

Metabolism of bradykinin in aorta of hypertensive rats

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Alterations in the formation and metabolism of bradykinin (Bk) are hypothesized to play a role in the pathophysiology of hypertension, atherosclerosis and vascular complications of diabetes. However, despite its prominent role in cardiovascular regulation, studies on bradykinin have been limited by various difficulties in accurate measurements of this peptide in biological samples. In this study, using the LC-ESI-MS method we estimated the conversion of exogenous Bk to its main metabolites — Bk-(1–5) and Bk-(1–7) — in endothelial cell culture and in fragments of aorta of normotensive (WKY) and hypertensive rats (SHR). The effects of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP) inhibitors were more pronounced in SHR: perindoprilat inhibited Bk-(1–5) formation by 49 % and 76 % in WKY and SHR rats, respectively, and tiorphan tended to decrease formation of Bk-(1–5) in both groups of animals. The degradation of bradykinin and generation of both metabolites were significantly higher in the aorta of SHR rats than in WKY controls. Our results show that even in relatively early hypertension (in 4-month old SHR rats) inactivation of Bk by aorta wall is enhanced.

Keywords: bradykinin, angiotensin converting enzyme, mass spectrometry, bioactive peptides

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INTRODUCTION

Bradykinin (Bk) is a small peptide involved in a variety of cardiovascular effects, e.g., endothelium-dependent vasodilatation (by stimulation of endothelial nitric oxide, prostacyclin and endothelial hyperpolarizing factor release), natriuresis, cardioprotection and regulation of smooth muscle cell proliferation. Alterations in the formation and/or metabolism of Bk are hypothesized to play a role in the pathophysiology of hypertension, atherosclerosis and vascular complications of diabetes (Mombouli & Vanhoutte, 1995; Erdos Marcic, 2001; Bryant & Shariat-Madar, 2009; Sharma, 2009). However, despite the prominent role of Bk in cardiovascular regulation, its studies have been limited by various difficulties in accurate measurements of this peptide in biological samples: the concentrations of Bk in plasma or extracellular fluid are very low and its half-life is very short (17 s) (Ferreira & Vane, 1967). Moreover, with its rapid degradation, it is reasonable to assume that the vascular action of Bk occurs mostly locally and tracking its metabolism within the vessel wall seems more relevant than measurements done in the plasma (Campbell *et al.*, 1993; Mombouli & Vanhoutte, 1995; Erdos & Deddish, 2002).

It is known that Bk is rapidly cleaved by various proteases to yield several products — mainly Bk-(1–5) and Bk-(1–7) — in this regard collectively called kininases, and that angiotensin converting enzyme (ACE) shows high kininase activity (Fig. 1) (Campbell *et al.*, 1993; Bryant & Shariat-Madar, 2009). It has also been demonstrated that increased Bk bioavailability represents an important mechanism of beneficial cardiovascular actions of ACE inhibitors (ACEi) (Tom *et al.*, 2002). Notably, overactivation of the renin-angiotensin system is a hallmark of hypertension (Ramírez-Expósito & Martínez-Martos, 2008). Thus, the aim of our study was to determine the vascular metabolism of bradykinin in hypertension.

Recently, we developed an LC-ESI-MS method for *in vitro* and *ex vivo* study of angiotensin metabolism in various rat tissues (Bujak-Giżycka *et al.*, 2007; Olszanecki *et al.*, 2009). Here, we adapted this method to compare the metabolism of Bk to its main metabolites, Bk-(1–5) and Bk-(1–7) in aortas of normotensive and hypertensive rats and to assess Bk degradation in a culture of endothelial cells.

MATERIALS AND METHODS

Animals. Male Wistar-Kyoto rats (WKY, n=5) and spontaneously hypertensive rats (SHR, n=5) at 4 months of age and 230–270 g of weight were kept under controlled conditions (temperature, humidity and 12 h/12 h light/dark cycle), with a free access to food and water. Animals were administered fraxiparine (2850 IU, i.p.) and anaesthetized with 50 mg of thiopentone (50 mg/ml, i.p.). Fragments of aorta were excised through abdominal incision. All procedures were approved by the Ethical Committee of the Jagiellonian University.

Preparation of aorta fragments and organ-bath procedure. Tissue fragments were washed with cold standard Krebs-Henseleit solution (glucose 10 mM, pyruvate 2 mM, Hepes 10 mM, EDTA 0.03 mM, NaCl 118 mM, KCl 4.7 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, NaHCO₃ 15 mM) and cleaned of thrombi and tissue remnants. Blood vessels were cut into a suitable number of rings and opened flat. Tissue fragments were incubated before further procedures for 30 min at 37 °C in Krebs-Henseleit solution continuously bubbled with 95 % O₂/5 % CO₂.

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Abbreviations: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme-related carboxypeptidase; APP, aminopeptidase P; Bk, bradykinin; CPN, carboxypeptidase; DPP IV, dipeptidylpeptidase IV; ECE, endothelin converting enzyme; HUVEC, human umbilical vein endothelial cells; NEP, neprilysin, membrane-bound neutral endopeptidase; POP, prolyl oligopeptidase

Aorta fragments were individually transferred into Eppendorf tubes filled with 0.4 ml of bubbled Krebs-Henseleit solution at 37°C. Sample of 50 µl of buffer was removed to provide information on background production of Bk metabolites. Subsequently, 50 µl of Krebs-Henseleit solution or perindoprilat or tirophan (to achieve a final concentration of 10 µM) were added to each tube. After another 5 min of incubation, Bk was added to a final concentration of 100 nM. After 15 minutes a second sample of 50 µl of incubation buffer was removed. Each sample was promptly frozen at -70°C until further analysis with mass spectrometry. Tissue samples were dried overnight at 60°C to allow estimation of bradykinin metabolite production per mg of dry tissue.

Cell culture experiments. Hybridoma EA.hy926 cell line, formed by the fusion of HUVEC with the human lung carcinoma cell line A549 was kindly provided by Dr. C.-J. Edgell (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) (Edgell *et al.*, 1990). EA.hy926 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma Chemical Co., USA) supplemented with HAT Media Supplement (Sigma Chemicals, USA), penicillin G sodium sulphate (100 units/ml), streptomycin sulphate (100 µg/ml), amphotericin B (0.25 µg/ml) and 10% FBS. Cells were grown in humidified atmosphere of 5% CO₂/ 95% air at 37°C and were subcultured every 3–4 days as described previously (Olszanecki *et al.*, 2006).

The cells were seeded in wells of six-well plates (1 × 10⁶ per well) and serum-starved (appropriate medium containing one-tenth of the normal FBS concentration for 6 hours before experiment). The cells were incubated with Bk (10 nM, Sigma Chemicals, USA) for up to 24 hours. Supernatants were collected either at time „0” (immediately after administration of Bk) or at indicated time points and frozen at -70°C.

Measurement of bradykinin peptide concentration by LC-MS. Frozen supernatants from tissue samples and cell culture samples were thawed at room temperature and injected onto a chromatographic column. Separation of bradykinins was performed on a reversed-phase, high performance liquid chromatography (HPLC) system Ultimate 3000 (Dionex, USA) with a PepMap 100 C18 column (150 mm × 1 mm, 5 µm particle size) in combination with an appropriate guard column (5 mm × 1 mm; 5 µm particle size) (Dionex, USA). The optimized mobile phase solvents were: 5% acetonitrile in a buffer

of 4 mM ammonium formate with 4 mM formic acid (phase A) and 90% acetonitrile in same buffer (phase B). Bk and its metabolites were separated at a flow rate of 0.045 ml/min with a linear gradient of B in A.

Mass spectrometric detection was performed using an LCQ ion-trap mass spectrometer (Finnigan, San Jose, USA) equipped with an ESI source (electrospray). High purity nitrogen used as a sheath gas was generated with a Claind nitrogen generator. All experiments were carried out in the positive ion mode. Bradykinins solutions were infused directly into the mass spectrometer at 3 µl/min. The main working parameters of the mass spectrometer were as follows: nitrogen (sheath gas) flow rate 65 arb, ion spray voltage 5 kV, capillary temperature 200°C, capillary voltage 46 V, tube lens offset 40 V. For optimization of ESI conditions the scan mode in the range 100–2000 Da was used. For bradykinin detection, selected ion monitoring (SIM) mode was used with total microscans = 1 and maximum inject time of 500 ms. Scanned mass range was set at 530–760 Da. LCQ data were analyzed by Xcalibur Software, version 1.0 (Finnigan). Concentrations of bradykinins were calculated using standard calibration curves constructed by linear regression analysis of peak area *versus* bradykinin concentration. The calibration curves were prepared for each peptide examined — Bk, Bk-(1–7) and Bk-(1–5) — at a concentration range of 20 pM to 100 nM.

Chemicals. Bradykinin (Bk), Bk-(1–7) and Bk-(1–5) were purchased from Sigma Chemicals (USA). Formic acid (99%) (Riedel de Haen, Germany), acetonitrile (Baker), ammonium formate (Fluka) were HPLC grade. Deionized water was obtained using a MilliPore (MilliQ) system.

Statistics. Concentrations of bradykinin peptides were expressed in pmol/mg dry tissue and in pM (cell culture). All values in the figures and text are expressed as mean ± S.E. of n = 15 observations. Concentrations of the analytes studied were compared using non-parametric Wilcoxon's test. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Measurement of concentrations of Bk and its metabolites

The LC-ESI-MS method proved to be a valuable tool for assessment of exogenous Bk metabolism *ex vivo*, in organ-bath of tissue fragments excised from rats, and *in vitro* in a culture of endothelial cells. The separation conditions allowed easy determination of Bk and its two main metabolites in both types of samples (Fig. 2). The molecular mass of Bk, Bk-(1–7) and Bk-(1–5) are 1059 Da, 756 Da and 572 Da, respectively (Table 1). The ions monitored in the samples by ESI-MS in the SIM mode are presented in Table 1. The detection limit for Bk, Bk-(1–5) and Bk-(1–7) was 0.1 pM.

Bradykinin degradation by cultured endothelial cells

Ea.hy926 endothelial cells were able to metabolize exogenous Bk both to the Bk-(1–7) and Bk-(1–5) peptides. The proportion of these metabolites changed over the time of incubation with Bk: in the beginning, in the presence of excess substrate, much more Bk-(1–7) than Bk-(1–5) was produced. This is in agreement with our previous results on angiotensin I metabolism, showing that neutral endopeptidase (NEP) and/or ACE2 over-

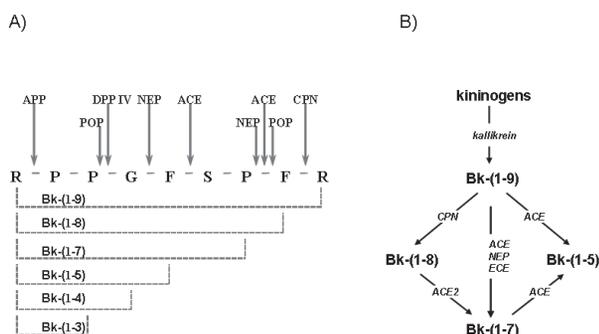


Figure 1. Main bradykinin degradation pathways

ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme-related carboxypeptidase; APP, aminopeptidase P; CPN, carboxypeptidase; DPP IV, dipeptidylpeptidase IV; ECE, endothelin converting enzyme; NEP, neprilysin, membrane-bound neutral endopeptidase; POP, prolyloligopeptidase

Table 1. Molecular characteristics of bradykinin and its metabolites

Peptide name	Amino-acid sequence	Molecular mass [g/mol]	Monitored ion (<i>m/z</i>)	Ion charge (<i>z</i>)
Bradykinin	R-P-P-G-F-S-P-F-R	1060.22	531	2
Bradykinin-(1-7)	R-P-P-G-F-S-P	756.86	757	1
Bradykinin-(1-5)	R-P-P-G-F	572.66	573	1

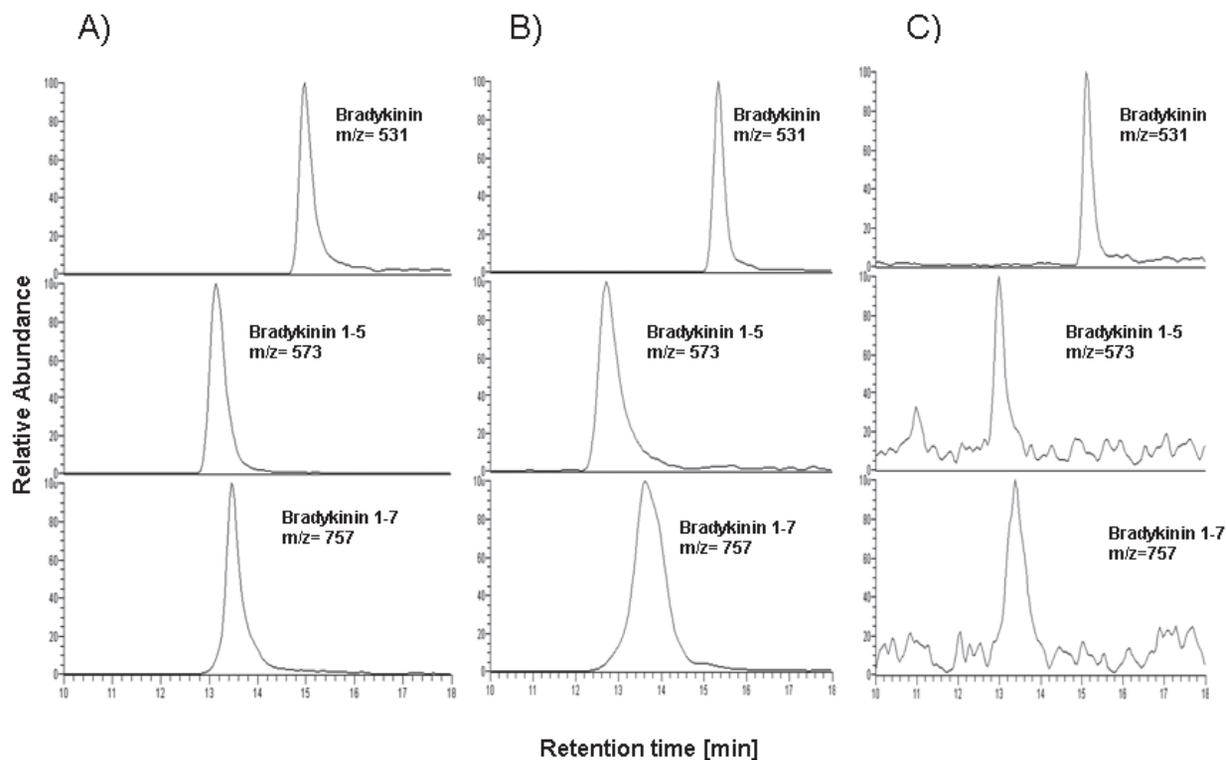
whelm ACE activity in these cells (Bujak-Gizycka *et al.*, 2007). Importantly, Bk-(1-7) may serve as a substrate only for ACE (Fig. 1), so as expected, starting from the 4th hour of incubation, Bk-(1-5) became the prevalent metabolite of Bk slowly accumulating in culture medium (concentrations after 24 h: 233.1 pM of Bk-(1-5) *vs.* 21.5 pM of Bk-(1-7)). Thus, our data suggest sequential degradation of Bk in cell culture: first to Bk-(1-7) and subsequently to Bk-(1-5). It has been demonstrated that in primary endothelial cell cultures activities of ACE and NEP may vary and strongly depend on the vascular origin (cells from pulmonary artery express much more ACE than from other vascular beds) and time of cultivation (ACE in HUVEC is downregulated with subsequent passages) (Baudin *et al.*, 1997; Balyasnikova *et al.*, 1998). Our study confirms that the LC-MS method is a valuable tool for assessment of kinin metabolism in cell culture setting.

Bradykinin metabolism in aorta of hypertensive rats

Incubation of aorta of WKY and SHR rats with exogenous Bk for 15 min resulted in the generation of a roughly equimolar mixture of Bk-(1-5) and Bk-(1-7) (at that time the substrate was still present in excess in incubation buff-

er). Importantly, the degradation of bradykinin and generation of both metabolites were significantly higher in the aorta of SHR rats than in the WKY controls (Table 2). Accordingly, the effects of ACE and NEP inhibitors were more pronounced in SHR: perindoprilat inhibited Bk-(1-5) formation by 49% and 76% in WKY

and SHR rats, respectively, and tiotropin tended to decrease formation of Bk-(1-5) in both groups of animals, but only in SHR its action reached statistical significance (Table 2). Although some studies have shown higher ACE activity in spontaneously hypertensive rats (Sivieri, Jr. *et al.*, 2007), previous reports, focused mainly on Bk metabolism in the plasma, did not reveal any differences between WKY and SHR groups (Dendorfer *et al.*, 2001). Here, differences in the rate of Bk degradation directly in the artery wall are demonstrated between WKY and SHR rats for the first time. Our results demonstrated that even in relatively early hypertension (in 4-month old SHR rats) decomposition of Bk by the vessel wall is enhanced. It is widely recognized that Bk is one of the most potent stimulators of endothelial cells to release nitric oxide and prostacyclin (Mombouli & Vanhoutte, 1995; Gryglewski *et al.*, 2002). Whether enhanced Bk degradation in hypertensive rats may contribute to the endothelial dysfunction seen in these animals (Bernatova *et al.*, 2009) is still a matter of controversy. Interestingly, young hypertensive rats seem not to suffer from lower levels of Bk in tissues — on the contrary, Campbell and coworkers (1995) observed elevated levels of Bk as well as its metabolites (reflecting over activation of kalikrein) in tissues of these animals. There are limited data concerning similar

**Figure 2. Chromatograms of Bk and its main metabolites**

(A) Mixture of standards; (B) in EA.hy926 cell culture (4 h of incubation with 10 nM Bk); (C) in aorta of WKY rats (15 min of incubation with 100 nM Bk).

Table 2. Production of bradykinin metabolites in aorta of WKY and SHR rats

Tissue fragments were incubated for 15 min with 100 nM bradykinin (Bk) with or without perindoprilat (10 µM) or tiorphan (10 µM). All values are mean ± S.E.M. pmol/mg dry tissue. **P*<0.05 vs. control; #*P*<0.05 vs. WKY.

	WKY		SHR	
	Bk-(1-7)	Bk-(1-5)	Bk-(1-7)	Bk-(1-5)
Control	3.3±0.65	4.9±1.34	5.3±0.76 [#]	6.6±0.84 [#]
Perindoprilat	3.4±0.47	2.5±0.31*	4.0±0.77	1.6±0.79*
Tiorphan	4.3±0.76	3.8±0.30	4.24±0.73	2.8±0.58*

changes in Bk metabolism in humans. Notably, some authors showed that concentration of Bk-(1-5) in human circulation system was significantly higher in hypertensive patients (Murphey *et al.*, 2004). It is tempting to speculate that because of the more intense Bk degradation, prehypertensive and hypertensive patients could benefit more from the “bradykinin-sparing” effect of ACE and NEP inhibitors.

In contrast to the generation of Bk-(1-5), which was mainly ACE-dependent, our study confirmed an involvement of numerous proteases in the formation of Bk-(1-7) in the rat aorta: neither ACE nor NEP inhibition influenced significantly formation of Bk-(1-7) in the SHR or WKY rats (Table 2). This is in agreement with previous reports, identifying Bk-(1-5) as the main product of direct Bk degradation by ACE, and direct and/or indirect involvement of ECE, CPN, POP and ACE2 in tissue formation of Bk-(1-7) (Dendorfer *et al.*, 2001). Clearly, a thorough analysis of Bk degradation pathways would require not only a pharmacological approach, but also estimation of particular protease activity in aortic tissue. Our study opens the question of differences between WKY and SHR rats regarding Bk metabolism at the molecular level for further research.

In comparison to angiotensins, studies exploring the bioactivities of Bk metabolites are limited. It has been shown that Bk-(1-5) (according to amino-acids sequence sometimes presented as RPPGF; thrombostatin) can inhibit thrombin-induced platelet activation by blocking protease-activated receptor type 1 (PAR-1), thereby preventing occlusion in a canine coronary thrombosis model (Hasan *et al.*, 1996; 1999). There is no data about biological activity of Bk-(1-7).

CONCLUSION

Our study shows differences between metabolism of bradykinin in young hypertensive and normotensive rats. We demonstrated that degradation of Bk to Bk-(1-5) and Bk-(1-7) is enhanced in the aorta of hypertensive rats probably due to higher ACE activity in these animals. The functional significance of this finding requires further investigation.

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