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Bj-PRO-5a, a natural angiotensin-converting enzyme inhibitor, promotes vasodilatation mediated by both bradykinin B_2 and M1 muscarinic acetylcholine receptors

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ABSTRACT

Bradykinin-potentiating peptides (BPPs) or proline-rich oligopeptides (PROs) isolated from the venom glands of *Bothrops jararaca* (*Bj*) were the first natural inhibitors of the angiotensin-converting enzyme (ACE) described. *Bj*-PRO-5a (<EKWAP), a member of this structurally related peptide family, was essential for the development of captopril, the first site-directed ACE inhibitor used for the treatment of human hypertension. Nowadays, more *Bj*-PROs have been identified with higher ACE inhibition potency compared to *Bj*-PRO-5a. However, despite its modest inhibitory effect of ACE inhibition, *Bj*-PRO-5a reveals strong bradykinin-potentiating activity, suggesting the participation of other mechanisms for this peptide. In the present study, we have shown that *Bj*-PRO-5a induced nitric oxide (NO) production depended on muscarinic acetylcholine receptor M1 subtype (mAchR-M1) and bradykinin B_2 receptor activation, as measured by a chemiluminescence assay using a NO analyzer. Intravital microscopy based on transillumination of mice cremaster muscle also showed that both bradykinin B_2 receptor and mAchR-M1 contributed to the vasodilatation induced by *Bj*-PRO-5a. Moreover, *Bj*-PRO-5a-mediated vasodilatation was completely blocked in the presence of a NO synthase inhibitor. The importance of this work lies in the definition of novel targets for *Bj*-PRO-5a in addition to ACE, the structural model for captopril development.

1. Introduction

Bradykinin-potentiating peptides (BPPs) found in the venom gland of the South American pit viper *Bothrops jararaca* are typically proline-rich oligopeptides (PROs) composed of 5–14 aminoacid residues with a pyroglutamate and a proline residue in their N- and C-termini, respectively [1,2]. These peptides named as *Bj*-PROs or *Bj*-BPPs were the first naturally occurring angiotensin-converting enzyme (ACE) inhibitors to be described [1]. The pentapeptide *Bj*-PRO-5a, one of the first BPPs, those sequence was determined and produced by chemical synthesis, was shown to be active in inhibiting angiotensin I conversion and bradykinin (BK) degradation *in* rat lungs [3]. The work of Cushman et al. [4] suggested that the C-terminal domain of this peptide as well as of the related peptide *Bj*-PRO-9a specifically interacted with the catalytic site of ACE. These pioneering studies resulted in the development of the first site-directed ACE inhibitor, named Captopril, which as oral-administered drugs has been used in humans since the 1980s for treatment of systemic arterial hypertension [5,6].

Although the intensive characterization of *Bj*-PRO-5a as an efficient potentiating agent of BK effects [1,7,8], this peptide is not a high-affinity inhibitor of C-terminal active site of ACE [9]. In fact, *Bj*-PRO-5a revealed much more affinity for the N-terminus active

Abbreviations: ACE, angiotensin-converting enzyme; ASS, argininosuccinate synthase; *Bj*, *Bothrops jararaca*; BK, bradykinin; BPPs, bradykinin-potentiating peptides; *Bj*-PROs, proline-rich oligopeptides from the venom glands of *Bothrops jararaca* venom glands; HEK, human embryonic kidney; CHO, Chinese hamster ovarian; $[Ca^{2+}]_i$, intracellular calcium concentration; eNOS, endothelial nitric oxide synthase; L-NMMA, N^G-methyl-L-arginine acetate salt; mAchR, muscarinic acetylcholine receptor; mAchR-M1, muscarinic acetylcholine receptor M1 subtype; NO, nitric oxide; PLC-γ, phospholipase C-γ.

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site of ACE, demonstrated to be less efficient to catalyze the formation of angiotensin II and to degrade BK [10,11], giving more support to the suggestion of a potential existence of other targets for *Bj*-PRO-5a action. Despite the enormous contribution of *Bj*-PRO-mediated ACE inhibition on science and public health, we have demonstrated that ACE inhibition does not fully explain vasodilatation and anti-hypertensive actions exerted by *Bj*-PROs *in vivo* [12–14]. In fact, we have recently shown that *Bj*-PRO-promoted vasodilatation is not only due to a decrease of angiotensin II formation and/or to the increase of BK concentration, but it also involves nitric oxide (NO) production [13]. NO principally participates in the regulation of local and systemic vascular resistance and sodium balance and consequently in the control of arterial pressure [15]. NO diffuses through smooth muscles, where it activates guanylate cyclase, stimulating cGMP production and therefore promoting blood vessel relaxation [16–18]. In endothelium, NO originates from L-arginine by action of endothelial NO synthase (eNOS), whose activity is supposed to increase when cytosolic calcium levels ($[Ca^{2+}]_i$) are increased by receptor activity [19–21]. Both BK B_2 receptors and muscarinic acetylcholine receptors (mAChRs) can induce $[Ca^{2+}]_i$ transients that results in eNOS activation and NO production [16,22].

In the present work, we have provided evidence that *Bj*-PRO-5a increased NO production in human embryonic kidney (HEK293) cells, which is mediated by BK B_2 receptor as well as by mAChR-M1 activation. *Bj*-PRO-5a–induced mAChR-M1 activity was confirmed by $[Ca^{2+}]_i$ measurements in Chinese hamster ovarian (CHO) cells expressing recombinant rat mAChR-M1 (CHO-M1). Moreover, intravital microscopy based on transillumination of mice cremaster muscle revealed that both BK B_2 receptor and mAChR-M1 contribute to the vasodilatation induced by *Bj*-PRO-5a *in vivo*, and that this effect was completely blocked by the NO synthase inhibitor L-NMMA.

2. Materials and methods

2.1. Synthesis and purification of Bj-PRO-5a

Bj-PRO-5a (<EKWAP) was synthesized essentially as previously described by Gomes et al. [23]. Briefly, the synthesis of *Bj*-PRO-5a was performed on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by a stepwise solid-phase method using N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Novabiochem-EMD Chemicals, San Diego, CA). Cleavage of the peptide from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA)/1,2-ethanedithiol/ethyl methyl sulfide for 2 h at room temperature. After removal of the resin by filtration and washing twice with tri-fluoracetic acid (TFA), the crude synthetic peptide was purified by preparative reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu Corp.) on a YMC-Pack ODS column 20 mm × 150 mm (YMC, Kyoto, Japan), using a linear gradient from 3% to 20% CH_3CN in 0.1% TFA, at a flow rate of 7 ml/min. Both, the purity and primary structure of each synthetic peptide were confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Amersham Biosciences, Uppsala, Sweden). The sample was frozen in liquid nitrogen and then freeze-dried (Edwards Freeze Dryer Super Modulyo Pirani 1001, Thermo Fisher Scientific, Waltham, MA) for 48 h at −50 °C under vacuum. Following this procedure [14], the counter ion TFA was completely exchanged as determined mass spectrometry.

2.2. Animals

Experiments were performed using 12 male BALB/c mice (25–30 g body weight), which were bred in animal care facility of the Instituto Butantan (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 handled under ethical conditions according to international rules of animal care, stated by the International Animal Welfare Recommendations, and in accordance with the guidelines established by our local institutional animal welfare committee (CEUAIB/Instituto Butantan, protocol 653/09).

2.3. Cell culture

HEK293 (Human Embryonic Kidney 293) cells [ATCC No. CRL-1573] were cultured in DMEM, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin sulfate and 100 U/ml penicillin G. Wild type Chinese hamster ovarian (CHO-K1) cells [ATCC No. CCL-61], as well as mAChR-M1 expressing CHO cells (CHO-M1) [ATCC No. CRL-1984] and mAChR-M3 expressing CHO cells (CHO-M3) [ATCC No. CRL-1981], were all cultured in F-12 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin sulfate and 100 U/ml penicillin G. Geneticine (100 μg/ml) was added to the media of transfected cells for selection of mAchR expressing cells. HEK293 and CHO cells were kept at 37 °C in 5% CO_2 atmosphere. The medium was changed after 12 h and then every three days.

2.4. Chemiluminescence assay for measurement of nitric oxide products

For the NO quantification, 1×10^6 HEK293 cells were placed on 6-wells plates with culture medium supplemented with fetal bovine serum. After the period of cell adhesion, the culture medium was replaced by medium without serum, adding to the cell culture *Bj*-PRO-5a in the range final concentration of 0.03–3 μM, for a period of 24 h. Then the culture medium was collected and centrifuged at 90 g for 5 min to eliminate possible debris and the supernatant was collected for analysis of nitrate. The cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, Nonidet P-40 (NP-40) 1% sodium deoxycholate 0.5%, 0.1% SDS, 1 mM DTPA and 10 mM N-ethylmaleimide). Then, the cells were incubated on ice for 30 min for cell lysis and then were centrifuged for 15 min at 21,000 × g and 4 °C. The methodology and instrumentation for the measurements was as described by Feelisch et al. [24]. The intracellular medium and extracellular medium were directly injected into a vessel containing a saturated solution of vanadium III chloride in 1 N HCl maintained at 90 °C. Under these conditions, all nitric oxide-derived products (nitrate, nitrite, nitrosothiol, nitrosamines, and iron-nitrosyl complexes) were reduced and compared with those of standard solutions of nitrate under the same experimental conditions.

2.5. Measurements of changes in free intracellular calcium concentration ($[Ca^{2+}]_i$) measurements by microfluorimetry

Changes in $[Ca^{2+}]_i$ were determined by microfluorimetry using the FlexStation III (Molecular Devices Corp., Sunny Valley, CA), following the same protocol described in Lameu et al. [14]. Briefly, cells were seeded a night before starting the experiment at a density of 5×10^4 cells/well in 96-well black walled and clear bottomed microplates, with 100 μl of cell culture medium per well. Cells were incubated for 60 min at 37 °C with the FlexStation Calcium Assay Kit (Molecular Devices Corp.) containing 2.5 mM probenecid in a final volume of 200 μl/well. Before and after addition of *Bj*-PRO-5a, fluorescence of samples was excited at 485 nm, and fluorescence emission was detected at 525 nm. Samples were read at 1.52 s intervals for 120 s with a total of 79 read-outs per well. Responses were measured at the peak intensity of fluorescence compared to baseline fluorescence intensity.

2.6. Intravital microscopy

The dynamics of alterations in the microcirculatory network were determined using intravital microscopy by trans-illumination of the mice cremaster muscle after intravenous application of the peptide (71 nmol/Kg) dissolved in saline sterile. Administration of the same amount of sterile saline was used as a control. Before beginning the experiments, mice were injected with a muscle relaxant drug (0.4% Xilazin) (Coopazine1, Schering-Plough) and then anaesthetized with 0.2 g/kg chloral hydrate and the cremaster muscle was exposed for microscopic examination *in situ* as previously described [25,26].

The animals were maintained on a special designed board controlled thermostatically at 37 °C, which included a transparent platform on which the trans-illuminated tissue was placed arteriolar diameter was measured in different times after peptide injection (5, 10, 15, 20, 25 and 35 min). The study of the microvascular system of trans-illuminated tissue was performed using an optical microscope (Axiolab, Carl Zeiss, Oberkochen, Germany) coupled to photographic camera (AxioCam ICc1) using a 10/0.3 longitudinal distance objective/numeric aperture and 1.6 optovar.

2.7. Statistical analysis

Comparisons were made by one-way analysis of variance (ANOVA) with the Bonferroni post-test using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). The criteria for statistical significance were set at $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Bj-PRO-5a-induced NO production in HEK293 cells

Previous work has suggested that *Bj*-PRO-promoted vasodilatation does not only result from ACE inhibition [12,14], but also involves sustained NO production [13]. Therefore, aiming to measure the sustained NO production in HEK293 cells induced by *Bj*-PRO-5a, NO_x (nitrate, nitrite, nitrosothiol, nitrosamines, and iron-nitrosyl complexes) levels in total (intracellular and extracellular) media were determined following 24 h of incubation with several concentrations of this peptide. NO_x concentration in HEK293 cells was increased in the presence of 0.3–3 μM *Bj*-PRO-5a dose-dependently. Maximal stimulations of 137% (13.68 ± 1.84 nmol/10^6 cells) over basal values (4.75 ± 0.68 nmol/10^6 cells) were obtained in the presence of 3 μM *Bj*-PRO-5a (Fig. 1).

HEK293 cells express functional BK B_2 receptors as well as mAChRs [27,28], and both play a role in the NO production of the endothelium. NO_x levels were also determined in cells pretreated with HOE-140, an antagonists of BK B_2 receptors [29], or with pirenzepine, gallamine or 4-DAMP being at the concentration used specific inhibitors of mAChRs subtypes M1, M2 or M3, respectively [30], prior to addition of 0.3 μM *Bj*-PRO-5a. The used concentrations of the membrane receptor antagonists were sufficient to inhibit subtype-specific receptor responses elicited by 1 μM BK or 50 μM muscarine (data not shown). Inhibition of BK B_2 receptor or mAchR-M1 activity resulted in a reduction of *Bj*-PRO-5a-induced NO production by 40% and 60%, respectively (Fig. 2).

3.2. Bj-PRO-5a-induced $[Ca^{2+}]_i$ transients in HEK293 and CHO-M1 cells

Bj-PRO-5a-induced $[Ca^{2+}]_i$ transients were measured by microfluorimetry after stimulation of HEK293 cells, showing an effect dose–response curves (Fig. 3A). The *Bj*-PRO-5a showed potency

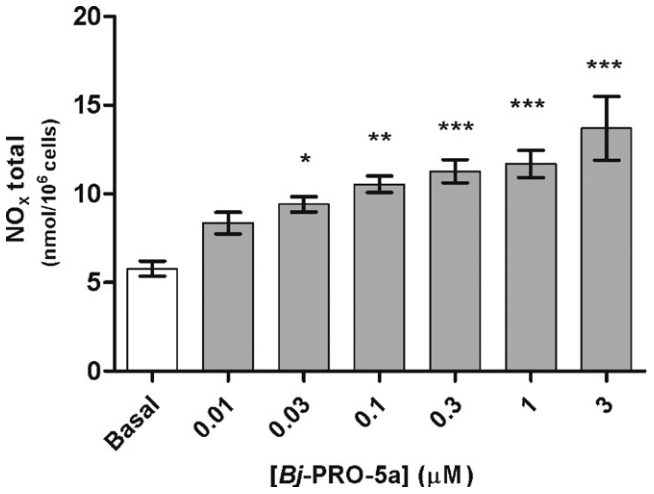

Fig. 1. Dose-dependent induction of NO production by *Bj*-PRO-5a in HEK293 cells. HEK293 cells were seeded into Petri dishes in appropriate medium and cultured for 24 h. Then, cells were incubated for 24 h periods in serum-free medium with increased concentrations of *Bj*-PRO-5a. The medium was collected (extracellular medium), and the cells were lysed with RIPA buffer to measure intracellular NO products by a chemiluminescence assay. The shown data are expressed as mean values ± S.E. of three independent assays. $^{*}P < 0.05$; $^{**}P < 0.01$ or $^{***}P < 0.001$ compared to basal NO production by HEK293 cells.

(pD_2 values) of 7.93 ± 0.12. Moreover, we observed that the increase of $[Ca^{2+}]_i$ was instantaneous and transient, i.e., the maximal peak response of $[Ca^{2+}]_i$ was reached immediately after the stimulation of HEK293 cells with the *Bj*-PRO-5a, returning to the baseline values within 120 s (data not shown). Although, *Bj*-PRO-5a has been primarily described as a potentiate of BK, the *Bj*-PRO-5a-induced $[Ca^{2+}]_i$ transients are not mediated by BK B_2 receptor, since pretreatment of HEK293 cells with a specific antagonist of this receptor, HOE-140, did not affect its response, showing pD2 values of 8.05 ± 0.21 similar to that obtained by *Bj*-PRO-5a in HEK293 cells without any pretreatment (Fig. 3A). These $[Ca^{2+}]_i$ transients contributing to NOS activation may be due to mAchR-M1 activation, since *Bj*-PRO-5a-promoted $[Ca^{2+}]_i$ transients were completely inhib-

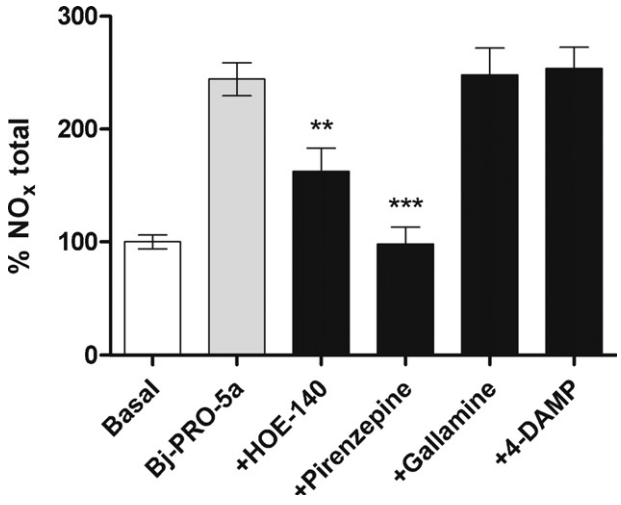

Fig. 2. Involvement of BK B2 receptors and mAChRs in *Bj*-PRO-5a-induced NO production by HEK293 cells. NO production by HEK293 cells was determined following treatment for 24 h with 0.3 (M *Bj*-PRO-5a in the absence or presence of specific antagonists of the BK B2 receptor (1 (M HOE-140), the mAChR-M1 (1 mM pirenzepine), mAChR-M2 (100 (M gallamine) and mAChR-M3 (10 (M 4-DAMP). NO peak values obtained in the presence of *Bj*-PRO-5a alone were considered as 100% activation. The shown data are expressed as mean values ± S.E. of three independent assays. $^{**}P < 0.01$ or $^{***}P < 0.001$ compared to the control measurements in the presence of *Bj*-PRO-5a alone.

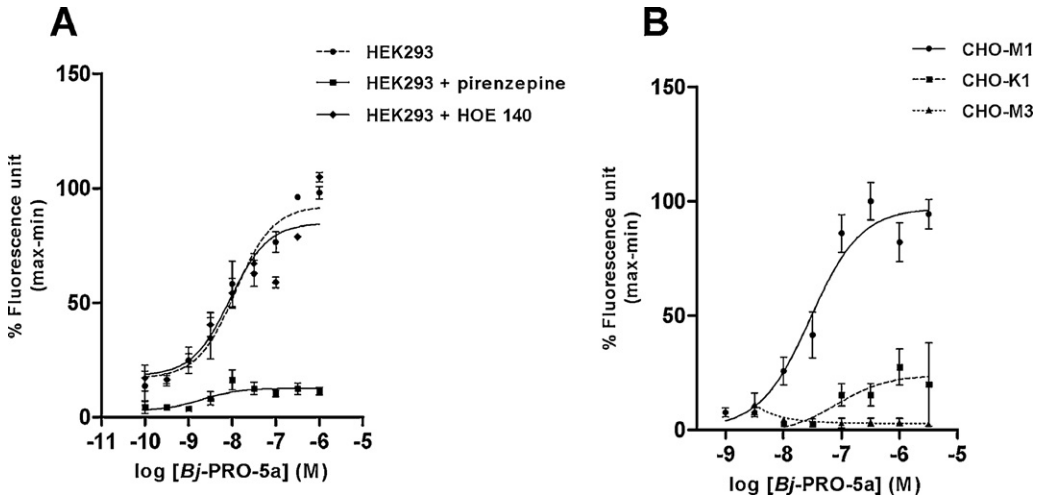

Fig. 3. *Bj*-PRO-5a-induced $[Ca^{2+}]_i$ elevations in HEK293 and CHO cells expressing recombinant rat m1AChR. Changes of maximal peak heights of $[Ca^{2+}]_i$ responses induced by several concentrations of *Bj*-PRO-5a (0.003–1 μM) were measured in (A) HEK293 cells and HEK293 cells pretreated with 1 μM HOE140 or 1 mM pirenzepine for 30 min prior to addition of *Bj*-PRO-5a and (B) CHO-K1 cells; CHO-K1 cells expressing recombinant mAChR-M1 (CHO-M1), CHO-K1 cells expressing mAChR-M3 (CHO-M3) receptor by microfluorimetry using FlexStation III (Molecular Devices Corp.). The data shown are mean values ± S.E. of three independent assays.

ited when HEK293 cells were pretreated with an antagonist this subtype receptor, pirenzepine (Fig. 3A). *Bj*-PRO-5a induced $[Ca^{2+}]_I$ elevations observed in CHO-M1 were never detected in CHO-M3 or in wild-type CHO-K1 cells (Fig. 3B). *Bj*-PRO-5a effects on the mobilization of the $[Ca^{2+}]_i$ in CHO-M1 cells revealed pD_2 values of 7.54 ± 0.14.

3.3. *Bj-PRO-5a-promoted vasodilatation in the microvascular system of BALB/c mice*

Based on the observation of NO production in HEK293 cells in the presence of *Bj*-PRO-5a, we analyzed the effect of the peptide in the microvasular system of BALB/c mice. For this, intravital microscopy of mice cremaster muscle was performed. These experiments revealed that 71 nmol/Kg of *Bj*-PRO-5a promoted vasodilatation in the arterioles medium caliber, showing discrete effect after 5 min of peptide administration and reaching the maximum effect (change of 49% in diameter) at 25 min (Fig. 4). Moreover, the *Bj*-PRO-5a-provoked vasodilator action significantly decreased when the animals were pretreated with 100 nmol/Kg of HOE-140 and 175 nmol/Kg of pirenzepine (Fig. 5), but was totally abolished by pretreatment with 100 mg/Kg N^G-methyl-L-arginine acetate salt (L-NMMA), a specific inhibitor of NOS [31].

4. Discussion

Recently published works have suggested that the mechanism responsible for the enhancement of BK activity by *Bj*-PROs cannot be entirely explained by the inhibition of ACE enzymatic activity [9,23,32,33]. Furthermore, ACE inhibition as the only explanation for the antihypertensive effects caused by inhibitory molecules of this enzyme has been challenged in the literature [34–38]. Thus, the hypothesis was raised that each *Bj*-PRO might act through distinct mechanism of action, probably involving different target proteins [9,23]. Recently, our group demonstrated that *Bj*-PRO-7a is a mAchR-M1 agonist [39], and *Bj*-PRO-10c action involves argininosuccinate synthase (ASS) activation, a key enzyme to provide substrate for NOS [13].

Nitric oxide is a key-mediator for the maintenance of integrity and functionality of the endothelium including regulation of the vascular tonus, prevention of leukocyte filtration, thrombus

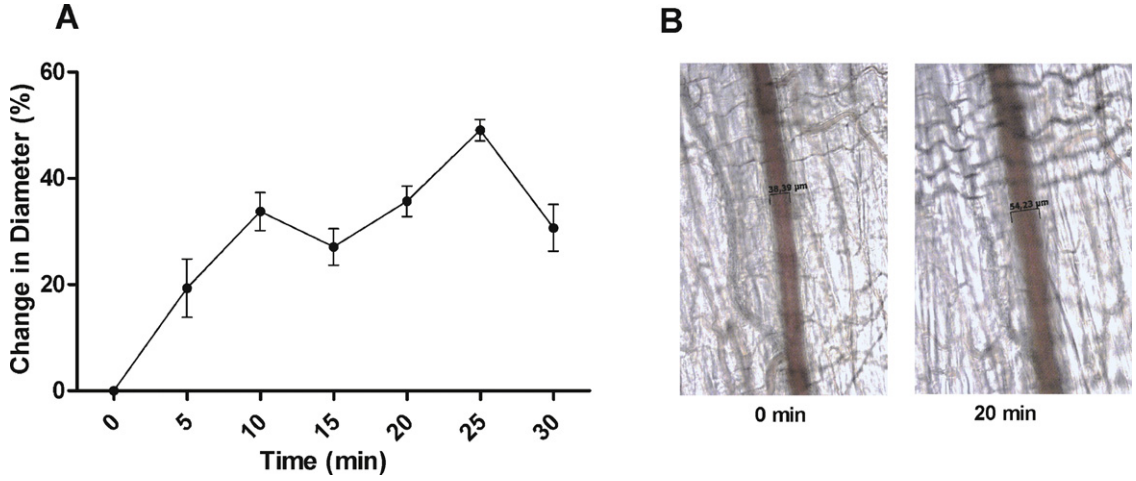

Fig. 4. *Bj*-PRO-5a produced relaxation of blood vessels of the Balb-c mouse observed by intravital micrographs of cremaster muscle. (A) Representative graph showing percentages of vasodilatation in different times (5, 10, 15, 20, 25 and 30 min) after intravenous application of 71 nmol/Kg *Bj*-PRO-5a. (B) Intravital micrographs of cremaster muscle before (0 min) and after 20 min of intravenous application of 71 nmol/Kg *Bj*-PRO-5a. Microphotographs were obtained from computer digitalized images. The data shown are mean values ± S.E. of three independent experiments.

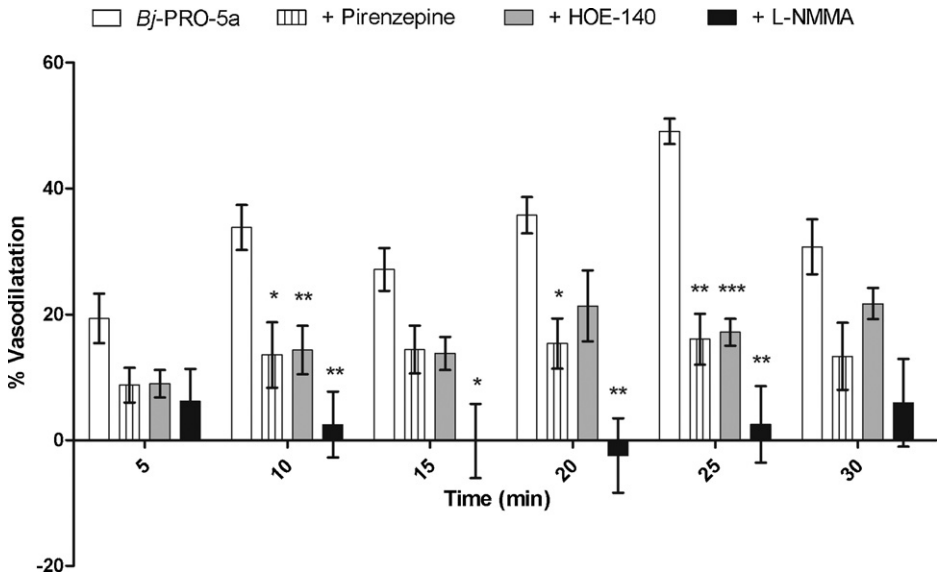

Fig. 5. *Bj*-PRO-5a-provoked vasodilatation effect is mediated by NO production following BK B2 receptor and m1AChR activation. The figure shows percentages of vasodilatation at different times after intravenous application of 71 nmol/Kg *Bj*-PRO-5a (white bars) and after administration of *Bj*-PRO-5a in mouse pretreated with 100 mg/Kg L-NMMA, a specific inhibitor of NOS (black bars) or 175 nmol/Kg pirenzepine or 100 nmol/Kg HOE-140, which are m1AChR and BK B2 receptor antagonists, respectively. The data shown are mean values ± S.E. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control (percentage of *Bj*-PRO-5a-promoved vasodilatation).

formation and angiogenesis [40]. Although, *Bj*-PRO-5a is not an ASS activator (data not shown), this peptide also induced NO production in a dose-dependent manner. Attempting to elucidate molecular mechanisms of *Bj*-PRO-5a-induced NO production, we provide evidence that BK B_2 receptors and mAchR-M1 affect NO production produced by *Bj*-PRO-5a in HEK293 cells, since in the presence of specific antagonists of these receptors *Bj*-PRO-5a-evoked NO accumulation was reduced to 40% and 60% of respective control values found in the absence of these inhibitors.

Stimulation of BK B_2 receptors by BK leads to transients increases in $[Ca^{2+}]_i$. This BK-induced $[Ca^{2+}]_i$ elevation activates NOS for NO generation [16,41]. Many other receptors, when activated, generate intracellular signals to induce NO production; among these are the mAchR-M1 and mAchR-M3, which are also involved in blood pressure regulation [22,42–44]. Muscarinic AChR-M1-promoted NO production is also known to depend on $[Ca^{2+}]_i$ mobilization involving formation of cGMP by soluble guanylate cyclase [45]. The signal transduction exerted by the mAchR-M1 involves G_q protein activation followed by participation of various signaling molecules including phospholipases, mitogen-activated protein kinases and NOS and result in physiological actions depending on the localization of the receptor [46]. In the endothelium, for instance, mAchR-M1-mediated increases in $[Ca^{2+}]_i$ provokes eNOS activation and NO production for promotion of vasodilatation. Regulation of vascular tonus by the mAchR-M1 produced vasodilatation effect was described by Ryberg et al. [22], who showed that the mAChR-M1 is expressed in the endothelium, and stimulation of this receptor promotes NO-dependent relaxation in arterial preparations from rat submandibula. In agreement with the activation of mAchR-M1-specific signal transduction, vasodilatation induced by *Bj*-PRO-5a in mouse microvasculature was completely blocked by a specific NOS inhibitor, L-NMMA. Furthermore, this vasodilatation effect was compromised when animals had been pretreated BK B_2 receptor or mAchR-M1 antagonists, confirming the participation of these receptors in *Bj*-PRO-5a-mediated actions.

The participation of the BK B_2 receptor in *Bj*-PRO-5a-promoted actions leading to NO production might result from BK accumulation following ACE inhibition; however *Bj*-PRO-5a is a very modest inhibitor of this enzyme important for BK degradation [9,47]. Moreover, ACE is absent in HEK293 cells [48,49], and the endogenous expression of the BK B_2 receptor and ACE is very low or absent in CHO cells [50]. Therefore, the *Bj*-PRO-5a mechanism of action may not involve a direct action on BK B_2 receptors and/or BK accumulation. In view of that and having in mind that *Bj*-PRO-5a potentiates BK-induced effects [9,23], our data could be explained by a *Bj*-PRO-5a-promoted crosstalk between BK B_2 receptors and mAChR-M1 expressed in the same cell sharing signaling pathways and resulting in a synergistic regulation of NO signaling. This synergistic action should be independent from oligomerization [51], since *Bj*-PRO-5a-induced $[Ca^{2+}]_i$ mobilization was not observed in HEK293 cells pretreated with an antagonist of BK B_2 receptors, but was completely abolished by an antagonist of mAchR-M1. Moreover, *Bj*-PRO-5a did not exert effects on cells not expressing mAChR-M1, such as CHO-K1 (wild type) cells and CHO-M3 cells (expressing recombinant M3 receptors), suggesting that *Bj*-PRO-5a is a mAChR-M1 agonist, such as *Bj*-PRO-7a [14,34]. Activation of mAChR-M1 is supposed to activate a cross-talk with the BK B_2 receptor, thereby potentializing BK-mediated effects [23] and producing vasodilatation *in vivo*.

We have also observed that the non-peptide ACE inhibitor, captopril, does not induce $[Ca^{2+}]_i$ mobilization in cells responsive to *Bj*-PRO-5a, such as human neuroblastoma and HEK293 cells, which express functional mAchR (data not shown). We might also consider that non-peptide ACE inhibitors are usually dramatically smaller than the peptide toxin, even when compared to *Bj*-PRO-5a and, therefore, it would not be expected to observe similar docking features [52,53]. In fact, as discussed in this work, *Bj*-PRO-5a does not seem to act on ASS in the same way as described for *Bj*-PRO-10c [13].

Many studies have previously suggested different mechanisms of BK-potentiation by ACE inhibitors [48,50,54]. Marcic et al. demonstrated that enalaprilat preserved the activity of the high-affinity BK B_2 receptor by abolishing of ligand-induced receptor desensitization and decreasing receptor endocytosis rates by inducing crosstalk between ACE and the receptor [54]. Another work performed by Bachvarov et al. showed that captopril may limit BK B_2 receptor independently from ACE involvement [48].

The conclusions of our paper are in agreements with those actions of ACE inhibitors of the cited previous and contribute to the elucidation of involved mechanisms. In addition to the identification of a novel putative target underlying the vasodilatation promoted by the *Bj*-PRO-5a, possible future therapeutic applications of this peptide are not limited to the treatment of cardiovascular diseases. Much effort has been undertaken in the search of mAchR-M1 agonists to treat cognitive disorders including the Alzheimer disease [55–57], since the observed cognitive deficiencies have been shown to correlate with the loss of mAchR-M1 functionality [58]. Furthermore, this peptide could become an important tool to study cross-talk mechanisms between the two Gq-protein coupled metabotropic receptors.

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