# Re-evaluation of Cyclooxygenase-2-inhibiting Activity of Vanillin and Guaiacol in Macrophages Stimulated with Lipopolysaccharide

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**Abstract.** *Phytophenols such as para-substituted 2-methoxyphenols exhibit antioxidant and anti-inflammatory activities, however, their biological activities are concentration-dependent, possibly due to their dual property of being both antioxidant and prooxidant. Eugenol (2-allyl-2-methoxyphenol) and isoeugenol(4-propenyl-2-methoxyphenol) did not reveal cyclooxygenase-2 (COX-2)-inhibiting activity in macrophages stimulated with lipopolysaccharide (LPS). In contrast, vanillin (2-hydroxy-3-methoxybenzaldehyde) and guaiacol (2-methoxyphenol), especially the former, inhibited LPS-stimulated nuclear factor kappa B (NF-κB) activation and cyclooxygenase (COX)-2 gene expression in cells of the RAW 264.7 murine macrophage cell line. Among the 2-methoxyphenols, vanillin demonstrated a potent anti-inflammatory activity. The phenolic O-H bond dissociation enthalpy (BDE) and molecular orbital energies (chemical hardness [eta], electronegativity [chi], and electrophilicity [omega]) were examined to clarify the mechanism responsible for inhibition of COX-2 expression. The BDE, chi, and omega values for vanillin were significantly higher than the corresponding values for the other 2-methoxyphenols. The anti-inflammatory activity of 2-methoxyphenols depended on the BDE and the phenol function was crucial for eliciting this activity. In addition, the anti-inflammatory activity depended on the chi and omega. These findings make vanillin attractive as a candidate therapeutic agent.*

*Abbreviations:* COX, cyclooxygenase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; IκB-α, inhibitor kappa B alpha.

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Phenols have previously been demonstrated to have a non-steroidal anti-inflammatory activity in an animal model (1). Also, phenolic compounds such as phenol, eugenol, guaiacol and vanillin have previously been reported to inhibit sheep vesicular gland prostaglandin cyclooxygenase, as indicated by their 50% inhibition concentrations, which declined in the following order: phenol (1600 μM) > vanillin (>500 μM) > guaiacol (72 μM) > eugenol (12 μM) (2). These compounds act weakly as prostaglandin cyclooxygenase inhibitors (1, 2). The antimicrobial activity of flavouring agents (vanillin, peppermint oil and a fruit flavouring) has recently been reported (3). The biological research on phenolic compounds as non-steroidal anti-inflammatory drug (NSAD)-like compounds has been greatly expanded over the last decade. We have previously shown that the 2-methoxyphenols, eugenol and isoeugenol, did not inhibit lipopolysaccharide (LPS)-stimulated cyclooxygenase-2 (COX-2) gene expression in RAW 264.7 cells (4-6). A re-examination of the effect and mechanism of action of the phenolic compounds in the light of current developments in medical biology is hence warranted, since phenolic compounds such as eugenol in zinc-oxide eugenol cements are used as endodontic therapeutic agents in dental practice.

Using a series of *p*-substituted 2-methoxyphenols, in the present study we investigated whether or not vanillin and guaiacol could inhibit LPS-stimulated COX-2 expression and nuclear factor-kappa B (NF-κB) activation in RAW 264.7 cells. NF-κB, which is activated by phosphorylation-dependent proteolysis of the inhibitor κB-α (IκB-α), is an important transcriptional factor involved in the expression of various inflammatory mediators including COX-2. The 2-methoxyphenols might also act as inhibitors of NF-κB. The mechanism of the anti-inflammatory activity of the 2-methoxyphenols is discussed on the basis of their bond dissociation enthalpy (BDE) and molecular orbital energies.

## Materials and Methods

*Reagents. o*-Vanillin was obtained from Tokyo Kasei Co., Tokyo, Japan; and guaiacol, from Wako Pure Chemical Industries, Ltd.,

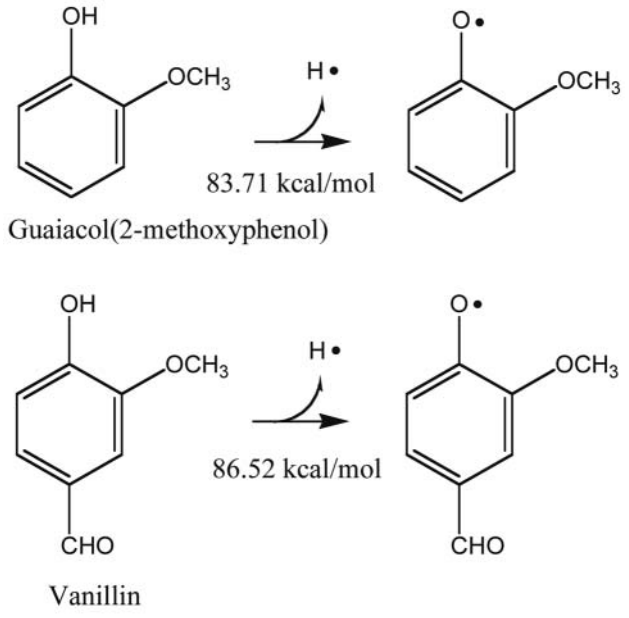


Figure 1. *Chemical structures and BDE values of vanillin and guaiacol.*

Osaka, Japan. The chemical structures of guaiacol and vanillin are shown in Figure 1. The megaprime DNA labeling systems, 5' end labeling system, 5'-[α-$^{32}$P]dCTP, and [γ-$^{32}$P]ATP were purchased from Amersham Biosciences Co. (Piscataway, NJ, USA). Phospho-specific anti-inhibitor kappa B alpha (IκB-α) antibody (recognizing phospho-Ser 32) and anti-IκB-α, both rabbit polyclonal antibodies, as well as horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and a Phototope-HRP Western blot detection kit were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). β-Actin rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and RPMI 1640, from Invitrogen Corp. (Carlsbad, CA, USA). FBS was purchased from HyClone (Logan, UT, USA). Escherichia coli O111 B4-derived LPS was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA).

*Cell culture.* Cells of the murine macrophage cell line RAW 264.7 were obtained from the Riken Cell Bank. They were cultured to the subconfluent state in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% $CO_2$ in air, washed, and then incubated overnight in serum-free RPMI 1640. They were then washed further and treated with the test samples.

*Assay for cytotoxic activity.* The cytotoxic activity was determined by an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorometric method. In brief, near-confluent RAW 264.7 cells were incubated for 48 hours with or without various concentrations of each sample. The cells were washed once with medium and incubated with 0.1 ml of fresh medium containing 0.2 mg/ml MTT and incubated for 4 hours at 37˚C. After removal of the medium, the cells were lysed with 0.1 ml of DMSO, and the absorbance at 540 nm of the lysate was determined using a microplate reader (Bichromatic Labsystem, Helsinki, Finland). The viability was defined as the ratio (percent) of the absorbance in the experimental well to that of the control well which contained 1% DMSO (no test compound).

*cDNA hybridization probe.* The COX-2 cDNA probe was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). A 25-mer β-actin oligonucleotide (single- stranded DNA) probe was purchased from GeneDetect.com Ltd. (Bradenton, FL, USA).

*Northern blot analysis.* After the cells ($10^6$ cells) in Falcon 5-cm-diameter dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) had been treated with the test samples, the total cellular RNA was extracted from them by the acid guanidine phenol chloroform (AGPC) procedure (7). As described previously (8), the RNA was electrophoresed in 1% agarose gels with 0.2 M sodium phosphate as running buffer and blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with the COX-2 cDNA probe labeled with 5'-[α-$^{32}$P]dCTP by use of the megaprime DNA labeling system or with the β-actin oligonucleotide probe labeled with [γ-$^{32}$P]ATP by use of the 5'-end labeling system. After hybridization, the membranes were washed, dried, and exposed overnight to Kodak x-ray film (Eastman Kodak Co., Rochester, NY, USA) at –70˚C. β-Actin was used as an internal standard for quantification of total RNA in each lane of the gel.

*Western blot analysis.* The cells in Falcon 5-cm-diameter dishes ($10^6$ cells per dish) were treated with the test samples. Then the cells were solubilized with lysis buffer containing 20 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 10 mM NaF, 1 mM β-glycerolphospate, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein concentrations were measured by the method of Smith *et al.* (9). Each sample (10 μg of protein) was subjected to SDS-PAGE on a 12.5% polyacrylamide gel and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA, USA). Then, the blots were blocked with 5% skimmed milk, washed and incubated with x2,000 diluted primary antibodies, (anti-IκB-α, and phospho-specific anti-IκB-α antibody) in the working solution (5% bovine serum albumin [BSA], 1x Tris buffered saline [TBS] [50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl] and 0.1% Tween 20) at 4˚C. β-Actin antibody was used at 0.1 μg/ml after dilution with the working solution. After incubation, the blots were treated with a 4,000 times-diluted HRP-conjugated secondary antibody at room temperature. Proteins were detected with a Phototope-HRP Western blot detection kit (Cell Signaling Technology, Inc.) and the blots were exposed to Kodak X-ray film for 10 min. β-Actin was used as a loading control in each lane of the gel.

*Preparation of nuclear extract and gel mobility shift assay.* The nuclei were isolated and prepared for the gel mobility shift assay as described previously (8). The cells in Falcon 15-cm-diameter dishes ($10^7$ cells per dish) were treated with the test samples. Thereafter, the cells were scraped into the phosphate-buffered saline, pelleted, and suspended in lysis buffer containing 10 mM Tris-HCl (pH7.4), 3 mM $MgCl_2$, 10 mM NaCl, 0.5% Nonidet P-40). The nuclei were separated from the cytosol by centrifugation at 3,000 xg for 15 min. The nuclei were then treated with buffer A (10 mM HEPES, [pH7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT])

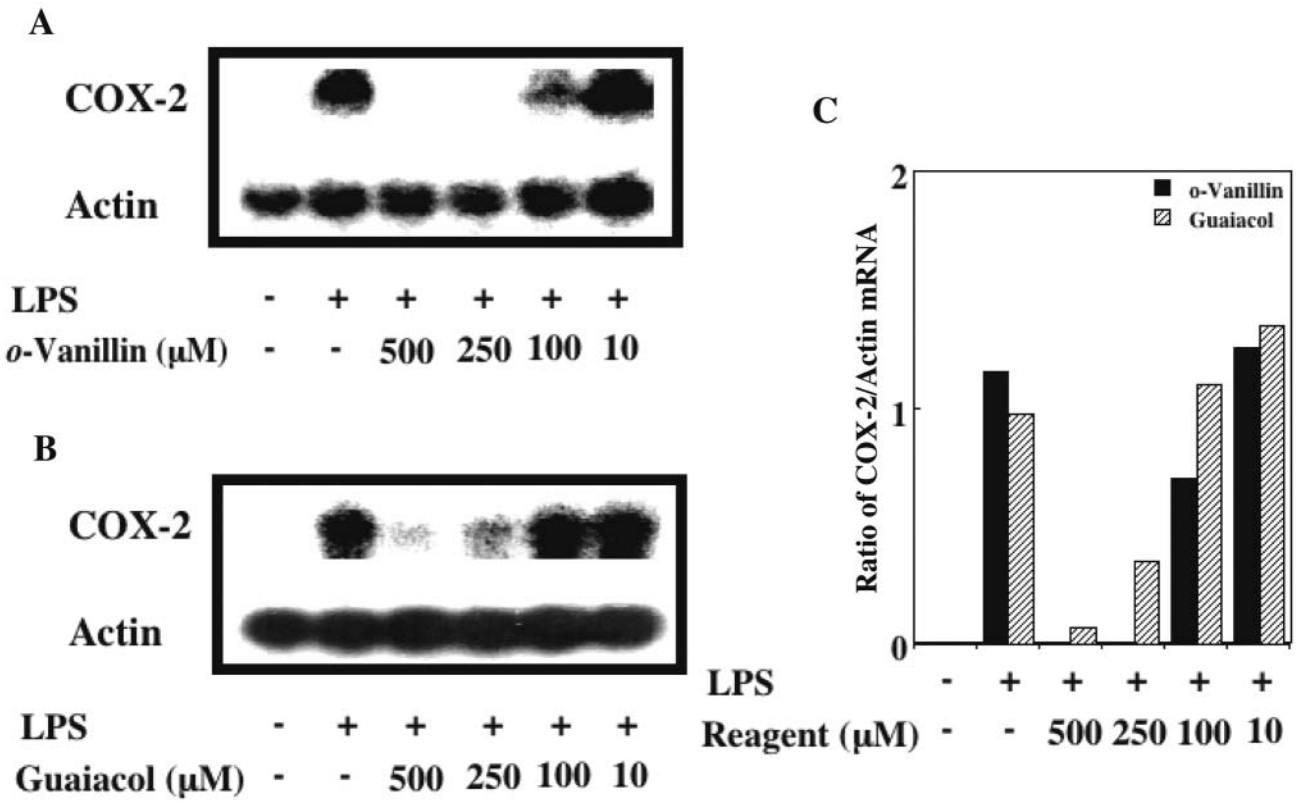


Figure 2. *Regulatory effect of vanillin and guaiacol on LPS-stimulated expression of COX-2 gene in RAW 264.7 cells. The cells were pretreated for 30 min with the indicated dosages of vanillin (A) or guaiacol (B). They were then treated with or without LPS at 100 ng/ml. Thereafter, their total RNA was prepared 3 h after the LPS addition. Northern blot analysis was performed with radio-labeled COX-2 cDNA and β-actin antisense oligonucleotide probes. The experiment was independently performed in triplicate with similar results. C) Quantification of COX-2 gene expression shown in "A" and "B" was by densitometry and the data are expressed as the ratio of COX/Actin mRNA. Bars show the mean of three independent experiments. Standard errors <15%. There was a significant difference between vanillin and guaiacol at 500 μM and 250 μM ($p<0.001$), and 100 μM ($p<0.01$) for inhibition of COX-2 gene expression.*

and further treated by stirring for 60 min at 4˚C in buffer B (20 mM HEPES, [pH7.9], 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.42 M NaCl, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Nuclear extracts were obtained by centrifugation for 60 min at 25,000xg and dehydrolyzed by passage through a Sephadex G-25 column equilibrated with buffer C [20 mM HEPES, (pH7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF]. The protein concentrations were measured by the method of Smith *et al.* (9). Binding reactions were performed for 20 min at room temperature with 10 μg of the nuclear proteins in 2 mM Tris (pH7.5) containing 8 mM NaCl, 0.2 mM EDTA, 0.8% (v/v) glycerol, 0.2 mM DTT, 0.5 mM PMSF, 1 μg of poly (dI-dC) and 20,000 cpm of a $^{32}$P-labeled NF-κB oligonucleotide in a final volume of 20 μl. Poly (dI-dC) and nuclear extract were incubated at 4˚C for 10 min before addition of the labeled oligonucleotide. The double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the binding site, -GGGGACTTTCCC-, for NF-κB was end-labeled by the T4 polynucleotide kinase-[γ-$^{32}$P] ATP method. DNA-protein complexes were electrophoresed in native 6% polyacrylamide gel in 0.25 x Tris borate EDTA (TBE) buffer [22 mM Tris-HCl (pH 8.0), 22 mM boric acid, 0.6 mM EDTA]. The gel was dried, and then exposed to Kodak X-ray film at –70˚C.

*Bond dissociation enthalpy (BDE) calculation.* The structures of vanillin and guaiacol were fully optimized by density functional theory (B3LYP level) using Spartan 04 for Windows program package (WaveFunction Inc., Irvine, CA, USA). The BDE value was expressed as kcal/mol.

## Results

The results of Northern blot assay, to examine the inhibitory activity of vanillin and guaiacol against LPS-stimulated COX-2 gene expression in the RAW 264.7 cells are shown in Figure 2. The density of the 4.1 kb band indicating LPS-induced COX-2 mRNA was clearly reduced by vanillin at the concentration of 250 μM, whereas it was only slightly inhibited by guaiacol at this concentration (Figure 2 A-C). However, when this concentration of guaiacol was doubled, the inhibition was almost complete. None of the compounds tested caused notable cytotoxicity at concentrations up to 1 mM in these experiments. These results demonstrated that vanillin was a potent inhibitor of LPS-induced COX-2 gene expression.

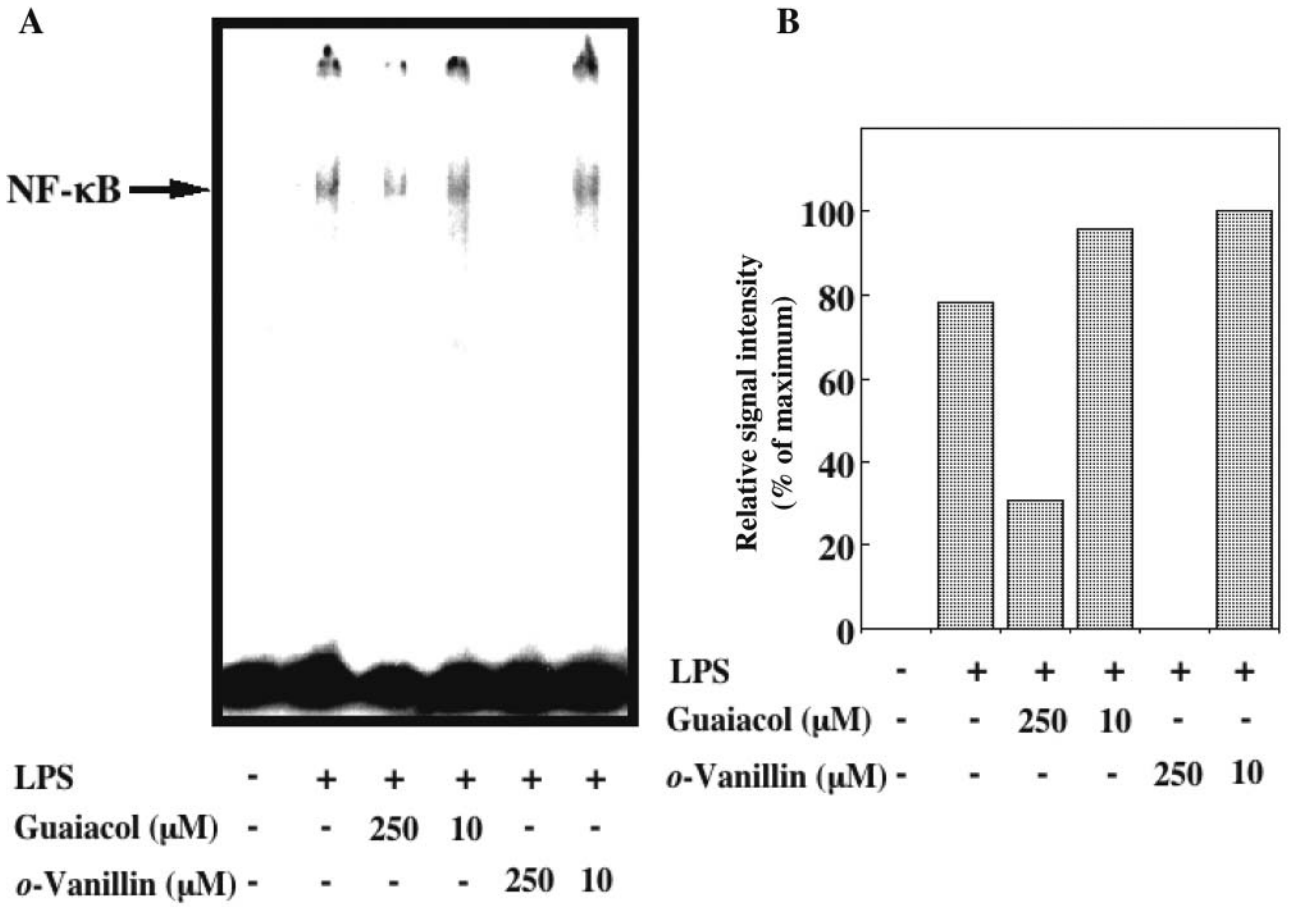


Figure 3. *Inhibit of LPS-stimulated NF-κB binding by vanillin and guaiacol in RAW 264.7 cells. A) The cells were pretreated for 30 min with o-vanillin or guaiacol at 0, 250 or 10 μM and then treated with LPS at 100 ng/ml for 1 h. A gel mobility shift assay was performed with the nuclear proteins and $^{32}$P-labeled oligonucleotide containing the NF-κB consensus sequence. The experiment was independently performed in triplicate and gave similar results. B) Quantification of the NF-κB binding in "A" by densitometry, the data are expressed as the relative signal intensity (percentage of the maximum). Bars show the mean of three independent experiments. Standard errors <15%. There was a significant difference between o-vanillin and guaiacol at 250 μM ($p<0.01$).*

As shown in Figure 3 A and B, 250 μM vanillin clearly inhibited LPS-stimulated NF-κB binding to its consensus sequence. In contrast, guaiacol only slightly inhibited it at this concentration. These results suggest that the inhibitory effects of vanillin on the LPS-stimulated NF-κB binding in RAW 264.7 cells may have been due to the suppression of IκB-α degradation, which would prevent NF-κB activity.

The results of Western blot assay to examine the effect of vanillin and guaiacol on the LPS-stimulated phosphorylation-dependent proteolysis of IκB-α in RAW cells are shown in Figure 4. LPS markedly stimulated both phosphorylation and degradation of IκB-α 30 minutes after the start of treatment, and these activities were significantly inhibited by vanillin treatment at the concentration of 100 μM, whereas neither was inhibited by the guaiacol treatment (Figure 4 A, B). Vanillin was a potent inhibitor of LPS-stimulated NF-κB. From these findings it is clear that vanillin exhibited anti-inflammatory activity.

## Discussion

Cyclooxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin (PG), and it is mainly induced in response to the many pro-inflammatory stimuli such as cytokines, growth factors, LPS and mitogens *via* activation of *cis*-acting transcription factors such as NF-κB in a variety of cells (10-14). Such factors are closely involved in inflammation and cancer (15-18). Thus, it is very important to investigate agents that selectively inhibit COX-2 expression. Compounds with COX-2-inhibiting activity possess anti-inflammatory properties. We recently reported that eugenol ortho dimer (*bis*-EUG): (3,3'-dimethoxy-5,5'-

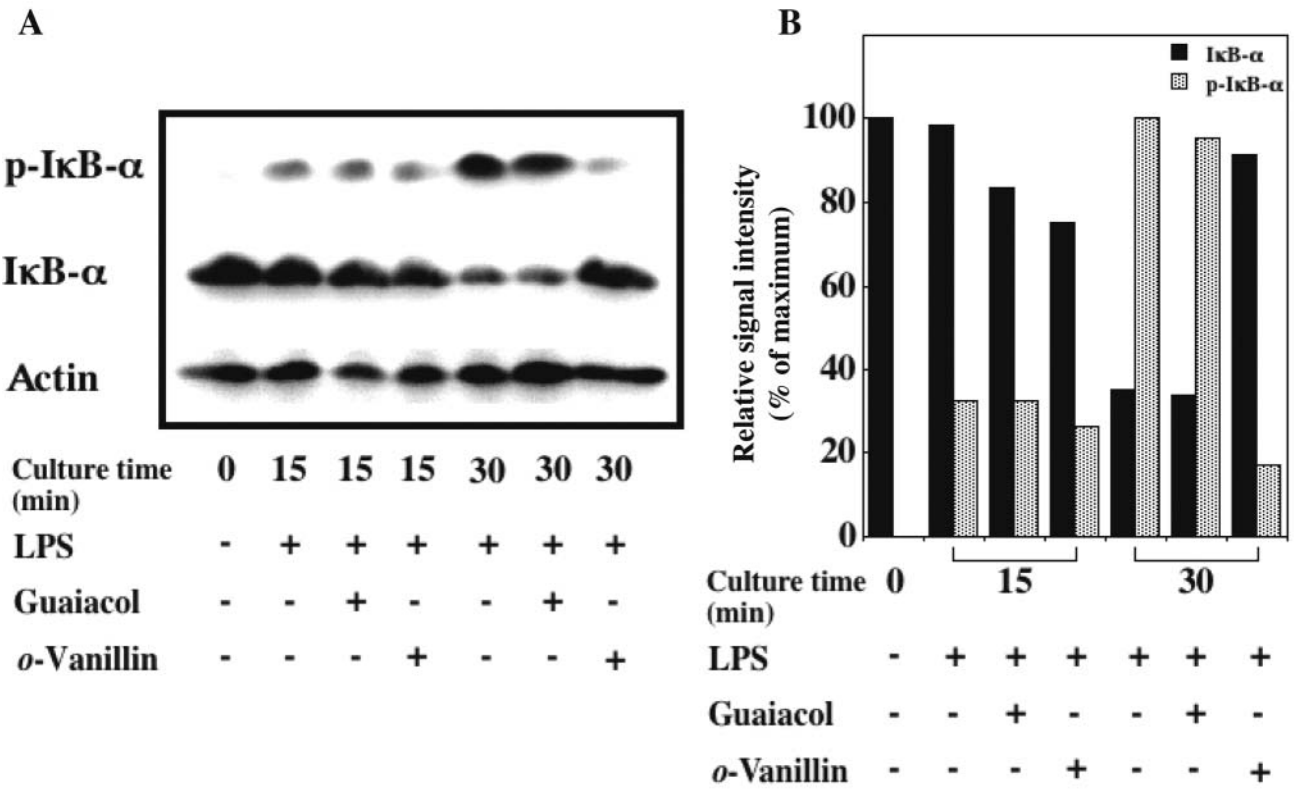


Figure 4. *Vanillin inhibition of LPS-stimulated IκB-α degradation and phosphorylation in RAW 264.7 cells. A) The cells were pretreated for 30 min with or without vanillin or guaiacol at 100 μM and then treated with or without the LPS at 100 ng/ml. Equal amounts of cell lysates were analyzed by Western blotting after SDS-PAGE with phospho-specific anti-IκB-α antibody, anti-IκB-α antibody or anti-β-actin antibody. The experiment was independently performed in triplicate with similar results. B) Quantification of expression of IκB-α and phospho-specific IκB-α shown in "A" was by densitometry, the data are expressed as the relative signal intensity (percentage of the maximum). Bars show the mean of three independent experiments. Standard errors <15%. There was a significant difference between vanillin and guaiacol at 30 min ($p<0.01$) for both IκB-α and phospho-specific IκB-α.*

di-2-propenyl-1,1'-biphenyl-2,2'-diol), and isoeugenol dimer (dehydrodiisoeugenol): (2(3-methoxy-4-hydroxyphenyl)-3-methyl-5-(1-propenyl)-7-methoxy-2,3-dihydrobenzofuran), especially the latter, both possessed potent COX-2-inhibiting activity in LPS-stimulated RAW 264.7 cells (4-6). Conversely, their parent monophenols, eugenol and isoeugenol, did not inhibit COX-2 expression in LPS-stimulated RAW 264.7 cells (4-6). The discrepancy in activity between monomer and dimer may be interpreted as being due to the dual antioxidant/prooxidant property of these compounds. In the oxidation of the monomer, but not the dimer, the superoxide-generating (prooxidant) properties could be much more prevalent than the peroxyl-radical-scavenging (antioxidant) or superoxide-scavenging (antioxidant) ones, suggesting a marked effect of substitution in the para position of the phenolic benzene ring. In p-substituted 2-methoxyphenols, the antioxidant/prooxidant activity is associated with substitutions at the para position. In order to estimate the antioxidant/prooxidant activity, the phenolic O-H BDE and anti-DPPH (2,2'-diphenyl-1-picrylhydragyl) radical activity for the 2-methoxyphenols, eugenol, isoeugenol, vanillin and guaiacol were determined. The results are summarized in Table I. The radical-scavenging activity of eugenol and isoeugenol was greater than that of vanillin, guaiacol or phenol, possibly due to their lower BDE value.

The COX-2-inhibiting activity of the 2-methoxyphenols may be related to their thermodymic properties characterized by the BDE. Vanillin, with BDE and $IC_{50}$ values higher than those of guaiacol, eugenol or isoeugenol, showed a potent COX-2 inhibiting activity, suggesting that the potent anti-inflammatory activity of vanillin may be derived from its phenol function characterized by the higher BDE value (19). We recently reported that eugenol and isoeugenol treated with horseradish peroxidase preferentially acted as prooxidants, and this activity was accompanied by a decrease in intracellular glutathione levels (20). The catalytic and prooxidative activities of

Table I. *Experimentally determined inhibition of COX-2 and anti-DPPH (2,2'-diphenyl-1-picrylhydrazyl) activity ($IC_{50}$), and calculated molecular descriptors, phenolic O-H bond dissociation energy, BDE;1-octanol-water partition coefficient, log P; chemical hardness, $\eta=(ELUMO–EHOMO)/2$; electronegativity, $\chi=–(ELUMO+EHOMO)/2$ , and electrophilicity, $\omega=\chi^2/2\eta$ for 2-methoxyphenols.*

| Compound | BDE[a], kcal/mol | $IC_{50}$[a], mM | COX-2 inhibition[a] | Log P[b] | $\eta$[b] eV | $\chi$[b] eV | $\omega$[b] eV |
|---|---|---|---|---|---|---|---|
| Eugenol | 84.00 | 0.08 | negative | 2.40 | 9.055 | 4.135 | 0.944 |
| Isoeugenol | 82.96 | 0.05 | negative | 2.45 | 8.426 | 4.207 | 1.050 |
| Vanillin | 86.52[c] | 27.40[c] | positive[c] | 1.28 | 8.650 | 4.802 | 1.333 |
| Guaiacol | 83.71[c] | 0.51[c] | partially positive[c] | 1.32 | 9.234 | 4.353 | 1.025 |
| Phenol | 87.05 | 100> | negative | 1.48 | 9.512 | 4.358 | 0.998 |

[a]from ref. (4, 5, 19), [b]from ref. (25), and [c]for the present work.

eugenol and isoeugenol have been reported previously (21). From these findings it was inferred that the COX-2-inhibiting activity of phenolic compounds may not be induced by their prooxidant state, even though these were NSAID-like compounds. A chain-breaking mechanism has been proposed to explain the anti-inflammatory action of phenolic compounds on the basis of their antioxidant properties. The anti-inflammatory activity of the phenolic compound is increased by the stability of its derived phenoxyl radical (22). However, eugenol, with a lower BDE, was not an active COX-2 inhibitor, possibly due to the catalytic and prooxidative activity demonstrated in the present study; although its derived phenoxyl radical is very stable (22). The prooxidant state of the phenolic compounds may cause cellular pro-inflammatory effects. On the other hand, phenolic compounds do not contain an ionized function at physiological pH; and, therefore, vanillin with log P=1.28 (Table I) can readily penetrate cellular lipid membranes. NSAIDs are competitive inhibitors of COX, the enzyme that mediates the biosynthesis of prostaglandins and thromboxanes from arachidonic acid. Since the COX-inhibitory potency of NSAID-like compounds mainly originates from their interactions with prostaglandin-endoperoxide H synthase-1 (PGHS-1) and prostaglandin-endoperoxide H synthase-2 (PGHS-2) (23), it is expected that the chemical hardness concept would provide important insight into their anti-inflammatory behavior (24). The antifungal activities of phenolic compounds have previously been evaluated in terms of their molecular orbital energies by using the chemical hardness concept (25). Therefore, we evaluated the COX-2- inhibiting activity of the 2-methoxyphenols on the basis of molecular properties such as chemical hardness (eta), electronegativity (chi), and electrophilicity (omega) (Table I). The chi and omega values of vanillin were noticeably greater than those of the other 2-methoxyphenols or phenol. Chi refers to the interaction with enzymes (24). Omega is the index that measures the propensity to absorb electrons (26). This index also relates to nucleophilic, electrophilic and radical attacks. The high chi and omega values for vanillin suggest that its anti-inflammatory activity may be controlled by these descriptors. The chemical hardness concept may be useful for analyzing the origin of inhibition by COX inhibitors.

COX-2 and inducible nitric oxide synthesis (iNOS) are two major inflammatory mediators (27). Recently, a physiological binding interaction between iNOS and COX-2 has been reported to be related to inflammatory responses (28), selective disruption of iNOS-COX-2 binding prevented the NO-mediated activation of COX-2, which is possibly associated with the S-nitrosylation of COX-2 by NO radicals. Drugs that block the iNOS-COX-2 interaction may be anti-inflammatory, synergizing with COX-2 inhibitors.

In conclusion, vanillin, a 2-methoxy monophenolic compound, was found to possess anti-inflammatory activity, possibly due to a phenol function. Following this hypothesis, quantitative structure-activity relationships could be the subject of a future mechanistic study.

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