The American Journal of Pathology, Vol. 189, No. 9, September 2019

ELSEVIER

The American Journal of
PATHOLOGY

ajp.amjpathol.org

GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Involvement of α-Melanocyte—Stimulating Hormone—Thromboxane A_2 System on Itching in Atopic Dermatitis

Tsugunobu Andoh,* Chihiro Akasaka,* Kyoko Shimizu,† Jung-Bum Lee,‡ Yoko Yoshihisa,† and Tadamichi Shimizu†

From the Departments of Applied Pharmacology and Dermatology† and the Laboratory of Medicinal Bio-resources,‡ Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan*

Accepted for publication May 16, 2019.

Address correspondence to Tsugunobu Andoh, Ph.D., Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan. E-mail: andoht@pha.u-toyama.ac.jp.

α-Melanocyte—stimulating hormone (α-MSH) is an endogenous peptide hormone involved in cutaneous pigmentation in atopic dermatitis (AD) with severe itching. α-MSH elicits itch-related responses in mice. We, therefore, investigated whether α-MSH was involved in itching in AD. In the skin of AD patients and mice with atopy-like dermatitis, α-MSH and the prohormone convertase 2, which is the key processing enzyme for the production of α-MSH, were distributed mainly in keratinocytes. In the skin of mice with dermatitis, melanocortin receptors (MC1R and MC5R) were expressed at the mRNA level and were distributed in the dermis. In the dorsal root ganglion of mice with dermatitis, mRNAs encoding MC1R, MC3R, and MC5R were also expressed. MC1R antagonist agouti-signaling protein inhibited spontaneous scratching in mice with dermatitis. In healthy mice, intradermal α-MSH elicited itch-associated responses, which were inhibited by thromboxane (TX) A_2 receptor antagonist ONO-3708. In mouse keratinocytes, α-MSH increased the production of TXA_2, which was inhibited by adenylyl cyclase inhibitor SQ-22536 and Ca^{2+} chelator EGTA. In mouse keratinocytes treated with siRNA for MC1R and/or MC5R, α-MSH—induced TXA_2 production was decreased. α-MSH increased intracellular Ca^{2+} ion concentration in dorsal root ganglion neurons and keratinocytes. These results suggest that α-MSH is involved in itching during AD and may elicit itching through the direct action of primary afferents and TXA_2 production by keratinocytes. *(Am J Pathol 2019, 189: 1775—1785; https://doi.org/10.1016/j.ajpath.2019.05.017)*

Atopic dermatitis (AD) is a chronic inflammatory skin disease that causes severe itching.[1] Scratching induced by itching further exacerbates dermatitis and itching.[2] Therefore, the control of itching in AD is important. Histamine is a well-known itch mediator, and histamine H_1 receptor antagonists are used as the first-line treatment for itching. However, the treatment is not effective for AD-associated itching,[3] suggesting that histamine is unlikely to be pruritogenic in AD. Therefore, the precise mechanisms and mediators of itching in AD remain unclear.

α-Melanocyte—stimulating hormone (α-MSH) is a neuropeptide generated by cleaving a precursor protein proopiomelanocortin with prohormone convertases 1 and 2 (PC2).[4] Melanocortin receptors (MCRs) have five subtypes [MC1R, MC3R, MC4R, and MC5R for α-MSH and MC2R for adrenocorticotropic hormone (ACTH)].[4,5] Activation of these receptors increases the production of cAMP[5] and intracellular Ca^{2+} ion concentrations.[6] It has been reported that MC1R and MC5R are mainly expressed in the skin,[7] and α-MSH is known to be involved in cutaneous pigmentation,[8] including that by AD.[9] An intradermal injection of α-MSH elicits itch-related behaviors in naïve mice [Institute of Cancer Research (Philadelphia, PA) strain], and the histamine released from epidermal keratinocytes is partly involved in scratching.[10] However, it is unclear whether α-MSH is involved in AD-associated itching. Therefore, this study investigated the

Supported by the Japan Agency for Medical Research and Development Practical Research Project for Allergic Diseases and Immunology (Research on Allergic Diseases and Immunology) grant 16ek0410011h0003 (T.A.) and JSPS KAKENHI grant JP16K15337 (T.A.).

Disclosures: None declared.

Copyright © 2019 American Society for Investigative Pathology. Published by Elsevier Inc. All rights reserved.
https://doi.org/10.1016/j.ajpath.2019.05.017

involvement of α-MSH and underlying mechanisms in AD-associated itching.

Materials and Methods

Animals

Male Nishiki-nezumi Cinnamon/Nagoya (NC/Nga) mice (14 to 21 weeks old) were used in this study. NC mice were bred in a specific pathogen-free environment at the Division of Animal Resource and Development of the Life Science Research Center, University of Toyama (Toyama, Japan). The mice were housed in a room under controlled temperature (21°C to 23°C), humidity (45% to 65%), and light (lights on from 8:00 AM to 8:00 PM). Food and water were freely available. These mice were kept healthy and without dermatitis. Some mice were transferred to a conventional environment at 4 to 5 weeks of age and cohoused with mite-infected mice experiencing chronic dermatitis for 2 weeks.[11] A few months later, these mice showed severe dermatitis. Animal experiment procedures were approved by the Committee for Animal Experiments at the University of Toyama and were conducted in accordance with the Guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Subjects

A skin biopsy was performed for patients with AD (27 to 42 years old) enrolled at Toyama University Hospital. Healthy control skin used for this experiment was obtained from an age- and sex-matched healthy volunteer. This study was approved by the Medical Ethics Committee of the University of Toyama (approval number 27-66).

Materials

α-MSH was synthesized (J.-B.L.) using standard solid-phase techniques with N-α-9-fluorenylmethoxycarbonyl chemistry on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan). The free amino group of the peptide was acetylated with acetic acid anhydride/dichloromethane. Cleavage of the peptide from the resin was achieved by treatment with a mixture of trifluoroacetic acid/1,2-ethanedithiol/triisopropylsilane/water (94/2.5/1/2.5, by volume) at room temperature for 2 hours. After removal of the resin by filtration and washing twice with trifluoroacetic acid, the combined filtrate was added dropwise to diethyl ether at 0°C and centrifuged at 1500 × *g* for 10 minutes. The obtained crude synthetic peptide was purified by semipreparative reverse-phase high-performance liquid chromatography using Cosmosil 5C18 ARII (20 × 300 mm; Nacakai Tesque, Inc., Kyoto, Japan) in a linear gradient system of 0.1 mol/L HCl with acetonitrile at a flow rate of 7 mL/minute. The homogeneity was confirmed by high-performance liquid chromatography equipped with a Cosmosil 5C18 ARII (4.6 × 150 mm) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Autoflex T1; Bruker Daltonics Inc., Billerica, MA) (Supplemental Figure S1). The materials used for synthesizing α-MSH are described as follows: N-α-9-fluorenylmethoxycarbonyl—protected amino acids, N-α-9-fluorenylmethoxycarbonyl—NH-SAL resin, 1H-benzotriazol-1-yloxy-tri(pyrrolidino)phosphonium hexafluorophosphate, 1-hydroxy-1H-benzotriazole hydrate, and piperidine were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Peptide synthesis grade N, N-dimethylformamide and N-methylmorpholine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Sigma-Aldrich (St. Louis, MO), respectively.

Histamine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). α-MSH and histamine were dissolved in physiological saline. These agents were injected intradermally in the rostral back skin in a volume of 50 μL. Mouse recombinant agouti-signaling protein (CUSABIO TECHNOLOGY LLC, College Park, MD) was dissolved in phosphate-buffered saline (PBS). Recombinant agouti-signaling protein (2.5 μg) was injected intraperitoneally in a volume of 50 μL once per day for 6 days. ONO-3708 (7-[2α, 4α-(dimethylmethano)-6β-(2-cyclohexyl-2β-hydoxyacetamino)-1α-cyclohexyl]-5[Z]-heptanoic acid; Ono Pharmaceutical, Osaka, Japan) was dissolved in tap water and administered orally in a volume of 0.1 mL/10 g body weight 1 hour before α-MSH injection. SQ-22536 (Tocris Bioscience, Bristol, UK) and EGTA (Dojindo Laboratories, Kumamoto, Japan) were dissolved in CnT-Prime Basal medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) containing 0.1% dimethyl sulfoxide. These agents were treated 30 minutes before the application of α-MSH.

siRNA Treatment in Primary Cultures of Mouse Keratinocytes

Primary cultures of mouse keratinocytes[12] were performed in CnT-Prime medium (CELLnTEC Advanced Cell Systems AG) in a glass-bottom culture dish or 24-well culture plates.

As a part of the experiment, keratinocytes were treated with *siRNA*.[10] siRNAs (control, *MC1R*, and *MC5R*; Santa Cruz Biotechnology Inc., Dallas, TX) were transfected in keratinocytes using Lipofectamine RNAi MAX reagent (Life Technologies, Carlsbad, CA). These transfections were performed according to the manufacturer's protocol (Life Technologies).

Immunostaining

Human skin samples were fixed in 10% neutral-buffered formalin and then embedded in paraffin. The paraffin sections were prepared using a microtome. After deparaffinization, these sections were treated at 120°C for 60 minutes using PASCAL (Dako, Carpinteria, CA). Next, the sections were treated with methanol containing 0.3% hydrogen peroxide/methanol for 10 minutes and washed with PBS. After

treatment with Protein Block (Dako) to block Ig binding, the sections were incubated with rabbit anti−α-MSH (Bioss Antibodies Inc., Woburn, MA: bs-1848R) or goat−anti-PC2 (Santa Cruz Biotechnology Inc.: sc-22891) at 4°C overnight, followed by biotin-conjugated anti-goat or anti-rabbit IgG antibody (Dako) and then horseradish peroxidase−conjugated avidin (Dako). The sections were subsequently treated with 3,3′-diaminobenzidine tetrahydrochloride (Dako) and counterstained with hematoxylin. After staining, the slides were dehydrated in graded ethanol and cleared in xylene. Preparations were mounted with Canada balsam and observed using a light microscope (BX-61; Olympus, Tokyo, Japan) with a charge-coupled device camera (DP70; Olympus).

Mouse skin samples were fixed in 4% paraformaldehyde and immersed in 30% sucrose solution for 2 days. The tissue was embedded in Tissue-Tek1 O.C.T. Compound (Sakura Fineteck Co, Ltd, Tokyo, Japan), and frozen samples were divided into sections (10 μm thick) with a cryostat (Leica, Wetzlar, Germany). The sections were washed with PBS and then treated with 1.5% fetal bovine albumin (Sigma-Aldrich), followed by 0.3% Triton X-100 in PBS. The sections were treated with rabbit anti−α-MSH (Bioss Antibodies Inc.: bs-1848R), goat anti-PC2 (Santa Cruz Biotechnology Inc.: sc-22891), goat anti-MC1R (Santa Cruz Biotechnology Inc.: sc-19485), or goat-MC5R (Santa Cruz Biotechnology Inc.: sc-7644) antibody at 4°C overnight. After washing with PBS, the preparations were incubated with Alexa Fluor 488−conjugated anti-rabbit or anti-goat IgG (Life Technologies) for 1 hour at room temperature. Fluorescence signals were observed using a fluorescence microscope (BX-61/DP70; Olympus).

Primary cultures of keratinocytes were fixed in 4% paraformaldehyde and treated with 0.3% Triton X-100 in PBS. The cells were treated with 1.5% fetal bovine albumin (Sigma-Aldrich) and then incubated with rabbit-antithromboxane synthase (160715; Cayman Chemical, Ann Arbor, MI), goat anti-MC1R (sc-19485; Santa Cruz Biotechnology Inc.), or goat-MC5R (sc-7644; Santa Cruz Biotechnology Inc.) antibody at 4°C overnight. After washing with PBS, the preparations were incubated with Alexa Fluor 488−conjugated anti-rabbit or anti-goat IgG (Life Technologies) for 1 hour at room temperature. Fluorescence signals were observed using a fluorescence microscope (BX-61/DP70; Olympus).

For a portion of the immunostaining for MC1R and MC5R, the antibodies preabsorbed with the antigenic peptides (Santa Cruz Biotechnology Inc.) were used as a negative control. The antibodies preabsorbed with the antigen peptides were prepared following the manufacturer's protocol (Santa Cruz Biotechnology Inc.). The relevant results are shown in Supplemental Figure S2.

RT-PCR

The isolated rostral back skin and dorsal root ganglion (DRG) were immediately placed in RNAlater stabilization solution (Thermo Fisher Scientific, Waltham, MA) and stored at −80°C after overnight incubation at 4°C. *RNA* samples were extracted using the TRIzol reagent (Invitrogen Co., Carlsbad, CA). The total *RNA* was treated with DNase I (Takara Bio Inc., Otsu, Japan) in 50 μL of reaction buffer. After purification, total *RNA* (1 μg) was added to the PrimerScript RT Master Mix (Takara Bio Inc.) and reverse transcribed at 37°C for 1 hour. A portion (1 μL) of the cDNA was mixed with SYBR Premix Ex Taq II (Takara Bio Inc.) containing 10 μmol/L of sense and antisense primer pairs. The primer sequences used for RT-PCR are shown in Table 1. The cycling conditions were 30 seconds at 95°C and then 36 cycles (for skin) or 40 cycles (for DRG) of 5 seconds at 94°C, 10 seconds at 58°C, and 30 seconds at 72°C. After PCR, the amplified products were analyzed by 2% agarose gel electrophoresis.

Real-Time PCR

The extraction of total *RNA* and the preparation of *cDNA* were performed following the method mentioned in *RT-PCR*. Quantitative PCR using a portion (2.5 μL) of the *cDNA* and specific primers was performed using the Mx3000PTM real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA). The cycling conditions were 30 seconds at 95°C and then 40 cycles of 5 seconds at 94°C, 10 seconds at 58°C, and 30 seconds at 72°C. The primer sequences for *MC1R* to *MC5R* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) used for real-time PCR are shown in Table 1. The mRNA expression of *GAPDH* and *MCR* was analyzed using Mx Pro QPCR software version 4.10 (Agilent Technologies, Inc.). The relative amount of *MCR* mRNA was first normalized to the level of *GAPDH* mRNA in each sample and then to the average of expression levels in healthy mice.

Behavioral Experiments

When α-MSH or histamine was injected intradermally, the animal's hair over the rostral part of the back was clipped on the day before the behavioral experiments. When spontaneous scratching was evaluated, the mouse hair was not removed. Mice were kept individually in an acrylic cage composed of four cells (13 × 9 × 35 cm) for >1 hour to allow acclimation. Immediately after intradermal injection, mice were placed back in the same cells and their behaviors were video recorded for 1 hour. During some of the experiments, after the acclimation periods, the spontaneous scratching behaviors were video recorded for 1 hour. During the recording of their behaviors using a digital video camera (HDC-TM25; Panasonic Co., Osaka, Japan), personnel were not present in the observation room. Playback of the video was used to determine hind paw scratching as an index of itching. Scratching of any region of the body by the hind paw was recorded for mice with dermatitis. For α-MSH− or histamine-injected mice, scratching of the injected site with the hind paw was recorded. A series of several scratching

Table 1 Sequences of Primers for PCR

MCR name	Forward primer	Reverse primer
MC1R	5′-GCCCACATGTTCACGAGAGC-3′	5′-AGTTACCCTTTCTCCTGGCCC-3′
MC2R	5′-GCCAGCAGGAAAAAATGAAGC-3′	5′-TGTTAGGGTGATGATGGTGCG-3′
MC3R	5′-TGCCTGTCTTCTGTTTCTCCG-3′	5′-ACCGAAGGGCATAGAAGATGG-3′
MC4R	5′-GGCAGATATGCTGGTGAGCG-3′	5′-TGGTCAAGGTAATCGCCCC-3′
MC5R	5′-CTCGGATGCAAGACCAGAGC-3′	5′-AGCAAACTGCACATCGAGGC-3′
GAPDH	5′-CCAAGGTCATCCATGACAAC-3′	5′-TTACTCCTTGGAGGCCATGT-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCR, melanocortin receptor.

movements, lasting approximately 1 second each, were considered a single bout of scratching.[13] All behavioral experiments were performed in a blinded manner (T.A.).

Enzyme-Linked Immunosorbent Assay for TXB_2

Primary cultures of mouse keratinocytes treated with or without siRNA were washed with Opti-MEM (Life Technologies). The medium was collected 10 minutes after α-MSH application, and the concentration of thromboxane (TX) B_2 was measured using an enzyme-linked immunosorbent assay kit for TXB_2 (Cayman Chemical, Ann Arbor, MI). After collecting all media, keratinocytes were washed with PBS and treated with 1% Triton X-100 to solubilize the cell proteins. The concentration of proteins was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). The concentration of TXB_2 in keratinocytes was normalized to the protein amount. The treatment methods involving siRNA, SQ-22536, and EGTA were described previously.[10]

Measurements of Intracellular Ca^{2+} Concentrations

Primary cultures of murine keratinocytes and DRG neurons[12] were washed with Opti-MEM (Life Technologies) and incubated with fluo-3/AM (10 μmol/L; Dojindo Laboratories) in Opti-MEM containing poloxamer (0.05%; Calbiochem, Darmstadt, Germany) at 37°C for 30 minutes in a 5% CO_2 incubator. Cells were washed with buffer (115 mmol/L NaCl, 5 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 0.4 mmol/L KH_2PO_4, 0.4 mmol/L Na_2HPO_4, 20 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4) for calcium imaging. The intracellular Ca^{2+} concentration was measured fluorometrically at an excitation of 488 nm and an emission between 515 and −545 nm using a laser-scanning microscope system (MiCAM02; Brainvision Inc., Tokyo, Japan).

Statistical Analysis

Data are presented as means ± SEM. The statistical significance of differences in means among groups was analyzed using the *t*-test; one-way analysis of variance, followed by a post-hoc Holm-Šidák test; or two-way repeated-measures analysis of variance, followed by a post-hoc Holm-Šidák test. $P < 0.05$ was considered significant.

Results

Expression and Distribution of α-MSH and PC2 in the Skin of AD Patients and Mice with Atopy-Like Dermatitis

α-MSH was expressed in the skin of both AD patients and healthy subjects (Figure 1A), and expression mainly occurred in epidermal keratinocytes (Figure 1A). In addition, the expression level was higher in AD patients than in healthy subjects (Figure 1A). PC2 was expressed in epidermal keratinocytes of AD patients (Figure 1A); however, it was barely expressed in the skin of healthy subjects (Figure 1A). These expression levels were significantly higher in AD patients than in the healthy subjects (Supplemental Figure S3, A and B).

In the skin of mice with dermatitis and healthy mice, α-MSH and PC2 were expressed in epidermal keratinocytes (Figure 1B), with higher expression in mice with dermatitis than in healthy mice (Supplemental Figure S3, C and D).

Expression of Melanocortin Receptors in the Skin and Dorsal Root Ganglia of Mice with Atopy-Like Dermatitis

The skin of both healthy mice and those with dermatitis expressed mainly *MC1R*, *MC2R*, and *MC5R* mRNAs (Figure 2A). *MC3R* and *MC4R* mRNA was expressed at low levels in the skin of healthy mice (Figure 2A). However, mRNA of *MC4R* was expressed at low levels, and *MC3R* was barely expressed in the skin of mice with dermatitis (Figure 2A). In the DRG of both healthy mice and those with dermatitis, all subtypes of *MCR* mRNA were expressed (Figure 2A).

The expression of *MCR5* mRNA was significantly higher in the skin of mice with dermatitis than in healthy mice (Figure 2B). Expression of *MC1R* mRNA in the skin of mice with dermatitis tended to be higher than that in the healthy mice (Figure 2B). Expression of *MC3R* mRNA in the DRG of mice with dermatitis was significantly higher than that in healthy mice (Figure 2B). Expression of *MC1R* mRNA in the DRG of mice with dermatitis tended to be

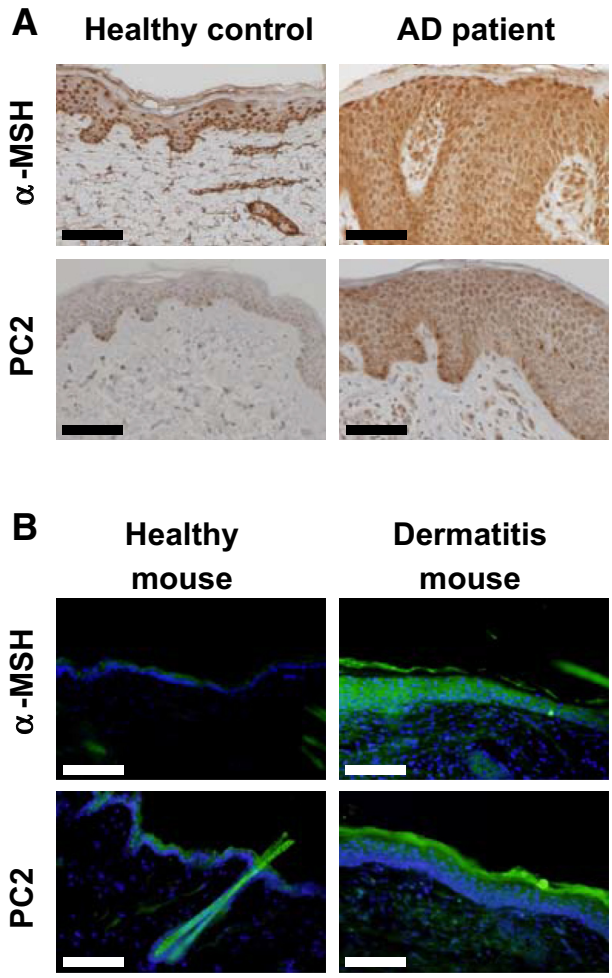


Figure 1 Distribution of α-melanocyte—stimulating hormone (α-MSH) and prohormone convertase 2 (PC2) in the skin of an atopic dermatitis (AD) patient and a mouse with atopy-like dermatitis. α-MSH and PC2 immunoreactivities of the skin of an AD patient or healthy control (paraffin sections; **A**) and an AD-like mouse with dermatitis or a healthy mouse (frozen sections; **B**). The data on immunoreactive intensities of α-MSH and PC2 in epidermis of AD patients, AD-like mice, and healthy controls are shown in Supplemental Figure S3. $n = 3$ (**A**); $n = 4$ (**B**). Scale bars = 100 μm.

higher than that in healthy mice (Figure 2B). However, the expressions of *MC4R* and *MC5R* mRNA in the DRG of mice with dermatitis were significantly lower than those in healthy mice (Figure 2B).

In the skin of both healthy mice and those with dermatitis, MC1R and MC5R were mainly expressed in epidermal keratinocytes (Figure 2C), with higher expression in mice with dermatitis than in the healthy mice (Supplemental Figure S3, E and F). In the skin of mice with dermatitis, MC1R and MC5R were also expressed in the dermal cells (Figure 2C).

Effects of MC1R Antagonist Agouti-Signaling Protein

In mice with dermatitis treated repetitively with vehicle, spontaneous scratching increased gradually (Figure 3). However, repetitive administration of MC1R antagonist recombinant agouti-signaling protein[14,15] significantly inhibited the increase in spontaneous scratching in mice with dermatitis compared with vehicle treatment in these mice (Figure 3).

Scratching Induced by α-MSH in Healthy Mice and Effects of TXA_2 Receptor (TP Receptor) Antagonist on Behavior

An intradermal injection of α-MSH (100 nmol/site) elicited hind paw scratching in healthy mice, compared with vehicle-treated mice; the effect peaked during the first 10 minutes and almost subsided by 40 minutes (Figure 4A). The intradermal injection of α-MSH at a dose of 1 to 100 nmol/site induced scratching in a dose-dependent manner. It was found that α-MSH (100 nmol/site) elicited significant scratching compared with vehicle treatment (Figure 4B). In addition, this dose (100 nmol/site) of α-MSH did not induce any abnormal behavior except scratching. On visual examination of the injected skin, no signs of inflammation, flare, and hair removal at a couple of hours or days after the injection were observed. Interestingly, in this strain of mice, histamine (1 to 100 nmol/site) did not induce scratching (Figure 4B).

In mice with dermatitis, it has been reported that TXA_2 released from epidermal keratinocytes can induce scratching.[16] Because α-MSH and its receptors (MC1R and MC5R) were expressed in epidermal keratinocytes, the inhibitory effect of TP receptor antagonist ONO-3708 was demonstrated for α-MSH—induced scratching. ONO-3708 (100 mg/kg) significantly suppressed α-MSH—induced scratching compared with vehicle for ONO-3708 (Figure 4C). ONO-3708 did not affect 5-hydroxy tryptamine—induced scratching in mice (Supplemental Figure S4), suggesting that the inhibitory effect of ONO-3708 on α-MSH—induced scratching is not a sedative effect.

Production of TXA_2 Induced by α-MSH in Primary Cultures of Mouse Keratinocytes

The primary cultures of mouse keratinocytes showed immunoreactivity for TX synthase, MC1R, and MC5R (Figure 5A).

An *in vivo* study showed that intradermal α-MSH (100 nmol/50 μL = 2000 μmol/L) elicited scratching (Figure 4B). However, intradermal α-MSH (10 nmol/50 μL = 200 μmol/L) induced slight, but not significant, scratching compared with vehicle-injected mice (Figure 4B). Because α-MSH acted directly on cells in an *in vitro* study, 200 μmol/L of α-MSH was used as the final concentration in this study. α-MSH significantly induced the production of TXA_2, as determined by an increase in TXB_2 concentration, in mouse keratinocytes treated with control siRNA compared with those treated with control siRNA without α-MSH (Figure 5B). Treatment with MC1R siRNA or MC5R siRNA in mouse keratinocytes tended to inhibit α-MSH—induced TXA_2 production (Figure 5B and

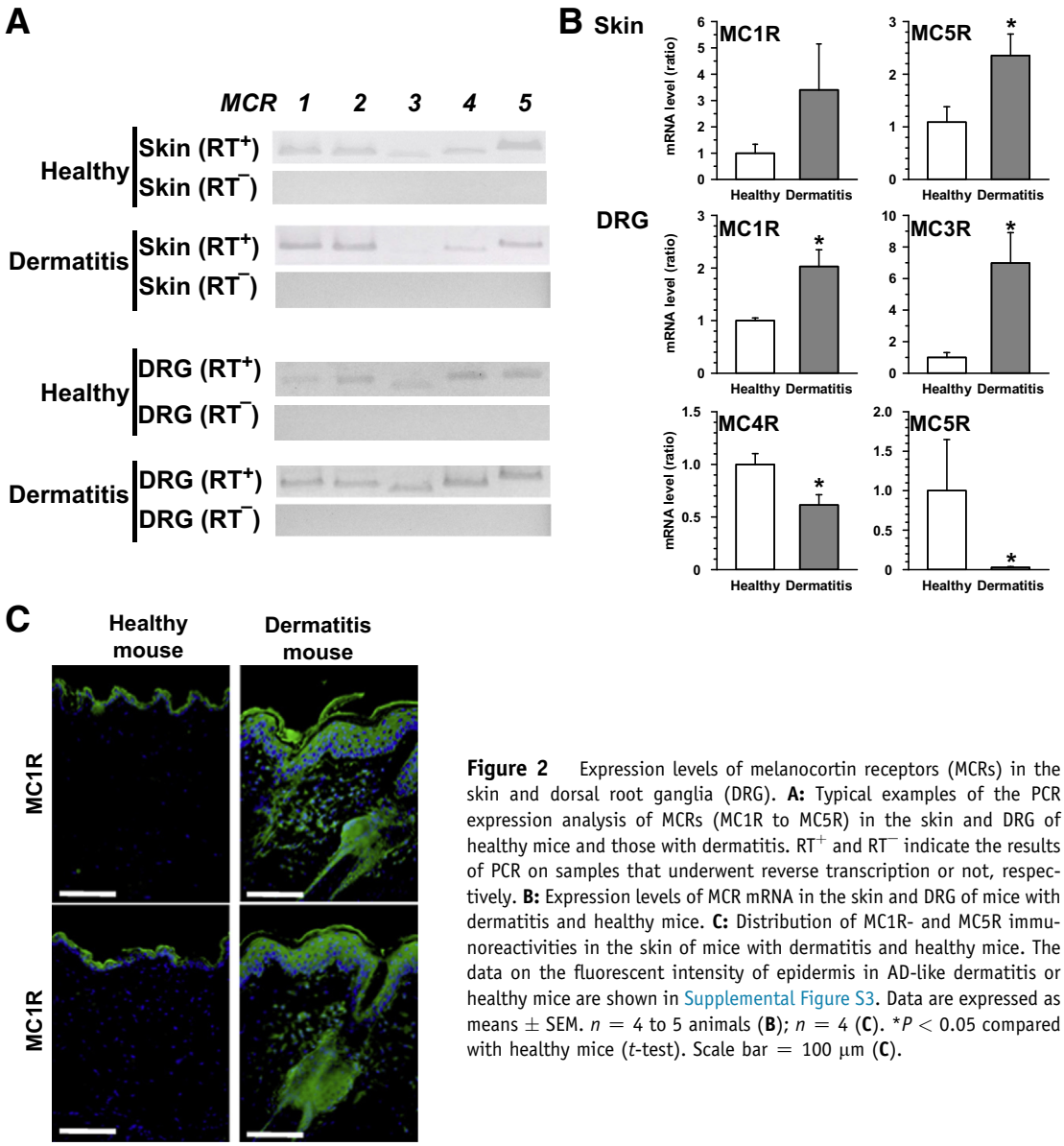


Figure 2 Expression levels of melanocortin receptors (MCRs) in the skin and dorsal root ganglia (DRG). **A:** Typical examples of the PCR expression analysis of MCRs (MC1R to MC5R) in the skin and DRG of healthy mice and those with dermatitis. RT^+ and RT^- indicate the results of PCR on samples that underwent reverse transcription or not, respectively. **B:** Expression levels of MCR mRNA in the skin and DRG of mice with dermatitis and healthy mice. **C:** Distribution of MC1R- and MC5R immunoreactivities in the skin of mice with dermatitis and healthy mice. The data on the fluorescent intensity of epidermis in AD-like dermatitis or healthy mice are shown in Supplemental Figure S3. Data are expressed as means ± SEM. $n = 4$ to 5 animals (**B**); $n = 4$ (**C**). $^*P < 0.05$ compared with healthy mice (*t*-test). Scale bar = 100 μm (**C**).

Supplemental Figure S5). α-MSH–induced TXA_2 production was significantly inhibited in mouse keratinocytes treated with both MC1R siRNA and MC5R siRNA, compared with mouse keratinocytes treated with control siRNA (Figure 5B and Supplemental Figure S5). Adenylyl cyclase inhibitor SQ-22536 (100 μmol/L) and calcium chelator EGTA (1 mmol/L) also significantly inhibited α-MSH–induced TXA_2 production (Figure 5B).

α-MSH–Induced Increase in Intracellular Ca^{2+} Ion Concentrations in Primary Cultures of Mouse DRG Neurons and Mouse Keratinocytes

α-MSH (200 μmol/L) markedly induced the concentration of intracellular Ca^{2+} ions in primary cultures of mouse DRG neurons (Figure 6A) and mouse keratinocytes (Figure 6B). The effects peaked at approximately 3 minutes after administration of α-MSH. Compared with vehicle treatment, α-MSH (200 μmol/L) significantly increased the concentration of intracellular Ca^{2+} ions in both DRG neurons and keratinocytes (Figure 6).

Discussion

In the skin of humans with AD and mice with dermatitis, α-MSH and PC2, which are related to the production of α-MSH, were mainly expressed in the epidermis. The immunoreactive intensities of α-MSH and PC2 were also higher than those of healthy controls. It is suggested that not only the increase in number of keratinocytes but also the

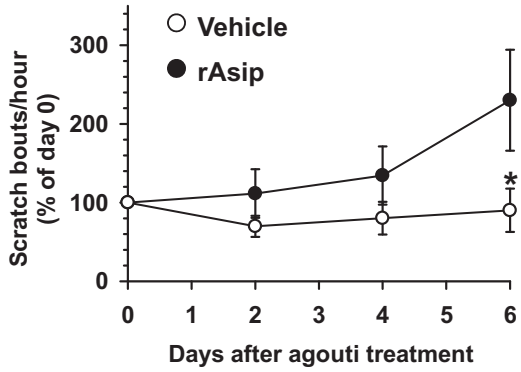


Figure 3 Effect of recombinant agouti-signaling protein (rAsip) on spontaneous scratching in mice with dermatitis. rAsip (2.5 μg; closed circle) or vehicle (open circle) was injected intraperitoneally in a volume of 50 μL once a day for 6 days. The behavioral observation was recorded before rAsip injection. Data are expressed as means ± SEM. $n = 7$ to 8 animals. $*P < 0.05$ compared with vehicle (Holm-Šidák multiple comparisons).

amount of their protein level in epidermis were increased in AD patients and mice with dermatitis. PC2 is an important enzyme for the production of α-MSH.[4] The cutaneous levels of PC2 were higher in AD patients and mice with dermatitis than in healthy controls. PC2 is activated by an increase in cAMP and Ca^{2+} levels.[17] cAMP and Ca^{2+} in keratinocytes are increased by some itch-related factors (such as prostaglandin E_2 and IL-31),[18,19] which are produced in the skin during atopic dermatitis.[20,21] In addition, α-MSH increases the production of cAMP[5] and intracellular Ca^{2+} through MCRs,[6] suggesting that α-MSH itself also enhances their production through the activation of PC2. Taken together, in AD, it is suggested that PC2 is activated. In the present study, an intradermal injection of α-MSH elicited scratching in healthy mice. Furthermore, spontaneous scratching in mice with dermatitis was inhibited with an MCR antagonist. Therefore, these findings suggest that α-MSH is involved in AD-associated itching.

Our recent study showed that α-MSH induces scratching through histamine from keratinocytes, but not from mast cells, in Institute of Cancer Research mice.[10] In NC/Nga mice, histamine did not induce scratching, as shown in this study and in another report.[22] Spontaneous scratching in mice with dermatitis was also not inhibited by an antihistamine.[11] In addition, H_1 receptor antagonist terfenadine and H_4 receptor antagonist JNJ7777120 did not inhibit α-MSH−induced scratching in NC/Nga mice (Supplemental Figure S6). Therefore, it is suggested that the role of histamine in α-MSH−induced and spontaneous scratching in mice of this strain (NC/Nga) is small. Therefore, mediators other than histamine may be involved in α-MSH−mediated scratching in both healthy mice and those with dermatitis.

TXA$_2$ is an itch mediator.[12] Furthermore, TXA$_2$ produced from epidermal keratinocytes is involved in spontaneous scratching in mice with dermatitis.[16] In this study, α-MSH−induced scratching was inhibited by a TP receptor antagonist. Therefore, it is suggested that TXA$_2$ plays an important role in α-MSH−mediated scratching in mice with dermatitis. TX synthase and α-MSH receptors (MC1R and MC5R) were colocalized in epidermal keratinocytes and

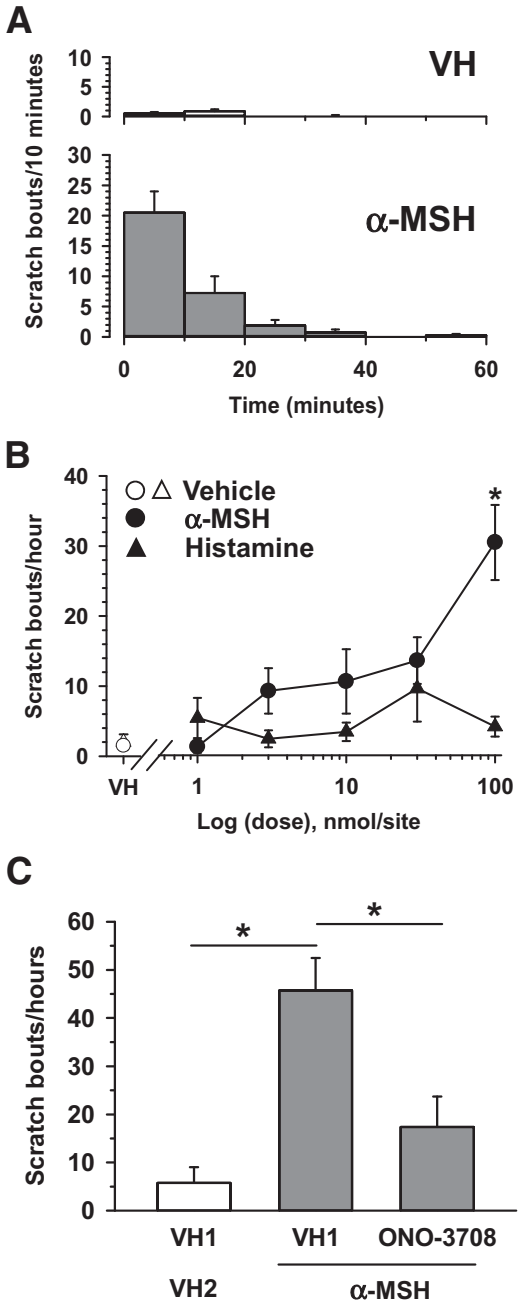


Figure 4 Scratching after intradermal injection of α-melanocyte−stimulating hormone (α-MSH) in healthy mice and the effects of TP receptor antagonist ONO-3708 on scratching. **A:** Time course of scratching after vehicle (VH; **top panel**) and α-MSH (100 nmol/site; **bottom panel**) injection. **B:** Dose-response curves for the scratching-induced effects of α-MSH and histamine. Healthy mice were administered an intradermal injection of α-MSH (closed circle), histamine (closed triangle), or VH (open circle and triangle). **C:** Suppressive effects of ONO-3708 on α-MSH−induced scratching. ONO-3708 (100 mg/kg) or VH1 was administered orally 1 hour before α-MSH (100 nmol/site) or VH2 injection. Data are expressed as means ± SEM (**A**−**C**). $n = 8$ animals (**A**−**C**). $*P < 0.05$ compared with VH (Holm-Šidák multiple comparisons).

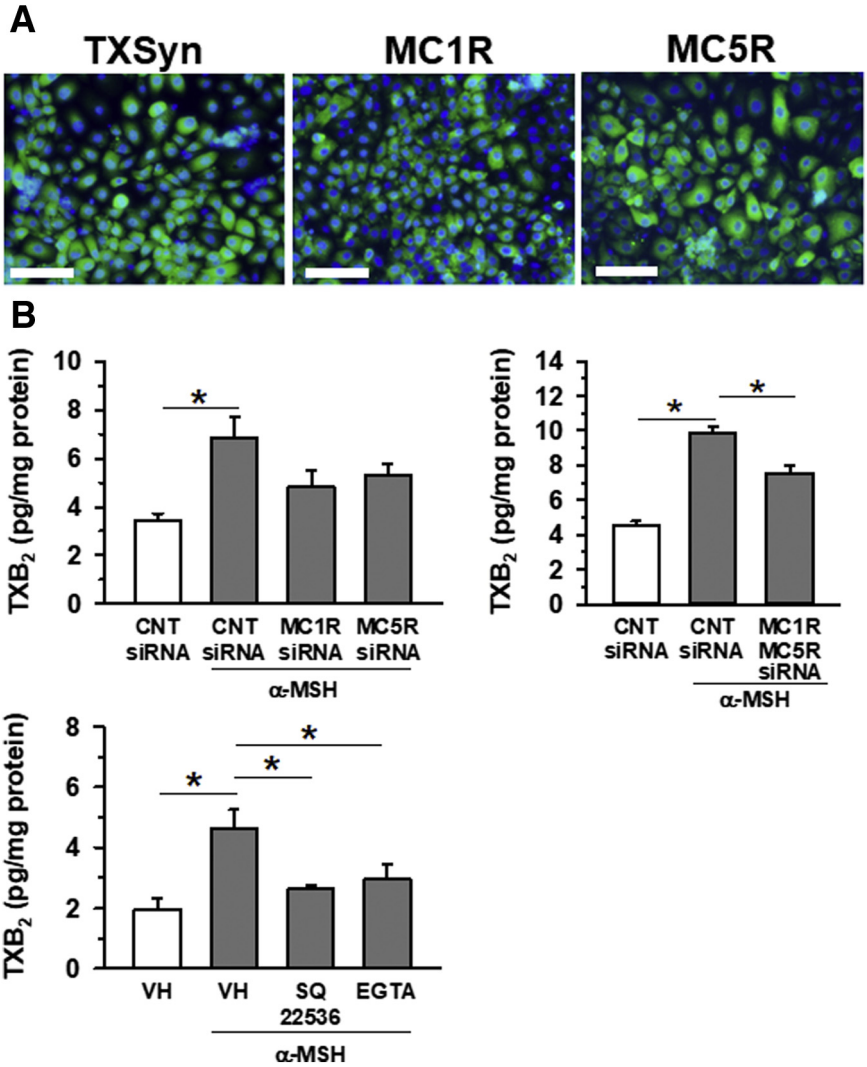


Figure 5 α-Melanocyte–stimulating hormone (α-MSH)–induced thromboxane (TX) A_2 production in primary cultures of mouse keratinocytes. **A:** Immunoreactivity of TX synthase (TXSyn) and melanocortin receptors (MC1R and MC5R) in mouse keratinocytes. **B:** α-MSH–induced TXA_2 production in mouse keratinocytes. α-MSH (200 μmol/L) was applied to mouse keratinocytes treated with siRNA for MC1R, MC5R, or nonspecific control (CNT) siRNA. SQ-22536 (100 μmol/L), EGTA (1 mmol/L), and vehicle (VH; 0.1% dimethyl sulfoxide) were administered 30 minutes before α-MSH (200 μmol/L) application. The culture medium was collected 10 minutes after α-MSH treatment. Because TXA_2 alters spontaneously to form inactive TXB_2, the TXB_2 content in the assay medium was measured by an enzyme-linked immunosorbent assay. The TXB_2 content was normalized to the protein extracted from keratinocytes. Data are expressed as means ± SEM. $n = 6$ to 12 wells (**B**). $^*P < 0.05$ (Holm-Šidák multiple comparisons). Scale bar = 100 μm (**A**).

α-MSH–induced TXA_2 production through MC1R and MC5R on the basis of the results of siRNA inhibition. Therefore, it is suggested that keratinocytes are the main TXA_2-producing cells in the skin, and that both MC1R and MC5R play an important role in the production of TXA_2. However, α-MSH–induced TXA_2 production was not suppressed, despite the complete suppression of *MC1R* and *MC5R* mRNA expression by siRNA (Supplemental Figure S5). Because primary cultures of keratinocytes also expressed *MC3R* and *MC4R* mRNA, these receptors may be involved in the actions of α-MSH. However, in the skin of mice with dermatitis, MC1R and MC5R for α-MSH are mainly expressed and the binding affinity to these receptors for α-MSH is high.[4] Therefore, it has been suggested that these receptors are important for TXA_2 production by α-MSH under pathologic conditions. However, the mechanisms of α-MSH–induced TXA_2 production are not completely understood. Prostaglandin H_2, which is produced through the oxidation of arachidonic acid catalyzed by cyclooxygenase, is a substrate of TX synthase for the production of TXA_2. An increase in intracellular Ca^{2+} ions activates phospholipase A_2 and accelerates the production of arachidonic acid.[23] In this study using primary cultures of keratinocytes, α-MSH increased intracellular concentration of Ca^{2+} ions, and α-MSH–induced TXA_2 production was inhibited by the Ca^{2+} ion chelator EGTA. Therefore, our findings suggest that increased intracellular levels of Ca^{2+} ions are involved in α-MSH–induced TXA_2 production through MC1R and MC5R. The activation of MC1R and MC5R also increases the production of cAMP.[5] α-MSH–induced TXA_2 was also inhibited by adenylyl cyclase inhibitors, which reduces the production of cAMP. However, the mechanisms of cAMP-dependent TXA_2 production remain unclear. Therefore, underlying mechanisms will be demonstrated in a future study.

α-MSH elicits scratching through the production of TXA_2, which, in turn, activates TP receptors.[24] TP receptors are expressed in primary afferent neurons, and the activation of TP receptors increases the concentration of intracellular Ca^{2+} ions.[12,16] Therefore, TXA_2 produced from α-MSH–stimulated keratinocytes directly acts on primary afferent neurons and elicits scratching.

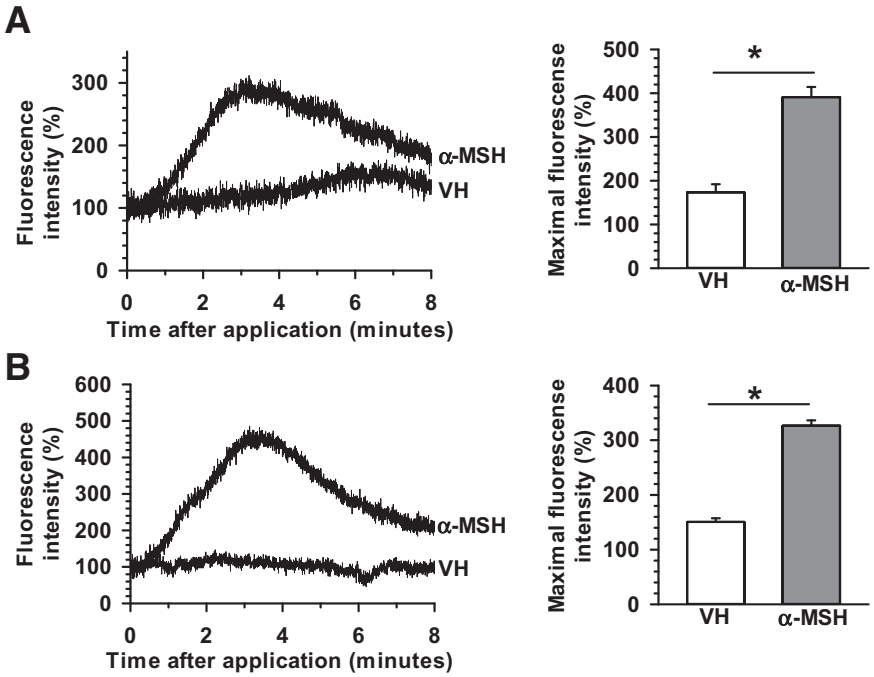


Figure 6 α-Melanocyte−stimulating hormone (α-MSH) acts on dorsal root ganglion (DRG) neurons and keratinocytes to increase intracellular Ca^{2+} ions. Typical examples of the time courses of vehicle (VH)−induced or α-MSH−induced changes in the concentrations of intracellular Ca^{2+} ions (fluorescence) in DRG neurons (**A**) and keratinocytes (**B**). Maximal change in VH- or α-MSH−induced increase in intracellular Ca^{2+} ions in DRG neurons and keratinocytes. Primary cultures of mouse DRG neurons and keratinocytes were preloaded with fluo-3. α-MSH was added to the culture medium at a final concentration of 200 μmol/L. Data are expressed as means ± SEM. $n = 32$ (**A**, VH group); $n = 49$ (**A**, α-MSH group); $n = 43$ (**B**, VH group); $n = 102$ (**B**, α-MSH group). $^*P < 0.05$ (*t*-test).

The DRG is an accumulation of cell bodies of primary afferent neurons. α-MSH increased the concentration of intracellular Ca^{2+} ions in primary cultures of DRG neurons. It is possible that α-MSH also acts directly on primary afferents and elicits scratching. All subtypes of *MCR* mRNA were expressed in the DRG of mice with dermatitis. Expressions of *MC1R* and *MC3R* mRNA, but not *MC4R* and *MC5R* mRNA, in the DRG of mice with dermatitis were higher than those in healthy mice, suggesting that MC1R and MC3R expressed in primary afferents may be involved in α-MSH−mediated scratching in mice with dermatitis. However, in addition to the DRG in mice with atopy-like dermatitis, all subtypes of *MCR* mRNA were expressed in the primary cultures of DRG neurons, which were obtained from healthy mice (Supplemental Figure S7). Therefore, when using healthy mice, α-MSH may elicit scratching through all subtypes of melanocortin receptors expressed in primary afferents. Recently, it has been reported that two types of primary afferents are related to the transmission of itching, such as histaminergic (eg, expressions of histamine H_1 and H_4 receptors) and nonhistaminergic (eg, expressions of Mas-related G-protein−coupled receptor A3 and proteinase-activating receptor 2) neurons.[25] However, in this study, the expression of melanocortin receptor subtypes in these itch-related primary afferents was not observed. Therefore, the distribution of MCRs in these primary afferents should be determined in a future study.

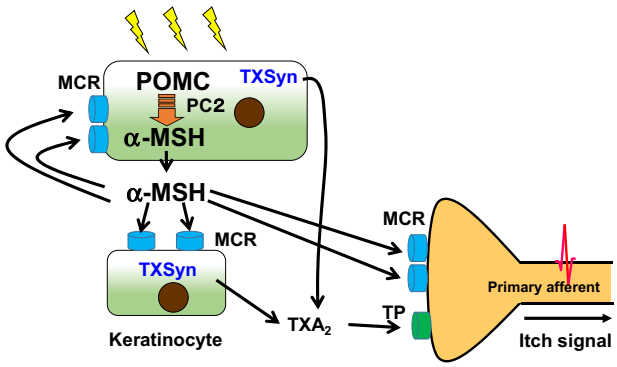


Figure 7 Schematic of the possible mechanisms of α-melanocyte−stimulating hormone (α-MSH)−mediated itching in atopic dermatitis. Keratinocytes express prohormone convertase 2 (PC2) related to the processing of proopiomelanocortin (POMC) and produce α-MSH. α-MSH binds to melanocortin receptors (MCRs; mainly MC1R and MC5R) in keratinocytes, which express thromboxane synthase (TXSyn) and produce TXA$_2$. TXA$_2$ acts on TP receptors in primary afferents to produce itch signals. α-MSH also binds directly to MCRs (MC1R and MC3R) on primary afferents to produce itch signals.

Immunoreactivities of MC1R and MC5R were detected not only in the epidermis but also in the dermis of mice with dermatitis. Mast cells, T cells, and macrophages are increased in the dermis of mice with dermatitis.[26−28] It has been reported that MC1R and MC5R are expressed in these immune cells: (MC1R) T cells,[29] macrophages,[29] mast cells,[30] and (MC5R) T cells.[31] MC1R and/or MC5R expressed in these immune cells may contribute to α-MSH−mediated itching. A future study will demonstrate the role of MCR-expressing cells in AD-related itching.

TXA$_2$ from keratinocytes is involved in spontaneous scratching in mice with dermatitis.[16] This study demonstrated, for the first time, that α-MSH, an endogenous factor, was involved in TXA$_2$ production in keratinocytes of mice with dermatitis. However, the degree of inhibition of itch-related responses was different between α-MSH−induced

scratching in healthy mice and spontaneous scratching in mice with dermatitis. The antipruritic effect of ONO-3708 against α-MSH−induced scratching in healthy mice was more effective than that against spontaneous scratching in mice with dermatitis. Therefore, these findings suggest that not only α-MSH but also other endogenous factors in the skin are involved in TXA_2 production related to spontaneous scratching in mice with dermatitis.

In mice with dermatitis (NC/Nga), not only α-MSH but also proopiomelanocortin-derived ACTH, known as α-MSH precursor-peptide, are increased in plasma, compared with healthy mice.[9] It was reported that ACTH acts mainly through MC2R.[4,5] In this study, *MC2R* mRNA was expressed in the skin and DRG of mice with dermatitis. In addition, primary cultures of keratinocytes and DRG neurons also expressed *MC2R* mRNA (Supplemental Figure S7). Therefore, ACTH may induce itching through keratinocytes and/or through direct action on primary afferents. However, it is not clear whether ACTH itself elicits itching. This point of view will be investigated in our future studies.

The endogenous factors related to the production of ACTH and α-MSH remain unclear in mice with dermatitis. As a possible endogenous factor, nitric oxide is known.[32] Nitric oxide is also involved in spontaneous scratching in mice with dermatitis (NC mice).[33] Therefore, nitric oxide may be involved in ACTH- and/or α-MSH−mediated scratching in mice with dermatitis.

In summary, this study demonstrated that α-MSH, produced from keratinocytes, was involved in spontaneous scratching in a mouse model of dermatitis, although the mechanisms of production and activation of PC2 related to the production of α-MSH remain unclear. It is unclear whether the results of *in vitro* experiments using cells directly lead to the mechanisms of itching. However, because α-MSH acts on keratinocytes and DRG neurons, it is suggested that the scratching may be elicited through the direct activation of primary afferents and the production of TXA_2 via the autocrine/paracrine system in keratinocytes (Figure 7). Considering the distribution of α-MSH and the inhibitory action of TP antagonist on α-MSH−induced scratching, we suggest that the action of α-MSH on keratinocytes may be more important than that on primary afferents. These findings further suggest that α-MSH production−related enzymes and receptors may be target molecules for the development of new antipruritic drugs.

Supplemental Data

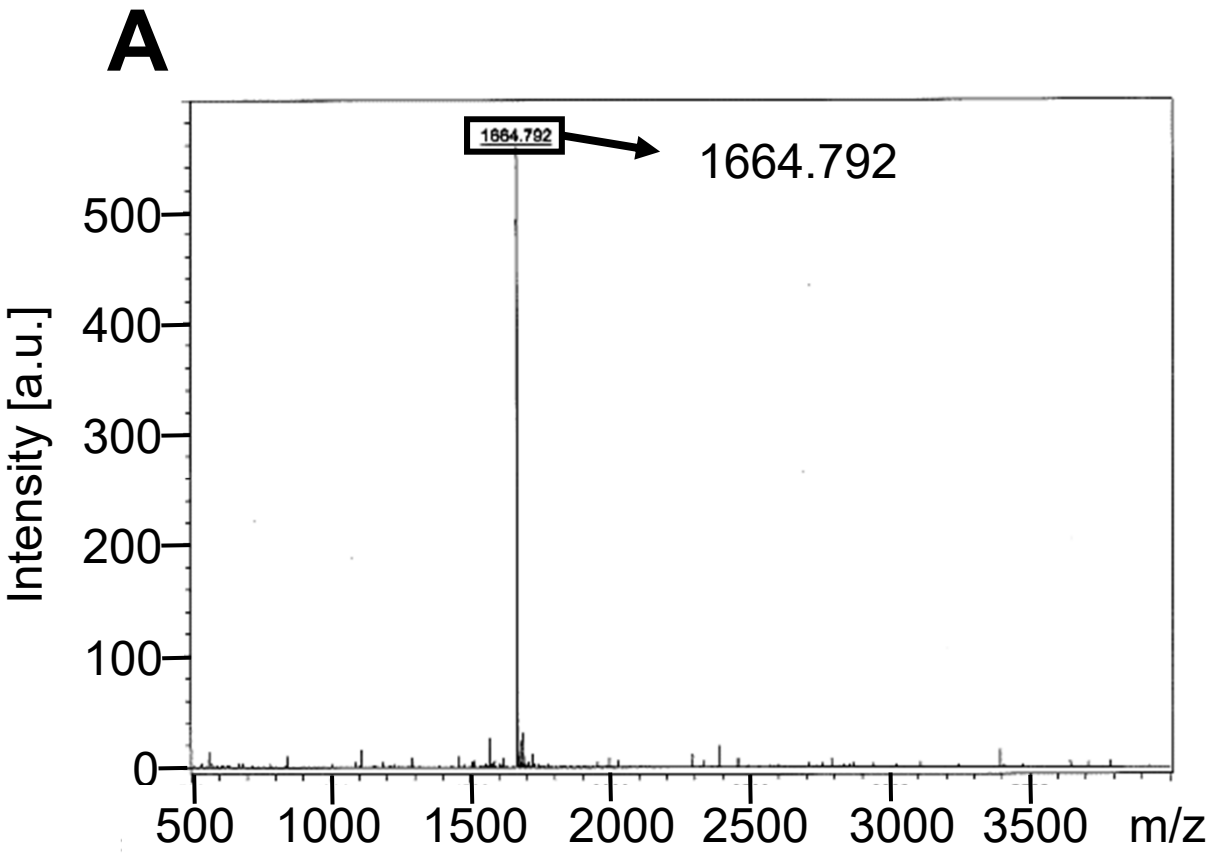
Supplemental material for this article can be found at *http://doi.org/10.1016/j.ajpath.2019.05.017*.

References

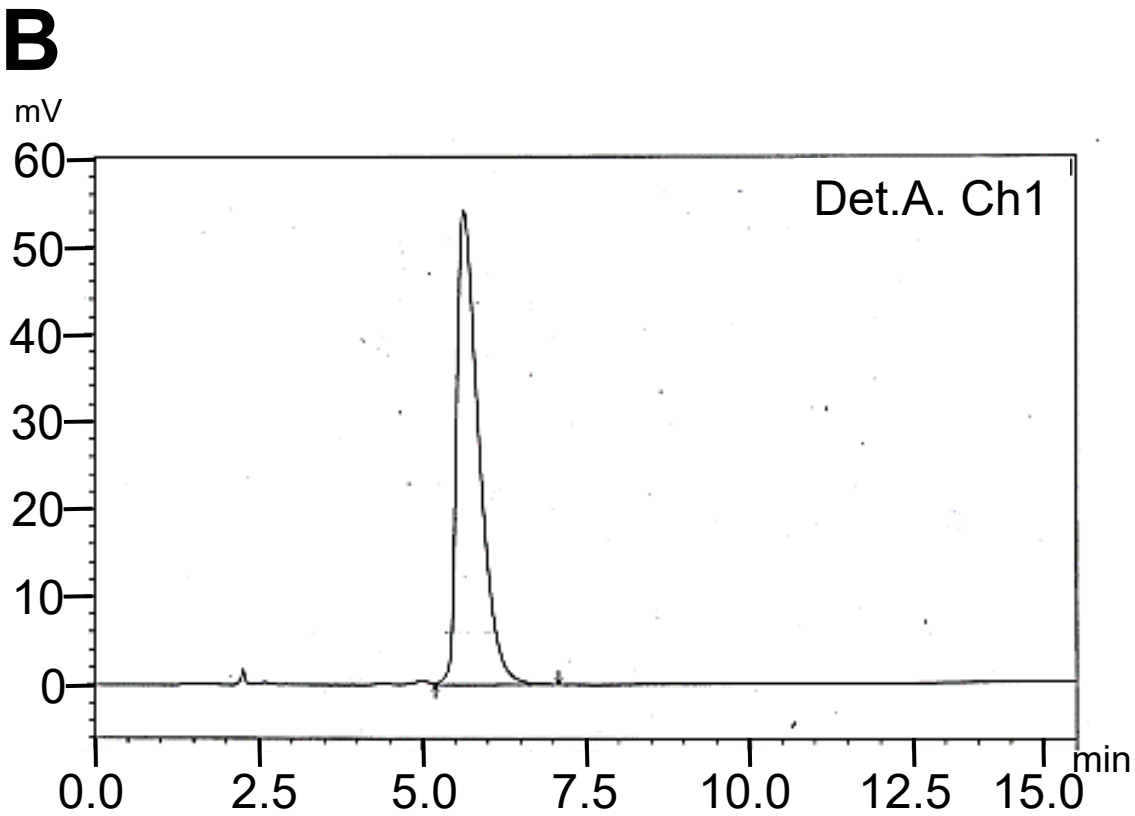
1. Bos JD, Brenninkmeijer EE, Schram ME, Middelkamp-Hup MA, Spuls PI, Smitt JH: Atopic eczema or atopiform dermatitis. Exp Dermatol 2010, 19:325−331
2. Wahlgren CF: Itch and atopic dermatitis: an overview. J Dermatol 1999, 26:770−779
3. Wahlgren CF: Itch and atopic dermatitis: clinical and experimental studies. Acta Derm Venereol Suppl (Stockh) 1991, 165:1−53
4. Millington GW: Proopiomelanocortin (POMC): the cutaneous roles of its melanocortin products and receptors. Clin Exp Dermatol 2006, 31:407−412
5. Cooray SN, Clark AJ: Melanocortin receptors and their accessory proteins. Mol Cell Endocrinol 2011, 331:215−221
6. Mountjoy KG, Kong PL, Taylor JA, Willard DH, Wilkison WO: Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. Physiol Genomics 2001, 5:11−19
7. Böhm M, Luger TA, Tobin DJ, García-Borrón JC: Melanocortin receptor ligands: new horizons for skin biology and clinical dermatology. J Invest Dermatol 2006, 126:1966−1975
8. Gantz I, Fong TM: The melanocortin system. Am J Physiol Endocrinol Metab 2003, 284:E468−E474
9. Hiramoto K, Kobayashi H, Ishii M, Sato E, Inoue M: Increased alpha-melanocyte-stimulating hormone (alpha-MSH) levels and melanocortin receptors expression associated with pigmentation in an NC/Nga mouse model of atopic dermatitis. Exp Dermatol 2010, 19:132−136
10. Shimizu K, Andoh T, Yoshihisa Y, Shimizu T: Histamine released from epidermal keratinocytes plays a role in α-melanocyte-stimulating hormone-induced itching in mice. Am J Pathol 2015, 185:3003−3010
11. Yamaguchi T, Maekawa T, Nishikawa Y, Nojima H, Kaneko M, Kawakita T, Miyamoto T, Kuraishi Y: Characterization of itch-associated responses of NC mice with mite-induced chronic dermatitis. J Dermatol Sci 2001, 25:20−28
12. Andoh T, Nishikawa Y, Yamaguchi-Miyamoto T, Nojima H, Narumiya S, Kuraishi Y: Thromboxane A_2 induces itch-associated responses through TP receptors in the skin in mice. J Invest Dermatol 2007, 127:2042−2047
13. Kuraishi Y, Nagasawa T, Hayashi K, Satoh M: Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. Eur J Pharmacol 1995, 275:229−233
14. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD: Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 1994, 371:799−802
15. Suzuki I, Tada A, Ollmann MM, Barsh GS, Im S, Lamoreux XL, Hearing VJ, Nordlund JJ, Abdel-Malek ZA: Agouti signaling protein inhibits melanogenesis and the response of human melanocytes to α-melanotropin. J Invest Dermatol 1997, 108:838−842
16. Andoh T, Yamamoto A, Haza S, Yuhki KI, Ushikubi F, Narumiya S, Kuraishi Y: Thromboxane A_2 is involved in itch-associated responses in mice with atopic dermatitis-like skin lesions. Acta Derm Venereol 2016, 96:899−904
17. Li QL, Naqvi S, Shen X, Liu YJ, Lindberg I, Friedman TC: Prohormone convertase 2 enzymatic activity and its regulation in neuroendocrine cells and tissues. Regul Pept 2003, 110:197−205
18. Kanda N, Mitsui H, Watanabe S: Prostaglandin E_2 suppresses CCL27 production through EP2 and EP3 receptors in human keratinocytes. J Allergy Clin Immunol 2004, 114:1403−1409
19. Andoh T, Li S, Uta D: Involvement of thromboxane A_2 in interleukin-31-induced itch-associated response in mice. Pharmacol Rep 2018, 70:251−257
20. Fogh K, Herlin T, Kragballe K: Eicosanoids in skin of patients with atopic dermatitis: prostaglandin E2 and leukotriene B4 are present in biologically active concentrations. J Allergy Clin Immunol 1989, 83:450−455
21. Cevikbas F, Wang X, Akiyama T, Kempkes C, Savinko T, Antal A, Kukova G, Buhl T, Ikoma A, Buddenkotte J, Soumelis V, Feld M, Alenius H, Dillon SR, Carstens E, Homey B, Basbaum A, Steinhoff M: A sensory neuron-expressed IL-31 receptor mediates T helper cell-dependent itch: involvement of TRPV1 and TRPA1. J Allergy Clin Immunol 2014, 133:448−460

22. Inagaki N, Nagao M, Igeta K, Kawasaki H, Kim JF, Nagai H: Scratching behavior in various strains of mice. Skin Pharmacol Appl Skin Physiol 2001, 14:87−96
23. Clark JD, Milona N, Knopf JL: Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. Proc Natl Acad Sci U S A 1990, 87:7708−7712
24. Narumiya S, Sugimoto Y, Ushikubi F: Prostanoid receptors: structures, properties, and functions. Physiol Rev 1999, 79:1193−1226
25. Green D, Dong X: The cell biology of acute itch. J Cell Biol 2016, 213:155−161
26. Matsuda H, Watanabe N, Geba GP, Sperl J, Tsudzuki M, Hiroi J, Matsumoto M, Ushio H, Saito S, Askenase PW, Ra C: Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 1997, 9:461−466
27. Oshio T, Sasaki Y, Funakoshi-Tago M, Aizu-Yokota E, Sonoda Y, Matsuoka H, Kasahara T: Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment. Int Immunopharmacol 2009, 9:403−411
28. Tsujii K, Andoh T, Ui H, Lee JB, Kuraishi Y: Involvement of tryptase and proteinase-activated receptor-2 in spontaneous itch-associated response in mice with atopy-like dermatitis. J Pharmacol Sci 2009, 109:388−395
29. Lipton JM, Catania A: Anti-inflammatory actions of the neuroimmunomodulator alpha-MSH. Immunol Today 1997, 18:140−145
30. Adachi S, Nakano T, Vliagoftis H, Metcalfe DD: Receptor-mediated modulation of murine mast cell function by alpha-melanocyte stimulating hormone. J Immunol 1999, 163:3363−3368
31. Taylor A, Namba K: In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH). Immunol Cell Biol 2001, 79:358−367
32. Orita K, Hiramoto K, Kobayashi H, Ishii M, Sekiyama A, Inoue M: Inducible nitric oxide synthase (iNOS) and α-melanocyte-stimulating hormones of iNOS origin play important roles in the allergic reactions of atopic dermatitis in mice. Exp Dermatol 2011, 20:911−914
33. Tsukumo Y, Andoh T, Yamaguchi T, Nojima H, Kuraishi Y: Involvement of nitric oxide in itch-scratch response of NC mice. Nihon Yakurigaku Zasshi 1999, 114:17P−21P [Japanese: abstract in English]

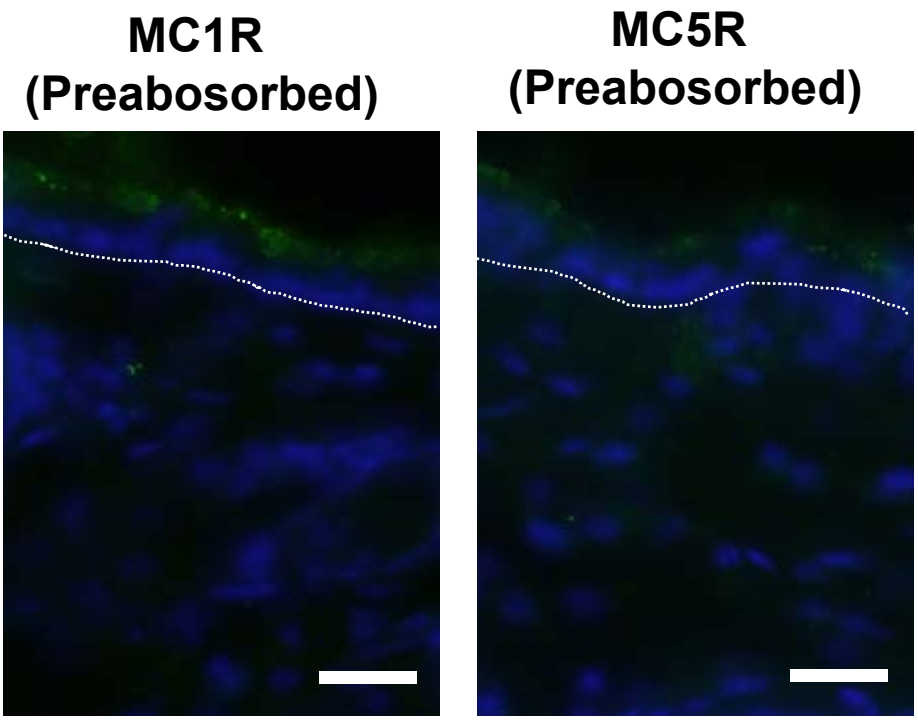
Supplemental Figure S1 (Andoh et al.)



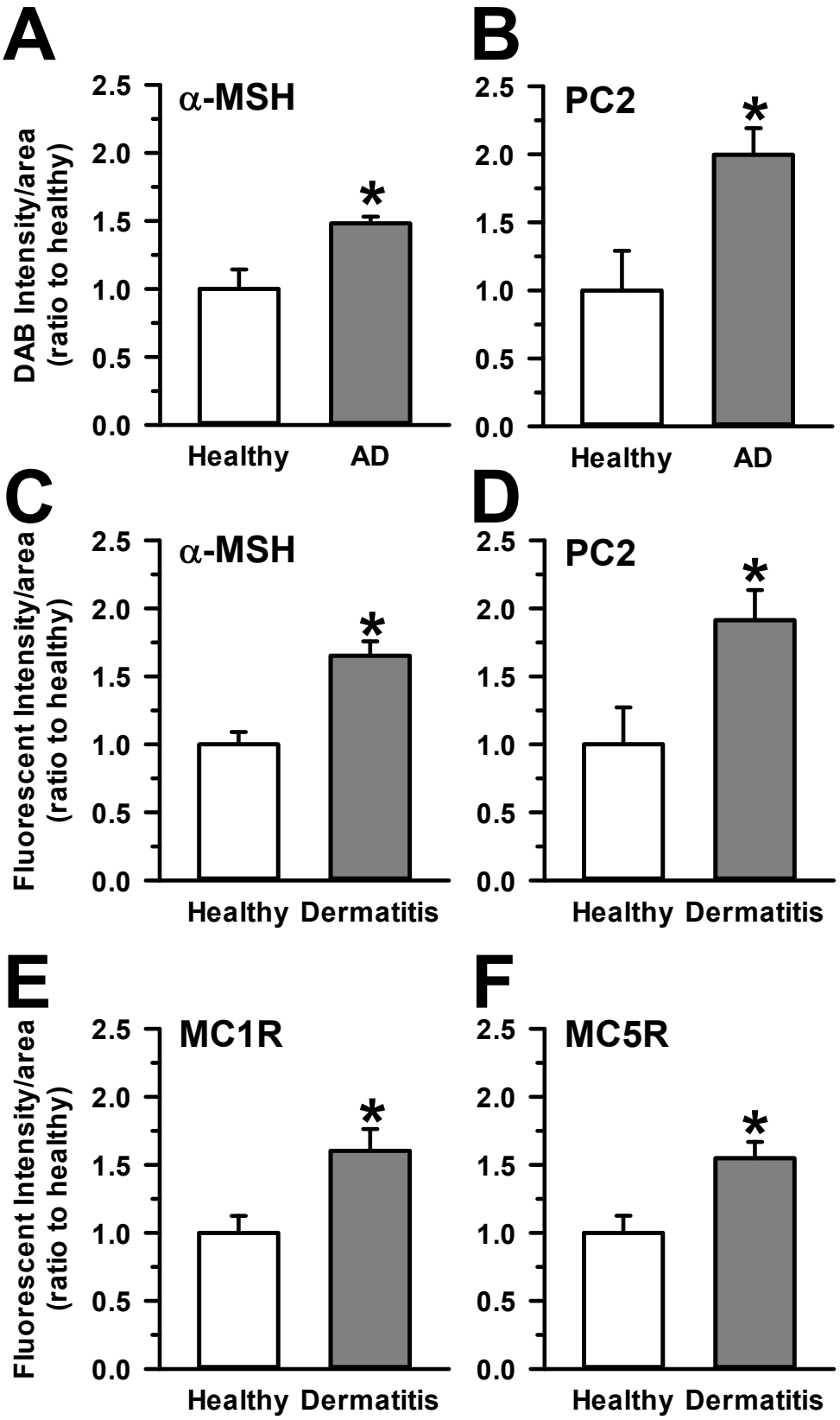
m/z	S/N	Quality Fac.	Res.	Intens.	Area
166.792	22	42581	5958	558	469



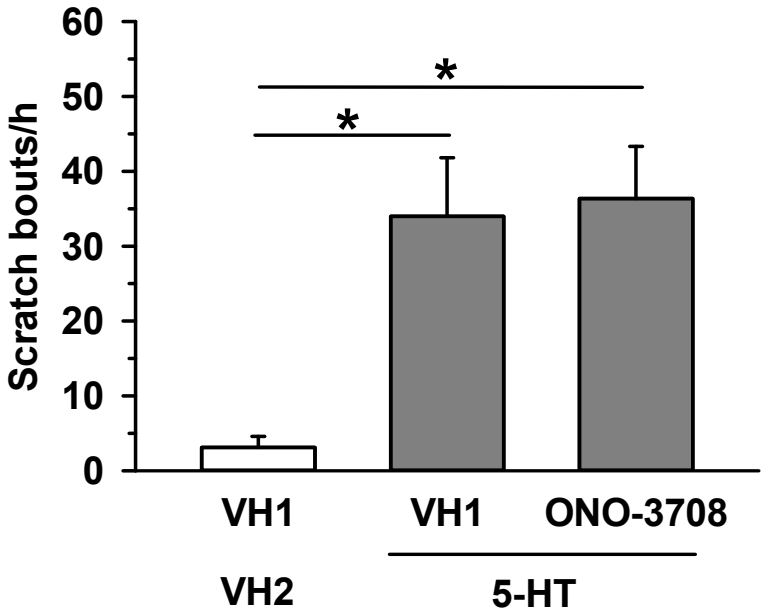
Supplemental Figure S2 (Andoh et al.)



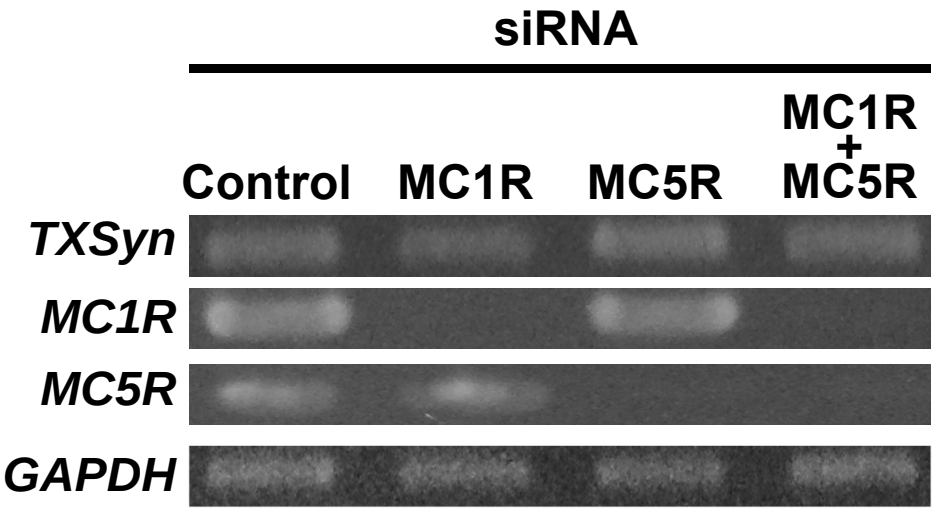
Supplemental Figure S3 (Andoh et al.)



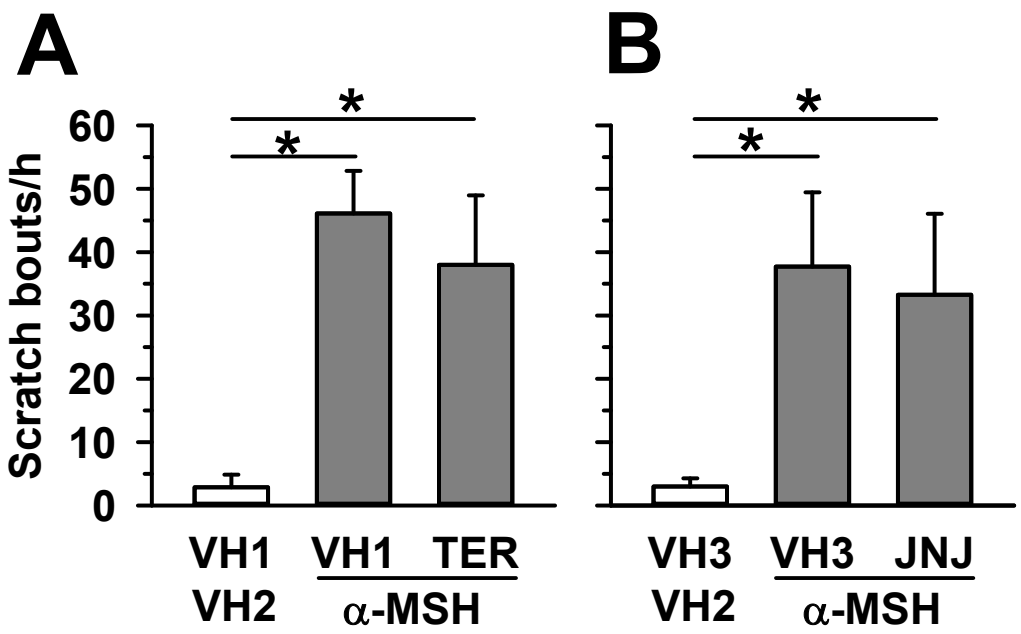
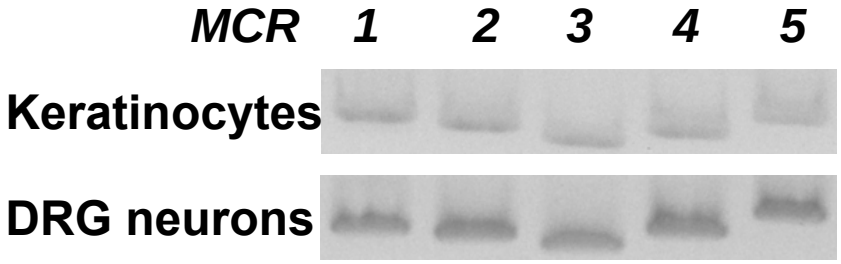
Supplemental Figure S4 (Andoh et al.)



Supplemental Figure S5 (Andoh et al.)



Supplemental Figure S6 (Andoh et al.)



Supplemental Figure S7 (Andoh et al.)