RAPID COMMUNICATION

Regulation of corticotropin-releasing hormone receptor-2 expression in human cord blood-derived cultured mast cells

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Abstract

Corticotropin-releasing hormone (CRH) is secreted under stress and regulates the hypothalamic-pituitary-adrenal (HPA) axis; it is also secreted outside the brain where it exerts proinflammatory effects, possibly through mast cell activation. Mast cells are necessary for allergic reactions, but are increasingly implicated in acquired immunity and inflammatory diseases worsened by stress. Acute stress and intradermal CRH induced murine skin mast cell activation and increased vascular permeability that was absent in W/W^v mast cell deficient mice. The presence of functional CRH receptors (CRH-R) was recently reported on human mast cells. Here, we studied the expression of CRH-R1 and CRH-R2 by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescent immunocytochemistry in human umbilical cord blood-derived cultured mast cells (hCBMCs) treated with Interleukin (IL)-1, IL-4 or lipopolysaccharide (LPS). Ten week-old hCBMCs cultured in the presence of Stem cell factor (SCF) and IL-6 were positive for both CRH-R1 and CRH-R2. However, the expression of only CRH-R2 mRNA and protein was induced by priming hCBMCs with IL-4 for the last three weeks of culture. Further analysis of the CR-H R2 mRNA expression showed that addition of IL-1 or LPS for 6 h increased only CRH-R2 gene expression. CRH had negligible effect on IL-6 secretion from non-primed hCBMCs, but induced release from IL-4 primed cells. Interestingly, LPS alone increased IL-6 release in non-primed cells, but lost this effect in primed cells. These results further implicate mast cells and CRH in either initiating or potentiating inflammatory diseases, especially those affected by stress.

Journal of Molecular Endocrinology (2005) **35,** R1–R8

Introduction

Stress activates the hypothalamic-pituitary-adrenal (HPA) axis through hypothalamic secretion of corticotropin-releasing hormone (CRH) leading to suppression of immune responses indirectly via stimulation of glucocorticoid secretion from the adrenal glands (Chrousos 1995). CRH exerts its effects by binding to two specific G protein coupled cell surface receptors, CRH-R1 and CRH-R2 (Chen *et al.* 1993, Liaw *et al.* 1996).

Mast cells are ubiquitous in the human body and are critical for allergic reactions during which they secrete numerous vasoactive molecules, cytokines and proteases (Kobayashi *et al.* 2000, Marone *et al.* 2002, Huang *et al.* 2003). Mast cells are also involved in acquired and innate immunity to fungi and parasites (Marone *et al.* 2002, Puxeddu *et al.* 2003), but more recently also in immune defenses against bacteria (Malaviya & Abraham 2001, Galli & Nakae 2003) through expression of Toll-like receptors 2 (TLR2) and TLR4 (Varadaradjalou *et al.* 2003, Okumura *et al.* 2003). Increasing evidence also implicates mast cells in inflammatory diseases exacerbated by stress (Theoharides TC & Cochrane DE 2004).

Acute restraint stress induced rat intracranial mast cell activation that was CRH-dependent, as it was blocked by the CRH-R1 antagonist Antalarmin (Theoharides *et al.* 1995). Moreover, acute stress increased blood-brain barrier (BBB) permeability in rats, an action dependent on mast cells and CRH (Esposito *et al.* 2002). Such findings have implicated stress and mast cells in brain inflammation, including multiple sclerosis (Goodin *et al.* 1999, Mei-Tal *et al.* 1970, Theoharides & Cochrane 2004), but did not prove that CRH had a direct action on mast cells.

DOI: 10.1677/jme.1.01833
0952–5041/05/035–R1
Online version via http://www.endocrinology-journals.org

Human skin expresses primarily CRH-R1, with CRH-R2 protein found only in hair follicles and eccrine glands (Slominski *et al.* 2004). Overexpression of CRH-R2 was reported in inflamed subcutaneous tissue (Mousa *et al.* 2003) and in stress-induced alopecia (Katsarou-Katsari *et al.* 2001). Mast cells in the joints of rheumatoid arthritis (RA) patients were shown to express CRH-R (McEvoy *et al.* 2001). CRH binding sites were previously noted on immune cells (Webster *et al.* 1990, Singh & Fudenberg 1988, Audhya *et al.* 1991). CRH-R1 mRNA expression had been reported in human leukemic mast cells (HMC-1) (Theoharides TC *et al.* 1998), which were recently shown to express a number of CRH-R1 isoforms, but no CRH-R2 mRNA, in contrast human umbilical cord blood-derived cultured mast cells (hCBMCs) expressed CRH-R1 isoforms and CRH-R2α (Cao *et al.* 2005). Activation of CRH-R1 led to selective release of vascular endothelial growth factor (VEGF) without degranulation (Cao *et al.* 2005). Interestingly, hCBMCs were recently shown to be particularly rich in both CRH and the structurally related peptide urocortin (Ucn), which they can secrete upon immunologic stimulation, implying that they could both secrete and respond to these peptides (Kempuraj *et al.* 2004).

Here, we show for the first time that hCBMCs upregulate only CRH-R2 in response to IL-1, IL-4 and LPS.

Materials and methods

Recombinant human stem cell factor (rhSCF) was kindly donated by Amgen Inc. (Amgen Center, Thousand Oaks, CA, USA). Cytokines were obtained from Chemicon International (Temecula, CA, USA). LPS was obtained from Sigma.

Culture and stimulation of human mast cells

Human cord blood was obtained from placentas during normal deliveries in accordance with established institutional guidelines. hCBMCs were derived by the culture of $CD34^+$ progenitor cells as previously described (Kempuraj *et al.* 2004) with minor modifications. Briefly, mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (Biochemicals Biomedical, Aurora, OH, USA). $CD34^+$ progenitor cells were isolated from mononuclear cells by positive selection of AC133 ($CD133^+/CD34^+$) cells by magnetic cell sorting (Miltenyi Biotech, Auburn, CA, USA). For the first four weeks, $CD34^+$ cells were cultured in Isove's Modified Dulbecco's Medium (IMDM) (Gibco BRL) supplemented with $5{\cdot}5 \times 10^{-5}$ M 2-mercaptoethanol (Gibco BRL), 100 mg/L Insulin-Transferin-Selenium (ITS; GIBCO BRL), 0·1% bovine serum albumin (BSA; Sigma), 100 ng/ml rhSCF (Amgen) and 50 ng/ml IL-6 (Chemicon) at 37 °C in 5% CO_2 balanced air. After four weeks of culture, BSA and ITS in the culture medium were substituted with 10% Fetal Bovine Serum (FBS) (Bio Whittaker, Walkerville, MD, USA). By 10 weeks, 100% of the cells in culture were identified as mast cells by immunostaining for tryptase (Fig. 1). For stimulation, hCBMCs were washed once in Dulbecco's Phosphate Buffered Saline (Gibco BRL) and resuspended in fresh medium containing rhSCF (100 ng/ml) alone that was included in stimulation medium in all experiments for optimal mast cell viability, except when IL-6 release was measured following stimulation. For some experiments, hCBMCs were primed with IL-4 (10 ng/ml, Chemicon) for the last three weeks of culture (Toru H *et al.* 1998). During the last day of culture, cells primed with or without IL-4 were treated for 6 h with either IL-1α (50 ng/ml; Chemicon) IL-1β or lipopolysaccharide (LPS 100 ng/ml; Sigma), and collected for RNA extraction. Cytospin smears were prepared using Cytospin 3 (Shandon, Pittsburg, PA, USA).

Immunocytochemistry for tryptase

Immunostaining for tryptase was performed as reported previously (Kempuraj D *et al.* 1999). Briefly, cytospin smears were prepared, air dried and stored at –80°C until staining. Slides were air dried for 1 h at room temperature and fixed with Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 3 min. The cytospin smears were then processed for mast cell tryptase by the alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure using the Dako APAAP Kit system (Dako Corp, Carpinteria, CA, USA). The cytospin smears were incubated overnight at 4 °C with mouse anti-human tryptase monoclonal antibody (Chemicon) diluted to a working dilution of 1 μg/ml in Tris–HCl-PBS, pH 7·6,+10% FBS. The smears were then brought to room temperature and incubated with rabbit anti-serum (Ig fraction) to mouse immunoglobulins for 30 min. The samples were then incubated with the APAAP immune complex for 30 min. Between each incubation, cytospin smears were rinsed in Tris–buffered saline (pH 7·6) for 10 min. The reaction was finally developed with substrate solution (naphthol AS-MX phosphate, Fast Red, and Levamisole) for 20 min and then rinsed briefly in a water bath. Negative controls were performed either by the omission of the primary antibody or by using an isotype-matched mouse IgG1 antibody instead of the primary antibody. Positive staining resulted in the formation of a bright red precipitate at the site of the target antigen tryptase.

Immunocytochemistry for CRH receptors

Cytospin smears of unfixed hCBMCs were prepared using Cytospin 3 and were fixed with Carnoy's solution for 2 min. The primary antibodies used were polyclonal goat anti-human/mouse/rat CRH-R1 (V-14 sc-12381, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal goat anti-human/mouse/rat CRH-R2 (C-15 sc-20550, Santa Cruz) at 1:50 and 1:100 dilutions, respectively; incubation was carried out at 4 °C overnight. All antibody dilutions and washes were performed with 3% BSA-PBS. The next day samples were incubated with fluorescein conjugated donkey anti-goat IgG for 60 min. Slides were washed 4 times, 10 min each, stained with DAPI, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and kept in the dark at 4 °C until observation using a 'cool snap' epifluorescent microscope equipped with fluorescein and UV (for DAPI visualization) filters and connected to camera and computer operating Image Pro Plus 4·1 for Windows (Media Cybernetics, Silver Spring, MD, USA). Negative controls contained samples in which the slides were incubated with a mixture of CRH-R1 (V-14P) and CRH-R2 (C-15P) blocking peptides (Santa Cruz) at equal ratio, but 50 times excess of equivalent antibody, as well as omission of the primary antibody in order to account for any background non-specific fluorescence signal.

Total RNA extraction and RT-PCR analysis

Total RNA was extracted from 10^6 hCBMCs using the Qiagen RNeasy mini kit (Qiagen). Contaminating DNA was removed from all RNA preparations using Turbo DNase treatment (Ambion Inc., Austin, USA). RNA (1μg) was then used to perform first-strand cDNA synthesis with 200 units/μl Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), 50 pmol/μl random hexamer oligonucleotide primer, 40 units/μl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen) and 10 mM deoxynucleotide triphosphates (Roche). The reaction (20 μl) was run at 25 °C for 10 min, 37 °C for 50 min, and inactivated by heating at 70 °C for 15 min. A portion of the reaction (10%) was used for PCR amplification in a 50 μl reaction. Oligonucleotide primers for CRH-R1, CRH-R2 and β-actin were used from previous studies (Pisarchik & Slominski 2001, Florio *et al.* 2000). Samples underwent 25, 30, 35 and 40-cycle amplifications in order to obtain results from the exponential phase of the reaction. At 35 to 40 cycles, samples would reach the linear phase of amplification. Visualization of the PCR products was done on a 2% agarose gel, stained with ethidium bromide, and viewed on an ultraviolet light box. Positive controls included human brain pituitary cDNA for CRH-R1 and human brain cerebral cortex cDNA for CRH-R2 (Clontech. Negative controls included (a) sample without the reverse transcriptase (RT) to control for genomic DNA contamination, (b) sample with water instead of template to check for external contamination.

All PCR images were analyzed by densitometry using the Stratagene Eagle Eye II System (La Jolla, CA, USA). The results were normalized against the housekeeping gene β-actin, expression that was not altered by any treatment. Experiments were repeated at least three times using RNA extracted from two different primary hCBMC cultures and cDNA synthesized at least twice from each RNA preparation. Results shown are representative of these experiments.

Sequence analysis

Each PCR product was purified from unwanted dNTPs and remaining primers using 2μl ExoSAP-IT (United States Biochemicals, Cleveland, OH, USA) with every 5μl PCR product. The PCR products were then sequenced with the appropriate CRH receptor forward primers using the automated ABI 3100 DNA sequencers at the Tufts University Core Facility, Boston, MA, USA. The results confirmed the specificity of the amplified product.

Statistics

Comparisons of the densitometry data were performed using a two tail Student's *t*-test. Significance is denoted by $P<0{\cdot}05$.

Results

Homogeneity of hCBMCs

In order to determine purity, hCBMCs were stained immunocytochemically for the unique mast cell protease tryptase; by 10 weeks, 100% of hCBMCs were identified as mast cells, based on the bright red staining for tryptases (Fig. 1). RNA was extracted from 10 week-old hCBMCs, whether they had been primed with IL-4 for the last three weeks of culture or not.

Immunocytochemical detection of CRH-R

Ten week-old hCBMCs cultured only in the presence of SCF and IL-6 were stained with either CRH-R1 or CRH-R2 antibodies to determine whether the protein for each subtype was expressed. Both CRH-R1 (Fig. 2A) and CRH-R2 (Fig. 2B) proteins were expressed in these untreated hCBMCs. When hCBMCs were primed with IL-4 for the last 3 weeks of culture, there was no apparent change in the fluorescent staining for CRH-R1

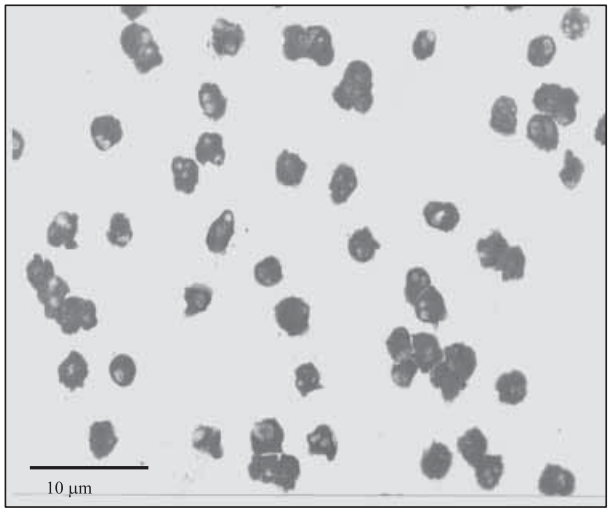

Figure 1 Photomicrograph of hCBMCs stained immunocytochemically for tryptase. Cytospins of hCBMCs were fixed and stained for tryptase. Positive staining resulted in bright red color confirming that the culture consisted 100% of mast cells. Scale bar=10 μm.

(Fig. 2C), but considerably more CRH-R2 protein (Fig. 2D); Negative controls included omission of the primary antibody to check for background fluorescence (Fig. 2E) and incubation with excess amounts CRH-R1 and CRH-R2 blocking peptides, together or in separate incubations (Fig. 2F).

RT-PCR analysis of mRNA for CRH-R1 and CRH-R2 with different treatments

RT-PCR analysis showed that 10 week-old hCBMCs cultured in the presence of SCF and IL-6 expressed mRNA for CRH-R1 (Fig. 3A). They also expressed a considerable amount of CRH-R2 mRNA (Fig. 4). A PCR amplification product using primers for β-actin as a housekeeping gene was used to normalize for any variation in the starting template among these samples, after amplification of the PCR product (Fig. 5). The results shown are representative of 2–4 similar experiments. Negative controls included a sample without RT to check for genomic DNA contamination and one without template, but water instead, to check for external contamination.

Effect of IL-4, IL-1 and LPS on CRH-R expression

Addition of IL-1α or IL-β to 10 week-old hCBMCs for 6 h (Fig. 4) decreased basal CRH-R1 expression but increased CRH-R2 expression (1·5 fold), (Fig. 4). The expression of CRH-R2, but not CRH-R1 was increased 1·5 fold when hCBMCs were cultured in the presence of IL-4 (10 ng/ml) for the last 3 weeks of culture. (Figs 3 & 4). Addition of IL-1α or IL-β (50 ng/ml) to these IL-4-treated cultures for 6 h more augmented (6 fold) the effect of IL-4 on CRH-R2 expression (Fig. 4).

Addition of LPS (100 ng/ml) for 6 h to hCBMCs cultured in the absence of IL-4 induced considerably only the CRH-R2 expression (Fig. 4), as determined

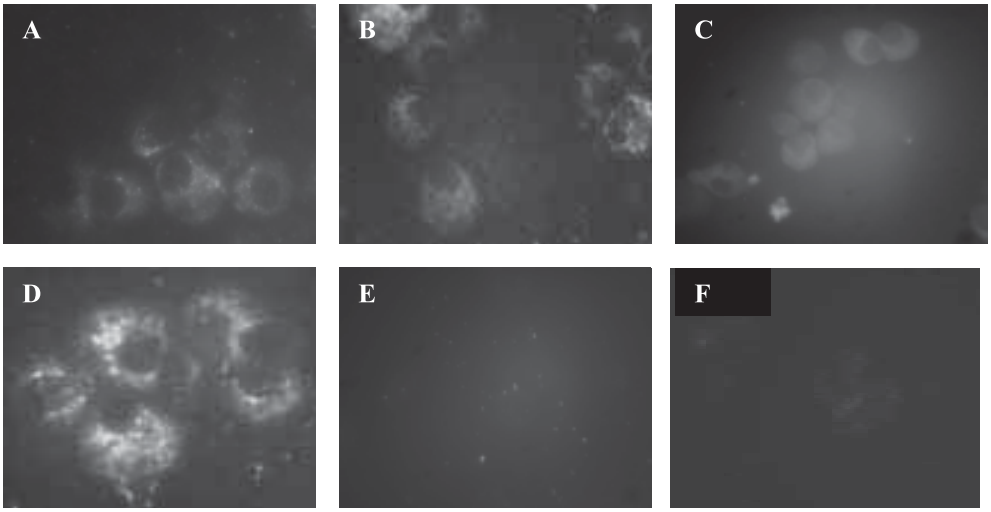

Figure 2 Photomicrographs of hCBMCs showing expression of CRH-R1 and CRH-R2 proteins by immunofluorescence. (A) Ten week-old hCBMCs cultured with SCF and IL-6 stained for CRH-R1 (B) CRH-R2 proteins (C) CRH-R1 and (D) CRH-R2 protein expression in hCBMCs primed with IL-4; Negative controls included (E) omission of the primary antibody and (F) incubation with excess amounts of CRH-R1 and CRH-R2 blocking peptides, together or in separate incubations to check for background fluorescence.

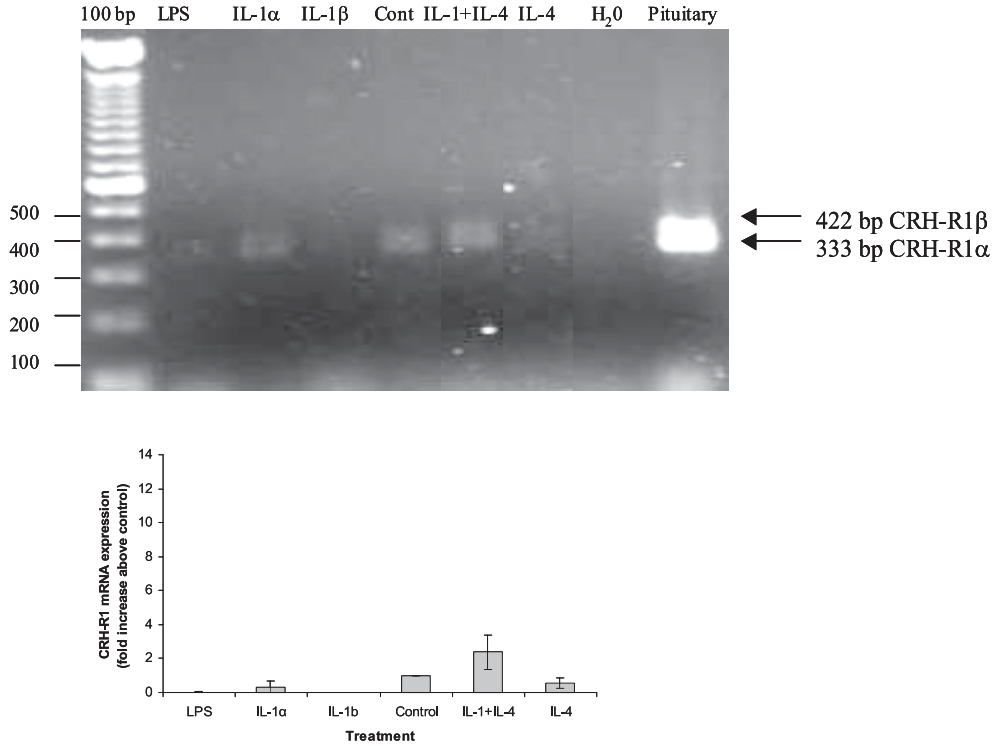

Figure 3 RT-PCR analysis of hCBMCs expression of mRNA for CRH-R1 (Upper Panel) CRH-R1 expression under different treatments of hCBMCs culture. (Lower Panel) Densitometric analysis of the PCR products shows the mean ±S.D. of at least two independent experiments.

from densitometry of the bands from at least three different RT-PCR experiments, normalized against β-actin (Fig. 5).

Due to the fact that hCBMCs synthesize both CRH and Ucn, we examined their respective mRNA expression during the experimental conditions described above. The expression of mRNA for CRH or Ucn was not affected by either IL-4, IL-1 or LPS (results not shown).

Stimulation of mast cell secretion by CRH and LPS

Ten weeks-old hCBMCs had 3·8 ± 1·3 pg/ml basal IL-6 release (Table 1). Priming the cells with IL-4 for the last 3 weeks of culture increased IL-6 to 19·5 ± 2·2 pg/ml (n=5, P<0·05). Addition of CRH (100 nM) for 6 h to non-primed cells, had a negligible effect on IL-6 release (n=4, 11·2 ± 6·6 pg/ml); however, addition of CRH (100 nM) increased IL-6 significantly to 33·3 ± 3·5 pg/ml (n=5, P<0·05). Addition of LPS to non-primed cells induced considerable IL-6 release, while addition of LPS to primed hCBMCs decreased the IL-6 release observed in untreated IL-4 primed cells (data not shown).

Discussion

This is the first report to our knowledge that normal human mast cells can upregulate both mRNA and protein expression only for CRH-R2 in response to IL-4 and LPS. Neither CRH nor Ucn mRNA expression varied between the different treatments. IL-4 had previously been shown to induce chymase expression in hCBMCs (Toru *et al.* 1998) and, together with SCF, to induce functional neurokinin-1 receptor (NK-1) expression on mouse bone marrow-derived mast cells (van der Kleij *et al.* 2003). IL-4 also enhanced mediator release, including FcεRI-independent activation, from purified human intestinal mast cells (Bischoff *et al.* 1999). Moreover, IL-4 priming was recently shown to significantly augment selective IL-6 release from hCBMCs stimulated by IL-1 (Kandere-Grzybowska *et al.* 2003). IL-4 is known to play an important role in allergic inflammation because it increases IgE production from B cells (Snapper *et al.* 1991, Spiegelberg 2005); moreover, allergic patients have increased IL-4 production from T cells (Wosinska-Becler *et al.* 2004).

The ability of IL-4 and LPS to induce CRH-R2 expression by hCBMCs paralleled their ability to trigger selective IL-6 release without tryptase. Interestingly,

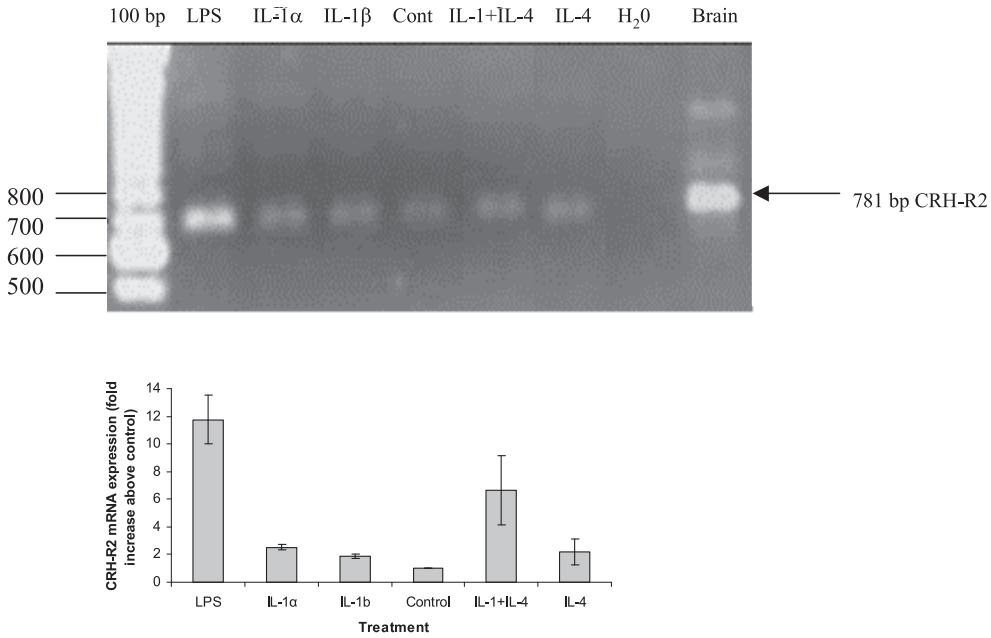

Figure 4 RT-PCR analysis of hCBMCs expression of mRNA for CRH-R2 (Upper Panel) CRH-R2 expression under different treatments of hCBMCs; the control lane corresponds to untreated cell preparation. (Lower Panel) Densitometric analysis of the PCR products shows the mean ±S.D. of at least two independent experiments.

addition of LPS to IL-4 primed cells abrogated their effects. In contrast, CRH acting through CRH-R1 activation was recently shown to stimulate selective release of vascular endothelial growth factor (VEGF) without histamine, tryptase or IL-6 (Cao *et al.* 2005). LPS had previously been reported to stimulate IL-6 secretion without histamine from mast cells (Leal-Berumen *et al.* 1994). Moreover, CRH was reported to enhance LPS-induced tumor necrosis factor (TNF-α) and IL-6 production from mouse macrophages (Agelaki *et al.* 2002). The ability of LPS to induce CRH-R2 expression is particularly interesting in view of the fact that mast cells express TLR-4 suggesting that activation of such receptors may affect CRH-R regulation (Varadaradjalou *et al.* 2003, McCurdy *et al.* 2003). It had previously been suggested that LPS may reduce CRH-R2 expression in the heart, but this effect could be indirect as neither LPS nor IL-1 had any direct effect on cultured cardiomyocytes *in vitro* (Coste *et al.* 2001).

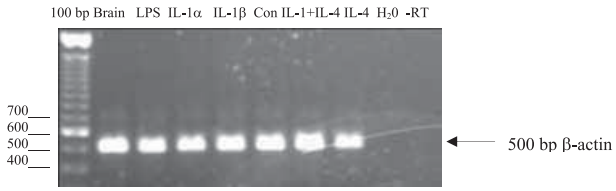

Figure 5 PCR for the housekeeping gene β-actin was used to normalize the CRH-R results obtained in figures 3 & 4. The last lane corresponds to the cDNA synthesis negative control reaction minus reverse transcriptase (-RT).

Table 1 Effect of priming of hCBMCs on IL-6 release

	IL-6 (pg/ml)	
	Minus IL-4	Plus IL-4
Conditions		
Spontaneous	3·8±1·3	19·5±2·2
CRH (100 nM)	11·2±6·6	33·3±3·5*

*$P<0.05$.

CRH regulates the HPA axis and typically suppresses inflammatory processes via induction of glucocorticoid release (Chrousos 1995). CRH, however, is also secreted in peripheral sites where it has proinflammatory actions (Karalis *et al.* 1991). Skin expresses different CRH-R isoforms, (Pisarchik & Slominski 2001) all of which were also identified on hCBMCs, activation of which led to selective release of VEGF without degranulation (Cao *et al.* 2005). However, it was reported that soluble CRH-R1e isoform inhibited the cAMP response element (Pisarchik & Slominski 2004), while a soluble mouse brain variant of CRH-R2α was recently shown to neutralize CRH (Chen *et al.* 2005). These findings indicate that there may be innate ways of regulating CRH-R activation. So far, skin CRH-R2 was shown to be overexpressed at affected areas of alopecia areata (Katsarou-Katsari *et al.* 2001), while skin CRH-R1 was

overexpressed in chronic urticaria (Papadopoulou *et al.* 2005).

Intradermal injection of CRH had been previously reported to induce skin mast cell degranulation and increase vascular permeability, effects absent in W/W^v mast cell deficient mice (Theoharides *et al.* 1998). However, CRH has never been shown to cause mast cell degranulation directly. Recent evidence indicates that intradermal effect of CRH on rodent skin mast cell degranulation depends on neurotensin (Donelan *et al.* 2005). Mast cell stimulation by CRH, with or without neuroinflammatory triggers, could lead to secretion of proinflammatory mediators that recruit circulating immune cells to the inflammation site, activate local immune accessory cells and stimulate peripheral nerves (Theoharides *et al.* 2004). Such findings strengthen the notion that, in addition to their role in allergic reactions and asthma, mast cells participate in acquired and innate immunity (Marone *et al.* 2002, Puxeddu *et al.* 2003, Malaviya & Abraham 2001, Galli & Nakae 2003), especially in inflammatory diseases exacerbated by stress (Theoharides & Cochrane 2004). Mast cells, themselves, are actually rich sources of CRH and Ucn, both of which could be released upon allergic stimulation (Kempuraj *et al.* 2004), leading to further autocrine actions.

In summary, human mast cells are shown to express functional CRH-R, of which CRH-R2 was upregulated by inflammatory conditions.

Acknowledgements

We thank Dr. James Marchand (Dept. of Anatomy and Cell Biology, Tufts University) for his help with the fluorescence imaging. Thanks are also due to Amgen, Inc. (Thousand Oaks, CA) for the human rSCF. We also thank Ms. Jessica Christian for her patience and word processing skills. This work was supported in part by NIH grant No. AR47652 to T C T. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 16 August 2005
Accepted 24 August 2005