RAPID COMMUNICATION

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

Nikoletta G Papadopoulou¹, Lauren Oleson¹, Duraisamy Kempuraj¹, Jill Donelan¹, Curtis L Cetrulo4 and Theoharis C Theoharides¹,²,³

Departments of ¹Pharmacology and Experimental Therapeutics, ²Biochemistry and ³Internal Medicine, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA

4Department of Obstetrics and Gynecology, Tufts-New England Medical Center, Boston, MA 02111, USA

(Requests for offprints should be addressed to T C Theoharides at Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111, USA. Email: theoharis.theoharides@tufts.edu)

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/Wv 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.

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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.
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. 2003) and in stress-induced alopecia (Katsarou-Katsari et al. 2001). CRH-R2 was reported in inflamed subcutaneous tissue (Kempuraj et al. 2004) with minor modifications (Kempuraj 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 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 from 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 Isole’s Modified Dulbecco’s Medium (IMDM) (Gibco BRL) supplemented with 5·5 × 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% CO₂ 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, Chemikon) 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 antialkaline 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 slides 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 10⁶ 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 $P<0.05$.

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.
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-1β 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-1β (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

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(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).

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**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.

**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.
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 FceRI-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 trypstatin. Interestingly,
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).

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-R1 isoform inhibited the cAMP response element (Pisarchik & Slominski 2004), while a soluble mouse brain variant of CRH-R2 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

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

<table>
<thead>
<tr>
<th>Conditions</th>
<th>IL-6 (pg/ml)</th>
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<tbody>
<tr>
<td>Minus IL-4</td>
<td>Plus IL-4</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>3.8±1.3</td>
</tr>
<tr>
<td>CRH (100 nM)</td>
<td>11.2±6.6</td>
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*P<0.05.
overexpressed in chronic urticaria (Papadopoulo 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/Wv 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.

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