Identification of snake bradykinin-potentiating peptides (BPPs)-simile sequences in rat brain – Potential BPP-like precursor protein?

Joana D'Arc Campeiro [a], Izabella P. Neshich [b], Osvaldo A. Sant'Anna [c], Robson Lopes [d], Danielle Ianzer [d,1], Marina T. Assakura [d], Goran Neshich [b], Mirian A.F. Hayashi [a,*]

[a] Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil
[b] EMBRAPA Informática Agropecuária, Computational Biology Research Group, Campinas, Brazil
[c] Laboratory of Immunochemistry, Instituto Butantan, São Paulo, Brazil
[d] Center for Applied Toxicology, Instituto Butantan, São Paulo, Brazil

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ABSTRACT

Bradykinin-potentiating peptides (BPPs) from the South American pit viper snake venom were the first natural inhibitors of the human angiotensin I-converting enzyme (ACE) described. The pioneer characterization of the BPPs precursor from the snake venom glands by our group showed for the first time the presence of the C-type natriuretic peptide (CNP) in this same viper precursor protein. The confirmation of the BPP/CNP expression in snake brain regions correlated with neuroendocrine functions stimulated us to pursue the physiological correlates of these vasoactive peptides in mammals. Notably, several snake toxins were shown to have endogenous physiological correlates in mammals. In the present work, we expressed in bacteria the BPPs domain of the snake venom gland precursor protein, and this purified recombinant protein was used to raise specific polyclonal anti-BPPs antibodies. The correspondent single protein band immune-recognized in adult rat brain cytosol was isolated by 2D-SDS/PAGE and/or HPLC, before characterization by MS fingerprint analysis, which identified this protein as superoxide dismutase (SOD, EC 1.15.1.1), a classically known enzyme with antioxidant activity and important roles in the blood pressure modulation. *In silico* analysis showed the exposition of the BPP-like peptide sequences on the surface of the 3D structure of rat SOD. These peptides were chemically synthesized to show the BPP-like biological activities in *ex vivo* and *in vivo* pharmacological bioassays. Taken together, our data suggest that SOD protein have the potential to be a source for putative BPP-like bioactive peptides, which once released may contribute to the blood pressure control in mammals.

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1. Introduction

Extreme low levels of blood pressure (BP) can determine an insufficient blood flow for adequate nutrition of all tissues of an organism body. On the other hand, chronic severe high BP can overload the heart, accelerate the aging process of the arteries, and increase the risk of stroke, and consequently the maintenance of adequate BP is highly desirable [1]. BP is controlled by several systems, including the central nervous system (CNS) mechanisms, which control the cardiac chronotropy and inotropy, and also vasomotion, mediated by autonomic nervous supplies to organs. In special, the sympathetic outflow to microvasculature is a key regulator of the vascular resistance, which is important to maintain the BP levels [2,3].

The availability of reactive oxygen species (ROS) in neurons located in brain areas involved in the control of sympathetic

activity is an important factor for the maintenance of the peripheral sympathetic tone and BP control in health and disease [4–6]. The oxidative stress is the result of excessive generation of oxidizing agents, and the inadequate removal of ROS and consequent imbalance between the antioxidant and the pro-oxidant mechanisms were shown to be involved in the pathogenesis of hypertension [7–9]. Any molecule presenting oxygen with a high oxidative capacity is considered as potential ROS, as for instance the superoxide ion (O_2^-), the hydroxyl radical (OH^-), and the hydrogen peroxide (H_2O_2). The identification of specific activators and inhibitors of ROS is a challenge for a better understanding of the underlying mechanism(s) of action involved in the central BP control, besides having the potential value for the development of new treatment strategies and representing today a major target chosen by the pharmaceutical industry to develop new drugs for several diseases [10].

Angiotensin II (Ang II) also controls vascular tone, growth and apoptosis by multiple mechanisms, including the signaling mediated by ROS [11,12]. Ang II has hypertensive activity by promoting vasoconstriction [13] as well by acting on oxidative stress [14,15]. Therefore, Ang II decapeptide antagonizes the effects of the bradykinin (BK), which is a vasodilator peptide [16]. Displaying contradictory actions, these humoral factors are both crucial for the regulation of BP, and they are both modulated by the action of the angiotensin I-converting enzyme (ACE), a key enzyme for the renin–angiotensin system (RAS) [17]. The first reported natural inhibitors of ACE, coined as BK-potentiating peptides (BPPs), were originally identified in the venom of a South American pit viper [18,19]. They not only contributed to the development of the first site-directed inhibitor drug captopril used in humans since a long time [18–22], as they were also essential for the discovery of the humoral peptide BK [23], which is also know to play roles in the CNS [24]. BPPs were therefore the inspiring template and the perfect basis for the development of the antihypertensive drug captopril, which may act primarily decreasing the production of the hypertensive peptide Ang II and decreasing the degradation of the hypotensive peptide BK [18–21]. However, it is of worth mentioning that the effects of captopril drug class on oxidative stress markers and antioxidant enzymes have also been suggested more recently [25]. Moreover, it is also well known that BK action through BK B2 receptor induces NO release and upregulates the activity and expression of the antioxidants Cu/Zn-SOD and Mn-SOD, with subsequent inhibition of ROS production and suppression of the oxidative stress [26].

The cloning and sequence analysis of the cDNA encoding the BPPs precursor from the venom gland of the South American pit viper *Bothrops jararaca* (*Bj*) revealed that this precursor protein is composed by seven tandem aligned BPPs forming a 'rosary-type' structure, followed by a C-type natriuretic peptide (CNP). Natriuretic peptides (NPs) also play a fundamental role in the cardiovascular homeostasis by modulating the fluid and electrolyte balance, and the vascular tone [27]. Interestingly, the NPs were also recently suggested to be the natural antagonists of the BK receptor-signaling pathway, acting *via* regulation of G-protein signaling proteins [28]. The expression of BPPs/CNP precursor in snake tissues, besides the gland venom (namely spleen, pancreas and brain) was demonstrated by our group in late 90s [29,30], and this stimulated us to suggest that these peptides were endogenously expressed by snakes, and that they could also be expressed in mammals. In addition, the cloning and sequencing of the BPPs precursor cDNA from *Bj* brain not only confirmed the co-expression of the CNP in the same precursor protein in the CNS in the same was as in the venom gland, as well as *in situ* hybridization studies showed the expression of BPPs/CNP precursor in snake CNS regions associated to the regulation of neuroendocrine functions [30]. In addition, we believe that the recent demonstration of the effects of snake BPPs on central BP control of rats reinforces the hypothesis of the BPPs/CNP roles as neuropeptide hormones [31–33].

Many isoforms of BPPs isolated from the venom glands of several snakes, and also from other venomous animals such as scorpions, and spiders, also have the ability to potentiate the hypotensive effect of BK [34,35]. However, although unavoidably displaying the canonical proline-rich domain and the pyro-Glu residue at the C- and N-terminus extremities, respectively, not necessarily all BPP-like peptides are effective BK-potentiator and/or have the ability to decrease the BP or inhibit ACE activity *in vitro* [34–37]. As some potential BPP-like precursor proteins from other snakes [34,35] or identified based on primary structural similarity searches in the human genome database did not necessarily show BK-potentiating activity (data not shown).

In this work, we describe for the first time the recombinant expression of the BPPs domain of the snake venom gland precursor protein in bacteria. This recombinant protein was characterized and used to raise highly specific polyclonal antibody against the BPPs (anti-BPPs). The anti-BPPs antiserum was able to recognize both the natural and recombinant snake BPPs precursor protein, as well as they were also capable to recognize a single protein band in the homogenates of both snake venom gland and adult rat brain, as demonstrated by Western blot (WB) assays. This cytosolic protein from rat brain was immune-traced during the chromatography purification steps, and it was biochemically isolated and characterized, allowing the identification of a protein with well-known key roles in the regulation of BP and ROS pathway. The possible structural and/or functional relationships between the BPPs and this rat brain cytosolic protein, led us to suggest this protein as a potential precursor for endogenous BPP-like peptides in mammals, as it will be discussed herein.

2. Materials and methods

2.1. Chemicals and drugs

HPLC grade acetonitrile (AcN), trifluoroacetic acid (TFA) and all other chemical reagents were from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA), captopril and bradykinin (BK), as well any other chemical not specified in the text, were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MI, USA) including the amino acids and other reagents for N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The skim milk used was MOLICO® (Nestlé Brasil Ltda., SP, Brazil).

2.2. Animals

Genetically selected high antibody producer mice [H_{III} mice] [38] were used for the production of the antibodies, and they were maintained at the animal facilities of the Special Laboratory of Microbiology of the Instituto Butantan (SP, Brazil). Male guinea pig (180–200 g body weight) and male Wistar rats (250–280 g body weight) were bred in animal care facility of the Instituto Butantan (SP, Brazil). Male spontaneously hypertensive rats (SHRs) (250–280 g body weight) were bred in animal care facility of the Biological Science Institute, University of Sao Paulo (SP, Brazil). The animals had free access to food and water and were submitted to a light/dark cycle (12 h each) before the preparation for the experiments. All animals were caged and handled under ethical conditions according to international rules of animal care, stated by the International Animal Welfare Recommendations [39], and in accordance with the guidelines established by our local institutional animal welfare committee (CEUAIB/Instituto Butantan, protocol N° 443/2008).

2.3. Expression of the recombinant protein

The recombinant bradykinin-potentiating peptide (BPP) was expressed by subcloning into the expression vector pProEx-HT (Invitrogen/Life Technology, Carlsbad, CA, USA) the BPP precursor protein cDNA fragment coding for the domain containing the seven BPPs. Escherichia coli BL21(DE3) strain was then transformed with this expression vector and, after amplification of this clone, the expression was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) followed by incubation at 37 °C for 4 h with agitation at 200 rpm. The bacteria were recovered from this culture by centrifugation and the bacteria pellet was frozen at −20 °C before sonication, recovery, and purification of the recombinant protein by affinity chromatography with Ni-NTA agarose (Qiagen, Hilden, Germany). The obtained sample was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) followed by Coomassie Blue and/or silver staining or followed by Western blot (WB) analysis. To assure a higher purity, the obtained recombinant protein was further subjected to a preparative SDS/PAGE, and the protein band of interest was then cutout and electro-eluted from the gel before the primary sequence of the purified protein was confirmed by MALDI-TOF mass spectrometry (TofSpec-E, Micromass, Waters Corp., Milford, MA, USA).

2.4. Immunological assays

2.4.1. Production of specific antibodies

Twenty μg of recombinant BPP precursor protein emulsified with Incomplete Freund's Adjuvant (IFA) was given to each H$_{III}$ Balb/C mice divided into three batches of six 4-weeks old females. In the first batch, the emulsified sample was injected subcutaneously (SC). Subsequent boosters were given biweekly at the same dose by intraperitoneal (IP) immunizations. The second batch was initiated 44 days after the first application of the first batch. Only 5 of 6 mice received a dose SC, one of the mice was used as controls. The following applications were performed every month by SC injection.

Testing another immunization protocol, the third batch, was immunized SC. For this batch, the following applications were made every three months by SC injection. After every bled, the sera were collected after centrifugation and stored at −20 °C until use.

2.4.2. Determination of the antibody titers of antiserum

The recombinant BPP precursor protein was used as antigen for the determination of the antibody titers of antiserum by enzyme-linked immunosorbent assay (ELISA). Initially, 5 μg/mL of purified recombinant BPP precursor protein was added to 96-wells plate (Costar #3690, Corning, NY, USA), which was incubated at 4 °C overnight for antigen fixation. BSA was also used to increase the fixation on plate, and also served as negative control. Unbound antigen was removed and the plate was incubated at 37 °C for 1 h with the blocking solution (3% of skim milk in phosphate-buffered saline – PBS). Then, the blocking solution was replaced with a serial two-fold dilution of serum, starting at an appropriate dilution for each serum. The pre-immune serum was also added in appropriate dilution and used as control of binding. Wells were washed 6 times with PBS to remove unbound antibodies, and a 1/5000 dilution of alkaline phosphatase conjugated anti-mouse-IgG (H + L) (Promega, Madison, WI, USA) was used as secondary antibody. Then the plate was incubated at 37 °C for 1 h with 1 mg/mL of p-nitrophenylphosphate in 1.5 M NaCl and 0.1 mM Tris pH 9.8, as substrate, for the color development. The reaction was stopped by the addition of 2 mol/L NaOH, and the absorbance was read at 405 nm using a SpectraMax 1900 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). The antibody titers were expressed as the log$_2$ of maximum antiserum dilution giving an absorbance twice as high as the absorbance of the control. The control corresponds to addition of pre-immune serum instead of antiserum dilution.

Immunoglobulin isotypes IgG1 and IgG2a were evaluated from antisera of mice primed with carrier protein and unprimed mice. For this purpose, 1:2000 dilution of alkaline phosphatase conjugated rat anti-mouse-IgG1 or anti-mouse IgG2a (BD Biosciences Pharmigen, San Diego, CA, USA) was used as secondary antibody. Antibody titer was determined as described above.

2.4.3. Western blot of cytosol of rat tissues using the anti-BPPs

After electrophoresis on SDS/PAGE, samples were transferred by electrophoresis onto a nitrocellulose membrane using transfer buffer (25 mM Tris–HCl pH 8.3, 0.2 M glycine, 20% methanol), under constant voltage of 30 V for 16 h. Membrane was then blocked with a buffer containing 5% solution of BSA in TBST buffer [150 mM NaCl, 20 mM Tris–HCl pH 7.5, and 0.05% Tween-20], for 1 h, followed by three washes with TBST. Membrane was incubated with the primary antibody (anti-BPPs) diluted in TBST for 1 h. After this period, the membrane was washed with TBST solution three times for 10 min each, on shaker at room temperature (25 °C). After washing, membrane was incubated with the secondary antibody anti-mouse IgG conjugated with alkaline phosphatase (Promega) diluted 1:7500 in TBST buffer for 1 h. After incubation, membrane was washed three times in TBST buffer for 10 min each. Finally, the proteins bands were revealed with a buffer containing 0.10 M Tris–HCl pH 9.5, 0.02 M NaCl, 0.005 M MgCl$_2$ containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

2.5. Preparation of rat brain cytosol proteins and separation by chromatography

2.5.1. Preparation of cytosolic extract from rat tissues

Wistar rats were anesthetized, heparinized and subjected to intracardiac perfusion with saline 0.9% NaCl. Brain tissue was homogenized with 3 volumes (v/w) of buffer containing 0.1 M Tris–HCl pH 7.5 and 0.25 M sucrose. The samples were then centrifuged at 3000 rpm for 15 min at 4 °C. Each supernatant was ultra-centrifuged tube at 100,000 × g for 1 h at 4 °C. An aliquot of the cytosol obtained was submitted to qualitative analysis through electrophoresis in SDS/PAGE, and the remainder was freeze-dried or concentrated in a speed-vac (Savant Instruments Inc., Holbrook, NY, USA) and stored at 4 °C for later use.

2.5.2. Isolation of proteins from rat brain cytosol by gel filtration

Cytosolic fraction of rat brain was obtained as described above. An aliquot of 3.0 mL of this sample was applied to a column chromatography Sephadex G-50 (3.5 cm × 93.0 cm) in order to obtain a separation of the fractions containing the proteins recognized by anti-BPPs. Fractions were eluted with the buffer 0.2 M ammonium acetate pH 6.3. We collected 5 mL fractions and absorbance reading at 280 nm was monitored in the spectrophotometer. The fractions containing protein were selected and analyzed through SDS/PAGE and WB using anti-BPPs.

2.5.3. Isolation of rat brain proteins between 10 and 100 kDa

The chromatography fractions containing the protein of interest (revealed by WB) were pooled. Each pool formed was concentrated using filtration system (CENTRICON, Amicon, Millipore Corporation, Billerica, MA, USA), primarily with 100 kDa membrane, and then with 10 kDa membrane. Further analysis in SDS/PAGE and WB were performed to verify the losses of target protein during the filtration process.

2.5.4. Isolation and purification of the cytosol fractions of rat brain by 2D SDS/PAGE or liquid chromatography

For 2D SDS/PAGE, the first-dimension isoelectrophoresis was performed in IPGphor (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to manufacturer's using a using a pre-cast immobilized pH 3–10 strip, and after the isoelectric focusing (IEF), the samples were further resolved in a second dimension by SDS/PAGE 12.5%, prepared in duplicate. After electrophoresis, one gel was stained with Coomassie Blue while other was transferred by electrophoresis onto a nitrocellulose membrane as described above (Section 2.4.3). The protein spot of interest was then cutout and digested overnight with trypsin for mass spectrometry (MS) analysis.

High performance liquid chromatography (HPLC) was performed using a Shimadzu (Shimadzu Corp., Kyoto, Japan) system (model CBM-101), equipped with two pumps (model LC-10Advp), Rheodyne injector (model 7725i) with loop injection of 100 μL, UV-visible detector (model SPD 10AV) coupled with an analytical C-18 column (Ultrasphere ODS, Beckman – 4.6 mm × 250 mm/Ø 5 mm). The sample was resuspended in solvent A (0.1% TFA/H$_2$O) and loaded into the system. The fractions were eluted in a gradient of 25–75%, solvent B (AcN/TFA 0.1%) over 30 min according to their absorbance at 214 nm, under a constant flow rate of 1 mL/min. Each peak was independently collected and analyzed by one dimension SDS/PAGE, prepared in duplicate. After electrophoresis, one gel was stained with Coomassie Blue while other was transferred by electrophoresis onto a nitrocellulose membrane as described above (Section 2.4.3). The protein band of interest was then cutout and digested overnight with trypsin for mass spectrometry (MS) analysis.

2.6. Peptide synthesis

Peptides synthesis was performed on an automated PSSM-8 peptide synthesizer (Shimadzu Corp.), by a stepwise solid-phase method, using N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry [40]. Cleavage of the peptide from the resin was achieved by treatment with a mixture of TFA/1,2-ethanedithiol/ethyl methyl sulfide for 2 h at room temperature. The resin was removed by filtration and twice washing with TFA. The crude synthetic peptide was purified by preparative reversed-phase HPLC (Shimadzu Corp.) on YMC-Pack ODS column (20 mm × 150 mm; YMC, Kyoto, Japan), using a linear gradient from 3% to 20% AcN in 0.1% TFA, at a flow rate of 5 mL/min. Both the purity and primary structure of each synthetic peptide were confirmed by analytical HPLC and MALDI-TOF mass spectrometry (TofSpec-E, Micromass, Waters Corp., Milford, MA, USA). Samples were frozen in liquid nitrogen and freeze dried for 48 h, at −50 °C, under vacuum (Edwards Freeze Dryer Super Modulyo Pirani 1001, Thermo Fisher Scientific, Waltham, MA).

2.7. Biological assays for synthetic peptides

2.7.1. Contractile effect on smooth muscles

Potentiating activity of the putative peptides of superoxide dismutase (SOD) on the contractile effect of bradykinin (BK) on smooth muscle was measured using preparations of isolated guinea pig ileum. After 18–24 h fasting period, approximately the distal ileum of guinea pigs was removed immediately after death and washed thoroughly with Tyrode's solution (137 mM NaCl, 2.70 mM KCl, 1.36 mM CaCl$_2$, 0.49 mM MgCl$_2$, 0.36 mM NaH$_2$PO$_4$, 11.9 mM NaHCO$_3$, 5.04 mM D-glucose). Cut segments of 2.0 cm of the isolated ileum were mounted at isotonic condition, under 1 g load, in 5 mL of muscle bath containing Tyrode's solution maintained at 37 °C and bubbled with air.

2.7.2. Systolic blood pressure (BP) recording in anaesthetized rats

BK-potentiation on systolic blood pressure (BP) recording in anaesthetized rats assays were performed as previously described [41]. Before the experiment, male Wistar rats (250–300 g) were anesthetized with urethane 12% (1.0 mL/100 g weight body) IP, and a polyethylene catheter (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery for arterial pressure measurements. For intravenous (IV) bolus injections, polyethylene catheter was implanted into the femoral vein. The cannulas were closed by a metallic pin and filled with isotonic saline solution. Pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were continuously monitored by a solid-state strain gauge transducer connected to a computer using a data acquisition system (MP 100; BIOPAC Systems Inc., Santa Barbara, CA). The rats were kept anesthetized during the experiments. After BP stabilization, the BK hypotensive response on BP was standardized using doses of 0.5 and 1.0 μg. For testing and comparisons of the effect of injection of the synthetic peptides, different doses (30, 60 and 120 nmol) of SODa and 60 nmol of SODb peptide were injected in bolus, in each animal, followed by 0.5 μg of BK injections in 5, 10, 15, 20, 25 and 30 min. The number of animals (N) used for each peptide was: SODa 30 nmol (N = 6); SODa 60 nmol (N = 6); SODa 120 nmol (N = 5); and SODb 60 nmol (N = 5). Peptides were dissolved in sterile isotonic saline (0.9% NaCl) immediately before use. Statistical comparisons were made using the effect of 0.5 μg of BK before the drug injection as reference.

2.7.3. BP and heart rate (HR) recording in non-anaesthetized rats

SHR were anesthetized by IP injection of ketamine (150 mg/kg) and xylazine (11.5 mg/kg) before stereotactic surgery, performed to implant the cannula in the cerebral lateral ventricle (LV). The animals recovered from the interventions for at least 4 days before any measurements were recorded. One day before the experiments, animals were anesthetized by IP injection of ketamine (150 mg/kg) and xylazine (11.5 mg/kg) for the catheter implantation (Tygon Flexible Plastic Tubing – Saint-Gobain PPL Corp.) in the femoral artery. The catheter was exteriorized and fixed on the upper back of the animal. One day after cannulation, the femoral catheter was connected to a pressure transducer. The BP and HR were recorded in non-anaesthetized animals with a Biopac System (Biopac Systems Co.). Both mean BP (MAP) and mean HR (MHR) were recorded continuously for 30 min after stabilization of BP. The animals were divided into three groups, and on the first day after cannulation, the rats in group 1 received the injection, into the LV, of SODa peptide (1.4 nmol/kg); in group 2 received SODb peptide (1.4 nmol/kg) and, in group 3, the rats received injections of the vehicle, and they were considered as a control group. BP and HR were both monitored for 6 h, and the values were recorded at every 15 min. After experiments, the rats were deeply anesthetized, and submitted to vascular perfusion through the heart with a 10% buffered formalin solution, after injection of Evans blue (1 μL) through the cerebral cannula. After sacrifice by decapitation, the brains were removed, fixed in 10% formalin and stored in 30% sucrose solution. Transverse slices of the brain were prepared (50 mm-thick) for the analysis and confirmation of the presence and distribution of the injected compounds in the lateral ventricle (LV) by visual observation of the presence of Evans blue dye.

2.8. Structural analysis by bioinformatics

2.8.1. Rattus norvegicus SOD1 modeling procedures

Cu, Zn SOD (EC 1.15.1.1), also known as SOD1, from R. norvegicus (UniProt ID: Q6LDS4_RAT) is a protein composed by 152 amino acids. The SOD1 has no experimental three-dimensional (3D) structure defined and it was used in this work as a target in a

homology modeling procedure. We used BLASTp [42] to identify homologous proteins in the PDB database, and then Modeller 9 v.6 program [43] for protein modeling. The human Cu, Zn SOD (PDB code: 1HL5) has 83% of amino acids identical and 89% of similarity to rat SOD1, and therefore, its 3D structure was used here as the template for homology modeling of rat SOD1. Then the obtained 3D model was evaluated by both Ramachandran plot analysis [44] and ProSA-web program [45]. The model created was labeled here as SOD1.

2.8.2. R. norvegicus SOD1 structural analysis

Structural analysis was initiated by studying the accessibility of BPP-similar peptides named as SODa and SODb. For this purpose, we used SurfV program [46] to calculate solvent accessibility area *per* residue, which allowed us to analyze if SODa (residues 21–27) and SODb (residues 54–61) regions are located at molecular surface and exposed to a solvent above a selected threshold: residues exposed above 25% of their total surface area. Additionally, we used STING server [47] to generate the "TGZ" file containing all STING structure descriptors. The TGZ file was used as input to Java Protein Dossier [48], in order to analyze other physical–chemical features of amino acids for a given 3D configuration. PyMol [49] was then used to generate the molecular images.

2.8.3. Homologous sequences and cleavage sites analysis

In order to check the presence of the BPP-like peptides in SOD proteins from other organisms, the primary sequences of *R. norvegicus* SOD (SODC_RAT) homologous proteins were selected using BLASTp [42] against SwissProt, and they were then aligned using ClustalW 2.1 [50]. Primary sequences from mammals with more than 70% identity and having similar regions to BPP-like peptides, identified by BLASTp, were selected to be used in this analysis. Therefore, the selected sequences for analysis were (with SwissProt codes): SOD from the primates *Cebus apella*, capuchin monkey (SODC_CEBAP), *Callithrix jacchus*, common marmoset (SODC_CALJA), *Macaca mulatta*, *Rhesus* monkey (SODC_MACMU), *Hylobates lar*, lar gibbon (SODC_HYLLA) and *Homo sapiens*, human (SODC_HUMAN); the Gilres *Oryctolagus cuniculus*, rabbit (SODC_RABIT), *Cavia porcellus*, domestic guinea pig (SODC_CAVPO), *Mus musculus*, house mouse (SODC_MOUSE); and the carnivore *Canis lupus familiaris*, dog (SODC_CANFA).

Additionally, aiming to identify known proteases that could recognize and cleave peptide bonds neighboring the BPP-like sequences, theoretical protease cleavage sites analysis was performed using the program PeptideCutter [51], which contains information about known cleavage target sites of several proteases (including commercially available enzymes). This database has a module of prediction cleavage sites into a query sequence. The sequence used in this approach was SODC_RAT. Besides, the database CutDB was used to predict the known cleavage sites of rat proteases, as this bank includes the enzymes from rat and other organisms. A search was conducted using *R. norvegicus* proteases, which returned a set of 172 proteolytic events (cleavage sites identified onto known proteins), and for *M. musculus*, a total of 402 events. The cut sites were also compared manually to neighboring regions of BPP-like sequences of SOD protein sequence.

2.9. Statistical analysis

Comparisons were made by one-way analysis of variance (ANOVA) with Newman–Keuls post-test using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM. The criteria for statistical significance were set at $p < 0.05$.

3. Results

3.1. Expression and characterization of the recombinant protein

The cDNA fragment coding for the precursor protein domain containing the seven bradykinin-potentiating peptides (BPPs) was subcloned in frame into the expression vector pProEx-HT (Invitrogen) and allowed the expression of a predominant protein band that migrated close to the region of 20 kDa, as observed by denaturing polyacrylamide gel electrophoresis (SDS/PAGE) analysis followed by Coomassie Blue and/or silver staining (Fig. 1A), although the theoretical expected MW was estimated as 18,169.5 Da.

Mass spectrometry analysis of this affinity purified protein sample, before and after tripsinization, confirmed that the expressed recombinant protein corresponded to the expected theoretical deduced amino acid sequence of the BPPs domain of the snake precursor protein (Table 1).

3.2. Production of specific antibodies to BPPs by immunization with recombinant protein

The antibodies against the BPPs (anti-BPPs) were raised by three different immunization protocols. Three cohorts of Balb/c immunized as described in Methods, using the obtained recombinant protein (as described above), allowed raising antisera with similar titers, regardless of the employed immunization protocol. Essentially, the following protocols were evaluated here: fortnightly, monthly or quarterly immunizations (Fig. 2). The biweekly immunization protocol yielded a larger volume of serum with elevated titer in a shorter period. Despite requiring a longer period for the obtainment of more specific sera against the BPPs, the monthly or quarterly immunization protocols needed less amount of the recombinant protein for the immunizations procedures, as fewer booster injections were necessary in these later protocols. We believe that the monthly or quarterly immunization protocols may also determine a lower animal suffering, as they were less manipulated along all immunization process, and that the final obtained antisera were qualitatively and quantitatively very similar for the several immunizations frequencies evaluated here, as demonstrated by WB and ELISA assays, with no significant differences in quality or specificity for the antibodies. However, specifically the 8th and 9th bleedings of the fortnightly/biweekly
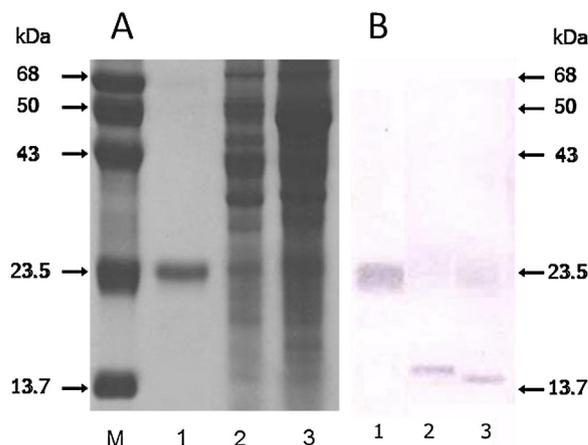
Fig. 1. Analysis of the specificity of anti-BPPs. (A) Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS/PAGE). (B) Western blot (WB) analysis using a pool of anti-BPPs serum and the secondary antibody anti-mouse IgG conjugated with alkaline phosphatase. For (A) and (B): (1) recombinant BPP precursor protein (5 μg), (2) cytosol of *Bothrops jararaca* brain (20 μg), (3) cytosol of adult Wistar rat brain (50 μg) and (MW) protein size marker (in kDa).

Table 1
Mascot search results for ESI-TRAP mass spectrometry analysis of trypsin digested samples.

Mascot search results for ESI-TRAP analysis of trypsin digested samples					
Protein band	Match to	Seq coverage	Score for each analysis	No. of unique peptides	No. of match queries
~17 kDa	Q6LDS4 (Mr 15871 Da)	43%			
			427	6	39
			355	7	29
			355	7	31
~34 kDa	Q6LDS4 (Mr 15871 Da)	65%			
			450	8	13
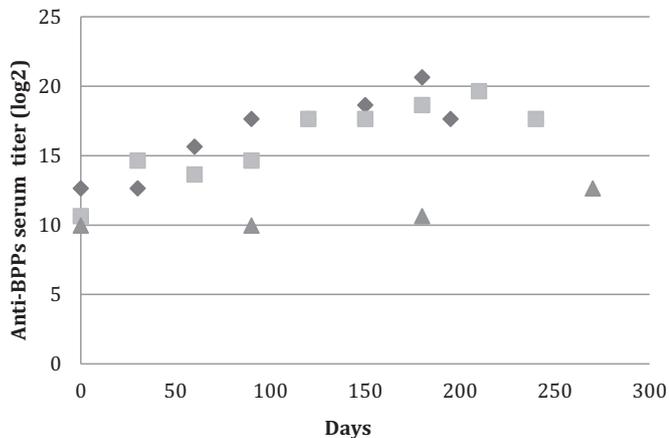
Fig. 2. Production of anti-BPP by immunization of Balb/C mice with the recombinant BPPs domain of the precursor protein. Mice were immunized with 20 μg of protein emulsified with IFA. Day 0 corresponds to the first immunization, and the bled was performed 3 days after each booster. Titers of batch of animals immunized biweekly (square), monthly (diamond) and quarterly (triangle) on a plate coated with recombinant BPP precursor protein-BSA. (B) The antibody titer was determined comparing to the binding level of preimmune serum ($\leq log_2$).

protocol showed the highest specificity in the WB analysis (lower degree of cross-reactivity). These sera aliquots allowed detecting a clear single protein band of about 17 and 16 kDa in the total cytosol of snake and rat brain homogenate, respectively, in addition to a very intense and specific recognition of the recombinant BPPs domain precursor protein with theoretical expected MW estimated as 18,169.5 Da that migrated close to the region of about 20 kDa (Fig. 1B), even after long exposition periods. Therefore, these serum aliquots were chosen for use in the following steps of this work.

3.3. Isolation and purification of the BPPs immune-related protein from the rat brain cytosol

Considering that the immune-recognized protein from the adult rat brain has a MW of about 16 kDa, as demonstrated by WB analysis (Fig. 1B), the total proteins from a whole cytosol homogenate of adult rat brains were initially fractionated by gel filtration using a Sephadex G-50 resin, and all obtained fractions were analyzed by SDS/PAGE and WB, allowing the identification of the fractions that contain the proteins immuno-recognized by the anti-BPPs serum, which at this step were basically two bands of about 16 and 32 kDa (Fig. 3).

The chromatography fractions containing the protein(s) of interest, as determined by WB, were then pooled and size sorted using microcentrifuge columns with cut-off for 100 kDa, which
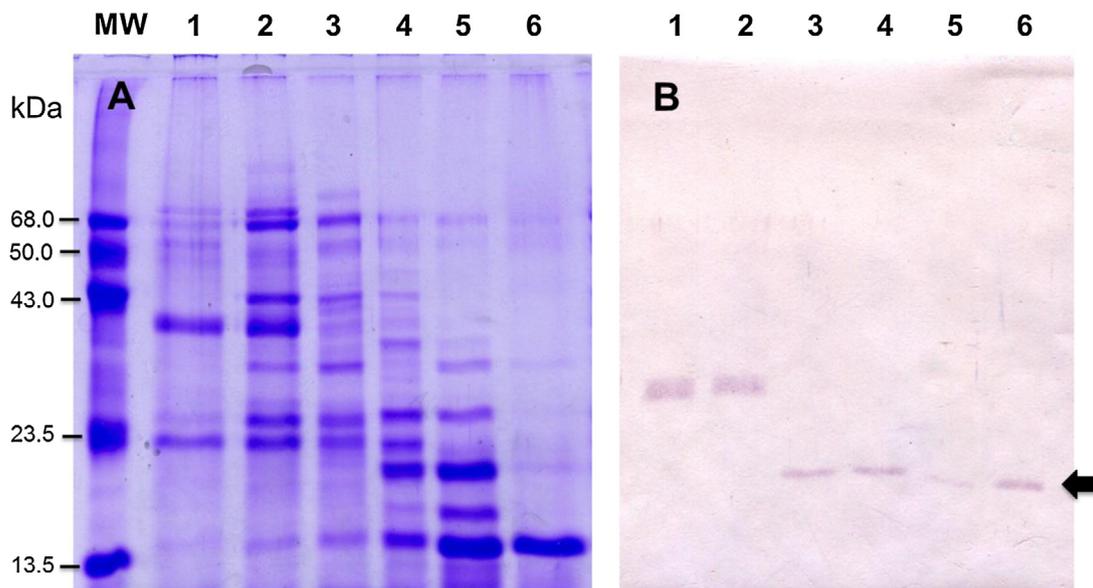
Fig. 3. SDS/PAGE analysis for the fractions from the gel filtration chromatography of adult rat brain cytosolic homogenate. The homogenate was applied into a Sephadex G-50 column and the eluted fraction were monitored at λ = 260 nm, and the obtained fractions were pooled as 16–30 (1), 31–40 (2), 41–50 (3), 51–58 (4), and 59–90 (5). Aliquots of each pool and total brain homogenate (6) were submitted to 12.5% SDS/PAGE followed by staining with Coomassie brilliant Blue (A) or Western blot analysis using the anti-BPP antiserum (B). The molecular weight standards (M) are indicated on the left for (A) and (B), and the arrow in panel (B) indicate the protein band of about 17 kDa, immune-recognized by the anti-BPPs antiserum.
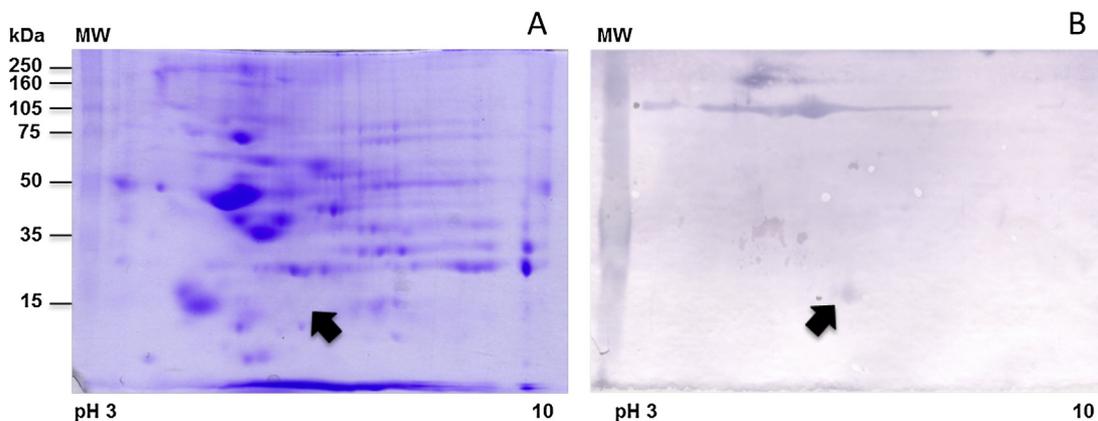
Fig. 4. 2D SDS/PAGE of the fraction containing the immune-recognized protein of interest. First-dimension isoelectrophoresis was performed in IPGphor (GE Healthcare) according to manufacturer's using a using a pre-cast immobilized pH 3–10 strip, and after the isoelectric focusing (IEF), the samples were further resolved in a second dimension by SDS/12.5% PAGE, prepared in duplicate. One gel was stained with Coomassie brilliant Blue (A) and the other was analyzed by Western blot using the anti-BPP antiserum (B) The collected protein spot is indicated by the arrow.

allow the removal of proteins of higher molecular weight (above ~100 kDa). And then, the low molecular weight fraction of the sample containing the protein of interest was concentrated by filtration in Centricon columns with cut-off for 10 kDa.

For further isolation and biochemical characterization/identification of the immune-recognized protein, two-dimensional electrophoresis in polyacrylamide gel (2D/PAGE) and high performance liquid chromatography (HPLC) fractionation were employed. The 2D/PAGE analysis followed by Coomassie Blue and/or silver staining allowed to observe the total protein composition of the concentrated low molecular weight fraction sample (Fig. 4), and WB analysis confirmed the presence of ~16 kDa protein recognized by the anti-BPPs antibody (Fig. 4). The immune-recognized spots were then cutout from the 2D/PAGE gels stained with Coomassie Blue, and the sample was trypsinized before the mass spectrometry (MS) fingerprint analysis.

Aiming a more refined isolation and to confirm the 2D/PAGE results, the same concentrated low molecular weight fraction sample containing the protein of interest was also fractionated by HPLC, and the observed peaks were individually collected and once more analyzed by SDS/PAGE and WB using the anti-BPPs antiserum. A single protein band of about 16 kDa was observed only in the fraction eluted at 16 min (data not shown), and this immunorecognized protein band was excised from the Coomassie Blue stained one dimension SDS/PAGE, before trypsinization and MS fingerprint analysis.

The identification by MS of these proteins (of about 16 and 32 kDa), isolated by either 2D/PAGE or HPLC, consistently confirmed that the immune-recognized rat brain proteins are in fact the endogenous antioxidant enzyme Cu, Zn superoxide dismutase (SOD), which plays a key role in the blood pressure (BP) regulation and has a molecular weight of 15,871 Da and isoelectric point of 5.88. The about 32 kDa protein was shown to correspond to dimers of the SOD (Table 1).

3.4. Homologous sequences and cleavage sites analysis

The selected sequences of mammalian SOD sequences were aligned using ClustalW in order to highlight the conservation of BPP-like regions within distinct groups of several animals. The alignment is presented in Fig. 5A, and SODa and SODb peptides are colored in blue and green, respectively. The identifiers of the SOD protein sequences are colored accordingly to the taxonomic group of the organism source: yellow for order Primates, red for group Gilres and gray for order Carnivora. The first BPP-like peptide

[rat: QKASGEP] had the Gln[21], Lys[22] and Pro[27] fully conserved. The primates group have identical region in the SOD sequence and a particular feature is observed: the presence of a glutamic acid at the corresponding position of the rat Ala[23] and a Gly residue in the correspondent position of rat Glu[26]. This means that there is a change of a non-polar to a charged residue in the first site, and the reverse situation in the second site, which might conserve the electrostatic potential of the region. Additionally, in rat sequence, this region is identical in mouse, and there is a loss of the charged residue at BPP-like regions in the SOD from rabbit, guinea pig, and dog. The second BPP-like region, comprising the SODb sequence is more conserved, and all residues (unique exception for the conservative change between Thr[58] for a Ser) are present in the SOD sequences from all analyzed animals. The human SOD sequence has an Ala residue at the first position of SODb region, but all remaining SODb sequence is identical to that found in rat SOD protein.

The program PeptideCutter was used here to search for known protease cleavage sites neighboring the BPP-like regions. The enzyme Asp-N endopeptidase + N-terminal Glu was suggested to be able to cleave the F[19]–E[20] bond preceding the BPP-like SODa peptide and glutamyl endopeptidase could potentially cleave the E[20] peptide bond. Other enzyme with potential interest is the thermolysin, which would be able to cut and release the exact sequence of BPP-like SODa peptide, acting at positions 20 and 29 of rat SOD protein. Unfortunately, these enzymes are originally from bacteria and obviously nobody would expect these enzymes could be the responsible for the cleavage and processing of a mammal cytosolic SOD, except if other enzyme with similar catalytic specificity of thermolysin exists in the cell cytosol providing the appropriate scenario for this endogenous cleavage. Thus, the database CutDB was used to search for cut sites of known rat and mouse proteases. After a manual check, it was possible to notice that ADAM10 peptidase may be able to recognize and cleave a similar sequence to that found at BPP-like peptides C-terminal region. The cleavage site of ADAM10 at p75 neurotrophin receptor is SSQP-VVT, which is similar to the region found at SOD (i.e. SGEP-VVV). Interestingly it is discussed that the active sites of ADAM10 peptidase and thermolysins may be similar to each other [52]. No obvious cleavage sites similar to the regions neighboring the BPP-like sequences of SODa could be found in rat database. On the other hand, in mouse database, a cleavage site between a glutamate and a glutamine residue (similar to E[20]–Q[21] site found in SOD) is described in the Trim28 transcription intermediary factor

A
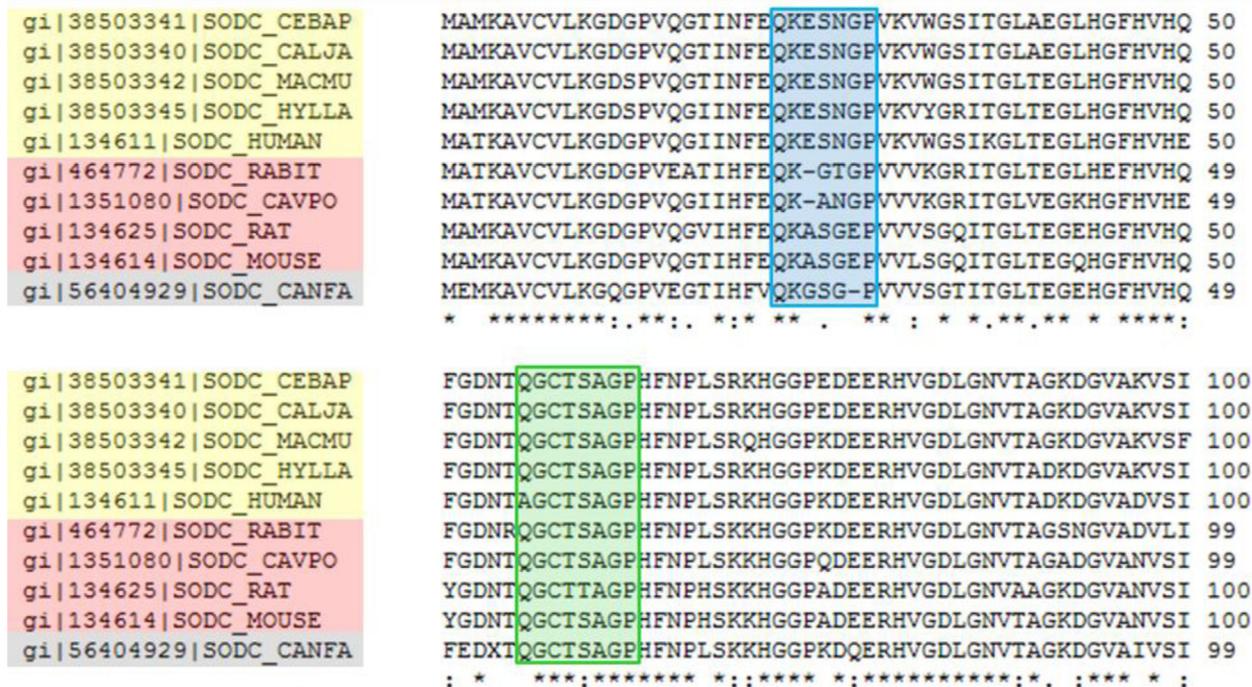
B
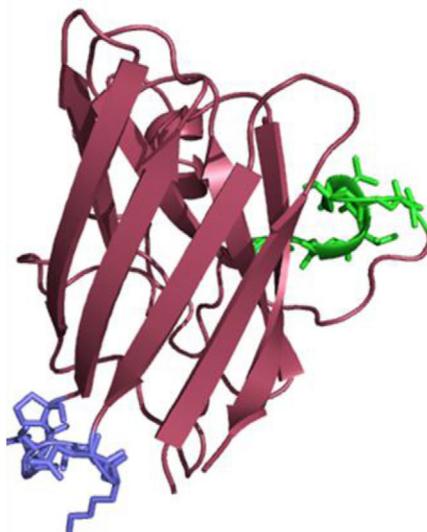
Fig. 5. Sequence alignment of mammalian SODs and 3D structure of modeled rat SOD1. (A) Primary sequence alignment of mammalian SODs by ClustalW. The codes are displayed with NCBI identifier (gi) and SwissProt identification from organisms defined in materials and methods. (B) *Rattus norvegicus* 3D structure of modeled SOD1. The BPP-like peptides SODa and SODb are highlighted in blue and green, respectively, showing that they are exposed to a solvent, located at the molecular surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

1-beta, but this site may be recognized and cleaved by a still unknown protease [53]. It is clear that it is not possible for us to suggest here that these enzymes are involved in the cleavage and processing of the SOD protein, but the conceivable existence of enzymes that can potentially cleave proteins in similar recognition sites, as the one neighboring the BPP-like peptides found in rat SOD protein, may support the hypothesis of a possible existence of endogenous proteases with ability to cleave SOD and to release the active BPP-like peptides. Experimental works are still required to confirm this hypothesis, but the chemical synthesis of the expected mature BPP-like peptides may also be an important step to first confirm the potential pharmacological effects of these peptides, once released from the precursor protein.

3.5. R. norvegicus SOD1 modeling

The modeled SOD1 structure was evaluated by Ramachandran plot analysis and validated as good because 147 residues (98% of residues) are located at most favored regions. ProSA web service confirmed the high quality of model having a compatible z-score (-7), located within the range of scores typically found for native proteins of similar size. ProSA also showed a good local model quality since the average energies (for 40 and 10 residues window size) are predominantly negative.

Then, we used SOD1 coordinate file to perform SurfV calculation. The output was used to analyze whether BPP-similar regions containing the "SODa" and "SODb" peptides are located at surface and if they are exposed to the solvent above the threshold

Table 2
Accessible area and percentage of exposure to a solvent for BPP-similar peptide of rat SOD1 peptide SODa and SODb.

	Position	Amino acid	Accessibility	% of total amino acid area at surface
	BPP-like peptides from modeled rat SOD1			
SODa	21	Q	20.08	6.27%
	22	K	178.30	49.78%
	23	A	56.56	23.67%
	24	S	104.35	41.72%
	25	G	61.21	29.20%
	26	E	45.69	13.68%
	27	P	38.90	14.46%
		Average	72.16	25.54%
SODb	54	Q	121.53	37.95%
	55	G	32.25	15.39%
	56	C	20.53	7.60%
	57	T	94.00	33.58%
	58	T	36.36	12.99%
	59	A	0.00	0.00%
	60	G	14.72	7.02%
	61	P	86.50	32.14%
		Average	50.74	18.33%

of at least 10% of their respective total surface area. The data showed that SODa peptide [QKASGEP], located at position 21–27 of rat SOD1, is exposed to a solvent with an average of accessible area per residue of 72.16 Å^2 (25.54% of respective total surface area), as is shown in Table 2. These residues are located on a loop between two anti-parallel β-strains (Fig. 5B). The most exposed residues are those polar: e.g. Lys^{22} and Ser^{24} (178.30 Å^2 and 104.35 Å^2 of accessible area, respectively).

The BPP-like peptide SODb [pQGCTTAGP] located at position 54–61 of rat SOD1 was also evaluated with respect to solvent exposure, and we found that although also exposed, it is less exposed than the SODa peptide. There, an average of 50.74 Å^2 of accessible area was found (18.33% of respective total surface area), as is shown in Table 2. These residues are located in a loop with polar Gln^{54} and Thr^{57} (121.53 Å^2 and 94.00 Å^2 of accessible area, respectively), as most exposed amino acids. It is important to emphasize that the Cys^{56} (of the SODb peptide) is establishing a disulfide bridge with Cys^{145}, what approximates the SODb region to protein core and creates a small α-helix like conformation, which hides Ala^{59} (which is not exposed). Modeling and 3D structure analysis also demonstrated the high exposure of these peptides in SOD1 protein surface as follows (Fig. 5B).

3.6. Synthesis of SOD peptides

As mentioned, the SOD enzyme is an endogenous antioxidant with a key role in regulating BP, which is in line with the activities described up to now for the BPPs [35]. However, a comparison of SOD primary sequence to the BPPs precursor protein showed little or no primary sequence similarity (data now shown). On the other hand, knowing that the BPPs are typically composed by 5–17 amino acid residues per molecule, with an invariable pyroglutamic acid (after post-translational modification of the glutamine residue at N-terminus of the peptide [29,30]) and a proline residues at the N- and C-terminal extremities, respectively [54,55], two peptide sequences presenting these features were identified in the rat SOD1 primary amino acid sequence (Fig. 5). These peptides were synthesized and named as SODa [pQKASGEP] and SODb [pQGCTTAGP]. In other words, these peptide fragments of the rat SOD sequence were chosen due to the presence of the glutamine and proline residues spaced by less than 7 amino acid residues, and also due the presence of arginine and/or tryptophan residues, often observed in many BPPs isolated from snakes and other animals [35]. Moreover, aiming to better mimic the native

BPPs found in snake venom glands these SOD peptide fragments were also synthesized with a pyro-Glu [pQ] residue at their N-terminus.

3.7. Pharmacological assays with the SOD-derived synthetic peptides

To determine if the SODa [pQKASGEP] and SODb [pQGCTTAGP] peptides synthesized based on the sequence of rat SOD1 have the same pharmacological functional characteristics described for the BPPs, their ability to potentiate the contractile effect of BK on smooth muscle was evaluated using preparations of isolated guinea pig ileum. Under the conditions tested in this assay, no potentiation of BK activity in isolated smooth muscle was observed for the peptides studied here (Table 3).

3.8. Systolic blood pressure recording in anaesthetized rats

The activity after peripheral injection of synthetic SODa [pQKASGEP] and SODb [pQGCTTAGP] peptides on the BP of anesthetized rats was also evaluated.

As observed, although the statistical analysis showed no significant BK-potentiating effect in the presence of low doses of SOD peptides, it was possible to observe for SODa a trend to potentiate the hypotensive effects of BK compared to the control. This difference was not significant as determined by the variation in the maximum peak response for each animal, but it is important to note that, for each animal, the maximum response was observed at different time lapses, i.e. at 5, 10, 15, 20, 25 or 30 min after peptide IV bolus injection (Fig. 6A and B). On the other hand, if the maximum response was considered regardless of the response time lapse, a clear significant increase of the hypotensive effects of BK compared to the control could be noticed only for the SODa peptide with a dose–concentration response (Table 4 and Fig. 6C–F). However, in contrast to the SODa peptide (Table 4 and Fig. 6C–F) and the ACE inhibitory activity described for the snake BPPs [56], for concentrations up to 120 nmol, the SODb peptide showed no obvious ACE inhibition, evaluated by in vivo potentiation of the BK hypotensive effects in anaesthetized rats (data now shown).

3.9. Blood pressure (BP) and heart rate (HR) recording in unanesthetized spontaneously hypertensive rats (SHRs)

Taking into account that these BPP-like peptides were isolated from the adult rat brain, we decided to evaluate whether the SODa

Table 3
EC$_{50}$ values for captopril (control) and rat SOD1 derived putative peptides (namely SODa and SODb). Potentiating activity of the putative peptides of SOD on the contractile effect of BK on isolated guinea pig ileum using captopril as positive control. The number of experiments (N) and the standard error of the mean (S.E.M.) are presented.

Drug	EC$_{50}$ (nM)
BK (N = 10)	1.93 ± 0.40
BK + captopril 15 nM (N = 10)	0.20 ± 0.15*
BK + captopril 45 nM (N = 10)	0.34 ± 0.10*
BK + captopril 150 nM (N = 10)	0.33 ± 0.06*
BK (N = 10)	1.78 ± 0.38
BK + SODa 45 nM (N = 6)	1.45 ± 0.47
BK + SODa 150 nM (N = 10)	1.19 ± 0.25
BK + SODa 450 nM (N = 10)	1.09 ± 0.22
BK + SODa 1500 nM (N = 4)	1.18 ± 0.30
BK (N = 10)	2.59 ± 0.43
BK + SODb 45 nM (N = 6)	2.04 ± 0.45
BK + SODb 150 nM (N = 10)	1.91 ± 0.33
BK + SODb 450 nM (N = 10)	1.37 ± 0.33
BK + SODb 1500 nM (N = 4)	3.12 ± 0.83

* $p < 0.05$.
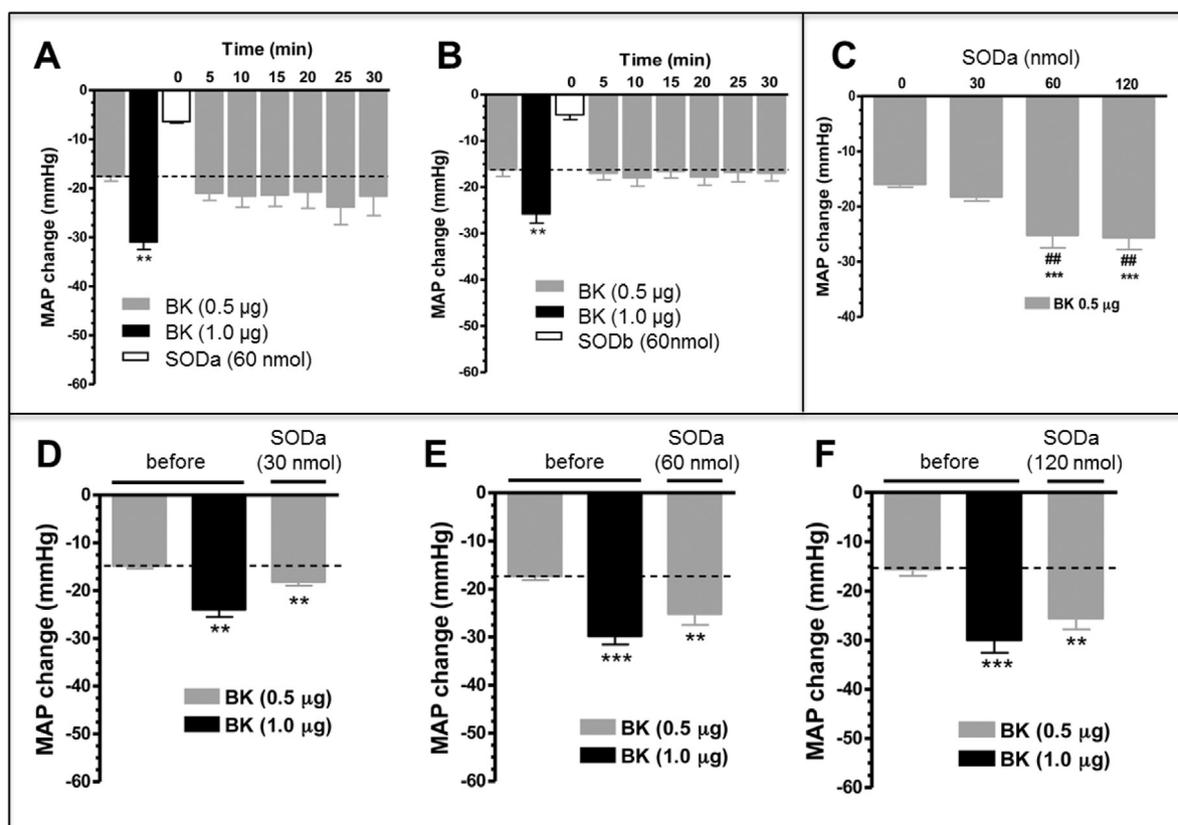
Fig. 6. Hypotensive effect of intravenous bolus injections of BK before and after SOD peptides administration in the blood pressure of anesthetized Wistar rats. (A) SODa, 60 nmol ($N = 6$), (B) SODb, 60 nmol ($N = 6$). (C) Average of maximal response on blood pressure in the anesthetized Wistar rats evoked by intravenous bolus injections of BK before and after different doses of SODa, namely (D) SODa, 30 nmol ($N = 6$), (E) SODa, 60 nmol ($N = 6$) and (F) SODa 120 nmol ($N = 5$). Results extracted of each experiment independent of time record (until 30 min after SODa injection). Data are expressed as mean ± SEM. ***$p < 0.001$ SODa doses in comparison of the values obtained with 0.5 μg of BK before SODa injection and ##$p < 0.01$ comparison between SODa doses 60 nmol vs. 30 nmol and 120 nmol vs. 30 nmol. (D–F) Data are expressed as mean ± SEM. **$p < 0.01$ and ***$p < 0.001$ in comparison to the values obtained with 0.5 μg of BK before SODa injection.

and SODb peptides could present similar functional characteristics as described for the snake BPPs in CNS, after intraventricular (ICV) brain injections in spontaneously hypertensive rats (SHRs) [36,57], for which an increased baroreflex sensitivity and decreased mean arterial pressure (MAP) and heart rate (HR) were described [32,33].

The ICV injection of SODa and SODb (Fig. 7) produced a maximum hypotensive effect (ΔMAP) of about -28 ± 4 and -23 ± 3 mmHg, respectively, which are both more intense than that observed for the control (-12 ± 2 mmHg) and also described for BPP-5a (i.e. ΔMAP: -38 ± 4 mmHg) [57]. The maximum delta heart rate (ΔHR) for SODb (-112 ± 13 bpm) were also more intense than the control (-50 ± 4 bpm) and also BPP-5a (ΔHR: -71 ± 17 bpm) values [57], although for SODa (-86 ± 17 bpm) only a trend to a smaller HR was observed, as this difference was not statically different ($p = 0.0622$).

In other words, the bradycardia was more intense and longer than that observed for BPP-5a [57], with a maximum delta HR (of about-197 bpm) achieved from 1 to 2 h after injection of SODa or

Table 4
Percentage of maximal potentiation of BK hypotensive effect on rat blood pressure after intravenous bolus injection of SODa peptide. Results extracted of each experiment independent of time record (until 30 min after SODa injection). Data are expressed as mean ± S.E.M.

SODa dose (nmol)	N	Maximal potentiation (%)
30	6	24.9 ± 4.5
60	6	46.2 ± 13.5
120	5	65.6 ± 11.8

N is the number of animals used for each peptide.

SODb. In turn, SODb injection produced a maximum hypotensive effect less intense than SODa, but that remained for longer (monitored up to 360 min) compared to the fast decrease of the MAP in the first hour after injection of the peptide SODa followed by a gradual recovery of the BP (data now shown). These effects could be attributed to a reduction in the peripheral sympathetic activity, and further studies are being conducted by the group to clarify the eventual neural mechanism underlying the central effects of these peptides.

Taken together, this assay allowed us to observe a decrease in MAP, for SODa and SODb, both accompanied by a significant decrease in HR, but with a faster effect for SODa compared with SODb, which showed a slower and long lasting effect. In other words, the maximum hypotensive and bradycardic effects of SODa and SODb were more severe and remained for longer than for BPP-5a (Fig. 7).

4. Discussion

Efforts to search for bradykinin-potentiating peptides-like (BPP-like) sequences in available databanks, by employing different bioinformatics tools, were very frustrating, leading us to identify couple of sequences from mammals with BPP-like structure based on the primary structure and/or amino acid composition, but with no effective in vitro and in vivo pharmacological effects for the respective identified peptide. Taking into account that the 3D structure should be conserved for the maintenance of the biological functions, we have concentrated our efforts on strategies that allow the search for topologically

Fig. 7. Cardiovascular parameters changes in response to the ICV injection of SODa and SODb into unanesthetized SHRs brain. (A) Delta mean arterial pressure (ΔMAP), and (B) delta heart rate (ΔHR) compared to initial values, *i.e.* mean BP (MAP) and mean HR (MHR) recorded continuously for 30 min after stabilization of BP, before the ICV injection into the lateral ventricle (LV) of 1.4 nmol/kg of SODa (*N* = 7) and 1.4 nmol/kg SODb (*N* = 7). $^*p < 0.005$ in comparison to the control. (C) Exemplary transverse slices of the rat brain prepared (50 mm-thick) for the analysis and confirmation of the presence and distribution of the injected compounds in the LV by visual observation of the presence of Evans blue dye.

homogenate was used for the screening and tracking an immune-recognized protein during the protein isolation process by chromatography.

The fractionation of rat brains proteins using Sephadex G-50 chromatography column followed by size cut-off filtration system (Centricon) enabled the subsequent enrichment of the immune-recognized protein in selected fractions (*e.g.* containing the protein of interest), which underwent a final isolation processes using either two-dimensional denaturing polyacrylamide gel electro-phoresis (2D SDS/PAGE) or high performance liquid chromatography (HPLC). Our results revealed that these both methods were effective in generating samples of the protein of interest with enough amount and purity for further characterization steps. Mass spectrometry (MS) fingerprint analysis identified the $\sim$16 kDa protein band as the Cu, Zn superoxide dismutase (SOD), which is an enzyme classified as an endogenous antioxidant with a key role in regulating blood pressure (BP). SOD has molecular weight of 15,871 Da and isoelectric point equivalent to 5.88 [61]. MS analysis also allowed the characterization of the 32 kDa band, also recognized by the anti-BPP antiserum in the rat brain cytosol, as dimers of SOD. In fact, the trend of SOD to form dimers in high concentrations is well known as was already described in the literature [62].

Analysis of the primary sequence of rat SOD allowed the identification of two peptide sequences with glutamine and proline residues spaced by less than 7 amino acid residues, and also detected the presence of arginine and/or tryptophan residues, often observed in many BPPs isolated from snakes and other animals [35,54]. The modeling and 3D structure analysis of rat SOD demonstrated the high exposure of these peptides, named as SODa [pQKASGEP] and SODb [pQGCTTAGP], in the protein surface. Modeled rat SOD1 structure was evaluated by Ramachandran plot analysis and that was validated as good, as 147 residues (98% of residues) are located at most favored regions. ProSA web service also confirmed the high quality of this model. The program PeptideCutter allowed searching for known protease cleavage sites neighboring the BPP-like regions, and the enzyme Asp-N endopeptidase + N-terminal Glu was suggested to cleave the F^{19}–E^{20} bond preceding the BPP-like SODa peptide sequence, and glutamylendopeptidase would be able to cleave the E^{20} peptide bond. Another interesting enzyme identified was thermo-lysin, which would be able to cut the flanking regions of the BPP-like SODa peptide, cleaving at positions 20 and 29, suggesting the existence of proteases with potential to cleave rat SOD and to release the identified peptides. However, we are conscious that experimental works are still required to confirm this hypothesis, as our efforts to identify these peptides by MS analysis of the low molecular fraction of adult rat whole brains under physiological conditions were unfruitful up to now.

Preliminary tests performed in anaesthetized rats with these two peptides (SODa and SODb) synthesized with a pyro-Glu [pQ] residue at the N-terminus, based on excerpts from the rat SOD sequence, indicated that these BPP-like peptides have bradykinin (BK) potentiating activity *in vivo* (Figs. 6 and 7), although *ex vivo* assays using isolated guinea pig ileum did not show any potentiating activity (Table 3). The difference in results obtained in *ex vivo* and *in vivo* assays suggests that these synthetic peptides may be able to potentiate the effect of BK, but not in isolated tissues experiments (Table 3 and Figs. 6 and 7). This might suggest that the mechanism of action of these putative peptides of SOD requires pathways or effectors not available in isolated guinea pig tissues, as it was demonstrated also for other BPPs from snake venom [35]. It is well know that the BK action is dependent of its binding and activation of the BK B2 receptor, which triggers intracellular signaling through G-protein activation and consequent increase in the SOD expression [63,26], which could be then the source for an

conserved regions. The use of specific antibodies for this purpose is classically employed [58,59], although the high content of proline residues characteristic of the snake bradykinin-potentiating peptides (BPPs) may represent the principal limitation for this approach due to the risk of eventual immune cross-reactivity. In fact, previous attempts to raise specific antibodies against snake BPPs demonstrated the challenging limitations imposed by using short proline-rich peptides (PRPs) as antigen [60]. The employ-ment of several innovative strategies culminated in the production of anti-BPPs antiserum able to recognize a single band correspond-ing to the BPP precursor protein in the cytosol of the *B. jararaca* venom gland [56], but which was not able to specifically recognize proteins in any other snake or rat tissue homogenates (data not shown). In the present work, we demonstrate the high specificity of anti-BPPs antiserum raised in mice by several immunization protocols using purified recombinant BPP precursor protein expressed in bacteria, which was shown to be able to recognize a single protein band in the whole cytosol of snake and rat brain, independent of the immunization protocol used. The antiserum aliquot presenting the highest specificity against the adult rat brain

increased release of these putative peptides. This suggestion is in line with the recent scientific discovery trend suggesting the importance of the production of bioactive peptides derived from recycling and/or metabolism of proteins after their primary function [64].

However, it is important to consider that the SOD was identified in the brain cytosol and one would expect to see the effect of these putative peptides in the central nervous system (CNS), taking into account its hypothetical existence in the free form in the brain. Interestingly, infusion of these synthetic SOD peptides into spontaneously hypertensive rats (SHRs) brain showed bradycardic and hypotensive effects in these animals, in similar fashion as already described for several BPPs isolated from snakes [65], suggesting a potential physiological central role for these peptides in rodents and mammals. A significant decrease in mean arterial pressure (MAP) accompanied by a significant decrease in heart rate (HR) was observed for both SODa and SODb, in a similar way as recently described also for the snake BPPs [31,32,36,57,65], but with a slower and long lasting effect for SODb compared to the faster effect of SODa (data not shown). Considering that the rat SOD enzyme has a key role in regulating the BP, acting as an endogenous antioxidant [66], it would be expected that peptide fragments derived from SOD protein sequence could be biologically effective only in conditions suitable for the antioxidant system's triggering. It is well-known that SOD, as an antioxidant, acts in the CNS by reducing the levels of reactive oxygen species (ROS), which in turn increases the excitability of sympathetic premotor neurons, subsequently reducing the sympathetic tone [67], although vagal-mediated increase in parasympathetic tone could not be ruled out [68,69,67,70], as angiotensin-converting enzyme (ACE) inhibitors can significantly increase vagal tone [67,70]. Therefore, it would be conceivable that after this primary action, SOD could also be the source of bioactive peptides that could further decrease the BP by a mechanism of action that still need to be unraveled. Although the potential involvement of NO synthesis and Ca^{2+} influx should also be considered for BPP-like peptides [31–33,57].

Unfortunately, as mentioned, attempts to isolate these peptides from adult rat brains under physiological conditions were unsuccessful up to now. However we need to consider that it is possible that these peptides can be found in very low levels/concentrations or even be completely absent in physiological conditions. So forth, pharmacological manipulations that could potentially favor the release of these peptides are under study now. The identification of potential proteases with ability to release these peptides from the SOD sequence, as those suggested by bioinformatics analysis performed here, may open new insights for novel experimental strategies to solve this question, not only for rat brain protein, but also for the snake venom gland precursor protein, as the BPPs production mechanism and bioactive peptide release process still remain unknown.

Moreover, it is of note that despite the recognized and overestimated importance of the canonical PIPP sequence in the C-terminus of the snake BPPs for their biological activity on potentiating the BK effects in isolated smooth muscles [34,35], the recent find of the group showed that the N-terminal variable region plays a more decisive role for the in vivo effects of BPPs in the BP, and a dissociation of antihypertensive and bradycardic effects of snake BPPs [65]. Interestingly, among the various biological functions endowed by PRPs present in many tissues and biological fluids of humans, we highlight the antioxidant properties and the potential therapeutic role of PRPs as a promising source of new peptide based novel drugs [71].

The demonstrated significant degree of ligand–receptor coevolution with the evolution of peptidergic signaling should also be considered here as this partnership may contribute in fine tuning orchestrated multi-organ physiological outcomes, and consequently, this may be subjected to selection pressures in the evolutionary process supporting eventual pleiotropic interactions among closely related ligands [72] as the BPP-like or PRPs family, which are composed by a wide variety of structurally related members [34,35,37].

The identification of these peptides in their free form from CNS preparations is under consideration for the continuation of the present research project.

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