Full Paper

Antioxidant, Free Radical–Scavenging, and NF- κ B–Inhibitory Activities of Phytosteryl Ferulates: Structure–Activity Studies

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Abstract. Some of the pharmacological properties of phytosteryl ferulates may be linked to their antioxidant potential. In this study, 2,2-diphenyl-1-picrylhydrazyl (DPPH), electron spin resonance (ESR), and thiobarbituric acid–reactive substances (TBARS) assays demonstrated that phytosteryl ferulates such as cycloartenyl ferulate (CAF), 24-methylenecycloartanyl ferulate (24-mCAF), and β -sitosteryl ferulate (β -SF) and ferulic acid (FA) each exerted strong free radical scavenging and antioxidation of lipid membrane, which were comparable to α -tocopherol. However, the sterol moiety alone, such as cycloartenol (CA), had neither activity. Since, the reactive oxygen species (ROS) production in the cell complex defense mechanism cannot be ruled out with the cell free system, we measured ROS production in NIH 3T3 fibroblast cells induced by H₂O₂. CAF and ethyl ferulate (eFA) greatly decreased the ROS level in this system. CA also significantly inhibited the ROS level, suggesting that CA could inhibit ROS production in living cells. Besides these, CAF, 24-mCAF, β -SF, as well as eFA and CA, all these chemicals significantly inhibited the NF- κ B activity as analyzed by measuring translocation of NF- κ B p65 in LPS-stimulated RAW 264.7 macrophages. These observations revealed that phytosteryl ferulates are responsible for the antioxidant and anti-inflammatory activity via ROS scavenging and inhibition of ROS production.

Keywords: phytosteryl ferulate, cycloartenyl ferulate, NF- κ B, antioxidant, reactive oxygen species (ROS)

Introduction

There has been considerable public and scientific interest in the field of free radical chemistry and biology. During the past 30 years, the fields of free radical chemistry and biology have risen from relative obscurity to become mainstream elements of biomedical investigation and pharmaceutical development (1). Mitochondria are an important source of reactive oxygen species

(ROS), and uncontrolled and/or sustained increase in ROS production by mitochondria causes oxidative damage to biological molecules, which could be implicated in the pathogenesis of cancer, diabetes mellitus, atherosclerosis, neurodegenerative diseases, rheumatoid arthritis, ischemia/reperfusion injury, obstructive sleep apnea, and other diseases (2). Therefore, the antioxidant therapies could provide a potential means to treat conditions in which the formation of reactive oxygen species exceeds the capability of natural protective mechanisms (3).

Identification of oxidant and antioxidant bioactive compounds is important, not only for predicting and

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reducing health risks, but also for evaluating possible combinatory beneficial effects of bioactive ingredients in phyto-therapeutic formulations (4). Polyphenols have attracted attention as a functional food with various bioactivities including anticancer, antimutagenic, antimicrobial, and antiviral activities (5). Caffeic acid, one of the most commonly occurring phenolic acids in fruits, grains, and dietary supplements, has shown strong antioxidant efficacy against oxidative damage (6); and gallic acid, one of the major constituents of grape seed extracts, exhibited anticancer efficacy against human prostate cancer cells and transgenic adenocarcinoma of mouse prostate via a strong suppression of cell cycle progression and cell proliferation and an increase in apoptosis (7). Moreover, curcumin, the medicinal constituent of turmeric (*Curcuma longa* rhizomes) has received much attention recently as a promising dietary supplement for its anti-inflammatory, anti-angiogenic, and anti-oxidant effects; wound healing effects; and anti-cancer effects (8).

Chemopreventive and chemoprotective phytochemicals and phytonutrients can alter or correct undesired cellular functions caused by abnormal pro-inflammatory signal transmissions mediated by NF- κ B (9). This key regulator of inflammation as well as transcription factor exists mainly as a heterodimer comprised of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex due to its binding with inhibitors of κ B (I κ Bs) in unstimulated cells; activation of NF- κ B involves the phosphorylation of I κ Bs, and the resulting free NF- κ B is then translocated to the nucleus, where it binds to κ B-binding sites in the promoter regions of target genes and regulates the expression of crucial mediators including pro-inflammatory cytokines, chemokines, adhesion molecules, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) involved in chronic inflammatory disease and cancer (10). It is well documented that ROS activate NF- κ B, which leads to the generation of pro-inflammatory cytokines and inducible enzymes, such as COX-2 and iNOS, in leukocytes and macrophages. Conversely, the pro-inflammatory cytokines cause oxidative stress by promoting the release of ROS by immune cells and also by non-immune cells. Thus inflammation and oxidative stress are involved in the spiraling vicious inflammatory cycle (11, 12).

Rice bran is a component of raw rice that is obtained when it is removed from the starchy endosperm in the rice milling process. Rice bran oil derived from rice bran has been found to possess promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease (13). Rice bran oil is a rich

source of γ -oryzanol (γ -ORZ), which contains a number of phytosteryl ferulates such as 24-methylenecycloartanyl ferulate (24-mCAF), cycloartenyl ferulate (CAF), campesteryl ferulate, β -sitosteryl ferulate (β -SF), and campestanyl ferulate; and the mixture of these is called as γ -ORZ (14). It is of particular importance that γ -ORZ exhibits antioxidant properties in *in vitro* systems (15). The antioxidant potency of γ -ORZ makes it a good candidate for pharmaceutical drugs, cosmetic formulations, and health food (16).

Notably, we have recently reported that a phytosteryl ferulates mixture, γ -ORZ, and its component CAF inhibited NF- κ B activity in dextran sulfate-induced colitis in mice (17) and macrophages (18). In addition, *trans*-ferulic acid (FA) and γ -ORZ also ameliorate ethanol-induced liver injury in mice through enhancing superoxide dismutase activity (19). Therefore, it seems reasonable to assume that γ -ORZ components could also be used for pharmaceutical purposes. Antioxidant activity of steryl ferulates (γ -ORZ) has been shown to be similar to that of non-esterified FA, indicating that the FA moiety is responsible for the antioxidant properties (20). The aim of this study was to investigate the structure–activity relationships of FA and sterol moieties of phytosteryl ferulates in relation to antioxidant and NF- κ B activities in a cell-free system and living cultured cells.

Materials and Methods

Drugs and reagents

Drugs and reagents used in the experiment were, 2-thiobarbituric acid, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and α -tocopherol (Sigma-Aldrich, Tokyo); 2,2'-azobis(2-methylpropionamide) dihydrochloride, ferulic acid (FA, *trans*-4-hydroxy-3-methoxycinnamic acid), and ethyl ferulate (eFA, ethyl *trans*-4-hydroxy-3-methoxycinnamate) (Wako Chemical, Osaka). γ -ORZ was purified from Japanese rice, Koshi-hikari, as reported earlier (21).

CAF, β -SF, and 24-mCAF were purified from γ -ORZ by repeated recrystallization as reported earlier (19). Cycloartenol (CA) was prepared by alkaline hydrolysis of CAF.

Determination of free radical-scavenging ability with DPPH assay

The free radical-scavenging capacity of the phytosteryl ferulates, sterol, and FA were determined by using DPPH according to the previously described procedure (22). Antioxidant solution to be tested was added in a final concentration of 1 – 60 μ M to the reaction mixture.

Absorbance of the DPPH-solution (0.45 mg/L in methanol) was recorded at 517 nm using a spectrophotometer (V-550; JASCO, Tokyo). α -Tocopherol was used as a positive control.

Determination of antioxidation activity of lipid membrane with thiobarbituric acid-reactive substances (TBARS) assay

Modified thiobarbituric acid-reactive assay was used to measure the potential antioxidant capacity using egg yolk homogenates as lipid rich media. Malondialdehyde, a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of thiobarbituric acid, yielding a pinkish red chromogen with an absorbance maximum at 532 nm (23). Briefly, 0.1 mL of 10% (w/v) egg yolk homogenate and 1–60 μ M of sample to be tested was added to a test tube. A 0.01-mL aliquot of 2,2'-azobis(2-methylpropionamidine) dihydrochloride solution (0.07 M) in water was added to induce lipid peroxidation. Then 0.3 mL of 20% acetic acid (pH 3.5) and 0.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% (w/v) sodium dodecyl sulphate solution was added and the mixture adjusted to a final volume of 1.0 mL by adding distilled water. The resulting mixture was vortexed and then heated in a water bath at 95°C for 60 min. After cooling, 1.0 mL of 1-butanol was added to each tube, extensively vortexed, and centrifuged at 1,200 $\times g$ for 10 min. Absorbance of the organic upper layer was measured using a spectrophotometer (V-550 or EMC-418; JASCO, Tokyo) set at 532 nm. All the values were calculated on the basis of the percentage in antioxidant index (AI %):

$$AI \% = (1 - A_T/A_C) \times 100$$

Where A_C is the absorbance value of the fully oxidized control and A_T is the absorbance of the test sample.

Cell culture and treatment

The NIH 3T3 (mouse embryonic fibroblast cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Cells were seeded at 1×10^6 cells/mL and grown at 37°C in 5% CO₂ for 2–4 days in DMEM supplemented with 10% fetal bovine serum. Treatment with test compounds and H₂O₂ were carried out under serum-free conditions.

Measurement of intracellular ROS with DCFH-DA assay

Intracellular ROS levels were measured by the dichlorofluorescein assay, as described by T Teissier et al. (24). Briefly, NIH 3T3 cells were pretreated with 10 μ M CAF, eFA, and CA overnight followed by 2 h of 100 μ M H₂O₂ stimulation. Afterwards, the cells were

washed twice with calcium-magnesium-free phosphate-buffered saline (PBS) and incubated with 100 μ M DCFH-DA containing medium for 30 min. Medium was removed; cells were washed with calcium-magnesium free PBS and then solubilized in 1 mL of 1 M NaOH. 2',7'-Dichlorodihydrofluorescein (DCF) fluorescence was read at λ_{exc} = 485 nm and λ_{em} = 530 nm using a photospectrofluorometer (EMC-418, JASCO).

Immunohistochemical analysis of NF- κ B p65 nuclear translocation in RAW 264.7 macrophages

Immunofluorescence was employed to detect translocation of the p65 component of NF- κ B to the nucleus as described by Lebron et al. (25). Briefly, RAW 264.7 macrophages were seeded onto sterile 22 $\times$ 22-mm glass cover slips in 6-well tissue culture plates and pretreated with 10 μ M CAF, 24-mCAF, β -SF, eFA, or CA overnight, followed by LPS for 2 h prior to fixation with 3.7% paraformaldehyde for 30 min at room temperature. After fixation, the cells were subsequently permeabilized with 0.2% Triton X-100 for 30 min at room temperature and rinsed with PBS. Blocking of non-specific binding sites was performed with 3% goat serum along with 1% BSA in PBS for 60 min at room temperature. Rabbit polyclonal IgG against NF- κ B p65 (Santa-Cruz Biotechnology, Tokyo), diluted 1:200 in PBS containing 3% goat serum and 1% BSA, was added and incubation carried out for 90 min at room temperature in a humidified chamber. The cover slips were washed extensively and incubated for 60 min with a 1:500 dilution of Alexa Fluor 568-conjugated anti-rabbit antibody. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI, 0.1 μ g/mL; Sigma-Aldrich, Tokyo). Following extensive washing, the cover slips were mounted on slides and analyzed by confocal fluorescence microscopy (C1 Plus; Nikon, Tokyo). Randomly, five fields of each slide were counted to obtain the percentage of activated and non-activated cells.

Hydroxyl radical-scavenging activity measured by electron spin resonance spectroscopy (ESR)

The ESR spectra were recorded on a JEOL Model JES-FA100 ESR spectrometer (JEOL, Tokyo) in accordance with the previous study by Matsuki et al. (26). Hydroxyl radical ($\cdot$ OH)-scavenging activity was determined by quantification of 5,5-dimethyl-1-pyrroline N-oxide (DMPO)-OH spin trap adducts resulting from the reaction of DMPO with the radicals generated by the Fenton reaction. The experimental parameters were as follows: temperature, ambient; microwave power, 1.00 mW; modulation frequency, 100 kHz; modulation width, 0.16 mT; sweep width, 5.0 mT; sweep time,

30.0 s; center field, 335.070 mT; and time constant, 0.03 s. A 40- μ l aliquot of DMPO (1/10 diluted with ultra pure water, UPW) was mixed with 76 μ l of 0.2 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 134 μ l of UPW, and 0.4 μ l of vehicle (90% ethanol and 10% dimethyl sulfoxide mixture) or 40 mM stock chemical solution to make the final concentration 40 μ M each. The mixture was stirred, added with 150 μ l of 1 mM H_2O_2 , and again stirred. The solution was then transferred to a capillary tube placed in the cavity of the ESR spectrometer for measurement. After 5 min, the ESR signal was taken to measure the inhibition of $\cdot\text{OH}$ radical by chemicals. The inhibition was determined by the ratio of the peak height of the second signal from the DMPO-OH spin adduct.

Statistical analyses

Numerical data are expressed as the mean $\pm$ S.E.M. Statistical evaluation was carried out by Student's *t*-test and, where appropriate, one-way ANOVA; Tukey's multiple comparison test was applied.

Results

FA and sterol moieties in phytosteryl ferulates

In the early period, γ -ORZ was originally considered as a single compound but later it has been characterized as a mixture of FA esters of several sterols and triterpene alcohols called α , β , and γ -ORZ, of which γ -ORZ has been the most commonly mentioned compound. All components of γ -ORZ contain one unit of FA, that is, a FA moiety, and the residual moiety is a triterpene alcohol or sterol similar to cycloartenol. According to the nomenclature of the sterol moieties, steryl ferulates differ principally in 1) presence or absence of a double bond between C-5 and C-6; 2) the number of methyl groups attached to C-4 classified as 4-desmethylsterols; normal phytosterols without methyl group at C-4 position; 3) 4-monomethyl; 4) 4,4-dimethylsterols, also known as triterpene alcohols; and 5) the structure of the side chain attached to the tetracyclic ring system (27). To investigate the structure–activity relationship of phytosteryl ferulates in relation to antioxidant, free radical–scavenging, and anti-inflammatory activity, we selected 4,4-dimethylsterol ferulates such as CAF and 24-mCAF; a 4-desmethylsterol ferulate such as β -SF; a sterol such as CA; and non-sterols such as FA and eFA (Fig. 1).

Effects of phytosteryl ferulates and sterol on DPPH free radical–scavenging ability

To evaluate the structural and functional relationship of FA and sterol moieties in phytosteryl ferulates for free radical–scavenging ability, we investigated CAF,

24-mCAF, β -SF, FA, and CA (1 – 60 μ M) in comparison with that of α -tocopherol. The bleaching of DPPH absorption is considered the representative capacity of test compounds to scavenge free radicals independently from any enzymatic activity. Our results demonstrated that all phytosteryl ferulates such as CAF, 24-mCAF, and β -SF and also FA strongly scavenged the free radical dose-dependently (comparable to α -tocopherol); however, CA, the sterol moiety alone, completely failed to scavenge the free radical (Fig. 2a). Rice bran phytosteryl ferulates contain one unit of FA acid and the residual moiety is triterpene alcohol or a sterol similar to CA (Fig. 1). The 4-hydroxyl group of FA is an active unit in the free radical–scavenging and antioxidant reactions. Therefore, the hydroxyl group of phytosteryl ferulates (equivalent to the 4-hydroxyl group of FA) plays a pivotal role in the free radical–scavenging reaction.

Effects of phytosteryl ferulates and sterol on cholesterol oxidation accelerated by 2,2'-azobis(2-methylpropionamide) dihydrochloride

Free radical scavengers are considered to be inhibitors of lipid peroxidation. Therefore, we next evaluated the structure–activity relationship of FA and sterol moieties of phytosteryl ferulates using CAF, 24-mCAF, β -SF, FA, and CA (1 – 60 μ M) by estimating the free malondialdehyde by the TBARS assay. As we expected, phytosteryl ferulates (CAF, 24-mCAF, β -SF, and FA) exhibited strong antioxidant potency (comparable to α -tocopherol), whereas, sterol CA completely failed to exhibit any antioxidant potency in the cholesterol oxidation system (Fig. 2b). In accordance with the DPPH assay, the FA moiety of the phytosteryl ferulates might be responsible for these compounds having an antioxidant potency like FA does.

Antioxidative effect of phytosteryl ferulates and sterol on H_2O_2 -induced oxidative stress in NIH 3T3 fibroblast cells

To investigate the pharmacological action of CAF, ethylester FA, and CA against oxidative stress, we investigated H_2O_2 -induced oxidative stress in NIH 3T3 cells using the latent fluorescent probe DCFH-DA. The non-fluorescent molecule DCFH-DA freely permeates into the cells. After incorporation into the cells, its acetate moiety is rapidly hydrolyzed with esterase followed by oxidation by cellular oxidative substances affording fluorescent DCF. Therefore, DCFH-DA reveals the intracellular production of redox-active substances to investigate oxidative damage in intact cells (28). Expectedly, 10 μ M CAF and eFA exhibited strong antioxidant potency; surprisingly, however, 10 μ M CA, a

Fig. 1. Structural nomenclature of phytosteryl ferulate showing ferulic acid and the sterol moiety (IUPAC-IUB 1989) (I), cycloartenyl ferulate (CAF, principal components of γ -oryzanol), 24-methylenecycloartenyl ferulate (24-mCAF), and β -sitosteryl ferulate (β -SF) showing ferulic acid and sterol moieties (II – IV); cycloartenol (CA) showing side chains and the tetracyclic ring system (V); α -tocopherol (VI); ferulic acid (FA) (VII); and ethyl ferulate (eFA) (VIII).

sterol moiety alone, also significantly ($P < 0.05$) suppressed the H_2O_2 -induced oxidative stress (Fig. 3).

Effects of phytosteryl ferulates and sterol on LPS-induced NF- κ B p65 translocation in RAW 264.7 macrophages

Degradation of cytosolic I κ B α enables NF- κ B sub-

units to migrate to the nuclear compartment, where they promote gene transcription (25). Since the p65 subunit has been demonstrated to exert critical activity in the transcription of many inflammatory genes, we investigated the effects on LPS-induced translocation of NF- κ B p65 in RAW 264.7 macrophages. Our results demonstrated that LPS (10 ng/mL) exhibited NF- κ B p65 trans-

Fig. 2. Free radical-scavenging and antioxidant potency of phytosteryl ferulates and sterol in solution. a) Radical-scavenging ability of CAF, 24-mCAF, β -SF, FA, and CA (1, 20, 40, and 60 μ M) in comparison with α -tocopherol. Scavenging activity was measured using the 2,2-diphenyl-1-picrylhydrazyl assay (see Materials and Methods). b) Antioxidant potency of CAF, 24-mCAF, β -SF, FA, and CA (1, 20, 40, and 60 μ M) in comparison with that of α -Toco. Antioxidant potency was evaluated by estimating the free malondialdehyde using the TBARS assay (see Materials and Methods). Values are expressed as the mean $\pm$ S.E.M. from three individual experiments. α -Toco, α -tocopherol; CAF, cycloartenyl ferulate; 24-mCAF, 24-methylenecycloartanyl ferulate; β -SF, β -sitosteryl ferulate; FA, ferulic acid; CA, cycloartenol.

location from the cytoplasm into the nuclei of the macrophages. According to the results in the cell based-oxidative stress assay (Fig. 3), CAF, 24-mCAF, β -SF, CA, or eFA at 10 μ M each significantly inhibited nuclear translocation of NF- κ B p65 in macrophages (Figs. 4: a and b).

Effects of phytosteryl ferulates and sterol on the inhibition of hydroxyl radical

The $\cdot$ OH-scavenging activities of rice bran constituents purified from γ -ORZ were assessed by ESR spectroscopy. The ESR-spin trapping technique is widely used and is a precise method for the measurement of $\cdot$ OH-scavenging activity. In Fig. 5, the relative signal intensity of DMPO-OH spin trap adducts resulting from the reaction of DMPO with the radicals generated by the Fenton reaction in the presence or absence of phytosteryl ferulates, sterol, or α -tocopherol have been demon-

strated. The height of the second peak of the spectrum represented the relative amount of DMPO-OH adduct. Addition of the phytosteryl ferulate compound: CAF, 24-mCAF, or β -SF; FA, or α -tocopherol, at 40 μ M each, significantly ($P < 0.05$ and $P < 0.01$) decreased the amount of DMPO-OH adduct. It is to be noted that, β -SF and FA strongly ($P < 0.01$) decreased the DMPO-OH adduct. On the other hand, the sterol compound CA did not decrease the DMPO-OH adduct significantly (Fig. 5).

Discussion

There is emerging interest in the use of naturally occurring antioxidants for the management of a number of pathophysiological conditions, most of which involve free radical damage (29). Dietary antioxidant is one of the defense mechanisms protecting the body against the damaging effects of ROS and may reduce the risk of

Fig. 3. Antioxidative effects of CAF, eFA and CA on H₂O₂-induced oxidative stress in NIH 3T3 fibroblast cells. Cells were pretreated with 10 μ M CAF, eFA, or CA overnight followed by 100 μ M H₂O₂ stimulation for 2 h. Afterwards, the cells were washed twice with calcium-magnesium free PBS and incubated with 100 μ M DCFH-DA-containing medium for 30 min. Medium was removed; the cells were washed with calcium-magnesium free PBS and then solubilized in 1 mL of 1 M NaOH. DCF fluorescence was read at λ_{exc} = 485 nm and λ_{em} = 530 nm. The data represent the mean $\pm$ S.E.M. (n = 5–8). * P < 0.05, compared to the control and # P < 0.05, compared to the treatment groups. CAF, cycloartenyl ferulate; eFA, ethyl ferulate; CA, cycloartenol; AFU, arbitrary fluorescent unit.

coronary heart disease, cancer, and other aging-associated diseases (30). To explore the mechanism underlying the antioxidant, free radical, and anti-inflammatory activity, we investigated the effects of phytosteryl ferulates on inflammation-related macrophages, fibroblasts, and chemical reactions.

In this study, we initially measured antioxidant and free radical-scavenging activities using TBARS and DPPH assays, respectively. The results demonstrated that CAF, 24-mCAF, and β -SF and also FA had strong free radical-scavenging and antioxidant potencies, which were comparable to those of α -tocopherol. However, the sterol moiety, like CA, had neither antioxidant nor free radical-scavenging activity. It is known that the antioxidant activity of phenols depends on the electronic and steric effects of the ring, substituents, and the strength of hydrogen-bonding interactions between the phenol and the solvent (31). Therefore, it is suggested that the common FA-derived structure of CAF, 24-mCAF, and β -SF with a phenolic hydroxyl group under the similar situation exhibited both antioxidant potency and scavenging activity (Fig. 2: a and b). To confirm the ROS-scavenging activity of γ -ORZ and the related compounds, we further analyzed ROS-scavenging activity by using ESR. Our results indicated that phytosteryl ferulates compounds such as CAF, 24-mCAF, β -SF, and FA significantly decreased the amount of DMPO-OH adduct (Fig. 5), suggesting direct evidence that these

Fig. 4. Effects of phytosteryl ferulates and sterols on NF- κ B p65 nuclear translocation in RAW 264.7 macrophages. a) Inhibitory effect of CAF, 24-mCAF, β -SF, CA, and eFA, 10 μ M each, on NF- κ B activation in LPS-stimulated RAW 264.7 macrophages. The anti-NF- κ B p65-bound cells were incubated with Alexa Fluor 568-conjugated anti-rabbit IgG and analyzed using a fluorescence microscope. Experiments were repeated three times and similar results were obtained. Arrows indicate the nuclear translocation of NF- κ B p65. b) Graphical representation of % NF- κ B-activated cells per field. Values are expressed as percent mean $\pm$ S.E.M. from three individual experiments. **Significantly different between LPS and LPS + treatment group. CAF, cycloartenyl ferulate; 24-mCAF, 24-methylenecycloartanyl ferulate; β -SF, β -sitosteryl ferulate; CA, cycloartenol; eFA, ethyl ferulate.

Fig. 5. Hydroxyl radical-scavenging potency of phytosteryl ferulates and sterol. Relative signal intensity of DMPO-OH spin trap adducts resulting from the reaction of DMPO with the radicals generated by the Fenton reaction was evaluated by ESR assay. Comparison of the $\cdot$ OH-scavenging ability of CAF, 24-mCAF, β -SF, FA, CA, and α -toco at 40 μ M concentration (details described in Materials and Methods). Each point represents the mean $\pm$ S.E.M. * P <0.05 and ** P <0.01: significantly different from the vehicle-treated control (n =3). α -Toco, α -tocopherol; CAF, cycloartenyl ferulate; 24-mCAF, 24-methylenecycloartanyl ferulate; β -SF, β -sitosteryl ferulate; FA, ferulic acid; CA, cycloartenol.

compounds can scavenge hydroxyl radicals.

It has been reported that rice bran extract significantly inhibits xanthine oxidase activity and formation of superoxide anions, scavenges hydroxyl radicals in a cell-free system (32), and enhances superoxide dismutase (SOD) activity in a cell-based system by using RAW 264.7 macrophages stimulated with LPS (18). In the present study, we also determined effects of phytosteryl ferulates compounds in a cell-based ROS production system. It was found that CAF and eFA, a membrane permeable FA, greatly inhibited the ROS production in NIH 3T3 fibroblast cells induced by H_2O_2 (Fig. 3). This result supports our data obtained in the cell-free systems and also indicates the possibility that γ -ORZ potentially protects activities of glutathione, SOD, catalase, and glutathione peroxidase, similar to the action of FA (33). Further investigation is required to clarify this point.

In the cell-free system to measure antioxidant and free radical-scavenging activities using TBARS and DPPH assays, the sterol moiety of γ -ORZ, CA, had no antioxidant and free radical-scavenging activities. To support these data, we also confirmed that CA did not affect the amount of DMPO-OH adduct in the ESR assay system. However, it is notable that CA significantly inhibited the intracellular ROS production in living cells stimulated with H_2O_2 . Sultana et al. (34) reported that topical application of CA significantly inhibits epidermal ornithine decarboxylase activity, DNA synthesis, lipid peroxidation, xanthine oxidase activity, and protects glutathione and phase II metabolizing enzymes in benzoyl peroxide

and UVB radiation-induced tumor promotion and oxidative stress in murine skin. Several lines of evidence suggest that cholesterol protects the phospholipids bilayer from oxidative stress in living cells (35). Intracellular ROS production is a complex enzymatic phenomena involving a number of events including NAD(P)H oxidase, xanthine oxidase, and the mitochondrial respiratory chain (36). There are numerous sources of ROS production in the cell complex defense mechanism that cannot be ruled out with chemical reactions in solution. During the course of radical oxidation, cholesterol may play an important role in lipid bilayer stabilization as cholesterol-modified cell membrane provoked the effects on peroxidation, with corresponding increases in oxidative damage in the cell, possibly as a consequence of lipid bilayer destabilization (37). As CA, the sterol moiety of γ -ORZ, has a similar chemical structure to cholesterol, it will be possible to induce radical-scavenging ability in living cells. Further investigations are required to clarify this point.

Recently, great attention has been focused on the relationship between NF- κ B and ROS. Reactive oxygen species are involved in the initiation of the NF- κ B-activating cascade to trigger a series of inflammatory genes (38). Inflammatory stimuli such as LPS triggers ROS production via the activation of the NADPH-oxidase system in macrophages (39). In addition, there is evidence that an exogenous oxidant such as H_2O_2 induces NF- κ B activation in some cell lines (40), and high concentration of antioxidants abolish NF- κ B activation in response to LPS or hypoxia, suggesting that ROS is involved in NF- κ B activation (11). However, the relationship between NF- κ B activation and ROS is still controversial (11, 12, 41). In this study, we finally analyzed NF- κ B activity by measuring translocation of NF- κ B p65 in LPS-stimulated RAW 264.7 macrophages. It is to be noted that 10 μ M α -tocopherol partially inhibited NF- κ B p65 nuclear translocation in macrophages stimulated with higher concentration of LPS (1 μ g/ml) (data not shown), indicating the possibility that LPS-stimulation may produce ROS, and α -tocopherol may scavenge generated ROS. In this cell system, we found that CAF, 24-mCAF, and β -SF as well as eFA and CA significantly inhibited the NF- κ B activity (Fig. 4: a and b). The NF- κ B-inhibitory activity of CA could not be explained precisely, but several lines of scientific evidence suggest that CA, the free triterpene alcohol, has strong anti-inflammatory potency, inhibits leucocyte infiltration against carrageenan-induced peritonitis in mice, and inhibits phospholipase A_2 (42). Similarly, Akihisa et al. (43) also reported that CA acts as a strong anti-inflammatory compound against 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation

in mice. The CA-induced inhibitory action of NF- κ B p65 nuclear translocation may be partially contributed by the antioxidant potency of CA, similar to the mechanism of cholesterol.

Together with our previous report demonstrating an anti-inflammatory effect of phytosteryl ferulates on the intestinal inflammation model (17) and ethanol-induced liver injury model (19), the present study suggested that the modulation of cellular signaling involved in the chronic inflammatory response by anti-inflammatory phytochemicals may comprise a rational and pragmatic strategy in molecular target-based chemoprevention.

In conclusion, our results suggest that phytosteryl ferulates are responsible for the antioxidant and anti-inflammatory activity in living cells via ROS scavenging and inhibition of ROS production. We found that not only the FA but also the sterol moiety exerts antioxidative and anti-inflammatory activity in the complex cell system.

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