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EDITORIAL COMMENT

Pleiotropic, Cellular Effects of Rivaroxaban on Autophagy Explain Atheroprotective Effects*

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Thrombotic occlusion of vital arteries, following rupture and/or erosion of an atherosclerotic plaque, is the main event leading up to ischemic clinical events such as stroke or myocardial infarction. Rivaroxaban is a direct oral anticoagulant inhibiting activated coagulation factor X (FXa) and is widely used in the clinic to prevent and treat thrombosis. In the COMPASS (Cardiovascular Outcomes for People Using Anticoagulation Strategies) trial, patients with stable coronary artery disease or peripheral arterial disease treated with a low dose of rivaroxaban in addition to traditional antiplatelet therapy (aspirin) had significantly fewer cardiovascular events and mortality than patients treated with either aspirin or high-dose rivaroxaban (1). The additive, mild antithrombotic effect of low-dose rivaroxaban with antiplatelet therapy might explain the superior effects of the combination therapy over monotherapy. However, recent animal work suggests that inhibition of FXa by rivaroxaban has protective cardiovascular properties beyond the scope of its anticoagulant function.

Zhou et al (2) showed that inhibition of FXa by rivaroxaban attenuates the development of atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) mice fed a high-fat diet (2). The protective effect of rivaroxaban on atherogenesis was associated with decreased inflammation and a more stable phenotype of the plaque. FXa can exert pleiotropic actions via the g-coupled protein receptor 2 (PAR2). Hara et al (3) proposed that decreased FXa-mediated activation of hematopoietic PAR2 contributed to the observed atheroprotective phenotype. The underlying mechanisms, however, are not clear.

The study by Ito et al (4) in this issue of *JACC: Basic to Translational Science* proposes a potential mechanism by which inhibition of FXa exerts its beneficial effects on atherogenesis. The authors used $ApoE^{-/-}$ mice on a high-fat diet supplemented with the FXa inhibitor rivaroxaban for 20 weeks. In agreement with previous work (2,3), the authors found that rivaroxaban treatment inhibited atherogenesis and inflammasome activation. Dampening of the inflammasome indeed offers protection to atherogenesis. However, the authors also observed changes in another cell process. Ultrastructural examinations of the lesions revealed that plaque-resident macrophages from rivaroxaban-treated mice had significantly more autophagosomes than control mice. Autophagy is a crucial, beneficial process in atherosclerosis because it removes toxic proteins, excessive lipids, and apoptotic cells.

To unravel how rivaroxaban could affect these 2 cellular stress pathways, the authors (4) exposed macrophages in vitro to 7-ketocholesterol (7KC), a known inducer of autophagy and the inflammasome. Subsequent treatment with FXa further enhanced inflammasome activation in 7KC-exposed macrophages, as reflected by increased accumulation of the proteins NLR family pyrin domain containing 3, procaspase-1, and pro-interleukin-1β. More interestingly,

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FXa treatment fully reversed 7KC-induced autophagy. The cell-signaling target of FXa, PAR2, has been implicated in autophagy. To test whether FXa modulates the inflammasome and autophagy pathways via PAR2, the authors generated $PAR2^{-/-}$ macrophages and exposed them to FXa. Removal of the PAR2 gene abolished FXa-induced inflammasome activation and inhibition of autophagy, thus supporting a role for FXa/PAR2 signaling in these pathways. Because PAR2 can be activated by other proteases as well, it is currently unclear to what extent PAR2 signaling plays a role in modulating the inflammasome and autophagy pathways following rivaroxaban treatment in vivo. To further address this issue, the authors used $PAR2^{-/-}$ mice crossed on an $ApoE^{-/-}$ background, which were protected from atherosclerosis. The authors extended these observations, as treatment of $PAR2^{-/-}/ApoE^{-/-}$ double knockout mice with the autophagy inhibitor chloroquine abolished the protective effect of rivaroxaban. This finding suggests a role for autophagy in the rivaroxaban/PAR2-mediated effects in mice in vivo. Nevertheless, as discussed by the authors, chloroquine is not a specific autophagy inhibitor. This antiviral, anti-inflammatory agent increases endosomal pH and thus blocks binding of autophagosomes to lysosome and hence autophagosomal flux. Future work with genetic deficiency of key autophagy genes such as beclin-1, autophagy gene (ATG) 5 or 7 would provide a final decisive answer if autophagy alleviation mediates the effect of rivaroxaban on atherogenesis. In addition, because PAR2 can be activated by proteases other than FXa, the in vivo experiment does not provide conclusive evidence on the role of FXa in PAR2-mediated autophagy. Unfortunately, FXa deficiency results in lethality. Thus, treatment of PAR2/autophagy mutants with a compound that would selectively inhibit FXa PAR2 signaling without affecting hemostasis would provide the proof in the pudding. Obviously, a quadruple knockout strategy is a time- and money-consuming strategy, difficult in the current funding climate and not in line with the Replacement, Reduction, and Refinement (3R) principle of animal ethical conduct.

This study (4) highlights another interesting finding: the addition of rivaroxaban on top of $PAR2^{-/-}$ enhances the protective effects of PAR2 deletion, suggesting that other proteins downstream of FXa modulate atherosclerosis as well. The current study thus shows that FXa-PAR2-signaling ameliorates inflammasome formation and autophagy in vitro, with PAR2 signaling (FXa dependent or independent) protecting against atherogenesis via autophagy in vivo. However, this remains an observation in a mouse model, with known limitations. The authors provide pilot data of 2 coronary endarterectomy specimens treated with rivaroxaban or vehicle ex vivo showing enhanced autophagosomes following rivaroxaban treatment. Although this supports the start of a well-powered study, thus far the translation of this murine observation by Ito et al is lacking.

Another hurdle in the mouse to human translation lies in the low comparability of rivaroxaban dose across species. The murine dose exceeds the human dose by 3- to 7-fold. Ito et al (4) discuss that percent FXa inactivation is, however, comparable to human. If genetic studies would be pursued, including a more comparable dose would certainly add to the translational value. Nevertheless, low-dose rivaroxaban provided protection against major clinical cardiovascular effects as shown by the COMPASS trial (1), no matter the mechanism. It is imperative, however, to monitor the mild additive antithrombotic effects in patients on human plaque composition and stability after a treatment lasting longer than the 2 years in the COMPASS trial. Specifically, a potential increase in frequency or extent of intraplaque hemorrhages (IPH) by long-term treatment could be an undesired side effect of anticoagulant effects of rivaroxaban. IPH is an independent predictor of future clinical cardiovascular events. Because mice models do not recapitulate this phenomenon particularly well, microscopic analysis and/or noninvasive imaging of rivaroxaban treatment in human subjects is warranted. Notably, a correlation of IPH, measured by magnetic resonance imaging, was reported previously with other anticoagulants (ie, vitamin K antagonists and antiplatelet therapy) (5). Also, longer use of vitamin K antagonists (>3 months) strengthened the chance of detecting IPH. However, it should be taken into consideration that retrospective analysis of human longitudinal cohorts may be confounded by increased frequency of anticoagulant use in patients with existing cardiovascular disease, who also inherently exhibit more IPH. In a subgroup analysis of patients with no history of cardiovascular disease, the association weakened, and only a nonsignificant trend remained (5). However, clinical trials thus far with direct anticoagulant therapy report a beneficial safety profile.

In conclusion, rivaroxaban may have pleiotropic effects, explaining protection of atherosclerosis, much like statins. However, the old paradigm “mice are not man” is as true as ever, and both mechanistic refinement and long-term safety aspects related to atherosclerotic plaque composition after rivaroxaban treatment deserve future translational studies.

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