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]

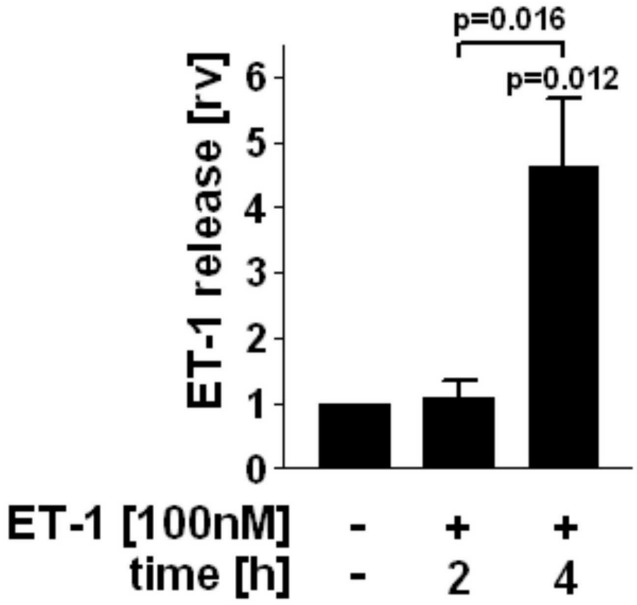
Induction of the ET-1 autoregulatory feedback mechanism by TNFα in order to maintain GM-CSF expression. ET-1 release induced by ET-1 itself and by TNFα as well as ET-1-induced GM-CSF transcription all are very rapid processes with measurable outcomes after 2-4 hours of stimulation (Fig. 1D; 3B; 4C). 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 REFERENCES

1. Marsault R, Feolde E, Frelin C. Receptor externalization determines sustained contractile responses to endothelin-1 in the rat aorta. *Am J Physiol* 1993;**264:**C687-C693.
2. Goldie RG. Endothelin receptor subtypes: distribution and function in the lung. *Pulm Pharmacol Ther* 1998;**11**:89-95.
3. Bermek H, Peng KC, Angelova K, *et al.* Endothelin degradation by vascular smooth muscle cells. *Regul Pept* 1996; **66**:155-162.
4. Chun M, Liyanage UK, Lisanti MP, Lodish HF. Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A* 1994;**91**:11728-32.
5. Chun M, Lin HY, Henis YI, *et al.* Endothelin-induced endocytosis of cell surface ETA receptors. Endothelin remains intact and bound to the ETA receptor. *J Biol Chem* 1995;**270**:10855-10860.
6. Dupuis J, Hoeper MM. Endothelin receptor antagonists in pulmonary arterial hypertension. *Eur Respir J* 2008;**31**:407-415.
7. Ivey ME, Osman N, Little PJ. Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. *Atherosclerosis* 2008. 199:237-247.
8. Song HJ, Min YS, Shin CY, *et al.* Activation of p38 MAPK is involved in endothelin-1-stimulated COX-2 expression in cultured Feline esophageal smooth muscle cells. *Mol Cells* 2006. 22:44-50.
9. Kohan DE. Production of endothelin-1 by rat mesangial cells: regulation by tumor necrosis factor. *J Lab Clin Med* 1992;119:477-484.
10. Oltmanns U, Issa R, Sukkar MB, *et al.* Role of c-jun N-terminal kinase in the induced release of GM-CSF, RANTES and IL-8 from human airway smooth muscle cells. *Br J Pharmacol* 2003;**139**:1228-1234.



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.