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Title page

Do the cardiovascular effects of ACEI involve ACE-independent mechanisms? New insights from proline-rich peptides of *Bothrops jararaca*.

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Running title page

a)

Antihypertensive effects of two BPPs from *Bothrops jararaca*

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Abbreviations:

ACE, angiotensin-I converting enzyme; ACEI, angiotensin-I converting enzyme inhibitors; Ang I, angiotensin I; Ang II, angiotensin II; *Bj*, *Bothrops jararaca*; BK, bradykinin; BPPs, bradykinin potentiating peptides; C-, carboxy-; CNP, C-type natriuretic peptide; DpaOH, N3(2,4-dinitrophenyl)L-2,3-diaminopropionyl; HR, heart rate; I.V., intravenous; MAP, mean arterial pressure; McaAla, Mca-Ala-Ser-Asp-Lys-DpaOH; McaSer, Mca-Ser-Asp-Lys-DpaOH; N-, amino-; PAP, pulsatil arterial pressure; sACE, somatic angiotensin converting enzyme; U, potentiation unit.

e)

Cardiovascular

Abstract

ACE inhibitors were developed based on proline rich oligopeptides found in the venom of *Bothrops jararaca* previously known as bradykinin-potentiating peptides (BPPs). However, the exact mechanism of action of BPPs remains unclear. The role of the ACE in the cardiovascular effects of two of naturally proline-rich oligopeptides (*Bj*-BPP-7a and *Bj*-BPP-10c) was evaluated *in vitro* and *in vivo*. *Bj*-BPP-7a does not potentiate the cardiovascular response to bradykinin and is a weak inhibitor of ACE C- and N-site (Ki = 40,000 and 70,000 nM, respectively), while *Bj*-BPP-10c is a strong bradykinin potentiator and inhibitor of ACE C-site (Ki = 0.5 nM vs. 200 nM for N-site). Strikingly, both peptides in doses ranging from 0.47 to 71 nmol/Kg, produced long-lasting reduction (> 6 hours) in the mean arterial pressure of conscious SHR (maximal change 45 ± 6 and 53 ± 6 mmHg for *Bj*-BPP-7a and *Bj*-BPP-10c, respectively). The fall in blood pressure was accompanied by variable degrees of bradycardia. In keeping with the absence of relationship between ACE inhibitory and antihypertensive activities, no changes in the pressor effect of Ang I or in the hypotensive effect of bradykinin were observed at the peak of the cardiovascular effects of both peptides. Our results indicate that the antihypertensive effect of two *Bj*-BPPs containing the motif Ile-Pro-Pro, is unrelated to their ability for inhibiting ACE or potentiating BK indicating as a major component, ACE and BK-independent mechanisms. These results are in line with previous observations suggesting ACE inhibition-independent mechanisms for ACEI.

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Introduction

In 1967, John Vane, as consultant to The Squibb Institute for Medical Research, suggested that the angiotensin-converting enzyme (ACE) should be studied as a possible target for the treatment of cardiovascular diseases, and its inhibitors found. One should benefit from the work of his colleague Sergio Ferreira, who had shown that the venom from the Brazilian pit viper *Bothrops jararaca* (*Bj*) contained peptides that greatly enhanced the smooth-muscle contracting activity of bradykinin (BK) (Ferreira and Rocha e Silva, 1965).

Subsequently, a pentapeptide and a nonapeptide were isolated from the *Bj* venom and fully characterized (Ferreira et al., 1970; Ondetti et al., 1971; Stewart et al., 1971). The corresponding synthetic peptides displayed transient potentiation of BK *ex vivo* and *in vivo*. In addition, one of these peptides, SQ 20.881 (*Bj*-BPP-9a) produced an antihypertensive effect, as shown in animal models and human subjects (Bianchi et al., 1973; Gavras et al., 1975). Vane's suggestion implied that the antihypertensive activity and the potentiation of BK by this *Bj*-BPP were a consequence of sACE (somatic Angiotensin I-converting Enzyme) inhibition, since the *in vivo* experiments showed that the *Bj*-BPPs blocked bradykinin degradation and inhibited the conversion of Ang I to Ang II (Stewart et al., 1971). The *Bj*-venom peptides raised enormous interest and only the lack of oral activity prevented their general therapeutic use (Ondetti and Cushman, 1981; Hayashi et al., 2003). Thus, the development of a specific non-peptidic sACE inhibitor, effective by oral route, became mandatory for pharmaceutical application.

The structure-activity studies by Cushman and Ondetti, from The Squibb Institute, suggested that the C-terminal proline of the *Bj*-BPPs specifically interacted with putative subsites, or pockets, at the active site of the sACE. Extensive studies on the binding to the Zn^{2+} of the active site led to the generation of non-peptide compounds that combined proline with a sulfhydryl group, increasing the inhibitory potency. The best compound was

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named Captopril, a very potent ACE inhibitor, the first truly useful antihypertensive drug designed to bind to the active site of ACE (Ondetti and Cushman, 1981). Captopril was shown to reproduce all known pharmacological and enzymatic features of the *Bj*-BPPs, consequently strongly reducing the interest in the *Bj*-peptides themselves (Case et al., 1978; Antonaccio et al., 1981).

However, recent results revived the interest in these peptides. Three reasons explain the renewed interest: (i) a number of the *Bj*-BPPs can distinguish between the N- and the C-active site of sACE (Cotton et al., 2002). In fact, sACE has two homologous and functionally distinct active sites (Wei et al., 1991), one of which, the active site at the C-domain (C- site), is slightly more effective in hydrolysing some vasoactive peptides, like bradykinin and angiotensin I (Perich et al., 1992; Jaspard et al., 1993). (ii) the isolation and identification of novel *Bj*-BPPs in the crude venom (Ianzer et al., 2004). (iii) the presence of several *Bj*-BPPs in the CNP-precursor of the snake brain may reveal novel neuropeptides (Murayama et al., 1997; Hayashi et al., 2003).

We have previously described the isolation of a cDNA coding for a *Bj*-BPP precursor protein from the venom gland and found in the neuro-endocryne areas of the *Bj*-brain, containing seven tandemly arranged *Bj*-BPP sequences at the N-terminus, and one C-type natriuretic peptide (CNP) sequence at the C-terminus (C-site) (Murayama et al., 1997; Hayashi et al., 2003). Among the endogenous, BPP-9a and BPP-10c, turned out to be the most efficient inhibitors of the sACE, displaying high selectivity toward the C-active site (Cotton et al., 2002; Hayashi et al., 2003).

It has been suggested that *Bj*-BPPs may act by an ACE-independent mechanism (Camargo and Ferreira, 1971; Green et al., 1972; Mueller et al., 2005; Mueller et al., 2006). A similar possibility has been raised for conventional ACEI (Marks et al., 1980; Mittra and Singh, 1998; Houben et al., 2000; Ignjatovic et al., 2002). In the present study, we tested whether

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this possibility is true by selecting two peptides, a heptapeptide and a decapeptide, among 19 *Bj*-BPP sequences according to their totally distinct properties concerning BK potentiation in isolated guinea pig ileum (Ianzer et al., 2004). We observed a sustained antihypertensive activity of both *Bj*-BPPs in SHR. Strikingly, this activity was not related to the inhibition of sACE *in vitro* or to the bradykinin potentiation or blockade of the pressor effect of Angiotensin I *in vivo.*

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Methods

Drugs

Human wild-type somatic ACE was obtained through stable expression in Chinese hamster ovary cells transfected with appropriate ACE cDNA. The expression and purification of ACE were performed as previously described (Wei et al., 1991) and was kindly provided by Pierre Corvol from the Institut National de la Santé et de la Recherche Médicale (France). Mca-Ala-Ser-Asp-Lys-DpaOH (Mca-Ala) and Mca-Ser-Asp-Lys-DpaOH (Mca-Ser) were prepared following the procedure previously described (Dive et al., 1999). BPP-7a (PyroGlu-Asp-Gly-Pro-Ile-Pro-Pro-OH), BPP-10c (PyroGlu-Asn-Trp-Pro-His-Pro-Gln-Ile-Pro-Pro-OH), Bradykinin, Captopril, Angiotensin I and Angiotensin II were purchased from Bachem Chemical Co (USA). The peptides were dissolved in isotonic sterile saline (0.9% NaCl) (Asterflex, Brazil) just before use *in vivo*.

In vitro assay: Enzyme assays for selective inhibition of the ACE active sites

The inhibitions of somatic ACE determined for *Bj*-BPP-7a were performed as previously described (Cotton et al., 2002). In summary, the synthetic BPP was dissolved in buffer containing either the Mca-Ala or Mca-Ser substrate. The reactions were initiated by addition of the recombinant human ACE. The assays were carried out at 25º C in 50 mM HEPES pH 6.8, 200 mM NaCl and 10 μM $ZnCl_2$. Continuous assays were performed by recording the fluorescence increase at 390 nm (λ_{ex} = 340 nm) induced by the cleavage of Mca-Ala and Mca-Ser substrates by ACE, monitored using a Biolumin 960 photon counter spectrophotometer (Molecular Dynamics, USA) equipped with a device control and a plate shaker. The substrate and enzyme concentrations for the experiments were chosen so as to remain well below 10% of substrate utilization and observe initial rates. Reported inhibition percentages corresponded to the average of three independent experiments.

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Cell Culture. Commercially available cell lines Chinese hamster ovary (CHO), obtained from the American Type Culture Collection, Manassas, VA, were used for cell culture experiments. Cells were cultured 3 days or more as a monolayer (94/16-mm Petri dish) in culture medium recommended for each cell type. Cells stably transfected with AT_1 receptors cDNA driven by a cytomegalovirus promoter and selected by neomycin (Pesquero et al., 1994), were obtained from Dr. JE Krieger.

Binding Studies in cell cultures. *Competition experiments.* AT_1 receptor-transfected cells were incubated with ^{125}I-Ang II (0.4 nM) in 24-well plates for 60 min at 4°C in 300 µL of serum-free medium (DMEM) supplemented with 0.2% BSA, 0.005% bacitracin, 100 µM phenylmethylsulfonyl fluoride, and 500 µM *o*-phenanthroline. Competition experiments were performed by pre-incubating the cells with Ang II (1 µM) (0.01 to 1 µM) or *Bj*-BPP-7a and *Bj*-BPP-10c (1 pM to 10 µM). After two washes with ice-cold serum-free DMEM, cells were disrupted with 0.1% Triton X-100 in water at 22–24°C. Bound radioactivity in the cell lysate was measured in a γ-counter (1275 MINIGAMMA LKB Wallac). Experiments were made in duplicate (n = 3-6 for each peptide). Ang II was labeled with ^{125}I by the chloramine T method and purified by HPLC, as described (Pinheiro et al., 2004).

***Ex vivo* assay: BK-potentiation on isolated guinea pig ileum**

Animals: Experiments were carried out in 5 female guinea pigs (160–200 g) bred at the Instituto Butantan (Sao Paulo, Brazil). The animals had free access to food and water and were submitted to a light-dark cycle (12 hours each) before the preparation for the experiments. All experimental protocols were performed in accordance to the guidelines for the human use of laboratory animals of our institute and approved by local authorities.

Experimental protocol: The BK-potentiation assays on isolated guinea pig ileum were performed as previously described (Ianzer et al., 2006). After a 20 hours fasting period, approximately 15 cm of the distal ileum of the female guinea pigs were removed

immediately after death and washed throughly with Tyrode solution (137 mM NaCl; 2.7 mM KCl; 1.36mM $CaCl_2$; 0.49 mM $MgCl_2$; 0.36 mM NaH_2PO_4; 11.9 mM $NaHCO_3$; 5.04 mM D-glucose). 2.5 cm segments of the ileum were isotonically mounted under a 1 g load in a 10.5 mL muscle bath containing Tyrode solution at 37 ^oC and bubbled with air. Muscular contraction was recorded on a Gould 2600 polygraph (USA). One unit of BK-potentiation (U) is defined as the amount of potentiator (nanomoles) necessary to transform the effects of the single dose of BK into that produced by the double dose.

***In vivo* assays: Blood pressure recording in rats**

Animals: Experiments were carried out in 50 male Spontaneously Hypertensive Rats (SHR) (280 to 350 g) and 33 Wistar rats (250 to 320 g) bred at the animal facility of the Biological Science Institute (CEBIO, Universidade Federal de Minas Gerais, Minas Gerais, Brazil). The animals had free access to food and water and were submitted to a light-dark cycle (12 hours each) before the preparation for the experiments. All experimental protocols were performed in accordance to the guidelines for the human use of laboratory animals of our institute and approved by local authorities.

Arterial Pressure Measurements: The cardiovascular parameters, pulse arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) were monitored by a solid-state strain gauge transducer connected to a computer through a data acquisition system (MP 100; BIOPAC Systems, Inc, USA). The PAP, MAP and HR were monitored jointly during experiments in different monitor channels and recorded in the computer hard disk for late analysis.

Experimental Protocols

Protocol 1: Inhibition of the pressor effect of Angiotensin I on blood pressure in anaesthetized Wistar rats: The surgery was performed under anesthesia with urethane 12% (1.0 mL/100g of body weight) intraperitoneally. A polyethylene catheter (PE-10 connected

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to PE-50) was introduced into abdominal aorta through the left femoral artery for measurements of cardiovascular parameters and into right femoral vein for intravenous injection. During the experimental procedure, the temperature of the animal was maintained in 36 - 37º C (rectal temperature) through an electrical heating blanket.

Before drug administration, the cardiovascular parameters of the rat were monitored during 10 minutes. After this period, intravenous injections of Ang I (20 and 40 ng) were made. After administration of *Bj*-BPP (45nmoles), Ang I (40 ng) was injected in regular intervals (5, 10, 20, 30, 40, 50, 60, 70 and 90) (n = 4 for each *Bj*-BPP). ACE inhibition was estimated by the time (min) needed for 50% recovery of the response to 40 ng of Ang I before the administration of *Bj*-BPPs.

Protocol 2: Potentiation of the hypotensive effect of Bradykinin by Bj-BPP in anaesthetized Wistar rats: This assay was performed according to (Ianzer et al, 2006), as follows: After surgery (performed according to protocol 1), the cardiovascular parameters were monitored during 10 minutes. After this period, intravenous injections of BK (0.5 and 1.0 µg) were made. After administration of *Bj*-BPP, BK (0.5 µg) was injected in regular intervals (5, 10, 15, 20, 25, 30, 40, 50, 60, 75 and 90 minutes). Different doses of *Bj*-BPP were injected in order to achieve the dose corresponding to the potentiating unit (U): dose necessary to transform the effects of the single dose of BK (0.5 µg) into that produced by the double dose. *Bj*-BPP-7a (n = 7) and *Bj*- BPP-10c (n = 5).

Protocol 3: Effect of Bj-BPP on blood pressure of conscious SHR and Wistar rats: 20 hours before the experiment, under anesthesia with tribromoetanol 2.5% (1.0 mL/100g of body weight) intraperitoneally, a polyethylene catheter (PE-10 connected to PE-50) was introduced into abdominal aorta through a femoral artery for measurements of cardiovascular parameters and into a femoral vein for intravenous injection. After recovery

from anesthesia, the rats were kept in individual cages with free access to water and chow until the end of the experiments.

Before drug administration, the cardiovascular parameters were monitored during 1 hour (baseline period). After this period, intravenous bolus injection of the *Bj*-BPPs or vehicle (NaCl 0.9%) in a total volume of 0.5 mL was made. *Bj*-BPPs doses of 71; 14.2; 2.37 and 0.47 nmol/Kg and Captopril dose of 71 nmol/Kg were used in SHR rats (n = 5-8 for each dose); *Bj*-BPPs doses of 71 and 0.47 nmol/Kg were used in Wistar rats (n = 5-6 for each dose). The cardiovascular parameters were monitored continuously during 6 hours after drug administration. MAP and HR values were computed at 2 min interval for the entire recording period. Each animal received two randomically selected treatments [drugs *Bj*-BPPs or Captopril) or vehicle]. In the second experimental day, all animals presented MAP and HR levels similar to the basal period of the first day.

Protocol 4: Potentiation of bradykinin hypotensive effects and inhibition of angiotensin I pressor effect during cardiovascular effects of the Bj-BPPs in conscious SHR: The surgery was performed according to protocol 3. Before drug administration, the cardiovascular parameters were monitored during 40 minutes (baseline period). After this period, intravenous bolus injection of BK (0.5 μg and 1.0 μg) and Ang I (20 ng and 40 ng) were made to obtain the control responses. *Bj*-BPP-5a and Captopril (71 nmol/Kg), *Bj*-BPP-10c (2.37 and 71 nmol/Kg) or saline (0.9%) in a total volume of 0.5 mL were administrated in SHR (n = 4-5 for each BPP). Injections of BK (0.5 μg) were made at 10 and 210 minutes after *Bj*-BPP; injections of Ang I (40 ng) were made at 20 and 220 minutes after *Bj*-BPP. MAP and HR values were computed at 2 min interval for the entire recording period.

Statistical analysis

Comparisons were made by Student unpaired *t* test or one-way ANOVA with Dunnett post-test when appropriate. GraphPad Prism 4.0, GraphPad Software, Incorporation software

program (USA) was used in all statistical analysis. The criteria for statistical significance was set at $p < 0.05$.

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Results

In vitro and *in vivo* inhibition of sACE, and *ex vivo* and *in vivo* potentiation of BK.

In vitro, Bj-BPP-7a presented a weak inhibition of sACE and showed no clear preference for either active site of ACE. Its inhibition constants for either site (μM range) was 4 orders of magnitude higher than that of *Bj*-BPP-10c (nM range). The potency of sACE inhibition by the two *Bj*-BPPs, both, *in vitro* and *in vivo*, was proportional to the degree of BK potentiation in both bioassays. Indeed, *in vivo*, *Bj*-BPP-7a neither inhibited ACE nor potentiated BK. On the other hand, *in vivo*, the ACE inhibition by *Bj*-BPP-10c presented a half-life of about 30 minutes (Table 1).

Effect of *Bj*-BPPs on rat blood pressure

The cardiovascular effects of intravenous injections of *Bj*-BPPs were evaluated in conscious SHR and Wistar rats. Both *Bj*-BPPs showed potent and long-lasting antihypertensive activity, which was sustained for more than 6 hours in all doses administered to SHR. Figure 1, panels A and C, illustrates the effect of the peptides at the dose of 0.47 nmol/Kg. Interestingly, for the *Bj*-BPPs in all doses assayed, the fall in blood pressure was associated with a reduction of the HR (Figure 1, panels B and D).

Figure 2, panels A and C, shows the peak changes in MAP produced by *Bj*-BPP-7a and *Bj*-BPP-10c in SHR. Figure 3, panels A and C, shows the mean changes in MAP and HR in the 6 hours following administration of both peptides. In sharp contrast with its weak potency as ACE inhibitor or bradykinin potentiator, *Bj*-BPP-7a produced a marked and sustained fall in the MAP of SHR even at doses as low as 0.47nmol/Kg (peak change: -33 ± 5 mmHg vs. -11 ± 3 mmHg in vehicle treated SHR, $p < 0.05$ and mean change over a six hour period: -14 ± 3 mmHg vs. -1 ± 3 mmHg in vehicle treated SHR, $p < 0.01$). The maximum change in MAP was obtained with the dose of 14.2nmol/Kg (peak change: -45 ± 6 mmHg, $p < 0.01$ and mean change -22 ± 4 mmHg, $p < 0.001$). However, this effect was

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not statistically different from that obtained with the lowest dose tested (0.47nmol/Kg) (Figures 2A and 3A). The changes in MAP were associated with significant reductions in HR (peak change: -78 ± 15 bpm for 0.47 nmol/Kg and -89 ± 18 bpm for 14.2 nmol/Kg, $p < 0.05$) (Figures 2B and 3B).

For *Bj*-BPP-10c, a dose-dependent effect was observed in the range of 0.47 nmol/Kg to 71 nmol/Kg (peak change: -36 ± 3 mmHg to -53 ± 6 mmHg and mean change: -14 ± 2 mmHg to -26 ± 5 mmHg) (Figures 2C and 3C). An inverse relationship between the changes in HR and the doses used was also observed. The maximum response was observed with the dose of 0.47 nmol/Kg (peak change: -89 ± 9 bpm, $p < 0.05$ and mean change: -41 ± 8 bpm, $p < 0.01$) (Figures 2D and 3D).

The maximum changes in MAP and HR usually occurred after 160 minutes of the *Bj*-BPP injection (Table 2). However, no clear relationship could be established between the changes in MAP and HR.

In Wistar rats, no significant changes in MAP and HR were observed after intravenous administration of *Bj*-BPPs with the dose of 0.47 nmol/Kg, considering either the peak change or mean change (Figures 4 and 5). At the dose of 71 nmol/Kg, both *Bj*-BPPs caused a significant decrease in MAP when the peak changes were considered (-28 ± 4 mmHg for *Bj*-BPP-7a and -20 ± 1 mmHg for *Bj*-BPP-10c, $p < 0.05$ and $p < 0.01$ respectively; Figure 4 panels A and C). The maximal changes in HR were -50 ± 9 bpm for *Bj*-BPP-7a (71 nmol/Kg) and -69 ± 8 bpm for *Bj*-BPP-10c (0.47 nmol/Kg), however these changes were not statistically significant when compared with vehicle (-40 ± 17 bpm). On the other hand, considering the average of the changes in the entire period of observation (6 hours) no noticeable effect on MAP or HR was observed (Figure 5). Similarly to SHR, the maximum changes in cardiovascular parameters started after 160 minutes of the *Bj*-BPP injection (Table 2).

Effect of BK and Ang I in the arterial blood pressure of conscious SHR before and after *Bj*-BPP administration.

The results presented in Figure 6 (panel A) show that the extent of hypotention following the injection of BK was not affected by the administration of *Bj*-BPP-7a (71 nmol/Kg), either after 10 min or 210 min of administration. Accordingly, the extent of the increase in blood pressure following administration of Ang I, was not affected by the injection of *Bj*-BPP-7a (71 nmol/Kg). On the other hand, *Bj*-BPP-10c at 71 nmol/Kg produced a modest, but statistically significant, potentiation of BK without interfering with the Ang I pressor effect, at both time points (Figure 6, panel C). Both *Bj*-BPPs produced a significant decrease in MAP (Figure 6, panels B and D). A similar but more intense BK potentiation was observed with Captopril at the same molar dose (71nmol/Kg or 15.4μg/Kg). As observed with both *Bj*-BPPs no significant change in the pressor effect of Ang I were observed (Figure 6, panel E). In sharp contrast with the *Bj*-BPPs, despite changing the BK hypotensive effect, Captopril did not affect blood pressure levels (Figure 6, panel F). With a lower dose of *Bj*-BPP-10c (2.37 nmol/Kg) no effect was observed in the BK response at both time points (10 and 210 minutes after administration) while a significant reduction in MAP was observed for up to 6 hours (Figure 7, panels A and B).

Binding studies

In order to test whether the dissociation of the ACE inhibitory activity of *Bj*-BPPs from their antihypertensive effect could be due to blockade of AT_1 receptors, we determine the effect of both peptides on the binding of ^{125}I-Ang II to AT_1 receptor-transfected cells. No significant displacement was observed with both peptides in concentrations ranging from 10^{-12} to 10^{-5} M (Figure 8).

Discussion

The snake venom contains a number of strong inhibitors of the Ang I-converting enzyme (Ferreira et al., 1970; Chi et al., 1985; Ianzer et al., 2004). They were useful not only to validate ACE as a target to treat human hypertension, but also as lead molecules for the development of the active-site directed inhibitors of ACE (Ondetti et al., 1977; Ondetti and Cushman, 1981). It was demonstrated that once sACE is inhibited, the concentration of Ang II decreases, while the concentration of BK increases, both reactions concurring to reduce hypertension (Ondetti and Cushman, 1981). Indeed, a direct relationship between the duration of the ACE inhibition and the antihypertensive actions of the active-site directed inhibitors of ACE has been suggested (Sweet et al., 1981). Experimental and clinical trials have also raised the possible importance of the inhibition of BK degradation, since it increases the endothelial production of NO and prostacyclin, promoting vasodilation (Zhang et al., 1999). Accordingly, a similar mechanism has been applied to explain why *Bj*-BPPs display antihypertensive activity (Vanhoutte et al., 1995).

However, the mechanisms of the bradykinin potentiation as well as the mechanism of the antihypertensive effect of ACEI appears to be more complex than previously suspected (Mittra and Singh, 1998; Marcic et al., 1999; Houben et al., 2000). Bradykinin potentiation by ACEI might involve beside ACE inhibition, induction of a cross-talk mechanism between ACE and B_2 receptors (Marcic et al., 1999; Mittra and Singh, 1998; Houben et al., 2000; Mueller et al., 2006). Indeed more than 30 years ago, an ACE-indepentent mechanism for BK potentiation by ACEI of *Bothrops jararaca* venom was suggested (Camargo and Ferreira, 1971; Greene et al., 1972).

Likewise, there are scarce but consistent reports showing that ACEI can produce vasodilation by mechanism, that cannot be ascribe to ACE inhibition (Marks et al., 1980; Antonaccio et al., 1981; Mittra and Singh, 1998; Houben et al., 2000; Mueller et al., 2006).

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For example, Houben and co-workers (2000) have found that quinaprilat but not enaprilat produced significant vasodilation with 15 minutes of administration. At this time both drugs completely blocked the Ang I pressor effect. Nakamura and co-workers (1992) have observed that although enalaprilat was without effect on basal forearm blood flow or vascular resistance, it significantly augmented the increase in blood flow and reduction in forearm resistance induced by acetylcholine. Captopril lowers blood pressure of animals with different models hypertension including situations where the renin-angiotensin system is not responsible for blood pressure maintenance (Marks et al., 1980). In addition, Captopril has been shown to produce vasodilation in vitro in conditions that lisinopril was without effect (Mittra and Singh, 1998). Our data are in line with these previous observations. Taken all together, the current available data show that an ACEI can produce: 1) BK-potentiation without blood pressure lowering effect (captopril in our study); 2) antihypertensive effect without changing the hypotensive action of BK or the hypertensive effect of Ang I or Ang II (*Bj*-BPPs 7a and 10c in our study); 3) blockade of the pressor effect of Ang I without producing vasodilation (Houben et al., 2000); 4) vasorelaxation which can be dissociated from ACE inhibition (Captopril) (Mittra and Singh, 1998); 5) facilitation of NO release induced by acethylcholine without producing vasodilation directly (Nakamura et al., 1992). Furthermore, the antihypertensive effect of ACE inhibitors does not correlate with Ang II plasma levels (Marks et al., 1980; Duncan et al., 1999) and a similar dissociation has been described for bradykinin (Stanziola et al., 1999). In addition, it has been suggested that ACEI can act as anti-oxidant (de Cavanagh et al., 1997) or can also act as B1 receptor agonist (Ignjatovic et al., 2002). It should be also pointed out that Proline-rich peptides which were the basis for the development of ACEI (Ondetti et al., 1977) having the same C-terminal motif (Ile-Pro-Pro) produced similar and potent antihypertensive effects, despite the fact that their ACE inhibitory potency differ in about

80,000-fold (*Bj*-BPP-7a as compared to *Bj*-BPP-10c) (Table I, this study). Therefore although, ACE inhibition is apparently the main mechanism of the antihypertensive effects of ACEI, other mechanisms unrelated to interference with the hydrolytic activity of ACE are obviously involved. More important, our study and the available data strongly support the hypothesis that ACEIs are not a single (uniform) class of antihypertensive drugs. Actually, they may present completely distinct mechanisms of action. In this regard, the contribution of other factors such as the increase of the vasodilator Ang-(1-7) should be considered in future studies (Duncan et al., 1999; Stanziola et al., 1999).

As mentioned above, the present study clearly demonstrates that, at least in SHR, ACE inhibition cannot be applied to describe the antihypertensive effect of the proline-rich oligopeptides since the peptide concentrations used were far below those required to inhibit ACE *in vivo*. In fact, the hypotensive effect of the *Bj*-BPPs was also independent of BK potentiation, whether or not this effect is a consequence of the ACE inhibition. Therefore, in contrast to what have been suggested for currently used ACEI, our results strongly suggests that the reduced formation of Ang II and BK potentiation are not essential mechanisms involved in the antihypertensive action of ACEI. We have also shown that the antihypertensive effect of *Bj*-BPPs can not be ascribed to direct effects on AT_1 receptors.

Interestingly, *Bj*-BPPs caused a significant reduction instead of an increase of HR, as would be expected by the unloading of baroreceptors due to hypotension or if it was mediated by BK (Bunag et al., 1975). Whether this reflects a consequence of the tendency to a decrease in the locomotion activity following administration of the two *Bj*-BPPs (data not shown) or a direct effect of these peptides on the heart, such as interference in the autonomic activity modulating the heart pacemaker and/or changes in the baroreflex control of heart rate, remains to be clarified. It should be pointed out that our current data do not allow to discard a contribution of heart rate (cardiac output) changes to the blood pressure effects of *Bj*-

BPPs, although a clear relationship between changes in BP and HR could not be established.

We would like to direct the attention to one interesting result presented herein: the hypotensive effect of *Bj*-BPPs especially in low doses was observed in SHR but not in normotensive animals. This observation suggests that the antihypertensive effect produced in SHR is not due to a nonspecific effect of both peptides on blood vessels or in the heart.

In conclusion, synthetic compounds, displaying properties similar to these *Bj*-BPPs, which produced antihypertensive effect without affecting the complex physiological role of ACE, could represent an attractive alternative for the treatment of human hypertension and other cardiovascular diseases.

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Footnotes

a)

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Legends for figures

Figure 1. Time course of the effect of intravenous administration of *Bj*-BPPs on the mean arterial pressure (MAP) and heart rate (HR) of spontaneously hypertensive rats (SHR). The I.V. injections of BPPs (0.47 nmol/Kg) and vehicle were made at the time zero, indicated by the solid line.

Figure 2. Maximal changes in MAP and HR produced by *Bj*-BPPs in SHR. *Bj*-BPPs were administered in doses ranging from 0.47 to 71 nmol/Kg. The data show the mean ± SEM of maximal (peak) changes observed in each rat with each dose. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the change observed with vehicle.

Figure 3. Average of the changes in MAP over six-hour following administration of *Bj*-BPPs in SHR. *Bj*-BPPs were administered in doses ranging from 0.47 to 71 nmol/Kg. The data are presented as mean ± SEM of averaged changes of the period of observation (6 hours) * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to the change with vehicle.

Figure 4. Maximal changes in MAP and HR of *Bj*-BPPs in normotensive Wistar rats. *Bj*-BPPs were administered in two different doses: 0.47 and 71 nmol/Kg. The data are presented as mean ± SEM of maximal (peak) changes observed in each rat with each dose. * $p < 0.05$ and ** $p < 0.01$ compared to the change observed with vehicle.

Figure 5. Average of the changes in MAP over six-hour following administration of *Bj*-BPPs in in normotensive Wistar rats. *Bj*-BPPs were administered in two different doses: 0.47 and 71 nmol/Kg. The data are presented as mean ± SEM of averaged changes of period of observation (6 hours).

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Figure 6. Effect of intravenous administration of *Bj*-BPPs or Captopril (71 nmol/Kg) on the mean arterial pressure and on the blood pressure change produced by intravenous injection of BK and Ang I in SHR. Panels A, C, E and G show the influence of *Bj*-BPPs, Captopril and vehicle on the depressor effect of BK and the pressor effect of Ang I. Gray bars: hypotensive effect of bradykinin (0.5 μg); black bars: pressor response of angiotensin I (40 ng). Panels B, D, F and H show the time course of the effect of *Bj*-BPPs, Captopril and vehicle on the MAP of SHR (white bars). The data are presented as mean ± SEM, (n = 4-6), * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Figure 7. Effect of intravenous administration of *Bj*-BPP-10c (2.37 nmol/Kg) on the mean arterial pressure and on the blood pressure change produced by intravenous injection of BK and Ang I in SHR. Panel A shows the influence of *Bj*-BPP-10c on the depressor effect of BK and the pressor effect of Ang I. Gray bars: hypotensive effect of bradykinin (0.5 μg); black bars: pressor response of angiotensin I (40 ng). Panel B shows the time course of the effect of *Bj*-BPP-10c on the MAP of SHR (white bars). The data are presented as mean ± SEM, (n = 5), * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

Figure 8. Competition for ^{125}I-Ang II binding to AT_1-transfected CHO cells by Ang II and *Bj*-BPPs. Competition curves were generated by adding increasing concentrations of Ang II (10^{-11} to 10^{-6} M) or *Bj*-BPP-7a or *Bj*-BPP-10c (10^{-12} to 10^{-5} M) to the incubation buffer containing 0.4 nmol/L of 125I-Ang II. Data are presented as mean ± SEM of three to six independent experiments.

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Tables

Table 1 – Inhibition studies with sACE and biological activities of the synthetic BPPs.

	ACE inhibition			BK Potentiation - U (nmol)	
	K_i (nM)		Blood pressure Time for 50% recovery (min)	Isolated ileum	Blood pressure
	N-site	C-site			
***Bj*-BPP-7a**	70,000	40,000	0	10.77 ± 0.6	> 200
***Bj*-BPP-10c**	200 [a]	0.5 [b]	20-30	0.48 ± 0.02 [b]	5.85

The *in vitro* inhibition of ACE correspond to the average value of three independent $K_{i(app)}$ values determined according to Cotton et al. (2002). The *in vivo* inhibition of ACE was estimated by the time required for 50% recovery of the response to Ang I (40 ng) before administration of 45 nmol of BPPs. The BK potentiation unit (U) is described in methods.

a Cotton et al., 2002

b Hayashi et al., 2003

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Table 2: Time for maximal cardiovascular effect produced by *Bj*-BPPs at different doses in SHR and Wistar rats.

Strain	*Bj*-BPP	Time for maximal change (min)			
		0.47 nmol/Kg	**2.37 nmol/Kg**	**14.2 nmol/Kg**	**71 nmol/Kg**
SHR	**BPP-7a**	257 ± 10 (range: 224–290)	308 ± 14 (range:272-348)	248 ± 23 (range:170-342)	323 ± 13 (range:278-354)
	BPP-10c	248 ± 28 (range:160–334)	294 ± 25 (range:274-350)	285 ± 29 (range:192-358)	345 ± 7 (range:304-358)
Wistar	**BPP-7a**	250 ± 73 (range:168-324)	ND	ND	283 ± 13 (range:242-310)
	BPP-10c	230 ± 33 (range:178-356)	ND	ND	246 ± 26 (range:222-324)

Figure 1

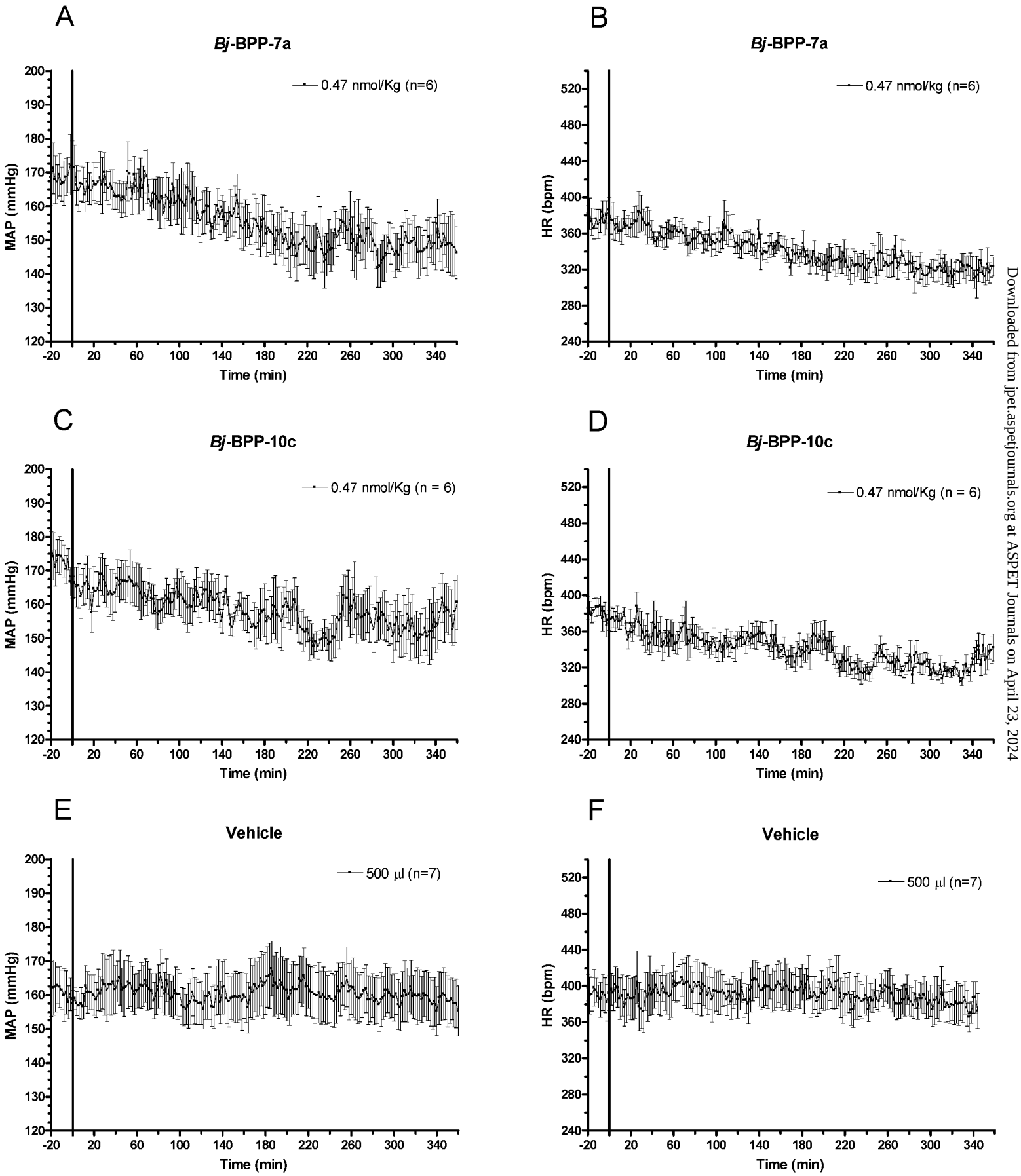


Figure 2

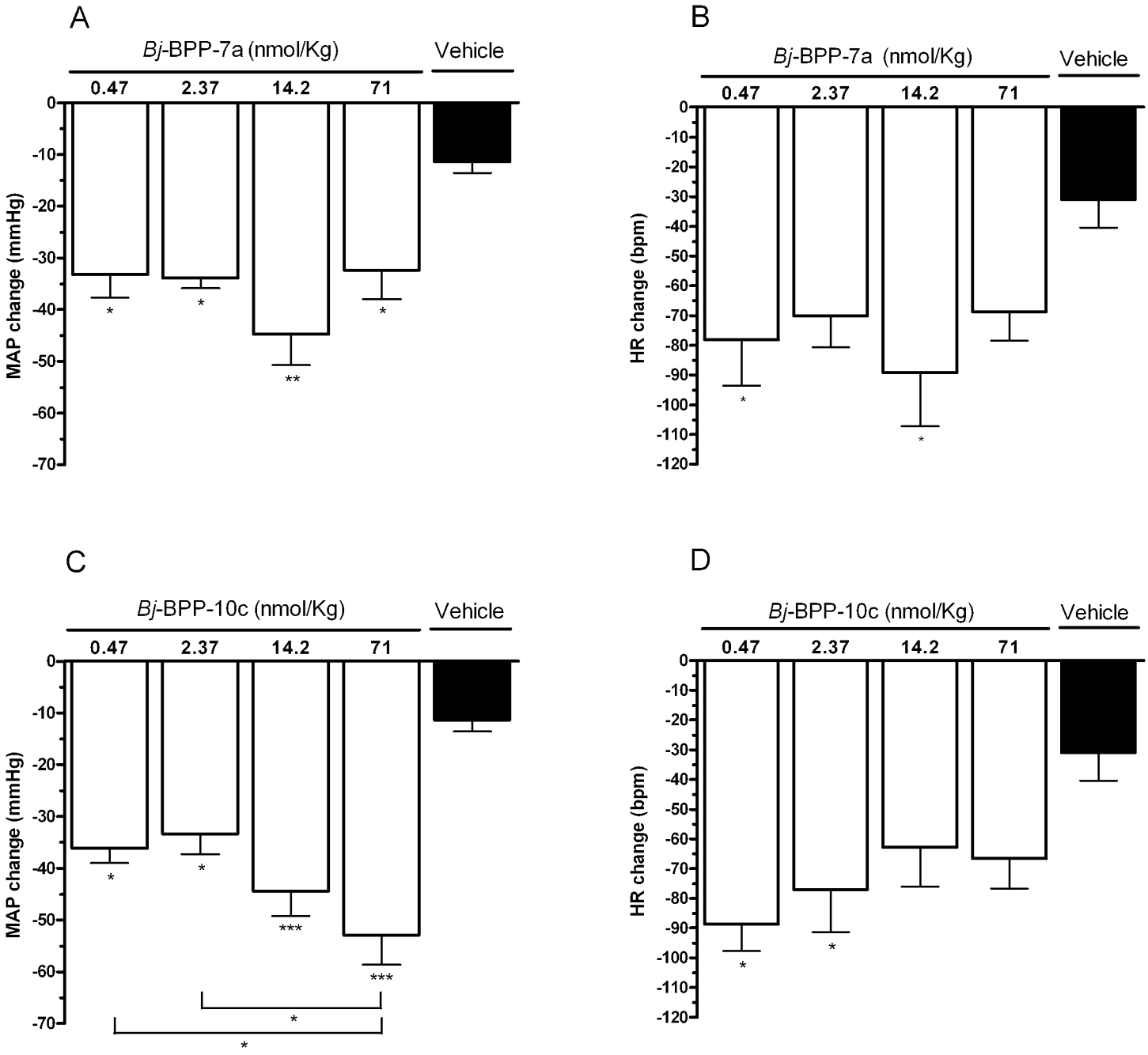


Figure 3

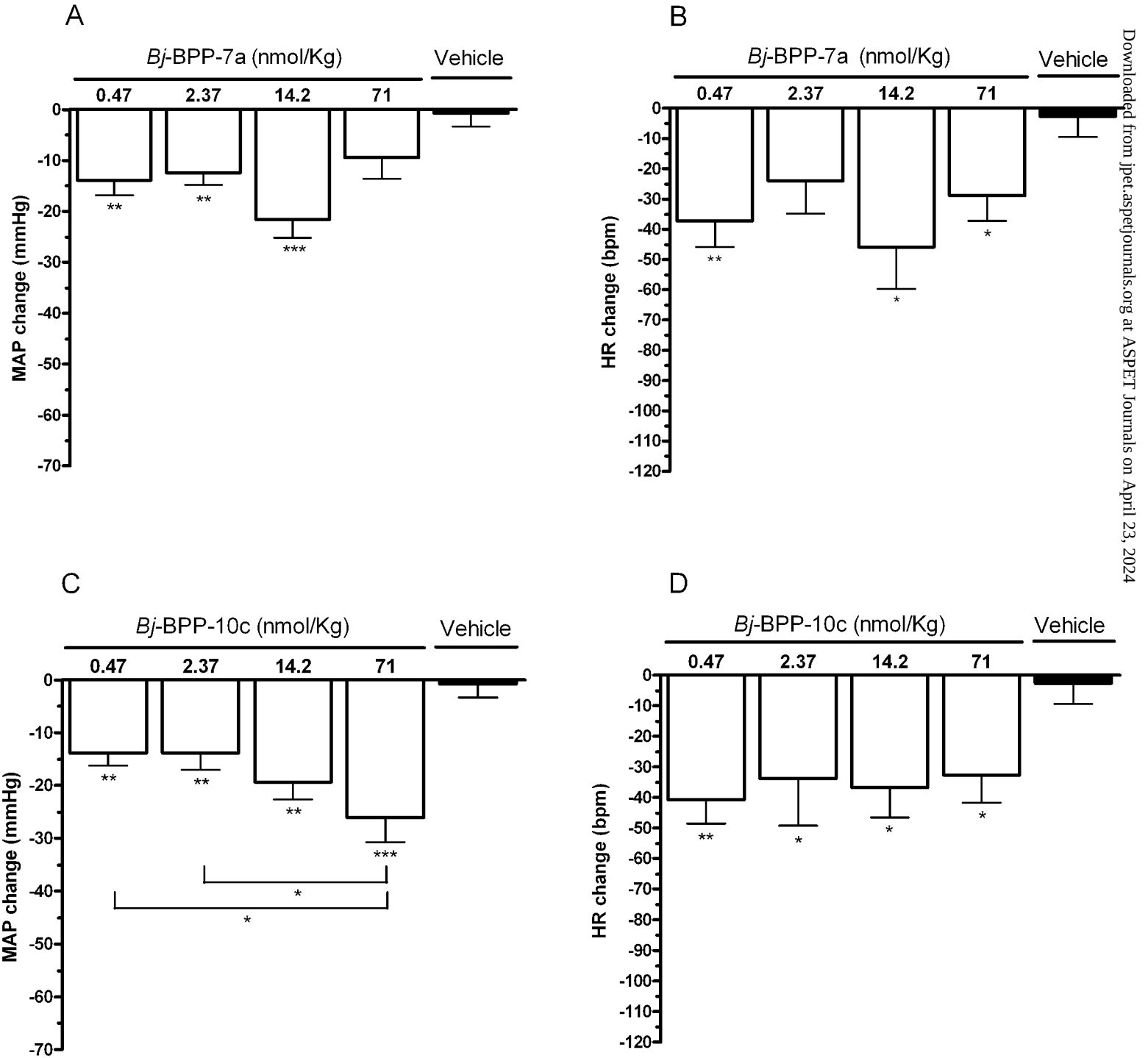


Figure 4

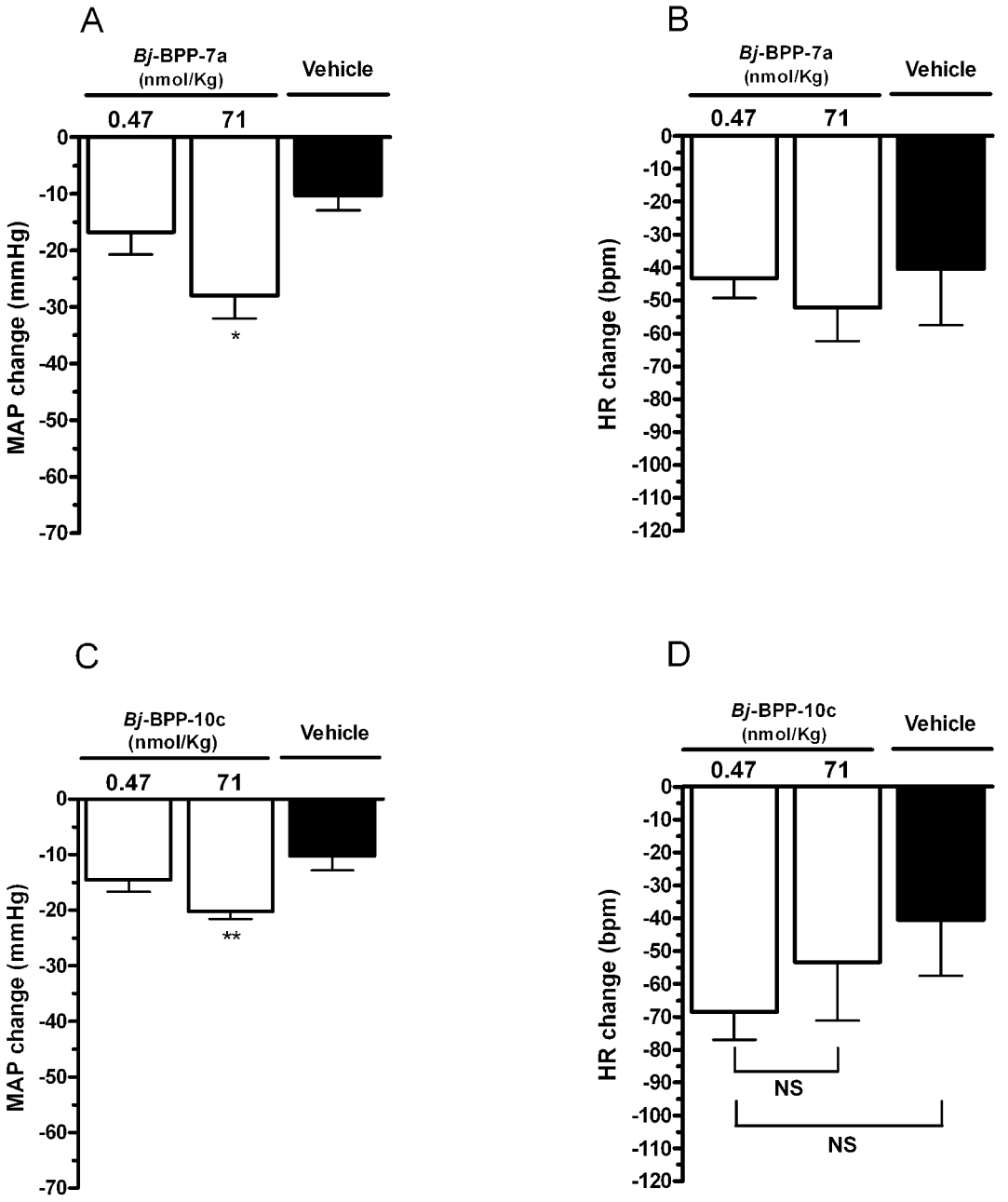

A
Bj-BPP-7a (nmol/Kg)
Vehicle
0.47
71
MAP change (mmHg)
0
-10
-20
-30
-40
-50
-60
-70
*
B
Bj-BPP-7a (nmol/Kg)
Vehicle
0.47
71
HR change (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
C
Bj-BPP-10c (nmol/Kg)
Vehicle
0.47
71
MAP change (mmHg)
0
-10
-20
-30
-40
-50
-60
-70
**
D
Bj-BPP-10c (nmol/Kg)
Vehicle
0.47
71
HR change (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
NS
NS

Figure 5

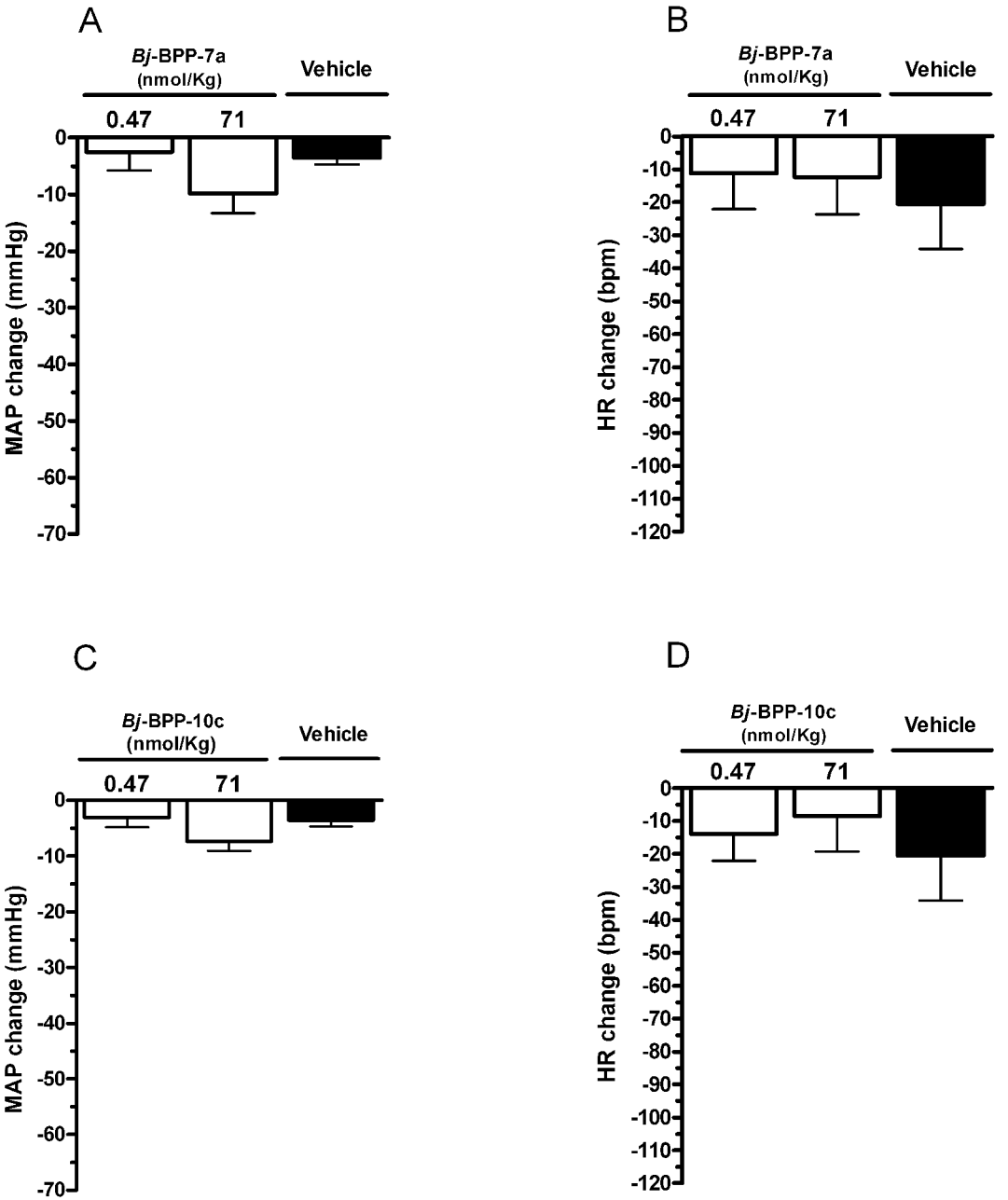

A
Bj-BPP-7a (nmol/Kg)
Vehicle
0.47
71
MAP change (mmHg)
0
-10
-20
-30
-40
-50
-60
-70
B
Bj-BPP-7a (nmol/Kg)
Vehicle
0.47
71
HR change (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120
C
Bj-BPP-10c (nmol/Kg)
Vehicle
0.47
71
MAP change (mmHg)
0
-10
-20
-30
-40
-50
-60
-70
D
Bj-BPP-10c (nmol/Kg)
Vehicle
0.47
71
HR change (bpm)
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
-110
-120

Figure 6

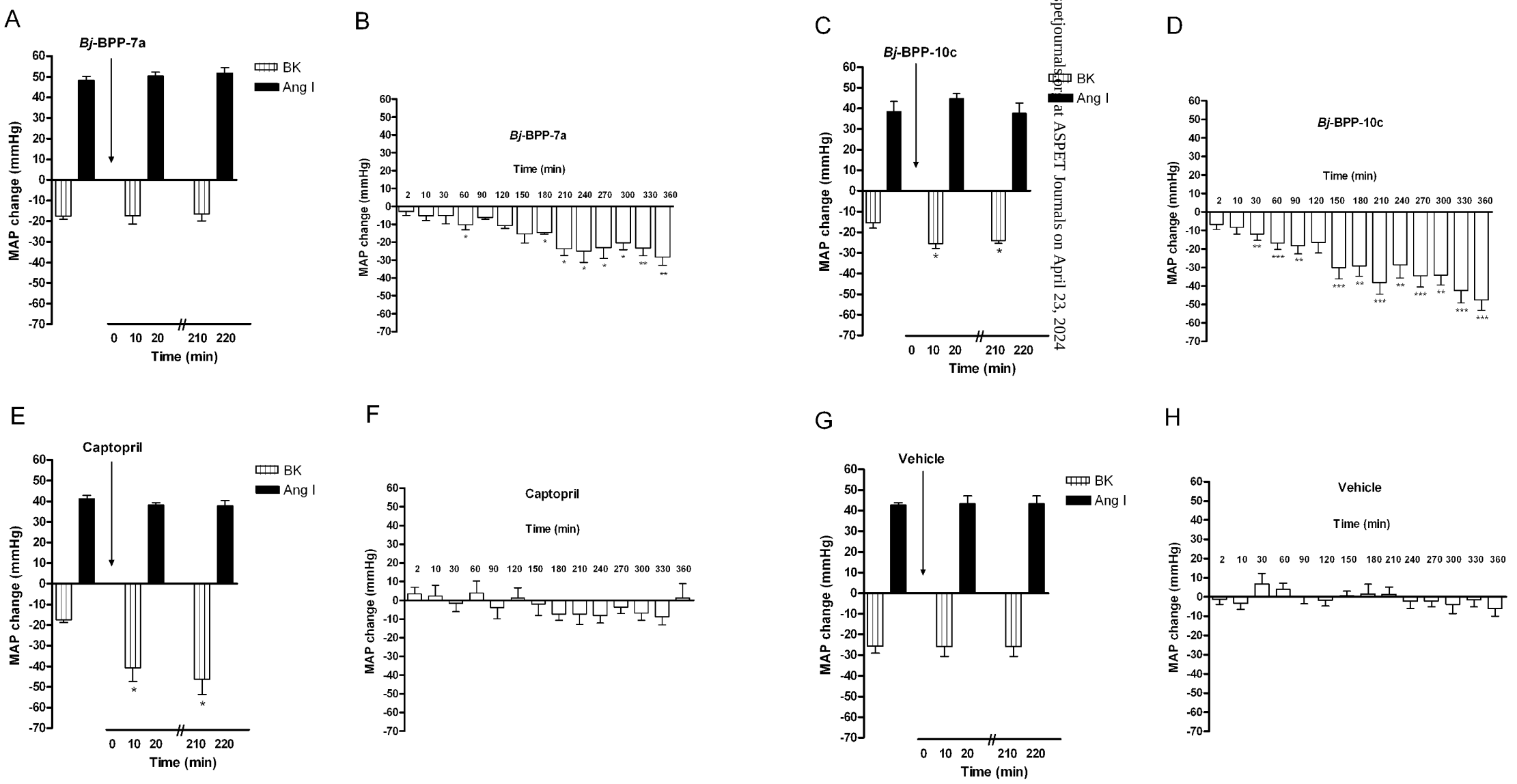
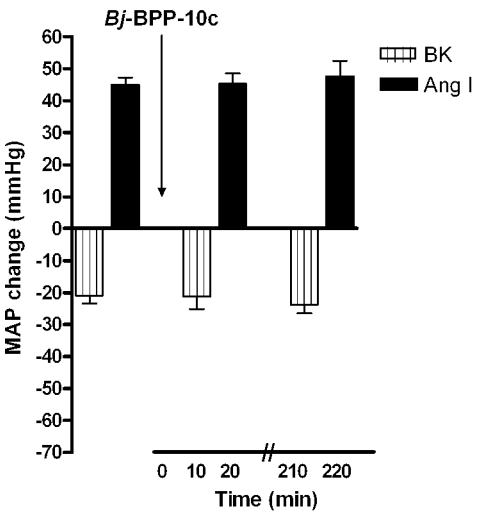


Figure 7

A


Bj-BPP-10c
BK
Ang I
MAP change (mmHg)
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
0 10 20 210 220
Time (min)

B

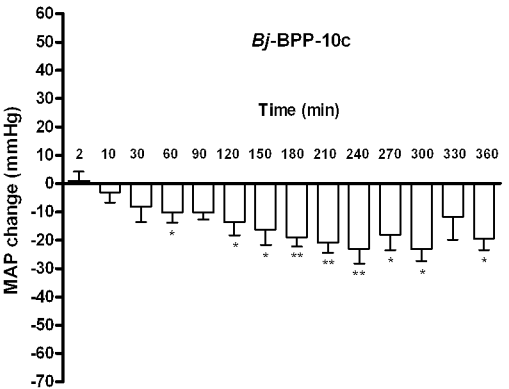
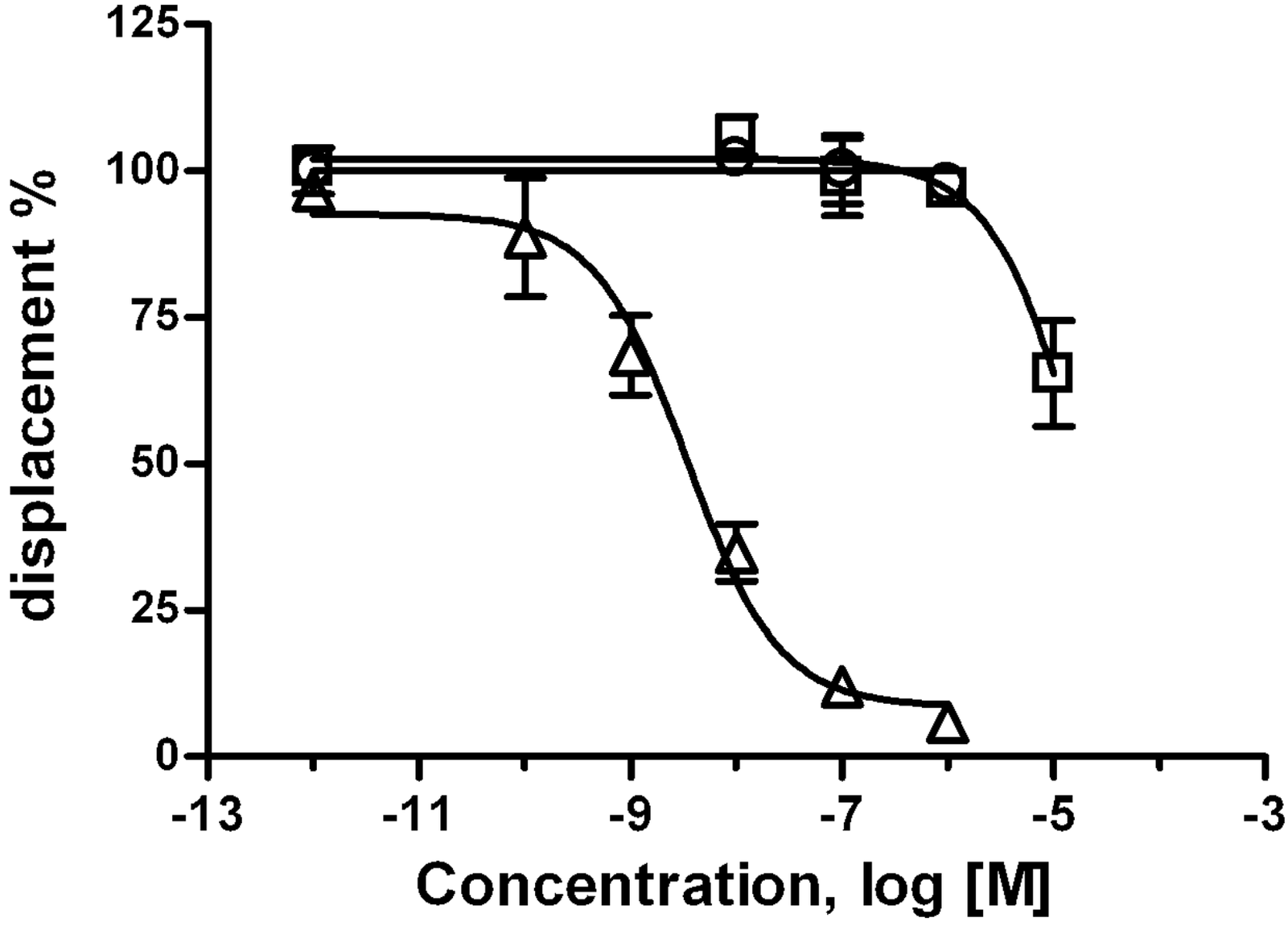

Bj-BPP-10c
Time (min)
MAP change (mmHg)
2 10 30 60 90 120 150 180 210 240 270 300 330 360
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70

Figure 8



△ Ang II, EC50 = 3.41×10^{-9} M (n = 9)

□ *Bj*-BPP-7a, EC50 > 2.06×10^{-5} M (n = 5)

○ *Bj*-BPP-10c, EC50 > 10^{-6} (n = 5)