ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 9 & 10, 2005
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Mini Review

Redox Regulation of the Coagulation Cascade

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ABSTRACT

Enhanced coagulation and thrombosis are linked to a variety of cardiovascular and metabolic diseases, as well as to cancer. Many of these diseases are also associated with enhanced levels of reactive oxygen species (ROS). Indeed, ROS have been made responsible for promoting many of these diseases. They have been shown not only to be cytotoxic, but also to serve as signaling molecules in a variety of cells. Recently, evidence accumulated that ROS and the redox state are also important in the control of blood coagulation and thrombosis. *Antioxid. Redox Signal.* 7, 1398–1404.

REACTIVE OXYGEN SPECIES (ROS) AND THE CELLULAR REDOX STATE—FROM TOXICITY TO SIGNALING

Transfer of one electron to O_2 results in the generation of superoxide anion radicals ($O_2^{-\bullet}$), which can be transformed further to other ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$). The formation of ROS is counterbalanced by a tightly regulated antioxidant defense system consisting of superoxide dismutases (SODs), glutathione peroxidases (GPXs), catalase, peroxiredoxins, thioredoxin, and exogenously taken up micronutrients and vitamins (20, 60). In mammalian cells, ROS can be formed in response to toxic reagents or as (by-)products of O_2-utilizing enzymes, such as NADPH oxidases, enzymes of the mitochondrial respiratory chain, the arachidonic acid pathway, the cytochrome p450 family, glucose oxidase, amino acid oxidases, xanthine oxidase, and uncoupled nitric oxide (NO) synthases (20, 60) (Fig. 1). In recent years, NADPH oxidases have gained increasing interest as sources of $O_2^{-\bullet}$ and its derivatives not only in phagocytes, where they are part of the innate immune system, but also in many nonphagocytic cells, including vascular cells, tumor cells, and platelets, where they contribute to low-level ROS production (38). These ROS and reduction–oxidation (redox) reactions play an important role not only in the intermediary metabolism, but also in the control of gene regulation by transcription factors, protein–protein interactions, and DNA synthesis (20). Indeed, modulation of ROS production has been shown to regulate a variety of genes, most of them linked to proliferation, migration, growth, and development, as well as to inflammation and chemotaxis (31, 49). In recent years, evidence accumulated that ROS and the redox state are able to affect the delicate balance between procoagulant, anticoagulant, and fibrinolytic systems and thus are actively involved in thrombotic diseases (28, 31, 34).

THE BLOOD COAGULATION CASCADE—A TIGHT BALANCE BETWEEN BLEEDING AND THROMBOSIS

The intact endothelial cell layer provides a nonthrombogenic surface that helps to prevent undesired hemostasis. Disruption of endothelial integrity leads to the development of an adhesive surface and the formation of intercellular gaps that allow the passage of soluble plasma and blood cells out of the vascular lumen into the underlying tissue (41). Platelets rapidly adhere to collagen fibrils in the vascular subendothelium, a process mediated mainly by integrin $\alpha 2\beta 1$ and glycoprotein (GP) Ib/IX. Subsequently, they aggregate and form an initial plug, thereby exposing cell-surface phospholipids that allow the assembly of blood-clotting enzyme complexes. When blood comes in contact with tissue factor (TF) in the subendothelial space, the extrinsic pathway of blood coagulation is initiated. TF binds to activated factor VII, re-

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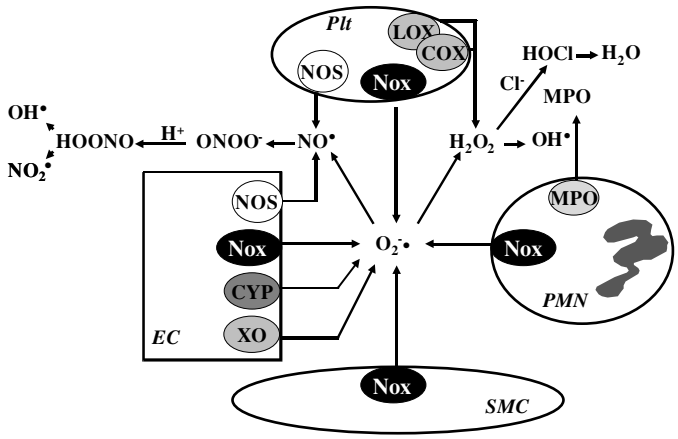

FIG. 1. ROS are generated by various sources in vascular cells. NADPH oxidases (Nox) contribute to superoxide anion radical ($O_2^{-\bullet}$) formation in polymorphonuclear neutrophils (PMN), platelets (Plt), endothelial cells (EC), and smooth muscle cells (SMC). In addition, the conversion of xanthine dehydrogenase to xanthine oxidase (XO), as well as cytochrome p450 monooxygenases (CYP), also generates ROS in EC, whereas lipoxygenases (LOX) and cyclooxygenases (COX) form ROS in platelets. Myeloperoxidase (MPO) is required for the generation of HOCl. NO synthases (NOS) produce NO ($NO^{\bullet}$), which readily reacts to peroxynitrite ($ONOO^-$) with $O_2^{-\bullet}$. NOS in the uncoupled state also generates ROS.

sulting in the activation of a cascade of coagulation factors leading to the conversion of prothrombin to thrombin. Thrombin cleaves fibrinogen to generate fibrin monomers, which polymerize and form a chemically stable clot. Thrombin also feeds back to activate cofactors VIII and V, thereby amplifying the coagulation process. Moreover, by binding to thrombin receptors on platelets, it contributes to the activation of platelets (52). This potentially explosive cascade is offset by different anticoagulant mechanisms. The maintenance of adequate blood flow and the regulation of cell-surface activity limit the local accumulation of activated blood-clotting enzymes and complexes. Moreover, antithrombin III inhibits the activity of the serine proteases of the intrinsic and common coagulation pathways, whereas thrombomodulin, which is bound to endothelial cells, allows thrombin activation of protein C. Finally, tissue-factor-pathway inhibitor (TFPI) is a lipoprotein-associated plasma protein that forms a complex with TF and activates factors VII and X, thereby inhibiting the extrinsic coagulation pathway (14).

Excess thrombus growth is also prevented by proteolytic degradation of fibrin (fibrinolysis), which is critical for preventing and restoring blood flow following thrombotic vascular occlusion. Fibrinolysis is mediated by plasminogen and its activators, tissue-type plasminogen activator (tPA), and urokinase (uPA) and is controlled by plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) superfamily that acts as the principal inhibitor of tPA and uPA in the fibrinolytic system (6). Thus, any disturbance to the fine balance between procoagulant and anticoagulant activities will lead to a prothrombotic state or a bleeding disorder, making the control of this balance a delicate physiological and pharmacological task.

ROS AND THE REDOX STATE IN THE CONTROL OF PLATELETS

Upon vascular injury and disruption of the endothelial cell layer, platelets adhere to the underlying extracellular matrix via two main adhesion receptors, GPIb-IX–V and GPVI, that bind von Willebrand factor and collagen, respectively, and that are primarily responsible for regulating this initial platelet adhesion and activation in flowing blood (2). Platelet aggregates then form by fibrinogen binding to the membrane receptor GPIIb/IIIa. When platelets become activated, they secrete a variety of mitogenic and growth factors and chemokines to promote tissue repair, including platelet-derived growth factor, transforming growth factor-β1, epidermal growth factor, and vascular endothelial growth factor. These factors can stimulate ROS production within the vessel wall mainly by activating vascular NADPH oxidases (3, 31). Enhanced ROS generation subsequently promotes adhesion and activation of neutrophils and platelets, as well as endothelial and smooth muscle cell proliferation. Moreover, the secretion products of activated platelets can induce TF expression via activation of the vascular NADPH oxidases and thus stimulate the extrinsic coagulation cascade (29).

Redox control of platelet functions

Platelets themselves provide important targets for ROS produced or released in the vascular lumen. *In vitro* and *in vivo* studies showed that low levels of oxidants may promote aggregation, whereas exposure to high concentrations of exogenous H_2O_2 may result in platelet inhibition (23). H_2O_2 can thereby induce Ca^{2+} release from agonist-sensitive stores by oxidation of sulfhydryl groups in the sarcoendoplasmic reticulum Ca^{2+}-ATPase and the inositol 1,3,5-trisphosphate (IP_3) receptors independently of IP_3 generation. In addition, H_2O_2 can also induce Ca^{2+} release from mitochondria (48). ROS can act on platelet function also indirectly via oxidative modification of low-density lipoproteins (LDL) or oxidation of lipids and their derivatives, as well as by interacting with NO, leading to the formation of $ONOO^-$ (23). Recently, it was shown that LDL that was oxidized by HOCl (HOCl-LDL) induced platelet function, aggregation, and α-granule secretion, suggesting that HOCl-LDL, which can be exposed during atherosclerotic plaque rupture, could contribute to the formation of thrombi (12). In contrast, oxidized LDL (oxLDL) concentration-dependently inhibited platelet aggregation in human platelets stimulated by collagen and arachidonic acid, but not by thrombin. This response was accompanied by markedly reduced $OH^{\bullet}$ formation in collagen-activated platelets, suggesting that oxLDL may induce radical–radical termination reactions by

promoting interaction of lipid radicals with free radicals (such as $OH^{\bullet}$) released from activated platelets. This would lower intracellular Ca^{2+} mobilization and inhibit thromboxane A_2 (TXA_2) formation and platelet aggregation (9). However, antioxidants, as well as resveratrol and other polyphenols that can antagonize lipoprotein oxidation, have been shown to prevent platelet aggregation in response to thiols, $ONOO^-$, peroxynitrite, metals, and other prooxidative agents (9, 45, 46). Moreover, treatment with resveratrol significantly reduced the size of laser-induced thrombi in apolipoprotein E−/−/LDL receptor−/− mice on a high-fat diet (26), further supporting the view that redox activation of platelets may play an important role under pathological conditions associated with oxidative stress such as hypercholesterolemia, a major risk factor of atherosclerosis (23). Indeed, it was recently found that hypercholesterolemia promoted a P-selectin-dependent interaction between platelets and the vessel wall in the brain that was dependent on ROS formation by the NADPH oxidase and exacerbated by ischemia/reperfusion (35).

Redox activation of platelets has also been found in hyperglycemia associated with diabetes (24). Recently, in thalassemia, platelets have been found to undergo a state of oxidative stress, which leads to their activation and potentially to thromboembolic consequences (1).

Moreover, it was recently shown that *in vivo* lipid peroxidation and platelet activation were enhanced in dyspeptic individuals with *Helicobacter pylori* infection. This was mediated at least in part by F_2-isoprostane formation and cyclooxygenase 1 activation, suggesting that enhanced generation of bioactive isoeicosanoids may transduce the oxidant signal associated with a variety of cardiovascular risk factors into a functional platelet response, possibly contributing to an enhanced thrombotic risk (15).

In contrast, heme oxygenase-1 (HO-1), which produces carbon monoxide (CO), has been shown to prevent platelet aggregation under oxidative stress conditions (47) and has been suggested also to act as an antioxidant. These observations were confirmed by a study in microvessels of mouse cremaster muscle preparations where ferric chloride-induced thrombus formation was delayed in mice pretreated with an intraperitoneal injection of hemin, which induces HO-1. Blockade of HO-1 induction prevented delayed thrombus formation, whereas coadministration of the vitamin E analogue Trolox in HO-1-blocked animals almost completely restored the delay in thrombus formation, implying that, besides CO, the HO-1 pathway metabolite bilirubin mainly contributes to the antithrombotic property of HO-1 (39).

The importance of the redox state for platelet activation was further supported by findings where reduced glutathione (GSH) or a mixture of GSH/glutathione disulfide (GSSG) potentiated platelet aggregation. GSSG added to platelets alone also potentiated platelet aggregation because most of the GSSG was converted to GSH by a flavoprotein-dependent platelet surface mechanism that provided an appropriate redox potential for platelet activation. The addition of GSSG to platelets generated sulfhydryls in the β-subunit of the $\alpha_{IIb}\beta_3$ fibrinogen receptor, which may facilitate platelet activation (22).

One important mechanism of how the redox state controls platelet activation and adhesion is via the redox cofactor protein disulfide isomerase (PDI), which is involved in disulfide-bond formation and isomerization. PDI and sulfhydryl groups are present on the platelet surface and provide redox-sensitive sites that regulate platelet aggregation and secretion, as well as activation of platelet integrin receptors (21). PDI has recently been shown to be sensitive to *S*-nitrosothiols (RSNOs). PDI can denitrosate RSNOs, thereby releasing NO that, via the guanylate cyclase/G-kinase route, attenuates platelet activation. In addition, RSNOs are denitrosated at the same PDI-active site that catalyzes the disulfide bond formation between integrins and their ligands, thereby attenuating activation, adhesion, and aggregation (50). Recently, an additional thiol isomerase enzyme, endoplasmic reticulum protein 5 (ERP5), has been characterized in platelets (36). ERP5 is resident mainly on platelet intracellular membranes, although it is rapidly recruited to the cell surface in response to a range of platelet agonists. ERP5 becomes physically associated with the integrin β_3 subunit during platelet stimulation. Blocking cell-surface ERP5 leads to decreased platelet aggregation, fibrinogen binding, and P-selectin exposure possibly due to the disruption of integrin function.

Many members of the integrin family play a central role in hemostasis, because they control not only adhesion and aggregation of platelets, but also the contractility and barrier function of endothelial cells, thereby acting as key elements in the interplay between the coagulation system and the vascular wall. Whereas on the one hand integrin activity can be controlled by ROS, these receptors have also been shown to generate ROS—possibly via activation of vascular NADPH oxidases and lipoxygenases—and to activate redox-sensitive signaling cascades, thereby modulating the thrombotic balance (33). Recently, it was also found that the cross-talk between different integrins on monocytes, which is crucial for a coordinated regulation of the cellular adhesion during the complex process of transendothelial migration, is mediated by ROS and the NADPH oxidase (10).

Platelets generate ROS via an NADPH oxidase

In addition to the role of ROS and the redox state on platelet activation and adhesion, platelets themselves have been shown to produce ROS. Platelets contain, similar to neutrophils, a gp91phox-containing NADPH oxidase (8), which is involved in the control of TXA_2 production and thrombin-induced platelet aggregation. Activation of platelet NADPH oxidase requires phosphatidylinositol 3-kinase and protein kinase Cζ (PKCζ) with subsequent translocation of the cytosolic subunit p47phox to the plasma membrane (11, 59). Platelet ROS formation was shown to potentiate neutrophil ROS production, suggesting that the proinflammatory effects frequently associated with coagulation (13) may be at least partially due to the promotion of neutrophil ROS production (8). In contrast, strenuous, acute exercise suppressed platelet-promoted oxidative burst of neutrophils possibly by reducing phosphorylation of PKCζ, p47phox phosphorylation and membrane translocation, thus inhibiting the assembly and activation of the NADPH oxidase in neutrophils. These findings provide an explanation for the beneficial effects of physical exercise on cardiovascular function (59). On the other hand,

platelet NADPH oxidase activity increased vascular cell apoptosis (59). Interestingly, compared with healthy individuals, patients with hypertension showed a greater production of $O_2^{-\bullet}$ by platelet NADPH oxidase, which was not correlated with blood pressure, but was mediated by activation of the angiotensin I receptor (27).

Moreover, tamoxifen, which is used to prevent and treat breast cancer, is known to enhance the risk of thrombosis. In a recent study, it was shown that tamoxifen increased platelet $O_2^{-\bullet}$ release and enhanced the functional activation of NADPH oxidase as determined by phosphorylation of its subunits p47phox and p67phox, thus providing an explanation for previous oncological studies demonstrating tamoxifen-dependent increase in ROS generation (56). In addition, GPIIb/IIIa inhibitors abolished not only platelet aggregation, but also platelet $O_2^{-\bullet}$ release, whereas stimulation-dependent NO release was significantly enhanced. Preincubation with GPIIb/IIIa inhibitors also modified aggregation-induced membrane translocation of the platelet endothelial NO synthase and the NADPH oxidase subunits p67phox and p47phox. These observations suggest that GPIIb/IIIa antagonists may not only inhibit aggregation, but also limit platelet functions related to ROS generation (7). In contrast, a recent study showed that a platelet GPIIIa antibody activated platelet NADPH oxidase via the 12-lipoxygenase product, 12(*S*)-hydroxyeicosatetraenoic acid, resulting in thrombocytopenia and ROS-induced platelet fragmentation (42).

ROS AND THE REDOX STATE IN THE CONTROL OF THE EXTRINSIC COAGULATION CASCADE

Activation of the extrinsic coagulation cascade is mediated by TF. The expression of TF has been shown to be redox-sensitive in different cell types (34). Moreover, thrombin, the downstream product of activated TF, can activate ROS production in vascular cells by stimulating NADPH oxidases (31). These ROS serve as signaling molecules mediating a variety of responses in thrombin-stimulated vascular cells. In a recent report, we could show that thrombin induces a biphasic increase in ROS production in endothelial cells whereby ROS derived from thrombin-stimulated NADPH oxidase could further up-regulate the expression of the NADPH oxidase subunit p22phox in a kinase-dependent manner (19). These findings suggest that thrombin activation of NADPH oxidases may contribute to prolonged ROS generation at sites of vascular injury. Moreover, thrombin itself can induce and activate TF in a ROS-dependent manner involving NADPH oxidases in vascular cells (34). NADPH oxidase-mediated TF up-regulation by thrombin was shown to be mediated by the redox-sensitive transcription factor nuclear factor-κB (NFκB) (17). A role for NFκB in TF expression has also been shown in response to activated liver X receptors, which suppressed TF expression induced by proinflammatory stimuli in monocytes (54). In contrast, in human endothelial cells stimulated simultaneously with tumor necrosis factor-α and thrombin, the up-regulation of TF was mediated by c-Jun and c-Fos, but not by NFκB, indicating that cell- and tissue-specific pathways are involved in the regulation of TF (40).

An *in vitro* study demonstrated that CD40L-stimulated monocytes from patients with hypercholesterolemia expressed more TF and thrombin than monocytes from controls, and that co-incubation of monocytes with an inhibitor of NADPH oxidases significantly reduced CD40L-mediated clotting activation. Interestingly, a marked inhibition of CD40L-mediated clotting activation was also observed in two male patients with hereditary deficiency of the NADPH oxidase subunit gp91phox, thus confirming the pivotal role of NADPH oxidases in the control of coagulation (51).

Moreover, enhanced ROS production and NADPH oxidase expression were found to be accompanied by elevated levels of TF, as well as of the endogenous TF inhibitor TFPI, in atherosclerotic plaques in patients with coronary artery disease, suggesting a balance between pro- and anticoagulant factors (3, 5, 16, 37). However, when oxLDL was present, the anticoagulant activity of TFPI was impaired due to direct interaction of oxidized phospholipids with the protein (44), thus providing a further mechanism of how an oxidizing environment can lead to a prothrombotic state in the vasculature.

PAI-1–A REDOX-SENSITIVE INHIBITOR OF FIBRINOLYSIS

In addition to increased procoagulant activity, also decreased fibrinolytic activity may result in a thrombotic phenotype. Enhanced levels of the fibrinolysis inhibitor PAI-1 have been associated with thrombosis, many cardiovascular diseases, and cancer. ROS and reduced oxygen levels as observed in ischemic or thrombotic diseases have been shown to play an important role in regulating the expression of PAI-1 in different cell systems (16). Antioxidants and depletion of NADPH oxidase subunits inhibited PAI-1 expression in smooth muscle cells in response to thrombin (30), as well as to the vasoactive peptide urotensin-II (18). Redox sensitivity of PAI-1 expression was also found in response to angiotensin II in vascular smooth muscle cells (61) and to oxLDL in endothelial cells (43). However, under hypoxia, overexpression of a constitutively active mutant of Rac, known to stimulate the NADPH oxidase, prevented hypoxia-induced PAI-1 expression in HepG2 hepatoma cells (32), further supporting an important role of ROS and the NADPH oxidases in the regulation of PAI-1 by various stimuli.

Redox sensitivity of PAI-1 expression was also confirmed *in vivo*. In aged OLETF (Otsuka Long–Evans Tokushima Fatty) rats, which model obese type 2 diabetes, enhanced levels of advanced glycation end products (AGE), the lipid peroxidation product 4-hydroxy-2-nonenal (HNE), and PAI-1 were observed. *In vitro* studies confirmed redox sensitivity of PAI-1 induction in response to HNE and AGE in adipocytes (55). Consistently, overexpression of Cu/Zn SOD, which has been shown to increase ROS levels (4), in mouse hepatocytes enhanced PAI-1 expression compared with wild-type mice. However, overexpression of GPX, which has been shown to decrease ROS levels (4), up-regulated PAI-1 expression in

mouse hepatocytes (25). Although the reasons for these conflicting observations are not completely clarified, one may speculate that reduced ROS production, as with enhanced GPX levels, may mimic the situation under hypoxia. However, a recent study showed that treatment with vitamins C and E decreased malondialdehyde levels as markers of lipid oxidation, whereas PAI-1 levels remained unchanged (53). In contrast, in premature rat lungs exposed to prolonged hyperoxia during the saccular stage of development, oxidative stress was associated with increased expression of PAI-1, suggesting a complex regulatory role of ROS in PAI-1 expression *in vivo* (58).

Whereas redox-sensitive PAI-1 expression in response to hypoxia and thrombin has been related to activation of the hypoxia-inducible transcription factor HIF-1 (30, 32), oxidative stress and insulin enhanced PAI-1 transcription via activation of activator protein-1 in GH4 cells (57), further confirming a complex, cell type-specific mechanism of ROS activation of PAI-1.

In summary, the redox state and ROS generation are importantly involved in controlling the coagulation system at multiple sites in a cell type- and stimulus-dependent manner. Platelet activation and function are modulated by ROS, and platelets themselves are able to generate ROS, thus contributing to the pathogenesis of many disorders associated with oxidative stress. Moreover, ROS can also be generated by vascular cells in response to coagulation factors and activated platelets, thereby affecting expression and interaction of proteins of the coagulation cascade. Thus, ROS act as key players in mediating the interaction between the coagulation system and the vessel wall. This interaction may promote a hypercoaguable state in the blood, but may also provide an essential mechanism of how coagulation factors and platelets activate the vessel wall or other organs by modulating gene expression, inflammatory or chemotactic responses, proliferation, or angiogenesis. The elucidation of the complex involvement of ROS on the diverse functions associated with hemostasis, thrombosis, and fibrinolysis is still not complete, but should provide insights to target thrombosis and thrombosis-related diseases.

ACKNOWLEDGMENTS

This work was supported by Deutsche Forschungsgemeinschaft GO 709/4-1 and by the 6th framework program of the European Commission (EUROXY).

ABBREVIATIONS

AGE, advanced glycation end product; CO, carbon monoxide; ERP5, endoplasmic reticulum protein 5; GP, glycoprotein; GPX, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; HNE, 4-hydroxy-2-nonenal; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; IP_3, inositol 1,3,5-trisphosphate; LDL, low-density lipoprotein; NFκB, nuclear factor-κB; NO, nitric oxide; $O_2^{-\bullet}$, superoxide anion radical; $OH^{\bullet}$, hydroxyl radical; $ONOO^-$, peroxynitrite; oxLDL, oxidized low-density lipoprotein; PAI-1, plasminogen activator inhibitor-1; PDI, protein disulfide isomerase; Phox, phagocyte oxidase; PKC, protein kinase C; ROS, reactive oxygen species; RSNO, *S*-nitrosothiol; SOD, superoxide dismutase; TF, tissue factor; TFPI, tissue-factor-pathway inhibitor; tPA, tissue-type plasminogen activator; TXA_2, thromboxane A_2; uPA, urokinase.

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Received for publication April 11, 2005; accepted April 11, 2005.

This article has been cited by:

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