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Identification of enzymes responsible for dantrolene metabolism in the human liver: A clue to uncover the cause of liver injury

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ABSTRACT

Dantrolene is used for malignant hyperthermia during anesthesia, and it sometimes causes severe liver injury as a side effect. Dantrolene is metabolized to acetylaminodantrolene, which is formed via the reduction of dantrolene to aminodantrolene and subsequent acetylation. Formation of hydroxylamine during the metabolic process may be associated with liver injury. We identified the enzymes responsible for dantrolene metabolism in humans to elucidate the mechanism of liver injury. Dantrolene reductase activity was not detected in human liver microsomes, but it was detected in cytosol. Formation was increased in the presence of N^1-methylnicotineamide, which is an electron donor to aldehyde oxidase 1 (AOX1). Potent inhibitors of AOX1 and a correlation study with a marker of AOX1 activity, namely phthalazine oxidase activity, in a panel of 28 human liver cytosol samples supported the role of AOX1 in dantrolene reduction. Acetylaminodantrolene formation from aminodantrolene was highly detected in recombinant *N*-acetyltransferase (NAT) 2 rather than NAT1. A glutathione trapping assay revealed the formation of hydroxylamine via an AOX1-dependent reduction of dantrolene but not via hydroxylation of aminodantrolene. In conclusion, we found that AOX1 and NAT2 were responsible for dantrolene metabolism in humans and that AOX1-dependent metabolism determines dantrolene-induced liver injury.

1. Introduction

Dantrolene is a skeletal muscle relaxant that is used for the treatment of malignant hyperthermia and malignant syndrome. Dantrolene was approved for sale in the US and the UK in 1974 [1–3]. However, the US Food and Drug Administration ordered pharmaceutical companies to include a warning for liver injury in a black box on the package insert in 1976. Nineteen of 1044 patients (1.8%) who received dantrolene for 60 days or more developed liver injury, 6 of 19 patients developed hepatitis accompanied by jaundice, and 3 patients died [3]. However, the mechanism of the onset of dantrolene-induced liver injury is not known. Reactive metabolites formed via metabolic reactions are likely involved in drug-induced toxicity [4]. The metabolic pathways and enzymes responsible for this injury should be determined to clarify whether reactive metabolite(s) are formed during dantrolene metabolism.

Acetylaminodantrolene and 5-hydroxydantrolene are major metabolites of dantrolene in humans (Fig. 1). Intravenous administration of 25 mg of a ^{14}C-labelled dantrolene sodium hydrate preparation to adults resulted in the excretion of 5-hydroxydantrolene and acetylaminodantrolene in urine at 21.6% and 0.5% of the administered dose, respectively, and 2.2% and 10.8% of the dose, respectively, were excreted in the feces. Jayyosi et al. [5] reported that CYP1A1, CYP1A2, and CYP3A were involved in the formation of 5-hydroxyldantrolene in rats. We previously evaluated the cytotoxicity of dantrolene using HepG2 cells overexpressing human CYP3A4 and assumed that human

Abbreviations: AKR, aldo-keto reductase; AOX1, aldehyde oxidase 1; 11β-HSD, 11β-hydroxysteroid dehydrogenase; CBR, carbonyl reductase; CYP, cytochrome P450; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase; GSH, glutathione, reduced form; HLC, human liver cytosol; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MNA, N^1-methylnicotinamide; MRM, multiple reaction monitoring; NADPH, nicotinamide adenine dinucleotide phosphate; NAT, *N*-acetyltransferase; NQO1, NAD(P)H: quinone oxidoreductase 1; RA, rapid acetylator; SA, slow acetylator; SIM, single ion monitoring; SNP, single nucleotide polymorphism; XOR, xanthine oxidoreductase

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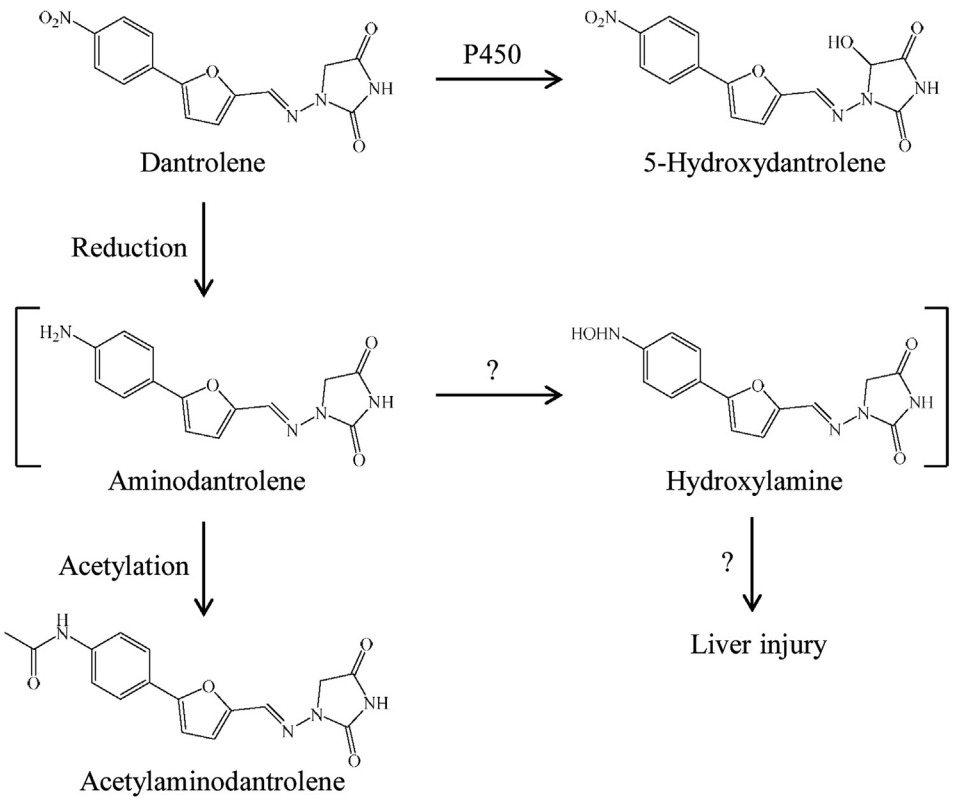

Fig. 1. Proposed metabolic pathways of dantrolene in humans.

CYP3A4 would catalyze the 5-hydroxylation of dantrolene. However, CYP3A4 overexpression did not produce dantrolene cytotoxicity [6]. These results suggested that 5-hydroxydantrolene was unlikely to be involved in dantrolene-induced liver injury. Another metabolite, acetylaminodantrolene, is likely formed via the reduction of dantrolene to aminodantrolene with subsequent acetylation [7], but this reduction was not experimentally demonstrated. Aminodantrolene is a source for hydroxylamine production, which is generally recognized as a perpetrator of adverse reactions because of its instability and binding of macro molecules [8].

The present study elucidated the metabolic pathway for acetylaminodantrolene formation from dantrolene and the metabolic reactions involved in hydroxylamine formation to identify the mechanisms of dantrolene-induced liver injury. We also identified the enzymes responsible for dantrolene metabolism.

2. Materials and methods

2.1. Materials

Dantrolene sodium salt, phthalazine, reduced glutathione (GSH), and acetyl-CoA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Human liver microsomes (HLM) (pooled donors, n = 50), human liver cytosol (HLC) (pooled donors, n = 150), and Supersomes human *N*-acetyltransferase (NAT) 1 and NAT2 that are cytosolic fractions of insect cells infected with baculovirus containing human NAT cDNAs were obtained from Corning (Corning, NY). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and the oxidized form of β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Oriental Yeast (Tokyo, Japan). N^1-Methylnicotinamide (MNA) was purchased from Cosmo Bio (Tokyo, Japan). Phthalazone was purchased from Tokyo Chemical Industry (Tokyo, Japan). A mouse anti-human AOX1 antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). IRDye680-conjugated goat anti-mouse IgG antibody and Odyssey blocking buffer were obtained from LI-COR Biosciences (Cambridge, UK). All other chemicals were of the highest quality or analytical grade that could be obtained commercially.

2.2. Human tissues

Human liver samples from 28 donors (19 Caucasians, 6 Hispanics, 2 Asians, and 1 Black; 17 males, 11 females) were supplied by the National Disease Research Interchange (Philadelphia, PA) via the Human and Animal Bridging Research Organization (Chiba, Japan). HLC was prepared according to a previously reported method [9]. The ethics committees of Kanazawa University (Kanazawa, Japan) approved the use of human tissue samples.

2.3. Synthesis of aminodantrolene

HCl (2.5 mL or 4 M) was added to 168 mg (0.50 mmol) dantrolene sodium salt, and the mixture was stirred for 30 min at room temperature. The resultant precipitation was collected via filtration and washed with diethyl ether (3 mL × 3) to obtain dantrolene as a yellow solid. Palladium (5%) on carbon (6.0 mg) was added to the solution of dantrolene (31 mg, 0.10 mmol) in absolute ethanol (5.0 mL), and the mixture was vigorously stirred for 1 h at room temperature under a hydrogen atmosphere (balloon). The mixture was filtered through a Celite® pad, and the solvent was removed under reduced pressure. The resultant crude product was washed with methanol (3 mL × 3) to yield aminodantrolene as a brown solid. An ^{1}H NMR spectrum was recorded on a JEOL JNM ECA600 (600 MHz) spectrometer. Chemical shifts (δ) are quoted relative to tetramethylsilane (δ 0 ppm). Coupling constants (*J*) are given in Hz. A mass spectrum was recorded on a JEOL JMS-T100TD spectrometer using direct analysis in real-time (DART) mode: ^{1}H NMR (600 MHz, DMSO-d_6, 293 K) δ 11.21 (1H, s), 7.65 (1H, s), 7.43 (2H, app. d, J = 8.4 Hz), 6.85 (1H, d, J = 3.6 Hz), 6.72 (1H, d, J = 3.6 Hz), 6.61 (2H, app. d, J = 8.4 Hz), 5.50 (2H, br-s), 4.32 (2H, s); HRMS (DART–) *m/z*: calcd for $C_{14}H_{11}N_4O_3$ $[M-H]^-$ 283.0831, found