ANTICANCER RESEARCH *25*: 3263-3270 (2005)

Induction of Cytotoxicity and Apoptosis and Inhibition of Cyclooxygenase-2 Gene Expression by Eugenol-related Compounds

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Abstract. *Induction of cytotoxicity and internucleosomal DNA fragmentation by 4-allyl-2-methoxyphenol (eugenol, EUG), 2-methoxy-4-methylphenol (MMP), 3,3'-dimethoxy-5,5'-di-2-propenyl-1,1'-biphenyl-2,2'-diol (bis-EUG) and 3,3'-di-methoxy-5,5'-dimethyl-1,1'-biphenyl-2,2'-diol (bis-MMP) were investigated in HL-60 leukemia cells. The 50% cytotoxic concentrations (CC_{50}) for EUG, MMP, bis-EUG and bis-MMP were 0.38 mM, 0.38 mM, 0.18 mM and 0.20 mM, respectively. DNA fragmentation was induced most strongly by bis-EUG, followed by EUG, MMP and bis-MMP. The expression of MnSOD and, less strongly, Cu/ZnSOD activity, as assessed by acrylamide gel electrophoresis, was inhibited by EUG, suggesting mitochondrial dysfunction. The expression of the mRNAs for MnSOD and Cu/ZnSOD in HL-60 cells, as assessed by RT-PCR, was significantly inhibited by treatment with 1 mM EUG for 1 hour. Furthermore, inhibition of SOD mRNAs expression by EUG was strongly potentiated by the addition of 5 mM N-acetyl cysteine (NAC) or glutathione (GSH), whereas NAC or GSH alone did not affect the expression of SOD mRNAs. The cytotoxicity of EUG was significantly enhanced by high concentrations of NAC or GSH, which may be attributed to the inhibition of SOD mRNAs expression by the synergistic action of EUG and GSH or NAC. The regulatory effects of eugenol-related compounds on lipopolysaccharide (LPS)-induced cyclooxygenase-2 (COX-2) gene expression in RAW 264.7 cells were investigated by Northern blot analysis. Bis-EUG, MMP and bis-MMP inhibited COX-2 gene expression at concentrations of 300 μM, 500 μM and 500 μM, respectively. In contrast, no inhibitory effect of EUG was found over the wide concentration range of 10-500 μM, possibly as a result of the extensive mitochondrial dysfunction induced by this compound, which possesses potent pro-oxidative activity. Eugenol-related compounds, particularly bis-EUG, may act as nonsteroidal anti-inflammatory drug (NSAID)-like compounds.*

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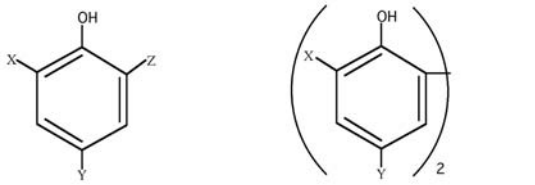
Key Words: Eugenol-related compounds, cytotoxicity, apoptosis, HL-60 cells, LPS, COX-2, RAW cells.

We have previously reported the antioxidant and pro-oxidant activities and cytotoxicity of eugenol-related compounds, showing that dimers of eugenol-related compounds have much more potent antioxidant activity than do the original monomers (1, 2). We have also previously reported that methoxyphenols, such as 2,6-di-*tert*-butyl-4-methoxyphenol (BHA), induce tumor-specific cytotoxicity and internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in HL-60 leukemic cells (3). These findings prompted us to investigate the apoptosis-inducing effects of eugenol-related compounds and methoxyphenols that might have greater cytotoxic activity against tumor cells. There are few studies of the apoptosis-inducing effects of eugenols in cancer cells (4, 5). Thus, as an extension of our previous studies (1-3), we investigated the induction of apoptosis by eugenol and related compounds (MMP, bis-EUG and bis-MMP) in HL-60 cells and the effect of *N*-acetyl cysteine, glutathione and glutathione ethyl ester (GSHee) on these activities. Recent reports have described the role of *COX-2* and prostaglandins in apoptosis, particularly of cancer cells (6, 7), leading us to ask whether the apoptosis-inducing activity of eugenol-related compounds is dependent on *COX-2* inhibition. Thus, the regulatory effects of eugenol, MMP, bis-EUG and bis-MMP on LPS-induced *COX-2* gene expression in RAW 264.7 cells were investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: eugenol (4-allyl-2-methoxyphenol) and 2-



EUG X=OMe Y=Allyl Z=H *bis*-EUG X=OMe Y=Allyl

MMP X=OMe Y=Me Z=H *bis*-MMP X=OMe Y=Me

Figure 1. *Chemical structures of eugenol-related compounds.*

methoxy-4-methylphenol (MMP) (Tokyo Kasei Kogyo Ltd., Tokyo, Japan). The dimers of eugenol (bis-EUG; 3,3'-dimethoxy-5,5'-di-2-propenyl-1,1'-biphenyl-2,2'-diol) and of MMP (bis-MMP; 3,3'-dimethoxy-5,5'-dimethyl-1,1'-biphenyl-2,2'-diol) were synthesized (2). The chemical structures of the eugenol-related compounds are shown in Figure 1. The biological materials used were as follows: Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA); fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA); *N*-acetyl cysteine, glutathione, glutathione ethyl ester, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), riboflavin and nitro blue tetrazolium (NBT) (Sigma Chemical Co., St. Louis, MO, USA); dimethyl sulfoxide (DMSO), RNase A and proteinase K (Sigma Chemical Co.); Megaprime DNA labeling system and 5'-[α-^{32}P]dCTP (Amersham Biosciences Co., Piscataway, NJ, USA); *Escherichia coli* O111 B4-derived LPS (List Biological Laboratories, Inc., Campbell, CA, USA); RAW 264.7 cells (Riken Cell Bank, Saitama, Japan); COX-2 cDNA probes (Cayman Chemical Co., Ann Arbor, MI, USA); plasmid containing β-actin cDNA (Japanese Cancer Research Bank, Tokyo, Japan).

Cell culture. The HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Cells of the murine macrophage cell line RAW 264.7 were cultured to the subconfluent state in RPMI 1640 medium supplemented with 10% FBS at 37˚C under 5% CO_2 in air, washed and then incubated overnight in serum-free RPMI 1640. They were then washed further and treated with the test samples.

Assay for cytotoxic activity. Near-confluent HL-60 cells were incubated for 24 h without (control) or with various concentrations of EUG, MMP, bis-EUG or bis-MMP. The numbers of viable HL-60 cells were determined by trypan blue exclusion (8).

Assay for DNA fragmentation. DNA fragmentation was assayed by agarose gel electrophoresis. The cells were washed once with phosphate-buffered saline (PBS), then lysed in 20 μL of lysis buffer [50 mM Tris-HCl, pH 7.8, 10 mM EDTA and 0.5% (w/v) sodium *N*-lauroyl sarcosinate]. This solution was incubated sequentially with 0.5 mg/mL RNase A at 50˚C for 60 min and 0.5 mg/mL proteinase K at 50˚C for 60 min. DNA was extracted with chloroform/isoamyl alcohol, precipitated and subjected to 2% agarose gel electrophoresis. DNA from apoptotic HL-60 cells (apoptosis induced by UV irradiation) was run in parallel. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay of superoxide dismutase (SOD) activity. MnSOD and Cu/ZnSOD activities were detected by acrylamide gel electrophoresis with a modification of the method of Beauchamp and Fridovich (9). The cells were dissolved in sample buffer (1% Triton X-100, 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4) and used as an enzyme source. An aliquot equivalent to 1x10^6 cells was subjected to 9% polyacrylamide gel electrophoresis (PAGE) for 60 min. The gel was stained first with 4 mg/mL NBT, then with riboflavin (10 μg). It was left in the dark for 15 min after the addition of each staining agent, then illuminated overnight. During illumination, the gel became uniformly blue, except for the areas containing SOD. The gel was destained by washing in water and subjected to image processing with a scanner (Canon N656U; Canon Inc., Tokyo, Japan).

Determination of MnSOD and Cu/ZnSOD mRNAs by the reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated with a Pure Script RNA Isolation kit (Gentra Systems, Minneapolis, MN, USA). HL-60 cells (1 x 10^6) were lysed in 300 μL of cell lysis solution and then 100 μL of Protein-DNA precipitation solution was added. The lysis fluid was centrifuged at 15,000 x g for 3 min, and then the pellet was washed in 300 μL of 75% isopropanol. After further centrifugation at 15,000 x g for 1 min, the pellet was air-dried for 15 min, then dissolved in diethyl pyocarbonate (DEPC)-treated water.

RT-PCR was performed on 1.0 μg of total RNA with the Rever Tra Ace system (Toyobo Co. Ltd., Osaka, Japan). Single-strand cDNA, obtained from the RT reaction with an oligo(dT)$_{20}$ primer, was amplified with the KOD Plus system (Toyobo) with MnSOD-specific primers (5'-TCCCCGACCTGCCCTACGAC-3' and 5'-CATTCTCCCAGTTGATTACAT-3'), Cu/ZnSOD-specific primers (5'-ATGGCCACGAAGGCCGTGTG-3' and 5'-GGAATGTTTA TTGGGCGATCCA-3') and G3PDH-specific primers (5'-TCCAC CACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCAT CAC-3'), according to the protocol. The RT-PCR products were applied to 2% agarose gels, stained with ethidium bromide and photographed under UV light.

Plasmid preparation. The methods used for plasmid preparation were described previously (10).

Northern blot analysis. RAW 264.7 cells (1 x 10^6 cells) were treated with the test samples and their total cellular RNA was then extracted by the AGPC procedure (11). As described previously (11), the RNA was subjected to 1% agarose gel electrophoresis and blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with cDNA probes that had been labeled with 5'-[α-^{32}P]dCTP by use of a Megaprime DNA labeling system. After hybridization, the membranes were washed, dried and exposed overnight to Kodak X-ray film (Eastman Kodak Co., Rochester, NY, USA) at –70˚C. β-Actin was used as internal standard for quantification of total RNA in each lane of the gel.

Results

Cytotoxicity. Treatment with eugenol-related compounds caused a dose-dependent decrease in the viability of HL-60 cells. The concentrations of EUG, MMP, bis-EUG and bis-MMP at which each compound was cytotoxic to 50% of cells (CC_{50}) were 0.38 mM, 0.38 mM, 0.18 mM and 0.20 mM,

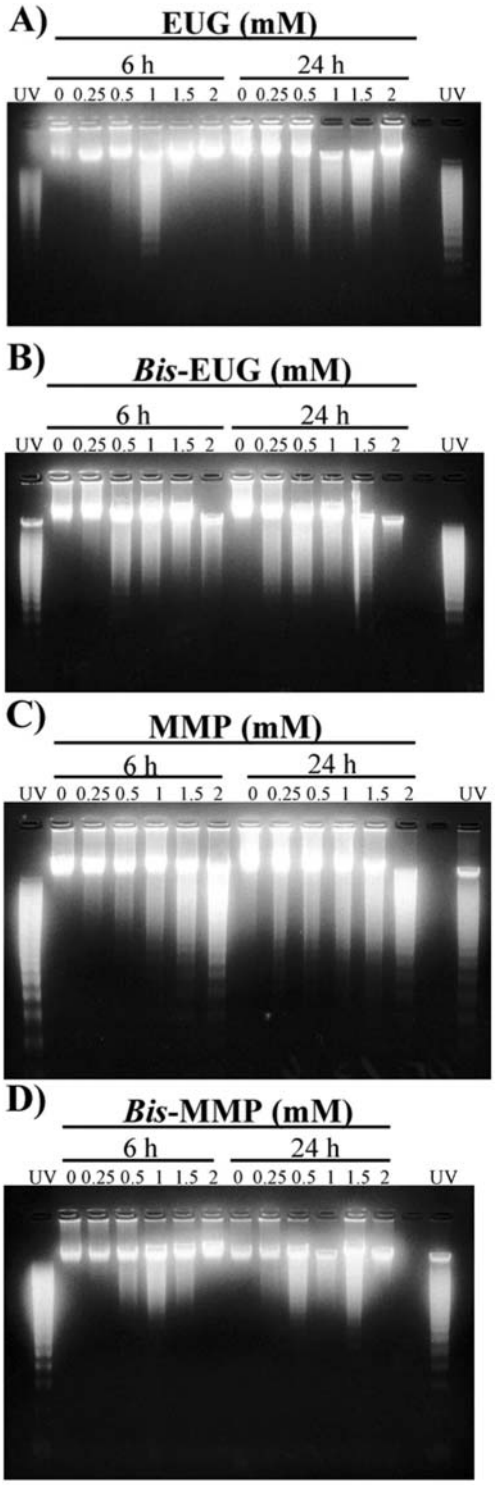


Figure 2. *Induction of DNA fragmentation by EUG (A), bis-EUG (B), MMP (C) and bis-MMP (D) in HL-60 cells. Near-confluent HL-60 cells were incubated with the indicated concentrations of each compound for 6 and 24 hours at 37˚C. The cells were lysed and DNA was extracted and applied to agarose gel electrophoresis, as described in Materials and Methods. UV represents DNA from apoptotic cells induced by UV irradiation.*

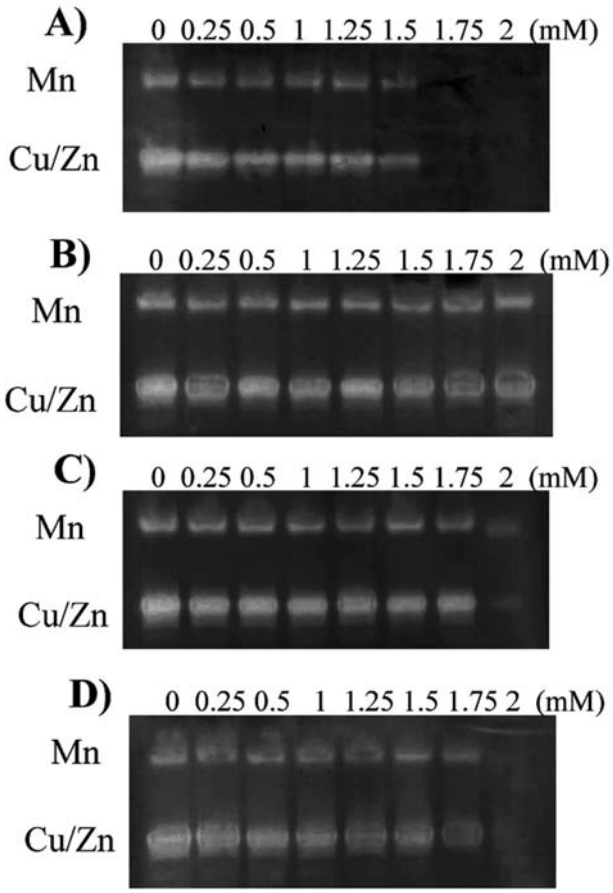


Figure 3. *Electrophoretic separation of MnSOD and Cu/ZnSOD in EUG (A), bis-EUG (B), MMP (C) and bis-MMP (D)-treated HL-60 cells. The activities of the two forms of SOD detected by activity staining after separation by polyacrylamide gel electrophoresis, as described in Materials and Methods.*

respectively. The dimers bis-EUG and bis-MMP showed higher cytotoxicity than did the corresponding monomers.

Induction of internucleosomal DNA fragmentation. The induction of DNA fragmentation was subsequently examined in EUG (A), bis-EUG (B), MMP (C) and bis-MMP (D)-treated HL-60 cells (Figure 2). After 6 h, EUG, MMP and bis-MMP induced DNA fragmentation at 1 mM, whereas bis-EUG induced DNA fragmentation at 0.5 mM. For each compound, the concentration required to promote the induction of apoptosis at 6 h was higher than the CC_{50}, whereas the concentration required to promote the induction of apoptosis at 24 h was close to the CC_{50}.

Expressions of MnSOD and Cu/ZnSOD by acryamide gel electrophoresis. To assess biological functions during apoptosis, the effect of the eugenol-related compounds on

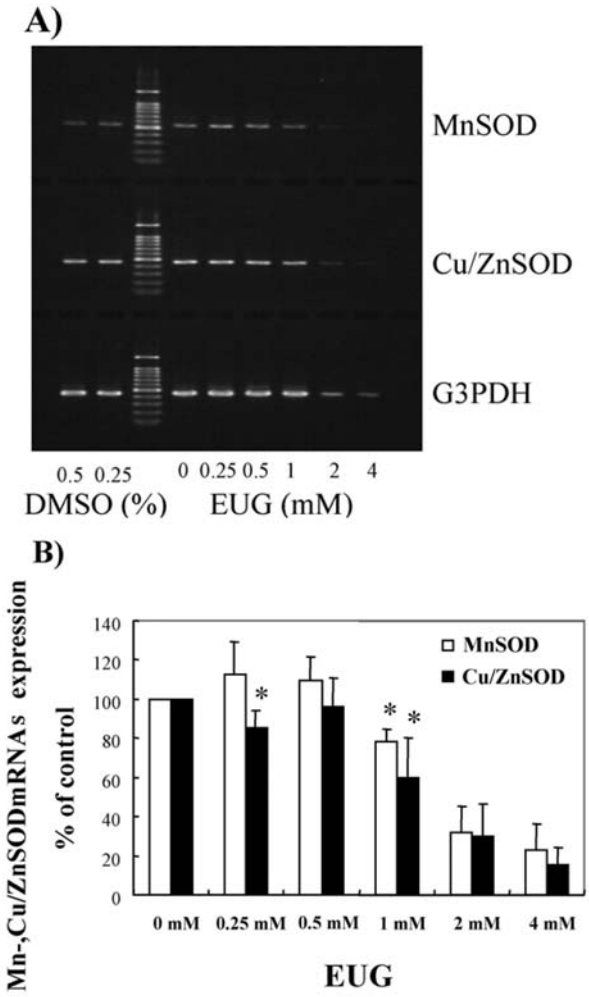


Figure 4. *Expression of MnSOD and Cu/ZnSOD mRNAs in DMSO- and EUG-treated HL-60 cells by RT-PCR (A) and as a function of the concentration of EUG, expressed as a percentage of the control value (B). Each value represents the mean of three independent experiments. The bar indicates the mean and standard deviation. A significant difference (*p<0.05) in Cu/ZnSOD mRNA expression was found between controls and 0.25 mM EUG or 1 mM EUG. Similarly, a significant difference (*p<0.05) in MnSOD mRNA expression was found between controls and 1 mM EUG. Total RNA was prepared and the RT-PCR reaction was performed, as described in Materials and Methods. Statistical analysis was performed by Student's t-test.*

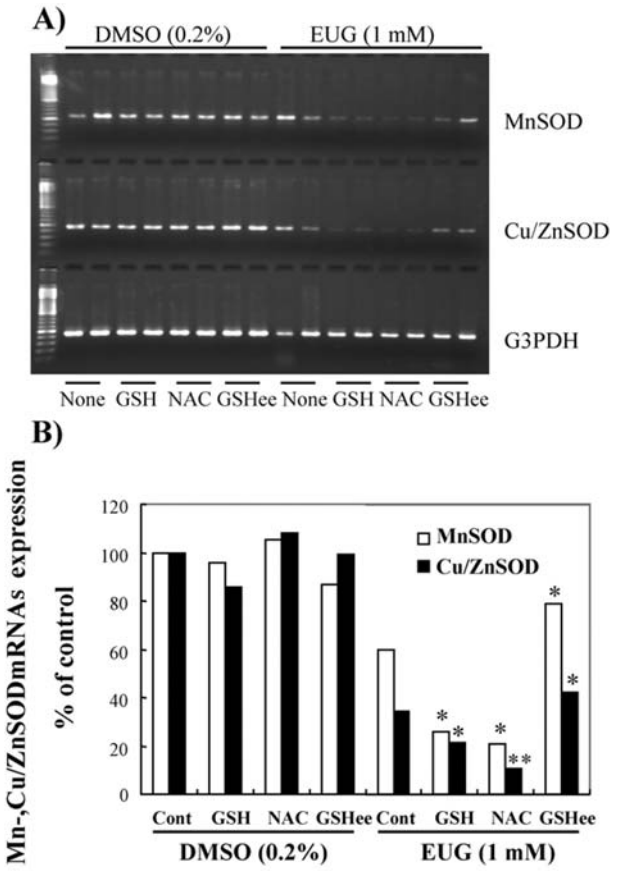


Figure 5. *Expression of MnSOD and Cu/ZnSOD mRNAs in 0.2% DMSO- and 1 mM EUG-treated HL-60 cells in the absence and presence of GSH, NAC or GSHee by RT-PCR (A) and as a function of the indicated thiol antioxidants, GSH, NAC and GSHee, expressed as a percentage of the control value (B). The reaction was conducted for 1 h. Each value represents the mean of three independent experiments. Standard deviations were <15%. A significant difference (*p<0.05, **p<0.01) between control (none) and GSH or NAC was found for the expression of both MnSOD and Cu/ZnSOD mRNAs. Total RNA was prepared and the RT-PCR reaction was performed, as described in Materials and Methods. Statistical analysis was performed by Student's t-test.*

the two forms of SOD in HL-60 cells was examined (Figure 3). The cytoplasmic Cu/ZnSOD and the mitochondrial MnSOD were identified as bands of higher and lower mobility, respectively. Compared with Cu/ZnSOD, MnSOD migrated faster after treatment with the monomers, EUG and MMP, than with the dimers, bis-EUG and bis-MMP. The expressions of both MnSOD and Cu/ZnSOD were completely abolished by EUG at 1.75 mM and by bis-MMP at 2 mM, and were greatly reduced by MMP at 2 mM. In contrast, bis-EUG, even at 2 mM, failed to abolish the expression of SOD. Inhibition of the expressions of MnSOD and Zn/CuSOD by EUG was greater than that by the other compounds, suggesting that EUG has a potent ability to cause mitochondrial dysfunction.

Expression of MnSOD and Cu/ZnSOD mRNAs. The results shown in Figure 3 suggest that treatment with EUG can cause alterations in mitochondrial function. Consequently, changes in the expression of MnSOD and Cu/ZnSOD mRNAs in the presence of EUG, with or without the

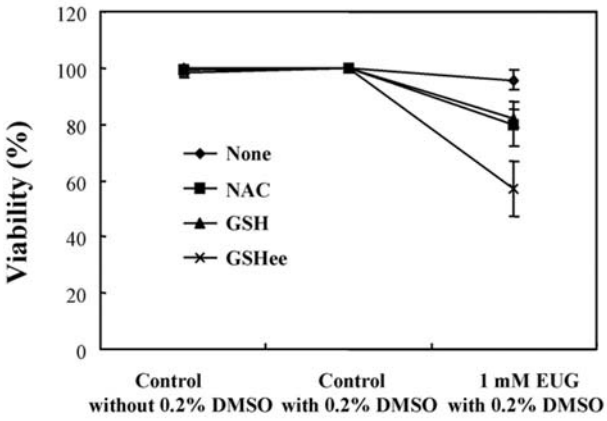


Figure 6. *Cell viability (%) as a function of controls of 0 mM EUG with or without 0.2% DMSO and 1 mM EUG with 0.2% DMSO in the absence (None) or presence of the antioxidants NAC, GSH and GSHee at 5 mM. Each value represents the mean of three independent experiments. Comparisons between None and NAC, GSH or GSHee were performed by Student's t-test ($p<0.05$). The effect of each compound on cell viability was tested for 2 h, as described in Materials and Methods.*

antioxidants GSH, NAC and GSHee, were examined. The results for EUG alone are shown in Figure 4. The expression of Cu/ZnSOD mRNA, but not of MnSOD mRNA, was slightly but significantly suppressed by 0.25 mM EUG, a concentration close to the CC_{50}. EUG at 1 mM significantly suppressed the expression of both MnSOD and Cu/ZnSOD mRNAs. At concentrations above 1 mM, EUG greatly reduced the expression of the mRNAs for both SODs, but statistical analysis could not be performed, possibly because of cytotoxicity at these concentrations.

Changes in the expression of MnSOD and Cu/ZnSOD mRNAs in the presence of EUG, with or without GSH, NAC or GSHee, are shown in Figures 5A and B. For both MnSOD and Cu/ZnSOD, the inhibition of mRNA expressions by EUG was strongly potentiated by the addition of GSH or NAC. Conversely, the addition of GSHee caused a recovery of the reduced mRNA expressions in EUG-treated cells. Furthermore, the EUG-induced reduction in expression of SOD mRNAs was reversed to the control level by low concentrations (equimolar or less with EUG) of GSH (data not shown). GSH, NAC or GSHee alone did not affect the expression of the mRNAs for MnSOD and Cu/ZnSOD, which was similar to that of controls.

The cytotoxicity of 1 mM EUG against HL-60 cells was subsequently examined in the presence of the thiol antioxidants NAC, GSH and GSHee at 5 mM for 2 h, and the results are provided in Figure 6. The cytotoxicity declined in the order GSHee > GSH > NAC > control. GSHee enhanced EUG-induced cytotoxicity to a significantly greater extent than did NAC or GSH. The

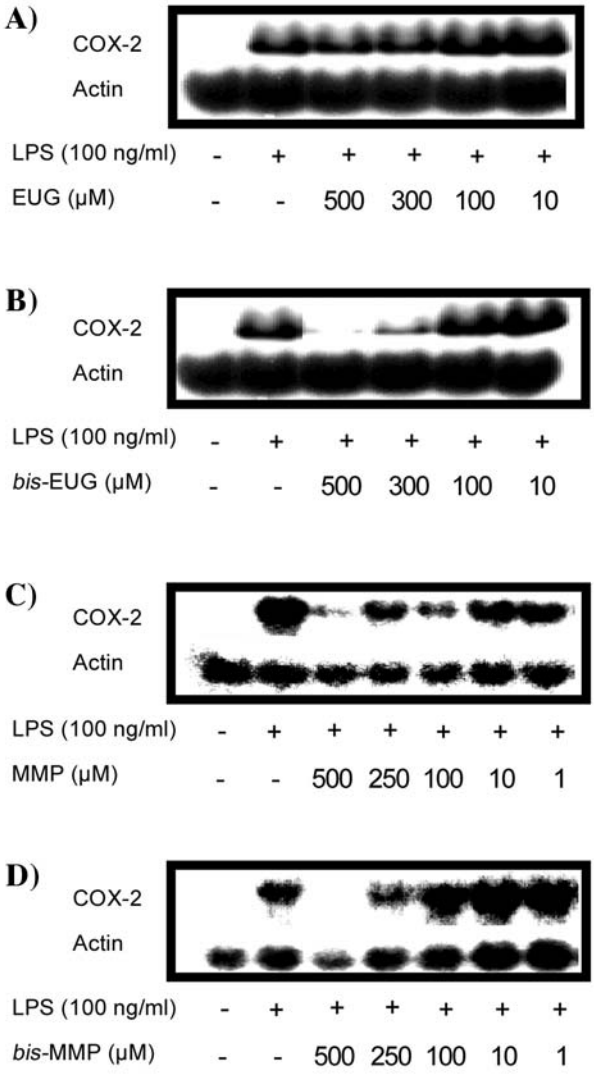


Figure 7. *Inhibition of LPS-induced COX-2 gene expression by EUG (A), bis-EUG (B), MMP (C) or bis-MMP (D) in RAW 264.7 cells. Northern blot assay was performed, as described in Materials and Methods. Two independent experiments were conducted and similar results were observed.*

apparent discrepancy between the effects of GSHee on the biological activities of EUG, reversing its inhibition of SOD mRNA expression (Figure 5) but strongly enhancing its cytotoxicity (Figure 6), may be attributable to the different durations of these experiments, at 1 h and 2 h respectively, because GSHee, is, to a large extent, de-esterified to GSH during the longer reaction time.

Inhibitory effect on COX-2 expression. A Northern blot assay was carried out in order to investigate the inhibitory effects of eugenol-related compounds on the LPS-induced expression of the *COX-2* gene in RAW cells and the results

are shown in Figure 7. LPS-induced expression of the *COX-2* gene was clearly inhibited by bis-EUG at a concentration of 300 μM. Similarly, the expression of *COX-2* was inhibited by MMP and bis-MMP at concentrations of 500 μM. Potent *COX-2* inhibition was found for bis-EUG but not for EUG. This may be a result of the mitochondrial dysfunction induced by EUG.

Discussion

We previously found that eugenol did not induce apoptosis in human squamous carcinoma (HSC-2) or salivary gland tumor (HSG) cells (data not shown). The present study demonstrated that eugenol induced apoptosis in HL-60 cells, implying that the nuclear chromatin DNA of human oral tumor cells is more resistant to attack. This suggests the possibility that different signal transduction systems are involved in apoptosis in different cell lines. Eugenol-treated RBL-2H3 cells have recently been reported to show typical apoptotic manifestations and translocation of *p53* into mitochondria (4). Eugenol has also been found to be a potent inhibitor of the proliferation of melanoma cells and to induce apoptosis (5). In the present study, bis-EUG was a better inducer of apoptosis in HL-60 cells than was EUG. Bis-EUG may have tumor specificity for HL-60 cells, since the cytotoxicity of bis-EUG was the highest among the present series of eugenol-related compounds. Conversely, bis-EUG has previously been reported to be less cytotoxic against HSC-2 and HSG cells (1). In contrast, MMP and bis-MMP had identical abilities to induce apoptosis, although MMP was much less cytotoxic than bis-MMP.

The marked inhibitory effect of EUG on SOD expression suggests that EUG causes much more mitochondrial dysfunction than the other compounds. We previously reported that eugenol preferentially acts as a pro-oxidant under light, alkaline or oxygen-rich conditions (12). The high level of mitochondrial dysfunction induced by EUG may be a result of the pro-oxidative activity of eugenol involving reactive oxygen species (ROS) (12). The synergistic activity of a combination of EUG and GSH or NAC significantly reduced the expression of MnSOD and Cu/ZnSOD mRNAs when high concentrations of GSH or NAC were added to EUG-treated HL-60 cells, accompanied by an enhancement of cytotoxicity (Figures 5 and 6). On the other hand, lower concentrations of GSH led to the recovery of expression of SOD mRNAs in EUG-treated cells, suggesting that the ability of GSHee to enhance the expression of MnSOD and Cu/ZnSOD mRNAs in EUG-treated cells is attributable to small quantities of GSH derived from the de-esterification of GSHee. The cytotoxicity of EUG was previously reported to be reduced by low concentrations of the antioxidants GSH and NAC (1). The increased cytotoxicity of the combination of eugenol and GSHee could be the result of the lag time for de-esterification of GSHee in cells, because treatment with GSHee was for 2 h in the study of cytotoxicity as opposed to 1 h in the study of SOD mRNAs expression. GSHee enters cells more readily than do NAC or GSH, and is subsequently hydrolyzed by intracellular esterases and converted to GSH. We recently reported that activation of caspase-3 by hydroquinone (HQ) was suppressed by a low concentration of NAC but enhanced by a higher concentration, suggesting that NAC, a precursor for intracellular glutathione synthesis, acts as a co-catalyst during HQ-induced apoptosis in HL-60 cells (8). In addition, we reported the mechanism of the interaction between eugenol and thiols during radical oxidation, suggesting the formation of a new product resulting from the Michel reaction (13). Furthermore, GSH has previously been reported to reduce the phenoxyl radical of EUG back to EUG or to interact directly with EUG quinone methide to produce a GSH adduct (14). The high cytotoxicity of eugenol in the presence of a high concentration of GSH may result from the formation of such an adduct.

Recent reports have outlined the role of *COX-2* and prostaglandins in carcinogenesis (15), metastatic properties of cancer cells (16), induction of apoptosis (6, 7) and immune suppression (17). Overexpression of the *COX-2* gene protects cancer cells from apoptosis, and drugs that inhibit *COX-2* have potent anti-inflammatory and anticancer activities. In the present study, we demonstrated that bis-EUG and, to a lesser extent, MMP and bis-MMP, are potent inhibitors of LPS-induced *COX-2* expression. Eugenol did not have any inhibitory activity. The failure of eugenol to inhibit *COX-2* expression may be a result of its high pro-oxidative activity, in turn causing mitochondrial dysfunction. We previously reported that bis-EUG, but not eugenol, showed potent inhibitory activity on LPS-stimulated NFkappaB activation and inflammatory cytokines in RAW264.7 cells (18). Analogously, MMP and bis-MMP may also inhibit LPS-stimulated NFkappaB activation and inflammatory cytokines in RAW264.7 cells. Together, these results suggest that bis-EUG, MMP and bis-MMP may have chemopreventive properties similar to those of NSAIDs.

Acknowledgements

This study was supported, in part, by a Grant-in-Aid from the Minister of Education, Science, Sports and Culture of Japan (Fujisawa S, No. 14571859; Okada N, No.13672119; and Murakami Y, No. 15592133).

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Received April 25, 2005
Accepted May 30, 2005