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Self-assembled micellar formulation of chafuroside A with improved anti-inflammatory effects in experimental asthma/COPD-model rats

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ABSTRACT

Chafuroside A (CFA), a poorly water-soluble flavone C-glycoside, was firstly isolated from oolong tea, and it acts as a potent anti-inflammatory agent. The present study was undertaken to develop a water-soluble formulation of CFA using a self-assembled micellar (SAM) system, with the aim of improved dissolution behavior and potent anti-inflammatory effects. The SAM formulation of CFA (CFA/SAM) was characterized in terms of its morphology, particle size distribution, crystallinity, and dissolution behavior. In dissolution testing, the CFA/SAM exhibited marked improvement in dissolution behavior when compared with crystalline CFA, and then, nano-micellar particles were constituted with a mean diameter of 84 nm. The therapeutic potential of the crystalline CFA and CFA/SAM was assessed using an experimental asthma/chronic obstructive pulmonary disease (COPD)-like model. Orally-administered CFA at 0.5 mg/kg or higher could attenuate inflammatory symptoms in a dose-dependent manner, as evidenced by decreases of infiltrated granulocytes, including macrophages and neutrophils, and myeloperoxidase, a specific biomarker for neutrophilia. Biomarker profiling demonstrated that the CFA/SAM at 0.1 mg CFA/kg was equipotent to CFA at 1.0 mg/kg in ameliorating antigen-induced airway inflammation, suggesting the better pharmacological effect of CFA/SAM due to improved dissolution behavior. From these observations, the SAM formulation might be an efficacious approach for enhancing the therapeutic potential of CFA for treatment of inflammatory diseases.

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1. Introduction

Tea beverages and tea polyphenols have drawn considerable attention from researchers and the general public because of their health benefits for a variety of disorders, ranging from cancer to obesity (Hursel and Westerterp-Plantenga, 2010; Zaveri, 2006). A number of *in vitro* and *in vivo* studies have demonstrated the therapeutic potential of oolong tea and its extract for the treatment of various diseases, such as recalcitrant atopic dermatitis, obesity, and cancer (Aoki et al., 2007; Uehara et al., 2001), even though their detailed mechanisms and major contributing constituents have not been fully elucidated. Recently, a new flavone derivative, chafuroside A [CFA; (2*R*,3*S*,4*S*,4a*S*,11b*S*)-3,4,11-trihydroxy-2-(hydroxymethyl)-8-(4-hydroxyphenyl)-3,4,4a,11b-tetrahydro-2*H*,10*H*-pyrano[2′,3′:4,5]furo[3,2-g]chromen-10-one], was isolated as a bioactive component from oolong tea leaves (Ishikura et al., 2004). CFA has been believed to serve as a free-radical scavenger and anti-inflammatory agent (Tammariello and Milner, 2010), and CFA orally-administered at dose of 1.0–10 mg/kg exhibited potent suppression on inflammatory responses in dinitrofluorobenzene-sensitized mice.

In spite of these attractive biological functions, CFA was found to be poorly soluble in water (equilibrium solubility in water (37 °C): 3.3 μg/mL, unpublished data), possibly leading to limited *in vivo* efficacy. In this context, as observed in biopharmaceutics classification system class II and IV materials, solubilization technology could be a key consideration for improving the biological activity of CFA. Currently, self-assembled micellar (SAM) formulation is accepted as a potential oral dosage form for improving solubility and dissolution of poorly soluble chemicals (Torchilin, 2007). Application of the SAM formulation approach to CFA might also provide better pharmacological outcomes, although no efforts have been made to prepare and characterize SAM formulation of CFA so far. The purpose of the present study was to develop a novel SAM formulation of CFA (CFA/SAM) with improved dissolution behavior and pharmacological effects. The newly prepared CFA/SAM, a polymeric mixture containing CFA, PEG400, propylene glycol, and

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ethanol, self-assembled to form micellar nanoparticles in aqueous media. The CFA/SAM was characterized in terms of its morphology, particle size distribution, solubility, and dispersibility. In experimental asthma/chronic obstructive pulmonary disease (COPD)-like rats, the therapeutic potential of CFA/SAM was assessed on the basis of histochemical analyses and inflammation-related biomarkers.

2. Materials and methods

2.1. Chemicals

CFA (Fig. 1A) was chemically synthesized from isovitexin by modified Mitsunobu reaction with triphenylphosphine and diethyl azodicarboxylate as reported previously (Ishida et al., 2009). The synthesized CFA was characterized by Fourier transform infrared (FT-IR) and ultraviolet (UV) spectra, LC-MS/MS, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra according to previous report (Ishida et al., 2009). Ammonium acetate, *o*-phenylenediamine (OPD), polyethylene glycol 400 (PEG400), propylene glycol, sodium dodecyl sulfate (SDS), and triton X-100 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Horseradish peroxidase, ovalbumin (OVA), and sodium pentobarbital were purchased from Sigma (St. Louis, MO). 3,3′-Diaminobenzidine (DAB) and 3,3′,5,5′-tetramethylbenzidine (TMBZ) were bought from Tokyo Chemical Industry (Tokyo, Japan) and Dojindo (Kumamoto, Japan), respectively. All other chemicals were purchased from commercial sources.

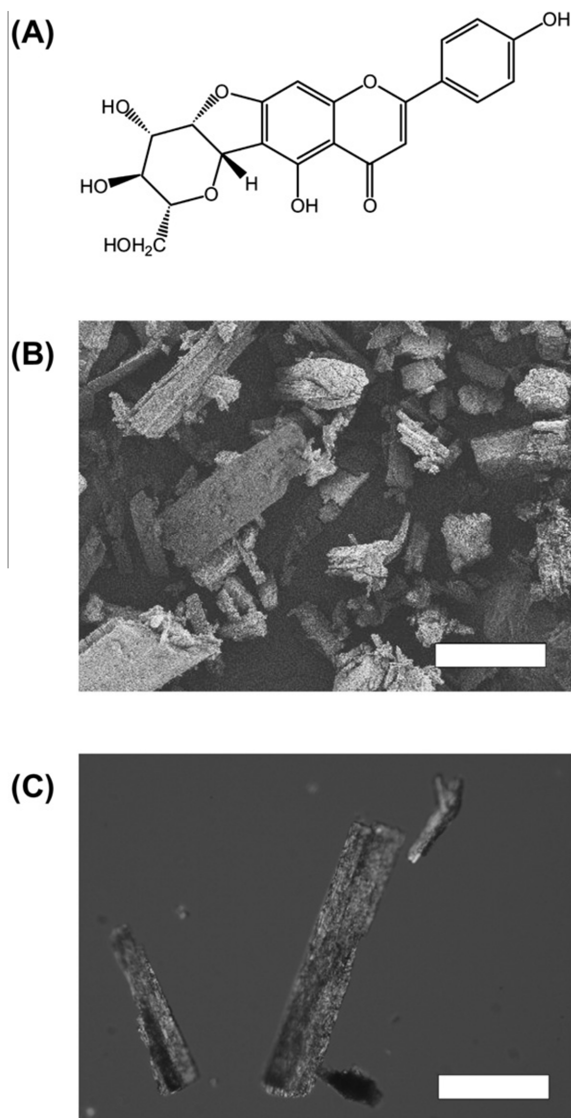

Fig. 1. Chemical structure (A) and morphology (B and C) of CFA. Surface morphology of crystalline CFA was evaluated by scanning electron microscopy (B) and polarized light microscopy (C). White bar represents 100 μm.

2.2. Self-assembled micellar formulation of CFA

After preliminary studies on the solubility of CFA in micelle components, CFA (1 mg) was solubilized in 160 μL of transparent structured mixture of PEG400/propylene glycol/tween 80/ethanol (1:1:1:1). Final formulations were prepared 30 min before dosing through addition of ca. 125-fold amount of distilled water (final concentration, 0.05 mg CFA/mL), then self-assembled to form micelles. The amount of CFA in the formulations was determined by an absolute calibration curve method using the Waters Acquity UPLC system (Waters, Milford, MA) equipped with electrospray ionization mass spectrometry (UPLC/ESI-MS). An Acquity UPLC BEH C 18 column (particle size: 1.7 μm, column size: 2.1 mm × 50 mm; Waters) was used, and column temperature was maintained at 40 °C. Samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 5 mM ammonium acetate (B) with a flow rate of 0.25 mL/min, and the retention time of CFA was 2.21 min. The gradient condition of the mobile phase was 0–0.5 min, 15% A; 0.5–3.0 min, 15–30% A; and 3.0–4.0 min, 90% A. Analysis was carried out using selected ion recording (SIR) for specific *m/z* 413 for CFA $[M–H]^-$.

2.3. Microscopic experiments

2.3.1. Scanning electron microscopy (SEM)

Representative scanning electron microscopic images of CFA samples were taken using a VE-7800 scanning electron microscope (Keyence Corporation, Osaka, Japan). For the SEM observations, each sample was fixed on an aluminum sample holder using double-sided carbon tape.

2.3.2. Transmission electron microscopy (TEM)

An aliquot of CFA/SAM dispersed in water was placed on a carbon-coated Formvar 200 mesh nickel grid. The sample was allowed to stand for 15–30 s, and then any excess solution was removed by blotting. The samples were negatively stained with 2% (w/v) uranyl acetate and allowed to dry. The samples were then visualized under an H-7600 transmission electron microscope (Hitachi, Tokyo, Japan) operating at 75 kV.

2.3.3. Polarized light microscope (PLM)

Representative PLM images of CFA samples were taken using a CX41 microscope (Olympus Co. Ltd., Tokyo, Japan). CFA samples were examined under various conditions including differential interference contrast, slightly uncrossed polars, and using a red wave compensator.

2.4. Dissolution test

Dissolution tests were carried out in 900 mL of acidic solution (pH 1.2) and a phosphate buffer solution (pH 6.8) with constant stirring at 100 rpm in a dissolution test apparatus NTR 6100A (Toyama Sangyo, Osaka, Japan) at 37 °C. Each CFA sample (0.9 mg of CFA) was weighed in the dissolution vessel. The collected samples were centrifuged at 15,000 rpm for 5 min, and the supernatants were diluted with an equal amount of methanol. The concentrations of CFA were determined by Waters UPLC/ESI-MS as described in Section 2.2 CFA.

2.5. Dynamic light scattering (DLS)

Mean particle size of the CFA/SAM suspended in water at a final concentration of 10 mg/mL was measured in triplicate by dynamic light scattering (DLS) using a Zetasizer Nano ZS (MALVERN, Worcestershire, UK). All measurements were performed at 25 °C at a measurement angle of 90°.

2.6. Animals and antigen-sensitization

Male Sprague–Dawley rats (8–11 weeks of age; Japan SLC, Shizuoka, Japan), weighing ca. 400 g, were housed three per cage in the laboratory with free access to food and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). Animals were fasted for 12 h before experiments. Rats were sensitized by the intraperitoneal injection of 100 μg ovalbumin (OVA) with 5 mg alum on days 0, 7, and 14. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and received intratracheal administration of OVA (100 μg/rat) powder at 24 h after the last OVA sensitization. Before the final OVA challenge, crystalline CFA (0.1, 0.5, or 1.0 mg/rat, once a day) or CFA/SAM (0.1 mg CFA/rat, once a day) suspended in water was repeatedly administered orally for 7 days. All procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of University of Shizuoka.

2.7. Total cell count in bronchoalveolar lavage fluid (BALF)

At 24 h after OVA challenge, rats were exsanguinated via the descending aorta under anesthesia. A bronchoalveolar lavage was performed immediately using 5 mL of PBS. Recovery rate of BALF was approximately 70%. The number of cells in BALF was counted using a Burker-Turk counting chamber after the addition of an equal amount of 0.2% trypan blue. The collected BALF samples were subjected to a flow cytometric hematology system (XT-2000iV, Sysmex Corporation, Kobe, Japan) and Diff-Quick staining (Behring Ag, Dudingen, Switzerland) to classify the type of inflammatory cells.

2.8. Biomarkers in BALF

Enzymatic detection of MPO and EPO in BALF was performed in accordance with a previous report (Misaka et al., 2010; Onoue et al., 2011). For MPO measurement, assay mixtures consisted of 40 μL of H_2O_2 (final concentration 0.3 mM) in 80 mM sodium phosphate buffer (pH 5.4) and 50 μL of plasma samples. The reaction was initiated by the addition of 10 μL of TMBZ (final concentration 1.6 mM) in dimethyl sulfoxide at 37 °C, and stopped after 2 min by the addition of 0.18 M H_2SO_4. Subsequently, optical density was determined at 450 nm. For detection of EPO activity in plasma, the reaction mixture was prepared by adding 500 μL of OPD (50 mM) to 24.25 mL of Tris buffer (pH 8.0), 3 μL of 30% H_2O_2, and 25 μL of Triton X-100. Then, 100 μL of reaction mixture was added to 50 μL of biological fluid sample in a 96-well plate and incubated for 30 min at room temperature. The reaction was stopped by the addition of 2 M H_2SO_4, and absorbance at 490 nm was measured. A titration curve of horseradish peroxidase was used for the calculation of MPO and EPO activities, which are expressed in arbitrary units. All samples were assayed in duplicate, and optical densities in all assays were measured using a microplate reader, Safire (Tecan, Männedorf, Switzerland).

2.9. Statistical analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher's least significant difference procedure was carried out using Statcel software (OMS publishing Inc., Saitama, Japan). A P value of less than 0.05 was considered significant for all analyses.

3. Results and discussion

3.1. Physicochemical characterization of CFA/SAM

In SEM images, crystalline CFA showed the particles to be predominantly dispersed and irregularly shaped, with sizes ranging over about 10–200 μm (Fig. 1B). According to PLM appearance (Fig. 1C), intense birefringence was observed in CFA powder, suggesting its high crystallinity. The PLM observation was consistent with the result from powder X-ray diffraction analysis on CFA powder, in which several intense peaks were observed (data not shown). In general, solubility and wettability of crystalline chemicals tend to be less than those of their amorphous forms, and poor dissolution and high variations in bioavailability and pharmacodynamics were often observed in some crystalline forms of bioactive chemicals. For improvement of the solubility of poorly soluble chemicals, popular and commercially viable formulation approaches can be used, such as formation of emulsions or micelles and amorphization. Amorphous formation of poorly water-soluble chemicals would serve to enhance the intrinsic solubility of bioactive chemicals, resulting in a faster dissolution rate and then a supersaturated solution in dissolution media. However, despite the development of extensive expertise with amorphous formulations, they are not widely used in commercial products, mainly because there is a possibility that the amorphous state may undergo crystallization during processing or storage, eventually resulting in limited dissolution behavior (Vasconcelos et al., 2007). Recently, the preparation of submicron micelles has emerged as a promising alternative dosage form (Strickley, 2004) because of their long-term stability, ease of preparation and high solubilization of drug molecules. The SAM approach was chosen for solubilization of CFA in the present study.

A micellar preconcentrate of CFA was prepared consisting of CFA, PEG400, Tween 80, and propylene glycol, which formed a spontaneous and fine dispersion upon contact with the aqueous media. To clarify possible enhancement of dissolution behavior by SAM approaches, dissolution testing for CFA formulation was carried out in both acidic solution (pH 1.2) and a phosphate buffer solution (pH6.8) at 37 °C (Fig. 2A). Poor dissolution behaviors of crystalline CFA powders were seen in both dissolution media, and there was no significant difference in terms of the release of CFA between them. In contrast, SAM formulation of CFA showed fast dispersion in both conditions. For at least 2 h after suspension of CFA/SAM, no significant increase of turbidity was observed without any precipitation at room temperature (data not shown). Morphology of water-suspended CFA/SAM was characterized by TEM observation, indicating that all the micellar particles were evenly distributed and spherical (Fig. 2B). The micellar preconcentrate of CFA formed a nano-sized micelle where particle sizes were less than 300 nm. In DLS analysis of water-suspended CFA/SAM, the mean particle size of CFA micelle was found to be 84 nm with polydispersity index of 0.28 (Fig. 2C), indicating that the micellar formulation was moderately homogeneous. The particle size estimated from DLS analysis seemed to be slightly smaller than the diameters observed by TEM, and the differences in experimental condition might be part of reason for the data discrepancy. On the basis of the dissolution profiles, as well as physicochemical

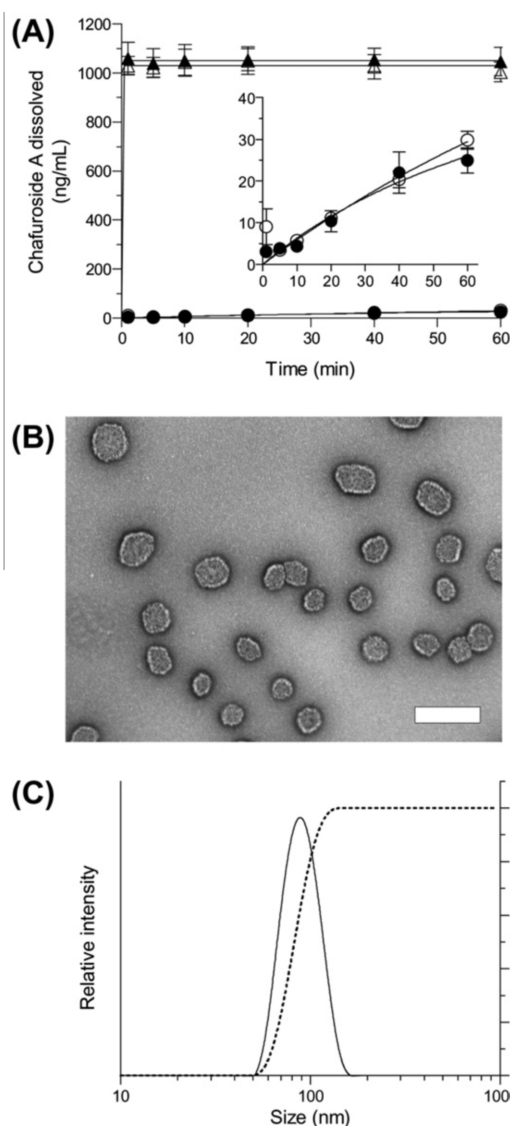

Fig. 2. Dissolution behaviors of CFA samples. (A) *In vitro* dissolution behavior of CFA samples in acidic solution (pH 1.2) and a phosphate buffer solution (pH 6.8) at 37 °C. ●, CFA at pH 1.2; ○, CFA at pH 6.8; ▲, CFA/SAM at pH 1.2; and △, CFA/SAM at pH 6.8. Data represent mean ± SE of 3 experiments. (B) Transmission electron microscopic image of the CFA/SE dispersed in distilled water. Bar represents 500 nm. (C) Particle size distributions of the CFA/SE after suspension in water. Solid line, intensity; and dashed line, accumulation.

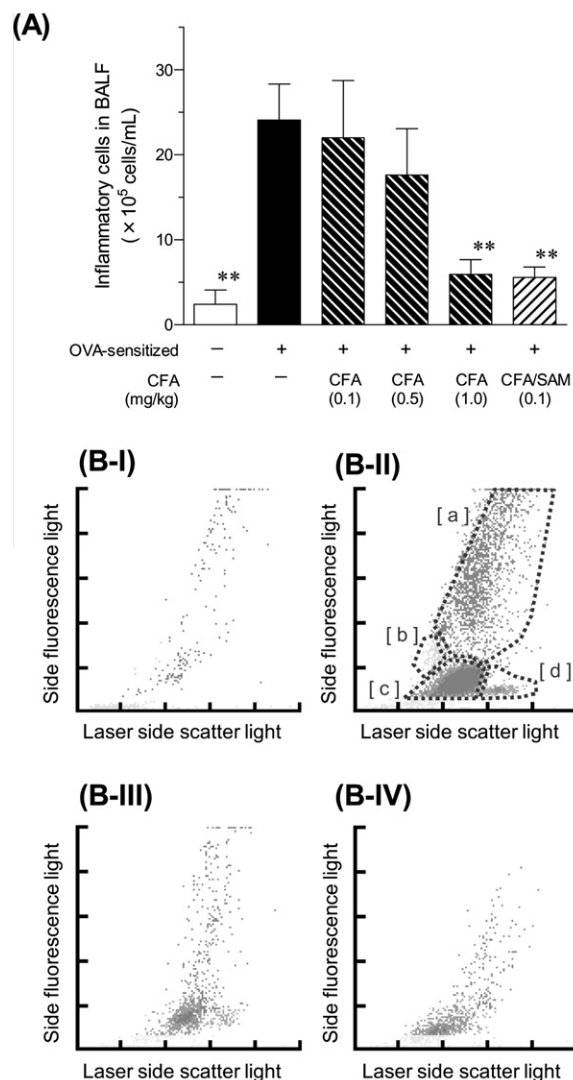

Fig. 3. Anti-inflammatory effects of CFA samples in antigen-sensitized rats. (A) Inflammatory cells recruited in BALF. Data represent the mean ± SE of 4–6 rats. $^{**}p < 0.01$ with respect to the antigen-sensitized rats. (B) Cytogram of BALF from antigen-sensitized rats. (I) Non-sensitized rat; (II) OVA-sensitized rat; (III) OVA-sensitized rat with CFA (0.5 mg/kg); and (IV) OVA-sensitized rat with CFA (1.0 mg/kg). Cells were classified as monocytes (a), lymphocytes (b), neutrophils (c), and eosinophils (d).

characteristics of water-suspended CFA/SAM, the SAM formulation of CFA might be a suitable approach for oral use because of the improved dissolution property under both acidic and neutral conditions.

3.2. Anti-inflammatory effects of orally-administered CFA formulations in lung

To elucidate the therapeutic potential of CFA/SAM, anti-inflammatory effects of orally-administered crystalline CFA and CFA/SAM were assessed using experimental asthma/COPD-model rats that we developed previously (Misaka et al., 2009). Both COPD and asthma can be defined as airway inflammation with some differences in inflammatory cells, mediators, response to inflammation, and anatomical distribution. For clinical investigation of these inflammatory pulmonary diseases, BALF has been frequently used as a reliable biological source (Tzortzaki et al., 2006). In the present study, BALF was obtained at 24 h after the last antigen challenge, and the inflammatory cells in BALF were counted (Fig. 3). The antigen challenge caused a marked recruitment of inflammatory cells in BALF as evidenced by ca. 10-fold increase of cell numbers (Fig. 3A). Treatment of antigen-exposed rats with CFA at 0.5 and 1.0 mg/kg resulted in a dose-dependent reduction of inflammatory cells in BALF by ca. 30% ($P = 0.26$) and ca. 84% ($P < 0.01$), respectively, although CFA at 0.1 mg/kg found to be less effective.

For further characterization, types of recruited cells in BALF were analyzed using a Sysmex XT-2000iV system (Fig. 3B). DIFF scattergram of BALF from the antigen-sensitized rats suggested that the recruited cells mainly consisted of monocytes and neutrophils, and a smaller number of eosinophils were also observed (Fig. 3: B-II). Activated macrophages tend to produce and secrete matrix metalloproteases, and activated neutrophils also produce matrix-degrading proteases and reactive oxygen free radicals, leading to damage of the epithelium and underlying basement membrane in pulmonary tissues (Barnes, 2004). Interestingly, oral administration of the CFA at 0.5 and 1.0 mg/kg resulted in

Table 1
Recruited immune cells in BALF.

	Recoverable cells in BALF		
	Macrophages ($\times 10^5$ cells/mL)	Neutrophils ($\times 10^5$ cells/mL)	Eosinophils ($\times 10^4$ cells/mL)
Non-sensitized rats	2.08 ± 0.15**	0.23 ± 0.13**	0.06 ± 0.03**
OVA-sensitization with			
Vehicle (Control)	10.23 ± 1.97	10.13 ± 1.07	3.72 ± 1.06
CFA (0.1 mg/kg)	6.11 ± 1.72*	10.36 ± 1.08	5.54 ± 0.87
CFA (0.5 mg/kg)	7.53 ± 0.38	7.45 ± 0.51*	2.59 ± 0.42
CFA (1.0 mg/kg)	3.03 ± 0.11**	2.15 ± 0.12**	0.76 ± 0.06**
CFA/SAM (0.1 mg CFA/kg)	2.44 ± 0.40**	2.44 ± 0.32**	0.69 ± 0.19**

Each BALF sample was stained by Wright-Giemsa method to classify the type of inflammatory cells. Data represent mean ± SE (n = 4–6).
* $p < 0.05$ with respect to OVA-sensitized rat (control).
** $p < 0.01$ with respect to OVA-sensitized rat (control).

decreases of both macrophages and neutrophils in the antigen-sensitized rats (Fig. 3: B-III/IV). These pathological transitions might be attributable to the anti-inflammatory effects of orally-administered CFA.

In addition to the CFA itself, the CFA/SAM also exhibits potent anti-inflammatory effects in antigen-sensitized rats, as evidenced by ca. 85% decrease of inflammatory cells in BALF (Fig. 3A). There was no significant difference in the number of granulocytes between antigen-sensitized rats with CFA at 1.0 mg/kg and that with CFA/SAM at 0.1 mg CFA/kg, suggesting a better anti-inflammatory effect of CFA/SAM compared with CFA. For quantitative analysis, the cells recovered from BALF were counted after Wright-Giemsa staining (Table 1). The numbers of macrophages, neutrophils, and eosinophils in BALF retrieved from OVA-exposed rats were higher than those in non-sensitized rats by 4.9-, 44-, and 62-fold, respectively. On the other hand, pretreatment with the CFA/SAM (0.1 mg CFA/kg) led to ca. 70–80% reductions in the numbers of these inflammatory cells, whereas it did not completely reach levels similar to those in the non-sensitized group in the present experimental condition. In contrast, CFA at the same dose (0.1 mg/kg) exhibited only slight reduction of macrophages, and no significant decrease in neutrophils and eosinophils was observed. On the basis of these findings, the CFA/SAM at 0.1 mg CFA/kg might be equipotent to CFA at 1.0 mg/kg, and the better pharmacological effect of CFA/SAM might be attributable to the improved dissolution behavior compared with crystalline CFA.

3.3. Profiling of pro-inflammatory biomarkers

In the development of asthma and COPD, MPO and EPO act as pro-inflammatory and pro-oxidant mediators, mainly released from activated neutrophils/macrophages and eosinophils, respectively (Onoue et al., 2009). These enzymatic activities have thus been suggested as inflammation biomarkers of granulocyte activation, possibly reflecting the degree or type of inflammation. In our investigation, MPO activity in BALF was markedly increased after OVA challenge (Fig. 4A; 0.81 ± 0.09 and 8.26 ± 2.70 mUnit/mL in non-sensitized and OVA-sensitized rats, respectively), whereas no significant elevation of EPO level in BALF was seen (Fig. 4B; 0.49 ± 0.07 and 0.62 ± 0.05 mUnit/mL in non-sensitized and OVA-sensitized rats, respectively). These observations were consistent with the results from the Wright-Giemsa staining of BALF cells in the OVA-sensitized rats, in which the neutrophils were 27-fold more numerous than the eosinophils. From these data, OVA challenge in the airway system resulted in significant elevation of MPO level in the lung, suggesting development of pulmonary neutrophilia in OVA-exposed rats. In contrast, there appeared to be significant decreases of MPO level in the BALF of OVA-exposed rats pretreated with CFA/SAM (0.1 mg CFA/rat) or CFA (1.0 mg/kg) by 90% and 69%, respectively, compared with that in the OVA-exposed rat. Not surprisingly, no significant changes in EPO level were seen even after pre-treatment with CFA or CFA/SAM. The data suggested that the orally-administered CFA/SAM could attenuate the neutrophilic inflammatory symptoms, in agreement with the marked inhibition on the recruitment of immune cells in the BALF. The improved dissolution behavior of SAM formulation would explain in part the marked differences in anti-inflammatory effects between CFA and CFA/SAM. The present observations could be indicative of the therapeutic potential of CFA/SAM for treatment of asthma, COPD, and other airway inflammatory diseases.

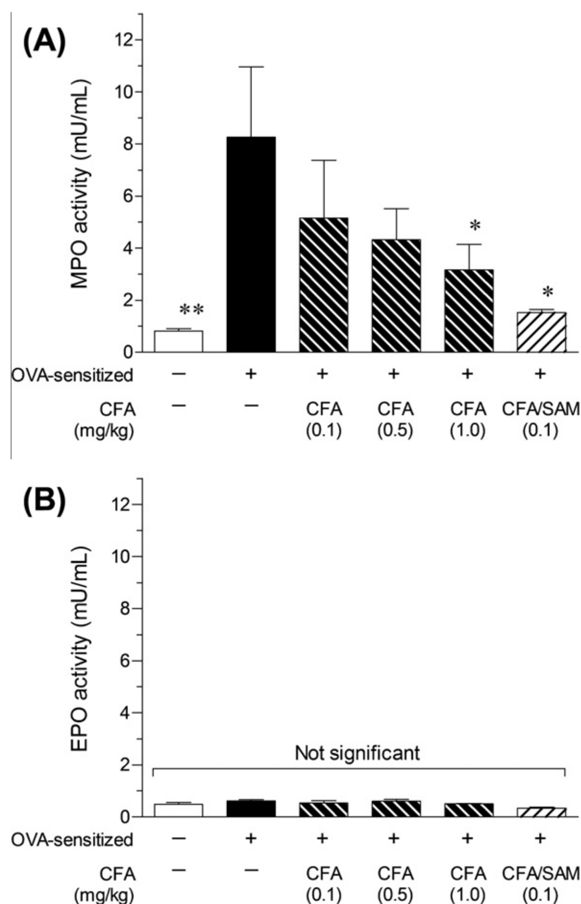

Fig. 4. Biomarker profiling in antigen-sensitized rats. (A) Myeloperoxidase (MPO) and (B) eosinophil peroxidase (EPO) activities in BALF recovered from antigen-sensitized rat with or without CFA treatment. Data represent the mean ± SE of 4–6 determinations. *$p < 0.05$; **$p < 0.01$ with respect to the antigen-sensitized rats without CFA treatment.

4. Conclusion

In the present study, water-soluble formulation of CFA was newly prepared using a SAM system. There appeared to be marked improvement in the dissolution behavior of the CFA/SAM compared with that of crystalline CFA, and this was consistent with outcomes from *in vivo* pharmacological studies in experimental asthma/COPD model rats. From these observations on dissolution and anti-inflammatory effect, SAM approach should be efficacious to enhance the pharmacological effects of CFA, and this might be advantageous for CFA-based clinical treatment of several inflammatory diseases.

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