Review

Reactive oxygen metabolites and arterial thrombosis

Giuseppe Ambrosio a,*, Isabella Tritto a, Paolo Golino b

a Division of Cardiology, University of Perugia School of Medicine, Perugia, Italy
b Federico II School of Medicine, Naples, Italy

Received 14 October 1996; accepted 27 March 1997

Abstract

Arterial thrombus formation is the result of complex events which require the interaction of damaged vessel walls with blood cellular elements and coagulation factors, and in which several mediators may play a role. In this context, the role of ‘classical’ chemical mediators such as thrombin, thromboxane or serotonin in initiating and/or amplifying intravascular thrombus formation is well established. However, it is now being recognized that certain chemical species formed in the metabolism of oxygen may also be involved in the process of arterial thrombosis. This review will focus on recent evidence in this field.

1. Characteristics of reactive oxygen metabolites

Aside from H₂O₂ and CO₂, which can be considered chemically ‘inert’, metabolism of O₂ can give rise to various species which instead are extremely reactive [1]. One major category is represented by oxygen radicals, which are chemical species characterized by the presence of an unpaired electron. Oxygen radicals can attack and oxidize most cell components. This, in turn, may have profound effects on cell structure and function. Small amounts of superoxide radicals (‘O₂⁻’) are continuously formed in vivo, a major source being mitochondrial respiration [1,2]. Generation of superoxide has also been associated with certain steps in prostaglandin synthesis, particularly with the activity of prostaglandin hydroperoxidase [3]. Once formed, superoxide radicals can give rise to hydrogen peroxide (H₂O₂), a non-radical yet oxidant species, and to the powerful oxidant hydroxyl radical (‘OH). Leukocytes are another major source of oxidants, because upon activation they generate H₂O₂, ‘O₂⁻, and hypochlorous acid [4].

In addition to being produced in small amounts under physiologic conditions, large amounts of reactive oxygen metabolites can be released in the vascular lumen following restoration of flow after a period of ischemia [5,6]. It has been proposed that this oxidant load might be responsible for a specific form of reperfusion-mediated tissue injury, secondary to lipid peroxidation and other irreversible alterations of cell constituents [7–9]. However, it is now appreciated that, apart from inducing gross cell damage, oxidants might also exert other effects which may be mediated through activation or inhibition of various enzymes [10]. Some of these effects may play a role in intravascular thrombosis, and oxidants may influence the process of thrombus formation by interfering at multiple steps with its various components (i.e., platelets, vessel wall, and coagulation factors), as discussed below.

2. Effects of oxidants on platelet function

Platelets, a major player in thrombus formation, are obviously a prime target for oxidants produced or released in the vascular lumen and, at the same time, they are also capable of endogenous generation of oxidants [11,12]. Thus, it is important to characterize the effects of oxidants on platelet function. Earlier studies investigating the effects of oxidants on platelet aggregation in vitro reached conflicting conclusions. While several investigators described an inhibitory effect of oxidants on platelets, other

* Corresponding author. Sezione di Cardiologia ‘R’, Dipartimento di Medicina Clinica, Via Euginia 42, 06122 Perugia, Italy. Tel.: +39-75-5853842; fax: +39-75-5858840; e-mail: cardiopg@unipg.it

Time for primary review 28 days.
reports suggest that reactive oxygen species might actually enhance aggregation. Which oxidant species was responsible for the reported effects was also debated. Much of the controversy is probably related to differences in the experimental conditions employed in those studies, such as concentration and chemical nature of oxidants tested, or the effects of plasma components. Furthermore, it is now clear that oxidants can affect several key steps of platelet function, and therefore their net effect on the final process of aggregation may depend on various factors.

2.1. Role of different oxidant species

As already mentioned, earlier studies have investigated the effects of reactive oxygen metabolites on platelet aggregation. Several investigators observed inhibition of platelet function upon exposure to $\text{H}_2\text{O}_2$ [13–15], while others suggested that it might actually enhance aggregation [12,16,17]. Furthermore, studies can also be found that indicate that exposure to $\text{H}_2\text{O}_2$ has no effects on platelets, and that instead superoxide radicals may enhance aggregation [18–20]. Much of the controversy seems attributable to important differences in the experimental protocol, as discussed below.

In the attempt to clarify these discrepancies, we have recently conducted experiments aimed at systematically assessing the effects of different oxidant species on platelet function under pathophysiologically relevant conditions [21]. To this goal, we studied platelets in the presence of plasma, since this experimental model more closely resembles the physiological condition. In this system we investigated separately the effects of either superoxide radicals or $\text{H}_2\text{O}_2$, at biologically relevant concentrations. Platelet function was studied after a 60 s exposure to exogenously generated oxidants at a flux of 15–30 nmol/min. This protocol was designed to reproduce the duration and magnitude of oxidative stress as it may occur in vivo [5,22]. It was found that $\text{H}_2\text{O}_2$, but not superoxide radical, can profoundly yet reversibly impair platelet aggregation [22] (Fig. 1). With respect to previous studies, our data confirm that $\text{H}_2\text{O}_2$ has an inhibitory role when platelets are exposed to concentrations of this oxidant comparable to those achieved during oxidative stress [13–15]. As for the lack of effect of superoxide radical, it should be pointed out that studies showing a pro-aggregatory role of this species typically used washed or gel-filtered platelets resuspended in buffer [18–20], that is a system lacking the endogenous scavengers which are physiologically present in plasma. Our data [21] and experiments by Salvemini et al. [20] demonstrate instead that the aggregatory action of superoxide radical is lost when platelets are resuspended in plasma, thus indicating that the effects of superoxide radical on platelets may have limited pathophysiological relevance.

2.2. Role of cyclic nucleotides

A major mechanism controlling platelet aggregation is represented by the activity of adenylate cyclase and of guanylate cyclase. Exposure to oxidants of plasma-resuspended platelets had no effects on basal cAMP levels [21]. Similarly, cAMP concentration showed the expected decrease following stimulation [21]. In contrast, impaired aggregation by $\text{H}_2\text{O}_2$ was accompanied by a > 10-fold increase in platelet concentration of cGMP [21], consistent with the notion that $\text{H}_2\text{O}_2$ can stimulate guanylate cyclase in other cell types [23] and that stimulation of cGMP...
production can exert inhibitory effects on platelets in response to various agents, such as prostacyclin and nitrates [20,24,25]. That stimulation of guanylate cyclase was responsible for the inhibitory effect of H₂O₂ seen in our study is further supported by the observation that exposure to H₂O₂ of platelets that had been preincubated with the inhibitor of guanylate cyclase LY-83583 was associated with normal aggregatory response [21].

2.3. Role of cyclooxygenase

The activity of cyclooxygenase (the key enzyme of prostanoid metabolism) can be importantly affected by oxidants. This enzyme has the peculiarity of catalyzing two different reactions: oxygenation of arachidonic acid to oxidants. This enzyme has the peculiarity of catalyzing prostanoid metabolism can be importantly affected by oxidants on platelet cyclooxygenase activity.

Under our experimental conditions, superoxide radicals had no effects on platelet synthesis of thromboxane. In contrast, H₂O₂ played an important role. In fact, arachidonic acid-dependent aggregation was almost completely suppressed in the presence of catalase (a scavenger of H₂O₂) [21]. Furthermore, exogenously administered hydrogen peroxide markedly shortened the lag phase that precedes aggregation by arachidonic acid [21]. This lag phase corresponds to the lag phase between addition of arachidonate and synthesis of prostanoids demonstrated in partially purified preparation of cyclooxygenase, which can similarly be abolished by catalase or enhanced by peroxides [3,26]. Low-level production of hydrogen peroxide seems therefore necessary to promote thromboxane synthesis, and hence platelet aggregation, in response to arachidonic acid stimulation. This conclusion is also supported by data by Del Principe et al. [12,16] and by Praticò et al. [17], who showed that low concentrations of hydrogen peroxide could potentiate the aggregatory response to arachidonic acid or collagen.

In summary, the effects of reactive oxygen metabolites on in vitro platelet function are complex. Low levels of H₂O₂ may promote thromboxane synthesis and aggregation, whereas exposure to larger (albeit not toxic) concentrations of exogenous H₂O₂ may inhibit aggregation to several agonists via stimulation of guanylate cyclase and increased cyclic GMP formation.

2.4. Role of other platelet agonists

Another possible mechanism by which oxidants may influence platelet aggregation is through potentiation of the effects of platelet-activating factor (PAF). PAF is an autacoid released by platelets and other cell types (e.g., endothelium, leukocytes), which acts on platelets at extremely low concentrations (10⁻¹¹–10⁻¹⁰ M). We have recently shown that superoxide and hydroxyl radicals can rapidly (i.e., within seconds) and irreversibly inactivate plasma PAF-acetylhydrolase, the enzyme that catabolizes PAF [27] (Fig. 2). Once PAF is formed or released in the blood, inhibition of PAF-acetylhydrolase would enhance concentrations and prolong half-life of this powerful agonist. Consistent with this hypothesis is the observation that neutrophil recruitment and activation induced by PAF in the mesenteric circulation is substantially reduced by administration of scavengers [28]. Thus, it may be speculated that oxygen radicals may indirectly enhance platelet aggregation, through local increases in PAF concentrations and in vivo breakdown of PAF. In vivo this hypothesis is indirectly supported by data derived from a dog study reviewed below [29], in which administration of superoxide dismutase was associated with a significant reduction in PAF-mediated aggregation of platelets suspended in plasma, consistent with preserved activity of plasma acetylhydrolase.

2.5. Comparison of in vitro vs. in vivo effects of oxidants on platelets

The complexity of the effects of reactive oxygen metabolites on platelets observed in vitro is reflected also in experiments conducted in vivo. The role of endogenous oxidants on intravascular thrombus formation has recently been examined in a canine model of coronary artery stenosis and endothelial injury [29,30]. In this model, which mimics the situation potentially encountered in patients with unstable angina, episodes of gradual decrease in flow develop cyclically as a consequence of recurrent platelet aggregation/thrombus formation at the site of
stenosis. Administration of the scavengers superoxide dismutase and catalase abolished these cyclic flow reductions in the majority of the animals. Compared with our in vitro data, these observations would confirm that under certain circumstances low concentrations of endogenously produced oxidants are required to promote platelet aggregation. However, it should also be noted that in one study administration of superoxide dismutase to those animals in which catalase failed, worsened cyclic flow reductions, indicating a more complex role of oxidants in intracoronary thrombus formation [29].

The role of exposure to exogenously administered oxidants on in vivo platelet aggregation has also been recently evaluated. In the same study by Willerson and associates [29], when a coronary artery stenosis was created without inducing a concomitant injury to the arterial wall, cyclic flow reductions could not be spontaneously induced. Yet, infusion of an enzymatic system that generates both superoxide anion and hydrogen peroxide promptly induced cyclic flow reductions [29]. However, it is also possible that those thrombogenic effects were related to oxidant-mediated injury of the endothelial cell layer (as suggested by histological examination of the vessels), rather than to a direct stimulating effect on platelets. A possible inhibitory role of exogenous hydrogen peroxide on platelet aggregation/thrombus formation in vivo is suggested by observations performed in our laboratory. In a rabbit model of carotid artery stenosis and endothelial injury, similar to the canine model previously described, cyclic flow reductions spontaneously developed at the site of endothelial injury. These spontaneous episodes of intravascular platelet aggregation could be abolished in all animals by local infusion of \( \text{H}_2\text{O}_2 \) [31] (Fig. 3).

![Fig. 3. Effects of hydrogen peroxide infusion on blood flow in rabbit carotid arteries with a stenosis and endothelial injury. Data are from 31. Open bars represent baseline values. Hatched bars are data obtained during intracarotid infusion of hydrogen peroxide (average dose 6 \( \mu \text{mol/min} \)). Solid bars are data obtained 10 min into washout. Left panel: Effects of hydrogen peroxide on the number of episodes of flow reduction, measured as cycles/hour. Right panel: Effects of hydrogen peroxide on peak carotid flow, measured as percent of normal values. Note that \( \text{H}_2\text{O}_2 \) infusion abolished cyclic flow reductions (caused by intravascular platelet thrombus formation) and restored normal flow. This antiaggregatory effect was lost on washout. * \( P < 0.05 \) vs. baseline and washout.]

Taken together, the in vivo data are consistent with two major observations obtained in in vitro platelet studies, namely that low levels of oxidants may promote aggregation [12,16–21] whereas exposure to high concentrations of exogenous \( \text{H}_2\text{O}_2 \) may result in platelet inhibition [13–15,21]. However, the in vivo situation is clearly more complex, since the net effect of oxidants on intravascular thrombosis is also dependent on the integrity of the endothelium, as well as on oxidant-mediated alterations of other major players of thrombosis, such as endothelial-derived relaxing factor and coagulation factors. These issues will also be briefly reviewed.

3. Effects of oxidants on the vessel wall

Under physiologic conditions the vascular endothelium plays a pivotal role in inhibiting intravascular thrombus formation, since a dynamic interaction exists at the endothelial cell surface between mechanisms which respectively inhibit or promote thrombus formation. Endothelial cells can inhibit intravascular thrombosis by synthesizing various substances, such as thrombomodulin, tissue factor-pathway inhibitor, prostacyclin, tissue plasminogen activator [32], which inhibit the coagulation cascade and platelet function, or which activate fibrinolysis. Disruption of endothelial integrity by oxidant attack will grossly impair these activities. However, oxidants may also influence the anti-thrombotic properties of the endothelium in a more specific way, even at relatively low concentrations. This may happen through interaction with components of the coagulation system (as described in the next section), or with endothelial-derived relaxing factor (EDRF). Endothelial cells release EDRF also in the vascular lumen. In vitro, EDRF has been shown to exert antiplatelet effects via stimulation of guanylate cyclase [25]. That endogenous EDRF is important in counteracting platelet aggregation has been directly demonstrated in vivo by studies in which stimulation of EDRF release by intra-arterial infusion of acetylcholine inhibited intravascular thrombus formation at sites of arterial stenosis and endothelial injury [33,34]. Furthermore, in one of these studies we were able to show that administration of L-NMMA (an inhibitor of endogenous EDRF production) to animals in which cyclic flow reductions had been previously abolished with aspirin was associated with restoration of cyclic flow reductions [33], thus demonstrating the importance of EDRF in modulating intravascular platelet aggregation in vivo. There are reasons to believe that EDRF could be another potential target for the effects of oxidants.

Superoxide anions have been implicated in the breakdown of EDRF. In vitro, the half-life of EDRF is significantly shortened in the presence of superoxide radicals and, conversely, superoxide dismutase has been demonstrated to prolong EDRF half-life [35,36]. Indirect support to the involvement of oxygen radicals in reducing the
effects of EDRF in vivo comes from the finding that formation of active oxygen species during reperfusion of ischemic myocardium causes vessel dysfunction and that superoxide dismutase protects the vasodilator ability of coronary vessels [37], and from the observation that release of active EDRF depends on preserved endothelial superoxide dismutase activity [38]. Taken together, these findings would suggest that active oxygen species might facilitate intravascular thrombus formation by reducing the antiplatelet effects of EDRF.

4. Effects of oxidants on the coagulation cascade

As previously stated, under normal conditions endothelial cells synthesize a variety of substances which may inhibit intravascular thrombus formation. In addition, since the endothelium is in contact with circulating blood, synthesis and expression of one important component of the coagulation pathway, ‘tissue factor’ (TF), is normally suppressed in these cells [32]. The result of these regulatory mechanisms is a tight control of the coagulation system, such that unwanted intravascular thrombus formation is normally inhibited. TF is a 47-kDa membrane-bound glycoprotein essential for activation of the extrinsic coagulation pathway. TF forms a complex with coagulation factors VII and VIIa, allowing enzymatic activation of factors X and IX, the substrates for factor VIIa [39], ultimately leading to the generation of thrombin. The importance of TF in triggering intravascular thrombus formation in vivo has been directly shown in a recent study in which endothelial disruption at the site of arterial stenosis, with its attendant exposure of TF present in the subendothelium, resulted in intravascular thrombus formation via direct activation of the extrinsic coagulation pathway [40].

To protect against this unwanted intravascular activation of the coagulation system, normal endothelium lacks TF activity [32,41]. However, several stimuli can affect anticoagulant properties of the endothelium by inducing TF expression on the membrane of endothelial cells [42,43]. Endothelial cells represent both a source and a possible target of oxidants released in the vasculature [44]. At the same time, oxidants are known to activate nuclear transcription factors [45]. Thus, we felt it was important to determine whether oxygen radicals would also promote de novo synthesis of TF activity by endothelial cells. To test this hypothesis, endothelial cells were exposed to exogenously generated oxygen radicals. A brief period of exposure to oxygen radicals resulted in a significant increase in TF mRNA levels, accompanied by appearance of large TF procoagulant activity [46] (Fig. 4). This phenomenon was not confined to endothelial cells in vitro. In rabbits subjected to coronary artery occlusion and reperfusion, a condition associated with endogenous production of large amounts of oxygen radicals, we detected a marked increase in tissue factor activity in the coronary circulation [46]. Importantly, this phenomenon was accompanied by a significant reduction of myocardial perfusion, and it could be abolished by oxygen radical scavengers [46]. We speculate that this oxygen radical-mediated TF expression by endothelial cells, with its attendant activation of the extrinsic coagulation pathway, may have important consequences as it might impact on the pathophysiology of post-ischemic reperfusion.

5. Effects of oxidants on anti-coagulant factors

In addition to inducing TF expression in endothelial cells, active oxygen species might promote intravascular thrombus formation also by interfering with mechanisms that normally inhibit activation of the coagulation pathway. Earlier studies have shown that lipid peroxides can increase the amount of thrombin produced and can slow down the rate of thrombin decay [47]. Both effects are

![Fig. 4. Effects of oxidants on tissue factor mRNA levels and activity in cultured endothelial cells (data are from [46]). Oxidants were generated by 5 min incubation with xanthine/xanthine oxidase (X/XO; 400 μM and 20 mU/ml, respectively). Left panel: Northern blot of RNA isolated from control (left) and oxygen radical-treated (right) cells. TF mRNA was almost undetectable in control cells, but it markedly increased following exposure to oxygen radicals. Glyceraldehyde-phosphate dehydrogenase (GADPH) mRNA was used as internal control. Right panel: TF activity, measured by the amount of factor Xa generated. TF activity was almost undetectable in controls cells. Incubation of cells with X/XO for 1 or 5 min resulted in a marked increase in TF activity. Cells simultaneously incubated with AP-1, a monoclonal antibody against TF, showed values similar to controls. ND = undetectable. *P < 0.05 vs. controls.](image-url)
consequent to inhibition of plasma antithrombin by lipid peroxides formed as a consequence of oxygen radical attack to circulating lipoproteins [48]. Similar susceptibility to oxidant-mediated inactivation has been reported for other key anti-thrombotic factors, such as alpha-2-antiplasmin [49], plasminogen activator [50], and thrombomodulin [51]. More recently, in a preliminary study we have shown that endothelial cell exposed to oxygen radicals exhibit, in addition to the induction of TF procoagulant activity, a concomitant marked decrease of tissue factor-pathway inhibitor (TFPI) activity to almost undetectable levels [52]. TFPI is a protein synthesized by endothelial cells that inhibits the extrinsic coagulation pathway.

6. Leukocytes and thrombus formation

It has long been known that leukocytes could be found within thrombi. However, the prevailing theory was that leukocytes take no part in the coagulation process, but they are merely trapped by the polymerization of fibrin. This ‘mechanical’ theory to explain the association of leukocytes with thrombi has recently been challenged. In an elegant experiment, Palabrica et al. [53] demonstrated that leukocyte accumulation within thrombi is in fact a cellular adhesion-mediated event. According to the results of that study, platelets which accumulate in the thrombus rapidly express the adhesion molecule P-selectin on their surface, thereby inducing adherence of circulating monocytes and neutrophils. This would lead to a chain of events culminating in tissue factor-mediated local fibrin deposition.

The study by Palabrica et al. [53] was concerned with the interaction between leukocytes and platelets already trapped in the thrombus. However, these two cell types can adhere and ‘cross-talk’ to each other also under dynamic conditions, both in vitro [54] and in vivo [55]. The net effect of this phenomenon on the coagulation system is difficult to predict, as certain secretory products of activated neutrophils can inhibit platelet aggregation (e.g., hydrogen peroxide [13–15,21], nitric oxide [25], lipoxynase metabolites [56]), while others have stimulatory effects (PAF [27], cathepsin G [57]). However, it is possible that under certain conditions intravascular leukocyte activation may contribute to thrombus formation. In the clinical setting, leukocyte-platelet aggregates have been found in the blood of patients with unstable angina [55], and it has been suggested that activation of leukocytes may contribute to intravascular coagulation in patients with septicemia [58].

7. Conclusions

In summary, reactive oxygen metabolites might affect thrombus formation within the vasculature through several mechanisms. Oxidants may enhance the activity of the extrinsic coagulation cascade, ultimately leading to thrombin formation, via their combined effects on stimulation of tissue factor activity and inhibition of fibrinolytic pathways. At the same time, oxidants may have complex effects of platelets, since while hydrogen peroxide acts to inhibit aggregation superoxide radical may inactivate EDRF and PAF-acetylhydrolase, and it may also enhance thrombin formation (via TF), all events which would promote aggregation. The net result of all these effects may depend on a number of factors, the relative importance of which is not easily dissected out and will require much further investigation. Similarly, further studies are warranted to investigate whether the oxidant load which may occur upon postischemic reflow might contribute at least in part to the high incidence of reocclusion frequently seen in patients with acute myocardial infarction undergoing thrombolytic therapy, and whether enhanced oxidative stress is part of the picture of patients with unstable angina.

Acknowledgements

Supported in part by grant No. 96.03489.CT04, from Consiglio Nazionale delle Ricerche, Italy.

References


Barrowcliffe TW, Gutteridge JMC, Dormann TL. The effects of


