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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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ABSTRACT

Background: There is an urgent need to inhibit endothelin (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: We investigated ET-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in response to tumor necrosis factor-α (TNFα) and ET-1 stimulation, and studied the impact of mitogen-activated protein (MAP) kinase pathways in this context. 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), we investigated its effect on the TNFα/ET-1/GM-CSF network.

Methods: ET-1 and GM-CSF expression and activation of MAP-kinases were investigated via quantitative RT-PCR, Western blotting and ELISA.

Main results: Both, TNFα and ET-1 activated $p38^{MAPK}$ and 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 our data suggest therapeutic utility for Bosentan in treating particularly early stages of chronic inflammatory airway diseases.

INTRODUCTION

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 characterized by elevated tumor 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, 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 pro-inflammatory and pro-fibrotic cytokine with key functions in the establishment of airway inflammation,[10,11] and recent studies predict therapeutic utility for GM-CSF neutralization in chronic inflammatory airway diseases and pulmonary fibrosis. GM-CSF deficiency/neutralization 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 pulmonary fibrosis patients, stimulates macrophages to release pro-fibrotic 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, ET_BR), both belonging to the G-protein superfamily, and both 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 pointing out the role of ET-1 as a pro-inflammatory cytokine. ET-1 is known to prime neutrophils, activate mast cells, and stimulate monocytes to produce a variety of pro-inflammatory cytokines.[16] The hypothesis that ET-1 might contribute to airway inflammation in chronic inflammatory airway diseases finds support by studies demonstrating increased ET-1 levels in exhaled breath condensates of asthma patients (in correlation to disease severity) and in sputum of COPD patients.[17,18] ET-1 also has pro-fibrotic 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 ET-1 effects on vascular smooth muscle cells,[20] there is no information about ET-1 expression in HASMCs and 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 to ET_AR and ET_BR and thus commonly referred to as a dual blocker.[20] Concerning the inflammatory and pro-fibrotic 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 leukocyte number and/or a decrease in the level of pro-inflammatory cytokines in the BALF and in lung tissue.[21,22]

The aim of this study was to elucidate for the first time 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 to temporarily induce their own expression to rapidly potentiate inflammation (autoregulatory positive feedback mechanism), we investigated the auto-regulation 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- (MAP) kinase 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 characterization of HASMCs was performed as described elsewhere.[24] For details see the Supplementary Online Material.

Stimulation of HASMCs

Before stimulation, sub-confluent cell monolayers were deprived from serum for 24 hours as previously described.[24] Details for the media used for serum withdrawal and stimulation are given in the Supplementary Online Material. HASMCs were stimulated with ET-1 (Sigma, Hamburg, Germany; cat#-E7764) at 100nM or with human TNFα (R&D systems, Minneapolis, MN, USA; cat#-210-TA) at 20ng/ml for times as indicated. Bosentan (Actelion Pharmaceuticals, Freiburg, Germany), BQ123 (Sigma; cat#-B150) or BQ788 (Sigma; cat#-B157) were added at 10^{-6}M or indicated concentrations 60 or 120 minutes before ET-1 or TNFα-stimulation, respectively. Pre-treatment with monoclonal blocking antibodies to TNFR1 (clone H398; Alexis, Lausen, Switzerland; cat#-ALX-804-200) and TNFR2 (clone 80M2; Alexis; cat#-ALX-804-450) each at 10µg/ml or with PD098059 (10µM; Calbiochem/VWR, Darmstadt, Germany; cat#-513000) and SB203580 (10µM, Calbiochem/VWR; cat#-559389) was done 60 or 30 min before stimulation, respectively.

Semi-quantitative 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 analyze the output in the linear range) was performed as described before.[25] GAPDH was used as a standard. PCR conditions and primer sequences are given in the Supplementary Online Material.

ELISA

Quantitative enzyme-linked immunosorbent assays (ELISA) for GM-CSF, ET-1 or big-ET-1 were done with supernatants of sub-confluent HASMCs on a 6-well plate. Intracellular ELISA for phosphorylated (active) $p38^{MAPK}$ with total $p38^{MAPK}$ as a reference was carried out on sub-confluent HASMCs on a 96-well plate. GM-CSF ELISA was performed as described before.[26] ET-1, big-ET-1 (Biomedica, Vienna, Austria; cat#-BI-20052; cat#-BI-20082) and phosphorylated-$p38^{MAPK}$ ELISA (R&D Systems; cat#-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) ERK-1/-2 and of GAPDH was 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 ANOVA with 95% confidence intervals. For separate comparisons of each stimulation post-hoc Bonferroni-Holm tests were performed. A *p*-value below 0.05 was considered as statistically significant.

RESULTS

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

In dose- and time-response experiments ET-1 induced its own transcription in HASMCs at the highest level (about 3-fold above unstimulated control) at 100nM after two hours of stimulation (Fig. 1A,B). These conditions were used in the further experiments. Notably, the ET-1-inducing effect on ET-1 transcription was maintained for eight hours (Fig. 1B). A minimum concentration of 10^{-6}M of 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 three to eight hours of stimulation, and this effect was completely blocked by pre-treatment 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 of ET_AR in this process.

ET-1 at 1, 10 (data not shown) and 100nM (Fig. 2A) rapidly activated ERK-1/-2 after five to 30 minutes of stimulation in a concentration-independent manner. ET-1 transiently activated $p38^{MAPK}$ with four peak levels: after five to 30 minutes, two, four and 6.5 hours of stimulation (Fig. 2B). ET-1-induced ERK-1/-2 and $p38^{MAPK}$ activation were almost completely impaired by pre-treatment 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 not detectable in the supernatant anymore after two hours of stimulation, however, we detected ET-1 four hours after stimulation (Supplementary Fig. 1), indicating ET-1 de novo synthesis. Post-incubation with Bosentan three hours after ET-1 stimulation reduced both, the release of big-ET-1 after eight hours of ET-1 stimulation and the four-hour $p38^{MAPK}$ activation peak (Fig. 1E, 2D). We conclude that the four-hour peak level results from the re-activation of $p38^{MAPK}$ through *de novo* synthesized ET-1 and contributes to the big-ET-1

expression observed after eight hours 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- and time-response experiments ET-1 induced GM-CSF transcription in HASMCs at the highest level (5 to 8-fold above unstimulated control) at 100nM after four hours of stimulation (Fig. 3A,B). Notably, the inducing effect on GM-CSF transcription was detected at two to eight hours 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 1ng/ml (data not shown). However, at 10ng/ml (data not shown) and 20ng/ml TNFα (Fig. 4A) strongly induced ET-1 transcription after two to eight hours 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 four to 72 hours of stimulation (Fig. 4C). Surprisingly, TNFα-induced ET-1 transcription after four hours and ET-1 release after six hours 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 50ng/ml (Fig. 5A, data not shown) after five to 30 minutes of stimulation in a concentration-independent manner. TNFα activated $p38^{MAPK}$ at three time-peaks: after five to 45 minutes, five and nine hours 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 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 two and eight to twelve hours of stimulation (Fig. 6A). Whereas the two-hour peak was completely reduced by specific blocking of TNFR1 but not of TNFR2 blockade, the eight-hour peak was partially reduced by individual blocking of both receptor subtypes (Fig. 6B,C). Bosentan did not modulate GM-CSF transcription after two hours of TNFα stimulation, however, the drug clearly impaired the induction of GM-CSF transcription after eight hours of TNFα stimulation (Fig. 6D). Both BQ123 and BQ788 individually blocked TNFα-induced GM-CSF transcription after eight hours with a similar efficiency as 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 subtypes.

TNFα induced a strong GM-CSF release after 72 hours, and this effect was reduced by about 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 to 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 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, e.g. lung fibroblasts,[16] but we were first able to demonstrate this ET-1-effect in HASMCs thereby underlining ET-1 pro-inflammatory functions. In contrast to ET-1 auto-regulation, 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 about 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 three hours 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 up-regulation 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 a 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 prominent pro-inflammatory MAP-kinase pathways are substantially involved in this process. We showed that TNFα induces GM-CSF transcription at two peak levels after two and eight to twelve hours of stimulation. While the short-term effect exclusively depends 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 and GM-CSF expression 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 auto- and/or paracrinally 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 MAP-kinases by TNFR1. This hypothesis is supported by our data showing a rapid increase of $p38^{MAPK}$- and ERK-1/-2 activity as early as five minutes 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 up-regulation 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 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/pro-fibrotic 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 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 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 diseases and lung fibrosis.

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COMPETING INTERESTS

The authors declare to have no competing interests.

ETHICS COMMITTEE APPROVAL

This study has been approved by the ethics committee of the University of Cologne, Cologne, Germany.

FIGURE LEGENDS

Figure 1. ET-1 induces its own expression in HASMCs via ET_AR. HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B-G) for two hours (A, C, F, G) or for times as indicated (B, D, E). In (C), (F), and (G) Bosentan or selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) were added to the medium one hour before ET-1 stimulation at concentrations as indicated, in (E) Bosentan at 10^{-6} M was added at times as 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 (sq-) RT-PCR with ET-1-specific primers. GAPDH was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. sq-RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalized 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 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 values as indicated 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 2. ET-1 autoregulatory positive feedback loop depends on $p38^{MAPK}$. HASMCs were stimulated with ET-1 at 100 nM for times as indicated (A, B, D), for 5 min (C) or for two hours (E). Bosentan at 10^{-6} M was added to the medium one hour before ET-1 stimulation (C) or at times as indicated before (pre-incubation) or after (post-incubation) ET-1 addition (D). In (E) PD 098059 (10 µM) or SB 203580 (10 µM) was added 30 minutes before ET-1 stimulation. (A, C) After incubation, proteins were extracted and subjected to quantitative immunodetection of activated (phosphorylated) ERK-1/-2 (pERK-1/-2) with a pERK-1/-2 specific antibody. GAPDH was used as a standard. (B, D) After incubation, 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 sq-RT-PCR with ET-1-specific primers. GAPDH was used as a standard. sq-RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2 and ET-1 or for $p\text{-}p38^{MAPK}$ were normalized 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 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 values as indicated 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 up-regulations compared to the solvent control are indicated by black bars; ##, $p<0.01$; ###, $p<0.001$ related to values as indicated; rv, relative values

Figure 3. ET-1 induces GM-CSF expression in HASMCs via ET_AR, ET_BR, ERK-1/-2 and $p38^{MAPK}$. HASMCs were stimulated with ET-1 at the indicated concentrations (A) or at 100 nM (B-F) for four hours (A, C-F) or for times as indicated (B). In (C) Bosentan at 10^{-6} M and in (D) and (E) selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) at concentrations as indicated were added to the medium one hour before ET-1 stimulation. In (F) PD 098059 (10 µM) or SB 203580 (10 µM) was added 30 minutes before ET-1 stimulation. After incubation, the RNA was extracted and subjected to sq-RT-PCR with GM-CSF-specific primers. GAPDH was used as a standard. In (B, top) one representative example of a time-course set of RT-PCRs is shown. sq-RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalized 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 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 values as indicated 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 4. TNFα induces ET-1 expression in HASMCs via ET_AR. HASMCs were stimulated with human TNFα at 20 ng/ml for times as indicated (A, C) for four (B, D, F, G) or six hours (E). (B) Monoclonal blocking antibodies specific for TNFR1 (αTNFR-I, 10 µg/ml) or TNFR2 (αTNFR-II, 10 µg/ml) were individually added 60 min prior to TNFα stimulation to the medium. Bosentan (10^{-6} M) (D, E) or selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) (at concentrations as indicated) (F, G) were individually added two hours prior to TNFα stimulation to the medium. (A, B, D, F, G) After incubation, the RNA was extracted and subjected to sq-RT-PCR with ET-1-specific primers. GAPDH was used as a standard. sq-RT-PCR signals were evaluated by densitometry. Values for ET-1 were normalized 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 normalized to solvent controls. A value of 1 corresponds in 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 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 values as indicated 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 5. TNFα induces ET-1 expression in HASMCs via $p38^{MAPK}$. HASMCs were stimulated with human TNFα at 20 ng/ml for times as indicated (A,B) or for four hours (C). In (C) SB 203580 (10 µM) was added 30 minutes before TNFα stimulation. (A) After incubation, proteins were extracted and subjected to quantitative immunodetection of phosphorylated (p-) ERK-1/-2. 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 sq-RT-PCR with ET-1-specific primers. GAPDH was used as a standard. sq-RT-PCR signals were evaluated by densitometry. Values for pERK-1/-2, p-$p38^{MAPK}$ or ET-1 were normalized 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 ANOVA: p<0.0001 (A-C, in C values for ERK-1 and ERK-2 were separately analysed). 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

Figure 6. The maintenance of TNFα-induced GM-CSF expression depends on signalling through TNFR1 and -2, ET_AR and ET_BR, and $p38^{MAPK}$- and ERK-1/-2. HASMCs were stimulated with human TNFα at 20 ng/ml for times as indicated (A-F) or for 72 hours (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 60 min prior to TNFα stimulation to the medium. Bosentan (BO; 10^{-6} M) (D, G) or selective inhibitors for ET_AR (BQ123) or ET_BR (BQ788) (at concentrations as indicated) (E, F) were individually added two hours prior to TNFα stimulation to the medium. In (G) PD 098059 (10 µM) or SB 203580 (10 µM) were added 30 minutes before TNFα stimulation. (A-F) After incubation the RNA was extracted and subjected to sq-RT-PCR with GM-CSF-specific primers. GAPDH was used as a standard. One representative example of a set of RT-PCRs is shown in (D, top). sq-RT-PCR signals were evaluated by densitometry. Values for GM-CSF were normalized to GAPDH and related to solvent controls (-). (G) After incubation the absolute amount 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 ANOVA: $p<0.0001$ (A, C, D [values for 2h and 8h were separately analysed], 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 values as indicated 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 TNFα-induced GM-CSF expression and release. TNFα is purposed to initially activate the ET-1 gene via the $p38^{MAPK}$-pathway (indicated in black). As a consequence, the ET-1 auto-regulatory positive feedback loop (indicated in blue) is activated, which depends on 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 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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SUPPLEMENTARY ONLINE MATERIAL

METHODS

Cultivation of HASMCs

HASMCs were maintained in Dulbecco´s modified Eagle medium (DMEM, Invitrogen, Karlsruhe, Germany; cat#-31885-023) with 10% FCS (Sigma; cat#-N-4637), sodium pyruvate (1 mM; Invitrogen; cat#-11360-039), L-glutamine (2 mM; Sigma cat#-G-7513), non-essential amino acids (1%; Invitrogen; cat#-11140-035), penicillin (100 U/ml), streptomycin (100 µg/ml; Sigma; cat#-P-4333), and amphotericin B (1.5 µg/ml; Sigma cat#-A-2942) in a humidified atmosphere at 37° C, 5% CO_2. Subconfluent cells were passaged with trypsin (0.25%; Sigma; cat#-T-4674) and 1 mM EDTA. HASMCs were used for experiments at passages 2-7. Cells were plated at equal density into 6-well plates. HASMCs were characterized by positive immunostaining for calponin, smooth muscle α-actin, and myosin heavy chain.

Stimulation of HASMCs: Media

Serum withdrawal: serum-free and low glucose (1g/l) DMEM (Invitrogen; cat#-41966-029) supplemented with 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin, 1.5µg/ml amphotericin B, 1mM insulin, 5mg/ml apo-transferrin and 100µM ascorbic acid. *Stimulation*: serum-free and low glucose (1g/l) DMEM supplemented with 1mM sodium pyruvate, 2mM L-glutamine, non-essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin, and 1.5µg/ml amphotericin B.

Semiquantitative RT-PCR (PCR conditions and primer sequences)

PCR conditions for ET-1: denaturation, 94°C, 30 sec; annealing, 61°C, 30 sec; extension, 72°C, 30 sec; 39 cycles; ET-1 forward primer 5´-GAG AGG TCC ATT GTC ATC CCC-3´, ET-1 reverse primer 5´-TGT GGC GAC TCT GCA CTC-3´. PCR conditions for GM-CSF: denaturation, 94°C, 30 sec; annealing, 55°C, 30 sec; extension, 72°C, 30 sec; 35 cycles; GM-CSF-primer pairs were purchased from R&D systems (cat#-RDP-34). PCR conditions for GAPDH: denaturation, 94°C, 45 sec; annealing, 60°C, 45 sec; extension, 72°C, 45 sec; 27 cycles; GAPDH forward primer 5´-TCT AGA CGG CAG GCT AGG TCC ACC-3´, GAPDH reverse primer 5´-ACG GTA CCT TAA ACG GTA CCC ACC-3´.

ELISA protocols

ET-1 ELISA was performed according to the instructions of the manufacturer (Biomedica, Vienna, Austria cat#-BI 20052). Briefly, 50 µl of supernatants together with monoclonal mouse anti-human-endothelin antibody were incubated in 96-wells (coated with polyclonal anti-human ET-1 antibody) for 16 hours at room temperature. Following washing, HRP-coupled anti-mouse IgG was added for one hour at room temperature. After washing, detection was performed with TMB as a substrate and measuring extinction at 450 nm with 620 nm as reference. Quantification was done by interpolation from a standard curve with the lower limit of detection of 0.3 fmol/ml.

Big-ET-1 ELISA (that does not crossreact with ET-1) was performed according to the instructions of the manufacturer (Biomedica; cat#-BI 20082). Briefly, 50 µl of supernatants of HASMC cultures together with HRP-coupled monoclonal mouse anti-human big-ET-1 antibody were incubated in 96-wells (coated with polyclonal sheep anti-human big-ET-1 antibody directed against amino acids 22-38) for 4 hours at room temperature. After washing, detection was performed with TMB as a substrate and measuring extinction at 450 nm with

620 nm as reference. Quantification was done by interpolation from a standard curve with the lower limit of detection of 0.02 fmol/ml.

Intracellular ELISA for active $p38^{MAPK}$ (R&D Systems; cat#-KBC869) on sub-confluent HASMCs on a 96-well plate was performed according to the instructions of the manufacturer. This assay is based on the simultaneous detection of phosphorylated (active) $p38^{MAPK}$ and total $p38^{MAPK}$ as a reference. Briefly, cells were fixed and lysed with 4% formaldehyde for 20 minutes at room temperature. After washing, fixed cells were treated with quenching buffer (20 minutes at room temperature), and, after additional washing, with 10% fetal bovine serum for blocking (one hour, room temperature). After washing, a mixture of rabbit anti-human-phospho-$p38^{MAPK}$ (T180/Y182) antibody and mouse anti-human-total-$p38^{MAPK}$-antibody was added for 16 hours at 4° C. In order to distinguish between phosphorylated $p38^{MAPK}$ and the total cellular $p38^{MAPK}$ content the respective specific antibodies added have to be derived from different species. After washing, a secondary antibody mixture (HRP-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG) was added for two hours at room temperature. After washing, HRP-fluorogenic substrate was added for 60 minutes and, afterwards, AP-fluorogenic substrate was added for 5 minutes. Phospho-$p38^{MAPK}$ was measured using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm, total $p38^{MAPK}$ was measured with excitation at 360 nm and emission at 450 nm. Values for phospho-$p38^{MAPK}$ were normalized to values for total $p38^{MAPK}$.

Western Blotting

The activation status of ERK-1 and ERK-2 was assessed by Western immunoblot analysis using antibodies that recognize the dual phosphorylated (activated) form of the enzymes. After treatment, HASMCs were lysed in lysis buffer (20 mM Tris base, pH 7.4; 1% Triton X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM NaCl, 5 mM EDTA), supplemented with the proteinase inhibitors PMSF (500 µM), Na-orthovanadate (2 mM), leupeptin (10 µg/ml), aprotinin (25 µg/ml), pepstatin (10 µg/ml), NaF (1.25 mM), and Na-pyrophosphate (1 mM). Insoluble protein was removed by centrifugation at 12,000 x *g* for 5 min and aliquots of the resulting supernatant were diluted 1:4 in Laemelli buffer (62.5 mM Tris-HCl, 10% v/v glycerol, 1% w/v SDS, 1% β-mercaptoethanol, 0.01% w/v bromophenol blue, pH 6.8) and boiled for 5 min. Denatured proteins (40 µg) were subsequently separated by SDS/PAGE upon 10% vertical slab gels and transferred to Hybond ECL membranes (Amersham, Buckinghamshire, UK) in blotting buffer (Tris base 50 mM, pH 8.3; glycine 192 mM) supplemented with 20% vol/vol methanol. The filter was incubated for 1 h in TBS-Tween-20 (25 mM Tris-base, 150 mM NaCl, pH 7.6 and 0.1% Tween 20, 10% [w/v] nonfat milk and incubated 2 h in TBS-Tween 20 containing 3% BSA and either primary antibodies raised against pERK-1/-2 (Cell Signaling Technology/New England Biolabs, Frankfurt, Germany; cat#-9101) or GAPDH (each diluted 1:1,000). Following 3 x 10-min washes in TBS-Tween 20, the membranes were incubated for 60 min with a goat anti-rabbit peroxidase–conjugated IgG antibody diluted 1:5,000 in TBS-Tween 20 supplemented with 1% nonfat milk for ERK-1/-2, and for 60 min with a rabbit, anti-mouse peroxidase–conjugated IgG antibody diluted 1:4,000 in TBS-Tween 20 supplemented with 5% nonfat milk for GAPDH and then washed again (3 x 10 min). Antibody-labeled proteins were subsequently visualized by enhanced chemiluminescence (Amersham).

DISCUSSION

Hypothesis: TNFα-induced GM-CSF release depends on activation of an $p38^{MAPK}$–dependent ET-1 autoregulatory positive feedback mechanism – additional support.

ET-1 autoregulatory positive feedback mechanism. It has been reported for other cell types that large amounts of ET-1 rapidly (within a few minutes) and pseudo-irreversibly bind to its receptors.[1,2] Due to the rapid internalization of the ET-1/endothelin-receptor complexes (again within a few minutes) followed by their intracellular dissociation and an externalization of unoccupied and functional endothelin receptors to the plasma membrane, free (unbound) ET-1 has an extremely short half-life time in the extracellular milieu.[1,3] This might also apply to HASMC culture since we were not able to detect significant ET-1 amounts in the supernatant anymore two hours after stimulation with 100nM ET-1 (Supplementary Fig. 1). Three experiments strongly support the hypothesis that stimulation with ET-1 induces *de novo* synthesis and release of big-ET-1 followed by its maturation to active ET-1 (autoregulatory positive feedback): (1) the rapid increase of ET-1 mRNA levels in response to ET-1 stimulation (Fig. 1B), (2) the release of big-ET-1 after ET-1 stimulation (Fig. 1D), and (3) the presence of ET-1 in the culture medium four hours after exogenous ET-1 addition although two hours after stimulation the exogenously added ET-1 was not detectable in the supernatant anymore (Supplementary Fig. 1).

The ET-1 autoregulatory positive feedback depends on $p38^{MAPK}$ activity. The induction of big-ET-1 release by ET-1 is maintained for up to eight hours (Fig. 1D), and ET-1 induces four peaks of $p38^{MAPK}$-activation: after five to 30 minutes, two, four and 6.5 hours of stimulation (Fig. 2B). Pre-treatment with Bosentan would be expected to abolish big-ET-1 release and all $p38^{MAPK}$-activation peaks. Indeed, we demonstrated this for short- (3h) and long-term (8h) big-ET-1 release (Fig. 1E) and exemplary for the initial (5-30 min) $p38^{MAPK}$-activation peak (Fig. 2D). We hypothesize that the long-term $p38^{MAPK}$-activation peaks are the consequence of $p38^{MAPK}$ re-activation through *de novo* synthesized ET-1. Strong support comes from the experiment showing that the third (4h) $p38^{MAPK}$-activation peak was attenuated through the addition of Bosentan to the culture medium at a time (3 hours after ET-1 addition) when the exogenously added ET-1 is not detectable anymore (see above) and the second (2h) peak of $p38^{MAPK}$-activation has already receded (Fig. 2D). Notably, addition of Bosentan at the same time also partially impaired the long-term (8h) expression of big-ET-1 in response to ET-1 stimulation (Fig. 1E). This indicates that $p38^{MAPK}$ re-activation is required for the maintenance of ET-1 *de novo* synthesis and thus for the maintenance of the autoregulatory positive ET-1 feedback mechanism in HASMCs. As Bosentan inhibits endothelin signalling through extracellular binding to endothelin receptors these data also exclude the possibility that continued signalling via internalized ET-1/endothelin receptor complexes (as it has been proposed for other cell types [4,5]) governs the re-activation of $p38^{MAPK}$ or the maintenance of big-ET-1 release. As Bosentan added after the first (5-30 min) peak of $p38^{MAPK}$-activation did not abolish the second (2h) peak, this early re-activation of $p38^{MAPK}$ might be independent of *de novo* synthesised ET-1. In agreement we did not find ET-1 in the HASMC supernatant after two hours of ET-1 stimulation (Supplementary Figure 1). Instead, this second $p38^{MAPK}$-activation peak could be induced by other, yet unidentified, factors that are rapidly up-regulated by ET-1 stimulation or by signalling from internalized ET-1/endothelin-receptor complexes.

The ET-1 autoregulatory positive feedback depends on ET_AR but not on ET_BR. As selective ET_AR- but not ET_BR-antagonism blocks ET-1-induced ET-1 transcription, our data demonstrate that ET-1-induced ET-1 expression is triggered by ET_AR-mediated $p38^{MAPK}$

signalling but not by ET_BR. This raises the question why ET_BR activation does not contribute to ET-1 expression? ET-1 binding to its receptors results in the activation of complex intracellular signalling networks that include besides MAP-kinases numerous other signalling pathways, e.g. depending on phospholipase C or Rho kinase.[6,7] The ET_AR-triggered signalling network might contain besides $p38^{MAPK}$ at least one other, yet unidentified, pathway with a key role for ET-1 gene induction, which, however, is not activated by ET_BR. As the differences between the ET_AR- and ET_BR-triggered signalling networks and targeted transcription factors are not well understood for HASMCs or any other cell type, the mention of candidate pathways would remain highly speculative. Nevertheless, this theory can just as well explain the failure of activated ET_BR to induce ET-1 expression as the hypothesis that the $p38^{MAPK}$ pathway itself might not be associated with ET_BR in HASMCs. However, the latter assumption might be rather unlikely as ET_BR-dependent $p38^{MAPK}$ activation has been reported for cell types closely related to HASMCs.[8]

Induction of the ET-1 autoregulatory feedback mechanism by TNFα in order to maintain GM-CSF expression. ET-1 release induced by ET-1 itself and by TNFα as well as ET-1-induced GM-CSF transcription all are very rapid processes with measurable outcomes after 2-4 hours of stimulation (Fig. 1D; 3B; 4C). A comparable rapid release of *de novo* synthesised ET-1 in response to TNFα has been demonstrated for cultured rat mesangial cells.[9] The reducing effect of endothelin receptor inhibitors on TNFα-induced GM-CSF transcription in HASMCs after eight hours of stimulation could sufficiently be explained by a cascade of TNFα-induced ET-1 release with subsequent ET-1-induced GM-CSF transcription. However, there are two major arguments for an involvement of the ET-1 feedback mechanism as a substantial part of this cascade maintaining GM-CSF expression. First support is found when matching the ET-1- with the TNFα-induced $p38^{MAPK}$ activation patterns (compare Fig. 2B with 5B). Besides the expected short-term activation peak (5-45 min) in response to TNFα, $p38^{MAPK}$ re-activation was observed after five and nine hours of stimulation. Given that TNFα induces (low but measurable) ET-1 release after four and six hours (Fig. 4C) and that initial $p38^{MAPK}$ activation by ET-1 occurs within five to 30 minutes (Fig. 2B) it is reasonable to suggest that the TNFα-induced five hour $p38^{MAPK}$ re-activation peak reflects an "initial" $p38^{MAPK}$ activation through ET-1 that is expressed and released in response to TNFα. Moreover, exogenous ET-1 addition leads to $p38^{MAPK}$ re-activation via *de novo* synthesized ET-1 about four hours after initial $p38^{MAPK}$ activation (Fig. 2B,D). This time lag matches with the time lag between the five and the nine hour $p38^{MAPK}$ re-activation peaks in response to TNFα underlining the hypothesis of an induction of the ET-1 autoregulatory positive feedback by TNFα.

Further and strong support for this hypothesis comes from the experiments in Figures 4D-F showing that TNFα-induced ET-1 expression can partially be abolished by ET_AR inactivation with BQ123 and by Bosentan. As the literature does not provide any evidence for a direct TNFα/ET_AR interaction the only reasonable explanation for the effects of BQ123 and Bosentan is that ET-1 released in response to TNFα-stimulation activates ET_AR to further maintain ET-1 expression implying the activation of the ET-1 autoregulatory positive feedback by TNFα. Bosentan already blocks ET-1 release after six hours of TNFα-stimulation (Fig. 4E) and ET-1 can induce GM-CSF transcription within two hours (Fig. 3B). Thus, a cascade of TNFα-induced ET-1 release with subsequent ET-1-induced ET-1 release and ET-1-induced GM-CSF transcription would also coincide with the long-term effect (eight to twelve hours, Fig. 6A) of TNFα on GM-CSF transcription. Moreover, the activation of the ET-1 positive feedback mechanism by TNFα can also explain (1) the increasing TNFα-induced ET-1 release for up to 72 hours, and (2) the maintenance of TNFα-induced GM-CSF release over a 48 hour-period (after 24 to 72 hours of stimulation, Fig. 6G; [10]).

Summarized, our data provide strong evidence for an activation of the ET-1 autoregulatory positive feedback mechanism by TNFα in HASMCs to maintain ET-1 and GM-CSF expression.

ONLINE FIGURE LEGENDS

Supplementary Figure 1. ET-1 is rapidly removed from the extracellular milieu in HASMC culture and induces its own release. HASMCs were stimulated with ET-1 at 100nM for times as indicated. After incubation concentrations of ET-1 in supernatants were measured by ELISA. ET-1 values were normalized to solvent controls. The graph represents the mean ± SEM of n=4 individual experiments. One-way repeated measures ANOVA: p=0.0034. Post hoc Bonferroni-Holm tests: exact p-values are indicated in the graph related to non-stimulated controls if placed on top of a bar or to values as indicated; rv, relative values.

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Fig. 1

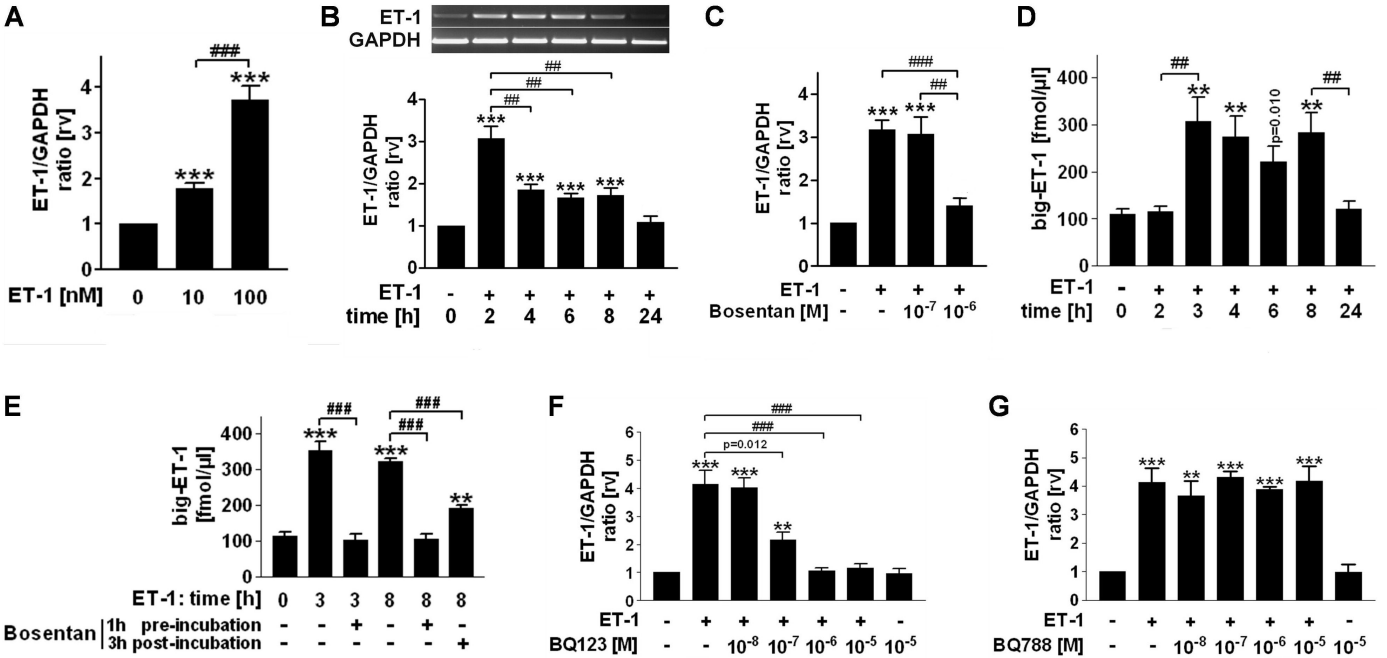

A
ET-1/GAPDH ratio [rv]
ET-1 [nM] 0 10 100
B
ET-1
GAPDH
ET-1/GAPDH ratio [rv]
ET-1 - + + + + +
time [h] 0 2 4 6 8 24
C
ET-1/GAPDH ratio [rv]
ET-1 - + + +
Bosentan [M] - - 10-7 10-6
D
big-ET-1 [fmol/µl]
p=0.010
ET-1 - + + + + + +
time [h] 0 2 3 4 6 8 24
E
big-ET-1 [fmol/µl]
ET-1: time [h] 0 3 3 8 8 8
Bosentan 1h pre-incubation - - + - + -
3h post-incubation - - - - - +
F
ET-1/GAPDH ratio [rv]
p=0.012
ET-1 - + + + + + -
BQ123 [M] - - 10-8 10-7 10-6 10-5 10-5
G
ET-1/GAPDH ratio [rv]
ET-1 - + + + + + -
BQ788 [M] - - 10-8 10-7 10-6 10-5 10-5

Fig. 2

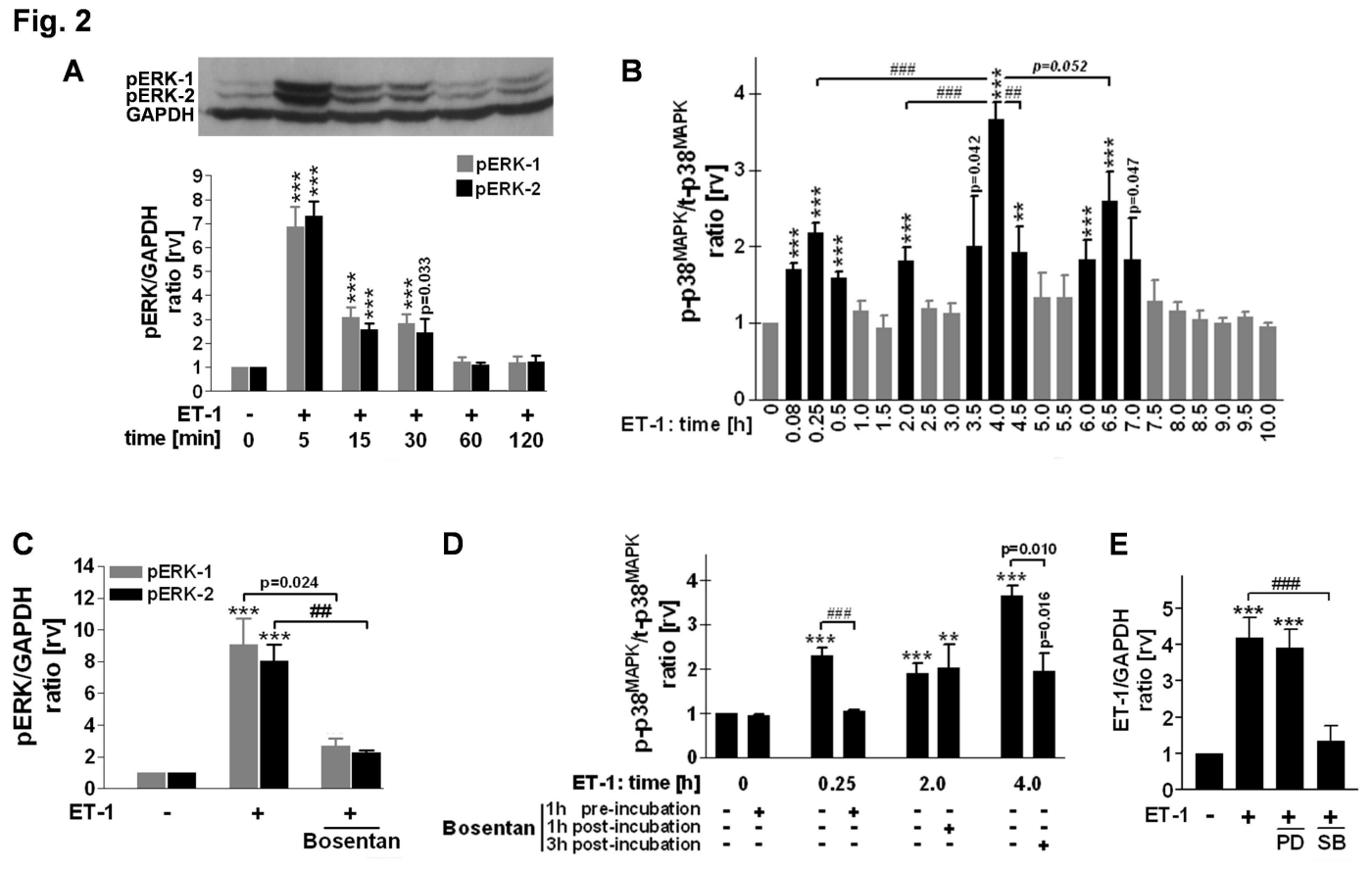

A
pERK-1
pERK-2
GAPDH
pERK-1
pERK-2
pERK/GAPDH ratio [rv]
ET-1 - + + + + +
time [min] 0 5 15 30 60 120
B
p-p38MAPK/t-p38MAPK ratio [rv]
p=0.052
p=0.042
p=0.047
ET-1 : time [h]
C
pERK-1
pERK-2
pERK/GAPDH ratio [rv]
p=0.024
ET-1 - + +
Bosentan
D
p-p38MAPK/t-p38MAPK ratio [rv]
p=0.010
p=0.016
ET-1: time [h] 0 0.25 2.0 4.0
Bosentan
1h pre-incubation
1h post-incubation
3h post-incubation
E
ET-1/GAPDH ratio [rv]
ET-1 - + + +
PD SB

Fig. 3

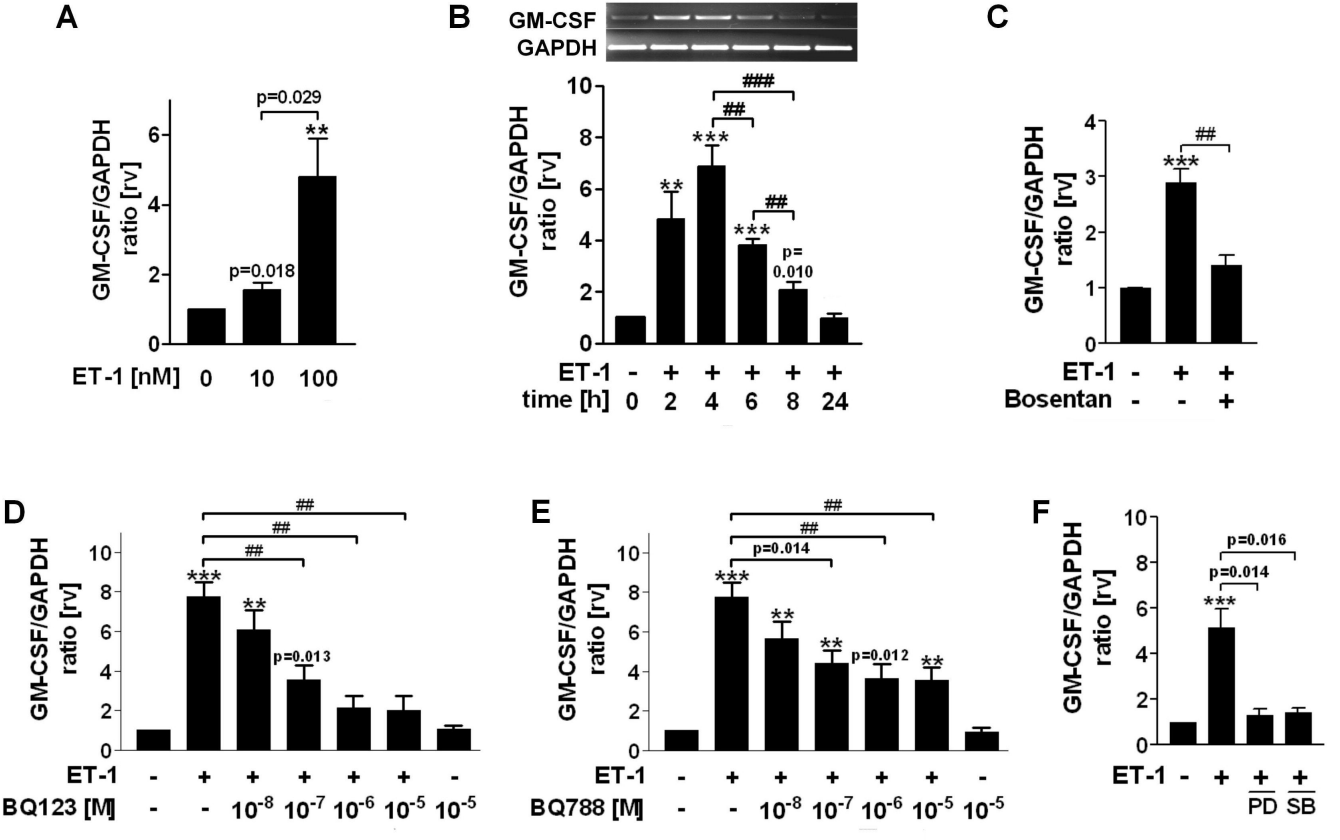

A
GM-CSF/GAPDH ratio [rv]
p=0.029
p=0.018
**
ET-1 [nM] 0 10 100
B
GM-CSF
GAPDH
GM-CSF/GAPDH ratio [rv]
###
##
##
**
***
***
p= 0.010
ET-1 - + + + + +
time [h] 0 2 4 6 8 24
C
GM-CSF/GAPDH ratio [rv]
##
***
ET-1 - + +
Bosentan - - +
D
GM-CSF/GAPDH ratio [rv]
##
##
##
***
**
p=0.013
ET-1 - + + + + + -
BQ123 [M] - - 10-8 10-7 10-6 10-5 10-5
E
GM-CSF/GAPDH ratio [rv]
##
##
p=0.014
***
**
**
p=0.012
**
ET-1 - + + + + + -
BQ788 [M] - - 10-8 10-7 10-6 10-5 10-5
F
GM-CSF/GAPDH ratio [rv]
p=0.016
p=0.014
***
ET-1 - + + +
PD SB

Fig. 4

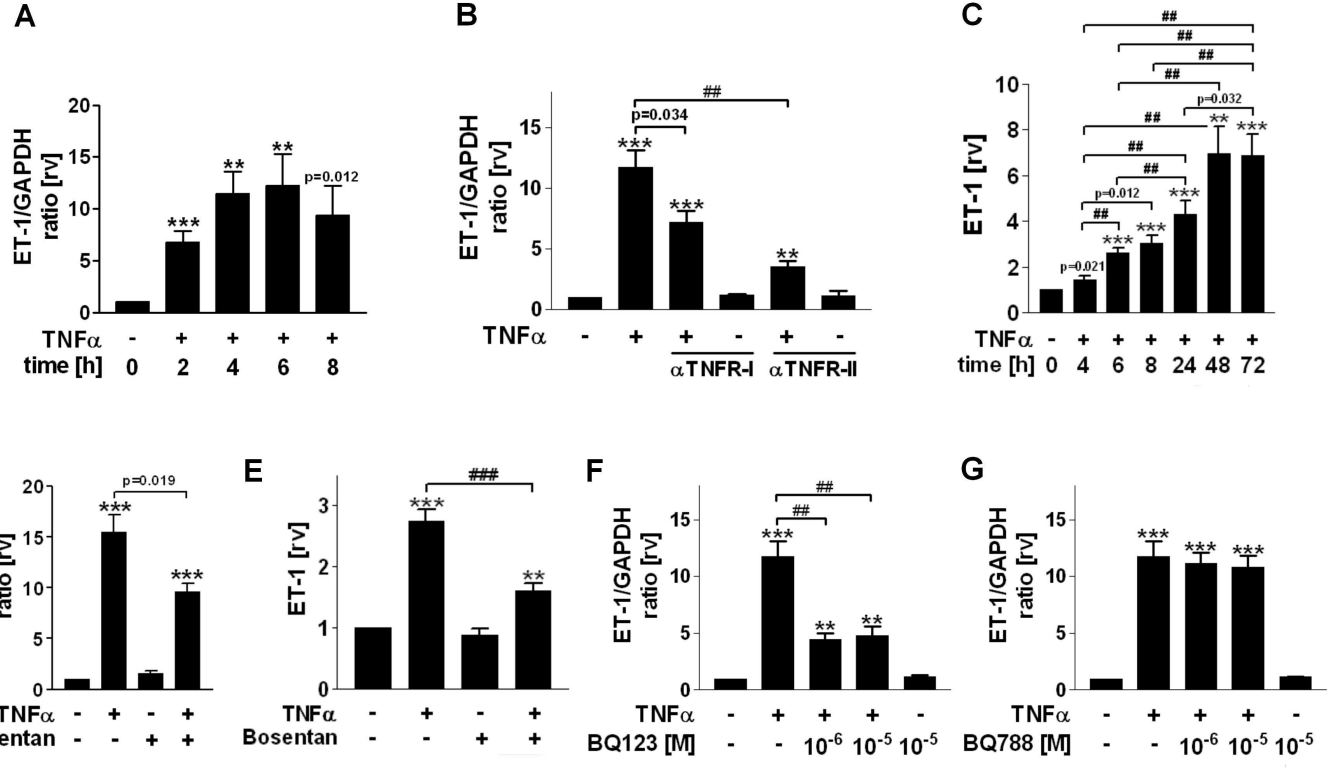

A
ET-1/GAPDH ratio [rv]
TNFα - + + + +
time [h] 0 2 4 6 8
p=0.012
B
ET-1/GAPDH ratio [rv]
p=0.034
TNFα - + + - + -
αTNFR-I αTNFR-II
C
ET-1 [rv]
p=0.021
p=0.012
p=0.032
TNFα - + + + + + +
time [h] 0 4 6 8 24 48 72
D
ET-1/GAPDH ratio [rv]
p=0.019
TNFα - + - +
Bosentan - - + +
E
ET-1 [rv]
TNFα - + - +
Bosentan - - + +
F
ET-1/GAPDH ratio [rv]
TNFα - + + + -
BQ123 [M] - - 10-6 10-5 10-5
G
ET-1/GAPDH ratio [rv]
TNFα - + + + -
BQ788 [M] - - 10-6 10-5 10-5

Fig. 5

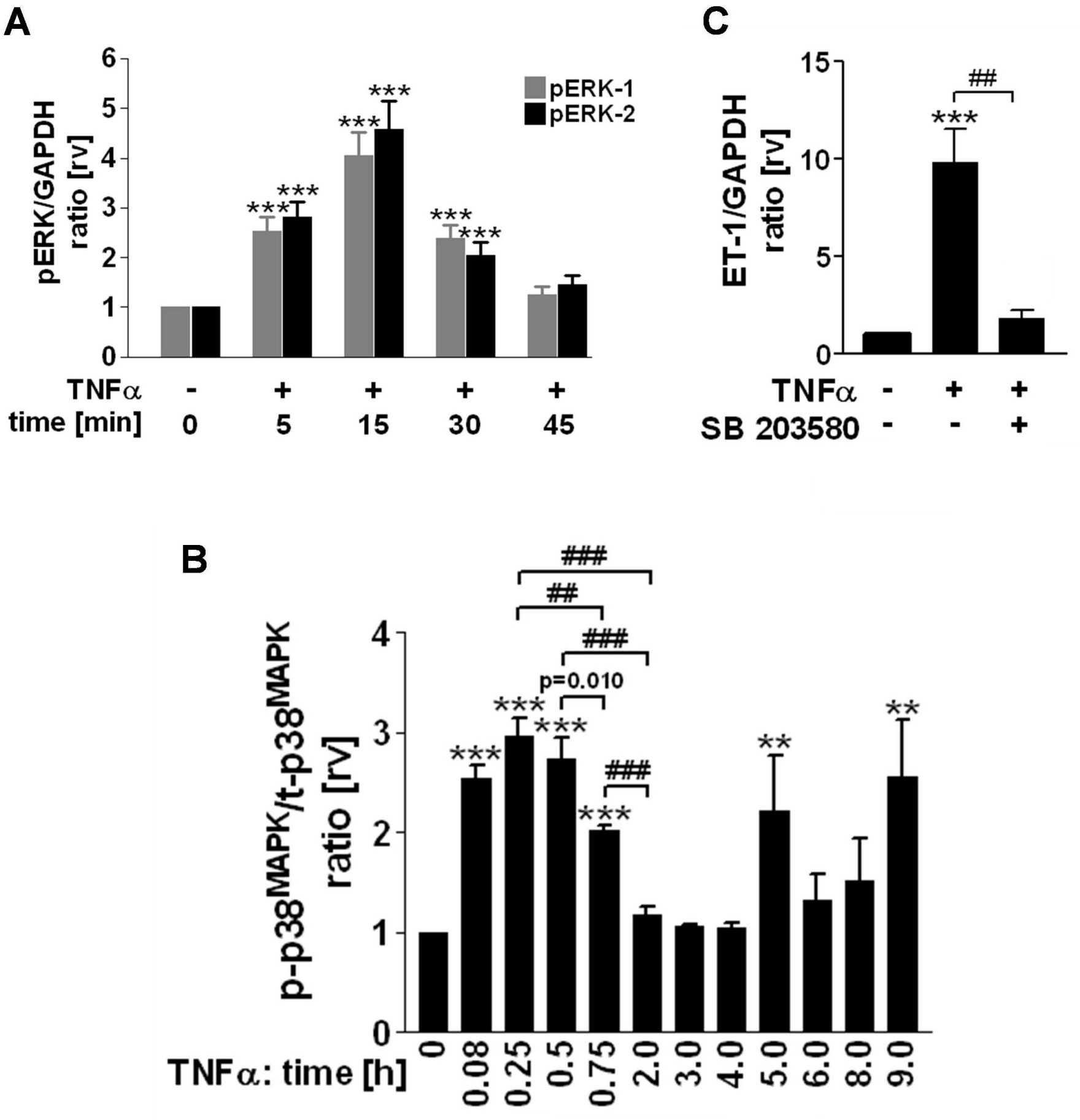

A
pERK/GAPDH ratio [rv]
pERK-1
pERK-2
TNFα - + + + +
time [min] 0 5 15 30 45
C
ET-1/GAPDH ratio [rv]
TNFα - + +
SB 203580 - - +
B
p-p38MAPK/t-p38MAPK ratio [rv]
p=0.010
TNFα: time [h]
0 0.08 0.25 0.5 0.75 2.0 3.0 4.0 5.0 6.0 8.0 9.0

Fig. 6

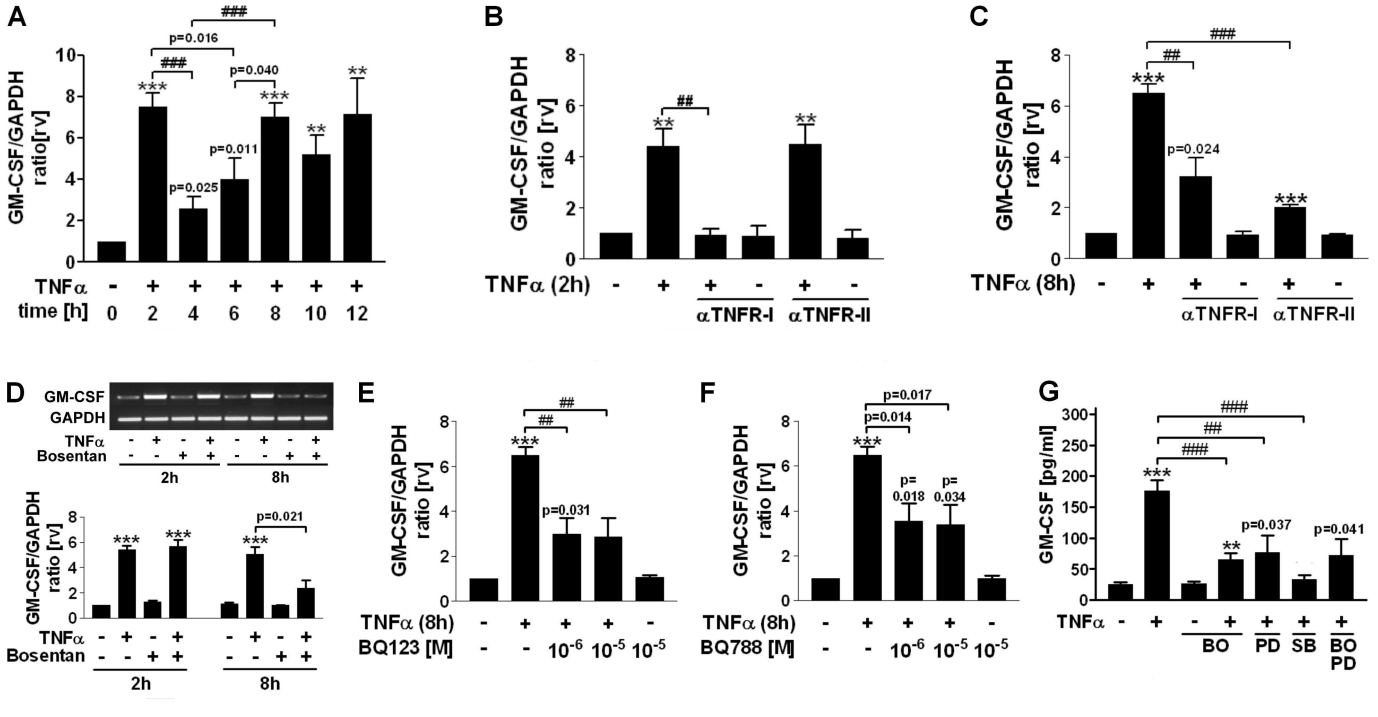
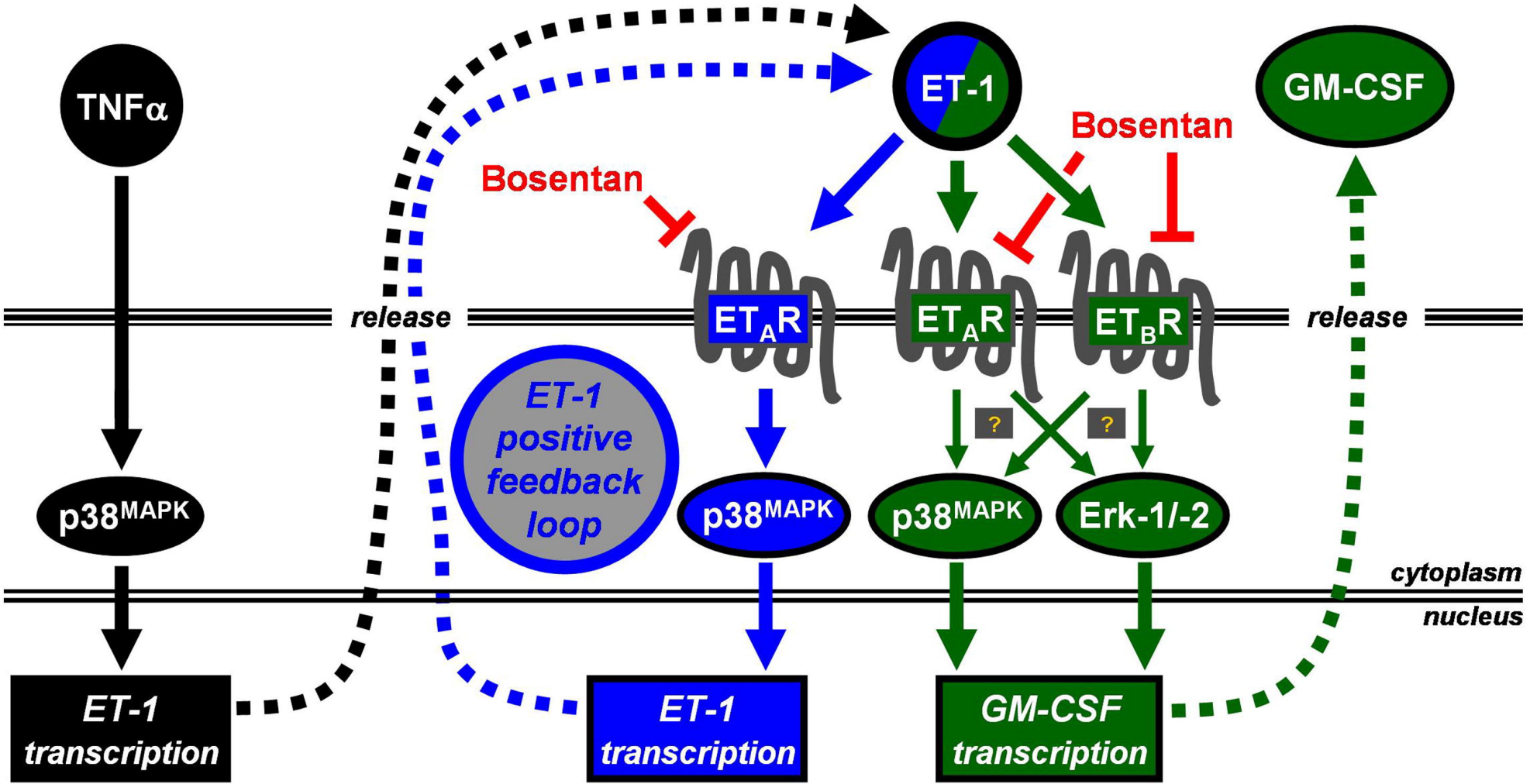

A
GM-CSF/GAPDH ratio[rv]
TNFα - + + + + + +
time [h] 0 2 4 6 8 10 12
B
GM-CSF/GAPDH ratio [rv]
TNFα (2h) - + + - + -
αTNFR-I αTNFR-II
C
GM-CSF/GAPDH ratio [rv]
TNFα (8h) - + + - + -
αTNFR-I αTNFR-II
D
GM-CSF
GAPDH
TNFα - + - + - + - +
Bosentan - - + + - - + +
2h 8h
GM-CSF/GAPDH ratio [rv]
E
GM-CSF/GAPDH ratio [rv]
TNFα (8h) - + + + -
BQ123 [M] - - 10-6 10-5 10-5
F
GM-CSF/GAPDH ratio [rv]
TNFα (8h) - + + + -
BQ788 [M] - - 10-6 10-5 10-5
G
GM-CSF [pg/ml]
TNFα - + - + + + +
BO PD SB BO PD

Fig. 7


TNFα
ET-1
GM-CSF
Bosentan
Bosentan
release
release
ET$_A$R
ET$_A$R
ET$_B$R
ET-1 positive feedback loop
?
?
p38^{MAPK}
p38^{MAPK}
p38^{MAPK}
Erk-1/-2
cytoplasm
nucleus
ET-1 transcription
ET-1 transcription
GM-CSF transcription

Supplementary Figure 1