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Suppressive Effects of Mioga Ginger and Ginger Constituents on Reactive Oxygen and Nitrogen Species Generation, and the Expression of Inducible Pro-Inflammatory Genes in Macrophages

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ABSTRACT

We previously conducted screening tests of the chloroform extracts from a total of 89 species of Japanese plant food items for their suppressive effects on superoxide (O_2^-) generation through both NADPH oxidase and xanthine oxidase, and reported that mioga ginger (*Zingiber mioga* Roscoe) indicated the strongest suppressive activities. In this study, the suppressive effects of mioga ginger constituents, aframodial, and galanal B, together with [6]-gingerol and galanolactone occurring in ginger, on free radical generation and inducible proinflammatory gene expressions were investigated. Of these constituents, aframodial (20 μ*M*) exhibited marked suppressive effects on 12-*O*-tetradecanoylphorbol-13-acetate-induced O_2^- generation in HL-60 cells and lipopolysaccharide (LPS)/interferon-γ-induced nitric oxide (NO) generation in RAW264.7 cells (inhibition rates [IRs] = 84.6% and 95.9%, respectively). Aframodial also strongly suppressed the stimulated HL-60 cell-induced mutagenicity in AS52 cells (IR = 95.9%). The LPS-induced expression of inducible pro-inflammatory genes such as inducible NO synthase, interleukin (IL)-1β, IL-6, and granulocyte-macrophage colony-stimulating factor was significantly abolished (IRs = 99.1%, 74.6%, 74.0%, and 64.4%, respectively) by aframodial. In addition, degradation of the inhibitor of nuclear factor κB was suppressed by this compound (IR = 100%), suggesting that the suppression of nuclear factor κB activation, at least in part, is involved. Taken together, these results suggest that aframodial has potent antioxidative and anti-inflammatory potentials, and may be a promising candidate in prevention and/or therapy for chronic inflammation-associated carcinogenesis. *Antioxid. Redox Signal.* 7, 1621–1629.

INTRODUCTION

Excess production of reactive oxygen and nitrogen species (RONS) generated from activated inflammatory leukocytes, especially under conditions of chronic inflammation, may have an important role in tumor initiation and promotion, and inhibitors that block the generation of RONS may inhibit or delay carcinogenic processes (6). Suppression of superoxide (O_2^-) generation is thought to be important in reducing and mitigating oxidative stress, because O_2^- is one of the initial RONS, *i.e.*, reactive oxygen species-producing pathways, and reacts rapidly with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), which is thought to be more toxic than other types of reactive oxygen (36). We previously

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conducted screening tests of the chloroform extracts from a total of 89 species of Japanese plant food items for their suppressive effects on O_2^- generation in both HL-60 (human promyelocytic leukemia cells generating O_2^- through NADPH oxidase) and AS52 (Chinese hamster ovary cells generating O_2^- through xanthine oxidase), and reported that mioga ginger (*Zingiber mioga* Roscoe), a perennial herb with pungently aromatic flower buds native to Eastern Asia, was the most effective (12). We have previously reported that the methanol extract from mioga ginger showed a potent suppressive effect on the ability to generate NO in lipopolysaccharide (LPS) and interferon (IFN)-γ-stimulated RAW264.7 mouse macrophages (14). Thus, constituents of mioga ginger are anticipated to be promising candidates for cancer chemopreventive agents.

Abe *et al*. (2) recently isolated volatile constituents, aframodial, and galanal A and B, from ethyl acetate extracts of mioga ginger (Fig. 1). These labdane-type diterpene dialdehydes are known to be pungent principles in mioga ginger (2). In addition, it is well-known that a ginger preparation (*Zingiber officinale* Roscoe, Zingiberaceae) is a promising material for cancer prevention, as it has been shown to inhibit 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse skin tumor formation (6), TPA-induced epidermal ornithine decarboxylase activity (7), tumor necrosis factor (TNF)-α production in mouse skin (25), and azoxymethane-induced intestinal tumorigenesis (38), as well as pulmonary metastasis in mice implanted with B16F10 melanoma cells (30). [6]-Gingerol was reported to be the major pharmacologically active component of ginger (3, 28). However, it is important to note that aframodial and galanolactone are also present in ginger as well as mioga ginger (Table 1), and there are no known reports that have evaluated their physiological and cancer chemopreventive activities except for [6]-gingerol. Inducible NO synthase (iNOS) is a key enzyme that produces NO in response to inflammatory stimuli. Multiple lines of evidence support the notion that iNOS plays important roles in the development of tumors (31, 32), and iNOS inhibitors are regarded as candidates for chemoprevention involved with chronic inflammation-associated carcinogenesis. One nuclear target of the intracellular signaling pathways responsible for the induction of iNOS expression is the transcription factor nuclear factor κB (NF-κB) (37), which has been shown to be responsible for the expression of not only iNOS, but also a variety of pro-inflammatory genes, such as *cyclooxygenase (COX)-2* (7, 17), interleukin (IL)-1β (1), IL-6 (16), TNF-α (33), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (4). In an unstimulated cell, NF-κB is normally present in the cytoplasmic compartment bound with its inhibitor, IκB (9). Once stimulated with various endogenous and exogenous agents, IκB is phosphorylated and subsequently degraded via ubiquitination. Releasing IκB from NF-κB leads to the activation and nuclear translocation of NF-κB subunits, including p50 and p65 (8). Therefore, degradation of IκB is regarded as a key step for NF-κB-induced transcription of certain pro-inflammatory genes.

TABLE 1. CONSTITUENTS OF GINGER AND MIOGA GINGER

Constituent	*Ginger*	*Mioga ginger*
[6]-Gingerol	+	−
Aframodial	+	+
Galanal A/B	−	+
Galanolactone	+	−

In the present study, the suppressive effects of mioga ginger constituents, aframodial, and galanal B, together with [6]-gingerol and galanolactone occurring in ginger, on TPA-induced O_2^- generation in differentiated HL-60 cells, and on LPS/IFN-γ-stimulated RAW264.7 cells, as well as on the expression of inducible pro-inflammatory genes such as iNOS, COX-2, IL-1β, IL-6, TNF-α, and GM-CSF were examined. In addition, to investigate the suppressive mechanism of pro-inflammatory gene expressions, the involvement of NF-κB was examined by investigating the degradation of IκB.

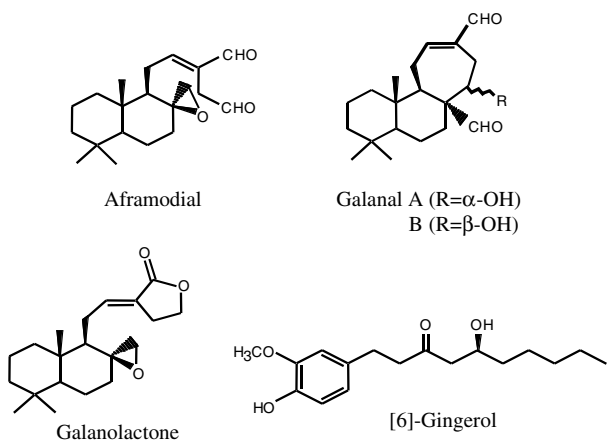

FIG. 1. Chemical structures of aframodial, galanal A/B, galanolactone, and [6]-gingerol.

MATERIALS AND METHODS

Chemicals

Aframodial, galanal B, and galanolactone were purified as described previously (2). [6]-Gingerol was obtained from Wako Pure Chemicals (Osaka, Japan). Rabbit polyclonal anti-IκB and goat polyclonal anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-rabbit IgG was from Dako (Glostrup, Denmark).

Measurement of O_2^- generation in TPA-stimulated, differentiated HL-60 cells

The O_2^- level in medium from TPA-treated HL-60 cells was determined as described previously with some modifications (21). HL-60 cells were preincubated with 1.25% dimethyl sulfoxide at 37°C in a 5% CO_2 incubator for 6 days, differentiating them into granulocytes. Differentiated HL-60 cells were suspended in RPMI 1640 medium at a density of 1×10^6 cells/ml, and incubated at 37°C for 15 min. After differentiated HL-60 cells were stimulated with TPA (100 n*M*),

the cell suspension was incubated for another 15 min. Cytochrome *c* solution (1 mg/ml) was added to the cell suspension 15 min before measurement. The reaction mixture was centrifuged at 4,000 *g* for 1 min, and visible absorption at 550 nm was measured. The level of O_2^- production was calculated from the formula: O_2^- (nmol/ml) = 47.7 $A_{550\ nm}$. The concentration of O_2^- in cell culture medium without cells was measured as a background control and subtracted.

Measurement of NO generation in LPS/IFN-γ-stimulated RAW 264.7 cells

RAW264.7 cells were suspended in Dulbecco's modified Eagle's medium (DMEM) at a density of 2×10^5 cells/ml, and then treated with LPS (100 ng/ml) and IFN-γ (100 U/ml). HL-60 cells were differentiated as described above, and suspended at a density of 2×10^5 cells/ml before addition of TPA (100 n*M*). Cells were treated for 1–24 h, and NO synthesis was determined using the Griess reaction. Briefly, 0.5 ml of the cell medium supernatant was added to a solution (0.5 ml) of the Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% H_2PO_4), and visible absorption at 543 nm was monitored. The amount of NO production was indirectly measured by the level of nitrite (NO_2^-), one of the stable NO metabolites in media. The NO_2^- concentration was calculated by the formula: NO_2^- (μ*M*) = ($A_{543\ nm}$ −0.042)/0.091. The concentration of NO_2^- in cell culture medium without cells was measured as a background control and subtracted.

Mutation assay in AS52 cells co-cultured with HL-60 cells

A co-culture mutation assay was determined as described previously (13). Briefly, AS52 cells were plated at a density of 5×10^5 cells per 100-mm diameter dish in 6 ml of Ham's F-12 medium containing 5% fetal bovine serum 12 h prior to co-culturing with differentiated HL-60 cells. On day 0, differentiated HL-60 cells were co-cultured with AS52 cells at a ratio of 1:1 in the presence or absence of test compounds, and then treated with TPA (100 n*M*). After incubating at 37°C for 1 h, HL-60 cells were removed by decantation. AS52 cells were washed three times with Hanks' Balanced Salt Solution, and then subcultured for cytotoxicity and mutagenesis studies. On days +3 and +6, the cells were subcultured at 10^6 cells per dish. On day +9, cells were cultured in 100-mm-diameter dishes with 2×10^5 cells per dish in Ham's F-12 medium containing 5% fetal bovine serum and 10 μ*M* 6-thioguanine. Cells were incubated for another 10 days and examined for the development of 6-thioguanine-resistant clones. Cells were fixed with a solution containing methanol, acetic acid, and water (50:7:43 by volume), and then stained with a 1% crystal violet solution. Only those colonies containing at least 50 cells per colony were counted. Mutation frequency was expressed as mutants per 10^6 clonable cells.

Cytotoxicity was determined on day +4 and plating efficiency on day +7. Briefly, AS52 cells were plated at 200 cells per 60-mm-diameter dish in Ham's F-12 medium, and incubated at 37°C for 7 days (three to five dishes). Only those clones containing at least 50 cells per colony were counted.

Reverse transcription-polymerase chain reaction (RT)

RAW264.7 cells (10^6) were preincubated in 3 ml of DMEM for 12 h, and then treated with each sample for 30 min in a 60-mm-diameter dish, after which LPS (100 μg/ml) was added. After being incubated for 8 h, the cells were harvested, and total RNA was extracted using kits (RNeasy® and QI-Ashredder®, Qiagen, Valencia, CA). One microgram of total RNA was reverse-transcribed using an RNA PCR Kit® (Takara, Kyoto, Japan) with an oligo (dT)-adaptor primer, as recommended by the supplier. PCR was carried out using aliquots of the cDNA preparations (1 μl each), with 40 μl of Platinum PCR SuperMix® (Invitrogen, Carlsbad, CA). The sequence of each primer used is listed in Table 2. Amplifications of cDNA using a thermal cycler (PTC-0100, MJ Research, Watertown, MA) were performed as shown in Table 3. The PCR products were then separated on 2% NuSieve® 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME), and each band was visualized using 0.01% SYBR Gold® stain (Molecular Probes, Leiden, The Netherlands). The amplified products were photographed with a digital camera (ATTO Bioinstrument, Tokyo, Japan), and band intensities were analyzed using NIH Image software.

Western blotting

RAW264.7 cells (3×10^6) were preincubated in 3 ml of DMEM for 12 h, and then treated with each sample for 30 min in a 60-mm dish, after which LPS (100 ng/ml) was added. After incubation for another 30 min, the cells were harvested and lysed with lysis buffer [10 m*M* Tris-HCl, pH 7.4; 150 m*M* NaCl; 1% Triton X-100; 1% sodium dodecyl sulfate (SDS); and 1 m*M* sodium vanadate], and the lysates were centrifuged at 3,200 *g* for 5 min. The lysates were then centrifuged at 12,000 *g* for 20 min, and the supernatants were boiled in an SDS sample loading buffer for 10 min prior to electrophoresis on 12% SDS-polyacrylamide gels. Twenty micrograms of protein was used for western blotting. Samples were separated on a 12% SDS-polyacrylamide gel, and transferred to Immobilon-P membranes (Millipore, Billerica,

TABLE 2. LIST OF PRIMERS USED FOR REVERSE TRANSCRIPTION-PCR

IL-1β	5′-ATGGCAACTGTTCCTGAACTCAACT-3′
	5′-CAGGACAGGTATAGATTCTTTCCTTT-3′
IL-6	5′-TGCTGGTGACAACAACGGCC-3′
	5′-GTACTCCAGAAGACCAGAGG-3′
TNF-α	5′-TTGACCTCAGCGCTGAGTTG-3′
	5′CCTGTAGCCCACGTCGTAGC-3′
GM-CSF	5′TGTGGCTGCAGAATTTAC-3′
	5′-GCTGTCTATGAAATCCGC-3′
GAPDH	5′-AGCCTTCTCCATGGTGGTGGTGAAGAC-3′
	5-′-CGGAGTCAACGGATTTGGTCGTAT-3′
iNOS	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′
	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′
COX-2	5′-GCATTCTTTGCCCAGCACTT-3′
	5′-AGACCAGGCACCAGACCAAAGA-3′

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

TABLE 3. PCR CONDITIONS FOR EACH GENE AMPLIFICATION

IL-1β	94°C 3 min	(× 25)
IL-6	60°C 30 s	
TNF-α	72°C 30 s	
GM-CSF	94°C 2 min	(× 25)
	94°C 45 s	
	65°C 45 s	
	72°C 2 min	
	72°C 10 min	
iNOS	94°C 2 min	(× 25)
	94°C 45 s	
	65°C 45 s	
	72°C 2 min	
	72°C 10 min	
COX-2	94°C 30 s	(× 25)
	59°C 30 s	
	72°C 30 s	
GAPDH	94°C 2 min	(× 25)
	94°C 1 min	
	60°C 30 s	
	72°C 7 min	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

MA). After blocking with Block Ace (Snow Brand Milk Products, Tokyo) overnight at 4°C, the membrane was incubated with rabbit polyclonal anti-IκB (1:1,000) or goat polyclonal anti-actin (1:1,000), and then with horseradish peroxidase-conjugated secondary antibody (1:1,000). The transferred proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RESULTS

Suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on TPA-induced O_2^- generation in differentiated HL-60 cells

We examined the suppressive effects on O_2^- generation in TPA-stimulated, differentiated HL-60 cells (Fig. 2). [6]-Gingerol, aframodial, and galanal B markedly suppressed O_2^- generation at a concentration of 20 μ*M* (inhibitory rates [IRs] = 96.1%, 100%, and 100%, respectively) with moderate cytotoxicity (cell viability = 60.6%, 47.6%, and 53.1%, respectively). When the sample concentration was decreased to 4 μ*M*, aframodial maintained marked O_2^- suppressive activity (IRs = 79.3%; cell viability = 81.4%), whereas [6]-gingerol and galanal showed only moderate effects (IRs = 38.3% and 45.9%, respectively). Galanolactone was significantly less active than the other constituents tested.

Suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on LPS plus IFN-γ-induced NO generation in RAW264.7 cells

Figure 3 shows the suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on NO generation in LPS plus IFN-γ-stimulated RAW264.7 cells. Aframodial exhibited the strongest suppressive effect at a concentration of 20 μ*M* (IR = 84.6%), and the effect of galanal B was also significant (IR = 74.0%). In contrast, [6]-gingerol and galanolactone were less active (IRs = 45.9% and 37.3%, respectively). No marked cytotoxicity was observed under any of the experimental conditions.

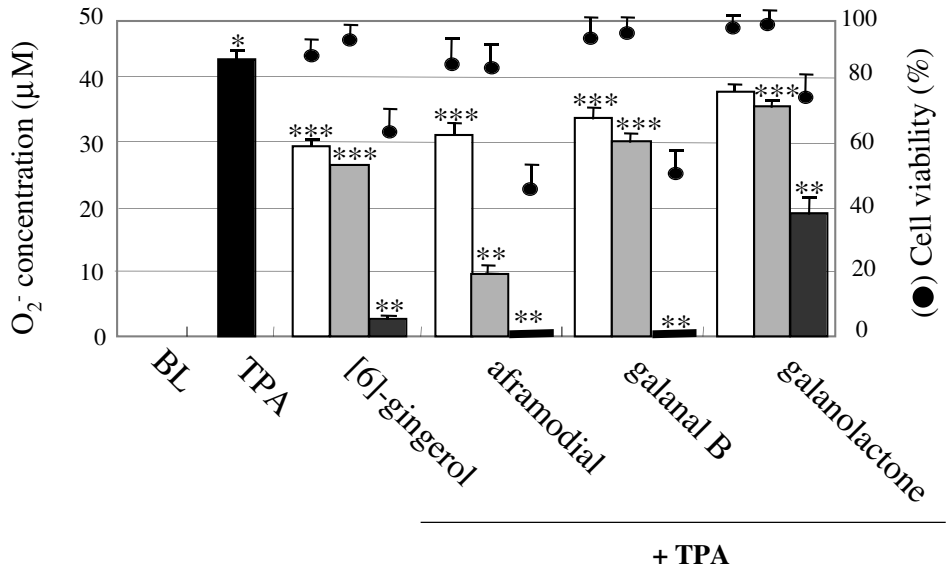

FIG. 2. Suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on O_2^- generation from TPA-stimulated, differentiated HL-60 cells. (□) 0.8 μ*M*, (▩) 4 μ*M*, and (■) 20 μ*M*. HL-60 cells were differentiated by incubation in RPMI 1640 medium containing 1.25% dimethyl sulfoxide for 6 days. For the determination of O_2^- production, cells (10^6) were incubated with 100 n*M* TPA at 37°C for 30 min. Cytochrome *c* (1 mg/ml) was added to the medium 15 min before measurement. The extracellular O_2^- was determined using a cytochrome *c* reduction method. **p* < 0.001 versus BL; ***p* < 0.001, ****p* < 0.01 versus TPA. BL, only vehicle-treated.

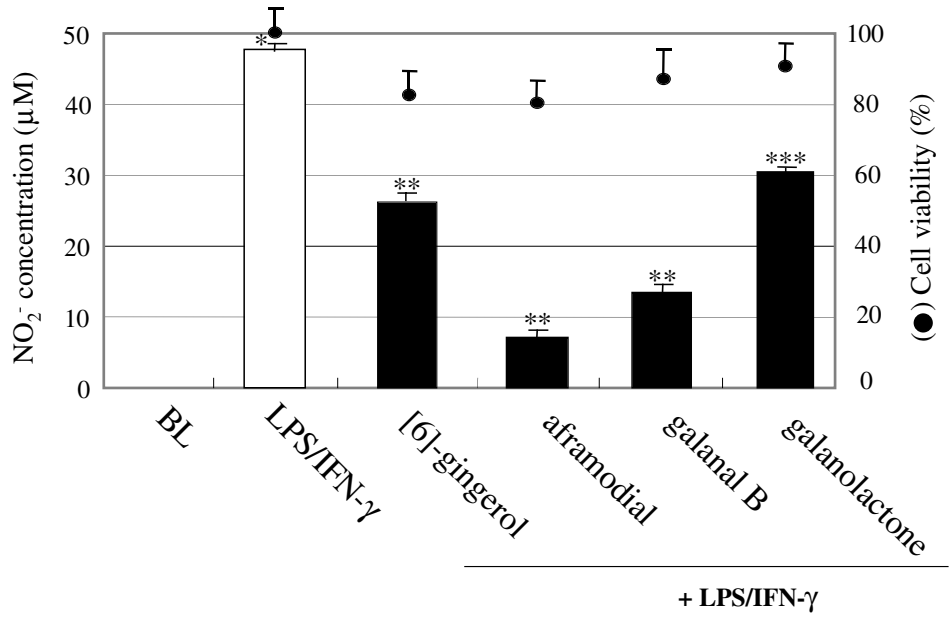

FIG. 3. Suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone (20 μ*M* each) on NO generation from LPS/IFN-γ-stimulated RAW264.7 cells. For the determination of NO, the cells were incubated with LPS (100 ng/ml) and IFN-γ (100 U/ml) at 37°C for 24 h. The amount of NO_2^- produced was determined using the Griess method. *$p < 0.001$ versus BL; **$p < 0.001$, ***$p < 0.05$ versus LPS/IFN-γ. BL, only vehicle-treated.

Suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on mutagenesis in AS52 cells following co-culture with stimulated HL-60 cells

Table 4 shows the suppressive effects on mutagenesis in AS52 cells following co-culture with TPA-stimulated, differentiated HL-60 cells. Aframodial showed the strongest suppressive effect at a concentration of 20 μ*M* (IR = 95.9%), and the effect of galanal B was also remarkable (IR = 60.9%). However, [6]-gingerol and galanolactone were less active (IR = 35.1%) and inactive (IR = 16%), respectively. No marked cytotoxicity was observed under any of the experimental conditions.

TABLE 4. SUPPRESSIVE EFFECTS OF [6]-GINGEROL, AFRAMODIAL, GALANAL B, AND GALANOLATONE ON MUTAGENESIS OF AS52 CELLS FOLLOWING CO-CULTURE WITH STIMULATED HL-60 CELLS

Culture, treatment	*6-Thioguanine-resistant mutants/ 10^6 clonable cells IR, %)*	*Cell viability (%)*
AS52 alone	23.2 ± 3.4	97.4 ± 4.3
AS52/HL-60	24.3 ± 4.3	91.2 ± 3.4
None		
TPA	51.4 ± 5.2[a]	83.3 ± 3.5
+[6]-gingerol	41.9 ± 3.7 (35.1)[b]	87.4 ± 5.6
+ aframodial	25.4 ± 4.2 (95.9)[c]	81.9 ± 3.2
+ galanal B	34.9 ± 3.8 (60.9)[b]	82.5 ± 6.4
+ galanolactone	44.7 ± 4.5 (16.0)	88.4 ± 5.3

HL-60 cells (5×10^5), co-cultured with AS52 cells at a ratio of 1:1, were treated with TPA (100 n*M*) alone or with TPA and [6]-gingerol, aframodial, galanal B, and galanolatone (20 μ*M* each) for 1 h. Mutagenesis analyses were performed as described in Materials and Methods.

[a]$p < 0.01$ versus AS52/HL-60 without TPA.

[b]$p < 0.05$, [c]$p < 0.01$ versus TPA.

Aframodial dramatically suppressed the expression of inducible pro-inflammatory genes in LPS-stimulated RAW264.7 cells

Next, we investigated the suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on inducible pro-inflammatory gene expression including iNOS in LPS-stimulated RAW264.7 cells. As shown in Fig. 4, LPS-induced iNOS expression was abolished by pretreatment with aframodial or galanal (IRs = 99.1% and 98.4%, respectively), while the effect of [6]-gingerol was moderate (IR = 43.2%), and galanolactone was virtually inactive. LPS-induced IL-1β expression was strongly suppressed by treatment with aframodial (IR = 74.6%), but not by that with [6]-gingerol, galanal, or galanolactone. Aframodial also potently suppressed the expression of IL-6 and GM-CSF (IRs = 74.0% and 64.4%, respectively) when compared to [6]-gingerol, galanal, and galanolactone (IRs = 25.9–41.2%). The effects of all constituents tested on TNF-α and COX-2 expressions were not remarkable, though [6]-gingerol slightly increased the level of LPS-induced COX-2 expression by 29%, which was unexpected.

Aframodial, galanal B, and galanolactone, but not [6]-gingerol, significantly suppressed LPS-induced IκB degradation in RAW264.7 cells

To explore the involvement of suppression of the NF-κB pathway on the attenuation of pro-inflammatory gene expression

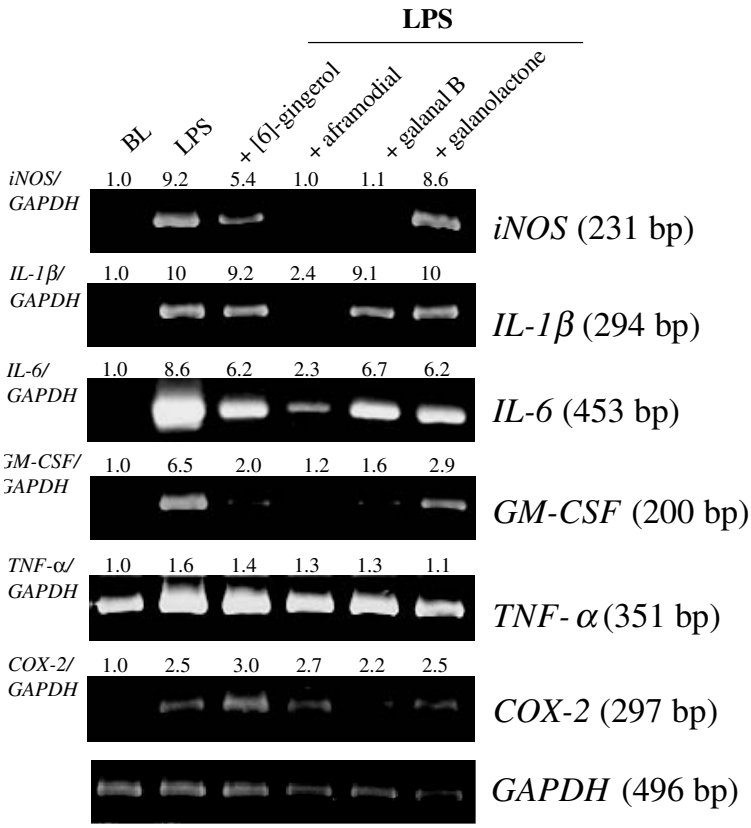

FIG. 4. Alterations of pro-inflammatory gene expression levels by [6]-gingerol, aframodial, galanal B, and galanolactone (20 μ*M* each) in LPS-stimulated RAW264.7 cells. Cells (10^6) were preincubated in 3 ml of DMEM for 12 h, and then treated with each sample for 30 min in a 60-mm-diameter dish, after which LPS (100 μg/ml) was added. After being incubated for 8 h, the cells were harvested, and pro-inflammatory gene expression levels were examined by reverse transcription-PCR. BL, only vehicle-treated. Gene expression levels were expressed by fold change that was standardized by the each gene/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratio in BL as 1.0.

by each constituent, the inhibitory effects of [6]-gingerol, aframodial, galanal B, and galanolactone on LPS-induced IκB degradation, the key step for NF-κB activation, were investigated. RAW264.7 cells were treated with samples (20 μ*M*) for 30 min, and then stimulated with LPS (100 ng/ml) for another 30 min. IκB degradation was examined by western blot analysis. Marked IκB degradation, observed 30 min after LPS treatment (46% reduction), was completely suppressed by pretreatment with aframodial, galanal B, and galanolactone, but not by that with [6]-gingerol (Fig. 5).

DISCUSSION

Mioga ginger (*Z. mioga* Roscoe) native to Eastern Asia is a perennial herb with pungently aromatic flower buds. It has been consumed as a spice or in pickles since ancient times in Japan, where it is now consumed in amounts exceeding 6,000 tons each year. Recently, Miyoshi *et al*. (22) demonstrated that galanal A and B had potent cytotoxic effects in human T lymphoma Jurkat cells, and exposure of Jurkat cells to galanals resulted in the induction of apoptotic cell death, as characterized by DNA fragmentation, caspase-3 activation, cytochrome *c* release, and down-regulation of anti-apoptotic Bcl-2 protein. Those results suggest that mioga ginger constituents, including galanals, are promising candidates for cancer chemoprevention. Further, the present study demonstrated for the first time the antioxidative and anti-inflammatory effects of aframodial, supporting the hypothesis that mioga ginger constituents are useful for cancer preventive and therapeutic strategies, though their *in vivo* efficacy remains to be demonstrated.

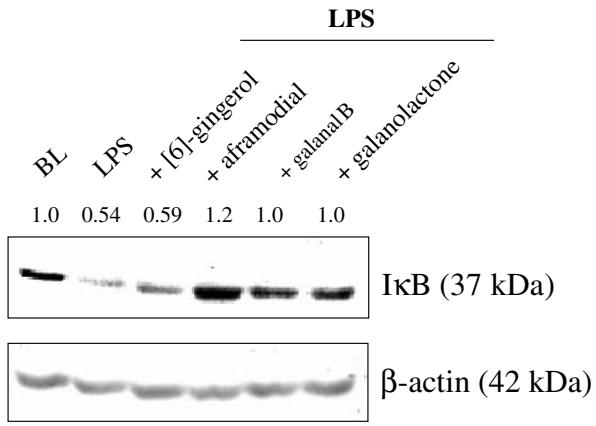

FIG. 5. Suppressive effects of aframodial, galanal B, and galanolactone (20 μ*M* each) on LPS-induced IκB degradation in RAW264.7 cells. RAW264.7 cells (3×10^6) were preincubated in 3 ml of DMEM for 12 h, and then treated with each sample for 30 min in a 60-mm-diameter dish, after which LPS (100 ng/ml) was added. After incubation for another 30 min, the cells were harvested, and IκB degradation levels were analyzed by western blotting. BL, only vehicle-treated. IκB degradation levels were expressed by fold changes that were standardized by the IκB/β-actin ratio in BL as 1.0.

We examined the suppressive effects of mioga ginger and ginger constituents, [6]-gingerol, aframodial, galanal B, and galanolactone, on NO and O_2^- generation. Aframodial and galanal B showed significant suppressive effects on NO generation in LPS/IFN-γ-stimulated RAW264.7 cells, and on O_2^- generation in TPA-stimulated, differentiated HL-60 cells, whereas galanolactone exhibited only weak suppressive effects, suggesting that aframodial and galanal B are the major antioxidative constituents of mioga ginger. It is of interest that [6]-gingerol suppressed O_2^- generation in HL-60 cells, but not NO generation in RAW264.7 cells. In addition, aframodial strongly suppressed the activated HL-60 cell-induced mutagenesis in AS52 cells, possibly through inhibition of O_2^- generation from HL-60 cells.

The suppressive effects of [6]-gingerol, aframodial, galanal B, and galanolactone on expression of inducible pro-inflammatory genes in LPS-stimulated RAW264.7 cells were also investigated. Pro-inflammatory genes including iNOS are expressed by early infiltrating inflammatory cells in innate immune responses (18, 29). In the present study, iNOS expression was strongly suppressed by treatment with aframodial or galanal B, which paralleled to the inhibitory effects on NO generation. Aframodial also potently suppressed the expression of IL-1β, IL-6, and GM-CSF, but not that of TNF-α or COX-2. Interestingly, IL-1β expression was abolished only by treatment with aframodial, suggesting that this compound was the most capable food phytochemical tested in the present study to attenuate pro-inflammatory gene expression in macrophages.

Numerous studies have demonstrated that activation of NF-κB is essential for induction of COX-2 (7, 17), IL-1β (1) , IL-6 (16), TNF-α (33), and GM-CSF (4), and that degradation of IκB is a critical process for NF-κB activation, and thus the induction of a variety of inflammation signaling pathways (5, 19). In the present study, aframodial, galanal B, and galanolactone significantly suppressed IκB degradation, indicating that suppression of pro-inflammatory gene expression was possibly through, at least in part, inhibition of the NF-κB signal transduction pathway. By not inhibiting IκB degradation, [6]-gingerol is considered to suppress the expression of iNOS, IL-6, and GM-CSF *via* other signaling pathways. There are possible alternative molecular mechanisms caused by [6]-gingerol, *e.g.*, inactivation of NF-κB subunits p50 and p65 following IκB degradation (8), or blockade of the mitogen-activated protein kinase signal transduction pathway that leads to inhibition of the activator protein-1 activity, another important transcriptional factor for the expression of pro-inflammatory genes (10, 35).

Previous studies have shown that ginger and its constituents are potent inhibitors of immune cell activation and cytokine secretion (22, 25). Various formulations of ginger have been shown to act as dual inhibitors of both COX and lipooxygenase, inhibit leukotriene synthesis (15), and reduce carrageenan-induced rat-paw and skin edema (26). Among ginger constituents, [6]-gingerol is believed to be a major constituent with cancer chemopreventive effects, including inhibition of DMBA/TPA-induced mouse skin tumor formation (11), TPA-induced epidermal ornithine decarboxylase activity (25), TNF-α production in mouse skin (25), and azoxymethane-induced intestinal tumorigenesis (38). Furthermore, ginger has been reported to abrogate pulmonary metastasis in mice implanted with B16F10 melanoma cells (30). However, there are few reports of the physiological effects of aframodial, galanals, or other ginger and mioga ginger constituents. In the present study, aframodial was found to show much higher suppressive effects on RONS generation and expression of pro-inflammatory genes than [6]-gingerol, leading to the hypothesis that aframodial, rather than [6]-gingerol, may be the major antioxidative and anti-inflammatory constituent of ginger.

Aframodial and galanals bear two aldehyde groups in their structures, whereas galanolactone does not. Since aframodial and galanal B exhibited much stronger suppressive activities toward both RONS generation and pro-inflammatory gene expression as compared to galanolactone in the present study, the dialdehyde groups in these diterpenoids may play some important roles in suppression of RONS generation and inflammatory genes expression. The dialdehyde groups in aframodial and galanals belong to the non-hindered α,β-unsaturated carbonyl group that is known as a Michael reaction acceptor. Previously, Maria *et al*. (20) investigated the relationship between the structure of several compounds containing the α,β-unsaturated carbonyl group and the activity of the gastric anti-ulcer effect, and demonstrated that the presence of a non-hindered α,β-unsaturated carbonyl group is a prerequisite for anti-ulcer activity. In addition, Rossi *et al.* (27) discovered that anti-inflammatory cyclopentenone prostaglandins directly inhibited IκB kinase by modifying the cysteine residue, thereby attenuating IκB phosphorylation to abolish NF-κB activity. Murakami *et al*. (23) also reported that the α,β-unsaturated carbonyl group in zerumbone plays pivotal roles in the interactions with unidentified target molecules that participate in free radical generation and pro-inflammatory gene expression in macrophages as well as the proliferation of cancer, but not normal, cells. Using structural analogy, aframodial and galanal B, but not galanolactone, are recognized to have chemical properties resembling that of cyclopentenone prostaglandins, supporting the hypothetical ability of aframodial and galanal B to directly suppress IκB kinase activity. In addition, compounds that contain the α,β-unsaturated aldehyde group are already known to induce phase II detoxification enzymes, such as glutathione *S*-transferase, which catalyze the conjugation of reactive chemicals with glutathione and play a major role in protecting cells from exogenous and endogenous stimuli (24, 34). In any case, the chemical properties of aframodial and galanal B to conjugate biological nucleophiles are considered to be essential for exertion of their biological activities.

Further studies are needed to elucidate the molecular mechanisms underlying the suppressive effects of these compounds on pro-inflammatory gene expression; however, the results of the present study strongly suggest that mioga ginger and ginger constituents, especially aframodial, have potent antioxidative and anti-inflammatory potentials, and may be promising candidates in prevention and/or therapy for chronic inflammation-associated carcinogenesis.

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ABBREVIATIONS

COX, cyclooxygenase; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMEM, Dulbecco's modified Eagle's medium; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IκB, inhibitor of nuclear factor κB; iNOS, inducible nitric oxide synthase; IR, inhibitory rate; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; NO, nitric oxide; O_2^-, superoxide; $ONOO^-$, peroxynitrite; PCR, polymerase chain reaction; RONS, reactive oxygen and nitrogen species; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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