

# FSAP, a new player in inflammation?

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## Keywords

Factor VII-activating protease, inflammation, hyaluronic acid binding protein 2, nucleosomes

## Summary

Factor VII-activating protease (FSAP) is a serine protease in plasma that has a role in coagulation and fibrinolysis. FVII could be activated by purified FSAP in a tissue factor independent manner and pro-urokinase has been demonstrated to be a substrate for purified FSAP in-vitro. However, the physiological role of FSAP in haemostasis remains unclear. More recently FSAP is suggested to be involved in inflammation. It modulates vascular permeability directly and indirectly by the generation of bradykinin. Furthermore, FSAP is activated by dead cells induced by the inflammatory response and subsequently removes nucleosomes from apoptotic cells. FSAP activation can be detected in sepsis patients as well. However, whether FSAP activation upon inflammation is beneficial or detrimental remains an open question.

In this review the structure, activation mechanisms and the possible role of FSAP in inflammation are discussed.

## Schlüsselwörter

Faktor-VII-aktivierende Protease, Entzündung, Hyaluronsäure-bindendes Protein-2, Nukleosome

## Zusammenfassung

Die Faktor-VII-aktivierende Protease (FSAP) ist eine Serinprotease im Plasma, der eine Rolle bei Koagulation und Fibrinolyse zukommt. FVII konnte, unabhängig von Gewebefaktor, durch gereinigte FSAP aktiviert werden und es wurde gezeigt, dass Pro-Urokinase in vitro ein Substrat für gereinigte FSAP ist. Jedoch ist die physiologische Rolle von FSAP bei der Hämostase weiterhin unklar. Neuerdings wird eine Beteiligung von FSAP an Entzündungen vermutet. Durch die Bereitstellung von Bradykinin moduliert es direkt und indirekt die Gefäßpermeabilität. Darüber hinaus wird FSAP, induziert durch die entzündliche Reaktion, durch tote Zellen aktiviert und entfernt anschließend Nukleosomen aus apoptotischen Zellen. Die Aktivierung von FSAP kann bei septischen Patienten ebenfalls festgestellt werden. Es bleibt jedoch offen, ob die Aktivierung von FSAP bei einer Entzündung von Vorteil oder von Nachteil ist.

In dieser Übersicht werden Struktur, Aktivierungsmechanismus und die mögliche Rolle des FSAP bei Entzündungen diskutiert.

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## FSAP, ein neuer Akteur bei Entzündungen?

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tributed to FSAP, its in-vivo functions are not clear yet.

In this review we will describe the structure, activation mechanisms and the possible role of FSAP in inflammation.

## Structure and function

FSAP consists of 560 amino acids including a 23 amino acid pro-peptide at the amino-terminal part of the molecule (1). It is synthesized in the liver and has a plasma concentration of 12 µg/ml. FSAP consists of

- three epidermal growth factor (EGF) domains
- a kringle domain
- a serine protease domain at its C-terminus

It has a high homology with urokinase, plasminogen, FXII or hepatocyte growth factor-activator (HGF-A) (1, 4). In plasma, FSAP circulates as an inactive single-chain molecule of 78 kDa that can be converted in its active two-chain form consisting of a 50 kDa heavy and a 28 kDa light chain connected by a disulfide bond.

Sequence analysis revealed that the first cleavage site of FSAP occurs at Arg290-Ile291 (5) resulting in the active two-chain form. The light chain harbors the proteolytic domain consisting of a catalytic triad formed by His239, Asp388 and Ser486 (1). At the N-terminal part, two possible N-linked glycosylation sites (Asn31 and Asn284) have been described (1). The isoelectric point of FSAP was determined to be 4.9–5.5 (6). Two single nucleotide polymorphisms (SNPs) were found in the FSAP gene (7), named

- Marburg I (G511E)
- Marburg II (G380Q)

The presence of the Marburg I polymorphism results in diminished proteolytic activity towards pro-urokinase (pro-UK) whereas the activity towards FVII remains

FSAP was firstly described as a plasma hyaluronan acid binding protein (HABP2), purified from plasma by affinity chromatography on hyaluronan-conjugated sepharose (1). Later, when purifying vitamin-K dependent coagulation factors from cryoplasma, Hunfeld et al. found a protease-activity towards a thrombin-sensitive chromogenic substrate which could

not be inhibited by hirudin to be HABP2 (2). At the same time another group identified a protease which was able to activate factor VII (FVII) in-vitro in the absence of tissue factor (TF) (3). In view of this activity the protease was termed as “factor VII-activating protease (FSAP)” which finally turned out to be identical to HABP2. Although many in-vitro functions are at-

unaffected (7). The Marburg II (E393Q) variant is not associated with an altered FSAP function.

### Activation and regulation of FSAP

Single-chain FSAP (scFSAP) in purified systems is reported to be susceptible to autoproteolysis. Kannemeier et al. showed that this autoactivation was enhanced in the presence of heparin, whereas  $\text{Ca}^{2+}$  ions stabilized scFSAP (6). Negatively charged polyanions with a high charge-to-size ratio enhance autoactivation of FSAP resulting in its active two-chain form (8). Besides heparin, polyphosphates, DNA and RNA have been demonstrated to accelerate autoproteolysis of purified scFSAP (3, 5, 8–10). The EGF3 domain containing a positively charged cluster was shown to be essential for this polyanion binding and for heparin-induced acceleration of autoproteolysis (8, 9). Urokinase has been demonstrated to activate purified FSAP (6). Furthermore, an acidic milieu has been shown to reduce the activation and degradation of scFSAP (6). Polycations, such as polyamine have been demonstrated to activate scFSAP as well (11).

Based on their findings Yamamichi et al. suggested an interesting model on how polyanions and -cations might promote autoactivation of purified FSAP. They suggest that an intramolecular interaction between the N-terminus containing acidophilic amino acids and positively charged basophilic clusters within the EGF3 domain prevent autoproteolysis. Polycations are suggested to interfere with that intramolecular interaction, thereby allowing intermolecular binding of the N-terminus to the EGF3 domain of an adjacent FSAP molecule to form an autoactivation complex. In contrast, polyanions offer a scaffold to which scFSAP molecules can bind via their EGF3 domain and when located in juxtaposition may lead to autoactivation (► Fig. 1) (11).

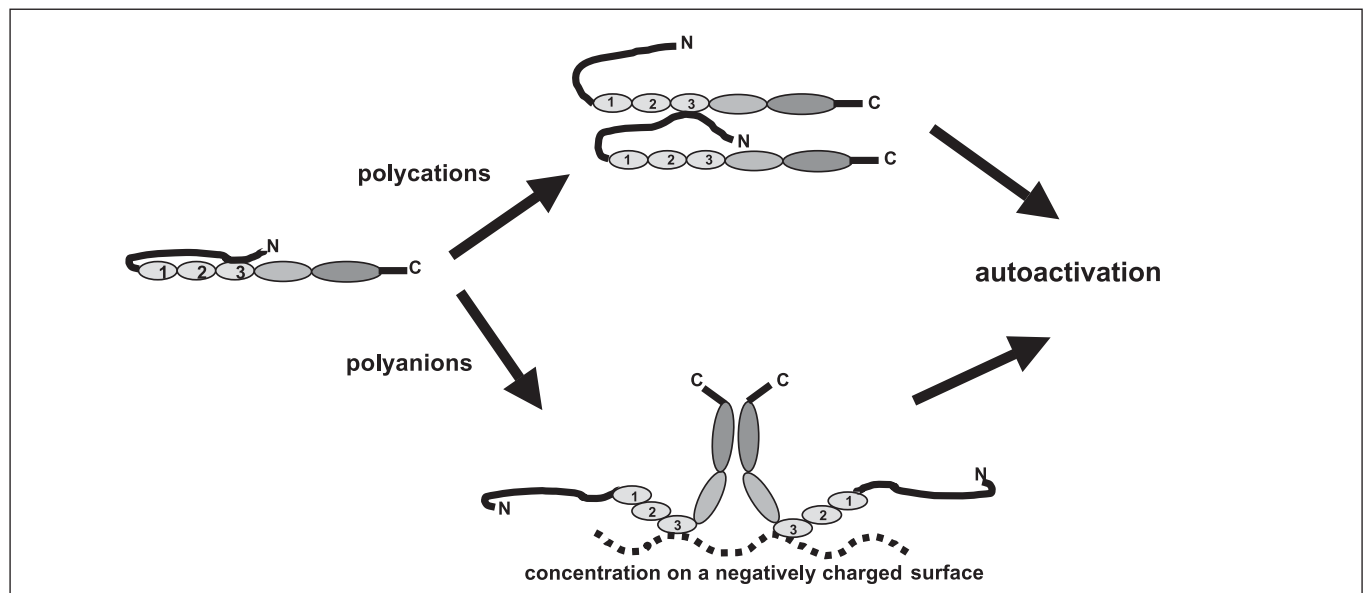
Interestingly, FSAP in plasma is resistant to autoactivation (12). Polycations, such RNA do not activate FSAP in plasma (12). The same holds for heparin. In contrast to purified FSAP, heparin does not activate FSAP in plasma (Stephan and Zeerleder, unpublished observations). The scFSAP in plasma is converted in its two-chain form upon contact with either apoptotic or necrotic cells (12). Recently histones have been shown to activate FSAP in plasma (13).

In purified systems, several serpins such as C1-inhibitor (C1inh),  $\alpha_2$ -antiplasmin (AP) and antithrombin III (AT-III) (2, 5, 14, 15) have been reported to inhibit the amidolytic activity of two-chain FSAP (tcFSAP). In plasma, C1inh has been reported to be the main inhibitor of activated tcFSAP (14). It has been demonstrated that upon contact with dead cells FSAP is activated and complexes with AP as well as with C1inh are formed in plasma (12). In bronchoalveolar fluid (BALF) of patients suffering from adult respiratory distress syndrome (ARDS) complexes of FSAP with plasminogen activator inhibitor-1 (PAI-1) can be detected suggesting that PAI-1 might be involved in the regulation of FSAP activity as well (16).

### Clinical relevance

#### FSAP in cardiovascular disease

Römisch et al. reported that in a purified system FVII could be activated by tcFSAP in a tissue factor independent manner (3). The same effects of tcFSAP on FVII could be observed in plasma although FSAP concentrations above the physiological con-



**Fig. 1** Autoactivation of FSAP by polyanions and polycations; adapted from Yamamichi et al. (11): An intramolecular interaction between the N-terminus containing acidophilic amino acids and positively charged basophilic clusters within the epidermal growth factor-3 (EGF3) domain prevent autoproteolysis. Polycations interfere with the respective intramolecular in-

teraction thereby allowing intermolecular binding of the N-terminus to the EGF3 domain of an adjacent FSAP molecule to form an autoactivation complex. Polyanions offer a scaffold to which single-chain FSAP (scFSAP) molecules can bind via their EGF3 domain and when located in juxtaposition may lead to autoactivation.

centration of 12 µg/ml were used and FVIIa generation was below the lowest FVIIa levels generated in the purified system (3). Therefore it remains doubtful whether FVII activation in plasma by FSAP is relevant. FSAP was reported to contribute to fibrinolysis as well. Pro-UK has been demonstrated to be a substrate for purified tcFSAP in-vitro (15). Since the Marburg I polymorphism results in diminished proteolytic activity towards pro-UK while the activity towards FVII remains preserved, the Marburg I polymorphism was suggested to be a risk factor for thrombosis (7). The role of FSAP Marburg I polymorphism in deep venous thrombosis is not clear yet. In contrast, there seems to be an association between the presence of FSAP Marburg I polymorphism and the cardiovascular risk and the risk for late complications of carotid stenosis (17,18).

This effects are hardly due to the pro-coagulatory and pro-fibrinolytic properties of FSAP. Interestingly, FSAP was demonstrated to inactivate platelet derived growth factor BB (PDGFBB), thereby inhibiting proliferation and migration of smooth muscle cells (19, 20). Moreover, FSAP has been identified in atherosclerotic plaques (21). In a mouse model Sedding and colleagues demonstrated that local application of wild-type plasma-derived FSAP reduced neointima formation after vascular damage, whereas local application of the Marburg I variant did not have any effect on this process (19, 22).

These findings suggest a role of FSAP in the pathogenesis of neointima formation and hence a possible role in the pathogenesis of cardiovascular disease.

### FSAP and circulating cellular fragments in inflammation

Sepsis is an inflammatory response characterized by activation of immune cells and plasmatic cascade systems such as the coagulation, contact phase and complement system leading to the production of proinflammatory mediators. Release of these mediators into the circulation has been suggested to mediate lethality. Therapies designed to neutralize these proinflammatory

mediators have been efficient in animal models (23–26). However, large randomized controlled trials in sepsis patients have mostly been disappointing without showing convincing efficacy (23, 25, 26). There is recent evidence that more downstream effects of the inflammatory response seem to be crucially involved in the pathophysiology of sepsis (27, 28). The inflammatory response leads to the induction of widespread cell death which is evidenced by extensive lymphocyte apoptosis in septic animals and sepsis patients (27). Circulating nucleosomes, a measure for cell death, were demonstrated to correlate with disease severity and fatality in sepsis patients (29, 30). Circulating nucleosomes and DNA-binding proteins such as histones, released either by dead or activated cells were reported to induce a potential fatal inflammatory response in sepsis (31, 32). Histone release contributes to death induced by inflammatory injury or chemical-induced cellular injury in mouse models, mediated in part through Toll-like receptors (TLRs) (33). Proteolytic cleavage of histones by recombinant activated protein C might explain the beneficial effects of APC in sepsis models in animals as well as in human sepsis (24, 32).

The origin of nucleosomes detected upon inflammation is not yet clear. Whether parenchymal cells, haematopoietic cells or both are the source of these nucleosomes remains to be established. Another explanation might be that neutrophils activated during inflammation might be the source of circulating nucleosomes. Upon activation neutrophils are reported to form neutrophil externalized traps (NETs). This NET formation is characterized by the exposition of DNA in complex with histones on the surface of activated neutrophils, dedicated to kill invading bacteria.

How nucleosomes are released into the circulation is not entirely clear. Plasma was described to release nucleosomes from late apoptotic cells (34) and the nucleosome releasing factor was identified to be FSAP (35). FSAP is demonstrated to bind to apoptotic and necrotic cells and is consequently activated (12). Although efficient in removing nucleosomes from apoptotic cells, DNA is not removed from necrotic cells by FSAP. A probable explanation for this difference

could be that necrosis induction does not necessarily leads to sufficient DNA fragmentation. Identification of the proteolytic target of FSAP leading to the release of nucleosomes is a crucial step in understanding the mechanism how FSAP releases nucleosomes from apoptotic cells. This mechanism still remains to be established.

Upon inflammation, FSAP in plasma is activated. FSAP-serpin complexes, a measure for FSAP activation, are increased in post-surgery patients, patients suffering from severe sepsis, septic shock and meningococcal sepsis (12).

The levels of FSAP activation correlated with nucleosome levels, disease severity and mortality in these patients, suggesting that FSAP is a sensor for cell death in the circulation (12).

However, strong correlations between FSAP and nucleosome levels do not necessarily implicate causality, *viz.* that FSAP is responsible for nucleosome release upon inflammation. Interestingly, low dose inflammation, such as LPS administration in healthy volunteers does not lead to FSAP activation although there is a transient nucleosome release (S. Zeerleder and T. van de Poll, unpublished observation). Taken together these findings suggest that a certain inflammatory threshold is needed for FSAP activation.

### FSAP and endothelial function in inflammation

Endothelial cells are key players during inflammation. Activation by proinflammatory stimuli results in an upregulation of pro-coagulatory molecules and to a fundamental change of the composition and contents of glycosaminoglycans on endothelial cells (36, 37). Moreover, these stimuli may also increase vascular permeability leading to vascular leakage, which is a crucial event in the pathogenesis of sepsis (38). FSAP was demonstrated to regulate endothelial cell function. Proteolytic release of basic fibroblast growth factor (bFGF) by FSAP was shown via stimulation of the FGF receptor 1 to activate ERK1/2 kinases in endothelial cells finally resulting in phos-

phorylation of the transcription factor c-Myc (39). Etscheid and colleagues demonstrated that FSAP proteolytically cleaves high molecular weight kininogen (HMWK) similar to kallikrein resulting in the release of the highly vasoactive bradykinin (BK) (40). Stimulation of the bradykinin receptor 2 (B2R) by BK induces an intracellular calcium flux finally resulting in an increase of vascular permeability (41, 42). Indeed, there is indirect evidence that FSAP is activated during increased permeability of the pulmonary vasculature since FSAP-PAI-1 complexes could be demonstrated in the BALF of patients suffering from ARDS (16). Interestingly, histones are able to induce FSAP activation in plasma resulting in the liberation of BK (13). Among others, BK is an important mediator of vasodilatation in severe sepsis and septic shock contributing to the development of the potentially fatal septic hypotension (43).

Therefore, BK formation due to FSAP activation by circulating histones might be critically involved in the development of hypotension in severe sepsis and septic shock.

Blocking factor XIIa in a baboon model for sepsis attenuated sepsis-related hypotension most probably via the inhibition of the FXII-kallikrein mediated BK formation but did not improve lethality (44). The contribution of FSAP in that system is not clear yet. In contrast, FSAP was shown to negatively regulate vascular permeability via activation of protease-activated receptor signaling/RhoA/Rho kinase signaling. In a murine LPS- and acute lung injury models, FSAP was demonstrated to reduce the permeability of the pulmonary vasculature (44, 45). Whether this observation is a general phenomenon or is restricted to the lung vasculature, is not known yet. Therefore, the net effect of FSAP on the vascular

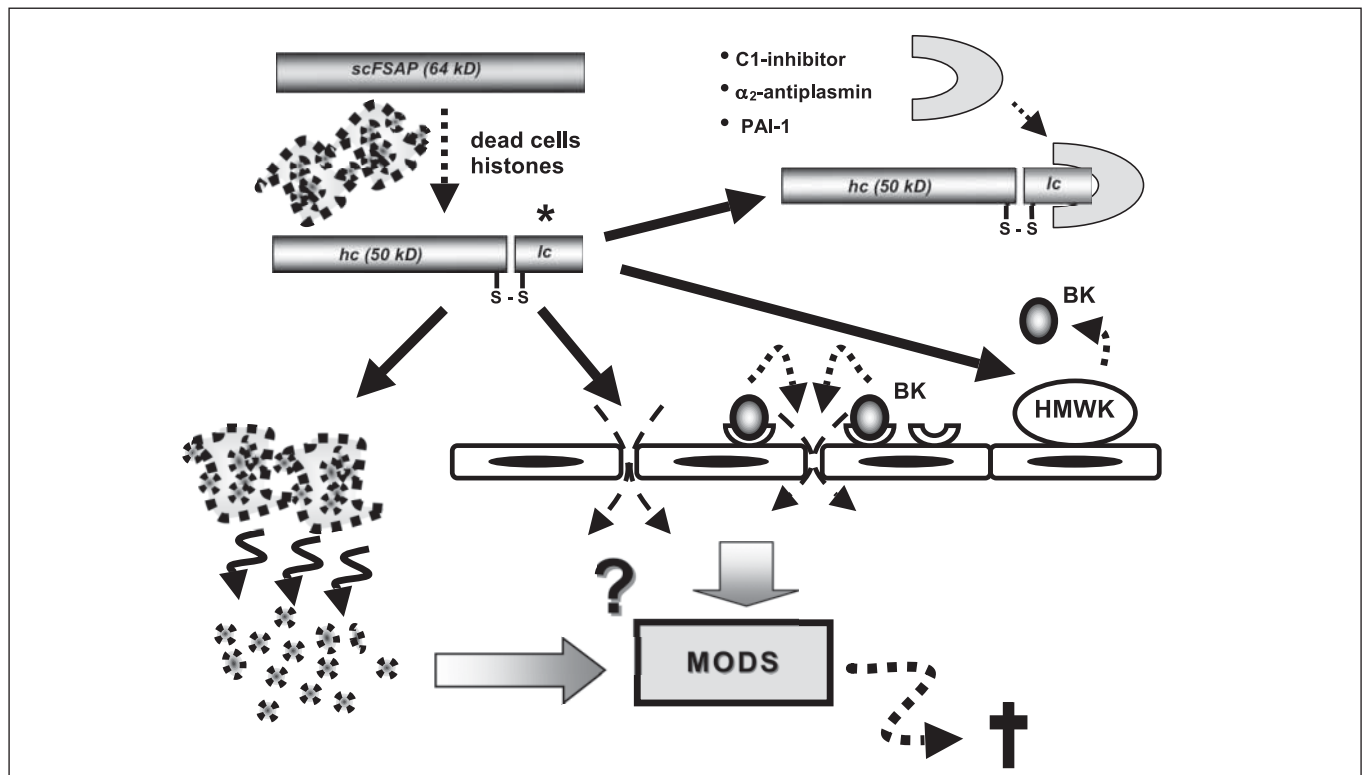
permeability during inflammation remains to be established.

## Concluding remarks

The role of FSAP in inflammation is still not well understood. FSAP seems to be involved in the pathogenesis of sepsis since

- it is activated by cellular debris resulting from the proinflammatory response
- FSAP removes nucleosomes from apoptotic cells
- it modulates vascular permeability both, directly and indirectly (► Fig. 2).

However, whether FSAP activation upon inflammation is beneficial or detrimental remains an open question. One might speculate that the release of nucleosomes by FSAP upon inflammation might be an epiphenomenon reflecting the severity of



**Fig. 2** Role of FSAP in inflammation: FSAP is activated by cellular debris resulting from the pro-inflammatory response and can remove nucleosomes from apoptotic cells. It can modulate vascular permeability both, directly and indirectly via proteolytic cleavage of high molecular weight kininogen (HMWK) resulting in the release of the highly vasoactive bradykinin (BK). Bradykinin is an important mediator of vasodilatation in severe sepsis and

septic shock contributing to the development of the potentially fatal septic hypotension. FSAP-induced nucleosome release might be harmful propagating the proinflammatory response.

MODS: multiorgan dysfunction system; *scFSAP*: single-chain FSAP; *hc*: heavy chain; *lc*: light chain

the inflammatory response. On the other hand FSAP-induced nucleosome release might be harmful as propagating the pro-inflammatory response. If FSAP contributes significantly to the hypotension observed in severe sepsis or septic shock its activation might be a fatal event in the pathogenesis. In contrast, improvement of vascular integrity by FSAP in sepsis might be beneficial. In conclusion, intervention studies in animals are warranted to elucidate the role of FSAP in the inflammatory response.

### Conflict of interest

The authors declare, that there is no conflict of interest.

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