Antihemostatic Molecules from Saliva of Blood-Feeding Arthropods

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Key Words

Vasodilators · Anticoagulants · Platelet aggregation inhibitors · Saliva · Blood-feeding · *Rhodnius prolixus* · Nitrophorin

Abstract

The ability to feed on vertebrate blood has evolved many times in various arthropod clades. Each time this trait evolves, novel solutions to the problem posed by vertebrate hemostasis are generated. Consequently, saliva of blood-feeding arthropods has proven to be a rich source of antihemostatic molecules. Vasodilators include nitrophorins (nitric oxide storage and transport heme proteins), a variety of peptides that mimic endogenous vasodilatory neuropeptides, and proteins that catabolize or sequester endogenous vasoconstrictors. A variety of platelet aggregation inhibitors antagonize platelet responses to wound-generated signals, including ADP, thrombin, and collagen. Anticoagulants disrupt elements of both the intrinsic and extrinsic pathways. Molecular approaches (termed 'sialomics') to characterize the full inventory of mRNAs transcribed in salivary glands have revealed a surprising level of complexity within a single species. Multiple salivary proteins may be directed against each component of hemostasis, resulting in both redundancy and in some cases cooperative interactions between antihemostatic proteins, as in

the case of the *Rhodnius prolixus* apyrase (which hydrolyzes ADP) and *Rhodnius* platelet aggregation inhibitor 1 (which sequesters ADP). The complexity and redundancy of saliva ensures an efficient blood meal for the arthropod, but it also provides a diverse array of novel antihemostatic molecules for the pharmacologist.

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Introduction

Feeding on vertebrate blood requires that arthropods be able to overcome a series of obstacles. Firstly, the vertebrate host must be located, not a trivial task as hosts are generally widely scattered across complex habitats. Once a suitable host is located, the skin must be penetrated to access the blood meal within. During this process, and while acquiring the meal, stealth and speed are of the essence, as discovery is likely to result in expulsion at best and an untimely demise at worst. Finally, access to the blood is defended by a complex series of responses at the feeding (i.e., injury) site, including platelet activation and aggregation, local vasoconstriction, and coagulation (clotting). These hemostatic responses on the part of the vertebrate host demand a counterattack by our blood-feeding arthropod. The counterattack takes the form of a series of components present in the saliva; most blood-feeders produce vasodilators, platelet aggregation

inhibitors, and anticoagulants, a rich pharmacopia of interacting molecules. Despite the obstacles to blood-feeding, blood is a rich food resource and the ability to utilize it has evolved multiple times. At least 20 currently extant arthropod families, distributed over two classes (Arachnida and Insecta) and numerous orders, have independently evolved blood-feeding [1]. These multiple evolutionary experiments have given rise to an amazing diversity of antihemostatic molecules [2]. Throughout this brief review, the diversity of antihemostatic molecules will be the central theme.

Vasodilators

Vasodilators facilitate feeding in two ways: injected into the skin as the arthropod probes and searches for blood, they enlarge the blood vessels and accelerate discovery, and subsequently they enhance blood flow to the feeding site, decreasing the time needed to acquire the meal. A surprising range of molecules, and biochemical strategies to accomplish vasodilation have been described.

The Chaga's disease vector *Rhodnius prolixus* was found to contain a salivary nitrovasodilator [3]. Pharmacological evidence included potentiation of the vasodilatory effect of saliva by superoxide dismutase, and inhibition with methylene blue. Subsequently the presence of heme proteins that bound and released nitric oxide (NO) in a pH-dependent manner was demonstrated [4]. The reversible interaction was shown to be due to a ferric (Fe^{3+}) heme. Later, four NO-binding heme proteins were isolated, partially characterized, and named nitrophorins [5]. Initially these proteins were thought to belong to a novel family, as database searches failed to identify homologous sequences. Following the development of an expression strategy [6], the X-ray crystal structures of these proteins have been determined to a high degree of resolution [7–9]. Surprisingly, the structures revealed a classic lipocalin fold, and subsequent analysis demonstrated that the nitrophorins are indeed highly derived lipocalins, modified to bind heme and function as specialized NO carriers. The heme iron coordinates with a proximal histidine, and on the distal side a large pocket is available for NO binding. The mechanism underlying the pH-dependent release of NO was determined by solving crystal structures at acid and neutral pH (5.0 and 8.0 respectively). At either pH, in the absence of ligated NO, the heme pocket is large and open, permitting easy access by NO [10, 11], and in fact NO binding is rapid and ap-

parently only limited by diffusion [11]. The distal pocket also contains several loosely bound water molecules. However, at pH 5, this distal pocket is somewhat hydrophobic, and as NO itself is a hydrophobic molecule, NO binding to the heme results in a net environment in the distal pocket sufficiently hydrophobic to result in the expulsion of the solvent molecules and collapse of the AB and GH random coil loops onto the heme, trapping the NO molecule in place. This is the pH environment that is maintained in the salivary glands [12], which explains how *Rhodnius* is able to store pharmacological amounts of NO and avoid potential problems resulting from the toxic nature of NO. When saliva is injected into the skin during blood-feeding, the pH shifts to about 7.4. In this environment, several residues on the AB and GH loops become charged, permitting entry of water into the distal pocket and resulting in movement of the loops away from the heme, restoring the opening and permitting easy egress of the NO. Sequence analysis revealed that the four nitrophorins (NPs) fall into two subfamilies that share only 56% sequence identity, with NP1 and NP4 in one group and NP2 and NP3 in the other [11]. Stopped-flow experiments demonstrated that NP1/NP4 release NO readily, with off rates of $2.2\text{--}2.6\text{ s}^{-1}$ at pH 8.0, and release is slower from NP2/NP3, with off rates of $0.08\text{--}0.12\text{ s}^{-1}$ [11]. This suggests a possible functional significance underlying the diversity of nitrophorins present in one insect: NP1/NP4 dissociate NO (resulting in vasodilation) in the immediate environment of the bite, and NP2/NP3 must be diluted much more, implying diffusion from the bite site, before NO release occurs. Collectively, this system results in vasodilation along a greater length of the blood vessel than would be achieved if all the NO was immediately unloaded at the bite site.

It is noteworthy that *Rhodnius* saliva contains a potent antihistamine activity, which was shown to be due to a factor binding histamine rather than an effect on the histamine receptor [13]. This effect is also due to the NPs, as they bind histamine with an affinity sufficient to out-compete the histamine receptor [14]. Binding is accomplished in the same site as NO binding [7], and histamine can accelerate NO release from NP under physiological conditions [11, 14]. This antihistamine activity prevents the host from detecting histamine released from mast cells at the feeding site, at least while the insect is present and injecting saliva containing fresh NP.

A similar system of NO storage and release by an NP was also found in another hemipteran, the bed bug *Cimex lectularius* [15]. Sequence analysis demonstrated that the single NP is a homolog of inositol polyphosphate 5-phos-

phatase, with no relationship to the *Rhodnius* NPs. Remarkably, it appears that two hemipteran families have independently evolved closely analogous (but not homologous) systems for accomplishing vasodilation in the vertebrate host. The crystal structure of the *Cimex* NP revealed a fold similar to other inositol polyphosphate 5-phosphatases, and a remarkable mechanism for reversibly binding two NO molecules [16]. The ferric heme is coordinated with a proximal cysteine, Cys60. At lower NO concentrations, NO enters the relatively small, slightly hydrophobic distal pocket and binds to heme through the ferric iron center. This nitrosyl-heme interaction pulls the iron towards the distal side, reducing the strength of the interaction with the proximal Cys. In the presence of higher concentrations of NO, another NO can enter to the proximal side of the heme and form a nitrosyl-thiol bond with the sulfur of Cys60, breaking the interaction between the heme and Cys60 and in the process reducing the iron, which now forms a strong and stable ferrous Fe^{2+} -NO bond. In the skin, NO dissociates from the Cys60, which then reforms the proximal interaction with the heme, returning the electron and oxidizing the Fe back to ferric heme, which then permits the release of the second NO molecule. This remarkable system for storing and transporting two NO molecules is not known to occur in any other organism.

Several blood-feeding arthropods use peptides or small proteins to interact with endogenous receptors in their vertebrate hosts. In the yellow fever mosquito, *Aedes aegypti*, sialokinins mimic the endogenous tachykinin substance P, interacting with endothelial tachykinin receptors to stimulate NO production [17, 18]. Although the sialokinins are closely similar to vertebrate tachykinins, especially at the C-terminal region that specifically interacts with the receptor, analysis of the gene encoding sialokinin reveals that these peptides are not homologous with vertebrate tachykinins, and must have arisen through a process of convergent evolution [19]. Phlebotomine sandflies in the genus *Lutzomyia* contain a peptide, maxadilan, with both vasodilatory and immunomodulatory activity [20, 21]. Indeed, maxadilan is about 500-fold more active than calcitonin gene-related peptide (CGRP), the most potent known vertebrate vasodilatory peptide. These activities arise from the interaction between maxadilan and the pituitary adenylate cyclase activating peptide (PACAP) receptor [22]. Remarkably, there is no obvious sequence or structural similarity between maxadilan and PACAP. Further, the role of PACAP in the regulation of vascular tone in peripheral blood vessels was not recognized prior to the discovery of the target for

maxadilan. Yet another peptide, *Simulium vittatum* erythema protein (SVEP), was isolated from salivary glands of the blackfly *Simulium vittatum* [23]. This peptide, which apparently is comparable to maxadilan in its activity, has no sequence similarity to other polypeptides, and its mechanism of action involves interaction with ATP-dependent potassium channels.

Several examples serve to illustrate the diversity of antihemostatic strategies that exist even within taxa (i.e., families) that are thought to have evolved blood-feeding as a single evolutionary event. *Aedes* mosquitoes (family Culicidae, subfamily Culicinae) use tachykinin peptides described above. In contrast, *Anopheles* mosquitoes (Culicidae: Anophelinae) use a myeloperoxidase with catechol oxidase/peroxidase activity to drive H_2O_2 -dependent destruction of noradrenalin and serotonin, important endogenous vasoconstrictors [24, 25]. This strategy removes the host's ability to maintain vascular tone at the bite site, as endogenously produced NO and other vasodilators are not balanced by vasoconstrictors, resulting in a slow but persistent local vasodilation. New world *Lutzomyia* sandflies use maxadilan, as described above. In contrast, old world sandflies (*Phlebotomus*) lack maxadilan, instead accumulating pharmacological amounts of the vasodilatory amines adenosine and AMP in their saliva [26]. Although *Rhodnius* uses nitrophorins to deliver NO to their hosts, related insects in the genus *Triatoma* lack NPs and instead rely on (as yet uncharacterized) peptide vasodilators [27]. Hard ticks (Acari: Ixodidae) use salivary prostaglandins, including prostacyclin, PGE_2 , and $\text{PGF}_{2\alpha}$, to accomplish vasodilation in their hosts [28]. Although the vasodilators from soft ticks (Acari: Argasidae) have yet to be characterized, they apparently do not produce prostaglandins [29]. These examples suggest that the diversity of vasodilators awaiting discovery may be even larger than what might be predicted from the number of families with the blood-feeding habit.

Platelet Aggregation Inhibitors

Platelet activation, shape change, and aggregation is driven by receptor-mediated responses to wound-associated signals, including ADP, collagen, and thrombin. As platelet responses can be extremely rapid, they are particularly relevant to the blood-feeding process, and arthropods have a variety of strategies to inhibit platelet function. Most widespread is the presence of a salivary apyrase (ATP/ADP diphosphohydrolase). This enzyme depletes ADP (and ATP) from the lesion produced by the

probing mouthparts, so that these molecules are largely removed by the time the blood vessel is penetrated. The first apyrase to be characterized at the molecular level was a homolog of vertebrate 5'-nucleotidases, isolated from saliva of *Aedes aegypti* [30]. Subsequently, homologous apyrases were found in saliva of *Anopheles* [31] and the hemipteran *Triatoma* [32]. A completely unrelated apyrase, belonging to a novel protein family, was characterized from *Cimex lectularius* [33]. Subsequently the apyrase from *Phlebotomus* was shown to also be a member of this family [34]. Homologs of this apyrase, encoding proteins of previously unknown function, were found in a cDNA library from a related blood-feeding insect, *Lutzomyia longipalpus*, and in the human, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Cryptosporidium parvum* genomes [35]. The two apyrases differ in their pharmacological properties: the 5'-nucleotidase type works with either Ca^{2+} or Mg^{2+} , and the pH optimum is about 9 (although the enzyme is still very active at physiological pH), and the *Cimex* apyrase works with Ca^{2+} only and at a more neutral pH optimum. Pharmacological characterization of apyrases from a range of blood-feeding arthropods [36] suggests that further apyrases remain to be characterized.

In *Rhodnius prolixus*, a novel lipocalin, named *Rhodnius* platelet aggregation inhibitor 1 (RPAI1), also inhibits platelet aggregation [37, 38]. This protein inhibits platelet responses to low but not high concentrations of a range of agonists, including ADP, collagen, thrombin, convulxin, and thromboxane A2 mimics. At low concentrations these agonists stimulate degranulation and ADP release from platelets, and this platelet-derived ADP largely drives the subsequent response. RPAI1 efficiently binds ADP and sequesters it from the platelet receptor, thus abrogating the effect of the agonists. RPAI1 appears to work cooperatively with the *Rhodnius* apyrase: apyrase lowers ADP concentrations at the feeding lesion to nanomolar levels, but it is not efficient at hydrolyzing substrate at very low ADP levels, and the RPAI1 sequesters the remaining ADP (as well as platelet-derived ADP), bringing ADP concentrations down to subphysiological levels. The existence of this system in *Rhodnius* leads to questions about analogous systems in other insects, but if they exist they have yet to be characterized.

Also in *Rhodnius prolixus*, a protein related to the nitrophorins, but without bound heme, has been shown to bind a variety of biogenic amines [39]. This protein, named amine binding protein, inhibits serotonin- and epinephrine-mediated effects on platelets, including synergy of the effects of collagen and ADP. In addition to its

antiplatelet activity, this protein acts as a vasodilator by sequestering serotonin, epinephrine, and norepinephrine. A functionally similar lipocalin has been described from saliva of the tick *Dermacentor reticulatus* [40], but this protein is not closely related to ABP and significant differences (including two amine-binding sites) indicated that its amine-binding properties were evolved independently.

Anticoagulants

Arthropod salivary anticoagulants have been reviewed recently [2], and only selected examples will be discussed here. In general, many arthropod anticoagulants target either thrombin or fXa (or sometimes fX), which doubtless reflects the central role of fX/fXa at the nexus of the intrinsic and extrinsic pathways, as well as the ultimate role of thrombin in driving production of fibrin from fibrinogen. Overall antithrombin molecules are most common, but sixteen species are known to produce antifXa, including four species that produce an antifXa in addition to an antithrombin.

Mosquitoes again illustrate the diversity that can occur with a single arthropod clade. Culicine mosquitoes universally produce an antifXa [41], which was shown to be a highly derived 54-kDa member of the serpin superfamily [42]. In contrast, anopheline mosquitoes produce an antithrombin [41]. In *Anopheles albimanus*, a 6.5-kDa peptide was characterized, named anophilin, and shown to bind to both the active cleft and the anion-binding exosite 1 of thrombin [43, 44].

In *Rhodnius prolixus*, a salivary anticoagulant was long ago named as prolixin-S (to distinguish it from a distinct anticoagulant, prolixin-G, expressed only in the gut) [45]. Prolixin-S was described as having antifVIII activity, unique in the animal world. Subsequently prolixin-S was shown to be identical to nitrophorin 2 [12, 46, 47]. NP1 and NP4 lack anticoagulant activity entirely, and NP3 is about 300-fold less active than NP2, despite a high degree (143 out of 180 residues) of sequence identity. NP2 was shown to be a hyperbolic mixed-function inhibitor of fIXa-catalyzed activation of fX [48]. Ordinarily fIXa interacts with fVIIIa on phospholipid membranes to form the Xase complex; this complex is highly efficient at activating fX, compared to free fIXa. Various assays, including surface plasmon resonance, indicate the NP2 binds to fIXa, preventing formation of the Xase complex and thereby inhibiting fIXa production [49]. This interaction occurs through the Gla domain, as fIXa lack-

ing this domain does not bind NP2 [49]. The interaction is complex, with two-phase kinetics possibly indicative of a conformational change involving domain swapping [50]. Mutation analysis has shown that the interaction involves a series of residues at the apex of the EF loop [50].

Examination of a library of cDNAs representing mRNAs expressed in *Rhodnius prolixus* salivary glands revealed a previously unknown nitrophorin, designated NP7, distinguished by a series of basic lysine residues in the C-terminal tail [51]. The presence of this basic tail suggested that NP7 might bind to anionic phospholipid membranes. In fact NP7 does inhibit thrombin activation by competing for binding sites for the prothrombinase complex. NP7 binds heme and functions as a NO carrier, in common with the other NPs. In addition, this protein inhibits platelet responses to collagen and ADP, likely by binding to activated platelets and releasing NO. Further basic-tail NPs and other lipocalins are present in the *Rhodnius* sialome, suggesting that direction of antihemostatic molecules to the platelet surface may be a more general strategy, although the function of these molecules has not yet been determined.

***Rhodnius prolixus*: A Model of Things to Come?**

Analysis of the sialome (the collection of all salivary-gland expressed mRNAs and proteins) of *Rhodnius prolixus* revealed 71 distinct sequence clusters with signal

peptides, indicative of secretion [52]. Of these, the majority encode members of the lipocalin family. The enormous expansion of this single protein family into a diverse array of antihemostatic functions is surprising, but it probably reflects the functional flexibility of the basic lipocalin form. Several homologs of the NPs are present. These include the amine-binding protein and NP7 described above, and many sequences encoding proteins of unknown function, including truncated versions of several NPs. Many homologs of triabin, an antithrombin molecule from *Triatoma pallidipennis* [53], are present, but *Rhodnius* saliva lacks antithrombin activity and the function of these triabin homologs is unknown. A similar situation applies to homologs of pallidipin, an inhibitor of collagen-mediated platelet activation also cloned from *Triatoma pallidipennis* [54]. Much work remains before the activity of *Rhodnius* saliva is fully understood. The sialome of several other blood-feeding arthropods has been examined [e.g., 55–60]. Although few contain the diversity seen in *Rhodnius*, all contain dozens of sequences, most of unknown function. Analysis of these sialomes seems certain to contribute many new compounds to the pharmacopia of antihemostatic molecules. Further, a more complete understanding of the function of saliva as a whole in these species may contribute to novel strategies to interfere with their ability to vector disease. Finally it should be recognized that attention has to this point focused on blood-feeding arthropods that are disease vectors, but many non-vector species, and even whole families (such as the Hippoboscidae), await investigation.

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