

# Inhibitory Effects of *p*-Cresol and *p*-Hydroxy Anisole Dimers on Expression of the Cyclooxygenase-2 Gene and Lipopolysaccharide-stimulated Activation of Nuclear Factor- $\kappa$ B in RAW264.7 Cells

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**Abstract.** *Background/Aim:* Phenolic compounds, particularly dihydroxybiphenyl-related compounds, possess efficient anti-oxidative and anti-inflammatory activity. We investigated the anti-inflammatory activity of 2,2'-dihydroxy-5,5'-dimethylbiphenol (*p*-cresol dimer), 2,2'-dihydroxy-5,5'-dimethoxybiphenol (*p*HA dimer), *p*-cresol, *p*-hydroxyanisole (*p*HA) and 2-*t*-butyl-4-hydroxyanisole (*BHA*). *Materials and Methods:* The cytotoxicity of the investigated compounds against RAW264.7 cells was determined using a cell counting kit (CCK-8). Their inhibitory effects on cyclooxygenase-2 (*Cox2*) mRNA expression stimulated by lipopolysaccharide (*LPS*) were determined using northern blot analysis, and their inhibition of *LPS*-stimulated nuclear factor-kappa B (*Nf- $\kappa$ B*) activation was evaluated using enzyme-linked immunosorbent assay-like microwell colorimetric transcription factor activity assay. The molecular orbital energy was calculated on the basis of density function theory BLYP/6-31G\*. *Results:* The cytotoxicity of the compounds declined in the order *p*HA dimer > *p*-cresol dimer > *BHA* > *p*-cresol > *p*HA. The inhibitory effect on *Cox2* expression and *Nf- $\kappa$ B* activation was enhanced by *p*-cresol dimer and *p*HA dimer, particularly the former, suggesting potent anti-inflammatory activity, whereas *p*-cresol and *p*HA showed weak activity, and *BHA* no activity. Both *p*-cresol dimer and *p*HA dimer were highly electronegative, as determined by quantum chemical calculations. *Conclusion:*

*Dimerization of p-cresol and pHA enhances their anti-inflammatory activity. p-Cresol dimer and pHA dimer, particularly the former, are potent anti-inflammatory agents.*

Phenolic compounds possess efficient antioxidative activity and are potentially useful bioactive agents. *p*-Cresol (4-methylphenol; Figure 1) is a natural product present in many foods, crude oil, and coal tar, and also detectable in animal and human urine. In addition to its industrial uses, *p*-cresol is also used as an antiseptic and disinfectant because of its bactericidal and fungicidal properties, but there are still insufficient data to support its safety for use in cosmetics (1). *p*-Hydroxyanisole (Figure 1) is also an acceptable anti-oxidant, but is known to be carcinogenic, being structurally similar to the known forestomach carcinogen, *BHA* (2-*t*-butyl-4-hydroxyanisole, Figure 1) (2, 3). We previously investigated the anti-oxidative activity, cytotoxicity and anti-inflammatory activity of *BHA* and 3,3'-di-*t*-butyl-5,5'-dimethoxy-1,1'-biphenyl-2,2'-diol (*bis-BHA*), a *BHA* orthodimer, and demonstrated that, unlike *BHA*, it exerted potent anti-oxidative and anti-inflammatory activity, although the molecular mechanism responsible for the biological activities of these compounds was not completely clarified (4). We also previously synthesized 2,2'-dihydroxy-5,5'-dimethylbiphenol, (*p*-cresol dimer) and 2,2'-dihydroxy-5,5'-dimethoxybiphenol (*p*HA dimer) by *ortho-ortho* coupling reactions with the parent monomers, *p*-cresol and *p*HA, respectively, and investigated their anti-oxidative activity and cytotoxicity toward the murine macrophage-like cell line, RAW264.7, demonstrating that like *BHA*, *p*-cresol dimer and *p*HA dimer possessed effective anti-oxidative activity and also acceptable cytotoxicity, although their anti-inflammatory activity was not investigated (5).

Lipopolysaccharide (*LPS*)-activated macrophage cell lines are routinely used to evaluate the anti-inflammatory activities of natural and artificial phenolic antioxidants. Activation of macrophages by bacterial *LPS* induces local inflammation,

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antibody production, and other phenomena through the secretion of numerous proinflammatory cytokines, chemokines, and inflammatory mediators, such as reactive oxygen species (ROS) including nitric oxide (NO), as well as tumoricidal activity (6). Inhibition of cyclooxygenase-2 (COX2) expression is the major parameter used for assessing the effects of anti-inflammatory drugs, as COX2 is usually specific to inflamed tissues. Nuclear factor-kappa B (Nf- $\kappa$ B), a major transcription factor, is closely related to the expression of proinflammatory genes. The most abundant activated form of Nf- $\kappa$ B is a heterodimer composed of a p50 subunit and p65 subunit, which functions predominantly as a transcriptional activator (7, 8). Also, LPS is strong inducer of CD-40 expression in macrophages, which involves the activation of the transcription factor of Nf- $\kappa$ B (9).

In the present study, we investigated the inhibitory effects of *p*-cresol, *p*-cresol dimer, pHA, pHA dimer and BHA (Figure 1) on Cox2 expression in RAW264.7 cells stimulated by exposure to LPS. Since Nf- $\kappa$ B activation requires nuclear translocation of dimerized Nf- $\kappa$ B components such as p50, p52, p65 and RelB, we examined the inhibitory effect of these phenolic compounds on the nuclear translocation of each component in the presence of LPS.

Quantum chemical calculations can provide a more detailed insight into the molecular mechanisms underlying the biological activities of phenolic antioxidants. We previously demonstrated that honokiol and magnolol inhibit *Porphyromonas gingivalis* fimbria-stimulated Cox2 expression and also inhibit fimbria-stimulated Nf- $\kappa$ B activation in RAW264.7 cells. We also found that these compounds possessed high electronegativity ( $\chi$ ), suggesting a causal link between the anti-inflammatory activity of phenolic antioxidants and their  $\chi$  value (10). These findings also suggest that quantum chemical calculations are useful for interpreting the mechanism responsible for the anti-inflammatory activity of phenolic antioxidants. Therefore, in the present study, we used the density functional theory (DFT) and B3LYP method with the 6-31G\* basis set as a computational tool. The relationships between the anti-inflammatory activity of these compounds and their molecular properties, chemical hardness ( $\eta$ ) and  $\chi$  value, are discussed.

## Materials and Methods

**Materials.** *p*-Cresol and pHA were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. *p*-Cresol dimer and pHA dimer were synthesized from their monomers by the CuCl(OH)-catalyzed *ortho* coupling reaction described previously (11, 12). BHA was purchased from Tokyo Kasei Co. (Tokyo, Japan). The chemical structures of these phenol-related compounds are shown in Figure 1. Their solutions were prepared by dissolving each of them in dimethyl sulfoxide, and they were then diluted to the required concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. A Megaprime DNA labeling system, 5'-[ $\alpha$ - $^{32}$ P]dCTP, and [ $\gamma$ - $^{32}$ P]ATP

were purchased from Amersham Biosciences Co. (Piscataway, NJ, USA). A 5'-end labeling system was purchased from Promega Co. (Madison, WI, USA). A mouse *Cox2* cDNA probe with a length of approximately 1.2 kbp was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). A 25-mer  $\beta$ -actin oligonucleotide (single-stranded DNA) probe was purchased from GeneDetect.com Ltd. (Bradenton, FL, USA). RPMI-1640 was purchased from Invitrogen Corp. Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). *Escherichia coli* O111 B4-derived LPS was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA).

**Cell culture.** The murine macrophage-like cell line RAW264.7, obtained from Dainippon Sumitomo Pharma Biomedical Co. Ltd. (Osaka, Japan), was used. The cells were cultured to a subconfluent state in RPMI-1640 medium supplemented with 10% FBS at 37°C and 5% CO<sub>2</sub> in air, washed, and then incubated overnight in serum-free RPMI-1640. They were then washed again and treated with the test samples.

**Cytotoxicity.** The relative number of viable cells was determined using a Cell Counting Kit-8 (CCK-8) (Dojindo Co., Kumamoto, Japan) (13). In brief, RAW264.7 cells (3×10<sup>4</sup> per well) were cultured in NUNC 96-well plates (flat-well-type microculture plates) for 48 h, after which the cells were incubated with test samples for 24 h. CCK-8 solution was added to each well and then the absorbance was measured at 450 nm with a microplate reader (Biochromatic, Helsinki, Finland). The 50% lethal cytotoxic concentration (LC50) was determined from the dose-response curves. Data are expressed as the means of three independent experiments. Statistical analyses were performed using the Student's *t*-test.

**Northern blot analysis and Cox2 mRNA inhibition.** The procedure employed was similar to that reported previously (14). Briefly, 10<sup>6</sup> cells were placed in Falcon 5-cm-diameter dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and pre-treated for 30 min with each of the phenol-related compounds at a concentration of 10<sup>-6</sup> M, 10<sup>-5</sup> M or 10<sup>-4</sup> M. They were then incubated in the presence or absence of LPS (100 ng/ml), and their total RNA was prepared 3 h later by the acid guanidine-phenol-chloroform procedure (15). The RNA was electrophoresed in 1% agarose gels with 0.2 M sodium phosphate as a running buffer, and then blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with a *Cox2* cDNA probe labeled with 5'-[ $\alpha$ - $^{32}$ P]dCTP using the Megaprime DNA labeling system (Amersham Biosciences Co.) and a  $\beta$ -actin oligonucleotide probe labeled with [ $\gamma$ - $^{32}$ P]ATP using a 5'-end labeling system purchased from Promega Co. After hybridization, the membranes were washed and dried, then exposed overnight to Kodak X-ray film (Carestream Health Inc., Rochester, NY, USA) at -70°C.  $\beta$ -Actin was used as an internal standard for quantification of total RNA in each lane of the gel. Quantification of *Cox2* expression was carried out by densitometry. The data were expressed as the relative signal intensity (percentage of maximum). Data are expressed as means of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Inhibition of LPS-stimulated *Cox2* mRNA expression by *p*-cresol, *p*-cresol dimer, pHA, pHA dimer and BHA in RAW264.7 cells was carried out as follows: The cells were pretreated for 30 min with or without addition of 10<sup>-5</sup> M *p*-cresol, pHA, *p*-cresol dimer, pHA dimer or BHA, and then incubated with or without LPS at 100 ng/ml for 3 h. Total RNA was prepared and *Cox2* mRNA expression was confirmed by northern blot analysis.

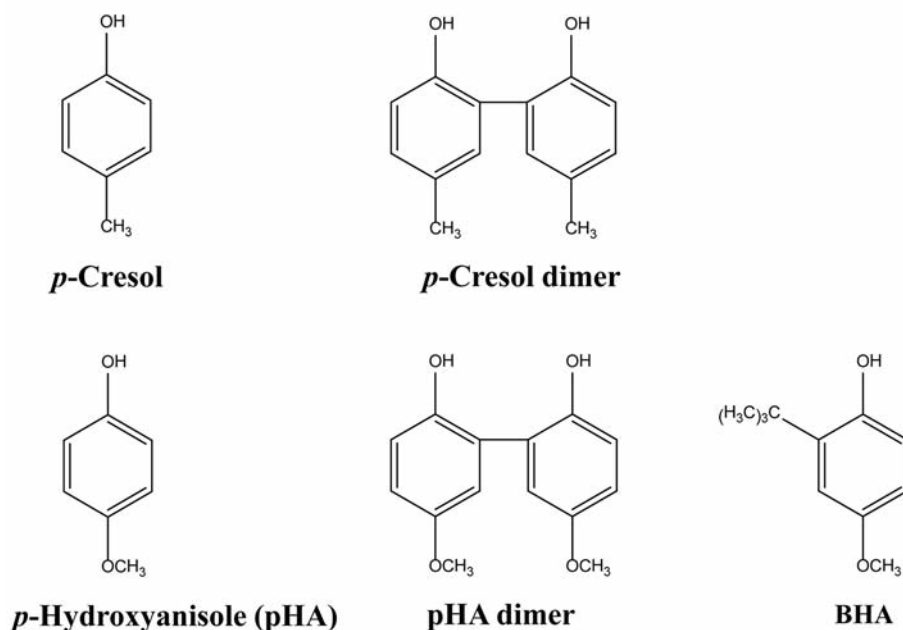


Figure 1. The chemical structures of *p*-cresol, *p*-cresol dimer, *p*-hydroxyanisole (pHA), pHA dimer and 2-*t*-butyl-4-hydroxyanisole (BHA).

**Preparation of nuclear extract and microwell colorimetric NfκB assay.** Nuclei were extracted and prepared for the microwell colorimetric NfκB assay. In brief, the cells in 5-cm-diameter Falcon dishes (10<sup>6</sup> cells per dish) were pretreated for 30 min with or without the indicated doses of the compounds, and then treated with LPS at 100 ng/ml for 1 h. Thereafter, nuclear extracts were prepared using a Nuclear Extraction kit (Active Motif Co., Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The microwell colorimetric NfκB assay was performed as described previously (16) using the Trans-AM NfκB family transcription factor assay kit (Active Motif Co.). Briefly, cell extracts were incubated in a 96-well plate coated with an oligonucleotide containing the NfκB consensus binding site (5'-GGGACTTTCC-3'). Activated transcription factors from extracts that bound specifically to each respective immobilized oligonucleotide were detected in an enzyme-linked-immuno-sorbent assay (ELISA)-like assay using antibodies against the Nf-κB p50, p52, p65 and RelB subunits, followed by a secondary antibody conjugated to horseradish-peroxidase. Optical density was measured at 450 nm with a microplate reader (Biochromatic). The specificity of the assay was validated by including both the wild-type and mutated oligonucleotides in the reaction. Raji cell nuclear extract was used as a positive control. Data are expressed as the means of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Inhibition of LPS-stimulated Nf-κB activation by *p*-cresol, *p*-cresol dimer, pHA, pHA dimer or BHA in RAW264.7 cells was carried out as follows: The cells were pretreated for 30 min with or without addition of 10<sup>-5</sup> M *p*-cresol, pHA, *p*-cresol dimer, pHA dimer or BHA, and then incubated with or without the LPS at 100 ng/ml for 1 h. Nuclear extracts were prepared and used in a TransAM (Active Motif) ELISA-like assay to quantify the NfκB p50, p52, p65 and RelB DNA-binding activity.

**Computation.** The lowest unoccupied molecular orbital (LUMO) energy (ELUMO) and highest unoccupied molecular orbital (HOMO) energy (EHOMO) were obtained from ground state equilibrium geometries with density functional theory calculated DFT B3LYP 6-31G\* in vacuum from 6-31G\* initial geometry (10, 17).

The absolute value of HOMO energy was adopted as an approximate ionization potential (IP) value according to Koopman's theorem (18). All calculations were performed with Spartan'10 (Wave Function Inc., Irvine, CA, USA).

The  $\eta$  and  $\chi$  values were calculated as follows:

$$\eta = (E_{\text{LUMO}} - E_{\text{HOMO}})/2 \quad (\text{Eq. 1})$$

$$\chi = -(E_{\text{LUMO}} + E_{\text{HOMO}})/2 \quad (\text{Eq. 2})$$

## Results

**Cytotoxicity.** The cytotoxicity of *p*-cresol, *p*-cresol dimer, pHA, pHA dimer and BHA towards RAW264.7 cells was investigated using CCK-8. The results are shown in Figure 2. The 50% lethal cytotoxic concentration (LC<sub>50</sub>) for *p*-cresol, *p*-cresol dimer, and pHA, pHA dimer and BHA was 850, 400, 1,100, 310 and 420 μM, respectively. pHA dimer showed the highest cytotoxicity, followed in order by *p*-cresol dimer and BHA. pHA exhibited the lowest cytotoxicity. The cytotoxicity of *p*-cresol dimer was double that of *p*-cresol monomer, whereas that for pHA dimer was three-times greater than for the pHA monomer.

**Cox2 inhibition.** Five compounds belonging to two representative chemical classes, *p*-cresol and the *p*-hydroxyanisole group, were tested at different concentrations

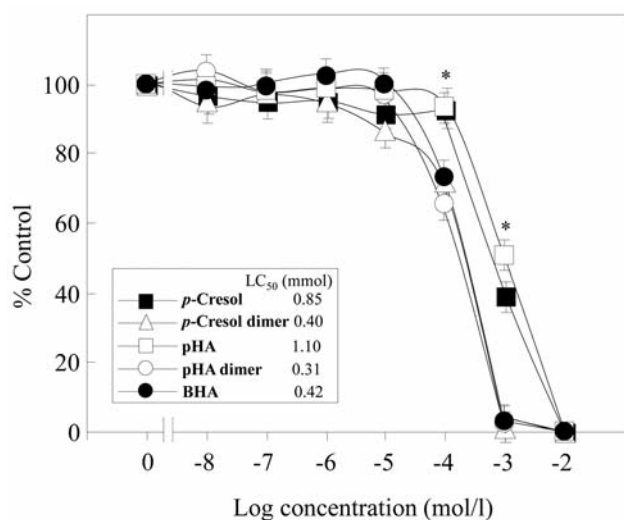


Figure 2. The cytotoxicity of *p*-cresol, *p*-cresol dimer, *p*-hydroxyanisole (*p*HA), *p*HA dimer and 2-*t*-butyl-4-hydroxyanisole (*BHA*) towards RAW264.7 cells. The procedures employed are described in Materials and Methods. The results are presented as the mean  $\pm$  standard error (SE) for three independent experiments. SE < 15%. There was a significant difference at  $10^{-4}$  M and  $10^{-3}$  M between the monomer and dimer for each of *p*-cresol and *p*HA. \* $p < 0.01$ .

up to  $10^{-4}$  M with regard to their effect on Cox2 transcriptional activity in the presence of LPS. No significant decrease in cell viability was observed in cultures with LPS and the indicated phenols at a concentration of less than  $10^{-4}$  M. The inhibitory effects of these compounds on LPS-stimulated Cox2 production in RAW264.7 cells were investigated within the concentration range of  $10^{-6}$  M to  $10^{-4}$  M. The results are shown in Figure 3. *p*-Cresol dimer and *p*HA dimer, particularly the former, dramatically inhibited the LPS-induced gene expression of *Cox2* at  $10^{-5}$  M. In contrast, the effective inhibitory activity for *p*-cresol and *p*HA was not attained at  $10^{-5}$  M, but the inhibitory activity for *p*-cresol appeared to be greater than that for *p*HA. *BHA* exerted no inhibitory effect over a wide range of concentrations. Interestingly, no inhibitory activity was observed for any of the compounds tested at the high concentration of  $10^{-4}$  M. This may have been attributable to the formation of intermediate phenolic compounds (quinones) through exposure to LPS; the intrinsic antioxidative properties of phenolic compounds may have decreased in the presence of mixtures of relatively high concentrations of phenols, radical scavengers and LPS that produce intercellular ROS. *p*-Cresol dimer and *p*HA dimer showed potent anti-inflammatory activity, whereas the activity of their monomers was weak. *BHA* had no activity.

**Inhibition of *Nf-kB* activation and calculations.** The results of inhibition of *Nf-kB* activation are shown in Figure 4, and the

Table I. Calculated quantum mechanical parameters for *p*-cresol and *p*-hydroxy anisole related compounds

Compound	LUMO e'	HOMO e'	$\eta$ e'	$\chi$ e'	IP <sub>Koopman</sub> e'
<i>p</i> -Cresol	0.0678	-5.7414	2.9046	2.8316	5.7414
<i>p</i> -Cresol dimer	-0.5204	-5.8071	2.6433	3.1637	5.8071
<i>p</i> -Hydroxy anisole ( <i>p</i> HA)	0.0064	-5.3443	2.6753	2.6689	5.3443
<i>p</i> HA dimer	-0.5792	-5.4526	2.4369	3.0159	5.4526
2- <i>t</i> -Butyl-4-hydroxy anisole ( <i>BHA</i> )	0.0903	-5.2768	2.6835	2.5932	5.2768
<i>Bis</i> - <i>BHA</i> a	-0.563	-5.365	2.402	2.964	5.365

a3,3'-Di-*t*-butyl-5,5'-dimethoxy-1,1'-biphenyl-2,2'-diol. The lowest unoccupied molecular orbital (LUMO) energy ( $E_{\text{LUMO}}$ ) and highest unoccupied molecular orbital (HOMO) energy ( $E_{\text{HOMO}}$ ) were calculated using Spartan 10 software (Wavefunction Inc. Irvine, CA, USA). Chemical hardness ( $\eta$ ) was calculated as  $\eta = (E_{\text{LUMO}} - E_{\text{HOMO}})/2$  and electronegativity ( $\chi$ ) as  $\chi = -(E_{\text{LUMO}} + E_{\text{HOMO}})/2$ . IP: Absolute  $E_{\text{HOMO}}$  value.

calculated HOMO, LUMO,  $\eta$ ,  $\chi$  and IP values for phenols are shown in Table I. As a reference compound, the calculated values of *bis*-*BHA*, a *BHA* dimer have been also added to Table I. This compound was previously reported to have potent anti-inflammatory activity (4). LPS-stimulated binding of *Nf-kB* subunits such as p50, p52, p65 and RelB to its consensus sequences was markedly inhibited by *p*-cresol dimer and also by *p*HA dimer, but was weakly inhibited by monophenols, *p*-cresol and *p*HA, but not by *BHA*, as observed using an ELISA-like microwell colorimetric transcription factor assay kit. For each of p50, p52, p65 and RelB, the LPS-stimulated binding activity for *p*-cresol dimer was significantly greater than that for *p*-cresol at  $10^{-6}$  M or  $10^{-5}$  M. Similarly, the activity for *p*HA dimer was significantly lower than that for *p*HA at  $10^{-6}$  M or  $10^{-5}$  M. The LPS-stimulated binding of *Nf-kB* was also completely inhibited by an excess amount of wild-type consensus oligonucleotide provided in the kit (data not shown). These findings indicated that *p*-cresol dimer and *p*HA dimer are potent inhibitors of LPS-triggered cellular signaling in RAW264.7 cells.

**Quantum molecular properties.** The results are shown in Table I. Molecules with a relatively large  $\eta$  value are generally unreactive, while those with a relatively small  $\eta$  value are generally reactive (17). *p*HA dimer and *bis*-*BHA* had small  $\eta$  values, possibly reflecting their bioactive nature. We previously reported a close relationship between phenol-induced cytotoxicity and  $\eta$  value for 2-methoxy phenols and 2-alkyl phenols, and found that their cytotoxicity increased as  $\eta$  decreased (19). *p*HA dimer and *p*-cresol dimer, with relatively small  $\eta$  values, had greater cytotoxicity than *p*HA or *p*-cresol. In contrast, the  $\chi$  value declined in the order *p*-cresol dimer >

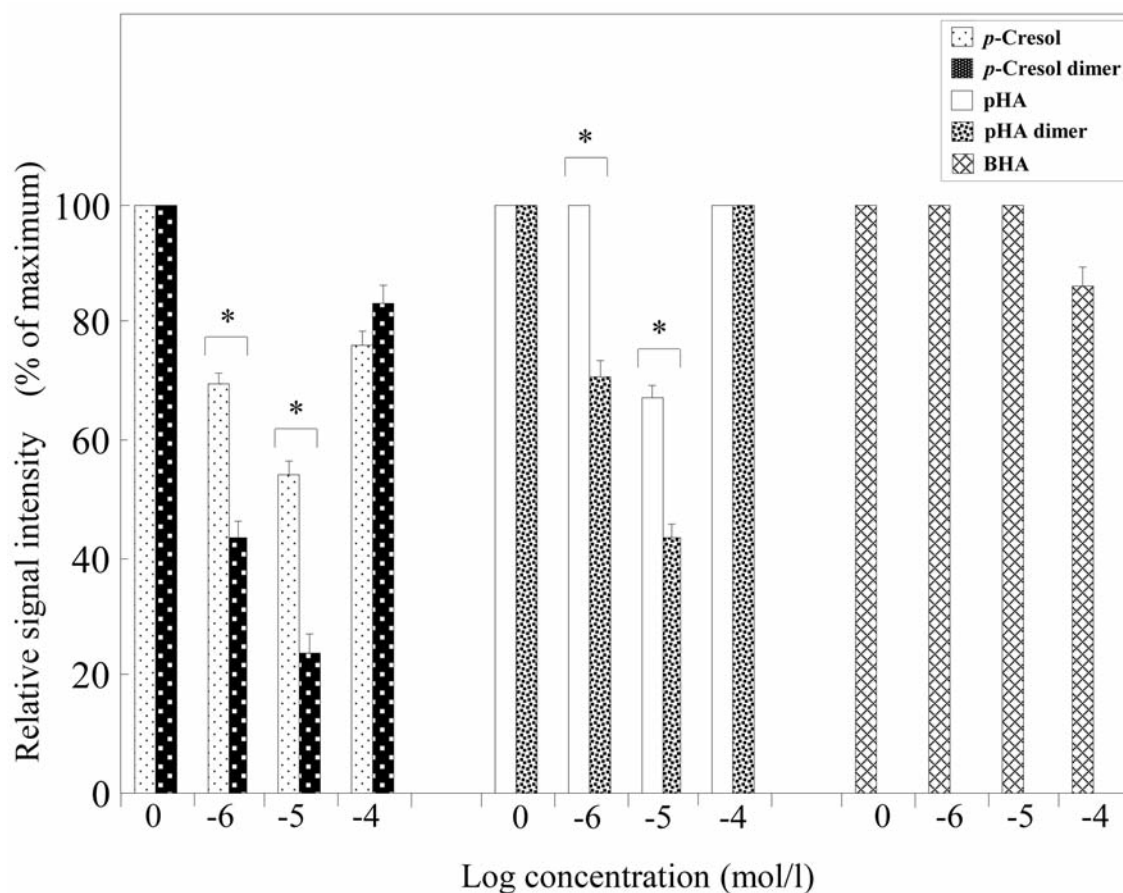


Figure 3. Inhibitory effect of *p*-cresol, *p*-cresol dimer, *p*-hydroxyanisole (*p*HA), *p*HA dimer and 2-*t*-butyl-4-hydroxyanisole (*BHA*) on lipopolysaccharide (*LPS*)-stimulated *Cox2* gene expression in RAW264.7 cells \* $p < 0.01$ . The mRNA levels for each compound were normalized to that of beta-actin and are expressed as a vertical bar graph indicating the mean  $\pm$  SE of three independent experiments. SE  $< 15\%$ . There was a significant difference at  $10^{-6}$  M and  $10^{-5}$  M between the monomer and dimer for each of *p*-cresol and *p*HA. The bar graph for controls (*LPS* alone in the absence of each compound) was defined as 100.

*p*HA dimer > *bis*-*BHA* > *p*-cresol > *p*HA > *BHA*. The three dimers, *p*-cresol dimer, *p*HA dimer and *bis*-*BHA*, which possessed a high  $\chi$  value, showed potent anti-inflammatory activity. It has been reported that *bis*-*BHA* has potent anti-inflammatory activity (20). The IP value declined in the order *p*-cresol dimer > *p*-cresol > *p*HA dimer > *bis*-*BHA* > *p*HA > *BHA*. Thus it is clear that *BHA* can act as a pro-oxidant, but not as an antioxidant, due to its small IP value.

## Discussion

*COX2* is the key enzyme that catalyzes the two sequential steps responsible for the biosynthesis of prostaglandins from arachidonic acid. *COX2*, the inducible isoform of *COX*, plays a critical role in renal function, maintenance of gastrointestinal integrity, ovarian and uterine function, bone metabolism, various inflammatory responses, rheumatoid arthritis, and Alzheimer's disease, and its overexpression has been

associated with several types of pathologies, including neurodegenerative diseases and various types of cancers (10). The transcription of *COX2* may be modulated by NF- $\kappa$ B, and the *COX2* gene also contains numerous cis-acting promoter elements, including NF- $\kappa$ B sites (7), and NF- $\kappa$ B plays a critical role in mediating *COX2* expression (21). NF- $\kappa$ B regulates the expression of genes involved in the control of proliferation/growth, inflammatory responses, cell adhesion, and other processes including other effector elements in a variety of cells (22). Functionally active NF- $\kappa$ B exists mainly as a heterodimer consisting of subunits of the Rel family (*e.g.* p50 p52, p65, RelB) which normally form an inactive cytoplasmic complex by binding to an inhibitory protein, I $\kappa$ B. Exposure of cells to external stimuli such as bacterial *LPS*, inflammatory cytokines and ROS is thought to cause rapid phosphorylation of I $\kappa$ B, with subsequent degradation by proteasomes. We previously found that honokiol and magnolol, phenolic antioxidants with potent anti-inflammatory

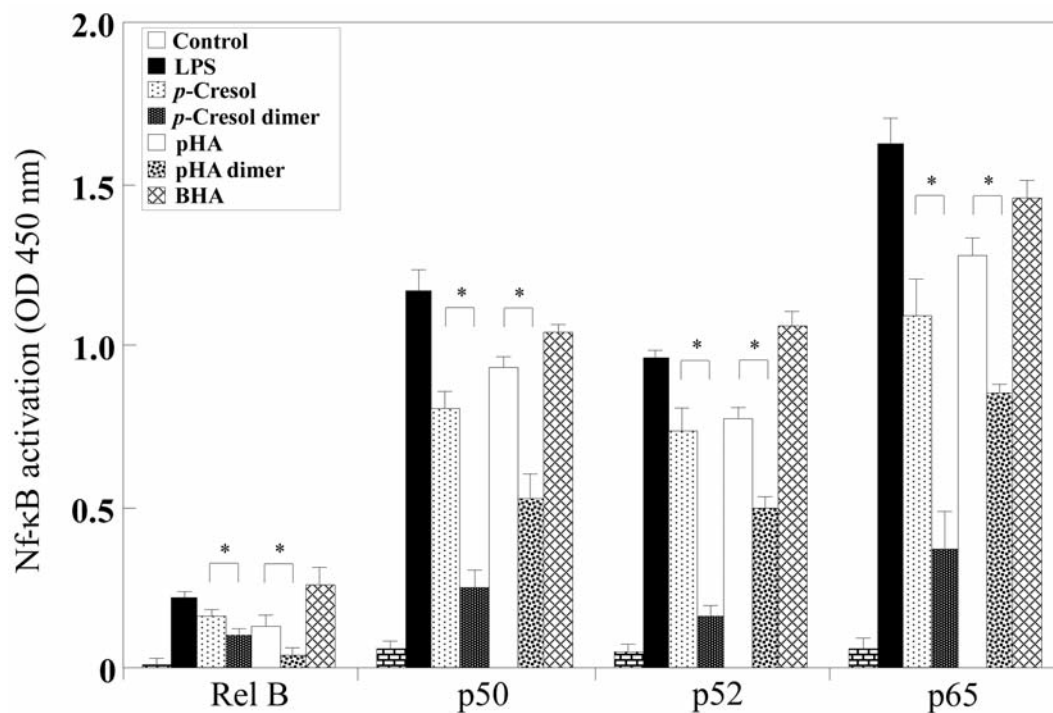


Figure 4. Inhibition of lipopolysaccharide (LPS)-stimulated nuclear factor-kappa B (NfκB) activation by addition of  $10^{-5}$  M *p*-cresol, *p*-cresol dimer, *p*-hydroxyanisole (pHA), pHA dimer or 2-*t*-butyl-4-hydroxyanisole (BHA) in RAW264.7 cells. The inhibition of Nf-κB subunit binding for each phenol as functions of RelB, p50, p52 and p65 are presented as a vertical bar graph indicating the mean±SE of three independent experiments. SE <15%. A significant difference between *p*-cresol and *p*-cresol dimer, and also between pHA and pHA dimer, was observed. \* $p < 0.01$ .

activity, have a relatively large  $\chi$  value, whereas eugenol, a 2-methoxy-4-allyl phenol that possesses no anti-inflammatory activity, had a smaller  $\chi$  value than the former two compounds, suggesting that the anti-inflammatory activity was proportional to the difference in  $\chi$  (10). Together with the findings of the present study, it can be assumed that the  $\chi$  value of phenolic compounds controls their targeting enzymes and proteins involved in COX2 expression and NF-κB activation.

Putz *et al.* reported that the  $\chi$  principle can be applied at the level of ligand-receptor binding in order to predict the genotoxicity and carcinogenicity of various chemicals (23). The molecular  $\chi$  is first equalized with that of the receptor, leading to selection of a molecular fragment with  $\chi$  complementary to that of the receptor, or adjustment of the receptor pocket to fit with the ligand  $\chi$ . From these hypotheses it is assumed that COX2 enzyme and NF-κB proteins activated by inflammatory stimuli such as LPS and ROS may be controlled by the  $\chi$  value of phenolic compounds. On the other hand, the radical-scavenging activity of phenolic compounds is well known to be related to their anti-inflammatory activity, as they can scavenge harmful free radicals. We previously reported that 2-*tert*-butylphenols, such as BHA, scavenged intracellular ROS (24). *p*-cresol dimer and pHA dimer also scavenged alkyl radicals to a much greater degree than *p*-

cresol or pHA, as a result of the relatively high stoichiometric factor (number of free radicals trapped by one mole of phenolic antioxidant moiety) of these compounds: *p*-cresol dimer and pHA dimer scavenge about four radicals, whereas *p*-cresol and pHA scavenge about two radicals (5).

Furthermore, the IP is the most important energetic factor for evaluation of scavenging ability. It is known that a relatively high IP value decreases the rate of electron transfer between a phenolic antioxidant and oxygen, and therefore phenolic compounds with a higher IP value have reduced pro-oxidative potency, whereas compounds with a smaller IP value have stronger pro-oxidative properties (25). From these findings, it is assumed that BHA and pHA, with smaller IP values, may act as pro-oxidants. We have previously reported that BHA-induced cytotoxicity was enhanced by horseradish peroxidases due to pro-oxidant activity, suggesting that BHA is oxidizable and produces cytotoxic BHA radicals within the cell; consequently, cytotoxic quinone intermediates may be produced, and therefore BHA shows no anti-inflammatory activity (24). Quantum chemical calculations might provide a closer insight into the molecular mechanisms of anti-inflammatory activity of these phenolic compounds, and the  $\eta$ ,  $\chi$  and IP values may be useful for prediction of their modes of action.

## References

- Andersen A: Final report on the safety assessment of sodium *p*-chloro-*m*-cresol, *p*-chloro-*m*-cresol, chlorothymol, mixed cresols, *m*-cresol, *o*-cresol, *p*-cresol, isopropyl cresols, thymol, *o*-cymen-5-ol, and carvacrol. *Int J Toxicol* 25(Suppl 1): 29-127, 2006.
- Asakawa E, Hirose M, Hagiwara A, Takahashi S and Ito N: Carcinogenicity of 4-methoxyphenol and 4-methylcatechol in F344 rats. *Int J Cancer* 56: 146-152, 1994.
- Shibata MA, Hirose M, Masuda A, Kato T, Mutai M and Ito N: Modification of BHA forestomach carcinogenesis in rats: inhibition by diethylmaleate or indomethacin and enhancement by a retinoid. *Carcinogenesis* 14: 1265-1269, 1993.
- Murakami Y, Shoji M, Hirata A, Tanaka S, Hanazawa S, Yokoe I and Fujisawa S: An ortho dimer of butylated hydroxyanisole inhibits nuclear factor kappa B activation and gene expression of inflammatory cytokines in macrophages stimulated by *Porphyromonas gingivalis* fimbriae. *Arch Biochem Biophys* 449: 171-177, 2006.
- Kadoma Y, Murakami Y, Ogiwara T, Machino M, Yokoe I and Fujisawa S: Radical-scavenging activity and cytotoxicity of *p*-methoxyphenol and *p*-cresol dimers. *Molecules* 15: 1103-1112, 2010.
- Aldridge C, Razzak A, Babcock TA, Helton WS and Espat NJ: Lipopolysaccharide-stimulated RAW264.7 macrophage inducible nitric oxide synthase and nitric oxide production is decreased by an omega-3 fatty acid lipid emulsion. *J Surg Res* 149: 296-302, 2008.
- Chen FE, Huang DB, Chen YQ and Ghosh G: Crystal structure of p50/p65 heterodimer of transcription factor NF- $\kappa$ B bound to DNA. *Nature* 391: 410-413, 1998.
- Beg AA and Baltimore D: An essential role for NF- $\kappa$ B in preventing TNF- $\alpha$ -induced cell death. *Science* 274: 782-784, 1996.
- Qin H, Wilson CA, Lee SJ, Zhao X and Benveniste EN: LPS induces CD40 gene expression through the activation of NF- $\kappa$ B and STAT-1 $\alpha$  in macrophages and microglia. *Blood* 106: 3114-3122, 2005.
- Murakami Y, Kawata A, Seki Y, Koh T, Yuhara K, Maruyama T, Machino M, Ito S, Kadoma Y and Fujisawa S: Comparative inhibitory effects of magnolol, honokiol, eugenol and *bis*-eugenol on cyclooxygenase-2 expression and nuclear factor- $\kappa$ B activation in RAW264.7 macrophage-like cells stimulated with fimbriae of *Porphyromonas gingivalis*. *In Vivo* 26: 941-950, 2012.
- Asakura K, Honda E and Osanai S: Selective oxidative coupling of *p*-cresol producing an *ortho-ortho* direct-linked dimer. *Chem. Lett* 24: 583-584, 1995.
- Kadoma Y, Murakami Y, Ogiwara T, Machino M, Yokoe I and Fujisawa S: Radical-scavenging activity and cytotoxicity of *p*-methoxyphenol and *p*-cresol dimers. *Molecules* 15: 1103-1112, 2010.
- Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y and Ueno K: A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. *Talanta* 44: 1299-1305, 1997.
- Hanazawa S, Takeshita A and Kitano S: Retinoic acid suppression of c-fos gene inhibits expression of tumor necrosis factor- $\alpha$ -induced monocyte chemoattractant JE/MCP-1 in clonal osteoblastic MC3T3-E1 cells. *J Biol Chem* 269: 21379-21384, 1994.
- Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159, 1987.
- Renard P, Ernest I, Houbion A, Art M, LeCalvez H, Raes M and Remacle J: Development of a sensitive multi-well colorimetric assay for NF $\kappa$ B. *Nucleic Acids Res* 29: E21, 2001.
- Lopachin RM, Gavin T, Decaprio A and Barber DS: Application of the hard and soft, acids and bases (HSAB) theory to toxicant-target interactions. *Chem Res Toxicol* 25: 239-251, 2012.
- Koopmans T: The distribution of wave function and characteristic value among the individual electrons of an atom. *Physica I*: 104-113, 1993.
- Fujisawa S and Kadoma Y: Relationship between phenol-induced cytotoxicity and experimental parameter. *Mini Rev Medic Chem* 12: 477-490, 2012.
- Murakami Y, Shoji M, Ogiwara T, Tanaka S, Yokoe I and Fujisawa S: Preventive effect of ortho dimer of butylated hydroxyanisole on activator protein-1 activation and cyclooxygenase-2 expression in macrophages stimulated by fimbriae of *Porphyromonas gingivalis*, an oral anaerobe. *Anticancer Res* 26: 2915-2920, 2006.
- Xiao G and Fu J: NF $\kappa$ B and cancer: a paradigm of Yin-Yang. *Am J Cancer Res* 1: 192-221, 2011.
- Klapproth K, Sander S, Marinkovic D, Baumann B and Wirth T: The IKK2/NF- $\kappa$ B pathway suppresses MYC-induced lymphomagenesis. *Blood* 114: 2448-2458, 2009.
- Putz MV, Ionaşcu C, Putz AM and Ostafe V: Alert-QSAR. Implications for electrophilic theory of chemical carcinogenesis. *Int J Mol Sci* 12: 5098-5134, 2011.
- Saito M, Sakagami H and Fujisawa S: Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). *Anticancer Res* 23: 4693-4701, 2003.
- Zhang HY, Sun YM and Wang XL: Substituent effects on O--H bond dissociation enthalpies and ionization potentials of catechols: a DFT study and its implications in the rational design of phenolic antioxidants and elucidation of structure-activity relationships for flavonoid antioxidants. *Chemistry* 9: 502-508, 2003.

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