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Venom Bradykinin-Related Peptides (BRPs) and Its Multiple Biological Roles

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1. Introduction

The kallikrein-kinin system is an extensively studied biological pathway and involves a multi-protein complex, which includes serine proteinases from tissue and plasma. These proteinases act on substrates as kininogens (high and low molecular weight), releasing the active kinins. The main kinin is the nonapeptide bradykinin (BK).

Several studies aiming to evaluate the biological activities of the kinins revealed that this peptide is implicated in diverse physiological processes as regulation of blood pressure, cardiac, and renal function. Due to its ability to increase the vascular permeability by acting on endothelial cells, BK is correlated to several pathological processes including inflammation. These actions have been observed and described in both mammals and rodents [1].

The knowledge on the role of BK in various biological pathways as coagulation cascade, blood pressure regulation, and central nervous system modulation and signaling has been significantly improved, leading to the identification of BK receptors and posterior development of drugs targeting its pathways [2].

This research was mainly driven by scientific studies on animal venoms, which lead to the identification of the BK-related peptides (BRPs). The best, and maybe also the first, example of such contribution was the discovery of the bradykinin-potentiating peptides (BPPs), first described in *Bothrops jararaca* venom [3, 4]. The BPPs are proline-rich oligopeptides that inhibit the angiotensin-converting enzyme (ACE), and that are responsible for the hypotensive effect of the *Bothrops* genus snake venoms. The pharmacological effects of these peptides have been studied since 70's [3, 4], and allowed not only to the discovery of the neuropeptide BK [5], but also to the development of the first active site-directed inhibitor of ACE as drug for the

treatment of human hypertension [6]. In fact, several other drugs derived from venom toxins, with or without modifications, are also commercially available (*e.g.* Captopril, Ancrod, and Prialt) [7]. Moreover, the study of toxins has widely contributed to the identification of new targets with therapeutic potential in mammals, as well as it has allowed to the understanding and discovery of the biochemical pathways involving these targets.

Since then, the BPPs/BRPs have been found in several snake venoms, and also in wasps and frogs, by using either biochemical or/and recombinant DNA techniques [8-11]. For instance, molecular cloning studies using cDNA libraries of four species of snakes from Crotalinae family showed evidences that these bioactive peptides are expressed by orthologous genes [12]. The cloning of orthologous precursors from different snakes from *Bothrops* and *Crotalus* genus allowed the identification of several new BPPs sequences [13-15], and some of them was shown to display different specificity toward each active sites of the somatic ACE ectoenzyme [16]. This was believed to be a great opportunity for the development of a new generation of antihypertensive drugs.

The employment of recombinant DNA techniques were also fundamental to first determine the structure of the precursor protein of BPPs, which was found to contain several sequences of BPPs distributed as tandem repeats, followed by a C-type natriuretic peptide (CNP) at the C-terminus of this precursor molecule [15]. In contrast to other members of the natriuretic peptide (NP) family, CNP is synthesized in the brain and has hypotensive effect with no significant diuretic or natriuretic actions [17]. Moreover, Northern blot analysis of several snake tissues demonstrated the presence of similar BPPs-CNP precursor mRNA in non-venomous tissues, such as the central nervous system (CNS) [14]. *In situ* hybridization studies also detected the presence of the BPP/CNP-precursor mRNA in regions of snake brain correlated with neuroendocrine functions, such as the ventromedial hypothalamus, paraventricular nuclei, paraventricular organ, and subcommissural organ [14]. Analogous CNP precursor mRNAs was also described in similar regions in rat and human brains [18].

These studies suggesting the potential expression of BPPs in snake CNS stimulated us to investigate the putative target(s) of these peptides. Based on the *in vivo* biodistribution studies showing the preferential accumulation of BPPs in the rat kidney, and also a significant presence in the brain, the first studies were conducted in these tissues leading to the description of several completely new potential targets and pathways, as the nicotinic acetylcholine receptors [19], L-argininosuccinate synthase [20], and an orphan G protein-coupled receptor (GPCR) [20]. The importance of both NO release for the antihypertensive effects of BPPs [20-22], and also the involvement of the GPCRs, namely B2 receptor and M1 muscarinic receptor (mACh-M1), in vasodilation were demonstrated [23].

Together all these data collected during the last decade showed the pharmacological significance of the BPPs and, more importantly, that the biological effects of these peptides, although first believed, are not limited to the inhibition of the somatic ACE [2]. The high variability of molecular structures of these peptides reflecting in different specificities is an indicative that there are still more to be discovered regarding the biological effects of this peptides family.

In this book chapter, we intend to gather the most important results obtained up to now, thanks to the isolation and characterization of BPPs from diverse organisms and to the knowledge accumulated, while searching for new targets for these molecules.

2. The discovery of the snake venom BK and BRPs

The main function of snake venoms is still believed to be the immobilization of preys to ensure feeding. The snake venoms are composed of a complex mixture of proteins and biologically active peptides [24, 25]. The study of the pathophysiological mechanisms of poisoning and molecular characterization of toxins from the venom of *Bothrops jararaca* resulted in many scientific contributions of great importance, and among them, stand out the discovery of BK [5] and the discovery of the first BRPs, more specifically the BPPs produced by the snake venom glands, [4, 26] whose synergistic action is capable of causing a sharp drop in blood pressure of small animals, for instance mammalian preys.

The BPPs are molecules able to enhance some pharmacological activities of BK, as the action of contractile smooth muscle of guinea pig ileum evaluated in *ex vivo* assays [26], and also *in vivo*, acting in the CNS, cardiovascular, and antinociceptive systems [27, 28]. The isolation of the first BPPs expressed by the *Bothrops jararaca* venom glands was described in the early 60's, and they were initially coined as Bradykinin Potentiating Factors (BPF) due to their ability to potentiate the effects of BK ignoring at that time the fact that these molecules were composed by amino acid residues [26]. Only in early 70's, when their primary sequence were determined, which allowed to characterize them as peptide molecules, they were re-named as BPPs [3]. Since then, several peptides presenting similar structural characteristics have been identified from the venom of these snakes and also from other snakes belonging to several different genus [12, 13, 29-31]. Interestingly, they had also been described in wasps and frogs [8-11]. Typically, the BPPs are peptides of 5-14 amino acid residues [32]. In general, all known naturally occurring BPPs could be classified into two groups: (i) peptides of small molecular size like BPP-5a from the venom of *Bothrops jararaca*, whose structural characteristic is a pyroglutamic acid at the N-terminal and a proline residues at the C-terminal of molecule, and (ii) peptides consisting of about ten amino acid residues, with a pyroglutamic acid at the N-terminal and a notable high content of proline residues [32], which gives to them some resistance to hydrolysis by amino-peptidases, carboxypeptidases, and also endopeptidases [33].

2.1. cDNA cloning, identification and characterization of BRPs

The BK and its related peptides, *e.g.* the BRPs, are widely found in venomous animals, for instance in snakes, lizards, frogs, and insects [10, 13, 34]. In general, they include several sequences, either showing only one single amino acid substitution compared to BK or, in some cases, presenting just a frugal sequence similarity, but with unquestionable biological/functional correlation, for instance, acting on the same pathway or even same target protein. In fact, these sequence variations were verified either by *de novo* sequencing of several BRPs found in snake venoms [32] or by analysis of the deduced amino acid sequences of cDNAs cloned from venomous glands [12, 14, 15], and in some cases by using both strategies [30, 34].

The pharmacological evaluations revealed that even acting in the same pathway, they can show distinct biological activities compared to BK, including potentiating its effects by inhibition of its degradation or by acting on receptors and/or molecules involved in the BK signaling pathway, including activating or blocking the BK receptors [10, 35]. As such, the BRPs also include BPPs and the Bradykinin Inhibitor Peptides (BIPs) [13, 29, 32, 36].

2.2. BPPs and BIPs

Helokinestatsins are a family of proline-rich peptides (PRPs) found originally in the lizard venom (*Heloderma suspectum*) that display the function of inhibiting the BK actions on the vascular smooth muscle [35]. Synthetic replicates of all helokinestatsins were found to antagonize the relaxation effect observed following BK application to a rat arterial smooth muscle preparation, and hence, represent a family of BRPs also known as BIPs [34].

In contrast, BPPs firstly described and isolated from the venom of the Brazilian snake *Bothrops jararaca* are mainly known due to their ability to potentiate the biological effects of BK [3, 26]. These BRPs are one of the most outstanding group of PRPs, as they were used as structural and functional template/model for the development of a drug employed up to now for the treatment of human hypertension [6].

Although functionally related to the BK and also present with the NPs in the same precursor protein, the helokinestatsins are quite different from the snake venom BPPs [12, 30, 31, 37-39]. PRPs with the same BK inhibitory characteristics have also been described in the 'venomous' secretion of two species of anguillid lizards, the *Texas alligator* (*Gerrhonotus infernalis*) and the *Giant Hispanolian galliwasp* (*Celestus warreni*) [38]. Although the primary structural variation of the peptides from these species, they share several common features [34]. For instance, they are peptides rich in prolyl residues (30-50%), which confer rigidity and order to the spatial structure features, and also a measure of resistance to generalized proteolysis. They all possess a Pro-Arg dipeptide motif at the C-terminus, which is quite different from the C-terminal Ile/Val-Pro-Pro motif present in most BPPs C-terminus extremity [12]. The high degree of conservation of these structural core features across phylogeny suggest a fundamental biological function for this group of peptides in the lizards venoms. Among the two closely-related species of helodermatid lizards, several helokinestatsins have fully-conserved primary structure, while several others present different sequences. Similarly to the BRPs from amphibian skin [40] and snakes venom [13, 14, 30], helokinestatsins compose tandem repeat domains in their respective precursor proteins, probably reflecting discrete exons within the genomic DNA. As already mentioned, some tandem repeats are composed by identical primary structure, while some others exhibit significant amino acid substitutions. prominent And this process of exon multiplication might facilitate the molecular diversity, by permitting the expression of site-mutated isoforms, which is a phenomenon often described for bioactive peptide-encoding genes, as also observed for the glucagon gene in vertebrates [41].

Cloning and alignment of cDNAs encoding BRPs precursors from the venom gland and brain of a pit viper have allowed observing a higher degree of sequence conservation for the regions not including the bioactive peptides, and a higher variation in the primary structure of these biological active peptides [14]. These results were shown to be in good agreement with the

accelerated evolution hypothesis suggested by Ohno and colleagues [42]. According to this hypothesis, the more frequent occurrence of nucleotide nonsynonymous substitutions in the coding regions compared to the untranslated regions (UTRs) of the genes allows specific genes to evolve in an accelerated fashion to attain unique physiological activity. On the other hand, despite the consequent changes in the BRPs sequences observed, both the high content of proline residues and the biological activities correlated to BK effects are still maintained (Figure 1). Another highly conserved region involves the sequence of the NPs always present in C-terminus extremity of all known BRPs precursor proteins [12-15, 30, 31, 34, 36].

2.3. BRPs and NPs

The NP system consists of three types of hormones [atrial NP (ANP), brain or B-type NP (BNP), and C-type NP (CNP)], and three types of receptors [NP receptor (R)-A, NPR-B, and NPR-C]. Both ANP and BNP are circulating hormones secreted from the heart, whereas CNP is basically a neuropeptide. The NP system plays pivotal roles in cardiovascular and body fluid homeostasis. The ANP is secreted in response to an increase in blood volume, and acts on various organs to decrease both water and Na⁺, resulting in restoration of blood volume. The family of NPs were originally Na⁺-extruding hormones in fishes; however, they evolved to be volume-depleting hormones promoting the excretion of both Na⁺ and water in tetrapods, in which both are always regulated in the same direction. Vertebrates expanded their habitats from fresh water to the sea or to land during evolution. The structure and function of osmoregulatory hormones have also undergone evolution during this ecological evolution [43].

Members of the NPs family have been detected in several snake venoms, and they have been shown to be located in the same precursor protein containing multiple BRPs sequences. While this organization was demonstrated for many species of viperid snakes, including members of the genera *Bothrops*, *Crotalus*, *Lachesis*, *Agkistrodon*, and *Trimeresurus* [12, 13, 15, 30, 31], it may extend to some other taxa such as *Bitis gabonica*, that was also shown to have BPPs in their venom [12, 13, 15, 30, 31, 44]. The presence of NPs in some elapid snakes venom (*Dendroaspis*) has also been described [45]. It has been shown that the venom-derived NP precursors from helodermatid lizard have a structural organization similar to that found in many BRPs precursors from viperid snake venoms. However, the additionally encoded tandem-repeat peptides are non-canonical BPPs, based on their primary structural characteristics or in terms of the amino acid cleavage site, presenting characteristics of a recognition site typical of propeptide convertase enzymes, that eventually might be the potential responsible for the release of the mature BIPs from the respective biosynthetic precursors [36]. However, the BPP/CNP biosynthetic precursors of the bushmaster (*Lachesis muta*), the tropical rattlesnake (*Crotalus durissus terrificus*), and the massasauga rattlesnake from desert (*Sistrurus catenatus edwardsi*) showed that, in addition to the classical BPPs and a NP sequence, they all also encode single copies of a BIP exhibiting a closer structural similarity, and a propeptide convertase cleavage site that allows the release of the BIP helokinestatsins, whose sequences are [TPPAGPDVGPR] or [TPPAGPDGGPR] [36] (Figure 1). On the other hand, putative helokinestatsins peptides could not be identified in the BPP/CNP precursor of snakes as *Bothrops jararaca*, *Bothrops jararacussu*, and *Agkistrodon blomhoffi* (Figure 1). The phylogenetic analysis

presented here separates NPs precursor of different species into three distinct groups, those which contain in their precursor sequence (i) only BPPs, (ii) only BIPs, and/or (iii) both BRPs, *i.e.* BPPs and BIPs (Figure 1 and 2), suggesting that mutations in the coding regions of BRPs were important for the adaptative changes along evolution of the venom system [40].

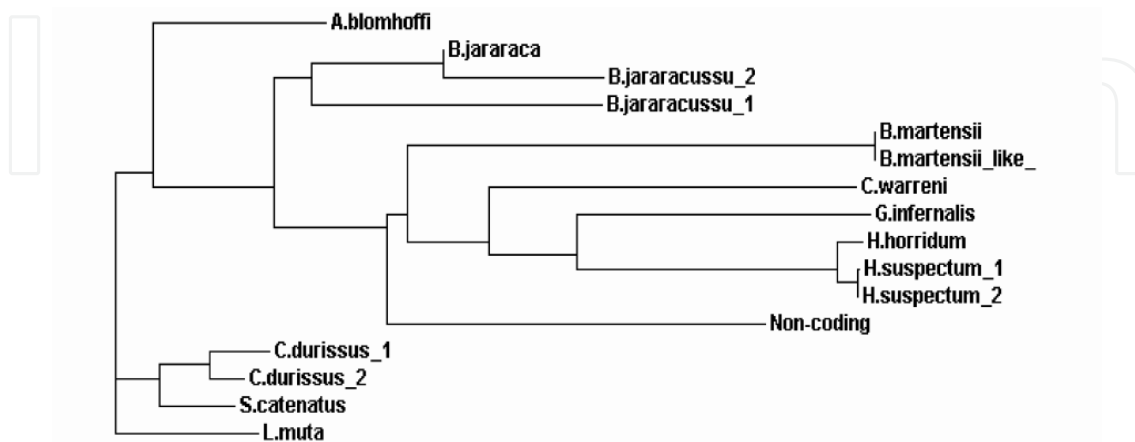


Figure 2. Phylogenetic tree based on BRP/NPs precursors. The phylogenetic analysis was performed using T-Coffee - Multiple Sequence Alignment available at <http://www.ebi.ac.uk/Tools/msa/tcoffee/>. In this analysis, protein sequences of BRP/CNP precursors from reptiles were used. The NPs precursor of *A. blomhoffi*, *B. jararaca*, and *B. jararacussu* contain only BPP sequences; *C. warreni*, *G. infernalis*, *H. suspectum*, and *H. horridum* contain only BIP sequences and, *C. durissus*, *L. mutta*, and *S. catenatus* contain both BRPs sequences, *i.e.* BPP and BIP. Despite the aligned *B. martensii* sequence was partial, and therefore does not contain the sequence coding for BRPs, this species was found to express the closest related precursor sequence to those containing only BIPs and to the *B. jararaca* non-coding RNA homologous to BPP/CNP precursor.

2.4. Molecular evolution of genes encoding BRPs

Here, we take BPP/CNP and helokinestatin/CNP precursors as examples to illustrate the evolution of a gene, since BPP/CNP precursor is also expressed in other tissues of *Bothrops jararaca* besides the venom gland, including brain and spleen [14, 15]. A high similarity was observed for the BPP/CNP cDNAs isolated from brain and venom gland, although they are not identical to each other as it should be expected [14]. Three out of the five BPP isoforms present in the brain precursor (BPPs of 5, 10 and 13 amino acid residues) were identical to those found in the venom gland precursor [14]. Moreover, most of insertions/deletions and point mutations were observed within the BPP/CNP coding region, suggesting an effect of a Darwinian-type accelerated evolution frequently observed intra-specie [42, 46]. This process has been widely observed, since a number of neuropeptides and hormones, such as the NPs [15, 47] and the vascular endothelium growth factor [48] evolved into toxins in the venom gland of poisonous animals.

It is believed that BRPs from the venom may be considered the toxin counterparts of endogenous peptides. It has also been suggested that both CNP and BPPs could be physiologically associated, to perform fluid homeostasis and regulation of the vascular tonus, since BPP/CNP precursor are present in regions of the snake brain showed to be involved in the control of these activities, as described for the mammalian CNS [14].

It is known that in the process of evolution, several mutations may occur in the genes, some of which not affecting the mature protein sequence, while others might lead even to the generation of messenger RNAs that are not translated into proteins. In 2000's it was first shown that non-coding RNAs can be involved in several roles including repression of genes, catalysis, regulation of the development process, among others [49]. A non-coding mRNA showing a sequence similarity to the BPPs precursor of the pit viper *Bothrops jararaca* was cloned by us from the venom gland [Genbank Acc. No. AY310916.1]. This long RNA sequence does not encode a protein, since several stop codons were observed for all possible reading frames (Figure 3).

[illegible]

Figure 3. Potential deduced amino acid sequences from the all possible frames of the non-coding RNA homologous to the BPP/CNP precursor coding RNA both from *Bothrops jararaca*. Total mRNA obtained from *Bothrops jararaca* venom gland higher than 2 Kb length was used to construct the long cDNA library. The complete cDNA sequence of the BPP/CNP precursor (clone NM 96) was used as template to screen about 2×10^6 clones, allowing the identification of four independent positive clones containing identical inserts of approximately 3.5 Kb [15]. All potential frames of the *Bothrops jararaca* BPP/CNP-related pseudogene mRNA presents a high content of stop codons (bold), as shown by the representative amino acid deduced sequence from one of the possible reading frames. No signal peptide could be observed, although several methionine (Met) residues (underlined) that does not seem to represent an initial of translation were found.

These non-coding RNAs are usually transcribed by a gene known as a pseudogene, which are often found in the genomes of several life forms, including bacteria, plants, insects, and vertebrates [50]. The pseudogene is a sequence that is present in the genome, and it is typically characterized by presenting high similarity with one or more functional gene paralogs. The pseudogene can be derived from gene duplication occurred by two different pathways: retrotransposition or duplication of genomic DNA [50].

The regulation of the expression of a functional gene showing sequence similarity to a pseudogene has been reported [51]. Generally, the sequence similarity between functional genes and pseudogenes is observed at the 5' UTR fragment. However, for the comparison of the non-coding RNA and the RNA coding for BPP/CNP precursor, a high similarity was observed only for the 3' UTR sequence (Figure 4). Moreover, the non-coding RNA is of approximately 3.5 Kb, while the RNA coding for the BPPs precursor is of about 1.8 Kb, and its RNA expression was found to be about 6-fold higher than that of the 3.5 Kb non-coding RNA.

Nevertheless, it is also possible that non-coding RNA of 3.5 Kb [GenBank Acc. No. AY310916.1] may also ensures the stability of the functional coding messenger RNA, since the stability of messenger RNAs is preferably controlled by factors present in the 3' UTR region [52].

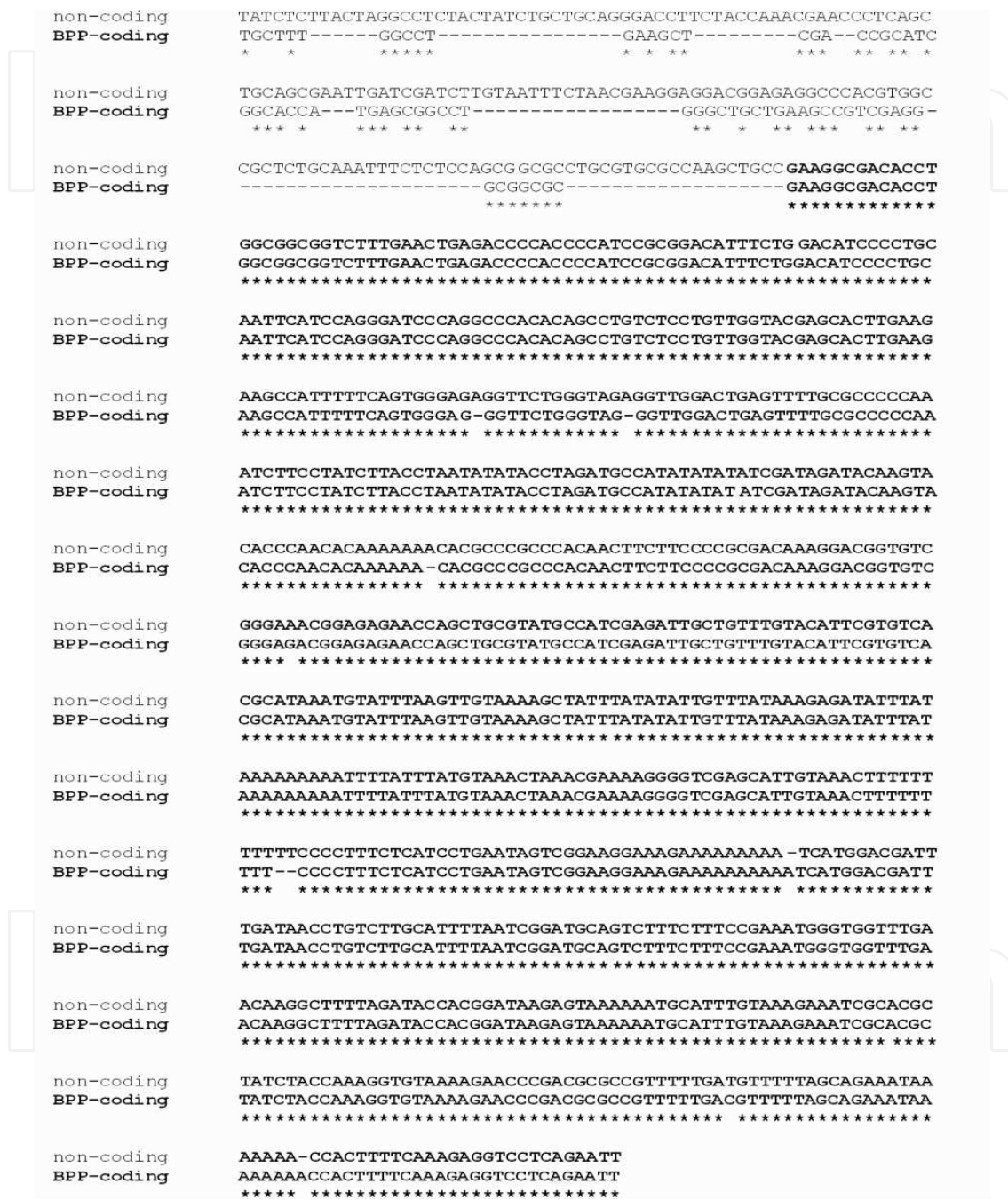


Figure 4. Partial sequence alignment of the *Bothrops jararaca* BPP/C NP-related pseudogene mRNA and mRNA coding for BPP/CNP precursor. Alignment of the nucleotide sequences of a segment of the pseudogene mRNA (non-coding: upper sequence) and the mRNA coding for BPP/CNP precursor (BPP-coding: lower sequence) was performed using the Clustaw W program, available at <http://www2.ebi.ac.uk/clustalw/>. Identical nucleotides are indicated by "*" and insertions or deletions are represented by gaps (-). The boldface type letters indicate the region with higher similarity between the RNA sequences, corresponding to about 97% identity in this region.

In the BPP/CNP precursor of *Bothrops jararaca*, the pentapeptide BPP-5a [QKWAP] that was used as template for the development of the antihypertensive drug captopril, is found duplicated, *i.e.*, there are two copies of the same peptide in a single precursor protein. It is believed that this peptide might have a special importance in the venom of snakes belonging to the *Bothrops* genus, since it is also found repeated three times in isoform 1 [GenBank Acc. No. AY310914.1], and four times in isoform 2 [Genbank Acc. No. AY310915.1] of the precursors isolated from *Bothrops jararacussu* venom glands (Figure 5). In fact, BPP-5a is a potent potentiator of the BK effects in isolated guinea pig ileum, and also *in vivo* [29].

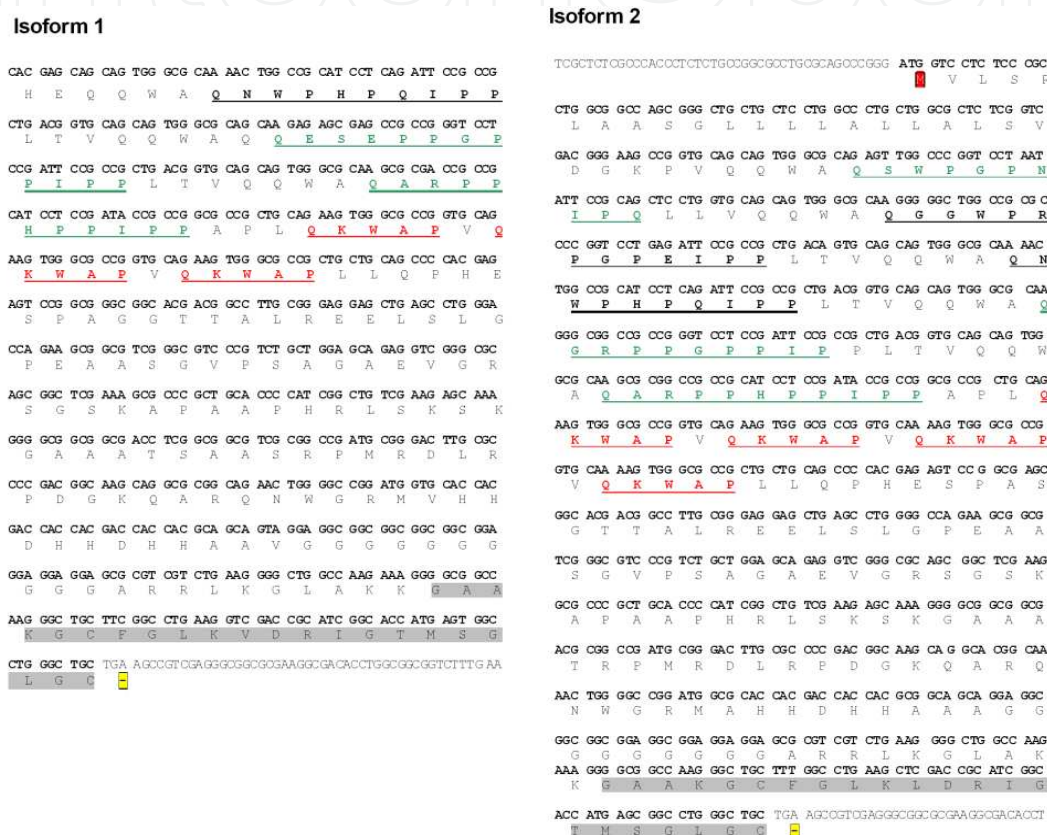


Figure 5. Partial nucleotide and amino acid sequences of the BPP/CNP of precursor from *Bothrops jararacussu* (isoform 1 and 2). Shaded in grey, the amino acid sequences of the C-type natriuretic peptide (CNP). Sequences of new putative BPPs are shown in green, and the underlined sequences correspond to other previously known BPPs. In red, the pentapeptide BPP-5a that was found in duplicate in the pit viper precursor and, triplicate and quadruplicate in the isoforms 1 and 2, respectively, of the precursor from *Bothrops jararacussu*. Symbol "M" represents the initial methionine and (-) the stop codon.

3. BRPs as structural model for drug development

The discovery of the potential inhibitory action of BPPs on ACE brought a great interest in these natural peptides, since the importance of ACE in blood pressure control and the urge to develop a therapy for cardiovascular disease, as hypertension, was imminent [2].

At that time, among the identified peptides were BPP-9a, under the generic name of teprotide, and the BPP-5a, which was also one of first BPP to be characterized. Assays using these peptides showed that BPP-9a was more effective and had a longer lasting effect in blood pressure compared to BPP-5a [53]. Therefore, BPP-9a was used in the first clinical demonstration of the potential use of BPPs for the hypertension control in humans, showing a significant antihypertensive effect [54, 55].

However, on that time it was demonstrated that the therapeutic utility of BPP-9a was limited by the lack of activity by oral administration and the high cost of its synthesis [54, 56, 57]. Therefore, the pharmaceutical development of a non-peptide inhibitor of ACE orally effective was essential. Thus, molecular structure of the BRPs, namely BPP-5a and BPP-9a, were studied by Cushman and Ondetti [3, 58], who suggested specific interaction of the proline, present at the C-terminal of these peptides, with the ACE active site [59]. Thus, captopril was synthesized by simple addition of a chelator radical to a dipeptide containing a proline residue (BPP carboxy-terminal amino acid) [59]. Undoubtedly captopril was a blockbuster drug that inspired the creation of generations of mimetic antihypertensive compounds [2].

4. Biological activities of BRPs

4.1. Interference of BRPs in the renin-angiotensin and kallikrein-kinin system

The ACE (EC 3.4.15.1) is mainly expressed in vascular endothelium in epithelial cells of the proximal tubules of the kidney, brain, and intestinal cells [60]. This enzyme is responsible for conversion of angiotensin I (Ang I) to angiotensin II (Ang II), and for the degradation of BK. Therefore, this enzyme has roles in both renin-angiotensin and kallikrein-kinin system [61].

The renin-angiotensin system (RAS) is composed by a set of peptides, enzymes, and receptors, that are involved in the control of the extracellular fluid and blood pressure [62]. The formation of the effector peptide of this system occurs initially by the action of the renin released by the kidneys [62] that acts on the angiotensinogen produced in the liver [63]. This leads to the generation of the decapeptide Ang I, which then is cleaved by ACE to form the octapeptide Ang II, a potent antihypertensive molecule [64]. Ang II actions is mediated by the angiotensin receptors AT1 and AT2. The binding of Ang II to the AT1 receptor triggers several cellular processes, among them vasoconstriction, protein synthesis, cell growth, regulation of renal function, and electrolyte balance [65]. Ang II also acts as a neurotransmitter and as a neuroregulator, modulating the central control of the blood pressure, influencing the sympathetic activity, salt appetite, and thirst [65].

The kallikrein-kinin system (KKS) is a metabolic cascade in which the tissue and plasma kallikrein release vasoactive kinins from both high and low molecular weight kininogens. The nonapeptide BK, derived from the cleavage of the high molecular weight kininogen by kallikrein, is the major plasma kinin playing a role in the KKS [66].

Kinins are involved in various physiological and pathological processes, including vasodilation, increased vascular permeability, release of plasminogen activator of tissue type (t-PA),

and nitric oxide (NO) and arachidonic acid metabolism, mainly due to their ability to activate endothelial cells [66]. Thus, the kinins participate in the physiological regulation of blood pressure, cardiac and renal functions, and also in pathological processes as inflammation [66].

The several pharmacological activities of kinins are mediated basically by their binding to two types of specific receptors (B1 and B2 receptors), prior to their fast metabolism by various peptidases [67].

Actions such as vasodilation and hypotension are mediated by the B2 receptor by releasing of NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). On the other hand, the actions mediated by the B1 receptors include important roles in angiogenesis, inflammation, and septic shock [68]. Moreover, unlike B2 receptor, B1 receptor is not constitutively expressed, and its expression is induced by mediators of inflammation in conditions of injury [68].

The primarily responsible for the degradation of BK are the peptidases (zinc metallopeptidases) including ACE [67]. Since the early 90's, it is well known that somatic ACE has two active sites, the N-terminus (N-site) and the C-terminus (C-site) active sites [69]. Although *in vitro*, the two active sites are equally effective to convert Ang I to Ang II, as well as to degrade the BK into BK₁₋₇ and BK₁₋₅ [70], the N-site is several times more effective to hydrolyze other bioactive peptides, such as the AcSDKP, a negative regulatory factor for differentiation and proliferation of hematopoietic stem cells [71].

Thus, ACE inhibitors as BPPs inhibit not only the generation of Ang II, but also potentiate the effects of BK, by inhibiting its degradation. Therefore, the physiological effects of the angiotensin system are decreased (since there is no formation of Ang II), and the physiological effects of KKS are potentiated (due to inhibition of the BK degradation). In contrast, the BIPs, most known as helokinestatsins, inhibit KKS by blocking the B2 receptor (Figure 6).

4.2. Mechanisms of action underlying the antihypertensive effect of BRPs

Although ACE inhibition is a relevant mechanism to explain the activity of most BPPs, and despite of their high primary sequence similarity [53, 72], as previously suggested, the BPPs show remarkable wide variety of mechanistic pathways that could explain the antihypertensive activity of BPPs at molecular level [13, 14, 19, 20, 22, 23, 29, 73-76]. Definitely the biological effects of BPPs and the consequent pharmacological importance of their activity are not limited to and it cannot be explained solely based on their ability to inhibit ACE [2].

The differences were first observed when comparing the selectivity of the BPPs encoded by the neuronal BPP/CNP precursor protein [*e.g.* BPP-5a, BPP-10c, BPP-11e, BPP-12b, and BPP-13a] [14] by the different active sites of the somatic ACE and the corresponding biological activity of these peptides evaluated by their ability to potentiate the contractile effect triggered by BK in isolated guinea pig ileum. For instance, the BPP-5a was shown to be much less effective ACE inhibitor compared to BPP-13a, although presenting one of the most potent potentiator effects of BK in *ex vivo* experiments. In contrast, BPP-10c is an excellent selective inhibitor of the C-terminal active site of somatic ACE, and its BK potentiating effect is very similar to that observed for both BPP-5a and BPP-12b, which were shown to be selective for

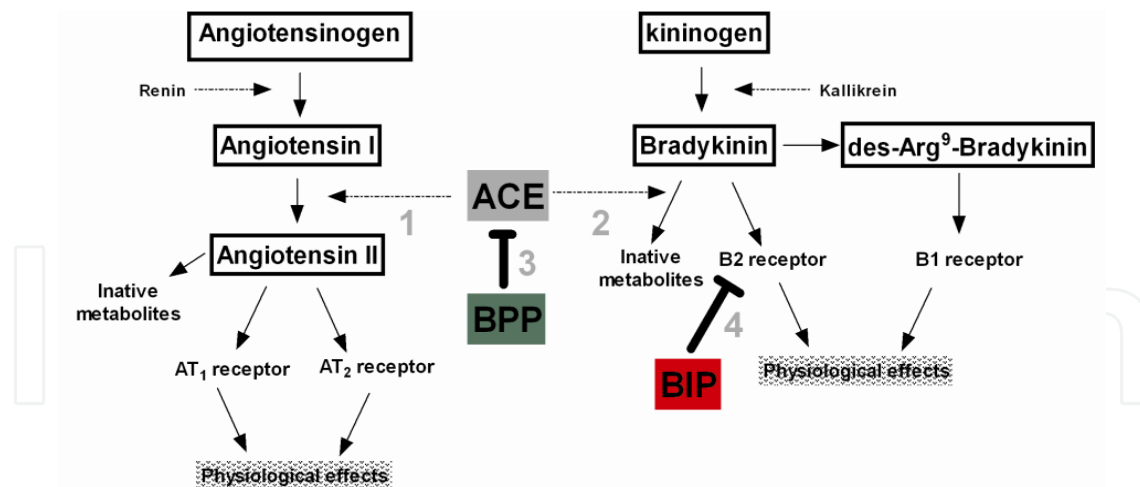


Figure 6. Schematic representation of ACE roles on the renin-angiotensin and kallikrein-kinin systems, and the potential sites for interference by BRPs (BPPs and BIPs). A) Conversion of angiotensin I into angiotensin II, 2) BK degradation 3) ACE inhibition by BPPs. 4) B2 receptor antagonism by BIPs. Physiological effects on the renin-angiotensin system mediated by AT₁ receptors include vasoconstriction, sodium and water retention, release of aldosterone, increased sympathetic nerve activity, among others, while those mediated by AT₂ receptors include cell differentiation, vasodilation, among others. The effects on the kallikrein-kinin system, mediated by kinins action on B2 receptor include vasodilation and hypotension via release of NO, prostacyclins and endothelium-derived hyperpolarizing factor (EDHF). Due to the ACE inhibition by BPPs, the physiological effects of angiotensin system are decreased (with no formation of angiotensin II) and the physiological effects of kallikrein-kinin are potentiated (by inhibition of BK degradation). In contrast, BIPs action on B2 receptor blocked BK effects. Adapted from [132-134].

the ACE N-terminal active site. In the same way, besides the weak BK potentiation effects of BPP-11e, it is also not among the best inhibitors of ACE, and no preference for any of the active sites of ACE was observed for this peptide [29].

Later on, in 2007, molecular studies of the antihypertensive activity of the BPPs, namely BPP-7a and BPP-10c, brought noteworthy information on the molecular mechanism underlying the action of these peptides at cellular level. In fact, these BPPs have a strong and sustained antihypertensive activity in awake spontaneously hypertensive rats (SHRs), but they do not prevent the formation of Ang II from Ang I *in vivo*, showing that they do not need to affect the physiological functions of ACE to promote the decrease of the blood pressure in these animals. Furthermore, for BPP-10c, we have also shown that the dose necessary to produce the antihypertensive effect is lower than that required to inhibit ACE *in vivo* [77], suggesting the participation of other putative targets determining this particular pharmacological effect.

This finding was reinforced by the studies conducted to clarify the biological distribution of BPP-10c using a I¹²⁵ labeled analog, which showed that this peptide accumulated in various rat organs such as brain, liver, testis, and kidney, even after pre-saturation of the potential active sites of ACE with a specific inhibitor of this enzyme, namely captopril [78].

This stimulated us to conduct studies aiming to identify new potential molecular targets for snake BPPs. So, it was shown that at least three BPPs, namely BPP-10c, BPP-12b, and BPP-13a, are able to bind to the enzyme argininosuccinate synthase (AsS) modulating positively its activity [20, 75].

The AsS is the rate-limiting step enzyme responsible for providing the substrate for the nitric oxide synthase (NOS) that produces NO [79, 80]. Guerreiro and colleagues also demonstrated that blood pressure decrease promoted in SHRs by BPP-10c administration is due to the increased bioavailability of L-arginine required for the production of NO [20], which is a potent vasodilator agent [81]. Later it was demonstrated that other BPPs also induces NO production to determine the antihypertensive effect [21, 75].

Moreover, at least for the BPP-5a-induced NO production, the involvement of both B2 receptor and mACh-M1, without any involvement of AsS, was recently demonstrated [23]. BPP-13a induces NO production through a mechanism that involves activation of subtype M3 muscarinic receptor (mACh-M3), triggering the raise of the free intracellular calcium concentration ($[Ca^{2+}]_i$) that is able to activate NOS and to provide the substrate for NO production by modulating the AsS activity [75].

Both BPP-11e and BPP-12b do not stimulate NO production, but the $[Ca^{2+}]_i$ mobilization assays suggest that these peptides are agonists of a membrane receptor involved in the release of EDHF, and other functions involving the modulation of gene expression and activation of different NOS enzymes is expected [82]. As BPP-12b modulates positively AsS activity only at very high concentrations, this should not be its main mechanism of action [75].

Since the BPP-9a has ACE as main target for its biological actions, based on its potent inhibitory activity against this enzyme also showing selectivity for the C-terminal active site [73], we suggest that it is possible to suggest this pathway to explain the antihypertensive effect and BK potentiation of BPP-9a (teprotide). Moreover, it has no effect on the AsS induced intracellular calcium and it also does not interfere with NO production.

Apparently all BPPs share the ability to decrease arterial pressure [21, 56, 75, 77, 83], through the amplitude of the antihypertensive effect caused by BK, each related peptides is different. But, unfortunately, the mechanisms of action of other BPPs are still less understood up to now [75].

4.3. Peripheral and central biological activities of BPPs

Changes in mean arterial pressure (MAP) promoted by some BPPs are accompanied by a significant reduction in heart rate (HR) [23, 75, 77] rather than by an HR increase, as it would be expected by the response of the baroreceptors to the hypotension [84]. The fact is that *in bolus* injections of BPPs decrease both MAP and HR of awake SHRs, and BPPs expression in the same precursor protein of a brain expressed peptide as CNP suggests a CNS role for these peptides. In fact, recently it was shown that the BPP-10c is able to promote the release of the neurotransmitters GABA and glutamate, which are known to participate in the regulation of cardiac and vascular autonomic systems, leading to decline MAP and HR of SHRs [22]. According to Lameu and collaborators, BPP-10c-induced decrease of MAP results from this BRP-induced interference in the autonomic nervous system, provoking subsequent changes in HR and baroreflex control [22, 74].

Arterial baroreflex is one of the most important regulatory mechanisms in the cardiovascular system, mainly by triggering a coordinated sympathetic and parasympathetic tone response on the heart and vessels [85-90].

The CNS is connected to the heart through two different groups of nerves, the parasympathetic and sympathetic systems. Stimulation of parasympathetic nerves determines the decrease of HR, of contraction force of atrial muscle, and of conduction of impulses through the atrioventricular node, and at the same time, it also causes the increase of the time delay between the atrial and ventricular contraction, and the reduction of blood flow through the coronary arteries, which maintains the nutrition of the myocardium. All these effects can be summarized by saying that the parasympathetic stimulation decreases all the activities of the heart. On the other hand, the stimulation of sympathetic nerves has exactly the opposite effects on the heart, leading to an increased HR, increased contraction force, and increased blood flow through the blood vessels [87, 91].

It was observed that BPP-11e causes a slight reduction in MAP, but surprisingly with a strong reduction in HR [75], suggesting a BPPs action in specialized muscle cells located in the sinoatrial region (pacemaker) of the heart, which is a special region of the heart that controls the cardiac frequency [92]. Although the heart has its own intrinsic control systems, it can operate under neural influences, therefore effectiveness of the cardiac action can be significantly modified by regulatory pulses from the CNS [92]. Thus there is also possible that the BPP-11e has an effect on the stimulation of the parasympathetic system and/or in the decreasing of the sympathetic system stimulation, leading to a reduction of the HR and a slight decrease in MAP, observed after *in vivo* injection of this peptide [75].

A more detailed study of the BPPs effects on the CNS was performed for the BPP-10c, in which intracerebroventricular administration was shown to produce similar effects to those observed for higher doses injections of this peptide by intravenous route. In our interpretation, this data suggested the involvement of the CNS in the pathway underlying these biological effects [74].

Aiming to explain the BPPs effects on CNS, *Lameu et al.* have also conducted studies to demonstrate that the BPP-10c acts through activation of an unidentified $G_{i/o}$ -coupled receptor present in neuronal cells, and that this effect was independent of both ACE inhibition and B2 receptor activation. Peptide-receptor binding resulted in the activation of calcium influx and release of intracellular calcium by calcium-induced calcium release (CICR) mechanism, which was shown to involve the activation of the ryanodine- or IP3-sensitive calcium stores and also the inhibition of adenylate cyclase [74]. However the specific target GPCR could not be identified yet.

On the other hand, affinity chromatography, using immobilized BPP-10c, associated with mass spectrometric and immunoblot analyses, allowed the identification of two important targets of BPP-10c, namely the AsS in the kidney cytosol [20] and the synapsin in the brain (Figure 7) [93]. AsS, together with argininosuccinate lyase (AsL), is part of the urea cycle in the liver and of the arginine-citrulline cycle, the major source of arginine in the renal cells and citrulline-NO cycle, which is the main source of NO in other cells, including endothelial and neuronal cells [94].

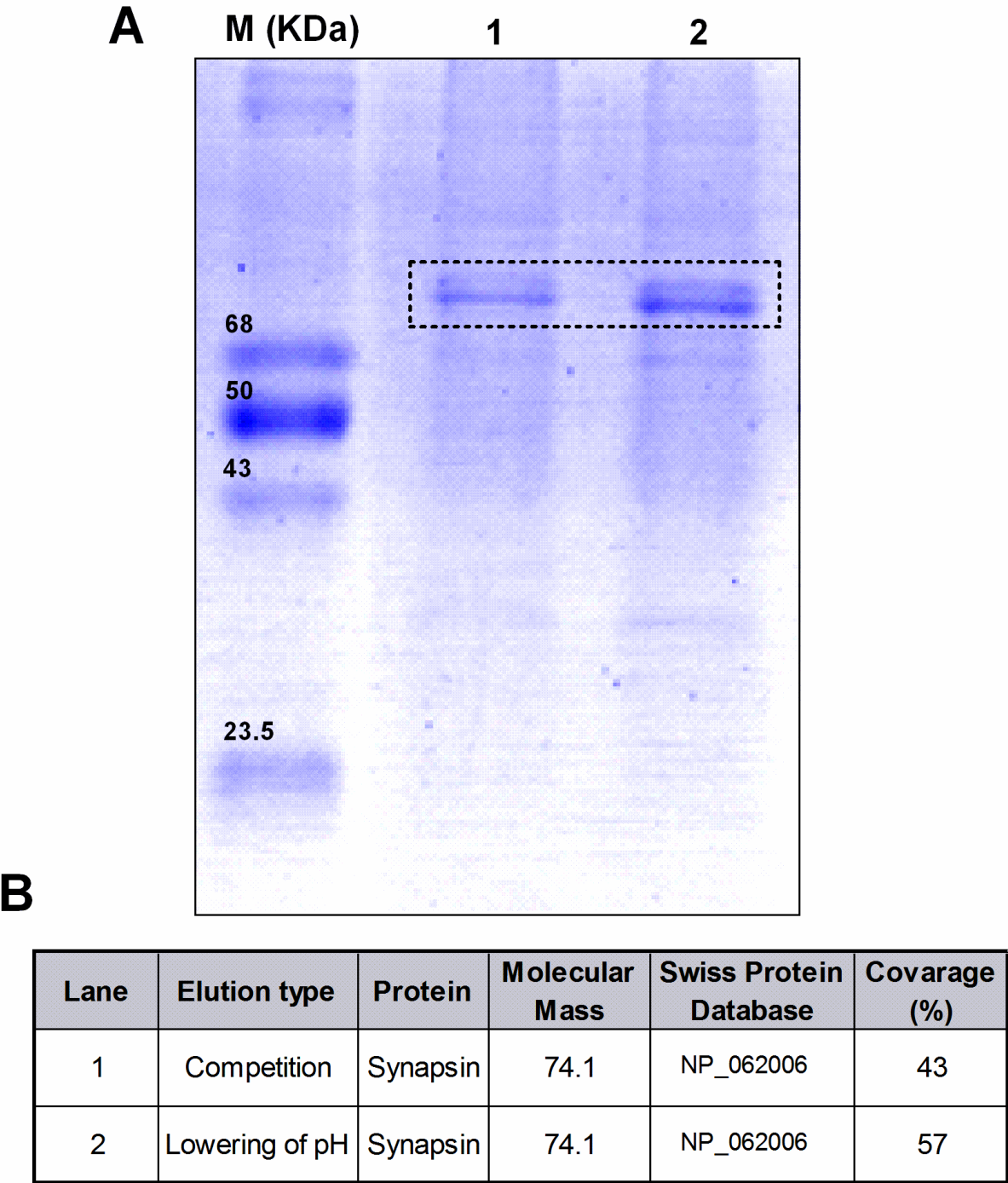


Figure 7. Synapsin binds to BPP-10c. (A) SDS-PAGE analysis of brain rat cytosolic proteins submitted to HiTrap-BPP-10c affinity chromatography. Rat brain cytosol preparation to affinity chromatography using the HiTrap NHS-activated HP resin to which BPP-10c was immobilized by chemical conjugation **M (KDa)**, molecular mass markers; **lane 1**, protein eluted by competition using 5 mg of BPP-10c; **lane 2**, protein eluted with 100 mM glycine, 0.5 M NaCl pH 3.0 (elution buffer: by lowering the pH). (B) protein identification by mass spectrometric analysis of the bands enclosed in the box in the panel A. The 74-kDa major protein that bounds to BPP-10c was identified as synapsin, by trypsin digestion and peptide mass fingerprint analysis.

AsS is a ubiquitously expressed enzyme, present in many tissues, including brain and kidney [94]. *In vivo* BPP-10c administration in SHRs animal models results in increase of plasma arginine level [20] and augmented NO production in brain tissues, as well as in neuronal and endothelial cells [20, 22].

NO is generated in the citrulline-NO cycle by NO synthase (NOS) using L-arginine as a substrate. Three isoforms of NOS have been described: Ca^{2+} -dependent endothelial (eNOS) and neuronal (nNOS) isoforms [95] and inducible NOS. The expression and activity of the latter are induced by inflammatory stimuli, independent of the cytosolic Ca^{2+} concentration [96].

NO is mainly involved in the regulation of local and systemic vascular resistance in sodium balance, and hence in blood pressure control [97], since it is one of the smooth muscle relaxing factors released by the endothelium, which diffuses to the adjacent smooth muscle cells promoting vasodilatation [98, 99].

Nevertheless, NO has been attributed to other various functions, including non-cholinergic and non-adrenergic smooth muscle relaxation, reduction of arterial pressure, and signal transmission in the CNS [100]. NO-mediated actions in the CNS include central vascular regulation [101] and baroreflex control of HR [102]. Antihypertensive activity, based on the facilitated release of both GABA and glutamate in the CNS and NO production, is suggested to result in the diminished transmission of sympathetic tone to the periphery [101, 103].

Treatments with BPP-10c also induced an increase in AsS gene expression [22]. In contrast, nNOS was not found differentially expressed in the brains of SHRs treated with BPP-10c compared to vehicle-treated animals. On the other hand, the gene expression levels of eNOS, similarly to those of AsS, were found increased in the brains of SHRs animals treated with BPP-10c [22]. This data is in line with the results obtained by Kishi and colleagues [104] who were able to show that the overexpression of eNOS in the rostral ventrolateral medulla and the nucleus of the solitary tract of hypertensive rats results in reduced systolic arterial pressure and reduced HR.

However, the specificity of NO reactions with neuronal targets is determined in part by the precise localization of NOS within the cell. The targeting of NOS to discrete nuclei of neurons, mediated by adapter proteins, allowed to suggest that both synapsin and NOS participate of a ternary complex, which changes in the subcellular localization of NOS [105].

Knowing that the BPP-10c binds to synapsin in the CNS, we hypothesized the formation of a quaternary complex upon binding of BPP-10c with synapsin. The formation of this complex would direct the reactions of NO in neural targets, which would be determined in part by the location of this complex and by targeting NOS to specific sites of neurons [105].

We were able to show that BPP-10c is capable to induce intracellular Ca^{2+} signaling that involves the activation of GPCRs, NO production, and release of neurotransmitters, such as GABA and glutamate [22, 74]. The amino acid glutamate is the major excitatory neurotransmitter in the CNS of mammals, whereas GABA is the main mediator of sympathetic inhibitory currents. Both glutamate and GABA play key roles in the control of cardiovascular function

in the CNS [103]. Excitatory amino acid neurotransmitters, like glutamate and aspartate, generally cause pressure responses and tachycardia, while inhibitory amino acid neurotransmitters, namely GABA and glycine, are responsible for depressing bradycardia [104]. It is well established that the excitatory amino acid glutamate is considered the main neurotransmitter of primary afferent fibers of baroreceptors to the nucleus tractus solitarii (NTS) [106]. Furthermore, an excitatory projection from NTS to the caudal ventrolateral medulla (CVLM) is an essential part of the circuit of baroreflex control. The CVLM communicates with the rostral ventrolateral medulla (RVLM) by secretion of GABA. In addition to GABAergic inhibition of RVLM, excitatory amino acids are also known to exert important roles in cardiovascular regulation [107]. These neurotransmitters can regulate vasodilatation through reduction of both sympathetic activity and baroreflex sensitivity control, by acting on regulation of both sympathetic and parasympathetic systems. Therefore, the augmented baroreflex sensitivity by i.v. injection of BPP-10c is attributed to the release of these neurotransmitters [22, 74]. These data is summarized in Figure 8.

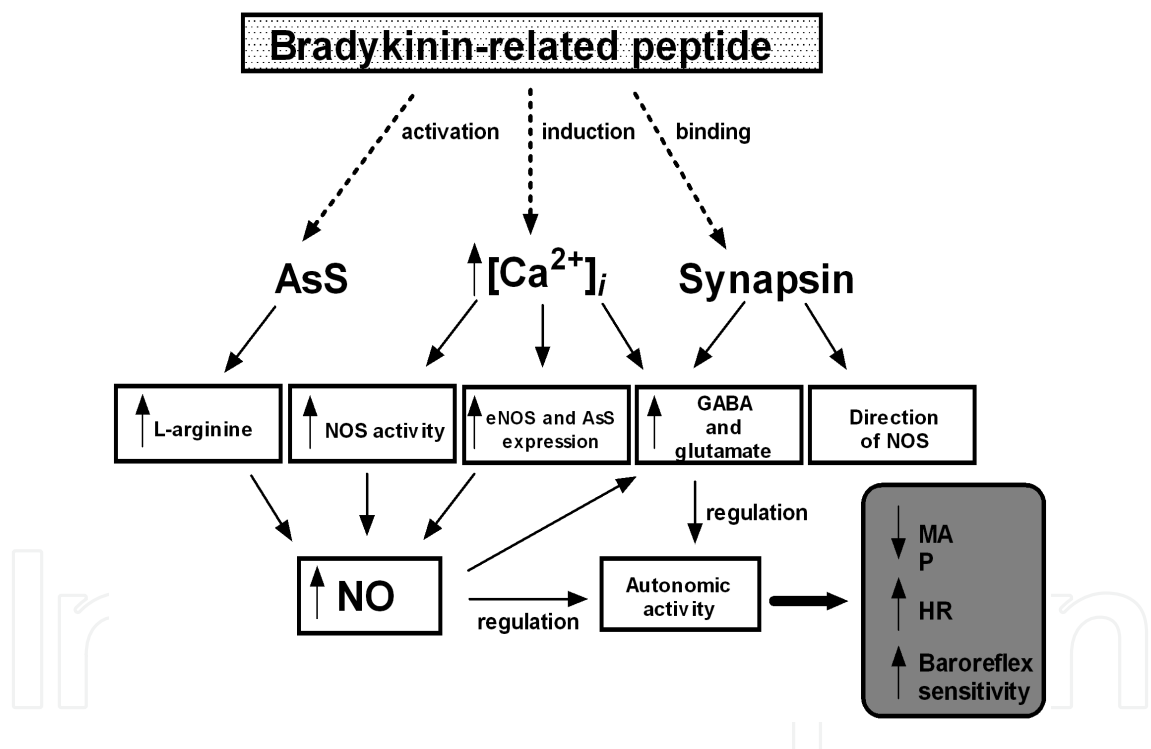


Figure 8. Schematic representation of BPP-10c mechanism of action in the CNS. According to [22, 74, 93], the proposed mechanism to explain the BPP-10c effects on the blood pressure (BP) and heart rate (HR) in spontaneously hypertensive rats (SHRs) was summarized in this figure. First, BPP-10c-induced $[Ca^{2+}]_i$ elevations activates signal transduction pathways responsible for the increased nitric oxide synthase (NOS) activity and the expression of the enzymes, namely endothelial NOS (eNOS) and argininosuccinate synthase (ASS). It also triggers the release of the neurotransmitters GABA and glutamate. After the BPP-10c internalization by neuronal cells, this peptide binds to synapsin to control the release of GABA and glutamate, and to direct the NOS to discrete cores of the neurons. Furthermore, BPP-10c can positively activate the AsS functions to increase the levels of L-arginine. NO production due to increased concentration of L-arginine, NOS activation, and increased expression of eNOS and AsS should contribute for the release of neurotransmitters and also for the regulation of autonomous activity. Likewise GABA and glutamate determine the reduction of both blood pressure and heart rate, and the increases of the baroreflex sensitivity in SHRs.

The fact of BPPs decrease the HR does not mean that its action is limited to the CNS. Taking the example of BPP-10c, *in vivo* biodistribution studies showed a significant presence of this peptide in the brain, however accumulation was also observed in the rat kidney [78]. Considering its high accumulation in kidneys with the fact that BPP-10c induces NO production in endothelial cells [20, 108] and the increase of plasma L-arginine level *in vivo* [20], we conclude that this peptide, and also potentially the other BPPs, may display both peripheral and central actions.

Moreover, there might exist BPPs with exclusive peripheral action. As suggested for BPP-9a that promotes discrete decrease of MAP and does not affect HR [75], whose effects were possible to be explained solely based on the classical mechanism of action suggested for the BPPs, *i.e.*, relying in the selective inhibition of the somatic ACE [73].

The pharmaceutical compositions for the applications in chronic-degenerative diseases and hypertension of the BPPs and their structural and/or conformational analogs, as well as the isolation and purification of BRPs secreted by snake venom glands are protected by the patents US20050031604 and US20080199503. The inventions further refer to pharmaceutical compositions that increase the bioavailability and efficacy of BRPs peripheral and central biological activities. BRPs allowed the development a successful oral drug to treat human hypertension, but they also have the potential to become a drug by itself or a drug model to develop compounds devoted to treat central nervous system diseases, once pharmaceutical compositions that allow efficient delivery *in vivo* of these BRPs is achieved.

4.4. Mechanism of action that underlies the hyperalgesia and inflammatory responses

For many years it has been known that BK is an inflammatory mediator involved in the nociceptive process [109]. BK and also the BRPs produce pain and hyperalgesia due to their ability to excite and/or sensitize nociceptors [10].

In particular, two novel BRPs named fulvonin [SIVLRGKAPFR] and cyphokinin [DTRPPGFTPFR] were recently described in wasp (*Cyphononyx fulvognathus*). They could be structurally and functionally considered as BRPs, since they both are able to inhibit ACE as well as to induce the hyperalgesic effect in living rats after intraplantar injection, mostly due to the agonist action of these peptides on distinct B2 or B1 receptors, respectively [10].

5. Potential and effective pharmaceutical applications

5.1. Application of BPPs to treat CNS disorders and hypertension

In the last few years, as presented here, it was possible to describe a number of new mechanisms of action for BRPs previously known only as potent ACE inhibitors. Taking this into account, many pharmaceutical applications could be possible suggested for these peptides solely based on the treatment of pathologies related with their targets, for instance the somatic ACE, AsS and so on.

Regarding AsS as a novel potential target for the development of new drugs based on BPPs structural model, it is worth mentioning that its action on L-arginine metabolims contributes to three main functions in the organism, depending on the cell or tissue type involved, including effects on detoxification of ammonia in the liver, production of L-arginine in the kidney to be distributed to the whole organism, and the synthesis of L-arginine for the production of NO in several other cells [94]. In addition to these three major functions, it has also been suggested that AsS plays an important role in neuromodulation by producing argininosuccinate [110].

Due to its involvement in biochemical processes that generate physiological impacts on the organism, AsS is of great clinical value since its deficiency or excessive expression has been associated with some diseases, such as citrullinemia [111], hypertension [112, 113], and Alzheimer's disease [114, 115].

Since, AsS also participates in the L-arginine recycling, which contributes to the maintenance of NOS substrate, and AsS catalytic activity is considered the limiting step for NO production [116], the upregulation of this enzyme restores the balance of that system with consequent reduction in blood pressure [20].

Moreover, the identification of acetylcholine receptors as novel putative targets responsible for the vasodilatation promoted by the BRPs [19, 23, 75] also opens new avenues to the development of possible future therapeutic applications of BRPs related compounds for CNS diseases treatment.

Different experimental approaches demonstrate that acetylcholine muscarinic receptors are present in virtually all organs, tissues and cell types. The muscarinic receptors in the CNS are involved in the regulation of an extraordinary number of cognitive, behavioral, sensory, motor, and autonomous functions. Reduced or increased signalling of different subtypes of muscarinic receptors are involved in the pathophysiology of several diseases of the CNS including Alzheimer's and Parkinson's diseases, depression, schizophrenia, and epilepsy [117].

The contribution of the muscarinic acetylcholine receptor, mAChR-M1, in the NO production stimulated by BPP-5a is therapeutically and scientifically interesting, since much effort has been undertaken in the search for mAChR-M1 agonists to treat cognitive disorders including the Alzheimer's disease [118, 119].

On other hand, the mAChR-M3 is mainly involved in the control of vascular tone. The main actions mediated by these peripheral muscarinic receptors include the reduction of HR, stimulation of glandular secretion, and smooth muscle relaxation [117]. Compounds that activate this receptor to promote vasorelaxation, such as pilocarpine, are used for the treatment of glaucoma, ocular hypertension [120, 121]. However, the BRPs as BPP-13a is not only able to activate mAChR-M3, but in the same time it acts modulating the AsS activity. Since both pathways contribute to the antihypertensive effects by controlling the NO production, BPP-13a represents a potent vasodilator compound with potential broader applications in the medicine [75].

Taking together, the comparison of the biological actions of BRPs found in the venom and brain of the pit viper *Bothrops jararaca* with those from different species, allowed us to describe

that, contrary to what was thought for several decades, these peptides have different biological effects and therefore are an inexhaustible source of powerful biological tools not only for the study and discovery of new physiological pathways, but also as potentially useful compounds for new drug development.

Therefore, a patent protecting the use of these oligopeptides capable of binding to diverse targets, determining the increase and the sustenance of nitric oxide (NO) production in mammalian cells by potentiating the endogenous argininosuccinate synthase activity present in animal cells and/or by increasing the intracellular bivalent free calcium ion in the cytosol of cells was filed (US20100035822). Pharmaceutical compositions containing one or more of these peptides is also described and also disclosed in this same patent.

5.2. Application of BIPs in the treatment of pain and inflammation

NO is involved in vasodilatation and in many other physiological processes. Several lines of evidence have indicated that NO plays a complex and diverse role in the modulation of pain [122]. It has been shown that NO mediates the analgesic effect of opioids and other analgesic substances, opening opportunities of potential use of molecules able to regulate NO production in pain therapy. Modification of pre-existing analgesic and anti-inflammatory drugs by addition of NO-releasing moieties has been shown to improve the analgesic efficacy of these drugs, and also to reduce their side effects [123].

NO donors have also been used with opioids to reduce pain in patients with cancer [124]. This strategy enhances the analgesic efficacy of morphine in patients with cancer pain, delaying the morphine tolerance and decreasing the incidence of the adverse effects of opioids [88, 125].

Nevertheless, the use of NO donors should be carefully evaluated, since the excessive levels of NO production can be deleterious to the organism [126]. Soon, keeping NO production at a safe level, avoiding the deleterious threshold, is of particular interest.

Therefore, the BRPs that modulate AsS activity [20, 75] could be considered for pain treatment. The fact that this AsS activity interferes with L-arginine metabolism, a source of NO synthesis that has its own levels subjected to very precise mechanisms of physiological control, represents the most likely target able to regulate NO production without generating undesired reactive by-products (review by [127]).

Other family of BRPs, which should be considered in the treatment of pain and inflammation, are the antagonists of BK receptors (BIPs), namely helokinestatsins. Although these BRPs were described as vasoactive peptides, due to their ability to antagonize the relaxation effect induced by BK [35, 36], they could potentially be employed in the therapy of hyperalgesia.

Kinins formed following tissue trauma and in inflammatory processes, acting by means of the activation of B2 receptors, are among the most potent endogenous algogenic mediators. Kinins through action on BK receptors can release a large number of inflammatory mediators, such as prostaglandins and neuropeptides such as neurokinins [128], that in turn amplify the nociceptive response. Therefore, these receptors play an important role in pain

transmission. BK produces a short-lived hyperalgesia, while des-Arg⁹-BK causes a long-lasting hyperalgesia [1].

However, most of the B₂ receptor antagonists present partial agonist activity and fail to produce antinociception when given orally [1]. Non-peptide B₂ receptor antagonists, although they are generally less potent when compared with Hoe 140, for instance, produce long-lasting oral antinociception with no evidence of partial agonistic activity [129-131]. In this way, further studies of BRPs applied in the pain treatment could provide valuable information for the development of novel peptidic or non-peptidic molecules to effectively relieve the pain of human patients.

The BRPs isolated from toad (*Bombina maxima*) defensive skin secretion, and their analogs thereof, prodrugs including the peptides, pharmaceutical compositions are protected by patent WO2004/068928. These BRPs and analogs thereof are antagonists of B₂ receptor and they can be used to treat and/or prevent disorders associated with BK, including cardiovascular disorders, inflammation, asthma, allergic rhinitis, angiogenesis, pain and related pathologies.

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