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Short Communication

Cycloartenyl ferulate, a component of rice bran oil-derived γ -oryzanol, attenuates mast cell degranulationT. Oka^a, M. Fujimoto^a, R. Nagasaka^b, H. Ushio^b, M. Hori^{a,*}, H. Ozaki^a^a Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan^b Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo 108-8477, Japan

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ABSTRACT

IgE-targeting therapy could provide significant progress in the treatment of allergic inflammation. In this study, we examined the effect of cycloartenyl ferulate (cycloartenol ferulic acid ester; CAF), a natural product from rice bran oil-derived γ -oryzanol, on allergic reaction. When CAF and γ -oryzanol were injected intradermally with anti-DNP IgE into the dorsal skin of rats, the passive cutaneous anaphylaxis reaction induced by DNP-HSA was attenuated. CAF and γ -oryzanol also inhibited the degranulation of DNP-IgE sensitized RBL-2H3 mast cells stimulated with anti-DNP-HSA. IgE conjugated with CAF could not be detected by anti-IgE antibody in the ELISA analysis. Although incubation of IgE with CAF did not decrease the amount of IgE, it was possible to precipitate IgE by centrifugation. These results demonstrate that CAF captures IgE, prevents it from binding to Fc ϵ RI, and attenuates mast cell degranulation.

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Introduction

Components extracted from rice bran oil have been attracting scholarly attention. For instance, ferulic acid (4-hydroxy-3-methoxycinnamic acid), a component of a variety of plants as well as of rice bran oil, exhibits a variety of effects including anti-inflammatory activity (Fernandez et al., 1998), anti-neurotoxicity (Ono et al., 2005; Yan et al., 2001), and anti-proliferation activity (Hou et al., 2004). Most recently, we reported that γ -oryzanol strongly ameliorates inflammation in sodium dextran sulfate-induced colitis in mice (Islam et al., 2008) and alcohol-induced acute liver injury (Chotimarkorn and Ushio, 2008) via inhibition of NF κ B activity (Nagasaka et al., 2007; Islam et al., 2008). On the other hand, it has also been suggested that γ -oryzanol, a generic term for ferulate esters (ferulic acid ester) (Xu and Godber, 1999), possesses the ability to lower blood cholesterol (Seetharamaiah and Chandrasekhara, 1989). We found that serum adiponectin concentration increased in mice administered γ -oryzanol, suggesting the possibility that hydroxycinnamic acid derivatives may be effective in ameliorating type 2 diabetes (Ohara et al., 2008). However, no reports have demonstrated that γ -oryzanol possesses the ability to suppress allergic reaction.

In this study, we investigated the effect of cycloartenyl ferulate, a major component of rice bran-derived γ -oryzanol, as an anti-allergic agent in passive cutaneous anaphylaxis (PCA) reaction and

mast cell degranulation, and we further demonstrated that this component had a novel effect on IgE.

Materials and methods

Purification and analysis of γ -oryzanol

Rice bran oil was extracted from domestic Japanese rice according to the method of Bligh and Dyer (1959). The γ -oryzanol fraction was isolated by flash column chromatography on silica gel 60 (spherical 40–50 μ m) using chloroform plus methanol (1:1, v/v) as an eluent.

PCA reaction

PCA reaction was performed as the allergic model using a method described previously (Oka et al., 2005). Eight-week-old, male Sprague-Dawley rats (Japan Charles River, Yokohama, Japan) were used for the experiment. Animal care and treatment were conducted in conformity with the institutional guidelines of the University of Tokyo, and are consistent with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Anti-DNP IgE was applied to the dorsal skin of rats as follows: anti-DNP IgE (200 ng/ml; Sigma-Aldrich Tokyo, Japan) was incubated at the indicated concentrations of γ -oryzanol or cycloartenyl ferulate for 60 min, and injected into the dorsal skin of rats by intradermal injection

* Corresponding author. Tel.: +81 3 5841 5393; fax: +81 3 5841 8183.
E-mail address: ahori@mail.ecc.u-tokyo.ac.jp (M. Hori).

under anesthesia. Two hours later, 1% Evans blue (Sigma-Aldrich Tokyo, Japan) and antigen, 1 mg/ml DNP-human serum albumin (DNP-HSA; Sigma-Aldrich Tokyo, Japan), in 1 ml of normal saline was injected via the tail vein (i.v.). Thirty minutes later, the rats were sacrificed by exsanguination while under anesthesia, and the skin was removed, turned over, and photographed. Identical circular skin areas (approximately 1 cm²) were then excised, and the extravasated Evans blue was extracted by incubating the skin samples in 99% *N,N*-dimethyl formamide (Wako, Tokyo, Japan) for 24 h at 55 °C. The supernatant was collected by centrifugation at 15,000 rpm for 15 min and OD (650 nm) was measured using a multilabel counter (Wallac ARVO SX, Perkin Elmer Japan, Tokyo, Japan). The percentage of extravasation was calculated, taking those induced by 0 and 200 ng/ml IgE as 0 and 100%, respectively.

β-Hexosaminidase degranulation

The release of β -hexosaminidase was measured as an index of mast cell degranulation using a method described previously (Oka et al., 2004). Briefly, RBL-2H3 cells (American Type Culture Collection, Manassas, VA, USA) cultured on a 24-well plate were stimulated at 37 °C under gentle rotation. Anti-DNP IgE (50 ng/ml) was incubated with the indicated concentrations of CAF for 60 min. RBL-2H3 cells were sensitized with IgE plus CAF for 15 min, and then stimulated with 10 ng/ml DNP-HSA for 15 min. The enzyme in the supernatants and 0.5% triton X-100-released enzyme were incubated with *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide (Sigma-Aldrich Tokyo, Japan) in 0.04 M sodium citrate, and the reaction was stopped using 0.2 M glycine adjusted to pH 10.0 with NaOH. The OD (405 nm) was measured using a multilabel counter. Percentage degranulation was calculated using the following formula,

$$\% \text{ Degranulation} = \text{OD}_{\text{supernatant}} / (\text{OD}_{\text{supernatant}} + \text{OD}_{\text{tritonX-100}}) \times 100$$

ELISA

The concentration of IgE was measured using a method from BD Biosciences. Briefly, a 96-well plate was coated with anti-mouse IgE capture monoclonal antibody (Japan BD Biosciences-Pharmingen, Tokyo, Japan), and blocked with PBS containing 1% BSA. Purified mouse anti-TNP IgE standard (Japan BD Biosciences-Pharmingen, Tokyo, Japan) was diluted in a series of two-fold dilutions, or incubated with γ -oryzanol or cycloartenyl ferulate as indicated in the figure legends. The samples were incubated with the IgEs, and then with biotinylated anti-mouse IgE (AbD Serotec, Raleigh, NC, USA). The plate was washed by PBS, incubated with SA-*HRP*, and then with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich Tokyo, Japan). The color reaction was stopped by the addition of 1 M sulfuric acid and OD (405 nm) was measured.

SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect the light and heavy chains of anti-DNP (Sigma-Aldrich Tokyo, Japan) or anti-TNP (Japan BD Biosciences-Pharmingen, Tokyo, Japan) IgE. Briefly, the centrifuged IgEs (20,000 × g, 4 °C, 10 min) were treated with materials as described in the figure legends. The light and heavy chains of IgEs were separated using 10% SDS-PAGE and detected with silver staining procedure (Silver staining II kit, Wako, Tokyo, Japan). The stained gels were saved as images using a digital camera, and protein intensity was quantified using an image-processing program (Scion image, Scion Corporation, Frederick, MD, USA). The optical density of

the bands was determined, and the data were normalized to control (100%).

Results and discussion

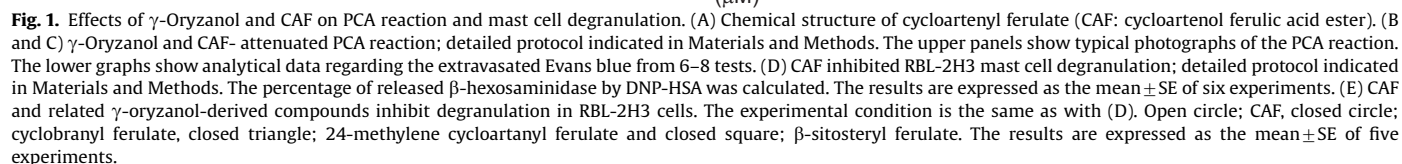
We first identified the chemical components contained in the γ -oryzanol fraction derived from rice bran oil. Results indicated that the predominant ferulates in the γ -oryzanol fraction were cycloartenyl ferulate (CAF, 28.2%), 24-methylene cycloartanyl ferulate (22.4%), campesteryl ferulate (17.8%), β -sitosteryl ferulate (12.3%), and cyclobranlyl ferulate (<10.0%). Ferulic acid could not be detected in the extracted γ -oryzanol (<1%). CAF was determined to be the major component of γ -oryzanol (Fig. 1A). We next examined the effect of γ -oryzanol on the PCA reaction, and found that the heterogeneous extract drastically inhibited the reaction. Because CAF is one of the main components of γ -oryzanol, we next tested the effect of CAF on the PCA reaction. As shown in Fig. 1B, CAF also inhibited the PCA reaction in a concentration-dependent manner. As γ -oryzanol contains >90% compounds related to CAF (M.W. 602.89), 10 μ g/ml of γ -oryzanol was estimated to be approximately 10 μ M. Therefore, CAF has a similar potency of anti-allergic reaction to γ -oryzanol. In addition, CAF and other related compounds may not have synergistic effects on one another.

To confirm that the inhibitory effect of CAF on the PCA reaction was due to the inhibition of mast cell degranulation, we further examined the effect of CAF on RBL-2H3 mast cell degranulation (Fig. 1C). Anti-DNP IgE-sensitized RBL-2H3 cells released β -hexosaminidase after DNP-HSA stimulation. The anti-DNP IgE incubated with CAF was applied to RBL-2H3 cells. The degranulation induced by subsequent DNP-HSA stimulation was inhibited by CAF in a concentration-dependent manner (Fig. 1D). The inhibitory effect was also dependent on the incubation time of CAF with anti-DNP IgE ($n = 6$, data not shown). Taken together, these results suggest that γ -oryzanol has an anti-allergic reaction effect by inhibiting mast cell degranulation.

We further investigated the effects of other compounds related to CAF on mast cell degranulation. We succeeded in purifying 24-methylene cycloartanyl ferulate, β -sitosteryl ferulate and cyclobranlyl ferulate. 24-methylene cycloartanyl ferulate and cyclobranlyl ferulate, but not β -sitosteryl ferulate, inhibited mast cell degranulation in a concentration-dependent manner. Cyclobranlyl ferulate was more potent than CAF in inhibiting degranulation, whereas 24-methylene cycloartanyl ferulate and β -sitosteryl ferulate were less potent, as shown in Fig. 1E.

As mentioned above, CAF significantly inhibited mast cell degranulation. We also found that once IgE bound to mast cells, CAF failed to inhibit the degranulation ($n = 4$, data not shown). These results suggest that CAF might have some effect on the ability of IgE to bind to Fc ϵ RI. To clarify how CAF prevents IgE binding, we measured the concentrations of IgE by ELISA. After the incubation of anti-TNP IgE with γ -oryzanol or CAF for 60 min, the IgE concentration in the incubation medium decreased in a concentration-dependent manner (Fig. 2A and B). The effect of CAF on IgE concentration was also dependent on the incubation time ($n = 4$, data not shown). These results suggest that, after incubation with CAF, the anti-IgE antibody used in ELISA was unable to detect the IgE.

There are at least two possibilities for the effect of CAF on IgE; the sequestration of IgE from the anti-IgE antibody, or IgE configuration change. To investigate these possibilities, the IgE molecules were analyzed using SDS-PAGE. The high and low molecular weight bands were detected (Fig. 2C control). The upper band, which was undetected after treatment with papain (Fig. 2F), is the heavy chain of IgE, and the lower one is its light



CAF may form large clusters with IgE molecules, suggesting the possibility that the hydrophobic interaction between CAF and IgE is required for the inhibition of the allergic reaction. Indeed, ferulic acid, which does not have the sterol chemical structure of CAF, had no effects on mast cell degranulation using the PCA reaction test and the IgE detection test by ELISA ($n = 4$ each, data not shown). In addition, from the analysis of related compounds' effects on mast cell degranulation (Fig. 1E), only β -sitosteryl ferulate has a very weak inhibitory effect on degranulation.

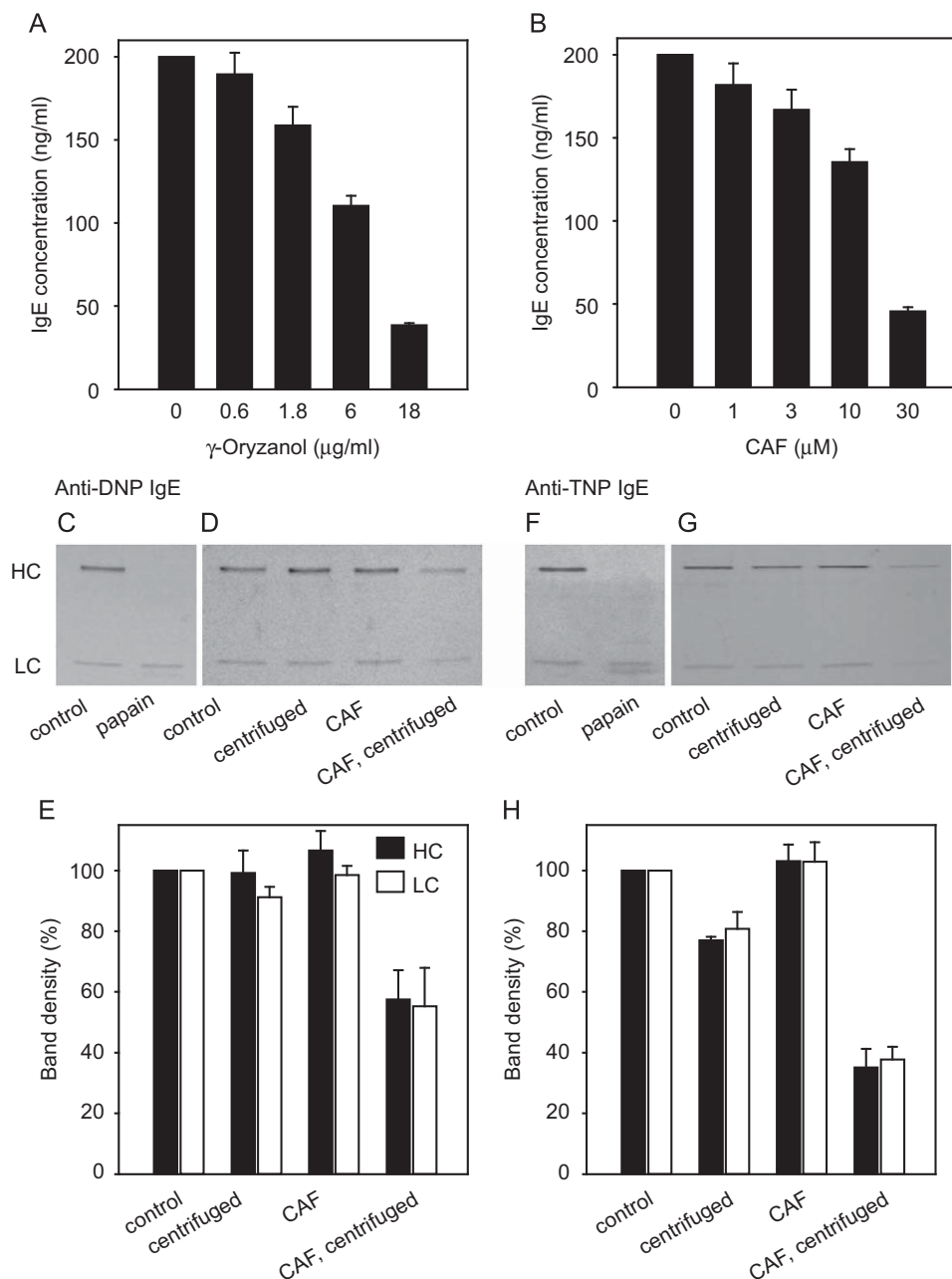


Fig. 2. γ -Oryzanol and CAF decreased IgE concentration as detected by ELISA and western blot. Anti-TNP IgE (200 ng/ml) was incubated with the indicated concentrations of γ -oryzanol (A) or CAF (B) for 60 min. IgE concentrations were detected by ELISA. The results are expressed as the mean $\pm$ SE of at least four experiments. The centrifuged anti-DNP IgE (10 μ g/ml, C-E) or anti-TNP IgE (10 μ g/ml, F-H) were incubated without (control) or with 2 μ M papain (C and F) or 30 μ M cycloartenyl ferulate (CAF, D and G) for 60 min. Subsequently, the solutions were either centrifuged again (20,000 $\times$ g, 4 $^{\circ}$ C, 10 min, B and E) or not. Each band was detected by western blot. The band densities of the heavy chain (HC) and the light chain (LC) in D and G were calculated and shown in E and H, respectively.

As there is no negative charge structure in the sterol structure of β -sitosterol ferulate as compared to other related compounds, the negative charge moiety of the sterol structure may be important in capturing IgE. Further study will be required to clarify the details of the mechanism involved in IgE capture.

The recent appearance of several reports on IgE in allergic diseases indicates that its importance is being recognized (Galli et al., 2005; Holgate et al., 2005; Poole et al., 2005). Therefore, IgE could become a key target in the therapy of allergic diseases. In fact, the recombinant humanized monoclonal antibody against IgE, Omalizumab, represents a novel therapeutic approach for allergic asthma (Hendeles and Sorkness, 2007). However, anti-IgE therapy is inconvenient and involves high costs. In this study, we demonstrated that a natural product, CAF, found in γ -oryzanol

extracted from rice bran oil, captures IgE and prevents it from binding to Fc ϵ RI, resulting in the attenuation of allergic reaction. In addition, we found that CAF inhibited NF κ B activity (Nagasaka et al., 2007), indicating that these natural products may also prevent the late phase of allergic inflammation. Taken together, we expect that the use of these low molecular weight natural products, including rice bran, has great potential for the treatment of allergic diseases. In an acute hepatitis animal model, we found that orally administered γ -oryzanol induced a high hepatoprotective effect (Chotimarkorn and Ushio, 2008). Orally administered γ -oryzanol and CAF also ameliorated colonic inflammation induced by sodium dextran sulfate (Islam et al., 2008), indicating that orally applied γ -oryzanol can enter the bloodstream. Therefore, it is possible that orally administered γ -oryzanol in the blood

might capture IgE. CAF and related molecules may be the seed compounds required to establish a new therapy using IgE as an alternative to anti-IgE therapy. However, further investigation is required to address the IgE specificity of these compounds, as well their effects in animal models of allergic diseases.

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