# The involvement of histamine released from epidermal keratinocytes in α-melanocyte-stimulating hormone-induced itching

α−メラノサイト刺激ホルモン誘発掻痒発生への
表皮ケラチノサイト遊離ヒスタミンの関与

富山大学大学院　医学薬学教育部（博士課程）
東西統合医学専攻　皮膚科学講座
清水教子

## CONTENTS

## INTRODUCTION

Itch is an unpleasant sensation associated with the immediate desire to scratch, thereby making the cutaneous symptoms worse. Several skin and general conditions are associated with both pigmentation and itch. In addition, itch associated with sunburn is widely recognized to be the result of an inflammatory reaction to ultraviolet (UV) irradiation, which increases the amount of itch and erythema, after which the healing process results in later skin pigmentation [1]. Gilchrest *et al.* [2] reported that the histamine level rises immediately after the onset of UV-induced erythema. Itch and cutaneous pigmentation also occur in association with chronic wounds and hypertrophic scars. Paul *et al.* [3] noted that wound-related itch is more frequently observed in patients with severe wounds. Itch may also be caused by dry skin or serious internal diseases, such as chronic renal failure requiring hemodialysis, which is usually accompanied by cutaneous pigmentation. Taken together, it is still unclear if the underlying mechanisms of itch in these pruritic diseases involve the interaction between cutaneous pigmentation and itch.

α-Melanocyte–stimulating hormone (α-MSH) is one of the neuropeptides that is generated through the cleavage of a precursor protein called proopiomelanocortin (POMC), which is produced through the production of corticotropin-releasing hormone

(CRH), also known as corticotropin-releasing factor (CRF) after various stressors, such as UV irradiation [4-7]. α-MSH receptors are known as melanocortin receptors (MC1R, MC3R, MC4R, and MC5R) [4]. These receptors belong to G protein-coupled receptor family and the activation of these receptors increases the production of cAMP [8]. In addition, it has been also reported that α-MSH increases intracellular free $Ca^{2+}$ concentration in HEK cells expressing these melanocortin receptors [9]. In the skin, MC1R and MC5R are mainly expressed [10]. α-MSH produced by external stimuli, such as UV irradiation, induces cutaneous pigmentation through the activation of MC1R [4,6,7,10,11]. α-MSH is also increased in plasma of patients with chronic hemodialysis [12] and in epidermal keratinocytes during cutaneous wound repair [13]. However, there are no previous reports regarding whether α-MSH is also involved in the itching that results from these factors.

Here, we investigated whether α-MSH elicited itch in mice. Furthermore, we performed a series of experiments to elucidate the mechanism underlying the development of α-MSH–induced itch, focusing on the involvement of histamine, which is known to play an important role in the pathogenesis of itch.

## MATERIALS AND METHODS

### Animals

Male ICR mice (four to nine weeks old), mast cell–deficient WBB6F1 *W/W*$^{v}$ (six to nine weeks old), and the normal littermates (WBB6F1$^{+/+}$, six to nine weeks old) were used in this study. These mice were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a room with controlled temperature (21 to 23 °C), humidity (45% to 65%), and light (7:00 AM to 7:00 PM) conditions. Food and water were available *ad libitum*. Procedures for animal experiments were approved by the committee for animal experiments at the University of Toyama.

### Cell culture and siRNA treatment

The murine epidermal cell line (Pam212) was cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum as well as 100 U/mL of penicillin and 100 μg/mL of streptomycin under standard cell culture conditions (37 °C, 5% $CO_2$ in a humidified incubator).

In a part of the experiment, siRNA-treated cells were used. siRNAs as a control, MC1R and MC5R were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). These siRNAs were transfected with Lipofectamin$^{TM}$ RNAi MAX reagent (Life

Technology, Inc., Carlsbad, CA). The transfection was performed according to the manufacturer's protocol (Life Technology, Inc.).

**Agents**

α-MSH was purchased from Peptide Institute, INC (Osaka, Japan). For *in vivo* experiments, α-MSH was dissolved in physiological saline and was injected intradermally in a volume of 50 μL into the rostral skin of mice. For *in vitro* experiments, α-MSH was dissolved in OPTI-MEM (Thermo Fisher Scientific Inc., Waltham, MA). Naltrexon hydrochloride (Sigma-Aldrich, St. Louise, MO) was dissolved in physiological saline and injected subcutaneously 15 minutes before α-MSH injection. Terfenadine (Sigma-Aldrich) was suspended in 0.5% sodium carboxy methylcellulose (Wako Pure Chemical Ind., Osaka, Japan) and was administered 30 min before α-MSH injection. SQ 22,536 (Tocris Bioscience, Bristol, UK) and ethylene glycol tetraacetic acid (EGTA) (Dojindo Lab., Kumamoto, Japan) were dissolved in dimethyl sulfoxide and diluted with OPTI-MEM (final concentration DMSO: 0.1%). These agents were treated 30 min before the application of α-MSH.

**Behavioral experiments**

The day before the experiment, the hair was clipped over the rostral part of mouse back. Prior to behavioral observation, the animals were placed individually in an acrylic cage composed of four compartments (13×9×35 cm) for at least 1 hour for acclimation. Immediately after intradermal injection, mice were put back into the same cells and their behaviors were recorded using a digital video camera (HDC-TM25, Panasonic Co., Osaka, Japan) for 1 hour with personnel kept out of the observation room. Playback of the digital recording allowed for counting of injection site scratching by hind paw. The series of movements scratched several times for about 1 sec were considered as one bout of scratching [14].

**Immunostaining**

Under anaesthesia with pentobarbital (80 mg/kg, intraperitoneal), mice was transcardially perfused with PBS and then 4% paraformaldehyde (PFA). The skin of the rostral back was isolated, postfixed with 4% PFA, and immersed in 30% sucrose solution for 2 days. The tissue was embedded in Tissue-Tek® O.C.T. Compound (Sakura Fineteck Co., Ltd., Tokyo, Japan) and kept at -80 °C until use. The frozen samples were sectioned at 20 μm with a cryostat (Leica, Wetzlar, Germany). In keratinocyte cell line Pam212, cells cultured on glass-bottomed dishes were washed

twice with phosphate-buffered saline (PBS) and fixed with 4% PFA. After being washed three times with PBS, the sections or cells were treated with 0.3% Triton X-100 in PBS and then with 0.25% fetal bovine serum to block non-specific immunoglobulin binding. The sections or cells were treated with the first antibodies at a dilution of 1/100 at 4°C overnight; the antibodies used were rabbit antibodies against HDC (Santa Cruz Biotechnology Inc.) and histamine (Abcam., Cambridge, UK), and goat antibodies against MC1R, MC5R, and mast cell protease 5 (Santa Cruz Biotechnology Inc.). After washing, the preparations were incubated with Alexa Fluor 555-conjugated anti-goat IgG (Life Technologies, Carlsbad, CA) for 1 hour at room temperature. The sections and cells were rinsed in PBS after each treatment. Finally, the sections and cells were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Immunofluorescence was visualized using a laser scanning confocal microscope (Leica Microsystems, Tokyo, Japan) or a fluorescent microscope (Olympus Co., Tokyo, Japan). After scanning, the slide glass was washed with PBS. The skin section was stained with toluidine blue and observed using light microscope (Olympus Co.).

For a portion of the immunostaining, we used the antibody preabsorbed with the antigen peptides as a negative control. The antigen peptides for HDC, MC1R, and MC5R were purchased from Santa Cruz Biotechnology, Inc. The preparation of the antibody preabsorbed

with the antigen peptides was performed according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc.).

**Reverse transcription- PCR**

Total RNA was extracted from cultured Pam212 cells. The cell samples were lyzed with the TRIzol® reagent (Invitrogen) for RNA preparation. Total RNA (0.4 μg/sample) was used for cDNA synthesis with oligo $(dT)_{16}$ primers and reverse transcriptase (Reverscript III, Wako Pure Chemical Ind.). cDNA was amplified using the following primers: MC1R 5'-GCCCACATGTTCACGAGAGC -3' (forward) and 5'- AGTTACCC TTTCTCCTGGCCC -3' (reverse); MC5R: 5'- AAATCCGATGCCAAGAAGTG -3' (forward) and 5'-GGTAGCGCAAGGCATAGAAG -3' (reverse); HDC: 5'-AGCACAA GCTGTCGTCCTTT -3' (forward) and 5'-GTGGATCACGAAGACCCTGT-3' (reverse). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control. The primers used for GAPDH were 5-ACCCAGAAGACTGTGGAT-3 (forward) and 5'-TCGTTGAGGGCAATGCCA-3' (reverse). The cycling conditions were 5 min at 94 °C, 35 cycles of 30 sec at 94 °C, 30 sec at 58 °C, and 30 sec at 72 °C, and 7 min at 72 °C. After PCR, the amplified products were analyzed by 2% agarose gel electrophoresis.

**Western blot analysis**

Proteins were extracted from cultured Pam 212 cells with a lysis buffer (20 mm Tris–HCl [pH 7.5], 137 mm NaCl, 1% NP-40, 10% glycerol, 1 mm phenylmethyl sulphonyl fluoride, 10 μg/mL aprotinin, and 1 μg/mL leupeptin). The protein lysates were denatured at 95°C for 5 minutes, and applied on an SDS-polyacrylamide gel for electrophoresis and transferred to nitrocellulose membranes. After blocking with 1% skim milk in PBS containing 0.1% Tween 20, the membrane were reacted with goat polyclonal anti-HDC, anti-MC1R, anti-MC5R, and anti-β-actin antibodies (1/1,000 each), respectively, overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-labeled donkey anti-goat IgG antibody (1/1,000) (Bethyl Laboratories, Inc., Montgomery, TX) for 2 h at room temperature. These membranes were then scanned using lumino image analyzer Image Quant LAS-4000(Fujifilm, Tokyo, Japan).

**Measurement of histamine released from murine cell line Pam212 and intracellular cAMP**

α-MSH (200 μM) was dissolved in OPTI-MEM, as a reaction medium, and applied to

the cells. Five and 10 minutes later, the reaction medium was collected. In the experiments using the cells treated with siRNA, SQ 22,536, or EGTA, the reaction medium was collected 5 min after α-MSH application. The concentration of the released histamine was measured using histamine enzyme immunoassay kit (Bertin Pharma, Montigny-le-Bretonneux, France) according to the manufacturer's recommendation. The protein of Pam212 cells was extracted by the application of 1% triton X-100 and the concentration was measured by using protein assay reagent (Bio-Rad). The released histamine concentration in the reaction medium was normalized to the protein concentration of Pam212 cells. The amount of intracellular cAMP 5 minutes after α-MSH application was measured with the enzyme immunoassay kit (GE Healthcare Bio-Sciences Co., Piscataway, NJ).

**Statistical analysis**

All values are expressed as the means ± SEM of the respective test or control group. Statistical significance was evaluated using either Student's *t*-test or Holm–Šidák multiple comparisons. $P < 0.05$ were considered to be significant.

## RESULTS

### Behavioral effects of α-MSH

An intradermal injection of α-MSH (100 nM/site) induced marked scratching of the injected site by the hind paws compared with the vehicle (VH). The effect peaked in the initial 10 min period, and almost completely subsided within 60 min (Figure 1A). The administration of α-MSH at intradermal doses of 10 to 100 nmol per site elicited scratching in a dose-dependent manner (Figure 1B). In the following *in vivo* experiments, a dose of 100 nmol per site of α-MSH was used.

### Effects of various agents on the α-MSH–induced scratching

Subcutaneous pretreatment with 1 mg/kg selective μ-opioid receptor antagonist, naltrexone hydrochloride [15] inhibited the α-MSH-induced scratching (Figure1C). Oral pretreatment with 30 mg/kg $H_1$ histamine receptor antagonist, terfenadine [16] also suppressed the α-MSH-induced scratching (Figure 2A).

### Effects of mast cell deficiency on the α-MSH-induced scratching

An intradermal injection of α-MSH elicited significant scratching in both mast cell-deficient mice (WBB6F1 $W/W^v$) and their normal littermates (WBB6F1$^{+/+}$),

compared with vehicle (VH)-injected mice (Figure 2B). The number of scratches was almost the same between these mice. Interestingly, the administration of 30 mg/kg $H_1$ histamine receptor antagonist terfenadine significantly inhibited the α-MSH–induced scratching in both mast cell-deficient mice (WBB6F1 $W/W^v$) and their normal littermates (WBB6F1$^{+/+}$), compared with vehicle (VH)-treated mice (Figure 2C).

**Distribution of HDC, MC1R, MC5R, and histamine in mouse skin**

Immunohistochemical staining showed that HDC, MC1R, and MC5R were mainly expressed in both epidermal keratinocytes and dermal cells stained with toluidine blue (Figure 3A). Histamine was similarly expressed in both epidermal keratinocytes and dermal cells seen by the immunoreactivity of mast cell protease 5 (Figure 3B).

**Expression of HDC, MC1R, MC5R, and histamine in the mouse keratinocyte cell line Pam212 cells**

Pam212 cells showed immunoreactivity for HDC, MC1R, MC5R, and histamine (Figure 4A). In addition, reverse transcription-PCR and western blot analysis also showed the expression of HDC, MC1R, and MC5R in Pam212 cells (Figure 4B and C).

**Release of histamine from Pam212 cells stimulated with α-MSH**

In the present *in vivo* study, an intradermal injection of α-MSH (100 nmol/50 μL = 2 mM) elicited scratching (Figure 1A). On the other hand, α-MSH (10 nmol/50 μl = 200 μM) led to a slight, but not significant, increase in scratching, compared with the vehicle (VH)-injected group (Figure 1B). Since α-MSH acts directly on the cells *in vitro*, α-MSH was administered at a final concentration of 200 μM in these cell culture studies. Since α-MSH–induced scratching was observed mainly during the initial 10 min period, the release of histamine was measured for 10 min after α-MSH stimulation. Treatment with 200 μM α-MSH significantly increased the concentration of histamine in the culture medium five and 10 min after the application of α-MSH, compared with the medium of cells treated without α-MSH (Figure 4D). The effect peaked after the initial five minute period (Figure 4D). α-MSH did not elicit the release of histamine in the cells treated with MC1R or MC5R siRNA (Figure 5A and Figure 6). In addition, 100 μM adenylyl cyclase inhibitor SQ 22,536 [17] (Figure 7 [18]) and 1 mM calcium chelator EGTA [19] also inhibited the release of histamine induced by α-MSH (Figure 5B).

Figure 1

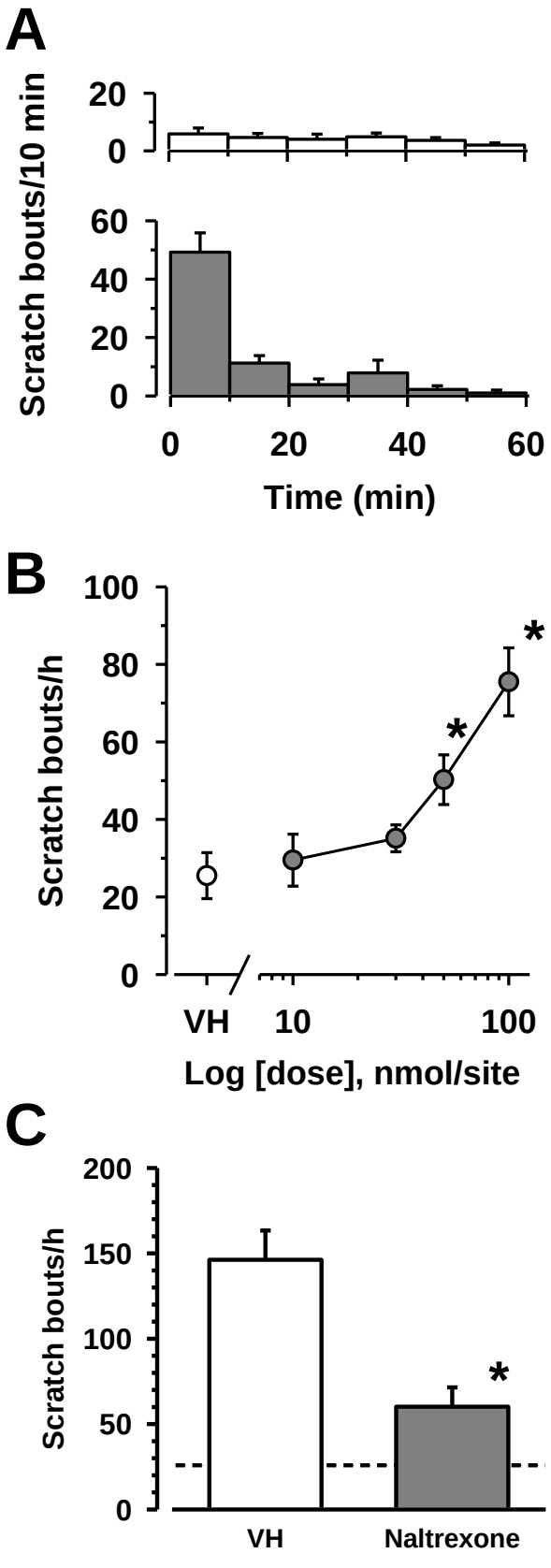
A
Scratch bouts/10 min
20
0
60
40
20
0
0
20
40
60
Time (min)
B
Scratch bouts/h
100
80
60
40
20
0
*
*
VH
10
100
Log [dose], nmol/site
C
Scratch bouts/h
200
150
100
50
0
*
VH
Naltrexone

**Figure 1.** Scratching after intradermal injections of α-MSH in ICR mice and effects of naltrexone. Hind-paw scratching of the injection site was counted for 1 hour after intradermal injection of α-MSH or the vehicle (VH1). **A:** Time course of scratching after VH1 (upper panel) and α-MSH (100 nM/site, lower panel) injections. **B:** Dose–response curve for the scratching effects of α-MSH and VH1 (representative experiment of two independent experiments). **C:** Naltrexone hydrochloride (1 mg/kg) or vehicle (VH) was injected subcutaneously 15 min before α-MSH (100 nM/site) injection. The dotted line represents the average value of scratching bouts after intradermal injection of VH1. Values represent the mean ± SEM for eight animals. *$P$ <0.05 when compared with VH (**B**, Holm–Šidák multiple comparisons; **C**, Student's $t$-test).

Figure 2

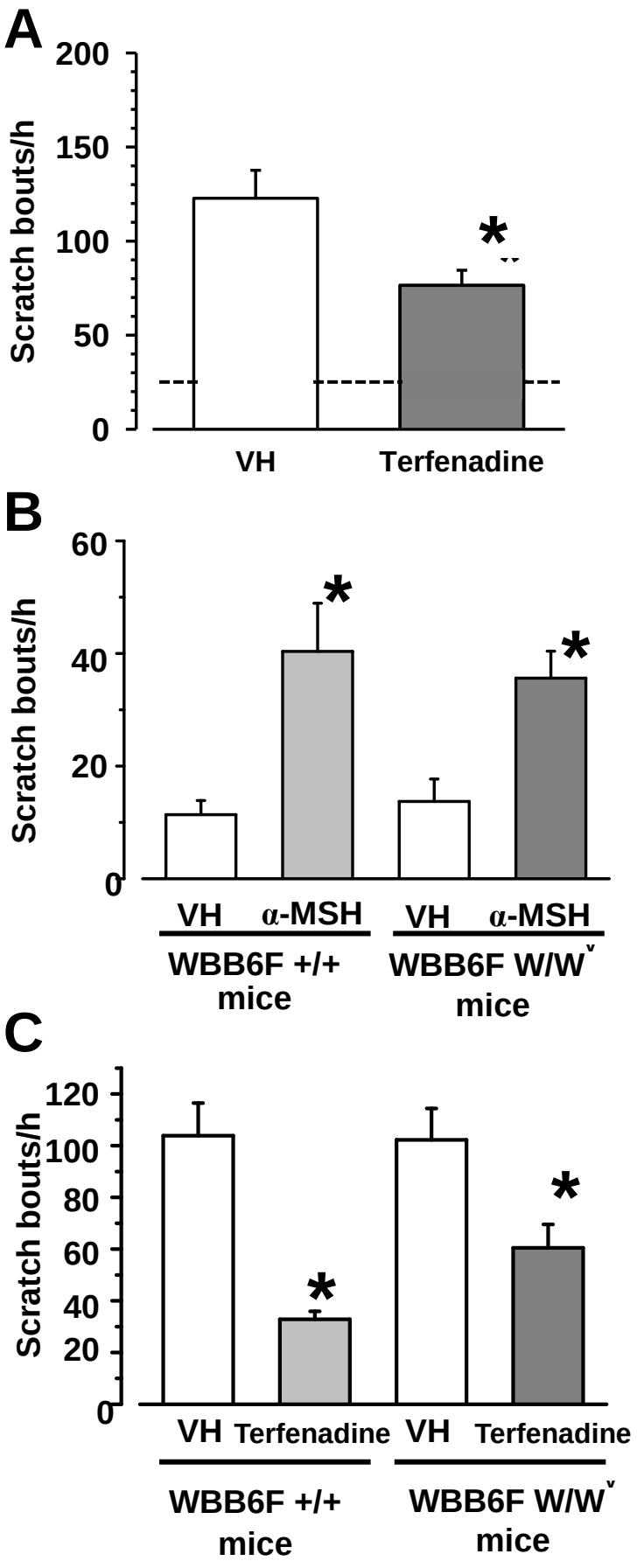
A
200
150
100
50
0
Scratch bouts/h
*
VH
Terfenadine
B
60
40
20
0
Scratch bouts/h
*
*
VH
α-MSH
VH
α-MSH
WBB6F +/+ mice
WBB6F W/Wv mice
C
120
100
80
60
40
20
0
Scratch bouts/h
*
*
VH
Terfenadine
VH
Terfenadine
WBB6F +/+ mice
WBB6F W/Wv mice

**Figure 2.** Effect of terfenadine and mast-cell deficiency on α-MSH–induced scratching. Terfenadine (30 mg/kg) or vehicle (VH1) was injected orally 30 min before α-MSH (100 nM/site) injection. Hind-paw scratching of the injection site was counted for 1 hour after intradermal injection of α-MSH or the vehicle (VH2). **A:** Effect of terfenadine on α-MSH–induced scratching in ICR mice. The dotted line represents the average value of scratching bouts after intradermal injection of VH2. **B:** Effect of mast-cell deficiency on α-MSH–induced scratching in mast cell-deficient WBB6F1-$W/W^v$ mice and in normal littermates (WBB6F1$^{+/+}$). **C:** Effect of terfenadine on α-MSH–induced scratching in WBB6F1-$W/W^v$ and WBB6F1$^{+/+}$ mice. Values represent the mean ± SEM for seven to eight animals. *$P$ <0.05 when compared with VH1 or VH2 (**A**, Student's $t$-test; **B**, **C**, Holm–Šidák multiple comparisons).

Figure 3

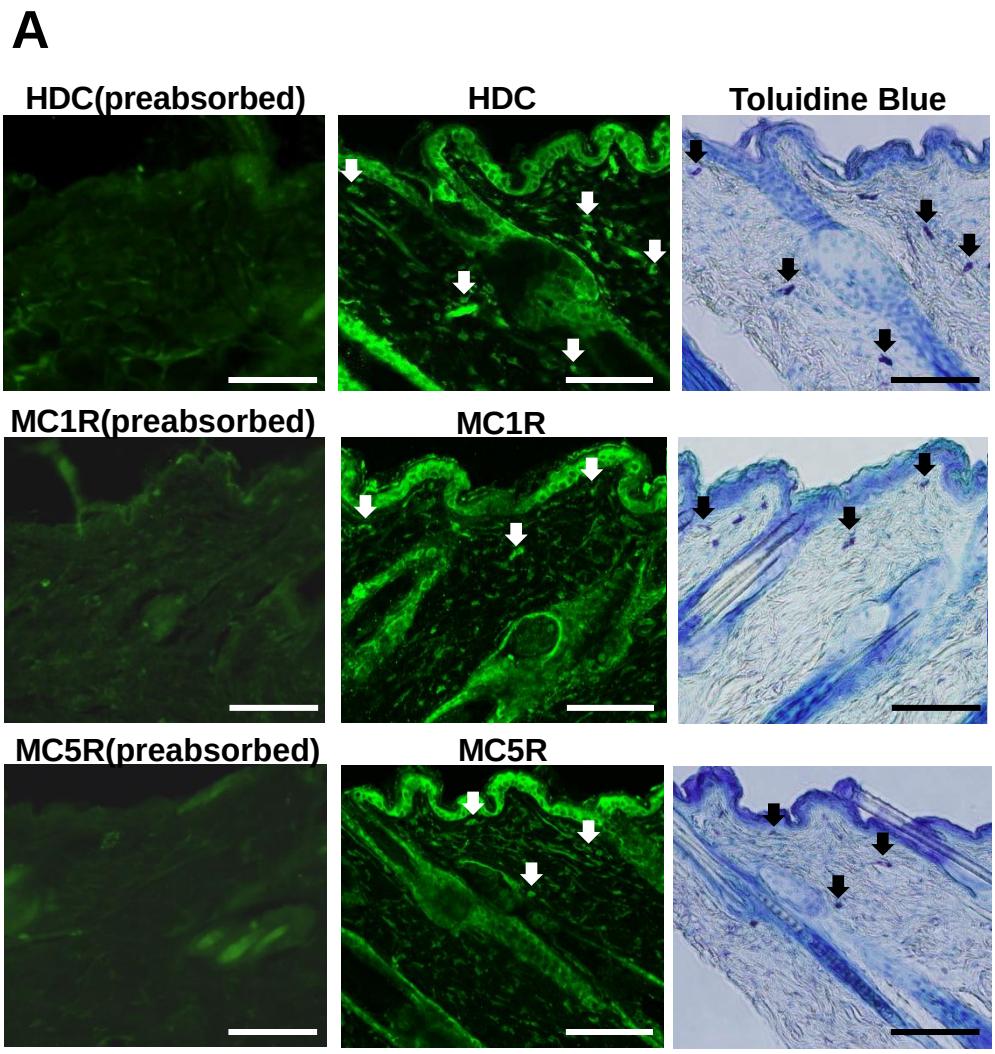
A
HDC(preabsorbed)
HDC
Toluidine Blue
MC1R(preabsorbed)
MC1R
MC5R(preabsorbed)
MC5R
B
Histamine
DAPI
MCP-5
Merge

**Figure 3.** Cutaneous distribution of HDC, melanocortin receptors (MC1R and MC5R), and histamine in ICR mice. **A:** Center panels show HDC and melanocortin receptors (MC1R and MC5R) immunoreactivity in the skin of the ICR mice. Left panels show the images obtained using the antibody preabsorbed with the antigens for HDC, MC1R, and MC5R (negative control). Right panels show the light microscopy images for toluidine blue-staining. **B:** Expression of histamine (green) and mast cell protease 5 (red). Arrow shows mast cell. Bar = 100 μm.

Figure 4

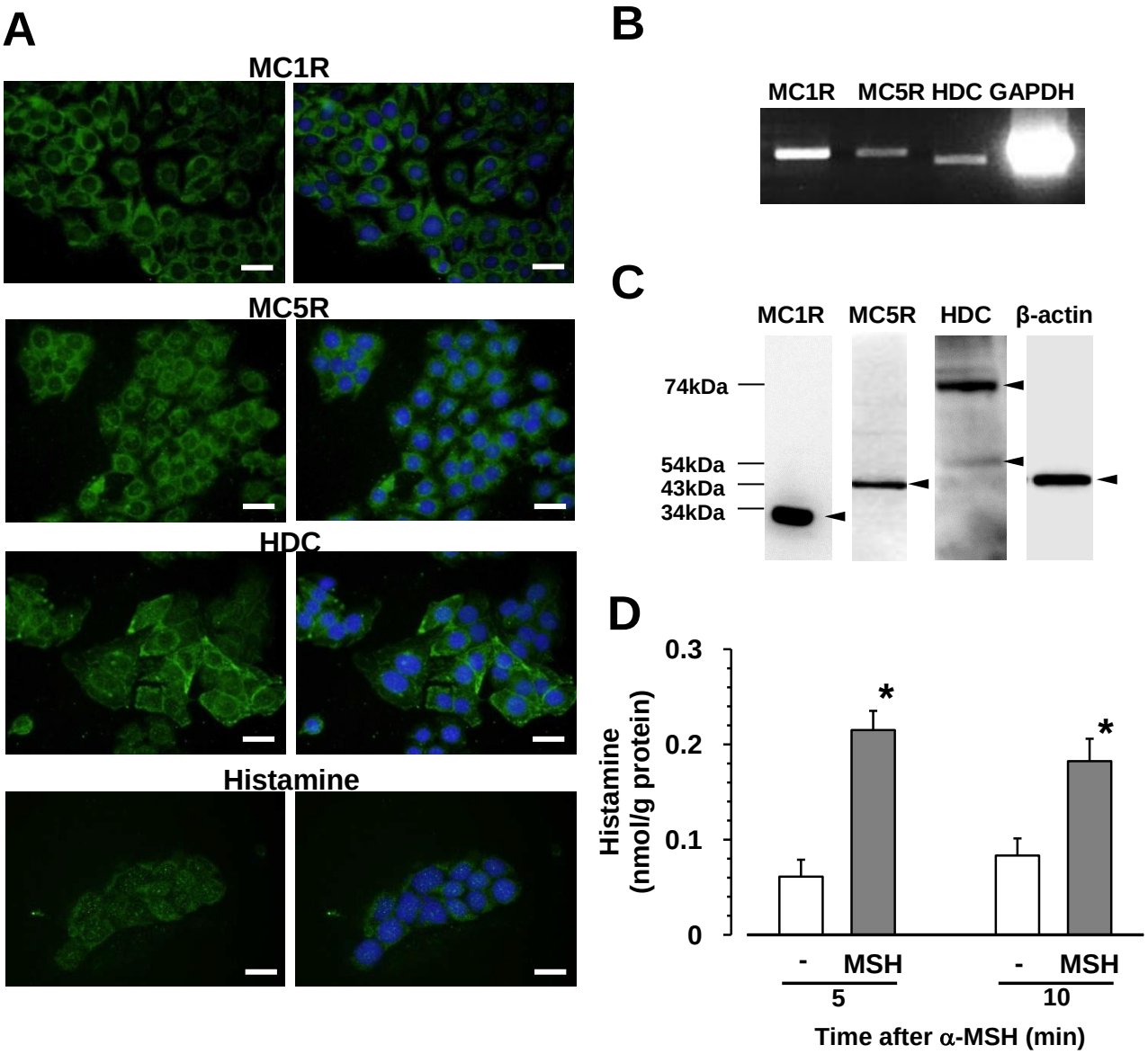
A
MC1R
MC5R
HDC
Histamine
B
MC1R MC5R HDC GAPDH
C
MC1R MC5R HDC β-actin
74kDa
54kDa
43kDa
34kDa
D
Histamine (nmol/g protein)
0.3
0.2
0.1
0
*
*
- MSH
- MSH
5
10
Time after α-MSH (min)

**Figure 4.** Expression of HDC, melanocortin receptors (MC1R and MC5R), and histamine in murine keratinocyte cell line Pam212 and release of histamine after α-MSH treatment. **A:** HDC, melanocortin receptors (MC1R and MC5R), and histamine immunoreactivity in Pam212 cells. Bar = 20 μm. **B, C**: Reverse transcription-PCR and a Western blot analysis shows the expression of HDC and melanocortin receptors (MC1R and MC5R) in the Pam212 cells. Representative data from two independent experiments are shown. The arrowhead indicates the protein levels of MC1R (34 kDa), MC5R (43 kDa), and HDC (74 and 54 kDa). **D:** Release of histamine after 200 μM α-MSH treatment. All results are representative of two independent experiments. Values represent the mean ± SEM for six wells. *$P$ <0.05 when compared with the culture Medium only (Holm–Šidák multiple comparisons).

Figure 5

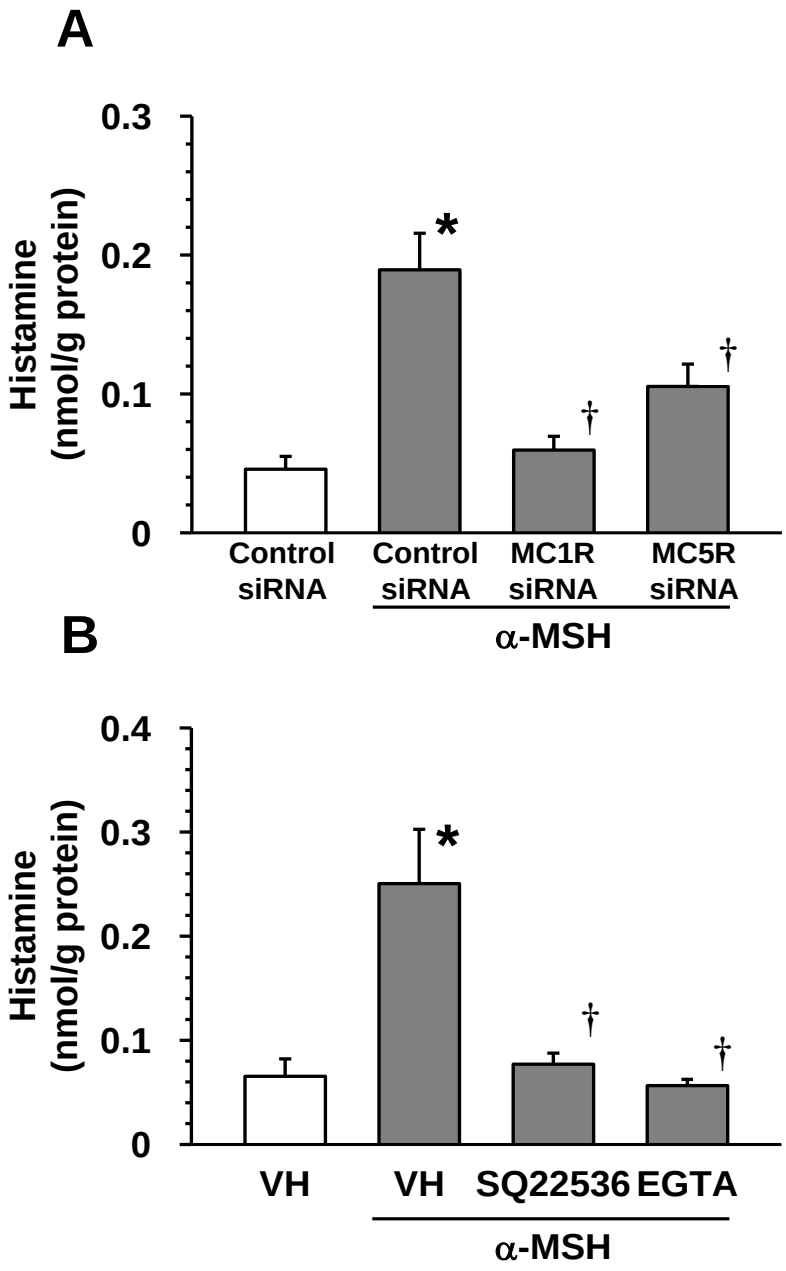
A
Histamine (nmol/g protein)
0.3
0.2
0.1
0
*
†
†
Control siRNA
Control siRNA
MC1R siRNA
MC5R siRNA
α-MSH
B
Histamine (nmol/g protein)
0.4
0.3
0.2
0.1
0
*
†
†
VH
VH
SQ22536
EGTA
α-MSH

**Figure 5.** Effects of siRNA on melanocortin receptors (MC1R and MC5R) and cell signaling-regulated agents. **A:** α-MSH (200 μM) was applied to Pam212 cells treated with siRNA for melanocortin receptor (MC1R or MC5R) or nonspecific control siRNA. Values represent the mean ± SEM for six wells. *$P$ <0.05 when compared with the reaction medium in the Pam212 cells treated with control siRNA. $^{\dagger}P$ <0.05 when compared with the reaction medium applied with α-MSH in the Pam212 cells treated with control siRNA (Holm–Šidák multiple comparisons). **B:** SQ 22,536 (100 μM), EGTA (1 mM), or vehicle (VH: 0.1% dimethyl sulfoxide) was treated 30 min before 200 μM α-MSH application. The amount of histamine in the reaction medium was measured 5 min after 200 μM α-MSH application using an enzyme immunoassay kit and was normalized to the amount of protein in the cells. Values represent the mean ± SEM for six wells. *$P$ <0.05 when compared with the reaction medium containing VH in the Pam212 cells. $^{\dagger}P$ <0.05 when compared with the reaction medium applied with α-MSH in the Pam 212 cells treated with VH (Holm–Šidák multiple comparisons).

Figure 6

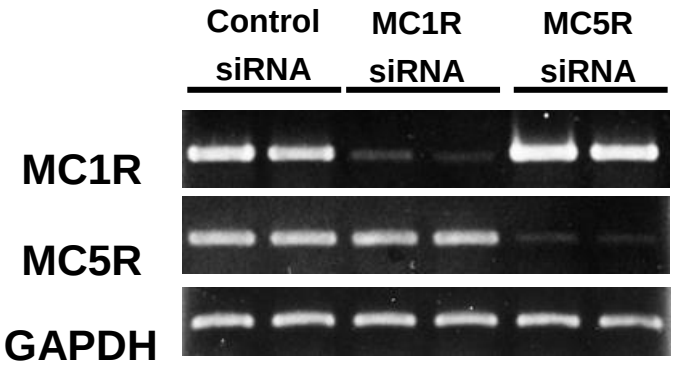


Figure 7

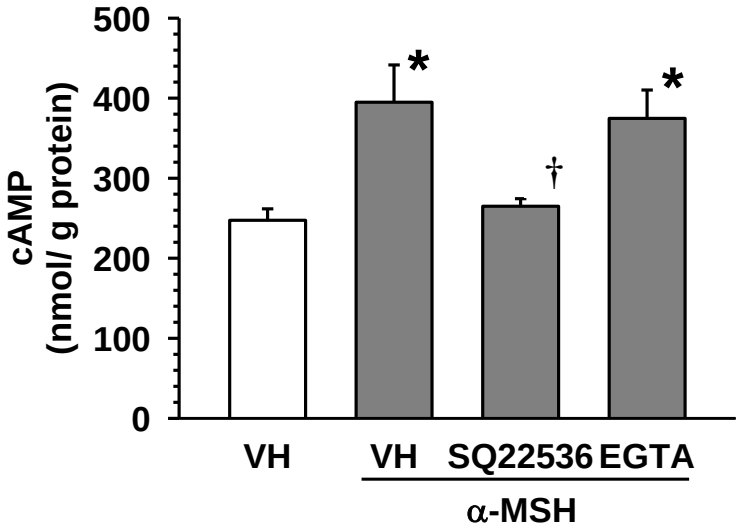

**Figure 6.** Expression of MC1R, MC5R, and GAPDH mRNA in the Pam212 cells treated with siRNA. Typical examples of the products obtained from the RT-PCR (two examples each). The method is described in Materials and Methods.

**Figure 7.** Concentration of intracellular cAMP in the Pam212 cells. SQ 22,536 (100 mM), EGTA (1 mM), or VH (0.1% dimethyl sulfoxide) was treated 30 minutes before 200 mmol/L α-MSH application. 18 Data are expressed as means ± SEM. n = 6 wells. $*P < 0.05$ versus Pam212 cells treated with VH; $†P < 0.05$ versus Pam212 cells treated with VH and α-MSH (Holm–Šidák multiple comparisons).

## DISCUSSION

Intradermal injections of α-MSH into the rostral part of the skin elicited hind-paw scratching of the injection site in mice. The α-MSH–induced scratching was inhibited by treatment with the μ-opioid receptor antagonist naltrexone. It has been reported that μ-opioid receptor antagonists inhibit scratching induced by pruritogens [15,20-22] and dermatoses in rodents [16,23-25], and itching and scratching in humans with pruritic diseases [26-29]. However, μ-opioid receptor antagonists attenuate itch-related, but not pain-related behavior [30-33]. Taking into account these findings in humans and rodents, our results showing that the action of α-MSH was inhibited by μ-opioid receptor antagonists is consistent with the idea that α-MSH–induced scratching is due to pruritogenic, but not algesiogenic, stimulation of the treated skin.

In this study, we also demonstrated that α-MSH–induced scratching was inhibited partially, but significantly, by treatment with a $H_1$ histamine receptor antagonist terfenadine at a dose that almost completely inhibited the histamine-induced scratching [16], suggesting that histamine is involved in α-MSH–induced scratching. α-MSH is involved in the pigmentation due to sunburn [10,11], hemodialysis [12], and wound repair [13]. Antihistamines are effective for treating pruritus in the patients with the above causes of pigmentation [34-36]. Therefore, these reports support our findings.

It is well known that histamine is mainly produced by the mast cells in skin.

However, in this study, α-MSH elicited scratching in both mast cell-deficient mice and their normal littermates. Interestingly, terfenadine inhibited the α-MSH–induced scratching in both the mast cell-deficient mice and normal littermates. Taken together, these findings suggest that histamine is involved in the α-MSH–induced scratching, and mast cells may not contribute to the release of histamine involved in this scratching.

HDC is a key enzyme in the biosynthesis of histamine [37]. In this study, we observed the immunoreactivity to HDC and histamine not only in mast cells, but also keratinocytes in mouse skin. In addition, the mouse keratinocyte cell line, Pam212, also showed the immunoreactivity to HDC and histamine. Human epidermal cells and keratinocytes also express HDC [38,39]. We also detected low (54 kDa)- and high (74 kDa)-molecular weight protein bands of HDC in the Pam212 cells. 74-kDa HDC is a precursor protein that exhibits a low enzyme activity. Furthermore, 74-kDa HDC is post-translationally cleaved to a 53 to 55-kDa species and histamine is synthesized mainly by 53 to 55-kDa HDC [37]. Treatment with α-MSH induced histamine release from Pam212 cells. Thus, keratinocytes may play an important role in the production of histamine, and this appears to be related to the α-MSH–induced scratching.

In mast cells, histamine is stored in granules and is released by several types of stimuli (such as immune reactions) [37]. Macrophages also express HDC and

spontaneously release histamine without it being stored intracellularly (these cells do not contain histamine-storing granules) [37,40]. Since keratinocytes also do not have histamine-storing granules, the histamine would be released immediately after its biosynthesis. In this study, keratinocytes slightly produced histamine. Recent report has shown histamine induces proliferation in keratinocytes through H4 histamine receptors [41]. Thus, in normal skin condition, histamine is involved in the proliferation in keratinocyte as one of the actions. However, over production of histamine induced by several stimulations, such as α-MSH (present study) and surfactant [38], may be involved in the induction of itching.

The application of α-MSH induced the release of histamine from the mouse keratinocytes cell line Pam212 in this study. Pam212 cells also expressed MC1R and MC5R receptors. The cells treated with siRNA for MC1R and MC5R showed no release of histamine after stimulation with α-MSH, suggesting that at least MC1R and MC5R are involved in α-MSH–induced histamine release. The mechanism underlying the production of histamine after the stimulation with α-MSH is still unclear. Miyazaki *et al.* [42] have shown that the activity of HDC was increased by $N^6$,$O^2$-dibutyryl cAMP plus $Ca^{2+}$ ionophore A23187. The activation of MC1R and MC5R receptors by α-MSH increases both the production of cAMP [8] and the intracellular free $Ca^{2+}$ concentration

[9]. Here, α-MSH–induced histamine release was inhibited by the adenylyl cyclase inhibitor, which suppressed cAMP production, and a calcium chelator. Therefore, our findings suggest that increased cAMP (Figure 7) and intracellular calcium levels may be involved in the production of histamine through melanocortin receptors.

In the present study, the treatment with a $H_1$ histamine receptor antagonist did not completely inhibit the α-MSH–induced scratching. The cutaneous distribution of melanocortin receptors (MC1R, MC3R, MC4R and MC5R) for α-MSH is not fully understood. It is known that in the mouse dorsal root ganglia, MC1R, MC3R, and MC4R, but not MC5R, are expressed [43], suggesting that MC1R, MC3R, and MC4R are presented on primary afferents. Thus, α-MSH may act directly via primary afferents to induce itch. Recently, it has been reported that there are two main types of itch-related primary afferents; $H_1$ histamine receptor-expressing neurons and mas-related G-coupled protein receptor A3-expressing neurons [44]. A future study should be performed to determine the distribution of melanocortin receptors in these primary afferents.

## CONCLUSION

In conclusion, α-MSH is an itch mediator, and the histamine released from keratinocytes, but not mast cells, may be involved in this α-MSH–induced itching. Additionally, this study revealed that α-MSH links pigmentation and itching in skin. Based on results of this study, α-MSH can be a new target of treatment for pruritic diseases.

**REFERENCES**


1 Han A, Maibach HI: Management of acute sunburn. Am J Clin Dermatol 2004, 5:39-47

2 Gilchrest BA: The human sunburn reaction: Histologic and biological studies. Am Acad Dermatol 1981, 5:411-422

3 Paul J: Characteristics of chronic wounds that itch. Adv Skin Wound Care 2007, 26:320-332

4 Slominski A, Tobin DJ, Shibahara S, Wortsman J: Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev 2004, 84: 1155-228

5 Slominski A, Wortsman J, Luger T, Paus R, Solomon S: Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev 2000, 80: 979-1020

6 Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J: Key role of CRF in the skin stress response system. Endocr Rev 2013, 34: 827-884

7 Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD: Sensing the environment: regulation of local and global homeostasis by the skin's neuroendocrine system. Adv Anat Embryol Cell Biol 2012, 212:v, vii, 1-115

8 Cooray SN, Clark AJ: Melanocortin receptors and their accessory proteins. Mol

Cell Endocrinol 2011, 331:215-221

9 Mountjoy KG, Kong PL, Taylor JA, Willard DH, Wilkison WO: Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. Physiol Genomics 2011, 5:11-19

10 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

11 Oren M, Bartek J (2007) The sunny side of p53. Cell 128:826-8.

12 Airaghi L, Garofalo L, Cutuli MG, Delgado R, Carlin A, Demitri MT, Badalamenti S, Graziani G, Lipton JM, Catania A: Plasma concentrations of alpha-melanocyte stimulating hormone are elevated in patients on chronic haemodialysis. Nephrol Dial Transplant 2000, 15:1212-1216

13 Muffley LA, Zhu KQ, Engrav LH, Gibran NS, Hocking AM: Spatial and temporal localization of the melanocortin 1 receptor and its ligand α-melanocyte-stimulating hormone during cutaneous wound repair. J Histochem Cytochem 2011, 59:278-288

14 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

15 Andoh T, Kuwazono T, Lee JB, Kuraishi Y: Gastrin-releasing peptide induces itch-related responses through mast cell degranulation in mice. Peptides 2011, 32:2098-2103

16 Ohtsuka E, Kawai S, Ichikawa T, Nojima H, Kitagawa K, Shirai Y, Kamimura K, Kuraishi Y. Roles of mast cells and histamine in mosquito bite-induced allergic itch-associated responses in mice. Jpn J Pharmacol 2001, 86:97-105

17 Turcato S, Clapp LH: Effects of the adenylyl cyclase inhibitor SQ22536 on iloprost-induced vasorelaxation and cyclic AMP elevation in isolated guinea-pig aorta. Br J Pharmacol 1999, 126: 845-847.

18 Andoh T and Kuraishi Y: Antipruritic mechanisms of topical E6005, a phosphodiesterase 4 inhibitor: inhibition of responses to proteinase-activated receptor 2 stimulation mediated by increase in intracellular cyclic AMP, J. Dermatol. Sci. 2014, 76:206-213.

19 Sun FC, Shyu HY, Lee MS, Lee MS, Lai YK: Involvement of calcium-mediated reactive oxygen species in inductive GRP78 expression by geldanamycin in 9L rat brain tumor cells. Int J Mol Sci 2013, 14: 19169-19185.

20Andoh T, Nagasawa T, Satoh M, Kuraishi Y: Substance P induction of itch-associated response mediated by cutaneous NK1 tachykinin receptors in mice. J Pharmacol

Exp Ther 1998, 286:1140-1145

21 Andoh T, Saito A, Kuraishi Y: Leukotriene B(4) mediates sphingosylphosphorylcholine-induced itch-associated responses in mouse skin. J Invest Dermatol 2009, 129:2854-2860

22 Yamaguchi T, Nagasawa T, Satoh M, Kuraishi Y: Itch-associated response induced by intradermal serotonin through 5-HT2 receptors in mice. Neurosci Res 1999, 35:77-83

23 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

24 Miyamoto T, Nojima H, Shinkado T, Nakahashi T, Kuraishi Y: Itch-associated response induced by experimental dry skin in mice. Jpn J Pharmacol 2002, 88:285-292

25 Fujii M, Nabe T, Tomozawa J, Tomozawa J, Kohno S: Involvement of skin barrier dysfunction in itch-related scratching in special diet-fed hairless mice. Eur J Pharmacol 2006, 530:152-156

26 Bernstein JE, Swift R: Relief of intractable pruritus with naloxone. Arch Dermatol 1979, 115:1366-1367

27 Monroe EW: Efficacy and safety of nalmefene in patients with severe pruritus caused by chronic urticaria and atopic dermatitis. J Am Acad Dermatol 1989,

21:135-136

28 Bergasa NV, Alling DW, Talbot TL, Swain MG, Yurdaydin C, Turner ML, Schmitt JM, Walker EC, Jones EA: Effects of naloxone infusions in patients with the pruritus of cholestasis. A double-blind, randomized, controlled trial. Ann Intern Med 1995, 123:161-167

29 Bergasa NV, Talbot TL, Alling DW, Schmitt JM, Walker EC, Baker BL, Korenman JC, Park Y, Hoofnagle JH, Jones EA: A controlled trial of naloxone infusions for the pruritus of chronic cholestasis. Gastroenterology 1992, 102:544-549

30 Maekawa T, Yamaguchi-Miyamoto T, Nojima H, Kuraishi Y: Effects of naltrexone on spontaneous itch-associated responses in NC mice with chronic dermatitis. Jpn J Pharmacol 2002, 90:193-196

31 Nojima H, Simons CT, Cuellar JM, Carstens MI, Moore JA, Carstens E: Opioid modulation of scratching and spinal c-fos expression evoked by intradermal serotonin. J Neurosci 2003, 23:10784-10790

32 Akiyama T, Carstens MI, Carstens E: Differential itch- and pain-related behavioral responses and μ-opoid modulation in mice. Acta Derm Venereol 2010, 90:575-581

33 Gotoh Y, Andoh T, Kuraishi Y: Noradrenergic regulation of itch transmission in the spinal cord mediated by α-adrenoceptors. Neuropharmacology 2011, 61:825-831

34 Han A, Maibach HI: Management of acute sunburn. Am J Clin Dermatol 2004, 5: 39-47

35 Matsui C, Ida M, Hamada M, Morohashi M, Hasegawa M: Effects of azelastine on pruritus and plasma histamine levels in hemodialysis patients. Int J Dermatol 1994, 33: 868-871

36 Matheson JD, Clayton J, Muller MJ: The reduction of itch during burn wound healing. J Burn Care Rehabil 2001, 22: 76-81

37 Ichikawa A, Sugimoto Y, Tanaka S: Molecular biology of histidine decarboxylase and prostaglandin receptors. Proc Jpn Acad Ser B Phys Biol Sci 2010, 86:848-866

38 Inami Y, Andoh T, Sasaki A, Kuraishi Y: Topical surfactant-induced pruritus: involvement of histamine released from epidermal keratinocytes. J Pharmacol Exp Ther 2013, 344:459-466

39 Gutowska-Owsiak D, Greenwald L, Watson C, Selvakumar TA, Wang X, Ogg GS: Histamine synthesizing enzyme, histidine decarboxylase, is upregulated by keratinocytes in atopic skin. Br J Dermatol 2014, 171:771-8

40 Hirasawa N, Murakami A, Ohuchi K: Expression of 74-kDa histidine decarboxylase protein in a macrophage-like cell line RAW 264.7 and inhibition by dexamethasone. Eur J Pharmacol 2001, 418:23-28

41 Glatzer F, Gschwandtner M, Ehling S, Rossbach K, Janik K, Klos A, Bäumer W, Kietzmann M, Werfel T, Gutzmer R: Histamine induces proliferation in keratinocytes from patients with atopic dermatitis through the histamine 4 receptor. J Allergy Clin Immunol 2013, 132:1358-1367

42 Miyazaki T, Ohgoh M, Ohmori E, Yamamoto J, Emoto S, Yatsunami K, Ichikawa A: Synergistic effects of cyclic AMP and Ca2+ ionophore A23187 on de novo synthesis of histidine decarboxylase in mastocytoma P-815 cells. Biochim Biophys Acta 1992, 1133:179-186

43 Tanabe K, Gamo K, Aoki S, Wada K, Kiyama H: Melanocortin receptor 4 is induced in nerve-injured motor and sensory neurons of mouse. J Neurochem 2007, 101:1145-1152

44 Akiyama T, Carstens E: Neural processing of itch. Neuroscience 2013 250:697-714

**Acknowledgements**

I would like to show my greatest appreciation to Professor Tadamichi Shimizu (Department of Dermatology, Graduate school of Medicine and Pharmaceutical Science, University of Toyama) and Assistant professor Tsugunobu Andoh (Department of Applied Pharmacology, Graduate school of Medicine and Pharmaceutical Science, University of Toyama) for their helpful guidance and giving a chance of this research work.

I am deeply grateful to Dr. Yoko Yoshihisa (Department of Dermatology, Graduate school of Medicine and Pharmaceutical Science, University of Toyama) for excellent technical assistance and generous support.

My heartfelt appreciation goes to all members of Department of Dermatology, Graduate school of Medicine and Pharmaceutical Science, University of Toyama.