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High molecular mass kininogen inhibits metalloproteinases of *Bothrops jararaca* snake venom

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Abstract

High molecular mass kininogen (HK) purified from *Bothrops jararaca* (Bj) plasma was tested on activities of the Bj venom in vivo and in vitro. Results showed that, when incubated with BjHK, the Bj venom presented inhibition on hemorrhagic, edema forming, myotoxic, and coagulant activities. It is well known that metalloproteinases are directly or indirectly involved in these activities. Similarly, human HK inhibits the hemorrhagic effect of the Bj venom as well as hemorrhagic and enzymatic effects of jararhagin, a hemorrhagic metalloproteinase isolated from Bj venom. Complex between HK and jararhagin was not detected by gel filtration. Nevertheless, the inhibitory effect of the hemorrhagic activity of the venom was only partial when HK was pre-incubated with 0.4 mM $ZnCl_2$ or with 0.45 mM $CaCl_2$. These data suggest that the inhibitory effect depends, at least partially, on the competition for ions between kininogen and metalloproteinases of the venom.

Keywords: *Bothrops jararaca*; Snake venom; Metalloproteinase; Proteinase inhibitors; High molecular mass kininogen

Venom from snakes of the genus *Bothrops* induces local reactions such as edema, pain, hemorrhage, and necrosis, besides systemic reactions, mainly represented by blood coagulation disturbances [1]. Systemic effects of the venom are efficiently neutralized by specific antivenom. Nevertheless, this type of treatment is not effective in restraining the outcome of local reactions [1–3], probably due to the immediate effect of the venom on the microcirculation [4–6].

The mechanisms that underlie snake venom-induced local reactions are not fully understood, although they are attributed to proteinases found in these venoms. Among them, Zn^{2+}-dependent metalloproteinases were isolated and characterized. Some of them are named hemorrhagic factors or hemorrhagins due to their local hemorrhagic effect [7].

Some animals are naturally resistant to snake envenoming. This natural resistance has been attributed to serum components that neutralize the noxious effects of snake venoms. It has been suggested that proteinase inhibitors are responsible for this effect [8].

A high concentration of proteinase inhibitors that interferes with coagulation and fibrinolytic systems of the *Bothrops jararaca* (Bj) plasma has been reported [9]. In this direction, a powerful cysteine-proteinase inhibitor, related to mammalian high molecular weight kininogen (HK), was described in Bj plasma [10]. Preliminary studies demonstrated that this BjHK was able to inhibit the hemorrhagic activity of the venom from snakes of the same specie [11]. Based on these finding, it was investigated about the possible influence of human and BjHK on different activities of the Bj venom. Results showed that both human and BjHK have a remarkable inhibitory effect on some metalloproteinase-dependent activities of the Bj venom, and that human HK inhibits hemorrhagic as well as enzymatic activities of jararhagin, a hemorrhagic metalloproteinase isolated from Bj venom.

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Materials and methods

Materials

DEAE–Sephadex A-50, CNBr activated Sepharose 4B, and Superose 12 were from Amersham–Pharmacia Biotech (Uppsala, Sweden). Papain, Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), hexadimethrine bromide (polybrene), phenylmethanesulfonyl fluoride (PMSF), and creatine kinase kit C-520 were from Sigma Chemical (St. Louis, MO, USA). Human single chain high molecular mass kininogen was from Enzyme Research (South Bent, IN, USA). Jararhagin, a snake venom metalloproteinase from *B. jararaca* venom, was purified according to Paine et al. [12]. Human kininogen and jararhagin used in this study presented single bands of, respectively, 120,000 and 52,000 Da in SDS–PAGE. The chromogenic substrate Ac-Phe-Arg-pNan (APANA) and the fluorogenic substrate Abz-L-V-E-A-L-Y-Q-EDDnp were from Department of Biophysics, UNIFESP-EPM (São Paulo, Brazil). All other chemicals used were of a proper analytical grade.

Isolation of *B. jararaca* kininogen (BjHK)

The *B. jararaca* (Bj) blood was obtained from the abdominal aorta after anesthesia with sodium pentobarbital and laparotomy as described in Chudzinski-Tavassi et al. [9]. Blood was collected into 3.8% sodium citrate (9:1) and plasma was obtained by centrifugation at 2000*g* for 15 min, at 4 °C. The plasma obtained was immediately mixed (1:1 v/v) with a 0.05 M Tris–HCl, pH 6.8, buffer, containing 0.1 mM EDTA, 100 μg/ml polybrene, 1.0 mM PMSF, 0.01% sodium azide, and 0.03 M NaCl.

The BjHK was purified according to what was previously described by Chudzinski et al. [10]. Briefly, plasma samples were submitted to an ion-exchange chromatography in DEAE–Sephadex A-50 resin. The resin was equilibrated with the 0.05 M Tris–HCl, pH 6.8, buffer containing the proteinase inhibitors mentioned above. The retained material was eluted by stepwise (0.03–0.3 M NaCl). The protein content was followed by $A_{280\,nm}$ and the active fraction was detected by the capacity of inhibiting the hydrolysis of APANA by papain [13]. Active fractions eluted with 0.3 M NaCl were submitted to an affinity chromatography in a carboxy-methyl papain Sepharose resin equilibrated with 0.05 M Tris–HCl, pH 7.0. This affinity resin was prepared coupling carboxy-methylated papain by reacting the active thiol group of the enzyme with iodoacetic acid to CNBr-activated Sepharose 4B [14]. The retained material (BjHK) was eluted with 0.5 M KCl–HCl, pH 2.0, and fractions obtained were immediately neutralized. After that, the purified material was dialyzed overnight against 0.03 M NaCl. The homogeneity of the purified kininogen was evaluated by SDS–PAGE [15] and gels stained with Coomassie brilliant blue R250 revealed bands of molecular mass estimated in 110 kDa.

Venom

A pool of lyophilized venom was obtained from adult specimens of *B. jararaca* snakes at the Laboratory of Herpetology, Butantan Institute. The venom was kept at −20 °C and venom solutions were prepared with sterile saline (0.15 M NaCl) at the moment of use.

Animals

Outbred Swiss male mice with weight ranging from 18 to 22 g, supplied by the Central Animal House of Butantan Institute, were used. Animals were maintained at the laboratory with free access to food and water for at least two days, to prevent stress. All experiments followed the animal experiments' ethical standards for toxinological researches recommended by the International Society of Toxinology [16].

Effect of BjHK on venom activities

Activities were evaluated basically in two groups: one containing Bj venom and the other containing Bj venom and BjHK. In all cases these solutions were incubated at 37 °C for 30 min, except in some experiments of hemorrhagic activity in which the incubation time was changed. Control groups injected only with BjHK were also performed.

Hemorrhagic activity

It was evaluated according to Kondo et al. [17], with some modifications. A dose of 2.5 μg of venom (0.1 ml) was injected by the subcutaneous route on the shaved abdominal region of mice. Two hours later, animals were sacrificed in ether chamber. Skin was removed and the hemorrhagic spot was measured at the inner surface of the skin. The area (mm^2) of the lesion obtained in this group was compared with the ones obtained when injecting the same concentration of the venom after incubation with different concentrations of BjHK. In other set of experiments, hemorrhagic action of 2.5 μg of the venom was evaluated after 5, 15, and 30 min incubation at 37 °C with or without the same BjHK concentration.

Edema forming activity

The paw edema was evaluated by plethysmography [18]. Groups of mice were injected with 2.5 μg venom with 2.5 μg BjHK or with the same concentration of Bj venom and BjHK. The injections were into the subplantar surface of the left paw and the contralateral paw received the same volume (50 μl) of sterile saline. The edema was measured at 0.5, 1, 3, 5, 7, and 22 h after the injection and results were expressed in microliters as the difference between the volume of the paw injected with Bj venom minus the volume of the paw injected with saline.

Myotoxic activity

Every mouse experienced an injection in the center of their anterior tibial muscle with 25 μg Bj venom, 25 g BjHK, or with a mixture containing 25 μg Bj venom and the same concentration of BjHK. Six hours later, blood was collected from retroorbital plexus. The myotoxicity was estimated by the serum creatine kinase (CK) (EC 2.7.3.2.) levels measured using the 520-C kit (Sigma). The enzyme activity was expressed in units/ml, being one unit the result of the phosphorylation of one mole of creatine per min at 25 °C.

Coagulant activity

The minimum coagulant dose (MCD) [19] was determined in human and rabbit plasmas. The MCD is the minimum concentration of venom that clots standard plasma in about 60 s. The clotting time was recorded in a fibrometer (BBL) adding the MCD with or without the same concentration of BjHK (0.1 ml of total volume) to 0.2 ml of the respective plasma. Results were expressed in seconds and were the average of three determinations in each plasma.

Verification of complex formation between BjHK and jararhagin

The BjHK, purified jararhagin or a mixture containing both of them (100 μg of each), was separately chromatographed on a gel filtration Superose 12 column in a FPLC System (Amersham–Pharmacia Biotech, Uppsala). The column was eluted with 0.05 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl, at a flow rate of 60 ml/h and the protein content was followed by $A_{280\,nm}$.

Effects of the human high molecular mass kininogen on jararhagin activities

Jararhagin hemorrhage. An in vivo assay for hemorrhagic activity was performed to evaluate the effect of human HK on jararhagin hemorrhagic activity as described above. Mice received 1 μg of jararhagin through injections into their subcutaneous tissues of abdominal regions, incubated or not with human HK in a molar ratio of 1:2, 1:10 or 1:20 (jararhagin: human HK, respectively) at 37 °C for 30 min. Two hours later, animals were sacrificed and the skin of the abdominal region was removed. The area of each hemorrhagic spot was measured in mm^2.

Hydrolytic activity of jararhagin. The inhibition of the hydrolytic activity of jararhagin by human HK was also tested using the Abz-L-V-E-A-L-Y-Q-EDDnp fluorogenic substrate. The hydrolysis of the substrate was conducted at 37 °C in 100 mM Tris–HCl buffer, pH 8.8, containing 1 mM $CaCl_2$, under stirring. The hydrolysis was monitored by measuring the fluorescence at emission and excitation wavelengths of 420 and 320 nm, respectively, in a Hitachi F-2000 spectrofluorimeter. The enzyme was incubated with different concentrations of human HK for 30 min at 37 °C. The mixture was added to the substrate and the hydrolysis was monitored during 5 min. Final concentrations of enzyme and substrate were, respectively, 50 nM and 1.3 μM.

Influence of Zn^{2+} and Ca^{2+} ions. Human kininogen was pre-incubated for 15 min at room temperature with 0.4 mM $ZnCl_2$ or 0.45 mM $CaCl_2$. After this time, Bj venom was added and the mixtures were incubated at 37 °C for 30 min. The final concentrations of Bj venom and HK were 2.5 μg each and the final volume was 0.1 ml. These mixtures were injected in mice and hemorrhage was evaluated 2 h later, as described above. Control groups were performed with Bj venom (100% of hemorrhagic activity), venom with HK (100% of hemorrhagic activity inhibition) or venom with ions, being incubated as the same way.

Statistics. Statistical analysis was carried out by ANOVA and differences among means were evaluated by Duncan test. A value of $P < 0.05$ was considered statistically significant.

Results

Inhibitory effect of human and BjHK on venom activities

Hemorrhage

High molecular mass kininogen from Bj plasma was able to inhibit the hemorrhagic activity of the Bj venom (Fig. 1). The inhibitory activity of BjHK started after 5 min and reached a complete inhibition after 30 min incubation at 37 °C (Fig. 2).

Edema formation

The Bj venom induces a significant edema when injected into footpad of mice. Their maximal values were observed 1 h after taking the injections. The lesion persists for up to 22 h. BjHK induced a mild fast-lasting edema. When the mixture of BjHK and Bj venom was injected in the mouse footpad, a significant lower edema was observed when compared with the venom-induced one. This edema had also a peak at the first hour but disappeared 7 h after venom was injected (Fig. 3).

Myotoxic activity

The intramuscular injection of Bj venom induced detectable amounts of CK into the bloodstream. This effect was significantly decreased when the venom was previously incubated with BjHK. CK levels found in BjHK and in saline injected groups were the same (Fig. 4).

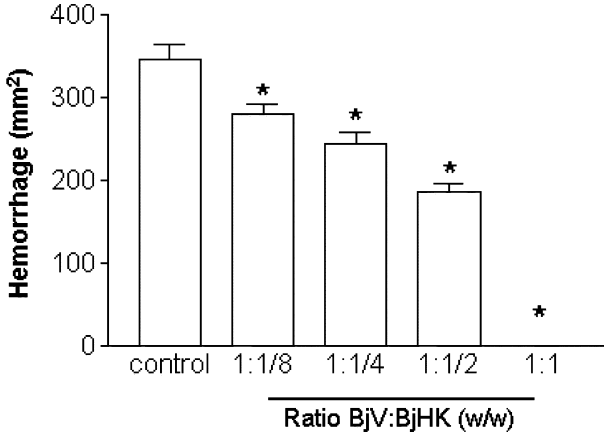

Fig. 1. Inhibition of hemorrhagic activity of Bj venom by Bj kininogen. Bj venom (2.5 μg) was incubated with different concentrations (0.31, 0.62, 1.25, and 2.5 μg) of Bj kininogen, for 30 min at 37 °C and then injected into the subcutaneous tissue of mice. Weight/weight proportions of Bj venom and Bj kininogen were 1:1/8, 1:1/4, 1:1/2, and 1;1. The hemorrhagic area was evaluated at the inner surface of the mouse skin, 2 h after the injection and compared to a control group injected with 2.5 μg Bj venom ($n = 4$/group). Results were expressed as means ± SEM of three determinations. *$P < 0.05$.

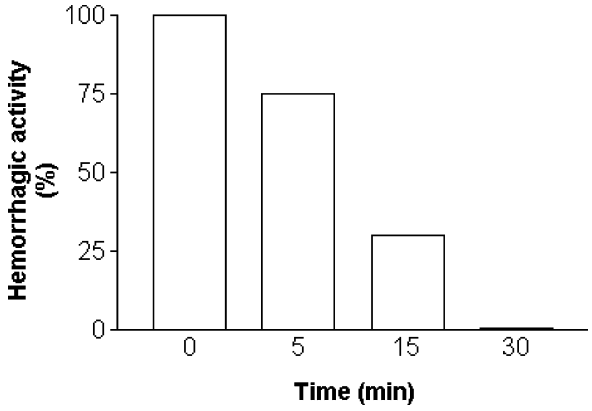

Fig. 2. Time-course of inhibitory activity of Bj kininogen on hemorrhagic activity of Bj venom. Bj venom and Bj kininogen (2.5 μg of each) incubated at 37 °C for different time intervals. The mixture was injected into the subcutaneous tissue of mice and the hemorrhagic activity was evaluated 2 h later comparing to control groups injected with 2.5 μg Bj venom incubated by the same way ($n = 4$/group).

Coagulant activity

The MCD determined for human and rabbit plasmas were 5 and 1.5 μg, respectively. As shown in Fig. 5, the mixture of BjHK and Bj venom prolonged significantly the coagulation time induced by the venom. A more pronounced effect was detected in rabbit plasma. The BjHK alone had no effect on the coagulation time of human or rabbit plasmas (data not shown).

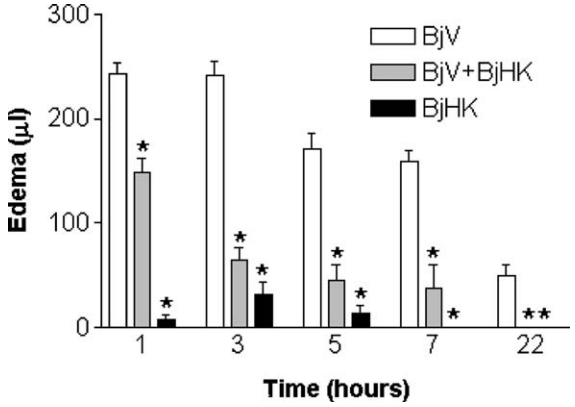

Fig. 3. Effect of the Bj kininogen on the edema forming activity of the Bj venom. Bj kininogen alone (2.5 µg) or Bj venom (2.5 µg) incubated for 30 min at 37 °C with or without Bj kininogen (2.5 µg) and injected into the foot pad of mice. The edema was evaluated by plethysmography at different time intervals ($n = 5$/group). Results were expressed as means ± SEM of three determinations. $^*P < 0.05$.

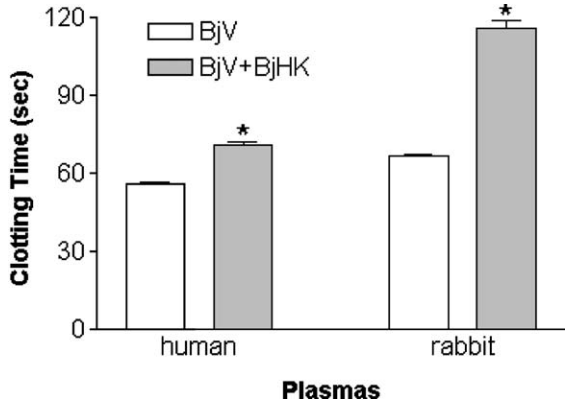

Fig. 5. Effect of Bj kininogen on coagulant activity of Bj venom on human and rabbit plasmas. The minimum coagulant doses of the Bj venom estimated for human or rabbit plasmas (1.5 and 5.0 µg, respectively) were incubated with the same concentration of Bj kininogen during 30 min at 37 °C. The coagulation times were determined and compared to those obtained only with the venom in each plasma. Results were expressed as means ± SEM of three determinations. $^*P < 0.05$.

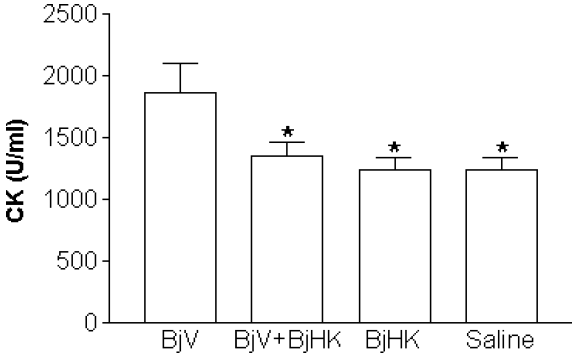

Fig. 4. Influence of Bj kininogen on the increase of serum CK levels induced by Bj venom. Intramuscular injection of 25 µg Bj venom was incubated for 30 min at 37 °C with or without 25 µg of Bj kininogen. CK levels were evaluated 6 h later. Mice injected with 25 µg Bj kininogen or saline were control groups ($n = 4$/group). Results were expressed as means ± SEM of three determinations. $^*P < 0.05$.

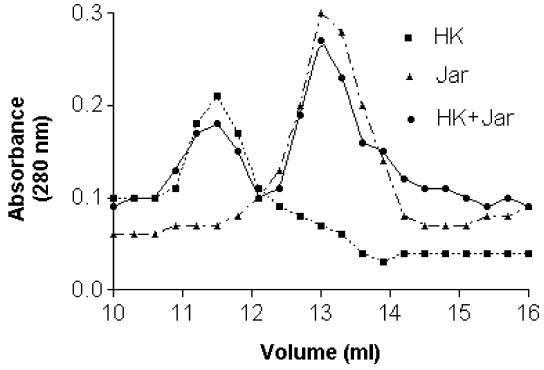

Fig. 6. Kininogen and jararhagin interaction. Mixture containing 100 µg Bj kininogen and the same concentration of jararhagin was chromatographed in a Superose 12 resin (FPLC System) and eluted at a flow rate of 60 ml/h, with 0.05 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl. The interaction between the proteins was evaluated by comparison of the elution profile of the mixture with the elution profiles of single proteins.

Mechanisms of inhibition

Lack of BjHK and jararhagin complex formation

As shown in Fig. 6, the elution of a mixture containing equal concentrations of BjHK and jararhagin in a FPLC gel filtration (Superose 12) did not present any modification of profile elution when compared with profiles obtained with the isolate proteins, suggesting that complexes between BjHK and jararhagin were not formed.

Influence of ions in the inhibitory activity

The hemorrhagic activity of the Bj venom was completely inhibited after incubation with human HK. Nevertheless, this inhibition was only partial when HK was pre-incubated with 0.4 M $ZnCl_2$ or with 0.45 M $CaCl_2$ (Fig. 7). The venom incubated with the same concentrations of $ZnCl_2$ or $CaCl_2$ did not present any changes in its hemorrhagic activity when compared to a venom not incubated with those ions (data not shown).

Inhibition of human kininogen on the enzymatic activity of jararhagin

The hemorrhage induced by jararhagin into the subcutaneous tissue of mice was significantly inhibited by human kininogen (Fig. 8). This inhibition was also evident when kininogen was assayed for the hydrolytic activity of jararhagin on Abz-L-V-E-A-L-Y-Q-EDDnp fluorogenic substrate (Table 1).

Discussion

High molecular mass kininogen, a multifunctional protein present on the plasma of different species, is the

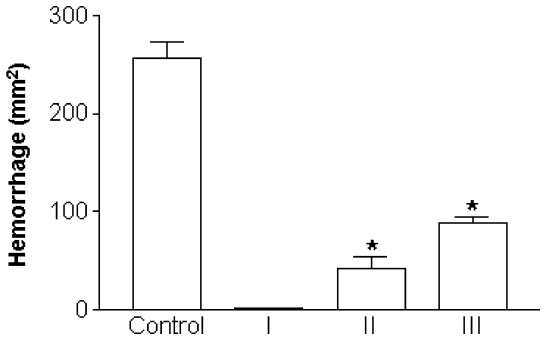

Fig. 7. Effect of Ca^{2+} and Zn^{2+} on the inhibitory activity of Bj venom by human kininogen. Human kininogen (2.5 μg) pre-incubated with (II) $ZnCl_2$ (0.4 M) or (III) $CaCl_2$ (0.45 M) during 15 min at room temperature and then with 2.5 μg Bj venom for 30 min at 37 °C. The hemorrhagic activity was evaluated 2 h after the injection into the subcutaneous tissue of mice and compared to a mixture in which kininogen was not pre-incubated with divalent salts before incubation with 2.5 g Bj venom (I, 100% inhibition) and to 2.5 μg Bj venom (Control, 100% hemorrhagic activity). Results were expressed as means ± SEM, $n = 4$/group. $^*P < 0.05$ against results observed in control and in I groups.

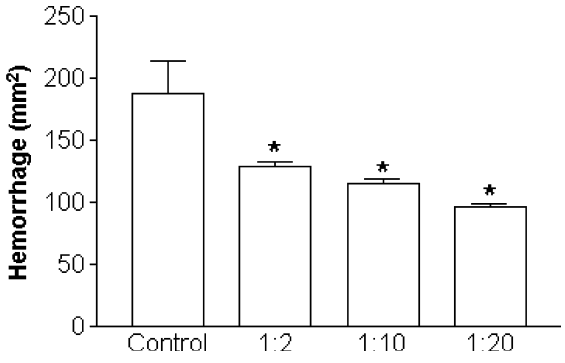

Fig. 8. Effect of human kininogen on hemorrhagic activity induced by jararhagin. Mice were injected with 1 μg jararhagin incubated or not with different concentrations of kininogen resulting in molar ratios [jararhagin:kininogen] of 1:2; 1:10, and 1:20. The hemorrhagic spot was measured 2 h after the injection. Results were expressed as means ± SEM, $n = 4$/group. $^*P < 0.05$.

Table 1
Inhibition of the hydrolytic activity of jararhagin by human high molecular mass kininogen

Mixture	AVG[b]	% inhibition
Jar	37.2 ± 1.38	—
Jar + HK (1:1)[a]	31.6 ± 0.95	15.1
Jar + HK (1:5)	18.4 ± 0.91	50.5
Jar + HK (1:10)	9.97 ± 0.88	73.2
Jar + HK (1:20)	14.6 ± 0.55	60.8

[a] Molar ratio.
[b] Average of hydrolysis (arbitrary unity).

most potent plasma cysteine proteinase inhibitor [20]. The plasma of *B. jararaca* snakes is rich in proteinase inhibitors [9] and has a protein related with mammalian high molecular mass kininogens [10] which inhibits the hemorrhagic activity of Bj venom [11].

Inhibitors of snake venom have been isolated from the blood of some snakes and marsupial mammals [8]. There are evidences that these inhibitors act on venom metalloproteinases [8], in some cases forming proteinase-inhibitor complexes [8,21–23]. In the present study, an inhibitory activity of the BjHK on metalloproteinase-dependent activities of the Bj venom and inhibition of activities of a purified snake venom metalloproteinase are also demonstrated.

BjHK inhibited venom activities in which metalloproteinases are directly or indirectly involved such as local hemorrhage, edema forming, coagulant, and myotoxic activities.

Hemorrhage locally induced by venoms of different snake species is caused by Zn^{2+}-dependent metalloproteinases [7]. In Bj venom, hemorrhagic factors with this profile were described [12,24,25].

During the multifactorial and multimediated phenomenon that leads to the edematogenic process by Bj venom, hemorrhagic factors have an important role [26]. Contrary to what could be supposed, bradykinin apparently should not participate in the genesis of the inflammatory edema induced by *B. jararaca* venom [27].

The Bj venom has two types of coagulant toxins: serine proteinases that directly act on fibrinogen and metalloproteinases that activate factors X and II [28]. BjHK inhibited the coagulant activity of the Bj venom in both plasmas but this inhibitory activity was higher in rabbit plasma. The human plasma is susceptible to both coagulant toxins of the Bj venom but only pro-coagulant metalloproteinases act on rabbit plasma [29]. It is important to note that BjHK had no effect on Bj venom hydrolytic activity on BAPNA chromogenic substrate (data not shown), indicating that venom serine proteinases were not inhibited.

All bothropic venoms are able to induce skeletal muscle damage. This effect can be caused either by direct activity of phospholipase A_2 myotoxins, or indirectly, by ischemic injury as a result of vascular lesions. As myotoxins are not found in Bj venom [27] and BjHK considerably inhibited the liberation of creatine kinase induced by the Bj venom, it is quite evident that this effect was due to less severe vascular lesions determined by the inhibitory effect of the kininogen on the hemorrhagic activity of the venom.

The mechanism of this inhibition is not clear. Complex formed by BjHK and jararhagin could not be detected by gel filtration. These data agree with observations of Kamiguti et al. [30] who showed that the only plasma protein that binds to this hemorrhagic metalloproteinase is α2-macroglobulin.

Two proteins that inhibit some activities of Bj venom were isolated from Bj plasma [21,22]. One of them differs from the BjHK in two important parameters: the lower

molecular weight (45 kDa) and the absence of inhibition of cysteine-proteinases [21]. The latter, better characterized, presents homology with members of the cystatin superfamily [22].

Antihemorrhagic factors can be grouped in three classes: which contains cystatin domain-like, α1B glycoprotein-like, and α2-macroglobulin [8].

The human HK inhibited the hemorrhagic effect as well as the enzymatic activity of jararhagin, a hemorrhagic Zn^{2+}-dependent metalloproteinase isolated from Bj venom. Human HK is an acidic glycoprotein belonging to the cystatin superfamily and presenting the sequence QVVAG in domains 2 and 3. This sequence is highly preserved in cysteine proteinase inhibitors such as cystatin and is responsible for the inhibition of cysteine proteinases [31].

Despite the fact that inhibition on cysteine-proteinases is an important characteristic of kininogens it seems not to be involved in the inhibition of the local hemorrhage induced by the Bj venom. Other cysteine-proteinase inhibitors (from plants and cystatin, isolated from egg yolk) which have the same sequence in the reactive site (QVVAG) failed in inhibiting it (data not shown).

Snake venom metalloproteinases have Zn^{2+}-dependent activities [7], but some of them are more active in the presence of Ca^{2+} ions [32]. Moreover, calcium-binding proteins were described in Bj venom [33]. As kininogen needs Zn^{2+} and Ca^{2+} in some of its activities [34,35], the hypothesis based on the competition for ions as responsible for the inhibition of the venom was tested. In agreement with this hypothesis, kininogen showed a significant lower inhibition when previously incubated with Zn^{2+} and a remarkable effect was noticed when it was pre-incubated with Ca^{2+} alone. These data can implicate domains 1, which bind Ca^{2+}, and domain 5, which bind Zn^{2+}, in this inhibitory activity.

In conclusion, the high molecular mass kininogen of the human as well as of the Bj plasma cause a significant inhibition of Bj venom metalloproteinases activities. This inhibition was clearly demonstrated when purified jararhagin was used and is possibly by a competition for ions.

These results are the first evidence that high molecular mass kininogen can affect metalloproteinase activities. This observation could involve this protein in pathophysiologic processes in which metalloproteinases have a central role.

Acknowledgments

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