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Original Contribution

Structure-activity relationship of C6-C3 phenylpropanoids on xanthine oxidase-inhibiting and free radical-scavenging activities

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Abstract

We employed the techniques of DNA relaxation, DPPH (1,1-diphenyl-2-picrylhydrazyl hydrate), and DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide)-electron spin resonance (ESR), to study the effects of reactive oxygen species (ROS) suppression by 11 selected C6-C3 phenylpropanoid derivatives under oxidative conditions. We also investigated the effects of the derivatives on the inhibition of xanthine oxidase (XO) activity, and the structure-activity relationships (SARs) of these derivatives against XO activity were further examined using computer-aided molecular modeling. Caffeic acid was the most potent radical scavenger among the 11 test compounds. Our results suggest that the chemical structure and number of hydroxyl groups on the benzene ring of phenylpropanoids are correlated with the effects of ROS suppression. All test derivatives were competitive inhibitors of XO. The results of the structure-based molecular modeling exhibited interactions between phenylpropanoid derivatives and the molybdopterin region of XO. The *para*-hydroxyl of phenylpropanoid derivatives was pointed toward the guanidinium group of Arg 880. The phenylpropanoid derivatives containing the *meta*-or *ortho*-hydroxyl formed hydrogen bonds with Thr 1010. In addition, *meta*-hydroxyl formed hydrogen bonds with the peptide bond between the residues of Thr1010 and Phe1009. CAPE, the phenylenethyl ester of phenylpropanoids, had the highest affinity toward the binding site of XO, and we speculated that this was due to hydrophobic interactions of the phenylethyl ester with several hydrophobic residues surrounding the active site. The hypoxanthine/XO reaction in the DMPO-ESR technique was used to correlate the effects of these phenylpropanoid derivatives on enzyme inhibition and ROS suppression, and the results showed that caffeic acid and CAPE were the two most potent agents among the tested compounds. We further assessed the effects of the test compounds on living cells, and CAPE was the most potent agent for protecting cells against ROS-mediated damage among the tested phenylpropanoids.

Keywords: Caffeic acid; CAPE; Molecular modeling; Phenylpropanoid; ROS; Xanthine oxidase

Introduction

Oxidative stress has been implicated in an enormous variety of physiological and pathological processes. An oxidation-reduction imbalance in a healthy living system leads to malfunctioning of cells that can ultimately result in various

Abbreviations: CAPE, caffeic acid phenethyl ester; DHR123, dihydrorhodamine 123; DMEM, Dulbecco's modified Eagle's medium; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl hydrate; FBS, fetal bovine serum; LPS, lipopolysaccharide; MEM, minimum essential medium; ROS, reactive oxygen species; SARs, structure-activity relationships; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XPH, hypoxanthine.

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diseases, including aging, cancer, neurological degeneration, and arthritis. The toxicity ascribed to the superoxide radical is believed to be caused by superoxide's direct interaction with biological targets. Reactive oxygen species (ROS) can initiate a wide range of toxic oxidative reactions [1]. ROS released by phagocytic cells are involved in the link between inflammation and cancer. Excessive and persistent formation of ROS by inflammatory cells is thought to be a key factor in genotoxic effects.

Xanthine oxidase (XO) is an important source of free radicals and has been reported in various physiological and pathological models. XO causes gout and is responsible for oxidative damage to living tissues. This enzyme reduces molecular oxygen, leading to the formation of $O_2^{\cdot-}$ and hydrogen peroxide. Regulation of XO activity is important during inflammation [2]. XO is a secreted enzyme which is formed in the liver as xanthine dehydrogenase (XDH) and binds to the vascular endothelium. High expression of XDH is found in the liver, and it is converted to the radical-forming XO on atherosclerosis development in mice. Treatment with an XO inhibitor largely prevents the development of endothelial dysfunction and atherosclerosis in mice [3]. XO catalyzes the oxidation of hypoxanthine and xanthine to uric acid yielding superoxide radicals and raises the oxidative level in an organism. Hydroxylation takes place at the molybdopterin center (Mo-pt) via a Mo-OH oxygen forming a bond with a carbon atom of the substrate such that the oxygen atom is derived from water rather than molecular oxygen [4]. The active form of XO is a homodimer with a molecular weight of 290 kDa with each of the monomers acting independently during catalysis. Each subunit contains one molybdopterin cofactor, two distinct [2Fe-2S] centers, and one FAD cofactor [5]. The cocrystalline structure of salicylate-XO was first reported by Enroth et al. [6]. Several amino acid residues, including Arg 880, Phe 914, Phe 1009, Thr 1010, and Glu 1261, are important for salicylate binding via hydrogen and electrostatic interactions. Although salicylate itself does not bind to the Mo-pt cofactor, it blocks the approach of substrates toward the metal complex.

Natural polyphenols can be divided into several different classes depending on each one's basic chemical structure, which ranges from simple molecules to highly polymerized compounds. Phenylpropanoid derivatives (C6-C3) are an important group of low-molecular-weight phenolics [7]. The most important phenylpropanoids are the hydroxycinnamic acids and their derivatives. Phenylpropanoid inhibition of XO has been reported. Structure-activity relationships (SARs) of caffeic acid analogues interacting with this enzyme have also been discussed [8]. However, the influence of enzyme-substrate binding by phenylpropanoids and the stereochemistry with XO have not been characterized. In this study, the protective effects of some C6-C3 phenylpropanoids against ROS and their influence on binding to the active site of XO according to various substitution groups and positions on phenylpropanoids were investigated. We also combined the role of ROS scavenging and XO inhibition of phenylpropanoids in order to identify which compounds are more vital for therapeutic applications.

Materials and methods

Materials

Xanthine oxidase (EC 1.2.3.2.), xanthine, allopurinol, cinnamic acid [(E)-3-phenyl-2-propenoic acid], caffeic acid, ferulic acid, isoferulic acid, *p*-hydroxycinnamic acid (*p*-coumaric acid), *o*-coumaric acid, *m*-coumaric acid, *p*-methoxycinnamic acid, *o*-methoxycinnamic acid, *m*-methoxycinnamic acid, caffeic acid phenethyl ester (CAPE), and lipopolysaccharide (LPS) (*Escherichia coli* O127:B8) were purchased from Sigma (St. Louis, MO) (Table 1). All of the solvents used in this study were from E. Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). β-Amyloid peptide ($A\beta_{25-35}$ fragment) was purchased from Jerini Peptide Technologies (Berlin, Germany).

Cell culture

The mouse monocyte-macrophage cell line, RAW 264.7 (ATCC TIB-71; American Type Culture Collection, Manassas, VA), was cultured in DMEM supplemented with 10% heat-inactivated FBS. Cells were plated at a density of 1.0×10^6/ml for 18–24 h before activation by LPS (50 ng/ml). Neuro 2A neuroblastoma cells (BCRC 60026) were purchased from CCRC (Culture Collection and Research Center, Hsinchu, Taiwan). Cells were grown in MEM containing 10% FBS, 1% nonessential amino acid, and 100 μg/ml penicillin-streptomycin. Conditions were maintained in a humidified 95% air/5% CO_2 incubator at 37°C.

Supercoiled DNA-relaxation assay

The inhibitory effect of phenylpropanoids on supercoiled DNA strand breakage caused by the Fenton reaction was evaluated [9]. pUC-19 plasmid DNA (200 ng) was incubated at 37°C for 30 min in TE buffer (10 mM Tris and 1 mM EDTA; pH 8.0) containing 100 mM H_2O_2 and 50 μM ferrous sulfate in the presence or absence of 5.0 μM flavonoids in a final volume of 20 μl. The conversion of the covalently closed circular double-stranded supercoiled DNA to a relaxed open-circle form was used to evaluate DNA strand breakage induced by the Fenton reaction. DNA strand breaks induced by the Fenton reaction occurred rapidly, with most of the supercoiled pUC-19 NDA converted to the relaxed form after a 30-min incubation at 37°C. Then, the samples were loaded onto a 1% agarose gel, and electrophoresis was performed in a TAE buffer (40 mM Tris-acetate and 1 mM EDTA) in the presence of 0.5 μg/ml of ethidium bromide. After electrophoresis, the gel was photographed under transmitted ultraviolet light.