Biomedicine & Pharmacotherapy 66 (2012) 256–265

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Original article

Characterization of two vasoactive peptides isolated from the plasma of the snake *Crotalus durissus terrificus*

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ARTICLE INFO

Article history:
Received 25 May 2011
Accepted 31 October 2011

Keywords:
Hypotensive
Kinin
Vasoactive
Angiotensin
Peptide
Snakes
Vascular

ABSTRACT

Incubation of plasma from the snake *Crotalus durissus terrificus* (CDTP) with trypsin generated two hypotensive peptides. The primary structure of the peptides was established for two sequences as: (Ser – Ile – Pro – Gln – Ala – Pro – Thr – Ser – Asn – Leu – Ile – Glu – Ala – Thr – Lys) and (Lys – Pro – Asp – Ala – Asn – Gln – Val – Leu – Ile – Gln – Val – Ile – Gly – Val). These peptides display homology with fragments of albumin from *Trimeresurus flavoviridis*. Bolus intra-arterial injection of the purified or the synthetic peptide produced a strong and sustained vasopressor response in the anaesthetized snake (CDT) and rats (*Wistar*); this hypotensive effect was also potentiated by captopril–an angiotensin-converting (0.1 mg/kg) enzyme inhibitor.

1. Introduction

Snake venoms are composed of several compounds and, very often, many of them are toxins.

The action site of these toxins includes cardiac muscle, vascular smooth muscle and the capillary vascular bed. Some snake venoms, for example, contain peptides that inhibit angiotensin-converting enzyme and potentiate the biological actions of bradykinin (BK).

The kallikrein-kinin system in mammals involves the sequential action of a series of well-characterized proteolytic enzymes. Activation of factor XII (Hageman factor) in blood at the site of tissue injury or, in vitro, by contact with a charged surface results in activation of plasma prekallikrein and generation of BK by the cleavage of high molecular mass kininogen [1]. Other snake venoms contain structural and functional equivalents of mammalian natriuretic peptides. Sarafotoxins are short peptide toxins found in the venoms of snakes from Atractaspis spp. which display potent vasoconstriction properties. These peptides, which share a high degree of sequence identity with endothelins, recognize and bind to endothelin receptors. Snakes have also evolved toxins which block L-type Ca(2+) currents (e.g. calciseptine, FS2 toxins, C(10)S(2)C(2) and S(4)C(8)). Snake venom proteins have also been shown to increase vascular permeability. One such protein, increasing capillary permeability protein (ICPP) has recently been isolated from the venom of *Vipera lebetina*. ICPP is an extremely potent permeability factor with a structure similar to vascular endothelial growth factor (VEGF). Thus there is a vast array of snake toxins with potent cardiovascular activity. Some of these proteins and peptides have proven to be highly selective tools in the study of physiological processes. Others have been used as potential therapeutic targets or as lead compounds in the development of therapeutic agents. Therefore these and other related snake venom proteins hold great promise in the future understanding and treatment of cardiovascular diseases [2].

In reptiles, infusion of the species-specific BK consistently causes a systemic vasodilatation, but the changes in blood pressure and heart rate vary among species. Besides the research on Kallikrein-Kinin system in mammals, including man, the analysis of the different aspects of the role of kinins between different species in zoological scale is of interest, particularly in snakes, where BPPs and kininogenases are important components of the venoms in order to understand different kinds of cardiovascular regulating systems, particularly those mediated by vasoactive peptides. The blood of reptiles contains some, but not necessarily all, components of the kallikrein-kinin system present in mammals. Very few aspects were studied concerning the kallikrein-kinin system in snakes, but it is known that, it is probably deficient of Factor XII (Hageman's factor), an activator of the prekallikrein in mammals. These peptides display homology with fragments of albumin from *Trimeresurus flavoviridis* too. The

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doi:10.1016/j.biopha.2011.10.001

new peptides displayed biological activity similar to that already described through BK-potentiating effect and ACE inhibitors (ACEIs) on the blood arterial pressure in rats and snakes [3]. Similarly kallikrein-like esterolytic activity was demonstrated in the plasma of the Brazilian snakes *Waglerophis merremii* and *Crotalus durissus* using synthetic chromogenic substrates [7].

The angiotensin-converting enzyme (ACE, EC 3.4.15.1) is the cytoplasmic membrane peptidase of endothelial cells responsible both for the conversion of angiotensin I into angiotensin II and for BK degradation [4,11]. This enzyme has been the critical metabolic target used by the pharmaceutical industry to generate antihypertensive drugs through the development of specific ACEIs. Several ACEIs are currently used to treat human hypertension [12,13].

The antihypertensive effect of the ACEIs is not only explained by the preclusion of the hypertensive effect of angiotensin II but also by the potentiating hypotensive effect of the circulating BK [11].

In the present study, we describe the purification and structural characterization of vasoactive peptides from plasma of the Brazillian snake *Crotalus durissus terrificus* (CDT): two current methodologies for BKG determination with trypsin as enzyme (Diniz and Carvalho (D&C) and Uchida and Katori (U&K) methods), and further chromatographic steps for isolation of the generated peptides and bioassays to characterize the effects were used and the possible physiologic role is discussed.

2. Methods

2.1. Animals

The CDT snakes (Serpentes, Viperidae, Crotalinae), adults, males or females, weighing 150–600 g, used in this work were collected from nature; maintained in a quarantine of at least 15 days after experiments under controlled environmental conditions, with a photoperiod of 12 h of light and 12 h of dark, at a temperature of 26 °C and 65% relative humidity.

Wistar rats (*Rattus norvegicus*), males weighing 350–500 g from the Central Animal Farm of Institute Butantan.

Spontaneous hypertensive rats (SHR), males, weighing 350–400 g, provided by Central Animal Farm of Federal University of Sao Paulo (UNIFESP) were used to measure the biological action of the isolated peptides from plasma on the arterial blood pressure.

Rats were maintained under controlled environmental conditions with a photoperiod of 12 h of light and 12 h of darkness and supplied with "ad libitum" water and food.

All animal management, surgical and postsurgical proceedings from approved by the Butantan Institute Ethic Commission for Animal Experimentation–CEUAIB (Protocol 094/2002), and was in accordance to the international rules for animal care and experimental management.

2.2. Plasma collection

Blood was collected after decapitation of the snakes (CDT) into polyethylene tubes containing sodium citrate (3.8 mg/mL of blood) as an anticoagulant and immediately centrifuged at 9.8 × g for 15 min [3].

2.3. Assays for kinin release from snake plasma

The CDT plasma was treated with trypsin as described previously by Diniz and Carvalho [5] or Uchida and Katori [6]. The released peptide(s) was (were) freeze dried, and ressuspended in 2 mL of 0.9% NaCl and assayed [5].

2.4. Determination of plasma kininogen contents [6]

Briefly, 0.2 mL of plasma were incubated with 1.8 mL of 0.03 N HCl at 37 °C for 15 min. After neutralizing with 0.05 mL of 1 N NaOH, 0.5 mL of 0.2 M Tris-HCl buffer pH 7.8 and 0.1 mL of trypsin (2 mg/mL) were added and the samples incubated at 37 °C for 30 min [6].

2.5. Determination of plasma kininogen contents [5]

Briefly, 0.2 mL of plasma are transferred with a siliconed pipette to 18 × 50 tubes containing 1.8 mL of 0.2% (v/v) of acetic acid and heated in a boiling water bath for 30 min. Denatured plasma can be kept in the refrigerator 2–3 days without any loss of BKG. For the release of BK, the denatured plasma is adjusted to pH 7.4–7.8 (by addition of 0.06 mL of NaOH (1 N) and the mixture buffered to pH 7.8 by the addition of 0.5 mL of 0.2 M Tris buffer. The tubes were incubated with a solution containing 200 μg of crystalline trypsin (Sigma Chem. Co., USA) dissolved in 0.10 mL of saline for 30 min at 37 °C [5].

2.6. Protein determination

The protein content was determined by the Bradford method by using Bio-Rad assay reagent (Bio-Rad, Munich, Germany) and bovine serum albumin used as standard [3].

2.7. High Performance Liquid Chromatography (HPLC) analysis

Reversed-phase binary HPLC system (Amersham) was used to the sample separation. The SPE eluted fraction was loaded in a Shimadzu, C_{18} column (4.6 mm × 250 mm) in a two-solvent system: (A) trifluoroacetic acid (TFA)/H_2O (1:1000) and (B) TFA/acetonitrile (ACN)/H_2O (1:900:100). The column was eluted at a flow rate of 1.0 mL/min with a 5–95%, 20–50% and 10–30% gradient of solvent B over 45 and 55 min. The HPLC column eluates were monitored by their UV absorbance at 214 nm. For peptides purification, further chromatographic steps were necessary, using the same column with optimized gradients over 45 and 55 min.

Steps:

- 1st step: gradient of 5–95% of B in 45 min 1 mL/min;
- 2nd step: gradient of 20–50% of B in 45 min 1 mL/min;
- 3rd step: gradient of 10–30% of B in 55 min 1 mL/min;
- 4th step: gradient of 10–30% of B in 55 min 1 mL/min.

The HPLC analysis was separated in different pools and analyzed on CDTs and rat mean arterial blood pressure (MAP) [3].

2.8. Blood arterial pressure (mean arterial blood pressure) in anaesthetized snakes

The snakes were anaesthetized with sodium pentobarbitone, a gift from Abbott Laboratories of Brazil Ltd.; (30 mg/kg) i.p and heparinized (sodium heparin liquemine, Roche Laboratories, Brazil), a surgical proceeding was done to insert a polyethylene tube (PE 50) in the carotid artery for the register of MAP by a (TSE) system polygraph. Administration of drugs was made through a polyethylene catheter (PE 10) introduced into the renal artery of the snake [7].

2.9. Blood arterial pressure in anaesthetized rats

The rats were anaesthetized with sodium pentobarbitone, a gift from Abbott Laboratories of Brazil Ltd.; (70 mg/kg) and heparinized, (sodium heparin liquemine, Roche Laboratories, Brazil).