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Forum Review

Insights into the Redox Control of Blood Coagulation: Role of Vascular NADPH Oxidase-Derived Reactive Oxygen Species in the Thrombogenic Cycle

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ABSTRACT

Various cardiovascular diseases including thrombosis, atherosclerosis, (pulmonary) hypertension and diabetes, are associated with disturbed coagulation. Alterations in the vessel wall common to many cardiovascular disorders have been shown to initiate the activity of the coagulation system, but also to be the result of an abnormal coagulation system. The primary link between the coagulation and the vascular system appears to be tissue factor (TF), which is induced on the surface of vascular cells and initiates the extrinsic pathway of the blood coagulation cascade, leading to the formation of thrombin. Thrombin can also interact with the vascular wall via specific receptors and can increase vascular TF expression. Such a "thrombogenic cycle" may be essentially involved in the pathogenesis of cardiovascular disorders associated with an abnormal coagulation. Therefore, the identification of the signaling pathways regulating this cycle and each of its relevant connecting links is of fundamental importance for the understanding of these disorders and their putative therapeutic potential. Reactive oxygen species (ROS) and the ROS-generating NADPH oxidases have been shown to play important roles as signaling molecules in the vasculature. In this review, we summarize the data supporting a substantial role of ROS in promoting a thrombogenic cycle in the vascular system. *Antioxid. Redox Signal.* 6, 765–776.

INTRODUCTION

VARIOUS CARDIOVASCULAR DISORDERS such as thrombosis, atherosclerosis, and (pulmonary) hypertension, but also diseases that are characterized by disturbances of wound healing like diabetes, are frequently associated with alterations of blood coagulation (9, 45, 53, 77).

In recent years, increased understanding of major regulatory elements of coagulation has resulted in a better appreciation of the complex interactions between humoral factors of the coagulation and fibrinolytic systems, constituents of the blood stream (*e.g.,* platelets), and cells of the vascular wall, and thus the physiology and the mechanisms of disorders associated with a disturbed coagulation. Moreover, several clinical investigations have shown that the development of abnormalities in coagulation is caused by fundamental pathophysiological alterations inside the vessel wall and on its surface that represent the initiation point for, but also simultaneously the result of, changes in factors of the coagulation system (30, 45, 82).

The primary link for the coupling between both systems is tissue factor (TF), a single-transmembrane receptor induced on the surface of vascular cells that acts as the essential cofactor of the coagulation factors VII/VIIa, resulting in the initiation of the extrinsic pathway of the blood coagulation cascade (71). 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, has been suggested to play a role in the pathogenesis of atherosclerotic and thrombotic diseases (40, 65, 66). Various studies have proved an up-regulation of TF in medial vascular smooth muscle cells of injured arteries, as well as in

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atherosclerotic plaques, and characterized alterations in the vascular bioavailability of TF as a primary event for coagulation disorders (67, 68, 101). The coupling between the vascular and the coagulation system is clearly demonstrated by the findings that thrombin, which cleaves fibrinogen to fibrin resulting in the initiation of clot formation, also potently induces TF expression in the vascular wall (44, 82).

Simultaneously, the formation of thrombin is also the consequence of an increased bioavailability of TF on the surface of vascular cells (43, 65, 66). Such a "thrombogenic cycle" as a positive feedback system may be essentially involved in the pathogenesis of various cardiovascular disorders associated with an abnormal coagulation. Therefore, the identification of the underlying signaling pathways regulating this cycle and each of its relevant connecting links is of fundamental importance for understanding these disorders and their putative therapeutic potential.

Reactive oxygen species (ROS), which result from the incomplete reduction of oxygen and include superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals, were initially considered to lead to tissue damage (26). However, more recently, evidence has been accumulated that they exert a prominent role as signaling molecules and second messengers in the vascular wall connecting receptor-mediated agonist stimulation and modulation of gene expression (26, 55, 69, 102). Characteristically, increased ROS production is observed at sites of vascular injury and has been related to the development of restenosis and atherosclerosis, but also to (pulmonary) hypertension and other disorders associated with a prothrombotic state (8, 36, 37, 41, 106, 112), and enhanced generation of ROS has also been linked to a procoagulant state (42, 44, 87). Thus, ROS appear to be key players in promoting a procoagulant and thrombogenic state. In this review we will therefore summarize the data supporting a substantial role of ROS in promoting a thrombogenic cycle in the vascular system.

NADPH OXIDASES AND VASCULAR GENERATION OF ROS

Vascular ROS production has been associated with a number of systems, including the mitochondrial electron transport chain, lipoxygenases, cyclooxygenase, xanthine and glucose oxidase, NADPH oxidases, nitric oxide (NO) synthases, the cytochrome P450s, peroxidases and various hemoproteins (15, 26). However, NADPH oxidases that catalyze the one-electron reduction of oxygen using NADH or NADPH as electron donor, resulting in the formation of O_2^-, have now been identified as probably the most prominent sources of agonist-induced ROS generation in vascular cells, as well as in platelets (15, 54, 57). The structure and function of the NADPH oxidases were initially characterized in neutrophils where two membrane components, p22phox and gp91phox, comprise the cytochrome b558 (3). Other important components include the cytoplasmic subunits p40phox, p47phox, p67phox and the small GTP-binding protein Rac (ras-related C3 botulinum toxin substrate). When phagocytic cells are activated, the cytosolic subunits translocate to the cytochrome b558 at the membrane leading to the activation of the oxidase and the well characterized oxidative burst (3). Vascular NADPH oxidases differ from the neutrophil NADPH oxidase in several important aspects. The neutrophil oxidase releases large amounts of O_2^- in bursts, whereas the vascular NADPH oxidases continuously produce low levels of O_2^-. In addition to the ubiquitously expressed p22phox and Rac proteins, p47phox and p67phox also appear to be present in vascular cells (57). In contrast, gp91phox expression seems to be more restricted, because a functional gp91phox-containing NADPH oxidase has been found in endothelial cells, but not in smooth muscle cells (34), although recently small amounts of gp91phox have also been detected in adventitial fibroblasts and vascular smooth muscle cells from resistance arteries (103).

In the past few years, a family of gp91phox-like proteins, termed the nonphagocytic NADPH oxidase (NOX) proteins, has been discovered (5, 18, 97). Based on homologies with each other and their apparent evolution from an ancestral NOX, these are named NOX1, NOX2 (the former gp91phox), NOX3, NOX4, and NOX5 (7, 18, 57). Two related proteins, called dual oxidase 1 (DUOX1) and DUOX2, have NOX-homologous regions, as well as regions with peroxidase activity (56). As stated before, NOX2 is expressed mainly in phago-

TABLE 1. AGONISTS OF VASCULAR NADPH OXIDASES AND REDOX-SENSITIVE TF EXPRESSION

	NADPH oxidase activity	*Vascular TF expression*	*NADPH-derived ROS in TF expression*
Thrombin	+	+	+
Ang II	+	+	ND
Activated platelets	+	+	+
Aldehyde	+	+	ND
PDGF	+	+	+
TNF-α	+	+	ND
IGF-1	+	—	—
IL-1α	+	+	ND
bFGF	+	+	ND
VEGF	+	+	ND
TGF-β	+	+	+
Serum	+	+	ND
H_2O_2	+	+	ND
LDL, oxLDL	+	+	ND
Endothelin	+	ND	ND
Mechanical forces	+	+	ND
Histamine	+	ND	ND
Hyperoxia	+	ND	ND
Glucose	+	ND	ND
CMV	+	ND	ND
Lipopolysaccharide	+	+	ND
Ceramide	+	ND	ND
Arachidonic acid	+	—	—
Lysophosphatidylcholine	+	—	—
Phorbol esters	+	+	ND
Arsenite	+	—	—

Symbols: +, increase; —, no effect; ND, not determined.

cytes and endothelial cells, whereas NOX1 and NOX4 are expressed in endothelial and smooth muscle cells (7, 34, 57). The reasons for this apparently redundant expression of NOX isoforms in vascular cells are not clear to date. In atherosclerotic arteries, NOX4, but not NOX1, expression was enhanced in α-actin-positive cells in the plaque shoulder (94). On the other hand, in the injured carotid artery, NOX1 expression preceded the expression of NOX4, suggesting that NOX1 is more involved in growth-dependent processes, whereas NOX4 may contribute to differentiation (98). These variations may be related to the recent findings that NOX1 was localized in focal adhesions, whereas NOX4 was found in caveolae, suggesting that due to their localization in different signaling domains, the NOX proteins are regulated by different signaling pathways and may respond differentially to agonist stimulation (46). In addition, two proteins with homology to p47phox and p67phox, termed NOX organizer 1 and NOX activator 1, have been discovered and shown to regulate NOX1 activity (6, 17, 99). These newly identified proteins might also modulate enzyme activity in a tissue- and/or stimulus-specific fashion.

Although to date a variety of agonists have been identified to regulate NADPH oxidase activity in vascular cells (see Table 1 and Fig. 1), the mechanisms of NADPH oxidase activation have been best characterized in response to angiotensin II (Ang II), which elicits a biphasic oxidase activation involving multiple pathways (Fig. 2). The initial and critical steps in the activation of NADPH oxidase after challenging with Ang II are mediated by c-Src, which induces p47phox phosphorylation, probably by protein kinase C (PKC) and/or other serine kinases, followed by membrane translocation and association with p22phox (60, 105). Moreover, a delayed phase of activation seems to be mediated by c-Src-dependent transactivation of receptor tyrosine kinases, such as the epithelial growth factor (EGF) receptor leading to phosphatidylinositol 3-kinase (PI3K)-dependent activation and translocation of the GTPase Rac (91). As c-Src can be activated by H_2O_2, the dismutation product of O_2^-, amplification of the response to Ang II may occur resulting in the sustained stimulation of NADPH oxidases (91, 104, 105). In addition, the expression of several NADPH oxidase subunits has been shown to be regulated at the transcriptional and/or posttranscriptional level in the vessel wall (57, 58, 94, 105). Thus, NADPH oxidase-dependent ROS production in response to Ang II and other stimuli is regulated at the level of activation, as well as at the expression level (Figs. 1 and 2).

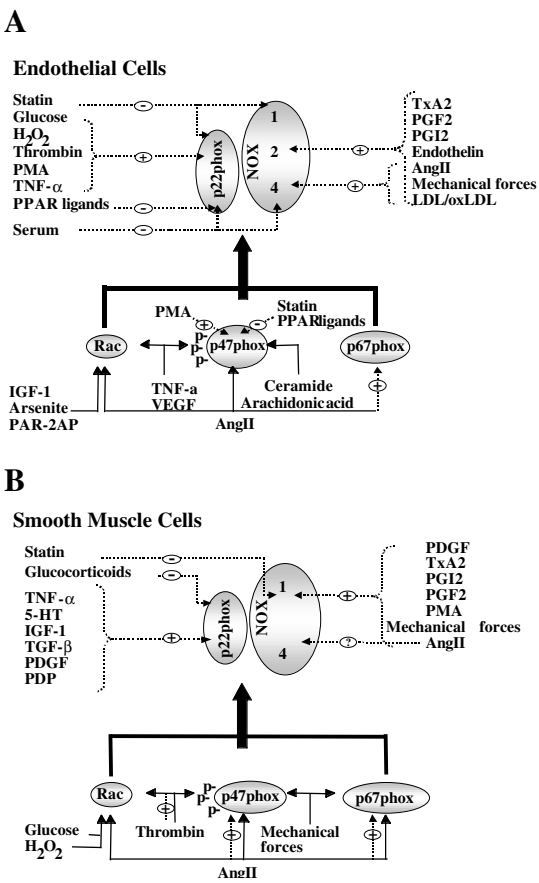

FIG. 1. NADPH oxidase expression and activation in the vasculature. ROS generation by vascular NADPH oxidases is regulated at the level of activation, as well as by regulation of the expression of NADPH oxidase subunits. The mechanisms of activation and the expression pattern of NADPH oxidase subunits differ between endothelial cells (**A**) and smooth muscle cells (**B**). Shown are agonists activating NADPH oxidases (**bottom**) and agonists regulating the expression levels of the different subunits (**top**). Symbols: (→), induces translocation; (- - - →), inhibits (−) or activates (+) the expression; (?), contradictory data.

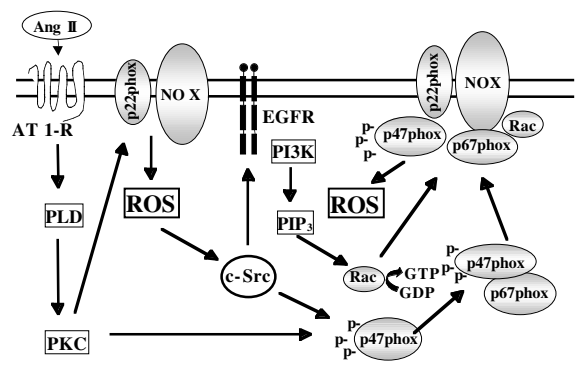

FIG. 2. Model of stimulation of vascular NADPH oxidases in response to angiotensin II. Activation of Ang II receptor (AT 1-R) leads to the phosphorylation of the p47phox subunit and the rapid generation of ROS through the induction of the enzyme complex via the activation of PKC. Initial production of ROS further activates c-Src tyrosine kinase that transactivates EGFR leading to the PI3K/PIP_3-mediated activation and translocation of Rac1, p47phox and p67phox. This answer results in sustained stimulation of the vascular NADPH oxidase that is even amplified through further activation of c-Src by H_2O_2.

THROMBIN STIMULATES THE PRODUCTION OF ROS: ROLE OF NADPH OXIDASES

Thrombin is a multifunctional serine protease that determines the fate of the blood coagulation cascade by affecting a series of factors, cofactors, and proteins inside the plasmatic hemostatic system and elicits biological responses in a variety of cells (65, 81). Thrombin is produced predominantly on the surface of circulating platelets as a result of proteolytic activation of the 72-kDa zymogen prothrombin, which is constitutively synthesized in the liver and released into the circulation (65, 66). In the blood plasma, thrombin as the last in a chain of membrane-bound factors and cofactors cleaves fibrinogen into fibrin, thus triggering rapid fibrin deposition and clot formation (79, 81). Thrombin also potently activates platelets and is able to stimulate various procoagulant and proinflammatory responses in vascular cells (11, 19, 20, 81). Interestingly, at sites of vascular injury and during the development of various cardiovascular diseases, increased amounts of thrombin are frequently associated with elevated ROS levels. Indeed, thrombin has been shown to stimulate the production of ROS in vascular cells and platelets (11, 35, 43, 48, 80, 110). In vascular smooth muscle cells, thrombin-induced ROS production has been prevented by addition of antioxidants such as vitamin C, vitamin E, *N*-acetylcysteine (NAC), and pyrrolidine dithiocarbamate (PDTC) or the flavin inhibitor diphenyleneiodonium (DPI), which acts as a nonspecific inhibitor of NADPH oxidases (35, 44). The involvement of vascular NADPH oxidases in thrombin-induced ROS production has been demonstrated because depletion of the subunit p22phox prevented ROS production in smooth muscle cells (11, 35, 44). Exposure to thrombin also increased p47phox phosphorylation and translocation to the cell membrane via a so far unknown mechanism (80), and thrombin-induced ROS production was increased in smooth muscle cells from wild-type, but not from p47phox−/− mice (12). Finally, the GTPase Rac is rapidly translocated to the cell membrane after challenging with thrombin and has been shown to contribute to thrombin-induced ROS production in smooth muscle cells (24, 44). In endothelial cells, inhibitors of the arachidonic acid metabolism or of phospholipase A2 or DPI prevented thrombin-induced ROS production (48). Furthermore, depletion of p22phox or expression of a dominant-negative Rac mutant inhibited thrombin-induced ROS production (T. Djordjevic and A. Görlach, unpublished observations). These findings suggest that NADPH oxidases are the main sources of ROS production in the vascular wall in response to thrombin. In contrast to the situation with Ang II, however, only limited information is available with regard to the activation mechanisms of NADPH oxidases elicited by thrombin (Fig. 3).

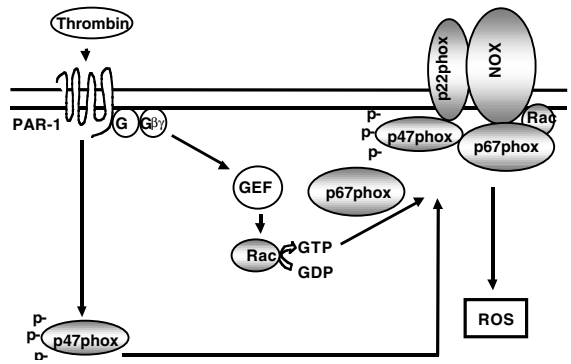

FIG. 3. Model of thrombin-mediated activation of vascular NADPH oxidases. Thrombin binds to its GPCR (PAR-1), leading to the activation of heterotrimeric G proteins (Gαβγ) that are linked to a number of signaling pathways. Gβγ dimer, in turn, activates guanine nucleotide exchange factor p114RhoGEF (GEF), leading to the activation of Rac1 and p67phox, their interaction, and finally their translocation to the membrane. In addition, phosphorylation of p47phox in response to thrombin leading to its membrane translocation is a critical step that promotes activation of the vascular NADPH oxidase.

PROTEASE-ACTIVATED RECEPTORS: EFFECTORS OF THROMBIN IN THE VASCULATURE

Thrombin exerts most of its actions on vascular target cells via an irreversible proteolytic activation of the seven-transmembrane G protein-coupled receptor (GPCR) protease-activated receptor-1 (PAR-1) (20, 21, 49, 61). Thrombin thereby binds to PAR-1's N-terminal exodomain and cleaves it after Arg41 to generate a new receptor N-terminus. The first six amino acids of this new N-terminus, SFLLRN, then serve as a tethered ligand that binds intramolecularly to the receptor's heptahelical bundle to effect transmembrane signaling and G-protein activation.

Three of the four currently identified PAR family members (PAR-1, PAR-3, and PAR-4) have been shown to be cleaved and activated by thrombin. The fourth PAR family member, PAR-2, is cleaved and activated by trypsin and tryptase, or transactivated by the tethered ligand from PAR-1 (61). PARs are expressed by a variety of cell types in and around blood vessels. In both humans and mice, PAR-1 appears to be the most widely expressed of the three thrombin-responsive PAR family members and to mediate most of the effects of thrombin in vascular cells (21, 47, 61).

The signaling pathways activated by PARs resemble the classic paradigm established for other GPCRs (20, 49, 107). PAR-1 couples to inhibition of cyclic AMP accumulation through G_i and stimulates phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides to enhance production of inositol trisphosphate and diacylglycerol through G_q, leading to mitogen-activated protein kinase (MAPK) phosphorylation and receptor tyrosine kinase transactivation. Gβγ interactions activate PI3K, which promotes the release of Ca^{2+}. PAR-1 also couples to G12/13 and modulates G12/13 effectors, such as Rho guanine exchange factors (GEFs) (25, 73, 75). The extent to which PAR-1 couples to each of these pathways in a particular cell type presumably depends on the G protein and effector repertoire expressed in the cell. Moreover, the precise mechanism(s) by which PAR-1 desensitizes to these distinct G-protein subtypes remains to be determined.

PARs are irreversibly activated by thrombin, and thus the mechanisms that contribute to termination of signaling are critical determinants of the magnitude and kinetics of the protease response in cells. This suggests that each activated PAR-1 signals, generates a defined amount of second messenger, and then shuts off (at least in terms of G_q activation). The rapid termination of PAR-1 signaling appears to be regulated at least in part by G-protein receptor kinase-mediated phosphorylation and by PKC-dependent mechanisms (21, 49, 114).

Moreover, PAR-1 down-regulation by receptor internalization and lysosomal sorting is required to terminate signaling by irreversibly activated receptors that remain at or return to the cell surface (107, 111). A major consequence of this unusual mechanism of activation and desensitization of PAR-1 is that the responsiveness of cells to thrombin is critically dependent on the constant supply of new receptor molecules to the plasma membrane.

In the case of endothelial cells, a reservoir of intact receptors is capable of replenishing the cell surface with receptors in 1–2 h, consistent with the idea that the endothelial cells play a primary role in mediating the vascular effects of PARs under physiological conditions (47). However, this pool has a limited and short-lived ability to resensitize endothelial cells to thrombin, and further thrombin responsiveness requires *de novo* PAR-1 synthesis via a heterotrimeric G_i-activated Ras (rat sarcoma oncogene)/MAPK signaling pathway involving PI3K and Src (avian sarcoma virus oncogene)-like protein tyrosine kinase activity (25). This indicates that it is necessary to activate the synthesis of PAR-1 to regenerate fully the cell-surface PAR-1 population once the preformed receptor pool has been depleted. In the smooth muscle, PAR expression is only rarely found under physiological conditions, but is induced under pathological conditions predominantly by *de novo* synthesis of the receptor (47, 90). This is supported by findings that in healthy arteries PAR-1 expression was detected only in endothelial cells, whereas in the smooth muscle PAR-1 expression was found only after endothelial injury, predominantly in areas of active cell proliferation (38). Similarly, PAR-1 expression was increased in human atheroma in regions rich in macrophages and smooth muscle cells (70). The advantages to be gained by such diverse and complicated mechanisms of activation and expressional regulation of PARs are not yet explained, although they probably contribute to the diversity of thrombin-mediated responses and allow for cell type–specific activation sequences to occur (81).

PROTEASE-ACTIVATED RECEPTORS AND GENERATION OF ROS

It is most likely that thrombin-caused ROS production in smooth muscle cells—similar to most of the effects induced by thrombin in this cell type—is mediated by PAR-1. Recently, Gβγ subunits of heterotrimeric G proteins or thrombin have been shown to activate the guanine nucleotide exchange factor p114RhoGEF, leading to the activation of Rac1 and subsequent induction of ROS formation and activation of the NADPH oxidase by promoting the membrane translocation of p67phox and its interaction with activated Rac1 (75) (Fig. 3). Indeed, thrombin and the peptide SFLLRN have been shown to rapidly activate Rac1 in platelets and in aortic and pulmonary artery smooth muscle cells (2, 83; T. Djordjevic, R.S. BelAiba, and A. Görlach, unpublished observations).

In contrast, in human umbilical vein endothelial cells, exposure to a PAR-1-activating peptide decreased Rac1 activation, whereas a PAR-2-activating peptide slightly increased Rac1 activity (109), although stimulation with thrombin is able to increase ROS production in this cell type, suggesting that thrombin and PAR signaling is different in endothelial and smooth muscle cells (47).

Although ROS generation via vascular NADPH oxidases plays a necessary role in thrombin-induced effects in vascular cells, the precise signaling events mediated by these ROS are still unclear. It seems that ROS are relatively proximal events in the thrombin signaling pathway. In smooth muscle cells, thrombin-induced activation of p38 MAPK and of protein kinase B/Akt, but not of extracellular signal-regulated kinase 1/2 (ERK1/2), is prevented by antioxidants and is dependent on NADPH oxidases because depletion of p22phox or overexpression of dominant-negative Rac inhibited these responses (35, 44). Moreover, thrombin or GPCR-stimulated Rac activity resulting in the binding to p67phox and the subsequent generation of ROS are necessary for activating tyrosine phosphorylation of janus kinases and for STAT (signal transducer and activator of transcription)-dependent transcription (83). Intriguingly, ROS may feed back on G proteins themselves because ROS directly activate G_i and G_o without receptor activation (74). The increase in the GTP-bound form of Gαi caused by ROS leads to liberation of Gβγ from G_i and G_o, thus activating MAPK and PI3K, which in turn leads to activation of Akt, suggesting that ROS may themselves contribute to oxidative stress responses by promoting or potentiating PAR signaling even after withdrawal of thrombin. Furthermore, thrombin induces PAR-1 *de novo* expression in a Src-dependent manner involving G proteins, PI3K, and p38 MAPK, suggesting that redox-sensitive pathways are also involved in the regulation of PAR-1 expression (27). Indeed, in smooth muscle cells, up-regulation of PAR-1 by cyclic strain or oxidative agents was prevented by DPI or antioxidants (59, 72). These observations suggest an important role for oxidant-mediated mechanisms in regulating PAR-1 expression, which may be mediated by several antioxidant response element-like consensus sequences described in the PAR-1 promoter (59). As potentiated thrombin generation and a procoagulant state are accompanied by increased production of ROS in various cardiovascular pathologies, PAR-1 could be of high importance for the regulation of vascular redox homeostasis.

TISSUE FACTOR: THE LINK BETWEEN THE VASCULAR AND HEMOSTATIC SYSTEM

The primary link between the vessel wall and the plasmatic hemostatic system is provided by the transmembrane human TF protein consisting of 263 amino acids: 219 amino acids form the large extracellular domain, 23 amino acids are integrated membranously, and the cytoplasmic tail is only

21 amino acid residues in length (62, 71). The major role of this 47-kDa glycoprotein is the initiation of blood coagulation by binding factor VII/VIIa with high affinity. This complex promotes the activation of factor IX and factor X with subsequent thrombin generation resulting in the formation of fibrin monomers, fibrin polymerization, and its rapid deposition finally leading to the formation of a stable fibrin clot (65, 66).

TF is differentially expressed in the various vascular and blood cell types (62, 71). In adult blood vessels, TF is abundant in adventitial fibroblasts, whereas quiescent endothelial cells *in vivo* do not express TF, possibly due to promoter elements in the TF gene that repress its transcription under basal conditions (50, 62). In addition, little or no TF is constitutively expressed in the intima or media, as well as in peripheral blood monocytes and macrophages (63, 76). Thus, cellular initiation of TF-dependent blood coagulation seems to require induction of TF expression on the surface of cells, which are normally or pathophysiologically in contact with plasmatic factors of hemostasis. Nevertheless, an increased expression of TF is not inevitably associated with an enhanced biological activity of TF because TF is regulated not only at the transcriptional, but also at a functional level. Whereas basal expression of TF is controlled by the transcription factor Sp1, inducible expression is regulated by the redox-sensitive transcription factors c-Fos/c-Jun, c-Rel/p65, and early growth response-1 (Egr-1) (22, 62, 76, 84). Functional TF is dependent on expression of a biologically active form on the cell surface. In smooth muscle cells and monocytes, only 10–20% of total cellular TF is available on the surface and reflects the biological activity, whereas the remainder is contained in intracellular pools (~30%) and as latent surface TF (50–60%) (89). The induction of TF expression and activity in smooth muscle cells is dependent on mobilization of intracellular Ca^{2+} (100). Moreover, increased TF activity also seems to be attributed, in part, to a process of decryption, whereby enhanced intracellular Ca^{2+} levels cause a change in TF accessibility on the cell surface, exposing previously encrypted and inactive TF molecules (113).

Enhanced expression of TF plays a pivotal role in the procoagulant activity of disrupted atherosclerotic plaques or of acutely injured arteries as a consequence of balloon angioplasty or coronary atherectomy (7, 31, 67). TF expression is also associated with life-threatening thrombosis in a variety of diseases, including sepsis, cancer, and atherosclerosis (28, 39, 101). Indeed, inflammatory toxins and cytokines can induce TF expression in monocytes/macrophages and endothelial cells (63). Increased TF expression is also found in foam cells, as well as in smooth muscle cells in atherosclerotic plaques and plays an important role in determining their thrombogenicity (101). In addition, non–cell-bound TF has been reported that is associated with microparticles derived from platelets, monocytes, lymphocytes, and endothelial cells (96). TF-containing microparticles may play a critical role in thrombus formation *in vivo* (10). In addition, enrichment of circulating TF in human atherosclerotic plaques has been linked to plaque disruption (64).

In addition, at sites of vascular injury, TF expression and activity are induced in smooth muscle cells (95). In animal models of balloon injury, TF expression increased rapidly in medial smooth muscle cells after endothelial denudation and markedly accumulated in the developing neointima (101). Thus, induction of TF in smooth muscle cells appears to be primarily responsible for the prolonged vascular procoagulant activity after endothelial denudation *in vivo* (62). These effects may be mediated by increased availability of mitogens, growth factors, vasoactive agonists, and clotting factors under these conditions. It has been shown that TF expression is rapidly and markedly induced in cultured smooth muscle cells by platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), EGF, Ang II, thrombin, and activated platelets (33, 88, 100). Moreover, the TF gene is also induced by serum, phorbol esters, and the Ca^{2+} ionophore ionomycin in smooth muscle cells (89, 100). Interestingly, most of these factors have been shown to also induce ROS production and activate the NADPH oxidase in vascular cells (4, 11, 15, 33, 34, 44, 57), suggesting a close link between ROS production and TF expression and/or activity (Table 1).

REDOX-SENSITIVE REGULATION OF TISSUE FACTOR

TF has been recognized as a redox-sensitive gene in several cell types, including endothelial and smooth muscle cells (42). Increased ROS levels appear to regulate TF at the transcriptional level, as well as at the functional level. Application of H_2O_2 induced TF mRNA and protein expression in aortic and pulmonary artery smooth muscle cells (44; R.S. BelAiba, T. Djordjevic, and A. Görlach, unpublished observations), whereas in endothelial cells exposure to xanthine/ xanthine oxidase (X/XO) resulted in enhanced TF mRNA levels (32).

On the other hand, application of H_2O_2 or exposure to X/XO stimulated TF activity in monocytes (13). In endothelial cells, exposure to X/XO resulted in enhanced TF activity (32). Similarly, a flux of X/XO induced TF activity in isolated rabbit hearts. TF activity was also significantly up-regulated in rabbit hearts subjected to ischemia followed by reperfusion (32), whereas administration of superoxide dismutase at the moment of reperfusion decreased TF activity in these hearts, indicating that ROS contributed to TF activation in response to reperfusion. In smooth muscle cells, H_2O_2 has been shown to be essential for activating the encrypted TF protein complex (85).

The involvement of ROS in agonist-induced TF activity and mRNA and/or protein expression has been mainly related to the inhibitory action of various antioxidants. In monocytes, TF mRNA and protein expression, as well as TF activity, was enhanced in response to AGE (advanced glycation end product)-albumin, a factor increased in diabetes (52). This response was inhibited in the presence of antioxidants such as NAC, thiourea, and dimethylthiourea, suggesting a role for ROS in diabetes-induced coagulopathies. Redox-sensitive TF activity has also been observed in monocytes in response to activated polymorphic neutrophils, suggesting an interaction between these blood cells in promoting coagulation during sepsis (13, 51).

In endothelial cells, TF mRNA and protein expression, as well as TF activity, in response to various cytokines including

interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), to lipopolysaccharide, an important mediator of endotoxic shock, to proatherosclerotic factors such as remnant lipoprotein, or to phorbol ester [phorbol 12-myristate 13-acetate (PMA)] is sensitive to antioxidant treatment (14, 42, 78). On the other hand, in smooth muscle cells, low-density lipoprotein (LDL) increased TF expression, but not its activity on the cell surface. However, H_2O_2 treatment of LDL stimulated smooth muscle cells, resulting in increased TF activity, supporting the idea that oxidative stress is required to mediate activation of the cell-surface TF complex (22, 33, 85, 86). Moreover, redox-sensitive regulation of TF expression and activity was observed in smooth muscle cells, but also in endothelial cells, in response to activated platelets, because treatment with antioxidants such as PDTC, NAC, or 11,12-*o*-phenanthroline abolished this response (33, 92). In addition, whereas inhibitors of cyclooxygenase (diclofenac) and xanthine oxidase (allopurinol) had no effect on TF mRNA induction by activated platelets in smooth muscle cells, the flavin inhibitor DPI abolished platelet-induced TF mRNA expression, suggesting that an NADPH oxidase was involved in this response (33).

NADPH OXIDASES: THE LINK BETWEEN TISSUE FACTOR AND THROMBIN

Besides its essential function as coagulation factor activated by both the intrinsic and the extrinsic pathway of the plasmatic hemostatic system, thrombin seems to fulfill a central role in inducing TF expression and therefore in the promotion of coagulation. Increased thrombin formation and TF expression are observed at sites of vascular injury and TF up-regulation after balloon angioplasty in rabbit femoral and porcine coronary arteries was attenuated by the thrombin inhibitor hirudin (31, 89). Intriguingly, thrombin has been shown to elevate TF mRNA and protein levels, as well as TF activity, in smooth muscle and endothelial cells (1, 24, 29, 31, 43, 44, 89). Up-regulation of PAR-1 bioavailability thereby appears to be sufficient to remarkably sensitize the vascular smooth muscle to increase the expression of TF and subsequently TF-dependent procoagulant surface activity in response to thrombin or the peptide SFLLRNP (43). Consistently, a neutralizing antibody directed against PAR-1 abolished this effect, indicating that thrombin, which is produced during activation of the coagulation cascade, is, via activation of PAR-1, able to rapidly and markedly induce and activate TF, the main initiator of this cascade. This raises the possibility that a positive feedback system exists whereby thrombin can help to propagate the clot by inducing TF expression in smooth muscle or endothelial cells, thus increasing the thrombogenicity of the injured vascular wall.

ROS may play an important role in regulating this potential thrombogenic cycle, because treatment with antioxidants such as vitamin C, NAC, and PDTC prevented thrombin-induced TF expression and activity in smooth muscle cells (44). Inhibition of thrombin-induced TF expression and activity by DPI or the *Clostridium difficile* toxin B, which inhibits Rho GTPases including the NADPH oxidase regulator Rac, further points toward the involvement of NADPH oxidases in this response. Indeed, depletion of p22phox or overexpression of a dominant-negative Rac mutant decreased thrombin-induced TF expression and activity in smooth muscle cells (24, 44). Moreover, thrombin-induced TF expression was diminished in smooth muscle cells derived from p47phox−/− mice when compared with smooth muscle cells from wild-type mice (12). Inhibition of thrombin-induced TF expression by statins, which inhibit Rho GTPases, suggests that ROS and NADPH oxidases may also be involved in endothelial TF expression by thrombin (29). Interestingly, thrombin-induced TF expression was mediated by NADPH oxidase-dependent activation of p38 MAPK and Akt, but not by ERK1/2 (44). These findings show that thrombin activates the NADPH oxidases via PAR-1 to generate ROS, which subsequently activate p38 MAPK and Akt, finally leading to enhanced TF expression and TF activity, indicating that these enzymes may indeed play a prominent role in promoting this thrombogenic cycle (Fig. 4).

Moreover, the redox-sensitive transcription factor nuclear factor-κB (NFκB) is involved in the regulation of TF expression in response to thrombin in pulmonary artery smooth muscle cells (24). A role for NFκB in regulating TF expression has also been shown previously for endothelial cells (84). In addition, NFκB-mediated TF expression was induced by activated Rac, whereas the thrombin response was abrogated in the presence of inactive Rac. As Rac is an activator of the NADPH oxidase, these findings add NFκB as a further signaling element into the thrombogenic cycle promoted by the NADPH oxidase. Whether other redox-sensitive transcription factors, such as activator protein-1 (AP-1)

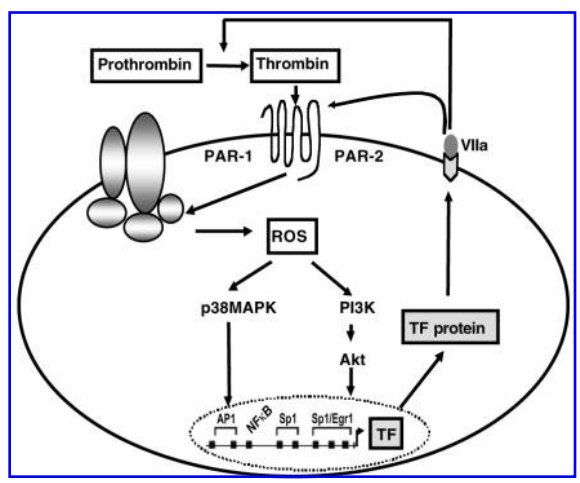

FIG. 4. A thrombogenic cycle is mediated via NADPH oxidases. Thrombin binds to its GPCR (PAR-1) that activates vascular NADPH oxidases. NADPH oxidases generate ROS, leading to the activation of the p38 MAPK and PI3K signaling pathways and subsequently to the activation of transcription factors in the nucleus, inducing TF expression. TF exposure to the cell surface allows factor VIIa binding that will result, on the one hand, in the activation of PAR-2 and possible transactivation of PAR-1 and, on the other hand, in the generation of thrombin.

and Egr-1, which are known to regulate TF expression in response to several agonists, are also linked to NADPH oxidase-dependent ROS generation or to the thrombogenic cycle remains to be elucidated.

Moreover, recent studies showed that the formation of the TF/VIIa complex not only leads to the generation of thrombin, but also exerts its own cellular effects. Thus, the TF/VIIa complex has been suggested to interact with PAR-2 and may influence PAR-1 activity, which may lead to its own up-regulation and thus to further potentiation of the thrombogenic cycle (16, 93). Indeed, activation of the NFκB pathway has been shown to be mediated via PAR-2 signal transduction. Furthermore, factor VIIa fixation on TF can increase intracellular Ca^{2+} levels and activate Src-like kinases, subsequently leading to the activation of Rac and the downstream p38 MAPK pathway (108). As TF/VIIa has been shown to activate ROS generation in monocytes (23), NADPH oxidase-derived ROS production may also be initiated by the TF/VIIa complex in vascular cells. Thus, NADPH oxidases as sources of vascular ROS production appear to play a pivotal role in promoting the thrombogenic cycle by affecting all essential controlling elements, suggesting that they effectively contribute to enhanced thrombogenicity of the injured vascular wall.

Remarkably, impaired bioavailability and bioactivity of the endothelium-derived anticoagulant NO caused by elevated levels of O_2^- have also been associated with an altered procoagulant state of the vascular system (14). Moreover, many effects of NO and ROS themselves occur in an opposite way allocating NO and ROS the function as contrary players also in blood coagulation. Moreover, the interaction of O_2^- with NO leads to the rapid formation of peroxynitrite, which may have pro- or antithrombotic effects. Under physiological conditions, endogenous antioxidant mechanisms minimize this interaction and maintain what seems to be a tenuous balance between ROS and NO. This balance is disturbed in a variety of cardiovascular diseases where a hypercoagulant state is associated with high levels of thrombin accompanied by an extended imbalance between ROS and NO (14, 41). Thrombin could be critically involved in the mechanisms of this dysregulated balance because it stimulates not only the production of ROS in vascular cells, but also the formation of NO. Dependent on the amount and timely release of NO in response to thrombin, this molecule, by diminishing TF expression and activation, may contribute to limit the progression of the thrombogenic cycle in the vessel wall.

CONCLUSION

TF has been shown to be the primary connecting link between vascular cells and the plasmatic hemostatic system, because surface exposure of active TF leads to the formation of the TF/VIIa complex, which promotes the generation of thrombin. Thrombin activates ROS generation by vascular NADPH oxidases, subsequently initiating and activating ROS-dependent signaling cascades that promote a thrombogenic cycle via up-regulation of TF. This cycle is further fueled by the sustained generation of ROS, explaining the occurrence of a prothrombotic state together with enhanced levels of ROS in many cardiovascular disorders. Although to date specific interventions to modulate NADPH oxidase activity *in vivo* are not available, they may gain increasing interest as novel therapeutic strategies in disorders associated with enhanced procoagulant activity and thrombogenicity of the vascular wall.

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ABBREVIATIONS

Ang II, angiotensin II; AP-1, activator protein 1; AT 1-R, angiotensin receptor 1; bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; DPI, diphenyleneiodonium; DUOX, dual oxidase; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; Egr-1, early growth response 1; ERK, extracellular signal-regulated kinase; GEF, guanine exchange factor; GPCR, G protein-coupled receptor; H_2O_2, hydrogen peroxide; 5-HT, 5-hydroxytryptamine; IGF, insulin-like growth factor; IL-1α, interleukin-1α; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NFκB, nuclear factor-κB; NO, nitric oxide; NOX, NADPH oxidase; O_2^-, superoxide anion radical; oxLDL, oxidized low-density lipoprotein; PAR, protease-activated receptor; PAR-2AP, protease-activated receptor 2 activator peptide; PDGF, platelet-derived growth factor; PDP, platelet-derived products; PDTC, pyrrolidine dithiocarbamate; PGF2, prostaglandin F2; PGI2, prostaglandin I2; Phox, phagocyte oxidase; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PPAR, peroxisome proliferator-activated receptor; Rac, ras-related C3 botulinum toxin substrate; Ras, rat sarcoma oncogene; ROS, reactive oxygen species; Src, avian sarcoma virus oncogene; TF, tissue factor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TxA2, thromboxane A2; VEGF, vascular endothelial growth factor; X/XO, xanthine/xanthine oxidase.

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