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Inhibition of nitric oxide by phenylethanoids in activated macrophages

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Abstract

Nitric oxide (NO) is one of the pro-inflammatory molecules. Some phenylethanoids have been previously shown to possess anti-inflammatory effects. Seven phenylethanoids from the stems of *Cistanche deserticola*, viz. isoacteoside, tubuloside B, acteoside, 2′-*O*-acetylacteoside, echinacoside, cistanoside A and tubuloside A, were tested for their effect on NO radical generation by activated murine macrophages. At the concentration of 100–200 μM, all the phenylethanoids reduced (6.3–62.3%) nitrite accumulation in lipopolysaccharide (0.1 μg/ml)-stimulated J774.1 cells. At 200 μM, they inhibited by 32.2–72.4% nitrite accumulation induced by lipopolysaccharide (0.1 μg/ml)/interferon-γ (100 U/ml) in mouse peritoneal exudate macrophages. However, these compounds did not affect the expression of inducible nitric oxide (iNOS) mRNA, the iNOS protein level, or the iNOS activity in lipopolysaccharide-stimulated J774.1 cells. Instead, they showed a clear scavenging effect (6.9–43.9%) at the low concentrations of 2–10 μM of about 12 μM nitrite generated from an NO donor, 1-propanamine-3-hydroxy-2-nitroso-1-propylhydrazino (PAPA NONOate). These results indicate that the phenylethanoids have NO radical-scavenging activity, which possibly contributes to their anti-inflammatory effects.

Keywords: Phenylethanoid; Macrophage; Nitric oxide (NO); Nitric oxide (NO) synthase; Inducible; 1-propanamine-3-hydroxy-2-nitroso-1-propylhydrazino (PAPA NONOate)

1. Introduction

During inflammatory reactions, the nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) in cells, such as macrophages, hepatocytes and renal cells, under the stimulation of lipopolysaccharide, tumor necrosis factor (TNF)-α, interleukin-1 or interferon-γ, acts as a defense and regulatory molecule with homeostatic activities (Kuo and Schroeder, 1995). However, NO is also pathogenic when excessively produced. NO per se, as a reactive radical, directly damages functional normal tissues. It can also react with superoxide anion radical to form the even stronger oxidant peroxynitrite ($ONOO^-$) (DeRojas-Walker, 1995; Szabó et al., 1996). iNOS expression in human glomeruli correlates positively with the degree of inflammatory injury (Furusu et al., 1998). Endotoxin-induced NO initiates the inflammatory response in the liver (Hierholzer et al., 1998). Therefore, effective inhibition of NO accumulation induced by inflammatory stimuli may represent a beneficial therapeutic strategy.

Phenylethanoids are a class of polyphenolic compounds distributed in many medicinal plants. Some of them are reported to have cytostatic (Saracoglu et al., 1995), cardioactive (Pennacchio et al., 1996), hepatocyte protective (Xiong et al., 1998) effects, as well as antibacterial, anti-stress, anti-oxidative, enzyme inhibitory and immuno-suppressant properties (Jimènez and Riguera, 1994). In particular, acteoside (also called verbascoside), a representative phenylethanoid, possesses anti-inflammatory effects against arachidonic acid-induced ear edema in mouse (Murai et al., 1995) and crescentic-type anti-glomerular basement membrane nephritis in rats (Hayashi et al., 1996). We recently found that acteoside had an anti-inflammatory effect against D-galactosamine/lipopolysaccharide-induced hepatitis in mice (Xiong et al., 1999). Although the exact amount or cellular environment needed for NO to exert

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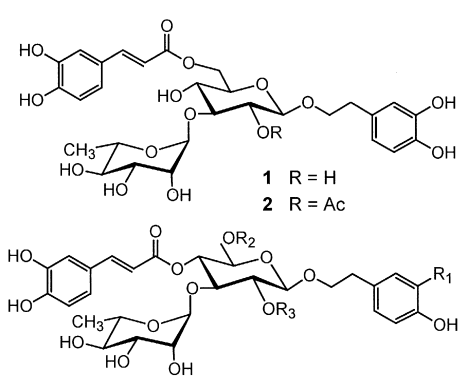
cytoprotective or cytotoxic effects is obscure in some cases, NO has been implicated in the signal transduction of the pathophysiology of immunoglobulin E-mediated allergic cutaneous reaction (Musoh et al., 1998), nephritis (Hortelano et al., 1997; Furusu et al., 1998) and hepatitis (Gardner et al., 1998; Takayama et al, 1999). Thus, study of the interaction of phenylethanoids with NO should be helpful for understanding the mechanisms underlying their anti-inflammatory actions.

In the present study, we investigated the effect of seven phenylethanoids isolated from *Cistanche deserticola* on NO production in lipopolysaccharide-stimulated murine J774.1 cells, as well as in lipopolysaccharide/interferon-γ-stimulated mouse peritoneal exudate macrophages, and the scavenging effect on NO radical released by an NO donor, 1-propanamine-3-hydroxy-2-nitroso-1-propylhydrazino (PAPA NONOate). The phenylethanoids have been proven to scavenge NO radicals without inhibiting iNOS mRNA expression, iNOS protein level, or iNOS activity.

2. Materials and methods

2.1. Reagents

Phenylethanoids (**1**–**7**, Fig. 1) were isolated from the stems of *C. deserticola*, with high purity as evidenced by NMR analysis (Xiong et al., 1996). Lipopolysaccharide (*Escherichia coli* 055:B5), pepstatin A, (*p*-amidinphenyl)methanesulfonylfluoride (APMSF), calmodulin, (6*R*)-5,6,7,8-tetrahydro-l-biopterin dihydrochloride (BH_4), bovine serum albumin and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Heat-inactivated fetal calf serum and trypsin were from GibcoBRL (Grand Island, NY, USA) and interferon-γ was from Genzyme (Cambridge, MA, USA). N^G-Monomethyl-L-arginine citrate was from Funakoshi (Tokyo, Japan). Phosphate-buffered saline (PBS) and RPMI 1640 medium were purchased from Nissui Pharmaceutical (Tokyo, Japan). NADPH, 2-mercaptoethanol, FAD, (±)-dithiothreitol, polyacrylamide, agarose, marker-4 (φX174/Hae III digest, 72–1353 bp) and thioglycollate medium I were from Wako (Osaka, Japan). The PVDF membrane for protein blotting and Bradford protein dye reagent were purchased from Bio-Rad Laboratories (Hercules, CA, USA). PAPA NONOate was from Cayman Chemical Company (Ann Arbor, MI, USA). An iNOS-specific antibody (rabbit polyclonal IgG) was from Upstate Biotechnology (Lake Saranac, NY, USA) and peroxidase-conjugated swine anti-rabbit immunoglobulins were from DAKO (DAKO A/S, Denmark). L-[*U*-^{14}C]arginine monohydrochloride (278 mCi/mmol) was obtained from Amersham (Buckinghamshire, England) and Dowex 50W-X4 (H^+ form, 200–400 mesh) was obtained from Muromachi Kagaku Kogyo (Tokyo, Japan). RNAzol B was from TEL-TEST, USA. For the reverse transcription-polymerase chain reaction (RT-PCR), the 1st strand RT buffer, and RNAse H-reverse transcriptase were from GibcoBRL. PCR buffer, dNTP mix and Taq DNA polymerase were from Takara Shuzo (Kyoto, Japan). Other reagents were of the highest grade available.



	R_1	R_2	R_3
3	OH	H	H
4	OH	H	Ac
5	OH	glc	H
6	OMe	glc	H
7	OH	glc	Ac

Fig. 1. Chemical structures of phenylethanoids (**1**–**7**).

2.2. Animals

Male 6-week-old ICR mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). The animals were housed in standard plastic cages in an air-conditioned room at 24°C and were given a commercial diet (CE-2, Kureha, Tokyo, Japan) and water ad libitum. The experiments were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University.

2.3. Stimulation of J774.1 macrophages by lipopolysaccharide

The cell line J774.1 was obtained from Riken Cell Bank, Tsukuba, Japan. The cells were propagated in 75-cm^2 plastic culture flasks (Falcone, Becton Dickinson, NJ, USA), containing RPMI-1640 medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% fetal calf serum. Confluent cells were harvested with trypsin and diluted to a suspension of 5×10^5 cells/ml in fresh medium. The cells were seeded in 24-well plastic plates with 5×10^5 cells/well and allowed to adhere for 2 h at 37°C in a humidified atmosphere containing 5% CO_2. Then the medium was replaced with fresh

medium, containing lipopolysaccharide (0.1 μg/ml) and test compounds at the indicated concentrations, and the cells were incubated for 24 h.

2.4. Stimulation of mouse peritoneal exudate macrophages by lipopolysaccharide and interferon-γ

Mouse peritoneal exudate macrophages were obtained from mice by lavage with 8.0 ml of cold RPMI-1640 per mouse, 4 days after i.p. injection of 2.0 ml of 3.0% thioglycollate (Lin and Lin, 1997). Cells were seeded in 96-well cluster plates at a density of 5×10^5 cells/ml (7.5×10^4 cells/well). Two hours later, the non-adherent cells were removed by washing with PBS and the remaining cells were incubated with medium containing various concentrations of test compounds and/or lipopolysaccharide (0.1 μg/ml)/interferon-γ (100 U/ml).

2.5. Determination of NO production in macrophages

NO production was determined by measuring the accumulation of nitrite, a stable end-product (Lin and Lin, 1997), in the culture supernatant. Equal volumes of culture supernatant and Griess reagent (0.5% sulfanilamide and 0.05% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) were mixed and left for 10 min. Absorbance was read at 560 nm and nitrite concentration in the medium was determined with sodium nitrite as standard.

2.6. Determination of cell viability

Cell viability was assessed by the mitochondrial respiration-dependent MTT reduction method (Szabó et al., 1996). After sampling of supernatant for the NO assay, 100 μl of fresh medium containing 0.5 mg/ml of MTT was added to each well and incubated for 2 h at 37°C. Then the medium was removed and the violet crystals of formazan in viable cells were dissolved in dimethyl sulfoxide. The absorbance of each well was then read at 540 nm using HTS-7000 Bio Assay Reader (Perkin Elmer, Norwalk, CT, USA).

2.7. RT-PCR analysis of iNOS mRNA

The J774.1 macrophages (5×10^6 cells/58.1 cm^2 culture dish) were cultured with 0.1 μg/ml lipopolysaccharide and 200 μM test compounds for 24 h. Total RNA was extracted from the cell pellet with RNAzol B, using the acid guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). The RT reaction mixture (10 μl), containing 2 μg of total RNA, 2 μl of RT buffer (5×), 0.5 mM dNTP mix, 10 mM dithiothreitol, 5 μM oligo-dT primer, 0.02 U of RNAse inhibitor and 1000 U of RNAse H-reverse transcriptase, was incubated at 42°C for 45 min. The reaction was stopped by heating at 70°C for 10 min. RT-generated cDNA encoding iNOS and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (as an internal standard) genes was amplified using PCR. The iNOS primer (sense 5′-CAACCAGTATTATGGCTCCT-3′, antisense 5′-GTGACAGCCCGGTCTTTCCA-3′) and GAPDH primer (sense 5′-TCCCTCAAGATTGTCAGCAA-3′, antisense 5′-AGATCCACAACGGATACATT-3′) were purchased from Nissinseihun (Kanagawa, Japan). The 50-μl reaction mixture contained 5 μl of PCR buffer (10×), 0.2 mM dNTP mix, 0.5 μl each of iNOS and GAPDH cDNA primers, 0.25 U of recombinant Taq DNA polymerase and 1 μl of RT product. The reaction was performed for 27 cycles (1 min denaturation at 94°C, 1 min annealing at 58°C and 2 min extension at 72°C) on a programmable thermal controller (MJ Research, Watertown, MA, USA). The PCR products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. The luminescence intensity of DNA bands was measured with a densitometer with Macintosh analysis software (ATTO, Tokyo, Japan).

2.8. Western blotting of iNOS protein

The iNOS protein level was analyzed by western blotting. The J774.1 macrophages (5×10^6 cells) were incubated for 24 h with 200 μM of test compounds in the presence of 0.1 μg/ml lipopolysaccharide. The cells were rinsed with PBS, scraped into 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 5 μM leupeptin, 2 μM pepstatin and 20 μM APMSF. The cell suspension was sonicated on ice and centrifuged for 20 min at $10\,000 \times g$ to obtain the supernatant. Aliquots of supernatants were mixed with an equal volume of 50 mM Tris–HCl buffer (pH 6.8) containing 4% SDS, 12% glycerol and 2% 2-mercaptoethanol, and denatured at 95°C for 3 min. For each sample, equal amounts of total cell protein were electrophoresed on 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane. Nonspecific antibody binding was blocked by incubating the membrane for 1 h with 3% bovine serum albumin, containing 0.05% Tween 20. Then, the membrane was incubated for 1 h with a 1:1000 dilution of the iNOS-specific antibody followed by a 1.5-h incubation with a 1:2000 diluted horseradish-peroxidase-conjugated anti-rabbit secondary antibody. iNOS was visualized on the blots using a chemiluminescence-based detection kit and X-ray film (ECL Western Blotting kit, Amersham Life Sciences). The band density was analyzed with Macintosh software (ATTO). Protein content in the supernatant of cell homogenates was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

2.9. Assay of iNOS activity

J774.1 cells (10^7 cells/dish, total 15 dishes) were homogenized in a glass tube on ice with 1 ml of 50 mM

Tris–HCl buffer (pH 7.4), containing 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 5 μM leupeptin, 2 μM pepstatin and 20 μM APMSF. The homogenate was centrifuged at $10\,000 \times g$ for 20 min to obtain a supernatant as the crude iNOS fraction. iNOS activity was determined by measuring the conversion of radiolabeled L-arginine to L-citrulline, as described previously (Fujiwara et al, 1996). Briefly, 10 μl of the supernatant was incubated for 10 min at 37°C in a solution comprising 50 mM HEPES, 1 mM dithiothreitol, 1 mM EGTA, 0.1 mM BH_4, 1 mM NADPH, 10 μg/ml calmodulin, 10 μM FAD, 1.55 μM L-[*U*-^{14}C]arginine, and 200 μM phenylethanoids, in a final volume of 100 μl. The reaction was terminated by the addition of 200 μl of stop solution (pH 5.2), containing 100 mM HEPES and 10 mM EDTA. The whole reaction mixture was then applied to a 0.3-ml Dowex 50W-X4 column (Na^+ form) that had been equilibrated with the stop solution. L-Citrulline was eluted with 0.5 ml of stop solution, and then, its radioactivity was determined with a liquid scintillation counter. Enzyme activity is expressed as percent inhibition of the maximum activity (taken as 100%) of the reaction mixture, in which H_2O was added instead of tested compounds.

2.10. Scavenging effect on PAPA NONOate-released NO radicals

PAPA NONOate (20 μM) was dissolved in serum-containing RPMI-1640 medium (pH 7.64), ready for cell culture or in PBS (pH 7.68), and incubated with various concentrations of test compounds at 37°C for 3 h. After incubation, the concentration of nitrite was measured with the Griess method as described above.

2.11. Statistical evaluation

Values are expressed as means ± S.D. One-way analysis of variance followed by Student–Newman–Keuls multiple comparison test was used to assess the statistical significance of differences. A *P*-value less than 0.01 was considered statistically significant.

3. Results

Stimulation of J774.1 macrophages with 0.1 μg/ml of lipopolysaccharide elicited the accumulation of 15.32 ± 0.48 μM of nitrite, the stable end-product of NO, in medium after a 24-h incubation (Table 1). This concentration of endotoxin hardly affected cell viability (92.1 ± 2.4% of untreated normal cells). The untreated normal cells had only a small amount of nitrite accumulation (3.09 ± 0.56 μM). Polymyxin B, a lipopolysaccharide inhibitor, was used as the positive control substance. It reduced nitrite accumulation to 9.64 ± 0.41 μM at 5 μg/ml. At the concentrations of 100 and 200 μM, phenylethanoids (**1**–**7**) dose dependently reduced the lipopolysaccharide-stimulated nitrite accumulation to between 7.70 ± 0.11 and 14.36 ± 0.8 μM (6.3–62.3% inhibition). Isoacteoside (**1**), tubuloside B (**2**), acteoside (**3**) and 2′-*O*-acetylacteoside (**4**) caused inhibition even at 50 μM (nitrite from 12.31 ± 0.46 to 13.81 ± 0.62 μM). The MTT reaction in **1**–**4** treated cells showed no difference in cell viability (89.5 ± 3.5 to 95.1 ± 1.6% of normal) from that of lipopolysaccharide-treated cells (Table 1), suggesting that the decrease in nitrite accumulation was not due to the cytotoxicity of the phenylethanoids. In addition, 200 μM phenylethanoids did not affect the Griess reaction when measured in cell culture medium (data not shown), excluding the possibility that the inhibition of the nitrite accumulation was due to their inhibition of the Griess reaction.

The mouse peritoneal exudate macrophages were inert to stimulation with 0.1 μg/ml of lipopolysaccharide. Instead, they were stimulated with a combination of 0.1 μg lipopolysaccharide and 100 U/ml interferon-γ to generate

Table 1
Inhibitory effects of phenylethanoids (**1**–**7**) on NO generation in lipopolysaccharide-stimulated murine J774.1 macrophages

Compounds	200 μM		100 μM		50 μM	
	Nitrite (μM)	Cell viability (%)	Nitrite (μM)	Cell viability (%)	Nitrite (μM)	Cell viability (%)
Normal			3.09 ± 0.56	100.0 ± 3.0		
Control			15.32 ± 0.48	92.1 ± 2.4		
Polymixin B (5 μg/ml)			9.64 ± 0.41*			
1	7.70 ± 0.11*	91.0 ± 2.5	12.17 ± 0.36*	92.6 ± 1.7	13.65 ± 0.45*	90.9 ± 0.3
2	8.16 ± 0.27*	95.1 ± 1.6	11.51 ± 0.92*	92.9 ± 1.8	13.66 ± 0.37*	94.8 ± 3.6
3	8.06 ± 0.51*	90.7 ± 1.7	12.11 ± 0.66*	89.5 ± 3.5	13.81 ± 0.62*	90.9 ± 3.5
4	8.00 ± 0.86*	94.6 ± 1.6	11.01 ± 0.14*	93.1 ± 2.8	12.31 ± 0.46*	94.8 ± 3.5
5	11.02 ± 0.65*		13.49 ± 0.73*		13.82 ± 0.68*	
6	12.27 ± 0.26*		14.36 ± 0.80**		15.75 ± 0.65	
7	11.57 ± 0.49*		14.16 ± 0.68**		15.63 ± 0.41	

J774.1 macrophages (5×10^5 cells/well) were incubated with lipopolysaccharide (0.1 μg/ml) and test compounds in 24-well culture plates for 24 h. Nitrite formation in medium, which reflects NO generation, was measured by the Griess reaction. Cell viability was measured by MTT method. Data are expressed as means ± S.D, $n = 4$; for normal (untreated cells) and control (cells treated with lipopolysaccharide alone), $n = 12$.

* $P < 0.001$, significantly different from control.

** $P < 0.01$, significantly different from control.

21.84 ± 1.75 μM nitrite in the culture medium (Table 2). The nitrite accumulation was decreased by the two positive control substances, polymyxin B (5 μg/ml) and dexamethasone (20 μM), to 9.03 ± 1.40 and 16.04 ± 2.79 μM, respectively. Phenylethanoids (**1**–**7**) at 200 μM reduced nitrite accumulation to between 8.63 ± 0.99 and 15.96 ± 1.17 μM (32.2–72.4% inhibition). At the concentration of 50 μM, only **2** caused significant inhibition (nitrite 15.01 ± 3.12 μM).

We further investigated whether these phenylethanoids affected iNOS gene expression. RT-PCR, using specific primers for iNOS, amplified a predicted 807 bp sequence, based on the reported sequence for iNOS cDNA of mouse macrophages (Lyonst et al., 1992). To confirm the identity of the PCR product as iNOS, it was digested with *Eco*RI to give the fragments 340, 307 and 160 bp. The 309 bp cDNA sequence of GAPDH (Fort et al., 1985) was also amplified as an internal standard for competitive PCR. As shown in Fig. 2, unstimulated J774.1 macrophages had barely detectable iNOS mRNA. In contrast, 24 h incubation with 0.1 μg/ml lipopolysaccharide induced a drastic increase in iNOS mRNA expression. In the densitometric analysis of the gel photograph, the cDNA ratio of iNOS/GAPDH was 1.6 in cells treated with lipopolysaccharide alone and 1.2–2.3 in cells treated with lipopolysaccharide plus 200 μM of **1**–**7** (Fig. 2A). At the same time, lipopolysaccharide-treated cells exhibited a clear iNOS band on western blot analysis. The proportion of the iNOS band density of phenylethanoid (in the presence of lipopolysaccharide)-treated cells to that of lipopolysaccharide-treated cells ranged between 0.9 and 1.0 (Fig. 2B). These data indicated that phenylethanoids (**1**–**7**) had no apparent inhibitory effect on the iNOS mRNA expression stimulated by lipopolysaccharide, nor did they affect the increase in iNOS protein. We then tested whether phenylethanoids affected iNOS activity. The radiolabeled arginine-to-citrulline conversion assay was performed to measure the activity of iNOS. As shown in Fig. 3, 50 μM of N^G-monomethyl-L-arginine, a positive control substance, inhibited 80.0% of the activity of iNOS prepared from lipopolysaccharide-stimulated J774.1 macrophages, while phenylethanods at 200 μM caused no inhibition of iNOS activity.

Thus, the inhibition of NO generation by these phenylethanoids was considered to be due to direct scavenging of NO radicals. This was confirmed by scavenging NO radicals released from PAPA NONOate. PAPA NONOate, a water-soluble NO/nucleophile complex, is capable of spontaneously dissociating to the free amine

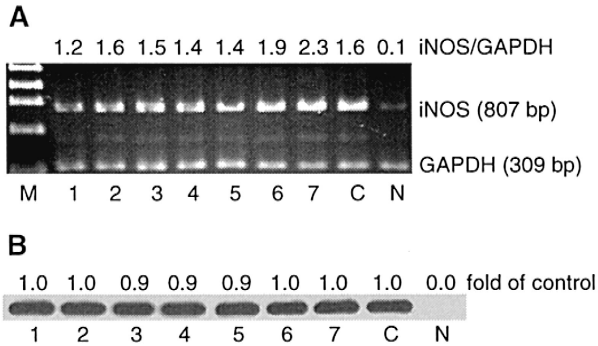


Fig. 2. RT-PCR of iNOS mRNA expression (A) and western blotting of iNOS protein (B) in J774.1 macrophages. Cells were incubated for 24 h in the presence of lipopolysaccharide (0.1 μg/ml) with or without phenylethanoids (**1**–**7**, 200 μM). Band intensity was quantified by densitometry. These experiments were repeated three times with similar results. M: 1 kbp marker, C: lipopolysaccharide-treated control cells, N: untreated normal cells.

Table 2
Inhibitory effects of phenylethanoids (**1**–**7**) on NO generation in mouse peritoneal machrophages stimulated by lipopolysaccharide/interferon-γ

Compounds	Nitrite (μM)	
	200 μM	50 μM
Normal	3.59 ± 0.22	
Control	21.84 ± 1.75	
Polymixin B (5 μg/ml)	9.03 ± 1.40 *	
Dexamethone (20 μM)	16.04 ± 2.79 *	
1	8.63 ± 0.99 *	19.54 ± 1.21
2	9.34 ± 1.94 *	15.01 ± 3.12 *
3	11.59 ± 1.56 *	19.75 ± 0.45
4	10.62 ± 2.42 *	20.06 ± 0.39
5	14.25 ± 1.52 *	21.75 ± 2.55
6	15.96 ± 1.17 *	21.81 ± 1.69
7	13.17 ± 1.98 *	21.77 ± 1.13

Mouse peritoneal exudate macrophages (7.5×10^4 cells/well) in 96-well plates were incubated with lipopolysaccharide (0.1 μg/ml)/interferon-γ (100 U/ml) and test compounds for 24 h. Nitrite accumulation in medium, which reflects NO generation, was measured by the Griess reaction. Data are expressed as means ± S.D., $n = 4$; for normal (untreated cells) and control (cells treated with lipopolysaccharide/interferon-γ alone) $n = 8$.

* $P < 0.001$, significantly different from the control.

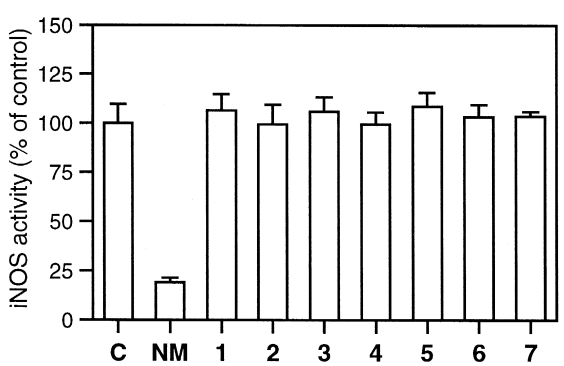


Fig. 3. Effect on iNOS activity. The iNOS activity was determined by the conversion of radiolabeled L-arginine to L-citrulline using a cytosolic preparation from 0.1 μg/ml lipopolysaccharide-stimulated J774.1 cells. Phenylethanoids (**1**–**7**) were added to the assay mixture at 200 μM. N^G-monomethyl-L-arginine (NM) was present in the assay at 50 μM. All values are expressed as percentages of the control (C, 100%: 67 ± 4 pmol L-citrulline/mg protein/min) and represent means ± S.D. of three independent determinations.

Table 3
Effects of phenylethanoids (**1**–**7**) on PAPA NONOate-released NO radical (measured in serum-containing medium)

Compounds	200 μM	100 μM	50 μM	10 μM	5 μM	2 μM
Control			22.68 ± 1.69			
1	35.72 ± 0.37 *	34.04 ± 0.60 *	31.64 ± 0.14 *	24.67 ± 0.84	22.43 ± 0.50	21.55 ± 1.39
2	37.24 ± 0.96 *	29.39 ± 0.37 *	24.75 ± 0.83	24.51 ± 0.83	22.27 ± 0.60	22.99 ± 1.45
3	37.80 ± 0.37 *	35.08 ± 0.42 *	24.91 ± 0.97	23.71 ± 1.21	22.25 ± 0.14	22.11 ± 0.24
4	36.76 ± 0.87 *	35.72 ± 1.00 *	25.07 ± 1.74	24.11 ± 0.50	22.35 ± 0.24	22.43 ± 1.39
5	37.48 ± 1.20 *	37.80 ± 3.09 *	24.19 ± 1.32	23.55 ± 0.42	23.15 ± 0.91	22.27 ± 0.84
6	26.75 ± 0.60 *	24.43 ± 1.08	23.79 ± 2.14	21.79 ± 0.69	21.55 ± 0.73	20.91 ± 0.83
7	34.76 ± 1.23 *	24.59 ± 1.60	24.67 ± 2.23	23.15 ± 2.82	22.67 ± 0.28	21.47 ± 0.91

Phenylethanoids were incubated with PAPA NONOate (in serum-containing culture medium, pH 7.64) at 37°C for 3 h. Nitrite concentration, which reflects NO generation, was measured with the Griess reagent. Data are means ± S.D. of three determinations (for control, $n = 8$). At the concentration 100–200 μM, all the phenylethanoids increased the amount of nitrite generated from PAPA NONOate. Control: 20 μM PAPA NONOate.

* $P < 0.01$, significantly different from control.

and NO radical in a pH-dependent manner following first-order kinetics (Morley and Keefer, 1993). One molecule of PAPA NONOate generates two molecules of NO. It has been reported that antioxidants, such as a thiol compound, *N*-acetylcysteine, and the phenolic compounds, resveratrol and quercetin, when tested in serum-containing cell culture medium, enhance the production of nitrite from the NO donor, sodium nitroprusside (Wadsworth and Koop, 1999), though the mechanism is unknown. In the present study, phenylethanoids (**1**–**7**) at the concentration of 50–200 μM, dose-dependently enhanced the generation of NO (measured as nitrite) from PAPA NONOate in cell culture medium (pH 7.64, Table 3). However, when tested in serum-free PBS solution (pH 7.68), NO generation was clearly inhibited (Table 4). The nitrite generated from 20 μM PAPA NONOate in PBS solution was 38.87 ± 0.49 μM after a 3-h incubation at 37°C, attaining a steady plateau in a time-course study. Even at the concentration of 2–10 μM, phenylethanoids (**1**–**7**) significantly reduced (6.9–43.9%) nitrite generation to between 36.07 ± 0.07 and 21.82 ± 0.06 μM (Table 4). These data indicated that phenylethanoids (**1**–**7**) can directly scavenge the NO radical.

4. Discussion

In the present study, we demonstrated that seven phenylethanoids (**1**–**7**), isolated from the stems of *C. deserticola*, reduced the amount of NO radical generated by activated macrophages. This was not due to their attenuation of iNOS mRNA expression, nor due to their blockage of iNOS protein synthase or iNOS activity, but was due to their scavenging of NO radical. In both J774.1 cells and mouse peritoneal exudate macrophages, **1**, **2**, **3** and **4**, which had a disaccharide in each molecule, showed a better inhibitory potency than echinacoside (**5**), cistanoside A (**6**) and tubuloside A (**7**), which had a trisaccha-

Table 4
Scavenging effect of phenylethanoids (**1**–**7**) on PAPA NONOate-released NO radical

Compounds	Nitrite (μm)					
	200 μM	100 μM	50 μM	10 μM	5 μM	2 μM
Control	38.87 ± 0.49	38.87 ± 0.49	38.87 ± 0.49	38.87 ± 0.49	38.87 ± 0.49	38.87 ± 0.49
1	16.20 ± 0.12	16.23 ± 0.17	16.27 ± 0.09	22.95 ± 0.09	30.90 ± 0.30	36.17 ± 0.03
2	16.04 ± 0.07	16.50 ± 0.12	16.87 ± 0.12	23.42 ± 0.11	31.05 ± 0.12	36.19 ± 0.13
3	16.52 ± 0.00	16.66 ± 0.18	16.95 ± 0.23	21.84 ± 0.07	26.94 ± 0.41	33.54 ± 0.27
4	15.98 ± 0.18	16.23 ± 0.21	16.60 ± 0.15	21.82 ± 0.06	27.99 ± 0.06	33.75 ± 0.21
5	16.02 ± 0.07	16.13 ± 0.23	16.44 ± 0.09	22.05 ± 0.06	27.32 ± 0.13	32.46 ± 0.09
6	15.75 ± 0.07	16.04 ± 0.41	20.25 ± 0.18	27.51 ± 0.53	32.99 ± 0.07	36.07 ± 0.07
7	15.98 ± 0.15	16.11 ± 015	16.81 ± 0.21	23.00 ± 0.09	28.92 ± 0.14	34.00 ± 0.16
Caffeic acid	–	–	25.16 ± 0.27	31.09 ± 0.26	32.11 ± 0.24	34.49 ± 0.10

Compounds were incubated with PAPA NONOate solution (in PBS, pH 7.68) at 37°C for 3 h. Nitrite concentration, which reflects NO generation, was measured with the Griess method. Data are expressed as means ± S.D. of three determinations. For control, $n = 12$. The nitrite generated from 20 μM PAPA NONOate was detected as 26.49 ± 0.13 μM and 38.87 ± 0.49 μM, before and after a 3-h incubation, respectively. Phenylethanoids (**1**–**7**) at each of the above tested concentrations showed scavenging effect on PAPA NONOate-released NO ($P < 0.001$, significantly different from control). Control: 20 μM PAPA NONOate. –: not measured.

ride. These results suggest that an increase in the number of monosaccharide units in glycosylated sugar attenuates the scavenging activity of phenylethanoids for macrophage-generated NO radical. **6**, with one phenol group methylated, exhibited weaker activity than the other six phenylethanoids in both the cell and cell-free systems. Caffeic acid, a positive control substance with only two phenol groups, showed weaker scavenging activity than all the phenylethanoids (**1**–**7**) that have four phenol groups, on PAPA NONoate-released NO radical. These results are in accordance with the scavenging activity of phenylethanoids on superoxide anion radical and hydroxyl radical (Wang et al, 1996; Xiong et al., 1996), that is, the increased number of phenol groups in a molecule enhances its NO radical-scavenging activity.

In the cell system, 50–200 μM phenylethanoids were used to achieve statistically significant reduction in about 20 μM nitrite, while in the cell-free system, only 2–10 μM were enough to show significant reduction in about 12 μM nitrite generated from PAPA NONOate in PBS (nitrite generated from 20 μM PAPA NONOate was detected as 26.49 ± 0.13 and 38.87 ± 0.49 μM, before and after a 3-h incubation, respectively). This discrepancy may be due to the following considerations.

Under the stimulation of lipopolysaccharide, macrophages generate not only the NO radical, but also other oxygen radicals, such as the superoxide anion radical and the hydroxyl radical (DeRojas-Walker et al, 1995). Especially, the hydroxyl radical is the most reactive radical among those that attack other molecules (Barber and Harris, 1994). Phenylethanoids are known as potent scavengers of the superoxide anion radical and the hydroxyl radical (Wang et al, 1996; Xiong et al, 1996), which possibly act as competitors of the NO radical for scavenging by the tested phenylethanoids. Therefore, phenylethanoids do not have the chance to scavenge NO and, thus, high concentrations are needed to show apparent inhibition. While in the cell-free PAPA NONOate system, only NO radicals were released and were immediately scavenged by phenylethanoids. Therefore, even a low concentration (2–10 μM) of phenylethanoids can exert apparent scavenging activity on PAPA NONOate-generated NO radicals. Moreover, phenylethanoids (**1**–**7**) enhanced the Griess reaction when incubated with the NO donor PAPA NONOate in serum-containing medium. Although the mechanism has not been clarified, a similar behavior possibly exists in cell culture, which blunts the inhibitory effects of phenylethanoids on nitrite accumulation in activated macrophages.

Inoue et al. (1998) reported that acteoside at 1–1000 ng/ml (about 0.0016–1.6 μM) possesses a lipopolysaccharide-like action to induce the production of TNF-α, interleukin-1 and interleukin-6 in interferon-γ-pretreated J774.1 macrophages. It is known that lipopolysaccharide-activated macrophages simultaneously produce NO and inflammatory cytokines, such as TNF-α, interleukin-1, and interleukin-6 (Wadsworth and Koop, 1999). The cytokines in turn accelerate macrophages to produce NO (Hortelano et al., 1997). Therefore, NO also may be produced and, thus, high concentrations of acteoside and its analogous phenylethanoids are needed to cause apparent inhibition of nitrite formation. Phenylethanoids (**1**–**7**) at 200 μM did not enhance either TNF-α induction (data not shown) or iNOS mRNA expression induced by lipopolysaccharide (Fig. 2), but significantly inhibited nitrite formation in the medium. These data suggest that phenylethanoids have a biphasic behavior in their interaction with macrophages, depending on their concentrations and cellular environment.

The onset of the cascade of NO production induced by lipopolysaccharide or cytokines requires a number of steps including the activation of nuclear factor (NF)-κB and subsequent iNOS mRNA expression. NF-κB activation can be inhibited by various antioxidants, such as curcumin (Kang et al, 1999), (−)-epigallocatechin-3-gallate (Lin and Lin, 1997) and quercetin (Wadsworth and Koop, 1999). As the migration of NF-κB to the nucleus and binding to the iNOS promoter located upstream of the transcription of iNOS mRNA (Lin and Lin, 1997), and phenylethanoids (**1**–**7**) had no apparent inhibitory effect on iNOS mRNA expression, it is unlikely that these phenylethanoids affect NF-κB activation. Therefore, unlike those antioxidants that inhibit NF-κB activation and the following events, such as the expression of iNOS mRNA and the increase of iNOS protein, phenylethanoids appear to be a unique class of natural antioxidants, which at high concentrations can specifically scavenge NO radicals induced by inflammatory stimuli, without inhibiting NF-κB activation, iNOS mRNA expression, iNOS protein level or iNOS activity. The interaction of phenylethanoids with NO in activated macrophages possibly plays a role in their anti-inflammatory effects against arachidonic acid-induced ear edema in mouse (Murai et al., 1995), crescentic-type anti-glomerular basement membrane nephritis in rats (Hayashi et al., 1996) and D-galactosamine/lipopolysaccharide-induced hepatitis in mice (Xiong et al., 1999), in which the signal transduction of NO is involved (Musoh et al., 1998; Hortelano et al., 1997; Furusu et al., 1998; Gardner et al., 1998).

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