TNFα-induced GM-CSF release from human airway smooth muscle cells depends on activation of an ET-1 autoregulatory positive feedback mechanism

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► Additional details of the methods and results, and an additional figure are published online only at http://thorax.bmj.com/content/vol64/issue12

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ABSTRACT

Background: There is an urgent need to inhibit endothelin-1 (ET-1) induced chronic inflammatory processes in early stages of lung diseases in order to prevent untreatable irreversible stages often accompanied by lung fibrosis and pulmonary hypertension. Nothing is known about the airway inflammation-inducing and/or maintaining role of ET-1 in human airway smooth muscle cells (HASMCs).

Objective: ET-1 and granulocyte–macrophage colony-stimulating factor (GM-CSF) expression in response to tumour necrosis factor α (TNFα) and ET-1 stimulation was investigated, and the impact of mitogen-activated protein kinase (MAPK) pathways in this context was studied. To elucidate the anti-inflammatory properties of the dual endothelin receptor antagonist bosentan that targets both endothelin receptor subtypes A (ET_AR) and B (ET_BR), its effect on the TNFα/ET-1/GM-CSF network was investigated.

Methods: ET-1 and GM-CSF expression and activation of MAPKs were investigated via quantitative reverse transcription-PCR (RT-PCR), western blotting and ELISA.

Main results: Both TNFα and ET-1 activated $p38^{MAPK}$ and extracellular signal-regulated kinase (ERK)-1/-2 signalling. ET-1 expression was induced by TNFα and by ET-1 itself. Both effects were inhibited by bosentan and by specific ET_AR or $p38^{MAPK}$ blockade. ET-1- and TNFα-induced GM-CSF expression were both reduced by bosentan as well as by specific inhibition of either ET_AR, ET_BR, $p38^{MAPK}$ or ERK-1/-2.

Conclusion: TNFα activates an ET_AR- and $p38^{MAPK}$-dependent ET-1 autoregulatory positive feedback loop to maintain GM-CSF release from HASMCs. Since bosentan impairs ET-1 autoregulation and TNFα-induced ET-1 release, as well as TNFα- and ET-1-induced GM-CSF release, the present data suggest therapeutic utility for bosentan in treating particularly the early stages of chronic inflammatory airway diseases.

Airway smooth muscle cells (ASMCs) are believed to play a critical role in the establishment of inflammation in chronic inflammatory airway diseases and also in pulmonary fibrosis for which current literature suggests airway inflammation as an important factor for disease genesis and progression.[1 2] An inflammatory environment can induce ASMCs to secrete chemokines, cytokines and growth factors. Hence, ASMCs are suggested to perpetuate inflammation within the airway wall by promoting recruitment, activation and trafficking of inflammatory cells.[1] This can lead to airway wall and airway vessel remodelling, which is understood as the main cause of irreversible airway obstruction or pulmonary hypertension of patients with chronic inflammatory airway diseases.[3] Thus, the therapeutic counter-regulation of inflammatory mechanisms of ASMCs might be an auspicious strategy to combat airway inflammation-associated diseases in early reversible stages.

Chronic inflammatory airway diseases and pulmonary fibrosis are characterised by elevated tumour necrosis factor α (TNFα) levels in sputum or bronchoalveolar lavage fluid (BALF), indicating a central role for TNFα in the establishment and/or maintenance of pathogenesis.[4–6] As TNFα is believed to act as an initiating cytokine in airway inflammation regulating infiltration/recruitment of immune effector cells into the lung,[7] TNFα-mediated processes are of interest in terms of therapeutically intervening airway inflammation at early disease stages. TNFα signals through TNF receptors 1 and 2 (TNFR1 and 2) which both are expressed on human ASMCs (HASMCs).[8]

In ASMCs, TNFα induces the release of granulocyte–macrophage colony-stimulating factor (GM-CSF).[9] GM-CSF is a proinflammatory and profibrotic cytokine with key functions in the establishment of airway inflammation,[10 11] and recent studies predict therapeutic utility for GM-CSF neutralisation in chronic inflammatory airway diseases and pulmonary fibrosis. GM-CSF deficiency/neutralisation impairs airway inflammation in murine models of allergic asthma and chronic obstructive pulmonary disease (COPD).[12–14] GM-CSF is increased in the BALF of patients with pulmonary fibrosis, stimulates macrophages to release profibrotic cytokines and might induce fibrosis by direct stimulation of ASMCs.[10]

Endothelin-1 (ET-1), a secreted peptide, signals through ET_A and ET_B transmembrane receptors (ET_AR and ET_BR), both belonging to the G-protein superfamily, and both are expressed on HASMCs.[15] Activation of the ET-1 gene leads to the release of big-ET-1, an inactive ET-1 precursor extracellularly processed into active ET-1 (hereafter referred to as ET-1).[16] Accumulating evidence is indicating the role of ET-1 as a proinflammatory cytokine. ET-1 is known to prime neutrophils, activate mast cells and stimulate monocytes to produce a variety of proinflammatory cytokines.[16] The hypothesis that ET-1 might contribute to airway inflammation in chronic inflammatory airway diseases finds support from studies demonstrating increased ET-1 levels in exhaled breath condensates of patients with asthma (in correlation with disease severity) and in sputum of patients with COPD.[17 18] ET-1 also has profibrotic properties and its levels are

elevated in sputum, BALF and fibrotic tissue of patients with cystic or idiopathic pulmonary fibrosis (IPF).[17 19] ET-1 is abundantly expressed in the vascular endothelium of the lung and, to a lesser extent, by other cell types, including pulmonary artery smooth muscle cells and lung fibroblasts.[20] Whereas many studies have focused on the effects of ET-1 on vascular smooth muscle cells,[20] there is no information about ET-1 expression in HASMCs and the ASMC inflammatory response to ET-1 stimulation.

Bosentan, approved as a treatment for pulmonary arterial hypertension combating vasoconstriction, is an endothelin receptor antagonist with almost equal affinity for ET_AR and ET_BR, and thus commonly referred to as a dual blocker.[20] Concerning the inflammatory and profibrotic properties of ET-1, bosentan might also be useful as a treatment for chronic inflammatory airway diseases and pulmonary fibrosis in early disease stages. In rat models of emphysema and eosinophilic airway inflammation, application of bosentan results in a decrease of inflammation due to a substantial reduction in leucocyte number and/or a decrease in the level of proinflammatory cytokines in the BALF and in lung tissue.[21 22]

The aim of this study was to elucidate for the first time the expression and inflammatory properties of ET-1 in HASMCs. To this end, we investigated ET-1 expression in response to TNFα stimulation. Since secreted inflammatory factors have been known temporarily to induce their own expression to potentiate inflammation rapidly (autoregulatory positive feedback mechanism), we investigated the autoregulation of ET-1 expression in cultured HASMCs. We studied the release of GM-CSF in response to TNFα and ET-1. To elucidate the anti-inflammatory properties of bosentan in HASMCs, we studied its effect on the TNFα/ET-1/GM-CSF network. Finally, we investigated the impact of endothelin receptor subtypes and mitogen-activated protein kinase (MAPK) pathways in these processes.

METHODS

Isolation and cultivation of HASMCs

HASMCs were dissected from lobar or main bronchus tissue obtained from patients undergoing lung resection as previously described.[23] Cultivation and characterisation of HASMCs was performed as described elsewhere.[24] For details see the Supplementary online material.

Stimulation of HASMCs

Before stimulation, subconfluent cell monolayers were deprived of serum for 24 h as previously described.[24] Details of the media used for serum withdrawal and stimulation are given in the Supplementary online material. HASMCs were stimulated with ET-1 (Sigma, Hamburg, Germany; catalogue no. E7764) at 100 nM or with human TNFα (R&D systems, Minneapolis, Minnesota, USA; catalogue no. 210-TA) at 20 ng/ml for the indicated times. Bosentan (Actelion Pharmaceuticals, Freiburg, Germany), BQ123 (Sigma; catalogue no. B150) or BQ788 (Sigma; cat catalogue no. B157) were added at 10^{-6} M or at the indicated concentrations 60 or 120 min before ET-1 or TNFα stimulation, respectively. Pretreatment with monoclonal blocking antibodies to TNFR1 (clone H398; Alexis, Lausen, Switzerland; catalogue no. ALX-804-200) and TNFR2 (clone 80M2; Alexis; catalogue no. ALX-804-450) each at 10 μg/ml or with PD098059 (10 μM; Calbiochem/VWR, Darmstadt, Germany; catalogue no. 513000) and SB203580 (10 μM, Calbiochem/VWR; catalogue no. 559389) was carried out 60 or 30 min before stimulation, respectively.

Semi-quantitative reverse transcription-PCR (RT-PCR)

RNA was isolated with the RNeasy spin column chromatography (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Semi-quantitative RT-PCR for ET-1 and GM-CSF (with PCR cycle conditions to analyse the output in the linear range) was performed as described before.[25] Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. PCR conditions and primer sequences are given in the Supplementary online material.

Enzyme-linked immunosorbent assays

Quantitative ELISAs for GM-CSF, ET-1 or big-ET-1 were done with supernatants of subconfluent HASMCs on a 6-well plate. Intracellular ELISA for phosphorylated (active) $p38^{MAPK}$ with total $p38^{MAPK}$ as a reference was carried out on subconfluent HASMCs on a 96-well plate. GM-CSF ELISA was performed as described before.[26] ET-1, big-ET-1 (Biomedica, Vienna, Austria; catalogue nos BI-20052 and BI-20082) and phosphorylated $p38^{MAPK}$ ELISAs (R&D Systems; catalogue no. KBC869) were performed according to the instructions of the manufacturer. ELISA protocols are given in the Supplementary online material.

Western blotting

Protein extraction from HASMCs and immunodetection of phosphorylated (active) extracellular signal-regulated kinase (ERK)-1/-2 and of GAPDH were performed as previously described.[24] Details are given in the Supplementary online material.

Statistical analysis

Statistical analyses were performed to examine the effects of TNFα and ET-1 alone or in combination with enzyme inhibitors on gene transcription, enzyme activation and cytokine release by HASMCs. The Gaussian distribution for each data set was confirmed by histogram analyses and Kolmogorov–Smirnov test. Results are expressed as mean (SEM). Comparisons over time or across different stimulations on matched HASMC samples of one subject for n independent subjects were analysed by one-way repeated measures analysis of variance (ANOVA) with 95% CIs. For separate comparisons of each stimulation, post hoc Bonferroni–Holm tests were performed. A p value <0.05 was considered as statistically significant.

RESULTS

ET-1 induces its own expression in HASMCs via ET_AR and $p38^{MAPK}$

In dose–repsonse and time–response experiments ET-1 induced its own transcription in HASMCs at the highest level (~3-fold above the unstimulated control) at 100 nM after 2 h of stimulation (fig 1A,B). These conditions were used in the subsequent experiments. Notably, the ET-1-inducing effect on ET-1 transcription was maintained for 8 h (fig 1B). A minimum concentration of 10^{-6} M bosentan was required to block ET-1-induced ET-1 transcription almost completely (fig 1C). ET-1 induced the release of big-ET-1 (the inactive ET-1 precursor) after 3–8 h of stimulation, and this effect was completely blocked by pretreatment with bosentan (fig 1D,E). ET-1-induced ET-1 transcription was completely blocked by BQ123, a highly selective ET_AR inhibitor,[15 27] but not by BQ788 (fig 1F,G), a highly selective ET_BR inhibitor,[15 27] demonstrating a predominant role for ET_AR in this process.

ET-1 at 1, 10 (data not shown) and 100 nM (fig 2A) rapidly activated ERK-1/-2 after 5–30 min of stimulation in a concentration-independent manner. ET-1 transiently activated $p38^{MAPK}$

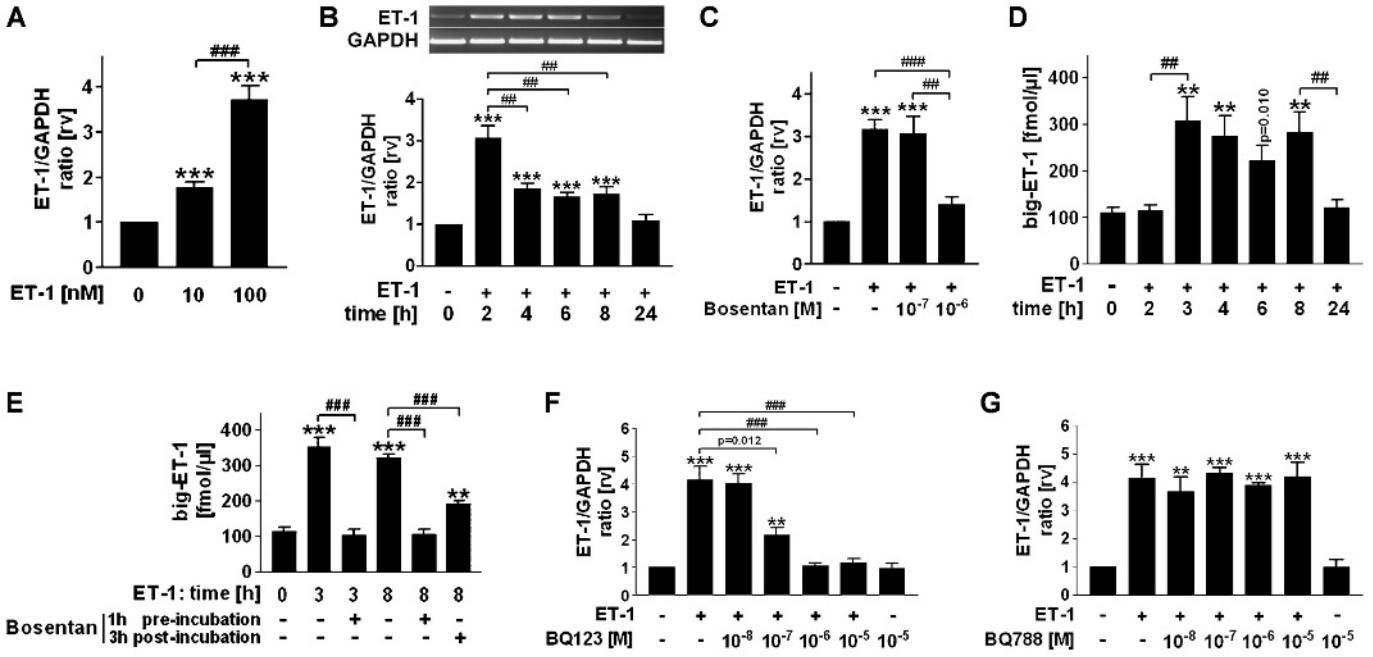

Figure 1 Endothelin-1 (ET-1) induces its own expression in human airway smooth muscle cells (HASMCs) via the enothelin receptor ET_AR. HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B–G) for 2 h (A, C, F, G) or for the times indicated (B, D, E). In (C), (F) and (G) bosentan or selective inhibitors of ET_AR (BQ123) or ET_BR (BQ788) were added to the medium 1 h before ET-1 stimulation at the concentrations indicated; in (E) bosentan at 10^{-6} M was added at the times indicated before (pre-incubation) or after (post-incubation) ET-1 addition. (A–C, F, G) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalised to GAPDH and related to solvent controls (0 nM, –). (D, E) After incubation, concentrations of big-ET-1 in supernatants were measured by ELISA. Each graph represents the mean (SEM) of n = 4 (E–G) or n = 5 (A–D) individual experiments. One-way repeated measures analysis of variance (ANOVA): $p<0.0001$ (A–G). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); **$p<0.01$; ***$p<0.001$ related to solvent controls; ##$p<0.01$; ###$p<0.001$ related to values as indicated; rv, relative values.

with four peak levels: after 5–30 min, and 2, 4 and 6.5 h of stimulation (fig 2B). ET-1-induced ERK-1/-2 and $p38^{MAPK}$ activation was almost completely impaired by pretreatment with bosentan (fig 2C,D). In contrast to PD098059, a specific inhibitor of the ERK-1/-2 pathway, the $p38^{MAPK}$-specific inhibitor SB203580 almost completely inhibited ET-1-induced ET-1 transcription (fig 2E). These data provide evidence for an ET_AR- and $p38^{MAPK}$-dependent ET-1 autoregulatory positive feedback loop in HASMCs.

This hypothesis was supported by the following data: the exogenously added ET-1 was no longer detectable in the supernatant after 2 h of stimulation; however, we detected ET-1 4 h after stimulation (Supplementary fig 1), indicating ET-1 de novo synthesis. Postincubation with bosentan 3 h after ET-1 stimulation reduced both the release of big-ET-1 after 8 h of ET-1 stimulation and the 4 h $p38^{MAPK}$ activation peak (figs 1E, 2D). We conclude that the 4 h peak level results from the re-activation of $p38^{MAPK}$ through de novo synthesised ET-1 and contributes to the big-ET-1 expression observed after 8 h of stimulation and thus to the maintenance of big-ET-1 release (for a detailed discussion see Supplementary online material).

ET-1 induces GM-CSF expression via ET_AR, ET_BR, ERK-1/-2 and $p38^{MAPK}$ signalling

In dose–response and time–response experiments ET-1 induced GM-CSF transcription in HASMCs at the highest level (five- to eightfold above the unstimulated control) at 100 nM after 4 h of stimulation (fig 3A,B). Notably, the inducing effect on GM-CSF transcription was detected at 2–8 h of stimulation (fig 3B). As individual treatments with bosentan, BQ123, BQ788, PD098059 and SB203580 all abolished ET-1-induced GM-CSF transcription (fig 3C–F) we conclude that ET-1 induces GM-CSF expression via ET_AR, ET_BR, ERK-1/-2 and $p38^{MAPK}$ signalling.

TNFα induces ET-1 expression via ET_AR and $p38^{MAPK}$

TNFα did not influence ET-1 mRNA levels at 1 ng/ml (data not shown). However, at 10 ng/ml (data not shown) and 20 ng/ml, TNFα (fig 4A) strongly induced ET-1 transcription after 2–8 h of stimulation without statistically significant differences between these concentrations and times. This effect was reduced by specific blocking of TNFR1 or TNFR2 (fig 4B). TNFα induced ET-1 release from HASMCs after 4–72 h of stimulation (fig 4C). Surprisingly, TNFα-induced ET-1 transcription after 4 h and ET-1 release after 6 h were both partially blocked by bosentan (fig 4D,E). TNFα-induced ET-1 transcription was also reduced by BQ123 but not by BQ788 (fig 4F,G).

TNFα activated ERK-1/-2 at 1, 20 and 50 ng/ml (fig 5A, data not shown) after 5–30 min of stimulation in a concentration-independent manner. TNFα activated $p38^{MAPK}$ at three time peaks: after 5–45 min, and after 5 and 9 h of stimulation (fig 5B). Bosentan did not significantly reduce ERK-1/-2-or $p38^{MAPK}$ activation in response to 15 min TNFα stimulation (data not shown). TNFα-induced ET-1 transcription was almost

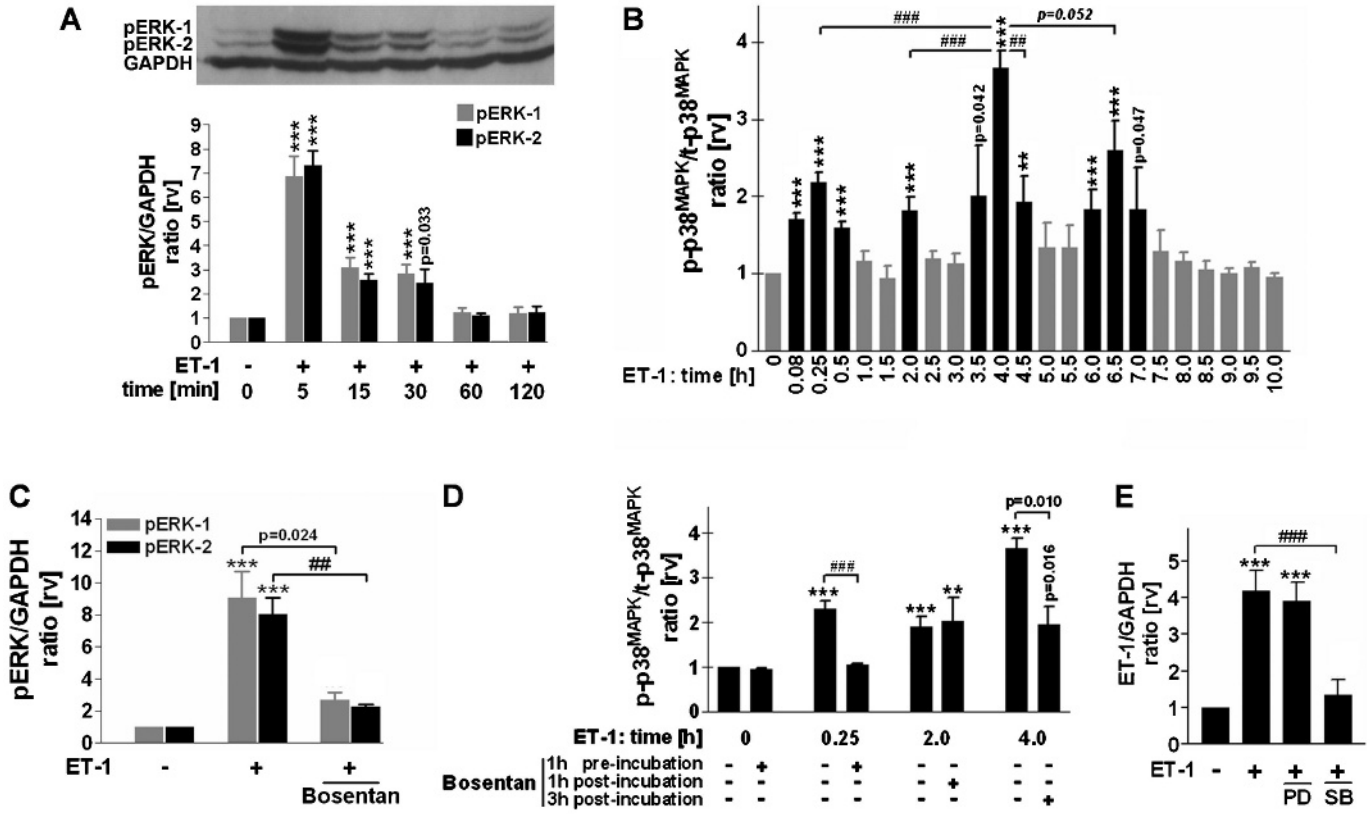

Figure 2 The endothelin-1 (ET-1) autoregulatory positive feedback loop depends on p38 mitogen-activated protein kinase ($p38^{MAPK}$). Human airway smooth muscle cells (HASMCs) were stimulated with ET-1 at 100 nM for the indicated times (A, B, D), for 5 min (C) or for 2 h (E). Bosentan at 10^{-6} M was added to the medium 1 h before ET-1 stimulation (C) or at the times indicated before (pre-incubation) or after (post-incubation) ET-1 addition (D). In (E) PD098059 (10 μM) or SB203580 (10 μM) was added 30 min before ET-1 stimulation. (A, C) After incubation, proteins were extracted and subjected to quantitative immunodetection of activated phosphorylated extracellular signal-regulated kinase-1/-2 (pERK-1/-2) with a pERK-1/-2-specific antibody. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. (B, D) After incubation, the activity of $p38^{MAPK}$ was measured by an intracellular ELISA for phosphorylated $p38^{MAPK}$ ($p\text{-}p38^{MAPK}$) with total $p38^{MAPK}$ ($t\text{-}p38^{MAPK}$) as a reference. (E) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. GAPDH was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2 and ET-1 or for $p\text{-}p38^{MAPK}$ were normalised to GAPDH or to $t\text{-}p38^{MAPK}$, respectively, and related to solvent controls (0 h, –). Each graph represents the mean (SEM) of n = 4 (B, D), n = 5 (E), n = 6 (C) or n = 7 (A) individual experiments. One-way repeated measures analysis of variance (ANOVA): $p<0.0001$ (A, B, D, E); $p = 0.0014$ (C, ERK-1); $p = 0.0001$ (C, ERK-2). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); $^{**}p<0.01$; $^{***}p<0.001$ related to solvent controls; in (B) statistically significant upregulations compared with the solvent control are indicated by black bars; $\#\#p<0.01$; $\#\#\#p<0.001$ related to values as indicated; rv, relative values.

completely blocked by SB203580 (fig 5C) but not by PD098059 (data not shown). Thus, our data demonstrate that TNFα-induced ET-1 transcription depends on ET_AR and $p38^{MAPK}$ signalling.

TNFα induces GM-CSF release via activation of endothelin signalling

TNFα induced GM-CSF transcription at two peak levels: after 2 and 8–12 h of stimulation (fig 6A). Whereas the 2 h peak was completely reduced by specific blocking of TNFR1 but not of TNFR2, the 8 h peak was partially reduced by individual blocking of both receptor subtypes (fig 6B,C). Bosentan did not modulate GM-CSF transcription after 2 h of TNFα stimulation; however, the drug clearly impaired the induction of GM-CSF transcription after 8 h of TNFα stimulation (fig 6D). Both BQ123 and BQ788 individually blocked TNFα-induced GM-CSF transcription after 8 h with an efficiency similar to bosentan (fig 6E,F). This indicates that unlike the short-term effect, the long-term effect of TNFα on GM-CSF expression depends on the activation of each endothelin receptor subtype.

TNFα induced a strong GM-CSF release after 72 h, and this effect was reduced by ~65% by bosentan (fig 6G). TNFα-induced GM-CSF release was also partially reduced by PD098059 and completely impaired by SB203580 (fig 6G). The combined treatment of TNFα-stimulated HASMCs with bosentan and PD098059 did not result in an additional reduction of GM-CSF expression compared with the individual treatments (fig 6G). These data demonstrate that TNFα-induced GM-CSF release is mediated by ERK-1/-2 and $p38^{MAPK}$ signalling, and depends, at least in part, on endothelin receptor activity.

DISCUSSION

We found that ET-1 rapidly induces its own expression in HASMCs, suggesting an ET-1 autoregulatory positive feedback loop (fig 7). In contrast to blocking ET_BR and ERK-1/-2, inhibiting ET_AR and $p38^{MAPK}$ activity almost completely

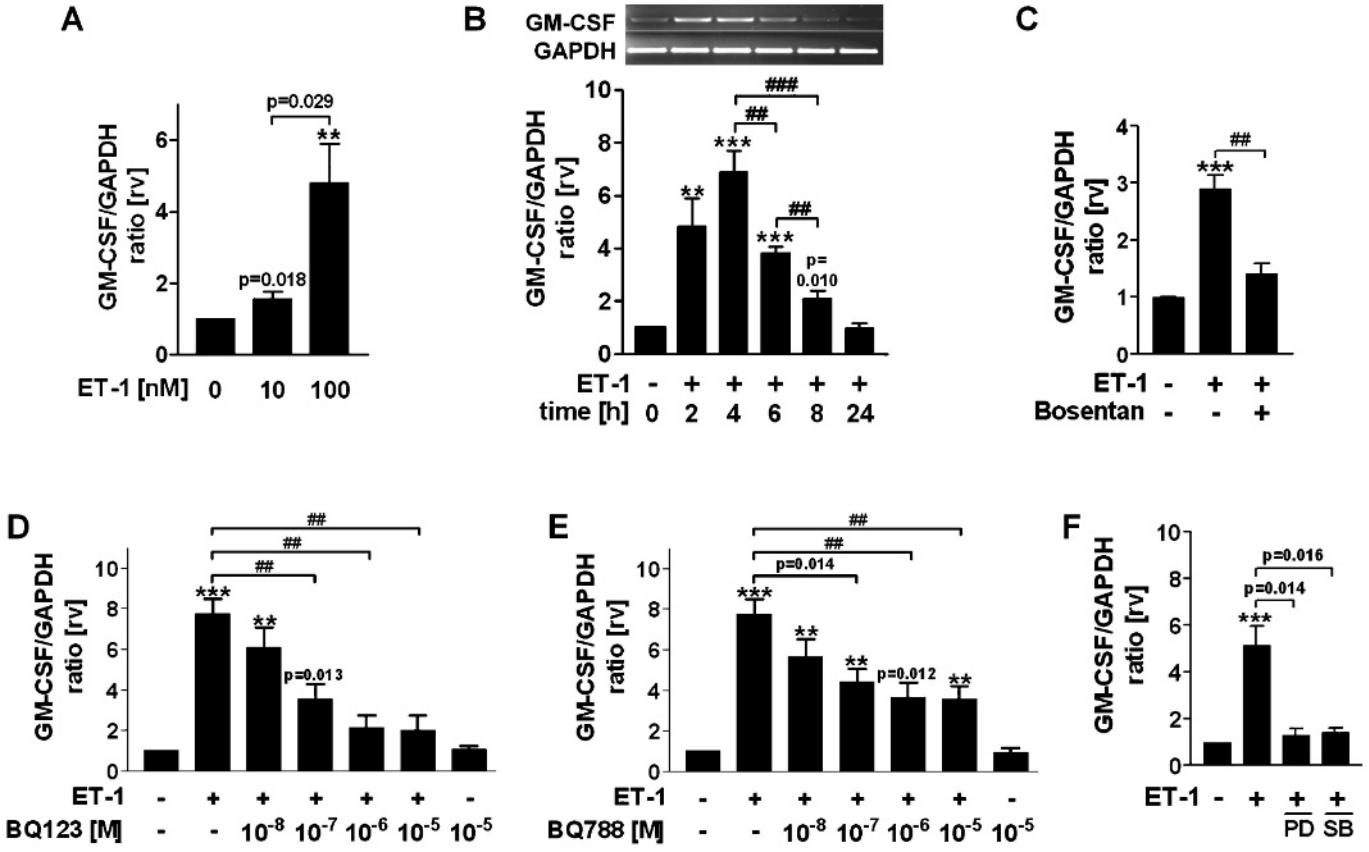

Figure 3 Endothelin-1 (ET-1) induces granulocyte–macrophage colony-stimulating factor (GM-CSF) expression in human airway smooth muscle cells (HASMCs) via the endothelin receptors ET_AR and ET_BR, extracellular signal-regulated kinase (ERK)-1/-2 and p38 mitogen-activated protein kinase ($p38^{MAPK}$). HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B–F) for 4 h (A, C–F) or for the indicated times (B). In (C) bosentan at 10^{-6} M and in (D) and (E) selective inhibitors of ET_AR (BQ123) or ET_BR (BQ788) at the indicated concentrations were added to the medium 1 h before ET-1 stimulation. In (F) PD098059 (10 μM) or SB203580 (10 μM) was added 30 min before ET-1 stimulation. After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with GM-CSF-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalised to GAPDH and related to solvent controls (0 nM, –). Each graph represents the mean (SEM) of n = 4 (D–F) or n = 5 (A–C) individual experiments. One-way repeated measures analysis of variance (ANOVA): $p = 0.0029$ (A); $p<0.0001$ (B–F). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); **$p<0.01$; ***$p<0.001$ related to solvent controls; ##$p<0.01$; ###$p<0.001$ related to values as indicated; rv, relative values.

impaired ET-1-induced ET-1 transcription, demonstrating that activation of the $ET_AR/p38^{MAPK}$ pathway is necessary to initiate and/or maintain this ET-1 feedback mechanism (for further discussion, see the Supplementary online material). The induction of GM-CSF expression by ET-1 has been described for other cell types—for example, lung fibroblasts,[16] but we were first able to demonstrate this ET-1 effect in HASMCs, thereby underlining ET-1 proinflammatory functions. In contrast to ET-1 autoregulation, its effect on GM-CSF transcription depends on the activation of both endothelin receptor subtypes and on $p38^{MAPK}$ and ERK-1/-2 pathways. Individual ET_AR or ET_BR blockade is sufficient to impair ET-1-induced GM-CSF transcription by ~75% or 50%, respectively, indicating that the combined activation of both receptor subtypes is required for maximum effects.

Here, we demonstrate for the first time that TNFα induces rapid ET-1 transcription and release by HASMCs. In a rat model of airway inflammation, increased ET-1 release in the lungs was observed after 3 h of provocation prior to the main inflammatory response.[28] Since both TNFα and ET-1 are suggested as initial cytokines in airway inflammation,[7 28] ET-1 release by HASMCs in response to TNFα might be an initial event in the amplification of inflammation in the airways. Mechanistic insights have been described for cerebrovascular endothelial cells, where the effect of TNFα on ET-1 mRNA upregulation depends on the generation of reactive oxygen species and on the activation of ERK-1/-2, $p38^{MAPK}$ and their common downstream target mitogen- and stress-activated protein kinase (MSK).[29] Consistently, we have shown here the complete reduction of TNFα-induced ET-1 transcription by a $p38^{MAPK}$-specific inhibitor, but, in contrast, that experimental blocking of ERK-1/-2 has no effect. This indicates that TNFα activates ET-1 transcription in HASMCs via the $p38^{MAPK}$/MSK rather than the ERK/MSK pathway. However, our data cannot rule out an MSK-independent signalling mechanism.

It has previously been described that TNFα-induced GM-CSF release from HASMCs depends on the activation of the c-Jun N-terminal kinase pathway.[23] We have extended these studies by demonstrating that ERK-1/-2 or $p38^{MAPK}$ blockade also impairs TNFα-induced GM-CSF release, suggesting that all three

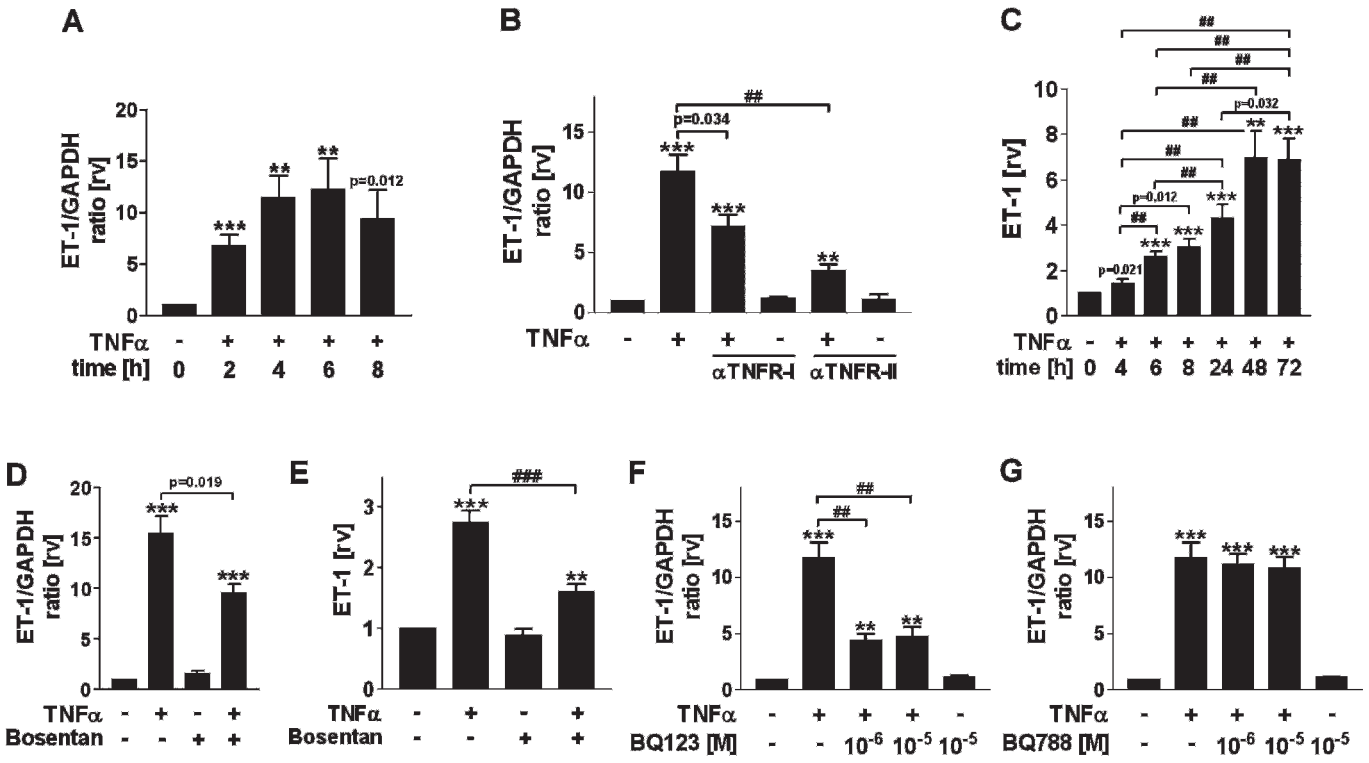

Figure 4 Tumour necrosis factor α (TNFα) induces endothelin-1 (ET-1) expression in human airway smooth muscle cells (HASMCs) via the endothelin receptor ET_AR. HASMCs were stimulated with human TNFα at 20 ng/ml for the indicated times (A, C) for 4 h (B, D, F, G) or for 6 h (E). (B) Monoclonal blocking antibodies specific for TNF receptor 1 (TNFR1; αTNFR-I, 10 μg/ml) or TNFR2 (αTNFR-II, 10 μg/ml) were individually added to the medium 60 min prior to TNFα stimulation. Bosentan (10^{-6} M) (D, E) or selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) (at the indicated concentrations) (F, G) were individually added to the medium 2 h prior to TNFα stimulation. (A, B, D, F, G) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalised to GAPDH and related to solvent controls (–). (C, E) After incubation, ET-1 concentrations in supernatants were measured by ELISA, and ET-1 values were normalised to solvent controls. A value of 1 corresponds on average to an ET-1 concentration of 0.45 fmol/ml. Each graph represents the mean (SEM) of n = 4 (B, F, G) or n = 5 (A, C–E) individual experiments. One-way repeated measures analysis of variance (ANOVA): p = 0.0069 (A); p<0.0001 (B–G). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); **p<0.01; ***p<0.001 related to solvent controls; ##p<0.01; ###p<0.001 related to values as indicated; rv, relative values.

prominent proinflammatory MAPK pathways are substantially involved in this process. We showed that TNFα induces GM-CSF transcription at two peak levels after 2 and 8–12 h of stimulation. While the short-term effect depends exclusively on TNFR1, both TNFα receptor subtypes mediate the long-term effect and—notably—also TNFα-induced ET-1 expression, indicating a link between these two signalling events. In addition, bosentan partially inhibited the long-term (but not the short-term) TNFα effect, demonstrating that endothelin receptor activity is necessary to obtain complete induction and/or to maintain the complete level of GM-CSF expression in response to TNFα. Consistently, bosentan reduced TNFα-induced GM-CSF release from HASMCs.

These observations raise the question of a mechanistic link between TNFα and endothelin receptor activation. Since TNFα induces ET-1 release, and ET-1 induces its own expression and that of GM-CSF, we suggest the following model for the long-term effect of TNFα on GM-CSF expression: TNFα activates the ET-1 autoregulatory positive feedback loop by initial activation of the ET-1 gene via the $p38^{MAPK}$ pathway. As a consequence, ET-1 autocrinally maintains the increased level of ET-1 expression via ET_AR and $p38^{MAPK}$, and concurrently induces the expression and release of GM-CSF via both endothelin receptor subtypes and $p38^{MAPK}$ and ERK-1/-2 signalling (fig 7). Our data demonstrating that blocking ET_AR (but not ET_BR) activity attenuates TNFα-induced ET-1 expression strongly support the hypothesis of an activation of the ET-1 feedback loop in response to TNFα (for a detailed discussion see the Supplementary online material). In contrast, the short-term TNFα effect on GM-CSF expression is bosentan insensitive and thus independent from endothelin signalling, and might include the canonical and rapid activation of MAPKs by TNFR1. This hypothesis is supported by our data showing a rapid increase of $p38^{MAPK}$ and ERK-1/-2 activity as early as 5 min after TNFα stimulation.

HASMCs are believed to contribute to the establishment of inflammation in chronic inflammatory airway diseases and lung fibrosis,[1] and our data suggest that this could be mediated by ET-1 and GM-CSF upregulation in response to pathogenically elevated TNFα levels. Accordingly, the inhibiting effects of bosentan on the TNFα/ET-1/GM-CSF network in HASMCs suggest therapeutic utility for bosentan in the treatment of inflammation in chronic inflammatory airway diseases and lung fibrosis, particularly in early stages. Maximal TNFα-induced GM-CSF expression requires the activity of both ET_AR and ET_BR, suggesting that a dual endothelin receptor blocker might

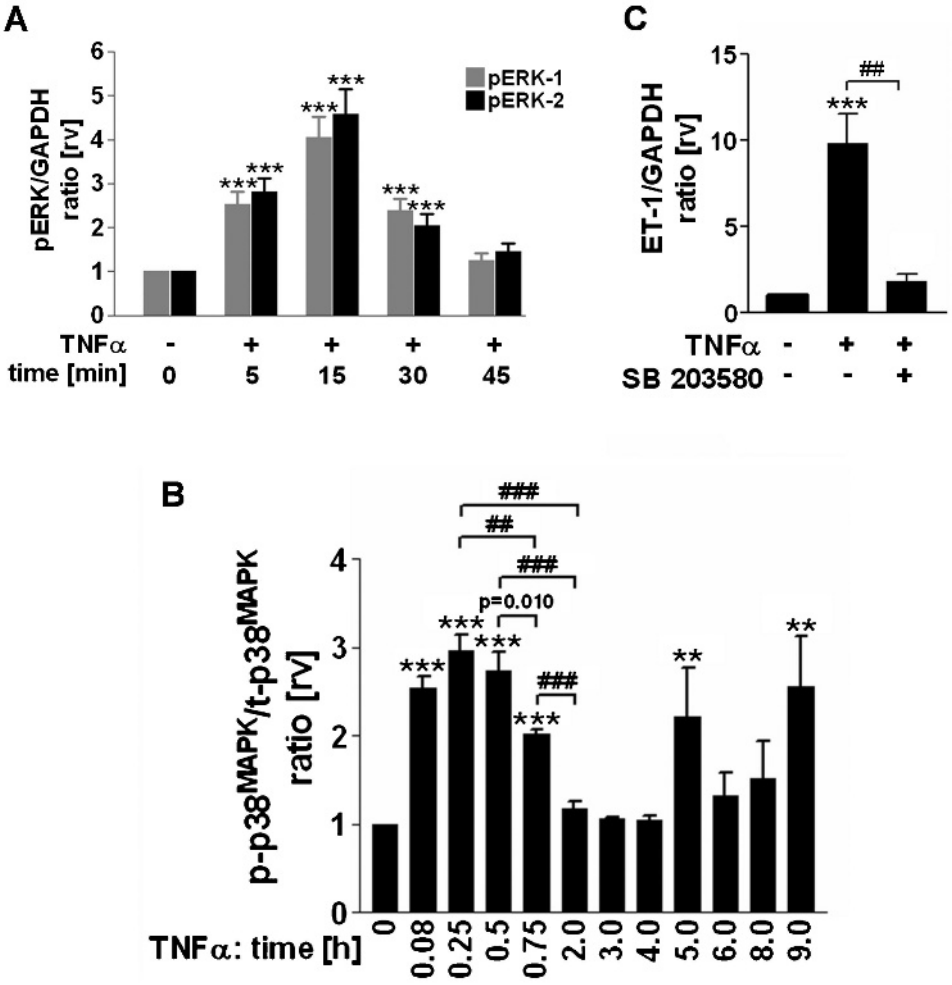

Figure 5 Tumour necrosis factor α (TNFα) induces ET-1 expression in human airway smooth muscle cells (HASMCs) via p38 mitogen-activated protein kinase ($p38^{MAPK}$). HASMCs were stimulated with human TNFα at 20 ng/ml for the indicated times (A, B) or for 4 h (C). In (C) SB203580 (10 μM) was added 30 min before TNFα stimulation. (A) After incubation, proteins were extracted and subjected to quantitative immunodetection of phosphorylated extracellular signal-regulated kinase-1/-2 (p-ERK-1/-2). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. (B) After incubation, the amount of phosphorylated (p-) $p38^{MAPK}$ was determined in relation to total (t-) $p38^{MAPK}$ by intracellular ELISA. (C) After incubation, the RNA was extracted and subjected to semi-quantitative reverse transcription-PCR (RT-PCR) with ET-1-specific primers. GAPDH was used as a standard. Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2, p-$p38^{MAPK}$ or ET-1 were normalised to GAPDH (A, C) or to t-$p38^{MAPK}$ (B), respectively, and related to solvent controls (0 h, –). Each graph represents the mean (SEM) of n = 5 (B, C) or n = 7 (A) individual experiments. One-way repeated measures analysis of variance (ANOVA): $p<0.0001$ (A–C, in A values for ERK-1 and ERK-2 were analysed separately). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph; $^{**}p<0.01$; $^{***}p<0.001$ related to solvent controls; $\#\#p<0.01$; $\#\#\#p<0.001$ related to values as indicated; rv, relative values.

be advantageous in therapy over selective blockers. The current study was designed to elucidate a novel signalling pathway with relevance for airway inflammation in order to provide a basis for developing new therapeutic strategies. The efficiency of dual versus selective endothelin receptor antagonists available for therapy in inhibiting the TNFα/ET-1/GM-CSF network or other inflammatory/profibrotic cytokines whose expression is mediated by TNFα/ET-1 has to be deciphered in further studies.

The BUILD-1 study evaluated the efficacy of bosentan in IPF. Bosentan was associated with a trend toward delayed time to disease progression or death, and improvement in quality of life, both of which were more pronounced (and reached statistical significance in a post hoc analysis) in a subgroup of patients with a biopsy-confirmed IPF diagnosis.[19] These observations are currently being investigated in the BUILD-3 trial. Our data suggest that this beneficial effect of bosentan might be a result of antagonising airway inflammation associated with the TNFα/ET-1/GM-CSF network.

In summary, we demonstrated that TNFα induces GM-CSF release in HASMCs via the activation of an ET-1 autoregulatory positive feedback loop. Moreover, we provided evidence for the anti-inflammatory properties of the dual receptor antagonist bosentan in HASMCs, and our data predict therapeutic utility for bosentan in the treatment of chronic inflammatory airway diseases and lung fibrosis in the early stages. In this context, treatment with bosentan might be an auspicious therapy helping to reduce the application of high doses of corticosteroids and other immunmodulatory drugs. Furthermore, treatment with bosentan in early disease stages might be useful to avoid the induction of pulmonary hypertension as a late and serious complication of chronic inflammatory airway diseases and lung fibrosis.

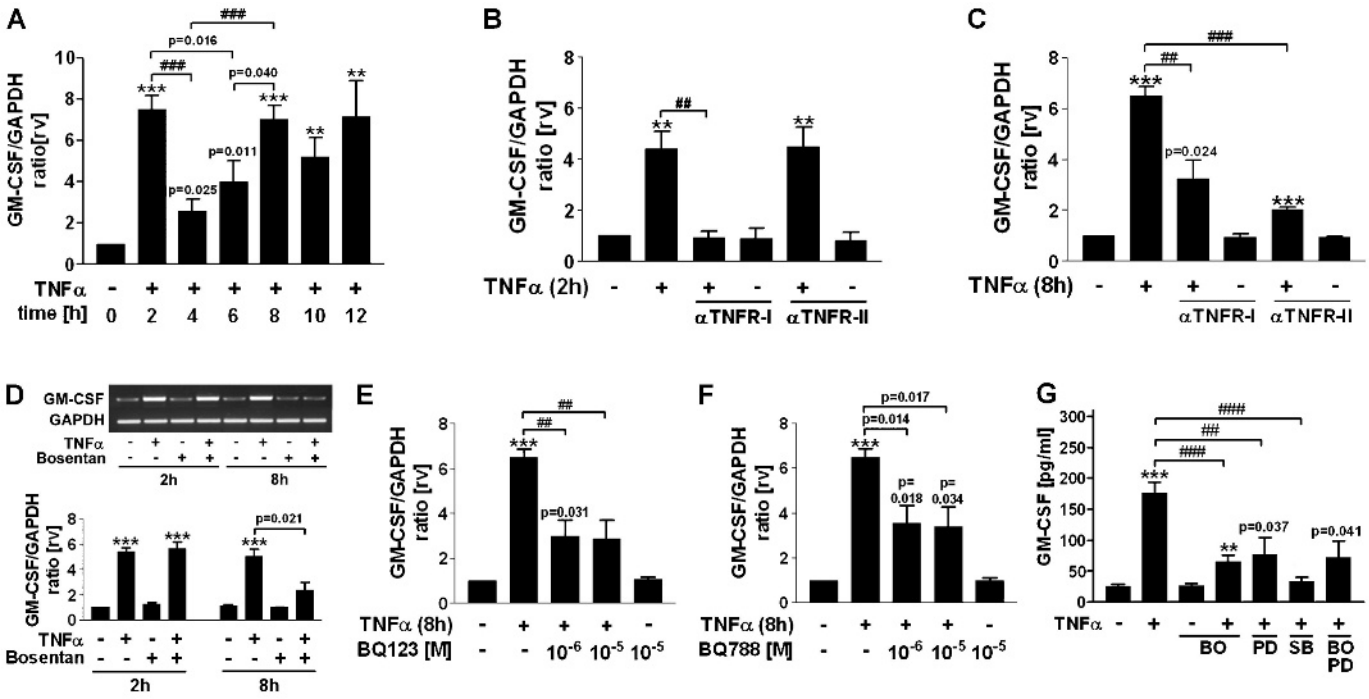

Figure 6 The maintenance of tumour necrosis factor α (TNFα)-induced granulocyte–macrophage colony-stimulating factor (GM-CSF) expression depends on signalling through TNF receptor 1 (TNFR1) and 2, endothelin receptors ET_AR and ET_BR, and p38 mitogen-activated protein kinase ($p38^{MAPK}$) and extracellular signal-regulated kinase (ERK)-1/-2. Human airway smooth muscle cells (HASMCs) were stimulated with human TNFα at 20 ng/ml for the indicated times (A–F) or for 72 h (G). In (B) and (C) monoclonal blocking antibodies specific for TNFR1 (αTNFR-I, 10 μg/ml) or TNFR2 (αTNFR-II, 10 μg/ml) were individually added to the medium 60 min prior to TNFα stimulation. Bosentan (BO; 10^{-6} M) (D, G) or selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) (at the indicated concentrations) (E, F) were individually added to the medium 2 h prior to TNFα stimulation. In (G) PD098059 (10 μM) or SB203580 (10 μM) were added 30 min before TNFα stimulation. (A–F) After incubation the RNA was extracted and subjected to semi-quantitative reverse transcription PCR (RT-PCR) with GM-CSF-specific primers. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a standard. One representative example of a set of RT-PCRs is shown in (D, top). Semi-quantitative RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalised to GAPDH and related to solvent controls (–). (G) After incubation the absolute amounts of GM-CSF protein in supernatants were determined by ELISA. Each graph represents the mean (SEM) of n = 4 (B–F), n = 5 (A) or n = 9 (G) individual experiments. One-way repeated measures analysis of variance (ANOVA): $p<0.0001$ (A, C, D (values for 2 h and 8 h were analysed separately), E, G); $p = 0.0001$ (B, F). Post hoc Bonferroni–Holm tests: exact p values down to 0.01 are indicated in the graph (related to the indicated values or to solvent controls if placed on top of a bar); $^{**}p<0.01$; $^{***}p<0.001$ related to solvent controls; $\#\#p<0.01$; $\#\#\#p<0.001$; related to values as indicated; rv, relative values.

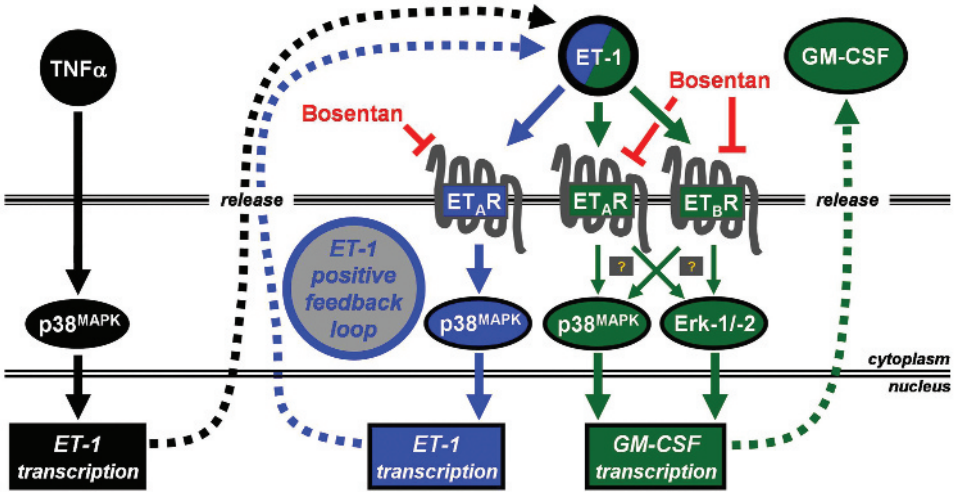

Figure 7 Model to explain the involvement of endothelin signalling in tumour necrosis factor α (TNFα)-induced granulocyte–macrophage colony-stimulating factor (GM-CSF) expression and release. TNFα is purposed initially to activate the enothelin-1 (ET-1) gene via the p38 mitogen-activated protein kinase ($p38^{MAPK}$) pathway (indicated in black). As a consequence, the ET-1 autoregulatory positive feedback loop (indicated in blue) is activated, which depends on the endothelin receptor ET_AR and $p38^{MAPK}$. In turn, elevated ET-1 levels induce and maintain GM-CSF expression and release via ET_AR, ET_BR, $p38^{MAPK}$ and extracellular signal-regulated protein kinase (ERK)-1/-2 (indicated in green). The individual impact of the endothelin receptor subtypes on the activation of $p38^{MAPK}$ and ERK-1/-2 signalling is unclear (indicated by question marks). Inhibition of endothelin receptor activity by bosentan (indicated in red) impairs the long-term effect of TNFα on GM-CSF expression.

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