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Novel bioassay system for evaluating anti-oxidative activities of food items: Use of basolateral media from differentiated Caco-2 cells

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Abstract

Reactive oxygen and nitrogen species, including superoxide (O_2^-) and nitric oxide (NO), are known to be mediators of oxidative stress and play pivotal roles in the onset of numerous life style-related diseases. While a number of studies have shown that naturally occurring anti-oxidants may be applicable for prevention and therapy for those diseases, most *in vitro* anti-oxidation tests reported have not provided significant insight into the absorption efficiency or metabolism of dietary anti-oxidants in the gastrointestinal tract. In the present study, we established a novel assay system by focusing on the bioconversion of food constituents using differentiated Caco-2 cells as a model of human intestinal epithelial cells. Various fresh food preparations [ginger, garlic, *shimeji* (*Hypsizigus marmoreus*), onion, carrot] were added to the apical side of differentiated Caco-2 monolayers. After incubation, the medium was recovered and tested for its inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced O_2^- generation in differentiated HL-60 cells, and on combined lipopolysaccharide (LPS)- and interferon (IFN)-γ -induced NO generation in RAW 264.7 macrophages. The garlic preparation (25% v/v) basolateral medium abolished O_2^- generation without any cytotoxicity toward HL-60 cells, though it was cytotoxic to Caco-2 cells. In the NO generation tests, all of the food preparations showed notable inhibitory activity, while the garlic preparation (5% v/v) basolateral medium inhibited NO generation with substantial cytotoxicity toward RAW 264.7 cells. Interestingly, the carrot preparation (1% v/v) basolateral medium inhibited NO generation in both a concentration- and time-dependent manner without any cytotoxicity toward RAW 264.7 or Caco-2 cells, and its activities were higher than those of the carrot preparation alone (1% v/v). Our results indicate that the present assay system is appropriate and reliable for determination of the anti-oxidative efficacy of dietary phytochemicals *in vivo*.

Keywords: *Caco-2, absorption, superoxide, nitric oxide, antioxidant*

Introduction

Reactive oxygen and nitrogen species (RONS) are considered to play important roles in the onset of a variety of diseases [1–3]. Superoxide anion (O_2^-) and nitric oxide (NO) are precursors of several types of RONS [4], and it is widely recognized that suppression of excessive O_2^- and NO generation is effective and promising for both prevention and therapy for oxidative stress-related diseases. We previously reported the inhibitory properties of several food phytochemicals towards O_2^- generation in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-activated dimethylsulfoxide (DMSO)-differentiated human promyelocytic HL-60 cells and NO generation in lipopolysaccharide (LPS)/interferon (IFN)-γ -activated RAW 264.7 macrophages [5–9], and some compounds shown to be active in *in vitro* experiments have also shown remarkable cancer chemopreventive activities in rodent models, as well as anticipated anti-oxidative properties [8–12].

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In order to more accurately determine the *in vivo* anti-oxidative activities of food items and their components, their bioavailability and metabolism in the gastrointestinal tract must be taken into account, because most plant secondary metabolites are scarcely absorbed, metabolized, or chemically decomposed. However, only a limited number of *in vitro* methods for precisely investigating small intestinal absorption and gut wall extraction have been reported. Further, most studies regarding the absorption of food phytochemicals in humans and experimental animals have required long periods of time for the experiments and a large number of samples. Although some models have been used to determine the intestinal uptake of food components, as well as to investigate absorption and intestinal and presystemic metabolism [13,14], they are complex, time-consuming, and expensive, and occasionally require sophisticated equipment.

The Caco-2 cell line, derived from human colon adenocarcinomas, exhibits enterocyte-like characteristics, such as the expression of brush-border enzymes [15,16], nutrient transporters [17,18], and an intercellular tight junction [16,19], and has been used widely as an *in vitro* model for studies of the small intestine, as well as for studying drug metabolism and transport [20,21]. In addition, many researchers have recently utilized these cells to investigate the absorption and metabolism of dietary carotenoids (β-carotene, lutein, lycopene and fucoxanthin) [22–24], polyphenols (quercetin, apigenin, genistein, daidzein, epicatechin and resveratrol) [18,25–29], and chlorophyll [23], as well as others [30,31].

In the present study, we aimed to establish a novel assay system that reflected *in vivo* anti-oxidative activity by focusing on the bioconversion of food constituents using differentiated Caco-2 cells. Selected food preparations were added to Caco-2 monolayers on the apical side, and then the inhibitory effects of conditioned media taken from the basolateral side toward O_2^- and NO generation by differentiated HL-60 cells and RAW 264.7 cells, respectively, were measured (Figure 1). In addition, transepithelial electrical resistance (TEER) values of the Caco-2 monolayers were determined to examine the effects of the food preparations on the tight junction. Our results showed that a carrot preparation was promising, as the basolateral medium from Caco-2 cells exposed to it highly suppressed NO generation at relatively low concentrations.

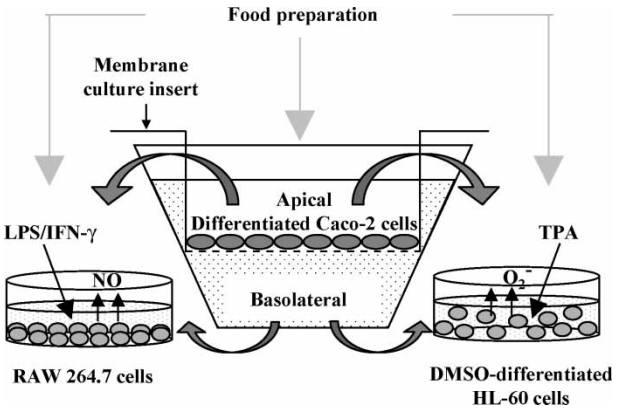

Figure 1. Illustrative outline of the present novel anti-oxidative assay system using differentiated Caco-2 cell monolayers. Caco-2 cells were seeded on cell culture inserts in 6-well plates at a density of 2×10^5 cells/insert and fully differentiated. Each fresh food preparation was added to the apical side of the monolayer at various final concentrations and incubated at 37°C for the designated times. After incubation, the TEER values of the Caco-2 monolayers were measured. Each conditioned medium and food preparation were separately tested for their inhibitory effects on TPA-induced O_2^- generation in differentiated HL-60 cells and on combined LPS/IFN-γ -induced NO generation in RAW 264.7 macrophages. The viability of Caco-2, RAW 264.7, and HL-60 cells was determined by MTT and Trypan Blue dye exclusion tests, respectively.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), and IFN-γ were purchased from Gibco BRL (Grand Island, NY). LPS (*E. coli* serotype 0127, B8) was obtained from Difco Labs (Detroit, MI). Cytochrome C was from Sigma Chemical (St Louis, MO). All other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise specified.

Cell cultures

Caco-2 cells, HL-60 human promyelocytic leukemia cells, and RAW 264.7 mouse macrophages were obtained from the American Type Culture Collection (Rockville, MA). The Caco-2 and RAW 264.7 cells were maintained in DMEM, and the HL-60 cells in RPMI 1640. Each medium contained 300 ng/ml of L-glutamine supplemented with 10% FBS, 100 U/ml of penicillin, and 100 ng/ml of streptomycin. The cells were incubated under a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C, and maintained at 80–90% confluence.

For each experiment, Caco-2 cells were seeded on cell culture inserts (PET track-etched membrane, 23 mm in diameter, pore size 0.4 μm, Becton Dickinson Labware, Franklin Lakes, NJ) in 6-well plates at a density of 2×10^5 cells/insert. The basolateral and apical compartments contained 2.0 and 3.0 ml of culture medium, respectively. Medium was replaced freshly two or three times a week. The cell culture inserts were used for the experiments 14–21 days after seeding, when TEER exceeded 300 Ωcm^2.

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Measurement of TEER

The TEER value of the Caco-2 monolayers was measured according to the method of Hidalgo et al. using a Millicell-ERS instrument (Millipore Co., Bedford, MA) [16]. The measurements were performed prior to and at the end of each experiment to assess the integrity of the Caco-2 monolayers. Monolayer resistance, obtained by subtracting intrinsic resistance (membrane alone) from total resistance (membrane plus monolayer), was corrected for the surface area and expressed as Ωcm^2. The effect of each assay sample on TEER was expressed as the relative value, obtained by correcting the control value (PBS-treated values as 100%).

Sample preparation

Fresh food items [ginger, garlic, *shimeji* (*Hypsizigus marmoreus*, a mushroom), onions, and carrots] were purchased from a local supermarket in Kyoto, Japan. Ten grams of each were cut into small pieces, suspended in 10 ml of PBS, and subjected to a homogenizer (Ultra Turrax T25 basic, IKA Labortechnik, Staufen, Germany) for 30 s at room temperature. Then, the homogenates were centrifuged at 5000 × *g* for 5 min and filtrated through filter paper (P3801 No. 2 Advantec Tokyo Japan). Each resulting food preparation was aliquoted and frozen immediately at − 80°C until use.

Food preparation-treated media from Caco-2 monolayer

Fully differentiated Caco-2 cells, prepared as described above, were used for the experiments. The medium from each side was removed and the inserts were washed with Hank's balanced salt solution (HBSS) twice. Phenol-red free DMEM medium was then added to the apical (2 ml) and basolateral (3 ml) sides. Then, one of the food preparations or PBS at various volumes (0–25%, v/v) was added to the apical side of the Caco-2 monolayers and incubated at 37°C for the designated time periods. After incubation, the medium from both sides was independently collected and the anti-oxidative activity of the each resultant medium preparation was evaluated as described below. The viability of the Caco-2 cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [32].

TPA-induced O_2^- generation in differentiated HL-60 cells

An inhibitory test of TPA-induced O_2^- generation was performed as previously reported with some modifications [8]. HL-60 cells were pre-incubated with 1.25% dimethylsulfoxide (DMSO) in RPMI 1640 medium for 6 days and differentiated into granulocytes. Differentiated HL-60 cells (1×10^6) were incubated in 1 ml of each food preparation, or apical or basolateral medium at 37°C for 15 min. Following the pre-incubation, the cell suspension was centrifuged and washed with HBSS twice, then suspended in 1 ml of HBSS. Ninety seconds after stimulation with 100 nM of TPA, cytochrome C (1 mg/ml) was added to the reaction mixture and incubated for 15 min at 37°C. Visible absorption by the supernatant at 550 nm, due to reduced cytochrome C, was measured for O_2^- generation. The viability of HL-60 cells was determined using a Trypan Blue dye exclusion test. Cells treated with only the vehicle and those with TPA alone were used as negative and positive controls, respectively.

LPS/IFN-γ -induced NO generation in RAW 264.7 Cells

Inhibitory tests of LPS/IFN-γ -induced NO generation were performed as previously reported with some modifications [7]. RAW 264.7 mouse macrophages (2×10^5 cells/ml) in 1 ml of DMEM medium on a 24-well plate were pre-incubated at 37°C for 18 h. After the cells were washed with PBS twice, 1 ml of each food preparation, or medium from the apical or basolateral side incubated with each food preparation, was added to the cells. Following incubation for 30 min, LPS (100 ng/ml), IFN-γ (100 units/ml), and L-arginine (2 mM) were added to the medium. After 24 h, the levels of nitrite (NO_2^-) and cytotoxicity were measured using Griess [33] and MTT assays, respectively. Cells treated with only the vehicle and with LPS/IFN-γ alone were used as negative and positive controls, respectively.

Statistical analysis and inhibitory rate (IR)

Each experiment was done independently at least 3 times and the data are expressed as the mean ± standard deviation (SD). The statistical significance of differences between groups in each assay was assessed by a Student's *t*-test (two-sided) that assumed unequal variance. The IR in each assay was calculated using the following equation: $IR(\%) = \{1 - [(\text{test sample data}) - (\text{negative control data})][(\text{positive control data}) - (\text{negative control data})]^{-1}\} \times 100$.

Results

Suppressive effects of food preparations, and conditioned apical and basolateral media on O_2^- generation

The ginger, garlic, *shimeji*, onion, and carrot preparations were selected as test samples, based on the results of a previous screening test [34], after being made from a homogenate at a concentration of 1 g/ml of fresh PBS followed by filtration. Each preparation at a concentration of 25% (v/v) showed 90% or greater inhibition toward TPA-induced O_2^- generation in

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differentiated HL-60 cells (Figure 2A). Further, the garlic [cell viability (CV) = 0%] and *shimeji* (CV < 20%) preparations showed substantial cytotoxicity toward HL-60 cells. Next, each preparation was added to the apical side of the Caco-2 monolayers at a concentration of 25% (v/v), and the apical and basolateral media were separately collected after 24 h of incubation. The apical medium samples treated with garlic, *shimeji* and carrot preparations showed significant inhibitory effects [IR > 80%] (Figure 2B), while the ginger- and onion-conditioned apical media (IR < 20 and <40%, respectively) were less active.

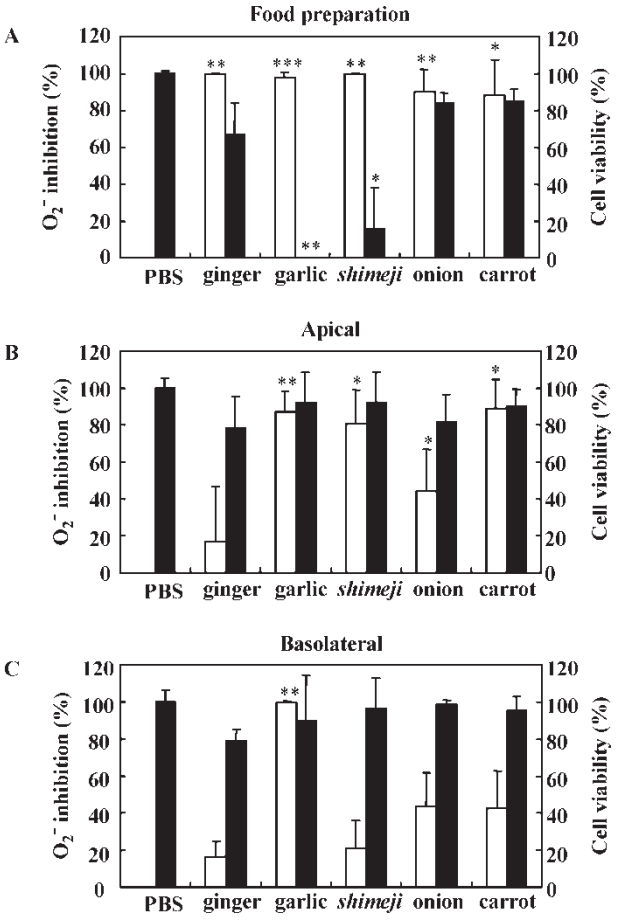

Figure 2. Suppressive effects of food preparations (A), and apical (B) and basolateral (C) medium samples on TPA-induced O_2^- generation in differentiated HL-60 cells. Open bars; inhibitory rates, closed bars; HL-60 cell viability. HL-60 cells were pre-incubated with 1.25% DMSO in RPMI 1640 medium for 6 days and allowed to differentiate into granulocytes. Differentiated HL-60 cells (1×10^6) were incubated with 1 ml of each food preparation (25% v/v), or apical or basolateral medium samples for 15 min at 37°C. Following that pre-incubation, the suspension was centrifuged and washed with HBSS twice, then the cells were suspended in 1 ml of HBSS. Ninety seconds after stimulation with TPA (100 nM), cytochrome C (1 mg/ml) was added to the reaction mixture and incubated for 15 min at 37°C. Visible absorption by the supernatant was measured at 550 nm to determine O_2^- generation. HL-60 cell viability was determined by a Trypan Blue dye exclusion test. Values are shown as the means ± SD ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

In addition, no notable cytotoxicity was observed (CV > 80%) for any medium sample from the apical side of Caco-2 cells treated with the preparations for 24 h (Figure 2B). It was notable that only the garlic-conditioned basolateral media abolished O_2^- generation without any marked cytotoxicity toward HL-60 cells (IR = 100, CV > 85%) (Figure 2C). Both the onion and carrot-conditioned basolateral medium showed moderate inhibition (IR ~ 40%), and that treated with ginger or *shimeji* had weaker activity (IR ~ 20%).

Suppressive effects of food preparations, and conditioned apical and basolateral media on NO generation

Each preparation (25% v/v)-conditioned basolateral medium suppressed LPS/IFN-γ -induced NO generation in RAW 264.7 cells by 90% or more (data not shown), therefore, they were retested at a concentration of 5% (v/v). Although all of the preparations (5% v/v) highly suppressed NO generation (IR > 70%), the ginger, garlic, and *shimeji* preparations were cytotoxic (CV < 30%) (Figure 3A). Further, it is interesting that apical medium collected from Caco-2 cells treated separately with those 3 preparations were not cytotoxic (CV > 95%) (Figure 3B). In addition, all five food preparations maintained their potent NO suppressive effects (IR > 70%) when exposed to the Caco-2 monolayers for 24 h (Figure 3B). As shown in Figure 3C, the ginger- (IR < 40%), *shimeji*- (IR < 20%) and onion- (IR < 20%) conditioned basolateral media showed weak suppression, while the garlic-conditioned medium was cytotoxic (IR = 71.2%, CV < 50%) toward RAW 264.7 cells. It should be also be noted that the carrot-conditioned basolateral medium exhibited pronounced suppression without any cytotoxicity (IR = 90%, CV = 100%).

Effects of food preparations on Caco-2 cell viability and TEER

The cytotoxicity and TEER values of the Caco-2 cell monolayers were determined to investigate the effects of the tested food preparations on CV and permeability through the tight junction, respectively, of Caco-2 cells. As shown in Figure 4A, the values for CV were 90% or more when the cells were exposed to the ginger, *shimeji*, or carrot preparations (25% v/v) for 24 h, whereas their relative TEER values decreased significantly by 40–50% as compared with the control. On the other hand, a garlic preparation at the same concentration was highly cytotoxic toward Caco-2 cells (10% > CV) and also decreased the relative TEER value by 50%. It is notable that an onion preparation did not significantly affect the values for CV and TEER. When the sample concentrations were reduced to a concentrations of

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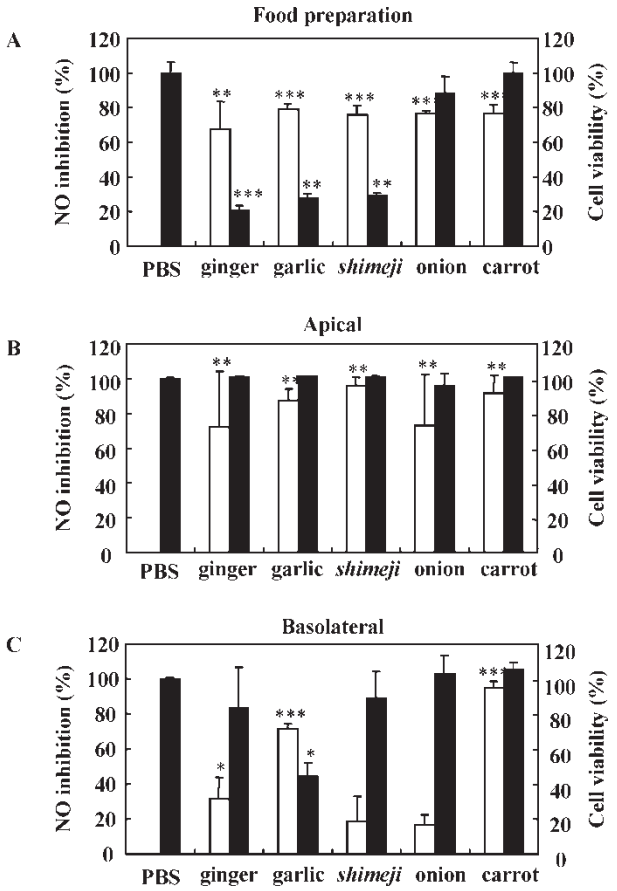

Figure 3. Inhibitory effects of food preparations, and apical and basolateral medium samples on LPS/IFN-γ -induced NO generation in RAW 264.7 cells. Open bars; inhibitory rates, closed bars; cell viability. RAW 264.7 cells (2×10^5) were incubated in 1 ml of the food preparations (25% v/v) (A), or apical (B) or basolateral (C) medium samples for 30 min at 37°C. Following the pre-incubation, the cells were treated with LPS (100 ng/ml), IFN-γ (100 units/ml), and L-arginine (2 mM) for 24 h at 37°C. After 24 h, the levels of NO_2^- were measured using a Griess assay. Cell viability was determined using MTT assays. Values are shown as the means ± S.D. ($n = 3$). $^{\star}P < 0.05$, $^{\star\star}P < 0.005$, $^{\star\star\star}P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

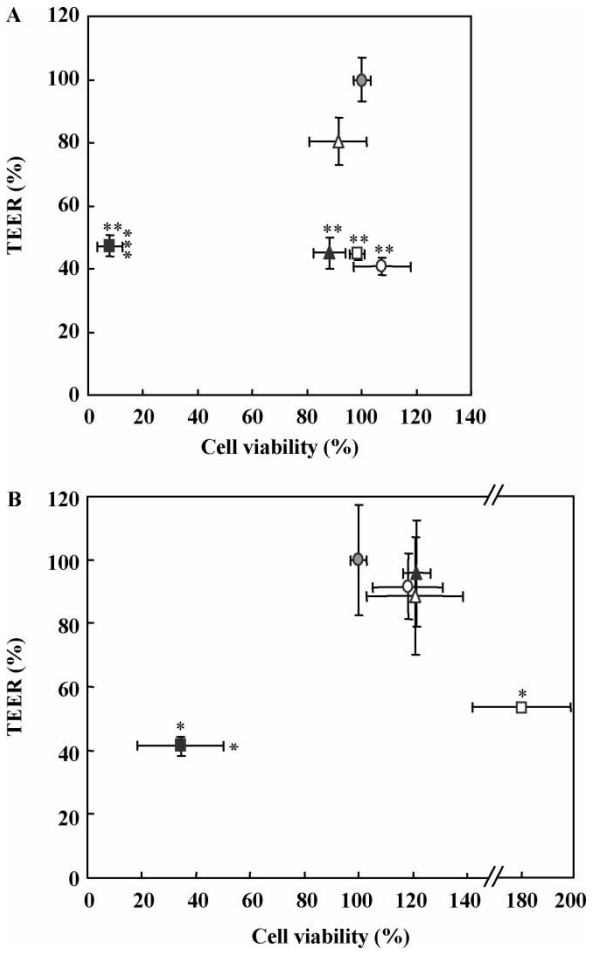

Figure 4. The effects of food preparations on TEER value and Caco-2 cell viability. Caco-2 cells monolayers were incubated with the food preparations at 25% (v/v) (A) and 5% (v/v) (B) concentrations for 24 h. The TEER values are presented as relative to the value of the control (PBS). Values are shown as the means ± S.D. ($n = 3$). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test, •: PBS, ▲: ginger, ■: garlic, ○: *shimeji*, △: onion, □: carrot

5% (v/v) (Figure 4B), the garlic preparation still exhibited substantial cytotoxicity (CV < 35%) and reduced the TEER value by 60%, as compared with the vehicle-added medium. In addition, the carrot preparation decreased the TEER value by 50% and increased CV by 80%. In contrast, the other preparations did not significantly change the CV or TEER values when compared with the control.

Concentration- and time-dependent suppressive effects of carrot preparation-treated basolateral medium on NO generation

Since the effects of carrot-conditioned basolateral medium were found to be promising, it was used for further investigations of concentration- and time-dependent activities toward NO suppression. When the Caco-2 cell monolayer was exposed to 0.04–1.0% (v/v) of the carrot preparation, the inhibitory effect of the apical medium was remarkable (IR ~ 80%), though it declined at a concentration of 0.008% (v/v). On the other hand, the inhibitory effects of the carrot (0.2–1.0%, v/v)-conditioned basolateral medium were high (IR > 84%), and then became drastically diminished at concentrations of 0.04% or lower (v/v) (Figure 5A). In our time-course results (Figure 5B), the value at 0 h indicates the IR of the carrot preparation itself (1%, v/v) without incubation with Caco-2 cells. There was no time-dependency for the NO generation suppressive effect of the apical medium. In contrast, there was a linear increase in the IR results of carrot (1%, v/v)-conditioned basolateral medium samples from 6 to 12 h, which was sustained until 24 h. Interestingly, the inhibitory effects of the carrot-conditioned apical and basolateral media (IR > 93%) at 12 and 24 h, respectively, were

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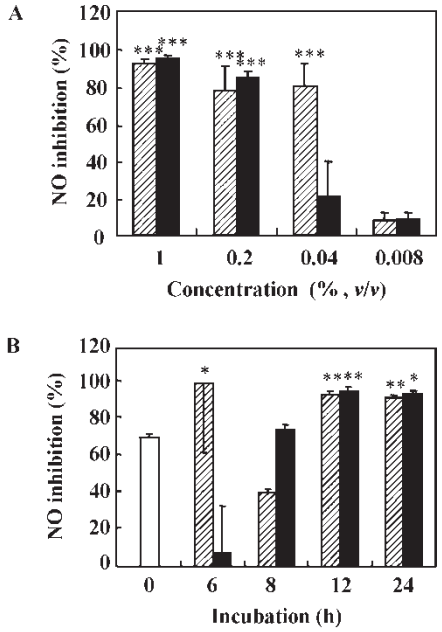

Figure 5. Concentration- and time-dependency of carrot preparation, and apical and basolateral medium treated with the carrot preparation on NO generation in RAW 264.7 cells. (A) Carrot preparations at concentrations ranging from 0.008 to 1.0% (v/v) were added to the apical side of the Caco-2 monolayers for 24 h. After incubation, apical (bias bars) and basolateral (solid bars) media were collected separately, and their inhibitory effects on LPS/IFN-γ -induced NO generation in RAW 264.7 cells were measured using Griess assay. (B) Carrot preparations at a concentration of 1% were added to the apical side of the Caco-2 monolayers for 0, 6, 8, 12, and 24 h. At each incubation time point, the preparation without the Caco-2 monolayer (open bar), and apical (bias bars) and basolateral (closed bars) media were collected, and their inhibitory effects toward LPS/IFN-γ -induced NO generation in RAW 264.7 cells were measured using a Griess assay. Values are shown as the means ± S.D. ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.001$ versus equivalent volume of PBS-treated control using Student's *t*-test.

significantly higher than those of the preparation itself (IR = 70%) (Figure 5B).

Discussion

The activated leukocytes induce oxidative stress via the activation of NADPH oxidase and iNOS to generate O_2^- and NO, respectively. Dietary factors have biological potentials to alternate these free radicals generation through several modes of actions including: (1) alternation of stimuli-induced activation of signal transduction pathways for NADPH oxidase and iNOS; (2) induction of anti-oxidant enzymes such as SOD and catalase; and (3) scavenging ROS. Because our experimental systems estimate the sum of these effects [(1)–(3)], detailed modes of anti-oxidation of active food preparations remain to be clarified in the near future.

For the present study, we selected five food items for testing based on previous data showing that their chloroform extracts demonstrated notable suppressive activities toward phorbol ester-induced O_2^- generation in differentiated HL-60 cells [34]. The food preparations were prepared from homogenates, not extracts, which is in contrast to most of the reported anti-oxidative assay systems that have been used to determine the activity of pure chemicals or samples prepared as alcohol extracts, whereas the chemical compositions of the extracts with water may resemble to those of the food preparations in this study. We considered that the present experimental approach utilizing food preparations may be more practical, because epithelial cells in the small intestinal tract are exposed to considerably high concentrations of food digestive compounds after ingestion, the characteristics of which is more resemble to food preparations than chemicals or alcoholic extracts. In addition, a recent study found that the complex mixture of phytochemicals contained in fruits and vegetables provides a more beneficial effect towards health promotion and disease prevention through overlapping or complementary effects than isolated phytochemicals [35]. However, the present *in vitro* assay system did not integrate some of the metabolic and digestive aspects that are important for mimicking *in vivo* situations, as ingested meals are subjected to a two-phase process in humans. Namely, following the gastric phase, which includes acidification and treatment with pepsin, the intestinal phase is initiated by neutralization, then continues with the addition of pancreatin, lipase and bile acids. It would be desirable to utilize those factors to improve the validity and predictive ability of our assay system. This notion is supported by a report by Ferruzzi et al., who showed that the small intestinal uptake of carotenoids and chlorophyll derivatives from spinach puree was significantly promoted by use of an *in vitro* digestion method with the Caco-2 model [23,36].

Interestingly, the inhibitory activities of the apical medium samples were decreased, while cell viability increased, after 24 h of incubation, as compared to the corresponding preparations alone. For example, the O_2^- generation inhibitory effects of apical medium from ginger (IR = 100–17%) and onion (IR = 90–44%) were largely decreased (Figure 2A and B). Additionally, the viability of HL-60 cells was increased when garlic (CV = 0–90%) and *shimeji* (CV = 16–90%) were incubated with the Caco-2 monolayers (Figure 2A and B). In the NO generation experiment, the cytotoxicity of apical medium disappeared when the cells were treated with ginger (CV = 20–100%), garlic (CV = 27–100%), or *shimeji* (CV = 30–100%) preparations (Figure 3A and 3B). These results imply that certain components responsible for anti-oxidative activity and cytotoxicity drastically changed by chemical degradation and/or biological effects of the Caco-2 monolayer. Further, our findings showed that conventional anti-oxidation tests do not have the ability to detect such changes,

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some of which may occur as a part of metabolic processes in the small intestine. In the present experiment condition, we could find the inhibitory effect on ROS generation of basolateral medium after incubation with differentiated Caco-2 cells, however, we have to investigate the mechanism point.

The efficiency of intestinal absorption of non-nutrients is controlled by a variety of factors, including gastrointestinal secretion, peristaltic activity in the intestine, the physiological condition of the epithelial membrane, and the mucosal immune system. Various regions of the gastrointestinal tract are normally exposed to a wide range of diverse molecules and microorganisms. In our assay system, we speculated that some components existed in the basolateral medium after incubation with differentiated Caco-2 cells. These might be classified into: (1) food components that penetrate the paracellular pathway or tight junction with an intact structure; (2) food component metabolites, including conjugates with glucuronic and sulfuric acids and/or methylated derivatives; (3) chemically degraded products; and (4) secreted factors, including hormones and cytokines from Caco-2 cells in response to stimulation from the food preparation stimuli; as well as others.

It is important to note that differentiated Caco-2 cells have been reported to express UDP-glucuronosyl-transferase [37,38] and sulfotransferase [39–41] and that quercetin-4′-glucoside, present in onions, is hydrolyzed by β -glucosidase [25,27,42] or lactase phlorizin hydrolase in this cell line. Further, Caco-2 cells are known to release various cytokines, including interleukin-6 (IL-6) or IL-8, in response to a number of different food factors, such as human milk factors, oleic acid, capric acid, butyrate and *Bacillus subtilis* present in *natto*, as well as others [43–46]. Kuo et al. reported that the level of the metal binding antioxidant protein, metallothionein, increased when genistein and biochanin A were added to Caco-2 cells [47]. These findings may help to identify the active components responsible for the suppression of O_2^- and NO generation, which not be components of the original food.

The paracellular pathway is thought to provide a highly dynamic transport route for certain ions and macromolecules, thereby contributing to the intestinal transport of various nutrients. Food components that are orally ingested and present in the intestinal lumen are also likely to participate in regulating tight junction permeability. TEER, a highly sensitive parameter for membrane permeability, is regulated by membrane-perturbing toxicants and a decrease in TEER is a clear indication of an increase in cell permeability caused by toxins that enhance tight-junction permeability [48,49]. In the present study, only the carrot preparation decreased TEER without cytotoxicity to Caco-2 cells at a lower concentration (Figure 4B). Ginger and *shimeji* preparations had a propensity to decrease TEER with increasing concentration of these preparations (Figure 4A and B). Along a similar line, Xu et al. evaluated the effects of lime, lemon, grapefruit, and pummelo juices on a Caco-2 cell line, and found that TEER decreased with increasing concentrations of the lime and lemon juices, while the grapefruit and pummelo juices increased TEER at high concentrations [50]. Hashimoto et al. also reported that a sweet pepper extract enhanced tight junction permeability [51]. Additionally, various food factors, such as chitosan [52], saponin [53] and capric acid [54], as well as others, have shown similar effects, whereas piperine [55] and eicosapentaenoic acid [56] have been demonstrated to have contrasting effects.

One of the intriguing findings of the present study was that the inhibitory activity of the carrot-conditioned basolateral medium toward NO generation after 12 and 24 h was higher than that of the carrot preparation itself at the same concentration (Figure 5B). This may indicate that unknown factors responsible for NO suppression were newly produced in the Caco-2 cell monolayer in response to the carrot preparation. We are currently attempting to identify the active component present in carrot preparation-conditioned basolateral medium. In addition, the viability of the carrot preparation-treated Caco-2 cells exceeded 180%, as measured by an MTT test (Figure 4B), suggesting that the carrot preparation might stimulate the mitochondria in Caco-2 to generate ROS, which are known to reduce MTT [57]. The viability of the carrot preparation-treated Caco-2 cells should be measured by other systems including the release of cytosolic lactate dehydrogenase (LDH) and Trypan Blue-dye exclusion tests.

In conclusion, when exposed to Caco-2 cells, five different food preparations exhibited a wide range of antioxidant activities, cytotoxicity, and effects on the tight junction, suggesting that the present novel bioassay system may be appropriate to determine the *in vivo* anti-oxidative efficacy of dietary anti-oxidants following ingestion.

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References

[1] Witztum JL. The oxidation hypothesis of atherosclerosis. Lancet 1994;344:793–795.
[2] Markert M, Andrews PC, Babior BM. Measurement of O_2^- of NADPH oxidase-containing particles from human neutrophils. Methods Enzymol 1984;105:358–365.
[3] Nathan C, Xie QW. Nitric oxide synthases: Roles, tolls, and controls. Cell 1994;78:915–918.

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[4] Ischiropoulous H, Zhu L, Beckman JS. Peroxynitrite formation from macrophage-derived nitric oxide. Arch Biochem Biophys 1992;298:446–451.
[5] Murakami A, Ohura S, Nakamura Y, Koshimizu K, Ohigashi H. 1′-Acetoxychavicol acetate, a superoxide anion generation inhibitor, potently inhibits tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in ICR mouse skin. Oncology 1996;53:386–391.
[6] Nakamura Y, Murakami A, Koshimizu K, Ohigashi H. Identification of pheophorbide α and its related compounds as possible anti-tumor promoters in the leaves of *Neptunia oleracea*. Biosci Biotechnol Biochem 1996;60:1028–1030.
[7] Kim OK, Murakami A, Nakamura Y, Ohigashi H. Screening of edible Japanese plants for nitric oxide generation inhibitory generation inhibitory activities in RAW 264.7 cells. Cancer Lett 1998;125:199–207.
[8] Murakami A, Kuki W, Takahashi Y, Yonei H, Nakamura Y, Ohto Y, Ohigashi H, Koshimizu K. Auraptene, a citrus coumarin, inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in ICR mouse skin, possibly through suppression of superoxide generation in leukocytes, Jpn. J Cancer Res 1997;88:443–452.
[9] Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S, Ohigashi H. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. Cancer Res 2000;60:5059–5066.
[10] Ohnishi M, Tanaka T, Makita H, Kawamori T, Mori H, Satoh K, Hara A, Murakami A, Ohigashi H, Koshimizu K. Chemopreventive effect of a xanthine oxidase inhibitor 1′-acetoxychavicol acetate, on rat oral carcinogenesis. Jpn J Cancer Res 1996;87:349–356.
[11] Tanaka T, Makita H, Kawamori T, Kawabata K, Mori H, Murakami A, Satoh K, Hara A, Ohigashi H, Koshimizu K. A xanthine oxidase inhibitor 1′-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Carcinogenesis 1997;18:1113–1118.
[12] Nakamura Y, Murakami A, Koshimizu K, Ohigashi H. Inhibitory effect of pheophorbide α, a chlorophyll-related compound on skin tumor promotion in ICR mouse. Cancer Lett 1996;108:247–255.
[13] Lennernas H, Ahrenstedt O, Hallgren R, Knutson L, Ryde M, Paalzow LK. Regional jejunal perfusion, a new *in vivo* approach to study oral drug absorption in man. Parm Res 1992;9:1243–1251.
[14] Lennernas H. Human intestinal permeability. J Pharm Sci 1998;87:403–410.
[15] Pinto M, Robine-leon S, Appay MD, Kedinger M, Triadou N, Dussaulx E, Lacroix E, Simon-Assmann P, Haffen K, Fogh J, Zwibaum A. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 1983;47:323–330.
[16] Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology 1989;96:736–749.
[17] Wang H, Dutta B, Huang W, Devoe LD, Leibach FH, Ganapathy V, Prasad PD. Human Na(+)-dependent vitamin C transporter 1 (hSVCT1): Primary structure, functional characteristics and evidence for a non-functional splice variant. Biochim Biophys Acta 1999;1461:1–9.
[18] Walgren RA, Lin JT, Kinne RK, Walle T. Cellular uptake of dietary flavonoid quercetin 4′-beta-glucoside by sodium-dependent glucose transporter SGLT1. J Pharmacol Exp Ther 2000;294:837–843.
[19] Hashimoto K, Kawagishi H, Nakayama T, Shimizu M. Effect of capsianoside, a diterpene glycoside, on tight-junctional permeability. Biochim Biophys Acta 1997;1323:281–290.
[20] Artursson P, Karlsson J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal (Caco-2) cells. Biochem Biophys Res Commun 1991;175:880–885.
[21] Pade V, Stavchansky S. Link between drug absorption solubility and permeability measurements in Caco-2 cells. J Pharm Sci 1998;87:1604–1607.
[22] During A, Hussain MM, Morei DW, Harrison EH. Carotenoid uptake and secretion by CaCo-2 cells: beta-carotene isomer selectivity and carotenoid interactions. J Lipid Res 2002;43:1086–1095.
[23] Ferruzzi MG, Failla ML, Schwartz SJ. Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an *in vitro* digestion and Caco-2 human cell model. J Agric Food Chem 2001;49:2082–2089.
[24] Sugawara T, Baskaran V, Tsuzuki W, Nagao A. Brown algae fucoxanthin is hydrolyzed to fucoxanthinol during absorption by Caco-2 human intestinal cells and mice. J Nutr 2002;132:946–951.
[25] Murota K, Shimizu S, Chujo H, Moon JH, Terao J. Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Arch Biochem Biophys 2000;384:391–397.
[26] Liu Y, Hu M. Absorption and metabolism of flavonoids in the Caco-2 cell culture model and a perused rat intestinal model. Drug Metab Dispos 2002;30:370–377.
[27] Murota K, Shimizu S, Miyamoto S, Izumi T, Obata A, Kikuchi M, Terao J. Unique uptake and transport of isoflavone aglycones by human intestinal Caco-2 cells: Comparison of isoflavonoids and flavonoids. J Nutr 2002;132:1956–1961.
[28] Vaidyanathan J, Walle T. Transport and metabolism of the tea fravonoid (−)-epicatechin by the human intestinal cell line Caco-2. Pharm Res 2001;18:1420–1425.
[29] Kaldas MI, Walle UK, Walle T. Resveratrol transport and metabolism by human intestinal Caco-2 cells. J Pharm Pharmacol 2003;55:307–312.
[30] Murakami A, Wada K, Ueda N, Sasaki K, Haga M, Kuki W, Takahashi Y, Yonei H, Koshimizu K, Ohigashi H. *In vitro* absorption and metabolism of a citrus chemopreventive agent, auraptene, and its modifying effects on xenobiotic enzyme activities in mouse livers. Nutr Cancer 2000; 36:191–199.
[31] Murakami A, Kuwahara S, Takahashi Y, Ito C, Furukawa H, Ju-Ichi M, Koshimizu K. *In vitro* absorption and metabolism of nobiletin, a chemopreventive polymethoxyflavonoid in citrus fruits. Biosci Biotechnol Biochem 2001;65:194–197.
[32] Sladowski D, Steer SJ, Clothier RH, Balls M. An improved MTT assay. J Immunol Methods 1993;157:203–207.
[33] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–138.
[34] Kim HW, Murakami A, Nakamura Y, Ohigashi H. Screening of edible Japanese plants for suppressive effects on phorbol ester-induced superoxide generation in differentiated HL-60 cells and AS52 cells. Cancer Lett 2002;176:7–16.
[35] Murakami A, Takahashi D, Koshimizu K, Ohigashi H. Synergistic suppression of superoxide and nitric oxide generation from inflammatory cells by combined food factors. Mutat Res 2003;523–524:151–161.
[36] Garrett DA, Failla ML, Sarama RJ. Development of an *in vitro* digestion method to assess carotenoid bioavailability from meals. J Agric Food Chem 1999;47:4301–4309.
[37] Abid A, Bouchon I, Siest G, Sabolovic N. Glucuronidation in the Caco-2 human intestinal cell line: Induction of UDP-glucuronosyltransferase 1*6. Biochem Pharmacol 1995;50: 557–561.
[38] Paine MF, Fisher MB. Immunochemical identification of UGT isoforms in human small bowel and in Caco-2 cell

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monolayers. Biochem Biophys Res Commun 2000;273: 1053–1057.
[39] Tamura H, Matsui M. Inhibitory effects of green tea and grape juice on the phenol sulfotransferase activity of mouse intestines and human colon carcinoma cell line, Caco-2. Biol Pharm Bull 2000;23:695–699.
[40] Baranczyk-Kuzma A, Garren JA, Hidalgo IJ, Borchardt RT. Substrate specificity and some properties of phenol sulfotransferase from human intestinal Caco-2 cells. Life Sci 1991;49:1197–1206.
[41] Prueksaritanont T, Gorham LM, Hochman JH, Tran LO, Vyas KP. Comparative studies of drug-metabolizing enzymes in dog, monkey, and human small intestines, and in Caco-2 cells. Drug Metab Dispos 1996;24:634–642.
[42] Ioku K, Pongpiriyadacha Y, Konishi Y, Takei Y, Nakatani N, Terao J. beta-Glucosidase activity in the rat small intestine toward quercetin monoglucosides. Biosci Biotechnol Biochem 1998;62:1428–1431.
[43] Claud EC, Savidge T, Walker WA. Modulation of human intestinal epithelial cell IL-8 secretion by human milk factors. Pediatr Res 2003;53:419–425.
[44] Tanaka S, Saitoh O, Tabata K, Matsuse R, Kojima K, Sugi K, Nakagawa K, Kayazawa M, Teranishi T, Uchida K, Hirata I, Katsu K. Medium-chain fatty acids stimulate interleukin-8 production in Caco-2 cells with different mechanisms from long-chain fatty acids. J Gastroenterol Hepatol 2001;16: 748–754.
[45] Fusunyan RD, Quinn JJ, Ohno Y, MacDermott RP, Sanderson IR. Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1beta and lipopolysaccharide. Pediatr Res 1998;43:84–90.
[46] Hosoi T, Hirose R, Saegusa S, Ametani A, Kiuchi K, Kaminogawa S. Cytokine responses of human intestinal epithelial-like Caco-2 cells to the nonpathogenic bacterium *Bacillus subtilis* (natto). Int J Food Microbiol 2003;82: 255–264.
[47] Kuo SM, Leavitt PS, Lin CP. Dietary flavonoids interact with trace metals and affect metallothionein level in human intestinal cells. Biol Trace Elem Res 1998;62:135–153.
[48] Okada T, Narai A, Matsunaga S, Fusetani N, Shimizu M. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. Toxicol In Vitro 2000;14:219–226.
[49] Karyekar CS, Fasano A, Raje S, Lu R, Dowling TC, Eddington ND. Zonula occludens toxin increases the permeability of molecular weight markers and chemotherapeutic agents across the bovine brain microvessel endothelial cells. J Pharm Sci 2003;92:414–423.
[50] Xu J, Go ML, Lim LY. Modulation of digoxin transport across Caco-2 cell monolayers by citrus fruit juices: Lime, lemon, grapefruit, and pummelo. Pharm Res 2003;20:169–176.
[51] Hashimoto K, Matsunaga N, Shimizu M. Effect of vegetable extracts on the transepithelial permeability of the human intestinal Caco-2 cell monolayer. Biosci Biotech Biochem 1994;58:1345–1346.
[52] Kotze AF, Luessen HL, de Boer AG, Verhoef JC, Junginger HE. Chitosan for enhanced intestinal permeability: Prospects for derivatives soluble in neutral and basic environments. Eur J Pharm Sci 1999;7:145–151.
[53] Cho SY, Sim JS, Kang SS, Jeong CS, Linhardt RJ, Kim YS. Enhancement of heparin and heparin disaccharide absorption by the Phytolacca americana saponins. Arch Pharm Res 2003;26:1102–1108.
[54] Lindmark T, Kimura Y, Artursson P. Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells. J Pharmacol Exp Ther 1998;284:362–369.
[55] Jensen-Jarolim E, Gajdzik L, Haberl I, Kraft D, Scheiner O, Graf J. Hot spices influence permeability of human intestinal epithelial monolayers. J Nutr 1998;128:577–581.
[56] Usami M, Muraki K, Iwamoto M, Ohata A, Matsushita E, Miki A. Effect of eicosapentaenoic acid (EPA) on tight junction permeability in intestinal monolayer cells. Clin Nutr 2001;20:351–359.
[57] Alvarez G, Ramos M, Ruiz F, Satrustegui J, Bogonez E. Pyruvate protection against β -amyloid-induced neutonal death: Role of mitochondrial redox state. J Neurosci Res 2003;73:260–269.

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