British Journal of Pharmacology (2009), 157, 436–450

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Transcriptional regulation of monocyte chemotactic protein-1 release by endothelin-1 in human airway smooth muscle cells involves NF-κB and AP-1

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Background and purpose: Endothelin-1 (ET-1) is implicated in airway inflammation in asthma, but the mechanisms of its effects are poorly understood. We studied the effect of ET-1 on expression of the chemokine, monocyte chemotactic protein-1 (MCP-1), in primary cultures of human airway smooth muscle cells.
Experimental approach: MCP-1 release was measured by ELISA. Pharmacological antagonists/inhibitors, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting were used to study ET receptors and kinase cascades. Transcriptional regulation was studied by real-time RT-PCR, transient transfection studies and chromatin immunoprecipitation assay. Major findings were confirmed in cells from three donors and mechanistic studies in cells from one donor.
Key results: ET-1 increased MCP-1 release through an ET_A and ET_B receptor-dependent mechanism. ET-1 increased MCP-1 mRNA levels but not mRNA stability suggesting it was acting transcriptionally. ET-1 increased the activity of an MCP-1 promoter–reporter construct. Serial deletions of the MCP-1 promoter mapped ET-1 effects to a region between −213 and −128 base pairs upstream of the translation start codon, containing consensus sequences for activator protein-1 (AP-1) and nuclear factor-κB (NF-κB). ET-1 promoted binding of AP-1 c-Jun subunit and NF-κB p65 subunit to the MCP-1 promoter. Blocking the inhibitor of κB kinase-2 with 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide (TPCA-1) decreased ET-1-stimulated MCP-1 production. p38 and p44/p42 mitogen-activated protein kinases were involved in upstream signalling.
Conclusions and implications: ET-1 regulated MCP-1 transcriptionally, via NF-κB and AP-1. The upstream signalling involved ET_A, ET_B receptors, p38 and p44/p42 mitogen-activated protein kinases. These may be targets for novel asthma therapies.
British Journal of Pharmacology (2009) **157**, 436–450; doi:10.1111/j.1476-5381.2009.00143.x; published online 9 April 2009

Keywords: endothelin-1; monocyte chemotactic protein-1; asthma; inflammation; G protein-coupled receptor; mitogen-activated protein kinase; transcription factor; activator protein-1; nuclear factor-κB

Abbreviations: AP-1, activator protein-1; ASM, airway smooth muscle; β2M, β2 microglobulin; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, gamma-activated site; GPCR, G protein-coupled receptor; HASM, human airway smooth muscle; HASMC, human airway smooth muscle cells; IKK-2, inhibitor of κB kinase-2; IRIS, interferon response inhibitory sequence; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue); NF-1, nuclear factor-1; NF-κB, nuclear factor-κB; PI3 kinase, phosphatidyl inositol 3-kinase; qRT-PCR, quantitative real-time reverse transcriptase PCR; RT-PCR, reverse transcriptase-polymerase chain reaction; Sp1, small protein-1; TPCA-1, 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide

Introduction

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,

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Received 30 May 2008; revised 24 November 2008; accepted 5 December 2008

manifest as an increase in airway smooth muscle (ASM) mass, mucous gland hyperplasia, an increase and alteration in extracellular matrix and subepithelial fibrosis (Vignola *et al.*, 2000). The increased thickness of the ASM layer is a key feature of the remodelled asthmatic airway. This is highly significant, as 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 (Bowie and O'Neill, 2000) or Th-2 (Kisselera *et al.*, 2002) cytokines or inflammatory mediators acting at G protein-coupled receptors (GPCRs) (Ye, 2001). Activation of gene expression by GPCRs provides an important means of local production of cytokines and growth factors, which contribute to inflammation and remodelling (Ye, 2001). Although nuclear signalling by cytokines has been extensively studied, the nuclear signalling cascades used by GPCRs have been less well characterized. Human airway smooth muscle cells (HASMC) are a useful model system to study GPCR signalling in primary cells as they express the GPCRs for many important inflammatory mediators (Barnes *et al.*, 1998; Hay, 1999).

We have previously shown that the inflammatory mediator bradykinin (BK) can actively signal to the nucleus to mediate gene transcription events by using complex prostanoid-dependent signalling pathways involving activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and CCAAT/enhancer binding protein (C/EBP) (Nie *et al.*, 2003; Zhu *et al.*, 2003). 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 (receptor nomenclature follows Alexander *et al.*, 2008). ET-1 is implicated in asthma through several lines of evidence: ET-1 levels are elevated in bronchoalveolar lavage fluid, bronchial biopsies and peripheral blood of asthmatics (Hay, 1999), and inhaled ET-1 causes bronchoconstriction in asthmatic patients (Chalmers *et al.*, 1997). ET-1 is a potent contractile agonist of isolated human bronchus (Hay *et al.*, 1993); it potentiates mitogenic responses in cultured ASM cells (Panettieri *et al.*, 1996) and induces expression of matrix proteins by pulmonary fibroblasts (Xu *et al.*, 2004), suggesting a role in airway remodelling. In animal models, over-expression of ET-1 in the lungs of transgenic mice induces chronic pulmonary inflammation (Hocher *et al.*, 2000) and in a rat model, induction of pulmonary eosinophilic inflammation caused a significant increase in ET-1 mRNA and protein (Finsnes *et al.*, 2000). Furthermore, the ET-1 receptor antagonist SB-217242 inhibits airway eosinophilia and hyperresponsiveness to methacholine in Der P1-sensitized mice (Henry *et al.*, 2002). Lastly, ET-1 and ET_A receptor gene polymorphisms are linked with asthma and atopy respectively (Mao *et al.*, 1999; Immervoll *et al.*, 2001).

There has been a wealth of studies of the effects of ET-1 on ASM contraction. However, despite the implication of ET-1 in airway inflammation through animal studies, there are virtually no 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 two chemokines, interleukin-8 and monocyte chemotactic protein-1 (MCP-1) from several other cell types *in vitro* (Mullol *et al.*, 1996; Chen *et al.*, 2001) and has been implicated in induction of chemokine release *in vivo* (Chen *et al.*, 2001; Finsnes *et al.*, 2001). The ET_A and ET_B receptors activate calcium and inositol phosphate second messenger pathways (Vichi *et al.*, 1999; Fehr *et al.*, 2000). ET-1 can also activate the mitogen-activated protein kinase (MAPK) family of signalling proteins. For example, ET-1 activates extracellular signal-regulated kinase-2 (ERK-2) and c-Jun NH_2-terminal kinase (JNK) in rat tracheal smooth muscle cells (Shapiro *et al.*, 1996; Vichi *et al.*, 1999). Similarly, ET-1 induces the MEK (MAPK/ERK kinase)/ERK pathway in human lung fibroblasts (Xu *et al.*, 2004), and the ERK 1/2, p46 and p54 JNKs and p38 MAPKs in cardiac myocytes (Clerk and Sugden, 1999). However, there is a paucity of data on nuclear signalling by ET-1 and, furthermore, the transcriptional and post-transcriptional regulation of chemokine expression by ET-1 has not been studied.

Monocyte chemotactic protein-1 is a chemokine of the CC subgroup, which is chemotactic for monocytes (Zachariae *et al.*, 1990) and T-lymphocytes (Loetscher *et al.*, 1994). Levels of MCP-1 are elevated in the epithelium and subepithelial tissues of bronchial biopsies from asthmatic subjects, including the bronchial smooth muscle layer (Sousa *et al.*, 1994). Allergen challenge significantly increased MCP-1 levels in bronchoalveolar lavage fluid of asthmatic patients (Holgate *et al.*, 1997). 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 immunoneutralization (Rose *et al.*, 2003). Interestingly, a polymorphism in the MCP-1 gene is associated with the presence of atopic asthma and its severity in children (Szalai *et al.*, 2001). These observations suggest that MCP-1 is an important chemokine contributing to airway inflammation in asthma. We and others have shown that HASMC express MCP-1 and that its level of expression is up-regulated by cytokines (Watson *et al.*, 1998; Nie *et al.*, 2005). However, there have been few studies in ASM of the effects on MCP-1 expression by asthma mediators acting at GPCRs, although, in human lung fibroblasts, MCP-1 secretion is induced by BK (Koyama *et al.*, 2000).

Here we have explored the mechanisms used by ET-1 to induce MCP-1 expression. We found that ET-1 regulates MCP-1 expression transcriptionally via AP-1 and NF-κB. The signalling pathway involves ET_A and ET_B receptors, p38 and p44/p42 MAPKs but not JNK or phosphatidyl inositol 3-kinase (PI3 kinase). This study provides insight into nuclear signalling by GPCR ligands such as ET and adds to the growing body of evidence suggesting that such common downstream signalling moieties may be promising targets for the development of novel therapies for asthma.

Methods

Cell culture

The studies involving human cells were approved by the Nottingham City Hospital Research Ethics Committee. HASMC from normal subjects were prepared as described previously (Hall *et al.*, 1992; Pang *et al.*, 2006). Cells were

cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mmol·L^{-1} L-glutamine, 2.5 μg·mL^{-1} amphotericin B, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin and 10% heat-inactivated foetal calf serum in humidified 5% CO_2-95% air at 37°C. Cells at passage 6–7 were used for all experiments. The principal findings of this study were confirmed in tissue from three different donors. Subsequent mechanistic studies were performed on cells from one donor. Experiments were performed on cells at 95–100% confluency.

Experimental protocols

Confluent HASMC in 24 well plates were growth-arrested for 24 h in serum-free medium and then incubated at 37°C with ET-1 (0–10 ng·mL^{-1}) for 24 h in concentration–response experiments or with ET-1 (10 ng·mL^{-1}) in kinetic experiments. Immediately before each experiment, the cells were treated for 24 h with fresh serum-free medium containing ET-1 at the concentrations indicated, and supernatants assayed for MCP-1. In inhibitor studies, cells were pre-incubated for 30 min with inhibitors prior to treatment with ET-1 (10 ng·mL^{-1}) for 24 h. Vehicle (DMSO, dimethyl sulphoxide) was added to control wells at equivalent concentrations (maximum concentration 0.2%).

MCP-1 assay

Monocyte chemotactic protein-1 concentrations in cell culture supernatants were measured by using ELISA kits according to the manufacturer's instructions as previously described (Nie *et al*., 2005).

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Confluent and growth-arrested HASMC were treated with ET-1 (10 ng·mL^{-1}) for the times indicated. Total RNA was isolated by using the RNeasy mini kit following the manufacturer's protocol with on-column DNase digestion. Reverse transcription and PCR were performed as described previously (Zhu *et al*., 2003). The primers used for PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (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′. Amplification was carried out in a PTC 100 thermal cycler (MJ Research, Inc., Waltham, MA, USA), by using the following temperature and time profiles, GAPDH: 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 30 s; ET receptors: 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. Both programmes included an initial denaturation step at 95°C for 1 min, and a final chain extension at 72°C for 10 min.

Quantitative real-time RT-PCR

Monocyte chemotactic protein-1 mRNA expression was determined by quantitative real-time RT-PCR (qRT-PCR) as described previously (Bradbury *et al*., 2005). β2 Microglobulin was used as the housekeeping gene (Pallisgaard *et al*., 1999). MCP-1 expression was normalized to the housekeeping gene by dividing the MCP-1 triplicate values by the mean of the β2 microglobulin triplicate values. Primer sequences used for qRT-PCR were: β2 microglobulin sense, 5′-GAG TAT GCC TGC CGT GTG-3′; antisense, 5′-AAT CCA AAT GCG GCA TCT-3′. MCP-1 sense, 5-GAT CTC AGT GCA GAG GCT CG-3′; antisense, 5′-TGC TTG TCC AGG TGG TCC AT-3′.

Cell viability (MTT) assay. The toxicity of inhibitors and the vehicle DMSO to HASMC was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue)] assay as described previously (Pang and Knox, 1997). Viability was compared with that of control cells, with viability of controls defined as 100%.

Western blotting analysis. Western blotting was performed as described by Clarke *et al*. (2004) to assess the phosphorylation of MAPKs in response to ET-1.

Vectors and transient transfections

Monocyte chemotactic protein-1 enhancer and MCP-1 promoter vectors consisted of the pGL3-basic plasmid vector containing either the wild-type human MCP-1 enhancer or promoter regulatory sequences driving a luciferase reporter gene. The MCP-1 enhancer construct contained the region –2802 to –2573 relative to the human MCP-1 translational start codon, which harbours two NF-κB binding sites. The MCP-1 promoter construct contained the proximal section of the wild-type human MCP-1 promoter region (–167 to –1), which harbours a number of different transcription factor binding sites (Figure 1A). These constructs have previously been described in detail (Nie *et al*., 2005). The MCP-1 promoter construct deletion series was a gift from Dr Garzino Demo (University of Maryland, USA) (Lim and Garzino-Demo, 2000) and consisted of the PGL2-basic vector containing the 486, 213 and 128 bp upstream of the translational start codon of the human MCP-1 promoter, driving a luciferase reporter gene (Figure 1B). 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 (Bergmann *et al*., 1998). 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) (Kieser *et al*., 1996). All transient transfections were conducted by using FuGene 6 transfection reagent according to the manufacturer's recommended protocol as described previously with some modifications (Nie *et al*., 2003; Pang *et al*., 2003). HASMC were seeded into 24 well plates at a concentration of 2.5 × 10^4 mL^{-1}. Cells at 50–60% confluence were transfected with 0.4 μg DNA : 1.2 μL FuGene 6 per well in serum-free,

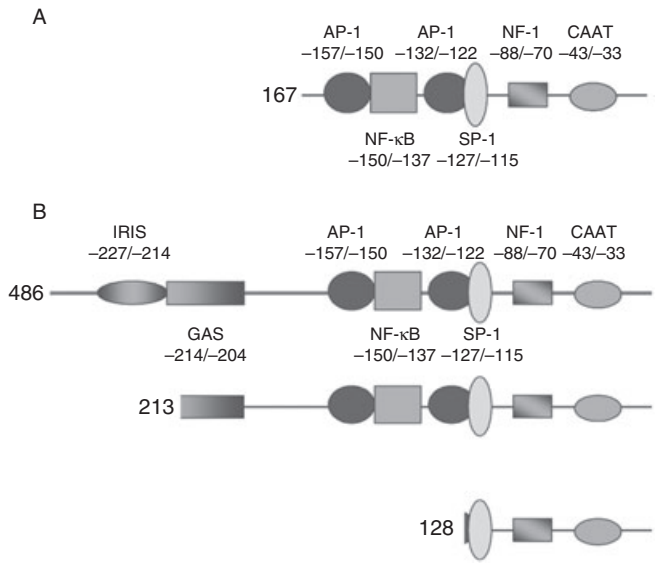

Figure 1 The human MCP-1 gene is regulated by a distal enhancer region containing two NF-κB consensus sequences (not shown), and a more complex proximal promoter region from –486 to –32. Numbers refer to the 5′ nucleotide relative to the translation start site. (A) MCP-1 promoter. The MCP-1 promoter construct used in this study consisted of the 167 bp upstream of the translational start codon driving a luciferase reporter gene. (B) MCP-1 promoter deletion series. The MCP-1 promoter deletion series consisted of the 486, 213 and 128 bp upstream of the translational start codon driving a luciferase reporter gene. AP-1, activator protein-1; GAS, gamma-activated site; IRIS, interferon response inhibitory sequence; MCP-1, monocyte chemotactic protein-1; NF-1, nuclear factor-1; NF-κB: nuclear factor-κB; Sp1, small protein-1.

antibiotic-free DMEM containing 4 mmol·L^{-1} L-glutamine. After 16 h transfection, cells were treated with ET-1 (10 ng·mL^{-1}) for the indicated times. Cells were then lysed and firefly luciferase activity determined as described previously (Nie *et al*., 2003).

Chromatin immunoprecipitation (ChIP) assay

Confluent, serum-deprived HASMC in 75 cm^2 dishes were incubated with ET-1 (10 ng·mL^{-1}) for the times indicated. In inhibitor studies, cells were pre-incubated with inhibitors for 30 min prior to stimulation for 1 h with 10 ng·mL^{-1} ET-1. ChIP assay was performed by using the ChIP-IT Express kit according to the manufacturer's recommended protocol. DNA-bound NF-κB p65 subunit, or AP-1 c-Jun subunit, was immunoprecipitated by incubating each sample with 4 μg of antibody directed against NF-κB p65/AP-1 c-Jun overnight at 4°C with rotation. After reversal of cross links and digestion of bound proteins, the recovered DNA was quantified by PCR using the following specific primers spanning the regulatory region of the human MCP-1 promoter containing the NF-κB and AP-1 consensus sequences: sense, 5′-CCC ATT TGC TCA TTT GGT CTC AGC-3′; antisense, 5′-GCT GCT GTC TCT GCC TCT TAT TGA-3′ (Ritchie *et al*., 2004).

Data analysis

Monocyte chemotactic protein-1 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. *N* values indicate the number of primary smooth muscle cell donors from which the data are derived. 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, by 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 with ET-1-stimulated cells, were assessed by using Dunnett's post hoc correction for multiple comparisons. A *P*-value of <0.05 was regarded as statistically significant.

Materials

Human ET-1, DMEM, penicillin/streptomycin, L-glutamine, amphotericin B, actinomycin D, LY294002, wortmannin, DMSO, MTT and RedTaq DNA polymerase 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. 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide (TPCA-1) was a gift from Dr Rick Williamson, GlaxoSmithKline, Uxbridge, Middlesex, UK. MCP-1 ELISA kits 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 firefly luciferase assay system were purchased from Promega, Southampton, Hampshire, UK. Primers were purchased from Sigma Genosys, Haverhill, Suffolk, UK. Excite Real Time Mastermix with sybr green was from Biogene, Cambridge, Cambridgeshire, UK. Nitrocellulose membrane for Western blotting was purchased from Bio-Rad, Hemel Hempstead, Hertfordshire, UK. Anti-p38 MAPK and anti-p44/p42 MAPK antibodies (total and phospho-) were from Cell Signaling/New England Biolabs, Hitchin, Hertfordshire, UK. Horseradish peroxidase-conjugated secondary antibodies were from DakoCytomation, Ely, Cambridgeshire, UK. ECL Western blotting detection reagent and Hyperfilm-ECL 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. Anti-NF-kB p65 and anti-c-Jun antibodies were from Santa Cruz/Insight, Wembley, Middlesex, UK.

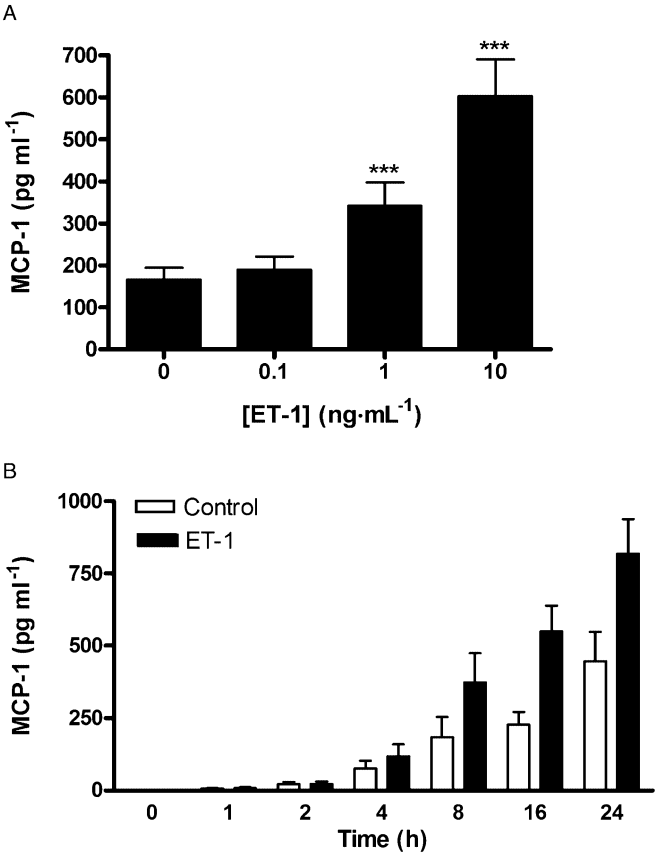

Figure 2 (A) Concentration–response of ET-1-stimulated MCP-1 production by HASMC. Cells were treated for 24 h with ET-1 at the concentrations indicated. ET-1 significantly increased MCP-1 release ($P < 0.001$). ***$P < 0.001$ compared with unstimulated cells. Each bar represents group mean (SE) derived from 13 replicates in four independent experiments ($n = 3$ different primary donors). (B) Time–course of ET-1-stimulated MCP-1 production by HASMC. Cells were treated with ET-1 (10 ng·mL^{-1}) 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 seven independent experiments ($n = 3$ different primary donors). ET-1, endothelin-1; HASMC, human airway smooth muscle cells; MCP-1, monocyte chemotactic protein-1.

Results

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. Growth-arrested, cultured HASMC were treated for 24 h with increasing concentrations of ET-1 up to 10 ng·mL^{-1}. ET-1 stimulated MCP-1 release in a concentration-dependent manner (Figure 2A). In other experiments, higher concentrations of ET-1 were used and no further increase in effect was observed (data not shown). We therefore used 10 ng·mL^{-1} ET-1 in all subsequent experiments. HASMC were next treated with 10 ng·mL^{-1} 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 2B). These findings were confirmed in cells from three different donors. Subsequent mechanistic studies were performed in cells from one donor. Experiments were repeated at least twice.

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

Two ET receptors have been cloned and sequenced, the ET_A and ET_B receptors. RT-PCR demonstrated the presence of mRNA for both receptors in HASMC (Figure 3A). We 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 (Figure 3B–D). 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 3E), but this was of borderline statistical significance ($P = 0.05$).

Effects of ET-1 on MCP-1 production are mediated by p44/p42 and p38 MAPKs but not JNK or PI3 kinase

PD98059 (20 μmol·L^{-1}), a selective inhibitor of mitogen-activated protein kinase kinase (MEK, immediately upstream of p44/p42 MAPK), inhibited ET-1-stimulated MCP-1 production (Figure 4A) suggesting a role for p44/p42 MAPK. Consistent with this, Western blotting demonstrated a time-dependent increase in phosphorylation of p44/p42 MAPK following stimulation with ET-1 (Figure 4B). The p38 MAPK inhibitor SB203580 (20 μmol·L^{-1}) also inhibited ET-1-stimulated MCP-1 production (Figure 4C). Furthermore, ET-1 caused an increase in phosphorylation of p38 on Western blotting (Figure 4D). In contrast, the selective JNK inhibitor SP600125 (10 μmol·L^{-1}) and the PI3 kinase inhibitors wortmannin (100 nmol·L^{-1}) and LY294002 (1 μmol·L^{-1}) had no effect on ET-1-stimulated MCP-1 production (Figure 4E,F).

ET-1 up-regulates MCP-1 expression transcriptionally

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^{-1}). MCP-1 mRNA levels were normalized to those of the housekeeping gene, β2 microglobulin. ET-1 significantly increased MCP-1 mRNA levels relative to control (unstimulated) cells (Figure 5A). The increase was detectable at 2 h, peaking at 4 h and tailing off over the 24 h period studied. We performed mRNA stability studies with the transcription inhibitor actinomycin D to determine whether ET-1's effects on MCP-1 mRNA levels were due to stabilization of the MCP-1 mRNA. MCP-1 mRNA is constitutively expressed in HASMC. Cells were incubated for 0–24 h with 1 μg·mL^{-1} of actinomycin D alone (which blocks the production of new transcripts), or actinomycin 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 stabilize MCP-1 mRNA (Figure 5B).

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

Next, vectors encoding the wild-type MCP-1 enhancer or promoter regions upstream of a luciferase reporter gene were

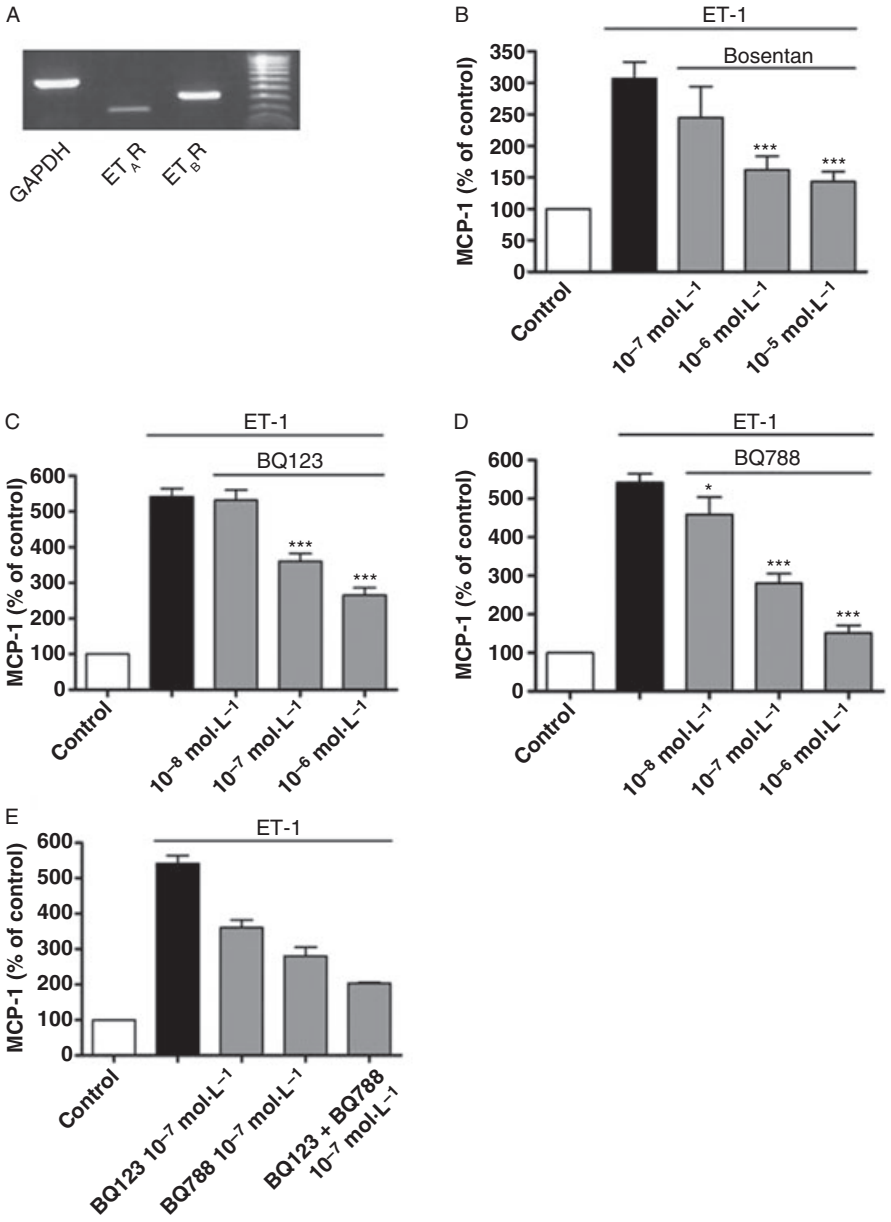

Figure 3 (A) ET_A and ET_B receptor mRNA is expressed in HASMC. Representative gel from one of three independent experiments ($n = 1$). (B) The dual selective ET receptor antagonist bosentan concentration-dependently inhibited ET-1-stimulated MCP-1 production ($P < 0.001$). ***$P < 0.001$ compared with ET-1-stimulated cells. Each bar represents group mean (SE) derived from 11 replicates in three independent experiments ($n = 1$). (C) 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. (D) 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. (E) BQ123, BQ788 and both inhibitors in combination (10^{-7} mol·L^{-1}) significantly inhibited ET-1-stimulated MCP-1 production ($P < 0.001$). For interaction $P = 0.053$. (C–E) each bar represents group mean (SE) derived from six replicates in two independent experiments ($n = 1$). ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HASMC, human airway smooth muscle cells; MCP-1, monocyte chemotactic protein-1.

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 6A). 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 6B), although this did not reach statistical significance ($P = 0.1$). 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, as 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

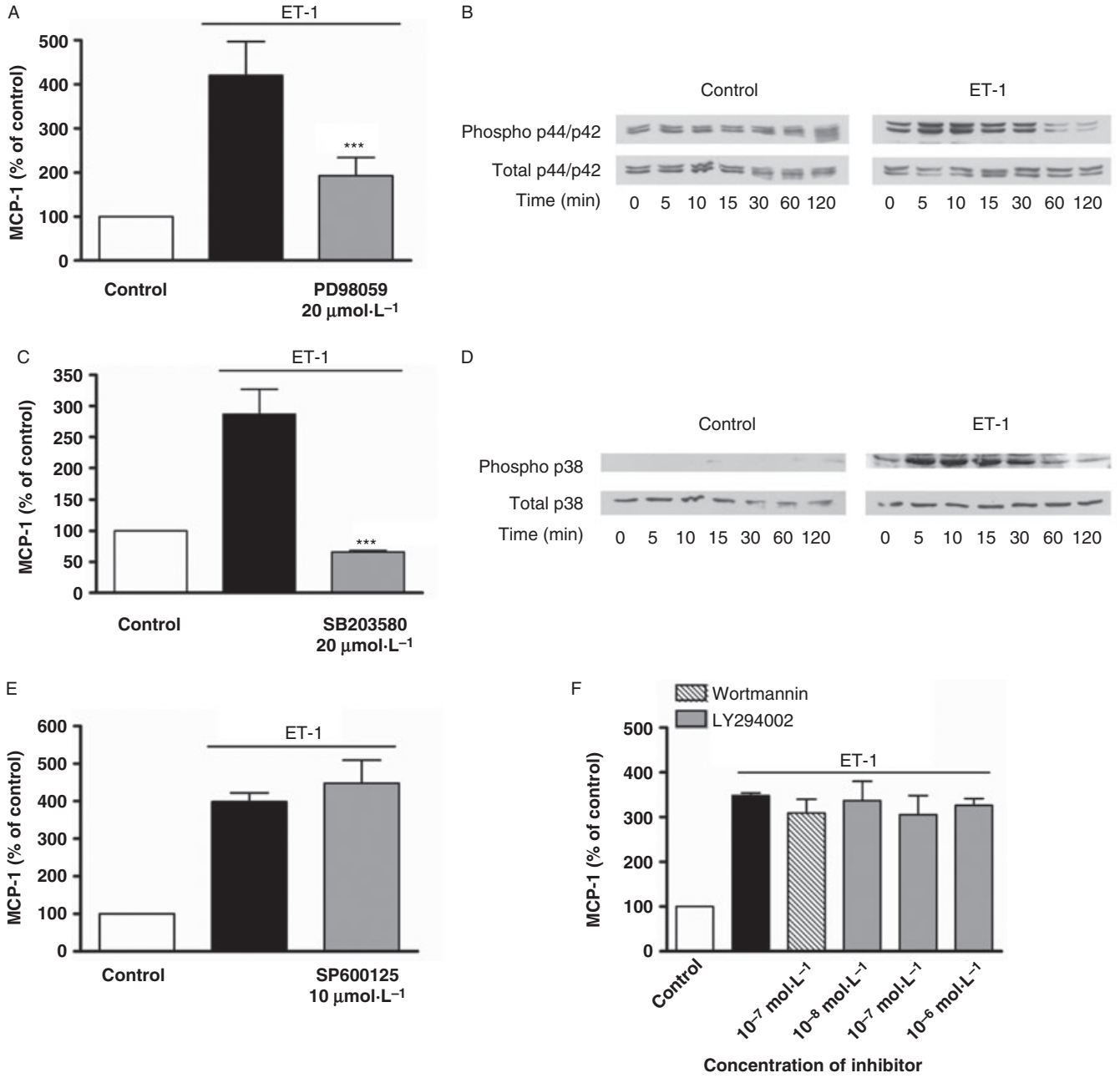

Figure 4 (A) Effect of PD98059 (MEK inhibitor) on ET-1-stimulated MCP-1 production. PD98059 (20 μmol·L^{-1}) inhibited ET-1-stimulated MCP-1 production. ***$P < 0.001$ compared with cells treated with ET-1 alone. Each bar represents group mean (SE) derived from 11 replicates in three independent experiments ($n = 1$). (B) Western blotting showing time-dependent phosphorylation of p44/p42 MAPK by ET-1. An increase in phosphorylation of p44/p42 MAPK is seen at 5 min and is sustained until 30 min. Representative blot from one of four independent experiments ($n = 1$). (C) Effect of SB203580 (p38 MAPK inhibitor) on ET-1-stimulated MCP-1 production. SB203580 (20 μmol·L^{-1}) 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 16 replicates in two independent experiments ($n = 1$). (D) Western blotting showing time-dependent phosphorylation of p38 MAPK by ET-1. An increase in phosphorylation of p38 MAPK is seen at 5 min and is sustained until 30 min. Representative blot from one of three independent experiments ($n = 1$). (E) The JNK inhibitor SP600125 (10 μmol·L^{-1}) had no effect on ET-1-stimulated MCP-1 production. Each bar represents group mean (SE) derived from 10 replicates in three independent experiments ($n = 1$). (F) The PI3 kinase inhibitors wortmannin (10^{-7} mol·L^{-1}) and LY294002 (10^{-6} mol·L^{-1}) had no effect on ET-1-stimulated MCP-1 production. Each bar represents group mean (SE) derived from eight replicates in two independent experiments ($n = 1$). (A, C, E and F) MCP-1 production is shown as a percentage of MCP-1 production by control (untreated) cells. ET-1, endothelin-1; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MEK, mitogen-activated protein kinase kinase; PI3 kinase, phosphatidyl inositol 3-kinase.

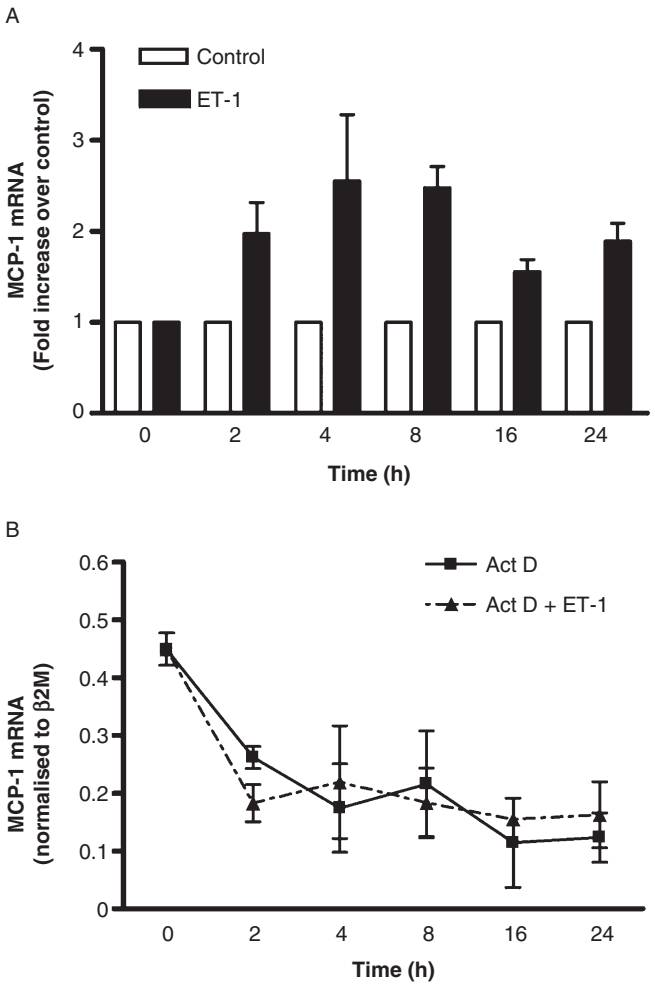

Figure 5 (A) ET-1 increased expression of MCP-1 mRNA measured by qRT-PCR ($P < 0.001$ compared with control cells). The effect was time-dependent with the maximal effect of ET-1 observed at around 4 h stimulation (for interaction between ET-1 and time, $P = 0.003$). MCP-1 mRNA was normalized to the housekeeping gene β2M. The results are expressed as fold increase over unstimulated controls at the same time point. Each bar represents group mean (SE) derived from 12 replicates in four independent experiments ($n = 1$). (B) Effect of ET-1 on MCP-1 mRNA stability. ET-1 treatment had no effect on the rate of decay of MCP-1 transcripts, after treatment with actinomycin D (Act D). MCP-1 mRNA was normalized to the housekeeping gene β2M. Each point represents group mean (SE) derived from six replicates in three independent experiments ($n = 1$). β2M, β2 microglobulin; ET-1, endothelin-1; MCP-1, monocyte chemotactic protein-1; qRT-PCR, quantitative real-time reverse transcriptase-polymerase chain reaction.

and 128 bp upstream of the translational start codon is required for the effects of ET-1 on MCP-1 transcriptional activation (Figure 6C). This region contains consensus sequences for the transcription factors AP-1 and NF-κB. 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 6 h. ET-1 caused a 1.7-fold increase in luciferase activity of the NF-κB construct (Figure 6D) and a 1.6-fold increase in activity of the AP-1 construct (Figure 6E). As ET-1 increased the activation of both the MCP-1 promoter, which harbours two AP-1 binding sites, and the AP-1 reporter construct, we tested the effect of a JNK inhibitor SP600125 on ET-1-stimulated MCP-1 promoter activity. Interestingly, SP600125 had no effect on ET-1-stimulated MCP-1 promoter activity (Figure 6F). In contrast, blocking the inhibitor of κB kinase-2 with TPCA-1 concentration-dependently inhibited ET-1-stimulated MCP-1 production with a $-\log IC_{50}$ of 6.192 ± 0.109 (IC_{50} 0.709 ± 0.192 μmol·L^{-1}, Figure 6G), which further supports the hypothesis that NF-κB is involved in ET-1-stimulated MCP-1 production.

ET-1 promotes in vivo *binding of NF-κB p65 subunit and AP-1 c-Jun subunit to the MCP-1 promoter and this effect is inhibited by PD98059 and SB203580*

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 c-Jun to the MCP-1 promoter, suggesting that both transcription factors are involved (Figure 7A,B). 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 c-Jun binding to the MCP-1 promoter was observed, with a 2.2-fold increase in c-Jun binding seen at 1 h that returned to basal levels by 2.5 h. Binding of p65 and c-Jun to the MCP-1 promoter was inhibited by the MEK inhibitor PD98059 (20 μmol·L^{-1}) and the p38 MAPK inhibitor SB203580 (20 μmol·L^{-1}, Figure 7C). 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.

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 (data not shown).

Discussion

The major finding here is that ET-1 induces MCP-1 expression in HASMC via transcriptional activation of the MCP-1 enhancer and promoter regions. This involves ET_A and ET_B receptors and both p44/p42 and p38 MAPK-dependent pathways, but not JNK or PI3 kinase.

Monocyte chemotactic protein-1 is released from HASMC constitutively and, 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 (Figure 2B). Of note, a previous study in HASMC did not show induction of MCP-1 in response to ET-1 (Watson *et al*., 1998). However, the concentration of ET-1 used in that study was 1 μmol·L^{-1}, or 2492 ng·mL^{-1}, 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

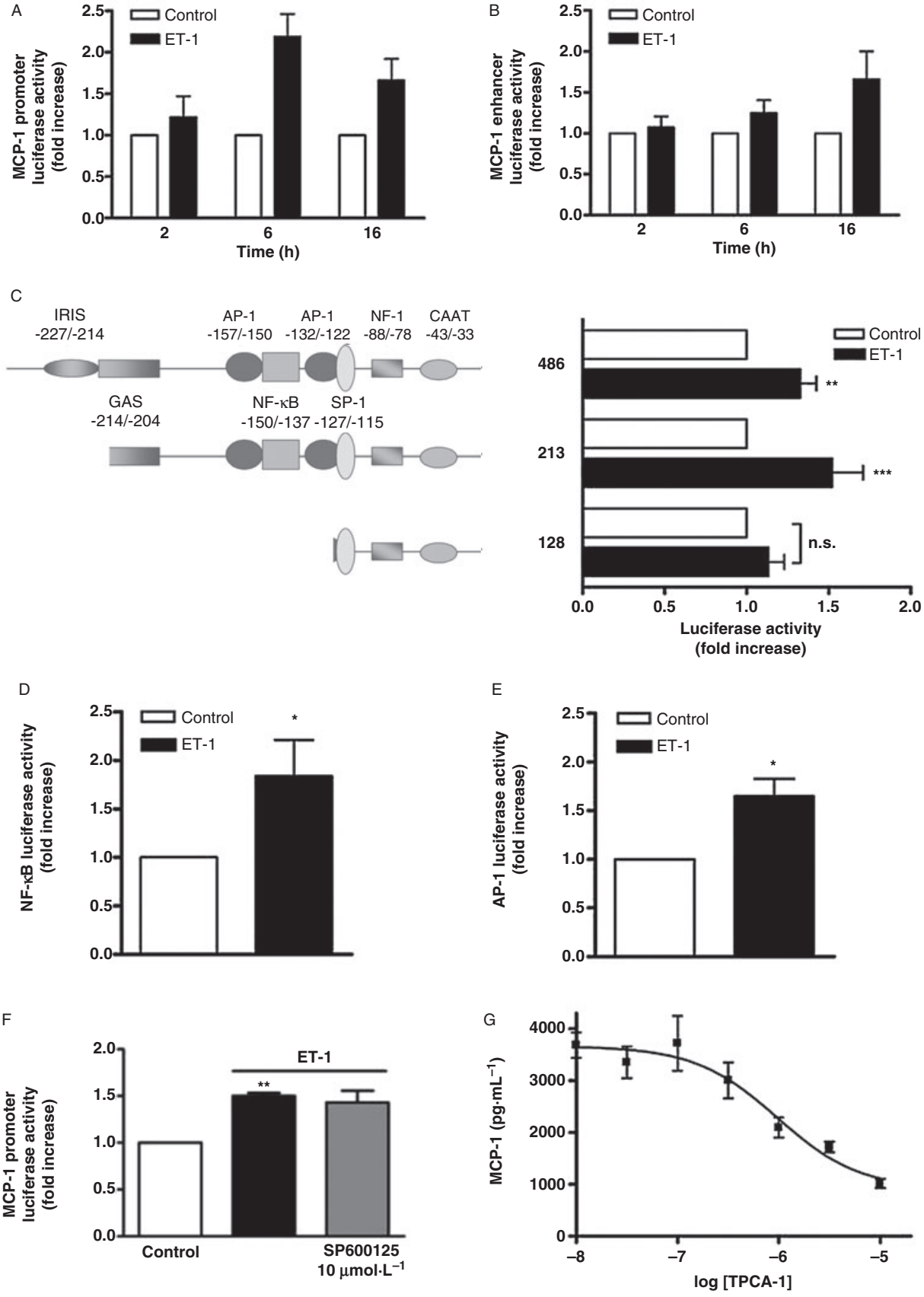
A
MCP-1 promoter luciferase activity (fold increase)
Control
ET-1
Time (h)
B
MCP-1 enhancer luciferase activity (fold increase)
Control
ET-1
Time (h)
C
IRIS -227/-214
AP-1 -157/-150
AP-1 -132/-122
NF-1 -88/-78
CAAT -43/-33
GAS -214/-204
NF-κB -150/-137
SP-1 -127/-115
486
213
128
n.s.
Luciferase activity (fold increase)
D
NF-κB luciferase activity (fold increase)
E
AP-1 luciferase activity (fold increase)
F
MCP-1 promoter luciferase activity (fold increase)
Control
SP600125 10 μmol·L⁻¹
G
MCP-1 (pg·mL⁻¹)
log [TPCA-1]

Figure 6 (A) Effect of ET-1 on MCP-1 promoter activity. ET-1 stimulated MCP-1 promoter-driven luciferase activity ($P = 0.03$ compared with controls). Each bar represents group mean (SE) derived from 18 replicates in three independent experiments ($n = 1$). (B) 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 with controls). Each bar represents group mean (SE) derived from 16–26 replicates in three to six independent experiments ($n = 1$). (C) 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 three independent experiments ($n = 1$). (D and E) Effect of ET-1 on NF-κB (D) and AP-1 (E) reporter-driven luciferase activity following 6 h treatment with ET-1. $^{*}P = 0.03$ compared with control. Each bar represents group mean (SE) derived from 18 replicates in three independent experiments (D) or 12 replicates in two independent experiments (E, $n = 1$). (F) Effect of the JNK inhibitor 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 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 two independent experiments ($n = 1$). (A–F) expressed as fold increase in luciferase activity in ET-1-treated cells compared with control (untreated) cells. (G) 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 (four-parameter logistic regression). Each point represents group mean (SE) derived from 12 replicates in four independent experiments ($n = 1$). AP-1, activator protein-1; ET-1, endothelin-1; HASMC, human airway smooth muscle cells; IKK-2, inhibitor of κB kinase-2; JNK, c-Jun NH_2-terminal kinase; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor-κB; n.s., not significant; TPCA-1, 2-[(aminocarbonyl)amino]-5-[4-fluorophenyl]-3-thiophenecarboxamide.

isolated reports of ET-1 increasing MCP-1 production in endothelial and mesangial cells (Chen *et al*., 2001; Ishizawa *et al*., 2004), 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.

Two ET receptors have been identified and cloned, ET_A and ET_B receptors, both of which are expressed by HASMC (Goldie *et al*., 1995; Fukuroda *et al*., 1996). We confirmed the presence of mRNA for both receptors in HASMC (Figure 3A). Our studies with antagonists of differing ET receptor specificity indicated that both receptor subtypes are involved in ET-1-stimulated MCP-1 release (Figure 3B–D). Both antagonists inhibited ET-1-stimulated MCP-1 production at concentrations equivalent to and 10-fold higher than their respective pA_2 values [6.9–7.4 and 6.9 for BQ123 and BQ788 respectively (Davenport and Battistini, 2002)]. The antagonist concentrations were chosen to be selective for the relevant receptor, as both inhibitors are at least three orders of magnitude more selective for their respective receptors (Ihara *et al*., 1992; Ishikawa *et al*., 1994). These observations are interesting as they contrast with ET-1-potentiated HASMC proliferation, which appears to be mediated solely by ET_A receptors (Panettieri *et al*., 1996) and ET-1-mediated human airway smooth muscle (HASM) contraction, which is predominantly ET_B-mediated (Goldie *et al*., 1995; Adner *et al*., 1996).

We next turned our attention to downstream signalling pathways. Studies using kinase inhibitors suggested that ET-1-stimulated MCP-1 production by HASMC involved p44/p42 and p38 MAPKs, but not JNK (Figure 4A, C and E). Although there is evidence to suggest that SB203580 can inhibit certain kinases other than p38 *in vitro* (Bain *et al*., 2007) we have previously found, in tumour necrosis factor-α-stimulated HASMC, that SB203580 (30 $\mu mol \cdot L^{-1}$) did not inhibit phosphorylation of p44/p42 MAPK and, similarly, PD98059 (30 $\mu mol \cdot L^{-1}$) did not inhibit phosphorylation of p38 MAPK, confirming the selectivity of these inhibitors for their respective targets in these cells (DL Clarke and AJ Knox, unpubl. obs.). Consistent with the inhibitor studies, ET-1 activated p44/p42 and p38 MAPKs (Figure 4B,D). ET-1 activates MAPKs in a number of systems (Shapiro *et al*., 1996; Clerk *et al*., 1998; Vichi *et al*., 1999; Shi-Wen *et al*., 2004). p44/p42 MAPK can be activated by ET-1 in ASM from other species and has been implicated in ET-1-mediated proliferative responses (Shapiro *et al*., 1996; Fujitani and Bertrand, 1997; Whelchel *et al*., 1997; Vichi *et al*., 1999). Interestingly, p44/p42 and p38 MAPKs are involved in interleukin-1β-stimulated MCP-1 production in HASMC (Wuyts *et al*., 2003), suggesting that crosstalk could occur between cytokine- and GPCR-mediated pathways of chemokine expression. MAPKs are involved in many inflammatory pathways (Dong *et al*., 2002), and MAPK inhibitors can attenuate the inflammatory disease process in animal models of rheumatoid arthritis (Han *et al*., 2001; Nishikawa *et al*., 2003). Interestingly, ET-1 also induces expression by human lung fibroblasts of connective tissue growth factor, an important profibrotic protein, 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.

ET_A and ET_B receptors are $G\alpha_q$-coupled receptors that activate calcium and inositol phosphate second messenger pathways (Hay *et al*., 1999; Vichi *et al*., 1999; Fehr *et al*., 2000). Furthermore $G\alpha_q$ can activate PI3 kinase (Clerk and Sugden, 1999; Ye, 2001), although this pathway is less well characterized. However 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 independent of PI3 kinase (Figure 4F). The inhibitor concentrations were selected to be equal to or higher than their published IC_{50} values (Arcaro and Wymann, 1993; Vlahos *et al*., 1994).

We found that MCP-1 mRNA is expressed by HASMC under resting conditions, and that ET-1 increased MCP-1 mRNA (Figure 5A). mRNA stability studies confirmed that this was not through stabilization of the MCP-1 message (Figure 5B). Transient transfection studies with wild-type MCP-1 promoter and enhancer luciferase reporter constructs supported the hypothesis that ET-1 regulates MCP-1 expression transcriptionally (Figure 6A,B). There is one previous report of ET-1 increasing MCP-1 mRNA [in human brain-derived endothelial cells (Chen *et al*., 2001)] 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 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

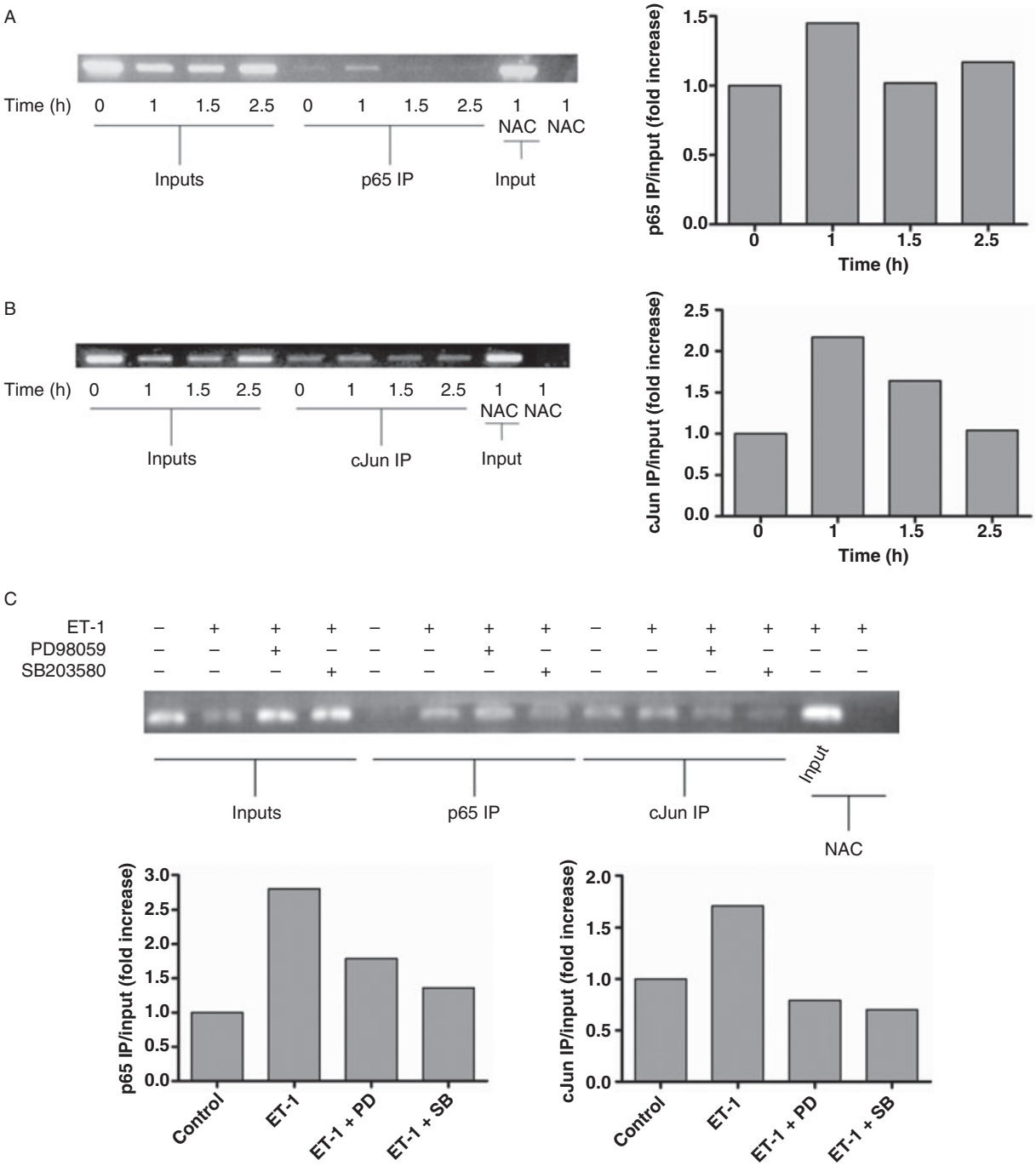

Figure 7 ChIP assays showing effect of ET-1 on *in vivo* binding of NF-κB p65 subunit (A) and AP-1 c-Jun subunit (B) to the MCP-1 promoter. ET-1 promoted *in vivo* binding of p65 and c-Jun to the MCP-1 promoter. (C) ET-1-stimulated binding of p65 and c-Jun to the MCP-1 promoter at 1 h was inhibited by PD98059 (20 μmol·L^{-1}) and SB203580 (20 μmol·L^{-1}). (A–C) Representative gels, with accompanying densitometry, from one of two independent experiments ($n = 1$). The density of the immunoprecipitated band (IP) was normalized to that of the input control at the same time point, and expressed as fold increase over time zero (time–course) or unstimulated cells (inhibitor studies). AP-1, activator protein-1; ChIP, chromatin immunoprecipitation; ET-1, endothelin-1; MCP-1, monocyte chemotactic protein-1; NAC, no antibody control; NF-κB, nuclear factor-κB.

regulation of MCP-1 by ET-1. We therefore focused our subsequent mechanistic studies on the promoter region.

There is little information on the transcription factors activated by ET-1. AP-1, NF-κB and small protein-1 (Sp1) have been implicated in ET-1-stimulated responses in other cell systems (Yin *et al.*, 1992; Liang *et al.*, 2000; Chintalgattu and Katwa, 2004), but 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 nuclear factor-1 binding sites and a CCAAT box (Figure 1A). Serial deletion

studies showed that a region between 213 and 128 base pairs upstream of the transcriptional start site was both necessary and sufficient for maximal activation of the MCP-1 promoter by ET-1 (Figure 6C). This region harbours consensus sequences for NF-κB and AP-1, suggesting that either or both of these transcription factors may be involved. Truncation of the promoter to –213 bp disrupts the sequence for the gamma-activated site (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. Our studies with the NF-κB and AP-1 luciferase reporter constructs showed that ET-1 activated both of these transcription factors in HASM (Figure 6D,E), consistent with a role for these transcription factors in regulation of MCP-1 by ET-1. Furthermore the results of the ChIP assays (Figure 7A,B) and of NF-κB pathway inhibition by TPCA-1 (Podolin *et al.*, 2005) (Figure 6G) strongly suggest 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 c-Jun to the MCP-1 promoter, suggesting that these kinases mediate ET-1-stimulated MCP-1 production transcriptionally, via NF-κB and AP-1 (Figure 7C). It was beyond the scope of the current study to determine the precise mechanisms by which p38 and p44/p42 MAPKs alter p65 and c-Jun binding to the MCP-1 promoter. However this could be brought about through phosphorylation of components of the NF-κB- and c-Jun-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. We were surprised that the JNK inhibitor SP600125 had no effect on either MCP-1 protein release or promoter activation (Figures 4E and 6E respectively), as JNK is an upstream activator of c-Jun and, taken together, our transfection and ChIP studies strongly suggest that c-Jun mediates transcriptional regulation of MCP-1 by ET-1. However, there are reports of JNK-independent activation of c-Jun (Ibarz *et al.*, 2006), including by p44/p42 MAPK (Pulverer *et al.*, 1991). Thus ET-1-mediated activation of c-Jun in HASMC may be occurring by a JNK-independent pathway.

There have been a number of studies of MCP-1 transcriptional regulation in the context of inflammation, demonstrating involvement of various transcription factors, including STAT-1, Sp1, C/EBP and NF-κB, in an apparently stimulus- and tissue-specific manner (Teferedegne *et al.*, 2006; Harvey *et al.*, 2007; Sato *et al.*, 2007). While several studies have indirectly implied involvement of AP-1 in the transcriptional regulation of MCP-1, for example through correlation of levels of MCP-1 expression with transcription factor expression or activation (Dragomir *et al.*, 2006; Ichihara *et al.*, 2006), or through electrophoretic mobility shift assay studies (Jaramillo *et al.*, 2004; Cullen *et al.*, 2005), ours 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 (Tsuchiya *et al.*, 2006) and the first to demonstrate *in vivo* binding of NF-κB and c-Jun to the MCP-1 promoter in response to ET-1 in any biological system.

Nuclear factor-κB inhibition reduces the release of other inflammatory cytokines and chemokines from HASMC and in the lungs of rodent ovalbumin asthma models (Birrell *et al.*, 2005). Blockade of the NF-κB pathway also inhibited the late asthmatic response in the ovalbumin-challenged animals (Birrell *et al.*, 2005). 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 confirmed the principal finding of this study, namely that ET-1 stimulates MCP-1 release from HASMC, in cells from three different primary donors. The subsequent mechanistic studies were performed in cells from one of these donors. In view of this, although it seems likely that the mechanism by which ET-1 produces its effects will be common to all donors, a degree of caution should be exercised if extrapolating our findings to HASM generally. The situation is however no different to studies in which cell lines are used as these are usually transformed cell lines obtained from one donor. We have shown that ET-1 regulates MCP-1 by a transcriptional mechanism 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 MAPKs. This study adds to the growing body of evidence implicating a number of common downstream targets, including MAPKs and the pro-inflammatory transcription factors NF-κB and AP-1, 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.

Acknowledgements

AMS was funded in part by a Wellcome Trust VIP award. DLC was funded by a Wellcome Trust project grant. DAB was funded by a British Heart Foundation project grant. The authors would like to thank the following people for their generous donation of materials: Dr Marc Iglarz (Actelion Pharmaceuticals, Allschwil, Basel, Switzerland) for bosentan; Dr Rick Williamson (GlaxoSmithKline, Uxbridge, Middlesex, UK) for TPCA-1; Dr Garzino Demo (University of Maryland, USA) for the MCP-1 promoter construct deletion series; Dr Robert Newton (University of Calgary, Canada) for the NF-κB reporter construct 6NF-κBtkluc and Dr Arnd Kieser (GSF National Research Centre, Munich, Germany) for the AP-1 reporter construct pRTU14. We thank Professor Sarah Lewis for assistance with the statistical analyses, and Dr Karl Deacon for assistance with sequencing of the immunoprecipitated MCP-1 promoter (ChIP studies).

Conflict of interest

None to declare.

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