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Bidirectional Pathologic Effects of Thrombin

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Abstract

Hemostasis is an essential reaction in organisms with circulatory systems. To stop bleeding as soon as possible, reactions of the coagulation system proceed rapidly and are regulated by positive feedback loops. However, coagulation can be harmful when induced at inappropriate sites, potentially resulting in local thromboembolisms in vital organs such as the heart and brain and systemic microthrombi in conditions such as disseminated intravascular coagulopathy. Increasingly, reports indicate that coagulation-related factors can cause serious diseases without obvious thromboembolism-associated ischemia, for example, atherosclerosis and Alzheimer disease. Thrombin is an essential coagulation factor that can also cause tissue injury, particular to endothelial cells. However, thrombin can also function as a tissue-protective factor depending on conditions. Endothelial protein C receptor (EPCR) and protease-activated receptors (PARs) regulate thrombin activity, but many details regarding the mechanisms remain unknown. This short review summarizes the bidirectional effects of thrombin signaling via EPCR and PAR1 and discusses points relevant to translating the tissue-protective effects of thrombin into clinical benefits.

Introduction

Blood coagulation must be strictly regulated, as genetic or pharmacologic inhibition of the coagulation system can induce life-threatening bleeding. Coagulation occurring at the wrong time or wrong location can cause perfusion disturbances that result in organ failure. For example, infarctions occurring in the heart or brain are major causes of death in developed countries. Additionally, excessive and expanded coagulation beyond physiologic control can lead to disseminated intravascular coagulopathy (DIC) with sepsis. An increasing number of reports indicate that the coagulation system dysfunction could be involved in many other diseases, including atherosclerosis, cancer, neurodegenerative diseases, and bronchial asthma [1-3]. In each of these diseases, deleterious activity of a major coagulation factor, thrombin, is thought to worsen the medical condition, independent of thromboembolism.

The coagulation process involves positive feedback reactions that are essential for rapid and sufficient hemostasis; however, these reactions can occasionally proceed in an uncontrolled manner, which has stimulated significant research and therapeutic interest [4]. Despite continual additions to the list of approved anti-coagulation drugs, abnormal coagulation and associated diseases are still clinically important. Over the past few decades, an increasing number of studies have begun to target cellular responses to coagulation instead of the coagulation system per se. The most well-studied coagulation factor is thrombin (activated coagulation factor II), which is vitamin K dependent. After receptors for thrombin were identified on platelets and endothelial cells, researchers began to intensively examine the effects of thrombin on cells [5]. Thrombin increases the permeability of the endothelial cell layer and induces apoptosis of endothelial cells [6-8]. These functions play an important role in wound healing in conjunction with hemostasis. Specific proteins in plasma exudate facilitate cell migration and apoptosis to promote tissue reconstruction. However, when activated inappropriately, these functions of thrombin can negatively impact the blood vessels.

Protease-activated receptors (PARs), which are members of the G-protein-coupled receptor (GPCR) family, mediate thrombin signaling (Figure 1). PAR1 plays a particularly pivotal role in thrombin signaling. Similar to other GPCRs, PAR1 forms complexes with many different plasma membrane proteins to transmit differential signals. Endothelial cells express endothelial protein C receptor (EPCR) in the plasma membrane. EPCR serves as a receptor for activated protein C, which has anti-coagulation activity. Binding of PAR1 to EPCR in the membrane reportedly induces thrombin activities that are beneficial and protective for endothelial cells. This 'switching' mechanism is thus an attractive therapeutic target and has been the focus of numerous studies [8].

This short review will discuss the current state of knowledge regarding thrombin signaling via PAR1 and its regulatory mechanism. The effect of thrombin on endothelial cells is of particular focus due to the associations between thrombin and a number of serious diseases involving injury to endothelial cells, such as atherosclerosis and sepsis. Furthermore, from the perspective of therapeutic development, potential future targets in the thrombin signaling pathway are discussed. Although other PARs are involved in thrombin signaling and other ligands activate PAR1, these are not discussed here; excellent reviews of these subjects are available elsewhere, however [2,9,10]. Among cells affected by thrombin, platelets are the most well-studied to date. Nieman and Jamasbi have written excellent reviews concerning PARs in platelets [11,12].

Coagulation and its Regulation

Coagulation is a sequential reaction involving various proteases and co-factors known as coagulation factors [13]. Coagulation begins with the binding of coagulation factor VII (VII) in the blood with tissue factor derived from injured tissue and ends with the formation of fibrin clots catalyzed by thrombin. Several vitamin K–dependent enzymes produced in the liver play key roles in coagulation: factors II (prothrombin), VII, IX, and X. These enzymes have a Gla domain that

requires vitamin K for normal function. The Gla domain binds to phosphatidylserine residues on the cell surface to initiate the coagulation process. Except for prothrombin, VII, IX, and X share an EGF motif that is essential for enzymatic activity. VII, IX, and X require cofactors for maximal activity. Tissue factor, activated factor VIII (VIIIa) and activated factor V (Va) are cofactors for activated VII (VIIa), activated factor X (Xa), and activated factor IX (IXa), respectively. These cofactors amplify the activity of the coagulation factors 30,000- to 9,000,000-fold.

Amplification of responses is essential for coagulation to produce rapid and effective hemostasis. Thrombin is localized downstream in the coagulation cascade and serves as a key molecule in the positive feedback loop. When a small amount of thrombin is generated by coagulation, it forms a positive feedback loop via three reactions, the activation of XI, V, and VIII. In addition to this feedback loop, concentration of coagulation factors on the cell membrane via the Gla domain accelerates these reactions.

Coagulation is strictly regulated via an inhibitory system comprised of four primary components: 1) protein C-thrombomodulin, 2) antithrombin (AT)-heparin, 3) tissue factor pathway inhibitor (TFPI)-protein S, and 4) Protein Z-protein Z–dependent protease inhibitor (ZPI). Although thrombin is a potent coagulation factor, it plays an inhibitory role in conjunction with thrombomodulin on the endothelial cell membrane, leading to activation of the anti-coagulation factor, protein C. Activated protein C (aPC) inactivates Va and VIIIa, whereas AT inactivates thrombin and Xa in conjunction with heparin. TFPI inactivates VIIa and Xa in cooperation with protein S. Finally, ZPI inactivates Va, IXa, and XIa in conjunction with protein Z.

Thrombin Signaling via PARs

Thrombin signaling is mediated by PARs, which are members of the GPCR family. The GPCRs constitute a large family of proteins that share various characteristics in terms of signal transduction. Desensitization is one of the most important functions of GPCRs, protecting cells from excessive and toxic reactions [14]. Desensitization has been intensively studied with a prototypical GPCR, beta-2 adrenergic receptor. Desensitization involves receptor-transducer uncoupling in the short-term and down-regulation of GPCR in the long-term. Receptor-transducer uncoupling is caused by phosphorylation of the GPCR, binding of GPCR to beta-arrestin, and subsequent clathrin-dependent endocytosis. Down-regulation is caused by GPCR endocytosis and degradation and a decrease in mRNA levels.

Another important characteristic of GPCR is "biased ligand," a term that refers to different agonists initiating differential signaling via the same GPCR via distinct mechanisms [3,15]. G-proteins consist of three subunits, each of which can be one of several types. Different G-proteins recognize their own specific effectors, resulting in a variety of possible signaling pathways via one GPCR. Additionally, signaling via phosphorylated GPCRs is mediated via beta-arrestin instead of G-proteins, although details of the signaling mechanism remain unclear [16]. Some receptor cofactors appear to play significant roles in signal transduction, however.

To date, four PARs have been identified: PAR1-4. Each of these PARs can mediate thrombin signaling. PAR2 and PAR4 respond to high concentrations of thrombin [4,17]. The extracellular domain of the PARs has its ligand at the C-terminus and a partial ligand block at the N-terminus (Figure 1). Upon cleavage of the partial ligand block by a protease such as thrombin, the ligand becomes free to bind to its receptor and remains tethered to the receptor after binding. This mechanism enables a single thrombin molecule to activate more than one receptor.

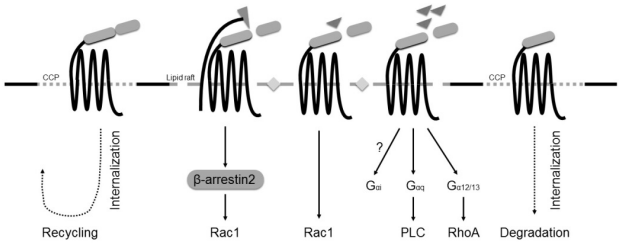

Figure 1: Membrane localization and signaling of PAR1. PAR1 is a member of seven-transmembrane GPCRs. Upon cleavage of the partial ligand block by a protease such as thrombin and aPC, the ligand becomes free to bind to its receptor and remains tethered to the receptor. PAR1 with EPCR and PAR1 activated by low concentration of thrombin mediate the cell protective signals. PAR1 activated by high concentration of thrombin is involved in cytotoxic signaling. Ligand-free PAR1 is internalized and recycled via clathrin-coated pits (CCP). Ligand-stimulated PAR1 is endocytosed via clathrin-mediated process and degraded in lysosomes. Lipid raft is essential for the cytoprotective and cytotoxic signaling via PAR1.

PAR1 functions as a major thrombin receptor. Ligand binding induced by thrombin causes binding of G-protein to the receptor. Ga12/13, Gaq, and Gai bind PAR1. Signaling via Ga12/13 activates the RhoA-ROCK (rho-associated coiled coil–containing protein kinase) axis and induces stress fiber formation. In endothelial cells, activation of the RhoA-ROCK axis increases endothelial permeability and induces apoptosis. Signaling via Gaq activates phospholipase C, which increases intracellular calcium levels and stimulates PKC activity, resulting in increased secretion of cell adhesion molecules and inflammatory cytokines. Signaling via Gai suppresses adenylyl cyclase, leading to decreased levels of cAMP. In addition to signaling via G-protein, PAR1 signaling also involves a pathway mediated by beta-arrestin. Thrombin signaling via Ga12/13 and Gaq can damage tissues, as it induces hyper-permeability, apoptosis, and inflammation, whereas thrombin signaling via Gai and beta-arrestin is protective. Therapeutic targeting of PAR1 thus necessitates that its beneficial protective activity be preferentially stimulated.

Bidirectional Activities of Thrombin

The bidirectional activities of thrombin are regulated via two mechanisms. One mechanism involves interaction between EPCR and aPC on the cell membrane, whereas the other mechanism is dependent upon the concentration of thrombin (Figure 1). Interaction between

EPCR and aPC in conjunction with low thrombin concentrations has a protective effect on cells. The bidirectional activities of thrombin are thought to involve biased ligands.

As mentioned above, thrombin activates protein C in the presence of thrombomodulin. aPC affects endothelial cells independent of coagulation. Like thrombin, aPC is a vitamin K–dependent serine protease that cleaves the extracellular domain of PAR1 to extricate its ligand. However, aPC cleaves a different site in PAR1 than doe's thrombin, and this difference was thought to be the reason for the cytoprotective effects of aPC. However, more recent research has revealed that the cytoprotective effects are associated with binding between PAR1, EPCR, and aPC, even if thrombin has cleaved PAR1 [18]. EPCR acts as a switch for the bidirectional activities of PAR1 [8]. EPCR-dependent cytoprotective signaling is mediated by PAR1 phosphorylation by GRK5 through beta-arrestin, resulting in reduced endothelial permeability due to Rac1 activation [16,19]. The contribution of Gai to these reactions is not well understood, however.

Thrombin reportedly exhibits opposing effects depending on its concentration in a variety of cell types, including endothelial cells, neurons, and glia cells [20-22]. At concentrations less than 50 pM, thrombin exhibits cytoprotective effects. At concentrations greater than 1 nM, by contrast, thrombin acts as a cytotoxic protein. The bidirectional activities of thrombin have been demonstrated not only through *in vitro* and *in vivo* experiments but also in human clinical studies [23,24]. A 1-year observational study of patients with acute coronary syndrome demonstrated that patients with a thrombin concentration between 1.5 and 1.9 nM have the lowest incidence of coronary events. Thrombin concentrations above or below this range were associated with a higher rate of coronary events.

Thrombin signaling is regulated by its membrane localization. PAR1 and its cofactors, including G-proteins and EPCR, are localized in lipid rafts, which are specialized cell surface microdomains with or without caveolin [18,19,25]. Endothelial cells are rich in lipid raft. Lipid rafts are thought to be platforms for signal transduction involving many different ligands [26,27]. Caveolin 1 plays pivotal roles in signal transduction regulation [28]. In the absence of ligand, Ga12/13 binds caveolin1. Signaling via Ga12/13 is suppressed by inhibition of raft formation, but not by targeting caveolin1. Raft formation is essential for signaling involving the Ga12/13-Rho axis, which appears to be regulated by caveolin1. The cytoprotective effects of the EPCR and PAR1 complex are abolished by targeting caveolin1 [8,29]. However, in signaling mediated by aPC, binding between EPCR and caveolin1 is reversed.

Ligand-stimulated PAR1 is endocytosed via a clathrin-mediated process and degraded in lysosomes [30,31]. PAR1 internalization plays a role in desensitization of PAR1. By contrast, although ligand-free PAR1 is also internalized, it is recycled to the plasma membrane [32]. These two distinct internalization mechanisms are regulated by rab11A and B [33]. In many GPCRs, beta-arrestin is involved in internalization; however, it is not involved in internalization PAR1 [34]. Instead, beta-arrestin plays a role in cytoprotective signaling involving non-internalized EPCR [29].

Signal transduction via PAR1 is regulated by its localization and trafficking. Studies to elucidate the mechanism through which PAR1 signaling is regulated are in early stages. The role of caveolin1 in switching between cytotoxic and cytoprotective signaling is poorly understood, as is the mechanism of PAR1 translocation from rafts to clathrin pits. Post-translational ubiquitination and palmitoylation could be important steps in these processes [35-37]. As describe below, deleterious effects of thrombin have been linked to numerous serious diseases. PARs could be therapeutic targets in treating these diseases, but further investigations of PAR localization and trafficking are necessary.

Clinical Aspects of Thrombin and PAR1

Under normal circumstances, the blood coagulation process is slightly activated and thrombin does not cause injury. However, infection-associated inflammation or metabolic disorders can lead to dysregulation of thrombin activity, resulting in adverse effects in numerous organs and tissues. Continuous activation of thrombin can cause serious diseases even in the absence of obvious thrombotic events. The bidirectional effects of thrombin appear to play pivotal roles in the pathogenesis of these diseases.

Blood vessels

Thrombin induces the expression of various adhesion molecules (P-selection, E-selection, ICAM-1, VCAM-1) and chemokines (IL-1, IL-6, IL-8, TNF-alpha) in endothelial cells, inducing platelets and leukocytes to adhere to the surface of endothelial cells, resulting in local inflammation [1,4]. Inflammation increases the expression of VEGF receptors and matrix metalloproteinases (MMPs) [2]. VEGF and MMPs accelerate the proliferation and migration of endothelial cells, resulting in angiogenesis. Thrombin stimulates endothelial cells to reconstruct the cytoskeleton, thus altering the cell form and degrading intercellular adhesion mediated by VE-cadherin [38]. Thrombin also induces the apoptosis of endothelial cells [6-8]. These responses decrease endothelial permeability, leading to thrombin localization under endothelial cells. As in endothelial cells, thrombin induces apoptosis and expression of MMPs in smooth muscle cells. These processes reportedly affect the structure of blood vessels in a manner that promotes atherosclerosis [39-43]. In atherosclerotic lesions, PAR1 expression is increased [1]. While thrombin promotes atherosclerotic lesion formation, it can simultaneously suppress apoptosis and decrease endothelial permeability. These cytoprotective effects involve complex interactions between EPCR and PAR1 [44].

Central nervous system

Recent research has focused on the role of thrombin in the pathogenesis of neurodegenerative disorders such as Alzheimer disease and Parkinson disease in addition to brain infarction and bleeding [45]. Most thrombin is generated in the liver. However, the brain is now thought to be a source of prothrombin and PAR1 [46,47]. The thrombin-PAR system in the brain may play significant roles in various brain-related diseases. In animal models of brain ischemia, traumatic brain and spinal cord injury, aPC significantly suppressed brain edema by protecting the integrity of the blood brain barrier, thus improving the prognosis [48]. This signaling is mediated via complex interaction between EPCR and PAR1. aPC also protects neural cells via PAR1 and PAR3. Clinical trials of therapies targeting aPC in brain infarction are ongoing [49].

Dermis

The cornified epithelium expresses thrombin, protein C, EPCR, thrombomodulin, and PARs [50,51]. The barrier function of keratinocytes is promoted by the EPCR-PAR1 complex. Tie2 and epidermal growth factor receptor (EGFR), which are cross-activated

with PAR1, are also involved in the protective effects of thrombin. Signaling via EPCR-PAR1 and EGFR also stimulates the proliferation of keratinocytes [52,53].

Others

Bidirectional effects of thrombin have also been observed in podocytes under high glucose stress [54]. Low concentrations of thrombin are protective, whereas high concentrations lead to cell death in high-glucose culture. In many disease models, such as diabetic nephropathy, ischemia-reperfusion, and acute tubular injury, aPC signaling via EPCR and PAR1 reportedly restores renal function, protecting glomerular endothelial cells and renal tubular cells [55-57].

The opposing effects of thrombin and aPC via PAR1 and EPCR reportedly control hematopoietic stem cell differentiation [58,59]. Thrombomodulin, which is essential for the activation of protein C, maintains hematopoietic stem cells in a quiescent state and causes retention of them in the bone marrow. Administration of aPC or soluble thrombomodulin improves hematopoietic progenitor activity after total body irradiation [60].

Concluding Remarks

Among therapies for diseases involving pathologic coagulation, tissue plasminogen activator has shown tremendous promise for use in the treatment of brain and heart infarctions due to formation of massive thrombi. However, limited effects of anticoagulation therapy have been reported for sepsis and DIC involving systemic microthrombi [61,62]. One reason for this limited efficacy could be endothelial cell dysfunction in these diseases. Therapies to restore endothelial function should be provided in addition to anti-thrombotic therapy. From this perspective, the protective effects of thrombin are attractive in the development of therapeutic approaches. EPCR is thought to be a key molecule in mediating the protective effects of thrombin, and therapeutic methods employing recombinant EPCRs are being developed [44,63,64]. However, the mechanisms leading to switching between the protective and deleterious signaling via thrombin remain poorly understood. Considering that the signaling via EPCR is intrinsically overcome by deleterious signaling, elucidation of the switching mechanism could lead to improved efficiency of therapies employing recombinant EPCRs. Intracellular trafficking of EPCR and PAR1 likely play pivotal roles in the activation, inactivation, and down-regulation of those receptors, because they function in lipid rafts and are degraded via clathrin-dependent endocytosis. Additionally, lipid rafts are rich in cholesterol and phosphatidylserine, an essential lipid for activation of coagulation factors, including thrombin, FVII, FIX, and FX. Development of therapies targeting the regulation of lipid raft formation in endothelial cells during the coagulation process is a worthy goal [65].

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