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Substance P (SP) induces expression of functional corticotropin-releasing hormone receptor-1 (CRHR-1) in human mast cells

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Abstract

Corticotropin-releasing hormone (CRH) is secreted under stress and regulates the hypothalamic-pituitary-adrenal (HPA) axis. However, CRH is also secreted outside the brain where it exerts pro-inflammatory effects through activation of mast cells, which are increasingly implicated in immunity and inflammation. Substance P (SP) is also involved in inflammatory diseases. Human LAD2 leukemic mast cells express only CRHR-1 mRNA weakly. Treatment of LAD2 cells with SP (0.5–2 μ M) for 6 hr significantly increases CRHR-1 mRNA and protein expression. Addition of CRH (1 μ M) to LAD2 cells “primed” with SP for 48 hr and then washed, induces synthesis and release of IL-8, tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) 24 hr later. These effects are blocked by pretreatment with an NK-1 receptor antagonist. Treatment of LAD2 cells with CRH (1 μ M) for 6 hr induces gene expression of NK-1 as compared to controls. However, repeated stimulation of mast cells with CRH (1 μ M) leads to downregulation of

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

CRHR-1 and upregulation in NK-1 gene expression. These results indicate that SP can stimulate mast cells and also increase expression of functional CRHR-1, while CRH induces NK-1 gene expression. These results may explain CRHR-1 and NK-1 expression in lesional skin of psoriatic patients.

Introduction

Mast cells are ubiquitous in the human body and are critical for allergic reactions (Theoharides and Kalogeromitros, 2006), but are also involved in innate (Abraham and St John, 2010) and acquired immunity (Galli *et al.*, 2005) during which they secrete numerous vasoactive molecules, cytokines and proteases. Increasing evidence implicates mast cells in inflammatory diseases, especially those exacerbated by stress (Theoharides and Cochrane, 2004). Corticotropin-releasing hormone (CRH) is secreted under stress and activates the hypothalamic-pituitary-adrenal (HPA) axis (Chrousos, 1995). CRH, CRH receptors (CRHR), other neuropeptides such as substance P (SP), mast cells and other cells communicate as part of a local network mimicking the HPA axis in the skin (Slominski and Wortsman, 2000).

SP exerts its effects by binding to two specific G protein-coupled cell surface receptors, CRHR-1 and CRHR-2 (Chen *et al.*, 1993; Liaw *et al.*, 1996). Human mast cells express CRHR-1, stimulation of which by CRH leads to selective release of VEGF without degranulation from human mast cells (Cao *et al.*, 2005).

SP was originally isolated and characterized from the brain (Carraway and Leeman, 1973), but is now known to be widely distributed in many tissues, and is induced in many inflammatory processes (O'Connor *et al.*, 2004). SP was known to be a potent mast cell trigger of rodent mast cells (Fewtrell *et al.*, 1982), but less was known of its effect on human mast cells. We have shown that SP induces release of vascular endothelial growth factor (VEGF) from human mast cells, an action augmented by IL-33 (Theoharides *et al.*, 2010b).

Here we report that SP induces expression of functional CRHR-1, while CRH induces NK-1 gene expression on LAD2 human leukemic mast cells. Repeated stimulation of mast cells with CRH leads to downregulation of CRHR-1 and upregulation of NK-1 genes.

Results

Effect of SP on CRHR-1 expression on LAD2 cells

LAD2 mast cells show weak gene expression of CRHR-1, but CRHR-2 gene expression was below detection by the method used. Addition of SP (0.5, 1, 2 μ M) to LAD2 cells for 6 hr leads to statistically significant increase in CRHR-1 mRNA (Fig. 1a). FACS analysis shows that 48 hr following treatment with SP (0.5, 1 μ M) expression of CRHR-1 protein is also increased (Fig. 1b). In order to investigate if these findings are limited to the leukemic nature of LAD2 mast cells, we repeated some of the experiments using ten week-old hCBMCs. (Gene expression of CRHR-1 > CRHR-2 is significantly induced (Fig. 1c,d) following 6 hr treatment with SP 10 μ M, with human placenta used as a positive control

because it expresses both CRHR-1 and CRHR-2). In contrast, human basophils do not express any CRHR (results not shown).

CRH stimulates LAD2 cell secretion of VEGF, TNF, and IL-8

Addition of CRH (1 μ M; lower concentration gave inconsistent results) for 24 hr to LAD2 cells “primed” with SP (2 μ M) for 48 hr leads to statistically significant ($p < 0.05$) more VEGF release (429.4 ± 47 pg/ 10^6 cells by CRH alone to 700.2 ± 37 pg/ 10^6 cells in SP primed cells). This increase could not be due to any residual effect of the original SP stimulation, because parallel tubes with cells treated with SP alone washed at 48 hr and then incubated for another 24 hr without CRH had much less VEGF release compared to the samples to which CRH was added (Fig. 2a). The effect of SP on VEGF release is blocked by an NK-1 antagonist added 30 min before SP (Fig. 2a). Moreover, CRH (1 μ M) increases TNF (Fig. 2b) and IL-8 (Fig. 2c) release from LAD2 cells (2.9 ± 0.14 pg/ 10^6 cells and 0 pg/ 10^6 cells to 86.4 ± 11.1 pg/ 10^6 cells, 41.7 ± 5.1 pg/ 10^6 cells in SP (2 μ M) primed cells, respectively), and these effects are also blocked by the NK-1 antagonist (Fig. 2b, 2c).

CRH stimulates truncated NK-1 gene expression

CRH and SP may be released together in inflammatory diseases that worsen by stress. We therefore, investigated if CRH could affect NK-1 gene expression. Addition of CRH (1, 10 μ M) to LAD2 cells for 6 hr results in statistically significant increase in gene expression of only truncated NK-1 mRNA (Fig. 3). The full length NK-1 was not expressed (data not shown).

Effect of repeated stimulation of LAD2 cells with CRH on CRHR-1 and truncated NK-1 gene expression

We then investigated if repeated stimulation with CRH would affect CRHR-1 or NK-1 gene expression. Repeated stimulation of mast cells every 6 hr up to 24 hr with CRH (1 μ M) downregulates CRHR-1 mRNA expression (Fig. 4a), but upregulates NK-1 mRNA expression (Fig. 4b).

Discussion

This is the first report to our knowledge that SP can increase both mRNA and protein expression of CRHR-1 on human mast cells, activation of which leads to IL-8, TNF and VEGF secretion. This effect of SP could not be due to the leukemic nature of LAD2 cells because it was also present in hCBMC cells. Moreover, the effect of SP could not be nonspecific because contrary to our findings, SP was previously reported to decrease Fc ϵ RI expression on human mast cells (McCary *et al.*, 2010). SP had previously been shown to induce CRHR-1 expression in human astrocytoma 4373 MG cells (Hamke *et al.*, 2006).

CRH also induced mRNA expression of only the truncated NK-1 on LAD2 cells which is known to be at least ten times less sensitive than the full length receptor (Tuluc *et al.*, 2009) and was recently shown to be associated with pathological states (Tuluc *et al.*, 2009). These findings could be relevant for the pathogenesis of skin diseases that worsen by stress

(Slominski, 2009), (Slominski *et al.*, 2007), such as psoriasis, where both SP (Remröd *et al.*, 2007) and CRH (Tagen *et al.*, 2007), (Slominski *et al.*, 2000) have been implicated.

We also report that overstimulation with CRH leads to downregulation of CRHR-1 on LAD2 cells. This finding may explain the fact that CRHR-1 gene expression is decreased in lesional psoriatic skin, possibly due to chronic overstimulation (Tagen *et al.*, 2008). Human skin expresses primarily CRHR-1, while CRHR-2 protein is found only in hair follicles and eccrine glands (Slominski *et al.*, 2004). It was previously shown that IL-1, IL-4, and LPS could increase CRHR-2 expression on hCBMCs (Papadopoulou *et al.*, 2005b). These results suggest that activation of CRHR-1 and CRHR-2 may have different functions on the same or different cells or tissues. For instance, activation of these receptors was shown to have different actions on keratinocytes (Zbytek *et al.*, 2005; Zbytek *et al.*, 2004) and macrophages (Tsatsanis *et al.*, 2007; Agelaki *et al.*, 2002). Moreover, CRHR-1 was responsible for skin (Agren *et al.*, 1976), while CRHR-2 for bladder (Boucher *et al.*, 2010) and cardiac inflammation (Huang *et al.*, 2002). Moreover, while skin CRHR-1 was overexpressed in chronic urticaria (Papadopoulou *et al.*, 2005a), skin CRHR-2 was overexpressed in affected areas of alopecia areata (Katsarou-Katsari *et al.*, 2001). It is, therefore, interesting that hair root progenitor cells were recently shown to develop into mature mast cells in response to CRH (Paus *et al.*, 1994).

CRH secreted outside the brain has pro-inflammatory actions (Karalis *et al.*, 1991). Such pro-inflammatory activities could mediate the action of lipopolysaccharide (Zbytek and Slominski, 2007), or through mast cell activation leading to increased vascular permeability (Theoharides *et al.*, 1998). CRH stimulates CRHR-1-dependent selective release of VEGF without histamine from human mast cells (Cao *et al.*, 2005). Mast cell stimulation by SP and CRH, would result in secretion of pro-inflammatory cytokines that would recruit and activate local immune accessory cells that would also stimulate peripheral nerves (Theoharides *et al.*, 2004). Some of these cytokines may have synergistic action with either SP and / or CRH. For instance, we recently reported that the ability of SP to stimulate VEGF release from human mast cells is augmented by IL-33 (Theoharides *et al.*, 2010a). Mast cells had been previously shown to release VEGF in response to IgE (Boesiger *et al.*, 1998; Grutzkau *et al.*, 1998), and CRH (Cao *et al.*, 2005).

SP could be involved in the pathogenesis of inflammatory skin disorders, such as psoriasis (Remröd *et al.*, 2007; Farber *et al.*, 1986), which worsens by acute stress (Fortune *et al.*, 2005; Harvima *et al.*, 1996). SP-positive nerve fibers are more dense in psoriatic lesions compared to normal skin and have an increased number of contacts with mast cells (Jiang *et al.*, 1998; Chan *et al.*, 1997; Al'Abadie *et al.*, 1995; Naukkarinen *et al.*, 1996), which are also increased in lesional psoriatic skin (Harvima *et al.*, 1993; Özdamar *et al.*, 1996). SP-positive nerve fibers making contact with mast cells were increased by acute stress in mice, (Peters *et al.*, 2005) leading to dermal mast cell degranulation (Kawana *et al.*, 2006; Fortune *et al.*, 2005; Paus *et al.*, 1995).

The ability of CRH and SP to induce VEGF release from mast cells is also relevant to psoriasis. Psoriatic plaques contain increased levels of VEGF compared to normal skin (Heidenreich *et al.*, 2009; Yalcin *et al.*, 2007; Simonetti *et al.*, 2006), and the VEGF 121

isoform also causes vascular permeability (Zhang *et al.*, 2005; Creamer *et al.*, 2002). Several VEGF polymorphisms are associated with an increased risk of developing psoriasis (Detmar, 2004; Barile *et al.*, 2006) and epidermal overexpression of VEGF in transgenic mice led to a phenotype nearly identical to that of psoriasis (Xia *et al.*, 2003).

Mast cells, CRH, and SP have been implicated in a brain-skin connection (Paus *et al.*, 2006), and SP was shown to be involved in stress-induced hair loss in mice (Arck *et al.*, 2005). In addition, to the role of CRH in a local skin “hypothalamic-pituitary-adrenal axis” (Slominski *et al.*, 2000), the present results indicate that CRH and SP have proinflammatory actions not only on their own, but also by augmenting the expression of their respective receptors on human mast cells.

Materials and methods

Recombinant human stem cell factor (rhSCF) was kindly donated by Biovitrum AB (Stockholm, Sweden). SP, CRH were obtained from Sigma-Aldrich (St. Louis, MO). Anti-human CRHR-1-Allophycocyanin (APC) for FACS analysis and Enzyme-Linked Immunosorbent Assay (ELISA) kits were from R&D Systems (Minneapolis, MN).

Human mast cell culture

LAD2 human mast cells (obtained from NIH) were cultured in serum-free media (StemPro-34; Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml rhSCF. For optimal cell growth, LAD2 cell density was maintained between 0.5×10^6 and 1×10^6 cells/ml.

In order to obtain human cord blood mast cells (hCBMCs), human cord blood was obtained from placenta during normal deliveries in accordance with established institutional guidelines (Kempuraj *et al.*, 2004). Briefly, mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, OH). CD34⁺ progenitor cells were isolated from mononuclear cells by positive selection of AC133 (CD133⁺/CD34⁺) cells by magnetic cell sorting (Miltenyi Biotech, Auburn, CA). hCBMCs were derived by the culture of CD34⁺ progenitor cells with minor modifications. For the first six weeks, CD34⁺ cells were cultured in AIM medium (Gibco, Grand Island, NY) supplemented with 100 ng/ml rhSCF and after six weeks 50 ng/ml IL-6 (Chemicon) was added and cultured at 37°C in 5% CO₂ balanced air. Mast cell viability was determined by trypan blue (0.3%) exclusion.

Human basophils were obtained from ATCC (Manassas, VA) and were cultured in Eagle's Minimum Essential Medium from ATCC.

Human placenta, while express both CRHR-1 and CRHR-2 (Florio *et al.*, 2000; Grammatopoulos, 2008) was obtained from the division of maternal-fetal medicine Tufts medical center, under exemption #4 of H1RB.

IL-8, TNF, and VEGF assays

LAD2 cells were washed with DPBS and suspended in complete culture medium. LAD2 cells (2×10^5 cells/200 μ l/well) were plated in 96 well flat bottom Falcon cell culture plates from Becton Dickinson (Franklin Lakes, NJ), and then cells were incubated with the SP for 48 hr at 37°C in 5% CO₂ incubator. CRH was added for 24 hr and the plates were centrifuged. IL-8 or VEGF were measured in the supernatant fluid by ELISA. The minimum detectable level of VEGF was 5 pg/ml. Control cells were treated with equal volume of only culture medium.

FACS analysis for CRH receptors

Mast cells were Fc-blocked by treatment with 1 μ g of human IgG/ 10^5 cells for 15 min at room temperature prior to staining. APC-conjugated CRHR-1 reagent was added to the Fc-blocked cells and were incubated for 30 min at 4°C. Following the incubation, unreacted CRHR-1 detection reagent we removed by washing. Finally, the cells were resuspend in PBS buffer for final flow cytometric analysis. As a control for analysis, cells in a separate tube were treated with APC-labeled mouse IgG2A antibody. Analysis was performed using the FACS analyzer Amnis Image Stream (Amnis, Seattle, WA).

Total RNA extraction and real time PCR analysis

Total RNA was extracted from LAD2 mast cells or hCBMCs (106) using the Qiagen RNeasy mini kit (Valencia, CA). RNA was then used to perform first-strand cDNA synthesis. The reaction (20 μ l) was run at 25 °C for 10 min, 37°C for 50 min, and inactivated by heating at 70°C for 15 min. In order to measure CRHR-1, 2, and truncated NK-1 receptor gene expression, quantitative real time PCR was performed using Taqman gene expression assays. The following probes obtained from Applied Biosystems (Carlsbad, CA): CRHR-1 (Hs00366363_m1), CRHR-2 (Hs00266401_m1), NK-1 full length (Hs00185530_m1), NK-1 truncated (AIV13FD); the sequences are proprietary and not released by the company.

The cycling conditions consisted of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec, 1 cycle at 60°C for 1 min, 1 cycle at 95°C for 15 sec, 1 cycle at 60°C for 30 sec and 1 cycle at 95°C for 15 sec. Relative mRNA quality was determined from standard curves run with each experiment, and the expression was normalized to GAPDH used as the endogenous control. Positive control included cDNA for CRH-R1 and 2 from hCBMCs and human placenta. Negative control included the sample with water instead of template to check for external contamination.

Statistical analysis

All conditions were performed in triplicate, and all experiments were repeated at least three times (n=3). Results are presented as mean \pm SD. Data from stimulated and control samples were compared using the unpaired 2-tailed, Student's t-test. Significance of comparisons is denoted by p<0.05.

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Abbreviations

CRH	corticotropin-releasing hormone
hCBMC	human umbilical cord blood-derived mast cell
VEGF	vascular endothelial growth factor
TNF	tumor necrosis factor

Reference List

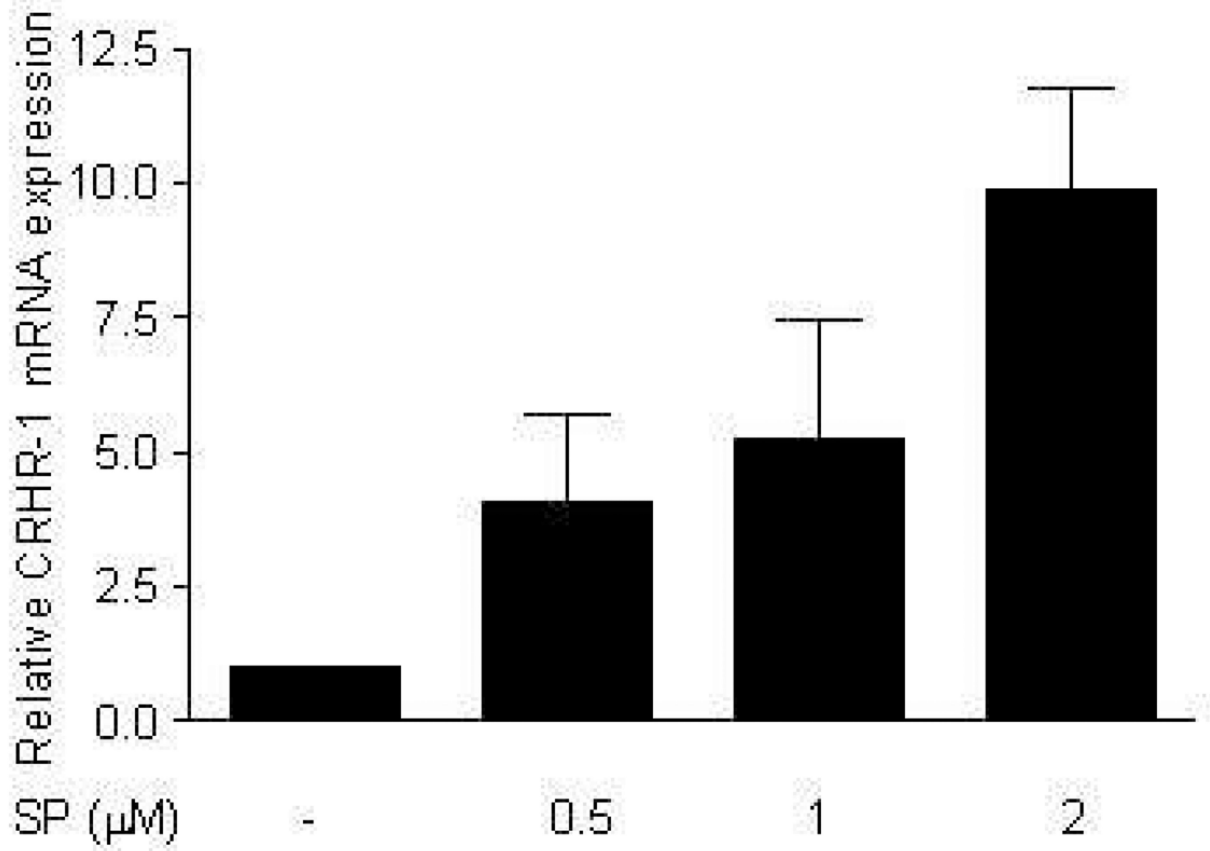
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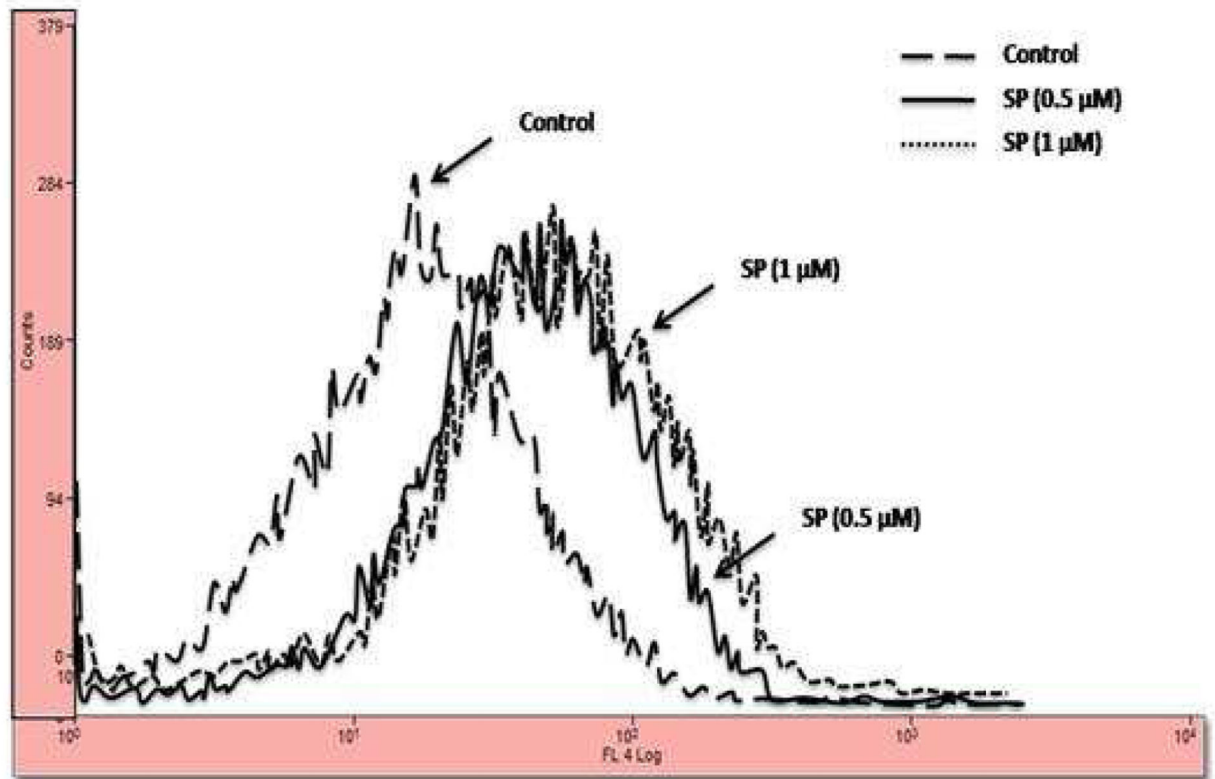
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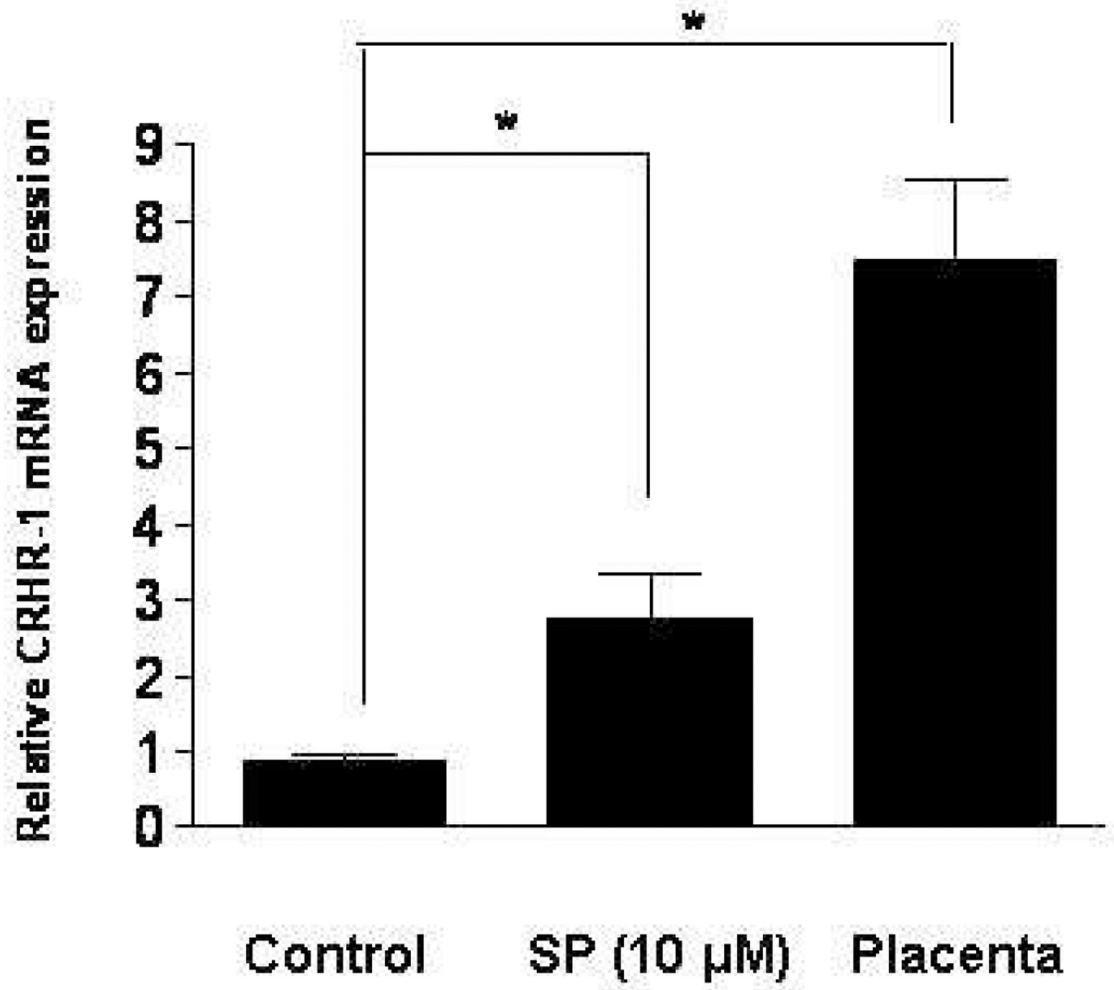
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b



C



d

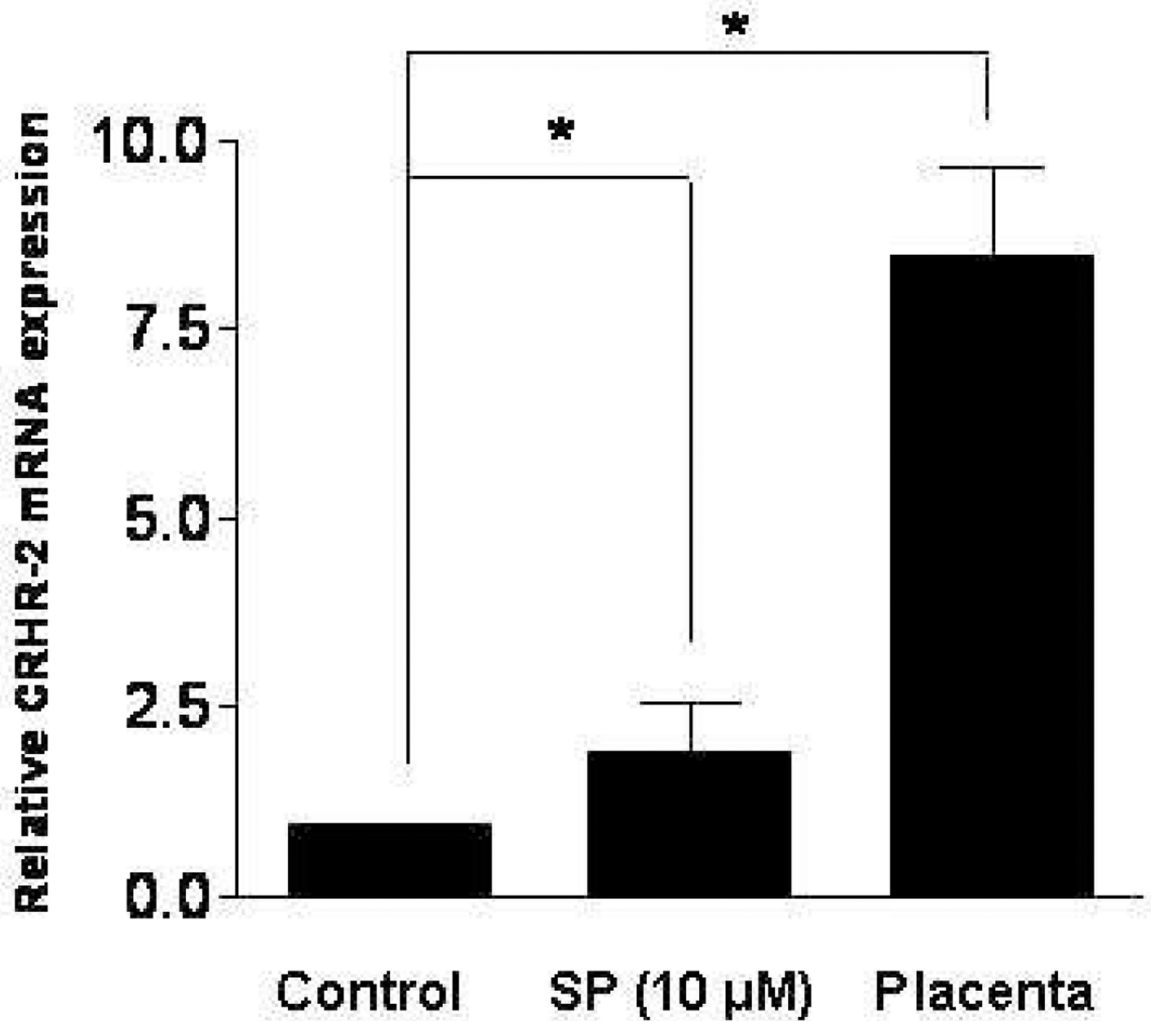
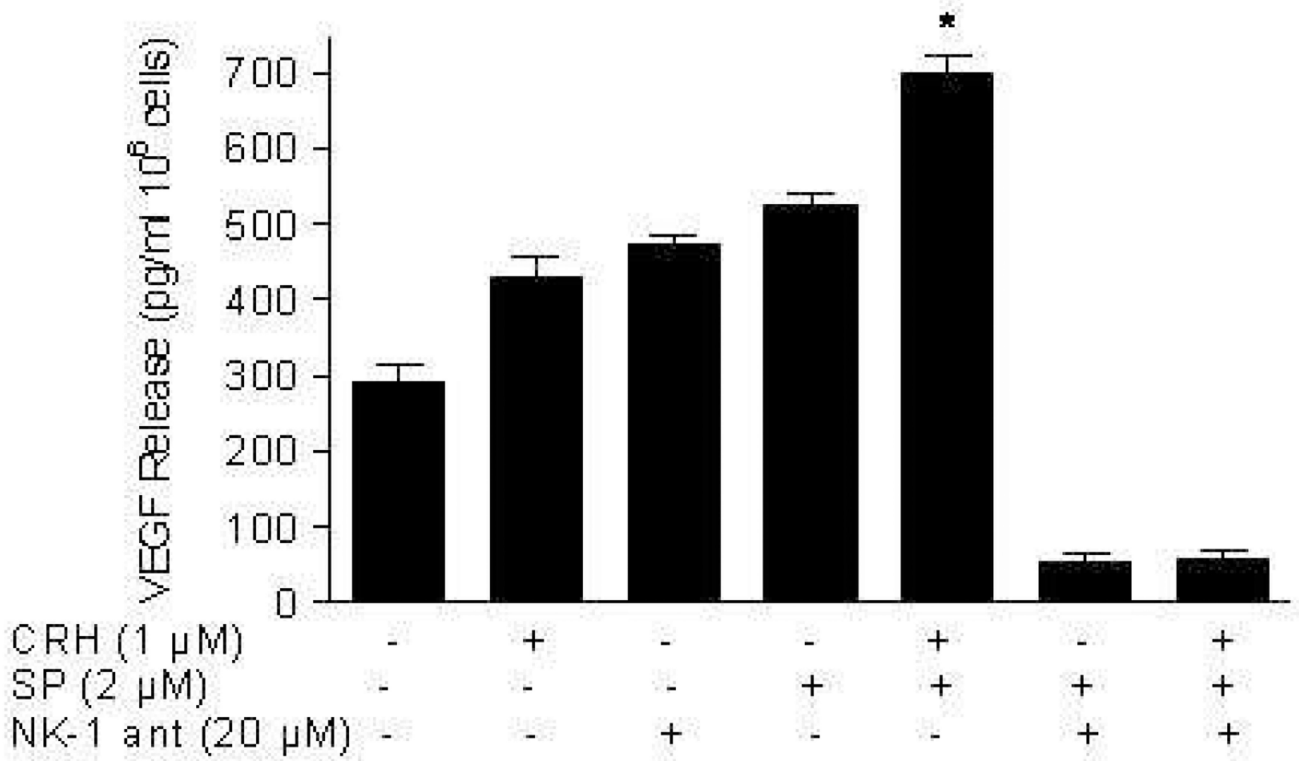
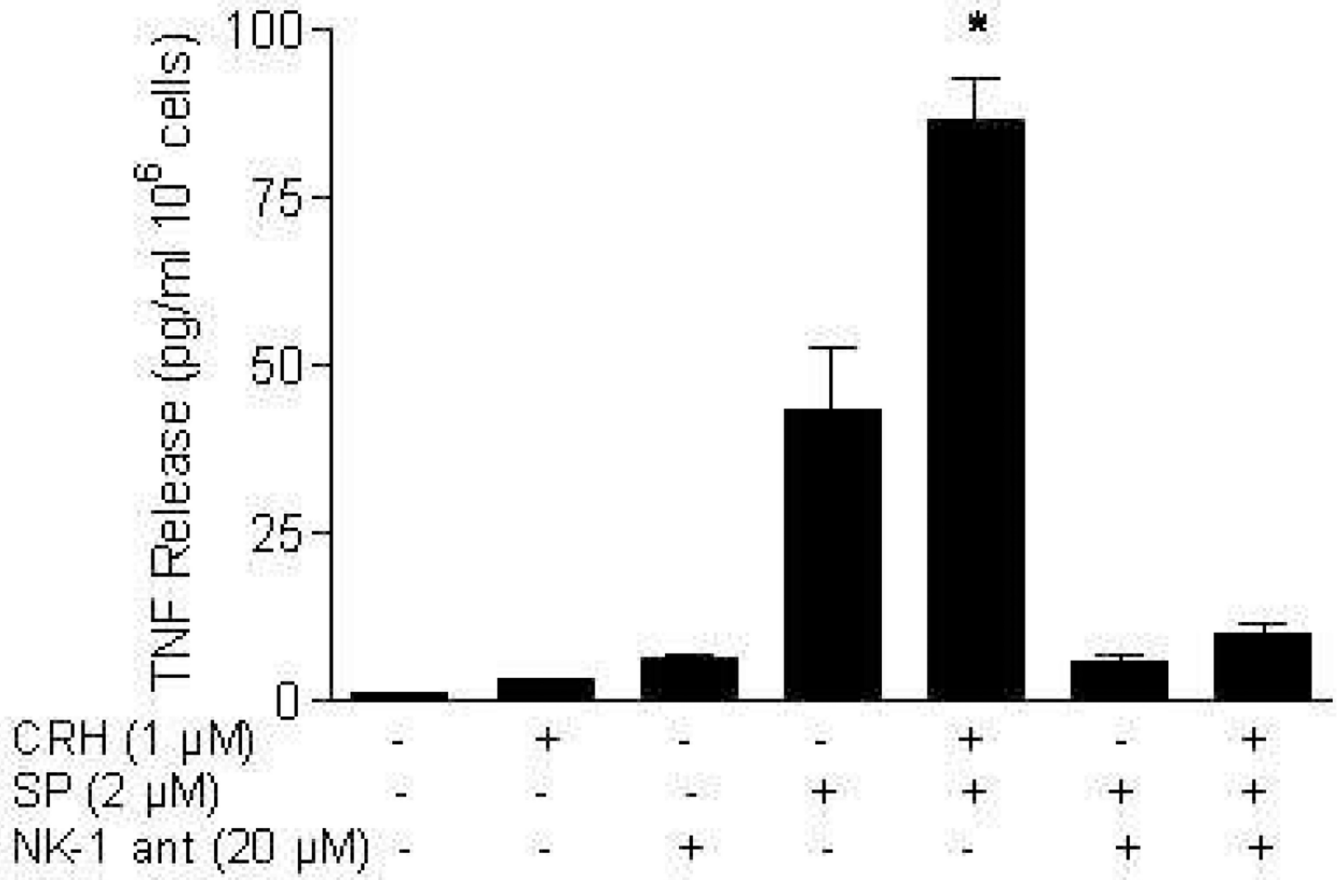


Figure 1. Effect of SP on CRHR-1 mRNA expression in human mast cells

LAD2 cell expression of CRHR-1 (a) mRNA following treatment with SP (0.5, 1, 2 μ M) for 6 hr and (b) protein following treatment with SP (0.5, 1 μ M) for 48 hr; y-axis indicates “counts” of cells, while the x-axis indicates log fluorescence intensity (n=3, p<0.05). (c,d) Ten week-old hCBMCs expression of CRHR-1 and CRHR-2 mRNA, before and after 6 hr incubation with SP (10 μ M) at 37°C. Human placenta was used as a positive control for CRHR-1 and CRHR-2. Gene expression was analyzed by RT-PCR and expression is indicated relative to control (n=3, p<0.05).

a



b

C

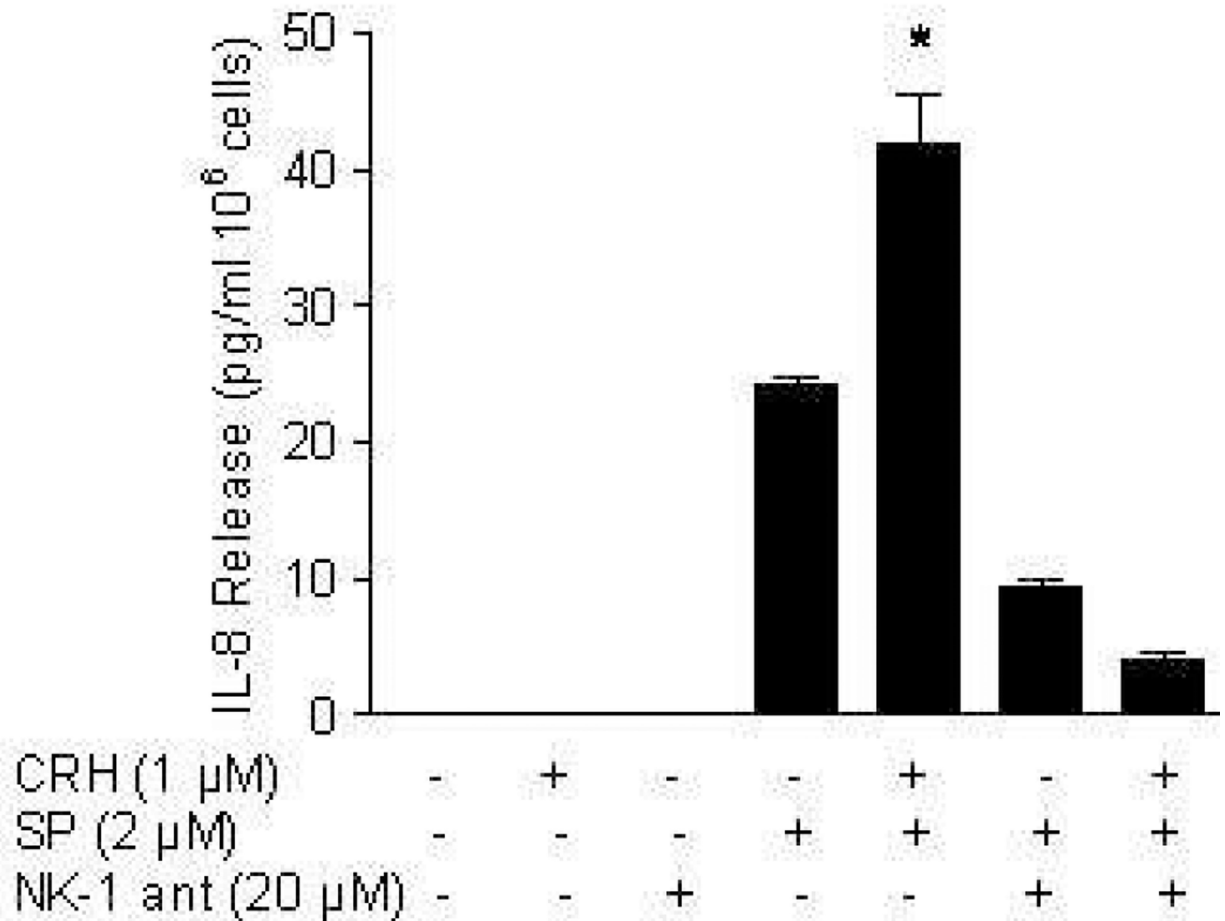


Figure 2. CRH stimulates LAD2 cell secretion of VEGF, TNF, and IL-8

Release of (a) VEGF, (b) TNF, and (c) IL-8 following stimulation with CRH (1 μ M) for 24 hr after pretreatment with SP (2 μ M) for 48 hr; in certain experiments mast cells were pretreated with NK-1 antagonist (ant, 20 μ M for 30 min prior to SP) (n=3, p<0.05).

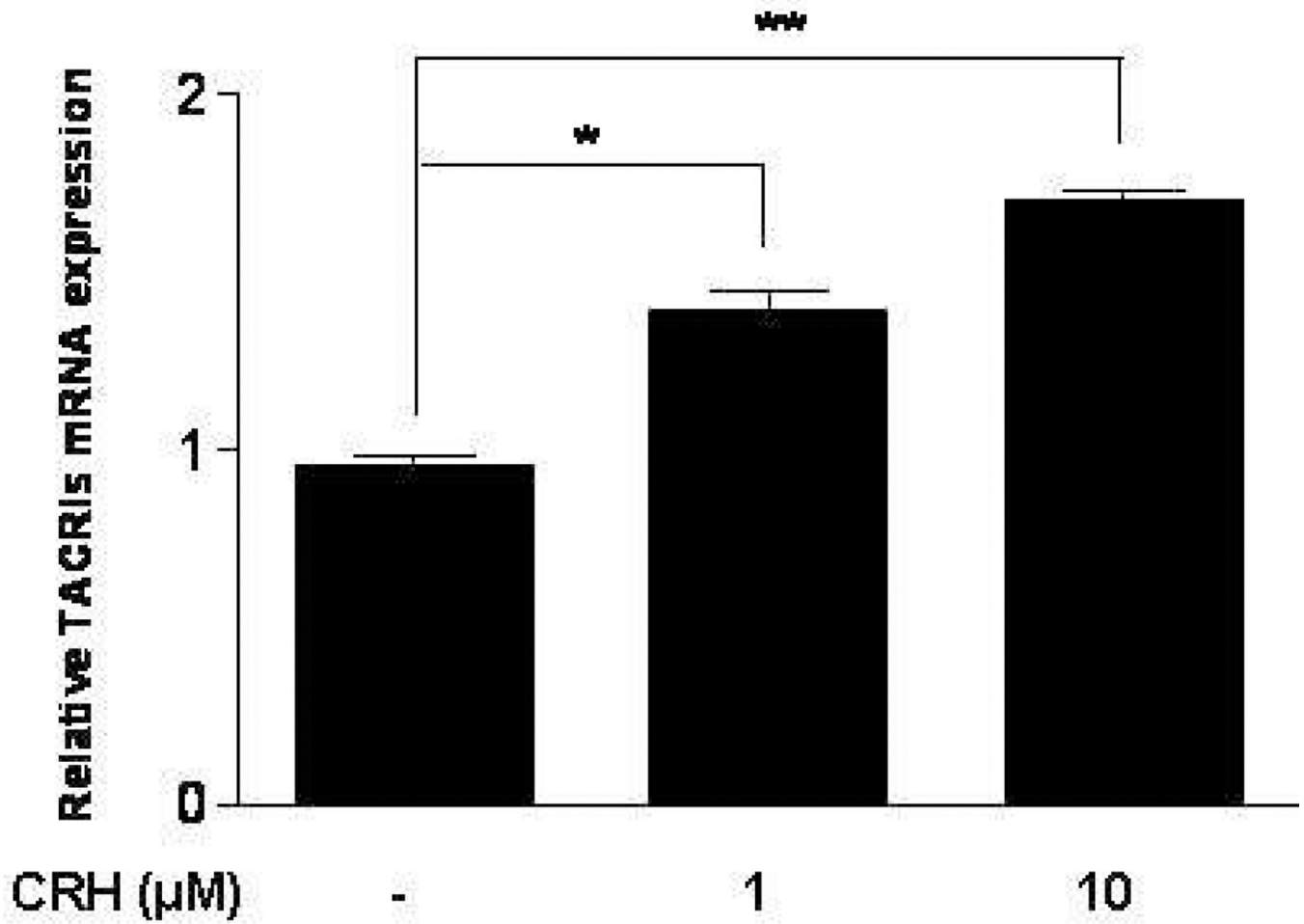
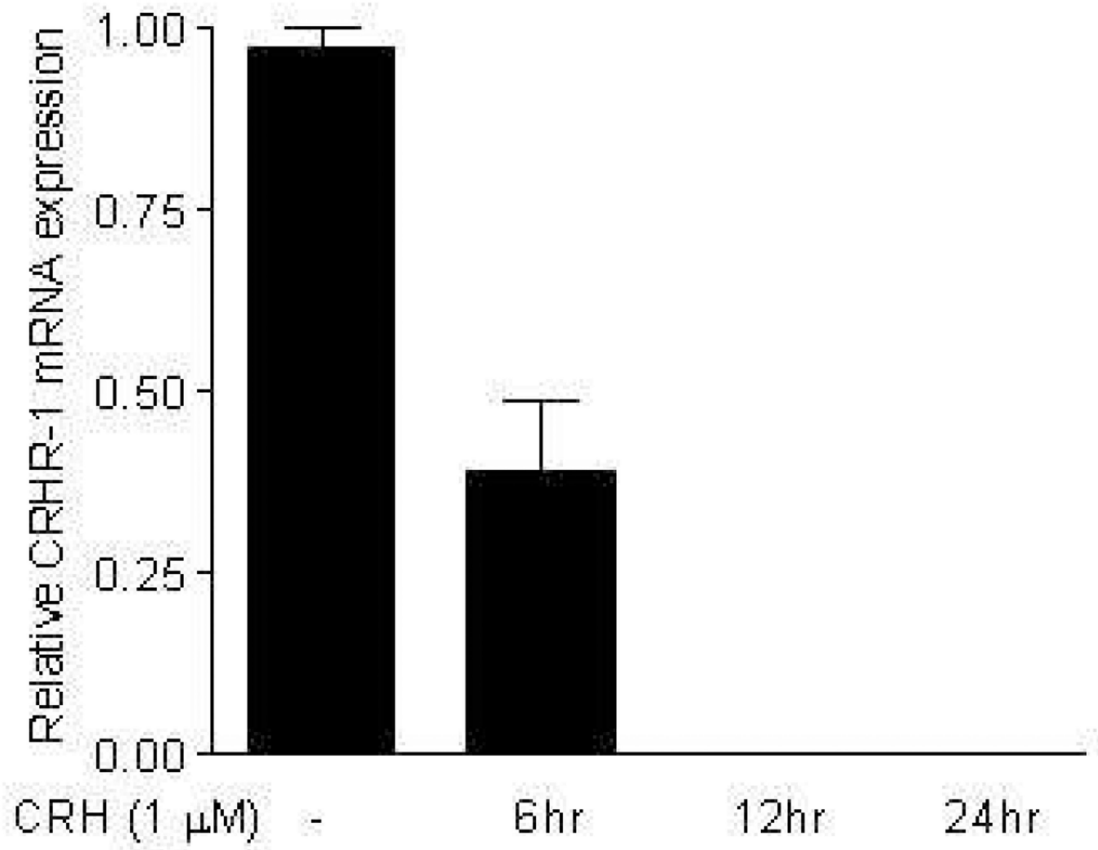


Figure 3. CRH stimulates mRNA expression of NK-1 receptor
Treatment of LAD2 cells with CRH (1, 10 μM) for 6 hr induces gene expression of truncated NK-1 (n=3, p<0.05).

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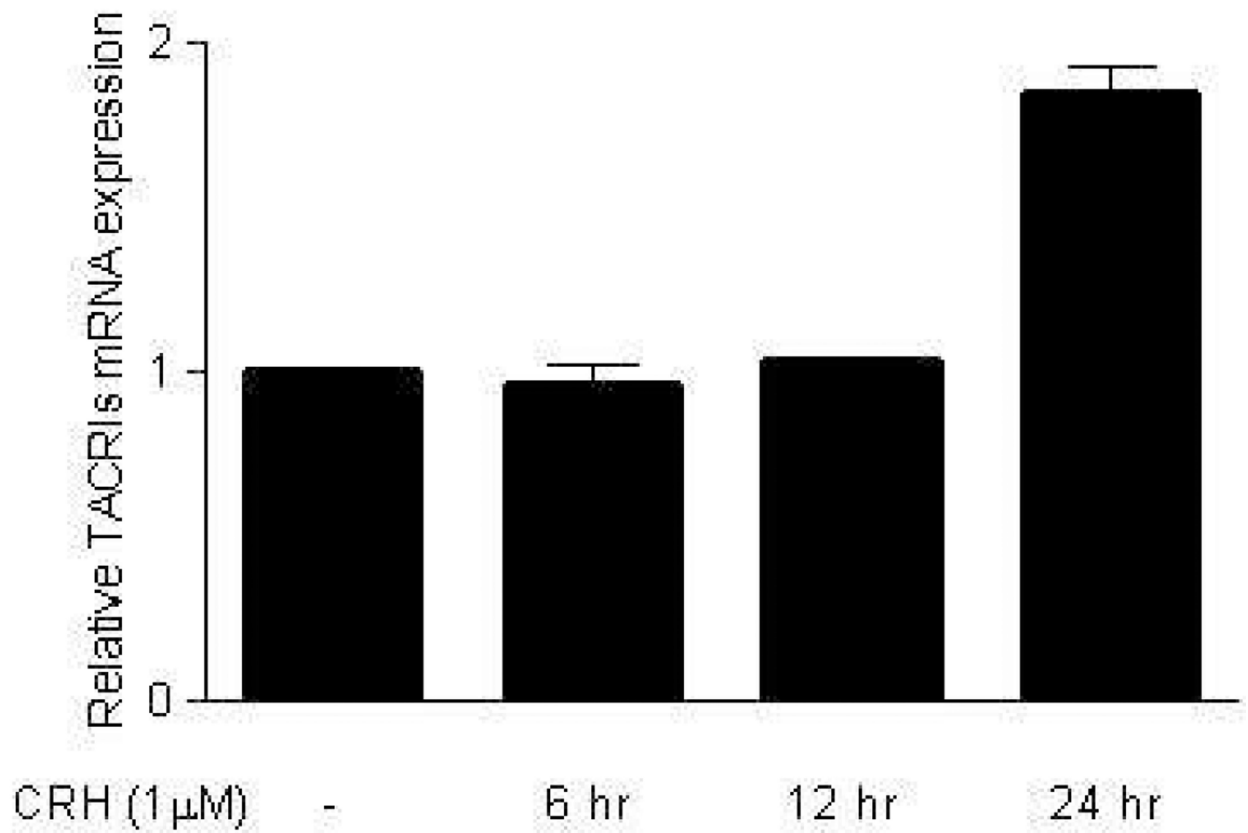
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Figure 4. Effect of CRH on CRHR-1 and NK-1 receptor mRNA expression

Repeated stimulation of mast cells with CRH every 6 hr up to 24 hr downregulates CRHR-1

(a), but upregulates truncated NK-1 receptor gene expression (b).