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Anti-allergic activity of emodin on IgE-mediated activation in RBL-2H3 cells

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Abstract:
Background: Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a Chinese herbal anthraquinone derivative from the rhizome of rhubarb (*Rheum palmatum* L.) that exhibits numerous biological activities, such as antitumor, antibacterial, antiinflammatory, and immunosuppressive. In the present studies, the anti-allergic activities of emodin were investigated to elucidate the underlying active mechanisms.
Methods: The inhibitory effects of emodin on the IgE-mediated allergic response in rat basophilic leukemia (RBL-2H3) cells were evaluated by measuring the release of granules and cytokines. The Ca^{2+} mobilization in RBL-2H3 cells loaded with the Ca^{2+}-reactive fluorescent probe Fluo-4 AM was also measured by laser scanning confocal microscope.
Results: Emodin inhibited the release of β-hexosaminidase (β-HEX; IC_{50} = 5.5 μM) and tumor necrosis factor (TNF)-α (IC_{50} = 11.5 μM) from RBL-2H3 cells induced by 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) and displayed stronger inhibition of β-HEX release than ketotifen fumarate salt (IC_{50} = 63.8 μM). Emodin at a concentration of 12.5 μM also inhibited the DNP-BSA-induced influx of extracellular Ca^{2+} in RBL-2H3 cells.
Conclusions: These results suggested that emodin likely exhibits anti-allergic activities *via* increasing the stability of the cell membrane and inhibiting extracellular Ca^{2+} influx.

Key words:
emodin, β-HEX, TNF-α, RBL-2H3 cells

Abbreviations: β-HEX – β-hexosaminidase, TNF-α – tumor necrosis factor-α, IgE – immunoglobulin E

Introduction

Allergy, a serious health problem worldwide, is due to immune dysfunction. Substances that cause allergic reactions are called allergens, including dust mites, pollen, cosmetics, food, and mold spores. Immediate hypersensitivity (type I allergy), is an immunoglobulin E (IgE)-mediated immune response, resulting in conditions such as food allergies, hay fever, asthma, and drug-induced allergies. The number of patients with these conditions is increasing worldwide [8]. Mast cells and basophils are well-known as critical participants in various biologic allergic disease processes [2, 18]. These cells express receptors on their surface membranes that have high affinity and specificity for IgE. Interactions between multivalent antigens and surface-bound IgE release histamine, prostaglandins, leukotrienes, and cytokines [16, 17]. These cytokines activate chemotaxis and phagocytosis of neutrophils and macrophages. Finally, the cytokine-

induced reaction causes tissue inflammation. Anti-allergic agents, such as antihistamines, steroids, and immunosuppressants, have been used to treat allergic diseases including allergic rhinitis, atopic dermatitis, asthma, and food allergies [19–21]. However, many of these medications have undesirable side effects and adverse reactions. Instead, bioactive constituents from herbal medicines have been used for treatment of allergic diseases, and their effectiveness has received increasing attention.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone) is a major active component of a herbal medicine derived from rhubarb, the rhizome of *Rheum palmatum* L., aloe, senna leaves, and *Polygonum multiflorum* roots. Rhubarb has shown mild laxative properties in traditional Chinese medicine [22] in pharmacological studies. Additionally, emodin was found to have antitumor, antibacterial, diuretic, and vasorelaxant effects [9, 24]. Moreover, emodin has shown antiinflammatory and immunosuppressive effects [7, 10]. The underlying mechanisms, however, are still not fully understood.

RBL-2H3 cells were previously used to study comprehensive events on mast cells induced by multivalent allergens [12]. In this study, we selected the 2,4-dinitrophenyl (DNP)-specific immunoglobulin E (IgE) to sensitize RBL-2H3 cells and the antigen DNP-bovine serum albumin (DNP-BSA) to stimulate cells, which is a classic way to study the effects of unknown compounds on antigen-induced activation of mast cells. We examined the effects of emodin on the degranulation and release of tumor necrosis factor-α (TNF-α). In addition, the effect of emodin on extracellular Ca^{2+} influx inhibition was examined to elucidate the underlying mechanism.

Materials and Methods

Materials

Emodin was purchased from Shaanxi ZhongXin Biotechnology Co. Ltd. (Xi'an, China; purity > 98%). Monoclonal anti-DNP IgE (#D8406), 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide (#N9376, purity > 98%), thiazolyl blue tetrazolium bromide (MTT, #M2128), and ketotifen fumarate salt (#K2628) were obtained from Sigma. DNP-BSA (#A23018), Minimum Essential Medium (MEM, #41500-034), Fluo-4 AM (#F14201), 0.25% trypsin-EDTA (#25200), and the rat TNF-α ELISA kit (#KRC3011) were obtained from Invitrogen. Fetal bovine serum (FBS) superior (#S0615) was purchased from Biochrom AG (Germany).

Cell cultures

RBL-2H3 cells, obtained from the American Type Culture Collection (ATCC, #CRL-2256), were cultured in MEM with 15% heat-inactivated FBS at 37°C in a humidified atmosphere of 5% CO_2 and subcultured after trypsinization (0.25% trypsin-EDTA).

MTT assay

Cells grown in 96-well plates (1.8×10^5 cells/well) were incubated for 44 h with and without emodin solution at final concentrations of 3.12, 6.25, 12.5, 25, 50, and 100 μM. Control samples were cultured with 0.1% dimethyl sulfoxide (DMSO) culture medium. Twenty microliters of MTT (5 mg/ml) solution was added to each well, and the plates were incubated for another 4 h at 37°C. The medium was removed, and 150 μl DMSO was added to each well to solubilize formazan crystals formed in viable cells before measuring absorbance at 492 nm (TECAN Genios). The density of formazan formed in control (medium alone) cells was taken as 100% viability.

β-Hexosaminidase (β-HEX) secretion assay

The amount of β-HEX, a marker of degranulation of RBL-2H3 cells, released into the medium was determined as described previously [13, 14]. Briefly, RBL-2H3 cells grown in 24-well plates (2.5×10^5 cells/well) were sensitized with 0.5 μg/ml of DNP-specific IgE overnight. After two washes with Siraganian buffer (119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-*N*,*N'*-bis[2-ethanesulfonic acid], and 40 mM NaOH, pH 7.2) supplemented with 5.6 mM D-glucose, 1 mM $CaCl_2$, and 0.1% bovine serum albumin (BSA; incubation buffer), cells were incubated in 160 μl buffer for 10 min at 37°C. Cells were then treated with 20 μl of test sample solution for 10 min and stimulated with 20 μl DNP-BSA (10 μg/ml) as an antigen for 10 min. The supernatant (50 μl) was then transferred to a 96-well plate and incubated with 50 μl substrate (1 mM 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer

(pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 200 µl stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured at 405 nm by a microplate reader. The emodin test sample was dissolved in DMSO, and the solution was added to incubation buffer (0.1% final DMSO concentration).

The inhibition (%) of β-HEX release by emodin was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{inhibition (\%)} = [1 - (T - B - N)/(C - N)] \times 100\%.$$

Control (C) was DNP-BSA (+), test sample (–); test (T) was DNP-BSA (+), test sample (+); blank (B) was DNP-BSA (–), test sample (+); and normal (N) was DNP-BSA (–), test sample (–). Ketotifen fumarate salt was used as a reference compound [15].

β-HEX activity assay

To clarify that the anti-allergic effects of these samples were attributable to the inhibition of β-HEX release but not false positives from the inhibition of β-HEX activity, RBL-2H3 cells were lysed by 0.1% Triton X-100, and the lysate was centrifuged. The supernatant was then diluted with Siraganian buffer and adjusted to the equivalent enzyme activity of the degranulation tested above. The enzyme solution (45 µl) and test sample solution (5 µl) were transferred to a 96-well plate, and enzyme activity was examined as described above.

Measurement of TNF-α release

RBL-2H3 cells (2.8×10^6 cells/well) were sensitized overnight with anti-DNP IgE as described above. The cells were washed twice with 500 ml of MEM containing 10% FCS, penicillin and streptomycin, then exchanged with 320 ml of the fresh medium. Forty microliters of the test sample solution and 40 µl DNP-BSA (10 µg/ml final concentration) were added to each well and incubated at 37°C for 4 h. The supernatant (50 µl) was transferred to a 96-well ELISA plate. The amount of TNF-α was determined using ELISA kits according to the manufacturer's instruction. The emodin test sample was dissolved in DMSO, and the solution was added to MEM (0.1% final DMSO concentration). The inhibition (%) of TNF-α release by emodin was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{inhibition (\%)} = [1 - (T - N)/(C - N)] \times 100\%.$$

Control (C) was DNP-BSA (+), test sample (–); test (T) was DNP-BSA (+), test sample (+); blank (B) was DNP-BSA (–), test sample (+); and normal (N) was DNP-BSA (–), test sample (–). Luteolin was used as a reference compound [14].

Measurement of Ca^{2+} mobilization

Ca^{2+} mobilization in RBL-2H3 cells loaded with the Ca^{2+}-reactive fluorescent probe Fluo-4 AM was measured using a laser scanning confocal microscope (Nikon ECL IPSE TE2000-E). RBL-2H3 cells in a Confocal Dish (Coverglass Bottom Dish, Nikon) were sensitized with 0.5 µg/ml anti-DNP IgE overnight. After two washes with HBSS buffer (5.33 mM KCl, 0.44 mM KH_2PO_4, 137.93 mM NaCl, 0.41 mM $MgSO_4 \cdot 7H_2O$, 5.56 mM D-glucose, 4.17 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.49 mM $MgCl_2 \cdot 6H_2O$, and 0.34 mM Na_2HPO_4, pH 7.2), cells were incubated with 4.5 µM Fluo-4 AM solution in HBSS buffer for 30 min at 37°C. Cells were then washed twice with HBSS buffer to remove free Fluo-4 AM in solution and incubated in HBSS buffer for 40 min. Ten microliters of the test sample solution were added for incubation for 10 min. The fluorescence of the solution was monitored before and after DNP-BSA stimulation (10 µg/ml final concentration). Fluo-4-AM-loaded RBL-2H3 cells were excited at 488 nm, and the fluorescence emission was observed at 515 nm. Fluorescence images were collected at 6 s intervals, and the changes in Ca^{2+} levels were analyzed with NIS-Elements AR.

Statistical analysis

Values are expressed as the mean ± SEM. The IC_{50} values were calculated using Prism 5.0 software. Statistical significance was calculated by one-way analysis of variance (ANOVA) using SPSS 15.0 software. Values of $p < 0.01$ were considered statistically significant.

Results

Cytotoxicity effects of emodin on RBL-2H3 cells

The cytotoxic effects of emodin in RBL-2H3 cells were measured by the MTT assay. As shown in Figure 1,

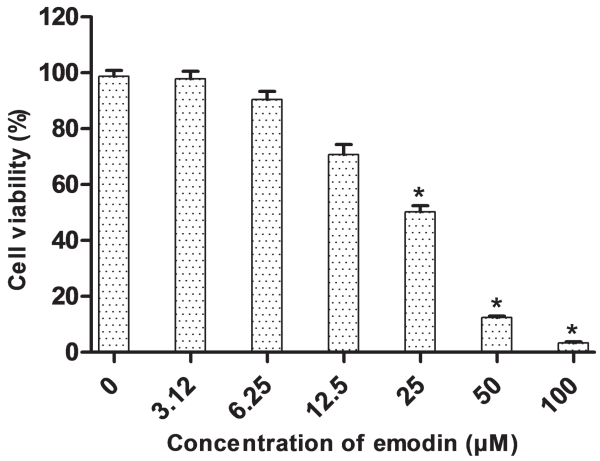


Fig. 1. Effects of emodin on the proliferation of RBL-2H3 cells. Values represent the mean ± SEM (n = 8); * $p < 0.01$, significantly different from control

emodin at concentrations of 3.12, 6.25, and 12.5 μM did not significantly affect cell proliferation. The IC_{50} value was calculated as 22 μM. Thus, the concentrations of emodin treated with RBL-2H3 cells ranged from 3.12 to 25 μM during the subsequent experiments.

Inhibitory effects on β-HEX release from RBL-2H3 cells

β-HEX has been used as a marker of the degranulation of RBL-2H3 cells. β-HEX release into the medium was determined. The inhibitory effects of emodin on antigen-induced degranulation in sensitized RBL-2H3 cells were examined. As shown in Figure 2A, emodin inhibited the release of β-HEX from the cells stimulated by DNP-BSA, with an IC_{50} value of 5.5 μM and a significant difference between the test group and control group ($p < 0.01$). Compared with the reference compound, ketotifen (IC_{50} = 63.8 μM; Fig. 2B), our results revealed that the activity of emodin (25 μM) was similar to ketotifen (1,000 μM). Thus, the potent inhibitory effect of emodin against β-HEX release indicated that emodin may be a promising new antihistamine agent.

Inhibitory effects of β-HEX on enzyme activity

The effects of emodin on β-HEX were examined to clarify whether its effects were attributable to the inhibition of enzyme activity or degranulation. Figure 3

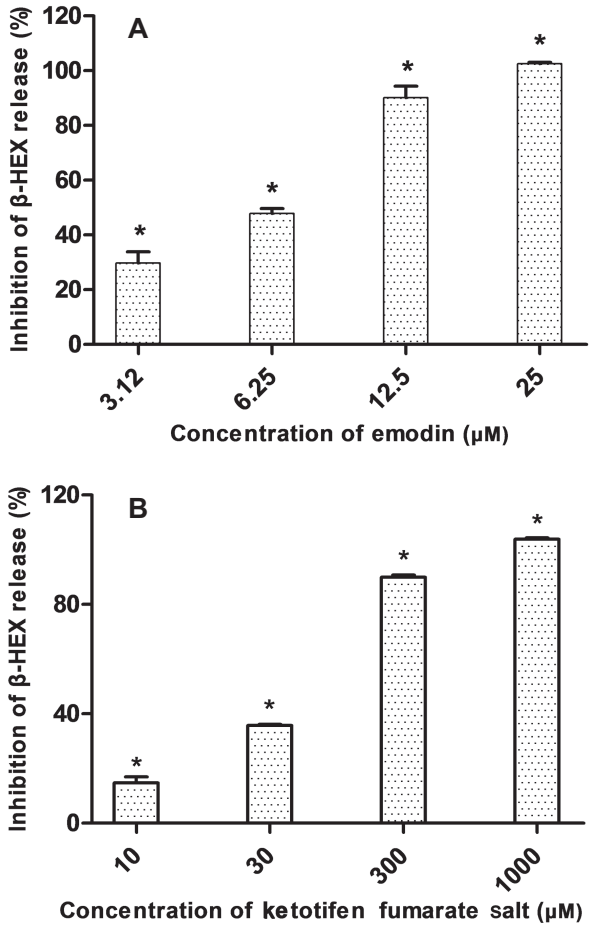
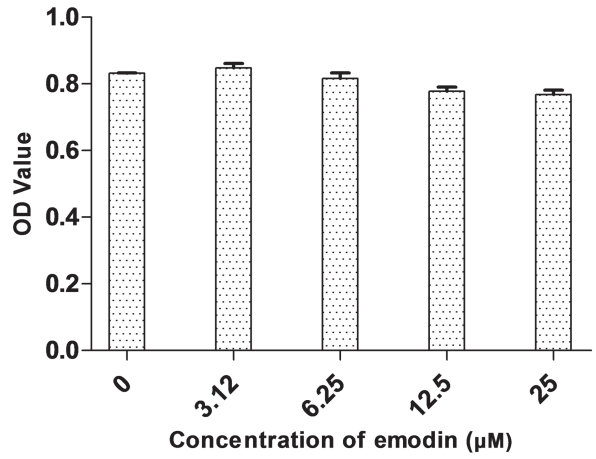


Fig. 2. Effects of samples on β-HEX release from RBL-2H3 cells. (**A**) treated with emodin. (**B**) treated with ketotifen fumarate salt. Each value represents the mean ± SEM (n = 4); * $p < 0.01$, significantly different from control

shows that emodin had less inhibition against the enzyme activity of β-HEX at concentrations of 3.12, 6.25, 12.5, and 25 μM. The results demonstrated that the anti-allergic effects of emodin were attributable to the inhibition of β-HEX release and not a false positive from inhibition.

Inhibitory effects of emodin on TNF-α secretion

The inhibitory effects of emodin on TNF-α release were measured in sensitized RBL-2H3 cells stimulated by DNP-BSA using ELISA. The results demonstrated that emodin exhibited marked activity against TNF-α release, with an IC_{50} of 11.5 μM. Emodin at concentrations of 12.5 and 25 μM significantly inhibited TNF-α secretion with inhibition rates of 61.6 ± 5.1% and 79.3 ± 6.6%, respectively, which were



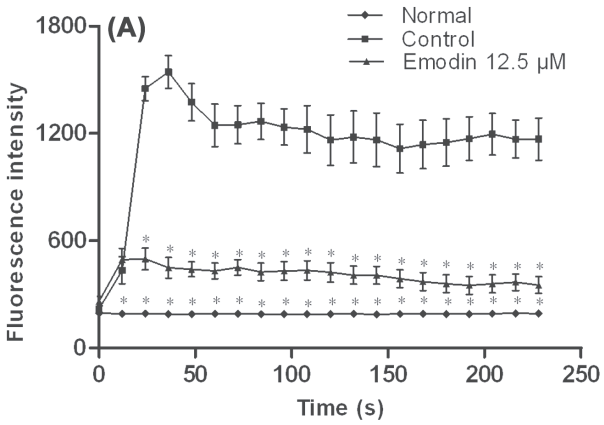


Fig. 3. Inhibitory effects of emodin on β-HEX enzyme activity. Each value represents the mean ± SEM (n = 4)

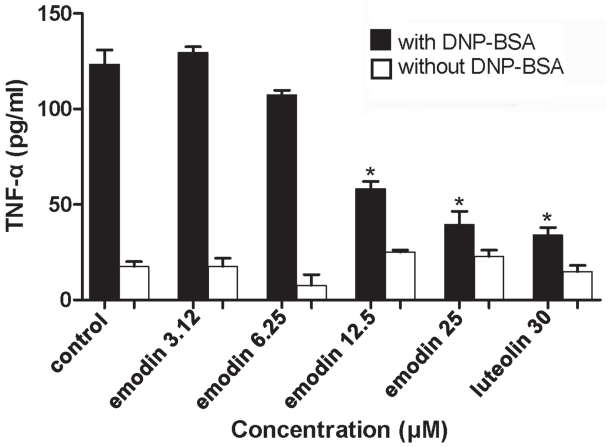
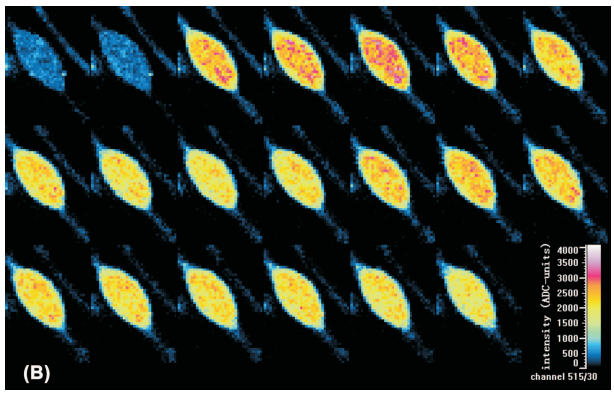


Fig. 4. Inhibitory effects of emodin on TNF-α secretion in RBL-2H3 cells. Each value represents the mean ± SEM (n = 2); * p < 0.01, significantly different from control

significantly different from the control group ($p < 0.01$; Fig. 4). The suppressive effects of 25 μM emodin approached the effects of the reference compound luteolin at 30 μM.

Inhibitory effects of emodin on extracellular Ca^{2+} influx

Increased levels of intracellular Ca^{2+} is important for degranulation in the Ca^{2+}-dependent pathway. Therefore, the effects of emodin on extracellular Ca^{2+} influx induced by DNP-BSA in sensitized RBL-2H3 cells were analyzed using a laser scanning confocal micro-



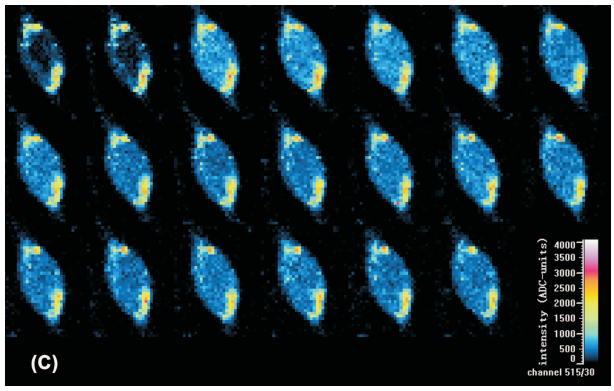


Fig. 5. (**A**) Effects of emodin on changes in Ca^{2+} level induced by DNP-BSA in RBL-2H3 cells. Fluorescence images were collected at 6 s intervals. Each value represents the mean ± SEM (n = 3). * p < 0.01, significantly different from control. (**B**, **C**) Confocal fluorescence images of Fluo-4-AM-loaded RBL-2H3 cells: (**B**) stimulated with DNP-BSA and (**C**) stimulated with DNP-BSA after pretreatment with 12.5 μM emodin. Sequential fluorescence images of antigen-stimulated cells are shown on top, from left to right. Fluorescence images were collected at 6 s intervals. DNP-BSA was added at the time of the first frame

scope with the Ca^{2+} probe Fluo-4 AM. In Figure 5A, the results indicated that the level of intracellular Ca^{2+} in sensitized RBL-2H3 cells was stable (mean fluorescence intensity = 191.0 ± 5.2) under normal conditions. Typical examples of the confocal fluorescence images of Fluo-4-AM-loaded RBL-2H3 cells are shown in Figure 5B and C. Sequential Fluo-4 AM fluorescence images in antigen-stimulated RBL-2H3 cells with and without emodin treatment are shown. When sensitized cells were stimulated with 10 μg/ml DNP-BSA, the intracellular Ca^{2+} level increased dramatically, which was approximately eight-fold greater than responding in the normal group within the first 30 s (Fig. 5A, B). Pretreatment with 12.5 μM emodin inhibited extracellular Ca^{2+} influx in sensitized RBL-2H3 cells stimulated with DNP-BSA (Fig. 5A, C). Significant differences ($p < 0.01$) were found in the test group and normal group compared with the control group.

Discussion

Type I hypersensitivity is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. Histamine release increases vascular permeability and recruits inflammatory leukocytes [5]. Recently, biphasic early-phase and late-phase reactions have been reported in type I allergy. The early-phase reaction of allergy occurs within minutes after allergen exposure, whereas the late-phase reaction occurs hours later and involves cytokine secretion, such as TNF-α and IL-4. β-HEX is also stored in secretory granules of mast cells and basophils and released along with histamine when mast cells and basophils are activated. Therefore, this enzyme is commonly used as a marker for degranulation in RBL-2H3 cells [1]. This convenient assay can be used for monitoring the capacity of potential new drugs to block mast cell activation and degranulation [4].

A previous study reported that emodin had antiinflammatory and immunosuppressive effects [7, 10] The present study found that emodin markedly decreased β-HEX release (Fig. 2A) and displayed stronger inhibition of β-HEX release (IC_{50} = 5.5 μM) than ketotifen fumarate salt (IC_{50} = 63.8 μM). The significant inhibitory activity of emodin against β-HEX production indicates that emodin may be a promising new anti-histamine agent.

In RBL-2H3 cells, tyrosine kinase Syk recruited by aggregated FcεRI phosphorylates phospholipase Cγ (PLCγ), which leads to the generation of inositol 1,4,5-triphosphate (IP3). IP3 causes the release of Ca^{2+} from intracellular Ca^{2+} stores and activates Ca^{2+} influx *via* Ca^{2+} release-activated Ca^{2+} channels (CRAC) to replenish the depleted Ca^{2+} stores. Ca^{2+} influx is an important event for degranulation and cytokine production [3, 13]. The increase in Ca^{2+} influx is followed by the degranulation of mast cells and production of inflammatory mediators, such as prostaglandins and arachidonic acid [6, 10]. In this study, we found that pretreatment with 12.5 μM emodin markedly inhibited the influx of extracellular Ca^{2+} in sensitized RBL-2H3 cells stimulated with DNP-BSA, although pretreatment with 3.12 and 6.25 μM emodin had a less extended effect or unstable effect. These findings suggest that 12.5 μM emodin influences degranulation *via* the Ca^{2+}-dependent pathway because it could inhibit extracellular Ca^{2+} influx in sensitized RBL-2H3 cells stimulated with DNP-BSA.

The late-phase reaction occurs within 4–6 h after the early-phase reaction in type I allergy. Mediators such as cytokines (TNF-α, IL-4, etc.) from the cells are involved in the late phase. TNF-α is an important pro-inflammatory cytokine that plays a critical role in late-phase hypersensitivity reactions. TNF-α is mainly produced by activated macrophages and T cells in response to infection, although it is also formed and secreted by mast cells in response to Ig-E challenge. Previous studies have demonstrated that emodin could suppress the release of cytokines, such as TNF-α and interleukin-6 (IL-6), in severe acute pancreatitis in rats [11, 23]. Therefore, we examined whether emodin suppressed TNF-α secretion in antigen-stimulated RBL-2H3 cells. Emodin exhibited concentration-dependent effects against TNF-α, with an IC_{50} of 11.5 μM (Fig. 4), indicating that emodin is also effective against late-phase reactions.

In summary, emodin suppressed degranulation and cytokine production in antigen-induced activation of sensitized RBL-2H3 cells. Our results indicate that the mechanism of action of emodin on degranulation (the early-phase reaction) is somewhat similar to its effects on TNF-α release (the late-phase reaction), perhaps by increasing the stability of the cell membrane. Although emodin inhibited extracellular Ca^{2+} influx, future experiments are necessary to elucidate the effects of emodin-induced blockade of extracellular Ca^{2+} influx pathways on signal transduction.

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