

Corticotropin-Releasing Hormone Receptor-1 and Histidine Decarboxylase Expression in Chronic Urticaria

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Certain skin disorders, such as contact dermatitis and chronic urticaria, are characterized by inflammation involving mast cells and worsen by stress. The underlying mechanism of this effect, however, is not known. The skin appears to have the equivalent of a hypothalamic–pituitary–adrenal (HPA) axis, including local expression of corticotropin-releasing hormone (CRH) and its receptors (CRH-R). We have reported that acute stress and intra-dermal administration of CRH stimulate skin mast cells and increase vascular permeability through CRH-R1 activation. In this study, we investigated the expression of CRH-R1, the main CRH-R subtype in human skin, and the mast cell related gene histidine decarboxylase (HDC), which regulates the production of histamine, in normal and pathological skin biopsies. Quantitative real time PCR revealed that chronic urticaria expresses high levels of CRH-R1 and HDC as compared to normal foreskin, breast skin and cultured human keratinocytes. The lichen simplex samples had high expression of CRH-R1, but low HDC. These results implicate CRH-R in chronic urticaria, which is often exacerbated by stress.

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Certain skin disorders, such as contact dermatitis, chronic urticaria and psoriasis, are characterized by inflammation and mast cell activation (Maurer *et al*, 2003), they are also reportedly triggered or exacerbated by stress (Katsarou-Katsari *et al*, 1999), but the mechanism remains unknown. These conditions are associated with itch that involves histamine and possibly other triggers/mediators.

Corticotropin-releasing hormone (CRH) and CRH receptor (CRH-R) gene expression has been documented in rodent and human skin (Slominski *et al*, 2001), which has been proposed to have the equivalent of the hypothalamic–pituitary–adrenal (HPA) axis (Slominski *et al*, 2000a). CRH in mice is, however, released from nerve endings (Slominski *et al*, 2001), whereas in humans it is synthesized by skin cells (Slominski *et al*, 1998). The contribution of CRH to cutaneous inflammatory processes has generated significant interest, but had not been shown to be overexpressed in any inflammatory skin disease so far. CRH regulates HPA axis (Chrousos, 1995) through activation of CRH receptor-1 (CRH-R1), but both CRH-R1 and CRH-R2 have been also identified outside the brain. CRH, itself, is also found outside the brain (Chalmers *et al*, 1996) and has been postulated to have pro-inflammatory actions. CRH can be

synthesized by a variety of immune cells (Chrousos, 1995; Karalis *et al*, 1997). Human mast cells, were recently shown to be particularly rich in both CRH and the structurally related peptide Urocortin (Ucn), both of which could be secreted in response to immunologic stimulation (Kempuraj *et al*, 2004). These stress peptides could, therefore, have autocrine effects.

CRH and CRH-R expression in skin is associated with the hair cycle (Roloff *et al*, 1998). Skin mast cells are juxtaposed to nerve endings during hair follicle formation and are located close to CRH-positive nerve endings (Rozniecki *et al*, 1999), suggesting that they could have functional associations. We hypothesized that skin inflammatory disorders exacerbated by stress may be characterized by increased numbers of skin CRH-R and mast cells, so that activation of CRH-R by stress peptides would lead to increased mast cell stimulation and inflammation.

Here we studied the expression of CRH-R1 and histidine decarboxylase (HDC), as a marker of mast cells, in skin from controls and from patients with chronic urticaria.

Results

The characteristics of the samples used are presented in Table I. Controls included normal foreskin from a male infant, unaffected breast skin from a mastectomy and HaCaT human keratinocytes. There was also unaffected skin from one post-menopausal female with poikiloderma of undetermined

Abbreviations: CRH, corticotropin-releasing hormone; HDC, histidine decarboxylase; HPA, hypothalamic–pituitary–adrenal; Ucn, urocortin

Table I. Characteristics of different human skin samples used for real-time quantitative PCR

Sample #	Age (y)	Sex	Clinical condition ^a	Pre-menopausal	Post-menopausal
1	1	Male	Normal foreskin	N/A	N/A
2	42	Female	Breast skin	N/A	N/A
3	72	Female	Unaffected skin from poikiloderma	–	+
4	60	Female	Lichen simplex—A	–	+
5	45	Male	Lichen simplex—B	N/A	N/A
6	34	Female	Chronic urticaria—A	+	–
7	44	Female	Chronic urticaria—B	–	+ ^b
8	42	Male	Chronic urticaria—C	N/A	N/A
9	N/A	N/A	Human keratinocyte cell line	N/A	N/A

^aSamples were obtained from patients who had not received any medication for 15 days prior to biopsy.

^bPost-hysterectomy.

etiology. There was one pre-menopausal female, one post-hysterectomy female, as well as one male with chronic urticaria (Table I). These patients reported that their symptoms worsened with emotional stress. One post-menopausal female and one male with lichen simplex were also included. Obviously, the patients from whom the biopsies were obtained were not matched for sex or age and it is difficult to make any direct comparisons. Unfortunately, there was not sufficient biopsy material for both quantitative PCR and immunohistochemistry.

There was low expression of CRH-R1 mRNA in normal foreskin and it was used to compare expression in all other samples. There was about 4-fold induction of CRH-R1 mRNA expression in human cultured keratinocytes (4.1 ± 0.6) and in unaffected skin from the patient with poikiloderma (3.8 ± 0.4) used as additional “controls”, however, these results were not statistically significant (Fig 1). In normal breast skin there was 7.85 ± 0.016 -fold increase in CRH-R1 mRNA expression compared to foreskin. CRH-R1 mRNA expression was increased 24.6 ± 0.9 fold in the female and 11 ± 0.2 fold in ($p < 0.001$) the male with lichen simplex. The expression was 17.9 ± 0.4 -fold higher in one patient with chronic urticaria, whereas the second one had 18.7 ± 0.2 ($p < 0.01$) fold increase and the third one had 12.7 ± 0.0008 -fold increase ($n = 3$, $p < 0.01$).

HDC mRNA expression was very low in the normal human foreskin and normal breast skin (1.84 ± 0.05 -fold) and was absent in HaCaT human keratinocytes. It was about 8.3 ± 0.1 -fold higher in unaffected skin from the patient with poikiloderma ($p < 0.01$) and 5.18 fold in the female with lichen simplex, whereas it was 1.9-fold in the male with lichen simplex ($p < 0.01$). HDC mRNA expression was, however, 59.5 ± 0.5 -fold higher ($p < 0.001$) in one patient with chronic urticaria, 19.8 ± 0.7 -fold $p < 0.01$ in the other and 9.76 ± 0.09 -fold in the third one as compared to normal foreskin ($n = 3$, $p < 0.001$).

Discussion

CRH-R1 mRNA is shown to be overexpressed in any human inflammatory skin disorder. Human skin, squamous cell

carcinoma and melanoma cells had been previously reported to express CRH and CRH-R1 (Slominski *et al*, 1998; Slominski *et al*, 2001). Normal foreskin had low expression as did unaffected skin from a patient with poikiloderma of unknown etiology and normal breast tissue. It is obvious that this latter sample is not a healthy true control because there may be some systemic immunologic abnormality even though none was uncovered. The biopsies showing the highest expression of CRH-R1 and HDC were obtained from patients who reported that their symptoms worsened by stress. The lichen simplex biopsies did show increased CRH-R1 mRNA expression, which may not be surprising given the fact that it has been associated with higher levels of stress (Chaudhary, 2004). Unfortunately, there was not sufficient biopsy material to perform immunocytochemistry for CRH-R1 protein. CRH-R1 overexpression in chronic urticaria may derive from keratinocytes, where it was originally described (Slominski *et al*, 2000b). Alternatively, skin mast cells may, themselves, express CRH-Rs since normal umbilical cord-derived human mast cells express mRNA and protein for CRH-R1 (Cao *et al*, 2005).

Acute restraint stress was shown to induce increased skin vascular permeability; (Singh *et al*, 1999) this effect was mimicked by CRH, was inhibited by a CRH-R receptor antagonist and was absent in mast cell deficient mice (Theoharides *et al*, 1998). CRH-R1 was also involved in stress-induced exacerbation of chronic contact dermatitis in rats (Kaneko *et al*, 2003). CRH increased vascular permeability in human skin shown by micro-iontophoresis, an effect dependent on CRH-R1 and mast cells (Crompton *et al*, 2003).

Many dermatoses, such as chronic urticaria, contact dermatitis and psoriasis, are reportedly triggered or exacerbated by stress (Katsarou-Katsari *et al*, 1999). Eczema and acne, have also been reported to worsen by examination stress (Chiu *et al*, 2003). Chronic urticaria involves skin inflammation mast cell activation and TH1 processes; however, its pathogenesis, especially the reason they are exacerbated by stress, is not clear. Chronic urticaria may involve sensitivity to foods, temperature, drugs and possible autoimmune processes.

Chronic stress typically attenuates immune processes, whereas acute stress appears to enhance antigen-specific

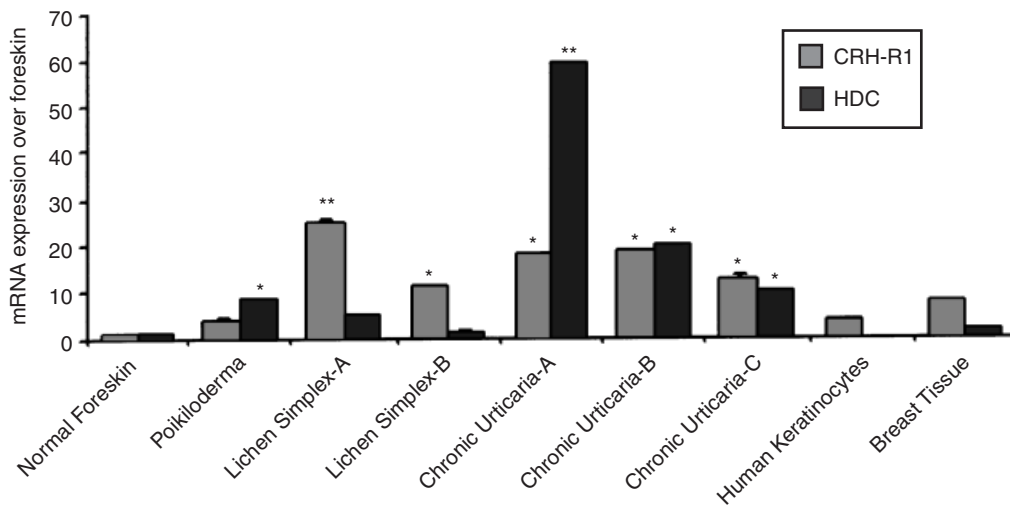


Figure 1
Differential gene expression of corticotropin-releasing hormone receptor-1 and histidine decarboxylase in human skin samples (n=3) by real-time quantitative PCR. Relative quantities were normalized against 18S internal control and results are expressed as fold induction above mRNA expression in normal foreskin. TaqMan runs were performed with cDNA reverse transcribed from 50 ng RNA from each sample. (* $p < 0.01$, ** $p < 0.001$). F, female; M, male.

cell-mediated immunity and causes a significant decrease in circulating lymphocytes and monocytes with a shift to the skin (Dhabhar and McEwen, 1999). It was further recently shown that acute stress induces local release of CRH in the skin (Lyttinas *et al*, 2003).

Mast cells derive from stem cells in the bone marrow and are prominently located just below the dermal-epidermal junction; they mature under the influence of stem cell factor (SCF), IL-3, IL-4 and IL-9 and are involved in allergic reactions, but also in innate immunity (Maurer *et al*, 2003) and inflammation (Theoharides and Cochrane, 2004). The increased expression of HDC we report indicates either increased mast cell enzyme activity or mast cell accumulation (Conti *et al*, 1998). Mast cell infiltration and/or proliferation may be triggered by SCF and by nerve growth factor (NGF), which are also secreted from mast cells, as well as by RANTES released from other immune cells (Conti *et al*, 1998). Histamine was recently shown to induce secretion of NGF from normal keratinocytes (Kanda and Watanabe, 2005). CRH has also been shown to stimulate NF- κ B in HaCaT human keratinocytes (Zbytek *et al*, 2004), which may then release cytokines or neuropeptides that could further activate mast cells (Paus *et al*, in press). In addition to histamine, which is well known to stimulate c-fibers, other triggers and mediators may be involved in the itching associated with contact dermatitis and chronic urticaria. Mast cells are known to be activated by neuropeptides, such as substance P (SP), neurotensin (NT), and pituitary adenylate cyclase activating polypeptide (PACAP) released from human dermal neurons (Theoharides *et al*, 2004).

The present findings suggest that CRH-R and mast cells may participate in the pathogenesis of chronic urticaria, especially when worsened by stress (Theoharides and Cochrane, 2004). Additional studies with more patients and immunocytochemistry of affected lesions for CRH-R1 protein expression would be required to provide conclusive evidence for CRH-R1 involvement.

Materials and Methods

Methods Expression of CRH-R1 and HDC was investigated using real-time quantitative PCR in human skin samples (Table I) as follows: (a) two females and one male with chronic urticaria, (b) one female with lichen simplex, (c) one male with lichen simplex, (d)

unaffected skin from one female with poikiloderma of unknown origin, (e) unaffected skin from a mastectomy, (f) one normal infant foreskin, as well as (g) human cultured keratinocytes (HaCaT).

All skin biopsies requiring two stitches were collected in Greece for diagnostic purposes. The Medical Ethics Committee of Attikon Hospital HIRB approved all described studies. All participants gave their written informed consent according to the Declaration of Helsinki Principles. All biopsy samples were obtained from patients who had not received any medication for 15 d prior to biopsy; these patients were free from any other medical problems, but reported exacerbations of their skin symptoms by emotional stress. The foreskin was obtained from the pediatric Urology Division of Tufts-New England Medical Center under Exemption 4 (discarded material without any identifying information). All biopsies were immediately placed in RNAlater solution (Ambion, Austin, Texas) and stored at -20°C .

HaCaT human keratinocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (GIBCO, Grand Island, New York), plus 1% antibiotic/antimycotic (GIBCO) solution (Slominski *et al*, 1998).

Total RNA extraction and cDNA synthesis Total RNA was extracted using the Qiagen RNeasy mini kit (Valencia, California) following the manufacturer's specifications of RNA isolation from skin tissue. Contaminating DNA was removed from all RNA preparations using Turbo DNase treatment (Ambion). RNA (500 ng) from each sample was then used to perform first-strand cDNA synthesis with 200 Units per μL Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 50 pmol per μL random hexamer oligonucleotide primer, 40 units per μL RNaseOUT recombinant ribonuclease inhibitor (Invitrogen Life Technologies, Carlsbad, California) and 10 mM deoxynucleotide triphosphates (Roche Diagnostics, Mannheim, Germany). The reaction (20 μL) was run at 25°C for 10 min, 37°C for 50 min, and was inactivated by heating at 70°C for 15 min.

Real-time quantitative PCR For real-time quantitative PCR amplification, the cDNA preparation was diluted 1:5 and 25 μL reaction was prepared using the iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Hercules, California) together with the CRH-R1 (Sehringer *et al*, 2004) and HDC (Laszlo *et al*, 2001) oligonucleotide primers. The CRH-R1 primers recognized all of the R1, but not the R1g, isoforms that were recently reported to be expressed both by human keratinocytes (Slominski *et al*, 2000b) and mast cells (Cao *et al*, 2005). The specificity of the CRH-R1 PCR primers was tested under conventional PCR conditions, and shown to amplify a single product of 66 bp length. Ribosomal RNA control primers (18S) were used for endogenous control with the same SYBR Green reaction cocktail. 100 nM of each primer was used in the final reaction. PCR was carried out in a 7300 sequence detector (Applied Biosystems, Foster City, California). Thermal

cycling proceeded at 95°C for 15 s and 60°C for 1 min, for 40 cycles. Input RNA amounts were normalized against 18S and the control (foreskin) cDNA. Experiments were performed in quadruplicate for each data point.

Normal positive controls included foreskin, unaffected breast skin as well as HaCaT cells that have both been previously reported to express CRH-R1 (Slominski *et al*, 2000b). Negative controls included; (a) sample without RT to control for genomic DNA contamination which gave no product in regular RT-PCR amplification for 30 cycles in our cDNA preparations, and (b) sample with water instead of template to check for external contamination.

Statistics Results were normalized against an 18S internal control and are expressed as induction over mRNA expression of normal foreskin. Results are presented as mean \pm SD ($n=3$) and were analyzed using the two-tailed Student's *t* test. Significance is denoted by $p < 0.05$.

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