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원 저

Inhibition of Cyclooxygenase and Prostaglandin E2 Synthesis by Crude Methanolic Extract from *Euonymus Alatus* (Thunb.) Sieb in SKBR3 Human Breast Cancer Cell Line

Joong-Oh Kim, Tae-Hyun Jang, Min-Sung Kim, Dong-Il Kim, Tae-Kyun Lee[1)]

Department of Gynecology, College of Oriental Medicine, Dongguk University.
Dr. Lee's Traditional Korean Medical Clinic[1)]

In the present study, we examined the effect of crude methanolic extract (CME) from *Euonymus alatus* (Thunb.) Sieb on arachidonic acid (AA) cascade in SKBR3 human breast cancer cell line. CME had a potent inhibitory activity of prostaglandin E2 (PGE_2) release induced by A23187, a Ca^{2+} ionophore. The inhibition was concentration-dependent, with the 50 value of about 5 M. CME had no inhibitory effect on A23187-induced phosphorylation of p42/p44 extracellular signal regulated kinase/mitogen-activated protein kinase or on the liberation of [14C]-AA from the cells labeled with [14C]-AA. However, CME concentration-dependently inhibited the conversion of AA to PGE_2 in microsomal preparations, showing its possible inhibition of cyclooxygenase (COX). In enzyme assay in vitro, CME inhibited the activities of both constitutive COX (COX-1) and inducible COX (COX-2) in a concentration-dependent manner, with the 50 values of about 0.8 and 2 M, respectively. Lineweaver-Burk plot analysis indicated that CME competitively inhibited the activities of both COX-1 and -2. This study is a first demonstration that CME directly inhibits COX activity.

Key Words: CME cyclooxygenase, PGE_2, SKBR3 human breast cancer cell line

Introduction

Breast carcinoma (BC) is the commonest cancer among women and the second highest cause of cancer death[1)]. Most cases occur during age 45-55. It also occurs in men but is more than 100-fold less frequent than in women[2)]. At present, the cancer treatment by chemotherapeutic agents, surgery and radiation have not beneficially effective against the high incidence or low survival rate of most the cancers. The development of new therapeutic approach to breast cancer remains one of the most challenging area in cancer research.

Euonymus alatus (Thunb.) Sieb (EA), known as 'Gui jun woo' in Korea, was used in folk medicine to regulate *qi* (bodily energy) and blood circulation, relieve pain, eliminate stagnant blood, and treat dysmenorrhea in eastern asia. It can increase tolerance to oxygen deprivation, and has a significant, albeit temporary, hypotensive effect. It acts as a depressant on the CNS and can lengthen barbiturate-induced sleeping times. Its effects on metabolism include a reduction of blood sugar levels via stimulation of the beta cells of

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· 채택 : 2004년 12월 9일
· correspondence to : Dong-Il Kim, Gangnam Oriental Medical Hospital, Dongguk University 37-21 Nonhyun-Dong, Gangnam-Gu, Seoul 135-010, Korea; Tel: 82-2-3416-9737, Fax: 82-2-3416-9770, E-mail: obgykdi@netian.com

pancreatic islets. The antimetastatic and cytotoxic activity of the crude extract or the isolated compounds, however, have not yet been demonstrated. The stems of EA, commonly known as winged euonymus, have been used in traditional medicine for cancer treatment. Previous phytochemical and biological studies on winged euonymus have resulted in the isolation of cardenolides[3]. Substances isolated from EA have been documented to exhibit antioxidant capabilities, and recent studies also indicated that EA has anti-tumor potential[4]. It was reported that the crude extract of EA markedly prolonged the survival period of cervical carcinoma-bearing mice, and methanol extract from this plant. Methanol and buthanol extracts were also found to have anti-tumor activity in mice[5]. Moreover, there are some reports on the action of EA extract on transformed cells in vitro[4]. It was recently found that the methanolic extract of EA exhibited a significant anti-proliferation effect against cultured human cancer cell lines[6]. Our recent findings also suggest that EA is a potent antioxidant in protecting primary hepatocytes from oxidative damage induced by aflatoxin B1, a well recognized hepatocarcinogen [unpublished results]. In our preliminary study, EA inhibited ULMC proliferation with an increased PKC activity.

In the cells, prostaglandin E_2 (PGE_2) levels are very low or undetectable in normal conditions, but can rise during inflammatory processes and multiple sclerosis[7]. High levels of PGE_2 can affect the activities of several cell types such as endothelial cells, and can regulate macrophage and lymphocyte functions during inflammatory and immune processes[8]. Therefore, the interplay between PGE_2 and other local factors, including pro- and anti-inflammatory cytokines, is likely to influence the outcome of inflammatory and immune responses in the cellular system. There is considerable evidence linking the generation of prostaglandins (PGs) with inflammation, pain, and fever. It is suggested that regulation of arachidonic acid (AA) metabolism, particularly PGE_2 production, appears beneficial in patients with inflammatory conditions[9].

Phospholipase A_2 (PLA_2) is subdivided into several groups based on their structures and enzymatic characteristics[10,11,12]. Secretory (s)PLA_2 is a family of low-molecular-mass (~14 kDa) enzymes that require millimolar concentration of Ca^{2+} for enzymatic activity. Cytosolic ($cPLA_2$), or group IV PLA_2, is a ubiquitously distributed 85 kDa enzyme, the activation of which is tightly regulated by postreceptor transmembrane signaling. $cPLA_2$ is activated by p42/p44 extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) under cytosolic Ca^{2+} concentration of the submicromolar or micromolar range, and p42/p44 ERK/MAPK was activated by dual phosphorylation of tyrosine/threonine residues[11,13].

Cyclooxygenase (COX) is the rate-limiting enzyme in PG synthesis and exists as two isoforms, constitutive (COX-1) and inducible (COX-2). These isoforms originate from distinct genes, but are structurally conserved[14,15]. COX-1 is regarded as a constitutive enzyme whose expression is developmentally regulated. PGs produced by COX-1 primarily function in fluid and electrolyte homeostasis, gastric acid secretion, and platelet aggregation. In contrast, COX-2 is expressed in response to inflammatory stimuli and is active in physiological responses to growth factors and glucocorticoids[16].

In this study, we examined the effect of CME from EA on AA cascade in breast cancer cells.

Materials and Methods

1. Materials

Fetal bovine serum was obtained from Cell Culture Laboratory (Cleveland, OH, USA); horse serum was

purchased from Jeil Biotech (Daegu, Korea) and also obtained from GIBCO BRL). Eagle' s minimum essential medium (EMEM) was purchased from Nissui Pharmaceutical Co. Ltd. PGE_2 was a generous from Ono Pharmaceuticals. Anti-PGE_2 antibody was obtained from Chemicon International Inc. [3H]-PGE_2 (200 Ci/mmol) and [14C]-AA (51 mCi/mmol) were from NEN/DuPont. Anti-phospho-p44/p42 ERK/MAPK antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG were obtained from New England Biolabs. COX-1 and -2 were purchased from Cyman Chemical. AA was obtained from Sigma Chemical Co., Ltd. AA and [14C]-AA were diluted by dimethyl sulfoxide. A final concentration of less than 0.5% dimethyl sulfoxide was kept. Other chemicals and drugs were of reagent grade or of the highest quality available.

2. Plant material of methanol extracts

The plant samples were extracted three times with methanol at 70℃ for 5 hours. The extracts were filtered through a 0.45 ㎛ filter and lyophilized. The w/w yield of the methanol extracts was about 2.25%[7]. The extracts were filtered and concentrated to remove the solvent at 75℃ for 4 and 100 of CME was yielded eventually. The CME was kept at 4℃ and dissolved with 10% DMSO in RPMI 1640 medium containing 10% FCS for further experiment. The stems of EA (Thunb.) Sieb were collected in Kyungju city, the Republic of Korea, and the sample and voucher specimen (number 4-99-221) are kept in the herbarium of the College of Oriental Medicine, Dongguk University.

3. Cell culture

SKBR3 human breast cancer cell line wasgrown in F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37℃ humidified incubator in an atmosphere of 5% CO_2 in air.

4. Assay of PGE_2

SKBR3 human breast cancer cells were seeded into 12-well plates at the density of 1.0×10^5 cells per well. The experiment was performed 2 days after seeding of cells. The cells were washed twice with EMEM buffered with 20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; EMEM-HEPES), pH 7.35, and were pre-incubated with or without CME-EA for 10 min. The cells were further incubated with or without 10 M A23187 for an additional 10 min. The medium was acidified to pH 4.0 by addition of 1 N HCl, and PGE_2 was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N_2 gas, the sample was dissolved in 10 mM Tris-HCl (pH 7.6). PGE_2 was determined by radioimmunoassay, as described previously[17,18].

5. Immunoblotting

SKBR3 human breast cancer cells were seeded into 6-well plates at the density of 2.0×10^5 cells per well. Two days after seeding, the cells were washed twice with EMEM-HEPES and pre-incubated with or without CME-EA for 10 min at 37℃. After the cells were incubated with or without 10 M A23187 for an additional 2 min, the medium was aspirated. The cells were solubilized by the addition of Laemmli sample buffer[19], the composition of which was Tris-HCl 187.5 mM, sodium dodecyl sulfate 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8. The sample was boiled at 95℃ for 5 min. Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto Immobilon polyvinylidene difluoride membranes (Millipore) by the semi-dry blotting method[20]. The immunoblots were blocked for 2 h with 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 25℃ and incubated with anti-phospho-p42/p44 ERK/MAPK antibody (rabbit) at 1 g/mL for 2 h at 25

℃. The immunoblots were washed several times and incubated with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG in TBST containing 2% BSA overnight at 4℃. Blots were developed by using a chemiluminescence assay kit (Bio Rad) and visualized by exposing the membrane to Hyper-film ECL (Amersham).

6. Analysis of AA liberation

SKBR3 human breast cancer cells were seeded into 12-well plates at the density of 1.0×10^5 cells per well. Two days after seeding, the medium was changed to Dulbecco' s modified Eagle' s medium containing 0.3 Ci/ml of [14C]-AA, and the cell were incubated for 18 h. The cells were washed twice with EMEM-HEPES-3 mg/mL albumin solution (pH 7.35) and pre-incubated with or without CME-EA for 10 min at 37℃. After the cells were incubated with or without 5 M A23187 for 5 min, the reaction was terminated by removing the medium to tubes[21]. After acidifying the medium (0.6 mL) to pH 4.0 by 1N HCl, it was mixed with chloroform (1.2 mL) and water (0.6 mL). The lower phase was dried by the stream of nitrogen gas and applied to a thin layer chromatography (TLC) plate (LK5D, Whatman). The developer used was the upper phase of benzene-isooctane-acetic acid (60:30:3, v/v)[21]. [14C]-AA metabolites were visualized as radioluminogram with a Molecular Imager (GS363, Bio-Rad).

7. Assay of AA conversion by microsomes of cells

SKBR3 human breast cancer cells were seeded into 150-mm dishes at the density of 5.0×10^5 cells per dish. Three days after seeding, cells were homogenized in 50 mM Tris-buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid. Microsomes were prepared by centrifugation at 3,000 g for 5 min at 4℃ followed by centrifugation of the supernatant at 100,000 g for 60 min at 4℃. Microsomes (90 g of protein/tube) were pre-incubated in 50 mM Tris-buffer containing 2 M hematin and 5 mM tryptophan with or without CME-EA for 10 min at 37℃, and they were incubated with 0.1 Ci/mL of [14C]-AA for 40 min at 37℃. After the cells were incubated, the reaction was terminated by the addition of ice-cold diethyl ether/methanol/1 M citric acid (30:4:1, v/v). The ether layer was dried by a stream of nitrogen gas and applied to a TLC plate (LK5D, Whatman). The developer used was the upper phase of ethyl acetate/water/isooctane/acetic acid (110:100:50:20, v/v). [14C]-AA metabolites were visualized as radioluminogram with a Molecular Imager (GS363, Bio-Rad).

8. Assay of the enzyme activities of COX-1 and -2

Activities of COX-1 and -2 were determined according to the procedure described.[22]. COX-1 or -2 enzyme protein (each 0.5-1.0 unit) was dissolved in 150 μl of 50 mM Tris-buffer containing 2 M hematin and 5 mM tryptophan (reaction mixture). The reaction mixture was pre-incubated with or without drugs for 10 min at 37℃ and further incubated with 50 μl AA for 10 min at 37℃. Then, the reaction was terminated by the addition of 20 μl of 1N HCl. AA metabolites were extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of nitrogen gas, the sample was dissolved in 10 mM Tris-HCl (pH 7.6). PGE_2 was determined by radioimmunoassay, as described previously[17].

9. Data analysis

50 values were calculated from non-linear regression analysis of the data. The statistical differences ($p < 0.05$) of values was determined with ANOVA.

Results

1. Effect of CME from CME-EA on PGE_2 synthesis stimulated by A23187 in SKBR3 human breast cancer cells

A23187, a Ca^{2+} ionophore, is known to stimulate PG synthesis mediated through an activation of $cPLA_2$ following by AA liberation in glial cells[23]. In SKBR3 human breast cancer cells, A23187 potently stimulated PGE_2 release (2.3 ng/well from 0.05 ng/well), and CME inhibited the release in a concentration-dependent manner, with the value of about 5 M (Fig. 1).

2. Effect of CME-EA on p42/p44 ERK/MAPK phosphorylation and AA liberation

To determine the site of action of CME-EA, we first examined upstream of AA liberation. The p42/p44 ERK/MAPK is known to be involved in a wide range of cellular functions, including $cPLA_2$ activation.[24,25]. CME-EA (110 ug/ml) slightly augmented, but do not inhibited A23187-induced phosphorylation of p42/p44 ERK/MAPK (data not shown), indicating that the acting site of CME-EA for inhibition of PGE_2 release is downstream of p42/p44 ERK/MAPK.

Because CME-EA did not cause the inhibition of phospholyration of p42/p44 ERK/MAPK, we examined the effect of CME-EA on AA liberation from [14C]-AA-labeled cells (Fig. 2). CME-EA slightly augmented, but did not inhibited A23187-induced AA liberation, suggesting that CME-EA inhibits PGE_2 synthesis at the downstream of AA liberation. Interestingly, CME-EA alone at a concentration of 20 μg/ml did activate rather than inhibit the liberation of AA.

3. Effect of CME-EA on the conversion of AA to PGE_2 in microsomal fraction

COX, existing in microsomes, is the rate-limiting enzyme in the conversion of AA to prostanoids.

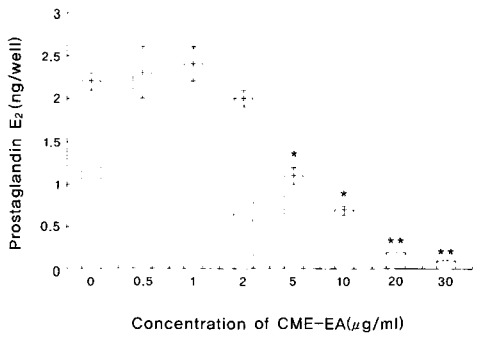

Fig. 1. Effect of CME-EA on PGE_2 release from SKBR3 human breast cancer cells.

The cells were preincubated with indicated concentrations of CME-EA for 10 min, and they were stimulated by 5 M A23187 for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. * and **, Significant difference from A23187 alone ($p<0.05$; $p<0.01$).

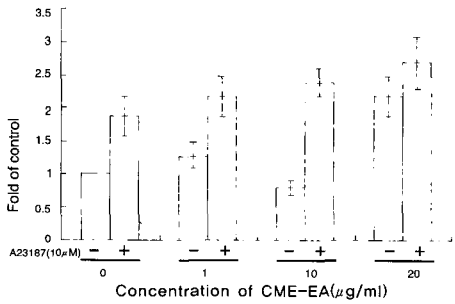

Fig. 2. Effect of CME-EA on liberation of AA.

The cells were labeled with 0.3 Ci/mL of [14C]-AA for 18 h. After washing out, the cells were pre-incubated with indicated concentrations of CME-EA for 10 min, and they were stimulated by 5 M A23187 for 5 min. [14C]-AA was analyzed in radioluminogram with a molecular imager (GS363, Bio-Rad) after separation by TLC (upper figure). Densitometric analysis of radioluminogram (lower figure). Open column: basal liberation, hatched column: A23187-induced liberation. The results are shown as the 1.5-3 fold increase from control (without A23187). Each column represents the mean with S.E. of three determinations.

Therefore, we examined the conversion of AA to PGE_2 in the microsomal fraction. Western blotting analysis revealed that COX-1, but not COX-2, was expressed in SKBR3 human breast cancer cells under this experimental condition (data not shown). Incubation of the microsomes with [14C]-AA resulted in the productions of [14C]-eicosanoids. Among them, [14C]-PGE_2 was a main metabolite. CME inhibited [14C]-PGE_2 production from [14C]-AA in a concentration-dependent manner, with the IC50 value of about 17 μl /ml (Fig. 3).

4. Effect of CME-EA on enzyme activities of COX-1 and -2 in vitro

Because CME-EA inhibited the conversion of AA to PGE_2 in microsomes, we examined the direct effect of CME-EA (Fig. 4A,B)on COX-1 or -2 enzyme in vitro, comparing the effects of indomethacin, a non-selective inhibitor of COX, and NS398, a selective inhibitor of COX-2. Indomethacin strongly inhibited both the COX-1 and -2 activities in a concentration-dependent manner (Fig. 5A and B). On the other hand, NS-398 inhibited the COX-2 activity in a concentration-dependent manner, but not COX-1 activity (Figs. 7A and B). CME-EA inhibited both the COX-1 and -2 activities in a concentration-dependent manner, with the IC_{50} values of about 1.0 μg/ml and 20 μg/ml, respectively (Figs. 4A and B).

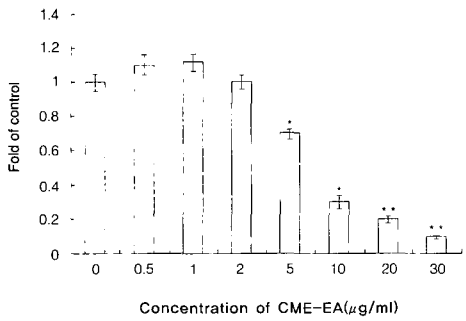

Fig. 3. Effect of CME-EA on COX activity of microsomes of SKBR3 human breast cancer cells.

Microsomes of SKBR3 human breast cancer cells were pre-incubated with indicated concentrations of CME-EA for 10 min and were incubated with [14C]-AA for 40 min. [14C]-AA metabolites were analyzed in radioluminogram with a molecular imager (GS363, Bio-Rad) after separation by TLC. Densitometric analysis of radioluminogram (lower figure). The results are shown as the 0.2-1 fold increase as without CME of 1. Each point represents the mean with S.E. of three determinations. * and **, Significant difference from without CME-EA ($p<0.05$; $p<0.01$).

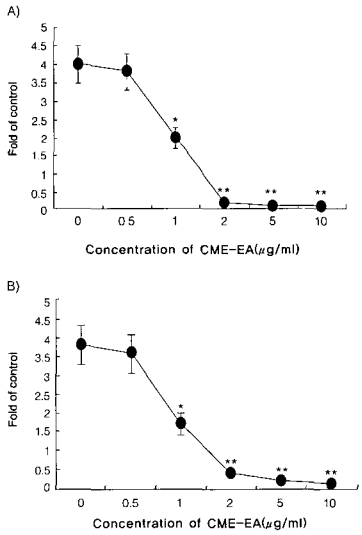

Fig. 4. Effects of CME-EA on enzyme activities of COX-1 and -2.

The reaction mixture with COX-1 (A) or -2 (B) were pre-incubated with indicated concentrations of CME-EA for 10 min and were incubated with AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. *Significant difference from without treatment.

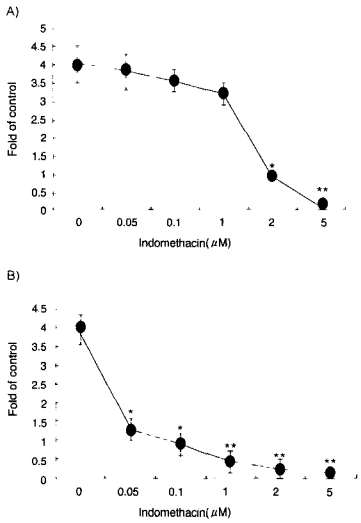

Fig. 5. Effects of indomethacin on enzyme activities of COX-1 and -2.

The reaction mixture with COX-1 (A) or -2 (B) were pre-incubated with indicated concentrations of indomethacin for 10 min and were incubated with AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. *Significant difference from without treatment.

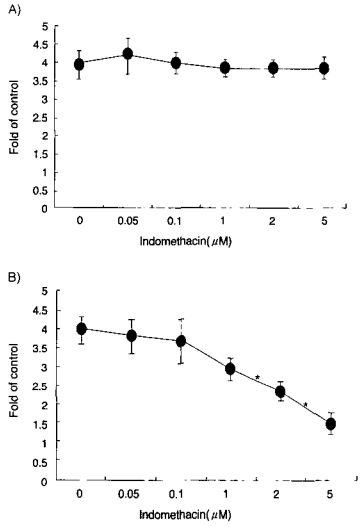

Fig. 6. Effects of NS-398 on enzyme activities of COX-1 and -2.

The reaction mixture with COX-1 (A) or -2 (B) were pre-incubated with indicated concentrations of NS-398 for 10 min and were incubated with AA for 10 min. The released PGE_2 in the medium was determined by radioimmunoassay. Each point represents the mean with S.E. of three determinations. *Significant difference from without treatment.

Discussion

EA has been widely used as an anti-inflammatory agent for the treatment of skin infections, wounds, and diarrhea for many years. Although EA has long been served as traditional medicines, very few authentic scientific studies in field of cancer therapy are available. Recent in vitro studies have shown that many constituents from EA have a wide range of biological actions including antibacterial, antifungal, antihelmith and insecticidal activities. We selected CME-EA to examine its activity of PGE_2 synthesis. In the analysis using SKBR3 human breast cancer cells, we show that CME-EA has a potent inhibitory effect on PGE_2 synthesis. In the present study, we showed for the first time that CME-EA directly binds to COX and inhibits its activity. This effect of CME-EA may contribute to its anti-inflammatory activity.

Because a high PGE_2 level is observed in some diseases, such as multiple sclerosis and AIDS-associated dimension, drugs reducing PGE_2 synthesis in SKBR3 human breast cancer cells have a possibility to improve the diseases with inflammation in the gynecological region. Thus, CME-EA is one of the candidates of the drugs for treatment brain diseases accompanied with inflammation, although further detailed analysis is necessary, such as metabolism and distribution of the drug in vivo.

Because CME-EAhas an inhibitory effect of PGE_2 synthesis, we examined its site of action. However,

CME-EA slightly augmented, but not inhibited, the phosphorylation of p42/p44 ERK/MAPK. Furthermore, the A23187-induced AA liberation was slightly augmented, but not inhibited, by CME-EA. Interestingly, CME-EA alone at a concentration of 20 ㎍/ml did activate rather than inhibit the liberation of AA, although it inhibited PGE_2 synthesis. Because the acting site of CME-EA may be downstream of AA liberation, we examined the effect of CME-EA on the conversion of AA to PGE_2 in microsomal preparations. The conversions of AA to PGE_2 was potently inhibited by CME-EA, suggesting that the site of CME-EA was COX. In the analysis of enzyme activity in vitro, we found that CME-EA directly interacted with COX-1 and -2 and inhibited their activities. There is a certain difference in the 50 values of CME-EA between intact cells and microsomal preparations. However, the 50 values of CME-EA in purified COX enzymes are similar to the 50 values in intact cells. Because - CME-EA inhibited both the COX-1 and -2, the drug seems to be similar to indomethacin in its selectivity to COX. Indomethacin is known as a competitive, time-dependent, reversible COX inhibitor[34]. Aspirin is a competitive, irreversible COX inhibitor. Like indomethacin, CME-EA does not have a site of covalent modification (e.g. acetoxyl group). Then, CME-EA may be a reversible COX inhibitor.

In conclusion, we showed for the first time that CME-EA reduces PG generation through its direct inhibition of COX.

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