Peptides 36 (2012) 206–212

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Vascular effects and electrolyte homeostasis of the natriuretic peptide isolated from *Crotalus oreganus abyssus* (North American Grand Canyon rattlesnake) venom

S.L. Da Silva[a,*], C.A. Dias-Junior[b], P.A. Baldasso[c], D.C.S. Damico[c], B.M.A. Carvalho[a], A. Garanto[a], G. Acosta[f], E. Oliveira[e], F. Albericio[e,f,g,h], A.M. Soares[c], S. Marangoni[d], R.R. Resende[i,**]

[a] *Federal University of São João Del Rei Campus Alto Paraopeba, 36420-000 Ouro Branco, Minas Gerais, Brazil*
[b] *Universidade Estadual Paulista Júlio de Mesquita Filho, 18618-970 Botucatu, São Paulo, Brazil*
[c] *Oswaldo Cruz Foundation, Federal University of Rondonia, Porto Velho, Rondônia, Brazil*
[d] *Department of Biochemistry of Biology Institute, State University of Campinas, Campinas 13083-970, São Paulo, Brazil*
[e] *Proteomics Platform, Barcelona Science Park, University of Barcelona, 08028 Barcelona, Spain*
[f] *Institute for Research in Biomedicine, Barcelona Science Park, University of Barcelona, 08028 Barcelona, Spain*
[g] *CIBER-BBN, Networking Centre on Bioengineering Biomaterials and Nanomedicine, Barcelona Science Park, 08028 Barcelona, Spain*
[h] *Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain*
[i] *Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, 31270-901 Belo Horizonte, Minas Gerais, Brazil*

ARTICLE INFO

Article history:
Received 7 May 2012
Received in revised form 10 May 2012
Accepted 11 May 2012
Available online 19 May 2012

Keywords:
Crotalus oreganus abyssus
Hypotensive agents
Snake venoms
Natriuretc peptides
Nitric oxide

ABSTRACT

Crotalus oreganus abyssus is a rattlesnake that is usually found in the Grand Canyon, United States of America. Knowledge regarding the composition of *C. o. abyssus* venom is scarce. New natriuretic peptides (NPs) have been isolated and characterized from the venoms of members of the *Crotalinae* family. The NP family comprises three members, ANP (atrial natriuretic peptide), BNP (b-type natriuretic peptide) and CNP (c-type natriuretic peptide), and has an important role in blood pressure regulation and electrolyte homeostasis. The aim of the present study was to characterize a novel natriuretic-like peptide (Coa_NP2), isolated from *C. o. abyssus* venom. The Coa_NP2 presents an average molecular mass of 3419.88 Da (theoretical average molecular mass 3418.94 Da, monoisotopic molecular mass 3416.66 Da and theoretical PI 7.78) and its amino acid sequence presents the loop region that is characteristic of natriuretic peptides. The peptide has 32 amino acids and its complete sequence is SYGISSGCFGLKLDRIGTMSGLGCWRLLQDSP. Coa_NP2 is a natriuretic peptide of the ANP/BNP-like family, since the carboxyterminal region of CNP has its own NP domain. We demonstrate, herein, that Coa_NP2 produces a dose-dependent decrease in mean arterial pressure in rats, followed by significant increases in concentrations of markers of nitric oxide formation measured in the plasma and vasorelaxation in a thoracic aortic ring bath. The structural and biological aspects confirm Coa_NP2 as a new natriuretic peptide, isolated from snake venom.

1. Introduction

Snake venoms are protein mixtures that act in several physiological systems of their prey or victims. Some effects related to venomous snake bites can promote tissue damage, myotoxicity, hemorrhagic effects, and inflammation amongst others [2,6,8]. Many snake venoms contain toxins that produce interesting cardiovascular effects, such as hypotension, or bradykinin-potentiating peptides [15,28], or renal effects [10,39]. Natriuretic peptides (NPs) are body fluid volume modulators that play important roles in natriuresis and dieresis [23]. The three mammalian NPs, atrial natriuretic peptide (ANP), brain or b-type natriuretic peptide (BNP) and cardiac or c-type natriuretic peptide (CNP), have been extensively investigated for their use as therapeutic agents in the treatment of cardiovascular diseases [1,3,18,22,23,30,31].

Human NPs form a family of structural-similar polypeptides. They have a highly conserved 17-residue intra-molecular disulfide loop (CFGxxxDRIxxxSGLGC), which is important for their biological activity. Within this cyclic structure, nine amino acids are identical in all three classes of NPs. However, they differ from each

* Corresponding author at: Universidade Federal de São João Del Rei, Campus Alto Paraopeba, Rodovia MG 443 Km 7, Fazenda do Cadete, Caixa Postal 131, Ouro Branco CEP 36420-000, MG, Brazil.
** Corresponding author at: Cell Signaling and Nanobiotechnology Laboratory, Federal University of Minas Gerais – UFMG, Department of Biochemistry and Immunology, Institute of Biological Sciences, Av. Antônio Carlos, 6627, Pampulha, Caixa Postal 486, 31270-901 Belo Horizonte, MG, Brazil. Tel.: +55 013 3409 2627.
E-mail addresses: biomol2@hotmail.com (S.L. Da Silva), rrresende@hotmail.com (R.R. Resende).

0196-9781/
http://dx.doi.org/10.1016/j.peptides.2012.05.005

other in that they have different numbers of amino acid residues at the N- and C-terminal portion of the peptide [11,20,23]. In 1992, Schweitz et al. [33] identified the first venom NP from the green mamba snake, *Dendroaspis angusticeps*, and named it as the Dendroaspis natriuretic peptide (DNP). Although DNP shares similarity with ANP, it has a distinctly different C-terminal tail. Ho et al. [17] identified and characterized a NP from the South American coral snake, *Micrurus corallines*, and reported that it shows some similarities with DNP. Recently, another NP isolated from the venom of the *Lachesis muta* snake (Lm-CNP) was identified with a similar structure to human CNP [35]. In addition, a cDNA sequence of Bj-CNP from *Bothrops jararaca* has also been shown to encode a CNP-like NP and seven bradykinin potentiation [28]. On the other hand, knowledge regarding the composition of *Crotalus oreganus abyssus* venom is scarce [24]. From previous experiments in our laboratory in which we have studied the effects of peptides isolated from *C. o. abyssus* venom, we showed the presence of a natriuretic peptide in its venom (now called Coa_NP1) that produced hypotensive and vasorelaxation effects [5]. The aim of the present study was to identify and investigate the systemic and vascular effects of a new natriuretic peptide isolated from *C. o. abyssus* venom (Coa_NP2).

2. Materials and methods

All reagents were purchased from Aldrich or Sigma Co. (USA). *C. o. abyssus* (Coa) venom was obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University-Kingsville (Kingsville, TX, USA).

2.1. Isolation and purification of the natriuretic peptide from C. o. abyssuss

C. o. abyssus (Coa) whole venom was submitted to an FPLC molecular exclusion chromatographic column packed with Sephadex 75 (Akta Primer, GE, USA), exactly as described in [5]. In this separation, five peaks were observed (I–V) (Fig. 1A). Only fraction V presented a hypotensive effect. To perform a better study and for separation of peptides and proteins, fraction V was then submitted to ultra-filtration using the MidJet apparatus (Ge Healthcare, USA), equipped with the UFP-10-C-MM01A cartridge, and a superficial area of 26 cm^2, cut off: 10,000 Da (Ge Healthcare, USA). The filtrate presented hypotensive effects and was lyophilized and stored at −20 °C, until use.

The filtrate was subjected to reverse phase HPLC (model 2010, Shimadzu, Japan) using an analytical C5 column (Supelco, 250 mm × 4.6 mm), which was previously equilibrated with buffer A (0.1% TFA). The filtrate (10 mg) were dissolved completely in buffer A (0.1% TFA), centrifuged at 5000 × *g* and then loaded onto a reverse-phase column. The peptides were purified using a linear gradient of buffer B concentration (66% acetonitrile in buffer A) and the chromatographic UV monitoring was carried out at 216 nm [6,7]. For electrophoresis, Tricine PAGE-SDS was used for characterization of low molecular weight proteins and peptides [32]. Each peak or peak group was tested for its action on blood pressure and Coa_NP2 showed positive results.

2.2. Sequencing of the Coa_NP

Two milligrams of the purified peptide were dissolved in 200 μl of a 6 mol/l guanidine chloride solution containing 0.4 mol/l of Tris–HCl and 2 mmol/l EDTA (pH 8.15). Nitrogen was blown over the top of the protein solution for 15 min, before reducing with DTT (6 M, 200 μl). This solution was incubated in the dark at 37 °C for 1 h and desalted using a Sephadex G25 column (0.7 cm × 12 cm) with 1 mol/l acetic acid buffer. The reduced peptide was sequenced using an automatic peptide sequencer (890C automatic sequencer, Beckman, USA). The phenylthyoidantoin (PTH) amino acids were identified by comparing their retention times to the 20 PTH amino acid standards [2].

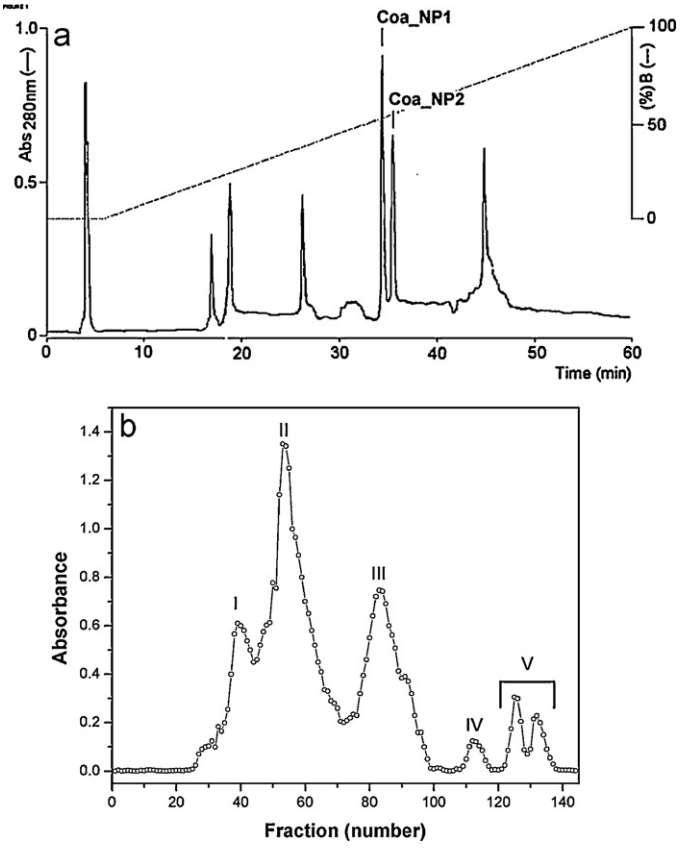

Fig. 1. (A) Chromatographic profile from the whole venom fraction of *C. o. abyssus* eluted by Sephedex G 75 FPLC molecular exclusion. (B) RP-HPLC (C5) chromatogram of filtrate from peak V from *C. o. abyssus*. The peaks containing the natriuretic peptide (Coa_NP1 and Coa_NP2) are indicated with an arrow.
Fig. 1 was modified from [21]

2.3. MALDI-TOF mass spectrometric analysis (MS)

The molecular mass of isolated Coa-NP was analyzed by MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF apparatus (Applied Biosystems, USA). One microliter of sample in 0.1% TFA was mixed with 2 μl of 3,5-dimethoxy-4-hydroxycinnamic acid (matrix sinapinic acid). The matrix was prepared with 30% acetonitrile and 0.1% TFA. Conditions of analysis: (1) acceleration of voltage 25 kV; (2) laser fixation at 2890 mJ/com2; (3) delay of 300 ns; and (4) linear analysis mode [2].

2.4. Animals: rats, conditions and ethical statements

Male Wistar rats (250–300 g) were used in this study and were maintained under specific pathogen-free conditions. The animals were housed in laminar-flow cages maintained at a temperature of 22 ± 2 °C and a relative humidity of 50–60%, under a 12:12 h light–dark cycle. Animal experiments were performed in accordance with the ethical guidelines of Helsinki Declaration (1975), the Institutional Animal Care and Use Ethical Committee of State University of Campinas (UNICAMP) and the Federal University of São João Del Rei (UFSJ), both Brazilian universities.

2.5. Blood pressure measurements

Male Wistar rats were anesthetized with 50 mg/kg pentobarbital and, thereafter, the right carotid artery was cannulated with a polyethylene tube (PE50), under anesthetic conditions (50 mg/kg of pentobarbital). Mean arterial pressure (MAP) was continuously recorded for 30 min using a pressure transducer (P23 Gould Statham, USA) connected to a polygraph (Narco Biosystems,). After this time, solutions used as controls and tests (Coa_NP2; 0.25 or 0.50 μg/ml) were injected (every 15 min) through a catheter implanted in the jugular vein Each measurement was compared with an isovolumetric injection of saline [10]. Mean arterial pressure (MAP) was calculated by the following formula:

$$\mathrm{MAP} = \frac{\mathrm{DP} + (\mathrm{SP} - \mathrm{DP})}{3}$$

where MAP is the mean arterial pressure; SP is the systolic pressure and DP is the diastolic pressure.

2.6. Aortic ring experiments

Male Wistar rats (250–300 g) were killed and the descending thoracic aorta was rapidly removed and flushed with physiological solution. After removal of adhering fat and connective tissue, 5 mm rings were obtained from preparations of endothelium-intact (e^+) and endothelium-denuded (e^-). This latter preparation was carried out by gently rubbing the vessel's lumen and mounting the aortic rings under 1 g resting tension, between two stainless steel hooks, in organ baths (37 °C, pH 7.4) and bubbling with a carbogenated (95% O_2 and 5% CO_2) physiological salt solution of the following composition (mM): NaCl: 118.4; $NaHCO_3$: 25; glucose: 11; KCl: 4.7; $MgSO_4$: 1.2; KH_2PO_4: 1.2; and $CaCl_2$: 2.5. The lower hook was attached to a tissue holder and the upper hook was connected to an isometric force displacement transducer (F-60, Narco Biosystems, Houston, TX, USA); the responses were recorded though a 4-channel polygraph (Narco BioSystems, TX, USA). The aortic rings were submitted to a tension of 1 g during a 60-min equilibration period and were considered to have an intact functional endothelium when acetylcholine (1 μmol/l) produced a relaxation of more than 80%. Relaxation was calculated as a percentage of the contraction induced by phenylephrine (PE; 0.1 μM) and concentration–response curves were performed for NP isolated by *C. o. abyssus*, Coa_NP2 (10^{-11} to 10^{-7}M), in both e^+ and e^- aortic rings. In another set of experiments, concentration–response curves for Coa_NP2 (10^{-11} to 10^{-7}M) were compared with phenylephrine precontracted endothelium-intact tissues in the absence or presence of ISATIN (1 μM, a potent guanylate cyclase-coupled atrial natriuretic peptide receptor type A antagonist) [13,25] and incubated for 30 min. In the last set of experiments, concentration–response curves for Coa_NP2 (10^{-11} to 10^{-7}M) were performed on aortic rings precontracted with isosmotic high-potassium (K^+ 80 mM) Krebs–Henseleit solution [10,12].

2.7. Determination of plasma NOx (nitrate + nitrite) concentrations

After the infusion of Coa_NP2 extracted from *C. o. abyssus* and the blood pressure assessment, levels of plasma nitrite were measured by colorimetric Griess methods. In these assays, 50 μl of the samples were incubated with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid). Nitrite levels were determined by comparison with a standard curve obtained by incubating sodium nitrate (10–200 μM) with reductase, buffered and measured at 550 nm in a multiwell plate reader (HIDEX, Shimadzu, Japan). The results were reported as micromolar concentrations (μM) of NO_2 [26].

2.8. Measurement of plasma nitrite concentrations

After the infusion of Coa_NP2 extracted from *C. o. abyssus* and the blood pressure assessment, levels of plasma nitrite were analyzed in duplicate for their nitrite content using an ozone-based reductive chemiluminescence assay as previously described [27]. Briefly, to measure nitrite concentrations in plasma, 100 μl of plasma samples were injected into a solution of acidified tri-iodide, purging with nitrogen in-line with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO). Approximately 8 ml of tri-iodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was placed in the purge vessel, into which plasma samples were injected. The tri-iodide solution reduces nitrites to NO gas, which is detected by the NO analyzer.

2.9. Theoretical model: computational methods

After the release of the primary sequence of Coa_NP2, a set of homology modeling studies was carried out in order to obtain tertiary structure information following a previously described protocol [14]. Initially, a template search was performed by SWISS-MODEL workspace, which identified several close homologue-resolved structures by SWISS-MODEL Template Library (SMTL). As the alignment between the target and the templates sequences showed high similarity, the automated mode was chosen to build the tridimensional structure target. The models were then refined with the AMBER 9.0 package. The models built were prepared using Leap and submitted to Sander software for geometry refinement. The models were fully optimized with 1000 steps of steep descent followed by 4000 steps of conjugate gradient. The minimization processes were performed with a cutoff value of 14 Å for non-bonded interactions, implicit solvent generalized Born model, and using a ff03 force field [9]. The figures for 3D structure were prepared using the Discovery Studio Visualizer v 2.5, Accelrys Software Inc. 2009.

2.10. Statistical analysis

Data were analyzed with Student's *t*-test for variance. Experimental values are expressed as means ± S.D. The level of statistical significance was set at a level of $p < 0.05$.

3. Results

3.1. Isolation and purification of the natriuretic peptide from C. o. abyssus

Fractionation of the whole venom using gel filtration (Sephadex G-75) produced the elution profile shown in [5]. After ultrafiltration (cartridge UFP-10-C-MM01A, GE Healthcare), the filtrate was analyzed by Tricine SDS-PAGE electrophoresis and presented protein and peptides bands with molecular masses of around or smaller than 10 kDa. The filtrate decreased blood pressure and was further purified by C5-HPLC (Fig. 1B). The RP-HPLC chromatography of filtrate demonstrated seven different peaks (or peak groups); all of these fractions were tested and just two showed activity by affecting blood pressure. One peak was identified as the Coa_NP1 (natriuretic peptide 1 from *C. o. abyssus*) described by [5]. The second peak selected was denominated as Coa_NP2 (Fig. 1B). Both peptides are identified in the RP-HPLC chromatogram shown in Fig. 1.

Table 1
Multiple alignments using the amino acid sequence of Coa_NP2 with other natriuretic peptides.

Consensus	CFG XXXDRI XXXSGLGC	Ref
Coa_NP2	SYGISSG**CFG**LKL **DRI**GTM **SGLGC**WRLLQDSP	
Coa_NP	SKRLSNG**CFG**LKL **DRI**GAM **SGLGC**WRLINESK	(1)
NP2_Cdc	VSTSRGSQG**CFG**LKL **DRI**GAA **SGLGC**WRRIVDS	(2)
BNP_Bj	EVKYDP**CFG**HKL **DRI**NEV **SGLGC**PSLRDPRPNAPSTSA	(3)
CNP_Bj	GAAKG**CFG**LKL **DRI**GTM **SGLGC**	(3)
CNP_Lm	RVGDG**CFG**LKL **DRI**GSM**SGLGC**	(4)
VNOC_Om2	SDPKIGDG**CFG**LPL **DRI**GSV **SGLGC**NRPVQNRPKK	(5)
VNOC_Om	SDSKIGNG**CFG**FPL **DRI**GSV **SGLGC**NRPVQNRPKK	(5)
H-ANP	SLRRSS**CFG**GRM**DRI**GAQ**SGLGC**NSFRYRR	(6)
H-BNP	SPKMVQGSG**CFG**RKM**DRI**SSS**SGLGC**KVLRRH	(6)
H-CNP	GLSKG**CFG**LKL **DRI**GSM**SGLGC**	(6)

(1) Coa_NP1 (natriuretic peptide 1 from *C. o. abyssus*) (Da Silva et al. [5]); (2) NP2_Cdc (natriuretic peptide from the *Crotalus durissus cascavella*) (Evangelista et al. [10]); (3) BNP_Bj (b-type natriuretic peptide from *Bothrops jararaca* gland) and CNP_Bj (c-type natriuretic peptide from *Bothrops jararaca* gland) (Hayashi and Camargo [15]); (4) CNP_Lmuta (*Lachesis muta* CNP) (Soares et al. [35]); (5) VNOC_Om and VNOC_Om2 (natriuretic peptide from the *Oxyuranus micropidotus*) (Graham et al. [41]); (6) H-ANP (human ANP), H-BNP (human BNP) and H-CNP (human CNP) (Schweitz et al. [33]).

The complete amino acid sequence of Coa_NP2 was carried out by Edman degradation (Table 1) and average molecular mass (3419.88 Da) was confirmed by mass spectrometry (Fig. 2). The theoretical average molecular mass was 3418.94 Da, monoisotopic molecular mass was 3416.66 Da and PI was 7.78.

The amino acid sequence of Coa_NP presented the loop region that is characteristic of natriuretic peptides (17 amino acids – NP domain consensus = CFGxxxDRIxxxSGLGC) and presented 8 amino acid residue extensions following the NP domain in the sequence (Table 1). The amino acid sequence of Coa_NP2 was identified as:

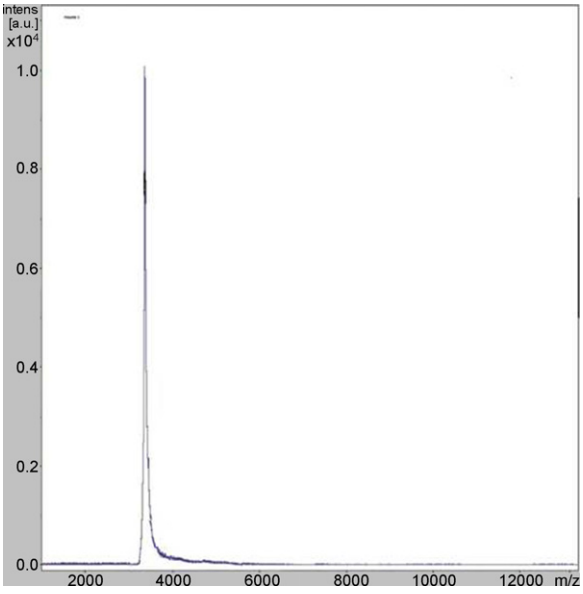

Fig. 2. The Coa_NP2 MALDI-TOF mass spectrometry profile. The average molecular mass determined is 3419.88 Da. The molecular mass of isolated Coa_NP2 from *C. o. abyssus* venom was analyzed using a Voyager-DE PRO MALDI-TOF. (Conditions of analysis: acceleration of voltage to 25 kV; laser fixed at 2890 mJ/com2; delay 300 ns; and linear analysis mode.)

SYGISSGCFGLKLDRIGTMSGLGCWRLLQDSP (underlined sequence represents the domain consensus of the NPs). As expected for natriuretic-like peptides, the primary structure revealed two half cysteines, suggesting the presence of one disulfide bridge (Table 1) and belongs to the ANP/BNP-like family, since the carboxyterminal regions of c-natriuretic peptides (CNP) end in NP domains. The experimental results obtained in this study support the hypothesis that Coa_NP2 is really a peptide of either the ANP or BNP families.

3.2. Rat blood pressure, nitric oxide plasma concentrations, and aortic rings natriuretic peptide isolated from C. o. abyssus venom (Coa_NP2) effects

The natriuretic peptide isolated from *C. o. abyssus* venom (Coa_NP2) caused a dose-dependent decrease in the median arterial pressure after its intravenous infusion (Fig. 3).

We observed an increase in the production of plasma NO*x* (nitrate + nitrite) concentrations after the infusion of the Coa_NP2, isolated from the *C. o. abyssus* venom (Fig. 4).

An increase in the production of plasma nitrite concentrations was also observed after Coa_NP2 infusion, isolated from the *C. o. abyssus* venom (Fig. 5).

Coa_NP2 induced a relaxant effect on endothelium-intact thoracic aortic rings that were precontracted with phenylephrine in the absence and presence of ISATIN. Coa_NP2 failed to relax endothelium-intact aortic rings that were precontracted with an isosmotic potassium Krebs–Henseleit solution (Fig. 6).

3.3. Theoretical model: computational methods

Unfortunately, when searching for homologous sequences (for the structural model of Coa_NP2), using the Blast tool, no adequate sequences for ANP or BNP were found stored in the RCSB Protein Data Bank. The best homology (95%) was achieved with a CNP complexed with its receptor (1JDP) [16]. To better understand our structure, we removed the receptor from the global complex and used the CNP (GLSKGCFGLKLDRIGSMSGLGC) as a

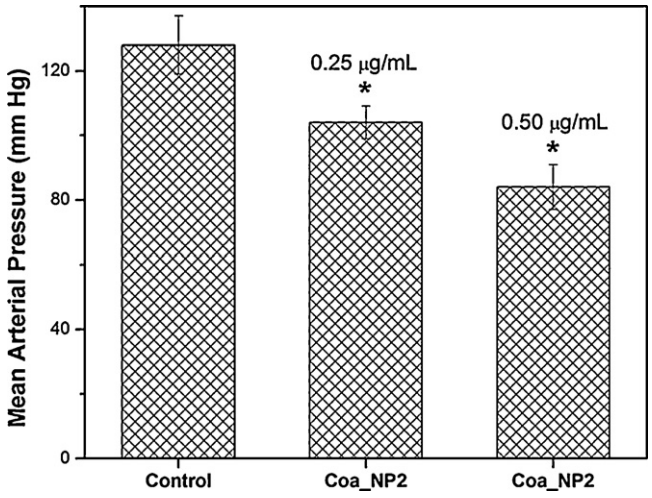

Fig. 3. Effects of two doses (0.25 μg/ml or 0.50 μg/ml) of natriuretic peptide 2 isolated from *C. o. abyssus* (Coa_NP2 venom) on mean arterial pressure (mmHg). Results are presented as the means ± S.D. of six animals in each group for three independent experiments. $^{*}p < 0.05$ for Coa_NP2 group compared to the control group.

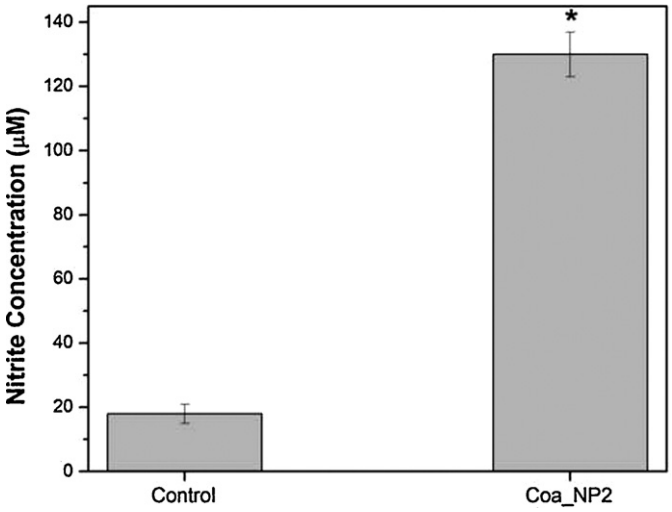

Fig. 4. Plasma NO*x* (nitrate + nitrite) concentrations (μM) after infusion of natriuretic peptide 2, isolated from *C. o. abyssus* (Coa_NP2 venom). $^{*}p < 0.05$ for Coa_NP2 group, compared to the control group.

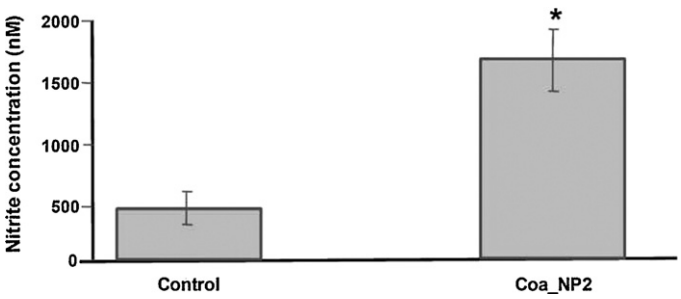

Fig. 5. Plasma nitrite concentrations (nM) after infusion of natriuretic peptide 2, isolated from *C. o. abyssus* (Coa_NP2 venom). $^{*}p < 0.05$ for Coa_NP2 group, compared to the control group.

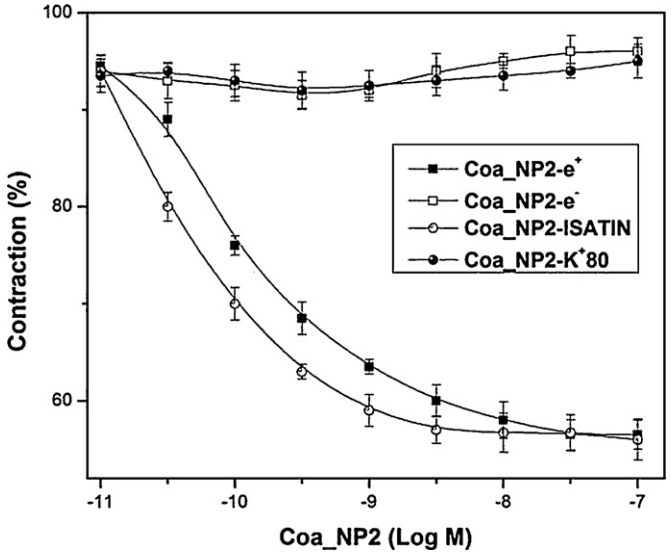

Fig. 6. Concentration–response curves (%) after infusion of natriuretic peptide 2 isolated from *C. o. abyssus* (Coa_NP2 venom) on intact-endothelium (e^+) or denuded-endothelium (e^-) thoracic aortic rings precontracted with phenylephrine (PE; 0.1 μM). The relaxant effect evoked by Coa_NP2 (10^{-11} to 10^{-7}M) was also compared on endothelium-intact tissues precontracted with PE (0.1 mM) and incubated with ISATIN (1 μM), a natriuretic receptor A antagonist, 15 min before performing concentration–response curves to Coa_NP2. The relaxant response was also evaluated in endothelium-intact rings precontracted with an isosmotic 80 mM potassium Krebs–Henseleit solution (K^+80). The results are expressed as the means ± S.D. of six independent experiments. $^{*}p < 0.05$ vs. Coa_NP2 intact endothelium group (Coa_NP2 e^+).

model, because its consensus domain is similar to that of the Coa_NP2 (SYGISSGCFGLKLDRIGTMSGLGCWRLLQDSP). After applying the methods of molecular modeling and energy refinement, the most probable structure for Coa_NP2 is shown in Fig. 7A.

The overlap of Coa_NP2 after refinement and use of the CNP model is shown in Fig. 7B. The structural differences (RMSD 1.79 Å) should be noted; these are probably related to the extensions of the portions C and N terminal Coa_NP2 in relation to CNP. The overlap (RMSD 0.54 Å) of Coa_NP1 and Coa_NP2 shows that both

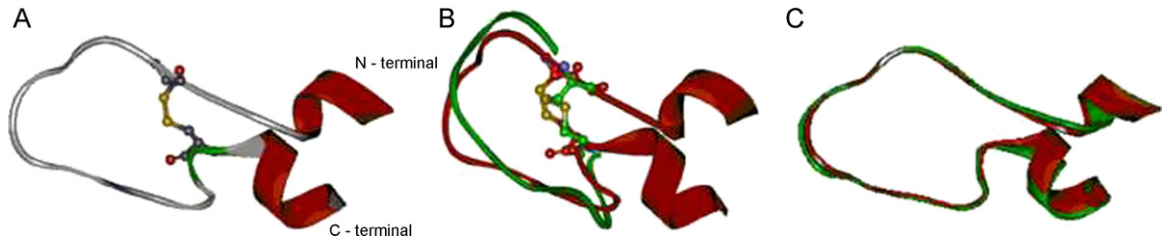

Fig. 7. (A) Structure of Coa_NP2 from *C. o. abyssus* venom. (B) Coa_NP (red) superimposed on CNP (Green). CNP template (RMSD 1.79 Å) was obtained from the RCSB Protein Data Bank (1JDP). The alignment between the target and the template sequences showed a high similarity (95%). The models were then refined with the AMBER 9.0 package (optimized with 1000 steps of steep descent followed by 4000 steps of conjugate gradient). (C) Coa_NP1 (red) superimposed on Coa_NP2 (Green). The calculated RMSD is 0.54 Å and this shows high similarity between both natriuretic peptides from *C. o. abyssus*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Aligment of Coa_NP1 and Coa_NP2 sequences. Both natriuretic peptides from *C. o. abyssus* venom presented approximately 90% of homology. The symbols denoted: (*) identical aminoacids and (:) homologous aminoacids.

Coa_NP1	SKRLSNGCFGLKLDRIGAMSGLGCWRLINESK
Coa_NP2	SYGISSGCFGLKLDRIGTMSGLGCWRLLQDSP
	* :* **********:*********:::*

molecules have very similar structures (Fig. 7C). The comparative analysis between the sequences of Coa_NP1 and Coa_NP2 show a 90% homology approximately (Table 2).

4. Discussion

This study describes the isolation of a new natriuretic peptide from *C. o. abyssus* venom (Coa_NP2), whose primary structure was determined as SYGISSGCFGLKLDRIGTMSGLGCWRLLQDSP. Its purity and average molecular mass were confirmed by mass spectrometry as being 3419.88 Da (Fig. 2) (the theoretical average molecular mass is 3418.94 Da, monoisotopic molecular mass is 3416.66 Da and PI is 7.78). The primary structure revealed two half cysteines, suggesting the presence of one disulfide bridge.

Tertiary structure of Coa_NP2 prevision, when compared to CNP (human), revealed a RMSD difference of 1.79 Å and this effect is probably caused by the extension of the C-terminal of Coa_NP2, but when compared to the structure of Coa_NP1 [5], the RMSD difference is only 0.54 Å. It was expected because Coa_NP1 and Coa_NP2 sequences have homologies of around 90%.

We found that the natriuretic peptide isolated from *C. o. abyssus* venom (Coa_NP2) presented a homologous structure to ANP and BNP. Furthermore, the mean functional findings of this present study were (i) the Coa_NP2 produced a dose-dependent decrease in the mean arterial pressure (Coa_NP2 infusion of 0.25 or 0.50 μg/ml; Fig. 3); (ii) this hypotensive effect occurred along with a significant increase of nitric oxide formation in plasma (Figs. 4 and 5); and (iii) the vasorelaxation produced by the natriuretic peptide, Coa_NP2, in thoracic aortic rings precontracted with phenylephrine was endothelium-dependent. As it was demonstrated by the vasorelaxation abolition in endothelium-denuded ring preparations (Fig. 6), and (iv) Coa_NP2 failed in induce aortic rings relax after precontract with 80 mM potassium (Fig. 6).

Snakes venoms contain peptides with structural and functional equivalents of mammalian NPs (ANP, BNP and CNP), which present dose-dependent hypotensive effects [10,34,40]. In addition to natriuretic peptide studies, a 38-amino acid residues peptide (DNP) was isolated from the venom of *Dendroaspis angusticeps* (the green mamba snake), demonstrating properties that are similar to both human ANP and BNP [33]. Other NPs from snake venoms were identified from *Lachesis muta* (Lm-CNP), *Bothrops jararacusu* (Bj-CNP) and other snakes presenting a homologous structure for the human CNP [28,35].

The hypotensive effect of Coa_NP2, presented herein, occurred in association with a significant increase in plasma nitrite levels, corroborating with previously data suggesting that NPs are able to stimulate nitric oxide (NO) production [4]. Together, a NO-dependent hypotensive effect was identified with a peptide isolated from the snake venom of *Agkistrodon acutus* [34], and it was shown that infusion of NP isolated from *Crotalus durissus cascavella* venom was responsible for the increased nitrite levels [10]. Thus, these findings support the notion that Coa_NP2 exerts its hypotensive action, at least in part, through stimulation of NO production.

As such, there are three different receptor isoforms for the NPs, namely, natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B), and natriuretic peptide receptor C (NPR-C), in which the human NP family have been shown natriuretic, diuretic, hypotensive and vasodilator actions [20,22]. It has recently been suggested that BNP exerts its vascular effects through the same pathway as ANP, i.e. the NPR-A. This guanylate cyclase-coupled receptor is located both on endothelial and vascular smooth muscle cells [37]. Activation of NPR-A generates the second messenger cyclic guanosine monophosphate (cGMP) which, in turns, activates Ca^{2+} channels and ATP-sensitive K^+-channels leading to vasorelaxation [21,29]. However, CNP binds to the NPR-B, a specific guanylate cyclase-coupled receptor, and it is located on the vascular smooth muscle cell, also leading to vasodilatation through hyperpolarization [19].

To evaluate the possible mechanisms responsible for these dose-dependent hypotensive effects, we used endothelium-denuded rings preparations (Coa_NP2-e$^-$). It was observed that vasorelaxation produced by the Coa_NP2 in thoracic aortic rings precontracted with phenylephrine was endothelium-dependent, as evidenced by its abolition when it was used Coa_NP2-e$^-$. (Coa_NP2-e$^+$ or Coa_NP2-e$^-$ group, respectively, Fig. 5). Similar findings were revealed by other NPs originated from different snake venoms [10,38].

The vasorelaxant effect caused by Coa_NP2 seems not to be involved in the NP receptor type A (NPR-A). As the action verified of the Coa_NP2 is not modified by addition of ISATIN (a potent guanylate cyclase-coupled NPR-A antagonist) in the aortae bath (Fig. 6), the failure of Coa_NP2 to relax aortic rings precontracted with 80 mM potassium suggested a possible role for voltage-dependent ion channels that may include potassium channels; however, the primary mediator could be calcium influx, which activates a calcium-activated potassium channel and/or NO release [13]. Supporting this affirmation, the potassium-channel blocker, tetraethylammonium has been found to reduce the BNP-induced dilatation of brachial humans arteries [36]. As such, our findings demonstrate that the hypotension and vasodilatation caused by Coa_NP2 is consistent with the hypothesis that both NPR-B pathways activate and stimulate NO production in parallel.

In conclusion, we isolated and characterized a new NP-like peptide from *C. o. abyssus* venom (Coa_NP2), and we also report a dose-dependent hypotensive effect of this peptide in association with increased nitrite production, as well as vasodilatory endothelium-dependent effects. Therefore, these data suggest that the NO-release dependent vasodilator action of Coa_NP2 may occur by stimulation of potassium channels.

Conflicts of interest

The authors report no conflicts of interest in this work.

Acknowledgments

We would like to thank CAPES, CNPq, FAPEMIG and FAPESP (Brazilian agencies) for financial support.

References

[1] Almenar Bonet L, Martinez-Dolz L. Natriuretic peptides in heart failure. Rev Esp Cardiol 2006;6(Suppl. F):15–26.
[2] Calgarotto AK, Damico DC, Ponce-Soto LA, Baldasso PA, Da Silva SL, Souza GH, et al. Biological and biochemical characterization of new basic phospholipase A(2) BmTX-I isolated from *Bothrops moojeni* snake venom. Toxicon 2008;51:1509–19.
[3] Cardarelli R, Lumicao Jr TG. B-type natriuretic peptide: a review of its diagnostic, prognostic, and therapeutic monitoring value in heart failure for primary care physicians. J Am Board Fam Pract 2003;16:327–33.
[4] Chatterjee PK, Hawksworth GM, McLay JS. Cytokine-stimulated nitric oxide production in the human renal proximal tubule and its modulation by natriuretic peptides: a novel immunomodulatory mechanism. Exp Nephrol 1999;7:438–48.
[5] Da Silva SL, Almeida JR, Resende LM, Martins W, Henriques FA, Baldasso PA, et al. Isolation and characterization of a natriuretic peptide from *Crotalus oreganus*

abyssus (Grand Canyon Rattlesnake) and its effects on systemic blood pressure and nitrite levels. Int J Peptide Res Ther 2011;17:165–73.
[6] Da Silva SL, Calgarotto AK, Chaar JS, Marangoni S. Isolation and characterization of ellagic acid derivatives isolated from *Casearia sylvestris* SW aqueous extract with anti-PLA(2) activity. Toxicon 2008;52:655–66.
[7] Da Silva SL, Calgarotto AK, Maso V, Damico DC, Baldasso P, Veber CL, et al. Molecular modeling and inhibition of phospholipase A2 by polyhydroxy phenolic compounds. Eur J Med Chem 2009;44:312–21.
[8] de Alvarenga ES, Silva SA, Barosa LC, Demuner AJ, Parreira AG, Ribeiro RI, et al. Synthesis and evaluation of sesquiterpene lactone inhibitors of phospholipase A2 from *Bothrops jararacussu*. Toxicon 2011;57:100–8.
[9] Duan Y, Wu C, Chowdhury S, Lee MC, Xiong G, Zhang W, et al. A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. J Comput Chem 2003;24:1999–2012.
[10] Evangelista JS, Martins AM, Nascimento NR, Sousa CM, Alves RS, Toyama DO, et al. Renal and vascular effects of the natriuretic peptide isolated from *Crotalus durissus cascavella* venom. Toxicon 2008;52:737–44.
[11] Fry BG, Roelants K, Champagne DE, Scheib H, Tyndall JD, King GF, et al. The toxicogenomic multiverse: convergent recruitment of proteins into animal venoms. Annu Rev Genomics Hum Genet 2009;10:483–511.
[12] Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373–6.
[13] Glover V, Medvedev A, Sandler M. Isatin is a potent endogenous antagonist of guanylate cyclase-coupled atrial natriuretic peptide receptors. Life Science 1995;57:2073–9.
[14] Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 1997;18:2714–23.
[15] Hayashi MA, Camargo AC. The bradykinin-potentiating peptides from venom gland and brain of *Bothrops jararaca* contain highly site specific inhibitors of the somatic angiotensin-converting enzyme. Toxicon 2005;45:1163–70.
[16] He X, Chow D, Martick MM, Garcia KC. Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone. Science 2001;293: 1657–62.
[17] Ho PL, Soares MB, Maack T, Gimenez I, Puorto G, Furtado MF, et al. Cloning of an unusual natriuretic peptide from the South American coral snake *Micrurus corallinus*. Eur J Biochem 1997;250:144–9.
[18] Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A. Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. Circulation 2004;110:1231–5.
[19] Honing ML, Smits P, Morrison PJ, Burnett Jr JC, Rabelink TJ. C-type natriuretic peptide-induced vasodilation is dependent on hyperpolarization in human forearm resistance vessels. Hypertension 2001;37:1179–83.
[20] Joseph R, Pahari S, Hodgson WC, Kini RM. Hypotensive agents from snake venoms. Curr Drug Targets Cardiovasc Haematol Disord 2004;4:437–59.
[21] Kubo M, Nakaya Y, Matsuoka S, Saito K, Kuroda Y. Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K+ channels in cultured vascular smooth muscle cells. Circ Res 1994;74:471–6.
[22] Lee CY, Burnett Jr JC. Natriuretic peptides and therapeutic applications. Heart Fail Rev 2007;12:131–42.
[23] Levin ER, Gardner DG, Samson WK. Natriuretic peptides. N Engl J Med 1998;339:321–8.
[24] Mackessy SP. Evolutionary trends in venom composition in the western rattlesnakes (*Crotalus viridis sensu lato*): toxicity vs. tenderizers. Toxicon 2010;55:1463–74.
[25] Medvedev A, Buneeva O, Glover V. Biological targets for isatin and its analogues: implications for therapy. Biologics 2007;1:151–62.
[26] Montenegro MF, Neto-Neves EM, Dias-Junior CA, Ceron CS, Castro MM, Gomes VA, et al. Quercetin restores plasma nitrite and nitroso species levels in renovascular hypertension. Naunyn Schmiedebergs Arch Pharmacol 2010;382:293–301.
[27] Montenegro MF, Pessa LR, Gomes VA, Desta Z, Flockhart DA, Tanus-Santos JE. Assessment of vascular effects of tamoxifen and its metabolites on the rat perfused hindquarter vascular bed. Basic Clin Pharmacol Toxicol 2009;104:400–7.
[28] Murayama N, Hayashi MA, Ohi H, Ferreira LA, Hermann VV, Saito H, et al. Cloning and sequence analysis of a *Bothrops jararaca* cDNA encoding a precursor of seven bradykinin-potentiating peptides and a C-type natriuretic peptide. Proc Natl Acad Sci U S A 1997;94:1189–93.
[29] Price JM, Hellermann A. Inhibition of cGMP mediated relaxation in small rat coronary arteries by block of CA++ activated K+ channels. Life Science 1997;61:1185–92.
[30] Rose RA, Giles WR. Natriuretic peptide C receptor signalling in the heart and vasculature. J Physiol 2008;586:353–66.
[31] Saito Y. Roles of atrial natriuretic peptide and its therapeutic use. J Cardiol 2010;56:262–70.
[32] Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.
[33] Schweitz H, Vigne P, Moinier D, Frelin C, Lazdunski M. A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). J Biol Chem 1992;267:13928–32.
[34] Shen D, Xu X, Zhang L, Wu H, Peng L. Identification of a nitric oxide-dependent hypotensive effect of anticoagulation factor II from the venom of *Agkistrodon acutus*. Biochem Pharmacol 2010;79:498–506.
[35] Soares MR, Oliveira-Carvalho AL, Wermelinger LS, Zingali RB, Ho PL, Junqueira-de-Azevedo IL, et al. Identification of novel bradykinin-potentiating peptides and C-type natriuretic peptide from *Lachesis muta* venom. Toxicon 2005;46:31–8.
[36] van der Zander K, Houben AJ, Kroon AA, De Mey JG, Smits PA, de Leeuw PW. Nitric oxide and potassium channels are involved in brain natriuretic peptide induced vasodilatation in man. J Hypertens 2002;20:493–9.
[37] Woodard GE, Rosado JA. Natriuretic peptides in vascular physiology and pathology. Int Rev Cell Mol Biol 2008;268:59–93.
[38] Yamazaki Y, Morita T. Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. Curr Pharm Des 2007;13:2872–86.
[39] Yuan K, Jin X, Gao S, Shah A, Kim SY, Kim SZ, et al. Osmoregulation of natriuretic peptide receptors in bromoethylamine-treated rat kidney. Peptides 2009;30:1137–43.
[40] Zhang Y, Wu J, Yu G, Chen Z, Zhou X, Zhu S, et al. A novel natriuretic peptide from the cobra venom. Toxicon 2011;57(1):134–40.
[41] Graham RL, Graham C, McClean S, Chen T, O'Rourke M, Hirst D, et al. Identification and functional analysis of a novel bradykinin inhibitory peptide in the venoms of New World Crotalinae pit vipers. Biochem Biophys Res Commun 2005;338(3):1587–92.