Molecular Immunology 47 (2010) 1426–1437

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Histamine modulation of peripheral CRH receptor type 1α expression is dependent on Ca^{2+} signalling and NF-κB/p65 transcriptional activity

Davide Zocco[a,b], Jason P. McMorrow[a,b], Evelyn P. Murphy[a,b,*,1]

[a] College of Life Sciences, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland
[b] UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

ARTICLE INFO

Article history:
Received 16 December 2009
Received in revised form 16 February 2010
Accepted 19 February 2010
Available online 15 March 2010

Keywords:
Histamine
Corticotropin-releasing hormone receptor 1
Inflammatory arthritis
Calcium
NF-κB
Promoter activity
Gene expression

ABSTRACT

Histamine promotes immune complex-induced vascular leakage *in vivo*, a critical and early event that leads to joint-specific autoimmune damage. Initial assessment, using explanted human synovial tissue (ST), indicates that histamine can modulate local expression of type 1 alpha CRH receptors (CRH-R1α). The objective of this study was to elucidate the signalling events and transcriptional mechanism(s) controlling histamine-dependent regulation of CRH-R1α expression in human inflammatory arthritis. Histamine significantly promotes CRH-R1α mRNA and protein expression in a time- and concentration-dependent manner in human endothelial and synoviocyte cells. Transactivation of the human CRH-R1 promoter is significantly enhanced by histamine which can be mimicked by treatment with a Ca^{2+} ionophore and completely diminished in the presence of a Ca^{2+} chelator. Histamine-mediated responses involve enhanced activation and nuclear localisation of transcription factors including CREB, NF-κB and NR4A2. Functional consequences of enhanced CREB, NF-κB and NR4A2 activity confirm that NF-κB/p65 selectively controls CRH-R1 promoter activity. Co-transfection of NF-κB/p65 potently transactivates the CRH-R1 promoter while co-expression of a dominant negative IκBα kinase inhibits endogenous and histamine-induced promoter activity. Bioinformatic analysis identifies three putative κB consensus binding sites at proximal and distal positions and 5′ deletional analysis identifies promoter region(s) required for activation by histamine and NF-κB/p65. We observe direct NF-κB/p65 interaction within the promoter region and site-directed mutagenesis reveals that all three κB sites are required to mediate histamine and NF-κB/p65 regulation of CRH-R1 promoter activity. These findings confirm that histamine, via enhanced Ca^{2+} signalling and NF-κB/p65 activity, contributes to changes in ST inflammation by promoting CRH-R1α-mediated responses.

1. Introduction

In rheumatoid arthritis (RA) mast cells are central players in perpetuating inflammatory responses, particularly in early stages of disease where these cells may contribute to the initiation of the inflammatory process (Nigrovic and Lee, 2007). The leading edge of pannus, and particularly the sublining layer of active RA synovium, is populated with excessive numbers of mast cells when compared to normal synovial tissue (ST). Mast cell-deficient mice are resistant to serum-induced inflammatory arthritis in the K/BxN mouse model (Ditzel, 2004; Lee et al., 2002). Stem cell factor (SCF), a potent mast cell chemotaxin and ligand for c-Kit, is a key regulator of mast cell proliferation, survival, differentiation and degranulation (Reber et al., 2006). SCF is up-regulated in chronic inflammatory conditions and inhibition of the SCF/c-Kit pathway leads to a significant reduction in histamine levels and mast cell infiltration both *in vitro* and *in vivo* (Reber et al., 2006). Recent targeting of SCF/c-Kit pathway in RA using Masitinib, an inhibitor of c-Kit, provides emerging evidence of significant therapeutic and clinical benefits *in vivo* (Tebib et al., 2009).

Mast cell activation leads to degranulation with the concomitant release of mediators including histamine, corticotropin-releasing hormone (CRH) and pro-inflammatory cytokines. These agents promote inflammation by inducing vascular leakage, leukocyte recruitment, and activation of synovial macrophage and fibroblast-like synoviocyte cells (Nigrovic and Lee, 2007). IL-33, a member of the IL-1 family of cytokines, is over-expressed in RA ST and has recently been shown to promote inflammation via mast cell activation (Xu et al., 2008). Following IL-33 stimulation, activated mast cells enhance the production of pro-inflammatory cytokines including IL-1β, IL-6, IL-13 and GM-CSF. IL-33-induced mediators drive local hyper-inflammation via recruitment of further immune cells into ST providing evidence that mast cells may play an early

* Corresponding author at: UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland. Tel.: +353 1 7166215; fax: +353 1 7166219.
E-mail address: evelyn.murphy@ucd.ie (E.P. Murphy).
[1] Supported by grants from the Health Research Board of Ireland and Science Foundation Ireland to EPM.

0161-5890/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.molimm.2010.02.012

and critical role in promoting a transition from an acute to a chronic phase of joint disease (Xu et al., 2008).

In RA, the effects of histamine are associated with histamine receptors HR1, HR2 and more recently HR4 (Adlesic et al., 2007; Binstadt et al., 2006; Grzybowska-Kowalczyk et al., 2007). In the K/BxN model of serum-induced inflammatory arthritis, histamine promotes joint-localised vascular permeability leading to organ-specific autoimmune damage and recent results confirm that histidine decarboxylase deficiency protects against K/BxN-induced arthritis (Ditzel, 2004; Nigrovic and Lee, 2007; Rajasekaran et al., 2009). Histamine, *via* HR1 and intracellular Ca^{2+} release, can induce changes in the adherent junctions and cytoskeleton of microvascular endothelial cells, leading to vascular leakage and HR1-specific antagonists have been shown to potently alter vascular permeability (Tiruppathi et al., 2002). Histamine modulates, via enhanced activation of several transcription factors including NF-κB, NF-AT, AP-1 and CREB, the expression of adhesion molecules, cytokines and prostaglandins, exacerbating microvasculature inflammation (Adlesic et al., 2007; Bakker et al., 2001b; Matsubara et al., 2005). Our recent findings reveal that, in an *ex vivo* model of RA, histamine through HR1, modulates CRH receptor subtype 1 alpha (CRH-R1α) expression suggesting that histamine may promote vascular permeability in a CRH receptor-mediated fashion (Ralph et al., 2007).

The role of CRH and CRH receptors in regulating peripheral inflammation has been established both *in vitro* and *in vivo* (Karalis et al., 1999; McEvoy et al., 2001; Webster et al., 2002). Linkage and association results for CRH provide evidence that genetic variation of the CRH locus may be a risk factor for the development of RA (Fife et al., 2000). Elevated levels of CRH mRNA and protein are produced locally and regulated by pro-inflammatory cytokines in synovial cells (Murphy et al., 2001). CRH-R1-mediated responses in human endothelial and synoviocyte cells involve enhanced transcriptional activity of several transcription factors, including CREB, NF-κB and NR4A2 (McEvoy et al., 2004, 2002a). Interestingly, in early RA, aberrant CRH-R1α expression co-localises with E-selectin and PECAM-1, markers of activated endothelium and CRH-R1α expression promotes CRH-dependent production of endothelial nitric oxide (Ralph et al., 2007). Studies using a rat model of adjuvant-induced arthritis demonstrate that chronic treatment with a CRH-R1 antagonist significantly reduces inflammation-induced degeneration of synovia, cartilage and bone in arthritic joints (Webster et al., 2002). However, despite the growing evidence that CRH-R1α expression is associated with the development and clinical symptoms of RA, the signalling events and transcriptional mechanisms regulating peripheral CRH-R1α expression in the inflamed joint remain unknown.

In this study, we establish that CRH-R1α is a molecular target of histamine action in human endothelial and synoviocyte cells and demonstrate, for the first time, that histamine modulation of CRH-R1α expression is dependent on Ca^{2+} signalling and enhanced NF-κB transcriptional activity. These findings indicate that, during the early phase of inflammation, histamine regulates changes in ST through enhanced CRH-R1-mediated responses.

2. Materials and methods

2.1. Tissue culture

HMEC-1 cells are an established human microvascular cell line immortalized with SV40 antigen (Ades et al., 1992). Cells were maintained in EBM® media supplemented with 5% FCS (10 ng/mL), gentamicin (50 μg/mL), amphotericin-B (50 μg/mL), and hydrocortisone (Biowhittaker Inc., Clonetics Products). K4 IM synoviocyte cells are an established human synoviocyte cell line derived from a healthy donor and immortalized with SV40 antigen. The K4 IM synoviocyte cell line was provided by Professor H. Eibel (Clinical Research Unit for Rheumatology, University Hospital, Freiburg, Germany) and grown as previously described (Haas et al., 1997).

2.2. Reagents and cell treatments

K4 IM synoviocyte and HMEC-1 cells were cultured in T25-cm^2 culture flasks (Greiner) and grown to 80% confluency and maintained in serum free medium for 24 h before treatments. Histamine (Sigma) was resuspended according to the manufacturer's instructions and included at the concentrations indicated. For transient transfection experiments, K4 IM synoviocyte and HMEC-1 cells were grown in 12-well plates and stimulated with histamine, ionomycin or phorbol 12-myristate 13-acetate (Calbiochem) for 24 h. To block calcium signalling, cells were left untreated or treated with the calcium chelator 2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA; Calbiochem) for 45 min and left untreated or treated with histamine or ionomycin. For calcium-free experiments, cells were left untreated or treated with histamine or ionomycin for 6 h in calcium-free media. Vehicles used for these treatments were water or DMSO which showed no effect on CRH-R1 transcription or promoter activity.

2.3. Synthesis of cDNA and polymerase chain reaction (PCR)

Complementary DNA (cDNA) was prepared by reverse transcription of 1 μg of each total RNA sample as described previously (McEvoy et al., 2001). PCR was performed in 50 μl volumes containing 2 μl of cDNA reaction mixture, 1× PCR buffer II, 1.25 mM each of dATP, dCTP, dGTP, dTTP and 2.5 U AmpliTaq Gold (Perkin Elmer), 1.0 mM $MgCl_2$ (for CRH-R1α and GAPDH) and 200 ng of sense and antisense primers (for CRH-R1α) and 100 ng of sense and antisense primers (for GAPDH). The concentrations of $MgCl_2$ and primer, along with annealing temperature have been previously validated and reported (Lammi et al., 2004; McEvoy et al., 2001). Each PCR sample underwent a 30-cycle amplification, to ensure that the reactions had not reached the plateau phase of amplification. PCR products were electrophoresed on a 1.5% agarose gel and visualised using ImageMaster system (ImageMaster VDS, Amersham Biosciences). Quantitative real-time RT-PCR was performed in triplicate reactions of 10 μl volumes with 1 μl SYBR Green I Master Mix (Roche), 0.25 μM of each primer and 40 ng of cDNA using a Roche Lightcycler or an ABI 7300 thermocycler (Applied Biosystems). NR4A1-2 expression levels were measured using TaqMan Assay-on-Demand real-time primer sets (Applied Biosystems). Relative expression levels of target genes were determined using the $2^{-(\mathrm{ddc}(t))}$ method with GAPDH as a control.

2.4. Immunofluorescence analysis

After the treatment, HMEC-1 cells were air dried for 15 min, fixed in 1% paraformaldehyde and blocked with diluted normal horse serum (Vector Laboratories) for 1 h. Cells were incubated overnight with an anti-CRH-R1 goat polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 in normal horse serum. Following primary antibody incubation, cells were washed with 1× PBS and diluted biotinylated anti-goat secondary antibody (Vector Laboratories) was added for 25 min. Following removal of the secondary antibody, a Cy3 fluorochrome conjugated anti-biotin antibody (BN-134, 1.2 mg/mL, 1:100, Sigma) was added and incubated in the dark for 30 min, to allow binding to the biotinylated secondary antibody. Cells were washed in 1× PBS and mounted using VECTASHIELD® mounting media with DAPI. Images were acquired using a Zeiss Axioplan 2 imaging microscope at 100×.