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The metabolisms of agaritine, a mushroom hydrazine in mice

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Abstract

The mushroom hydrazine agaritine was measured in mouse plasma and urine using LC/MS/MS, which is highly specific. Agaritine concentration peaked 20 min after oral administration to mice (4.0 and 40 mg/kg). The concentration gradually decreased and returned to the basal level in 100 min. The maximum concentration, the time to the maximum concentration, and the half life were 0.37 μg/ml plasma, 0.33 h, and 0.71 h, respectively after administration of agaritine at 40 mg/kg body weight. One agaritine metabolite was found in the plasma and the urine from agaritine-administered mice. The structure of metabolites of agaritine by γ-GT was next investigated using LC/MS. HMPH proved to be generated from agaritine. The oxidative stress marker 8-OHdG was detected in agaritine-administered mouse urine. After administration, the 8-OHdG level immediately tripled, and then decreased to the control level over 48 h. Its level then elevated again and remained high for 11 days. These results suggest that agaritine quickly metabolizes and disappears in the plasma, whereas DNA damage lasts for a long time after a single administration of agaritine to mice.

Keywords: Agaritine; Mushroom; Plasma; Urine; LC/MS/MS; Phenylhydrazine; Metabolism

1. Introduction

Agaricus mushrooms (*Agaricus* spp.) including the cultivated mushrooms, *Agaricus bisporus*, contain substantial amounts of aromatic hydrazines. The most abundant is genotoxic agaritine, [β-*N*-(γ-L(+)-glutamyl)-4-(hydroxymethyl)phenylhydrazine] (Kelly et al., 1962; Gigliotti and Levenberg, 1964). This L-glutamic acid-containing phenylhydrazine is susceptible to oxidation. Toth and Erickson demonstrated that the administration of uncooked mushrooms to mice induced a significant increase in the number of bone and forestomach tumors in both sexes, and in the occurrence of lung tumors in males (Toth and Erickson, 1986; Toth et al., 1998). Bladder implantation of methanol extracts of fresh mushrooms induced cancer in the bladder epithelium (Hashida et al., 1990). Toth et al. reported that backed mushrooms could induce tumors (Toth et al., 1997). Ethanolic and aqueous extracts from *A. bisporus* demonstrated mutagenicity in the Ames test (von Wright et al., 1982). The carcinogenicity and mutagenicity of these mushrooms can be attributed to agaritine and 4-(hydroxymethyl)phenylhydrazine (HMPH) or the 4-(hydroxymethyl)benzenediazonium ions (HMBD), both of which are believed to be formed by the enzymatic degradation of agaritine, resulting in the loss of the γ-glutamyl group (Toth et al., 1978, 1981; Toth and Nagel, 1981; Walton et al., 1997). It has been previously reported that HMPH and HMBD are highly unstable and exhibit carcinogenicity (Toth et al., 1978, 1981; Ross et al., 1982; Walton et al., 1997). HMPH and HMBD are thought to be potent components that exert genotoxicity. However, they are virtually impossible to detect, because of their instability. The formation of HMPH was speculated by a UV absorption shift from 273 to 325 nm in the presence of sodium glyoxylate (Gigliotti and Levenberg, 1964).

Ross et al. revealed the recovery of agaritine from the gastro-intestinal tract of 3 mg-agaritine-administered mice using the HPLC–UV method. They also showed the agar-

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itine contents in the stomach, small intestine, caecum, and large intestine were successfully determined, but failed to detect agaritine in the blood because of the low sensitivity of the UV method (Ross et al., 1982). Walton et al. reported an investigation of the agaritine content in blood and urine using a scintillation counting method after ^{14}C-labeled agaritine had been administered to rats and mice. In the mouse, radioactivity in the blood peaked after 30 min, and then gradually decreased over a period of 12 h (Walton et al., 2000). The ^{14}C-labeled-agaritine-based experiment, however, has a limitation in that the radioactivity detected in the blood cannot distinguish between intact agaritine and degradative products including ^{14}C-labeled moieties. The fate of agaritine has thus up to now remained unclear.

More recently, we have established a new method for agaritine determination in mouse plasma and urine using HPLC coupled with tandem mass spectrometry (LC/MS/MS) that has high sensitivity and specificity (Kondo et al., 2006a,b). In the present study, the agaritine content in the plasma and the urine from agaritine-administered mice was determined using this method in addition to LC/MS/MS investigation of agaritine metabolism and DNA adduct (Singh and Farmer, 2006), the structure of the degraded products, DNA damage by agaritine in mice. This should facilitate the assessment of the health risk to humans.

2. Materials and methods

2.1. Chemicals and LC/MS/MS conditions

Agaritine and agaritine-COOH ([β-*N*-(γ-L(+)-glutamyl)-4-carboxyphenylhydrazine]) were synthesized according to the methods of Wallcave et al. and Hoesch et al. with minor modifications (Wallcave et al., 1979; Hoesch and Datta, 1987). Briefly, reaction mixtures in the reduction reaction of carboxylic acid to hydroxymethyl group in *p*-hydrazinobenzoic acid moiety were recrystallized from acetone/diethyl ether (1:4) to remove byproducts. Total yield was 17%.

The purities of the synthetic compounds were >95% by HPLC (254 nm) and >95% by ^1H NMR (500 MHz, DMSO-d_6 and DMSO-d_6-D_2O). The synthetic agaritine and agaritine-COOH were stored at −80 °C under a N_2 gas atmosphere. Standard stock solutions were prepared in methanol and stored at below −20 °C before use. The both compounds were stable in methanol for 1 week at −20 °C.

The structures of synthetic agaritine and agaritine-COOH were examined using 2-D NMR and high-resolution (HR) mass analysis. The results of ^1H, ^{13}C, and 2D NMR (HMBC and HMQC) were recorded using an ECA 500-MHz FT NMR spectrometer (JEOL, Japan).

Chemical shifts (δ) are described in ppm using tetramethylsilane (TMS) as a reference. Coupling constants (J) are given in Hz. HR-FABMS was performed in positive mode to identify them described previously (Kondo et al., 2006).

LC/MS/MS measurements were performed using a PE SCIEX model API 3000 triple–quadrupole mass spectrometer coupled to an Agilent 1100 HPLC system, including a G1315 photodiode array detector and a 3-μm Shiseido Capcell Pak AQ column (2.0 × 250 mm). Gradient conditions ranged from 99% water containing 0.01%AcOH–1%MeOH to 90% MeOH–10% water containing 0.01% AcOH. The analyte was detected using electrospray ionization in negative mode. Multiple-reaction-monitoring (MRM) was performed using characteristic fragmentation ions (m/z 266 > 248 and 266 > 122) for agaritine. A full scan analysis (m/z 50–1000) was carried out to analyze agaritine metabolites in plasma and urine. The parameters for the LC/MS/MS analysis of agaritine were as follows. Ionspray voltage (IS) = −4500 V; collision gas = 6; focusing and entrance potentials (FP and EP) = −60 V and −20 V, and temperature (T) = 500 °C, respectively. A switching valve led the column eluents to the mass spectrometer while the analytes were being eluted. The data were acquired and calculated using Analyst 1.4.1 software (PE SCIEX). Metabolite ID 1.3 was used to analyze agaritine metabolites.

2.2. Animals

Male ddY mice (7 weeks, 26–30 g, average = 28.07 ± 0.81 g) were purchased from SLC (Shizuoka, Japan) and were housed for one week. Each cage contained three or four mice. The animals were kept on a 12 h light/dark cycle, at room temperature of 24 ± 1 °C, humidity of 55 ± 5%, with free access to food (CRF-1, Oriental Yeast Co Ltd.) and water. The animals were used according to the guidelines of National Institute of Health Sciences and the Ministry of Health, Labour and Welfare of Japan.

2.3. Agaritine in plasma and pharmacokinetic study

The synthetic agaritine in MilliQ water (4.0 and 40.0 mg/kg) was administered to male ddY mice (8 weeks) by gavage after overnight starvation. The 33 mice were used for the time course of agaritine content in mouse plasma. Three mice were anesthetized with diethyl ether and the blood samples were withdrawn from them to prepare the plasma every 20 min until 180 min after agaritine-administration. MilliQ water was administered to the control mice. The pharmacokinetic parameters were calculated using the PK program @PKANS (D Three, Japan).

Mouse plasma from agaritine-administered mice was prepared as follows; blood was collected 20 min after oral administration of agaritine to mice and immediately placed on ice before centrifugation (10,000 rpm, 2 min). After centrifugation, the plasma (200 μL) was deproteinized by acetonitrile (750 μL), and then evaporated the solvent. The residue was dissolved with mobile phase (600 μL).

2.4. Agaritine in urine

For the analysis of agaritine and its metabolites in mouse urine, 20 mice were divided into five groups (one control and four agaritine-administration groups). Each group of four mice was housed in a metabolic cage immediately after oral administration of agaritine or MilliQ water to mice, and then urine was collected in 50 ml Falcon tubes every 12 or 24 h. Urine and fecal matter were separated by metabolic cages. Falcon tubes were covered with aluminum foil to avoid oxidative degradation.

2.5. Oxidative stress

Forty mice were divided into 5 control and 5 agaritine administration groups. Each group comprised 4 mice. The synthetic agaritine was dissolved in MilliQ water prior to this experiment. Mice were put in metabolic cages immediately after agaritine or MilliQ water administration. Mouse urine was then collected at indicated times (12, 24, 48, 72, 216, 264 h). Urine samples were diluted to 1:10 for ELISA. Creatinine contents were also measured by an enzymatic method. Oxidative damage to mice was evaluated by quantifying an oxidative stress marker 8-OHdG (8-hydroxy-2′-deoxyguanosine) according to the manufacturer's instruction. This marker was measured using a high sensitivity 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan). MDA (malondialdehyde) levels were also measured using HPLC after the reaction of MDA with thiobarbituric acid (TBA).

2.6. Agaritine degradation by γ-glutamyltranspeptidase (γ-GT)

Sodium glyoxylate (23.5 mg, Sigma, St. Louis, MO) and bovine γ-GT (29.2 mg, Sigma EC 2.3.2.2) were added to agaritine (3.6 mg) in 2.0 ml of