REGULATION OF CHEMOKINE EXPRESSION IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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ABSTRACT

Asthma is an inflammatory disease of the airways characterised by airway remodelling and hyperresponsiveness. New treatments are needed for patients with severe asthma whose disease is not controlled with currently available therapies. Asthma pathophysiology is complex, however, accumulating evidence suggests multiple inflammatory pathways in asthma converge onto a relatively small number of downstream targets that may be of therapeutic interest. These include mitogen activated protein kinases (MAPKs), the pro-inflammatory transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) and transcriptional regulators such as histone acetyl transferases (HATs) and histone deacetylases (HDACs). Chemokines are molecules secreted at sties of inflammation, attracting inflammatory cells and perpetuating the inflammatory response. Here we studied the mechanisms by which the pro-inflammatory mediator endothelin-1 (ET-1) and the cytokine tumour necrosis factor-α (TNF-α) promoted expression by primary human airway smooth muscle cells (HASMC) of two important chemokines, monocyte chemotactic protein-1 (MCP-1) and eotaxin. Further, we studied the mechanisms by which existing asthma therapies (long acting beta agonists (LABA) and glucocorticoids) modulated TNF-α-stimulated eotaxin expression. Endothelin-1 stimulated MCP-1 release through a transcriptional mechanism involving NF-κB and AP-1; the upstream signalling pathway involved p38 and p44/p42 MAPKs. Previously, this lab showed that TNF-α-induced eotaxin release is also NF-κB-dependent, involving histone H4 acetylation at the eotaxin promoter. Here we found that TNF-α-induced eotaxin release does not involve histone H3 acetylation, and that TNF-α-dependent histone H4

acetylation does not occur through alterations in total HDAC activity or levels of the key HDACs -1 and -2. Similarly, modulation of TNF-α effects on eotaxin expression by glucocorticoids and LABA is independent of total HDAC activity and HDAC-1 and -2 levels. These studies support the body of evidence suggesting that multiple inflammatory pathways in asthma converge onto a small number of downstream targets, and are relevant to the understanding and treatment of asthma.

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Sutcliffe AM*, Corbett LM, Pang L and Knox AJ. Endothelin-1 stimulates interleukin-8, eotaxin and monocyte chemotactic protein-1 release by cultured human airway smooth muscle cells. 2004 American Thoracic Society Conference (poster).

* My maiden name was Sutcliffe

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CHAPTER 1: INTRODUCTION

1.1 Asthma

Asthma is an inflammatory disease of the airways characterised by airway hyperresponsiveness (AHR) and airway remodelling. AHR leads to variable airflow obstruction, manifest clinically by symptoms such as cough, wheezing, breathlessness and chest tightness. The disorder can range in severity through a continuum from mild and intermittent to persistent and severe asthma that may be life-threatening. Typically, inhaled spasmogens trigger an early asthmatic response characterised by bronchoconstriction, oedema of the airway wall and increased mucus secretion, which typically resolves within 2 to 3 hours. This is often followed by a late asthmatic response characterised by a longer period of bronchospasm, maximal between 6 and 12 hours and associated with an influx of inflammatory cells in the airways.

Asthma affects around 5.2 million adults in the UK, and its prevalence is increasing (1). It costs the NHS £889 million and the UK economy a total of £2.3 billion per annum. Over 1400 people died from asthma in the UK in 2002 (1). The causes for asthma remain obscure but it is known that both genetic and environmental influences play a part. At the airway level, the clinical syndrome of asthma is brought about by a complex interplay between a network of multiple inflammatory and structural airway cells. These cells produce a vast array of biologically active molecules, including cytokines, chemokines, mediators, angiogenic factors, growth factors and matrix modifying enzymes, that act in an autocrine and paracrine manner to initiate and perpetuate airway inflammation and remodelling.

1.2 The immune response in asthma

Airway inflammation is fundamental to the pathogenesis of asthma. The initial trigger to this cascade of inflammatory responses is sensitisation to inhaled allergen. Allergen is taken up and processed by antigen presenting dendritic cells residing under the airway epithelium, via IgE bound to high affinity cell surface receptors. Following engagement with antigen, dendritic cells migrate to local lymphoid tissue. There, they present processed antigen, loaded onto MHC class II HLA molecules, to naïve T cells residing within the lymphoid follicle. This results in activation of the T-cell specific to that antigen, through co-stimulatory signals mediated by the interaction of CD28 on the surface of the T cell with CD80 (HLA B 7.1) or CD86 (B7.2) on the antigen presenting cell (APC) (2, 3). The activated T-cell subsequently expresses IL-2 receptors, and proliferation (clonal expansion) is triggered by release of IL-2 from the APC (4). Activated T-cells migrate back to the asthmatic airway under the influence of chemokines (5).

Activation of T cells by antigen leads to their maturation into one of several different functional subsets, described below, dependent on their cluster differentiation (CD) marker status, and the inflammatory milieu. $CD4^+$ cells have the capacity to differentiate into T helper 1 (Th1), T helper 2 (Th2), regulatory T cells (Treg) or Th17 cells. $CD8^+$ cells develop into cytotoxic T cells (Tc). The level of IL-12 secretion by dendritic cells affects the balance of Th1 versus Th2 responses, IL-12 shifting the balance towards the Th1 response (6). Asthma is characterised by a predominantly Th2 phenotype (7).

Th2 cells are characterised by the production of IL-4, IL-5 and IL-13, and the presence of CCR4 and CCR8 receptors on their cell surface (4, 8). Their physiological role is to protect against extracellular parasites through interaction with, and stimulation of antibody production by, B cells. Human and animal studies support the concept of a Th2 dominant response in asthmatic airway inflammation. Th2 cytokines are elevated in asthmatic patients at baseline and after subsegmental allergen challenge ((8) and references therein). The Th2 cytokine IL-13 is overexpressed in patients with asthma (9, 10) and, furthermore, its expression is related to the degree of eosinophilic inflammation (11). In murine models of asthma, IL-13 was both necessary and sufficient to cause airway hyperresponsiveness (AHR) (12), and depletion of $CD4^+$ lymphocytes abolished AHR (13).

B lymphocytes, like T-cells, express surface receptors specific for a particular antigen. When a B-cell encounters its specific antigen, it internalises, processes and presents it to activated Th2 cells. Again, in association with specific co-stimulatory signals (ie CD40 ligand on the T-cell surface and CD40 on the B cell), IL-2 stimulation promotes clonal expansion of the B-cell. The B-cell then differentiates into a plasma cell and secretes large amounts of soluble antibody corresponding to its specific surface receptor. IL-4 and IL-13, secreted by Th2 cells, promote isotype switching of the B-cell to release IgE (4), which can then bind to high affinity receptors on the surfaces of mast cells. Subsequent exposure to the relevant inhaled allergen cross links the IgE on the mast cell surface, causing degranulation and activation of asthmatic airway inflammation.

1.3 Role of other immune cells in asthma

1.3.1 T helper 1 cells

Th1 cells are defined by the secretion of interferon γ (IFNγ) and tumour necrosis factor β (TNF β) (4). Their role in asthma is unclear, with conflicting evidence as to whether they augment or attenuate asthmatic airway inflammation. For example, some studies found that levels of INFγ and TNFα were elevated in the bronchoalveolar lavage fluid (BALF) of patients with asthma (14) and correlated with disease severity (15) suggesting that Th1 cells may contribute to the asthma phenotype and may be associated with more severe disease. In support of this hypothesis, adoptive transfer of Th1 cells in one mouse model of asthma caused severe airway inflammation. In contrast, however, similar experiments in different animal models demonstrated inhibition of airway inflammation and AHR by Th1 cells and IL-16 (which promotes differentiation of $CD4^+$ cells towards a Th1 phenotype) (16-18). In keeping with a potential negative regulatory effect of Th1 cells, Krug *et al* found a reduction in IFNγ-producing cells in asthmatic patients after segmental allergen challenge (19). Perhaps most importantly, in asthmatic patients, inhaled IFNγ had no effect on symptoms, lung function or BALF eosinophil counts (20).

1.3.2 Regulatory T cells

Tregs are important regulators of autoimmunity. They are naturally generated immune cells thought to be part of the host defence mechanism limiting the pathogen-triggered immune response, before it becomes overwhelming and

causes excessive tissue damage. Thus, it has been hypothesised that Treg recruitment into the asthmatic airway could suppress allergic inflammation. In support of this hypothesis, Treg levels were reduced in BALF of children with asthma (21), normal patients had a higher frequency of peripheral blood Tregs compared to atopic patients, whereas atopic patients had higher peripheral blood levels of Th2 cells (22) and levels of Tregs were reduced in individuals with allergic rhinitis (23). Furthermore, the Tregs of atopic patients were less able to suppress allergen-induced T-cell proliferation *in vitro* (23). Interestingly though, in another study, whilst Tregs were again found to be decreased in atopic versus non-atopic children, in the atopic group, the levels of Tregs were actually higher in those with more severe disease compared to those with mild disease. Functional assays confirmed the competence of the Treg cells from this group (24). The authors speculate that Tregs may be generated as a response to atopy and disease severity. The concept that Tregs may downregulate allergic inflammation is supported by animal studies showing that adoptive transfer of Tregs into allergen sensitised mice prevented allergic airway inflammation and AHR (25, 26). However, their role in asthma remains unclear.

1.3.3 Th17 cells

Little is known about the role of these recently described $CD4^+$ T cells in asthma. Th17 cells and their main product, IL-17, are important in inflammatory and autoimmune diseases (27). IL-17 has been linked to neutrophilic lung inflammation and AHR in a mouse model (28), and release of neutrophil chemokines from airway epithelial cells *in vitro* (29). IL-17 mRNA

levels were increased in the sputum of asthmatic patients, and correlated with sputum neutrophil numbers (30). Th17 cells can also secrete IL-21, which inhibits Treg development (31) and IL-22, which has multiple actions on non-immune cells in various organs, including the lungs (reviewed in (32)). However, more work is needed to fully understand the role of Th17 cells in asthma.

1.3.4 Natural Killer T Cells

Natural killer T (NKT) cells are a sub-population of T lymphocytes that function to recognise and kill virally infected cells and tumour cells. Their role in asthma is controversial. Akbari *et al* have proposed an important role for NKT cells in asthma based on their observations that these cells formed more than 60% of the $CD4^+$ lymphocyte population in the BALF of patients with asthma (33) and that, in a mouse model, NKT cells were essential to the development of allergen induced AHR (34), a finding supported by others (35). However, others, who have criticised the methodology used by Akbari *et al*, showed the proportion of NKT cells in BALF of asthmatic patients to be less than 2.5% of the total lymphocyte population (36, 37) and, furthermore, these cells were <u>not</u> required for the development of allergic airway inflammation in a different mouse model (38). The role of NKT cells in asthma remains uncertain.

1.3.5 Cytotoxic T cells

Like Th17 and NKT cells, there is uncertainty surrounding the role of $CD8^+$ Tc cells in asthma. Sputum $CD8^+$ cells from asthmatic patients were found to

release higher levels of IL-4, IL-5 and IFNγ compared to those from non-asthmatic controls (39) suggesting a role for these cells in asthma pathogenesis. In a $CD8^+$ deficient mouse model, ovalbumin-stimulated AHR and airway inflammation was significantly reduced, and adoptive transfer of $CD8^+$ cells restored these responses (40). In contrast, Das *et al* found that depletion of $CD8^+$ cells had no effect on the development of allergic airway inflammation (38). Several studies have found correlations between the number or activity of Tc cells and asthma severity (39, 41, 42) and viral asthma exacerbations, particularly fatal exacerbations (reviewed in (43)), suggesting these cells may assume more importance in severe disease. However, their exact role remains unclear.

1.3.6 Mast cells

Mast cells are tissue cells housing numerous granules containing a host of pre-formed mediators, including leukotrienes, histamine, tryptase and some cytokines. They bear high affinity FCεRI IgE receptors on their surface. Crosslinking of these receptors, on binding of antigen-associated IgE, triggers mast cell activation and degranulation, with the release of both preformed and newly synthesised (PGD_2, thromboxaneA_2) mediators. These mediators can both trigger bronchoconstriction and contribute, through their effects on other immune cells and airway structural cells, to ongoing airway inflammation. As described in section 1.4.6.1, there is current interest in the observation that mast cells migrate into airway smooth muscle (ASM) bundles in asthma, where it is thought that they may also contribute to the remodelling response through effects on smooth muscle cell proliferation and matrix deposition (44, 45).

Tryptase released from mast cells may also contribute to airway remodelling through its action on protease-activated receptor type 2 (PAR2). PAR2 is present on many cell types, including epithelial cells, fibroblasts, ASM, and inflammatory cells (5) and its activation induces ASM proliferation (46).

As well as its effect on the early asthmatic response, mast cell activation is also thought to contribute to the late asthmatic response. Mast cell activation leads to the release from mast cell granules of certain cytokines, including TNF-α, IL-4 and IL-5 (47, 48) as well as *de novo* synthesis, and thus sustained release, of certain of these cytokines (49), thereby contributing to continued inflammation. In support of the mast cell's dual role in the early and late asthmatic responses, is the observation that the anti-IgE monoclonal antibody omalizumab inhibits both responses (50).

1.3.7 Eosinophils

Eosinophils are terminally differentiated polymorphonuclear granulocytes whose physiological function is to protect the host against large parasitic infections. Maturation of granulocyte precursors in the bone marrow into eosinophils is brought about through the sequential action of IL-3 and GM-CSF, which commit the immature granulocyte to the eosinophil lineage, followed by maturation and recruitment to the asthmatic airway under the influence of IL-5 and eotaxin (51, 52). Airway eosinophilia is a consistent finding in patients with asthma (53), with the exception of a subgroup of patients with severe asthma who may demonstrate mixed eosinophilic and neutrophilic inflammation, or neutrophilic inflammation alone (54).

Eosinophil numbers correlate with disease severity (53), and the dramatic reduction in sputum and tissue eosinophils on treatment of asthma with inhaled or oral glucocorticoids, associated with clinical improvement, is the central facet of the idea that eosinophils are fundamental to airway dysfunction in asthma (55, 56). Eosinophils contribute to airway inflammation and remodelling through the release of several chemokines, cytokines and mediators. Release of chemokines such as regulated on activation, normal T-cell expressed and secreted (RANTES) and IL-8 promotes recruitment of more eosinophils and other inflammatory cells. A host of cytokines may be released by eosinophils (57), which not only have proinflammatory actions on other structural and inflammatory cells within the airway, but may also enhance eosinophil survival (58). Perhaps more important, though, are the multiple mediators released by eosinophils that can contribute to airway remodelling through their direct effects on airway tissues. Eosinophils synthesise a range of cytotoxic mediators, including major basic protein (MBP), eosinophil peroxidise (EPO), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN), that are stored in granules and released on activation. Eosinophils and their mediators have been shown to cause direct damage to the epithelial layer (59, 60), causing epithelial detachment and cell lysis and thereby contributing to airway remodelling (see section 1.4.1.1). Furthermore, MBP causes increased ASM hyperreactivity *in vitro* (61), and its levels in BALF correlate with AHR (62).

The central paradigm that eosinophils are the key cells mediating asthmatic airway inflammation and AHR has been challenged recently by the observation

that administration of a monoclonal antibody against IL-5, which is essential to eosinophil maturation, had negligible effects on airflow and AHR despite a greater than 80% reduction in circulating and sputum eosinophils (63). However, despite the reduction in blood and sputum eosinophil count, lung parenchymal eosinophil levels were reduced to a much lesser degree, leading to the suggestion that the remaining eosinophils could account for the continuing symptoms (64). Furthermore, eosinophils entering the airway lumen lose their surface receptors for IL-5, but display enhanced levels of receptors for GM-CSF, another eosinophil activating cytokine, suggesting that eosinophil activation in the airways may be IL-5-independent (65). Interestingly, a recent study demonstrated a significant reduction in exacerbations following anti IL-5 therapy in a population of patients with refractory asthma and eosinophilic airway inflammation (66), whilst confirming earlier observations of a lack of effect on airflow, symptoms and AHR.

There is certainly evidence to support a role for eosinophils in airway remodelling, in that eosinophil depletion significantly reduced deposition of several matrix proteins in the subepithelial layer (67). Further support for a role of eosinophils in airway remodelling, but not AHR, comes from the observation that in eosinophilic bronchitis, a disease that shares many features with asthma but lacks AHR, subepithelial and intraepithelial eosinophil infiltration is observed, in association with subepithelial fibrosis (68). Undoubtedly eosinophils play an important role in the asthmatic airway, but the exact nature of their role remains unclear.

1.3.8 Neutrophils

A subgroup of patients with asthma demonstrate a marked airway neutrophilia and this appears to be associated with a more severe phenotype (54, 69). It has been argued that neutrophilic inflammation in severe asthmatics is a consequence of treatment, as the majority of these patients take long term oral glucocorticoids, which enhance neutrophil survival. A counterargument to this is that the relative steroid resistance of neutrophils may explain the poor response to corticosteroids seen in refractory disease. Interestingly, Ordoñez *et al* found significantly elevated levels of neutrophils, and the neutrophil chemokine IL-8, in the tracheal aspirate of patients intubated for acute severe asthma, compared to patients intubated electively for non-pulmonary surgery, yet, perhaps surprisingly, only five of the ten patients studied were taking inhaled corticosteroids or maintenance oral steroids (70), supporting the concept that neutrophilic inflammation is a primary feature of severe asthma rather than a consequence of therapy. Furthermore, neutrophil numbers in severe asthma correspond to indices of airway damage and reduced glucocorticoid responsiveness.(71, 72) It is thought that this neutrophilic phenotype is associated with more aggressive disease resulting in greater tissue destruction and airway remodelling. As described in section 1.4.1.2, mucus hypersecretion may contribute to airway narrowing and accelerated decline in FEV_1 in chronic asthma. Neutrophils may contribute to this through secretion of neutrophil elastase, a potent secretagogue for submucosal gland and goblet cells (73).

Evidence that neutrophils contribute to airway remodelling comes from a number of observations. Neutrophils from patients with asthma release higher levels of the profibrotic cytokine transforming growth factor β (TGFβ) (74). In severe asthma, high levels of a neutrophil-specific form of MMP-9, HMW MMP-9, were elevated in BALF and correlated with neutrophil numbers. Glucocorticoids were significantly less effective in suppressing HMW MMP-9 mRNA and protein in severe asthmatics than in controls (75). Furthermore, the ratio of MMP-9 to its endogenous inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1) was reduced in severe asthma, and correlated positively with FEV_1 (76). Thus it seems likely that these cells play a more prominent role in severe asthma associated with airway remodelling.

1.3.9 Monocyte macrophages

Macrophages are derived from circulating monocytes which migrate to the lungs in response to chemokines such as monocyte chemotactic protein-1 (MCP-1), and differentiate into macrophages under the influence of GM-CSF. Macrophage numbers are elevated within the airway mucosa of asthmatics (77), yet their role in asthma is unclear. Macrophages are APCs that, when activated, can release several mediators that induce bronchoconstriction, such as prostaglandins and leukotrienes (78). They are also a source of tissue damaging reactive oxygen species and lysosomal enzymes (5, 78). Interestingly, a recent study in a mouse model of asthma found that macrophages were the dominant source of the cytokine IL-17 (79). This cytokine is thought to be important in neutrophilic inflammation in asthma (discussed in section 1.3.3) and, by inference, this study supports an important

role for macrophages in this disease. In a recent study alveolar macrophages were found to be dysfunctional in children with steroid resistant asthma, suggesting these cells may be important in corticosteroid-resistant disease (80). Overall, however, little is known about the role of macrophages in asthma and more studies are required.

1.4 Structural changes in the asthmatic airway

Chronic inflammation in asthma leads to structural changes in the airways in asthma collectively termed airway remodelling. These changes include changes in the epithelium and subepithelial layers, the latter including matrix abnormalities and alterations of the airway smooth muscle layer.

1.4.1 Epithelial abnormalities

1.4.1.1 Epithelial desquamation

Epithelial denudation has been reported to be increased in biopsy specimens and post mortem specimens from patients with asthma compared to healthy controls, leading to the hypothesis that disruption of the protective epithelial layer results in alterations in host defences and responses to exogenous stimuli (81-84). Further, Chanez *et al* found that epithelial shedding occurred in untreated asthmatics but not in corticosteroid-dependent asthmatics treated with oral corticosteroids, or healthy controls (85). The observation that epithelial desquamation occurs in asthma is supported by observations of increased numbers of epithelial cells in BALF of patients with asthma (62). It has been proposed that an intrinsic weakness of the epithelial layer in patients with asthma contributes to epithelial desquamation. This hypothesis is

supported by the observation that the columnar epithelium lining the bronchi of asthmatic patients has less contact area with the basal lamina than in healthy controls (86). Furthermore, electron microscopy studies have also shown a reduction in epithelial cell desomosome length in asthmatic patients compared with healthy controls (87).

It has been suggested that epithelial desquamation may be an artefact of tissue sampling, that is, the biopsy itself may cause damage to the epithelium. This is because some studies of the cellular composition of BALF and sputum have shown no difference between numbers of epithelial cells from asthmatic patients and healthy controls (88, 89). Furthermore, one study found both epithelium-denuded areas and intact epithelium in bronchial biopsies from healthy volunteers and asthmatic patients, with no differences observed between the two groups (90). However, two observations support the hypothesis that epithelial damage occurs *in vivo*. CD44 is an adhesion molecule which is found on basal epithelial cells remaining after damage. CD44 immunoreactivity is higher in areas of damaged epithelium. Lackie *et al* found that expression of $CD44^{+}$ cells was increased in the epithelium of asthmatic patients compared with controls. Other studies have found elevated levels of staining for epidermal growth factor receptor (EGFR), known to be involved in epithelial repair processes, in the bronchial epithelium of asthmatic patients compared to healthy control subjects (91, 92). These observations support the concept of *in vivo* epithelial damage in asthma. Proposed mechanisms of bronchial epithelial shedding in asthma include direct toxicity

of eosinophil major basic protein to epithelial cells (93, 94) and intrinsic weakness of epithelial attachment as discussed above.

Although the widely accepted paradigm in asthma is that it is chronic inflammation that leads to airway remodelling, Federov *et al* have proposed that epithelial stress and injury may lead to collagen deposition and subepithelial fibrosis, based on their finding of increased EGFR staining in the bronchial epithelium of asthmatic children (a marker of epithelial activation/injury) associated with increased collagen deposition and thickness of the lamina reticularis. These changes were seen in the absence of any increase in eosinophil numbers (92). *In vitro* models of epithelial injury have been shown to cause increased fibroblast proliferation through release of growth factors including fibroblast growth factor (FGF), platelet derived growth factor (PDGF), ET-1 and TGF-β (95, 96). Thus the authors speculate that airway remodelling in asthma may occur as a result of epithelial stress, independently, to some degree, of inflammation, particularly as the structural changes were seen in children, including those with only moderately severe asthma.

1.4.1.2 Goblet Cell Hyperplasia

Asthma exacerbations are frequently characterised by sputum production and, furthermore, fatal asthma is commonly associated with mucus plugging of the airways (97). The mechanism of mucus hypersecretion is probably multifactorial, but one consistent finding is that of goblet cell hyperplasia in the airway epithelium of patients with asthma. Some studies have found goblet

cell hyperplasia to be much more marked in patients who have died from acute severe asthma (98), but a more recent study found similar changes in the airway epithelium of patients with mild to moderate asthma (99). The same study found that the volume of stored mucin in goblet cells in the airway epithelium was higher in asthmatic patients overall than healthy controls, that stored mucin levels were (non-significantly) higher in mild than moderate asthmatics, and that secreted mucin was significantly higher in moderate than mild asthma (99). This may have important clinical implications, since mucus hypersecretion is associated with accelerated decline in forced expiratory volume in one second (FEV_1) in both non-smokers and smokers with asthma (100). As well as mucus plugging in acute severe asthma, mucus hypersecretion may contribute to airway narrowing, meaning that a more pronounced effect may be produced for a given degree of smooth muscle contraction (bronchoconstriction).

1.4.1.3 Subepithelial fibrosis

Subepithelial fibrosis refers to the thickening observed in asthmatic airways of the layer immediately below the basement membrane. Multiple studies have shown an increase in subepithelial fibrosis in asthma (101-103). The degree of subepithelial fibrosis correlates with the severity (104) but not the duration (105) of disease. Interestingly, although one study found that treatment with an inhaled corticosteroid (ICS) for four weeks had no effect on thickness of the subepithelial layer (101), another study in which treatment with ICS was continued for 12 months showed significant reductions in the thickness of the

subepithelial layer (106), suggesting that subepithelial fibrosis is a consequence of airway inflammation.

1.4.2 Increased vascularity of the airways in asthma

Several studies have shown that there is an increase in bronchial wall vascularity in asthma. This has been observed at the macroscopic and microscopic level. Hashimoto *et al* studied the number of vessels and extent of vascularity of the small and medium airways of patients with asthma compared to healthy controls (107). They found that the number of vessels and vascularity of the airways was increased in asthmatics. This difference was most marked in the medium airways, where there was also a significantly greater degree of vascularity in moderate compared to mild asthmatics. Furthermore, vascularity of the medium (but not small) airways was inversely correlated with FEV_1. Interestingly, treatment with ICS for one year had no effect on vascularity of the asthmatic airways. This contrasts with other studies that found a reduction in airway vascularity following treatment with ICS (108-110), although the same effect was not observed with a low dose of ICS (109). The differences may be due to differences in study populations or in the dose of ICS.

A number of candidate angiogenic factors are thought to promote the vascular changes seen in asthmatic airways, perhaps the most important of which are vascular endothelial growth factor (VEGF) and the angiopoietins, Ang1 and Ang2. These are thought to play distinct but coordinated roles in the angiogenic process. In embryonic development, VEGF is involved in primitive

new vessel formation, whereas Ang1, acting at its receptor Tie2, is essential for vessel maturation (reviewed in (111)). Ang2 is in some contexts a natural antagonist of Ang1. Whereas Ang1 is widely expressed in normal tissues, Ang2 is expressed mainly at sites of vascular remodelling (112). Levels of VEGF and Ang1, but not the VEGF receptors, VEGFR1 and 2, were shown to be significantly higher in bronchial biopsies from patients with asthma compared to controls. VEGF levels were also elevated in the BALF of asthmatic subjects (113). Furthermore, ICS reduced VEGF but not Ang1 staining in the airways of asthmatics treated with ICS compared with placebo (110).

Increased airway vascularity may contribute to the asthma phenotype through several mechanisms. The microvascular bed is a substantial component of the airway wall, and even small alterations to the thickness of the inner airway wall can contribute to enhanced airway narrowing (114). Further, the increased vasculature may facilitate influx of inflammatory cells and exudation of inflammatory mediators, particularly if there is an associated increase in vascular permeability. Finally, the increase in vasculature may contribute to AHR by supporting the airway smooth muscle layer, which is thickened in asthmatic airways. An alternative hypothesis is that the increased vasculature may have a protective effect by increasing trafficking of inflammatory cells and mediators away from sites of inflammation. The former hypothesis is supported by studies in a mouse model of asthma. Levels of VEGF were elevated in the lungs of these mice, and VEGF receptor antagonists inhibited inflammatory cell influx and AHR in this model. Furthermore, some (108,

115), but not all (110, 116), studies of ICS and airway vascularity in humans have shown an inverse correlation between measures of lung function and airway vasculature.

1.4.3 Airway smooth muscle layer

It is generally accepted that there is an increase in ASM mass in the airways of patients with asthma compared to control subjects. The first study examining the amount of ASM in asthmatic airways dates back to 1922 (117). This study compared the airways of patients with fatal asthma to those of patients who had died suddenly of non-respiratory conditions. The authors found an increase in smooth muscle layer thickness in the asthmatic group. Subsequent studies have confirmed this finding in patients with fatal asthma (118, 119). However, in cases of non-fatal asthma, increased ASM thickness is a less consistent finding with some (118, 120, 121) but not all (119, 122) studies showing an increase. Although such studies are fraught with theoretical technical limitations (reviewed in (123)), it is likely that an increase in ASM thickness forms part of the remodelling process observed in asthmatic airways, at least in severe/fatal asthma.

Similarly, the literature is conflicting regarding whether the increased ASM observed in asthmatic airways is due to hypertrophy or hyperplasia of the ASM layer. Woodruff *et al* found an increase in cell number but not size in the airways of patients with mild to moderate asthma compared to normal controls (120). In contrast, another study found that ASM cells were increased in size across all asthma groups, that is, those with intermittent, persistent mild-to-

moderate and persistent severe asthma (121). A further study defined two subgroups of asthmatic patients, one in whom only hyperplasia of the larger bronchi was observed, with no evidence of cellular hypertrophy, and a second group in whom hypertrophy predominated and was seen at all levels of the bronchial tree, with only mild hyperplasia seen (124).

The amount of ASM in the airway is determined by the balance between cell proliferation and cell death. A number of growth factors have been shown to promote ASM proliferation *in vitro*, several of which are found in elevated amounts in the asthmatic airway (125). A less well studied area is ASM cell apoptosis. Freyer *et al* showed that various components of the extracellular matrix (ECM), which is known to be deposited in increased amounts in asthmatic airways (126), inhibit ASM cell apoptosis. The authors postulate that this may contribute to airway remodelling in asthma by promoting ASM survival and hence contributing to the increased amount of ASM found in the asthmatic airway (127).

One further mechanism which may contribute to ASM accumulation in the airway is cell migration. This function of ASM has only recently been identified. Migratory responses of ASM have been observed *in vitro* (128, 129) and, furthermore, ASM migration can be modulated by a variety of factors, including matrix components (130), growth factors (131), lipid mediators (132), glucocorticoids and β2 adrenoceptor agonists (133). On this basis, two hypotheses have been proposed (125). The first is that ASM cells might migrate out of the muscle bundles towards the lumen, thus contributing

to the appearance of myofibroblasts in the submucosal space; the second that myofibroblasts outside the muscle bundles may migrate toward the bundles, precipitating a change of phenotype to that of mature smooth muscle, thus adding to its overall content. Studies of ASM migration are still in their infancy and it remains unclear what if any contribution migration makes to overall ASM accumulation in the asthmatic airway. However, if cell migration does indeed make a significant contribution to airway remodelling, this would provide an exciting new target for therapeutic intervention in asthma.

1.4.4 Functional significance of increased ASM in asthma

There are several potential functional consequences of an increase in ASM amount in asthmatic airways.

1.4.4.1 Reduction in airway calibre and increased force generation

Early studies concluded that the increase in smooth muscle mass in the airways of people with asthma could account for the increased resistance in response to bronchoconstrictor stimuli through two mechanisms. First, the ASM bulk in itself, as well as other components of airway remodelling including subepithelial fibrosis, would cause a reduction in airway calibre, thus a greater degree of airflow limitation would be produced for a given degree of smooth muscle contraction (114). Second, the greater amount of ASM would result in a greater degree of bronchoconstriction in response to stimulation (134, 135). However, most *in vitro* studies have not shown an increase in force production in response to various stimuli in bronchial preparations from asthmatic patients ((136) and references therein). It has been postulated that *in vitro* force

production may not reflect the ability of ASM to shorten, which is thought to cause airway narrowing *in vivo* (137). There is evidence from animal models to support this, in that force generation does not necessarily correlate with ability of ASM to shorten (138). Finally, it has been argued that increased shortening velocity, rather than force generation, may be the more important determinant of bronchoconstriction *in vivo* (139).

1.4.4.2 Altered contractility of ASM

Another possibility is that ASM in the remodelled airway has altered contractile properties. Two potential mechanisms may affect ASM contractility: (1) length adaptation, or (2) altered expression or phenotype of the smooth muscle contractile apparatus. The phenomenon of length adaptation refers to reduced stretch/length of the muscle, which may occur as a consequence of airway wall thickening. This would allow the muscle to chronically adapt to a shorter length, resulting in increased shortening and luminal closure upon exposure to bronchoconstrictor stimuli (140). Many investigators have explored the possibility of altered expression or function of the contractile apparatus in asthmatic ASM. Antonissen *et al* showed an increase in the amount and velocity of ASM shortening in a canine model of asthma, compared to unsensitised controls (141). In keeping with this observation, canine ASM of sensitised animals was found to have increased myosin light chain kinase (MLCK) content and actomyosin ATPase activity (142). More recently, it has been shown that human ASM from asthmatic patients expresses greater quantities of MLCK mRNA than controls, with an associated increase in shortening capacity and velocity (143). Similarly, in

another study, tissue from asthmatic patients expressed increased MLCK compared to controls and levels correlated with asthma severity (121). In contrast, a further study found no difference between asthmatics and controls in expression of genes related to a hypercontractile phenotype (120). However, there is *in vitro* evidence to suggest inflammatory stimuli may augment ASM contraction by affecting intracellular calcium signalling, or other mechanisms including increased RhoA/Rho kinase pathway activation, persistent β2 adrenoceptor activation or increased phosphodiesterase 4 expression (reviewed in (144)). It remains unclear whether asthmatic ASM has an intrinsic difference in contractile properties, or whether it has normal contractility but shortens to a greater extent due to the increased levels of contractile agonists and inflammatory mediators liberated in the asthmatic airway. However, what has become clear is that, in addition to its contractile properties, ASM has important synthetic functions, which in themselves may contribute in an autocrine and paracrine manner to ASM contraction and bronchoconstriction, as well as contributing to airway inflammation and remodelling, discussed below.

1.4.5 Synthetic functions of ASM

Over the last decade or so, it has become clear that ASM cells are key contributors to the inflammatory and remodelling processes in the asthmatic airway (145-147). This group and others have shown that ASM has important synthetic functions and can contribute to chronic inflammation and remodelling in chronic asthma through production of mediators (148-151), chemokines (152-157), cytokines (158), growth factors (159), matrix

metalloproteinases (160, 161) and angiogenic factors (162). These factors have the capacity to contribute to inflammation and remodelling through a variety of mechanisms. The release of chemokines by ASM contributes to the recruitment of inflammatory cells including eosinophils, T-lymphocytes, neutrophils, and monocyte-macrophages. The production of cytokines (158) and pro-inflammatory mediators, such as PGE_2 (149), provides a mechanism by which ASM can act not just on other structural and inflammatory cells in the airway, but also in an autocrine manner to further increase release of biologically active molecules contributing to inflammation and remodelling (152). Growth factors released by ASM may contribute to remodelling by promoting proliferation of structural airway cells. MMPs selectively degrade ECM components; thus, as discussed in section 1.3.8, imbalances between MMPs and their inhibitors, TIMPs, may contribute to tissue damage and some of the remodelling features seen in asthma. For example, MMP-9 can degrade various components of the ECM thus disrupting the basement membrane and potentially increasing the migration of inflammatory cells (163). MMPs are also known to play a role in the trafficking of inflammatory and structural cells (164, 165). Finally, as discussed in section 1.4.2, there is an increase in bronchial wall vascularity in patients with asthma (115) suggesting that vascular dilatation and proliferation are important components of airway remodelling in chronic asthma. The release of angiogenic factors such as VEGF from ASM likely contributes to this process.

1.4.6 Differences in ASM between asthmatic and non-asthmatic patients

Recently, there has been increasing interest in whether there are intrinsic differences in ASM from asthmatic patients compared to those without asthma. Studies on asthmatic-derived human ASM cells (HASMC) *in vitro* have supported the notion that there are indeed intrinsic differences that persist when these cells are cultured *in vitro*, away from the altered environment within the asthmatic airway.

1.4.6.1 Cytokine and mediator release

A number of studies have explored the release of various cytokines and mediators from asthmatic HASMC. Oliver *et al* found that rhinovirus-induced release of IL-6 and IL-8 was elevated in asthmatic HASMC compared to normal controls (166). This may explain, in part, why many asthma exacerbations are triggered by respiratory infections. Brightling *et al* found that the mast cell chemokine CXCL10 (IP-10) was more frequently expressed in the ASM layer of bronchial biopsies of patients with asthma compared to non-asthmatic patients. Furthermore, *in ex-vivo* cell culture, the asthmatic HASMC released significantly greater quantities of CXCL10 following stimulation with pro-inflammatory cytokines (44). This was associated with an elevated number of CXCR3 expressing mast cells within the ASM layer in bronchial biopsies of patients with asthma. Mast cells migrated towards CXCL10-releasing HASMC in chemotactic assays *in vitro*, suggesting a potentially important mechanism through which mast cell/HASM interactions occur in asthma. The authors suggest that this is of direct relevance to the asthma phenotype, since mast cell infiltration into the ASM layer is not

observed in eosinophilic bronchitis, a condition related to asthma that presents with a glucocorticoid responsive cough, but lacks the variable airflow obstruction observed in asthma. Since mast cells are a source of a number of factors that can induce ASM proliferation, hyperresponsiveness and contraction, the differential production of CXCL10 by asthmatic HASMC may be an important determinant of the asthma phenotype. Another study comparing HASMC from asthmatic individuals and normal controls found that trypsin and bradykinin-induced PGE_2 release was reduced in proliferating, but not quiescent, asthmatic ASM (167). The authors speculate that, since PGE_2 can inhibit HASM proliferation, this could partly explain increased ASM proliferation observed in asthmatic cells. Furthermore, PGE_2 has anti-inflammatory properties, so defective release from proliferating asthmatic ASM could contribute to airway inflammation.

1.4.6.2 ASM proliferation

Ex-vivo, cultured HASMC from asthmatic patients have been shown to proliferate significantly more rapidly than those from normal controls (168), which may explain, in part, the increase in smooth muscle mass in the asthmatic airway. There is evidence that the mechanism of disordered proliferation is due to a deficiency of the transcription factor CCAAT/enhancer binding protein-α (C/EBPα) in these cells (169). In ASM from non-asthmatic subjects, C/EBPα mediates the antiproliferative effects of glucocorticoids. In HASMC from asthmatic patients, C/EBPα was not expressed, and glucocorticoids failed to inhibit cell proliferation. Transfection of asthmatic HASMC with a C/EBPα expression vector restored glucocorticoid

antiproliferative effects. There is some evidence to suggest that the cell signalling pathways responsible for ASM proliferation also differ between asthmatics and non-asthmatics. Burgess *et al* found that ASM cell proliferation was mediated by both extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) in asthmatic and non-asthmatic ASM. However, in the presence of a strong mitogenic stimulus, signalling in asthmatic ASM shifted towards a PI3K-mediated pathway, raising the possibility of specific targeting of this pathway in the prevention or treatment of airway remodelling (170). Interestingly, elevated levels of the nuclear hormone receptor peroxisome proliferator activated receptpor γ (PPARγ) have been demonstrated in the bronchial submucosa, the airway epithelium, and ASM of steroid-untreated asthmatics, and this is associated with enhanced proliferation and apoptosis of airway epithelial and submucosal cells, but not ASM (171). The authors speculate that PPARγ may have pro-inflammatory and fibrotic actions, in contrast to the prevalent view of PPARs as anti-inflammatory.

1.4.6.3 ASM extracellular matrix interactions

The ASM is surrounded by the ECM, which consists of an array of structural matrix proteins, including collagens, laminins, elastin and chondroitin sulphate, matrix-degrading MMPs and TIMPs. The ECM is a dynamic structure, with a turnover rate of around 10 – 15% per day. Degradation of the ECM releases factors which can feed back on ASM. The complex interaction between ASM the ECM is exemplified by the observation that certain ECM components inhibit whereas others increase growth factor-stimulated ASM proliferation

((172) and references therein). As mentioned above, the ECM is deposited in increased amounts in the asthmatic airway. In keeping with this, asthmatic ASM cells *in vitro* produced altered amounts of ECM components compared to non-asthmatic cells, and this altered ECM enhanced proliferation of both asthmatic and non-asthmatic ASM (173). Furthermore, like ASM proliferation, PI3K was involved in TGF-β-stimulated fibronectin and collagen I production in asthmatic but not non-asthmatic ASM, again suggesting this pathway may be of therapeutic interest (174).

1.4.7 Airway smooth muscle/T-cell interactions

In section 1.3 we discussed the roles of T-cells in asthma. Interestingly, activated T-cells can bind to ASM cells *in vitro* via CD44 expressed on the ASM cell surface and may stimulate ASM proliferation (175). ASM also express other surface receptors with the potential to bind to T-cells (176, 177). These observations suggest that ASM-T-cell interactions may be important in asthmatic airway inflammation. However, *in vivo* evidence for such interactions is currently lacking.

1.5 Mediator component of asthma

As has been alluded to in our discussion of the roles of inflammatory and structural cells in asthma, a range of mediators are secreted by these cells including histamine, adenosine, 5-hydroxytriptamine, lipid mediators (prostaglandins, thromboxanes), leukotrienes, and peptide mediators such as endothelin-1 (ET-1) and bradykinin (BK). These mediators have multiple overlapping actions, including triggering bronchoconstriction, promitotic

effects on ASM and other airway structural cells, pro-inflammatory effects and effects on airway remodelling. It is beyond the scope of this introduction to review this vast area in detail; this has been done elsewhere (178). In this thesis, we have focused on the role of the peptide mediator ET-1.

1.5.1 Mediator signalling

Many of the important mediators in asthmatic airway inflammation signal through seven-transmembrane G-protein coupled receptors (GPCRs). On their cytoplasmic domain, GPCRs are associated with heterotrimeric G proteins composed of a G_{α} subunit and a $G_{\beta\gamma}$ subunit. Binding of an agonist to the GPCR results in a change in receptor conformation, triggering exchange of bound GDP on the G_{α} surface for GTP, and dissociation of $G_{\beta\gamma}$ from G_{α}. This promotes interaction of the G_{α} subunit with its specific effector, resulting in generation of second messengers and triggering an intracellular signalling cascade and cellular response.

G_{α} proteins are classified depending on the second messenger system with which they interact. $G_{\alpha s}$ activates adenylyl cyclase (AC) resulting in generation of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) activation. $G_{\alpha i}$ inhibits AC, $G_{\alpha q}$ activates calcium and inositol phosphate dependent pathways via phospholipase C, and can also activate PI3K, and $G_{\alpha 12/13}$ couples to the Rho family of guanine nucleotide exchange factors. The classic example of a GPCR-mediated response is that of the β-adrenergic receptor. Binding of a β-adrenergic agonist such as salbutamol

results in activation of AC via $G_{\alpha s}$, generation of cAMP and, in the airway, relaxation of bronchial smooth muscle.

It is clear that many mediators important in asthma can upregulate the expression of inflammatory response genes. For example, the Knox group has shown that bradykinin induces cyclooxygenase 2 (COX-2), VEGF and IL-8 expression in HASMC (152, 162, 179), others have shown that leukotrienes induce chemokine and cytokine expression in mononuclear cells (180, 181). However, the signalling pathways leading to inflammatory response gene expression in response to GPCR agonists have not been well characterised and, in particular, the nuclear signalling events are poorly understood.

Inflammatory gene expression is regulated at several levels. These include activation of transcription factors, such as nuclear factor κB (NF-κB), via kinase cascades, binding of transcription factors to specific recognition elements in gene promoters, by chromatin remodelling, and through post-transcriptional modification. Transcriptional activation is particularly complex. DNA in its native state exists as chromatin and is wound around four core histones (H2A, H2B, H3, H4). These histones undergo a number of covalent modifications (acetylation, phosphorylation, methylation) which regulate chromatin unwinding thereby allowing access of transcription factors to their binding sites on DNA, recruitment of essential co-factors and activation of transcription. Histone acetylation is brought about by histone acetlyltransferases (HATs) such as cAMP response element binding protein (CREB) binding protein (CBP), p300 and p300/CBP associated factor (pCAF).

This process is reversed through the actions of a class of counterregulatory proteins, the histone deacetylases, or HDACs.

The Knox group has explored some of the signalling events involved in induction of IL-8 and COX-2 expression by BK. NF-κB, activator protein-1 (AP-1) and NF-IL-6 are all involved in IL-8 induction by BK (182). In contrast BK induced COX-2 via the cAMP response element (CRE) with no involvement of NF-κB or NF-IL-6 (183). In this latter study, BK-induced COX-2 transcription was found to involve acetylation of promoter-associated histones on lysine residues 5, 8 and 16 (183). Complex autocrine loops involving endogenous prostanoids seem to be responsible for some of these effects but not others (182, 183). The Knox group also showed that another important inflammatory mediator in asthma, PGE_2, transcriptionally upregulates VEGF production in HASMC through a cAMP dependent mechanism. This effect was mediated through binding of the transcription factor small protein 1 (Sp1) to the VEGF promoter, demonstrated by chromatin immunoprecipitation (ChIP) assay (184).

A number of studies have shown that the pro-inflammatory transcription factors NF-κB and AP-1 may be activated by a variety of inflammatory mediators, including leukotrienes (181, 185), histamine (186) and prostaglandins (187). However, the majority of these studies used strategies such as transcription factor mRNA levels, reporter gene assays and electrophoretic mobility shift assays to infer involvement of these transcription factors. Very few studies have gone on to demonstrate *in vivo* binding of

transcription factors to the relevant gene promoters using, for example, ChIP assays. Furthermore, more complex transcriptional mechanisms involving recruitment of cofactors (such as p300) to gene promoters, and chromatin remodelling in the form of histone modifications, have not been explored, and the upstream signalling pathways are not well characterised. Aside from the studies of BK effects from this lab, an exception to this is in the field of endocrinology, where binding of histone-modifying proteins and alterations in histone acetylation status of relevant promoters have been demonstrated in prostaglandin-regulated ovarian luteolysis and oestrogen biosynthesis (188, 189).

It is well established that mediators acting at GPCRs can activate various intracellular signalling pathways downstream of second messenger systems. For example, in pulmonary artery smooth muscle cells, PI3K and p38 MAPK regulate BK-stimulated PGE_2 release (190) and ET-1 can activate the MEK (MAPK/ERK kinase)/ERK, p38 MAPK and cJun N-terminal kinase (JNK) pathways in various systems (191, 192). Other intracellular targets include protein kinase C (PKC) and phospholipase A_2 (PLA_2) (193). Furthermore, $G_{\alpha s}$-stimulated PKA activation may have inhibitory effects on MAPK dependent pathways via phosphorylation and inhibition of the upstream intermediate raf-1 (193). However, there have been few studies directly linking these signalling cascades with nuclear gene expression events.

1.5.2 Endothelin-1

ET-1 is a 21 amino acid vasoactive and pro-inflammatory peptide that acts at $G_{\alpha q}$-coupled ET_A and ET_B receptors. ET-1 is implicated in asthma through several lines of evidence: 1) ET-1 levels are elevated in BALF, bronchial biopsies and peripheral blood of asthmatics (194); 2) inhaled ET-1 causes bronchoconstriction in asthmatic patients (195); 3) ET-1 is a potent contractile agonist of isolated human bronchus (196); 4) ET-1 potentiates mitogenic responses in cultured ASM cells (197); 5) ET-1 induces expression of matrix proteins by pulmonary fibroblasts (191), suggesting a role in airway remodelling; 6) in animal models, overexpression of ET-1 in the lungs of transgenic mice induces chronic pulmonary inflammation (198) and in a rat model, induction of pulmonary eosinophilic inflammation caused a significant increase in ET-1 mRNA and protein (199). Furthermore, the ET-1 receptor antagonist SB-217242 inhibits airway eosinophilia and hyperresponsiveness to methacholine in Der P1 sensitised mice (200). 7) ET-1 and ET_A receptor gene polymorphisms are linked with asthma and atopy respectively (201, 202). Despite the implication of ET-1 in airway inflammation through animal studies, there have been very few studies of ET-1's effects on release of inflammatory cytokines and mediators from ASM. Furthermore, there have been few studies of nuclear signalling by ET-1 and the transcriptional and post-transcriptional regulation of chemokine expression by ET-1 has not been studied. The current understanding of cell signalling and transcriptional regulation by ET-1 is described in more detail in chapter 4. In that chapter, we have explored the transcriptional regulation of inflammatory genes by ET-1 in more detail, focusing on the chemokine MCP-1.

1.6 Cytokines

The immune system is regulated by a bewildering array of cytokines. Cytokines are small proteins that act in an autocrine and paracrine manner to regulate local and systemic immune and inflammatory responses. They function in a complex network where production of one cytokine will influence the production of, or response to, several others, often in a stimulus and tissue-specific manner. Their many actions include effects on growth, mobility, differentiation or function of target cells (178). Cytokines act by binding to specific cell surface receptors. Receptor binding leads to activation of intracellular signalling pathways and downstream effects, such as induction of inflammatory response genes (148, 203). Levels of many cytokines thought to be important in asthma are elevated in the airways of asthmatic patients (9, 10, 14, 30) and, in earlier sections of this chapter, we have touched on the functions of some of these. We focus in this thesis on the role of TNF-α. A review of the functions of other individual cytokines in asthma has been performed by Barnes (178).

1.6.1 Tumour necrosis factor-α

TNF-α is a 17 kDa protein which acts via two receptors, TNFR1 or p55, and TNFR2 or p75. It is produced predominantly by macrophages, but also T-lymphocytes, dendritic cells, mast cells, neutrophils and eosinophils as well as structural cells including fibroblasts, epithelial cells and smooth muscle cells (178, 204). Release of TNF-α may be triggered by pathogens, physical or chemical stimuli, and a range of cytokines such as IL-1, GM-CSF and IFN-γ

(178). The physiological role of TNF-α is to modulate the growth, differentiation and proliferation of a range of cell types as well as mediating apoptosis (205). It also triggers the release of various other members of the cytokine network such as IL-8 (154), RANTES (206) and eotaxin (203), thereby triggering a "second wave" of inflammatory responses.

TNF-α levels are elevated in the BALF and bronchial biopsies of asthmatic patients (207, 208). Furthermore, cells from BALF of asthmatic patients release significantly greater amounts of TNF-α (14). As mentioned above, once released in the airways, TNF-α can activate the release of multiple inflammatory mediators, chemokines and cytokines, thus promoting ongoing inflammation. There is also evidence for a direct role of TNF-α in airway remodelling in that it stimulates the release of MMP-9 and the matrix protein tenascin from bronchial fibroblasts (209). TNF-α promotes AHR; TNF-α increased maximal isotonic contraction to methacholine of guinea pig tracheal preparations *in vitro* (210). In animal studies, TNF-α has been shown to mediate LPS and allergen-induced inflammation and AHR (211, 212). Furthermore, TNF-α inhalation increases airway responsiveness and induces sputum neutrophilia in normal controls (213) and asthmatic patients (214), the latter group also showing an increase in sputum eosinophilia. Importantly, there is evidence of upregulation of the TNF-α pathway and increased mucosal TNF-α expression in severe/refractory asthma (215, 216) which has led to increasing interest in the use of anti-TNF-α treatments for patients with severe asthma whose disease cannot be controlled with conventional therapies (see section 1.9.2).

Most cytokine receptors are associated with molecules called Janus kinases (JAKs). Receptor binding causes tyrosine phosphorylation and subsequent activation of JAKs. JAKs may then activate various signalling proteins, including signal transducers and activators of transcription (STATs), through tyrosine phosphorylation. STAT dimers then translocate to the nucleus where they bind to the promoters of and activate cytokine responsive genes. TNF-α does not signal through the JAK-STAT pathway. Binding of TNF-α to its receptor initiates one of many potential intracellular signalling cascades depending on the exact tissue and the expression pattern of associated signalling machinery (217). There are two main pathways of signal transduction between the TNFRs and subsequent intracellular events. TNFR1 contains a "death domain" (DD) which, under quiescent conditions, is bound to the silencer of death domain (SODD). On binding of TNF-α, SODD dissociates from the receptor, allowing other DD-interacting proteins to bind and trigger a cascade of reactions primarily involved in signalling cell death. However, it is the second pathway that is probably more relevant in the activation of inflammatory response genes. Both TNFR1 and TNFR2 contain sequences that bind TNF receptor-associating factors (TRAFs). Upon receptor occupation by TNF-α, TRAFs transduce this signal to activate a variety of intracellular signalling pathways, including activation of MAP kinase pathways (p38, MEK/ERK and JNK pathways), activation of phospholipase C (PLC) and thence PKC, and activation of the pro-inflammatory transcription factors NF-κB and AP-1 (217-219).

In contrast to GPCR-mediated inflammatory gene regulation, transcriptional regulation of inflammatory genes by cytokines such as TNF-α has been more extensively studied. There have been many studies of the roles of various transcription factors in inflammatory gene regulation by cytokines. More recently, a number of groups have begun to extend these studies to explore the role of histone modifications such as phosphorylation and acetylation and the roles of the transcriptional co-activators that bring these modifications about. For example, IL-1β induced COX-2 transcription in HASMC through binding of p65, CREB and C/EBPβ, and acetylation of lysine 8 associated with the COX-2 promoter (183). Similarly, IL-1β caused histone acetylation at the GM-CSF promoter, accompanied by an increase in GM-CSF release from A549 cells (220). Several studies have investigated the transcriptional regulation of the chemokines MCP-1 and eotaxin in various systems. Transcriptional regulation of MCP-1 by the cytokines IL-1β and TNF-α has been shown to be NF-κB-dependent in several different tissues (221-223). Interestingly, in NIH 3T3 cells, TNF-α-stimulated MCP-1 transcription involves a cooperative interaction between NF-κB and Sp1 that is dependent upon CBP and p300-mediated histone acetylation at the MCP-1 promoter (222, 223). The Knox group have shown that transcriptional regulation of eotaxin by TNF-α in HASMC is brought about through acetylation of histone H4 and binding of NF-κB p65 to the eotaxin promoter (224).

Although inflammatory response gene regulation by cytokines is better characterised than that mediated by GPCRs, and although there is a wealth of studies on TNF-α-signalling (217), the exact kinase cascades mediating

complex gene transcription events are less clear. There is evidence, in various different biological systems, for involvement of JNK, ERK and p38 MAP kinases in these pathways (225, 226), as well as for involvement of the PI3K/Akt pathway (227) and of PKC (228, 229). Indeed, the Knox group have shown that transcriptional regulation of eotaxin by TNF-α involves PKCβ-mediated phosphorylation of pCAF, facilitating acetylation of histone H4 and thence p65 association with the eotaxin promoter (228). This is a novel action of PKCβ that has not been previously observed in other systems. MAP kinase cascades can also lead to histone phosphorylation through the direct action of downstream kinases such as mitogen- and stress-activated protein kinase (MSK).

1.7 Inflammatory gene regulation: cross talk between cytokine- and GPCR-mediated pathways

It is clear that transcriptional gene regulation by inflammatory mediators and cytokines is extremely complex. The involvement of common signalling moieties to both GPCR- and cytokine-mediated pathways implies that extensive cross talk may occur. Indeed there is evidence that this is the case. Histamine, which couples to AC and PI3K pathways through H2 and H1 receptors respectively, may regulate release of the cytokine IL-13 in T cells through cross-talk with the JAK-STAT pathway (230). As previously discussed, PGE_2, which acts via $G_{\alpha s}$-coupled receptors, modulates cytokine-stimulated induction of VEGF and COX-2 (183, 231). Furthermore, mitogenic signals acting at receptor tyrosine kinases (epidermal growth factor) and GPCRs (thrombin, acting at the $G_{\alpha q}$-coupled PAR) both signal through PI3K in

HASMC (232). Finally, BK-stimulated IL-6 release in HASMC is mediated by p38 MAPK and ERK, and is downregulated by the Th2 cytokines IL-4 and IL-13, implying that MAP kinases may also mediate cross talk between GPCR- and cytokine-stimulated responses (233).

In addition to the complex cross talk between different pro-inflammatory stimuli, evidence is accumulating that existing asthma therapies, including glucocorticoids (GC) and long acting β-adrenergic agonists (LABA) can modulate these inflammatory pathways. This is discussed in detail in section 1.9.1 and chapter 5. A simplified representation of GPCR- and cytokine-mediated inflammatory gene regulation, potential points of cross-talk, and of modulation by existing asthma therapies is shown in Figure 1.1. In chapter 5 we have further explored the mechanisms of TNF-α-stimulated eotaxin expression, and its modulation by glucocorticoids and long acting β-adrenergic agonists (LABA).

1.8 Chemokines

Chemokines are a subgroup of cytokines that attract inflammatory cells (**chemo**tactic cyto**kines**). Examples include MCP-1 (chemotactic for monocytes and T-lymphocytes), RANTES (T-cells, eosinophils and basophils), eotaxin (eosinophils) and IL-8 (neutrophils). Chemokines exert their biological actions by binding to specific receptors expressed on the surfaces of target cells. Cells that are attracted by chemokines follow a signal of increasing chemokine concentration towards the source of the chemokine through a complex process involving interaction of adhesion molecules expressed on the

surfaces of inflammatory and endothelial cells (234). Thus at sites of inflammation such as the asthmatic airway, release of chemokines from inflammatory and structural cells results in further influx of inflammatory cells and perpetuation of inflammation. Chemokines are classified on the basis of their structural characteristics, specifically the distance between the first two cysteine residues in their amino acid chain (235). Thus the two cysteine residues at the N-terminus of the CC chemokines are adjacent to one another, those of the CXC chemokines are separated by one amino acid, and those of the CX_3C group by three amino acids. A fourth group, with one cysteine residue at their N-terminus and one downstream are termed the C chemokines. This has led to a revised nomenclature based on these structural characteristics (235). Thus for the chemokines listed above, the alternate names would be CCL2 (MCP-1), CCL5 (RANTES), CCL11 (eotaxin) and CXCL8 (IL-8). For the purpose of readability, the classical names will be used in this thesis. The chemokine receptors are GPCRs divided into four families based on the class of chemokine they bind; CXCR that bind CXC chemokines, CCR that bind CC chemokines, XCR1 that binds the two C chemokines and CX3R1 that binds the sole CX_3C chemokine. An individual receptor may bind several different chemokines and vice versa. In this thesis we have explored the regulation of two key chemokines in the asthmatic airway, MCP-1 and eotaxin.

1.8.1 MCP-1 and asthma

MCP-1 is a chemokine of the CC subgroup which is chemotactic for monocytes (236, 237) and T-lymphocytes (238, 239). It is produced by a variety of cell types, including mononuclear phagocytes, epithelial cells,

fibroblasts, smooth muscle cells and endothelial cells (240-244). Levels of MCP-1 are elevated in the epithelium and subepithelial tissues of bronchial biopsies from asthmatic subjects, including the bronchial smooth muscle layer (245). Allergen challenge significantly increased MCP-1 levels in BALF of asthmatic patients (246). Furthermore, there is evidence from animal models that MCP-1 expression is increased in asthmatic airways and that the disease process is attenuated by MCP-1 immunoneutralisation (247). Interestingly, a polymorphism in the MCP-1 gene is associated with the presence of atopic asthma and its severity in children (248). These observations suggest that MCP-1 is an important chemokine contributing to airway inflammation in asthma. The Knox group and others have shown that HASMC express MCP-1 and that levels of expression are upregulated by cytokines (242, 249, 250). However, there have been few studies in ASM of the effects on MCP-1 expression by other types of asthma mediators. In chapter 4 we explore the mechanisms used by the pro-inflammatory mediator ET-1 to upregulate MCP-1 expression in HASMC.

1.8.2 Eotaxin and asthma

As described in section 1.3.7, eosinophils are important cells in asthmatic airway inflammation. Eotaxin is chemotactic for eosinophils *in vivo* and *in vitro* (251, 252). It also exerts chemotactic actions on basophils (253), Th2 lymphocytes (254) and mast cells (255), through its specific receptor CCR3. Sources of eotaxin include monocyte-macrophages, T-cells, eosinophils, airway epithelial cells and ASM cells (256-258). Several lines of evidence support a critical role for eotaxin in asthmatic airway inflammation. Levels of

eotaxin are elevated in the bronchial mucosa and BALF of asthmatic subjects compared with normal controls (256-258), eotaxin expression correlates with the number of airway eosinophils, markers of disease severity and AHR (257, 258), and the time course of eotaxin expression in the asthmatic airway following allergen challenge correlates with the early phase of eosinophil recruitment (259). Furthermore, eotaxin release from airway epithelial and smooth muscle cells is increased *in vitro* by the pro-inflammatory cytokines IL-1β and TNF-α, the levels of which are elevated in the asthmatic airway (207, 208). The Knox group and others have shown that eotaxin expression by HASMC is downregulated by existing asthma therapies, namely β2 adrenergic agonists and glucocorticoids (203, 260). We have begun to explore the mechanisms of TNF-α-stimulated eotaxin expression in HASMC and its modulation by glucocorticoids and LABA in more detail (224). In chapter 5 we have extended these studies to investigate these mechanisms further.

1.9 Current asthma treatments

Asthma treatments can be divided into those that primarily affect bronchoconstriction, used in the short term relief of symptoms and for managing severe bronchospasm in the acute setting, and those that are primarily aimed at controlling airway inflammation. Those in the first group include short-acting β2 adrenergic agonists (SABA), such as salbutamol, formotorol and terbutaline, and anticholinergics such as ipratropium bromide. SABA promote smooth muscle relaxation through binding to the β2 adrenergic GPCR, activation of AC and generation of intracellular cAMP. This acts via

PKA to inhibit MLCK. Anticholinergics block muscarinic cholinergic receptors, thus reducing ASM tone by reducing parasympathetic stimulation.

Other classes of drugs used to treat asthma include LABA such as salmeterol and formotorol (which has both short and long acting properties), xanthines, namely theophylline, leukotriene antagonists, such as montelukast and zafirlukast and chromones including sodium chromoglicate and necrodomil. The LABA act like SABA to relax ASM but, as the name suggests, have a longer duration of action. However, they also likely have anti-inflammatory actions; the anti-inflammatory effects of LABA are discussed in more detail in chapter 5. The current British Thoracic Society guidelines on the management of asthma recommend that LABA are prescribed in addition to inhaled corticosteroids (ICS) (261). They should not be prescribed alone due to concerns over safety (262). Theophylline relaxes airway smooth muscle through elevation of intracellular cAMP and cyclic guanosine monophosphate via inhibition of phosphodiesterases (PDE) 3, 4 and 5 (263). Unfortunately it has limited efficacy and, due to its narrow therapeutic window, has an extensive side effect profile at doses that inhibit PDE, meaning its usefulness in the management of asthma has been limited. However, there is emerging interest in more recently recognised anti-inflammatory effects of low dose theophylline, and this drug may yet prove to have therapeutic utility in the management of asthma and COPD in certain circumstances (263-265). Leukotrienes are potent mediators of bronchoconstriction (266) as well as having multiple pro-inflammatory actions *in vivo*, including inflammatory cell recruitment and increases in vascular permeability and mucus secretion (267-

270). Leukotriene antagonists improve baseline lung function in asthmatic patients (178), but are probably most useful in the clinical setting as an add on therapy for patients with exercise induced asthma (178, 261), or those with disease with a prominent leukotriene-driven component, that is those with aspirin induced asthma or asthma associated with allergic rhinitis (271). Sodium chromoglicate and necrodomil are said to be “mast cell stabilisers”, but their mechanism of action is not fully understood. Their role in adults is limited to the management of exercise induced asthma (261).

1.9.1 Glucocorticoids

Inhaled corticosteroids are the mainstay of asthma treatment. They are highly effective and are recommended for the management of any patient with asthma who has symptoms requiring more than just occasional use of a SABA (261). They have potent anti-inflammatory effects, thereby inhibiting ongoing inflammation in the asthmatic airway and the late allergic response. The mechanisms through which glucocorticoids (GC) achieve their effects are complex. They can both activate and inhibit transcription of GC-responsive genes (transactivation and transrepression, respectively).

As described in section 1.5.1, inflammatory gene regulation may involve activation of transcription factors, such as NF-κB, via kinase cascades, binding of transcription factors to gene promoters, chromatin remodelling brought about through specific covalent modifications to histone proteins, and post-transcriptional modification. Transactivation by GC is brought about through binding of GC to a ubiquitously expressed glucocorticoid receptor (GR)

localized to the cytoplasm of target cells. Binding of GC to GR results in dissociation of the molecular chaperone protein hsp90 from GR, allowing nuclear translocation of the activated GC-GR complex and binding to specific sequences within DNA termed glucocorticoid response elements (GRE). Within the nucleus, the DNA binding domain of the GR directs dimerisation and activation of gene transcription. The expression of a number of anti-inflammatory genes such as annexin 1 and IL-10 has been shown to be increased by GC (272, 273). However, the majority of anti-inflammatory effects of GC are thought to be brought about through gene repression.

Switching off of gene transcription by GR can occur through binding of GR to negative GREs in the regulatory region of certain genes. This is thought to disrupt transcription as a result of the GRE being positioned across the transcriptional start site or the binding sites for other transcription factors (274). However, although a functional negative GRE has been identified in the IL-1β promoter (275), the genes of most inflammatory mediators that are suppressed by GC in asthma do not have GREs in their promoters (276).

The inhibitory effect of GC in inflammation appears to be largely due to modulation of transcription of AP-1- and NF-κB-responsive genes. These transcription factors mediate the expression of multiple inflammatory response genes (277, 278). Alterations in AP-1 and NF-κB-dependent transcription can be brought about at several levels. GR can recruit nuclear corepressors, such as HDACs (220); it may alter RNA polymerase II phosphorylation (279); it may bind to transcriptional coactivators such as CBP thus competing with

transcription factor binding to these coactivators (280); or GR may inhibit the activity of HATs such as CBP (220). The effects of GC on HAT and HDAC activity and associated effects on chromatin structure and transcriptional activation are discussed further in chapter 5. Several other mechanisms of GR suppression of NF-κB and AP-1 have been described. GR can induce expression of inhibitor of κBα (IκBα) in certain cell types (281, 282). AP-1-DNA binding can be prevented through induction of the glucocorticoid inducible gene GILZ (glucocorticoid inducible leucine zipper) (283). Finally, GC may inhibit activation of AP-1 and NF-κB through inhibitory effects on mitogen activated protein kinases (MAPK) (276). Whilst most of the effects of GC are mediated by changes in gene transcription, they may also have post transcriptional, translational and non-genomic actions (274, 284).

1.9.2 Limitations of current asthma treatments

Whilst for the majority of patients with asthma, adequate control of disease can be achieved with the inhaled therapies described above, a minority of patients require long term oral corticosteroids to control their disease, or have corticosteroid resistant (CR) disease. These patients are at increased risk of death from asthma and from side effects of chronic systemic steroid therapy, and account for around 50% of the total health care costs of asthma (274). As well as their anti-inflammatory effects, steroids have a role in many metabolic processes. Thus long term steroid treatment in those with steroid dependent disease can cause serious systemic side effects including adrenal insufficiency due to suppression of the hypothalamic-pituitary-adrenal axis, osteoporosis,

impaired glucose tolerance/diabetes mellitus, cataracts and, in children, growth retardation.

There is much research interest in mechanisms of steroid resistance in asthma. A number of different potential mechanisms have been described, that are likely to differ between patients. Certain cytokines, such as IL-2, IL-4 and IL-13, which show increased expression in the airways of patients with CR asthma (274), may induce reduced GR affinity in inflammatory cells, causing local resistance to the anti-inflammatory effects of GC (285, 286). This effect appears to be mediated by p38 MAPK, raising the possibility that p38 MAPK inhibitors may have a role in restoring steroid sensitivity in CR asthma (287). Furthermore, MAP kinase phosphatase-1 (MKP-1), which dephosphorylates and inactivates p38 MAPK, is rapidly induced by GC (288). Thus p38/MKP-1 homeostasis might be important in contributing to steroid insensitivity. Changes in GR nuclear translocation or its ability to interact with HATs may contribute to steroid resistance. In one subgroup of CR patients, nuclear translocation of GR was defective (289). In another group, GR was unable to promote acetylation of lysine 5 on histone H4, leading the authors to speculate that this defect reduces the ability of GR to activate certain anti-inflammatory genes (289). Increased levels and/or activation of the proinflammatory transcription factor AP-1 may contribute to steroid insensitivity in CR asthma. Expression of the AP-1 subunit cFos is enhanced in bronchial biopsies of CR asthmatics (290). In keeping with a role for AP-1 in CR asthma, AP-1 DNA binding and cFos expression were increased in peripheral blood mononuclear cells (PBMC) from CR patients, and cFos was shown to mediate reduced GR-

GRE binding in response to dexamethasone (291, 292). Further support for a role of AP-1 comes from the observation that using a tuberculin skin response model of mononuclear cell inflammation, there was significantly greater expression of cFos, phosphorylated cJun and phosphorylated JNK in CR asthmatics, and, whilst CS suppressed cJun and JNK phosphorylation in steroid sensitive asthmatics, they enhanced it in the CR group (293). As discussed in section 1.4.6.2, defective expression of the transcription factor C/EBP in asthma may prevent the antiproliferative effects of GC on ASM (169). Finally, cigarette smoking is known to abolish the therapeutic response to inhaled and oral corticosteroids, (294, 295). Cigarette smoke causes oxidative stress and, interestingly, markers of oxidative stress are also increased in severe CR asthma (296), suggesting that oxidative stress may contribute to steroid resistance. In support of this hypothesis, the level in exhaled breath condensate of one marker of oxidative stress, 8-isoprostane, was unaltered by treatment with ICS in children with asthma (297). Another possible mechanism of reduced steroid responsiveness in association with cigarette smoking is through alterations in the levels and activity of HDACs. In smokers with COPD, a disease characterised by little clinical response to GC, HDAC2 expression and activity is reduced (298) and restoration of HDAC2 expression by transfection into alveolar macrophages from BALF of COPD patients restored GC function (299).

New biological therapies, such as the anti-IgE antibody omalizumab, anti IL-5 therapy and anti-TNFα therapy may be of benefit for some patients with steroid dependent or CR asthma, however they have significant limitations.

Omalizumab is only of benefit for a very small subgroup of patients with a highly atopic phenotype (261); anti-IL-5 similarly has only shown benefit in particular subgroups and is currently only available within the context of clinical trials (66), whilst trials of anti-TNFα therapy have had variable results. Early trials with the synthetic soluble TNF-α receptor etanercept showed promising results, with significant improvements in lung function and symptom scores (215, 216). However, a more recent trial showed less dramatic results, with a small improvement in Asthma Control Questionnaire score but no significant difference between treatment and placebo groups in Asthma Quality of Life Questionnaire Score or measures of lung function (300). Furthermore, there are significant concerns over the safety profile of anti-TNFα treatments (204). Thus, whilst the mechanisms of CR remain unclear, for the small proportion of asthmatic patients with steroid dependent or CR asthma, it is clear that new therapeutic options are urgently needed.

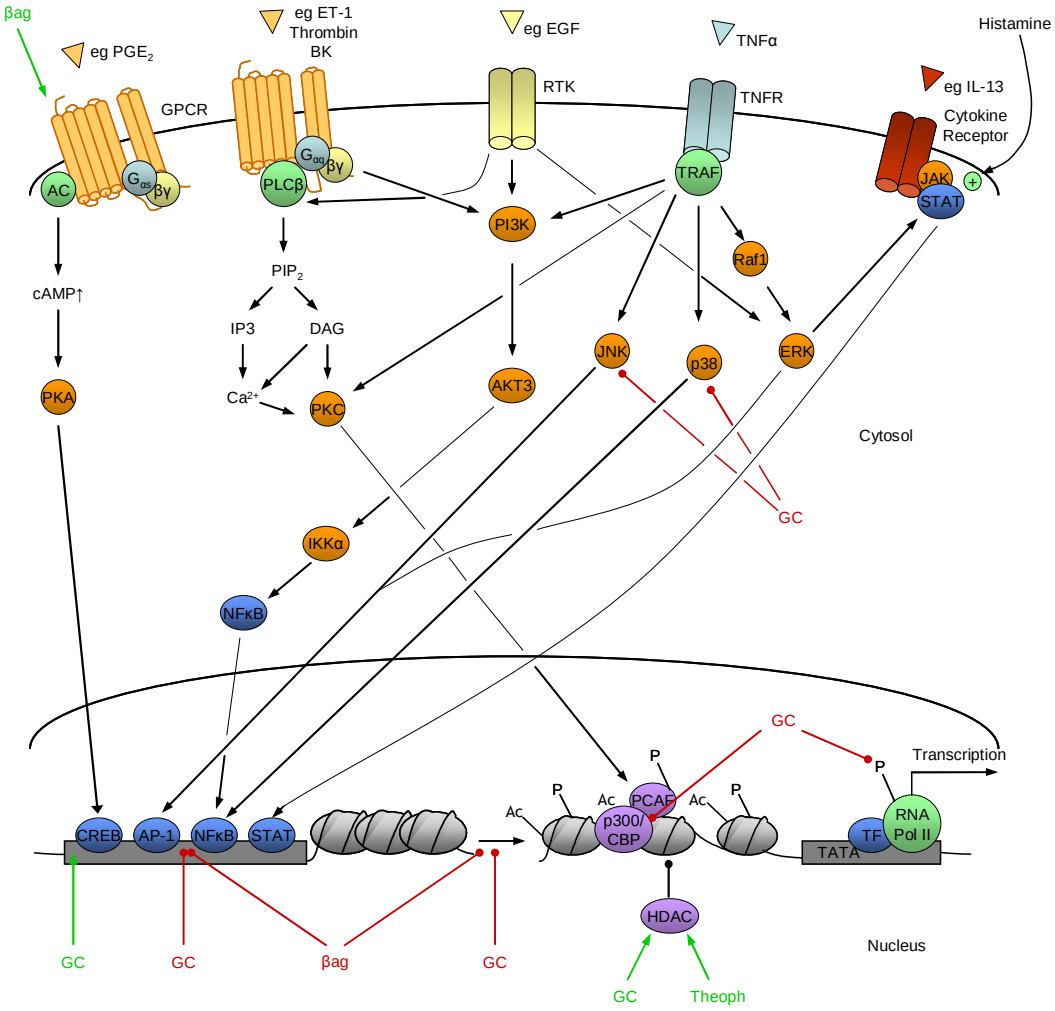

Figure 1.1 Cross talk between cytokine- and GPCR-mediated pathways. Positive regulatory pathways are indicated by arrows, stopped arrows represent inhibitory pathways. Points of interaction between steroids (GC) and β-agonists (βag) are shown. Activating actions of these drugs are shown in green, inhibitory effects in red. Note that steroids have both positive and negative effects on gene transcription. They predominantly switch off transcription of inflammatory genes, but may also increase transcription of a number of anti-inflammatory genes. Kinases are shown in orange, non-kinase signalling proteins in green, transcription factors in blue and other transcriptional regulatory proteins in lilac.

CHAPTER 2: AIMS OF THIS THESIS

2.1 Aims of this thesis

In chapter 1 we have summarised current knowledge of the roles of various immune cells, structural cells, mediators and cytokines in airway inflammation and remodelling in asthma. We have discussed how, in addition to their critical role in bronchoconstriction of the asthmatic airway, airway smooth muscle cells play a key part in these inflammatory and remodelling responses. Further, that differences beginning to be identified between asthmatic and normal ASM are helping to shed light on the causes of airway dysfunction in asthma. We have also discussed current treatment modalities and where these fall short for the management of patients with more severe disease. The intense research interest in novel mechanisms of action of existing therapies, and how these actions differ between steroid responsive and CR asthmatics, is starting to highlight potential new targets for treatment for this subgroup of patients whose disease cannot be managed effectively with existing therapies. We hypothesised, therefore, that inflammatory mediators and cytokines important in driving asthmatic airway inflammation may signal through common downstream signalling moieties which may act as promising new targets for asthma therapy.

Using primary human airway smooth muscle cells as a model, we therefore set out

1) To determine the signalling pathways used by inflammatory mediators in asthma, using the important pro-inflammatory mediator ET-1 as an example, to upregulate the expression of inflammatory response genes.

2) To determine the mechanisms of action of cytokine-mediated inflammatory response gene signalling, focusing on mechanisms of TNF-α-stimulated eotaxin expression.
3) To study the mechanisms by which existing asthma therapies, namely GC and LABA, affect inflammatory response gene expression.
4) Through (1), (2) and (3), to identify potential common downstream signalling moieties shared by inflammatory mediator-mediated and cytokine-mediated pathways that may have therapeutic potential as new drug targets for asthma.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Human ET-1, DMEM, penicillin/streptomycin, L-Glutamine, amphotericin B, actinomycin D, LY294002, Wortmannin, Salmeterol, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue [MTT]), RedTaq DNA polymerase and NuCLEAR extraction kits were purchased from Sigma, Poole, Dorset, UK. Foetal calf serum was from Seralab, Loughborough, Leicestershire, UK. SB203580, PD98059, SP600125 and BQ788 were purchased from Tocris, Bristol, Avon, UK and BQ123 was purchased from Merck Biosciences, Nottingham, Nottinghamshire, UK. Bosentan was a gift from Dr. Marc Iglarz, Actelion Pharmaceuticals, Allschwil, Basel, Switzerland. Fluticasone was a gift from Dr. Malcolm Johnson and 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide (TPCA-1) was a gift from Dr. Rick Williamson, both of GlaxoSmithKline, Uxbridge, Middlesex, UK. MCP-1 and eotaxin ELISA kits and recombinant human TNF-α were purchased from R&D Systems, Abingdon, Oxfordshire, UK. RNeasy mini kits were from Qiagen, Crawley, West Sussex, UK. All reagents for reverse transcription and the Dual Luciferase Reporter Assay System were purchased from Promega, Southampton, Hampshire, UK. GAPDH, ET_A receptor, ET_B receptor, MCP-1 and β2 microblobulin primers were purchased from Sigma Genosys, Haverhill, Suffolk, UK. Primers for ChIP assay, spanning the MCP-1 and eotaxin promoters, were purchased from MWG, Ebersberg, Germany. Excite Real Time Mastermix with SYBR Green was from Biogene, Cambridge, Cambridgshire, UK. Nitrocellulose membrane for Western blotting and Bradford assay dye reagent were purchased from Bio-Rad, Hemel Hempstead,

Hertfordshire, UK. Anti-p38 MAP kinase and anti-p44/42 MAP kinase antibodies (total and phospho-) were from Cell Signaling/New England Biolabs, Hitchin, Hertfordshire, UK. Horseradish peroxidase-conjugated secondary antibodies were from DakoCytomation, Ely, Cambridshire, UK. ECL Western blotting detection reagent, Hyperfilm-ECL and rainbow coloured molecular weight markers were from Amersham, Buckinghamshire, UK. Fugene 6 transfection reagent was from Roche Molecular Biochemicals, Lewes, East Sussex, UK. ChIP-IT Express kit was from Active Motif, Rixensart, Belgium. NF-κB p65 and cJun antibodies were from Santa Cruz/Insight, Wembley, Middlesex, UK. Total HDAC activity kit, acetyl histone H3, HDAC-1 and HDAC-2 antibodies were from Upstate/Millipore, Watford, Hertfordshire, UK. GAPDH and α-tubulin antibodies were from AbD Serotec, Kidlington, Oxford, UK.

3.2 Human airway smooth muscle cell culture

Human airway smooth muscle cells (HASMC) were prepared as described previously (148, 301). Human trachea was obtained from post mortem individuals within 12h of death. Clinical and demographic characteristics of the donors (where available) are shown in Table 3.1. Tissue was transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 2.5μg/ml amphotericin B, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated foetal calf serum (FCS, CM^+ media). The trachealis smooth muscle was dissected free of epithelium and connective tissue under sterile conditions. Small 2 x 2 mm explants of airway smooth muscle were then excised and several explants placed in small petri

dishes. Explants were then bathed in media and incubated in CM^+ in humidified 5% CO_2-95% air at 37°C. Medium was changed every three days, and when cells were approaching confluence, the explants were removed. Once confluent, cells were trypsinized with 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS), centrifuged, resuspended in CM^+ and plated out in 75 cm^2 flasks. Once cells had again reached confluence, the passaging procedure was repeated and cells resuspended in 90% FCS + 10% dimethyl sulphoxide (DMSO), frozen in liquid nitrogen and stored until required. The cultured tissue was positively identified as HASMC through the combination of characteristic light microscopic appearances, positive staining for smooth muscle actin, desmin and myosin heavy chain, and negative staining for cytokeratin. Cells were cultured in CM^+ in humidified 5% CO_2-95% air at 37°C. Cells at passage 6-7 were used for all experiments. The studies were approved by the Nottingham Local Research Ethics Committee.

Donor	**Experimental chapter(s) in which donor's cells used**	**Age (years)**	**Sex**	**Notes**
1	4	44	Male	No evidence of airway disease
2	4	52	Female	No evidence of airway disease
3	4 and 5	No demographic or clinical data available		
4	5	34	Female	No clinical data available
5	5	42	Male	No clinical data available

Table 3.1: Demographic and, where available, clinical characteristics of airway smooth muscle cell donors. All donor tissue was tracheal in origin.

3.3 Time course, concentration response and inhibitor studies

Confluent HASMC in 24 well plates were growth arrested for 24 h in serum free medium and then stimulated at 37°C with ET-1 or TNF-α (0 – 10 ng/ml) for 24 h in concentration response experiments, or with ET-1 or TNF-α (10 ng/ml) for the times indicated in kinetic experiments. After incubation for 24 h, cell culture supernatants were transferred to separate microfuge tubes and stored at -20°C until determination of MCP-1 or eotaxin content.

In inhibitor studies, cells were pre-incubated for 30 min with inhibitors or vehicle prior to treatment with ET-1 or TNF-α for the times indicated. The vehicle was dimethylsulphoxide (DMSO) for all inhibitors except for Bosentan, which was dissolved in water. Vehicle was added to control wells at equivalent concentrations (maximum concentration DMSO 0.2%).

3.4 MCP-1 and eotaxin release

MCP-1 or eotaxin concentrations in cell culture supernatants were measured by enzyme-linked immunosorbant assay (ELISA) using commercially available sandwich ELISA kits according to the manufacturers instructions as previously described (250). 96 well mirotitre plates were coated with human MCP-1 or eotaxin-specific capture antibody at 1 μg/ml (MCP-1) or 2 μg/ml (eotaxin) in phosphate buffered saline (PBS) and incubated overnight at room temperature. Plates were then washed in 0.05% Tween 20 in PBS and blocked for 1 h at room temperature with 1% bovine serum albumin (BSA)/5 % sucrose in PBS, to prevent non-specific binding. An eight point standard curve was generated

by serial two-fold dilution of recombinant human MCP-1 or eotaxin (supplied) in 1% BSA in PBS, with a top standard of 1000 pg/ml. Standards or samples (100 μl) were then added to wells in duplicate and incubated at room temperature for 2h. Plates were washed again, 100 μl of detection antibody (100 ng/ml in 1% BSA) added to each well and samples incubated for 2 h at room temperature. After a further wash, samples were incubated for 20 min at room temperature with 100 μl streptavadin-conjugated horseradish peroxidase (streptavidin-HRP, supplied), diluted 1:200 in 1% BSA in PBS. Plates were then washed again before addition of 100 μl per well of substrate solution (a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine) for 20 min at room temperature. The resulting colorimetric reaction was stopped by addition of 50 μl per well of 1M sulphuric acid. The optical density of the wells was read at 450 nm with reference wavelength 570 nm. The concentration of MCP-1 or eotaxin in samples was determined with reference to the standard curve using a computer-generated 4-PL curve fit.

3.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

3.5.1 RNA extraction

Confluent, growth arrested HASMC in 24 well plates were treated with serum-free medium (controls) or serum-free medium containing ET-1 or TNF-α for the times indicated. Three wells of a 24 well plate were used for each condition. Following aspiration of cell culture supernatants, cells were washed with phosphate buffered saline (PBS). PBS was removed and discarded and RNA was extracted and purified using the RNEasy mini kit according to the manufacturer's instructions. The concentration of RNA in the purified samples

was measured spectrophotometrically. The absorbance at 260 nm (RNA content) and 280 nm (protein content) of a 1:20 dilution of purified RNA was measured. RNA concentration in μg/ml was calculated by multiplying A_{260} by 20 (dilution factor) x 38 (extinction coefficient for RNA).

3.5.2 Reverse transcription

An equal quantity of RNA (typically 0.5 – 1 μg) from each sample was used for the RT step. RNA was reverse transcribed in a total reaction volume of 25 μl of 1x Moloney murine leukaemia virus (M-MLV) RT buffer, containing 130 units of M-MLV reverse transcriptase, 26 units of RNase inhibitor, 0.6 μg oligo$(dT)_{15}$ primer and a 2 mM concentration of each dNTP. The reaction was incubated at 42°C for 90 min.

3.5.3 Polymerase chain reaction

Aliquots of RT products were subsequently used for polymerase chain reaction (PCR). RT products (2μl) were amplified in a total reaction volume of 50 μl 1x PCR buffer containing 1.5 mM magnesium chloride, 0.2 mM of each dNTP and 0.5 μM of both the sense and antisense primers. Reactions were "hot started" by addition of 2.5 units of RedTaq DNA polymerase during the initial denaturation step at 95°C. Amplification was carried out in a PTC 100 thermal cycler (MJ Research, Inc). Primer sequences and amplification time and temperature profiles are described under experimental protocols in chapter 4. The PCR products were visualized by eletrophoresis on 1% agarose gel in 0.5x TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) after staining with 0.5 μg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were

photographed, and the densitometry was analyzed using the GeneGenius gel documentation and analysis system (Syngene, Cambridge, UK).

3.6 Quantitative real time RT-PCR

MCP-1, HDAC-1 and HDAC-2 mRNA expression was determined by quantitative real time RT-PCR (qRT-PCR). β-2-microglobulin was used as the housekeeping gene (302). Reverse transcribed cDNA was subjected to real time PCR using Excite Real Time Mastermix with SYBR Green and the ABI Prism 7700 detection system (Applied Biosystems, Warrington, Cheshire, UK). Each reaction consisted of 1x Excite mastermix, SYBR Green (1:60,000 final concentration), 40nM of both sense and antisense primers, 1.6µl DNA (or dH_2O) and H_2O to a final volume of 20µl. Thermal cycler conditions included incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minute. Integration of the fluorescent SYBR Green into the PCR product was monitored after each annealing step. Amplification of one specific product was confirmed by melt curve analysis where a single melting peak eliminated the possibility of primer-dimer association. For melting curve analysis to be performed the products were heated from 60°C to 95°C over 20 min after the 40 cycles.

To enable the levels of transcripts to be quantified standard curves were generated using serial dilutions of HASM cDNA. Negative controls consisting of no template were included and all reactions were set up in triplicate. MCP-1 or HDAC expression was normalised to the housekeeping gene by dividing the MCP-1 or HDAC triplicate value by the mean of the β-2-microglobulin

triplicate value. Primer sequences used for qRT-PCR are described under experimental protocols in chapters 4 and 5.

3.7 Western blot analysis

HASM cells were grown to confluence in 6 well plates and growth arrested for 24 h. Two wells were used for each condition. Immediately before each experiment, media was changed to fresh serum-free media and cells were treated with ET-1 (10 ng/ml) or TNF-α (10 ng/ml) for the times indicated. In inhibitor studies, cells were pre-incubated with vehicle or inhibitor for 30 min prior to stimulation. The medium was removed, the cells were washed with PBS and lysed with 100µl lysis/protein extraction buffer consisting of 25 mM Tris·HCl, pH 6.8, 75 mM NaCl, 5 mM EDTA, 0.25% wt/vol sodium deoxycholate, 0.05% wt/vol sodium dodecyl sulphate (SDS) and 0.5% vol/vol Triton X-100 supplemented with PMSF (0.1 mg/ml), leupeptin (10 µg/ml), and aprotinin (25 µg/ml). The protein content of the extracts was determined by Bradford assay (303), and the samples were then diluted 1:4 in Laemmli buffer (0.125 M Tris·HCl, 20% vol/vol glycerol, 0.2% wt/vol SDS, 6% vol/vol β-mercaptoethanol and 0.2% wt/vol bromophenol blue) and boiled for 5 min. Denatured proteins (25 µg) were separated by SDS-PAGE using an 8 cm x 8 cm 7.5% SDS gel and electroblotted onto an Immuno-Blot PVDF membrane in Tris buffer (20 mM Tris base, pH 8.3, 192 mM glycine, 20% vol/vol methanol). The membrane was blocked overnight in Tris-buffered saline-Tween (TBS-T, 20 mM Tris-base, pH 7.4, 150 mM NaCl, 0.1% vol/vol Tween 20) containing 5% wt/vol non-fat dry milk (hereafter referred to as 5% milk). After washing in TBS-T, the membrane was then incubated with 1:1000

dilution of primary antibody overnight at 4°C in TBS-T containing 5% wt/vol BSA (for phosphokinase or total kinase antibodies) or 1 h at room temperature in 5% milk (for all other primary antibodies). Blots were washed with TBS-T and incubated with 1:3000 dilution of the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody in 5% milk for 1 h at room temperature. The blot was washed in TBS-T and staining was achieved with enhanced chemiluminescence (ECL) Western blotting detection reagent. The blot was incubated with ECL reagent for 45 sec and finally exposed to high performance chemiluminescence film. The position and molecular weight of the protein of interest was validated by reference to rainbow coloured molecular weight markers. Reprobing of the blot for the loading controls GAPDH or α-tubulin was carried out by blocking the membrane for 1 h at room temperature in 5% milk then following the above steps to detect GAPDH/α-tubulin with anti-GAPDH antibody (1:20000 dilution) or anti-α-tubulin antibody (1:5000) in 5% milk.

3.8 Vectors and transient transfections

3.8.1 Vectors

MCP-1 promoter/enhancer vectors consisted of the pGL3-basic plasmid vector containing either the wild-type human MCP-1 enhancer region (-2802 to -2573 relative to the translation start codon) or the proximal section of the wild type human MCP-1 promoter region (-167 to -1, Figure 3.1) upstream of a luciferase reporter gene. These constructs have previously been described in detail (250).

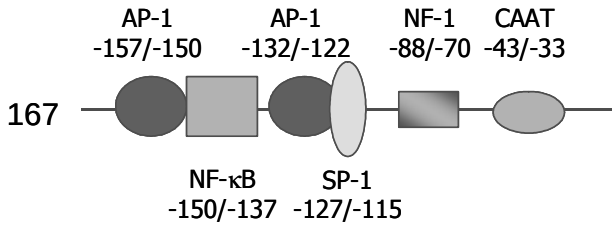

Figure 3.1 The human MCP-1 gene is regulated by a distal enhancer region containing two nuclear factor kappa B (NF-κB) consensus sequences (not shown), and a more complex proximal promoter region. The MCP-1 promoter construct used in this study consisted of the 167 bp upstream of the translational start codon driving a luciferase reporter gene. Numbers refer to the 5' nucleotide relative to the translation start site. AP-1: activator protein-1; NF-κB: nuclear factor-κB; Sp1: small protein 1; NF-1: nuclear factor-1.

The MCP-1 promoter construct deletion series was a gift from Dr. Garzino Demo (University of Maryland, USA) (304) and consisted of the PGL2-basic plasmid vector containing the 486, 213 or 128 bp upstream of the translational start codon driving a luciferase reporter gene (Figure 3.2).

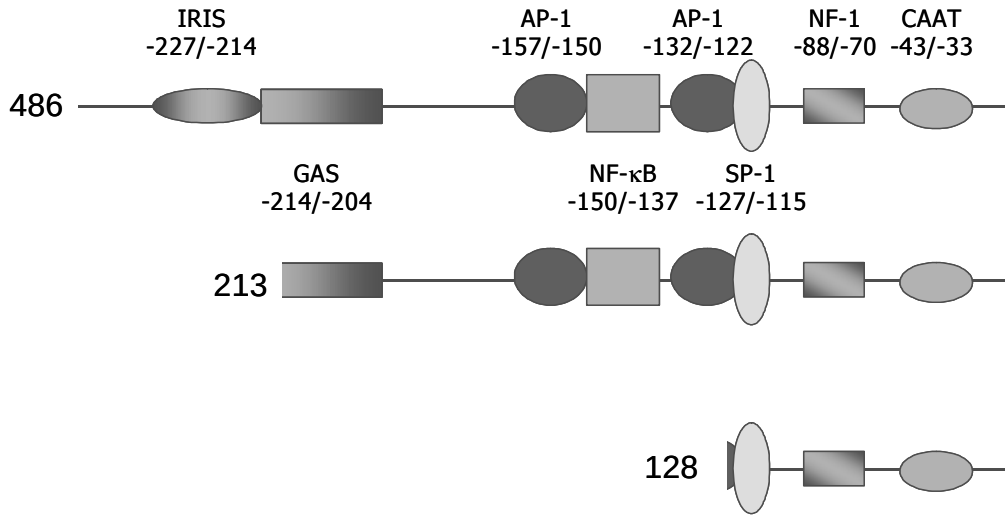

Figure 3.2 MCP-1 promoter deletion series. The MCP-1 promoter deletion series consisted of the 486, 213 or 128 bp upstream of the translational start codon driving a luciferase reporter gene. IRIS: interferon response inhibitory sequence; GAS: gamma activated site.

The NF-κB reporter construct 6NF-κBtkluc was a gift from Dr. Robert Newton (University of Calgary, Canada) and contains three tandem repeats of the sequence 5'-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3', which harbours two copies of the NF-κB binding site (underlined) upstream of a minimal thymidine kinase promoter driving a luciferase reporter gene (305). The AP-1 reporter construct pRTU14 was a gift from Dr. Arnd Kieser (GSF National Research Centre, Munich, Germany) and consists of a luciferase gene under the control of a minimal promoter and four TREs (12-*O*-tetradecanoate-13-acetate responsive element, to which the AP-1 transcription factor binds) (306).

3.8.2 Transfection protocol

All transient transfections were conducted using FuGene 6 transfection reagent according to the manufacturer's recommended protocol. HASMC were seeded into 24-well plates at a concentration of 2.5 x 10^4/ml. When cells reached 50 to 60% confluence they were growth arrested for 8 h in serum-free, antibiotic free DMEM containing 4 mM L-glutamine. After 8 h, media was changed for 400 µl of fresh, serum-free, antibiotic-free DMEM. DNA was complexed with FuGene 6 in serum-free, antibiotic free DMEM at a ratio of 1 µg DNA:3 µl FuGene 6. 100 µl of DNA:FuGene 6 complex was added to each well of the 24 well plate to give a final amount of 0.4 µg DNA:1.2 µl FuGene 6 per well. After 16 h of incubation, the media was changed and the transfected cells were treated with ET-1 (10 ng/ml) or TNF-α at the concentrations indicated, for the indicated times. The cells were then washed with PBS and lysed in 100 µl of passive lysis buffer. Firefly luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega) with a MicroLumatPlus LB96V Automatic Microplate Luminometer (Berthold Technologies, Herts, United Kingdom).

3.9 Chromatin immunoprecipitation assay

ChIP was performed using the ChIP-IT™ Express kit (Active Motif). HASMC in 75cm^2 dishes at 80-90% confluence were growth arrested for 24 h and incubated with ET-1 (10 ng/ml) or TNF-α (10 ng/ml) for the times indicated. Cells were then fixed by incubation for 10 min at room temperature with 1% formaldehyde. The 1% formaldehyde media were removed and the cells washed in ice-cold PBS. The reaction was stopped with 0.1 M glycine (5 min

room temperature) and the cells washed again with PBS. The cells were then collected in 2 ml PBS supplemented with PMSF and pelleted for 10 min at 2500 rpm at 4°C. The cells were re-suspended in lysis buffer (supplied) containing PMSF and a protease inhibitor cocktail (PIC) and incubated on ice for 30 min. Cells were centrifuged at 5000 rpm for 10 min at 4°C and the pellet re-suspended in shearing buffer (supplied) supplemented with protease inhibitors. Samples were subsequently sonicated (5 x 10 sec pulses with incubation on ice between each cycle of sonication) into chromatin fragments of an average length of 500 - 800 bp. The sheared chromatin was centrifuged at 13,000 rpm at 4°C for 12 min and the supernatants, containing the sheared chromatin, retained. Ten µl of chromatin from each sample was transferred to a fresh microfuge tube and reserved as "input" DNA. The remaining chromatin was aliquoted and incubated with protein G magnetic beads and antibody (4 µg) directed against the transcription factor or cofactor of interest overnight at 4°C with rotation. Immunoprecipitated material was washed 3 times with wash buffer (supplied), and eluted from the magnetic beads by incubation in elution buffer (supplied) for 15 min at room temperature with rotation. Cross-links were reversed by addition of reverse cross link buffer (supplied) followed by incubation at 65°C for 2.5 h. Input samples were subject to the same incubation to reverse cross links after dilution 1:10 into ChIP buffer 2 (supplied) and addition of NaCl to a final concentration of 0.1 M. Recovered material was treated with proteinase K and incubated at 37°C for 60 min to digest the proteins. Input samples were subject to an additional purification step prior to PCR analysis. Input samples were treated with RNAse A and incubated at 37°C for 30 min prior to phenol chloroform

extraction. The recovered DNA was quantified by PCR as described in section 3.5.3, using the specific primers spanning the regulatory region of the human MCP-1 or eotaxin promoters and the following time and temperature profiles: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 20 sec, primer annealing at 59°C for 30 sec, primer extension at 72°C for 30 sec.

3.10 Cell viability (MTT) assay

The toxicity of inhibitors and the vehicle DMSO to HASMC was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue [MTT], Sigma). After incubation with the chemicals for the indicated times, the cell culture supernatants were aspirated and 250 µl of 1 mg/ml MTT in serum-free medium was added to each well of a 24 well plate and incubated for 20 min at 37°C. The MTT medium was removed and 250 µl of DMSO was added to solubilize the blue-coloured tetrazolium. Optical densities were read at 550 nm in a microplate reader. Viability was compared to that of control cells, with viability of controls defined as 100%.

CHAPTER 4: REGULATION OF MONOCYTE CHEMOTACTIC PROTEIN-1 EXPRESSION BY ENDOTHELIN-1

4.1 Introduction

As described in chapter 1, asthma is an inflammatory disease of the airways. Resolution of inflammation normally results in restoration of normal tissue structure and function. In chronic asthma, these processes become disordered, leading to airway remodelling, manifest as an increase in ASM mass, mucous gland hyperplasia, an increase and alteration in extracellular matrix (ECM) and subepithelial fibrosis (126). The increased thickness of the ASM layer is a key feature of the remodelled asthmatic airway. This is highly significant, since in addition to their contractile properties, ASM cells are a rich source of cytokines, mediators, chemokines, growth factors and matrix modifying enzymes that contribute to airway inflammation and remodelling.

Inflammatory response genes can be switched on by Th-1 (307) or Th-2 (308) cytokines or inflammatory mediators acting at 7 transmembrane GPCRs (309). Activation of gene expression by GPCRs provides an important means of local production of cytokines and growth factors which contribute to inflammation and remodelling (309). As we discussed in chapter 1, in contrast to gene expression events mediated by cytokines, the nuclear signalling cascades used by GPCRs have not been well characterised. Human ASM cells (HASMC) are a useful model system to study GPCR signalling in primary cells as they express the GPCRs for many important inflammatory mediators (178, 310).

The Knox lab have previously shown that the inflammatory mediator BK can actively signal to the nucleus to mediate gene transcription events using complex prostanoid dependent signalling pathways involving AP-1, NF-κB

and C/EBP (311, 312). These signals are transduced by the $G_{\alpha q}$-coupled B2 BK receptor. It is not clear whether these nuclear signalling pathways are unique to BK or shared by other $G_{\alpha q}$-linked GPCRs. Endothelin-1 (ET-1) is a 21 amino acid vasoactive and pro-inflammatory peptide that acts at $G_{\alpha q}$-coupled ET_A and ET_B receptors. As described in chapter 1, there is extensive evidence implicating ET-1 in the pathogenesis of asthma. There has been a wealth of studies of the effects of ET-1 on ASM contraction but, despite animal studies implicating ET-1 in airway inflammation, there have been very few previous studies of ET-1's effects on release of inflammatory cytokines and mediators from ASM. ET-1 has been shown to increase the release of the chemokines IL-8 and MCP-1 from several other cell types *in vitro* (241, 313, 314) and has been implicated in induction of chemokine release *in vivo* (241, 315, 316).

The ET_A and ET_B receptors activate calcium and inositol phosphate second messenger pathways (317-319). ET-1 can also activate the MAPK family of signalling proteins. For example, ET-1 activates ERK-2 and JNK in rat tracheal smooth muscle cells (319, 320). Similarly, ET-1 induces the MEK/ERK pathway in human lung fibroblasts (191), and the ERK 1/2, p46 and p54 JNKs and p38 MAPKs in cardiac myocytes (192). However, there have been few studies of nuclear signalling by ET-1 and, furthermore, the transcriptional and post-transcriptional regulation of chemokine expression by ET-1 has not been studied.

As described in chapter 1, MCP-1 is a chemokine of the CC subgroup which is chemotactic for monocytes (236, 237) and T-lymphocytes (238, 239) and is, like ET-1, implicated in the pathophysiology of asthma through multiple lines of evidence. The Knox group and others have shown that HASMC express MCP-1 and that levels of expression are upregulated by cytokines (242, 249, 250). However, there have been few studies in ASM of the effects on MCP-1 expression by asthma mediators acting at GPCRs, although it is known that bradykinin induces MCP-1 secretion from human lung fibroblasts (321). The aim of the experiments described in this chapter was to determine the mechanisms used by ET-1 to induce MCP-1 expression. We used a variety of molecular and pharmacological tools to characterise the signalling pathways involved. Specifically we tested the hypotheses that:

1. ET-1 stimulates MCP-1 release from HASMC.
2. This occurs through a transcriptional mechanism.
3. The downstream signalling pathways are MAP kinase dependent.

4.2 Experimental protocols

HASMC were grown in 6 well plates (Western blotting experiments), 75cm^2 flasks (ChIP assay) or 24 well plates (all other experiments) as described in chapter 3. Cells were growth arrested for 24 h prior to each experiment. The principle findings of this study were confirmed in tissue from three different donors. Subsequent mechanistic studies were performed on cells from one donor.

4.2.1 MCP-1 release from HASMC

Human MCP-1 release from HASMC was measured using a commercially available ELISA kit (R&D) as described in chapter 3.

4.2.2 Determination of ET receptor mRNA expression by polymerase chain reaction

RNA extraction, reverse transcription and PCR were carried out as described in chapter 3. The primer sequences used were as follows: GAPDH sense, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; GAPDH antisense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'; ET_A receptor sense, 5'-TGG CCT TTT GAT CAC AAT GAC TTT-3'; ET_A receptor antisense, 5'-TTT GAT GTG GCA TTG AGC ATA CAG GTT-3'; ET_B receptor sense, 5'-ACT GGC CAT TTG GAG CTG AGA TGT-3'; ET_B receptor antisense, 5'-CTG CAT GCC ACT TTT CTT TCT CAA-3' (182, 322). PCR was carried out using the following temperature and time profiles. GAPDH: initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, primer

annealing at 60°C for 30 sec, primer extension at 72°C for 30 sec. A final chain extension was carried out at 72°C for 10 min. ET receptors: initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, primer extension at 72°C for 3 min. Final chain extension was carried out at 72°C for 10 min.

4.2.3 Determination of MCP-1 mRNA expression by real time polymerase chain reaction

Real time PCR was carried out as described in chapter 3. Primer sequences used were as follows: MCP-1 sense, 5- GAT CTC AGT GCA GAG GCT CG -3'; MCP-1 antisense, 5'- TGC TTG TCC AGG TGG TCC AT -3' (250); β2 microglubulin sense, 5'- GAG TAT GCC TGC CGT GTG-3'; β2 microglobulin antisense, 5'- AAT CCA AAT GCG GCA TCT-3' (302);

4.2.4 Chromatin immunoprecipitation assay

ChIP assay was performed as described in chapter 3. Primer sequences used to amplify the immunoprecipitated products by PCR were as follows: MCP-1 promoter sense: 5'-CCC ATT TGC TCA TTT GGT CTC AGC-3'; MCP-1 promoter antisense: 5'-GCT GCT GTC TCT GCC TCT TAT TGA-3' (243).

4.2.5 Data Analysis

MCP-1 protein or mRNA or luciferase levels were expressed as the means of the individual technical replicates for that experiment. The experiments were repeated at least twice, and the results shown represent group means +/- SE. Absolute MCP-1 levels are presented for the initial concentration response and

time course experiments. In subsequent mechanistic studies, the data are expressed as fold increase or percentage of control. The number of independent experiments and technical replicates from which the data are derived is also indicated in the figure legends. Analysis of variance (ANOVA) of the raw data was used to determine statistically significant differences, using the statistical software package SPSS version 14.0. In time course experiments, the terms of the ANOVA included experiment, time and ET-1. Overall p-values for the effect of time and ET-1 are presented. Time dependence of the effect of ET-1 was determined by fitting an interaction between ET-1 and time. In concentration response experiments, the terms of the ANOVA included experiment and concentration. Overall p values for the effect of ET-1 or inhibitor are presented. Comparisons between individual concentrations of ET-1 and control, or inhibitor compared to ET-1-stimulated cells, were assessed using Dunnet's post-hoc correction for multiple comparisons. A p value of <0.05 was regarded as statistically significant.

4.3 Results

4.3.1 ET-1 stimulates the release of MCP-1 from cultured HASMC

We first studied the effect of ET-1 on the release of MCP-1 from HASMC. Sub-confluent, growth arrested cultured HASMC were treated for 24 h with increasing concentrations of ET-1 up to 10 ng/ml. ET-1 stimulated MCP-1 release in a concentration dependent manner (Figure 4.1). In other experiments where higher concentrations of ET-1 were used, no further increase in effect was observed (data not shown). Ten ng/ml of ET-1 was therefore used in all subsequent experiments. HASMC were next treated with 10 ng/ml of ET-1 for 0, 1, 2, 4, 8, 16 and 24 h. ET-1 caused a time dependent increase in MCP-1 release (Figure 4.2).

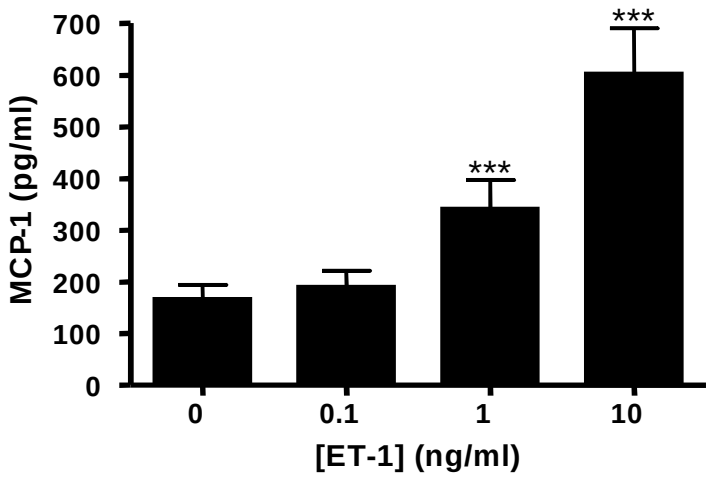

Figure 4.1 Concentration response of ET-1-stimulated MCP-1 production by HASMC. Cells were treated for 24 h with ET-1 at the concentrations indicated. ET-1 significantly increased MCP-1 release ($p<0.001$). ***$P<0.001$ compared with unstimulated cells. Each bar represents group mean (SE) derived from 13 replicates in 4 independent experiments (n=3 different primary donors).

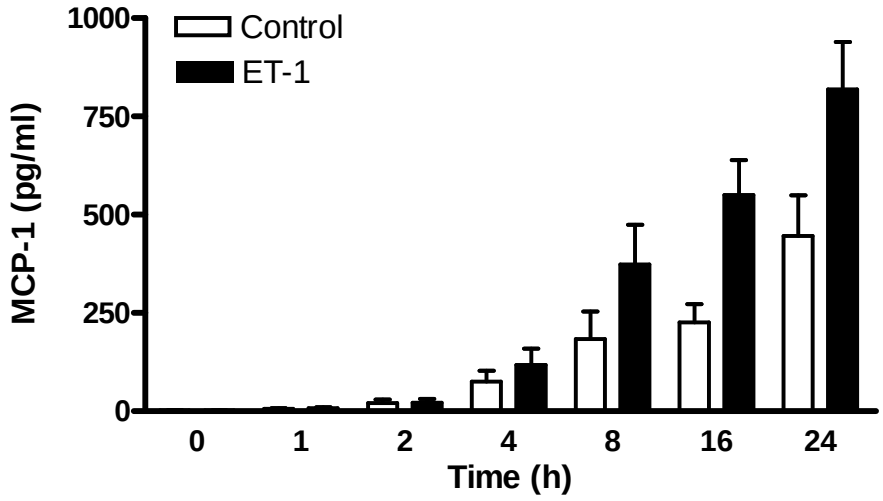

Figure 4.2 Time course of ET-1-stimulated MCP-1 production by HASMC. Cells were treated with ET-1 (10 ng/ml) for the indicated times. ET-1 significantly increased MCP-1 release from HASMC in a time-dependent manner (ET-1 vs control: $p<0.001$. Interaction between ET-1 and time: $p<0.001$). Each bar represents group mean (SE) derived from 18 replicates in 7 independent experiments (n=3 different primary donors).

4.3.2 HASMC express endothelin A and endothelin B receptor mRNA

Two endothelin receptors have been cloned and sequenced, the ET_A and ET_B receptors. To determine whether HASMC expressed these receptors we used RT-PCR to look at their RNA. RT- PCR demonstrated the presence of mRNA for both receptors in HASMC (Figure 4.3).

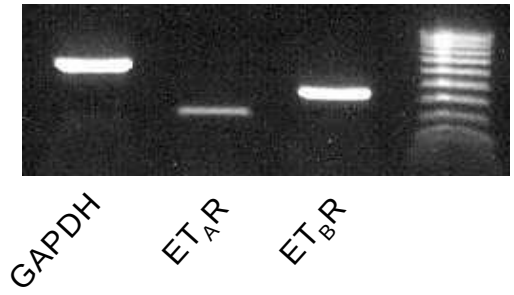

Figure 4.3 ET_A and ET_B receptor mRNA is expressed in HASMC. Representative gel from one of 3 independent experiments.

4.3.3 Effects of ET-1 on MCP-1 release are mediated by ET_A and ET_B receptors

We next performed pharmacological studies to explore the relative contributions of the two receptor subtypes to ET-1-mediated MCP-1 expression. The dual-selective ET receptor antagonist bosentan and the selective ET_A and ET_B receptor antagonists, BQ123 and BQ788 respectively, inhibited ET-1-stimulated MCP-1 production in a concentration-dependent manner (Figures 4.4 to 4.6). Both antagonists inhibited ET-1-stimulated MCP-1 production at concentrations equivalent to and ten-fold higher than their respective pA_2 values (6.9 - 7.4 and 6.9 for BQ123 and BQ788 respectively (323)). The antagonist concentrations were chosen to be selective for the relevant receptor, as both inhibitors are at least 3 orders of magnitude more selective for their respective receptors (324, 325). These data suggest that both ET_A and ET_B receptors are involved in this response. An additional effect was observed when BQ123 and BQ788 were used in combination at a submaximal concentration (Figure 4.7) but this was of borderline statistical significance ($p=0.05$).

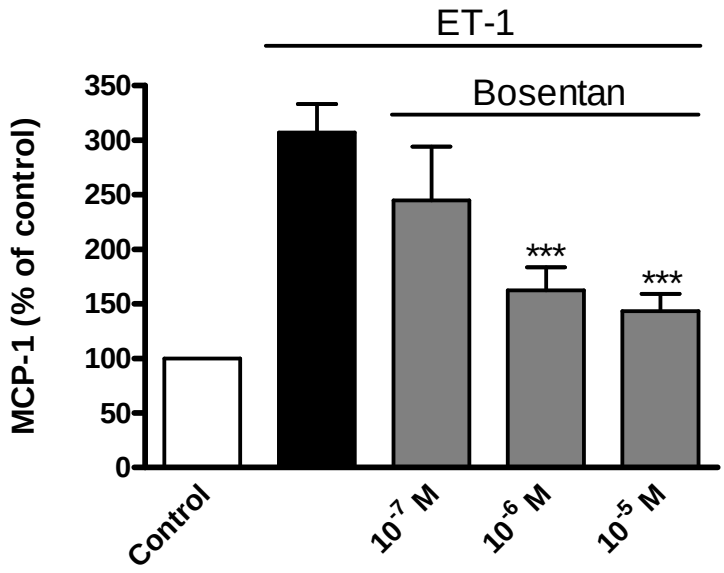

Figure 4.4 The dual selective ET receptor antagonist bosentan concentration-dependently inhibited ET-1-stimulated MCP-1 production ($p<0.001$). ***$P<0.001$ compared with ET-1-stimulated cells. Each bar represents group mean (SE) derived from 11 replicates in 3 independent experiments.

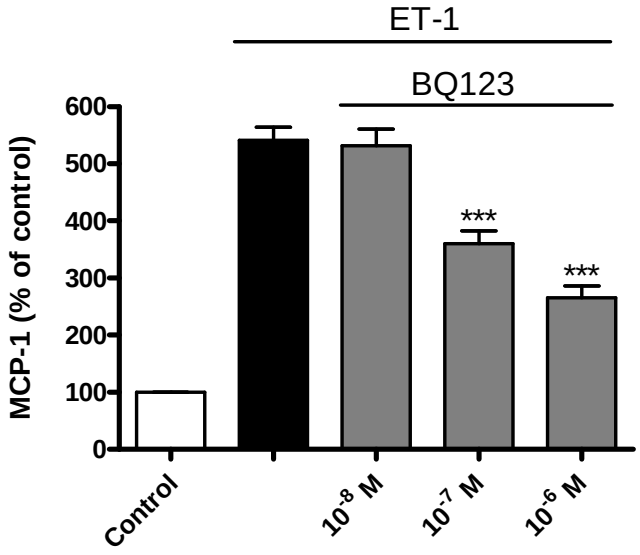

Figure 4.5 The selective ET_A receptor antagonist BQ123 concentration-dependently inhibited ET-1 stimulated MCP-1 production ($p<0.001$). ***P<0.001 compared with ET-1-stimulated cells.

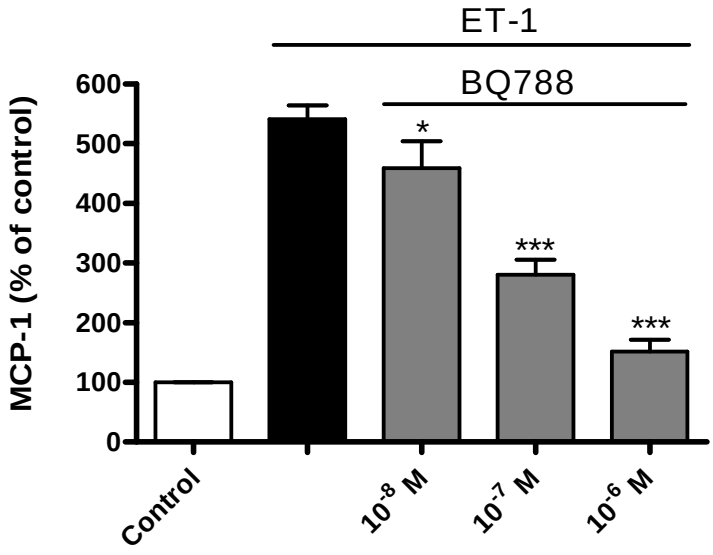

Figure 4.6 The selective ET_B receptor antagonist BQ788 concentration-dependently inhibited ET-1 stimulated MCP-1 production ($p<0.001$). *P=0.03; ***p<0.001 compared with ET-1-stimulated cells.

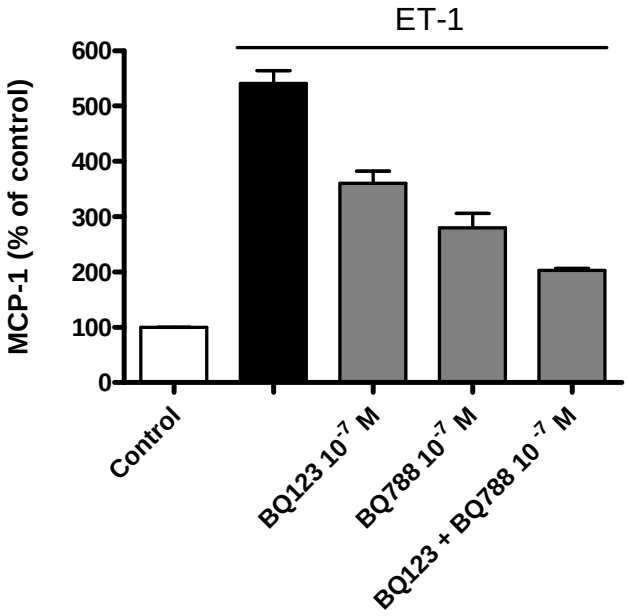

Figure 4.7 BQ123, BQ788 and both inhibitors in combination (10^{-7} M) significantly inhibited ET-1-stimulated MCP-1 production ($p<0.001$). P for interaction = 0.053.

Figures 4.5 – 4.7: each bar represents group mean (SE) derived from 6 replicates in 2 independent experiments.

4.3.4 Effects of ET-1 on MCP-1 release are mediated by p44/p42 and p38 MAP kinases but not by JNK or PI3 kinase

MAP kinases are involved in multiple inflammatory pathways, including airway inflammation (326) and, furthermore, it is known that ET-1 can activate MAP kinases in ASM and other biological systems (191, 319, 320, 327). We therefore hypothesised that ET-1 may stimulate MCP-1 release through MAP kinase dependent pathways. PD98059 (20 μM), a selective inhibitor of MEK, immediately upstream of p44/p42 MAPK, or ERK (328, 329), inhibited ET-1 stimulated MCP-1 production (Figure 4.8) suggesting a role for p44/p42 MAP kinase. Consistent with this, Western blotting demonstrated a time dependent increase in phosphorylation of p44/p42 MAP kinase following stimulation with ET-1 (Figure 4.9).

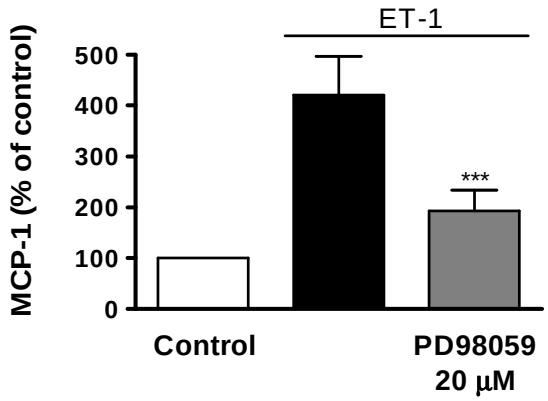

Figure 4.8 Effect of PD98059 on ET-1-stimulated MCP-1 production. PD98059 (20 μM) inhibited ET-1-stimulated MCP-1 production. ***p<0.001 compared with cells treated with ET-1 alone. Each bar represents group mean (SE) derived from 11 replicates in 3 independent experiments.

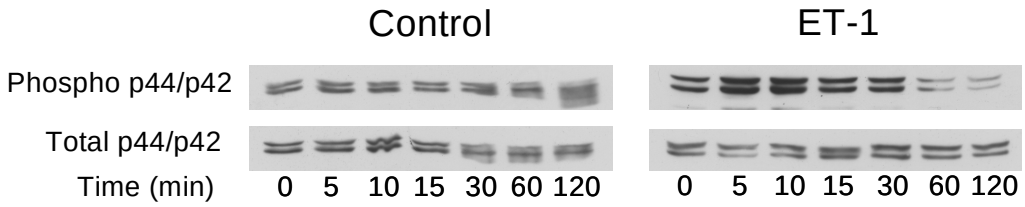

Figure 4.9 Western blot showing time-dependent phosphorylation of p44/p42 MAPK by ET-1. An increase in phospho-p44/p42 MAPK is seen at 5 minutes and is sustained until 30 minutes. Representative blot from one of 4 independent experiments.

The p38 MAP kinase inhibitor SB203580 (20 μM) also inhibited ET-1-stimulated MCP-1 production (Figure 4.10) (330). Furthermore, ET-1 caused an increase in phosphorylation of p38 on Western blotting (Figure 4.11).

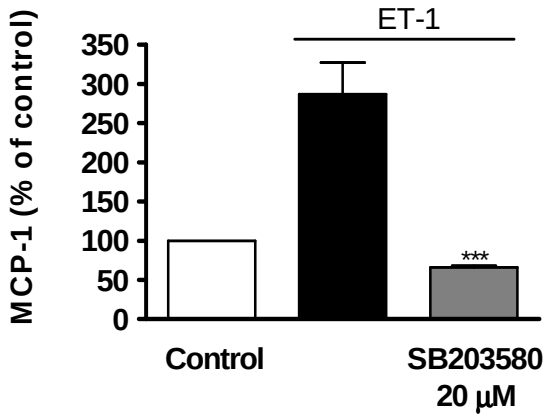

Figure 4.10 Effect of SB203580 on ET-1-stimulated MCP-1 production. SB203580 (20 μM) inhibited ET-1-stimulated MCP-1 production. ***P<0.001 compared with cells treated with ET-1 alone. Each bar represents mean (SE) of 16 replicates in 2 independent experiments.

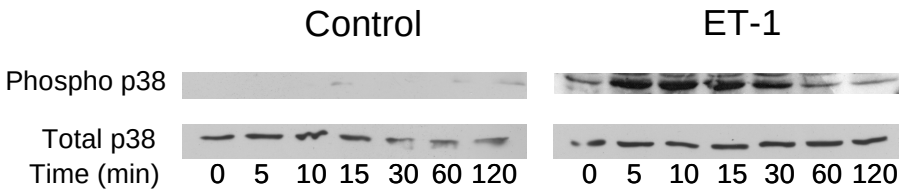

Figure 4.11 Western blot showing time-dependent phosphorylation of p38 MAPK by ET-1. An increase in phospho-p38 MAPK is seen at 5 minutes and is sustained until 30 minutes. Representative blot from one of 3 independent experiments.

In contrast to our observations with PD98059 and SB203580, the selective JNK inhibitor SP600125 (10 μM) (331) and the PI3 kinase inhibitors Wortmannin (100 nM) and LY294002 (1 μM) (332, 333) had no effect on ET-1-stimulated MCP-1 production (Figures 4.12 and 4.13).

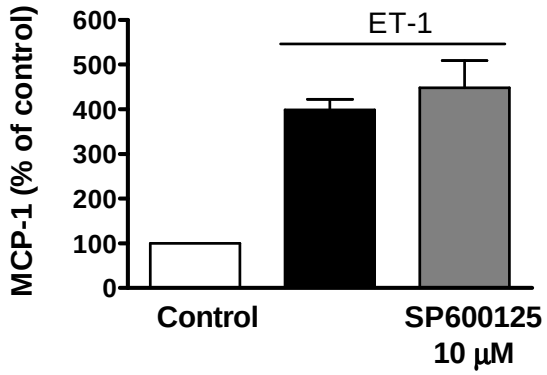

Figure 4.12 The JNK inhibitor SP600125 (10μM) had no effect on ET-1-stimulated MCP-1 production. Each bar represents group mean (SE) derived from 10 replicates in 3 independent experiments.

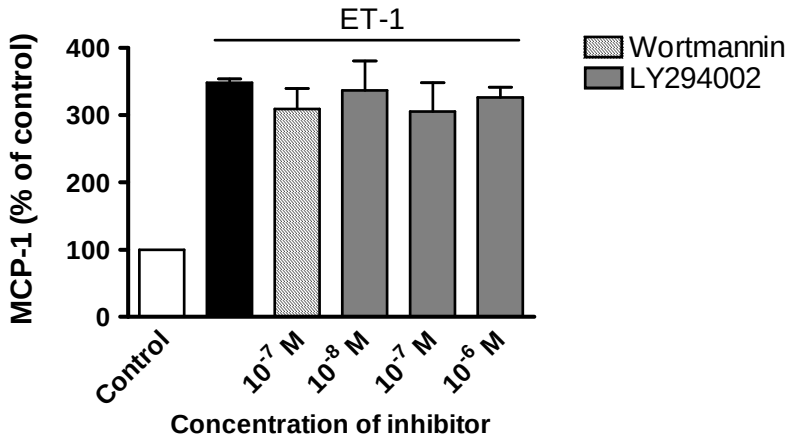

Figure 4.13 Effect of the PI 3 kinase inhibitors wortmannin and LY294002 on ET-1-stimulated MCP-1 production. Wortmannin (10^{-7} M) and LY294002 (10^{-6} M) had no effect on ET-1-stimulated MCP-1 production. Each bar represents group mean (SE) derived from 8 replicates in 2 independent experiments.

4.3.5 ET-1 increases MCP-1 mRNA levels

We next tested the hypothesis that the regulation of MCP-1 expression by ET-1 occurs at a transcriptional level. We used qRT-PCR to determine the levels of MCP-1 mRNA following treatment of HASMC with ET-1 (10 ng/ml). MCP-1 mRNA levels were normalised to the house keeping gene β2 microblogulin. ET-1 significantly increased MCP-1 mRNA levels relative to control (unstimulated) cells (Figure 4.14). The increase was detectable at 2 h, and was sustained throughout the 24 hour period studied.

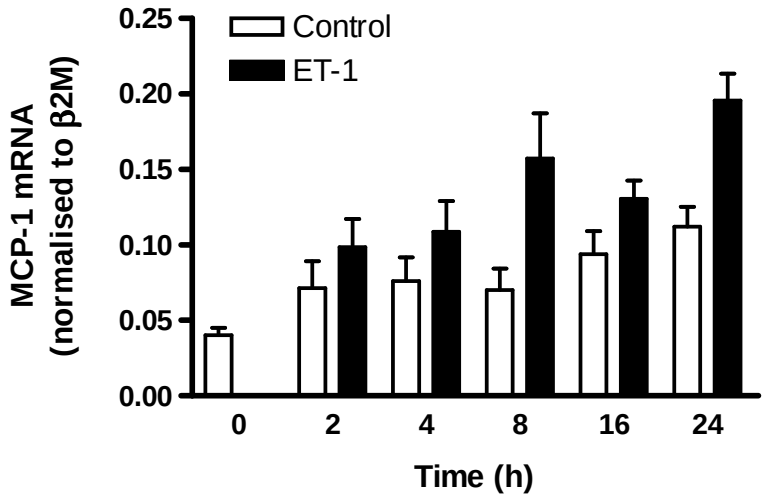

Figure 4.14 ET-1 increased expression of MCP-1 mRNA measured by qRT-PCR ($p<0.001$ compared to control cells). The effect was time dependent (p for interaction between ET-1 and time = 0.003). MCP-1 mRNA was normalised to the housekeeping gene β2 microglobulin. Each bar represents group mean (SE) derived from 12 replicates in 4 independent experiments.

4.3.6 ET-1 does not affect MCP-1 mRNA stability

We performed mRNA stability studies with the transcription inhibitor actinomycin D (Act D) to determine whether ET-1's effects on MCP-1 mRNA levels were due to stabilisation of the MCP-1 mRNA. MCP-1 mRNA is constitutively expressed in HASMC. Cells were incubated for 0 to 24 h with 1 μg/ml of Act D alone (which blocks the production of new transcripts), or Act D and ET-1 in combination. ET-1 had no effect on the rate of decay of MCP-1 transcripts, indicating that ET-1 was not acting to stabilise MCP-1 mRNA (Figure 4.15).

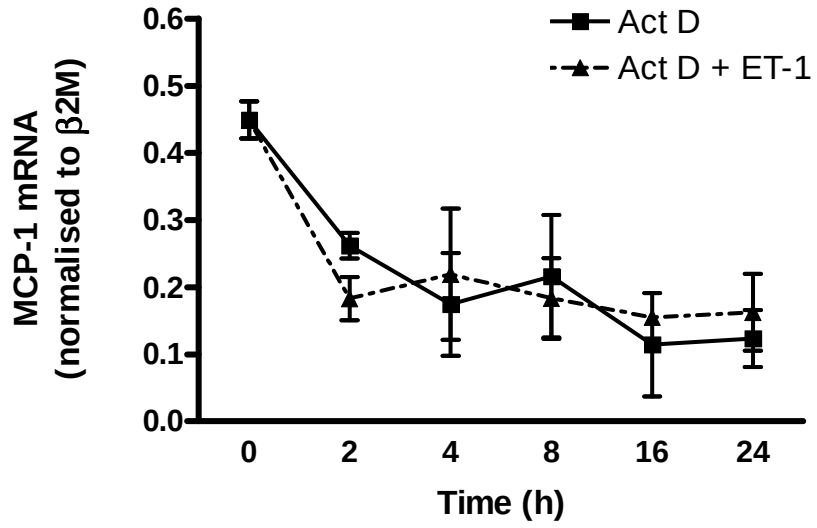

Figure 4.15 Effect of ET-1 on MCP-1 mRNA stability. ET-1 treatment had no effect on the rate of decay of MCP-1 transcripts. Each point represents group mean (SE) derived from 6 replicates in 3 independent experiments.

4.3.7 ET-1 increases MCP-1 promoter activity

Next, vectors encoding the wild type MCP-1 promoter or enhancer regions upstream of a luciferase reporter gene were transiently transfected into HASMC. Consistent with the qRT-PCR results, ET-1 caused a 2.2-fold increase in MCP-1 promoter-driven luciferase reporter gene expression at 6 h. This effect was still detectable at 16 h as a 1.7-fold increase (Figure 4.16). There was a trend towards a smaller and later stimulation of MCP-1 enhancer-driven reporter gene expression, with a 1.7-fold increase detected following 16 h incubation with ET-1 (Figure 4.17), although this did not reach statistical significance (p=0.1).

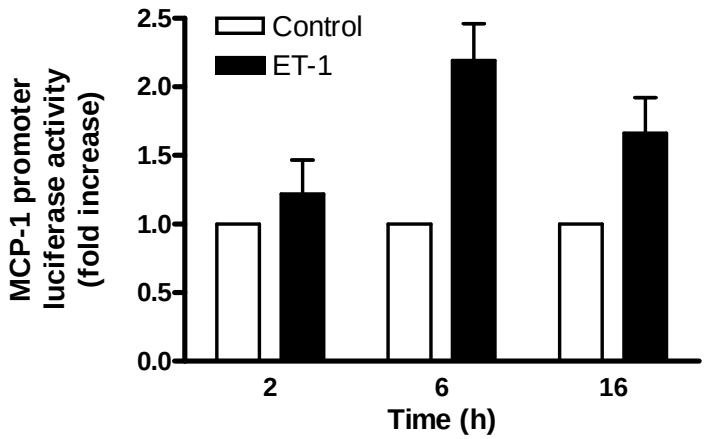

Figure 4.16 Effect of ET-1 on MCP-1 promoter activity. ET-1 stimulated MCP-1 promoter-driven luciferase activity (p=0.03 compared to controls). Each bar represents group mean (SE) derived from 18 replicates in 3 independent experiments.

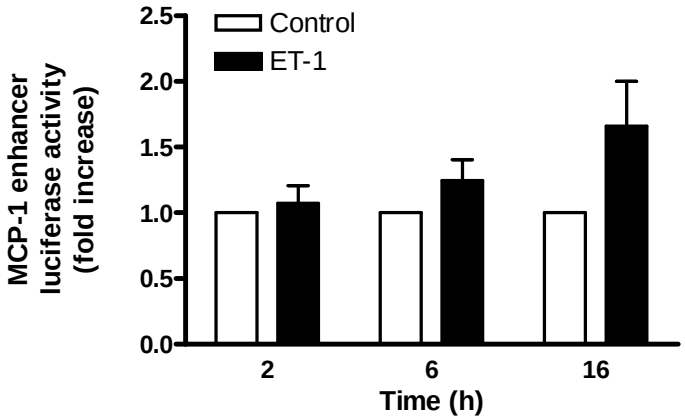

Figure 4.17 Effect of ET-1 on MCP-1 enhancer activity. Although there was a trend towards an effect of ET-1 on MCP-1 enhancer-driven luciferase activity, this was not statistically significant (p=0.106 compared to controls). Each bar represents group mean (SE) derived from 16-26 replicates in 3-6 independent experiments.

4.3.8 Effects of ET-1 on the MCP-1 promoter map to a region between 213 and 128 base pairs upstream of the translational start codon

In order to determine which region of the MCP-1 promoter was responsible for ET-1's effects, HASMC were transiently transfected with constructs expressing serial deletions of the human MCP-1 promoter, consisting of the 486, 213 or 128 bp upstream of the translational start codon. Cells were stimulated with ET-1 for 6 h, since maximal transactivation of the wild-type promoter was observed at this time. ET-1 significantly up-regulated activity of the 486 and 213 constructs but had no effect on activity of the 128 construct, suggesting that the region between 213 and 128 bp upstream of the translational start codon is required for the effects of ET-1 on MCP-1 transcriptional activation (Figure 4.18).

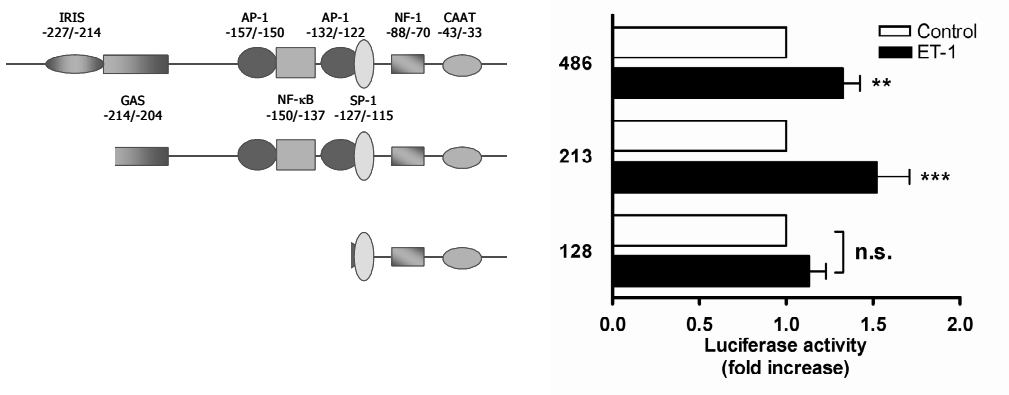

Figure 4.18 HASMC were transiently transfected with serially deleted MCP-1 promoter constructs consisting of the 486, 213 or 128 bp upstream of the translational start codon and stimulated with ET-1 for 6 h. ET-1 stimulated luciferase activity driven by the 486 and 213 constructs but had no effect on the 128 construct. **p=0.008; ***p<0.001. Each bar represents group mean (SE) derived from 18 replicates in 3 independent experiments. IRIS = interferon regulated inhibitory sequence; GAS = gamma activated site; AP-1 = activator protein-1; NF-κB = nuclear factor-κB; Sp1 = small protein-1; NF-1 = nuclear factor 1.

4.3.9 ET-1 stimulates activity of an NF-κB and an AP-1 reporter construct

The region between 128 and 213 bp upstream of the MCP-1 translational start codon contains consensus sequences for the transcription factors NF-κB and AP-1. To confirm that ET-1 can activate these transcription factors in HASMC, we transiently transfected HASMC with NF-κB and AP-1 reporter constructs and stimulated with ET-1 for 6h. ET-1 caused a 1.7-fold increase in luciferase activity of the NF-κB construct (Figure 4.19) and a 1.6-fold increase in activity of the AP-1 construct (Figure 4.20).

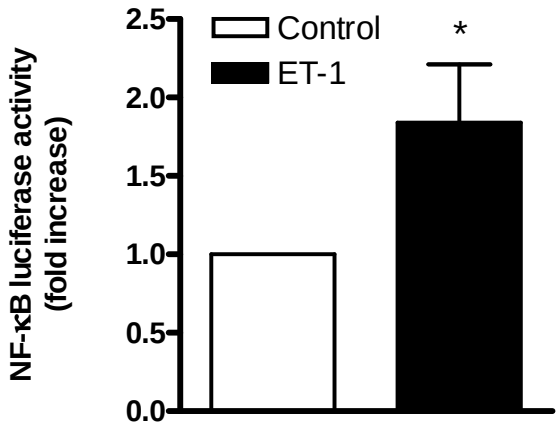

Figure 4.19 Effect of ET-1 on NF-κB reporter-driven luciferase activity following treatment with ET-1 for 6 h. *P=0.03 compared with control. Each bar represents group mean (SE) of 18 replicates in 3 independent experiments.

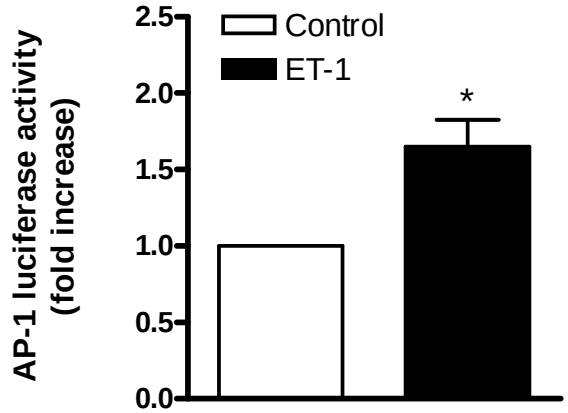

Figure 4.20 Effect of ET-1 on AP-1 reporter-driven luciferase activity following treatment with ET-1 for 6 h. *P=0.03 compared with control. Each bar represents group mean (SE) of 12 replicates in 2 independent experiments.

4.3.10 JNK inhibition has no effect on ET-1-stimulated MCP-1 promoter activity

Since ET-1 activated both the 128 MCP-1 promoter construct (containing two AP-1 consensus sequences) and the AP-1 reporter construct, we hypothesised that inhibition of JNK, an upstream activator of AP-1, would inhibit ET-1-stimulated MCP-1 promoter activity. Interestingly, however, the JNK inhibitor SP600125 (10μM) had no effect on ET-1-stimulated MCP-1 promoter activity (Figure 4.21).

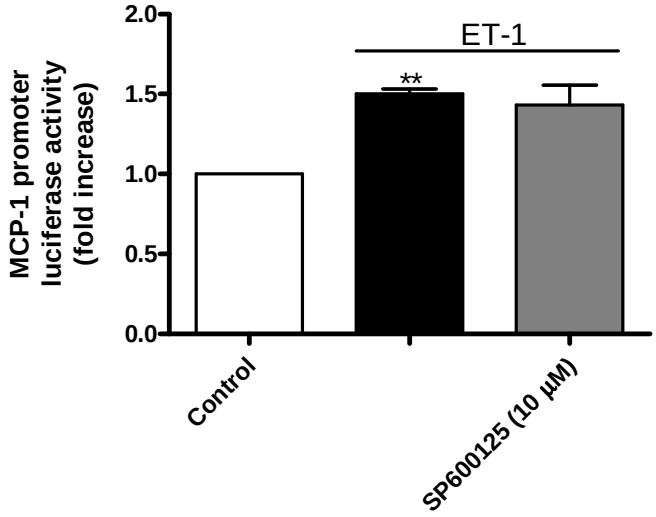

Figure 4.21 Effect of SP600125 on ET-1-stimulated MCP-1 promoter activity. HASMC transiently transfected with the wild-type MCP-1 promoter construct were stimulated with ET-1 for 6 h in the absence or presence of the JNK inhibitor SP600125. SP600125 had no effect on ET-1-stimulated MCP-1 promoter activity. **p=0.009 compared with controls. Each bar represents group mean (SE) derived from 12 replicates in 2 independent experiments.

4.3.11 Inhibitor of κB kinase-2 (IKK-2) blockade inhibits ET-1-stimulated MCP-1 release

Our findings with the transiently transfected MCP-1 promoter and NF-κB reporter constructs suggested that NF-κB was involved in ET-1-stimulated MCP-1 release. We next studied the effect of the inhibitor TPCA-1 (which blocks IKK-2, thereby inhibiting the NF-κB activation pathway) on ET-1-stimulated MCP-1 release. TPCA-1 concentration-dependently inhibited ET-1-stimulated MCP-1 production with a –log IC_{50} of 6.2 +/- 0.1 (Figure 4.22), further supporting the hypothesis that NF-κB is involved in ET-1-stimulated MCP-1 production.

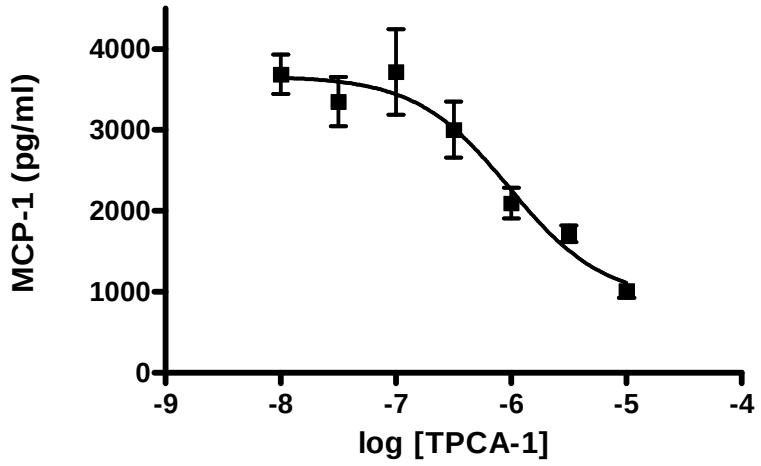

Figure 4.22 Cells were pre-incubated with the IKK-2 inhibitor TPCA-1 for 30 min prior to 24 h stimulation with ET-1. TPCA-1 concentration-dependently inhibited ET-1-stimulated MCP-1 production (4 parameter logistic regression). Each point represents group mean (SE) derived from 12 replicates in 4 independent experiments.

4.3.12 ET-1 promotes in-vivo *binding of NF-κB p65 subunit and AP-1 cJun subunit to the MCP-1 promoter*

To confirm whether NF-κB, AP-1, or both were involved in ET-1's effects at the MCP-1 promoter, we studied the *in vivo* binding of these transcription factors to the MCP-1 promoter by ChIP assay. We found that ET-1 stimulated binding of both p65 and cJun to the MCP-1 promoter, suggesting that both transcription factors are involved (Figures 4.23 and 4.24). We observed a 1.5-fold increase in p65 binding to the MCP-1 promoter at 1 h, with a return to basal levels by 1.5 h. A similar transient rise in cJun binding to the MCP-1 promoter was observed, with a 2.2-fold increase in cJun binding seen at 1 h that returned to basal levels by 2.5 h.

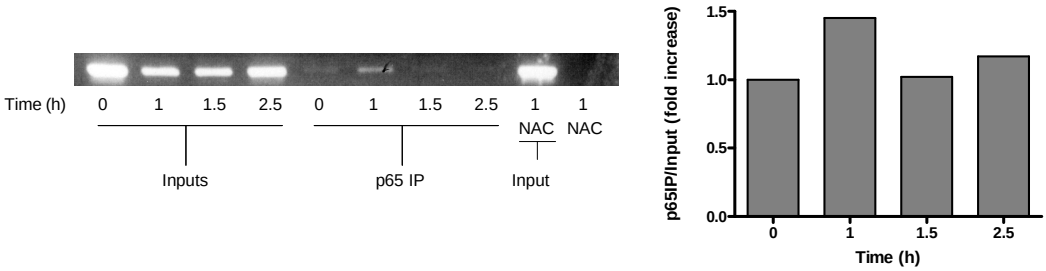

Figure 4.23 ChIP assay, with accompanying densitometry, showing effect of ET-1 on *in vivo* binding of NF-κB p65 subunit to the MCP-1 promoter. ET-1 promoted *in vivo* binding of p65 to the MCP-1 promoter. The density of the immunoprecipitated band (IP) was normalised to that of the input control at the same time point, and expressed as fold increase over time zero. Representative gel from one of two independent experiments. NAC = no antibody control.

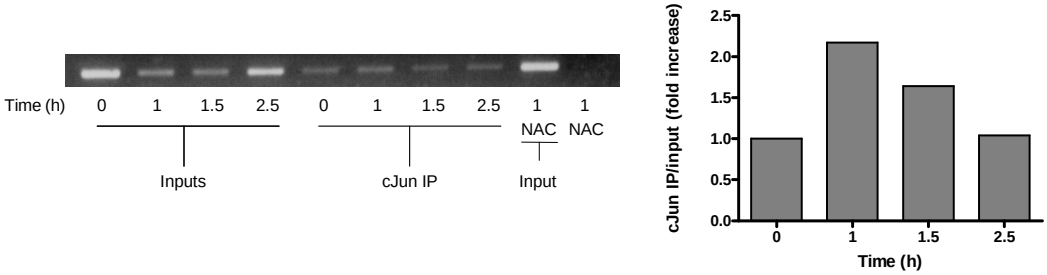

Figure 4.24 ChIP assay, with accompanying densitometry, showing effect of ET-1 on *in vivo* binding of AP-1 cJun subunit to the MCP-1 promoter. ET-1 promoted *in vivo* binding of cJun to the MCP-1 promoter. The density of the immunoprecipitated band (IP) was normalised to that of the input control at the same time point, and expressed as fold increase over time zero. Representative gel from one of two independent experiments. NAC = no antibody control.

4.3.13 PD98059 and SB203580 inhibit ET-1-stimulated binding of p65 and cJun to the MCP-1 promoter

We next performed ChIP assay to study the effects of the MEK inhibitor PD98059 (20 μM) and the p38 MAPK inhibitor SB203580 (20 μM) on ET-1-stimulated binding of p65 and cJun to the MCP-1 promoter. PD98059 and SB203580 inhibited p65 and cJun binding to the MCP-1 promoter (Figure 4.25).

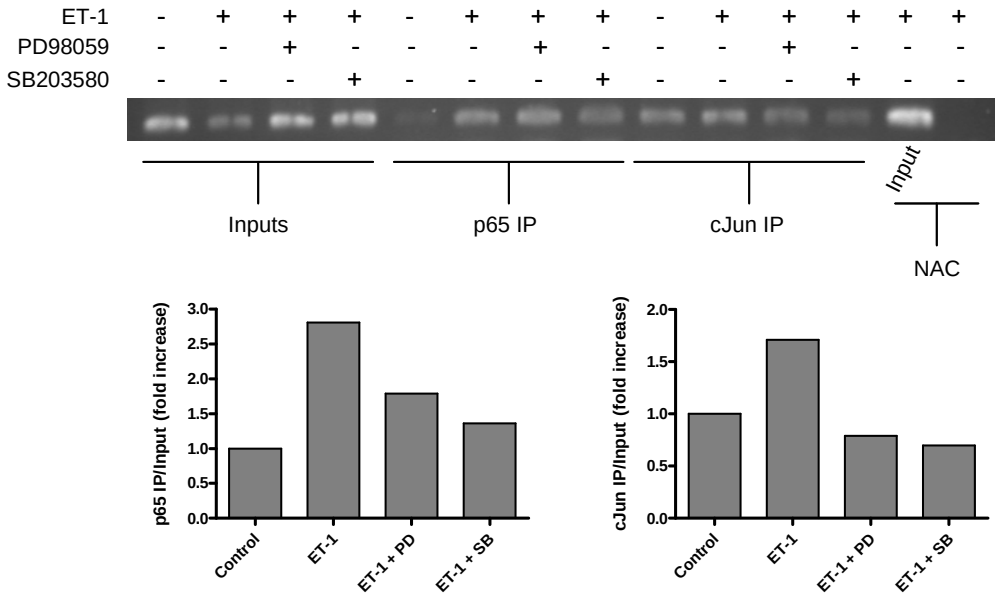

Figure 4.25 ET-1-stimulated binding of p65 and cJun to the MCP-1 promoter at 1h was inhibited by PD98059 (PD, 20 μM) and SB203580 (SB, 20 μM). The density of the immunoprecipitated band (IP) was normalised to that of the input control of the same condition, and expressed as fold increase over unstimulated cells. Representative gels, with accompanying densitometry, from one of two independent experiments. NAC = no antibody control.

4.3.14 Cell viability

Cell viability with all chemicals/inhibitors used in this study was greater than 90 % of that of control cells as determined by MTT assay (see Appendix).

4.4 Discussion

The major finding of this study is that ET-1 induces MCP-1 expression in HASMC and that this occurs through a transcriptional mechanism involving activation of the MCP-1 promoter region. Furthermore, the regulation of MCP-1 by ET-1 involves ET_A and ET_B receptors and both p44/p42 MAPK and p38 MAPK dependent pathways, but not JNK or PI3 kinase dependent pathways.

We found that ET-1 increased MCP-1 production in a concentration and time dependent manner (Figures 4.1 and 4.2). MCP-1 is released from HASMC constitutively, thus even under unstimulated conditions (control cells), MCP-1 accumulates in the culture medium over time. However, there is a clear increase in MCP-1 release from the ET-1 stimulated cells. Of note, a previous study in HASMC did not show induction of MCP-1 in response to ET-1 (242). However, the concentration of ET-1 used in that study was 1 μM, or 2492 ng/ml, some 250-fold higher than the concentration used in our study. It may be that ET-1's effects are biphasic, with a loss of effect at this higher concentration. There are isolated reports of ET-1 increasing MCP-1 production in endothelial and mesangial cells (241, 334), but none from HASMC. Furthermore, the mechanisms used by ET-1 to up-regulate MCP-1 expression have not been studied in any biological system.

We next determined the endothelin receptor(s) responsible for its effects. Two endothelin receptors have been identified and cloned, ET_A and ET_B receptors,

both of which are expressed by HASMC (335, 336). We confirmed the presence of mRNA for both receptors in HASMC by RT-PCR (Figure 4.3). Our studies with antagonists of differing ET-receptor specificity indicated that both receptor subtypes are involved in ET-1 stimulated MCP-1 release (Figures 4.4 to 4.6 respectively). Both antagonists inhibited ET-1-stimulated MCP-1 production at concentrations equivalent to and ten-fold higher than their respective pA_2 values (6.9 - 7.4 and 6.9 for BQ123 and BQ788 respectively (323)). The antagonist concentrations were chosen to be selective for the relevant receptor, as both inhibitors are at least 3 orders of magnitude more selective for their respective receptors (324, 325). Our observations using the ET_A and ET_B receptor antagonists are interesting as they contrast with ET-1-potentiated HASMC proliferation, which appears to be mediated solely by ET_A receptors (197) and ET-1-mediated HASM contraction, which is predominantly ET_B mediated (335, 337) (although one study suggested that both receptors contribute (336)).

Inhibition of the effects of ET-1 by each of the selective ET receptor antagonists strongly implies the presence of both receptor subtypes at the protein level. However, our findings would be strengthened by confirming receptor expression at the protein level. Potential techniques that could be used to demonstrate the presence of ET_A and ET_B receptors include flow cytometry, immunofluorescent labelling coupled with confocal microscopy, or Western blotting. Although, as discussed above, the ET receptor antagonists BQ123 and BQ788 should be selective for their respective targets at the concentrations used in this study, it is possible that the observed effects could be brought

about through non-selective effects on the opposite receptor. Demonstrating the presence of both receptors at the protein level would support the inference that both receptor subtypes are involved in ET-1-stimulated MCP-1 expression.

To further probe the signalling pathways involved we turned our attention to kinase cascades. Studies using kinase inhibitors suggested that ET-1-stimulated MCP-1 production by HASMC involved p44/42 and p38 MAP kinases, but not JNK (Figures 4.8, 4.10 and 4.12 respectively) (328-331). Consistent with the inhibitor studies, ET-1 was shown to activate p44/p42 and p38 MAP kinases by Western blotting (Figures 4.9 and 4.11 respectively).

ET-1 activates MAP kinases in a number of systems (319, 320, 327, 338). P44/p42 MAP kinase is known to be activated by ET-1 in ASM from other species and has been implicated in ET-1-mediated proliferative responses (319, 320, 339, 340). Furthermore, p44/p42 MAPK and p38 MAPK are involved in IL-1β-stimulated MCP-1 production in HASMC (341). This is interesting as it suggests that cross talk could occur between cytokine- and GPCR-mediated pathways of chemokine expression. MAP kinases are involved in multiple inflammatory pathways (342) and it has been shown that MAPK inhibitors can attenuate the inflammatory disease process in animal models of rheumatoid arthritis (343, 344). Interestingly, ET-1 induces expression by human lung fibroblasts of connective tissue growth factor, an important profibrotic protein that induces collagen synthesis, via a p44/p42 MAPK dependent pathway, suggesting that ET-1 may be involved in both the inflammatory and

remodelling components of asthmatic disease and that both components involve p44/p42 MAPK.

The ET-1 receptors, ET_A and ET_B, are seven transmembrane GPCRs which couple to $G_{\alpha q}$ and activate calcium and inositol phosphate second messenger pathways (317-319). There is also some experimental evidence that $G_{\alpha q}$ can activate PI3 kinase (192, 345), although this pathway is not well characterised. Pharmacological studies using wortmannin and LY294002 implicated PI3 kinase in the development of an enhanced contractile phenotype of pulmonary fibroblasts by ET-1(338). In contrast, mitogenic signalling in response to ET-1 in bovine ASM appeared to be independent of PI3 kinase (346). We found that the PI3 kinase inhibitors wortmannin and LY294002 had no effect on ET-1-stimulated MCP-1 production, suggesting that ET-1's effects are not mediated by PI3 kinase (Figure 4.13). The concentrations of these inhibitors were selected to be equal to or higher than their published IC_{50} values (332, 333).

We found that MCP-1 mRNA is expressed by HASMC under resting conditions, and that ET-1 increased MCP-1 mRNA (Figure 4.14). Studies of MCP-1 mRNA stability confirmed that this was not an effect on stabilisation of the MCP-1 message (Figure 4.15).

To confirm that ET-1 was acting transcriptionally, we transiently transfected cells with wild-type MCP-1 promoter and enhancer luciferase reporter constructs. ET-1 significantly increased MCP-1 promoter-luciferase reporter activity and there was a trend towards activation of the MCP-1 enhancer

construct (Figures 4.16 and 4.17 respectively), supporting the hypothesis that ET-1 regulates MCP-1 expression by HASMC transcriptionally. There is one previous report of ET-1 increasing MCP-1 mRNA (in human brain-derived endothelial cells (241)) but, to our knowledge, this is the first study to show such an effect in HASMC and the first to demonstrate direct activation by ET-1 of the MCP-1 promoter/enhancer in any biological system. The magnitude of ET-1's effects on the MCP-1 promoter and enhancer constructs suggested that the promoter was more important in the transcriptional regulation of MCP-1 by ET-1. We therefore focused our subsequent experiments on determining which transcription factors were most important in activation of the MCP-1 promoter by ET-1.

There is little information on the transcription factors activated by ET-1. In glioma cells, AP-1 is involved in ET-1-stimulated proenkephalin expression (347) and, in rat cardiac myocytes, ET-1-stimulated brain natriuretic peptide transcription involves NF-κB (348) and a ternary complex of GATA proteins and serum response factor-activated atrial natriuretic factor (349). ET-1 has also been shown to increase Sp1 expression in rat cardiac myofibroblasts (350). However, there are no published studies on the transcription factors activated by ET-1 in HASMC. The wild type MCP-1 enhancer region contains two NF-κB binding sites; the MCP-1 promoter construct used in these studies includes two binding sites for AP-1, single Sp1, NF-κB and NF-1 binding sites and a CCAAT box (Figure 3.1). Given that previous studies have implicated AP-1, NF-κB and Sp1 in ET-1-stimulated responses in other systems, any or all of these transcription factors could potentially be important in ET-1-induced

MCP-1 expression. Using serially deleted MCP-1 promoter constructs, we found that a region between 213 and 128 base pairs upstream of the translational start site was both necessary and sufficient for maximal activation of the MCP-1 promoter by ET-1 (Figure 4.18). This region harbours consensus sequences for the NF-κB and AP-1 transcription factors. Truncation of the promoter to -213 bp disrupts the GAS found at -214 to -204 bp, therefore it is unlikely that the GAS is involved in ET-1's effects, although it is possible that this region retains some functionality. Site directed mutagenesis or additional deletion analysis of the GAS would be required to clarify this further.

The magnitude of the effect of ET-1 on the MCP-1 promoter deletion constructs was modest, and smaller that that on the MCP-1 promoter construct (Figures 4.16 and 4.18 respectively). The deletion series was a gift from another laboratory; the serially deleted promoters were cloned into the commercially supplied PGL2 basic plasmid (Promega). In contrast, the wild-type MCP-1 promoter used in this study was cloned into the newer PGL3 basic plasmid vector (Promega). These plasmids have been sequentially optimised by the supplying company to improve, amongst other characteristics, reporter gene expression in response to stimulation of cloned regulatory elements. This may in part explain the smaller effects of ET-1 seen with the deletion constructs. Although a formal quantification of the efficiency of our transfection system (Fugene 6, Roche Molecular Biochemicals) was not specifically performed for this study, other researchers in the Knox lab have demonstrated an overall efficiency of transfection in HASMC of 10 – 15% when transfecting a green fluorescent protein-expressing plasmid using the

Fugene 6 system (K. Deacon, personal communication). The relative advantages and disadvantages of this system are discussed in more detail in Chapter 6 (Methodological Considerations)

Our studies with the NF-κB and AP-1 luciferase reporter constructs showed that ET-1 activated both of these transcription factors in HASM (Figures 4.19 and 4.20 respectively). This is consistent with a role for these transcription factors in transcriptional regulation of MCP-1 by ET-1. Furthermore the results of ChIP assays (Figures 4.23 and 4.24) and of NF-κB pathway inhibition by TPCA-1 (Figure 4.22) (351) support a role for NF-κB and AP-1 in ET-1-stimulated MCP-1 expression. The MEK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 inhibited binding of p65 and cJun to the MCP-1 promoter, suggesting that these kinases mediate ET-1-stimulated MCP-1 production transcriptionally, via NF-κB and AP-1 (Figure 4.25). It was beyond the scope of this study to determine the precise mechanisms by which p38 and p44/42 MAPK alter p65 and cJun binding to the MCP-1 promoter. However this could be brought about through phosphorylation of components of the NF-κB and cJun activating pathways, or by affecting chromatin accessibility. To our knowledge, this is the first study to delineate the kinase cascades used by ET-1 to activate specific transcription factors.

The effect of ET-1 on NF-κB reporter luciferase activity was relatively small (1.7-fold increase). Although the ChIP studies and the effect of NF-κB pathway inhibition on ET-1-stimulated MCP-1 expression provide additional evidence of a role for NF-κB, additional techniques could have been employed

to strengthen these findings. These include studies of NF-κB nuclear translocation, through immunofluorescence studies or Western blotting of nuclear and cytosolic fractions before and after stimulation with ET-1, or studies of NF-κB phosphorylation or IκB degradation by Western blotting.

We were surprised that the JNK inhibitor SP600125 had no effect on either MCP-1 protein release or promoter activation (Figures 4.12 and 4.21 respectively), as JNK is an upstream activator of cJun and, taken together, our transfection and ChIP studies strongly suggest that cJun mediates transcriptional regulation of MCP-1 by ET-1. However, there are reports of JNK-independent activation of cJun (352), including by p44/42 MAPK (353). Thus ET-1-mediated activation of cJun in HASMC may be occurring by a JNK-independent pathway.

In the ChIP assays, binding of p65 and of cJun to the MCP-1 promoter was transient and relatively short lived, maximal at 1 hour in both cases, and returning to baseline by 1.5 hours in the case of p65, and by 2.5 hours for cJun. In contrast, an increase in MCP-1 mRNA was first seen at 2 hours, peaking later, at 8 hours. A similar phenomenon was seen in the transcriptional regulation of eotaxin release by TNF-α, where p65 binding and histone acetylation at the eotaxin promoter was maximal at 30 - 60 min, waning by 2.5 hrs, whereas eotaxin mRNA peaked later, at 4 hours, suggesting that the two processes are not temporally contiguous (224). Similar responses have been observed in the Knox lab in the regulation of other chemokines by TNF-α and IL-1 (K Deacon, personal communication). A possible explanation for this

observation is that, once transcriptional activation has taken place through transcription factor binding and chromatin remodelling at promoter regions, the transcriptional apparatus is free to continue transcribing the coding region of the gene, even after transcription factor dissociation from the promoter.

The magnitude of ET-1's effects on p65 and cJun binding to the MCP-1 promoter, as measured by semi-quantitative PCR and densitometry, is modest. For the ChIP studies, we would ideally have liked to quantify the immunoprecipitated material by qPCR. However, we encountered a number of technical difficulties in optimising the qPRC assay for the region of interest of the MCP-1 promoter. This is discussed in more detail in chapter 6.

Our transcriptional studies of ET-1-stimulated MCP-1 release (mRNA studies, reporter gene assays and ChIP assays) all showed relatively modest effects of ET-1 relative to the more pronounced effect on MCP-1 protein release. This may be for a number of reasons. First, as described above, transcriptional activation *in vivo* is in part regulated through chromatin remodelling and the epigenetic code. In the reporter gene assays, the MCP-1 promoter and enhancer plasmid vectors lack the native chromatin environment of the chromosomal MCP-1 gene. Thus transcriptional activation of the reporter gene vectors is likely to be less efficient in the absence of the normal chromatin structure. Secondly, ET-1's effects may be amplified between the mRNA level and the level of protein expression through signalling cascades. Thirdly, other than mRNA stability studies, we did not explore any post-transcriptional mechanisms. It may be that ET-1 exerts its effects on MCP-1 expression, in

part, through post-transcriptional mechanisms. However, a detailed exploration of post-transcriptional mechanisms was beyond the scope of this study.

There have been a number of studies of MCP-1 transcriptional regulation in the context of inflammation. These have demonstrated involvement of various transcription factors in MCP-1 regulation in an apparently stimulus- and tissue-specific manner. For example, STAT-1 and Sp1 were found to interact with the MCP-1 promoter in response to interferon gamma (IFN-γ) in macrophages (354). In contrast, in rat aorta cells hyperinsulinaemia induced C/EBP binding to the MCP-1 promoter (355). Intriguingly, Teferedegne *et al* defined an ordered sequence of events in the TNF-α-regulated activation of the MCP-1 promoter in a human fibroblast cell line (223). TNF-α induced binding of NF-κB to the MCP-1 enhancer, triggering recruitment of the transcriptional co-activators and histone acetyltransferase enzymes CBP and p300. This facilitated interaction of the distal MCP-1 enhancer with the proximal promoter region, enabling Sp1 binding to the promoter region and subsequent activation of transcription. Whilst a number of studies have indirectly implied involvement of AP-1 in MCP-1 transcriptional regulation, for example through correlation of levels of MCP-1 expression with transcription factor expression or activation (356, 357), or through electrophoretic mobility shift assay studies (358, 359), to our knowledge this is the first study to directly demonstrate *in vivo* binding of AP-1 to the MCP-1 promoter by ChIP assay. This is one of very few reports of MCP-1 regulation by mediators acting at GPCRs (360) and,

to our knowledge is the first to demonstrate *in vivo* binding of NF-κB and cJun to the MCP-1 promoter in response to ET-1 in any biological system.

Inhibition of the NF-κB pathway has been shown to inhibit the release of other inflammatory cytokines and chemokines from HASMC and to inhibit cytokine expression and inflammatory cell accumulation in the lungs of a rodent model of asthma following ovalbumin challenge (361). NF-κB pathway blockade also inhibited the late asthmatic response in the ovalbumin-challenged animals (361). Our results with ET-1 add to the body of evidence suggesting that targeting common downstream signalling moieties, such as NF-κB, may have therapeutic potential by simultaneously inhibiting multiple inflammatory pathways.

In conclusion, this study is the first to delineate the signalling pathways used by ET-1 to increase production of MCP-1, an important chemokine strongly implicated in asthmatic airway inflammation. We have shown that ET-1 regulates MCP-1 by transcriptional mechanisms involving NF-κB and AP-1, and that the upstream signalling pathways involve both ET_A and ET_B receptors and p38 and p44/p42 MAP kinases. Our propsed mechanism of MCP-1 regulation by ET-1 is summarised in Figure 4.26. This study adds to the growing body of evidence implicating a number of common downstream targets, including MAP kinases and the pro-inflammatory transcription factor NF-κB, onto which multiple inflammatory signalling pathways converge. Such targets are likely to be relevant in the development of new therapeutic modalities for the treatment of asthma.

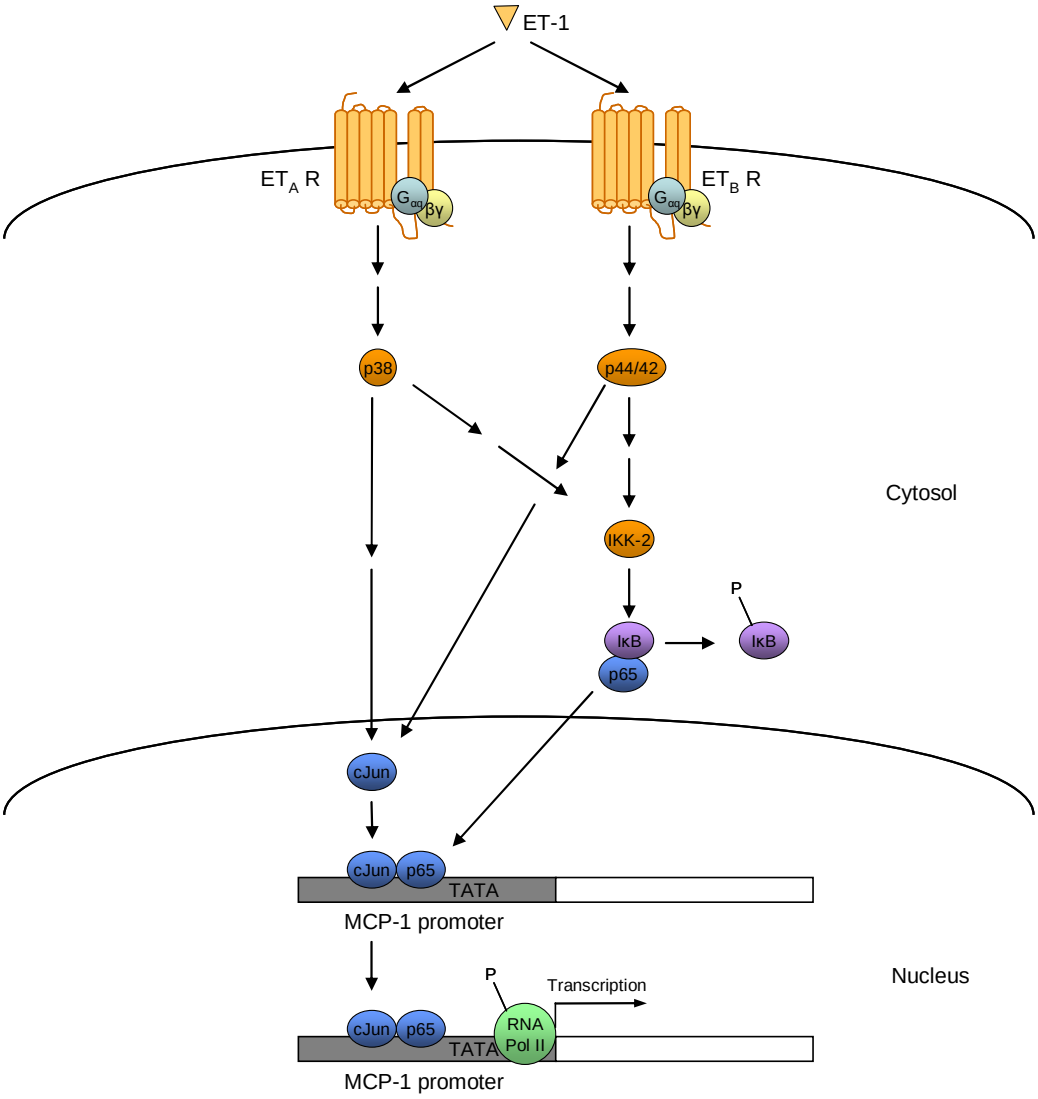

Figure 4.26 Proposed sequence of events in the transcriptional regulation of MCP-1 by ET-1. ET-1, acting at ET_A and ET_B receptors, activates p38 and p44/42 MAP kinases. Activation of these kinases leads on to activation of cJun and p65, the latter through activation of IKK-2, leading to phosphorylation and dissociation of I-κB, allowing translocation of p65 from the cytosol to the nucleus. Upon activation, cJun and p65 bind to the MCP-1 promoter, allowing RNA polymerase II to bind at the transcriptional start site and synthesise MCP-1 mRNA. Multiple arrows indicate probable multiple steps in the signalling pathways that were not fully characterised in this study. It is not clear whether the two ET receptors activate distinct kinase cascades, or whether both receptors can activate p38 and p44/42.

CHAPTER 5: REGULATION OF EOTAXIN EXPRESSION BY TNF-α AND ITS MODULATION BY GLUCOCORTICOIDS AND LONG ACTING BETA AGONISTS: ROLE OF NF-κB, HISTONE H3 ACETYLATION AND HISTONE DEACETYLASES

5.1 Introduction

Allergic airway inflammation in asthma is characterised by accumulation of eosinophils within local tissues. Eotaxin is an important CC chemokine that is a potent chemoattractant for eosinophils and plays an important role in eosinophilic inflammation (362). As described in Chapter 1, airway smooth muscle cells are critically involved in inflammation and remodelling in the asthmatic airway, and studies from this lab and others have previously shown that eotaxin is released from these cells in response to proinflammatory cytokines such as IL-1β and TNF-α (203, 363).

The main two classes of drugs used in the treatment of asthma in the clinical setting are ICS and β2-adrenoceptor agonists (β2-agonists). Clinical studies have shown that treatment of patients with moderate to severe asthma with a combination of ICS and LABA provides superior benefit compared to treatment with either class of drug alone (364, 365). It is currently unclear whether the superiority of the LABA/ICS combination is due to their complementary mechanisms of action or due to additive or synergistic effects on airway inflammation. The mechanisms of the anti-inflammatory effects of GC have been discussed in detail in chapter 1. By contrast, the potential anti-inflammatory effects of LABA are less well understood, but may involve effects on mast cell stabilisation, plasma exudation or inflammatory cell trafficking, independent of the actions of GC (366). However, there is increasing interest in the interactions between LABA and GC, and a number of observations support the possibility of additive or synergistic effects of these agents on inflammatory gene expression. For example, β2 agonists caused

nuclear translocation and DNA binding of the GR in a fibroblast cell line (367). Furthermore, salmeterol enhanced the ability of dexamethasone to suppress proliferation and cytokine release from allergen-stimulated human peripheral blood mononuclear cells (368). Similar additive effects of LABA and GC on cytokine release were observed in cultured human airway epithelial cells (369) and, in fibroblasts, salmeterol also enhanced the suppressive effect of fluticasone on the expression of intercellular adhesion molecule-1 (370).

In keeping with these clinical and laboratory observations, this group has shown previously that GC and β2-agonists both inhibit TNF-α-stimulated eotaxin and IL-8 release from HASMC and that, when used in combination, the two classes of drug have additive or synergistic effects (154, 203, 224). This is interesting, as it may explain, in part, the clinical observations when these drugs are administered in combination. We have begun to explore the mechanisms involved.

Inflammatory gene expression is regulated at several levels. These include activation of transcription factors (such as NF-κB) via kinase cascades, binding of transcription factors to specific recognition elements in gene promoters, by chromatin remodelling, and through post-transcriptional modification. As described in chapter 1, transcriptional regulation of genes in their native chromatin environment is highly complex, involving covalent modifications to histone proteins, resulting in a more open chromatin structure, and facilitating binding of transcription factors and their co-factors and activation of transcription. Acetylation of histone proteins is brought about by HATs, and

deacetylation by HDACs. Our previous studies showed that TNF-α increased eotaxin release through a transcriptional mechanism. The effects of TNF-α at the eotaxin promoter were largely mediated by the transcription factor NF-κB, and the mechanism involved acetylation of histone H4 associated with the eotaxin promoter (224). This same study went on to show that the GC, fluticasone propionate (FP) and the LABA salmeterol (salme) inhibited TNF-α-induced p65 binding and histone H4 acetylation at the eotaxin promoter (224). Again, the two drugs in combination had additive effects. However, the mechanisms by which FP and salme repressed histone H4 acetylation, and thereby NF-κB recruitment to the eotaxin promoter remain unclear. Furthermore, it is not known whether TNF-α alters acetylation status of other histones at the eotaxin promoter, and whether, therefore, part of the effects of FP and salme may be mediated through effects on acetylation status of other histone proteins.

Recently, there has been interest in the roles of HDACs in inflammatory airways diseases including asthma and COPD. In studies of bronchial biopsies of patients with asthma, levels of HDACs 1 and 2 were reduced compared to control subjects, whereas HDAC 2 levels in asthmatic patients treated with ICS were the same as control subjects. Furthermore, total HDAC activity in bronchial biopsies of patients with asthma was also reduced compared to control subjects, and was significantly higher (although still reduced compared to controls) in patients treated with ICS (371). Similar observations were noted in alveolar macrophages isolated from asthmatic patients and normal controls (372). Similarly, HDAC 2 expression and total HDAC activity was reduced in

peripheral lung tissue and alveolar macrophages of patients with COPD compared to normal controls (298). The possibility that targeting HDACs may be of therapeutic potential has been raised by the observation that the existing PDE4 inhibitor, theophylline, used to treat COPD and asthma, increased HDAC activity in bronchial biopsies of patients with asthma, and that the increase in HDAC activity showed a positive correlation with PC_{20} for methacholine and correlated inversely with sputum eosinophilia (373). There is also evidence that, in response to glucocorticoid stimulation, the GR can interact with HDACs to inhibit histone acetylation induced by inflammatory cytokines (220). However, the mechanism through which TNF-α induced histone H4 acetylation at the eotaxin promoter is not fully understood, furthermore, the mechanisms used by FP and salme to reduce histone H4 acetylation and p65 recruitment to the eotaxin promoter are not known. In the experiments described in this chapter we sought to explore these mechanisms in more detail. Using a variety of cellular and molecular assays, we tested the hypotheses that:

1. TNF-α causes other histone acetylation events (namely H3 acetylation) at the eotaxin promoter.
2. FP and salme modulate TNF-α-stimulated NF-κB activation.
3. TNF-α, FP and salme alter the expression of the key anti-inflammatory HDACs 1 and 2 in HASMC.
4. TNF-α, FP and salme alter total HDAC activity in HASMC.

5.2 Experimental protocols

HASMC were grown in 6 well plates (Western blotting experiments, HDAC activity assay), 75cm^2 flasks (ChIP assay) or 24 well plates (all other experiments) as described in chapter 3. Cells were growth arrested for 24 h prior to each experiment. The principle findings of these studies were confirmed in tissue from three different donors. Subsequent mechanistic studies were performed on cells from one to three donors.

5.2.1 Eotaxin release from HASMC

Human eotaxin release from HASMC was measured using a commercially available ELISA kit (R&D) as described in chapter 3.

5.2.2 Determination of HDAC 1 and 2 mRNA expression by real time polymerase chain reaction.

Real time PCR was carried out as described in chapter 3. Primer sequences used were: HDAC-1 sense, 5'- ACC GGG CAA CGT TAC GAA T-3'; HDAC-1 antisense, 5'- CTA TCA AAG GAC ACG CCA AGT G -3'; HDAC-2 sense, 5'- TCA TTG GAA AAT TGA CAG CAT AGT -3'; HDAC-2 antisense, 5'- CAT GGT GAT GGT GTT GAA GAA G -3'; β2 microglubulin sense, 5'- GAG TAT GCC TGC CGT GTG-3'; β2 microglobulin antisense, 5'- AAT CCA AAT GCG GCA TCT-3' (302).

5.2.3 Nuclear extractions for total HDAC activity assay

Nuclear extractions were performed using the NuCLEAR™ (Sigma) extraction kit according to the manufacturers protocol with some modifications. HASMC

were grown to confluence in 90 mm Petri dishes. One dish was used per condition. Cells were washed twice in ice-cold PBS prior to transfer to microcentrifuge tubes and centrifugation at 450g for 5 min at 4°C. The supernatants were discarded and the cells resuspended in hypotonic lysis buffer (supplied) supplemented with PIC and 1μM dithiothreitol (DTT) at a ratio of 5μl lysis buffer : 1μl packed cell volume. Samples were incubated on ice for 15 min prior to addition of 10% Igepal (6 μl Igepal to 100 μl lysate). The lysates were centrifuged at 10,000g for 30 sec at 4°C. The supernatants containing the cytoplasmic fraction were removed and the pellet containing the nuclear fraction washed with lysis buffer. The pellets were resuspended in extraction buffer (supplied) supplemented with PIC and 1 μM DTT (0.66 μl extraction buffer to 1 μl packed cell volume) and vortexed for 15 min. The samples were then centrifuged at 20,000g for 5 min at 4°C and the supernatants containing the nuclear fractions snap frozen in liquid nitrogen prior to storage at -70°C until use.

5.2.4 Histone deacetylase assay

HDAC assay was performed using a colorimetric assay kit according to the manufacturer's protocol (Upstate). HASMC were treated with TNF-α (10 ng/ml) for the times indicated. In inhibitor studies, cells were pre-incubated with inhibitors or vehicle for 30 min prior to treatment with TNF-α (10 ng/ml). Following stimulation, nuclear extracts were prepared as described in section 5.2.3. Thirty μg of nuclear extract in a volume of 20 μl was added to 10μl of 2 x HDAC assay buffer (supplied) in duplicate into wells of the supplied 96 well plate and allowed to equilibrate to 37°C. Ten μl of 4 mM HDAC assay

substrate (supplied) was added to each well, the plate mixed and incubated at 37°C for 60 min to allow deacetylation of the colorimetric substrate by HDAC enzymes contained in the test samples. To release the colorimetric molecule from the deacetylated substrate, 20 µl of activator solution containing 4 µM trichostatin A (TSA) was added to each well. The plate was mixed and incubated at room temperature for 20 min prior to reading absorbance at 405 nm in a microplate reader (Tecan GENios). A standard curve was generated by serial two-fold dilution of the provided 1 mM HDAC assay standard. The standards were incubated with the test samples and treated identically from addition of activator solution. Twenty µl of HeLa nuclear extract (supplied), or 20 µl of HeLa nuclear extract plus TSA were assayed alongside the test samples as positive and negative controls respectively. Results were expressed as absorbance at 450 nm A_{450} (arbitrary units). Effects of drugs (FP, salme) were expressed as percentages of control values.

5.2.5 Chromatin immunoprecipitation assay

ChIP assay was performed as described in chapter 3. Primer sequences used to amplify the immunoprecipitated products by PCR were as follows: eotaxin promoter sense: 5'-CTT CAT GTT GGA GGC TGA AG-3'; eotaxin promoter antisense: 5'-GGA TCT GGA ATC TGG TCA GC-3'.

5.2.6 Data analysis

Results were expressed as the means of the individual technical replicates for that experiment. The experiments were repeated at least twice, and the results shown represent group means +/- SE. Absolute eotaxin levels are presented for

the initial concentration response and inhibitor experiments. In subsequent mechanistic studies, the data are expressed as fold increase or percentage of control. The number of independent experiments from which the data are derived is also indicated in the figure legends. Analysis of variance (ANOVA) of the raw data was used to determine statistically significant differences. A p value of <0.05 was regarded as statistically significant.

5.3 Results

5.3.1 TNF-α stimulates the release of eotaxin from cultured HASMC

We first studied the effect of TNF-α on eotaxin release from HASMC. HASMC were stimulated for 24 h with 0 – 100 ng/ml TNF-α. TNF-α concentration-dependently stimulated eotaxin release from HASMC (Figure 5.1).

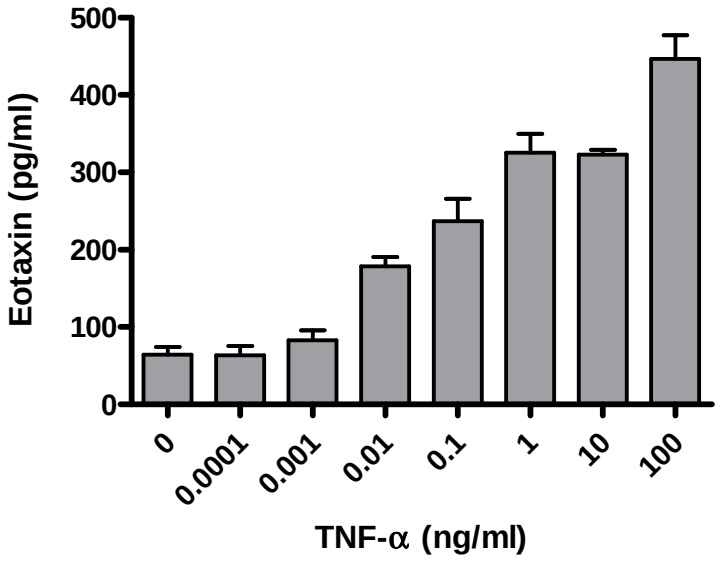

Figure 5.1. Effect of TNF-α on eotaxin release from HASMC. HASMC were incubated for 24 h with TNF-α at the concentrations indicated. TNF-α caused a concentration-dependent increase in eotaxin release from HASMC ($p<0.001$). Each bar represents group mean (SE) derived from 8 replicates in 3 independent experiments performed on cells from 3 different primary donors.

5.3.2 Fluticasone and salmeterol inhibit TNF-α-stimulated eotaxin release

We next studied the effect of the glucocorticoid fluticasone propionate (FP) and the long-acting beta agonist salmeterol (salme) on TNF-α-stimulated eotaxin release. Both FP (10^{-7} and 10^{-6} M) and salme (10^{-7} and 10^{-6} M) inhibited TNF-α-stimulated eotaxin release (Figure 5.2).

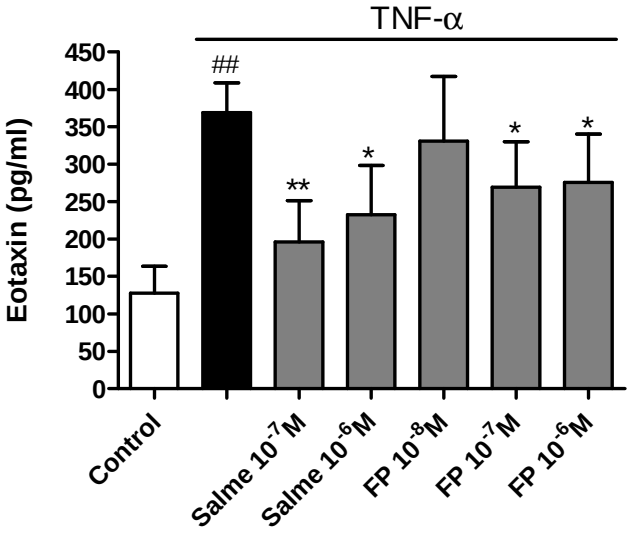

Figure 5.2. Fluticasone (FP, 10^{-7} and 10^{-6} M) and salmeterol (Salme, 10^{-7} and 10^{-6} M) significantly inhibited eotaxin release from cells stimulated with TNF-α (10 ng/ml). ## $p < 0.01$ compared with unstimulated (control) cells. * $p< 0.05$, ** $p < 0.01$ compared with TNF-α-stimulated cells. Each bar represents group mean (SE) derived from 9 replicates in 3 independent experiments.

5.3.3 TNF-α does not cause acetylation of histone H3 associated with the eotaxin promoter

Previous work the Knox group has shown that TNF-α increases histone H4 acetylation and thence p65 recruitment to the eotaxin promoter and that FP and salme downregulate H4 acetylation and p65 binding. The mechanisms by which FP and salme reduce p65 binding are not clear. We initially sought to determine, by ChIP, whether other histones, namely histone H3 associated with the eotaxin promoter were acetylated in response to TNF-α. However, we found that TNF-α had no effect on H3 acetylation at the eotaxin promoter (Figure 5.3). Since the lack of upregulation of histone H3 acetylation makes it unlikely that FP and salme were exerting their effects on p65 binding through effects on H3 acetylation, we did not explore this further.

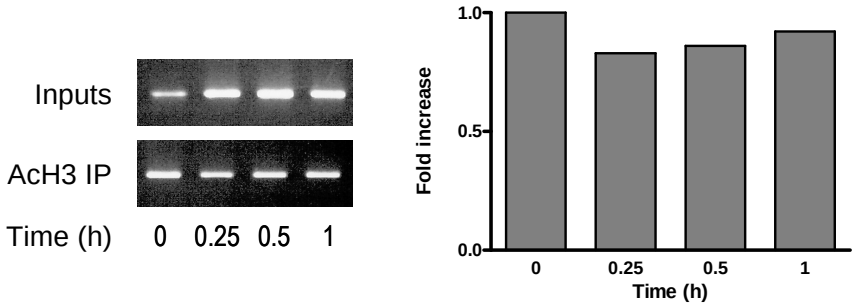

Figure 5.3. ChIP assay, with accompanying densitometry, showing effect of TNF-α on histone H3 acetylation at the eotaxin promoter. HASMC were treated with TNF-α (10 ng/ml) for the times indicated. The density of the immunoprecipitated band (IP) was normalised to that of the input control at the same time point, and expressed as fold increase over time zero. Representative gel from one of two independent experiments.

5.3.4 TNF-α stimulates NF-κB reporter activity in HASMC: fluticasone and salmeterol do not alter this response

Since our previous studies showed that FP and salme down-regulated TNF-α-induced NF-κB p65 binding to the eotaxin promoter, we went on to study the effect of TNF-α, FP and salme on a transiently transfected NF-κB reporter construct. TNF-α (10 ng/ml, 6 h) concentration-dependently stimulated NF-κB reporter activity in HASMC (Figure 5.4). However, FP and salme had no effect on TNF-α-stimulated NF-κB reporter activity (Figures 5.5 and 5.6), suggesting that the effect of these drugs on p65 binding to the eotaxin promoter is not through altering NF-κB activation.

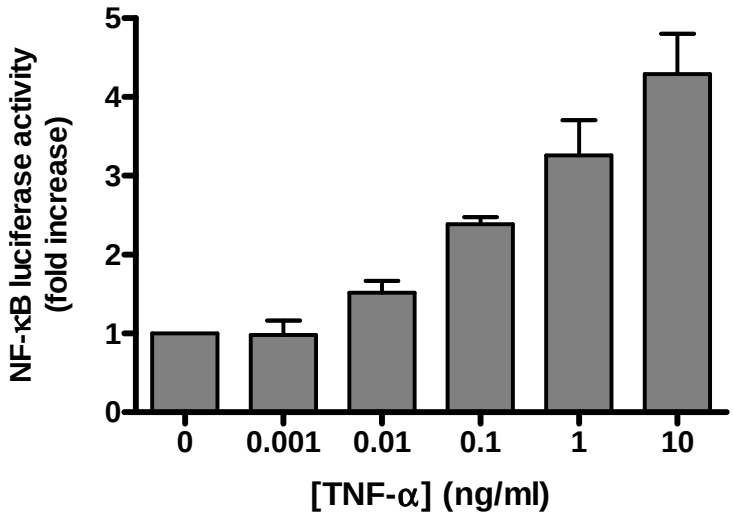

Figure 5.4. Concentration response of TNF-α-stimulated NF-κB reporter activity. HASMC were transiently transfected with an NF-κB reporter construct and stimulated with TNF-α for 6 h at the concentrations indicated. TNF-α concentration-dependently stimulated NF-κB reporter activity ($p<0.001$). Each bar represents group mean (SE) derived from 12 determinates in 3 independent experiments.

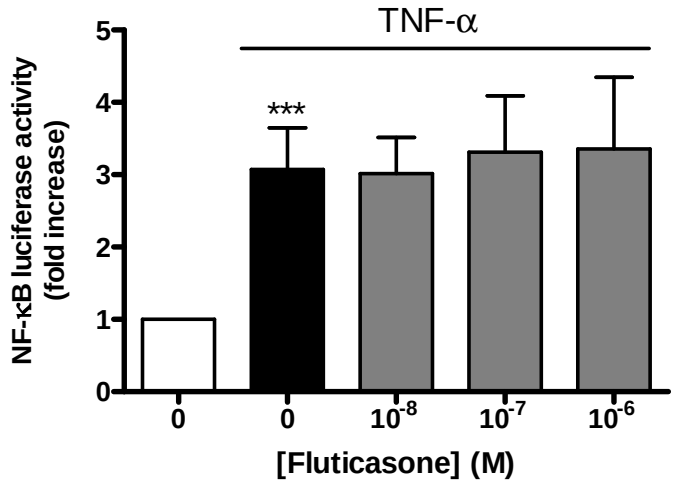

Figure 5.5. Fluticasone had no effect on TNF-α-stimulated NF-κB reporter activity when cells were stimulated with a sub-maximal concentration of TNF-α (1 ng/ml). Each bar represents group mean (SE) derived from 20 determinates in 5 independent experiments. *** p < 0.001 compared to unstimulated (control) cells. Overall p for effect of fluticasone = 0.425.

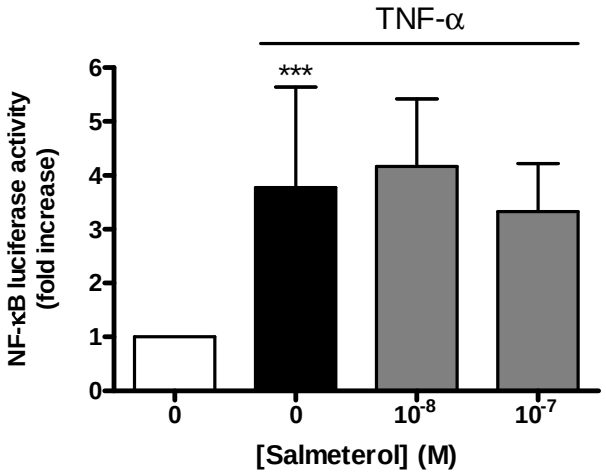

Figure 5.6. Salmeterol had no effect on TNF-α-stimulated NF-κB reporter activity when cells were stimulated with a sub-maximal concentration of TNF-α (1 ng/ml). Each bar represents group mean (SE) derived from 12 determinates in 3 independent experiments. *** p < 0.001 compared to unstimulated (control) cells. Overall p for effect of salmeterol = 0.269.

5.3.5 TNF-α does not affect HDAC 1 and 2 mRNA levels

Alterations in histone acetylation and transcription factor recruitment may be brought about by altered global HDAC or HAT activity, altered HDAC or HAT expression, or altered recruitment of specific HATs or HDACs to the gene promoter. We therefore went on to study the effect of TNF-α (10 ng/ml) on total HDAC 1 and 2 mRNA levels. We quantified HDAC 1 and 2 mRNA levels by qRT-PCR. HDAC mRNA levels were normalised to the housekeeping gene β2-microglobulin (β2M). We found that there was a decay of HDAC 1 and 2 mRNA levels over the 24 h studied, but there was no significant difference between control (unstimulated) and TNF-α-stimulated cells (Figures 5.7 and 5.8 respectively).

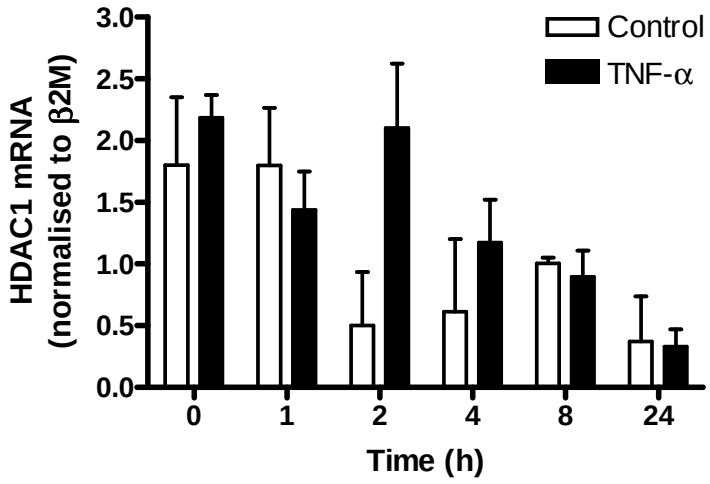

Figure 5.7. Effect of TNF-α (10 ng/ml) on HDAC 1 mRNA levels. There was a time-dependent decay in HDAC 1 mRNA levels but no differences were observed between control and TNF-α-stimulated cells (p=0.15). Each bar represents mean (SE) derived from 4 independent experiments.

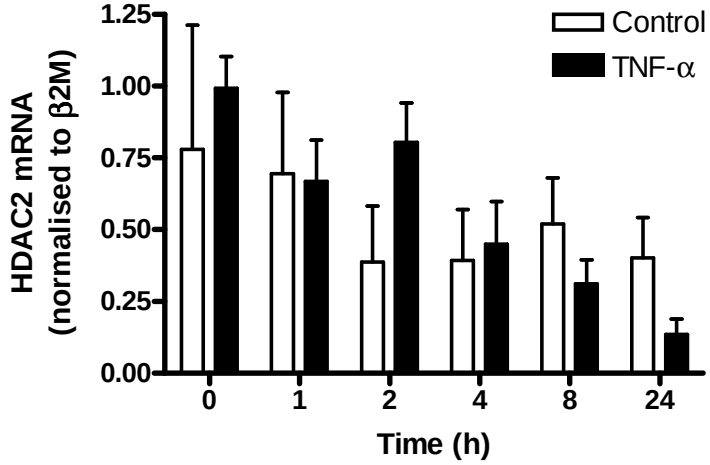

Figure 5.8. Effect of TNF-α (10 ng/ml) on HDAC 2 mRNA levels. There was a time-dependent decay in HDAC 2 mRNA levels but no differences were observed between control and TNF-α-stimulated cells (p=0.76). Each bar represents mean (SE) derived from 4 independent experiments.

5.3.6 TNF-α does not affect HDAC 1 and 2 protein levels

As there were no differences in HDAC 1 and 2 mRNA levels between control and TNF-α-stimulated cells, we speculated that TNF-α may alter levels of HDAC expression at the protein level. HDAC 1 and 2 protein levels were therefore studied by Western blot of whole cell lysates (Figures 5.9 and 5.10 respectively). There was no change in HDAC 1 and 2 protein levels in response to TNF-α stimulation over the time course studied.

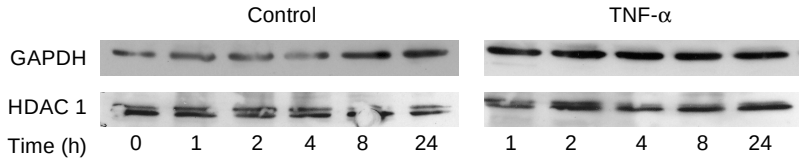

Figure 5.9. TNF-α (10 ng/ml) had no effect on total HDAC 1 protein levels by Western blotting. Representative blots from one of two independent experiments.

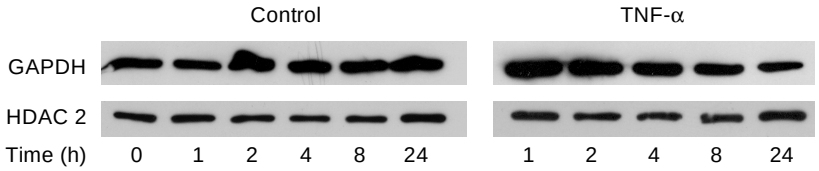

Figure 5.10. TNF-α (10 ng/ml) had no effect on total HDAC 2 protein levels by Western blotting. Representative blots from one of two independent experiments.

5.3.7 Fluticasone and salmeterol do not affect HDAC 1 and 2 protein levels

Since our group's previous studies have shown that FP and salme downregulate TNF-α-stimulated H4 acetylation and p65 recruitment to the eotaxin promoter (224), but our NF-κB reporter gene assays suggested that this was not due to an effect on TNF-α stimulated NF-κB activation (Figures 5.5 and 5.6), we hypothesised that FP and salme's effects may be mediated through altered levels of HDAC expression. We were most interested in their effects under TNF-α-stimulated conditions, since the aim of these experiments was to determine the mechanism by which these drugs altered TNF-α-stimulated H4 acetylation and p65 recruitment to the exotaxin promoter. However, since TNF-α itself did not affect levels of HDAC 1 and 2 expression, we also tested the effects of FP and salme on unstimulated cells. We found that FP and salme at 1μM had no effect on HDAC 1 and 2 levels in either unstimulated or TNF-α-stimulated cells (Figures 5.11 to 5.14).

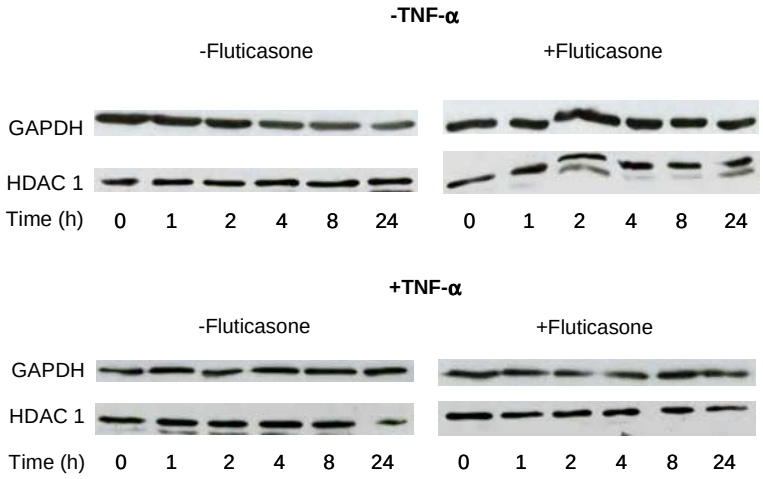

Figure 5.11. Fluticasone (1 μM) had no effect on total HDAC 1 protein levels in either unstimulated or TNF-α-stimulated cells. Representative blots from one of two independent experiments.

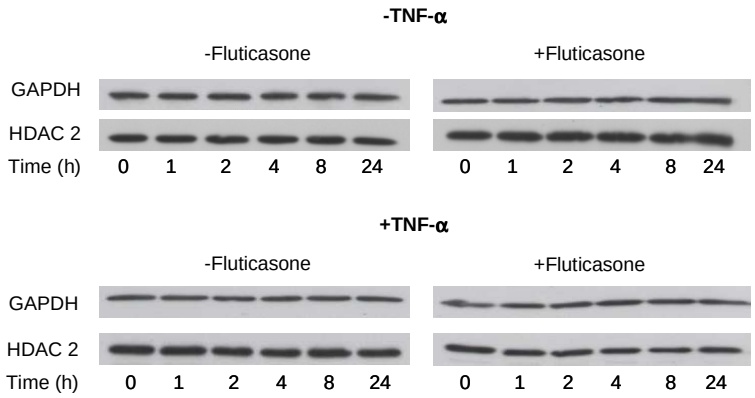

Figure 5.12. Fluticasone (1 μM) had no effect on total HDAC 2 protein levels in either unstimulated or TNF-α-stimulated cells. Representative blots from one of two independent experiments.

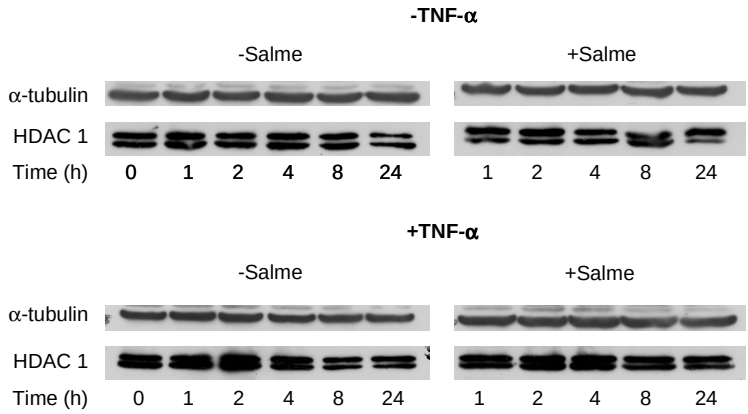

Figure 5.13. Salmeterol (1 μM) had no effect on total HDAC 1 protein levels in either unstimulated or TNF-α-stimulated cells. Representative blots from one of two independent experiments.

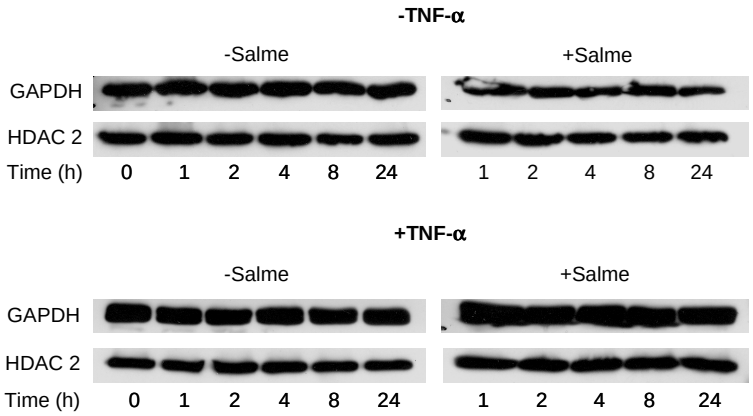

Figure 5.14. Salmeterol (1 μM) had no effect on total HDAC 2 protein levels in either unstimulated or TNF-α stimulated cells. Representative blots from one of two independent experiments.

5.3.8 TNF-α does not alter total HDAC activity

Since TNF-α had no effect on levels of HDAC 1 and 2 at either the mRNA or protein level, we hypothesised that TNF-α might increase HDAC activity without modifying mRNA/protein levels. To test this hypothesis we measured HDAC activity using a commercially available assay. HASMC were incubated with TNF-α (10 ng/ml) for the times indicated. TNF-α had no effect on total HDAC activity over the time course studied (Figure 5.15).

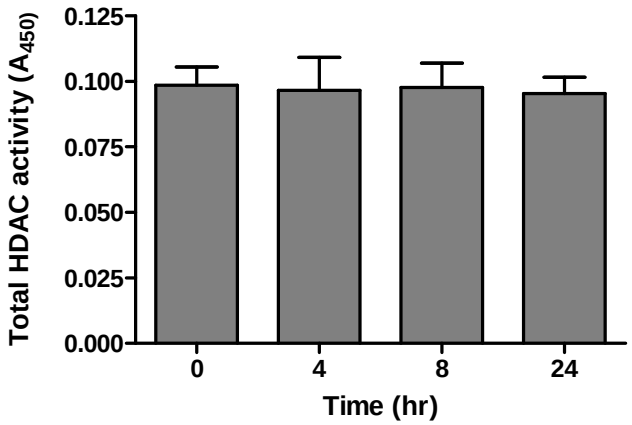

Figure 5.15 Effect of TNF-α on global HDAC activity in HASMC. HASMC were stimulated with TNF-α (10 ng/ml) for the times indicated. Nuclear extracts were assayed for total HDAC activity, measured by colorimetric assay as absorbance at 450 nm (A_{450}). TNF-α had no effect on total HDAC activity in HASMC. Each bar represents mean (SE) derived from 4 independent experiments.

5.3.9 Fluticasone and salmeterol do not affect total HDAC activity

Similarly, since FP and salme had no effect on levels of HDAC protein expression, we speculated that these drugs may alter total HDAC activity. However, FP and salme had no effect on total HDAC activity in either unstimulated or TNF-α-stimulated conditions at 4 and 24 h (Figures 5.16 and 5.17).

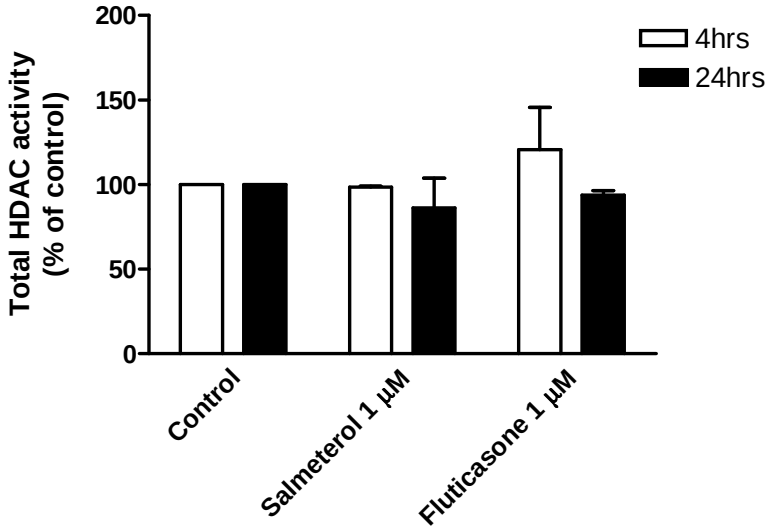

Figure 5.16 Effect of salmeterol (1 μM) and fluticasone (1μM) on total HDAC activity in unstimulated HASMC. Cells were incubated for 4 or 24 h in the presence of salmeterol or fluticasone. Salmeterol and fluticasone had no effect on total HDAC activity. Each bar represents mean (SE) derived from 2 independent experiments.

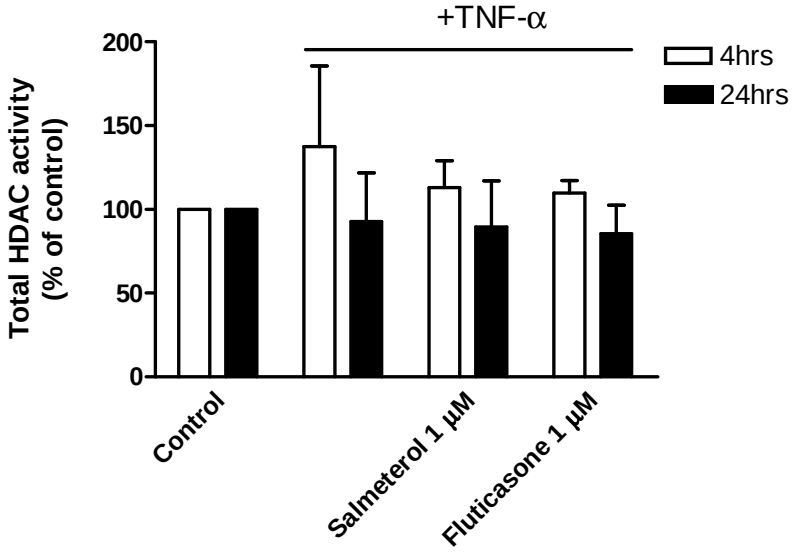

Figure 5.17 Effect of salmeterol (1 μM) and fluticasone (1μM) on total HDAC activity in TNF-α stimulated HASMC. Cells were pre-incubated for 30 min with vehicle, salmeterol or fluticasone then stimulated for 4 or 24 h with TNF-α (10 ng/ml). Salmeterol and fluticasone had no effect on total HDAC activity. Each bar represents mean (SE) derived from 2 independent experiments.

5.4 Discussion

The human eotaxin promoter has been cloned and a number of putative regulatory elements identified, including binding sites for C/EBP, AP-1, NF-κB and STAT-6 (374-376). Eotaxin gene regulation is predominantly mediated by NF-κB and STAT-6, in a tissue- and stimulus-specific manner. In human airway epithelial cells, NF-κB mediates TNF-α-induced transcription, whereas IL-4-stimulated transcription is mediated by STAT-6. Neither stimulus promoted binding of C/EBP or AP-1 to eotaxin promoter elements (376). In contrast, in fibroblasts, TNF-α-induced eotaxin transcription is mediated by both NF-κB and STAT-6 (377). In previous studies from this group, the mechanism of TNF-α-induced eotaxin expression was explored in more detail in HASMC. We found that TNF-α regulated eotaxin expression transcriptionally and, furthermore, TNF-α's effects were predominantly mediated by NF-κB. TNF-α promoted binding of NF-κB p65 to the eotaxin promoter and acetylation of histone H4 associated with the eotaxin promoter (224).

We have previously shown that TNF-α-stimulated eotaxin release from HASMC is modulated by FP and salme (203). Subsequent studies showed that TNF-α regulates eotaxin transcriptionally and that FP and salme inhibit TNF-α-induced eotaxin transactivation. Furthermore, *in vivo* ChIP studies showed that FP and salme repressed TNF-α-stimulated eotaxin transcription through inhibition of histone H4 acetylation and p65 binding at the eotaxin promoter (224). In this study we set out to determine the mechanisms by which FP and

salme might modulate histone acetylation events and NF-κB-dependent activation of the eotaxin promoter.

We first confirmed the original observations by our group that TNF-α concentration-dependently stimulated eotaxin release from HASMC (Figure 5.1) and that FP and salme significantly inhibited TNF-α-stimulated eotaxin release (Figure 5.2). The results of these experiments were broadly in agreement with the previous reports from our group (203, 224).

We initially turned our attention to histone acetylation events, exploring whether TNF-α affected acetylation of other histone proteins, specifically histone H3, at the eotaxin promoter, by ChIP assay. Although the role of histone H4 acetylation has been studied quite extensively in inflammatory gene regulation (183, 220, 224, 250, 378), histone H3 modifications in association with inflammatory gene promoters are less well studied, although have been described (379-381). We found that TNF-α (10 ng/ml) had no effect on histone H3 acetylation (Figure 5.3). This makes it unlikely that histone H3 acetylation events contribute to FP and salme effects on TNF-α-stimulated p65 recruitment to the eotaxin promoter. We therefore did not pursue this line of enquiry further.

The previous study by Nie *et al* showed that FP and salme inhibited *in vivo* NF-κB p65 binding to the eotaxin promoter. We therefore went on to determine if the effects of salme and FP were through NF-κB activation. To do this we transiently transfected HASMC with an NF-κB luciferase reporter

construct. The advantage of this approach is that it is a relatively straightforward assay system which can give useful information on transcription factors or genes activated or downregulated in response to a particular stimulus. A potential disadvantage is that the transfected vectors lack the chromatin environment of native DNA, so results need to be interpreted with caution, since the lack of a chromatin environment may alter some responses. We found that TNF-α concentration-dependently stimulated the activity of a transiently transfected NF-κB reporter construct (Figure 5.4). Interestingly, however, FP and salme had no effect on TNF-α-stimulated NF-κB reporter activity (Figures 5.5 and 5.6 respectively). As described in chapter 1, the binding of transcription factors and their co-factors to gene promoters, and activation of transcription, involves covalent modifications to histone proteins, promoting a more open chromatin structure. We know from previous studies in our laboratory that FP and salme inhibition of binding of NF-κB to the eotaxin promoter was dependent on the chromatin environment and associated with acetylation of histone H4. It was not seen when EMSA assays were used which lack this chromatin environment (224). The results with the NF-κB reporter assay are probably compatible with this lack of effect of FP and salme, reflecting the lack of a chromatin environment when reporter assays are used. The observation that neither FP nor salme had any effect on TNF-α-stimulated NF-κB reporter activity suggests that their effects are not mediated through direct inhibition of NF-κB or its upstream activating pathway, but that some other, perhaps chromatin-dependent mechanism is involved.

We speculated that the effects of TNF-α on NF-κB binding and histone H4 acetylation at the eotaxin promoter may be mediated through an effect on histone deacetylase (HDAC) levels. Since HDACs act to remove acetyl groups from acetylated histone proteins, a down-regulation of HDACs might be expected to increase total acetylation at the eotaxin promoter. Further, since transcription factor binding is facilitated by histone acetylation, downregulation of HDACs could also explain the increase in NF-κB binding to the eotaxin promoter in response to TNF-α. The aforementioned studies on bronchial biopsies of patients with asthma (371, 372), would support the hypothesis that alterations in the amounts of HDACs 1 and 2 may be important in promoting inflammatory gene expression in asthma. We therefore studied the effect of TNF-α (10 ng/ml) on total HDAC mRNA and protein levels, focusing our studies on HDAC 1 and 2. We found that there was a decay of HDAC 1 and 2 mRNA levels over the 24 h studied, but there was no difference between control (unstimulated) and TNF-α-stimulated cells (Figures 5.7 and 5.8). HDAC 1 and 2 protein levels were studied by Western blotting (Figures 5.9 and 5.10 respectively). HDACs 1 and 2 belong to the class I HDACs, which are exclusively localised to the nucleus and do not shuttle between nucleus and cytoplasm (382). For this reason we studied their levels in whole cell lysates rather than nuclear and cytoplasmic fractions. There was no change in HDAC 1 and 2 protein levels in response to TNF-α stimulation over the time course studied, suggesting that alterations in levels of HDAC expression are not responsible for the effects of TNF-α on NF-κB binding and histone acetylation at the eotaxin promoter.

Although TNF-α had no effect on HDAC 1 and 2 levels, we hypothesised that FP and/or salme may upregulate total HDAC 1 and 2 levels, which may explain the effects of these drugs on H4 acetylation and NF-κB binding at the eotaxin promoter. As our experiments with TNF-α alone had shown no effect on HDAC 1 and 2 levels, but any effect of FP or salme would be most relevant in TNF-α stimulated cells (since we were interested in effects on the eotaxin promoter under stimulated conditions), we performed the experiments in both unstimulated and TNF-α-stimulated cells. We found that FP and salme at 1μM, concentrations that this group has previously shown reduce acetylation of H4 and NF-κB binding at the eotaxin promoter (224), had no effect on HDAC 1 and 2 levels in either unstimulated or TNF-α-stimulated cells (Figures 5.11 to 5.14).

In some of the Western blots depicting HDAC1 levels under various conditions and at various time points, a second, slightly lower molecular weight band was observed. This band was also noted in the other experiments of which the data presented are representative. This second band did not have a consistent relationship to time, presence or absence of TNF-α or presence or absence of fluticasone. It most likely represents a non-specific band with a similar molecular weight to HDAC-1. To our knowledge, HDAC1 is not expressed as splice variants. An alternative explanation may be post-transcriptional modification of HDAC-1, which is subject to various of these, including phosphorylation, acetylation, sumoylation and ubiquitylation (383). SUMO (small ubiquitin-like modifier) proteins consist of around 100 amino acids, with a molecular weight of approximately 12kDa. Ubiquitin consists of around

76 amino acids, with a molecular weight of around 8.5 kDa. Such modifications would therefore alter the molecular weight of HDAC1 by an amount consistent with the additional band observed in some, but not all of the HDAC1 Western blots. However, these post translational modifications are tightly regulated, having important effects on cellular functions. Thus it is difficult to reconcile this possibility with the inconsistent relationship of the additional band with the various conditions and time points studied, and non-specific binding remains a more likely explanation.

Despite the lack of effect of TNF-α, FP and salme on HDAC 1 and 2 mRNA or protein levels, it is possible that their effects on histone H4 acetylation and NF-κB binding to the eotaxin promoter may be mediated through alterations of HDAC activity, particularly as the previously described studies in bronchial biopsies and alveolar macrophages from asthmatic patients showed reduced HDAC activity in these patients compared to normal controls (371, 372). We therefore studied the effects of TNF-α, FP and salme on total HDAC activity in HASMC. TNF-α had no effect on HDAC activity in HASMC (Figure 5.15). This contrasts with the findings of Keslacy *et al*, who found an increase in total HDAC activity in HASMC stimulated with TNF-α (384). We are unsure why this should be the case, since the conditions for preparation, culture and treatment of HASMC used in our laboratory were very similar to those described by Keslacy *et al*. Similarly, FP and salme had no effect on HDAC activity in either unstimulated or TNF-α-stimulated conditions (Figures 5.16 and 5.17).

In summary, taken together, these data suggest that the mechanisms by which TNF-α, FP and salme bring about their effects on histone H4 acetylation at the eotaxin promoter is not through alterations in HDAC levels or activity. Furthermore, since TNF-α had no effect on histone H3 acetlylation at the eotaxin promoter, it is unlikely that alterations in histone H3 acetylation contribute to FP and salme's effects on p65 recruitment to the eotaxin promoter. This begs the question, what other mechanisms may be responsible for the effects of FP and salme?

We focused our studies on HDACs 1 and 2 as these have been shown to have altered levels in inflammatory lung diseases (298, 371). However, it is possible that alterations in levels of other HDACs may have been responsible. An alternative explanation is that FP and salme either inhibit recruitment of essential HATs to the eotaxin promoter, or promote recruitment of counterregulatory HDACs. A recently published study by this group demonstrated that TNF-α increased p300 and pCAF recruitment to the eotaxin promoter (228). It would be interesting to extend these studies to determine whether FP or salme modulate TNF-α-stimulated p300 or PCAF recruitment. Similarly, ChIP assays could be employed to study the association of HDACs with the eotaxin promoter, and whether their association is up- or downregulated by TNF-α/FP/salme. There is certainly evidence from co-immunoprecipitation studies in A549 transformed alveolar epithelial cells that another GC, dexamethasone, in association with the GR, can recruit HDAC 2 to the activated p65/CBP complex. This observation was associated with an

inhibition of IL-1β-induced histone acetylation at the granulocyte-macrophage colony stimulating factor promoter (220).

Salme is a LABA that acts at β-adrenergic receptors at the cell surface to increase intracellular levels of cAMP. Cyclic AMP activates cAMP-responsive genes through binding of cAMP response element binding protein (CREB) to the cAMP response element (CRE). It is possible that the effects of salme are mediated indirectly through induction of cAMP-responsive genes. It is tempting here to draw parallels with the effects of theophylline on HDAC activity since theophylline inhibits PDE4, thus resulting in elevated intracellular cAMP levels. However, the study by Ito *et al* found the effects of theophylline to be independent of PDE4 inhibition (373).

In addition to acetylation of histones, it is now known that other cellular proteins may be targets for both HATs and HDACs. These include transcription factors such as NF-κB (385), the GR (299) and molecular chaperone proteins critical to normal GR function (386). It would be interesting to determine whether FP or salme alter acetylation status of these other regulatory elements in HASMC, and whether this has any bearing on regulation of inflammatory response genes such as eotaxin.

In conclusion, we have shown that FP and salme do not inhibit TNF-α induced histone H4 acetylation or p65 binding at the eotaxin promoter through alterations in H3 acetylation status, alterations in levels of HDAC 1 and 2 expression, or changes in total HDAC activity. Other possible mechanisms

include effects on HAT or HDAC binding to the eotaxin promoter, changes in expression of HDACs other than 1 or 2, or perhaps alterations in acetylation status of other important nuclear regulators.

CHAPTER 6: METHODOLOGICAL CONSIDERATIONS

6.1 Introduction

Several methodological considerations relating to the studies presented in this thesis are worthy of mention. These are discussed below.

6.2 Transcriptional regulation

In these studies, we explored transcriptional regulation using real time PCR, assays of transiently transfected reporter genes, and ChIP assays.

6.2.1 Polymerase chain reaction

In chapter 4, in our studies of endothelin receptor expression, we were interested solely in whether HASMC expressed the mRNA for the endothelin receptors, ET_A and ET_B. We therefore used semi-quantitative RT-PCR to demonstrate the presence of ET_AR and ET_BR mRNA. In contrast, in our studies of MCP-1 mRNA expression, we wanted to quantify MCP-1 mRNA levels to determine any changes in expression in response to ET-1. We therefore used quantitative real time reverse transcriptase PCR (qRT-PCR) to measure MCP-1 mRNA. With this technique, amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle, either through quantification of fluorescent dyes, such as SYBR Green, that intercalate with double-stranded DNA (as used in this study), or fluorescent probes that hybridise to a specific DNA target sequence. This allows for more accurate quantification of transcripts than the semi-quantitative RT-PCR. Potential pitfalls of dye-based qRT-PCR include non-specific amplification and primer dimer formation, particularly at very low target concentrations. In these

studies, dissociation curve analysis was undertaken and no secondary peaks suggestive of primer dimer formation or non-specific products were observed.

6.2.2 Transient transfections

We used FuGene 6 transfection reagent for all transfections. FuGene 6 is a proprietary blend of lipids and other components supplied in 80% ethanol that is said to transfect eukaryotic cells with high efficiency and minimal cytotoxicity (387). We found that this method produced acceptable levels of transfection of plasmid DNA into HASMC, as reflected in detectable levels of luciferase activity, consistently above background levels. A formal quantification of transfection efficiency was not performed. Other methods of transfection include the use of liposomal transfection reagents, such as Lipofectamine (Invitrogen), or the use of nucleofection systems such as that produced by Amaxa. Liposomal reagents probably have similar efficacy to Fugene 6. Indeed, in published studies from this lab we have found similar levels of efficacy using Lipofectamine to transfect HASMC (184). We have some experience in our laboratory of using the Amaxa nucleofection system. This method combines electroporation with a cell-type specific Nucleofector solution, and is useful for those applications that require very high transfection efficiencies, for example gene knock-down protocols utilising small interfering RNA. However, we observed higher levels of cytotoxicity using this method than with standard transfection reagents (LM Corbett, unpublished observations). Furthermore, the Amaxa system is prohibitively expensive for applications such as reporter gene assays, where such high transfection efficiencies are not essential.

Reporter gene assays such as those used in this study place a gene with a readily measurable product (in this case, firefly luciferase) under the control of the promoter (or other regulatory sequence) of the target gene of interest. Following transfection of the cells with the reporter plasmid, cells are treated with stimuli and/or drugs/inhibitors of interest. The level of luciferase expression provides a measure of the effect of the treatment on activity of the gene promoter and, by extrapolation, expression of the target gene. Reporter gene assays are a relatively simple, convenient means of studying gene regulation. However, this technique assumes that the process of transfection does not alter the response of the cells to the treatment under study. Furthermore, eukaryotic DNA in its native form exists as chromatin, a complex structure consisting of DNA wound around 4 core histones (H2A, H2B, H3, H4). These histones undergo a number of covalent modifications (acetylation/phosphorylation/methylation) which regulate chromatin unwinding thereby allowing access of transcription factors to their binding sites on DNA, recruitment of essential co-factors and activation of transcription. Since eukaryotic genes are normally regulated, in part, through this "epigenetic" code, and since reporter plasmids lack this chromatin structure, reporter gene assays can only give a partial insight into gene regulation events *in vivo*.

6.2.3 Chromatin immunoprecipitation assay

In contrast to reporter gene assays, ChIP assays study gene regulation events "*in vivo*" (in this context, *in vivo* refers to DNA in its native environment in the form of chromatin). Following treatment with the stimulus under study, DNA

is covalently cross linked to bound proteins (transcription factors, histones, essential cofactors) through a fixing procedure. After shearing the DNA into approximately 500bp fragments, DNA bound to the transcription factor (or other protein) of interest is immunoprecipitated with an antibody directed against the relevant protein. After reversal of the cross links, the target region of interest is amplified by PCR using specific primers spanning the regulatory region of the gene under study. Thus if a stimulus triggers binding of a particular transcription factor to the gene promoter, then the amount of PCR product will be increased. ChIP assays are therefore more physiological than some other techniques used to study gene regulation, such as reporter gene assays and electrophoretic mobility shift assays.

We used ChIP assays in the studies presented in chapters 4 and 5, to study transcription factor binding to the MCP-1 promoter, and to study histone H3 acetylation at the eotaxin promoter in response to TNF-α. Disadvantages of ChIP include the need for large amounts of starting material (two 75cm^2 flasks of cells per condition in this study) and the number of steps involved in the procedure, which may potentially lead to some loss of sample and therefore some inter-sample variability.

In chapter 4, the magnitude of ET-1's effects on p65 and cJun binding to the MCP-1 promoter, as measured by semi-quantitative PCR and densitometry, is modest. For the ChIP studies, we would ideally have liked to quantify the immunoprecipitated material by qPCR. However, we encountered a number of technical difficulties in optimising the qPRC assay for the region of interest of

the MCP-1 promoter. In ChIP, the quantity of DNA immunoprecipitated is small, thus the amount of starting material for the PCR assay is lower than in some other PCR applications. Although it is important that the reaction efficiency should be as high as practicably possible for all qPCR applications, a high reaction efficiency and specificity is even more critical where the quantity of starting material is very small. This requires the design of effective primers, however, ChIP and qPCR both place certain constraints on primer design. First, for qPCR applications it is best to aim for as short an amplicon as possible (max 200 bp) to ensure efficient denaturation during thermal cycling. Second, in studies of transcription factor binding by ChIP, the region of interest is in the gene promoter, and must span the binding sites for the transcription factors under study. Together, these two constraints limit the region of DNA to which one can design primers to hybridize. Ideally the resulting amplicon should have a GC content of less than 60%, again to ensure efficient denaturation during thermal cycling, yet promoter regions are often GC rich. Within these constraints, using freely available on-line primer design tools (CyberGene), we found that most possible primer pairs that could potentially amplify the region of interest of the MCP-1 promoter had potential for secondary structure (hairpin loop) formation. Despite this, we tested a number of primer pairs, against a standard curve generated through serial dilutions of human genomic DNA (hgDNA) but the resulting qPCR reaction was not sufficiently efficient for accurate quantification of immunoprecipitated products.

We therefore used semi-quantitative PCR to analyse our ChIP data. For each sample, an aliquot of DNA was removed as an input control, prior to addition of antibody (immunoprecipitation step), thus acting as a control for any variability in the total DNA recovered from each sample. Results were then quantified by densitometry of the immunoprecipitated bands, normalised to the corresponding input and compared with time zero (time course experiments) or untreated samples. To confirm that the PCR product generated in the ChIP studies was indeed from the MCP-1 promoter, the band was excised and sequenced. The sequence products aligned with the MCP-1 promoter, with no other hits by NCBI BLAST for the whole human genome, confirming that the PCR product was from the MCP-1 promoter.

Another technique that could have been employed to support the ChIP data is EMSA (electrophoretic mobility shift assay), however this technique suffers from the limitation that transcription factors are binding to synthetic oligonucleotides, not to native DNA in the form of chromatin. Thus EMSA is a less physiological assay than ChIP, meaning that results must be interpreted with caution.

6.3 Receptor studies

In our studies of ET-1-stimulated MCP-1 release in chapter 4, the combination of the presence of mRNA for both ET receptors, and inhibition of ET-1 mediated responses by both ET_A and ET_B receptor antagonists, provides strong evidence of the presence and involvement of both receptor subtypes. To provide further evidence of the presence of both receptors at the protein level,

antibody detection strategies, such as flow cytometry or immunofluoresecence studies could be employed. Although the ET_A and ET_B receptor antagonists were used at concentrations that should be selective for their respective targets (324, 325), to rule out non-selective effects and provide further evidence of the involvement of both receptors, molecular approaches such as knock down of receptor expression with siRNA could have been employed.

6.4 Kinase studies

There are conflicting reports in the literature regarding the selectivity of the kinase inhibitors SB203580, PD98059 and SP600125 for their targets. Whilst the concentrations of inhibitors used have previously been reported to be selective for these pathways (328-331), subsequent studies have suggested that these inhibitors may have non-selective effects in some systems (388). However, we have previously found, in TNF-α-stimulated HASMC, that SB203580 (30 μM) does not inhibit phosphorylation of p44/p42 MAPK and, similarly, PD98059 (30 μM) does not inhibit phosphorylation of p38 MAPK, suggesting that these inhibitors are selective for their respective targets in these cells (Clarke DL and Knox AJ, unpublished observations).

Other methods that could have been used to confirm the involvement of these kinases in this pathway include knock-down of kinase effects using, for example, siRNA, or dominant negative studies in which cells are transfected with a vector encoding a non-functional variant of the relevant kinase thereby knocking out the kinase function (389).

No experimental system is 100% physiological, and all have their shortcomings. The studies presented in this thesis were performed with the methodological considerations discussed above in mind. For this reason, we have used a range of techniques in our mechanistic studies (eg transient transfections as well as ChIP assays for studies of transcriptional regulation). Consequently, the conclusions drawn from those studies and presented here are, we feel, valid and strongly supported by the data presented.

CHAPTER 7: CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

7.1 Conclusions

The purpose of these studies was to explore the mechanisms of inflammatory response gene regulation in HASMC by mediators and cytokines important in asthmatic airway inflammation. Specifically, we aimed to identify common pathways onto which multiple inflammatory pathways converge, that may be targets for future drug development for asthma. We studied ET-1 and TNF-α as examples of an important inflammatory mediator and cytokine respectively, and focused on regulation of chemokine expression, using the two important chemokines MCP-1 and eotaxin as examples.

We chose primary HASMC for our studies. These cells secrete a variety of biologically active substances important in asthmatic airway inflammation and remodelling. They express receptors for cytokines such as TNF-α and IL-1β, as well as G-protein coupled receptors for many inflammatory mediators (178). They are thus a useful model system to study regulation of these pathways and, as primary cells, are more physiologically representative than transformed cell lines.

The main findings of these studies were that ET-1 regulates MCP-1 release from HASMC transcriptionally, by activating the MCP-1 promoter through binding of the pro-inflammatory transcription factors NF-κB and AP-1. ET-1's effects were mediated via ET_A and ET_B receptors, and the downstream signalling pathway involved p38 and p44/p42 MAP kinases. Similarly, the effects of TNF-α on eotaxin expression were also NF-κB-dependent. NF-κB-dependent eotaxin expression in response to TNF-α has been previously shown

to involve histone H4 acetylation at the eotaxin promoter (224). In these studies, we found that TNF-α-mediated eotaxin release did not involve acetylation of histone H3 at the eotaxin promoter, and that alterations in the levels of HDACs 1 and 2, or of total HDAC activity, did not account for TNF-α effects on H4 acetylation. Further, that although FP and salme downregulate H4 acetylation and NF-κB p65 binding, this also was not mediated by changes in HDAC levels or activity.

These findings are novel, in that although there are isolated reports of ET-1 increasing MCP-1 production in endothelial and mesangial cells (241, 334), the mechanisms used by ET-1 to up-regulate MCP-1 expression have not been studied in any biological system. This is also one of very few studies that have demonstrated *in vivo* binding of transcription factors to inflammatory gene promoters in response to GPCR-activating mediators. Similarly, the mechanisms regulating modulation of NF-κB binding and histone acetylation in response to TNF-α at the eotaxin promoter have not been previously investigated.

The findings of these studies have implications for understanding the pathophysiology of asthma. There is an emerging body of evidence that the myriad of intracellular signalling events triggered by inflammatory mediators and cytokines may converge on a small number of downstream pathways that are key to the inflammatory response. These potential "key players" include the MAP kinase pathways (390) and the NF-κB-activating pathway (277). ET-1 is found in elevated levels in the asthmatic airway and, as discussed in

chapter 1, plays a significant role in airway inflammation and bronchoconstriction in asthma. However, it is generally accepted that the vast numbers of cytokines and mediators at play in the asthmatic airway leads to a complex network of overlapping inflammatory responses; this explains why single mediator approaches to treating asthma (using drugs targeting, for example, histamine or leukotriene-mediated pathways) have limited efficacy. This has stimulated the research effort aimed at identifying the "key players" referred to above. We showed that ET-1 stimulates MCP-1 release via p38 and p44/p42 MAPK, enzymes known to also mediate a number of cytokine-driven responses (217). Such findings suggest that targeting MAP kinases may be of benefit in the management of inflammatory diseases such as asthma. There is evidence from animal models that such a strategy may be beneficial (391).

Like TNF-α-mediated eotaxin release, we found that NF-κB was involved in the transcriptional regulation of MCP-1 by ET-1. AP-1 was also involved in this response. This is interesting, as it adds to the body of evidence showing that these two particular transcription factors are key regulators of multiple inflammatory responses (277, 278). The NF-κB pathway in particular has been the subject of intense study, and inhibition of this pathway has been shown to reduce inflammation and the late asthmatic response in animal models (361).

Although we did not explore potential synergistic effects in these studies, the fact that TNF-α and ET-1 can both activate common pathways such as the MAP kinases and NF-κB suggests that they may be able to synergise to further increase inflammatory responses, when present together in the complex

inflammatory milieu of the asthmatic airway. This further supports the concept that targeting these common pathways may be beneficial in treating human disease.

Recently, there has been increasing interest in the role in inflammatory gene regulation of chromatin remodelling and the regulatory proteins that bring this about. Histone acetylation has received the most attention in this context. Cytokines are known to promote histone acetylation at the promoters of a number of inflammatory response genes, including acetylation of histone H4 at the eotaxin promoter by TNF-α (183, 220, 224). In these studies, we have explored this mechanism in more detail, finding that levels of HDAC1 and 2 expression and global HDAC activity were not the responsible mechanisms. Furthermore, alterations in HDAC expression/activity were not responsible for the effects of FP and salme on TNF-α-stimulated eotaxin expression. These studies suggest that targeting HDACs therapeutically may not be the most effective anti-inflammatory strategy, although it would be important to explore whether HDACs are key to regulating other inflammatory response genes before discounting them as potential therapeutic targets.

In conclusion, we have delineated the signalling pathway used by ET-1 to increase MCP-1 expression in HASMC. We have found that this pathway shares some common features with cytokine-mediated signalling, in particular that of TNF-α. We have extended earlier studies to characterise the mechanisms by which TNF-α, GC and LABA modulate transcriptional regulation at the eotaxin promoter, and shown that HDACs do not mediate

these events. These studies are likely to be of relevance to the understanding and treatment of asthmatic airway inflammation.

7.2 Suggestions for future studies

As discussed in chapter 1, nuclear signalling by mediators acting at GPCRs is less well characterised than cytokine-mediated signalling. Our studies with ET-1 therefore focused on intracellular signalling cascades, promoter activation and binding of transcription factors to the MCP-1 promoter. It would be interesting to extend these studies in the future to determine whether activation of transcription at the MCP-1 promoter by ET-1 involves HAT-mediated chromatin remodelling events similar to those seen with TNF-α (222-224). HATs and HDACs have been proposed as exciting new targets for future asthma treatments (382), and further characterising their roles in inflammatory gene regulation will be important in clarifying which of these are the key regulatory proteins in asthmatic airway inflammation.

In contrast to GPCR-mediated signalling, studies of nuclear signalling by cytokines are at a more advanced stage. Our studies with TNF-α therefore focused more on chromatin remodelling events. Although we showed that global HDAC activity and total HDAC1 and 2 levels were not responsible for the effects of TNF-α, FP and salme at the eotaxin promoter, it is possible that alterations in HDAC recruitment to the eotaxin promoter and thence changes in promoter acetylation status may be responsible. ChIP assays could be used to explore this possibility.

In our studies of MCP-1 regulation by ET-1, we used inhibitor studies to show that MAP kinases were involved in the signalling pathway. Although we have data from our lab to show that these inhibitors are selective, at the concentrations used, for their respective targets in HASMC (Clarke DL and Knox AJ, unpublished observations), it would strengthen our observations to confirm these findings using molecular strategies such as siRNA or dominant negative kinases. It would also be of interest to delineate the signalling cascades in more detail. Our studies suggested that ET-1 activates AP-1 by a JNK-independent mechanism in these cells. This is a recognised (352, 353), but not commonly reported phenomenon, and it would be interesting to know exactly which upstream kinases were responsible. It would also be appealing to determine whether, like TNF-α-mediated events, steroids and LABA can alter the expression of MCP-1 in response to ET-1, and to explore the mechanisms involved.

Finally, our studies were performed in HASMC derived from non-asthmatic donors. As discussed in section 1.4.6, a number of functional differences have been identified between cells derived from normal and asthmatic donors. The phenomena described in this thesis are worthy of study in asthmatic cells. It is possible that there are significant differences that may be of pathophysiological relevance. Comparative studies in asthmatic cells would give the most accurate assessment of the relevance of the effects we describe here.

APPENDIX: CELL VIABILITY STUDIES

The effect on cell viability of the stimulants and inhibitors used in these studies was assessed by MTT assay. In all of the following figures, MTT assay was performed after 24 h treatment with stimulants/inhibitors. Optical densities were read at 550 nm in a microplate reader. Viability was compared to that of control cells, with viability of controls defined as 100%. Cell viability with all chemicals/inhibitors used in this study was greater than 90% of that of control cells as shown in the following figures.

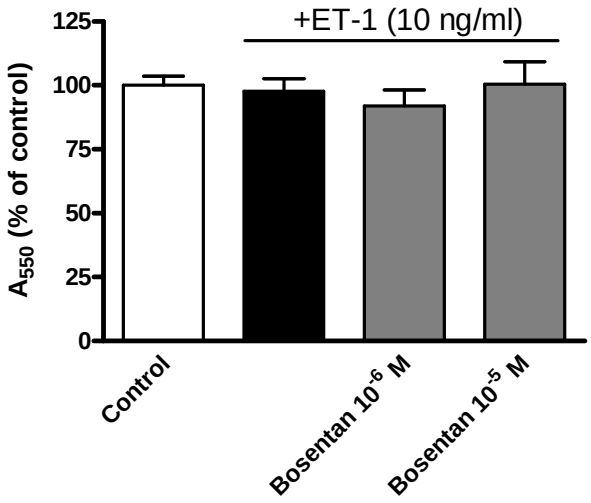

Figure A.1 Effect of Bosentan on HASMC viability

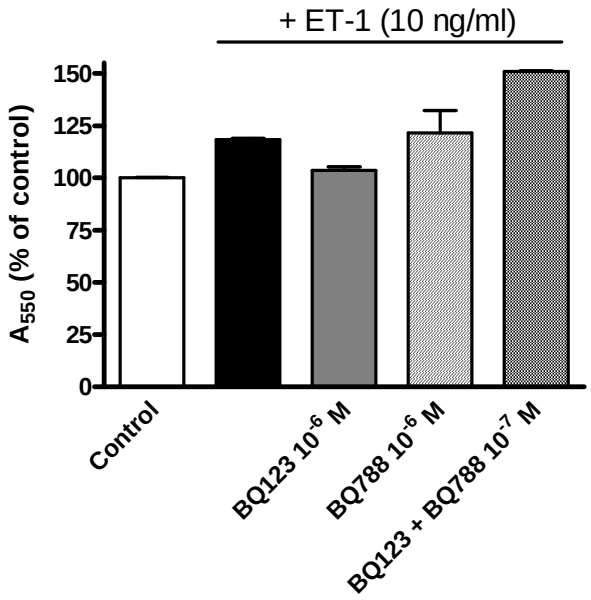

Figure A.2 Effect of BQ123 and BQ788 on HASMC viability

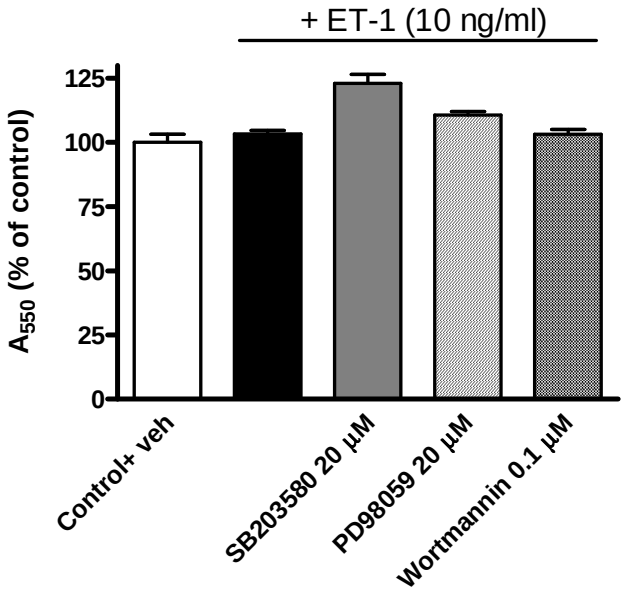

Figure A.3 Effect of SB203580, PD98059 and Wortmannin on HASMC viability

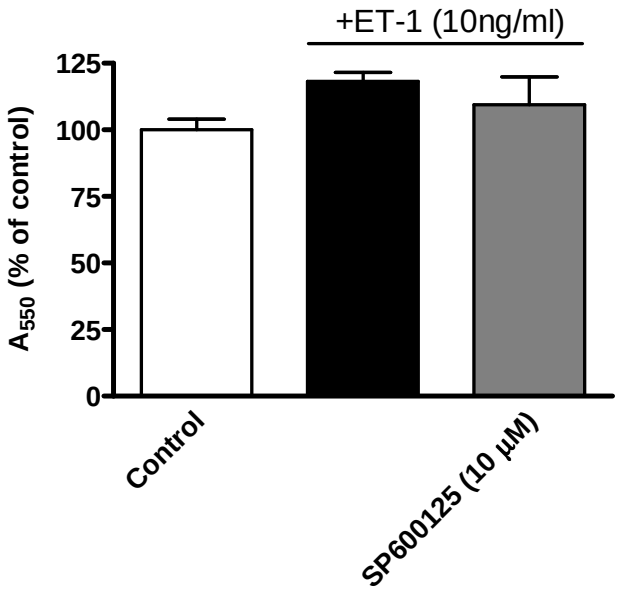

Figure A.4 Effect of SP600125 on HASMC viability

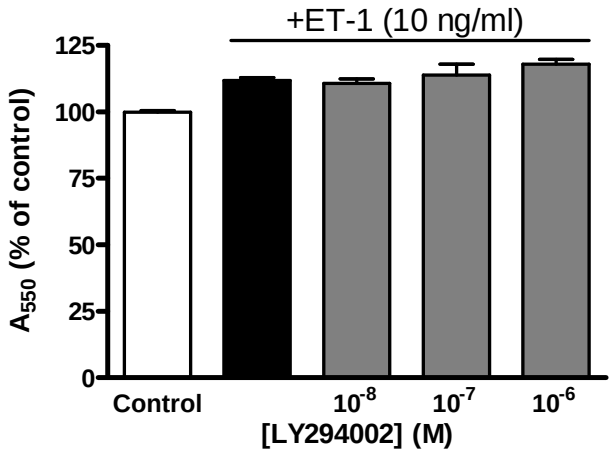

Figure A.5 Effect of LY294002 on HASMC viability

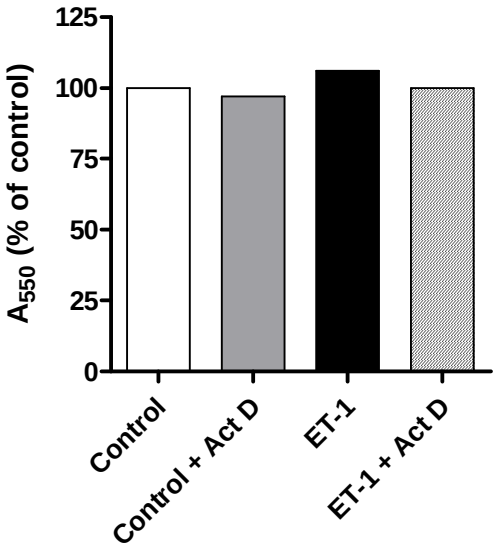

Figure A.6 Effect of ET-1 (10 ng/ml), Act D (5μg/ml) and ET-1 and Act D in combination on HASMC viability

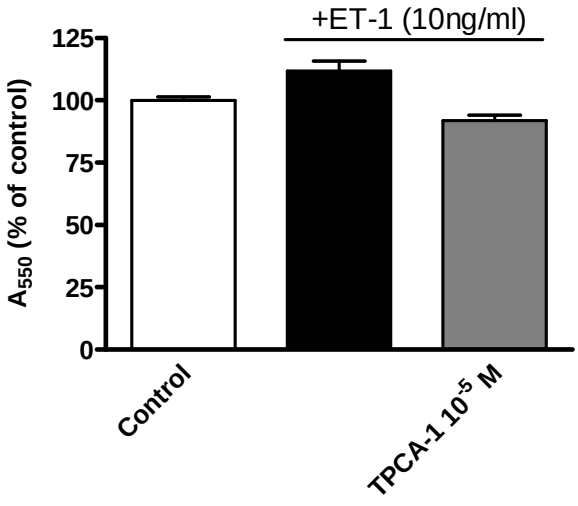

Figure A.7 Effect of TPCA-1 on HASMC viability

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