Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma

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1Wihuri Research Institute, FIN-00140 Helsinki; 2Institute of Biotechnology, University of Helsinki Biocentrum, FIN-00710 Helsinki; and 3Division of Cardiology, Helsinki University Hospital, FIN-00290 Helsinki, Finland

Kuoppala, Antti, Ken A. Lindstedt, Juhani Saarinen, Petri T. Kovanen, and Jorma O. Kokkonen. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. Am J Physiol Heart Circ Physiol 278: H1069–H1074, 2000.—Because bradykinin (BK) appears to have cardioprotective effects ranging from improved hemodynamics to antiproliferative effects, inhibition of BK-degrading enzymes should potentiate such actions. The purpose of this study was to find out which enzymes are responsible for the degradation of BK in human plasma. Human plasma from healthy donors (n = 10) was incubated with BK in the presence or absence of specific enzyme inhibitors. At high (micromolar) concentrations, BK was mostly (>90%) degraded by carboxypeptidase N (CPN)-like activity. In contrast, at low (nanomolar) substrate concentrations, at which the velocity of the catalytic reaction is equivalent to that under physiological conditions, BK was mostly (>90%) converted into an inactive metabolite, BK-(1–7), by angiotensin-converting enzyme (ACE). BK-(1–7) was further converted by ACE into BK-(1–5), with accumulation of this active peptide. A minor fraction (<10%) of the BK was converted into another active metabolite, BK-(1–8), by CPN-like activity. The present study shows that the most critical step in plasma kinin metabolism, i.e., inactivation of BK, is mediated by ACE. Thus inhibition of plasma ACE activity would be cardioprotective by elevating the concentration of BK in the circulation.

BK is thought to act as a local tissue hormone. Thus the local concentration of BK in the vascular bed of tissues is likely to be critical for the BK-mediated hemodynamic effects. This concentration is partly determined by the degradation of BK by local kininases present in plasma and on the endothelium. However, the relative contributions of these kininases in the local degradation of BK are not known. In a recent study, the relative roles of plasma ACE and ACE bound to the endothelium of human heart tissue were compared (5). It was found that in the human heart, the tissue-to-plasma ratio for ACE was 54%, i.e., the plasma ACE activity was about twofold higher than that in cardiac tissue (mostly located on the coronary endothelial surfaces). This finding suggests that the plasma compartment may have an important role in the local regulation of BK concentration in the heart.

Several enzymes may be involved in plasma degradation of BK. Figure 1 shows the structure of BK and the potential sites of degradation by carboxypeptidase N (CPN; EC 3.4.17.3, kininase I), ACE (EC 3.4.15.1, kininase II), and neutral endopeptidase (NEP; EC 3.4.24.11). CPN degrades BK to BK-(1–8), and ACE and NEP degrade BK to BK-(1–7). ACE readily degrades BK-(1–7) to BK-(1–5) (8).

The results of earlier studies on the enzymatic degradation of BK in human plasma or serum have been controversial. Direct measurements of the concentrations of kinin peptides in the circulation are difficult because of their very low physiological (pico- or nanomolar) concentrations. In one report (20), the major kinin peptide in human plasma was BK-(1–8), suggesting that BK was mostly degraded by CPN. In studies by Marceau et al. (17) and Sheikh and Kaplan (23), incubation of plasma with synthetic BK revealed that CPN was the major BK-degrading enzyme, with ACE playing only a minor role. In contrast, in a recent report in which a chemiluminescent enzyme immunoassay was used to measure the changes in BK concentration, Decaré et al. (6) suggested that about two-thirds of the BK-degrading activity in human serum is due to ACE activity.

In an attempt to solve the apparent controversy concerning the major BK-metabolizing enzyme in human plasma, we investigated the enzymatic degradation of BK by human plasma in vitro by using radiolabeled BK.
beled BK, which enabled us to test a wide range of BK concentrations. Both BK and its degradation products were directly detected by reverse-phase HPLC (RP-HPLC) and NH₄-terminal sequencing. We found that degradation of BK in human plasma in vitro depends strongly on its concentration. At high (micromolar) BK concentrations, the major degrading enzyme is CPN. In striking contrast, at low (nanomolar) concentrations, at which the velocity of the catalytic reaction is equivalent to that under physiological conditions, BK is metabolized mainly by ACE, with CPN playing only a minor role.

MATERIALS AND METHODS

Materials. Synthetic kinin peptides were purchased from Bachem, [prolyl2,3,4(n)-3H]bradykinin (71 Ci/mmol) from Amersham, captopril from Sigma, dl-2-mercaptomethyl-3-guanidino ethylthiopropanoic acid (MGEA) from Calbiochem, Dulbecco’s PBS from Gibco, and dalteparin (Fragnimin) from Kabi Pharmacia. A specific NEP inhibitor, Sch-39370, was a kind gift from Schering-Plough.

Preparation of human plasma. Plasma was prepared from 10 apparently healthy persons, 5 males and 5 females, from 24 to 52 yr of age. Blood (5 ml) was withdrawn by venipuncture in tubes containing 100 IU of dalteparin (final concentration 20 µl/ml) containing 1 mg/ml BSA, 20 IU/ml dalteparin, and 2.5 pmol of [3H]BK was incubated with PBS containing 20 IU/ml of dalteparin and used immediately for the experiments.

Determination of kinin degradation. The standard assay was conducted at 37°C in 25 µl of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH₂PO₄, 0.9 mM CaCl₂, 11 mM KH₂PO₄, and 0.5 mM MgCl₂, pH 7.3) containing 1 mg/ml BSA, 20 µl/ml dalteparin, 2.5 µl of diluted plasma (30% vol/vol), and 20 µl/ml of ACE and NEP (CPN) activities were indicated in legends to Figs. 2–4 and Tables 1 and 2. When analyzed by RP-HPLC, the elution profiles of labeled BK was diluted with unlabeled BK to give the specific activities indicated in legends to Figs. 2–4 and Tables 1 and 2. When analyzed by RP-HPLC, the elution profiles of labeled BK was diluted with unlabeled BK to give the specific activities indicated in legends to Figs. 2–4 and Tables 1 and 2.

RESULTS

Degradation of BK by human plasma. We first studied degradation of BK by human plasma as a function of time. For this purpose, [3H]BK was incubated with diluted plasma (3% vol/vol) derived from a healthy person at 37°C. Figure 2 shows a typical RP-HPLC analysis. For RP-HPLC analysis, the supernatants containing kinin peptides were evaporated to dryness and finally dissolved in 100 µl of 0.1% trifluoroacetic acid. The samples were analyzed by RP-HPLC as described previously (14). Fractions of 250 µl (30 s) from the RP-HPLC eluate were collected and measured for their 3H radioactivity. Kinin peptides were identified by comparing the retention times of the peaks with those of synthetic standards and by NH₄-terminal sequencing of the eluted material. Formation of kinin peptides was quantitated by counting the radioactivity in each peak area. The results are expressed as nanomoles of BK peptides formed per minute per liter of plasma. The recoveries of the eluted [3H]-labeled material averaged >90% of the radioactivity applied to the column.

NH₄-terminal sequence analysis. The kinin peptide fractions obtained from RP-HPLC analysis were subjected to automatic sequence analysis with an Applied Biosystems Procise 494 protein sequencing system and a model 610 data analysis system.

Table 1. Degradation of bradykinin by human plasma

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Sex</th>
<th>Age</th>
<th>BK-(1–5) Converted, nmol·min⁻¹</th>
<th>BK-(1–7) Converted, nmol·min⁻¹</th>
<th>BK-(1–8) Converted, nmol·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>25</td>
<td>15.7 ± 0.7</td>
<td>9.6 ± 0.6</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>36</td>
<td>16.8 ± 2.6</td>
<td>10.4 ± 1.6</td>
<td>0.51 ± 0.19</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>52</td>
<td>8.7 ± 0.6</td>
<td>6.9 ± 0.5</td>
<td>1.15 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>24</td>
<td>13.5 ± 0.9</td>
<td>9.5 ± 0.3</td>
<td>0.47 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>41</td>
<td>7.9 ± 0.9</td>
<td>6.7 ± 0.4</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>12.4 ± 1.8</td>
<td>8.6 ± 0.8</td>
<td>0.71 ± 0.14</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>43</td>
<td>14.7 ± 2.4</td>
<td>10.4 ± 2.3</td>
<td>1.25 ± 0.28</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>26</td>
<td>8.8 ± 0.9</td>
<td>6.2 ± 0.6</td>
<td>0.41 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>32</td>
<td>9.8 ± 1.1</td>
<td>6.6 ± 0.8</td>
<td>1.27 ± 0.23</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>52</td>
<td>13.1 ± 1.6</td>
<td>9.7 ± 2.0</td>
<td>1.25 ± 0.27</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>36</td>
<td>18.4 ± 2.1</td>
<td>11.5 ± 1.6</td>
<td>1.10 ± 0.28</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>13.0 ± 1.7</td>
<td>8.9 ± 1.1</td>
<td>1.06 ± 0.16</td>
</tr>
<tr>
<td>Total average</td>
<td></td>
<td></td>
<td>12.7 ± 1.2</td>
<td>8.7 ± 0.6</td>
<td>0.88 ± 0.12</td>
</tr>
</tbody>
</table>

Human plasma (30% vol/vol, 2.5 µl) derived from 5 healthy males and 5 healthy females was incubated at 37°C in 25 µl of PBS containing 1 mg/ml BSA, 20 µl/ml dalteparin, and 2.5 pmol of [3H]BK (final concentration 100 nM). After 10 min of incubation, amounts of BK-(1–5), BK-(1–7), and BK-(1–8) were analyzed by reverse-phase HPLC (RP-HPLC). Values are expressed as nanomoles of BK-(1–5), BK-(1–7), and BK-(1–8) formed per minute per liter of plasma; each value is a mean ± SE of triplicate incubations. Possible differences in angiotensin-converting enzyme (ACE) and carboxypeptidase N (CPN)-like activities between the two groups (male and female) were analyzed using a logistic regression model (see MATERIALS AND METHODS). No significant effect was detected with either variable (P = 0.9249 for sex; P = 0.7233 for age) using likelihood ratio statistics.
analysis of [3H]BK-derived peptides after incubation for 8 min. The elution profile displayed, in addition to [3H]BK (elution time 35 min), three other peptide peaks, the first of these eluting at 24 min and the other two at 26 and 38 min, respectively. NH₂-terminal sequence analysis disclosed that the peptide eluting at 35 min was BK and that the peptides eluting at 24, 26, and 38 min were three degradation products of BK, BK-(1–5), BK-(1–7), and BK-(1–8), respectively.

The time courses of formation of these peptides are illustrated in Fig. 3. As shown in Fig. 3A, [3H]BK was rapidly degraded by human plasma. The rate of BK degradation was closely followed by formation of the three degradation products, BK-(1–5), BK-(1–7), and BK-(1–8) (Fig. 3B). It seems evident that degradation of BK leads to accumulation of a pentapeptide, BK-(1–5). The rates of formation of the major BK-derived metabolites BK-(1–5), BK-(1–7), and BK-(1–8) were closely linear for 8 min. Accordingly, in subsequent experiments the incubation time was 8 min.

The rates of formation of BK-derived peptides by plasma prepared from healthy persons (n = 10, 5 males and 5 females) are summarized in Table 1. In all tested plasmas, the major degradation products were BK-(1–5) and BK-(1–7) (total averages 12.7 and 8.7 nmol·min⁻¹·l⁻¹, respectively), and BK-(1–8) was a minor degradation product (total average 0.88 nmol·min⁻¹·l⁻¹). The plasmas derived from males and females did not differ significantly in their ability to degrade BK. In additional experiments, we found that degradation of BK was similar in citrated plasma and serum (data not shown).

Inhibition of BK degradation by enzyme inhibitors. The similar degradation patterns of BK in every plasma sample suggested that the same enzymes were responsible for the degradation in all these samples. To study the contribution of the enzymes potentially involved, the degradation of BK was studied in the presence of various enzyme inhibitors. We assessed the degradation of BK in three different plasma preparations. In all three preparations, the results were closely similar (Table 2). Conversion of BK to BK-(1–5) and BK-(1–7) was effectively inhibited by captopril, a specific ACE inhibitor. In contrast, captopril did not inhibit the formation of BK-(1–8). However, formation of BK-(1–8) was effectively inhibited by MGEA, a widely used but not fully specific inhibitor of CPN (24). MGEA did not inhibit the formation of BK-(1–5) or BK-(1–7). Because MGEA is not fully specific for CPN, this MGEA-inhibitable activity in human plasma will hereafter be referred to as CPN-like activity. In additional experiments, we found that Sch-39370, the specific inhibitor of NEP (27), had no effect on the formation of BK-(1–5), BK-(1–7), or BK-(1–8), indicating that NEP is not involved in BK metabolism in plasma (data not shown).

It was recently shown that aminopeptidase P (APP; EC 3.4.11.9) and dipeptidylaminopeptidase IV (DPAP; EC 3.4.14.5) contribute to the regulation of BK concentration in the rat (7, 12). APP removes the amino-terminal arginine to produce BK-(2–9), which is rapidly de-
varying concentrations of $[^3]$H]BK ranging from 30 nM to 100 µM. As shown in Fig. 4, at a high BK concentration of 100 µM, the major (>90%) BK-degrading enzyme was ACE in all samples tested (n = 10, data not shown).

**DISCUSSION**

Figure 5 summarizes the enzymatic degradation pathways of BK by human plasma. The major pathway consisted of conversion of BK by ACE to BK-(1–7), an inactive metabolite. BK-(1–7) was further converted to BK-(1–5) by ACE, leading to accumulation of this active peptide. Less than 10% of BK was converted to the active metabolite BK-(1–8). The inhibition profile of this enzyme activity by MGEA is consistent with the enzyme responsible for this degradation being CPN-like activity (24). In addition, our preliminary findings suggested that CPN-like activity plays an insignificant role in the metabolism of kinins in human plasma, although in several studies it has been reported to be the major BK-degrading enzyme in plasma and serum (17, 23). However, in these studies the BK concentration was well above the values of the Michaelis-Menten constant ($K_m$) of the competing enzymes ACE and CPN. Because the physiological concentrations of kinins in plasma are well below the $K_m$ values of the competing enzymes and BK has a higher affinity for ACE than for CPN (11, 25), we suspected that the BK concentration used affected the degradation profile of BK by plasma. To test this hypothesis, we incubated plasma with varying concentrations of $[^3]$H]BK ranging from 30 nM to 100 µM. As shown in Fig. 4, at a high BK concentration of 100 µM, the major (>90%) BK-degrading enzyme was CPN-like activity. With decreasing BK concentrations, there was a gradual shift in the relative activities of the enzymes in favor of ACE over CPN-like activity. At a BK concentration of 100 nM, which was used throughout this study, the major (>90%) BK-degrading enzyme was ACE in all samples tested (n = 10, data not shown).
show that BK-(1–8) is also slowly degraded to BK-(1–5) by the endopeptidase activity of ACE (data not shown). No differences in ACE and CPN-like activities were found between the analyzed plasma samples (Table 1).

Contributions of ACE and CPN to BK degradation. The results of earlier observations of the relative contributions of ACE and CPN to degradation of BK in plasma and/or serum have been discrepant (6, 17, 20, 23). In vitro experiments may include pitfalls that lead to artificially low ACE activities. The results presented in Fig. 4 offer a plausible explanation for these findings. At BK concentrations below the $K_m$ values of both ACE (0.2–1 µM) and CPN (6–19 µM) (11, 24–26), the hydrolysis of BK follows first-order kinetics. The velocity of the catalytic reaction is determined by the ratio of $k_{cat}$ to $K_m$ values (where $k_{cat}$ is the catalytic constant) and by the absolute concentrations of ACE, CPN, and BK in plasma. Thus, at low substrate concentrations ($S << K_m$, where $S$ is substrate concentration), the affinity of BK for ACE and CPN becomes rate limiting for the velocity of the catalytic reaction, and the $k_{cat}/K_m$ values for ACE (500–3,300 µM $^{-1} \cdot$min$^{-1}$) and CPN (3.1–7.2 µM $^{-1} \cdot$min$^{-1}$) determine their catalytic efficiency. In contrast, at high (micromolar) substrate concentrations ($S >> K_m$), the hydrolysis of BK follows zero-order kinetics, i.e., the velocity of the reaction is determined by the $k_{cat}$ values for ACE (500–600 min$^{-1}$) and CPN (43–59 min$^{-1}$) and their absolute concentrations in plasma. Because the physiological concentration of BK in plasma is in the picomolar range ($S << K_m$), it is evident that experiments performed at picomolar concentrations of BK ($S > K_m$) obey different kinetic rules and may lead to an underestimation of the role of ACE in BK degradation in plasma (17, 23). Indeed, as shown in Fig. 4, with decreasing BK concentration there was a gradual shift from zero-order to first-order kinetics, causing a change in the relative activity of the enzymes. At a BK concentration ≤100 nM, the velocity of the catalytic reaction was equivalent to that under physiological conditions in plasma, and the major BK-degrading enzyme was ACE.

Interestingly, the $k_{cat}/K_m$ value for ACE for the BK degradation product BK-(1–7) (2,063 µM $^{-1} \cdot$min$^{-1}$) is similar to that for BK (3,300 µM $^{-1} \cdot$min$^{-1}$), suggesting that both BK and BK-(1–7) are excellent substrates for ACE. In contrast, BK-(1–7) is not a substrate for CPN. Although ACE has two competing substrates with similar $k_{cat}/K_m$ values [BK and BK-(1–7)] present at the same time, this does not significantly affect its catalytic efficiency to degrade BK compared with that of CPN.

Accumulation of BK-(1–5). A novel finding in this study is that degradation of BK by plasma enzymes in vitro leads to accumulation of a pentapeptide, BK-(1–5). Until recently, this kinin was considered to be an inactive metabolite. However, in a recent report, BK-(1–5) was found to be a selective inhibitor of thrombin-induced platelet activation (10). This finding suggests that BK-(1–5) may contribute to the constitutive anticoagulant nature of the intravascular compartment and thus may contribute to the cardioprotective nature of kinins independently of BK receptor-mediated effects (10). However, the concentrations of BK-(1–5) used in these in vitro experiments were very high (millimolar), challenging the physiological significance of this finding.

The role of CPN in BK metabolism. Our results imply that CPN (kininase I) is not the major BK-degrading enzyme in the circulation. However, depending on BK receptor expression, even small concentrations of BK-(1–8) may exert important effects on hemodynamics. BK-(1–8) is not bound to BK-2-type receptors, but it is an agonist for the BK-1-type receptor. However, the BK-1-type receptor is not normally expressed in vascular tissues (22). Thus, normally, BK-(1–8) is an inactive metabolite, and BK-(1–8) formation in plasma represents the termination of kinin activity. BK-1-type receptors are induced in pathological circumstances such as tissue trauma, inflammation, and anoxia (18, 22). The effects mediated by BK-1-type receptors are largely unknown, and the results of studies concerning their cardioprotective effects are inconsistent (2, 4, 15, 22).

However, in vitro evidence obtained with isolated, perfused rat hearts implies that stimulation of BK-1-type receptors, similar to that of BK-2-type receptors, may contribute to the cardioprotective effects of kinins (4).

The role of ACE in BK metabolism. Several lines of evidence suggest that BK metabolism in the interstitial space and in the vascular bed of tissues may differ. In the rat, levels of BK are at least 10-fold higher in the interstitial space than in plasma, a finding that suggests that BK is not derived from plasma but is formed locally in the interstitial space (3). It has been suggested that NEP plays a significant role in BK metabolism in the interstitial space of the heart. Findings with rat heart tissue indicated that NEP was exclusively localized in the interstitial space (7). Furthermore, our own findings with human heart tissue indicated that the major BK-degrading enzyme was NEP, with ACE being of little importance (13). Thus both the work cited above and the present findings suggest that the effect of ACE on kinin metabolism is restricted to the vascular bed of tissues and that ACE located both on the endothelium and in the plasma is responsible for BK degradation in this compartment. Furthermore, it can be speculated that the circulatory kinins (and ACE inhibitors) mostly affect the hemodynamics but have little direct effect on the growth regulation of target cells (e.g., heart myocytes and fibroblasts) within the interstitial spaces.

In conclusion, the present in vitro study estimated the relative contributions of the two major BK-degrading enzymes, CPN and ACE, to kinin metabolism in plasma. We found that the use of high substrate concentrations led to gross overestimation of the role of CPN in BK metabolism. By using a (nanomolar) substrate concentration at which the velocity of the catalytic reaction is equivalent to that under physiological conditions, we showed that >90% of the BK is sequentially degraded by plasma ACE, leading to accumulation of BK-(1–5). Inhibition of BK-degrading enzyme(s) has been suggested as one strategy for increasing the...
beneficial effects of BK, and it is generally believed that this can be achieved with ACE inhibitors. Our data support this important aspect of the pharmacological control of kinins, which may induce cardioprotective effects by elevating the concentration of BK in plasma.

We thank J. aana Tuomikangas for excellent technical assistance and Dr. Nisse Kalkkinen, Institute of Biotechnology, University of Helsinki, Biocentrum, for performing the NH₂-terminal sequencing analyses.

This study was supported by grants from the Aarne Koskelo Foundation, Helsinki, and the Finnish Heart Association (to J. O. Kokkonen and A. Kuoppala) and from the Finnish Academy of Science and the Ella and Georg Ehrnrooth Foundation (to K. A. Lindstedt).

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Received 17 May 1999; accepted in final form 19 October 1999.

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