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The Radical Scavenging Activity and Cytotoxicity of Resveratrol, Orcinol and 4-Allylphenol and their Inhibitory Effects on *Cox-2* Gene Expression and Nf-κb Activation in RAW264.7 Cells Stimulated with *Porphyromonas gingivalis*-fimbriae

YUKIO MURAKAMI[1], AKIFUMI KAWATA[1], SHIGERU ITO[2], TADASHI KATAYAMA[1] and SEIICHIRO FUJISAWA[1]

[1]*Division of Oral Diagnosis, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado-City, Saitama, Japan;*
[2]*Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-Ku, Tokyo, Japan*

Abstract. *Background/Aim: Resveratrol is a polyphenol with efficient anti-oxidative and anti-inflammatory activity. To clarify the molecular mechanism responsible for its anti-inflammatory action, we investigated the radical scavenging activity, cytotoxicity and anti-inflammatory activity of resveratrol and its related compounds, orcinol and 4-allylphenol. Materials and Methods: The radical scavenging activities of these compounds were determined by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) assay and their cytotoxicities against RAW264.7 cells were determined using a cell-counting kit (CCK-8). The inhibitory effects of these compounds on cyclooxygenase-2 (Cox2) expression in RAW264.7 cells stimulated with Porphyromonas gingivalis (Pg) fimbriae were also determined using real-time polymerase chain reaction and western blot analysis, while inhibition of the fimbria-stimulated activation of nuclear factor-kappa B (Nf-κb) was evaluated using western blot analysis and enzyme-linked immunosorbent assay-like microwell colorimetric transcription factor activity assay, respectively. The quantum chemical parameters were calculated on the basis of the density function theory (DFT) BLYP/6-31G*. Results: DPPH radical scavenging activity declined in the order resveratrol > orcinol > 4-allylphenol. The cytotoxicity of the compounds was in the order 4-allylphenol > resveratrol > orcinol. The inhibitory effect on Pg fimbria-stimulated Cox2 expression and Nf-κb activation was enhanced by resveratrol-alone. Resveratrol showed high electronegativity (χ) and softness (σ) values, as determined by quantum chemical calculations. Conclusion: Resveratrol exerts potent anti-inflammatory activity in RAW264.7 cells stimulated with Pg-fimbriae and may be applicable as a therapeutic agent for inflammatory periodontal disease as a manifestation of systemic disease.*

Correspondence to: Dr. Yukio Murakami, Division of Oral Diagnosis, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado City, Saitama 350-0283, Japan. Tel: +81 492855511, Fax: +81 492876657, e-mail: ymura@dent.meikai.ac.jp

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Activation of macrophages induces local inflammation through secretion of numerous pro-inflammatory cytokines, chemokines and inflammatory mediators, such as reactive oxygen species (ROS), including nitric oxide (NO), and prostaglandins (PGs). Cyclooxygenase (COX) is a key enzyme responsible for conversion of arachidonic acids to PGs. It consists of two different isoforms, designated COX1 and COX2. COX2 is induced by growth factors, cytokines and bacterial lipopolysaccharides (LPS) that are closely involved in inflammation and tumorigenesis (1). Inhibition of COX2 is useful for the treatment of inflammation and prevention of cancer (2). Therefore, agents that interfere with the signaling mechanisms governing COX2 production can inhibit inflammation and tumorigenesis. Furthermore, nuclear factor-kappa B (NFκB), a major transcription factor, is closely related to the expression of pro-inflammatory genes in macrophages. Functionally-active NF-κB exists mainly as a heterodimer consisting of subunits of the Rel family (*e.g.* c-Rel or p50, p52, p65 and RelB), which is normally sequestered in an active cytoplasmic complex by binding to an inhibitory protein, IκBα. Exposure of cells to external stimuli, such as inflammatory cytokines, ultraviolet irradiation, bacterial LPS and ROS causes rapid phosphorylation of Iκbα with subsequent degradation by

proteasomes (3, 4). The regulatory effect of non-steroidal anti-inflammatory drug (NSAID)-like phenolic compounds was previously evaluated in terms of inhibitory IκBα, p65 and p50 levels after exposure of RAW264.7 cells to LPS (5).

Fimbriae of *Poryphyromonas gingivalis* (Pg), in addition to LPS, are also involved in the pathogenesis of periodontal disease through production of various pro-inflammatory cytokines leading to differentiation of osteoclasts (6-8). We previously investigated the inhibitory effects of several natural and artificial phenolic antioxidants on *Cox2* mRNA in RAW264.7 cells stimulated with LPS or Pg-fimbriae and found that biphenolic compounds, such as *bis-tert*-butyl hydroxyanisole, curcumin and honokiol inhibited Cox2 expression and Nfκb activation (9-12). Pg-fimbriae are peritrichous filamentous appendages, whereas LPS is a major component of the outer membrane of Pg and capable of host activation mediating the adhesion of bacteria to both host cells and a variety of oral substrates and molecules (13). The bi-phenols described above have been shown to act as NSAID-like compounds (9-12).

Resveratrol (*trans*-3,4',-5-trihydroxystilbene) is a dietary anti-oxidant polyphenol found in various plants, such as grapes, mulberries and peanuts, and also red wine. Growing evidence suggests that resveratrol plays a role in the prevention of human pathological processes, such as inflammation, atherosclerosis and carcinogenesis (14). The anti-inflammatory ability of resveratrol has also recently been highlighted for possible application to the treatment of periodontitis (15). Rizzo *et al*. demonstrated that resveratrol decreases the production of pro-inflammatory cytokines, such as interleukin (IL)-1 beta (β), IL-6, IL-8, IL-12 and tumor necrosis factor alpha (TNFα), and also NO, using a human periodontal ligament cell (HPLC) model stimulated with Pg-LPS (16).

Vincent *et al*. have also demonstrated that murine osteoclast progenitor RAW264.7 cells readily differentiate into osteoclast-like cells in defined serum-free conditions and that this process is enhanced by addition of 1 alpha, 25-dihydroxyvitamin D_3 (17). Resveratrol was previously reported to prevent receptor activator nuclear factor kappa-B ligand (RANKL)-induced differentiation of RAW264.7 cells through inhibition of ROS production (18). These results suggest that resveratrol inhibits the periodontal tissue destruction caused by bacterial fimbriae *via* exertion of anti-oxidant activity.

In the present study, using a series of both natural and artificial phenolic compounds, including resveratrol, orcinol and 4-allylphenol, we investigated their diphenylpicrylhydrazyl (DPPH) radical scavenging activity, cytotoxicity and inhibitory effects on the *Cox2* gene in RAW264.7 cells stimulated with Pg-fimbriae. Also, since Nf-κb activation requires nuclear translocation of Nf-κb components, such as p50, p52, p65 and RelB, we examined the inhibitory effect of resveratrol, orcinol and 4-allylphenol on the nuclear translocation of each component resulting from Nf-κb activation in this Pg-fimbria-stimulated model. We also discuss the relationship between the radical scavenging activity, cytotoxicity or anti-inflammatory activities and quantum chemical parameters for resveratorol, orcinol, 4-allylphenol and 4,4'-biphenol (19).

Materials and Methods

Materials. Resveratrol (*trans*-1,2-(3,4',5-trihydroxydiphenyl)ethylene) was purchased from Wako Pure Chemical Industries (Osaka, Japan), 4-allylphenol (4-(2-propenyl)-phenol) was obtained from Parkway Scientific Co. (New York, NY, USA) and orcinol (5-methylbenzene-1,3-diol) and 4,4'-biphenol were from Tokyo Kasei Co. (Tokyo, Japan). The chemical structures of these compounds are shown in Figure 1, while the possible oxidation mechanisms of resveratrol and orcinol are illustrated in Figure 2. Solutions of these compounds were prepared by dissolving each of them in dimethyl sulfoxide, followed by dilution to the indicated concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. A goat polyclonal antibody against Cox2, a rabbit polyclonal antibody against β-actin, horseradish peroxidase (HRP)-conjugated mouse anti-goat IgG and HRP-conjugated goat anti-rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phospho-specific anti-Ikbα (recognizing phospho-Ser32) and anti-Ikbα, both rabbit polyclonal antibodies, as well as HRP-conjugated goat anti-rabbit IgG and a Phototope-HRP Western blot detection kit were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). RPMI-1640 was purchased from Invitrogen Corp. Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) was purchased from Tokyo Kasei Co. (Tokyo, Japan).

DPPH-radical scavenging activity. The radical scavenging activities were determined using 0.1 mM DPPH as a free radical. In brief, each of the resveratrol-related compounds was used at various concentrations in ethanol. The decrease in absorbance was determined at 540 nm for 1 h at room temperature. Radical absorbance activity was defined from the dose-response curves as the amount of inhibitor necessary to decrease the initial DPPH-radical concentration (EC_{50}). Data are expressed as means of three independent experiments. Statistical analyses were performed using the Student's *t*-test.

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_2 in air, washed and, then, incubated overnight in serum-free RPMI-1640. Cells 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) (20). In brief, RAW264.7 cells (3×10^4 per well) were cultured in NUNC 96-well plates (flat-well-type microculture plates) for 48 hours, after which the cells were incubated with test samples for 24 hours. The 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 (LC_{50}) was determined from the dose-response curves. Data are expressed as means of three independent experiments. Statistical analyses were performed using the Student's *t*-test.

Preparation of Porphyromonas gingivalis fimbriae. *P. gingivalis* ATCC33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura *et al*. (6). As documented previously, purified fimbria-induced biological activities were not attributable to lipopolysaccharide (LPS) contaminants in the preparation (7, 8). Viability of the cells after exposure to the fimbriae at the concentrations used was over 90%, as determined using the Cell Counting Kit-8 (CCK-8) (Dojindo Co.) (20). The protein content of the fimbriae was measured by the method of Smith *et al*. (21).

Preparation of total RNA and real-time polymerase chain reaction (PCR). The preparation of total RNA and the procedure for real-time PCR have been described previously (12). In brief, RAW264.7 cells in NUNC 96-flat-well-type microculture plates (10^5 cells per well) were treated with the test samples. Total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen Japan Co. Ltd., Tokyo, Japan) in accordance with the instruction manual. cDNA was synthesized from total RNA (2 μg) of each sample by random priming using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan, Tokyo, Japan). Reaction mixtures without the reverse transcriptase were used as a negative control. An aliquot of each cDNA synthesis reaction mixture was diluted and used for quantification by real-time PCR. An equal-volume aliquot of each cDNA was mixed, serially diluted and used as a standard. TaqMan probes/primers for *Cox2* and *18S* rRNA and the PCR enzyme mix for real-time PCR were purchased from Life Technologies Japan. Real-time PCR quantification was performed in triplicate using the GeneAmp Sequence Detection System 5700 software (Life Technologies Japan) in accordance with the instruction manuals. The relative amount of target gene was calculated from standard curves generated in each PCR and quantitative data with a coefficient of variance (CV) of less than 10% were used for further analyses. Each calculated amount of mRNA was standardized by reference to *18S* rRNA. Data are expressed as means of three independent experiments. Statistical analyses were performed using the Student's *t*-test.

Western blot analysis. RAW264.7 cells in 5-cm-diameter Falcon dishes (10^6 cells per dish) were treated with the test samples. The cells were then solubilized with lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v), Triton X-100, 2.5 mM sodium pyrophosphate, 2 mM Na_3VO_4, 10 mM NaF, 1 mM β-glycerophosphate, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Protein concentrations were measured by the method of Smith *et al*. (21). Each sample (10 μg of protein) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 12.5% polyacrylamide gel and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA, USA). The blots were then blocked with 5% skim milk, washed and incubated with anti-Cox2 antibody, phospho-specific anti-IκBα antibody and anti-IκBα as the primary antibody diluted 1:2000 in working solution (5% bovine serum albumin, 1 × TBS (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) and 0.1% Tween 20) at 4˚C. Antibody against β-actin was used at 0.1 μg/ml after dilution with working solution. After incubation, the blots were treated at room temperature with HRP-conjugated secondary antibody diluted 1:4,000. 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 microwell colorimetric Nfκb assay. Nuclei were extracted and prepared for the microwell colorimetric Nfκb assay. In brief, RAW264.7 cells in 5-cm-diameter Falcon dishes (10^6 cells per dish) were pretreated for 30 min with or without the indicated doses of the compounds and, then, treated with fimbriae at 4 μg/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 (23) 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 the respective immobilized oligonucleotide were detected in an enzyme-linked immnosorbent assay (ELISA)-like assay using antibodies against the Nfκb p50, p52, p65 and RelB subunits, followed by a secondary antibody conjugated to HRP. 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.

Computation. The phenolic O-H bond dissociation enthalpy (BDE) for the indicated phenolic compounds was calculated as follows: First, the lowest and second-lowest energy conformers of both the phenol derivatives and their phenoxyl radical species were identified as candidates for geometry optimization using the conformer search procedure by MMFF (Merck Molecular Mechanics) force fields calculation. The tentative conformers were then geometrically optimized by *ab initio* molecular orbital calculation at the HF/6-31G* level for the phenols and with a UHF/6-31G* basis set for the phenoxyl radicals *in vacuo* to afford the respective energetic minimized structures. The electronic energy was further preceded by single point calculation involving density functional theory (DFT) using the B3LYP functional at the 6-31G*level: BDE=Hr + Hh - Hp, where Hr is the enthalpy of the phenoxyl radical generated by H- abstraction, Hh is the enthalpy of the hydrogen radical and Hp is the enthalpy of the parent phenol. The lowest unoccupied molecular orbital (LUMO) energy (E_{LUMO}) and highest occupied molecular orbital (HOMO) energy (E_{HOMO}) were obtained from the ground state equilibrium geometries with density functional theory calculated DFT B3LYP 6-31G* *in vacuo* from 6-31G* initial geometry (12, 23). The absolute value of HOMO energy was adopted as an approximate ionization potential (IP) value according to Koopman's theorem (24). All calculations were performed using Spartan'10 (Wave Function Inc., Irvine, CA, USA).

The η , σ and χ values were calculated as follows:

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

$$\sigma= 1/\eta \quad \text{(Eq. 2)}$$

$$\chi= -(E_{LUMO} + E_{HOMO})/2 \quad \text{(Eq. 3)}$$

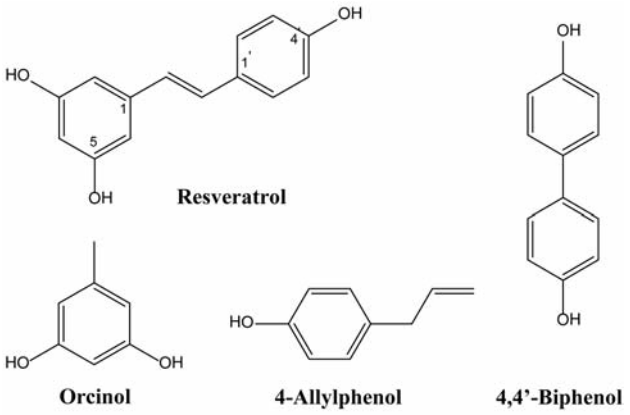

Figure 1. *The chemical structures of resveratrol, 4-allylphenol, orcinol and 4,4'-biphenol.*

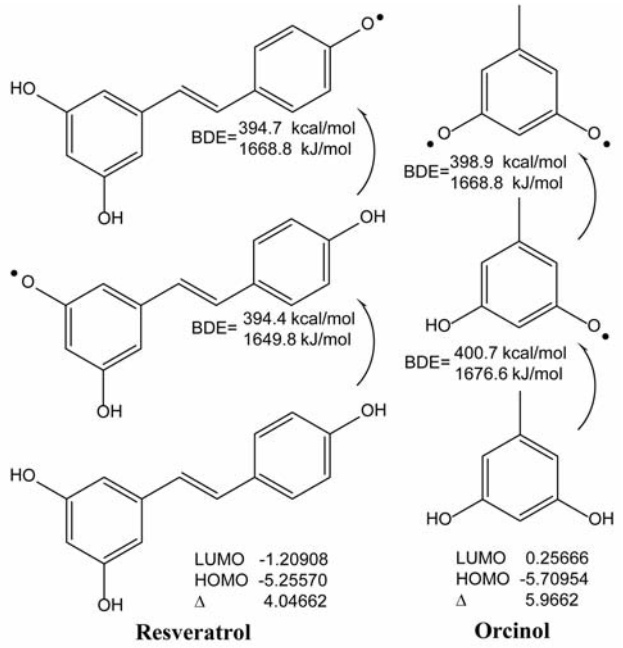

Figure 2. *The possible mechanism of resveratrol and orcinol oxidation. BDE, phenolic O-H dissociation enthalpy; LUMO, lowest unoccupied molecular orbital (LUMO) energy (eV); HOMO, highest occupied molecular orbital (HOMO) energy (eV) Δ, HOMO-LUMO gap (eV).*

Results

DPPH-radical scavenging activity. Resveratrol, 4-allylphenol and orcinol are shown in Figure 3. The EC_{50} values (μM) for resveratrol, orcinol, 4,4'-biphenol and 4-allylphenol were 45, 80, 90 and 7,500, respectively (Table I). Resveratrol was the highest, whereas 4-allylphenol was the lowest.

Cytotoxicity. The cytotoxicity of resveratrol, 4-allylphenol and orcinol towards RAW264.7 cells was investigated using CCK-8 and the results are shown in Figure 4. The 50% lethal cytotoxic concentration (LC_{50}, μM) for resveratrol, 4-allylphenol and orcinol was 430, 290 and 3,000, respectively (Table I). Together with the LC50 for 4,4'-biphenol (19), the cytotoxicity declined in the order 4,4'-biphenol > 4-allylphenol > resveratrol > orcinol.

Cox2 inhibition. The inhibitory effects of resveratrol, 4-allylphenol and orcinol on *P. gingivalis* fimbria-stimulated expression of *Cox2* mRNA in RAW264.7 cells were investigated using real-time PCR and the results are shown in Figure 5. Resveratrol strongly inhibited the fimbria-induced expression of *Cox2* mRNA at a non-cytotoxic concentration of 50 μM, whereas 4-allylphenol and orcinol did not have an inhibitory effect at the same concentration. Furthermore, using Western blot analysis, we tested whether resveratrol inhibited fimbria-induced expression of Cox2 protein. The results are shown in Figure 6. Resveratrol also markedly inhibited the expression of Cox2 protein at a concentration of 50 μM, whereas 4-allylphenol and orcinol had no effect. These compounds did not induce expression of Cox2 protein in the cells. These results indicate that resveratrol exerted a stronger inhibitory effect on Cox2 expression than did 4-allylphenol and orcinol.

Inhibition of Nfκb activation. The results are shown in Figure 7. Resveratrol clearly inhibited both the phosphorylation and degradation of IκBα in RAW264.7 cells when stimulated by the fimbriae. However, 4-allylphenol and orcinol had no effect. The inhibitory effects of resveratrol on the binding of Nf-κb to its consensus sequence in fimbria-stimulated RAW264.7 cells were also investigated using an ELISA-like microwell colorimetric transcription factor assay kit. The results are shown in Figure 8. The fimbria-stimulated binding of Nfκb subunits, such as p50, p52, p65 and RelB to its consensus sequences were markedly inhibited by resveratrol but not by 4-allylphenol and orcinol. The fimbria-stimulated binding of Nfκb was also completely inhibited by an excess amount of wild-type consensus oligonucleotide provided in the kit (data not shown). These findings indicate that resveratrol is a potent inhibitor of fimbria-triggered cellular signaling in RAW264.7 cells.

Quantum chemical calculation. The η, σ and χ values for resveratrol and its related compounds are shown in Table I, together with data for BDE, ionization potential (IP) and the octanol-water partition coefficient (log P). The η value (eV) declined in the order resveratrol (0.50) > 4,4'-biphenol (0.40) > 4-allylphenol (0.35) > orcinol (0.34), whereas the IP value (eV) declined in the order 4-allylphenol (5.80) > orcinol (5.71) > 4,4'-biphenol (5.35) > resveratrol (5.26). Investigation of the

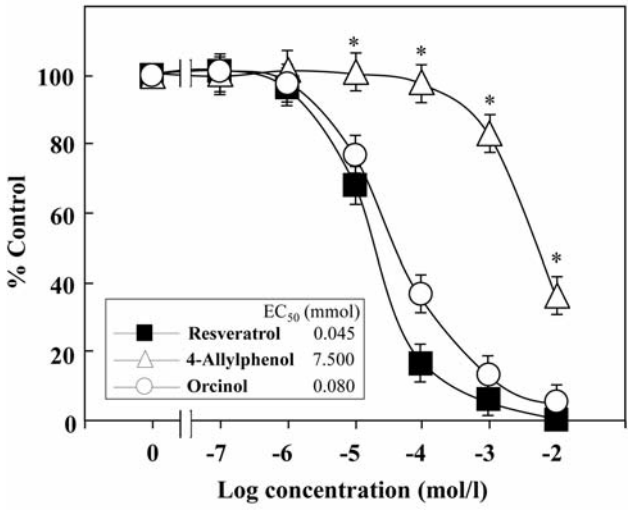

Figure 3. *Anti-radical effects of resveratrol, 4-allylphenol and orcinol. The procedures employed are described in Materials and Methods. DPPH, 0.1 mM. The antiradical activity was determined as the amount of inhibitor necessary to decrease the initial DPPH concentration by 50% (EC_{50}, mol/l). Values are the means of three separate measurements. The results are presented as the means±standard error (SE). SE <15%. There was a significant difference between 4-allyphenol and resveratrol or orcinol within the range of 10 μM to 10 mM. *p<0.01.*

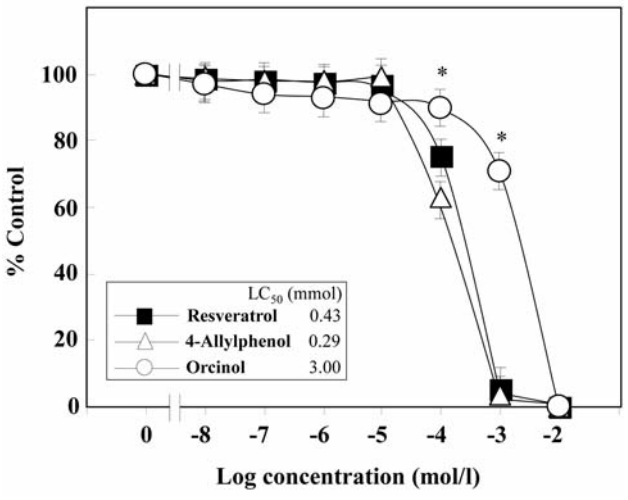

Figure 4. *The cytotoxicity of resveratrol, 4-allylphenol and orcinol on RAW264.7 cells. The procedures employed are described in Materials and Methods. The results are presented as the means±standard error (SE). SE <15%. There was a significant difference between orcinol and resveratrol or 4-allyphenol within the range 100 μM to 1 mM. *p<0.01.*

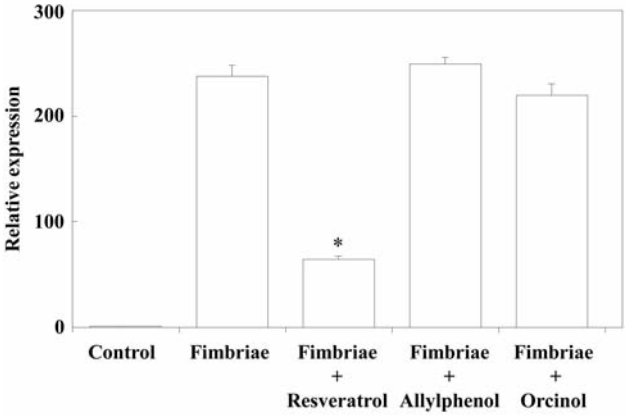

Figure 5. *Regulatory effects of resveratrol, 4-allylphenol and orcinol on fimbria-stimulated expression of the Cox2 gene in RAW264.7 cells. The cells were pretreated for 30 min with resveratrol, 4-allylphenol or orcinol at 50 μM, respectively. They were then incubated for 3 h with or without fimbriae at 4 μg/ml and their total RNAs were then prepared. Each cDNA was synthesized and the expression levels of Cox2 mRNA were determined by real-time PCR and standardized against the expression of 18S rRNA. The results are presented as means±standard error (SE) of three independent experiments. SE <15%. Significant differences between samples for resveratrol, 4-allylphenol and orcinol were observed for inhibition of Cox2 gene expression. *p<0.01.*

structure-activity relationship between the cytotoxicity and log P, IP or σ value revealed no significant linear relationship. The high cytotoxicity of 4-allylphenol may account for the formation of intermediates and metabolites derived from oxidation in biological systems, because eugenol (2-methoxy-4-allylphenol) elicits skin sensitization in mice through a possible metabolic pathway involving *ortho*-quinone and quinone methide intermediates (25).

Investigation of the relationship between anti-inflammatory activity and quantum chemical parameters revealed that resveratrol, which has potent anti-inflammatory activity, had a large χ value but also a small η value, *i.e.* a HOMO-LUMO gap (Δ). The σ value can be calculated as the inverse of η value (Eq. 2). The potent anti-inflammatory activity of resveratrol was considered to be associated with its high χ and σ values. Also, since resveratrol had the smallest IP value, it had the strongest pro-oxidative properties.

Discussion

It is generally recognized that the anti-inflammatory activity of NSAID-like phenolic compounds is related to their high radical scavenging activity. It is well known that ROS, such as the superoxide anion (O_2^-), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and peroxy radical ($ROO^\bullet$) play major roles in the development of oxidative stress that can lead to inflammation, cancer and neurodegenerative disorders. As shown in Table I, resveratrol had the strongest radical scavenging activity (EC_{50}) among the compounds tested. The anti-oxidant activity of resveratrol

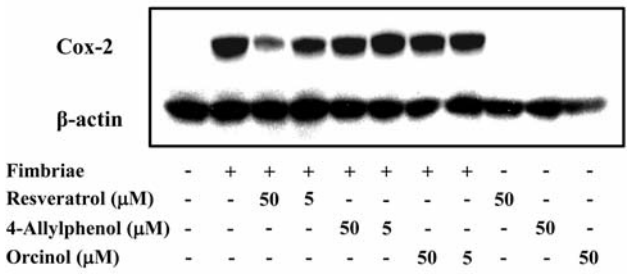

Figure 6. *Regulatory effects of resveratrol, 4-allylphenol and orcinol on fimbria- stimulated expression on Cox2 protein in RAW264.7 cells. The cells were pretreated for 30 min with or without the indicated doses of resveratrol, 4-allylphenol and orcinol and, then, incubated with or without the fimbriae at 4 μg/ml. The cells were solubilized with lysis buffer at 6 h after the start of treatment. Equal amounts of cell lysates were analyzed by western blotting after SDS-PAGE with anti-Cox2 antibody or anti-β-actin antibody. Blotting analysis was performed to determine the level of Cox2 protein. Three independent experiments were performed and similar results were obtained.*

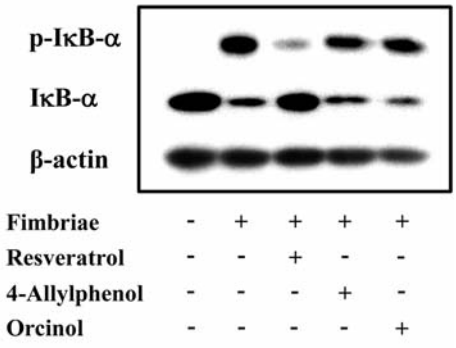

Figure 7. *Inhibitory effects of resveratrol, 4-allylphenol and orcinol on the phosphorylation-dependent proteolysis of fimbria-stimulated IκBα. The cells were pre-treated for 30 min with or without resveratrol, 4-allylphenol or orcinol at 50 μM and, then, incubated with or without the fimbriae at 4 μg/ml. Equal amounts of cell lysates were analyzed by western blotting after SDS-PAGE with phospho-specific anti-IκBα antibody, anti-IκBα antibody or anti-β-actin antibody. Three independent experiments were performed and similar results were obtained.*

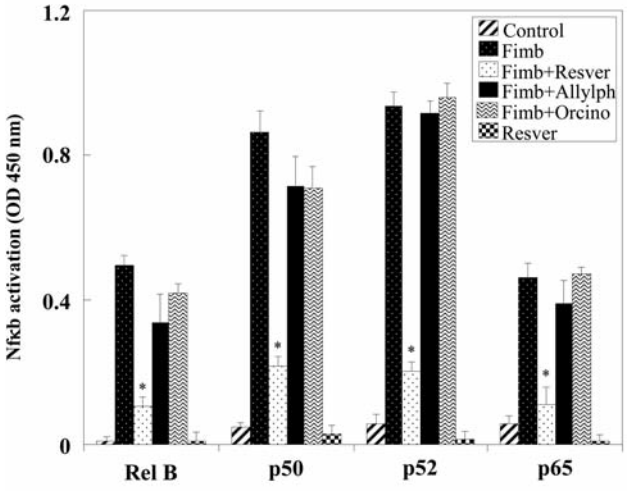

Figure 8. *Inhibition of fimbria-stimulated Nfκb activation by resveratrol, 4-allylphenol and orcinol in RAW264.7 cells. The cells were pre-treated for 30 min with or without resveratrol, 4-allylphenol or orcinol at 50 μM and, then, incubated with or without the fimbriae at 4 μg/ml for 1 h. Nuclear extracts were prepared and used in a TransAM (Active Motif) ELISA-like assay to quantify the Nfκb p50, P65, P52 and RelB DNA-binding activity. The results are presented as means±standard error (SE) of three independent experiments. SE <15%. Significant differences between samples for resveratrol, 4-allylphenol and orcinol were observed for inhibition of Nfκb subunit binding. *p<0.01. Fimb, Fimbriae; Resver, resveratrol; Allylph, 4-allylphenol; Orcino, orcinol.*

lies in its hydroxyl (OH) groups, which can scavenge free radicals produced *in vivo*. We calculated the stoichiometric factor (*n*, number of free radicals trapped by one mole of phenolic antioxidant moiety) using DPPH data (Table I) and found that the *n* values for resveratrol, orcinol, 4,4'-biphenol and 4-allylphenol were approximately 2, 1.3, 1.1 and 0.01, respectively. The *n* value of nearly 1 for orcinol may be due to dimer formation. Also, the value of 1.3 for 4,4'-biphenol suggests formation of a dimer. In general, for phenolic compounds, a *n* value of 1 indicates dimerization, whereas fully oxidized monophenols show a *n* value of 2. Oxidation of orcinol is known to yield a C-C dimer but not a C-O dimer. The very small *n* value for 4-allylphenol may be due to its large BDE value.

We previously investigated the radical scavenging activity of resveratrol using the induction period method with a peroxy radical at 70˚C and found that the stoichiometric factor (*n*) was above 2.7 (26) suggesting that the radical scavenging activity of this compound involves the 5-OH (or 3-OH) group in the A ring, as well as the 4'-OH group in the B ring, and consequently the formation of quinone (27).

In general, however, the cytotoxicity of phenolic compounds is known to be related to log P (octanol-water partition coefficient), *i.e.* hydrophobicity. Also, the cytotoxicity of phenolic compounds is reported to be nonspecific due to the log P value and formation of phenoxyl radicals expressed by BDE (28). In the present study, although the number of samples was limited, the cytotoxicity of resveratrol and its related compounds showed no linear relationship to log P or the BDE value. Quantum chemical calculation might provide a closer insight into the molecular mechanisms of the anti-inflammatory activity. Molecules with a relatively large HOMO-LUMO gap, *i.e.* a large η value, are generally unreactive, whereas those with a relatively small η value are generally reactive (23). The

Table I. *Quantum chemical parameters. E_{HOMO}, Highest occupied molecular orbital energy; E_{LUMO}, lowest unoccupied molecular orbital energy; η, chemical hardness; σ, softness; χ, electronegativity; IP, ionization potential, BDE, phenolic O-H bond dissociation enthalpy; log P, octanol-water partition coefficient; cytotoxicity (LC_{50}), DPPH-radical scavenging activity (EC_{50}) and anti-inflammatory activity for resveratrol, orcinol, 4-allylphenol and 4,4'-biphenol.*

Parameters	Resveratrol	Orcinol	4-Allylphenol	4,4'-Biphenol
E_{HOMO}, eV	–5.256	–5.709	–5.80	–5.35
E_{LUMO}, eV	–1.209	0.26	–0.02	–0.29
η, eV (σ, eV)	2.02 (0.495)	2.98 (0.336)	2.89 (0.346)	2.53(0.395)
χ, eV	3.23	2.73	2.91	2.82
IP, eV	5.26	5.71	5.80	5.35
BDE, kcal mol^{-1}	394.7(4'-OH) 394.4 (5-OH)	400.7(3-OH), 398.9(5-OH)	403.15	346.14
Log P	3.1[a]	1.88[b]	2.52[c]	2.8[d]
LC_{50}, mM	0.43	3.00	0.29	0.25[e]
EC_{50}, mM	0.45	0.08	7.5	0.09
Anti-inflammatory activity	potent	none	none	none[e]

The chemical parameters were calculated with DFT, using BLYP/6-31G* basis set. [a]www.drugbank.ca/drugs/DB02709. [b]www.chemicalize.org/structure/#!mol=Cc1cc(O)cc(O)c1. [c]www.springermaterials.com/docs/.../LQPGRLWPPYVTYMHE.html. [d]chemicalland21.com/specialtychem/nh/4,4'-BIPHENOL.htm. [e]Taken from Murakami *et al*. (19).

softness (σ) can be calculated as the inverse of hardness (η) (Eq. 2). The high anti-inflammatory activity of resveratrol may be related to its large σ value.

Also, the IP value is the most important energetic factor for evaluation of scavenging ability. It is known that a relatively high IP value reflects a decreased rate of electron transfer between a phenolic antioxidant and oxygen; therefore, phenolic compounds with a higher IP value have reduced pro-oxidative potency, whereas those with a smaller IP value have stronger pro-oxidative properties (29). The relatively small IP values for resveratrol and 4,4'-biphenol may reflect pro-oxidative activity. The pro-oxidant effect of resveratrol has been supported by other observations (30, 31). Quinone formation has been reported to be one mechanism for the observed pro-oxidant effect of resveratrol involving redox cycling with oxygen leading to ROS formation (27). Indeed, it has been considered that the high cytotoxicity of 4,4'-biphenol and 4-allylphenol may reflect possible metabolic pathways involving *ortho*-quinone intermediates. By contrast, the cytotoxicity and σ value of orcinol showed the smallest values among the tested compounds. Although resveratrol is known for its antioxidant properties, it has been considered to exert cytotoxic and pro-oxidant effects (32).

In the present study, the cytotoxicity of resveratrol, which had in the highest log P, σ and χ values, was lower than that of 4,4'-biphenol and 4-allylphenol. The anti-inflammatory activity of resveratrol in different cancer cell lines stimulated with LPS has been widely reported (2). Similarly, the present study, using RAW264.7 cells stimulated with Pg-fimbriae, demonstrated that resveratrol strongly inhibited the expression of *Cox2* mRNA and also suppressed Nfκb activation, as reflected in phosphorylation-dependent proteolysis of IκBα and binding of p50, p52, p65 and RelB to its consensus sequence. On the other hand, orcinol, 4-allylphenol and 4.4'-biphenol had no effect. From these results, it was assumed that the anti-inflammatory activity of resveratrol may involve its polyphenol B-ring (4'-OH) (Figure 1). Quantum chemical analysis of resveratrol has previously revealed that this 4'-OH is more reactive than the corresponding moieties at the 3'- and 5'-positions (33). In the present study, although the BDE of the 5'-OH moiety was slightly lower than that of 4'-OH (Table I), the latter was most reactive (Figure 2). The presence of 4'-OH in resveratrol, together with stereoisometry, has been reported to be an absolute requirement for inhibition of cell proliferation, as well as antioxidant activity (34). Since the pro-inflammatory activity in RAW264.7 cells stimulated with Pg-fimbriae was inhibited by resveratrol, the latter may exert a beneficial action against inflammation associated with periodontitis, which causes alveolar bone resorption.

We demonstrated a possible relationship between the anti-inflammatory activity and specific electronegativity (χ) of NSAID-like phenolic compounds using the chemical-associated frontier principle and found that phenolic compounds with a large χ value possess potent anti-inflammatory activity (12, 35, 36). Indeed, resveratrol with a large χ value exhibited potent anti-inflamatory activity, whereas orcinol, 4-allylphenol and 4, 4'-biphenol, all having a small χ value, exhibited no activity. Putz *et al*. have reported that the χ principle can be applied at the level of ligand-receptor binding in order to predict the genotoxicity and carcinogenicity of various chemicals (37). The molecular χ is first equalized with that of the receptor

leading to selection of a molecular fragment with χ complementary to that of the receptor or adjustment of the receptor pocket to fit with the χ of the ligand. Based on this hypothesis, it is assumed that Cox2 enzymes and Nfκb proteins activated by pro-inflammatory stimuli, such as LPS, ROS and bacterial fimbriae, may be controlled by the χ value of phenolic antioxidants, such as biphenols and polyphenols.

Although resveratrol exerts a beneficial anti-inflammatory action, its systemic bioavailability is reportedly very low (38), being rapidly and extensively metabolized and excreted (39). Our results suggest that the low bioavailability of resveratrol may be attributable to its large σ, 1/η value (Table I). To improve the bioavailability of resveratrol, a number of proposals have been reported, including the use of nano- and micro-formulations for encapsulation (40).

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