 Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study

CHARLES BLAIS, JR.,1 GUY DRAPEAU,2 PHILIPPE RAYMOND,1 DANIEL LAMONTAGNE, NICOLE GERVAIS,1 INGRID VENNEMAN,2 AND ALBERT ADAM1

1Faculté de Pharmacie, Université de Montréal, Montreal H3C 3J7; 2Centre de Recherche (Université Laval), Hôpital-Dieu de Québec, Québec, Canada G1R 2J7; and Service d’Anesthésie-Réanimation, Centre Hospitalier Universitaire de Liège, B.35-B 4000, Liège, Belgium

Blais, Charles, J r., Guy Drapeau, Philippe Raymond, Daniel Lamontagne, Nicole Gervais, Ingrid Venneman, and Albert Adam. Contribution of angiotensin-converting enzyme to the cardiac metabolism of bradykinin: an interspecies study. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2263–H2271, 1997.—The role of angiotensin-converting enzyme (ACE) in the metabolism of bradykinin (BK) has been studied in several tissues. However, and contrary to angiotensin I, the metabolism of BK at the cardiac level has not been investigated. In this study, we define the participation of ACE in the carboxy-terminal degradation of BK in heart membranes of the dog, human, rabbit, and rat. The calculation of the kinetic parameters characterizing the metabolism of BK and the generated des-Arg9-BK can be summarized as follows: the half-life (t1/2) of BK [dog (218 ± 32 s) > human (143 ± 9 s) > rat (150 ± 4 s) > rabbit (22 ± 2 s)] and of des-Arg9-BK [dog (1,042 ± 40 s) > human (891 ± 87 s) > rat (621 ± 65 s) > rabbit (89 ± 8 s)] both showed significant differences according to species. Enalaprilat, an ACE inhibitor, significantly prevented the rapid degradation of BK and des-Arg9-BK in all species studied, whereas retrothiorphan, a neutral endopeptidase inhibitor, and losartan, an angiotensin II type I receptor antagonist, did not affect this metabolism. The relative importance of ACE in the cardiac metabolism of BK was species related: dog (68.4 ± 3.2%) > human (72.2 ± 2.0%) > rabbit (47.7 ± 5.0%) = rat (45.3 ± 3.9%). ACE participation in the metabolism of des-Arg9-BK was as follows: rabbit (57.0 ± 4.0%) > dog (39.9 ± 8.8%) = human (25.4 ± 5.5%) = rat (36.0 ± 7.0%). The participation of cardiac kininase I (carboxypeptidase M) in the transformation of BK into des-Arg9-BK was minor: human (2.6 ± 0.1%) = dog (0.9 ± 0.1%) = rabbit (1.0 ± 0.1%) = rat (1.0 ± 0.1%). These results demonstrate that ACE is the major BK-degrading enzyme in cardiac membranes. However, the metabolism of exogenous BK by heart membranes is species dependent. Our observations could explain some discrepancies regarding the contribution of kinins in the cardioprotective effects of ACE inhibitors.

angiotensin-converting enzyme inhibitor; bradykinin metabolism; cardioprotection

ANGIOTENSIN-CONVERTING ENZYME (ACE) inhibitors have clearly been shown to be cardioprotective in humans. Numerous clinical trials have demonstrated the therapeutic benefits of ACE inhibitors that can improve survival in patients with severe heart failure, delay the development of heart failure in patients with asymptomatic left ventricular dysfunction, and reduce short- and long-term mortality in patients surviving myocardial infarction (19). Moreover, ACE inhibition induces regression of left ventricular hypertrophy in patients with hypertension (24) and in survivors of myocardial infarction (19). These beneficial effects are due to inhibition of local cardiac formation of angiotensin II (ANG II), a potent vasoconstrictor and myocyte growth factor, but could also be attributed to a protective effect of ACE inhibitors on the metabolism of bradykinin (BK; Arg1-Pro2-Pro3-Gly4-Phe5-Ser6-Pro7-Phe8-Arg9), a powerful vasodilator and antiproliferative peptide (15).

ACE, also known as kininase II, converts angiotensin I (ANG I) to ANG II and degrades kinins (37). ACE inactivates BK by cleaving the carboxy-terminal Phe8-Arg9 dipeptide. Besides ACE, at least two other classes of enzymes are involved in vivo in the metabolism of the carboxy-terminal end of BK. Endopeptidases, such as neutral endopeptidase 24.11, also inactivate BK by splitting the same Pro7-Phe8 bond as ACE (14). Carboxypeptidases N and M (kininase I (KIN I)) hydrolyze the carboxy-terminal Phe8-Arg9 bond of BK to produce its active metabolite des-Arg9-BK (31). Moreover, ACE inactivates des-Arg9-BK by hydrolyzing the Phe5-Ser6 bond. Aminopeptidases are also potentially involved in the metabolism of BK. Among these latter, aminopeptidase P, which cleaves the Arg1-Pro2 bond, has been shown to be physiologically relevant in the rat pulmonary vascular bed (28).

Only indirect, confusing, and often contradictory evidence exists for a role of BK in the cardioprotective effect of ACE inhibitors (30). Until now, only partial evidence has been obtained for a potential effect of ACE inhibitors on the cardiac content of endogenous BK (3). These contradictions are due to the fact that the contribution of ACE in the cardiac metabolism of BK remains unknown. Also, the effect of ACE inhibition on the kinetic parameters characterizing the metabolism of BK in the cardiac tissue remains to be established.

Recently, Décarié et al. (9) carefully defined the effect of ACE inhibition on the metabolism of BK in the serum of three animal species and of humans. They clearly showed important interspecies differences not only in the half-life (t1/2) of BK but also in the different metabolic pathways involved in the metabolism of this peptide. On the basis of this previous work, the objectives of the present study were to characterize the metabolism of exogenous BK when incubated with a preparation of total heart membranes from dogs, rabbits, and rats in the presence or absence of the ACE inhibitor enalaprilat and to compare the results obtained for these three animal species, which are often used to study the cardioprotective effect of BK and ACE inhibitors, with those obtained from human atrial membranes.

Because neutral endopeptidase 24.11 has been shown to play a role in the metabolism of BK (14), the effect of...
retrotiophorphan, a specific inhibitor of this enzyme, was
also tested. Finally, the acute effect of losartan on the
kinetic parameters characterizing the metabolism of
BK was also defined because Schieffer et al. (29) reported a
decrease in cardiac ACE activity in rats treated chronically with this ANG II type I (AT1)
receptor antagonist.

In the first part, this study will focus on the enzymes
involved in the carboxy-terminal metabolism of BK. For
this purpose, residual immunoreactive BK and
generated immunoreactive des-Arg8-BK will be quanti-
fied by chemiluminescent enzyme immunoassays
(CLEIA) after different periods of incubation. Both
immunologic methods use highly specific polyclonal
antibodies raised against the carboxy-terminal amino
acid sequence of BK (8) and des-Arg8-BK (26). This
experimental approach will be completed by the mea-
surement of the ACE activity. In the second part, the
role of aminopeptidases potentially involved in the
metabolism of BK will be studied with high-perfor-
manoe liquid chromatography (HPLC) followed by an
immunoactivity profile of the amino-truncated me-
tabolites of BK. This approach will allow us to confirm
that, at the t1/2 value, the measured immunoreactivity
corresponds to the native BK because it has been shown
that the truncated amino-terminal BK has no pharma-
cological activity (28).

MATERIALS AND METHODS
Drugs, Peptides, and Reagents
BK and des-Arg8-BK were purchased from Peninsula Labo-
ratories (Belmont, CA). Enalaprilat and losartan, an ACE
inhibitor and an AT1-receptor antagonist, respectively, were a
generous gift from Merck Frosst Canada (Kirkland, Canada).
Retrotiophorphan (27), a specific inhibitor of neutral endopepti-
dase 24.11, was generously supplied by Dr. Bernard P. Roques
(Laboratoire de Pharmacochimie Moleculaire et Structurale, Univer-
site Rene Descartes, Paris, France). Pentobarbital
sodium was obtained from MTC Pharmaceuticals (Missis-
sauga, Canada). 3-[3-Cholamidopropyl]dimethylammonio]-1-
propanesulfonic acid (CHAPS) and hippuryl-l-histidyl-l-leu-
cine were from Sigma-Aldrich (Mississauga, Canada). Ethanol of
HPLC grade was purchased from J. T. Baker
(Phillipsburg, NJ). Heptfluorobutyric anhydride (HFBA)
and trifluoroacetic acid (TFA) were from Pierce (Rockford, IL).
Acetonitrile (HPLC grade) and all other chemicals of analytic
grade were obtained from Fisher Scientific (Montreal,
Canada).

All BK amino-truncated peptides were synthesized by one
of us (G. Drapeau) using solid-phase synthesis. Peptide
chains were assembled on Wang (p-alkoxybenzyl alcohol)
resins from Fmoc-protected amino acids (Bachem, Torrance,
CA) activated with the benzotriazolyl-N-oxy-tris(dimethylami-
no)phosphonium hexafluorophosphate (BOP) reagent (5). Pe-
tides were cleaved from the resin by treatment with TFA.
Crude synthetic peptides were purified by preparative chro-
matography as previously described (12). The purity of the
final products was assessed by HPLC analysis performed
with a Waters system equipped with a 746 data module and a
486 ultraviolet detector set at 214 nm. Separation was
performed with a Vydac 10-μm (3.9 × 300 mm) reverse-phase
C18 column with a linear gradient of 5–65% acetonitrile-
0.05% TFA in water at 2 ml/min over a 20-min period.

Animal Heart Tissues

Male Sprague-Dawley rats (300–350 g; n = 8; Charles
River, St. Constant, Canada) were exposed to a CO2-enriched
atmosphere until unconsciousness and killed by decapitation.
The thorax was rapidly opened, and the heart was excised,
immersed in ice-cold modified Krebs-Henseleit buffer solu-
tion, and installed within 2 min on the perfusion setup. New
Zealand White rabbits (1.0–1.5 kg; n = 7; Charles River) of
either sex were anesthetized with pentobarbital sodium
(30–60 mg/kg iv). The animals were exsanguinated by a transect
of the carotid artery, and the heart was rapidly
perfused in situ through a cannula inserted into the aortic
stump. The heart was then excised and installed on the
perfusion setup without any interruption in perfusion. Mon-
ogel dogs (n = 3) were anesthetized with thiopental sodium
(25–30 mg/kg iv) and ventilated artificially with room air
through an endotracheal tube by means of a Harvard pump
(model 607). A left thoracotomy in the fifth intercostal space
was performed to gain access to the heart. Ventricular
fibrillation was induced by the application of a direct current
(directly from a 9-V alkaline battery) on the epicardium, after
which the aorta was clamped and the heart was rapidly
excised and immersed in ice-cold buffer. The heart was then
installed within 2 min on the perfusion setup.

All animal hearts were perfused retrogradely at a pressure
of 75–80 mmHg with a modified Krebs-Henseleit buffer
trough the aorta (Langendorff model) for 15 min to wash
away all traces of blood in the coronary bed. The modified
Krebs-Henseleit buffer solution contained (in mmol/l) 118
NaCl, 24 NaHCO3, 4 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1 MgSO4, 5
d-glucose, and 2 pyruvate. The buffer was gassed with 95%
O2–5% CO2 (pH 7.4) and kept at a constant temperature
of 37°C. The perfusate did not recirculate. After a 15-min
perfusion period, the hearts were cut and rapidly frozen in
liquid nitrogen. All animal procedures conformed to the
Canadian Council on Animal Care and were approved by the
Universite de Montreal Committee for Animal Research
(Montreal, Canada).

Human Heart Tissue

Right atrial pieces (≈20 mg) were taken from patients (n = 34;
23 men and 11 women; age 55–74 yr) who underwent a
first surgical operation for a coronary or valvular insuffi-
ciency. The subjects of this experimental group had received
pharmacological treatments with calcium-blocking agents,
β-adrenergic blocking agents, and nitrate preparations.
The only exclusion criterion was the use of ACE inhibitors. All
patients were anesthetized according to a single protocol with
opiates, benzodiazepines, and nondepolarizing neuromuscu-
lar blocking agents. The hemodynamic parameters were
stabilized by means of vasoactive agents such as ephedrine
and calcium-blocking agents or by inotropic agents such as
dobutamine. The sampling of these tissues was approved by
the Centre Hospitalier Universitaire de Liege (Belgium),
and consent was obtained from the donors. Tissue fragments
were sampled at the end of the extracorporeal circulation after
the removal of the atrial cannula. These pieces were immediately
frozen at −80°C until used for biochemical investigations.

Preparation of Membranes From Animal Hearts
and From Human Atria

The total heart tissue was cut into 3- to 4-mm pieces, and
membranes were prepared as described to measure the effect
of ACE inhibition on the metabolism of ANG I (18). Briefly,
the pieces were placed in 50 mM tris(hydroxymethyl)amino-
methane (Tris)-HCl buffer, pH 7.4, at 4°C (10 ml/g) and
homogenized with a Polytron homogenizer (Brinkmann Instrument, Rexdale, Canada) at setting 8 for 15 s. The homogenate was centrifuged at 40,000 g for 20 min at 4°C. The tissue pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.4, containing 100 mM NaCl at 4°C; a hand-driven glass-glass homogenizer was used for this procedure. Membrane protein concentration was determined by the biocinchonic acid protein assay (Pierce) with bovine serum albumin as the standard.

For the three animal species, the total homogenate before centrifugation and the supernatant after centrifugation, which represents the cytosol, were also used in preliminary assays for incubation procedures as described.

For the human atria only, eight to nine samples of equal weight were pooled in four groups before preparation of the membranes.

For the dog, the hearts were also dissected into right and left atria and right and left ventricles. These four heart parts were homogenized separately as described above. The membranes were used to study the metabolism of BK in these four different heart compartments.

Carboxy-Terminal Metabolism of BK by Membrane Preparations

Experimental conditions to assess the contribution of ACE in the metabolism of BK. INCUBATION PROCEDURE. The metabolic profile of BK was measured at 37°C by the addition of synthetic BK (10 ml) and a saline solution (10 µl) to 980 ml of the heart membrane preparation to obtain a final concentration of BK equal to 471 nM. After various incubation periods of between 30 s and 50 min at 37°C, the reaction was stopped by adding cold anhydrous ethanol at a final concentration of 80% (vol/vol). The samples were then incubated for 1 h at 4°C and centrifuged (2,000 g for 15 min at 4°C) to allow a complete precipitation of the proteins. The clear supernatant was collected and evaporated to dryness in a Speed Vac concentrator (Savant, Farmingdale, NY). The residues were stored at −80°C until quantification of both immunoreactive kinins was performed. In some experiments, enalaprilat (10 µl; final concentration 130 nM (25)), retrothiorphan (10 ml; final concentration 25 nM (27)), and losartan (10 ml; final concentration 10 mM (32)), were preincubated with the heart membrane preparation (980 µl) for 15 min at 37°C before the addition of exogenous BK (10 µl). The same incubation conditions were applied to dog, human, rabbit, and rat heart membrane preparations.

In a series of preliminary experiments, the metabolism of BK was also tested using pooled (n = 3 animals) total homogenates, cytosols, and membranes from dogs, rabbits, and rats. In this case, the effect of both enzyme inhibitors (enalaprilat and retrothiorphan) and the AT1 antagonist (losartan) was defined.

QUANTIFICATION OF BK AND DES-ARG9-BK. Residual immunoreactive BK and formed immunoreactive des-Arg9-BK were quantified in the residue of the evaporated ethanolic extracts resuspended in 400 µl of 50 mM Tris·HCl buffer, pH 7.4, containing 100 mM NaCl and 0.05% Tween 20. The diluted samples were separated in two parts of 200 µl for each peptide quantification. Competitive CLEIA enabled the quantification of BK (8) and des-Arg9-BK (26). Both assays used highly specific polyclonal rabbit immunoglobulins (Ig) raised against the carboxy-terminal end of BK and des-Arg9-BK, digoxigenin-labeled peptides as tracers, and alkaline phosphatase-labeled IgG anti-digoxigenin with the chemiluminescent substrate LUMI-PHOS 530 (Boehringer Mannheim) to detect and quantify the immune complexes. Each sample was measured in triplicate. On a molar basis, the polyclonal anti-BK-purified IgG exhibited no cross-reactivity with des-Arg9-BK. Otherwise, anti-des-Arg9-kallidin IgG showed no cross-reactivity with BK. Typical calibration curves were characterized by half-maximal saturation values of 0.78 pmol/ml for BK and 1.53 pmol/ml for des-Arg9-BK. Both methods were precise and accurate.

Kinetic parameters analysis. The kinin hydrolysis rate constant (k) was evaluated with a first-order equation, S = S0 × e−kt, where S is the concentration of substrate (kinin) and S0 is S at time (t) = 0, whereas t1/2 was represented as ln (2)/k (23). The different t1/2 values were expressed for 1 mg of protein. ACE relative activity (ACE% = 100 × [1 − k(+)k(−)]) was estimated from k without [k(−)] or with [k(+)] enalaprilat. The effect of retrothiorphan and losartan on the BK t1/2 was also determined. KI relative participation was expressed as the percentage of added BK transformed into des-Arg9-BK.

Measurement of ACE Activity

Membrane fractions were prepared as described in Preparation of Membranes From Animal Hearts and From Human Atria. After solubilization in 8 mM CHAPS (6), ACE activity was measured with the method of Cushman and Cheung (7).

Amino-Terminal Metabolism of BK by Membrane Preparations

Cross-reactivity of polyclonal anti-BK antibodies with amino-terminal BK metabolites. The specificity of polyclonal anti-BK antibodies used for CLEIA of BK was assessed by testing the cross-reactivity to six BK amino-truncated peptides [BK-(7–9), BK-(6–9), BK-(5–9), BK-(4–9), BK-(3–9), and BK-(2–9)] at concentrations ranging from 150 nM to 25 µM (Table 1). The cross-reactivity (in percent) was defined as described previously (1).

Separation and identification of amino-terminal BK metabolites, INCUBATION PROCEDURE. Under the conditions described in Experimental conditions to assess the contribution of ACE in the metabolism of BK, BK was incubated with a heart membrane preparation of each animal species for a period corresponding to the calculated t1/2 in the presence and absence of enalaprilat. After precipitation of the proteins with cold anhydrous ethanol and centrifugation, the ethanolic extracts were separated into two parts. After evaporation, the first part was used for the quantification of immunoreactive BK. The second part was dissolved in 0.025% (vol/vol) HFBA in distilled water before HPLC separation.

HPLC ANALYSIS. An HPLC system (Waters, Milford, MA) consisting of a model 600 multisolvent delivery system and a
model 484 tunable absorbance detector was employed for HPLC analysis. BK and four products of the amino-terminal enzymatic cleavage of BK were separated on a reverse-phase column (Vydac C18 5 µm, 4.6 × 250 mm; Hesperia, CA) at a constant flow rate of 0.7 ml/min with a 45-min linear gradient from 80% solvent A-20% solvent B to 65% solvent A-35% solvent B. Solvent A was 0.025% (vol/vol) HFBA in distilled water and solvent B was 0.025% (vol/vol) HFBA in 90% acetonitrile:10% distilled water. The column effluent was monitored continuously at 214 nm. Fractions of 0.7 ml were collected, evaporated to dryness in a Speed Vac concentrator, and then frozen at −80°C until immunoreactivity profile determination. BK and the metabolites were identified by comparing their retention times with those of reference peptides.

Statistical Analysis

All results are expressed as means ± SE for n values. Statistical analysis of data was performed with a paired Student’s t-test to test the effect of enalaprilat within species and an analysis of variance followed by a post hoc Tukey test for multiple comparisons between species. A difference was accepted as significant at \( P < 0.05 \).

RESULTS

Preliminary Assays

Metabolism of BK by total homogenate, cytosol, and membrane preparations in the absence and presence of enalaprilat. The \( t_{1/2} \) of BK was determined in the presence of the different pooled heart preparations from dogs, rabbits, rats, and humans. Within the same animal species (dog, rabbit, and rat) compared with the same concentration of proteins, the total homogenates and cytosols metabolize BK faster than the membranes, leading for each species to a BK \( t_{1/2} \) of <10 s. Moreover, preincubation of the total homogenates and cytosols with enalaprilat has no significant effect on the \( t_{1/2} \) of BK. The BK degradation with the three heart preparations from the rabbits was at least threefold faster than that of the other species. For the four membrane preparations, however, the ACE inhibition has a significant effect on the \( t_{1/2} \) of BK. Because the inhibition of ACE was observed for the membranes only, further experiments were performed with this sole preparation.

Relationship between the metabolism of BK and the peptide concentration. The linear relationship between the hydrolysis rate of immunoreactive BK and increasing concentrations of exogenous BK when incubated in the presence of a constant concentration of membrane proteins (rabbit, 1.5 mg/ml; rat, 10 mg/ml) is illustrated at Fig. 1A. The hydrolysis of BK increases linearly for concentrations ranging from 74 to 1,179 nM. From these experiments and the results obtained previously for serum metabolism (9), a 471 nM (500 ng/ml) concentration of BK was selected for further experiments.

Linearity of the metabolism of BK according to the membrane protein concentration. Figure 1B shows a linear relationship between \( k \) and the increasing concentrations of heart membrane proteins (1.1–14.1 mg in 1 ml of incubation buffer) on BK hydrolysis rate constant (\( k \)). Dog, human; rabbit; rat. Each point represents 1 experiment of pools of 3–6 animal hearts and 1 pool of 8–9 human atria quantified in triplicate.

Carboxy-Terminal Metabolism of BK by Heart Membrane Preparations

Kinetics of disappearance of BK by heart membrane preparation in the absence and presence of losartan and retrothiorphan. Both losartan and retrothiorphan did not significantly modify the \( t_{1/2} \) of BK when incubated in the absence and presence of enalaprilat with the membrane preparations.

Kinetics of disappearance of BK in the absence and presence of enalaprilat. The \( t_{1/2} \) of BK presented significant differences among species: dog (218 ± 32 s) > human (143 ± 9 s) = rat (150 ± 4 s) > rabbit (22 ± 2 s).
The preincubation of heart membrane preparations with enalaprilat significantly increases the \( t_{1/2} \) of BK in these species with, however, some differences. Indeed, the \( t_{1/2} \) values of BK for dogs and humans were enhanced 3.2- and 3.6-fold, respectively, whereas, for rabbits and rats, the \( t_{1/2} \) values were increased 2.0- and 1.8-fold, respectively. Thus, in the presence of enalaprilat, the order of \( t_{1/2} \) values for BK was similar to that measured in the absence of enalaprilat: dog (695 \( \pm \) 90 s) > human (515 \( \pm \) 19 s) > rat (282 \( \pm \) 17 s) > rabbit (44 \( \pm \) 3 s) (\( F = 95.6; \ P < 0.001; \) Fig. 2A).

Kinetics of metabolism of formed des-Arg<sup>9</sup>-BK in absence and presence of enalaprilat. The \( t_{1/2} \) values of des-Arg<sup>9</sup>-BK were significantly higher than those measured for BK (\( P < 0.01; \) Fig. 2). They also varied significantly according to species: dog (1,042 \( \pm \) 40 s) > human (891 \( \pm \) 87 s) > rat (621 \( \pm \) 65 s) > rabbit (89 \( \pm \) 8 s) (\( F = 48.0; \ P < 0.001; \) Fig. 2B). As with BK, the ACE inhibitor prevented the degradation of des-Arg<sup>9</sup>-BK, but the degree of inhibition differed among species. When dog, human, and rat membranes were preincubated with enalaprilat, \( t_{1/2} \) values of des-Arg<sup>9</sup>-BK exhibited a similar increase (1.7-, 1.3-, and 1.6-fold, respectively), whereas, in rabbits, the \( t_{1/2} \) of des-Arg<sup>9</sup>-BK was increased 2.3-fold. In the presence of enalaprilat, the order of \( t_{1/2} \) values for des-Arg<sup>9</sup>-BK was dog (1,786 \( \pm \) 194 s) > human (1,188 \( \pm \) 46 s) = rat (1,019 \( \pm \) 87 s) > rabbit (209 \( \pm \) 14 s) (\( F = 53.7; \ P < 0.001; \) Fig. 2B).

These results clearly show that the effect of enalaprilat was more marked on BK metabolism than on that of des-Arg<sup>9</sup>-BK in dogs and humans. For rabbits and rats, enalaprilat had a similar effect on BK and des-Arg<sup>9</sup>-BK metabolism (Fig. 2).

Importance of ACE in the metabolism of BK and des-Arg<sup>9</sup>-BK. The degradation of BK and des-Arg<sup>9</sup>-BK by ACE was species related. The relative importance of ACE in the metabolism of BK by heart membrane preparations was dog (68.4 \( \pm \) 3.2\%) = human (72.2 \( \pm \) 2.0\%) > rabbit (47.7 \( \pm \) 5.0\%) = rat (45.3 \( \pm \) 3.9\%) (\( F = 8.4; \ P = 0.001 \)). On the other hand, the relative participation of ACE in the metabolism of des-Arg<sup>9</sup>-BK was rabbit (57.0 \( \pm \) 4.0\%) > dog (39.9 \( \pm \) 8.8\%) = human (25.4 \( \pm \) 5.5\%) = rat (36.0 \( \pm \) 7.0\%) (\( F = 4.2; \ P < 0.05 \)). The ACE participation in the hydrolysis of BK at the membrane level was more important than that of des-Arg<sup>9</sup>-BK with the exception of the rabbit.

Importance of KI in the metabolism of BK. The KI metabolic pathway was evaluated by the percentage of transformation of the total BK added to the membrane preparations into des-Arg<sup>9</sup>-BK (Table 2). The formation of des-Arg<sup>9</sup>-BK represented a minor metabolic pathway among all species studied. The quantity of immunoreactive des-Arg<sup>9</sup>-BK formed was \(<3\%\) of the total amount of BK added. However, human heart membranes transformed significantly more BK (2.6 \( \pm \) 0.1\%) into des-Arg<sup>9</sup>-BK than other species (\( F = 28.1; \ P < 0.001 \)).

The inhibition of ACE with enalaprilat significantly increased the relative participation of the KI pathway in the metabolism of BK in humans and rabbits (Table 2). In dogs and rats, the ACE inhibitor pretreatment had no significant effect on the participation of the KI pathway in the degradation of BK into des-Arg<sup>9</sup>-BK.

Metabolism of BK by dog atrial and ventricular membranes in the absence and presence of enalaprilat.

### Table 2. Percentage of BK transformation into des-Arg<sup>9</sup>-BK with or without enalaprilat when incubated with various heart membranes

<table>
<thead>
<tr>
<th>Species</th>
<th>Without enalaprilat</th>
<th>With enalaprilat</th>
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<tbody>
<tr>
<td>Dog</td>
<td>0.9 ( \pm ) 0.1</td>
<td>2.2 ( \pm ) 0.7</td>
</tr>
<tr>
<td>Human</td>
<td>2.6 ( \pm ) 0.1*</td>
<td>6.1 ( \pm ) 0.5†</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.0 ( \pm ) 0.1</td>
<td>1.6 ( \pm ) 0.2*</td>
</tr>
<tr>
<td>Rat</td>
<td>1.0 ( \pm ) 0.1</td>
<td>1.2 ( \pm ) 0.2</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE; \( n = 3–8 \) samples. Final concentration of BK, 471 nM. *\( P < 0.01 \) vs. all other values in same column (by analysis of variance followed by post hoc Tukey test). †\( P < 0.01 \) and ‡\( P < 0.001 \) vs. without enalaprilat (by paired Student’s t-test).

![Fig. 2](http://ajpheart.physiology.org/)
The calculated t_{1/2} of BK when incubated with dog membranes of the four cardiac chambers exhibited significant differences (F = 33.7; P < 0.001; Fig. 3A): right ventricle (292 ± 21 s) > left ventricle (182 ± 22 s) > right atrium (58 ± 13 s), but left ventricle (182 ± 22 s) = left atrium (129 ± 9 s) and left atrium (129 ± 9 s) = right atrium (58 ± 13 s) (P = 0.071; n = 3 dogs). Enalaprilat significantly prevented the degradation of BK by both atrial and ventricular membrane preparations (Fig. 3A) and allowed us to calculate the relative lower importance of ACE in the metabolism of BK for the right ventricle (62.4 ± 5.5%; F = 12.9; P < 0.01; Fig. 3B).

ACE Activity of Heart Membrane Preparations

ACE activity of the membranes also presented significant differences among species: rabbit (0.305 ± 0.015 mU/mg protein; n = 5) > rat (0.068 ± 0.009 mU/mg protein; n = 6) = human (0.068 ± 0.011 mU/mg protein; n = 4) > dog (0.012 ± 0.004 mU/mg protein; n = 3) (F = 128.5; P < 0.001).

Amino-Terminal Metabolism of BK by Heart Membrane Preparations

Cross-reactivity of polyclonal anti-BK antibodies with amino-terminal BK metabolites. On a molar basis, the cross-reactivity of the anti-BK antibodies with BK-(7—9) and BK-(6—9) was <0.01%. Cross-reactivity toward BK-(5—9), BK-(4—9), BK-(3—9), and BK-(2—9) was 0.16, 1.4, 5.3, and 100%, respectively (Table 1). Due to the absence of immunoreactivity of BK-(7—9) and BK-(6—9), only BK-(5—9), BK-(4—9), BK-(3—9), and BK-(2—9) peptides were selected for HPLC analysis and immunograms.

HPLC analysis. HPLC analysis applied to residues of the incubation mixture of BK with membrane preparations in the absence and presence of enalaprilat showed clearly that, at the t_{1/2} value, immunoreactivity was mainly detected at the retention time corresponding to that of native BK (Fig. 4). For dogs, humans (Fig. 4), rabbits, and rats, this native BK represents >95% of the measured immunoreactivity; only a minor peak (<5%) eluting in the position of BK-(2—9) was detected.

DISCUSSION

The role of ACE and other peptidases, mainly KI and neutral endopeptidase 24.11, in the metabolism of BK has been studied specifically in different tissues [kidney (33), lung (10), and skeletal muscle (35)], in several cultured cells [synovial fibroblasts (2), endothelial cells (16), and vascular smooth muscle cells (22)], and in plasma (17). More recently, this metabolism has been extensively defined in serum (9). Until now, and surprisingly, the role of ACE in the metabolism of BK at the cardiac level has not been investigated. However, the contribution of ACE to the cardiac metabolism of exogenous BK must be clearly established to clarify whether BK and its active metabolite des-Arg^9-BK are responsible for the cardioprotective effect of ACE inhibitors (30).

In the present study, we have clearly defined the role of ACE and other enzymes in the metabolism of exogenous BK incubated at a nanomolar concentration in vitro in the presence of cardiac membranes from dogs, rabbits, and rats, three animal species often used to evaluate the cardioprotective effect of ACE inhibitors and BK in acute and chronic experimental models (21). The kinetic parameters obtained in these animal species were compared with those measured for membranes prepared from human atria.

Membrane preparations from the total heart and from cardiac chambers were previously used to investigate the role of cardiac ACE in the ANG I-ANG II pathway (18). They are representative of cardiac muscle because they are composed of 75% cardiomyocyte membranes (36). Before this experimental approach was
applied to individual preparations, it was validated with total homogenates, cytosols, and membranes from pooled cardiac tissues. This approach allowed us to calculate a linear relationship between the hydrolysis of BK and the membrane protein concentration. Moreover, we observed that ACE activity is located in the membranes because enalaprilat has no effect on the t1/2 of BK when incubated in the presence of homogenate and cytosol. This observation confirms that ACE is a transmembrane enzyme (14) at the cardiomyocyte level and not only at the endothelial level. The degradation of BK by homogenate and cytosol is very rapid and could be attributable to nonspecific proteases released from the different subcellular compartments.

We have clearly demonstrated that the metabolism of exogenous BK by total heart membranes is species dependent. The calculated t1/2 of BK for rabbits is very short compared with the values measured for dogs, humans, and rats and for the same concentration of membrane proteins. When cardiac membranes were preincubated with the ACE inhibitor, the t1/2 of BK increased in the different species, but this effect was more marked in dogs and humans.

Our results indicate that ACE is the major BK-degrading enzyme in the hearts of all species. In fact, ACE is the main enzyme (70%) responsible for the metabolism of BK in dog and human cardiac membranes. However, in rabbits and rats, the ACE pathway participated in ~50% of the BK degradation. The importance of ACE in the metabolism of BK can be compared with some results obtained for the metabolism of ANG I. In a recent study, Urata et al. (34) demonstrated that ACE is not a major ANG II-forming enzyme in human left ventricular membrane preparations. Captopril inhibited ANG II formation by only 11%, whereas soybean trypsin inhibitor and phenylmethylsulfonyl fluoride, serine proteinase inhibitors, suppressed it up to 80%. Heart chymase appears to be this specific ANG II-forming cardiac serine proteinase in the human heart (34). If ACE does not constitute a major cardiac ANG II-forming pathway, the use of ACE inhibitors could have a weak impact on the cardiac ANG II formation because the chymase-dependent ANG II-forming pathway is not blocked by ACE inhibitors. In the present study, we have clearly shown that BK is mainly metabolized by ACE because an ACE inhibitor has a substantial inhibiting effect on the degradation of BK. Our observations could plead for a role of BK in the cardioprotective effect of ACE inhibitors. Thus the cardioprotective effect of ACE inhibitors could be explained by BK accumulation rather than by suppression of ANG II formation.

The use of specific antibodies allowed us to explore the kinetics of des-Arg9-BK after the metabolism of BK by heart membrane preparations. In this case too, some interspecies differences could be measured. The t1/2 of des-Arg9-BK calculated for rabbits is markedly shorter than that measured for other species. In each case, the t1/2 of des-Arg9-BK is at least fourfold higher than the values measured for BK within the same species. Furthermore, human cardiac membranes metabolize BK up to six times more rapidly than des-Arg9-BK. These results can be explained by a lower affinity of des-Arg9-BK for purified ACE (Michaelis-Menten constant = 148 mM) (11) compared with that of BK (Michaelis-Menten constant = 8.8 mM) (11). Preincubation of the membrane preparations with enalaprilat also increased the t1/2 of des-Arg9-BK, and participation of ACE in the hydrolysis of des-Arg9-BK is an important metabolic pathway.

Fig. 4. A: retention times for reference peptides of amino-terminal truncated metabolites of BK: BK-(5–9), 28.5 min (1); BK-(4–9), 32.0 min (2); BK-(3–9), 35.3 min (3); BK-(2–9), 36.5 min (4), and BK, 42.3 min (5). B and C: immunoreactivity profiles after reverse-phase high-performance liquid chromatography of incubation medium after BK was incubated with human atrial membranes without and with enalaprilat, respectively. Extraction and chromatographic conditions are described in MATERIALS AND METHODS.
Membrane ACE activity also exhibits significant interspecies differences. The measured activities correlate with the $t_{1/2}$ values of BK and to a lesser extent with those of des-Arg$^9$-BK.

The KI metabolic pathway of BK was evaluated by the percentage of added BK transformed into des-Arg$^9$-BK in heart membranes, as described previously for serum (9). The participation of KI in the metabolism of BK represents a minor pathway in this experimental approach with normal hearts. However, des-Arg$^9$-BK-like immunoreactivity overflowing rat ischemic hearts on reperfusion in the presence of an ACE inhibitor has recently been reported by our group (20). Therefore, formation of des-Arg$^9$-BK in cardiac diseases may well be different from that of normal hearts.

The kinetic parameters of BK and des-Arg$^9$-BK measured for human tissues must be compared with those for animal species with caution. In fact, the human heart pieces were from the atria, and at this time, we cannot exclude differences between atrial and ventricular metabolism as observed in dogs. Second, the heart pieces were sampled from patients with coronary or valvular insufficiency, which could alter the BK metabolism compared with normal hearts.

Because our results show the participation of enzymes other than KI and ACE in the metabolism of BK in all species membrane preparations (Fig. 5), we evaluated the contribution of neutral endopeptidases in the degradation of BK. The addition of retrothiorphan, a selective neutral endopeptidase inhibitor, to the cardiac membranes did not modify the $t_{1/2}$ of BK. This observation suggests that neutral endopeptidases could not be implicated in the cardiac metabolism of BK with this experimental approach. These results contrast with those of Dumoulin et al. (13), who used isolated Langendorff rat hearts perfused through the coronary bed with BK. These authors showed that perfusion of retrothiorphan alone was without effect on the recovery of immunoreactive BK. However, when this inhibitor was coperfused with enalaprilat, the recovery of BK was potentiated by 36% compared with enalaprilat alone (recovery of 59.9 ± 2.6%). This apparent discrepancy can be explained by the difference in the experimental approach. Moreover, Piedimonte et al. (25) found evidence of neutral endopeptidase immunoreactivity in cultured 1-day-old rat ventricle myocytes but apparently not in adult cardiomyocytes. Taken together, all these observations show that neutral endopeptidase does not play a role in the metabolism of BK at the myocyte level, confirming the endothelial localization of the enzyme in the normal adult heart.

Campbell et al. (4) did not observe a significant effect of a chronic treatment (8 days) with losartan on the circulating and cardiac levels of BK in rats. We demonstrated that an in vitro incubation of heart membranes in the presence of this AT$_1$ antagonist does not interfere with the metabolism of BK and particularly with the ACE pathway. Direct interference of the AT$_1$ blocker in the metabolism of BK had to be rejected before future in vivo studies. Schieffer et al. (29), however, reported that a chronic treatment of rats with losartan diminished the activity of cardiac ACE. In that case, these authors hypothesized an indirect effect of the AT$_1$ blocker on the gene expression of ACE.

Our experimental approach uses antisera that specifically recognize the carboxy-terminal end of BK or des-Arg$^9$-BK. Because it has been established that amino-terminal truncated peptides are devoid of pharmacological activity (28), it was important to verify whether the measured carboxy-terminal immunoreactivity corresponds to native and pharmacologically active peptides. Among the different aminopeptidases potentially involved in the metabolism of BK, aminopeptidase P is physiologically relevant in the rat pulmonary vascular bed (28). This latter can remove a variety of amino-terminal amino acids bound to proline, like the Arg$^1$ residue in BK (28). HPLC separation followed by an immunoreactivity profile clearly shows that the measured immunoreactivity at $t_{1/2}$ corresponds to >95% of native BK, which has a B$_2$ pharmacological activity.

In conclusion, we have clearly established that 1) ACE is the major cardiac BK-degrading enzyme because the ACE inhibitor significantly increases the $t_{1/2}$ values of BK and des-Arg$^9$-BK, 2) KI is a minor BK metabolic pathway, and 3) important interspecies differences exist among the $t_{1/2}$ values of BK and des-Arg$^9$-BK using an in vitro experimental approach. These differences could explain some discrepancies in the literature regarding the contribution of kinins in the cardioprotective effects of ACE inhibitors. Moreover, the predominance of ACE in BK degradation by human cardiac tissue is compatible with an important contribution of kinins in the cardioprotection reported from the clinical use of ACE inhibitors. Further experiments are needed to define the effect of ACE inhibitors on the metabolism of BK in experimental models of acute myocardial infarction and chronic left ventricular hypertrophy.

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**Fig. 5.** Synoptic representation of relative contribution of different kininases to hydrolysis of BK in this experimental model.
References


