Superoxide Scavenging Activity of Rosmarinic Acid from *Perilla frutescens* Britton Var. *acuta* f. *viridis*

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Rosmarinic acid (RoA) has been isolated in high yield (0.1%) from the leaves of *Perilla frutescens* Britton var. *acuta* f. *viridis*, one of the most common garnishes in Japan, as a superoxide scavenger in a xanthine/xanthine oxidase system. The scavenging activity of RoA was significantly higher than that of ascorbic acid (AA). The structure−activity relationships using RoA, caffeic acid (CaA), and its related compounds indicated that the presence of an ortho dihydroxyphenyl group is essential for the scavenging effect. In addition, the conjugated double bond in the C3 carbon chain is important for enhancing the effect. The activity of RoA was then shown to be due to the additive effect of both CaA and (3,4-dihydroxyphenyl)lactic acid units, both of which are hydrolyzed products of RoA. RoA significantly inhibited both intracellular superoxide and peroxide formation in differentiated HL-60 cells, suggesting that RoA effectively exhibited antioxidative activity in the biological systems through the scavenging of superoxide.

Keywords: *Perilla frutescens; rosmarinic acid; superoxide; xanthine oxidase; HL-60*

INTRODUCTION

Reactive oxygen species (ROS) have been considered to play some important roles in the carcinogenesis process including tumor promotion (Perchellet et al., 1995). In particular, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type tumor promoters are reported to trigger superoxide (O_2^-) generation in epithelial cells and leukocytes through the xanthine/xanthine oxidase (XA/XOD) and NADPH oxidase systems, respectively. Indeed, ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens (Ji and Marnett, 1992; Kensler et al., 1987) and the increased levels of oxidized DNA bases (Wei and Frenkel, 1993). Among the ROS, O_2^- is one of the precursors of some types. O_2^- is readily converted to H_2O_2 nonenzymatically or by function of superoxide dismutase in biological systems. The hydroxyl radical (•OH), formed subsequently from H_2O_2, randomly reacts with biological components such as lipids or DNA bases intracellularly.

A body of epidemiological surveys and animal experiments has demonstrated that ingestion of some constituents occurring in vegetables and fruit may contribute to the reduction of cancer incidence in humans (Bertram et al., 1987; Wattenberg, 1985). At present, when a reliable method for cancer therapy has not yet been established, cancer chemoprevention by food phytochemicals is accepted as one of the most attractive and promising avenues for cancer control (Lippman et al., 1994). Vegetables and fruits all contain a variety of microconstituents thought to protect against cancer. Especially, antioxidative vitamins, carotenoids, and flavonoids may play important roles in the basis of the results of animal carcinogenesis experiments and have attracted much attention in the field of chemoprevention study. In the ongoing studies of food phytochemicals for chemoprevention from these viewpoints (Murakami et al., 1996a; Ohigashi et al., 1997), we have focused on O_2^- formation inhibitors as effective and promising candidates for prevention of cancer, because we and other groups have recently reported that O_2^- formation inhibitors effectively inhibit oxidative stress in vivo and tumorigenesis (Murakami et al., 1996b, 1997; Nakamura et al., 1996, 1998; Wei et al., 1993, 1995). In the preliminary screening study of Japanese edible plants, the methanol extract of *Perilla frutescens* Britton var. *acuta* f. *viridis*, one of the most common garnishes in Japan, has shown potent superoxide scavenging activity in an XA/XOD system. The leaf of *P. frutescens* is usually consumed and well-known to be a folk medicine for use as a detoxicant, antitussive, and antibiotic and for treating intestinal disorders and allergies in Japan and China. Okuyama et al. (1997) reported that perilla oil with a high α-linolenic acid ($n - 3$, ∼60%) prevents chemical carcinogenesis and metastasis in animal experiments. Recently, many antioxidants were isolated from plants in the Labiatae family (Nakatani and Inatani, 1984; Miura and Nakatani, 1989; Cuvelier et al., 1994; Tada et al., 1996), suggesting that antioxidative constituents other than α-linolenic acid may contribute to the chemopreventive potential of perilla.

This paper describes the inhibitory effects on O_2^- formation in the XA/XOD of rosmarinic acid (RoA, Figure 1), which is mainly responsible for the potent superoxide scavenging activity of *P. frutescens*. Recently, RoA was isolated as an inhibitor of arachidonate lipoxygenase from defatted perilla seed, suggesting that RoA may have the potential to regulate or prevent

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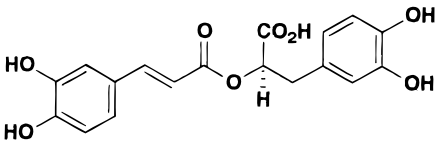

Figure 1. Chemical structure of RoA.

inflammatory diseases including carcinogenesis (Yamamoto et al., 1998). In the present study, to further evaluate RoA as a promising chemopreventive food phytochemical, the inhibitory effect of RoA on TPA-induced ROS formation in a differentiated HL-60 cell system is determined. In addition, to discuss the structure−activity relationships, inhibitory effects of caffeic acid (CaA) derivatives were also estimated.

MATERIALS AND METHODS

General Remarks. Analytical instruments used were as follows: HPLC, Hitachi 655A-11; $[\alpha]_D$, Jasco DIP-1000; UV, Shimadzu UV 200 and UV 2200AI; IR, Shimadzu IR-435; MS, JEOL JMS-DX 300 and JMS600; ^{1}H NMR, Bruker ARX500 and AC300 (ref TMS); and flow cytometer, CytoACE 150 (Jasco, Tokyo, Japan). Chromatographic materials used were as follows: Wako gel C-100 and Wako gel C-200 from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan), YMC I-40/64 gel from Yamamura Chemical Laboratory (Kyoto, Japan), Nova Pak C_{18} (4.6 mm i.d. × 150 mm, particle diameter = 4 μm, Waters Associates, Milford, MA), and μBondasphere C_{18} (3.9 mm i.d. × 150 mm, particle diameter = 5 μm, Waters Associates).

Materials. *Perilla frutescens* Britton var. *acuta* f. *viridis* was purchased at a market in Kyoto in November 1996. TPA was obtained from Research Biochemical International, Natick, MA. RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL, Grand Island, NY. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes, Inc., Leiden, The Netherlands. Cytochrome *c* and ferulic acid were obtained from Sigma, St. Louis, MO. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka, Japan. Human promyelocytic leukemia HL-60 cells (Collins et al., 1977) were obtained from the Health Science Research Resources Bank (Osaka, Japan).

O_2^- Scavenging Test in the XA/XOD System. O_2^- scavenging activity was measured by an XA/XOD system using SOD Test Wako (Murakami et al., 1996b). In this system, O_2^- scavenging activity was estimated by measuring the nitroblue tetrazolium (NBT) reduction and XOD inhibitory activities as previously reported (Ohnishi et al., 1985). O_2^- scavenging (SOS) activity is given by the following equation:

$$\text{SOS activity (\%)} = \text{NBT reduction inhibitory activity (\%)} - \text{XOD inhibitory activity (\%)}$$

The value of 50% inhibition concentration (IC_{50}) was determined for the comparison with the inhibitory activities.

Isolation and Identification of Active Constituents. Fresh leaves of *P. frutescens* (92 g) were cut into small pieces and extracted with MeOH (300 mL) at room temperature for 1 week, and the filtrate was concentrated in vacuo to give a green oily syrup. This syrup was stored at −20 °C before it was used for the experiments. The fractionation was carried out by monitoring NBT reduction activity in the XA/XOD system. The extract (2.1 g) was partitioned between EtOAc (500 mL) and water (500 mL). The active water layer (1.6 g) was partitioned between *n*-butanol (500 mL) and water (500 mL). The *n*-butanol layer (550 mg) was chromatographed on preparative HPLC on Nova Pak C_{18} (volume of injection = 10 mg/25 μL of MeOH solution; flow rate = 1.0 mL/min; mobile phase = acetonitrile/H_2O/AcOH = 28:72:0.1) to give rosmarinic acid (RoA, 91 mg): $[\alpha]^{19}_D$ +69.4° (*c* 0.41, MeOH); UV λ_{max} (MeOH) (log ϵ) 290 (3.97), 330 (4.08) nm; IR ν_{max} (KBr) 3400−2200, 1690, 1600 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.00 (1H, dd, J = 8.0, 14.0 Hz, 11α-CH_2), 3.10 (1H, dd, J = 4.0, 14.0 Hz, 11β-CH_2), 5.18 (1H, dd, J = 4.0, 8.0 Hz, 10-CH), 6.26 (1H, d, J = 16.0 Hz, 8-H), 6.61 (1H, dd, J = 2.0, 8.0 Hz, 17-H), 6.69 (1H, d, J = 8.0 Hz, 16-H), 6.75 (1H, d, J = 2.0 Hz, 13-H), 6.77 (1H, d, J = 8.0 Hz, 5-H), 6.95 (1H, dd, J = 2.0, 8.0 Hz, 6-H), 7.04 (1H, d, J = 2.0 Hz, 2-H) 7.54 (1H, d, J = 2.0 Hz, 7-H).

Hydrolysis of RoA. RoA (10 mg, 27.8 μmol) was treated with 200 μL of hydrochloric acid (1 M). The mixture was heated at 100 °C for 24 h, then neutralized with a saturated $NaHCO_3$ solution, and concentrated by a rotary evaporator. The final purification was done by preparative HPLC on μBondasphere C_{18} (volume of injection = 2 mg/20 μL of MeOH solution; flow rate = 1.0 mL/min; mobile phase = acetonitrile/H_2O/AcOH = 28:72:0.1) to afford (3,4-dihydroxyphenyl)lactic acid (DHLA; 1.6 mg, 8.1 μmol, 29% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.73 (1H, dd, J = 8.0, 13.8 Hz), 2.94 (1H, dd, J = 3.8, 14.0 Hz), 4.22 (1H, dd, J = 4.2, 7.7 Hz), 6.57 (1H, dd, J = 1.7, 8.1 Hz), 6.66 (1H, d, J = 8.0 Hz), 6.71 (1H, d, J = 1.6 Hz).

Inhibitory Test of TPA-Induced Superoxide Generation. Inhibitory test of TPA-induced O_2^- generation was done as previously reported (Nakamura et al., 1998). Briefly, human promyelocytic leukemia HL-60 cells were preincubated with 1.25% (v/v) dimethyl sulfoxide (DMSO) at 37 °C in a 5% CO_2 incubator for 6 days to differentiate into granulocyte-like cells. To determine the inhibitory effect of O_2^- generation, the test compound dissolved in 5 μL of DMSO was added to the cell suspension and the mixture thus obtained was incubated at 37 °C for 15 min. The cells were washed with PBS twice for removal of extracellular test compounds to avoid direct scavenging effect. Ninety seconds after stimulation with 5 μL of a TPA solution (20 μM), 50 μL of a cytochrome *c* solution (20 μg mL^{-1}) was added to the reaction mixture and incubated for another 15 min. The visible absorption at 550 nm was measured. The inhibitory effects were expressed by a relative decreasing ratio of the absorbance of test compound to a control experiment. The experiment was done in duplicate.

Detection of intracellular O_2^- was carried out using NBT (Kobayashi et al., 1990). Fifteen minutes before the test compound was added, NBT (610 μM) was incorporated at 37 °C. After stimulation with TPA, the reaction mixture was incubated for another 15 min and then the reaction was terminated by placing the mixture on ice. The cells were washed with PBS twice for excessive NBT. The ratio of blue-stained (O_2^- positive) cells was compared to that of a control experiment with only TPA, for which the ratio of positive cells was almost 100%.

Inhibitory Test of Intracellular Peroxide Formation in Differentiated HL-60 Cells. Intracellular peroxides were detected using DCFH-DA as an intracellular fluorescence probe (Nakamura et al., 1998). Five microliters of DCFH-DA solution (200 μM) was added to the cell suspension, and cells were incubated at 37 °C for 15 min. After the test compound dissolved in 5 μL of DMSO was added to the cell suspension, the mixture was incubated at 37 °C for 15 min, followed by the addition of 5 μL of a TPA solution (10 μM). After another 15 min, the reaction was stopped by adding 50 μL of a EDTA solution (800 mM). After washing with PBS, 2′,7′-dichlorofluorescein (DCF) formed by the reaction of DCFH with intracellular peroxides was detected by a flow cytometer (CytoACE 150). Experiments were repeated two times. The data are expressed as one representative histogram.

RESULTS AND DISCUSSION

O_2^- Scavenging Activities of RoA and Ascorbic Acid (AA). It has been accepted that ROS such as O_2^- and hydroxyl radicals are agents causing oxidative damage, and much attention has been focused on ROS scavenging agents such as tocopherols, natural phenolics such as flavonoids, and tannins. First, inhibitory activity of RoA from perilla against O_2^- generation in an XA/XOD system was examined. As shown in Figure

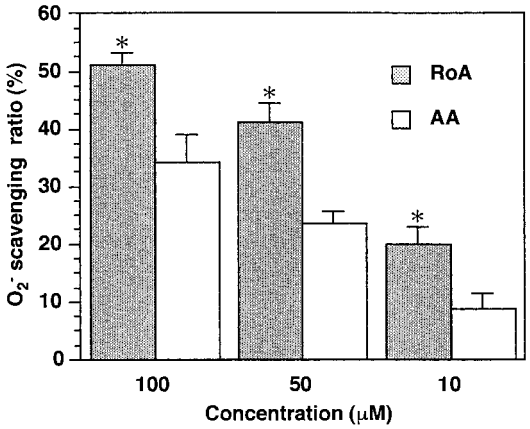

Figure 2. O_2^- scavenging activities of RoA (dotted bars) and AA (open bars) in an XA/XOD system. Values show mean ± standard deviation from two experiments. *P < 0.05 versus AA at the same concentration (Student's t test, n = 2).

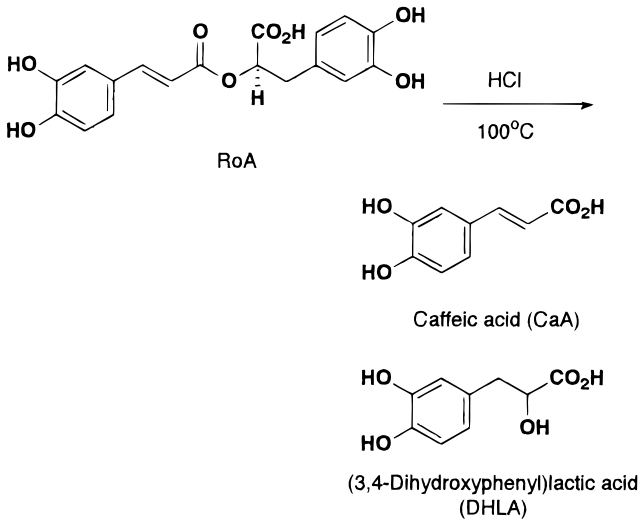

Figure 3. Chemical structures of CA and DHLA, afforded by HCl hydrolysis of RoA.

2, RoA showed NBT reduction inhibitory effect in a concentration-dependent manner, as did AA. The activity of RoA was significantly higher than that of AA, a well-known water-soluble radical scavenger. On the other hand, RoA and AA showed no inhibitory effect on XOD enzyme activity even at a concentration of 100 μM. Thus, their NBT reduction inhibitory activities are due to O_2^- scavenging effects.

Structure−Activity Relationships. RoA has two *o*-diphenol moieties that were considered to contribute to scavenging free radicals by functioning as a proton donor (Brand-Williams et al., 1995). RoA is an ester formed by CaA and a carboxylic acid carbinol, DHLA. We therefore examined which moiety of RoA was responsible for the O_2^- scavenging effect. Hydrolysis of RoA by HCl at 100 °C gave CaA and DHLA (Figure 3), and we determined the O_2^- scavenging activities of these two molecules and completed the study by the analysis of five other phenolcarboxylic acid derivatives (Figure 4; Table 1). Ferulic acid (FA), *p*-coumaric acid (*p*-CoA), and cinnamic acid (CiA) showed no scavenging effect even at concentrations of 250 μM, whereas the acids having *o*-diphenol such as CaA or protocatechuic acid (PA) showed significant scavenging effect, suggesting that the *o*-diphenol moiety is essential for scavenging activity, although the monophenol has been reported

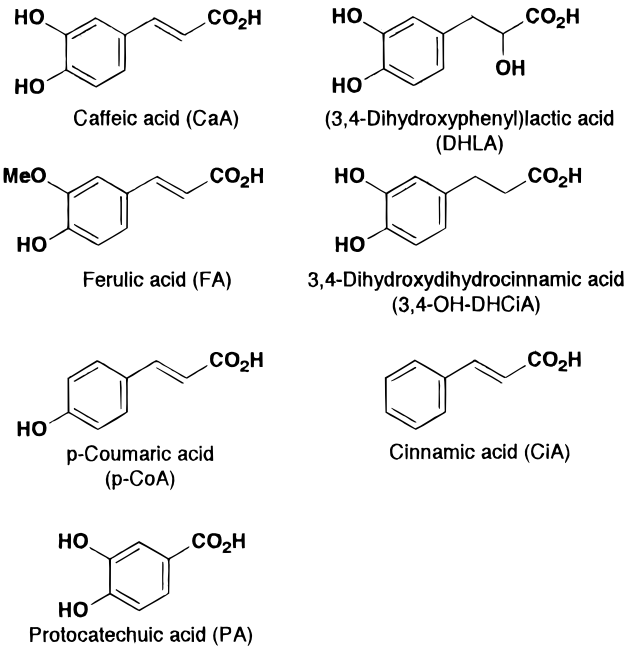

Figure 4. Chemical structures of phenolcarboxylic acid derivatives.

Table 1. O_2^- Scavenging Activities of the Phenolcarboxylic Acid Derivatives in an XA/XOD System

	O_2^- scavenging ratio (%) at concentration of[a]				
compound	10 μM	50 μM	100 μM	250 μM	IC_{50} (μM)
RoA	20	41	51	84	95
CaA	10	26	40	53	200
DHLA	10	11	28	47	>250
FA	0	0	0	0	inactive
3,4-OH-DHCiA	1	17	23	38	>250
p-CoA	0	0	0	0	inactive
CiA	0	0	0	0	inactive
PA	5	14	18	32	>250

[a] The maximal SD for each experiment was 5% (n = 2).

to scavenge other free radicals (Brand-Williams et al., 1995) or to inhibit lipid peroxidation (Toda et al., 1991). The antioxidative study by Tsuda et al. on anthocyanin pigments has clearly demonstrated that as the number of hydroxyl substituents on the phenolic B-ring of anthocyanins was increased, greater O_2^- scavenging activity was achieved (Tsuda et al., 1996). The inhibitory activity of CaA (IC_{50} = 200 μM) was significantly higher than those of DHLA (IC_{50} > 250 μM) and 3,4-dihydroxydihydrocinnamic acid (3,4-OH-DHCiA, IC_{50} > 250 μM). The conjugated double bonds on the carbon chain should therefore enhance the scavenging effect, possibly by delocalization and stabilization of the trapped radical. The activity of RoA resulted from the additive effect of both CaA and DHLA, suggesting that RoA may have a greater advantage for the radical scavenging effect than simple phenolics because RoA is a dimer of *o*-diphenol, essential for scavenging of the O_2^- radical. All compounds showed no inhibitory effect on XOD activity even at a concentration of 250 μM except for the case of CaA, which exhibited slight inhibition of XOD at 250 μM [inhibitory effect (IE) = 18%].

Inhibitory Effect on TPA-Induced O_2^- Generation in Differentiated HL-60 Cells. Human promyelocytic leukemia HL-60 cells were incubated in medium containing 1.25% DMSO for 6 days to differentiate them into granulocyte-like cells which are able to generate

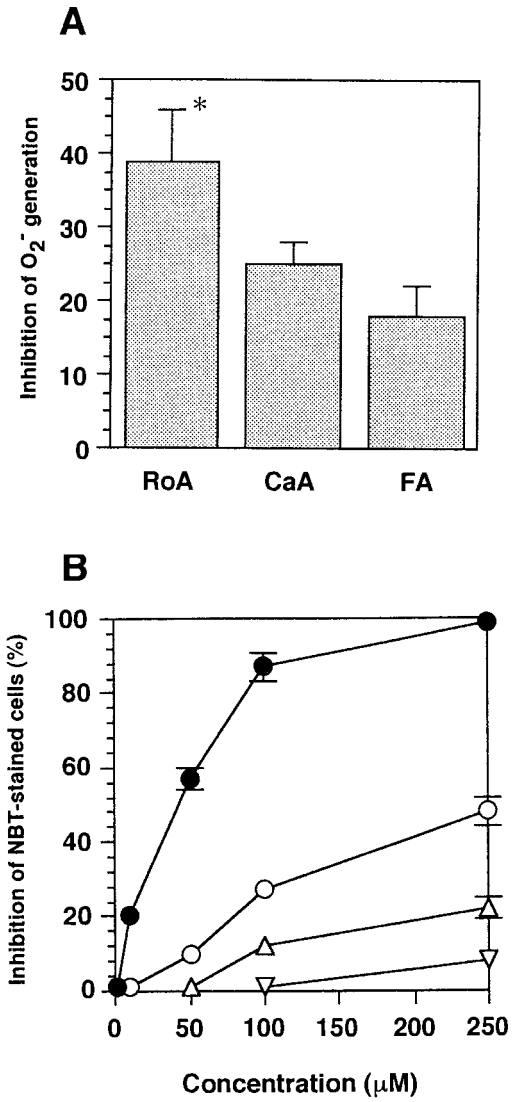

Figure 5. Inhibitory effects on O_2^- generation in differentiated HL-60 cells. (A) Inhibitory effect on extracellular O_2^- level was detected by the cytochrome *c* reduction method. The concentrations of both tested compounds were 250 μM. *P < 0.005 versus CaA or FA (Student's *t* test, $n = 2$). (B) Inhibitory effect on intracellular O_2^- level was detected by the NBT reduction method. Values show mean ± standard deviation ($n = 2$): RoA (●); CaA (○); FA (△); AA (▽).

O_2^- with stimulation of TPA through the NADPH oxidase system (Henderson and Chappell, 1996). In the extracellular O_2^- assay, after preincubation with test compounds, the cells were washed with PBS twice for removal of extracellular test compounds. Their O_2^- scavenging effect in the medium, thus, should be neglected. The concentrations of extracellular O_2^- were measured by the cytochrome *c* reduction method. Figure 5A shows the inhibitory effect of RoA, CaA, and FA for suppressive activity toward extracellular O_2^- generation in differentiated HL-60 cells. RoA at a concentration of 250 μM significantly inhibited O_2^- generation by 39%, whereas CaA and FA showed little inhibitory potential (IE = 25 and 18%, respectively). The inhibitory activity of RoA was weaker than that of genistein ($IC_{50} = 102$ μM; Murakami et al., 1996), a well-known antitumor promoter from soybean. Takeuchi et al. (1996) have indicated that extracellular O_2^- of HL-60 cells may be generated by plasma membrane-bound NADPH oxidase and diffuse into the cells through the anion channel. Lundqvist et al. (1996) also confirmed the origin of intracellular O_2^- and H_2O_2 by the observation that differentiated HL-60 cells, lacking specific granules in which all membrane components necessary for NADPH oxidase activity are present, showed much lower intracellular chemiluminescence response than the isolated neutrophils having specific granules. These findings support the idea that RoA suppresses extracellular O_2^- formation due to inhibition of plasma membrane-bound NADPH oxidase.

Next we examined whether RoA can reduce the intracellular O_2^- level. Figure 5B shows the inhibitory effect on an NBT-stained cell population evaluating the intracellular O_2^- level. Intracellular O_2^- molecules were detected according to the NBT reduction method. RoA effectively inhibited the formation of NBT-positive cells in a dose-dependent manner ($IC_{50} = 40$ μM). Similar to the results of extracellular O_2^-, inhibitory activities of CaA and FA ($IC_{50} > 250$ μM) were weaker than that of RoA. AA showed no inhibitory effect even at a concentration of 250 μM. Because the test compound existed in the reaction mixture after TPA stimulation in this intracellular O_2^- assay, the inhibitory effect of RoA may be mainly due to its scavenging effect on extracellular O_2^- diffused from inside the cells.

Inhibitory Effect on TPA-Induced H_2O_2 Formation in Differentiated HL-60 Cells. As mentioned above, O_2^- is converted to hydrogen peroxide (H_2O_2) in biological systems. The hydroxyl radical ($OH^•$), formed subsequently from H_2O_2, has an extremely short half-life time (<1 μs) and thus randomly reacts with biological components. Recently, Takeuchi et al. (1996) reported that $OH^•$ may directly induce formation of 8-hydroxydeoxyguanosine in DMSO-differentiated HL-60 cells. Alternatively, $OH^•$ reacts with membrane lipids to form hydroperoxides, which are then decomposed and converted to mutagenic, reactive carbonyl compounds such as malondialdehyde. Significant inhibitory activity of RoA toward O_2^- generation in differentiated HL-60 cells led us to address the inhibitory efficacy against intracellular peroxide (H_2O_2, etc.) formation by using DCFH-DA as an intracellular fluorescence probe (Bass et al., 1983; Nakamura et al., 1998).

Figure 6 shows the cytograms in which differentiated HL-60 cells were treated with or without 100 nM TPA. The hydroperoxide positive rate HPR indicates the rate of TPA-pretreated cells exhibiting the fluorescence levels that are equal to or higher than (mean + 3 ×standard deviations) those in the control cells. As shown in panel A, most of the cells were estimated to produce intracellular peroxides (HPR = 90%) with TPA stimulation alone. RoA at a concentration of 250 μM (panel B) significantly inhibited peroxide formation (HPR = 12%, IE = 87%). Intracellular H_2O_2 may be formed extracellularly from O_2^-, being diffused into the cells through the membrane. The significant inhibitory activity of RoA may be due mainly to scavenging extracellular O_2^- and/or H_2O_2 rather than the inhibition of NADPH oxidase. To evaluate RoA as a chemopreventive food phytochemical, further studies such as in vivo modification of oxidative damage and pharmacokinetics of RoA are needed.

Conclusion. RoA has been isolated from the leaves of perilla as a superoxide scavenger in a xanthine/xanthine oxidase system. The scavenging activity of

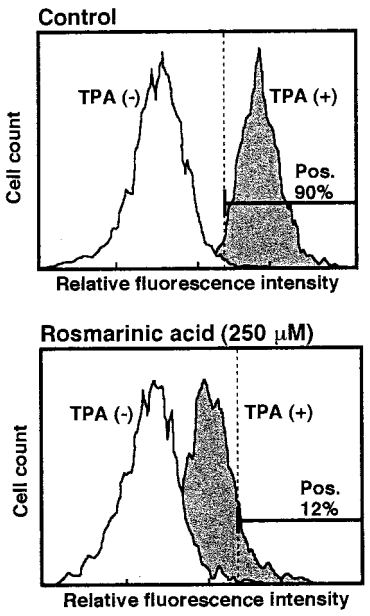

Figure 6. DCF fluorescence distribution in DMSO-differentiated HL-60 cells. (A) Cells were preincubated with 50 μM DCFH-DA at 37 °C for 15 min. After being treated with DMSO at 37 °C for 15 min, the cells were treated with EtOH [unstimulated control, TPA(−)] or 100 nM TPA [positive control, TPA(+)]. (B) To determine the inhibitory effect of RoA on TPA-induced intracellular peroxide formation, cells were preincubated with 50 μM DCFH-DA at 37 °C for 15 min. After being treated with 250 μM RoA at 37 °C for 15 min, the cells were treated or not with 100 nM TPA [TPA(+) and TPA(−), respectively]. The DCF fluorescence was monitored on a flow cytometer (CytoACE 150) with excitation and emission wavelengthes at 488 and 512 nm, respectively.

RoA was greater than that of AA or other phenolcarboxylic acids. The activity of RoA was the additive effect of both CaA and DHLA. In the cell system, RoA significantly inhibited intracellular O_2^- and peroxide formation in differentiated HL-60 cells. These results led us to the conclusion that RoA effectively exhibited antioxidative activity in the biological systems through the scavenging of O_2^-, one of the precursors of ROS. Recently, RoA and luteolin have been isolated from defatted perilla seed as in vitro lipoxygenase inhibitors (Yamamoto et al., 1998), suggesting that RoA may be capable of preventing atherosclerotic, inflammatory, and tumor metastatic processes. In addition to biological activities, the industrial waste defatted perilla seed has thus been developed into a useful source of these lipoxygenase inhibitors (Yamamoto et al., 1998). However, the fresh leaves are more useful for the source of RoA because the quantity of RoA in fresh leaves (yield = 0.1%) is 10 times larger than in the seed (yield = 0.01%; Yamamoto et al., 1998).

ABBREVIATIONS USED

AA, ascorbic acid; CaA, caffeic acid; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DHLA, (3,4-dihydroxyphenyl)lactic acid; CiA, cinnamic acid; *p*-CoA, *p*-coumaric acid; FA, ferulic acid; HPR, hydroperoxide positive rate; NBT, nitroblue tetrazolium; PA, protocatechuic acid; 3,4-OH-DHCiA, 3,4-dihydroxydihydrocinnamic acid; RoA, rosmarinic acid; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; XA/XOD, xanthine/xanthine oxidase.

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