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Identification of glutathione and related cysteine conjugates derived from reactive metabolites of methyleugenol in rats☆

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ABSTRACT

Methyleugenol (ME), an alkenylbenzene compound, is a constituent of many foods and is used as flavoring agent in foodstuffs and as fragrance in cosmetics. It has been reported that exposure to ME can cause carcinogenicity, cytotoxicity, and genotoxicity. Metabolic activation is suggested to play an important role in ME-induced toxicities. Electrophilic metabolites of ME have been reported to covalently bind to proteins and nucleic acids. The objective of this study was to identify GSH and related cysteine conjugates derived from these reactive metabolites *in vivo*. Five biliary GSH (M1-M5) and four urinary cysteine conjugates (M6-M9) were detected in rats given ME. M1 and M2 were GSH conjugates derived from the epoxide of ME. M3, M4, and M5 were GSH conjugates possibly generated from the corresponding α,β-unsaturated aldehyde, carbonium ion, and quinone methide, respectively. The structures of the GSH conjugates were verified by chemical synthesis. Cysteine conjugates M6, M7, M8, and M9 were found to correspond to the respective M1/M2, M3, M4, and M5. The data obtained from the present *in vivo* work facilitate the understanding of mechanism action of ME toxicities and may provide information suitable for use as biomarkers of exposure to ME.

1. Introduction

Methyleugenol (ME, 4-allyl-1,2-dimethoxybenzene, **1**, Scheme 1) is a naturally occurring constituent of essential oils of a number of plants [1], and is widely used as spice and medicine. The National Toxicology Program (NTP) reported carcinogenic activity of ME in F344/N rats and B6C3F1 mice [2]. The European Union Scientific Committee on Food also concluded that ME and the related herb-based alkenylbenzenes estragole and safrole were carcinogenic and genotoxic and their use should be restricted [3–6]. Studies have demonstrated ME-derived DNA adduction in rats [7] and in cultured human HepG2 cells [8] after exposure to ME, using ^{32}P-postlabeling technique. In addition, ME and several metabolites were found to induce DNA strand breaks by comet assay [9,10]. Cartus et al. reported a virtually complete pattern of microsomal (rat, bovine, and human) and hepatocellular (rat) metabolites of ME, suggesting the formation of several reactive metabolites possibly involved in carcinogenicity, organ toxicity, and immune reactions [11,12]. Numbers of studies have demonstrated that ME and its proximate carcinogenic metabolite 1′-hydroxy ME

Abbreviations: ME, methyleugenol; GSH, glutathione; GS-1′OME, 3′-(glutathione-*S*-yl)-1′-oxo-2′,3′-dihydromethyleugenol; GSTs, glutathione-*S* -transferases; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; RLMs, rat liver microsomes; MRM, multiple-reaction monitoring; CE, collision energy; CXP, cell exit potential; DP, declustering potential; EP, entrance potential; EPI, enhanced product ion; *m*-CPBA, *meta*-chloroperbenzoic acid; THF, tetrahydrofuran; DDQ, 2,3-dichloro-5,6-dicyanoquine; GS-3′OME, 1′-(glutathione-*S*-yl)-3′-oxo-1′,2′-dihydromethyleugenol; NAC, *N*-acetylcysteine.

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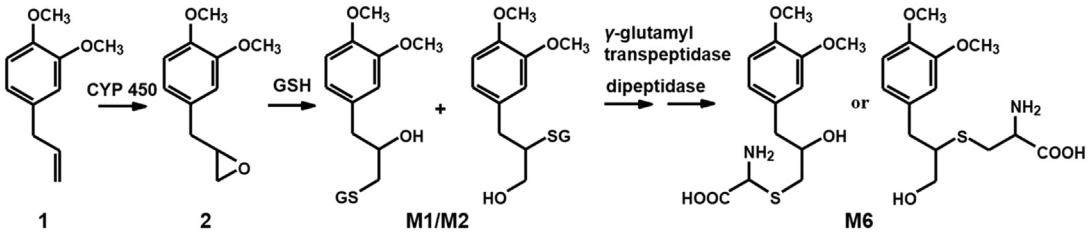

Scheme 1. Proposed pathways for the formation of M1/M2 and M6.

induced liver tumors in mice and rats [2,13].

There are three possible metabolic options available for ME, including 1) *O*-demethylation of ME; 2) 1′-hydroxylation of ME to 1′-OH ME widely recognized as the primary toxification pathway [13–15]; and 3) epoxidation of the side chain alkene to 2′,3′-epoxide. Cartus et al. identified nine phase I metabolites of ME in microsomes and primary rat hepatocytes [11,12]. Al-Subeihi et al. concluded that 1′-OH ME could be metabolized to 1′-oxomethyleugenol and 1′-sulfooxymethyleugenol by monitoring the formation of 3′-(glutathione-*S*-yl)-1′-oxo-2′,3′-dihydromethyleugenol (GS-1′OME) and GS-1′-methyleugenol in rat/human microsomal incubations [16,17]. GSH conjugation is usually considered as a detoxification mechanism and plays an important role against toxicity of electrophilic agents. Previous studies have shown that GSH plays an important role in the detoxification of safrole [18], estragole, and allylbenzene [19,20]. ME may also be detoxified through glutathione *S*-transferase-mediated or non-enzymatic pathways to form GSH conjugates *in vivo* mainly excreted into bile. GSH conjugates can be considered as biomarkers to exposure to reactive intermediates of ME. Moreover, the characterization of GSH conjugates may assist us to elucidate the structures of the corresponding reactive intermediates. Cysteine conjugates are generally considered to be derived from GSH conjugates through subsequent hydrolytic cleavage of glutamic acid and glycine residues [21–23] and excreted through urine. These GSH and cysteine conjugates derived from the reactive metabolites of ME have not been well studied to serve biomarkers for ME exposure and to infer ME metabolism *in vivo*. This study aimed to identify ME-derived GSH and related cysteine conjugates in bile and urine of rats.

2. Materials and methods

2.1. Chemicals and materials

Methyleugenol (purity, ≥ 99.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Eugenol (purity, ≥ 98.0%) was supplied by Beijing Hua-Mai-Ke Biotech Co., Ltd (Beijing, China). Glutathione (GSH) and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). All organic solvents were purchased from Fisher Scientific (Springfield, NJ). Ascorbic acid was offered by the Pharmacy Warehouses of Shenyang Pharmaceutical University (Shenyang, China). *meta*-chloroperbenzoic acid (*m*-CPBA), *N*-bromosuccinimide, and 2,3-dichloro-5,6-dicyanoquine (DDQ) were acquired from Aladdin Reagent Co., Ltd (Shanghai, China). All reagents and solvents were either analytical or HPLC grade.

2.2. Animal experiment

Animal studies were approved by the Ethics Review Committee for Animal Experimentation of Shenyang Pharmaceutical University. Male Sprague-Dawley rats (200–220 g, age of 6–7 weeks), purchased from the Animal Center of Shenyang Pharmaceutical University, were allowed free access to standard laboratory chow and water. They were housed in regular cages in a controlled housing environment with a 12-h cycle of light and darkness. One group of the rats were anesthetized with chloral hydrate, and the bile ducts were cannulated with PE-10 tubing. After collection of the bile for 20 min, ME dissolved in corn oil was administered intraperitoneally at a moderate dose of 100 mg/kg according to reported literature [16,17], and the bile was collected for 4 h. After cannulating the bile duct, animals were used immediately, then killed without regaining consciousness. The other group of rats treated (i.p.) with the same dose of ME were placed in metabolic cages. Urine samples were collected at room temperature for 24 h after the treatment with ME. The rats were allowed free access to water during the whole experiments. Blank urine samples were collected prior to the ME treatment. The bile and urine samples were stored at −80 °C until analysis.

2.3. Sample preparation for LC-MS/MS analysis

To the bile or urine samples were triple volumes of acetonitrile added, and the resulting samples were vortexed for 3 min and centrifuged at 16,000 rpm (4 °C) for 10 min. The supernatants were concentrated to dryness under a stream of N_2 at 40 °C. The resulting residues were reconstituted with 200 μL of acetonitrile/water (50/50, *v/v*), followed by centrifugation. An aliquot (5 μL) of the supernatants was injected into a LC-MS/MS system for analysis.

2.4. Microsomal incubations

Male SD rat liver microsomes (RLMs) were prepared, according to a procedure reported by our laboratory [24]. ME (100 μM) was mixed with RLMs (1.0 mg protein/mL) in 145 μL buffer (pH 7.4) supplemented with GSH (1.0 mM) as a trapping agent. The total volume was 500 μL. The reactions were started by addition of NADPH (1.0 mM). Control samples containing no NADPH were included. After 2 h incubation at 37 °C, the reactions were terminated by mixing with double volumes of ice-cold acetonitrile. The mixtures were vortexed and centrifuged, and the supernatants were concentrated to dryness, reconstituted, centrifuged, and analyzed by LC-MS/MS.

2.5. Chemical synthesis of 3′-(glutathione-S-yl)-2′-hydroxy-2′,3′-dihydromethyleugenol and 2′-(glutathione-S-yl)-3′-hydroxy-2′,3′-dihydromethyleugenol (M1 & M2, m/z 502)

The synthesis started with epoxidation of ME, followed by reaction with GSH. The epoxy intermediate was prepared using a reported method [11]. Briefly, ME (17.8 mg, 0.1 mmol) dissolved in CH_2Cl_2 (1 mL) was mixed with a saturated solution of sodium bicarbonate (0.5 mL), followed by addition of *m*-chloroperbenzoic acid (*m*-CPBA, 80%, 0.12 mmol) dissolved in CH_2Cl_2 (1 mL). The resulting mixture was vigorously stirred at room temperature for 4 h. The organic layer was collected, and the aqueous phase was extracted three times with CH_2Cl_2, and the combined organic phases were concentrated to dryness under vacuum. The residues