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Full Length Article

Changes in coumarin kinetics and subcellular localization of CYP2E1 contribute to bile duct damage and reduce hepatocellular damage after repeated administration of coumarin in rats

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ABSTRACT

Coumarin exhibits different hepatotoxicity in rats depending on the administration frequency. To investigate the underlying mechanisms for the differences, we administered coumarin to rats as a single dose or repeatedly for 4 weeks. We found large increases in blood levels of liver enzymes and noticeable centrilobular hepatic necrosis after a single dose of coumarin. After repeated administration, enzyme levels mildly increased, while those of γ-GTP and total bilirubin significantly increased, suggesting bile duct damage. In the control group, cytochrome P450 2E1 (CYP2E1) showed a diffuse subcellular distribution but accumulated within the hepatocyte endoplasmic reticulum after repeated coumarin administration. The maximum blood concentrations of coumarin and its metabolites were significantly lower upon repeated administration. The results suggest that changes in coumarin pharmacokinetics and CYP2E1 subcellular distribution contribute to resistance to coumarin-induced hepatic necrosis, while cytotoxicity of metabolic conjugates shown in vitro may contribute to bile duct damage upon repeated coumarin administration.

1. Introduction

Coumarin is a natural compound found in various plants and essential oils (Abraham et al., 2010; Wang et al., 2013). It is known to cause acute and chronic hepatotoxicity in rats (Den Besten et al., 1990; Fentem et al., 1992, 1993; Lake, 1984, 1999). Coumarin is metabolized by cytochrome P450 1A (CYP1A) and 2E1 (CYP2E1) in rats, producing reactive metabolites, coumarin 3,4-epoxide and hydroxyphenylacetaldehyde (Born et al., 1997, 2000; Cohen, 1979; Lake et al., 1979, 1994). These reactive metabolites are detoxified and eliminated by conjugation with compounds such as glucuronic acid, sulfates, and glutathione. However, if their metabolic pathways are saturated, the metabolites become toxic for the liver. The risk of severe hepatotoxicity has been reported to be low in humans because the metabolic pathway of coumarin in humans is different from that in rats (Born et al., 2002; Vassallo et al., 2004).

During the acute phase of coumarin toxicity in rats, centrilobular hepatic necrosis occurs. However, during the chronic period, the toxicity target changes from the centrilobular region to the bile duct, with symptoms such as bile duct hyperplasia and cholangiofibrosis (Carlton et al., 1996; Evans et al., 1989; Hagan et al., 1967; Lake, 1984; Lake et al., 1989; Lake and Grasso, 1996). Hepatocellular carcinoma and bile duct tumor have also been reported upon long-term administration of large amounts of coumarin (Carlton et al., 1996). The centrilobular hepatocyte damage observed in the acute toxicity period and not found during the chronic period is attributed to cytochrome P450, which contributes to the production of active metabolites and is inhibited after repeated administration of coumarin. Detoxification is enhanced by the induction of conjugating enzymes, such as glutathione-*S*-transferase and UDP-glucuronic acid transferase, which promote metabolite conjugation, thereby contributing to detoxification (Lake and Grasso, 1996; Lake et al., 2002). Bile duct toxicity frequently observed in the chronic period has been attributed to unstable metabolites in the bile duct, which might demonstrate local toxicity; however, the details are unknown (Lake and Grasso, 1996).

The purpose of this study was to obtain more information about the differences in coumarin-induced hepatotoxicity between the acute and chronic periods and the mechanisms involved. In the present study, we

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCK-8, cell counting kit-8; CYP1A, cytochrome P450 1A; CYP2E1, cytochrome P450 2E1; GLDH, glutamate dehydrogenase; H & E, hematoxylin and eosin; *o*-HPAA, *o*-hydroxyphenylacetic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PKA, protein kinase A; TBIL, total bilirubin; TBS, Tris-buffered saline; UDPGA, UDP glucuronic acid; UFLC, ultrafast liquid chromatography

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used rats showing significant coumarin-induced hepatopathy. HepG2 cytotoxicity experiments were performed using the S9 mix, which is a liver extract containing metabolic enzymes and cofactors for glucuronic acid and sulfate conjugation, such as UDP glucuronic acid (UDPGA) and 3′-phosphoadenosine-5′-phosphosulfate (PAPS), to mimic the conditions of metabolic activation and evaluate the toxicity of coumarin metabolites, including conjugated forms.

2. Materials and methods

2.1. Chemicals

Coumarin, the lithium salt of PAPS, and the trisodium salt of UDPGA were obtained from Sigma–Aldrich Japan (Tokyo, Japan). Cell counting kit-8 (CCK-8) for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was obtained from Wako Pure Chemical Industries (Osaka, Japan). NADPH regeneration system solutions A and B were obtained from Corning (New York, NY). The rat liver S9 mix was obtained from Ieda Trading Corporation (Tokyo, Japan).

2.2. Animals

Five-week-old male Sprague–Dawley rats were purchased from Charles River Laboratories Japan, Inc., Hino, Japan. The animals were used in experiments after an acclimation period of one week. They were kept at a temperature and humidity of 23.5 ± 2.0 °C and 55.0 ± 10.0%, respectively, and a 12-h light/dark cycle. Feed and water were freely available. All protocols for animal procedures were approved by the Ethics Committee of Animal Experiment in accordance with the Internal Regulations on Animal Experiments at Suntory, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 30 May 2014).

2.3. Experimental design of in vivo study

The animals were randomly assigned to four experimental groups, with six rats in each group. Coumarin was administered by oral gavage at 200 mg/kg, either as one dose or for 28 consecutive days. Corn oil (5 mL/kg) was administered by a similar method to control groups. Table 1 shows the experimental design. We also set up satellite groups to draw blood over time, with nine rats per group.

2.4. Sample collection

Blood was drawn from the abdominal aorta, with the animal under anesthesia, 24 h after the single-dose or repeated administration of coumarin. After the blood was drawn, the liver was excised, macroscopically examined, weighed, and analyzed.

2.5. Blood chemistry

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), alkaline phosphatase (ALP), γ-GTP, and total bilirubin (TBIL) were measured as markers of liver function using a Hitachi 7180 or TBA-200FR chemistry analyzer (Toshiba Corporation).

Table 1
Experimental design.

Group 1	Corn oil	–	24 h after single administration
Group 2	Coumarin	200 mg/kg	24 h after single administration
Group 3	Corn oil	–	24 h after 28-day treatment
Group 4	Coumarin	200 mg/kg	24 h after 28-day treatment

2.6. Histopathological examination

Excised liver tissue was fixed with 10% formalin solution and embedded in paraffin. Sections (2 μm) were prepared and stained with hematoxylin and eosin (H & E). The stained sections were observed under a microscope.

2.7. Immunohistochemical examination

Dual immunofluorescence labeling of CYP2E1 and calnexin (an endoplasmic reticulum marker) was performed to investigate the subcellular localization of CYP2E1 in rats from groups that received single-dose or repeated administration of coumarin. Routinely processed, formalin-fixed, paraffin-embedded tissue specimens were cut into 2-μm sections. The sections were deparaffinized in xylene and rehydrated with graded ethanol at room temperature. For each section, antigen retrieval was performed in a pressure cooker for 15 min with a citrate-buffered solution (pH 6.0). All the slides were rinsed with 0.05 M Tris-buffered saline (TBS, pH 7.6), treated with 1% hydrogen peroxide in methanol, and rinsed again with TBS. The slides were incubated with bovine serum albumin for 5 min and overnight at 4 °C with an antibody mixture that included a goat polyclonal anti-calnexin antibody (diluted 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX) and a rabbit polyclonal anti-CYP2E1 antibody (diluted 1:200, Merck Millipore, Darmstadt, Germany). The sections were exposed for 60 min to appropriate fluorochrome-conjugated secondary antibodies, goat anti-rabbit-Alexa 488 (Invitrogen, Carlsbad, CA) and donkey anti-goat-Alexa 594 (Invitrogen), respectively. The slides were mounted with a mounting medium containing 4′,6-diamino-2-phenylindole (Invitrogen). As negative controls, primary antibodies were omitted and isotype-specific immunoglobulin was also used.

2.8. Measurement of blood concentrations of coumarin and coumarin metabolites

Plasma samples for toxicokinetics were obtained from blood collected from the subclavian vein and placed into heparinized centrifuge tubes. Blood was collected at 0 min (before the administration) and at 15, 30, 60 min, 4 h, and 12 h after the administration. Blood was collected either two or four times from the same rat, at 0, 15, 30, and 60 min or at 4 and 12 h after the administration. Plasma was separated by centrifugation at 10,000 × *g* for 3 min and stored at −70 °C until analysis. To determine the concentration of coumarin, 7-hydroxycoumarin, *o*-hydroxyphenylacetic acid (HPAA), and hydroxyphenylethanol, the supernatant was subjected to ultrafast liquid chromatography (UFLC, Shimadzu Corporation, Kyoto, Japan). The UFLC conditions were as follows: an Inertsil ODS-3 column (3 μm, 2.1 mm × 100 mm; GL Sciences, Inc., Tokyo, Japan), column temperature of 40 °C, mobile phase of 0.1% formic acid in methanol, flow rate of 0.2 mL/min, and injection volume of 5 μL.

2.9. Cell culture

The HepG2 cell line was obtained from the American Type Culture Collection (Manassas, VA). HepG2 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum in a 5% CO_2 atmosphere at 37 °C.

2.10. Preincubation

Dimethyl sulfoxide was used to adjust the coumarin concentration. Preincubation was performed in 24-well plates using 5 μL of the coumarin solution or the vehicle without metabolic activation. For Phase I activation, 10 μL of the S9 mix was added to the coumarin solution or the vehicle. For Phase I and II activation, 5 μL each of UDPGA and PAPS was added to the mixtures used for Phase I activation. The culture

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