



Article

Chronic Histamine Exposure Promotes Melanogenesis via ORAI1-STIM1-Mediated Calcium Signaling Remodeling

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Abstract

Post-inflammatory hyperpigmentation (PIH) is a common pigmentary disorder characterized by excessive melanin production following skin inflammation. Histamine, a key inflammatory mediator, is known to stimulate melanogenesis via H₂ receptors; however, the underlying calcium (Ca²⁺) signaling mechanisms remain largely unexplored. In this study, we investigated the role of the ORAI1-STIM1 complex in histamine-induced melanogenesis using B16F10 melanoma cells and normal human epidermal melanocytes (NHEMs). Histamine (10–30 μM) significantly increased melanin content (2.5–2.8-fold), an effect specifically abolished by the H₂ antagonist famotidine. Notably, while acute histamine application failed to trigger immediate Ca²⁺ influx, chronic exposure significantly enhanced store-operated Ca²⁺ entry (SOCE) capacity by approximately 2.8-fold, providing evidence for a functional remodeling of the Ca²⁺ signaling machinery. Histamine-induced melanogenesis was significantly suppressed by intracellular Ca²⁺ chelation, pharmacological inhibition of ORAI1 (BTP-2 or Synta-66), and siRNA-mediated silencing of ORAI1 or STIM1, but not ORAI2, ORAI3, or STIM2. Our findings demonstrate that chronic histamine exposure drives hyperpigmentation through ORAI1-STIM1-mediated SOCE remodeling, establishing this complex as a promising therapeutic target for the treatment of PIH and related inflammatory pigmentary disorders.



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1. Introduction

Melanin is the primary pigment determining the coloration of skin, hair, and eyes, while simultaneously providing essential protection against DNA damage induced by ultraviolet (UV) radiation [1]. Melanogenesis, the biosynthetic pathway producing melanin, involves an intricate enzymatic cascade initiated by the tyrosinase-catalyzed conversion of

L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), followed by progressive oxidation and polymerization into melanin macromolecules [2,3]. The microphthalmia-associated transcription factor (MITF) serves as the master regulator of this process, governing the transcriptional activation of key melanogenic enzymes, including tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and dopachrome tautomerase (DCT/TRP-2) [2,4,5]. Multiple intracellular signaling cascades, notably the cAMP-dependent protein kinase A (PKA), MAPK, and Wnt/ β -catenin pathways, converge to modulate MITF activity in response to external stimuli such as UV radiation, hormones, and inflammatory mediators [2,6–8]. While photoprotective, excessive melanin deposition characterizes hyperpigmentation disorders such as melasma, solar lentigines, and post-inflammatory hyperpigmentation (PIH), which present substantial aesthetic and psychological burdens to affected individuals [9,10].

PIH represents one of the most common acquired pigmentary disorders, characterized by darkened patches of skin that develop following inflammatory or traumatic insults to the epidermis and dermis [11]. PIH can arise from diverse triggers, including acne vulgaris, atopic dermatitis, infections, and cosmetic procedures, significantly impacting patients' quality of life [11]. The pathogenesis of PIH involves a complex interplay between inflammatory mediators and melanocyte activity. During the inflammatory response, keratinocytes, fibroblasts, and immune cells (including mast cells and macrophages) release various mediators such as prostaglandins, cytokines (IL-1 α , IL-6, TNF- α), reactive oxygen species, and histamine. These mediators stimulate melanocyte activity, leading to increased melanin synthesis and transfer to surrounding keratinocytes [12]. Despite its clinical prevalence, current therapeutic options primarily targeting tyrosinase activity or epidermal turnover frequently yield slow or incomplete responses and carry risks of adverse effects [11]. Among these inflammatory mediators, histamine is a potent stimulator of melanogenesis, particularly in pruritic inflammatory conditions that lead to pronounced PIH [13,14]. Early investigations demonstrated that histamine induces morphological changes, including cell enlargement and increased dendricity, alongside elevated tyrosinase activity and melanin content [13]. Subsequent studies confirmed that histamine also enhances melanocyte proliferation and migration [15]. Mechanistically, H₂ receptor activation increases intracellular cyclic AMP (cAMP) accumulation, which activates PKA, ultimately upregulating MITF and its downstream targets [13].

Intracellular calcium (Ca²⁺) serves as another critical second messenger in melanogenesis, directly influencing melanin synthesis by modulating cAMP and tyrosinase activity [16–19]. Elevated intracellular Ca²⁺ enhances melanin production, while disruption of Ca²⁺ homeostasis impairs pigmentation [16,17,20]. Ca²⁺-dependent kinases, such as CaMKII and PKC, transduce Ca²⁺ signals by phosphorylating key regulators like CREB and MITF [21]. Among various Ca²⁺ entry mechanisms, store-operated Ca²⁺ entry (SOCE) has emerged as a key regulator. Previous studies have established that ORAI1 mediates melanin synthesis triggered by endothelin-1 (ET-1) and UV, where pharmacological inhibition or siRNA-mediated ORAI1 downregulation significantly reduces Ca²⁺ entry and tyrosinase activity [22–24].

Despite these advances, critical gaps remain regarding how inflammatory mediators drive sustained melanin overproduction in PIH. While histamine signals via H₂ receptors and ORAI1 mediates ET-1-induced melanogenesis, the potential crosstalk between these systems remains unexplored. Significantly, in other pathological models, chronic exposure to inflammatory stimuli has been shown to induce 'SOCE remodeling', a phenotypic shift that enhances the Ca²⁺ signaling machinery.

Building on these observations, we hypothesized that chronic histamine exposure might remodel the SOCE machinery in melanocytes, thereby enhancing Ca²⁺ entry to drive sustained melanin production. In this study, we investigated the role of ORAI1 channels in

histamine-induced melanogenesis. We aimed to determine the effects of histamine on SOCE capacity and the specific contribution of ORAI1 to melanin production in both B16F10 melanoma cells and normal human epidermal melanocytes (NHEMs). Our findings identify ORAI1 as a potential therapeutic target for inflammation-associated pigmentary disorders.

2. Results

2.1. Histamine Induces Melanin Production in Melanocytes

To investigate the effect of histamine on melanogenesis, we first evaluated its impact on B16F10 cell viability. After 24 h, histamine (0–100 μ M) showed no significant effect on cell viability. However, at 48 h, treatment with 100 μ M histamine reduced cell viability to 76.8% compared to the control. At 72 h, treatment with 30 μ M and 100 μ M histamine decreased cell viability to 72.8% and 71.8% of the control, respectively, while lower concentrations remained non-toxic (Figure 1A). Based on these findings, we selected 0–30 μ M histamine with 48 h treatment for the next experiments. Visual examination of B16F10 cell pellets revealed progressive darkening with increasing histamine concentrations (Figure 1B). Quantitative analysis revealed a dose-dependent stimulation of melanin synthesis by histamine. While 1 μ M histamine showed no significant effect, 3 μ M histamine significantly increased melanin production approximately 1.42-fold compared to control ($p < 0.05$). At higher concentrations, histamine substantially enhanced melanin synthesis, with 6 μ M, 10 μ M, and 30 μ M inducing approximately 2.08-fold, 2.93-fold, and 2.90-fold increases relative to untreated controls, respectively ($p < 0.001$) (Figure 1B). Therefore, 10 μ M histamine for 48 h was used in subsequent experiments. To validate these findings in primary cells, we examined NHEMs. Histamine (10 μ M) induced progressive darkening of NHEM pellets over 8 days (Figure 1C). Quantitative analysis showed that histamine significantly increased melanin production from day 4 (approximately 1.95-fold, $p < 0.001$), reaching peak levels at days 6 and 8 (approximately 2.51-fold and 2.70-fold, $p < 0.001$), while controls maintained relatively stable melanin levels (Figure 1D). Collectively, these results demonstrate that histamine potently stimulates melanin production in both immortalized melanoma cells and primary human melanocytes.

2.2. Histamine Induces Melanogenesis in Melanocytes Through Histamine H_2 Receptors

To identify the histamine receptor subtype mediating melanin synthesis, we examined the effects of selective receptor antagonists in B16F10 cells (Figure 2A) and NHEMs (Figure 2B). Cells were treated with histamine (10 μ M) alone or in combination with pyrilamine (H_1 antagonist, 10 μ M), famotidine (H_2 antagonist, 10 μ M), or thioperamide (H_3 antagonist, 10 μ M). Visual inspection revealed that histamine-induced darkening was maintained with pyrilamine and thioperamide but markedly attenuated by famotidine in B16F10 cells (Figure 2A) and NHEMs (Figure 2B). Quantitative analysis confirmed that histamine significantly increased melanin content approximately 2.5-fold in B16F10 cells and 2-fold in NHEMs compared to controls ($p < 0.001$). Co-treatment with pyrilamine or thioperamide did not significantly affect histamine-induced melanin production ($p > 0.05$). In contrast, famotidine completely abolished the stimulatory effect of histamine in both cell types, reducing melanin levels to control values ($p < 0.001$, Figure 2). These results demonstrate that histamine-induced melanogenesis is mediated specifically through H_2 receptor activation, with no significant contribution from H_1 or H_3 receptors in both melanoma and primary human melanocytes.

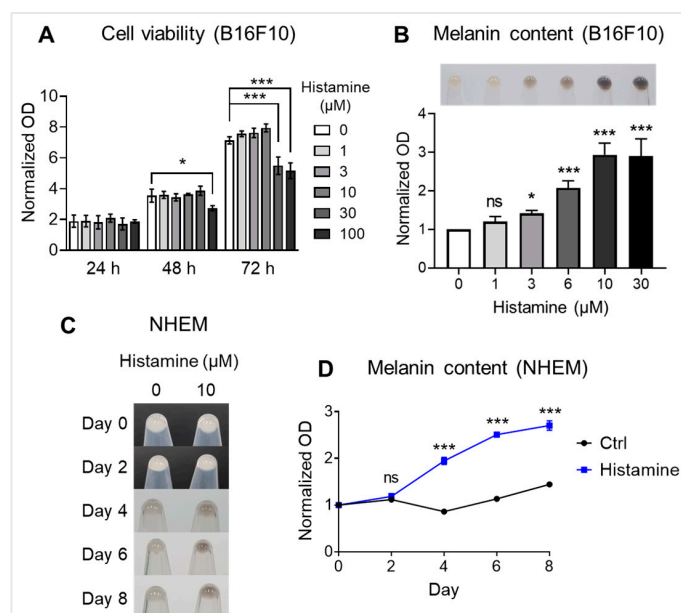


Figure 1. Histamine induces melanin production in melanocytes. **(A)** Cell viability was assessed over 72 h following treatment with increasing concentrations of histamine (0–100 μM) in B16F10 cells. Cell density was measured by optical density (OD) and normalized to the value of day 0. **(B)** Representative images showing B16F10 pellets treated with histamine (0–30 μM) for 48 h and quantification of melanin content normalized to total protein in B16F10 cells. Data are expressed as fold change relative to untreated control (0 μM). **(C)** Representative images of NHEM pellets showing melanin accumulation at indicated time points (days 0, 2, 4, 6, and 8) in the absence (0 μM) or presence (10 μM) of histamine. **(D)** Quantification of melanin content normalized to total protein over the 8-day culture period in NHEMs. Data are expressed as fold change relative to control at day 0. Data are presented as mean ± SD ($n = 3$ –6). One-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test, was used for multiple comparisons. * $p < 0.05$, *** $p < 0.001$, ns: not significant.

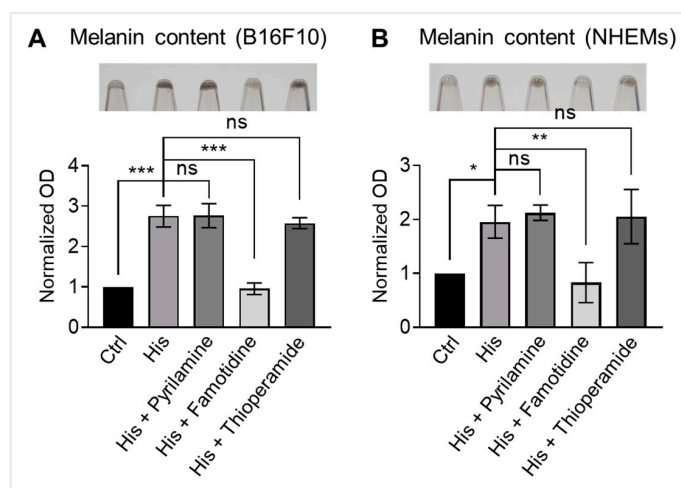


Figure 2. Histamine induces melanogenesis in melanocytes through histamine H_2 receptors. Representative images and quantification of melanin content in B16F10 cells **(A)** and NHEMs **(B)**. Cells were treated with vehicle control (Ctrl), histamine (His, 10 μM), or histamine in combination with selective histamine receptor antagonists: pyrilamine (H_1 antagonist, 10 μM), famotidine (H_2 antagonist, 10 μM), or thioperamide (H_3 antagonist, 10 μM) for 48 h (B16F10) or 6 days (NHEMs). Melanin content was normalized to total protein. Data are expressed as fold change relative to the control group and presented as mean ± SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett’s post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns: not significant compared to His group.

2.3. Chronic Histamine Exposure Enhances SOCE Capacity in Melanocytes

To investigate whether histamine modulates Ca^{2+} signaling in melanocytes, we first examined whether histamine acutely activates SOCE or other Ca^{2+} channels. In B16F10 cells, we compared Ca^{2+} responses under three conditions: control (Ctrl) representing baseline Ca^{2+} influx, histamine (10 μM) application, and thapsigargin (Tg)-induced SOCE as a positive control. While Tg robustly activated SOCE, histamine application followed by Ca^{2+} readdition produced a Ca^{2+} increase comparable to control, with no significant difference between the two conditions (Figure 3A). Similarly, in NHEMs, acute histamine application failed to induce SOCE above baseline Ca^{2+} influx levels (Figure 3B). These results demonstrate that histamine does not immediately activate the SOCE pathway or other Ca^{2+} channels in either cell type.

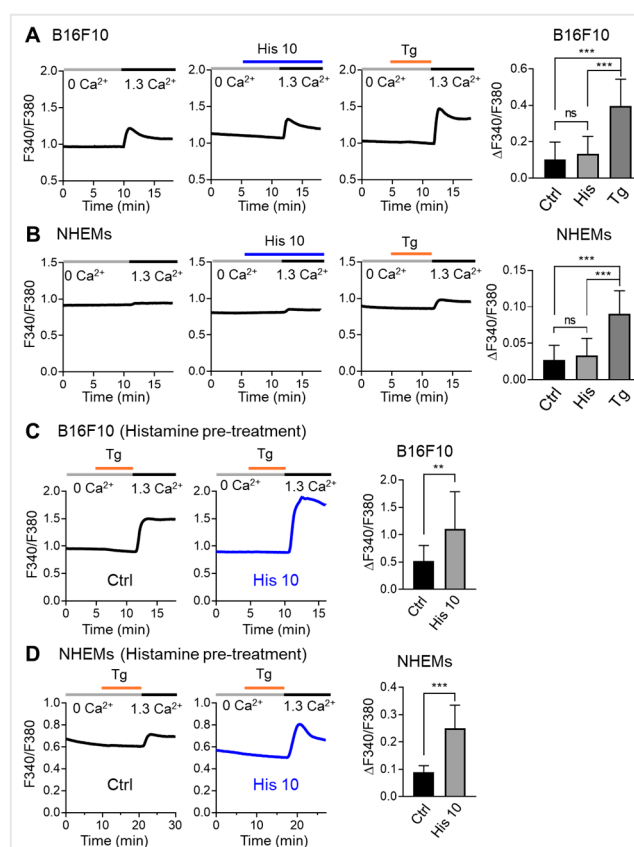


Figure 3. Histamine enhances store-operated Ca^{2+} entry (SOCE) through long-term pretreatment but not acute application. (A,B) Representative traces and quantification showing Ca^{2+} responses in B16F10 cells (A) and NHEMs (B). Cells were bathed in Ca^{2+} -free solution (0 Ca^{2+}) for 5–10 min, then treated with histamine (His, 10 μM) or thapsigargin (Tg, 1 μM) for 5–10 min, followed by readdition of 1.3 mM Ca^{2+} . $\Delta\text{F340}/\text{F380}$ represents the change in cytosolic Ca^{2+} level before and after Ca^{2+} readdition. Tg serves as a positive control for SOCE activation, while Ctrl represents baseline Ca^{2+} influx without SOCE activation. (C,D) Representative traces and quantification showing SOCE in B16F10 cells (C) pretreated with 10 μM histamine (His 10) for 48 h and NHEMs (D) pretreated for 6 days. Cells were bathed in Ca^{2+} -free solution (0 Ca^{2+}) for 5–10 min, then Tg (1 μM) was applied to deplete intracellular Ca^{2+} stores for 5–10 min, followed by Ca^{2+} readdition (1.3 mM Ca^{2+}) to measure SOCE. $\Delta\text{F340}/\text{F380}$ represents the change in cytosolic Ca^{2+} level before and after Ca^{2+} readdition. Data are presented as mean $\pm$ SD ($n = 3$). One-way analysis of variance (ANOVA), followed by Dunnett's post hoc test, was used for multiple comparisons. Student's t -test was used for comparison between two groups. ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

We next examined whether prolonged histamine exposure influences SOCE in melanocytes. B16F10 cells were pretreated with histamine (10 μM) for 48 h, followed by

SOCE measurement using Tg to deplete ER Ca^{2+} stores and trigger SOCE. Representative traces showed that histamine pretreatment markedly enhanced SOCE amplitude compared to control cells (Figure 3C). Quantitative analysis revealed that histamine increased SOCE amplitude ($\Delta\text{F}340/\text{F}380$) approximately 2.1-fold compared to control ($p < 0.001$) (Figure 3C). Similar experiments were performed in NHEMs pretreated with vehicle or histamine (10 μM) for 6 days. Consistent with B16F10 results, histamine pretreatment significantly enhanced SOCE in NHEMs (Figure 3D). Quantification demonstrated that histamine increased SOCE amplitude approximately 2.7-fold compared to control ($p < 0.001$) (Figure 3D). The marked contrast between the lack of acute response and the significant enhancement following long-term exposure provides strong evidence that histamine does not simply trigger an immediate Ca^{2+} signal but rather remodels the Ca^{2+} signaling machinery over time to sustain hyperpigmentation.

2.4. Intracellular Ca^{2+} Is Required for Histamine-Induced Melanogenesis

To determine whether intracellular Ca^{2+} plays a role in histamine-induced melanin production, we examined the effects of BAPTA-AM (an intracellular Ca^{2+} chelator, 10 μM) on melanogenesis in both B16F10 cells and NHEMs. Kojic acid (KA, 50 μM), a well-known tyrosinase inhibitor, was used as a positive control. In both B16F10 cells and NHEMs, histamine significantly increased melanin content compared to control ($p < 0.001$). Co-treatment with BAPTA-AM significantly reduced histamine-induced melanin production ($p < 0.001$), while kojic acid completely abolished the effect (Figure 4). These findings demonstrate that histamine-induced melanogenesis requires intracellular Ca^{2+} signaling in both B16F10 cells and primary human melanocytes, directly linking the observed SOCE potentiation to pigment production.

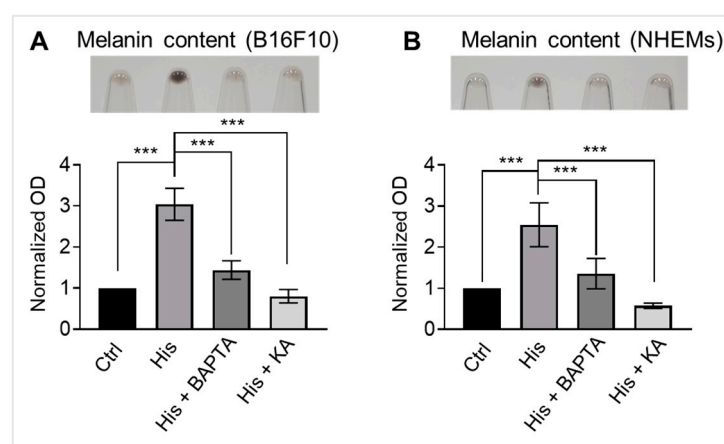


Figure 4. Intracellular Ca^{2+} is required for histamine-induced melanogenesis. Representative images and quantification of melanin content in B16F10 cells (A) and NHEMs (B). Cells were treated with vehicle control (Ctrl), histamine (His, 10 μM), histamine plus BAPTA-AM (intracellular Ca^{2+} chelator, 10 μM), or histamine plus kojic acid (KA, tyrosinase inhibitor, 50 μM) for 48 h (B16F10) or 6 days (NHEMs). Melanin content was normalized to total protein. Data are expressed as fold change relative to the control group and presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. *** $p < 0.001$ compared to His group.

2.5. ORAI1 and STIM1 Are the Essential Molecular Components of SOCE in Melanocytes

To determine the specific molecular mediators of SOCE in melanocytes, we performed siRNA-mediated knockdown of the ORAI and STIM family members in NHEMs. The knockdown efficiency was confirmed by RT-PCR analysis. The results demonstrated an 80–90% reduction in target mRNA expression for each respective siRNA with high specificity and statistical significance ($p < 0.001$) (Supplementary Figure S1). In control cells,

thapsigargin-induced store depletion followed by the addition of extracellular Ca^{2+} elicited a robust SOCE response. Strikingly, silencing of either ORAI1 or STIM1 significantly abrogated the SOCE response compared to the control. In contrast, siRNA-mediated silencing of ORAI2, ORAI3, or STIM2 had no significant effect on the SOCE amplitude (Figure 5). These findings demonstrate that ORAI1 and STIM1 are the primary functional units mediating SOCE in primary human melanocytes, while other ORAI or STIM family members do not contribute significantly to this pathway.

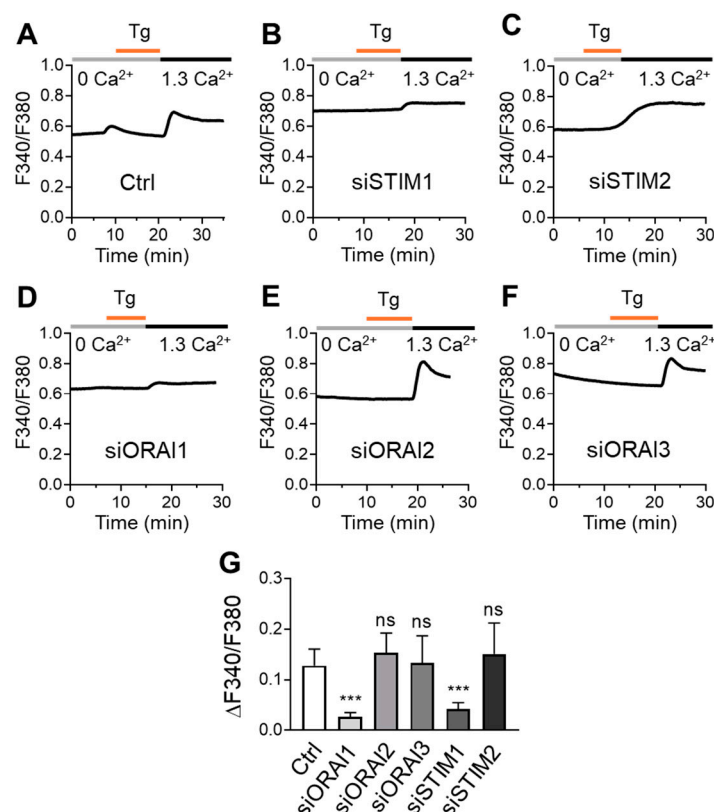


Figure 5. ORAI1 and STIM1 are the essential molecular components of store-operated Ca^{2+} entry (SOCE) in melanocytes. (A–F) Representative traces showing SOCE in NHEMs. Cells were transfected with siRNAs targeting ORAI1, ORAI2, ORAI3, STIM1, or STIM2 for 6 days. Cells were bathed in Ca^{2+} -free solution (0 Ca^{2+}) for 5–10 min, then Tg ($1 \mu\text{M}$) was applied to deplete intracellular Ca^{2+} stores for 5–10 min, followed by Ca^{2+} readdition (1.3 mM Ca^{2+}) to measure SOCE. (G) $\Delta\text{F340/F380}$ represents the change in cytosolic Ca^{2+} level before and after Ca^{2+} readdition. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. *** $p < 0.001$, ns: not significant.

2.6. Histamine-Induced Melanogenesis Is Specifically Mediated Through the ORAI1-STIM1-Dependent SOCE Pathway

To investigate whether ORAI1-STIM1 complex-mediated SOCE is functionally linked to histamine-induced melanogenesis, we employed both pharmacological inhibitors and genetic silencing approaches. In B16F10 melanoma cells, histamine ($10 \mu\text{M}$) significantly increased melanin content to approximately 2.77-fold compared to untreated control ($p < 0.001$). Co-treatment with the SOCE inhibitor BTP-2 ($5 \mu\text{M}$) or the ORAI1-selective inhibitor Synta-66 ($10 \mu\text{M}$) substantially attenuated this histamine-induced melanogenesis, reducing melanin levels to 1.44-fold and 1.10-fold of control, respectively ($p < 0.01$ and $p < 0.001$, compared to histamine alone) (Figure 6A). Similar inhibitory patterns were observed in primary human melanocytes (NHEMs), where histamine increased melanin content to approximately 2.14-fold of control ($p < 0.001$), and this enhancement was signifi-

cantly suppressed by BTP-2 and Synta-66 to 1.14-fold and 1.04-fold of control, respectively ($p < 0.001$ compared to histamine alone) (Figure 6B).

To validate the specific molecular components of the SOCE machinery responsible for melanogenesis, we performed siRNA-mediated knockdown experiments. Transfection with non-targeting siRNA (siNT) did not alter histamine-induced melanin production compared to histamine treatment alone in either cell type, confirming that the transfection procedure itself does not interfere with melanogenesis. Using histamine-treated siNT cells as the reference control, silencing of either *ORAI1* or *STIM1* effectively abolished histamine-induced melanin synthesis in both B16F10 cells and NHEMs, reducing melanin levels to near-baseline ($p < 0.001$ compared to His + siNT) (Figure 6C,D). In marked contrast, knockdown of *ORAI2*, *ORAI3*, or *STIM2* showed no significant effect on histamine-induced melanogenesis in either cell type, demonstrating the specific and non-redundant requirement for ORAI1 and STIM1 in this pathway. Collectively, these pharmacological and genetic data establish that histamine-induced melanin overproduction is critically dependent on the ORAI1-STIM1-mediated SOCE pathway in both melanoma cells and primary human melanocytes.

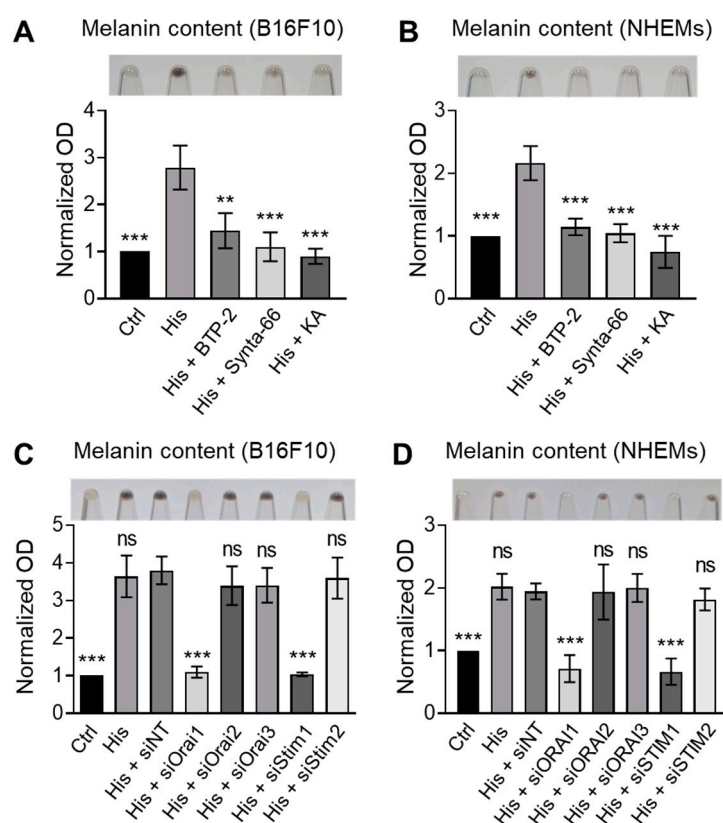


Figure 6. Histamine-induced melanogenesis is specifically mediated through the ORAI1-STIM1-dependent SOCE pathway. (A,B) Representative images showing cell pellets and quantification of melanin content in B16F10 cells (A) and NHEMs (B). Cells were treated with vehicle control (Ctrl), histamine (His, 10 μ M), histamine and BTP-2 (CRAC channel inhibitor, 5 μ M), or Synta-66 (ORAI1 inhibitor, 10 μ M), or kojic acid (KA, tyrosinase inhibitor, 50 μ M) for 48 h (A) or 6 days (B). (C,D) Representative images showing cell pellets and quantification of melanin content in B16F10 cells (C) and NHEMs (D). Cells were treated with histamine (His, 10 μ M) and transfected with non-targeting control siRNA (siNT) or siRNAs targeting Orai1/ORAI1, Orai2/ORAI2, Orai3/ORAI3, Stim1/STIM1, or Stim2/STIM2 for 48 h (C) or 6 days (D). Melanin content was normalized to total protein. Data are expressed as fold change relative to vehicle control and presented as mean $\pm$ SD ($n = 3-6$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. ** $p < 0.01$, *** $p < 0.001$, ns: not significant.

2.7. Histamine-Induced Melanogenesis Is Mediated by PKA-Dependent Signaling

To elucidate the downstream signaling pathways linking H₂ receptor activation to melanogenesis and SOCE remodeling, we investigated the involvement of key intracellular signaling mediators. B16F10 cells and NHEMs were treated with histamine in the presence or absence of H-89 (PKA inhibitor, 10 μ M) or U73122 (PLC inhibitor, 10 μ M), and melanin production was assessed. Kojic acid (KA, 50 μ M) was used as a positive control for melanogenesis inhibition. As shown in Figure 7A,B, PKA inhibition with H-89 significantly suppressed histamine-induced melanin synthesis in both B16F10 cells and NHEMs ($p < 0.001$), reducing melanin content to levels comparable to or below control. In contrast, PLC inhibition with U73122 did not significantly affect histamine-induced melanin production, with melanin levels remaining comparable to histamine treatment alone in both B16F10 cells and NHEMs. These findings indicate that histamine-induced melanogenesis is primarily mediated through PKA-dependent signaling pathways rather than PLC-dependent mechanisms.

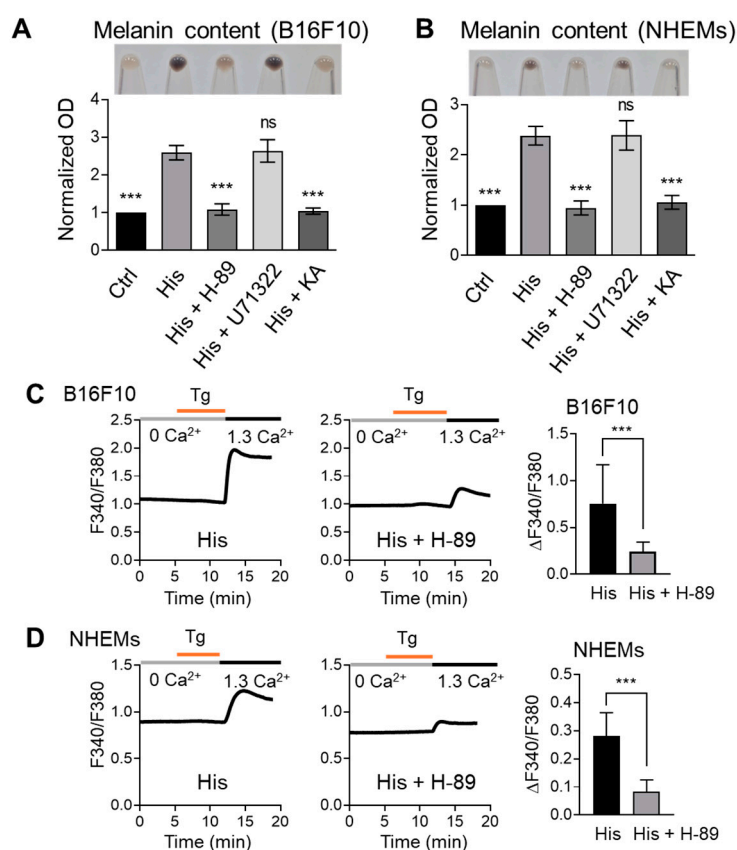


Figure 7. Histamine-induced melanogenesis and SOCE enhancement are mediated by PKA-dependent signaling. (A,B) Melanin content in B16F10 cells (A) and NHEMs (B) treated with vehicle control (Ctrl), 10 μ M histamine (His), or histamine in combination with 10 μ M H-89 (PKA inhibitor), 10 μ M U73122 (PLC inhibitor), or 50 μ M kojic acid (KA, tyrosinase inhibitor) for 48 h (B16F10) or 6 days (NHEMs). Representative images of cell pellets are shown above the graphs. Melanin content was normalized to total protein. Data are presented as mean $\pm$ SD ($n = 3$). *** $p < 0.001$; ns, not significant compared to His group (one-way ANOVA followed by Dunnett's post hoc test). (C,D) Representative traces and quantification showing SOCE in B16F10 cells (C) and NHEMs (D) pretreated with 10 μ M histamine alone or histamine plus 10 μ M H-89 (His + H-89) for 48 h (B16F10) or 6 days (NHEMs). Cells were bathed in Ca²⁺-free solution (0 Ca²⁺) for 5–10 min, then Tg (1 μ M) was applied to deplete intracellular Ca²⁺ stores, followed by Ca²⁺ readdition (1.3 mM Ca²⁺) to measure SOCE. $\Delta F_{340}/F_{380}$ represents the change in cytosolic Ca²⁺ level before and after Ca²⁺ readdition. Data are presented as mean $\pm$ SD ($n = 3$). *** $p < 0.001$ (Student's *t*-test).

To further investigate whether PKA signaling is involved in histamine-mediated SOCE remodeling, we examined the effect of H-89 on thapsigargin-induced Ca^{2+} influx in histamine-pretreated cells. Ca^{2+} imaging experiments revealed that co-treatment with H-89 significantly attenuated the histamine-induced enhancement of SOCE in both B16F10 cells (Figure 7C, $p < 0.001$) and NHEMs (Figure 7D, $p < 0.001$), demonstrating that PKA activity is required for histamine-induced functional remodeling of the SOCE machinery. Collectively, these results demonstrate that histamine promotes melanogenesis through a PKA-dependent signaling cascade that enhances SOCE function, leading to increased intracellular Ca^{2+} signaling and subsequent activation of melanogenic pathways.

3. Discussion

In this study, we investigated the role of ORAI1 Ca^{2+} channels in histamine-induced melanogenesis and uncovered a novel Ca^{2+} -dependent mechanism linking inflammatory mediators to melanin production. Our key findings demonstrate that: (1) histamine significantly increases melanin content in both B16F10 melanoma cells and primary human melanocytes (NHEMs) through H_2 receptor activation; (2) while acute histamine application does not trigger immediate Ca^{2+} influx, chronic histamine pretreatment significantly enhances the capacity for SOCE; (3) intracellular Ca^{2+} is essential for histamine-induced melanogenesis; (4) ORAI1 and STIM1 serve as the primary molecular mediators of SOCE in melanocytes and pharmacological inhibition or genetic silencing of ORAI1 or STIM1 effectively suppresses histamine-induced melanin production; and (5) histamine-induced melanogenesis and SOCE enhancement are mediated by PKA-dependent signaling. These findings reveal ORAI1-STIM1-mediated Ca^{2+} signaling as a critical pathway in histamine-induced melanogenesis and establish ORAI1 channels as a potential therapeutic target for PIH.

A notable observation from our study is the marked differential temporal response to histamine between B16F10 melanoma cells and normal human melanocytes. While B16F10 cells demonstrate robust melanin production within 48–72 h, NHEM cells require 6–8 days to achieve comparable melanogenic responses. This temporal difference reflects multiple interconnected aspects of malignant transformation. First, the accelerated proliferation rate of melanoma cells (doubling time ~18–24 h vs. 48–72 h for normal melanocytes) enables faster accumulation of melanogenic enzymes and melanin intermediates. Second, malignant transformation is associated with constitutive overexpression and activation of MITF [25], leading to an increased expression of its downstream targets, including tyrosinase, TRP-1, and TRP-2, positioning melanoma cells to produce melanin more rapidly compared to primary melanocytes when exposed to histamine. Third, melanoma displays remodeled Ca^{2+} homeostasis with upregulated SOCE machinery. Indeed, our Ca^{2+} imaging experiments reveal that B16F10 cells exhibit markedly higher SOCE amplitude compared to NHEM cells, with approximately 3-fold greater Ca^{2+} influx following thapsigargin-induced store depletion, regardless of histamine treatment (Figure 3). This enhanced ORAI1/STIM1-mediated Ca^{2+} entry in B16F10 cells facilitates more efficient Ca^{2+} signaling in response to histamine, thereby accelerating downstream melanogenic responses. Finally, melanoma cells produce dramatically elevated endogenous histamine levels (280-fold higher than normal melanocytes) [26], creating sustained autocrine signaling that primes cells for enhanced responsiveness. Furthermore, histamine H_2 receptor expression positively correlates with melanoma progression [27]. Despite these temporal differences, the fundamental mechanistic pathway is conserved in both cell types, as demonstrated by the comparable efficacy of ORAI1/STIM1 knockdown in blocking histamine responses (Figures 5 and 6).

The 10 μM histamine concentration employed in this study reflects pathophysiologically relevant conditions in the skin microenvironment. Under basal conditions, hu-

man tissues contain histamine ranging from less than 1 $\mu\text{g/g}$ to more than 100 $\mu\text{g/g}$ (approximately 9–900 μM), with the highest concentrations found in mast cell-rich tissues, including skin (72 μM total tissue pool) [28,29]. Critically, following mast cell degranulation during inflammatory responses, local extracellular histamine concentrations in the interstitial fluid can rise dramatically to 10–1000 μM [28]. Such elevations have been documented in various inflammatory skin conditions, including atopic dermatitis and allergic contact dermatitis [30,31]. Therefore, our experimental concentration models the chronic pathophysiological histamine exposure that melanocytes encounter during inflammatory conditions.

Our results confirm previous findings that histamine-induced melanogenesis is mediated specifically through H_2 receptors, as demonstrated by the complete blockade with the H_2 antagonist famotidine, while H_1 and H_3 antagonists showed no effect. This is consistent with earlier reports by Yoshida et al., who established that histamine stimulates melanogenesis via H_2 receptor-cAMP/PKA signaling in human melanocytes [13]. However, our study reveals a previously unrecognized role for Ca^{2+} signaling in this process.

A pivotal finding of this study is the temporal distinction in histamine's effect on Ca^{2+} signaling. Acute histamine application (1–100 μM) failed to trigger immediate Ca^{2+} influx, yet chronic histamine pretreatment (48 h in B16F10 cells, 6 days in NHEMs) significantly enhanced SOCE capacity by approximately 2.8-fold. This time-dependent effect suggests that histamine does not directly activate Ca^{2+} channels but rather induces a functional remodeling of the Ca^{2+} signaling machinery over time. The requirement for chronic exposure aligns with the clinical observation that PIH develops gradually during sustained inflammation rather than appearing immediately after inflammatory insults [10]. This distinguishes the histamine- H_2 receptor pathway from other stimuli like ET-1, which acutely triggers Ca^{2+} influx through ORAI1 channels.

Our study identifies PKA as the key signaling mediator linking H_2 receptor activation to SOCE enhancement and melanogenesis. Our results demonstrate that PKA inhibition with H-89 effectively suppressed both histamine-induced melanin production and the enhanced SOCE capacity observed in histamine-pretreated cells, while PLC inhibition with U73122 had no significant effect (Figure 7). This finding reveals that histamine activates SOCE through a non-canonical, PKA-dependent mechanism rather than the classical PLC- IP_3 -mediated store depletion pathway typically associated with SOCE activation. This PKA-dependent mechanism is particularly suited for sustained SOCE modulation during the prolonged histamine exposure periods required for melanogenesis, as continuous PLC-dependent store depletion would be energetically unsustainable. Notably, our Western blot analysis revealed that chronic histamine treatment did not alter the total protein expression levels of ORAI1 or STIM1 in either B16F10 melanoma cells or NHEMs (Supplementary Figure S2). This finding suggests that the functional enhancement of SOCE observed in histamine-treated melanocytes is not mediated by upregulation of SOCE protein components. Instead, histamine-induced functional remodeling may involve post-translational modifications of ORAI1/STIM1 proteins, altered subcellular localization to ER-plasma membrane junctions, or enhanced protein–protein interactions that increase channel coupling efficiency. Further investigation into these mechanisms will provide deeper insights into how histamine chronically enhances SOCE-mediated Ca^{2+} signaling in melanocytes to promote melanogenesis.

The pivotal role of ORAI1 in melanogenesis has been increasingly recognized across diverse stimuli. Previous studies have shown that ORAI1 mediates ET-1-induced pigmentation, where its knockdown significantly reduces both Ca^{2+} influx and tyrosinase activity [22]. Similarly, ORAI1 inhibition has been shown to block UV-induced melanogenesis, with ORAI1-targeting agents suppressing melanin production by up to 60% [24].

Interestingly, recent reports on echinochrome A (Ech A) further support this paradigm; Ech A not only downregulates the cAMP/PKA-CREB-MITF cascade but also potently inhibits ORAI1/STIM1 channels at similar concentrations [32,33]. This dual inhibitory action suggests a synergistic interplay between cAMP and Ca^{2+} signaling pathways, a concept that aligns with our identification of the ORAI1-STIM1 complex as a central hub for histamine-induced signaling.

Our findings extend these observations by identifying histamine as a novel upstream stimulus that converges on the ORAI1-STIM1 machinery. However, we reveal a distinct temporal signature: while ET-1 and UV typically trigger immediate Ca^{2+} influx through direct channel activation, chronic histamine exposure enhances SOCE capacity without eliciting an acute response [22]. This suggests that different melanogenic stimuli engage ORAI1 through specialized mechanisms, either through rapid gating or through the functional remodeling we observed here. Despite these upstream differences, the convergence of multiple pathways (UV, ET-1, and histamine) on ORAI1-mediated Ca^{2+} entry marks it as a critical and universal step in melanogenesis. From a clinical perspective, this makes ORAI1 an ideal therapeutic target. Unlike conventional tyrosinase inhibitors that often interfere with basal pigmentation [34], ORAI1-targeted therapy could specifically suppress stimulus-induced hypermelanosis while potentially preserving the skin's natural baseline coloration.

While this study provides compelling evidence for ORAI1's role in both B16F10 cells and NHEMs, certain aspects warrant further investigation. Although we focused on the functional outcomes of melanin content, future studies mapping the precise temporal expression dynamics of MITF and tyrosinase proteins would complement our current findings. Furthermore, while two-dimensional cultures established the fundamental signaling logic, validating these pathways in three-dimensional skin models or in vivo PIH models will be essential to account for the complex interactions within the skin microenvironment.

Ultimately, the development of topically applicable ORAI1 inhibitors presents a promising translational opportunity. By targeting the ORAI1-STIM1 complex locally, it may be possible to mitigate inflammatory hyperpigmentation with high specificity and minimal systemic exposure. These findings establish a strong foundation for advancing ORAI1-targeted interventions as a next-generation treatment strategy for PIH and related pigmentary disorders.

4. Materials and Methods

4.1. Chemicals and Cell Lines

Histamine, pyrilamine, famotidine, thioperamide, BTP-2, Synta-66, BAPTA-AM, kojic acid, and thapsigargin were procured from Sigma-Aldrich (St. Louis, MO, USA). H-89 and U73122 were procured from MedChemExpress (Monmouth Junction, NJ, USA). Both the B16F10 cell line and Normal Human Epidermal primary Melanocytes (NHEMs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

4.2. Cell Culture

Experiments were performed on B16F10 and NHEM cells. B16F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Welgene Inc., Daegu, Korea) with 10% fetal bovine serum (FBS, Welgene Inc.) and 1% penicillin/streptomycin (P/S, Life Technologies, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 incubator. Melanoma cells with passage 5–15 were used. NHEMs were cultured in dermal cell basal medium (ATCC, Cat# PCS-200-030) supplemented with adult human melanocyte growth supplement (ATCC, Cat# PCS-200-013) and Penicillin-Streptomycin-Amphotericin B (ATCC, Cat# PCS-999-002) at 37 °C in a 5% CO_2 incubator. The primary melanocytes with passage 2–6 were used.

4.3. Cell Viability

B16F10 cells were cultured in a 96-well plate at a density of 1×10^4 cells per well. After 24 h, various concentrations of histamine were added. Following 24, 48, and 72 h of incubation, cells were exposed to 10 μ M CCK-8 (Dojindo Laboratories, Kumamoto, Japan) for 120 min. The optical density (OD) was measured at 450 nm and normalized to the value of blank and control cells to calculate viability.

4.4. Melanin Content Measurement

B16F10 cells were seeded into a 6-well plate at a density of 1×10^5 cells per well. After culturing overnight, cells were treated with various concentrations of histamine with or without other chemicals for 48 h. NHEMs were seeded into 100 mm plates at a density of 1×10^6 cells per plate. After culturing overnight, cells were treated with histamine (10 μ M) with or without other chemicals for 6 days. To maintain consistent drug exposure during this extended treatment period, the culture medium containing fresh chemicals was replaced every 48 h.

Following treatment, cells were harvested and washed twice with phosphate-buffered saline (PBS). The pelleted cells were lysed in 1N sodium hydroxide (NaOH) and then heated at 80 °C for 1 h. Cell lysates were transferred to a 96-well plate, and the absorbance was measured at 405 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Relative melanin content was normalized to total protein measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.5. Small Interfering RNA Transfection

B16F10 cells were seeded into a 6-well plate at a density of 1×10^5 cells per well. After culturing overnight, cells were transfected with each siRNA (100 nM) using TurboFect™ Transfection Reagent (Thermo Fisher Scientific) for 48 h. NHEMs were seeded into 100 mm plates at a density of 1×10^6 cells per plate. After culturing overnight, cells were transfected with siRNA (300 nM) using TurboFect™ Transfection Reagent for 6 days; the medium containing siRNA was changed every 48 h. Pools of three or five target-specific siRNA directed against *ORAI1*, *ORAI2*, *ORAI3*, *STIM1*, and *STIM2* for human and mouse were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The knockdown efficiency of siRNA-mediated gene silencing was validated by RT-PCR analysis as described in Supplementary Materials. Primer sequences used for RT-PCR are provided in Supplementary Table S1.

4.6. Ca^{2+} Imaging

Ca^{2+} influx measurements were performed with B16F10 and NHEM cells. B16F10 cells were seeded into a 6-well plate at a density of 1×10^5 cells per well. After culturing overnight, cells were treated with histamine (10 μ M) for 48 h. NHEMs were seeded into 100 mm plates at a density of 1×10^6 cells per plate. After culturing overnight, cells were treated with histamine (10 μ M) for 6 days. To maintain consistent drug exposure during this extended treatment period, the culture medium containing fresh chemicals was replaced every 48 h.

Ca^{2+} imaging experiments were performed using two Normal Tyrode's (NT) solutions. The Ca^{2+} -free NT solution contained 145 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 1 mM MgCl_2 , 1 mM EGTA, 5 mM glucose (pH 7.4 adjusted with NaOH). The Ca^{2+} -containing NT solution contained 145 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 1 mM MgCl_2 , 1.3 mM CaCl_2 , 5 mM glucose (pH 7.4 adjusted with NaOH). B16F10 and NHEM cells were washed twice using PBS, then incubated with Fura-2 acetoxymethyl ester (Fura-2 AM, Thermo Fisher Scientific) at a final concentration of 2 μ M for 30 min at 37 °C. Then, these cells

were loaded onto 14 mm poly-L-lysine-coated coverslips. Cells were first perfused with Ca^{2+} -free NT solution for 5–10 min to establish a baseline. Store-operated Ca^{2+} entry (SOCE) was then induced by perfusing 1 μM thapsigargin in Ca^{2+} -free NT solution for 5–10 min to deplete ER Ca^{2+} stores and activate ORAI1 channels. Subsequently, the Ca^{2+} -containing NT solution was applied to measure Ca^{2+} influx. Changes in intracellular Ca^{2+} concentration were monitored by measuring the fluorescence intensity ratio (F340/F380). Fura-2 fluorescence was excited alternately at 340 nm and 380 nm, and emission was collected at 510 nm using a digital imaging system comprising an illuminator (pE-340 fura; CoolLED, Andover, UK) and a sCMOS camera (pco.edge 4.2; PCO, Kelheim, Germany). The F340/F380 fluorescence ratio was acquired every 10 s, and changes in intracellular Ca^{2+} concentration were analyzed using NIS-Elements AR Version 5.00.00 (Nikon Instruments Inc., Melville, NY, USA).

4.7. Statistical Analysis

The results were analyzed using GraphPad Prism 8.0 (GraphPad Software, Boston, MA, USA) and Origin 2021b (OriginLab Corporation, Northampton, MA, USA). Shapiro–Wilk tests were performed to assess data normality prior to applying parametric statistical analyses. All data are shown as the mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test, was used for multiple comparisons. Student’s *t*-test was used for comparison between two groups. Statistical significance was defined as *p*-value < 0.05 .

5. Conclusions

This study demonstrates that the ORAI1-STIM1-mediated SOCE pathway is essential for histamine-induced melanogenesis. We reveal that chronic, rather than acute, histamine exposure induces functional remodeling of the Ca^{2+} signaling machinery, significantly enhancing SOCE capacity to drive sustained melanin overproduction. Pharmacological or genetic inhibition of this complex effectively suppresses histamine-induced hyperpigmentation. These findings establish the ORAI1-STIM1 complex as a promising therapeutic target for treating PIH and related inflammatory pigmentary disorders.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27042055/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

PIH	Post-inflammatory hyperpigmentation
NHEMs	Normal Human Epidermal Melanocytes
SOCE	Store-operated Ca^{2+} entry
UV	Ultraviolet
ET-1	Endothelin-1
MITF	Microphthalmia-associated transcription factor

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