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# **Corticotropin-releasing hormone (CRH) induces vascular endothelial growth factor (VEGF) release from human mast cells via the cAMP/protein kinase A/p38 MAPK pathway**

**Jing Cao, Curtis L. Cetrulo, Theoharis C. Theoharides**

Departments of Biochemistry and Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston, MA; Department of Obstetrics and Gynecology, Tufts-New England Medical Center, Boston, MA; Departments of Pharmacology and Experimental Therapeutics and Internal Medicine, Tufts University School of Medicine, Tufts-New England Medical Center, Boston, MA, 02111, USA

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**Correspondence and reprints to:**

T.C. Theoharides, Ph.D., M.D.  
Department of Pharmacology and Experimental Therapeutics  
Tufts University School of Medicine  
136 Harrison Avenue  
Boston, MA 02111, USA  
Phone: (617) 636-6866  
Fax: (617) 636-2456  
E-mail: [theoharis.theoharides@tufts.edu](mailto:theoharis.theoharides@tufts.edu)

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**Abbreviations:** CRH, corticotropin-releasing hormone; HMC-1, human leukemic mast cell line; hCBMC, human umbilical cord blood-derived mast cell; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase

## ABSTRACT

Mast cells are involved in allergic reactions, but also in innate immunity and inflammation. Corticotropin-releasing hormone (CRH), the key regulator of the hypothalamic-pituitary-adrenal axis, also has proinflammatory effects, apparently through mast cells. We showed recently that CRH selectively stimulates human leukemic mast cells (HMC-1) and human umbilical cord blood-derived mast cells (hCBMC) to release newly synthesized vascular endothelial growth factor (VEGF) without release of either preformed mediators or cytokines. This effect was mediated through activation of CRH receptor-1 and adenylate cyclase with increased intracellular cAMP. However, the precise mechanism by which CRH induces VEGF secretion has not yet been defined. Here, we show that CRH-induced VEGF release was dose-dependently inhibited by the specific protein kinase A inhibitor H89 or the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, but not by the specific inhibitor PD98059 of MEK, the upstream kinase of the extracellular signal-regulated protein kinase (ERK) or the c-Jun N-terminal kinase (JNK) inhibitor SP600125. Furthermore, CRH significantly increased protein kinase A activity, which could be mimicked by the cell-permeable cAMP analogue 8-bromo-cAMP, and was blocked by H89 or the adenylate cyclase inhibitor SQ22536. CRH also induced rapid phosphorylation of p38 MAPK, which was mimicked by 8-bromo-cAMP and was inhibited by H89 or SB203580. CRH did not stimulate ERK or JNK phosphorylation and did not increase intracellular calcium levels. These results indicate that CRH induces VEGF release in human mast cells via selective activation of the cAMP/protein kinase A/p38 MAPK signaling pathway, thereby providing further

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insight into the molecular mechanism of how CRH affects the release of a key proinflammatory mediator.

## INTRODUCTION

Corticotropin-releasing hormone (CRH), mainly produced in hypothalamus, is a 41-amino acid peptide, which regulates endocrine and behavioral responses to stress through activation of the hypothalamic-pituitary-adrenal axis that down-regulates immune responses (Karalis et al., 1997). CRH, however, is also secreted peripherally and has proinflammatory effects possibly mediated through stimulation of mast cells (Theoharides et al., 1998). The cellular effects of CRH are initiated by binding and activation of two main types of receptors, CRH-R1 and CRH-R2, which belong to G protein-coupled seven-transmembrane receptors (Wiesner et al., 2003; Grammatopoulos and Chrousos, 2002). We showed recently that mast cells express CRH-R1, stimulation of which by CRH leads to selective release of vascular endothelial growth factor (VEGF) through activation of adenylate cyclase and increased cAMP (Cao et al., 2005). The exact mechanisms of VEGF release, however, have not yet been fully clarified.

CRH receptors are linked to a number of intracellular signaling pathways, including ligand-dependent increase of intracellular cAMP and calcium. In most cells, binding of CRH to CRH-R1 leads to activation of adenylate cyclase, and increased protein kinase A activity, which in turn phosphorylates and activates its downstream targets (Grammatopoulos and Chrousos, 2002). It was shown in several cell types that cAMP/ protein kinase A pathway plays a key role in VEGF production (Hoper et al., 1997; Cheng et al., 1998; Amano et al., 2001). In addition, CRH receptor-mediated activation of mitogen-activated protein kinase (MAPK) signal transduction pathways has also been reported (Kovalovsky et al., 2002; Dermitzaki et al., 2002). The release of

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inflammatory mediators is believed to be mediated through signaling pathways involving MAPKs. The family of MAPKs consists of at least three different subgroups including extracellular signal-regulated protein kinase (ERK1/2 or p44/p42), the p38 MAPK, the c-Jun N-terminal kinase (JNK1/2 or p46/p54), and some more distantly related kinases (Johnson and Lapadat, 2002). The p38 MAPK regulates the expression of many cytokines. In addition to its recognized role in IL-1 and TNF- $\alpha$  production (Lee et al., 1994), p38 MAPK is also involved in the production of IL-4 (Schafer et al., 1999), IL-6 (Wery-Zennaro et al., 2000), IL-8 (Marie et al., 1999), IL-12 (Lu et al., 1999) and VEGF (Tokuda et al., 2003b). In addition, ERK and JNK have also been shown to be involved in VEGF production in some cell types (Tanabe et al., 2003; Wang et al., 2002). The present study was undertaken to elucidate the role of protein kinase A and MAPK signaling pathways in CRH-mediated VEGF release in human mast cells.

We report that CRH triggers activation of protein kinase A and p38 MAPK in human leukemic mast cells (HMC-1) and human umbilical cord blood-derived mast cells (hCBMC). Using highly selective inhibitors of adenylate cyclase, protein kinase A and p38 MAPK, we found that stimulation of the cAMP/ protein kinase A /p38 MAPK pathway is crucial in the mechanism of CRH-mediated VEGF release in human mast cells.

## MATERIALS AND METHODS

**Reagents.** CRH was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA) and was dissolved in deionized H<sub>2</sub>O on the same day of the experiments. SQ22536, H89, PD98059, SB203580 and SP600125 were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and were dissolved in DMSO on the day of the experiments.

**Culture of human mast cells.** HMC-1 cells were kindly supplied by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN) and were cultured in IMDM (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% (v/v) bovine calf serum (Hyclone, Logan, UT), 100 U/ml penicillin/streptomycin and 2  $\mu$ M  $\alpha$ -thioglycerol. Cells were kept in 5% CO<sub>2</sub> balanced air at 37°C.

Human cord blood was obtained from human placentas from normal vaginal deliveries in accordance with established institutional guidelines. hCBMC were derived by the culture of CD34<sup>+</sup> progenitor cells as previously described (Kempuraj et al., 1999) with minor modifications. Briefly, mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, OH). CD34<sup>+</sup> progenitor cells were isolated from mononuclear cells by selection of cells positive for the AC133 Ag (CD133<sup>+</sup>/CD34<sup>+</sup>) by magnetic cell sorting (Miltenyi Biotec, Auburn, CA). For the first 4 wk, CD34<sup>+</sup> cells were cultured in IMDM supplemented with 0.55  $\mu$ M 2-ME, 100 mg/liter insulin-transferin-selenium, 0.1% BSA (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin/streptomycin, 100 ng/ml of human recombinant stem cell factor (Amgen, Thousand Oaks, CA), and 50 ng/ml IL-6 (Chemicon Inc., Temecula, CA) at 37°C in 5% CO<sub>2</sub> balanced air. After 4 wk of culture, BSA and insulin-transferin-

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selenium in the culture medium were substituted with 10% FBS (Invitrogen Life Technologies).

**VEGF release assay.** For stimulation of VEGF production, HMC-1 ( $2 \times 10^5$  cells/200  $\mu$ l) and hCBMC ( $10^5$  cells/200  $\mu$ l) were distributed to 96-well microtiter assay plates in duplicate or triplicate and stimulated in complete culture medium with the indicated concentrations of CRH. Optimal concentrations were determined from the dose-response experiments. VEGF was determined in cell-free supernatants with a commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. VEGF secretion data are expressed as picograms per  $10^6$  cells.

**Measurement of protein kinase A activity.** Cells were plated in 48-well plates ( $3 \times 10^5$  cells/300  $\mu$ l) in each well and serum-starved overnight. Next day, cells were stimulated with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for 3 min. Stimulation was terminated by addition of ice-cold PBS. The cells were collected and sonicated on ice in cold sample preparation buffer (50 mM Tris-HCl, 50 mM  $\beta$ -mercaptoethanol, 10 mM EGTA, 5 mM EDTA, 1 mM PMSF, and 10 mM Benzamidine (pH 7.5)). Cell lysates were prepared after centrifugation of the sonicated samples at 100,000 x g for 1 h at 4°C. Protein concentration of the supernatant was determined by the Bio-Rad protein assay. The protein kinase A activities were measured by ELISA (Calbiochem-Novabiochem Corp., La Jolla, CA) according to the manufacturer's instructions.



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**Western blot analysis of MAPKs phosphorylation.** HMC-1 cells were plated in 6-well plates ( $2 \times 10^6$  cells/2 ml/well) in serum-free medium and serum-starved overnight. Next day, cells were stimulated with CRH (100 nM) for the indicated time points, or stimulated with PMA (10 nM) for 5 min as a positive control for ERK activation, or IL-1 (10 ng/ml) for 10 min as a positive control for JNK activation. Stimulation was terminated by addition of ice-cold PBS and cells were washed once with PBS. Cells were then lysed in lysis buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin and 1 mM  $\text{Na}_3\text{VO}_4$ . Cell lysates were cleared by centrifugation and supernatants were subjected to 10% SDS-PAGE with antibodies against the phosphorylated form of p38 MAPK, ERK or JNK at a dilution of 1:1000. HRP-conjugated antibody against rabbit IgG was used at 1:2000 dilution. The reaction was detected using the ECL reagent (Amersham). The membrane was then stripped and reprobed with the antibody that reacts with total p38 MAPK, total ERK or total JNK.

**ELISA of phosphorylated p38 MAPK.** HMC-1 cells and hCBMC were washed twice in PBS, distributed to 48-well plates ( $10^5$  cells/200  $\mu$ l/well) in duplicate in serum-free culture medium and serum-starved overnight. Next day, hCBMC were stimulated with 8-bromo-cAMP (1mM) for 5 min or with CRH (1  $\mu$ M) for indicated times; HMC-1 cells were stimulated with 8-bromo-cAMP (1 mM) for 5 min. Stimulation was terminated by addition of ice-cold PBS. Cells were washed twice in PBS and lysed in 50  $\mu$ l of cell extraction buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate,

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1mM PMSF and protease inhibitor cocktail) (Sigma) for 30 min on ice. Activation of p38 MAPK was determined by quantifying amounts of phosphorylated and total form of kinase using ELISA (Biosource International, Camarillo, CA). Samples were diluted at least 1:10 for the assay. Data in each sample was normalized against total amount of kinase and expressed as fold induction.

**Measurement of intracellular calcium.** Cells ( $2 \times 10^6$  cells/ml) were resuspended in Tyrode's buffer (133 mM NaCl, 4 mM KCl, 0.64 mM  $\text{KH}_2\text{PO}_4$ , 10 mM HEPES, 1g/L glucose, 1 mM  $\text{CaCl}_2$ , 0.6 mM  $\text{MgCl}_2$ , 0.03% human serum albumin, pH 7.2) containing 2.5 mM probenecid and 2  $\mu\text{M}$  cell-permeant fluorescent calcium indicator Calcium Green-1-AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. Cells were then washed and resuspended in fresh Tyrode's buffer ( $10^6$  cells/ml). After two washes,  $2 \times 10^6$  cells were placed in a cuvette with constant stirring at  $37^\circ\text{C}$ , maintained by a closed circuit temperature regulated water pump. Samples were excited at 506 nm and fluorescence was recorded at 530 nm using a Perkins-Elmer fluorescence spectrophotometer (LS-5B, Shelton Chuck, CT). Cells were stimulated with CRH, anti-IgE (Chemicon), or Br-A23187 (Sigma). For anti-IgE stimulation, hCBMC were resuspended ( $10^6$  cells/ml) and passively sensitized by incubation with human myeloma IgE (2  $\mu\text{g}/\text{ml}/10^6$  cells; Chemicon) for 48 h before stimulation with anti-IgE. Data are presented in arbitrary fluorescence units.

**Statistical analysis.** Data represent the mean  $\pm$  SEM from three or more experiments, each in duplicate or triplicate; for hCBMC, each experiment was done with

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a culture from a different donor. Data were analyzed with the Student's *t* test, Mann-Whitney test and ANOVA, followed by the Holm-Sidak test or Newman-Keuls test as appropriate. Statistical significance was considered for  $p < 0.05$ .

## RESULTS

**Inhibition of CRH-stimulated VEGF release by the protein kinase A inhibitor H89.** As protein kinase A is a direct target of cAMP in many cells, we examined if protein kinase A is involved in CRH-stimulated VEGF production. The specific protein kinase A inhibitor H89 significantly blocked CRH-induced VEGF release in a dose-dependent manner in HMC-1 cells and hCBMC (Fig. 1a,b). H89, when added to cell cultures for 30 min prior to the addition of CRH, completely inhibited CRH-induced VEGF release at a concentration of 10  $\mu$ M in HMC-1 cells (Fig. 1a) and 1  $\mu$ M in hCBMC (Fig. 1b). H89 alone at the concentrations used did not alter the basal level of VEGF production and did not affect cell viability (data not shown). This result suggests that protein kinase A is essential for CRH-mediated VEGF release.

**Inhibition of CRH-stimulated VEGF release by the p38 MAPK inhibitor SB203580.** To test whether MAPK signaling pathways are involved in the effect of CRH on VEGF release, HMC-1 cells and hCBMC were treated with the specific p38 MAPK inhibitor SB203580, the upstream inhibitor of ERK, PD98059, or the JNK inhibitor SP600125 at various concentrations for 30 min prior to treatment with CRH. IL-1 (10 ng/ml) was used as a positive stimulus for MAPKs activation and VEGF release. Both PD98059 (50  $\mu$ M) and SP600125 (1  $\mu$ M) partially blocked IL-1-induced VEGF release in both cells, but had no effect on CRH-induced VEGF release; other concentrations of both inhibitors yielded similar results (data not shown). The p38 MAPK inhibitor SB203580 dose-dependently inhibited CRH-induced VEGF release (Fig. 2a,b). SB203580 alone at the concentrations used did not alter the basal level of VEGF release

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and did not affect cell viability (data not shown). These results indicate that ERK and JNK are not involved in CRH-induced VEGF production, whereas p38 MAPK is critical in this process.

**CRH induces activation of PKA and p38 MAPK.** To further investigate the role of protein kinase A in CRH-induced VEGF release, protein kinase A activity was measured in both cells. Incubation of HMC-1 cells with CRH (100 nM) or hCBMC with CRH (1  $\mu$ M) increased protein kinase A activity significantly ( $n=3$ ,  $p<0.05$ ) by  $2.5 \pm 0.6$  fold and  $1.8 \pm 0.3$  fold, respectively (Fig. 3a,b). The protein kinase A activity in both cells was inhibited by H89 in a dose-dependent manner (Fig. 4a,b). H89 alone at the concentrations used did not alter the basal level of protein kinase A activity. To further study the role of p38 MAPK in CRH-induced VEGF release, HMC-1 cells were treated with CRH (100 nM) for different time periods and the phosphorylation status of p38 MAPK was analyzed by Western blotting using a commercially available antibody specific for the phosphorylated form of p38 MAPK. The maximal activation of p38 was observed within 5 to 15 min and returned to basal level at 30 to 60 min (Fig. 5a). We failed to detect any ERK or JNK phosphorylation in response to CRH stimulation by Western blot analysis in HMC-1 cells (Fig. 5b,c). Due to the shortage of hCBMC, p38 MAPK activation was analyzed using phospho-specific p38 MAPK ELISA. Phosphorylation of p38 MAPK was enhanced significantly above baseline ( $n=3$ ,  $p<0.05$ ) from 5 to 30 min and decreased to basal level within 60 min (Fig. 5d). The phosphorylation of p38 MAPK in HMC-1 and hCBMC was dose-dependently inhibited by SB203580 (Fig. 6a,b). SB203580 alone at the concentrations used did not alter the

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basal level of p38 MAPK phosphorylation and did not affect cell viability (data not shown). Our results strongly suggest that protein kinase A and p38 MAPK activation is critical for CRH-mediated VEGF release in human mast cells.

**CRH-induced VEGF release is mediated through cAMP/protein kinase A/p38 MAPK signaling pathway.** We investigated whether protein kinase A is a downstream target of cAMP in HMC-1 and hCBMC, and whether CRH-stimulated p38 MAPK activation requires protein kinase A activity. HMC-1 cells and hCBMC were preincubated with SQ22536 for 30 min before stimulation with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) and protein kinase A activity was measured at 3 min. SQ22536 at a concentration of 10  $\mu$ M completely abrogated protein kinase A activity in both cells (Fig. 7a,b). SQ22536 alone at the concentrations used did not alter the basal level of protein kinase A activity and did not affect cell viability (data not shown). Moreover, the effects of CRH on protein kinase A activation and p38 MAPK phosphorylation were mimicked by the cAMP analog, 8-bromo-cAMP (1  $\mu$ M) in both cells (Fig. 8a,b; Fig. 9a,b), suggesting protein kinase A and p38 MAPK as downstream targets of cAMP. Next, we showed that H89 dose-dependently inhibited p38 MAPK phosphorylation in both cells (Fig. 10a,b). H89 alone at the concentrations used did not alter basal level of p38 MAPK phosphorylation and did not affect cell viability (data not shown). Taken together, these results suggest that the cAMP/ protein kinase A/p38 MAPK signaling pathway is key in CRH-induced VEGF release in human mast cells.

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**Effects of CRH on intracellular calcium levels in HMC-1 and hCBMC.** In addition to increased production of cAMP, activation of CRH has also been shown to increase hydrolysis of phosphatidylinositol 4,5-bisphosphate (Xiong et al., 1995; Kiang, 1997), leading to elevated levels of intracellular calcium (Fazal et al., 1998; Wiesner et al., 2003) that was also reported to be involved in VEGF production (Tokuda et al., 2000). Therefore, we examined if CRH could increase calcium in human mast cells. HMC-1 cells were stimulated with 100 nM of CRH followed by 0.5  $\mu\text{g/ml}$  Br-A23178. Only Br-A23187 resulted in an increase of calcium (Fig. 11a). Sensitized hCBMC were stimulated by CRH (1  $\mu\text{M}$ ), followed by anti-IgE (10  $\mu\text{g/ml}$ ). Only anti-IgE resulted in a sustained increase in calcium level (Fig. 11b). These results were confirmed using different concentrations of CRH at different time points (data not shown). Results are presented as arbitrary fluorescence units.

## DISCUSSION

We recently demonstrated that CRH selectively stimulates HMC-1 cells and hCBMC to release newly synthesized VEGF without the release of preformed mediators or other newly synthesized cytokines. This effect was mediated through activation of CRH-R1 and adenylate cyclase with increased intracellular cAMP (Cao et al., 2005). In the present study, we showed that activation of protein kinase A and p38 MAPK are essential steps in CRH-induced VEGF release with the cAMP/protein kinase A/p38 MAPK pathway being the key signaling pathway, without concomitant increase of intracellular calcium levels. To our knowledge, this is the first report clarifying the molecular mechanism of CRH-induced VEGF release in human mast cells. What is particularly unique in these findings is the apparent ability of CRH to stimulate only one signaling pathway without involving other pathways necessary for degranulation and cytokine production.

The effect of CRH on cytokine release has been reported in various cell types. CRH could stimulate IL-1, IL-2 and IL-6 production in human mononuclear cells (Singh and Leu, 1990; Salas et al., 1997), as well as IL-6 and IL-11 release from human HaCaT keratinocytes (Zbytek et al., 2002). CRH binding to CRH-R1 typically activates adenylate cyclase, which leads to increased intracellular concentrations of cAMP and activation of protein kinase A (Grammatopoulos and Chrousos, 2002). For instance, the antiproliferative effects of CRH in human endometrial adenocarcinoma cells is via CRH-R1-mediated activation of the cAMP-protein kinase A pathway (Graziani et al., 2002). CRH regulates the proopiomelanocortin gene expression in AtT20 cells (Aoki et al.,



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1997) and NURR1 gene expression in primary microvascular endothelial cells (McEvoy et al., 2002) through the cAMP-protein kinase A pathway. Our results showed for the first time that CRH induces protein kinase A activation in human mast cells and the specific protein kinase A inhibitor H89 significantly inhibited CRH-induced VEGF release. Moreover, the activation of protein kinase A could be blocked by the adenylate cyclase inhibitor SQ22536 or mimicked by the cell-permeable cAMP analog, 8-bromo-cAMP, supporting the involvement of a cAMP-protein kinase A pathway in VEGF release. Indeed, the role of cAMP-protein kinase A pathway in VEGF production has been reported in other cell types. Prostaglandin E2 induced VEGF expression in a human monocytic cell line (Hoper et al., 1997) and in cultured rat Muller cells (Cheng et al., 1998) via the cAMP-protein kinase A pathway. Leydig cells produced VEGF through protein kinase A activation by a process under gonadotropic control (Anand and Hickey, 1992).

In addition to protein kinase A, it has been demonstrated that CRH can activate MAPK-dependent signaling pathways. CRH was reported to induce the proliferation and release of TNF- $\alpha$  in cultured rat microglia by activation of ERK and p38 MAPK (Wang et al., 2003). CRH downregulated IL-18 expression in human HaCaT keratinocytes by activation of p38 MAPK pathway (Park et al., 2005). Our findings showed for the first time that CRH-induced VEGF release in human mast cells could be abolished by the specific p38 MAPK inhibitor SB203580, but not by the specific inhibitor of MEK PD98059, the upstream kinase of ERK, or the JNK inhibitor SP600125. SB203580 was able to inhibit CRH-induced rapid phosphorylation of p38 MAPK, suggesting a critical

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role of p38 MAPK in CRH-mediated VEGF release. Indeed, other groups have reported the involvement of the p38 MAPK signaling pathway in VEGF production in other cell types, including human ovarian carcinoma cells in response to hypoxia (Xu et al., 2004), as well as osteoblasts in response to TGF- $\beta$  (Tokuda et al., 2003a) or bone morphogenetic protein-4 (Tokuda et al., 2003b). The p38 MAPK pathway controls the expression of many genes by phosphorylating and enhancing the activity of multiple transcription factors, such as ATF-2, CREB, Sp-1 and NF- $\kappa$ B (Kyriakis and Avruch, 2001). Multiple regulatory elements have been found in the promoter region of human VEGF gene, including the binding sites for transcription factors Sp-1, AP-1, AP-2, NF- $\kappa$ B and CREB (Loureiro and D'Amore, 2005). Therefore, p38 MAPK could regulate VEGF gene expression at the transcriptional level by activation of one or some of these transcription factors. Interestingly, VEGF can also be regulated at the level of mRNA stability (Dibbens et al., 1999). It was shown that activation of the p38 MAPK increased the stability of the VEGF mRNA in some cell types, an action presumably mediated through the AU-rich region of its 3' untranslated region (Pages et al., 2000). Therefore, p38 MAPK could also regulate VEGF gene expression by increasing VEGF mRNA stability in human mast cells. Further investigation is undergoing in our laboratory to address these questions.

MAPKs have been shown to be substrates of protein kinase A in osteoblast-like cells (Kakita et al., 2004) and PC12 cells (Park et al., 2000). In this study, we extended the findings to human mast cells and showed that protein kinase A is required for CRH-induced p38 MAPK activation and CRH-induced VEGF release is mediated through the

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cAMP/protein kinase A/p38 MAPK signaling pathway. It has been reported that CRH could also increase intracellular levels of calcium in some cell types (Fazal et al., 1998; Wiesner et al., 2003). However, we failed to detect any increase of calcium in both HMC-1 and hCBMC. The selective activation of p38 MAPK without the involvement of ERK, JNK or calcium by CRH differs from the mast cell signaling mediated by the high-affinity IgE receptor; in the latter case there is broad activation of the ERK, JNK and p38 MAPK, as well as increased levels of intracellular calcium that leads to mast cell degranulation, as well as *de novo* synthesis and release of both cytokines and arachidonic acid metabolites (Siraganian, 2003). Therefore, our results, could explain, at least in part, why CRH selectively stimulates VEGF production in human mast cells without degranulation and other cytokine production. The potential mechanism for the selective activation of p38 MAPK could involve activation by CRH of other CRH receptor isoforms that inhibit other signaling pathways. For instance, a soluble CRH-R1 isoform, CRH-R1e, was shown to attenuate CRH-R1 $\alpha$ -coupled cAMP production stimulated by CRH in COS cells (Pisarchik and Slominski, 2004). A soluble splice variant of CRH-R2 $\alpha$  (sCRH-R2 $\alpha$ ) dose-dependently inhibited the cAMP response to CRH and its structurally related peptide urocortin in HEK293T cells transfected with mouse CRH-R2 $\alpha$  (Chen et al., 2005). Moreover, sCRH-R2 $\alpha$  inhibited the activation by urocortin of ERK in CATH.a cells, which endogenously express CRH-R1 and CRH-R2 $\alpha$  (Chen et al., 2005).

It should be pointed out that CRH induced a relative modest, but significant increase (~30% increase) of VEGF release from hCBMC compared to that from HMC-1 cells (~100% increase). The extent of VEGF increase in this study is in agreement with

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the previous report showing that immunologic stimulation of hCBMC through FcεRI cross-linking by anti-IgE resulted in a small, but significant induction of VEGF secretion as compared with untreated controls (Boesiger et al., 1998). Hence, CRH stimulation of hCBMC for VEGF release parallels that of immunologic stimulus. In addition, our recent findings show that forskolin and 8-bromo-cAMP mimic the effect of CRH on VEGF release in hCBMC by inducing a modest increase of VEGF secretion (~40% increase), whereas both agents induce much higher increase of VEGF release (~10 fold) from HMC-1 cells (Cao et al., 2005). These observations indicate that the VEGF production by neoplastic mast cell lines in response to some stimuli can greatly exceed those produced by the corresponding nonneoplastic cells. This property of neoplastic mast cells could explain why patients with mastocytosis are more susceptible to develop vascular abnormalities which can be worsen during inflammation (Escribano et al., 2002). The VEGF release from other cell types and in response to other stimuli have been documented. Human eosinophils also spontaneously release VEGF that is upregulated (~100% increase) by granulocyte-macrophage colony-stimulating factor and IL-5 (Horiuchi and Weller, 1997). Macrophage colony-stimulating factor induces freshly isolated normal human monocytes to produce and release VEGF in a dose-dependent manner, with a maximum of 5-fold more VEGF release at 5 days in culture (Eubank et al., 2003). UV radiation and prostaglandin E2 can increase VEGF release from cultured human dermal fibroblasts by 3-fold after treatment for 24 h (Trompezinski et al., 2001). The extent of the increase of VEGF release varies from cell to cell and depends on specific stimulus.

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This is the first report showing that cAMP/protein kinase A/p38 MAPK pathway is the key signaling pathway mediating the effect of CRH on selective VEGF release in human mast cells, thereby providing insight into the molecular mechanism of how CRH affects the release of a key proinflammatory mediator. There has been increasing evidence indicating that mast cells are involved in inflammatory diseases worsened by stress where degranulation is not necessarily present (Bienenstock, 2002; Theoharides and Cochrane, 2004), many of which are associated with increased VEGF expression. For instance, increased and activated mast cells have been documented in the bladder of patients with interstitial cystitis (Theoharides et al., 2001), an inflammatory condition worsen by stress recently associated with increased bladder VEGF expression (Tamaki et al., 2004). Mast cells are also increased in psoriasis, the symptoms of which worsen by stress (Katsarou-Katsari et al., 1999). There is increased VEGF expression in psoriatic skin (Detmar et al., 1994) and VEGF levels from psoriatic plaques correlated with the extent of clinical symptoms (Bhushan et al., 1999). Moreover, overexpression of VEGF in transgenic mice led to a skin condition resembling psoriasis (Xia et al., 2003). Our findings, therefore, provide the molecular basis for explaining and possibly developing new approaches for the treatment of inflammatory diseases, especially those affected by stress (Theoharides et al., 2004).

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## FOOTNOTES

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### Correspondence and reprints to:

T.C. Theoharides, Ph.D., M.D.  
Department of Pharmacology and Experimental Therapeutics  
Tufts University School of Medicine  
136 Harrison Avenue  
Boston, MA 02111, USA  
Phone: (617) 636-6866  
Fax: (617) 636-2456  
E-mail: [theoharis.theoharides@tufts.edu](mailto:theoharis.theoharides@tufts.edu)

## LEGENDS TO FIGURES

**Figure 1. Effect of H89 on CRH-induced VEGF release in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 96-well plates and preincubated with protein kinase A inhibitor H89 at the indicated concentrations for 30 min and were then stimulated with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for another 6h (HMC-1) or 24 h (hCBMC) in the presence or absence of H89 in complete culture media. Data are the mean  $\pm$  SEM (n=4). \*  $p$ <0.05 versus control, \*\*  $p$ <0.05 versus CRH.

**Figure 2. Effect of SB203580 on CRH-induced VEGF release in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 96-well plates and preincubated with the p38 MAPK inhibitor SB203580 at the indicated concentrations for 30 min and were then stimulated with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for another 6h (HMC-1) or 24 h (hCBMC) in the presence or absence of SB203580 in complete culture media. SB, SB203580. Data are the mean  $\pm$  SEM (n=4). \*  $p$ <0.05 versus control, \*\*  $p$ <0.05 versus CRH.

**Figure 3. CRH induces activation of protein kinase A in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 48-well plates and serum-starved overnight. Next day, cells were stimulated with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for 3 min. Cell lysates were prepared as described in *Materials and Methods*. The protein kinase A activities were measured by ELISA according to the manufacturer's instruction. Data are the mean  $\pm$  SEM (n=3). \*  $p$ <0.05 versus control.

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**Figure 4. Effect of H89 on CRH-stimulated activation of protein kinase A in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 48-well plates and serum-starved overnight. Next day, cells were preincubated with the protein kinase A inhibitor H89 at the indicated concentrations for 30 min before stimulation with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for 3 min in the presence or absence of H89 in serum-free culture media. Cell lysates were prepared as described in *Materials and Methods*. The protein kinase A activities were measured by ELISA according to the manufacturer's instruction. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control.

**Figure 5. CRH induces p38 MAPK phosphorylation in HMC-1 and hCBMC.** HMC-1 cells were plated in 6-well plate and serum-starved overnight. Next day, cells were stimulated with CRH for indicated times (100 nM), or stimulated with PMA (10 nM) for 5 min as a positive control for ERK activation, or IL-1 (10 ng/ml) for 10 min as a positive control for JNK activation. Cell lysates were subjected to 10% SDS-PAGE and immunoblotted with phospho-p38 (Thr180/Tyr182) (a, upper panel), phospho-ERK (Thr202/Tyr204) (b, upper panel; +, PMA) or phospho-JNK (Thr183/Tyr185) (c, upper panel; +, IL-1) specific antibody. Membranes were stripped and reprobed with total p38 (a, lower panel), total ERK (b, lower panel) or total JNK (c, lower panel) antibody. The results are representative of at least three independent experiments. (d) hCBMC were distributed to 48-well plates and serum-starved overnight. Next day, cells were stimulated for indicated times with CRH (1  $\mu$ M). Cell lysates were then prepared and activation of p38 MAPK was determined by quantifying the amounts of the phosphorylated and total form of kinase using ELISA. Data in each sample were normalized against the total

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amount of kinase and expressed as fold induction. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control.

**Figure 6. Effect of SB203580 on CRH-induced p38 MAPK phosphorylation in HMC-1 and hCBMC.** (a) HMC-1 cells were plated in 6-well plate and (b) hCBMC were distributed to 48-well plates and serum-starved overnight. Next day, cells were pretreated with SB203580 at the indicated concentrations for 30 min before stimulation with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) in the presence or absence of SB203580 for another 5 min. Cell lysates were subjected to 10% SDS-PAGE (HMC-1) or ELISA (hCBMC). SB, SB203580. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control, \*\*  $p < 0.05$  versus CRH.

**Figure 7. Effect of SQ22536 on CRH-stimulated protein kinase A activation in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 48-well plates and serum-starved overnight. Next day, cells were preincubated with SQ22536 at the indicated concentrations for 30 min before stimulation with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) for 3 min in the presence or absence of SQ22536 in serum-free culture media. Cell lysates were then prepared and assayed for protein kinase A activity using ELISA. SQ, SQ22536. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control, \*\*  $p < 0.05$  versus CRH.

**Figure 8. 8-bromo-cAMP induces activation of protein kinase A in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 48-well plates and serum-starved



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overnight. Next day, cells were stimulated with 8-bromo-cAMP (1 mM) for 3 min in the serum-free culture media. Cell lysates were then prepared and assayed for protein kinase A activity. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control.

**Figure 9. 8-bromo-cAMP induces p38 MAPK phosphorylation in HMC-1 and hCBMC.** (a) HMC-1 and (b) hCBMC were plated in 48-well plates and serum-starved overnight. Next day, cells were stimulated with 8-bromo-cAMP (1 mM) for 5 min in the serum-free culture media. Cell lysates were then prepared and activation of p38 MAPK was determined by quantifying the amounts of the phosphorylated and total form of kinase using ELISA. Data in each sample were normalized against the total amount of kinase and expressed as fold induction. Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control.

**Figure 10. Effect of H89 on CRH-induced p38 MAPK phosphorylation in HMC-1 and hCBMC.** (a) HMC-1 cells were in 6-well plate and (b) hCBMC were distributed to 48-well plates and serum-starved overnight. Next day, cells were pretreated with H89 at the indicated concentrations for 30 min before stimulation with CRH (100 nM for HMC-1 or 1  $\mu$ M for hCBMC) in the presence or absence of H89 for another 5 min. Cell lysates were then prepared and subjected to 10% SDS-PAGE (HMC-1) or ELISA (hCBMC). Data are the mean  $\pm$  SEM (n=3). \*  $p < 0.05$  versus control.

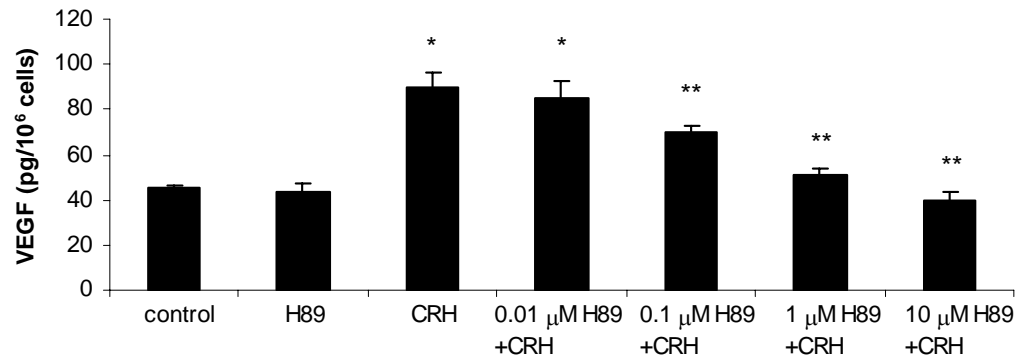
**Figure. 11. Effect of CRH on intracellular calcium levels.** (a) HMC-1 cells were stimulated with 100 nM CRH followed by 0.5  $\mu$ g/ml Br-A23187. (b) hCBMC were

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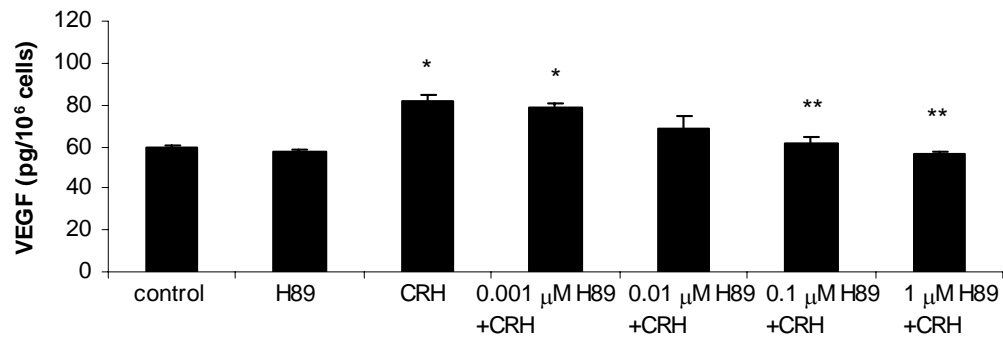
stimulated by CRH (1  $\mu$ M) followed by anti-IgE (10  $\mu$ g/ml). Results are presented as arbitrary fluorescence units.

**Figure 1.**

**a HMC-1**

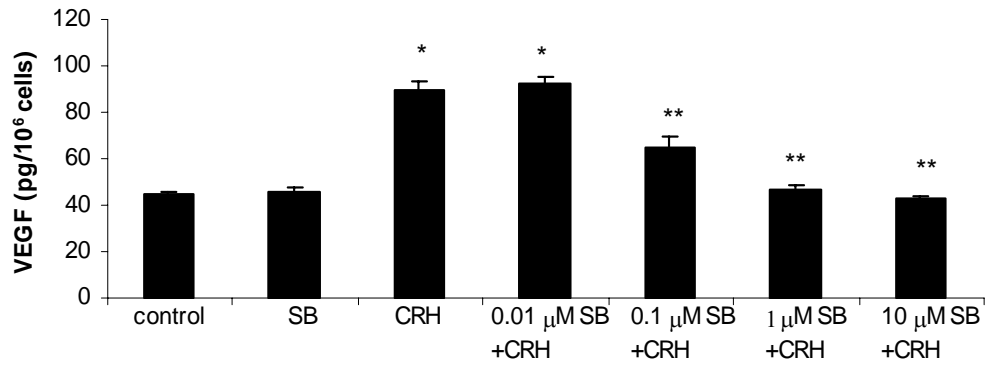


**b hCBMC**

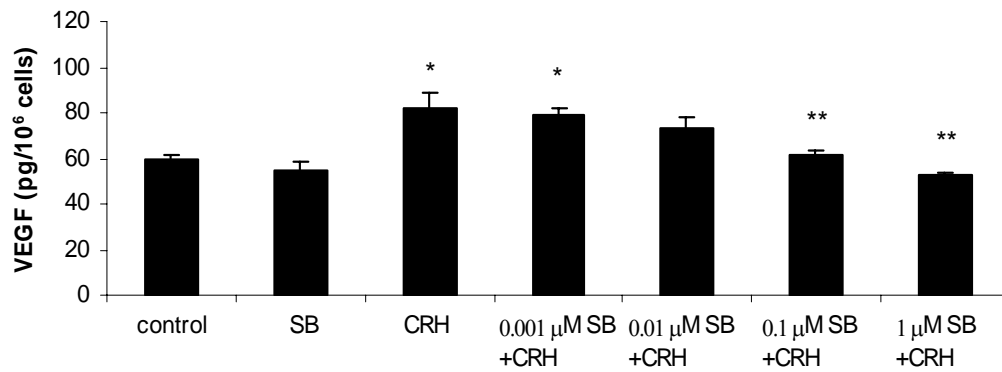


**Figure 2.**

**a HMC-1**

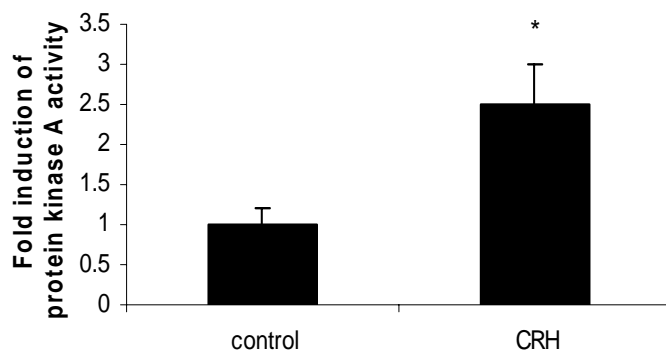


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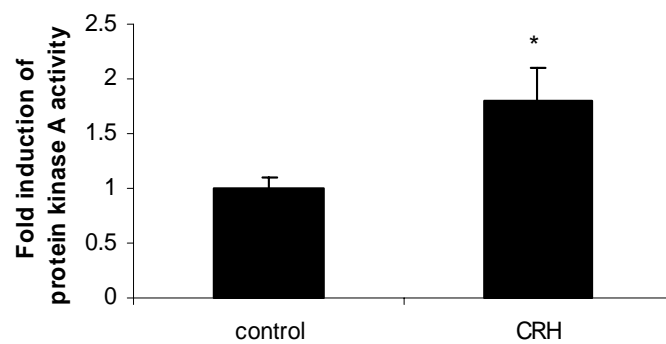


**Figure 3.**

**a HMC-1**

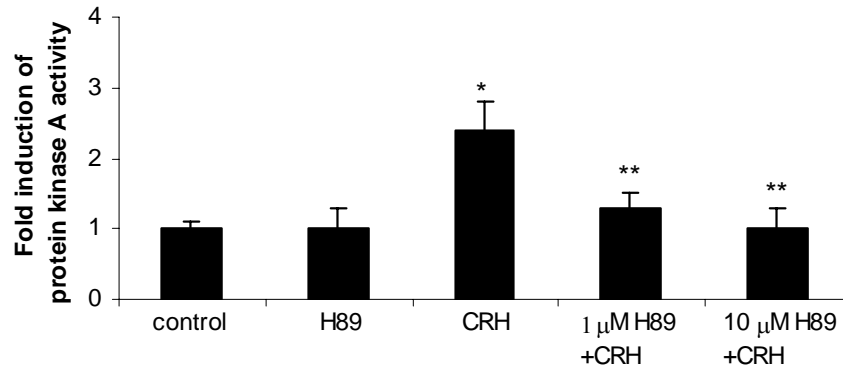


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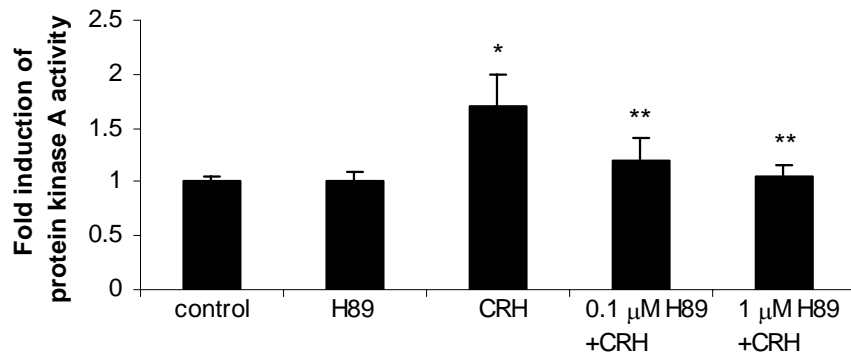


**Figure 4.**

**a HMC-1**



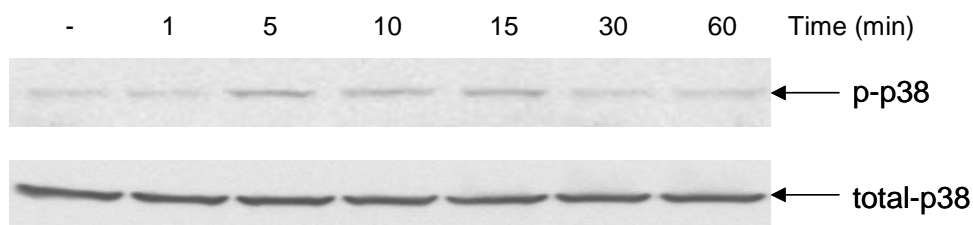
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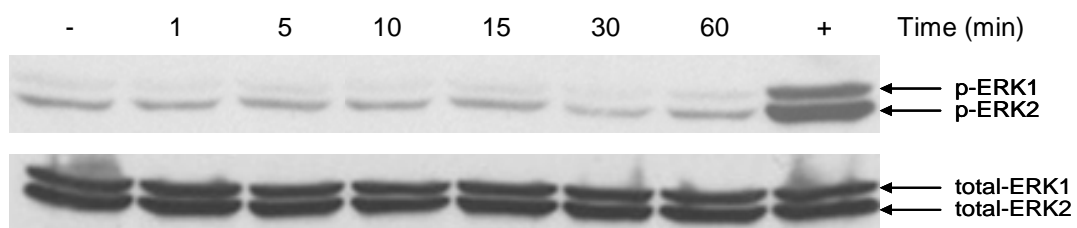
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**Figure 5.**

**a HMC-1**

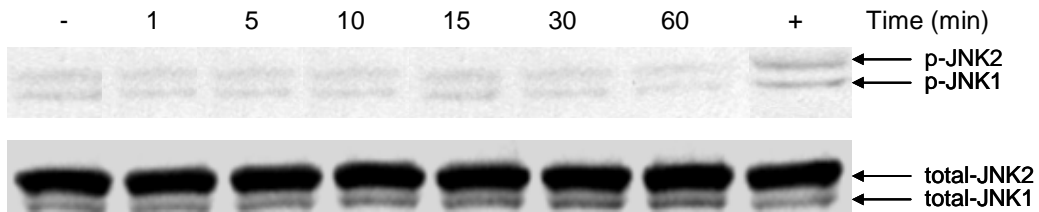


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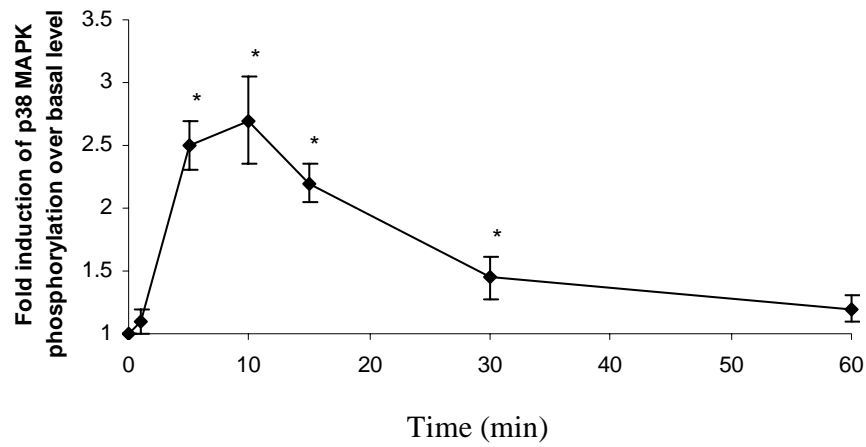


**Figure 5.**

**c HMC-1**



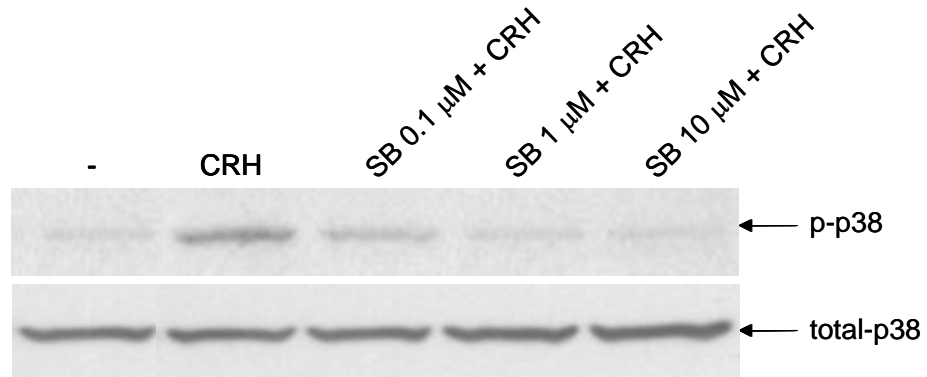
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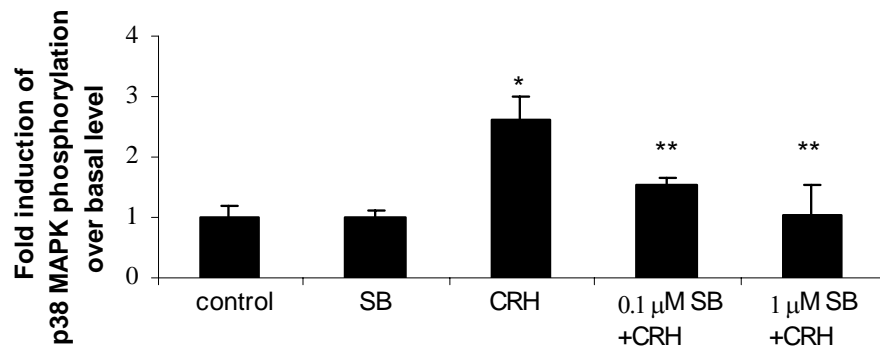


**Figure 6.**

**a HMC-1**

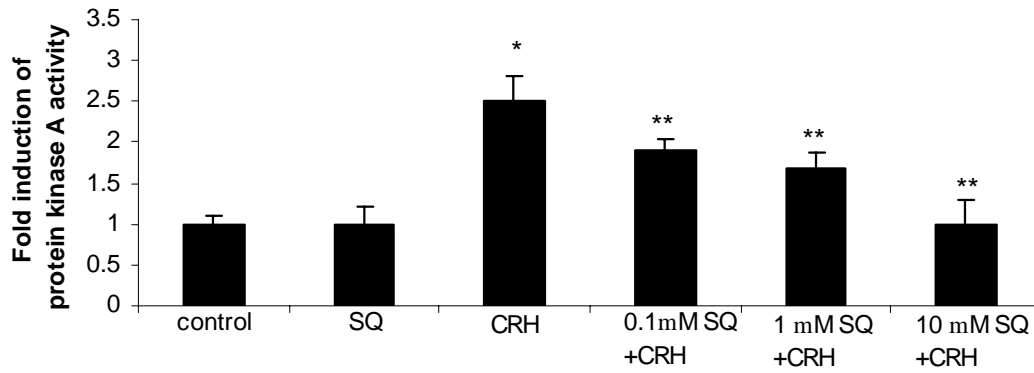


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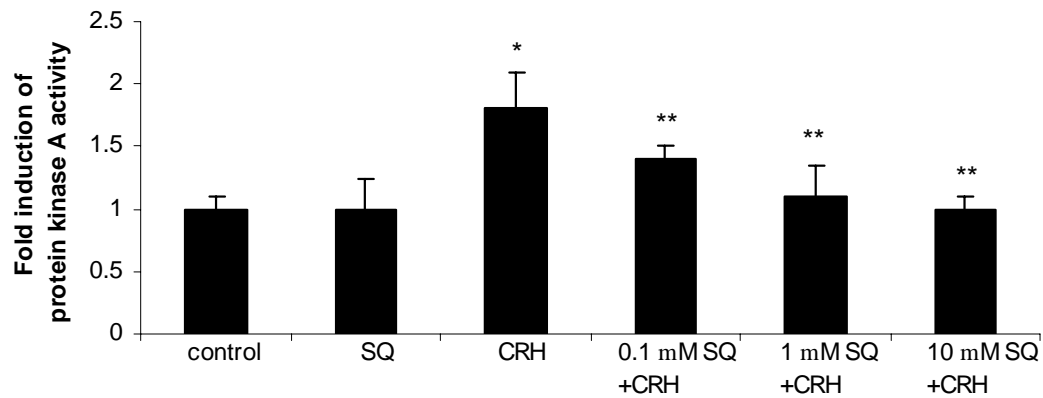


**Figure 7.**

**a HMC-1**

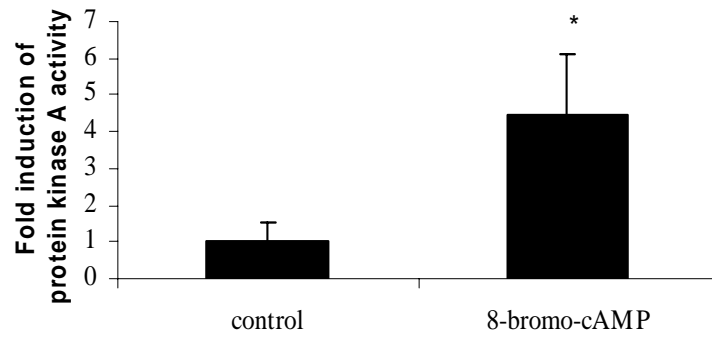


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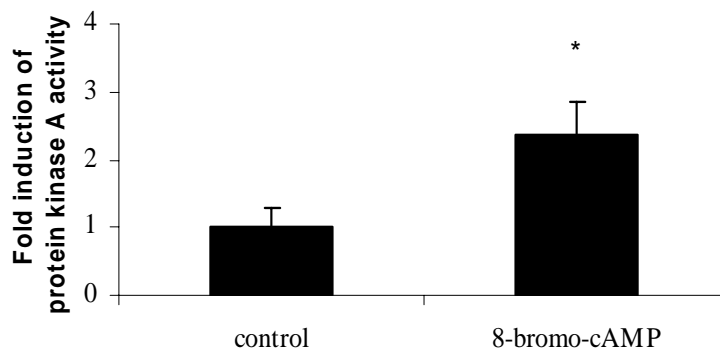


**Figure 8.**

**a HMC-1**

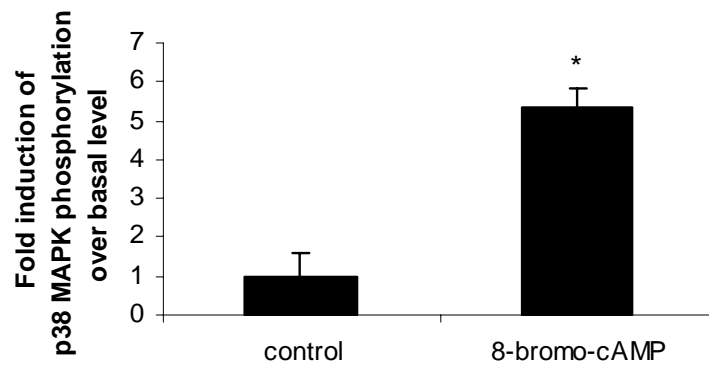


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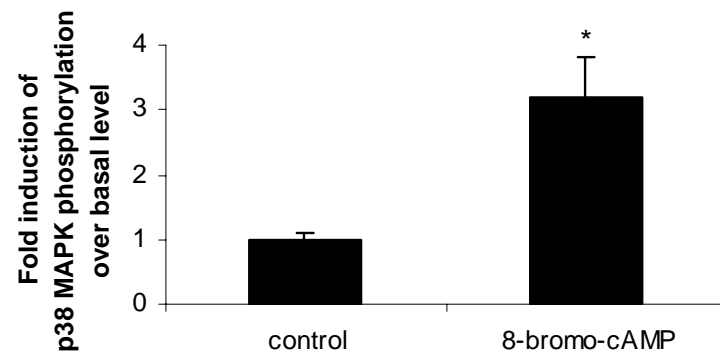


**Figure 9.**

**a HMC-1**

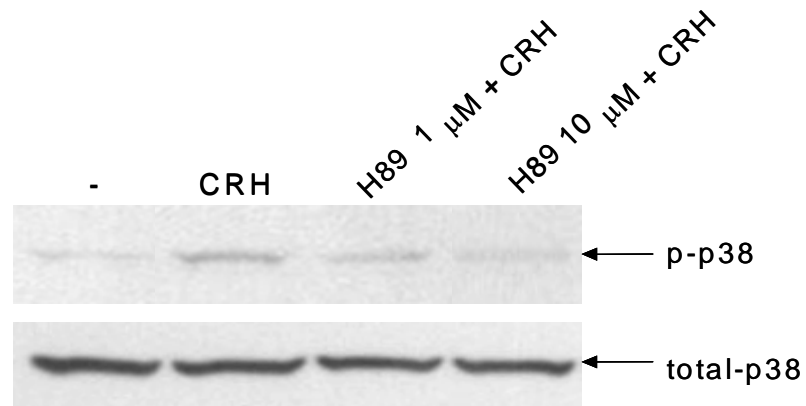


**b hCBMC**

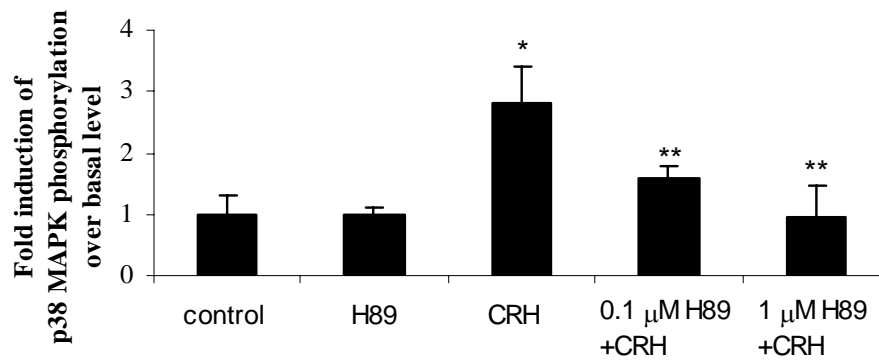


**Figure 10.**

**a HMC-1**

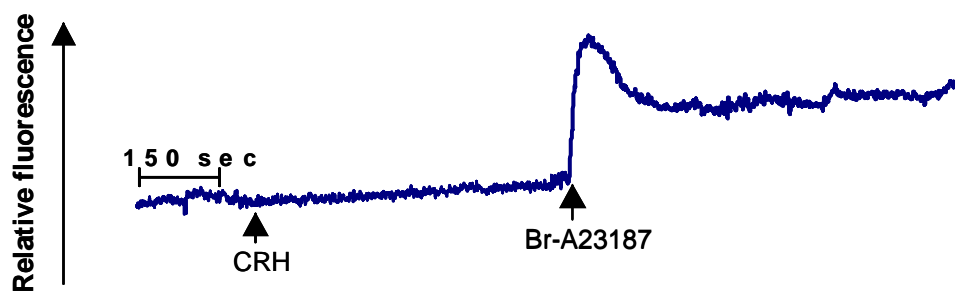


**b hCBMC**



**Figure 11.**

**a HMC-1**



**b hCBMC**

