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Short Communication

Suppression Effect of *Curcuma longa* Rhizome-Derived Components against Nitric Oxide Synthase

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The inhibitory effects of *Curcuma longa* rhizome-derived materials against nitric oxide (NO) production were assessed. The inhibitory effect (57%) on NO production was evidenced by the methanol extract of *C. longa* at 1 μg/mL. In the fractionation of the methanol extract, the ethyl acetate fraction evidenced an inhibitory effect greater than 62.1% at 1 μg/mL. The active constituent was identified as curcumin. Curcumin exerted potent inhibitory effects of 78.7 and 65.7% at concentrations of 1 and 0.5 μg/mL, respectively. Furthermore, the inhibitory effect of *ar*-turmerone was measured as 31.3 and 15.8% at 1 and 0.5 μg/mL, respectively. The iNOS expression-suppressive effects of curcumin were assessed via western blot analysis. Our results suggest that curcumin and *ar*-turmerone may prove useful in the development of new types of NO inhibitors.

Key words: Curcuma longa, *curcumin, inducible nitric oxide synthase, NO production, suppression*

As a component of the broad search for anti-inflammatory natural products, an extract of the *Curcuma longa* L. (Zingiberaceae) rhizome in East Asia was selected for analysis in this study. This substance has long been considered to have medicinal properties, including analgesic properties in the treatment of menstrual disorders, rheumatism, and traumatic diseases, as it harbors a number of monoterpenoids, sesquiterpenoids, and curcuminoids [Tang and Eisenbrand, 1992]. Furthermore, the extract of *C. longa* rhizomes has also been shown to have insecticidal [Chander *et al*., 1991], repellent [Su *et al*., 1982], and antifeeding activities against certain insects that infest stored products [Chowdhury *et al*., 2000]. The insect repellent and antifeeding constituents from *C. longa* rhizomes have been identified as turmerone and *ar*-turmerone [Su *et al*., 1982; Lee *et al*., 2001] and curcuminoids [Chowdhury *et al*., 2000], respectively. However, relatively little research has thus far addressed the inhibitory responses of turmerone derivatives and curcuminoids against iNOS, in spite of their insecticidal and pharmacological activities [Kim *et al*., 2003]. As a component of a broader effort to develop safer iNOS inhibitors, this study attempted to characterize the inhibitory effects of *C. longa* rhizome-derived materials on iNOS in murine peritoneal macrophages after stimulation with lipopolysaccharide (LPS).

Chemicals. Acrylamide, ε-amino-*n*-caproic acid, borneol, bovine serum albumin, brilliant blue G-250, ethylene diamine acid, 1,8-cineole, leupeptin, lipopolysaccharide, *N,N'*-methylene-bis-acrylamide, sabinene, sodium dodecyl sulfate, sodium nitrite, sulfanilamide, and trypsin inhibitor type II (soybean) were purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum, penicillin, RPMI 1640, skim milk dehydrate, and streptomycin were obtained from Gibco (Gaithersburg, NM). Goat anti-rabbit IgG(H+L)-AP conjugate was acquired from Bio-rad Laboratories (Hercules, CA) and rabbit anti-mouse macNOS was purchased from Transduction Laboratories (Rockville, MD). All other chemicals used in this study were of reagent grade.

Murine peritoneal macrophage cells. Macrophages were purified from peritoneal exudate cells in accordance with established protocols [Kirikae *et al*., 1996; Lee *et al*., 2000; Lee *et al*., 2002; Lee, 2009]. Thioglycollate-elicited peritoneal exudate cells were acquired from 8- to 10-week old ICR male mice via intraperitoneal injection of 1 mL Brewer Thioglycollate broth (4.05% w/v) and lavage of the peritoneal cavity with 5 mL of Roswell Park Memorial Instituete-1640 (RPMI-1640, GibcoBRL, USA) medium 3 days later. The cells were washed twice and resuspended in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM glutamine, penicillin (200 IU/mL), and streptomycin (200 IU/mL). Peritoneal exudate cells were seeded at densities of 1×10^5 cells/well in 96-well tissue culture plates or at 1×10^7 cells/dish on 6 cm tissue culture dishes, and the

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Abbreviations: iNOS, inducible nitric oxide synthase; IFN-γ, interferon-γ; LPS, lipopoly saccharide; NO, nitric oxide

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macrophages were allowed to adhere for 2 h in a 5% humidified atmosphere. The nonadherent cells were removed by pouring off the medium and rinsing the wells twice with pre-warmed medium. The adherent cells were incubated under the appropriate conditions for each of the individual experiments.

Treatment of macrophages with LPS. Peritoneal macrophages were incubated in 96-well tissue culture plates (1×10^6 cells/mL) or on 6 cm tissue culture dishes (1×10^7 cells/dish) with LPS (1 μg/mL) for 24 h at 37°C in a 5% CO_2-air incubator [Lee *et al*., 2000; 2002; Lee, 2009]. The supernatant was harvested and assayed for nitrite production.

Cell stimulation and samples co-treatment. RAW 264.7 cells were obtained from the American Type Culture Collection (ATTC, Rockville, MD). RAW 264.7 cells were stimulated with LPS (1 μg/mL) and IFN-γ (10 U/mL) with or without samples [Lee *et al*., 2000; 2002; Lee, 2009]. The supernatants were subsequently harvested and assayed for nitrite production. The cells incubated on 6 cm culture dishes were scrapped and collected. The cells were then resuspended with 500 μL of sonication buffer. The cells were disrupted via 10 sec of sonication, and the sonicates were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were used as cytosol fractions in Western blot analysis.

Nitrite and protein determination. NO production was assessed by estimating the stable NO metabolite, nitrite, in conditioned medium via the Griess reaction [Lee *et al*., 2000; Lee *et al*., 2002; Lee, 2009]. Cell-free supernatants (100 μL) were mixed with 100 μL Griess reagent (1% sulfanilamide in 5% phosphoric acid: 0.1% naphthylethylenediamine dihydrochloride =1:1) and incubated for 15 min at room temperature. Following incubation, the absorbance of the wells was determined with a microplate reader (Multiscan MCC/340 P version 2.3, Labsystems, Finland) equipped with a 540 nm filter. Nitrite concentrations were determined via a linear regression analysis of standards (sodium nitrite) generated for each plate. Protein contents in the cytosol were measured via the Bradford method, using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and western blot analysis. The sonicated cells were subjected to electrophoresis on 1.5 mm thick 15% polyacrylamide gels. The separated proteins were then transferred to PVDF membranes using Trans-Blot [Lee *et al*., 2000; 2002; Lee, 2009]. The membranes were blocked for 30 min at room temperature with 5% skim milk in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20, and were subsequently incubated with anti-iNOS antibody (1:2000 dilution) in blot buffer (50 mM Tris-HCl, ph 7.5, 200 mM NaCl, 5% skim milk and 0.05% Tween 20) overnight at 4°C. The membranes were then washed twice in blot buffer, and incubated for 2 h with alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000 dilution) for 2 h at room temperature, followed by three washings in blot buffer for 3 min, and one in TBS for 20 min. The membranes were incubated for an additional 1-10 min with alkaline phosphatase substrate, and the immunoreactive bands were identified as iNOS protein, with a molecular weight of 130 kDa.

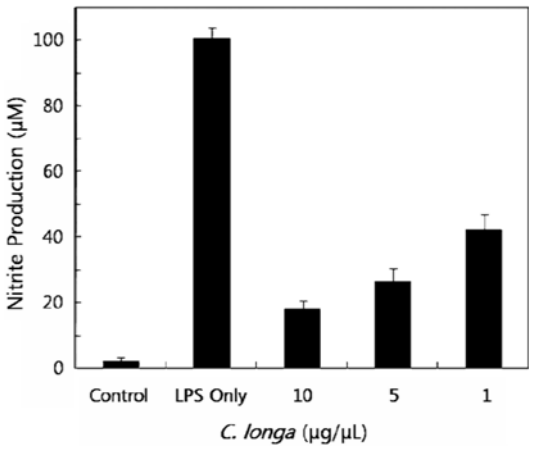

Fig. 1. Inhibitory effect of *C. longa* rhizome extracts on nitrite release from LPS-treated macrophages. Macrophages (1×10^5 cells/dish) were incubated for 24 h with LPS (1 μg/mL) and IFN-γ (10 U/mL) in 96-well tissue culture plates in the absence or presence of *C. longa* rhizome extracts. After incubation, NO contents in the supernatant (100 μL) were measured using Griess reagent. The results are expressed as the means±SE of 3 separate experiments.

Extraction and isolation. The rhizomes (4 kg) from *C. longa* were purchased from a medicinal herb shop, Kyungdong Market, Seoul (September, 2006) and identified by Prof. Sang-Hyun Lee (Forestry Department, Chonbuk National University, South Korea). It was finely powdered, extracted twice with methanol (10 L) at room temperature (25°C) for 2 days, and filtered. The combined filtrate was concentrated *in vacuo* at 35°C to obtain a yield of approximately 10%, based on the weight of the dried rhizome. In routine screening, the inhibitory responses of the methanol extracts of *Curcuma longa* rhizomes on NO production in RAW 264.7 cells after stimulation by LPS (1 μg/mL) and IFN-γ (10 units/mL) were assessed at 10, 5, and 1 μg/mL using the Griess assay (Fig. 1). In a test with 5 μg/mL, a more inhibitory effect (73.5%) on NO production was exhibited by the methanol extract. Furthermore, this inhibitory effect (57.7%) was also detected at 1 μg/μL. Owing to the potent activity of the methanol extract derived from the *C. longa* rhizomes, the isolation of the active component was pursued. First of all, the methanol extract (20 g) was then sequentially partitioned into hexane (6.9 g), chloroform (5.1 g), ethyl acetate (4.4 g), butanol (0.5 g), and water (3.1 g) for subsequent bioassays. The organic solvent portions were concentrated to dryness via rotary evaporation at 40°C, while the water portion was freeze-dried. And then we conducted a bioassay-guided fractionation of the methanol extracts and the inhibitory responses of each fraction from the methanol extract were

Table 1. Inhibitory effects of various fractions of methanol extract from *C. longa* L. rhizomes on nitrite release from LPS-treated macrophages

Fractions	Mean±SE (%) Nitrite production (% Control)	
	1 μg/μL	2 μg/μL
Hexane	61.2±4.7	43.2±3.8
Chloroform	100.6±3.9	100.5±4.7
Ethyl-acetate	37.9±4.2	28.8±2.8
Butanol	100.1±3.4	101.3±4.7
Water	100.3±5.9	100.8±5.1

Macrophages (1×10^5 cells/dish) were incubated with LPS (1 μg/mL) and IFN-γ (10 U/mL) at 96-well tissue culture plate in absence or presence of five fractions of *C. longa* rhizome extracts for 24 h. Results are the means±SE of three separate experiments.

Table 2. Inhibitory effects of *C. longa* rhizome-derived components on nitrite release from LPS-treated macrophages

Compounds	Mean±SE (%) Nitrite production (% Control)		
	0.5 μg/μL	1 μg/μL	2 μg/μL
Curcumin	34.3±4.8	21.3±3.0	15.8±4.1
ar-Turmerone	84.2±3.9	68.7±4.2	45.9±5.1
Borneol	101.4±4.7	100.9±3.5	100.1±3.3
1,8-Cineole	102.2±5.8	101.9±3.9	101.7±6.2
Sabinene	101.4±2.9	101.9±5.2	100.8±3.4

Macrophages (1×10^5 cells/dish) were incubated for 24 h with LPS (1 μg/mL) and IFN-γ (10 U/mL) in 96-well tissue culture plates in the absence or presence of materials derived from *C. longa* rhizome. The results are expressed as the means±SE of three separate experiments.

evaluated at 2 and 1 μg/μL (Table 1). In fractionation, guided by the inhibitory effects of NO production, the hexane and ethyl acetate fractions of methanol extract exhibited inhibitory effects of 56.8 and 71.2% at 2 μg/μL and 38.8 and 62.1% at 1 μg/μL, respectively. Little or no activity was noted with the chloroform, butanol, and water fractions. These results showed that the active NO production-inhibitory component may have been present in the hexane and ethyl acetate fractions of the methanol extract of *C. longa* rhizomes.

Based on the inhibitory effects of the ethyl acetate fraction of *C. longa* rhizomes, the ethyl acetate fraction (4.4 g) was purified via silica gel column chromatography and HPLC. The ethyl-acetate fraction was chromatographed on a 8×95 cm silica gel column (Merck 230-400 mesh, 700 g), and successively eluted with 20%, 30%, and 40% ethyl acetate/hexane (4 L each) followed by 10% methanol/acetone (5 L). The column fractions were analyzed via TLC (silica gel G), and fractions with a similar TLC pattern were pooled. The CLEA3 fraction (2.8 g) exerted inhibitory effects against NO production and were successively rechromatographed on a 4×100 cm column with 20% and 25% ethyl acetate/hexane (4 L each) used as the eluent. Next, CLEA33 fraction (1.7 g) exhibited inhibitory effect against NO production, therefore this fraction was noted in the subfractions, which were chromatographed further on a 2.5×70 cm column with 20% (800 mL) and 25% (2 L) acetone/hexane as an eluent. During this step, the active fraction 333 (1.07 g) was obtained. The CLEA333 fraction was further purified by HPLC (System P2000, Thermo Separation Products, San Jose, CA) was utilized for the further separation of the constituents. The column was a 19×300 mm Porasil silica (Waters, Milford, MA) using ethyl acetate/hexane (3:1, v/v) at a flow rate of 2 mL/min, and detection was performed at 242 nm. Finally, the principal active component CLEA3334 (125 mg) was isolated. Structural determination of the active isolate was conducted via spectroscopic analysis techniques, including UV, EI-MS, ^{13}C NMR and ^{1}H NMR, ^{1}H-^{13}H COSY, ^{1}H-^{13}H DEPT, and directly compared with authentic reference compounds. ^{1}H- and ^{13}C-NMR spectra were recorded in deuterochloroform using a Bruker AM-500 spectrometer at 400 and 100 MHz, respectively. Chemical shifts were reported as δ values downfield from an internal standard of Me_4Si. Mass spectra were acquired with a JEOL GSX 400 spectrometer.

Based on the results of these analyses, the biologically active constituent was identified as curcumin. The compound was identified on the basis of the following evidence: Orange-yellow solid; mp 182-184°C; elemental analysis calculated for $C_{21}H_{20}O_6$; EI-MS (70 eV) *m/z* (% rel. int.): 368 (63) $[M]^+$, 350 (58), 320 (22), 272 (20), 232 (23), 217 (27), 177 (100), 145 (49), 137 (48), 117 (18); CIMS *m/z* 369 $[M+1]^+$; IR (ν KBr) cm^{-1}: 3427 (O-H str); 2950-3000 (α,β-unsaturated and aryl C-H str), 1287, 1207 (C-O str). ^{1}H NMR (400 MHz, acetone-d_6): δ 9.25 (2H, Ar-OH), 7.54 (2H, d, *J*=16 Hz, 1,7-H), 7.10 (2H, d, *J*=2 Hz, 2',2"-H aromatic), 7.04-7.07 (2H, dd, *J*=2 and 8Hz, 6',6"-H), 6.87 (2H, d, *J*=8 Hz, 5',5"-H, aromatic), 6.53 (2H, d, *J*=16 Hz, 2,6-H), 5.88 (1H, s, -C(OH)=CH-, enol form), 3.89 (6H, s, 2× -OCH_3); ^{13}C NMR (100 MHz, acetone-d_6) δ 184.5, 150.0, 148.8, 141.4, 128.2, 123.8, 122.3, 116.2, 111.6, 101.6, 56.3. The spectroscopic analyses of curcumin in this study are identical to the data of curcumin isolated from *C. longa* [Kim *et al*., 2003].

In order to further assess the inhibitory effects of curcumin and other components derived from *C. longa* L., four commercially available compounds derived from this plant species [Negi *et al*., 1999], were measured for their activity against NO production (Table 2). Curcumin exhibited inhibitory effects of 84.2 and 78.7% at 2 and 1 μg/μL, respectively, and an inhibitory effect of 65.7% was observed at 0.5 μg/μL. *ar*-Turmerone evidenced inhibitory effects of 54.1 and 31.3% at 2 and 1 μg/μL, respectively, and an inhibitory effect of 15.8% was observed at 0.5 μg/μL. These results demonstrate that the observed inhibitory effects of *C. longa* extract against NO production were attributable to curcumin and *ar*-turmerone. However, minimal or no activity was observed for borneol, 1,8-

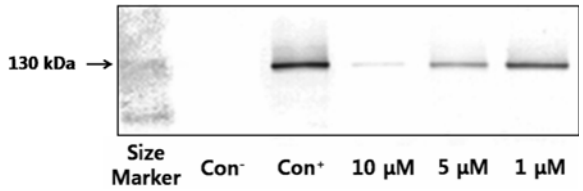

Fig. 2. Western blot analysis of iNOS in RAW 264.7 cells treated with curcumin isolated from *C. longa*. Immunoblot. The cells were stimulated with LPS (1 μg/mL) plus IFN-γ (10 U/mL) and incubated for 16 h in the presence of curcumin. $Cont^-$: unstimulated, $Cont^+$: stimulated.

cineole, and sabinene at 2 μg/μL. The extract of *C. longa* L. is frequently utilized in foods, as a condiment. It is also employed as an essential ingredient in medicines: it has been used as a carminative, anthelminthic, laxative, and as a cure for liver ailments [Srimal, 1997]. *C. longa* has also traditionally been utilized as an insect repellent, and insecticide, and an antibacterial agent [Su *et al*., 1982; Negi *et al*., 1999; Lee *et al*., 2001]. Among its active components, turmeric oils and curcuminoids constitute a major group of secondary metabolites. Derivatives of turmerone have been previously reported to repel *Tribolium castaneum*, and have been shown to exhibit a potent insecticidal activity against *Nilaparvata lugens* and *Plutella xylostella* [Su *et al*., 1982; Lee *et al*., 2001]. Furthermore, curcuminoids including curcumin, demethoxycurcumin, and bismethoxycurcumin also evidence profound antibacterial, antioxidant, and antitumorigenic properties [Srimal *et al*., 1997; Negi *et al*., 1999]. In order to clarify the mechanism underlying curcumin's NO inhibitory activity, iNOS protein expression was assessed in cellular extracts generated from RAW 264.7 cells stimulated with LPS and IFN-γ with or without this inhibitor (Fig. 2). In general, iNOS was expressed to a sufficient degree by stimulation with either LPS or IFN-γ. Fig. 2 shows that the combination of LPS and IFN-γ was a strong inducer of iNOS protein expression in RAW 264.7 cells. However, curcumin induced a significant and dose-dependent reduction of iNOS expression in LPS/IFN-γ-stimulated RAW 264.7 cells. Curcumin blocked iNOS protein expression almost completely at 10 μM.

In conclusion, it seems clear that excessive NO induces disruptions in energy metabolism and oxidative stress. Furthermore, NO interacts with mitochondria in several ways central to its functions in cell death [Lee *et al*., 2002]. In the majority of cases, the relevant pathologies are ameliorated via the inhibition of NO production, which is achieved by the administration of NO inhibitors. In this study, curcumin was shown to potently interfere with excess NO production via the activity of inhibitory responses against iNOS expression. On the basis of our limited data, the inhibitory activities of curcumin suggest that it may be a superior iNOS-inhibitory agent for use in the development of medicinal foods, although its *in vivo* efficacy and clinical usefulness remain to be clearly assessed.

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