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Oxidative metabolism of curcumin-glucuronide by peroxidases and isolated human leukocytes

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ABSTRACT

Conjugation with glucuronic acid is a prevalent metabolic pathway of orally administrated curcumin, the bioactive diphenol of the spice turmeric. The major in vitro degradation reaction of curcumin is autoxidative transformation resulting in oxygenation and cyclization of the heptadienedione chain to form cyclopentadione derivatives. Here we show that curcumin-glucuronide is much more stable than curcumin, degrading about two orders of magnitude slower. Horseradish peroxidase-catalyzed oxidation of curcumin-glucuronide occurred at about 80% of the rate with curcumin, achieving efficient transformation. Using LC–MS and NMR analyses the major products of oxidative transformation were identified as glucuronidated bicyclopentadione diastereomers. Cleavage into vanillin-glucuronide accounted for about 10% of the products. Myeloperoxidase and lactoperoxidase oxidized curcumin-glucuronide whereas tyrosinase and xanthine oxidase were not active. Phorbol ester-activated primary human leukocytes showed increased oxidative transformation of curcumin-glucuronide which was inhibited by the peroxidase inhibitor sodium azide. These studies provide evidence that the glucuronide of curcumin is not an inert product and may undergo further enzymatic and non-enzymatic metabolism. Oxidative transformation by leukocyte myeloperoxidase may represent a novel metabolic pathway of curcumin and its glucuronide conjugate.

1. Introduction

Conjugation with glucuronic acid or sulfate is the major pathway of phase II metabolism of curcumin following oral administration [1,2]. Concurrent with conjugation is the stepwise reduction of the double bonds of the heptadienedione chain of curcumin to yield di-, tetra-, hexa-, and octahydrocurcumin [3]. Rapid metabolism by conjugation and reduction together with poor intestinal absorption result in low plasma levels of free curcumin in man, even when large oral doses (8–12 g) are ingested [4]. The near absence of free curcumin in human and animal plasma makes it difficult to rationalize its biological and therapeutic effects [5].

An attractive hypothesis, therefore, is to invoke metabolites of curcumin as mediators of its biological effects. Such has been suggested for the glucuronic acid conjugate (Fig. 1A) [6], for the reduced metabolites, especially tetra- and hexahydrocurcumin [7], for the degradation products formed via chain cleavage (vanillin, ferulic acid, and feruloylmethane) [8,9], and, lastly, for its oxidative metabolites, the formation of which has been recognized only recently [10–12]. Few studies have tested the biological effects of curcumin-glucuronide in cell culture based assays, and these implied that the glucuronide is biologically inert or less active in the growth inhibition of cancer cells and suppression of inflammatory markers and cytokines [13,14]. The reduced metabolites appear to be able to recapitulate some but not all biological activities of curcumin [15–18]. For example, tetrahydrocurcumin was active in azoxymethane-induced colon carcinogenesis [15–18] but less active in the inhibition of COX-2 in human colonic

Abbreviations: BCP, bicyclopentadione; ESI, electrospray ionization; HRP, horseradish peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; PMA, phorbol-12-myristate-13-acetate; SRM, elected reaction monitoring.

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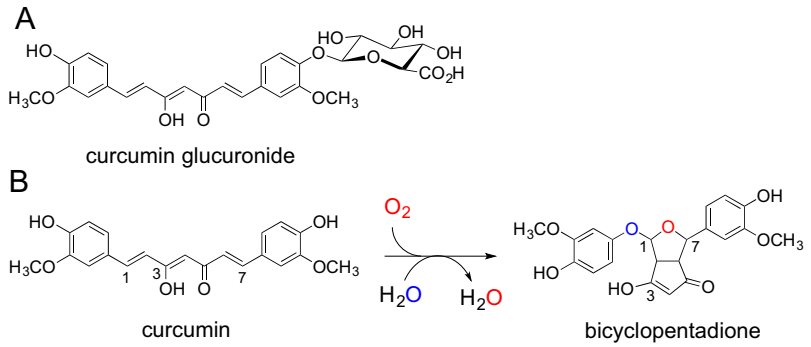

Fig. 1. (A) Structure of curcumin-glucuronide. (B) Oxidative transformation of curcumin to its bicyclopentadione derivative. The transformation occurs as a spontaneous autoxidation as well as catalyzed by peroxidases and proceeds with the incorporation of O_2 followed by an exchange of water [10,21].

epithelial cells [15–18]. The cleavage products vanillin and ferulic acid show greater overlap with the activities of curcumin [9,19] – the relevance of these observations, however, has been contested when the initial finding of Wang and co-workers that vanillin and ferulic acid are minor degradation products of curcumin was confirmed, and the mechanism of the degradation reaction was elucidated [20–22]. The major pathway of non-enzymatic degradation of curcumin at physiological pH is an oxidative transformation leading to dioxygenated bicyclopentadione (BCP) diastereomers (Fig. 1B) [21]. Oxidative transformation of curcumin occurs spontaneously as autoxidation and can also be catalyzed by the peroxidase activity of cyclooxygenase-2 [10].

We sought to determine whether curcumin-glucuronide undergoes non-enzymatic and enzymatic oxidative transformation. We describe conditions for oxidative transformation, identify products formed, and tested different peroxidases for in vitro catalysis. Oxidative transformation catalyzed by activated human leukocytes indicated that this may represent a novel metabolic pathway of curcumin.

2. Materials and methods

2.1. Materials

Curcumin was synthesized from vanillin and acetylacetone as described [23]. The isotopic standards d_6-curcumin and d_6-bicyclopentadione were synthesized as described [24]. Horseradish peroxidase (P8250; Type-II, 5 kU/ml; 25.9 mg/ml), myeloperoxidase (M6908), lactoperoxidase (61328), and tyrosinase (T3824) were purchased from Sigma (St. Louis, MO), xanthine oxidase (682151) was from EMD Millipore (Billerica, CA). β-Glucuronidase was from MP Biomedicals (Santa Ana, CA). Chemicals were purchased from Sigma (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA) and used at highest grade available.

2.2. Synthesis of curcumin-glucuronide

Curcumin-glucuronide was synthesized following a protocol by Moon et al. [25]. Curcumin (100 mg, 0.27 mmol) and acetobromo-α-D-glucuronic acid methyl ester (500 mg, 1.26 mmol) were dissolved in 5 ml dimethylformamide. K_2CO_3 (100 mg, 0.72 mmol) was added, and the solution was stirred for 2 h at room temperature. Then 30 ml cooled H_2O were added and the solution was acidified using formic acid. Acidification resulted in the formation of a precipitate which was collected by centrifugation (4 °C, 5000 rpm, 20 min). The precipitate was washed with 5 ml 0.2% formic acid and dissolved in 10 ml MeOH/$CHCl_3$ (1:1, by vol.). The solvent was evaporated and the residue was dissolved in 5 ml dry MeOH. In order to remove the acetyl moieties 150 µl NaOH (28% in MeOH) were added, and the solution was stirred for 30 min at 4 °C. The methyl ester was hydrolyzed by treatment with 5 ml H_2O for 30 min at room temperature. Then 2 N HCl (370 µl) and a few drops of formic acid were added to acidy the solution. The solution was filtered, and the solvent evaporated. The product was purified by RP-HPLC (Econosil C18 column, 250 mm × 10 mm) eluted with a solvent of MeCN/H_2O (45/55) with 0.01% acetic acid at a flow rate of 4 ml/min.

2.3. Transformation of curcumin-glucuronide

Curcumin-glucuronide (25 µM) was added to 500 µl 20 mM Na-phosphate buffer pH 7.5. The reaction was monitored in a UV/Vis spectrophotometer by repetitive scanning from 700 to 220 nm every 1 or 2 min or by following the disappearance of the chromophore at 430 nm in the time drive mode. To some reactions $K_3Fe(CN)_6$ (15 µM final; from a 5 mM stock solution in water) or horseradish peroxidase (0.01 U/ml) and H_2O_2 (40 µM) were added. For the initial chromatographic analyses, an HRP-oxidized samples was hydrolyzed with β-glucuronidase, conducted at pH 4 for one hour at 37°.

Lactoperoxidase (0.444 U) was added to curcumin-glucuronide (40 µM) and H_2O_2 (40 µM) in 500 µl 100 mM K-phosphate buffer pH 6 containing 0.25% BSA. Myeloperoxidase (0.64 U) was added to curcumin-glucuronide (40 µM) and H_2O_2 (20 µM) in 500 µl 50 mM K-phosphate buffer pH 7 with 0.25% BSA. Tyrosinase (1162 U) was added to curcumin-glucuronide (40 µM) in 500 µl 50 mM K-phosphate buffer pH 6.5 with 0.25% BSA. Xanthine oxidase (0.2 U) was added to curcumin-glucuronide (40 µM) in 500 µl 50 mM K-phosphate buffer pH 7.5 with 0.25% BSA. The enzymatic reactions were monitored in a UV/Vis spectrophotometer by repetitive scanning from 700 to 220 nm every 1 or 2 min.

A large scale incubation of curcumin-glucuronide (1 mg) with 50 µM $K_3Fe(CN)_6$ was conducted in 40 ml of buffer for 20 min. Products were extracted using a Waters HLB cartridge and products were eluted with MeOH. Samples were analyzed by HPLC using a Waters T3 column (250 mm × 4.6 mm). Products were eluted using a linear gradient of 20%– 80% MeCN in H_2O containing 0.05% acetic acid within 20 min at a flow rate of 1 ml/min.

2.4. Leukocyte isolation and incubations

The study was approved by the Vanderbilt University Medical Center IRB. Normal healthy volunteers were enrolled and gave written informed consent. Blood was drawn (45 ml) from a forearm vein into a syringe that contained 10 ml of a 6% dextran solution and 4.5 ml of sodium citrate. The syringe was kept upright for 60 min to allow red cells to settle. The upper layer containing leukocytes was removed, centrifuged, and the pellet was washed with PBS. Remaining red blood cells were lysed by incubation with H_2O for 30 s. Tonicity was restored by adding 10× PBS. Leukocytes