Pleiotropic effects of factor Xa and thrombin: what to expect from novel anticoagulants

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Factor Xa and thrombin are well-known components of the coagulation cascade and have been proven to be viable targets for effective anticoagulation treatment. However, accumulating evidence suggests that these serine proteases are also crucial modulators of other cellular mechanisms through the activation of protease-activated receptor (PAR)-mediated signalling. The involvement of factor Xa, thrombin, and PARs in normal biological and pathophysiological processes has been recognized, and their potential implications have been explored in recent years. Both factor Xa and thrombin play significant roles in mediating cellular signalling effects associated with the initial development of atherosclerosis: a chronic inflammatory vascular disease. In addition, increased expression and activation of PARs may be associated with atrial fibrillation (AF) and AF-associated thromboembolism hypercoagulability. Both pathologies are associated with hypercoagulability, suggesting that the role of cellular effects of factor Xa and thrombin and of their specific inhibitors should be studied in relation to the prevention of thrombotic and pro-arrhythmic changes. This review examines the role of factor Xa-mediated and thrombin-mediated PAR activation in modulating cellular processes involved in atherosclerosis and AF and discusses the potential implication of direct factor Xa and thrombin inhibition on effects outside coagulation.

Keywords Anticoagulants • Atherosclerosis • Receptors • Thrombin

1. Introduction

Adequate blood flow results from the balanced interplay between haemostasis and fibrinolysis. Haemostasis depends on the interplay between the vessel wall (vasoconstriction), platelet aggregation, and coagulation, and is based on a cascade of sequential proteolytic reactions undertaken by serine proteases, the enzymatic activity of which is tightly regulated. Fibrinolysis involves the proteolytic degradation of fibrin by a serine protease, plasmin, to prevent excessive clot formation and subsequent thrombosis.1,2 Factor Xa, produced by both the extrinsic tenase and intrinsic tenase complexes, represents the point of convergence of the extrinsic and intrinsic pathways of the coagulation cascade and converts prothrombin into thrombin (Figure 1)—the serine protease responsible for fibrin formation and the subsequent growth and stabilization of thrombi.2,3 Factor Xa and thrombin are viable targets for anticoagulation4 (Figure 2), but have also been shown to participate in other biological and pathophysiological processes.1,3 Therefore, the properties of oral, direct inhibitors of factor Xa (e.g. apixaban and rivaroxaban) and thrombin (e.g. dabigatran) have started to be examined outside the realm of haemostasis and thromboembolism management. In addition, the view that factor Xa-mediated signalling would be similar to that of thrombin has recently been revised. Although both factors signal through protease-activated receptors (PARs), PAR1-induced responses differ according to the nature of the ligand, whereas PAR2 (a receptor for factor Xa but not for thrombin) seems to be a key player in the progression of fibroproliferative disorders.3 Consequently, unrestrained coagulation activity and/or excessive PAR activation may be involved in a range of conditions, including arthritis, fibrotic lung disease, cancer, and atherosclerosis.3,5,6 The direct cellular effects of factor Xa are responsible for promoting inflammation, leucocyte trans-endothelial migration, angiogenesis, and narrowing of blood vessels, which are ultimately the basis of atherosclerotic plaque development.1

Thrombin activity has also been recognized as playing a role in the development of atherosclerotic plaques.1 This review will focus on the non-haemostatic functions of factor Xa and thrombin, and their role as essential molecules for modulating processes in different cell types involved in atherosclerosis and atrial fibrillation (AF). Therefore, we will discuss the existing pre-clinical data on the effects of...
Figure 1  Schematic overview of the blood coagulation cascade. The model is divided into initiation, propagation, amplification, and clot formation phases. Blood coagulation factors are denoted in Roman numerals, and the active forms are indicated by a small ‘a’. II indicates prothrombin and IIa thrombin. vWF, von Willebrand factor.

Figure 2  Inhibitors of thrombin and factor Xa. Factor Xa interacts with factor Va to convert prothrombin into thrombin. Besides their activity in coagulation, factor Xa and thrombin can signal through activation of PAR1 and PAR2 by proteolytic cleavage of a distinct N-terminal cleavage site in these receptors, which generates a new bound peptide that acts as a tethered signal-inducing ligand in an autocatalytic activation process. Therefore, anticoagulation not only will attenuate fibrin clot formation, but also can lead towards diminished cellular signalling. Blood coagulation factors are denoted in Roman numerals, and the active forms are indicated by a small ‘a’. II indicates prothrombin and IIa thrombin.
2. Non-haemostatic functions of factor Xa and thrombin

Cross-talk between coagulation and inflammatory pathways has been well documented. The endothelium, platelets, pro-inflammatory cytokines and chemokines, and several serine proteases [e.g. tissue factor (TF), factor Xa, thrombin], via activation of PARs, are major points of contact between these two pathways.\(^5\)

2.1 Signalling pathways mediated via PARs

The PAR family comprises four members (PAR1 to PAR4) with a proteolytic cleavage-based activation mechanism.\(^5\) With the exception of PAR2, all PARs are cleaved by, and signal directly in response to, thrombin. However, thrombin-cleaved PAR1 can donate its tethered ligand to transactivate PAR2, which, in turn, has been shown to modulate the PAR1-induced hyperplastic response to arterial damage preceding stenosis.\(^7\) The simultaneous activation of PAR2 and PAR1 by thrombin is also involved in PAR1-mediated tumour cell migration and metastasis.\(^8\) In addition, thrombin-induced signalling responses through PAR1 activation or transactivation of PAR1 and PAR2 heterodimers seem to be different.\(^9\) Formation of PAR1 and PAR4 heterodimers dependent on thrombin-induced cleavage of both receptors has also been reported.\(^10\) Factor Xa-initiated cellular responses occur through PAR1 and/or PAR2 cleavage, and these processes may depend on the receptor-specific cell expression pattern, ligand concentration, solubility, or association with other coagulation factors.\(^5\) Factor Xa and thrombin activity may also be more efficiently directed towards PAR2 and PAR1 cleavage, respectively, owing to specific differences in the amino acid sequences of these receptors.\(^11\) The proteolytic cleavage of PARs by thrombin or factor Xa results in the activation of a canonical G-protein pathway and, consequently, of downstream signalling pathways that trigger multiple transcription-regulated, cell-specific events.\(^5,12\) PAR1 and PAR2 expression occur in almost all tissues and cell types, including in the cardiovascular system and in immune cells.\(^5\) Factor Xa-induced cellular responses would, therefore, be dependent on this extravascular presence of the factor due to vascular injury, macrophage-mediated binding and migration into sites of ischaemia or inflammation, and tissue ectopic expression of factor Xa, which may occur in both normal and distinct pathophysiological conditions, including tumour and fibrotic tissues or even in atherosclerotic tissues.\(^3,13\)

2.2 Pleiotropic functions of factor Xa and thrombin in development, inflammation, remodelling, and fibrosis

Gene-knockout studies in animals have demonstrated a role for factor Xa in normal physiology, with factor X deficiency resulting in death either during embryonic development or soon after birth; a phenotype similar to that of prothrombin- or PAR2-null mice. These three phenotypes, in contrast to that resulting from fibrinogen deficiency, led to the hypothesis that the role of factor Xa and thrombin during development depends on their signalling-mediated effects rather than effects on coagulation,\(^5\) which are also important for other biological processes. Moreover, abnormal tissue remodelling and aberrant activation of the coagulation pathway have also been reported in a number of fibroproliferative disorders.\(^3\) Inadequate thrombin- and factor Xa-dependent activation of PARs may contribute to pathological fibrotic remodelling and inflammation.

Activation of factor X during haemostasis as a consequence of vascular damage, inflammation, or fibrosis seems to play a key role in these tissue remodelling and fibrosis events, mostly due to the mitogenic properties of factor Xa and its ability to induce the expression of chemokines and profibrotic cytokines.\(^3,13\) Consistently, factor Xa-mediated mitogenic effects were found to affect coronary artery smooth muscle cells (SMCs)\(^14\) and to be mediated via PAR1 in heart- and lung-resident fibroblasts, whereas PAR2 signalling was shown to be involved in lung and renal fibrosis.\(^5\) Factor Xa acts as a potent in vitro inducer of pro-inflammatory cytokine expression [interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1] by fibroblasts, lymphocytes, and endothelial cells in addition to adhesion molecules in monocytes.\(^5\) Thrombin is capable of promoting the expression of adhesion molecules, growth factors, and cytokines, for instance, by mononuclear leucocytes and endothelial cells.\(^15\) In addition, thrombin increases the expression of adhesion molecules on leucocytes and their activation, and thrombin-activated platelets can potentiate CD40 ligand-mediated stimulation.\(^17\) Conversely, inflammatory cytokines are known to initiate coagulation by promoting the expression of cellular membrane-bound TF and fibrinogen.\(^19\) The cross-talk activation and regulation between the coagulation and inflammation processes via PAR activation are now seen as potentially relevant for a number of clinical conditions, such as atherosclerosis.\(^3,13\)

3. Pathophysiology of the vascular wall and atherosclerosis

Atherosclerosis is a chronic inflammatory vascular disease and is described as the pathological basis of coronary artery diseases (such as myocardial infarction or unstable angina), stroke, and peripheral arterial disease.\(^20\) Although coagulation proteins, such as TF and factor VII, are known to contribute to atherothrombosis, the relationship between progression of atherosclerosis and thrombogenic potential in these lesions because of the presence of these factors has only been addressed recently.\(^11\) TF, thrombin, factor Xa, and factor Xlla proteolytic activities were found to be significantly increased in early atherosclerotic lesions compared with lesions at a later stage.\(^11\) This supports an important role for coagulation-mediated cellular effects in the initial development of atherosclerosis (Figure 3) rather than involvement limited to thrombus formation in unstable, advanced plaques. Vascular remodelling associated with atherosclerosis is induced by factor Xa-dependent PAR activation or factor Xa-mediated TF expression in vascular endothelial cells and SMCs, which leads to narrowing of the blood vessel lumen because of abnormal cell proliferation and extracellular matrix accumulation.\(^3\) Several studies seem to suggest that PAR2 activation may play a prominent role in vascular remodelling and atherosclerosis.\(^3\) Factor Xa participation in the atherosclerotic process is likely to result from the orchestration of several signalling pathways in distinct cell types that are either part of the vascular system (namely endothelial cells and vascular SMCs) or immune cells that contribute to atherosclerotic plaque progression.\(^3\) The contribution of factor Xa to atherosclerosis is, therefore, either directly via binding and activation of PAR1 and/or PAR2 or indirectly through the generation of thrombin. The role of thrombin in vascular lesions and in the atherosclerotic process has been attributed not only to thrombus formation, but also...
to its ability to act as a potent activator of platelets. Thrombin also induces the expression of an array of cytokines (such as IL-6 and IL-8), chemokines (such as MCP-1), and cell adhesion molecules (such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1), which promote the recruitment of monocytes to the vessel wall. Thrombin-mediated regulation of platelet-derived growth factor influences the migration and proliferation of vascular SMCs leading to plaque formation. Although thrombin’s pro-atherogenic role involving the arterial vessel wall is mediated via PARs, the early development of atherosclerotic plaques does not seem to be dependent on thrombin-mediated platelet activation via PAR4 signalling.

Thrombin has been shown to induce the synthesis of MCP-1 in vascular SMCs, and this chemokine seems to be abnormally expressed in the walls of atherosclerotic vessels. Moreover, thrombin inhibition by binding to a recombinant fraction of thrombomodulin impaired PAR1 internalization and reduced the expression of adhesion molecules and MCP-1 in endothelial cells while increasing the permeability of these cells. Thrombin has been shown to be active in atherosclerotic vessel walls, and thrombin inhibition is responsible for reducing the development of atherosclerotic plaques in apolipoprotein E-deficient mice, which was accompanied by a reduction in the expression of PAR1 and adhesion molecules and the number of infiltrating macrophages.

A number of studies suggest that inflammation induced by coagulation effectors has a detrimental impact in acute coronary syndromes, as suggested by the contribution of factor Xa to the severity of acute myocardial infarction via stimulation of IL-8 production in human endothelial cells and mononuclear leukocytes, and the predictive nature of high levels of inflammatory cytokines (e.g. IL-8, IL-6, and MCP-1) in recurrent plaque instability. The end stage of atherosclerosis is characterized by rupture of an atherosclerotic plaque and subsequent (occlusive) thrombus formation. Arterial thrombosis in coronary arteries causes myocardial ischaemia, often followed by reperfusion injury. Ischaemia/reperfusion-associated cell death initiates an acute inflammatory response, generation of reactive oxygen species, neutrophil and monocyte recruitment, and apoptosis within and around the infarct area. Given this relationship between inflammation and coagulation, as well as the cellular processes induced by thrombin and factor Xa through distinct PAR activation, anticoagulants might be beneficial in attenuating ischaemia/reperfusion injury. Anticoagulant therapy has previously been shown to have protective properties in ischaemia/reperfusion injury. Besides the classical anticoagulant heparin, inhibition of coagulation through active site-inhibited factor VIIa or activated protein C attenuated ischaemia/reperfusion injury in mouse models of acute myocardial infarction. Inhibition of both thrombin and factor Xa might contribute to attenuating PAR1- or PAR2-mediated ischaemia/reperfusion-related pathology. The involvement of PAR1 in ischaemia/reperfusion injury stems from experiments using mice lacking PAR1. Deficiency in PAR1 reduced infarct size after cerebral ischaemia, reduced left ventricle dilation, and improved left ventricle function in this mouse model. PAR2 activation seemed to have an opposite effect in ischaemia/reperfusion, because activation of PAR2 using an activating peptide caused enhanced recovery of myocardial function and a decrease in oxidation at reperfusion. Whether the novel direct thrombin and factor Xa inhibitors have equal protective characteristics in ischaemia/reperfusion injury is currently not known and needs to be investigated.

4. Contribution of hypercoagulability and PAR activation to AF

AF is the most common cardiac arrhythmia. Atrial remodelling has been described in patients with AF and in animal models of AF and includes
structural changes such as atrial dilation, cellular hypertrophy, dedifferentiation, and fibrosis.\textsuperscript{36} These structural alterations are known to cause conduction disturbance\textsuperscript{37} and may contribute to the progressive nature of AF, a phenomenon initially coined as ‘AF begets AF’ (Figure 3).\textsuperscript{38} It has been known for many decades that AF is associated with the activation of local and circulating coagulation factors (hypercoagulability). This AF-related hypercoagulability significantly increases the risk of clot formation and stroke in patients with AF.\textsuperscript{39} However, little is known about the potential role of this AF-associated hypercoagulability in atrial tissue remodelling and particularly the contribution of thrombin or factor Xa.

Both PAR1 and PAR2 are present in the heart. PAR1 is mainly expressed by myocytes, fibroblasts, endothelial cells, and SMCs.\textsuperscript{40} Although PAR2 is also expressed by myocytes, endothelial cells, and SMCs, its expression by fibroblasts has not been confirmed.\textsuperscript{40} In cardiac fibroblasts, PAR1 has been shown to be the most highly expressed G-protein coupled receptor, out of a panel of 190 expressed receptors, and thrombin treatment induced a profibrotic response.\textsuperscript{41} A proliferative effect of both PAR1 and PAR2 activation has been demonstrated in ventricular fibroblasts from chick embryos.\textsuperscript{42} In neonatal rat ventricular cardiomyocytes, PAR1 and PAR2 activation leads to different patterns of mitogen-activated protein kinase activation and cellular hypertrophy.\textsuperscript{43} In experiments in vivo, it has been shown that mice overexpressing PAR1 develop cardiac hypertrophy and dilated cardiomyopathy.\textsuperscript{34} Reducing coagulation activity in these mice by interbreeding with mice expressing a low level of TF resulted in reduced hypertrophy.\textsuperscript{34} PAR2 deficiency leads to reduced infarct sizes in ischaemia/reperfusion mouse models and reduced inflammatory responses.\textsuperscript{44} In addition to effects related to tissue remodelling, an arrhythmogenic potential has been suggested for thrombin in ventricular myocytes.\textsuperscript{45,46} Thrombin prolonged the action potential duration and enhanced early after depolarizations induced by caesium in canine Purkinje fibres.\textsuperscript{46} In addition, thrombin increased ventricular arrhythmias in reperfusion experiments performed in adult rat hearts.\textsuperscript{45} Moreover, in rabbit pulmonary vein preparations, thrombin enhanced arrhythmogenesis by reducing the spontaneous beating rate and inducing delayed after depolarizations and burst firing.\textsuperscript{47} Besides an arrhythmogenic potential, thrombin also enhanced the release of atrial natriuretic peptide from adult rat atrial cardiomyocytes.\textsuperscript{48} as well as from rat ventricular myocytes.\textsuperscript{49} In addition, a decreased contractile force was observed upon incubation with thrombin in rabbit atrial preparations.\textsuperscript{47}

Thrombin and PAR1 have been detected in human autopsied hearts, and their expression levels were higher in left atrial tissue compared with left ventricular tissue.\textsuperscript{50} Thrombin-activated PAR1 also induced arrhythmogenic effects in atrial preparations and increased sodium influx by increasing the persistent sodium current in human atrial cardiomyocytes.\textsuperscript{51} Direct signalling effects of factor Xa have also been observed. Factor Xa induced the expression of PARs and inflammatory molecules in human atrial tissue slices.\textsuperscript{52} and tachyarrhythmia alone also increased the expression of PAR1.\textsuperscript{52} Simultaneous stimulation with factor Xa and tachyarrhythmia synergistically increased PAR expression and induced inflammatory signalling.\textsuperscript{52} PAR1 expression was also elevated in patients with ischaemic heart disease or heart failure cardiac.\textsuperscript{53} The atrial effects of PAR signalling are currently being explored. The cellular effects of this stimulation are likely to contribute to structural remodelling in fibrillating and dilated atria.\textsuperscript{53} Inflammatory changes, tissue fibrosis, and cellular hypertrophy significantly contribute to loss of electrical conductivity between myocytes and thus conduction disturbances in fibrillating and dilated atria. Overall, the role of PAR activation and hypercoagulability in the development of AF might be important for the notion that specific coagulation inhibitors may prevent arrhythmogenic cellular changes.

5. Pre-clinical data for the anti-inflammatory effects of direct factor Xa and factor IIa inhibition

The vitamin K-dependent coagulation factors (e.g. prothrombin; factors VII, IX, and X; protein C, and protein S) are mainly synthesized by hepatocytes in the liver. The activity of these proteins is dependent on their membrane-binding capacity, which is provided by the gamma-carboxyglutamic acid (Gla)-rich domain. The Gla domain is a well-defined part of the protein in which certain glutamic acid residues are altered by means of a post-translational modification step involving vitamin K as a cofactor for the enzyme gamma-glutamyl carboxylase.\textsuperscript{54} Administration of vitamin K antagonists (warfarin and coumarin derivatives) interferes with the cofactor activity of vitamin K, thereby causing inhibition of the post-translational modification, resulting in improper formation of the Gla domain and attenuation of binding of the coagulation factor to the negatively charged cell membranes. Coagulation largely depends on the vitamin K-dependent proteins binding to negatively charged cell membranes, as provided by activated platelets, thereby enhancing the velocity of the enzyme reaction.\textsuperscript{55,56} Coagulation factors synthesized without the Gla domain are called proteins induced by vitamin K antagonism or absence (PIVKAs).\textsuperscript{57} Therefore, factor X produced in the presence of vitamin K antagonists is called PIVKA-X and its active form, PIVKA-Xa, cannot bind to negatively charged cell membranes and will only convert minimal amounts of prothrombin into thrombin (Figure 4). However, activation of PARs by coagulation factors has been suggested to occur in a membrane-binding or Gla domain-independent way.\textsuperscript{58} Therefore, PIVKA-Xa will most likely activate PAR1 and/or PAR2. Indeed, our own data (unpublished) showed activation of PAR1 and/or PAR2 by both native factor Xa (containing the Gla domain) and a PIVKA-Xa (Gla domain-free factor Xa) on cultured fibroblasts. Inhibition of factor Xa using a direct inhibitor, such as rivaroxaban, will inhibit both the conversion of prothrombin to thrombin and factor Xa-mediated activation of PAR1 and PAR2. Similarly, a direct thrombin inhibitor will attenuate the known activities of this enzyme, including activation of PAR1; however, it will not inhibit factor Xa, which will still be available for the activation of PAR1 or PAR2 (Figure 4). In summary, in patients receiving VKAs, coagulation is attenuated but the synthesized PIVKAs retain the capability to activate PARs, whereas in patients who receive direct oral factor Xa or thrombin inhibitors both coagulation and activation of PARs are inhibited.

Thrombin inhibition by the direct thrombin inhibitor melagatran was shown to limit the dimensions of atherosclerotic plaques and also the instability of lesions in an advanced stage of progression in an apolipoprotein E-deficient mouse model of atherosclerosis, although no effect was observed during the early stage of atherosclerosis progression in wild-type mice.\textsuperscript{59} Other studies conducted in an apolipoprotein E-deficient mouse model using dabigatran have also found that direct thrombin inhibition impaired the formation and size of atherosclerotic plaques in addition to preventing progression of the disease and associated stenosis.\textsuperscript{27} Apolipoprotein E-deficient mice with genetically reduced levels of thrombin have decreased atherosclerosis, increased plaque stability, and a decreased pro-inflammatory profile.\textsuperscript{27} In rabbits, selective inhibition of factor Xa by using recombinant antistatin or tick anticoagulant peptide was shown to ensure the reduction of restenosis after balloon...
angioplasty of atherosclerotic femoral arteries and limit the narrowing of these vessels (Figure 5).61

The effect of rivaroxaban on factor Xa-induced thrombin generation was assessed, and a comparison was established with the thrombin inhibitor dabigatran and a PAR1 antagonist (vorapaxar) using primary human umbilical endothelial cells. Rivaroxaban was able to prevent thrombin generation and thus down-regulate the thrombin-mediated pro-inflammatory cytokine expression, as were dabigatran and vorapaxar, supporting the potential anti-inflammatory properties of rivaroxaban.62 Another study demonstrated an in vitro ability of rivaroxaban to impair thrombin-induced activation of human platelets obtained from patients with coronary artery disease, suggesting that this inhibitor could be beneficial in the context of atherosclerotic plaque rupture.63

The role of factor Xa as a key player in the context of atherosclerosis becomes more evident because rivaroxaban administration has been shown to increase the stability of advanced atherosclerotic plaques in apolipoprotein E-deficient mice, as verified by the presence of thicker protective fibrous caps and decreased erosion of the plaques. In addition, significant impaired transcription of pro-inflammatory agents was also reported in mice receiving the specific factor Xa inhibitor, in

Figure 4 Potential effects of anticoagulants on cellular signalling. Thrombin and factor Xa contain Gla domains, which are necessary for proper coagulation. However, during VKA treatment, Gla domain-less thrombin or factor Xa is generated, which diminishes coagulation activity, but has unaltered signalling potential. Treatment with a direct thrombin inhibitor will cause both attenuated coagulation and cellular signalling through PAR1 with unaltered signalling capacity for factor Xa. Only with the use of a direct factor Xa inhibitor will both coagulation and PAR1 and PAR2 signalling be altered. Blood coagulation factors are denoted in Roman numerals, and the active forms are indicated by a small ‘a’. II indicates prothrombin and IIa thrombin.
addition to a non-significant tendency to reduce the size of lesions (Figure 5).6

6. Conclusions

Contrary to what was thought previously, factor Xa- and thrombin-mediated cellular effects may differ, even though both proteases bind to PAR1 and can contribute to the pathophysiology of several disorders such as atherosclerosis.3 The existing data support the view that both factor Xa and thrombin are viable targets for effective anticoagulation. However, additional studies are needed to firmly establish the involvement of pro-coagulant factors in the pathogenesis of atherosclerotic plaques because of the signalling-mediated cellular events in atherosclerosis and other fibroproliferative disorders. Factor Xa may be a preferred target because of its upstream position in the coagulation cascade and its role in PAR-mediated cellular functions, either through direct activation of PARs or by producing thrombin, another activator of these receptors. Although inhibiting factor Xa and thrombin may provide additional therapeutic potential, such as in the context of different fibroproliferative diseases, the data are still limited to pre-clinical studies and are preliminary. There is still much to learn about the pleiotropic functions of these serine proteases, and further investigation is needed to fully understand the complexity and possible long-term complications of targeting factor Xa and thrombin.

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