Protective Effect of Omapatrilat, a Vasopeptidase Inhibitor, on the Metabolism of Bradykinin in Normal and Failing Human Hearts

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ABSTRACT

Because part of the cardioprotective effects of angiotensin-converting enzyme (ACE) inhibitors results from their protective effects on cardiac bradykinin (BK) metabolism, the purpose of this study was to define the metabolism of BK in normal and failing human hearts and to compare the effect of omapatrilat, a vasopeptidase inhibitor (VPI), which simultaneously inhibits both neutral endopeptidase (NEP) and ACE, with that of an ACE inhibitor. Exogenous BK at a nanomolar concentration was incubated alone, in the presence of an ACE inhibitor (ramiprilat, 36 nM), or in the presence of a VPI ( omapatrilat, 61 nM) with left ventricular membranes prepared from normal donor hearts (n = 7), and hearts from patients with an ischemic (n = 11) or dilated (n = 12) cardiomyopathy (DCM). The half-lives calculated for BK alone (199 ± 60, 224 ± 108, and 283 ± 122 s; P = NS) exhibited similar values for normal, ischemic, and DCM heart tissues, respectively. Ramiprilat significantly increased the half-life of BK (P < .01), but the effect was similar for the three kinds of tissues (297 ± 104, 267 ± 157, and 407 ± 146 s, respectively; P = NS). The potentiating effect of the VPI omapatrilat on the kinetic parameter of BK (478 ± 210, 544 ± 249, and 811 ± 349 s, respectively) was greater than that of the ACE inhibitor (P < .01). Moreover, omapatrilat had a more important potentiating effect with DCM than normal heart membranes (P < .05). These results show that not only ACE but also and mainly NEP play an important role in the degradation of BK in human heart membranes. Omapatrilat, a VPI, has a greater protective effect on BK metabolism than that of a pure ACE inhibitor. Thus, inhibition of both ACE and NEP with omapatrilat could be more cardioprotective than ACE inhibition alone.

Angiotensin-converting enzyme (ACE; EC 3.4.15.1) inhibitors have been shown to improve long-term survival in patients with severe congestive heart failure (CHF), to prevent the development of heart failure and recurrent myocardial infarction (MI) in patients surviving MI, and to improve endothelial dysfunction in patients with coronary artery disease [The CONSENSUS Trial Study Group, 1987; Pfèffer et al., 1992; The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators, 1993; Mancini et al., 1996]. Different mechanisms have been proposed to explain these beneficial effects of ACE inhibitors. The major local effect was initially thought to be the result of partial inhibition of the cardiac conversion of angiotensin I to angiotensin II; all of the components of the renin-angiotensin system being present in the cardiac tissue (Dzau, 1988). The presence of a local kallikrein-kinin system has also been demonstrated in the heart (Nolly et al., 1994; Minshall et al., 1995; Yayama et al., 2000). The activation of that system leads to the release of bradykinin (BK) that exerts its pharmacological effects by stimulating B2 receptors (Linz et al., 1995; Matoba et al., 1999). Mounting evidence suggests that inhibition of the degradation of BK by ACE inhibitors may be as, if not more, important than the inhibition of angiotensin II formation (Linz et al., 1995). The increased formation of nitric oxide, cGMP, and prostaglandins mediates the vasodilator, anti-ischemic, and antiproliferative effects of BK (Linz et al., 1995). Although some information regarding the effects of ACE inhibitors on the production of angiotensin II in the human heart is available (Urata et al., 1990), there is little information regarding the effects of ACE inhibitors on the metabolism of BK in the human heart.

In previous studies, we described the metabolism of BK in card...
different normal and pathological animal models (Blais et al., 1997; Dumoulin et al., 1998; Raut et al., 1999). In normal hearts, we demonstrated that important differences exist in the metabolism of BK among the different animal species most often used to evaluate the role of BK in the cardioprotective effects of ACE inhibitors (Blais et al., 1997). Nevertheless, we found that rat and human heart membrane preparations degrade BK at a similar rate. In rat hearts, Dumoulin et al. (1998) have demonstrated that both ACE and neutral endopeptidase (NEP; EC 3.4.24.11) play an important role in the metabolism of BK by the endothelium of the coronary vascular bed. More recently, Raut et al. (1999), using a model of MI induced in the rat by coronary ligation, have shown that the cardiac metabolism of BK by representative membrane preparations of the cardiomyocytes is profoundly modified in the acute and chronic postinfarction state and that the relative importance of ACE and NEP in that metabolism changes markedly.

The development of a new class of cardiovascular agents, the vasopeptidase inhibitors (VPIs), single molecules that simultaneously inhibit both NEP and ACE (Robl et al., 1997), may be expected to increase cardiac BK \( k_{1/2} \) much more than ACE inhibitors alone. The purpose of the present study was thus to define the effects of simultaneous inhibition of NEP and ACE with the new VPI omapatrilat that inhibits both enzymes with similar nanomolar inhibitory constants \( (K_I) \) (Robl et al., 1997; Trippodo et al., 1998) and to compare the protective effect of omapatrilat to that of a pure ACE inhibitor, ramiprilat. The effects of omapatrilat and ramiprilat on BK metabolism were compared not only in normal hearts but also in hearts with either ischemic or nonischemic end-stage heart failure.

**Materials and Methods**

**Drugs, Peptides, and Reagents**

BK was purchased from Peninsula Laboratories (Belmont, CA). The ACE inhibitor ramiprilat was a generous gift from Hoechst Marion Roussel Canada (Laval, Québec, Canada). The VPI omapatrilat was kindly provided for research purposes by Bristol-Myers Squibb (Princeton, NJ). Actinomycin, apstatin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, hippuryl-L-histidyl-L-leucine, and Pro-Ille were from Sigma-Aldrich (Oakville, Ontario, Canada). 7-Amino-4-methylcoumarin (AMC) and succinyl-alanyl-alanil-phenylalanyl-AMC were from Bachem (Torrance, CA). Phenylmethylsulfonyl fluoride (PMSF), \( p \)-chloromercuri phenyl sulphonate (PCMS), \( p \)-anthonathine, alkaline phosphatase-labeled anti-digoxigenin Fab fragments, and \( p \)-nitrophenyl phosphate were purchased from Boehringer Mannheim (Laval, Québec, Canada). \( n \)-(1(R),S)-Carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP-AAP-pAB) was kindly provided by Dr. M. Orlowski (Department of Pharmacology, Mount Sinai School of Medicine, New York, NY). Ethanol of HPLC grade was obtained from American Chemicals (Montréal, Québec, Canada). All other chemicals of analytical grade were from Fisher Scientific (Montréal, Québec, Canada).

**Human Heart Samples**

Human heart tissues were obtained from the Réseau de la fonction ventriculaire (Montréal Heart Institute, Montréal, Québec, Canada). Metabolism of BK was assessed in 30 human hearts. The failing hearts \( (n = 23) \) were harvested during cardiac transplantation. The explanted hearts from patients with CHF were rinsed in a cardioprotective solution and immediately frozen in liquid nitrogen and stored at \(-80^\circ \)C. The normal hearts \( (n = 7) \) were hearts that were harvested for the purpose of cardiac transplant but could not be used for various technical or logistic reasons. Once the decision not to use the heart was made it was rapidly frozen in liquid nitrogen and stored at \(-80^\circ \)C. The explanted hearts were classified as follows: normal donor hearts \( (n = 7); 53 \pm 6 \) years; 4 men and 3 women, and hearts from patients with ischemic (ICM; \( n = 11; 57 \pm 5 \) years; 10 men and 1 woman) or dilated (DCM; \( n = 12; 57 \pm 6 \) years; 5 men and 7 women) cardiomyopathy. The use of these tissues was approved by the ethics committees on human subjects of the Montreal Heart Institute and the University of Montreal (Montréal, Québec, Canada).

**Preparation of Total Heart Membrane Suspensions**

To assess the metabolism of BK by enzymes located on cardiac cell membranes, membranes were prepared from the hearts using the method previously used to define the metabolism of BK in normal and pathological rat hearts (Blais et al., 1997; Raut et al., 1999). The left (LV) and right (RV) ventricular portions of each human heart were cut into 3- to 4-mm pieces, and then homogenized (10 ml/g tissue) at \( 4^\circ \)C in a 50 mM Tris-HCl buffer, pH 7.4, using a polytron homogenizer (Brinkmann Instruments, Reedale, Ontario, Canada) at setting 8 for 15 s. After centrifugation (40,000g, 20 min at \( 4^\circ \)C), the cytosolic supernatant was discarded, and the pellet of membranes was resuspended in a 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl at \( 4^\circ \)C. The fibrous tissue was discarded with a Wheaton potter-Elvehjem tissue grinder (setting 8 for 60 s) (Fisher Scientific, Pittsburgh, PA). The protein concentration of the membrane suspensions was determined by the bicinchoninic acid method (Pierce, Rockford, IL) using bovine serum albumin as the standard.

**Metabolism of BK**

**Incubation of BK with Heart Membrane Suspensions.** The metabolic profile of BK was measured at \( 37^\circ \)C as described previously (Blais et al., 1997; Raut et al., 1999) using the membrane preparations diluted in a 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl. Ten microliters of a BK solution was added to 990 \( \mu l \) of the membrane suspension (protein concentration \( \sim 5 \) mg/ml) to obtain a final concentration of 471 nM BK. After various incubation periods at \( 37^\circ \)C, ranging between 2 and 20 min, the reaction was stopped by the addition of cold ethanol (\( 4^\circ \)C) at a final concentration of 80% \( \nu/v \). In two sets of parallel experiments, and before adding the synthetic BK, the membrane suspensions were preincubated for 15 min at \( 37^\circ \)C with either ramiprilat (36 nM) or omapatrilat (61 nM). In preliminary studies, we have determined that these concentrations of ramiprilat and omapatrilat inhibit totally ACE activity, and ACE and NEP activities, respectively. The precipitated samples were centrifuged 15 min at \( 4^\circ \)C and 2000g. The clear supernatants containing BK and its metabolites were evaporated to dryness in a Speed Vac concentrator (Savant, Farmingdale, NY). The residues were stored at \(-80^\circ \)C until quantification of the residual BK was performed.

**Quantification of BK.** Immunoreactive BK was quantified in the residues of the evaporated ethanolic extracts using a highly specific enzyme immunoassay developed in our laboratory (Décarie et al., 1994; Blais et al., 1997). This assay uses highly specific polyclonal rabbit IgG raised against the carboxy-terminal end of BK, digoxigenin-labeled peptide as tracer, and alkaline phosphatase-labeled anti-digoxigenin Fab fragments with the substrate \( p \)-nitrophenyl phosphate to detect and quantify the immune complexes (Décarie et al., 1994). Each sample was measured in triplicate.

**Kinetic Parameters Analysis.** BK hydrolysis rate constant \( (k) \) was evaluated with the first order equation \( [BK] = [BK]_0 e^{-kt} \), where \([BK]_0\) is the concentration of BK at a given time and \([BK]_0\) is the BK at time \( t = 0 \). The BK \( k_{1/2} \) was represented at \( t_{1/2} = \ln(2)/k \) (Moore and Pearson, 1981). The different \( t_{1/2} \) values were expressed for 1 mg of protein.
Measurement of ACE and NEP Activities

The membranes used for BK metabolism were solubilized in 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid as described by Costerousse et al. (1994). ACE activity was measured using the colorimetric method of Cushman and Cheung (1971). NEP activity was assessed using the fluorimetric assay described by Nortier et al. (1995). Each sample was quantified in duplicate for both assays. ACE activity was expressed in nanomoles of hippuric acid per minute per milligram of protein, and NEP activity was expressed in nanomoles of AMC per minute per milligram of protein.

Contribution of Other Peptidases in Metabolism of BK at Cardiac Membrane Level

To approach the potential role of other peptidases, besides ACE and NEP, that may be involved in the metabolism of BK, pooled membranes from the three groups of heart tissues were preincubated in the above-mentioned conditions with omapatrilat (final concentration 61 nM) and then further incubated with specific inhibitors for different classes of enzymes: PMSF, inhibitor of serine proteinases; PCMS, inhibitor of cysteine proteinases; and o-phenanthroline, inhibitor of metalloproteases, at a final concentration of 1 x 10^{-3} M.

In a second set of experiments, and after an identical preincubation of membranes from the three groups of heart tissues were preincubated with specific inhibitors for different classes of enzymes: PMSF, inhibitor of serine proteinases; PCMS, inhibitor of cysteine proteinases; and o-phenanthroline, inhibitor of metalloproteases, at a final concentration of 1 x 10^{-3} M.

Statistical Analysis

All data were expressed as means ± S.D. for n values. A paired Student’s t test was used to test the effect of ramiprilat and omapatrilat on BK t_{1/2} within a same group of hearts. An unpaired Student’s t test was used to assess statistical significance between the normal, ICM, and DCM heart groups. All pairwise comparisons were examined using Tukey’s method of post hoc testing. A difference was accepted as significant at P < .05.

Results

Metabolism of BK by Human Heart Membranes Prepared from LV and RV. Figure 1 represents the t_{1/2} value of BK degradation when incubated in presence of membranes prepared from the LV of normal, ICM, and DCM hearts. The degradation rate of BK was similar for normal and pathological tissues; the calculated t_{1/2} for normal hearts being 199 ± 60 s (n = 7), for ICM 224 ± 108 s (n = 11), and for DCM 283 ± 122 s (n = 12; P = NS).

For ICM and DCM hearts, BK metabolism was also defined for RV. Again, similar values of BK t_{1/2} were calculated for both types of tissues (ICM 169 ± 73 s, n = 5; DCM 259 ± 93 s, n = 11). These values were no different from those measured for the corresponding LV.

Effect of Ramiprilat and Omapatrilat on BK t_{1/2}. Preincubation of LV membranes from the three types of patients with ramiprilat significantly increased BK t_{1/2} (P < .01 for all comparisons). The potentiating effect of ACE inhibition on BK t_{1/2} was similar in all three groups, normal and both pathological heart tissues (Fig. 2).

The effect of simultaneous inhibition of NEP and ACE by omapatrilat increased BK t_{1/2} of LV membranes more than pure ACE inhibition (P < .01 for all comparisons). In normal hearts, omapatrilat increased BK t_{1/2} by 140% compared with 49% with ramiprilat. A similar effect of omapatrilat was measured in membranes from ICM hearts (143 versus 19% for ramiprilat). In ICM, the t_{1/2} value calculated in the presence of omapatrilat (544 ± 249 s) was not statistically different from that measured for normal hearts (478 ± 210 s). The greatest effect of omapatrilat was in LV membranes from DCM hearts. Here, preincubation with omapatrilat increased the t_{1/2} of BK by 187%, whereas the effect of ACE inhibition was only 44%. The potentiating effect of omapatrilat in the DCM LV was significantly higher than that measured for normal LV (P < .05) (Fig. 2).

ACE and NEP Activities. As shown in Fig. 3, the activities of the ACE and NEP enzymes were similar in membrane preparations from all three groups of hearts.

Participation of Other Enzymes in Metabolism of BK at Membrane Level. The preincubation of DCM membranes (pools of six membrane preparations; n = 3) in the presence of omapatrilat plus different enzyme inhibitors allowed us to evaluate the contribution of other peptidases potentially responsible for the metabolism of BK once ACE and NEP were inhibited. Neither PMSF (inhibitor of serine proteinases) nor PCMS (inhibitor of cysteine proteinases) was found to inhibit the metabolism of BK. However,
-phenanthroline (inhibitor of metallopeptidases) totally inhibited the degradation of BK (Fig. 4). These results exclude the participation of serine and cysteine proteinases in the metabolism of BK by human heart membranes, and show that, besides ACE and NEP, only metallopeptidases are responsible for the metabolism of BK in this preparation. Identical results were found with normal and ICM heart tissues (data not shown).

Among the different specific metallopeptidase inhibitors tested, only two inhibitors (cFP-AAF-pAB and actinonin) totally prevented the degradation of BK, suggesting the participation of endopeptidase 24.15 and endopeptidase 24.18, besides ACE and NEP, in the degradation of BK (Fig. 5). The effect of Pro-Ile and apstatin indicates a lower contribution of endopeptidase 24.16 and aminopeptidase P, respectively, in the degradation of BK in the present experimental model. Similar results were found with normal and ICM heart tissues (data not shown).

**Discussion**

In this article, we demonstrate that the VPI omapatrilat inhibits the metabolism of BK by human cardiac membrane preparations more than the pure ACE inhibitor ramiprilat. Omapatrilat simultaneously inhibits NEP and ACE with similar nanomolar inhibitory constants (9 and 6 nM, respectively) (Trippodo et al., 1998). Because of the cardioprotective effects of BK (Linz et al., 1995; Emanueli et al., 1999; Matoba et al., 1999), it is therefore possible that a VPI may prove to be more cardioprotective than a pure ACE inhibitor under certain pathological conditions. This is particularly true considering that we found superior protective effects of VPI on BK degradation in membranes prepared from pathological human hearts (DCM).

The membrane preparation used in the present study is similar to that used previously to define the importance of ACE in the cardiac metabolism of angiotensin I (Kinoshita et al., 1993), but also to study the metabolism of BK in normal rat hearts (Blais et al., 1997) and to define the influence of MI and LV hypertrophy on the cardiac metabolism of BK in rats (Raut et al., 1999). This membrane preparation is representative of cardiomyocytes because this is the cell type that forms the overwhelming surface of the heart (Weber and Brilla, 1991). Recently, other authors have also used a similar membrane preparation from human hearts to study the influence of single ACE, NEP, and aminopeptidase M inhibition on the formation of the metabolites of BK and Lys-BK (kallidin) after incubations for a fixed period of time (2 h) (Kokkonen et al., 1999).

As method of detection of residual BK at the different
incubation times, we used a highly specific enzyme immunoassay developed in our laboratory (Décarie et al., 1994; Blais et al., 1997). This assay characterized by a sensitivity level of 0.1 pM allowed the definition of the kinetic profile of disappearance of BK when incubated in a biological milieu. These subpicomolar residual concentrations measured with our approach contrast with the micromolar concentrations needed for the physicochemical detection of BK and its metabolites after a HPLC chromatography (Kokkonen et al., 1999). However, because BK is considered as an autocrine and a paracrine mediator being metabolized locally at the site of its synthesis, rather than a hormone, the concentration of BK used in our experimental approach would be closer to the in vivo reality.

Under the conditions of this study, we measured similar \( t_{1/2} \) values for normal and failing hearts, whether the CHF was due to ischemic heart disease (ICM) or not (DCM). The values are in the range of those we previously described for human atria (143 ± 18 s) (Blais et al., 1997). Moreover, these values are also similar to those recently calculated for normal rat hearts (150 ± 11 s) and hypertrophied rat LV (137 ± 34 s) (Blais et al., 1997; Raut et al., 1999). The relationship of rat to human metabolism is interesting because rat is the species most often used in experimental models to demonstrate the cardioprotective effects of ACE inhibitors and the effect of ACE inhibitors on the metabolism of exogenous BK. Our finding of no significant difference in the \( t_{1/2} \) of BK degradation by membranes prepared from normal and pathological hearts complete the findings of Kokkonen et al. (1999) who found no significant difference in the rate of formation of BK \( \text{I-7I} \), an inactive metabolite of BK, between normal human hearts and hearts obtained at the time of transplant for end-stage heart failure.

When membranes were preincubated with ramiprilat, the \( t_{1/2} \) of BK was increased similarly in the three kinds of membrane preparations. This modest but significant potentiating effect found (19–49%) would not have been predicted by the study of Kokkonen et al. (1999) who showed that ACE had little or no effect on cardiac metabolism of BK despite the fact that the affinity of ACE for BK \( (K_m = 0.18 \mu M; \frac{k_{cat}}{K_m} = 3667 \mu M^{-1} \cdot min^{-1}) \) is higher than that of other metallopeptidases susceptible to metabolize BK (Erđos and Skidgel, 1997). The most likely explanation for this discrepancy probably results from methodological differences between the two studies, the major being the difference of concentrations of BK measured between both protocols (pM versus \( \mu M \)) and the sensitivity of both methods of detection. Our results are nevertheless similar to those in the postinfarction rat heart except that in the rat, the potentiating effect of the ACE inhibitor on the \( t_{1/2} \) of BK was higher than that measured for human hearts. This difference of effect between rats and humans during ACE inhibition could be theoretically attributed to the interference of endogenous ACE inhibitor in ICM and DCM hearts. However, such interference was excluded because no difference could be measured for the \( t_{1/2} \) of BK when the metabolism was tested in absence of ramiprilat before and after extensive dialysis of the membranes against a 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl (data not shown). Moreover, the ACE activity was similar in the normal, ICM, and DCM human hearts.

We found that the VPI omapatrilat had a much greater protective effect on cardiac BK metabolism compared with an ACE inhibitor alone. Kokkonen et al. (1999) using a specific NEP inhibitor (SCH 39370) found a similar degradation pattern regardless of whether the membranes were from normal or failing hearts. In our study, the effect of the simultaneous inhibition of ACE and NEP on the metabolism of BK by human cardiac membrane preparations was similar in normal and ICM hearts but appeared to be greater in DCM hearts. The reason for this could be the result of the simultaneous inhibition of both enzymes rather than a pure ACE or NEP inhibition. In fact, Dumoulin et al. (1998) using 10 nM BK could not measure an inhibiting effect of retrothiophan, a specific NEP inhibitor, on the rate of degradation of BK \( (V_{max}/K_m) \) by the coronary vascular bed of rat hearts. The potentiating effect of retrothiophan becomes evident only in the presence of ACE inhibition. These former observations and those presented in this article agree with the kinetic constants for hydrolysis of BK by both ACE \( (K_m = 0.18 \mu M; \frac{k_{cat}}{K_m} = 3667 \mu M^{-1} \cdot min^{-1}) \) and NEP \( (K_m = 120 \mu M; \frac{k_{cat}}{K_m} = 39.8 \mu M^{-1} \cdot min^{-1}) \) (Erđos and Skidgel, 1997). The values of ACE and NEP activities measured with synthetic substrates are difficult to put in relation with the metabolism of BK, an endogenous substrate for both metallopeptidases. Previously, we reported similar observations for hypertrophied rat LV (Raut et al., 1999).

ACE and NEP are not the only enzymes responsible for the metabolism of BK. In fact, besides ACE and NEP, different purified aminopeptidases, carboxypeptidases, and endopeptidases are potential candidates for the use of BK as a substrate. Their nature and their kinetic parameters have been reviewed recently (Erđos and Skidgel, 1997). In this study, we showed at the membrane level that only metallopeptidases are involved in the metabolism of BK. Moreover, the use of specific inhibitors of endopeptidase 24.15 (cFP-AAP-PAB; Orlowski et al., 1988) and endopeptidase 24.18 (actinonin; Choudry and Kenny, 1991) pleads for the participation of both enzymes, besides ACE and NEP, in the metabolism of BK. These results show clearly that other metallopeptidases that have been defined to have a membrane localization are also involved in the metabolism of BK.

In conclusion, the simultaneous inhibition of NEP and ACE with the VPI omapatrilat that has similar nanomolar inhibitory constants for both metallopeptidases has a greater protective effect on BK metabolism by human cardiac membranes than an ACE inhibitor alone. This is true not only for membranes from normal hearts but also for membranes from hearts with end-stage heart failure. Indeed, the protective effect of the VPI omapatrilat on BK in cardiac membranes appears to be the result of their protective effects on BK degradation, there is reason to believe that VPI could have cardioprotective effects that are even greater than those of ACE inhibitor alone.

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References


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