

Original article

Investigation of the molecular mechanism underlying the inhibitory activities of ethanol extract of *Bombyx mori* pupa-incubated *Cordyceps militaris* fruiting bodies toward allergic rhinitis

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ARTICLE INFO

Keywords:

Cordyceps militaris
Allergic rhinitis
CM-EE
Ca²⁺ ion mobilization
PKC
Syk

ABSTRACT

Cordyceps militaris has been widely studied for its various pharmacological activities such as antitumor, anti-inflammation, and immune regulation. The binding of an allergen to IgE-sensitized mast cells in nasal mucosa triggers allergic rhinitis. We found that oral administration of 300 mg/kg of the ethanol extract prepared from silkworm pupa-cultivated *Cordyceps militaris* fruiting bodies significantly alleviated the symptoms of ovalbumin-induced allergic rhinitis in mice, including sneeze/scratch, mast cell activation, eosinophil infiltration, and Syk activation. The treatment of ethanol extract significantly suppressed the release of β -hexosaminidase (a degranulation marker) and mRNA expression levels of various cytokines, including IL-3, IL-10, and IL-13 in activated RBL2H3 cells. The ethanol extract and β -sitosterol, which was purified from the extract, could respectively reduce the Ca²⁺ ion mobilization in activated RBL-2H3 cells. Furthermore, results collected from western immunoblotting demonstrated that ethanol extract significantly retarded Ca²⁺ ion mobilization-initiated signaling cascade, which provoked the expression of various allergic cytokines. Also, the extract incubation interfered with P38 as well as NF- κ B activation and Nrf-2 translocation. Our study suggested that ethanol extract possessed some natural constituents which could inhibit immediate degranulation and de novo synthesis of allergic cytokines via inhibition of Ca²⁺ ion mobilization in mast cells in the nasal mucosa of allergic rhinitis mice.

1. Introduction

An allergy, one kind of type I hypersensitivity disorder, is caused by an abnormal immune system and can evoke allergic disorders such as allergic rhinitis, asthma, and atopic eczema [1,2]. The IgE-bound mast cells' or basophils' stimulation by the allergen-specific to an IgE antibody contributes to an allergy. Allergens provoke the synthesis of antigen-specific IgE antibodies in B cells, and then the antibodies bind to the high-affinity IgE receptor (Fc ϵ RI) on the cell membrane of mast cells or basophils [1,2]. The allergens binding to IgE antibodies result in

IgE/Fc ϵ RI complex cross-linking. Fc ϵ RI cross-linking causes the prompt liberation of pre-produced inflammatory factors from the granules through degranulation of activated mast cells or basophils, such as histamine, leukotriene C4, β -hexosaminidase, prostaglandin, chemokines, and cytokines, which are responsible for various allergic symptoms. Aside from immediate degranulation, IgE/allergen-activated mast cells also release de novo synthesized cytokines, chemokines, and growth factors in addition to de novo produced lipid-originated mediators, such as prostaglandin D2, leukotriene B4, and cysteinyl leukotriene [1,2]. Cross-linkage of IgE/Fc ϵ RI complexes by allergens induces

Abbreviations: Akt, protein kinase B; BTK, B cell progenitor tyrosine kinase; CM, *C. militaris* aqueous extract; CMP, submerged fermented broth of *C. militaris* mycelia; CM-EE, ethanol extract of silkworm pupa-cultivated *C. militaris* fruiting bodies; DAG, diacylglycerol; DNP, 2,4-dinitrophenol; EGFR, epidermal growth factor receptor; EtOAc, ethyl acetate; Erk1/2, extracellular signal-regulated kinases 1/2; Fc ϵ RI, high affinity IgE receptor; GSK3 β , glycogen synthase kinase 3 β ; HO-1, heme oxygenase-1; HSA, human serum albumin; IKK, I κ B kinase; IL, interleukin; IP3, inositol 1,4,5-trisphosphate; JNK, c-Jun N-terminal kinase; MEK1, mitogen-activated protein kinase kinase 1; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf-2, nuclear factor erythroid 2-related factor 2; o.g., oral gavage; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, phosphatidylinositol 1,4,5-trisphosphate; PKC, protein kinase C; PLC- γ , phospholipase C- γ ; RBL-2H3, rat basophilic leukemia-2H3; Syk, spleen tyrosine kinase.

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<https://doi.org/10.1016/j.bioph.2021.111248>

Received 30 September 2020; Received in revised form 22 December 2020; Accepted 3 January 2021

Available online 1 February 2021

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Lyn to phosphorylate spleen tyrosine kinase (Syk). Phosphorylated Syk activates the Ras/Raf1/Erk 1/2 signaling cascade via LAT induction, responsible for producing lipid factors and cytokines. Phosphorylated Syk can also evoke the Rac/MEKK1/JNK pathway, which controls cytokines' de novo synthesis. Besides, active Syk causes phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) to evoke phospholipase C- γ (PLC- γ) through phosphorylation or B cell progenitor tyrosine kinase (BTK) activation. Active PLC- γ results in Ca^{2+} ion entering the cytoplasm from the endoplasmic reticulum or from outside the cells to augment Ca^{2+} ion level, which causes degranulation and activates the signaling cascade to promote de novo synthesis of cytokines in mast cells [1,2].

Cordyceps militaris (L.) Link is a well-known medicinal fungus and has been extensively utilized as a herbal medicine source and a health-promoting food in East Asia. A variety of its pharmacological activities have been explored. Various extracts prepared from *C. militaris* are reported to possess a wide range of anti-cancer properties established upon cell-based models such as breast cancer [3], colon cancer [4], lung cancer [5], and renal cell carcinoma [6]. In addition to the above tumors, Park et al. (2017) [7] reported that the treatment of ethanol extract prepared from *C. militaris* could induce apoptosis via Akt inhibition and activation of GSK and caspase-3 in xenografted leukemia tumor in mice. Hsu et al. (2017) [8] also demonstrated that CMP (submerged fermented broth of *C. militaris* mycelia) administration could suppress oral tumor growth in xenografted mice, possibly through the induction of apoptosis via inhibition of EGFR and IL-17A receptor signaling. Furthermore, *C. militaris* exhibits anti-melanoma activities in cancer cell lines and xenografted animal models [9]. Also, various extracts prepared from *C. militaris* demonstrate the effectiveness of anti-inflammation [10], immunomodulatory effects [11], and anti-asthma [12]. Recently Song et al. (2016) [13] reported that the 4-week administration of *C. militaris* aqueous extract (CM) reduces 24-h urine protein and serum creatinine in membranous glomerulonephritis rats. CM treatment restores the kidney histological architecture and glutathione peroxidase and superoxide dismutase levels in MGN rats. Besides, inflammatory cytokines and nuclear transcriptional factor NF- κ B subunit p65 recover to normal levels. The above results imply that CM might be an effective renoprotective agent.

In a documented studies, Oh et al. has shown that the ethyl acetate portion of the methanol extract of germinated soybean cultivated *Cordyceps militaris* fruiting bodies can attenuate passive cutaneous anaphylaxis in mice, likely through the inhibition of both the degranulation and the Syk/LAT/Map kinase pathways initiated by IgE/Fc ϵ R aggregation in mast cells [14]. The previous findings imply that *C. militaris* might be one of the alternative therapies for allergic rhinitis. This study examines the molecular mechanism underlying the ethanol extract's inhibitory efficacies (CM-EE) of silkworm pupa-cultivated *C. militaris* fruiting bodies toward allergic rhinitis induced in mice. We found that ethanol extract treatment significantly suppressed degranulation and mRNA expression levels of various cytokines in activated RBL2H3 cells, including IL-3, IL-10, and IL-13. Oral gavage of mice with 300 mg/kg of ethanol extract significantly alleviated ovalbumin-induced allergic rhinitis and could inhibit mast cell activation, eosinophil infiltration, and Syk activation in the noses of allergic mice. Ethanol extract incubation retarded significantly Ca^{2+} ion mobilization-initiated signaling cascade in activated RBL2H3 cells. Also, it interferes with p38 as well as NF- κ B activation and Nrf-2 translocation.

2. Material and methods

2.1. Production of the ethanol extract of *C. militaris* fruiting bodies (CM-EE)

Silkworm pupa-incubated *C. militaris* fruiting bodies were bought from Phytomedicine Biotechnology Co., Ltd of Nan Pao Resins Chemical

Co., Ltd. Group, as demonstrated in Fig. S1a. The fungus strain had been identified as *Cordyceps militaris* (L.) Link by the Food Industry Research and Development Institute of Taiwan. They exploited mycelia's characteristics and the micro-structures of conidiogenous cells and conidia spores to identify the fungus. Also, they used the rDNA ITS1-5.8S-ITS2 sequence data to confirm the identification. The identified fungal strain had been deposited in the Bioresource Collection and Research Center (BCRC) with the number BCRC930194. Dried *C. militaris* fruiting bodies (Fig. S1a) were ground into powder. The resulting powder was filtered through a 200-mesh metal sieve. Then 50 g of the fine powder was soaked in 95 % (v/v) ethanol at a 1:10 (w/v) partition and shaken at room temperature for 24 h. After incubation, the suspension was filtered using filter paper (ADVANTEC NO.2, 5 μ m). The filtrate was vacuum-dried afterward to obtain 13.16 g of brown syrup marked as CM-EE (Fig. S1b) and stored at -80 °C for later studies.

2.2. Cell line

Rat basophilic leukemia-2H3 (RBL-2H3) cells were obtained from American Type Culture Collection (ATCC) and incubated in a minimum essential medium enriched with 15 % (v/v) fetal bovine serum (FBS) at 37 °C with 5 % CO_2 in a humidified incubator. RBL-2H3 cells have been implemented to investigate Fc ϵ RI-mediated signaling cascade in mast cells [15].

2.3. β -hexosaminidase secretion assay

RBL-2H3 cells were first sensitized with 2 μ g/mL of 2,4-dinitrophenol (DNP)-specific IgE antibody for 16 h within a 24-well plate loaded with 4×10^5 cells/well. Then sensitized cells were washed twice with PIPES buffer [25 mM PIPES, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1 % (w/v) BSA, pH 7.2] and incubated in PIPES buffer with 0.01 % (v/v) DMSO or 0.01 % (v/v) DMSO-dissolved CM-EE (50 or 100 μ g/mL) or 0.01 % (v/v) DMSO-dissolved β -sitostone (0.12 or 0.24 μ M) for 60 min at 37 °C. After the above treatment, cells were provoked with 100 ng/mL of human serum albumin conjugated with DNP (DNP-HSA) for 2 h at 37 °C. After that, 30 μ l of the conditioned medium was combined with the same volume of substrate solution (2 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate, pH 4.5) and stood for 1 h at 37 °C to detect β -hexosaminidase activity. The reaction was stopped by mixing with 250 μ l of stop buffer (0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.0). The absorbance was calculated using a microplate reader at 405 nm (BioTEK Instrument, Inc., Winooski, VT, USA) and analyzed with the Student's *t*-Test (SigmaPlot, Systat Software, Inc. San Jose, CA, USA). For each sample, triplicate wells were implemented for data consistency. In terms of PMA + A23187 stimulation, 25 nM PMA combined with 1 μ M A23187 was added directly onto CM-EE-treated or un-treated RBL2H3 cells to activate cells and incubated for 2 h. Then β -hexosaminidase activity was measured again following the same procedure as described above. After activation and CM-EE incubation, cells were also harvested to detect proteins of interest for Western immunoblotting.

2.4. Allergic rhinitis induced in mice

According to internationally accepted principles for laboratory animal use and care, animal studies were performed following the institutional guidelines. Institutional Animal Care and Use Committee (IACUC) at the Southern Taiwan University of Science and Technology had approved this animal study (approved case number: 106-8). Like humans, Balb/c mice primarily produce IgE antibodies when induced by allergens [16], which was the reason why Balb/c mice were used in this study. Specific pathogen-free (SPF) male Balb/c mice were provided by the National Laboratory Animal Center, Taiwan. New coming male Balb/c mice (the mice were five weeks age, and the weight was 18–20 g) were caged in groups of six on wood chips for one week at 23 ± 2 °C and

30–70 % relative humidity with 12-h light/12-h darkness cycle (lighting time: 08:00 to 20:00) for environmental accustomization before the experiments. Mice intake rodent Lab Diet 5001 and sterile water ad libitum.

Allergic rhinitis induced by OVA in mice was carried out similarly, as reported by Hong et al. with some modifications [17]. Mice were randomly assigned into five groups, with a total of 32 mice. In group 1 each mouse (control, N = 4) was fed oral gavage (o.g.) with 500 μ l olive oil every day from the 21 st to the 30th day. At 0th, 7th, and 14th days, in group 2, each mouse (sham, N = 4) was injected intraperitoneally (i. p.) with 1 mg Al(OH)₃ in 200 μ l saline while in groups 3 (N = 8), 4 (N = 8), and 5 (N = 8), each mouse was injected i.p. with 50 μ g OVA mixed with 1 mg Al(OH)₃ in 200 μ l saline. Starting from the 21 st to the 30th day, every day in groups 2 and 3, 500 μ l olive oil was used to feed each mouse o.g. while in groups 4 and 5, 500 μ l olive oil containing 300 and 500 mg/kg of CM-EE was fed o.g. to each mouse. After that, in group 2, each of the mice's nostrils was intranasally inoculated with 10 μ l saline 30 min after olive oil feeding. In groups 3, 4, and 5, each nostril was intranasally inoculated with 0.5 μ g OVA in 10 μ l saline 30 min after CM-EE serving. After 5-minute incubation, each mouse was counted for the numbers of sneezing and scratching within 10 min. However, on the 30th day, the numbers of sneezing and scratching were counted respectively within 30 min. Two observers, blinded to the experimental mice, were responsible for the counting. The results were analyzed using Student's *t*-Test (SigmaPlot, Systat Software, Inc. San Jose, CA, USA). After the experiments, mice were sacrificed by injecting intraperitoneally 0.5–1 mL of 500 mg/mL urethane. After injection, the blood was not withdrawn from the hearts until the experimental mice had no response to their hindfeet stimulus.

2.5. Western immunoblotting

RBL2H3 cells were first activated and treated with CM-EE, as mentioned in the Results section. Then RBL2H3 cells were collected and lysis buffer [10 mM Tris (pH 8.0), 0.32 M sucrose, 1 % (v/v) Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM PMSF] was used to lyse the cells. After protein concentration was measured using Bio-Rad DC protein assay kit, the same volume of 2 $\times$ sample buffer [0.1 M Tris (pH 6.8), 2 % (w/v) SDS, 0.2 % (v/v) β -mercaptoethanol, 10 % (v/v) glycerol, and 0.0016 % (w/v) bromophenol blue] was mixed with the protein lysate. Proteins present in suitable quantities of the lysates were resolved by electrophoresis at 100 V with 10 % (w/v) SDS-PAGE, and proteins on the gels were transferred onto a PVDF membrane. After blocked for 1 h in 3 % (w/v) bovine albumin serum (BSA) at room temperature, membranes were hybridized overnight at room temperature with primary antibodies listed in Materials. The membranes were washed, and the primary antibodies binding on the membranes were detected with appropriate secondary antibodies for 1 h at room temperature. Secondary antibodies present on the membranes were observed using the chemiluminescence ECL detection system under ImageQuant LAS 4000 (Fujifilm super CCD area type chip) (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The intensity of each protein band was counted by PDQUEST Quantity One software (Bio-Rad Laboratory, Hercules, CA, USA) and normalized with actin protein quantity. The statistic results were evaluated with Student's *t*-Test (SigmaPlot, Systat Software, Inc., San Jose, CA, USA).

All the sources of materials and additional methods were listed in the supplementary data.

3. Results

3.1. Alleviation of allergic rhinitis induced on mice by CM-EE treatment

Our previous results demonstrated that CM-EE has no harm to RBL2H3 cells. Most constituents of CM-EE are weakly polar, as shown in HPLC profiles. Further analyses using GC/MS showed that CM-EE components belong to fatty acid, ester, and ketone [18]. We also

found that CM-EE treatment could inhibit Syk/JNK/c-jun pathway and decrease mice's allergic symptoms with passive cutaneous anaphylaxis [18]. We anticipated that CM-EE therapy might improve the indications of allergic rhinitis. To assess our anticipation, we examined the CM-EE's effects on diminishing allergic rhinitis induced in Balb/c mice, as described in the Methods section and Fig. 1A. As indicated in Figs. 1a and S2a, after OVA induction, the numbers of nasal rubbing and sneezing increased, and even wounds could be observed on mouse noses. However, CM-EE feeding reduced the numbers of nasal rubbing/sneezing in mice, suggesting that CM-EE treatment could compromise allergic rhinitis symptoms. Extract treatment also attenuated the nasal septum's thickness (Figs. 1B and S2B). To further characterize the CM-EE's therapeutic activities, nasal specimens of allergic rhinitis mice were stained with toluidine blue. In this metachromatic staining, the cytoplasm of mast cells in nasal specimens was stained to become purple. The staining results demonstrated that the number of mast cells in nasal specimens of OVA-induced allergic mice was higher than those of control and sham mice. However, CM-EE treatment decreased the number of mast cells in nasal biopsies of allergic mice compared to those of un-treated counterparts (Figs. 1C and S2C), implicating that CM-EE could alleviate mast cell infiltration into the nasal mucosa. Eosinophil staining also demonstrated that eosinophil infiltration was relieved in CM-EE-treated allergic mice (Figs. 1D and S2D). Furthermore, IHC of nasal specimens with p-Syk antibody and hematoxylin counterstaining showed that the number of p-Syk positive mast cells in the nasal tissues of CM-EE treated-allergic mice was lower than those of the uncured counterparts. (Figs. 1E and S2E). The results in Fig. 1F and G demonstrated that CM-EE treatment could reduce IgE and histamine amounts in sera withdrawn from allergic mice. The above results implicated that the CM-EE cure could lower allergic rhinitis symptoms by inhibiting mast cell activation in nasal tissues. There was no weight difference between control, sham, and CM-EE administrated mice, suggesting that CM-EE treatment would not affect the animal's metabolism (Fig. S3).

3.2. Reduction of the cytokine secretion from activated RBL2H3 cells by CM-EE incubation

To explore the mechanism underlying the suppressive activities of CM-EE administration in allergic rhinitis mice, the inhibitory efficacy of degranulation was investigated first in the DNP-HSA/IgE-irritated RBL-2H3 cells. β -hexosaminidase released from challenged RBL-2H3 cells into the media is deemed as a sign of degranulation [14]. Data of β -hexosaminidase measurement indicated that incubation of DNP-HSA/IgE-activated RBL-2H3 cells with 50 or 100 μ g/mL CM-EE could dwindle the β -hexosaminidase activity in cell media, suggesting that CM-EE could inhibit the degranulation (Fig. 2A).

To further examine the influences of CM-EE treatment on the secretion of cytokines and chemokines, a cytokine array was carried out to investigate the exudation of various cytokines, and chemokines from CM-EE-treated activated RBL-2H3 cells. The results in Fig. 2B showed that CM-EE incubation could significantly suppress the secretion of IL-13, thymus chemokine, IL-3, VEGF, sICAM-1, and IL-10. Real-time PCR data confirmed that CM-EE administration could substantially lower the amount of IL-3, IL-10, and IL-13 in active RBL-2H3 cells (Fig. 2C). The above results implicated that CM-EE might inhibit the release of various allergic mediators through the hindrance of degranulation and de novo synthesis.

3.3. Inhibition of the augmentation of intracellular calcium ion concentration in activated-RBL2H3 cells by CM-EE treatment

In allergen-binding mast cells, MAP kinase activation happens, and Ca²⁺ ions enter the cytoplasm from the endoplasmic reticulum or outside of the cells to augment Ca²⁺ ion level, leading to degranulation and subsequently the de novo synthesis of cytokines/chemokines [1,2]. To further shed light on the molecular mechanism of anti-allergic

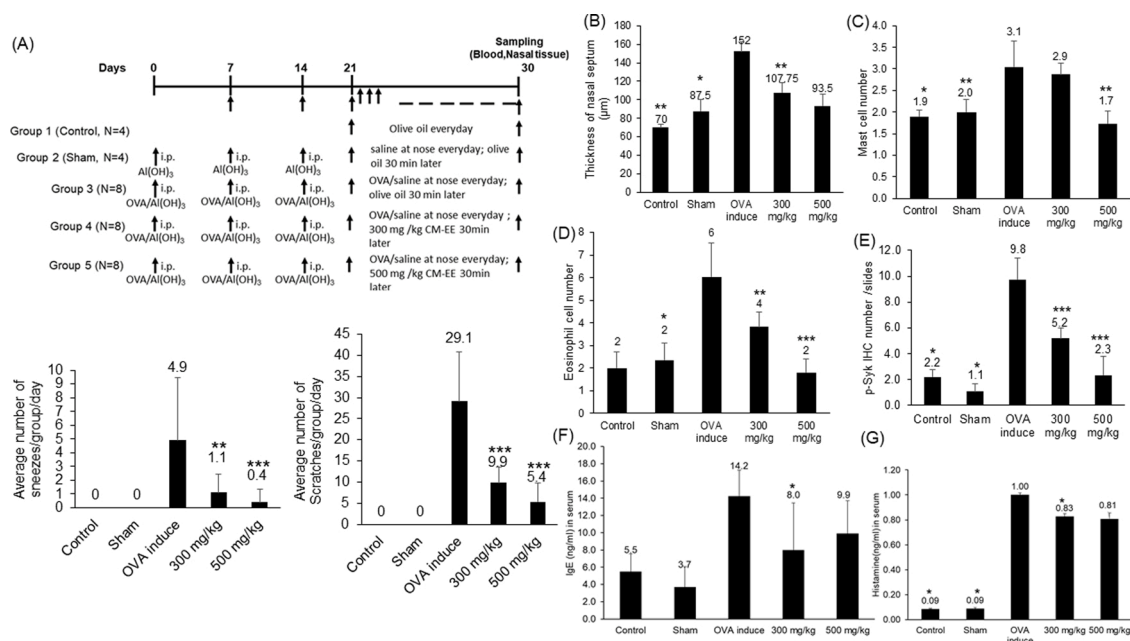


Fig. 1. Alleviation of OVA-induced allergic rhinitis in mice by CM-EE treatment. (A) The numbers of nasal rubbing/sneezing of allergic mice per day; (B) the average number of the thickness of nasal septum on each slide; (C) the average number of mast cells on each slide; (D) the average number of eosinophil cells on each slide; (E) the average of the number of Syk(+) mast cells on each slide; (F) IgE amount in the blood of allergic mice; (G) Histamine amount in the blood of allergic mice. In (A), results were indicated by the average number of total sneeze or scratching of each group for ten consecutive days. In (C), (D), and (E), the average cell number in each slide was counted as described in the Methods section. OVA induce, allergic rhinitis mice; 300 mg/kg and 500 mg/kg, allergic mice fed with 300 and 500 mg/kg of CM-EE, respectively. The results were indicated as the mean $\pm$ SD. The number above each bar was the average number. *, ** and *** denoted $p < 0.05$ and $p < 0.01$ and $p < 0.001$ respectively as compared to OVA-induced control using Student's *t*-test.

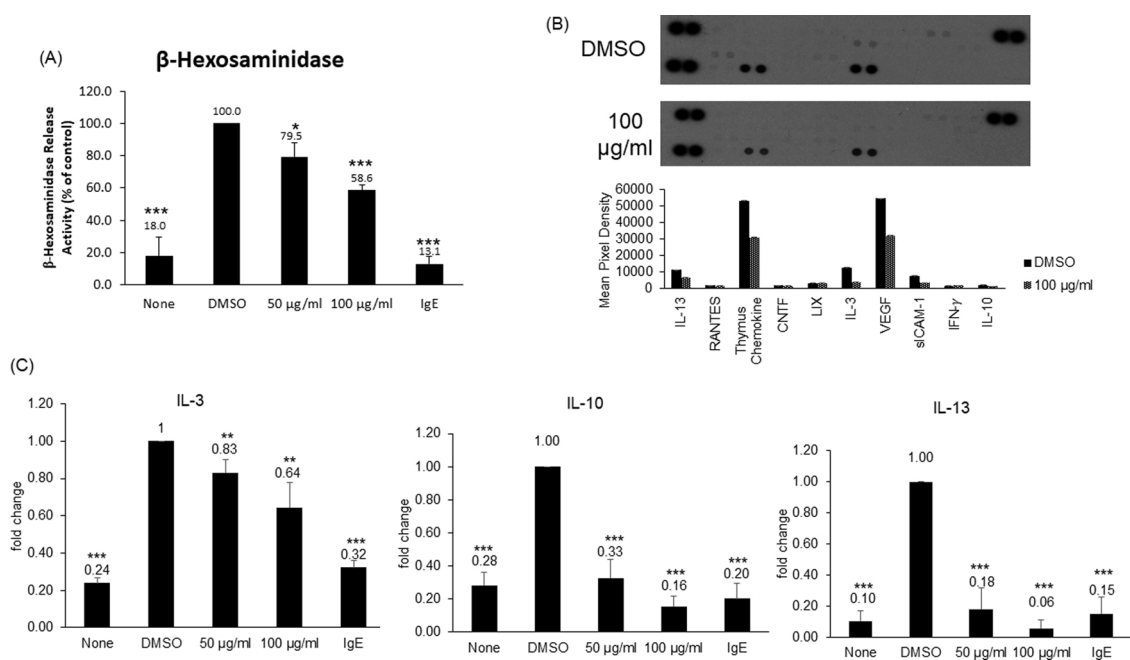


Fig. 2. The impacts of CM-EE treatment on cytokine production in irritated RBL2H3 cells. (A) β-hexosaminidase secretion in RBL2H3 cells; (B) Cytokine array; (C) The mRNA synthesis of IL-3, IL-10, and IL-13 in activated RBL2H3 cells. Sensitization/activation, CM-EE incubation, and β-hexosaminidase secretion from RBL-2H3 cells were mentioned in the Methods section. The procedures of cytokine array and real-time PCR were indicated in the Methods section. The cytokine array blot was the representative result of two independent experiments in Fig. 2B. None, RBL2H3 cells; DMSO, 0.01 % (v/v) DMSO-treated activated RBL2H3 cells, 50 and 100 μg/mL, activated RBL2H3 cells treated respectively with 0.01 % (v/v) DMSO-dissolved CM-EE (50 and 100 μg/mL); IgE, IgE-sensitized RBL2H3 cells. CM-EE-exposed active RBL2H3 cells were compared to DMSO control using Student's *t*-test. The denotation of the *p*-value is the same as those in Fig. 1.

rhinitis capabilities of CM-EE therapy, we surveyed if CM-EE treatment could lower the effects of calcium ion concentration in activated RBL2H3 cells. The results of Fig. 3A demonstrated that CM-EE

incubation could lower the increase of Ca^{2+} ion concentration within the cytoplasm of DNP-HSA/IgE-challenged RBL2H3 cells. It could also decrease the degranulation in RBL2H3 cells, owing to PMA + A23187 (a

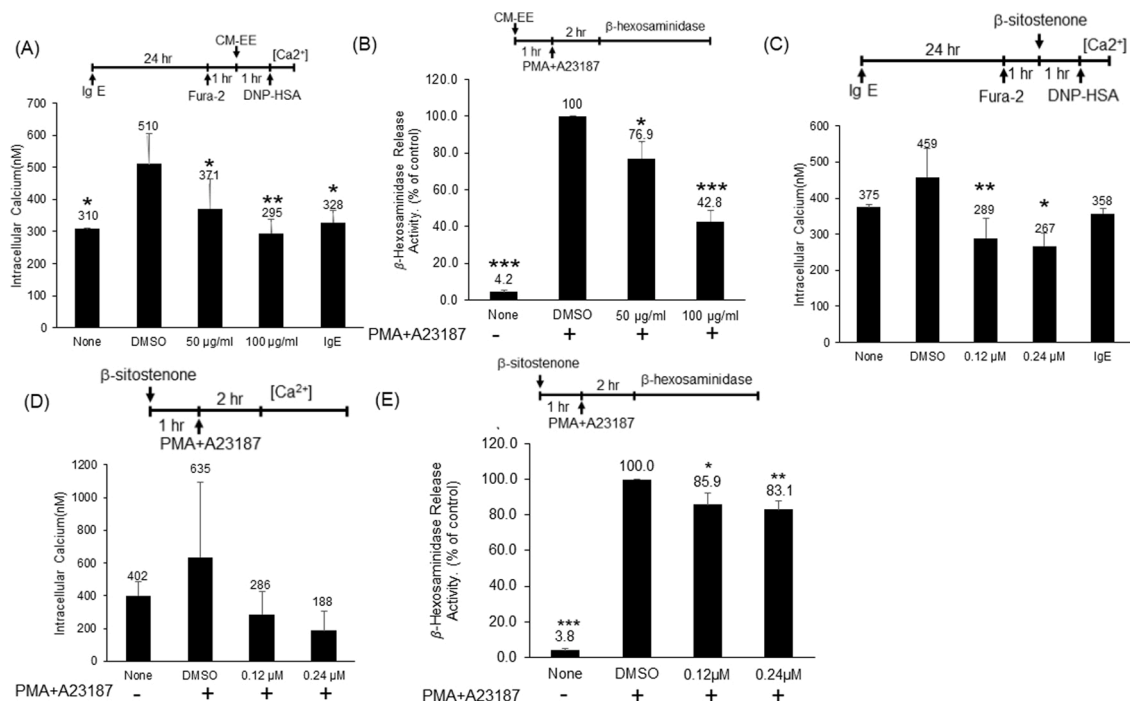


Fig. 3. The influences of CM-EE or β -sitosterone on Ca^{2+} ion mobilization in activated RBL2H3 cells. (A) Intracellular Ca^{2+} ion level in DNP-HSA/IgE-activated RBL2H3 cells incubated with CM-EE; (B) β -hexosaminidase secretion from PMA/A23187-activated RBL2H3 cells treated with CM-EE; (C) Intracellular Ca^{2+} ion level in DNP-HSA/IgE-activated RBL2H3 cells administrated with β -sitosterone; (D) Intracellular Ca^{2+} ion level in PMA/A23187-evoked RBL2H3 cells treated with β -sitosterone; (E) β -hexosaminidase secretion from PMA/A23187-activated RBL2H3 cell exposed to β -sitosterone. Sensitization/activation, CM-EE or β -sitosterone treatment of RBL-2H3 cells, and Ca^{2+} ion mobilization were indicated in Methods section. 0.12 and 0.24 μM , 0.01 % (v/v) DMSO-dissolved β -sitosterone (0.12 and 0.24 μM)-incubated aroused RBL2H3 cells.

specific Ca^{2+} ion mobilization agent) treatment (Fig. 3B). Besides, previous studies had shown that the treatment of β -sitosterone isolated from CM-EE could inhibit the degranulation in activated RBL2H3 cells [18]. In this study, Fig. 3C and D results demonstrated that β -sitosterone administration could distinctly decrease Ca^{2+} ion concentration in the cytoplasm of DNP-HSA/IgE or PMA + A23187 evoked-RBL2H3 cells. It also could reduce the degranulation in PMA + A23187 stimulated-RBL2H3 cells (Fig. 3E). The above results implicated that CM-EE might alleviate allergic rhinitis symptoms by restricting calcium ion mobilization into the cytoplasm in activated mast cells.

3.4. Inhibition of the signaling cascade leading to Ca^{2+} release in activated RBL2H3 cells by CM-EE therapy

Our previous documented results suggested that the restraint of de novo synthesis of various cytokines/chemokines and lipid mediators by

CM-EE probably attributes to the inhibition of MAP kinase pathways, e.g., Syk/PI3K/MEKK4/JNK/c-jun, Syk/Erk1/2 pathways, and PLC- γ in irritated RBL-2H3 cells [18]. PLC- γ splits phosphatidylinositol biphosphate (PIP2) to generate diacylglycerol (DAG) and phosphatidylinositol 1,4,5-trisphosphate (IP3). DAG activates PKC and IP3 stimulates the movement of Ca^{2+} ions out of the endoplasmic reticulum to facilitate the downstream biochemical pathways [19]. In this study, to further explore the CM-EE-induced impacts on Ca^{2+} ion mobilization, CM-EE's effects on the signaling pathways related to the Ca^{2+} ion trafficking were examined.

We found that CM-EE exposure could inhibit the activation of BTK, PKC α , PKC μ , and PKC δ by phosphorylation in DNP-HSA/IgE incited RBL-2H3 cells (Fig. 4A–D and Fig. 5). Additionally, it could lower the expression of phosphatidylinositol 1,4,5-trisphosphate (IP3) receptor (IP3-R) in the activated RBL-2H3 cells (Figs. 4E and 5). The above outcomes intimated that CM-EE incubation in activated RBL-2H3 cells

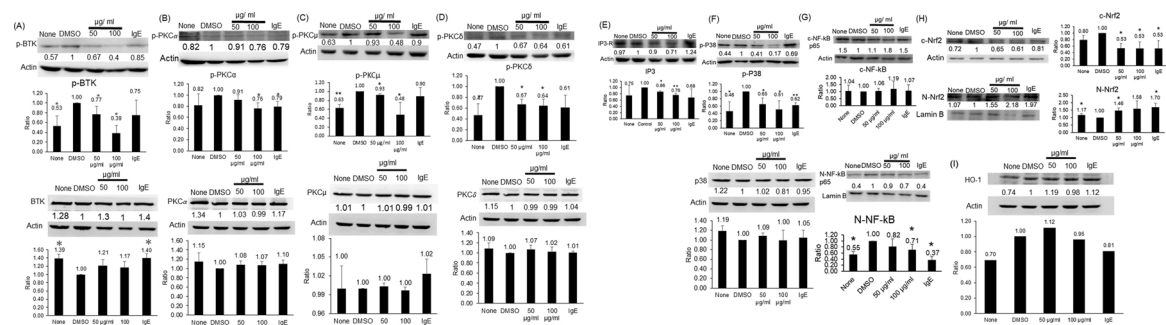


Fig. 4. Effects of CM-EE on DNP-HSA/IgE-initiated signaling pathway in RBL-2H3 cells. Sensitization/activation, CM-EE incubation of RBL-2H3 cells, and Western immunoblotting were mentioned in the Methods section. (A) p-Btk; (B) p-PKC α ; (C) p-PKC μ ; (D) p-PKC δ ; (E) IP3-R; (F) p-P38; (G) NF- κ B; (H) Nrf2; (I) HO-1. The blots in individual figures were the symbolic data of at least three independent inspections except for the HO-1 examination, which was two independent experiments. The proportion (mean $\pm$ SD) was indicated as the normalized intensity ratio (protein under inspection/actin) at each dose divided by DMSO control.

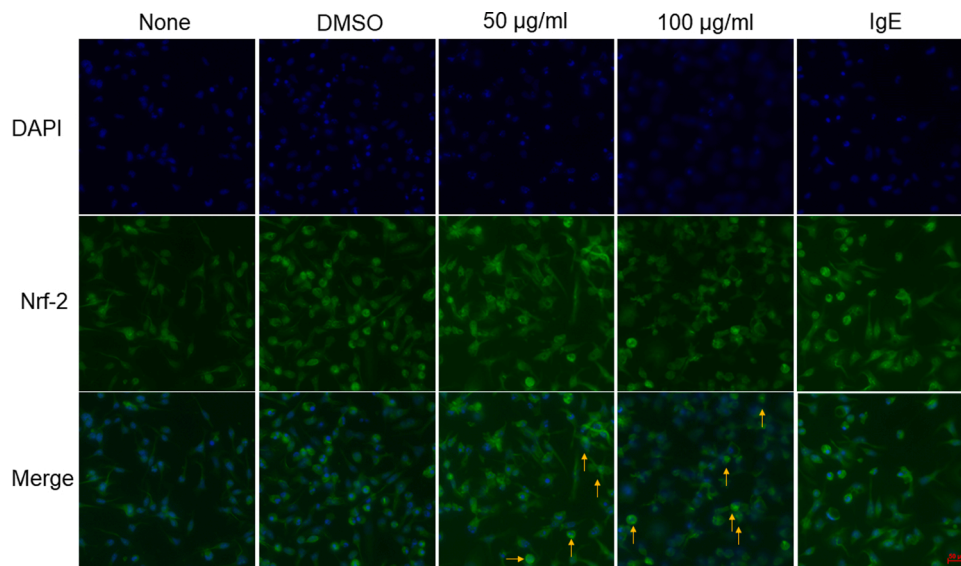


Fig. 5. The immunofluorescent staining of IgE/DNP-HSA activated RBL2H3 cells treated with CM-EE. RBL2H3 cells were evoked with DNP-HSA and DNP-specific antibody and treated with DMSO (vehicle control), 50 or 100 µg/ml CM-EE as described in the Methods section. Arrows indicated the Nrf-2 staining in the nucleus in RBL2H3 cells.

could inhibit degranulation and de novo synthesis of cytokines/chemokines due to the regulation of the Syk/BTK/PLC- γ /IP $_3$ receptor/ Ca^{2+} ion mobilization pathway. In addition to Ca^{2+} ion movement, our recent documented findings [18] and data in Fig. 4F and G indicated that CM-EE administration down-regulated the expression of MAP kinases – Erk1/2, JNK as well as p38 and also inhibited the translocation of NF- κ B subunit p65 to the nucleus (Fig. 5), which alluded that CM-EE treatment could decrease the de novo synthesis of a variety of cytokines/chemokines via the control of NF- κ B activation and MAP kinases in the provoked-RBL-2H3 cells. Documented findings demonstrated that the activities of NF- κ B-mediated inflammatory pathways are antagonistic to those of Nrf-2/heme oxygenase-1 (HO-1) signaling pathways [20]. Nrf-2 plays a vital role in cellular defense against the oxidative stress provoked by many chemical insults [21]. Under normal circumstances, Nrf-2 remains in the cytoplasm, while under oxidative stress, it travels to the nucleus, where it initiates the transcription of various anti-oxidative genes, including HO-1. In line with the above findings, we also found

that CM-EE treatment elevated the Nrf-2 protein level in the activated RBL-2H3 cells' nuclei while the Nrf-2 protein amount decreased in the cytoplasm (Figs. 4H and 6). Immunofluorescent staining data showed that the Nrf-2 protein moved into the nuclei of activated RBL2H3 cells under CM-EE influence (Fig. 5).

To further elucidate the nuclear movement of Nrf-2, immunofluorescent staining was carried out on activated RBL2H3 cells in the presence of CM-EE. The observation in Fig. 5 demonstrated that the fluorescent intensity of the nuclei of CM-EE-affected active RBL2H3 cells was more robust than those of the DMSO counterpart, suggesting that CM-EE treatment contributed to the Nrf-2 translocation into the nuclei. Also, to ensure the effects of CM-EE on the Nrf-2 expression, the results presented in Fig. 4I showed that 50 µg/mL CM-EE incubation increased the HO-1 production in RBL2H3 cells as compared to that of the DMSO counterpart. The above results implicated that CM-EE incubation might reduce allergic rhinitis through enhancing the Nrf-2-mediated anti-oxidative status and suppressing the NF- κ B-mediated inflammatory

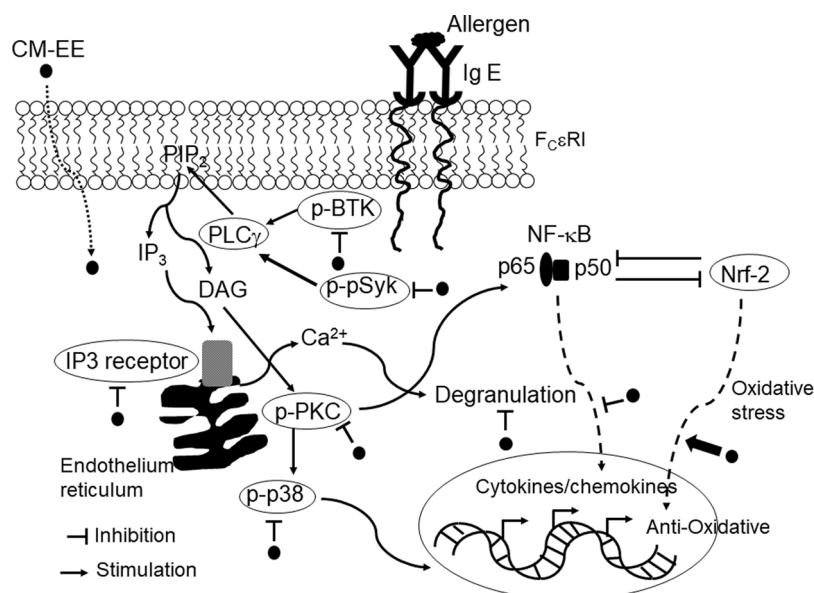


Fig. 6. The proposed pathway for the impacts of CM-EE on IgE-initiated signaling pathway in RBL2H3 cells.

pathways in active mast cells.

4. Discussion

The incidences of allergic rhinitis attributing to type I hypersensitivity disorder surge recently in Asia, including Taiwan, mostly because of aggravating air pollution. Allergic rhinitis routine therapies are mostly small-molecule inhibitors on leukotrienes or histamine receptors and steroid cure [22,23]. These typical therapeutic methods contribute to annoying side effects, e.g., stomach upset, dry mouth, and drowsiness [22]. To date, anti-allergic therapies resulting from medicinal chemicals or extracts purified from traditional medicinal herbs or fungi [24,25] with far less or no side effects are getting attention. Previous findings have demonstrated that *Cordyceps* spp. have anti-allergy effectiveness [14,26]. Thus the natural ingredients present in *C. militaris* fruiting bodies may be alternative therapies for allergic rhinitis.

In this study, *C. militaris* fruiting bodies cultured on silkworm pupa were implemented to explore the possible efficacies against allergic rhinitis. The results of animal studies demonstrated that CM-EE administration could compromise allergic rhinitis symptoms initiated by allergen-activated mast cells. CM-EE treatment could reduce the numbers of nasal rubbing/sneezing, the thickness of nasal septum, mast cell infiltration, and eosinophil infiltration in mice with OVA-triggered allergic rhinitis. However, there are some drawbacks when the above model was applied to human disease. Gross structural differences exist between mice and the human nose and also, distribution of epithelium. The variation of nasal concha shape between mice and humans gives rise to apparent discrepancies in airflow patterns [16].

Mast cells are the cardinal effector cells that initiate IgE-mediated allergic responses through secreting inflammatory mediators and cytokines/chemokines. The alleviation of symptoms of allergic mice by CM-EE administration might attribute to the silencing of mast cells. Per our anticipation, the cytokine array data demonstrated that CM-EE treatment could inhibit degranulation (β -hexosaminidase freed) and the release of various cytokines/chemokines from activated RBL2H3 cells.

As stated previously, allergen-evoked PLC- γ activation results in Ca^{2+} ion movement in mast cells. This study showed that CM-EE treatment could compromise the increase of Ca^{2+} ion concentration in PMA + A23187- or DNP-HSA/IgE-provoked RBL2H3 cells. The therapeutic effects of CM-EE on allergic rhinitis might be plausible owing to the dwindling of Ca^{2+} ion concentration augment, and thus the degranulation and de novo synthesis of various cytokines/chemokines in mast cells within the nasal mucosa. Furthermore, the treatment of β -sitosterone identified in our previous studies also could inhibit the Ca^{2+} ion mobilization in PMA + A23187- or DNP-HSA/IgE-stimulated RBL2H3 cells, suggesting that β -sitosterone might be one of the active components present in CM-EE against allergic rhinitis.

Cross-linkage of Fc ϵ RI by allergen triggers Syk to provoke BTK/PLC- γ /IP3-R to increase intracellular calcium ion concentration. Following the above pathway, PIP2 is hydrolyzed by PLC- γ to produce diacylglycerol (DAG) and IP3. DAG is subsequently to activate protein kinase C (PKC), which stimulates I κ B kinase (IKK) [26], and IP3 binds to the IP3 receptor to trigger extracellular calcium influx and calcium release from the endoplasmic reticulum. PKC can provoke IKK and thus regulates the translocation of NF- κ B, which starts the transcription of various pro-inflammatory cytokine genes (Fig. 6). In our previous studies, we found that CM-EE treatment significantly dwindled Syk/PI3K/MEKK4/JNK/c-jun pathway as well as Erk activation and thus inhibited the de novo synthesis of various cytokines/chemokines and lipid mediators [18]. In this study, we examine the influences of CM-EE incubation on the biochemical cascade of Ca^{2+} ion mobilization to decipher the CM-EE mechanism at the molecular level and provide a more detailed explanation for the cure of allergic rhinitis by CM-EE administration. Our signaling studies demonstrated that CM-EE treatment could inhibit the activation of BTK, PKC α , PKC μ , PKC δ as well as p38 and interferes with NF- κ B translocation (Fig. 6).

Further, the treatment could also lower the expression of the IP3 receptor in DNP-HSA/IgE evoked RBL2H3 cells. The above results shed light on why, in addition to Syk/JNK/c-jun pathway, CM-EE incubation could dwindle the scale of Ca^{2+} ion concentration and the de novo synthesis of cytokines/chemokines in activated RBL2H3 cells. The results mentioned above provided the molecular clue to the attenuation of allergic rhinitis by CM-EE application. Through disturbing Ca^{2+} ion mobilization in mast cells, CM-EE treatment might inhibit the immediate degranulation at an early stage. It might profoundly interfere with IgE/Fc ϵ R-commenced signaling pathway, including de novo synthesis of cytokines/chemokines and lipid mediators to alleviate the symptoms at a late stage.

As mentioned above, the ethyl acetate portion of the methanol extract of germinated soybean cultivated *Cordyceps militaris* fruiting bodies can alleviate passive cutaneous anaphylaxis in mice [14]. Their results suggest that the ingredients within the EtOAc fraction facilitate the anti-allergic activities. Thus, an anti-allergic compound, genistein 4'-O- β -D-glucoside 4''-O-methylate, is further purified from the methanol extract of GSCM [28]. The inhibitory activity of this compound toward degranulation is better than that of genistein or genistin, both of which are main isoflavones in most soybean products and are helpful to regulate immune responses. More data shows that it can also inhibit IL-4 and TNF- α expressions and IgE/Fc ϵ R-initiated biochemical cascade.

In contrast to Park et al.'s examination, we found that CM-EE possesses anti-allergic effectiveness not only to passive cutaneous anaphylaxis [18] but, more importantly, to allergic rhinitis. We further investigated more details regarding CM-EE's effects on the signaling pathway contributing to Ca^{2+} ion mobilization in irritated RBL2H3. No other partition of CM-EE was needed to recognize the anti-allergic portion. Furthermore, β -sitosterone, a triterpenoid purified from CM-EE in our previous studies, inhibited Ca^{2+} ion distribution in activated RBL2H3 cells, suggesting that β -sitosterone might be one of the active ingredients in CM-EE that contributes to allergic cure. So far, no documented findings report the above activity. However, a previous study reported two triterpenoids, β -sitosterol, and β -sitosterone purified from *Leucosidea sericea* (Rosaceae), demonstrated anti-inflammatory activities through down-regulation of COX-1 and COX-2 [29].

5. Conclusion

Our study implicated that CM-EE treatment could alleviate allergic rhinitis symptoms, probably because CM-EE possessed the chemicals that could interfere with prompt degranulation and restrict Ca^{2+} mobilization and thus inhibit de novo synthesis of cytokines/chemokines in IgE-triggered allergic mast cells. However, except β -sitosterone, the chemicals responsible for the anti-allergic rhinitis activities are not identified yet in CM-EE. More efforts may be spent to locate and purify more effective chemicals from ethyl acetate fraction of ethanol extract in the following studies. Meanwhile, the anti-allergic rhinitis activities of β -sitosterone and the underlying molecular mechanism may be examined by cell and animal studies shortly. Besides, the toxicity studies of CM-EE may be investigated in the future. The toxicity examination may include the biochemical parameters and biopsies for the liver and kidney and the critical parameters of whole blood.

Funding

This research was funded by the Ministry of Science and Technology of Taiwan, grant number MOST 105-2320-B-218-001-MY3 and MOST 108-2320-B-218-001. The Ministry of Science and Technology of Taiwan has not participated in the design of the study, data collection/analysis, data interpretation, and writing the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

The authors would like to thank Litzung Biotechnology Inc. Taiwan for helping H/E and IHC staining.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2021.111248>.

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Supplementary Materials

Supplementary Materials and Methods

Materials

Minimum essential medium and fetal bovine serum were supplied by GIBCO (Life technologies), Grand Island, NY, USA. Tris base, DTT, NaCl, PMSF, SDS, β -mercaptoethanol and bromophenol blue were bought from aMResco, Solon, OH, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, glucose, Na_2CO_3 , EDTA and Triton X-100 were purchased from Merck Millipore, Darmstadt, Germany. DMSO, dinitrophenol-specific IgE antibody, dinitrophenol-human serum albumin, Evans blue, NaHCO_3 , p-nitrophenyl-N-acetyl- β -D-glucosaminide, and bovine serum albumin were provided by Sigma-Aldrich, St. Louis, MO, USA. PIPES, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and sucrose were bought from Avantor Performance Materials, Center Valley, PA, USA. KCl was from Scharlau, Sentmenat, Barcelona, Spain. DTT was from USB Corporation, Cleveland, OH, USA. Glycerol was from Kanto Chemical, Oregon, USA. Chemiluminescence ECL detection system was from GE Healthcare Bio-Sciences AB, Uppsala, Sweden. Phosphor-BTK(1:1000), phosphor-PKC α (1:1000), phosphor-PKC μ (1:1000), phosphor-PKC δ (1:1000), phosphor-p38 (1:1000) and NF- κ B/p65 (1:1000) antibodies were bought from Cell Signaling Technology, Danvers, MA, USA. Nrf2 (1:1000) antibody was purchased from St. John's Laboratory, London, United Kingdom and actin antibody (1: 5000) was from GeneTex, Irvine, CA, USA. Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (1:1000) was from Albumin from chicken egg white (OVA) was obtained from Sigma Aldrich, St. Louis, MO, USA. Rat Cytokine Antibody Array Kit was bought from R&D Systems, Minneapolis, MN, USA. Mouse IgE ELISA kit, histamine ELISA Kit and Rabbit specific HRP/DAB Detection IHC Detection kit were purchased from Abcam Cambridge, MA, USA. SYBR Green master mix kit was from BioGenesis, Westminster, CO, USA. RNeasy Mini kit was from Qiagen, MD, USA. High-capacity cDNA reverse transcription kit was from Applied Biosystems, Waltham, MA, USA. PVDF membrane was from Stratagene, La Jolla, CA, USA. Lab Diet 5001 was from Lab Supply, Fort Worth, TX, USA.

Fura-2, AM ester (Biotium, Ferment, CA, USA). Formalin was obtained from Sigma. 4.

Primers	Sequences
Rat IL-3	Forward 5'-ACAATGGTTCTTGCCAGCTCTAC-3' Reverse 5'-AGGAGCGGGAGCAGCAT-3'
Rat IL-10	Forward 5'-GGCCCTTTGCTATGGTGTCT-3' Reverse 5'-GTAGGGGAACCTCTGAGCTGC-3'
Rat IL-13	Forward 5'-CTCAGGGAGCTTATCGAGGA-3' Reverse 5'-TGCAACTGGAGATGTTGGTC-3'
Rat GAPDH	Forward 5'-CCCCCAATGTATCCGTTGTG-3' Reverse 5'-TAGCCCAGGATGCCCTTTAGT-3'

Cytokine array

RBL2H3 cells were activated and treated with CM-EE, as described above. The conditioned media were collected from DMSO- and CM-EE-administrated IgE/DNP-HSA activated RBL2H3 cells and evaluated for cytokine secretion using Rat cytokine antibody array (R&D Systems) according to the manufacturer's protocol.

Reverse transcriptase-real time polymerase chain reaction (RT-real time PCR)

A 24-well plate was loaded with 4×10^5 RBL-2H3 cells/well, and cells were sensitized/activated as aforementioned. Total RNA was purified from RBL2H3 cells, IgE-sensitized cells, DMSO, 50, or 100 $\mu\text{g/ml}$ CM-EE-incubated IgE/DNP-HSA provoked cells using the RNeasy Mini kit according to the manufacturer's suggested protocols. cDNA was produced using a high-capacity cDNA reverse transcription kit according to the manufacturer's suggestion. Real-time PCR to measure mRNA amount of IL-3, IL-10, and IL-13 were conducted with Applied Biosystems 7500 Real-Time PCR System using the correspondent primers listed in the supplementary documents. Rat GAPDH was used as an internal control. 2 μl of cDNA was used in each real-time PCR reaction using the SYBR Green master mix kit with 25 μM of each primer. PCR amplifications were carried out at 50 °C for 2 minutes and 95 °C for 10 minutes for one cycle as the first stage, followed by 50 cycles at 95 °C for 15 seconds, 50 °C for 10 seconds and 60 °C for 40 seconds as the second stage and then one cycle at 95 °C for 15 seconds, 50 °C for 1 minute and 95 °C for 15 seconds as the last stage. C_T results were acquired at the end of the last stage. Relative level variations were counted with the comparative C_T method. C_T is the cycle number at which fluorescent intensity first exceeds the threshold level. ΔC_T

$=C_T$ (IL-3, IL-10 or IL-13) - C_T (GAPDH). $\Delta\Delta C_T$ is ΔC_T (CM-EE-treated evoked RBL2H3)- ΔC_T (DMSO-incubated provoked RBL2H3). $2^{-[\Delta\Delta C_T]}$ represented fold change between IL-3, IL-10, or IL-13 amount of CM-EE-treated evoked RBL2H3 cells, and that of DMSO-treated stimulated cells. $2^{-[\Delta\Delta C_T]}$ data were evaluated using the Student's t-Test (SigmaPlot, Systat Software, Inc. San Jose, CA, USA).

Toluidine blue staining of nasal tissues of mice

The 5 μ m nasal tissue slides were de-waxed with xylene for 2 minutes for three times. Then the de-waxed slides were washed in absolute alcohol for 2 minutes twice, in 90% (v/v) alcohol for 2 minutes, in 70% (v/v) alcohol for 2 minutes, and lastly in running water for 2 minutes. After wash, the slides were stained with toluidine blue working solution for 2-3 minutes and then washed in distilled water for 2 minutes. Then slides were dehydrated quickly in 95% (v/v) alcohol and 100% alcohol for ten dips twice. Then slides were cleared in xylene for 2 minutes three times. In each slide, the number of purple-stained mast cells was counted in 5 randomly selected fields, and the sum of mast cell number of 5 field area was deemed as mast cell number of each slide. The sum of the positive cell number of 6 slides was divided by 6 to gain the average number of positive cells per slide. The individuals who counted the cells were blinded to the experiments.

Histamine release assay

50 mL plasma was precipitated by an addition of 60 % perchloric acid and the resulting precipitate is removed by centrifugation. The supernatant was assayed for histamine release using a histamine ELISA kit.

Syk immunohistochemistry of nasal tissues of mice

IHC was conducted on tissue sections dissected from formalin-fixed, paraffin-embedded mouse nasal blocks at 3- μ m thickness. Xylene was first exploited to deparaffinize the slides. Then, the slides were rehydrated with ethanol and heated using a microwave in a 10 mM citrate buffer (pH 6)

for 7 min to retrieve the antigen epitopes. After antigen retrieval, 3% (v/v) H₂O₂ was used to quench endogenous peroxidase, and slides were washed in Tris-buffered saline for 15 minutes. Then slides were hybridized with a phosphor-Syk (p-Syk) antibody for 1 hour, followed by antibody detection using a ChemMate EnVision™ kit. IHC specimens were subsequently stained with toluidine blue. In each slide, the number of p-Syk-positive and purple-stained mast cells was counted in 5 randomly selected fields. The sum of the Sky-positive mast cell number of 5 field area was regarded as a Sky-positive mast cell number of each slide. The sum of the positive cell number of 6 slides was divided by 6 to obtain the average number of positive cells per slide. The persons who counted the cells were blinded to the experimental conditions.

Calcium ion mobilization assay

For the measurement of Ca²⁺ ion concentration in DNP/IgE activated RBL2H3 cells, RBL-2H3 cells were first sensitized with 2 µg/ml of 2,4-dinitrophenol (DNP)-specific IgE antibody for 16 hours at 37 °C. Then IgE-sensitized RBL2H3 cells were incubated at 37 °C for 1 hour with 2 µM Fura-2/AM in modified tyrode-hepes medium-1 (MTH-1) which was tyrode-hepes medium (134 mM NaCl, 2.9 mM KCl, 4 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5 mM dextrose, 0.2 mg/ml fatty-acid-free BSA, 10 mM Hepes, pH 7.4) plus 1 mM CaCl₂ and 1 mM MgCl₂. After incubation, sensitized cells were collected from MTH-1 medium, and cells were re-suspended in MTH-2 medium, which was tyrode-hepes medium with the addition of 1 mM CaCl₂ and 0.1 mM MgCl₂. The re-suspended cells were then treated with DMSO, 50, or 100 µg/ml of CM-EE at 37 °C for 1 hour. 1×10⁶ of resultant cells were re-suspended in 2 ml MTH-2 medium within a cuvette and put in SPEX CMT1 11 spectrofluorometer (Hitachi) for fluorescence detection. After that 100 ng/ml DNP-HSA was added onto the sensitized cells for activation, and fluorescent intensity was immediately measured for 3 minutes with 340/380 nm excitation waves/505 nm emission wave. After the detection of fluorescent intensity, 1 µl of 50 µM digitonin was applied to the activated cells, and then fluorescent intensity was measured for 1 minute. At last, 1 µl of 20 mM EGTA/Tris buffer (pH 8.0) was added on the irritated cells, and the fluorescent intensity was again detected for 3 minutes. Ca²⁺ concentration was

calculated using the built-in program (FL Solutions 2.0) based upon the fluorescent intensity. Regarding the PMA+A23187 stimulation experiment, a similar protocol was carried out as above except that the naïve RBL2H3 cells were incubated with Fura-2/AM ester in MTH-1 medium and then 25 nM PMA+1 μ M A23187 was used to provoke RBL2H3 cells in MTH-2 medium in spectrofluorometer for fluorescence detection.

All the sources of materials were listed in the supplementary information.

In situ fluorescent hybridization of CM-EE treated and activated RBL2H-3 cells with Nrf-2 antibody

1×10^4 DNP-HAS/IgE activated RBL2H3 cells treated with DMSO, 50 μ g/ml or 100 μ g/ml CM-EE were loaded onto each well of a 4-well culture slide (Millicell EZ SLIDE 4-well Glass) and cultivated overnight. After culture, cells were incubated with 10 % (v/v) formalin (Sigma) for 15 minutes at room temperature and then washed with 1 $\times$ PBS (1.36 M NaCl, 0.024 M KCl, 0.1 M Na₂HPO₄, 0.016 M KH₂PO₄) for 5 minutes three times. Then cells were permeablized with 0.1% (v/v) Triton X-100 for 10 minutes at room temperature and washed with 1 $\times$ PBS for 5 minutes three times. Permeablized cells were then treated with 1 % (w/v) BSA for 60 minutes at room temperature. After blocking, cells were incubated with Nrf-2 antibody (1:200 in 1 % BSA) for 2 hours at room temperature and then washed with 1 $\times$ PBS 5 minutes three times. After Nrf-2 antibody treatment, cells were incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (1:1000 in 1 % BSA) and 1 μ g/ml DAPI at the same time for 1 hour at room temperature and then washed for 5 minutes three times. After immunostaining, the stained coverslips were mounted onto the slides for the observation under Nikon ECLipseTi/iNU_TOKAZHIT.

Supplemenatry Materials and Methods—Figures

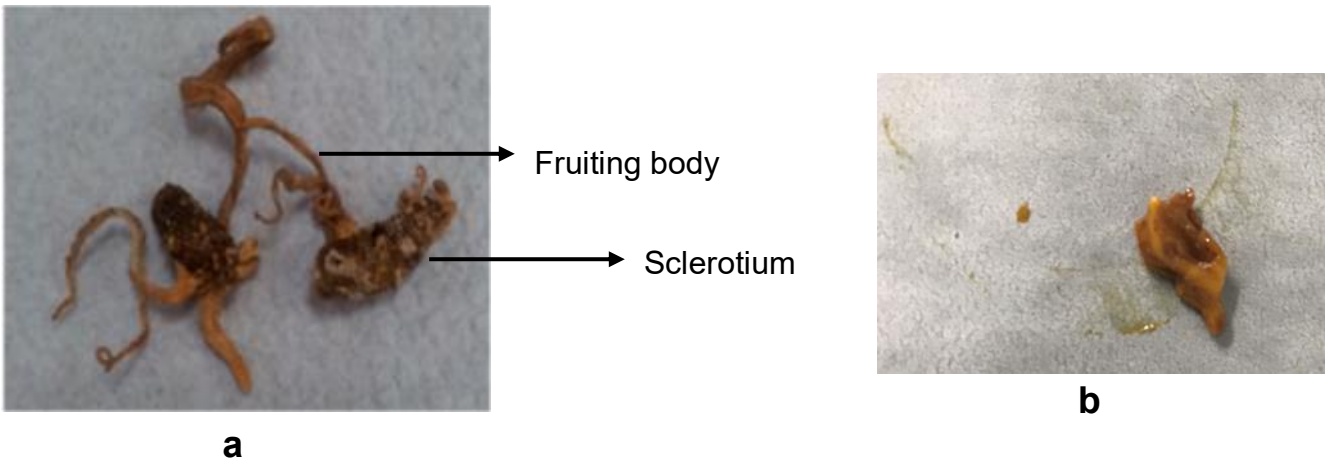


Fig. S1 The pictures of **(a)** Silk worm pupa-cultured *Cordyceps militaris* fruiting bodies; **(b)** CM-EE.

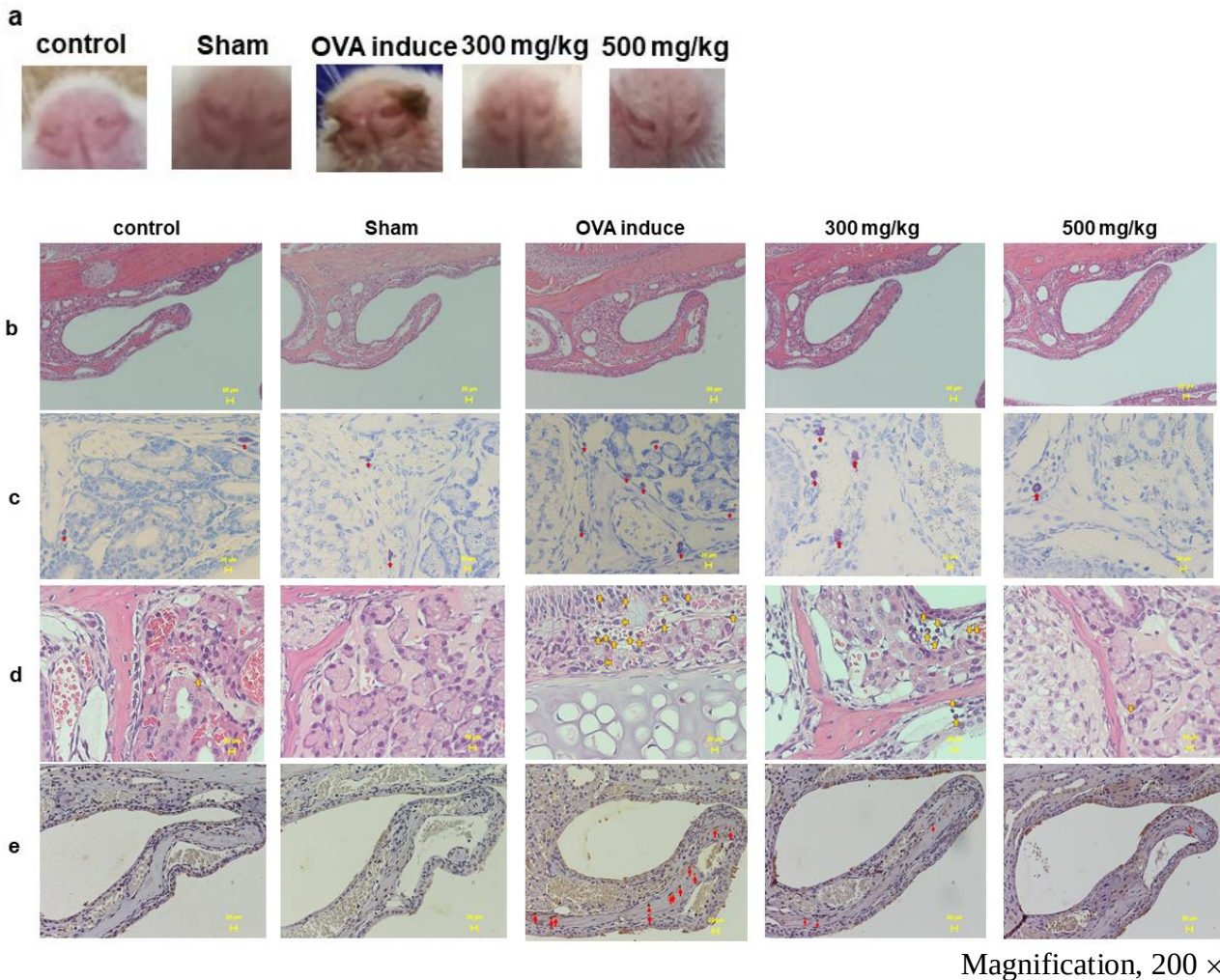


Fig. S2 The IHC results of nasal specimens of allergic rhinitis mice.

(a) The nasal symptoms of allergic rhinitis mice; **(b)** the typical hematoxylin/eosin (H/E) staining of the nasal septum; **(c)** the typical toluidine blue staining of nasal specimens; **(d)** the representative hematoxylin/eosin (eosinophil) staining of nasal tissues; **(e)** the symbolic p-Syk IHC staining of nasal

specimens; In (c), (d), and (e), mast cells, eosinophil cells, and p-Syk(+) cells were indicated respectively by red arrows, yellow arrows, and red arrows. OVA induce, allergic rhinitis mice; 300 mg/kg and 500 mg/kg, allergic mice fed with 300 and 500 mg/kg of CM-EE respectively.

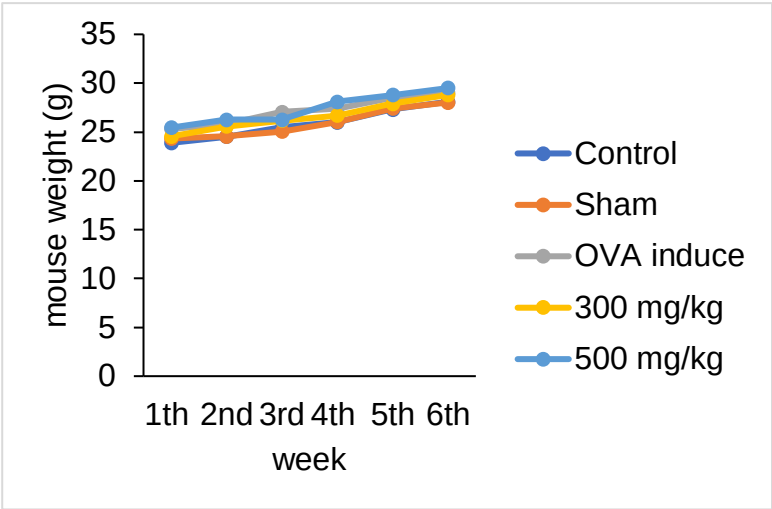


Fig. S3 The effects of CM-EE on the weight of mice during the experimental period.

Fig 4A

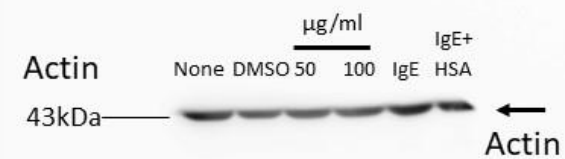
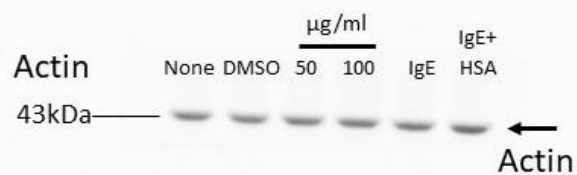
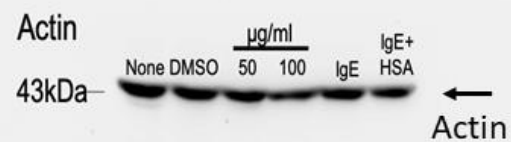
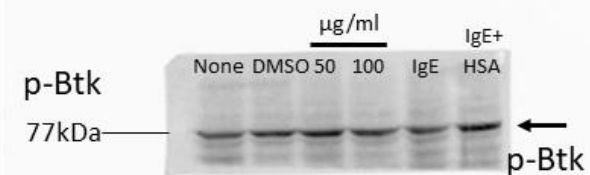
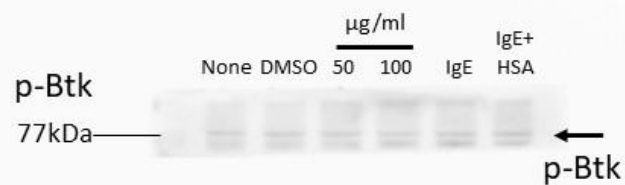
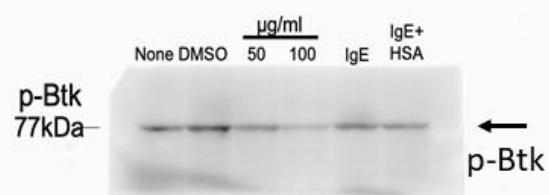


Fig 4A

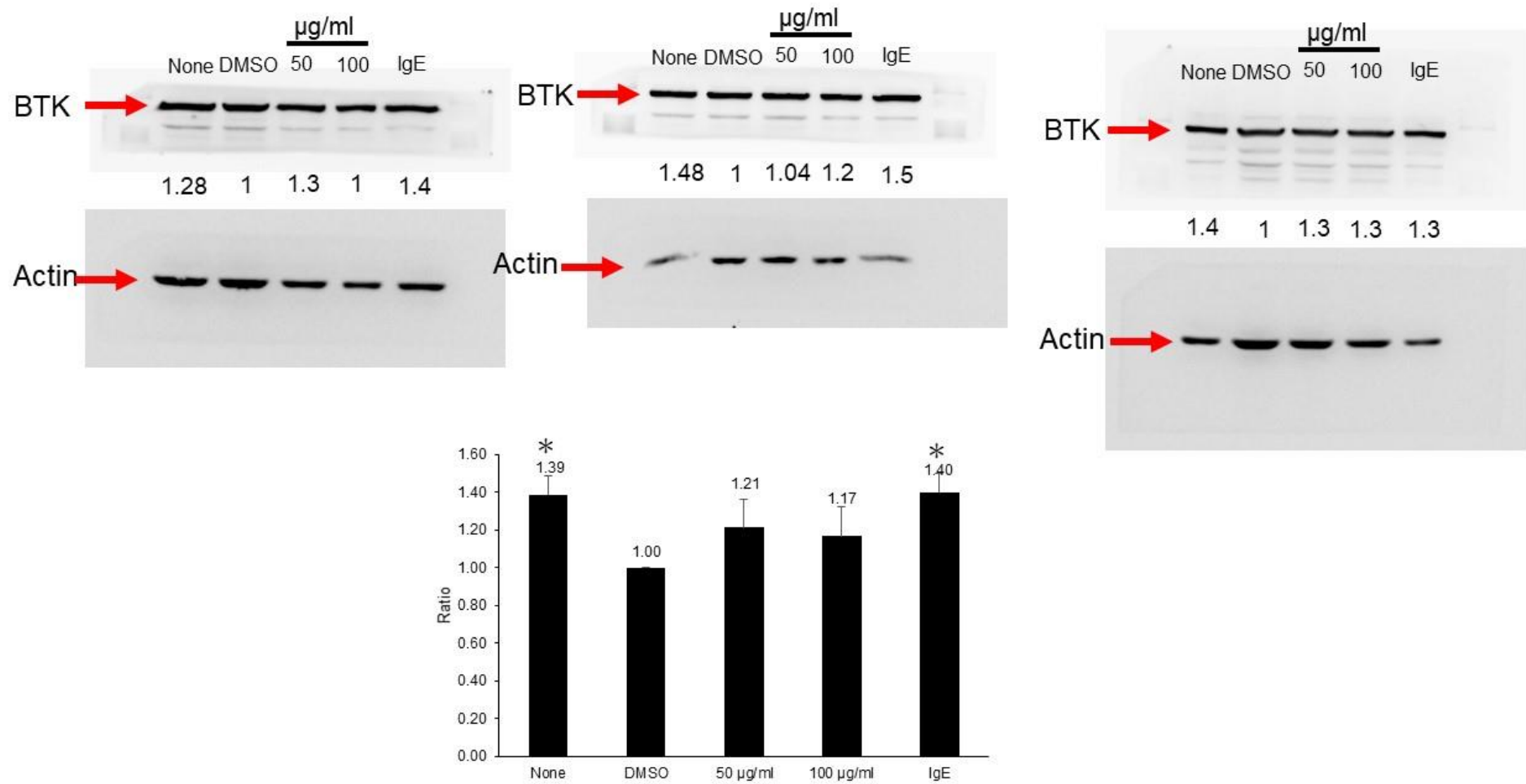


Fig 4B

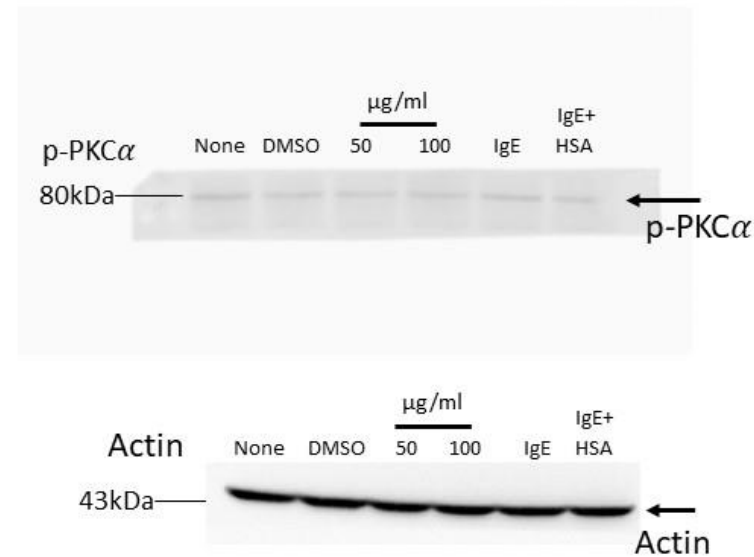
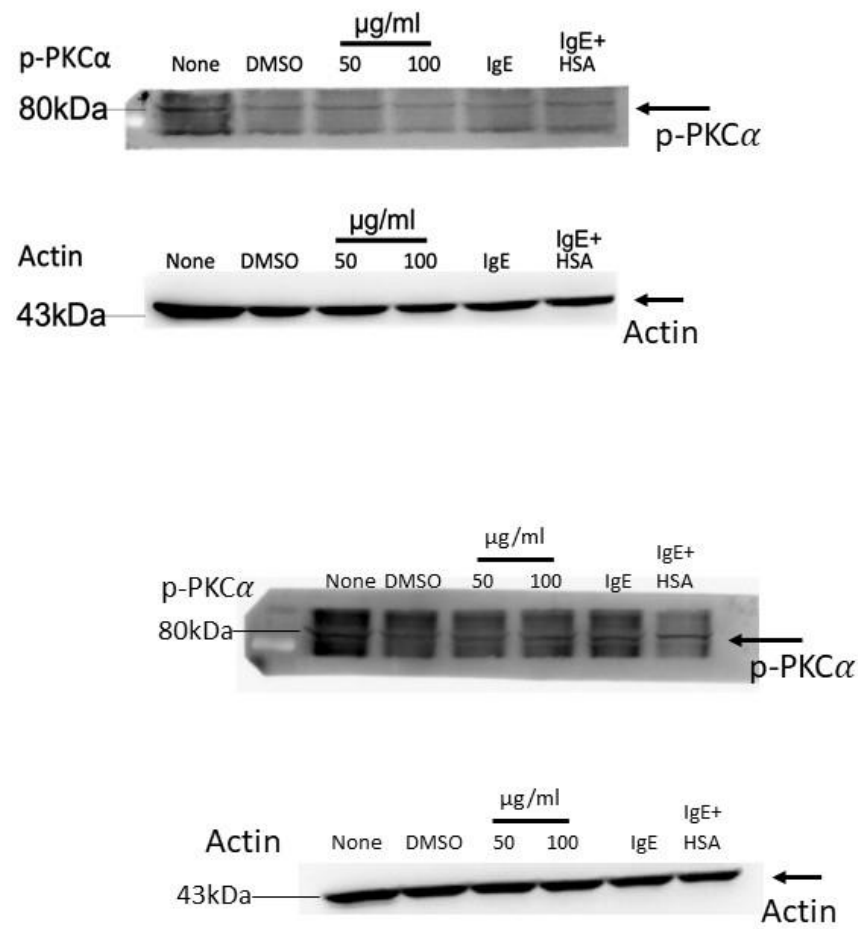


Fig 4B

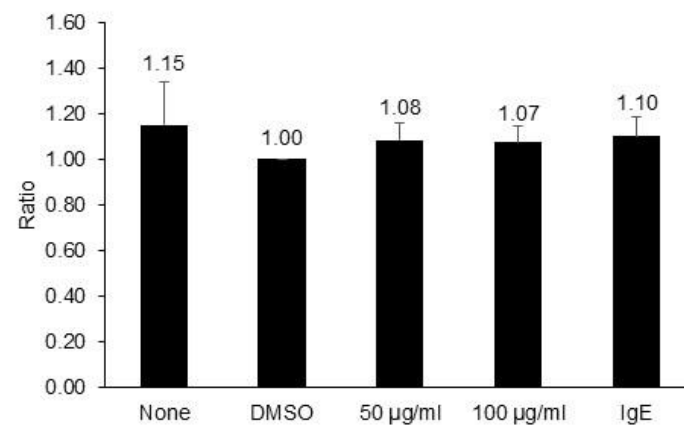
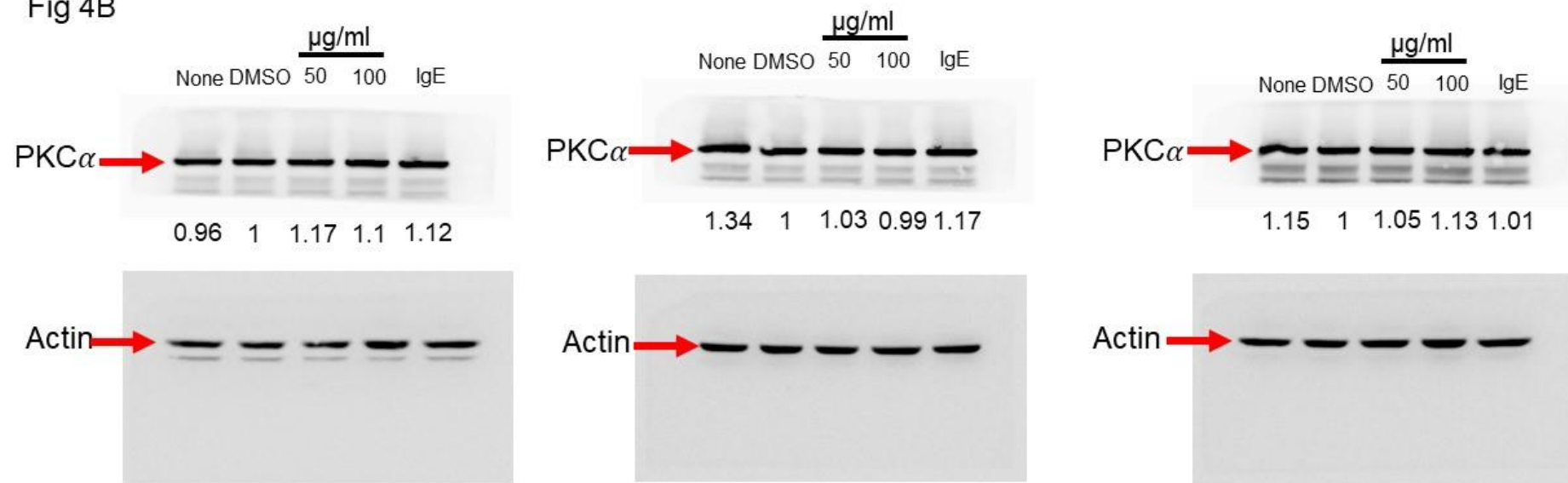


Fig 4C

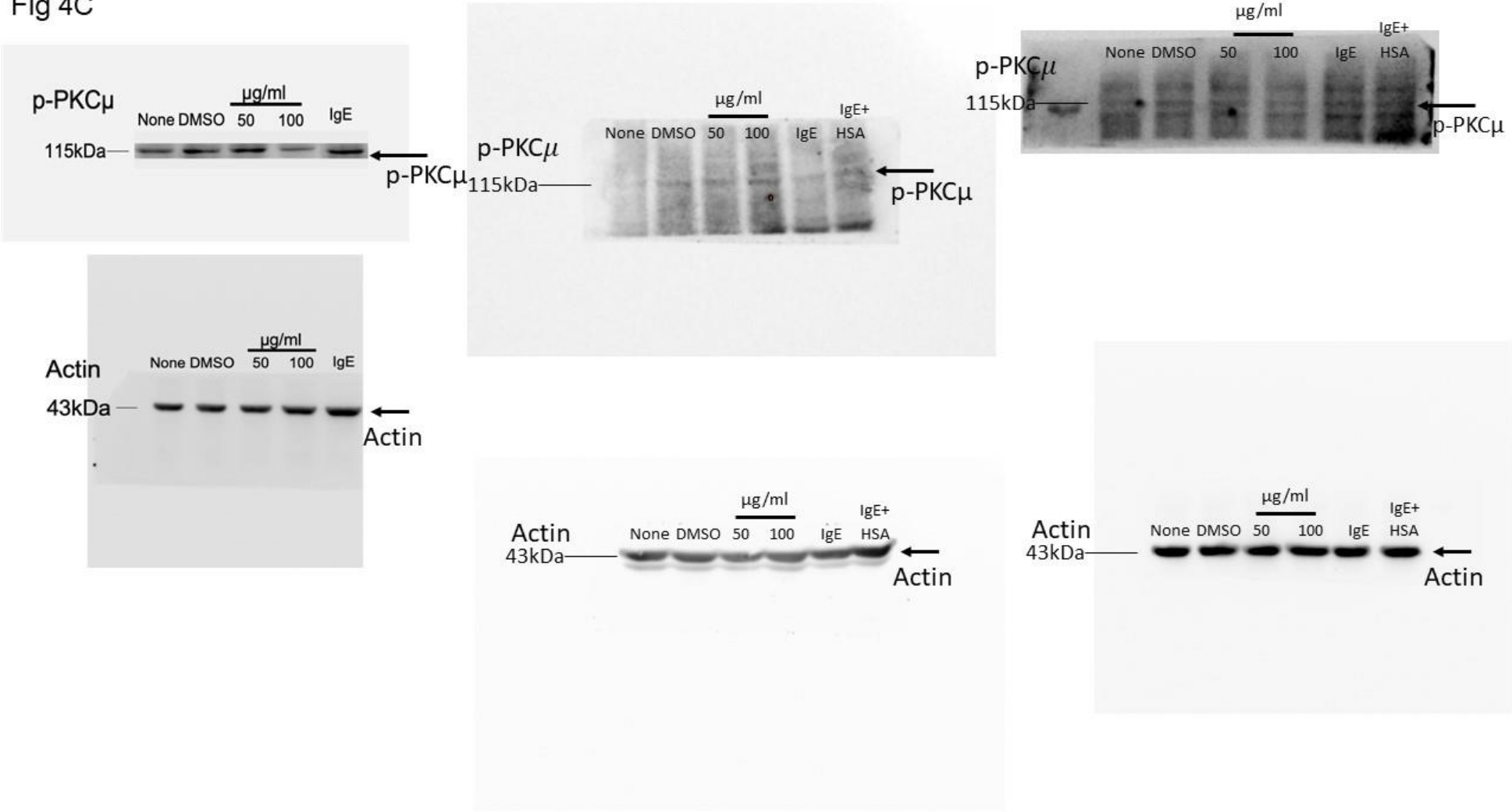


Fig 4C

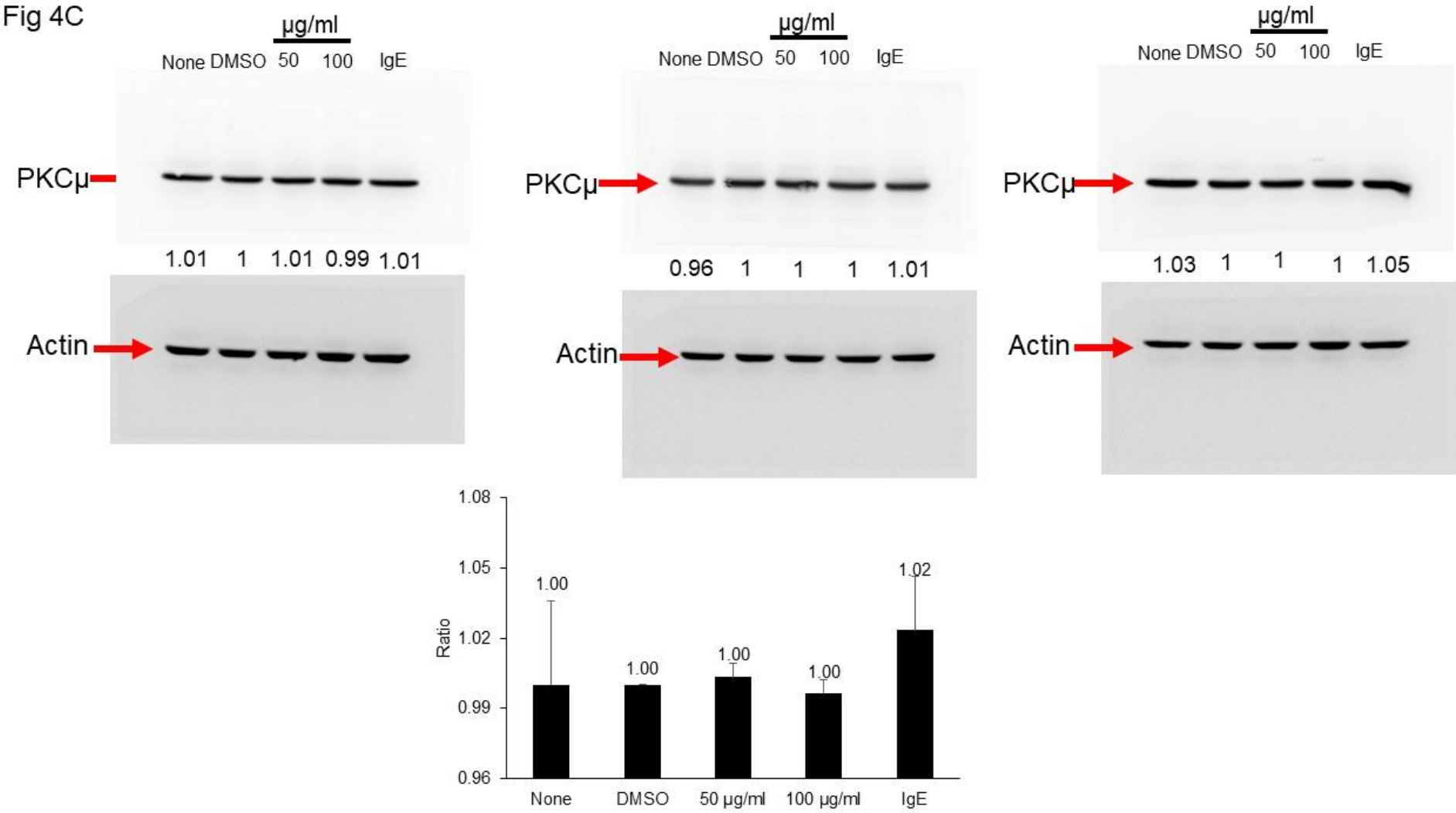


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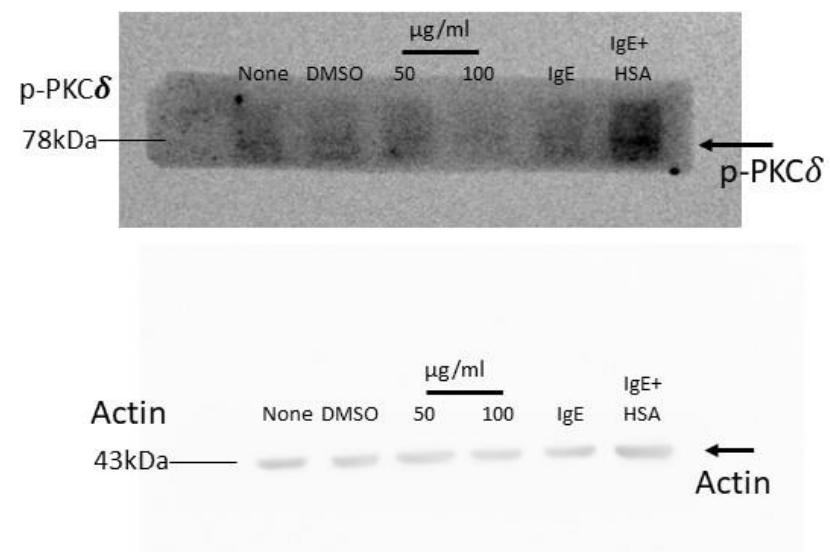
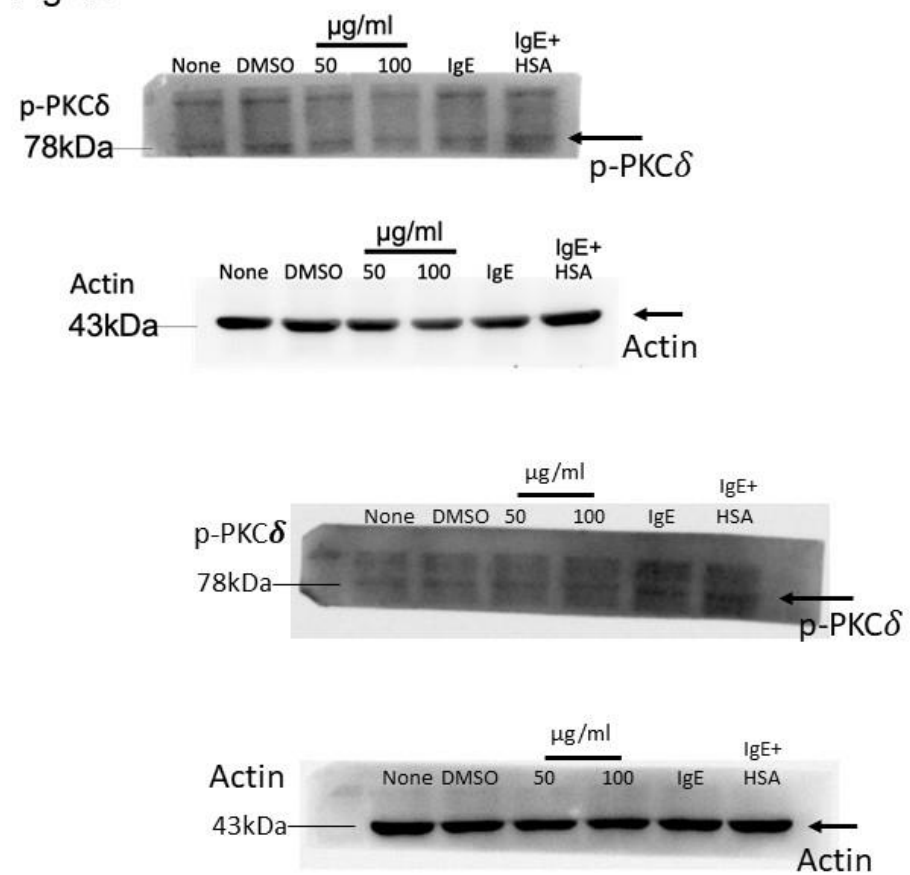


Fig 4D

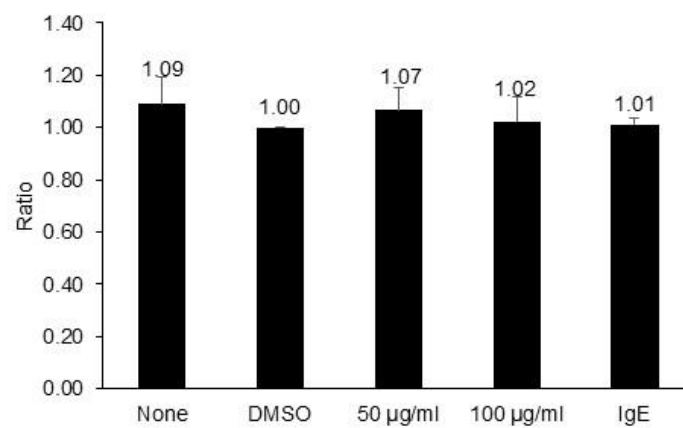
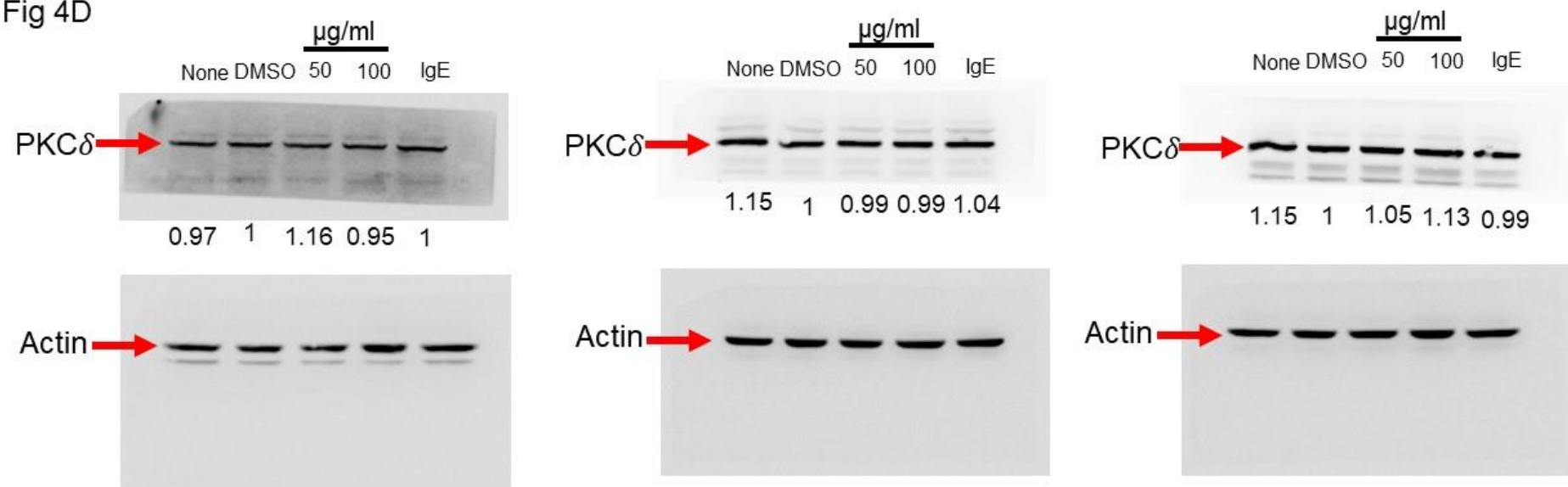


Fig 4E

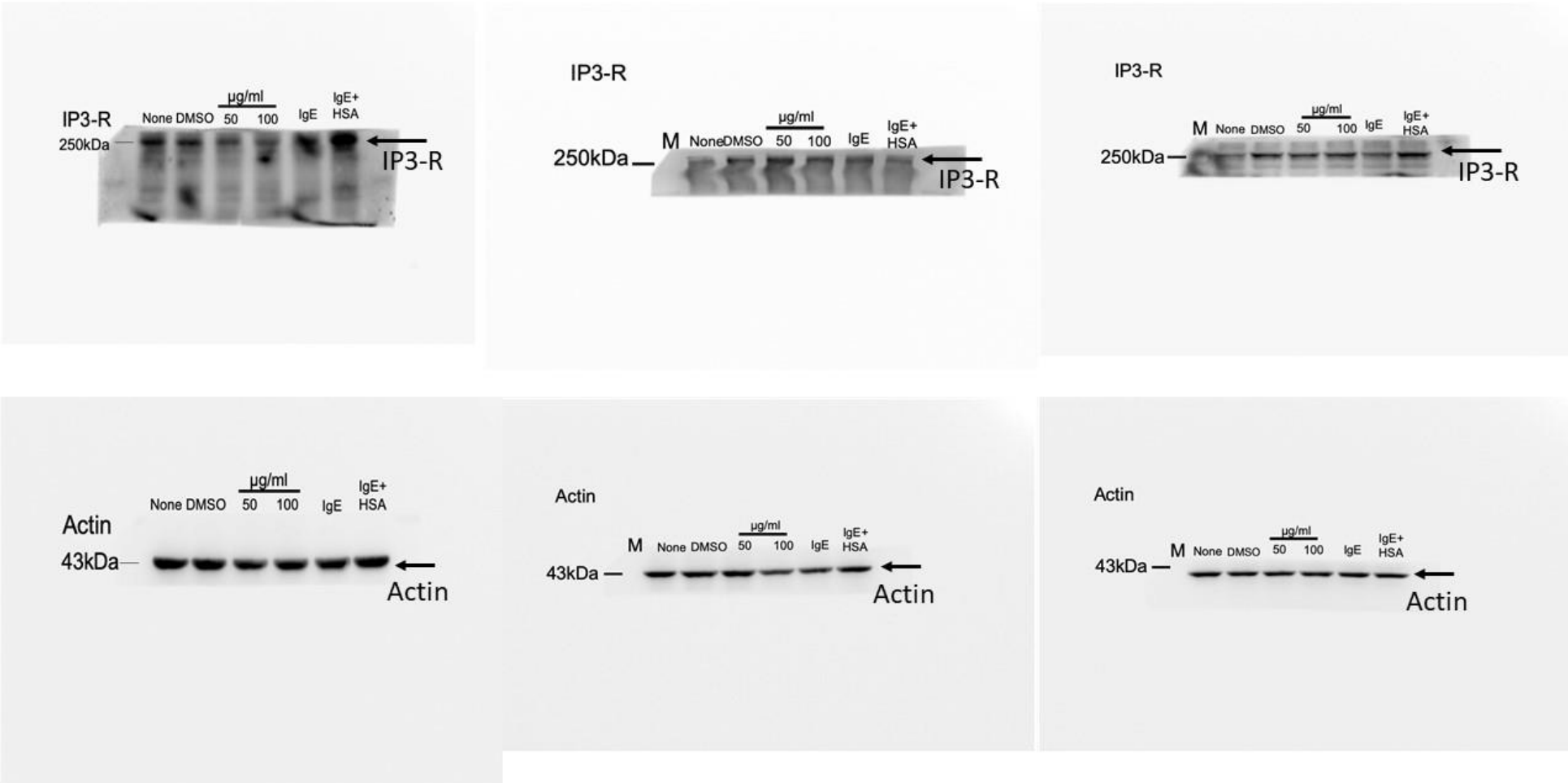


Fig 4F

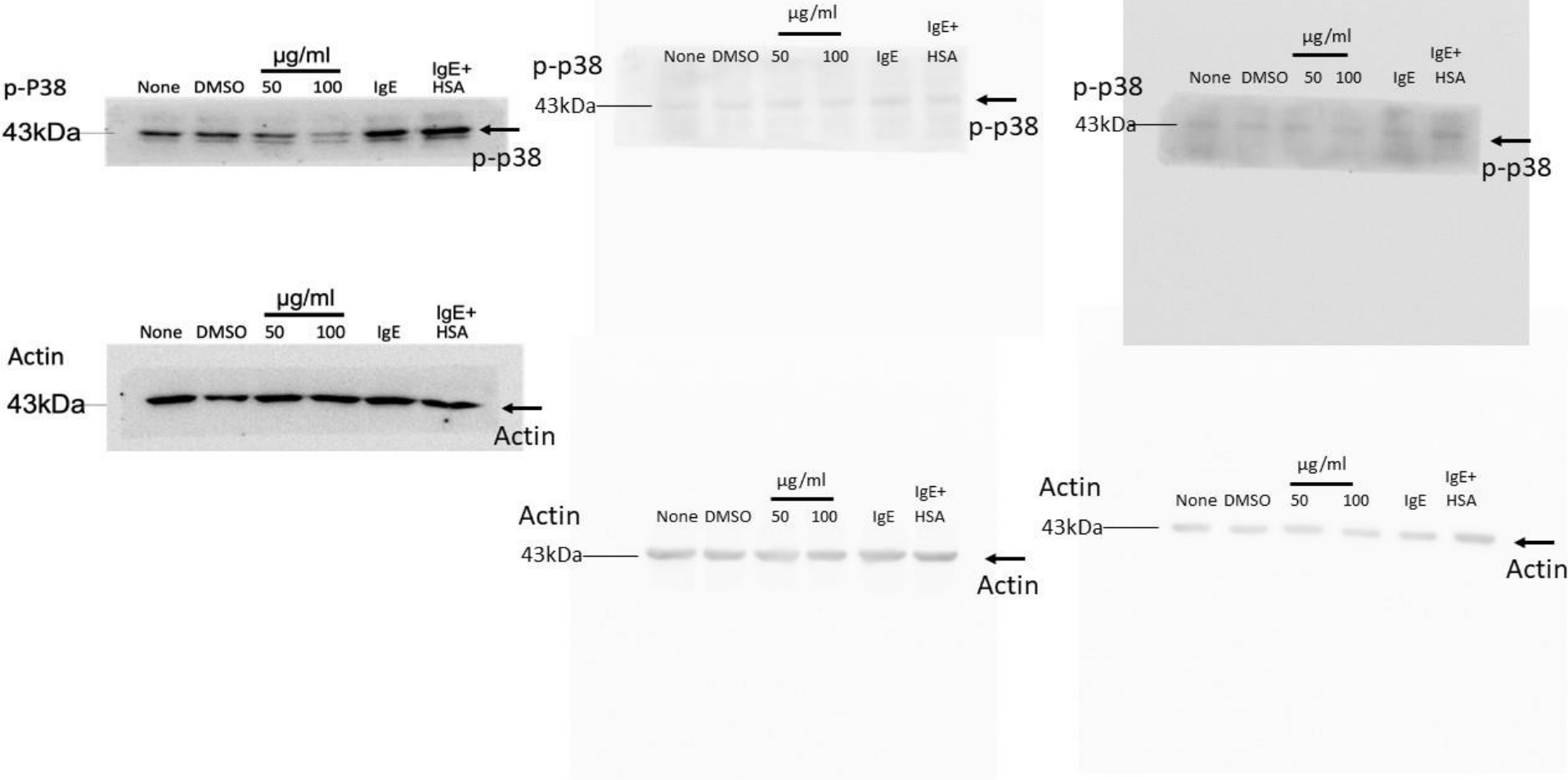


Fig 4F

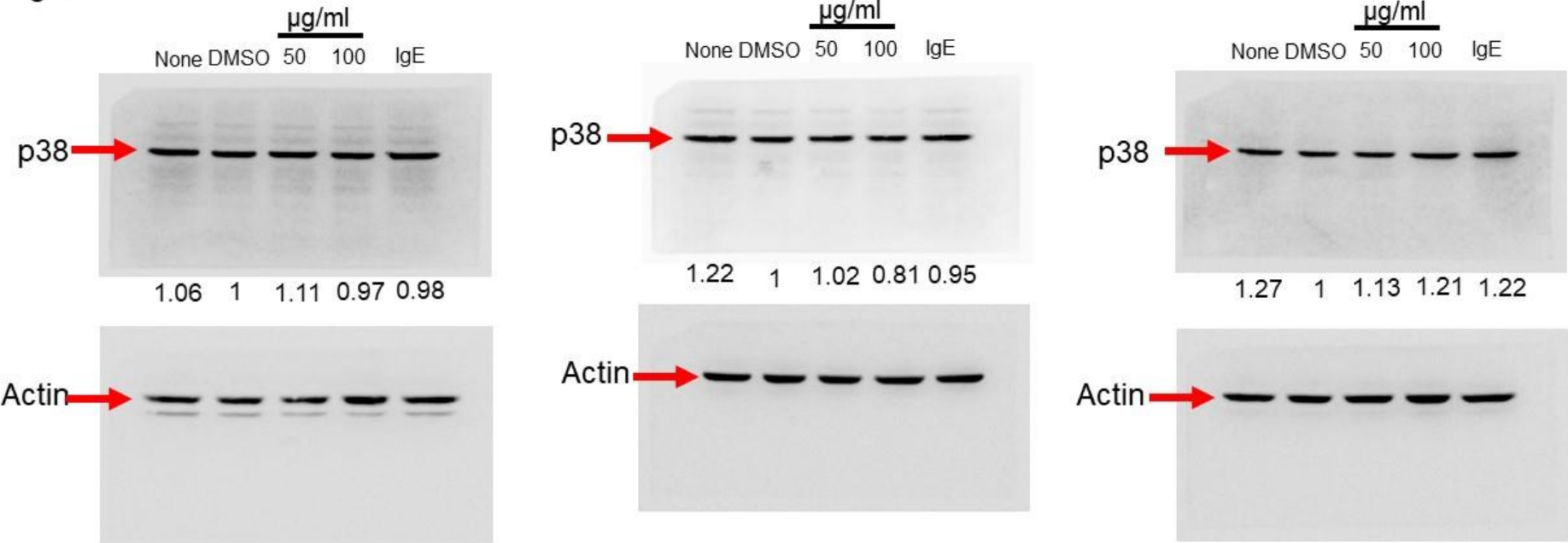
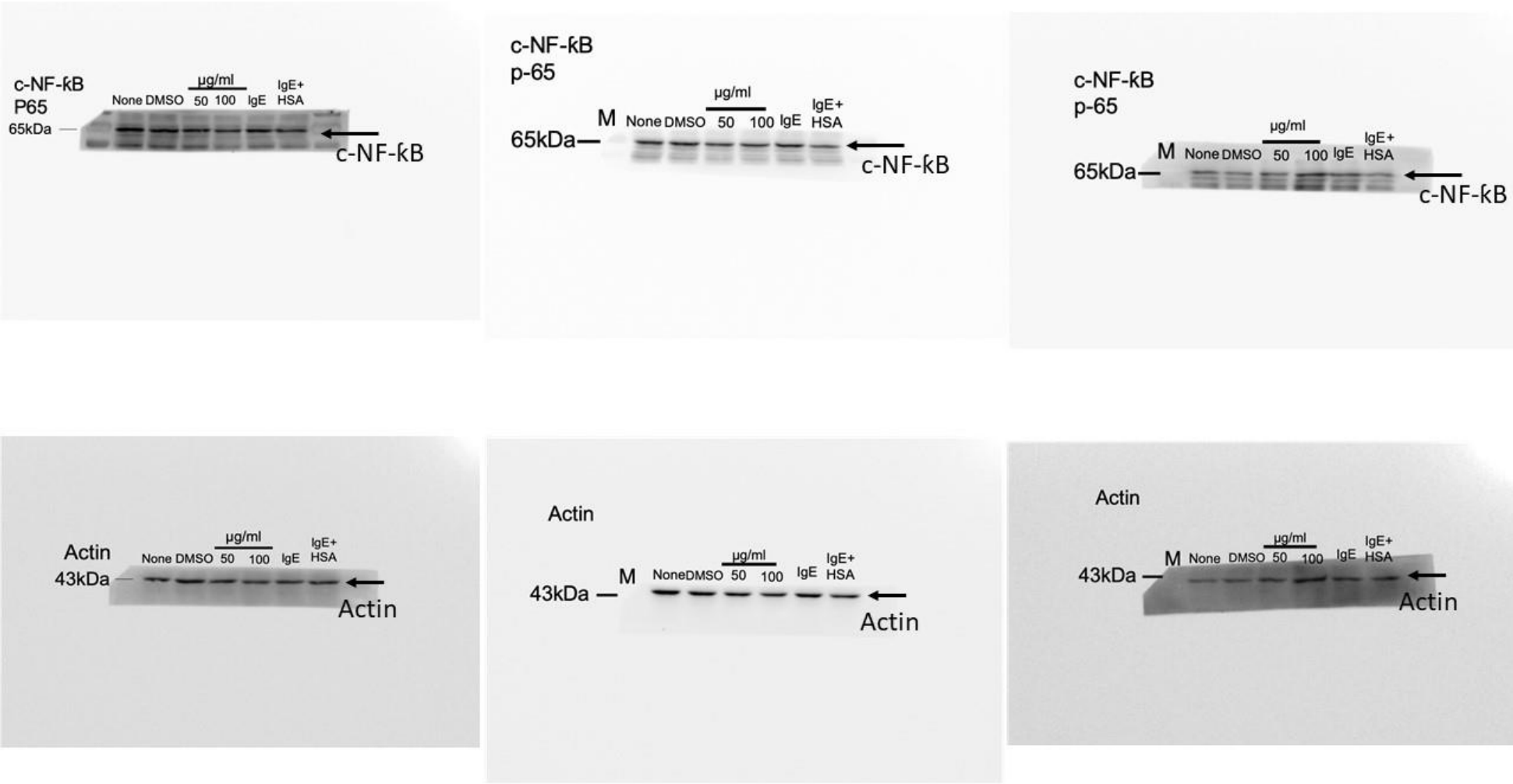


Fig 4G



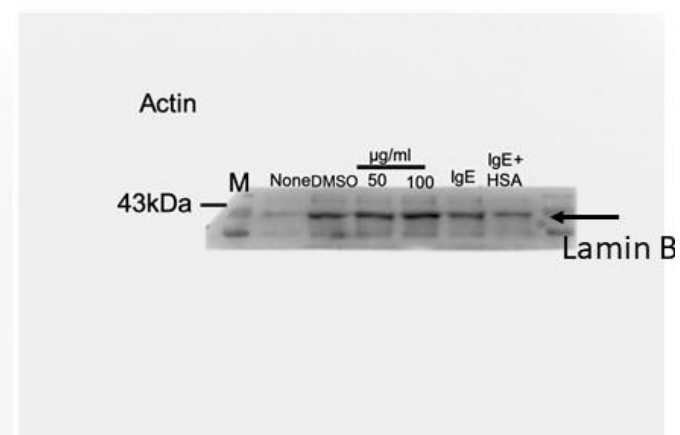
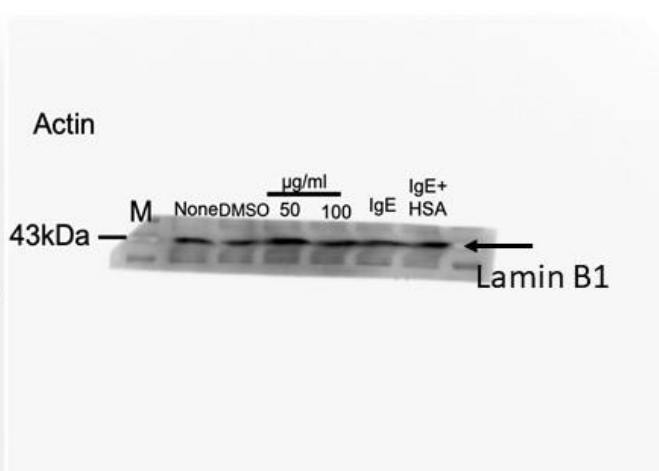
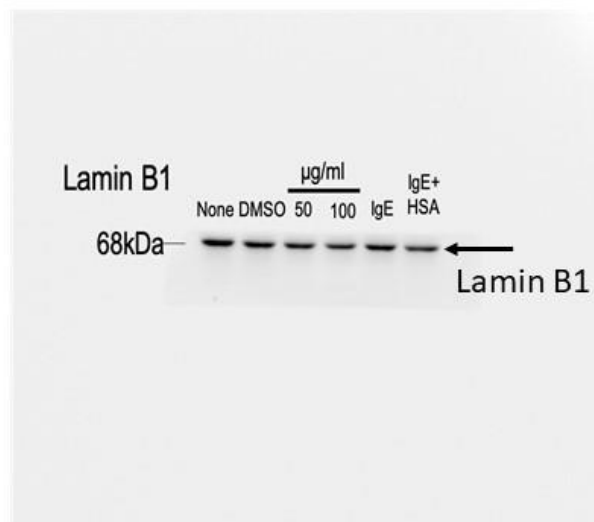
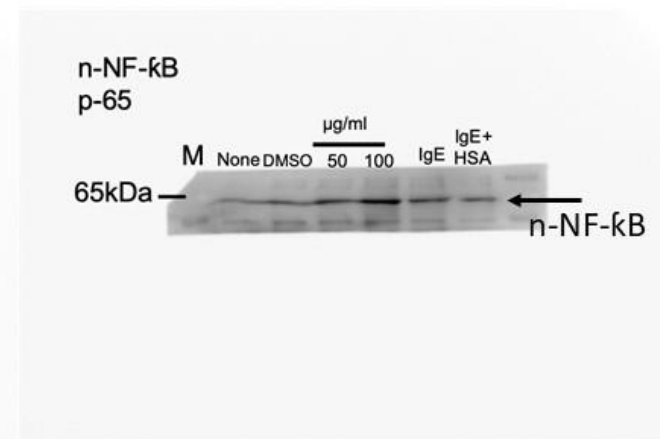
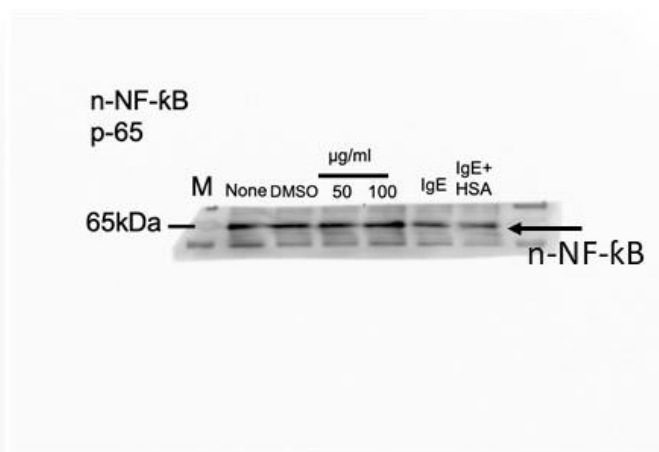
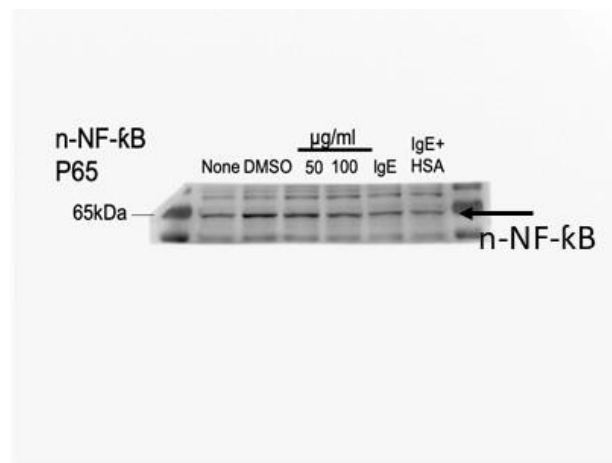
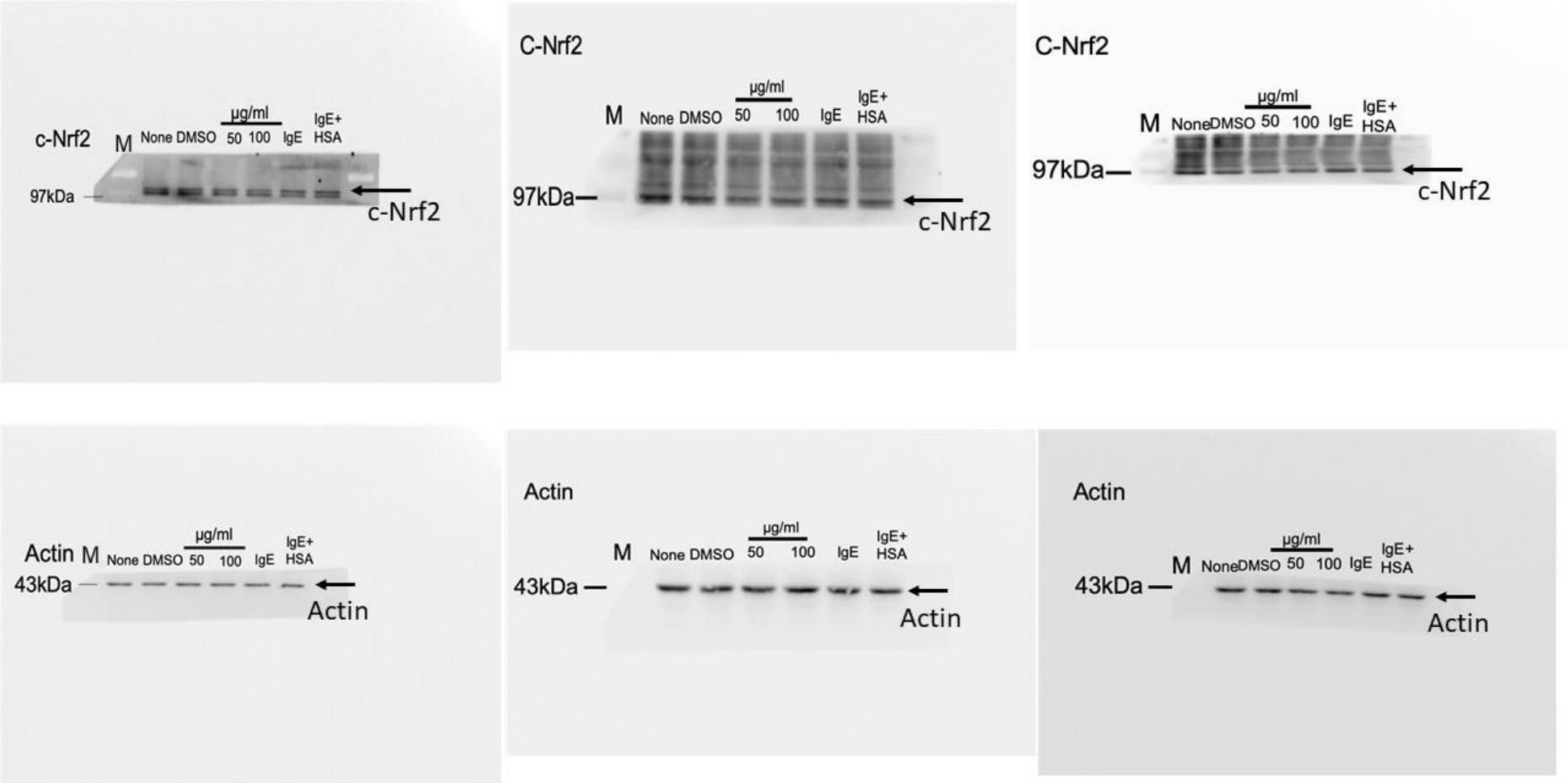


Fig 4H



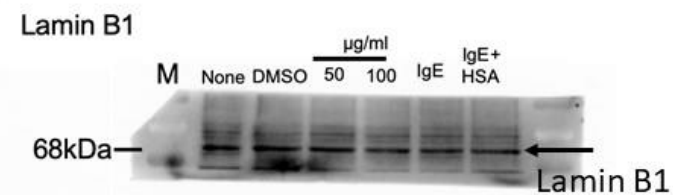
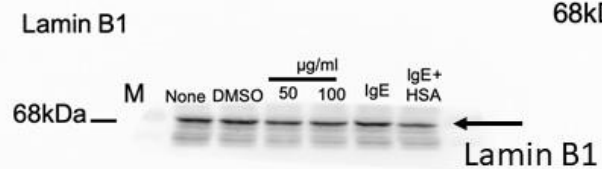
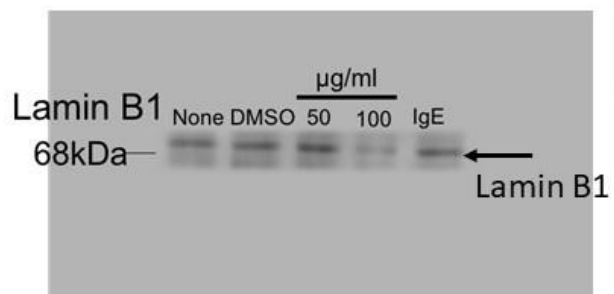
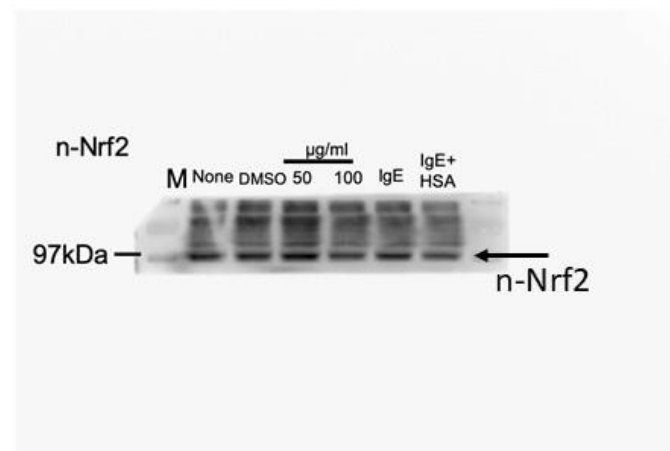
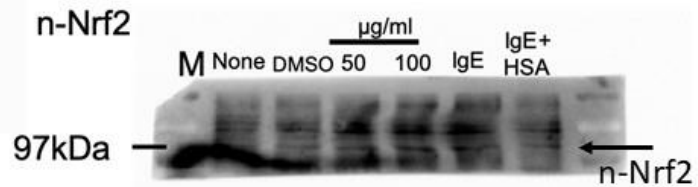
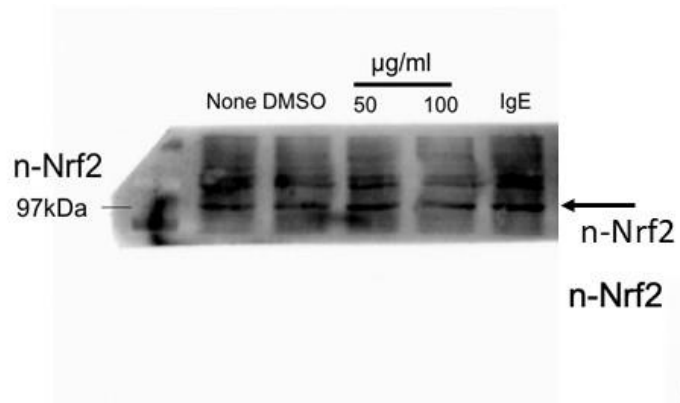


Fig 4I

