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Title

Inhibition of mast cell degranulation by melanin

Category: Inflammation and Immunopharmacology

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Abstract

Melanin is a dark naturally occurring pigment produced in nature and in many organisms. Although several reports have demonstrated applications for melanins in various therapeutic treatments, to date, no research has examined the anti-allergic effect of melanin. In this study, we for the first time found that solubilized or synthesized soluble melanin acts as a potent inhibitor of the degranulation of mast cells. We found that squid-ink-derived melanin significantly inhibited antigen-IgE-Fc ϵ RI-mediated degranulation of the mucosal mast cell line RBL-2H3. A homogenized melanin nanoparticle prepared by laser ablation also clearly suppressed antigen-induced mast cell degranulation. We also successfully solubilized synthetic melanin in a neutral biochemical buffer and found that it also significantly inhibited IgE-sensitized mast cells. The anti-degranulation activity of synthesized melanin was abolished in the melanin fraction below 50-kD molecular weight. All melanins used in this study did not exert significant cell death. Signal transduction analysis revealed that melanin suppressed antigen-triggered phosphorylation of signaling molecules as well as calcium influx. Transmission electron microscopy revealed that homogenized melanin nanoparticles partially attached to the cell surface and some nanoparticles were internalized to the cell. Flow cytometry revealed that the number of Fc ϵ RI-bound IgE molecules was decreased by melanin. Fluorescence recovery after photobleaching analysis indicated that melanin attenuated both plasma membrane and

cytoplasmic fluidity, implying that melanin increased their viscosities. *In vivo* experiments using passive systemic anaphylaxis (PSA) and passive cutaneous anaphylaxis (PCA) mouse models demonstrated that oral administration of melanin accelerated the recovery of decreased body temperature after antigen infection in PSA, and combination sensitization of IgE with melanin attenuated antigen-induced extravasation in PCA. These findings indicated that melanin exhibits preventative effects against IgE-mast cell-mediated anaphylaxis. This study provides the first evidence that homogenized melanin may be a potential therapeutic agent for diseases involving mast cells.

Keywords:

sepia, melanin, allergy, hypersensitivity, mast cell, degranulation

1. Introduction

Allergies are an immunological disease, and the number of patients with allergies in developed countries has increased in recent years [1-3]. Various over-the-counter medicines or prescription medicines (e.g., antihistamines, leukotriene receptor antagonists, corticosteroids, chemical mediator release inhibitors) are available to manage allergic symptoms. Although various anti-allergic pharmaceuticals have been developed and show low side effects, their effectiveness varies among patients, due to differences in single nucleotide polymorphisms in the histamine receptor or cytochrome P450 genes [4-6]. Therefore, it is important to develop safe and more effective drugs to broaden the range of patient options.

Mast cells and basophils play essential roles in the pathogenesis of type I hypersensitivity reactions such as allergic rhinitis (hay fever), atopic dermatitis, asthma and some food allergies [7]. Both mast and basophils express the Fc epsilon receptor I (FcεRI), a high-affinity receptor for the antigen-specific immunoglobulin E (IgE) [8]. Allergic reactions are induced when an allergen (or antigen) cross-links with IgE, which has bound to FcεRI on mast cells, leading to the secretion of inflammatory mediators [9, 10]. The process of releasing these mediators from the secretory granules within mast cells is known as degranulation. Since the inhibition of mast cell activation or degranulation contributes to the regulation of various IgE-mediated hypersensitivities, mast cells are one of the ideal targets for exploring anti-allergic agents. Rat basophilic leukemia (RBL)-2H3 cells are mucosal mast cells that have been used for studies of degranulation, intracellular Ca^{2+} response and signal transduction induced by stimulation with antigens or calcium ionophore [11, 12].

Melanin is a polymer compound that is biosynthesized by the oxidative polymerization

of tyrosine, and its molecular weight is not fixed [13]. Organic melanins are naturally occurring pigments present in most organisms, including bacteria, fungi, plants and animals. Melanins are commonly divided into four main types: eumelanin, pheomelanin, neuromelanin and allomelanin. Eumelanin (brown-black) and pheomelanin (yellow-red) are responsible for the coloration of skin, hair and eye. Neuromelanin (dark black) is found in the *substantia nigra* neurons of the human brain and inner ear of human, whereas allomelanin (brown-black) is present in plants and micro-organisms. These melanins serve a variety of functions that vary among different organisms. In humans, melanins play a critical role in protecting the skin against ultraviolet (UV) light [14]. In plants, melanins act as cell wall strengtheners [15], while in microbes, they act as protective agents against environmental stresses such as antibiotics or act as a virulence factor in bacteria by protecting bacteria from several host defense mechanisms [16, 17]. Cephalopod ink contains high concentrations of eumelanin and is used as a smokescreen or a decoy to escape from predators [18, 19]. Since melanins play diverse biological roles, these compounds have been explored for use in various medical, cosmetic and pharmacological applications. However, since melanin is usually a highly insoluble substance that is only dissolved in a strong alkaline solution, biochemical tests using melanin have not been attempted thus far.

Several papers have investigated the possibility of using melanin for therapeutic treatments. Montefiori et al. found that soluble melanin synthesized from L-tyrosine or L-dopa inhibited replication of HIV [20]. Mohaghehpour et al. demonstrated that synthetic soluble melanin suppressed lipopolysaccharide-induced TNF- α , IL-1 β , IL-6 and IL-10 in human peripheral blood monocytes [21]. Huang et al. reported that melanin extracted from black tea has antivenin activity in mice [22]. Furthermore, Fan et al.

proposed the use of water-soluble melanin nanoparticles as a novel multimodality imaging nanoplatform [23]. To date, however, no studies have examined the potential anti-allergic effects of melanin.

In this study, we evaluated the potential efficacy of soluble or solubilized melanin as an anti-degranulation agent and the underlying molecular mechanisms. We also examined the cytotoxicity of melanin in RBL-2H3 cells and the potential utility as an anti-allergic agent using a passive anaphylaxis model.

2. Materials and methods

2.1 Chemicals and antibodies

Synthetic melanin was purchased from MP Biomedicals (Solon, OH). Anti-DNP-IgE (monoclonal anti-dinitrophenyl antibody produced in mouse, IgE isotype, SPE-7, #D8406), anti-rabbit IgG (produced in goat, horseradish peroxidase-conjugated, #A6154), DNP-HSA (dinitrophenyl-human serum albumin), 3,4-dihydroxy-L-phenylalanine (L-dopa), *p*-nitrophenyl-N-acetyl- β -D-glucosaminide and calcium ionophore A23187 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho(Tyr⁷⁸³)-PLC γ 1 (#2821), anti-phospho(Tyr⁷⁵⁹)-PLC- γ 2 (#3874), anti-phospho(Thr²⁰²/Tyr²⁰⁴)-ERK1/2 (#4370), anti-phospho(Thr^{638/641})-PKC α/β II (#9375) antibodies and secondary horseradish peroxidase-conjugated antibody (#7074) for western blotting were obtained from Cell Signaling (Danvers, MA, USA). Anti-phospho(Tyr³⁴⁸)-Syk (#CSB-PA000697) antibody was obtained from CUSABIO TECHNOLOGY (Houston, TX, USA). CellBrite™ Cytoplasmic Membrane Staining Kit (NeuroDiO) was obtained from Biotium (Hayward, CA, USA). All reagents for biochemical buffers and ketotifen fumarate were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2 Cell lines and culture

The RBL-2H3 rat cell line derived from basophilic leukemia cells was provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The cells were maintained in Eagle's minimum essential medium (EMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C and

5% CO₂ in a humidified atmosphere. Cells from passages 3–10 from their arrival were used for experiments.

2.3 Mice

Twelve-week-old male SPF BALB/c mice (for the passive systemic anaphylaxis model; n = 18) with body weights ranging from 26 to 32 g or seven-week-old female SPF BALB/c mice (for the passive cutaneous anaphylaxis model; n = 18) with body weights ranging from 17 to 22 g were purchased from Charles River Laboratories Inc., Japan, and maintained in a humid environment (50±10%) under a 12-h light/dark cycle in a temperature-controlled (22±2°C) pathogen-free animal facility. Three to five mice were housed in each cage; mice were fed a standard chow diet (CRF-1; Oriental Yeast Co., Ltd., Japan) and provided with tap water filtered by a polypropylene string wound cartridge (Organo Co., Ltd., Japan). The mice were allowed to acclimate for 1 to 2 weeks before the experiments. The Animal Care and Use Committee in Chubu University approved this study (approval no. 2910043 and 3010039).

2.4 Preparation of dispersed-melanin

Commercially obtained melanin was suspended in 5 mL PBS at 0.1% (w/v). The completely homogeneously dispersed-melanin was produced by pulse laser irradiation (wavelength: 1064 nm; full width at half maximum: 5 ns, 1.2 J/cm² pulse, 10 Hz, 2.5 mmφ, 60 min), supplied by HAMAMATSU Nano Technology Inc., Japan.

2.5 Preparation of squid-ink-derived melanin

An ink sac from a squid (*Todarodes pacificus*) was surgically removed and the ink was

carefully extracted. For crude squid ink preparation, the squid ink was weighed and PBS was added to yield a 50% solution. The crude solution was heated for 10 min at 100°C and centrifuged at $10,000 \times g$ for 10 min, and then the supernatant was collected. The absorbance was read at 405 nm, and melanin content was determined by comparison with a standard curve using the dispersed-melanin. The squid-ink-derived melanin was finally adjusted to 2 mg/ml by adding PBS. Homogenized Sepia-melanoprotein was obtained from KANKIYOUSOUKEN (Hakodate, Japan). A dried squid ink sac from a cuttlefish (*Sepia esculenta*) was crushed and then digested with an alkaline protease (pH 7.0) at 50°C for 24 h. The enzyme additive was adjusted to 0.25% based on the dry weight of the sample. After digestion, the sample was subjected to ultrafiltration using 1,000 kD cut off membrane. The concentrated squid ink was digested with alkaline protease (pH 10) at 50°C for 24 h. The enzyme additive was adjusted to 10% based on the dry weight of the sample. After the second digestion, the sample was again subjected to ultrafiltration using 1,000 kD cut off membrane. The highly purified samples were monodispersed protein-bound melanin particles (Sepia-melanoprotein). To remove protein from melanoprotein, samples were treated with 1.25 N NaOH at 80°C for 24 h. The sample was neutralized with HCl, desalted by a Float-A-Lyzer G2 dialysis device (MWCO 20 kDa; Spectrum Laboratories, CA, USA) and lyophilized (deproteinized sepia-melanin).

2.6 Preparation of HEPES-dissolved melanin

Commercially obtained melanin was dissolved at 50 mg/ml in 500 mM HEPES-KOH (pH 7.2–7.8) and then filtered through 0.22 μm sterile syringe filters (Merck Millipore, MA, USA). For size fractionation, HEPES-dissolved melanin was fractionated by molecular size using a 50-kDa MWCO Amicon Ultra centrifugal ultrafilter (Merck

Millipore).

2.7 Chemical synthesis of water-soluble melanin

Water-soluble melanin was synthesized according to the previously disclosed protocol [24, 25], with slight modifications. Briefly, L-dopa was dissolved in 0.025 N NaOH and incubated for 3 days at room temperature with constant aeration. Melanin was precipitated by adding concentrated HCl. The moist precipitate was collected by centrifugation (1,000 × g, 5 min), dissolved in deionized water, and precipitated again with concentrated HCl. The melanin was purified this way a total of four times and then dissolved in 0.025 N NaOH. The solution was desalted by a Float-A-Lyzer G2 dialysis device (MWCO 20 kDa; Spectrum Laboratories) and lyophilized.

2.8 Cytotoxicity assay

RBL-2H3 cells (2×10^5) were transferred into 96 well plates and cultured for 24 h until confluent. Melanin samples or staurosporine were added and cultured for another 24 h. Cells were washed three times with PBS, and the viable cells were examined with a WST-8 assay using the Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's protocol.

2.9 β -Hexosaminidase secretion assay

Degranulation was determined by measuring the release of β -hexosaminidase. RBL-2H3 cells (2×10^5) were cultured in 96 well plates with DNP-specific IgE antibody (100 ng/ml) for 24 h. The IgE-sensitized cells were washed with PBS and pretreated with melanin at various concentrations (0, 8, 16, 31, 62, 125, 250, or 500 μ g/ml) in EMEM for 2 h at 37°C

in 5% CO₂. The cells were washed three times with pre-warmed Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 5.6 mM glucose, 12 mM NaHCO₃, 0.37 mM NaH₂PO₄·2H₂O, 25 mM HEPES, 0.49 mM MgCl₂, 0.1% BSA) and then stimulated with DNP-HSA (200 ng/ml) for 90 min in the cell culture incubator. To determine the total amount of β -hexosaminidase released, Tyrode's buffer containing 1% (v/v) Triton X-100 was added to three or four wells. Following stimulation, the supernatant of each well was transferred into new wells and incubated with an equal volume of substrate solution (1.29 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide in 150 mM Na₂HPO₄, 79.6 mM citric buffer) for 1 h at 37°C. The enzyme reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.7) and the reaction product was measured at 405 nm. The inhibitory effect of melanin on β -hexosaminidase secretion was compared with that of ketotifen fumarate (125–500 μ g/ml). Supernatant samples stimulated with IgE-antigen and not treated with melanin samples or ketotifen fumarate were used as a positive control of 100%.

2.10 Measurement of intracellular Ca²⁺

A fluorometric assay was used to determine the intracellular calcium levels according to the manufacturer's instructions (Calcium Kit Fura-2, Dojindo). RBL-2H3 cells were sensitized with 100 ng/ml of DNP-specific IgE antibody overnight. After washing with pre-warmed PBS three times, cells were treated with dispersed-melanin (62.5–250 μ g/ml) for 2 h. The cells were washed with pre-warmed PBS three times and then treated with loading buffer (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 13.8 mM glucose, 5 μ g/ml Fura-2 AM, 0.04% Pluronic F-127, 1.25 mM Probenecid, pH 7.4) for 1 h at 37 °C. After the Loading Buffer was removed from wells, cells were treated with pre-warmed Recording Medium (20 mM HEPES, 115 mM NaCl,

5,4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 13.8 mM glucose, 1.25 mM Probenecid, pH 7.4). Cells were set on an InCyt IM2™ microfluorescence imaging system (Intracellular Imaging Inc., Cincinnati, OH, USA) and the ratio of fluorescence was monitored with excitation at wavelengths of 340 nm and 380 nm with emission at 510 nm to measure the intracellular free Ca²⁺ concentration [26]. After 30 sec, the cells were challenged with DNP-HSA (200 ng/ml) for 4 min.

2.11 Western blotting

Western blotting was performed according to the previously described method [27]. Briefly, lysates of RBL-2H3 cells were separated by SDS-PAGE on an 8% polyacrylamide gel and proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore). The membrane was blocked with 5% nonfat milk or 2% BSA for 1 h at room temperature and then reacted with primary antibody (all antibodies used at 1:1000 dilution) for 18 h at 4°C, followed by reaction with the corresponding secondary horseradish peroxidase-conjugated antibody (all antibodies used at 1:1000 dilution) for 1 h at room temperature. The signals were detected by Western Lightning Plus-ECL (PerkinElmer, MA, USA). Chemiluminescence was captured using a cooled CCD Light-Capture camera system and analyzed using CS Analyzer software version 2.0 (ATTO, Tokyo, Japan).

2.12 Scanning electron microscope (SEM) and transmission electron microscopic (TEM) analysis

SEM analysis for measuring particle size of melanin was performed by HAMAMATSU Nano Technology Inc. (Hamamatsu, Japan). Briefly, the melanin sample was filled in a

glass tube (outer ϕ 40 μm , inner ϕ 24 μm). DC 2200 V was applied to the sample, and nanoparticles in the sample were dispersedly coated on the observation substrate made of silicon. The samples were observed using a Field Emission Scanning Electron Microscope (S-4200, Hitachi High-Technologies Corporation, Japan). For TEM analysis, RBL-2H3 cells were fixed with a fixative solution A containing 2% (vol/vol) paraformaldehyde, 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4 °C. Cells were then immersed in another fixative solution B containing 2% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. The cells were then fixed with a fixative solution C containing 2% (vol/vol) osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 4°C for 3 h. After rinsing the fixative solution from the cells, the cells were then dehydrated with a graded series of ethanol and embedded in epoxy resin (Quetol 812; Nisshin EM). Ultrathin sections (thickness: 70 nm) were observed under an electron microscope at 80 kV (JEM-1400Plus; JEOL, Japan).

2.13 Measurement of IgE-bound Fc ϵ RI

RBL-2H3 cells were sensitized with 100 ng/ml of DNP-specific IgE antibody overnight. After washing with pre-warmed PBS three times, cells were incubated with HEPES-melanin (0.125–0.5 mg/ml) for 2 h at 37°C in a CO₂ incubator. The cells were washed with PBS, removed from the dish by 0.25% trypsin and treated with Alexa Fluor 488 anti-mouse IgE antibody for 30 min. All cells were passed through a 70 mm cell strainer (BD Biosciences, USA) and subjected to flow cytometric analysis (FACS Canto™, BD Biosciences).

2.14. Fluorescence recovery after photobleaching (FRAP) analysis

For cytoplasmic fluidity analysis, the pmaxGFP plasmid vector (Lonza, NJ, USA) was transiently transfected into RBL-2H3 cells using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instruction. RBL-2H3 cells were seeded in a glass-bottom dish at a density of 4×10^4 cells/ml in EMEM. The following day, cells were pretreated with or without HEPES-melanin (0.125 and 0.5 mg/ml) for 2 h at 37°C in a CO₂ incubator. For plasma membrane fluidity analysis, cells were washed with pre-warmed PBS three times and then stained with the CellBrite™ Cytoplasmic Membrane Staining Kit, a NeuroDiO cell labeling solution, for 45 min at 37°C in a CO₂ incubator according to the manufacturer's instruction. GFP-transfected or NeuroDiO-stained cells were washed with pre-warmed Tyrode's buffer containing 0.5% BSA for 10 min three times and then examined by confocal laser scanning microscopy (FLUOVIEW FV1000, Olympus, Japan). For FRAP experiments, the fluorescence emission was bleached in a region of interest (ROI) with the 405 nm laser at 100% power (for GFP) or the 488 nm laser at 3.0% power (for Neuro DiO). Fluorescence recovery was followed for 34.9 (for GFP) or 51.9 sec (for NeuroDiO), exciting the sample with the 488 nm laser at 1.0% power (for both GFP and NeuroDiO). The time-sequential fluorescence intensity of each cell (n=8) was analyzed by Olympus FV10-ASW software (ver. 4.02).

2.15 Passive systemic anaphylaxis (PSA)

Eighteen male BALB/c mice (14 weeks old) were divided into three groups: the control group, positive control group and Dopa-melanin group (n = 6/group). Synthetic Dopa-melanin in water (100 mg/kg) was administered intragastrically to mice at 12 using a gastric sonde at every other day for ten days. PSA was induced based on the previously described method [28]. Briefly, mice were sensitized by intraperitoneal injection with 20

mg of DNP-specific IgE in 100 μ l PBS. After 24 h, the baseline temperature of mice was measured using a rectal probe (thermistor thermometer, Natsume Seisakusho, Japan) and then mice were immediately challenged by intraperitoneal injection with 1 mg of DNP-HSA in 100 μ l PBS. The body temperatures of mice were measured every 10 min for 2 h.

2.16 Passive cutaneous anaphylaxis reactions (PCA)

Mice were intradermally sensitized with 20 ng (20 μ l) of DNP-specific IgE antibody (1 μ g/ml), Dopa-melanin-containing DNP-specific IgE antibody, or vehicle (EMEM) only in the right ear pinna. After 48 h, aliquots (0.2 ml) of antigen solution (1 mg/ml DNP-HSA) mixed with 1% Evans blue dye (Wako Pure Chemical Industries, Osaka, Japan) were injected intravenously (i.v.). Mice were euthanized by cervical dislocation 30 min after antigen injection, and their ears were removed. The ear tissues were placed in 0.5 ml of 1.0 N KOH and incubated at 37°C overnight. Then the samples were mixed with 2 ml of 0.2 M phosphoric acid and acetone (1:13, v/v) solution and centrifuged at $900 \times g$ for 10 min. The supernatants were collected and the amount of dye was estimated by measuring the absorbance at 620 nm.

2.17 Statistical analysis

The results are presented as mean $\pm$ standard deviation (SD) from at least three independent experiments. Differences between two groups were analyzed by using the unpaired two-tailed Student's *t*-test or Mann-Whitney's *U*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Inhibition of degranulation in RBL-2H3 cells by crude squid ink and melanin

Because squid ink is one of the most accessible natural sources of melanin (eumelanin), we initially examined the effects of melanin derived from squid (*T. pacificus*) on cell degranulation using the RBL-2H3 cell line as an *in vitro* model for degranulation studies. RBL-2H3 cells sensitized with the DNP-specific IgE antibody were treated with various concentrations of the crude squid ink (25, 50, 100, or 200 µg/ml, as melanin equivalent) for 2 h. Cells were then washed and stimulated with an antigen DNP-HSA to induce degranulation, as measured by β-hexosaminidase secretion assays. As shown in Fig. 1A, we found that the degranulation of cells was significantly ($*P < 0.05$) suppressed by the crude squid ink in a dose-dependent manner (IC₅₀: 109 µg/ml).

Although the crude squid ink used in the experiment contained high concentrations of melanin, the crude ink also contained protein, amino acid, lipid, carbohydrates and many biogenic substances. To ascertain whether the melanin in the squid ink was the source of the inhibitory effect on cell degranulation, we next used commercial pure melanin that was chemically synthesized. Synthesized melanin is extremely insoluble in water or organic buffers. Therefore, we employed a laser ablation technique to prepare homogenized melanin nanoparticles. As shown in Fig. 1B, the commercially obtained melanin was insoluble in PBS before the laser treatment; however, the melanin solution was completely dispersed after the laser treatment. The diameter of the homogeneously dispersed-melanin was within 70–130 nm (Fig. 1C). We next used this laser-dispersed melanin of DNP-IgE-sensitized cells, followed by β-hexosaminidase secretion assays. Figure 1D showed that the dispersed-melanin clearly inhibited RBL-2H3 cell

degranulation in a dose-dependent manner (IC_{50} : 56.6 μ g/ml).

3.2 Solubilization of melanin in a biological buffer and anti-degranulation effects

The commercially obtained melanin is insoluble material. We next tested the potential solubility of the commercially obtained melanin in six well-known buffers that are frequently used for biochemical tests. To assess the solubility of samples, we placed the samples on a filter paper and visually compared diffusion. If the sample was not homogeneously dissolved in the buffer, the spot will form a multiple ring shape on the filter paper due to the presence of molecules with different degrees of polymerization. We found that the melanin was homogeneously dissolved only in the 500 mM HEPES-KOH buffer (pH 7.5) (Fig. 2A). Therefore, we next assessed the inhibitory effect of the melanin dissolved in HEPES buffer (hereinafter called “HEPES-melanin”) on DNP-HSA-induced degranulation of RBL-2H3 cells. Pretreatment of HEPES-melanin significantly ($*P < 0.05$, $**P < 0.01$) inhibited DNP-HSA-evoked degranulation of RBL-2H3 cells in a dose- and time-dependent manner (IC_{50} : 39.8 μ g/ml) (Fig. 2B, C).

The inhibitory effects of dispersed-melanin and HEPES-melanin were compared with that of ketotifen fumarate, a well-known stabilizer of mast cells [29]. Figure 2D shows that the anti-degranulation activities of both dispersed-melanin and HEPES-melanin in RBL-2H3 cells were comparable that of ketotifen at least at 125 μ g/ml. To examine whether the dispersed-melanin and HEPES-melanin exhibited cytotoxicity in RBL-2H3 cells, we next performed cell viability assays using the WST-8 assay kit. Although our previous assays involved pretreatment of dispersed-melanin or HEPES-melanin for 2 h, here we treated cells with dispersed-melanin or HEPES-melanin for 24 h. As shown in Fig. 2E, both dispersed-melanin and HEPES-melanin did not affect cell viability at any

concentration, indicating that these agents were not cell death-inducing agents under at least 24 h treatment. These results suggest that the dose-dependent decrease of β -hexosaminidase release from RBL-2H3 cells was not due to cytotoxicity.

3.3 Inhibition of degranulation in RBL-2H3 cells by chemically synthesized melanin

Next, we investigated soluble-melanin that we chemically synthesized *in vitro*. In nature, melanin biosynthesis starts with either tyrosine or L-dopa as the precursor (Fig. 3A). As previously described [24], we choose L-dopa as the precursor for the soluble synthetic melanin. We successfully prepared homogeneous melanin (hereinafter called “Dopa-melanin”) dissolved in both 500 mM HEPES buffer and ultrapure water at 50 mg/ml. Although a uniform structure or molecular weight of the synthetic melanin has not been identified, since it is thought to be a mixture of complex polymers, we show speculative structures of the synthetic melanin in Figure 3B, as reported previously [30]. We confirmed that 2 h of pretreatment of Dopa-melanin, whether dissolved in HEPES or water, potently and significantly ($\dagger P < 0.005$) inhibited DNP-HSA-induced degranulation in RBL-2H3 cells in a dose-dependent manner (IC_{50} : 74.6 μ g/ml, 60.2 μ g/ml, respectively) (Fig. 3C). Intriguingly, we also found that a melanin-like particle that was synthesized from L-dopamine also inhibited degranulation in RBL-2H3 cells at almost the same concentrations (data not shown). Figure 3D shows that the Dopa-melanin did not decrease cell viability at the concentrations that inhibit degranulation. These data suggest that chemically synthesized Dopa-melanin is a strong inhibitor of antigen-IgE-mediated degranulation of mast cells without causing significant cell damage.

Next, to investigate whether the size of the melanin molecule influences the anti-degranulation effect, we performed size fractionation using an ultrafiltration membrane.

We prepared three samples for evaluation: a control (no fractionation), a sample with molecules over 50 kD and a sample with molecules under 50 kD, all dissolved in HEPES buffer. As shown in Fig. 3E, the sample with molecules over 50 kD showed almost the same effect on inhibition of IgE-mediated mast cells activation compared with controls. In contrast, the sample with molecules under 50 kD completely lost the inhibitory activity.

To further investigate the differences of the melanin samples under and over 50 kD, we examined the samples under bright field microscopy. When cells were pretreated with HEPES-melanin (125 μ M, 2 h) over 50 kD, cells were clearly stained by the melanin, whereas cells treated with melanin under 50 kD melanin at the same concentration showed no staining and appeared similar to untreated controls (Fig. 3F). This result indicates that melanin over 50 kD can interact with cells to exert inhibitory effects, while melanin under 50 kD has little, if any, effect on mast cells. Taken together, these results suggest that the size of the molecule in HEPES-dissolved melanin that inhibits IgE-mediated mast cell activation is over 50 kD.

3.4 Pretreatment effects of purified sepia-derived melanin on degranulation in RBL-2H3 cells

Our results thus far have shown that pure melanin strongly suppressed antigen-IgE-mediated degranulation in RBL-2H3 cells. Next, we examined whether melanin from the cuttlefish *Sepia esculenta* could also inhibit mast cell degranulation. We obtained a highly homogeneous and purified protein-bound melanin that was concentrated by ultrafiltration over a 1,000 kD cutoff membrane from the ink sac of *S. officinalis*. This melanin was also dissolved in 500 mM HEPES buffer (hereinafter called “sepia-melanoprotein”). Interestingly, the sepia-melanoprotein was not able to inhibit antigen-IgE-mediated

degranulation in RBL-2H3 cells at any concentration (Fig. 4). In comparison, the deproteinized sepia-melanin significantly ($P < 0.005$) inhibited mast cell degranulation in a dose-dependent manner. These results suggest that purified protein-bound melanin cannot inhibit degranulation of RBL-2H3 cells and that melanin is bona fide mast cell suppressor.

3.5 Analysis of signal molecules activated by antigen-IgE stimulation in melanin-treated RBL-2H3 cells

Our results have demonstrated that all melanin solutions examined in this study (dispersed-melanin, HEPES-melanin, Dopa-melanin and squid-ink-derived melanin) significantly inhibited Fcε-RI-mediated degranulation of mast cells. Since these melanin solutions exhibited similar or identical functions in our assays, we selected commercially obtained melanin (dispersed-melanin and HEPES-melanin) for further *in vitro* analysis.

FcεRI signaling has been reviewed in detail by Sibilano *et al* [31]. FcεRI-mediated activation in mast cells causes activation of Src family kinases (Lyn, Syk), phospholipase C gamma (PLCγ), PI3K, protein kinase C (PKC), nonselective Ca²⁺ channels, members of the MAPK family, and transcription factors including NF-κB [32-36]. To examine the inhibitory mechanism of melanin on IgE-mediated degranulation, we next investigated the activation of intracellular signaling molecules in RBL-2H3 cells.

The antigen-induced phosphorylation of Syk (Y³⁴⁸), PI3K(Y^{467/199}), PLC-γ1 (Y⁷⁸³), PLC-γ2 (Y⁷⁵⁹) and ERK (Thr²⁰²/Tyr²⁰⁴) were suppressed by the pretreatment of HEPES-melanin in a dose-dependent manner (Fig. 5A). Phosphorylation levels of PKCα/β II (Thr^{638/641}) increased after antigen stimulation, whereas pretreatment of HEPES-melanin

gradually decreased basal levels of phosphorylation after antigen stimulation (Fig. 5B). In the absence of cofactors such as Ca^{2+} and diacylglycerol and activators such as phorbol esters (TPA, PMA), it is known that these bind to the substrate-binding sites of the catalytic domain to suppress activation [37]. This result indicated that the signal for PKC activation is suppressed to below the basal level. Transient ERK phosphorylation by antigen stimulation was significantly ($*P < 0.05$) attenuated by pretreatment with HEPES-melanin. We also examined activation of NF- κ B, a key transcription factor for cytokine expression. Pretreatment with HEPES-melanin significantly inhibited the phosphorylation of NF- κ B (p65/RelA) in a dose-dependent manner (data not shown). These results suggest that melanin suppresses at or immediately after the antigen binding stage.

3.6 Effect of melanin on intracellular calcium signaling

PLC- γ hydrates phosphatidylinositol-4, 5-bisphosphate to form membrane-bound diacylglycerol (DAG) and soluble inositol-1, 4, 5-trisphosphate (IP_3), which are second messengers. DAG kinases then catalyze the degradation of DAG to phosphatidic acid, which is necessary for mast cell degranulation [38-40]. The binding of IP_3 to its receptor causes the transient release of Ca^{2+} from endoplasmic reticulum stores. The massive Ca^{2+} influx is essential for the activation of NFAT, AP-1 and NF- κ B, which are crucial for the transcription of cytokines. Together these studies suggest that melanin may influence Ca^{2+} signaling. Therefore, we next investigated the effect of melanin on the intracellular Ca^{2+} levels in activated RBL-2H3 cells using Fura-2 AM, a calcium-specific fluorescent probe. The results showed that the release of Ca^{2+} from intracellular stores was effectively suppressed by pretreatment with dispersed-melanin in a dose-dependent manner (Fig. 6A).

These results led us to further analyze whether melanin can inhibit calcium ionophore-induced degranulation. The calcium ionophore A23187 is a potent inducer of mast cell degranulation that acts by increasing intracellular Ca^{2+} concentrations [41]. Intriguingly, HEPES-melanin significantly ($*P < 0.05$, $\ddagger P < 0.005$) inhibited A23187-induced degranulation in a dose-dependent manner although IC_{50} value was approximately ten times higher than melanin samples (IC_{50} : 950.8 $\mu\text{g/ml}$) (Fig. 6B). These results suggest that melanin may exert an influence on the intracellular dynamics and cytoplasmic fluidity to stabilize cells, thereby inhibiting Fc ϵ RI-mediated signaling as well as calcium ion channel-mediated cell signaling.

3.7 Incorporation of melanin in RBL-2H3 cells

The soluble melanin-treated RBL-2H3 cells gradually turned black according to the length of the treatment time (data not shown). In addition, the melanin could not be easily washed from cells by rinsing with buffer. Visual observation suggested that cells were completely covered with melanin, resulting in inhibition of antigen and Fc ϵ RI-bound IgE. To investigate this assumption, we performed transmission electron microscopic (TEM) analysis to observe the soluble melanin-treated cells. As shown Fig. 7A, the melanin bound to the cell on only a part of the cell surface; the cells were not entirely covered with melanin. Intriguingly, a part of the melanin was incorporated into the cell by endocytosis. In accordance with this observation, cytometric analysis demonstrated that Fc ϵ RI-bound IgE level on the cell surface was significantly ($**P < 0.01$) decreased by soluble melanin treatment in a dose-dependent manner (Fig. 7B).

Next, to examine whether melanin directly interacts with IgE, we performed a pre-absorption test by mixing melanin with IgE. We first prepared a solution of commercially

obtained melanin solution in EMEM, in which the melanin did not fully dissolve because the solution was not treated with sonication or laser-desorption. Next, DNP-specific IgE antibody was added and the solution was incubated for 2 h at 37°C. The solution was centrifuged at $10,000 \times g$, and almost all of the melanin particles were precipitated and removed. The supernatant containing melanin-treated IgE was used for cell sensitization of RBL-2H3 cells. Degranulation was clearly induced in cells exposed to melanin-treated IgE as well as control IgE (Fig. 7C), indicating that melanin did not interact with the IgE antibody.

Together these results suggest that incorporation of soluble melanin in cells by endocytosis independent of an interaction with IgE might be one of the mechanisms of mast cell inactivation.

3.8 Reduction of cytoplasmic and plasma membrane fluidity by melanin

Because onset of signal transduction and intracellular signaling is tightly associated with molecular interactions in the plasma membrane and cytosol, respectively, a decrease in the plasma membrane and/or cytoplasmic fluidity would confer attenuation of receptor-ligand signaling. Therefore, we next examined whether melanin exerted an influence on cytoplasmic and/or membrane fluidity. FRAP analysis can be used to determine the cytoplasm fluidity of cells in which a noninvasive probe such as green fluorescence protein (GFP) is expressed or the mobility of a fluorescence-labeled membrane by analyzing the recovery of the fluorescent signal in a specific ROI after photobleaching [42, 43]. To analyze cytoplasmic fluidity, GFP was transiently expressed in RBL-2H3 cells. Although transfection efficiency was low (about 20%), GFP was expressed uniformly in transfected cells. For plasma membrane fluidity analysis, NeuroDiO reagent,

an improved cytoplasmic membrane labeling reagent based on DiO (3,3'-dioctadecyloxacarbocyanine perchlorate), was used to specifically label the cytoplasmic membrane [44]. The fluorescence signals in HEPES-melanin treated or untreated cells were traced before and after FRAP. The fluorescence intensity of the ROI in GFP-expressing control cells was quickly recovered after photobleaching, whereas the intensities of the ROI in HEPES-melanin treated cells were significantly ($*P < 0.05$) lower than that of controls (Fig. 8A, B). Similarly, the fluorescence intensity of the ROI in NeuroDio-labeled control cells was gradually recovered after photobleaching, whereas the intensities of ROI in HEPES-melanin treated cells were also significantly ($**P < 0.01$) lower than controls (Fig. 8C, D). Fluorescence fading by photobleaching is not affected by cytoplasm or cell membrane fluidity, but the recovery of fluorescence is influenced by the fluidity of the cytoplasm or cell membrane. In both GFP and NeuroDiO bleaching, the degree of fluorescence loss after laser coagulation was small in cells pretreated with melanin compared with the negative control, which is probably due to a masking effect of melanin. Therefore, the relative comparison of the fluorescence value recovery was calculated as a value with the fluorescence value after photobleaching being 1. These results suggest that melanin attenuates both cytoplasm and plasma membrane fluidity, probably by increasing the viscosity of plasma membrane and cytoplasm, and may weaken any signal transduction that requires molecular associations at the plasma membrane and/or in the cytosol.

3.9 Effect of Dopa-melanin on the PSA and PCA mouse models

We next examined the anti-allergic effect of melanin using *in vivo* anaphylaxis models. To examine the effect of melanin on PSA and PCA mice, we used water-soluble Dopa-

melanin that was synthesized in-house. To first determine whether oral administration of this melanin would induce toxic effects in mice, Dopa-melanin was orally administered to Balb/c mice at a dose of 100 mg/kg every other day for 30 days (Fig. 9A). There was no difference in body weight compared with the control group, and thus we increased the concentration by five-fold (500 mg/kg) and treated the Dopa-melanin mice for an additional 30 days. There was no visible difference between the control group and experimental group, indicating that an oral dose of Dopa-melanin at least under 500 mg/kg was applicable for *in vivo* experiments using mice.

We next examined the effect of melanin *in vivo* using the mast cell-mediated PSA mouse model. Mice were first sensitized by DNP-specific IgE or vehicle. On the following day, 30 min before antigen injection, Dopa-melanin (500 mg/kg) or vehicle was orally administered to the sensitized mice. After antigen injection, the body temperature of the positive control group rapidly declined for 30 min, tended to recover once at 50 min, but continued to decline over 120 min (Fig. 9B). In contrast, in the experimental group preliminarily administered with Dopa-melanin, the body temperature did not decrease as much as the positive control group, and body temperature gradually recovered over 120 min. These results indicate that oral administration of Dopa-melanin has a prophylactic effect against IgE-mediated anaphylaxis.

To further confirm the inhibitory effect of melanin on mast cell activation *in vivo*, we elicited a FcεRI-mediated PCA reaction. Mice were intradermally sensitized with DNP-IgE, DNP-IgE admixed with Dopa-melanin, or vehicle only in the right ear pinna. Two days later, antigen solution containing 1% Evans blue dye was intravenously injected via the tail. After 30 min, the antigen-challenged ears of the IgE-sensitized mice turned completely blue by intravasation, whereas those sensitized with IgE with Dopa-melanin

turned partially blue. By contrast, only staining spots appeared on the ears of the control mice after challenge (Fig. 9C). Extravasation was quantified by measuring the amount of Evans blue dye in tissue biopsies from the IgE-treated ears. In the ears treated with IgE and Dopa-melanin, the quantity of dye was significantly ($*P < 0.05$) lower than that in the IgE-alone-treated ears (Fig. 9D). These results suggested that Dopa-melanin suppressed the mast cell-mediated PCA reaction *in vivo*.

4. Discussion

In this study, we demonstrated for the first time that squid-ink-derived and synthetic melanin had a potent inhibitory effect against antigen-IgE-Fc ϵ RI-mediated degranulation in RBL-2H3 cells without evident cytotoxicity (Fig. 1A, 1D, Fig. 2B-E, Fig. 3C, 3D). In mammalian skin, melanin functions as a protective biosynthetic component against sunlight or UV damage by forming melanin caps in suprabasal keratinocytes [45, 46]. Melanin is initially produced by melanocytes present in the epidermal basal layer and then accumulates in melanosome vesicles within the cells that are then passed to keratinocytes. After that, the keratinocytes are terminally differentiated to enucleated corneocytes, which are then continually eliminated through rubbing, skin washing or detergents. Due to the turnover of the skin, melanin does not remain in the dermal layer, and thus it is unlikely that melanin will affect cells in the dermal layer, including mast cells, under natural conditions. Therefore, there has never been any discussion on the interaction between melanin and mast cells.

Although synthetic melanin can be dissolved in water or PBS, natural melanin and commercial melanin are highly insoluble in almost all solvents, which is one reason why melanin has not been used for biological assays. Indeed, our results showed that a commercially available melanin did not dissolve in a variety of biological buffers. Squid ink is also known to be insoluble in both water and organic solvents, such as ethanol, hexane, acetone, benzene and chloroform [47].

Unexpectedly, we found that only HEPES buffer (500 mM, pH 7.4; note that the pH varied depending on production lot) was able to homogeneously dissolve the melanin at 50 mg/ml (Fig. 2A). However, the mechanism by which the melanin dissolves in this

solvent is unknown. In addition, we successfully prepared homogeneously dissolved melanin in PBS by a laser ablation technique (Fig. 1B, 1C), which is a more effective method to obtain a homogeneous solution compared with sonication. Because of the success of this technique, it was possible to accurately evaluate the effects of melanin *in vitro*. In this study, we confirmed that all melanin solutions, i.e., HEPES-dissolved melanin (commercially obtained), synthetic Dopa-melanin and squid-ink-derived melanin, significantly suppressed mast cell degranulation in the range of at least 31 to 500 $\mu\text{g/ml}$. Because the molecular weights of commercially available and natural melanin have not been clarified, in this study, the molecular weight of synthesized melanin was not defined (Fig. 3B). Based on our results showing that the HEPES-melanin fraction above 50 kD was responsible for inhibiting mast cell degranulation (Fig. 3E), this suggests that the 31–500 $\mu\text{g/ml}$ range corresponds to 0.6–10 μM . Our results in Fig. 3F showed that melanin under 50 kD does not interact with cells, indicating that melanin over 50 kD is necessary to attach or integrate to cells to exerting anti-degranulation effects.

The anti-degranulation effect of melanin was approximately equal to that of ketotifen fumarate, a commercially available medicine, without showing evident cytotoxicity (Fig. 2D, 2E). Because squid ink is used for food and safe for consumption, we presume that the ingestion or uptake of melanin may be safe for the human body. Consistent with our cytotoxicity assay results in RBL-2H3 cells, a previous report showed that synthetic melanin from L-dopa (Fig. 3A) showed no cytotoxicity to murine NIH/3T3 cells [48] and human HEP-2 cancer cells [49]. Furthermore, we confirmed here that oral administration of the synthetic Dopa-melanin of at least at 500 mg/ml to mice every other day over a 30-day period did not result in weight loss or other significant adverse effects (Fig 9A). We also found that although squid ink prepared from *T. pacificus* significantly inhibited IgE-

mediated degranulation in RBL-2H3 cells *in vitro*, the commercially obtained sepia-melanin from *Sepia officinalis* (Sigma-Aldrich, MO, USA) did not impact degranulation (data not shown). A plausible hypothesis is that because the sepia-melanin is bound with protein, the so-called “melanoprotein”, an important site in melanin that is responsible for cell binding or exerting inhibition of IgE-signaling may be covered by protein (Fig. 4). Although the crude squid ink might also contain melanoprotein, there may be a portion of melanin that is free from protein. This may explain the weaker anti-degranulation effect of squid ink compared with that of pure melanins (Fig. 1A). If this is the case, natural squid ink that has been sufficiently deproteinized may be a powerful degranulation inhibitor.

To investigate the mechanism underlying the process by which soluble melanin inhibits Fcε receptor-mediated degranulation of RBL-2H3 cells, we examined the phosphorylation status of several signaling molecules in mast cells. Pretreatment of the solubilized melanin appreciably inhibited the antigen-triggered phosphorylation of Syk, PI3K, PLC-γ1 and γ2, PKC-α/β II, p42/44 MAPK and PKCα/β II (Fig. 5A, B). In addition, pretreatment of the solubilized melanin also inhibited calcium ionophore-induced upregulation of the intracellular Ca²⁺ as well as degranulation in a dose-dependent manner (Fig. 6A, B). Syk, which interacts with immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domain of immune receptors including FcεRI [50], is the most upstream signaling molecule in FcεRI-triggered activation. Thus, our data indicate that the soluble melanin inhibits the degranulation just after binding of antigen to FcεRI-bound IgE antibody.

Our TEM data indicated that soluble melanin was taken up by endocytosis after treatment (Fig. 7A). Observation by light microscopy also showed that cells treated with

soluble melanin gradually turned black in a time-dependent manner (data not shown). Flow cytometric analysis of the Fc ϵ RI-IgE levels on the cell surface indicated that the levels were decreased after melanin treatment (Fig. 7B). Furthermore, soluble melanin did not directly interact with the IgE antibody (Fig. 7C), indicating that the melanin may downregulate the assembly of signaling molecules or inhibit the formation of the IgE-antigen-IgE bridge. These results led us to speculate that the Fc ϵ RI-IgE complex was internalized in cells after melanin treatment presumably by endocytosis. Previous studies showed that IgE internalization (loss of IgE molecules from the cell surface) is a mechanism in rapid desensitization [51-53]. Although we have not examined whether inhibition of degranulation by melanin treatment is abrogated if IgE-internalization is inhibited, it is worth considering this approach to elucidate the precise mechanism of the anti-degranulation effect of melanin. Hence, the partial blockade from antigen and/or the decrease of IgE-Fc ϵ RI molecules on the cell surface are feasible mechanisms of mast cell inhibition by solubilized or soluble melanin. However, these findings could not explain why melanin was able to inhibit mast cell activation by the calcium ionophore A23187 (Fig. 6B), as this molecule activates mast cells without an antigen-antibody reaction through the cell surface. Therefore, we examined the possibility that melanin negatively impacts the fluidity of intracellular molecules. As shown in Figure 8, FRAP analysis clearly demonstrated that both cytoplasmic and plasma membrane fluidities were dampened by melanin treatment, presumably by increasing plasma membrane and cytoplasm viscosity. This finding suggests that suppression of plasma membrane and/or cytoplasmic fluidity by melanin may lead to a delay in the rate of signaling, which may inhibit the degranulation reaction. Since the internalization of membrane-bound receptors depends on the mobility of the plasma membrane and cytoplasm, these two hypotheses

seem to be contradictory at first glance. However, we consider that both these hypotheses may be valid, as the attenuation of the fluidity of the plasma membrane and/or cytoplasm would occur after the internalization of melanin. To confirm these ideas, further approaches such as a membrane fluidity test using other membrane-specific probes [54, 55] or monitoring live cell viscosity using environment-sensitive dyes [56, 57] may be valuable. Identification of the target molecule(s) of the dispersed, solubilized or soluble melanin on the cell surface is critical for elucidating the molecular mechanism of melanin-mediated inhibition of mast cell activation. Our results demonstrated that dispersed, soluble or solubilized melanin behaves as a mast cell stabilizer. Although the precise mechanism of mast cell suppression remains unknown, it may be possible that multiple factors are involved and the mechanism may be complex.

PSA revealed that oral administration of Dopa-melanin significantly ($\dagger P < 0.1$, $*P < 0.05$, $**P < 0.01$,) suppressed the severe anaphylactic reaction (Fig. 9B). These findings may provide a foundation for new drug discovery for allergic diseases, but are also associated with some problems, as the precise mechanism by which orally administered melanin inhibits the anaphylactic reaction is unknown. We speculate that orally administered melanin was absorbed from the intestinal tract and circulated in blood vessels to act on the effector mast cells or may have inhibited the effects of histamine and leukotrienes. At present, there is no established method for measuring the blood concentration of orally administered melanin, and this should be examined in future studies. In addition, whether melanin interferes with the action of chemical mediators such as histamine or leukotrienes should be examined.

PCA induction is dependent mainly on the degranulation of activated mast cells, with rapid release of histamine and other chemical mediators resulting in locally increased

blood vessel permeability. Therefore, the FcεRI-mediated immune reaction in the IgE-sensitized ears of mice can be manifested by antigen injection via the tail vein with Evans blue dye. Eliciting a PCA reaction in the mouse model confirmed the inhibitory effect of Dopa-melanin on IgE-mediated extravasation (Fig. 9C, D), suggesting that the efficacy of melanin is tissue-resident mast cell-dependent.

Our findings are novel in clearly demonstrating the anti-allergic potential of melanin (eumelanin) and the biological effect of melanin on immune cells. Future studies should also examine whether other type of melanins, such as pheomelanin or allomelanin, have the same activities. Furthermore, comparison of results from other melanins may give clues as to structural elements in melanin required for the anti-degranulation effects. It is worth mentioning that in addition to the degranulation assays described in this paper, the homogeneous black solution can be tested in a variety of other *in vitro* and *in vivo* assays. Although evident toxicity was not observed using WST-8, which produces a formazan dye generated by dehydrogenases, safety evaluations such as reverse-mutation test, chromosome aberration test, and endotoxin tests (*in vitro*), as well as acute toxicity test, antigenicity test, skin irritation and sensitization test (*in vivo*) should be performed to confirm the materials are safe for humans.

So far, several studies have reported degranulation suppression effect by several natural products, such as procyanidin, O-methylated catechin, s-petasin, hesperetin and naringenin [58-61]. However, these natural compounds are usually quickly metabolized and excreted outside the body. In contrast, the melanins used in this study can remain in the cell for a relatively long time. In our observations, RBL-2H3 cells treated with melanin for 2 h maintain brown/black staining for at least 24 h, suggesting that melanin could exert a relatively long and strong inhibitory effect on degranulation.

In conclusion, our findings demonstrate that squid ink derived-melanin and synthetic dispersed, solubilized, and soluble melanins are candidates for novel and effective sources of medicinal agents for diseases involving mast cells.

Conflict of interest statement

There is no potential conflict of interest to disclose.

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Figure legends

Figure 1

Inhibition of FcεRI-mediated degranulation by crude squid ink and dispersed-melanin.

A. Dose-dependent effect of anti-degranulation by squid-ink-derived melanin. RBL-2H3 cells were sensitized with DNP-specific IgE overnight and then treated with squid-ink-derived melanin for 2 h at the indicated concentrations. Cells were washed and degranulation was induced by DNP-HSA treatment for 90 min. Results are expressed as mean $\pm$ SD ($n = 3$). NC: negative control (without DNP-HSA treatment); PC: positive control (DNP-HSA treatment only). B. The appearance of commercially obtained melanin in PBS before and after treatment of laser-ablation. The laser-treated solution was left standing for at least 24 h. C. The scanning electron microscopic analysis of laser-dispersed melanin. The diameter of melanin particles was around 70–130 nm. D. Dose-dependent inhibition of Fcε-RI-mediated degranulation in RBL-2H3 cells by dispersed-melanin. Results are expressed as mean $\pm$ SD ($n = 3$). * $P < 0.05$ compared with PC, analyzed by unpaired two-tailed Student's *t*-test.

Figure 2

Commercial melanin dissolved in HEPES buffer inhibited Fcε-RI-mediated degranulation in RBL-2H3 cells.

A. Comparison of the solubility of commercially obtained melanin in biochemical buffers. The melanin was dissolved in a variety of biochemical buffers (0.5 M) as indicated at 50 mg/ml. Solutions were centrifuged at $10,000 \times g$ for 30 min and then 10 μ l of supernatant was applied to a filter paper (a). After centrifugation, the solution was then vigorously

mixed and 10 μ l of the solution was immediately applied to the filter paper (b). B. Dose-dependent inhibition of Fc ϵ -RI-mediated degranulation in RBL-2H3 cells by HEPES-melanin. IgE-sensitized RBL-2H3 cells were treated with HEPES-melanin at the indicated concentrations for 2 h, followed by degranulation assays. C. Time-dependent inhibition of Fc ϵ -RI-mediated degranulation in RBL-2H3 cells by HEPES-melanin. IgE-sensitized RBL-2H3 cells were treated with 500 μ g/ml HEPES-melanin for the indicated times, followed by degranulation assays. D. Comparison of melanin solutions and an anti-allergic drug on inhibition of Fc ϵ -RI-mediated degranulation. IgE-sensitized RBL-2H3 cells were pretreated with melanin solutions or ketotifen fumarate at the indicated concentrations for 2 h, followed by β -hexosaminidase assays. E. Cell viability analysis of dispersed-melanin and HEPES-melanin. RBL-2H3 cells were treated with the melanin solutions for 24 h at the indicated concentrations. Staurosporine (STPO, 2.5 μ g/ml) was used as a toxic agent that induced apoptosis. Results are expressed as mean $\pm$ SD (n = 3). NC and PC stand for negative control (without DNP-HSA treatment) and positive control (DNP-HSA treatment only), respectively. * P < 0.05, ** P < 0.01, § P < 0.001 compared with PC (B, C, D) or NC (E), analyzed by unpaired two-tailed Student's t -test. n.s., not significant.

Figure 3

Preparation of Dopa-melanin by chemical synthesis and its anti-degranulation activity.

A. Chemical structure of L-dopa that was used for melanin synthesis as an initial reactant.

B. Putative structures of chemically synthesized Dopa-melanin. The arrows indicate direction of polymerization. C. Dose-dependent inhibition of Fc ϵ -RI-mediated

degranulation in RBL-2H3 cells by Dopa-melanin. RBL-2H3 cells were sensitized with

DNP-specific IgE and then treated with Dopa-melanin dissolved in HEPES or H₂O for 2 h at the indicated concentrations, followed by degranulation assays. NC and PC indicate negative control and positive control, respectively. Results are expressed as mean $\pm$ SE (n = 4). $\ddagger P < 0.005$ compared with PC, analyzed by unpaired two-tailed Student's *t*-test.

D. Cell viability assay. Dopa-melanins dissolved in HEPES or H₂O were applied to completely confluent cell cultures for 24 h at the indicated concentrations. Sodium arsenite (As, 1 mM) was used a toxic agent that induces apoptosis. E. Effect of HEPES-melanin fractionated by molecular size on RBL-2H3 cell degranulation. IgE-sensitized cells were pretreated with HEPES-dissolved melanin (no fractionation, over 50 kD or under 50 kD) for 2 h at the concentrations between 125 to 500 μ g/ml and β -hexosaminidase assay was performed. F. Microscopic observation of cells treated with HEPES-melanin over or under 50 kD. RBL-2H3 cells were treated with 125 μ g/ml of HEPES-melanin over 50 kD (left) or under 50 kD (right) for 2 h. Cells were washed with PBS three times and observed under bright-field microscopy. Data were collected by an Olympus IX71 with DP controller software version 3.1.1.267 (Olympus). Representative images are shown. Scale bar = 100 μ m. NC: negative control (without DNP-HSA treatment); PC: positive control (DNP-HSA treatment only). Results are expressed as mean $\pm$ SD (n = 3). $**P < 0.01$, $\ddagger P < 0.005$, compared with PC (C, E) or NC (D), analyzed by unpaired two-tailed Student's *t*-test. n.s., not significant.

Figure 4

Effect of purified melanin-bound protein or deproteinized melanin on RBL-2H3 cell degranulation. IgE-sensitized RBL-2H3 cells were pretreated with or without Sepia-melanoprotein or deproteinized sepia-melanin at the indicated concentrations for 2 h. The

melanin solutions were removed, antigen was added for 90 min, and β -hexosaminidase assay was performed. NC: negative control (without DNP-HSA treatment); PC: positive control (DNP-HSA treatment only). Results are expressed as mean $\pm$ SD ($n = 3$). $\ddagger P < 0.005$ compared with PC, as analyzed by unpaired two-tailed Student's *t*-test.

Figure 5

Downregulation of antigen-triggered phosphorylation level of cytoplasmic signaling molecules in solubilized-melanin-treated RBL-2H3 cells. A. Dose-dependent inhibition of phosphorylation of signal molecules by HEPES-melanin in RBL-2H3 cells. Anti-DNP-IgE-sensitized cells were treated with HEPES-melanin and then stimulated with DNP-HSA (200 ng/ml) for 5 min, followed by western blotting. β -actin served as the loading control. B. Time-dependent changes of the phosphorylation states of signal molecules after DNP-HSA stimulation. DNP-IgE-sensitized RBL-2H3 cells were pretreated with or without HEPES-melanin (500 μ g/ml, 2 h) and stimulated with DNP-HSA for 5, 15 or 30 min. β -actin served as the loading control. Band signals (left panel) were analyzed by ImageJ software and the fold increase of signal levels compared with the negative control was measured (right panel). NC: negative control (without DNP-HSA treatment). Results are expressed as mean $\pm$ SD ($n = 3$). $*P < 0.05$ as analyzed by unpaired two-tailed Student's *t*-test.

Figure 6

Inhibition of DNP-HSA-induced Ca^{2+} release and calcium ionophore-induced degranulation by dispersed-melanin. IgE-sensitized RBL-2H3 cells were pretreated with various concentrations of dispersed-melanin for 2 h. A. After exposure to melanin, cells

were treated with Fura-2 AM and then stimulated with DNP-HSA (200 ng/ml) for 270 sec. The F340/F380 ratio was analyzed by a fluorescence imaging system. B. Cells were stimulated with a calcium ionophore A23187 (1 μ M) for 2 h and then β -hexosaminidase release was measured. NC: negative control (without DNP-HSA treatment); PC: positive control (DNP-HSA treatment only). Results are expressed as mean $\pm$ SD ($n = 3$). $*P < 0.05$, $\ddagger P < 0.005$ compared with PC, as analyzed by unpaired two-tailed Student's *t*-test.

Figure 7

Mechanism of hyporesponsiveness to antigen in RBL-2H3 cells. A. Representative transmission electron microscope images of RBL-2H3 cells treated with dispersed-melanin (250 μ g/ml). Right panel is a magnified image of the section in the square in the left panel. Black arrowheads indicate melanin that attached to the cell surface, and white arrowhead indicates internalized melanin. B. Flow cytometric analysis of cell surface Fc ϵ RI that could bind with IgE. RBL-2H3 cells were sensitized with IgE overnight and treated with HEPES-melanin for 2 h at the indicated concentrations. Fc ϵ RI-bound IgE level was measured using Alexa Fluor 488 anti-IgE antibody. C. Examination of interaction between melanin and IgE. Melanin powder was placed in EMEM at 0.1% (w/v) and remained undissolved because it was not treated by sonication or laser desorption. DNP-specific IgE antibody (100 ng/ml) was added and the solution was incubated for 2 h at 37°C. After centrifugation at 10,000 $\times$ g for 10 min, the supernatant, containing free IgE antibody, was used for sensitization of RBL-2H3 cells. The mean value of the fluorescence intensity of each sample was used for comparison of the relative expression levels of antigen-accessible Fc ϵ RI-bound IgE. Results are expressed as mean $\pm$ SD ($n = 3$). $**P < 0.01$ from the only IgE antibody-treatment sample (no-melanin

pretreatment, white bar, right panel), as analyzed by unpaired two-tailed Student's *t*-test.

Figure 8

Suppression of plasma membrane fluidity by melanin. GFP-transfected or NeuroDiO-stained RBL-2H3 cells were used for cytoplasmic fluidity (A, B) or membrane fluidity (C, D) analysis, respectively, by fluorescence recovery after photobleaching (FRAP) analysis as described in Materials and Methods. RBL-2H3 cells were pretreated with HEPES-melanin (250 or 500 $\mu\text{g/ml}$) for 2 h at 37°C. A, C. Representative sequential changes of GFP-labeled cytoplasmic fluidity (A) and fluorescence-labeled membrane fluidity (C) by FRAP of cytoplasmic GFP or cell surface NeuroDiO regions shown as dotted circles. Images were taken at 3.24 sec intervals, starting 3.24 sec before photobleaching, followed by imaging for a total time of 34.9 (GFP) or 51.9 sec (NeuroDiO) sec after photobleaching. The specific regions of interest (ROI) were calculated using Olympus software. B, D. Corrected relative fluorescence intensities at the indicated time points. Results are expressed as mean $\pm$ SE (ROI, $n = 8$). $^{***}P < 0.01$ compared with the negative control (NC) sample (no-melanin pretreatment, white circles), as analyzed by unpaired two-tailed Student's *t*-test.

Figure 9

Anti-allergic effect of melanin evaluated by *in vivo* anaphylaxis models. A. Body weight changes of melanin-administered mice. Water or Dopa-melanin (100 mg/kg) was intragastrically administered to Balb/c mice (male, 14 weeks, $n = 6$ per group) every other day for 30 days. Dopa-melanin (500 mg/kg) was subsequently administered to the Dopa-melanin group every other day for an additional 30 days. The body weight was measured

every 2–3 days. B. Changes of body temperature by passive systemic anaphylaxis. After antigen challenge, body temperatures of mice were measured every 10 min for 2 h using a rectal thermometer. C, D. Passive cutaneous anaphylaxis. Twenty μ l of vehicle (EMEM), DNP-specific IgE antibody, or IgE with Dopa-melanin (500 μ g/ml) were injected into the right pinna of the ears of the mice. After 48 h, DNP-HSA in PBS containing 1% Evans blue was intravenously injected into the mice via the tail vein. After 30 min, the ears were photographed to show Evans blue extravasation and a representative image for each group is shown (C). The dye was extracted from the ear and the absorbance was measured at 620 nm (D). Results are expressed as mean $\pm$ SD ($n = 6$ per group). $\dagger P < 0.1$, $*P < 0.05$, $**P < 0.01$, compared with PC (positive control), as analyzed by Mann-Whitney's *U*-test.

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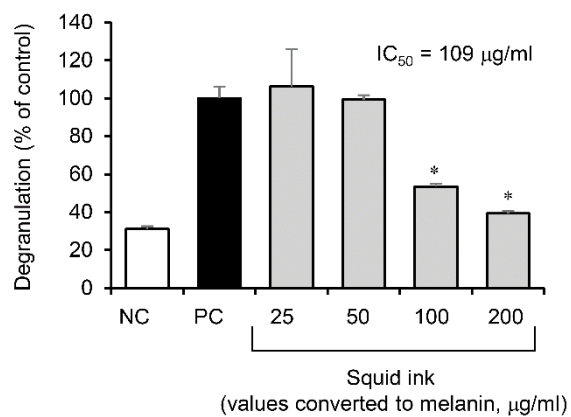
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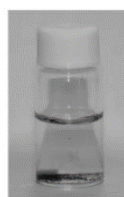
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Fig. 1

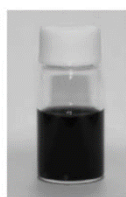
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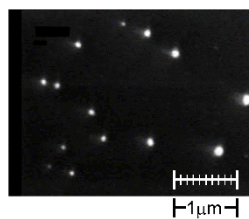


Before



After

C



1µm

D

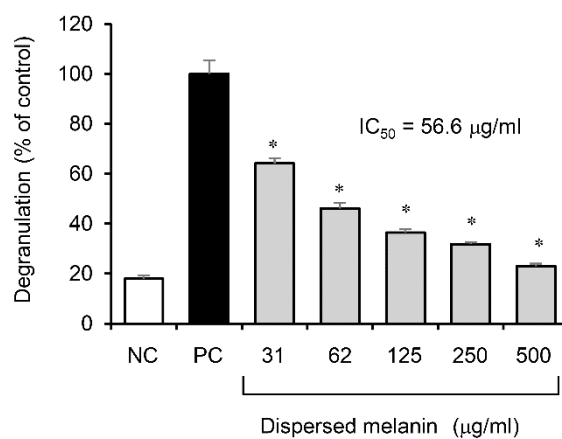
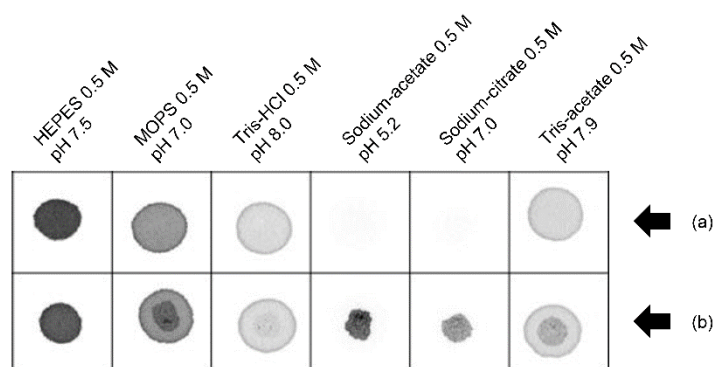
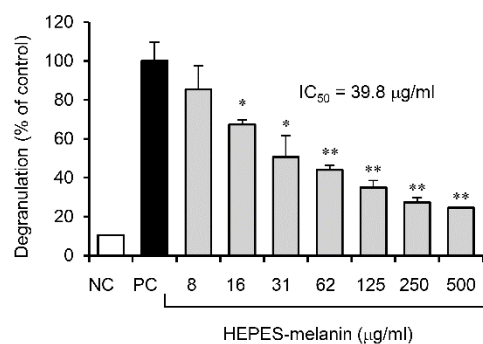


Fig. 2

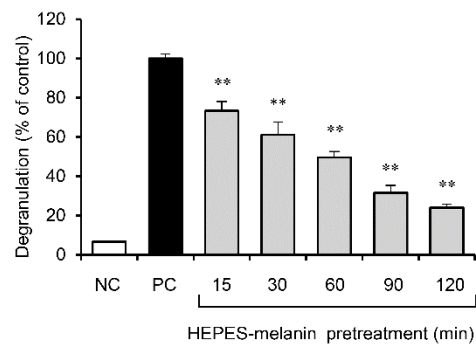
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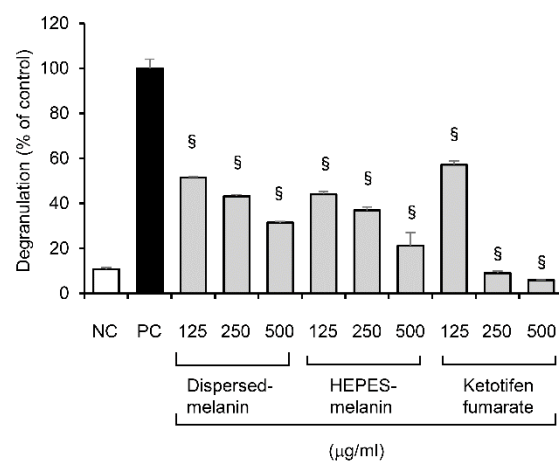
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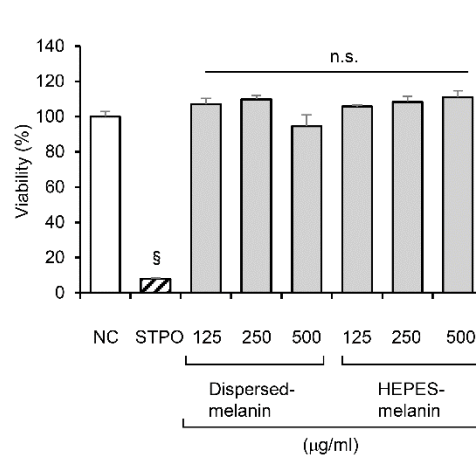


Fig. 3

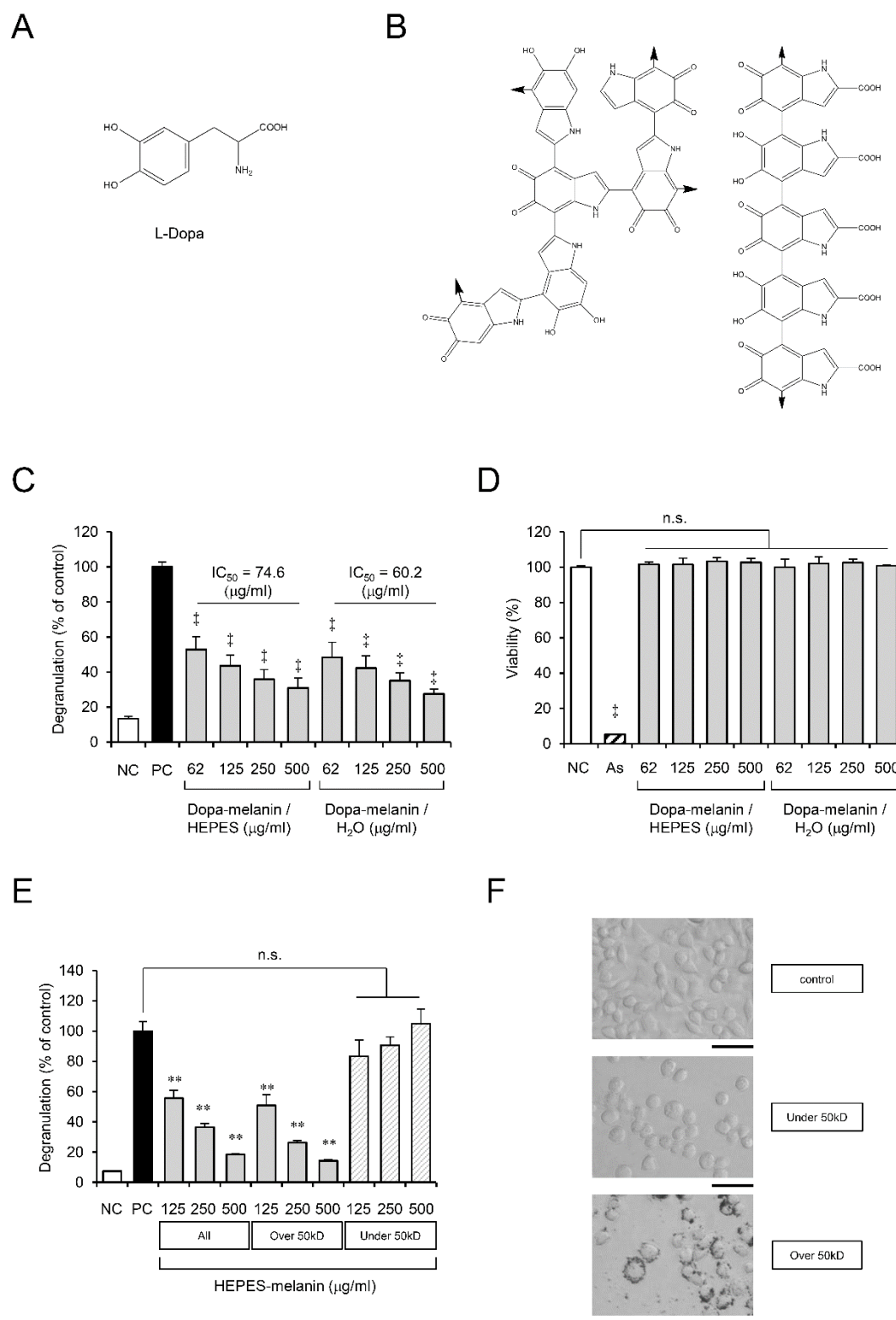


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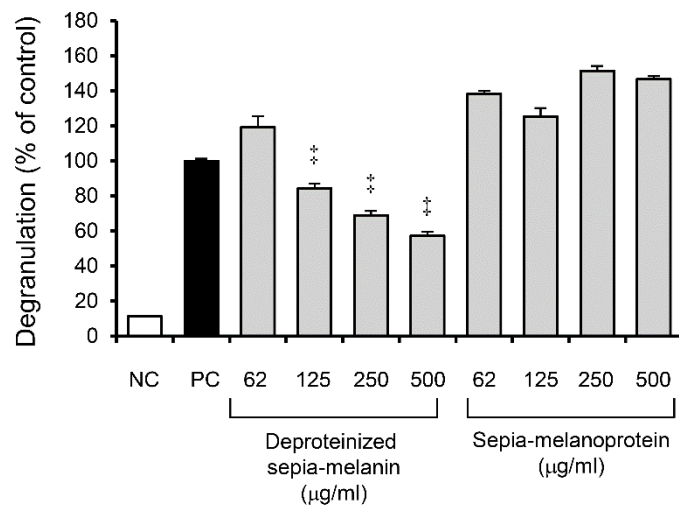
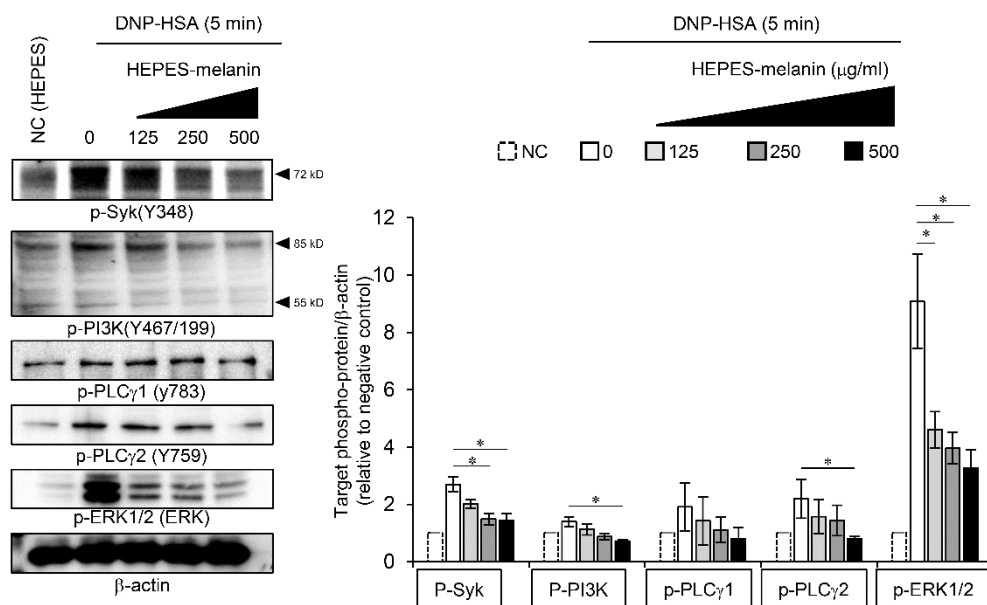


Fig. 5

A



B

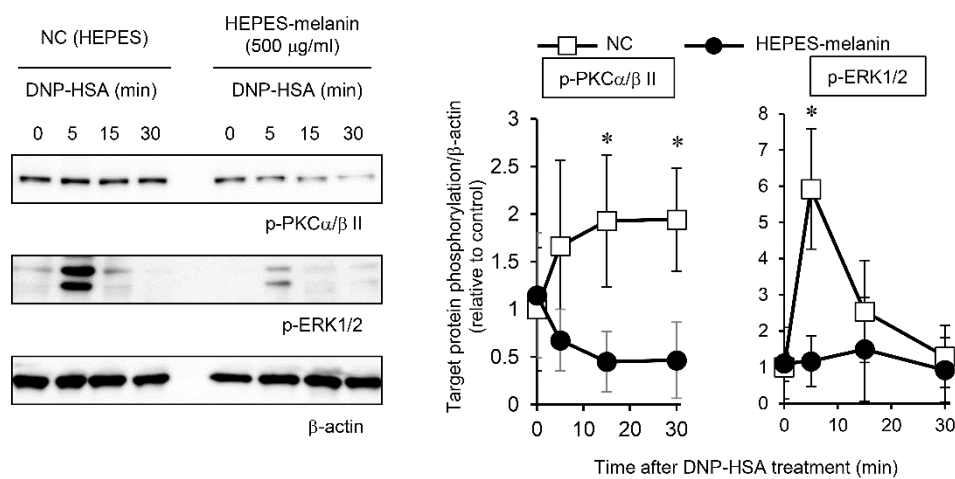
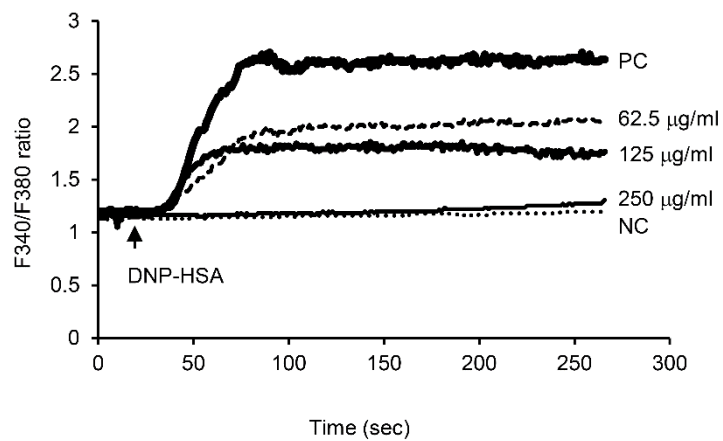


Fig. 6

A



B

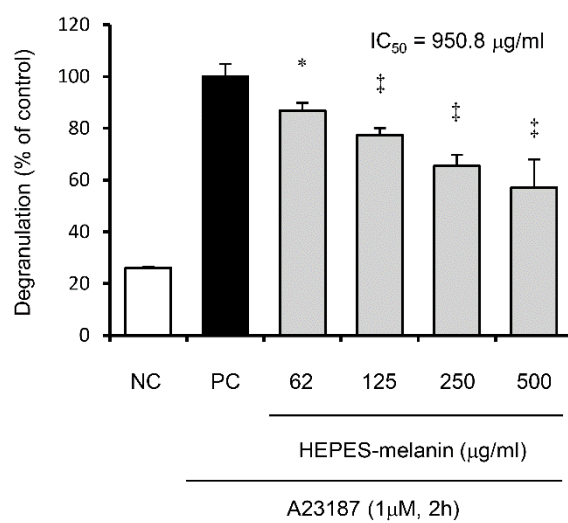


Fig. 7

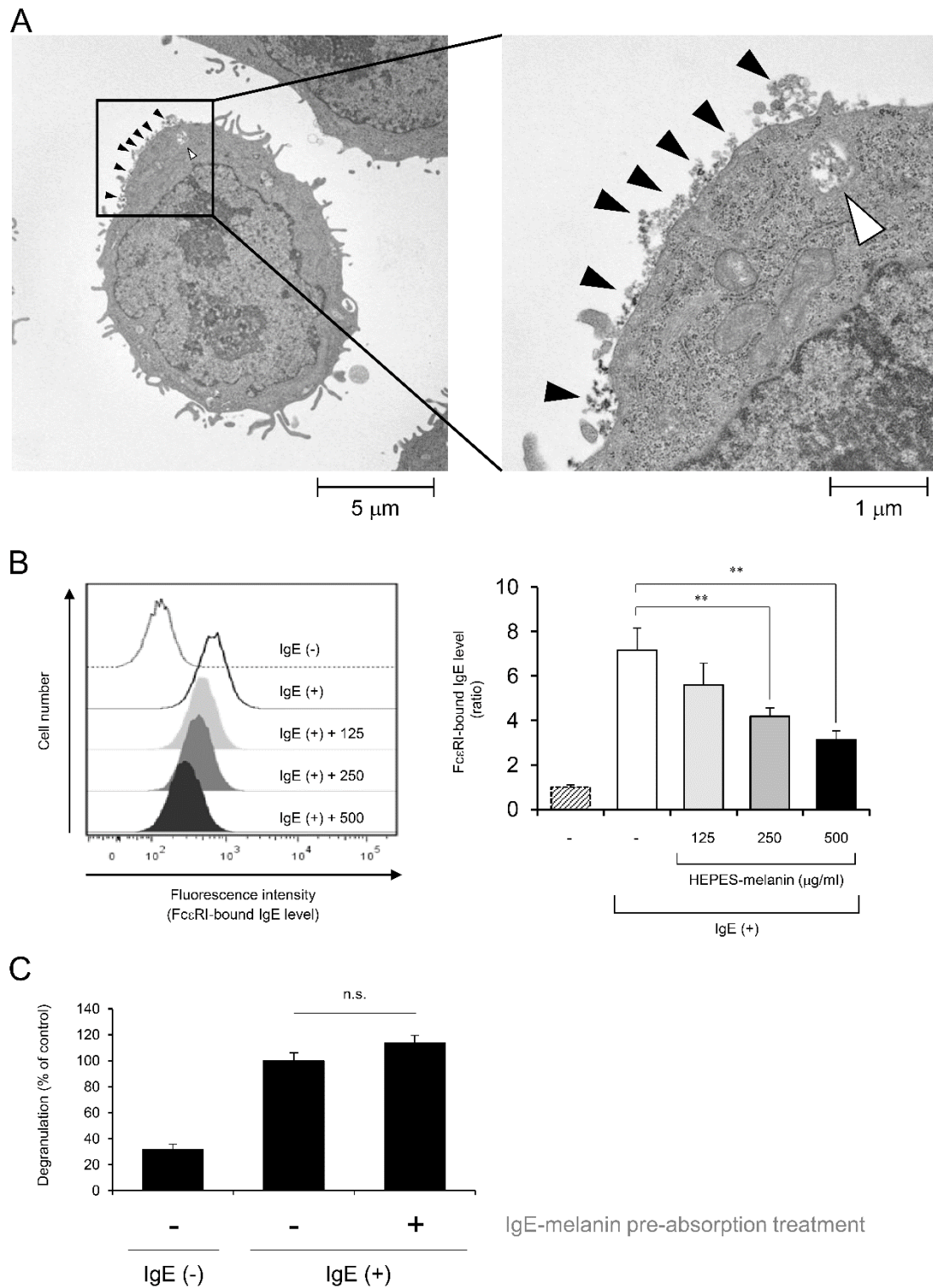


Fig. 8

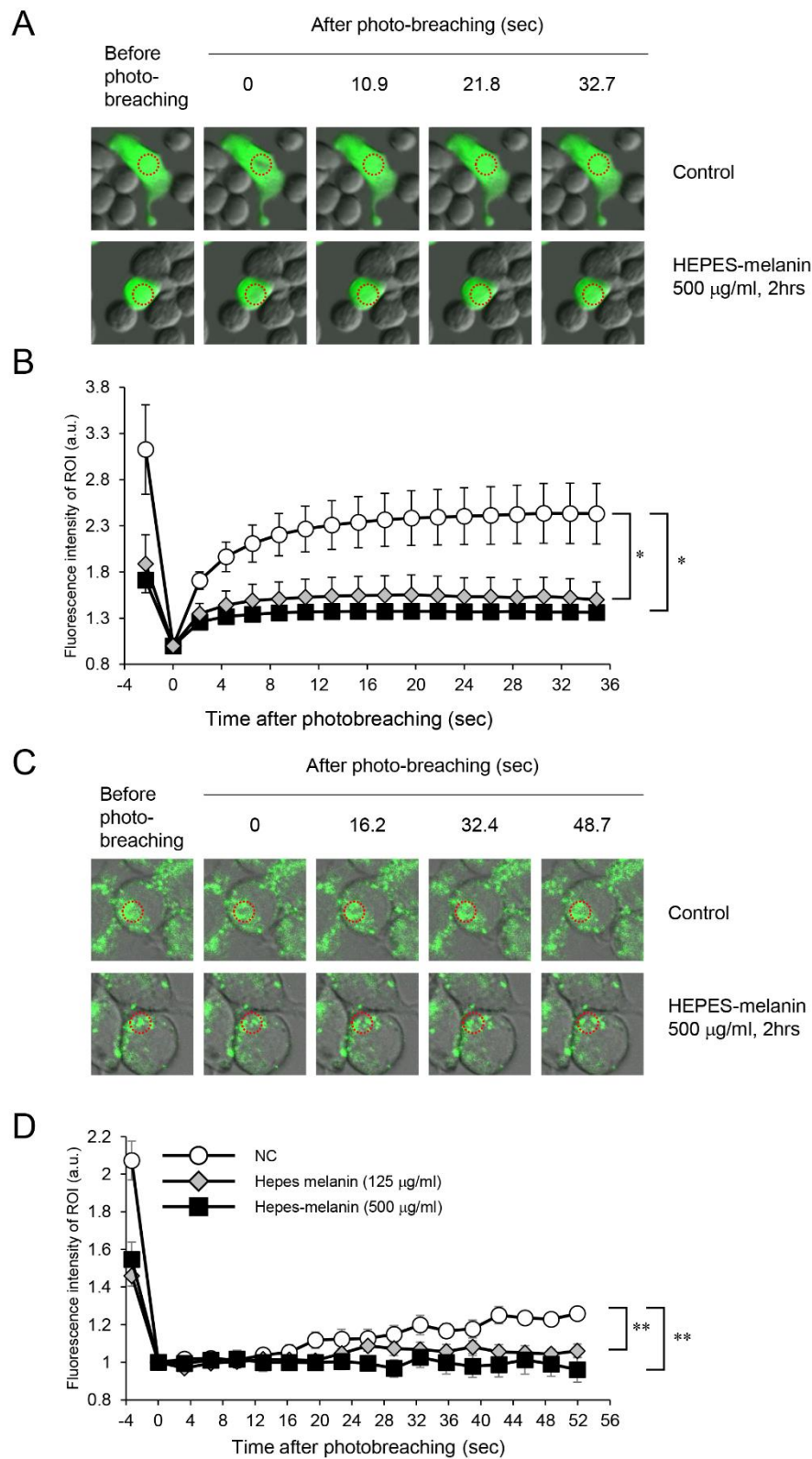


Fig. 9

