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Forum Review

Integrins and Coagulation: A Role for ROS/Redox Signaling?

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ABSTRACT

Integrin regulation and signaling play a central role in the hemostasis process, particularly at the level of endothelial cells by regulating the contractility and barrier function of these cells and in platelets by controlling adhesion and aggregation at the site of cell injury. Reactive oxygen species (ROS) have emerged as an important mediator both transducing the signals associated with integrin activation and modulating integrin function. Ligation of integrins in endothelial cells and platelets induces activation of the Ras/mitogen-activated protein kinase, nuclear factor-κB, and phosphatidylinositol 3-kinase and Rho-GTPases pathways. Following vessel-wall injury and associated with activation and recruitment of platelets, there is a production of ROS concomitant with the stimulation of the blood coagulation. Moreover, ROS are capable of inducing conformational changes in integrins to change their binding affinity and function. This review will explore how ROS have emerged as an important modulator of integrins in coagulation through both outside-in (integrins stimulating ROS production to effect intracellular events) and inside-out signaling (intracellular ROS altering integrin function). *Antioxid. Redox Signal.* 6, 757–765.

INTRODUCTION

THE REGULATION OF THROMBOSIS and hemostasis requires carefully orchestrated interactions between diverse cell types, including platelets, inflammatory cells, plasma proteins, and the vessel wall. Integrin and reactive oxygen species (ROS) signaling has emerged as a potentially important element contributing to the communication between the diverse cell types involved in thrombosis. Regulation of the integrin $\alpha_{2b}\beta_3$ has already proved a successful target for controlling pathologic thrombosis in myocardial infarction, and interest in ROS signaling continues to grow. Particular interest has focused on the small GTP-binding protein Rac, which through its regulation of NADPH-derived superoxide contributes to interactions between thrombin, platelets, endothelial cells, and leukocytes involved in thrombus formation (23). Moreover, regulation of Rac and ROS production may be regulated by the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCo-A) reductase inhibitors, a class of cholesterol-reducing medications, which exhibit pleiotropic effects, including reducing inflammation and thrombosis, and which may account for their success in preventing pathologic thrombosis that occurs in stroke and myocardial infarct (64).

There are two aspects involved in the generation of thrombus and clot formation: (a) platelet plugs and aggregation, and (b) coagulation to form a fibrin-rich network of clot in association with platelet plugs. Each is a unique process, but they interact with platelet activation contributing to activation of the clotting cascade. In normal physiology, this process works to achieve hemostasis, stopping bleeding from cuts or injuries; however, pathologic thrombosis may occur that leads to myocardial infarction or stroke. Vascular injury triggers a reparative process, which depends on a coordinated series of events, including platelet, leukocyte, and vascular cell adhesion and migration (45). A diverse group of plasma membrane integrin receptors, some of which trigger ROS synthesis, mediate cell adhesion and exhibit specialized ligand-binding properties to respond to injury and coordinate thrombus formation. For example, when blood flows through a damaged blood vessel, leukocytes slow down and roll on the endothelial surface through interaction of sialyl Lewis X-rich membrane glycoproteins on the leukocytes and selectins on

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the endothelial cells (46). Similarly, platelets roll through interactions with the platelet glycoprotein Ib–V–IX complex with von Willebrand factor (vWF) in the subendothelial matrix of denuded vascular surfaces or shear stress-perturbed endothelium (57). Once the rolling process has slowed down these blood cells, they are further targeted to the right location through regulated interactions between integrin receptors and either counterreceptors on endothelial cells or adhesive proteins in the matrix (33). Integrins and ROS also mediate responses necessary for eventual completion of the injury response, including leukocyte transmigration and platelet aggregation (23, 41). Capitalizing on this important role in coagulation, integrins have been successfully targeted to inhibit platelet aggregation and further coagulation through antagonism for $\alpha_{2b}\beta_3$ integrin. ROS production has been demonstrated during blood coagulation; it is mainly induced by the coagulation factors released by activated platelets and may, thus, provide an important secondary target to regulate thrombus formation (21). ROS, besides their toxic effect at high concentration, are now well recognized as regulators of cell proliferation, differentiation, cell stress, inflammation, cell death, and senescence (17). Recent data suggest that ROS production is induced by integrins and might participate in the signaling mediated by these proteins. This review discusses the relationship between ROS generation and integrin signaling in the regulation of blood coagulation.

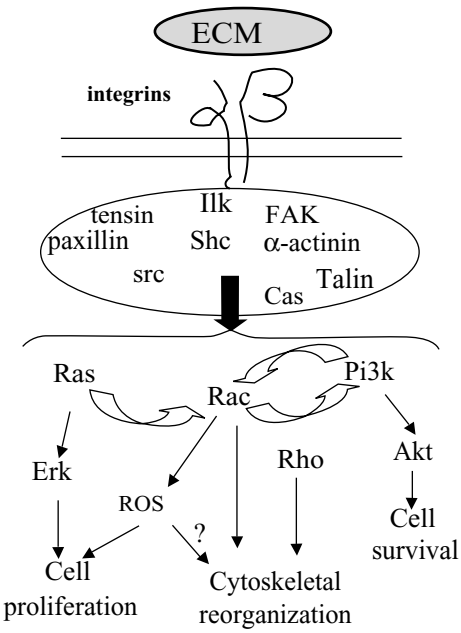

FIG. 1. Outside-in integrin signaling pathway.

ENDOTHELIAL CELLS AND PLATELETS IN BLOOD COAGULATION: ROLE OF INTEGRINS

Integrins are transmembrane heterodimeric proteins consisting of two noncovalently associated glycoproteins, α and β. They are the main receptors for extracellular matrix (ECM) proteins and mediate signaling events essential for proliferation, differentiation, spreading, migration, and survival. Four different signaling pathways that are also activated by growth factor receptors are controlled by integrin activation: Ras/mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI-3K)/Akt and Rho-GTPase pathways (56) (see Fig. 1). Integrins that, after activation by binding specific ECM proteins, induce signals leading to protein phosphorylation and protein synthesis are usually characterized as demonstrating "outside-in" signaling. On the other hand, if intracellular proteins bind to the intracytoplasmic tail of the integrins to modulate the conformation and affinity for the ECM of the extracellular part, this process is described as "inside-out" signaling (29). Integrin regulation and signaling play a central role in the hemostasis process, particularly at the level of endothelial cells by regulating the contractility and barrier function of these cells and in platelets by controlling adhesion and aggregation at the site of cell injury, and involve both outside-in and inside-out signaling. Endothelial cells are the main regulator of blood flow and in their basal state provide a nonthrombogenic surface that helps prevent undesired thrombosis. After blood vessel injury, the endothelium rapidly becomes an adhesive surface with intercellular gaps that allow the passage of soluble plasma and inflammatory cells from the vascular lumen to the underlying tissue (47). Brief exposure of endothelial cells to physiological concentrations of thrombin causes a marked inhibition of their ability to adhere to basement membrane collagen IV or to laminin (3). These endothelial changes are in part modulated by Rho-GTPases and ROS signaling through cytoskeleton changes in endothelial cells challenged with thrombin, and a Rho-GTPase-dependent pathway is involved in cell rounding and barrier dysfunction (68).

Additionally, activation of platelets by exposed collagen after vessel-wall injury involves two collagen receptors, integrin $\alpha_2\beta_1$ and glycoprotein VI (GPVI) (11), and ROS signaling. Integrin $\alpha_2\beta_1$ exerts primarily an adhesive function, recruiting platelets to collagen surfaces under arterial flow conditions (55). A reciprocal two-receptor model of collagen signaling in platelets has been suggested in which the nonintegrin receptor GPVI provides the primary collagen signal that activates and recruits the integrin receptor $\alpha_2\beta_1$ to further amplify collagen signals and fully activate platelets through a common intracellular signaling pathway (5). Furthermore, increased collagen-binding affinity correlates with disulfide rearrangement within $\alpha_2\beta_1$ on the intact platelet (19). Furthermore, GPVI serves a key signaling role leading to aggregation through activation of $\alpha_{2b}\beta_3$, which further augments platelet adhesion to collagen (69). In fact, one of the best studied integrins from the standpoint of acute regulation is $\alpha_{2b}\beta_3$, which interacts with Arg-Gly-Asp–containing ligands, such as fibrinogen and vWf, during platelet aggregation and spreading on vascular surfaces (58). $\alpha_{2b}\beta_3$ integrins are the primary mediators of platelet aggregation. Platelet agonists, such as thrombin and ADP, cause rapid changes in the adhesive function of $\alpha_{2b}\beta_3$ demonstrated by increases in the binding of soluble fibrinogen, vWf, and ligand-mimetic mono-

clonal antibodies, including PAC1 (58). Antagonists, such as prostacyclin and nitric oxide (NO), can inhibit and, under some conditions, reverse these acute changes (10). Interestingly, illustrating the fundamental function of this integrin in blood coagulation, a defect in $\alpha_{2b}\beta_3$ integrin binding to fibrinogen and fibrin results in an autosomal recessive disorder called Glanzmann thrombasthenia, which is characterized by platelet dysfunction and prolonged bleeding time (70). Integrin function must sometimes be regulated acutely over seconds to minutes to enable rapid changes in cell adhesion and migration required during immune responses, inflammation, and hemostasis. Several integrins in blood cells are targets of this type of inside-out signaling, including $\alpha_4\beta_1$, $\alpha_l\beta_2$, and $\alpha M\beta_2$ in leukocytes, and $\alpha_{2b}\beta_3$ and $\alpha_v\beta_3$ in platelets (1, 2, 58). Thus, platelet adhesion to collagen fibers through integrin $\alpha_2\beta_1$ induces tyrosine phosphorylation (27) and involves Rac GTPase and p-21 activated kinase (PAK) activation, which is regulated by Src and PI-3K (63). In the same way, it has been shown that human platelet Rac is rapidly activated by thrombin and collagen through a phospholipase (PL) C and calcium mobilization-mediated mechanism, independently of integrin $\alpha_{2b}\beta_3$ engagement (61). When platelets adhere to, and spread on collagen fibers, and finally form aggregates by recruiting other platelets through integrin $\alpha_{2b}\beta_3$–fibrinogen interaction, the activation of adherent platelets stimulates tyrosine phosphorylation of many of the proteins in the GPVI–FcR chain cascade, including Src, Syk, SLP-76, and PLC-2, as well as plasma membrane calcium ATPase and focal adhesion kinase (FAK) (30).

ROS AS MEDIATORS OF INTEGRIN SIGNAL TRANSDUCTION?

ROS, besides their known toxic effect, are now considered as potential messengers for signal transduction (65). During vessel-wall injury, after activation and deposition of platelets, there is production of ROS concomitant with the stimulation of blood coagulation (32). This stimulation of ROS production may result from coagulation factors released from activated platelets. The role of ROS production during coagulation is not fully described; however, ROS may affect protein function necessary for coagulation by oxidation of specific amino acids present on the protein. Oxidation of cysteine residues present at the active site of protein tyrosine phosphatase represents the prototypic mechanism of control of the signal transduction pathway by ROS. This reversible oxidation of cysteine residue supports the concept of oxidative signaling (8). On the other hand, oxidation of other types of amino acids (like arginine, proline, lysine, and threonine) leads to an irreversible formation of a carbonyl group targeting the protein to destruction. Irreversible oxidation is usually associated with oxidative stress and high ROS production rates. The regulation of many signal transduction proteins, such as IκB, (24) or Rho-GTPases (36), however, is performed through proteasomal degradation, suggesting that irreversible oxidation might represent a way to turn off some cellular signal transduction pathways. ROS production has been shown to affect numerous proteins involved in signal transduction, including receptor tyrosine kinases (epidermal growth factor receptor, insulin receptor), kinases (JAK/STAT, p38 MAPK, Src), transcription factors (AP1, Myb, Ets, HIF1α, PPARγ, and NF-κB), phospholipases (A_2, C, and D), and tyrosine phosphatases (for review, see 8, 17, 25, 65). Moreover, it has recently been reported that $\alpha_{2b}\beta_3$ integrin contains a redox site constituted of several unpaired cysteine residues (74). $\alpha_{2b}\beta_3$ integrin is regulated by an on/off switch that regulates its ligand binding affinity. Different reports show that treatment with the reducing agent dithiothreitol induces activation of this integrin (18, 51). *In vitro,* dithiothreitol reduces two disulfides bond within the integrin's cysteine-rich domain to induce a global conformational change of the $\alpha_{2b}\beta_3$ integrin, opening the RGD and fibrinogen binding sites (74). This effect might explain the inhibition of platelet aggregation observed with a high concentration of hydrogen peroxide (H_2O_2) and phagocyte-produced ROS. However, lower H_2O_2 concentrations are reported to induce aggregation (32). Interestingly, H_2O_2 in the presence of a phosphatase inhibitor ($NaVO_4$) has been shown to induce tyrosine phosphorylation of the β_3 subunit, leading to the priming of the $\alpha_{2b}\beta_3$ integrins. In these conditions, however, many other platelet proteins are tyrosine-phosphorylated, and this effect is independent of aggregation (31). Finally, a recent study found that integrin α4 undergoes redox regulation through exofacial thiol reduction. The reduction of surface protein disulfides in Jurkat cells increases cell adhesion through integrin (39).

Besides ROS regulation of integrin activation and stability, integrins may induce ROS production. Recent evidence suggests that integrin activation leads to ROS production in different cell types (for review, see 7) and that integrins regulate Rac subcellular localization (14, 15). It seems that three different sources of ROS production may be involved during cell adhesion to the ECM: the mitochondrial electron transport chain, the NADPH oxidase, and the arachidonic acid metabolism (9, 28, 71). All these pathways of ROS production have been shown to involve the Rho-GTPase family and particularly Rac1. Mitochondria represent the main source of ROS in mammalian cells. Mitochondrial ROS are a by-product of the oxidative metabolism, and increased mitochondrial ROS production is classically reported during cell apoptosis. Recent data suggest that mitochondrial ROS production may additionally lead to gene expression rather than apoptosis (71). Indeed, $\alpha_5\beta_1$ integrin engagement in fibroblasts induces Rac activation and ROS production leading to NF-κB activation and up-regulation of collagenase-1 expression (34). The ROS production induced by $\alpha_5\beta_1$ integrin engagement was mainly due to the mitochondria and is inhibited by Bcl2 (71). Besides mitochondria, NADPH oxidase represents another potential source of ROS production. Indeed, since the recent identification of different isoforms of the catalytic subunit of the NADPH oxidase (Nox1, -2, 3, -4, and -5) in nonphagocytic cells, the involvement of NADPH oxidase in cell signal transduction pathways has represented an intensive area of research (6, 38). At the level of integrin signal transduction, we recently found that adhesion of epithelial cancerous cells on collagen IV through integrin $\alpha_2\beta_1$ leads to an increase in $\alpha_2\beta_1$ integrin expression and increased proliferation that is dependent on NADPH-derived ROS production and subsequent p38 MAPK activation (28).

Another report shows that adhesion of fibroblasts through $\alpha_5\beta_1$ integrin on fibronectin leads to ROS production and subsequent low-molecular-weight (LMW) phosphatase inhibition with secondary FAK activation leading to cell adhesion and spreading onto fibronectin (9). This ROS production can be inhibited by diphenyleneiodonium (DPI) and by a lipoxygenase (Lox) inhibitor (nordihydroguaiaretic acid) and is dependent on Rac signaling. These results reflect that Rac may stimulate ROS through NADPH oxidase, as well as arachidonic acid metabolism through PLA_2 activation (52). Additionally, the inhibition of the LMW phosphate by ROS has been shown to be responsible for Rac-induced down-regulation of Rho signaling (50). The metabolism of arachidonic acid, as well as enzymes such as PLA_2, Lox, and cyclooxygenases (Cox), are also thought to have an important role in the regulation of blood coagulation and in the production of ROS. Many reports show that arachidonic acid modulates NADPH oxidase and mitochondrial ROS production to create a complex web of regulation for ROS production. Recent data suggest that, in addition to NADPH oxidase, Lox activation may be involved in integrin-induced ROS production (9). The mechanism of ROS production induced by integrin through Lox might result from direct production of a peroxide derivative or by an indirect production induced by Lox end products (*i.e.,* leukotrienes) (see Fig. 2). Indeed, leukotriene B4 has been shown to activate NADPH oxidase in different cell types (43, 44). The idea that NADPH oxidase acts downstream of Lox in integrin signaling is attractive, but not yet fully demonstrated by experimental data. In addition, although many reports show that Rac1 activates PLA_2 (59, 73), the mechanism of that activation remains elusive. However, the work from Chiarugi *et al.* (9) demonstrates a mechanism by which integrin-dependent ROS production might participate in cytoskeletal reorganization through LMW phosphatase inhibition and FAK activation in fibroblasts.

A more complicated adherence-dependent production of ROS is reported in nonfibroblastic cells. First, endothelial cell rounding and detachment lead to an increase in DPI-inhibitable ROS production compared with cells plated on gelatin (40). Second, deadhesion occurring at the leading edge of migrating endothelial cells induces DPI-inhibitable ROS production in these moving areas (48, 49). Third, Fig. 3 shows that epithelial cancer cells exhibit a higher level of ROS production in suspension or in spreading cells compared with cells adherent for a short time (30–60 min). Finally, in neutrophils, the initial time of adhesion to collagen leads to a delayed activation of Rac and to a decreased ROS production compared with a longer time of adhesion and spreading (76). Overall, integrin-dependent attachment of cells to the ECM and cell detachment from the ECM induced cytoskeletal reorganization with a concomitant production of ROS (see Fig. 4). In adherent cells, this ROS production participates in the actin cytoskeletal reorganization by increasing FAK activity during cell spreading and participates in the further control of gene expression and cell proliferation. Through these mechanisms, ROS contribute to the regulation of hemostasis by signaling endothelial changes that would provide a thrombogenic surface.

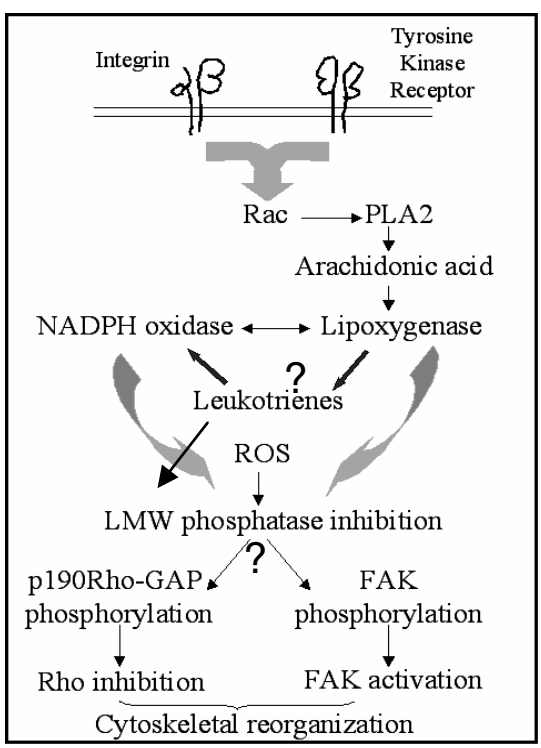

FIG. 2. Integrin-dependent ROS production pathway.

DO ROS MODULATE INTEGRIN REGULATION OF BLOOD COAGULATION?

Endothelials cells

Different reports have shown that integrin binding activates Rac GTPase in endothelium (16, 35, 66). Furthermore, endothelial cells express a neutrophil like NADPH oxidase (with the catalytic subunit Nox2/gp91phox), and different

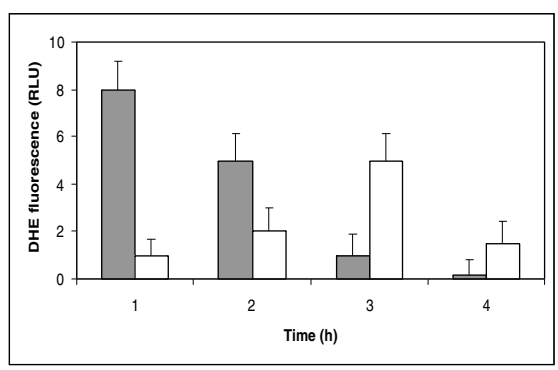

FIG. 3. Time dependence of ROS production in adherent (gray columns) versus nonadherent (white columns) Caco-2 cells to collagen IV. ROS production was assessed on fetal bovine serum-deprived cells by dihydroethidium (DHE) fluorescence. Results are the means ± SD of three different experiments.

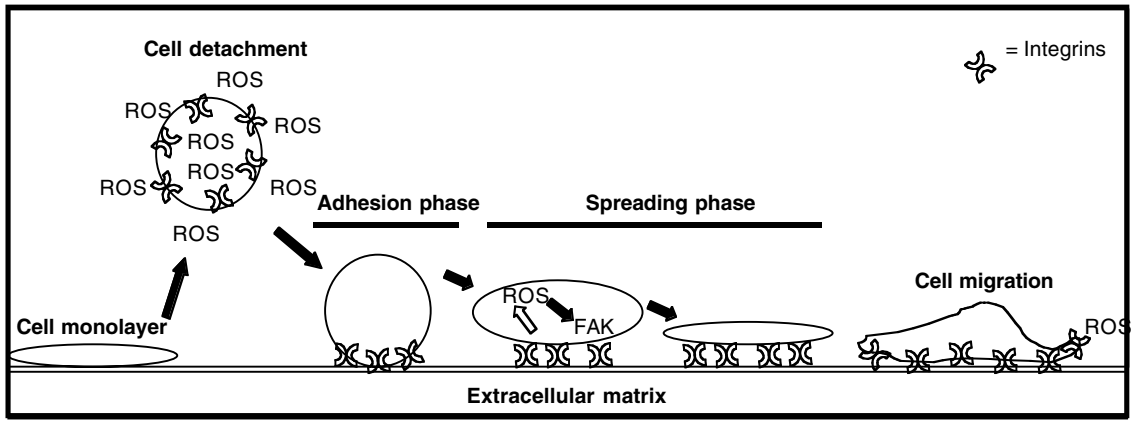

FIG. 4. ROS production during cell adhesion, detachment, and migration.

groups have reported increased ROS production by overexpression of a constitutive active mutant of Rac (13, 20, 36, 67, 75). Increased NADPH-derived superoxide may, in turn, decrease the level of NO (with an increase in peroxynitrite) and lead to the activation or inactivation of cytosolic proteins controlling morphology and gene expression, particularly integrin and ECM protein expression. This is illustrated by a recent report showing that NADPH oxidase-produced ROS modulate the availability of NO to inhibit platelet disaggregation (12).

Interestingly, cytosolic regulation by ROS may demonstrate a biphasic pattern illustrated by endothelial cell synthesis of prostaglandin I_2 (PGI_2), a known inhibitor of platelet aggregation, which increases with low ROS production, but is inhibited by high ROS production. Moreover, constitutive Rac expression has been shown to induce the synthesis of thrombospondin-2 (TSP2), a matricellular protein with antiangiogenic activity, by activation of ROS production in a similar biphasic pattern (42). Abnormal collagen fibrils have been observed in TSP2-null mice, leading to the idea that an abnormal subendothelial matrix could compromise platelet activation and/or adhesion. Indeed, mice that lack TSP2 display a bleeding diathesis, despite normal blood coagulation and the lack of thrombocytopenia. TSP2-null platelets are compromised in their ability to aggregate in response to denudation of the carotid artery endothelium, and *in vitro* following exposure to ADP. It has been suggested that the uptake of TSP2 by megakaryocytic cells in bone marrow is necessary to obtain functional platelets (4, 37). The increased expression of TSP2 at the site of cell injury by a Rac-dependent ROS production in endothelial cells might improve platelet aggregation and thus participate in a procoagulant effect.

Platelets

Rac and ROS involvement in platelet function has been documented for a long time (26). The impact of integrin signaling on ROS production, however, is less clearly established. Indeed, ROS-induced platelet activation might involve arachidonic acid metabolism and NADPH oxidase. Collagen has been shown to induce Rac and PAK activation through the integrin $\alpha_2\beta_1$ ligation (63), whereas this effect is not dependent on integrin $\alpha_{2b}\beta_3$ (61). In addition, collagen-induced platelet aggregation is associated with a burst of H_2O_2 (53, 54) activating arachidonic acid metabolism and the PLC pathway. This effect might be mediated by NADPH oxidase and Cox. Data show that the antiaggregative prostaglandin synthase product, PGI_2, inhibits Rac activation by collagen in platelets, suggesting that Rac activation of ROS production is procoagulant in these cells (61). Another study showed that a Lox product (15-hydroxyeicosatetraenoic acid) induces integrin $\alpha_{2b}\beta_3$ activation and expression at the membrane (62). Finally, proteins present in plasma or secreted during platelet aggregation, like fibronectin and thrombospondin-1, may stimulate endothelial cell integrins to induces ROS production and to initiate a thrombogenic cycle observed after wall injury.

Besides endothelial cells and platelets, other cells present at the site of vascular injury (vascular smooth muscle cells and phagocytes) have been reported to produce different factors affected by the redox status, like tissue factor and plasminogen activator inhibitor-1 (PAI-1), but the implication of integrins in these regulations is unknown (22). To summarize, Fig. 5 presents the different pathways that might involve integrin and ROS production in the control of blood coagulation at the site of vascular wall injury.

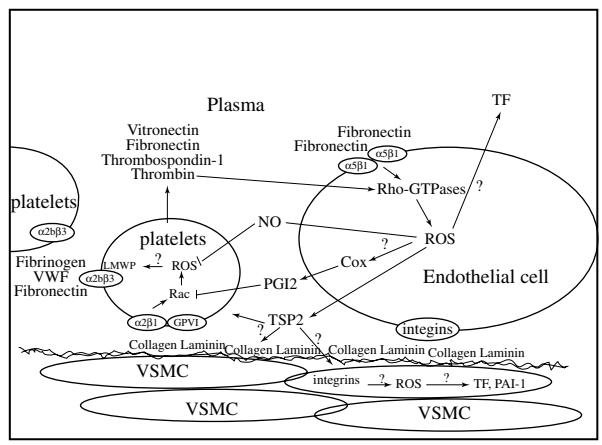

FIG. 5. Viewpoint of the involvement of ROS production in integrin-mediated aggregation and coagulation. LMWP, LMW phosphatase; TF, tissue factor; VSMC, vascular smooth muscle cells.

ROS and integrins display a complex interaction, including both inside-out and outside-in signaling during blood coagulation. Indeed, integrins orchestrate the spatiotemporal regulation of the Rho-GTPase family proteins, which are involved in different pathways of ROS production and lead to inside-out signaling. In endothelial cells, a thrombogenic effect seems to be mediated by Rho by limiting the barrier function (72), whereas it is mediated by Rac in platelets. In this situation, the specific role of ROS produced by different cells and stimulated by many circulating factors in addition to integrins is difficult to demonstrate fully. Additionally, the study of ROS is complex because they are small, quickly inactivated or diffused molecules and may stimulate different physiologic effects depending on their subcellular localization and concentration. Although available data relative to blood coagulation do not permit a complete understanding of the interaction of ROS and integrins, it represents an intriguing topic for further investigations. The involvement of ROS in the regulation of integrin during coagulation represents a promising target for therapeutic intervention suggested by initial success with integrin and ROS modulation with $\alpha_{2b}\beta_3$ antagonists and HMGCoA reductase inhibitors in myocardial infarction and stroke-related thrombosis. It thus seems evident that ROS production cannot be considered only as a product of the oxidative metabolism, but also as an important signaling model coordinating the cellular changes necessary for thrombosis and hemostasis.

ABBREVIATIONS

Cox, cyclooxygenase; DPI, diphenyleneiodonium; ECM, extracellular matrix; FAK, focal adhesion kinase; GPVI, glycoprotein VI; HMGCo-A, 3-hydroxy-3-methylglutaryl coenzyme A; H_2O_2, hydrogen peroxide; LMW, low molecular weight; Lox, lipoxygenase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PAK, p-21 activated kinase; PGI_2, prostaglandin I_2; PI-3K, phosphatidylinositol 3-kinase; PL, phospholipase; ROS, reactive oxygen species; TSP2, thrombospondin-2; vWf, von Willebrand factor.

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