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Increased Growth Inhibitory Effects on Human Cancer Cells and Anti-inflammatory Potency of Shogaols from *Zingiber officinale* Relative to Gingerols

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Ginger, the rhizome of the plant *Zingiber officinale*, has received extensive attention because of its antioxidant, anti-inflammatory, and antitumor activities. Most researchers have considered gingerols as the active principles and have paid little attention to shogaols, the dehydration products of corresponding gingerols during storage or thermal processing. In this study, we have purified and identified eight major components, including three major gingerols and corresponding shogaols, from ginger extract and compared their anticarcinogenic and anti-inflammatory activities. Our results showed that shogaols ([6], [8], and [10]) had much stronger growth inhibitory effects than gingerols ([6], [8], and [10]) on H-1299 human lung cancer cells and HCT-116 human colon cancer cells, especially when comparing [6]-shogaol with [6]-gingerol (IC_{50} of ~8 versus ~150 μM). In addition, we found that [6]-shogaol had much stronger inhibitory effects on arachidonic acid release and nitric oxide (NO) synthesis than [6]-gingerol.

KEYWORDS: Ginger; *Zingiber officinale*; [6]-gingerol; [6]-shogaol; inflammation; cancer

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), a member of the Zingiberaceae family that consists of 47 genera, including *Zingiber* and *Curcuma*, has been cultivated for thousands of years as a spice and for medicinal purposes. The gingerols, a series of homologues differentiated by the length of their unbranched alkyl chains, were identified as the major pungent components in the ginger oleoresin from fresh rhizome, with [6]-gingerol (**Figure 1**) being the most abundant (*1*). Gingerols are not stable during storage or thermal processing to generate the dehydration products, shogaols, which are predominant pungent constituents in the ginger oleoresin from dried ginger (*1*, *2*). It has been reported that shogaols were minor components in fresh ginger, and the ratio of [6]-shogaol/[6]-gingerol was about 1:1 in dried ginger (*1*, *2*). Other gingerol- or shogaol-related compounds have also been reported in ginger rhizome, such as [6]-paradol, [6]- and [10]-dehydrogingerdione, [6]- and [10]-gingerdione, [4]-, [6]-, [8]-, and [10]-gingerdiol, [6]-methylgingerdiol, zingerone, [6]-hydroxyshogaol, [6]-, [8]-, [10]-dehydroshogaol, and diarylheptanoids (*1*−*3*). However, these minor compounds only count for about 1−10% of those for the overall amount of gingerols and shogaols (*1*−*3*).

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Recently, ginger has received extensive attention as a botanical dietary supplement in the U.S.A. and Europe because of its antioxidative, anti-inflammatory, and antitumor activities (*4*, *5*). Although most of the animal studies with ginger extract showed antioxidative, anti-inflammatory, and antitumor activities, no report has considered the instability of gingerols during the thermal process and long-term storage will affect the chemical profile of the ginger extract used in their animal studies. They either did not quantify the levels of the active components in their raw material or simply used the total levels of gingerols as the standard.

The nature of the active components in ginger has not been fully explored. It has been reported that topical application of [6]-gingerol or [6]-paradol onto shaven backs of female ICR mice prior to each topical dose of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) significantly inhibited 7,12-dimethylbenz[α]anthracene (DMBA)-induced skin tumor incidence and tumor burden (*6*). The same group also found that [6]-gingerol inhibited TPA-induced cyclooxygenase-2 (COX-2) expression in mouse skin *in vivo* by blocking the p38 MAP kinase-NF-κB signaling pathway (*7*). [6]-Gingerol was found to decrease the number of lung metastasis in mice implanted with B16F10 melanoma cells (*8*). Jeong et al. reported that [6]-gingerol effectively suppressed *in vivo* tumor growth in HCT-116 cancer cell-bearing

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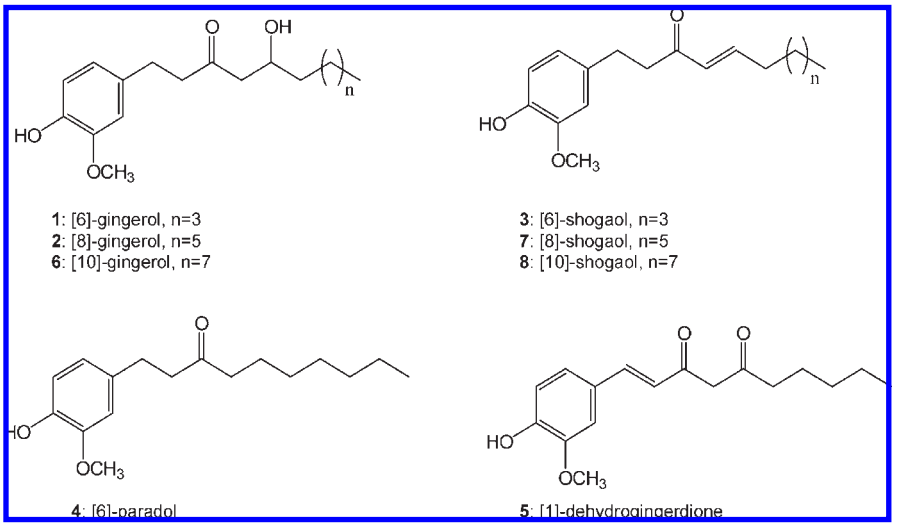

Figure 1. Structures of the eight major components purified from ginger extract.

nude mice (*9*). Several *in vitro* studies have found that shogaols also have anti-inflammatory and anticancer activities. It has been reported that [6]-shogaol significantly suppressed the expression of inducible nitric oxide sysnthesis and COX-2 in lipopolysaccharide (LPS)-induced macrophages (*10*). Wei et al. found that [6]- and [10]-shogaol could significantly inhibit the growth of HL-60 human leukemia cells. Rhode et al. reported that [6]-, [8]-, and [10]-gingerol had no effect and [6]-shogaol could significantly inhibit the growth of A-2780 ovarian cancer cells (*11*). Pan et al. found that [6]-shogaol inhibited the growth of human colon COLO-205 cells and induced apoptosis through modulation of mitochondrial functions regulated by reactive oxygen species (*12*). Kim et al. reported that [6]-shogaol had much stronger growth inhibitory effects on A-549 human lung cancer cells, SK-OV-3 human ovarian cancer cells, SK-MEL-2 human skin cancer cells, and HCT-15 human colon cancer cells than [4]-, [6]-, [8]-, and [10]-gingerol (*13*).

To further study whether shogaols have better anti-inflammatory and anticancer activities than corresponding gingerols, we purified and identified eight major components, including three major gingerols ([6]-, [8]-, and [10]-gingerol), corresponding shogaols, [6]-paradol, and [1]-dehydrogingerdione, from ginger extract and compared their growth inhibitory effects against human lung and colon cancer cells and the inhibition of arachidonic acid release and nitric oxide (NO) synthesis from LPS-stimulated RAW 264.7 cells.

MATERIALS AND METHODS

Materials. Ginger extract, which contains 20% gingerols and shogaols, was obtained from Sabinsa Corporation (Piscataway, NJ). RP C-18 silica gel, silica gel, Sephadex LH-20 gel, thin-layer chromatography (TLC) plates (250 μm thickness, 2−25 μm particle size), CD_3OD, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade solvents and other reagents were obtained from VWR Scientific (South Plainfield, NJ). HPLC-grade water was prepared using a Millipore Milli-Q purification system (Bedford, MA). H-1299 human lung cancer cells, HCT-116 human colon adenocarcinoma cells, and RAW 264.7 murine macrophages were obtained from American Type Tissue Culture (Manassas, VA).

Nuclear Magnetic Resonance (NMR). ^1H (400 MHz), ^{13}C (100 MHz), and all 2D NMR spectra were acquired on a Varian 400 instrument (Varian, Inc., Palo Alto, CA). Compounds were analyzed in CD_3OD, with tetramethylsilane (TMS) as the internal standard. $^1H-^{13}C$ heteronuclear multiple-quantum correlation (HMQC) and heteronuclear multiple-band correlation (HMBC) experiments were performed as described previously (*14*).

HPLC Analysis. HPLC analysis was carried out with a system consisting of a Waters 717 refrigerated autosampler, a HITACHI L-6200A intelligent pump, and an ESA 5600 coulochem electrode array system (CEAS). The potentials of the CEAS were set at 0, 200, 300, and 400 mV. The column used was a 150 × 4.6 mm inner diameter, 5 μm, Supelcosil RP-18. For binary gradient elution, mobile-phase A (1.75% acetonitrile and 0.12% tetrahydrofuran in 30 mM NaH_2PO_4 at pH 3.35) and B (58.5% acetonitrile and 12.5% tetrahydrofuran in 15 mM NaH_2PO_4 at pH 3.45) were used. The flow rate was maintained at 1 mL/min, and the mobile phase began with 100% A. It was followed by progressive linear increases in B to 65% at 15 min and 100% at 35 min. The mobile phase was maintained at 100% B for 10 min and then was re-equilibrated to 100% A at 46 min for another run. The HPLC profile of the ginger extract displaying these eight compounds is shown in **Figure 2**.

Isolation of the Major Constituents in the Ginger Extract. The ginger extract (50 g) was chromatographed on a Sephadex LH-20 column with 95% ethanol as an eluant to remove the nonphenolic compounds (Fraction 1, 21.8 g) and to generate the gingerol- and shogaol-enriched fraction (fraction 2, 28 g). Fraction 2 was then loaded into a Diaion HP-20 column, eluted first with water to remove the water-soluble compounds and then with 40% aqueous ethanol to obtain fraction A (9 g), followed by 95% aqueous ethanol to obtain fraction B (11 g). Fraction A (5 g) was subjected to a normal phase silica-gel column with a stepwise gradient of hexane/ethyl acetate [9:1; 8:2, and 7:3] to give pure [6]-gingerol (2 g), [8]-gingerol (0.5 g), and [10]-gingerol (0.4 g). Fraction B (5 g) was also subjected to a normal phase silica-gel column with a stepwise gradient of hexane/ethyl acetate (9:1 and 8:2) to generate 13 fractions. Fraction B5 (1 g) was subjected to a C-18 reverse-phase column eluted with a stepwise gradient of methanol/water [3:2, 7:3, and 4:1] to give [6]-paradol (40 mg), [1]-dehydrogigerdione (60 mg), and [10]-shogaol (120 mg). Following a similar procedure, fraction B7 (1.5 g) gave 200 mg of [8]-shogaol and 1 g of [6]-shogaol. The purification procedure was guided by TLC and HPLC analyses. The structures of these eight compounds were confirmed on the basis of their ^1H and ^{13}C NMR analyses (**Figure 1**).

Growth Inhibition against Human Lung and Colon Cancer Cells. Cell growth inhibition was determined by the MTT assay (*15*). The cells were plated in 96-well microtiter plates and allowed to attach for 24 h at 37 °C. The test compounds [in dimethylsulfoxide (DMSO)] were added to cell culture medium to desired final concentrations (final DMSO concentrations for control and treatments are 0.1%). After the cells were cultured for 24 h, the medium was aspirated and the cells were treated with 100 μL of fresh medium containing 2.41 mmol/L MTT. After incubation for 1−3 h at 37 °C, the MTT-containing medium was aspirated, 100 μL of DMSO was added to solubilize the formazan precipitate, and the plate was read at 550 nm on a microtiter plate reader. The reading reflected the number of viable cells and was expressed as a percentage of viable cells in the control. Both H-1299 and HCT-116 cells were cultured in McCoy's 5A

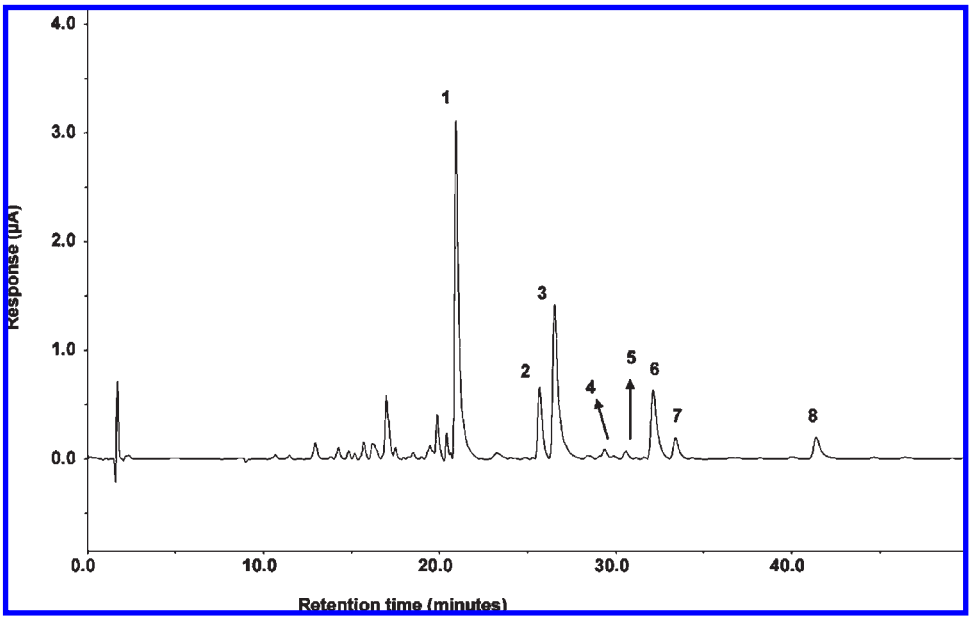

Figure 2. HPLC−electrochemical detection (ECD) profiles of ginger extract: peak 1, [6]-gingerol; peak 2, [8]-gingerol; peak 3, [6]-shogaol; peak 4, [6]-paradol; peak 5, [1]-dehydrogingerdione; peak 6, [10]-gingerol; peak 7, [8]-shogaol, and peak 8, [10]-shogaol.

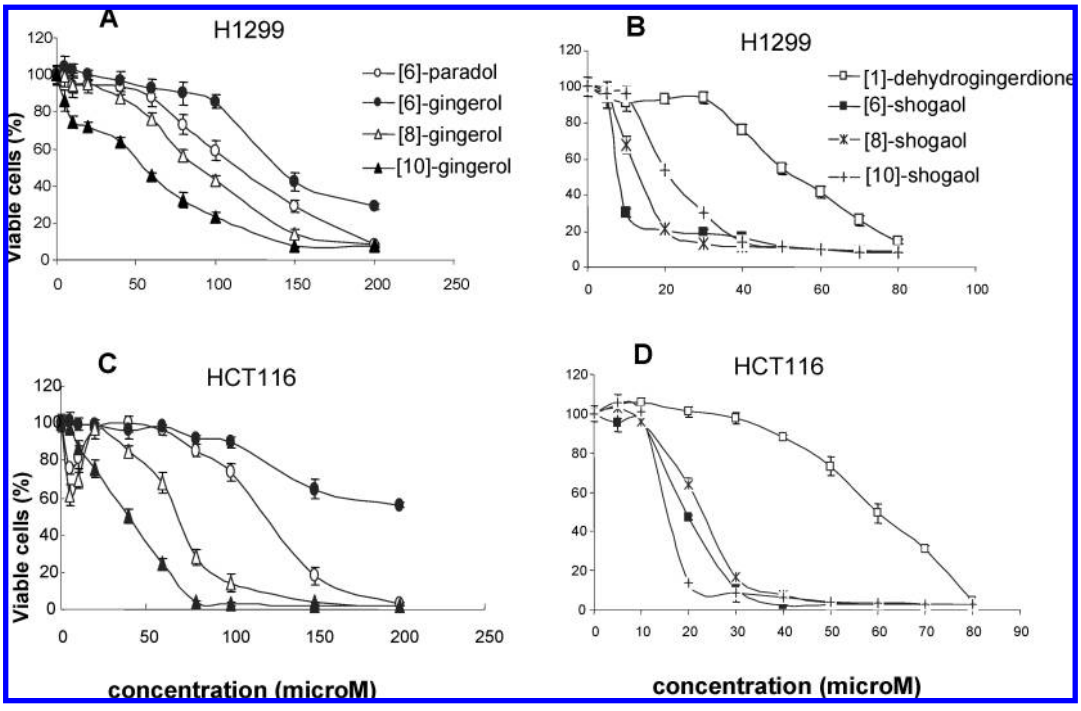

Figure 3. Effects of [6]-, [8]-, and [10]-gingerol, [6]-, [8]-, and [10]-shogaol, [6]-paradol, and [1]-dehydrogingerdione on the growth of H1299 human lung cancer cells and HCT-116 human colon adenocarcinoma cells. Each value represents the mean ± standard deviation (SD) ($n = 8$).

medium. All of the above media were supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine, and the cells were kept in a 37 °C incubator with 95% humidity and 5% CO_2.

Inhibition of Arachidonic Acid Release from LPS-Stimulated RAW 264.7 Cells. To determine the inhibition of arachidonic acid release, RAW 264.7 cells were plated into a 24-well plate (3×10^5 cells per well). After 24 h, the media were removed and replaced with 1 mL of serum-free Dulbecco's modified Eagle's medium (DMEM) containing 0.1 μCi/mL [5, 6, 8, 9, 11, 12, 14, and 15 ^{3}H] (N) arachidonic acid (NEN Life Science, Boston, MA). The cells were incubated overnight, resulting in over 90% arachidonic acid absorption, and washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). The cells were stimulated with 2 μg/mL LPS for 1 h, and the media were replaced with serum-free medium containing the test compounds with desired final concentrations. After incubation for 18 h, the media were collected and centrifuged for 10 min at 12 000 rpm. Radioactivity in the extracellular fluid was measured with a scintillation counter. RAW 264.7 cells were maintained in log-phase growth in DMEM.

Inhibition of NO Synthesis. RAW 264.7 cells were plated in 24-well plates (3.0×105 cells per well) and stimulated for 1 h with 1 μg/mL LPS and 10 ng/mL interferon γ (IFNγ). The media were then replaced with serum-free medium containing compounds with desired final concentrations, and cells were cultured for 24 and 30 h. NO production was determined spectrophotometrically using previously reported methods (*16*).

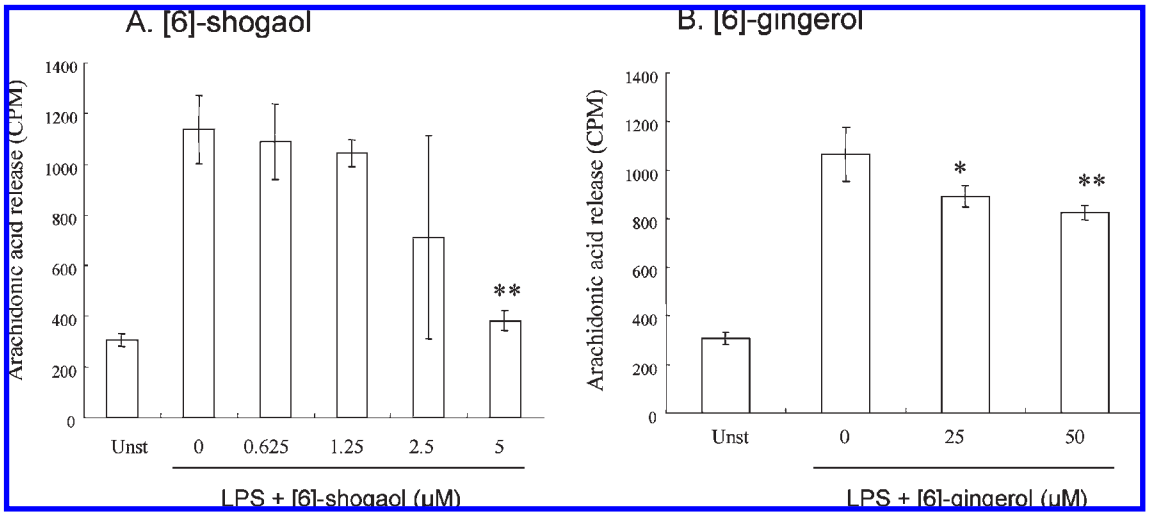

Figure 4. Effects of (A) [6]-shogaol and (B) [6]-gingerol on arachidonic acid release in LPS-stimulated RAW264.7 cells. Each bar represents the mean ± SD (n = 8). (∗ and ∗∗) Significantly different from the control according to Student's t test (∗, $p < 0.05$; ∗∗, $p < 0.01$).

Statistical Analysis. For simple comparisons between two groups, a two-tailed Student's t test was used. A p value of less than 0.05 was considered statistically significant in all of the tests.

RESULTS AND DISCUSSION

Purification and Structure Elucidation. Ginger extract was chromatographed successively on Sephadex LH-20, Diaion HP-20, normal phase silica-gel, and/or RP-C18 columns to afford [6]-, [8]-, and [10]-gingerol, [6]-, [8]-, and [10]-shogaol, [6]-paradol, and [1]-dehydrogingerdione (compounds **1**−**8**) (**Figure 1**). Their structures were determined by a comparison of their NMR and MS data to those reported in the literature (*13*, *17*, *18*). Then, we developed a HPLC method using an electrochemical array detector (ECD) to analyze those eight components (**Figure 2**). This method is about 50−100-fold more sensitive than the UV detection method reported in previous literature (*19*). The limit of detection is ∼1−2 ng/mL for all eight components, and the limit of quantification is ∼2−4 ng/mL for all eight components.

Inhibitory Effects on the Proliferation of Human Lung and Colon Cancer Cells. The growth inhibitory activities of compounds **1**−**8** were determined after treatment for 24 h in H-1299 human lung cancer cells and in HCT-116 human colon cancer cells. Our results indicated that [6]-, [8]-, and [10]-gingerol and [6]-paradol showed effective inhibition on H-1299 cells with [10]-gingerol > [8]-gingerol > [6]-paradol > [6]-gingerol. However, shogaols ([6], [8], and [10]) had much stronger growth inhibitory effects than gingerols ([6], [8], and [10]) on H-1299 human lung cancer cells, especially when comparing [6]-shogaol to [6]-gingerol (IC_{50} of ∼8 versus ∼150 μM) (panels **A** and **B** of **Figure 3**). The inhibitory effects of [6]-, [8]-, and [10]-shogaol exhibited an order of [6]-shogaol > [8]-shogaol > [10]-shogaol and were much stronger than the effects of [1]-dehydrogingerdione.

Similarly, we found that shogaols ([6], [8], and [10]) had much stronger growth inhibitory effects than gingerols ([6], [8], and [10]) on HCT-116 human colon cancer cells (panels **C** and **D** of **Figure 3**). HCT-116 cells were less sensitive to both [6]-shogaol and [6]-gingerol treatments than H-1299 cells.

Inhibitory Effects on Arachidonic Acid Release. After stimulation of the cells with 2 μg/mL LPS for 1 h, the release of arachidonic acid and its metabolites from RAW264.7 macrophage cells to the culture media increased ∼3-fold after 18 h of incubation. At 5 μM, [6]-shogaol significantly decreased the release of arachidonic acid and its metabolites during the 18 h incubation (∼90% inhibition) (**Figure 4A**). This is much more effective than 50 μM [6]-gingerol, which yielded ∼30% inhibition (**Figure 4B**).

Inhibitory Effects on NO Synthesis. After stimulation of RAW264.7 cells with 1 μg/mL LPS and 10 ng/mL IFNγ for 1 h, significant NO accumulation in culture medium was observed at 24 h and much higher accumulation of NO was observed at 30 h. Our results indicated that both [6]-shogaol and [6]-gingerol significantly inhibited NO accumulation ($p < 0.01$) (**Figure 5A**), whereas [6]-shogaol at 5 μM has much stronger inhibitory effects than [6]-gingerol at 35 μM (**Figure 5A**). The inhibitory effect of [6]-shogaol was concentration-dependent, with significant inhibition observed at a concentration as low as 1.25 μM (**Figure 5B**).

One of the challenges to study the *in vivo* efficacy of [6]-gingerol and [6]-shogaol is that both compounds are not commercially available with affordable prices. In this study, we found that the Diaion HP-20 column chromatogram is a useful tool to separate [6]-gingerol and [6]-shogaol in large quantities. We recently further polished our purification methods. We found that 1 kg of Diaion HP-20 resin could load 100 g of ginger extract. [6]-Gingerol with 90% purity could be eluted by 40% aqueous ethanol, and [6]-shogaol could be eluted out by 75% aqueous ethanol through a Diaion HP-20 column. Both [6]-gingerol- and [6]-shogaol-enriched fractions could be further purified using a RP C-18 column to generate [6]-gingerol and [6]-shogaol with more than 95% purity. Therefore, large quantities (10−20 g) of [6]-gingerol and [6]-shogaol can be purified from ginger extract within 4 weeks. Because both Diaion HP-20 resin and RP C-18 silica gel are reusable, the purification method developed in this study will be very practical to prepare large quantities of [6]-gingerol and [6]-shogaol in an academic lab or a botanical company to support future *in vivo* studies.

In this study, we purified eight major ginger components, including [6]-, [8]-, and [10]-shogaols and related gingerols from ginger extract, and compared their anticarcinogenic and anti-inflammatory effects. Our results indicated that shogaols ([6], [8], and [10]) had much stronger growth inhibitory effects than gingerols ([6], [8], and [10]) on H-1299 human lung cancer cells and HCT-116 human colon cancer cells, especially when comparing [6]-shogaol to [6]-gingerol. This is the first study to show that both shogaols and gingerols could significantly inhibit the growth of H-1299 human lung cancer cells. It has been reported that [6]-gingerol effectively suppressed *in vivo* tumor growth in HCT-116 human colon cancer cell-bearing nude mice. Our results indicated

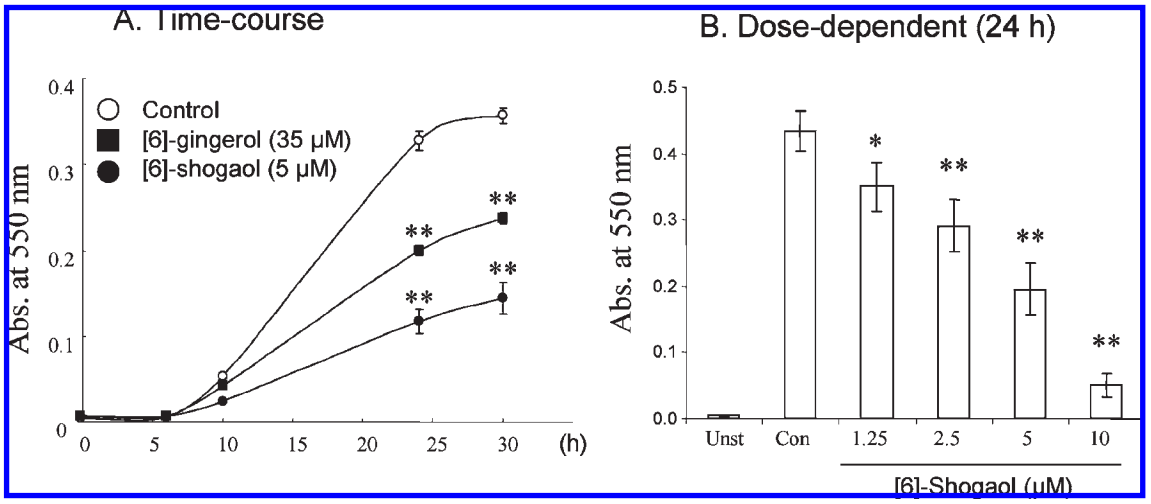

Figure 5. Effects of [6]-shogaol and [6]-gingerol on NO synthesis in LPS/IFNγ-stimulated RAW264.7 cells. (A) Levels of NO in the culture medium of LPS/IFNγ-stimulated RAW264.7 cell were analyzed at different time points after treatment with 5 μM [6]-shogaol and 35 μM [6]-gingerol. (B) Concentration-dependent effects of [6]-shogaol on NO release was analyzed after 24 h of incubation. ($*$ and $**$) Significantly different from the control according to Student's *t* test ($*$, $p < 0.05$; $**$, $p < 0.01$). Each bar represents the mean $\pm$ SD ($n = 8$).

that [6]-shogaol had much stronger inhibitory effects than [6]-gingerol on the growth of HCT-116 cancer cells *in vitro*. Therefore, it is worthwhile to further study whether [6]-shogaol has a stronger inhibitory effect to suppress *in vivo* tumor growth in HCT-116 colon cancer cell-bearing nude mice. In addition, we found that both [6]-shogaol and [6]-gingerol were extensively metabolized under cell culture conditions (unpublished data). We did not observe any conversion between [6]-shogaol and [6]-gingerol in the cell medium after 24 h of incubation. We are in the process of purifying and identifying the major metabolites of [6]-shogaol and [6]-gingerol. Whether their metabolites have anticancer activities merits further investigation.

The shogaols are the dehydration products of related gingerols during storage or thermal processing (*2*, *3*). Therefore, the contents of shogaols and gingerols in ginger preparations can vary greatly. This may have contributed to the inconsistencies in published effects of ginger preparations. Thus, ginger extracts with high levels of shogaols, such as extracts from dry ginger, may have stronger cancer-preventive effects than ginger extracts with high levels of gingerols, such as extracts from fresh ginger. It is important to identify the bioactive components in ginger and standardize the products for use in future laboratory studies and clinical trials. The development of a standardized and more active ginger extract preparation will facilitate future preclinical and clinical studies on the health benefits of ginger extracts.

Both arachidonic acid metabolites and NO are important mediators of oxidative stress and inflammation *in vivo*. Many studies have shown that arachidonic acid and its metabolites as well as NO play important roles in the development of cancer (*20*, *21*). An increasing number of studies have indicated that inhibitors of arachidonic acid cascade and NO synthesis have potential therapeutic value for cancer prevention (*22*−*28*). The present results demonstrate that [6]-shogaol is capable of inhibiting both of these processes in LPS-induced murine macrophages. The inhibition of arachidonic acid release may be caused by a blockage of phospholipase A_2 activation or activity, and further mechanistic studies are required. Similarly, the mechanism for the inhibition of NO synthesis by LPS/IFNγ-activated macrophages is not clear. [6]-Shogaol may inhibit either iNOS activity or LPS induction of the enzyme. Further studies are needed to determine the mechanism(s) of action of shogaols.

Supporting Information Available: NMR (^{1}H and ^{13}C) data for [6]-, [8]-, and [10]-gingerol, [6]-, [8]-, and [10]-shogaol, [6]-paradol, and [1]-dehydrogingerdione. This material is available free of charge via the Internet at http://pubs.acs.org.

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