Effects of a Novel Gaseous Antioxidative System Containing a Rosemary Extract on the Oxidation Induced by Nitrogen Dioxide and Ultraviolet Radiation

Yoshiro Saito,1,* Akira Shiga,2,* Yasukazu Yoshida,1,† Takuya Furuhashi,2 Yoji Fujita,2 and Etsuo Niki1

1 Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan
2 Living Environment Systems Laboratory, Mitsubishi Electric Corporation, 5-1-1 Ofuna, Kamakura, Kanagawa 247-8501, Japan

Received August 4, 2003; Accepted December 15, 2003

Rosemary is commonly used as a spice and a flavoring agent in food processing. Although the antioxidative properties of its extracts have been investigated, there have been few reports on the volatile components of rosemary. We designed a novel antioxidative system which can generate the volatile constituents in the gaseous phase from a rosemary extract and evaluated the gaseous antioxidative activities against both lipid peroxidation and cell death induced by nitrogen dioxide and ultraviolet radiation. The antioxidative effects of the major volatile components on the oxidation of linoleic acid induced by azo compounds were also investigated in a solution.

The volatile components in the novel antioxidative system suppressed the Jurkat cell death induced by nitrogen dioxide and the intracellular formation of reactive oxygen species in fibroblast cells induced by ultraviolet radiation. 1,8-Cineole among the volatile components exerted an antioxidative effect against the oxidation of linoleic acid in a solution induced by azo compounds and ultraviolet radiation. These data suggest that the volatile constituents of a rosemary extract had antioxidative properties and that gaseous exposure antioxidant is a promising method for promoting health.

Key words: antioxidant; free radical; lipid peroxidation; rosemary; nitrogen dioxide

There is increasing experimental and clinical evidence which suggests the involvement of oxidative stress induced by active oxygen and nitrogen species and ultraviolet radiation in the pathogenesis of various diseases, cancer, and aging. As a consequence, the role of antioxidants has been receiving considerable attention. Rosemary is commonly used as a spice and a flavoring agent in food processing and its extract has been investigated for antioxidative activities. The constituents of a rosemary extract vary with the origin and preparation method. It has been reported that the antioxidative activities were mainly attributable to polyphenolic and non-volatile compounds such as carnosol, carnosic acid, rosmanol, and rosmarinic acid. However, there are few reports on the antioxidative effects of the volatile constituents of rosemary.

One of the important functions of an antioxidant is for scavenging active radicals to inhibit lipid peroxidation, protein modification, and DNA damage. Numerous natural radical-scavenging antioxidants have been investigated, and novel synthetic antioxidants have also been tested. The antioxidative activities have often been measured in a homogeneous solution, where by the activity is primarily determined by the chemical reactivity toward an active radical. However, it is known that the potency of radical-scavenging antioxidants in vivo is determined not only by the chemical reactivity toward such radicals, but also by other factors such as the localization, concentration and mobility in the micro-environment. On the basis of these considerations, we designed a novel gaseous antioxidative system (Fig. 1(B)) containing a rosemary extract and emitting volatile components to evaluate its antioxidative activity against lipid peroxidation.

We investigated in this study, the oxidation of linoleic acid induced by nitrogen dioxide, and that of human T-leukemia Jurkat and normal human skin fibroblast cells induced by either ultraviolet radiation or nitrogen dioxide in the gaseous phase in order to elucidate the antioxidative effects of the volatile components of a rosemary extract.

1 To whom correspondence should be addressed. Tel: +81-72-751-8183; Fax: +81-72-751-9964; E-mail: yoshida-ya@aist.go.jp
* These authors equally contributed to this study.
Abbreviations: AMVN, 2,2’-azobis(2,4-dimethylvaleronitrile); DCF, 2’,7’-dichlorofluorescein; DCFH-DA, 2’,7’-dichlorofluorescin-diacetate; ROS, reactive oxygen species; VR, volatile constituents of the rosemary extract
Materials and Methods

Materials. The particulate containing the rosemary extract (50 wt.% volatile components) was obtained from Kankyokagaku Co. (Osaka, Japan). A headspace analysis showed five typical volatile compounds in the rosemary extract, 1,8-cineole, camphor, borneol, limonene and α-pinene (43:41:13:2.5:0.5, respectively, by molar ratio, the structures being shown in Fig. 1(A)), and several unknown compounds. Linoleic acid, which was used as an oxidizable substrate, was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Methyl linoleate, (+)-camphor, (−)-α-pinene, (R)-(+)−limonene, and 1,8-cineole, which were all obtained from Wako Pure Chemical Industry (Osaka, Japan) were used without further purification. The azo compound, 2,2′azobis-(2,4-dimethylvaleronitrile) (AMVN), which was obtained from Wako Pure Chemical Industry (Osaka, Japan), was used as a radical initiator. Nitrogen dioxide (99.93% vol./vol. in nitrogen) was purchased from Takachiho Chem. Ind. Co. (Tokyo, Japan). Human T-leukemia Jurkat E6-1 cells and normal human skin fibroblast NB1RGB (#RCB60222) were respectively obtained from American Tissue Type Collection (Rockville, MD, USA) and RIKEN cell bank (Ibaraki, Japan). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo laboratory (Kumamoto, Japan), and dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA). All other chemicals used were of the highest grade commercially available.

Oxidation of linoleic acid by nitrogen dioxide. Linoleic acid (1 ml) was exposed to nitrogen dioxide (10 ppm by volume) in a transparent chamber (15 l) equipped with an air-circulating fan (this apparatus is shown in Fig. 1(B)). To evaluate the antioxidative effect of the volatile constituents of the rosemary extract (VR), the particulate containing the rosemary extract in a stainless steel dish was heated at 80°C by a ceramic heater located in the chamber.

Cell culture and determination of the cell viability. Jurkat cells and NB1RGB fibroblasts were maintained in a basal medium containing 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B and 10% heat-inactivated fetal calf serum at 37°C in an atmosphere of 95% air and 5% CO₂ as previously described. An RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA) and MEMα medium (Gibco BRL, Rockville, MD, USA) were respectively used as

![Fig. 1. (A) Structures of the Volatile Constituents in Rosemary (VR) and (B) Apparatus Used in This Study.](image-url)
the basal medium for Jurkat cells and NB1RGB fibroblasts. To determine the cell viability, a trypan blue assay was conducted at the indicated times. The cells that excluded trypan blue after being incubated with PBS containing 0.04% trypan blue dye (Gibco BRL, Rockville, MD) were considered viable.

Irradiation by ultraviolet light. After washing 3 times with a PBS (pH 7.4) solution, confluent cells in a 6-well dish were loaded into the chamber, three wells were irradiated with black light at room temperature, while the other three were covered with aluminum foil to prevent irradiation. The light energy was 1 mW/cm² at 365 nm. The chamber was humidified and the air circulated to prevent water loss and heating of the air.

Determination of the intracellular reactive oxygen species (ROS). Intracellular ROS were detected by using DCFH-DA as previously described with a slight modification. The cells were washed with PBS and then incubated with DCFH-DA at a final concentration of 10 μM for 15 min at 37 °C. The cells were then washed once with PBS and incubated under UV irradiation for the specified times at room temperature. The fluorescence emission of 2',7'-dichlorofluorescein (DCF) in the cells was measured and recorded with a Fluoroscan Ascent CF fluorescence plate reader (Thermo Lab systems, Helsinki, Finland). The excitation and emission filters for this apparatus were set at 485 nm and 527 nm, respectively.

Oxidation of linoleic acid by the azo initiator in an acetonitrile solution. The oxidation of linoleic acid induced by radicals generated from the decomposition of AMVN was carried out in homogeneous acetonitrile solution in the absence or presence of the rosemary volatile compounds at 37 °C in air. The absorption spectra were measured at regular time intervals. The rate of oxidation of methyl linoleate due to conjugated diene was also measured from the increase in absorption at 234 nm.

Repeated experiments were carried out, and representative results are shown. The reproducibility was within ±10%.

Results

Oxidation of linoleic acid by nitrogen dioxide

Figure 2 shows the absorption spectra of linoleic acid oxidized by nitrogen dioxide in the absence and presence of VR. Oxidation was performed in the chamber shown in Fig. 1(B), and VR were concomitantly produced by heating the rosemary extract and dispersed in the gaseous phase of the chamber. As shown, an increase in absorption at 234 nm and the formation of oxidized linoleic acid such as hydroperoxides and hydroxides resulted from exposure to nitrogen dioxide (Fig. 2(A)). It was also observed that the absorption decreased with increasing VR concentration, although it is not clear at present whether VR scavenged nitrogen dioxide in the gas or liquid phase.

In order to clarify the effects of a practical concentration of nitrogen dioxide in the atmosphere, the oxidation of linoleic acid induced by 1 ppm of nitrogen dioxide was carried out in the presence of 10 mg/m³ VR (Fig. 3). Oxidation proceeded strongly after exposure to nitrogen dioxide for 1 hr, while it was significantly suppressed in the presence of VR.

Protective effect of VR on the cell death induced by nitrogen dioxide

To demonstrate the antioxidative effect of VR on the cells, we investigated the effects of VR on the cell injury induced by nitrogen dioxide. When Jurkat cells were exposed to 90 ppm of nitrogen dioxide for 2 hr at room temperature, the viability of the cells decreased, more than a 95% loss being observed (Fig. 4). In contrast,
when Jurkat cells were exposed to nitrogen dioxide with 200 ppm VR, 73% of the total cells remained alive.

Effect of VR on the intracellular formation of ROS induced by UV irradiation

We next examined the effect of VR on the formation of intracellular ROS in human skin fibroblast NB1RGB cells by using a DCFH-DA fluorescence probe. An increase in the intracellular ROS level was observed by irradiating with UV (1.0 mW/cm$^2$ at 365 nm) for 3 hr at room temperature. The inhibiting effect was calculated by the following equation:

$$\text{Inhibiting effect} = \frac{1 - \frac{[\text{with UV and no VR}]}{[\text{no UV and no VR}]}}{\frac{[\text{with UV and VR}]}{[\text{no UV and VR}]}}$$

where square brackets indicate the fluorescence emission from DCF (a.u.). In the presence of VR, the increase in DCF fluorescence was prevented in a concentration-dependent manner (Fig. 5). These results suggest that VR in the gaseous phase was protective against the accumulation of intracellular ROS caused by UV irradiation.

Oxidation of linoleic acid and methyl linoleate induced by azo compounds

In order to clarify the antioxidative effect of VR, we examined the main volatile components of rosemary (their structures are shown in Fig. 1(A)). The reactivity toward radicals is obviously one of the important factors that determines antioxidative activity. The oxidation of linoleic acid is known to quantitatively give four conjugated diene hydroperoxides, which can be measured from the strong absorption at 234 nm. Figure 6 shows typical results for the oxidation of linoleic acid induced by radicals formed from the decomposition of AMVN in the absence and presence of each constituent in a solution. Figure 6 (A) shows that linoleic acid hydroperoxides accumulated with time and that oxida-
tion was retarded by 1,8-cineole; however, little inhibiting effect was apparent from the other constituents, camphor, borneol, limonene, and C11-pinene (Fig. 6(B)).

The oxidation rate of methyl linoleate was calculated from the increase in absorbance at 234 nm over 30 minutes in the absence and presence of 1,8-cineole. As shown in Fig. 7, the oxidation rate in the presence of 1,8-cineole was decreased with its increasing concentration.

Discussion

Rosemary is widely used for its desirable flavor and is known to exert an antioxidative effect.13) Ai-Hsiang et al. have reported that carnosol, the phytopolyphenol component in rosemary, suppressed NO production and iNOS gene expression by inhibiting NF-κB activation in the mouse macrophage RAW 264.7 cell line.7) Rosemary contains flavonoids, phenols, volatile oil and terpenoids, and more than 90% of the antioxidative activity has been attributed to carnosol and carnosolic acid.14,15) It was found in this study that the volatile components of rosemary, especially 1,8-cineole, also exerted an antioxidative effect on the oxidation of linoleic acid induced by the azo radical initiator. The antioxidative properties of 1,8-cineole are still a debatable issue: some researchers have reported that there was little antioxidative effect of 1,8-cineole,16–18) whereas others have found a profound effect.19,20)

The reactivity of natural and synthetic radical-scavenging antioxidants has been extensively studied;2–4) however, relatively little attention has been paid to the dynamics of the antioxidative action in a heterogeneous system. It has been shown that the efficacy of radical scavenging by antioxidants varied markedly depending on the environment.8,9) For example, it has been accepted that vitamin C (ascorbic acid) is the primary hydrophilic radical-scavenging antioxidant in human whole blood21) and plasma.22) However, the efficacy of scavenging radicals within the lipophilic domain by vitamin C has been reported to be low and to decrease as the radical goes deeper into the interior of membranes or lipoproteins.23–25) Thus, the antioxidative capacity in a heterogeneous system are determined by several factors, of which structural hindrance significantly contributes. These issues have not received as much attention as they should. The volatile constituents of a rosemary extract, which are produced by heating the extracts, may dissolve into both the lipophilic and aqueous compartments. VR also inhibited the oxidation of cultured cells induced by both nitrogen dioxide and ultraviolet irradiation. It is interpreted from this result that either the volatile constituents of the rosemary extract directly scavenged gas-phase reactive oxygen and ultraviolet, or that they penetrated the cell membrane after dissolving...
in the aqueous solution. It is still unclear which suppressive effect is important in this system; however, this novel antioxidative system forming volatile components of rosemary may have an anti-deleterious effect on organisms in the living body as a chain-breaking antioxidant.

In conclusion, the results of the present study show that the volatile constituents of a rosemary extract had antioxidative properties and that volatile antioxidant exposure is a promising method for health promotion.

References