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Structural Features of Polyphenolic Compounds in Their NO Inhibitory Activities

Byung Hun Kim[1], Yong Gyu Lee[1], Tae-Woong Kim[2], and Jae Youl Cho[1],*

[1]*School of Bioscience and Biotechnology, and Institute of Bioscience and Biotechnology,*
[2]*Deprtment of Biochemistry, Kangwon National University, Chuncheon 200-701, Republic of Korea*

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Abstract − Polyphenolic compounds are reported to have various pharmacological activities such as anti-oxidative, anti-cancerous, anti-inflammatory and anti-aging effects. Although numerous papers explore their functional roles in many different cellular actions, not many studies handle their structural features in anti-inflammatory responses. In this study, therefore, we examined structural role of substituted *trans*-stilbenes in their NO inhibitory and NF-κB suppressive activities. Of 10 compounds tested, 4 compounds (cinnamic acid, resveratrol, piceatannol and curcumin) displayed NO inhibitory activities in a dose-dependent manner. Similarly, these compounds blocked LPS-induced cytotoxicity of RAW264.7 cells. All NO inhibitory compounds also inhibited IκBα phosphorylation, a hallmark for NF-κB activation. However, these inhibitory compounds exhibited distinct suppressive pattern in tumor necrosis factor (TNF)-α- or phorbol-12-myristate-13-acetate (PMA)-induced NF-κB and AP-1 activation. According to structure-activity relationship study, polarity and size of ring B seem to be important for diminishing NO production. Therefore, our data suggest that substituted *trans*-stilbenes can be developed as novel anti-inflammatory drug or further developed as lead compounds for another improvement.

Keywords: Polyphenolic compounds, Substituted *trans*-stilbenes, NO production, NF-κB, Structure-activity relationship study

INTRODUCTION

Chronic (or acute) inflammation is a complicate process which is managed by various immune cells. Macrophages in particular play an important role in mediating pathological events upon inflammation states through over-production of inflammatory mediators [nitric oxide (NO), prostaglandin E_2 (PGE_2) and some cytokines such as TNF-α]. Interaction between surface receptors [pattern recognition receptor (PPR)] such as toll-like receptor (TLR)-4 or TLR-2 and their ligands derived from bacterial products such as lipopolysaccharide (LPS) are very critical initiation point for the activation of macrophages (Palaniyar *et al.,* 2002). Indeed, the molecular interaction of these molecules upregulates intracellular signaling machinery including phosphoinositide-3-kinase (PI3K) and Akt (protein kinase B) to activate redox-sensitive transcription factors such as nuclear factor (NF)-κB or activator protein (AP)-1 (Butchar *et al.,* 2006; Yoshimura, 2006). The cellular responses of macrophages (e.g., primary cells, RAW264.7 and J774 cells) induced by LPS are now regarded as a useful *in vitro* model to screen potent anti-inflammatory drugs as well as their pharmacological mechanisms (Kobori *et al.,* 2007).

Polyphenolic compounds, characterized by the presence of phenol unit per molecule, are one of the well-known anti-oxidants generating a great deal of interest (Lull *et al.,* 2005). These are particularly enriched in green tea, cocoa and fruits (Lull *et al.,* 2005). Polyphenols are generally divided into hydrolyzable tannins and phenylpropanoids, such as stilbenoids, coumarins, lignins, flavonoids, and condensed tannins (Chen and Zhang, 2007). The main pharmacological merit of these chemicals is to protect the host from oxidative stress (Lee *et al.,* 2007), although these originally act as a defensive material for the plants from insect attack. These phytochemicals are reported to possess potent anti-oxidative property. In addition, these compounds negatively regulate numerous cel-

*Corresponding author
Tel: +82-33-250-6488 Fax: +82-33-253-6560
E-mail: jaecho@kangwon.ac.kr

lular responses such as inflammatory responses such as NO production, cytokine secretion, and PGE_2 releases from macrophages and endothelial cells by inhibiting MAPK such as p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) as well as transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1 (Kobori *et al.,* 2007). Even though numerous papers suggested anti-inflammatory role of polyphenolic compounds, so far not many studies are presented in terms of their structural features and molecular targets. In this study, therefore, we aimed to understand structural features of polyphenolic compounds in their NO inhibitory activities as well as in blockade of NF-κB.

MATERIALS AND METHODS

Materials

Compounds used in this study were generously donated from Aging Tissue Bank (Pusan National University, Busan, Korea). The purity of these compounds was more than 95% under HPLC analysis. Lipopolysaccharide (LPS, *E. coli* 0111:B4) was purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 (LY), wortmannin (Wort), parthenolide (Parth), and BAY11-7082 (BAY) were obtained from Calbiochem (La Jolla, CA). Fetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7 and HEK293 cells were purchased from the American Tissue Culture Center (Rockville, MD). Luciferase constructs containing NF-κB and AP-1 binding promoters were gifts from Prof. Chung, Hae Young (Pusan National University, Busan, Korea). All other chemicals were of Sigma grade. Phospho- or total antibodies to IκBα and β-actin were purchased from Cell Signaling (Beverly, MA).

Cell culture

RAW264.7 and HEK293 cells were maintained in RPMI1640 or DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2.

Determination of NO production

RAW 264.7 cells (1×10^6 cells/ml) were preincubated with each compound for 30 min and continuously activated with LPS (1 μg/ml) for 24 h (Lee *et al.,* 2008). Nitrite in culture supernatants was also measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples of medium for 10 min at room temperature. The OD at 570 nm (OD_{570}) was measured using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA). A standard curve of NO was made with sodium nitrite. The detection limit of the assay is 0.5 μM.

MTT assay (colorimetric assay) for measurement of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Cho *et al.,* 2004). RAW 264.7 cells (1×10^6 cells/ml) were cultured in flat bottom 96 well microtiter plates with testing compounds for 24 h. At 4 h prior to culture termination, 10 μl MTT solution (10 mg/ml in phosphate buffered-saline, pH 7.4) was added to culture in each well and cells continuously cultured until termination. The culture was stopped by addition of 15% sodium dodecyl sulfate (SDS) solved in 1.5N HCl into each well for solubilization of formazan. The optical density at 570 nm (OD_{570}) was measured by a microplate Spectramax 250 microplate reader.

Luciferase reporter gene activity assay

HEK293 cells (1×10^6 cells/ml) were transfected with 1 μg of plasmids with NF-κB-Luc or AP-1-Luc as well as β-galactosidase by using calcium phosphate method in a 12-well plate according to the manufacturer's protocol. The cells were used for experiments 48 h after transfection. Luciferase assays were performed using the Luciferase Assay System (Promega) (Lee *et al.,* 2008). Briefly, the transfected cells treated with testing compounds in the presence of PMA (20 ng/ml) or TNF-α (20 ng/ml) were lysed in the culture dishes with reporter lysis buffer. Lysates were centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge. Ten μl of the supernatant fraction were incubated with 50 μl of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent (Thermo Labsystems Oy, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

Preparation of cell lysates and Immunoblotting: RAW264.7 cells (5×10^6 cells/ml) were washed 3 times in cold PBS with 1 mM sodium orthovanadate and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzimide, and 2 mM phenylmethanesulphonylfluoride) for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 g for 10 min at 4°C and stored at −20°C until needed. Cell lysates were

then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidenedifluoride (PVDF) membrane. Membranes were blocked for 60 min in Tris-buffered saline containing 3% bovine serum albumin, 20 mM NaF, 2 mM EDTA, and 0.2% tween 20 at room temperature. The membrane was incubated for 60 min with specific primary antibody at 4°C, washed 3 times with the same buffer, and further incubated for an additional 60 min with HRP-conjugated secondary antibody. The total and phosphorylated levels of IκBα, Akt, and β-actin were visualized by ECL system (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistic analysis

The Student's *t*-test and an one-way ANOVA were used to determine the statistical significance between values of the various experimental and control groups. *p* values of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

Polyphenolic phytochemicals (Fig. 1) are of interest since their anti-oxidative effects could protect our body from numerous oxidative stress conditions (Lee *et al.,* 2007). In particular, because the oxidative stress caused inflammatory and cancer diseases, intake of these natural products is now widely recommended to elderly people (Chen and Zhang, 2007). Although many experiments allowed us to understand the pharmacological mechanism of polyphenolic compounds, exact molecular events regulated by these phytochemicals are largely unelucidated yet. In this study, we evaluated chemical feature of naturally occurring compounds in their inhibitory activities on NO production and NF-κB activation. Fig. 2 shows the NO inhibitory effects of polyphenolic compounds tested. Of compounds, curcumin displayed the highest suppressive

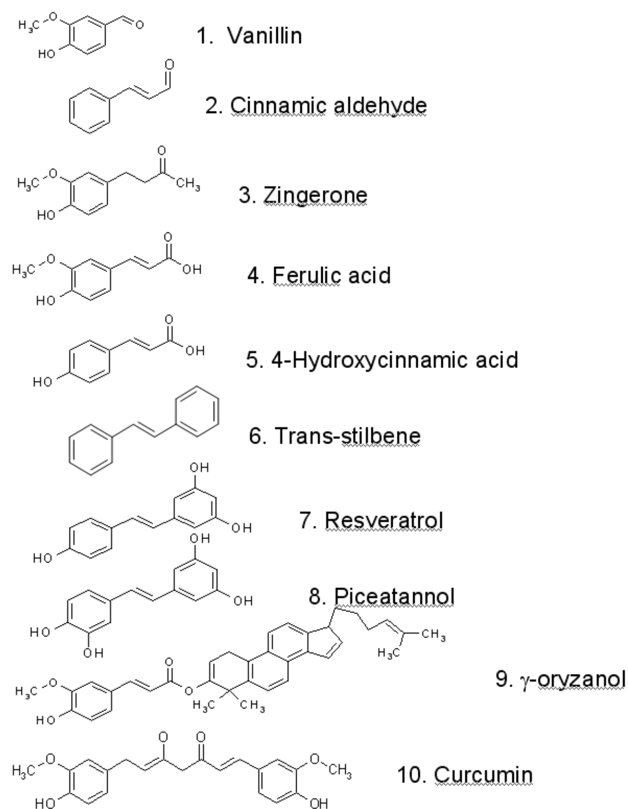
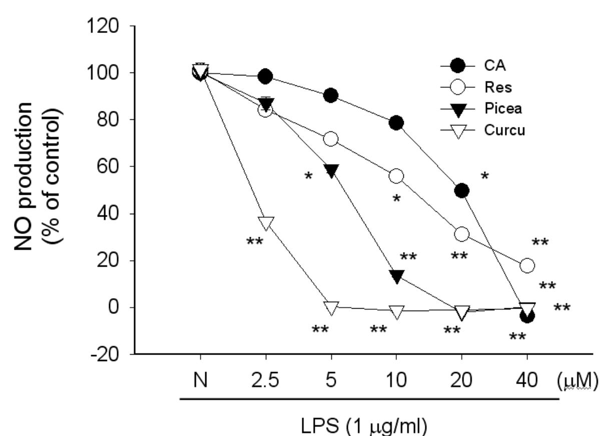


Fig. 1. Chemical structures of polyphenolic compounds tested.



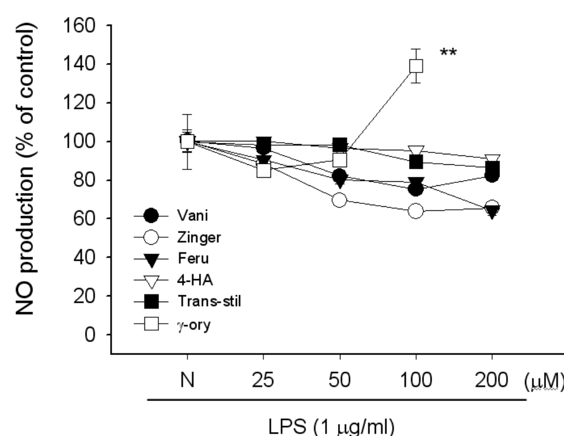


Fig. 2. The effect of polyphenolic compounds on NO production in LPS-activated RAW264.7 cells. RAW264.7 cells (1×10^6 cells/ml) were treated with polyphenolic compounds in the presence or absence of LPS (1 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. *$p<0.05$ and **$p<0.01$ compared to the control group.

activity with an IC_{50} value of 5.8 μM (Table I). In contrast, six compounds [vanillin (Vani), zingerone (Zinger), ferulic acid (Feru), trans-stilbene (Trans-stil), and γ-oryzanol (γ-ory)] never blocked NO release from LPS-activated RAW264.7 cells, while resveratrol (Res), piceatannol (Picea), and cinnamic aldehyde (CA) strongly diminished NO production in a dose-dependent manner (Fig. 2 and Table I).

Over-produced NO from macrophages in inflammatory process induces apoptosis of the cells or other neighbored cells via inhibiting JNK (Callsen and Brune, 1999). Indeed, RAW264.7 cells were killed by LPS up to 40% compared to normal level and this phenomenon was abolished by the treatment of N-monomethyl-L-arginine, an iNOS inhibitor (Fig. 3). By the reason, inhibition of NO production is related to cytoprotective activity of drugs by suppressing the apoptosis of macrophages themselves or even other cells (Lee *et al.,* 2008). Similarly, three of NO inhibitory com-

Table I. Inhibitory effects of polyphenolic compounds on NO production

Compounds	NO production [IC_{50} (μM)]
Vanillin	>200
Cinnamic aldehyde	20.1 ± 0.1
Zingerone	>200
Ferulic acid	>200
4-Hydroxycinnamic acid	>200
Trans-stilbene	>200
Resveratrol	13 ± 0.2
Piceatannol	7.2 ± 0.3
γ-Oryzanol	>100
Curcumin	5.8 ± 0.02

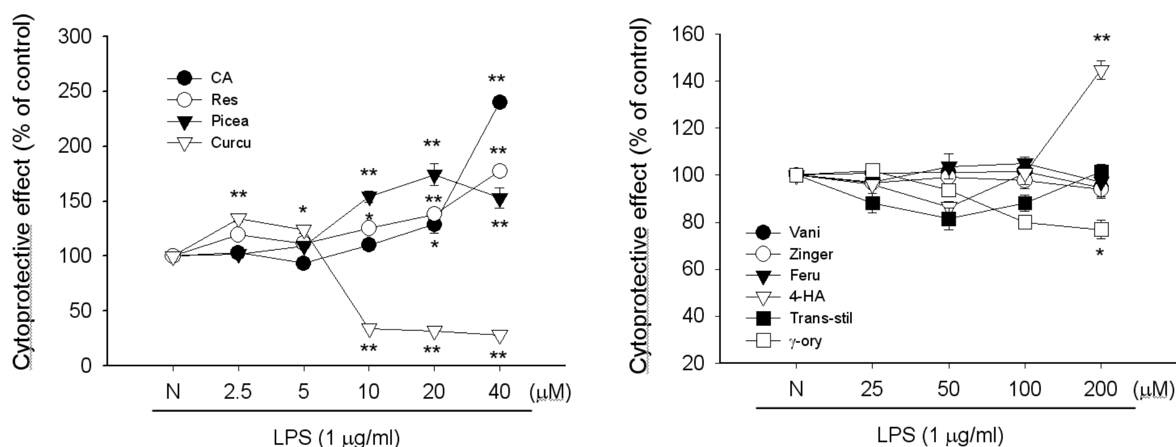


Fig. 3. The cytoprotective effect of polyphenolic compounds in LPS-activated RAW264.7 cells. RAW264.7 cells (1×10^6 cells/ml) were treated with polyphenolic compounds in the presence or absence of LPS (1 μg/ml) for 24 h. Cell viability was determined by MTT assay, as described in Materials and Methods. *$p<0.05$ and **$p<0.01$ compared to the control group.

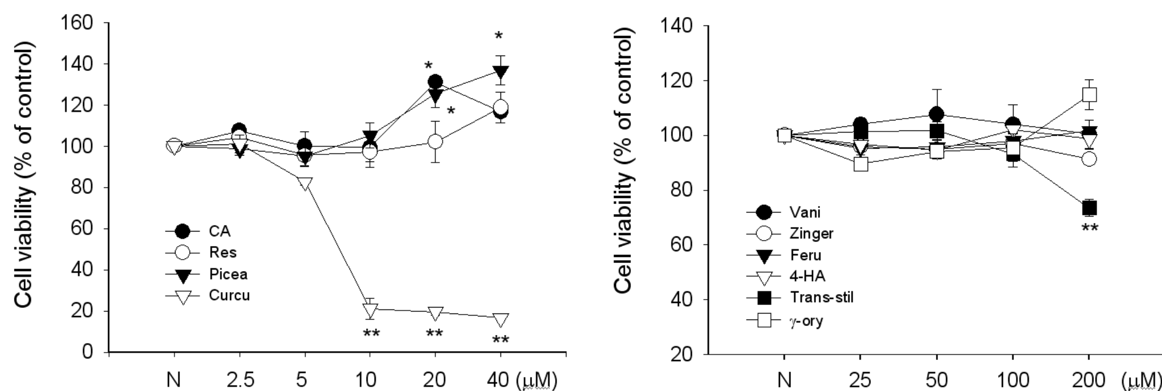


Fig. 4. The effect of polyphenolic compounds on viability of RAW264.7 cells. RAW264.7 cells (1×10^6 cells/ml) were treated with polyphenolic compounds for 24 h. Cell viability was determined by MTT assay, as described in Materials and Methods. *$p<0.05$ and **$p<0.01$ compared to the normal group.

pounds (CA, Res, and Picea) and one (4-HA) of non-inhibitory compounds displayed significant cytoprotective activity up to 2.5-fold. However, curcumin protected cell death at only lower concentrations (2.5 to 5 μM) up to 30 to 50% compared to LPS alone, due to its cytotoxic activity at higher concentrations (Fig. 4). Other NO inhibitory compounds as well as non-NO inhibitory compounds did not exhibit cytotoxic effect (Fig. 4).

These polyphenolic compounds are reported to block NF-κB and its upstream kinases such as ERK, p38 and Akt (Lee *et al.,* 2008). Therefore, we next investigated NF-κB regulatory activities of the compounds to see whether NF-κB inhibition was closely related to their inhibitory actions. To do this, we first examined regulatory role of these phenolic compounds on the phosphorylation of IκBα, a hallmark of its degradation and subsequent activation of NF-κB using LPS-treated macrophages (Doyle and O'Neill, 2006; Lee *et al.,* 2008). As Fig. 5A shows, all NO inhibitory compounds (curcumin, piceatannol, resveratrol, and cinnamic aldehyde) strongly blocked IκBα phosphorylation, while the rest did not (Fig. 6). The inhibitory compounds also down-regulated the phosphorylation of Akt (data not shown), an upstream kinase responsible for activation of IKK and IκBα pathway (Lee *et al.,* 2008), indicating that these pathways seem to be an important event in LPS-induced NO production. Indeed, inhibitors of PI3K/Akt and NF-κB displayed strong inhibitory effect on NO production from LPS-activated RAW264.7 cells (Fig. 5B).

To confirm NF-κB inhibitory activities of these compounds, we employed *in vitro* reporter gene assay with NF-κB- or AP-1-linked luciferase genes using HEK cells, displaying higher transfection efficiency, treated with PMA or TNF-α for NF-κB activation as well as PMA for AP-1. Strangely, we obtained that cinnamic aldehyde, zingerone, resveratrol and curcumin were able to block PMA-induced NF-κB activation up to 80%. In contrast, PMA-induced NF-κB- and AP-1 activation was significantly suppressed by cinnamic aldehyde and *trans*-stillbene up to 50 to 60%. We cannot exactly understand the reason why there is big difference, but these are presumably regarded as some

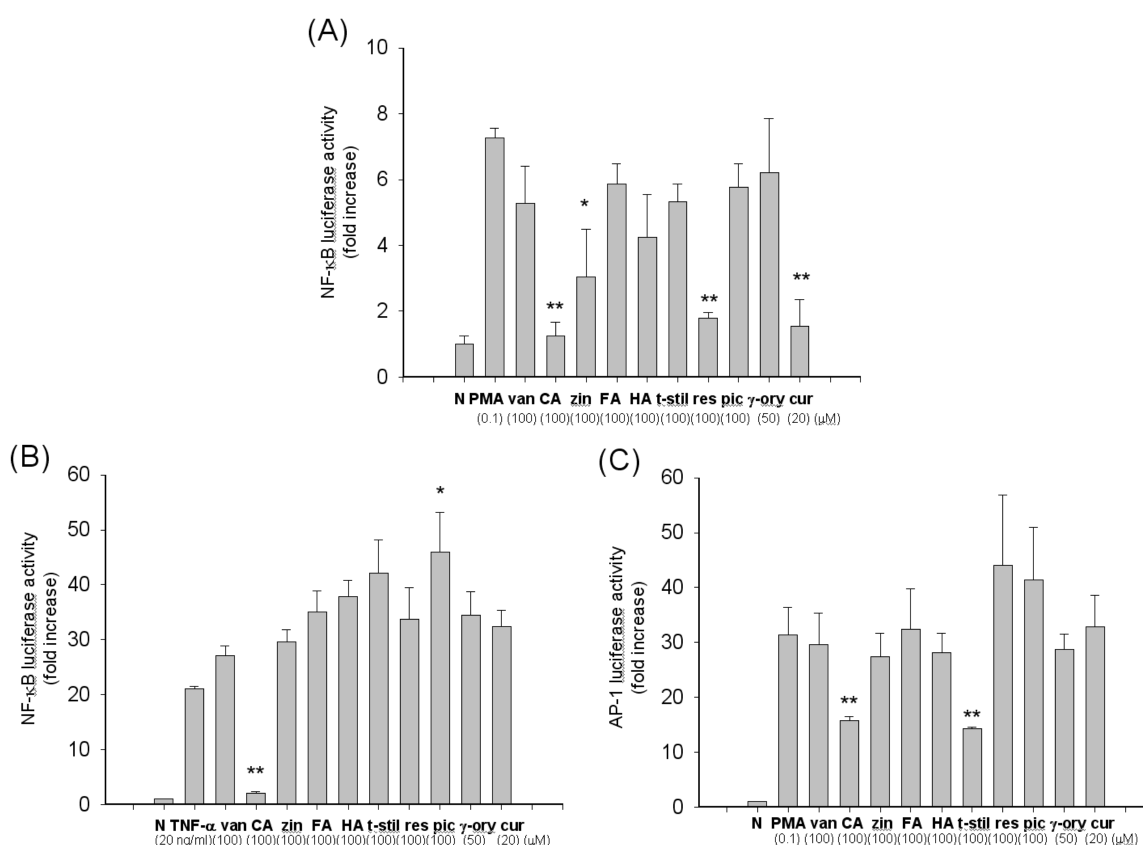


Fig. 5. The effect of polyphenolic compounds on NF-κB reporter gene assay. HEK293 cells transfected with plasmid constructs including NF-κB-Luc (1 μg/ml) or AP-1-Luc (1 μg/ml) as well as β-gal (0.5 μg/ml) were treated with polyphenolic compounds in the presence or absence of PMA (10 ng/ml) or TNF-α (15 ng/ml) for 6 h. Luciferase activity was determined by a luminometer as described in Materials and Methods . $^*p<0.05$ and $^{**}p<0.01$ compared to the control group.

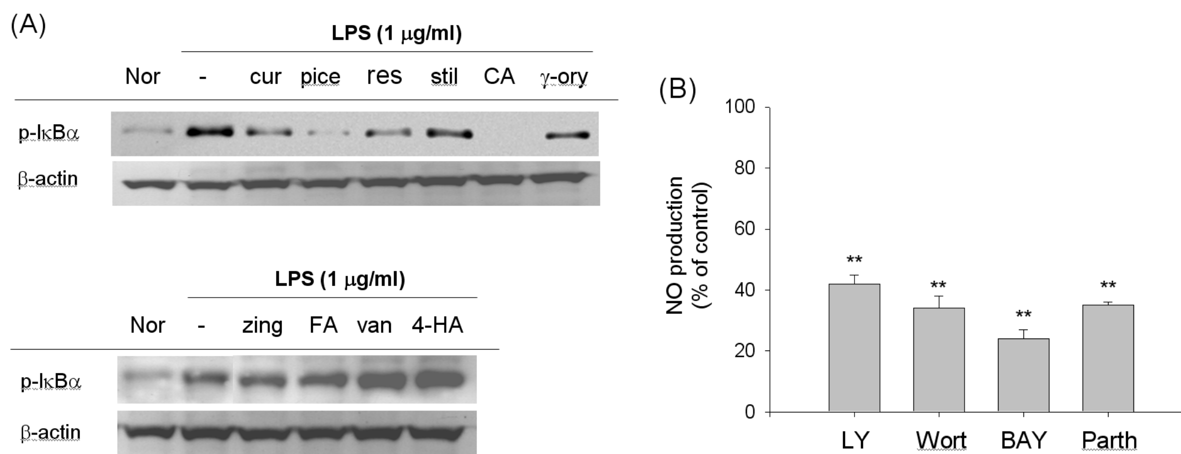


Fig. 6. The effect of polyphenolic compounds on AP-1 and NF-κB activation. (A) RAW264.7 cells (5×10^6 cells/ml) pre-treated with polyphenolic compounds were stimulated in the absence or presence of LPS (1 μg/ml) at the indicated times. After immunoblotting, the phosphorylation or total levels of IκBα and β-actin were identified by phospho-specific or total protein antibodies. The results show one experiment out of three. (B) RAW264.7 cells (1×10^6 cells/ml) were treated with various inhibitors [LY: LY294002 (25 μM), wort: wortmannin (30 μM), BAY: BAY11-7082 (10 μM) and Parth: parthenolide (10 μM)] in the presence or absence of LPS (1 μg/ml) for 24 h. Supernatants were collected and the nitrite (NO) concentration from the supernatants was determined by Griess reagent, as described in Materials and Methods. **$p<0.01$ compared to the control group.

different conditions such as stimuli and cell types (Lee *et al.,* 2008). To prove this discrepancy, further studies will be followed using macrophages or TLR4 (LPS receptor)-transfected cell lines.

Regardless, there are several considering points in inhibition of NO production by four compounds. Firstly, to suppress NO production, compound should have a benzene ring A and additional functional groups with two carbons. Secondly, methoxyl or hydroxyl groups seem not to be always necessary. This is because cinammic aldehyde maintained its inhibitory activity with IC_{50} value of 20 μM. Thirdly, large hydrophobic molecule with hydroxyl group appears to be important for enhancement of NO inhibitory activity. Indeed, resveratrol and piceatannol displayed higher inhibitory effects with IC_{50} value of 10 μM. In contrast, hydrophilic groups at ring B caused to abrogation of their suppressive activities. Thus, by the reason, structurally similar compounds such as vanillin, gingerone, and 4-hydroxycinnamic acid never blocked NO release, although these possess a functionally useful basic structure composed of ring A as well as hydroxyl or methoxyl groups. In addition, polarity and size of ring B appear to be also considering point. Curcumin exhibited the highest inhibitory activity, whereas the effect of γ-oryzanol has dramatically been abrogated. Thus, it is assumed that a drug binding space in target enzyme to interact with ring B seems to be critical for strong activity as indicated in Fig. 7. Therefore, these results suggest that polarity and size of ring B may play an important role in modulating NO production.

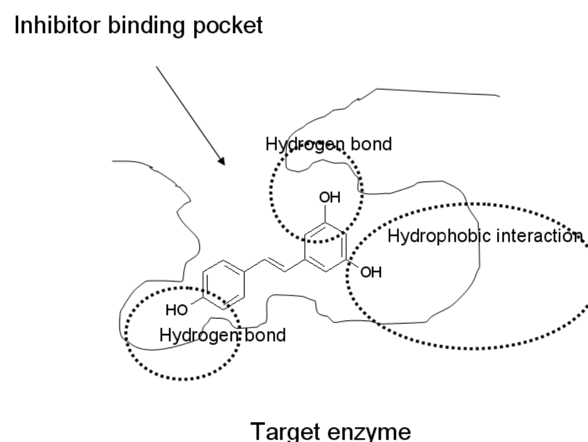


Fig. 7. Schematic draw of polyphenolic compounds on NO inhibitory pathways.

In conclusion, we found that NO inhibitory activities of stillbene analogs are dependent on its structural features. In particular, these compounds dose-dependently blocked NO production in LPS-treated RAW264.7 cells. The inhibition of NO release by some compounds seems to be due to suppression of IκBα phosphorylation. According to structure-activity relationship study, polarity and size of ring B may play an important role in modulating NO production. Therefore, our data suggest that stillbene ana-

logs can be developed as novel anti-inflammatory drug or can be a lead moiety to be further optimized for enhanced efficacy.

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