsorbed to form mercapturic acid (19, 20), which appears to be responsible for the lack of an increase in the GSH and mercapturic acid conjugates in plasma. Augmentation in the generation of an oxidative product of APAP is in agreement with the results showing an induction of CYP enzymes in the rats treated with a single dose of APAP before sacrifice. In the meantime, concentrations of APAP-sulfate in plasma were decreased significantly. Sulfation, an important phase II conjugation reaction, is catalyzed by sulfotransferases that transfer sulfate from the donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to a sulfate acceptor. Generally sulfation is considered to be a high-affinity and low-capacity reaction, whereas glucuronidation is a low-affinity, high-capacity reaction (19, 21). The low-capacity of the sulfation reaction is attributed to the limited availability of PAPS that is in turn determined by the availability of its precursor, inorganic sulfate. In fact, PAPS and inorganic sulfate were shown to decrease rapidly to 20% – 30% of normal levels in the rats treated with a moderate dose of APAP (22, 23). Therefore, the reduction of APAP-sulfate concentrations in this study is ascribed to the depletion of PAPS and inorganic sulfate by a prior dose of APAP. It is also suggested that the reduction of APAP-sulfate generation due to decreased availability of endogenous substrates cancels out the increase in conversion of APAP to an oxidative metabolite, thereby leaving the overall elimination of this drug unchanged.

APAP-induced hepatotoxicity is associated with intake of an excessively large dose of this drug. APAP at such a dose reduces the CYP activities in the liver (24 – 26). In contrast, a few studies have suggested that a low dose of APAP may enhance the metabolism and/or toxicity of other substances that are metabolically activated by CYP enzymes. It was shown that pretreatment of rats with a nontoxic dose of APAP potentiated the toxicity of model hepatotoxicants such as CCl_4, thioacetamide, bromobenzene, allyl alcohol, and dichloroethylene (27). Since these substances have diverse mechanisms of toxic actions, the authors suggested that various factors might be involved in the APAP-induced potentiation of hepatotoxicity. Also a low dose or concentration of APAP increased the total CYP contents in rat liver (28) and CYP3A4 contents and activity in transfected HepG2 cell line (29). More recently, a subtoxic dose of APAP was shown to increase the metabolic generation of carbon monoxide in rats challenged with dichloromethane (2). Expression of CYP2E1 and CYP3A enzymes was also induced, which is consistent with the present results.

In conclusion, the present study indicates that administration of APAP at a dose considered to be nontoxic may result in induction of hepatic enzymes involved in the metabolic activation of this drug. This suggests a possibility that a nontoxic dose of this drug could be unsafe when administered repeatedly. Likewise, administration of APAP would affect, via induction of CYP enzymes, the toxicological consequences resulting from exposure to various chemical substances that are metabolically activated by the same enzyme system. The wide use of APAP in humans suggests that unexpected drug–drug interactions could result, especially when APAP is administered repeatedly. Further studies are being conducted in this laboratory to determine the underlying mechanism and the extent of CYP induction by APAP.

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