NADPH Oxidase Mediates Tissue Factor–Dependent Surface Procoagulant Activity by Thrombin in Human Vascular Smooth Muscle Cells

Olaf Herkert, PhD; Isabel Diebold; Ralf P. Brandes, MD; John Hess, MD; Rudi Busse, MD, PhD; Agnes Görlach, MD

Background—Tissue factor (TF) initiates the extrinsic coagulation cascade leading to thrombin formation. Thrombin induces TF mRNA in vascular smooth muscle cells (VSMCs), thereby contributing to the prolonged procoagulant activity and enhanced thrombogenicity at sites of vascular injury. However, the signaling mechanisms mediating this thrombogenic cycle are unclear. Characteristically, vascular injury promotes the generation of reactive oxygen species (ROS). Because ROS exert signaling functions, we investigated whether the NADPH oxidase, an important source of ROS in VSMCs, contributes to upregulation of TF by thrombin.

Methods and Results—Thrombin not only stimulated TF mRNA expression, but also TF-dependent surface procoagulant activity in cultured human VSMCs. This response was attenuated by antioxidants; the flavin inhibitor diphenyleneiodonium, Clostridium difficile toxin B, which inhibits Rho GTPases, p22phox antisense oligonucleotides, or the dominant-negative RacT17N mutant. Inhibitors of p38 MAP kinase and phosphatidylinositol (PI) 3-kinase also prevented thrombin-stimulated TF mRNA expression. Furthermore, thrombin stimulated the phosphorylation of the PI 3-kinase target protein kinase B/Akt in a redox-sensitive and NADPH oxidase–dependent manner.

Conclusion—These findings indicate that the NADPH oxidase is essentially involved in the redox-sensitive induction of TF mRNA expression and surface procoagulant activity by thrombin. This response is mediated by NADPH oxidase–dependent activation of p38 MAP kinase and the PI 3-kinase/protein kinase B/Akt pathway. Given that active TF promotes thrombin formation, the NADPH oxidase may play a crucial role in perpetuating the thrombogenic cycle in the injured vessel wall. (Circulation. 2002;105:2030-2036.)

Key Words: free radicals | signal transduction | thrombosis | atherosclerosis

Prolonged procoagulant activity induced by vascular injury has been associated with restenosis after balloon dilatation or the development of atherosclerosis. The key player in regulating the hemostatic and thrombotic response to injury is the 47-kDa single-transmembrane glycoprotein tissue factor (TF), which is upregulated in medial vascular smooth muscle cells (VSMCs) of injured arteries and in atherosclerotic plaques.

TF acts as the primary link between vascular cells and the hemostatic system by binding factor VII/VIIa with high affinity, resulting in the activation of the extrinsic pathway of the blood coagulation cascade. The TF/VIIa complex promotes the activation of factor IX and factor X with subsequent thrombin formation. Thrombin, which is generated in abundance at sites of vascular injury, plays a central role in the pathogenesis of atherosclerotic and thrombotic diseases. Thrombin not only catalyzes the conversion of fibrinogen to fibrin, thus triggering rapid fibrin deposition and clot formation, but also induces the expression of different chemotactic and procoagulant factors in vascular cells.

Intriguingly, thrombin has been shown to elevate TF mRNA levels in VSMCs and TF upregulation after balloon angioplasty in rabbit femoral and porcine coronary arteries was attenuated by the thrombin inhibitor hirudin. Thrombin also increased TF surface activity in cultured VSMCs. Thus, this protein, which is produced during activation of the coagulation cascade, is capable of rapidly and markedly inducing and activating TF, the main initiator of this cascade. This raises the possibility that a positive feedback system exists whereby thrombin can help propagate the clot by inducing TF expression in VSMCs, thus increasing the thrombogenicity of the injured vascular wall. In spite of the potential clinical importance of this thrombogenic cycle, the signaling mechanisms mediating the TF-dependent surface procoagulant activity in response to thrombin are largely unknown.

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From the Institut für Kardiovaskuläre Physiologie (O.H., I.D., R.P.B., R.B., A.G.), Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, and Klinik für Kinderkardiologie und Angeborene Herzfehler (J.H., A.G.), Deutsches Herzzentrum München des Freistaates Bayern, Klinik an der Technischen Universität München, Germany.

Correspondence to Agnes Görlach, MD, Experimentelle Kinderkardiologie, Klinik für Kinderkardiologie und angeborene Herzfehler, Deutsches Herzzentrum München des Freistaates Bayern, Klinik an der Technischen Universität München, Lazaretstrasse 36, D-80636 München, Germany. E-mail goerlach@dhm.mhn.de

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Although initially considered to lead to tissue damage, reactive oxygen species (ROS) have recently been acknowledged to exert a prominent role as signaling molecules in the vascular wall connecting receptor-mediated agonist stimulation and modulation of gene expression. Characteristically, increased ROS production is observed at sites of vascular injury and has been related to the development of restenosis/atherosclerosis. Moreover, the enhanced generation of ROS promotes a procoagulant state.

In VSMCs, an NADPH oxidase has been identified to contribute considerably to agonist-induced ROS formation. This complex enzyme system is composed of several subunits including p22phox, p47phox, the GTPase Rac, and possibly the recently identified Nox1. The importance of p22phox in agonist-induced ROS production and gene expression in VSMCs in response to angiotensin II, tumor necrosis factor-α, and activated platelets as well as thrombin has been demonstrated using antisense oligonucleotides, antisense cDNA, or neutralizing antibodies. Thus, this enzyme appears to be an attractive candidate to promote thrombogenicity of the injured vessel wall.

Here, we studied the role of ROS and the NADPH oxidase as well as of redox-sensitive signaling cascades in the regulation of TF mRNA expression and TF-dependent surface procoagulant activity in response to thrombin in cultured human VSMCs.

**Methods**

**Reagents**
Wortmannin, LY294002, PD98059, SB202190, SB203580, and SB202025 were from CalBiochem. FCS was from Biochrom, and deoxyctydine 5′-α-thio-P-triphosphate (3000 Ci/mm) was from Hartmann Analytic. Human α-thrombin (thrombin-specific clotting activity 3261 U/mg) was from Hemochrom Diagnostika. All other chemicals were from Sigma.

**Cell Culture**
Human aortic smooth muscle cells (VSMCs) were from Clonetics and cultured as recommended. VSMCs (passages 3 to 13) were serum deprived for 24 to 48 hours before stimulation.

**Preparation of Rat Aortae**
The adventitia and intima from rat aortae were mechanically removed, and the smooth muscle cell layers were washed twice and incubated in MCDB131 containing 0.1% BSA for 30 minutes before stimulation.

**Plasmids, Oligonucleotides, and Transfections**
pexVrac and RacN17pc-myc (provided by Dr R.H. Cool, University of Groningen, the Netherlands) were digested with EcoRI, and the myc-tagged Rac-containing fragments were inserted into the vector pcDNA3.1 resulting in pcRacWT and pcRacT17N. Transfection of plasmids or phosphorothioate-modified p22phox antisense or scrambled oligonucleotides was performed as described. Transfection efficiency of the plasmids was controlled by Western blot analysis using an antibody against the c-myc epitope (Santa Cruz).

**Northern Blot Analysis**
Total RNA from human VSMCs was subjected to Northern blot analysis, and hybridizations were performed with a 32P-labeled human TF cDNA fragment (provided by Drs A. Bierhaus and P. Nawroth, University of Tubingen, Germany) as described.
determined in human VSMCs stimulated with thrombin (2 U/mL) for 6 hours. Compared with control cells, thrombin-treated VSMCs showed a significantly elevated procoagulant activity (Figure 1B). Addition of a neutralizing antibody directed against TF diminished procoagulant activity to values even lower than control levels, suggesting that basal as well as thrombin-stimulated surface procoagulant activity is dependent on the availability of membrane-bound TF.

Role of ROS on TF mRNA Expression and Surface Procoagulant Activity in Response to Thrombin

To investigate whether ROS are involved in thrombin-induced TF mRNA expression and TF-dependent surface procoagulant activity (Figure 1B). Addition of a neutralizing antibody directed against TF diminished procoagulant activity to values even lower than control levels, suggesting that basal as well as thrombin-stimulated surface procoagulant activity is dependent on the availability of membrane-bound TF.

FIGURE 2. ROS are involved in thrombin-induced TF mRNA expression and TF-dependent surface procoagulant activity. A, Human VSMCs were incubated with H2O2 (100 μmol/L) or treated with vitamin C (VitC, 100 μmol/L) or NAC (10 mmol/L) for 30 minutes before stimulation with thrombin (2 U/mL, 4 hours). Cumulative data represent TF mRNA as means±SEM of 5 different Northern blots. *P<0.05 vs control. #P<0.05 vs thrombin alone. B, TF-dependent surface procoagulant activity of human VSMCs was determined under control conditions (control), in the presence of vitamin C (VitC; 100 μmol/L), or after stimulation with thrombin (2 U/mL) for 6 hours in the absence or presence of vitamin C (VitC+thrombin) added 20 minutes before stimulation. Similar observations were made in 2 additional experiments.

FIGURE 3. Inhibition of flavin-containing enzymes or Rho GTPases attenuates thrombin-induced TF mRNA expression and TF-dependent surface procoagulant activity. A, Human VSMCs were treated with diphenyleneiodonium (DPI; 10 μmol/L), the C difficile toxin B (TxB, 100 pg/mL), allopurinol (Allo, 100 μmol/L), or indomethacin (Indo, 100 μmol/L) for 30 minutes before stimulation with thrombin (2 U/mL, 4 hours). Cumulative data represent TF mRNA as means±SEM of 5 different Northern blots. *P<0.05 vs control. #P<0.05 vs thrombin alone. B, TF-dependent surface procoagulant activity was determined under control conditions (control), in the presence of DPI (10 mol/L), or after stimulation with thrombin (2 U/mL) for 6 hours in the absence or presence of DPI added 20 minutes before stimulation. Similar observations were made in 2 additional experiments.

Effects of p22phox Antisense Oligonucleotides and RacT17N on TF mRNA Expression and Surface Procoagulant Activity in Response to Thrombin

To further delineate the role of the NADPH oxidase in TF signaling, human VSMCs were transfected with antisense oligonucleotides directed against the subunit p22phox. This
approach has been shown previously to specifically inhibit the NADPH oxidase.\textsuperscript{20–22} TF mRNA expression on stimulation with thrombin was significantly lower in p22phox antisense–transfected cells than in cells transfected with unrelated p22phox scrambled oligonucleotides (Figure 4A). Similarly, thrombin-induced TF-dependent surface procoagulant activity was inhibited in p22phox antisense–transfected cells compared with cells transfected with p22phox scrambled oligonucleotides (Figure 4B). Because the GTPase Rac is an essential component of the NADPH oxidase, human VSMCs were transfected with plasmids expressing either wild-type Rac (pcRacWT) or a dominant-negative Rac mutant (pcRacT17N), and stimulated with thrombin. Both TF mRNA expression and TF-dependent surface procoagulant activity were markedly reduced in cells transfected with pcRacT17N compared with cells transfected with pcRacWT (Figure 4, C and D).

**Effects of Different Kinase Inhibitors on TF mRNA Expression in Response to Thrombin**

To study the pathway(s) involved in thrombin-induced TF mRNA expression, human VSMCs were treated with PD98059 (50 \(\mu\)mol/L), an inhibitor of MEK1 and thus phosphorylation of ERK1/2; with SB203580 (20 \(\mu\)mol/L), a p38 MAP kinase inhibitor; or with the phosphatidylinositol (PI) 3-kinase inhibitor wortmannin (20 nmol/L) for 30 minutes before stimulation with thrombin (2 U/mL) for 4 hours. SB203580 as well as wortmannin significantly inhibited thrombin-induced TF mRNA expression, whereas PD98059 was only slightly effective (Figure 5). Moreover, 2 other p38

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**Figure 4.** Thrombin-induced TF mRNA expression and TF-dependent surface procoagulant activity is attenuated by inhibition of NADPH oxidase. A, Human VSMCs were transfected with p22phox scrambled (SC) or antisense (AS) oligonucleotides directed against the small subunit p22phox of the NADPH oxidase and stimulated with thrombin (2 U/mL, 4 hours). Northern blot analyses were performed using a human TF cDNA or ribosomal 18S probe. *\(P<0.05\) vs scrambled; \#\(P<0.05\) vs scrambled/thrombin; \(n=5\). B, TF-dependent surface procoagulant activity of human VSMCs transfected with p22phox scrambled or antisense oligonucleotides was determined under control conditions or after stimulation with thrombin (2 U/mL) for 6 hours. Similar observations were made in 3 additional experiments. C, Human VSMCs were transfected with the expression vectors pcRacWT (WT) or pcRacT17N (T17N) and stimulated with thrombin (2 U/mL) for 4 hours. Upper panels show representative (\(n=3\)) Northern blot analyses performed with a human TF cDNA or ribosomal 18S probe. Lower panel shows corresponding Western blot analysis using an antibody against the c-myc epitope. D, TF-dependent surface procoagulant activity of human VSMCs transfected with pcRacWT or pcRacT17N was determined under control conditions or after stimulation with thrombin (2 U/mL) for 6 hours. Similar observations were made in 2 additional experiments.
MAP kinase inhibitors, SB202190 and SB220025, as well as the PI 3-kinase inhibitor LY294002, showed similar effects (data not shown).

**Effects of the C difficile Toxin B on Phosphorylation of Akt, p38 MAP Kinase, and ERK1/2 in Response to Thrombin**

Stimulation of VSMCs with thrombin (2 U/mL) for 15 minutes resulted in the activation of p38 MAP kinase, ERK1/2, and the PI 3-kinase-target protein kinase B (Akt) as demonstrated by Western blot analysis (Figure 6). Pretreatment with the C difficile toxin B dose-dependently inhibited phosphorylation of Akt and p38 MAP kinase, but only slightly affected ERK1/2 phosphorylation in response to stimulation with thrombin, suggesting the involvement of Rho GTPases such as Rac in activation of Akt and p38 MAP kinase.

**Effects of Antioxidants and p22phox Antisense Oligonucleotides on Phosphorylation of Akt in Response to Thrombin**

Because we have previously shown that activation of p38 MAP kinase, but not of ERK1/2, by thrombin is specifically mediated by p22phox,21 we investigated whether ROS and the NADPH oxidase are also involved in thrombin-induced activation of Akt. Preincubation of human VSMCs with vitamin C (100 μmol/L), NAC (10 mmol/L), or DPI (10 μmol/L) for 30 minutes decreased phosphorylation of Akt in response to thrombin (Figure 7A). Moreover, transfection of p22phox antisense oligonucleotides attenuated thrombin-stimulated phosphorylation of Akt, implicating ROS and the NADPH oxidase in this response (Figure 7B).

**Discussion**

Increased thrombin formation and TF expression are observed at sites of vascular injury.1,4-7 The finding that thrombin itself is an agonist for TF induction in vitro and in vivo in VSMCs8–11 points toward an important role that these cells play in the perpetuation of the thrombogenic state of the injured vascular wall. In addition, the generation of ROS, which act as signaling molecules in the vascular system, is enhanced in response to injury and has been associated with a procoagulant state and the progression of atherosclerotic disease.12–14
The results of this study now provide a link between these observations. We demonstrate that (1) thrombin enhances TF mRNA expression and TF-dependent surface procoagulant activity in human VSMCs; (2) this response is redox-sensitive; and (3) the NADPH oxidase, a ROS-generating enzyme known to be sensitive to thrombin, is crucially involved in this response.

The importance of an NADPH oxidase–dependent signaling cascade leading to upregulation of TF expression and surface procoagulant activity was supported by the findings that (1) the flavin inhibitor DPI, known to inhibit the NADPH oxidase, diminished TF expression and surface activity; (2) the C difficile toxin B, which inhibits Rho GTPases including the NADPH oxidase regulator Rac,24 decreased TF mRNA expression; and (3) most importantly, p22phox antisense oligonucleotides as well as a dominant-negative Rac mutant abrogated thrombin-induced TF mRNA expression and surface activity. These observations show for the first time that the NADPH oxidase not only exerts a prominent role in the regulation of expression of TF, the key determinant of thrombogenic responses, but also is functionally involved in the modulation of TF-dependent cell surface procoagulant activity. This is even more critical because the identification of TF protein or mRNA is not inevitably correlated with the presence of TF activity on the cell surface.25

The NADPH oxidase has been identified as an important source of ROS in VSMCs,15 which is sensitive to stimulation with agonists, including thrombin.21,22,26 We have previously demonstrated that p22phox antisense cDNA and p22phox antisense oligonucleotides effectively downregulate p22phox mRNA expression and prevent thrombin-stimulated ROS generation.20–22 Consistently, inhibition of the NADPH oxidase subunit p47phox also diminished thrombin-stimulated ROS production,26 and a dominant-negative Rac mutant prevented growth factor–induced ROS production in fibroblasts.27 Moreover, thrombin induced membrane translocation of Rac in vascular smooth muscle cells, suggesting that this GTPase is an essential component of the NADPH oxidase in vascular smooth muscle cells.26

ROS-dependent signaling cascades are mediated by activation of different kinases. The NADPH oxidase exerts a prominent function in the activation of a specific signaling cascade in response to thrombin, involving the phosphorylation of p38 MAP kinase but not of ERK1/2.21,22 Because inhibition of p38 MAP kinase but not of ERK1/2 significantly reduces thrombin-induced TF mRNA expression, TF can be considered a novel target gene of NADPH oxidase-dependent activation of p38 MAP kinase by thrombin.

Furthermore, thrombin-induced TF mRNA expression is abrogated by inhibitors of PI 3-kinase. These reagents also prevent thrombin-induced phosphorylation of Akt in VSMCs (data not shown). Using antioxidants, DPI, the C difficile toxin B, and p22phox antisense oligonucleotides, we demonstrate that activation of Akt by thrombin is redox-sensitive and involves the NADPH oxidase. Consistently, antioxidants and DPI diminish Akt phosphorylation in VSMCs in response to angiotensin II, another potent activator of the NADPH oxidase,28 further supporting the idea that Akt is specifically activated via the NADPH oxidase. Similarly, in rat basophilic leukemic cells, C difficile toxin B has been shown to decrease phosphorylation of Akt but not of ERK1/2 in response to Fcε receptor stimulation,29 and in murine hematopoietic BaF3 cells, Rac was essentially involved in the activation of Akt and p38 MAP kinase.30 Because PI 3-kinase inhibitors attenuate TF mRNA expression and Akt phosphorylation in response to thrombin, the NADPH oxidase–mediated activation of the PI 3-kinase/Akt pathway may thus provide a novel mechanism contributing to thrombin-induced TF expression and surface procoagulant activity.

In summary, we demonstrate that the NADPH oxidase is a critical element regulating the redox-sensitive TF mRNA expression and TF-dependent surface procoagulant activity induced by thrombin in human VSMCs. This response involves the activation of p38 MAP kinase and PI 3-kinase. Moreover, phosphorylation of the PI 3-kinase target Akt by thrombin provides a novel redox-sensitive pathway mediated by the NADPH oxidase, which may also contribute to TF induction by thrombin. Because active TF leads to the generation of thrombin, our findings point toward a pivotal role of the NADPH oxidase in promoting the thrombogenic cycle leading to enhanced thrombogenicity of the injured vascular wall.

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References

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