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Tissue distribution in mice of BPP 10c, a potent proline-rich anti-hypertensive peptide of *Bothrops jararaca* ☆

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Abstract

The snake venom proline-rich peptide BPP 10c is an active somatic angiotensin-converting enzyme (sACE) inhibitors. Recently we demonstrated that the anti-hypertensive effect of BPP 10c is not related to the inhibition of sACE alone, thus suggesting that this enzyme is not its only target for blood pressure reduction. In the present work, a biodistribution study in Swiss mice of [^{125}I]-BPP 10c in the absence or in the presence of a saturating concentration of captopril, a selective active-site inhibitor of sACE, demonstrated that: (1) [^{125}I]-BPP 10c was present in several organs and the renal absorption was significantly high; (2) [^{125}I]-BPP 10c showed a clear preference for the kidney, maintaining a high concentration in this organ in the presence of captopril for at least 3 h; (3) The residual amount of [^{125}I]-BPP 10c in the kidney of animals simultaneously treated with captopril suggest that the peptide can interact with other targets different from sACE in this organ. We also showed that Cy3-labeled BPP 10c was internalized by human embryonic kidney cells (HEK-293T). Taken together, these results suggest that sACE inhibition by captopril affects the tissue distribution of [^{125}I]-BPP 10c and that the anti-hypertensive effects of BPP 10c are not only dependent on sACE inhibition.

Keywords: *Bothrops jararaca*; Proline-rich peptides; Angiotensin-converting enzyme inhibitors; Biodistribution; Internalization

Abbreviations: Ang-I, angiotensin I; Ang-II, angiotensin II; BK, bradykinin; Bj, *Bothrops jararaca*; ESI-MS, electrospray ionization-mass spectrometry; [^{125}I]-BPP 10c, iodinated BPP 10c; NEP, neprilysin; sACE, somatic angiotensin I converting enzyme.

☆ *Ethical statement*: We have obtained permission from all the authors. I declare that the material has not been published in whole or in part elsewhere, the paper is not currently being considered for publication elsewhere, and all experimental protocols were performed in accordance with the guidelines for the human use of laboratory animals of the Butantan Institute and approved by local authorities.

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1. Introduction

The primordial basis for the development of captopril, the first active-site inhibitor of somatic angiotensin-converting enzyme (sACE), was established in the late 1960s by John Vane, a consultant to the Squibb Institute for Medical Research. Dr. Vane associated the anti-hypertensive activity of the proline-rich oligopeptides of *Bothrops jararaca* (Bj) venom to the strong inhibition of sACE (Hayashi and Camargo, 2005). By inhibiting sACE, captopril blocks the conversion of angiotensin I (Ang-I) into Ang-II and increases the effects of bradykinin (BK) *in vivo*. The non-peptide inhibitor, captopril, displaying orally active anti-hypertensive activity, was made possible through the combination of the C-terminal proline present in all Bj-proline-rich oligopeptides with a sulfhydryl group (Ondetti and Cushman, 1982). Since captopril reproduced all the known pharmacological effects and sACE-inhibiting features of the proline-rich oligopeptides (Ng and Vane, 1970), the interest for the Bj-peptides themselves was substantially reduced.

In the last three decades, different explanations for the BPP-potentiating mechanism demonstrated that the enhancement of Bk activity cannot be entirely due to ACE inhibition (revised by Erdos and Marcic, 2001). Recently, studies using BK-potentiating peptide BPP-9a analogues, demonstrated the evidence that ACE inhibition and the BK-potentiating activity occur by different mechanisms (Mueller et al., 2005).

Two new discoveries, however, stimulated a renewed interest for these peptides: (a) a recent *in vivo* study showed that the C-site of the sACE plays the most important role in the metabolism of Ang-I and BK (Georgiadis et al., 2003); (b) the identification of a proline-rich decapeptide (BPP 10c) as the most potent and selective inhibitor of the C-site of the sACE, described so far (Cotton et al., 2002). Recently we showed in spontaneously hypertensive rats (SHR) that the strong and long-lasting anti-hypertensive effect of BPP 10c is independent of sACE inhibition (Ianzer et al., 2007). These results suggested that the active site of the sACE is not the only target for the BPP 10c (Ianzer et al., 2007).

In the present study we analyzed the biodistribution of radiolabeled BPP 10c alone or in combination with a saturating concentration of captopril in order to describe the organs and tissues that are targeted by the peptide in mice and also to get insights into the role of sACE in the strong anti-hypertensive effect of BPP 10c.

2. Materials and methods

2.1. Animals

Male Swiss mice (22–25 g) were bred at the Butantan Institute (São Paulo, Brazil). Animals had access to food and water *ad libitum* until 20 h before the bioassays. All animals were submitted to a light–dark cycle (12 h each). 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.

2.2. Reagents

All chemicals for peptide synthesis and iodination protocols were analytical reagent grade, purchased from Merck (USA) and Sigma-Aldrich (USA); captopril and BK were from Sigma Chemical Co.; sodium [^{125}I] iodine from Amersham (Buckinghamshire, UK); Sep-Pak μC_{18} columns from Waters (USA); Reagents for culture cell were purchased from Invitrogen (USA).

2.3. Cell culture

Human embryonic kidney cells (HEK-293T) were obtained from American Type Culture Collection and maintained in Dulbecco's modified medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 1 mM L-glutamine and 100 mg/mL streptomycin/penicilin at 37 °C in humidified air with 5% CO_2. Cells at 50–70% confluency were used in all experiments. Serum-starved cells were maintained overnight in DMEM supplemented with 100 mg/mL streptomycin/penicilin.

2.4. Peptide synthesis, purification, determination of concentration, and analysis of homogeineity of BPP 10c

BPP 10c (<ENWPHPQIPP) was synthesized using automated solid-phase synthesis by Fmoc strategy (Atherton and Sheppard, 1989), and the final deprotected peptides were purified by semi-preparative HPLC chromatography (Ianzer et al., 2004). The molecular mass and purity of the synthetic peptide were determined by MALDI-TOF mass spectrometry on an Ettan MALDI-TOF/Pro

system (Amersham Biosciences, Sweden) using cinnamic acid as the matrix. The peptide concentration was determined by amino acid analysis after acid hydrolysis for 22 h in 6 M HCl, at 110 °C, in glass tubes sealed in vacuum. The amino acid analysis of the hydrolysate was performed in a HPLC system by Shimadzu, after o-phthalaldehyde (OPA)-derivatization, and the effluent monitored by fluorescence emission at 450 nm after excitation at 350 nm (Bennett and Solomon, 1986).

2.5. Iodination of BPP 10c

The His^5 of BPP 10c was iodinated by chloramine T method as described by Hunter and Greenwood (1962), with minor modifications (Cardi et al., 1998). Briefly, BPP 10c (5 μg 10 μl^{-1}) in phosphate-buffered saline (PBS) was mixed with 1 mCi of sodium [^{125}I] iodine and 50 μg chloramine T. After 30 s, 100 μg sodium metabisulfite and 200 μg potassium iodine were added to the mixture and diluted in 50 mL water. The iodinated peptide was purified in a Waters Sep-Pak μC_{18} cartridge, and washed with 10 mL potassium iodine (5 mM) and 10 mL water. The [^{125}I]-labeled peptide was eluted with 2 mL methanol (Christophe et al., 2001). Tubes containing [^{125}I]-BPP 10c were counted in a gamma counter (Nuclear Chicago), 100–250 μCi/μg was routinely obtained. We adjusted the specific activity 1 μCi/nmol by dilution with non-radioactive BPP 10c and 22 nmol were injected per animal.

2.6. Labeling of BPP 10c with the fluorescent dye Cy3

One milligram of BPP-10c was covalently conjugated to the fluorescent dye Cyanine 3 (Cy3) by using the Fluorolink Cy3 reactive dye (GE Healthcare, UK). Labeling was performed according to the manufacturer instructions. Labeled BPP 10c was purified using RP-HPLC and analyzed by MALDI-TOF mass spectrometry (Lindahla et al., 2007).

2.7. [^{125}I]-BPP 10c, Cy3-BPP 10c, and BPP 10c biological activity

The biological activity of [^{125}I]-BPP 10c, Cy3-BPP 10c, and BPP 10c was assessed by the BK-potentiation assay on isolated guinea pig ileum, performed as previously described (Ianzer et al., 2004). After a 20 h fasting period, approximately 15 cm of the distal ileum of female guinea pigs were removed immediately after death and washed thoroughly with Tyrode solution (137 mM NaCl; 2.7 mM KCl; 1.36 mM $CaCl_2$; 0.49 mM $MgCl_2$; 0.36 mM NaH_2PO_4; 11.9 mM $NaHCO_3$; 5.04 mM D-glucose). Segments of 2.5 cm of the ileum were mounted under a 1 g load in a 10.5 mL muscle bath containing Tyrode solution at 37 °C and bubbled with air for isotonic bioassays. Muscular contraction was recorded on a Gould 2600 polygraph. One unit of BK-potentiation (U) is defined as the amount of potentiator (nmol) necessary to transform the effects of a single to a double dose of BK.

2.8. Biodistribution of [^{125}I]-BPP 10c

Groups of five male Swiss mice were killed by cervical dislocation at time intervals 5–180 min after i.p. injection of 22 nmol [^{125}I]-BPP 10c alone (normalized by specific activity), or associated with captopril (2200 nmol). Blood, kidney, heart, muscle, lung, liver, brain, and thyroid were collected, and washed with 10 volumes (v/w) of 0.15 M NaCl and weighed. The radioactivity of whole organs was measured in a gamma counter (Nuclear Chicago), and results were calculated as the percentage of injected dose per gram tissue (%ID/g). The standard source of [^{125}I] was also measured together with the samples in order to perform decay corrections.

2.9. Quantification of [^{125}I]-BPP 10c in mice urine

Twenty-two nanomoles of [^{125}I]-BPP 10c (normalized by specific activity) alone or associated with 2200 nmol of captopril were administered i.p. to two groups of 10 male Swiss mice. The animals were kept in stainless-steel metabolic cages adjusted to collect urine samples, which was done in time intervals of 15–1440 min after administration of the radioactive peptide. Radioactivity of the samples was counted and expressed as percentage of injected dose (%ID).

2.10. Analysis of BPP 10c stability

2.10.1. In vivo assay

About 22 nmol of BPP 10c was intra-peritonially injected into five mice kept in a metabolic cage. Pooled mice urine was collected for 6 h in 10 mL of 10% TFA solution and, subsequently purified in a Waters Sep-Pak μC_{18} cartridge (Waters, USA). Retained molecules were eluted with 2 mL of

acetonitrile, lyophilized, resuspended in 0.2 mL of acetonitrile, and submitted to HPLC chromatography using an Ultrasphere C-18 column (5 μm, 4.6 mm × 150 mm) coupled to an analytical HPLC system (Shimadzu, Japan). The peptides were eluted at a flow rate of 1 mL/min with a 10–80% gradient of trifluoroacetic acid (TFA)/H_2O [A] and TFA/acetonitrile(ACN)/H_2O (1:900:100) [B] over 20 min and absorbance at 220 nm were monitored.

Molecular masses and sequences of the eluted peptides were determined by mass spectrometric analysis using Q-TOF Ultima API (Micromass, UK), under positive ionization mode and/or by MALDI-TOF mass spectrometry on an Ettan MALDI-TOF/Pro system (Amersham Biosciences, Sweden), using cinnamic acid as matrix. The mass spectra corresponding to each signal from the total ion current (TIC) chromatogram were averaged, allowing an accurate molecular mass determination. External calibration of the mass scale was performed with horse heart myoglobin (Sigma) or NaI (Fluka). For MS/MS analysis on the Q-TOF, collision energy ranged 18–45 and the precursor ions were selected under a 1 m/z window.

2.10.2. In vitro assay

About 50 nmol of BPP 10c were incubated for 6 h at 37 °C in the buffer 0.1 M Tris–HCl, pH 7.0, 0.05 M NaCl, 10 μM $ZnCl_2$, and 0.2 mU of sACE (purified rabbit lung sACE purchased from Sigma, USA) or 0.05 M Tris–HCl, pH 7.5 and 0.2 mU of neprilysin (recombinant NEP kindly provided by Dr. Guy Boileau, Montreal, Canada). For this study, the activity of sACE and NEP were determined using BK as a substrate. One unit of enzymatic activity corresponds to the enzyme concentration that is able to hydrolyze 1 μM of BK/min. The reaction was interrupted by addition of 1% TFA and subjected to HPLC chromatography. The quantification of the peak corresponding to the elution of BPP 10c was estimated by comparing the elution time and height with the peak of a quantified standard peptide.

2.11. Internalization of BPP 10c by HEK-293T cells

HEK-293T cells were seeded on cover slips, allowed to settle, and serum starved overnight. Monolayers of HEK-293T cells were treated at 37 °C for 30 min with 1 μM Cy3-BPP 10c or control 1 μM Cy3 dye dissolved in a serum-free medium. After treatment, cell monolayers were washed with PBS. Cells on cover slips were fixed in 4% formaldehyde for 15 min and washed with sterile water. Cells were then incubated with anti-fading (Vector Laboratories, USA) for fluorescence maintenance. The images were obtained using a fluorescence microscope (Axiocam, Zeiss, Germany) and Cy3 filter sets (ex 550 nm, em 570 nm). Photomultiplier gain and laser power were kept constant throughout each experiment.

2.12. Statistical analysis

Tissue distribution results are expressed as mean ± SEM. Comparisons were made by Student's unpaired t test or one-way ANOVA with Dunnett post-test when appropriate (GraphPad Prism 4.0, GraphPad Software, Incorporation). The criteria for statistical significance were set at $p < 0.05$.

3. Results

3.1. Biological properties of [^{125}I]-BPP 10c, Cy3-BPP 10c, and BPP 10c

We initially tested the effect of iodination on BPP 10c. Iodination and Cy3-labeling of BPP 10c did not substantially affect the biological activity of the peptide as evaluated by the potentiation of BK on isolated guinea-pig ileum bioassay. The doses of [^{125}I]-BPP 10c (0.47 ± 0.02 nmol), Cy3-BPP 10c (0.45 ± 0.03 nmol), and of BPP 10c (0.48 ± 0.02 nmol), which doubled the contraction of the isolated guinea-pig ileum elicited by a single dose of BK were essentially the same.

3.2. Biodistribution of [^{125}I]-BPP 10c in mice tissues

The tissue distribution of [^{125}I] BPP 10c showed the kidney as the targeted tissue for the peptide. Five minutes after administration, [^{125}I]-BPP 10c selectively concentrated in the kidneys and to a much lesser extent in the lungs, heart, and liver (Figs. 1(A) and (B)), and, interestingly, after 60 min only the kidney sustained a high concentration of radiolabeled peptide. In the lungs, heart, and liver the peptide concentration rapidly decreased to about 30% of the maximal level after 60 min (Figs. 1(A) and (B)). The kidneys were able to maintain for at least 180 min about 50% of the maximal level of radiolabeled peptide (Fig. 1(C)). For the thyroid, the uptake was lower than 0.012%ID/g (data not shown).

Captopril drastically affected the biodistribution of [^{125}I]-BPP 10c, giving rise to two distinct results: (a) it

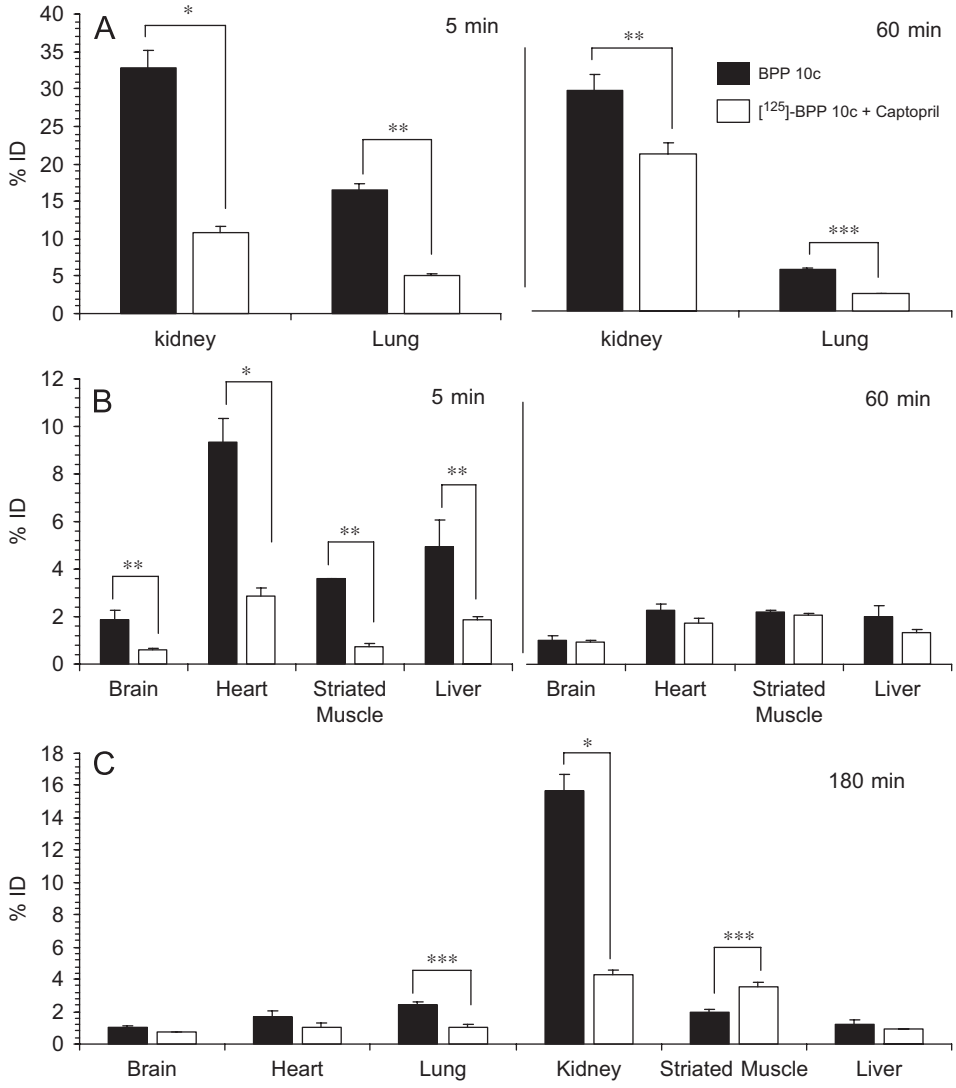

Fig. 1. Biodistribution of [^{125}I]-BPP 10c in the absence or in the presence of a saturating dose of captopril in Swiss mice organs. (A) In kidney and lung 5 and 60 min after injection; (B) in brain, heart, striated muscle, and liver 5 and 60 min after injection; (C) in brain, heart, lung, kidney, striated muscle, and liver 180 min after injection. Data are expressed as %ID/g. Data are reported as means ± SD of $n = 5$. *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$.

decreased the level of the radiolabeled peptide in all tissues; this effect was higher at 5 min (70–80% reduction) and decreased at 60 min (20–50% reduction, Figs. 1(A) and (B)); (b) from 60 to 180 min the level of the radiolabeled peptide decreased in all organs; however, it increased by 60% in striated muscle of mice treated with captopril (Fig. 1(B) and (C)).

3.3. Excretion of [^{125}I]-BPP 10c

About 20% of the radioactive peptide injected into mice appeared in the urine after 15 min and about 50% was eliminated after 3 h (Table 1). Captopril associated with [^{125}I]-BPP 10c increased twofold the amount of excreted radioactive peptide within 15–30 min, without substantially affecting the time and the maximum amount of radioactive peptide excretion (Table 1).

3.4. Stability of BPP 10c

In order to verify the stability of BPP 10c, a major prerequisite for the analysis of the tissue distribution of the [^{125}I]-labeled BPP 10c, we tested the

Table 1
Cumulative excretion of [^{125}I]-BPP 10c and [^{125}I]-BPP 10c supplemented with captopril in urine of Swiss mice

Time (min)*	Urine (%ID)	
	[^{125}I]-BPP 10c	[^{125}I]-BPP 10c + captopril
15	22.43 ± 1.67	49.06 ± 4.25**
30	37.91 ± 1.92	56.71 ± 5.45*
45	47.33 ± 2.36	57.89 ± 3.32*
60	47.35 ± 4.59	59.37 ± 4.47*
120	48.14 ± 4.99	64.13 ± 2.61*
180	52.16 ± 5.67	69.35 ± 3.33*
360	84.97 ± 5.54	71.34 ± 5.21*
1440	86.57 ± 6.35	78.43 ± 4.15

Time of urine collection was after administration of the radioactive peptide. Radioactivity of the samples was counted and expressed as percentage of injected dose (%ID). Data are means ± SD ($n = 5$). *$P < 0.05$; **$P < 0.01$.

integrity of the peptide by *in vivo* and *in vitro* assays. BPP 10c was injected into mice and the peptides were analyzed in the urine collected for 6 h. As shown in Fig. 2, ~70% of the peptide, recovered in the effluent of the RP-chromatography (HPLC), was identified by mass spectrometric analysis as the intact BPP 10c (Figs. 2(A) and (B)). The only metabolite identified by this analysis was BPP 10c missing the last Pro residue, corresponding to ~10% of the total injected amount (Fig. 2(C)). BPP 10c was resistant to hydrolysis by incubation for 6 h with either sACE and NEP (data not shown).

3.5. Internalization of BPP 10c by HEK-293T cells

To establish whether BPP 10c could be internalized by kidney cells, Cy3-BPP 10c or control Cy3 dye were incubated with human embryonic kidney cells HEK-293T for 30 min. The localization of labeled peptide in the cells was performed by employing fluorescence microscopy (Fig. 3). The internalization of Cy3-BPP 10c was detected as an almost homogeneous distribution of Cy3-BPP 10c in the cytosol of the cells (Figs. 3(A)–(C)). No fluorescence was detected in cells incubated with the Cy3 dye (Fig. 3(D)–(F)).

4. Discussion

One of the most successful examples of a peptidase as a drug target is the sACE inhibition for the treatment of cardiovascular pathologies. Captopril, the first of this class of active-site directed inhibitors of sACE, has been very effective in treating patients with hypertension, myocardial infarction, congestive heart failure, and diabetes nephropathy (Gavras and Gavras, 2004). However, a growing number of evidences suggests that the inhibition of sACE alone does not fully explain the effects of sACE inhibitors (Campbell et al., 2004). In fact, recently, Cotton et al. (2002) showed that BPP 10c, a potent sACE inhibitor, selective for the C-site of sACE, exerts strong and long-lasting anti-hypertensive effect in SHR, in doses which do not affect the metabolism of Ang-I and BK (Ianzer et al., 2007). Besides showing a clear distinction between BPP 10c and captopril, these results demonstrated that the anti-hypertensive effect of BPP 10c is not due to inhibition of sACE alone (Ianzer et al., 2007).

BPP 10c, found within the C-type natriuretic peptide precursor protein of Bj brain and venom gland (Hayashi et al., 2003), is a proline-rich oligopeptide from the same family of sACE inhibitors (Cushman et al., 1977). It is a very strong inhibitor of sACE (Kiapp = 5 nM; Ianzer et al., 2007, 2004; Hayashi et al., 2003; Cotton et al., 2002), and a weak inhibitor of the NEP neutral peptidase 24.11, (Kiapp = 250 nM, data not shown), thus suggesting that sACE is an important protein target in the plasma membrane of endothelial cells. This is also the case for captopril, whose inhibition constant for sACE is in the same order of magnitude (Kiapp = 3.98 nM; Michaud et al., 1997). The similar inhibition constants for BPP 10c and captopril make the active sites of sACE the major, if not the only binding sites for both inhibitors. Thus, it should be expected that a saturating concentration of captopril, associated with [^{125}I]-BPP 10c, would prevent the peptide to bind to the active site of sACE. Hence, it was not surprising that immediately after administration (5 min), captopril reduced by ~70% the accumulation of [^{125}I]-BPP 10c in all tissues, and consequently increased the renal excretion of [^{125}I]-BPP 10c. However, this effect was substantially reduced after 60 min, as the concentration of [^{125}I]-BPP 10c detected in all tissues was not affected by the saturating concentration of captopril. These results suggest that [^{125}I]-BPP 10c binds at a fast binding rate to sACE, and could bind at a slow rate to an yet unknown target (Skidgel and Erdös, 2004; Alhenc-Gelas et al., 1990).

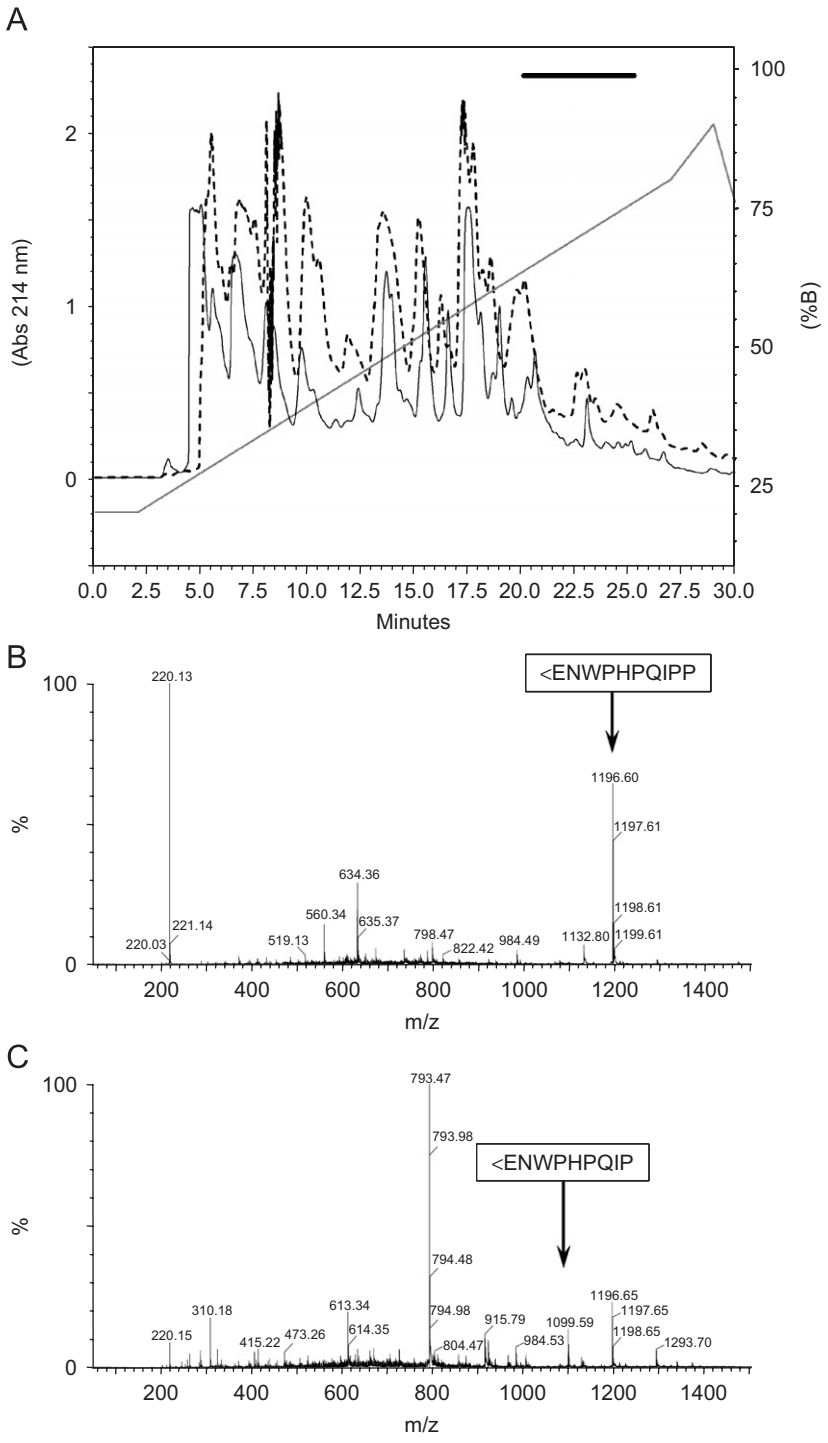

Fig. 2. Stability of BPP 10c in mice urine. (A) HPLC analysis of the urine of mice treated with BPP 10c (- - -) or 0.15 M NaCl (—) as described in Section 2. The black bar indicates the fractions of urine of BPP 10c-treated mice further analyzed by ESI-MS spectrometry shown in (B) and (C); the peak of 1196.60 Da corresponds to the full peptide, and the one of 1099.56 Da to the metabolite.

In favor of this hypothesis is the fact that the highest level of the radiolabeled peptide occurred in the kidneys and in the lungs, two tissues which express the highest level of sACE (Igic and Behnia, 2003). The possible binding to a slow-binding target was more evident in the kidneys, the brain, and the

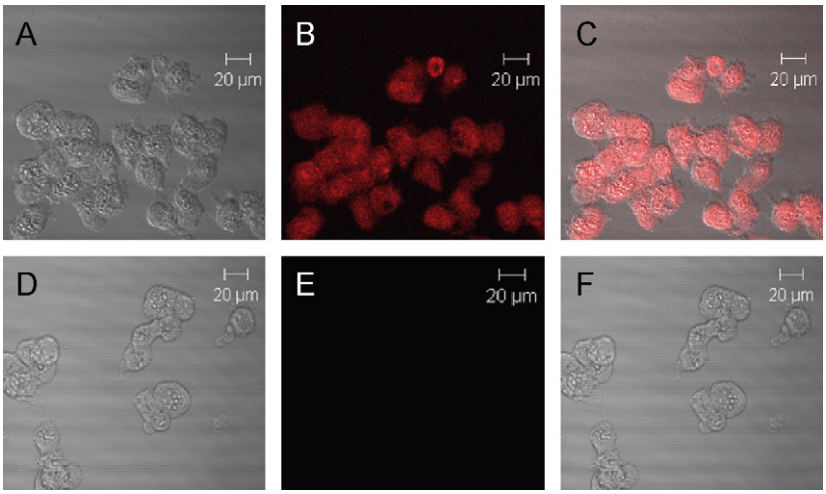

Fig. 3. Internalization assay of Cy3-BPP 10c examined with fluorescence microscopy. HEK-293T cells were incubated with Cy3-BPP 10c or Cy3 dye (1 μM) for 30 min at 37 °C and samples were analyzed as described in Section 2. Figures are representative of three individual experiments. (A) Phase contrast of the HEK-293T cells treated with Cy3-BPP 10c, (B) fluorescence of Cy3-BPP 10c, (C) merged images of (A, B), (D) phase contrast of the HEK-293T cells treated with Cy3 dye, (E) fluorescence of Cy3 dye, and (F) merged images of (D, E).

striated muscle, which showed increased radioactive peptide levels 1 h after administration of [^{125}I]-BPP 10c and captopril together. These results reinforce the hypothesis of the existence of other binding site(s) for BPP 10c in the plasma membrane of kidney cells. Alternatively, the peptide might undergo endocytosis. This hypothesis seems to represent a real possibility, since a growing number of peptides, including toxins, have been shown to penetrate cells (Kerkis et al., 2006; Joliot and Prochiantz, 2004). In fact, here we demonstrated that Cy3-BPP-10c was internalized by HEK-293T cells. On the other hand, in a recent study captopril was shown not to be internalized by kidney mesangial cells, which contain sACE (Singh and Leehey, 2007). The mechanism for the uptake of Cy3-BPP10c by HEK-293T cells is yet unknown; however, it could not be explained by interaction with sACE, because it is absent from HEK-293 cells (Bachvarov et al., 2001).

One important aspect concerning the use of BPP 10c as an anti-hypertensive compound is the striking stability of this peptide *in vivo*. Indeed, the two major putative plasma membrane peptidases, sACE and NEP, were unable to degrade BPP 10c after a 1-h incubation *in vitro* (data not shown). This is not surprising for a decapeptide containing four proline residues, including two at the C-terminus, and a pyroglutamic acid residue at the N-terminus. This specific structure makes this peptide resistant to endo- and exopeptidases (Barrett et al., 2001).

In conclusion, this study suggests that there might be a novel mechanism for the anti-hypertensive activity elicited by BPP 10c, in addition to the sACE inhibition. However, considering the high potency of *in vitro* sACE inhibition by BPP 10c, it cannot be ruled out that the effect of the peptide upon sACE can synergistically contribute to its anti-hypertensive *in vivo* activity (Ianzer et al., 2007). On the other hand, the biodistribution studies of [^{125}I]-BPP 10c in the presence and in the absence of captopril suggest novel target(s), characterized by a slow and sustained binding process, evident in several mice tissues. The identification of other target(s) besides sACE is in progress in our laboratory with the aim to explain the anti-hypertensive effect of BPP 10c at a concentration far below the one needed to inhibit sACE.

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