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Relationship between coumarin-induced hepatocellular toxicity and mitochondrial function in rats

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ABSTRACT

The manifestation of coumarin-induced hepatocellular toxicity may differ and depends on the frequency of administration to rats. A single coumarin dose induces hepatocellular necrosis while repeated doses induce only hepatocyte degeneration. However, the mechanism underlying these effects remains unclear. Therefore, we investigated the mechanism of coumarin-induced hepatotoxicity in rats. Coumarin was administered to male rats as a single dose or for 4 consecutive days, and samples were obtained 4 or 24 h after a single dose or 24 h after the repeated doses. A single coumarin dose significantly induced hepatocellular necrosis in rats; however, toxicity was attenuated after repeated dosing. With a single dose, hepatocellular necrosis was preceded by increased mitochondrial number and size and decreased mitochondrial function. An increased expression of granular cytochrome P450 (CYP) 2E1 protein was observed in the cytoplasm and mitochondria of coumarin-treated rats compared to the expression in the untreated controls. Nevertheless, repeated dosing showed mitochondrial function that was equivalent to that of the control while enlarged CYP2E1 protein droplets were distributed outside the mitochondria. These results suggest that mitochondrial function and CYP2E1 expression might be involved in coumarin-induced hepatocellular toxicity in rats. A reduction in mitochondrial CYP2E1 might be implicated in the acquisition of coumarin resistance after repeated doses.

1. Introduction

Coumarin is a naturally occurring substance present in various plants and essential oils (Abraham et al., 2010; Wang et al., 2013). Previous reports have shown that coumarin exhibits significant acute and chronic hepatotoxicity in rat (Den Besten et al., 1990; Fentem et al., 1992, 1993; Lake, 1984, Lake and Grasso, 1996). The risk of severe hepatotoxicity in humans is reported to be low because the metabolic pathway of coumarin is different from that of rats (Born et al., 2002; Vassallo et al., 2004). However, the tolerable daily intake is set at 0.1 mg kg^{-1} day^{-1} in Europe because the risk cannot be ignored, and moderate intake of cinnamon, which includes considerable levels of coumarin, is recommended (Abraham et al., 2010). In rats, centrilobular hepatocellular necrosis is a prominent toxic feature in the acute phase, which may be induced by the reactive coumarin metabolites 3,4-epoxide and *o*-hydroxyphenylacetaldehyde (HPA) (Born et al., 1997, 2000; Cohen, 1979; Lake et al., 1989, 1994). Normally, reactive metabolites are detoxified by phase II metabolic reactions such as glutathione (GSH) conjugation and glucuronidation reactions. However, substrate saturation of these proteins leads to the covalent binding of reactive metabolites to intracellular proteins and DNA, thereby inducing toxicity (Lake et al., 2002; Vassallo et al., 2004). *In vitro* studies have demonstrated the involvement of hepatocellular metallothionein and lipid metabolic pathways in the onset of coumarin-induced toxicity (Kienhuis et al., 2006, 2009). However, the intracellular targets of reactive coumarin metabolites and the initial reactions that induce hepatocyte necrosis are unclear.

Numerous studies have reported that mitochondrial function is closely associated with the onset of drug-induced liver injury. In addition to producing adenosine triphosphate (ATP), the

Abbreviations: ADP, adenosine diphosphate; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CYP, cytochrome P450; ER, endoplasmic reticulum; GLDH, glutamate dehydrogenase; GSH, glutathione; γ-GTP, γ-glutamyl transpeptidase; HPA, *o*-hydroxyphenylacetaldehyde; PKA, protein kinase A; PT, permeability transition; RCI, respiratory control index; ROS, reactive oxygen species; SRP, signal recognition particle.

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mitochondria have numerous functions that are required for cell survival including lipid metabolism and the reduction of reactive oxygen species (ROS). Several hepatotoxicants including troglitazone, acetaminophen, and tamoxifen, which are converted into reactive metabolites and induce idiosyncratic hepatotoxicity, are known to act either alone or as a complex to inhibit mitochondrial function, thereby inducing hepatocellular necrosis (Begriche et al., 2011; Masubuchi et al., 2005; Meyer et al., 2013; Pessayre et al., 1999, 2012).

Reactive coumarin metabolites are generated by cytochrome P450 (CYP) 1A and CYP2E1 in the endoplasmic reticulum (ER) and mitochondria (Born et al., 2002). Therefore, saturation of the GSH conjugate formation may lead to mitochondrial toxicity. Although previous reports have indicated that coumarin alters lipid metabolism-associated gene expression and induces centrilobular hepatocellular steatosis, its mitochondrial effects remain unknown (Grasso et al., 1974; Kienhuis et al., 2009). Therefore, in this study, we evaluated the oral toxicity of a single dose as well as consecutive 4-day repeated doses of coumarin administered to rats susceptible to coumarin-induced hepatotoxicity. In particular, we investigated the mechanism of hepatocellular toxicity, focusing on the mitochondria.

2. Material and method

2.1. Chemicals

Coumarin was procured from Sigma–Aldrich Japan (Tokyo, Japan).

2.2. Animals

Five-week-old male Sprague–Dawley rats were purchased from Charles River Laboratories Japan Inc., and were allowed to acclimate for 1 week before they were used in the experiments. The rats were maintained at a temperature and humidity of 23.5 ± 2.0 °C and 55.0 ± 10.0%, respectively, with a 12-h light–dark cycle while feed and water were provided *ad libitum*. The experiments were performed according to the Ethical Guidelines for the Care and Use of Laboratory Animals established by the Committee for the Use of Experimental Animals of Suntory Business Expert Limited.

2.3. Experimental design

Animals were randomly allocated into six groups of six rats each. Coumarin (300 mg/kg) was administered by gavage as a single dose or consecutively for 4 days. The vehicle control group received corn oil (5 mL/kg) by a similar method. Table 1 shows the experimental design.

2.4. Sample collection

Four and 24 h after single administration of coumarin or 24 h after repeated dosing, blood samples were collected from the animals via the abdominal aorta under isoflurane inhalation anesthesia. Blood samples were centrifuged at 3000 rpm for 15 min and stored at −20 °C until used. After blood collection had been completed, the liver was excised, observed macroscopically, and weighed.

Table 1
Experimental design.

Group	Compound	Dosage (mg/kg)	Sampling point
1	Corn oil	–	4 h after single dosing
2	Coumarin	300	4 h after single dosing
3	Corn oil	–	24 h after single dosing
4	Coumarin	300	24 h after single dosing
5	Corn oil	–	24 h after 4 times dosing
6	Coumarin	300	24 h after 4 times dosing

2.5. Blood chemistry analysis

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), and total bilirubin were measured as the liver function markers using a Hitachi 7180 chemistry analyzer (Hitachi, Tokyo, Japan).

2.6. Histopathological examination

The rat livers were fixed in 10% neutral-buffered formalin, embedded in paraffin, and 2-μm sections were cut and stained with hematoxylin and eosin (H&E). The stained specimens were examined histopathologically.

2.7. Liver function tests

2.7.1. Hepatic GSH measurement

Measurements were obtained using the GSH Glo Assay kit (Promega, Madison, WI, USA). The rat livers were homogenized in a buffer solution and added to 96-well plates in duplicate. An equal volume of the GSH-Glo Reagent was added, and the plates were incubated at room temperature for 30 min. Luciferin Detection Reagent was added, and the plates were incubated for an additional 15 min at room temperature. The luminescence intensity was measured using a multi-plate reader. Liver extracts were stored at −70 °C until analyzed.

2.7.2. Aconitase activity measurement

Measurements were obtained using an Aconitase Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The rat livers were homogenized in a buffer solution, centrifuged, sonicated, and the protein concentration was diluted to 1000 μg/mL with assay buffer. The homogenate, an NADP + reagent, isocitric dehydrogenase, an enzyme mixture, and a fluorometric detector were added in duplicate to 96-well plates, and a multi-plate reader was used to measure the fluorescence intensity (excitation and emission wavelengths, 530–540 and 585/595 nm, respectively).

2.7.3. Mitochondrial respiratory activity

Mitochondrial respiration was measured as described previously (Gatley and Sherratt, 1976; Roos et al., 1982). Briefly, the excised rat livers were collected and homogenized on ice, followed by centrifugation at 4 °C and 700 rpm to obtain the supernatant, which was centrifuged again at 4 °C and 7000 rpm. The precipitate obtained was the mitochondrial fraction, which was dissolved in 1 mL of measurement buffer containing 130 mM sucrose, 50 mM KCl, 5 mM HEPES, 5 mM KH_2PO_4, and 5 mM $MgCl_2$ (pH 7.4). Respiration was measured using a precision dissolved oxygen measurement system (SI928, Strathkelvin Instruments Ltd, North Lanarkshire, Scotland). Succinic acid was used to assess the complex II-dependent respiration, and glutamic acid and malic acid were used to assess the complex I-dependent respiration. Respiratory ability was assessed using the ratio (respiratory control index [RCI]) of the oxygen consumption (state 3) in the presence of adenosine diphosphate (ADP) and the oxygen consumption (state 4) in the absence of ADP.