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Preventive Effect of Methanol Extract from *Agrocybe cylindracea* (MEAC) Mushroom on the Cooking Oil Fumes-Induced Expressions of Cyclooxygenase-2 *via* Suppression of Nuclear Factor-kappaB (NF–*k*B) in CL-3 Cells

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ABSTRACT

Methanol extract from *Agrocybe cylindracea* strain B (MEAC) mushroom is reported to suppress reactive oxygen species (ROS) production and DNA oxidative damage induced by cooking oil fumes (COF). This study examined the effects of MEAC on COF-stimulated expression of the cyclooxygenase-2 (COX-2) and cytochrome P450 CYP1A1 genes in human lung adenocarcinoma CL-3 cells. MEAC did not alter COF-stimulated CYP1A1 mRNA expression. However, MEAC significantly inhibited COF (100 μg/mL) stimulation of COX-2 mRNA and protein levels in a dose-dependent manner. The concentration of MEAC that decreased COF-stimulated COX-2 protein expression by 50% was 500 μg/mL. These data suggest that MEAC selectively affects particular types of the COF-mediated genes. COX-2 is reported to be regulated by the nuclear factor-*kappa*B (NF-*k*B) pathway. The gel retardation assay was used and showed that MEAC alone did not alter the basal NF-*k*B DNA binding activity; in the presence of COF, it dose-dependently suppressed COF-increased activity of NF-*k*B. These data suggest that MEAC may exert its anti-COF action on COX-2 gene expression *via* the NF-*k*B pathway. The results of this study support that MEAC mediates the COF-stimulated ROS production and DNA damage in CL-3 lung cancer cells.

Key words: cooking oil fumes, *Agrocybe cylindracea*, COX-2, nuclear factor-*kappa*B

INTRODUCTION

Environmental exposure to cooking oil fumes (COF) may cause cervical intraepithelial neoplasm[(1)]. Epidemiological study has demonstrated that exposure to carcinogenic components formed in volatile fumes during cooking may be associated with lung cancer risk of Chinese women[(2)]. Our previous studies have shown that 2-amino-3-methyl imidazo [4,5-*f*] quinoxaline (MeIQx) and benzo[a]pyrene (BaP) contributed to the major mutagenic activity of COF in bacterial system and DNA adduct formation in human lung adenocarcinoma CL-3 cells, respectively[(3,4)]. Some studies suggested that BaP is metabolized by cytochrome P-450 (CYP450) enzyme family, especially CYP1A1, to anti-7β,8α-di-hydroxy-9α,10α-epoxy-7,8,9,10-tetra-hydro-benzo[a] pyrene, which can attack DNA directly and form a DNA adduct, benzo[a]pyrene 7,8-diol 9,10-epoxide *N*-2-deoxy-guanosine (BPDE-N_2-dG). In contrast, our recent studies indicated the absence of (BPDE)-N_2-dG in CL-3 cells after COF (100 μg/mL) treatment[(4)]. These results showed there

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may be alternative mechanisms surrounding the actions of COF in lung cancer cells.

BaP was found to induce cyclooxygenase-2 (COX-2) expression through the NF-*k*B pathway in vascular smooth muscle cells[5], and COX-2 could mediate DNA adduct formation in tumor cells through activating lipid peroxidation[6]. Our recent study has demonstrated that COF could induce ROS production and COX-2 expression in CL-3 lung cancer cells[7]. These observations suggest that the COX-2 appears to play an important role in mediating the actions of COF and BaP on CL-3 lung cancer cells. A number of reports have shown that the expression of COX-2 and CYP1A1 genes in cancer cells can be regulated by ROS, NF-*k*B, genetic, endocrine, inflammatory, nutritional, and environmental cues[8-15]. In particular, mushroom extracts from *Ganoderma lucidum*[16] and *Inonotus obliquus (Fr.) Pilát* (*Hymenochaetaceae*)[17] were found to reduce the stimulation of COX-2 gene expression by lipopolysaccharide. However, whether mushroom extracts regulate COF stimulation of COX-2 expression in lung cancer cells remain unanswered.

Agrocybe cylindracea (DC: Fr.) Mre. [syn. Agrocybe cylindracea (Briganti) Singer] strain B (ACB), also known as the "liu-sung-ku", is a cultured edible mushroom in Taiwan with brown color in caps. ACB has good bite texture and is delicious[18]. The effects of ACB on diseases have received increasing attention in Taiwan. Because bioactive components such as α-glucan, peptide, and heterodimer lectin of the ACB mushroom were found to possess hypoglycemic, mitogenic, antitumorgenic, and antioxidative activities[19-22]. Our recent data have shown that water extract from *A. cylindracea* strain B (WEAC) has greater antioxidant activity than shiitake mushroom in suppressing hydroxyl radical-induced DNA strand breakage[22]. In addition, methanol extract from *A. cylindracea* strain B (MEAC) has antioxidative activity, and it is able to inhibit COF- and BaP-induced DNA damage in human lung adenocarcinoma CL-3 cells[23]. Despite the demonstration that MEAC had an influence on the COF-stimulated DNA damage[23] and the fact that COF and BaP could mediate COX-2 expression[5,7], no study has demonstrated whether MEAC acted on the COX-2 expression and NF-kB activity for the COF effect in CL-3 lung cancer cells.

The present study aimed to understand the possible mechanism underlying MEAC inhibition of COF effects in CL-3 lung cancer cells. The treatment with MEAC was shown to prevent the effect of COF on COX-2, but not CYP1A1, expression. MEAC was found to inhibit the COF-stimulated NF-kB activity. Our results indicated that the NF-*k*B/COX-2-mediated pathway plays a role in regulating the anti-COF effects of MEAC in CL-3 lung cancer cells.

MATERIALS AND METHODS

I. *Chemicals and Enzymes*

Benzo[a]pyrene (BaP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, Mo). *Taq* DNA polymerase, oligo(dT)18 primer, deoxyribonuclease I were from Amersham Buchler (Braunschweig, Germany). Dimethyl sulfoxide (DMSO) and Folin-Ciocalteu reagent were from Merck Chemical. *Taq* SuperScript II reverse transcriptase (RTase), TRIzol kit, ribonuclease inhibitor, deoxynucleotide triphosphate (dNTP) and media for cell culture, penicillin/streptomycin, and fetal bovine serum (FBS) were from GIBCO BRL (Eggenstein, Germany). Anti-cyclooxygenase-2 (COX-2) was from Alexis Chem. Co. All other chemicals and biochemicals were of the highest quality available from commercial sources.

II. *Extraction and Sub-fractionation of Methanol Extracts*

The edible *A. cylindracea* strain B mushroom was obtained from Lung-Kuo Mushroom Cultivation Farm (Taichung, Taiwan). Methanol extract from *A. cylindracea* (MEAC) mushroom fruit bodies was prepared as followed. Fruit body (200 g) of mushroom was ground in methanol (400 mL) with Waring Blender for 3 min. The mixture was then stirred for 30 min at room temperature. The methanol-soluble fraction was separated by centrifugation (25,000 ×*g* for 30 min at 4°C) and filtration. The insoluble residue was re-extracted as described above. The soluble fractions were combined and methanol was removed by vacuum evaporator and the residue was re-dissolved in distilled water.

The MEAC was further sub-fractionated into two fractions corresponding to the molecular weight cutoff of 5,000 Daltons by the ultra-filtration (UF) method.

III. *Collection and Preparation of COF*

The details of COF preparation have been described previously[4]. Oil smog particulates from frying a pomfret fish were collected, filtered with high-purity glass filters (Whatman, EPM1000) and extracted with acetone. The acetone extracted cooking oil fumes from frying fish (i.e. the COF) were weighed, dried using a stream of nitrogen, dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C until assay.

IV. *Cell Culture and Treatment*

CL-3 lung cancer cells were obtained from Dr. P. C. Yang (Department of Internal Medicine, College of Medicine, National Taiwan University, Taiwan, R.O.C.). The cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 2 mM glutamine, 10 mM non-essential amino acids, 100 mM sodium pyruvate, 5.5% bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a standard culture incubator with humidified atmosphere containing 5% CO_2/95% air at 37°C. CL-3 cells of 30 - 35 passages were used in this study. The cells were sub-cultured every 3 to 4 days.

To induce gene expression and DNA damage, CL-3 cells (5×10^5) grown in 5 cm dishes were treated with

COF, BaP, or in the presence or absence of various concentrations of MEAC in DMEM for 2 - 24 h at 37°C in 5% CO2/95% air. The cells were washed twice with 1 × PBS, pH 7.4, and trypsinized. The cells were centrifugated at 3,500 rpm for 5 min at 4°C and then resuspended in PBS (pH 7.4) for assay. Respective controls were treated with an equal volume of DMSO.

V. *Cytotoxicity Assay*

As previously described by Lin *et al.*[7], cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, CL-3 lung cancer cells (~2600 cells/cm^2) were plated in triplicate wells of 96-well plates with 2 mL DMEM and 10% FBS. After allowing 24 h for attachment, the medium was then replaced with 2 mL of fresh FBS-supplemented DMEM containing COF (100 μg/mL) and various concentrations of MEAC (0 - 1000 μg/mL). After 24 h of treatment, the culture medium was removed from cell monolayers and each well was reconstituted with 0.2 mL supplemented DMEM containing 1 mg/mL MTT. Following incubation at 37°C for 4 h in a fully-humidified atmosphere at 5% CO_2 in air, MTT was taken up by active cells and reduced in the mitochondria to form insoluble purple formazan granules. Subsequently, the medium was discarded and the precipitated formazan was dissolved in DMSO (150 μL/well). The optical density of the solution was measured on a microplate spectrophotometer at a wavelength of 490 nm. All the results obtained from MTT assays were confirmed by repeating the experiment on at least three independent occasions and testing in triplicate wells each time. Cell viability was determined by the following formula: Cell viability(%) =OD (test well)/ OD (reference well) × 100%

VI. *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

Total RNA was prepared using an RNA isolation kit (GIBCO BRL) according to the manufacturer's instructions and then digested with ribonuclease free deoxy- ribonuclease I. For cDNA synthesis, 5 μg of total RNA were heated in a final volume of 12 μL with 0.5 ng of oligo(dT)$_{18}$ primer for 10 min at 70°C, chilled on ice, and reverse transcribed in a final volume of 20 μL containing 0.5 mM each dNTP, 4 μL of 5X first-strand buffer, 10 mM dithiothreitol, and 200 U of Super-Script II RTase (GIBCO BRL). Samples were incubated at 42°C for 50 min and subsequently denatured for 15 min at 70°C. The product was then subjected to the following amplification reactions. For CYP1A1, sequences of primers used in amplification were 5'-TAGACACTGATCTG-GCTGCAG-3' (sense) and 5'-GGGAAGGCTCC-ATCAGCATC-3' (antisense). Sequences of COX-2 specific primers were 5'-GGTCTGGTGCCTGGTCTGATGATG-3' (sense) and 5'-GTCCTTTCAAGGA -GAATGGTGC-3' (antisense). For the internal control-β-actin, sequences of primer pair used in amplification was 5'-CATGGCGTGATGGTGGGCA-3' (sense) and 5'-CAAACATGATCTGGGTCATCTTCTC-3' (antisense). Each amplification was carried out in a final volume of 50 μL containing 1 μL of RT sample, 5 μL of 10X *Taq* buffer, 200 μM each dNTP, 0.2 μM each primer, and 2 U of *Taq* DNA polymerase. The reaction was carried for the indicated cycles with the following profiles: 3 min at 94°C before the first cycle, 50 sec for denaturalization at 94°C, 30 sec for annealing at 60°C, 1 min for primer extension at 72°C, and 10 min at 72°C after the last cycle. PCR cycle number was 27 and 35 for CYP 1A1 and COX-2, respectively. PCR products were analyzed on a 2% agarose gel. Each data represents the means ± SD of three individual experiments.

VII. *Immunoblotting Analysis*

Immunoblotting analysis was performed according to previously described methods[24]. Samples with 20 μg of total protein were heated in a final volume of 20 μL with sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 40 mM Tris-HCl, pH 6.8) for 5 min at 95℃ and then chilled on ice. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis (5% acrylamide stacking gel and 10% acrylamide separating gel). For immunoblotting, proteins were transferred to a nitrocellulose membrane by means of a semidry blotting technique. After blocking with 5% blocking reagent [5% (w/v) nonfat dry milk powder and 0.1% Tween 20 in PBS], the membrane was probed with the antibody preparations. Immunoreactive proteins were then visualized by the enhanced chemiluminesence (ECL) detection method according to the manufacturer's protocol (NEN). Each data represents the means ± SD of three individual experiments.

VIII. *Gel Retardation Assay*

Nuclear extract was prepared and the extent of DNA binding was measured by a gel retardation assay[25]. A complementary pair of synthetic oligonucleotides containing the sequence of NF-*k*B (5'-AGTT-GAGGGGACTTTCCCAGGC-3') were labeled with [γ-^{32}P] dATP at the 5'-end using T_4-polynucleotide kinase. Nuclear extract (15 μg) was incubated with 25 mM Hepes, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 1 μg of poly[d (I-C)] at room temperature for 15 min, followed by addition of 0.5 ng of ^{32}P-labeled NF-*k*B at room temperature for another 20 min. The reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresized at 120V for 2.5 h in TG buffer (125 mM Tris-195 mM glycine, pH 8.5). The resulting gels were then dried and exposed on a X-ray film overnight.

IX. *Estimation of Total Phenolics Content*

The total phenolic contents of MEAC were determined colorimetrically by the Folin-phenol method. A sample aliquot of 100 μL was added to 900 μL water, 0.1 mL of 50% Folin-Ciocalteu reagent, and 2 mL of 2% sodium carbonate solution, mixed in a Vortex mixer, and incubated for 30 min

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at room temperature. The absorbance was measured at 750 nm on a Hitachi UV-visible spectrophotometer. The standard curve was plotted using 1.25 - 10 μg of gallic acid. The total phenolic content was expressed as gallic acid equivalent (GAE), which reflected the phenolic content as the amount of gallic acid in milligrams per gram of sample.

X. *Statistical Analysis*

Results are expressed as the mean ± SD, with n = 3. Statistical evaluations were performed using analysis of variance followed by Duncan's test for dose-response data. $p < 0.05$ was considered significant.

RESULTS

I. *No Cytotoxic Effect of MEAC*

To determine whether MEAC mediated the effects of COF on CL-3 cells through cytotoxic effects, we measured the mitochondrial NADH activity using the MTT assay in response to 24 h of MEAC treatment (data not shown). It was observed that the doses of MEAC (100 - 1000 μg/mL) and COF (100 μg/mL) used in our study were not cytotoxic when compared with the control.

II. *MEAC Did Not Alter COF-Stimulated Expression of CYP1A1 Gene*

In previous studies, we showed that COF induced CYP1A1 mRNA transcription in CL-3 lung cancer cells[7] and that COF-stimulated DNA damage was suppressed by MEAC[23]. Here, we further examined whether MEAC affected the stimulation of CYP1A1 mRNA expression by COF (Figure 1). Using RT-PCR, we found that MEAC did not significantly alter COF-stimulated levels of CYP1A1 mRNA in the concentration range of 100 - 1000 μg/mL. Treatment with MEAC alone did not alter CYP1A1 mRNA expression in CL-3 lung cancer cells when compared with the control.

III. *MEAC Inhibited COF-Stimulated Expression of Cox-2 Gene*

BaP, one of the major components of COF, was reported to induce COX-2 expression in vascular smooth muscle cells[5]. In parallel, our recent study has demonstrated that COF could induce ROS production and COX-2 expression in CL-3 lung cancer cells[7]. Accordingly, we examined the effect of MEAC on COF stimulation of COX-2 gene expression in CL-3 cells (Figures 2 and 3). First, we measured changes in the level of COX-2 mRNA (Figure 2). Indeed, either BaP or COF treatment significantly stimulated COX-2 mRNA expression. In contrast to the COF-stimulated COX-2 mRNA levels, MEAC alone significantly decreased the steady-state levels of COX-2 mRNA in a concentration-dependent manner when compared to the control (Figure 2). In the presence of

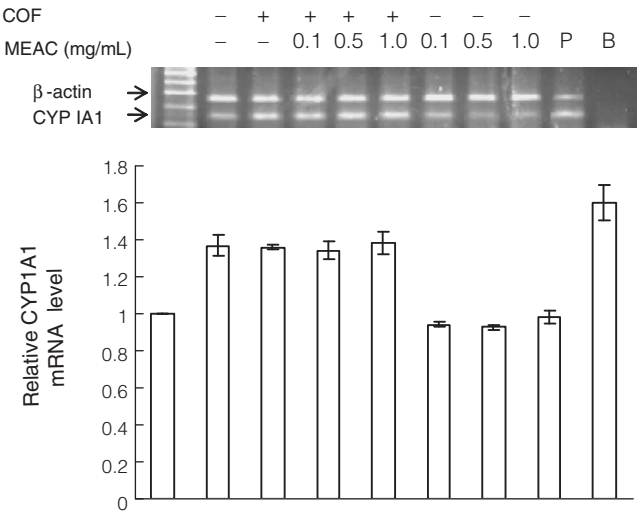

Figure 1. MEAC did not alter COF stimulation of CYP1A1 mRNA expression in CL-3 lung cancer cells. Cells were treated with or without COF (100 μg/mL) in the presence and absence of the various concentrations of MEAC (0.1 - 1.0 mg/mL) for 2 h. CYP1A1 mRNA levels were determined by RT-PCR as described in the text. The control experiment was that not treated with COF or MEAC, whereas the positive control experiment was that treated with BaP (5 μM). In the lower part of the figure, CYP1A1 mRNA levels were calculated after normalization to β-actin mRNA. Data are expressed as the means ± SD from three independent experiments. COF, cooking oil fumes; MEAC, methanol extract from *Agrocybe cylindracea*; P, BaP; B, blank.

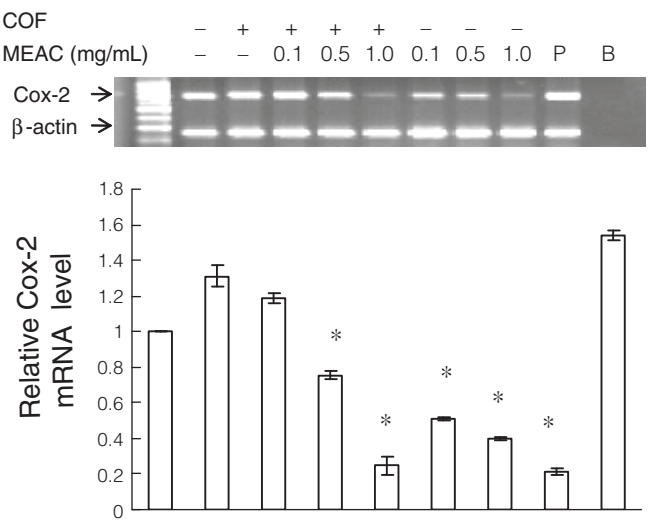

Figure 2. The inhibitory effect of MEAC on COF stimulation of cyclooxygenase-2 (COX-2) mRNA expression in CL-3 lung cancer cells was dose-dependent. Cells were treated with or without COF (100 μg/mL) in the presence and absence of the various concentrations of MEAC (0.1 - 1.0 mg/mL) for 2 h. COX-2 mRNA levels were determined by RT-PCR as described in text. The control experiment was that not treated with COF or MEAC, whereas the positive control experiment was that treated with BaP (5 μM). In the lower part of the figure, COX-2 mRNA levels were calculated after normalization to β-actin mRNA. Data are expressed as the means ± SD from three independent experiments. *: significant difference comparing to corresponding control ($p < 0.05$). COF, cooking oil fumes; MEAC, methanol extract from *Agrocybe cylindracea*; P, BaP; B, blank.

COF, MEAC also dose-dependently suppressed COF-stimulated levels of COX-2 mRNA expression. The concentration of MEAC that suppressed COF-stimulated COX-2 mRNA expression (IC_{50}) was in the ranges of 500 - 1000 μg/mL for 2 h of treatment.

The effect of MEAC on COF stimulation of COX-2 protein expression were further examined. Figure 3 showed that MEAC alone significantly reduced the basal levels of the COX-2 protein; in the presence of COF, it suppressed COF-stimulated levels of COX-2 protein in a dose-dependent manner (Figure 3A and 3C). The IC_{50} value of MEAC to suppress COF-stimulated COX-2 protein expression was 507 μg/mL. The percentage inhibition of 100 - 1000 μg/mL MEAC on COF-stimulated relative COX-2 protein levels increased from 22.48% to 84.99% (Figure 3C). MEAC also significantly suppressed BaP stimulation of COX-2 protein expression in a dose-dependent manner (Figure 3B). The IC_{50} value of MEAC to reduce BaP-increased levels of the COX-2 protein was 513 μg/mL.

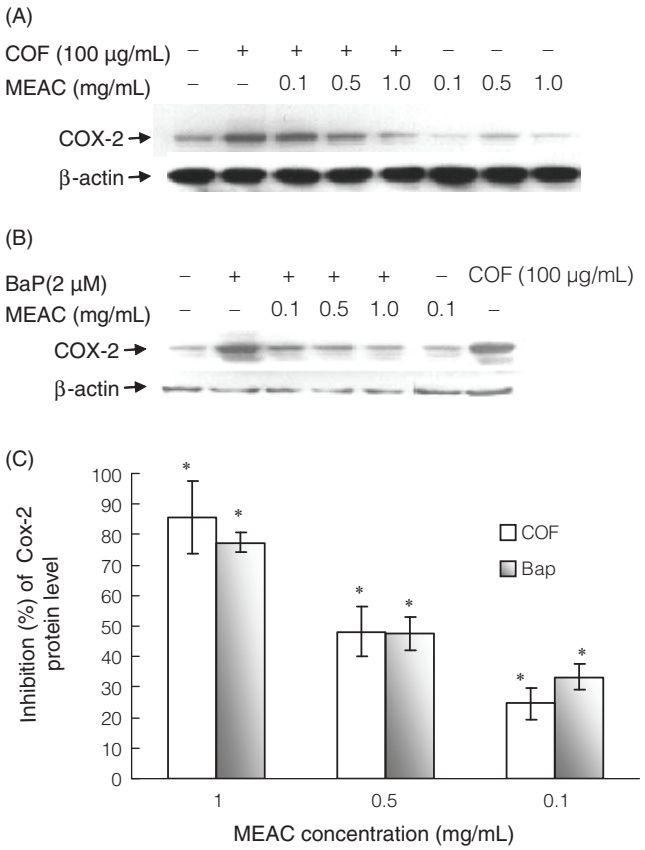

Figure 3. The inhibitory effect of MEAC on COF stimulation (A) of cyclooxygenase-2 (COX-2) protein expression in CL-3 lung cancer cells was dose-dependent. Cells were treated with or without COF (100 μg/mL), or BaP (2 μM), in the presence and absence of the various concentrations of MEAC (0.1 - 1.0 mg/mL) for 4 h. Western blot analysis was performed as described in the Materials and Methods section. The control experiment was that not treated with COF or MEAC, whereas the positive control in (B) was that treated with BaP. In (C), COX-2 protein levels were calculated as percentage of the control after normalization to β-actin protein. Each value represents the means ± SD from three independent experiments. *: significant difference comparing to corresponding control ($p < 0.05$). COF, cooking oil fumes; MEAC, methanol extract from *Agrocybe cylindracea*.

IV. *MEAC Inhibited COF-Stimulated NF-kB Activity*

Our previous data showed that NF-*k*B mediated COF stimulation of COX-2 gene expression in CL-3 lung cancer cells[7]. To further characterize the effect of MEAC on COF-stimulated COX-2 gene expression, we examined changes in the activity of NF-*k*B protein. Using the gel retardation assay, Figure 4 showed that MEAC alone did not significantly alter the NF-kB activity. However, in the presence of COF, MEAC significantly suppressed COF stimulation of the NF-*k*B DNA binding activity in a dose-dependent manner. The doses of MEAC at 500 and 1000 μg/mL used in our study could completely suppress the DNA binding activity of the NF-kB protein induced by COF.

V. *Different Sub-Fractions of MEAC Affected COF-Stimulated COX-2 Protein Expression*

Differences in regulating COF-stimulated COX-2 protein expression among sub-fractions of MEAC were also

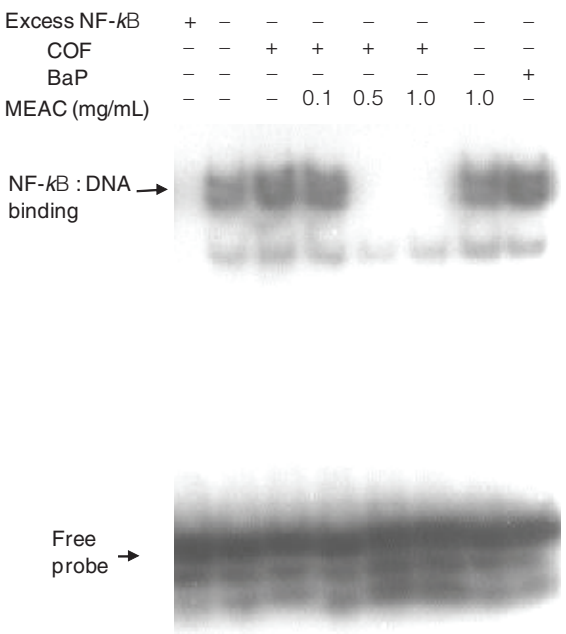

Figure 4. MEAC inhibited COF activation of the nuclear factor-*k*B (NF-*k*B) activity in CL-3 cells. Cells were treated with or without COF (100 μg/mL) in the presence and absence of the various concentrations of MEAC (0.1 - 1.0 mg/mL) for 2 h. Nuclear protein was extracted from the treated cells and gel retardation assay was performed when a consensus ^{32}p-labeled NF-*k*B binding oligonucleotide was used. The control experiment was that not treated with COF or MEAC, whereas the positive control experiment was that treated with BaP (2 μM). Nuclear extract of cells treated with BaP was incubated with an excess of unlabeled NF-*k*B probe to determine the specificity. COF, cooking oil fumes; MEAC, methanol extract from *Agrocybe cylindracea*.

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assessed. In Figure 5, we observed that a fraction of MEAC with a high molecular weight over 5,000 Daltons could inhibit COF-stimulated levels of the COX-2 protein. The other fraction of MEAC with a low molecular weight under 5,000 Daltons had similar effect in suppressing the COF-stimulated COX-2 protein expression.

VI. *Total Phenolics Content of MEAC*

Because edible mushrooms contain phenolic compounds, it is interesting to investigate the inhibition activity of MEAC on COF-induced DNA damage in relation to their total phenolic contents. Accordingly, we analyzed the total phenolic content among the different solvent extracts of *A. cylindracea* Strain B (Table 1). We found that the GAE values of acetone extract (AE), water extract (WE) and ether extract (EE) were higher than those of boiled water extract (BWE), ethyl acetate extract (EAE) and methanol extract (MEAC), with AE having the highest GAE value (3.454 ± 0.333 mg/g extract). In addition, MEAC had a significantly lower GAE value (1.576 ± 0.057 mg/g extract) than the other five solvent extracts.

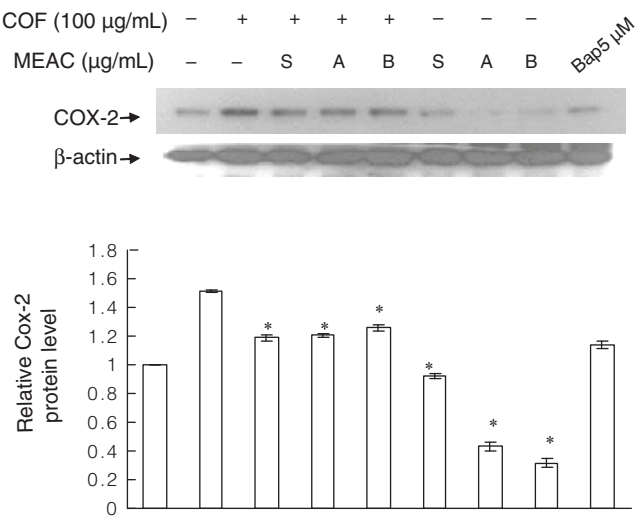

Figure 5. Different sub-fractions of MEAC with a molecular weight cutoff of 5,000 Daltons significantly inhibited COF stimulation of cyclooxygenase-2 (COX-2) protein expression in CL-3 lung cancer cells. Cells were treated with or without COF (100 μg/mL) in the presence and absence of the MEAC (0.5 mg/mL) for 4 h. Western blot analysis was performed as described in the Materials and Methods section. The control experiment was that not treated with COF or MEAC, whereas the positive control experiment was that treated with BaP (5 μM). COX-2 protein levels were calculated after normalization to β-actin protein. *: significant difference comparing to corresponding control ($p < 0.05$). COF, cooking oil fumes; MEAC, methanol extract from *Agrocybe cylindracea*; S, stock; A, a subfraction of MEAC with a high molecular weight above 5 kDa; B, a subfraction of MEAC with a low molecular weight under 5 kDa.

Table 1. Total phenolic content of the six solvent crude extracts from *Agro. cylindracea* strain B

Solvent	Total phenolic content (GAE, mg/g)[a]
Water	3.387 ± 0.227
Boiled water	2.828 ± 0.001
Methanol	1.576 ± 0.057
Acetone	3.454 ± 0.333
Ether	3.246 ± 0.168
Ethyl acetate	2.498 ± 0.065

[a] Total phenolic content is expressed as gallic acid equivalent (GAE;mg/g of sample). Each value is the mean ± SD of triplicate measurement.

DISCUSSION

Our previous studies have contributed to the proposal that MEAC is a chemo-preventive substance for COF-induced DNA damage of lung cancer cells[4,7,23]. To extend the findings of a preliminary report[23], this study provide an in-depth understanding of the mechanism underlying the effects of MEAC on COF regulation of CL-3 cancer cell activity. The COF-induced upregulation in the levels of COX-2 mRNA and protein were prevented by MEAC, and this preventive effect may be attributable to its inhibition of NF-kB activity. This conclusion is consistent with those observed for the anti-COX-2 and anti-NF-kB effects of the methanol extracts from other mushrooms, such as *Ganoderma lucidum*[16] and *Inonotus obliquus (Fr.) Pilát* (*Hymenochaetaceae*)[17]. In support of these observations, MEAC at 100 - 1000 μg/mL for 4 h was found to suppress the BaP-stimulated levels of the COX-2 protein. However, MEAC did not alter COF stimulation of CYP1A1 mRNA expression. These data suggest that MEAC selectively affects the particular types of the COF-mediated genes in CL-3 lung cancer cells. This contention also supported that MEAC did not alter COX-1 mRNA expression in CL-3 cells (data not shown).

Cellular actions of COF and BaP can be oxidative, ROS-productive, DNA-adductive, and gene-regulatory[7,22,23]. The ROS-productive mechanism through which BaP and COF induced ROS production is blocked by free radical scavengers, such as superoxide dismutase (SOD; a superoxide anion scavenger) and mannitol (a hydroxyl radical scavenger)[7]. The gene-regulatory mechanism through which COF induces increases in the levels of COX-2 and CYP1A1[7]. Our experiments showing that MEAC blocked the COX-2 activation by COF and BaP, support the possible involvement of the functional activity of MEAC in regulating COX-2 expression in CL-3 lung cancer cells. Due to that MEAC was also found to suppress COF-stimulated ROS production in CL-3 cells[7] and COX-2 gene expression could be upregulated by ROS[8], we cannot completely exclude a possible role of the antioxidant activity or ROS-protective pathway of MEAC in regulating COF or BaP effects on COX-2 gene of CL-3 lung cancer cells. This notion is also supported by the findings that MEAC was demonstrated to scavenge the free radicals[26] and that SOD and mannitol were respectively shown to suppress COF-stimulated COX-2 protein expression[7].

Interestingly, we reported herein that MEAC alone was found to suppress COX-2 expression in CL-3 cells, but it did not alter NF-kB activity. In the presence of COF, MEAC significantly suppressed COF stimulation of COX-2 expression and NF-kB activity. These data suggest that the way MEAC signaling in the absence of COF suppresses COX-2 gene expression in CL-3 cells is different from that by which it in the presence of COF suppresses COX-2 gene expression; it is likely mediated through both NF-*k*B-independent and NF-kB-dependent pathways. One possible explanation for the disparate findings studied here is that CL-3 lung cancer cells may respond to MEAC differently in the presence and absence of the COF. To support these observations, our data also showed that the inhibitory effect of MEAC on COX-2 gene expression in the absence of COF was greater than that observed in the presence of MEAC and COF. An alternative explanation is that the antioxidant activity of MEAC in CL-3 cells varies with the presence and absence of the COF.

In this study, COF at 100 μg/mL was found to significantly increase the level of the COX-2 protein of CL-3 lung cancer cells (Figure 3A). Our previous report demonstrated that the BaP content in 100 μg/mL COF was 0.074 μM[27]. We did find herein that BaP at 2 μM stimulated COX-2 protein expressions in CL-3 lung cancer cell and its stimulation could be blocked by MEAC treatment. These observations is in agreement with the fact that BaP is one of the major components of COF. However, the BaP-induced increases in the COX-2 levels was lower than those induced by COF (Figure 3B). This suggests that other components of the COF besides the BaP may act alone or synergistically with BaP on the COX-2 protein expression in CL-3 lung cancer cells. Our experiments showing that MeIQx contributed to the major mutagenic activity of COF in the bacterial system[3] support this contention.

Extracts of the ACB mushroom have numerous biological activities that provide various health benefits[19-22]. While WEAC has been shown to possess antioxidant activity and to suppress hydroxyl radical-induced DNA strand breakage[22], we showed that MEAC had antioxidative activity and inhibited COF- and BaP-induced DNA damage in CL-3 lung cancer cells[23]. In this study, we attempted to search the active components of MEAC required for the anti-COF effect of MEAC on COX-2 protein expression. We divided the MEAC into two fractions with the cutoff molecular weight of 5000 Daltons; one fraction had a higher molecular weight than 5000 Daltons, and the other had a lower molecular weight than 5000 Daltons. It is evident from these data that both fractions prevented the COF-induced increase in COX-2 protein levels. These results suggest that many ingredients of MEAC may contribute to the inhibitory effect of MEAC on COF stimulation of COX-2 protein expression in CL-3 lung cancer cells. This contention is supported by the findings that MEAC contained two indole derivatives with free radical scavenging activity[28]. Thus, it would be worthwhile in a future study to explore whether any of the indole derivatives is responsible for the anti-COF effect of MEAC on CL-3 lung cancer cells. Although our data also showed that MEAC contained the phenolic compounds, the total phenolic amounts in the MEAC, as expressed by gallic acid equivalent, were lower than those extracted by other solvents (Table 1). Whether the COF stimulation of COX-2 expression in CL-3 cells is altered by the phenolic compounds of the MEAC was not determined in this study.

Expression of the COX-2 gene is upregulated in many cancers[29-32]. Its metabolic product named prostaglandin H_2 can be converted by prostaglandin E_2 (PGE_2) synthase into PGE_2 which can subsequently cause cancer progression[31]. In non-small cell lung cancer, COX-2 was found to upregulate vascular endothelial growth factor *via* the protein kinase C pathway[30]. Thus, suppressing COX-2 may have benefit in the prevention and treatment of cancers [33-36]. In our study, we showed that MEAC inhibited COF stimulation of COX-2 gene expression in CL-3 lung cancer cells. Inhibition of the COF-stimulated levels of COX-2 gene expression by MEAC may be beneficial to prevent and treat these types of lung cancers and requires further exploration.

We conclude that MEAC inhibits COF stimulation of COX-2 gene expression in CL-3 lung cancer cells and its inhibitory effect is likely associated with the blockage of DNA binding activity of NF-*k*B. To our knowledge, this is the first report concerning the anti-COF actions of MEAC on COX-2 expression and NF-*k*B activity in CL-3 lung cancer cells. The results of this study support the findings that MEAC mediates the effects of COF on ROS production and DNA oxidative damage[7], as well as may be potentially utilized in the chemopreventive agent against environmental mutagen-associated lung cancer using ACB-based nutrients.

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