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Mutual upregulation of endothelin-1 and IL-25 in atopic dermatitis

Mst. Khudishta Aktar^{a#}, Makiko Kido-Nakahara^{a#*}, Masutaka Furue^{a,b}, and Takeshi Nakahara^b

^aDepartment of Dermatology, Graduate School of Medical Sciences, Kyushu University,
Fukuoka, Japan

^bDivision of Skin Surface Sensing, Department of Dermatology, Graduate School of Medical
Sciences, Kyushu University, Fukuoka, Japan

[#]These authors contributed equally to this manuscript.

Short title: **Correlation between ET-1 and IL-25 in keratinocytes**

*Corresponding author:

Makiko Kido-Nakahara

Department of Dermatology, Graduate School of Medical Sciences, Kyushu University,
Fukuoka, Japan

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Abstract

Background: Endothelin 1 (ET-1) has been reported to evoke histamine-independent pruritus in mammals. However, its association with pruritus or inflammation of atopic dermatitis (AD) has not been clarified. We sought to investigate the role of ET-1 in the skin inflammation of AD.

Methods: To examine the role of ET-1 in AD, we investigated the expression of ET-1 and IL-25 in the skin of an AD mouse model and AD patients, and examined the mutual regulatory relationship between ET-1 and IL-25, one of the important cytokines in AD, using the human HaCaT keratinocyte cell line.

Results: We immunohistochemically confirmed the upregulation of ET-1 and IL-25 expression in the epidermis of both the AD mouse model and AD patients. In vitro, IL-25 upregulated ET-1 mRNA and protein expression in a concentration- and time-dependent fashion in HaCaT cells. This IL-25-induced ET-1 expression was inhibited by ERK1/2 or JNK inhibitor. In a reciprocal manner, ET-1 also induced IL-25 upregulation. The enhancing effect of ET-1 on IL-25 was inhibited by an endothelin A receptor antagonist, ERK1/2 inhibitor or p38 inhibitor, but not by an endothelin B receptor antagonist or JNK inhibitor.

Conclusion: These findings suggest that mutual upregulation of ET-1 and IL-25 takes place in the epidermis in AD, which may be a future target for anti-pruritic agents.

Keywords: atopic dermatitis; endothelin-1; IL-25; keratinocyte; MAPK

Introduction

Atopic dermatitis (AD) is a chronic, inflammatory skin disease and a major public health problem worldwide. Most of the patients with AD can control their skin disease with topical therapy, but there is a considerable group of patients with severe patients who requires the use of systemic immunosuppressive drug application (1). AD is characterized by intense itch, inflammation, and skin barrier dysfunctions. Many peptides, proteinases, and cytokines have been shown to play important roles in AD (2). Endothelin-1 (ET-1), which induces pruritus in mice and humans, might be one of the mediators of AD. It is a 21-amino-acid peptide that is expressed by a variety of cell types, including immune cells, endothelial cells, neurons, and keratinocytes (3-6). ET-1 was found to be a potent vasoconstrictor through the activation-specific G-protein-coupled endothelin A receptor (ETAR) and endothelin B receptor receptor (ETBR) (7). Besides its vasoconstrictive properties, increased ET-1 expression is closely associated with a number of diseases, such as scleroderma, psoriasis, and asthma (8, 9). It has been reported that ET-1 levels were found to be elevated in bronchoalveolar lavage fluid, bronchial biopsies, and peripheral blood of asthmatic patients (10) and blocking ET-1 prevented airway remodeling and hyper-reactivity in a murine asthma model (11). In addition, ET-1 acts as a pruritogen by inducing itch, which is a major diagnostic criterion of AD and one of its most troublesome symptoms. It has been reported that ET-1 induces pruritus in mice (12, 13) and humans by a histamine-independent mechanism (14). The levels of vascular cell adhesion molecule-1 (VCAM-1) and IL-18 were found to be increased with increasing disease severity in AD-associated inflammation (15-17). In this context, ET-1 has been shown to upregulate VCAM-1 (18) and IL-18 expression (19), suggesting its indirect association with AD inflammation. However, the expression of ET-1 in AD has not been studied yet.

IL-25, a newly identified cytokine, was first described as a Th2-cell-derived cytokine and was found to be expressed in keratinocytes (20, 21). Recently, IL-25 has been identified as one of the initiators of Th2 responses (22). It has been suggested that mRNA and protein levels of IL-25 and its receptor are elevated in the skin of individuals with AD, and their expression is elevated in lesional skin compared with that in non-lesional skin. These results coincide with the fact that IL-25 augments Th2 immune responses (20, 23).

To reveal the relationship between ET-1 and IL-25, we investigated their in vivo expression in murine and human AD skin. We also examined the mutual regulatory mechanism involved in ET-1 and IL-25 expression in human keratinocytes.

Materials and methods

Materials

ET-1, actinomycin D, SP600125, and SB202190 were obtained from Sigma Aldrich (St. Louis, MO, USA). BQ610 and BQ788 were purchased from Peninsula Laboratories (Belmont, CA, USA) and U0126 was from Calbiochem (San Diego, USA), respectively. Anti-rabbit monoclonal antibodies for ERK1/2, pERK1/2, JNK, pJNK, p38, and p-p38 were obtained from Cell Signaling Technology (Billerica, MA, USA). Anti-ET-1 mouse monoclonal antibody and anti-IL-25 polyclonal antibody were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Abcam (Minneapolis, MN, USA), respectively. Biostir-AD reagent ointment including *Dermatophagoides farinae* antigen was prepared by Biostir Inc. (Tokyo, Japan).

Induction of atopic dermatitis-like skin lesion

NC/Nga mice (six weeks old) were purchased from Charles River Laboratories (Tokyo, Japan) and were maintained in specific pathogen-free conditions. The protocol was approved by the Committee of Ethics on Animal Experiments at the Graduate School of Medical Sciences, Kyushu University. AD-like skin lesions were induced in six-week-old male NC/Nga mice using Biostir-AD, as described by the manufacturer (24).

Cell culture

Human HaCaT keratinocytes (kindly provided by Dr. N.E. Fusenig, DKFZ Heidelberg) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamate and 1% penicillin-streptomycin and incubated in a humidified 5% CO₂ atmosphere at 37 °C in a 25-cm² flask.

Histological analysis

All formalin-fixed and paraffin-embedded tissues (7 samples from chronic uncured lesions of AD patients and 7 for normal skin) were obtained from the archives of the Department of Dermatology of Kyushu University Hospital, Japan. Skin samples (mouse and human) were fixed in 4% phosphate-buffered formalin solution, embedded in paraffin, sliced into 3-μm-thick sections, and stained with hematoxylin-eosin. Sections for the staining of ET-1 and IL-25 were deparaffinized, rehydrated, and the antigens were retrieved by pressure-cooking the sections in 10 mM sodium citrate buffer at pH 6.0 (Histofine PRO II) for 20 min. The sections were

immersed in 3% H₂O₂ in methanol for 30 min. After blocking with 10% goat serum for 30 min, the sections were incubated with either anti-ET-1 or anti-IL-25 antibody overnight at 4 °C. Then, the sections were incubated with secondary antibody for 30 min. Immunoreactivity was detected using the Liquid DAB+ Substrate Chromagen System (DaKo, Tokyo, Japan).

Reverse transcriptase and semi-quantitative RT-PCR analysis

Total RNA was extracted by using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed by using PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan). qRT-PCR (40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 20 s) was performed using the Mx3000p Real-Time System (Stratagene, La Jolla, CA, USA) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), as described by the manufacturer. The results were normalized against that of β -actin in the same sample. The sequences of the primers were as follows: for human ET-1 (forward): 5'-TCCTCTGCTGGTTCCTGACT-3', (reverse): 5'-CAGAAACTCCACCCCTGTGT-3'; for human IL-25 (forward): 5'-CGACCCAGATTAGGTGAGGA-3', (reverse): 5'-TCCATCTTCACTGGCCCTAC-3'; for human ETAR (forward): 5'-GGGATCACCGTCCTCAACCT-3', (reverse): 5'-CACGACTCCAGGAGGCAACT-3'; for ETBR (forward) 5'-TCAACACGGTTGTGTCCTGC-3', (reverse): 5'-ACTGAATAGCCACCAATCTT-3'; for human β -actin (forward): 5'-TCATGAAGTGTGACGTTGACATCCGT-3', (reverse): 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'; and for mouse ET-1 (forward): 5'-GAGTTCCATTTGCAACCGAGT-3', (reverse): 5'-CTGAGTTCGGCTCCCAAGAC-3'.

Western blotting analysis

Equal amounts of protein from cell lysates and mouse back skin were subjected to 4–12% NuPage Bis-Tris Gels (Invitrogen, CA, USA) at 200 V for 35 min. The proteins were then transferred onto polyvinylidene fluoride membranes (Invitrogen) and blocked in 2% BSA with 0.1% Tween-20 (Sigma Aldrich) in Tris-buffered saline. The membranes were probed with primary antibody overnight, followed by incubation in secondary antibody for 30 min; protein bands were detected using Western Breeze Kit (Invitrogen).

ELISA experiment

HaCaT cells (0.8×10^6 cells per well) were seeded onto 12-well plates in 1 ml of medium with 10% fetal bovine serum. Growth-arrested cells at 70% confluence were stimulated with ET-1 and IL-25 (R&D Systems, Minneapolis, USA) for the indicated time and supernatants were analyzed for IL-25 and ET-1 by ELISA (Peprotech, Rocky Hill, USA; R&D Systems, Minneapolis, USA), in accordance with the manufacturers' instructions.

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM) for three independent experiments and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

ET-1 and IL-25 expression increases in mouse and human AD skin

We first investigated ET-1 expression in a Biostir-AD-induced mouse AD model. In line with a previous study (25), Biostir-AD treatment clinically induced AD-like skin lesions (Fig. 1A and B). The mRNA and protein levels of ET-1 were significantly increased in skin with an AD-like phenotype (n = 6) (Fig. 1C and D). Increased ET-1 staining in the epidermis was observed in AD mouse skin compared with that in control mouse skin (n = 6) (Fig. 1E). Furthermore, ET-1 expression was enhanced in the epidermis of human AD patients (n = 7) compared with that of healthy individuals (n = 7) (Fig. 1F).

We next investigated the expression of IL-25 in AD. Immunohistochemical analysis showed clear upregulation of IL-25 in the epidermis of mouse AD skin compared with that in controls (Fig. 1G). Enhanced expression of IL-25 was also evident in the epidermis of AD patients (n = 7) compared with that in healthy controls (n = 7) (Fig. 1H), which suggests that IL-25 plays a significant role in AD (26).

IL-25 promotes ET-1 mRNA levels in HaCaT keratinocytes

To address the mechanism of elevated expression of ET-1 in the epidermis of AD lesions, we investigated the effect of IL-25 on the ET-1 expression in keratinocytes. We confirmed the expression of IL-25 and ET-1 in HaCaT keratinocytes by RT-PCR analysis (Fig. 2A). IL-25 upregulated the ET-1 mRNA expression in a dose- and time-dependent manner (Fig. 2A and

2B). ET-1 protein was detected in the supernatants of HaCaT keratinocytes and was significantly upregulated by IL-25 in a time-dependent fashion (Fig. 2D).

IL-25 increases ET-1 expression via ERK1/2 and JNK MAPK pathway

To examine the intracellular pathway involved in IL-25-induced ET-1 expression, we next investigated the activation of the MAPK pathway using IL-25-stimulated keratinocytes.

Phosphorylation of ERK1/2 and JNK was upregulated by IL-25 in western blot assay (Fig. 3A).

In contrast, IL-25 did not phosphorylate p38 MAPK. Preincubation of cells with U0126 (ERK1/2 inhibitor) or SP600125 (JNK inhibitor) was confirmed to diminish the activation of ERK1/2 or JNK (Fig. 3B and 3D). Pretreatment with U0126 and SP600125 significantly inhibited the IL-25-induced ET-1 mRNA expression in a concentration-dependent manner (Fig. 3C and E).

ET-1 induces the expression of IL-25 in HaCaT cells

To examine the relationship between IL-25 and ET-1, we next investigated whether ET-1 could stimulate IL-25 expression in HaCaT keratinocytes. Concentration- (Fig. 4A) and time-dependent (Fig. 4B) inductions of IL-25 mRNA and protein (Fig. 4C) were observed in the presence of ET-1. The regulation of IL-25 expression by ET-1 occurred at the transcriptional level because pretreatment with actinomycin D, a transcription inhibitor, inhibited the ET-1-induced change in IL-25 mRNA (Fig. 4D).

ET-1 induces IL-25 expression through ETAR

After confirming the expression of ETAR and ETBR in HaCaT cells by RT-PCR analysis (Fig. 5A), we investigated which subtype of receptor is involved in the ET-1-induced IL-25

upregulation. Pretreatment with BQ610 (ETAR antagonist), but not BQ788 (ETBR antagonist), attenuated the ET-1-induced change in IL-25 mRNA expression in a concentration-dependent manner (Fig. 5B and C), suggesting a pivotal role for ETAR in mediating the ET-1-induced upregulation of IL-25.

ET-1-induced IL-25 production is mediated by ERK1/2 and p38 MAPK but not JNK

To reveal intracellular pathway involved in ET-1-induced IL-25 expression, we then investigated whether ET-1 could activate MAPK pathways. Phosphorylation of ERK1/2, JNK, and p38 MAPK was induced by ET-1, as determined by western blot analysis (Fig. 6A). Preincubation of cells with U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), or SB202190 (p38 inhibitor) diminished the phosphorylation of ERK1/2, JNK, and p38, respectively (Fig. 6B, D, and F). Interestingly, significant dose-dependent inhibition of IL-25 expression was evident with U0126 and SB202190, but not with SP600125, in the presence of ET-1 (Fig. 6C, E, and G).

Discussion

Although ET-1 induces pruritus, which is an essential feature of AD, its actual role in AD remains largely unknown. Here, we report for the first time that the expression of ET-1, as well as of IL-25, was elevated in the epidermis of AD cases and that mutual positive feedback regulation between ET-1 and IL-25 was in operation.

In AD, pruritus causes an itch–scratch cycle, and intensive scratching promotes both the susceptibility to itching and the exacerbation of skin lesions (2, 27). Antihistamines have little

effect on alleviating itch in patients with AD, suggesting that histamine is not a major mediator of itch in AD patients (28, 29). IL-31 has been claimed to be critical for itch in AD. However, Hawro et. al. recently suggested the role of IL-31 in itch induction may be minor in human (30). ET-1 could be an important candidate cause of skin inflammation and itch in AD patients as it has been shown to induce histamine-independent itch in humans and mice (13, 14). We found that the expression of ET-1 was augmented in AD-like skin lesions in a murine model. Moreover, immunohistochemical analysis revealed that the expression of ET-1 was elevated together with that of IL-25 in the lesional epidermis of AD-like mice and AD patients, compared with those of controls. These results suggest that ET-1 is involved in the pathogenesis of AD and might interact with IL-25 in keratinocytes. Therefore, we examined the mutual regulatory effects of ET-1 and IL-25 using HaCaT keratinocytes.

First, we investigated whether IL-25 could induce ET-1 expression in keratinocytes. A critical role of IL-25 in the pathogenesis of AD, by skewing the microenvironment to a Th2 milieu, was emphasized in recent studies (26, 31). Hvid et al. reported an elevated level of IL-25 mRNA in the skin of AD patients in comparison with that in skin from healthy volunteers (23). We found that IL-25 significantly upregulated the gene and protein expression of ET-1 in keratinocytes. Since TNF- α was reported to upregulate the expression of ET-1 in an ERK1/2- and JNK-dependent manner in hepatic stellate cells (32), we next examined the involvement of MAPKs in IL-25-induced upregulation of ET-1. The expression of ET-1 by IL-25 was mediated via the phosphorylation of ERK1/2 and JNK pathways because it was inhibited by either ERK1/2 inhibitor or JNK inhibitor.

We then examined whether ET-1 modulates the expression of IL-25 in keratinocytes. The expression and secretion of IL-25 were significantly upregulated by ET-1 in HaCaT keratinocytes. These effects were mediated by ERK1/2 and p38 pathways, but not by JNK. A previous study by Yu et al. demonstrated that the IL-25 expression of murine pulmonary epithelial cells after protease allergen treatment was inhibited by inhibitors of three MAPK pathways, ERK1/2, JNK, and p38 MAPK (33, 34). The lack of JNK involvement in ET-1-induced IL-25 upregulation in the present study may have been due to the difference in the cell source or method of stimulating cells.

In various experimental settings, the involvement of ETAR, but not ETBR, in ET-1-induced cytokine production and the expression of adhesion molecules (35), as well as histamine release from mast cells (36), has consistently been evident, suggesting a critical role of ETAR in the signaling pathway of ET-1. Our results also reveal that ETAR is mainly responsible for upregulating IL-25 expression because an ETAR antagonist, but not an ETBR one, significantly inhibited ET-1-induced IL-25 expression.

In conclusion, we have demonstrated that the expression of ET-1 and IL-25 was coordinately upregulated in the lesional keratinocytes of AD patients and a murine AD model. Of note, IL-25 augmented ET-1 expression, and ET-1 reciprocally increased IL-25 expression in keratinocytes. This vicious circle of mutual positive feedback between ET-1 and IL-25 may be regarded as one of the therapeutic targets for the treatment of AD.

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Figure Legends

1. ET-1 and IL-25 expression induced in the skin of AD patients and mice with an AD-like phenotype. (A) AD-like skin lesions in NC/Nga mice 27 days after Biostir-AD treatment. (B) Histological changes of dorsal skin after Biostir-AD treatment. (C) ET-1 mRNA levels increased in skin with an AD-like phenotype. Results are expressed as mean $\pm$ SEM (n = 6). * $P < 0.05$ compared with the control group. (D) ET-1 protein levels increased in dorsal shaved skin of mice with an AD-like phenotype. (E) ET-1 expression in epidermal layers of mice with an AD-like phenotype visualized by immunohistochemistry. (F) Staining for ET-1 was significantly enhanced in the epidermis of AD human skin compared with that in healthy skin. Staining for IL-25 was enhanced in the epidermis of AD mouse (G) and AD human skin compared with that in the controls (H).
2. Effect of IL-25 on ET-1 expression in HaCaT cells. (A) IL-25 expression in HaCaT cells. Representative gel from one of three independent experiments. (B) Concentration-dependent effect of IL-25 on ET-1 expression. Cells were treated with increasing concentrations of IL-25 up to 500 nM for 24 h and mRNA levels were analysed by RT-PCR. ET-1 mRNA levels

were normalized using β -actin. (C) Time-dependent stimulation of ET-1 by IL-25 treatment in HaCaT cells. Cells were stimulated with 500 nM IL-25 for 0, 7, and 24 h and mRNA was analysed by RT-PCR. mRNA levels are expressed as fold induction compared with that in the control group. (D) Effect of IL-25 on ET-1 protein levels. Cells were stimulated with IL-25 for 0, 24, 48, and 72 h and supernatants were analysed by ELISA. Results are expressed as mean $\pm$ SEM of three experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (B, C, D) vs. control cells.

3. IL-25 induced ET-1 expression through ERK1/2 and JNK phosphorylation in HaCat cells. Western blot showing phosphorylation of ERK1/2, JNK, and p38 MAPK by IL-25. HaCaT cells were stimulated with 500 nM IL-25 for the indicated time and lysates were subjected to western blot analysis (A). Effect of U0126 on ERK1/2 phosphorylation by IL-25. Cells were stimulated with 500 nM IL-25 after pretreatment with U0126 at concentrations of 10 and 20 μ M and lysates were subjected to western blotting (B) and RT-PCR analyses (C). Effect of SP00125, a JNK inhibitor, on JNK phosphorylation and ET-1 expression induced by IL-25. Cells were stimulated with IL-25 after pretreatment with SP600125 and lysates were subjected to western blot (D) and RT-PCR analyses (E). Results are expressed as mean $\pm$ SEM (n = 3) of three experiments. $*P < 0.05$ compared with the ET-1-treated cells (C and D).
4. Effect of ET-1 on IL-25 mRNA expression in HaCaT cells. (A) HaCaT cells were treated with different (10–500 nM) concentrations of ET-1 for 24 h for RT-PCR analysis. (B) Time-dependent effect of ET-1 on IL-25 expression. Cells were stimulated with 100 nM ET-1 for different lengths of time (0–48 h) and the expression of IL-25 mRNA was analyzed by RT-PCR. (C) Effect of ET-1 on IL-25 secretion from HaCaT cells. HaCaT cells were treated

with different (0–200 nM) concentrations of ET-1 for 24 and 48 h. Concentrations of IL-25 in the culture medium were measured by ELISA. Data are the mean $\pm$ SEM of three

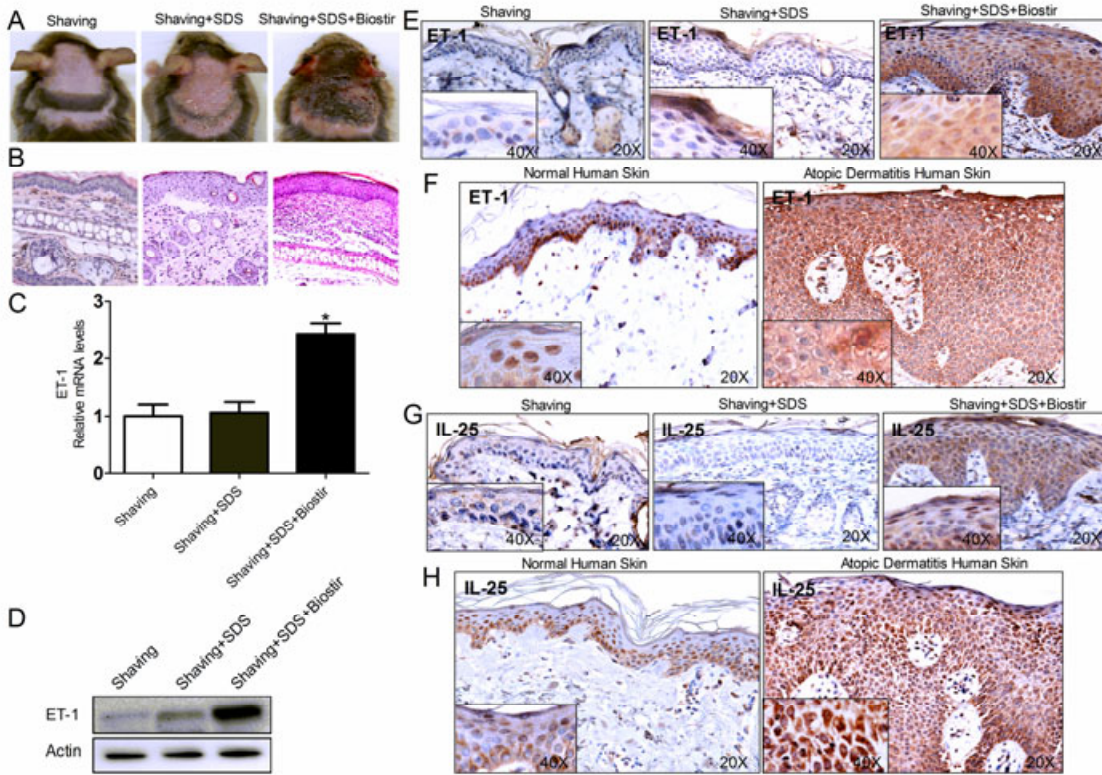
experiments. $*P < 0.05$ versus control. (D) Effect of actinomycin D on ET-1-induced IL-25 mRNA levels. Growth-arrested cells were stimulated with 100 nM ET-1 for 8 h.

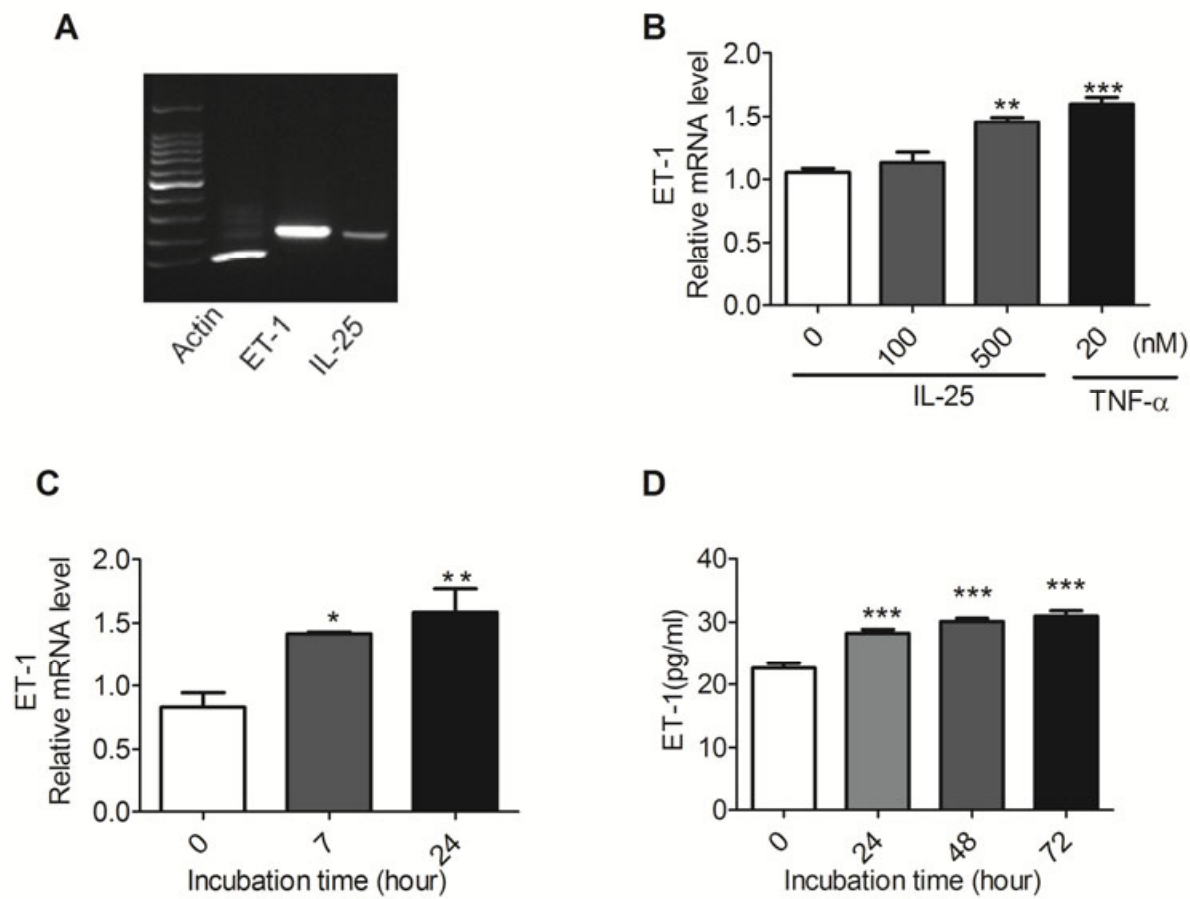
Actinomycin D (1, 5, and 10 μ M) was added to the medium 30 min before ET-1 treatments.

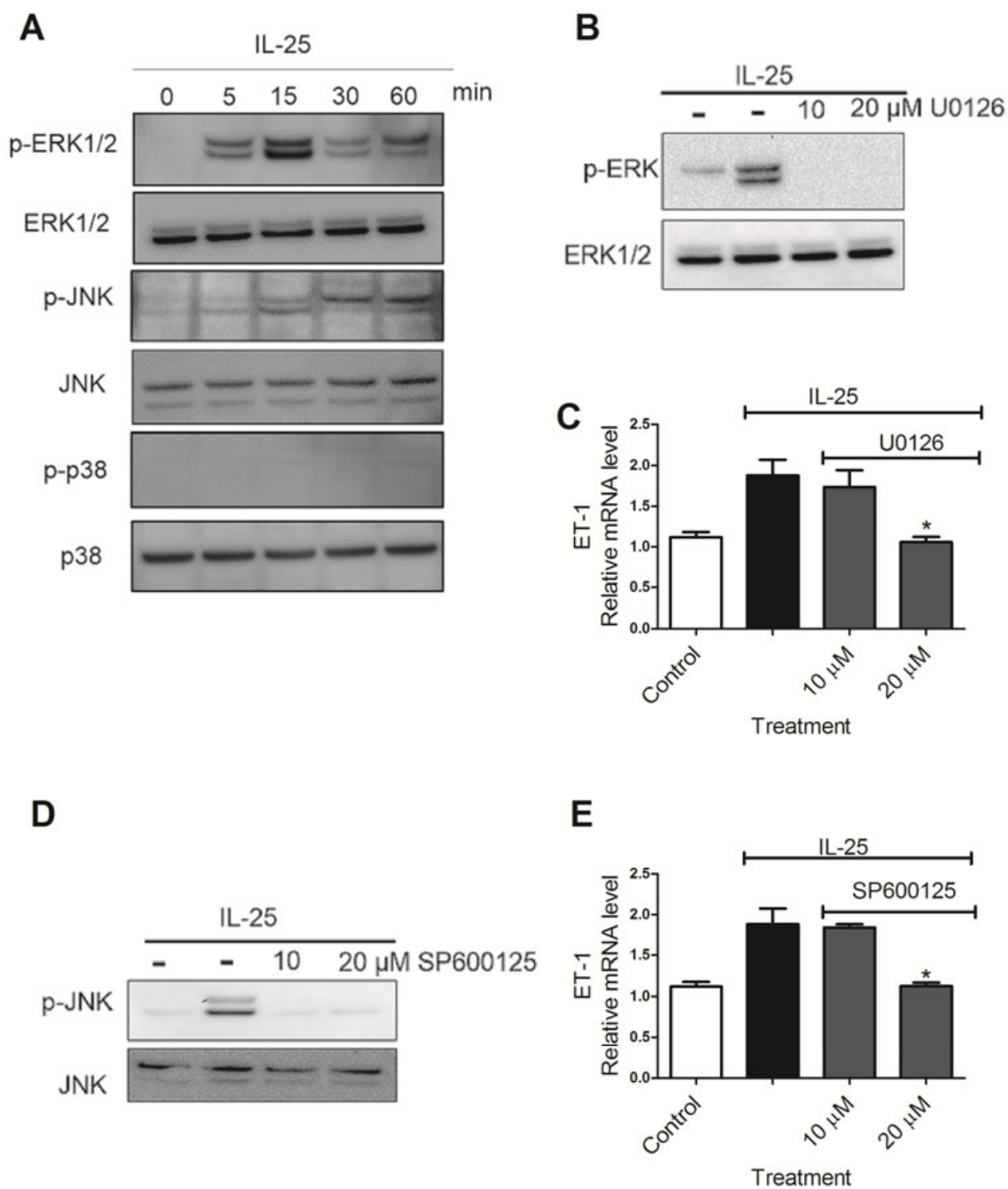
(D). Data are the mean $\pm$ SEM of three experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with the control (A, B, and C) and compared with the ET-1-treated cells (D).

5. ET-1 induced IL-25 expression through ETAR. (A) HaCaT cells were stimulated with 100 nM ET-1 for 24 h. BQ610 and BQ788 (ETAR and ETBR antagonists, respectively) were added 1 h before treatment with ET-1. Data are the mean $\pm$ SEM of three experiments. $**P < 0.01$ compared with ET-1-treated cells by one-way ANOVA followed by Dunnett's post hoc test.
6. ET-1 induced IL-25 expression through ERK/p38 MAPK. (A) Effect of ET-1 on ERK1/2, JNK, and p38 phosphorylation in HaCaT cells. Cells were stimulated with 100 nM ET-1 for different lengths of time (0, 5, 10, 15, and 30 min). Cell lysates were subjected to western blot analysis. (B and C) Effect of signal transduction inhibitor on ET-1-induced changes of IL-25 expression. After pretreatment with U0126 at concentrations of 10 and 20 μ M, cells were stimulated with ET-1 for 30 min (B) and 24 h (C) for western blot and RT-PCR analyses, respectively. (D and E) After pretreatment with SB2020190 at concentrations of 10 and 20 μ M, cells were treated with ET-1 for 30 min and 24 h. Cell lysates were subjected to western blot (D) and RT-PCR analyses (E). (F and G) Cells were treated with 100 nM ET-1 for 30 min and 24 h in the presence or absence of SP600125 (F) and subjected to RT-PCR

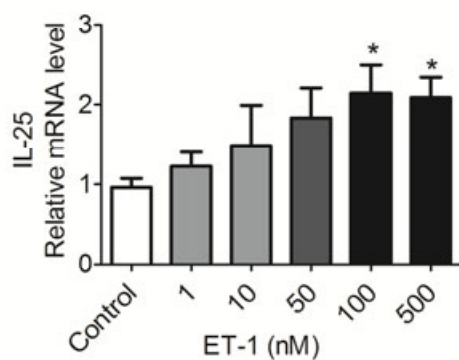
analysis (G). Data are the mean $\pm$ SEM of three experiments. $*P < 0.05$ and $**P < 0.01$ compared with ET-1-stimulated cells.



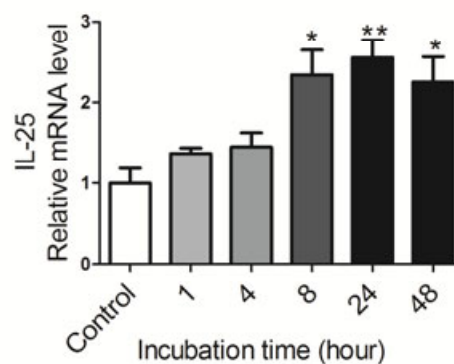




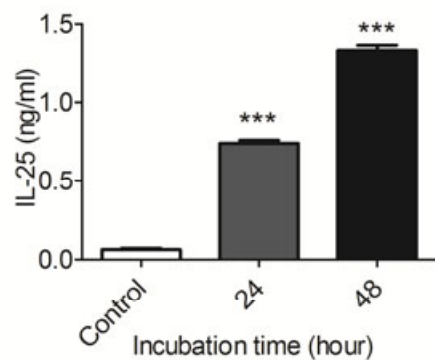
A



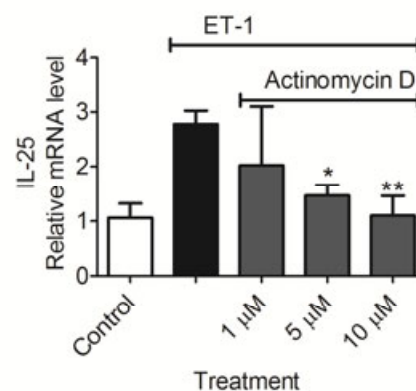
B



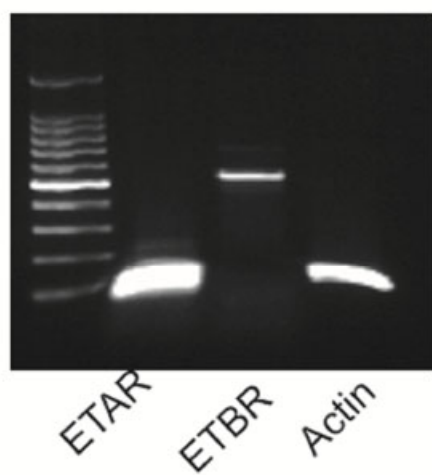
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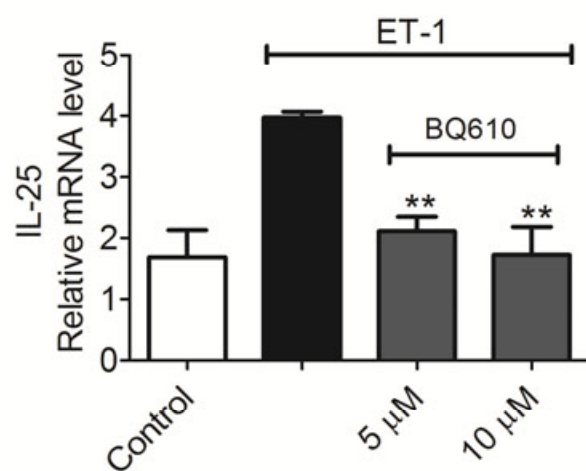
D



A



B



C

