

## Theme Issue Article

# Rac-1 promotes pulmonary artery smooth muscle cell proliferation by upregulation of plasminogen activator inhibitor-1: Role of NF $\kappa$ B-dependent hypoxia-inducible factor-1 $\alpha$ transcription

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### Summary

Pulmonary vascular remodeling is commonly associated with pulmonary hypertension and is characterized by media thickening and disordered cellular proliferation, often accompanied by fibrin deposition and thrombosis in situ. However, the signaling pathways linking these different processes are not well understood. Since the GTPase Rac-1 has been suggested to act as a signaling relay in various cell types we investigated whether Rac-1 could be the link between thrombin signaling, plasminogen activator inhibitor-1 (PAI-1), which inhibits fibrinolysis and promotes fibrin deposition, and proliferation of pulmonary artery smooth muscle cells (PASMC). Exposure to thrombin enhanced the levels of Rac-1 protein and increased PAI-1 mRNA and protein expression in dependence of the thrombin receptor PAR-1. Expression of dominant-negative Rac-1 (RacT17N) prevented thrombin-induced PAI-1 expression whereas constitutively active RacG12V enhanced PAI-1 levels. In the presence of

RacT17N thrombin-induced PAI-1 promoter activity was abrogated whereas RacG12V increased PAI-1 promoter activity, and this response was essentially dependent on the transcription factor hypoxia-inducible factor-1 (HIF-1). Subsequently, RacG12V not only increased HIF transcriptional activity but also HIF-1 $\alpha$  protein and mRNA levels, whereas RacT17N prevented these responses elicited by thrombin. In line, RacG12V enhanced HIF-1 $\alpha$  promoter activity, and this response was dependent on nuclear factor-kappaB (NF $\kappa$ B) binding to the HIF-1 $\alpha$  promoter. Finally, upregulation of PAI-1 by Rac-1 and HIF-1 was essential for thrombin-stimulated proliferation of PASMC. These findings indicate that Rac-1 is an important mediator of thrombin signaling and may contribute to pulmonary vascular remodeling via HIF-1-dependent upregulation of PAI-1 leading to enhanced proliferation of PASMC.

### Keywords

Thrombin, PAR-1, plasminogen activator inhibitor-1, Rac-1, hypoxia-inducible factor-1, pulmonary vascular remodeling, smooth muscle cells

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## Introduction

Chronic alterations in the structure and composition of pulmonary arteries, commonly referred to as remodeling, have been associated with pulmonary hypertension (PH). They affect phenotype and function of all cell types of the vascular wall and are associated with disordered proliferation and thickening of the vascular wall, inflammation, changes in the extracellular matrix as well as thrombosis and fibrin deposition due to disturbed fibrinolysis and decreased fibrin clearance (1).

Thrombin was first identified as a trypsin-like serine protease produced at sites of vessel injury or tissue damage, which plays a key role in blood coagulation by converting fibrinogen to fibrin. Subsequently, it was shown that thrombin can act on platelets and many other cells including vascular cells via protease-activated receptors (PARs) (2, 3), suggesting that it may have profound effects on modulating vessel wall homeostasis. Indeed, enhanced levels of thrombin have been described in patients with several forms of PH (4).

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Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor (serpin) which targets the two plasminogen activators tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (5). PAI-1 maintains normal haemostasis by limiting the fibrinolytic system, and enhanced PAI-1 levels have been shown to promote fibrin deposition in several animal models (6–8). In addition, PAI-1 has been suggested to be involved in many cellular processes associated with tissue remodeling, and PAI-1 expression was found to be increased in smooth muscle cells activated by different growth factors, hormones, cytokines, vasoactive peptides as well as by thrombin (9). However, the exact link between enhanced levels of thrombin and PAI-1, which both have been associated with pulmonary vascular remodeling in PH (4, 10–14), has not been completely resolved yet.

PAI-1 was identified as a target gene for the hypoxia-inducible transcription factor HIF-1 in the cellular response to hypoxia (15). This heterodimer consists of the constitutively expressed HIF-1 $\beta$  subunit and HIF-1 $\alpha$  which is regulated by the cellular O<sub>2</sub> concentration (16). In normoxia, HIF-1 $\alpha$  is hydroxylated within the oxygen-dependent degradation domain (ODD) in the N-terminal transactivation domain (TADN), allowing the physical interaction with the tumor suppressor von Hippel-Lindau protein (pVHL) which targets its degradation by the 26S proteasome (17). Upon exposure to hypoxia, hydroxylase activity is diminished and non-hydroxylated HIF-1 $\alpha$  escapes the proteasomal degradation, accumulates and dimerizes with HIF-1 $\beta$  to bind to specific hypoxia-responsive elements (HRE) in the promoters or enhancers of target genes such as PAI-1 (16, 17). In addition to hypoxia, HIF-1 $\alpha$  can also be induced and activated under normoxic conditions in response to several stimuli, including thrombin (18, 19), indicating that this transcription factor may contribute to a prothrombotic state also independently from hypoxia.

We have previously shown that the GTPase Rac-1 plays an important role in promoting a prothrombotic state by upregulating expression and activity of tissue factor, the activator of the extrinsic coagulation cascade which leads to the formation of thrombin (20–22). Rac-1 has also been implicated in signaling cascades regulating cytoskeletal remodeling, migration and proliferation (23, 24) although its exact role in pulmonary artery smooth muscle cells, the main cell type responsible for pulmonary vascular remodeling, is not clear. In addition, Rac-1 has been shown to play a controversial role in the cellular response to hypoxia in hepatoma cells (25, 26). However, it is not known whether Rac-1 is involved in the regulation of the HIF pathway by thrombin. In this study we therefore investigated whether Rac-1 could provide a link between thrombin signaling, the HIF pathway, PAI-1 expression and proliferation of pulmonary artery smooth muscle cells.

## Material and methods

### Materials

TRAP6 was from Calbiochem (San Diego, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA) if not stated otherwise.

### Cell culture

Human pulmonary artery smooth muscle cells (PASMC) were from Lonza (Wuppertal, Germany) and cultured as recommended. Cells were used up to passage 12 and deprived from serum for 16 hours (h) before stimulation. A7r5 rat smooth muscle cells (rSMC) were cultured in DMEM as described (22). Since PASMC do not sufficiently express luciferase constructs (22) reporter gene assays have been performed in rSMC.

### Plasmids

The expression vectors encoding myc-tagged RacT17N or RacG12V, as well as the luciferase constructs containing the human wildtype genomic 5' HIF-1 $\alpha$  sequence from –538 to +284 bp (HIF-1 $\alpha$ -538) or the same sequence mutated at a NF $\kappa$ B-binding element at –197/–188 bp (HIF-1 $\alpha$ -538m) as well as a luciferase construct containing three copies of the hypoxia responsive element (HRE) from the erythropoietin gene (pGL3-EPO-HRE-luc) have been described (27–29). The human PAI-1 promoter 5' flanking region from –796 to +13 was cloned into the pGL3Basic vector using the primers GATAGG-TACCCCTGGTCCCGT and ACAGCTGGATCAGGCTGCTGCAGATCTCCGA to give PAI-1–796. Site-directed mutagenesis was performed at the HRE-2 (–194/–187 bp) (29, 30) to give PAI-1–796m.

The expression vector encoding for PAI-1 has been kindly provided by Dr. T. Kietzmann (University of Kaiserslautern, Germany).

Specific short-hairpin RNA encoding siRNA against PAR-1 (siPAR-1) (AAGGGACTGCTGGGAGGTAA, gene bank NM-001992) were designed and created using siRNA Target Designer and the siSTRIKE U6 Hairpin Cloning System (Promega, Madison, WI, USA). The vectors encoding siRNA against HIF-1 $\alpha$  or an unspecific random sequence (siNONE) have been previously described (28).

### Transfections and luciferase assays

PASMC were cultured for 24 h to a density of 70% and transfections were performed using 6  $\mu$ g of plasmid DNA and 36  $\mu$ l of fuge reagent (Roche Diagnostics, Indianapolis, IN, USA) per 10-cm dish as described (20). Sixteen hours after transfection, medium was changed and cells were serum-deprived for 24 h prior to stimulation.

Luciferase activity assays were performed in rSMC which were transfected using Superfect reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions as described previously (20). Briefly, cells were plated in 24-well plates (25,000 cells/well) the day before transfection. A ratio of a total of 1  $\mu$ g of DNA to 5  $\mu$ l of Superfect per well was respected for each transfection. After 2 h, the medium was changed; cells were cultured for 7 h and then serum-starved for 16 h before thrombin stimulation for the indicated times. Transfection efficiency was controlled by cotransfection of 0.25  $\mu$ g of Renilla Luciferase expression vector (pRLSV40) (Promega).

### Northern blot analysis

Total RNA from PASMC was isolated as described (28). Ten  $\mu$ g RNA were subjected to Northern blot analysis. Hybridizations

were performed with digoxigenin-labeled antisense RNA probes for PAI-1 or HIF-1 $\alpha$  at 65°C (15, 28).

### Western blot analysis

Western blot analysis was performed using antibodies against HIF-1 $\alpha$  (1:500 in 5% milk; BD transduction laboratories; BD, Franklin Lakes, NJ, USA), Rac-1 (BD Transduction laboratories, 1:1000 in 5% milk), PAR-1 (1:100 in 5% milk; Santa Cruz Laboratories, Santa Cruz, CA, USA), PAI-1 (1:500 in 5% milk; American Diagnostica, Stamford, CT, USA) or  $\alpha$ -actin (1:500 in 5% milk, Sigma). Goat anti-mouse or anti-rabbit immunoglobulin G (1:10000 in 5% milk or 5% bovine serum albumin [BSA]; Calbiochem, San Diego, CA, USA) was used as secondary antibody. Blots were scanned and analyzed using GelDoc software (BioRad, Hercules, CA, USA).

### Proliferation assay

Proliferative activity of PASMC was evaluated by 5-bromo-2'-deoxyuridine (BrdU) labeling (Roche Diagnostics) according to the manufacturer's instructions as described (31). In some experiments, an inhibitory mouse monoclonal antibody against PAI-1 (1  $\mu$ M; Loxo, Dossenheim, Germany) or control mouse IgG (Santa Cruz) were added.

### Statistical analysis

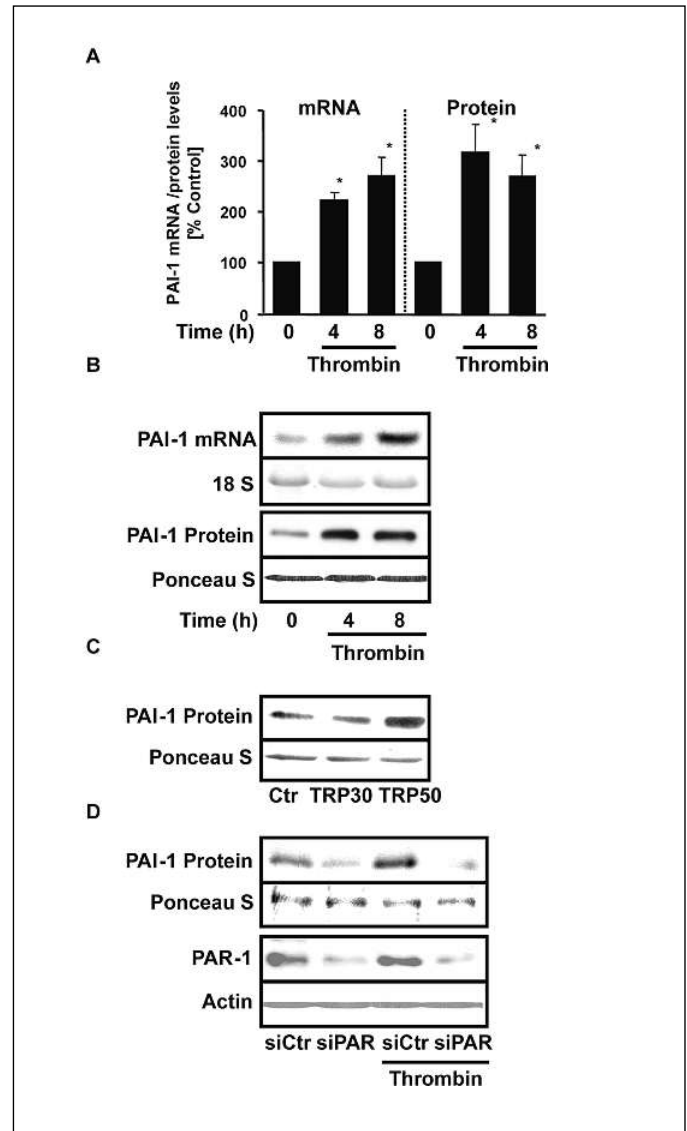
Values presented are means  $\pm$  standard deviation (SD). Results were compared by ANOVA for repeated measurements followed by Student-Newman-Keuls' t-test.  $P < 0.05$  was considered statistically significant.

## Results

### PAR-1 and Rac-1 increase PAI-1 expression by thrombin

Stimulation with thrombin resulted in the upregulation of PAI-1 mRNA and protein levels after 4 and 8 h of stimulation (Fig. 1A, B). To determine whether the thrombin receptor PAR-1 is involved in this response, PASMC were treated with the thrombin receptor activating peptide TRAP6 (Fig. 1C). This peptide is the tethered ligand of the protease activated receptor-1 (PAR-1) and is known to selectively activate PAR-1 (32). Exposure for 4 h to 50  $\mu$ M TRAP6 enhanced PAI-1 levels similar to the situation with thrombin, although lower doses were less effective. Subsequently, PASMC were transfected with siRNA targeting PAR-1. This treatment was effective in downregulating PAR-1 levels under control conditions as well as under thrombin stimulation (Fig. 1D). Similar to the situation in wild-type cells, thrombin enhanced PAI-1 levels in cells transfected with siCtr (Fig. 1D), although the relative increase in PAI-1 levels upon thrombin stimulation was somewhat lower than in untransfected cells, possibly due to the transfection process. Importantly, however, our data clearly show that depletion of PAR-1 decreased PAI-1 levels under control conditions and prevented PAI-1 upregulation by thrombin (Fig. 1D).

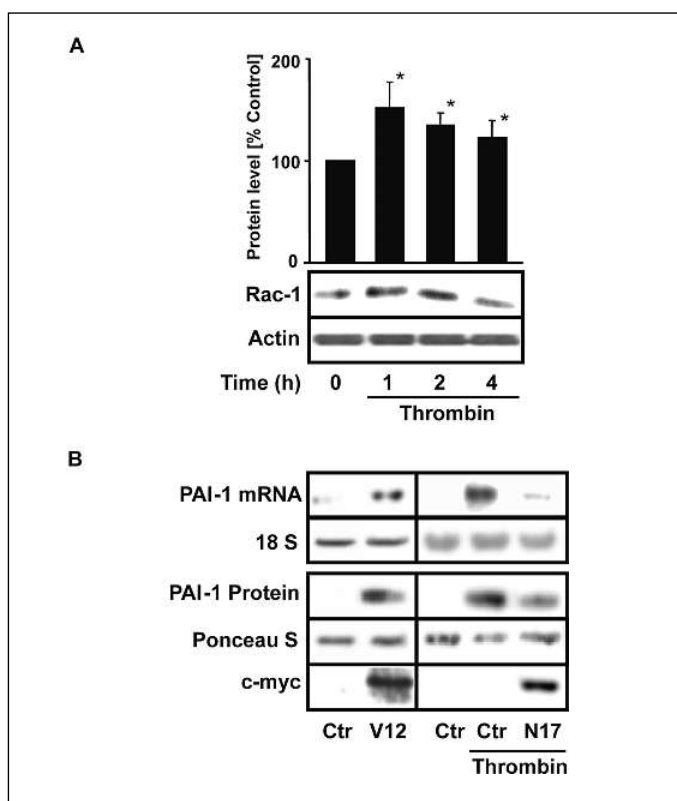
To evaluate the involvement of Rac-1 in the regulation of PAI-1 by thrombin, we first determined the levels of Rac-1 protein under our experimental conditions. Western blot analysis revealed that thrombin rapidly but transiently increased Rac-1 pro-



**Figure 1: Thrombin increases PAI-1 expression via PAR-1.**

A) Pulmonary artery smooth muscle cells (PASMC) were stimulated with thrombin (3 U/ml) for 4 and 8 h and mRNA levels were evaluated by Northern blot analyses using a PAI-1 probe. PAI-1 protein was determined from supernatants by Western blot analyses using an antibody against PAI-1. 18 S or Ponceau S staining, respectively, served as loading control. Quantification of all experiments is shown ( $n=7$  or  $n=4$ , respectively,  $*p < 0.05$  versus non-stimulated cells (0)). B) Representative Northern and Western blots are shown. C) PASMC were stimulated with 30  $\mu$ M TRAP6 (TRP30) or 50  $\mu$ M TRAP6 (TRP50) or left unstimulated (Ctr) or D) were transfected with siRNA against PAR-1 (siPAR) or control siRNA (siCtr) and stimulated with thrombin (3 U/ml) for 4 h. PAI-1 protein levels were determined in supernatants by Western blot analyses. Ponceau S staining served as loading control. Representative blots are shown ( $n=4$ ). PAR-1 protein levels were determined to assess transfection efficiency.

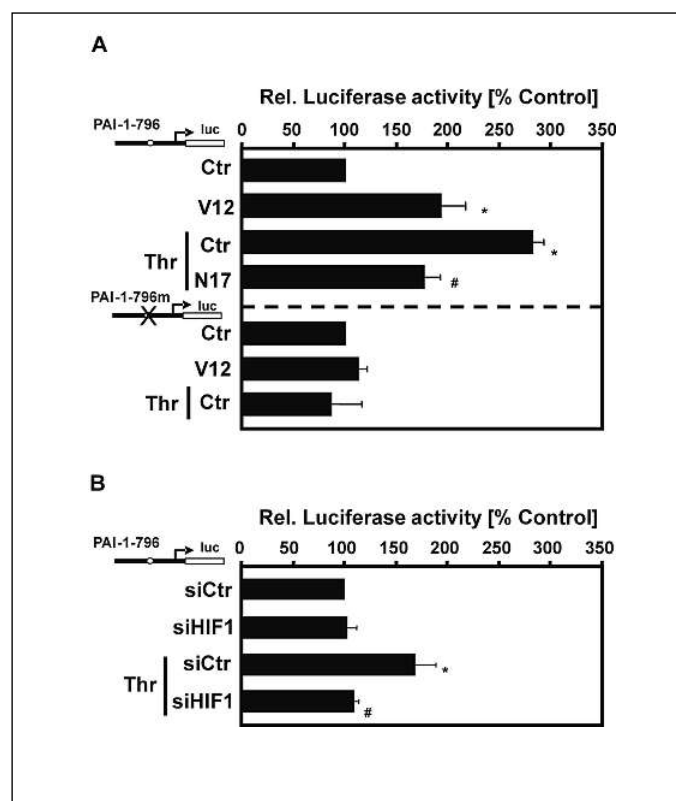
tein levels within 1 h of stimulation (Fig. 2A). Subsequently, PASMC were transfected with constitutively active (RacG12V) or dominant-negative (RacT17N) Rac-1 mutants. Active Rac-1 enhanced PAI-1 mRNA and protein levels, while thrombin-stimulated PAI-1 expression was diminished in PASMC express-



**Figure 2: Rac-1 regulates PAI-I expression by thrombin.** A) Pulmonary artery smooth muscle cells (PASMC) were stimulated with thrombin (3 U/ml) for 1, 2 and 4 h. Rac-1 protein was evaluated in cellular lysates by Western blot analyses. Actin staining served as loading control. Quantification of all experiments is shown ( $n=4$ ,  $*p<0.05$  versus non-stimulated cells (0)). B) PASMC were transfected with control vector (Ctrl) or vectors for RacG12V (V12) or RacT17N (N17) and left untreated or stimulated with thrombin (3 U/ml) for 4 h. PAI-I mRNA levels were determined by Northern blot analysis. 18 S served as loading control. Representative blots are shown ( $n=3$ ). PAI-I protein was evaluated in the supernatant by Western blot analyses ( $n=4$ ). Transfection efficiency of the c-myc-tagged Rac constructs was controlled by Western blot using an antibody against c-myc. Ponceau S served as loading control.

ing RacT17N (Fig. 2B). In addition, thrombin or RacG12V stimulated PAI-1 promoter activity, whereas RacT17N abrogated this response (Fig. 3A).

It has been previously shown that PAI-1 is regulated by the transcription factor HIF-1 under hypoxic conditions. In fact, a binding site for HIF-1 at the rat or human PAI-1 promoter has been described (15, 29, 30). To investigate whether PAI-1 induction by thrombin and Rac-1 is dependent on HIF-1, luciferase activity of a PAI-1 promoter construct where the HIF-1 binding site was mutated at bp -193/-185 as described (29, 30) was determined in the presence of thrombin or active RacG12V. In contrast to the situation with the wild-type construct, neither RacG12V nor thrombin increased luciferase activity of the mutated PAI-1 promoter construct compared to control (Fig. 3A). In line, depletion of HIF-1 $\alpha$  by siRNA diminished control and thrombin-induced PAI-1 promoter activity (Fig. 3B) indicating that Rac-1 is upregulating PAI-1 via HIF-1 $\alpha$ .



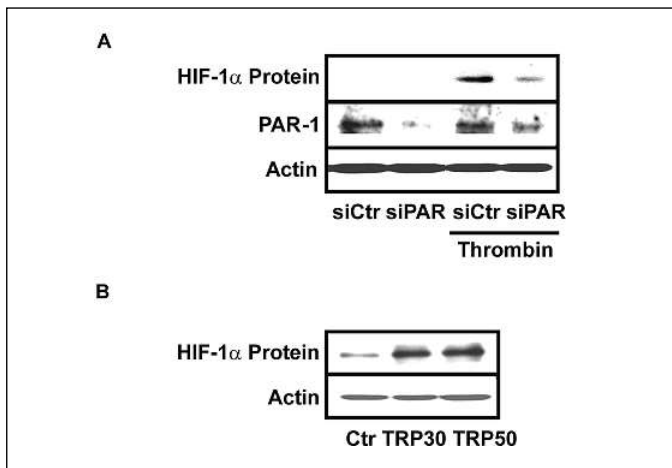
**Figure 3: Thrombin and Rac-1 increase PAI-I promoter activity.** A) rSMC were transfected with control vector (Ctrl) or vectors for active RacG12V (V12) or dominant-negative RacT17N (N17) and human PAI-1 promoter luciferase constructs harboring either the wild-type sequence (PAI-1-796) or a sequence mutated at the hypoxia-responsive element binding HIF (PAI-1-796m). After 24 h, cells were left untreated or stimulated with thrombin for 8 h, and luciferase activity was determined ( $n=6$ ;  $*p<0.05$  vs. Ctrl (unstimulated);  $\#p<0.05$  vs. Ctrl (thrombin-stimulated)). B) rSMC were transfected with control vector (siCtrl) or siRNA against HIF-1 $\alpha$  (siHIF1) and the wild-type human PAI-1 promoter luciferase construct (PAI-1-796). After 24 h, cells were left untreated or stimulated with thrombin for 8 h and luciferase activity was determined [ $n=3$ ;  $*p<0.05$  vs. Ctrl (unstimulated);  $\#p<0.05$  vs. Ctrl (thrombin-stimulated)].

### PAR-1 and Rac-1 are involved in activation of HIF-1 by thrombin

Since thrombin-induced PAI-1 promoter activity was dependent on HIF-1, we then investigated whether Rac-1 contributes to the upregulation of HIF-1 $\alpha$  by thrombin. First, we examined the involvement of PAR-1 in this response. Thrombin and TRAP6 increased HIF-1 $\alpha$  protein, whereas depletion of PAR-1 by siRNA prevented thrombin induction of HIF-1 $\alpha$  (Fig. 4) suggesting that PAR-1 is essential for the upregulation of HIF-1 $\alpha$  by thrombin.

Next, PASMC were transfected with the Rac-1 mutants. Indeed, active RacG12V increased HIF-1 $\alpha$  protein levels, whereas dominant-negative RacT17N prevented upregulation of HIF-1 $\alpha$  by thrombin (Fig. 5A).

Since we have previously shown that thrombin increases HIF-1 $\alpha$  at the transcriptional level (28) we determined whether Rac-1 acts via this mechanism. In the presence of RacG12V, HIF-1 $\alpha$  mRNA levels in PASMC as well as HIF-1 $\alpha$  promoter ac-



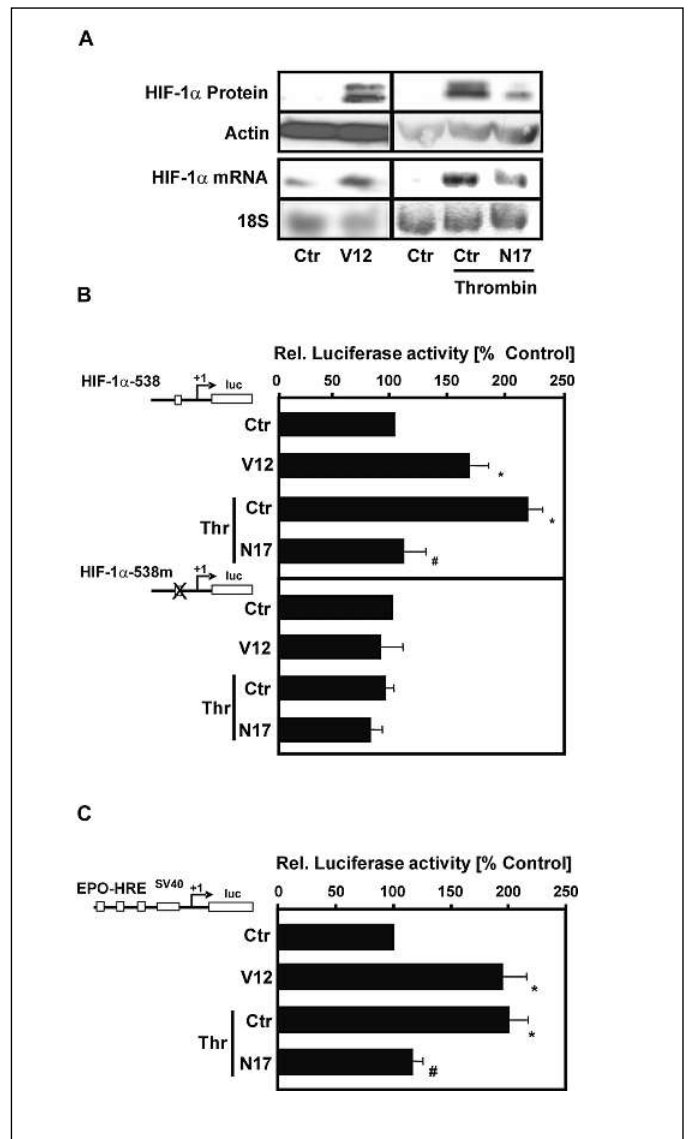
**Figure 4: PAR-1 is involved in the upregulation of HIF-1 $\alpha$  protein by thrombin.** A) Pulmonary artery smooth muscle cells (PASMC) were transfected with control vector (siCtr) or siRNA against PAR-1 (siPAR) and then left untreated or stimulated with thrombin for 2 h. B) PASMC were stimulated with 30  $\mu$ M TRAP6 (TRP30) or 50  $\mu$ M TRAP6 (TRP50) or left unstimulated. HIF-1 $\alpha$  protein levels were determined by Western blot analyses. Actin staining served as loading control. Representative blots are shown (n=3).

tivity in rSMC were enhanced, whereas RacT17N prevented induction of HIF-1 $\alpha$  mRNA and promoter activity by thrombin (Fig. 5A, B). This induction was completely abrogated when using a HIF-1 $\alpha$  promoter construct where a NF $\kappa$ B binding site at -197/-188 bp which we previously identified (28) was mutated. In line, expression of a dominant-negative mutant of inhibitor of NF $\kappa$ B (I $\kappa$ B) prevented induction of HIF-1 $\alpha$  by RacG12V (data not shown) indicating that Rac-1 induces HIF-1 $\alpha$  via activation of NF $\kappa$ B.

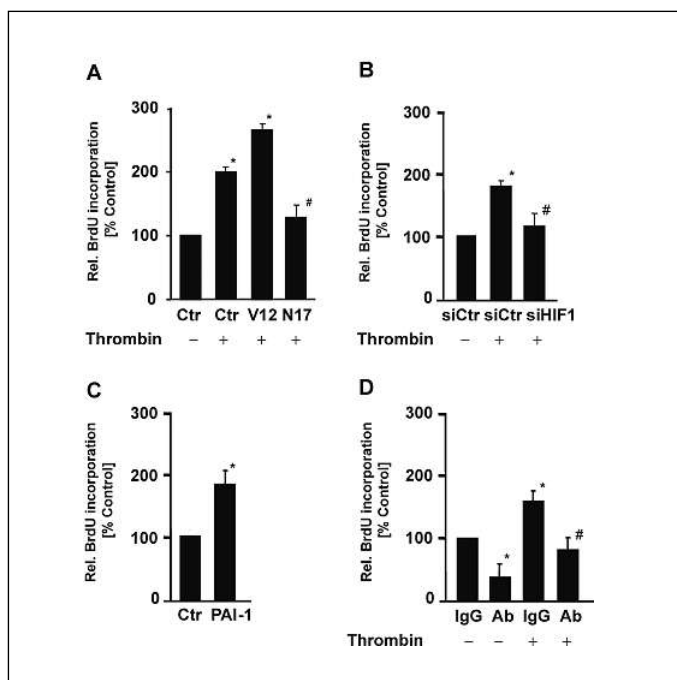
To determine whether Rac-1 would also mediate HIF transcriptional activity, cells were transfected with a luciferase construct containing three HRE in front of the SV40 promoter. Co-transfection of RacG12V increased HIF activity, whereas thrombin-induced HIF activation was diminished in the presence of RacT17N (Fig. 5C).

### Rac-1, HIF-1 and PAI-1 mediate the proliferative response by thrombin

Since pulmonary vascular remodeling is associated with increased proliferation of PASMC, we determined whether thrombin and Rac-1 signaling contributes to proliferation of PASMC using BrdU incorporation. Proliferation was induced by RacG12V and thrombin, whereas RacT17N diminished thrombin-stimulated proliferation of PASMC (Fig. 6A). Subsequently, depletion of HIF-1 $\alpha$  by siRNA decreased the proliferative response to thrombin (Fig. 6B). To investigate the role of PAI-1 in PASMC proliferation, cells were transfected with control vector or a PAI-1 expression vector. Interestingly, overexpression of PAI-1 resulted in increased proliferation (Fig. 6C). On the other hand, addition of an inhibitory antibody against PAI-1 to the media of thrombin-stimulated PASMC prevented the stimulatory effect of the control and thrombin-conditioned media on PASMC proliferation (Fig. 6D), indicating that PAI-1 is upregu-



**Figure 5: Rac-1 increases HIF-1 $\alpha$  at the transcriptional level.** A) Pulmonary artery smooth muscle cells (PASMC) were transfected with control vector (Ctr), RacG12V (V12) or RacT17N (N17) and then left untreated or stimulated with thrombin for 2 h. HIF-1 $\alpha$  mRNA levels were determined by Northern blot analysis. 18 S served as loading control (n=4). HIF-1 $\alpha$  protein was evaluated by Western blot analyses. Actin served as loading control. Representative blots are shown (n=3). B) rSMC were transfected with control vector (Ctr) or vectors for active RacG12V (V12) or dominant-negative RacT17N (N17) and human HIF-1 $\alpha$  promoter luciferase constructs harboring either the wild-type sequence (HIF-1 $\alpha$ -538) or a sequence mutated at a NF $\kappa$ B consensus sequence (HIF-1 $\alpha$ -538m) and stimulated with thrombin for 8 h. Luciferase activity from controls was set to 100% (n=6; \*p<0.05 vs. unstimulated control (Ctr); #p<0.05 vs. thrombin-stimulated control (Ctr)). C) rSMC were transfected with control vector (Ctr) or vectors for active RacG12V (V12) or dominant-negative RacT17N (N17) and a luciferase construct containing three hypoxia-responsive elements in front of the SV40 promoter and stimulated with thrombin for 8 h. Luciferase activity from controls was set to 100% (n=6; \*p<0.05 vs. unstimulated control (Ctr); #p<0.05 vs. thrombin-stimulated control (Ctr)).



**Figure 6: Rac-1, HIF-1 $\alpha$  and PAI-1 mediate proliferative responses of PSMC by thrombin.** Pulmonary artery smooth muscle cells (PSMC) were transfected with A) control vector (Ctr) or vectors encoding for RacG12V (V12), RacT17N (N17) or B) siRNA against HIF-1 $\alpha$  (siH1 $\alpha$ ) or control siRNA (siCtr) and stimulated with thrombin (3 U/ml) for 24 h. C) PSMC were transfected with PAI-1 expression vector (PAI-1) or control vector (Ctr). A-C) Proliferative activity was assessed by BrdU incorporation (n=3, \*p<0.05 vs. control cells (Ctr), #p<0.05 vs. thrombin-stimulated control cells (Ctr)). D) PSMC were stimulated with thrombin (3 U/ml) for 8 h. The conditioned medium was transferred to another set of serum-starved PSMC. An antibody directed against PAI-1 (Ab) or mouse IgG were added and BrdU incorporation was determined (n=3; \*p<0.05 vs. IgG control (unstimulated IgG treated); #p<0.05 vs. IgG control (thrombin-stimulated IgG treated)).

lated and secreted in response to thrombin and Rac-1 and promotes proliferation of PSMC.

## Discussion

In this study we demonstrated that Rac-1 plays an essential role in regulating proliferation of PSMC in response to thrombin, involving HIF-1 $\alpha$  and PAI-1. This is based on the following findings: a) Thrombin increased the levels of Rac-1 and PAI-1 dependent on PAR-1. b) PAI-1 upregulation was dependent on Rac-1 and was mediated by the transcription factor HIF-1. c) Rac-1 increased HIF activity due to promoting HIF-1 $\alpha$  expression by stimulating binding of NF $\kappa$ B to the HIF-1 $\alpha$  promoter. d) Rac-1, HIF-1 $\alpha$  and PAI-1 were required for thrombin-dependent stimulation of PSMC proliferation.

### Rac-1 regulates PAI-1

The GTPase Rac-1 has been shown to act as a signaling relay in various cellular responses. In this study we demonstrate that Rac-1 is upregulated by thrombin and is critically involved in the regulation of PAI-1 downstream of the thrombin receptor PAR-1.

In line with this study, we and others have shown that thrombin is able to enhance PAI-1 mRNA and protein levels in pulmonary artery and other smooth muscle cells (18, 28, 33, 34) although the involvement of PAR-1 in PAI-1 regulation has not been described so far in PSMC. However, in pericytes, but not in endothelial cells, thrombin increased the secretion of PAI-1 by activation of PAR-1 (35) suggesting that PAR-1 regulation of PAI-1 levels is cell-type specific.

Whereas upregulation of Rac-1 levels by thrombin has not been described so far, a role for Rac-1 in the regulation of PAI-1 has been suggested previously. Interestingly, whereas Rac-1 has been shown to mediate PAI-1 expression by transforming growth factor (TGF)- $\beta$ 1 in NIH 3T3 fibroblasts under normoxic conditions similar to the situation in our study (36), expression of dominant-negative RacT17N further increased PAI-1 levels under hypoxic conditions in HepG2 cells (27). Although the reasons for these conflicting data are not resolved, yet, they suggest that this GTPase plays a cell type- and/or stimulus-specific role in controlling PAI-1 levels. Since elevated levels of PAI-1 have been associated with vascular remodeling processes (9), our findings in PSMC, the major cell type involved in pulmonary vascular remodeling, suggest an important contribution of Rac-1 in linking enhanced haemostatic activity and deteriorated fibrin clearance as is observed in these conditions.

### Rac-1 regulates HIF-1 $\alpha$

Our findings further suggest that Rac-1 regulates PAI-1 expression via the transcription factor HIF since mutation of the HIF binding site in the PAI-1 promoter abolished induction of luciferase activity in the presence of active RacG12V. In line, our studies showed that expression of active Rac-1 resulted in transcriptional activation of HIF, whereas dominant-negative Rac-1 diminished thrombin-induced HIF activation. Consistently, RacT17N has been shown to prevent HIF activation by carbachol in HEK293 cells overexpressing a muscarinic acetylcholine receptor (25). In contrast, under hypoxic conditions, contradictory results have been observed with regard to the effects of Rac-1 mutants on HIF activity in Hep3B or HepG2 cell lines (26, 27), describing either diminished or increased HIF activity in the presence of inactive Rac-1. Although the reasons for these inconsistent results are not resolved to date, one may speculate that additional regulatory elements contribute to Rac-1 signaling under conditions of impaired oxygen availability which may also be cell type-specific. In support of the latter, RacG12V reduced HIF-1 $\alpha$  protein levels under conditions of mild hypoxia in HepG2 cells, whereas opposite effects were observed in PSMC under the same conditions (data not shown).

However, our data in PSMC clearly show that Rac-1 is required for upregulation of HIF-1 $\alpha$  by thrombin. Furthermore, active Rac-1 itself was able to induce HIF-1 $\alpha$  expression at the transcriptional level, since RacG12V increased HIF-1 $\alpha$  mRNA levels and HIF-1 $\alpha$  promoter activity. Since we could previously show that HIF-1 $\alpha$  is a direct transcriptional target of NF $\kappa$ B (28, 37) and NF $\kappa$ B is activated by thrombin and Rac-1 in PSMC (20, 28), it was conceivable to assume that NF $\kappa$ B may also play a role in mediating HIF-1 $\alpha$  upregulation by Rac-1. Indeed, RacG12V was unable to increase HIF-1 $\alpha$  promoter activity when the NF $\kappa$ B binding element was mutated, further indicating

that Rac-1 leads to activation of NF $\kappa$ B thus increasing HIF-1 $\alpha$  levels and the HIF pathway.

### Rac-1, HIF-1 $\alpha$ and PAI-1 stimulate proliferation of PASMC

Rac-1 is well-known as a regulator of cytoskeletal remodeling and cell motility, and can also promote transformation and tumor cell proliferation (23, 24, 38). In this study we show that Rac-1 is required for proliferation of PASMC since RacG12V increased, similar to thrombin, the proliferative response, whereas RacT17N diminished thrombin-stimulated proliferation.

In line, thrombin has been shown previously to increase proliferation of various types of smooth muscle cells (39). Consistently, it has been reported that PASMC expressing RacT17N had decreased proliferation due to cell cycle arrest (40). Interestingly, transgenic mice expressing constitutively active human Rac-1 were described to have accelerated wound healing associated with more efficient proliferation of vascular cells (41), while supernatants from Rac-1-deficient tumor cells inhibited proliferation of endothelial cells (42). These data would support a mechanism where Rac-1 regulates the secretion of proteins which may exhibit a paracrine effect on proliferation of vascular cells, such as in our study with PAI-1 (see below).

The present study also revealed that HIF-1 $\alpha$  knockdown by specific siRNA markedly reduced proliferation of PASMC in response to thrombin. These findings suggest that Rac-1-mediated upregulation of HIF-1 $\alpha$  may induce target genes which are essentially involved in PASMC proliferation. In line, in a recent study it was shown that HIF-1 $\alpha$  promotes proliferation of PASMC in response to PDGF, FGF-2 or EGF (43). Although in that study thrombin failed to stimulate PASMC proliferation due to unknown reasons, our present findings clearly show that thrombin increases proliferation of PASMC via HIF-1 $\alpha$ . A role for HIF-1 $\alpha$  in pulmonary vascular remodeling processes has been indicated in an experimental model of hypoxia-induced PH where muscularization of small pulmonary arteries in response to chronic hypoxia was delayed in heterozygous HIF-1 $\alpha$  mice, compared with wild-type mice (44). Moreover, HIF-1 $\alpha$  has been found in remodelled vessels in tissue sections from patients with different forms of PH (45) indicating that HIF-1 $\alpha$  may indeed play a role in promoting pulmonary vascular remodeling also independently from hypoxia.

The importance of Rac-1-dependent control of HIF-1 $\alpha$  for PASMC proliferation is further substantiated by our findings that PAI-1 is upregulated in a Rac-1- and HIF-1 $\alpha$ -dependent manner by thrombin, and that cell proliferation after addition of conditioned medium from PASMC stimulated with thrombin was attenuated when incubated with an inhibitory antibody against PAI-1, indicating that thrombin-mediated secretion of PAI-1 stimulates PASMC proliferation. Consistently, overexpression of PAI-1 increased proliferation of PASMC. In line with our observation, overexpression of PAI-1 has been previously shown to enhance proliferation of smooth muscle cells *in*

*vitro* (46), and several studies *in vivo* provided similar evidence (5–7, 46). However, also contrasting effects of PAI-1 have been reported with regard to the proliferative activity of different cell types. Addition of recombinant PAI-1 decreased PASMC proliferation (47), and migration of smooth muscle cells was inhibited in smooth muscle cells from transgenic PAI-1 mice (48), whereas vascular cells from PAI-1-deficient mice showed increased proliferation or migration (49, 50). Furthermore, ectopic expression of PAI-1 in proliferating HaCaT cells induced growth arrest (51).

Although the reasons for the contradictory effects of PAI-1 on proliferation and migration of vascular cells, sometimes referred to as the PAI-1 paradox, are not completely resolved to date they may be related to the multiple effects exerted by this serine protease inhibitor which exceed the sole inhibition of fibrinolysis. Additionally, the ability of PAI-1 to interact with a variety of molecules may also be responsible for its opposing effects on vascular proliferative activity and remodeling which may be differentially prominent dependent on cell type, culture conditions, genotypes and experimental design (52). In fact, whether PAI-1 promotes or prevents proliferation appears to depend on the vascular bed, type of lesion, and the experimental and clinical conditions which affect the various PAI-1 functions in one or more aspects.

However, in our study, PAI-1 overexpression increased proliferation and PAI-1 was clearly required for proliferation of PASMC under control conditions as well as in the presence of thrombin. In line, in a longitudinal study based on microarray analyses, PAI-1 expression was clearly increased during the development of vascular obliteration and PH in monocrotaline-treated rats (53) and enhanced PAI-1 levels have been found in vascular remodeling associated with pulmonary thromboembolism (54). Moreover, several studies have been performed to determine the plasma levels of PAI-1 in patients with different forms of PH. Although it was very early reported that PAI-1 activity did not differ between controls and patients with PH (13), studies in the following years showed elevated levels of PAI-1 in patients with different forms of PH (10–12).

Taken together our results clearly demonstrate that Rac-1 plays an important role in regulating PAI-1 expression and PASMC proliferation. This response requires increased expression of HIF-1 $\alpha$  involving a NF $\kappa$ B-dependent transcriptional mechanism. Furthermore, this GTPase is essential for inducing this pathway in response to thrombin and PAR-1. Since thrombin, HIF-1 $\alpha$  and PAI-1 have been associated with pulmonary vascular remodeling under different disease conditions, our findings suggest an important role of Rac-1 in linking thrombin signaling to HIF-dependent PAI-1 expression and increased proliferation in pulmonary vascular remodeling. Thus, additional studies have to show whether Rac-1 could be a target for the design of novel treatment strategies to combat disorders associated with pulmonary vascular remodeling.

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