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Interaction of *Bothrops jararaca* venom metalloproteinases with protein inhibitors

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ABSTRACT

Snake venom metalloproteinases (SVMPs) play important roles in the local and systemic hemorrhage observed upon envenomation. In a previous study on the structural elements important for the activities of HF3 (highly hemorrhagic, P-III-SVMP), bothropasin (hemorrhagic, P-III-SVMP) and BJ-PI (non-hemorrhagic, P-I-SVMP), from *Bothrops jararaca*, it was demonstrated that they differ in their proteolysis profile of plasma and extracellular matrix proteins. In this study, we evaluated the ability of proteins DM43 and α2-macroglobulin to interfere with the proteolytic activity of these SVMPs on fibrinogen and collagen VI and with their ability to induce hemorrhage. DM43 inhibited the proteolytic activity of bothropasin and BJ-PI but not that of HF3, and was not cleaved the three proteinases. On the other hand, α2-macroglobulin did not inhibit any of the proteinases and was rather cleaved by them. In agreement with these findings, binding analysis showed interaction of bothropasin and BJ-PI but not HF3 to DM43 while none of the proteinases bound to α2-macroglobulin. Moreover, DM43 promoted partial inhibition of the hemorrhagic activity of bothropasin but not that of HF3. Our results demonstrate that metalloproteinases of *B. jararaca* venom showing different domain composition, glycosylation level and hemorrhagic potency show variable susceptibilities to protein inhibitors.

1. Introduction

Manifestations of local and systemic damage, such as hemorrhage and coagulopathy, are among the most dramatic effects of envenomation by viperid snakes. In cases of less severe envenomation, the hemorrhagic effect is usually localized at the site of the bite. However, hemorrhage can be also found spread widely through a substantial area of the involved extremity and, in cases of severe envenomation, bleeding in organs distant from the site of bite, such as heart, lungs, kidneys and brain, may also occur (Gutierrez et al., 2005). Snake venom metalloproteinases (SVMPs) are abundant toxins in viperid venoms and play important roles in the severe local tissue damage, hemorrhage and profound consumption coagulopathy observed upon envenomation (Gutierrez et al., 2005; Fox and Serrano, 2008). They are classified in three classes according to their domain organization and the proteinase domain of hemorrhagic toxins is believed to function to degrade capillary basement membranes, endothelial cell surface proteins, and stromal matrix ultimately causing extravasation of capillary contents into the surround stroma (Baramova et al., 1989; Escalante et al., 2009; Paes Leme et al., 2012). Interestingly, the P-III class of SVMPs is typically much more potent in causing hemorrhage compared with the P-I and P-II classes that lack the

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disintegrin-like/cysteine-rich domains. Proteolytic degradation of capillary structures allied to effects on plasma proteins and platelet aggregation have been considered to be the key features underlying the hemorrhagic potency of P-III SVMP hemorrhagic toxins (Baldo et al., 2010; Serrano et al., 2007; Rucavado et al., 2005; Santoro and Sano-Martins, 2004; Wijeyewickrema et al., 2007).

Three SVMPs of different domain organization and glycosylation levels, isolated from *Bothrops jararaca* venom, were shown to display different proteolytic and hemorrhagic activities: HF3 (P-IIIa class) is highly glycosylated showing a 70 kDa molecular mass (Silva et al., 2004) and is an extremely hemorrhagic toxin that shows a minimum hemorrhagic dose of 240 fmol on the rabbit skin (Assakura et al., 1986); bothropasin (P-IIIb class), which contains a minor carbohydrate moiety, is ~80 times less hemorrhagic than HF3 but is a highly proteolytic enzyme (Assakura et al., 2003), and BJ-PI (P-I class) is a potent fibrinogenolytic enzyme but is devoid of hemorrhagic activity (Oliveira et al., 2009). In a recent comparative study of the structural elements important for the activities of HF3, bothropasin and BJ-PI, it was demonstrated that SVMPs of different domain composition, glycosylation level and hemorrhagic potency differ in their interaction affinities and proteolytic specificity upon plasma and extracellular matrix proteins (Oliveira et al., 2010).

The inhibition of SVMP activity may be an important factor in the control of local and systemic effects of envenomation. Despite the general success of antivenom therapy, it is still important to find natural inhibitors of SVMPs and to design synthetic inhibitors to be used as therapeutic tools. The anti-hemorrhagic protein DM43, isolated from the serum of the opossum *Didelphis marsupialis*, was able to inhibit the hydrolysis of casein, fibronectin and fibrinogen by *B. jararaca* venom proteinases and the hemorrhagic effect of jararhagin (Neves-Ferreira et al., 2000). The mechanism of such inhibition involves the formation of soluble inactive complexes between the serum protein and the toxin (Neves-Ferreira et al., 2002). On the other hand, the partial or complete resistance of some SVMPs to the inhibitory action of plasma proteinase inhibitors, especially the macroglobulins, is believed to be an important factor contributing to the variable capacity of SVMPs to induce local and systemic hemorrhage (Baramova et al., 1990; Kamiguti et al., 1994). Although α2-macroglobulin is able to form covalent complexes with the proteinases, a number of P-III class SVMPs are not inhibited by this protein (Baramova et al., 1990; Escalante et al., 2003).

In order to further analyze the features of P-I and P-III SVMPs involved in their proteolytic and hemorrhagic activities, this study focused on the inhibition HF3, bothropasin and BJ-PI by proteins DM43 and α2-macroglobulin. Our results demonstrate that HF3, bothropasin and BJ-PI have different susceptibilities to inhibition by proteins.

2. Materials and methods

2.1. SVMPs

HF3, bothropasin and BJ-PI were isolated from *B. jararaca* venom as described previously (Oliveira et al., 2009). Partially *N*-deglycosylated HF3 was prepared as described elsewhere (Oliveira et al., 2010).

2.2. Protein inhibitors

Protein DM43 was isolated according to Neves-Ferreira et al. (2000) and α2-macroglobulin was purchased from Sigma (St. Louis, MO).

2.3. Effect of DM43 and α2-macroglobulin on the fibrinogenolytic activity of HF3, bothropasin and BJ-PI

Native and partially *N*-deglycosylated HF3 (200 ng), or bothropasin (200 ng) were preincubated with protein DM43 (200 ng) and BJ-PI (200 ng) was preincubated with DM43 (400 ng) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 40 min at room temperature and then incubated with human fibrinogen (10 μg) for 1 h at 37 °C.

Native and partially *N*-deglycosylated HF3 (200 ng), bothropasin (200 ng) and BJ-PI (200 ng) were preincubated with α2-macroglobulin (2 μg) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 30 min at 37 °C and incubated with human fibrinogen (10 μg) for 1 h at 37 °C.

Human fibrinogen (Kabi Diagnostica, Sweden) was dissolved in 0.15 M NaCl at 10 mg/ml and incubated with and without enzyme under identical conditions as a control. Reactions were stopped by adding four fold concentrated Laemmli sample buffer and samples were submitted to SDS-PAGE using 9% or 12% polyacrylamide gels (Laemmli, 1970). Gels were silver stained.

2.4. Effect of DM43 and α2-macroglobulin on the proteolytic activity of HF3, bothropasin and BJ-PI on collagen VI

Native HF3 or bothropasin (100 ng) were preincubated with protein DM43 (100 ng) and BJ-PI (100 ng) was preincubated with DM43 (200 ng) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 40 min at room temperature and then incubated with collagen VI (5 μg) for 3 h at 37 °C.

Native and partially *N*-deglycosylated HF3 (200 ng), bothropasin (200 ng) and BJ-PI (200 ng) were preincubated with α2-macroglobulin (2 μg) in 50 mM Tris–HCl, pH 8.0, containing 1 mM $CaCl_2$ for 30 min at 37 °C and then incubated with collagen VI (5 μg) for 3 h at 37 °C.

Collagen VI (Sigma, Saint Louis, MO) was dissolved in 0.25% acetic acid at 2 mg/ml and the acidic pH was neutralized with 1.5 M Tris–HCl, pH 8.8, prior to the incubation with and without enzyme under identical conditions as a control. Reactions were stopped by adding four fold concentrated Laemmli sample buffer and then submitted to SDS-PAGE using 8% polyacrylamide gels (Laemmli, 1970). Gels were silver stained.

2.5. Proteolytic activity of HF3, bothropasin and BJ-PI on DM43 and α2-macroglobulin

DM43 (500 ng) was incubated with HF3, bothropasin or BJ-PI (500 ng) in 50 mM Tris–HCl, pH 8.0 containing 1 mM $CaCl_2$ for 1 h at 37 °C and the reactions were stopped by adding four fold concentrated Laemmli sample buffer and then submitted to SDS-PAGE under non-reducing