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Short communication

Gyroxin increases blood-brain barrier permeability to Evans blue dye in mice

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ABSTRACT

Gyroxin is a serine protease enzyme component of the South American rattlesnake (*Crotalus durissus terrificus*) venom. This toxin displays several activities, including the induction of blood coagulation (fibrinogenolytic activity), vasodilation and neurotoxicity, resulting in an effect called barrel rotation. The mechanisms involved in this neurotoxic activity are not well known. Because gyroxin is a member of a potentially therapeutic family of enzymes, including thrombin, ancrod, batroxobin, trypsin and kallicrein, the identification of the mechanism of gyroxin's action is extremely important. In this study, gyroxin was isolated from crude venom by affinity and molecular exclusion chromatography. Analysis of the isolated gyroxin via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single protein band with a molecular weight of approximately 28 kDa, confirming the identity of the molecule. Furthermore, intravenous administration of purified gyroxin (0.25 μg/g of body weight) to mice resulted in symptoms compatible with barrel rotation syndrome, confirming the neurotoxic activity of the toxin. Mice treated with gyroxin showed an increase in the concentration of albumin-Evans blue in brain extracts, indicating an increase in the blood-brain barrier (BBB) permeability. This gyroxin-induced increase in BBB permeability was time-dependent, reaching a peak within 15 min after exposure, similar to the time span in which the neurotoxic syndrome (barrel rotation) occurs. This work provides the first evidence of gyroxin's capacity to temporarily alter the permeability of the BBB.

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1. Introduction

Gyroxin is a serine protease component of the venom of the South American rattlesnake *Crotalus durissus terrificus* that has neurotoxic effects. It constitutes about 2.5% of the crude venom of this species. This toxin was partially characterized by Barrio (1961) who described gyroxin as a non-lethal neurotoxin responsible for the induction of a neurological syndrome in mice. This effect begins with a cataleptic stage followed by rolling movements similar to a barrel roll. This syndrome is associated with the intracerebral action of neuropeptides (Cohn and Cohn, 1975). Kruse et al. (1977) observed the same neurological effect after intracerebroventricular administration of arginine vasopressin in rats, and several studies were performed to better understand this pathological syndrome and also to define the location and mechanism involved in the genesis and development of the syndrome (Diamant et al., 1994; Kannan et al., 1994; Kawachi et al., 1998; Willcox et al., 1992; Wurpel et al., 1986a, b; Yoshizawa et al., 1990).

Alves da Silva et al. (2006) analyzed the biodistribution of gyroxin in mice and observed a very small proportion of the protease in the brains of exposed animals when compared to concentrations in other observed organs. In addition, Camillo et al. (2001) reported that gyroxin does not affect the release of the neurotransmitters dopamine

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and acetylcholine in the nervous system. Together, these facts suggest that the brain might not be the primary target of gyroxin, despite the fact that this toxin exhibits neurotoxic activity.

However, it is well known that serine proteases such as thrombin, alter the membrane permeability of endothelial cells and increase the permeability of the blood-brain barrier (BBB) (Déry et al., 1998; Guan et al., 2004; Kataoka et al., 2003; Kim et al., 2004; Misaki et al., 2006).

The BBB is relatively impermeable to ions, some amino acids, small peptides and proteins. The main function of the BBB is to regulate the transport of substances between the blood and the brain tissue in order to maintain a neural environment that is protected from neurotoxic substances and abrupt changes in blood composition (Bondan and Lallo, 1998; Laquintana et al., 2009). The BBB also acts to exclude inflammatory cells from the central nervous system (Leibowitz and Hughes, 1983; Sternberg et al., 2000). Despite this complex system protecting the brain, some pathological conditions, such as hypertension, radiation, multiple sclerosis, tumors, and some animal venoms (Carvalho et al., 2000; da Silva et al., 2004; Laquintana et al., 2009; Wilson et al., 2009) can alter the permeability of the BBB which brings medical risks such as altered interstitial concentrations of protein, water and electrolytes (Tétrault et al., 2008).

Although gyroxin has been studied in several recent works (Alves da Silva et al., 2006; Torrent et al., 2007; Yonamine et al., 2009) the mechanism of action responsible for the barrel rotation syndrome remains unknown and the possible effects of gyroxin on BBB permeability have not yet been determined. The thrombin-induced increase in vascular permeability and vasodilation (via thrombin's action on protease-activated receptors - PARs) lead us to hypothesize that gyroxin might exhibit some activity in the BBB and this knowledge could contribute towards understanding the mechanisms involved in its neurotoxic activity.

The aim of the present study is to analyze the integrity of the BBB after intravenous injection of gyroxin in mice using the albumin-Evans blue dye complex as a tracer.

2. Materials and methods

Crotalus durissus terrificus venom (dry form) was kindly provided by the Centro de Estudos de Animais Peçonhentos - CEVAP (UNESP, Botucatu, Brazil) and from the Divisão de Herpetologia do Instituto Butantan, São Paulo, Brazil.

2.1. Materials

Benzamidine sepharose 6B resin was obtained from GE Healthcare (Fairfield, CT, USA). All other reagents were obtained from commercial sources and were of analytical grade.

2.2. Swiss mice

The animals used in this study were obtained from the animal housing facility of IPEN/CNEN/SP and maintained in sterilized isolators with absorbent media and water *ad libitum*. All manipulations of these animals before or during the experiments were performed according to the "Principles of Laboratory Animal Care" (SBCAL/COBEA; NIH publ. No 86-23, revised 1985) and were approved by the Ethics Committee of the Animal IPEN-Project no. 27/CEPA-IPEN/SP.

2.3. Purification of gyroxin

Gyroxin was obtained by fractionating crude venom of the *Crotalus durissus terrificus* employing affinity chromatography and molecular exclusion. Approximately 300 mg of venom were dissolved in 4 mL of Tris–HCl 0.5 M pH 9.0, and centrifuged at 10 000 *g* for 1 min at 4 °C. Benzamidine Sepharose 6B resin was activated with 0.1 M Tris–HCl, pH 9.0, 0.5 M NaCl, and 0.1 M sodium acetate, pH 4.5, 0.5 M NaCl. The supernatant was fractionated on Benzamidine Sepharose 6B at 4 °C with a flow rate of 0.2 mL/min, as previously described (Camillo et al., 2001).

Next, the lyophilized fraction was dissolved in 1 mL of ammonium formate buffer (0.1 M pH 3.0) and applied to a Superdex 75 column (XK 1.6 $\times$ 70) in the same buffer. The elution was performed at 4 °C with a flow rate of 0.5 mL/min, and 1.0 mL fractions were collected. The absorbance of each fraction was measured at 280 nm. The collected fractions containing purified gyroxin were combined in a conical tube, measured for protein concentration, cooled, lyophilized and stored at −20 °C.

2.4. Protein concentration

The protein concentration of samples was determined by the modified Bradford method (Bollag et al., 1996). This method is based on the ability of the Coomassie Brilliant Blue G-250 dye to bind to proteins in a highly acidic environment, resulting in a proportionate change in color detectable at 595 nm.

2.5. Neurotoxicity assay

The activity of the isolated gyroxin was measured by a neurotoxicity assay (barrel rotation). Five Swiss male mice weighing 25–35 g were injected intravenously with 150 μL of gyroxin (0.25 μg/g body weight) dissolved in 0.15 M NaCl (Alexander et al., 1988). After injection, each animal was kept under observation for up to 60 min. Barrel rotation activity was observed and recorded.

2.6. Analysis on agarose gel electrophoresis

Electrophoresis was performed following the method described by Laemmli (1970) using denaturing polyacrylamide gels under non-reducing conditions. Proteins were resolved over stacking and resolving gels of 6% and 15% polyacrylamide, respectively.

2.7. Albumin-Evans blue dye extravasation method

Permeability of the BBB was quantified after intravenous injection of gyroxin using the modified albumin-Evans blue extravasation method as previously described (Kaya et al., 2004). Using this method, we quantified the concentration of albumin-Evans blue dye in brain homogenates.

For this assay, 25 Swiss male mice (weighing 23–30 g) were divided into 5 groups with 5 mice in each group (this assay was repeated 3 times). Mice were anesthetized with a dorsal subcutaneous injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}).

Briefly, albumin-Evans blue (2% in saline; 2 mL/kg) was intravenously administered 30 min before mice were euthanized. Intracardiac perfusion was then performed through the left ventricle with saline (this was coupled with a peristaltic pump with a flow rate of 10 mL/min) to remove intravascular albumin-Evans blue and continued until the fluid from the right atrium became colorless. Mice were then euthanized and their brains were quickly removed, weighed and homogenized in 2.5 mL of phosphate buffered saline (PBS) pH 7.2. After the first homogenization, 2.5 mL of 50% trichloroacetic acid were added, followed by vortex mixing for 2 min. The supernatants were cooled to 10 °C and centrifuged at 10 000 *g* for 30 min. The albumin-Evans blue concentrations were measured with a spectrophotometer at 610 nm and compared against a standard curve (serial dilutions of the stock dye solution, 1:2 dilution factor in the concentration range of 0.12–31.25 μg/mL). Albumin-Evans blue extravasation was analyzed by linear regression: $Y = aX + b$, where Y is the measured absorbance and X is the concentration of the dye. The results were expressed as micrograms of albumin-Evans blue/milligram of brain tissue.

- Group 1. Negative control. The first group received 100 μL of conjugated bovine albumin-Evans blue solution followed by 100 μL of 0.15 M NaCl and was euthanized within 10 min.
- Group 2. Positive control. This group received 100 μL of albumin-Evans blue followed by 100 μL of a mannitol suspension −0.5 g/kg intravenous bolus (Rapoport, 2000; Tétrault et al., 2008) - and euthanized within 10 min.
- Groups 3–4–5. Gyroxin. These groups received albumin-Evans blue at a dose of 2 mL/kg of body weight and were then treated with gyroxin at 0.25 μg/g 5 min later. Both treatments were administered intravenously. Animals treated with gyroxin were euthanized after 7 min (group 3), 15 min (group 4) or 30 min (group 5).

2.8. Statistical analysis

The concentration of albumin-Evans blue dye in each sample was obtained by comparison to a standard curve. For comparisons between groups, we used analysis of variance (ANOVA) followed by Dunnett's test. A value of $p < 0.05$ indicated significance. All statistical tests were performed using GraphPad Prism software (version 5.0).

3. Results

3.1. Purification of gyroxin

Affinity chromatography separated the venom into 2 fractions AP1 and AP2 (Fig. 1). The AP1 fraction consisted of toxins that did not bind to benzamidine and were therefore constituents of the poison that did not belong to the serine protease enzyme family. The AP2 fraction consisted of serine proteases that were only eluted after changing the pH from 9.0 to 4.5. The AP2 fraction was frozen and lyophilized. After lyophilization, the AP2 fraction was dissolved in ammonium formate buffer (0.1 M, pH 3.0) and applied to a molecular exclusion column. Fig. 2 shows the elution profile of the crude venom and the purified gyroxin (the AP2 fraction from the affinity chromatography) obtained from *Crotalus durissus terrificus* using a Superdex 75 Fast Flow molecular exclusion column. Both profiles were obtained using the same flow rate conditions, temperature, volume and concentrations of the samples. The fractionation profile of the crude venom was used as a reference to determine the elution volume (V_e) of gyroxin (as indicated by the gray bar).

3.2. Protein concentration

The determination of protein concentration at different stages of the purification process revealed a yield of 2.3% (i.e., 7 mg of gyroxin was obtained from 300 mg of crude venom).

3.3. Neurotoxicity assay

Treatment with purified gyroxin induced the barrel rotation syndrome in 80% of treated mice ($n = 10$). The barrel rotation phenotype was observed and recorded. The syndrome started from 3 to 5 min after gyroxin injection and this process occurred for 2 min approximately. Generally, 1 h after the injection the animals returned to their normal behavior.

3.4. Analysis by agarose gel electrophoresis

Gyroxin migrated by agarose gel electrophoresis as a single band with an estimated molecular mass of 28 kDa (Fig. 2) thus demonstrating the efficiency of the purification method and the integrity of the molecule.

3.5. Albumin-Evans blue extravasation method

Fig. 3A shows the standard curve used to determine the concentration of albumin-Evans blue in brain samples.

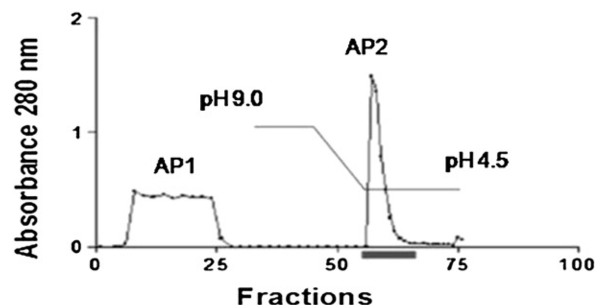

Fig. 1. Chromatographic profile obtained by fractionation of crude venom of *Crotalus durissus terrificus* on benzamidine sepharose 6B Fast Flow affinity chromatography. The initial buffer was 0.1 M Tris–HCl pH 9.0 and final buffer, ammonium acetate 0.5 M pH 4.5, flow rate 0.2 mL/min, fraction volume 1.0 mL, temperature 4 °C.

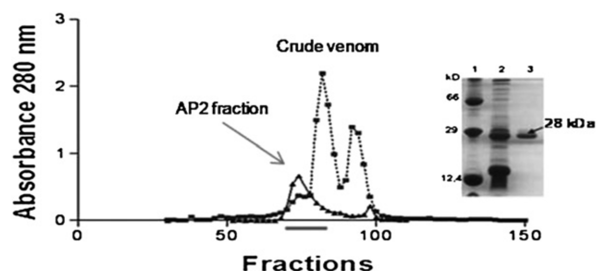

Fig. 2. Elution profile of crude venom and gyroxin obtained from *Crotalus durissus terrificus* on Superdex 75 Fast Flow molecular exclusion chromatography. Buffer was ammonium formate 0.1 M pH 3.0, flow rate 0.5 mL/min, fractions of 1 mL, temperature 4 °C. The detection was by absorbance at 280 nm. The gyroxin region of elution is indicated by the gray bar. In the SDA-PAGE photo: 1 molecular weight standard; 2 crude venom of *Crotalus durissus terrificus* and 3 fraction obtained from molecular exclusion, the arrow indicates the band of the gyroxin at the end of purification with determined molecular mass of 28 kDa.

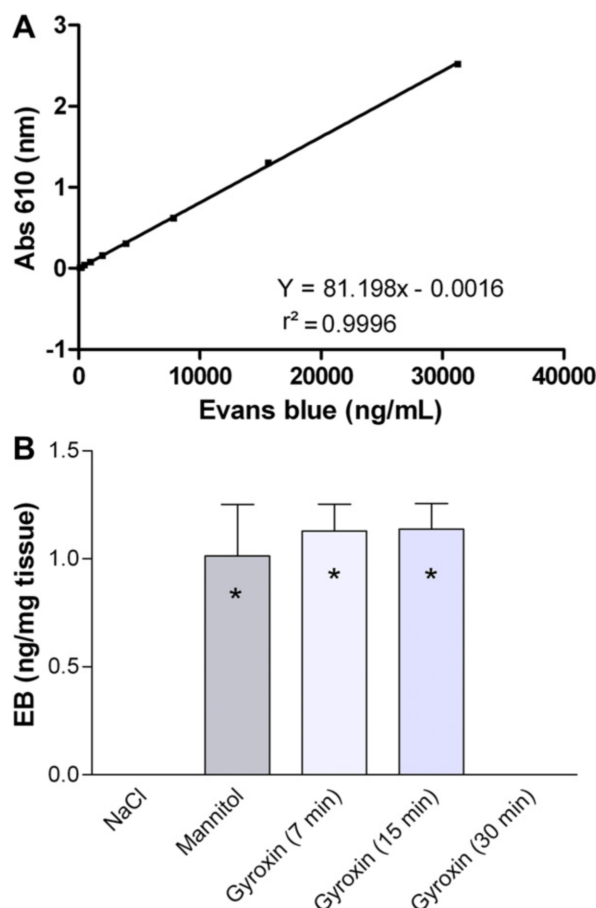

Fig. 3. A) Standard curve used to estimate the concentration of albumin-Evans blue in brain extracts. B) Determination of albumin-Evans blue in the supernatant of brain extract of Swiss mice with and without intravenous injection of 0.15 M NaCl (negative control), 20% mannitol, 0.5 g/kg (positive control) and gyroxin in different intervals (7, 15 and 30 min). The groups treated with gyroxin 0.25 μg/g and euthanized after 7 or 15 min showed a significant increase in the concentration of albumin-Evans blue in brain extracts ($p < 0.05$). The group euthanized after 30 min, had no significant difference compared with negative control.

The mannitol-treated group showed a significant increase ($p < 0.05$) in the concentration of albumin-Evans blue in brain extracts (Fig. 3B) confirming a change in the permeability of the BBB. The group treated with 0.15 M NaCl did not show any change in the permeability of the BBB. Brain homogenates from the groups treated with gyroxin and euthanized after 7 or 15 min (Fig. 3B) showed residues of albumin-Evans blue. This finding provides the first evidence that gyroxin can alter the permeability of the BBB. This alteration occurred rapidly following exposure, similar to the mannitol-induced BBB breakdown. Additionally, the change in BBB permeability was reversible, as the homogenates isolated after 30 min displayed no measurable levels of albumin-Evans blue.

4. Discussion

In this study, fractionation of crude venom was accomplished by first using an affinity column specific for serine proteases, rather than using the traditional method of molecular exclusion, followed by affinity chromatography (Alexander et al., 1988; Barrabin et al., 1978). Since gyroxin represents only 2.5% of the crude venom, this strategy allowed us to initiate the first step of purification with a higher amount of crude venom, avoiding the frequent differences in the enzymatic potency of gyroxin that is usually obtained after the purification, freezing, lyophilization and dilution steps.

Our experiments using Swiss mice uncovered a change in the permeability of the BBB in response to treatment with gyroxin. Animals treated with gyroxin displayed increased permeability of the BBB, allowing the entry of albumin-Evans blue into the brain. This gyroxin-induced increase in BBB permeability was time-dependent and displayed a peak within 15 min of exposure, similar to the time span in which the neurotoxic syndrome (barrel rotation) occurs. It is important to note that the dose of gyroxin (0.25 μg/g of body weight) used in the albumin-Evans blue extravasation assay was the same as the dose used for the gyroxin-induced barrel rotation assays.

Changes in permeability of the BBB are commonly associated with serine proteases that affect coagulation factors (Ay et al., 2008; Tétrault et al., 2008; Yepes et al., 2003), vascular tone, membrane receptors and the production of reactive species such as nitric oxide (NO). Previous reports have shown that gyroxin treatment of ECV304 cells results in the release of NO, an important vasorelaxant agent (Camillo et al., 2001; Hashizume and Camillo, 2005). Besides that, data from the literature have indicated that NO modulates the permeability of BBB in mice. Taking these two facts together, a contribution of NO to the increase in permeability of BBB caused by gyroxin could be considered.

The possible release of NO and the hypotension observed in rats (Alves da Silva, 2004) exposed to gyroxin provided evidence that gyroxin could act on the permeability of the BBB in a manner similar to that of other vasodilator agents, such as thrombin and mannitol (Rapoport, 2000). To verify this hypothesis a study concerning the correlation between NO, BBB and barrel

rotation was conducted and those results will be published elsewhere.

The present work provides the first evidence that gyroxin, a serine protease, can regulate the permeability of the BBB like other serine proteases. However, it is important to note that thrombin does not cause the barrel rotation syndrome (Alexander et al., 1988) and that there is no evidence that mannitol, NO donors, isoflurane and other BBB disruptor substances can induce barrel rotation after intravenous administration. Although the disruption of BBB was found to be associated with various neurological disorders such as septic encephalopathy (Davies, 2002), multiple sclerosis (Minagar and Alexander, 2003), cerebral edema formation (Kahle et al., 2009) and epileptic syndrome (Seiffert et al., 2004), BBB disruption may be just one stage of gyroxin's neurotoxic effects. Serine protease toxins isolated from other snake venoms such as ancrod®, gyroxin-like and crotalase® (by intravenous administration) can induce the rotational behavior in mice. It is likely that the barrel rotation syndrome is a special consequence of the intravenous administration of viperid snake venoms (Alexander et al., 1988; Camillo et al., 2001; Perez et al., 2008). Therefore, gyroxin, besides its effect on BBB permeability, could display other actions, affecting endogenous substrates present in the endothelium, nervous tissues or blood, releasing active forms of these substrates that ultimately lead to induction of this syndrome.

The disruption in BBB permeability induced by gyroxin occurs during the first few minutes after exposure to the toxin. Furthermore, similar to mannitol (Rapoport, 2000), the change induced by gyroxin is reversible, since the presence of albumin-Evans blue is no longer detectable in the brain 30 min after toxin exposure. This result correlates with the neurotoxic symptoms observed in mice; barrel rotations occurred in the first few minutes after the administration of gyroxin, most frequently between 3 and 5 min post-exposure. Usually the animals recovered after 60 min and displayed no further symptoms. The dose of gyroxin used in this study (0.25 μg/g body weight) was not lethal. In some cases, animals died during the first minute after injection, however those animals that survived did not show any observable symptoms after 1 h.

Further studies are needed to determine the specific mechanisms involved in the gyroxin-induced alteration of mouse BBB permeability. Particular emphasis should be focused on the role of membrane receptors and the possible neurological damage resulting from gyroxin exposure.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxicon.2010.06.027.

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