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A comparative study of the antioxidant/prooxidant activities of eugenol and isoeugenol with various concentrations and oxidation conditions

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Abstract

Eugenol (compound **1** in Fig. 1, 4-allyl-2-methyoxyphenol) and isoeugenol (compound **2** in Fig. 1, 4-propenyl-2-methoxyphenol), both used as a flavor agent in cosmetic and food products, have both prooxidant and antioxidant activities. Their adverse effects such as allergic and inflammatory reaction may be due to their prooxidant activity. To clarify the mechanisms of their cytotoxicity and the factors affecting their antioxidant/prooxidant activities, we investigated the cytotoxicity, ROS production, and cellular glutathione (GSH) levels induced by eugenol and isoeugenol in a human submandibular cell line. The cytotoxicity (MTT method) of eugenol was 1 order of magnitude lower than that of isoeugenol (CC_{50}: eugenol, 0.395 mM; isoeugenol, 0.0523 mM); and ROS production (CDF staining) was induced significantly by isoeugenol, but not by eugenol. Under treatment with H_2O_2 (100 μM) plus horseradish peroxidase (1 μg/ml) for 30 min or with visible light irradiation for 5 min, eugenol caused biphasic ROS production characterized by enhanced at lower eugenol concentrations (5–10 μM) and decreased at higher concentrations (500 μM). In contrast, isoeugenol enhanced ROS production over a wide range of concentrations (5–500 μM). Isoeugenol at 1000 μM significantly reduced GSH levels compared with eugenol at the same concentration. The high cytotoxicity of isoeugenol may be attributed to its induction of high ROS production and low GSH levels, possibly as a result of benzyl radical formation. In contrast, the cytotoxicity of eugenol is likely to be mediated by ROS-independent mechanisms, possibly involving phenoxyl radicals and/or eugenol quinone methide.

Keywords: Eugenol; Isoeugenol; Antioxidant; Prooxidant; ROS; Glutathione; Cytotoxicity

1. Introduction

Eugenol (compound **1** in Fig. 1) is a flavoring agent used in cosmetic and food products (Opdyke, 1975). This compound is also widely used in dentistry as a cement material with zinc oxide or as a sedative agent (Markowitz et al., 1992). On the other hand, isoeugenol (compound **2** in Fig. 1), an isomer of eugenol, is found in essential oils and soaps (Bergonzelli et al., 2003), wine (Cullere et al., 2004), and monsooned coffee (Variyar et al., 2003) and acts as a flavoring and storage agent. Eugenol and isoeugenol are well known to possess antioxidant activity. Oxidative stress is produced when the balance between oxidative stimulation and various antioxidant systems is impaired (Sies, 1991). Although eugenol and isoeugenol are used at low levels, these compounds were previously reported to have a skin-sensitizing ability (Barratt and Basketter, 1992) and to cause allergic reactions (Frosch et al., 1995), possibly due to the oxidative stress. Since these compounds make direct contact with the mucosa in the oral cavity or with

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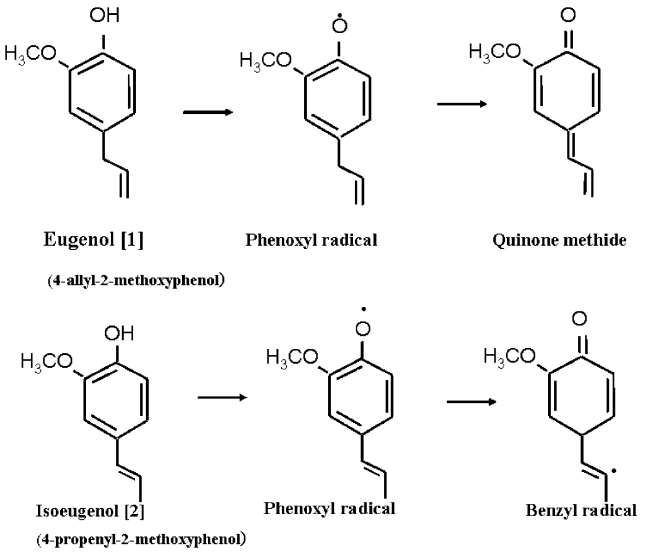

Fig. 1. Chemical structures of eugenol and isoeugenol and their intermediates.

skin, oxidative stress by light and molecular oxygen may play a crucial role in their cytotoxicity.

There have been many studies on the cytotoxicity of *ortho*-methoxyphenols such as eugenol and isoeugenol (Hume, 1984; Wright et al., 1995; Rauscher et al., 2001). These molecules possess prooxidant as well as antioxidant activities (Ogata et al., 2000; Atsumi et al., 2000; Fujisawa et al., 2002) under certain circumstances. In general, low concentrations of eugenol are thought to act as antioxidants, with beneficial anti-inflammatory effects; whereas high concentrations act as prooxidants, leading to tissue damage as a result of the formation of harmful phenoxyl radicals (Suzuki et al., 1985; Decker, 1997). Previously, we demonstrated that the antioxidant/prooxidant activities of *o*-methoxyphenols are dependent on their metal-reducing potential, chelating behavior, and pH and solubility characteristics and showed that their cytotoxicity could be attributed to the biological activity and stability of phenoxyl radicals (Atsumi et al., 2000; Fujisawa et al., 2000, 2002). Also, we recently demonstrated that eugenol produces intracellular ROS derived from photo-oxygenation by visible light (VL) irradiation and/or alkalization, resulting in enhanced cytotoxicity (Atsumi et al., 2001). However, the mechanisms determining the antioxidant/prooxidant activities of eugenol and isoeugenol are poorly understood.

The cytotoxicity of *o*-methoxyphenols appears to be associated with the production of phenoxyl radicals, which have a resonance stability and hydrophobicity (Fujisawa et al., 2002). In general, ROS are formed enzymatically or non-enzymatically as by-products of normal cellular metabolism. However, oxidative damage to biological systems is not reversible in vivo when ROS levels surpass the protective antioxidant defense systems of cells. In such a situation, reduced cellular glutathione (GSH) plays a crucial role in the rescue of cells from the induction of apoptosis by buffering endogenously induced oxidative stress (Hall, 1999; Thannickal and Fanburg, 2000). A number of authors have previously investigated the ability of ROS to cause damage to lipids, proteins, and DNA in mammalian cells (Green and Reed, 1998; Bhaumik et al., 1999). These findings led us to suppose that alteration of intracellular redox conditions by *o*-methoxyphenols can affect ROS production and consequently can result in cytotoxicity. In the present study, we determined the effects of eugenol and isoeugenol at various concentrations in terms of cytotoxicity, ROS production, and intracellular GSH levels in a human submandibular cell line (HSG cells), which is representative of oral cancer cell lines. Horseradish peroxide (HRP) with hydrogen peroxide (H_2O_2) and light irradiation have widely used as an experimental oxidative material for phenolic compounds. Thus, we used experimental oxidative stress induced by enzymatic treatment with H_2O_2/HRP or by non-enzymatic treatment with VL irradiation to investigate whether the cytotoxicity of *o*-methoxyphenols is related to levels of ROS. Our discussion focused on intermediates arising from a phenoxyl radical mechanism for eugenol and a benzyl radical mechanism for isoeugenol.

2. Methods and materials

2.1. Chemicals

The following chemicals and reagents were obtained from the companies indicated: eugenol **[1]** and isoeugenol **[2]**, from Tokyo Kasei Chem. Co., Tokyo, Japan; 5-(and 6-)carboxy-2′,7′-dichlorofluorescein diacetate (CDFH-DA), from Molecular Probes Inc., Eugene, OR and from Promega Co., Madison, WI; Glutathione Detection Kit, from Chemicon International Inc., Temecula, CA; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and Aqueous One Solution, from Promega Co., Madison, WI; and horseradish peroxidase (HRP) and glutathione, from Wako Pure Industrials, Ltd, Osaka. The chemical structures of eugenol and isoeugenol are shown in Fig. 1.

2.2. Cell survival

The HSG human adenocarcinoma submandibular gland cell line (Shirasuna et al., 1981) was donated by Dr. M. Satoh of Tokushima University. HSG cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 0.1 ml of minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air for 2 days. One hour before the addition of test compounds,