DMD Fast Forward. Published on July 9, 2007 as doi:10.1124/dmd.107.015545

Induction of hepatic CYP2E1 by a subtoxic dose of APAP in rats: Increase in dichloromethane metabolism and carboxyhemoglobin elevation

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DMD Fast Forward. Published on July 9, 2007 as DOI: 10.1124/dmd.107.015545
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a) Running Title: Induction of CYP2E1 and DCM metabolism by APAP

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c) Number of text pages: 20

Number of tables: 2

Number of figures: 3

Number of words in the Abstract: 239

Number of words in the Introduction: 379

Number of words in the Discussion: 1272

d) List of abbreviations: APAP, acetaminophen; DCM, dichloromethane; CYP, cytochrome P450; GSH, glutathione; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; COHb, carboxyhemoglobin; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase

Abstract

Dichloromethane (DCM) is metabolically converted to carbon monoxide mostly by CYP2E1 in liver, resulting in elevation of blood carboxyhemoglobin (COHb) levels. We investigated the effects of a subtoxic dose of acetaminophen (APAP) on the metabolic elimination of DCM and COHb elevation in adult female rats. APAP, at 500 mg/kg, i.p., was not hepatotoxic as measured by a lack of change in serum aspartate aminotransferase, alanine aminotransferase, and sorbitol dehydrogenase activities. In rats pretreated with APAP at this dose, the COHb elevation resulting from administration of DCM (3 mmol/kg, i.p.) was enhanced significantly. Also blood DCM levels were reduced and its disappearance from blood appeared to be increased. Hepatic CYP2E1-mediated activities measured with chlorzoxazone, *p*-nitrophenol and *p*-nitroanisole as substrates were all induced markedly in microsomes of rats treated with APAP. Aminopyrine *N*-demethylase activity was also increased slightly, but significantly. Western blot analysis showed that APAP treatment induced the expression of CYP2E1 and CYP3A proteins. Neither hepatic glutathione contents nor glutathione *S*-transferase activity was changed by the dose of APAP used. The results indicate that, contrary to the well-known hepatotoxic effects of this drug at large doses, a subtoxic dose of APAP may induce CYP2E1, and to a lesser degree, CYP3A expression. This is the first report that APAP can increase the CYPs-mediated hepatic metabolism and the resulting toxicity of a xenobiotic in the whole animal. The pharmacological/toxicological significance of induction of CYPs by a subtoxic dose of APAP is discussed.

Introduction

Acetaminophen (APAP), a widely used analgesic-antipyretic, may cause severe liver and kidney injuries at large or chronic doses. The APAP-mediated cellular injury is primarily initiated by metabolic conversion of this drug into a reactive intermediate, *N*-acetyl-*p*-benzoquinoneimine (NAPQI). It has been demonstrated that multiple forms of cytochrome P450 (CYP) including CYP2E1, 1A2 and 3A4 are implicated in the activation of APAP to the reactive metabolite (Patten et al., 1993; Raucy et al., 1989; Zaher et al., 1998), but most investigators accept that CYP2E1 has a principal role in the oxidative metabolism of this drug both in human and rodents. The reactive metabolite is normally detoxified by conjugation with glutathione (GSH). Therefore, the hepatotoxicity of APAP is mainly dependent on the metabolic activities responsible for the activation of this drug and the effectiveness of GSH conjugation reaction.

Dichloromethane (DCM; CH_2Cl_2) is widely used in industry as a degreaser, solvent, an extraction medium, and also as an important constituent of paint removers. Unlike its chemical congeners such as carbon tetrachloride and chloroform that are potent hepatotoxins, the liver toxicity of this substance is negligible (Nitschke et al., 1988). Instead, DCM is metabolically converted to carbon monoxide (CO) by CYP2E1 (Guengerich et al., 1991; Kim and Kim, 1996; Wirkner et al., 1997), leading to elevation of blood carboxyhemoglobin (COHb) levels. Accordingly hypoxia due to a decrease in O_2 carrying capacity constitutes a major toxicological problem associated with DCM exposure

(Horowitz, 1986; Mahmud and Kales, 1999).

While many studies have described the toxic consequences resulting from an excessively large dose of APAP, its potential adverse effects at a lower or therapeutic dose have hardly been explored. Considering the wide use of APAP in human, a better understanding of its effects at such a dose, presumed to be clinically nontoxic, seems to be relevant. In this study we investigated the effects of treating rats with a subtoxic dose of APAP on the metabolic disposition of DCM and COHb elevation. These two substances share common properties in that their toxic metabolites are generated via enzyme reactions mediated mostly by CYP2E1. Therefore, examination of the interaction between the two substances would provide valuable information regarding the effects of APAP administration on the metabolic fate and resulting consequences of various substances metabolically converted by the identical enzyme system.

Materials and Methods

Animals and Treatments

Female Sprague-Dawley rats (Dae-Han Laboratory Animal, Seoul, Korea) were acclimated in temperature (22 ± 2°C) and humidity (55 ± 5 %) controlled rooms with a 12 h light-dark cycle for at least three weeks before use. The use of rats was in compliance with the guidelines established by Animal Care Committee of this institute. Regular laboratory rat diet (Purina-Korea, Seoul, Korea) and tap water were allowed *ad libitum* until sacrifice. Rats, 10-14 weeks old and weighing 190-230 g, were treated intraperitoneally with APAP (500 mg/kg) dissolved in 10 % tween 80. The volume of injection was 15 ml/kg. After 18 h DCM (3 mmol/kg), mixed with corn oil, was administered to rats intraperitoneally. The volume of injection was 2 ml/kg. Control animals were treated with an identical volume of each vehicle.

For the experiments determining blood COHb and DCM concentrations, a jugular vein was catheterized with silicon tubing (Dow Corning, Midland, MI) 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 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. Eighteen hours following the treatment blood was sampled by cardiac puncture from each rat under light ether anesthesia. Serum sorbitol dehydrogenase (SDH) activity was determined spectrophotometrically according to the method described by Gerlach (1983). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum were determined using the method of Reitman and Frankel (1957).

Measurement of COHb and DCM Levels in Blood

Blood samples were obtained directly from the catheter inserted into a jugular vein. The COHb concentration in blood was determined using the method of Rodkey et al. (1979). In short, blood sample was diluted approximately 1500-fold with 0.01 M Tris solution containing sodium dithionite to prevent dissociation of COHb by oxygen. Absorbance measurements were made at 420 and 432 nm, and the fraction of the total hemoglobin present as COHb was calculated from these measurements and the molar absorptivities of hemoglobin and COHb determined in this laboratory.

The DCM levels in blood were determined using a modification of the method previously reported (Kim and Carlson, 1986). Each 100 μl blood sample in a sealed vial stood at room

temperature for 60 min. An aliquot (100 μl) of head-space vapor was sampled and injected into a gas chromatograph equipped with a flame ionization detector. A 2 m stainless steel column packed with 4 % OV-101 and 6 % OV-210 (Supelco, Inc., Bellefonte, PA) was used. Nitrogen was the carrier gas (30 ml/min), and air (300 ml/min) and hydrogen (30 ml/min) were utilized in the flame ionization detector. The column was set at 70°C, the detector at 150°C, and the injector at 130°C.

Measurement of Hepatic Glutathione Contents and Glutathione *S*-Transferase Activity

Hepatic total GSH contents were determined using an enzymatic recycling method of Griffith (1980). Whole liver was homogenized in ice-cold 1 M perchloric acid with 2 mM EDTA followed by centrifugation. An aliquot of the supernatant was mixed with 0.3 mM NADPH and 6 mM 5,5'-dithio-bis(2-nitrobenzoic acid). After addition of GSH reductase the absorbance was monitored at 412 nm. Both DCM and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (ENPP) are known to be substrates for the theta class GST (Meyer et al., 1991). Therefore, cytosolic GST activity was measured using ENPP as a substrate according to the method of Habig et al. (1974).

Measurement of Microsomal Enzyme Activities

Whole liver was homogenized in an ice-cold buffer of 0.154 M KCl/50 mM Tris-HCl, pH 7.4, with 1 mM EDTA. Homogenate was centrifuged at 10000 g for 20 min, and the

supernatant was further centrifuged at 104000 g for 60 min. The microsomal pellet was suspended in the homogenizing buffer followed by recentrifugation at 104000 g for 60 min. Protein contents were measured using the method of Lowry et al. (1951). The concentration of cytochrome P450 was estimated from the CO difference spectrum (Omura and Sato, 1964). The activity of NADPH-dependent CYP reductase was determined by using cytochrome c as a substrate (Phillips and Langdon, 1962). *p*-Nitrophenol hydroxylase activity was determined by measuring the formation of *p*-nitrocatechol (Koop, 1986). For measurement of chlorzoxazone 6-hydroxylase activity, reaction conditions identical to those described by Koop et al. (1997) were used. Quantification of 6-hydroxychlorzoxazone was conducted using a HPLC method (Baek et al., 2006). *p*-Nitroanisole *O*-demethylase was determined using the method of Shigematsu et al. (1976). The production of formaldehyde was quantified for measurement of aminopyrine *N*-demethylase activity (Nash, 1953).

Western Blot Analysis

The microsomal suspension (10 μg of protein per lane) was loaded, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Bio-Rad Inc., Hercules, CA) 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 (Detroit R&D, Detroit, MI) were used as probes. Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma-Aldrich, St. Louis, MO) and scanning densitometry was performed with a microcomputer imaging device (Model M1, Imaging Research, St. Catharines, Canada).

Data Analysis

All results expressed as the mean ± S.E. 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. The half-life of DCM was derived from a semilogarithmic plot of blood DCM concentrations *vs* time using the method of least squares.

Results

Elevation of Serum Enzyme Activities by APAP Treatment

Serum AST, ALT, and SDH activities was determined in female rats treated with APAP at various doses (250-1000 mg/kg) (Table 1). At 18 h following the treatment serum enzyme activities were not elevated by an APAP dose of up to 500 mg/kg. Starting from the APAP dose of 750 mg/kg the enzyme activities were increased, although the difference from control was not statistically significant until the dose reached 1000 mg/kg. In this study the maximal dose of APAP that did not influence the serum parameters in rats under normal feeding was determined to be 500 mg/kg. Therefore, all the subsequent experiments were conducted employing this dose level.

COHb Elevation and Disappearance of DCM from Blood

In rats treated with a dose of DCM (3 mmol/kg, i.p.) blood COHb levels were elevated rapidly, reaching a peak of 11 % at 3 h after the treatment (Fig. 1). A dose of APAP 18 h prior to the DCM challenge enhanced the increase in COHb levels significantly. Elevation of COHb levels seemed to be more rapid and the peak was higher in rats pretreated with APAP compared with rats treated with DCM only. The COHb levels of both groups returned to near-normal values in 6 h.

APAP pretreatment also affected the DCM levels and the rate of its elimination from blood in rats challenged with a dose of DCM (Fig. 2). The DCM levels in blood were lower in the APAP-treated rats than in the control rats. The blood DCM concentrations declined in a log-linear fashion. The mean half-life of DCM calculated from the disappearance curve of this solvent was reduced by approximately 20 % in rats pretreated with APAP.

Alterations in Metabolizing Enzyme System

Rats were treated with a dose of APAP (500 mg/kg, i.p.) and sacrificed 18 h later to determine the hepatic metabolizing enzyme activities at the time point when DCM was administered (Table 2). The total cytochrome P450 contents were not changed by APAP treatment. But chlorzoxazone 6-hydroxylase, *p*-nitrophenol hydroxylase and *p*-nitroanisole *O*-demethylase activities as well as NADPH-dependent CYP reductase activity were all increased in microsomes of rats treated with APAP. A small, but significant, increase in aminopyrine *N*-demethylase activity was also demonstrated. Hepatic GSH contents were not altered by a prior dose of APAP. There was no difference in the activity of GST measured with ENPP as a substrate between control and APAP-treated rats.

Expression of CYP2E1 and 3A proteins was determined in liver of rats that had been treated with APAP 18 h earlier (Fig. 3). Western blot analysis showed results comparable to those obtained from measurements of the hepatic microsomal metabolizing enzyme activities. An 18 h prior dose of APAP induced the expression of CYP2E1 protein in liver

by 65 %. CYP3A proteins were also increased significantly.

Discussion

In this study a dose of APAP up to 500 mg/kg did not affect the serum AST, ALT, or SDH activities when measured 18 h following the treatment. The serum enzyme activities were elevated in rats treated with larger doses of APAP, but a statistical difference from control was shown only at the highest dose used in this study. It has been recognized that the hepatotoxic potential of APAP shows large variations depending on animal species, sex, and feeding status. Generally humans and rats are less susceptible to APAP-induced hepatotoxicity compared with mice and hamsters (Green et al., 1984; Tee et al., 1987). Also APAP is more hepatotoxic to male than female rats (Raheja et al., 1983). In fact APAP, at an oral dose up to 2400 mg/kg, was shown to produce no significant change in serum ALT activity in fed male rats (Wright and Moore, 1991). Therefore, the dose of APAP, 500 mg/kg, selected in this study was considered to be subtoxic or nontoxic to female rats under normal feeding.

Pretreatment of rats with a subtoxic dose of APAP significantly increased the COHb elevation resulting from a DCM challenge in this study. The blood DCM levels were reduced and its disappearance from blood also seemed to be more rapid in rats pretreated with APAP. The results indicate that the metabolic elimination of DCM and its conversion to CO are enhanced by pretreatment with an APAP dose incapable of elevating the serum enzyme activities.

Two major pathways are involved in the metabolic disposition of DCM; a microsomal oxidation process and a GSH-dependent cytosolic pathway. The microsomal reaction involves oxidative dechlorination of this solvent resulting in production of CO and CO_2 via proposed intermediates chloromethanol and formyl chloride (Kubic and Anders, 1978). CYP2E1 was shown to be the major subtype of cytochrome P450 family responsible for the metabolic conversion of DCM to CO (Guengerich et al., 1991). This pathway has a high affinity for DCM with limited capacity (McKenna et al., 1982). The alternative metabolic pathway of DCM is a reaction with GSH mediated by GSTs, especially the theta class GST, which produces CO_2 via *S*-chloromethyl GSH ($GSCH_2Cl$) and formaldehyde (Blocki et al., 1994; Mainwaring et al., 1996). This metabolic reaction shows no indication of saturation, suggesting that the cytosolic pathway is low affinity, high capacity compared with the CYP-dependent reaction (Gargas et al., 1986). Therefore, the elevation of blood COHb resulting from a moderate dose of DCM is directly related to CYP2E1 activity in liver (Kim and Carlson, 1986; Kim and Kim, 1996).

In this study chlorzoxazone 6-hydroxylase, *p*-nitrophenol hydroxylase and *p*-nitroanisole demethylase activities were all induced significantly in liver microsomes derived from animals treated with APAP 18 h earlier. *p*-Nitrophenol and chlorzoxazone are generally considered to be a selective substrate for CYP2E1 (Koop, 1986; Peter et al., 1990), although it has been suggested that CYP3A also make a considerable contribution to hydroxylation of *p*-nitrophenol (Zerilli et al. 1997). Both CYP2E1 and 1A2 are involved in *p*-nitroanisole demethylase activity (Jones et al., 1997). The induction of chlorzoxazone 6-

hydroxylase, *p*-nitrophenol hydroxylase and *p*-nitroanisole demethylase activities (Table 2) is well correlated to the increase in CYP2E1 protein measured by Western blot analysis (Fig. 3). The results indicate that the increase in COHb elevation and DCM elimination in APAP-pretreated rats is associated with the induction of hepatic CYP2E1. Multiple forms of CYP isozyme including 2B1 and 3As are known to be involved in *N*-demethylation of aminopyrine (Imaoka et al., 1988). Therefore, the induction of aminopyrine *N*-demethylase activity was attributed to the increased expression of CYP3A enzymes.

In the cytosolic pathway that converts DCM into CO_2 via formaldehyde, GSH is required for formation of an intermediate, *S*-chloromethyl GSH, but this tripeptide is regenerated and not consumed in the metabolic reaction (Ahmed and Anders, 1978). But previous studies conducted in this laboratory and also by others showed that treatment with a GSH-depleting agent, such as 2,3-epoxypropane, buthionine sulfoximine, and phorone, increased the COHb elevation and DCM elimination (Gargas et al., 1986; Oh et al., 2002), indicating that the availability of GSH may affect the metabolism of DCM via the microsomal CYP-dependent reactions. The theta class GST is known to play a major role in the metabolism of DCM via the GSH-dependent cytosolic pathway (Blocki et al., 1994; Mainwaring et al., 1996). In this study neither hepatic GSH contents nor the theta class GST activity was influenced by the dose of APAP used, excluding a possibility that the increment in COHb elevation and DCM metabolism might be associated with a change in the GSH-dependent metabolic reaction.

It has been well established that the formation of a reactive metabolite of APAP by CYP activities is the critical event initiating various sequential cellular responses, ultimately leading to liver and kidney injuries. Because of the obvious role of CYP activities in APAP-mediated toxicity many studies have been directed to identification of exogenous and endogenous factors that may modulate the enzyme activities, and thereby alter the toxicological consequences resulting from an excessive dose of this drug. Since CYP activities are decreased by APAP at such a dose or level (Aikawa et al., 1977; Shayiq et al., 1999; Snawder et al., 1994), it is generally suspected that this drug would decrease the metabolism and activation of other toxic substances catalyzed by the same enzyme activities. However, the present results indicate that APAP, at a dose not capable of affecting the serum hepatotoxic parameters, may induce the CYP enzymes and metabolism of xenobiotics significantly. Induction of CYP enzymes by a subtoxic dose of APAP shown in this study was unexpected and seemed paradoxical. Extensive literature survey reveals that this issue has hardly been raised before. It was shown that the toxicity of several hepatotoxicants in rats was potentiated by a nontoxic dose of APAP (Wright and Moore, 1991). The authors suggested that this drug rendered hepatocytes susceptible to toxic insults via unknown mechanism(s). Plewka et al. (2000) demonstrated that treatment of rats with a low dose of APAP increased the total CYP contents, but expression of CYP2E1 and 1A2 appeared to be unaltered or reduced slightly in that study. The authors attributed the induction of total CYP to the need of liver cells to maintain the optimal CYP-dependent monooxygenase system in a critical situation. Meanwhile a low concentration of APAP was shown to increase CYP3A4 contents and activity in transfected HepG2 cell line (Feierman

et al., 2002). Its significance in whole animals was not assessed. Therefore, the present study is the first report demonstrating that a subtoxic dose of APAP to rats may induce the hepatic CYP enzymes and the metabolic reaction of a xenobiotic catalyzed by the CYP activities.

In conclusion the present study indicates that APAP, at a dose not affecting the serum hepatotoxic parameters, may induce the hepatic CYP2E1 and 3A contents and the metabolic activities involved in the activation of this drug. It is also shown that the altered CYP2E1 contents may actually influence the metabolism and the resulting toxicity of a xenobiotic which is a substrate for the enzyme. The induction of hepatic CYP2E1 might result in potentiation of the toxicities of various chemicals which are converted to toxic metabolites by this enzyme, including numerous drugs, industrial and environmental chemicals as well as natural substances. Considering the wide use of APAP as an analgesic-antipyretic, it is suggested that a greater concern should be expressed regarding the effects of acute or repeated dosing of this drug even at a therapeutic level, especially in combination with other medications. Further studies need to be undertaken to define the underlying mechanism and the extent of induction of the hepatic CYP enzymes by APAP.

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DMD Fast Forward. Published on July 9, 2007 as DOI: 10.1124/dmd.107.015545
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Figure Legends

Fig. 1. Effect of APAP pretreatment on blood COHb elevation by DCM. Rats were treated with APAP (500 mg/kg, i.p.) 18 h prior to DCM (3 mmol/kg, i.p.). Each value represents mean ± S.E. for six rats. *,**Significantly different from rats treated with DCM only (Student's *t*-test, $p < 0.05$, 0.01, respectively).

Fig. 2. Effect of APAP pretreatment on blood DCM levels. Rats were treated with APAP (500 mg/kg, i.p.) 18 h prior to DCM (3 mmol/kg, i.p.). Each value represents mean of three rats. Standard error bars were omitted for the sake of clarity. *,**Significantly different from rats treated with DCM only (Student's *t*-test, $p < 0.05$, 0.01, respectively). The half-life of DCM obtained from the elimination curve was 40.9 ± 4.0 min for control rats; 33.6 ± 1.2 min for APAP-pretreated rats (Student's *t*-test, $p = 0.15$).

Fig. 3. Expression of CYP2E1 and CYP3A4 in liver of rats treated with APAP. Rats were sacrificed 18 h after treatment with APAP (500 mg/kg, i.p.) (open bars) or with the vehicle only (filled bars). Bands resolved by Western blotting were quantified densitometrically for comparison. Each value represents mean ± S.E. for six rats. **.***Significantly different from rats treated with the vehicle only (Student's *t*-test, $p < 0.01$, 0.001, respectively).

DMD Fast Forward. Published on July 9, 2007 as DOI: 10.1124/dmd.107.015545
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Table 1. Elevation of serum enzyme activities in rats treated with APAP.

Rats were sacrificed 18 h following APAP treatment. Each value represents mean ± S.E. for six rats.

APAP (mg/kg, i.p.)	AST	ALT	SDH
	(units/ml)		
0	75.9 ± 3.9	30.7 ± 2.2	8.0 ± 3.6
250	77.4 ± 3.7	34.2 ± 4.6	10.5 ± 4.5
500	72.6 ± 3.6	34.6 ± 3.6	13.4 ± 5.5
750	183 ± 57	123 ± 54	202 ± 130
1000	426 ± 152*	433 ± 173*	791 ± 309*

*Significant differences from control were shown only in rats treated with a 1000 mg/kg dose of APAP (Student's *t*-test, $p < 0.05$).

Table 2. Effect of APAP treatment on hepatic metabolizing enzyme system.

Rats were treated with APAP (500 mg/kg, i.p.) and sacrificed 18 h later. Each value represents mean ± S.E. for six rats.

	Cytochrome P450	NADPH CYP reductase	Chlorzoxazone 6-hydroxylase	*p*-Nitrophenol hydroxylase	*p*-Nitroanisole demethylase	Aminopyrine *N*-demethylase	GSH	GST
	(nmol/mg protein)	(nmol/min/mg protein)					(μmol/g liver)	(nmol/min/ mg protein)
Control	0.84 ± 0.02	76.0 ± 1.4	1.13 ± 0.07	1.44 ± 0.07	1.13 ± 0.05	3.66 ± 0.16	5.8 ± 0.4	53.5 ± 4.1
APAP	0.81 ± 0.03	115.7 ± 8.7**	1.95 ± 0.08***	2.17 ± 0.06***	1.79 ± 0.18**	4.25 ± 0.21*	5.4 ± 0.6	45.4 ± 1.5
(% control)	(96 %)	(152 %)	(173 %)	(151 %)	(158 %)	(116 %)	(93 %)	(85 %)

*,**,***Significantly different from rats treated with the vehicle only (Student's *t*-test, $p < 0.05$, 0.01, 0.001, respectively).

Fig. 1

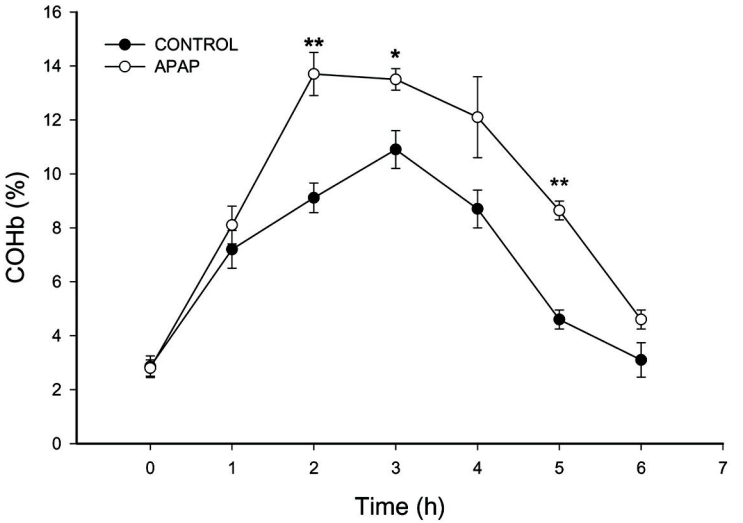


Fig. 2

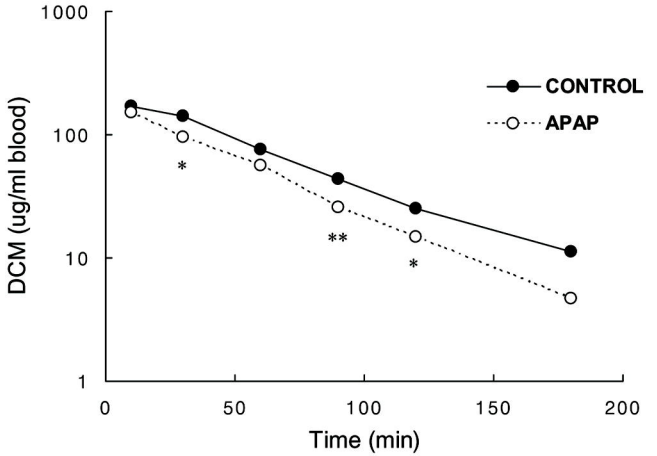
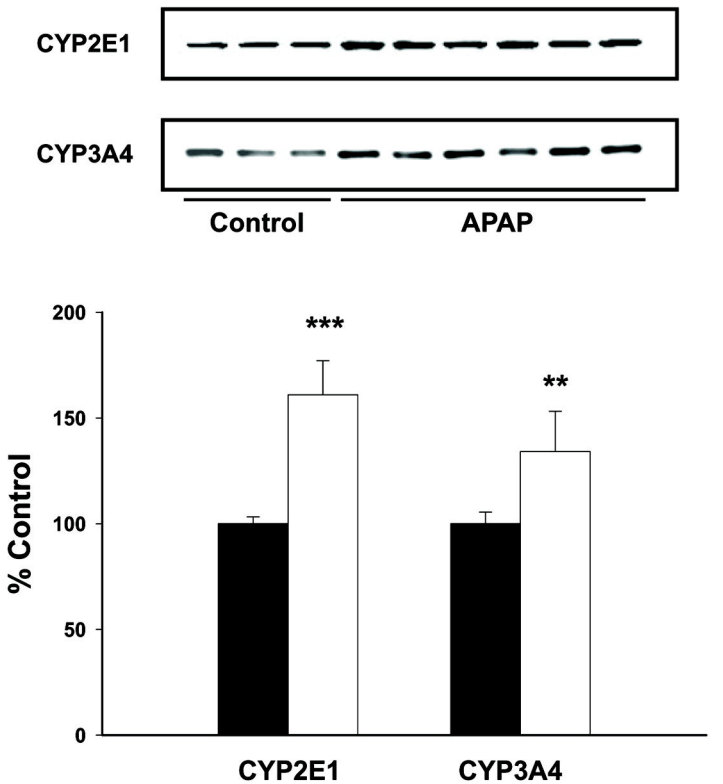

1000
100
10
1
DCM (ug/ml blood)
0
50
100
150
200
Time (min)
CONTROL
APAP
*
**
*

Fig. 3


CYP2E1
CYP3A4
Control
APAP
200
150
100
50
0
% Control
***
**
CYP2E1
CYP3A4