Changes in cytokine expression on T cells of patients with atopic dermatitis by corticotropin releasing hormone

Sang Ho Oh

Department of Medicine

The Graduate School, Yonsei University

Changes in cytokine expression on T cells of patients with atopic dermatitis by corticotropin releasing hormone

Directed by Professor Kwang Hoon Lee

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Sang Ho Oh

June 2010

This certifies that the Doctoral Dissertation of Sang Ho Oh is approved.

Thesis Supervisor : Prof. Kwang Hoon Lee

Thesis Committee Member : Prof. Choong-Rim Haw

Thesis Committee Member : Prof. Dong Soo Kim

Thesis Committee Member : Prof. Soo-Chan Kim

Thesis Committee Member : Prof. In Hong Choi

The Graduate School
Yonsei University

June 2010

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ABSTRACT

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Department of Medicine
The Graduate School, Yonsei University

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Corticotropin releasing hormone (CRH) is the central regulating hormone of the hypothalamic-pituitary-adrenal axis, which ultimately synthesizes glucocorticoid as a reaction to stress. CRH is not only released from the central nervous system, but also from various cells making up local tissues. Similarly, CRH receptors are distributed in both neurological and peripheral tissues. Furthermore, CRH is related to the development and aggravation of several cutaneous diseases including urticaria and allergic contact dermatitis. Although atopic dermatitis (AD) is known to be triggered by or exacerbated by stress, the mechanisms by which stress worsens symptoms of AD remain obscure.

CRH might directly affect immune cells, such as T cells, which are key effector cells of the immune system. The purpose of this study was to identify the isoforms of CRH receptors (CRH-R) that are located on T cells, to compare the differences in expression of these T cell receptors between AD patients and healthy controls (HCs), and to evaluate the direct effect of CRH on Th1, Th2, and regulatory T cells (Treg). CRH-R1/R2 proteins and mRNAs for CRH-R1α, 1β, and CRH-R2α were found to be

expressed on T cells. T cells from patients with AD expressed significantly lower levels of CRH-R1/R2 proteins than did those of HCs. In addition, CRH upregulated IL-4 production by Th2 cells and downregulated IFN-γ production by Th1 cells in HCs. However, the production of IFN-γ and IL-4 in T cells of AD patients did not show any statistical difference between with and without CRH treatment. And there were no significant changes in the polarization of T cells into Th1 and Th2 cells in both HCs and AD patients under 48 h incubation of CRH. CRH also negatively affected Treg cells producing IL-10 (IL-10-secreting Treg type 1 (Tr1) cells) in both HCs and AD patients, and IL-10 production significantly decreased after CRH treatment, especially in patients with AD. These results suggest that the decrease in IL-10 secretion through CRH-mediated Treg cell suppression could partially explain stress-related aggravation of AD.

Key words : corticotropin releasing hormone, Th2 cell, regulatory T cell, atopic dermatitis

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I. INTRODUCTION

Chronic stress can lead to exhaustion, distress, and altered immunity, which ultimately leads to disease. Atopic dermatitis (AD), which is a chronic, relapsing, inflammatory skin disease, is caused by a complex interaction of genetic predispositions, environmental factors, altered immunologic function, skin barrier dysfunction, and psychologic factors. Many skin diseases, including AD, are known to be triggered by or exacerbated by stress[1, 2]. Although the mechanisms by which stress worsens symptoms of AD remain obscure, several contributing factors have been suggested as causals, including a stress-induced immunologic shift toward type 2 helper T cells and neuropeptides, impaired hypothalamic-pituitary-adrenal (HPA) axis response to stress, and barrier dysfunction[3-6].

Stress stimulates the HPA axis through the release of corticotropin-releasing hormone (CRH), which leads to the secretion of glucocorticoids that

downregulate immune responses[7]. CRH is also secreted from peripheral sites, including endothelial cells, vascular smooth muscle cells, skin cells, and monocytes. CRH also functions as a CNS-independent autocrine or paracrine neurohormonal regulator[8]. Thus, CRH, produced within local tissues, can act as a regulatory element of local neuroendocrine interactions[9].

CRH secretion from the hypothalamus leads to the secretion of glucocorticoids through the HPA axis that can indirectly influence the expression of Th1 and Th2[10]. CRH may also directly affect immune cells in local tissues such as skin. This function is mostly accomplished by T cells, as CRH can bind to or be expressed in immune cells, including T cells, which are of key importance to the immune system and at the core of adaptive cell-mediated immunity[11-13].

CRH exerts its effects by binding to specific cell surface receptors, of which two receptor subtypes, CRH-R1 and CRH-R2, have been characterized in humans[14]. The distribution of the two subtypes of CRH receptors (CRH-R) differs according to body tissue. CRH-R1, which is the most prevalent isoform of CRH-R, is present in all major cell populations of the epidermis, dermis, and subcutis; the CRH-R2 gene is expressed in the dermis and in adnexal structures[15]. CRH-R1 and R2 mRNA and protein have been reported to be present in dendritic cells (DCs), and the exposure of CRH to DCs resulted in a decrease of IL-18 in both patients with AD and HCs[16]. However, the expressions of CRH-R subtypes and their isoforms in T cells are still poorly

characterized, and no direct effect of CRH on the cytokine profiles of Th1 and Th2 cells has been demonstrated. The immunosuppresive role of regulatory T (Treg) cells in the context of stress is also poorly understood.

The aim of this study was to identify the subtypes and isoforms of CRH receptors (CRH-R) that are located on T cells, to compare the differences in expression of these T cell receptors between AD patients and healthy controls (HCs), and to evaluate the direct effect of CRH on Th1, Th2, and Treg cells.

II. MATERIALS AND METHODS

1. Patients

Blood samples were collected after obtaining informed consent from 19 patients diagnosed with AD (male: 13, female: 6, ages: 16-30) according to the criteria of Hanifin and Rajka[17]. Fifteen non-atopic healthy controls (male: 12, female: 3, age: 15-25) were also included as HCs. The institutional review board of Severance Hospital approved this study. Patients with AD included in this study did not receive any systemic treatment, except for topical steroids, for at least four weeks before collection of blood samples. The patients had moderate to severe AD according to the eczema area and severity index (EASI) (>10).

2. Isolation of T cells and CD4+ T cells

Peripheral blood mononuclear cells (PBMCs) from AD patients and HCs were isolated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden), and allowed to adhere to plastic dishes for 1 h at 37℃. T cells were isolated from nonadherent floating cells by negative selection using CD14, CD19, and CD56 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) or positive selection with CD3 MicroBeads (Miltenyi Biotec).

For isolation of CD4+ T cells, 20 μl of CD4 MicroBeads (Miltenyi Biotec) were added to per 10^7 total non-adherent cells, and cells were incubated for 15

min at 4℃. LS column was placed in the magnetic field of a suitable magnetic cell sorting (MACS) separator and rinsed once with 3 ml of buffer solution. Cells were then resuspended in up to 500 μl of buffer and applied onto the column. The column was washed with 3 ml buffer 3 times, and the cells passing through were removed. The column was separated from the magnetic field, and 5 ml of buffer was pipetted onto the column. The flushed out fraction with the magnetically labeled cells was collected and cultured in 10% RPMI 1640 (Gibco, Invitrogen corporation, Carlsbad, CA, USA). The purity of CD4+ T cells was > 95% in all cases.

3. Western blot analysis of CRH-R

T cells were lysed and prepared for western blot analysis. Samples were resolved in 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with goat anti-human CRH-R1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). As a negative control, the primary antibodies were pre-absorbed with synthetic, receptor-blocking peptides (1 μM; Santa Cruz Biotechnology). Following stripping and washing, the membranes were incubated with mouse anti-goat horseradish peroxidase (HRP)-conjugated serum (Santa Cruz Biotechnology) at a dilution of 1/5,000. The protein bands were detected using an ECL reagent (Bio-Rad Laboratories, Hercules, CA, USA).

4. Immunofluorescence microscopy of CRH-R

Total PBMCs from HCs were stained with goat anti-human CRH-R1/2 polyclonal antibody (Santa Cruz Biotechnology) and mouse anti-human CD3 monoclonal antibody (BD Bioscience, San Jose, CA, USA) for 30 min. FITC-conjugated anti-goat IgG secondary antibody and PE-conjugated anti-mouse IgG secondary antibody were added after washing, and cells were incubated for 30 min at 4℃. CRH-R1/2 expression was examined in T cells using confocal microscopy.

5. Semi-quantitative RT-PCR for analysis of CRH-R mRNA

Total RNA was extracted from T cells using the RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. One microgram of the total RNA was used for cDNA synthesis with the RevertAid first strand cDNA synthesis kit (Fermentas Inc., Glen Burnie, MD, USA) For semi-quantitative RT-PCR, oligonucleotide primers were used for the CRH-R1 and the 2 isoforms, as described in Table 1.

Amplification was performed using a GeneAmp PCR system 2700 (Applied Biosystems, Mountain View, CA, USA). PCR was conducted under the following conditions: denaturation at 94℃ for 1 min, annealing at 67℃ for 1 min, and extension at 72℃ for 1 min, repeated for 40 cycles. Specific PCR fragments were separated on a 1% agarose gel and visualized using ethidium

bromide staining. Myometrial cells in primary culture was used as a positive control for CRH-R.

Table 1. PCR primers used for human CRH-R1 and R2

Receptor isoforms	PCR	Primer sequence (5' -3')
CRH-RIα	Sense	GGCAGCTAGTGGTTCGGCC
	Antisense	TCGCAGGCACCGGATGCTC
CRH-RIβ	Sense	GGCCAGGCTGCACCCATTG
	Antisense	TCGCAGGCACCGGATGCTC
CRH-RIIα	Sense	ATGGACGCGGCACTGCTCCA
	Antisense	CACGGCCTCTCCACGAGGG
CRH-RIIβ	Sense	GGGGCTGGCCAGGGTGTGA
	Antisense	CACGGCCTCTCCACGAGGG
CRH-RIIγ	Sense	CTGTGCTCAAGCAATCTGCC
	Antisense	CACGGCCTCTCCACGAGGG

6. Real-time RT-PCR

Quantitative, real-time PCR was performed three times for each sample using 2 μl of the cDNA supplemented with 0.3 μM of forward and reverse primers specific for CRH-R1, CRH-R2, and GAPDH. A Taqman probe and qPCR Master Mix in a reaction of 50 μl was used for the PCR study, which was

amplified in a 7,500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using an Exicycler Quantitative Thermal Block (Bioneer, Daejeon, South Korea) instrument. The primers and probes used were as follows: probe for CRH-R1, 5'-CGCCTCTGGACCTCGGTGATGCCTT-3'; sense primer for CRH-R1, 5'-GCCTCTGACTCACCACGATG-3'; and antisense primer 5'-TCTGATGATGACACCTGACTTCTG-3'; probe for CRH-R2, 5'-CGCACTCCCACTCCCTCTCCGCAC-3'; sense primer for CRH-R2, 5'-TCCACTCCCTCGCAGTCAC-3'; and antisense primer 5'-GCAGTTGGCCTCCAGCAG-3'; probe for GAPDH, 5'-CCTCCGACGCCTGCTTCACCACCTT-3'; sense primer for GAPDH, 5'-GGACCTGACCTGCCGTCTAG-3'; and antisense primer 5'-TGTAGCCCAGGATGCCCTTG-3'. Differences in the expression of CRH-R on T cells between HCs (n=5) and AD patients (n=5) were compared.

7. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed to analyze the effect of CRH on T cell metabolic activity according to varying concentrations (1, 10, 50 and 100 nM) and incubation times (1, 2 and 3 day) of CRH. Each well of a 96-well plate was seeded with 1×10^5 T cells and preincubated at 37℃ in a CO_2 incubator. The culture medium was removed and the cells were incubated for 3 h with 200 μl of 0.5 mg/ml MTT (Boehringer, Mannheim, Germany). The culture medium

was removed again and 200 μl of dimethyl sulfoxide (DMSO, Sigma, St Louis, MO, USA) was added into each well, and the optical density was measured using an ELISA reader with a wavelength of 570 nm.

8. Cytokine production in T cells and ELISA

T cells were seeded at the density of 4×10^5 per well to anti-CD3 and CD28-precoated plates; the plates were coated with 100 μl of 5 μg anti-CD3 (BD Bioscience) per ml plus 1 μg anti-CD28 (BD bioscience) per ml at 37℃ for 1 h. The optimal concentration and incubation time of CRH were determined from the dose and time-response experiments regarding IL-4 secretion. The cells were incubated for 48 h at 37℃ in the presence or absence of CRH (10 nM) in an atmosphere of 5% CO_2. The activities of IFN-γ, IL-4, and IL-10 in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (eBioscience, San Diego, CA, USA).

9. Carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assays

For the proliferation assay, 1×10^4 CFSE-labeled CD4+ T cells were suspended in a RPMI medium containing 10% fetal bovine serum, and were stimulated for 72 h in 96-well flat-bottom microtiter plates coated with anti-CD3 antibody (2 μg/ml). Differences in cell proliferation between plates

with and without CRH were measured using flow cytometry in triplicate.

10. Cytokine expression by flow cytometry analysis

Purified CD4+ T cells were stimulated using anti-CD3 and anti-CD28 in the presence or absence of CRH (10 nM) for 48 h. The cells were stained with anti-human CCR4-APC antibody (R&D Systems, Minneapolis, MN, USA) for 30 min at 4℃. Cells were fixed with fix/perm solution for 20 min and washed with perm/wash solution. Next, the cells were stained with mouse anti-human IL-4-PE antibodies (BD Bioscience) for 30 min at 4℃, and analyzed by fluorescence-activated cell sorting (FACS; FACStar, Becton-Dickinson, Lincoln, NJ, USA). In order to evaluate the expression of the Th1 cytokine, CD4+ T cells were stained with mouse anti-human CXCR3 antibody (BD Pharmingen, San Diego, CA, USA) for 30 min at 4℃, FITC-conjugated anti-mouse IgG secondary antibody was added after washing, and finally, cells were incubated for 30 min at 4℃. Cells were fixed with fix/perm solution for 20 min and washed with perm/wash solution. Cells were then stained with mouse anti-human IFN-γ-PE antibodies (BD Bioscience) for 30 min at 4℃ and analyzed by FACS. For intracytoplasmic IL-10 cytokine staining, CD4+ T cells were stained with PE-conjugated anti-human IL-10 antibodies (R&D Systems) and APC-conjugated anti-human FoxP3 antibodies (eBioscience) after fixation with fix/perm solution. For the quantification of Treg cells in PBMCs, blood

samples were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD25, and intracellular FoxP3 expression was detected using an APC-conjugated anti-human FoxP3 staining kit (eBioscience). Following antibody staining, the cells were analyzed by FACS. The population of Treg cells in PBMCs from HCs and AD patients was compared between with and without CRH.

11. Statistical analysis

All analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test and independent two samples *t*-test were used for the comparison of mean amounts in AD and HCs regarding the expression of CRH-R on T cells and cytokine production in T cells after CRH treatment. A p-value < 0.05 was accepted as statistically significant.

III. RESULTS

1. Expression of CRH-R1/2 protein in T cells of HCs

Western blot analysis was performed to detect the expression of CRH-R protein on the T cells of HCs. SDS-PAGE of whole T cell lysates was completed using an antibody that specifically recognizes CRH-R1/2. CRH-R1/2 showed two strong bands between 36 and 50 kDa. To confirm the specificity of the response, the membrane was preincubated with the corresponding receptor-blocking peptides. Preabsorption with this peptide made the bands disappear (Figure 1). CRH-R1/2 expression with green fluorescence was observed in PE-conjugated CD3+ T cells stained with red fluorescence (Figure 2A, B) using immunocytochemistry. Thus, the expression of the CRH-R1/2 protein in CD3+ T cells using western blot and confocal microscopy was confirmed.

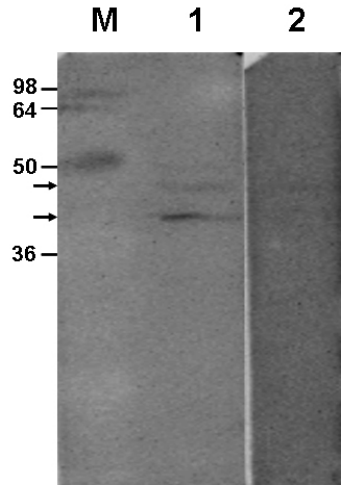


Figure 1. Western blot analysis of CRH-R1/2 in T cells of HCs. Whole T cell lysates were run on 10% SDS-PAGE, using antibodies that specifically recognize CRH-R1/2. CRH-R1/2 showed two strong bands between 36 and 50 kDa. The protein bands for CRH-R1/2 disappeared when the membrane was preincubated with receptor-blocking peptides. This result is the representative of three independent experiments. (M: marker, 1: anti-CRH-R1/2, 2: anti-CRH-R1/2 pre-absorbed with CRH-R blocking peptide)

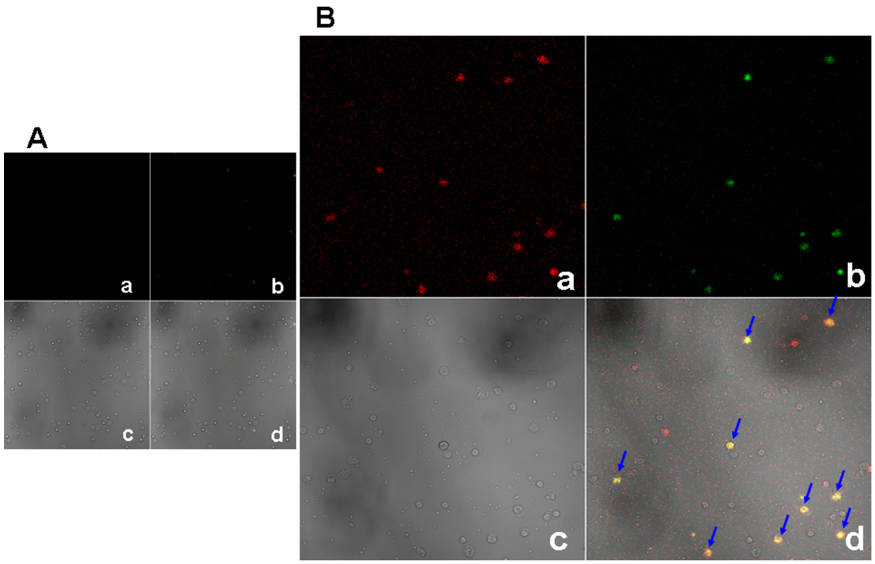


Figure 2. Immunocytochemistry of CRH-R1/2 in T cells from HCs. To identify the expression of CRH-R on T cells, PBMCs were stained with anti-CD3 antibodies reacting with PE-conjugated secondary antibodies, and anti-CRH-R1/2 antibodies reacting with FITC-conjugated secondary antibodies. (A) Isotype control, (B) Green fluorescence of CRH-R1/2 expression was observed in CD3+ T cells showing red fluorescence. Arrows indicate cells showing double staining when merging. (original magnification X200, a: anti-CD3 antibody with PE-conjugated secondary antibody, b: anti-CRH-R1/2 antibody with FITC-conjugated secondary antibody, c: cell morphology without staining, d: merge)

2. Expression of isoforms of CRH-R mRNA from T cells of HCs

RT-PCR analysis for CRH-R1 isoforms showed that T cells expressed specific mRNAs for CRH-R1α and 1β. Only CRH-R2α was expressed, while CRH-R2β and γ were not detected in the RT-PCRs of CRH-R2 isoforms (Figure 3). Myometrial cells in primary culture were used as positive controls. Treatment with CRH slightly decreased the expression of CRH-R mRNA in unstimulated, and anti-CD3 and anti-CD28 stimulated T cells of HCs.

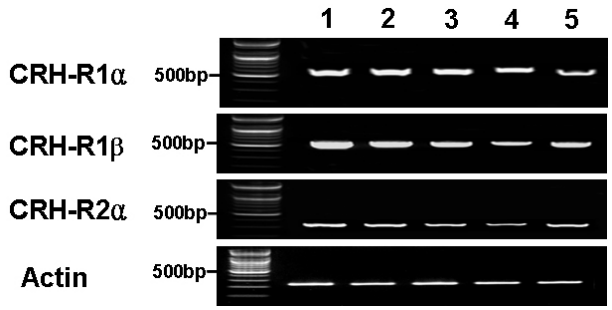


Figure 3. RT-PCR analysis of mRNA of CRH-R isoforms in T cells of HCs. RT-PCR analysis for CRH-R1 isoforms showed that T cells expressed specific mRNAs for CRH-R1α and 1β. Only CRH-R2α was expressed and CRH-R2β and γ were not detected in RT-PCR of CRH-R2 isoforms. Myometrium was used as a positive control. Treatment with CRH slightly decreased the expression of CRH-R mRNA in T cells and anti-CD3 and anti-CD28 stimulated T cells. (1: absence of CRH and no stimulation of anti-CD3/28 antibodies, 2: 10 nM CRH treatment and no stimulation of anti-CD3/CD28 antibodies, 3: absence of CRH and stimulation of anti-CD3/28 antibodies, 4: 10 nM CRH treatment

and stimulation of anti-CD3/28 antibodies, 5: myometrium as a positive control)

3. Differences in CRH-R mRNA expression between T cells of HCs and patients with AD

The difference in mRNA expression of CRH-R1/2 in T cells between HCs and AD patients was evaluated using real-time RT-PCR. CRH-R1 expression was significantly higher in T cells from HCs (mean: 0.178 ± 0.131) as compared to T cells from AD patients (mean: 0.034 ± 0.047; p=0.032) (Figure 4A). Similarly, T cells from HCs (mean: 0.276 ± 0.164) expressed significantly higher levels of CRH-R2 than T cells from AD patients (mean: 0.0198 ± 0.002; p=0.008) (Figure 4B). The significant decrease in CRH-R1 and CRH-R2 mRNA expression in T cells of patients with AD indicates that CRH might act differently on T cells in AD aggravated by stress than it does within normal T cells. Thus, we further investigated the immunoregulatory role of CRH on T cell subsets including Th1, Th2, and Treg cells in AD versus HC.

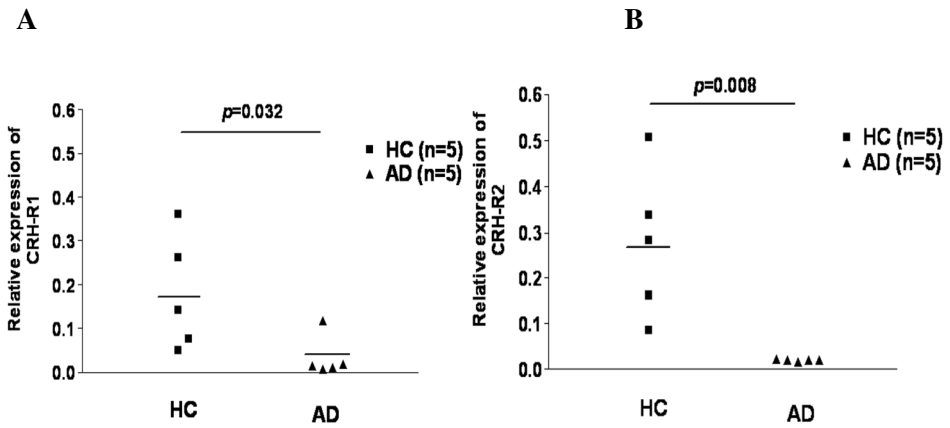


Figure 4. Real-time RT-PCR of mRNA expression for CRH-R in T cells between HCs and AD patients. The mRNA expression of CRH-R1/2 in T cells between HCs and AD patients was compared through real-time RT-PCR. In order to make comparisons between samples and groups, CRH-R1/2 gene expression was normalized to the endogenous control, GAPDH. (A) CRH-R1 expression was measured in T cells from HCs (n=5) and AD patients (n=5). (mean: 0.178 ± 0.131 vs 0.034 ± 0.047, p=0.032). (B) CRH-R2 expression was measured in T cells from HCs (n=5) and AD patients (n=5), (mean: 0.276 ± 0.164 vs 0.0198 ± 0.002, p=0.008).

4. Determination of appropriate concentration and incubation time of CRH on T cell activity and viability

An MTT assay was performed in order to analyze the effect of CRH on the viability of the T cells. The metabolic activities of the T cells were not altered according to CRH concentration (1, 10, 50 and 100 nM) or incubation time (1, 2

and 3 days; Figure 5). To determine the appropriate concentration and incubation time of CRH, IL-4 secretion was examined after stimulating T cells with anti-CD3 and anti-CD28 antibodies in the absence or presence of 1, 10, and 50 nM CRH, and after incubation for 24, 48 and 72 hours. Subsequent functional studies were performed using 10 nM CRH and 48 h incubation, because IL-4 secretion was the greatest after 48 hours of incubation and with 10 nM CRH (data not shown).

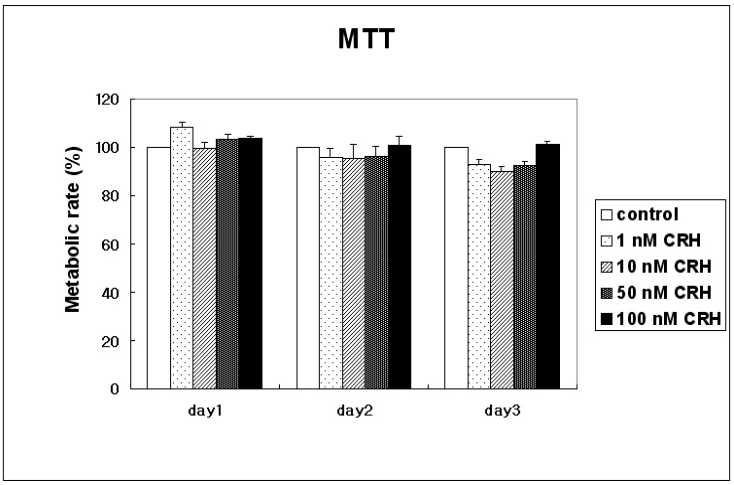


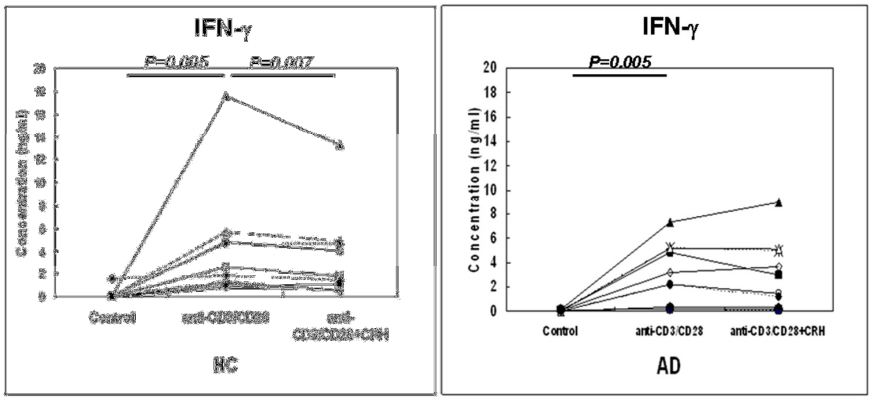
Figure 5. MTT assay to observe the effect of CRH on viability of T cells. Metabolic activities of T cells were not affected according to concentration (1, 10, 50 and 100 nM) or incubation time (1, 2 and 3 days) of CRH.

5. Cytokine changes in the supernatants of T cells after CRH treatment

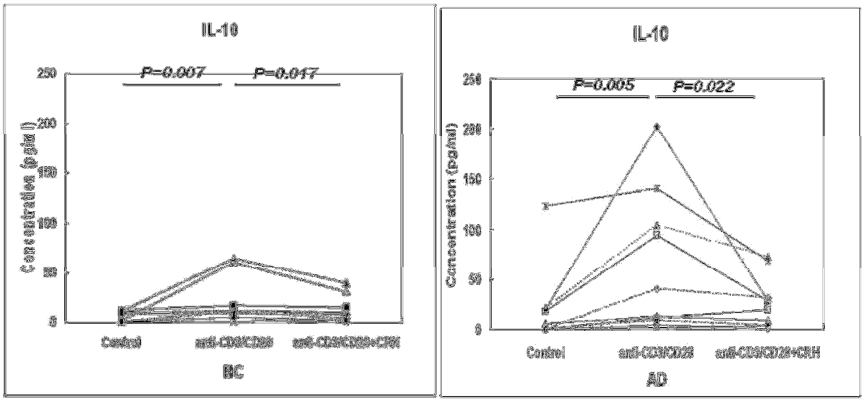
To evaluate the effect of CRH on T cells, isolated T cells from HCs and AD patients were stimulated with a combination of anti-CD3/28 antibodies and 10

nM CRH for 48 h. The cytokine levels were determined using ELISA kits. The production of IFN-γ and IL-10 in the supernatants of T cells from HCs were significantly decreased following CRH treatment (p=0.007 and 0.017, respectively), although IL-4 was significantly increased after CRH treatment (p=0.017). In the AD patients, only IL-10 was significantly decreased after CRH treatment (p=0.022), while the production of IFN-γ and IL-4 did not show any statistical difference between with and without CRH treatment (Figure 6A-F). The CRH-mediated immunoregulatory mechanism also appeared to be different between the control state and the stress-related disease state. This diverse response against stress might explain clinical exacerbations in stress-aggravated diseases such as AD. Finally, the direct effects of CRH on Th1, Th2, and Treg cells were investigated regarding the cytokine pattern of supernatants of CRH-treated T cells in AD patients and HCs.

A **B**


IFN-γ
P=0.005
P=0.007
Concentration (ng/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
HC
IFN-γ
P=0.005
Concentration (ng/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
AD

C **D**


IL-10
P=0.007
P=0.017
Concentration (pg/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
BC
IL-10
P=0.005
P=0.022
Concentration (pg/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
AD

E **F**

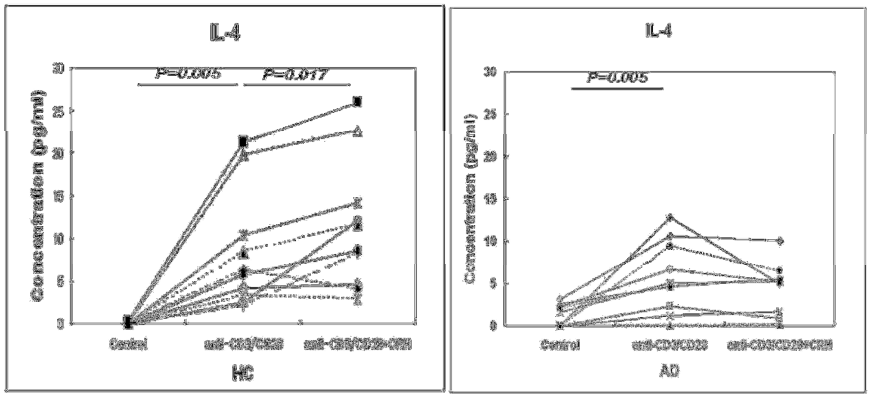

IL-4
P=0.005
P=0.017
Concentration (pg/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
HC
IL-4
P=0.005
Concentration (pg/ml)
Control
anti-CD3/CD28
anti-CD3/CD28+CRH
AD

Figure 6. Changes in cytokine expression after CRH treatment measured by ELISA. Peripheral blood T cells from HCs and AD patients were stimulated with a combination of anti-CD3/28 antibodies together with 10 nM CRH. (A, C, E: HCs, B, D, F: AD patients) The production of IFN-γ and IL-10 in T cells from HCs was significantly decreased by CRH treatment (p=0.007 and 0.017, respectively), but IL-4 was significantly increased by exposure to CRH (p=0.017). In the case of AD patients, only IL-10 was significantly decreased after CRH treatment (p=0.022), and the production of IFN-γ and IL-4 did not show any statistical difference between with and without CRH treatment.

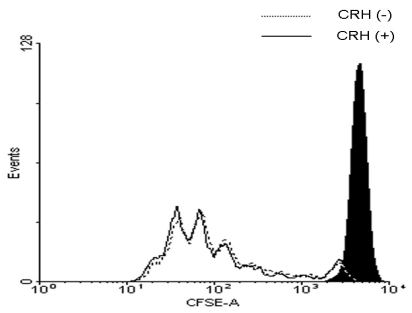
6. Effect of CRH on the proliferation of CD4+ T cells and on cytokine expression in Th1 and Th2 cells

To investigate differences in the proliferative capacity of CD4+ T cells according to CRH incubation, a CFSE-based proliferation assay was performed. CRH did not affect the proliferation response of CD4+ T cells when stimulated with anti-CD3 antibody (Figure 7A).

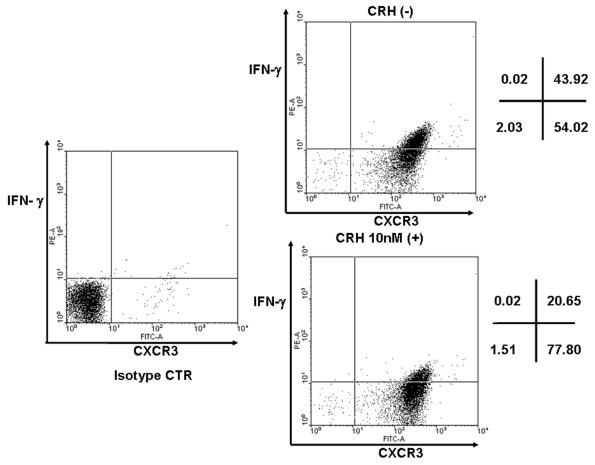
To confirm the patterns of cytokine secretion observed in the ELISA, chemokine receptors such as CXCR3 and CCR4 were examined together with cytokine secretion in isolated CD4+ T cells. The population of CXCR3-expressed CD4+ T cells from HCs was not significantly altered after CRH treatment, although the population of IFN-γ-secreting CXCR3-expressed CD4+ T cells was decreased after CRH treatment (Figure 7B). The number of

CCR4-expressed CD4+ T cells from HCs was also not significantly altered after CRH treatment, although the number of IL-4-producing CD4+ T cells was increased after CRH treatment (Figure 7C). However, CRH showed no remarkable effect on IFN-γ secretion of CXCR3-expressed CD4+ T cells or on IL-4 secretion of CD4+ T cells from AD patients (Figure 7D and E). Collectively, CRH upregulated IL-4 production by Th2 cells in HCs, while the Th2 cells in patients with AD (which are usually increased in number), did not properly respond to Th2 polarization after CRH treatment.

A



B



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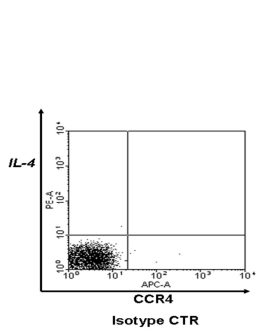
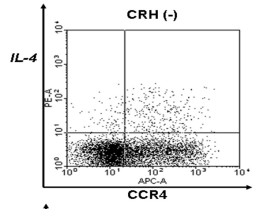
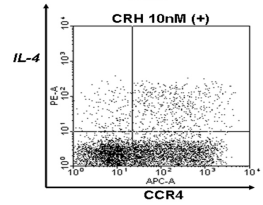


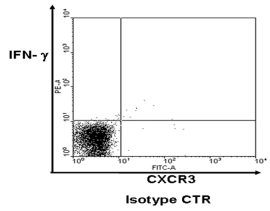


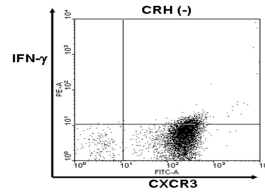
1.88	3.77
56.22	38.03



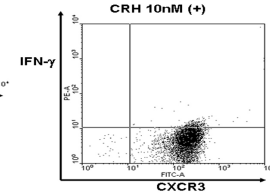
8.53	4.71
48.63	38.13

D



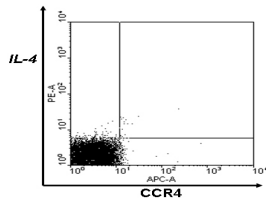


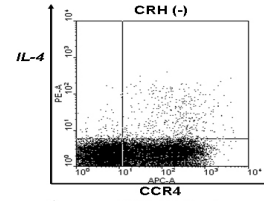
0.03	8.05
2.79	89.13



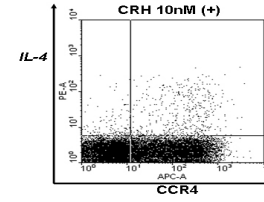
0.00	5.78
0.82	93.40

E





0.59	1.20
56.33	41.89



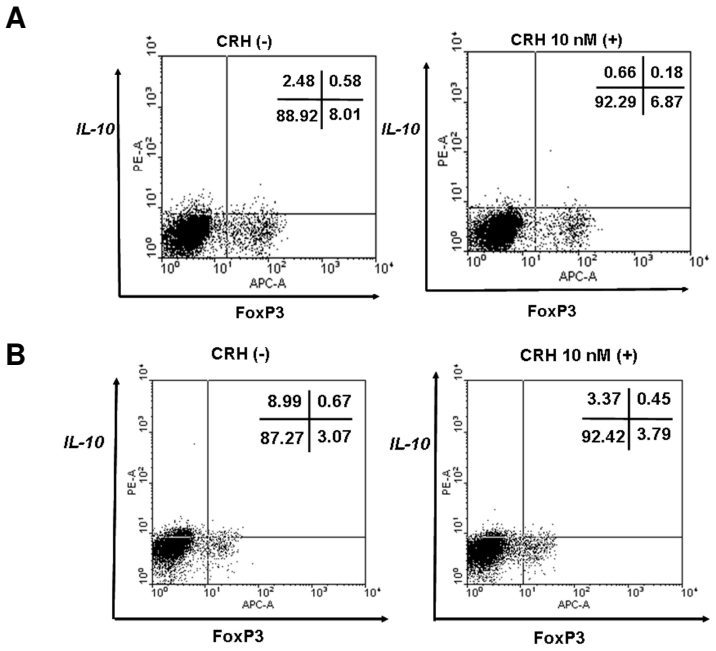
0.76	1.09
58.99	39.16

Figure 7. FACS analysis of proliferation and cytokine expression in T cells after CRH treatment. (A) CFSE-based proliferation assay showed that CRH did not affect the proliferation response of CD4+ T cells when stimulated with anti-CD3 antibody. Chemokine receptors such as CXCR3 and CCR4, which are expressed when T cells differentiate into Th1 or Th2 phenotypes, respectively, were examined together with cytokine secretion in isolated CD4+ T cells. (B) The population of CXCR3-expressed CD4+ T cells from HCs was not changed, but IFN-γ-secreting CXCR3-expressed CD4+ T cells were significantly decreased after CRH treatment. (C) The number of CCR4-expressed CD4+ T cells from HCs was not changed, but IL-4-producing CD4+ T cells were significantly increased after CRH treatment. (D-E) CRH showed no remarkable effect on IFN-γ secretion of CXCR3-expressed CD4+ T cells or on IL-4 secretion of CD4+ T cells from AD patients.

7. Effect of CRH on Treg cells

IL-10 expression in Treg cells was examined using a specific, natural Treg cell marker, FoxP3. Exposure to CRH decreased IL-10 secretion in FoxP3-negative T cells in both HCs and AD patients, but did not significantly affect IL-10 expression in FoxP3-positive T cells. The degree of decrease in IL-10 expression after CRH treatment was much greater in the patients with AD than in HCs (Figure 8A, B). Furthermore, the percentage of peripheral CD25+ FoxP3+ T cells found in CD4+ T cells from HCs and from patients with AD

slightly decreased after CRH treatment, although the degree of change was not statistically different (HCs: from 2.17 ± 0.13 to 1.85 ± 0.15%, p>0.05, n=4; AD patients: from 2.17 ± 0.62 to 1.95 ± 0.51%, p>0.05, n=4) (Figure 8C). CRH had a negative impact on Treg cells producing IL-10 in both HCs and AD patients, but the decrease in IL-10 production was more profound among the patients with AD. The population of FoxP3+ Treg cells was not greatly affected by CRH treatment, while IL-10 production in FoxP3-negative Treg cells significantly decreased after CRH treatment, especially among patients with AD. These results suggest that inducible FoxP3-negative Treg cells might be significantly affected by CRH among patients with AD.



C

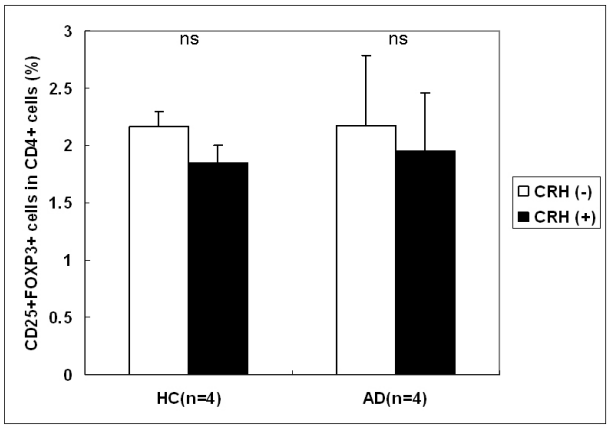


Figure 8. Effect of CRH on Treg cells. (A, B) IL-10 secretion in FoxP3-negative T cells from HCs and AD patients was decreased after CRH treatment, and the degree of decrease in IL-10 expression was much greater in the patients with AD. (C) The percentage of peripheral CD25+ FoxP3+ T cells in CD4+ T cells from HCs and AD patients decreased after CRH treatment without statistical significance (HCs: from 2.17 ± 0.13 to 1.85 ± 0.15%, $p>0.05$, n=4; AD patients: from 2.17 ± 0.62 to 1.95 ± 0.51%, $p>0.05$, n=4).

IV. DISCUSSION

Psychologic stress is undoubtedly an important modifying factor among patients with AD[5]. In addition, the chronicity of stress is closely associated with high levels of oxidative stress, increases of proinflammatory cytokines, and immunosenescence, such as a decrease of T cell proliferation or glucocorticoid sensitivity[18-20]. Therefore, chronic stress may alter development or aggravate disease through its modulation of the immune system, although the specific mechanism through which this might occur is unknown.

CRH is the central mediator of the HPA axis, which coordinates the body's response to stress. CRH not only has immunosuppressive effects via the HPA axis, but it also regulates behavioral, autonomic, endocrine, reproductive, cardiovascular, and gastrointestinal functions both centrally and peripherally[21, 22]. In contrast to its indirect immunosuppressive effects, the direct proinflammatory functions of peripheral CRH have been proven with systemic immunoneutralization of CRH in animal models of aseptic inflammation[23]. *In vitro* studies demonstrate that CRH stimulates lymphocyte proliferation, and that IL-1 and IL-2 production support this proinflammatory action[24, 25]. In addition, upregulation of CRH has been observed in peripheral tissues affected with acute or chronic inflammation, such as is caused by arthritis[26] and ulcerative colitis[27]. In addition, CRH has proinflammatory effects, as it stimulates mast cells to degranulate and release vascular endothelial growth factor[8, 28]. However, depending on receptor cell types, CRH can also lead to

anti-inflammatory activities, as it has been shown to decrease NF-κB activation in epidermal melanocytes and IL-18 expression in human HaCaT keratinocytes[29, 30]. According to previous work regarding the effect of CRH on DCs, CRH has been demonstrated to decrease the expression of IL-18, which functions as a promoter of the Th1 response and an inducer of the IFN-γ response in DCs[16]. In the present study, CRH stimulated IL-4 production by Th2 cells in HCs and decreased IFN-γ production by Th1 cells in HCs, but had neither effect among patients with AD. CRH also significantly downregulated IL-10 production by Treg cells in AD patients compared to HCs. Thus, CRH appears to have diverse actions according to receptor cell types, and the resulting response might influence the course of specific diseases through complex mechanisms.

This study is the first to describe mRNAs of CRH receptor isoforms expressed in T cells. mRNA for CRH-R1α and 1β as well as CRH-R2α (but not R2β or 2γ) were detected. CRH-R1/R2 protein was also identified in T cells by western blot and direct immunocytochemistry. Using semi-quantitative RT-PCR, T cells incubated with CRH from HCs resulted in a greater decrease in expression of CRH-R mRNA, as did the non-treated T cells. In addition, CRH-R1/R2 mRNA expression was significantly lower in T cells from AD patients than in T cells from HCs, as determined by real-time RT-PCR. These findings suggest that increased levels of local or systemic CRH resulting from chronic stress could lead to CRH-R1 downregulation on T cells. These results are in agreement with

a previous study, which found that psoriatic skin demonstrated decreased gene expression of CRH-R1 mRNA, and that the CRH level in psoriatic patients' serum was significantly higher than the CRH level in the HCs' serum[31]. A decrease in CRH-R expression in T cells from AD patients might therefore suggest that AD patients are exposed to chronic stress.

As skin expresses both CRH and CRH-R, it has been thought to be the equivalent of the HPA axis[32]. Several studies regarding the association between CRH or CRH-R and skin diseases have been reported. CRH stimulates skin mast cells and increases vascular permeability by releasing vascular endothelial growth factor through CRH-R1 activation[28, 33, 34]. This finding suggests that CRH is an important factor in skin disorders associated with mast cells, such as contact dermatitis, atopic dermatitis, and urticaria. Skin tissue from patients with chronic urticaria expresses high levels of CRH-R1 compared to normal skin tissue, and CRH-R1 is involved in the stress-induced exacerbation of chronic contact dermatitis in rats[35, 36]. Overall, CRH, which is secreted or accumulated in local tissue, might directly affect T cells that have infiltrated into the skin during exacerbations of inflammatory skin diseases including AD, resulting in aggravation of the exacerbation. Although the concentration of CRH (10 nM) used in this study is much higher than the concentration in the plasma (10^{-4} - 1 nM) of HCs, there have been inconsistent reports of the actual concentrations of plasma CRH, and CRH may be locally elevated within sites of inflammation.

Various factors that might have an effect on the response of Th1/Th2 cells have been investigated. There is a systemic shift of the immune system towards Th2 responses during the late, post-acute phase of ischemic strokes[37], which might be caused by the resulting oxidant stress that has been reported to promote polarization of human T cell differentiation toward a Th2 phenotype[38]. Glucocorticoids, or stress hormones, at concentrations mimicking the plasma cortisol concentration during cognitive stress, can also induce a shift towards a predominance of Th2 cells over Th1 cells and monocytes[39]. Peripheral CRH has been reported to have a proinflammatory effect, with a selective increase in Th1-type response in experimental models of autoimmune encephalomyelitis[40]. This study demonstrated that CRH increased IL-4 and decreased IFN-γ in T cells from HCs, implying that the CRH response increases the Th2 response in a normal state. These results were confirmed in the experiments with polarized T cells, including Th1 and Th2 cells of HCs using FACS analysis. However, the patients with AD did not show significant changes in IL-4 production after CRH treatment. It appears that the highly characteristic Th2 polarization observed in AD does not result in a unified response of Th2 cells to CRH. In addition, reduced CRH-R1/R2 expression within T cells of AD patients caused by the chronic stimulation of stress hormones might create an 'hyporesponsive state' in which the Th1/2 cells no longer respond to CRH simulation. And there was no significant changes in the polarization of T cells into Th1 and Th2 cells in both HCs and AD patients under 48 h incubation of CRH according to FACS

analysis. The CRH incubation time in this study, 48 h, could be too brief to allow us to confirm the polarization of T cells, therefore only the changes in the cytokine profiles of differentiated T cells were assessed. Further studies on the effect of longer incubation times of CRH, modeling the effect of chronic stress are needed.

The FACS analysis found that IL-10-producing Treg cells from both HCs and AD patients were decreased after CRH treatment, with the effect being greater among patients with AD. IL-10 expression in the FoxP3-positive CD4+ T cells from HCs and AD patients was not changed in the presence of CRH, while it was decreased in the FoxP3-negative CD4+ T cells after CRH exposure. This result suggests that CRH did not have an effect on IL-10 expression of naturally occurring CD4+ CD25+ Treg cells and CRH primarily influenced IL-10-secreting Treg type 1 (Tr1) cells, or inducible Treg cells, which have also been found to be increased in the peripheral blood of patients with AD[41]. These results imply that CRH, which is secreted locally or systemically under stress conditions may decrease the expression of IL-10 with major function of immune regulation through suppression of Tr1 cells. This study might explain the CRH-mediated immune mechanism in the pathogenesis of AD and stress-related exacerbations in AD. In short, CRH may preferentially increase the Th2 response in the normal state, but in cases of AD, CRH preferentially suppresses the IL-10 secretory function of Tr1 cells.

V. CONCLUSION

In conclusion, these results demonstrate that CRH, a stress hormone, can directly act on T cells, that CRH-R expression is different in T cells between patients with AD and HCs, and that CRH-mediated immunoregulatory mechanisms might be different in the normal state compared to the stress-aggravated disease state. The decrease in IL-10 expression through CRH-mediated Treg cell suppression could partially explain stress-related exacerbations especially in AD patients. Further research is needed to determine the specific components of the CRH pathway in AD, and to facilitate a better understanding of the stress mechanisms involved in this process.

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ABSTRACT (IN KOREAN)

Corticotropin releasing hormone에 의한 아토피피부염 환자의 T세포에서 사이토카인 발현의 변화

<지도교수 이광훈>

연세대학교 대학원 의학과

오 상 호

아토피 피부염은 스트레스에 의해 악화 또는 유발되는 피부 질환으로 아직까지 스트레스가 증상을 어떻게 악화시키는 지는 잘 밝혀져 있지 않다. Corticotropin releasing hormone (CRH)는 hypothalamic-pituitary-adrenal 축의 가장 상위에서 분비되는 호르몬으로 glucocorticoid의 합성을 통해 스트레스를 조절하는 것으로 알려져 있다. 최근 CRH가 중추신경계 뿐만 아니라 국소 조직을 구성하는 여러 세포에서도 분비되고 이에 대한 수용체도 말초 조직에 존재하는 것이 증명되었다. 또한 CRH는 두드러기, 알레르기 접촉 피부염 등 여러 피부질환의 발생과 악화에 연관이 있다는 것이 알려졌다. 따라서 이 연구에서는 CRH가 면역 체계에서 가장 중요 역할을 하는 세포인 T 세포에 직접적으로 영향을 줄 수 있다는 가설을 세우고 T 세포에 CRH 수용체가 발현되는지 확인하고 발현된다면 아토피 피부염과 아토피 피부염이 없는 건강인 사이에 T 세포에서 발현되는 CRH 수용체 발현량이 차이가 있는지 확인하며 CRH가 직접적으로 T세포의 사이토카인 발현에 미치는 영향을 확인하고자 하였다.

연구 결과 T세포에서는 단백질 수준에서는 CRH 수용체 제 1, 2 아형 모두가 발현되고 mRNA 수준에서는 CRH-R1α, 1β와 CRH-R2α의 아형이 발현되었다. 그리고 아토피 피부염 환자의

T세포에서는 건강인과 비교해서 CRH 수용체의 발현이 유의하게 낮았다. CRH를 건강한 사람의 T세포에 처리했을 때 Th2 세포에서는 IL-4의 발현이 증가하고 Th1 세포에서는 IFN-γ의 발현이 감소되는 소견이 관찰되었다. 하지만 CRH를 48시간 처리한 경우 T세포가 Th1/Th2세포로 각각 분화되는 정도에는 큰 영향이 없었다. 또한 CRH는 아토피 피부염과 건강인 모두에서 CD4+ FoxP3- T세포인 IL-10을 분비하는 Treg type 1 (Tr1) 세포에 영향을 주어 IL-10의 발현을 감소시켰으며 이는 아토피 피부염 환자에서 더 많이 감소되는 소견을 보였다. 결론적으로 이 연구에서는 스트레스에 의해 유도된 CRH가 Tr1 세포의 억제를 통해 IL-10의 감소를 유발함으로써 아토피 피부염을 악화시킬 수 있음을 보여주었다.

핵심되는 말 : corticotropin releasing hormone, Th2 세포, 조절 T 세포, 아토피 피부염