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Degradation of Bradykinin, a Cardioprotective Substance, during a Single Passage through Isolated Rat-Heart

M. Ahmad, I. J. Zeitlin, J. R. Parratt, and A. R. Pitt[1]

Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK and [1]Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, UK

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Angiotensin converting enzyme (ACE) inhibitors have cardioprotective effects in different species including human. This cardioprotective effect is mainly due to the inhibition of bradykinin (BK) degradation rather than inhibition of the conversion of angiotensin I to angiotensin II. Bradykinin, a nonapeptide, has been considered to be the potential target for various enzymes including ACE, neutral endopeptidase 24.11, carboxypeptidase M, carboxypeptidase N, proline aminopeptidase, endopeptidase 24.15, and meprin. In the present study, the coronary vascular beds of Sprague Dawley rat isolated hearts were perfused (single passage) with Krebs solution alone or with different concentrations of BK *i.e.* 2.75×10^{-10}, 10^{-7}, 10^{-6} and 10^{-5} M solution. Percent degradation of BK was determined by radioimmunoassay. The degradation products of BK after passing through the isolated rat-hearts were determined using RP-HPLC and mass spectroscopy. All the four doses of BK significantly decreased the perfusion pressure during their passage through the hearts. The percentage degradation of all four doses was decreased as the concentration of drug was increased, implying saturation of a fixed number of active sites involved in BK degradation. Bradykinin during a single passage through the hearts degraded to give [1-7]-BK as the major metabolite, and [1-8]-BK as a minor metabolite, detected on HPLC. Mass spectroscopy not only confirmed the presence of these two metabolites but also detected traces of [1-5]-BK and arginine. These findings showed that primarily ACE is the major cardiac enzyme involved in the degradation of bradykinin during a single passage through the coronary vascular of bed the healthy rat heart, while carboxypeptidase M may have a minor role.

Key words: Bradykinin, Angiotensin converting enzyme, Carboxypeptidase M, Rat heart

INTRODUCTION

Angiotensin-converting enzyme (ACE) inhibitors have been reported to be cardioprotective in rats, dogs and also in humans (Linz *et al.*, 1995). This cardioprotective effect is mainly due to the inhibition of bradykinin (BK) degradation by kininase, rather than inhibition of the conversion of angiotensin I to angiotensin II. ACE and neutral endopeptidase 24.11 (NEP) have been detected in rat hearts (Yamada *et al.*, 1991; Piedimonte *et al.*, 1994). In our previous studies, ACE was detected in rat heart homogenates and BK destroying activity was also observed in rat isolated heart perfusates. BK degradation in perfused rat heart effluents appeared to involve more than one enzyme (Ahmad *et al.*, 1996). After global ischaemia, release of the BK fragment [1-8]-BK was increased in rat isolated heart perfusates when ACE, which produces [1-7]-BK, was inhibited (Lamontagne *et al.*, 1995). Therefore, the functions of kininases may be altered under different pathophysiological conditions. The aim of this study was to evaluate the nature and extent of bradykinin metabolism during a single passage through the normally perfused heart by identifying and characterizing the bradykinin fragments formed during passage through rat isolated hearts. The study showed that ACE and possibly NEP are the major cardiac enzymes involved in the degradation of bradykinin during a single passage through healthy rat heart, whereas carboxypeptidase M has a minor role.

Correspondence to: Maqsood Ahmad, Department of Pharmacy, Bahauddin Zakariya University, Multan 60800, Pakistan
Tel: 0092-614-745830, Fax: 0092-61-9210089
E-mail: maqsood_mul@yahoo.com

MATERIALS AND METHODS

Materials

Bradykinin, [1-8]-BK, bovine serum albumin, normal pooled rabbit sera, sodium azide, aprotinin and trifluoroacetic acid were purchased from Sigma. Potassium dihydrogen phosphate and propan-2-ol were purchased from Fisons. Acetonitrile and dipotassium hydrogen phosphate were purchased from BDH. Rabbit anti-BK serum, [^{125}I-Tyr^{8}]-BK, [1-7]-BK, sodium chloride were purchased from Peninsula Laboratories, Dupont, Bachem, and M & B respectively. All the reagents used were of analytical grade or better.

Experimental animals

Male Sprague Dawley rats (supplied by BVK Universal, U.K.), weighing 250-300 g, were used. Before the day of experiment the animals were fed on CRM (P) pellets supplied by Special Diet Services (SDS), U.K. Feed and water were provided *ad libitum*. Animals were kept under 12 h light/12 h dark cycles in the animal unit of the department. All procedures conformed to UK legislation requirements.

Surgical procedure

Animals were anaesthetized by injecting pentobarbitone (60 mg kg^{-1}) intraperitoneally. Heparin (500 U) was injected through the dorsal penile vein. The skin was removed from the chest. The chest was opened after cutting the ribs from the left and right sides of the sternum. The heart was exposed, removed and quickly perfused by inserting a cannula through the aorta and attaching it to the Langendorff apparatus pre-calibrated for perfusion pressure and flow rate. Hearts were perfused with Krebs Henseleit solution at a rate of 10 mL per min (mM: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.23 and glucose 11), pH 7.4, aerated with O_2:CO_2, 95%:5%, filtered and maintained at 37°C. Perfusion pressure was recorded using a Gould P23 1D transducer *via* a side arm of the aortic cannula.

The experimental protocol and sampling times are given in Fig. 1. After a 60 min, stabilization period, an initial BK concentration of 2.75×10^{-10}M was infused into the perfusate (Harvard apparatus, syringe infusion pump 22) near the heart, at a flow rate of 0.5 mL/min and continued for 10 min. Perfusate samples (1 mL each) at 2 and 9 min after drug infusion were collected in chilled polypropylene tubes containing a mixture of EDTA and aprotinin (1 mg/mL and 500 KIU/mL final concentration respectively). The rest of the perfusates taken during the course of bradykinin infusion were pooled. Three other bradykinin concentrations (10^{-7}, 10^{-6} and 10^{-5} M) were also infused in a similar manner and perfusate samples were collected as described above (see Protocol, Fig. 1). Perfusate samples were analysed as follows.

Loss of bradykinin loss when perfused through rat isolated hearts

The assay range of the BK radioimmunoassay (Moshi *et al.*, 1992) was 4-2000 (pg per tube). To assay the perfusate samples, effluent samples produced by all four doses were diluted appropriately for the assay range and the percent loss of bradykinin in each dose was calculated.

Detection and characterization using RP-HPLC of bradykinin metabolites formed during a single passage through rat isolated hearts

For this purpose one dose (10^{-6} M) of bradykinin was used. The perfusate samples were extracted with 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) using Sep-Pak Vac C18 cartridges. The eluents were dried under a stream of nitrogen at 40°C and dried samples were run on RP-HPLC (Beckman System Gold Controller, model 166 variable Wavelength UV detector, at a Wavelength of 210 nm). The peaks were compared with synthetic bradykinin and its metabolites (chromatograms of reference standards are not shown). Eluted peaks were separated and dried under a stream of nitrogen and kept at -20°C until subjected to mass spectroscopy to identify the extracted peptides.

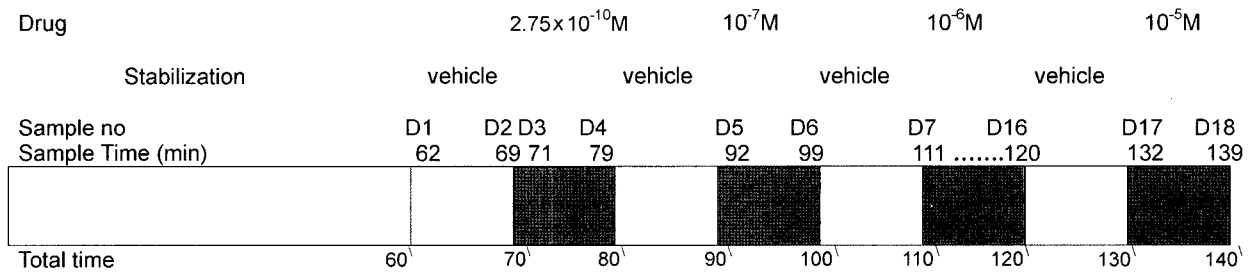

Fig. 1. Protocol for the infusion of different concentrations of bradykinin in rat isolated hearts. All the 'D' samples were taken in an EDTA and aprotinin mixture (1 mg/mL and 500 KIU/mL final concentrations, respectively). The figure shows time course of the experiment indicating the time points at which the samples (D1–D18) were taken from the coronary vascular effluent.

Confirmation of kinin metabolite identity by mass spectroscopy

Perfusate bradykinin (10^{-6} M) and its metabolites were extracted and dried as described above. Extracted bradykinin and its metabolites were subjected to mass spectroscopy. The formed fragments were separated using RP-HPLC and eluents were pooled and dried under a stream of nitrogen. The dried peaks were then subjected to electrospray mass spectrometry (Fenn *et al.*, 1989). Electrospray mass spectrometry was performed using a VG platform I mass spectrometer, with a quadrapole mass analyzer, standard lens stack and electrospray source, using the VG Mass Lynx and MaxEnt software. The carrier solvent was acetonitrile:water (50:50) and the solvent flow was 10 μL/min. Samples were dissolved in 50/50 acetonitrile/water containing 0.4% formic acid and injected directly into the carrier stream. Spectra were acquired every 4 seconds in the mass range 150-1200 atomic mass unit (amu). The masses quoted are for the peak heights for singly charged ions and the computer generated masses for multiply charged ion sets.

Statistical analysis

The results are presented as mean ± SEM. The two-tailed paired t-test was used to assess statistical significance. $P \leq 0.05$ was considered significant.

RESULTS

Perfusion pressure changes due to different concentrations of bradykinin

Infusion of 2.75×10^{-10} M BK caused a decrease ($P<0.01$) in perfusion pressure of 4.4±0.1 mm Hg (n=5) compared with vehicle (48.2±5.92 mm Hg, n=5). The decreases in perfusion pressure (mm Hg) produced by concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M BK were 6.4 ± 0.9, 5.8 ± 1.1 and 6.0 ± 1.8 respectively ($P<0.01$, $P<0.01$ and $P<0.05$ respectively, n=5, Fig. 2).

Percentage loss of bradykinin during perfusion through rat isolated hearts

Bradykinin was degraded during its passage through rat isolated hearts. Bradykinin (2.75×10^{-10} M) was degraded completely 100 ± 0% (n=5). The other three doses (10^{-7}, 10^{-6}, and 10^{-5} M) were degraded by 58.4 ± 2.07%, 40.8 ± 5% and 30.2 ± 5% respectively. The percentage degradation of bradykinin appeared to decrease as its dose increased (Fig. 3).

Detection of bradykinin metabolites using HPLC

On the HPLC chromatogram the bradykinin peak appeared with a retention time of 19.31 min (Fig. 4). The peaks, which appeared at a retention time of 9.03 min and 21.35 min were [1-7]-BK (major peak) and [1-8]-BK (minor peak) respectively, confirmed by running the reference standards (chromatograms not shown).

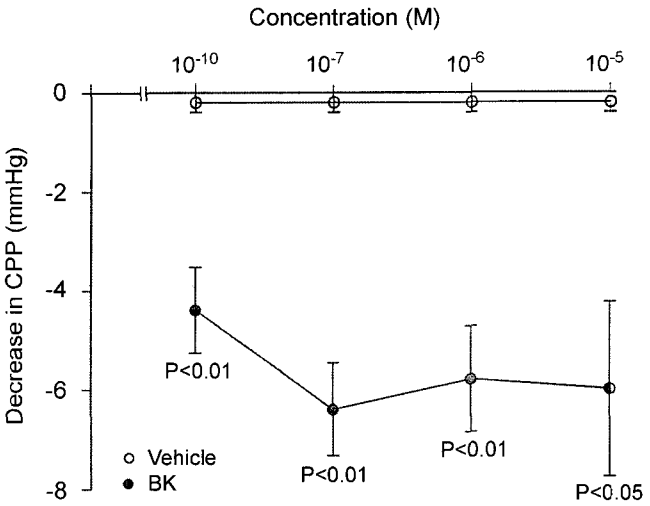

Fig. 2. Decrease in perfusion pressure (mm Hg) during the passage of different concentrations of bradykinin through rat hearts (Mean ± SEM, n=5). There was a significant decrease in perfusion pressure after the infusion of each of the four doses of BK compared with that produced by perfusion with vehicle alone.

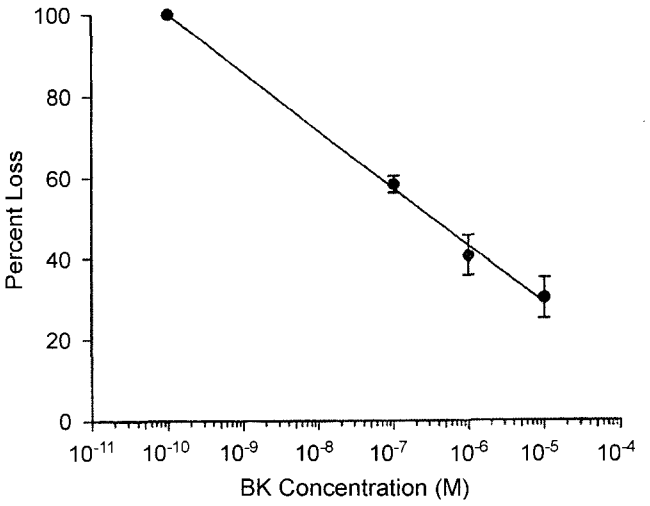

Fig. 3. Degradation (percent loss) of different concentration of BK during passage through rat isolated hearts (Mean±SEM, n=6). The percentage degradation of BK decreased as its dose increased. It compares apparent percent loss of bradykinin plotted against concentration of applied bradykinin in the perfusion fluid using the formula:
% age Loss = Conc. of BK applied – Conc. of BK in Effluent / Conc. of BK applied × 100

Confirmation of bradykinin and its fragments by mass spectroscopy

Mass spectroscopy showed that as previously determined using HPLC (Fig. 4), the rat isolated heart perfusates contained bradykinin (MW, 1060, Fig. 5). The peak

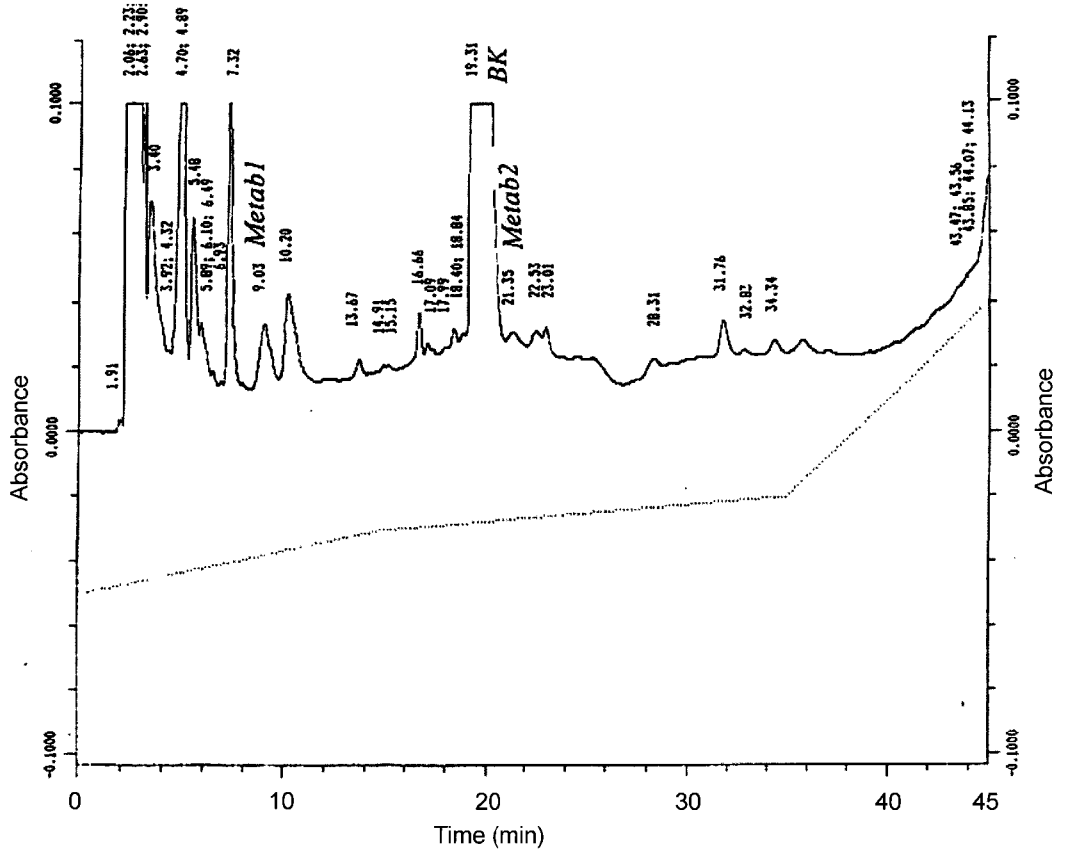

Fig. 4. HPLC chromatogram showing the formation of different fragments of BK after its passage through rat isolated hearts. Metabolite 1 & 2 (Metab 1 & Metab 2) are [1-7]-BK and [1-8]-BK, respectively. Bradykinin and its fragments were extracted using Sep-Pak Vac C18 cartridges. Samples were dried and redissolved in mobile phase and subjected to C18 (25 cm × 0.46 cm, 5 μ) column using a gradient of 30%-70% acetonitrile and water containing TFA. Peaks were detected at 210 nm.

at MW 530 was that of bradykinin^{2+}. The peak at MW 541 was that of bradykinin Na^+ H^+. The peaks with MW's of 757 and 452.7 were [1-7]-BK and [1-8^{2+}]-BK respectively (Fig. 5). Trace peaks at 573 and 175.2 were also detected and these were due to [1-5]-BK and arginine. The other trace peaks shown in the figure may be due to glucose and/or some other amino acids.

DISCUSSION

Bradykinin (BK) is a nine amino acid-containing peptide with the sequence Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9. Our results showed that it is mainly metabolized by angiotensin converting enzyme in rat heart during its single passage. Kininase is the generic term applied to enzymes responsible for the metabolism of BK (Skidgel 1992). The following BK-metabolic actions of the various kininases are summarised in Fig. 6. Kininase I (carboxypeptidase N) is present in plasma and acts on the COOH terminal of BK, removing Arg^9 and producing [1-8]-BK, a B1 receptor agonist (Bhoola *et al.*, 1992). In many tissues, carboxypeptidase M may also be responsible for the removal of the C-terminal Arg^9 of BK (Erdos, 1990). Kininase II (angiotensin converting enzyme, ACE) occurs in most tissues, but primarily in lung and removes the C-terminal dipeptide Phe^8-Arg^9 from BK. It further slowly cleaves the resulting [1-7]-BK to [1-5]-BK (Fig. 6). Endopeptidase 24.11 (neutral endopeptidase, NEP) cleaves BK to [1-7]-BK and further cleaves [1-7]-BK to form [1-4]-BK (Campbell *et al.*, 1998). Proline aminopeptidase (prolidase, aminopeptidase P, APP) cleaves the Arg^1-Pro^2 bond of BK (Bhoola *et al.*, 1992). Meprin is only detected in kidney and intestine. It cleaves the Gly^4-Phe^5 bond of BK (Skidgel, 1992). Endopeptidase 24.15 is found largely in brain, pituitary and testis and little in other tissues (Shrimpton *et al.*, 2002). It can cleave the Phe^5-Ser^6 bond of BK.

In the present study in which the BK was administered by single pass infusion, HPLC analysis of BK metabolism in the cardiac perfusates showed the formation of two fragments of BK. These were [1-7]-BK as the major fragment and [1-8]-BK as a minor fragment. Mass spectroscopy results not only confirmed the formation of these two

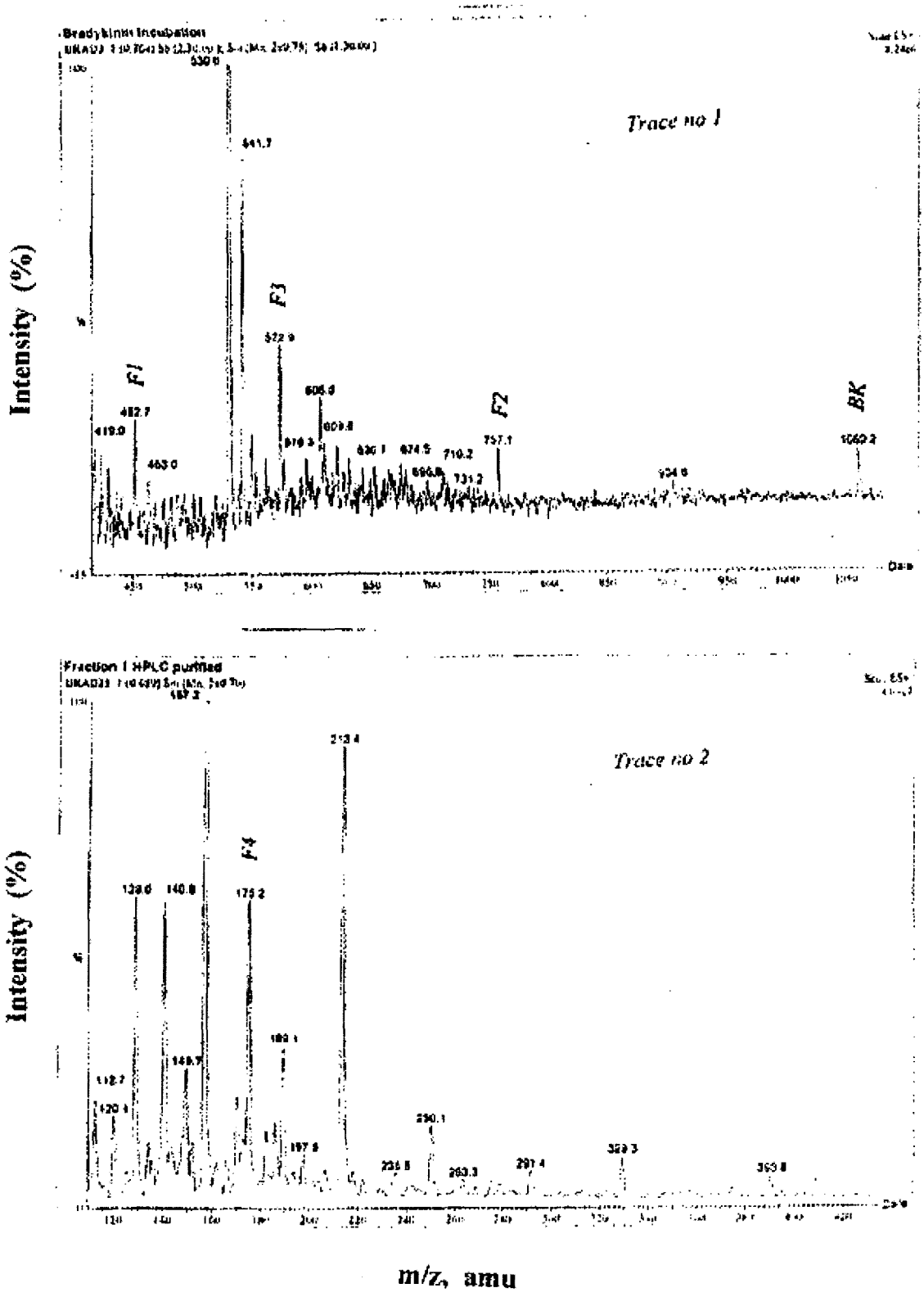

Fig. 5. Mass Spectrum of different metabolites formed by the degradation of BK after its passage through rat isolated heart. Trace no1 shows the mass spectrum of BK and its different fragments. The peaks indicated by F1, F2, and F3 correspond to the [1-8^{2+}]-BK, [1-7]-BK and [1-5]-BK respectively. Trace no 2 represents the mass spectrum of arginine (F4) detected after further purification by HPLC. Mass and charge ratio (m/z) was detected against % intensity.

	1 2 3 4 5 6 7 8 9	
A) Bradykinin Structure	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	
B) Coronary Intravascular Kininase (Present Study)		
Major Fragment	Arg-Pro-Pro-Gly-Phe-Ser-Pro	**(I)**
Minor Fragment	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	**(II)**
Trace	Arg	**(III)**
Trace	Arg-Pro-Pro-Gly-Phe	**(IV)**
C) Kininase I (Carboxypeptidase N)	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	**(II)**
	Arg	**(III)**
D) Carboxypeptidase M	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	**(II)**
	Arg	(III)
E) Kininase II (ACE)	Arg-Pro-Pro-Gly-Phe-Ser-Pro	**(I)**
	Phe-Arg	
	↓	
	Arg-Pro-Pro-Gly-Phe	**(IV)**
	Ser-Pro	
F) Neutral Endopeptidase	Arg-Pro-Pro-Gly-Phe-Ser-Pro	**(I)**
	Phe-Arg	
	↓	
	Arg-Pro-Pro-Gly	
	Phe-Ser-Pro	
G) Aminopeptidase P (Prolidase)	Arg	**(III)**
	Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	
H) Meprin	Arg-Pro-Pro-Gly	
	Phe-Ser-Pro-Phe-Arg	
I) Endopeptidase 24,15	Arg-Pro-Pro-Gly-Phe	**(IV)**
	Ser-Pro-Phe-Arg	

Fig. 6. Summary of bradykinin (BK) metabolism in coronary perfusate during a single passage through coronary vascular bed in the Krebs perfused rat isolated heart. The table compares the BK peptide fragments detected in the coronary effluent in the present study (Row B), with those known to be produced by tissue enzymes known to metabolise BK (Rows C–I). The Roman numerals in the results from the current study (Row B) are to aid identification of the equivalent BK fragments in rows C–I. The vertical arrows in rows E and F indicate a secondary metabolism of the same fragment carried out by the enzyme shown. Thus, the major fragment, (I) is produced by ACE (Row E) and NEP (Row F). However, NEP is found primarily in the interstitium and is unlikely to be found in significant concentrations intravascularly in non-traumatised, normally perfused hearts. Literature references for cited reactions are given in paragraph 1 of the discussion.

fragments but also detected the presence of a trace of arginine. A trace of [1-5]-BK was also detected by mass spectrometry. The presence of [1-7]-BK as the major metabolite indicates the intraluminal action of ACE or NEP on the infused BK (Fig. 6). However, NEP is primarily found in the myocardial interstitium (Skidgel, 1992). It is more likely to be found intravascularly following tissue trauma and is reasonably unlikely to affect a single pass infusion of BK in healthy heart. The presence of [1-8]-BK, as a minor peak possibly indicates the action of small amounts of carboxypeptidase N (CPN, kininase I) or carboxypeptidase M (CPM) (Fig. 6). However, CPN occurs primarily in the blood plasma and is not likely to be present to any extent in a Krebs-perfused heart. CPM, on the other hand has been found on arterial endothelium (Skidgel *et al.*, 1989) and could reasonably be responsible for the production of this peptide. This would also explain the appearance of trace amount of arginine in the perfusate (Fig. 6). The presence of trace amounts of [1-5]-BK in the perfusate is likely to be the result of secondary metabolism of [1-7]-BK by ACE (Fig. 6). It is highly unlikely to indicate the presence of endopeptidase 24,15, which

has so far only been found in brain, pituitary and testis, with little in any other tissues (Shrimpton *et al.*, 2002). Aminopeptidase P (APP, prolidase) and meprin did not appear to have any role in the degradation of BK in the present study, since none of the metabolites produced by these enzymes could be detected (Fig. 6). This was unsurprising in the case of meprin, which has only been detected in the kidney and intestine (Skidgel, 1992). It was less expected in the case of APP, since APP has been reported to be present on the luminal surface of rat coronary vascular endothelium (Dendorfer *et al.*, 1997). The present results accord with the findings of a number of other studies reporting that in a variety of species, at least 70% of the BK destruction in the normally perfused coronary circulation is due to ACE (Dendorfer *et al.*,1997; Ersahin and Simmons, 1997; Taylor-McCabe *et al.*, 2001).

The studies in healthy rats by Dendorfer and co-workers (1997) used five perfusion passages of a BK bolus through rat isolated heart to study the intracoronary metabolism of BK. Using this technique, they found that BK was metabolised to [1-7]-BK, [1-5]-BK and small amounts of [2-9]-BK. The fragment des-Arg-BK did not exceed 2.2%. Densdorfer *et al.* (1997) concluded that ACE and APP are the predominant kininases in the rat myocardium. As they perfused the same solution five times an initially cleaved fragment may be metabolised further to other smaller fragments, such as the reduction of [1-7]-BK to [1-5]-BK by ACE (Fig. 6). Multiple recycling in the isolated heart, permitting secondary metabolism of the primary peptide products, is likely to give a false picture of the relative activities of the major cardiac enzymes involved in intravascular BK-metabolism.

The present study was conducted using a single passage through healthy hearts and so the relevant BK-metabolic enzymes are likely to be intraluminal and have rapid and direct access to the perfused BK. However, tissue damage induced by brief or prolonged periods of myocardial ischaemia is likely to provoke the release of other BK-metabolising peptidases such as NEP or endo-peptidase 24,15 which are both present in the cytosol. Under these circumstances, inhibition of NEP has been reported to mediate cardioprotection after myocardial infarction of 35 days in rats (Raut *et al.*, 1999). Other workers have reported that inhibition of both ACE and NEP is involved in cardioprotection in rat heart produced by single passage infusion of omapatrilat, a dual inhibitor of ACE and NEP (Campbell *et al.*, 1998; Dumoulin *et al.*, 2001). The fact that NEP is exclusively present in the interstitial spaces (Dendorfer *et al.*, 1997), suggests that its involvement in BK metabolism would take place only after significant structural trauma. The present study is an attempt to understand the contribution of the cardiac enzymes to the metabolism of bradykinin under normally perfused physiological conditions. Our results showed that ACE is the major enzyme of bradykinin degradation in these circumstances. The cardioprotective role of ACE inhibitors can thus be exploited under physiological as well as pathophysiological conditions.

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