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Zingerone as an Antioxidant against Peroxynitrite

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Peroxynitrite ($ONOO^-$), formed from the reaction of superoxide ($^{\bullet}O_2^-$) and nitric oxide ($^{\bullet}NO$), induces cellular and tissue injury, resulting in several human diseases such as stroke, Alzheimer's disease, and atherosclerosis. Due to the lack of endogenous enzymes responsible for $ONOO^-$ scavenging activity, finding a specific $ONOO^-$ scavenger is of considerable importance. In this study we examined the scavenging effects of zingerone from ginger against $ONOO^-$, intracellular RS (reactive species), and $ONOO^-$. The data show that zingerone can efficiently scavenge native $ONOO^-$ as well as $ONOO^-$ derived from the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1). Zingerone inhibited the formation of $ONOO^-$-mediated tyrosine nitration through electron donation, nitration of bovine serum albumin (BSA) by $ONOO^-$, and intracellular RS and $ONOO^-$. The present study suggests that zingerone has an efficient $ONOO^-$ scavenging ability, which may be a potent $ONOO^-$ scavenger for the protection of the cellular defense activity against $ONOO^-$- involved diseases.

KEYWORDS: Antioxidant; electron donation; nitration; peroxynitrite; zingerone

INTRODUCTION

Concurrent generation of nitric oxide ($^{\bullet}NO$) and superoxide ($^{\bullet}O_2^-$) can produce a cytotoxic reaction product, peroxynitrite ($ONOO^-$) (*1*−*3*). $ONOO^-$ is a potent oxidant implicated in a number of neurodegenerative and pathophysiological processes (*4*). $ONOO^-$ can diffuse freely across phospholipids membrane bilayers to react with a wide variety of molecular targets including lipids, proteins, and DNA, leading to cell death via necrosis or apoptosis (*4*). Despite its nonradical nature, $ONOO^-$ is more reactive than its parent molecules and induces modification of proteins and has been connected with the development of several diseases, including Alzheimer's disease (*5*).

$ONOO^-$-induced protein modifications include protein oxidation (on methionine, cysteine, tryptophane, or tyrosine residues) and nitration (of tyrosine or tryptophane residues) (*6*). The nitration of tyrosyl residues in proteins may interfere with signaling pathways relying on tyrosine phosphorylation/dephosphorylation, which regulate cellular proliferation, apoptosis, and oxidation (*7*). Also, the nitration of proteins mediated by $ONOO^-$ may disrupt the tertiary structure of proteins and, at higher degrees of damage, render them inaccessible for degradation by the proteasome (*8*). Moreover, $ONOO^-$ ability to modify low-density lipoproteins (LDL) may initiate cellular signals that can lead to inflammation, mitosis, or cholesterol accumulation, thereby resulting in atherosclerosis (*9*). Because endogenous scavenging enzymes that can inactivate $ONOO^-$ are lacking, investigating specific scavengers to $ONOO^-$ is considerably important. The selenium-containing compounds, D-(−)-penicillamine (*10*) and ebselen (*11*), have been shown to be powerful scavengers against $ONOO^-$. Recently, there has been increasing interest in screening natural products for possible $ONOO^-$ scavengers. Studying the scavenging effect of zingerone (**Figure 1**) from nature products is a worthy endeavor.

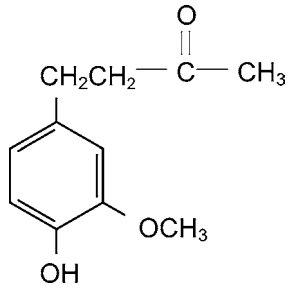

Figure 1. Chemical structure of zingerone.

Zingerone is a pungent pyrolytic product of ginger oleoresin found in ginger root (*12*) and a major component of several spice plants. Ginger (from the rhizomes of *Zingiber officinale* Roscoe) has been used for medicinal purpose for the treatment of a lot of diseases, including those affecting the digestive tract (*13*), is a household remedy for dyspepsia, flatulence, colic, and diarrhea, and also is used in foods as a spice around the world (*14*−*16*). Several studies have shown that ginger possesses antiemetic, antiinflammatory, anticancer, anxyolitic, antithrom-

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botic, and cardiovascular effects (*17*, *18*). Although its antioxidant capacity (*19*, *20*) has been shown against other reactive oxidants, almost no information is reported about zingerone's action against $ONOO^-$.

The purpose of this study is to define the effectiveness of zingerone as an antioxidant. In doing this, the possible regulation of $ONOO^-$ and its major production pathway by zingerone was checked using several in vitro models and *t*-BHP- (*tert*-butylhydroperoxide) stimulated YPEN-1 cells. Our results suggest that zingerone may be a selective regulator of $ONOO^-$-mediated diseases via its direct scavenging activity.

MATERIALS AND METHODS

Materials. Zingerone (vanillylacetone) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Aldrich Chemical Co. (Milwaukee, WI). DL-Penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid), bovine serum albumin (BSA), 3-morpholinosydnonine (SIN-1), sodium nitroprusside (SNP), and *tert*-butylhydroperoxide (*t*-BHP) were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) were purchased from Molecular Probes (Eugene, OR), and $ONOO^-$ was from Calbiochem Co. (San Diego, CA). 4,5-Diaminofluorescein (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Corp. (Billelica, MA), and the chemiluminescence detection system was from Amersham Life Sciences, Inc. (Arlington Heights, IL). Antibody to nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence (ECL) Western blotting detection reagents were from Amersham Life Science (Buckinghamshire, U.K.). All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO) or Junsei Chemical Co. (Tokyo, Japan).

Measurement of $ONOO^-$ Scavenging Activity. $ONOO^-$ scavenging was measured by monitoring the oxidation of DHR 123 by modifying the method of Kooy et al. (*21*). A stock solution of 5 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solution with 5 μM DHR 123 diluted from the stock solution was placed on ice in the dark immediately before the experiment. The buffer (90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride) was purged with nitrogen and placed on ice before use. Just before use 5 mM diethylenetriaminepentaacetic acid (DTPA) was added. The $ONOO^-$ scavenging ability, by the oxidation of DHR 123, was measured at room temperature on a microplate fluorescence Genious (Tecan, Austria) with excitation and emission wavelengths of 485 and 535 nm, respectively. The background and final fluorescent intensities were determined 5 min after treatment with or without authentic 10 μM $ONOO^-$ in 0.3 N sodium hydroxide or 20 μM SIN-1. Oxidation of DHR 123 by decomposition of SIN-1 gradually increased, whereas native $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time on the inhibition of DHR 123 oxidation by $ONOO^-$. Penicillamine was used as a positive control.

Measurement of $^{\bullet}O_2^-$ Scavenging Activity. H_2DCFDA (f.c. 2.5 mM) mixed with esterase (f.c. 1.5 units/mL) was incubated at 37 °C for 20 min and placed on ice in the dark prior to the study. H_2DCFDA was oxidized to fluorescent 2,7-dichlorofluorescein (DCF) by $^{\bullet}O_2^-$. A 50 mM phosphate buffer at pH 7.4 was used. The fluorescence intensity of DCF was measured for 1 h by using microplate fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively, with or without the addition of 50 mM menadione as an $^{\bullet}O_2^-$ source.

Measurement of $^{\bullet}$NO Scavenging Activity. $^{\bullet}$NO scavenging activity was determined by monitoring DAF-2 by modifying the method of Chung et al. (*22*). DAF-2, as a specific $^{\bullet}$NO indicator, selectively traps $^{\bullet}$NO between two amino groups in its molecule and yields triazolofluorescein, which emits green fluorescence when excited at 490−495 nm (*23*). In brief, 1 mg of DAF-2 in 0.55 mL of dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400-fold. A $^{\bullet}$NO donor, 2 mM SNP, and 3.14 μM DAF-2 were added to a 96-well black microplate. The fluorescence intensity was dependent on the amount of $^{\bullet}$NO trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with $^{\bullet}$NO was measured for 30 min by the fluorescence at excitation and emission wavelengths of 495 and 515 nm, respectively.

Inhibition of $ONOO^-$-Mediated Tyrosine Nitration by Zingerone. The ability of zingerone to inhibit the formation of 3-nitrotyrosine was quantified as the index of zingerone's inhibition of tyrosine nitration utilizing spectrophotometric and Western blot analyses.

(1) Spectrophotometric Analysis of Nitrated Proteins. The spectral change in the visible region of zingerone in the presence of $ONOO^-$ was monitored at 430 nm to detect the existence of nitration. $ONOO^-$ (200 μM) in 0.3 N NaOH was added to a solution containing a sample and tyrosine (200 μM) in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 mL. Each mixed solution was scanned between 190 and 600 nm on an Ultraspec 2000 UV−visible spectrophotometer (Pharmacia-Biotech, U.K.).

(2) Western Blotting Analysis. A 2.5 μL aliquot of zingerone dissolved in 10% v/v EtOH was added to 95 μL of BSA (0.5 mg of protein/mL) with 2.5 μL of $ONOO^-$ (100 μM). The mixed samples were incubated with shaking at 37 °C for 20 min.

The prepared samples in gel buffer, pH 6.8 (12.5 mM tris-[hydroxymethyl]aminomethane, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, and 0.2% bromophenol blue) in a ratio of 1:1 were boiled for 5 min. Total protein equivalents for each sample were separated on 8% SDS−polyacrylamide minigel at 100 V and transferred to a PVDF membrane at 100 V for 90 min in a wet transfer system (Bio-Rad, Hercules, CA). The membrane was immediately placed into a blocking solution (5% w/v skim milk powder in TBS-Tween buffer containing 10 mM Tris, 100 mM NaCl, and 0.1 mM Tween-20, pH 7.4) at room temperature for 1 h. The membrane was washed in TBS-Tween buffer for 30 min and then incubated with a monoclonal anti-nitrotyrosine antibody (1% w/v skim milk, diluted 1:2000 in TBS-Tween buffer) at room temperature for 3 h. After three 10 min washings in TBS-Tween buffer, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (1% w/v skim milk, diluted 1:2000 in TBS-Tween buffer) at room temperature for 2 h. After three 10 min washings in TBS-Tween buffer, antibody labeling was detected using ECL and exposed to radiographic film. Prestained blue protein markers were used for molecular-weight determination.

Cells and Cell Culture Conditions. YPEN-1 cells, rat prostatic endothelial cells, were obtained from ATCC (American Type Culture Collection, Rockville, MD). YPEN-1 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Nissui Co., Tokyo, Japan) supplemented with 5% heat-inactivated (56 °C for 30 min) fetal bovine serum (Gibco, Grand Island, NY), 233.6 mg/mL glutamine, 72 μg/mL penicillin−streptomycin, and 0.25 μg/mL amphotericin B and adjusted to pH 7.4−7.6 with $NaHCO_3$ in an atmosphere of 5% CO_2. The fresh medium was replaced after 1 day to remove nonadherent cells or cell debris.

Determination of Intracellular RS and $ONOO^-$ Scavenging Activity. *(1) Inhibition of Intracellular RS (Reactive Species).* YPEN-1 cells in a 96-well plate were preincubated for 24 h. After 1 day the medium was changed to fresh serum-free medium. The cells were treated with or without zingerone and incubated for 1 h. After treatment with 20 μM *t*-BHP for 30 min, the medium was replaced with fresh serum-free medium and 125 μM H_2DCFDA was added. The fluorescence intensity of DCF was measured for 30 min using the microplate fluorescence with excitation and emission wavelengths of 485 and 535 nm, respectively.

(2) Inhibition of Intracellular $ONOO^-$. YPEN-1 cells in a 96-well plate were preincubated for 24 h. After preincubation with or without zingerone for 1 h, cells were treated with 20 μM *t*-BHP for 30 min. Then the cells were added to the rhodamine solution (50 mM sodium phosphate buffer, 90 mM sodium chloride, 5 mM potassium chloride, 5 mM DTPA, and 5 μM DHR 123). The $ONOO^-$ scavenging ability by the oxidation of DHR 123 was determined using the microplate fluorescence for 30 min with excitation and emission wavelengths of 485 and 535 nm, respectively.

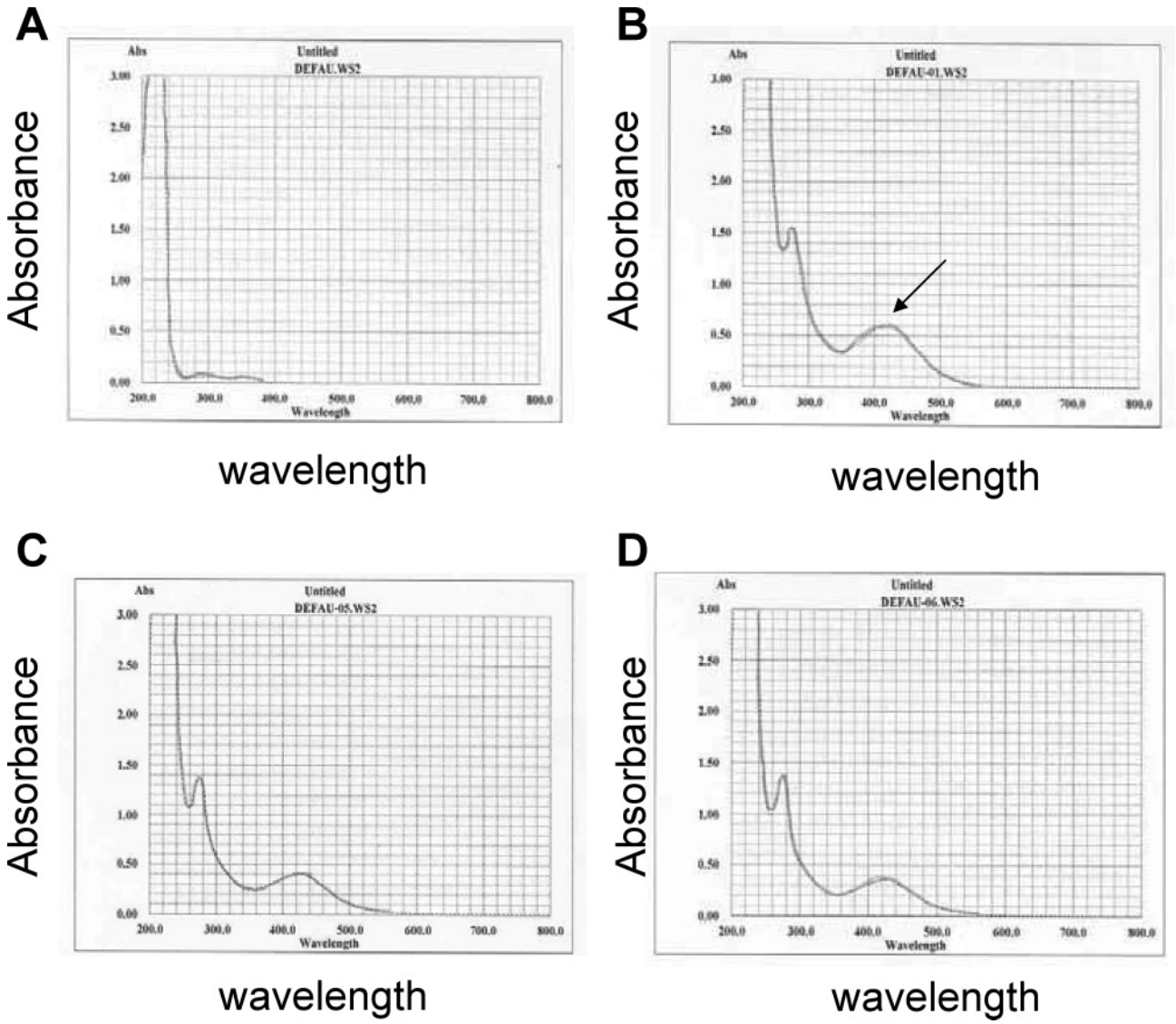

Figure 2. Interaction of zingerone with $ONOO^-$. Tyrosine (200 μM) was incubated without (A) or with $ONOO^-$ (200 μM) (B) in 50 mM phosphate buffer at pH 7.0 at room temperature for 10 min. Zingerone 15 μM (C) or 60 μM (D) was incubated with $ONOO^-$ (200 μM) at 37 °C for 10 min. The spectrophotometric analysis was performed as described in Materials and Methods. The arrow indicates a nitration peak at around 400–450 nm.

Table 1. Peroxynitrite ($ONOO^-$) Scavenging Activity of Zingerone

name	IC50 value (μM)	
	native $ONOO^-$	SIN-1-derived $ONOO^-$
zingerone	1.88 ± 0.12	1.81 ± 0.07
penicillamine[a]	1.60 ± 0.10	1.88 ± 0.12

[a] Used as a positive control. IC_{50}: 50% inhibition concentration.

Table 2. Effect of IC_{50} (μM) Zingerone on $^{\bullet}O_2^-$, and $^{\bullet}NO$ Scavenging Activities

name	$^{\bullet}O_2^-$	$^{\bullet}NO$
zingerone	49.14 ± 0.23	22.83 ± 0.31
trolox[a]	30.61 ± 0.11	
carboxy-PTIO[a]		15.92 ± 0.25

[a] Used as a positive control. IC_{50}: 50% inhibition concentration. Abbreviations: $^{\bullet}O_2^-$, superoxide; $^{\bullet}NO$, nitric oxide.

Statistical Analysis. Data are expressed as mean ± standard deviation (s.d.) of three or five experiments. Statistical analysis was confirmed by a Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

The $ONOO^-$ Scavenging Activity of Zingerone. The ability of zingerone to scavenge $ONOO^-$ was investigated using DHR 123 as shown in **Table 1**. The results showed that zingerone (IC_{50} 1.88 ± 0.12 μM) has a similar scavenging activity to penicillamine (IC_{50} 1.60 ± 0.10 μM), a well-known, strong $ONOO^-$ scavenger. In the case of the scavenging activity of $ONOO^-$ that was generated from SIN-1 reactions, zingerone showed an even a higher ability than penicillamine, as shown in **Table 1**. Their IC_{50} values are 1.81 ± 0.07 and 1.88 ± 0.12 μM for zingerone and penicillamine, respectively.

Scavenging Ability of Zingerone against $^{\bullet}O_2^-$ and $^{\bullet}NO$. Table 2 shows IC_{50} values of zingerone for $^{\bullet}O_2^-$ and $^{\bullet}NO$ scavenging abilities. Because $ONOO^-$ can be formed from reaction of $^{\bullet}NO$ with $^{\bullet}O_2^-$, we thought it would be interesting to compare the active components of $ONOO^-$, $^{\bullet}O_2^-$, and $^{\bullet}NO$. Compared with trolox (IC_{50} 30.61 ± 0.11 μM), a well-known antioxidant $^{\bullet}O_2^-$ scavenger, zingerone showed similar scavenging activity levels (IC_{50} 49.14 ± 0.23 μM). In addition, zingerone inhibitory action was considerately strong (IC_{50} 22.83 ± 0.31 μM) in $^{\bullet}NO$

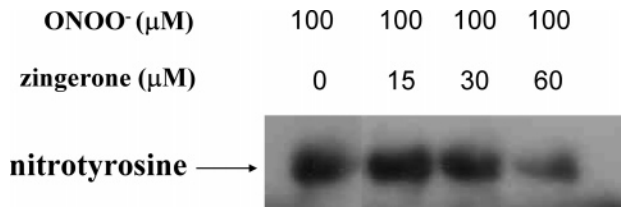

Figure 3. Effect of zingerone on the nitration of BSA by $ONOO^-$. Zingerone and $ONOO^-$ were added to BSA. The reaction samples were incubated with shaking at 37 °C for 20 min, which was resolved in 8% polyacrylamide-gel electrophoresis.

scavenging ability, compared with a $^{\bullet}NO$ scavenging standard carboxy-PTIO (IC_{50} 15.92 ± 0.25 μM).

Effect of Zingerone on $ONOO^-$ -Mediated 3-Nitrotyrosine Formation. To explore the scavenging mechanisms of zingerone by which it undergoes either nitration reaction or electron donation after the addition of $ONOO^-$, spectrophotometric analysis at 400−450 nm was employed. **Figure 2A** and **B** shows that tyrosine undergoes nitration by $ONOO^-$ because there is a peak around 430 nm. Incubation of zingerone with tyrosine prior to the addition of $ONOO^-$ resulted in the disappearance of the nitrotyrosine peak at 430 nm (**Figure 2C** and **D**) in a dose-dependent manner, implying that zingerone inhibited the formation of 3-nitrotyrosine.

Effect of Zingerone on BSA Nitration. Through Western blot analysis we tested the ability of zingerone to inhibit $ONOO^-$-mediated tyrosine nitration in common biological materials, such as BSA, using anti-3-nitrotyrosine antibody. The results indicated that zingerone at concentrations of 15, 30, and 60 μM attenuated the nitration of BSA in a dose-dependent fashion, as shown in **Figure 3**.

Effects of Zingerone on the Inhibition of Intracellular RS and $ONOO^-$ Generation. Overproduction of RS and $ONOO^-$ that outstrips antioxidant defenses generates oxidant stress that can lead to nitric oxide depletion and endothelial cell injury and contributes to the pathophysiology of cardiovascular disease and myocardial dysfunction. We wanted to test whether zingerone had an inhibitory effect on intracellular RS and $ONOO^-$. As shown in **Figure 4A**, the results revealed that preincubation of zingerone at concentrations of 5, 15, and 60 μM decreased the intracellular RS generation-induced *t*-BHP (20 μM) in a dose-dependent manner. Also, zingerone inhibited the intracellular $ONOO^-$ generation-induced *t*-BHP (20 μM) in a dose-dependent manner (**Figure 4B**).

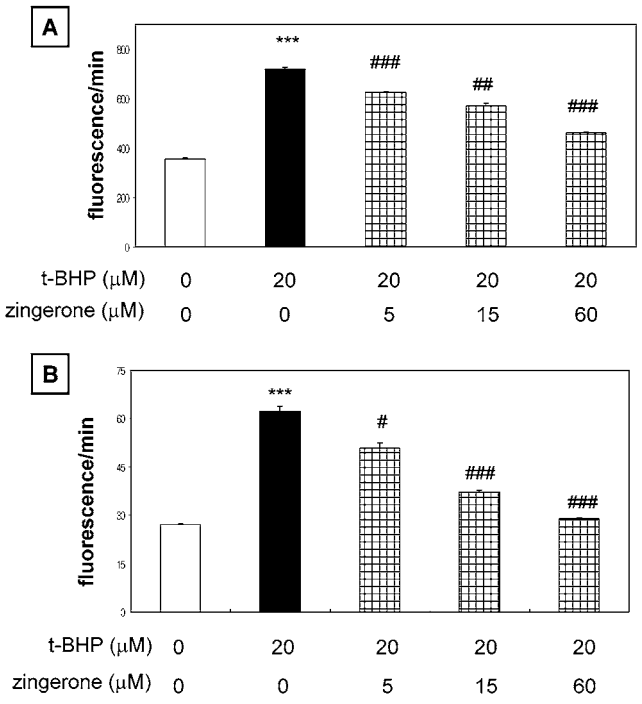

Figure 4. Zingerone inhibited intracellular free-radical generation induced *t*-BHP. Cells were pretreated with various concentrations of zingerone for 1 h and further treated with *t*-BHP (20 μM) for 30 min. (A) Detection of intracellular RS by staining with fluoremeter using DCFDA. (B) Detection of intracellular $ONOO^-$ by staining with fluoremeter using DHR 123. Each value is the mean ± SEM. Statistical results of one-factor ANOVA: (***) $p < 0.001$ vs not treated t-BHP, (#) $p < 0.05$, (##) $p < 0.01$, (###) $p < 0.001$ vs treated t-BHP.

DISCUSSION

$ONOO^-$ is a potent contributor to inflammatory conditions, including arthritis and endotoxic or septic shock, which is widely considered a systemic inflammatory disease, as well as being suspect in a variety of other models of inflammation (*24*). Moreover, $ONOO^-$ can nitrate tyrosine, a process specific to this species and not triggered by the hydroxyl radicals, $^{\bullet}NO$ or $^{\bullet}O_2^-$ (*25*). These are a variety of additive or synergistic cytotoxic processes triggered by $ONOO^-$, the combination of which can lead to acute and delayed cytotoxicity (*26*). Plant food-derived antioxidants and active principles, such as flavonoids, hydrocinnamates (chlorogenic acid, ferulic acid, vanillin, etc.), β-carotene, and other carotenoids, vitamin E, vitamin C, rosemary, sage, and tea are important dietary antioxidant substances (*27*) that may provide efficient $ONOO^-$ scavenger activities. Ginger is a widespread herbal medicine that has been reported to have an antioxidant property (*28*).

Our study shows that zingerone has a strong scavenging activity against $ONOO^-$. It would seem reasonable to use the well-known $ONOO^-$, $^{\bullet}NO$, and $^{\bullet}O_2^-$ scavenging agents penicillamine (*28*), carboxy-PTIO (*29*), and trolox (*30*) in a comparison study against the scavenging activity of zingerone. The most important findings from the present study are that the active component, zingerone, not only directly enabled $ONOO^-$ scavenging, but also was involved in the inhibition of $^{\bullet}O_2^-$ and $^{\bullet}NO$ radical formation. These phenomena might be explained in terms of chemical structures. Hydroxyl groups in the molecule are responsible for the $ONOO^-$ scavenging activity of the phenolic compound zingerone (*19*, *20*).

The detailed mechanisms of $ONOO^-$ scavenging actions by active components are not yet fully defined; thus far, two possible pathways, nitration or electron donation, have been suggested as a phenolic $ONOO^-$ scavenger interaction with $ONOO^-$ (*31*). The phenolic compound, especially with a monohydroxyl group such as the phenolic amino acid, tyrosine, is preferentially nitrated by $ONOO^-$. Whether nitration is derived from the breakdown of $ONOO^-$ to nitrogen dioxide radical ($^{\bullet}NO_2$) or the nitronium ion ($^{\bullet}NO_2^+$) has not been clarified (*28*).

In the present study we exposed zingerone to $ONOO^-$ to determine whether the zingerone scavenging mechanism involves the nitration reaction. The addition of $ONOO^-$ revealed no spectral change in the visible region, indicating that nitration of the aromatic ring did not occur. Furthermore, incubation of tyrosine and zingerone with $ONOO^-$ caused a decreased peak at 430 nm, which gave further evidence supporting the possibility of an electron-donation reaction between zingerone and $ONOO^-$.

The inhibitory action of zingerone was further tested using additional physiological substances such as BSA. We found that $ONOO^-$ could induce functional damage in some biological molecules, such as BSA and LDL, via nitrotyrosine (*22*). Protein tyrosine nitration by $ONOO^-$ may interfere with phosphorylation/dephosphorylation signaling pathways and alter enzyme functions (*32*−*34*). Nitrotyrosine has been reported in various hypertensive disorders, neurological disorders, and chronic renal disease as a footprint of $ONOO^-$ (*35*−*37*). In the current study evidence from Western blot analysis showed that zingerone, even at a concentration of 30 μM, could markedly reduce the nitrotyrosine present in BSA. With increases in zingerone concentrations, BSA nitration decreased, further suggesting the putative anti-$ONOO^-$ action in vivo.

t-BHP can be metabolized to free-radical intermediates by cytochrome P-450 or hemoglobin, which can subsequently initiate lipid peroxidation, affect cell integrity, and form covalent bonds with cellular molecules resulting in cell injury (*38*). Thus, in our study cells were treated with *t*-BHP to induce oxidative stress. When treated with zingerone we found it inhibited the generation of RS and $ONOO^-$ in a dose-dependent manner.

In summary, the present study suggested that the active phenolic component, zingerone, can scavenge $ONOO^-$ efficiently. Zingerone treatment led to an inhibition of $ONOO^-$-mediated nitration of tyrosine through electron donation, and it also showed significant dose-dependent inhibition of BSA nitration from ONOO. Zingerone was shown to induce *t*-BHP free radical equally well. In summary, the present results demonstrated that zingerone is an efficient $ONOO^-$ scavenger and therefore potentially useful in the prevention of the $ONOO^-$-related diseases.

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