

Theme Issue Article

Prothrombin/thrombin and the thrombin receptors PAR-1 and PAR-4 in the brain: Localization, expression and participation in neurodegenerative diseases

Elena Sokolova, Georg Reiser

Otto-von-Guericke-Universität Magdeburg, Medizinische Fakultät, Institut für Neurobiochemie, Magdeburg, Germany

Summary

Emerging evidence demonstrates that thrombin exerts physiological and pathological functions in the central nervous system. Both prothrombin and its active form thrombin have been detected locally in the brain. The cellular functions of thrombin are mainly regulated by G protein-coupled protease-activated receptors (PARs). Thrombin can signal via PAR-1, PAR-3 and PAR-4. Some neurological diseases (e.g. Alzheimer's disease or Parkinson's disease) are characterized by increased levels of both active thrombin and PAR-1. This indicates that thrombin

and its receptor may be closely involved in the development of neurodegenerative processes. The role of thrombin in brain injury can be either protective or deleterious, depending on the concentration of thrombin. Thrombin at high concentrations exacerbates brain damage. In contrast, low concentrations of thrombin rescue neural cells from death after brain insults. Also thrombin preconditioning has neuroprotective effects. Therefore, thrombin and thrombin receptors represent novel therapeutic targets for treating neurodegenerative diseases.

Keywords

Thrombin, PAR, central nervous system, neurodegeneration, neuroprotection

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Introduction

Thrombin (EC 3.4.21.5), a serine protease of the trypsin family, is a key enzyme of the blood coagulation system. It is generated from prothrombin by the cleavage of two peptide bonds (Arg₃₂₀-Ile and Arg₂₇₁-Thr) by prothrombinase (a complex of coagulation factors Xa and Va, Ca²⁺, and phospholipids) (1). Thrombin is a polyfunctional, but narrow-specificity proteinase. The high selectivity in substrate selection by thrombin arises from the enzyme structure. Thrombin consists of a light A-chain (49 amino acid residues) and a heavy catalytic B-chain (259 amino acid residues) connected by a disulfide bond. Besides the active site, the B-chain comprises additional extended substrate binding sites called exosites that increase substrate-enzyme affinity. The anion-binding exosite I (also called fibrinogen recognition site) recognizes fibrinogen and fibrin, factor V, thrombomodulin, membrane receptors, hirudin (a highly selective exogenous thrombin inhibitor), platelet integrin GP I β . The anion-binding exosite II is re-

sponsible for binding of thrombin to heparin, GP I β , and glycosaminoglycans (2–4).

Generation of thrombin leads to conversion of fibrinogen to fibrin, the main constituent of a clot. Thrombin also regulates the positive feedback, activating blood coagulation factors V, VIII, XI, and XIII, and the negative feedback, activating (in the complex with thrombomodulin) protein C, which limits thrombinogenesis by cleavage of factors Va and VIIIa (5). Thrombin takes part not only in blood coagulation, but it also interacts with specific cell receptors to induce signaling pathways which mediate inflammatory responses (6–9).

In the 1990s, the thrombin receptors, protease-activated receptors (PARs), were identified (10). Four members of PARs were cloned and characterized and named PAR-1, -2, -3, and -4. Discovery of the first protease-activated receptor (PAR-1, originally named thrombin receptor) allowed to explain direct cellular effects of thrombin on different cell types (11). PARs belong to a family of seven transmembrane domain, G protein-coupled receptors. Activation of PARs requires cleavage of a specific pep-

Correspondence to:

Prof. Dr. G. Reiser
Otto-von-Guericke-Universität Magdeburg
Medizinische Fakultät, Institut für Neurobiochemie
Leipziger Strasse 44, D-39120, Magdeburg, Germany
Tel.: +49 391 6713088, Fax: +49 391 6713097
E-mail: georg.reiser@med.ovgu.de

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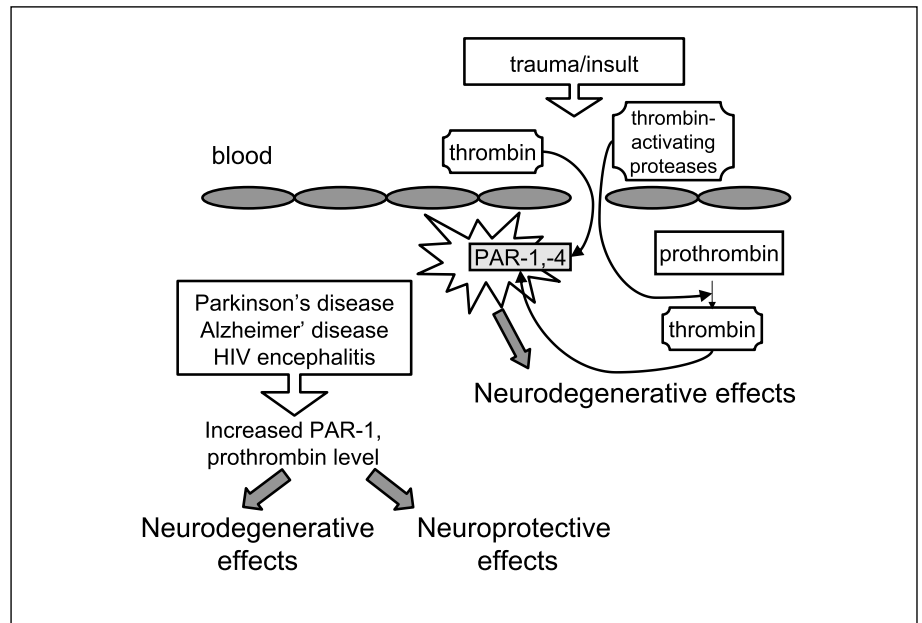
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Figure 1: Traumatic or ischemic brain injury results in disruption of the blood-brain barrier and subsequent infusion of thrombin and other blood-derived factors into the brain. Those factors activate local brain prothrombin giving rise to active thrombin. Bloodstream and locally formed thrombin activates PAR-1 and PAR-4 on neuronal and glial cells. Thrombin that accumulates at high levels is deleterious for neurons under ischemic conditions. Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease or HIV encephalitis, are characterized by increased levels of prothrombin and PAR-1 in the brain. Depending on the disease model, thrombin can show either neuroprotective or neurodegenerative effects (58, 65).



tide bond, called “activation site”, in the N-terminal extracellular part of the receptors that results in removal of the N-terminal peptide of 37–59 amino acids (the length varies depending on PAR subtype and species) and unmasking of a new N-terminus. The newly generated N-terminal end (so called “tethered ligand”) binds to the second extracellular loop of the same receptor thus activating it. The interaction of the hirudin-like sequences in PAR-1 and PAR-3 with exosite I of thrombin highly potentiates receptor activation by the enzyme. The lack of the hirudin-like sequence in the PAR-4 structure makes this receptor a rather poor substrate for thrombin (12). Nevertheless, PAR-4 can be activated by low concentrations of thrombin during the early phases of coagulation (13). PAR-2 is the only member of the PAR family that is not activated by thrombin due to the absence of the key Pro residue in the position P2 of the activation site (GTNRSSKGR/SLIGKV) of PAR-2. Its activation can be fulfilled by other coagulation proteases, factors Xa and VIIa, or by trypsin.

Accumulating evidence demonstrates a role of thrombin and thrombin receptors in neuroinflammatory and neurodegenerative processes in the central and peripheral nervous system (14–17). Thus, thrombin and its receptors represent valuable therapeutic targets for treating neurodegenerative disorders.

Localization of prothrombin/thrombin and PARs in brain

Thrombin at high concentrations can enter the brain as a result of increased permeability of the blood-brain barrier that takes place during closed head trauma, severe epilepsy and other pathologic situations. Additionally, thrombin can be formed locally from prothrombin which was shown to be expressed in rat and human brains (18–21). In rat brain, prothrombin is distributed in the olfactory bulb, cortex, colliculus superior and inferior, corpus striatum, thalamus and hippocampus (22). Prothrombin and throm-

bin have been localized on neurons and glial cells. Importantly, the expression of factor X, the prothrombin activator, is also observed in the brain (23). In addition, inhibitors that regulate thrombin activity, such as protease nexin-1 (PN-1) and anti-thrombin III, are locally expressed in the brain (24, 25). Much of the PN-1 in human brain is localized around blood vessels (26) where it can regulate extravasated thrombin following breakdown of the blood-brain barrier.

Prothrombin and thrombin receptors, PARs, are colocalized in the central nervous system (CNS) (18). Thrombin receptors are expressed in brains of rodent and human. In adult rats, PARs are widely distributed throughout the brain tissue (27–29). Immunoreactivity corresponding to PAR-1 was detected in normal human brain (20, 21, 30). PARs are expressed both on glial cells and neurons.

Thus, the expression in CNS of prothrombin, factor X, thrombin-specific inhibitor nexin-1 and PARs allows to suggest that thrombin can be produced and then mediate its effects via PAR activation under normal conditions in intact nervous tissue. However, this was questioned by Sinnreich et al. (31). Mice over-expressing prothrombin in neurons did not show any detectable thrombin enzymatic activity, unless thrombin-activating factors, e.g. blood-derived factors, were added.

Therefore, it is likely that the action of thrombin in the CNS becomes more prominent under pathological conditions when the blood-brain barrier is disrupted. In support of this, the levels of prothrombin and thrombin are increased on astrocytes, neurons and blood vessels in human brain of patients with neurodegenerative disorders, such as Parkinson's disease, human immunodeficiency virus (HIV) encephalitis, and Alzheimer's disease (20, 21, 32). Likewise, the prothrombin level was increased after transient global ischemia in rat brain (33). Concomitant elevation of PAR-1 in diseased human brain (20, 21) indicates that thrombin and PAR-1 may be closely involved in the development of neurological diseases.

Thrombin as a mitogenic and neuroinflammatory agent

Over the past years, thrombin has been increasingly considered as a potent mitogenic and proinflammatory agent, thereby regulating neuronal function. The proliferative effect of thrombin has been demonstrated in cultured glial cells, including microglia and astrocytes (34–37). Both studies *in vitro* and *in vivo* showed the involvement of PAR-1 in this effect of thrombin (35, 36, 38). Moreover, thrombin via PAR-1 activation participates in the process of gliotic scar formation after brain injury (38).

Activation of microglia, immune effector cells in the brain, is an important event in the development of neuroinflammation. Thrombin has been shown to induce microglia activation and mediate expression of a number of proinflammatory factors, such as nitric oxide (NO), interleukin (IL)-6, IL-1 α , IL-1 β , and tumor necrosis factor (TNF)- α *in vitro* and *in vivo* (39–42). On the other hand, in rat microglia, thrombin also induces the expression of the immunosuppressive cytokine IL-10, which in turn inhibits TNF- α release (43). The involvement of PARs in thrombin-induced microglial activation is still controversial. The indirect role of PAR-1 in microglia activation was shown by Suo et al. (35). Other reports demonstrated PAR-independent effects of thrombin (44–46). Moreover, it was suggested that thrombin does not require its proteolytic activity to activate microglia (44). Hanisch et al. further revealed that a high-molecular-weight contaminant of thrombin preparations, but not thrombin itself, induces cytokine release and proliferation in microglia (47).

Astrocytes, the major glial cell type in the brain, provide trophic factors and energy for neurons, and maintain extracellular ion homeostasis and neurotransmitter uptake. Besides this, astrocytes are also involved in inflammatory reactions in the brain. Thrombin induces the release of important proinflammatory mediators such as arachidonic acid (48, 49), the chemokine growth-regulated oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1), a counterpart of the human GRO and IL-8 (50), IL-1 β (20), and NO which is produced by iNOS (20, 51). Studies *in vivo* have shown that iNOS can be detected in astrocytes and microglia in mouse striatum injected with PAR-1 AP (20). Therefore, activation of astrocytes by thrombin also contributes to neuroinflammation in the brain.

Thrombin and PARs in neurodegenerative disorders

Ischemia

Thrombin has been demonstrated to modulate ischemic, haemorrhagic and traumatic brain injury. The ischemic rat brain is characterized by high levels of thrombin that results from increased local expression of prothrombin (33, 52) as well as from blood-brain barrier disruption and infusion of bloodstream thrombin into the brain parenchyma. On the other side, experimental ischemia conditions differentially regulate the expression of thrombin receptors in the rat brain. It was shown that the expression of PAR-1 is increased in hippocampal slices after exposure to experimental ischemia, i.e. oxygen-glucose deprivation (OGD) (28). Transient focal ischemia induced by microinjection

of endothelin-1 near the middle cerebral artery results in PAR-1 down-regulation (53). Focal ischemia induces expression of PAR-1 and PAR-3 on microglia and enhances PAR-4 labeling in the penumbra (54). These data strongly indicate the important role of thrombin and thrombin receptors in stroke.

Recent studies indicate that thrombin regulation by PN-1 is important following injury to the CNS. Several cytokines and growth factors produced in the brain following injury stimulate the synthesis and secretion of PN-1 by cultured brain cells (55, 56). On the other hand, PN-1 total expression level in the hippocampus was not changed after transient global ischemia (33). PN-1 was shown to maintain its constant level due to triggered *de novo* synthesis following transient forebrain ischemia (57). Therefore, the balance between PN-1 and thrombin may be important to optimize repair processes following brain injury.

Evidence accumulating over recent years demonstrates that low concentrations of thrombin are neuroprotective in the ischemic brain. Studies *in vitro* have shown that thrombin (10 pM–10 nM) protects hippocampal neurons and astrocytes from cell death in response to OGD, hypoglycemia, growth supplement deprivation, oxidative stress or C₂-ceramide (50, 58, 59). These effects of thrombin are mediated by PAR-1.

In studies *in vivo*, thrombin, given several days before a brain insult (the so-called thrombin preconditioning [TPC]), also exerted the neuroprotective effect. Intracerebral infusion of a low dose of thrombin attenuated the brain edema after intracerebral haemorrhage, a subtype of stroke (60, 61). Likewise, TPC reduced the infarct volume after focal cerebral ischemia (60, 62). The maximal effect of TPC on brain edema formation peaks at seven days after pre-treatment (61). The neurological behaviour test indicated that TPC enhances the capacity of mice on motor performance after transient focal cerebral ischemia (63). Activation of PAR-1 is required for thrombin-induced brain tolerance (64).

Although low concentrations of thrombin protect the brain from insults, thrombin at high concentrations (≥ 100 nM) causes brain damage (65). In the hippocampal slice cultures, high concentrations of thrombin exacerbate OGD-induced neuronal death. Moreover, thrombin at 500 nM alone induces more severe cellular damage than OGD alone (58). Thrombin also directly induces delayed neuronal injury in the cerebral cortex and shrinkage of the striatum in organotypic cortico-striatal slice cultures (66). Thrombin-induced shrinkage of the striatum is inhibited by the thrombin inhibitor argatroban and the PAR-1 antagonist FR171113, whereas thrombin-induced cortical injury is only partially attenuated by argatroban. This suggests that PAR-1 differently mediates the toxic effect of thrombin in the cortex and striatum. Thrombin-induced striatal neuronal death is further confirmed *in vivo* (42).

The detrimental effect of thrombin has been also demonstrated in other experimental ischemic models. Studies with knock-out mice revealed that PAR-1 increases the infarct volume and contributes to neuronal damage after transient focal cerebral ischemia (67) and combined cerebral hypoxia/ischemia (68). PAR-1-deficient mice have less neuronal death, reduced glial fibrillary acidic protein (GFAP) expression and smaller lesion volumes than wild-type mice after hypoxia/ischemia. Importantly, animal behavioral studies demonstrate that PAR-1 deficiency at-

tenuates the motor behavioral impairment in hypoxic/ischemic mice (68). Similarly, inhibition of thrombin by antithrombin reduced the infarct volume and neuronal loss, and prolonged animal survival after transient and permanent focal ischemia in rats and mice (69).

One of the mechanisms of thrombin-PAR-1-mediated excitotoxic neuronal injury in the brain has been proposed to involve release of glutamate from glial cells (70, 71). As a result, the elevated glutamate activates N-methyl-D-aspartate (NMDA) receptors on neighbouring neurons in the hippocampus that may mediate neuronal death. The neurotoxicity could be partially blocked by the NMDA receptor antagonist MK-801 (20).

Parkinson's disease

Recent studies demonstrate that thrombin is linked to an important neurodegenerative disease, Parkinson's disease. Parkinson's disease is characterized by the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (72) as well as by activated microglia (73, 74). The level of active thrombin is significantly increased in the vessel wall in SNpc of Parkinson's disease brains. The immunoreactivity of prothrombin and PAR-1 is strongly up-regulated in GFAP-positive astrocytes in SNpc of Parkinson's disease brains (21).

Emerging evidence demonstrates the deleterious effect of thrombin on dopaminergic neurons. Both studies *in vitro* (45) and *in vivo* (39–41, 75, 76) showed that neuronal death was a result of activation of microglia that was induced by thrombin. The catalytic activity of thrombin was shown to be necessary for microglia activation (39, 75). Thrombin-activated microglia released various proinflammatory mediators including NO, TNF- α , IL-1 α , IL-1 β , and IL-6 (39, 40, 45, 76) and produced reactive oxygen species (41). Subsequently, oxidative stress and inflammatory reactions were associated with apoptotic death of dopaminergic neurons (41, 75).

While the neurodegenerative role of thrombin in the rat model of Parkinson's disease is undoubtful, the utilization of PAR-1 by thrombin is unlikely (75). Moreover, in human SNpc of Parkinson's disease brains, microglia, neurons as well as oligodendrocytes were immunonegative for all thrombin receptors: PAR-1, PAR-3 and PAR-4 (21). This excludes the possibility of local PAR activation and the resultant loss of neurons. However, distant effects of PAR activation by thrombin were observed in rat brain (29). PAR-4 but not PAR-1 was shown to be responsible for the loss of dopaminergic neurons in substantia nigra when thrombin was injected into striatum. This highlights the importance of PAR-4 in mediating neurodegenerative effects of thrombin.

Conversely, PAR-1 after activation by thrombin has been shown to be protective in the rat 6-hydroxydopamine (6-OHDA) model of Parkinson's disease (77). By behavioral tests, it was detected that preconditioning with thrombin or with the PAR-1 agonist peptide attenuates neurological deficits in the animal model of Parkinson's disease. PAR-1 antagonists abolished this neuroprotective effect (77). Similarly, delayed thrombin administration, at one or seven days after 6-OHDA injection, also prevents 6-OHDA-induced behavioral and neurochemical deficits. However, thrombin or the PAR-1 agonist peptide, co-administered with 6-OHDA, significantly increases the behavioral defi-

cits (78). Therefore, the neuroprotective effect of thrombin depends on the time of administration in the 6-OHDA model of Parkinson's disease.

Alzheimer's disease

Thrombin is present in brains of patients with Alzheimer's disease (AD). It is accumulated in senile plaques, reactive microglial cells and neurofibrillary tangles in AD brains (19) and microvessels (32). Additionally, in an animal model of disease, the expression of PARs is modulated in the rat hippocampus. Following the administration of trimethyltin, that mimicks effects of AD, PAR-1, and to a lesser extent PAR-3 and PAR-4, are up-regulated in reactive hippocampal astrocytes (79). On the contrary, the levels of PN-1 around cerebral blood vessels and the number of blood vessels exhibiting PN-1 immunoreactivity were markedly reduced in the brains of patients with AD compared to age-matched controls (80). This suggests that an imbalance between PN-1 and thrombin may be a contributing factor in the pathology of AD.

Intracellular aggregates of the microtubule-associated protein tau are one of the pathological hallmarks of Alzheimer's disease. Thrombin is able to process tau *in vitro* (81). Interestingly, thrombin fails to degrade phosphorylated tau. Similarly, paired helical filament tau prepared from AD brain is more resistant to thrombin proteolysis than tau, which underwent dephosphorylation. Therefore, thrombin may be involved in the release of the N-terminal tau fragments to the cerebrospinal fluid, but may induce formation of intracellular aggregates of C-terminal tau fragments in the brain under pathological conditions. Indeed, thrombin has been shown to induce hyperphosphorylation and aggregation of tau in hippocampal neurons (82). Thrombin-induced tau aggregation is mediated by PAR-1 and PAR-4. Furthermore, it was shown that thrombin-induced tau aggregation is neurotoxic and contributes to apoptosis of hippocampal neurons (82). Taken together, thrombin might directly act on hippocampal neurons to induce neurofibrillary degeneration and contribute to the pathogenesis of AD.

Amyloid- β (A β) is significantly involved in initiation and progression of Alzheimer's disease. Thrombin appears to cleave amyloid precursor protein (APP) *in vitro*, thereby generating A β (83). However, several studies demonstrate that thrombin cannot induce APP processing in neurons and glioblastoma cells (84, 85). The effect of thrombin on A β -induced toxicity has been also investigated. It was shown that thrombin enhances A β -induced neurotoxicity via increased intracellular calcium levels and oxidative stress, whereas PN-1 protects neurons against A β toxicity (86). Conversely, thrombin was shown to attenuate A β -induced cell death of hippocampal neurons via PAR-1 activation (87). In addition, thrombin, through activation of PAR-1, reverses A β -induced astrocyte stellation.

Although studies *in vitro* have indicated the role of thrombin and PARs on tau aggregation and neuronal death, it is still unclear whether thrombin contributes to the formation of amyloid plaques and neurofibrillary tangles, and neurodegeneration *in vivo*. Further investigation in animal models may provide insights into the exact roles of thrombin and PARs in Alzheimer's disease.

Conclusions

Emerging evidence has revealed that thrombin at high concentrations contributes to the pathological processes in the brain during neurodegenerative diseases, including stroke, Alzheimer's disease, and Parkinson's disease. In contrast, low concentrations of thrombin and thrombin preconditioning can rescue cells to induce survival of neurons and astrocytes exposed to various brain insults. PAR-1 has been shown to extensively mediate thrombin effects in the brain (Fig. 1). In contrast, the func-

tions of PAR-3 and PAR-4 in the brain are still largely unknown; however, the evidence of neurodegenerative effects of PAR-4 is now accumulating (29, 75). Understanding PAR-3 and PAR-4 signaling would largely improve our knowledge of thrombin functions in the brain. For PAR-3, so far there is only scarce evidence that it can signal autonomously as a thrombin receptor (88). Overall, thrombin and PARs represent novel therapeutic targets for treating neurodegenerative disorders. Thrombin inhibitors, PAR agonists and antagonists will be invaluable pharmacological tools as therapeutics.

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