

## Oxidative Stress and Platelets

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**Abstract**—Platelet-dependent thrombus formation may be influenced by alteration of platelet or vascular redox state, the presence of endogenous or exogenous antioxidants, as well as the formation of reactive oxygen and nitrogen species. Specifically, settings and pathways that influence the formation of superoxide and nitric oxide, as well as their metabolism, may influence platelet function and thrombus formation. Although some antioxidant regimens have been associated with bleeding and hemorrhagic stroke, the therapeutic value of antioxidants in clinical syndromes that lead to platelet-dependent thrombosis is not clear, as supplemental antioxidants have not been generally associated with better cardiovascular outcome. (*Arterioscler Thromb Vasc Biol.* 2008;28:s11-s16)

**Key Words:** platelet ■ antioxidant ■ thrombosis ■ redox

*This article is part of a multi-part CME-certified activity titled Translational Therapeutics at the Platelet Vascular Interface. In order to achieve all of the activity's learning objectives, please read all of the components of the activity listed in the Table of Contents and follow the "Instructions for Participation and Obtaining CME Credit" outlined prior to the Introduction.*

**R**edox changes occur as a function of normal platelet activation; however, the introduction of additional oxidative stress in certain settings may be prothrombotic. Modulation of platelet or vascular redox status, the presence of reactive oxygen species, and the addition of exogenous antioxidants can alter platelet activation in vitro and in vivo and may have (patho)physiological ramifications.

The clinical role of oxidative stress in platelet function and thrombosis is not straightforward. Although earlier epidemiological studies found that dietary antioxidant consumption was inversely associated with the development of coronary artery disease, more recent studies of vitamin supplementation have presented conflicting or negative results.<sup>1</sup> Interestingly, some of the negative side effects of antioxidant therapies, such as enhanced hemorrhagic stroke, may be attributed to changes in the thrombotic response. Although the effects of antioxidants were attributed to the prevention of oxidative modification of low-density lipoprotein (LDL) and the inhibition of atherogenesis, other effects may be relevant, including regulation of platelet activation, which is dependent on the balance between oxidative stress and redox state. As platelet function has also been implicated in the development of atherosclerosis and in the acute occlusion of coronary vessels,<sup>2,3</sup> oxidative processes and platelet redox status may have far reaching effects on the homeostasis of vasculature.

### Platelet Activation, Cardiovascular Events, and the Role of Antioxidants

Thrombus formation within a coronary vessel is the precipitating event in myocardial infarction and unstable angina.<sup>4</sup> Normally, the intact endothelium prevents adhesion and activation of platelets. Adhesion of platelets to the endothelium is prevented by several mechanisms, including endothelial cell production of prostacyclin and nitric oxide (NO).<sup>5,6</sup> The occurrence of superficial intimal injury caused by endothelial denudation and deep intimal injury caused by plaque rupture expose collagen and von Willebrand factor (vWF) to platelets. Platelets then adhere directly to collagen or indirectly via the binding of vWF to the glycoprotein (GP) Ib/IX matrix. Local platelet activation stimulates further thrombus formation and additional platelet recruitment by supporting cell-surface thrombin formation and releasing potent platelet agonists such as adenosine diphosphate (ADP), serotonin, and thromboxane A<sub>2</sub>.<sup>7</sup> A thrombus forms as platelets aggregate via the binding of bivalent fibrinogen to GP IIb/IIIa (Figure 1).

Epidemiological studies initially suggested that antioxidants play a role in the prevention of cardiovascular disease. Plasma antioxidant levels were found to inversely correlate with the development of angina,<sup>8</sup> and dietary antioxidant consumption was inversely associated with the development of clinical coronary artery disease.<sup>9</sup> Because oxidative processes are important in the development of atherosclerosis, the use of antioxidant supplementation was proposed for the treatment and prevention of coronary disease. However, many studies of vitamin therapy have failed to show clinical benefit.<sup>1,10</sup>

Interestingly, studies have shown that despite lack of mortality benefit, supplemental antioxidants are associated

Original received November 9, 2007; final version accepted December 19, 2007.

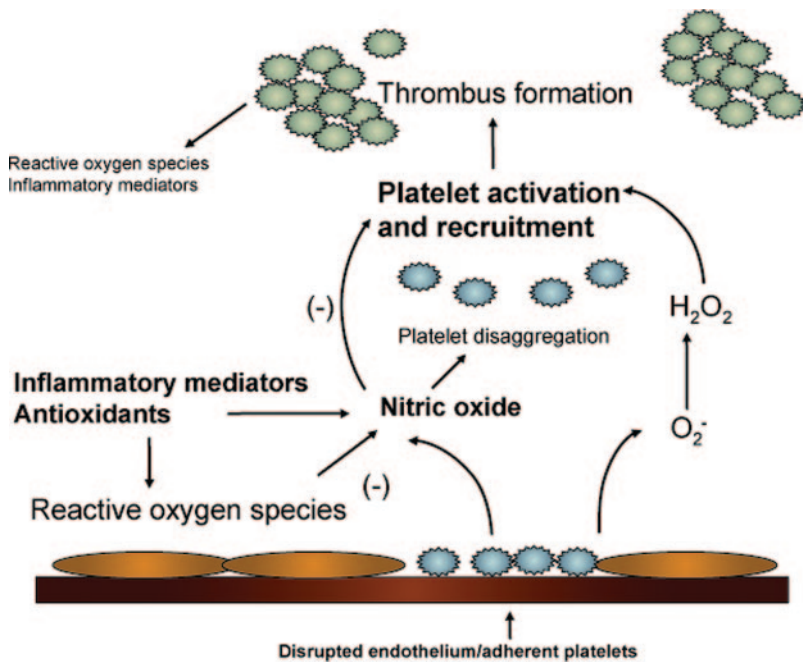
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*Arterioscler Thromb Vasc Biol* is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.107.159178



**Figure.** The role of oxidative stress, antioxidants and reactive oxygen, and nitrogen species in plaque disruption and thrombus formation.

with hemorrhagic stroke, suggesting platelet inhibition.<sup>11</sup> The precise mechanism(s) accounting for changes attributable to antioxidants in coronary disease remains unknown. Animal and cell culture data suggest that antioxidants preserve NO bioactivity in the face of oxidative stress. Because oxidative stress may alter platelet function, it is also conceivable that the effects of antioxidants may be a consequence of their enhancing or promoting the antiplatelet effects of NO derived from both endothelial cells and platelets. As discussed, the assumption that this leads to a decrease in acute coronary syndromes secondary to platelet-dependent thrombosis has not been borne out by large clinical trials.

### Reactive Oxygen Species and Platelet Function

Changes in redox status occur during normal platelet stimulation. Platelet aggregation is associated with a burst of oxygen consumption<sup>12</sup> and a marked rise in glutathione disulfide.<sup>13</sup> Although dramatic changes in redox status occur during normal aggregation, conditions that provoke oxidative stress without inducing a florid aggregation response may also be prothrombotic.

Reactive oxygen species derived from both platelets and other vascular sources have been shown to alter platelet responses. Superoxide is produced by platelets,<sup>14,15</sup> as are hydroperoxy derivatives of long-chain fatty acids (eg, 12-HpETE). Superoxide, in particular, is known to augment platelet aggregation responses.<sup>16</sup> Low ( $\mu\text{mol/L}$ ) concentrations of hydrogen peroxide, in the presence of plasma, inhibit platelet function.<sup>17</sup> Although high (mmol/L) concentrations of hydrogen peroxide have been shown to stimulate platelet aggregation, the physiological relevance of levels greater than 1 mmol/L is questionable.<sup>18</sup> Treatment of platelets with thrombin stimulates mitochondrial membrane potential depolarization and endogenous generation of hydrogen peroxide.

In addition, thrombin-induced apoptosis may be mediated by endogenous generation of hydrogen peroxide in platelets.<sup>19</sup>

Platelet-dependent reactive oxygen species appear to come from several sources. Platelets activated with different agonists may produce intracellular reactive oxygen species by nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidase.<sup>20</sup> NAD(P)H oxidase is present in platelets, and the activation of platelets is known to be associated with the activation of a gp91<sup>phox</sup>-dependent enzyme.<sup>21</sup> These studies have also shown that platelet production of reactive oxygen species enhances GP IIb/IIIa activation but not alpha or dense granule secretion.<sup>20</sup> NAD(P)H oxidase inhibitors and superoxide scavengers also reduce platelet aggregation and thrombus formation on collagen.<sup>22</sup> Collagen also induces NAD(P)H oxidase-dependent superoxide release in platelets, which in turn enhances availability of released ADP, resulting in increased platelet recruitment.<sup>23</sup> Consistent with these studies, platelets from gp91<sup>phox</sup>-deficient patients produce only a small amount of reactive oxygen species.<sup>24</sup>

### Nitric Oxide, Platelet Function, and Thrombosis

Activation and recruitment of platelets is tightly regulated, and whereas reactive oxygen species may be prothrombotic, reactive nitrogen species have primarily been shown to inhibit platelet function. Adhesion of platelets to the endothelium is prevented by several mechanisms, including endothelial cell production of prostacyclin and NO.<sup>5</sup> NO inhibits platelet activation<sup>25</sup> and prevents thrombosis.<sup>26</sup> Exogenous NO has been shown to inhibit the normal activation-dependent increase in the expression of platelet surface glycoproteins, including P-selectin and the integrin glycoprotein IIb/IIIa complex.<sup>27</sup>

Constitutive nitric oxide synthase (eNOS, cNOS, NOSIII) has been identified in both human platelets and megakaryoblastic cells.<sup>28</sup> Platelet aggregation is enhanced by incubation

with inhibitors of eNOS and inhibited by incubation with the eNOS substrate, L-arginine.<sup>29</sup> Interestingly, studies report NO release from resting<sup>30</sup> and aggregating platelets.<sup>31</sup> Platelet NO release influences platelet recruitment to the growing thrombus,<sup>32</sup> and impaired platelet-derived NO release is associated with acute coronary syndromes.<sup>33</sup> Coronary risk factors are also reported to be associated with decreased platelet-derived NO levels.<sup>33</sup> Impaired platelet NO responsiveness has also been shown to be an independent predictor of increased mortality and cardiovascular morbidity in patients with acute coronary syndromes.<sup>34</sup> However, in animal models, deficiency of eNOS is not associated with spontaneous thrombosis.<sup>35</sup> Interestingly, deficiency of eNOS is associated with enhanced fibrinolysis attributable to lack of NO-dependent inhibition of Weibel-Palade body release.<sup>36,37</sup> These compensatory processes highlight the complexity of NO-dependent regulation of vascular homeostasis. Such compensatory mechanisms may partially explain the lack of spontaneous thrombosis, minimally elevated baseline blood pressure, and normal life span that are seen in animals deficient in a pivotal regulator of vascular patency.<sup>36</sup>

### Superoxide and Platelet Function

Oxidative stress is an important mediator of both abnormal platelet function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular disease. Superoxide anion is an important source of oxidative stress, has direct effects, and limits the biological activity of NO. Excessive vascular superoxide production has been demonstrated in hypercholesterolemia as well as other disease states associated with endothelial dysfunction.<sup>38</sup> The production or release of reactive oxygen species by the vasculature or platelets can evoke an oxidative stress that supports lipid peroxidation, induces cellular activation, and promotes vascular dysfunction. Superoxide (in the presence of transition metals) promotes lipid peroxidation leading to the generation of lipid alkoxyl and peroxy radicals and causing lipid radical chain propagation reactions. This process can be terminated by NO through a reaction of NO with lipid peroxy radicals.<sup>39</sup> By reacting with superoxide or lipid peroxy radicals, NO can form peroxynitrite and lipid peroxynitrite, respectively, with a resultant loss in NO bioactivity. Thus, NO is readily inactivated by oxidative stress, specifically superoxide.

It is possible that NO bioactivity is dependent on platelet antioxidant status. Platelets have a number of antioxidant defenses, including superoxide dismutase (SOD). Human platelets contain approximately 1 femtogram of SOD/platelet, or about one fifth of that present in leukocytes. Approximately 77% of platelet SOD is believed to be Cu/Zn SOD, whereas the remainder is Mn SOD. SOD plays a role in normal platelet function and the prevention of thrombosis.<sup>40</sup> Studies suggest that dismutation of superoxide decreases platelet-dependent thrombus formation by potentiation of endogenous NO bioactivity.

### Antioxidants and Platelet-Mediated Thrombosis

Antioxidants may indirectly inhibit platelets through scavenging of reactive oxygen species, many of which alter

platelet function. Despite the different subcellular locations of water- and lipid-soluble antioxidants, these antioxidant pathways in platelets are closely linked. Glutathione depletion in platelets leads to attenuated glutathione peroxidase activity, decreased levels of  $\alpha$ -tocopherol, and increased lipid peroxidation.<sup>41</sup> Tocopherol oxidation in platelets can be blocked by preincubation of platelets with ascorbate.<sup>42</sup> In  $\alpha$ -tocopherol-depleted platelet lysates, the addition of either ascorbate or glutathione causes significant tocopherol regeneration.<sup>43</sup>

Evidence suggests that antioxidant status is an important determinant of platelet function. In normal individuals, selenium supplementation leads to increased plasma glutathione peroxidase activity and a prolongation of the bleeding time.<sup>44</sup> Decreased human platelet antioxidant content is associated with enhanced platelet activation responses, and normal aging is associated with increased platelet aggregation.<sup>45</sup> Smoking-induced platelet hyperactivity is associated with increased formation of lipid hydroperoxides and normalization of platelet aggregation with the addition of exogenous antioxidants.<sup>46</sup>

$\alpha$ -Tocopherol has been shown to be a platelet inhibitor causing dose-dependent inhibition of platelet aggregation and 5-hydroxytryptamine release in response to ADP, epinephrine, and collagen.<sup>47</sup> In men with previous coronary artery bypass graft surgery, vitamin E supplementation in the setting of colestipol-niacin treatment was associated with a reduction in coronary artery lesion progression.<sup>48</sup> However, enthusiasm for the use of supplemental vitamin E decreased in light of other trials demonstrating no beneficial effect.<sup>10</sup> The platelet inhibitory properties of vitamin E supplementation do not appear to be entirely irrelevant as supplementation is associated with increased hemorrhagic stroke.<sup>11</sup>

Antioxidant enzymatic mechanisms that metabolize these species also alter the prothrombotic effects of specific reactive oxygen species. One of these enzymes is glutathione peroxidase. Glutathione peroxidases are selenocysteine-containing enzymes that reduce hydrogen and lipid peroxides to their corresponding alcohols and use glutathione as an obligate cosubstrate. Hydroperoxides produced by the platelet (PGG<sub>2</sub>, 12-HpETE, and PLOOH) are metabolized by the selenium-dependent glutathione peroxidase enzyme that is also present in platelets. Glutathione peroxidase is tightly coupled to the hexose monophosphate shunt through reduced NAD(P)H which restores reduced glutathione concentrations and reestablishes the platelet thiol redox state via glutathione reductase. Glutathione depletion in platelets leads to attenuated glutathione peroxidase activity and increased lipid peroxidation. Antioxidants may indirectly inhibit platelets through the metabolism of reactive oxygen species, many of which alter platelet function. Glutathione peroxidase potentiates the inhibition of platelet function by NO by reducing LOOH.<sup>49</sup> Impairment of this process can lead to a clinical thrombotic disorder as shown in thrombotic strokes in childhood.<sup>50</sup>

### Inflammation, Platelets, and Oxidative Stress

Inflammation is linked with the evolution of cardiovascular disease and acute coronary syndromes. CD40 ligand (CD40L) is involved in the initiation of an inflammatory response at the vessel wall by inducing the expression of

adhesion molecules and the secretion of chemokines by vascular endothelial cells.<sup>51</sup> In addition to the trimeric membrane-bound form, CD40L also exists in a soluble, 18-kDa form (sCD40L) that is released from platelets after stimulation.<sup>51,52</sup> Platelets are the richest source of soluble CD40L (sCD40L) in circulation.<sup>53</sup> sCD40L mediates stimulation-induced platelet release of reactive oxygen and nitrogen species through activation of Akt and p38 mitogen activated protein kinase (MAPK) signaling pathways. These data suggest that sCD40L plays an important role in regulating platelet-dependent inflammatory and thrombotic responses.<sup>54</sup> Relative to oxidative stress in the platelet, redox-sensitive CD40-CD40L interactions specifically induce activation of Akt and p38 MAPK, leading to stimulation of nuclear factor kappaB and enhanced synthesis of CD40L and MCP1.<sup>55</sup> Increased CD40L and MCP1 also contribute to the adherence of CD40-positive cells, such as platelets, to the vessel wall, modulating atherothrombosis.<sup>55</sup>

### Platelets, Oxidative Stress, and Disease

In addition to cardiovascular syndromes, other diseases have been associated with redox imbalance, reactive oxygen species production, and altered platelet function. Intracellular Ca<sup>2+</sup> homeostasis in platelets of patients with non-insulin-dependent diabetes mellitus (NIDDM) has been reported to be altered, leading to an increased adhesiveness and spontaneous aggregation. Treatment of platelets from NIDDM patients with reactive oxygen species inhibitors can alter these processes.<sup>56</sup> It has also been shown that hydrogen peroxide and peroxy radicals are likely involved in the enhanced Ca<sup>2+</sup> mobilization observed in platelets from patients with type 2 diabetes, potentially leading to platelet hyperactivity and hyperaggregability.<sup>57</sup> In patients with hypercholesterolemia, platelet-associated NAD(P)H oxidase produces a thrombogenic phenotype and mediates the arteriolar dysfunction.<sup>20</sup>

Platelet superoxide production in patients with hypertension alone and in patients with coexistent diabetes mellitus has been examined. It was shown that eNOS can reside in the uncoupled state in patients with hypertension and, to a greater extent, in patients with coexisting hypertension and diabetes, and that this contributes significantly to increased superoxide production in these disease states.<sup>58</sup>

### The Effect of Therapeutics on Platelet-Dependent Oxidative Stress

Specific disease states are associated with platelet oxidative stress, and several relevant cardiovascular therapeutics have been found to alter oxidative-dependent platelet function. It has been shown that both aspirin and pravastatin inhibit lectin-like oxidized LDL receptor 1 (LOX-1) expression on platelets in part by favorably affecting reactive oxygen species and NO release from activated platelets.<sup>59</sup> 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibition also improves endothelial function, increases systemic NO bioavailability, and inhibits exaggerated platelet activation in an animal model of heart failure.<sup>60</sup> A study of GP IIb/IIIa inhibitors also showed that modified aggregation induced membrane translocation of the platelet proteins

eNOS and NAD(P)H oxidase (p67<sup>phox</sup> and p47<sup>phox</sup>), known to contribute to the generation of NO and superoxide, respectively.<sup>61</sup>

At therapeutically relevant concentrations, dipyridamole suppresses the formation of reactive oxygen species in platelets and endothelial cells and improves cellular redox status, with data suggesting that the redox-dependent properties of dipyridamole have a direct effect on vascular cells.<sup>62</sup> In addition, dipyridamole enhances platelet inhibition by amplifying the signaling of NO donors suggesting that enhancement of endothelium-dependent NO/cGMP-mediated signaling may be a relevant component of dipyridamole effect.<sup>63</sup>

Polymeric flavonoids, such as those found in red wine and purple grapes, contain antioxidant properties believed to be protective against cardiovascular events. Extracts from grape skins or seeds alter platelet release of reactive oxygen intermediates with enhanced NO release and attenuated superoxide production. Incubation with seed or skin extracts led to an immediate attenuation of release of the inflammatory mediator, sCD40L.<sup>64</sup> The polyphenols quercetin and catechin synergistically act in reducing platelet recruitment via inhibition of PKC-dependent NAD(P)H oxidase activation. This results in NO-mediated platelet GP IIb/IIIa downregulation.<sup>65</sup>

### Summary

When quiescent, platelet activation in the vasculature is limited by endothelial production of NO and prostacyclin but, in the atherosclerotic vessel, this process may be impaired. Numerous diseases including cardiovascular disease are associated with increased oxidative stress. This oxidant stress and decreased antioxidant levels found in cardiovascular disease are also associated with changes in platelet function. However, as demonstrated by the failure of antioxidant supplementation for the prevention of cardiovascular disease, the simple interpretation of redox balance/antioxidants versus oxidative stress in disease is likely simplistic. Although antioxidants, such as  $\alpha$ -tocopherol, inhibit platelet aggregation, the interaction between reactive oxygen species, antioxidants, and NO may contribute to platelet aggregability and thrombus formation although not always with the anticipated clinical response. In summary, the regulation of oxidative stress as well as reactive oxygen and nitrogen species plays an important role in platelet function and thrombosis and the clinical expression of thrombotic events.

### Disclosures

Dr. Freedman received research support from Boehringer Ingelheim Pharmaceuticals.

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