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Rac Regulates Thrombin-Induced Tissue Factor Expression in Pulmonary Artery Smooth Muscle Cells Involving the Nuclear Factor-κB Pathway

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ABSTRACT

Pulmonary hypertension is associated with enhanced thrombogenicity of the vessel wall contributing to vascular remodeling. However, the signaling mechanisms promoting this prothrombotic state are not resolved. Here we investigated the role of the GTPase Rac in the regulation of tissue factor (TF) expression and activity in response to thrombin in pulmonary artery smooth muscle cells (PASMC). TF mRNA and protein expression and surface procoagulant activity were increased by thrombin in PASMC. These responses were enhanced in the presence of the constitutively active Rac mutant RacG12V, but were abrogated in cells expressing dominant-negative RacT17N. Thrombin and RacG12V also increased human TF promoter activity primarily involving a sequence between −636 and −111 bp containing a distal, nuclear factor-κB (NFκB)-dependent enhancer element. Indeed, thrombin and RacG12V stimulated NFκB-dependent transcriptional activity, and overexpression of p50/p65 significantly increased human TF promoter activity. Moreover, in RacG12V-overexpressing cells, TF promoter activity was significantly decreased by coexpression of dominant-negative mutants of IκBα and IκBKα, which prevent NFκB activation. As enhanced NFκB activity has been observed in patients with pulmonary hypertension, Rac-dependent activation of the NFκB pathway may be a critical element promoting thrombin-induced TF expression and activity, and thus a prothrombotic state in pulmonary hypertension. *Antioxid. Redox Signal.* 6, 713–720.

INTRODUCTION

Pulmonary hypertension is characterized by hypertrophy and enhanced proliferation of vascular cells and is frequently associated with a prothrombotic state, which may play an important role in promoting vascular remodeling and thus the manifestation of dysfunction of the pulmonary vasculature (11, 14). The primary link between vascular cells and the hemostatic system is provided by the cell surface protein tissue factor (TF). TF serves as an essential cofactor for activated coagulation factor VII, finally allowing the formation of thrombin (19).

Thrombin has also been shown to act directly on vascular cells via specific receptors activating a number of downstream signaling pathways (22, 25). Although thrombin-induced proliferation is a well described phenomenon of smooth muscle cells, various other cellular programs that may contribute to the promotion of vascular remodeling, including procoagulant responses, are elicited by thrombin (22, 25). Intriguingly, thrombin itself has been shown to modulate the expression of TF in smooth muscle cells (12, 13, 15, 28). Thus, thrombin formation in response to activation of the coagulation cascade is capable of rapidly and markedly inducing and activating TF, the main initiator of this cascade, in

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smooth muscle cells. However, the signaling pathways underlying such a thrombogenic cycle are not completely understood yet. In cultured smooth muscle cells, thrombin has been shown to elevate TF expression by mobilizing intracellular calcium, or via a redox-sensitive mechanism involving the NADPH oxidases (12, 13, 28). In endothelial cells, thrombin-induced TF expression was mediated by Rho/Rho kinase, one of the effectors of the Rho family of small GTP-binding proteins such as Rho, Rac, and cdc42 (24). Rac has been shown to be essential for activation of the NADPH oxidase and subsequent production of reactive oxygen species (ROS), but can also act directly by activating downstream kinase cascades leading to modulation of transcription factor activity and gene expression (24, 26).

The expression of TF has been reported to be mediated by several transcription factors, including nuclear factor-κB (NFκB), activator protein 1 (AP-1), and early growth response 1 (Egr-1) (2, 15–17). NFκB is a member of *Rel*-related transcription factors and is involved in the control of inflammatory responses, cellular growth, or apoptosis, as well as in the development of cardiovascular diseases (18, 29). NFκB is composed of two subunits named p50 and p65. In resting cells, the NFκB complex is trapped in the cytosol by binding to the inhibitory κB proteins (IκBα or IκBβ). Phosphorylation of the IκB proteins by kinases (IκBKα or IκBKβ) marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NFκB complex. The activated NFκB complex translocates into the nucleus and binds DNA at specific motifs. NFκB is among the transcription factors recognized to respond directly to oxidative stress and to changes in the redox balance within the cell (18, 29).

As ROS have been shown to regulate thrombin-induced TF expression (12, 13), we investigated whether the GTPase Rac and the NFκB pathway participate in the control of such a thrombogenic cycle in pulmonary artery smooth muscle cells (PASMC), the primary cell type involved in promoting vascular remodeling in pulmonary hypertension.

MATERIALS AND METHODS

Reagents

Deoxycytidine 5′-[α-^{32}P]triphosphate (3,000 Ci/mmol) was from Amersham (Freiburg, Germany). Human α-thrombin (thrombin-specific clotting activity, 3.261 U/mg) and the chromogenic substrate S-2238 were from Haemochrom Diagnostika (Essen, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Cell culture

Human PASMC were purchased from Cambrex (Verviers, Belgium) and cultured in the medium provided as recommended. PASMC (passages 3–11) were grown to confluency and serum-deprived for 24 h prior to stimulation with thrombin. A7r5 rat smooth muscle cells (rSMC; kindly provided by Dr. H.H.H. Schmidt, Giessen) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were serum-starved for 16 h before experiments.

Plasmids

The expression vectors encoding myc-tagged racT17N or racG12V have been described previously (9, 13). The pTF111 construct contains the minimal human TF promoter (−111 to +121 bp) linked to the luciferase gene of the pgl2 basic vector and has been described previously (7). pTF636 was obtained by PCR from the full-length human TF promoter construct p19Luc (kindly provided by Dr. Nigel Mackman, La Jolla, CA, U.S.A.). The fragment encompassing the sequence from −636 to −111 bp was inserted into the *Sma*I site from pTF111. The plasmid pNFκB-Luc containing five NFκB sites in front of the SV40 promoter linked to the luciferase gene was from Clontech (Heidelberg, Germany) and has been described previously (9). The expression vectors p50 (pRSV-NFκB-1) and p65 (pRSV-RelA) encoding full-length p50 and p65/rel, respectively, have been described (4). The expression vector pCMV-IκBαdn (IκBdn) was purchased from Becton Dickinson (Heidelberg, Germany). The expression vector p-FlagCMV2-IκK-2 (IκKdn) encoding full-length IκK-2 mutated at S177A/S181A has been described previously (27).

Transfections and luciferase assay

PASMC were cultured for 24 h to a density of 70%, and transfections were performed using 6 μg of plasmid DNA and 36 μl of fugene reagent (Roche, Mannheim, Germany) per 10-cm dish. Sixteen hours after transfection, medium was changed and cells were serum-deprived for 24 h prior to stimulation. Transfection efficiency was controlled by Western blot analysis using an antibody against the c-myc epitope (Santa Cruz, Heidelberg, Germany).

For luciferase activity assays, rSMC were transfected using Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were plated in 24-well plates (25,000 cells/well) the day before transfection. A ratio of a total of 1 μg of DNA to 5 μl 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 μg of Renilla Luciferase expression vector (pRLSV40) (Promega, Mannheim, Germany).

Procoagulant activity assay

The surface procoagulant activity was evaluated in PASMC transfected with the expression vectors for racG12V or racT17N or with the control vector pcDNA3.1 as described previously (13).

Measurement of ROS production

The generation of ROS was measured using the fluoroprobe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Göttingen, Germany) as described (1). PASMC were seeded in 96-well plates, transfected, and serum-starved for 24 h. Cells were stimulated with thrombin (3 U/ml) for 2 h, washed with Hanks' balanced salt solution (HBSS), and incubated with CM-H_2DCFDA (8.5 μ*M*) for 5 min in the dark at 37°C. Cells were washed with HBSS, and fluorescence was

monitored using 480-nm excitation and 540-nm emission wavelength in a microplate reader (Tecan, Crailsheim, Germany). 2′,7′-Dichlorodihydrofluorescein (DCF) fluorescence was corrected to the number of viable cells using the Alamar Blue test according to the manufacturer's instructions (Biosource, Nivelles, Belgium). At the end of the experiment, cells were incubated with Alamar Blue in phosphate-buffered saline, pH 7.4, at 37°C to allow the indicator to change from the oxidized (blue) to the fully reduced (red) form. The absorbance was then measured at 580 nm.

Northern blot analysis

Total RNA from human PASMC was isolated as described (16). Ten micrograms of RNA was subjected to Northern blot analysis, and hybridizations were performed with a ^{32}P-labeled human TF cDNA fragment and an 18S cDNA fragment.

Western blot analysis

Western blot analysis was performed as described previously (8). The monoclonal antibody against human TF (kindly provided by Dr. Tom Zionchek, Genentech) was used in a 1:500 dilution. The antibody against smooth muscle cell actin (1:1,000 dilution) was from Sigma. Goat anti-mouse or goat anti-rabbit immunoglobulins G (Calbiochem, Darmstadt, Germany) were used as secondary antibodies. The enhanced chemiluminescent Western blotting system was used for detection.

Statistical analysis

Values presented are means ± SD. Results were compared by ANOVA for repeated measurements followed by Student–Newman–Keuls *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Thrombin enhances TF expression and promoter activity in PASMC

PASMC were exposed to thrombin for increasing time intervals, and TF expression was determined by Northern and Western blot analysis (Fig. 1). Thrombin transiently enhanced TF mRNA levels by fourfold, peaking after 2–4 h of incubation (Fig. 1A). Elevated TF mRNA levels were followed by enhanced TF protein levels, which were continuously increasing to levels about fourfold higher than in the respective controls after 16 h of exposure to thrombin (Fig. 1B).

To confirm further the transcriptional activation of TF by thrombin, cells were transfected with two human TF promoter luciferase constructs (Fig. 1C). The plasmid pTF111 contains the minimal promoter sequence (−111 to +121 bp) including a proximal enhancer with three overlapping Egr-1 and Sp1 sites (25). The plasmid pTF636 contains the sequence from −636 to +121 bp and harbors, in addition, a distal enhancer containing a NFκB and two AP-1 consensus sequences. Stimulation with thrombin increased luciferase activity of pTF636 by about fourfold, whereas pTF111-dependent luciferase activity was only elevated by about twofold, suggesting that

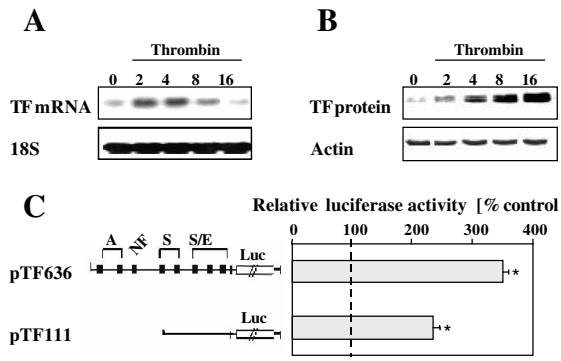

FIG. 1. Thrombin stimulates TF expression and promoter activity in PASMC. PASMC were stimulated with thrombin (3 U/ml) for different time points (in hours). (**A**) TF mRNA levels were evaluated by Northern blot analysis using a cDNA probe for human TF or for 18S to determine loading efficiency. These blots are representative of three independent experiments. (**B**) TF protein levels were determined by Western blot analysis using an antibody against human TF or against actin. These blots are representative of three independent experiments. (**C**) rSMC were transfected with the human TF promoter luciferase constructs pTF636 and pTF111. After 16 h of serum starvation, cells were stimulated for 8 h with thrombin (3 U/ml). In each experiment, the luciferase activity of the control vector measured under unstimulated conditions was set equal to 100% (represented by the dotted line). Values ± SD represent the % induction by thrombin of luciferase activity of three independent experiments. *$p < 0.05$ versus control (unstimulated). Abbreviations: A, AP-1; NF, NFκB; S, SP-1; E, Egr-1.

pTF636 contains thrombin-sensitive DNA binding consensus sequences that are not present in pTF111.

ROS production in response to thrombin is modulated by expression of Rac mutants

To investigate the role of Rac in thrombin-stimulated ROS production, PASMC were transfected with expression vectors encoding constitutively active racG12V (V12) or dominant-negative racT17N (N17) or with control vector (Ctr) and stimulated with thrombin for 2 h. Thereafter, generation of ROS was evaluated by DCF fluorescence (Fig. 2). Treatment with thrombin significantly enhanced levels of ROS in PASMC. Overexpression of the constitutively active mutant RacG12V further increased ROS production, whereas expression of RacT17N prevented thrombin-stimulated ROS generation, suggesting an important role of Rac in controlling ROS production in response to thrombin in PASMC.

Rac mutants modulate thrombin-induced TF expression and procoagulant activity in PASMC

To evaluate the role of Rac in thrombin-stimulated TF expression, human PASMC were transfected with expression vectors encoding constitutively active racG12V (V12) or dominant-negative racT17N (N17) or with control vector (Ctr) and stimulated with thrombin for 4 h for Northern blot analysis and for 16 h for Western blot analysis. In control

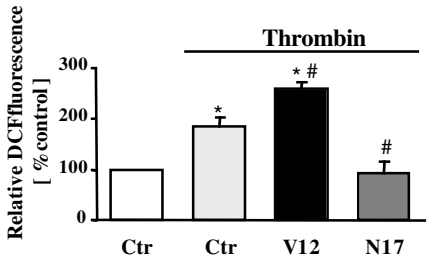

FIG. 2. Rac modulates thrombin-stimulated production of ROS in PASMC. PASMC were transfected with control vector (Ctr) or expression vectors for either a constitutively active (V12) or a dominant-negative (N17) form of rac and exposed to thrombin (3 U/ml) for 2 h. Cells were then washed with HBSS and incubated in the dark with CM-H_2DCFDA (8.5 μ*M*) for 5 min at 37°C. Thereafter, cells were washed with HBSS and fluorescence was monitored in a microplate reader using 480-nm excitation and 540-nm emission wavelength. DCF fluorescence was standardized to the number of viable cells using the Alamar Blue test. $n = 3$; *$p < 0.05$ versus Ctr (unstimulated); #$p < 0.05$ versus Ctr (thrombin-stimulated).

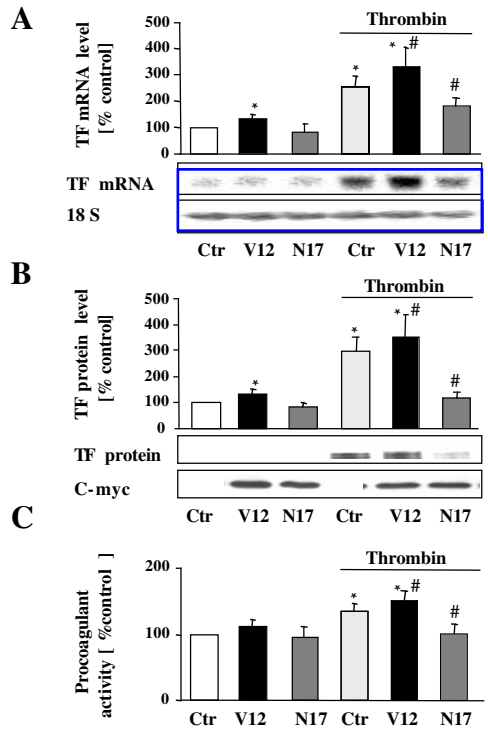

FIG. 3. Rac modulates thrombin-induced TF expression and activity in PASMC. PASMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active racG12V (V12) or the dominant-negative racT17N (N17). (**A**) Transfected PASMC were stimulated with thrombin (3 U/ml) for 4 h. Northern blot analyses were performed with a human TF cDNA or 18S cDNA probe. $n = 3$; *$p < 0.05$ versus Ctr (unstimulated); #$p < 0.05$ versus Ctr (thrombin-stimulated). (**B**) TF protein levels were determined in transfected PASMC stimulated for 16 h with thrombin (3 U/ml) by Western blot analysis using an antibody against human TF. The expression of the Rac constructs was controlled in the Western analysis with an antibody against the c-myc epitope. $n = 3$; *$p < 0.05$ versus Ctr (unstimulated); #$p < 0.05$ versus Ctr (thrombin-stimulated). (**C**) Surface procoagulant activity was investigated in human PASMC transfected with control vector (Ctr) or expression vectors encoding racG12V (V12) or racT17N (N17) and exposed to thrombin (3 U/ml) for 8 h. The formation of thrombin was evaluated using a chromogenic assay. $n = 3$; *$p < 0.05$ versus Ctr (unstimulated); #$p < 0.05$ versus Ctr (thrombin-stimulated).

cells, stimulation with thrombin resulted in a 2.8-fold increase in the TF mRNA levels (Fig. 3A). This response was further enhanced in RacG12V-expressing cells, but was significantly diminished in RacT17N-expressing PASMC. Similarly, TF protein levels were enhanced by threefold in control cells after thrombin stimulation, and were further elevated in RacG12V-expressing cells (Fig. 3B). By contrast, in RacT17N-expressing PASMC, TF protein levels were abrogated to levels similar to those from the unstimulated controls.

As TF protein levels in smooth muscle cells do not necessarily reflect the active TF, we evaluated the role of Rac in modulating surface procoagulant activity derived from PASMC under control conditions and in the presence of thrombin using a chromogenic assay (Fig. 3C). PASMC were transfected with racG12V or racT17N expression vectors or with control vector and exposed to thrombin for 8 h. Thrombin treatment resulted in a significant increase in surface procoagulant activity. In the presence of RacG12V, procoagulant activity was enhanced under control conditions and further potentiated in the presence of thrombin. In contrast, transfection of RacT17N abrogated thrombin-stimulated surface procoagulant activity. As addition of an inhibitory antibody against human TF inhibits basal, as well as thrombin-stimulated procoagulant activity as we have shown previously (21), these data suggest that Rac is involved in regulating TF expression, as well as TF activity, in response to thrombin in PASMC.

Rac modulates TF promoter activity: role of the NFκB pathway

To investigate the effect of Rac on TF promoter activity, reporter gene experiments were performed using the two TF promoter constructs pTF111 and pTF636. Cotransfection of the RacG12V mutant with pTF636 resulted in ~2.8-fold increased luciferase activity compared with control cells, whereas in RacT17N-expressing cells pTF636-derived luciferase activity was abrogated (Fig. 4A). pTF111-mediated luciferase activity was increased by only 1.5-fold in RacG12V-expressing cells and was not significantly decreased in the presence of RacT17N, suggesting that pTF636 contains DNA binding factor sequences sensitive to Rac that are not present in pTF111.

As the pTF636 construct, but not the pTF111 construct, contains a consensus sequence for the redox-sensitive transcription factor NFκB, we determined the role of this transcription factor in Rac-regulated human TF promoter activity.

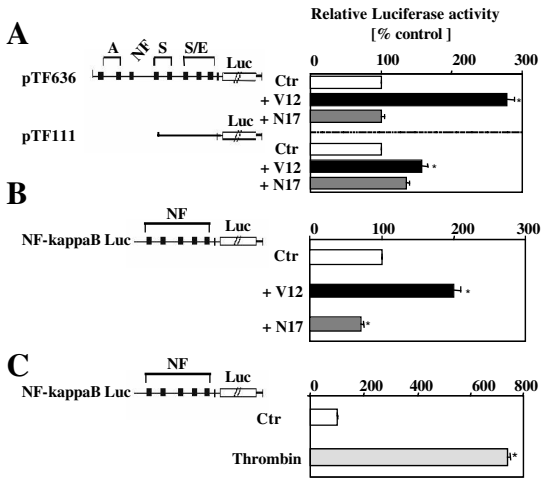

FIG. 4. Rac modulates TF promoter activity and NFκB-mediated transcriptional activity. (**A**) rSMC were cotransfected with expression vectors encoding constitutively active racG12V (V12) or dominant-negative racT17N (N17) or control vector (Ctr) and the human TF promoter luciferase constructs pTF636 or pTF111. Luciferase activity was measured 24 h after transfection. In each experiment, the luciferase activity determined in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments. $n = 3$; $*p < 0.05$ versus Ctr. (**B**) rSMC were cotransfected with expression vectors encoding constitutively active racG12V (V12) or dominant-negative racT17N (N17) or control vector (Ctr) and a luciferase construct containing five NFκB elements (NFkappaB-Luc) in front of the SV40 promoter. Luciferase activity was measured 24 h after transfection. In each experiment, the luciferase activity measured in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments. $n = 3$; $*p < 0.05$ versus Ctr. (**C**) rSMC were transfected with the NFkappaB-Luc reporter gene. Cells were then serum-deprived for 16 h and stimulated with thrombin for 8 h. In each experiment, the luciferase activity measured under unstimulated conditions was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments. $n = 3$; $*p < 0.05$ versus Ctr. Abbreviations: A, AP-1; NF, NFκB; S, SP-1; E, Egr-1.

First, we evaluated whether Rac is able to modulate NFκB-mediated transcriptional activity. To this date, a luciferase construct containing five NFκB consensus sites in front of the SV40 promoter was cotransfected with the mutant rac vectors (Fig. 4B). In the presence of active RacG12V, luciferase activity was significantly enhanced by 1.8-fold, whereas expression of dominant-negative RacT17N resulted in a significant decrease in reporter gene activity compared with control cells. Moreover, exposure to thrombin for 8 h resulted in about sevenfold increased NFκB-dependent luciferase activity (Fig. 4C). These findings demonstrate that thrombin and Rac are able to activate NFκB transcriptional activity in smooth muscle cells.

To evaluate whether NFκB contributes to TF promoter activity, cells were transfected with expression vectors encoding for the NFκB subunits p50 and p65 together with the TF promoter constructs pTF636 or pTF111, respectively. Coexpression of p50/p65 and pTF636 resulted in ~3.5-fold increased luciferase activity (Fig. 5A). Transfection of p50/p65, however, did not significantly influence reporter gene activity of the minimal TF promoter construct pTF111.

To evaluate whether Rac acts on the TF promoter via the NFκB pathway, cells were transfected with the racG12V expression vector and vectors encoding for either dominant-negative IκB (IKdn) or IκBK (IKKdn) as well as with pTF636 or pTF111 (Fig. 5B). RacG12V-stimulated luciferase activity of pTF636 was completely abolished in the presence of dominant-negative IκB or IκBK. In contrast, pTF111-dependent luciferase activity in RacG12V-expressing cells was not significantly reduced by dominant-negative IκB or IκBK. These findings indicate that the NFκB pathway is involved in thrombin-induced, Rac-dependent TF promoter activity.

DISCUSSION

In this study, we demonstrated that the GTPase Rac is involved in TF expression and activity in response to thrombin in PASMC, and that activation of the NFκB pathway largely contributes to Rac-dependent TF expression because (a) constitutively active and dominant-negative Rac mutants increased or decreased, respectively, thrombin-induced TF expression and TF-dependent procoagulant activity, (b) thrombin and Rac were able to activate NFκB-dependent transcriptional activity,

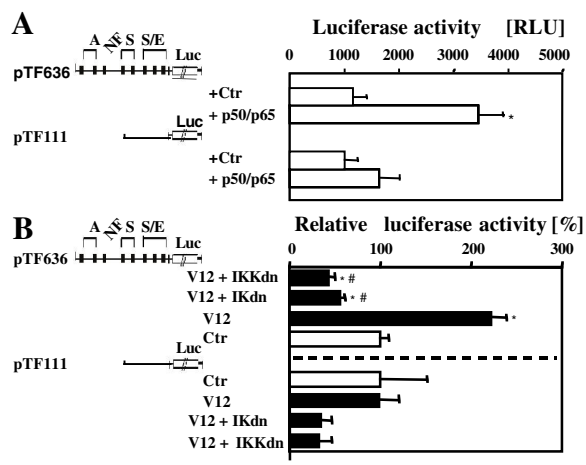

FIG. 5. The NFκB pathway is involved in Rac-dependent TF promoter activity. (**A**) rSMC were cotransfected with expression vectors encoding the NFκB subunits p50 and p65 or with control vector (Ctr) and the human TF promoter luciferase constructs pTF636 or pTF111. Luciferase activity was measured 24 h after transfection. $n = 3$; $*p < 0.05$ versus Ctr. (**B**) rSMC were cotransfected with the racG12V expression vector or control vector (pcDNA3) and the pTF636 or pTF111 human TF promoter luciferase constructs and expression vectors encoding for dominant-negative IκB (IKdn) or IκBK (IKKdn). Luciferase activity was measured 24 h after transfection. $n = 3$; $*p < 0.05$ versus Ctr (pcDNA3); $\#p < 0.05$ versus RacG12V transfected cells. Abbreviations: A, AP-1; NF, NFκB; S, SP-1; E, Egr-1.

(c) overexpression of NFκB enhanced TF promoter activity, and (d) inhibition of the NFκB pathway prevented Rac-dependent TF promoter activity.

Rac as mediator of cellular signaling processes

Pulmonary vascular remodeling is a common complication of pulmonary hypertension and is frequently associated with *in situ* thrombosis and increased procoagulant activity (11, 14). Thrombin and TF have been shown to interact directly with the vascular wall and to activate signaling pathways and gene expression, thus being key players in promoting vascular remodeling processes (19, 22). Indeed, enhanced levels of thrombin and TF have been associated with pulmonary hypertension and pulmonary vascular remodeling (11, 14). Thrombin-induced up-regulation of TF expression and TF-dependent procoagulant activity in PASMC may thus lead via the formation of thrombin to a prothrombotic state, thereby further promoting these remodeling processes.

In this study, we showed that thrombin-induced TF expression and activity are dependent on the GTPase Rac in PASMC because expression of a constitutively active Rac mutant increased TF expression and procoagulant activity in PASMC by thrombin, whereas this response was abolished in the presence of a dominant-negative mutant of Rac. Similarly, in aortic smooth muscle cells, RacT17N inhibited thrombin-induced TF mRNA expression and procoagulant activity (21). Moreover, in endothelial cells, TF expression by thrombin was decreased by treatment with statins that prevent activation of the RhoGTPases Rho, Rac, and cdc42 (5), further supporting an important role of Rac in the control of TF expression and activity by thrombin. Rac has been shown to be important not only as a regulator of cytoskeletal organization, but also as activator of the NADPH oxidase (24, 26). Thrombin-stimulated ROS production was potentiated in RacG12V-expressing PASMC, but was diminished in the presence of dominant-negative RacT17N. This indicates that Rac controls TF expression and activity in PASMC in response to thrombin via a redox-sensitive pathway and supports previous findings in aortic smooth muscle cells (13).

TF expression is mediated by Rac and the NFκB pathway

NFκB is one of the major transcription factors shown to be regulated in a redox-dependent manner (29). Functional studies of the human TF promoter identified a proximal enhancer containing three overlapping Egr-1 and Sp1 sites and a distal enhancer containing a NFκB and two AP-1 sites (15, 16). In our studies, thrombin-stimulated luciferase activity of pTF636 containing the distal and proximal enhancer was significantly higher than that of pTF111, which contained only the proximal enhancer, suggesting that the distal enhancer, is required for maximal TF promoter activity in response to thrombin. In contrast, in smooth muscle cells stimulated with activated platelets or in endothelial cells activated by vascular endothelial growth factor, luciferase activity of the pTF111 construct was not significantly different from the activity of promoter constructs containing also the distal enhancer (7, 17). However, in endothelial cells, pTF111-mediated luciferase activity in response to lipopolysaccharides, tumor necrosis factor-α, and interleukin-1β was significantly reduced compared with the luciferase activity of constructs containing also the distal enhancer (10, 20, 21). The lipopolysaccharide and cytokine-stimulated response in endothelial cells and in monocytes has been shown to require constitutive AP-1 DNA binding and inducible activation of the NFκB pathway (10, 21). Overexpression of the NFκB subunits p50 and p65 increased pTF636-, but not pTF111-derived luciferase activity, and thrombin potently stimulated NFκB-dependent luciferase activity, indicating that activation of NFκB by thrombin is able to induce TF expression in PASMC. Expression of active RacG12V further increased NFκB-dependent luciferase activity, similar to results obtained in fibroblasts or HepG2 cells (9, 26). Expression of RacG12V also preferentially activated pTF636-mediated luciferase activity which was completely abrogated in the presence of dominant-negative mutants of IκB or IκBK, consistent with the observation that Rac-induced transcriptional activation of NFκB involved phosphorylation of IκBα and translocation of NFκB to the nucleus (23). Together with the findings that active Rac and thrombin stimulate NFκB-dependent transcriptional activity, these data indicate that Rac increases pTF636-mediated luciferase activity primarily via a NFκB-dependent pathway. However, we cannot completely rule out a role for AP-1 in Rac-induced TF expression. Possibly, the interaction of AP-1 and NFκB is also required for maximal induction of TF in PASMC similar to the situation in endothelial cells and monocytes (15). Moreover, the modest induction of pTF111-derived luciferase activity by thrombin and RacG12V suggests that additional transcription factors, such as Sp1 and/or Egr-1, may also contribute to the regulation of TF expression by thrombin and Rac in PASMC.

In summary, this study shows that thrombin is able to increase TF expression and activity in PASMC via Rac involving activation of the NFκB pathway. As enhanced NFκB activity has been observed in patients with pulmonary hypertension (3) and statins that inhibit Rac and Rho kinases decrease procoagulant activity in the vascular wall (6), Rac-dependent activation of the NFκB pathway may be a critical element promoting thrombin-induced TF activity and thus *in situ* thrombosis and thrombogenictiy of the vascular wall in pulmonary hypertension.

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ABBREVIATIONS

AP-1, activator protein 1; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl

ester; DCF, 2′,7′-dichlorodihydrofluorescein; Egr-1, early growth response 1; HBSS, Hanks' balanced salt solution; IκB, inhibitory κB protein; IκBK, IκB kinase; NFκB, nuclear factor-kappa B; PASMC, pulmonary artery smooth muscle cells; Rac, ras-related C3 botulinum toxin substrate; ROS, reactive oxygen species; rSMC, rat smooth muscle cells; TF, tissue factor.

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