Kallidin- and Bradykinin-Degrading Pathways in Human Heart

Degradation of Kallidin by Aminopeptidase M–Like Activity and Bradykinin by Neutral Endopeptidase

Jorma O. Kokkonen, MD, PhD; Antti Kuoppala; Juhani Saarinen, MSc; Ken A. Lindstedt, PhD; Petri T. Kovanen, MD, PhD

Background—Since kinins kallidin (KD) and bradykinin (BK) appear to have cardioprotective effects ranging from improved hemodynamics to antiproliferative effects, inhibition of kinin-degrading enzymes should potentiate such effects. Indeed, it is believed that this mechanism is partly responsible for the beneficial effects of angiotensin-converting enzyme (ACE) inhibitors. In the heart, enzymes other than ACE may contribute to local degradation of kinins. The purpose of this study was to investigate which enzymes are responsible for the degradation of KD and BK in human heart tissue.

Methods and Results—Cardiac membranes were prepared from the left ventricles of normal (n=5) and failing (n=10) hearts. The patients had end-stage congestive heart failure as the result of coronary heart disease or idiopathic dilated cardiomyopathy. Heart tissue was incubated with KD or BK in the presence or absence of enzyme inhibitors. We found no difference in the enzymes responsible for kinin metabolism or their activities between normal and failing hearts. Thus KD was mostly converted into BK by the aminopeptidase M–like activity. When BK was used as substrate, it was converted into an inactive metabolite BK-(1-7) mostly (80% to 90%) by the neutral endopeptidase (NEP) activity, with ACE unexpectedly playing only a minor role. The low enzymatic activity of ACE in the cardiac membranes, compared with that of NEP, was not due to chronic ACE inhibitor therapy, because the cardiac ACE activities of patients, whether receiving ACE inhibitors or not, and of normal subjects were all equal.

Conclusions—The present in vitro study shows that in human cardiac membranes, the most critical step in kinin metabolism, that is, inactivation of BK, appears to be mediated mostly by NEP. This observation suggests a role for NEP in the local control of BK concentration in heart tissue. Thus inhibition of cardiac NEP activity could be cardioprotective by elevating the local concentration of BK in the heart. (Circulation. 1999;99:1984-1990.)

Key Words: bradykinin ■ growth substances ■ heart failure ■ peptides ■ remodeling

Several lines of evidence suggest that kinins, for example, kallidin (KD) and bradykinin (BK), have cardioprotective effects. Indeed, recent work in rats has shown that these kinins reduce left ventricular remodeling after myocardial infarction. The kinins appear to reduce interstitial fibrosis but not myocyte hypertrophy. Whether these cardioprotective effects are mediated by improved hemodynamics, by direct effects on target cells (eg, heart fibroblasts), or by both is not known. The cardioprotective effects of the kinins appear to be linked with the activity of the angiotensin-converting enzyme (ACE; EC 3.4.15.1), since bradykinin receptor blockers partially block the beneficial effects of ACE inhibitors on heart remodeling in the dog and the rat. Degradation of kinins is one mechanism for regulating their concentration, and, consequently their actions. It has been suggested that inhibition of ACE prevents the degradation of kinins and so elevates their concentration. Indeed, in vitro experiments have demonstrated that purified ACE readily degrades BK to BK-(1-7) and further to BK-(1-5). Moreover, inhibition of ACE has been shown to increase the blood levels of BK in rats and the outflow of BK from isolated perfused rat hearts. However, recent findings have suggested that part of the beneficial effects of ACE inhibitors can be attributed to their direct effects on bradykinin receptors rather than to their inhibitory effect on the degradation of kinins. Furthermore, direct measurements of kinin levels in rat heart tissue suggest that enzymes other than ACE may be responsible for BK degradation in the myocardium. Thus treatment of rats with an ACE inhibitor did not reduce the concentration of the major BK metabolite BK-(1-7) in heart tissue.

From the results of the above studies, we infer that the cardioprotective effects of kinins depend partly on their metabolism in the myocardium. However, no information is available on the enzymes responsible for local kinin degradation in the heart.
available on kinin metabolism in human heart tissue. Therefore we investigated the enzymatic degradation of the kinins KD and BK (Figure 1) by human cardiac membranes in vitro. We found that in the human cardiac membranes, these kinins are metabolized by both aminopeptidase M–like activity (APM; EC 3.4.11.2) and neutral endopeptidase (NEP; EC 3.4.24.11), with ACE playing only a minor role. The peptide product that accumulates through the combined action of these 2 peptidases is an inactive metabolite, BK-(1-7).

**Methods**

**Materials**

Synthetic kinin peptides furanacryloyl-Phe-Gly-Gly (FAPGG) and furanacryloyl-Phe (FAP) were purchased from Bachem; bestatin, captorpril, phosphoramidon, and thiorphan from Sigma; and Dulbecco’s PBS from Gibco. NEP inhibitor SCH 39370 was a kind gift from Schering-Plow.

**Preparation of Human Cardiac Membranes**

Human heart tissue from failing hearts was obtained from the excised hearts of patients (n=10) undergoing cardiac transplantation at the University Central Hospital, Helsinki. All patients were men and had end-stage congestive heart failure as the result of coronary heart disease (CHD) (n=6; age 57.7±1.5 years) or idiopathic dilated cardiomyopathy (IDC) (n=4; age 53.0±3.0 years). Before transplantation, all patients had been treated with ACE inhibitors. Normal heart tissue (n=5; age 40.4±5.6 years) was obtained from hearts that were unsuitable for donation from the University Central Hospital, Helsinki (n=2) and from the General Hospital of Vienna (n=3). The donors were 4 men and 1 woman. In the experiment presented in Table 4, ACE activity was also determined in the cardiac membranes from patients not treated with ACE inhibitors. These donors were 2 men and 2 women who had end-stage congestive heart failure as the result of CHD or IDC (n=4; age 37.2±8.7 years). The use of these tissues was approved by the Internal Review Committees of the corresponding hospitals. After excision, the heart was thoroughly flushed with ice-cold cardioplegia solution, and tissue pieces were cut from the left ventricles. The heart tissue was immediately frozen with liquid nitrogen and stored at −70°C. This tissue was then homogenized in PBS at 4°C (100 mg tissue/mL) with an Ultra-Turrax T25 homogenizer (IKA-Labotechnik) at 13 500 rpm for 1 minute. Cardiac membranes were prepared by centrifugation of the homogenates at 40 000g for 30 minutes at 4°C as described by Urata et al.11 The sediments were resuspended and centrifuged as described above and finally resuspended in PBS and stored at −70°C. The concentration of each cardiac membrane preparation is expressed in terms of its protein concentration. Protein was determined after solubilization with Triton-X-10012 by the method of Lowry, with bovine serum albumin as standard.13 The protein concentrations of the different preparations varied between 0.7 and 1.5 mg/mL.

**Determination of Kinin Degradation**

The standard assay was conducted at 37°C in 50 µL of PBS (137 mMNaCl, 2.7 mM KCl, 8.1 mM NaHPO4, 0.9 mM CaCl2, 1.1 mM KH2PO4, 0.5 mM MgCl2, pH 7.3) containing cardiac membranes (5 µg of protein), 5 nmol of kinins, and the indicated concentrations of inhibitors. After incubation for the indicated times, the reactions were stopped by adding 300 µL of ice-cold ethanol, and the preparations were incubated further at 4°C for 30 minutes to precipitate proteins. Finally, the mixtures were centrifuged at 15 000g for 10 minutes at 4°C to sediment the proteins. The supernatants were then collected for peptide analysis by reverse-phase high-performance liquid chromatography (RP-HPLC).

**RP-HPLC Analysis**

For RP-HPLC analysis, the supernatants containing kinin peptides or FAPGG were evaporated to dryness and finally dissolved in 100 µL of 0.1% trifluoroacetic acid. The samples were analyzed by RP-HPLC as described.14 Kinin peptides were identified by comparing the retention times of the peaks with those of synthetic standards and by N-terminal sequence analysis of the eluted material. Formation of kinin peptides or FAP was quantified by measuring peak area or peak height relative to known standards. The results are expressed as nmol of kinin peptides or FAP formed per minute per mg of cardiac membrane protein.

**N-Terminal Sequence Analysis**

The kinin peptide fractions obtained from RP-HPLC analysis were subjected to an automatic sequence analysis with an Applied Biosystems Procise 494 protein sequencing system and a model 610 data analysis system.

**Statistical Analysis**

The results are expressed as mean±SEM. Differences between groups were tested by use of the Kruskal-Wallis test, and a value of P<0.05 was considered statistically significant.

**Results**

**Degradation of Kinins by Human Cardiac Membranes**

We first studied the degradation of the 2 kinins KD and BK by human cardiac membranes as a function of time. For this purpose, KD was incubated with cardiac membranes derived from a normal heart for 2 hours at 37°C (Figure 2). Panel A shows a typical RP-HPLC analysis of KD-derived peptides after incubation for 2 hours. The elution profile disclosed, in addition to KD (elution time 29 minutes), 4 peptide peaks: 1 major peak eluting at 31 minutes and the other 3 at 21, 22, and 25 minutes, respectively. N-terminal sequence analysis disclosed that the peptide eluting at 31 minutes was BK and that eluting at 22 minutes was its degradation product, BK-(1-7). The peptides eluting at 21 and 25 minutes were 2 degradation products of kallidin, KD-(1-8) and KD-(6-10), respectively. The peaks eluting between 5 and 18 minutes represented non–kinin-derived material. The time courses of formation of these peptides are illustrated in panel B. The rates of formation of the major KD-derived metabolite BK and also KD-(6-10) and KD-(1-8) were linear for 90 minutes. After an initial lag phase of 30 minutes, the formation of the secondary degradation product BK-(1-7) proceeded linearly.
membrane preparation as in Figure 2. In addition to BK (elution time 31 minutes), the elution profile showed only 1 major peak, which eluted at 22 minutes. When identified by N-terminal sequence analysis, this peptide was found to be BK-(1→7). The peaks eluting between 5 and 18 minutes represented non-kinin-derived material. As shown in Figure 3B, during the 2-hour incubation period, the formation of BK-(1→7) was linear.

The rates of formation of KD- and BK-derived peptides by cardiac membranes prepared from normal hearts (n=5) and from hearts of patients with end-stage heart failure as the result of CHD (n=6) or IDC (n=4) are summarized in Table 1. The normal and the failing hearts did not differ significantly in their ability to degrade KD and BK, nor was a significant difference observed in the ability of CHD and IDC hearts to degrade KD and BK (Table 1).

### Inhibition of Kallidin and Bradykinin Degradation by Enzyme Inhibitors

The similar degradation patterns of kinins by cardiac membranes from normal and failing hearts suggested that the enzymes responsible for the degradation were the same in every membrane preparation. To study the contribution of the enzymes potentially involved, the degradation of KD and of BK were studied in the presence of various enzyme inhibitors. We first assessed the degradation of KD by using 3 different cardiac membrane preparations, 1 derived from a normal, 1 from a CHD, and 1 from an IDC heart. With all 3 preparations, the results were closely similar (Table 2). Conversion of KD to BK was effectively inhibited by amastatin and bestatin, indicating that this peptide was derived from BK. In sharp contrast, captopril had no significant effect on the formation of any of the peptides studied.

We then repeated the above experiments with BK as substrate (Table 3). As when KD was used as substrate, no differences were found between the normal and failing hearts. Unexpectedly, captopril, a specific ACE inhibitor, had no effect on BK-(1→7) formation. The experiment was repeated...
with another ACE inhibitor, lisinopril (10 μmol/L), and again no inhibition was found (data not shown).

However, formation of BK-(1-7) from BK was effectively (80% to 90%) inhibited by phosphoramidon, a widely used inhibitor of NEP.15 Phosphoramidon is not entirely specific for NEP because, at high concentrations, it may also inhibit endothelin-converting enzyme.18 Therefore we tested the effect of the specific NEP inhibitor SCH 3937017 on the formation of BK-(1-7). The results were very similar to those obtained with phosphoramidon. In addition, we were able to show that at the concentrations used, phosphoramidon and SCH 39370 were totally unable to inhibit the BK degradation by purified rabbit ACE and that captopril was totally unable to inhibit the degradation of BK by purified rabbit NEP (data not shown). In contrast, neither amastatin (10 μmol/L) nor bestatin (300 μmol/L; data not shown), two aminopeptidase inhibitors, had any effect on BK-(1-7) formation. These findings showed that in the cardiac membranes the conversion of BK to BK-(1-7) was mostly due to NEP, which is known to hydrolyze the Pro7-Phe8 bond in BK, producing the same metabolite, BK-(1-7), as ACE.19

Role of ACE in Bradykinin Metabolism

The above results suggested that ACE (EC 3.4.15.1) plays no significant role in the metabolism of kinins in the membrane fraction of heart tissue. The patients were all under chronic ACE inhibitor therapy, which, in the in vitro assays, may have inhibited the ACE activity in the cardiac membranes derived from their hearts. To rule out this possibility, we compared the ACE activities in the cardiac membranes of normal (n=5) and failing hearts from patients with chronic ACE inhibitor therapy (n=10) and in cardiac membranes from patients not treated with ACE inhibitors (n=4). These donors were 2 men and 2 women who had end-stage congestive heart failure as the result of CHD or IDC. ACE activity in cardiac membranes can be measured specifically

<table>
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<tr>
<th>Inhibitor</th>
<th>Conversion of KD into BK</th>
<th>Conversion of KD into KD-(6-10)</th>
<th>Conversion of KD into KD-(1-8)</th>
<th>Conversion of KD into BK-(1-7)</th>
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<tr>
<td>None</td>
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<td>100 (0.32±0.03)</td>
<td>100 (0.23±0.01)</td>
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Cardiac membrane preparations from 1 normal, 1 CHD, and 1 IDC heart were tested. Cardiac membranes (5 μg) were preincubated at 37°C for 15 minutes in 50 μL of PBS containing the indicated concentrations of inhibitors. Reactions were started by adding 5 nmol of kallidin. After incubation at 37°C for 60 minutes, amounts of bradykinin, KD-(6-10), KD-(1-8), and BK-(1-7) were determined. Control values are 100%. The 100% control values for cardiac membrane preparations from the normal, CHD, and IDC hearts are expressed as nmol of peptides formed per minute per milligram (values in parentheses). Each value is mean (±SEM) of triplicate incubations.

*Not detectable (<0.01 nmol·min⁻¹·mg⁻¹).
with FAPGG as substrate. As shown in Table 4, the cardiac ACE activities of patients receiving ACE inhibitors were found to be not lower than those of patients not receiving ACE inhibitors or those of normal subjects. Rather the ACE activities of patients receiving ACE inhibitors were somewhat higher, but the difference did not reach statistical significance. The degradation of FAPGG was strongly inhibited by captopril (10 μmol/L) but not by phosphoramidon (1 μmol/L) confirming the presence of ACE activity in the preparations (data not shown). This finding is in accord with that of Urata et al, who showed similar ACE activities in normal and failing hearts derived from patients with or without chronic ACE inhibitor therapy.

**Discussion**

Figure 4 summarizes the enzymatic degradation pathways of the two kinins, kallidin and bradykinin, by the membrane fraction of heart tissue. The major pathway consisted of conversion of KD to another metabolically active kinin BK by APM-like activity. BK was then converted by NEP to BK-(1-7), an inactive metabolite. The same enzymes were responsible for the degradation of the kinins in the cardiac membranes of normal and failing hearts and, moreover, the enzyme activities in the normal and failing hearts were equal.

**Local Formation of Kinins in Heart**

When formed locally, even in small amounts, kinins may exert important paracrine or autocrine effects in the myocardium, as opposed to the endocrine effects of the circulating kinins. Myocardial levels of BK and BK-(1-7) in the rat are at least 10-fold higher than those in plasma, a finding consistent with the notion that these peptides are formed locally in the heart. Further, it has been demonstrated that the rat heart contains an intrinsic kallikrein-kinin system. Thus tissue kallikrein mRNA is expressed in both the atria and the ventricles of the rat heart and, on incubation of heart tissue, kininogen and tissue kallikrein are released into the incubation medium. Although there have been no direct measurements of KD levels in heart tissue, it is conceivable that KD is formed as a result of the local action of tissue kallikrein on kininogen.

**Enzymatic Degradation of Kallidin and Bradykinin in Tissues**

Degradation of kinins is one mechanism for regulating their concentration, and, consequently, their actions. Therefore, to gain insight into the potential role of kinins in the pathogenesis of heart failure, it is important to learn about the local metabolism of these peptides in the heart. We showed that...
KD is rapidly degraded to BK by an aminopeptidase activity in the cardiac membranes. The inhibition profile of this enzyme activity by amastatin and bestatin is consistent with APM-like activity being the enzyme responsible for the degradation.\textsuperscript{15,16} APM is widely distributed in tissues, being present, for example, on the plasma membrane of cultured endothelial cells and on smooth muscle cells\textsuperscript{16} and human respiratory epithelial cells\textsuperscript{21} but, to our knowledge, there are no previous reports of the activity of this aminopeptidase in human heart tissue. The role of APM in the regulation of local BK concentrations in tissues is currently unknown. Local conversion of KD to BK may explain why the concentration of BK in heart tissue is far higher than in the circulation.

**Role of ACE in Bradykinin Metabolism**

The major BK-degrading enzyme is generally held to be ACE. Indeed, in vitro experiments have demonstrated that purified ACE readily degraded BK to BK-(1-7),\textsuperscript{5} and, in in vivo experiments, the use of ACE inhibitors increased the blood levels of BK in rats, suggesting that degradation of BK by ACE was reduced.\textsuperscript{6} Our results, in contrast, suggest that ACE plays only a minor role in the metabolism of kinins in the membrane fraction of heart tissue. However, in vitro experiments may include pitfalls that lead to artificially low ACE activities in tissues. To rule out these possibilities, we performed several control experiments, the results of which are as follows: (1) The ACE activities in total heart homogenates were the same as in cardiac membranes, indicating that no ACE activity is lost during preparation of the cardiac membranes (data not shown). (2) The results presented in Table 4 demonstrated that the cardiac ACE activities of patients receiving ACE inhibitors were no lower than those of patients not receiving ACE inhibitors or those of normal subjects. This finding, also shown by others,\textsuperscript{11} most probably reflects the release of ACE inhibitors from ACE during the preparation of cardiac membranes. (3) Since endogenous ACE inhibitors may be present in (rat) heart tissue,\textsuperscript{22} we performed an experiment in which the activity of human plasma ACE was measured in the absence and presence of human cardiac membranes. We found that ACE activity was not affected by the presence of cardiac membranes (5 µg/assay), thus ruling out the presence of significant amounts of endogenous ACE inhibitors in the cardiac membrane preparations used in this study (data not shown). (4) The physiological concentrations of kinins in the heart tissue are below the $K_{m}$ values of the competing enzymes NEP and ACE and thus lower than those used in our in vitro experiments. Because BK has a higher affinity for ACE than for NEP,\textsuperscript{23,24} we measured the degradation of BK by the cardiac membranes at a substrate concentration of 100 nmol/L, which is well below the $K_{m}$ values of both enzymes. At this concentration, the hydrolysis of BK follows first-order kinetics, that is, the substrate affinity may substantially affect the reaction kinetics. We could show that at this concentration NEP still was the major BK-degrading enzyme, with ACE playing a minor role (data not shown).

It is clear from our results that the enzymatic activity of ACE is low in human cardiac membranes compared with that of NEP. At least 2 possible explanations can be offered for these discrepant findings between in vivo and present in vitro experiments. First, recent findings have demonstrated that ACE inhibitors directly potentiate bradykinin receptor–mediated effects,\textsuperscript{8,9} making it possible that some of the observed effects of ACE inhibitors on BK metabolism are not due to inhibition of ACE. Second, several lines of evidence suggest that BK metabolism in tissues and in the circulation may differ. Thus in tissues such as the kidneys\textsuperscript{22} and lungs,\textsuperscript{26,27} NEP plays a significant role, along with ACE, in BK metabolism. Interestingly, in in vitro experiments with skeletal muscle, BK was degraded by NEP but not by ACE.\textsuperscript{28}

**Bradykinin Metabolism of the Heart**

What, then, has been the previous experience regarding the roles of ACE and NEP in the degradation of BK in heart tissue? In studies with rat hearts evidence has been found for both ACE-mediated and non–ACE-mediated degradation of BK. Thus in an isolated perfused rat heart, ACE inhibition increased the outflow of BK, suggesting that degradation of BK by ACE was reduced.\textsuperscript{7} Moreover, direct measurement of the levels of BK and its metabolites in the rat heart have shown that although BK-(1-7) is the major metabolite of BK,\textsuperscript{6} its concentration was not reduced by ACE inhibition, although in the same experiment, the concentration of angiotensin II was greatly reduced in the heart tissue, indicating effective inhibition of tissue ACE.\textsuperscript{10} Moreover, the BK concentration was increased, also suggesting involvement of ACE/ACE inhibitors in BK metabolism. Clearly, the results obtained so far are difficult to interpret and an experiment with a NEP inhibitor could clarify this issue. Interestingly, in one report, NEP inhibition prevented isoproterenol-induced myocardial hypoperfusion in the rat, and this effect was abolished by BK receptor blockers, suggesting that the effect was due to reduced degradation of BK by NEP.\textsuperscript{29} Our experiments with human cardiac membranes support the notion that the major BK-metabolizing enzyme in the heart tissue is NEP. NEP is a metalloendopeptidase that is widely distributed in tissues. Its activity is highest on the epithelial cells of kidneys,\textsuperscript{30} NEP activity has also been found in the cardiovascular system, on the plasma membranes of vascular endothelial cells,\textsuperscript{31} and on cultured rat myocytes.\textsuperscript{29}

**Conclusions and Clinical Implications**

The present in vitro study shows that metabolism of the kinins KD and BK by human heart membranes leads to accumulation of BK-(1-7) through the combined action of heart APM-like activity and NEP. The activities of these enzymes in normal and in failing hearts did not differ. Although ACE seems to be the principal enzyme responsible for BK degradation in the circulation, it appears to have little importance in the membrane fraction of the heart tissue. Because BK-(1-7) is an inactive metabolite, that is, one that does not bind to bradykinin receptors, the possibility exists that local BK-(1-7) formation represents the termination of kinin activity in the heart. Inhibition of BK-degrading enzyme(s) has been suggested as one strategy to potentiate the beneficial effects of BK, and it is generally believed that this can be achieved with ACE inhibitors. Our data suggest an additional perhaps even more important aspect of pharmaco-
logical control, namely inhibition of NEP in heart tissue, which may also induce cardioprotective effects by elevating the local concentration of BK.

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