Cytotoxicity and Pro-/Anti-inflammatory Properties of Cinnamates, Acrylates and Methacrylates Against RAW264.7 Cells

YUKIO MURAKAMI, AKIFUMI KAWATA, SEIJI SUZUKI and SEIICHIRO FUJISAWA

Division of Oral Diagnosis and General Dentistry, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Japan

Abstract. Background/Aim: Periodontitis is a chronic inflammatory disease linked to various systemic age-related conditions. It is known that α,β-unsaturated carbonyl compounds such as dietary cinnamates (β-phenyl acrylates) and related (meth)acrylates can have various positive and negative health effects, including cytotoxicity, allergic activity, pro-and anti-inflammatory activity, and anticancer activity. To clarify the anti-inflammatory properties of α,β-unsaturated carbonyl compounds without a phenolic group in the context of periodontal tissue inflammation and alveolar bone loss, we investigated the cytotoxicity and up-regulatory/down-regulatory effect of three trans-cinnamates (trans-cinnamic acid, methyl cinnamate, trans-cinnamaldehyde), two acrylates (ethyl acrylate, 2-hydroxyethyl acrylate), and three methacrylates (methyl methacrylate, 2-hydroxyethyl methacrylate, and triethyleneglycol dimethacrylate) using RAW264.7 cells. Materials and Methods: Cytotoxicity was determined using a cell counting kit (CCK-8) and mRNA expression was determined using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Pro-inflammatory and anti-inflammatory properties were assessed in terms of expression of mRNAs for cyclo-oxygenase-2 (Cox2), nitric oxide synthase 2 (Nos2), tumor necrosis factor-alpha (Tnfa) and heme oxygenase 1 (Ho1). Results: The most cytotoxic compound was 2-hydroxyethyl acrylate, followed by ethyl acrylate and cinnamaldehyde (50% lethal cytotoxic concentration, LC₅₀=0.2-0.5 mM). Cox2 mRNA expression was up-regulated by cinnamaldehyde and 2-hydroxyethyl acrylate, particularly by the former. In contrast, the up-regulatory effect on Nos2 mRNA expression was in the order: cinnamaldehyde >> ethyl acrylate ≈ triethyleneglycol dimethacrylate >> methyl methacrylate ≈ methyl cinnamate. On the other hand, cinnamic acid and 2-hydroxyethyl methacrylate had no effect on gene expression. The two acrylates, but not cinnamates and methacrylates, up-regulated the expression of Ho1 mRNA at a non-cytotoxic concentration of 0.1 mM. Expression of Cox2, Nos2 and Tnfa mRNAs induced by Porphyromonas gingivalis lipopolysaccharide was greatly suppressed by cinnamaldehyde, methyl cinnamate and the two acrylates at 0.1 mM (p<0.05), and slightly, but significantly suppressed by cinnamic acid and methacrylates at 0.1-1 mM (p<0.05). Conclusion: Cinnamaldehyde and acrylates exhibited both anti-inflammatory and pro-inflammatory properties, possibly due to their marked ability to act as Michael reaction acceptors, as estimated from the β-carbon 13C-nuclear magnetic resonance spectra. Methyl cinnamate exhibited potent anti-inflammatory activity with less cytotoxicity and pro-inflammatory activity, suggesting that this compound may be useful for treatment of periodontal disease and related systemic diseases.

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Correspondence to: Dr. Yukio Murakami, Division of Oral Diagnosis and General Dentistry, 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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when used in dentures, restorative resins and adhesives, thereby possibly having adverse effects such as cytotoxicity, skin sensitization, mutagenicity, carcinogenicity, respiratory allergy, and organ toxicity (8-11). The adverse effects of cinnamates and (meth)acrylates may be due to their ability to induce oxidative stress and their covalent interactions with cellular nucleophiles such as proteins, histidine, lysine, glutathione (GSH) and DNA bases (7, 8, 11, 12). The Michael addition of electrophilic cinnamates and (meth)acrylates to endogenous cysteine thiol plays a role in pathologies associated with oxidative stress (13), in the anti-inflammatory activity of cyclopentane prostaglandins (14), and in the induction of enzymes that protect against carcinogenesis (15-17).

Cinnamates, acrylates and methacrylates, which are thiol-reactive electrophiles, induce enzymes that are involved in their metabolism, particularly phase II detoxication enzymes such as glutathione-S-transferase, uridine diphosphate glucuronosyl transferase and nicotinamide adenine dinucleotide phosphate [NAD(P)H:quinone oxide reductase (NQOR1)] (15, 18-21). The NQOR1-inductive effects of α,β-unsaturated carbonyl compounds may be related to their anti-inflammatory activity in most cells and tissues.

In general, inflammatory activity is accompanied by overexpression of nitric oxide synthase 2 (NOS2), leading to production of nitric oxide, which enhances the catalytic activity of cyclooxygenase-2 (COX2) via formation of the peroxinitrite anion (22). COX2 is a downstream target of NOS2. In addition, heme oxygenase-1 (HO1), the inducible isofrom of HO, catalyzes the degradation of heme into biliverdin, iron, and carbon monoxide, and inhibits immune responses and inflammation in vivo. Biliverdin and bilirubin are potent antioxidants that attenuate oxidative stress (23), and HO1 has anti-inflammatory, antioxidant, and antiproliferative effects (22, 24, 25).

Chronic periodontal inflammation is a risk factor for systemic problems such as cardiovascular disease, diabetes mellitus and osteoporosis because of the transport of contributory factors via the blood circulation (26). The rod-shaped, gram-negative anaerobic bacterium Porphyromonas gingivalis is considered to be the major causative agent of periodontitis. P. gingivalis lipopolysaccharide (LPS), a component of the cell wall, acts as a powerful activator of macrophages through the production of pro-inflammatory cytokines (27). Therefore, inhibition of COX2, NOS2 and tumor necrosis factor-alpha (TNFa) protein or gene expression...
in *P. gingivalis* LPS-stimulated gingival fibroblasts and RAW264.7 cells may be one way to screen anti-inflammatory antioxidant agents for their effects on age-related chronic diseases such as periodontitis with systemic problems. In this context, HO1 expression may be useful as a target for anti-inflammatory antioxidant drugs and preventive agents against such age-related chronic diseases such as periodontitis (28-31). We previously reported that the inhibitory effects of phenylpropanoids, including eugenol, bis-eugenol (the ortho-dimer of eugenol), magnolol, honokiol and curcumin on *Escherichia coli* LPS-induced RAW264.7 cells in terms of the release of pro-inflammatory mediators and cytokines, suggested that these compounds had potent anti-inflammatory activities (30, 31).

In the present study, firstly we investigated the cytotoxicity of α,β-unsaturated carbonyl compounds, namely three cinnamates (cinnamaldehyde, methyl cinnamate, and cinnamic acid), two acrylates (2-hydroxyethyl acrylate, ethyl acrylate) and three methacrylates (methyl methacrylate, 2-hydroxyethyl methacrylate, triethylene glycol dimethacrylate) towards RAW264.7 cells using a Cell Counting Kit-8 (CCK-8). RAW 264.7 cells were used to elucidate implications of these compounds in periodontal tissue inflammation and alveolar bone loss. Secondly, we then investigated the stimulatory effects of these compounds on expression of Nos2, Cox2 and *Ho1* mRNA expression in this cell line. Subsequently, we investigated whether these compounds inhibited the expression of mRNAs for Nos2, Cox2 and *Tuba* in *P. gingivalis* LPS-stimulated RAW264.7 cells. On the basis of our results, we considered whether the cytotoxicity of cinnamates and (meth)acrylates is dependent on the pi-electron density of the α,β-carbon in these compounds. The higher the pi-electron density of the β-carbon, the higher the magnetic field at which the nuclear magnetic resonance (NMR) peak is observed, leading to a reduction of the NMR chemical shift. On this premise, we examined the relationship between the cytotoxicity, pro-inflammatory properties or anti-inflammatory properties and the NMR chemical shift of the β-carbons, and the site of electrophilic activity of cinnamates and (meth)acrylates.

**Materials and Methods**

*MATERIALS.* Acrylates (ethyl acrylate, 2-hydroxyethyl acrylate), methacrylates [methyl methacrylate, 2-hydroxyethyl methacrylate, triethylene glycol dimethacrylate (TEGDMA)] and N-acetyl-L-cysteine (NAC) were purchased from Tokyo Kasei Co. (Tokyo, Japan). Cinnamates (trans-cinnamic acid, methyl cinnamate, trans-cinnamaldehyde) were also purchased from Tokyo Kasei Co. The chemical structures of these compounds are shown in Figure 1. Solutions of these compounds were prepared by dissolving each of them in dimethyl sulfoxide, followed by dilution to the required concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). *P. gingivalis* ATCC33277 LPS was obtained from Wako Pure Chemical Industries (Osaka, Japan).

**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% CO2 in air, washed, and then incubated overnight in serum-free RPMI-1640. They were then washed again and treated with the test samples for cytotoxicity and real-time polymerase chain reaction.

**Cytotoxicity.** In brief, RAW264.7 cells (3×10⁴ per well) were cultured in NUNC 96-well plates (flat-well-type microculture plates) (100 μl) or 48 h, after which the cells were incubated with acrylates, methacrylates or cinnamates at a concentration of 0.001-100 mM for 24 h. The relative number of viable cells was then determined using a Cell Counting Kit-8 (CCK-8) (Dojindo Co., Kumamoto, Japan) (32). Ten microliters of CCK-8 solution was added to each well of the plate, which was then was incubated for 1 hour and then the absorbance was measured at 450 nm with a microplate reader (Biochromatic, Helsinki, Finland). LC₅₀ values were determined from the dose–response curves. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student’s *t*-test and one-way ANOVA.

**Effects of antioxidant NAC on ethyl acrylate or cinnamaldehyde effects.** The cells were cultured for 48 hours, and then incubated with 1 mM ethyl acrylate or cinnamaldehyde, with or without NAC at 1:1 molar ratio for 24 h. CCK-8 solution was added to each well and then the absorbance was measured at 450 nm with a microplate reader in a similar manner as described above.

**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 (33). In brief, RAW264.7 cells in NUNC 96-flat-well-type microculture plates (10⁵ cells per well) were pretreated for 30 min with or without acrylates, methacrylates and cinnamates at a concentration of 10-10,000 μM, and then incubated for 3.5 h with or without *P. gingivalis* LPS at 100 ng/ml. Total RNA was then isolated using an RNAeasy Plus Micro Kit (Qiagen Japan Co. Ltd., Tokyo, Japan) in accordance with the instruction manual. cDNA was synthesized from 2 μg total RNA 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 real-time PCR quantification. An equal-volume aliquot of each cDNA was mixed, serially diluted, and used as a standard. TaqMan probes/primer sets for *Cox2*, *Nos2*, *Ho1* 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 manual. The relative amount of target was calculated from standard curves generated in each PCR, and quantitative data with a coefficient of variance of less than 10% were used for further analyses. Each calculated amount of mRNA was standardized by reference to that for 18s rRNA. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student’s *t*-test and one-way ANOVA.
The cytotoxicity was closely positively correlated with the relative pi-electron density ($\delta C_\beta$) of the beta-carbon, i.e. the site of electrophilic activity. In addition, a linear relationship was observed between the LC$_{50}$ and $\delta C_\alpha$ values for these compounds ($r^2=0.87$) (Table II), as $\delta C_\alpha$ decreased, the cytotoxicity increased.

In a similar context, it was shown previously that when acrylates and methacrylates are separated, the LC$_{50}$ of an acrylate series against hepatocytes was linearly correlated with the partial charges of the carbon atoms that make up the $\alpha,\beta$-carbonyl structure; the partial charge of the carbon atoms was determined using semiempirical calculations. The hepatotoxicity of five acrylates in that study was linearly correlated with the increasing partial charge at the $\beta$-carbon ($p<0.05$) and more significantly with the negative partial charge on the $\alpha$-carbon ($p<0.01$) (10). However, the hepatotoxicity of acrylates was shown not to correlate with their lipophilicity (log P) (10). Organic chemistry has shown that nucleophilic addition to the electrophilic (electron-deficient) double bond generates a significant negative charge on the $\alpha$-carbon which is delocalized into the adjacent carbonyl moiety by pi-resonance (34). This suggests that the biological activity of acrylates without substituents at the $\alpha$-carbon may be more influenced by that at the $\beta$-carbon. Next, we investigated the relationship between the LC$_{50}$ and $\delta C_\beta$ or $\delta C_\alpha$ values for cinnamates, and a good positive linear relationship was demonstrated between the LC$_{50}$ and $\delta C_\alpha$ ($r^2=0.91$). The cytotoxicity of cinnamates may be attributed mainly to the pi-electron density at the alpha carbon. This was thought to be reasonable in view of the

### Results and Discussion

**Cytotoxicity and $^{13}$C-NMR chemical shifts.** LC$_{50}$ values and $^{13}$C-NMR chemical shifts of the $\alpha,\beta$-carbon ($\delta C_\alpha$, $\delta C_\beta$) for (meth)acrylates and cinnamates are shown in Tables I and II, respectively. The rank order of cytotoxicity potency for (meth)acrylates was 2-hydroxyethyl acrylate $>$ ethyl acrylate $>$ TEGDMA $>$ 2-hydroxyethyl methacrylates $>$ methyl methacrylate; that for cinnamates was cinnamaldehyde $>$ cinnamic acid $>$ methyl cinnamate, t-cinnamic acid.

The cytotoxicity of 2-hydroxyethyl acrylate and ethyl acrylate was markedly greater than that of methacrylates. The LC$_{50}$ value for 2-hydroxyethyl acrylate against RAW264.7 cells was similar to that against hepatocytes (10). Ethyl acrylate and methyl methacrylate had similar hydrophobicity (log P) but the cytotoxicity of the former was greater than that of the latter. As shown in Table II, the $\delta C_\beta$ value for ethyl acrylate was greater than that for methyl methacrylate, indicating that the electrophilicity of the former is greater than that of the latter. 2-Hydroxyethyl methacrylate was found to be far less cytotoxic than 2-hydroxyethyl acrylate, possibly due to the high electrophilic reactivity of the latter, resulting from the great difference of the $\delta C_\beta$ value between these compounds. Next, we investigated the relationship between the LC$_{50}$ and the $\delta C_\beta$ value for (meth)acrylates. A good linear relationship between the two parameters was observed, as shown below:

$$
\text{LC}_{50}=18.43 \pm 0.16 - 0.14 \pm 0.03 \delta C_\beta \\
(\text{n}=4, r^2=0.92, p<0.05)
$$

(Eq. 1)

2-Hydroxyethyl methacrylate was omitted from Eq. 1 because its cytotoxicity altered the reaction time.

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### Table I. 50% Lethal cytotoxic concentration (LC$_{50}$) and hydrophobicity (log P) for acrylates, methacrylates and cinnamates used in this study.

| Compound                  | LC$_{50}$ (mM) | Log P$^a$ |
|---------------------------|----------------|-----------|
| Experiment 1              |                |           |
| Methyl methacrylate       | 1.19 $\pm$0.04 | 1.38      |
| 2-Hydroxyethyl methacrylate | $>0.9$        | 0.47      |
| Triethylene glycol dimethacrylate | 0.89 $\pm$0.04 | 1.55      |
| Ethyl acrylate            | 0.48 $\pm$0.05 | 1.33      |
| 2-Hydroxyethyl acrylate   | 0.15 $\pm$0.04 | 0.17$^b$  |
| Experiment 2              |                |           |
| t-Cinnamic acid           | 2.87 $\pm$0.02 | 2.13$^c$  |
| Methyl cinnamate          | 12.07 $\pm$0.01 | 2.62$^c$  |
| t-Cinnamaldehyde          | 0.44 $\pm$0.02 | 1.91$^c$  |

$^a$Taken from Fujisawa and Kadoma (38); $^b$Taken from Chan and O’Brien (10); $^c$https://pubchem.ncbi.nlm.nih.gov/t-cinnamaldehyde, methyl cinnamate, t-cinnamic acid.

### Table II. $^{13}$C-NMR chemical shifts (δ) of β-carbon ($C_\beta$), α-carbon ($C_\alpha$) and electrophilicity (ω) for acrylates, methacrylates and cinnamates used in this study.

| Compound                  | $\delta C_\beta$ (ppm) | $\delta C_\alpha$ (ppm) | $\omega^c$ (eV) | $\omega^d$ (eV) |
|---------------------------|------------------------|-------------------------|-----------------|-----------------|
| Methyl methacrylate       | 125.23                 | 136.15                  | –               | 2.681           |
| 2-Hydroxyethyl methacrylate | 125.89                 | 135.96                  | –               | 2.725           |
| Ethyl acrylate            | 130.56                 | 128.15                  | 2.829           |                 |
| Ethyl acrylate            | 130.56                 | 128.15                  | 2.829           |                 |
| 2-Hydroxyethyl acrylate   | 131.30$^a$             | 127.50$^a$              | 1.533           | 3.065           |
| t-Cinnamic acid           | 141.55$^b$             | 126.83$^b$              | –               | –               |
| Methyl cinnamate          | 144.79$^b$             | 117.92$^b$              | –               | –               |
| t-Cinnamaldehyde          | 152.46$^b$             | 131.17$^b$              | 2.098           | –               |

$^a$Taken from Enoch et al. (53). $^b$Taken from Ishihara and Fujisawa (52).
similar molecular structures of acrylates and cinnamates (β-phenyl acrylates) but the LC50 for acrylates was found to be negatively correlated with their δCα value (10). The cytotoxicity of cinnamates and (meth)acrylates against RAW264.7 cells was also not affected by their hydrophobicity (log P).

In contrast, the cytotoxicity of methacrylates can be affected by a combination of electronic and steric factors introduced by methyl substitution on α-carbons. We previously investigated the possible link between this cytotoxicity and Ca2+ mobilization in a human salivary gland carcinoma cell line and human gingival fibroblasts by (meth)acrylates. This revealed that hydrophilic 2-hydroxyethyl methacrylate, as well as acrylic acid and methacrylic acid, have low cytotoxicity and elicit only a small elevation of intracellular calcium concentration [Ca2+]i, whereas hydrophobic (meth)acrylates are cytotoxic and elicit a large [Ca2+]i elevation (35). It is well established that variations in cytosolic calcium concentration, [Ca2+]i, trigger key cellular functions, and that cellular Ca2+ overload is highly toxic, being related to the induction of apoptosis (36). The cytotoxicity of (meth)acrylates against human salivary gland carcinoma cell line and human gingival fibroblasts is related to their log P (35), the cytotoxic mechanisms being dependent on the cell species and inducers involved. We also previously investigated the relationship between the in vivo toxicity of (meth)acrylates in mice (oral and intraperitoneal 50% lethal dose) and GSH reactivities predicted by their δCβ values, demonstrating a good relationship between these parameters in series of both acrylates and methacrylates (37, 38). Despite a possible discrepancy between the in vivo and in vitro toxicities, the cytotoxicity of (meth)acrylates in RAW264.7 cells is affected by the electrophilicity of the monomers.

Next, to clarify the cytotoxic effects of GSH reactivity, the cytotoxicity of ethyl acrylate and cinnamaldehyde with or without the antioxidant NAC was investigated and the effect of NAC was evaluated. The results are shown in Figure 2. The cytotoxicity of both compounds was greatly reduced by addition of NAC. Cinnamaldehyde and ethyl acrylate probably interact spontaneously and more rapidly in the presence of NAC. This interaction may involve Michael-type addition between the nucleophilic NAC and electrophilic cinnamaldehyde or ethyl acrylate, and NAC may block the induction of monomer-mediated DNA and apoptosis. The mechanism of cinnamaldehyde cytotoxicity was investigated previously in isolated F344 rat hepatocytes, and the results suggested that cinnamaldehyde, but not cinnamic acid, reacted spontaneously with reduced GSH in vitro (39). Taken together with our results, the evidence suggests that the toxicity of cinnamaldehyde and ethyl acrylate is attenuated through interaction between the β-carbons of these compounds and NAC in vitro. However, at the cellular level, cinnamaldehyde and ethyl acrylate may induce a variety of stress responses in RAW264.7 cells, including GSH depression, oxidative stress, and mitochondrial dysfunction, as well as release of inflammatory mediators and pro-inflammatory cytokines, resulting in cell death.

Cox2 mRNA expression in RAW264.7 cells stimulated with cinnamates and (meth)acrylates. It has been well documented that the inflammatory response to E. coli and P. gingivalis LPS or P. gingivalis fimbriae involves the production of COX2, NOS2 and proinflammatory cytokines such as TNFα, interleukin (IL)-6, and IL1β in RAW264.7 cells (28, 30, 40). Contrary to the pro-inflammatory effects of LPS, the inflammatory mediators COX2 and NOS2 and cytokines such as TNFα at both the protein and gene levels may also be expressed in macrophages stimulated by α,β-unsaturated carbonyl compounds.

Therefore, we investigated the expression of Cox2 mRNA in RAW264.7 cells stimulated by cinnamates, acrylates and methacrylates. The results are shown in Figures 3 and 4, respectively. Cinnamaldehyde stimulated Cox2 mRNA expression at 0.1 mM, whereas 2-hydroxyethyl acrylate stimulated its gene expression at 1 mM. Other compounds such as methyl cinnamates, ethyl acrylate and methacrylates had no effect on Cox2 expression. The concentration of cinnamaldehyde required for induction of Cox2 mRNA expression was approximately 10-fold higher than that of 2-hydroxyacrylate. In this study, despite the relatively high δCβ value for cinnamates, cinnamaldehyde was the most cytotoxic compound and potently elicited Cox2 gene expression, possibly reflecting the fact that aldehydes tend to have higher inductive potency. Among (meth)acrylates, 2-hydroxyethyl acrylate was the most cytotoxic and had the
highest $\Delta C_\beta$ value, possibly due to the induction of Cox2 gene expression. Macrophages and other activated inflammatory cells secrete high amounts of prostaglandin E$_2$ (PGE2), nitric oxide (NO), and cytokines such as IL6, TNF$\alpha$, and IL1$\beta$. COX2 is a key mediator of the inflammatory process, being responsible for production of PGE2 from arachidonic acid. Down-regulation of Cox2 is a condition for inhibition of PGE2, which is expressed in all processes that lead to major features of inflammation, i.e., swelling, redness, and pain (41, 42). On the other hand, it is suggested that up-regulation of Cox2 might lead to an increase of pro-inflammatory activity. It was shown that low concentrations (up to 1 $\mu$g/ml) of cinnamaldehyde induce a slight increase in nuclear factor-$\kappa$B (NF-$\kappa$B) activation (4), suggesting a degree of intrinsic pro-inflammatory activity. It has also occasionally been reported that cinnamaldehyde can induce intraoral allergic contact dermatitis; the most commonly implicated allergens are metals that are incorporated into dental appliances, but cinnamaldehyde is widely used as a flavoring agent in foods and dentifrices (43). Our results suggest that cinnamaldehyde ($\beta$-phenyl
acrolein) and 2-hydroxyethyl acrylate may exert cytotoxic/genotoxic cell damage in mammalian cells due to their high reactivity with cellular nucleophiles (e.g., Michael adduct formation with DNA bases and with GSH) (44-46).

In other contexts, cinnamaldehyde has been reported to exert marked antimutagenic effects against mutations induced by UV-mimic mutagens, but not those induced by N-methyl-N'-nitro-N-nitrosoguanidine or ethyl methanesulfonate, suggesting that cinnamaldehyde may interfere with the inducible error-prone DNA-repair pathway (47). In contrast, 2-hydroxyethyl acrylate, ethyl acrylate and TEGDMA have been listed as mutagenic (45).

**Stimulation of Nos2 mRNA expression by cinnamates and (meth)acrylates.** COX2 expression is selectively induced by pro-inflammatory cytokines at sites of inflammation, and Nos2 may be involved in the inflammatory process (48). Nos2 may be expressed in macrophages after induction of oxidative stress due to cinnamates and (meth)acrylates. Cinnamaldehyde elicited Nos2 mRNA expression at 0.1 mM, whereas methyl cinnamate did so at 10 mM, indicating that the inductive ability of the former is approximately two orders of magnitude greater than that of the latter. This may be due to the large electrophilicity of cinnamaldehyde, which has a reactive aldehyde moiety. 2-Hydroxyethyl acrylate, ethyl acrylate and TEGDMA elicited Nos2 mRNA expression at the cytotoxic concentration of 1 mM (Figure 5). Methyl methacrylate elicited Nos2 mRNA expression at a high cytotoxic concentration of 10 mM, whereas 2-hydroxyethyl methacrylate and cinnamic acid were ineffective over a wide concentration range of 1-10 mM. These findings suggest that the up-regulatory effect of monomers on Nos2 gene expression may not necessarily be controlled by their ω potency alone, and that the relative hydrophobic/hydrophilic balance of monomers is also important. Although methyl cinnamate and cinnamic acid have higher δCβ values than 2-hydroxyethyl or ethyl acrylate, they elicited weak Nos2 gene expression and were not effective. Up-regulation of Nos2 expression by TEGDMA, a dimethacrylate, may be attributable to the double Michael reaction acceptor. Production of PGE2 stimulated by TEGDMA, but not by 2-hydroxyethyl methacrylate, has been reported previously in RAW264.7 cells (49). TEGDMA and 2-hydroxyethyl methacrylate do not affect the expression of inducible nitric oxide synthase (iNos) mRNA (49).

We previously investigated the cytotoxicity mechanism of (meth)acrylates (50), and eugenol-related compounds, using computational methods (30, 51). The energy values of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energy are defined as \( E_{LUMO} \) and \( E_{HOMO} \), respectively. Chemical hardness (\( \eta \)), electronegativity (\( \chi \)), electrophilicity (\( \omega \)) and softness (\( \sigma \)) are calculated using equations 2-5, respectively:

\[
\eta = \frac{(E_{LUMO} - E_{HOMO})}{2} \quad (\text{Eq. 2})
\]

\[
\chi = \frac{-(E_{LUMO} + E_{HOMO})}{2} \quad (\text{Eq. 3})
\]

\[
\omega = \frac{\chi^2}{2\eta} \quad (\text{Eq. 4})
\]

\[
\sigma = \frac{1}{\eta} \quad (\text{Eq. 5})
\]

The log LC\(_{50}\) for phenyl propanoids, which are eugenol-related compounds, was reported to be linearly and positively related to their ω value calculated using the B3LYP/6-31G* level (\( p<0.001 \)) (30). In the present study, we also investigated the relationship between the ω and δCβ values for cinnamaldehyde, 2-hydroxy acrylates and ethyl acrylate using cited data (Table II), and found that the ω value of these compounds was markedly linearly negatively correlated with their δCβ value (\( n=3, r^2=0.999, p<0.01 \)). We also calculated the ω value of (meth)acrylates using Eq. 4 based on our previous data (52) (Table II), and examination of the relationship between the ω and δCβ values for these compounds revealed a moderate positive linear correlation (\( n=6, r^2=0.662, p<0.05 \)). These findings suggested that there is a linear relationship between the ω and δCβ value for cinnamaldehyde and (meth)acrylates. It may therefore be possible to estimate the electrophilicity (\( \omega \)) of α,β-unsaturated carbonyl compounds from their δCβ values. The ω value is a higher-order parameter that combines softness with electronegativity (\( \chi \)) and represents a sensitive measure of electrophilic reactivity. The ω value has been used as a parameter of allergic dermatitis and allergic sensitization associated with α, β-unsaturated carbonyl compounds (53), allergic potential increasing with the ω value. Induction of inflammatory mediators and cytokines by α, β-unsaturated carbonyl compounds may be related to other parameters in addition to the ω value. The ω value (HOMO-LUMO energy gap) for (meth)acrylates was also calculated from our previous data using Eq. 2 (38, 50); the rank order magnitude of ω (eV) is ethyl acrylate (5.495) > methyl acrylate (5.492) > methyl methacrylate (5.245) > 2-hydroxyethyl methacrylate (5.233) > 2-hydroxyethyl acrylate (5.174) > TEGDMA (5.092). A lower ω value for bioactive compounds indicates that the molecules are more easily excitable. Therefore, their σ values (softness) were calculated using Eq. 5.

The σ value (eV) for methyl methacrylates, 2-hydroxyethyl methacrylate, 2-hydroxyethyl acrylate and TEGDMA was 0.1907, 0.1911, 0.1932 and 0.1936, respectively, being grouped as having the most softness among the (meth)acrylates we tested. The σ value indicates the ease with which electron redistribution takes place during covalent bonding, i.e., donation of electrons by nucleophiles and acceptance by electrophiles. Therefore, with respect to electrophilic species, it is often the case that softness (i.e., a higher σ value) is correlated with the ease of adduct formation (54), that is, the σ energy serves as a measure of
molecular excitability. Up-regulation of Nos2 mRNA expression by TEGDMA and 2-hydroxyethyl acrylate may be related to their high softness (55). Although no stimulation of Nos2 mRNA expression by 2-hydroxyethyl methacrylate was observed in the present study, it may be induced under certain conditions in view of its relatively high softness.

Stimulation of H01 mRNA expression by cinnamates and (meth)acrylates. The results are shown in Figures 4 and 6. H01 expression is enhanced not only by free heme, but also by various pro-inflammatory agents such as NO, LPS, cytokines, heavy metals, and other oxidants (56-59). Cinnamaldehyde and methyl cinnamate did not elicit any H01 mRNA expression over a wide concentration range of 0.1-10 mM. In contrast,
2-hydroxyethyl acrylate and ethyl acrylate at 0.1 mM potently elicited expression of Ho1 mRNA whereas methyl methacrylate did so poorly at the high concentration of 1 mM. Cinnamates with phenyl substituents at the β-carbons (β-phenyl acrylates) and methacrylates with methyl substituents at the α-carbons did not elicit Ho1 gene expression at non-cytotoxic concentrations. In contrast, acrylates without any substituents at both the α- and β-carbons elicited potent Ho1 gene expression. Michael addition of methane thiol (CH₃SH), a model nucleophile of GSH, was performed previously at the B3LYP/6-31G* level for 47 Michael reaction acceptors, including α,β-unsaturated aldehydes, ketones and esters, focusing on the 1,2-olefin addition pathway without and with initial protonation. Michael reaction acceptors such as acrylates may be formed preferentially by direct 1,2 addition across the electron-poor double bond Cβ=Cα of Michael reaction acceptors (60). Induction of Ho1 gene expression by acrylates may be attributable to the high electrophilicity of their Cβ=Cα bond, which lacks substituents at the α- and β-carbons. These findings suggest that induction of Ho1 gene expression may be attributable to respective differences in the electrophilicity of β-arbon (δCβ) and steric interactions, as cinnamates with β-phenyl substituents and methacrylates with α-methyl substituents at the Cβ=Cα bond did not elicit Ho1 mRNA expression in the present study. Interestingly, methyl methacrylate weakly elicited Ho1 mRNA expression. Therefore, we also calculated the σ values for some (meth)acrylates using the B3LYP/6-31G* level (30), which yielded a σ value (eV) rank order of methyl methacrylates (0.329) > TEGDMA (0.327) > ethyl acrylate (0.323) > 2-hydroxyethyl methacrylates (0.322). Methyl methacrylate had a relatively high σ value. This compound may be an excitable molecule, although its σ value is relatively small (Table II).

Talalay et al. (15) reported that methyl acrylate is a potent inducer of NQOR1 in Hepa 1c1c7 cells, whereas cinnamaldehyde and methyl cinnamates are weakly effective and methyl methacrylate and cinnamic acid are inactive. Induction of NQOR1 expression in the human hepatoma cell line HepG2 was measured at both enzyme activity and RNA level after exposure to methyl acrylates and ethyl acrylate (61), the results suggesting that both compounds are potent inducers of HepG2 gene expression. The inductive potency is reliable, and may be related to anti-inflammatory activity via induction of Ho1 expression. Expression of the phase II detoxication gene NQOR1 is related to expression of the Ho1 gene and protein.

A PubMed search of recent articles on nuclear factor-erythroid related factor-2 (NRF2), HO and Ketch-like ECH-associated protein (KEAP1), an oxidative stress sensor, suggested that under normal physiological conditions, NRF2 in the cytoplasm is bound to its repressor, KEAP1. Upon activation, NRF2 is translocated to the nucleus and binds to the antioxidant response element located in the promoter region of some anti-oxidant genes, including that for the cytoprotective protein HO. Since the HO1 gene harbors a binding site for NRF2, mutual stimulatory and regulatory interactions between NRF2 and HO1 have been reported, and in fact the interaction between NRF2 and HO1 has been implicated in the regulation of many physiological antioxidants, including superoxide dismutase, catalase, glutathione S-transferase, peroxidase, NQOR1, and thioredoxin (62, 63).

In other contexts, research on HO1 induction by cinnamaldehyde has revealed that the latter up-regulated the cellular protein level of HO1 and promoted translocation of NRF2 to the nucleus in human dental pulp cells. Cinnamaldehyde-mediated NRF2/HO1 activation reduced the level of reactive oxygen species (ROS) and protected human dental pulp cells from H₂O₂-induced oxidative stress, which induced apoptosis (64). In contrast, it has been reported that overexpression of NFKB p65 mRNA induced by high levels of glucose was markedly and dose-dependently attenuated by cinnamaldehyde in an in vitro dorsal root ganglion neuron model of diabetic neuropathy, whereas the expression of NRF2 and HO1 was not up-regulated (65). NRF2/HO-1 activation and the signaling mechanisms involved may be dependent on the cell species, inducers employed and reaction time.

In the present study, 2-hydroxyethyl acrylate and ethyl acrylate potently elicited Ho1 mRNA expression at low non-cytotoxic concentrations, suggesting that these electrophilic compounds may be protective against inflammation, oxidative damage, and cell death. Carbon monoxide, another byproduct of heme degradation by HO, inhibits NO secretion and reduces inflammation. Up-regulation of Ho1 mRNA expression for α,β-unsaturated carbonyl compounds may be associated with induction of NQOR1 (15, 16). HO1 exerts a strong antioxidant and antiapoptotic effect favoring cancer cell growth (58). However, these active acrylates show marked cytotoxicity and pro-inflammatory activity and are well known to be toxic, allergic and mutagenic. Further studies of these compounds will be necessary to clarify the effects of Ho1 expression at the gene and protein level. Inhibitory effects on P. gingivalis LPS-stimulated expression of Cox2, Nos2 and Tnfa mRNA. LPS is known to induce high levels of ROS, thus promoting cytotoxicity, apoptosis, and pro-inflammatory activity. A high level of ROS modulates a number of cell signaling pathways and regulates the expression of multiple genes such as those for COX2 and NOS2 in vitro and in vivo (66). In general, inflammatory activity is accompanied by overexpression of inducible nitric oxide synthase, iNOS, leading to production of nitric oxide, which enhances the catalytic activity of COX2 via formation of the peroxyxinitrite anion (22). COX2 is a downstream target of NOS2.

The inhibitory activity of cinnamates against the expression of P. gingivalis LPS-stimulated Cox2, Nos2 or Tnfa mRNA is shown in Figure 7. Cinnamaldehyde
suppressed Cox2, Nos2 or Tnfa mRNA expression at a concentration of 0.1-1 mM, and methyl cinnamate did so at concentrations of 1-10 mM. In contrast, cinnamic acid had no effect. This may be due to the fact that free carboxyl groups weaken the efficiency of Michael reaction acceptors. Cinnamaldehyde, but not cinnamic acid, cinnamic alcohol and coumarin, inhibits the production of NO, TNFα and PGE2 by LPS-stimulated RAW264.7 cells (1). Cinnamaldehyde was also reported to inhibit the inflammatory activity of LPS-stimulated macrophages via suppression of mitogen-activated protein kinase (MAPK) phosphorylation and pro-inflammatory gene expression (67). Furthermore, it has been reported that when RAW264.7 macrophages were treated with cinnamaldehyde together with E. coli LPS, significant concentration-dependent inhibition of NO, TNFα, and PGE2 production was detected (1). It was also reported that cinnamaldehyde exerts a suppressive effect on toll-like-receptor-4 (TLR4)-mediated signaling and that this effect occurs through inhibition of receptor oligomerization (68). In contrast, cinnamic acid showed only low anti-inflammatory activity in a LPS-inducible inflammatory model in vitro (1), similarly to the findings of the present study.
Next, we investigated the effects of acrylates and methacrylates on *P. gingivalis* LPS-stimulated expression of *Cox2*, *Nos2* and *Tnfa* mRNA. The results are shown in Figure 8. Expression of *Cox2*, *Nos2* and *Tnfa* mRNA was markedly and significantly suppressed by 0.1 mM 2-hydroxyethyl acrylates and ethyl acrylate, particularly the former, but only weakly by TEGDMA and methyl methacrylate, even at a highly cytotoxic concentration of 1 mM, the inhibitory effect being less than 50%. Cell-surface antigens and cytokines in macrophages have been reported to be up-regulated after exposure to LPS, whereas TEGDMA causes significant down-regulation dependent on exposure time. LPS and TEGDMA act differently on MAPK (69). In the present study, 2-hydroxyethyl methacrylate did not suppress the expression of *Cox2* and *Tnfa* mRNA, but elicited overexpression of *Nos2* mRNA. The reason is not known. Comparison of the anti-inflammatory activity of cinnamates with that of (meth)acrylates in terms of suppression of *Cox2*, *Nos2* and *Tnfa* mRNA expression suggested that cinnamaldehyde and methyl cinnamate preferentially suppressed the expression of *Tnfa* in comparison with *Nos2* or *Cox2*, whereas 2-hydroxyethyl acrylate and ethyl acrylate appeared to preferentially suppress *Nos2* gene expression in comparison with *Cox2* or *Tnfa* (Figures 7 and 8). It is well known that LPS can directly activate macrophages, which trigger the production of inflammation mediators such as NO and TNFα at the protein level. Cinnamaldehyde suppresses LPS-induced production of NO and the expression of inflammatory protein products such as INOS, COX2 and TNFα. Pro-inflammatory cytokines such as TNFα are small secreted proteins which mediate and regulate immunity and inflammation. Production of TNFα is crucial for the synergistic induction of NO synthesis in LPS-stimulated macrophages in the presence of synapic acid (70). Cinnamates preferentially inhibit TNFα with LPS (71). When RAW264.7 cells are treated with cinnamaldehyde together with LPS for 24 h, the IC50 value required for inhibition of PGE2 production was about 37.7 μM, whereas that for TNFα production was about 30 μM (1). Cinnamaldehyde inhibited production of TNFα more strongly than that of PGE2 at the protein level. This marked ability of cinnamaldehyde to inhibit the production of TNFα at the protein level was similar to that of cinnamaldehyde at the gene level in the present study. Although cinnamaldehyde, 2-hydroxyethyl acrylate and ethyl acrylate had excellent anti-inflammatory properties, they also had potent pro-inflammatory properties. In contrast, methyl cinnamate had excellent anti-inflammatory properties together with slight cytotoxic and pro-inflammatory properties. Therefore, methyl cinnamate may have potential anti-inflammatory applications against periodontal disease and related systemic conditions.

α,β-Uunsaturated carbonyl compounds may exert dual pro-and anti-inflammatory properties. These compounds may not be ideal for drug design because of their tendency to undergo the Michael addition reaction leading to undesirable side-effects such as cytotoxicity, skin allergy, mutagenicity and carcinogenicity. Despite such side-effects, these agents may exert a range of beneficial effects similar to those of non-steroidal anti-inflammatory drugs, including anti-inflammatory and anticancer activity (72).

Cinnamaldehyde, a major chemical component of the cinnamon tree, has been shown to induce cellular ROS generation, leading to expression of the COX2 and NOS2 genes, and possibly apoptotic cell death. Cinnamaldehyde has an active Michael acceptor pharmacophore and is generally considered safe, with approval for use in the United States (73). It is noteworthy that some nucleophiles such as water, hydroxy anion (OH−), O2•− radical, peroxy radical (ROO•), nitric oxide (NO•) and GSH may be able to interact with the electrophilic β-carbon of cinnamates. LPS-treated cells generate a large amount of ROS/reactive nitrogen species (RNS) due to oxidative stress. It has been reported that cinnamaldehyde attenuates the release of ROS from RAW264.7 macrophages (71).

Comparison of the anti-inflammatory activity of cinnamates with that of (meth)acrylates in terms of suppression of *Cox2*, *Nos2* and *Tnfa* mRNA expression suggested that cinnamaldehyde and methyl cinnamate preferentially suppressed the expression of *Tnfa* in comparison with *Nos2* or *Cox2*, whereas 2-hydroxyethyl acrylate and ethyl acrylate appeared to preferentially suppress *Nos2* gene expression in comparison with *Cox2* or *Tnfa* (Figures 7 and 8). It is well known that LPS can directly activate macrophages, which trigger the production of inflammation mediators such as NO and TNFα at the protein level. Cinnamaldehyde suppresses LPS-induced production of NO and the expression of inflammatory protein products such as INOS, COX2 and TNFα. Pro-inflammatory cytokines such as TNFα are small secreted proteins which mediate and regulate immunity and inflammation. Production of TNFα is crucial for the synergistic induction of NO synthesis in LPS-stimulated macrophages in the presence of synapic acid (70). Cinnamates preferentially inhibit TNFα with LPS (71). When RAW264.7 cells are treated with cinnamaldehyde together with LPS for 24 h, the IC50 value required for inhibition of PGE2 production was about 37.7 μM, whereas that for TNFα production was about 30 μM (1). Cinnamaldehyde inhibited production of TNFα more strongly than that of PGE2 at the protein level. This marked ability of cinnamaldehyde to inhibit the production of TNFα at the protein level was similar to that of cinnamaldehyde at the gene level in the present study. Although cinnamaldehyde, 2-hydroxyethyl acrylate and ethyl acrylate had excellent anti-inflammatory properties, they also had potent pro-inflammatory properties. In contrast, methyl cinnamate had excellent anti-inflammatory properties together with slight cytotoxic and pro-inflammatory properties. Therefore, methyl cinnamate may have potential anti-inflammatory applications against periodontal disease and related systemic conditions.

In conclusion, α,β-unsaturated carbonyl compounds such as cinnamates (CMA, MC) and acrylates (2-HEA, EA) but not methacrylates (2-HEMA, MMA, TEGDMA) potently suppressed *P. gingivalis* LPS-stimulated *Cox2*, *Nos2* and *Tnfa* mRNA expression. Treatment with two acrylates up-regulated *Ho1* mRNA expression. The Michael addition in biological systems is a likely molecular mechanism for the toxicity and pro-/anti-inflammatory property of such compounds. MC had little cytotoxicity and anti-inflammatory activity.
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