



Beneficial effects of chelidonic acid on a model of allergic rhinitis

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ABSTRACT

Chelidonic acid (CA) is known as an inhibitor of the rat brain glutamate decarboxylase. However, the pharmacological effects of CA in allergic reactions have not yet been defined. Here, we show the effects and the mechanism of CA in the ovalbumin (OVA)-sensitized allergic rhinitis (AR) model. CA significantly decreased the number of nasal/ear rubs and increment of IgE levels in the AR mice. The level of interferon- γ was enhanced while the level of IL-4 was reduced on the spleen tissue of the CA-administered AR mice. Expressions of IL-1 β and cyclooxygenase-2 were inhibited by CA administration in the nasal mucosa tissues. Infiltration of eosinophils and mast cells was decreased in the CA-administered AR mice. Furthermore, CA decreased the caspase-1 activity in the same nasal mucosa tissue and human mast cell line, HMC-1. Our results indicate that CA may attenuate allergic reaction by inhibition of caspase-1 activity.

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1. Introduction

Allergic rhinitis (AR) is a common manifestation of allergic diseases, affecting approximately 500 million people worldwide [1]. Although not life threatening, AR can deteriorate the quality of life and can be a major risk. Despite its clinical and socioeconomic impact, advances in its treatment still have a long way to go [2]. Many of the symptoms of patients with AR, sneezing, itching, and respiratory obstruction cause a lot of pain. However, the symptoms of AR do not end here. If prolonged, AR, can cause problems in the nasal voice box, and can cause very severe eye and ear symptoms [3]. These symptoms are due to the release of histamine and other active substances by mast cells, which stimulate the dilation of blood vessels, irritate nerve endings and increase secretion of tears [4].

Since the discovery by Coffman and colleagues of two distinct types of Th in mice [5], mutual regulation between Th1 cells and Th2 cells has been considered important for homeostatic maintenance of the immune system in the whole body. Dysregulated Th1 and Th2 responses lead to excessive Th1 cell or Th2 cell activation, resulting in the development of autoimmune diseases associated with the accumulation of Th1 cells or in an induction of allergic diseases due to the accumulation of Th2 cells, respectively [6]. In response to exposure to allergens, patients with AR present an inflammatory IgE-mediated response characterized by a Th2 immunologic pattern with mast cells and eosinophils activation and release of inflammatory mediators, IL-1 β , IL-6, and TNF- α [7,8]. Leukotrienes and prostanoids produced by the 5-lipoxygenase and cyclooxygenase (COX)-2 path-

ways have potent pro-inflammatory and vascular actions that implicate them in allergic and inflammatory reactions [9]. Eosinophils are innate effector cells that are important in immune responses against helminth parasitic infections and contribute to the pathology associated with allergic inflammatory conditions. Mast cells contribute to induction and/or maintenance of eosinophilic inflammation by a variety of mechanisms, including IgE-dependent and IgE-independent processes [10]. The recruitment of these mast cells to inflammatory sites occurs in response to chemotactic and activation signals [11].

Caspase-1 is a member of the cystein-aspartic acid protease (caspase) family [12]. Caspase-1 is characterized by its ability to activate the inactive precursors of IL-1 β and IL-18 that are involved in inflammation. Caspase-1 contains an N-terminal caspase recruitment domain (CARD). This CARD promotes the proteolytic activation of the recruited caspase-1 in inflammation [13]. Caspase-1 is activated within inflammasome, a large cytosolic protein complex that is induced by a growing number of endogenous, microbial, chemical or environmental stimuli [14].

In this study, we investigated the regulatory activity of chelidonic acid (CA) on an ovalbumin (OVA)-induced AR mice model.

2. Materials and methods

2.1. Materials

CA, OVA, phorbol 12-myristate 13-acetate (PMA), A23187, O-phthalaldehyde (OPA), avidin peroxidase, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) tablets substrate (ABTS), bichononic acid (BCA), 3-(3, 4-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) and other reagents were purchased from Sigma (St.

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Louis, MO, USA). Fetal bovine serum (FBS), iscove's modified Dulbecco's medium (IMDM), and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Anti-mouse IgE/IL-1 β /IL-4/IFN- γ Ab, biotinylated anti-mouse IgE/IL-1 β /IL-4/IFN- γ Ab, and recombinant mouse (rm) IgE/IL-1 β /IL-4/IFN- γ were purchased from Pharmingen (San Diego, CA, USA). Ab for COX-2, Caspase-1, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The caspase-1 assay kit was supplied by R&D Systems Inc. (Minneapolis, MN, USA).

2.2. OVA-induced AR animal model

We maintained 6-week-old female BALB/c (Charles River Technology) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University. We sensitized mice on days 1, 5, and 14 by intraperitoneal (i.p.) injection of 100 μ g OVA

emulsified in 20 mg aluminum hydroxide (Sigma) and we challenged mice with 1.5 mg OVA. CA was administrated orally before intranasal (i.n.) OVA challenge for 10 days. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation at 10 days after challenge.

2.3. Histamine assay

The histamine content of serum was measured by the OPA spectrofluorometric procedure. The fluorescent intensity was measured at 460 nm (excitation at 355 nm) using a spectrofluorometer.

2.4. Enzyme-linked immunosorbent assay (ELISA)

A modified ELISA, as previously described, was used to measure the cytokine on tissue protein. The ELISA was performed by coating 96-well plates with 1 μ g/well of capture Ab. Before the subsequent

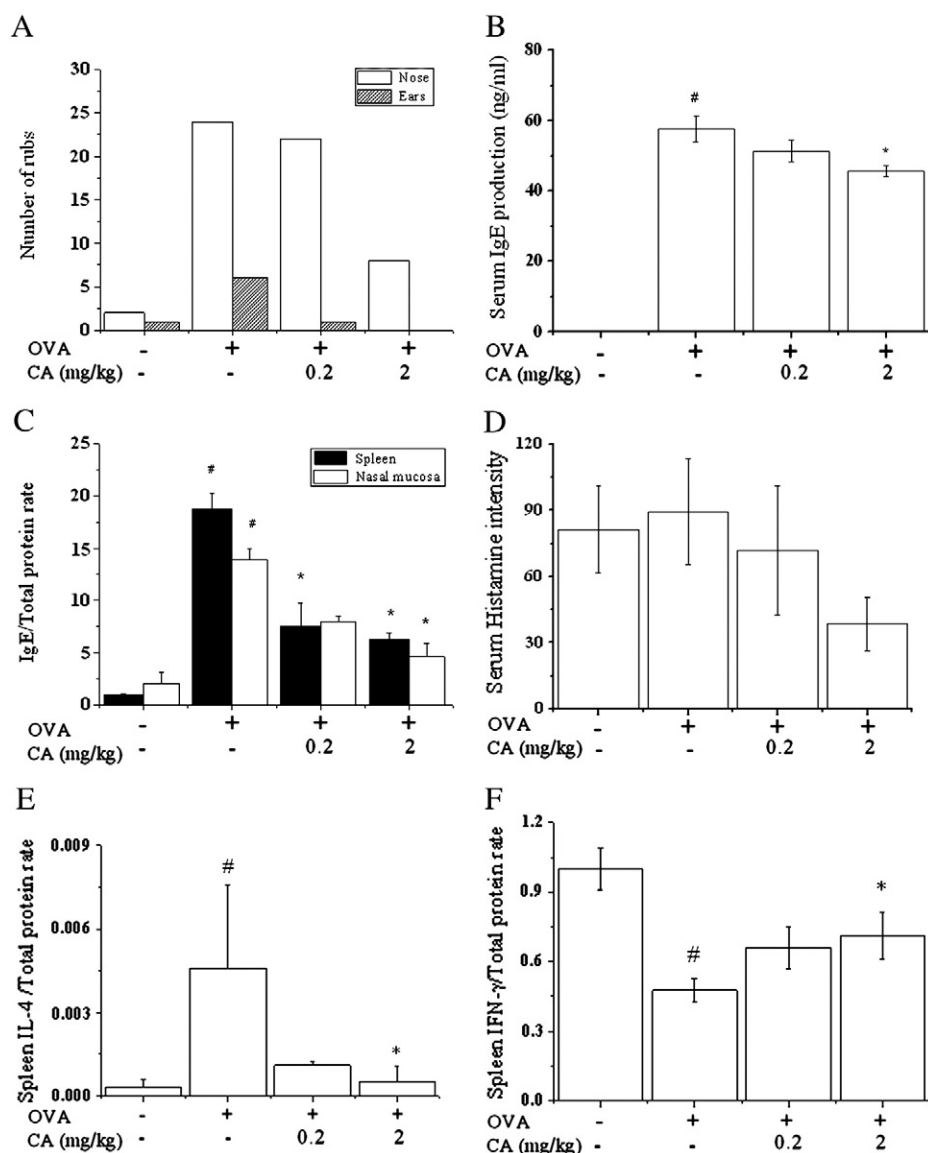


Fig. 1. Effect of CA on clinical symptoms, IgE level, histamine release, and IL-4 and IFN- γ level in the AR model. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received CA before the intranasal OVA challenge for 10 days. (A) The number of the nasal and ear rubs that occurred in the 10 min after the OVA intranasal provocation. (B and C) IgE, (E) IL-4, and (F) IFN- γ were measured by ELISA method. All parameters measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. (D) Serum was isolated from blood and then assayed about histamine: * $P < 0.01$, significantly different from the OVA-unsensitized mice. # $P < 0.01$, significantly different from the OVA-sensitized mice. $N = 5$.

steps in the assay, the coated plates were washed twice with PBS containing 0.05% tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, and avidin peroxidase, and ABTS substrate solution containing 30% H₂O₂. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Production of IgE/IL-1 β in spleen and nasal mucosa was divided according to the total protein. Protein was determined using a BCA.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells and nasal mucosa according to the manufacturer's specification using easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 μ g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with the following primers for IL-1 β (5' AGG CCA CAG GTA TTT TGT CG 3'; 5' GCC CAT CCT CTG TGA CTC AT 3'), GAPDH (5'TTC ACC ACC ATG GAG AAG GC 3'; 5vGGC ATG GAC TGT GGT CAT GA 3') was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 50 °C for IL-1 β and 60 °C for GAPDH respectively. Amplified fragment sizes for IL-1 β and

GAPDH were 545 bp and 446 bp, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.6. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following the centrifugation at 15,000g for 5 min, 50 μ g aliquots were resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycine, 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 $\times$ PBS containing 0.05% tween 20 containing 5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.7. Histological examination

Tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. Section of the nasal mucosa sample (4 μ m thick). Each section was stained with hematoxylin and eosin (H&E, for eosinophils), alcian blue and safranin O (A&S, for mast cells) or immunohistochemical stain (for IL-1 β) before dewaxing and dehydration. The number of eosinophils, mast cells, and IL-1 β on both sides of the septal mucosa was counted. Sections were coded and randomly analyzed by two blinded observers.

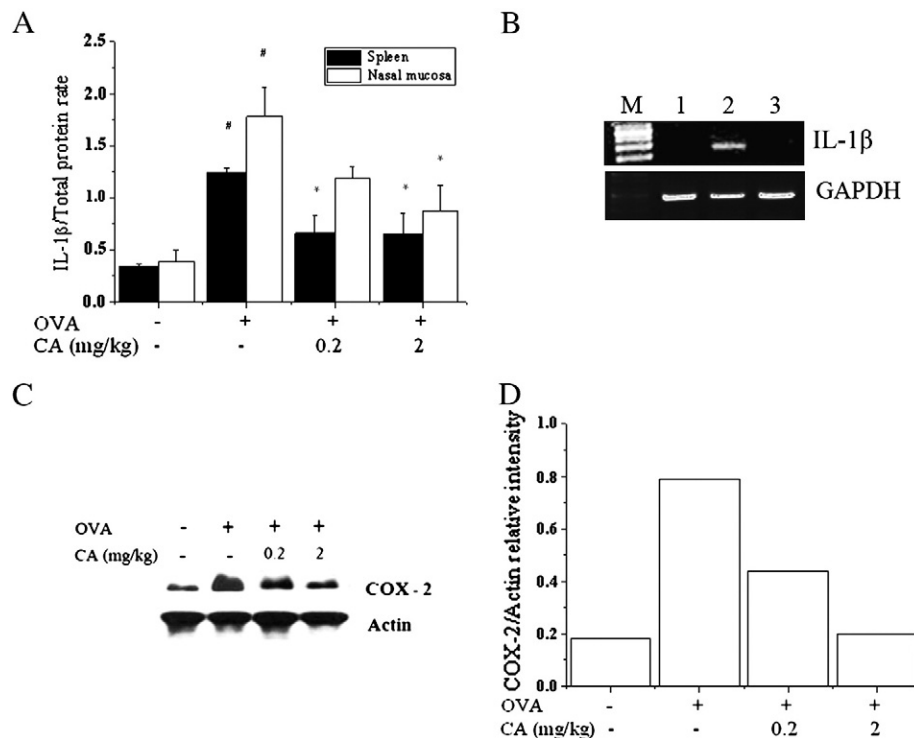


Fig. 2. Effect of CA on IL-1 β and COX-2 expression in the AR model. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received CA before the intranasal OVA challenge for 10 days. (A) IL-1 β was measured by ELISA method in the spleen and nasal mucosa tissue. All parameters measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. (B) Messenger RNA was measured using RT-PCR method. (C) COX-2 protein expression was evaluated by using Western blot analysis. (D) The protein levels were quantitated by densitometry. M, marker; 1, OVA-unsensitized; 2, OVA-sensitized; 3, CA + OVA-sensitized. * P <0.01, significantly different from the OVA-unsensitized mice. # P <0.01, significantly different from the OVA-sensitized mice. N =5.

2.8. Caspase-1 assay

Caspase-1 activity was measured according to the manufacturer's specification using caspase assay kit (R & D system). Equal amount of total protein was quantified by BCA protein quantifi-

cation kit (Sigma) in each lysate. Catalytic activity of caspase-1 from cell lysate was measured by proteolytic cleavage of WEHD-pNA for 4 h at 37 °C. The plates were read at 405 nm. A recombinant caspase-1 enzyme is available for use as a positive control.

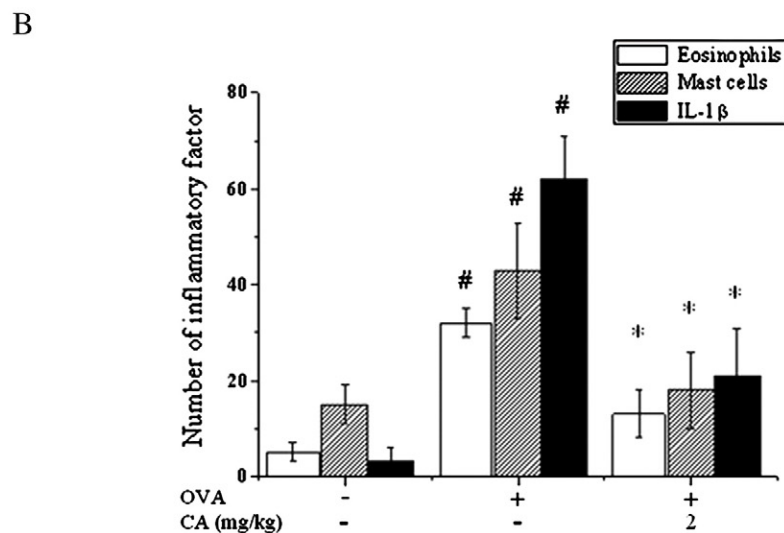
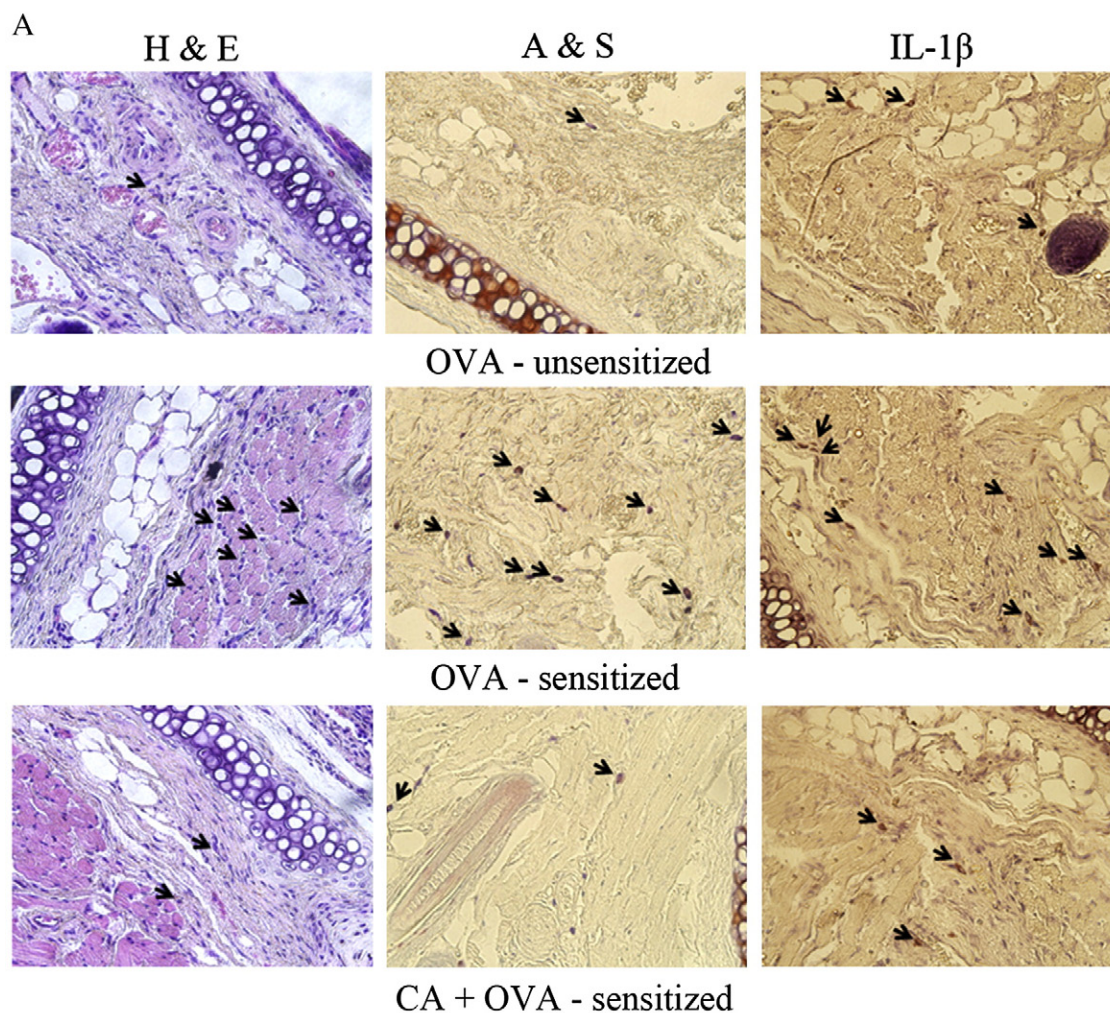


Fig. 3. Effect of CA on eosinophils and mast cells infiltration, and IL-1 β expression in the AR nasal mucosa tissue. (A) Nasal mucosa stained with H&E (for eosinophils), alcian blue and safranin O (for mast cells) and immunohistochemical DAB stain (for IL-1 β). Eosinophils, mast cells, and IL-1 β were indicated by arrows. (B) Eosinophil, mast cell, and IL-1 β were counted by two individuals. After five randomly selected tissue sections per mouse were counted. The absolute number of cells was counted as the mean $\pm$ standard error of the mean (S.E.M.). [#] $P < 0.01$, significantly different from the OVA-unsensitized mice. ^{*} $P < 0.01$, significantly different from the OVA-sensitized mice (original magnification $\times 400$).

2.9. Culture of HMC-1 cells

Human mast cell line (HMC-1) was grown in IMDM supplemented with 100 unit/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated FBS at 37 °C 5% CO₂ and 95% humidity.

2.10. MTT assay

Cell proliferation, an indicator of cell viability, was analysed by the mitochondrial dependent reduction of MTT to formazan, as described. The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density (OD) at 540 nm using an automated microplate reader.

2.11. Statistical analysis

The experiments shown are a summary of the data from at least three experiments and statistical analyses were performed using SPSS statistical software (SPSS 11.5, USA). Treatment effects were analyzed by one-way ANOVA, offered by Tukey's multiple range tests, and $P < 0.05$ was used to indicate significance.

3. Results

3.1. Effect of CA on clinical symptoms, IgE production, and histamine release in the AR model

To investigate the inhibitory effect of CA in the AR model, we sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 µg OVA emulsified in 20 mg aluminum hydroxide and challenged mice with 1.5 mg OVA. The number of nasal and ear rubs after the OVA

challenge in the OVA-sensitized mice was significantly higher than those in the OVA-unsensitized mice. Increased rub score was inhibited by treatment of CA (Fig. 1A). Levels of OVA-IgE in the AR mice were significantly higher than those in the serum, spleen, and nasal mucosa tissues of the OVA-unsensitized mice (Fig. 1B and C). Increased IgE levels in each tissue were reduced by CA. Histamine level in serum was reduced by CA (Fig. 1D). To identify the Th1/Th2 immune reaction in CA-administered mice, we measured IL-4 and IFN-γ production. As shown in Fig. 1E, the level of IL-4 in the AR mice was significantly increased compared to that in the normal mice. IL-4 level was significantly decreased in the CA-administered AR mice. However, IFN-γ level was significantly increased in the CA-administered AR mice (Fig. 1F).

3.2. Effect of CA on IL-1β and COX-2 levels in the AR model

To evaluate the regulatory effect of CA on IL-1β production, we measured the protein and mRNA levels of IL-1β in the AR model. The protein levels of IL-1β in the spleen and nasal mucosa tissue were increased in the AR mice compared to those levels in the control mice (Fig. 2A). However, protein levels of IL-1β were significantly reduced by CA administration ($P < 0.05$). CA inhibited the OVA-induced IL-1β mRNA expression (Fig. 2B). Expression of COX-2 was inhibited by CA in the nasal mucosa tissue (Fig. 2C and D).

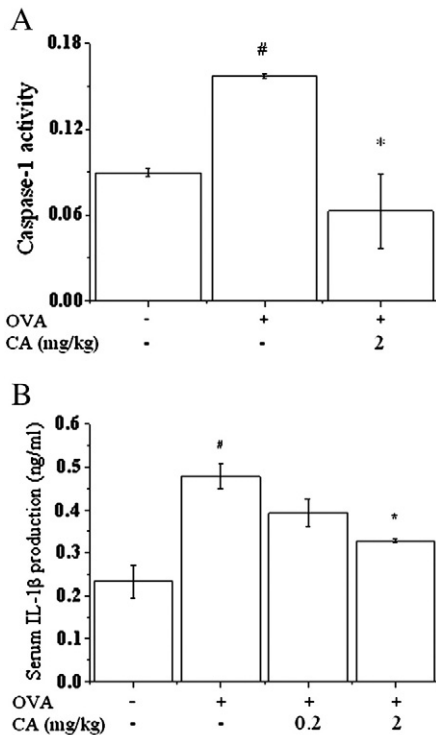


Fig. 4. Effect of CA on caspase-1 activation and IL-1β level in the AR mice. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 µg OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received CA before the intranasal OVA challenge for 10 days. (A) Protein was assayed about caspase-1 in nasal mucosa tissue. (B) IL-1β was measured by ELISA method in serum. [#] $P < 0.01$, significantly different from the OVA-unsensitized mice. ^{*} $P < 0.01$, significantly different from the OVA-sensitized mice. $N = 5$.

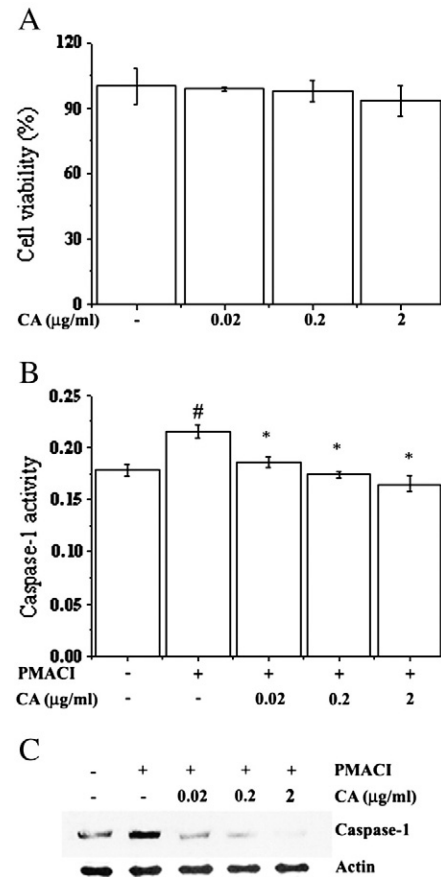


Fig. 5. Effect of CA on cell viability and PMACI-induced caspase-1 activation in HMC-1 cells. (A) HMC-1 cells were treated with CA (0.02, 0.2, and 2 µg/ml) for 24 h. Cell viability was evaluated by MTT colorimetric assay as described in the method. HMC-1 cells were pretreated with CA for 1 h prior to PMACI stimulation for 2 h. (B) The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. (C) The level of caspase-1 was assayed by Western blot analysis. Data represent mean $\pm$ S.E.M. of three independent experiments. [#] $P < 0.05$, significantly different from the unstimulated cells. ^{*} $P < 0.05$, significantly different from the PMACI-stimulated cells. PMACI, PMA plus A23187; Cas-1 inhi, caspase-1 inhibitor; Rec Cas-1, recombinant caspase-1.

3.3. Effect of CA on eosinophils and mast cells infiltration and IL-1 β expression in the nasal mucosa tissues

The respective numbers of inflammatory cells (eosinophils and mast cells) in the nasal mucosa in the AR mice were significantly higher than those in the control mice. In the CA-administered mice, eosinophils and mast cells infiltration increased by OVA sensitization was decreased (Fig. 3A and B). Immunohistochemical analysis of the nasal mucosa sections in the AR mice revealed that IL-1 β is highly expressed, whereas in the CA-administered mice it is decreased (Fig. 3A and B).

3.4. Effect of CA on caspase-1 activity in nasal mucosa tissues

Caspase-1 plays a key role in inflammatory responses by cleaving pro-IL-1 β into secreted pro-inflammatory cytokines. To investigate the effect of CA on caspase-1 activation, caspase-1 assay were performed with the nasal mucosa tissues. As shown in Fig. 4A, CA inhibited OVA-induced caspase-1 activation. CA also reduced the IL-1 β levels in the serum (Fig. 4B).

3.5. Effect of CA on cell viability in HMC-1 cells

To determine the effects of CA on viability of HMC-1 cells, we carried out MTT assay. When we treated the cells were treated with

CA at the concentrations of 0.02, 0.2, and 2 μ g/ml, CA had no effect on cell cytotoxicity (Fig. 5A).

3.6. Effect of CA on caspase-1 activation in HMC-1 cells

To evaluate whether CA regulated the caspase-1 activation in mast cells, we performed a caspase-1 assay using HMC-1 cells. As shown in Fig. 5B, caspase-1 activity increased by PMA plus A23187 (PMACI) was significantly inhibited by CA or caspase-1 inhibitor ($P < 0.05$). To confirm the effect of CA on caspase-1 activation induced by PMACI, Western blot analysis for caspase-1 was performed. The cells were pretreated with CA for 1 h and then treated with PMACI for 2 h. Caspase-1 activation was decreased by the treatment of CA (Fig. 5C).

3.7. Therapeutic effect of CA on AR-induced mice

To investigate the therapeutic effect of CA in the AR model, we sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μ g OVA emulsified in 20 mg aluminum hydroxide and challenged mice with 1.5 mg OVA for 10 days. And then, CA was administered orally for 5 days. The number of nasal rubs and level of IgE in the AR-induced mice was significantly higher than those in the OVA-unsensitized mice. Increased rub score and IgE level were diminished by treatment of CA (Fig. 6A and B). To investigate the effect of CA on caspase-1 activation, caspase-1 assay were performed with the nasal

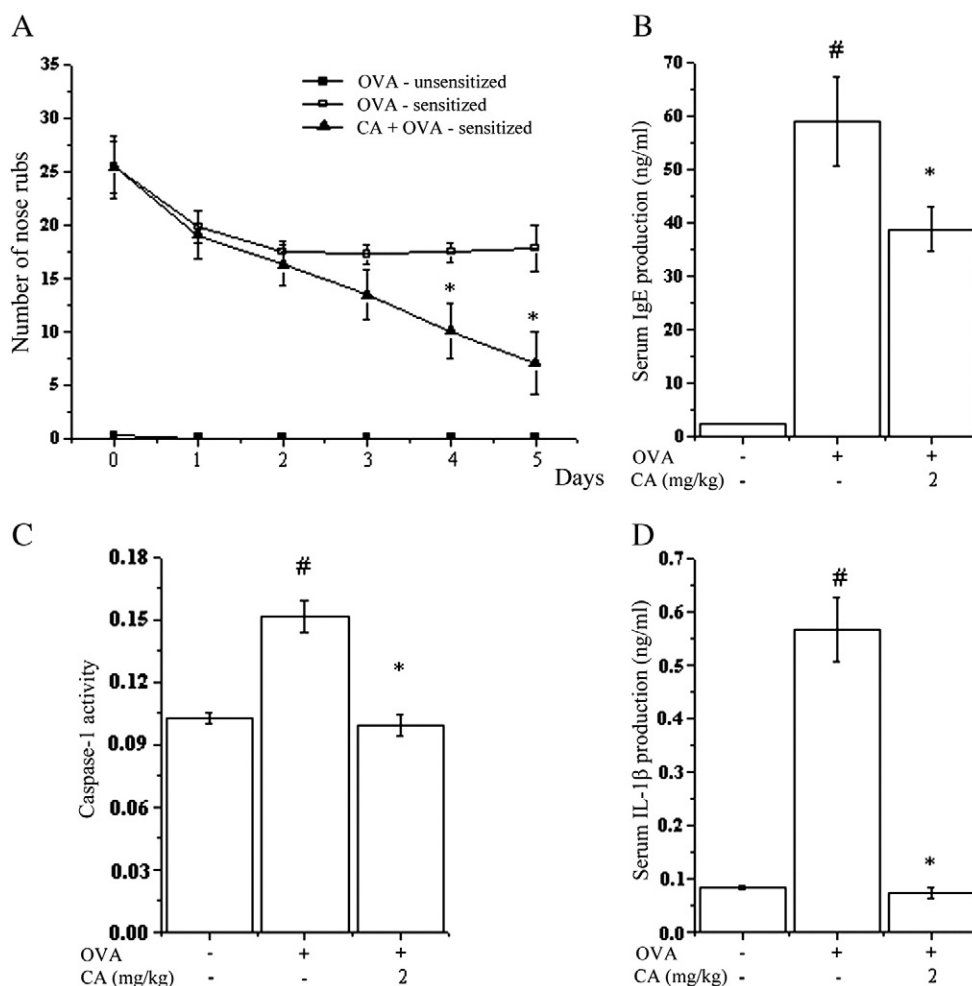


Fig. 6. Therapeutic effect of CA on AR-induced mice. We sensitized mice on days 1, 5, and 14 by intraperitoneal injections of 100 μ g OVA emulsified in 20 mg aluminum hydroxide and challenged mice with 1.5 mg OVA for 10 days. And then, CA was administered orally for 5 days. (A) The number of the nasal rubs that occurred in the 10 min after the treated CA. (B) IgE was measured by ELISA method. (C) Protein was assayed about caspase-1 in nasal mucosa tissue. (D) IL-1 β was measured by ELISA method in serum. # $P < 0.01$, significantly different from the OVA-unsensitized mice. * $P < 0.01$, significantly different from the AR-induced mice. $N = 5$.

mucosa tissues. As shown in Fig. 6C, CA decreased caspase-1 activation. CA also reduced the IL-1 β levels in the serum (Fig. 6D).

4. Discussion

In this study, CA reduced the allergic inflammatory reaction in the mice AR model.

AR is characterized by a two-phase allergic reaction. In the early-phase inflammatory response allergen-IgE dependent activation of mast cells and basophils results in the production of pharmacologically active mediators such as histamine, prostaglandins, leukotrienes, and cytokines which produce sneezing, rhinorrhea, and itching [15]. The late-phase of AR show accumulations of mast cells, eosinophils, and basophils in the epithelium and an accumulation of eosinophils in the deeper lamina propria [16]. Recruitment of inflammatory cells, including eosinophils, mast cells, basophils, and T cells, results in further release of histamine and leukotrienes, as well as in the release of other compounds including proinflammatory cytokines, COX-2, and chemokines, sustaining the allergic response and promoting the late phase response [16,17]. In previous studies, polyphenolic phytochemicals including rosmarinic acid have been shown to inhibit IgE response [18,19] and inflammation characterized by polymorphonuclear leukocytes (eosinophils, and neutrophils) infiltration [20,21]. Glucocorticosteroid (GC) is the most effective drug for AR. GC inhibits the function of infiltrating inflammatory cells and their recruitment into the nasal mucosa. GC inhibits the maturation, cytokine production, COX-2 expression, Fc ϵ R1 expression, and mediator release of mast cells [22,23]. We observed that CA inhibited the IgE production, inflammatory cytokine production, and COX-2 expression in the mice AR model. Our results suggest that there is a close analogy between CA and GC.

Inflammasomes are multiprotein cytoplasmic complexes that mediate the activation of inflammatory caspase-1 [24]. Inflammasome regulates the activation and secretion of caspase 1-regulated IL-1 β and IL-18. Caspase-1 $^{-/-}$ mice have decrease production of IL-6 after stimulation with lipopolysaccharide [25]. Grzegorzczuk et al. [26] have reported a significant increase in caspase-1 level in serum from allergic asthmatic patients as compared to a control group. Caspase-1 activity was increased in patients with Netherton syndrome [27]. In this study, we observed that CA regulated the caspase-1 activation in the AR mice and mast cells. We also performed the caspase-1 kinetic assay to determine binding affinity of CA and caspase-1 catalytic domain. Caspase-1 activity was increased after a treatment with WEHD-pNA (caspase-1 substrate), but CA did not affect on the binding of WEHD-pNA with the caspase-1 catalytic domain (data not shown). From this, we suggest that CA regulates the caspase-1 activation without influencing on interaction between substrate and caspase-1. We also postulate that the inhibitory effect of CA on inflammatory cytokine production might be derived from the regulation of caspase-1 activation. Further studies will be needed to clarify precisely the mechanism of CA on the relationship between caspase-1 activation and inflammatory cytokines production in the AR mice.

CA is a component of the herb celandine (*Chelidonium majus*). Porter et al. [28] have reported that CA is a potent inhibitors of rat brain glutamate decarboxylase (GAD) and that inhibitors of GAD would be useful as tools to study the regulation and role of γ -aminobutyric acid in the nervous system. However, the effect of CA and its mechanisms have not yet been examined. We observed for the first time that CA can regulate inflammatory cytokine expression and caspase-1 activation in the mice AR model. Taken together, our results suggest that the effect of the mechanism of CA may come about through the blockade of inflammatory cells infiltration in the AR nasal mucosa.

Acknowledgments

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