The Favorable Impact of Statins on Risk for Deep Vein Thrombosis May Reflect a Down-Regulation of NADPH Oxidase Activity that Suppresses Hypoxia-Mediated Activation of Venular Endothelium and Monocyte Tissue Factor Expression

Mark F. McCarty, Jorge Barroso-Aranda, and Francisco Contreras, Oasis of Hope Hospital, Tijuana, Mexico

Abstract

Statin therapy appears to be associated with a reduced risk for deep vein thrombosis (DVT). There is reason to suspect that this protection reflects a reduction in plasma tissue factor (TF) carried by leukocyte-derived microparticles, and possibly also a decrease in hypoxia-evoked P-selectin expression in venular endothelium. The ability of statins to down-regulate NADPH oxidase activity by opposing the isoprenylation of Rac1 and RhoA may be a key mediator of these effects, inasmuch as this activity can promote expression of both TF and P-selectin. Conversely, there is reason to believe that the increased risk for DVT associated with metabolic syndrome may reflect PKC-mediated activation of NADPH oxidase. These considerations suggest that the impact of pharmaceutical inhibitors of NADPH oxidase on stasis-evoked venous thrombosis in animal models should be evaluated. In light of recent evidence that biliverdin and phycobilins may have clinical potential as safe inhibitors of NADPH oxidase, resolution of these issues may be of some practical importance.

Statin Therapy Reduces Risk for Deep Vein Thrombosis

Several, though not all, epidemiological studies examining the impact of statin usage on risk for deep vein thrombosis (DVT) conclude that statins are protective in this regard.1-3 This protection was not observed in subjects using other lipid-lowering agents, suggesting that it was not mediated by serum lipid reduction (or hyperlipidemia) per se. One such study stratified their results by type of statin and found that simvastatin has a potent dose-dependent impact on DVT, whereas no such benefit was observed with pravastatin (which is water-soluble and thus has poor intracellular uptake).2 The protection associated with statin use appears to be relatively robust – adjusted odds ratios or relative hazards were calculated ranging from 0.30 to 0.78; thus, it may be quite worthwhile to understand the mechanism responsible for this phenomenon. Although one study failed to find an association between statin use and DVT risk,4 the statistical power of this study has been questioned.5 Thus, it seems likely that statin therapy does indeed decrease risk for DVT. What could be the basis for this effect?

Deep vein thrombosis is mechanistically quite different from arterial thrombosis. In the latter, thrombi form after the endothelial barrier is disrupted, exposing the contents of the blood to the sub-endothelial space; this contains collagen and von Willibrand factor – which activate platelets - as well as cell-bound tissue factor (TF), activator of the
extrinsic coagulation cascade. In sharp contrast, except when mechanical trauma initiates
the thrombus, venous thrombi form on the intact endothelial surface.6;7 This is
particularly remarkable inasmuch as endothelium is not believed to produce its own TF
except under conditions of extreme inflammation, such as lethal endotoxemia.8;9 In deep
venous thrombi, aggregated platelets are found some distance from the endothelial
surface; this suggests that platelet aggregation plays a secondary role in DVT, consistent
with the observation that platelet stabilizers such as aspirin only have a modest impact on
risk for this disorder.6;10 Thus, it appears that venous thrombi are initiated by triggering
of the coagulation cascade on the intact endothelial surface.

A Model for DVT Rationalizes the Protection Afforded by Statins

Recently, Conde and Lopez have proposed an elegant model for DVT which is credible
and appears consistent with current evidence.7 They note that blood contains
microparticles derived from the cell membranes of activated leukocytes and platelets that
express TF on their surfaces.11-14 Activated monocytes in particular are capable of
generating TF-rich microparticles, but other activated leukocytes also have this potential.
These microparticles also express P-selectin glycoprotein ligand-1 (PSGL-1), which
enables the microparticles to bind to cells which express P-selectin or E-selectin.15
Fusion of the microparticle membrane with that of the cellular plasma membrane thus
gives rise to cells expressing surface TF. When these cells are activated in a way that
induces phosphatidylserine to flip to the external membrane leaflet, this TF is rendered
active for initiation of coagulation.7 The ability of TF-bearing microparticles to bind to
activated platelets has been shown to play a key role in clot formation, helping to link
platelet activation to initiation of coagulation.16-18 Thus, in mice genetically deficient in
PSGL-1 or P-selectin, or which have been infused with P-selectin-blocking antibodies,
clots are unusually low in fibrin and TF.16

Conde and Lopez propose that a similar process occurs at the surface of activated venular
endothelium.7 They note that chronic venous stasis – a common predisposing factor for
DVT – subjects the venous endothelium to hypoxia, which for reasons that remain
somewhat unclear can lead to an activation state characterized by increased surface
expression of P-selectin, E-selectin, and phosphatidylserine. Venous inflammation owing
to other causes can likewise lead to endothelial expression of these ligands. Under these
circumstances, it should be feasible for TF-bearing microparticles to bind to the venous
endothelial surface and fuse with it, giving rise to an endothelial surface capable of
initiating the extrinsic coagulation pathway. This model thus suggests that risk for DVT
hinges on two key factors – the blood concentration of TF-bearing microparticles, and the
extent to which the venular endothelial surface expresses P-selectin, E-selectin, and
phosphatidylserine. Consistent with this thesis, a drug which inhibits P-selectin function
has been shown to decrease the formation and stability of venous thrombi in stasis-
induced thrombosis models employing rodents and baboons;19,20 moreover, induced
venous thrombi are smaller in mice that are genetically deficient in selectins.21

It is reasonable to expect that statin therapy can have a favorable impact on each of these
risk factors for DVT. Thus, both in vitro and in vivo, statins have been shown to
decrease expression of TF by various types of cells, including monocytes. Simvastatin therapy reduces plasma concentration of prothrombin fragments F1+2, suggesting that the impact of statins on TF expression may have clinical significance. Unfortunately, the impact of statin therapy on plasma concentrations of microparticle-borne TF has not yet been assessed, at least in published studies; nonetheless, the impact of statins on TF expression appears to be sufficiently general that it is probably safe to assume that statins can suppress the production of TF-rich microparticles.

Direct Endothelial Effects of Statins

With respect to endothelial activation, statins can have a favorable impact on this parameter as well. The direct anti-inflammatory effects of statins on endothelial function appear to reflect at least two phenomena – a suppression of endothelial oxidant stress, and up-regulated expression of the endothelial isoform of nitric oxide synthase (eNOS). These effects, in turn, appear to reflect a decrease in the levels of the isoprenylated forms of Rho family G-proteins. As is well known, statins can impede protein isoprenylation by suppressing the synthesis of geranylgeranylpyrophosphate, an obligate precursor for the isoprenylation of Rho family proteins. Lacking an isoprenyl group, Rho family proteins fail to migrate to membrane sites where they perform their regulatory roles.

The impact of statins on endothelial oxidative stress has been traced to the fact that Rac1 plays a key role in the membrane assembly of the NADPH oxidase complex. However, in the longer term, RhoA activity can also enhance the activity of this complex, by boosting the expression of many of its subunits via a mechanism dependent on Rho-kinase. The up-regulatory impact of statins on endothelial eNOS expression reflects increased stability of eNOS mRNA; conversely, RhoA activity decreases the half-life of eNOS mRNA, an effect that likewise is mediated by Rho-kinase.

Hypoxia-Induced Endothelial Activation – A Role for NADPH Oxidase?

Statins have been shown to suppress endothelial expression of P-selectin, both in vitro and in vivo. It is likely that a decrease in oxidant production by NADPH oxidase plays a role in this phenomena. Thus, there are many studies showing that activation of NADPH oxidase promotes P-selectin expression by endothelial cells, and is an obligate mediator of the impact of various pro-inflammatory cytokines or of hyperglycemia on such expression. Downstream activation of NF-kappaB by oxidant stress is a mediator of this effect, reflecting the presence of response elements for this transcription factor in the P-selectin promoter. Rapid translocation of preformed P-selectin to the endothelial surface is also promoted by oxidative stress, a mechanism which is independent of NF-kappaB or new protein synthesis. The up-regulatory impact of statins on eNOS expression can also promote a decrease in P-selectin expression, likely because nitric oxide can antagonize NF-kappaB activity by increasing transcription of the IkappaB-alpha gene, and possibly additional mechanisms. Concurrent
inhibition of NADPH oxidase could be expected to amplify the efficacy of this mechanism by suppressing the superoxide-mediated quenching of NO activity.

In contrast, the effects of statins on endothelial E-selectin are inconsistent, possibly because, while (as expected) E-selectin mRNA is decreased by statin exposure, the residence time of E-selectin on the membrane is increased, for reasons that remain unclear. It may be noted that inhibition of NADPH oxidase typically reduces E-selectin expression.

Consistent with the hypothesis of Lopez et al., chronic hypoxia has been shown to promote activation and leukocyte binding by human venular endothelium and endothelial cells. In particular, increased expression and membrane translocation of P-selectin has been observed in hypoxic endothelium. How this activation comes about is not clear. A number of studies have reported that hypoxia leads to rapid activation of various isoforms of PKC in endothelial cells; chronic hypoxia also leads to increased expression of PKC isoforms. Some evidence suggests that this hypoxic activation of PKC may reflect generation of diacylglycerol by a phospholipase C that specifically targets phosphatidylycholine. Many isoforms of PKC induce activation of NADPH oxidase by phosphorylating p47phox, which then translocates to the membrane surface to enable assemblage of the NADPH oxidase complex. Thus, it is reasonable to suspect that hypoxia, via PKC activation, promotes activation of NADPH oxidase in endothelial cells; if oxygen levels remain sufficiently high to enable superoxide production, an increase in oxidant stress would likely result, promoting endothelial activation – including increased expression of P-selectin and E-selectin. Statins could intervene in this process by abrogating the stimulatory impact of Rac1 and RhoA function on NADPH oxidase activity.

There is considerable evidence that activation of NADPH oxidase is a key mediator of the endothelial dysfunction associated with hypoxia/reoxygenation injury; in particular, this activation has been shown to be a mediator of the increased E-selectin expression observed in human umbilical vein endothelial cells (HUVECs) following anoxia/reoxygenation. The impact of hypoxia per se on this activity has been less studied. In retinal endothelial cells exposed to 1% oxygen for 6 hours, superoxide production increased significantly by about 50%; concurrent exposure of the cells to apocynin or an NADPH oxidase-inhibitory peptide completely abolished this increase. Other germane evidence derives from studies examining the phenomenon of hypoxia-evoked pulmonary hypertension. In chronically hypoxic mice, the resulting pulmonary hypertension is associated with increased oxidative stress in pulmonary arteries and endothelial dysfunction. In contrast, in gp91phox knock-out mice, hypoxia fails to evoke arterial hypertension or endothelial dysfunction. Of related interest is a report that expression of p47phox increases during anoxia in HUVECs.

There is however one report that total oxidant production – including oxidant production via NADPH oxidase – is decreased in HUVECS after 8 hours of exposure to mild hypoxia (30-35 mmHg). This study did not determine whether these cells were concurrently activated (that is, expressed increased levels of adhesion factors), and it is
hard to see how endothelial cells could be activated when oxidant stress is reduced. The disparity between this study and those previously cited likely reflects differences in experimental design.

In any case, there is reason to suspect that NADPH oxidase will be activated in venous endothelium during chronic hypoxia associated with static blood flow, that this activation will play a mediating role in the endothelial activation evoked by chronic hypoxia, and that statin therapy can oppose this process by down-regulating the function of Rac1 and RhoA. Perhaps it would be feasible to test this thesis in animal models of stasis-induced venous thrombosis, examining venous function ex vivo.

**Role of NADPH Oxidase in Tissue Factor Expression**

While the impact of statin therapy on the plasma concentration of TF-bearing microparticles has not been assessed, it is clear, as noted above, that statins can inhibit the expression of TF by monocytes and other cells, in vitro and in vivo. Moreover, it is clear that NADPH oxidase activation can promote TF expression in monocytes and other tissues. This latter effect may reflect the fact that the TF promoter contains response elements for NF-kappaB, AP-1, and egr-1. As is well known, oxidant stress often promotes activation of NF-kappaB-mediated transcription. Moreover, oxidant stress often provokes activation of the MAP kinases c-Jun N-terminal kinase (JNK) and p38, which can each boost AP-1 activity; JNK achieves this through phosphorylation of c-Jun, whereas p38 MAP kinase activates the ternary complex factor Sap-1a to boost c-fos transcription. Finally, p38 MAP kinase and JNK can also increase the transcription of the immediate-early gene egr-1. Thus, it is not surprising that oxidant stress stemming from NADPH oxidase activation tends to increase TF expression, and it is reasonable to postulate that the negative impact of statins on this expression stems, at least in part, from a down-regulation of NADPH oxidase activity. Indeed, there is a report that withdrawal of cerivastatin exposure boosts oxidant stress and TF expression in vascular smooth muscle cells, and that concurrent exposure to a Rac antagonist prevents these phenomena.

Whether statins or NADPH oxidase activity influence the propensity of monocytes or other leukocytes to shed microparticles does not seem to have been studied.

**Relevance to Increased DVT Risk in Metabolic Syndrome**

If NADPH oxidase activation does indeed play a key role in the induction of DVT, it becomes easy to explain the association between DVT and metabolic syndrome. Risk for DVT is elevated in individuals with metabolic syndrome, abdominal obesity, or diabetes. Consistent with this finding, plasma TF levels are increased in type 2 diabetics, and correlate with indices of insulin resistance. Conversely, substantial weight loss in patients with morbid obesity is associated with a notable decrease in plasma TF. Furthermore, an acute increase in plasma TF is observed in the hours following a fatty meal in healthy subjects. Monocyte TF levels tend to be higher in people habitually consuming diets high in saturated fat than in those that eat low-fat or
“Mediterranean” diets. All of these findings can be rationalized by the fact that adipocyte insulin resistance, particularly in conjunction with fatty meals, leads to postprandial increases in tissue exposure to free fatty acids. This results in de novo synthesis of diacylglycerol and activation of protein kinase C, which in turn promotes activation of NADPH oxidase – an effect which is triggered more intensely by saturated than unsaturated fatty acids. This mechanism presumably could induce NADPH oxidase activation both in monocytes and venular endothelium, increasing risk for DVT when venous stasis occurs. A recent study shows that an acute elevation of free fatty acids leads to activation of NF-kappaB in mononuclear cells – an effect that likely reflects NADPH oxidase activation, and that would be expected to induce increased monocyte TF expression.

Metabolic syndrome is also associated with elevated plasma levels of factor VII, fibrinogen, and PAI-1 – ancillary effects which evidently could promote extrinsic coagulation while suppressing fibrinolysis.

In the middle decades of the twentieth century, surgeons serving sub-Saharan black Africans still following their traditional diets and lifestyles noted that postsurgical thromboembolism was extremely rare in this population – far more so than in Euro-Africans. It is tempting to speculate that this phenomenon may have reflected the interaction of excellent insulin sensitivity and very-low-fat diets, which minimized free fatty acid exposure and NADPH oxidase activation.

**Summary and Future Prospects**

In brief, there is considerable indirect evidence favoring the hypothesis that the protective impact of statin therapy on DVT risk may be mediated in large part by a down-regulation of Rac1, RhoA, and hence NADPH oxidase activity, which leads to a decrease in the production of TF-bearing microparticles while concurrently limiting the ability of chronic hypoxia or venous inflammation to boost endothelial expression of P- and E-selectin. Conversely, the increased risk for DVT associated with metabolic syndrome and fatty diets may reflect increased activation of NADPH oxidase in monocytes and venular endothelium. If this hypothesis has merit, it should be possible to show that NADPH oxidase inhibitors (other than statins) can decrease plasma levels of microparticle-borne TF while also diminishing the activation state and adhesiveness of venular endothelium exposed to hypoxia in vitro or in vivo. In this regard, it should be noted that both biliverdin and phycocyanobilin have clinical potential as safe, orally active inhibitors of NAPDH oxidase that might complement or mimic the utility of statin therapy for prevention of vascular disorders, including possibly DVT.
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